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AMP-ACTIVATED PROTEIN KINASE REGULATES GLUT4 TRANSCRIPTION BY PHOSPHORYLATING HISTONE DEACETYLASE 5

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ABSTRACT

Objective: Insulin resistance associated with obesity and diabetes is ameliorated by specific overexpression of GLUT4 in skeletal muscle. The molecular mechanisms regulating skeletal muscle GLUT4 expression remained to be elucidated. The purpose of this study was to examine these mechanisms.

Research Design and Methods and Results: Here we report that AMPK regulates GLUT4 transcription through the HDAC5 transcriptional repressor. Overexpression of HDAC5 represses GLUT4 reporter gene expression and HDAC inhibition in human primary myotubes increases endogenous GLUT4 gene expression. In vitro kinase assays, site directed mutagenesis and site specific phospho-antibodies establish AMPK as a HDAC5 kinase that targets S259 and S498. Constitutively active, but not dominant negative AMPK and AICAR treatment in human primary myotubes, results in HDAC5 phosphorylation at S259 and S498, association with 14-3-3s and H3 acetylation. This reduces HDAC5 association with the GLUT4 promoter, as assessed through chromatin immunoprecipitation assays, and HDAC5 nuclear export, concomitant with increases in GLUT4 gene expression. Gene reporter assays also confirm that the HDAC5 S259 and S498 sites are required for AICAR induction of GLUT4 transcription.

Conclusions: These data reveal a signal transduction pathway linking cellular energy charge to gene transcription directed at restoring cellular and whole body energy balance and provide new therapeutic targets for the treatment and management of insulin resistance and type 2 diabetes.

ABBREVIATIONS. acetyl-H3, acetylated histone 3; AICAR, 5-aminoimidazole-4-carboxamide-1-beta-d-ribonucleoside; AMPK, AMP-activated protein kinase; BSA, bovine serum albumin; CA, constitutively active; CaMK, calcium/calmodulin dependent protein kinases; DMSO, dimethyl sulfoxide; DN, dominant negative; DSG, disuccinimidyl glutarate; ECM, extra cellular matrix; FBS, fetal bovine serum; GLUT4, glucose transporter protein isoform 4; H3, histone 3; H3K9, histone 3 lysine 9; HAT, histone acetyl-transferase; HDAC, histone deacetylase; HSM, human skeletal muscle; Leu, leucine; MARK2, MAP/microtubule affinity-regulating kinase 2; MEF2, myocyte enhancer factor 2; MHC, myosin heavy chain; PBS, phosphate buffered saline; PDH, pyruvate dehydrogenase; PIC, preinitiation complex; PKC, protein kinase C; PKD, protein kinase D; Pro, proline; RT, room temperature; WT, wild type.

Type 2 diabetes currently affects approximately 171 million people worldwide and this figure is predicted to rise to 366 million by 2030 (1). The pathogenesis of this disease involves insulin resistance in peripheral tissues, particularly in skeletal muscle (2). The insulin regulated glucose transporter isoform 4 (GLUT4) is highly expressed in skeletal muscle and adipose tissue (3). Although skeletal muscle GLUT4 expression is not compromised in diabetes and obesity (4), skeletal muscle specific overexpression of GLUT4 ameliorates insulin resistance associated with these diseases (5-7). Consequently, understanding how GLUT4 expression is regulated in skeletal muscle might provide new insights into therapeutic targets for the treatment and management of insulin resistance associated with type 2 diabetes and obesity.

Total GLUT4 levels in skeletal muscle are largely regulated at the level of transcription (8). Analysis of the GLUT4 gene promoter has revealed that a conserved binding site for the myocyte enhancer factor 2 (MEF2) transcription factor is essential for normal skeletal muscle GLUT4 expression (9). While other transcription factors such as MyoD, thyroid receptor α_1 (TRα_1; (10), Krűppel like factor 15 (KLF15; (11) and the GLUT4 enhancer factor (GEF; (12) are also involved, MEF2 appears to be the pivotal regulator of GLUT4 expression, as all of these factors physically interact with MEF2 to induce GLUT4 transcription. In support of this, reduced MEF2 expression correlates with reduced GLUT4 expression (9). In many cell types, MEF2 associates with the histone deacetylase 5 (HDAC5) transcriptional repressor following terminal differentiation. HDAC5 inhibits transcription recruiting histone deacetylase activity, resulting in a highly compact chromatin structure where the transcriptional preinitiation complex (PIC) does not have access to DNA (13). Dissociation of HDAC5 from MEF2 occurs following phosphorylation of HDAC5 on serines 259 and 498 and provides binding sites for the 14-3-3 chaperone proteins to escort HDAC5 from the nucleus (14). This permits the surrounding nucleosome to undergo chromatin remodeling through histone acetylation by histone acetyltransferases (HATs) at histone 3 lysine 9 (H3K9) and 14, which allows the PIC to assemble at the transcription start site (15) and allows MEF2-mediated transcription to proceed (13).

Although it is not clear if HDAC5 directly regulates the GLUT4 gene, we have previously observed that HDAC5 dissociation from MEF2 and subsequent HDAC5 nuclear export is associated with increased GLUT4 gene expression in human skeletal muscle (16). However, the putative HDAC5 kinase, the calcium/calmodulin dependent protein kinase IV (CaMKIV), is not expressed in adult skeletal muscle (16). Furthermore, HDAC5 phosphorylation is only partially blocked following pharmacological inhibition of the CaMK pathways (17). Another potential HDAC5 kinase is the AMP-activated protein kinase (AMPK). AMPK is a heterotrimer consisting of a catalytic α subunit and regulatory β and γ subunits and is activated in response to changes in cellular energy charge and cellular stress via increases in the AMP/ATP ratio. Full activation of AMPK requires phosphorylation of Thr172 in the catalytic α subunit activation T loop, which is mediated by a number of upstream kinases in a cell and context dependent fashion (18). Increased AMPK activity seen during exercise (19) is associated with increased GLUT4 gene expression (20). Furthermore, treatment with the AMPK activator 5-aminoimidazole-4-carboxamide-1-beta-d-ribonucleoside (AICAR), results in increased GLUT4 gene expression in skeletal

muscle (21). The underlying molecular mechanisms mediating this response are unknown.

In the present study, we provide evidence that a region of the GLUT4 gene promoter that includes the MEF2 binding domain is regulated by the HDAC5 transcriptional repressor in human skeletal muscle. Furthermore, we find that AMPK is a HDAC5 kinase and that AMPK regulation of HDAC5 can control GLUT4 gene expression in human skeletal muscle.

RESEARCH DESIGN AND METHODS

Human skeletal muscle biopsies. Muscle samples were obtained from the vastus lateralis of five male subjects (23±2 yrs: 75±5 kg; 21.9±0.3 $kg.cm^{-2}$) using the percutaneous needle biopsy technique with suction, after completing a medical questionnaire and giving their informed, written consent. All experimental procedures were approved by the Deakin University Human Research Ethics Committee.

