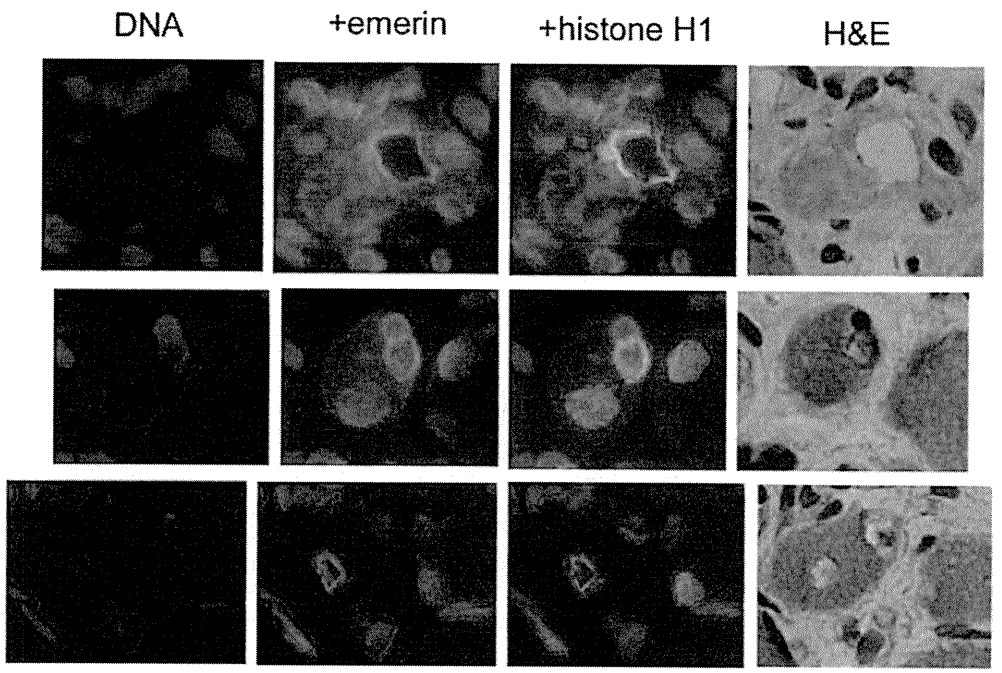

Fig. 4. Triple fluorescence studies in s-IBM vacuolated fibers. Blue: nuclear DNA; Green: emerin; Red: histone H1. Vacuoles usually appear to be more accentuated in H&E, probably due to dehydration process. The background green colour is purposely heightened to visualize muscle fibers.

releases histone H1 into the cytoplasm (Konishi et al 2003). Therefore, the cytoplasmic H1 release in s-IBM might indicate that some apoptotic stimuli causing DNA double strand breaks induce the s-IBM pathology. Apoptotic process exemplified by TUNEL revealed that it may scarcely operate in s-IBM muscle fibers (Hutchinson 1998). Nevertheless, several studies displayed some players of apoptosis in s-IBM muscle fibers (Behrens et al 1997, Li and Dalakas 2000b)

7. DNA double strand breaks (DSB) in s-IBM

7.1 The DNA damage responses

The primary structure of DNA is constantly exposed to cellular metabolites and extracellular DNA-damaging agents. These alterations can affect the cell to transcription of the genes. Other lesions induce potentially harmful mutations. Consequently, the DNA repair process must be constantly activated to respond to the damages in the DNA structure. Defects of the repair processes may cause genomic instability. To repair damage to one of the two paired molecules of DNA, there are many excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand. The examples of these are base excision repair, nucleotide excision repair, and DNA mismatch repair.

DNA double strand breaks (DSB), in which both strands in the double helix are severed, are particularly serious to the cell because they can lead to genome rearrangements. DSB are produced by reactive oxygen species, ionizing radiation, chemicals that generate reactive oxygen species and replication error. DSB are also a normal result of V(D)J recombination and immunoglobulin class-switching process. DSB are repaired either by homologous recombination (HR) or nonhomologous end-joining (NHEJ) mechanism (O'Driscol and Jeggo 2006). HR plays only in replicating cells, while NHEJ functions in both cells in the cell cycle and those terminally differentiated. Mature muscle cells are terminally differentiated cells, that is, the cells withdraw from the cell replication cycle. Terminally differentiated cells do not possess a replication-associated DNA repair mechanism (HR mentioned above). This lack makes the terminally differentiated cells particularly sensitive to DNA damage (Lee and McKinnon 2007). In a muscle cell culture study, the exposure of differentiated myocytes to hydrogen peroxide, which induces reactive oxygen species, resulted in the accumulation of foci of DSB. It is exemplified by immunolocalization of phosphorylated histone H2AX (γ-H2AX) (Narciso et al 2007). The detection of γ-H2AX is a sensitive marker of DSB (Nakamura 2006). Histone H2AX that is a variant of histone H2A is rapidly phosphorylated at Ser 139 in the chromatin region surrounding a DSB (Kinner 2008). Immunocytochemical staining of γ-H2AX has been broadly applied to reveal DNA damage caused by cancer and other cellular stresses (Nakamura 2006, Kinner 2008).

DSB is different from the apoptotic DNA fragmentation that has been residually detected in the s-IBM muscles (Hutchinson 1998). In DSB, DNA breaks are induced directly and randomly by radiation or other genotoxic agents, whereas apoptotic DNA fragmentation occurs at a late stage of programmed cell death, when endonucleases sever DNA strands at regular lengths, making a ladder formation in Southern blotting.

DNA-PK is an enzyme involved in the initial step of the DSB repair process NHEJ, which does not require DNA replication, and therefore NHEJ is the major DNA repair mechanism in terminally differentiated cells (O'Driscoll and Jeggo 2006, Mahaney et al 2009). DNA-PK consists of a catalytic subunit (DNA-PKcs) and two regulatory subunits (Ku70 and Ku80). The binding of hetero-duplexes of Ku70 and Ku80 to DSB sites initiates the repair process (Mari et al 2006, Weterings and Chen 2007).

We immunolocalized γ-H2AX in s-IBM and we also tested DNA-PK to see whether the repair mechanism is defective or not (Nishii et al 2011). In the study, vacuolar peripheries often showed strong immunoreactivity to γ-H2AX and the three components of DNA-PK (DNA-PKcs, Ku70, and Ku80). The percentage of positive nuclei for γ-H2AX was significantly higher in vacuolated fibers than non-vacuolated fibers in s-IBM, or fibers in polymyosits suggesting that nuclear breakdown occurs along with the accumulation of DSB in muscle cells in s-IBM. Moreover, a triple fluorescence study of Ku70, emerin, and DNA suggested impaired nuclear incorporation of Ku70. Nuclear translocation of Ku proteins is important for DBS repair, and a deficiency in nuclear translocation caused hypersensitivity against X-ray irradiation due to the lack of DBS repair in a cell culture study (Okui et al 2002). Therefore, we hypothesized that defects in Ku70 nuclear import accelerate DSB formation in s-IBM.

Despite DSB was the highest in s-IBM vacuolated fibers, DSB was sometimes found to be increased in myonuclei without nuclear breakdown. Therefore, additional factors may be involved in the nuclear breakdown detected in s-IBM. We consider that a dysfunction of nuclear envelope may explain all the alterations in s-IBM: 1) nuclear fragility; 2) DNA double-strand breaks; and 3) impaired nuclear transport in s-IBM.

Impaired DSB results growth arrest, senescence, and apoptosis (Rossetto et al 2010). Our earlier examination showed aberrant expressions of proteins associated with myogenic differentiation. In s-IBM, the accumulation of DSB could result in arrest of muscle fiber maturation.

