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Original Article

Myogenic differentiation 1 and transcription factor 12 activate the gene expression of mouse taste receptor type 1 member 1

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ABSTRACT

Objectives: Myogenic differentiation 1 (Myod1) is involved in the expression of taste receptor type 1 member 1 (*Tas1r1*) during myogenic differentiation. Further, the target genes of Myod1 participate in transcriptional control, muscle development, and synaptic function. We examined, for the first time, the function of Myod1 in the transcriptional regulation of *Tas1r1*.

Methods: ENCODE chromatin immunoprecipitation and sequencing (ChIP seq) data of myogenically differentiated C2C12 cells were analyzed to identify the Myod1 and transcription factor 12 (Tcf12) binding sites in the *Tas1r1* promoter region. Luciferase reporter assays, DNA affinity precipitation assays, and co immunoprecipitation assays were also performed to identify the functions of Myod1, Tcf12, and Krüppel like factor 5 (Klf5).

Results: Based on ENCODE ChIP seq, Myod1 bound to the *Tas1r1* promoter region containing E boxes 1 3. Luciferase reporter assays revealed that site directed E box1 mutations significantly reduced promoter activation induced by Myod1 overexpression. According to the DNA affinity precipitation assay and co immunoprecipitation assay, Myod1 formed a heterodimer with Tcf12 and bound to E box1. Further, Klf5 bound to the GT box near E box1, activating *Tas1r1* expression.

Conclusions: During myogenic differentiation, the Myod1/Tcf12 heterodimer, in collaboration with Klf5, binds to E box1 and activates *Tas1r1* expression.

1. Introduction

The basic helix loop helix transcription factor, myogenic differentiation 1 (Myod1), is expressed in skeletal muscles and is crucial for myogenic differentiation [1,2]. Myod1 functions as a heterodimeric partner of the E protein subfamily (transcription factor [Tcf] 4 and 12), and binds to the E box sequence element (CANNTG) in adult skeletal muscle tissue or C2C12 myoblasts [3]. The target genes of Myod1 participate in transcriptional control, muscle development, and synaptic function. The genes involved in myogenic differentiation have been identified via mouse promoter DNA microarray and chromatin immunoprecipitation (ChIP) on chip analysis. Myod1 regulates 61 genes in both myoblasts and myotubes, including taste receptor type 1 member 1 (Tas1r1) [4]. Tas1r1 and Tas1r3 combine to form a heterodimer (Tas1r1/Tas1r3) that binds to L amino acids [5].

Tas1r1/Tas1r3 functions as a taste receptor in taste buds and a direct amino acid sensor in other tissues, including muscle tissues [6,7]. Tas1r1/Tas1r3 detects extracellular amino acids and activates the mammalian target of rapamycin1 (mTORC1) to inhibit autophagy [7]. Satellite cells are regarded as the main stem cells responsible for muscle maintenance and repair in adult skeletal muscles [8]. Tas1r1/Tas1r3 expression in primary satellite cells

Abbreviations: ChIP-seq, chromatin immunoprecipitation and sequencing; DAPA, DNA affinity precipitation assay; Klf5, Krüppel-like factor 5; Myod1, myogenic differentiation 1; Tas1r1, taste receptor type 1 member 1; Tcf12, transcription factor 12.

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increases with their differentiation [9]. Furthermore, Tas1r1/Tas1r3 is expressed during C2C12 myogenic differentiation [10–12]. In particular, *Tas1r1* expression was found to markedly increase dur ing C2C12 myogenic differentiation [12]. Correct myogenic differ entiation requires finely tuned and balanced autophagy [13,14]. Tas1r1/Tas1r3 is therefore likely to participate in the regulation of autophagy during myogenic differentiation.

Although Myod1 regulates *Tas1r1* in both myoblasts and myo tubes during myogenic differentiation [4], its specific functions and heterodimeric partner in *Tas1r1* transcriptional regulation have not been previously described. Krüppel like factor 5 (Klf5) participates in the transcriptional regulation of *Tas1r1* during C2C12 myogenic differentiation by binding to the GT box (CCACCC) in the *Tas1r1* promoter, thereby activating *Tas1r1* expression [12]. Klf5 binds to Myod1 and directly regulates muscle specific genes involved in muscle differentiation [15]. To elucidate these mechanisms, we assessed the role of Myod1 in *Tas1r1* transcriptional regulation by determining whether Klf5 bound to Myod1 activates *Tas1r1* expression in myoblasts and myotubes.

2. Materials and methods

2.1. ChIP and sequencing (ChIP seq)

ChIP seq data for the Myod1 and Tcf12 binding sites in myo genically differentiated C2C12 cells (at 0, 24, and 60 h, and 6 d) were obtained from the ENCODE/Caltech database [16]. The UCSC genome browser was used to map the binding sites on the mm9 mouse genome [17,18].

2.2. Database search

The conservation of the E box1–3 sequences of various mammalian *Tas1r1* genes was assessed using the comparative ge nomics tool (UCSC genome browser; https://genome.ucsc.edu/cgi bin/hgGateway?redirect manual&source genome.ucsc.edu).

2.3. Cell culture

The RIKEN BioResource Research Center provided C2C12 myo blasts (RCB0987) via the National BioResource Project of the Min istry for Education, Culture Sports, Science and Technology (MEXT), Japan. C2C12 myoblasts were cultured in Dulbecco's modified Ea gle's medium (DMEM) (Wako, Osaka, Japan) containing 10% fetal bovine serum, and then differentiated into myotubes in DMEM containing 2% horse serum (Sigma–Aldrich, Saint Louis, MO). The differentiation medium was changed daily. C3H10T1/2 cells were cultured as previously described [10].

2.4. Plasmid construction

Mouse *Tas1r1* BAC clone (RP 23 37G1; Advanced GenoTechs, Tsukuba, Japan) genomic DNA was used to amplify the mouse *Tas1r1* promoter region (from 94 to +101 bp) via PCR; the primer set employed is shown in Table S1. The C2C12 transcriptional start site was indicated as +1. The PCR products were separated by gel elec trophoresis and purified. The purified DNA was digested with *Kpn*I and ligated into *Kpn*I/*Eco*RV digested pGL4.10 (Promega, Madison, WI), which was used as a template for site directed mutagenesis of a start codon (ATG to ATT) in *Tas1r1* to form pGL 94/+101. The mu tation was introduced using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan) with the primer set shown in Table S1. This mutation was confirmed using pGL 94/+101 sequence analysis.

