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THRAP3 interacts with and inhibits the transcriptional activity of SOX9 during chondrogenesis

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Abstract

Sex-determining region Y (Sry) - box (Sox)9 is required for chondrogenesis as a transcriptional activator of genes related to chondrocyte proliferation, differentiation, and cartilage-specific extracellular matrix. Although there have been studies investigating the Sox9-dependent transcriptional complexes, not all of their components have been identified. In the present study, we demonstrated that Thyroid hormone receptor-associated protein (THRAP)3 is a component of a SOX9 transcriptional complex by liquid chromatography mass spectrometric analysis of FLAG-tagged Sox9-binding proteins purified from FLAG-HA tagged *Sox9* knock-in mice. *Thrap3* knockdown in ATDC5 chondrogenic cells increased the expression of *Collagen type II alpha 1 chain* (*Col2a1*) without affecting *Sox9* expression. *THRAP3* and *SOX9* overexpression reduced *Col2a1* levels to a greater degree than overexpression of *SOX9* alone. The negative regulation of SOX9 transcriptional activity by THRAP3 was mediated by interaction between the proline-, glutamine-, and serine-rich domain of SOX9 and the innominate domain of THRAP3. These results indicate that THRAP3 negatively regulates SOX9 transcriptional activity as a cofactor of a SOX9 transcriptional complex during chondrogenesis.

Introduction

Sex-determining region Y -box (*Sox*)9 is required during sequential steps of chondrogenesis, mediating chondrocyte proliferation, differentiation, and extracellular matrix production [1, 2]. Sox9 binds to enhancer regions of and directly transactivates *Collagen type II alpha* Ⅰ *chain* (*Col2a1*), *Collagen type* ⅩⅠ *alpha* Ⅱ *chain* (*Col11a2)*, and *Aggrecan* genes. Mutations in human *SOX9* lead to campomelic dysplasia, which is characterized by skeletal malformation and sex reversal [3, 4]

Several proteins are known to modulate Sox9 transcriptional activity, including Sox5/6, thyroid hormone receptor-associated protein (TRAP)230/Med12, cAMP-response element-binding protein-binding protein/p300, mothers against decapentaplegic homolog (Smad)3, peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, protein inhibitor of activated signal transducer and activator of transcription (PIAS) proteins, Tat interactive protein (Tip)60, nuclear RNA-binding protein (p54nrb), Zinc finger protein (Znf)219, AT-rich interactive domain (Arid)5a, and WW domain containing E3 ubiquitin protein ligase (Wwp)2/Mediator complex subunit (Med)25 [5-14]. Sox9 induces *Sox5* and *Sox6* transcription, and the three proteins cooperatively regulate the expression of cartilage-specific extracellular matrix genes [1, 15-18). Tip60 and PIAS were identified by yeast-two-hybrid screening and found to modulate Sox9 transcriptional

activity by modifying Sox9. Arid5a, which was identified in a *Col2a1* promoter reporter assay as a transcriptional partner of Sox9, regulates chondrocyte differentiation by inducing histone H3 acetylation of chondrocyte-specific genes. Smad3 and PGC-1α regulate *Col2a1* expression by associating with Sox9 and p300; the latter stimulates Sox9-dependent transcription by modifying histone acetylation. p54nrb and Sox9 interact to regulate paraspeckle formation and mRNA processing, which are necessary for chondrocyte differentiation. In contrast, Trap230—a member of the TRAP complex—directly binds Sox9 and may interfere with the ability of T cell and Lymphoid enhancer-binding factors to bind β-catenin, thereby modulating Wnt signaling. Znf219 may function as an adaptor protein to recruit Sox9 binding partners. Med25, a component of the Mediator transcriptional coactivator complex, binds to and positively regulates Sox9 transcriptional activity in a Wwp2-dependent manner.

To identify the components of the SOX9 transcriptional complex during chondrogenesis, we generated FLAG-HA-tagged *Sox9* knock-in mice expressing FLAG-tagged Sox9 protein and identified a group of proteins that bind to Sox9 in chondrocytes in vivo by immunoprecipitation and liquid chromatography–tandem mass spectrometry (LC–MS/MS). One of these, Thyroid hormone receptor-associated protein (Thrap)3/TRAP150, is a multifunctional polypeptide and minor component of the

TRAP/Mediator complex that communicates regulatory signals from DNA-bound transcription factors to RNA polymerase II. It was previously reported that nuclear thyroid hormone receptor interactor 6 homodimers mediate the promoter recruitment of THRAP3, which acts as a co-activator for activator protein-1 [19]. Circadian-dependent Thrap3 was found to promote DNA binding of the CLOCK–Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 complex and its association with basic transcriptional machinery [20]. THRAP3 was found to directly bind helicase motifs in helicase with zinc finger 2β as an adaptor molecule for other transcriptional coregulators to enhance peroxisome proliferator-activated receptor (PPAR)γ-mediated gene activation [21]. In addition to its role in transcriptional regulation, THRAP3 is involved in precursor-mRNA alternative splicing [22] and the DNA damage response [23]. In the present study, we demonstrate that THRAP3 interacts physically with SOX9 to negatively regulate its transcriptional activity in chondrogenesis.

Materials and Methods

Generation of mutant mice

FLAG-HA-tagged *Sox9* knock-in mice were generated using a targeting vector spanning a 13-kb fragment of the 129SvEv mouse *Sox9* gene. FLAG and HA tags were introduced

immediately downstream of the first ATG of exon 1 of *Sox9*, while a *loxP*-flanked *PGK-neo-bpA* cassette was introduced into intron 1. The targeting vector was introduced into AB-1 embryonic stem (ES) cells [24]. Mouse chimeras were generated by injecting mutant ES cell clones into C57BL/6J host blastocysts, and were bred with C57BL/6J mice to obtain FLAG-HA-tagged *Sox9* knock-in heterozygous mice, which were confirmed by Southern blotting. Animal care and experimental procedures were approved by the Animal Research Committee of Kyoto University, and were in accordance with the Regulations on Animal Experimentation of Kyoto University.

Cell culture

Primary chondrocytes were isolated from the costal cartilage of neonatal FLAG-HA-tagged *Sox9* knock-in pups. Minced cartilage was incubated with a trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 25 min and subsequently digested with medium containing collagenase type 2 (1.5×10^4 U (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C for 3 h. Chondrocytes were grown in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories/GE Healthcare, Chicago, IL USA) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was replaced

every other day.

ATDC5 cells were cultured as previously described [25, 26]. Briefly, cells were maintained in log growth phase in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (Corning Inc., Corning, NY, USA) containing 5% FBS (Cambrex, Charles City, IA, USA), 10 μg/ml human transferrin (Wako Pure Chemical Industries, Osaka, Japan), and 3×10^{-8} M sodium selenite (Sigma-Aldrich) (maintenance medium) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Chondrogenesis was induced in maintenance medium supplemented with 10 μg/ml bovine insulin (Wako Pure Chemical Industries) (differentiation medium).

C3H10T1/2 [27] and HEK 293 [28] cells were grown in DMEM (Sigma-Aldrich) containing 10% FBS and 1% penicillin-streptomycin.

Plasmids

The pENTR221 human THRAP3 vector was purchased from DNAFORM (Yokohama, Japan). cDNA sequences encoding full-length and truncated human *THRAP3* were amplified using specific primers and inserted into pcDNA3.1 V5 His TOPO vectors (Thermo Fischer Scientific, Waltham, MA, USA). FLAG-tagged full-length and truncated human *SOX9* expression vectors were generated as previously described [2].

