Opposite Transcriptional Regulation in Skeletal Muscle of AMP-activated Protein Kinase γ3 R225Q Transgenic Versus Knock-out Mice*§

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AMP-activated protein kinase (AMPK) is an evolutionarily conserved heterotrimer important for metabolic sensing in all eukaryotes. The muscle-specific isoform of the regulatory γ-subunit of the kinase, AMPK γ3, has an important role in glucose uptake, glycogen synthesis, and fat oxidation in white skeletal muscle, as previously demonstrated by physiological characterization of AMPK γ3 mutant (R225Q) transgenic (TgPrkag3γ3Δ225Q) and γ3 knock-out (Prkag3−/−) mice. We determined AMPK γ3-dependent regulation of gene expression by analyzing global transcription profiles in glycolytic skeletal muscle from γ3 mutant transgenic and knock-out mice using oligonucleotide microarray technology. Evidence is provided for coordinated and reciprocal regulation of multiple key components in glucose and fat metabolism, as well as skeletal muscle ergogenics in TgPrkag3γ3Δ225Q and Prkag3−/− mice. The differential gene expression profile was consistent with the physiological differences between the models, providing a molecular mechanism for the observed phenotype. The striking pattern of opposing transcriptional changes between TgPrkag3γ3Δ225Q and Prkag3−/− mice identifies differentially expressed targets being truly regulated by AMPK and is consistent with the view that R225Q is an activating mutation, in terms of its downstream effects. Additionally, we identified a wide array of novel targets and regulatory pathways for AMPK in skeletal muscle.

AMP-activated protein kinase (AMPK) is a critical regulator of carbohydrate and fat metabolism in eukaryotic cells (reviewed in Refs. 1 and 2). AMPK is a heterotrimer that consists of α-, β-, and γ-subunits, all of which are required for its activity. The catalytic α-subunit contains a conventional serine/threonine protein kinase domain, and phosphorylation of Thr-172 residue within the activation loop of the α-subunit by upstream kinases is essential for the activity of the heterotrimer (3–6). Once phosphorylated at Thr-172, AMPK can be further activated by allosteric binding of AMP to the evolutionary conserved cytosine β-synthase domains in the regulatory γ-subunit (7). The AMPK β-subunit acts as a scaffold for binding of the α- and γ-subunits (8). The β-subunit also contains a glycogen-binding domain, and recent findings provide evidence that this motif is involved in targeting the AMPK complex to cellular glycogen stores (9, 10). The mammalian genome contains seven AMPK genes encoding for two α-, two β-, and three γ-isosforms. Thus, there are 12 possible combinations of heterotrimeric AMPK, and the physiological function of the AMPK holoenzyme depends on the particular isosforms present in the complex.

We have provided evidence that AMPK γ3 is the predominant γ-isof orm expressed in glycolytic (white, fast-twitch type II) skeletal muscle (11). In contrast, it is expressed at low levels in oxidative (red, slow-twitch type I) skeletal muscle and is undetectable in brain, liver, heart, or white adipose tissue (11). Thus, the AMPK γ3-subunit is the only isoform exhibiting tissue-specific expression. Furthermore, the AMPK γ3-subunit primarily forms heterotrimers with the α2- and β2-isoforms in glycolytic skeletal muscle (11).

The functional significance of the AMPK γ3-subunit has been demonstrated by phenotypic analysis of animal models carrying a mutated form of the gene. The dominant Rendement Napole (RN) phenotype identified in Hampshire pigs is caused by a single missense mutation (R225Q) in the AMPK γ3-subunit (12). RN pigs have a 70% increase in glycogen content in skeletal muscle, whereas liver and heart glycogen content remains unchanged (13). Furthermore, RN carriers are also characterized by a higher oxidative capacity in white skeletal muscle fibers (14, 15). Conversely, a second mutation (V224I) identified in pigs at the neighboring amino acid residue of the γ3-protein is associated with an opposite phenotype compared with the RN allele, resulting in reduced skeletal muscle glycogen content (16). Characterization of transgenic mice with skeletal muscle-specific expression of the mutant (R225Q) form of the mouse AMPK γ3-subunit, as well as AMPK γ3-subunit knock-out mice, provided further evidence that the γ3-subunit plays a key role in skeletal muscle carbohydrate and lipid metabolism. Glycogen resynthesis after exercise was impaired in AMPK γ3 knock-out mice but was markedly enhanced in transgenic mutant mice. An AMPK-activator failed to increase skeletal muscle glucose uptake in knock-out mice, whereas insulin-mediated glucose uptake was unaltered. When fed with a high fat diet, γ3 R225Q transgenic mice were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Table S1, and Fig. S1.

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2 The abbreviations used are: AMPK, AMP-activated protein kinase; RN, Rendement Napole; qRT-PCR, quantitative real-time PCR; EST, expressed sequence tag; MAPK, mitogen-activated protein kinase; AICAR, S-amino-4-imidazole-carboxamide riboside.
of the mutations in this isoform, we have studied AMPK γ3-dependent gene transcription by a systematic approach, using global analysis of the mRNA expression pattern in the skeletal muscle of γ3 R225Q mutant and γ3 knock-out mice. Here we describe distinct biomarker patterns, comprising AMPK γ3-dependent transcriptional changes of genes involved in glucose and lipid metabolism and skeletal muscle ergogenics.

