*Physiol Genomics* 32: 219–228, 2008.
First published October 23, 2007; doi:10.1152/physiolgenomics.00157.2007.

# Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation

**Adeel Safdar,[1,2] Nicholas J. Yardley,[2] Rodney Snow,[3] Simon Melov,[4] and Mark A. Tarnopolsky[2]**

*Departments of [1]Kinesiology and [2]Pediatrics and Medicine, McMaster University, Hamilton, Ontario, Canada; [3]School of Exercise and Nutrition Sciences, Deakin University, Burwood, Australia; and [4]Buck Institute for Aging Research, Novato, California*

Submitted 14 July 2007; accepted in final form 15 October 2007

**Safdar A, Yardley NJ, Snow R, Melov S, Tarnopolsky MA.** Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation. *Physiol Genomics* 32: 219–228, 2008. First published October 23, 2007; doi:10.1152/physiolgenomics.00157.2007.—Creatine monohydrate (CrM) supplementation has been shown to increase fat-free mass and muscle power output possibly via cell swelling. Little is known about the cellular response to CrM. We investigated the effect of short-term CrM supplementation on global and targeted mRNA expression and protein content in human skeletal muscle. In a randomized, placebo-controlled, crossover, double-blind design, 12 young, healthy, nonobese men were supplemented with either a placebo (PL) or CrM (loading phase, 20 g/day × 3 days; maintenance phase, 5 g/day × 7 days) for 10 days. Following a 28-day washout period, subjects were put on the alternate supplementation for 10 days. Muscle biopsies of the vastus lateralis were obtained and were assessed for mRNA expression (cDNA microarrays + real-time PCR) and protein content (Kinetworks KPKS 1.0 Protein Kinase screen). CrM supplementation significantly increased fat-free mass, total body water, and body weight of the participants ($P < 0.05$). Also, CrM supplementation significantly upregulated (1.3- to 5.0-fold) the mRNA content of genes and protein content of kinases involved in osmosensing and signal transduction, cytoskeleton remodeling, protein and glycogen synthesis regulation, satellite cell proliferation and differentiation, DNA replication and repair, RNA transcription control, and cell survival. We are the first to report this large-scale gene expression in the skeletal muscle with short-term CrM supplementation, a response that suggests changes in cellular osmolarity.

ergogenic aid; osmosensing; cell signaling; cDNA microarray; real-time PCR

CREATINE MONOHYDRATE (CrM) supplementation has a number of biochemical and physiological effects and enhances muscle performance in humans (89). Intracellular phosphocreatine (PCr) functions as an energy buffer to prevent ATP depletion in the skeletal muscle, especially during short-duration repetitive high-intensity exercise bouts (22, 43, 75, 89). Following the intake of 20 g CrM/day for 4–7 days (2, 29, 33) or 3 g CrM/day for 4–12 wk (4, 46, 91, 94), skeletal muscle total creatine and PCr increase by 10–20%. Short-term CrM supplementation increases muscle force and/or power (2, 3, 7, 8, 10, 18, 29, 59, 96), whereas chronic CrM supplementation in conjunction with weight training increases maximal muscle strength and power, fat-free mass (FFM), muscle fiber size, total body water, and total body weight (47, 91, 94) compared with placebo. The increase in FFM and total body weight is partly due to fluid retention in myocytes caused by the osmotic potential of high intracellular CrM abundance (45, 47, 60). Whether the aforementioned phenotypic effects are due to energy buffering, physiochemical attributes of the compound, cell volume regulation, or CrM supplementation's direct influence on cellular metabolism through changes in gene expression remains unclear.

Studies have found significant increases in total body water in men after both short-term (106) and long-term (47) CrM supplementation, and that this acute increase in fluid volume is limited to the intracellular compartment only (106). Our group has shown that 9 days of CrM supplementation decreased whole body protein breakdown and leucine oxidation in young men (66), and this was directionally similar to studies infusing a hypoosmotic solution to induce cell swelling (6, 34). Consequently, we hypothesized that CrM supplementation would induce cell swelling, which in turn would activate downstream cell volume-sensitive signaling cascades, and affect overall cellular metabolism (66). If cell volume changes modulate the anti-proteolytic effects of CrM supplementation, then this should involve the intrinsic and/or extrinsic signaling kinases.

Presently, little is known about the global changes in gene expression following short-term CrM supplementation in human skeletal muscle, independent of confounding factors such as exercise or disuse atrophy models. CrM supplementation enhances satellite cell differentiation in vitro (93) and satellite cell mitotic activity during compensatory hypertrophy in rat skeletal muscle (16). These observations are consistent with the results from an in vivo study showing an increase in the protein content of the myogenic transcription factors myogenic regulatory factor-4 (MRF4) and myogenin after recovery from cast-induced atrophy in the skeletal muscle of young subjects supplemented with CrM (36). Also, CrM supplementation in conjunction with strength exercise training increases satellite cell number, myonuclei concentration (63), and type II muscle fiber area (94).

Given the emerging interest in CrM supplementation in the management of neuromuscular disorders, including amyotrophic lateral sclerosis (44), Huntington's disease (56), Parkinson's disease (55), Alzheimer's disease (9), and muscular dystrophies (13, 85, 87, 88), our aim was to elucidate potential cellular and molecular mechanisms that underlie its therapeutic efficacy. The objectives of this study were *1*) to examine the intracellular global gene expression in human skeletal muscle following 10 days of CrM supplementation using cDNA microarray analysis, and *2*) to evaluate the effect of short-term CrM supplementation on targeted mRNA expression and pro-

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

Address for reprint requests and other correspondence: M. A. Tarnopolsky, Dept. of Pediatrics and Medicine, HSC-2H26, McMaster Univ. Medical Center, 1200 Main St. W., Hamilton, ON, Canada L8N 3Z5 (e-mail: tarnopol@mcmaster.ca).

tein content of kinases associated with cell volume regulation. In the present study, we analyzed muscle biopsies of the vastus lateralis from young men following 10 days of supplementation with placebo or CrM, without the superimposition of acute exercise.

## MATERIALS AND METHODS

### *Subjects*

Twelve young, healthy, nonsmoking, and nonobese men (age, 26 ± 3 yr; weight, 76 ± 9 kg; height, 178 ± 11 cm; regular physical activity ≤2× per wk) participated in the study. Before the study, potential subjects completed a health questionnaire to assess their health and fitness. All subjects provided written consent before their participation. The study was approved by the Hamilton Health Sciences Human Research Ethics Board and conformed to the guidelines outlined in the Declaration of Helsinki.

### *Experimental Protocol*

*Supplementation and tissue collection.* Following a block randomized, placebo-controlled, crossover, double-blind design, six participants consumed a placebo (PL; 75 g of dextrose), while the other six participants took a commercially available isoenergetic supplement predominantly containing CrM (CELL-Tech; 10 g of CrM, 75 g of dextrose, 2 g of taurine, 250 mg of ascorbic acid, 300 μg of chromium picolinate, 200 mg of α-lipoic acid, 100 mg of phosphorus, 150 mg of potassium, 60 mg of sodium, 70 mg of magnesium, and 20 mg of calcium; Iovate, Mississauga, ON, Canada) under the direct supervision of a research assistant. Subjects consumed the CrM supplement (CELL-Tech) for a total of 10 days: 10 g/day twice daily for the first 3 days (loading phase), followed by 5 g/day for the remaining 7 days (maintenance phase). Both CrM and PL had identical grape flavoring and were mixed into cold water. Because of direct supervision, compliance was 100% with supplement consumption. Following a 28-day washout period, which is sufficient for muscle PCr concentration to return to basal level (24, 39), subjects crossed over to consume the alternate supplement for 10 days. Four-day diet records were acquired from all subjects during the last 4 days of both treatments and were analyzed for daily total calorie and macronutrient composition using Nutritionist V (First Data Bank, San Bruno, CA) (86). FFM and body fat percentage were determined using dual-energy X-ray absorptiometry (QDR-1000W; Hologic, Waltham, MA) as previously described (86). Total body water was measured using bioelectric impedance analysis (BIA-101A; RJL Systems, Mt. Clemens, MI) (32).

