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Transcriptional regulation of chondrogenesis by coactivator Tip60 via chromatin association with Sox9 and Sox5

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ABSTRACT

Sox9 is a transcription factor of the SRY family required for several steps of chondrogenesis. It activates the expression of various chondrocyte-specific genes, but the mechanisms and role of cofactors involved in Sox9-regulated gene transcription are not fully understood. Here, we report on the characterization of a Tat interactive protein-60 (Tip60) as Sox9-associated protein identified in a yeast two-hybrid screen. Both *in vitro* and *in vivo* assays confirmed the specificity of interactions between Sox9 and Tip60 including the existence of an endogenous complex containing both polypeptides in chondrocytes. Gel shift assays showed the presence of a complex containing Sox9, Tip60 and the DNA of an enhancer region of the *Col2a1* promoter. Reporter assays using a *Col2a1* promoter with multimerized *Col2a1* Sox9-binding sites indicated that Tip60 enhanced the transcriptional activity of Sox9. A larger *Col2a1* promoter showed that Tip60 increased the activity of this promoter in the presence of both Sox9 and Sox5. Ectopic expression of Sox9 and transient-cotransfection with Tip60 in COS7 cells showed a more diffuse subnuclear colocalization, suggesting changes in the chromatin structure. Chromatin immunoprecipitation assays showed that Tip60, Sox9 and Sox5 associated with the same *Col2a1* enhancer region. Consistent with a role of Tip60 in chondrogenesis, addition of *Tip60* siRNA to limb-bud micromass cultures delayed chondrocyte differention. Tip60 enhances acetylation of Sox9 mainly through K61, 253, 398 residues; however, the K61/253/398A mutant of Sox9 still exhibited enhanced transcriptional activity by Tip60. Our results support the hypothesis that Tip60 is a coactivator of Sox9 in chondrocytes.

INTRODUCTION

Regulated changes in chromatin structure play a central role in the control of gene transcription (1). Posttranslational modifications of nucleosomal histones have been proposed to influence chromatin structure and to create a code that is interpreted by positive and negative transcriptional regulators recognizing specific histone modifications. Histone acetylation, catalyzed by histone acetyl transferase (HAT), promotes gene transcription by relaxing the chromatin structure, thereby facilitating access of the transcriptional machinery to DNA target sequences (1–3). The transcription-activating effect of histone acetylation is counterbalanced by histone deacetylation, which favors chromatin condensation and transcriptional repression (4).

Sox9, a transcription factor of the SRY (sex-determining region, Y-chromosome) family (5), is required for the establishment and differentiation of several cell lineages including chondrocytes (6–8), Sertoli cells of male gonads (9), glial cells of the nervous system in the spinal chord (10), Paneth cells in the intestine and others (11,12). During chondrocyte differentiation Sox9 is expressed abundantly in mouse chondroprogenitor cells and overtly differentiated chondrocytes. It regulates transcription of cartilage-specific extracellular matrix molecules such as collagen types II, IX, XI and aggrecan (6–8). Heterozygous mutations in the Sox9 gene cause

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campomelic dysplasia, a severe skeletal malformation syndrome characterized by a generalized hypoplasia of endochondral bones (13). Sox9 inactivation studies in mice indicate that Sox9 has an essential role at several steps of chondrogenic differentiation including mesenchymal condensations and overt differentiation of chondrocytes (14). In the absence of Sox9, no chondrocyte marker genes are expressed (7,13,14) but the precise mechanism of transcriptional activation of cartilage-specific genes by Sox9 is only poorly understood.

Members of the Sox family of transcription factors contain a high-mobility group (HMG) box DNA-binding domain that is at least 50% identical with an equivalent-domain in the sex-determining factor SRY (15–17). Sox9 also contains a potent transcription activation domain located at its carboxyl end (18) and a dimerization domain, needed for full activity in chondrocytes, located between the N-terminus and the DNA-binding domain (19,20). Previous experiments have shown that two other members of the Sox family, Sox5 and Sox6, cooperated with Sox9 to activate the *Col2a1* and *aggrecan* genes (7,21,22). In *Sox5* and *Sox6*, double mutants overt differentiation of chondrocytes is severely inhibited (23).

Because Sox9 is required for the differentiation of several cell lineages, which are characterized by very different genetic programs (10,24), other transcription factors should provide specificity to the function of Sox9 in each of these cell types. *In vivo*, this specificity needs additional factors besides Sox5 and Sox6 since these three Sox proteins are coexpressed in other cell types besides differentiating chondrocytes (9,25,26). In order to identify Sox9-associated polypeptides, a yeast two-hybrid screen was performed using a human chondrocytic cell line, HCS-2/8 (27). One of the factors identified as a Sox9 interacting protein was Tip60, originally described as a coactivator for the human immunodeficiency virus TAT protein (28). Tip60 is a member of the MYST family (2,29) of related chromatin polypeptides. It mainly acetylates histone H4, modulates DNA-damage response signaling, which is triggered by oncogenes, and controls cell cycle checkpoints and apoptosis(28). Haplo-insufficiency of Tip60 causes tumorigenesis through DNA-damage (30). Recent data suggest that Tip60 is a tightly regulated transcriptional coactivator for androgen- (31), estrogen- (32) and progesterone- (32) receptors. It also acts as a coactivator of p53 in activation of the p21 promoter and has been found on c-Myc and NFKB target genes (29,33,34). Tip60 is expressed transiently during early heart development (35). Our results indicate that Tip60 enhances Sox9 activation and Sox5-mediated Sox9 transactivation, and thus may be a key regulator of chondrogenesis.

