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Krüppel-like factor 5 (Klf5) regulates expression of mouse T1R1 amino acid receptor gene (*Tas1r1*) in C2C12 myoblast cells

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ABSTRACT

T1R1 and T1R3 are receptors expressed in taste buds that detect L-amino acids. These receptors are also expressed throughout diverse organ systems, such as the digestive system and muscle tissue, and are thought to function as amino acid sensors. The mechanism of transcriptional regulation of the mouse T1R1 gene (*Tas1r1*) has not been determined; therefore, in this study, we examined the function of *Tas1r1* promoter in the mouse myoblast cell line, C2C12. Luciferase reporter assays showed that a 148-bp region upstream of the ATG start codon of *Tas1r1* had a promoter activity. The GT box in the *Tas1r1* promoter was conserved in the dog, human, mouse, and pig. Site-directed mutagenesis of this GT box significantly reduced the promoter activation. The GT box in promoters is a recurring motif for Sp/KLF family members. RNAi-mediated depletion of Sp4 and Klf5 decreased *Tas1r1* expression, while overexpression of Klf5, but not Sp4, significantly increased *Tas1r1* expression. The ENCODE data of chromatin immunoprecipitation and sequencing (ChIP-seq) showed that Klf5 bound to the GT box during the myogenic differentiation. Furthermore, the *Klf5* knockout cell lines led to a considerable decrease in the levels of *Tas1r1* expression. Collectively, these results showed that Klf5 binds to the GT box in the *Tas1r1* promoter and regulates *Tas1r1* expression in C2C12 cells.

T1R1 and T1R3 (encoded by *Tas1r1* and *Tas1r3*) form the amino acid receptor that binds L-amino acids, including L-glutamate (17). Besides T1R1 and T1R3 expression in taste buds, these receptors are also expressed throughout diverse organ systems, including the digestive system and muscle tissue, and are considered as amino acid sensors (4, 11, 32). The heterodimer of T1R1 and T1R3 (T1R1/T1R3) is a direct sensor of the fed state and amino acid availability (32). Knockout of *Tas1r3* reduces the capacity of amino acids to signal to the mammalian target of rapamycin C1 (mTORC1) and induces autophagy. T1R1/T1R3 is also expressed at various differentiation periods of C2C12 cells (34). T1R1/T1R3-mediated amino acids regulate extracellular signal-regulated kinase 1 and 2 and mTORC1 through intracellular calcium increase in C2C12 myotubes. Furthermore, T1R1/T1R3 modulates amino acid-induced insulin secretion in pancreatic β-cells (19, 32).

Regulation of *Tas1r3* transcription has been investigated. Fushan *et al.* reported that a novel *cis*-acting element exists in a distal region of the human *TAS1R3* promoter (6), while human *TAS1R3* expression is regulated by the CCAAT-enhancer-binding protein β (C/EBPβ) in the cholangiocarcinoma cell

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Table 1 *Primers used for 5'-RACE and construction of luciferase assay plasmids*

Project/ primer name	Reverse strand (5'–3')	Forward strand (5'–3')
5'-RACE/ GRL	CAATGAGTGCCACCACCTTGGAGG	ACAGCGTGGTCAGTGTTATCAGGC
Luciferase assay plasmid/		
−886/+54	GGGGTACCGATCTCGCGAGACCGCGTCACC	GCTGGCCAGACGTCCCTCTGTCCAGCAT
−803/+54	GGGGTACCATCATCGGTGACTTCAGCC	GCTGGCCAGACGTCCCTCTGTCCAGCAT
−450/+54	GGGGTACCGCAAAGCCTGGTTTCAGGTTT	GCTGGCCAGACGTCCCTCTGTCCAGCAT
−94/+54	GGGGTACCACAGCCCGGAAGGCCAA	GCTGGCCAGACGTCCCTCTGTCCAGCAT
−37/+54	GGGGTACCAGGGGCATGCAGTTATGAA	GCTGGCCAGACGTCCCTCTGTCCAGCAT

line HuCCT1 (30). The myogenic regulatory factors MyoD and myogenin regulate *Tas1r3* promoter activity, and *Tas1r3* expression increases as myoblast C2C12 cells differentiate into skeletal muscle (14). Thus, several mechanisms have been elucidated for transcriptional regulation of *Tas1r3*; however, the mechanisms regulating *Tas1r1* transcription are unknown.

In this study, we investigated the mechanisms regulating the mouse *Tas1r1* gene using reporter assays, quantitative reverse transcription PCR (qRT-PCR), overexpression and RNAi knockdown assays, and chromatin immunoprecipitation sequencing (ChIP-seq) in C2C12 cells.

MATERIALS AND METHODS

Cell culture. The mouse myoblast cell line C2C12 (RCB0987) was provided by the RIKEN BioResource Center through the National BioResource Project of the MEXT, Japan. C2C12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. To induce myogenic differentiation, DMEM was replaced with DMEM containing 2% horse serum (Sigma-Aldrich, Saint Louis, MO, USA) when the density of the cells reached 90%. The medium was changed daily.

Rapid amplification of 5' cDNA ends (5'-RACE) analysis. Total RNA was extracted from C2C12 cells using a GenElute™ mammalian total RNA mini-prep kit (Sigma-Aldrich). Poly(A)$^+$-RNA was prepared from the total RNA of C2C12 cells using a GenElute™ mRNA Mini-prep Kit (Sigma-Aldrich). Two hundred and fifty nanograms of poly(A)$^+$-RNA was reverse transcribed and amplified using a GeneRacer™ Kit (Invitrogen, Carlsbad, CA, USA) with *Tas1r1* specific primers (Table 1). The PCR fragments were subcloned and sequenced.

Plasmids. The upper regions of mouse *Tas1r1* from −886 to +54 bp (Fig. 2A) were amplified by PCR using genomic DNA from a mouse *Tas1r1* BAC (RP-23-37G1; Advanced GenoTechs, Tsukuba, Japan) and the primers listed in Table 1. The PCR fragments were purified from agarose gels after electrophoretic separation, cleaved with *Kpn*I, and then ligated into *Kpn*I/*Eco*RV-linearized pGL4.10 (Promega, Madison, WI, USA) to form pGL−886/+54, pGL−803/+54, pGL−450/+54, pGL−94/+54, and pGL−37/+54. These plasmids were sequenced to check the correct sequences and orientations.

Human *Sp4* cDNA (40034749; Open Biosystems) was cleaved with *Kpn*I/*Xba*I, and ligated into *Kpn*I/*Xba*I-linearized pCMV-SPORT6 to form pCS6-hSp4. Human *KLF5* cDNA (BC042131; Open Biosystems) was cleaved with *Eco*RI/*Xho*I and inserted into *Eco*RI/*Xho*I-linearized pCMV-SPORT6 to form pCS6-hKLF5.