Additional site directed mutagenesis was then performed to generate pGLmEbox1, pGLmEbox2, pGLmEbox3, pGLmEbox1+3, and pGLmGT, using pGL 94/+101 as a template, and the primer set shown in Table S1. E box1, E box2, and E box3 (Fig. 1D) were deleted (CANNTG to NNTG), and the GT box (Fig. 1D) was mutated (TCCCACCCC to TCCCAAACC). Mouse *Myod1* cDNA (BC103613; Transomic Technologies, Huntsville, AL, USA) was used to amplify the *Myod1* coding region via PCR using the primer set shown in Table S1. Gel electrophoresis was then performed to separate and purify the PCR products. The purified DNA was then digested with *Eco*RI and *Xba*I, and ligated into *Eco*RI/*Xba*I digested pCMV SPORT6 to form pCS6 m*Myod1*. Human *TCF12* cDNA (BC050556; Transomic Technologies) was used to amplify the TCF12 coding region via PCR using the primer set shown in Table S1. The PCR products were separated by gel electrophoresis and purified. The purified DNA was ligated into *Eco*RI/*Xba*I digested pCMV SPORT6 using the NEBu ilder HiFi DNA Assembly Master Mix (New England Biolabs, Ips wich, MA) to form pCS6 h*TCF12*. To design *Myod1* specific shRNA, target site DNA sequences were selected using the siRNA Wizard software (https://www.invivogen.com/sirnawizard/). Annealed shRNA targeting Myod1 sequences (Table S2) were cloned into the pSilencer 2.1 U6 puro vector (Ambion, Austin, TX) between the *Hind*III and *Bam*HI sites to construct the pSilp *Myod1* plasmid. The plasmid was sequenced to validate its sequence and orientation.

2.5. Luciferase assays

C2C12 and C3H10T1/2 cells were co transfected with *Tas1r1* promoter reporter plasmids and the internal control reporter plasmid, pGL4.74 (Promega), using ViaFect Transfection Reagent (Promega) in 24 well plates. Luciferase activity was determined 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega) with passive lysis buffer (Promega).

2.6. Generation of C2C12 Myod1 knockdown derived cell lines

C2C12 cells were transfected with the *Myod1* shRNA containing plasmid, pSilp *Myod1*, and the negative control shRNA containing the pSilencer 2.1 U6 puro vector (Ambion), using ViaFect Trans fection Reagent (Promega) in 3.5 cm dishes. We used a pSilencer puro vector expressing an shRNA sequence with limited homology to any known mouse genome sequence as a negative control. Stable cell lines were selected using puromycin resistance (3 μg/mL). Total RNA and protein were then prepared from stable cell lines during myogenic differentiation.

2.7. RNAi based gene silencing

Two sequential steps of siRNA transfection were performed for Tcf12 knockdown [19]. C2C12 cell suspensions were transfected with 25 nmol/L MISSION Universal Negative Control (UNC) siRNA (SIC001) and mouse *Tcf12* siRNA (SASI_Mm01_00172489) (Sigma Genosys, Ishikari, Japan), using Lipofectamine RNAi MAX (Invi trogen, Carlsbad, CA) in 12 well plates. The medium was changed after 16 h and culture was carried out for another 8 h. The second transfection of siRNA was executed similar to the first transfection. In the analysis of differentiated myogenic C2C12 cells, the medium was replaced with DMEM containing 2% horse serum and incu bated for 24 h after re transfection. Total RNA and protein were then prepared at 24 h and 60 h, respectively, after the first differ entiation medium was changed.

2.8. Transfection and qRT–PCR

Myod1 and *TCF12* expression plasmids, or the empty expression vector, were transfected into C2C12 and C3H10T1/2 cells using ViaFect Transfection Reagent (Promega) in 12 well plates. After