8. Possible mechanism of nuclear breakdown

Nuclear envelope dysfunction can cause both mechanical fragility of the nucleus and DNA damage. Lamins are proteins of nuclear intermediate filaments that comprise the lamina, the meshwork supporting inner nuclear membranes. Mutations in the genes that encode lamins and emerin cause Emery-Dreifuss muscular dystrophy and a number of different diseases collectively called laminopathies (Capell and Collins 2006). In several laminopathies, blebbing of the nuclei in cultured fibroblasts can be seen, and it is hypothesized that such mutations result in fragile and mechanically unstable nuclei (Goldman et al 2004). Indeed, emerin mutations can cause myopathy with rimmed vacuoles (Paradas et al 2005, Fidziańska et al 2004). Besides structural integrity, the lamina is also involved in various other processes, such as replication and gene transcription, which are intimately associated with DNA damage repair. Accordingly, impaired DNA repair has been found in several laminopathies. Fibroblasts possessing a laminopathy mutation show an excessive amount of un-repaired DNA damage, as exemplified by γ-H2AX immunohistochemistry (Liu et al 2005). 3) Furthermore, lamins are important in the spatial rearrangement of nuclear pore complexes and therefore nuclear protein transport. Nuclear protein import is reduced in cells expressing lamin A mutants (Busch et al 2009). We repeatedly detected figures suggestive of impaired nuclear import of proteins, as has been described in our phosphorylated protein study.

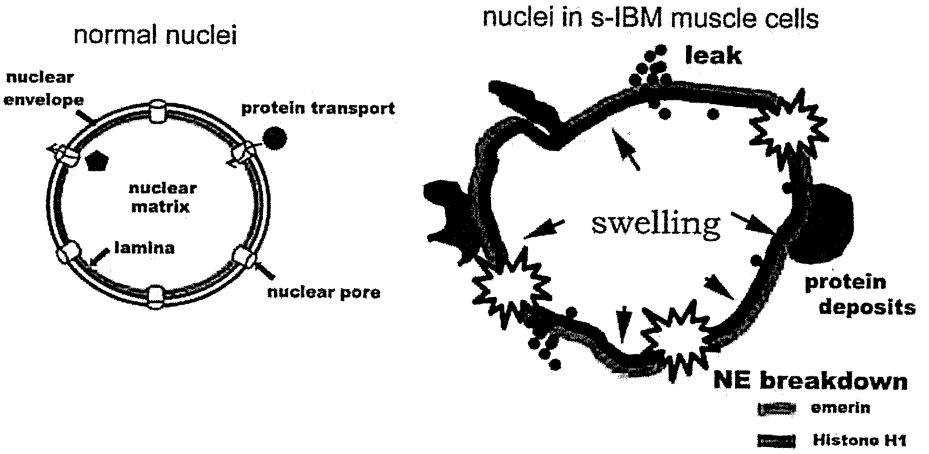

Fig. 5. Simplified schema of normal nuclei and nuclei in s-IBM muscle fibers. In s-IBM, nuclear proteins deposits occur in perinuclear regions due to inhibition of nuclear import, whereas histone H1 is released from nnclei. Finally, the nuclear envelopes break down to form rimmed vacuoles.

To summarize, dysfunctional lamins can explain the nuclear breakdown, accumulation of DSB, and impaired nuclear transport observed in s-IBM. As discussed in the section about perinuclear deposition of protein kinases, autoimmune mechanism could operate in the dysfunction of nuclear envelope. In addition, aging might increase the nuclear vulnerability and DNA damage. Nuclear pore complexes are not turned over in differentiated cells, and age-related alterations in nuclear pore complexes have been shown. Leaking of nuclear matrix proteins is dramatically accelerated during aging and that a subset of nucleoporins (components of nuclear pores) is oxidatively damaged in old cells (D'Angelo et al 2009). Moreover, several studies have indicated an age-dependent decline in DNA repair capacity (Gorbunova et al 2007). We suspect that these age-associated changes in nuclear envelope function and DNA repair mechanisms may predispose the muscles of the elderly to s-IBM pathology.

9. Similarity of s-IBM and DMRV/h-IBM

We found inclusions of a set of nucleus-oriented or nucleus-proper proteins in distal myopathy with rimmed vacuoles (DMRV)/hereditary inclusion body myopathy (h-IBM), a disorder in which the muscle biopsy displays rimmed vacuoles in muscle fibers as in s-IBM (Fig. 6) (Nakano et al 1999, 2001, 2003, 2008).

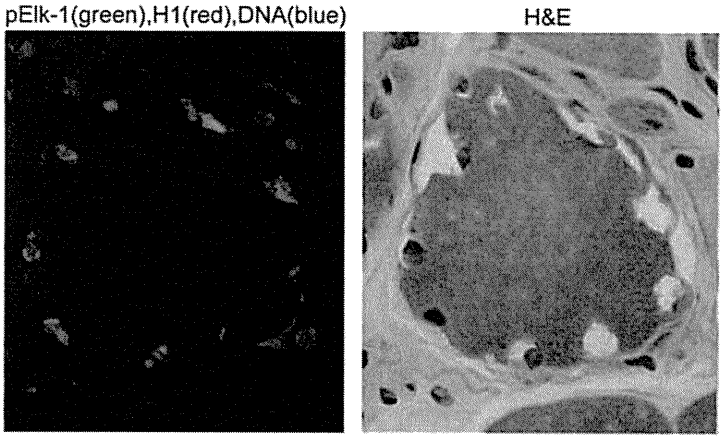

Fig. 6. Immunohistochemistry of pElk-1 and histone H1 in DMRV/h-IBM. Many pElk-1-positive deposits are seen in vacuoles .

Muscle pathology of DMRV/s-IBM shows rimmed vacuoles and tubulofilaments like s-IBM, but it lacks inflammation. The mutated gene for this autosomal recessive disease has been identified to be involved in glycosylation, named UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) (Eisenberg et al 2001, Nishino et al 2002). Remarkably, N-acetylglucosamine, the substrate of this enzyme is also a substrate of UDP-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyl transferase (OGT), the enzyme that adds O-linked-β-N-acetylglucosamine (O-GlcNAc) to a protein. A thousand of proteins including transcription factors, cytoskeletal proteins, kinases and nuclear pore proteins are modified with O-GlcNAc (Zachara and Hart 2004). O-GlcNAc is a highly dynamic process

and acts as a modulator of protein function, in a manner analogous to protein phosphorylation. Moreover, there is a complex crosstalk between O-GlcNAc modification and phosphorylation. The two post-translational modifications often regulate in an opposite manner by competitive attachment to the same serine/threonine residue, but they sometimes function co-operatively by binding at different sites of the same molecule (Zeidan 2010). In DMRV/h-IBM as well as in s-IBM, abnormal expression of proteins concerning to phosphorylation could be related to perturbation of the O-GlcNAc modification of proteins.

10. Conclusion

We have examined myonuclear dysfunction s-IBM. Similar degenerative mechanism may exist in DMRV/h-IBM that shows almost identical pathology and nuclear breakdown concerning to muscle fiber degeneration (Nonaka et al 1998). To reveal how GNE enzyme dysfunction affects myonuclei in this disorder may contribute to unveil the etiology of s-IBM. In addition, myofibrillar myopathy is a genetic disorder in which mutations of several Z-line associated proteins have been identified. Muscle biopsy studies have found congophilic inclusions (Selcen and Engel 2010). Players involved in excessive protein processing have been detected in s-IBM and myofibrillar myopathy (Ferrer et al 2004, 2005). Moreover, the disorder sometimes accompanies rimmed vacuoles (Shinde et al 2008). The comparative study of s-IBM and myofibrillar myopathy may also be helpful.
Concerning to the relationship between inflammation and nuclear breakdown, some immunological mechanism could operate in nuclear envelope dysfunction. Otherwise, nuclear aging and decrease of DNA repair capacity due to aging could induce the nuclear degeneration.

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The cathepsin L gene is a direct target of FOXO1 in skeletal muscle

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FOXO1 (forkhead box O1), a forkhead-type transcription factor whose gene expression is up-regulated in the skeletal muscle during starvation, appears to be a key molecule of energy metabolism and skeletal muscle atrophy. Cathepsin L, a lysosomal proteinase whose expression is also up-regulated in the skeletal muscle during starvation, is induced in transgenic mice overexpressing FOXO1 relative to wild-type littermates. In the present study, we conducted *in vivo* and *in vitro* experiments focusing on FOXO1 regulation of *Ctsl* (cathepsin L gene; *CTSL1* in humans) expression in the skeletal muscle. During fasting and refeeding of C57BL/6 mice, *Ctsl* was regulated in parallel with FOXO1 in the skeletal muscle. Fasting-induced *Ctsl* expression was attenuated in transgenic mice overexpressing a dominant-negative form of FOXO1 or in skeletal-muscle-specific *Foxo1*-knockout mice relative to respective wild-type controls. Using C2C12 mouse myoblasts overexpressing a constitutively active form of FOXO1, we showed that FOXO1 induces *Ctsl* expression. Moreover, we found FOXO1-binding sites in both the mouse *Ctsl* and human *CTSL1* promoters. The luciferase reporter analysis revealed that the mouse *Ctsl* and human *CTSL1* promoters are activated by FOXO1, which is abolished by mutations in the consensus FOXO1-binding sites. Gel mobility-shift and chromatin immunoprecipiation assays showed that FOXO1 is recruited and binds to the *Ctsl* promoter. The present study provides *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in the skeletal muscle, thereby suggesting a role for the FOXO1/cathepsin L pathway in fasting-induced skeletal muscle metabolic change and atrophy.