Site-directed mutagenesis. Site-directed mutagenesis was used to form pGLmEbox and pGLmGT1 using pGL−94/+54 as a template. Site-directed mutagenesis was also used to form pGLmGT2 using pGL−886/+54 as a template. The PrimeSTAR® Mutagenesis Basal Kit (TaKaRa, Otsu, Japan) and the primers listed in Table 2 were used to perform mutagenesis. The core sequence of a putative E box at bases −63 to −58 (shown in bold type) was deleted (**CAA**CTG to TG). The core sequence of a putative GT box at bases −50 to −41 was mutated (GTCCCACCCC to GTCCCA**A**ACC). After mutagenesis, sequence analyses of the mutated E box and GT box were performed to validate the mutation.

Luciferase assays. For reporter assays, C2C12 cells were plated in 24-well plates. The cells were co-transfected with the firefly luciferase reporter plasmids of

Table 2 *Primers used for site-directed mutagenesis and CRISPR-Cas9 mediated knockout study*

Project/ primer name	Reverse strand (5'–3')	Forward strand (5'–3')
Site-directed mutagenesis/		
Ebox del.	CCTTCCCATGGGCTCCAGTCCCACCC	GAGCCCATGGGAAGGCTGTTGGCCT
GT mut.	AGTCCCAAACCTTTCAGGGGCATGCAG	GAAAGGTTTGGGACTGGAGCCCAGTT
CRISPR-Cas9 mediated knockout study/		
*Klf5*KO1	CACCGTGCTCTGAAATTATCGGAAC	AAACGTTCCGATAATTTCAGAGCAC
CH1	CCTTCCTGACTGAGTCGGGTAGATTACGTG	AGGTGAGTGATGTCAGGGAGGAAGACGTTC
*Klf5*KO2	CACCGGAGGGGCCGTCGACTCGCTC	AAACGAGCGAGTCGACGGCCCCTCC
CH2	CCTGACATCACTCACCTGAGAACTGGCCTC	GATATTGTTCACCTCCTGTGGTGGCGCTGT

the *Tas1r1* promoter and the internal control *Renilla* luciferase reporter plasmid pGL4.74 (Promega) using Lipofectamine 3000 (Invitrogen). The cells were washed with phosphate-buffered saline (PBS) 24 h after transfection. Cell lysates were prepared with a passive lysis buffer (Promega). Luciferase activities were assayed using the Dual Luciferase Assay Kit (Promega).

RNAi-based gene silencing. We carried out two sequential steps of siRNA transfection to deplete Sp1, Sp3, Sp4, Klf2, Klf4, and Klf5 (29). Trypsinized C2C12 cell suspensions (2×10^5 cells) were transfected with 25 nM of each specific MISSION® siRNA (Sigma Genosys, Ishikari, Japan) using Lipofectamine RNAi MAX (Invitrogen). The transfected cells were seeded in 3.5-cm dishes. After being cultured for 16 h, the culture medium was replaced with fresh culture medium and the cells were incubated for 8 h. Re-transfection of siRNA was performed per abovementioned conditions. Total protein and total RNA were prepared 48 h after the second transfection.

The MISSION predesigned siRNAs were: MISSION siRNA Universal Negative Control (UNC; SIC001), mouse *Sp1* siRNA (SASI_Mm01_00145222), mouse *Sp3* siRNA (SASI_Mm01_00149247), mouse *Sp4* siRNA (SASI_Mm01_00067676), mouse *Klf2* siRNA (SASI_Mm02_00313949), mouse *Klf4* siRNA (SASI_Mm01_00104981), and mouse *Klf5* siRNA (SASI_Mm02_00316973).

Transfection and qRT-PCR. The expression plasmids of Sp4, Klf5 or the empty vector pCMV-SPORT6 were transfected to C2C12 cells using Lipofectamine 3000 (Invitrogen) in 6-well plates. Total RNA was prepared 24 h after the transfection. Following incubation with TURBO™ DNase (Ambion, Austin, TX, USA) to remove any contaminating genomic DNA, first-strand cDNA synthesis was performed by reverse transcription of 2 µg total RNA using a Superscript® VILO™ cDNA synthesis kit (Invitrogen). The cDNA was amplified by qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the Eco Real-Time PCR System (Illumina Inc., San Diego, CA, USA). TaqMan primers and probes (Applied Biosystems) were used for each of the following genes: *Tas1r1* (Mm00473433_m1), *Tas1r2* (Mm00499716_m1), *Tas1r3* (Mm00473459_g1), *Klf5* (Mm00456521_m1), *myogenin* (Mm00446194_m1), and *peptidylprolyl isomerase A* (*Ppia*) (Mm02342430_g1). The *Ppia* primers were used to normalize results obtained with gene-specific primers.

Immunoblot analysis. Transfected C2C12 cells or myogenically differentiated C2C12 cells were washed with PBS and lysed in sodium dodecyl sulfate sample buffer containing a protease inhibitor cocktail. The lysate was forced through a 20-gauge needle twenty times to reduce the viscosity of the solution. A 1/10 vol. 0.2 M dithiothreitol was added to each sample and heated at 99°C for 3 min prior to gel application. Aliquots containing 20 µg of protein were applied to 7.5% Tris–glycine extended-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA). The separated proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore Co., Bedford, MA, USA). The PVDF membranes were incubated with Blocking One (Nacalai Tesque) for 30 min and then incubated with anti-Sp1 rabbit polyclonal antibody (1 : 1,500 dilution; GTX110593; GeneTex, Irvine, CA, USA), anti-Sp3 rabbit polyclonal antibody (1 : 1,000 dilution; sc-644X; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sp4 rabbit polyclonal antibody (1 : 4,000 dilution; sc-645X; Santa Cruz Biotechnology), anti-KLF2 rabbit polyclonal antibody (1 : 1,000 dilution; 09-820; Millipore), anti-KLF4 rabbit polyclonal antibody (1 : 4,000 di-

lution; sc-20691X; Santa Cruz Biotechnology), anti-KLF5 rabbit polyclonal antibody (1 : 3,000 dilution; GTX103289; GeneTex), anti-Tas1r1 rabbit polyclonal antibody (1 : 2,000 dilution; sc-50308; Santa Cruz Biotechnology), or anti-β-actin mouse monoclonal antibody (1 : 10,000 dilution; sc-47778; Santa Cruz Biotechnology) for 12 h at 25°C. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 25,000 dilution; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. After three washes, the immunoreactive bands were made visible with an Immobilon Western HRP Substrate (Millipore) and detected using a luminescent image analyzer (LAS-4000 Mini; Fujifilm, Tokyo, Japan). The band intensities were quantified using the imaging software NIH Image J and normalized to β-actin.