**EXPERIMENTAL PROCEDURES**

**AMPK Knock-out (Prkag3γ3−/−) and R225Q Transgenic (TgPrkag3γ3R225Q) Mice**—The Prkag3γ3−/− and TgPrkag3γ3R225Q mice used in this study have been previously described (17). Prkag3γ3−/− mice were created by conventional gene targeting techniques. TgPrkag3γ3R225Q mice express the mutant γ3 R225Q subunit under the control of mouse myo- sin-light chain promoter and enhancer elements. Mice used in the study were bred into the C57BL/6 genetic background. Mice were maintained in a 12-h light-dark cycle and were cared for in accordance with regulations for the protection of laboratory animals. The study was performed after prior approval from the local ethical committee. Gene expression profiles were characterized in male mice fasted overnight (food was removed 16 h prior to study). The white portion of the gastrocnemius muscle was dissected from anesthetized mice, cleaned of fat and blood, and quickly frozen in liquid nitrogen as described (17).

**Preparation of Total RNA**—Total RNA was isolated from the white portion of the gastrocnemius muscle using the RNeasy Fibrous Mini Kit (Qiagen) applying Mixer Mill MM 301 (Retsch) followed by a DNase digestion step using RNase-Free DNase Set (Qiagen) according to the manufacturer’s instructions. The RNA yield was quantified by spectrophotometric analysis and the RNA purity was determined based on the A260/A280 ratio. The quality of the RNA was confirmed by Agilent 2100 Bioanalyzer analysis (Agilent Technologies) using the RNA 6000 Nano Assay Kit (Agilent Technologies).

**Preparation of cRNA, Gene Chip Hybridization**—10 μg of total RNA spiked with poly-A controls (pGIBS-TRP, -THR, and -LYS, American Type Culture Collection) was converted to cDNA utilizing a T7 promoter-poly-T primer (Affymetrix) and the reverse transcriptase SuperScript II (Invitrogen), followed by a second strand cDNA synthesis (Invitrogen). Double-stranded cDNA was in vitro transcribed to biotinylated cRNA (Enzo) and then fragmented (Invitrogen). The fragmented cRNA was mixed with control oligonucleotide B2 (Affymetrix) and a hybridization control cRNA mixture (BioB, BioC, BioD, and Cre, Affymetrix). Aliquots of each sample were hybridized (16 h at 45 °C) to GeneChip Mouse Expression Set 430A arrays (Affymetrix). The arrays were subsequently washed, stained, and scanned according to the manufacturer’s instructions (GeneChip Expression Analysis Technical Manual, Affymetrix).

**Data Analysis**—Data were analyzed using GeneTraffic UNO 3.2–11 (lobion Informatics) and Spotfire DecisionSite 8.1 (Spotfire Inc.). The TgPrkag3γ3R225Q dataset was analyzed separately from the Prkag3γ3−/− data set. For further details see the supplemental information.

**Quantitative Real-time PCR**—Quantification of mRNA levels for selected genes was performed by qRT-PCR as described (19) using acidic ribosomal phosphoprotein P0 (Arhp) as endogenous control (see supplemental Table SI for primer information). qRT-PCR was performed on extended set of samples including 7 Prkag3γ3−/− mice with 8 wild-type littermates and 13 TgPrkag3γ3R225Q mice with 10 wild-type littermates, while RNA from 6 animals in each group was used in gene array analysis.

**Histochemistry**—Enzyme activity staining for succinate dehydrogenase and cytochrome c oxidase was done on serial cross-sections (10-μm thickness) of frozen gastrocnemius muscle as described previously (20, 21). For succinate dehydrogenase activity staining, sections were incubated for 4 min in a 0.1 M phosphate buffer (pH 7.6) containing 5 mM EDTA, 45 mM disodium succinate, 1.2 mM nitro blue tetrazolium, 1 mM potassium cyanide, and 1 mM phenazine methosulfate. Cytochrome c oxidase activity staining was performed by incubating sections for 1 h in a 50 mM phosphate buffer (pH 7.6) containing 0.22 M sucrose, 2.3 mM 3,3’-diaminobenzidine tetrahydrochloride, 1 mM cytochrome c, and 1300 units of catalase.