MATERIALS AND METHODS

Yeast two-hybrid cDNA library screen and constructs

Yeast two-hybrid screen was performed as described previously (36). Full-length and truncated cDNAs of Sox9 were prepared as baits by PCR amplification and cloned into pGBKT7 vector containing the DNA-binding domain of GAL4. For screening, a cDNA library from the human chondrocytic cell line HCS-2/8 was constructed in the EcoRI site of the pGADT7 vector, which includes the GAL4-activation domain (AD). A total of 5×10^6 independent cDNA clones with an average insert size of ~2.4 kb (range: 2.0–4.0 kb) was obtained. Aliquots of 50 μg of the resulting cDNA library were then individually transformed into AH109 yeast cells that had been transformed with a Sox9-bait containing amino acids 26–189 or 26–415, and screened on selection-plates lacking leucine, tryptophan and adenine, and containing 3-AT and X-gal. After 3–5 days incubation, positive clones were picked, and plasmid DNA was re-transformed into *Escherichia coli* DH5α strain for further cloning and sequencing.

To confirm the binding specificity of Tip60, a pGADT7 vector expressing full-length Tip60 was re-transformed in the AH105 yeast strain together with the pGBKT7 vector expressing either full-length or truncated forms of *Sox9*. The empty pGADT7 vector was also re-transformed together with the same series of Sox9 truncations as control. All double transformants were cultured in SD/-Trp/-Leu selection medium, equal numbers of cells were collected and cellular proteins prepared by 3-fold freezing and thawing in yeast Z buffer (0.1 M Na-phosphate, pH 7.0, 20 mM KCl, 1 mM $MgSO_4$). To measure the binding strength between Tip60 and either full-length or truncated forms of Sox9, β-galactosidase activity of the reporter gene was measured, standardized to protein concentration and subtracted with the β-galactosidase activity obtained after cotransformation of the pGADT7 empty vector with the same series of Sox9 polypeptides.

A Sox9 expression vector (pcDNA/HA3Sox9) was kindly provided by Dr Shunichi Murakami (M.D. Anderson Cancer Center, TX, USA). Full-length Sox9 linked to a 3× multimerized hemaglutinin (HA) tag at its N-terminal was cloned into the pcDNA-5′UT vector (37). For generation of pEGFP-Tip60, Tip60 cDNA fragments were prepared by PCR using primer set of EGFPTip3′: 5′-gCCgAATTCTCACCACTTCCCCCTCT TgCTCCAgT-3′ and EGFPTip5′: 5′-CTCAgATCTATgg CggAggTgggggAgATAATCgAg-3′, and pGADT7/Tip60 vector as a template, and cloned into pEGFP-C1 vector (Clontech, CA, USA). A Sox5 (L-Sox5) expression vector was described previously (22). Wild-type and K61/253/398A Flag-Sox9-His expression vector were used previously (36).

Purification of recombinant proteins and production of antibody

For preparation of purified recombinant Sox9 protein, an HA-tag was coupled to the *Sox9* gene by PCR and subcloned into the pBACgus-2cp vector which carries a His and S-tag, and transfected into Sf-9 insect cells for expression of recombinant proteins. The expressed Sox9 protein was purified with Ni–NTA agarose (Qiagen, Valencia, CA, USA) (36). For Tip60 recombinant protein, Tip60 cDNA was amplified by PCR and subcloned into pBACgus-2cp vector, and transfected into Sf-9 cells: Expressed S- and His-tagged Tip60 protein was purified

with Ni–NTA agarose. Tip60 cDNA was also excised from pBACgus-2cp/Tip60 vector, and re-subcloned into pGEX-6P vector (GE Healthcare, NJ, USA). The pGEX-6P/Tip60 vector was transformed into *E. coli* BL21(DE3)pLysS Rosetta strain, and expressed GST-tagged Tip60 protein was immobilized onto glutathione–Sepharose-4B (GE Healthcare).

Cell culture and DNA transfections

COS7 monkey kidney cells, and HCS-2/8 human chondrosarcoma cells and human primary synovial cells were maintained in Dullbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transient transfection experiments were performed using Fugene 6 (Roche, Indianapolis, IN, USA). Knock-down experiments were performed with siRNAs for Tip60. This Tip60 siRNA, derived from mouse sequences, also recognize the corresponding human sequences. The sequences of the top strands of the siRNAs were as follows: Tip60-1: 5′-ACGGAAGGUGGAGGUGGUU-dTdT-3′ control-1: 5′-CAUGUCAUGUGUCACAUCU-dTdT-3′ (33). Transfection of siRNA was performed with X-tremeGENE (Roche, Penzberg, Germany) according to the manufacturer's instructions.