Preparation of whole cell lysates

Proteins were extracted from ATDC5 cells and C3H10T1/2 cells using cold radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitor cocktail (Sigma-Aldrich).

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Whole cell lysates of primary chondrocytes derived from costal cartilage of neonatal FLAG-HA-tagged *Sox9* knock-in mice were prepared in 50 mM Tris-HCl

(pH 7.9), 200mM NaCl, 1% NP-40 and complete protease inhibitor mixture (Roche Applied Science), and 150 mg of protein from the lysates were subjected to immunoprecipitation with anti-FLAG M2 antibody-conjugated agarose affinity gel (Sigma-Aldrich) overnight at 4°C. After washing, bound proteins were eluted with excess amounts of FLAG peptide (Sigma-Aldrich) and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To visualize the purified proteins, the gel was stained with silver nitrate [29]. To identify the proteins, the gel was stained with imidazole-zinc method [30] and bands were excised and analyzed by LC–MS/MS.

Western blotting

Costal cartilage of neonatal FLAG-HA-tagged *Sox9* knock-in mice was homogenized in RIPA lysis buffer; proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 for 30 min at room temperature. The membrane was incubated overnight at 4°C with rabbit anti-Sox9 (1:2000; Merck Millipore) and peroxidase-conjugated anti-FLAG M2 (1:3000; Sigma-Aldrich) antibodies. Immunoreactivity was detected using horseradish peroxidase (HRP)-conjugated swine anti-rabbit antibody (Dako, Carpinteria, CA, USA) diluted 1:3000 and enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific).

Short interfering (si)RNA and gene transfection

ATDC5 cells were transfected with siRNA specific for mouse *Thrap3* (MISSION siRNA; Sigma-Aldrich) using Lipofectamine 2000 (Thermo Fisher Scientific) and with V5-tagged THRAP3 expression plasmids using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's protocols. Total RNA was extracted 48 h later, and 100 ng of total RNA were used to synthesize cDNA with ReverTra Ace (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed on a Light Cycler instrument

(Roche Diagnostics, Indianapolis, IN, USA) with Thunderbird SYBR qPCR mix (Toyobo). Sequences of primers used for PCR are shown in Table 2.

Luciferase assay

A chondrocyte-specific p89/4×48 *Col2a1* reporter construct was used as previously described [14], with pcDNA-Renilla luciferase as an internal control. HEK293 cells were transfected with the plasmids using FuGENE HD reagent (Roche Diagnostics). Luciferase activity was assayed 48 h later.

Immunoprecipitation

C3H10T1/2 cells were transfected with tagged plasmids using the FuGENE HD transfection reagent (Roche Diagnostics); 24 h later, cells were lysed in buffer composed of 50 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100. Protein complexes were precleared with 40 µl protein G sepharose (GE Healthcare) for 1 h and immunoprecipitated overnight at 4°C using 60 µl EZview Red FLAG M2 Affinity Gel (Sigma-Aldrich), 60 µl agarose-conjugated anti-V5 tag polyclonal antibody (Medical and Biological Laboratories, Nagoya, Japan), and 60 µl of protein G agarose (Thermo Fisher Scientific) with 4 µg of rabbit anti-mouse IgG (2.5 mg ml^{-1}; Cell Signaling Technology,

Danvers, MA, USA). Immunoprecipitated proteins were detected by immunoblotting using HRP-conjugated mouse anti-V5 (1:5000; Invitrogen) and peroxidase-conjugated mouse anti-FLAG M2 (1:3000; Sigma-Aldrich) antibodies and visualized by ECL.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using a kit (Abcam, Cambridge, UK) according to the manufacture's protocol. Briefly, primary chondrocytes obtained from FLAG-HA-tagged Sox9 knock-in pups were cultured in a 100-mm dish, and cross-linked chromatin fragments were immunoprecipitated using 40 μl protein G agarose, 4 μg rabbit anti-mouse IgG (Cell Signaling Technology), and 4 μg anti-Sox9 antibody or 40 μl EZview Red FLAG M2 Affinity Gel. Co-precipitation of the *Col2a1* enhancer region was detected by PCR [14]. C3H10T1/2 cells cultured in 100-mm dishes were co-transfected with FLAG-tagged SOX9 and V5-tagged THRAP3 expression vectors. After 24 h, cross-linked chromatin fragments were immunoprecipitated with 4 μg anti-Sox9 antibody and 40 μl agarose-conjugated anti-V5 tag polyclonal antibody. Co-precipitation of the *Col2a1* enhancer region and the *Col11a1* promoter region were detected by PCR (Table 1) [14].

Immunohistochemical analysis

Limb buds of embryonic day 16.5 C57BL/6J mouse embryos were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin. Immunohistochemical analysis was carried out using SuperPicture HRP polymer conjugate diaminobenzidine (rabbit; Thermo Fisher Scientific), with hematoxylin used as a counterstain. We used anti-Sox9 and rabbit polyclonal anti-TRAP150 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies diluted 1:500 and 1:50, respectively, in Antibody Diluent Reagent Solution (Thermo Fisher Scientific).

Immunofluorescence analysis

ATDC5 cells were fixed with 10% formaldehyde for 12 min at room temperature. After washing, cells were blocked with fresh blocking solution composed of 4% donkey serum in PBS containing 0.1% Triton X-100 (PBST; Sigma-Aldrich) for 30 min and then incubated for 1 h at room temperature with rabbit polyclonal anti-TRAP150 antibodies diluted 1:50 in 1% bovine serum/PBST. Cells were washed and incubated for 1 h at room temperature with Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Abcam) diluted 1:1000. The procedure was repeated using a mouse anti-Sox9 antibody Alexa Fluor 488 (Santa Cruz Biotechnology) diluted 1:50. Nuclei were counterstained with 300nM of

DAPI (Thermo Fisher Scientific). Images were acquired on a fluorescence microscope (Keyence Corporation, Osaka, Japan). Transfected ATDC5 cells were fixed, blocked, and then incubated for 1 h at room temperature with anti-Sox9 antibody diluted 1:200 in 1% donkey serum/PBST. Cells were washed and incubated for 1 h at room temperature with Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Abcam) diluted 1:1000. The procedure was repeated using a goat anti-V5 antibody (Abcam) diluted 1:200 and Alexa Fluor 488-conjugated donkey anti-goat IgG (Abcam) diluted 1:1000. Nuclei were counterstained with TO-PRO-3 (Thermo Fisher Scientific) diluted 1:3000. Images were acquired on a confocal laser microscope (Nikon Instruments, Tokyo, Japan).

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Results

Generation of FLAG-HA tagged Sox9 knock-in mice

We generated mutant mice in which FLAG and HA tags were inserted into exon 1 of the *Sox9* gene by gene targeting in ES cells (Fig. 1a). Southern blot analysis of genomic DNA purified from recombinant ES cell clones revealed an 11.5-kb fragment corresponding to the mutant allele following digestion by *Bam*HI (Fig. 1b).

We also analyzed FLAG-tagged Sox9 purified from the rib cage of neonatal

FLAG-HA-tagged *Sox9* knock-in mice by western blot analysis (Fig. 1c). Hetero- and homozygous mutant mice were viable and fertile and showed no apparent phenotypic changes compared to controls (Fig. 1d), suggesting that the tagged Sox9 protein worked efficiently in vivo. ChIP using chondrocytes from FLAG-HA-tagged *Sox9* knock-in mice revealed specific binding of FLAG-tagged Sox9 protein to the *Col2a1* enhancer region (Fig. 1e).

