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Transcriptional regulatory network of SOX4 during myoblast differentiation

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ABSTRACT

The construction of transcriptional regulatory networks of transcription factors (TFs) has become more important and attractive to understand the alterations of binding protein-dependent transcriptional activity that governs the changes in spatiotemporal expression of TF target genes required in various cellular processes. Therefore, identification of new inner modules including target genes and protein interactions involved in unveiled TF-based transcription networks is currently in the research spotlight. In this study, we reveal a possible SOX4-centered transcriptional network by the identification of novel binding partners and target genes of the TF SOX4 using various screening techniques. Lamin B2, barrier to autointegration factor 1, and apolipoprotein C-III were identified as novel interacting partners of SOX4 by yeast two-hybrid screening, and the genes encoding lysosomal-associated membrane protein 1, ubiquitin-conjugating enzyme E2S, and Map2k2 were identified as putative target genes of SOX4. Differently from the computational networks of TFs, we revealed a SOX4-centered physical network during myoblast differentiation. These results will provide opportunities to better understand the SOX4-centered transcriptional regulation network and TF-based specific gene expression in various cellular environments.

1. Introduction

The transcriptional regulatory networks that are currently available are likely to be a small representation of all the interactions that occur *in vivo* [1]. To understand how differential gene expression is controlled in genome-wide or regulatory-system levels in various cellular processes, it is important to identify the involved trans-acting factors and all cis-acting regulatory sequences of downstream target genes [2]. In addition, it is necessary to know how and when transcription factors interact with binding partners to affect their target gene expression [2]. Therefore, identification of novel protein interactions and new target genes is crucial to establish transcriptional regulatory networks in order to obtain a comprehensive understanding of the regulation of transcription factor-specific gene expression in various cellular processes [1–3].

The transcription factor SOX4 is a member of the family of sex-determining genes on the Y chromosome (SRY) [4]. The SOX family shares a highly conserved high-mobility group (HMG) box domain that can bind to the minor groove of DNA [5]. According to sequence comparisons among the SOX family, SOX proteins are divided into eight groups, and SOX4 is included in the SOXC group together with SOX11 and SOX12 [4,5]. It has previously been shown that SOX4 plays important roles in many developmental processes, including embryonic, cardiac, thymocytic, and nervous system development [6–8]. Moreover, *SOX4* has dual functions, as an oncogene or tumor suppressor, depending on the specific cancer cell type [9–13]. Although we recently reported caldesmon 1 (*CaD1*) and *PUMA* genes as new SOX4 target genes [14,15], precise molecular mechanisms by which the SOX4-centered transcriptional network affects gene expression has not been fully elucidated.

In contrast to SOX4, the number of identified SOX-binding proteins and target genes in previous reports suggest that regulation of the SOX-mediated network, consisting of activators and repressors, by protein–protein interactions is important for controlling expression of tissue- or cell type-specific genes [4,16,17]. For example, SOX2 in the SOXB1 group, known as a master protein

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for embryonic development [18], directly activates its target genes together with interacting proteins including PAX6, OCT4, or BRN2 [19–21]. Moreover, the number of interacting proteins and target genes of SOX8 or SOX10 in the SOXE group, or SRY in SOXA group, were identified using various screening approaches, leading to better understanding of their functions based on transcriptional regulatory network construction [17]. In this regard, identification of novel SOX4 binding partners and new target genes is important for understanding the novel transcriptional network of SOX4.

In this study, we identified several SOX4 target genes and possible interacting proteins using DNA-protein interaction- and protein–protein interaction-based analyses. This study suggests an important role for the SOX4-centered regulatory network relevant to various cellular processes, including embryonic development and tumorigenesis.

