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# FOXO1 activates glutamine synthetase gene in mouse skeletal muscles through a region downstream of 3′-UTR: possible contribution to ammonia detoxification

**Yasutomi Kamei,[1,3] Maki Hattori,[2] Yukino Hatazawa,[2,3] Tomomi Kasahara,[2] Masanobu Kanou,[2] Sayaka Kanai,[2] Xunmei Yuan,[2] Takayoshi Suganami,[1] Wouter H. Lamers,[4] Tadahiro Kitamura,[5] and Yoshihiro Ogawa[2]**

[1]*Department of Organ Network and Metabolism and* [2]*Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan;* [3]*Laboratory of Molecular Nutrition, Graduate School of Environmental and Life Science, Kyoto Prefectural University, Kyoto, Japan;* [4]*Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and* [5]*Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan*

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**Kamei Y, Hattori M, Hatazawa Y, Kasahara T, Kanou M, Kanai S, Yuan X, Suganami T, Lamers WH, Kitamura T, Ogawa Y.** FOXO1 activates glutamine synthetase gene in mouse skeletal muscles through a region downstream of 3′-UTR: possible contribution to ammonia detoxification. *Am J Physiol Endocrinol Metab* 307: E485–E493, 2014. First published July 29, 2014; doi:10.1152/ajpendo.00177.2014.—Skeletal muscle is a reservoir of energy in the form of protein, which is degraded under catabolic conditions, resulting in the formation of amino acids and ammonia as a byproduct. The expression of FOXO1, a forkhead-type transcription factor, increases during starvation and exercise. In agreement, transgenic FOXO1-Tg mice that overexpress FOXO1 in skeletal muscle exhibit muscle atrophy. The aim of this study was to examine the role of FOXO1 in amino acid metabolism. The mRNA and protein expressions of glutamine synthetase (GS) were increased in skeletal muscle of FOXO1-Tg mice. Fasting induced FOXO1 and GS expression in wild-type mice but hardly increased GS expression in muscle-specific FOXO1 knockout (FOXO1-KO) mice. Activation of FOXO1 also increased GS mRNA and protein expression in $C_2C_{12}$ myoblasts. Using a transient transfection reporter assay, we observed that FOXO1 activated the GS reporter construct. Mutation of a putative FOXO1-binding consensus sequence in the downstream genomic region of GS decreased basal and FOXO1-dependent reporter activity significantly. A chromatin immunoprecipitation assay showed that FOXO1 was recruited to the 3′ region of GS in $C_2C_{12}$ myoblasts. These results suggest that FOXO1 directly upregulates GS expression. GS is considered to mediate ammonia clearance in skeletal muscle. In agreement, an intravenous ammonia challenge increased blood ammonia concentrations to a twofold higher level in FOXO1-KO than in wild-type mice, demonstrating that the capacity for ammonia disposal correlated inversely with the expression of GS in muscle. These data indicate that FOXO1 plays a role in amino acid metabolism during protein degradation in skeletal muscle.

forkhead box protein O1; 3′-untranslated region; skeletal muscle metabolism; gene regulation; transcription factors; amino acid; ammonia

FORKHEAD BOX PROTEIN O1 (FOXO1) is a forkhead-type transcription factor with opposite effects on the anabolic insulin pathway (22, 24). FOXO1 expression is markedly upregulated during energy deprivation (17, 24). We generated transgenic mice that selectively overexpress FOXO1 in skeletal muscles (FOXO1-Tg mice); these mice exhibited muscle atrophy (16). In skeletal muscle of FOXO1-Tg mice, the expression of lysosomal protease cathepsin L was markedly upregulated (35), suggesting that FOXO1 induces protein degradation in skeletal muscle.

Skeletal muscle is the largest organ in the human body and constitutes about 40% of body weight. Furthermore, it is a reservoir of energy in the form of protein (amino acids). During starvation, structural skeletal muscle proteins such as myosin and actin are catabolized and used as energy sources for other organs (26). Microarray analysis has shown that under various conditions causing muscle atrophy, a gene that is related to amino acid metabolism, glutamine synthetase (GS), is upregulated and accompanied with increased expression of FOXO1 (20, 21). In fact, we observed increased mRNA and protein expression of GS in FOXO1-Tg mice (6). Recently, it was also reported that FOXO3, a homologue of FOXO1, activated GS expression in an in vitro cellular study (31).

The only enzyme capable of glutamine synthesis in mammals is GS (14). Skeletal muscle is considered to be an important organ for glutamine synthesis (14). GS fixes ammonia during the conversion of glutamate to glutamine. He et al. (14) showed that skeletal muscle-specific GS-knockout (KO) mice exhibit a reduced capacity to detoxify ammonia during muscular protein degradation. GS expression is activated by glucocorticoids and suppressed by insulin (2, 9, 34). Stanulović et al. (28) showed that both the 5′ upstream enhancer and downstream regulatory sequences of the 3′-untranslated region (UTR) are important for GS expression in skeletal muscle. However, the regulatory sequences that mediate the expression of GS in skeletal muscle in vivo by FOXO1 have not yet been identified.

The aim of this study was to examine the effects of FOXO1 on muscle protein and amino acid metabolism. In this study, we hypothesized that FOXO1 regulates GS expression in skeletal muscle in vivo and may affect glutamine/ammonia metabolism. Thus we examined GS expression regulation by FOXO1 in skeletal muscle and investigated its physiological significance.

Address for reprint requests and other correspondence: Y. Kamei, Laboratory of Molecular Nutrition, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522 Japan (e-mail: kamei@kpu.ac.jp).

  

## MATERIALS AND METHODS

*Genetically modified animals.* Transgenic mice overexpressing FOXO1 specifically in skeletal muscles (FOXO1-Tg mice) under the control of the human skeletal muscle α-actin promoter were generated as described previously (16). To generate skeletal muscle-specific FOXO1 knockout mice (FOXO1-KO), we inactivated FOXO1 expression in skeletal muscles by crossing mice carrying a floxed FOXO1 allele with myogenin-Cre transgenic mice (35). FOXO1-KO were back-crossed with C57BL6 and were congenic (35). We used wild-type littermates of FOXO1-Tg and FOXO1-KO mice as controls. The mice were maintained at a constant temperature of 24°C with fixed artificial light (12:12-h light-dark cycle). All animal experiments were conducted in accordance with the guidelines of and approved by the Tokyo Medical and Dental University Committee on Animal Research (No. 0090041).