Key words: atrophy, cathepsin L, forkhead box O1 (FOXO1), forkhead transcription factor, muscle metabolism, starvation.

INTRODUCTION

The skeletal muscle is the largest organ in the human body, with important roles in exercise, glucose uptake and energy expenditure. Skeletal muscle metabolism is changed by the supply of nutrients and circulating hormones [1,2]. Starvation and disease states (such as diabetes and cancer cachexia) lead to a rapid reduction in skeletal muscle mass (atrophy) [2]. What is the physiological role of muscle atrophy? As the brain mainly uses glucose as an energy source, during starvation it needs to be supplied with glucose. Thus, for short periods of fasting, skeletal muscle increases utilization of lipids instead of glucose. On the other hand, for longer periods of fasting or starvation resulting in muscle atrophy, skeletal muscle protein is degraded and mobilized as a source of amino acids for gluconeogenesis that occurs mainly in the liver [3].

The FOXO (forkhead box O) members FOXO1, FOXO3a and FOXO4 belong to a subfamily of the forkhead transcription factors [4,5]. The FOXO family regulates a variety of biological processes such as metabolism, cell proliferation, apoptosis, stress response and longevity [6–9]. FOXO1 activates gluconeogenic enzyme genes in the liver, such as those for PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase). A dominant-negative form of FOXO1 (DN-FOXO1), which contains the DNA-binding domain, but lacks the transcriptional activation domain, suppressed the fasting-induced increase of *Pepck* and *G6Pase* expression in liver cells [10]. We showed previously that energy-deprived conditions in mice, such as fasting and diabetes, up-regulated expression of *Foxo1* in skeletal muscle of mice [11]. Several FOXO1 target genes have been identified in skeletal muscle. For instance, FOXO1 up-regulates *PDK4* (pyruvate dehydrogenase kinase 4), a kinase that suppresses glycolysis [12], and *LPL* (lipoprotein lipase), an enzyme that increases lipid incorporation [11], and down-regulates *SREBP1c* (sterol-regulatory-element-binding protein 1c), a master regulator of lipogenesis [13]. The FOXO1 target genes may be involved in the utilization of lipids instead of glucose in the skeletal muscle. On the other hand, forced expression of FOXO1 or FOXO3a up-regulates the expression of a variety of atrophy-related genes including the *MuRF1* and *atrogin/MAFbx* ubiquitin ligases [14,15], as well as *Bnip3* and *LC3*, important molecules for autophagy [16,17], thus inducing skeletal muscle atrophy *in vitro* and *in vivo*. We have created transgenic mice that overexpress FOXO1 in skeletal muscle (FOXO1 mice) and found

Abbreviations used: ChIP, chromatin immunoprecipitation; DBE, DAF16 (decay-accelerating factor 16)-binding element; DMEM, Dulbecco's modified Eagle's medium; DN-FOXO1, dominant-negative forkhead box O1; ER, oestrogen receptor; FBS, fetal bovine serum; FOXO, forkhead box O; GADD45α, growth-arrest and DNA-damage-inducible protein 45α; G6Pase, glucose-6-phosphatase; HEK, human embryonic kidney; PEPCK, phosphoenolpyruvate carboxykinase; PLSD, protected least-significant difference; TAM, tamoxifen.

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that they exhibit skeletal muscle atrophy [18]. Moreover, FOXO1 has been shown to induce *Gadd45a* (growth-arrest and DNA-damage-inducible protein 45α) [19], a suppressor of the cell cycle, thereby facilitating skeletal muscle atrophy. Thus identification and functional analysis of FOXO1 target genes will help facilitate a better understanding of skeletal muscle metabolism.

Cathepsin L is a lysosomal proteinase, whose expression is up-regulated during various forms of skeletal muscle atrophy including starvation [20–22]. In the skeletal muscle of the FOXO1 mice, *Ctsl* expression was markedly increased [18]. Earlier findings showed that lysosomal proteolysis is activated upon skeletal muscle atrophy [23,24]. Although circumstantial evidence suggests that cathepsin L is involved in skeletal muscle atrophy, to our knowledge, there are no reports on the regulation of *Ctsl* by FOXO1. In the present study, we provide *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in skeletal muscle.

EXPERIMENTAL

Genetically modified animals

The human skeletal muscle α-actin promoter [25] was kindly provided by Dr E.C. Hardeman and Dr K. Guven (Children's Medical Research Institute, Westmead, NSW, Australia). DN-FOXO1, a mutant version of FOXO1 containing amino acid residues 1–256, has been described previously [26]. Transgenic plasmid containing the cDNA for DN-FOXO1 (see Figure 2A) was excised and purified for injection (at 2 ng $\cdot$ μl^{-1}) [18]. Fertilized eggs were recovered from C57BL/6 females crossed with C57BL/6 males and microinjected at Japan SLC Inc. (Hamamatsu, Japan). To obtain skeletal-muscle-specific *Foxo1*-knockout mice, we inactivated *Foxo1* expression in the skeletal muscle by crossing mice homozygous for a floxed *Foxo1* allele with myogenin-cre transgenics. Myogenin-cre and *Foxo1*^{flox} mice were as described previously [27]. The mice were maintained at a constant temperature of 24 °C with fixed artificial light (12 h light/12 h dark). All animal experiments were conducted in accordance with the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0090041) and National Institute of Health and Nutrition (No. 0706).

C2C12 cells and cell cultures

C2C12 mouse myoblasts (RIKEN Cell Bank, Tsukuba, Japan) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS (fetal bovine serum) until the cells reached confluence. The medium was then replaced with DMEM containing 2 % (v/v) horse serum (differentiation medium) and incubated for 4 days to induce the formation of myotubes before each experiment. C2C12 myoblasts stably expressing FOXO1–ER (oestrogen receptor) fusion proteins were obtained as described previously [28]. In brief, C2C12 cells were stably transfected with the empty pBABE retrovirus or pBABE vectors expressing fusion proteins containing a constitutively active form of human FOXO1 [FOXO1(3A)] [26] [where three Akt phosphorylation sites (Thr^{24}, Ser^{256} and Ser^{319}) are replaced by alanine residues] in-frame with a modified TAM (tamoxifen)-specific version of the murine ER-ligand-binding domain. FOXO1–ER plasmid was provided by Dr Terry G. Unterman (Department of Medicine, University of Illinois at Chicago, U.S.A.) Cells were selected with puromycin and colonies were pooled for studies, as reported previously [13]. The fusion proteins are restricted to the cytoplasmic space until activation by treatment with TAM [28].

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously [13]. Total RNA was prepared using Sepazol. cDNA was synthesized from 5 μg of total RNA using ReverTra Ace® (TOYOBO) with random primers. Gene expression levels were measured with an ABI PRISM 7700 Sequence Detection System using SYBR Green PCR Core Reagents (Applied Biosystems). Levels of mRNA were normalized to those of *36B4* mRNA. The primers used were as follows. Cathepsin L: forward, 5′-TCTCACGCTCAAGGCAATCA-3′, reverse, 5′-AAGCAAAATCCATCAGGCCTC-3′; GADD45α: forward, 5′-CGTAGACCCCGATAACGTGGTA-3′, reverse, 5′-CGGATGAGGGTGAAATGGAT-3′; FOXO1: forward, 5′-ATTCGGAATGACCTCATGGA-3′, reverse, 5′-GTGTGGGAAGCTTTGGTTGG-3′; DN-FOXO1 (transgene specific): forward, 5′-GACTACAAGGACGACGATGA-3′, reverse, 5′-AGCGGCTCGAAGTCCGGGTC-3′, FOXO3a: forward, 5′-TCTGCGGGCTGGAAGAACT-3′, reverse, 5′-CTCTTGCCCGTGCCTTCAT-3′; FOXO4: forward, 5′-ATGGATGGTCCGCACGGTG-3′, reverse, 5′-CTTGCCAGTGGCCTCGTTG-3′; and 36B4: forward, 5′-GGCCCTGCACTCTCGCTTTC-3′, reverse 5′-TGCCAGGACGCGCTTGT-3′.