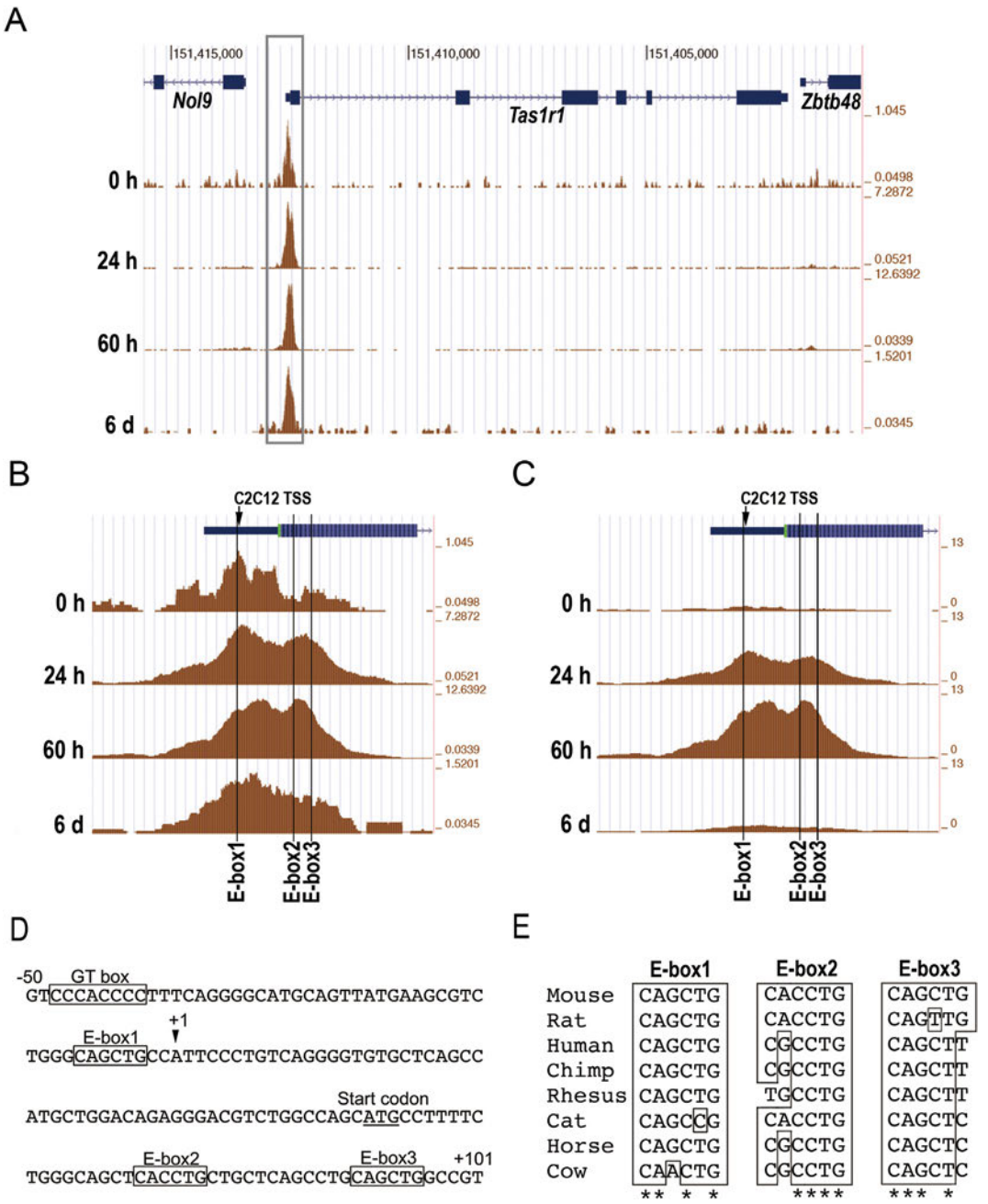

Fig. 1. ENCODE ChIP-seq data depict Myod1 binding to the *Tas1r1*-promoter E-box elements during C2C12 cell myogenic differentiation (at 0, 24, 60 h, and 6d). (A) UCSC genome browser illustration of Myod1 binding to the entire *Tas1r1* sequence and surrounding genes (*Nol9* and *Zbtb48*) during C2C12 myogenic differentiation. Rectangle: regions represented by (B) and (C). (B) UCSC genome browser illustration of Myod1 binding to *Tas1r1* exon 1 during C2C12 myogenic differentiation. Lines: E-box positions. Arrow: transcription start site of C2C12 cells. (C) The vertical lines in (C) were adjusted based on the maximum values of (B). (D) Distribution of E-box elements near the mouse *Tas1r1*-promoter start codon. Arrowhead: transcription start site (+1). The GT box and E-box elements are boxed. (E) Cross-species *Tas1r1*-promoter E-box conservation. Asterisks (*) indicate invariant bases in these genomes.

24 h of culture, total RNA was prepared using the FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan). Genomic DNA contaminants were removed using TURBO DNase (Ambion). Two micrograms of total RNA was converted to cDNA using the Superscript VILO cDNA synthesis kit (Invitrogen). qRT–PCR was then conducted using the Eco Real Time PCR System (Illumina Inc., San Diego, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and TaqMan probes targeting mouse *Tas1r1* and peptidylprolyl isomerase A (*Ppia*) genes were purchased from Applied Biosystems (*Tas1r1*, Mm00473433_m1; Ppia, Mm02342430_g1). The expression levels were normalized to those of *Ppia*.

2.9. DNA affinity precipitation assay (DAPA)

Nuclear C2C12 extracts were prepared as described by Hirata et al. (2019). Biotinylated DNA oligonucleotides containing the GT box and E box1 regions, with or without mutations, were

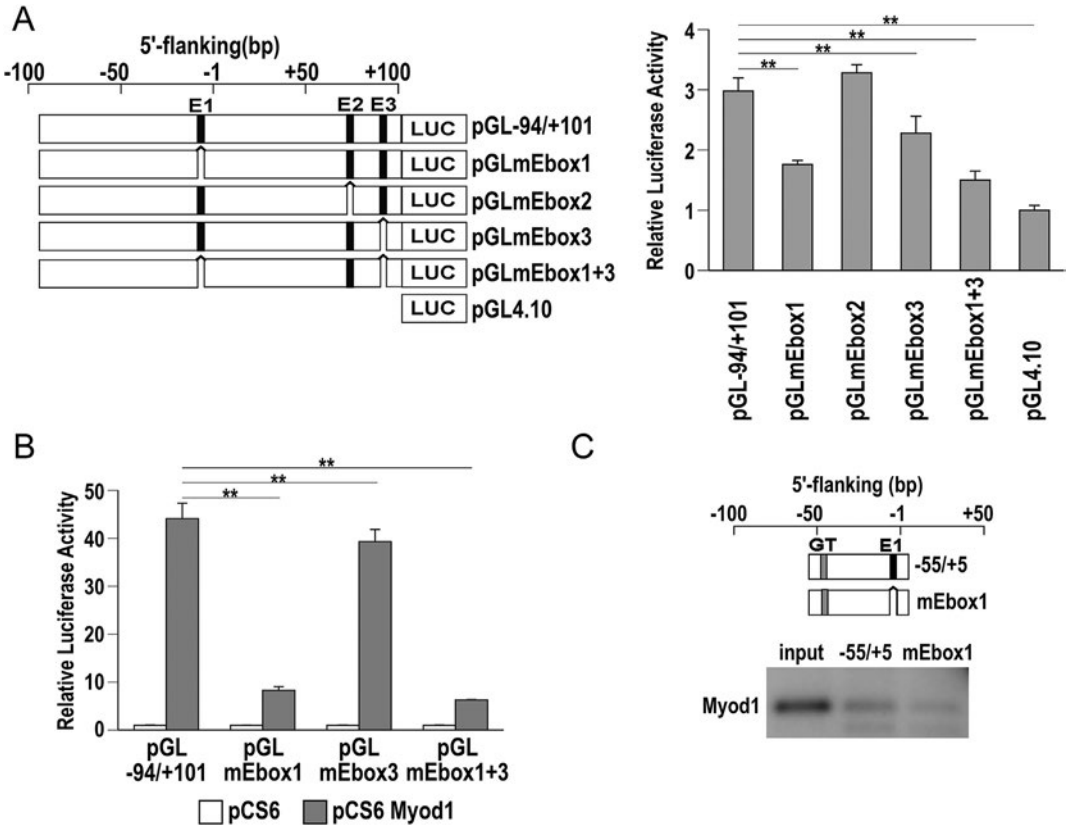

Fig. 2. Myod1 binds to *Tas1r1*-promoter E-box1. (A) Reporter-plasmid luciferase activity in the mouse *Tas1r1* promoter with deleted E-box elements. Results are expressed as mean ± SD of three biological replicates. $^{**}P < 0.01$. (B) Reporter-plasmid luciferase activity in the mouse *Tas1r1* promoter with deleted E-box elements, during Myod1 overexpression. pCS6: empty expression vector (pCMV-SPORT6). Results are expressed as mean ± SD of three biological replicates. $^{**}P < 0.01$. (C) DAPA analysis to confirm Myod1 binding to *Tas1r1*-promoter E-box1. Biotinylated double-stranded oligonucleotides containing E-box1 (−55/+5) or with E-box1 deletion (mEbox1) were used as probes. Myod1 binding to the oligonucleotide probes were examined using C2C12 cell nuclear extracts. DNA/Myod1 complex formation was analyzed by western blotting with anti-Myod1 antibodies. GT and E1: GT box and E-box1, respectively.

then annealed to produce the probes using the primer sets shown in Table S3. Thereafter, C2C12 nuclear extract (100 μg) was incubated with 2 μg wild type or mutant biotinylated probes in a binding buffer containing 20 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 10% glycerol, 0.2 mmol/L EDTA, 1.5 mM $MgCl_2$, 0.25% Triton X 100, 1 mmol/L dithiothreitol, 30 μg/mL poly(dI dC), and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) for 40 min at 4 °C, with gentle rotation. DNA−protein complexes were then incubated with 25 μL Streptavidin Mag neSphere Paramagnetic Particles (Promega) for Tcf4, Tcf12, and Klf5, or 25 μL MagCapture Tamavidin2 REV (Wako) for Myod1, for 30 min at 4 °C, with gentle rotation. Beads were then washed three times with the binding buffer for 10 min at 4 °C. There after, the beads were resuspended in 20 μL SDS sample buffer and boiled for 5 min. Proteins were separated by SDS PAGE and then Western blot analysis was performed using protein specific antibodies.