*Quantitative real-time RT-PCR analysis.* Total RNA was isolated from tissue and cell homogenates using TRIzol (Life Technologies Japan, Tokyo, Japan). cDNA was synthesized from 1 μg of each RNA sample using the QuantiTect Reverse Transcription Kit (Qiagen, Tokyo, Japan). Gene expression levels were measured as described previously (30). The following primers were used: FOXO1 forward, 5′-GCGGGCTGGAAGAATTCAAT-3′; FOXO1 reverse, 5′-TCCA-

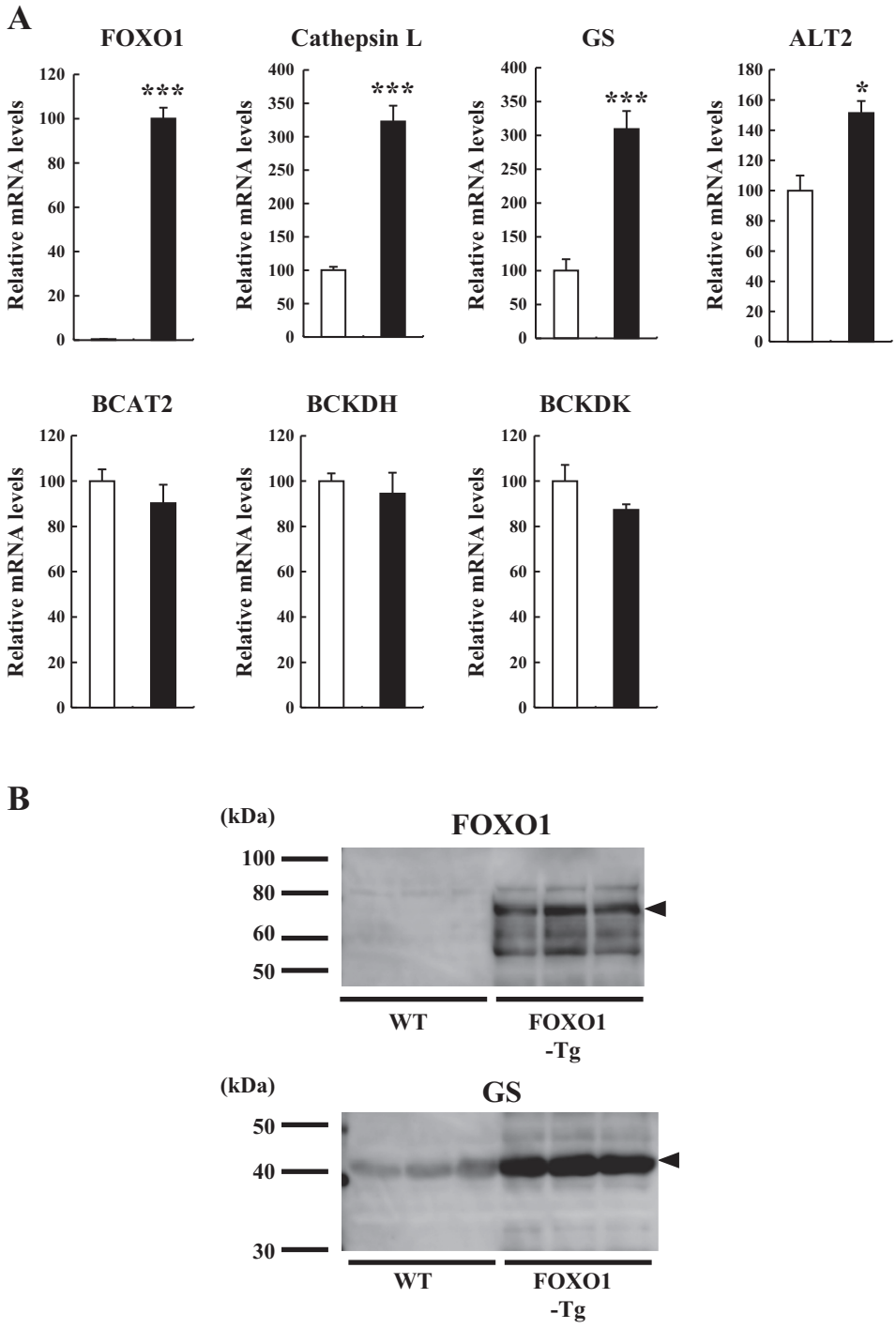


Fig. 1. Gene and protein expression in skeletal muscle of transgenic mice overexpressing forkhead box protein O1 (FOXO1) in skeletal muscles (FOXO1-Tg mice). *A*: FOXO1-Tg mice (12-wk-old males) and age- and sex-matched wild-type (WT) littermate mice were allowed ad libitum access to food. The nos. of animals used in each group were $n = 3$ (FOXO1-Tg) and $n = 4$ (WT). mRNA expressions of FOXO1, cathepsin L, glutamine synthetase (GS), alanine aminotransferase 2 (ALT2), branched-chain animo acid transferase 2 (BCAT2), branched-chain keto acid dehydrogenase (BCKDH), and branched-chain keto acid dehydrogenase kinase (BCKDK) in skeletal muscle (gastrocnemius) are shown. Open bars, WT; black bars, FOXO1-Tg (means ± SE). The data from quantitative real-time RT-PCR in WT mice were set at 100 arbitrary units. For the expression of FOXO1 transgene, the data from quantitative real-time RT-PCR in FOXO1 Tg mice, but not in WT mice, were set at 100 arbitrary units, as the endogenous FOXO1 mRNA level was very low. mRNA levels were normalized to those of 36B4 mRNA. $^{***}P < 0.001$ and $^{*}P < 0.05$ compared with the samples from WT mice. *B*: Western blot analysis. Total lysates from skeletal muscle were subjected to SDS-PAGE followed by Western blot analysis with anti-FOXO1 and anti-GS antibodies. Typical blots are shown. Molecular size marker is indicated on the *left* side of the blots. Arrowheads indicate FOXO1 transgene (*top*) and GS (*bottom*) signals. FOXO1-Tg and WT littermate mice were euthanized at 12 wk of age ($n = 3$ for FOXO1-Tg and $n = 3$ for WT mice). In these samples, we confirmed the increased mRNA expression of FOXO1 and GS, as observed in *A*.