Human primary skeletal muscle cell culture. Muscle samples were washed, trypsin digested before being filtered through a 100μm cell filter. Fibroblasts were removed by seeding the supernatant in an uncoated flask and incubating for 25 min at 37°C. Myoblasts were cultured in ECM coated flasks in growth media (α-MEM supplemented with 10% fetal bovine serum (FBS), 0.5% antibiotic and 0.5% anti-fungal) at 37°C and 5% CO_2. Myoblasts were grown to 80% confluence before being differentiated to myotubes via serum withdrawal, where media FBS was replaced with 2% horse serum.

Primary cell culture treatments. All experimental protocols were performed four days post differentiation following overnight incubation in serum free media. Cells were exposed to either 1mM AICAR, 10μM 6-(1,3-Dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) or an equivalent volume of vehicle (DMSO) as a control, for 60 min at 37°C. Cells designated for protein extraction were harvested immediately after treatment, while cells designated for RNA extraction were harvested 6 hr after treatment. For glucose uptake studies, cells were washed and incubated in fresh PBS containing 0.1% BSA /1μM insulin for 1hr at 37°C before incubation in 10 μM 2-deoxy-D-[1-^{3}H]-glucose (200μCi/mmol) for 15 mins. Cells were lysed in 0.3M NaOH and scintillation counted (Perkin Elmer, Wellesley).

Immunoblotting. Total and nuclear proteins were extracted, concentration determined and immunoblotted as previously described (16). Membranes were exposed to anti-GLUT4 (Biogenesis, Poole), MEF2, myosin heavy chain (MHC), myogenin, MyoD, 14-3-3 (Santa Cruz Biotechnology, Santa Cruz), HDAC5, pT172 AMPKα, AMPKα (Cell Signaling Technology, Beverly), histone 3 (H3) and acetylated H3K9 and 14 (acetyl-H3; Upstate Biotechnology, Lake Placid), pyruvate dehydrogenase (PDH; Molecular Probes, Eugene), α-tubulin and β-actin (Sigma, St. Louis) antibodies. HDAC5 phospho-Ser259 (raised against LRKTApSEPNLKC) and phospho-Ser498 (raised against LSRTQpSSPLPQ) antibodies were produced by immunizing rabbits with the aforementioned peptides coupled with keyhole limpet hemocyanin. Antisera was harvested and passed through dephosphorylated HDAC5 affinity purification columns followed by the relevant phospho-peptide affinity purification column. Antibodies were then eluted from the columns and dialyzed.

RNA extraction and real time RT-PCR. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia) and was reverse transcribed to cDNA. Real time RT-PCR was conducted with GLUT4 forward (CTT CAT CAT TGG CAT GGG TTT) and reverse (AGG ACC GCA AAT AGA AGG AAG A)

primers (5' to 3') using SYBR green chemistry (Applied Biosystems, Scoresby). Changes in GLUT4 gene expression were normalized to the housekeeping gene cyclophilin.

Expression plasmids. All cloning was performed using the Gateway cloning system (Invitrogen, Carlsbad). HDAC5 was subcloned into the pDEST26 expression vector. Wild type AMPK α_1, β_1 and γ_1 subunits were subcloned into the pDEST27 expression vector. Dominant negative (K45R) and constitutively active (T172D) mutations in pDEST27 AMPK α_1 and S259A and S498A mutations in pDEST26 HDAC5 were performed using the Quickchange kit (Stratagene, La Jolla).

HDAC5 phosphorylation assay. Subconfluent Cos7 cells were transiently transfected with HDAC5 wild type and mutant plasmids using FuGENE 6 (Roche Applied Science, Indianapolis), according to manufacturer's specifications. HDAC5 was immunopurified with 2ug of antibody and 30ul of protein A agarose beads before being incubated with 50mU purified AMPK (Upstate Biotechnology, Lake Placid) in the presence of γ^{32}P ATP (~ 5000cpm/pmol) for 60 min as previously described (22). Gels were dried and exposed to a phosphor imager (Fujifilm, Tokyo) before being reconstituted, transferred to a nitrocellulose membrane and immunoblotted for HDAC5.

Coimmunoprecipitation assays. Coimmunoprecipitation assays were performed as previously described (15) from transiently transfected subconfluent Cos7 cells.

Gene reporter assay. Gene reporter assays were performed as previously described (23) with minor modifications. Cos7 cells were transiently transfected with combinations of plasmids encoding the human GLUT4 promoter tagged to the firefly luciferase gene (hG4-Luc; 250ng), MEF2A plasmid (250ng), or various HDAC5 plasmids (250ng). To control for transfection efficiency, cells were cotransfected with *Renilla* luciferase (25ng) and total transfected DNA made up to 1μg with non expressing plasmid vector (pcDNA). Cells were AICAR or vehicle (DMSO) treated 24 hrs after transfection and harvested 48 hrs after transfection. Dual luciferase assays were performed using a kit (Promega, Madison) according to manufacturer's instructions and were measured using a manual luminometer (Hidex, Turku).

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed on vehicle (DMSO) and AICAR treated primary myotubes using a kit (Upstate Biotechnology, Lake Placid) according to manufacturer's instructions. Primary myotubes were double cross linked in 2mM DSG for 60 min at RT and in 1% formaldehyde for 12 mins before being quenched with 125mM glycine. Cells were lysed, input DNA stored and HDAC5 immunoprecipitated using a HDAC5 antibody (Active Motiff, Carlsbad), or unrelated antibody (mouse IgG) for controls. DNA was extracted and real time RT-PCR was conducted using SYBR green chemistry (Applied Biosystems, Scoresby) with primers designed to amplify a 75 bp fragment of the GLUT4 promoter surrounding the MEF2 binding region (Fwd: 5'-CCT GAC ATT TGG AGG CTC-3'; Rev: 5'-GGA GCA ATG CCC CAA AG-3'). To control for differences in initial DNA concentration, all ChIP cycle threshold (CT) values were normalized to the input CT value for that sample.

Statistical analyses. All data are presented as means ± standard error of the mean (SEM) with control samples assigned the arbitrary value of 1.0 and treatment samples expressed relative to control. For data from semi quantitive assays, values are designated as arbitrary units. Means were compared using a t-test or one way ANOVA with a significance level of $p < 0.05$.

RESULTS

HDAC5 regulates the GLUT4 gene. We have previously found that HDAC5 dissociation from MEF2 and subsequent HDAC5 nuclear export is associated with increased GLUT4 gene expression in human skeletal muscle (16). From these data it is not possible to determine if HDACs, or specifically HDAC5, directly regulates GLUT4 transcription. To test this, human skeletal muscle biopsies of the vastus lateralis were grown in culture and differentiated into human primary myotubes. These cells differentiated into elongated myotubes (Supplementary Figure 1 available at http://diabetes.diabetesjournals.org) concomitant with increased muscle specific myogenic marker expression, including myosin heavy chain (MHC), MyoD and myogenin (Figure 1A). GLUT4 and MEF2, a transcription factor required for GLUT4 expression, are expressed in these terminally differentiated myotubes, although GLUT4 was expressed at lower levels than in extracts from human skeletal muscle (Figure 1A). Furthermore, these myotubes displayed insulin dependent glucose uptake (Figure 1B).