2.10. Immunoprecipitation

C2C12 cells were lysed using a lysis buffer containing 50 mmol/L Tris HCl (pH 7.5), 100 mmol/L NaCl, 1% NP 40, and protease inhibitor cocktail (Nacalai Tesque) for 30 min at 4 °C, with rotation. Centrifugation (5 min at 14,000 ×*g* and 4 °C) was then used to clear the lysates, and 600 μg of lysate was incubated with 2 μg normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or specific rabbit IgG, in an IP buffer containing 50 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 0.05% NP 40, and protease inhibitor cocktail (Nacalai Tesque) for 30 min at 4 °C, with gentle rotation. The lysate was then incubated with 20 μL Dynabeads Protein G (Dynal, Oslo, Norway) for 30 min at 4 °C with gentle rotation. The beads were washed three times with IP buffer for 10 min at 4 °C, resuspended in 20 μL SDS sample buffer, and boiled for 5 min. The proteins were run on SDS PAGE gels, and Western blot analysis was subsequently performed with protein specific antibodies.

2.11. Western blotting

The cell lysate was prepared by lysis in SDS sample buffer and cleared by centrifugation. Aliquots containing 20 μg protein were separated using 7.5% Tris glycine extended polyacrylamide gel electrophoresis (Bio Rad, Hercules, CA) and transferred to poly vinylidene fluoride membranes using the Trans Blot Turbo Transfer System (Bio Rad). After blocking with Bullet Blocking One (Nacalai Tesque) for 5 min, the membranes were incubated with protein specific antibodies (Table S4) for 12 h at 25 °C. After three washes with Tris buffered saline Tween 20 (TBST, containing 20 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20) for 5 min, the membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti mouse IgG or HRP conjugated goat anti rabbit IgG (1:25,000; Jackson Immuno Research, West Grove, PA) for 1 h. After three washes with TBST for 5 min, immunolabeled bands were detected using an Immobilon Western HRP Substrate

(Millipore, Bedford, MA), and the signals were recorded using an ImageQuant LAS 4000 Mini chemiluminescence image analyzer (Fujifilm, Tokyo, Japan).

2.12. Statistical analyses

Significance analyses were performed using the Student's *t* test (for two groups) or one way ANOVA with the Tukey–Kramer test (for multiple groups). For all statistical analyses, differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of Myod1 binding sites

During C2C12 cell myogenic differentiation (at 0, 24, 60 h, and 6 d), Myod1 binding was mainly observed at the first exon of *Tas1r1* (Fig. 1A). Myod1 binding signals were higher during the early stages of myogenic differentiation (0–60 h) than the later stages of the process (Fig. 1C). Three E box elements (E box1–3) were identified in the Myod1 binding region of *Tas1r1* (Fig. 1B, D). Mammalian cross species conservation of these E box elements was evaluated using a comparative genomics tool (UCSC genome browser). The CANNTG E box1 sequence was conserved in the mouse, rat, human, chimpanzee, rhesus, cat, horse, and cow genomes (Fig. 1E). However, in the cat genome, a single nucleotide substitution (CANNTG to CANNCG) was observed in E box1. E box2 was conserved in the mouse, rat, and cat genomes (Fig. 1E) while E box3 sequences were only conserved in the mouse and rat genomes (Fig. 1E).

3.2. Functional analysis of Tas1r1 promoter E boxes

To examine *Tas1r1* promoter E box functions, luciferase reporter assays were performed using *Tas1r1* promoter reporter plasmids, with or without mutated E box elements (Fig. 2A). The reporter activity of pGL 94/+101 was 3.0 fold higher than that of pGL4.10 (Fig. 2A). Further, the reporter activity was found to be significantly reduced by E box1 and E box3 deletion (Fig. 2A, pGLmEbox1 and pGLmEbox3). These results indicate that E box1 and 3 act as binding sites for *trans* regulatory elements.

We proceeded to determine the effect of Myod1 overexpression on luciferase activity, driven by the mouse *Tas1r1* promoter (94/+101) with or without E box deletion. Myod1 overexpression markedly increased luciferase activity (by 44.1 fold) relative to that of cells transfected with the empty expression vector (pCMV SPORT6) (Fig. 2B, pGL 94/+101). Further, the effect of Myod1 overexpression was significantly reduced by E box1 and 3 deletion (Fig. 2B, pGLmEbox1, pGLmEbox3, and pGLmEbox1+3). DAPA analysis was carried out to detect Myod1 binding to E box1 using the biotinylated probe of the *Tas1r1* promoter (55/+5, including E box1). Myod1 bound to the biotinylated probe, and E box1 deletion affected this binding (Fig. 2C), ultimately revealing the specific binding of Myod1 to E box1.

3.3. Myod1 activates Tas1r1 expression during C2C12 myogenic differentiation

To investigate the function of Myod1 in *Tas1r1* expression during myogenic differentiation (at 0, 24, and 60 h), C2C12 cell lines stably expressing *Myod1* shRNA and the negative control shRNA were analyzed. Myod1 knockdown during myogenic differentiation, in the *Myod1* shRNA stable expression C2C12 cell line, was confirmed via western blotting: Myod1 knockdown reduced the expression of a muscle specific marker, myosin heavy chain (MHC) (Fig. 3A). Myod1 knockdown was then analyzed using qRT–PCR to determine its role in regulating *Tas1r1* transcription during myogenic differentiation; this knockdown was found to non significantly reduce *Tas1r1* expression by 35.1% at 24 h, and significantly reduce *Tas1r1* expression by 70.4% at 60 h, relative to that of the negative control shRNA (Fig. 3B).

3.4. Tcf12 interacts with Myod1 and binds to E box1

To identify the Myod1 heterodimer partner, DAPA analysis was performed using the biotinylated probe of the *Tas1r1* promoter (55/+5, including the E box1), specifically to determine how Tcf4 and Tcf12 interact with Myod1. DAPA revealed that Tcf12 (but not Tcf4) bound to the biotinylated probe, and this binding was reduced by E box1 deletion, indicating the specific binding of Tcf12 to E box1 (Fig. 4A). ENCODE ChIP seq analysis revealed that Tcf12 binding mainly occurred in the first exon of *Tas1r1* during C2C12 cell myogenic differentiation (at 60 h; Fig. 4B). E boxes1–3 were identified in the Tcf12 binding region (Fig. 4C). In addition, co immunoprecipitation analysis confirmed the direct binding of Myod1 and Tcf12 (Fig. 4D). To investigate the function of Tcf12 in *Tas1r1* expression during myogenic differentiation (at 24 h and 60 h), C2C12 cells were transfected with *Tcf12* siRNA and MISSION UNC siRNA. Thereafter, Tcf12 knockdown was confirmed by western blotting (Fig. 4E), and its effects on *Tas1r1* expression were