GTTCCTTCATTCTGCA-3′; cathepsin L forward, 5′-TCTCACGCTCAAGGCAATCA-3′; cathepsin L reverse, 5′-AAGCAAAATCCATCAGGCCTC-3′; GS forward, 5′-GCTGCAAGACCCGTACCCT-3′; GS reverse, 5′-TTCCACTCAGGTAACTCTTCCACA-3′; alanine aminotransferase 2 (ALT2) forward, 5′-GAAGGAAGTAGCCGCATCCA-3′; ALT2 reverse, 5′-AGGAAAAGCTGTAGACCGTCACA-3′; branched-chain amino acid aminotransferase 2 (BCAT2) forward, 5′-TGGATCTGGCCAGGACTTGG-3′; BCAT2 reverse, 5′-TGGTAGGTATGTGGAGTTGC-3′; branched-chain keto acid dehydrogenase (BCKDH) forward, 5′-CCCAGGGATCAAGGTGGTAAT-3′; BCKDH reverse, 5′-GAAGTCCCTTGGCCTGGAA-3′; branched-chain keto acid dehydrogenase kinase (BCKDK) forward, 5′-GATCCGAATGCTGGCTACTCA-3′; BCKDK reverse, 5′-GCCAACAAAATCAGGCTTGTC-3′; 36B4 forward, 5′-GGCCCTGCACTCTCGCTTTC-3′; 36B4 reverse, 5′-TGCCAGGACGCGCTTGT-3′.

*Western blot analysis.* Western blot analysis was performed as described previously (13). Densitometric analysis was done using ImageJ software (http://rsb.info.nih.gov/ij/index.html). The following primary antibodies were used: anti-FOXO1 (sc-11350; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GS (GTX109121; Gene Tex, Irvine, CA).

*$C_2C_{12}$ cells and cell cultures.* $C_2C_{12}$ mouse myoblasts (Riken Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM), which contains 4 mM glutamine supplemented with 10% fetal bovine serum (FBS), until the cells reached confluence. $C_2C_{12}$ cells stably expressing the FOXO1-estrogen receptor (ER) fusion protein were prepared as described previously (35). In brief, $C_2C_{12}$ cells were stably transfected with either the empty pBABE retroviral vector or pBABE vectors expressing fusion proteins containing a constitutively active form of human FOXO1 in which the Akt phosphorylation sites $Thr^{24}$, $Ser^{256}$, and $Ser^{319}$ are replaced with alanine [FOXO1(3A)] in frame with a modified tamoxifen-specific version of the ligand-binding domain murine ER (35). Cells were selected with puromycin, and the colonies were pooled for analysis. Fusion proteins were restricted to the cytoplasmic compartment until activation with tamoxifen, which caused FOXO1-ER to