To determine if the GLUT4 gene is regulated by HDACs, primary myotubes were exposed to a single, 60 min treatment with the HDAC inhibitor Scriptaid. Scriptaid possesses relatively low toxicity and is structurally similar to the class of hydroxamic acid-containing HDAC inhibitors (24) that inhibit HDAC activity by interacting with a zinc binding site found in a small polar pocket within the C-terminal deacetylase domain and do not appear to dissociated HDACs from their DNA binding partner or result in HDAC relocalization (25). Following Scriptaid treatment, GLUT4 mRNA was elevated 7 fold ($p<0.05$) when compared with control (Figure 1C). Expression of the housekeeping gene cyclophilin did not change (2^{-CT} values: $1.18x10^{-9} \pm 1.01x10^{-10}$ vs. $1.07x10^{-9} \pm 1.27x10^{-10}$; $p = 0.72$). Scriptaid treatment also increased H3 acetylation ~9 fold ($p <0.05$) when compared with control (Supplementary Figure 2), consistent with the increased GLUT4 transcription being a result of chromatin remodeling. There was no change in nuclear HDAC5 abundance following Scriptaid treatment (Supplementary Figure 3), confirming that Scriptaid does not alter HDAC localization.

To determine if the GLUT4 gene is directly regulated by HDAC5, a gene reporter assay using the human GLUT4 promoter coupled to the firefly luciferase gene (hG4-Luc) was performed. This promoter contains all the regulatory elements located within -895bp of the transcription start site that are required for normal GLUT4 expression *in vivo* (23). To control for transfection efficiency, cells were also transfected with a plasmid expressing *Renilla* luciferase. Cotransfection with MEF2A plasmid increased GLUT4 reporter gene expression ~4.5 fold ($p<0.05$), which was returned to control levels with the transfection of wild type (WT) HDAC5 (Figure 1D). Expression of the MEF2A and HDAC5 plasmids was verified by immunoblotting. Together, these data show that the GLUT4 gene is regulated by HDAC activity and identify HDAC5 as a repressor of GLUT4 gene expression.

AMPK regulates HDAC5. Since increases in AMPK activity are associated with increases in GLUT4 expression, we sought to determine if AMPK might regulate HDAC5. Specifically, phosphorylation of HDAC5 serines 259 and 498 causes HDAC5 dissociation from MEF2. To test if AMPK could act as a HDAC5 kinase, HDAC5 plasmids expressing either WT, a serine 259 to alanine mutant (S259A), a serine 498 to alanine mutant (S498A) or a dual serine 259 and 498 to alanine mutant (S259/498A) were transiently transfected into subconfluent Cos7 cells. HDAC5 was immunoprecipitated from lysates of these cells and incubated with purified AMPK in the presence of γ^{32}P ATP. Incubation of WT HDAC5 with AMPK

resulted in HDAC5 phosphorylation that was not observed when AMPK was absent (Figure 2A).

Mutation of serine 259 was sufficient to block AMPK phosphorylation of HDAC5 (Figure 2A), supporting previous observations that this site is a priming site required for the subsequent phosphorylation of other sites (26). This site is targeted by a number of kinases, including members of the family of AMPK related, LKB1 regulated kinases (26; 27). Mutation of serine 498 showed that AMPK only weakly phosphorylates serine 259 *in vitro* (Figure 2A). Differences in HDAC5 expression could not account for these results (Figure 2A). Although residual phosphorylation of HDAC5 is present in the HDAC5 mutants and when AMPK is not added to the reaction, this is likely due to endogenous HDAC5 and AMPK being pulled down during the immunoprecipitation. Both phosphorylation sites share features with other AMPK sites, namely a hydrophobic residue at -5 (Leu/Leu), a basic residue within -1 to -3 and a hydrophobic residue at +4 (Leu/Pro; LRKTAS^{259}EPNLKV and LSRTQS^{498}SPLPQ; (28).

Coimmunoprecipitation from lysates of transiently transfected Cos7 cells identified that both catalytic AMPK α_1 and α_2 subunits associate with HDAC5, consistent with kinase-substrate interactions (Figure 2B). Alanine mutations of S259 and S498 did not disturb this interaction, demonstrating that the reduction in AMPK mediated phosphorylation of these mutants was due to the mutation *per se*, and was not due to any alterations in protein/protein interactions between AMPK and HDAC5. It also appeared that HDAC5 S/A mutations increased its association with AMPK. While not examined in the present study, this could be due to disrupted phosphorylation-dissociation kinetics caused by the mutations.

From these data, it is clear that S259 and S498 are required for AMPK phosphorylation of HDAC5, however it is unclear if these are the sites directly phosphorylated by AMPK. To test this, antibodies were raised recognizing the sequences surrounding S259 and S498 when phosphorylated. Total HDAC5 was immunoprecipitated from lysates of Cos7 cells transiently transfected with WT, constitutively active (CA) or dominant negative (DN) AMPK (and regulatory β and γ subunits) and either WT or S259/498A HDAC5 and probed with these antibodies. Serine 259 showed high levels of basal phosphorylation (Figure 2C), consistent with the idea that this site is a hierarchical priming site targeted by a number of kinases and also potentially explaining the weak phosphorylation of this site by AMPK *in vitro*. Expression of CA AMPK induced phosphorylation of both S259 and S498 above that observed with the expression of WT AMPK, but increased S498 phosphorylation to a greater extent than the S259 site (Figure 2C). Furthermore, this was attenuated with expression of DN AMPK (Figure 2C). Phosphorylation of HDAC5 at these residues promoted HDAC5 association with 14-3-3 isoforms, as assessed by coimmunoprecipitation, and acetylation at H3K9 and 14. Importantly, these effects were blocked by expression of the S259/498A HDAC5 mutant (Figure 2C). It is well established that association of HDAC5 with 14-3-3 is required for disruption of the MEF2-HDAC5 complex and HDAC5 nuclear export (29). These results therefore indicate that AMPK phosphorylation of HDAC5 at S259 and S498 is sufficient to induce HDAC5 nuclear export via association with the 14-3-3 chaperone proteins, in turn shifting the chromatin state to one that favored histone acetylation.