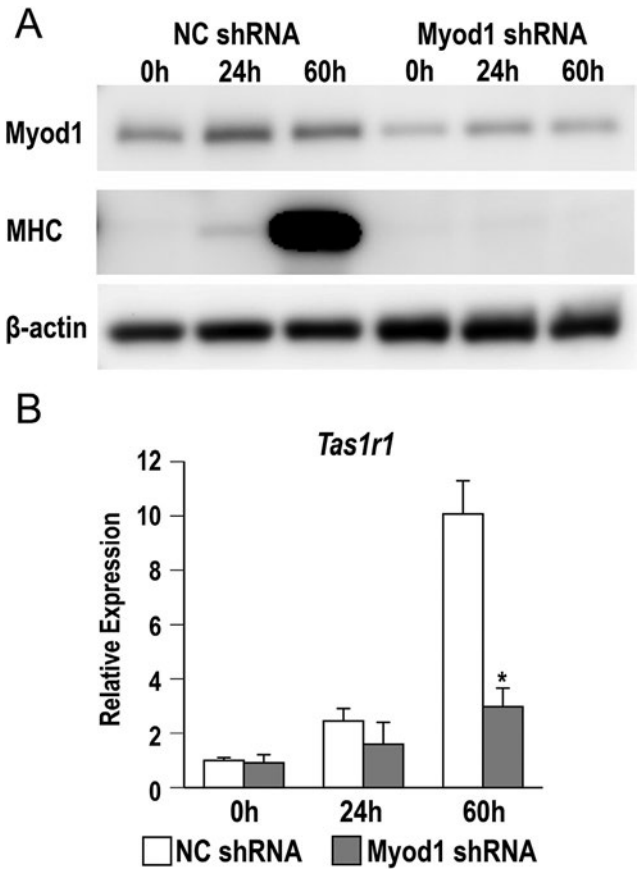

Fig. 3. Myod1 upregulates C2C12-cell *Tas1r1* expression during myogenic differentiation. (A) Western blotting of Myod1, myosin heavy chain (MHC), and β-actin in the negative control (NC) shRNA and *Myod1* shRNA stable-expression C2C12 lines during myogenic differentiation (at 0, 24, and 60 h). (B) qRT PCR of *Tas1r1* expression in the NC shRNA and *Myod1* shRNA stable-expression C2C12 cell lines during myogenic differentiation (at 0, 24, and 60 h). Results are expressed as mean ± SD of three biological replicates. *$P < 0.05$.