2. Materials and methods

2.1. Electrophoretic mobility shift assays (EMSA)

GST-SOX4 DBD fusion proteins and GST proteins were expressed in *Escherichia coli* strain BL21. Fusion proteins were purified using glutathione-Sepharose (GE Healthcare, Piscataway, NJ, USA), and their concentration was determined by Bradford assay using Bio-Rad protein assay kit (Bio-Rad, CA, USA) according to the manufacturer's instruction. Single-stranded complementary oligonucleotides were annealed and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The DNA sequences of the oligonucleotides corresponding to the conserved SOX4 element in the proximal promoter of CCNB1, LAMP1, UBE2S and Map2k2; for CCNB1, 5′-CGA TCA AAT GAA AGA ATG TCT ATT AAA GCC-3′ (sense) and 5′-GGC TTT AAT AGA CAT TGT TTC ATT TGA TCG-3′ (antisense); for LAMP1, 5′-CTC AAA AAA AAA AAC AAA GTG CTC GAC AGG-3′ (sense) and 5′-CCT GTC GAG CAC TTT GTT TTT TTT TTT GAG-3′ (antisense); for UBE2S, 5′-GGC GGC GTC TCA TTC AAA CGG TCC AAT CAG-3′ (sense) and 5′-CTG ATT GGA CCG TTT GAA TGA GAC GCC GCC-3′ (antisense); for Map2k2, 5′-TTT GTC AAT TAA AAT AAA TTT TAA AAA TGT-3′ (sense) and 5′-ACA TTT TTA AAA TTT ATT TTA ATT GAC AAA-3′ (antisense). EMSA was performed with 2 or 4 μg of GST or GST-fused SOX4 DBD protein in binding buffer (100 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 M KCl, 1 mM dithiothreitol, 50% glycerol and 100 ng μL^{-1} BSA). For competition or supershift assays, the indicated unlabeled oligonucleotide competitor or GST-antibody was added 30 min prior to addition of radiolabeled probe. After addition of the radiolabeled probe, the samples were incubated for 30 min at 30 °C and loaded on a 6.5% native polyacrylamide gel in 1 × Tris/Acetate/EDTA (TAE) buffer, electrophoresed, dried and exposed to X-ray film.

2.2. Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed following the protocol provided by Millipore (Temecula, CA). Briefly, the differentiating C2C12 cells for 4 days were cross-linked with 1% paraformaldehyde (#15710, Electron Microscopy Sciences, Hatfield, PA) in PBS for 10 min at 37 °C. The cells were then washed with ice-cold PBS and resuspended in 200 μl of SDS-sample buffer containing protease inhibitor mixture. The suspension was sonicated three times for 10 s with a 1-min cooling period on ice, after which it was precleared with 20 μl of protein A/G-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 °C. The beads were then removed, after which the chromatin solution of each experimental group was immunoprecipitated overnight with anti-SOX4 at 4 °C followed by incubation with 50 μl of protein A-agarose beads (Millipore) for an additional hour at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$), and formaldehyde cross-links were reversed by heating at 65 °C for 4 h. Proteinase K (P2308, Sigma) was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA were purified using the PCR purification kit (Qiagen, Valencia, CA) and then analyzed by quantitative PCR using promoter-specific primers of putative SOX4 target genes: for CCNB1, 5′-TCC GCG TTA GTG TTA CTG AAA A-3′ and 5′-GTC TGC CGG GCT TAG GTT TA-3′; for LAMP1, 5′-TCT TGA GTC TGA GGC CAA CC-3′ and 5′-TGG ACC ACG TGA TAT CTT GC-3′; for UBE2S, 5′-TCT GGC ACC CCT ACA GAA AC-3′ and 5′-GAG TTG ACC TGG GAG AAC CA-3′; for Map2k2, 5′-GCG GAG AAC TTC CTT GTG AG-3′ and 5′-CGT GTC CTA TAG GCG AGA GC-3′; for LAPTM4β, 5′-TCA GAA ATC CAC CTG CCT CT-3′ and 5′-CCC TGA GGG AGT CTC CAG AA-3′; for CLN3, 5′-TCA GGG TCC TGT CCT TCA GT-3′ and 5′-GGT TGC AGA AAT CGA ATG GT-3′; for PDCD4, 5′-GAC ACT GCA TGC AAT AGA CTC A-3′ and 5′-CTG CCT GTC TGG GTT CCT A-3′; for ARRβ2, 5′-ACT TCT GCC CAT CCT TCA GA-3′ and 5′-GCG TCC TCA CTC TTT CTC CT-3′.