Purification of Sox9 transcriptional complex

Proteins in whole cell lysates from the costal cartilage of neonatal FLAG-HA-tagged *Sox9* knock-in pups were immunoprecipitated using FLAG-M2 beads. The eluents were analyzed by gel electrophoresis and silver staining (Fig. 2a). A mass spectrometry analysis of the bands identified 24 transcriptional proteins associated with Sox9 (Fig. 2b and Table 2). We focused our analysis on Thrap3 which was present around 150 kDa.

Thrap3 *modulates* Sox9 *transcription of Col2a1*

We analyzed the expression of *Sox9*, *Col2a1*, *Col11a1* and *Thrap3* in chondrogenesis using ATDC5 cells. *Sox9* levels increased over the first 7 days of culture and then remained constant (Fig. 3a). *Thrap3* levels reached a peak on day 10 and decreased

thereafter. *Col2a1* levels gradually increased and reached a plateau after 3 weeks. *Col11a1* levels reached a peak on day 14 and decreased thereafter.

Thrap3 expression was silenced by siRNA in ATDC5 cells (Fig. 3b). *Thrap3* knockdown increased *Col2a1* expression without altering *Sox9* levels. In addition, overexpression of *SOX9* stimulated *Col2a1* expression, whereas *THRAP3* overexpression had no effect (Fig. 3c). Co-transfection of *THRAP3* and *SOX9* abolished the increase in *Col2a1* expression induced by *SOX9*.

A luciferase reporter assay in HEK293 cells using the p89/4×48 Col2a1 plasmid showed that co-transfection of *SOX9* and *THRAP3* reduced reporter activity as compared to transfection of *SOX9* alone (Fig. 3d).

THRAP3 *co-localizes with* SOX9 *in chondrocytes in the growth plate and in ATDC5 cells*

The humerus of a wild-type mouse was sectioned and labeled with anti-Sox9 or -Thrap3 antibody. Both Sox9 and Thrap3 were expressed in the nuclei of proliferating and pre-hypertrophic chondrocytes (Fig. 4a). To observe the subcellular localization of Thrap3, protein localization in ATDC5 cells was evaluated by immunofluorescence microscopy. Sox9 and Thrap3 immunoreactivities were detected in the nucleus (Fig. 4b and c), suggesting that they may interact to regulate transcription.

THRAP3 *interacts with* SOX9

To determine whether SOX9 and THRAP3 physically interact, we performed co-immunoprecipitation of C3H10T1/2 cells co-transfected with FLAG-tagged *SOX9* and V5-tagged *THRAP3*. We found that THRAP3 physically interacted with SOX9 (Fig. 5a). Similar experiments using deletion mutations of *SOX9* (Fig. 5b) and *THRAP3* (Fig. 5d) showed that this interaction was mediated by the proline-, glutamine-, and serine-rich domain of SOX9 (Fig. 5c) and by the innominate domain of THRAP3 (Fig. 5e). ChIP using C3H10T1/2 cells co-transfected with FLAG-tagged *SOX9* and V5-tagged *THRAP3* revealed specific binding of both THRAP3 and SOX9 to the *Col2a1* enhancer region and to the *Col11a1* promoter region (Fig. 5f). A luciferase reporter assay in HEK293 cells using the p89/4×48 Col2a1 plasmid showed that co-transfection of *SOX9* and C-terminus deleted *THRAP3* vector (1-597) reduced reporter activity as compared to transfection of *SOX9* alone. Co-transfection of *SOX9* and N-terminus of *THRAP3* vector (1-164) didn't show significant change in reporter activity compared with *SOX9* alone. (Fig. 5g).

Discussion

In this study, we identified THRAP3 as a component of the SOX9 transcriptional complex

in chondrocytes by LC-MS/MS analysis of proteins that co-precipitated with Sox9 from FLAG-HA-tagged *Sox9* knock-in mice. We confirmed that THRAP3 suppressed SOX9 transcriptional activity, while *Thrap3* knockdown increased the expression of the *Col2a1* gene. We also demonstrated by a co-immunoprecipitation assay that THRAP3 directly interacts with SOX9. Our results suggest that THRAP3 modulates SOX9 transcriptional activity in chondrocytes as a factor in the transcriptional complex.

Previous studies on the Sox9 transcriptional complex during chondrogenesis have mostly analyzed chondrocyte-specific gene rather than protein expression. Here we purified the SOX9 transcriptional complex from FLAG-HA-tagged *Sox9* knock-in mice, which is a more physiologically relevant approach than in vitro methods. Sox9 is an essential transcriptional factor in testicular formation, and binding of Nuclear receptor subfamily 5 group A member 1 and Sry to the testis-specific enhancer core element (TESCO) is required for *Sox9* transcription [31]. On the other hand, in the ovaries, Forkhead box L2 binds to TESCO to suppress enhancer activity [32]. *Wnt4* was found to block *Sox9* expression via activation of β-catenin [33]. This is in accordance with our previous report that β-catenin inhibited *Col2a1* reporter activity via inhibition of Sox9 [2]. THRAP3 activity is similar to that of β-catenin in that both proteins inhibit *Col2a1* expression. In resting T cells, glycogen synthase kinase phosphorylates the DNA/RNA

binding protein Polypyrimidine tract-binding protein-associated splicing factor and interacts with THRAP3 to block cluster of differentiation 45 exon skipping [34]. These findings imply that THRAP3 plays an indirect role in WNT signaling.

Smad nuclear interacting protein (SNIP)1 is a regulator of Cyclin D1 expression; THRAP3 was identified as a component of the SNIP1/SKI-interacting protein-associated RNA processing complex, which contributes to post-transcriptional *cyclin D1* mRNA stability [35]. Wilms' tumor 1-associating protein (WTAP) mediates G2/M transition by stabilizing *Cyclin A2* mRNA, and was found to be associated with THRAP3 in human umbilical vein endothelial cells. Double knockdown of *THRAP3* and B cell lymphoma 2-associated transcription factor 1 increased WTAP protein expression [36], demonstrating that THRAP3 is a component of the cell cycle-related gene expression complex. In addition, array-based comparative genomic hybridization analyses have revealed *THRAP3* deficiency in oral squamous cell carcinoma [37], while whole-genome sequencing analyses have detected somatic point mutations in the *THRAP3* gene in parathyroid carcinoma tissues as well as addition of intronic sequences to exons during splicing of the *THRAP3* gene in liver cancer [38]. THRAP3 phosphorylation may be associated with androgen-independent prostate cancer cell growth [39]. In mesenchymal cell lineages, THRAP3 was found to interact with PPARγ and activate diabetes-associated

genes in adipocytes [21]. Ours is the first report of a role for THRAP3 in chondrogenesis; we demonstrated that *Thrap3* knockdown and overexpression stimulated and inhibited chondrogenesis, respectively, which involved the regulation of *Sox9* transcriptional activity by THRAP3 in *Col2a1* transcription.

In preliminary experiments, we observed that thrap3b knockdown in zebrafish resulted in the abnormal somitogenesis and head formation, although thrap3a knockdown and overexpression had no effect on somitogenesis, chondrogenesis, or development (data not shown). However, loss of the WTAP complex resulted in *Cyclin A2* mRNA destabilization and embryonic lethality in the mouse [40]. Further study is needed in order to clarify the role of THRAP3 in somitogenesis.

