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Supplemental Data

Glucose Restriction Inhibits Skeletal Myoblast Differentiation by Activating SIRT1 through AMPK-Mediated Regulation of Nampt

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and Retroviral Constructs

Myc-AMPK-DN constructs were generated by subcloning PCR fragment corresponding to full-length cDNA of the α-2 catalytic subunit of rat AMPK, bearing the K45R mutation (Mu et al., 2001) (gift from R. Jones) restricted with EcoRI and XhoI into the pCS2MT plasmid. The BamH1 site in the AMPK-DN cDNA of pCS2MT-AMPK-DN was silently mutated (QuickChange kit, Stratagene) to allow its subcloning into the retroviral vector pHan. Myc-AMPK-DN was excised from the pCS2MT plasmid with BamHI and XhoI and ligated into BamHI/SalI restricted pHAN vector to generate pHan-myc-AMPK-DN. The construct was sequenced and the presence of nucleotides corresponding to the mutation K45R confirmed (AAG to AGG).

Nampt constructs were generated by subcloning PCR fragments corresponding to full-length cDNA of murine Nampt (gift from F. Andris) restricted with EcoRI/XhoI into the pCS2MT vector. Myc-Nampt was then excised from the pCS2MT vector using BamHI and XhoI restriction enzymes and ligated into BamHI/SalI restricted pHan retroviral vector to generate pHan-myc-Nampt. The mutants pHan-myc-Nampt A244M and pHan-myc-Nampt A244M-S314A were generated by site direct mutagenesis using the QuickChange kit from Stratagene. NNMT cloned into the pCS2MT plasmid was excised with BamHI/XhoI and subcloned into pHan restricted with BamHI/SalI to generated pHan-myc-NNMT. The pHan-Flag-Sirt1 vector has been previously described (Fulco et

al., 2003).To generate the retroviral vectors pSUPER.Retro.Puro sh Sirt1 and pSUPER.Retro.Puro sh Nampt, complementary oligonucleotides corresponding to specific regions of Sirt1 and Nampt mRNAs respectively (see Oligonucleotides) were ligated to the pSUPER.Retro.Puro vector (OligoEngine) digested with BglII and HindIII.

Antibodies, Immunoblotting and Immunofluorescence

C2C12 cells differentiated in either normocaloric or glucose restriction conditions for 48hr were harvested and lysed in RIPA buffer. Protein expression was assessed by Western blot analysis with chemiluminescence detection. Nampt was detected using a rabbit polyclonal anti-mouse Nampt antibody (Bethyl Laboratories). SIRT1, total ACC, phospho-ACC (pSer79), and myc antibodies were from Upstate Biotechnology. Total AMPK and phospho-AMPK (pThr172) rabbit monoclonal antibodies were from Cell Signaling Technology. Caveolin-3, Myf5, UCP-2, UCP-3, and myogenin antibodies were from Santa Cruz Biotechnologies. Anti-β-tubulin E7, MF20 (anti-myosin heavy chain), N2.261 (perinatal myosin), and Pax7 monoclonal antibodies were purchased from Developmental Studies Hybridoma Bank. Monoclonal anti-GAPDH antibody was from Abcam. For immunofluorescence analysis, C2C12 cells or mouse primary myoblasts were fixed in 3.7% paraformaldehyde. After permeabilization with 0.2 % TritonX, cells were incubated for an hour with the MF20 antibody. After incubation with fluoresceinated secondary antibody, cells were observed under inverted fluorescence microscope.

Nampt Enzymatic Activity

Nampt activity in cytoplasmic extracts was assayed as described in (Rongvaux et al., 2002) with minor modifications. Briefly, cytoplasmic extracts were obtained by lysing C2C12 cells in 0.01M NaH_2PO_4 (pH7.4) (Hasmann and Schemainda, 2003). 50-100 µg of cell extracts were used in a Nampt reaction mix (final volume 100 µl) containing 50 mM Tris (pH 8.8), 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM PRPP and 2.5 mM [carbonyl-^{14}C] nicotinamide (American Radiolabeled Chemicals Inc., St. Louis, MO). Reactions were incubated for 15 minutes at 37°C. Production of [^{14}C]-labeled NMN was analyzed by acetone precipitation-filtration assay (Elliott et al., 1980).

NAD^+/NADH Assays

NAD^+, NADH, and [NAD^+]/[NADH] ratio were measured from whole cells extracts using the NAD^+/NADH quantification kit from Biovision based on an enzymatic cycling reaction, according to manufacturer's instructions. In selected cases, the NAD^+ and NADH concentration measurement were repeated using an in house-developed method. NAD^+ was extracted from cell pellet by adding 7% perchloric acid and sonicated on ice for 5 mins. The sample was centrifuged for 3 mins at room temperature and neutralized with 1 M phosphate buffer and 3 M NaOH solution. The clear supernatant was used to determine NAD^+ concentration using enzymatic cycling assay. Typically, the sample was mixed with the cycling buffer containing 25 mM Tris-HCl (pH~8), 5 mM $MgCl_2$, 50 mM KCl, 2.25 mM lactate, 54 μM resazurin and 36 U/mL of diaphorase. The cycling reaction was initiated with the addition of lactate dehydrogenase and the increase in the resorufin fluorescence (with excitation at 560 nm and emission at 590 nm) was measured continuously using a fluorescent plate reader. Standard solutions of NAD^+ were prepared and taken through the same steps as the samples to obtain the standard curve. NADH was extracted from cell pellet by adding 0.05 M NaOH/1 mM EDTA and sonicated on ice for 5 mins. The alkali extract was incubated at 60°C for 30 mins and neutralized with 0.1 M HCl and 300 mM phosphate buffer (pH~4.4). The concentration of NADH was measured fluorometrically using the cycling assay described above. Standard curves were obtained by processing the standard NADH samples along with the biological samples.