ChIP-seq data. ChIP-seq data for Klf5 binding during myogenic differentiation (days 0, 2, and 5) in C2C12 cells (GSM2137967, GSM2137968, and GSM2137969) were downloaded from the Encyclopedia of DNA Elements (ENCODE) Consortium (2, 25). These ChIP-seq data were produced by the Department of Cellular and Molecular Medicine at the Tokyo Medical and Dental University (8). Reads were mapped to the mouse genome (mm9) using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTracks) (13, 23). We examined the loci proximal to *Tas1r1* for Klf5 during the myogenic differentiation process (days 0, 2, and 5) of C2C12 cells to identify Klf5-binding locations from the ChIP-seq datasets.

Generation of C2C12 Klf5 knockout-derived cell lines. CRISPRdirect (https://crispr.dbcls.jp) was used to analyze mouse *Klf5* (NM_009769.4) and design two different CRISPR-Cas guide RNAs with reduced off-target sites. The primers encoding the 25-nt guide sequences (Table 2) were annealed and ligated into a SpCas9(BB)-2A-Puro (pX459) V2.0 to form pX459-*mKlf5*KO1 and pX459-*mKlf5*KO2. The plasmid pX459 V2.0 was a gift from Feng Zhang (Addgene plasmid #62988). C2C12 cells were transfected with pX459-*mKlf5*KO1 or pX459-*mKlf5*KO2 using Lipofectamine 3000. After 24 h, cell selection was carried out using 3 μg/mL of puromycin. After a 2-day selection, 100 cells were plated into 10-cm dishes and left until colonies began to form (8 days) to obtain clones derived from a single cell. The genomic DNA of these clones was prepared using the HotSHOT method (31). The target regions of these clones were amplified by PCR using primers (CH1 and CH2) listed in Table 2. The PCR products were subcloned and sequenced to determine the mutated clones. The mutational efficiency was analyzed by immunoblot analysis of the cell extracts from the mutated clones.

Database searches. The 5'-flanking regions of the dog, human, mouse, pig, and rat *Tas1r1* genes were obtained via the Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=oryctolagus-cuniculus&group=glires). These regions were aligned using CLUSTALW (http://clustalw.genome.jp/).

Statistical analysis. For all studies, the Student's *t*-test was used for pair-wise comparisons. For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS

Determination of transcription start sites (TSSs)
The 5'-RACE analysis was performed to determine the TSSs of the *Tas1r1* gene. The amplified fragments acquired by 5'-RACE were subcloned and sequenced. Sequencing of 18 clones identified four distinct sites for the initiation of *Tas1r1* transcripts, starting at positions 54, 80, 95, and 116 bp upstream of *ATG* in the first exon (Fig. 1A). These sites were designated Sites 1–4, respectively. Site 2 is close to the previously reported TSS determined by cDNA cloning from tongue tissue (Site T; Fig. 1A) (9). Our results indicated that Sites 2 and 4 are the major TSSs, represented in 7 and 8 clones (Fig. 1A). The other two sites (Sites 1 and 3) are represented in 3 and 2 clones (Fig. 1A). Among these sites, Site 4 is indicated as +1.

The downstream promoter element (DPE) lies 30 bp downstream of the TSS. The initiator lies around the TSS. For Site 4, at nucleotide position 54 bp upstream of the ATG in the first exon, the initiator and DPE were found between −2 to +34 (Fig. 1A, B). In the other TSSs (Sites 1–3), the initiator and DPE were not found. The TATA box generally lies ~25–31 bp upstream of the TSS and has a consensus TATA(A/T)A(A/T) sequence. This DNA element was not located around these identified TSSs in the *Tas1r1* promoter (Fig. 1A).

The E box has a consensus CANNTG sequence and was located ~58–63 bp upstream of the Site 4 TSS (Fig. 1A). The GT box has a consensus CCACCC sequence and was located ~42–48 bp upstream of the Site 4 TSS (Fig. 1A). These boxes

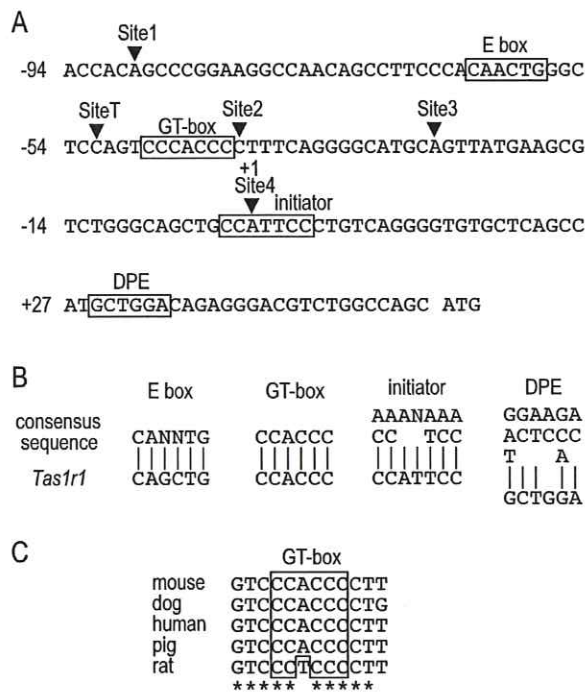

Fig. 1 Determination of the TSSs in the mouse *Tas1r1* in C2C12 cells. **(A)** The positions of TSSs were determined in the upstream region of the mouse *Tas1r1* using 5'-RACE. The arrowheads indicate the TSSs (Sites 1–4) and Site 4 is indicated as +1. The previously reported TSS, determined by cDNA cloning from tongue tissue, is designated as Site T. Putative DNA elements are boxed (E box, GT box, initiator, and downstream promoter element [DPE]). **(B)** Alignment of various regions in the mouse *Tas1r1* promoter with E box, GT box, initiator, and DPE consensus sequences. **(C)** Cross-species conservation of the GT box in the *Tas1r1* promoter. In the dog, human, pig, and rat species, the GT box (indicated by the box) is present in equivalent positions to that in the mouse *Tas1r1* promoter. In these GT boxes, only the rat GT box has the single nucleotide substitution (A to T). The asterisks show invariant bases in the five species.

were highly homologous to these consensus sequences (Fig. 1B). Next, we investigated whether the GT box in the mouse *Tas1r1* promoter is also present in upstream sequences of the *Tas1r1* gene from the dog, human, pig, and rat species. In these species, the GT box is present in equivalent positions to that in the mouse *Tas1r1* promoter. However, only the rat GT box has the single nucleotide substitution (A to T) (Fig. 1C).