To determine if activation of endogenous AMPK results in HDAC5 phosphorylation, human primary myotubes were treated with AICAR for 60 min. In these samples, AICAR treatment increased AMPK phosphorylation

~40% ($p<0.05$) and increased HDAC5 phosphorylation ~65% ($p<0.05$) and ~100% ($p<0.05$) at S259 and S498 respectively (Figure 3A). This again showed that AMPK increases phosphorylation at S498 than at S259. These results were associated with a ~55% increase in H3K9 and 14 acetylation (Figure 3A). To examine the functional relationship between AMPK activation and HDAC5 localization, human primary myotubes were exposed to an acute 60 min treatment of AICAR and nuclear and whole cell lysates were analysed for HDAC5 protein abundance. Following AICAR treatment, a 26% reduction ($p<0.05$) in nuclear HDAC5 was observed, without any change in total HDAC5 expression (Figure 3B), consistent with HDAC5 nuclear export. Immunoblotting for H3 and PDH confirmed nuclear fraction enrichment (Figure 3B). Taken together, these data suggest that AMPK is a HDAC5 kinase capable of regulating HDAC5 localization and chromatin remodeling by phosphorylating the key regulatory HDAC5 sites, serines 259 and 498.

AMPK regulates the GLUT4 gene through HDAC5. To investigate if AMPK can regulate the GLUT4 gene through HDAC5, further GLUT4 reporter gene assays were performed. Cos7 cells were transfected with hG4-Luc, MEF2A and WT HDAC5 plasmids and treated either with a vehicle control (DMSO) or with AICAR. AICAR treated cells showed ~40% higher ($p<0.05$) GLUT4 reporter gene expression than control cells (Figure 4A), indicating that activation of endogenous AMPK can reduce HDAC5 repression of the GLUT4 reporter gene due to HDAC5 overexpression. Cells transfected with hG4-Luc, MEF2A and S259/498A HDAC5 plasmids and treated with AICAR showed no increase in GLUT4 reporter gene expression when compared with control treated transfected cells (Figure 4B), indicating that serines 259 and 498 are required for the AICAR/AMPK mediated increase in GLUT4 reporter gene expression. Exclusive mutation of either site produced similar results (Supplementary Figure 4). Treatment of transfected cells with AICAR in the absence of HDAC5 expression had no influence on GLUT4 reporter gene expression when compared with control (Figure 4C) further supporting the idea that HDAC5 is required for AMPK induction of GLUT4 transcription and is not due to activation of endogenous transcriptional coactivators. This also supports previous observations that AMPK does not phosphorylate and transactivate MEF2 (30). Immunoblotting confirmed activation of AMPK in response to AICAR treatment (Supplementary Figure 5). Collectively, the results obtained from this series of reporter gene assays imply that HDAC5 phosphorylation on serines 259 and 498 by AMPK leads to an increase in GLUT4 gene expression.

To further examine this paradigm in a non reconstituted system *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed on human primary myotubes in the presence or absence of AICAR, using primers to amplify a 75 bp region of the GLUT4 promoter surrounding the MEF2 binding domain (Figure 5A). Using this method, HDAC5 was found associated with this region of the GLUT4 promoter in control cells, suggesting that HDAC5 regulates the GLUT4 gene *in vivo*. Furthermore, the amount of HDAC5 associated with the GLUT4 promoter was reduced ~70% ($p<0.05$) following AICAR treatment (Figure 5A). These results could not be attributed to non specific effects of the immunoprecipitating antibody, as an unrelated antibody (mouse IgG) immunoprecipitated vastly lower amounts of DNA (>100 fold), nor to any indirect effects of AICAR, as input DNA was not different with this treatment (Con; $6.2\text{x}10^{-9}\pm1.6\text{x}10^{-9}$ vs. AICAR; $8.2\text{x}10^{-9}\pm1.6\text{x}10^{-9}$). A reduction in HDAC5 associated with the GLUT4

promoter region was also associated with a ~ 2 fold increase in GLUT4 mRNA ($p<0.05$) in human primary myotubes treated with AICAR (Figure 5B). These results are consistent with those obtained from the gene reporter studies and suggest that AMPK mediated HDAC5 phosphorylation regulates the GLUT4 gene.

DISCUSSION

Given that overexpression of GLUT4 in skeletal muscle ameliorates many of the symptoms associated with type 2 diabetes (5-7), there has been considerable interest in understanding the regulation of GLUT4 expression. From results obtained in the present study, we propose that phosphorylation of HDAC5 on serines 259 and 498 by AMPK is sufficient to induce GLUT4 transcription. These data provide a molecular mechanism linking our previous observations that AMPK translocates to the nucleus (31) while HDAC5 is dissociated from MEF2 and exported from the nucleus (16) with associated increases in GLUT4 gene expression in human skeletal muscle. These findings also describe a signaling pathway linking cellular energy charge to gene transcription directed at restoring cellular and whole body energy balance. It is also well established that activation of AMPK is associated with enhanced expression of numerous metabolic genes involved in carbohydrate metabolism, lipid metabolism and the electron transport chain (32), suggesting that HDAC5 could be a key regulator of metabolic gene expression in skeletal muscle. Indeed, many of these genes possess conserved MEF2 binding domains on their gene promoter regions. In the heart, S259/498A mutation of HDAC5 leads to mitochondrial dysfunction and cardiac sudden death secondary to reduced mitochondrial enzyme expression (33). Preliminary work in cultured skeletal muscle cells suggests that HDAC5 is an important mediator of metabolism, as overexpression of HDAC5 represses numerous metabolic genes (SL McGee and K Baar, unpublished observations).

From data obtained in transgenic kinase dead AMPK expressing mice, it appears that AMPK is not the only kinase capable of regulating the GLUT4 gene. Although the AICAR induced increase in GLUT4 gene expression is abolished in these mice, they show similar increases in GLUT4 gene expression following exercise when compared with control mice (34). This apparent redundancy could be explained by recent studies identifying members of the family of AMPK related protein kinases (26; 27), as well as CaMKs and PKD (35) as kinases capable of phosphorylating serines 259 and 498 on HDAC5 and removing HDAC5 mediated repression of transcriptional activity. HDAC5 can also be regulated in a manner independent of kinase activity. Interaction of Ca^{2+}- calmodulin (36) and protein Gβγ (37) with HDAC5 inhibits HDAC5 and increases MEF2 transcriptional activity. A recent study also suggests that HDAC5 activity can be regulated by the ubiquitin proteasome pathway (38). Furthermore, we cannot exclude the possibility that other members of the class IIa HDACs regulate the GLUT4 gene, given that all isoforms are expressed in skeletal muscle to some degree. Together these data suggest that AMPK activation via perturbations in energy balance is one of a variety of stimuli that can regulate the GLUT4 gene. Further investigation of these mechanisms is currently underway.

In the present study, we propose that AMPK phosphorylation of HDAC5 relieves transcriptional repression of the GLUT4 gene. AMPK is also known to phosphorylate GEF *in vitro* (29). Although the specific site(s) on GEF have not yet been identified, nor is it known if this effect is preserved *in vivo*, phosphorylation of both HDAC5 and GEF by

AMPK could be a mechanism to exert further control over GLUT4 transcription. Supporting this, we have observed increases in DNA binding activity of both MEF2 and GEF during exercise in human skeletal muscle (39). However, further studies examining the relationship between HDAC5 and GEF regulation are required.