Cloning of the mouse *Ctsl* and human *CTSL1* promoters

The mouse *Ctsl* promoter has been described previously [29]. The 4-kb mouse *Ctsl* promoter region was excised with BamHI from pMEPCAT3 and cloned into a pGL3-basic luciferase vector (Promega Corporation). The 4-kb mouse promoter was sequenced. The human *CTSL1* promoter [30,31] was obtained by PCR from genomic DNA of HEK (human embryonic kidney)-293 cells. The PCR primers used were 5′-GTGGTGCGCGCCTGTAGTCC-3′ and 5′-GGCGCACTCCACGGATGCCG-3′. Mutations in the promoter sequences were introduced using a QuikChange® site-directed mutagenesis kit (Stratagene). Primers used were human DBE1: 5′-CTGGGACAGTCAGTGGGCAAGCCACGAACC-3′; human DBE2: 5′-GGGACAGTCAGTGGGCAAGCCACGAACC-3′; and mouse DBE: 5′-GTGATAGACTGAGTGGGCAAACATACAAAG-3′. DBE is DAF16 (decay-accelerating factor 16)-binding element, to which FOXO1 binds [32].

Transfection and luciferase assay

HEK-293 cells were plated at a density of 10^5 cells/12-well plate in DMEM containing 10 % (v/v) FBS. Luciferase gene constructs containing a *Ctsl* promoter fragment with or without mutations of putative FOXO1-binding sites were prepared. The luciferase reporter plasmid (0.8 μg), the expression plasmid [pCAG-FOXO1(3A) or empty pCAG, 0.8 μg], and a phRL-TK vector (25 ng; Promega) as an internal control for transfection efficiency, were transfected into HEK-293 cells using Lipofectamine™ 2000 (Invitrogen). After an overnight transfection period, 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 the average of triplicate experiments.

Gel mobility-shift assay

The gel mobility-shift assay was performed as described previously [33]. *In-vitro*-translated human FOXO1 was generated from pCMX-FOXO1, using the TNT® T7

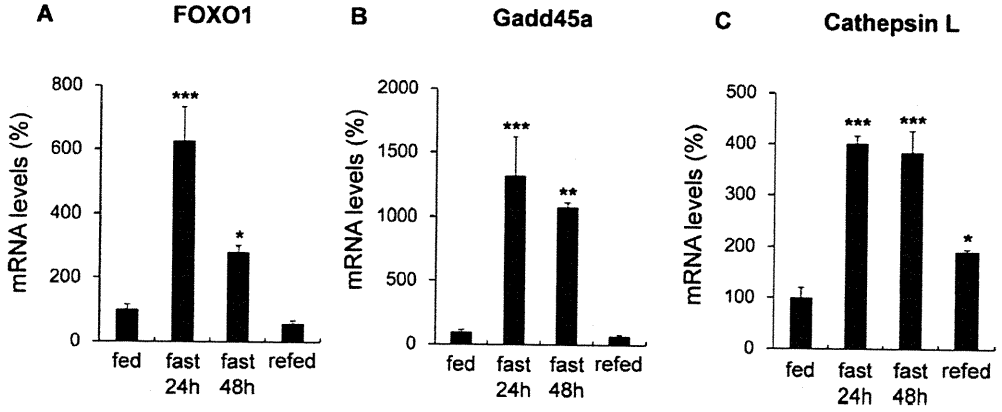

Figure 1 ***Foxo1*, *Gadd45a* and *Ctsl* expression in skeletal muscle of fasted and refed mice**

Mice (C57BL6, male, 8 weeks of age) were divided into four experimental groups of four mice each. They were either allowed to eat freely (fed), or subjected to a 24 or a 48 h fast. Others were subjected to a 48 h fast followed by 8 h of feeding (refed). Mice were killed, and relative mRNA levels of *Foxo1* (endogenous), *Gadd45a* and *Ctsl* in skeletal muscle (gastrocnemius) were analysed by quantitative real-time PCR. Levels of mRNA were normalized to those of *36B4* mRNA. Values of fed samples were set at 100. $*P < 0.05$, $**P < 0.01$; and $***P < 0.001$ compared with the fed group.

Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instruction. Double-stranded oligonucleotide probes used in gel mobility-shift assays were prepared by annealing both strands of each putative FOXO1-binding site in the human *CTSL1* promoter (DBE1: 5′-ATCTCCAAAATAGTAAACAAATTCCTGCAG-3′, −145 to −152, numbering the first nucleotide of exon 1 as +1; DBE2: 5′-GGGACAGTCAGTAAACAAGCCACGAACC-3′, −1400 to −1407, numbering the first nucleotide of exon 1 as +1; DBE1 mutant: 5′-ATCTCCAAAATAGTGGGCAA-ATTCCTGCAG-3′; and DBE2 mutant: 5′-GGGACAGTC-AGTGGGCAAGCCACGAACC-3′; underlining indicates sites of mutation) and labelling with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Roche Applied Science). The labelled probes (50000 d.p.m.) were incubated with extracts containing *in-vitro*-translated FOXO1 in a mixture (total volume of 25 μl) containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM DTT (dithiothreitol), 1 mM EDTA and 4.4 % glycerol with 1 mg of poly(dI-dC)·(dI-dC) for 30 min on ice and then separated by electrophoresis on a 6 % polyacrylamide gel in 45 mM Tris/HCl (pH 8.0), 45 mM borate and 1 mM EDTA. After electrophoresis, gels were dried and analysed with a BAS-2500 (Fuji Film).

ChIP (chromatin immunoprecipitation) assay

ChIP was carried out using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's guidelines [13,34]. Briefly, C2C12 myoblasts stably expressing FOXO1(3A)–ER were incubated for 24 h with or without 1 μM TAM. Proteins were cross-linked to DNA with the addition of formaldehyde (1 % final concentration). Cells were washed and lysed in SDS lysis buffer, sonicated for 10 s and allowed to recover for 30 s over ice (this was repeated seven times). Lysates were cleared with Protein A–agarose for 30 min, pelleted and incubated overnight with an anti-FOXO1 antibody (sc-11350; Santa Cruz Biotechnology). Before the incubation, input samples were removed from the lysate and stored at 4 °C until extraction. Following incubation with the antibody, protein–DNA complexes were eluted (1 % SDS and 0.1 M $NaHCO_3$), and the cross-links were reversed. DNA was purified by phenol/chloroform extraction. PCR primers were designed to locate DBE of the *Ctsl* promoter: forward, 5′-AAAAGACAAGAGGATGCCTT-3′, and reverse, 5′-CTGGTGTTCTCAGGTTAGTC-3′. The amplified region was −3670 to −3339, numbering the first nucleotide of exon 1 as +1. PCR primers were also designed to locate non-DBE of the *Ctsl* promoter: forward, 5′-CCACGAAAAGAATTTCTACCA-3′ and reverse, 5′-AGTTGTAGATTAAAATGTGCAG-3′. The amplified region was −439 to −289, numbering the first nucleotide of exon 1 as +1.

Statistical analysis

All results are expressed as means ± S.E.M. Statistical comparisons of data from experimental groups were made with a one-way ANOVA, and groups were compared using Fisher's PLSD (protected least-significant difference) test (Statview 5.0; Abacus Concepts, Berkeley, CA, U.S.A.). When differences were significant, groups were compared using Fisher's PLSD test. Statistical significance was defined as $P < 0.05$.