Promoter activity of the upstream region of Tas1r1 in C2C12 cells

To identify *cis*-acting elements of the *Tas1r1* promoter, reporter assays were performed using progressive 5' truncations of the *Tas1r1* promoter driving the expression of a luciferase reporter (Fig. 2). Transfection of C2C12 cells with pGL−803/+54 showed an 18.2-fold higher reporter activity compared with transfection with pGL4.10. Deletion of the 5'-end from −886 to −803 bp resulted in a 77.7% decrease in the reporter activity (Fig. 2A, pGL−803/+54). Though deletion of the 5'-end from −857 to −94 bp did not change the reporter activity, additional deletion of the 5'-end from −94 bp to −37 bp resulted in an 87.2% decrease in the reporter activity (Fig. 2A, pGL−37/+54). The luciferase reporter assay showed that the 148-bp upstream region of ATG had promoter activity. These results indicated that the two regions (−886 to −803 and −94 to −37 bp) included *cis*-acting elements that are critically involved in *Tas1r1* promoter activity.

The putative E box and GT box were identified in the region −94 to −37 bp (Fig. 1A). To elucidate the *cis*-acting element from −94 and −37 bp and its effect on modulating promoter activity, additional reporter plasmids were constructed by site-directed mutagenesis of the E box and GT box. Reporter activity was slightly increased by the deletion of the E box (Fig. 2B, pGLmEbox). On the other hand, mutation of the GT box resulted in a 61.9% decrease in reporter activity (Fig. 2B, pGLmGT1), and the mutation of GT box in pGL-886/+54 resulted in a 63.6% decrease in reporter activity (Fig. 2C, pGLmGT2). These reduction data indicated that the GT box may be a binding site for *trans*-acting factors.

Knockdown of Sp/KLF family members by siRNA represses transactivation of the Tas1r1 promoter

Site-directed mutagenesis analysis showed that *trans*-acting factors bind the GT box of the *Tas1r1* promoter. The Sp/KLF family of transcription factors regulate gene expression by binding to GC or GT boxes in regulatory regions (24). To assess the role of Sp/KLF family members in the regulation of the *Tas1r1* promoter, we transfected C2C12 cells with siRNAs targeting *Sp1, Sp3, Sp4, Klf2, Klf4,* or *Klf5*. Fig. 3A shows a significant decrease in the protein levels of these factors. Transfection with *Sp4* and *Klf5* siRNA resulted in a 57.6% and 47% decrease in the level of *Tas1r1* expression relative to UNC (Fig. 3B). While transfection with *Sp1, Sp3, Klf2,* and *Klf4* siRNA increased the level of *Tas1r1* expression relative to UNC (Fig. 3B).

Transactivation of the mouse Tas1r1 promoter by Sp4 and Klf5

Next, we examined whether Sp4 or Klf5 activates

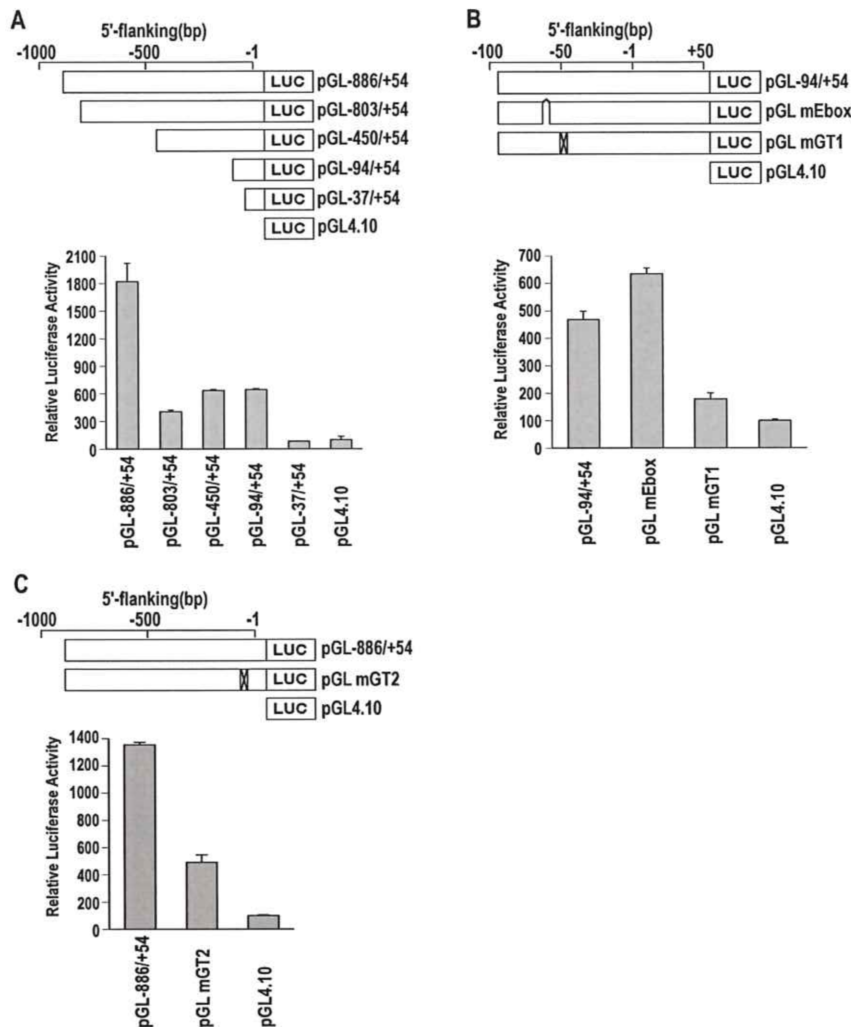

Fig. 2 Activities of the reporter plasmids of the mouse *Tas1r1* promoter in C2C12 cells. (**A**) Luciferase activities of the mouse *Tas1r1* promoter deletion plasmids. (**B**) Luciferase activities of the reporter plasmids (pGL-94/+54, pGLmEbox and pGLmGT1) of mouse *Tas1r1* promoter with mutated GT box or deleted E box. (**C**) Luciferase activities of the reporter plasmids (pGL-886/+54 and pGLmGT2) of mouse *Tas1r1* promoter with mutated GT box. The reporter plasmids were transfected into C2C12 cells. After 24 h transfection, the luciferase activity was measured. The plasmid pGL4.74 was co-transfected to monitor the transfection efficiency. The results are expressed as mean ± S.D. for three different experiments.