HPLC/MALDI /MS Determination of NAM

Standard O^{18}-NAM (1 nmol) was added to the whole cell pellet. After mixing well, NAM was extracted from cell pellet by adding 7% perchloric acid and sonicated on ice for 5 mins. The sample was centrifuged for 3 min at room temperature and neutralized with 1 M phosphate buffer (pH~9) and 3 M NaOH solution. Clear supernatant was taken out (100 μL) for HPLC injection to separate NAM from other cellular components. NAM peaks were collected, according to the retention time of a NAM standard, and dried on a lyophilizer. MALDI-TOF was used to detect distinct peaks (m/z = 123 or 125)

corresponding to isotopomers of NAM. Ratio of ion intensities (123/125) was multiplied by 1 nmol to determine total nmol NAM in sample. Corrections were applied for isotopic abundances.

SIRT1 Deacetylase Activity

Whole cell extracts were obtained using a mild lysis buffer (50 mM Tris-HCl (pH 8), 125 mM NaCl, 1mM DTT, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, and 0.1% NP40 supplemented with 1 mM PMSF and protease inhibitors mix) from cells cultured in DM in the presence of either high or low glucose, or AICAR (0.5mM). 30 μg of extracts were used in a *in vitro* deacetylation assay using the Fluor de Lys-SIRT1 substrate (Biomol). SIRT activity was determined as NAM-inhibitable, TSA-independent ability of cell extracts to deacetylate the specific fluorometric substrate. Released substrate was detected using a microplate-reading fluorimeter.

ATP Determination

ATP levels were measured according to Lamprecht and Trautschold (Lamprecht W, 1974) with some modifications. Triethanolamine was substituted by Tris buffer pH8.1 and a 96 well microplate The reagents and well concentrations were as follows: Tris pH8.1 (75mM);MgCl2 (7.5mM); NADP (0.45mM) and glucose (0.95mM). For the enzymes, G6PDH (0.04U/well) and HK (0.07U/well). The G6PDH was included in the solution of buffer, along with MgCl2, NADP and glucose. The ATP reaction was initiated by addition of hexokinase (HK) after establishing a baseline absorbance. The difference in absorbance before and after addition of HK was used to calculate the [ATP].

Expression Profiling and Data Analysis

Total RNA was extracted from C2C12 cells cultured in differentiation medium for 36 hrs in either normocaloric (25mM glucose) (n=3) or glucose restricted (5mM glucose)(n=3) conditions. Biotin-labeled cRNA was prepared and hybridized to GeneChip Murine Expression Genome Set 430 MOE430 (39,000 gene transcripts) (Affymetrix) as previously described (Zhao et al., 2002). Primary analysis was done

using Affymetrix Microarray Suite 5.0. Data derived from the Affymetrix analysis were input into GeneSpring (Agilent Technologies) for further analysis as described in (Zhao et al., 2002). The annotation of the transcripts identified as differentially regulated by 5mM glucose has been deposited in the GEO data base (http://www.ncbi.nlm.nih.gov/geo/ Accession number: GSE9756).

RT-qPCR

RNA from the gastrocnemii muscles of mice fed at libitum or subjected to a 48hr fast was extracted with TRIzol Reagent (Invitrogen) using a Polytron homogenizer. Total RNA from C2C12 cells or from mouse primary myoblats obtained from either wild-typeor Sirt1$^{+/-}$ heterozygous mice was extracted using TRIzol Reagent Total RNA was then treated with DNase I (Ambion) for 30 minutes at 37°C and
purified with RNeasy mini kit (Qiagen). cDNA was synthesized using Thermoscript II cDNA Synthesis kit (Invitrogen). 2 µL of a 1:5/1:10 dilution of the resulting cDNA were used for quantitative real time PCR. qPCR was performed using the Mx3000P Real-Time PCR System (Stratagene) with a SyberGreen PCR MasterMix (Applied Biosystems). All reactions were analyzed in triplicates.
Data were normalized to the signal obtained using primers that amplify the GAPDH transcripts using the comparative CT method for relative quantitation of gene expression.

siRNA

The mouse SIRT1 siRNA, a set of three siRNA oligonucleotides for mouse SIRT3 or SIRT4 were purchased from Invitrogen. Control siRNA (control med GC) was also from Invitrogen. Transfection of siRNAs (total 150pmoles) was performed with LIPOFECTAMINE 2000 (Invitrogen).