RESULTS

Co-ordinate regulation of *Foxo1* and *Ctsl* expression in the mouse skeletal muscle during fasting and refeeding

To analyse the *in vivo* relationship between *Foxo1* and *Ctsl* expression, we first examined their gene expression in the skeletal muscle of mice subjected to fasting and refeeding. Expression of mRNAs for *Foxo1* and *Gadd45a*, a *bona fide* FOXO1-target gene [19], was increased in skeletal muscle after 24 and 48 h of fasting (Figures 1A and 1B). *Ctsl* mRNA expression was also markedly increased in the skeletal muscle during fasting (Figure 1C). The effect of fasting was reversed by refeeding. *Foxo1* and *Ctsl* mRNA levels were increased in various regions of skeletal muscles, such as gastrocnemius (Figure 1), soleus, extensor digitorum longus and tibialis anterior (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270171add.htm). In other tissues, such as the brain, kidney and adipose tissue, fasting did not markedly alter *Foxo1* and *Ctsl* mRNA expression (Y. Kamei, unpublished work). These observations indicate that the expression of *Foxo1* and *Ctsl* is co-ordinately regulated in the skeletal muscle during fasting and refeeding.

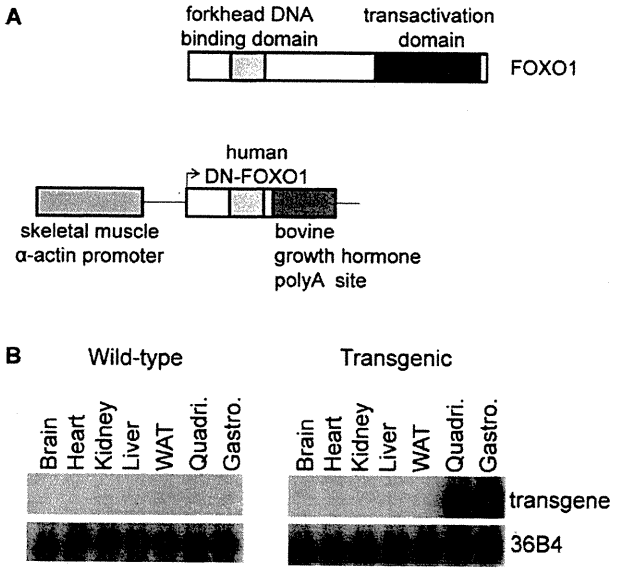

Figure 2 Creation of DN-FOXO1 transgenic mice

(**A**) The structure of FOXO1 and DN-FOXO1. DN-FOXO1 is described in the map of the 4.3-kb construct used for transgenic microinjection. The transgene was under the control of the human skeletal muscle α-actin promoter and included exon 1 and the intron of the human skeletal muscle α-actin gene as well as the bovine growth hormone polyadenylation (polyA) site. (**B**) Tissue distribution of transgene expression in DN-FOXO1 mice. RNA samples were prepared from various tissues in DN-FOXO1 and wild-type mice (male, 10 weeks of age). Northern blot analyses were conducted using the DN-FOXO1 probe and 36B4 reblotting was used as the loading control. Gastro., gastrocnemius; Quadri., quadriceps; WAT, white adipose tissue.

Transgenic mice overexpressing DN-FOXO1

Previously, we generated transgenic mice with skeletal-muscle-specific overexpression of human FOXO1 using the α-actin promoter (FOXO1 mice) [18]. Skeletal muscle in these FOXO1 mice showed an increase in *Ctsl* mRNA levels [18]. To examine the possible *in vivo* regulation of *Ctsl* by FOXO1, we also generated transgenic mice with skeletal-muscle-specific overexpression of DN-FOXO1 (Figure 2A), which suppresses FOXO1-mediated transcription. DN-FOXO1 contains the DNA-binding domain, but lacks the transcription activation domain, of FOXO1 [10,27,35]. DN-FOXO1 transgene expression was observed specifically in skeletal muscle (Figure 2B). Histologically, there was no appreciable difference in skeletal muscle between DN-FOXO1 and wild-type mice (results not shown).

Fasting-induced *Ctsl* expression is suppressed in the skeletal muscle of DN-FOXO1 mice

We used 16 DN-FOXO1 mice and 16 gender- and age-matched wild-type mice. Eight mice each were allowed to eat freely (fed) or were fasted for 24 h. *Foxo1*, *Gadd45a* and *Ctsl* expression was increased in the skeletal muscle from wild-type mice (Figure 3). In DN-FOXO1 mice, fasting-increased endogenous *Foxo1* expression was attenuated compared with wild-type mice (Figure 3A), suggesting that FOXO1 up-regulates its own gene expression. Moreover, induction of *Ctsl* as well as *Gadd45a* expression by fasting was markedly diminished in the DN-FOXO1 mice (Figures 3B and 3C). These observations indicate that FOXO1 significantly contributes to the up-regulation of *Ctsl* expression during fasting *in vivo*.

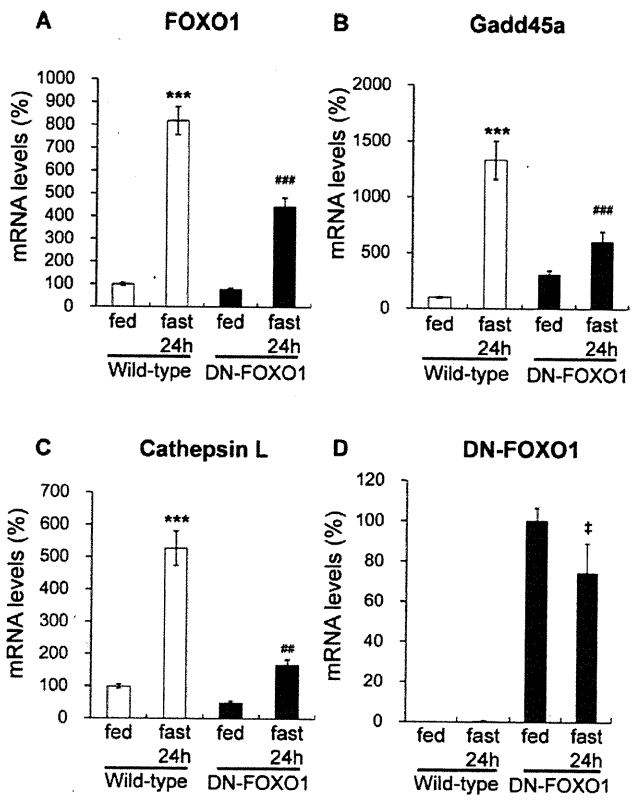

Figure 3 Gene expression in skeletal muscle of fed or fasted DN-FOXO1 mice

DN-FOXO1 mice (12 weeks of age) or age- and gender-matched wild-type mice were allowed to eat freely (fed) or subjected to a 24 h fast. The number of animals used in each group was eight. Expression of (**A**) *Foxo1* (endogenous), (**B**) *Gadd45a*, (**C**) *Ctsl* and (**D**) DN-FOXO1 (transgene) in skeletal muscle (gastrocnemius). (**A–C**) Values of wild-type mice with fed samples were set at 100. (**D**) Values of DN-FOXO1 mice with fed samples were set at 100 for DN-FOXO1 transgene. The transgene expression level was slightly decreased by fasting. Levels of mRNA were normalized to those of *36B4* mRNA. $***P < 0.001$ compared with samples of wild-type fed mice. $\#\#P < 0.01$ and $\#\#\# P < 0.001$ compared with samples of wild-type fasted mice. $\ddagger P < 0.05$ compared with samples of transgenic fed mice. Results are representative of three independent experiments with similar results.

Fasting-induced *Ctsl* expression is suppressed in the skeletal muscle of skeletal-muscle-specific *Foxo1*-knockout mice

To examine whether the induction of *Ctsl* expression in the muscles of fasting animals is dependent on FOXO1 or not, we used muscle-specific *Foxo1*-knockout mice (myogenin-cre/*Foxo1*^{flox}) [27]. Knockout and control mice were fed or were fasted for 24 h, and expression of FOXO family members (*Foxo1*, *Foxo3a* and *Foxo4*), *Ctsl* and *Gadd45a* was examined (Figure 4). In control mice, *Foxo1*, *Foxo3a*, *Ctsl* and *Gadd45a* expression was markedly up-regulated. The *Foxo1* expression in the knockout mice that were fed was much lower than that in the control mice that were fed. We did not observe marked induction of *Foxo1* expression in the fasted knockout mice relative to the fed knockout mice. In the knockout mice, the induction of *Ctsl* expression was suppressed (Figure 4C). Expression of *FOXO4* did not differ among groups (results not shown). These observations indicate that FOXO1 is important for the up-regulation of *Ctsl* expression during fasting. However, since *Ctsl* expression during fasting was not completely suppressed, there may be additional factor(s). FOXO3a may be such a factor, as its expression was up-regulated during fasting both in control and knockout mice (Figure 4D).