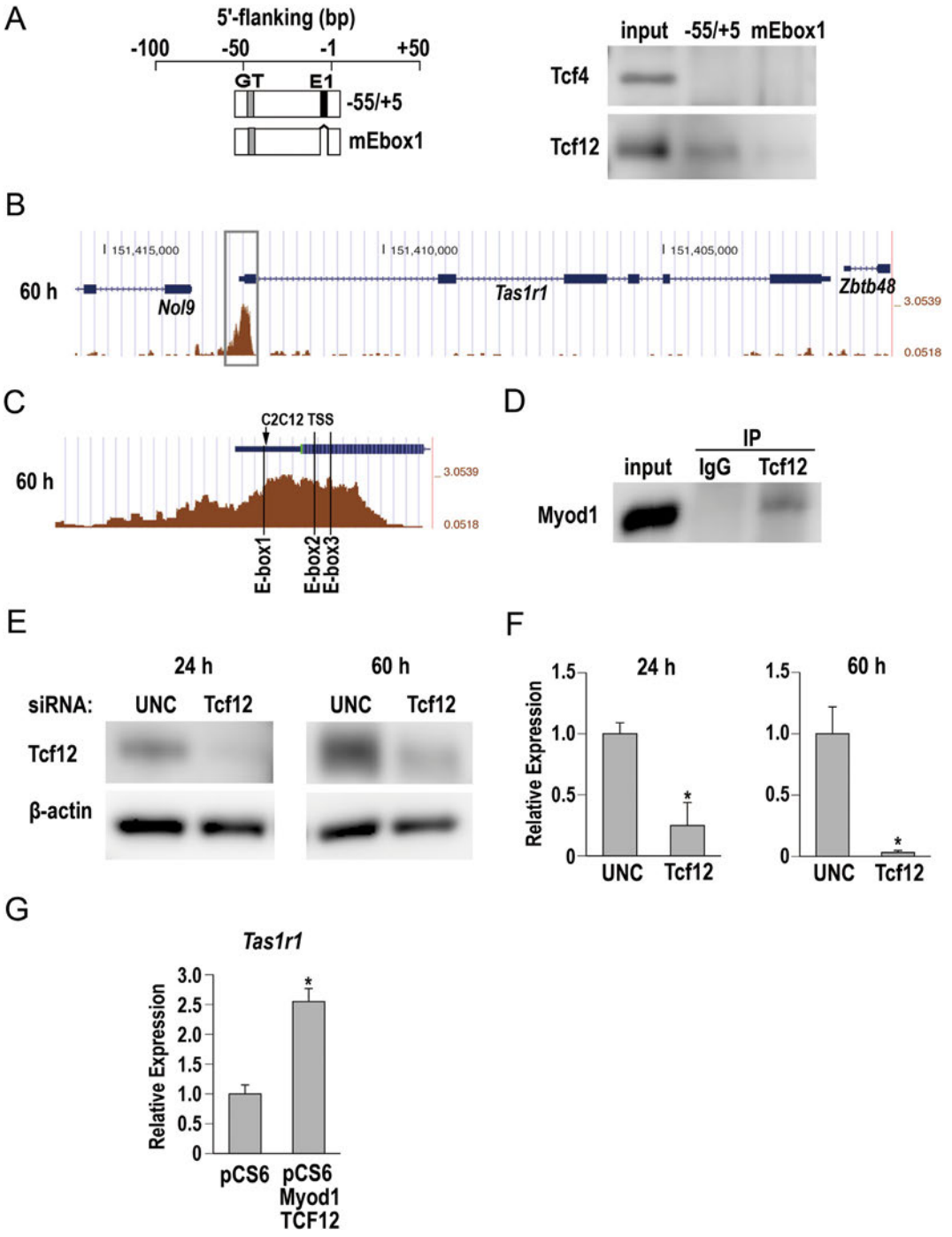

Fig. 4. The Myod1/Tcf12 heterodimer binds to *Tas1r1*-promoter E-box1. (A) DAPA confirmed Tcf4 and Tcf12 binding to *Tas1r1*-promoter E-box1. The biotinylated double-stranded oligonucleotides containing E-box1 (−55/+5), or without E-box1 (mEbox1), were used as probes. The binding of Tcf4 and Tcf12 to the oligonucleotide probe sequences was examined using C2C12 cell nuclear extracts. DNA/Tcf4 or DNA/Tcf12 complex formation was analyzed by western blotting with anti-Tcf4 or Tcf12 antibodies. GT and E1: GT box and E-box1, respectively. (B) UCSC genome browser illustration of Tcf12 binding to the entire *Tas1r1* sequence and surrounding genes (*Nol9* and *Zbtb48*), during C2C12 myogenic differentiation (at 60 h). Rectangle: region of (C). (C) UCSC genome browser illustration of Tcf12 binding to *Tas1r1* exon 1 during C2C12 myogenic differentiation (at 60 h). Lines: E-box positions. Arrow: transcription start site of C2C12 cells. (D) Coimmunoprecipitation of the Myod1/Tcf12 heterodimer in C2C12 cells. C2C12 whole-cell extracts were subjected to immunoprecipitation with anti-TCF2 antibodies. Input and coimmunoprecipitation fractions were subjected to western blotting with anti-Myod1 antibodies. (E) Western blotting of Tcf12 and β-actin in the universal negative control (UNC) and *Tcf12* siRNA-transfected cell lines cultured in differentiation medium, at 24 and 60 h. (F) qRT PCR analysis of *Tas1r1* expression in the UNC and *Tcf12* siRNA-transfected cell lines cultured in differentiation medium, at 24 and 60 h. Results are expressed as mean ± SD of three biological replicates. *$P < 0.05$. (G) qRT PCR of *Tas1r1* expression in Myod1-and Tcf12-overexpressing C2C12 cells. pCS6: empty expression vector (pCMV-SPORT6). Results are expressed as mean ± SD of three biological replicates. *$P < 0.05$.

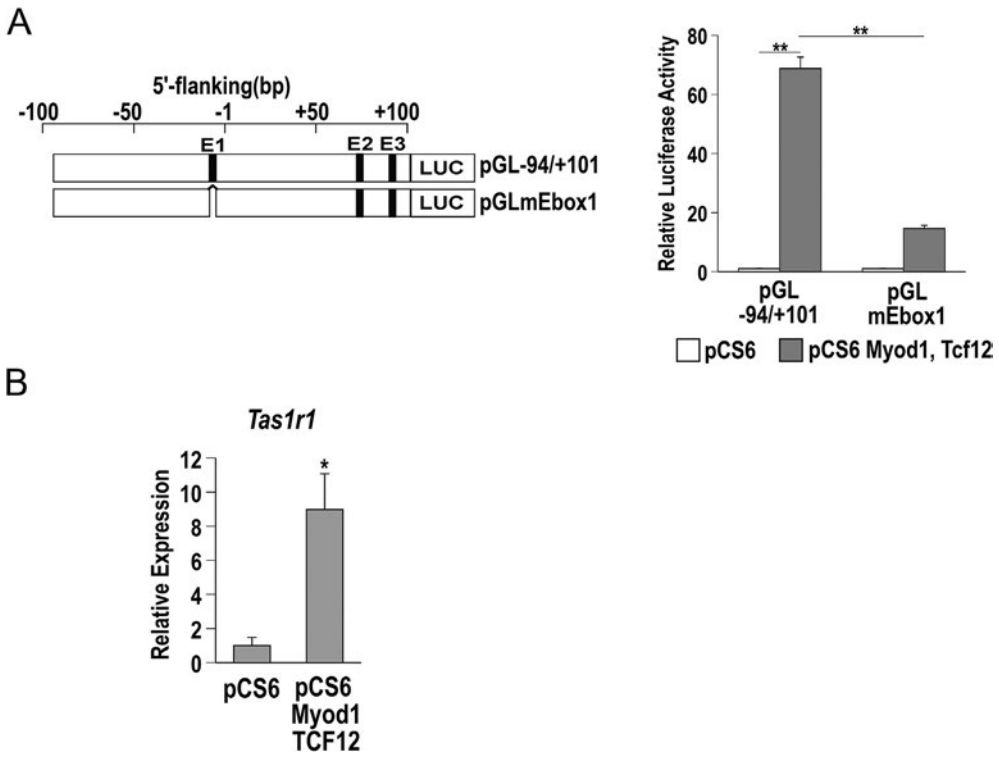

Fig. 5. Myod1 and Tcf12 bind to the *Tas1r1*-promoter E-box1 in C3H10T1/2 cells. (A) Luciferase activity of reporter plasmids in the mouse *Tas1r1* promoter with E-box1 deletion during Myod1 and Tcf12 overexpression. pCS6: empty expression vector (pCMV-SPORT6). Results are expressed as mean ± SD of three biological replicates. $^{**}P < 0.01$. (B) qRT PCR of *Tas1r1* expression in Myod1-and Tcf12-overexpressing C3H10T1/2 cells. pCS6: empty expression vector (pCMV-SPORT6). Results are expressed as mean ± SD of three biological replicates. $^{*}P < 0.05$.

analyzed using qRT−PCR. Tcf12 knockdown during myogenic dif ferentiation reduced *Tas1r1* expression by 75.2% at 24 h and 96.8% at 60 h, relative to that of the UNC siRNA (Fig. 4F). Myod1 and Tcf12 overexpression increased *Tas1r1* expression by 2.5 fold relative to that of the empty expression vector (pCMV SPORT6) transfected cells (Fig. 4G).

C3H10T1/2 cells have characteristics corresponding to multi potent mesenchymal progenitors [20] and can differentiate into myoblasts. C2C12 cells also endogenously express Myod1. However, Myod1 expression is low in C3H10T1/2 cells [21]. Therefore, we observed the transcriptional function of Myod1 in *Tas1r1* expression by overexpressing Myod1 and Tcf12 in C3H10T1/2 cells. Luciferase reporter assays were performed using *Tas1r1* promoter reporter plasmids with or without mutated E box1 elements (Fig. 5A). The effects of Myod1 and Tcf12 overexpression were significantly reduced by E box1 deletion (Fig. 5A). However, Myod1 and Tcf12 overexpression increased *Tas1r1* expression by 9.0 fold relative to that observed in pCMV SPORT6 transfected cells (Fig. 5B).

3.5. *Klf5 interacts with Myod1 and activates Tas1r1 expression*

To determine the function of the GT box and E box1 in the promoter activity of *Tas1r1*, luciferase reporter assays were per formed using *Tas1r1* promoter reporter plasmids with or without GT box mutation and E box1 deletion (Fig. 6A). The reporter activity was found to be substantially reduced by GT box mutation (Fig. 6A, pGLmGT) and E box1 deletion (Fig. 6A, pGLmEbox1), indicating that the GT box and E box1 act as binding sites for *trans* regulatory elements. Co immunoprecipitation analyses confirmed the direct binding between Myod1 and Klf5 (Fig. 6B). To assess the binding of Klf5 to the GT box, DAPA analysis was performed using the bio tinylated probe of the *Tas1r1* promoter (55/+5, including the GT box). Klf5 was found to bind to the probe; however, GT box deletion reduced this binding (Fig. 6C), indicating the specific binding of Klf5 to the GT box.