2.3. Yeast two-hybrid screening

The yeast two-hybrid assay was performed using the Matchmaker Gold Yeast Two-Hybrid System from Clontech according to the manufacturer's instructions. DBD domain of SOX4 was cloned into the pGBKT7 yeast expression vector, digested with EcoRI and SalI, and then ligated. The resulting plasmid containing the DBD domain of SOX4 (1-145 amino acids) was used as bait for the library screening after auto-activation and toxicity experiments. To confirm bait expression in the yeast system, yeast proteins were prepared, and the lysates were Western blotted using GAL4 antibodies. To assess SOX4-protein interactions, a human hepatocarcinoma cDNA library, constructed in the pGADT7 vector and pretransformed into the *Saccharomyces cerevisiae* Y187 strain, was used as prey for mating. The mated, diploid yeast cells were grown on different stringency-selection media to select for colonies that involved bait–prey interactions. The positive colonies were collected and yeast plasmid DNA was extracted using glass beads and pGADT7-positive plasmids were transformed into *E. coli* XL-1 Blue. Plasmids were isolated and sequenced, and analyzed by BLASTn.

2.4. Immunoprecipitation and western blotting

Transfected HEK293 cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 1 mM PMSF. The cell suspensions were incubated on ice for 20 min and centrifuged at 12 000 rpm at 4 °C for 20 min. The supernatants were precleaned with 20 μl of protein A/G agarose bead (50% slurry) and then incubated at 4 °C overnight with 40 μl of fresh protein A/G bead in the presence of GFP or FLAG antibodies. The beads were washed 3 times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. The protein samples were electrophoresed on a 10% or 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, PROTRAN). The membrane was blocked with 5% skim milk in a solution of 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 and incubated with appropriate dilutions of the primary antibody at room temperature for 3 h. Samples were analyzed by Western blotting using the appropriate antibodies to detect interacting proteins. Monoclonal antibodies against green fluorescence protein (GFP-1814 460) and FLAG-M2 (F3165) antibodies were purchased from Roche Diagnostics and Sigma, respectively.

2.5. Immunofluorescence microscopy

HEK293 cells were grown on a sterile coverslip in 60-mm dishes and transfected with indicated expression vectors using Lipofectamine 2000. Twenty-four hours after transfection, immunocytochemistry was performed following a protocol provided by Abcam. Primary antibodies were used at 1:1000 for anti-FLAG M2 antibody (Sigma). Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories) was used as secondary antibodies. DAPI (Sigma) was used for nuclear staining. Samples were incubated with 300 nM DAPI in PBS for 2 min at room temperature and visualized using a Nikon Eclipse 80i microscope (Melville, New York).

3. Results and discussion

3.1. Identification of novel target genes of SOX4

To investigate novel target genes of SOX4, we curated differentially expressed genes from previously studied microarray data [22]. We only selected genes that have SOX4-binding motifs within their promoter region (between 0 and −1 kb) using a binding motif search program (TRANSFAC database; http://motif.genome.jp, or Searching Transcription Factor Binding Sites, Version 1.3; http://www.cbrc.jp/research.db/TFSEARCH.html). Fifty-one genes were identified as differentially expressed (more than two-fold) by SOX4. Out of 51 genes, we found 9 genes that have SOX4-binding sequences in their promoter region (Fig. 1A). To determine whether SOX4 regulates transcription of the selected genes, HEK293 cells were transiently transfected with GFP-fused SOX4 expression plasmids, and mRNA levels of putative target genes were analyzed by quantitative real-time PCR. As shown in Fig. 1B, overexpression of SOX4 induced lysosomal-associated membrane protein 1 (*Lamp1*), ubiquitin-conjugating enzyme E2S (*Ube2s*), lysosomal protein transmembrane 4β (*Laptm4β*), programmed cell death 4 (*Pdcd4*), and mitogen-activated protein kinase kinase 2 (*Map2k2*) transcription. As previously reported, we detected *Cald1* expression by SOX4 overexpression [14]. However, transcription levels of cyclin B1 (*Ccnb1*), ceroid-lipofuscinosis neuronal 3 (*Cln3*), and arrestin β2 (*Arrβ2*) were not significantly changed in a SOX4-dependent manner. To further confirm the mRNA expression in C2C12 myoblast cells, we used published RNA-seq results and looked at gene expression values at UCSC genome browser (Fig. 1C). Lamp1, Ube2s and Map2k2 genes are expressed highly in differentiated myoblast cells. Altogether, our results show that SOX4 regulates the transcription of these putative target genes.