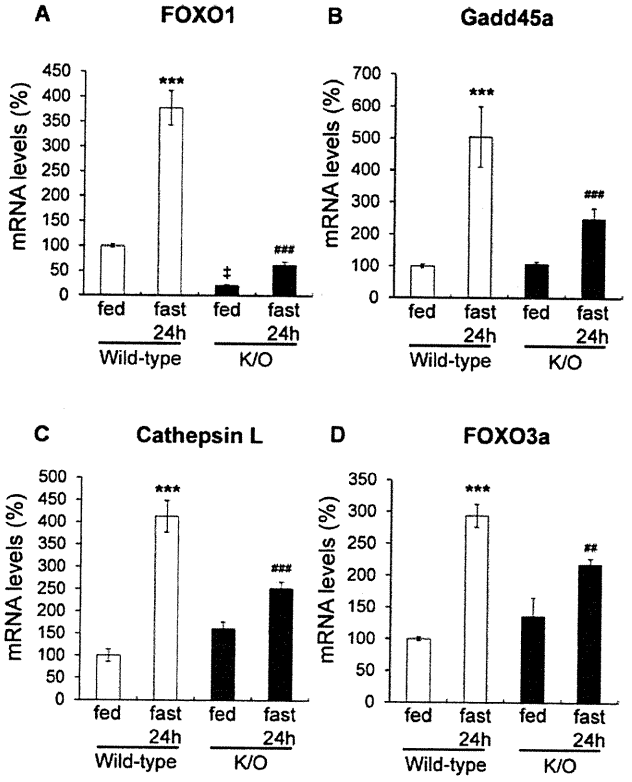

Figure 4 Gene expression in the skeletal muscle of fed or fasted muscle-specific *Foxo1*-knockout mice

Knockout (K/O) mice (4 weeks of age) or age-matched wild-type mice were allowed free access to standard chow (fed) or subjected to a 24 h fast. The number of animals used was: K/O fed, $n = 3$; K/O fast, $n = 3$; control fed, $n = 5$; control fast, $n = 4$. Expression of (**A**) *Foxo1* (endogenous), (**B**) *Gadd45a*, (**C**) *Ctsl* and (**D**) *FOXO3a* in the skeletal muscle (gastrocnemius). Values obtained in wild-type mice that were fed were set at 100. Levels of mRNA were normalized to those of *36B4* mRNA. ***$P < 0.001$, relative to wild-type fed mice. ## $P < 0.01$ and ###$P < 0.001$ relative to wild-type fasted mice. ‡ $P < 0.05$ relative to wild-type fed mice.

Activation of FOXO1 in C2C12 myocytes promotes *Ctsl* expression

To study the effects of FOXO1 on *Ctsl* expression in muscle cells, we first employed C2C12 cells stably expressing a constitutively active form of FOXO1 [FOXO1(3A)] in-frame with a modified form of the ER ligand-binding domain that responds selectively to TAM [28]. Previous studies with these cells have shown that fusion proteins are restricted to the cytoplasmic space in the absence of ligand and then rapidly translocate to the nucleus upon treatment with TAM [28]. Each mRNA signal in Figure 5(A) is the sum of endogenous *Foxo1* mRNA and retrovirus-derived FOXO1(3A)–ER mRNA. The endogenous *Foxo1* mRNA was very low in C2C12 cells. As expected, treatment with TAM did not change FOXO1(3A)–ER mRNA levels. Treatment with TAM resulted in a marked induction of *Gadd45a* expression, confirming that it successfully promoted the transcriptional activity of our FOXO1(3A)–ER-C2C12 myotubes. As shown in Figure 5, treatment with TAM also markedly increased the mRNA abundance of *Ctsl* as well as *Gadd45a*. No changes in *Ctsl* mRNA expression were observed in FOXO1(3A)–ER cells in the absence of TAM or in control C2C12 cells stably transfected with empty vector (Mock) (Figure 5C). These results suggest that the expression of *Ctsl* is up-regulated directly by the activation of FOXO1 in muscle cells.

The mouse *Ctsl* promoter is activated by FOXO1

The above data suggest that *Ctsl* is a direct transcriptional target of FOXO1 in muscle cells. FOXO1 is known to bind the sequence GTAAACAA or DBE [32]. We therefore examined using a transient transfection-reporter assay whether the mouse *Ctsl* and human *CTSL1* promoters are activated by FOXO1. The mouse *Ctsl* promoter has been cloned previously [29]. We sequenced the 4-kb mouse *Ctsl* promoter and found a single consensus FOXO1-binding site (GTAAACAA) (−3528 to −3535, numbering the first nucleotide of exon 1 as +1). Plasmid constructs linking the mouse *Ctsl* promoter including the putative FOXO1-binding site to the luciferase reporter gene were analysed. FOXO1 increased the mouse *Ctsl* promoter (−4000 to +10)-driven reporter activity (Figure 6A). Furthermore, mutation in the consensus FOXO1-binding site abolished the FOXO1-induced luciferase activity (Figure 6A). Consistent with *in vivo* transgenic mice data (Figure 3), in the *in vitro* transfection reporter assay, DN-FOXO1 dose-dependently suppressed the FOXO1(3A)-induced transcriptional activity of the mouse *Ctsl* promoter (Figure 6B). These observations suggest that FOXO1 up-regulates the mouse *Ctsl* expression via the DBE sequence in its promoter.

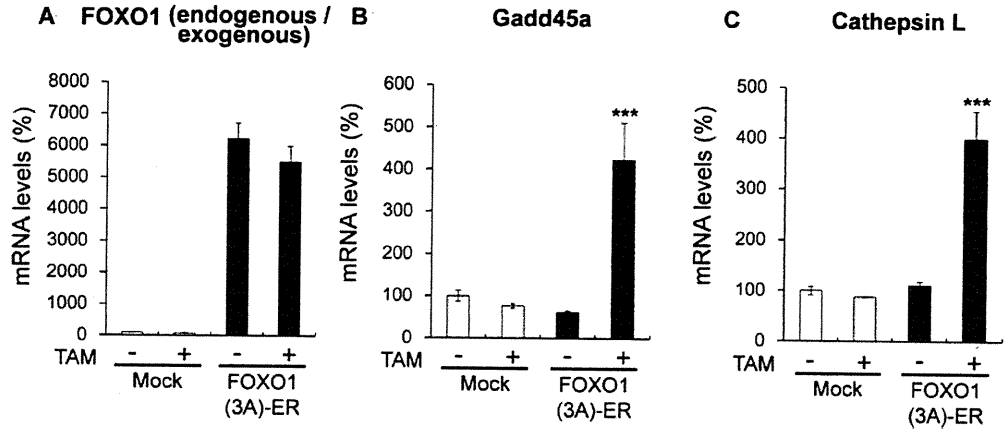

Figure 5 *Ctsl* expression by FOXO1 in C2C12 muscle cells

The abundance of mRNA transcripts for (**A**) *Foxo1*, (**B**) *Gadd45a* and (**C**) *Ctsl* in control (mock, open bars) and FOXO1(3A)–ER C2C12 cells (closed bars) treated with (+) or without (−) TAM for 24 h was analysed by quantitative real-time PCR. Levels of mRNA were normalized to those of *36B4* mRNA. ***$P < 0.001$ compared with samples without TAM.