On the last day of supplementation for both treatments, we collected muscle biopsies of the vastus lateralis that were taken from the same leg and were separated by ~6 cm to minimize the potential effect of the biopsy on gene expression (62). The sampling site was randomized with respect to the proximal vs. distal incision site. We took ~150 mg of wet muscle from each biopsy after quickly dissecting it of fat and connective tissue and sectioned the sample into four pieces. Two pieces of ~30 mg were immediately placed in separate RNase-free cryovials, flash-frozen in liquid nitrogen, and stored at −86°C until analysis (RNA and total protein extraction). A third piece of ~80 mg was lyophilized overnight and stored at −86°C for subsequent analysis of total creatine (TCr) content. The last piece of ~10 mg was mounted in Tissue-Tek optimal cutting temperature (OCT) compound embedding medium (EMS, Hatfield, PA) prechilled in isopentane that was cooled in liquid nitrogen, snap frozen, and stored at −86°C until subsequent analysis.

### *Biochemical Analyses*

Muscle samples frozen in liquid nitrogen were lyophilized, powdered, and extracted in 0.5 M perchloric acid-1 mM EDTA and neutralized using 2 M $KHCO_3$, as previously described (84). Extracts were analyzed for TCr using a fluorometric enzymatic method described previously by our group (86). The intra-assay coefficient of variation (CV) was 3.7% for TCr.

### *Histochemical Analyses*

The OCT-embedded biopsy samples were serially sectioned (7-μm thick) and were stained for myosin ATPase activity as previously described (86). A total of 150–500 fibers were available for analysis from each subject. Fiber analyses were performed using image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD) interfaced with a microscope (Olympus BX60, Melville, NY) and a digital camera (SPOT Diagnostics Instruments, Sterling Heights, MI). The intra-assay CVs were 1.5 and 2.6% for the analysis of percent total fiber area and mean fiber area, respectively.

### *RNA Analysis*

*RNA extraction.* The total RNA was extracted from the skeletal muscle biopsy followed by DNase I treatment as described previously in detail by our group (53). We assessed total RNA quality by measuring the size distribution on an Agilent Bioanalyzer (1.0–1.5 kb; Agilent Technologies, Santa Clara, CA) and by measuring the spectrophotometric 260/280 ratio (>1.8).

### *Microarray Analyses*

For analyses of CrM-induced global gene expression changes, muscle from all the participants ($n = 12$) from both phases of the study (PL and CrM) were analyzed to highlight a paired/repeated measures comparison. The study design (randomized, crossover, within subject) permitted the comparison of the CrM with the PL data for individual subjects, i.e., within-subject comparison. This allowed the examination of each individual subject's response to CrM vs. PL supplementation. Such a design minimizes interindividual variability in mRNA expression and recognizes that genetic influences remain identical on both treatments. This is a more powerful approach than comparing pooled data for all subjects during PL supplementation vs. pooled data for all subjects during CrM supplementation.

Two micrograms of total RNA were converted to cDNA and labeled with either cyanine-3 (Cy3; CrM condition) or Cy5 (PL condition) fluorescent dyes in preparation for in-house microarray analyses at the Genomics Facility of the Buck Institute for Age Research, as previously described (54). Detailed microarray experimental design and conditions are also found online on the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7877). The total number of analyzable genes per array after removal of PCR failures and genes with nondetectable expression was ~7,500 genes.

*RT and real-time PCR.* We performed RT (Roche Applied Science, Laval, QC, Canada) and real-time PCR on an iCycler real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) using SYBR Green chemistry, as described previously for cDNA microarray validation and targeted mRNA expression analysis (54). For these analyses, we used β2-microglobulin as a housekeeping gene, since our intervention did not influence the β2-microglobulin mRNA content in the skeletal muscle (data not shown). All samples were run in duplicate simultaneously with "no RNA" and RT negative controls. In addition, the melting point dissociation curve generated by the instrument was used to confirm the specificity of the amplified product. Please refer to Supplemental Table S1 for primer sequences used for real-time PCR (supplemental data are available at the online version of this article).

### *Kinexus Protocol*

The whole lysate protein samples were analyzed by Kinetworks KPKS 1.0 Protein Kinase screen (Kinexus Bioinformatics, Vancou-

ver, BC, Canada) as described previously (69, 70). Briefly, ~30 mg of skeletal muscle from each subject were lysed in 750 μl of homogenization buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, pH 7.2). The lysates were centrifuged for 10 min at 4°C at 600 *g*. Bradford assay was used to quantify the protein content. The samples were boiled in Laemmli sample buffer and shipped to Kinexus as per the company's instructions (http://www.kinexus.ca). *Subjects 1–6* for the CrM condition were pooled together and analyzed against themselves for the PL condition, and the same was done for the second pool containing *subjects 7–12*, due to the limited sample. The Kinetworks analysis involves resolution of a single pool of lysed samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting with panels of up to three primary antibodies per channel in a 20-lane Immunetics multiblotter, for the identification and activation assessment of a network of 78 protein kinases. The trace quantity units are arbitrary, based on the intensity of enhanced chemiluminescent fluorescence detection for target immunoreactive proteins recorded with a Fluor-S MultiImager and quantified using Quantity One software (Bio-Rad). The reproducibility of these signal transduction protein screens was within 15%.

### *Statistical Analysis*

Anthropometric measurements, muscle composition, and dietary data were analyzed using a Student's paired *t*-test. We used a one-tailed test when analyzing TCr concentration and body composition, because we a priori hypothesized that CrM supplementation would increase TCr concentration, FFM, total body water, and body weight but decrease percent body fat. For all other analyses, we used a two-tailed test. A $P$ value $\leq 0.05$ was considered statistically significant. Data are presented as means ± SD.

We analyzed microarray data using the significance analysis of microarrays (SAM; v.1.21) to identify statistically significant differential global gene expression in the CrM vs. PL group, using a false discovery rate of <5% (54). Real-time PCR data for both global and targeted gene expression were analyzed using a paired *t*-test on the linear $2^{-\Delta CT}$ data set (where $C_T$ is threshold cycle) (51). We analyzed Kinetworks KPKS 1.0 Protein Kinase screen data for protein content using a paired *t*-test on corrected band intensities and expressed it as fold change (CrM vs. PL).

## RESULTS

### *Body Composition, Dietary Analysis, and Muscle Fiber Characteristics*

Percent body fat was similar between the two interventions. CrM supplementation increased FFM by 1.6% ($P = 0.03$), total body water by 2.2% ($P = 0.04$), and body weight by 2.6% ($P = 0.002$) (Table 1). The total energy, carbohydrate, and protein intake of the participants did not change over the course of the study (Table 1). CrM supplementation increased total muscle creatine content by 14% ($P = 0.0009$), indicating excellent compliance (Table 2). Short-term CrM supplementation also resulted in a nonsignificant increase in type I, type IIa, and type IIx fiber area by ~9, 5, and 4%, respectively (Table 2).

Table 1. *Subject characteristics and diet composition*

| | PL | CrM | *P* Value |
|---|---|---|---|
| Weight, kg | 76±9 | 78±10 | $P = 0.002$ |
| FFM, kg | 64±7 | 65±7 | $P = 0.026$ |
| Total body water, liters | 45±6 | 46±6 | $P = 0.041$ |
| %Body fat | 18±3 | 18±4 | $P = 0.39$ |
| Energy, kcal/day | 2,542±615 | 2,604±592 | $P = 0.80$ |
| Energy, kJ/day | 10,623±2,570 | 10,882±2,474 | |
| CHO, g/day | 313±81 | 333±93 | $P = 0.36$ |
| PRO, g/day | 104±31 | 106±33 | $P = 0.75$ |

Values are means ± SD; $n = 12$. PL, placebo; CrM, creatine monohydrate; FFM, fat-free mass; CHO, carbohydrate; PRO, protein.