3.2. Direct interactions of SOX4 with target gene promoters in vitro and in vivo

To test whether SOX4 directly binds to the SOX4-binding sequences in the promoter region of the selected genes, an electrophoretic mobility shift assay (EMSA) was performed with glutathione S-transferase (GST)-fused recombinant SOX4 DNA-binding domain protein (SOX4-DBD) using *Lamp1*, *Ube2s*, and *Map2k2* probes containing SOX4-binding sequences. As shown in Fig. 2A, addition of SOX4 produced a more slowly migrating

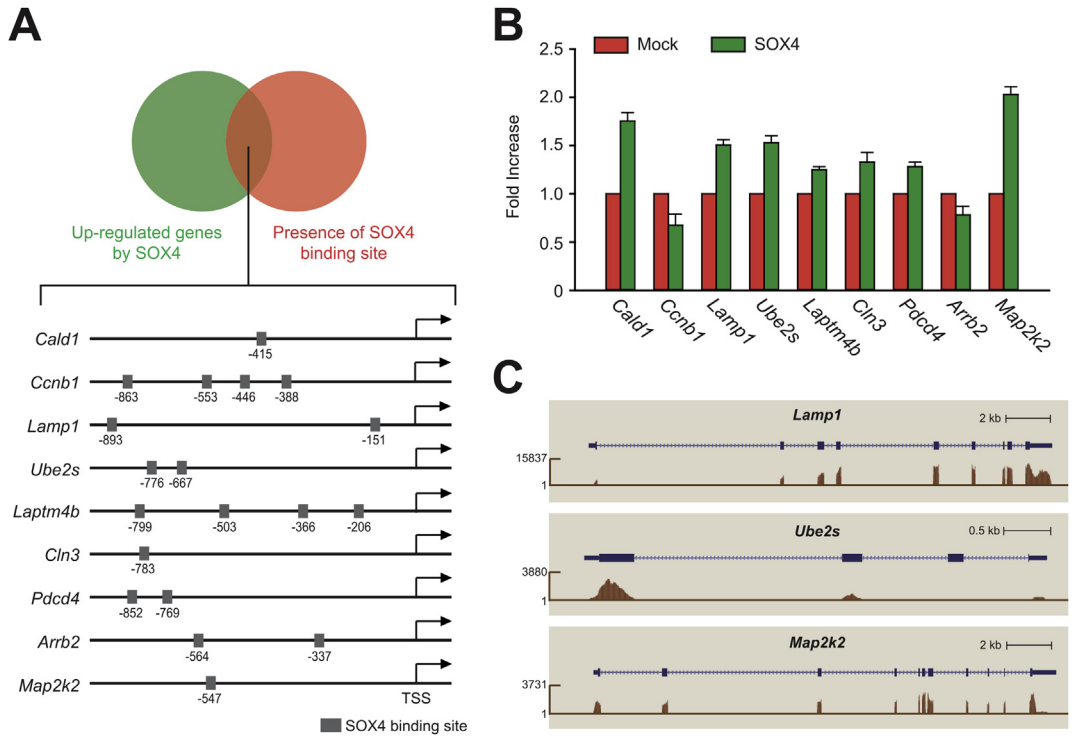

Fig. 1. Identification of possible new target genes of SOX4. (A) Schematic diagram of procedure of SOX4 response gene selection. Using published microarray data, transcriptionally upregulated target genes mediated by SOX4 expression were curated. And then, promoter regions of these genes were analyzed for searching SOX4 binding sequences using bioinformatic programs (top panel). In finally selected genes, possible SOX4 binding sites are shown with their distance from the transcription start site (TSS; arrow) (bottom panel). (B) SOX4 regulates transcription of possible SOX4 target genes. HEK293 cells were transfected with GFP-control vector or GFP-fused SOX4 expression constructs. After 24 h, cells were harvested for isolation of total RNA and used for reverse transcription and qRT-PCR. Transcription levels of selected genes were normalized by 18SrRNA gene. All data are representative of three independent experiments. (C) Gene expression patterns were further confirmed from mouse ENCODE project. RNA-seq signals were visualized on the UCSC genome browser and modified.