Oligonucleotide Sequences

Oligonucleotides Used in RT-PCR

Beta enolase 3
For: 5'CCATGCAGGAGTTCATGATTCTGC 3'

Rev: 5’ATTCAGACGCAGCTAGATCCATGC 3’

CD36
For: 5’CTTCCACATTTCCTACATGCAA 3’
Rev: 5’ATCCAGTTATGGGTTCCACATC 3’

CycD1
For: 5' CCGGCTTTGATCTCTGCTTA 3'
Rev: 5' CGCGGAGTCTGTAGCTCTCT 3’

CycE1
For: 5’GGAAAATCAGACCACCCAGA 3’
Rev: 5’AGACTTCGCACACCTCCA 3’

Epoxide hydrolase 1,microsomal
For: 5’CACGTGTTTGAAGTCATCTG 3’
Rev: 5’TTGTAGAAGATCCTCGCAGT 3’

GADD45 gamma
For: 5’AAAGTCCTGAATGTGGACC 3’
Rev: 5’CCTGAATCAACGTGAAATG 3’

Glutathione S-transferase, alpha 2
For: 5’ACCAGAGCCATTCTCAACTA 3’
Rev: 5’ATACATGTCAATCAGGGCTC 3’

GAPDH
For: 5’AACATCAAATGGGGTGAGGCC 3’
Rev: 5’GTTGTCATGGATGACCTTGGC 3’

Myf5
For: 5’GCTGAGGGAACAGGTGGAGAAC 3’
Rev: 5’CTGCTGTTCTTTCGGGACCAGAC 3’

Myh3 (MHC embryonic)
For: 5’CAATAAACTGCGGGCAAAGAC 3’
Rev: 5’ CTTGCTCACTCCTCGCTTTCA 3’

Myh8 (MHC perinatal)
For: 5’ GAACTTGAAGGAGAGGTCGA 3’
Rev: 5’ GAGCACATTCTTGCGGTCTT 3’

Myl6
For: 5’ TCCGTCATGTCCTAGTCACACT 3’
Rev: 5’CATAGTTGATGCAACCATTGCT 3’

MylpF
For: 5’CGGACCCGGAGGATGTG 3’
Rev: 5’TGGTGCCCTTCCCTTCTG 3’

Myogenin
For: 5’CTGGGGACCCCTGAGCATTG 3’
Rev: 5’ATCGCGCTCCTCCTGGTTGA 3’

Nampt
For: 5’AGCAGCAGAGCACAGTACCA 3’
Rev: 5’GCTATCGCTGACCACAGACA 3’

Pax7
For: 5'GTGTTTCTCATGGTTGTGTCTC 3'
Rev: 5'GGTTCTCTCTCTTATACTCCTCAA 3'

PCNA
For: 5' CGATTGGTCCTTGAGGAGAG 3'
Rev: 5' GGCCTACAGCGACAACTACC 3'

PDK4
For: 5’ CTTTGAGTGTGCAAAGATGCTC 3’
Rev: 5' GTACACAATGTGGATTGGTTGG 3’

Phosphofructokinase
For: 5’ATCTTTGACAGCAGGAAGAA
Rev: 5’CCTTAGCACCCATCTTAGTG

Phosphoglycerate mutase 2
For: 5’ACTACACCTCCATCAGCAAG
Rev: 5’ CAATGGTGTCCTTGAGACTT

SIRT1
For: 5’ CTTCAGTGTCATGGTTCCTT 3’
Rev: 5’ACCGAGGAACTACCTGATTA 3’

SIRT3
For: 5’CTTTTTCTTTCACAACCCCAAG 3’
Rev: 5’ GAGGAGCCTCAGGAAGTAGTGA 3’

SIRT4
For: 5’AACTGAGCTGAGCAGGAAAGAC 3’
Rev: 5’GTCCCAGAATCCTACGTGTCTC 3’

Tnnc2
For: 5'AAGAGGAACTGGCTGAGTGCTT 3'
Rev: 5'GCTAGCTCCTCAGCATCAATGTAG 3'

UCP2
For: 5' CTACAAGACCATTGCACGAGAG 3'
Rev: 5' TGATGAGGTCATAGGTCACCAG 3'

UCP3
For: 5' TAAACAGGTGAGACTCCAGCAACTT 3'
Rev: 5'ACTCCAGCGTCGCCATCAGGATTCT 3'

Oligonucleotides Employed to Generate pSUPER.Retro.Puro Sirt1 RNAi:

For: 5'GATCCCC***GACCAAGCAACAAACAACA***TTCAAGAGA***TGTTGT TTGTTGCTTGGTC***TTTTT GGAAA

Rev: 5'AGCTTTTCCAAAAA***GACCAAGCAACAAACAACA***T***CTCTTGAATGT TGTTTGTTGCTTGGTC***GGG

The 19 nt Sirt1 target sequences are indicated in bold italic in the oligonucleotide sequence. The resulting constructs were sequenced.

Oligonucleotides Employed to Generate pSUPER.Retro.Puro Nampt RNAi:

For: 5'GATCCCC***ATGGGTTGCAGTACATTCT***TTCAAGAGA***AGAATGTACT GCAACCCAT***TTTTTGGAAA

Rev: 5'AGCTTTTCCAAAAA***ATGGGTTGCAGTACATTCT***TCTCTTGAA***AGA ATGTACTGCAACCCAT***GGG

The 19 nt Nampt target sequences are indicated in bold in the oligonucleotide sequence.

The resulting constructs were sequenced.

SUPPLEMENTAL REFERENCES

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Zhao, P., Iezzi, S., Carver, E., Dressman, D., Gridley, T., Sartorelli, V., and Hoffman, E. P. (2002). Slug is a novel downstream target of MyoD. Temporal profiling in muscle regeneration. J Biol Chem *277*, 30091-30101.