Table 2. *Muscle fiber characteristics*

| | PL | CrM | *P* Value |
|---|---|---|---|
| TCr, mmol/kg dry mass | 127±11 | 143±8 | $P < 0.001$ |
| %Type I fiber | 43±5 | 41±8 | $P = 0.36$ |
| %Type IIa fiber | 22±6 | 24±9 | $P = 0.70$ |
| %Type IIx fiber | 35±10 | 36±5 | $P = 0.89$ |
| Type I fiber area, $\mu m^2$ | 5,416±1,580 | 5,909±1,948 | $P = 0.29$ |
| Type IIa fiber area, $\mu m^2$ | 7,282±2,620 | 7,621±2,911 | $P = 0.61$ |
| Type IIx fiber area, $\mu m^2$ | 8,713±2,451 | 9,093±3,267 | $P = 0.67$ |

Values are means ± SD; $n = 12$. TCr, total creatine content.

### *CrM Supplementation Altered mRNA Expression in the Skeletal Muscle*

cDNA microarray data showed that short-term CrM supplementation significantly upregulated the expression of 216 genes in the skeletal muscle of healthy young men (to view the complete data deposited in the NCBI Gene Expression Omnibus, visit http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7877). The mean fold increase in the mRNA for these genes was 1.7 ± 0.4-fold, with a minimum increase of 1.3-fold (activated RNA polymerase II transcription cofactor-4) and a maximum increase of 5.0-fold (sphingosine kinase-1) (Supplemental Table S2). We used real-time PCR to randomly confirm the expression of eight of the mRNA species whose expression changed by >2.0-fold in the cDNA microarray analyses (Fig. 1). In addition, CrM supplementation significantly downregulated the expression of 69 genes, with a mean decrease of 0.7 ± 0.1-fold (a minimal repression of 26% in $C_2H_2$ type zinc finger protein and a maximal repression of 70% in Frizzled-related protein) (Supplemental Table S3).

### *CrM Supplementation Altered the Protein and mRNA Contents of Skeletal Muscle Kinases*

The Kinetworks KPKS 1.0 Protein Kinase screen of 78 protein kinases showed that short-term CrM supplementation significantly upregulated the protein content of three protein-serine/threonine kinases, i.e., p38 MAPK, ERK6, and protein kinase Bα (PKBa/Akt1), in the skeletal muscle of healthy young men (mean increase of 2.8 ± 1.2-fold) (Supplemental Table S4). Also, there is a strong trend for an increase in the protein content of three other kinases [HPK1, focal adhesion kinase (FAK), and CDK9] with CrM supplementation (mean increase of 5.6 ± 2.4-fold). We also used real-time PCR to confirm the increase in the mRNA content in four of the aforementioned upregulated kinases (p38 MAPK, ERK6, PKBa/Akt1, and FAK) in the skeletal muscle of subjects supplemented with CrM (Fig. 2).

## DISCUSSION

In the present study, we observed an increase in total muscle creatine content, FFM, total body water, and body weight in healthy young men after 10 days of CrM supplementation with

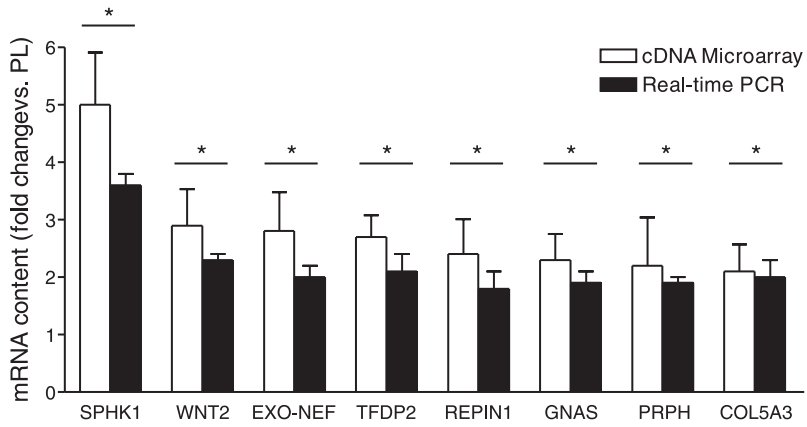


Fig. 1. mRNA content [mean fold change vs. placebo (PL)] of the 8 randomly selected genes significantly upregulated after creatine monohydrate (CrM) supplementation using cDNA microarray and real-time PCR. Pearson correlation analysis revealed a significant positive correlation between the cDNA array data and the real-time PCR data for expression changes >2.0-fold ($r = 0.94$, $P \leq 0.001$). Data are presented as means ± SD. SPHK1, sphingosine kinase-1; WNT2, wingless-type MMTV integration site family member-2; EXO-NEF, exonuclease NEF-sp; TFDP2, human transcription factor Dp-2; REPIN1, replication initiator-1; GNAS, GNAS complex locus; PRPH, peripherin; COL5A3, collagen, type V, α3. *Significant difference vs. PL ($P \leq 0.05$).

no changes in their dietary intake and muscle fiber characteristics. We used cDNA microarrays and Kinetworks KPKS 1.0 Protein Kinase screen to investigate differential gene expression in the skeletal muscle of men supplemented with CrM vs. PL. We found that CrM supplementation increased mRNA expression and protein content of genes involved in osmosensing and signal transduction [integrin-1 (ITGA1), FAK, wingless-type MMTV integration site family member-2 (WNT2), GNAS complex locus (GNAS), and calcium-sensing receptor (CASR)], cytoskeleton remodeling [collagen (type V, α3; COL5A3), filamin Aα (FLNA), Rho GTPase activating protein-8 (ARHGAP8), and peripherin (PRPH)], protein and glycogen synthesis regulation (PKBa/Akt), satellite cell proliferation and differentiation [sphingosine kinase-1 (SPHK1), PKBa/Akt, p38 MAPK, ERK6, and megakaryoblastic leukemia-1 (MKL1)], DNA replication and repair [replication initiator-1 (REPIN1), histone-1 (HIST1H2BK), and tyrosyl-DNA phosphodiesterase I (TDP1)], RNA transcription control [human transcription factor Dp-2 (TFDP2), CCAAT/enhancer binding protein-ζ (CEBPZ), PRP8 pre-mRNA processing factor-8 homolog (PRPF8), exonuclease NEF-sp, ROD1, RNA binding motif protein-4 (RBM4), and matrin-3 (MATR3)], cell survival [Fas apoptotic inhibitory molecule-2 (FAIM2) and angiopoietin-like-4 (ANGPTL4)], and other miscellaneous processes (Supplemental Tables S2 and S4). We are the first to report this novel differential gene expression in the skeletal muscle with 10 days of CrM supplementation, a response that appears to reflect a homeostatic adaptation to changes in cellular osmolarity.

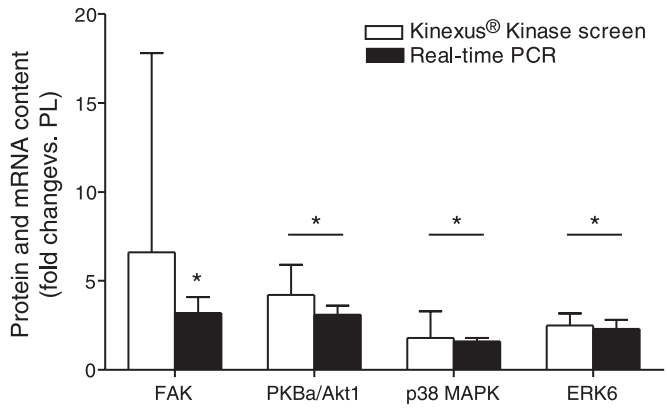


Fig. 2. Protein and mRNA content (mean fold change) of the 4 randomly selected genes significantly upregulated after CrM supplementation using Kinexus Kinase screen and real-time PCR. Data are presented as means ± SD. FAK, focal adhesion kinase; PKBa/Akt1, protein kinase Bα; p38 MAPK, p38 mitogen-activated protein kinase; ERK6, extracellular-regulated mitogen-activated protein kinase-6. *Significant difference vs. PL ($P \leq 0.05$).