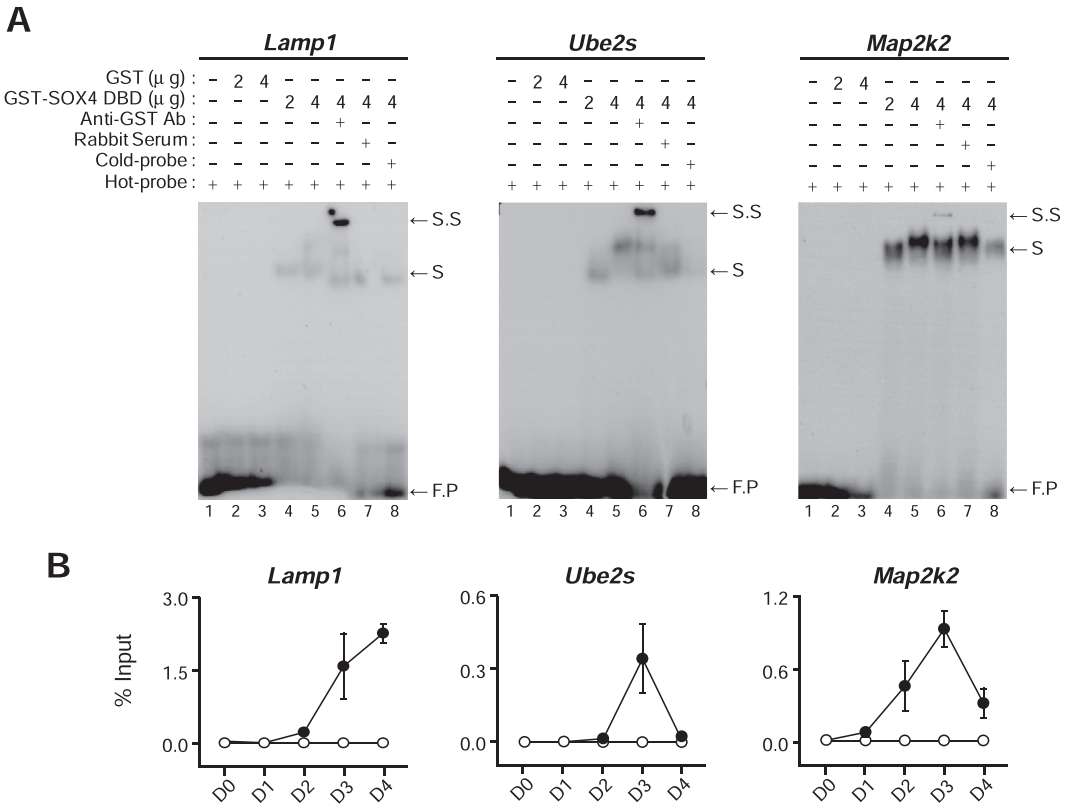

Fig. 2. SOX4 recruitment on possible SOX4 binding sites in putative target gene promoters *in vitro* and *in vivo*. (A) EMSA showing the binding of SOX4 to possible SOX4 binding sequences in the selected new target gene promoters. Lanes are as indicated below the autoradiograph. Two or four micrograms of purified proteins were used for EMSA. For the competition experiment, lane 8 included a 10-fold molar excess of unlabeled oligonucleotide. Lane 6 contains antibodies against GST, and lane 7 contains the same quantity of mouse serum as a negative control for GST antibody. Arrows represent the specific shifted band (S.S, supershifted band; S, shifted band by SOX4-DBD; F.P, free probes). These experiments were conducted at least three times, and similar results were obtained. (B) Recruitment of SOX4 to possible target gene promoter in myoblast differentiation. C2C12 myoblast cells were incubated under differentiation conditions for 4 days. SOX4 occupancy at the new target gene promoter was analyzed with ChIP assays using anti-SOX4 antibodies. The precipitated chromatin fragments were analyzed using primers specific to the selected gene promoter as described in Material and methods. All data are the mean ± SEM of three independent experiments performed in triplicate.

protein-DNA complex in a SOX4 dose-dependent manner (lanes 4 and 5), whereas the GST protein alone, used as the negative control for GST-SOX4-DBD, did not form a protein-DNA complex (lanes 2 and 3). The presence of SOX4 in the protein-DNA complex was verified by adding an anti-GST antibody. Addition of the anti-GST antibody resulted in supershifting by forming an antibody-protein-DNA complex (lane 6), whereas the negative-control IgG did not alter the shift pattern (lane 7). The SOX4-binding sequence containing the probe-SOX4 protein-antibody complex disappeared when cold probes were added as competitors of hot probes (lane 8). To further confirm SOX4 binding to the promoter region of selected genes, we performed a chromatin-immunoprecipitation assay (ChIP)with a differentiated C2C12 myoblast cells. To induce myoblast differentiation, confluent C2C12 cells were switched from proliferating medium to differentiation medium containing 2% horse serum for 4 days. As shown in Fig. 2B, SOX4 was recruited to specific gene promoters including those of *Lamp1*, *Ube2s*, and *Map2k2* during myoblast differentiation, but not during the proliferation stage. A recent report showed transcriptional induction of lysosome-related genes in enhanced myoblast differentiation conditions mediated by cholesterol depletion via methyl-β-cyclodextrin [23]. Moreover, the MEK1/2-dependent extracellular signal-regulated kinase (ERK) pathway is known as a crucial mechanism in late myoblast differentiation [24]. ARRβ2 is involved in ERK signaling by forming an ARRβ-ERK complex in smooth muscle [25].