HDAC5 belongs to the class IIa family of HDACs, which also includes isoforms 4, 7 and 9. Isoforms 4 and 7 are also regulated by phosphorylation dependent nuclear-cytoplasmic shuttling (14; 40) through interactions with 14-3-3s. Sequence analysis suggests that the phosphorylation sites involved in this mechanism are largely conserved between these isoforms. This raises the possibility that AMPK could also regulate HDAC4 and HDAC7. While these HDAC isoforms functionally overlap to regulate a number of common cellular process, there are also examples of specific functional regulation by these individual isoforms (41; 42) and suggest that AMPK might also be involved in such processes. There are other AMPK consensus sites (S279 and S661) in addition to S259 and S498 found within HDAC5 using the AMPK consensus motif. It is unknown if AMPK phosphorylates these sites *in vivo*. However, it is known that these sites have no effect on HDAC localization (14). The role, if any, that these sites play in HDAC5 regulation remains to be determined. Furthermore, the interaction between the 259 site, thought to be a priming site for subsequent phosphorylation (26), and the kinases that target this and other sites needs to be determined.

In conclusion, the results from the present study demonstrate that phosphorylation of HDAC5 on serines 259 and 498 by AMPK regulates the GLUT4 gene through a region containing the MEF2 binding site on the GLUT4 gene promoter in human skeletal muscle. These data provide further insights into potential therapeutic targets for the treatment and management of insulin resistance and type 2 diabetes.

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REFERENCES

1. Wild S, Roglic G, Green A, Sicree R, King H: Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27:1047-1053, 2004
2. Shulman GI: Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171-176, 2000
3. James DE, Brown R, Navarro J, Pilch PF: Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature* 333:183-185, 1988
4. Andersen PH, Lund S, Vestergaard H, Junker S, Kahn BB, Pedersen O: Expression of the major insulin regulatable glucose transporter (GLUT4) in skeletal muscle of noninsulin-dependent diabetic patients and healthy subjects before and after insulin infusion. *J Clin Endocrinol Metab* 77:27-32, 1993
5. Leturque A, Loizeau M, Vaulont S, Salminen M, Girard J: Improvement of insulin action in diabetic transgenic mice selectively overexpressing GLUT4 in skeletal muscle. *Diabetes* 45:23-27, 1996
6. Ren JM, Marshall BA, Mueckler MM, McCaleb M, Amatruda JM, Shulman GI: Overexpression of Glut4 protein in muscle increases basal and insulin-stimulated whole body glucose disposal in conscious mice. *J Clin Invest* 95:429-432, 1995
7. Tsao TS, Burcelin R, Katz EB, Huang L, Charron MJ: Enhanced insulin action due to targeted GLUT4 overexpression exclusively in muscle. *Diabetes* 45:28-36, 1996
8. Neufer PD, Carey JO, Dohm GL: Transcriptional regulation of the gene for glucose transporter GLUT4 in skeletal muscle. Effects of diabetes and fasting. *J Biol Chem* 268:13824-13829, 1993
9. Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL: Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem* 273:14285-14292, 1998
10. Santalucia T, Moreno H, Palacin M, Yacoub MH, Brand NJ, Zorzano A: A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. *J Mol Biol* 314:195-204, 2001
11. Gray S, Feinberg MW, Hull S, Kuo CT, Watanabe M, Sen S, DePina A, Haspel R, Jain MK: The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. *J Biol Chem* 277:34322-34328, 2002
12. Oshel K, Knight J, Cao K, Thai M, Olson A: Identification of a 30-base pair regulatory element and novel DNA binding protein that regulates the human GLUT4 promoter in transgeneic mice. *J Biol Chem* 275:23666-23673, 2000
13. McKinsey TA, Zhang CL, Olson EN: Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev* 11:497-504, 2001
14. McKinsey T, Zhang C, Olson E: Signal-dependent nuclear export of a histone deactylase regulates muscle differentiation. *Nature* 408:106-111, 2000
15. Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA: A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* 130:77-88, 2007
16. McGee SL, Hargreaves M: Exercise and myocyte enhancer factor 2 regulation in human skeletal muscle. *Diabetes* 53:1208-1214, 2004
17. McKinsey TA, Zhang CL, Olson EN: Signaling chromatin to make muscle. *Curr Opin Cell Biol* 14:763-772, 2002
18. Towler MC, Hardie DG: AMP-activated protein kinase in metabolic control and insulin signaling. *Circ Res* 100:328-341, 2007

19. Fujii N, Hayashi T, Hirshman M, Smith J, Habinowski S, Kaijser L, Mu J, Ljungqvist O, Birnbaum M, Witters L, Thorell A, Goodyear L: Exercise induces isoform-specific increase in 5' AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273:1150-1155, 2000
20. Kraniou Y, Cameron-Smith D, Misso M, Collier G, Hargreaves M: Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol* 88:794-796, 2000
21. Holmes B, Kurth-Kraczek E, Winder W: Chronic activation of 5' AMP-activated protein kinase increases GLUT4, hexokinase, and glycogen in muscle. *J Appl Physiol* 87:1990-1995, 1999
22. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE: AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443:285-289, 1999
23. Knight JB, Eyster CA, Griesel BA, Olson AL: Regulation of the human GLUT4 gene promoter: interaction between a transcriptional activator and myocyte enhancer factor 2A. *Proc Natl Acad Sci U S A* 100:14725-14730, 2003
24. Su GH, Sohn TA, Ryu B, Kern SE: A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. *Cancer Res* 60:3137-3142, 2000
25. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP: Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401:188-193, 1999
26. Dequiedt F, Martin M, Von Blume J, Vertommen D, Lecomte E, Mari N, Heinen MF, Bachmann M, Twizere JC, Huang MC, Rider MH, Piwnica-Worms H, Seufferlein T, Kettmann R: New role for hPar-1 kinases EMK and C-TAK1 in regulating localization and activity of class IIa histone deacetylases. *Mol Cell Biol* 26:7086-7102, 2006
27. Berdeaux R, Goebel N, Banaszynski L, Takemori H, Wandless T, Shelton GD, Montminy M: SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. *Nat Med* 13:597-603, 2007
28. Michell BJ, Stapleton D, Mitchelhill KI, House CM, Katsis F, Witters LA, Kemp BE: Isoform-specific purification and substrate specificity of the 5'-AMP-activated protein kinase. *J Biol Chem* 271:28445-28450, 1996
29. McKinsey TA, Zhang CL, Olson EN: Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A* 97:14400-14405, 2000
30. Holmes BF, Sparling DP, Olson AL, Winder WW, Dohm GL: Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 289:E1071-1076, 2005
31. McGee SL, Howlett KF, Starkie RL, Cameron-Smith D, Kemp BE, Hargreaves M: Exercise Increases Nuclear AMPK alpha(2) in Human Skeletal Muscle. *Diabetes* 52:926-928, 2003
32. Jorgensen SB, Treebak JT, Viollet B, Schjerling P, Vaulont S, Wojtaszewski JF, Richter EA: Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. *Am J Physiol Endocrinol Metab* 292:E331-339, 2007
33. Czubryt MP, McAnally J, Fishman GI, Olson EN: Regulation of peroxisome proliferator-activated receptor {gamma} coactivator 1{alpha} (PGC-1{alpha}) and mitochondrial function by MEF2 and HDAC5. *Proc Natl Acad Sci U S A*, 2003