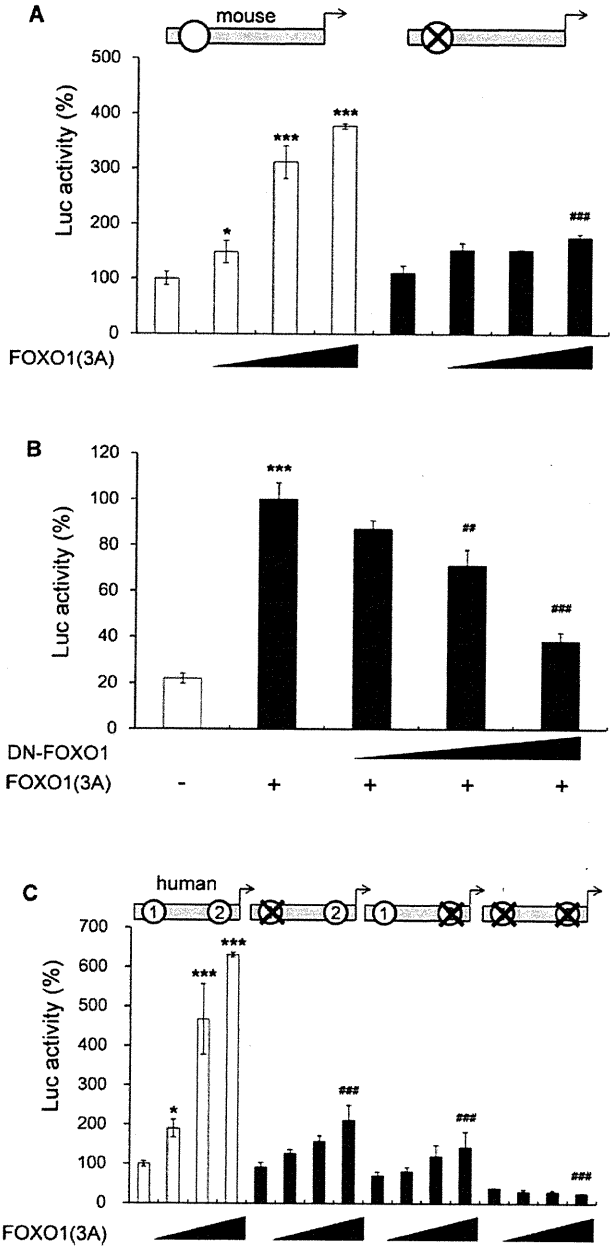

Figure 6 Transient transfection-reporter assay of the effect of FOXO1 on *Ctsl* promoters

The effect of increasing the amount of FOXO1 was examined by co-transfection with the reporter plasmids in HEK-293 cells. (**A**) The mouse *Ctsl* promoter (−4 kb) with mutations in putative FOXO1-binding sites. (**C**) The human *CTSL1* promoter (−1.6 kb) with mutations in the putative FOXO1-binding site. Activation of the luciferase reporter gene was measured in relative light units and was normalized to the dual-luciferase activity. Mean values from triplicate experiments are shown as the fold-induction, where the activity in the absence of FOXO1 is the reference value (set at 100). Schematic representations of *Ctsl* and *CTSL1* promoter constructs are shown above the histograms. Circles denote the putative FOXO1-binding sites and crosses denote mutations in the FOXO1-binding sites. Number in the circles: 1 is DBE1 (−1400 to −1407) and 2 is DBE2 (−145 to −152, numbering the first nucleotide of exon 1 as +1). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the value of wild-type promoter in the absence of FOXO1(3A). $^{\#\#\#}P < 0.001$ compared with the values of wild-type promoter with the largest amount of FOXO1(3A). (**B**) Transient transfection assay of the DN-FOXO1 construct used to suppress FOXO1-mediated transactivation *in vitro*. The effect of DN-FOXO1 was examined by co-transfection of the reporter plasmid containing the mouse *Ctsl* promoter with or without pCAG-FOXO1(3A). An increasing amount of DN-FOXO1 suppressed FOXO1(3A)-induced *Ctsl* promoter activity. Activation of the luciferase reporter gene was measured in relative light units and normalized to the dual-luciferase activity. Mean values from triplicate experiments are shown as the fold-induction, where the activity in the absence of DN-FOXO1 and in the presence of FOXO1(3A) is the reference value (set at 100). $^{***}P < 0.001$ compared with the value in the absence of DN-FOXO1 and FOXO1(3A). $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$ compared with the value in the absence of DN-FOXO1 and in the presence of FOXO1(3A).

The human *CTSL1* promoter is bound and activated by FOXO1

In the human *CTSL1* promoter [30,31], we also found two potential FOXO-binding sites; there are two perfect DBEs (−145 to −152 and −1400 to −1407, numbering the first nucleotide of exon 1 as +1). FOXO1 increased the human *CTSL1* promoter (−1600 to +10)-driven reporter activity in a transfection assay (Figure 6C). In addition, mutations in the consensus DBEs abolished the FOXO1-induced luciferase activity. Thus the results of the luciferase assay with the human *CTSL1* promoter were similar to those with the mouse *Ctsl* promoters.

We also examined the binding of FOXO1 to the DBEs in the human *CTSL1* promoter with a gel mobility-shift assay. FOXO1 that was synthesized *in vitro* clearly bound to oligonucleotides containing the putative FOXO1-binding sites of the human *CTSL1* promoter, and did not bind to oligonucleotides with mutations in the consensus DBEs (Figure 7A). Moreover, we performed a ChIP analysis using C2C12 cells expressing FOXO1(3A)–ER (as used in Figure 5), and found that FOXO1 was recruited to the mouse *Ctsl* promoter containing the DBE (Figure 7B). These observations, taken together, suggest that FOXO1 up-regulates the mouse *Ctsl* and human *CTSL1* expression via the DBE sequences of their promoters; *CTSL1* is a direct target of FOXO1 in the skeletal muscle.

DISCUSSION

FOXO1 signalling is important in linking nutritional and hormonal cascades to the regulation of skeletal muscle atrophy. As a transcriptional factor and/or cofactor, FOXO1 regulates many genes in a variety of biological processes. Identification and molecular analysis of FOXO1 target genes should help facilitate a better understanding of skeletal muscle metabolism. In the present study, we showed that FOXO1 directly activates *Ctsl* expression.

In the present study, we first conducted *in vivo* experiments focusing on FOXO1 regulation of *Ctsl* expression in skeletal muscle in the context of physiological nutritional change. During fasting and refeeding of C57BL6 mice, *Ctsl* was regulated in parallel with FOXO1 in skeletal muscle (Figure 1). Fasting-induced *Ctsl* expression was attenuated in DN-FOXO1 mice (Figure 3) and in skeletal-muscle-specific *Foxo1*-knockout mice (Figure 4) relative to respective wild-type controls. In this regard, we observed previously that *Ctsl* expression is markedly increased in skeletal muscle of FOXO1 mice [18]. Taken together, our results suggest that FOXO1 activates *Ctsl* expression *in vivo*. The increase in *Ctsl* mRNA is delayed compared with that of *Foxo1* (Figure 1). This could be explained as follows: (i) *Ctsl* mRNA may have a long half life, or (ii) *Ctsl* is activated by different transcription factors as well as FOXO1 during fasting. Indeed, it has been reported that addition of glucocorticoid, whose blood level is increased during fasting, has been reported to increase the level of *Ctsl* mRNA [20]. Cathepsin L is considered to play a major role in the terminal degradation of proteins delivered to lysosomes by endocytosis or autophagy [24,36]. A previous study shows that pharmacological inhibition in rats of both cathepsin L and calpain, an intracellular Ca^{2+}-dependent

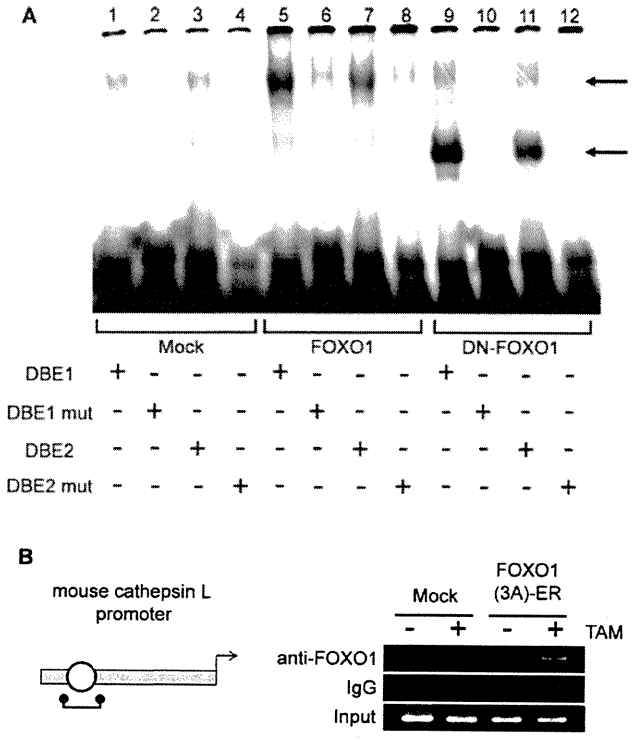

Figure 7 Recruitment of FOXO1 to the putative FOXO1-binding sites of the *Ctsl* promoter