Tas1r1 transcription. C2C12 cells were transfected with expression constructs for these transcription factors. The qRT-PCR results showed that expression of Klf5 increased the mRNA levels of *Tas1r1* in C2C12 cells (Fig. 3C). Klf5 had a significant activating effect, causing a 1.4-fold increase in *Tas1r1* expression level compared with that of the empty expression vector pCMV-SPORT6 (Fig. 3C), though Sp4 had no activating effect (Fig. 3C). These data indicated that Klf5, but not Sp4, plays a role in the transactivation of the *Tas1r1* promoter.

ChIP-seq

ChIP-seq peaks for Klf5 around *Tas1r1* were identified using the UCSC Genome Browser. Major Klf5 peaks were detected within the upstream region of the *Tas1r1* first exon during the myogenic differentiation process (days 0, 2, and 5) (Fig. 4A). However, other peaks were not detected in the *Tas1r1* gene without the first exon. The GT box of the *Tas1r1* promoter existed in the region of major Klf5 peaks in days 2 and 5 (Fig. 4B). Klf5 bound to the GT box in days 2 and 5, but with slightly different occupancies. Klf5 bound to the GT box more exten-

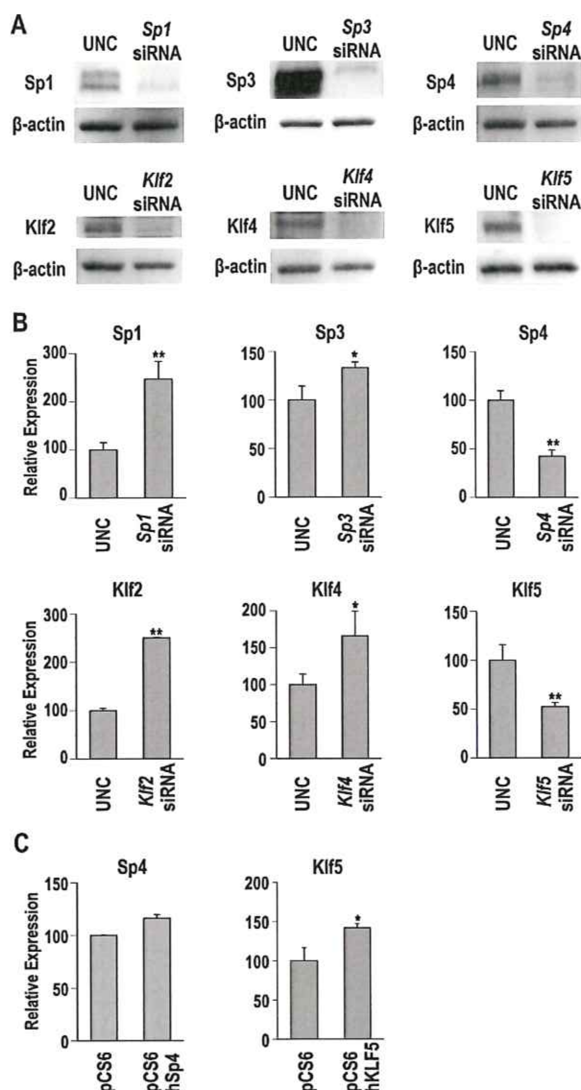

Fig. 3 Effects of knockdown and overexpression of Sp1, Sp3, Sp4, KLF2, KLF4, and KLF5 on the expression of *Tas1r1* in C2C12 cells. **(A)** C2C12 cells were transfected with *Sp1*, *Sp3*, *Sp4*, *Klf2*, *Klf4*, or *Klf5* siRNA, or with UNC, and the protein levels were analyzed by immunoblot analysis with the indicated antibodies. These siRNA significantly reduced the levels of these proteins. As a control, the expression of *β*-actin is shown. **(B)** The expression level of *Tas1r1* was measured 40 h after the second transfection using qRT-PCR. The results are expressed as mean ± S.D. for three different experiments. *$P < 0.05$ and **$P < 0.01$ when compared with UNC. **(C)** C2C12 cells were transfected with Sp4 or Klf5 expression plasmid. After 24 h transfection, the expression level of *Tas1r1* was quantified using qRT-PCR. The results are expressed as mean ± S.D. for three different experiments. *$P < 0.05$ when compared with the control vectors.

sively in day 2 than in day 0 (Fig. 4B).

qRT-PCR and immunoblot analyses of T1R family expression during the myogenic differentiation of C2C12 cells

C2C12 cells can be induced to differentiate into myoblasts by switching them to medium containing 2% horse serum. In this condition, the cells exhibited outstanding morphological changes over the course of 4–9 days, finally fusing into mature multinucleated myotubes (Fig. 5A). One of the myogenic regulatory factors, *myogenin*, was up-regulated during C2C12 myogenic differentiation. A 76.5-fold increase in *myogenin* mRNA levels was observed from day 0 to day 3 and a 125.4-fold increase from day 0 to day 9 (Fig. 5C). The morphological changes and the expression profile of *myogenin* during C2C12 myogenic differentiation were both in agreement with previous observations (10).

The *Tas1r1*, *Tas1r2*, and *Tas1r3* genes were expressed during C2C12 myogenic differentiation. *Tas1r1* and *Tas1r2* expression increased from day 0 to day 9 upon differentiation (Fig. 5B). A 23.1-fold increase in *Tas1r1* expression was observed from day 0 to day 3 and a 63.0-fold increase from day 0 to day 9 (Fig. 5B). A 1.7-fold increase in *Tas1r2* expression was observed from day 0 to day 9 (Fig. 5B).

Klf5 was also expressed during C2C12 myogenic differentiation. *Klf5* expression increased from day 0 to day 9 upon C2C12 myogenic differentiation (Fig. 5C). A 1.5-fold increase in *Klf5* expression was observed from day 0 to day 3 and a 3.3-fold increase from day 0 to day 9 (Fig. 5C).

Immunoblot analysis for Tas1r1 showed that expression of the Tas1r1 was detected in C2C12 cells at differentiation days 3 and 9 (Fig. 5D). Expression of Tas1r1 increased from day 0 to day 3 upon differentiation. This increase of the Tas1r1 protein level was in accordance with the increase of *Tas1r1* mRNA levels. Klf5 was expressed during C2C12 myogenic differentiation (Fig. 5D, E). The expression of Klf5 decreased from day 0 to day 3, in contrast to *Klf5* mRNA expression (Fig. 5D, E).

Knockout of Klf5 in C2C12 cells suppresses Tas1r1 expression at days 0 and 2 upon myogenic differentiation of C2C12 cells

To assess the role of Klf5 in the regulation of the *Tas1r1* promoter during myogenic differentiation of C2C12 cells, we generated the *Klf5* knockout cell lines (*Klf5*KO1 and *Klf5*KO2) in C2C12 cells using the CRISPR-Cas system (Fig. 6A). Fig. 6B shows a significant decrease in the Klf5 protein levels.