### *CrM Supplementation Causes an Increase in FFM, Total Body Water, and Body Weight*

Short-term CrM supplementation significantly increased FFM, total body water, and body weight, confirming previous research (45, 95, 106). It should be noted that the commercial CrM product (CELL-Tech) used in this study contained additional dietary constituents that have been previously shown not to provide any additional response to changes in body mass, body composition, maximal strength, and power output beyond those solely attributed to CrM supplementation (46). The primary purpose of these additional dietary constituents (i.e., dextrose, α-lipoic acid, ascorbic acid, etc.) with CrM supplementation was to enhance muscle TCr content gains in the short term compared with the ingestion of CrM alone (11). The increase in FFM and body weight following CrM supplementation may be due, at least in part, to water retention as previously suggested (106). These gains are attributed to the acute increase in fluid volume, limited to the intracellular compartment only, due to an osmotic load caused by cellular CrM accumulation (106). We speculate that the increase in cellular TCr and osmolarity following creatine supplementation leads to an increase in cell volume. Little is known about the molecular events following short-term CrM supplementation in human skeletal muscle, independent of exercise or disuse atrophy models.

Cell swelling has been identified as an anabolic proliferative signal (67, 72). We have previously shown that 9 days of CrM supplementation (similar to the present protocol) decreases whole body proteolysis and leucine oxidation in young men (66), and these results were directionally similar to studies showing cell swelling after infusion of a hypoosmotic solution (6, 34). In vitro studies have also described the stimulating effect of increased cell volume on glycogen synthesis (1, 72). On the basis of research from our group and others, we hypothesized that CrM-mediated cell swelling would activate cell volume-sensitive signaling cascades to adapt to the intracellular and extracellular changes in osmolarity by activating

signal transduction pathways for maintaining proper cell function (66).

### *CrM Activates Genes Involved in Osmosensing and Signal Transduction*

We observed a significant upregulation of ITGA1 and FAK mRNA with CrM supplementation. Both ITGA1 and FAK play a vital role in osmosensing, especially during the earlier phase of cell swelling, by promoting cell adhesion to the extracellular matrix and inducing intracellular signal transduction (77, 97). We also observed that CrM supplementation activates genes that constitute the cell signal transduction network including WNT2, GNAS, and CASR. In addition, CrM supplementation downregulates the expression of frizzled-related protein (FRZB), a negative regulator of WNT2 signaling (37).

WNT2 is a signal transducer and is implicated in several developmental processes, including regulation of cell fate and patterning during embryogenesis (98). Wassink et al. (99) suggested that WNT2 is a strong candidate gene for autism, a prototypical pervasive development disorder. Studies with autistic children have associated the severity of pathology with various markers including reduced brain creatine concentration (27) and increased brain choline-to-creatine ratio (80). Theoretically, CrM supplementation may hold potential therapeutic significance for autistic children. GNAS encodes the α-subunit of G proteins and is involved in modulating and transducing various transmembrane signaling systems and is also involved in hormonal regulation of adenylate cyclase in response to β-adrenergic stimuli (5, 12). CASR senses extracellular calcium concentration and assists in maintaining calcium homeostasis (35). The activity of this receptor is mediated by a G protein that activates a phosphatidylinositol-calcium second messenger system (28), which we speculate would be because of CrM-mediated cell swelling.

### *CrM Supplementation Activates Genes Involved in Cytoskeleton Remodeling*

We observed an increase in mRNA expression of collagen (COL5A3), FLNA, ARHGAP8, and PRPH. Cytoskeleton rearrangements have been previously implicated as a result of cell swelling, as an adaptive homeostatic response (68). COL5A3 encodes an α-chain for one of the low-abundance fibrillar collagens that plays a role in muscle development and cell adhesion (40). FLNA is a phosphoprotein that regulates reorganization of the actin cytoskeleton and links actin filaments to membrane glycoproteins by interacting with integrins, transmembrane receptor complexes, and second messengers (73). PRPH is a type III intermediate filament cytoskeletal protein that participates in the signal transduction events that play a role in the regulation of cell development, activation, and growth (90). We speculate that this CrM-mediated cytoskeleton organization and biogenesis is an early event that may be central to the modulation of cell shape.

### *CrM May Regulate Protein and Glycogen Synthesis via an Induction of PKBa/Akt1*

We observed a significant increase in PKBa mRNA (2.1-fold) and protein (4.2-fold) content in the skeletal muscle with CrM supplementation. PKBa is a serine-threonine protein kinase and is involved in upregulating general protein synthesis via an increase in the eukaryotic initiation factor-2B (31, 100). This finding, together with our previous observation that CrM supplementation decreases whole body proteolysis and amino acid oxidation in healthy young men (66), indicates a trend toward a net protein accretion.

Creatine supplementation increases glycogen content, but the cellular mechanisms supporting this observation are unclear (65). Creatine-fed rats increase muscle glycogen stores because of the upregulation of GLUT4 mRNA expression via AMP-activated protein kinase-dependent pathway (42), probably because of an increase in fasting plasma insulin levels (74). In contrast, Op't Eijnde et al. (64) reported that 5 days of creatine supplementation did not increase GLUT4 expression in rats. In humans, creatine supplementation prevented a decrease in muscle GLUT4 protein content during 2 wk of immobilization and increased it during a subsequent 10 wk of rehabilitation training in healthy subjects (65). Recently, van Loon et al. (92) observed that creatine supplementation in men increased skeletal muscle glycogen storage but had no effect on GLUT4 mRNA and protein content. Similarly, in our microarray analysis, we also observed no change in GLUT4 (SLC2A4; AA978042) mRNA expression ($P = 0.26$) with CrM supplementation (0.97-fold vs. PL). However, we observed a 21% decrease (0.79-fold) in both skeletal muscle phosphofructokinase and glycogen phosphorylase, but not hexokinase, mRNA content following CrM supplementation (refer to http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7877). Together with the GLUT4 data, these observations indicate that the intracellular glucose available for cellular functions is being directed toward glycogen accretion through a decrease in glycogen breakdown and glycolysis. This is in accordance with the observed increase in the potential energy provided by the phosphagen pathway (TCr content) and the subsequent lower dependence on glycolysis to furnish immediate energy sources.

In addition to protein synthesis, PKBa positively regulates glycogen synthesis by deactivating glycogen synthase kinase-β, thus increasing glycogen synthase activity (31). It is suggested that PKBa associates with GLUT4-containing vesicles, promoting their translocation to the plasma membrane to stimulate glucose transport (26). In vitro, L6 muscle cells overexpressing PKBa significantly increase glucose transport, to a level comparable to that elicited normally by insulin in nontransfected control cells, because of an increase in the recruitment of GLUT4 to the plasma membrane (30). On the basis of significant increases in PKBa and no change in GLUT4 mRNA content in the present study, we hypothesize that short-term CrM supplementation (in the absence of exercise and/or dietary intervention) increases muscle glycogen stores via an increase in GLUT4 recruitment to the sarcolemma, with no increase in GLUT4 mRNA and/or protein content.

### *CrM May Promote Satellite Cell Proliferation and Differentiation via an Induction of SPHK1 and MAPK Pathway*

In vitro and in vivo, CrM supplementation alone (41, 93) or in combination with progressive strength training or increased functional loading and compensatory hypertrophy (synergist ablation) (16) induces satellite cell (postnatal myogenic stem cells) proliferation and differentiation. This effect may be

mediated, at least in part, by CrM-induced cell swelling, an early cellular response to CrM supplementation. Cell swelling may act as an anabolic stimulus facilitating downstream myogenic regulatory factor (MRF) pathways that, in turn, may stimulate satellite cells to proliferate and fuse with existing myofibers (36, 63). In the present study, we did not observe an increase in mean fiber area as previously reported, which could be because of our short period of supplementation compared with longer periods in studies showing this effect (94). Nonetheless, we did observe an early transcriptional and translational response at the cellular level, which explains previously observed effects of creatine supplementation on satellite cell proliferation and differentiation (16, 36, 41, 63, 93).

We observed a substantial induction in SPHK1 mRNA expression (5.0-fold) in the skeletal muscle of subjects supplemented with CrM. SPHK1 is a lipid kinase that is greatly enhanced in differentiating C2C12 myoblasts via modulating signal transduction and catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P). S1P is a bioactive intracellular lipid mediator and a ligand for S1P receptors that trigger various key mitogenic signaling pathways (i.e., MAPK, phospholipase D, Rho, and cytosolic calcium increase) involved in the regulation of myoblast proliferation and differentiation and skeletal muscle fatigue prevention (17, 58, 82).