In conclusion, THRAP3 interacts with and inhibits the transcriptional activity of SOX9 during chondrogenesis. Our results provide insight into the mechanism underlying chondrocyte generation, which can serve as a basis for the development of therapeutic strategies to treat cartilage disorders.

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Table1 Sequences of primers used for RT-PCR and ChIP assay.

RT-PCR

Gapdh	forward	5’-TGTCCGTCGTGGATCTGAC-3’
	reverse	5’-CCTGCTTCACCACCTTCTTG-3’
Thrap3	forward	5’-GAGAAAACCCACAAAGGATCA-3’
	reverse	5’-GCTTTGTAGGAATGGGAGGA-3’
Sox9	forward	5’-AACATGGAGGACGATTGGAG-3’
	reverse	5’-TCCCCTCAAAATGGTAATGAG-3’
Col2a1	forward	5’-AGAACAGCATCGCCTACCTG-3’
	reverse	5’-CTTGCCCCACTTACCAGTGT-3’
Col11a1	forward	5’-GGCCAAAGGAGAAACCAGGAAG-3’
	reverse	5’- GGGCAGAGGCAGTCAGGAGCT-3’

ChIP

Col2a1	forward	5’-AACTGGTTCCTCGTGGAGAG-3’
	reverse	5’-GGCTGTGCATTGTAGGAGAG-3’
Col11a1	forward	5’- TCCTTTGAACCAATCTTTGGGTTT -3’
	reverse	5’- CAGTTCACTTTATACTCTTTCTCA -3’

Table2 Transcriptional proteins associated with Sox9 detected by LC-MS/MS.

Acidic nuclear phosphoprotein 32 family member B
Activated RNA polymerase II transcriptional coactivator p15
Chain A, X-ray Structure Of Nm23 Human Nucleoside Diphosphate Kinase B
General transcription factor IIIC polypeptide 5
General transcription factor IIIC, polypeptide 5 isoform 3
Heterogenous nuclear ribonucleoprotein U
High mobility group protein B1
Histone-binding protein Retinoblastoma-binding protein 7
Nascent polypeptide-associated complex alpha polypeptide
Ngg1 interacting factor 3- like 1
Nucleoside-diphosphate kinase B
Nucleosome assembly protein 1-like 1
Per1 protein
Polymerase I and transcript release factor
Prohibitin
Prohibitin 2
Protein arginine N-methyltransferase 5 isoform 1
Purine rich element binding protein B
Putative HLA DR Associated Protein II
RNA-binding protein 3
Staphylococcal nuclease domain-containing protein 1
Thyroid hormone receptor associated protein 3 (Thrap3)
Transcription intermediary factor 1-beta
V-rel reticuloendotheliosis viral oncogene homolog

Figure Legends

Fig.1. Generation of FLAG-HA tagged *Sox9* knock-in mice.

a) The structure of the *Sox9* genomic locus, targeting vector and targeted allele for FLAG-HA tagged *Sox9* knock-in mouse are shown. Exons are depicted as closed boxes and introns are shown as solid lines. FLAG-HA and *loxP*-flanked *PGK-neo-bpA* cassette are depicted as open boxes. B, *BamHI*; Bg, *BglII*; E, *EcoRI*; H, *HpaI*; P, *PstI*; X, *XbaI*.

b) Southern blot analysis of genomic DNA. Genomic DNA isolated from ES cell clones was digested with *BamHI* and then hybridized with the 3' probe. The wild-type and mutant alleles were detected as 13.0 kb and 11.5 kb bands, respectively.

c) Rib cartilages of neonatal wild-type pups or FLAG-HA tagged *Sox9* knock-in pups are lysed in RIPA buffer, and proteins are analyzed by western blotting using anti-Sox9 and anti-FLAG antibody.

d) Comparison between four-week-old FLAG-HA tagged *Sox9* knock-in homozygous mouse and a wild-type mouse. No phenotypic differences were observed.

e) Rib chondrocytes of FLAG-HA tagged *Sox9* knock-in pups are incubated and proteins of these cells are analysed by ChIP assay. Recruitment of Sox9 and FLAG to the chondrocyte specific enhancer region of the *Col2a1* gene was detected. Sox9-FLAG fusion proteins were successfully formed in vivo.

Fig.2. Silver stain of FLAG tagged Sox9 fusion proteins.

a) FLAG tagged Sox9 fusion proteins from rib cartilages of FLAG-HA tagged Sox9 knock-in pups were purified by FLAG beads. Then eluents were electrophoresed and stained. There were several bands around 150kDa. One of the bands turned out to be Thrap3 protein determined by a mass spectrometry.

b) Mass spectrometry revealed 732 proteins associated with Sox9 protein. Of these, 24 proteins were classified into transcriptional factors or transcription associated proteins.

Fig.3. THRAP3 blocks *Sox9* transcriptional activity in ATDC5 and HEK293 cells.

a) *Sox9*, *Col2a1,Col11a1* and *Thrap3* gene expression in ATDC5 cells. (mean±s.d.; n=5)

b) Knockdown of *Thrap3* in ATDC5 cells using siRNA led to the upregulation of *Col2a1*, without upregulation of *Sox9*. (mean+s.d.; Dunnett's test, **$p<0.01$, ***$p<0.001$; n=5).

c) Overexpression of *SOX9* in ATDC5 cells led to upregulation of *Col2a1*, which reduced by adding *THRAP3*. (mean+s.d.; Tukey Kramer's test, *$p<0.05$, **$p<0.01$; n=5)

d) Luciferase reporter assay using the p89/4×48 *Col2a1* reporter plasmid in HEK293 cells. Activation of the reporter construct was inhibited by *THRAP3*.(mean+s.d.; Tukey Kramer's test, ***$p<0.001$; n=6)

Fig.4. SOX9 and THRAP3 co-localized in nuclei of mouse chondrocytes and ATDC5

cells.

a) Hematoxylin and eosin (HE) stain, and immunohistochemical analysis of Sox9 and Thrap3 in the wild-type mouse humerus at E16.5 .

b) Immunofluorescent staining. V5-tagged THRAP3 overexpressed in ATDC5 cells. SOX9 (red) and THRAP3 proteins (green) are co-localized in the nucleus.

c) Immunofluorescent staining in ATDC5 cells. Sox9 (green) and Thrap3 proteins (red) are co-localized in the nucleus.

Fig.5. THRAP3 interacts physically with SOX9 and regulates *Sox9* transcriptonal activity.

a) Co-immunoprecipitation indicates that THRAP3 interacts physically with SOX9 in C3H10T1/2 cells. In the left lane, we used proteins from C3H10T1/2 cells into which V5 tagged *THRAP3* and empty vector were transfected. In the right lane, we used proteins from C3H10T1/2 cells into which V5 tagged *THRAP3* and FLAG tagged *SOX9* vector were transfected.

b) Schematic images of SOX9 deletion mutants. SOX9 contains a high-mobility group domain (HMG, residues 104-182); a proline, glutamine and alanine domain (PQA, residues 339-379); and a proline, glutamine and serine-rich domain (PQS-rich, residues

402-509).