Table S1. Genes Regulated by Glucose Restriction in Muscle Cells

Nucleotide	Gene	Transcript	Fold Change
		Muscle Sarcomere	
NM_080728.1	Myh7	Myosin, heavy polypeptide 7,cardiac muscle, beta	-3.2
NM_033268.1	Actn2	Actinin alpha2	-3.2
AJ002522.1	Myh1	Myosin, heavy polypeptide 1, skeletal muscle,adult	-2.8
NM_010861.1	Mylpc	Myosin light chain, phosphorylatable,cardiac ventricles	-2.7
BB011213	Myh8	Myosin, heavy polypeptide 8, skeletal muscle, perinatal	-2.7
NM_080728.1	Myh7	Myosin, heavy polypeptide 7, cardiac muscle, beta	-2.7
NM_010867.1	Myom1	Myomesin 1	-2.3
BC025840.1	Ttn	Titin	-1.5
		Mitochondrial energy production and respiration	
NM_007710.1	Ckmm	Creatine kinase, muscle	-1.5
BG967663	Ckb	Creatine kinase, brain	-2.3
NM_009802.1	Car6	Carbonic anydrase 6	3
AJ245857.1	Car9	Carbonic anydrase 9	2.2
NM_009801.2	Car2	Carbonic anydrase2	1.8
NM_008706.1	Nqo1	NAD(P)H dehydrogenase, quinone 1	1.9
		Xenobiotic Metabolism	
D87867.1	UGP1a1	UDP-glucuronosyltransferase 1 family, member 1	7.5
NM_010145.1	Ephx1	Epoxide hydrolase 1, microsomal	2
BC004037.1	Ung	Uracil DNA glycosylase	1.9
NM_008182.1	Gsta2	Glutathione S transferase, alpha2 (Yc2)	1.8
BC009155.1	Mgst1	Glutathione S transferase 1, microsomal	1.8
AK003232.1	Cbr3	Carbonyl reductase 3	1.7
NM_009994.1	Cyp1b1	Cytocrome P450, family 1, subfamilyb, polypeptide 1	1.6
NM_020010.1	Cyp51	Cytocrome P450, 51	1.5
		Metabolism	
		Glucose	
NM_018870	Pgam2	Phosphoglycerate mutase 2	-3
NM_021514.1	Pfkm	Phosphofructokinase, muscle	-2
NM_019703	Pfkp	Phosphofructokinase, platelet	-1.5
NM_008195.1	Gys3	Glycogen synthase3, brain	-1.9
NM_011224.1	Pygm	Muscle glycogen phosphorylase	-1.7
NM_008062.1	G6pdx	Glucose -6-phosphate dehydrogenase X linked	1.6
		Lipids	
NM_008642.1	Mttp	Microsomal triglyceride transfer protein	2.6
BM235734	Me2	Malic enzyme 2, NAD(+)-dependent , mitochondrial	2.8
NM_130450.1	Lce	Long chain fatty acid elongase	2.6
AK014742.1	LSS	Lanosterol synthase	2.3
BC024618.1	Cyb5r	Cytocrome B5 reductase	2.1
NM_007421.1	Adss1	Adenylosuccinate synthase1, muscle	-1.8
AW911807	Gda	Guanine deaminase	-2.7
BC006727.1	Pycr1	Pyrroline-5-carboxylate reductase 1	2
		Chaperones	
BM124741	Hspb7	Heat shock protein family , member 7	-4.9
AJ002387.1	Hspa5	Heat shock 70kD protein 5 (glucose related)	-3.3
NM_019960.1	Hspb3	Heat shock protein 3	-2.3
		Extracellular matrix	
NM_007739.1	Col8a1	Procollagen, type VIII, alpha1	-3.6
U65020.1	Dmp	Dentin matrix protein 1	-2.8
BB235530	Fmod	Fibromodulin	-2.3
U08020.1	Col1a1	Procollagen, type I, alpha1	-2
BC013560.1	Col4a2	Procollagen, type IV, alpha2	-1.9
BF158638	Col4a1	Procollagen, type IV, alpha1	-1.8
BM232384	Timp3	Tissue inhibitor of metalloproteinase 3	-2.2
		Protein turnover	
AF127766.2	Canp3	Calpain 3	-2.1
AV162459	Ube2	Ubiquitin-conjugating enzyne E2C	-2.7
AK014680.1	Risc	Retinoid -inducible serine carboxypeptidase	2.2
J02583.1	Ctsl	Catepsin L	1.5
		Excitation contraction	
NM_009813.1	Casq1	Calsequestrin 1	-2.1
X83932.1	RYR1	Ryanodine receptor 1, skeletal muscle	-1.5
		Cytokines and Growth Factors	
AK003506.1	Il17b	Interleukine 17B	-4.8
NM_020013.1	Fgf21	Fibroblast growth factor 21	-4.1
NM_008046.1	Fst	Follistatin	-3.7
NM_031380.1	Fstl3	Follistatin like 3	-3.6
NM_007556.1	Bmp6	Bone morphogenetic protein 6	-3.6
		Transcription factors	
BI110565	Osf2	Osteoblast specific factor 2	-2.4
NM_025282.1	Mef2c	Myocyte enhancer factor 2C	-2.9

RNA was extracted from C2C12 cultured for 36 hrs in differentiation medium in either normo caloric (25mM glucose) or calorie restricted (5mM glucose) conditions. Fold change indicates the extent of change in gene expression observed in cells cultured in 25mM compared to control cells cultured in 5mM glucose.