(**A**) Gel mobility-shift assay. Synthetic double-stranded oligonucleotides containing putative FOXO1-binding sites of the human *CTSL1* promoter were used. *In vitro* synthesized FOXO1 protein was incubated with ^{32}P-labelled double-stranded oligonucleotides (DBE1, −1400 to −1407; DBE2, −145 to −152, numbering the first nucleotide of exon 1 as + 1; or DBE1 mut and DBE2 mut, oligonucleotides with mutation in the consensus sequence) for 30 min on ice. The protein–DNA complexes were resolved on an 8% non-denaturing gel. FOXO1 and DN-FOXO1 were able to bind labelled *CTSL1* oligonucleotides (FOXO1, lanes 5 and 7; DN-FOXO1, lanes 9 and 11), but not mutated oligonucleotides (FOXO1, lanes 6 and 8; DN-FOXO1, lanes 10 and 12). The arrows indicate the specific protein–DNA complex. (**B**) ChIP assay. Primers specific to the region of the mouse *Ctsl* promoter containing the DBE were used for PCR analysis. FOXO1(3A)–ER was recruited to the *Ctsl* promoter in the presence of TAM. No signals were detected using control IgG. Primers corresponding to the region of non-DBE did not give any signals (not shown).

protease, prevents sepsis-induced bulk protein degradation [37] and suggests a role for cathepsin L in the degradation of various skeletal muscle proteins. It is therefore conceivable that FOXO1-induced transcriptional activation of *Ctsl* plays a role in fasting-induced autophagy and proteolysis. During fasting, a large number of genes show a change in their expression; some are changed directly as a physiological response, and others may be changed indirectly as secondary events. Nevertheless, *Ctsl* expression is likely to be regulated by FOXO1.

Using C2C12 myoblasts, we also showed that FOXO1 induces endogenous *Ctsl* expression *in vitro* (Figure 5). Moreover, we showed that FOXO1 can bind to and activate the *Ctsl* promoter (Figures 6 and 7). The promoter of *Ctsl* has been sequenced and analysed in humans, mice and rats. Transcription factors such as the specificity proteins Sp1/Sp3 have been reported to increase the basal activities of the promoter [31,38,39]. In the present study, we have provided the first evidence for transcriptional activation of the *Ctsl* promoter by an inducible transcription factor, FOXO1, thereby suggesting that *Ctsl* is a direct target gene of FOXO1. During fasting, among members of the cathepsin family, only *Ctsl* expression is markedly increased in skeletal muscle [21]. Indeed, there are no consensus DBEs in the putative promoter of other cathepsins (−1.5 kb from the transcription start sites of cathepsins B, C, D, E, G, H, J, K S and Z in humans and mice; Y. Yamazaki and Y. Kamei, unpublished work), indicating that FOXO1 specifically activates *Ctsl* during fasting. Therefore cathepsin L probably plays a role in fasting-induced adaptive responses including skeletal muscle atrophy.

FOXO1 has been shown to activate the expression of *Gadd45a*, *Pepck* and *G6Pase* via direct binding to DBE *in vitro* [19,40]. On the other hand, adenoviral introduction of DN-FOXO1 can suppress the gene expression of *Pepck* and *G6Pase* in the liver *in vivo* [10]. Previous studies suggest that FOXO1 can regulate gene expression in at least two different ways: (i) FOXO1 directly binds to and transactivates the promoter of its target genes [32], or (ii) FOXO1 interacts with other transcription factors via protein–protein interactions, without DNA binding, thereby regulating the expression of target genes [27,41]. Because FOXO1 interacts with nuclear receptors via its C-terminus [41], DN-FOXO1 can suppress the action of FOXO1 as a transcription factor without affecting its action as a transcriptional cofactor. Therefore FOXO1 appears to regulate *Ctsl* expression via a transcriptional mechanism.

We reported previously that FOXO1 mice have decreased skeletal muscle mass [18]. In the present study, there was no appreciable histological difference in skeletal muscle between DN-FOXO1 and wild-type mice. We also observed no marked difference in body weight and skeletal muscle mass between DN-FOXO1 and wild-type mice (results not shown). This may be because DN-FOXO1 does not suppress all the FOXO1 actions in a dominant-negative fashion, as described in the above. A detailed phenotypic analysis of DN-FOXO1 mice is ongoing in our laboratory.

In conclusion, the present study provides *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in skeletal muscle. The results provide important clues towards understanding the molecular mechanism underlying FOXO1-mediated transcriptional regulation of gene expression in skeletal muscle. Further studies will better clarify the physiological and pathophysiological implication of FOXO1-induced *Ctsl* expression in skeletal muscle.

AUTHOR CONTRIBUTION

Yasutomi Kamei, Tadahiro Kitamura, Takayoshi Suganami, Osamu Ezaki and Yoshihiro Ogawa led the design and overall implementation of the trial. Yasutomi Kamei wrote the initial draft of the paper in consultation with Yukio Hirata, Bruce Troen and Yoshihiro Ogawa. Yoshihiro Yamazaki, Satoshi Sugita, Fumiko Akaike, Sayaka Kanai, Shinji Miura and Ichizo Nishino were responsible for laboratory analyses. All authors contributed to interpretation of data and have seen and approved the final manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

The cathepsin L gene is a direct target of FOXO1 in skeletal muscle

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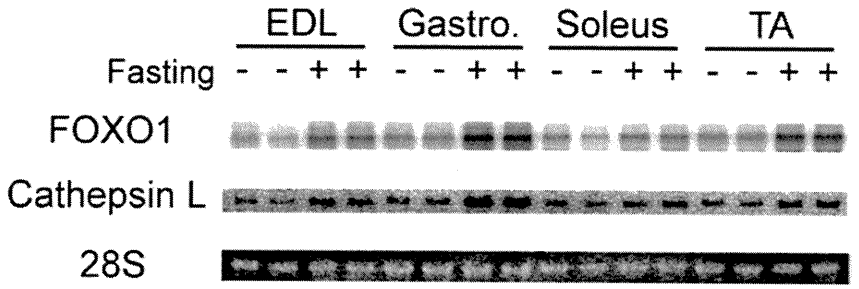

Figure S1 ***Foxo1*** **and** ***Ctsl*** **expression in various regions of skeletal muscle of fasted mice**

Mice (C57BL6, male, 8 weeks of age) were either allowed free access to standard chow (fed) or subjected to a 24 h fast. Mice were killed, and relative mRNA levels of *Foxo1* and *Ctsl* were examined by Northern blot analysis. RNA samples were prepared from indicated skeletal muscles. The 28S staining used as the loading control gave similar abundance. EDL, extensor digitorum longus; Gastro., gastrocnemius; TA, tibialis anterior.

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ORIGINAL PAPER

Defects in amphiphysin 2 (BIN1) and triads in several forms of centronuclear myopathies

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Abstract Myotubular myopathy and centronuclear myopathies (CNM) are congenital myopathies characterized by generalized muscle weakness and mislocalization of muscle fiber nuclei. Genetically distinct forms exist, and mutations in *BIN1* were recently identified in autosomal recessive cases (ARCNM). Amphiphysins have been implicated in membrane remodeling in brain and skeletal muscle. Our objective was to decipher the pathogenetic mechanisms underlying different forms of CNM, with a focus on ARCNM cases. In this study, we compare the histopathological features from patients with X-linked, autosomal recessive, and dominant forms, respectively, mutated in myotubularin (*MTM1*), amphiphysin 2 (*BIN1*), and dynamin 2 (*DNM2*). We further characterize the ultrastructural defects in ARCNM muscles. We demonstrate that the two BIN1 isoforms expressed in skeletal

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