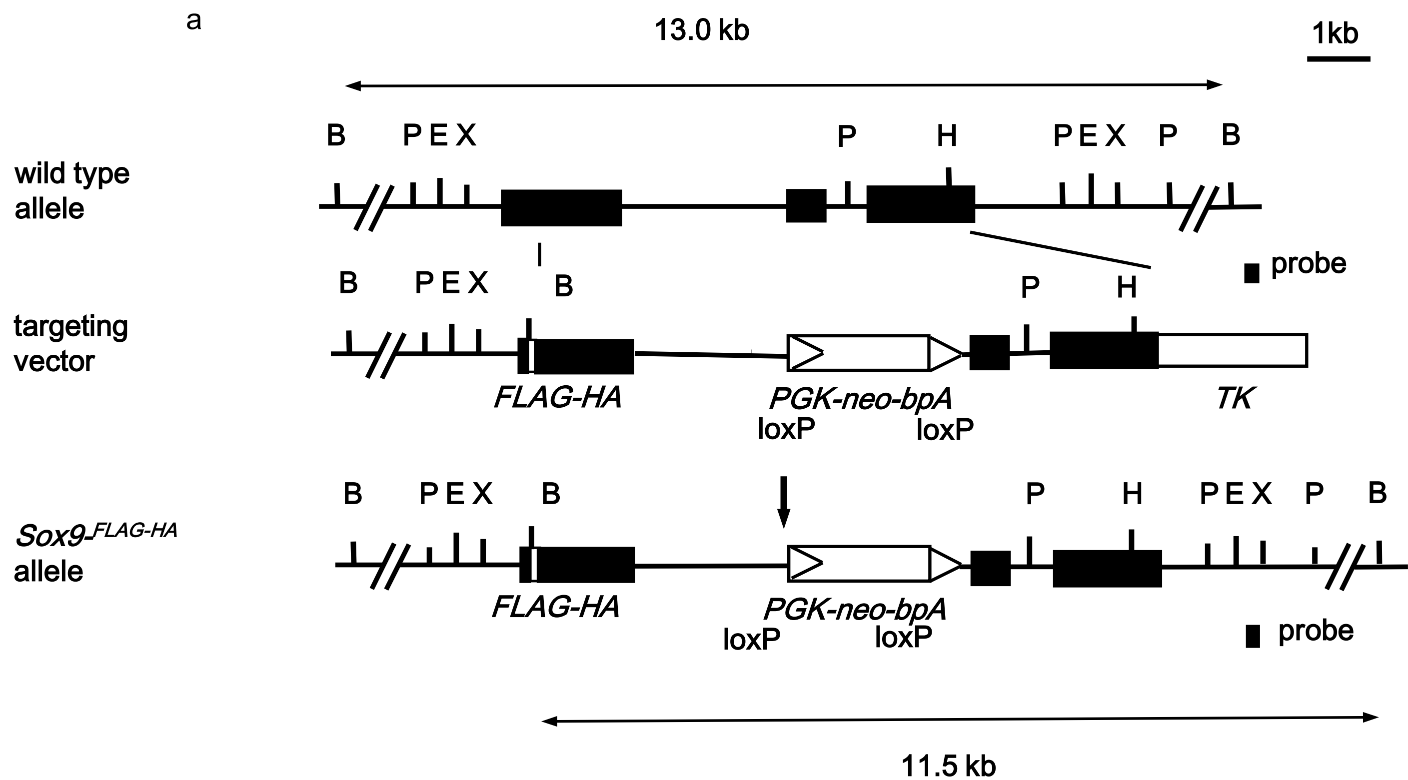
c) Co-immunoprecipitation assays between V5-tagged THRAP3 and FLAG-tagged SOX9 and deletion mutants.

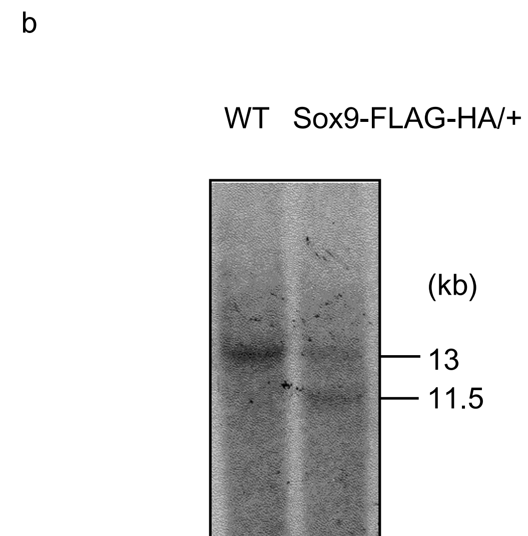
d) Schematic images of THRAP3 deletion mutants. THRAP3 contains a serine-arginine rich domain (SR, residues 1-164); and homologous domain to a BCL-2 associated transcription factor 1 (BCLAF1, residues 597-955).

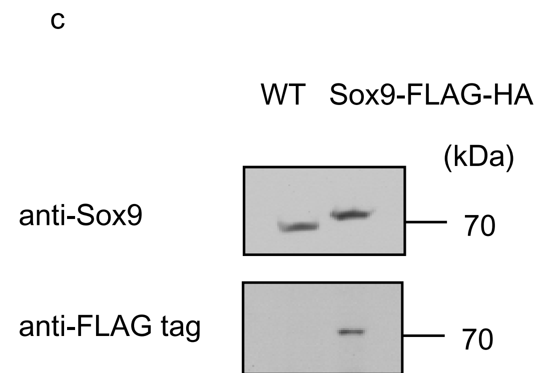
e) Co-immunoprecipitation assays between FLAG-tagged SOX9 and V5-tagged THRAP3 and deletion mutants.

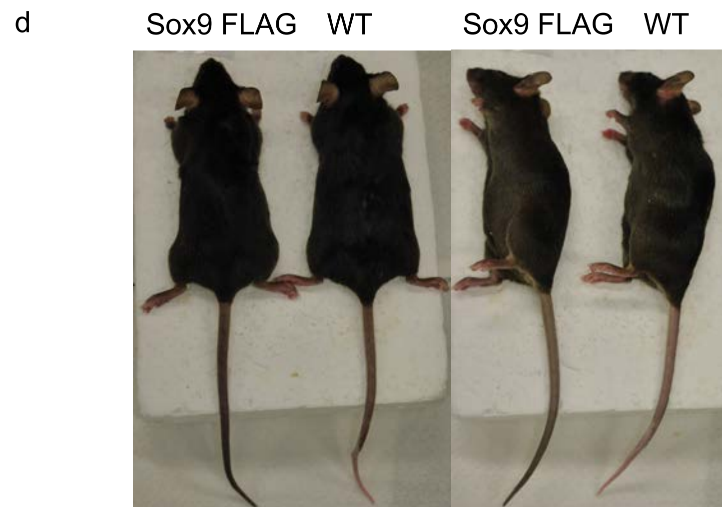
f) ChIP assay detected recruitment of SOX9 and THRAP3 to the chondrocyte-specific enhancer region of the *Col2a1* gene and to the promoter region of the *Col11a1* gene. Cell lysate extracted from C3H10T1/2 cells which were co-transfected with FLAG-tagged SOX9 and V5-tagged THRAP3 was immunoprecipitated with anti-SOX9 antibody or anti-V5 tag antibody.

g) Luciferase reporter assay using the p89/4×48 *Col2a1* reporter plasmid in HEK293 cells. Activation of the reporter construct was inhibited by C-terminus deleted THRAP3(1-597) . N-terminus THRAP3 (1-164) didn't affect the reporter activity. (mean+s.d.; Tukey Kramer's test, $*p<0.05$, $***p<0.001$; $n=3$)