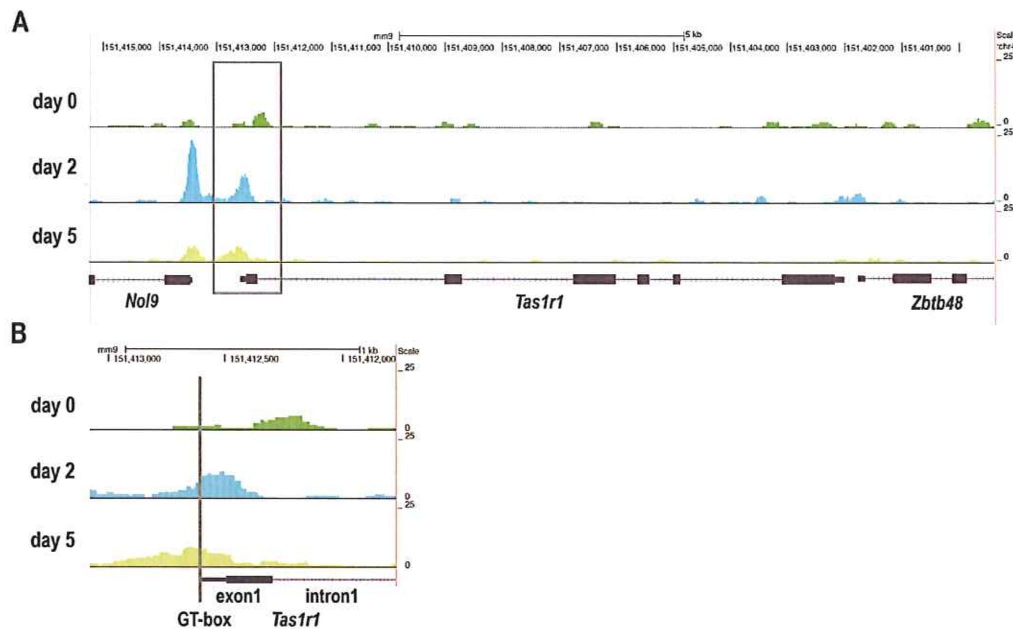

Fig. 4 ChIP-seq data from the ENCODE Consortium demonstrates Klf5 binding to the GT box in the *Tas1r1* promoter during the myogenic differentiation process (days 0, 2, and 5) of C2C12 cells. (**A**) UCSC genome browser view of Klf5 binding to the whole *Tas1r1* and surrounding genes (*Nol9* and *Zbtb48*) during the myogenic differentiation process of C2C12 cells is shown. The square shows the region of (B). (**B**) UCSC genome browser view of Klf5 binding to the exon 1 of *Tas1r1* during the myogenic differentiation process of C2C12 cells is shown. The line shows the position of the GT box.

Knockout of *Klf5* (*Klf5*KO1) resulted in a 79.0% and 83.6% decrease in the level of *Tas1r1* expression relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6C). Knockout of *Klf5* (*Klf5*KO2) also resulted in a 51.9% and 69.1% decrease in the level of *Tas1r1* expression relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6C). To confirm that the *Klf5* knockout decreases *Tas1r1* expression in *Klf5*KO1 and *Klf5*KO2, we examined the rescue of *Tas1r1* expression by forced expression of Klf5 in these clones (Fig. 6D). Klf5 had a significant activating effect, causing a 2.6- and 2.5-fold increase in *Tas1r1* expression levels in *Klf5*KO1 and *Klf5*KO2, compared with that of the empty expression vector pCMV-SPORT6. Taken together, these data indicated that Klf5 plays a role in the regulation of the *Tas1r1* expression.

To examine the effects on the myogenic differentiation of C2C12 by *Klf5* knockout, we examined the expression profiles of a myogenic regulatory factor, myogenin, at days 0 and 2 upon myogenic differentiation of *Klf5*KO1 by immunoblot analysis. Knockout of *Klf5* resulted in a 30.1% and 53.6% decrease in the protein level of myogenin relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation (Fig. 6E, F). These results showed that *Klf5* knockout effected on the myogenic differentiation of C2C12.

Immunoblot analysis for Tas1r1 in *Klf5*KO2 at differentiation days 2 showed that the Tas1r1 protein level was similar in C2C12 cells (Fig. 6G, H). This result showed that the decrease of the level of *Tas1r1* expression did not affect the Tas1r1 protein level in *Klf5*KO2.

DISCUSSION

Promoters can be classified in accordance with the distribution of the TSSs they use (16). Sharp promoters use only one or a few serial nucleotides as the TSSs, and often have a TATA box and an initiator. In contrast, a broad promoter can start transcription over a ~100 bp region, resulting in a population of mRNAs that have diverse sizes. Broad promoters usually regulate more widely expressed genes and are frequently TATA-less and CpG-island-enriched, while sharp promoters tend to regulate tissue-specific genes. The mouse *Tas1r1* promoter had four TSSs in a ~100 bp region and *Tas1r1* is also expressed in diverse tissues (32); therefore, it should be classified as a broad promoter.