**RESULTS**

**Microarray Analysis of the mRNA Expression in the Skeletal Muscle of AMPK γ3 Knock-out (Prkag3γ3−/−) and R225Q Transgenic (TgPrkag3γ3R225Q) Mice**—To determine the role of γ3-containing AMPK complexes in regulation of gene expression in the skeletal muscle, we utilized mouse models that either lack the AMPK γ3-protein (Prkag3γ3−/−) or express a R225Q mutant form of this protein in skeletal muscle (TgPrkag3γ3R225Q) (17). In Prkag3γ3−/− mice, AMPK γ3-protein expression is completely ablated, and importantly, no compensatory increase in γ1- or γ2-isofom is detected (17). Equally important, AMPK expression in TgPrkag3γ3R225Q mice resembles the expression pattern in wild-type mice, both with regard to tissue distribution and protein expression, with the mutant (R225Q) form replacing the endogenous AMPK γ3-protein (17). Global gene expression profiles in the white portion of gastrocnemius muscle of Prkag3γ3−/− and TgPrkag3γ3R225Q mice were compared with the corresponding wild-type littermates using oligonucleotide microarrays. The expression of 167 genes was significantly (p ≤ 0.05) changed by a factor of 20% or more, in TgPrkag3γ3R225Q and/or Prkag3γ3−/− mice, relative to the wild-type controls (Table 1). Applying the same filtering criteria on randomly created groups within the Prkag3γ3−/− dataset and TgPrkag3γ3R225Q dataset resulted in six genes determined as differentially expressed. This indicates that the rate of false positives is low. Consequently, the vast majority of the genes appearing differently expressed in Prkag3γ3−/− and/or TgPrkag3γ3R225Q mice can be considered as truly regulated.

Of the 167 differentially expressed transcripts, the identity of 148 genes is known and represents proteins of different functional classes, whereas 19 transcripts only show homology to sequences in the EST or genomic databases. Interestingly, the expression level of 16 genes was significantly changed in both AMPK γ3 R225Q transgenic and AMPK γ3 knock-out mice, compared with their respective wild-type littermates (Table 1). For these 16 transcripts, the direction of the observed change was the opposite in knock-out versus mutant transgenic mice. Furthermore, most of the genes that were significantly changed exclusively in Prkag3γ3−/− mice tended to be regulated in an opposite manner in TgPrkag3γ3R225Q mice, even though this difference did not reach statistical significance and/or meet the -fold change criteria. Correspondingly, the vast majority of transcripts, which were differentially regulated exclusively in R225Q transgenic mice, exhibited an opposite trend in knock-out mice. The striking pattern of opposing transcriptional changes in the AMPK γ3 R225Q transgenic versus knock-out mice, as compared with their wild-type littermates, is illustrated (Fig. 1).

Many of the genes, which were found to be differentially expressed in Prkag3γ3−/− and/or TgPrkag3γ3R225Q mice, are previously undescribed as being regulated by AMPK. Full functional significance of these changes in global transcriptional profile remains to be addressed in further experiments. To determine the possible mechanistic explanations for previously described physiological differences between Prkag3γ3−/− and TgPrkag3γ3R225Q mice (17, 18), we performed a more detailed analysis of gene expression changes for targets that are known to be involved in lipid and carbohydrate metabolism and muscle ergogenics. The expression of several genes involved in these functions depends on the skeletal
AMPK β3-dependent Transcriptional Responses

| Public ID       | Gene symbol | Gene title                                                                 | TgPrkag3225Q versus wild-type littermates | Prkg3−/− versus wild-type littermates |
|-----------------|-------------|----------------------------------------------------------------------------|------------------------------------------|--------------------------------------|
|                 |             |                                                                            | -Fold change    | p value        | -Fold change    | p value        |
| AU017649        | Gdap1a      | Ganglioside-induced differentiation-associated-protein 1                  | −1.82          | 0.004         | 1.27           | 0.003         |
| BB336256        | Mafa        | v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A           | −1.58          | 0.000008      | 1.29           | 0.002         |
| BC026377        | Rasd2a      | RASD family, member 2                                                      | 1.53           | 0.00005       | −1.21          | 0.048         |
| NM_010016       | Daf1a       | Decay accelerating factor 1                                                | 1.50           | 0.002         | −1.20          | 0.018         |
| M62838          | Scl7a2      | Solute carrier family 7, member 2                                          | 1.46           | 0.006         | −1.35          | 0.023         |
| BB445155        | Scl7a3      | Solute carrier family 2, member 3                                          | −1.42          | 0.002         | 1.22           | 0.035         |
| BB326929        | Slk3kbp1    | SH3-domain kinase binding protein 1                                        | 1.41           | 0.010         | −1.23          | 0.011         |
| NM_028800       | Gbe1        | Glucan branching enzyme 1                                                 | 1.40           | 0.012         | −1.28          | 0.011         |
| AJ787593        | Ugp2a       | UDP-glucose pyrophosphorylase 2                                            | 1.39           | 0.0001        | −1.21          | 0.008         |
| AF226613        | Sla9a1      | Solute carrier family 4, member 1                                          | 1.38           | 0.006         | −1.26          | 0.019         |
| NM_009976       | Cdkn1c      | Cyclin-dependent kinase inhibitor 1C                                        | −1.32          | 0.0005        | 1.20           | 0.022         |
| AV375971        | Xpr1        | Xenotropic and polytropic retrovirus receptor 1                           | −1.26          | 0.009         | 1.21           | 0.005         |
| BM111465        | Rap5        | Ribosomal protein L5                                                      | −1.24          | 0.015         | 1.21           | 0.024         |
| NM_009979       | Rap22       | Ribosomal protein L2                                                       | −1.24          | 0.007         | 1.20           | 0.00004       |
| AV306003        | 2010109N14Rik| RIKEN clone 2010109N14                                                        | −1.22          | 0.010         | 1.21           | 0.037         |
| BC011152        | Golph2      | Golgi phosphoprotein 2                                                   | −1.22          | 0.003         | 1.25           | 0.002         |