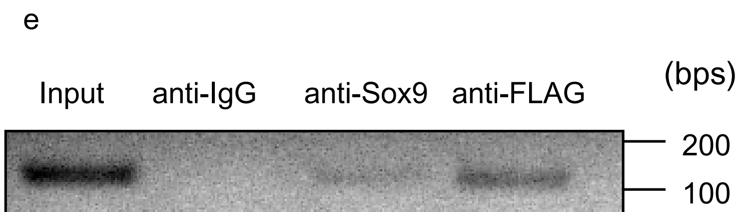
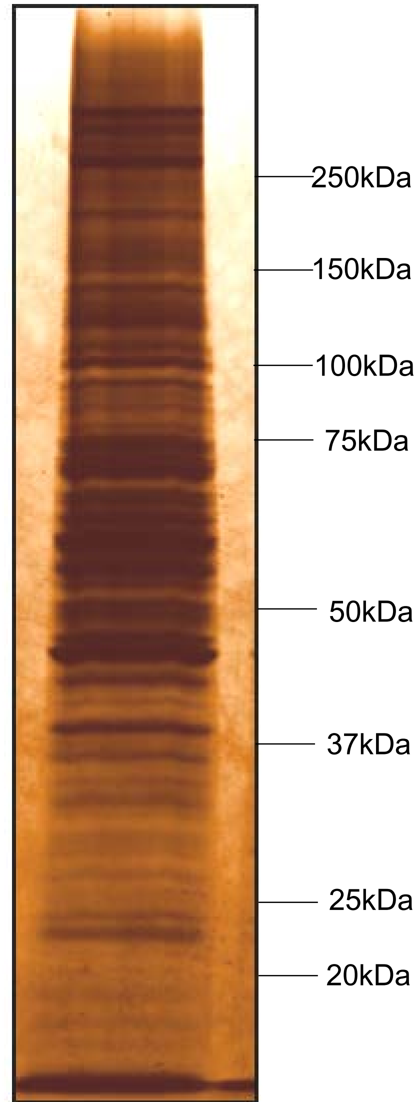


Figure 1

a



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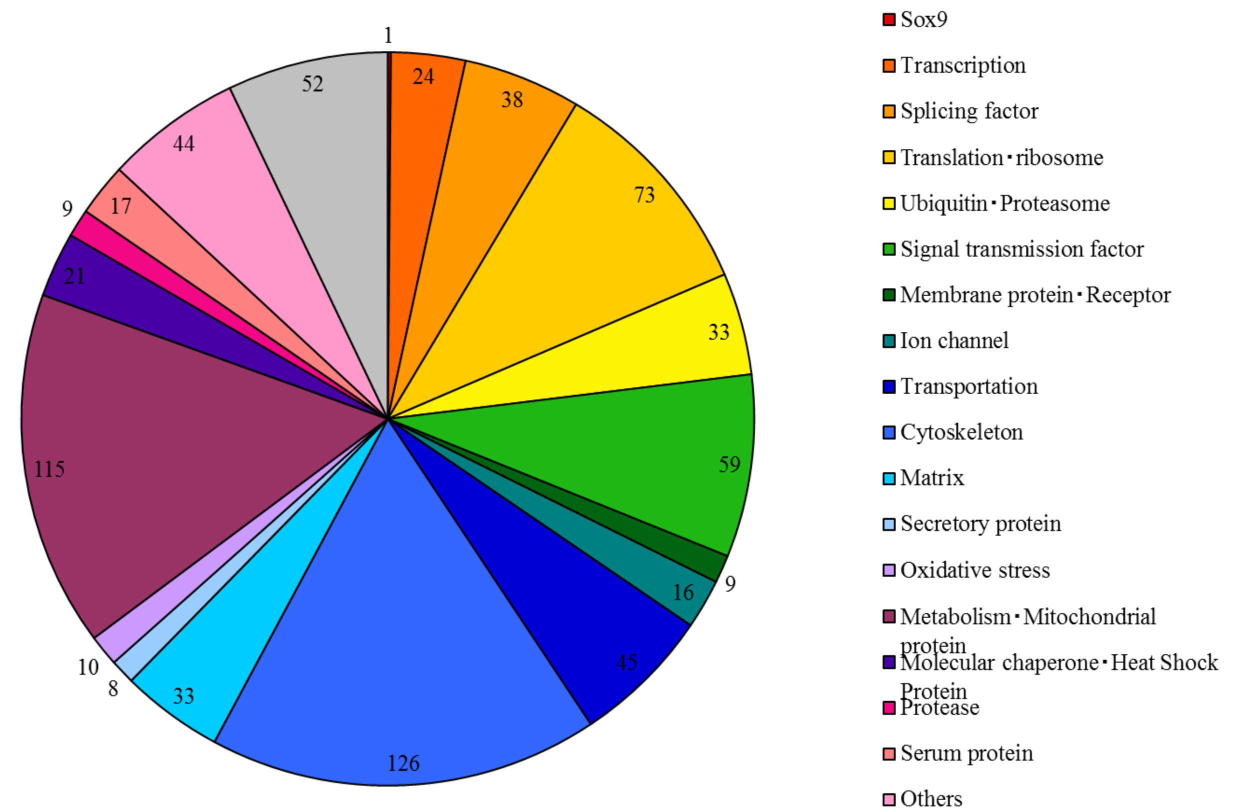