Myoblast proliferation and differentiation involve the activation of several key signaling pathways such as ERK1/ERK2, p38 MAPK, and PI3K/PKBa (14, 101). Donati et al. (21) observed that S1P exerts a prodifferentiation activity via p38 MAPK and PKBa induction (21). p38 MAPK, a stress-sensitive kinase, is upregulated in response to cell swelling (61). Indeed, we observed with CrM supplementation robust increases in p38 MAPK and PKBa mRNA and protein content, which are mandatory for myoblast differentiation and to enhance the expression of downstream myogenic differentiation markers, such as myogenin, myosin heavy chain, and caveolin-3, that may lead to muscle accretion (21). CrM supplementation increases actin and myosin protein and myosin mRNA content in vitro and myogenic transcription factors (MRF4, MEF2A, MEF2C, MEF2D, and myogenin) in vivo (41, 102). We suggest that, taken together, the SPHK1-mediated pathways play an important role in CrM-mediated myoblast differentiation.

Furthermore, we observed an upregulation of ERK6 mRNA and protein content and MKL1 mRNA expression in the skeletal muscle of CrM-supplemented subjects. In vitro studies have shown that the activation of ERK6, a serine/threonine stress-activated protein kinase, parallels that of p38 MAPK in response to osmotic shock (76). Lechner et al. (49) reported that ERK6 is highly expressed in human skeletal muscle and functions as a signal transducer during differentiation of myoblasts to myotubes (49). MKL1 is a potent transcriptional coactivator of serum response element-dependent genes through its direct binding to serum response factor and thus contributes to both striated and smooth muscle differentiation (78). The coordinated induction of these genes will regulate satellite cell proliferation and differentiation with CrM supplementation.

### *CrM Activates Genes Involved in Regulating DNA Replication and Repair*

Another novel finding is that CrM supplementation activated genes that are positive regulators of DNA replication and repair and nuclear chromatin organization, including REPIN1, HIST1H2BK, and TDP1. In vitro, REPIN1 possesses ATP-dependent DNA helicase activity and is involved in initiating chromosomal DNA synthesis in mammalian cells (15), which could explain the increase in DNA synthesis during satellite

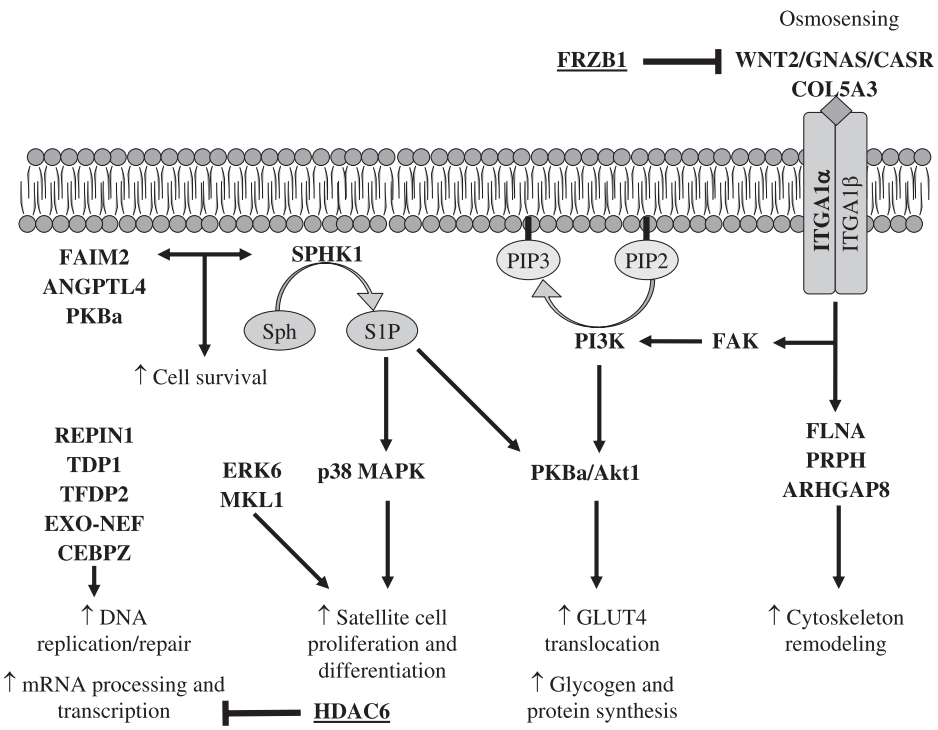


Fig. 3. The transcriptional response to short-term CrM supplementation in skeletal muscle. CrM supplementation induced the expression of genes involved in osmosensing, signal transduction, cytoskeleton remodeling, metabolism, cell survival, and genomic replication, repair, and transcription. Bold lettering denotes genes that were significantly upregulated, and underlined genes were significantly downregulated in this study. CASR, calcium-sensing receptor; FLNA, filamin Aα; ARHGAP8, Rho GTPase activating protein-8; PI3K, phosphoinositide kinase-3; PIP2, phosphotidylinositol 4,5-bisphosphate; PIP3, phosphotidylinositol 3,4,5-trisphosphate; PDK1/2, 3-phosphoinositide-dependent protein kinase-1/2; Sph, sphingosine; S1P, sphingosine-1-phosphate; MKL1, megakaryoblastic leukemia-1; FAIM2, Fas apoptotic inhibitory molecule-2; ANGPTL4, angiopoietin-like-4; TDP1, tyrosyl-DNA phosphodiesterase I; CEBPZ, CCAAT/enhancer binding protein-ζ; FRZB1, frizzled-related protein; HDAC6, histone deacetylase-6.

cell mitotic activity following creatine supplementation. HIST1H2BK gene encodes a member of the histone H2B family and is a core component of nucleosomes responsible for high-order chromatin stability and organization in the nucleus (20). TDP1 works in conjunction with topoisomerase I to repair DNA lesions and chromosomal single-strand breaks and removes glycolate from single-stranded DNA containing a 3′-phosphoglycolate, suggesting a role for TDP1 in the repair of free radical-mediated DNA double-strand breaks (23, 71). A deficiency in this DNA repair pathway in humans causes spinocerebellar ataxia with axonal neuropathy by affecting terminally differentiated neurons (83), and hence CrM supplementation may be a potential therapeutic agent for spinocerebellar ataxia patients.

### *CrM Activates Genes Involved in Regulating mRNA Processing and Transcription*

We also observed a significant upregulation (3.8- to 1.6-fold) of mRNA species collectively involved in regulating mRNA processing and transcription, including TFDP2, CEBPZ, PRPF8, exonuclease NEF-sp (EXO-NEF), RBM4, and MATR3.

TFDP2 is a transcription factor that heterodimerizes with E2F transcription factor-1 to regulate genes required for the progression of the S phase, such as DHFR and DNA polymerase-α, and thus is essential in cell cycle regulation and differentiation (103). CEBPZ interacts with the CCAAT motif present in the proximal promoters of numerous mammalian genes (57). In vitro, dominant-negative CEBPZ mutants reduce the expression of various genes including the α-2 collagen, E2F transcription factor-1, and topoisomerase IIα, resulting in fibroblast cell growth delay (38). Hence, CrM supplementation activates the transcription of genes that may play a role in downstream cellular proliferation and differentiation in addition to influencing DNA replication and repair regulation.

CrM supplementation also activated genes involved in mRNA processing and maturation, steps crucial for successful translation. PRPF8 is a component of both U2- and U12-dependent spliceosomes involved in mRNA processing (52). Exonuclease NEF-sp is involved in the rRNA and tRNA processing and maturation via 3′- to 5′-exonuclease activity. Similarly, RBM4 is involved in RNA processing (48). CrM supplementation also induced MATR3, a nuclear matrix protein, which is involved in nuclear retention of defective RNA and interacts with other nuclear matrix proteins to form the internal fibrogranular network (104).

### *CrM Supplementation Activates Genes That Promote Cell Survival*

CrM supplementation activated genes involved in cell survival and anti-apoptotic pathways, including PKBa, SPHK1, FAIM2, and ANGPTL4. Both PKBa and SPHK1 act as anti-apoptotic and cell survival signaling kinases, in addition to their role in regulating cellular metabolism (PKBa) and myogenesis (SPHK1) (50, 79). In cell culture, FAIM2 has an anti-apoptotic function that provides protection from Fas-mediated cell death (81). This is in parallel with previous findings that CrM supplementation has potential neuroprotective effects in a mouse model of Huntington's disease (19, 25) and cerebral ischemia (105), including buffering of intracellular energy reserves, stabilizing intracellular calcium, and inhibiting activation of the mitochondrial permeability transition pore, all of which have been linked to excitotoxic and apoptotic cell death.