3.3. Identification of novel SOX4-binding proteins

To identify proteins that interact with SOX4, we performed a yeast two-hybrid assay using the DNA-binding domain region (amino acids 1–145) of SOX4 as the bait. Screening about 2×10^6 yeast transformants led to the isolation of several positive clones. Nucleotide sequence analysis revealed that cDNAs in selected clones were identical to the annotated proteins (Table 1). Although we demonstrated interactions between SOX4 and proteins identified in the yeast two-hybrid assay, the environment in mammalian cells is markedly different from that in yeast, and there were many false-positive results in this assay. Therefore, to investigate whether two proteins could interact in mammalian cells, we selected three candidates including lamin B2 (LMNB2), barrier to autointegration factor 1 (BANF1), and apolipoprotein C-III (APOC3), and exogenously expressed them with SOX4 in HEK293 cells. Whole-cell lysates were immunoprecipitated with anti-FLAG or anti-GFP antibodies to precipitate FLAG- or GFP-tagged SOX4, followed by western blotting to determine co-precipitated interaction partners. As shown in Fig. 3, selected proteins were clearly co-immunoprecipitated with SOX4 (lane 2), and not with lysates of control, empty vector-transfected cells (lane 1). Furthermore, immunofluorescence staining showed that SOX4 and its binding proteins were clearly colocalized in HEK293 cells, primarily in the nucleus (Fig. 3, right panel). These results suggest that transcriptional activity of SOX4 may be regulated by

Table 1
Putative interaction partners of SOX4.

NCBI no.	Putative binding protein of SOX4	Gene symbol
NM_000363.4	TNNI3 troponin I type 3	TNNI3
NM_001039.3	Sodium channel, nonvoltage-gated 1, gamma	SCNN1G
NM_001178117.1	Multiple inositol-polyphosphate phosphatase 1	MINPP1
NM_000618.3	Insulin-like growth factor 1 (somatomedin C)	IGF1
NM_001031696.2	Phospholipase D family, member 3	PLD3
NM_005632.2	Small optic lobes homolog (Drosophila)	SOLH
NM_003923.2	Forkhead box H1	FOXH1
NM_001137669.1	Regulator of G-protein signaling like 1	RGSL1
NM_032737.2	Lamin B2	LMNB2
NM_014633.3	Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	CTR9
NM_001166434.1	Inter-alpha (globulin) inhibitor H1	ITIH1
NM_000040.1	Apolipoprotein C-III	APOC3
NM_003792.2	Endothelial differentiation-related factor 1	EDF1
NM_001145465.1	NANOG neighbor homeobox	NANOGNB
NM_020310.2	MAX binding protein	MNT
NM_001130082.1	Semaphorin receptor (plexin B1)	PLXNB1
NM_178857.5	Retinitis pigmentosa 1-like 1	RP1L1
NM_001143985.1	Barrier to autointegration factor 1, transcript variant 1	BANF1

the interaction with novel binding partners of SOX4, such as LMNB2, BANF1, and APOC3. Lamin B2 encoded by *LMNB2* is a major component of nuclear lamina together with A-type lamins, including lamins A and C, which are derived from a single gene (*LMNA*) via alternative splicing [26,27]. In addition to maintaining nuclear functions or the shape of the nuclear envelope, lamins are involved in a variety of chromatin-related functions. For example, a previous report showed that the expression of mutant lamins or depletion of lamins impairs the formation of DNA repair foci or DNA replication, suggesting that nuclear lamins are involved in DNA replication and repair [27]. Moreover, a close association between nuclear lamina and peripherally localized heterochromatin suggests that lamins may participate in anchoring or organizing chromosomes [28]. In addition, several transcription factors including Oct-1 and c-Fos can bind to lamins [29,30]. In particular, interaction between c-Fos and lamins A/C within the nuclear envelope inhibits AP-1 transcriptional activity by blocking DNA binding [30,31], suggesting that LMNB2 may regulate the transcriptional activity of SOX4 by alteration of chromatin locations involving SOX4 target genes or SOX4 anchoring. BANF1 is also considered an important component of the nuclear envelope, and is involved in chromatin structure and maintenance via binding to the core and tails of histones [32]. Moreover, BANF1 is known as a transcriptional repressor by associating with cone-rod homeobox factor (CRX) to inhibit CRX-dependent promoter activity [33]. Therefore, we propose that SOX4 transcriptional activity may be regulated by BANF1-dependent functions in common with LMNB2. Although there are relatively few reports concerning transcriptional regulation mediated by APOC3 interaction, future studies will elucidate the biological implications of the interaction between SOX4 and APOC3.