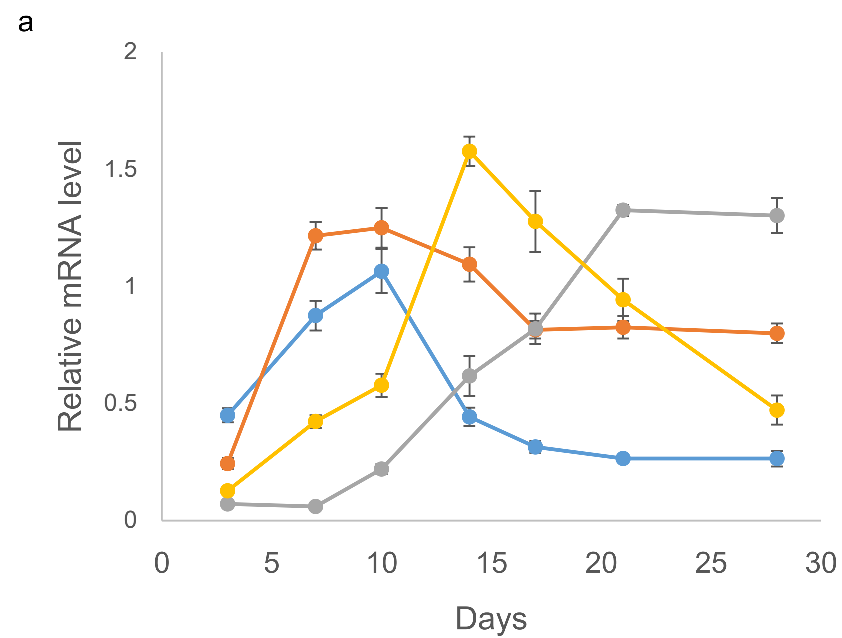
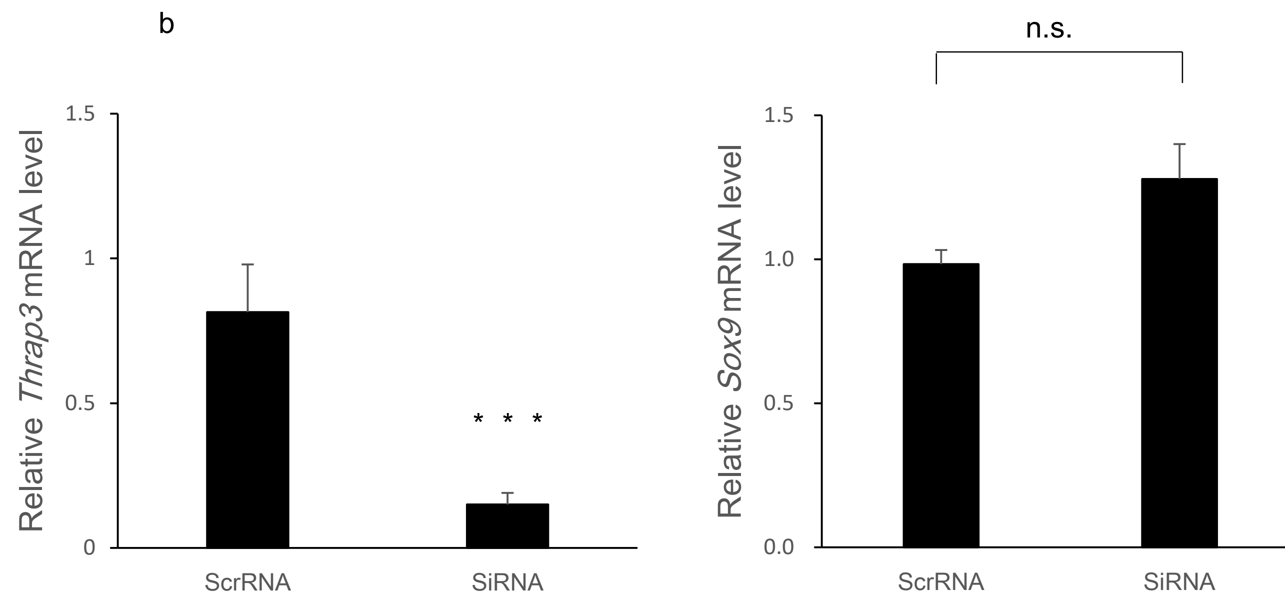
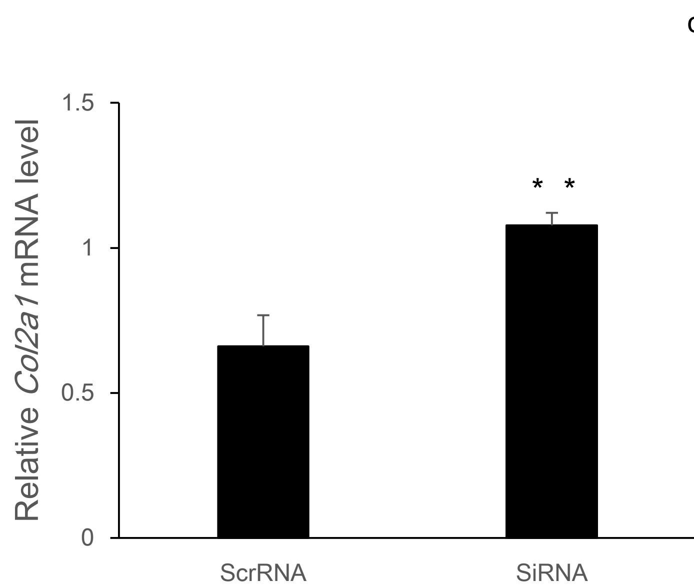
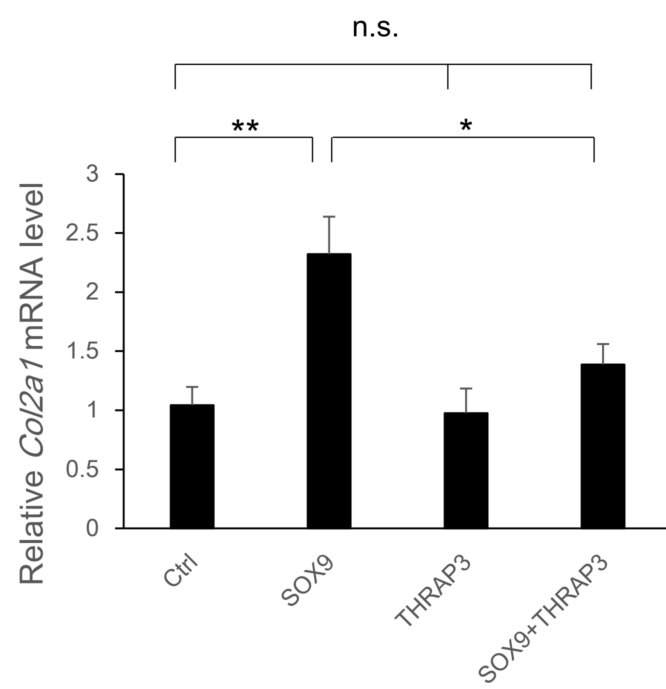