**TABLE 1**

Differentially expressed genes in TgPrkag3225Q and/or Prkg3−/− mice compared with wild-type littermates

Global mRNA expression pattern was characterized in the white portion of the gastrocnemius skeletal muscle in male mice of C57BL/6 genetic background. The filtering criteria were set to a mean absolute -fold change > 1.2 and a p value ≤ 0.05. In addition the mean intensity in the group showing highest expression should be > 75.
| Public ID | Gene symbol | Gene title |
|-----------|-------------|------------|
| NM_009523 | Wnt4        | Wingless-related MMTV integration site 4 |
| BB114336  | Bace1       | β-Site APP cleaving enzyme 1 |
| AK004781  | Sox17       | SRY-box containing gene 17 |
| BC014718  | Dnase1      | Deoxyribonuclease 1 |
| A.W.989981| 111008H02Rik| RIKEN cDNA 111008H02 gene |
| NM_011430 | Snog        | Synuclein, gamma 1 |
| NM_010240 | Ftil        | Ferritin light chain 1 |
| BC009165  | ThsP        | Thyroid hormone-responsive SPOT14 homolog (Rattus) |
| NM_008393 | Irx3        | Iroquois-related homeobox 3 (Drosophila) |
| NM_009379 | Thpo        | Thrombopoietin |
| NM_008285 | H1r         | Hematological and neurological expressed sequence 1 |
| BG070255  | Pde7a       | Phosphodiesterase 7A |
| NM_015797 | Fbxo6b      | F-box only protein 6b |
| BC010712  | Crc         | CREBBP/EP300 inhibitory protein 1 |
| BB261602  | Map2k6      | Mitogen-activated protein kinase 6 |
| U38384    | Shd1        | Inteptin of DNA binding 1 |
| BB278286  | 1810073P09Rik| mKIAA1760 |
| AK004874  | Rnf128      | Ring finger protein 128 |
| AK018605  | 4651408O11Rik| RIKEN cDNA 4651408O11 gene |
| BB039247  | C1qrl1      | Complement component 1q, subcomponent, receptor 1 |
| AV276428  | BC043118    | cDNA sequence BC043118 |
| AK018482  | Fbxo9       | F-box only protein 9 |
| BB066232  | Catna1      | Catenin α 1 |
| BI143942  | Sdt1*       | Sorbitol dehydrogenase 1 |
| BB359043  | 170007D05Rik| Transcription termination factor-like protein |
| BC021914  | Mnd         | Monocyte to macrophage differentiation-associated |
| AK020120  | Hrmt112     | Heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (S. cerevisiae) |
| AK010009  | Ooct1*      | 3-Oxoadic-CoA transferase 1 |
| BB035811  | Ift66       | Integrin α 6 |
| BB618702  | 493439F18Rik| RIKEN cDNA 493439F18 gene |
| BE968498  | Ppp1r144    | Protein phosphatase 1, regulatory subunit 14B |
| BI145101  | 111008E10Rik| Choline transporter-like proteins |
| BC019575  | Hist1h4i    | Histone 1, H4i |
| AV369098  | Dlat        | Dihydrolipoamide S-acetyltransferase |
| AB301049  | Rev3l       | REV-3-like |
| BB033733  | Trim16      | Tripartite motif protein 16 |
| AB302010  | Fxyd6       | FXYD domain-containing ion transport regulator 6 |
| NM_009214 | Smi         | Spermine synthase |
| AW534968  | Cedk5       | Cadherin 5 |
| BC023112  | Galnt2      | Chondroitin sulfate GalNACT-2 |
| NM_024493 | H47         | Histocompatibility 47 |
| NM_020581 | Angpot14    | Angiopoietin-like 4 |
| AF289490  | Asph*       | Aspartate-β-hydroxylase |
| BC008105  | Polk        | Polymorphic, kappa |
| NM_018833 | Pdzx        | PDZ domain containing, X chromosome |
| AP120617  | Glex1       | Glutaredoxin 1 (thioltransferase) |
| NM_007472 | Agpl1       | Aquaporin 1 |
| AW741495  | Eif4b       | Eukaryotic translation initiation factor 4B |
| NM_009076 | Rpl12       | Ribosomal protein L12 |
| CT7842    | Coq3        | Coenzyme Q3 homolog, methyltransferase (yeast) |
| BI739053  | Clcn3       | Chloride channel 3 |
| NM_008173 | Nrsc1       | Nuclear receptor subfamily 3, group C, member 1 |
| NM_002310 | Hspa5       | Heat shock 70-kDa protein 5 (glucose-regulated protein) |
| BG081851  | Actr1b      | ARPI actin-related protein 1 homolog B (yeast) |
| A.W.99410 | Phef1       | Pre-B-cell colony-enhancing protein |
| NM_138953 | Elz         | Elongation factor RNA polymerase II 2 |
| BB677778  | Neol        | Neogenin |
| NM_014347 | Hivep2      | Human immunodeficiency virus type 1 enhancer binding protein 2 |
| NM_016959 | Rps3a       | Ribosomal protein S3a |
| NM_007508 | Apnip1a12   | ATPase, H + transporting, V1 subunit A, isoform 1 |
| BC029959  | Acil6       | Acyl-CoA synthetase long-chain family member 6 |
| AK003350  | 573945F08Rik| Zinc finger CCCH-type domain containing 11A |
| BC003451  | Mat2a       | Methionine adenosyltransferase II, α |