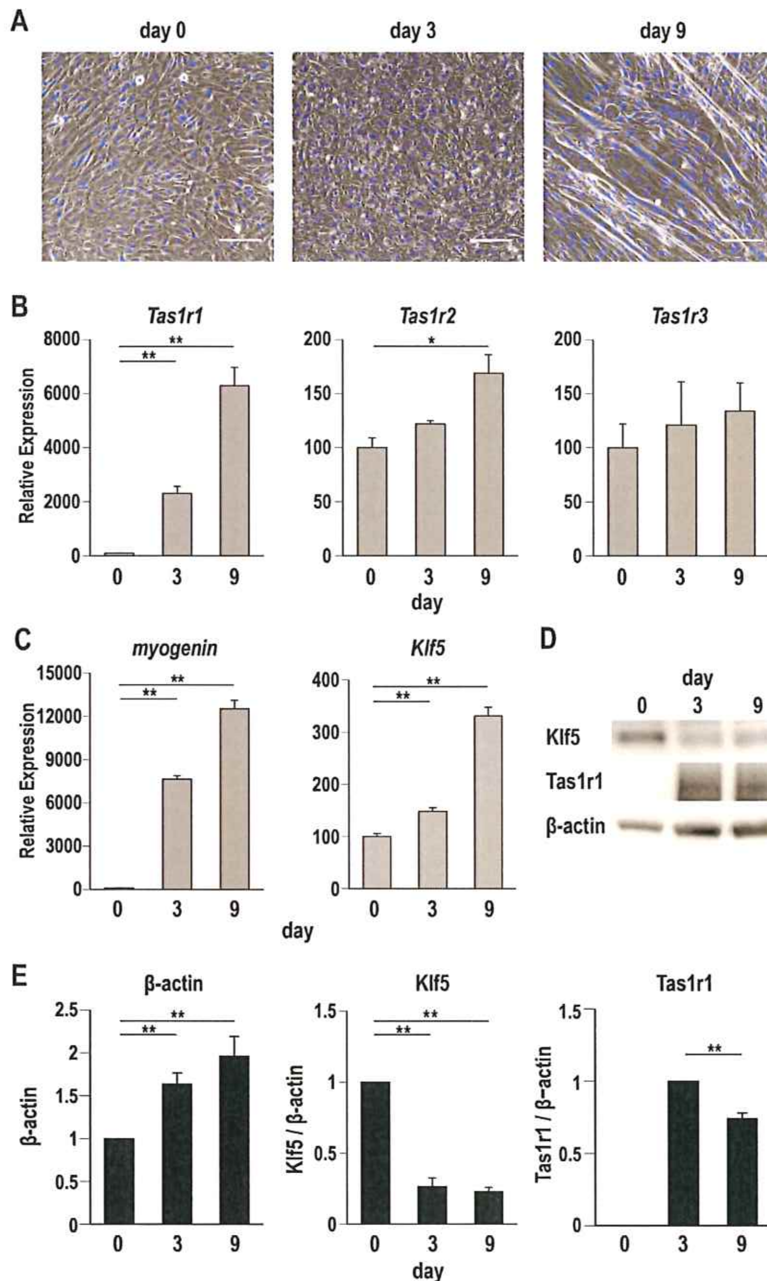

Fig. 5 Time-course changes in *Tas1r1*, *Tas1r2*, *Tas1r3*, *myogenin*, and *Klf5* expression during the myogenic differentiation process of C2C12 cells. C2C12 cells were cultured to confluence in growth medium, followed by culture in DMEM containing 2% horse serum. **(A)** The phase contrast images of the myogenic differentiation process of C2C12 cells are overlain with 4',6'-diamino-2-phenylindole counterstain. Scale bars denote 100 µm. **(B)** The expression levels of *Tas1r1*, *Tas1r2,* and *Tas1r3* were quantified by qRT-PCR. **(C)** The expression levels of *myogenin* and *Klf5* were quantified by qRT-PCR. The expression levels of these factors were normalized to *Ppia* expression. The results are expressed as mean ± S.D. for three different experiments. **P* < 0.05 and ***P* < 0.01 when compared with day 0. **(D)** The protein levels were analyzed by immunoblot analysis with the indicated antibodies. As a control, expression of β-actin is shown. **(E)** Immunoblot quantification of β-actin, Klf5, and Tas1r1 levels. The expression levels of Klf5 and Tas1r1 were normalized to β-actin. The results are expressed as mean ± S.D. for three different experiments. ***P* < 0.01 when compared with day 0. In the case of Tas1r1, ***P* < 0.01 when compared with day 3.

The luciferase reporter assay showed that the promoter region of mouse *Tas1r1* was located between −94 and +54 bp in C2C12 cells. The core promoter comprises elements that can elongate 35 bp upstream and/or downstream of the TSS (5, 26). Most core promoter elements seem to interplay directly with modules of the basal transcription machinery. The core promoter elements, an initiator, and a DPE but not the TATA box, were located in the *Tas1r1* promoter. Initiators can function independently of the TATA box. However in TATA-containing promoters, the initiator acts synergistically to enhance the efficiency of transcription initiation (1). In contrast to the TATA box, the DPE needs an initiator. Therefore, the initiator may function in collaboration with the DPE in the *Tas1r1* promoter.

The Sp/KLF family of transcription factors currently has 26 members (18). This family is charac-