### *Conclusion*

In this study, we have identified that short-term CrM supplementation in healthy young men activates genes in the skeletal muscle that are involved in various aspects of osmosensing, protein and glycogen synthesis regulation, satellite cell proliferation and differentiation, cell survival, DNA replication and repair, RNA transcription control, and cytoskeleton remodeling (Fig. 3). We propose that CrM supplementation induces rapid and coordinate induction of these regulatory proteins at the molecular level, resulting in increases in maximal muscle strength and power, FFM, total body water, and total body weight, independent of training and/or dietary intervention.

## ACKNOWLEDGMENTS

We acknowledge Dr. Mazen J. Hamadeh for gracious assistance with the preparation and critical review of the manuscript and Krysta Felkey for technical assistance in uploading the cDNA microarray data to the Gene Expression Omnibus repository.

## GRANTS

During this work, M. A. Tarnopolsky was supported by Iovate and the Natural Sciences and Engineering Research Council of Canada. Partial support came from the Hamilton Health Science Corporation, Department of Physical Medicine and Rehabilitation.

## REFERENCES

1. **al-Habori M, Peak M, Thomas TH, Agius L.** The role of cell swelling in the stimulation of glycogen synthesis by insulin. *Biochem J* 282: 789–796, 1992.
2. **Balsom PD, Harridge SD, Soderlund K, Sjodin B, Ekblom B.** Creatine supplementation per se does not enhance endurance exercise performance. *Acta Physiol Scand* 149: 521–523, 1993.
3. **Balsom PD, Soderlund K, Sjodin B, Ekblom B.** Skeletal muscle metabolism during short duration high-intensity exercise: influence of creatine supplementation. *Acta Physiol Scand* 154: 303–310, 1995.
4. **Barnett C, Hinds M, Jenkins DG.** Effects of oral creatine supplementation on multiple sprint cycle performance. *Aust J Sci Med Sport* 28: 35–39, 1996.
5. **Bastepe M, Gunes Y, Perez-Villamil B, Hunzelman J, Weinstein LS, Juppner H.** Receptor-mediated adenylyl cyclase activation through XLalpha(s), the extra-large variant of the stimulatory G protein alpha-subunit. *Mol Endocrinol* 16: 1912–1919, 2002.
6. **Berneis K, Ninnis R, Haussinger D, Keller U.** Effects of hyper- and hypoosmolality on whole body protein and glucose kinetics in humans. *Am J Physiol Endocrinol Metab* 276: E188–E195, 1999.
7. **Birch R, Noble D, Greenhaff PL.** The influence of dietary creatine supplementation on performance during repeated bouts of maximal isokinetic cycling in man. *Eur J Appl Physiol Occup Physiol* 69: 268–276, 1994.
8. **Bosco C, Tihanyi J, Pucspk J, Kovacs I, Gabossy A, Colli R, Pulvirenti G, Tranquilli C, Foti C, Viru M, Viru A.** Effect of oral creatine supplementation on jumping and running performance. *Int J Sports Med* 18: 369–372, 1997.
9. **Brewer GJ, Wallimann TW.** Protective effect of the energy precursor creatine against toxicity of glutamate and beta-amyloid in rat hippocampal neurons. *J Neurochem* 74: 1968–1978, 2000.
10. **Brose A, Parise G, Tarnopolsky MA.** Creatine supplementation enhances isometric strength and body composition improvements following strength exercise training in older adults. *J Gerontol A Biol Sci Med Sci* 58: 11–19, 2003.
11. **Burke DG, Chilibeck PD, Parise G, Tarnopolsky MA, Candow DG.** Effect of alpha-lipoic acid combined with creatine monohydrate on