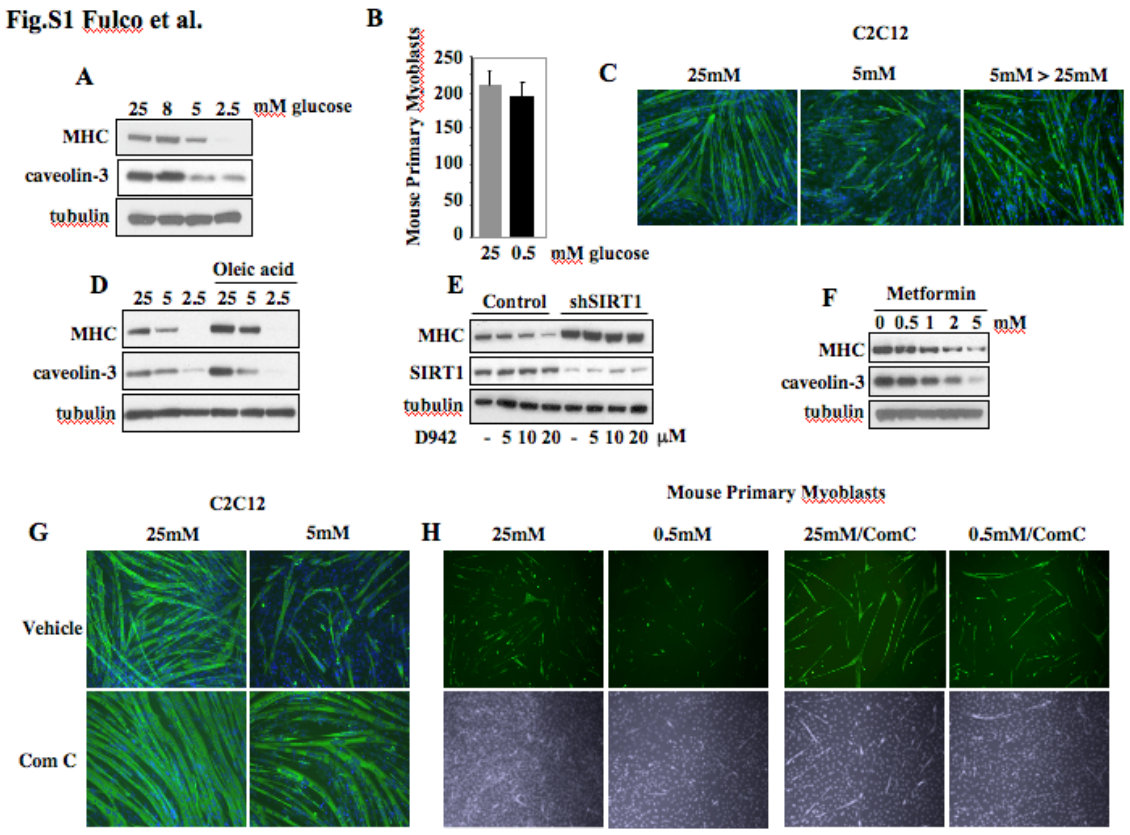

Figure S1. Effects of Glucose, Oleic Acid, and AMPK Modulators on Skeletal Muscle Differentiation

(A) C2C12 cells were differentiated in a medium (differentiation medium, DM) supplemented with 25mM, 8mM, 5mM, or 2.5mM glucose for 48 hours. Immunoblot was performed with MHC, caveolin-3, or tubulin antibodies. (B) Mouse primary myoblasts were cultured in DM with either 25 mM or 0.5mM glucose for 48 hours. The medium was then removed, cells washed twice with 1X PBS and counted in at least 10 microscopic fields. The total number of cells was divided by10 ($p< 0.01$). (C) MHC IF of C2C12 cells cultured in DM with either 25mM or 5mM glucose. After 48hr, the 5mM culture was switched to DM supplemented with 25mM glucose for additional 48 hr. (D) MHC, caveolin-3, and tubulin immunoblot of extracts from C2C12 cells cultured with decreasing glucose concentrations in the absence or presence of 0.1mM oleic acid. (E) MHC, SIRT1, and tubulin immunoblot of extracts from control or shSIRT1-expressing C2C12 cells exposed to increasing concentrations (5-20µM) of the AMPK activator D942. (F) MHC, caveolin-3, and tubulin immunoblot of extracts from C2C12 cells

cultured in DM with 25mM glucose with increasing concentrations of metformin. (G) MHC IF of C2C12 cells exposed to the AMPK inhibitor compound C (Com C, 1μM) in DM with 25mM or 5 mM glucose. (H) MHC IF (top panels) and DAPI staining (bottom panels) of mouse primary myoblasts exposed to compound C (1μM) in DM with 25mM or 0.5mM glucose.