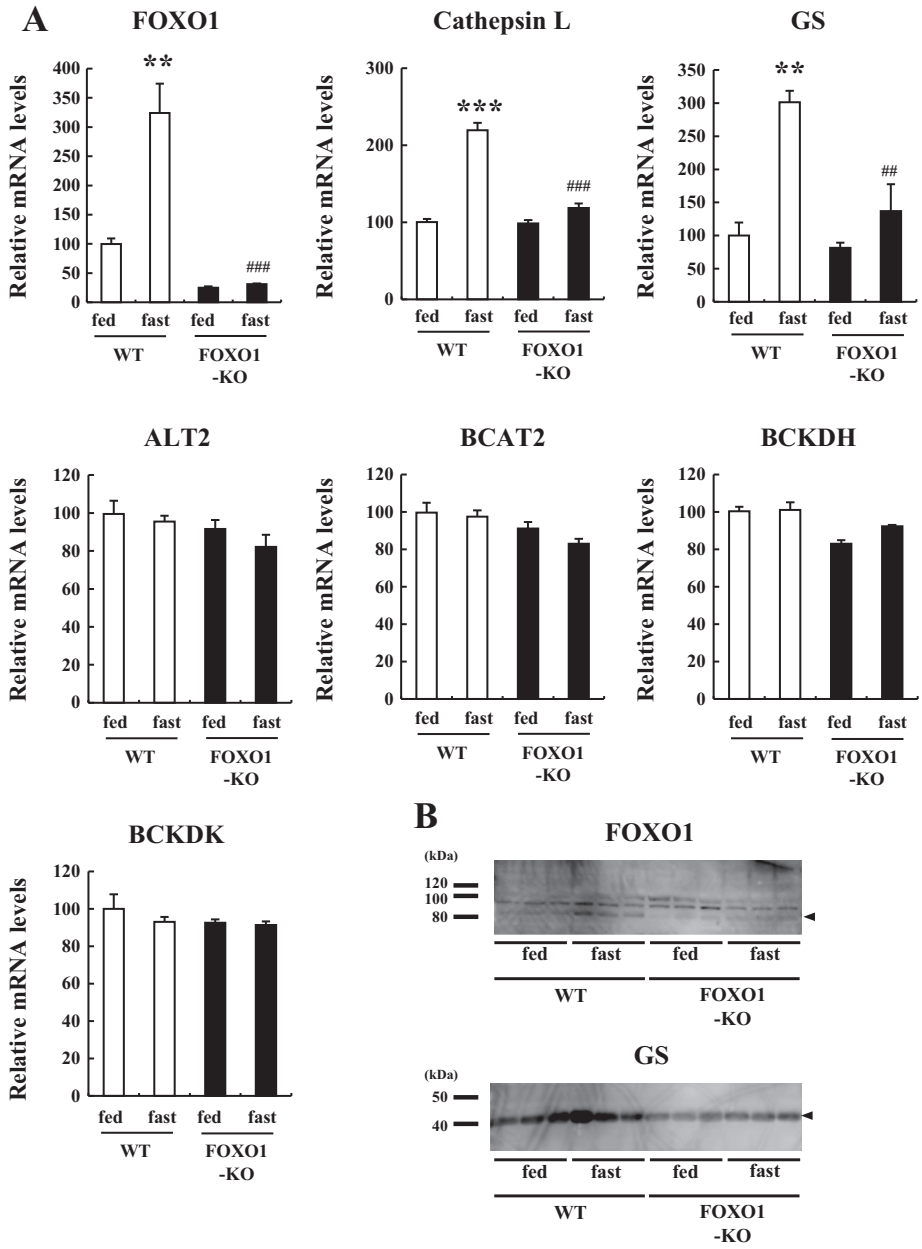


Fig. 2. Gene and protein expression in skeletal muscle of fed and fasted FOXO1-knockout (KO) mice. *A*: mice with FOXO1 deleted in skeletal muscle (FOXO1-KO mice; 18- to 22-wk-old males) and age- and sex-matched littermate WT mice were either allowed ad libitum access to food or subjected to a 24-h fast (KO fed, $n = 4$; KO fasted, $n = 4$; WT fed, $n = 3$; WT fasted, $n = 4$). Expressions of FOXO1, cathepsin L, GS, ALT2, BCAT2, BCKDH, and BCKDK in skeletal muscle (gastrocnemius) are shown. Quantitative real-time RT-PCR data from fed WT mice were set at 100 arbitrary units. mRNA levels were normalized to those of 36B4 mRNA. $***P < 0.001$ and $**P < 0.01$ relative to WT fed mice; $###P < 0.001$ and $##P < 0.01$ relative to WT fasted mice. *B*: Western blot analysis. Total lysates from skeletal muscle were subjected to SDS-PAGE followed by Western blot analysis with anti-FOXO1 and anti-GS antibodies. Molecular size marker is indicated on the *left* side of the blots. Arrowhead indicates FOXO1 (*top*) and GS (*bottom*).

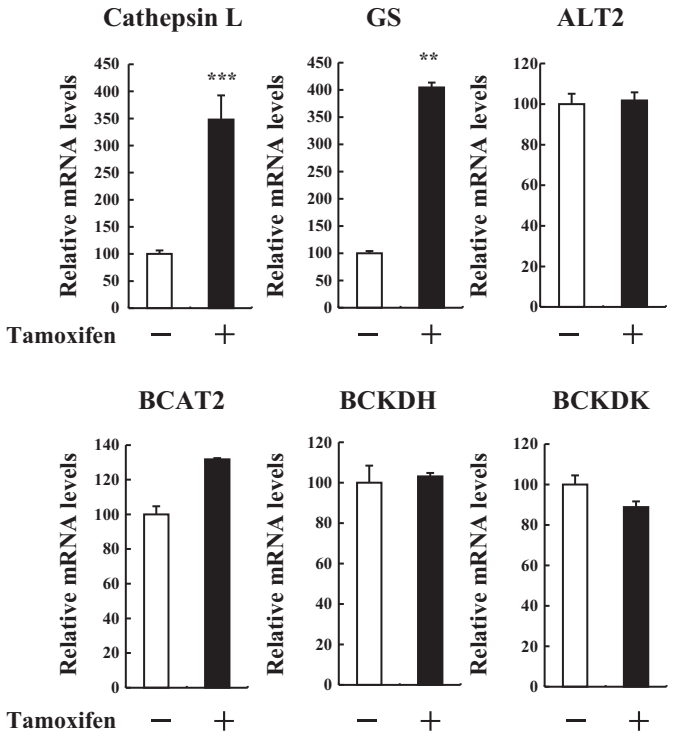


Fig. 3. Gene expression in $C_2C_{12}$ cells expressing FOXO1-ER. The abundance of mRNA transcripts of cathepsin L, GS, ALT2, BCAT2, BCKDH, and BCKDK in FOXO1-ER-expressing $C_2C_{12}$ cells in culture medium with 4 mM glutamine treated with vehicle (open bars) or tamoxifen (black bars) for 18 h was analyzed using quantitative real-time RT-PCR. mRNA levels were normalized to those of 36B4 mRNA. $***P < 0.001$ and $**P < 0.01$ compared with the samples without tamoxifen ($n = 3$; means ± SE).

relocate to the nucleus, where the FOXO1 moiety then functioned as a transcription factor (35). For Western blotting, cells at near-confluence were treated with DMEM, with or without 4 mM glutamine, supplemented with FBS for 24 h and treated with tamoxifen for another 24 h.

*Plasmid constructs.* As shown in Fig. 5, a serial set of reporter plasmids was constructed. Figure 5, *top*, shows mouse genomic DNA (GS gene). The constructs included a 3.4-kb genomic promoter region and the first exon (−3,397 to +140, from the transcription start site), the luciferase reporter gene, the GS transcription termination and polyadenylation signal (3′-UTR), and the immediate downstream sequence (GS tail). The longest construct contained the 5′ promoter region (−3,397 to +140) and GS tail (3′-UTR of 1,531 bp and GS tail of 526 bp). We constructed various deletion constructs from the longest plasmid, as shown in Fig. 5. In addition, the consensus FOXO1-binding sequence GTAAACAA [daf16-binding element (DBE)] (11) in the 3′ GS tail was mutated to GTGGGCAA (*bottom* construct in Fig. 5).

*Transfection and luciferase assay.* $C_2C_{12}$ cells were plated at a density of $1 \times 10^5$ cells/well in a 12-well plate in DMEM supplemented with 10% FBS. Luciferase gene constructs containing the GS promoter and 3′-UTR/GS tail fragments with or without mutations of putative FOXO1-binding elements were prepared as described above. The luciferase reporter plasmid (0.8 μg), expression plasmid [pCAG-FOXO1(3A): 0, 5, 10, or 25 ng], empty pCAG (≤0.8 μg), and phRL-TK vector (25 ng; Promega, Madison, WI) as an internal control of transfection efficiency were transfected into $C_2C_{12}$ cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were lysed and assayed for luciferase activity using the Dual Luciferase Assay kit (Promega). The activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity (internal control) and expressed as an average of triplicate experiments.

*Chromatin immunoprecipitation assay.* Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay kit (Upstate, Temecula, CA) according to the manufacturer's instructions and as described previously (35). In brief, $C_2C_{12}$ cells stably expressing FOXO1-ER were incubated for 24 h with or without 1 μM tamoxifen. Proteins were cross-linked to DNA with formaldehyde (final concentration, 1%). Cells were washed and lysed in SDS lysis buffer, sonicated for 10 s, and allowed to recover for 30 s on ice (7 cycles). Lysates were cleared using Protein A-agarose for 30 min, pelleted, and incubated overnight with an anti-FOXO1 antibody (sc-11350; Santa Cruz Biotechnology). Prior to incubation, input samples were taken from the lysate and stored at 4°C until extraction. Following incubation with the antibody, protein-DNA complexes were eluted (1% SDS in 0.1 M $NaHCO_3$), and cross-links were reversed. DNA was purified with phenol-chloroform extraction. PCR primers were designed for regions harboring putative FOXO1-binding elements in the 5′ and 3′ regulatory regions of the GS: forward 5′-GCCATCACTGCAGGGTTAAG-3′ and reverse 5′-GGACAACCAGGGTTTCACAG-3′ (the amplified region was −1,028 to −876, with the transcription start site being +1) and forward 5′-GGTGGTTCTTGTTTACGGACA-3′ and reverse 5′-CACTAGGACCCCGTCTCAAA-3′ (the amplified region was 55 to 148 bp downstream of the 3′-UTR of GS). PCR primers were also designed for GAPDH as a negative control: forward 5′-TCCTATAAATACGGACTGCAGCC-3′ and reverse 5′-ACAGGGAGGAGCAGAGAGCA-3′.