34. Holmes BF, Lang DB, Birnbaum MJ, Mu J, Dohm GL: AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. *Am J Physiol Endocrinol Metab* 287:E739-743, 2004
35. Chang S, Bezprozvannaya S, Li S, Olson EN: An expression screen reveals modulators of class II histone deacetylase phosphorylation. *Proc Natl Acad Sci U S A* 102:8120-8125, 2005
36. Berger I, Bieniossek C, Schaffitzel C, Hassler M, Santelli E, Richmond TJ: Direct interaction of Ca2+/calmodulin inhibits histone deacetylase 5 repressor core binding to myocyte enhancer factor 2. *J Biol Chem* 278:17625-17635, 2003
37. Spiegelberg BD, Hamm HE: G betagamma binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity. *J Biol Chem* 280:41769-41776, 2005
38. Potthoff MJ, Wu H, Arnold MA, Shelton JM, Backs J, McAnally J, Richardson JA, Bassel-Duby R, Olson EN: Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. *J Clin Invest* 117:2459-2467, 2007
39. McGee SL, Sparling D, Olson AL, Hargreaves M: Exercise increases MEF2- and GEF DNA-binding activity in human skeletal muscle. *Faseb J* 20:348-349, 2006
40. Grozinger CM, Schreiber SL: Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proc Natl Acad Sci U S A* 97:7835-7840, 2000
41. Chang S, Young BD, Li S, Qi X, Richardson JA, Olson EN: Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell* 126:321-334, 2006
42. Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN: Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* 119:555-566, 2004

FIGURE LEGENDS

Figure 1. Characterization of human primary myotubes and regulation of GLUT4 expression by HDACs and HDAC5. (A) Expression of GLUT4, MEF2 and myogenic markers in differentiating human primary muscle cell culture and in human skeletal muscle (HSM) (B) Basal and insulin stimulated glucose uptake in human primary skeletal muscle cells pre differentiation and following four days of differentiation (n=4). All values are reported as the means ± SEM. # significantly different from day 0 (p=0.001). + Significantly different from day 3 (p=0.001).* significantly different from basal at day four (p=0.027). (C) GLUT4 mRNA following 60 min treatment of 10μM Scriptaid (n=5). All values are calculated as the fold changes relative to control and reported as the means ± SEM. (D) Luciferase GLUT4 reporter gene activity and representative immunobolts following transfection of Cos7 cells with plasmids encoding MEF2A and WT HDAC5. All values are normalized to *Renilla* luciferase activity (n=4). All values are calculated as the fold changes relative to control and reported as the means ± SEM. * significantly different from control (p=0.010). + significantly different from control (p=0.014) # significantly different from control and MEF2 + HDAC5 (p=0.037 and 0.029 respectively).

Figure 2. AMPK regulates HDAC5. (A) Autoradiograph (top) and immunoblot (bottom) of *in vitro* phosphorylation assays with WT HDAC5 and serine to alanine HDAC5 mutants (S259A, S498A and S259/498A) incubated with or without purified AMPK in the presence of [γ^{32}P]-ATP. HDAC5 expression was verified by immunoblotting. (B) Western blot of WT HDAC5 and serine to alanine HDAC5 mutant (S259A, S498A and S259/498A) coimmunoprecipitates with AMPK α_1 and α_2 catalytic subunits. (C) Representative immunoblots of HDAC5 S259 and S498 phosphorylation, 14-3-3 association and H3 acetylation in response to transfection with WT, CA or DN AMPK.

Figure 3. AICAR regulates endogenous HDAC5 in human primary myotubes. (A) Phosphorylation of T172 AMPKα, S259 and S498 HDAC5 and acetylation of H3K9 and 14 and representative immunoblots following AICAR treatment for 60 min in human primary myotubes (n=6). All values are calculated as the fold changes relative to control and reported as the means ± SEM. * significantly different from control (p=0.03). † significantly different from control (p=0.004). Δ significantly different from control (p=0.002). ^ significantly different from control (p=0.007). (B) Total and nuclear HDAC5, H3 and PDH abundance and representative immunoblots following 60 min treatment of 1mM AICAR. All values are calculated as the fold changes relative to control and reported as the means ± SEM (n=5). # significantly different from control (p=0.030).

Figure 4. AMPK regulates the GLUT4 gene through phosphorylation of HDAC5. (A) Luciferase GLUT4 reporter gene activity and representative immunoblots following transfection of Cos7 cells with plasmids encoding MEF2A and WT HDAC5 in the absence or presence of AICAR. (B) Luciferase GLUT4 reporter gene activity and representative immunoblots following transfection of Cos7 cells with plasmids encoding MEF2A and S259/498A mutant HDAC5 in the absence or presence of AICAR. (C) Luciferase GLUT4 reporter gene activity and representative immunoblots in the absence or presence of AICAR and following co-transfection with a plasmid encoding MEF2A in the absence or presence of AICAR. All values are

normalized to *Renilla* luciferase activity and calculated as the fold changes relative to control and reported as the means ± SEM (n=4). * significantly different from control (p=0.032).

Figure 5. AMPK regulation of HDAC5 and the GLUT4 promoter *in vivo* (A) Schematic representation of the ChIP PCR amplification region surrounding the MEF2 binding region (B) HDAC5 associated GLUT4 promoter DNA following 60 min treatment with 1mM AICAR as determined through ChIP analysis (n = 3). (C) GLUT4 mRNA following 60 min treatment of 1 mM AICAR (n=5). All values are calculated as the fold changes relative to control and reported as the means ± SEM. + significantly different from control (p=0.001). # significantly different from control (p=0.021).