Genes differentially expressed in Prkag3<sup>−/−</sup> compared with wild-type littermates:

- **NM_021537**: Sdk25 (Serine/threonine kinase 25 (yeast))
- **NM_009099**: Aqpl1a12 (Asparagine repeat domain protein)
- **D87867**: Ugt1a12 (UDP-glycosyltransferase 1 family polypeptide members A12, A10, A5, A6, A1, and A2)

**AMPK δ-dependent Transcriptional Responses**
Table 1—Continued

| Public ID | Gene symbol | Gene title | TgPrkag3<sup>225Q</sup> versus wild-type littermates | Prkag3<sup>225Q/225Q</sup> versus wild-type littermates |
|-----------|-------------|------------|-----------------------------------------------|--------------------------------------------------|
| AV152334  | Ap1b1<sup>a</sup> | ATPase, Na<sup>+</sup>/K<sup>+</sup> transporting, β1 polypeptide | -1.14 0.395 | -1.21 0.004 |
| NM 009208 | Slc6a3       | Solute carrier family 4, member 3 | -1.04 0.653 | 1.34 0.010 |
| BB534670  | Cd36        | CD36 antigen | 1.08 0.658 | -1.13 0.008 |
| JA28061   | Clasp1      | CLIP associating protein 1 | 1.00 0.935 | 1.32 0.006 |
| NM 024264 | Cypl2a1     | Cytochrome P450, family 27, subfamily a, polypeptide 1 | -1.17 0.102 | 1.30 0.001 |
| AK004757  | Stk11p      | Serine/threonine kinase 11 interacting protein | 1.02 0.815 | -1.29 0.0003 |
| AK007410  | Gadd45g     | Growth arrest and DNA-damage-inducible 45γ | -1.13 0.617 | 1.27 0.010 |
| NM 016894 | Rampl       | Receptor activity modifying protein 1 | 1.02 0.780 | -1.26 0.005 |
| NM 013626 | Pam         | Peptidylglycine α-amidating monoxygenase | -1.04 0.272 | 1.26 0.001 |
| NM 013750 | Phlda3      | Pleckstrin homology-like domain, family A, member 3 | 1.16 0.003 | -1.25 0.026 |
| BM207588  | Slc2a1      | Solute carrier family 2 (facilitated glucose transporter), member 1 | -1.13 0.203 | 1.25 0.022 |
| BB085604  | 2610031L17Rik | Putative mitochondrial outer membrane protein import receptor | 1.01 0.965 | 1.25 0.008 |
| AA792094  | Got1        | Glutamate oxaloacetate transaminase 1, soluble | 1.17 0.016 | -1.14 0.011 |
| BC013271  | Annexin A8  | Annexin A8 | 1.07 0.211 | -1.24 0.009 |
| AF333325  | Ddit4l      | DNA-damage-inducible transcript 4-like | -1.21 0.087 | 1.23 0.013 |
| BF225802  | Igf8p5      | Insulin-like growth factor-binding protein 5 | -1.17 0.211 | 1.22 0.045 |
| AV337619  | Ppara-g1a   | Peroxisome proliferator-activated receptor γ, coactivator 1α | -1.06 0.531 | -1.22 0.007 |
| M64279    | Bmi1        | B lymphoma Mo-MLV insertion region 1 | -1.27 0.072 | 1.22 0.043 |
| BG060909  | Scd2        | Stearoyl-Coenzyme A desaturase 2 | 1.00 0.964 | 1.22 0.003 |
| M65053    | Fgr1        | Fibroblast growth factor receptor 1 | -1.20 0.096 | 1.22 0.008 |
| BB503267  | C35000S01L02Rik | Hypothetical mitochondrial energy transfer proteins | 1.10 0.210 | -1.21 0.015 |
| BB044517  | 5730551F12Rik | RIKEN cDNA 5730551F12 gene | 1.02 0.830 | 1.21 0.024 |
| AK004339  | Tmec2       | Transmembrane and coiled-coil domains 2 | -1.19 0.003 | 1.21 0.0002 |
| AV171622  | 3300001H21Rik | RIKEN cDNA 3300001H21 gene | -1.14 0.281 | 1.20 0.033 |
| BC024809  | Aebp3       | Amyloid β precursor protein-binding, family B, member 3 | 1.08 0.072 | -1.20 0.026 |
| AV023018  | Rpe<sup>a</sup> | Ribulose-5-phosphate-3-epimerase | -1.23 0.058 | 1.20 0.049 |
| AJ334936  | Rpl13a      | Ribosomal protein L13a | -1.13 0.002 | 1.20 0.005 |
| BR000904  | Mtap4       | Microtubule-associated protein 4 | -1.19 0.006 | 1.20 0.003 |
| BG060431  | Napl1<sup>a</sup> | Nucleosome assembly protein 1-like 1 | -1.17 0.041 | 1.20 0.014 |