*Ammonia challenge.* Mice fasted for 16 h [wild-type (WT) and FOXO1-KO mice; $n = 3$ each] were anesthetized and equipped for $NH_4HCO_3$ infusion into the external jugular vein as described (14). A

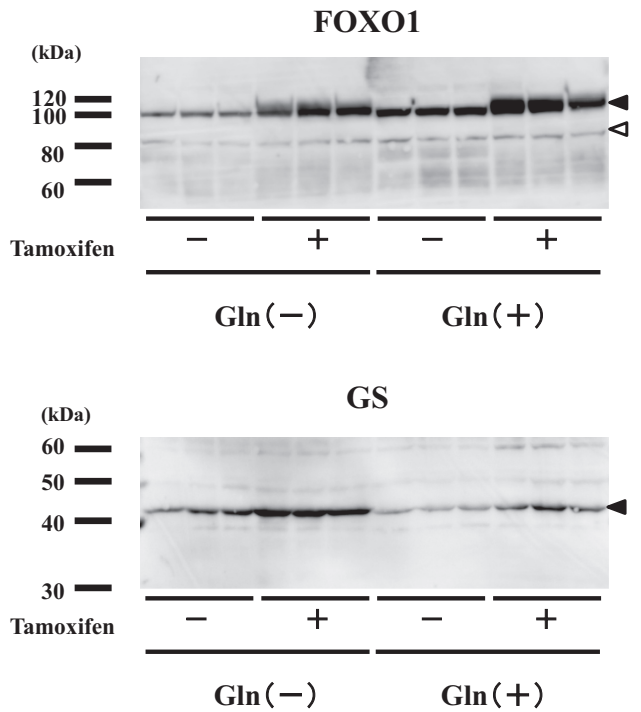


Fig. 4. Protein expression in $C_2C_{12}$ cells expressing FOXO1-ER. Total lysates from $C_2C_{12}$ were subjected to SDS-PAGE followed by Western blot analysis with anti-FOXO1 and anti-GS antibodies. Typical blots are shown. Molecular size marker is indicated on the *left* side of the blots. $C_2C_{12}$ cells in culture medium with (+) or without (−) 4 mM glutamine treated with vehicle (−) or tamoxifen (+) were analyzed ($n = 3$). In these samples, we confirmed increased mRNA expression of GS, as observed in Fig. 3. Open arrowhead indicates endogenous FOXO1; black arrowhead indicates FOXO1-ER signals (*top*).

solution containing $NH_4HCO_3$ was infused at a flow rate of 50 μmol/h, and 80 min after infusion, blood ammonia levels were examined using the Pocket Chem BA PA-4140 (Arkray, Tokyo, Japan).

*Statistical analyses.* We used Student's *t*-test and analysis of variance, followed by Scheffe's test. Data are expressed as means ± SE. $P < 0.05$ was considered significant.

## RESULTS

*Overexpression of FOXO1 changes expression of amino acid-related genes in skeletal muscle.* FOXO1 may affect amino acid metabolism. First, we examined amino acid metabolism-related gene expression in the skeletal muscles of FOXO1-Tg mice. The expression of cathepsin L and GS increased threefold in FOXO1-Tg compared with WT mice (Fig. 1*A*), whereas the expression of the branched-chain amino acid (BCAA)-metabolizing enzymes BCAT2, BCKDH, and BCKDK remained unchanged. In this experiment, the expression of ALT2 increased marginally (Fig. 1*A*). We confirmed increased protein expression of FOXO1 and GS in FOXO1-Tg mice. The protein expression of GS increased fourfold in FOXO1-Tg compared with WT mice (Fig. 1*B*). These data suggested a preferential increase in GS expression among amino acid metabolism-related genes due to the increased FOXO1 expression in skeletal muscle in FOXO1-Tg mice.

*FOXO1 deletion suppresses induction of GS expression by fasting in skeletal muscle.* In addition to a gain-of-function experiment (FOXO1-Tg mice), we performed a loss-of-function experiment to elucidate the physiological aspect of FOXO1 function in skeletal muscle. We also examined GS and other amino acid metabolism-related gene expressions in skeletal muscles of FOXO1-KO mice. Fasting induced threefold FOXO1 expression in WT mice but showed only residual expression in fed and fasted FOXO1-KO mice (Fig. 2*A*). Fasting induced 2.2-fold cathepsin L expression in WT mice but not in FOXO1-KO mice (Fig. 2*A*). Similarly, fasting increased GS expression threefold in WT mice, whereas this effect was markedly suppressed in FOXO1-KO mice (Fig. 2*A*). We observed increased twofold FOXO1 and GS protein levels by fasting WT mice. In KO mice, the FOXO1 protein level was very low, and the fasting-induced GS protein level observed in WT mice was suppressed (Fig. 2*B*). These data support the notion that GS is a target gene of FOXO1 in vivo.