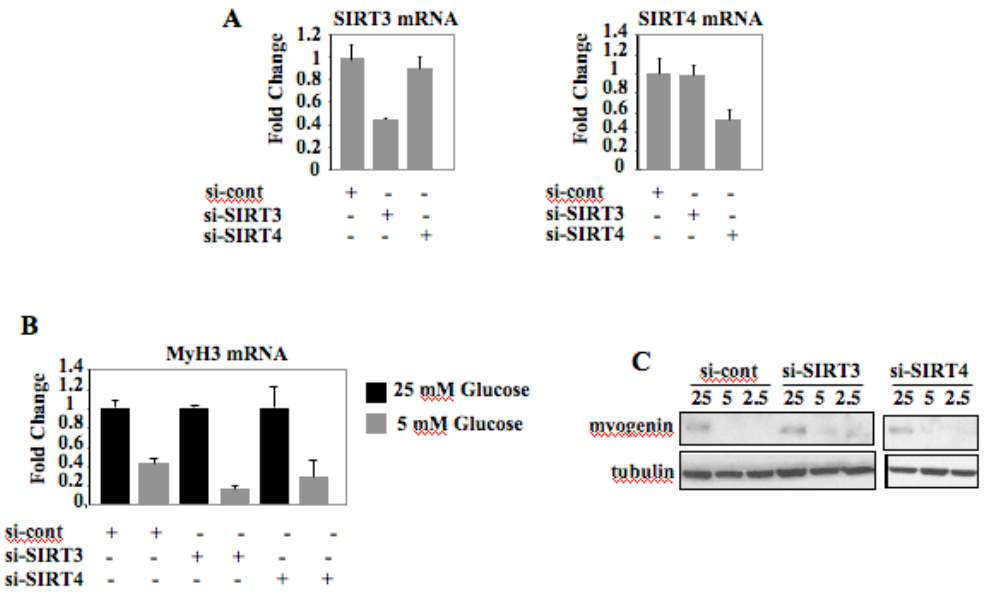

Figure S2. Reducing the Mitochondrial Sirtuin SIRT3 or SIRT4 Has No Effect on Glucose Restriction-Mediated Inhibition of Differentiation in Skeletal Muscle Cells
(A) RT-qPCR quantitative determination of SIRT3 and SIRT4 transcripts in C2C12 cells transfected with control, SIRT3, or SIRT4 small interfering (si) RNAs. (B) Embryonic myosin heavy chain (Myh3) transcripts in C2C12 cells cultured in DM with 25mM or 5mM glucose and transfected with control, SIRT3, or SIRT4 siRNA. (C) Myogenin and tubulin immunoblot of C2C12 cells described in (B).

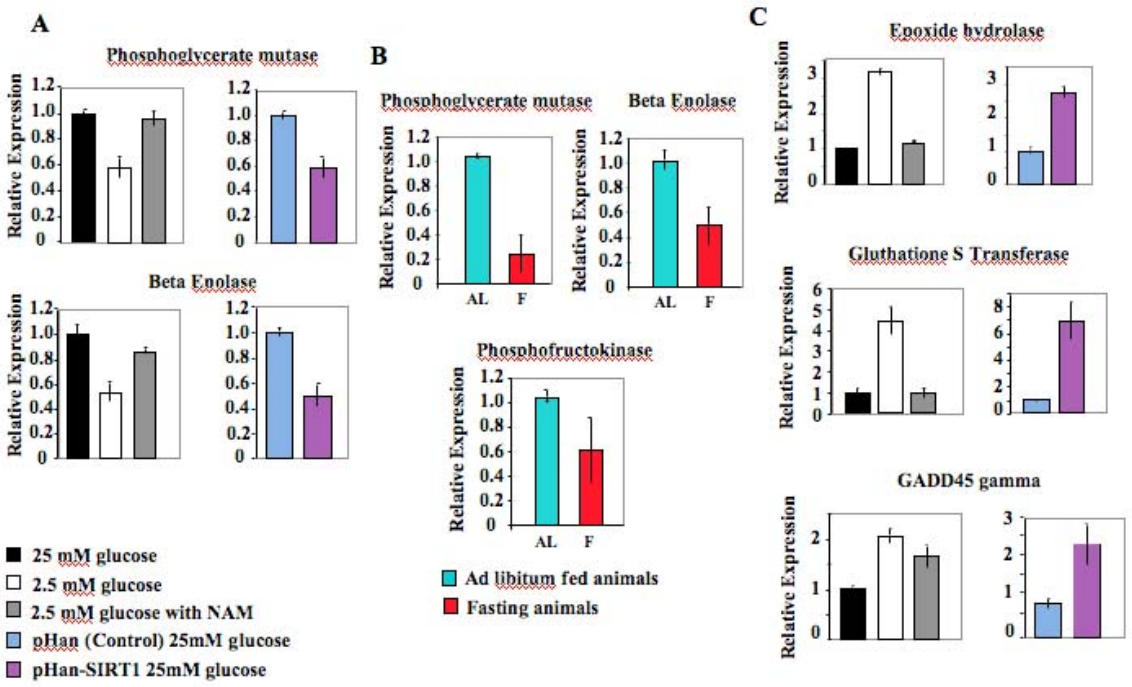

Figure S3. Glucose Restriction-Induced Modulation of Gene Expression in Skeletal Muscle Cells is SIRT1-Dependent

(A) RT-qPCR of the phosphoglycerate mutase and beta enolase transcripts in C2C12 cells cultured in DM with 25mM or 2.5mM glucose with or without NAM (5mM). The same transcripts were evaluated in control (pHan) or SIRT1-overexpressing (pHan-SIRT1) cells cultured in DM and 25mM glucose. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. (B) The indicated transcripts were evaluated by RT-qPCR in gastrocnemii muscle samples (n=3) from mice fed ad libitum (AL) or subjected to a 48 hr fast (F). The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. (C) The indicated transcripts were evaluated by RT-qPCR in C2C12 cells cultured in DM with 25mM or 2.5mM glucose with or without NAM (5mM) and in control (pHan) or SIRT1-overexpressing (pHan-SIRT1) cells cultured in DM and 25mM glucose. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations.