In combination with novel target genes and newly identified protein interactions of SOX4, we propose a putative transcriptional regulatory network of SOX4 (Fig. 3D). Although a few studies have investigated transcriptional regulation by SOX4, a comprehensive study of its transcriptional regulatory network is required to understand the molecular mechanisms of SOX4 in various cellular processes. Taken together, our findings shed light on the veiled molecular mechanism of SOX4 and will provide opportunities to understand the differences in the timing of specific gene expression according to interaction partner changes in a SOX4-centered transcriptional regulatory network.

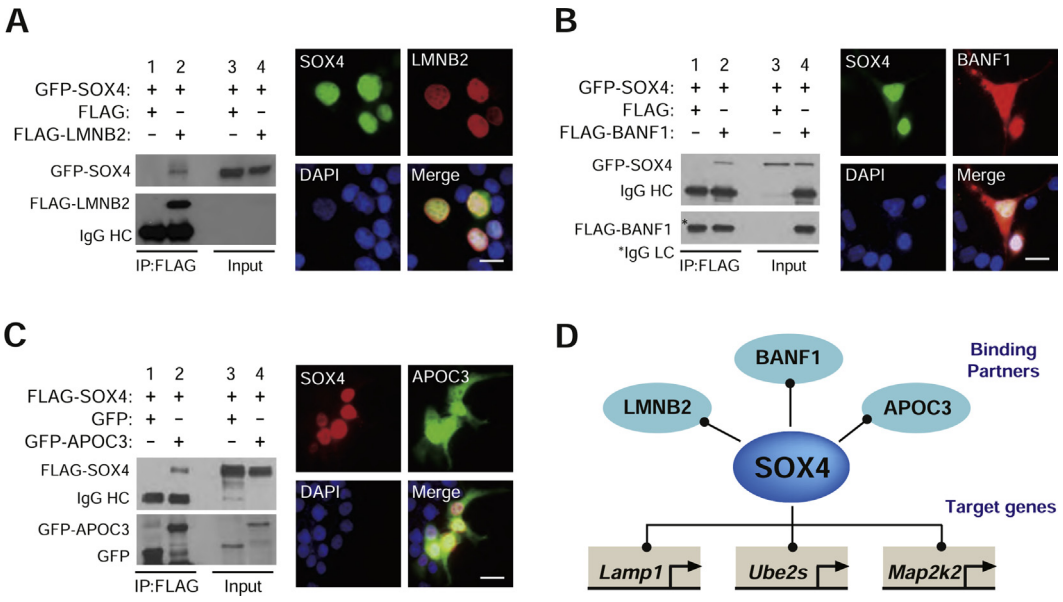

Fig. 3. Identification of SOX4 novel binding proteins and SOX4-centered transcriptional regulatory network. (A–C) Interactions between SOX4 and novel binding proteins of SOX4. HEK293 cells were transfected with plasmids expressing SOX4 with LMNB2 (A), BANF1 (B), or APOC3 (C), respectively. Total cell lysates were prepared and immunoprecipitated with anti-FLAG or anti-GFP antibodies. Co-precipitated proteins were detected by Western blot analysis using antibodies as indicated in figure (left panels in A-C). Co-localization of SOX4 and novel interaction proteins was analyzed by immunofluorescence analysis with anti-FLAG (red) and visualized with GFP fluorescence (green) or DAPI nuclear stain (blue) (right panels in A–C). Scale bar: 20 μm. (D) The model of SOX4-centered transcriptional regulatory network based on combinations between SOX4 interaction proteins and SOX4 target genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Conflict of interests

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.142.

Transparency document

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