<sup>a</sup> Genes having several probe sets supporting the regulation.
in Mapk14 phosphorylation remains obscure. Interestingly, two transcripts encoding proteins involved in regulation of Mapk14 activity, Map2k6 and Dusp10, were coordinately regulated in γ3 R225Q transgenic and knock-out mice, as shown by microarray analysis as well as qRT-PCR (Fig. 2). A protein encoded by Map2k6 (mitogen-activated protein kinase kinase 6) is known to activate Mapk14 by dual phosphorylation of specific threonine and tyrosine residues (31). Dusp10 (dual specificity phosphatase 10), on the other hand, down-regulates the enzymatic activity of MAPKs by dephosphorylating the threonine and tyrosine residues, with selectivity toward Mapk14 (32). Thus, the finding of an up-regulation of Map2k6 in combination with a suppression of the Dusp10 transcript in TgPrkag3225Q mice, and the reversed pattern of changes seen in Prkag3−/− mice, suggests that Mapk14 is a target of AMPK γ3-containing trimers in the skeletal muscle. An up-regulation of Map2k6 mRNA in TgPrkag3225Q muscle was accompanied by an increase in the protein level, as seen by Western blot analysis (data not shown).

γ3-Containing AMPK Heterotrimers Regulate Lipid Metabolism Gene Expression in Skeletal Muscle—The AMPK γ3-subunit has previously been shown to be involved in regulation of fat oxidation. Pigs and mice carrying the R225Q mutation in AMPK γ3-gene are characterized by increased lipid oxidation in skeletal muscle (14, 15, 17). In the microarray and qRT-PCR analysis, several genes involved in fat metabolism were differentially expressed in TgPrkag3225Q and Prkag3−/− mice, suggesting that γ3-containing AMPK complexes are involved in the regulation of lipid oxidation in skeletal muscle at the transcriptional level. mRNA for Srebf1 (sterol regulatory element binding factor 1), implicated in lipogenic gene expression (33), was down-regulated in γ3 R225Q mutant mice, whereas mRNA encoding for Ppargc1a ( Peroxisome proliferative-activated receptor, γ coactivator 1 α), known to increase the expression of both nuclear and mitochondrial-encoded genes of oxidative metabolism (34), was up-regulated. Additionally, a key gene integral to free fatty acid uptake (Cd36 (35)), as well as genes involved in use of fat-derived energy (3-oxoacid-CoA transferase 1, Oxct1, EC 2.8.3.5 and carboxylesterase 3, Ces3, EC 3.1.1.1) were up-regulated in TgPrkag3225Q mice. The opposite pattern of changes was observed in γ3 knock-out mice, with differential expression of mRNA for Cd36, Oxct1, and Ppargc1a reaching statistical significance (Fig. 2).
Possible Role for the AMPK γ3-Subunit in the Regulation of Cellular Iron Homeostasis—Expression of several genes involved in iron metabolism was coordinately changed in AMPK γ3 R225Q transgenic mice, compared with the wild-type littermates. The transferrin receptor, ferritin, and aminolevulinic acid synthase (a key enzyme for heme synthesis) regulate the uptake, storage, and use of iron in cells, respectively. Expression of these markers is known to be coordinately controlled by cellular iron supply such that, under conditions of iron starvation, the transferrin receptor is up-regulated, whereas ferritin and aminolevulinic acid synthase are down-regulated (36). In TgPrkag3γ3R225Q mice, the transcription of the transferrin receptor, Tfrc, was decreased, while mRNAs for ferritin light chain 1 and aminolevulinic acid synthase 1 were increased, suggesting the possibility of improved iron status compared with the wild-type mice (Fig. 2). Improved iron status may increase skeletal muscle capacity for aerobic metabolism (37, 38). In line with this, the level of skeletal muscle myoglobin, a heme-carrying protein, transporting oxygen to the mitochondria, was non-significantly increased in TgPrkag3γ3R225Q mice, as determined by Western blot analysis (data not shown).

AMPK γ3-Containing Complexes Regulate Transcription of the Nos1 Gene—Nitric oxide synthase 1 (Nos1) also known as nNOS was significantly up- and down-regulated by ~2-fold in γ3 R225Q transgenic and knock-out mice, respectively (Fig. 2). The family of Nos enzymes catalyzes formation of endogenous nitric oxide, a mediator implicated in regulation of skeletal muscle contractility, mitochondrial function, as well as glucose uptake (39, 40). Thus, the direction of differential expression of this gene was in complete agreement with the observed phenotype in the mouse models. Although Nos1 is considered a predominant isoform expressed in skeletal muscle fibers, endothelial Nos3 (Nos3 or eNOS) and inducible Nos2 (NOS2 or iNOS) are also expressed in this tissue (41–43). Interestingly, Nos2 and Nos3 were represented on oligonucleotide microarray and were detected in the skeletal muscle samples. However, mRNA levels for these two genes were unaltered.