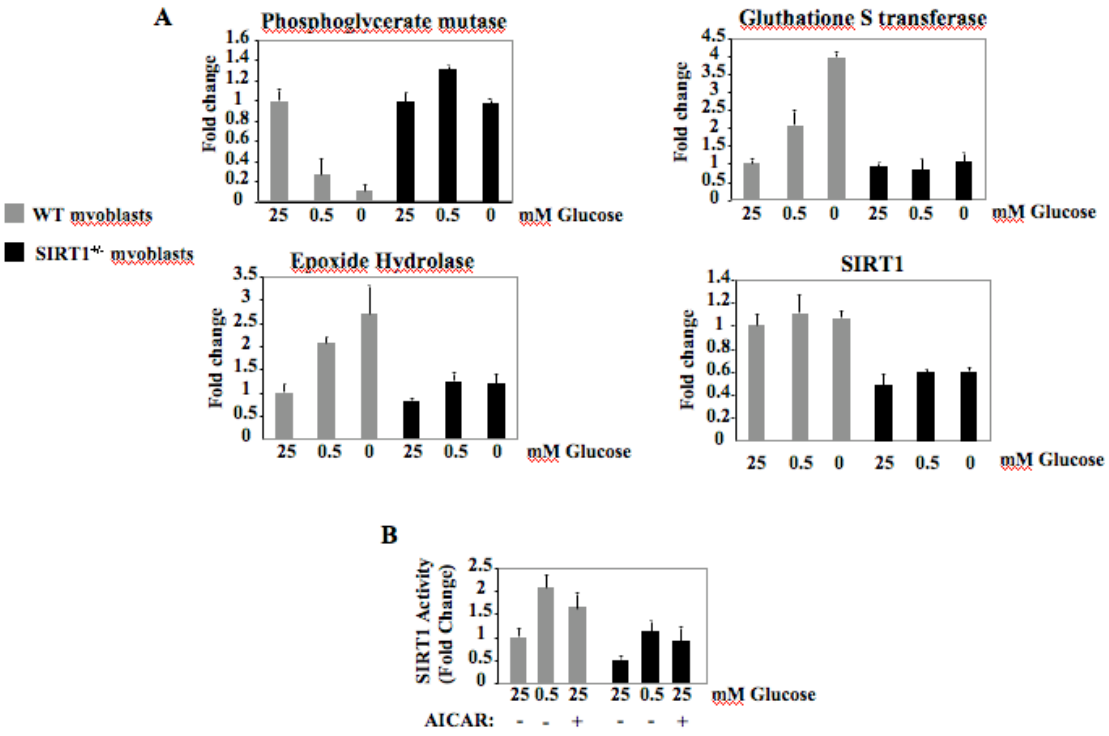

Figure S4. Gene Expression and SIRT1 Activity in Mouse Primary Myoblasts from WT or SIRT1$^{+/-}$ Heterozygous Mice

(A) The indicated transcripts obtained from RNAs isolated from mouse primary myoblasts of WT (n=3) or SIRT1$^{+/-}$ heterozygous mice (n=3) cultured in DM with 25mM, 0.5mM or, 0mM glucose were evaluated by RT-qPCR. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. (B) SIRT1 activity was evaluated in extracts derived from WT or SIRT1$^{+/-}$ myoblasts cultured in 25mM or 5mM glucose, or cultured in 25mM glucose with AICAR (0.5mM) for 24 hours.

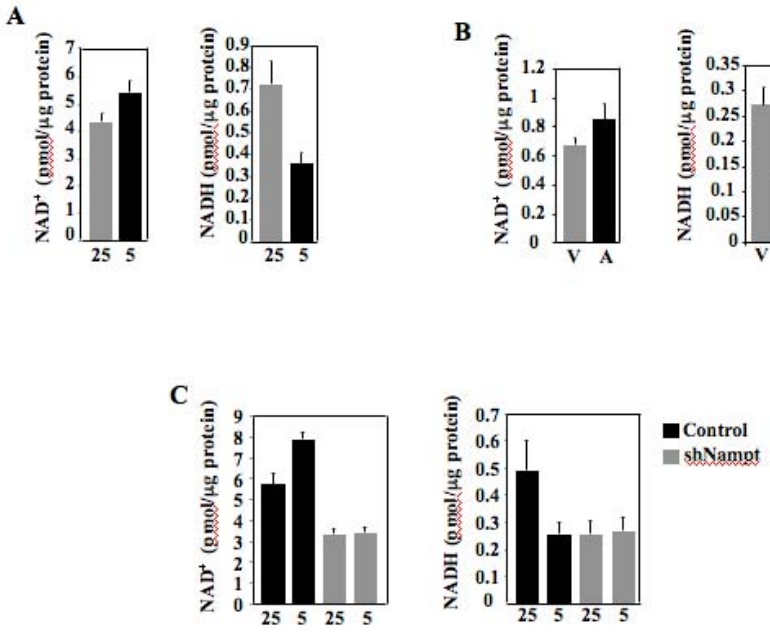

Figure S5. NAD^+ and NADH Levels in Skeletal Muscle Cells Cultured in Either High or Low Glucose, with AICAR, or with Reduced Levels of Nampt

(A) NAD^+ and NADH levels were determined in extracts of C2C12 cells cultured in DM with either 25mM or 5mM glucose. (B) NAD^+ and NADH levels were evaluated in C2C12 cells cultured in DM with 25mM glucose in the absence (vehicle, V) or presence of 0.5mM AICAR (A). (C) NAD^+ and NADH levels in control cells or cells expressing shNampt cultured in DM with either 25mM or 5mM glucose.