Figure 2









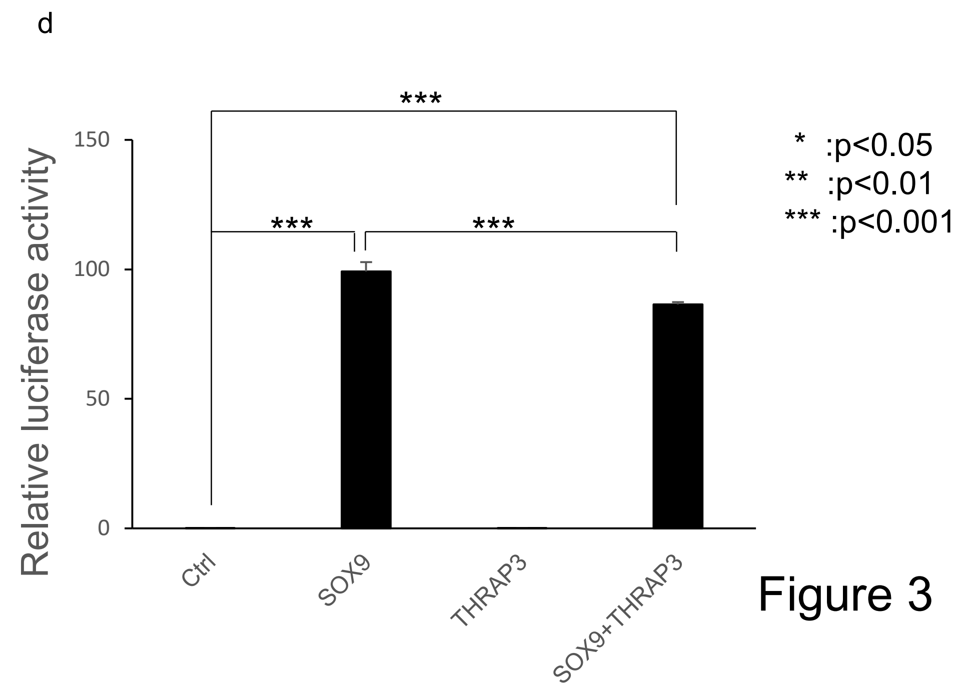


Figure 3

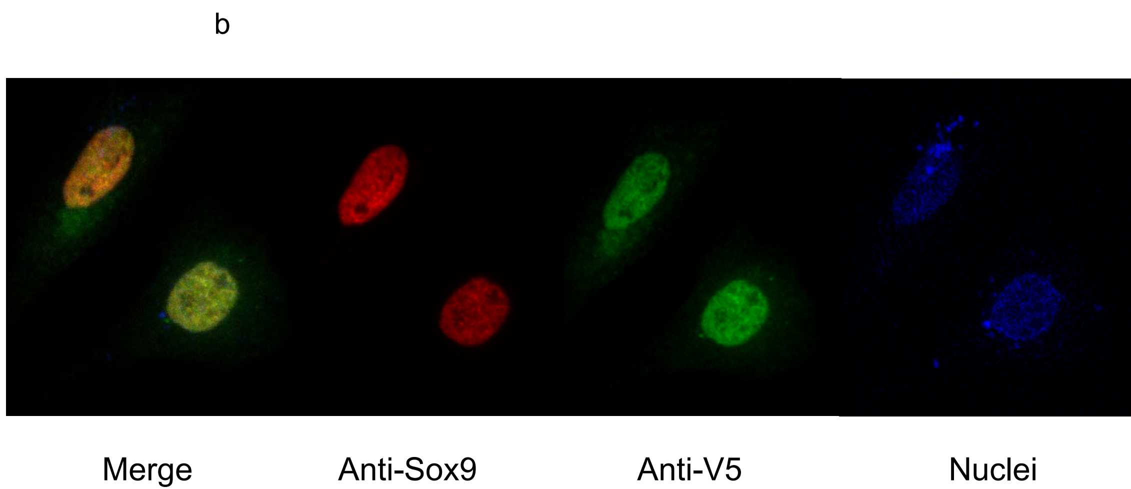
a

100μm HE

100μm Anti-Sox9

100μm Anti-Thrap3

b



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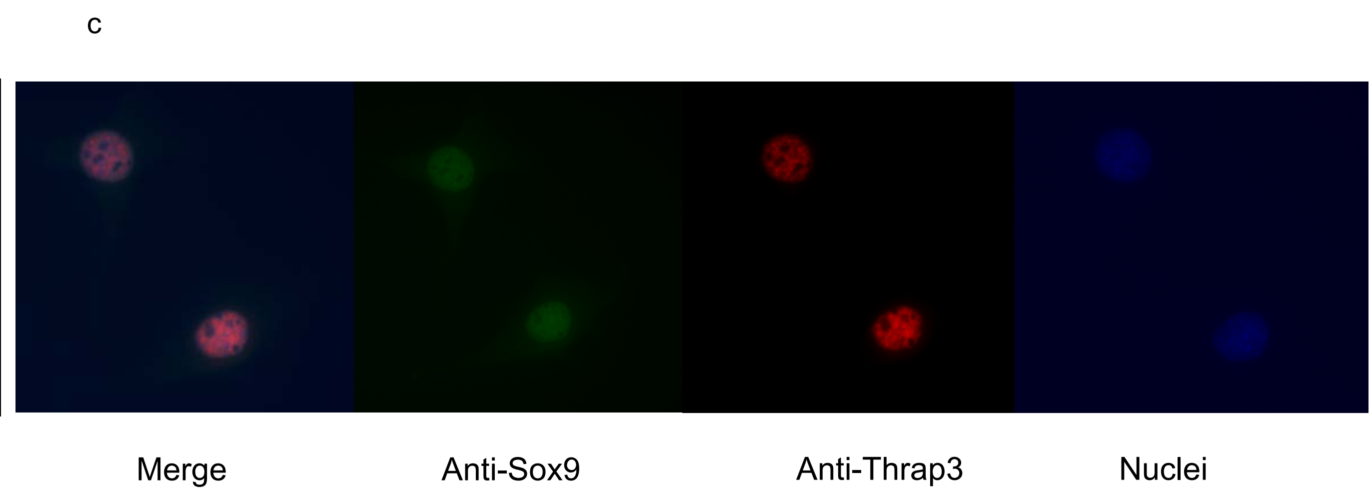

Figure 4

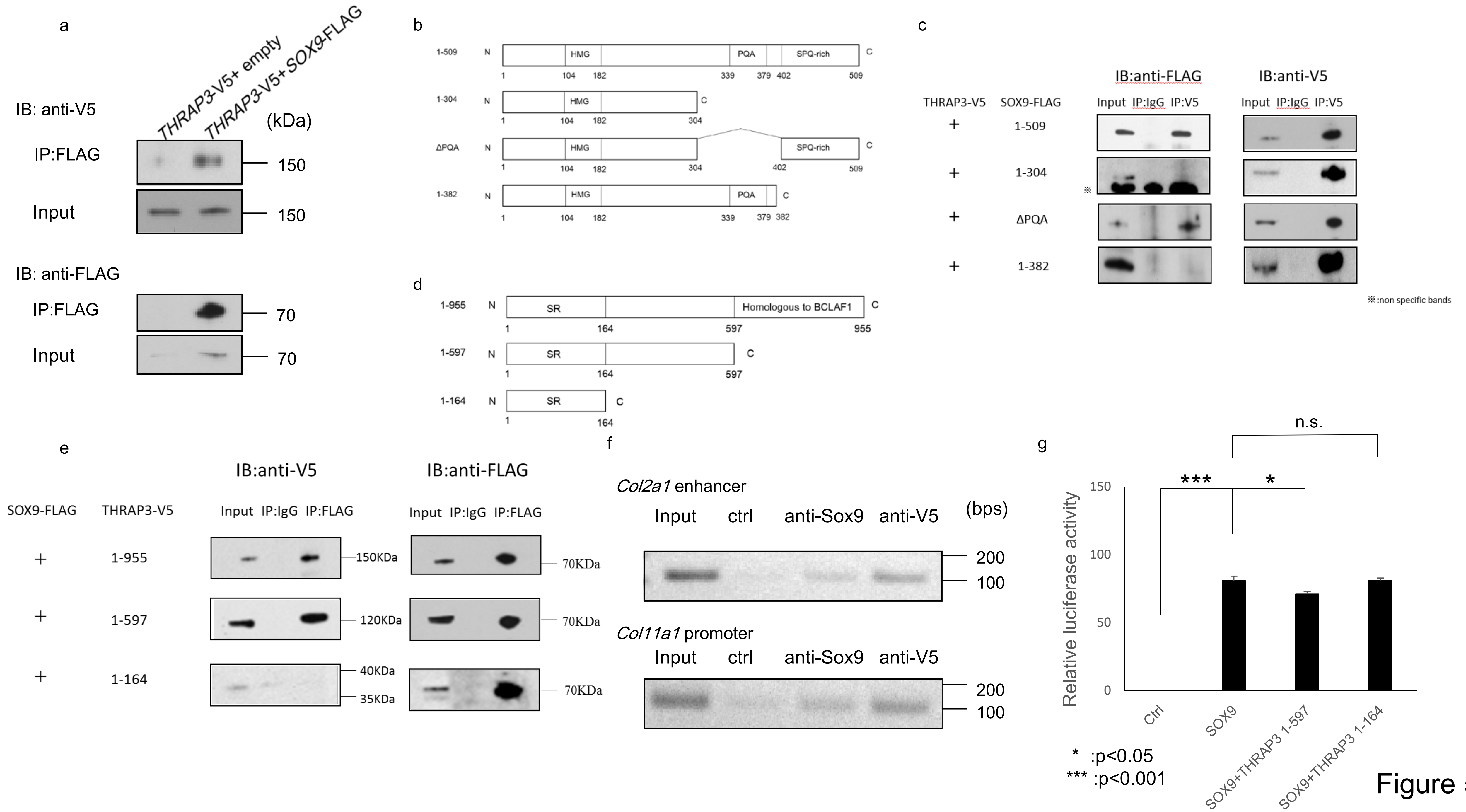

Figure 5