DISCUSSION

The present study provides the first systematic characterization of the role of the γ3-containing AMPK heterotrimers in transcriptional regulation in skeletal muscle. An oligonucleotide microarray technology was used to compare global transcriptional profiles in white skeletal muscle from AMPK γ3 R225Q transgenic (TgPrkag3γ3R225Q) and knock-out (Prkag3γ3−/−) mice and their respective wild-type littermates. Collectively, evidence is provided for an important role of the AMPK γ3-subunit in the transcriptional regulation of divergent groups of genes in glycolytic skeletal muscle. The expression of 167 genes was significantly changed (~fold change > 1.2 and p ≤ 0.05) in skeletal muscle from γ3 R225Q transgenic and/or knock-out mice. A number of genes from the same biological pathways was coordinately regulated. Changes in levels of mRNA of particular interest, including genes involved in glycolysis and lipid metabolism, as well as iron homeostasis and Mmpk14 signaling, were selected to be further verified by qRT-PCR (Fig. 2). For all of 13 selected genes, the qRT-PCR results were consistent with the gene array data, which emphasizes the quality of the microarray results. Of note is that most of the genes differentially regulated in TgPrkag3γ3R225Q mice were changed in an opposing manner in AMPK γ3 knock-out mice (Fig. 1). The reciprocal and coordinated expression pattern observed increases the confidence in the gene-array data and demonstrates that the majority of differentially expressed genes are true positives.

This reverse transcription pattern seen in TgPrkag3γ3R225Q versus Prkag3γ3−/− mice is intriguing, considering that the mechanism of action of R225Q substitution in AMPK γ3-subunit is still unresolved. A previous report provided evidence that the kinase activity of AMPK was reduced by ~3-fold in skeletal muscle of γ3 R225Q mutant pigs when measured in the presence and absence of the allosteric activator, AMP (12). Nevertheless, in resting muscle from TgPrkag3γ3R225Q mice, AMPK activity was unaltered (18). Excessive glycogen content characterizing skeletal muscle of mice and pigs carrying the γ3 R225Q mutation may inhibit AMPK activation by a feedback mechanism. Consistent with this hypothesis, AMPK activity and phosphorylation of the Thr172 residue in the α-subunit was elevated in Cos cells transiently transfected with plasmids encoding α2β2γ3 R225Q, compared with the cells transfected with plasmids encoding wild-type trimers (17). Interestingly, the introduction of the R225Q mutation at the corresponding site of the AMPK γ1 (R70Q) or γ2 (R302Q) leads to increased or decreased kinase activity, respectively, when measured in the presence of AMP (44, 45). Our data support a role for the γ3 R225Q as an activating mutation, as judged by its biological effects, because this substitution rendered the opposing changes in the gene expression profile compared with the changes resulting from genetic ablation of the AMPK γ3-subunit (Fig. 1).

The wide array of differentially expressed genes reported in this study has not previously been identified to be regulated by AMPK. Moreover, the biological function of a number of the regulated transcripts is poorly described in skeletal muscle and 19 of the differentially expressed genes remain unknown. Therefore, additional molecular and functional studies will be required to understand the full biological significance of the transcriptional changes described here. Importantly, the expression of several genes with known function in fat and carbohydrate metabolism and skeletal muscle ergogenics was coordinately and reciprocally changed in AMPK γ3 R225Q transgenic and knock-out mice. Thus, the transcriptional regulation by AMPK γ3-containing complexes could lead to at least some of the physiological differences observed in skeletal muscle from TgPrkag3γ3R225Q and Prkag3γ3−/− mice (Fig. 3).

FIGURE 3. Contrasting transcriptional and physiological responses in the skeletal muscle from AMPK γ3 R225Q transgenic and knock-out mice. A schematic illustration of the selected transcriptional changes (A) in TgPrkag3γ3R225Q (indicated by red arrows) and Prkag3γ3−/− mice (indicated by blue arrows), compared with wild-type littermates, and the predicted physiological response (B). The role of AMPKγ3 in regulation of functions like glycogen deposition, glucose uptake, oxidative metabolism, and muscle fatigue has previously been described (17, 18). The current study provides a transcriptional mechanism for the physiological differences between the genotypes.
One of the most distinct physiological differences between skeletal muscle from AMPK γ3 R225Q transgenic and knock-out mice is the change in glycogen metabolism (17). Glycogen content in skeletal muscle from TgPrkag3R225Q mice is 2-fold higher under either fed or fasted conditions. Furthermore, skeletal muscle glycogen re-synthesis after exercise is markedly enhanced in TgPrkag3R225Q mice. In contrast, glycogenesis after exercise was impaired in Prkag3−/− mice (17). Glycogen synthase catalyzes a rate-determining step in the glycogen biosynthesis pathway. However, at least in situations where glycogen synthase is activated, the reactions mediated by other enzymes in synthesis pathway can become rate-limiting (46). Interestingly, the microarray data and qRT-PCR analysis revealed a significant up- and down-regulation of Ugp2 and Gbe1 mRNAs (encoding two key enzymes in glycogen synthesis) in skeletal muscle from AMPK γ3 R225Q transgenic and knock-out mice, respectively. In agreement with these results, the protein level and enzyme activity of Ugp2 and Gbe1 is increased in RN pigs carrying an R225Q mutant form of the AMPK γ3-subunit (14, 47). Based on these observations, we hypothesize that the differences in the skeletal muscle glycogen re-synthesis rate displayed by γ3 R225Q transgenic and knock-out mice may be at least partly explained by coordinated differential transcription of Ugp2 and Gbe1.