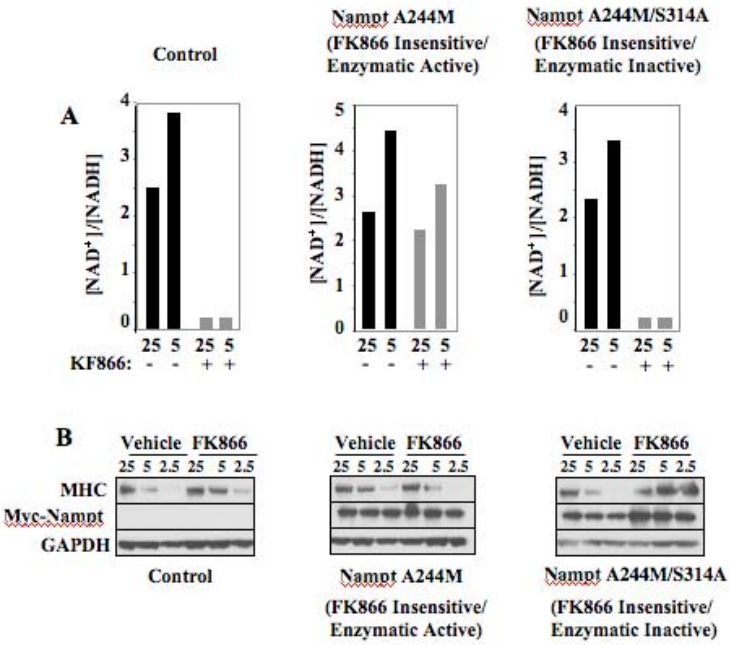

Figure S6. The Enzymatic Activity of Nampt Regulates the [NAD^+]/[NADH] Ratio and Cell Differentiation

(A) [NAD^+]/[NADH] ratio was measured in control, NamptA244M, or NamptA244M/S314-expressing C2C12 cells cultured in DM with 25mM or 5mM glucose in either the absence or presence of FK866 (10nM). (B) MHC, myc, and GAPDH immunoblots of extracts derived from the C2C12 cells described in (A).

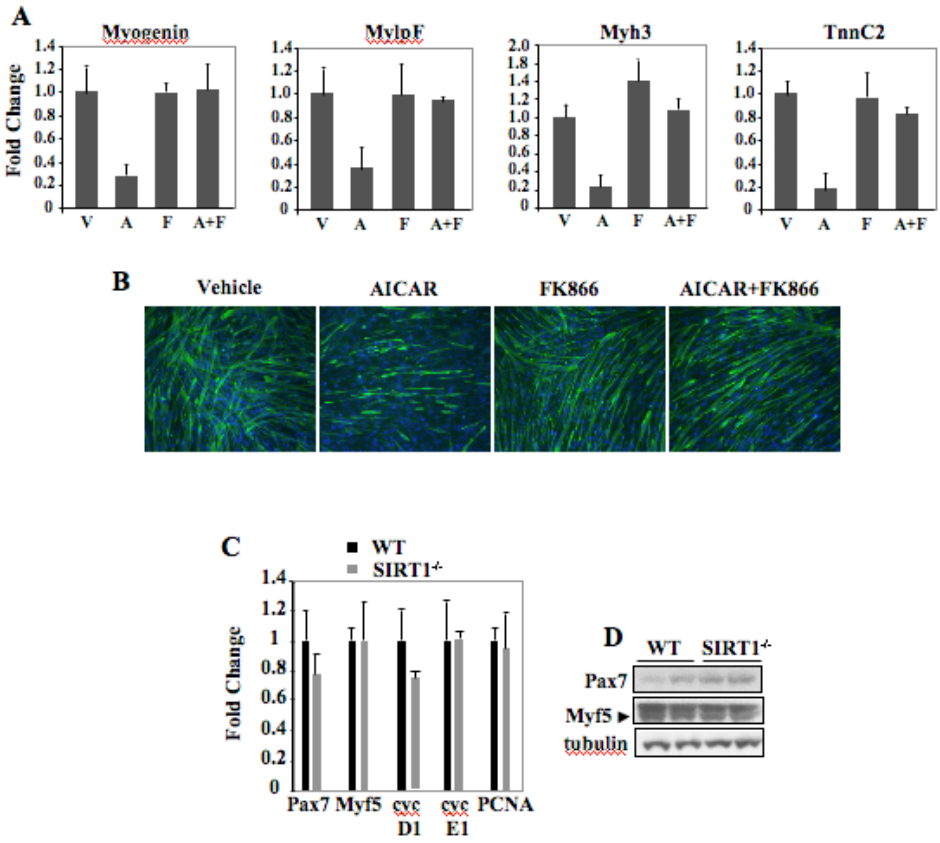

Figure S7. AMPK Activation Relies on Nampt to Inhibit Muscle Differentiation

(A) The indicated transcripts were evaluated by RT-qPCR after C2C12 cells were cultured in DM with 25mM glucose and exposed to vehicle control (V), AICAR (A, 0.5mM), FK866 (F, 10nM), or AICAR and FK866 (A+F). The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. MylpF, myosin light chain phosphorylatable fast; Myh3, embryonic myosin heavy chain; TnnC2, troponinC2. (B) MHC IF of C2C12 cells cultured as described in (A). DAPI marks the cell nuclei. (C) RT-qPCR of Pax7, Myf5, cyclins D1 and E1, and PCNA transcripts from hindlimb muscles of WT and SIRT1$^{-/-}$ mice. The RNAs obtained from three (n=3) animals per experimental group were analyzed. (D) Pax7, Myf5, and tubulin immunoblot of extracts derived from hindlimb muscles of two WT and two SIRT1$^{-/-}$ mice.