human skeletal muscle creatine and phosphagen concentration. *Int J Sport Nutr Exerc Metab* 13: 294–302, 2003.
12. **Chen M, Gavrilova O, Liu J, Xie T, Deng C, Nguyen AT, Nackers LM, Lorenzo J, Shen L, Weinstein LS.** Alternative Gnas gene products have opposite effects on glucose and lipid metabolism. *Proc Natl Acad Sci USA* 102: 7386–7391, 2005.
13. **Chetlin RD, Gutmann L, Tarnopolsky MA, Ullrich IH, Yeater RA.** Resistance training exercise and creatine in patients with Charcot-Marie-Tooth disease. *Muscle Nerve* 30: 69–76, 2004.
14. **Cuenda A, Cohen P.** Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J Biol Chem* 274: 4341–4346, 1999.
15. **Dailey L, Caddle MS, Heintz N, Heintz NH.** Purification of RIP60 and RIP100, mammalian proteins with origin-specific DNA-binding and ATP-dependent DNA helicase activities. *Mol Cell Biol* 10: 6225–6235, 1990.
16. **Dangott B, Schultz E, Mozdziak PE.** Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. *Int J Sports Med* 21: 13–16, 2000.
17. **Danieli-Betto D, Germinario E, Esposito A, Megighian A, Midrio M, Ravara B, Damiani E, Libera LD, Sabbadini RA, Betto R.** Sphingosine 1-phosphate protects mouse extensor digitorum longus skeletal muscle during fatigue. *Am J Physiol Cell Physiol* 288: C1367–C1373, 2005.
18. **Dawson B, Cutler M, Moody A, Lawrence S, Goodman C, Randall N.** Effects of oral creatine loading on single and repeated maximal short sprints. *Aust J Sci Med Sport* 27: 56–61, 1995.
19. **Dedeoglu A, Kubilus JK, Yang L, Ferrante KL, Hersch SM, Beal MF, Ferrante RJ.** Creatine therapy provides neuroprotection after onset of clinical symptoms in Huntington's disease transgenic mice. *J Neurochem* 85: 1359–1367, 2003.
20. **Dobner T, Wolf I, Mai B, Lipp M.** A novel divergently transcribed human histone H2A/H2B gene pair. *DNA Seq* 1: 409–413, 1991.
21. **Donati C, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P.** Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J* 19: 449–451, 2005.
22. **Earnest CP, Snell PG, Rodriguez R, Almada AL, Mitchell TL.** The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition. *Acta Physiol Scand* 153: 207–209, 1995.
23. **El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, Caldecott KW.** Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. *Nature* 434: 108–113, 2005.
24. **Febbraio MA, Flanagan TR, Snow RJ, Zhao S, Carey MF.** Effect of creatine supplementation on intramuscular TCr, metabolism and performance during intermittent, supramaximal exercise in humans. *Acta Physiol Scand* 155: 387–395, 1995.
25. **Ferrante RJ, Andreassen OA, Jenkins BG, Dedeoglu A, Kuemmerle S, Kubilus JK, Kaddurah-Daouk R, Hersch SM, Beal MF.** Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci* 20: 4389–4397, 2000.
26. **Foran PG, Fletcher LM, Oatey PB, Mohammed N, Dolly JO, Tavare JM.** Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptors in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2, and/or cellubrevin. *J Biol Chem* 274: 28087–28095, 1999.
27. **Friedman SD, Shaw DW, Artru AA, Richards TL, Gardner J, Dawson G, Posse S, Dager SR.** Regional brain chemical alterations in young children with autism spectrum disorder. *Neurology* 60: 100–107, 2003.
28. **Godwin SL, Soltoff SP.** Calcium-sensing receptor-mediated activation of phospholipase C-gamma1 is downstream of phospholipase C-beta and protein kinase C in MC3T3-E1 osteoblasts. *Bone* 30: 559–566, 2002.
29. **Greenhaff PL, Casey A, Short AH, Harris R, Soderlund K, Hultman E.** Influence of oral creatine supplementation of muscle torque during repeated bouts of maximal voluntary exercise in man. *Clin Sci (Lond)* 84: 565–571, 1993.
30. **Hajduch E, Alessi DR, Hemmings BA, Hundal HS.** Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. *Diabetes* 47: 1006–1013, 1998.
31. **Hajduch E, Litherland GJ, Hundal HS.** Protein kinase B (PKB/Akt)–a key regulator of glucose transport? *FEBS Lett* 492: 199–203, 2001.
32. **Hamadeh MJ, Devries MC, Tarnopolsky MA.** Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* 90: 3592–3599, 2005.
33. **Harris RC, Soderlund K, Hultman E.** Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci (Lond)* 83: 367–374, 1992.
34. **Haussinger D, Hallbrucker C, vom Dahl S, Lang F, Gerok W.** Cell swelling inhibits proteolysis in perfused rat liver. *Biochem J* 272: 239–242, 1990.
35. **Hendy GN, D'Souza-Li L, Yang B, Canaff L, Cole DE.** Mutations of the calcium-sensing receptor (CASR) in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. *Hum Mutat* 16: 281–296, 2000.
36. **Hespel P, Op't Eijnde B, Van Leemputte M, Urso B, Greenhaff PL, Labarque V, Dymarkowski S, Van Hecke P, Richter EA.** Oral creatine supplementation facilitates the rehabilitation of disuse atrophy and alters the expression of muscle myogenic factors in humans. *J Physiol* 536: 625–633, 2001.
37. **Hu E, Zhu Y, Fredrickson T, Barnes M, Kelsell D, Beeley L, Brooks D.** Tissue restricted expression of two human Frzbs in preadipocytes and pancreas. *Biochem Biophys Res Commun* 247: 287–293, 1998.
38. **Hu Q, Maity SN.** Stable expression of a dominant negative mutant of CCAAT binding factor/NF-Y in mouse fibroblast cells resulting in retardation of cell growth and inhibition of transcription of various cellular genes. *J Biol Chem* 275: 4435–4444, 2000.
39. **Hultman E, Soderlund K, Timmons JA, Cederblad G, Greenhaff PL.** Muscle creatine loading in men. *J Appl Physiol* 81: 232–237, 1996.
40. **Imamura Y, Scott IC, Greenspan DS.** The pro-alpha3(V) collagen chain. Complete primary structure, expression domains in adult and developing tissues, and comparison to the structures and expression domains of the other types V and XI procollagen chains. *J Biol Chem* 275: 8749–8759, 2000.
41. **Ingwall JS.** Creatine and the control of muscle-specific protein synthesis in cardiac and skeletal muscle. *Circ Res* 38: I115–I123, 1976.
42. **Ju JS, Smith JL, Oppelt PJ, Fisher JS.** Creatine feeding increases GLUT4 expression in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 288: E347–E352, 2005.
43. **Juhn MS, Tarnopolsky M.** Oral creatine supplementation and athletic performance: a critical review. *Clin J Sport Med* 8: 286–297, 1998.
44. **Klivenyi P, Ferrante RJ, Matthews RT, Bogdanov MB, Klein AM, Andreassen OA, Mueller G, Wermer M, Kaddurah-Daouk R, Beal MF.** Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 5: 347–350, 1999.
45. **Kraemer WJ, Volek JS.** Creatine supplementation. Its role in human performance. *Clin Sports Med* 18: 651–666, ix, 1999.
46. **Kreider RB, Ferreira M, Wilson M, Grindstaff P, Plisk S, Reinardy J, Cantler E, Almada AL.** Effects of creatine supplementation on body composition, strength, and sprint performance. *Med Sci Sports Exerc* 30: 73–82, 1998.
47. **Kutz MR, Gunter MJ.** Creatine monohydrate supplementation on body weight and percent body fat. *J Strength Cond Res* 17: 817–821, 2003.
48. **Lai MC, Kuo HW, Chang WC, Tarn WY.** A novel splicing regulator shares a nuclear import pathway with SR proteins. *EMBO J* 22: 1359–1369, 2003.
49. **Lechner C, Zahalka MA, Giot JF, Moller NP, Ullrich A.** ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc Natl Acad Sci USA* 93: 4355–4359, 1996.
50. **Leclercq TM, Pitson SM.** Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* 58: 467–472, 2006.
51. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* 25: 402–408, 2001.
52. **Luo HR, Moreau GA, Levin N, Moore MJ.** The human Prp8 protein is a component of both U2- and U12-dependent spliceosomes. *RNA* 5: 893–908, 1999.
53. **Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, Tarnopolsky MA.** Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226–231, 2004.