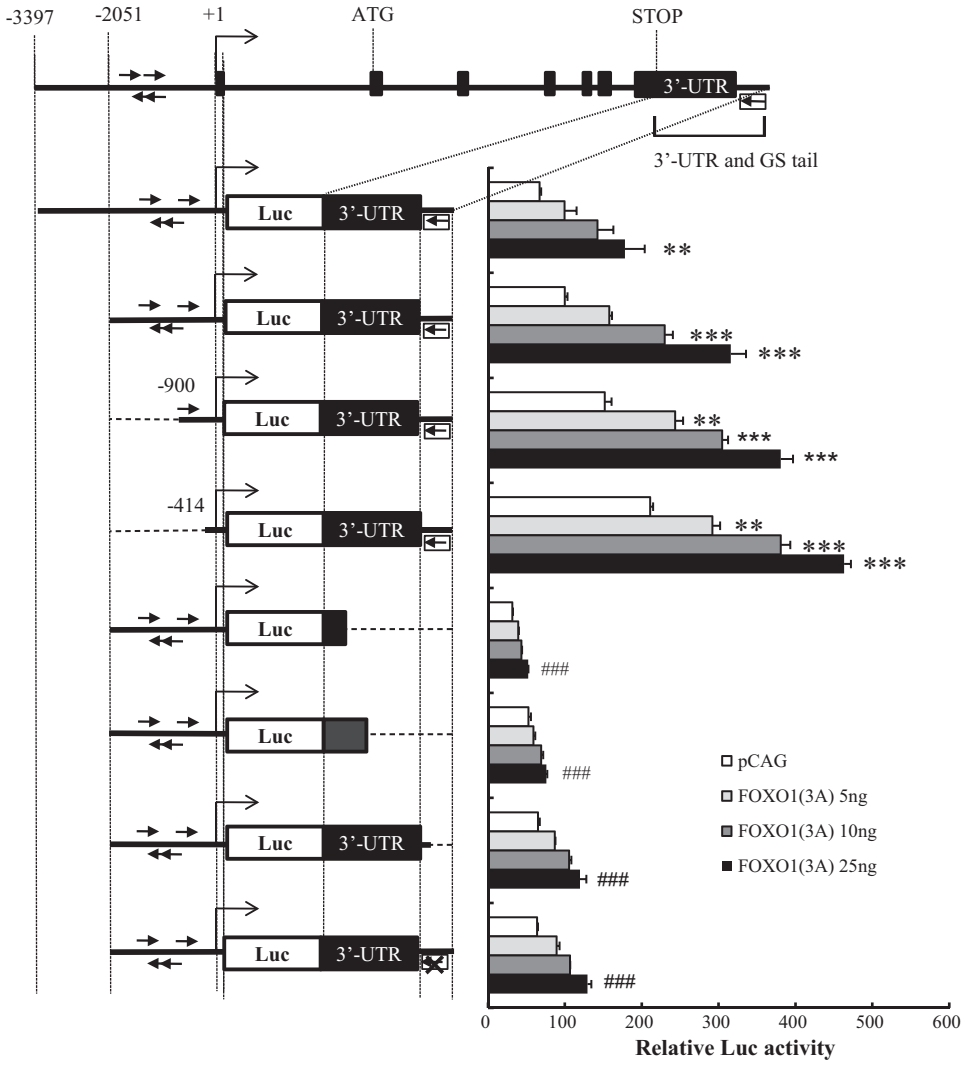


Fig. 5. Transient transfection reporter assay of the effect of FOXO1 on GS. The effect of increasing the expression of FOXO1 was examined by cotransfection with a reporter plasmid in $C_2C_{12}$ cells. *Top*: mouse genomic DNA (GS gene). Exons (filled boxes), transcription start site (+1), translation start site (ATG), and stop codon are shown. The constructs include a 3.4-kb genomic promoter region and the first exon (−3,397 to +140 from the transcription start site), the luciferase reporter gene, the GS transcription termination and polyadenylation signal [3′-untranslated region (UTR)], and the immediate downstream sequence (GS tail). A schematic representation of GS constructs is shown to the *left* of the histograms. Arrows indicate putative FOXO1-binding element (AAACAA or TTGTTT) (1, 8, 10, 18, 27). Boxed arrow is a putative FOXO1-binding element (TTGTTTAC), namely, daf16-binding element (DBE) (11). The DBE in the 3′ GS tail was mutated to GTGGGCAA (*bottom* construct). ***$P < 0.001$ and **$P < 0.01$ compared with the samples in the absence of FOXO1 expression vector; ###$P < 0.001$ compared with the samples of construct (−2,051 to +140 and 3′ UTR/GS tail). The data from the samples without FOXO1 expression vector with the construct (−2,051 to +140 and 3′ UTR/GS tail) were set at 100 arbitrary units.

*Activated FOXO1 induces GS expression in $C_2C_{12}$ cells.* We next examined FOXO1-dependent GS expression in mouse $C_2C_{12}$ cells expressing the tamoxifen-inducible fusion protein FOXO1-ER (35). Activated FOXO1-ER translocated to the nucleus and induced the expression of FOXO1 target genes. Endogenous FOXO1 mRNA was very low in $C_2C_{12}$ cells. As expected, treatment with tamoxifen did not change FOXO1 mRNA levels [the sum of endogenous FOXO1 mRNA and retrovirus-derived FOXO1(3A)-ER mRNA; data not shown]. In the presence of tamoxifen, cathepsin L mRNA expression was increased 3.5-fold and GS mRNA expression fourfold, suggesting that FOXO1 increased GS expression directly in muscle cells (Fig. 3). However, the mRNA expression of BCAA-metabolizing enzymes and ALT2 remained unchanged (Fig. 3). Then, we analyzed the GS protein level in the cells. The GS protein level is known to be downregulated by glutamine in the medium (15, 34). Thus, we performed experiments in both the presence and absence of glutamine in the medium. Endogenous FOXO1 protein levels were similar in all samples (Fig. 4). Tamoxifen treatment increased the FOXO1-ER fusion protein level in this experiment. Glutamine suppressed the GS level (0.5-fold), which is consistent with previous studies (15, 34). In the presence of tamoxifen, GS protein levels were increased twofold both in glutamine-free and glutamine-containing medium (Fig. 4). Thus, FOXO1 upregulated GS mRNA and the protein level in cells. These data show that the expression of GS is regulated similarly in vivo and in $C_2C_{12}$ cells.

*FOXO1 enhances transcriptional activity of GS via the 3′ region of the gene.* Because the GS gene appeared to be a direct transcriptional target of FOXO1 in myocytes in vivo and in vitro, we tested this hypothesis using a transient transfection reporter assay. Both the 5′ promoter region and 3′-UTR/GS tail are important for full gene activity in the rat GS gene (28). In the in vitro transfection assay, FOXO1 dose-dependently activated the rat GS reporter construct (data not shown). Therefore, we tested a Luc reporter construct containing the 5′ region (promoter) and 3′-UTR/GS-tail from mouse GS. FOXO1 expression vector enhanced Luc activity of mouse GS in a similar dose-dependent manner to that observed for the rat gene (data not shown). Then, we constructed a series of deletion mutants (Fig. 5). Deletion of the 5′ promoter region did not affect FOXO1-induced Luc activity. In contrast, deletion of the 3′ region (3′-UTR/GS tail) markedly decreased basal and FOXO1-dependent Luc activity. Mutation of a putative FOXO1-binding element [GTAAACAA; called DBE (11)] in the 3′ region markedly suppressed FOXO1-dependent Luc activity, suggesting that FOXO1 acts on the 3′ region of GS.