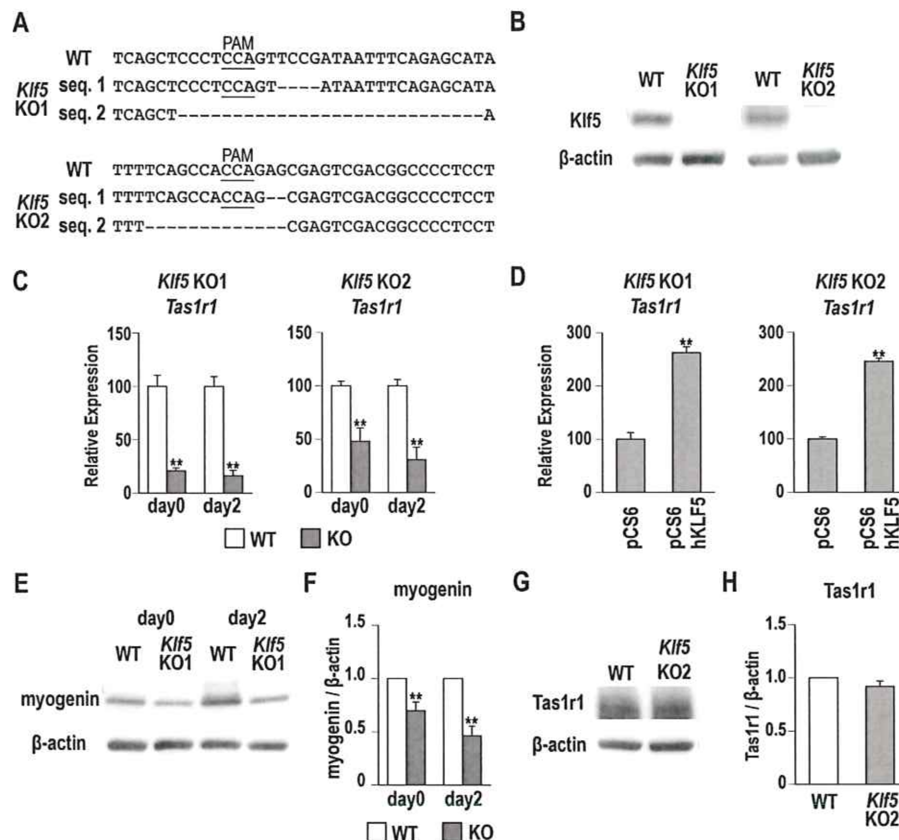

Fig. 6 Knockout of *Klf5* in C2C12 cells suppresses *Tas1r1* expression at days 0 and 2 of myogenic differentiation of C2C12 cells. (**A**) Sequencing data demonstrating the patterns of genomic editing in the *Klf5* knockout clones (*Klf5*KO1 and *Klf5*KO2). WT shows untreated C2C12 cells. Protospacer adjacent motif (PAM) underlined; the deletions are indicated by a hyphen (-). (**B**) The protein levels of the WT (C2C12 cells), *Klf5*KO1 and *Klf5*KO2 were analyzed by immunoblot analysis with the indicated antibodies. The level of Klf5 protein was significantly reduced in *Klf5*KO1 and *Klf5*KO2. As a control, expression of β-actin is shown. (**C**) The expression levels of *Tas1r1* were measured at days 0 and 2 of myogenic differentiation of WT (C2C12 cells), *Klf5*KO1, and *Klf5*KO2 using qRT-PCR. The expression levels were normalized to *Ppia* expression. The results are expressed as mean ± S.D. for three different experiments. $^{**}P < 0.01$ when compared with the wild-type clone. (**D**) Overexpression of Klf5 upregulates *Tas1r1* expression in *Klf5*KO1 and *Klf5*KO2. The expression levels of *Tas1r1* were quantified by qRT-PCR. The expression levels were normalized to *Ppia* expression. The results are expressed as mean ± S.D. for three different experiments. $^{**}P < 0.01$ when compared with the control vectors. (**E**) The protein levels of myogenin were analyzed at days 0 and 2 of myogenic differentiation of WT (C2C12 cells) and *Klf5*KO1 by immunoblot analysis with the indicated antibodies. (**F**) Immunoblot quantification of myogenin levels. The expression levels of myogenin were normalized to β-actin. The results are expressed as mean ± S.D. for three different experiments. $^{**}P < 0.01$ when compared with the wild-type clone. (**G**) The protein levels of the WT (C2C12 cells) and *Klf5*KO2 were analyzed by immunoblot analysis with the indicated antibodies. (**H**) Immunoblot quantification of Tas1r1 levels. The expression levels of Tas1r1 were normalized to β-actin. The results are expressed as mean ± S.D. for three different experiments.

terized by a highly conserved DNA binding domain near the C-terminus, which recognizes GC (consensus sequence: GGGGCGGGG) as well as GT/CACC (GGTGTGGGG) boxes. GC and GT boxes are important regulatory elements in the promoter region of many genes (20, 33). The *Tas1r1* promoter has a GT box located −48 to −42 bp upstream of the TSS. Important transcriptional regulatory elements often show conservation between species (15). Sequence alignment revealed conservation of the GT box in identical positions in the dog, human, mouse, and pig species. These results suggested that involvement of the GT box in *Tas1r1* transcription has been evolutionarily conserved.

Site-directed mutagenesis analysis showed that the *trans*-acting factors bound to the GT box of *Tas1r1* promoter. However, it was unclear which members of the Sp/KLF family bound to the GT

box of the *Tas1r1* promoter. Sp1, Klf2, and Klf4 all play a key role in the fusion process during skeletal muscle differentiation (27). In addition, Klf5 is an important factor in skeletal muscle regeneration and myogenic differentiation (8). In the Sp family, Sp1–4 have a very similar modular domain structure (33). Sp1, Sp3, and Sp4 can bind to GT and GC boxes; however, Sp2 has a weaker binding affinity to the GT box (12, 28). Therefore, we examined whether Sp1, Sp3, Sp4, Klf2, Klf4, or Klf5 activates *Tas1r1* transcription in C2C12 cells. RNAi-mediated depletion of Sp4 and Kllf5 led to a dramatic decrease in *Tas1r1* promoter activity. Overexpression of Klf5, but not Sp4, significantly increased *Tas1r1* expression. In addition, the ENCODE data of ChIP-seq showed that Klf5 binds to the GT box during myogenic differentiation. Furthermore, the *Klf5* knockout cell lines (*Klf5*KO1 and *Klf5*KO2) led to a considerable decrease in the levels of *Tas1r1* expression during the myogenic differentiation. These data indicated that Klf5 binds to the GT box of the *Tas1r1* promoter and regulates *Tas1r1* expression in C2C12 cells.

qRT-PCR analysis showed that *Tas1r1* expression was markedly increased after induction of C2C12 myogenic differentiation. In addition, immunoblot analysis for *Tas1r1* showed that protein expression increased from day 0 to day 3 upon differentiation. These expression patterns upon C2C12 myogenic differentiation were in agreement with previous observations (34). T1R1/T1R3 is an amino acid sensor and regulates mTORC1 and autophagy in a wide variety of tissues (32). mTOR is also a key regulator of skeletal myogenesis by governing multiple stages of myogenic differentiation (7). Therefore, the increased expression of *Tas1r1* may regulate mTORC1 in multiple stages of myogenic differentiation.

The CREB-binding protein (CBP/p300) is essential for muscle cell terminal differentiation, and CBP/p300 interacts with MyoD (21, 22). In addition, Klf5 and MyoD jointly modulate muscle differentiation by directly targeting muscle-specific genes in mice (8). Therefore, Klf5 may interact with CBP/p300 and MyoD in the activation of the *Tas1r1* promoter during C2C12 myogenic differentiation.

Tas1r1 expression was markedly increased during C2C12 myogenic differentiation. The knockout of *Klf5* (*Klf5*KO1 and *Klf5*KO2) resulted in a substantial decrease in the level of *Tas1r1* expression relative to that in C2C12 cells at day 0 and 2 upon myogenic differentiation. In addition, knockout of *Klf5* resulted in a significant decrease in the protein level of myogenin relative to the C2C12 cells at days 0 and 2 upon myogenic differentiation. This expression pattern of *Klf5* knockout clone upon C2C12 myogenic differentiation was in agreement with the previous report (8). Therefore, it is possible that the inhibition of C2C12 myogenic differentiation by *Klf5* knockout decreases the level of *Tas1r1* expression. The *Klf5* knockdown by *Klf5* siRNA in C2C12 myotubes will be necessary for revealing the exact functional role of Klf5 in the regulation of *Tas1r1* expression in a direct or indirect manner.

Klf5 was expressed during C2C12 myogenic differentiation; however, its expression decreased from day 0 to day 3. These results showed that Klf5 expression level was not consistent with that of *Tas1r1*. It is possible that *Tas1r1* expression during C2C12 myogenic differentiation is regulated by other *trans*-acting factors in addition to Klf5. Klf5 undergoes many post-translational modifications, which can alternatively regulate the transactivation capacity of Klf5 by changing its binding affinity for various co-regulators (3). Therefore, we deduced that the post-translational modifications of Klf5 modulated the regulation of *Tas1r1* expression during C2C12 myogenic differentiation.

In conclusion, we have demonstrated that Klf5 binds to the GT box and plays a role in the regulation of the transcriptional activity of *Tas1r1* in C2C12 cells. We also determined that the −886 to −803 bp region included *cis*-acting elements that are critically involved in *Tas1r1* promoter activity. However, it was unclear what kinds of the transcription factors bind to this region. Further studies are necessary to determine these *trans*-acting factors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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