Activation of AMPK either by physiological stimulation such as muscle contraction or by the pharmacological activator 5-amino-4-imidazole-carboxamide riboside (AICAR) leads to an increase in skeletal muscle glucose transport by promoting Glut4 translocation to the cell surface (48), as well as increasing Glut4 gene transcription (49). Importantly, the AICAR effect on glucose uptake is completely abolished in AMPK γ3 knock-out mice, suggesting that the γ3-subunit is essential for AICAR-induced glucose transport in skeletal muscle (17). Nonetheless, our microarray data did not detect any difference in the expression of the Glut4 gene when comparing skeletal muscle from TgPrkag3R225Q and Prkag3−/− mice. Mapk14 (also known as p38 MAPK) has been implicated as a downstream mediator of AMPK signaling to glucose transport in response to AICAR. In this context, it is interesting to note that mRNAs encoding two proteins involved in regulation of the phosphorylation pattern of Mapk14, Map2k6 and Dusp10, were coordinately and reciprocally regulated in AMPK γ3 R225Q and knock-out muscle. Another mediator implicated in AMPK-regulated glucose transport is nitric-oxide synthase, Nos (50). The microarray and qRT-PCR analyses revealed the mRNA encoding Nos1, the predominant nitric-oxide synthase isoform in skeletal muscle, was significantly up- and down-regulated by ~2-fold in TgPrkag3R225Q and Prkag3−/− muscle, respectively. Thus, the present study provides the first evidence of Nos1 being a transcriptional target of AMPK signaling.

Previously, the AMPK γ3-subunit has been shown to influence muscle ergogenics (18). In response to electrically stimulated muscle contraction, isolated EDL muscle from TgPrkag3R225Q mice is resistant to fatigue. Conversely, skeletal muscle from Prkag3−/− mice is fatigue prone (18). The increase in glycogen content in skeletal muscle of TgPrkag3R225Q mice may have a direct positive effect on muscle performance. Additionally, skeletal muscle is known to adapt to endurance exercise by increasing the oxygen carrying capacity. Our microarray data demonstrate a coordinated up-regulation of genes involved in the storage (Fli1) and use (Aldol) of cellular iron in TgPrkag3R225Q mice, which may indicate improved iron status and corresponding, increased oxidative capacity in skeletal muscle. Accordingly, the level of skeletal muscle oxygen carrying protein, myoglobin, was increased in TgPrkag3R225Q mice, compared with the wild-type mice. Endurance exercise is dependent upon oxidation of fatty acids as a major source of ATP. Two transcription factors implicated in the regulation of fatty acid homeostasis, Pparγ1a and Srebf1, as well as several genes necessary for the transport (Cd36) and utilization (Ces3 and Oxtc1) of fatty acids were differentially expressed in skeletal muscle from TgPrkag3R225Q mice, suggesting increased fatty acid availability and oxidation. In agreement with these observations, elevated reliance of fat-derived energy has been described in γ3 R225Q transgenic mice during exercise, as well as after challenge with high fat diet (17, 18).

We have applied global transcriptome analysis by oligonucleotide microarrays to systematically characterize the role of the AMPK γ3-subunit in the regulation of gene expression. Recently, a number of studies have used qRT-PCR analysis to characterize the role of AMPK complexes in transcription regulation (51, 52). However, gene array approaches allow for concurrent analysis of global mRNA profiles and offer an advantage of reducing bias in data collection, compared with the candidate gene-based approaches. Taken together, our data indicate that a number of genes involved in carbohydrate and fat metabolism, as well as skeletal muscle ergogenics are coordinately and reciprocally regulated in the skeletal muscle from AMPK γ3 R225Q transgenic and knock-out mice and provide a molecular mechanism for the previously described physiological differences between the models. Furthermore, the current study identifies that AMPK γ3-containing complexes play an important role in regulation of novel downstream targets and pathways in skeletal muscle.

AMPK has been identified as an attractive therapeutic target in the treatment of obesity and type II diabetes (53). However, the ideal therapeutic small molecule should modify the activity of AMPK heterotrimers in metabolic tissues such as skeletal muscle to increase fatty acid oxidation and glucose uptake without exerting any effect on organs including central nervous system, heart, pancreas, or lung, where AMPK activation potentially would lead to harmful consequences (54–57). Therefore, therapeutic agents that selectively modulate the activity of γ3-containing AMPK heterotrimers could potentially provide a way to specifically target skeletal muscle and thereby prevent any adverse effects in other organ types. The current study contributes to the understanding of the role of the AMPK γ3-subunit in the regulation of gene transcription and defines several sets of potential biomarkers to characterize the molecular effects in response to the administration of lead substances ideally mimicking the TgPrkag3R225Q phenotypes in mice and pigs.

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