*FOXO1 is recruited to the 3′ region of the GS gene.* We also performed ChIP analysis using $C_2C_{12}$ cells expressing FOXO1-ER (Figs. 3 and 4) and found that FOXO1 was recruited to the 3′ region of mouse GS, which encompasses the DBE, but only if FOXO1 was activated by the presence of tamoxifen (Fig. 6). No binding to putative FOXO1-binding elements (AAACAA or TTGTTT) (1, 8, 10, 18, 27) was found in the 5′ region (promoter) of GS (Fig. 6). The above observations suggest that GS is a direct target of FOXO1 in skeletal muscles and that FOXO1 upregulates GS expression through the 3′-UTR/GS tail.

*Physiological significance of FOXO1-mediated increase in GS expression in skeletal muscle.* To elucidate the physiological significance of FOXO1-induced GS expression, we examined blood ammonia levels in FOXO1-KO mice after ammonia infusion. Before the ammonia injection, blood ammonia levels were similar between WT and FOXO1-KO mice. The ammonia infusion increased blood ammonia concentration in both WT and FOXO1-KO mice, but the concentration in FOXO1-KO mice was twofold higher than in WT mice (Fig. 7*A*). Since GS mRNA and protein expressions in muscle were lower (0.3- and 0.5-fold, respectively) in FOXO1-KO than in WT mice (Fig. 7, *B* and *C*), this result suggests that the

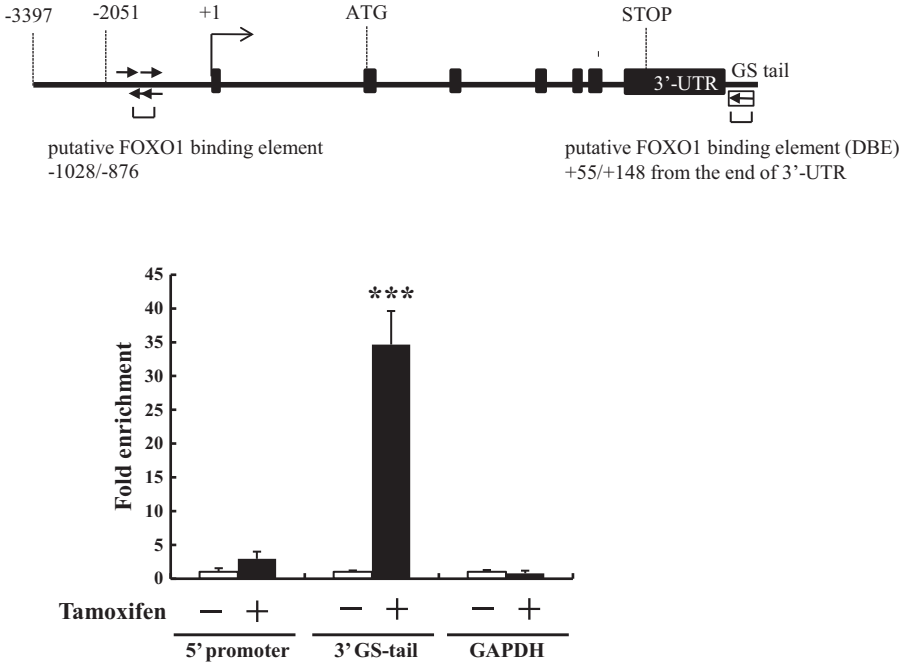


Fig. 6. Recruitment of FOXO1 to GS. Chromatin immunoprecipitation assay in $C_2C_{12}$ cells expressing FOXO1-estrogen receptor (ER) treated with vehicle (open bars) or tamoxifen (black bars) with a FOXO1 antibody. *Top*: mouse genomic DNA (GS gene). Primers specific to the regulatory regions of mouse GS were used for PCR analysis. FOXO1-ER was recruited to the 3′ region of GS when tamoxifen was added to the culture medium. No signals were detected with control IgG. Primers corresponding to the 5′ region (promoters) did not give strong signals. Primers designed for GAPDH as a negative control did not give signals. ***$P < 0.001$ compared with the samples without tamoxifen ($n = 7$; means ± SE).