54. **Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA.** Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *FASEB J* 19: 1498–1500, 2005.
55. **Matthews RT, Ferrante RJ, Klivenyi P, Yang L, Klein AM, Mueller G, Kaddurah-Daouk R, Beal MF.** Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Exp Neurol* 157: 142–149, 1999.
56. **Matthews RT, Yang L, Jenkins BG, Ferrante RJ, Rosen BR, Kaddurah-Daouk R, Beal MF.** Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J Neurosci* 18: 156–163, 1998.
57. **McNabb DS, Xing Y, Guarente L.** Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding. *Genes Dev* 9: 47–58, 1995.
58. **Meacci E, Donati C, Farnararo M, Bruni P.** Sphingosine 1-phosphate signal transduction in muscle cells. *Ital J Biochem* 52: 25–27, 2003.
59. **Mihic S, MacDonald JR, McKenzie S, Tarnopolsky MA.** Acute creatine loading increases fat-free mass, but does not affect blood pressure, plasma creatinine, or CK activity in men and women. *Med Sci Sports Exerc* 32: 291–296, 2000.
60. **Mujika I, Chatard JC, Lacoste L, Barale F, Geyssant A.** Creatine supplementation does not improve sprint performance in competitive swimmers. *Med Sci Sports Exerc* 28: 1435–1441, 1996.
61. **Niisato N, Post M, Van Driessche W, Marunaka Y.** Cell swelling activates stress-activated protein kinases, p38 MAP kinase and JNK, in renal epithelial A6 cells. *Biochem Biophys Res Commun* 266: 547–550, 1999.
62. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, Gustafsson T.** PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189–194, 2004.
63. **Olsen S, Aagaard P, Kadi F, Tufekovic G, Verney J, Olesen JL, Suetta C, Kjaer M.** Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training. *J Physiol* 573: 525–534, 2006.
64. **Op't Eijnde B, Richter EA, Henquin JC, Kiens B, Hespel P.** Effect of creatine supplementation on creatine and glycogen content in rat skeletal muscle. *Acta Physiol Scand* 171: 169–176, 2001.
65. **Op't Eijnde B, Urso B, Richter EA, Greenhaff PL, Hespel P.** Effect of oral creatine supplementation on human muscle GLUT4 protein content after immobilization. *Diabetes* 50: 18–23, 2001.
66. **Parise G, Mihic S, MacLennan D, Yarasheski KE, Tarnopolsky MA.** Effects of acute creatine monohydrate supplementation on leucine kinetics and mixed-muscle protein synthesis. *J Appl Physiol* 91: 1041–1047, 2001.
67. **Pasantes-Morales H, Lezama RA, Ramos-Mandujano G, Tuz KL.** Mechanisms of cell volume regulation in hypo-osmolality. *Am J Med* 119: S4–S11, 2006.
68. **Pedersen SF, Hoffmann EK, Mills JW.** The cytoskeleton and cell volume regulation. *Comp Biochem Physiol A Mol Integr Physiol* 130: 385–399, 2001.
69. **Pelech S, Sutter C, Zhang H.** Kinetworks protein kinase multiblot analysis. *Methods Mol Biol* 218: 99–111, 2003.
70. **Prudhomme W, Daley GQ, Zandstra P, Lauffenburger DA.** Multivariate proteomic analysis of murine embryonic stem cell self-renewal versus differentiation signaling. *Proc Natl Acad Sci USA* 101: 2900–2905, 2004.
71. **Raymond AC, Rideout MC, Staker B, Hjerrild K, Burgin AB Jr.** Analysis of human tyrosyl-DNA phosphodiesterase I catalytic residues. *J Mol Biol* 338: 895–906, 2004.
72. **Ritz P, Salle A, Simard G, Dumas JF, Foussard F, Malthiery Y.** Effects of changes in water compartments on physiology and metabolism. *Eur J Clin Nutr* 57, *Suppl* 2: S2–S5, 2003.
73. **Robertson SP, Twigg SR, Sutherland-Smith AJ, Biancalana V, Gorlin RJ, Horn D, Kenwrick SJ, Kim CA, Morava E, Newbury-Ecob R, Orstavik KH, Quarrell OW, Schwartz CE, Shears DJ, Suri M, Kendrick-Jones J, Wilkie AO.** Localized mutations in the gene encoding the cytoskeletal protein filamin A cause diverse malformations in humans. *Nat Genet* 33: 487–491, 2003.
74. **Rooney K, Bryson J, Phuyal J, Denyer G, Caterson I, Thompson C.** Creatine supplementation alters insulin secretion and glucose homeostasis in vivo. *Metabolism* 51: 518–522, 2002.
75. **Roy BD, de Beer J, Harvey D, Tarnopolsky MA.** Creatine monohydrate supplementation does not improve functional recovery after total knee arthroplasty. *Arch Phys Med Rehabil* 86: 1293–1298, 2005.
76. **Sabio G, Reuver S, Feijoo C, Hasegawa M, Thomas GM, Centeno F, Kuhlendahl S, Leal-Ortiz S, Goedert M, Garner C, Cuenda A.** Stress- and mitogen-induced phosphorylation of the synapse-associated protein SAP90/PSD-95 by activation of SAPK3/p38gamma and ERK1/ERK2. *Biochem J* 380: 19–30, 2004.
77. **Schliess F, Reissmann R, Reinehr R, vom Dahl S, Haussinger D.** Involvement of integrins and Src in insulin signaling toward autophagic proteolysis in rat liver. *J Biol Chem* 279: 21294–21301, 2004.
78. **Selvaraj A, Prywes R.** Megakaryoblastic leukemia-1/2, a transcriptional co-activator of serum response factor, is required for skeletal myogenic differentiation. *J Biol Chem* 278: 41977–41987, 2003.
79. **Simbula G, Columbano A, Ledda-Columbano GM, Sanna L, Deidda M, Diana A, Pibiri M.** Increased ROS generation and p53 activation in alpha-lipoic acid-induced apoptosis of hepatoma cells. *Apoptosis* 12: 113–123, 2007.
80. **Sokol DK, Dunn DW, Edwards-Brown M, Feinberg J.** Hydrogen proton magnetic resonance spectroscopy in autism: preliminary evidence of elevated choline/creatine ratio. *J Child Neurol* 17: 245–249, 2002.
81. **Somia NV, Schmitt MJ, Vetter DE, Van Antwerp D, Heinemann SF, Verma IM.** LFG: an anti-apoptotic gene that provides protection from Fas-mediated cell death. *Proc Natl Acad Sci USA* 96: 12667–12672, 1999.
82. **Squecco R, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E.** Sphingosine 1-phosphate induces myoblast differentiation through Cx43 protein expression: a role for a gap junction-dependent and -independent function. *Mol Biol Cell* 17: 4896–4910, 2006.
83. **Takashima H, Boerkoel CF, John J, Saifi GM, Salih MA, Armstrong D, Mao Y, Quiocho FA, Roa BB, Nakagawa M, Stockton DW, Lupski JR.** Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet* 32: 267–272, 2002.
84. **Tarnopolsky M, Martin J.** Creatine monohydrate increases strength in patients with neuromuscular disease. *Neurology* 52: 854–857, 1999.
85. **Tarnopolsky MA, Mahoney DJ, Vajsar J, Rodriguez C, Doherty TJ, Roy BD, Biggar D.** Creatine monohydrate enhances strength and body composition in Duchenne muscular dystrophy. *Neurology* 62: 1771–1777, 2004.
86. **Tarnopolsky MA, Parise G, Yardley NJ, Ballantyne CS, Olatinji S, Phillips SM.** Creatine-dextrose and protein-dextrose induce similar strength gains during training. *Med Sci Sports Exerc* 33: 2044–2052, 2001.
87. **Tarnopolsky MA, Raha S.** Mitochondrial myopathies: diagnosis, exercise intolerance, and treatment options. *Med Sci Sports Exerc* 37: 2086–2093, 2005.
88. **Tarnopolsky MA, Simon DK, Roy BD, Chorneyko K, Lowther SA, Johns DR, Sandhu JK, Li Y, Sikorska M.** Attenuation of free radical production and paracrystalline inclusions by creatine supplementation in a patient with a novel cytochrome b mutation. *Muscle Nerve* 29: 537–547, 2004.
89. **Terjung RL, Clarkson P, Eichner ER, Greenhaff PL, Hespel PJ, Israel RG, Kraemer WJ, Meyer RA, Spriet LL, Tarnopolsky MA, Wagenmakers AJ, Williams MH.** American College of Sports Medicine roundtable. The physiological and health effects of oral creatine supplementation. *Med Sci Sports Exerc* 32: 706–717, 2000.
90. **Thompson MA, Ziff EB.** Structure of the gene encoding peripherin, an NGF-regulated neuronal-specific type III intermediate filament protein. *Neuron* 2: 1043–1053, 1989.
91. **Vandenberghe K, Goris M, Van Hecke P, Van Leemputte M, Vangerven L, Hespel P.** Long-term creatine intake is beneficial to muscle performance during resistance training. *J Appl Physiol* 83: 2055–2063, 1997.
92. **van Loon LJ, Murphy R, Oosterlaar AM, Cameron-Smith D, Hargreaves M, Wagenmakers AJ, Snow R.** Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle. *Clin Sci (Lond)* 106: 99–106, 2004.
93. **Vierck JL, Icenoggle DL, Bucci L, Dodson MV.** The effects of ergogenic compounds on myogenic satellite cells. *Med Sci Sports Exerc* 35: 769–776, 2003.
94. **Volek JS, Duncan ND, Mazzetti SA, Staron RS, Putukian M, Gomez AL, Pearson DR, Fink WJ, Kraemer WJ.** Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. *Med Sci Sports Exerc* 31: 1147–1156, 1999.

95. **Volek JS, Kraemer WJ.** Creatine supplementation: its effect on human muscular performance and body composition. *J Strength Condit Res* 10: 200–210, 1996.
96. **Volek JS, Kraemer WJ, Bush JA, Boetes M, Incledon T, Clark KL, Lynch JM.** Creatine supplementation enhances muscular performance during high-intensity resistance exercise. *J Am Diet Assoc* 97: 765–770, 1997.
97. **vom Dahl S, Schliess F, Reissmann R, Gorg B, Weiergraber O, Kocalkova M, Dombrowski F, Haussinger D.** Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver. *J Biol Chem* 278: 27088–27095, 2003.
98. **Wassink TH, Piven J.** The molecular genetics of autism. *Curr Psychiatry Rep* 2: 170–175, 2000.
99. **Wassink TH, Piven J, Vieland VJ, Huang J, Swiderski RE, Pietila J, Braun T, Beck G, Folstein SE, Haines JL, Sheffield VC.** Evidence supporting WNT2 as an autism susceptibility gene. *Am J Med Genet* 105: 406–413, 2001.
100. **Welsh GI, Miller CM, Loughlin AJ, Price NT, Proud CG.** Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett* 421: 125–130, 1998.
101. **Wu Z, Woodring PJ, Bhakta KS, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY, Puri PL.** p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol Cell Biol* 20: 3951–3964, 2000.
102. **Young RB, Denome RM.** Effect of creatine on contents of myosin heavy chain and myosin-heavy-chain mRNA in steady-state chicken muscle-cell cultures. *Biochem J* 218: 871–876, 1984.
103. **Zhang Y, Venkatraj VS, Fischer SG, Warburton D, Chellappan SP.** Genomic cloning and chromosomal assignment of the E2F dimerization partner TFDP gene family. *Genomics* 39: 95–98, 1997.
104. **Zhang Z, Carmichael GG.** The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. *Cell* 106: 465–475, 2001.
105. **Zhu S, Li M, Figueroa BE, Liu A, Stavrovskaya IG, Pasinelli P, Beal MF, Brown RH Jr, Kristal BS, Ferrante RJ, Friedlander RM.** Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. *J Neurosci* 24: 5909–5912, 2004.
106. **Ziegenfuss TN, Lowery LM, Lemon PW.** Acute fluid volume changes in men during three days of creatine supplementation. *J Exerc Physiol* 1: 1–14, 1998.