FIGURE 1

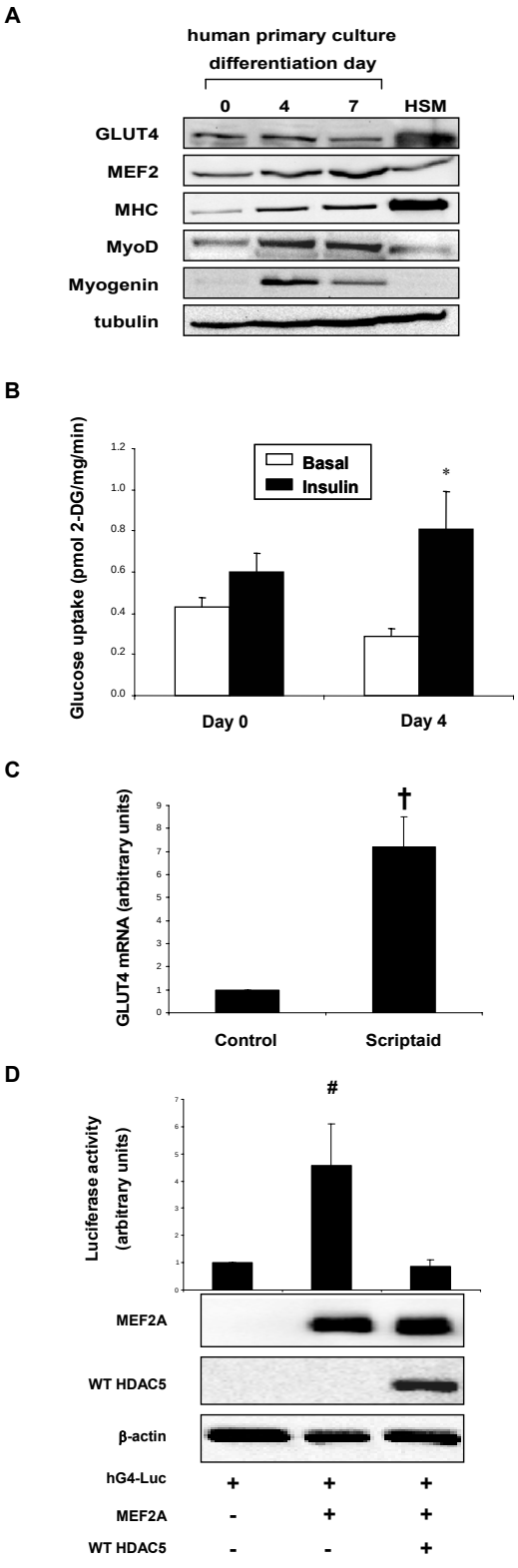

A
human primary culture
differentiation day
0
4
7
HSM
GLUT4
MEF2
MHC
MyoD
Myogenin
tubulin
B
Glucose uptake (pmol 2-DG/mg/min)
Basal
Insulin
*
Day 0
Day 4
C
GLUT4 mRNA (arbitrary units)
†
Control
Scriptaid
D
Luciferase activity (arbitrary units)
#
MEF2A
WT HDAC5
β-actin
hG4-Luc + + +
MEF2A - + +
WT HDAC5 - - +

FIGURE 2

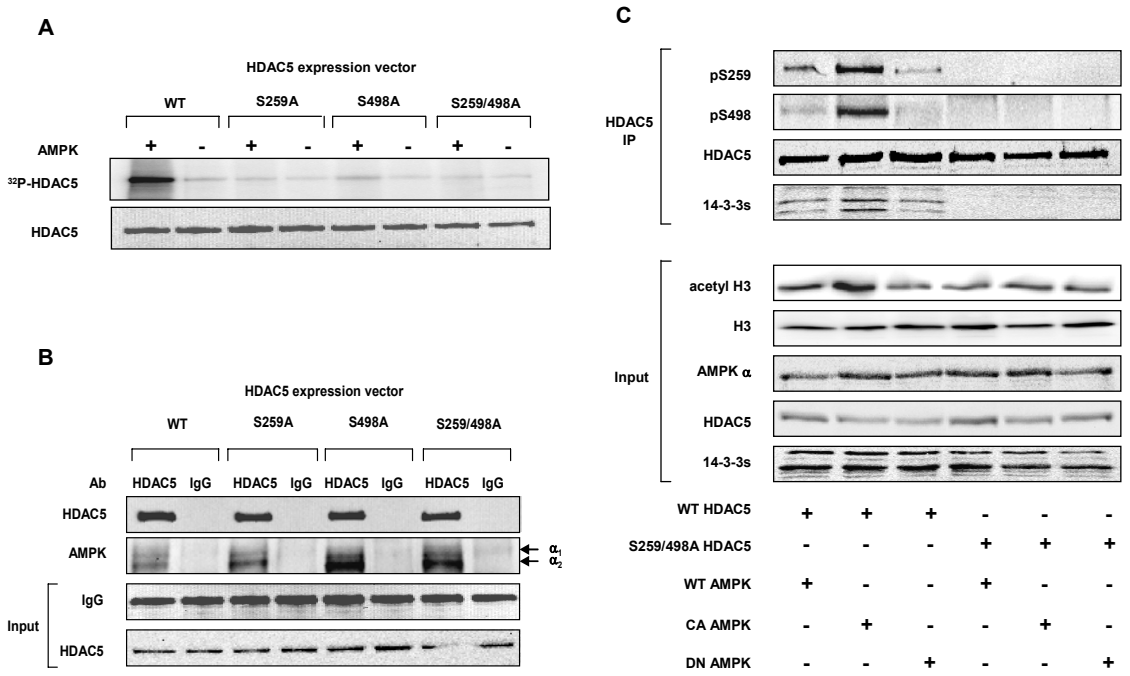

A
HDAC5 expression vector
WT
S259A
S498A
S259/498A
AMPK
+ - + - + - + -
32P-HDAC5
HDAC5
B
HDAC5 expression vector
WT
S259A
S498A
S259/498A
Ab
HDAC5 IgG HDAC5 IgG HDAC5 IgG HDAC5 IgG
HDAC5
AMPK
α1
α2
Input
IgG
HDAC5
C
HDAC5 IP
pS259
pS498
HDAC5
14-3-3s
Input
acetyl H3
H3
AMPK α
HDAC5
14-3-3s
WT HDAC5 + + + - - -
S259/498A HDAC5 - - - + + +
WT AMPK + - - + - -
CA AMPK - + - - + -
DN AMPK - - + - - +