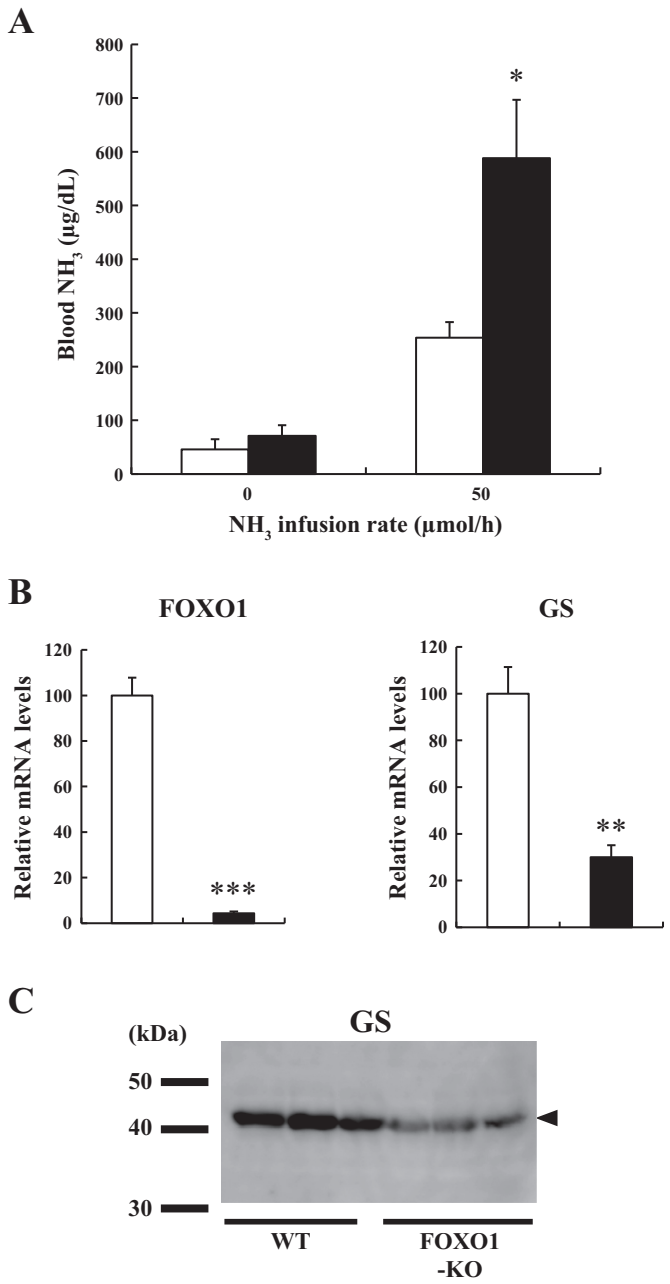


Fig. 7. Effect of $NH_4HCO_3$ infusion into the external jugular vein on circulating ammonia concentration in FOXO1-KO mice. *A*: WT (open bars; $n = 3$) and FOXO1-KO mice (black bars; $n = 3$) (means ± SE) were subjected to ammonia infusion. Ammonia levels in plasma, 80 min after infusion (flow rate of 50 μmol/h), are shown. The results represent 3 independent experiments. *B*: mRNA expression of FOXO1 and GS. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with WT mice. *C*: protein expression of GS. Immediately after the experiment in *A*, RNA and protein samples from skeletal muscle tissue were obtained and tested for FOXO1 and GS mRNA levels and GS protein level.

capacity for GS-mediated ammonia detoxification was attenuated in FOXO1-KO mice.

## DISCUSSION

Skeletal muscles account for 70% of the endogenous production of glutamine in humans (3, 25), ~15% of which arises from proteolysis and the remainder from de novo synthesis (3, 19). Glutamine is among the most abundant free amino acids in mammals (4) and can serve as an oxidative fuel for enterocytes and leukocytes, a precursor of purine and pyrimidine synthesis, a modulator of protein turnover, and an intermediate for gluconeogenesis and acid base balance as well as temporary storage of amino groups and ammonia transport (14). The only enzyme capable of glutamine synthesis is GS (14). Both FOXO1 and GS are upregulated in conditions associated with muscle atrophy (20, 21), such as fasting, diabetes, and advanced cancer (20, 21). Glutamine synthesized by GS may serve as a substrate for gluconeogenesis during fasting and diabetes (29, 32). In advanced cancer, glutamine serves as an energy source for tumor growth (7). Synthesized glutamine in skeletal muscle is released into the circulation and other organs (23). Consistently, in our study, the glutamine level was not increased in the skeletal muscle of FOXO1-Tg mice with an increased GS level (data not shown), suggesting that synthesized glutamine is secreted to other organs. Indeed, a previous study reported that during fasting the GS level was increased, but the muscle glutamine level was not increased (23). Thus, FOXO1-mediated GS upregulation appears to reflect a regulatory pathway that facilitates the role of muscle protein degradation if substrates are required for energy-requiring cells elsewhere in the body.

GS expression is known to be regulated by hormones and nutrition at mRNA (transcriptionally) and protein (posttranscriptionally and/or posttranslationally) levels. For example, glucocorticoid, a catabolic hormone, upregulates GS mRNA expression in various cells (2, 9, 34). On the other hand, insulin, an anabolic hormone, suppresses glucocorticoid-induced GS mRNA level in cells such as adipocytes (2, 34). Meanwhile, exogenous glutamine addition does not affect the GS mRNA level, but it decreases the GS protein level in cells (15, 34), which was also observed in the present study. FOXO1 is a catabolic transcriptional regulator that is known to have an opposite effect to insulin (24). In addition, it has been shown that FOXO1 and glucocorticoid receptor (GR) act synergistically in muscle (33). Thus, increased GS mRNA and protein expression by increased FOXO1 are consistent with the known direction of the physiological regulation of GS (increased by glucocorticoid and suppressed by insulin). Namely, FOXO1 may coactivate GR and increase the GS mRNA level. In the insulin/FOXO1 relationship, insulin-mediated kinase signaling phosphorylates and degrades FOXO1 protein (24). Thus, the insulin-mediated suppressed GS mRNA level may be involved in this process, which remains to be clarified. In this study, FOXO1 regulated GS at the mRNA level, and the in vitro reporter assay and ChIP assay indicated that FOXO1 regulates GS transcriptional activity. Thus, GS protein changed by glutamine is likely a differently regulated mechanism from the FOXO1 pathway.

Recently, van der Vos et al. (31), using a similar approach to our present study, demonstrated that a fusion protein of FOXO3a and ER also activates GS expression transcriptionally. In contrast to our finding that FOXO1 mediates its effect on GS expression in $C_2C_{12}$ muscle cells via the GS tail, van der Vos et al. (31) reported that FOXO3a regulates GS expression in human embryonic kidney-293 cells via the promoter region. Apart from the use of different cell types, the reason for this discrepancy is not clear at present. Although the issue of where

FOXO transcription factors interact with the GS gene may need additional study, our present study demonstrated clearly that FOXO1 regulates GS gene expression in skeletal muscle in vivo, in FOXO1-overexpressing and -depleted mice, and in vitro.

In this study, we observed a decreased capacity to detoxify ammonia concurrent with decreased GS expression in FOXO1-KO mice. This observation is consistent with the earlier finding that the capacity for ammonia detoxification decreases in skeletal muscle-specific GS-KO mice (14). Expressions of both FOXO1 and GS are increased during muscle degradation in various experimental models of muscle atrophy (20, 21). The induction of GS expression by FOXO1 shown here suggests that FOXO1 signaling may facilitate ammonia detoxification during skeletal muscle degradation. Conversely, the GS expression and ammonia detoxification capacity of muscle increased in a rat model of acute liver failure (ligation of the hepatic artery) with elevated blood ammonia levels (5, 19). We generated a liver failure model by injecting mice with α-naphthylisothiocyanate (12) and also observed a marked increase in FOXO1 and GS expression in skeletal muscles (data not shown), again suggesting that a FOXO1-mediated increase in GS expression may enhance the ammonia-detoxifying capacity of skeletal muscle.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Y.K., T.S., T. Kitamura, and Y.O. conception and design of research; Y.K., M.H., Y.H., T. Kasahara, M.K., S.K., and X.Y. performed experiments; Y.K., M.H., Y.H., T. Kasahara, M.K., S.K., and X.Y. analyzed data; Y.K., M.K., X.Y., T.S., W.H.L., T. Kitamura, and Y.O. interpreted results of experiments; Y.K. and W.H.L. drafted manuscript; Y.K., M.H., Y.H., T. Kasahara, M.K., S.K., X.Y., T.S., W.H.L., T. Kitamura, and Y.O. approved final version of manuscript; Y.H. prepared figures.

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