4. Discussion

Myod1 regulates *Tas1r1* in both myoblasts and myotubes during myogenic differentiation [4]; however, its binding site during the transcriptional regulation of *Tas1r1* has not been previously described. In the present study, Myod1 was found to bind to E box1 and activate *Tas1r1* expression during C2C12 myogenic differenti ation. Based on ChIP seq analysis, Myod1 binding to the *Tas1r1* region containing E box1−3 was higher during the early stages of myogenic differentiation of C2C12 than the late stages of the pro cess (Fig. 1C). *Tas1r1* expression was markedly elevated during C2C12 myogenic differentiation [12]. Moreover, Myod1 knockdown reduced *Tas1r1* expression during myogenic differentiation (Fig. 3B), and E box1 deletion reduced Myod1 transcriptional activation (Fig. 2B). Such findings suggest that *Tas1r1* expression increases proportionally with the binding of Myod1 to E box1 during early myogenic differentiation of C2C12 cells. E box ele ments regulate the expression of neuron , muscle , and pancreas specific genes [22]. Acetylcholine receptor genes, the myosin light chain gene, and the muscle creatine kinase gene require E box elements to enable full muscle cell activity. The function of E box1 in the transcriptional activation of *Tas1r1* expression is therefore similar to that of muscle specific gene E box elements. In addition, the presence of regulatory *cis* elements indicates evolutionary conservation between species [23]. Our findings indicate E box1 alignment conservation in the mouse, rat, human, chimpanzee, rhesus, cat, horse, and cow genomes, implying that the role of E box1 in *Tas1r1* expression is evolutionarily conserved.

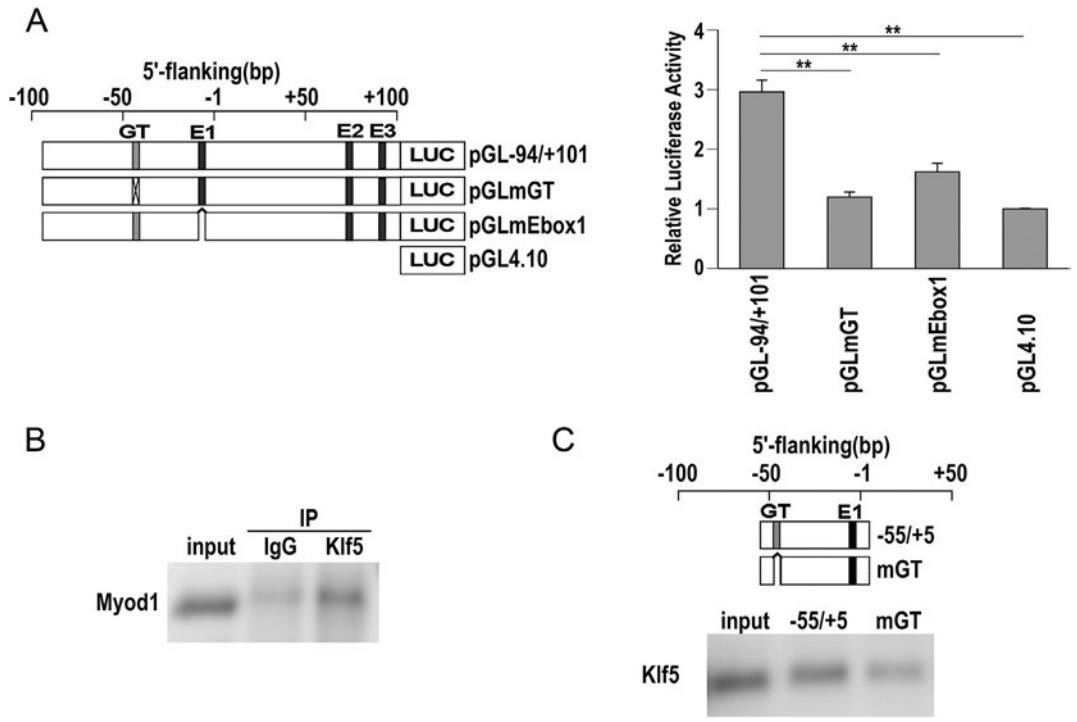

Fig. 6. Klf5 interacts with Myod1 and cooperatively activates *Tas1r1* expression. (A) Luciferase activity of the reporter plasmids in the mouse *Tas1r1* promoter with GT box mutation and E-box deletion. Results are expressed as mean ± SD of three biological replicates. $^{**}P < 0.01$. (B) Coimmunoprecipitation of Klf5 and Myod1 in C2C12 cells. C2C12 whole-cell extracts were subjected to immunoprecipitation using anti-KLF5 antibodies. Inputs and coimmunoprecipitation fractions were subjected to western blotting with anti-Myod1 antibodies. (C) DAPA confirmed Klf5 binding to the *Tas1r1*-promoter GT box. Biotinylated double-stranded oligonucleotides containing the GT box (−55/+5), or without the GT box (mGT), were used as probes. Klf5 binding to the oligonucleotide probe was examined using C2C12 nuclear extracts. DNA/Klf5 complex formation was analyzed by western blotting with anti-Klf5 antibodies. GT and E1: GT box and E-box1, respectively.

The Myod1 heterodimeric partner during the transcriptional regulation of *Tas1r1* in C2C12 myogenic differentiation has not been previously revealed. Herein, Myod1 and Tcf12 were found to form a heterodimer that binds to E box1 in the *Tas1r1* promoter during C2C12 myogenic differentiation. Moreover, DAPA analysis revealed the specific binding of Tcf12 to the *Tas1r1* promoter E box1 (Fig. 4A), and ENCODE ChIP seq analysis revealed the presence of E box1−3 sequences in the Tcf12 binding region of *Tas1r1* during C2C12 myogenic differentiation (at 60 h; Fig. 4C). Notably, co immunoprecipitation analyses revealed the direct binding of Myod1 to Tcf12 (Fig. 4D). Tcf12 knockdown reduced *Tas1r1* expression during myogenic differentiation (Fig. 4F), whereas Myod1 and Tcf12 overexpression increased *Tas1r1* expression in C3H10T1/2 cells, which usually express little Myod1, as well as C2C12 cells (Figs. 4G and 5B). These results confirm that the Myod1/Tcf12 heterodimer functions as a transcriptional activator of the *Tas1r1* promoter during myogenic differentiation. Myod1 and Tcf12 are expressed in C2C12 cells during early myogenic differentiation [24,25], with a Myod1/Tcf12 heterodimer binding to the E box in the myogenin (Myog) promoter, thereby leading to transcriptional activation [25]. Such findings indicate that the Myod1/Tcf12 heterodimer also plays a key role in early myogenic differentiation.