FIGURE 3

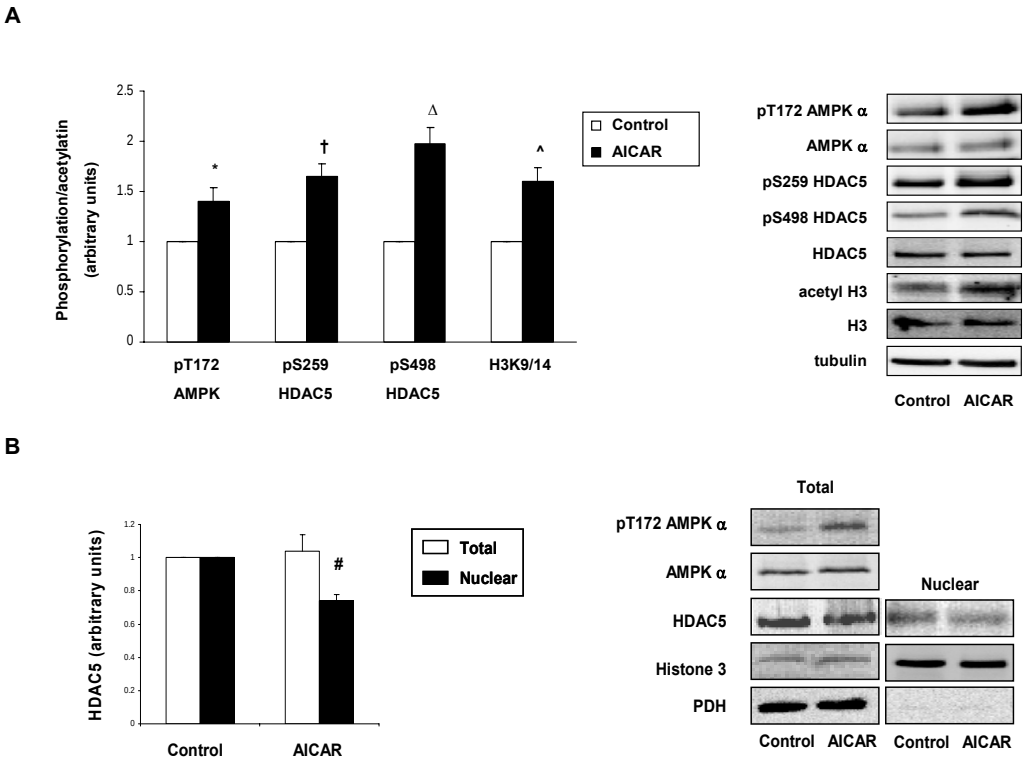

A
Phosphorylation/acetylatin (arbitrary units)
2.5
2
1.5
1
0.5
0
*
†
Δ
^
Control
AICAR
pT172 AMPK
pS259 HDAC5
pS498 HDAC5
H3K9/14
pT172 AMPK α
AMPK α
pS259 HDAC5
pS498 HDAC5
HDAC5
acetyl H3
H3
tubulin
Control AICAR
B
HDAC5 (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
#
Total
Nuclear
Control
AICAR
Total
pT172 AMPK α
AMPK α
HDAC5
Histone 3
PDH
Nuclear
Control AICAR Control AICAR

FIGURE 4

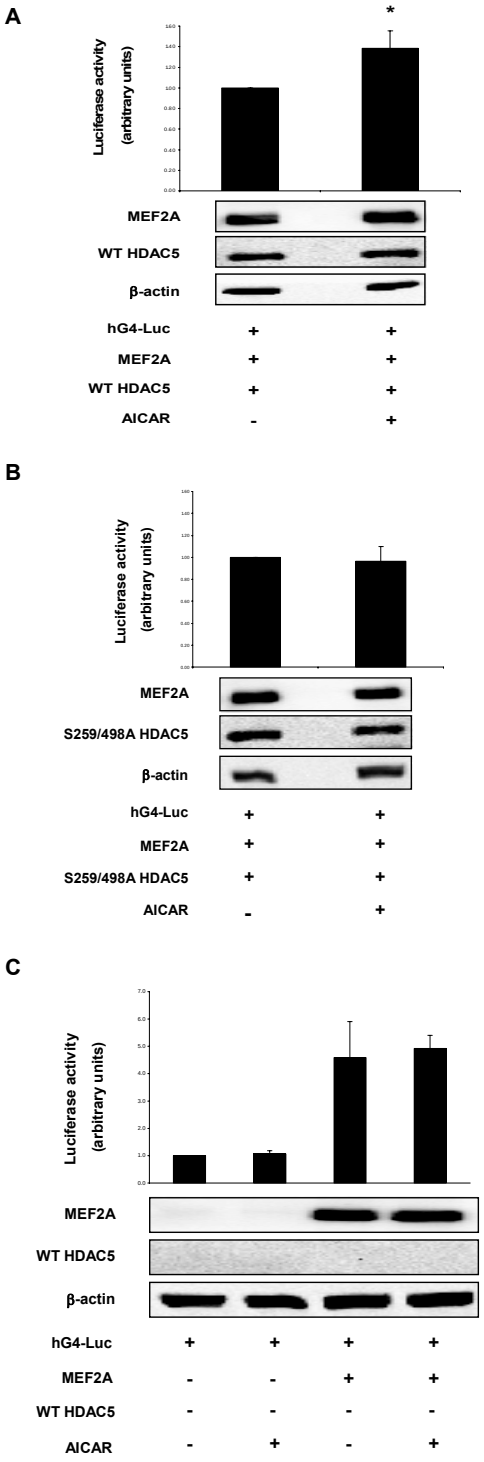
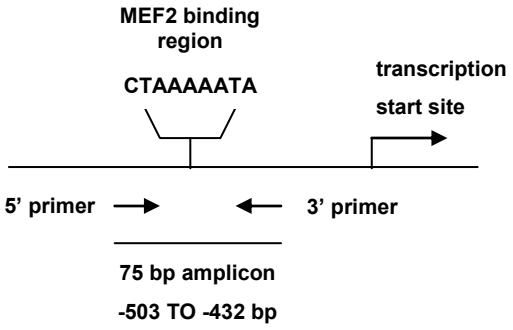
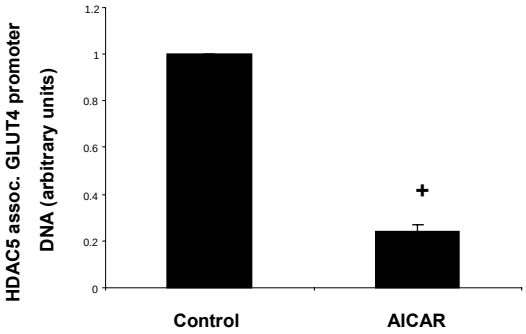

A
*
Luciferase activity (arbitrary units)
MEF2A
WT HDAC5
β-actin
hG4-Luc + +
MEF2A + +
WT HDAC5 + +
AICAR - +
B
Luciferase activity (arbitrary units)
MEF2A
S259/498A HDAC5
β-actin
hG4-Luc + +
MEF2A + +
S259/498A HDAC5 + +
AICAR - +
C
Luciferase activity (arbitrary units)
MEF2A
WT HDAC5
β-actin
hG4-Luc + + + +
MEF2A - - + +
WT HDAC5 - - - -
AICAR - + - +

FIGURE 5

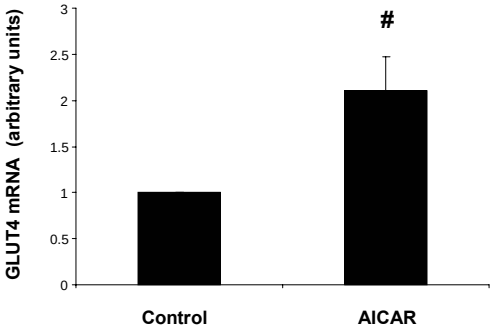
A


MEF2 binding region
CTAAAAATA
transcription start site
5' primer
3' primer
75 bp amplicon
-503 TO -432 bp

B


HDAC5 assoc. GLUT4 promoter DNA (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
+
Control
AICAR

C


GLUT4 mRNA (arbitrary units)
3
2.5
2
1.5
1
0.5
0
#
Control
AICAR