Klf5 regulates *Tas1r1* expression during C2C12 myogenic differentiation [12], recruits Myod1 to muscle specific target genes [15], and activates the expression of various genes that, together with Myod1, participate in myogenic differentiation. In the present study, Klf5 was found to bind to Myod1 to activate *Tas1r1* expression in C2C12 cells. *Tas1r1* promoter reporter assays revealed that GT box deletion reduced *Tas1r1* promoter activity in a similar manner to E box1 deletion (Fig. 6A). DAPA further revealed the specific binding of Klf5 to the *Tas1r1* promoter GT box (Fig. 6C), and co immunoprecipitation confirmed the direct binding of Myod1 to Klf5 (Fig. 6B). Collectively, these results indicate that Klf5 binds to Myod1 to activate *Tas1r1* expression.

In addition to Myod1, Myog also targets *Tas1r1* in C2C12 myotube cells [4]. Mouse promoter DNA microarray and ChIP on chip analysis were used to identify various overlapping and distinct targets of Myod1 and Myog in C2C12 myotube cells [4]. Myod1 and Myog regulate mouse *Tas1r3* expression in C2C12 cells [10]. Furthermore, Tas1r1 and Tas1r3 bind to form a heterodimer [5]. Therefore, the transcriptional regulation of *Tas1r1* and *Tas1r3* might occur concomitantly. Nonetheless, the functional role of Myog in *Tas1r1* transcriptional regulation remains to be elucidated.

5. Conclusions

In summary, this study revealed that the Myod1/Tcf12 heterodimer binds to E box1 and activates *Tas1r1* expression, in collaboration with Klf5. Furthermore, E box1 alignment was found to be conserved in several mammalian genomes, indicating that the role of E box1 in *Tas1r1* expression is evolutionarily conserved.

Ethics statement

Ethical approval was not required for this study.

Authorship contribution statement

Yui Obikane: Data acquisition, analysis and interpretation of data, drafting of the manuscript, and final approval of the manuscript. **Takashi Toyono:** Conception and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and

final approval of the manuscript. **Shoichiro Kokabu:** Conception and design, critical revision of the manuscript for important intel lectual content, and final approval of the manuscript. **Kae Matsuyama:** Acquisition of data, drafting of the manuscript, and final approval of the manuscript. **Shinji Kataoka:** Acquisition of data, drafting of the manuscript, and final approval of the manuscript. **Mitsushiro Nakatomi:** Acquisition of data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. **Ryuji Hosokawa:** Conception and design, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. **Yuji Seta:** Conception and design, critical revision of the manuscript for important intellectual con tent, and final approval of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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References

[1] Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 2005;132:2685 95.
[2] Yao Z, Fong AP, Cao Y, Ruzzo WL, Gentleman RC, Tapscott SJ. Comparison of endogenous and overexpressed MyoD shows enhanced binding of physiologically bound sites. Skelet Muscle 2013;3:8.
[3] Conway K, Pin C, Kiernan JA, Merrifield P. The E protein HEB is preferentially expressed in developing muscle. Differentiation 2004;72:327 40.
[4] Blais A, Tsikitis M, Acosta-Alvear D, Sharan R, Kluger Y, Dynlacht BD. An initial blueprint for myogenic differentiation. Genes Dev 2005;19:553 69.
[5] Yoshida R, Ninomiya Y. Taste information derived from T1R-expressing taste cells in mice. Biochem J 2016;473:525 36.
[6] Finger TE, Kinnamon SC. Taste isn't just for taste buds anymore. F1000 Biol Rep 2011;3:20.
[7] Wauson EM, Zaganjor E, Lee AY, Guerra ML, Ghosh AB, Bookout AL, et al. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol Cell 2012;47:851 62.
[8] Le Moal E, Pialoux V, Juban G, Groussard C, Zouhal H, Chazaud B, et al. Redox control of skeletal muscle regeneration. Antioxid Redox Signal 2017;27:276 310.
[9] Kobayakawa M, Nakajima T, Addison W, Matsubara T, Kokabu S. Tas1r1/Tas1r3 umami receptor expression levels are decreased in a serum starved muscle fiber model of atrophy. J Indian Prosthodont Soc 2018;18:S13.
[10] Kokabu S, Lowery JW, Toyono T, Seta Y, Hitomi S, Sato T, et al. Muscle regulatory factors regulate T1R3 taste receptor expression. Biochem Biophys Res Commun 2015;468:568 73.
[11] Zhou Y, Ren J, Song T, Peng J, Wei H. Methionine regulates mTORC1 via the T1R1/T1R3-PLCβ-Ca^{2+}-ERK1/2 signal transduction process in C2C12 cells. Int J Mol Sci 2016;17:1684.
[12] Hirata Y, Toyono T, Kokabu S, Obikane Y, Kataoka S, Nakatomi M, et al. Krüppel-like factor 5 (Klf5) regulates expression of mouse T1R1 amino acid receptor gene (*Tas1r1*) in C2C12 myoblast cells. Biome Res-Tokyo 2019;40:67 78.
[13] Fortini P, Ferretti C, Iorio E, Cagnin M, Garribba L, Pietraforte D, et al. The fine tuning of metabolism, autophagy and differentiation during *in vitro* myogenesis. Cell Death Dis 2016;7:e2168.
[14] Park S, Choi Y, Jung N, Kim J, Oh S, Yu Y, et al. Autophagy induction in the skeletal myogenic differentiation of human tonsil-derived mesenchymal stem cells. Int J Mol Med 2017;39:831 40.
[15] Hayashi S, Manabe I, Suzuki Y, Relaix F, Oishi Y. Klf5 regulates muscle differentiation by directly targeting muscle-specific genes in cooperation with MyoD in mice. eLife 2016;5:e17462.
[16] The Encode Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;489:57 74.
[17] Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res 2002;12:996 1006.
[18] Rosenbloom KR, Dreszer TR, Long JC, Malladi VS, Sloan CA, Raney BJ, et al. ENCODE whole-genome data in the UCSC Genome Browser: update 2012. Nucleic Acids Res 2012;40. D912 D7.
[19] Tapia O, Gerace L. Analysis of nuclear lamina proteins in myoblast differentiation by functional complementation. Methods Mol Biol 2016;1411:177 94.
[20] Taylor SM, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 1979;17:771 9.
[21] Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 1987;51:987 1000.
[22] Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol 2000;20:429 40.
[23] Liu Y, Liu XS, Wei L, Altman RB, Batzoglou S. Eukaryotic regulatory element conservation analysis and identification using comparative genomics. Genome Res 2004;14:451 8.
[24] Kontaridis MI, Liu X, Zhang L, Bennett AM. SHP-2 complex formation with the SHP-2 substrate-1 during C2C12 myogenesis. J Cell Sci 2001;114:2187 98.
[25] Parker MH, Perry RL, Fauteux MC, Berkes CA, Rudnicki MA. MyoD synergizes with the E-protein HEBβ to induce myogenic differentiation. Mol Cell Biol 2006;26:5771 83.