Luciferase reporter gene assay

Activities of the p89 (4×48) and p3000i3020 *Col2a1*-luciferase reporter (37,38) and that of the internal control pCMV/β-galactosidase or pGL3 (luciferase) were measured 24 h after transfection. Cells were harvested in lysis buffer containing 0.1 M potassium buffer, pH 7.8, 1 mM dithiothreitol and 0.2% TritonX-100 and luciferase and β-galactosidase activity measured as described previously (36,39).

Immunoblotting and immunoprecipitation

For both immunoblotting and immunoprecipitation, cells were washed with ice-cold phosphate-buffered saline (PBS) and lyzed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1 mM PMSF). After brief sonication, the lysates were centrifuged at 4°C, and protein concentration was measured. For immunoblotting, the cell lysates were boiled with 2 × SDS-sample buffer (0.125 M Tris–HCl, pH6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.02% bromphenol blue). For immunoprecipitation, the lysates were incubated with antibody and proteinG–Sepharose CL-4B (GE Heathcare) for 4 h at 4°C. The immunocomplexes were washed five times with the same lysis buffer, and the immunoprecipitated proteins were removed from the protein G agarose by boiling in 2 × SDS-sample buffer and loaded on SDS–PAGE. Polypeptides were transferred to PVDF membranes; membranes were treated with a primary antibody and then with a horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected by ECL chemiluminescene (ECL, GE Healthcare) and exposed to X-ray film.

GST pull-down assay

HA-tagged full-length Sox9 and truncated Sox9 cDNAs were transcribed and translated *in vitro* in 50 µl of reaction mixture using TnT-T7-Quick Coupled Transcription/translation kit (Promega, Madison, WI, USA). Ten percent of the reaction mixtures were mixed with SDS-sample buffer and directly analyzed by SDS–PAGE. The rest of the reaction mixtures were then used for pull-down assay with glutathione–Sepharose-bound purified GST-Tip60 (100 ng protein in each reaction). Reactions were performed in 10 vol of immunoprecipitation buffers containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1 mM PMSF, for 4 h at 4°C. The agarose-gel was washed five times with the same buffer, and HA-Sox9 proteins were released by boiling with 2 × SDS-sample buffer and fractionated by SDS–PAGE, and immunoblotted with anti-HA-antibody.

Electrophoretic mobility shift assays

An oligonucleotide probe of 48-bp enhancer, which bind to the HMG box was made with complementary oligonucleotides: 48 bp-1: 5′-ggCTGTGAATCGGGCTCTG TATGCGCTTGAGAAAAGCCCCATTCATGAGA-3′ and 48 bp-2: 5′-ggTCTCATGAATGGGGCTTTTCTC AAGCGCATACAGAGCCCGATTCACAG-3′. Upper case sequence contains the nearly consensus binding site for HMG protein including Sox9, and gg residues were added at 5′-end of each oligonucleotide for labeling with ^{32}P-dCTP. Protein–DNA reactions were carried out as previously described (40). Assays with recombinant proteins and antibodies were carried out with 1 ng of poly (dG–dC) and 20 µg of bovine serum albumin (BSA).

Indirect immunofluorescence and fluorescence deconvolution microscopy

COS7 cells transiently transfected with different expression vectors and HCS-2/8 cells were first fixed with 4% formaldehyde/PBS for 15 min at room temperature. The cells were then permeabilized with 20 mM Tris–HCl (pH 7.6), 137 mM NaCl and 0.1% Tween-20 (TBST) containing 5% skimmed milk for 30 min at room temperature, and incubated with primary antibodies for 90 min at 4°C. Anti-Sox9 rabbit antibody and anti-Tip60 goat antibody (Santa Cruz, CA, USA) were diluted to 1:100. After washing three times, the cells were incubated with secondary antibodies and 4′,6-diamidine-2-phenylindole dihydrochloride (2 µg/ml). The dilutions of the secondary antibodies were: Alexa Fluor 488-goat anti-rabbit IgG, 1:5000; Alexa Fluor 555 donkey-anti-goat IgG, 1:5000 (Molecular Probes, OR, USA). GFP-tagged Tip60 was directly detected. Images were obtained with a Zeiss Deconvolution Microscope (Zeiss, Oberkochen, Germany).

Micromass culture

Micromass cultures were prepared as described in (41,42). Briefly, anterior and posterior limb buds of 11.5-day mouse embryos (strain C57 black) were pooled and

digested by 2.5 mg/ml trypsin at 4°C for 3/4–1 h with gentle shaking. The cells were dissociated by pipetting and then centrifuged at 2000 r.p.m. for 5 min. The pellet was resuspended in culture medium F12 (Nissui, Tokyo, Japan)/DMEM (Nissui), 1:1, with 10% FCS (GIBCO) plus antibiotic/antimycotic (GIBCO, Pasuley, UK) at a density of 1–2 × 10^7 cells/ml. A total 1 × 10^5 cells/10 µl of cell suspension was placed in 24-well plate and placed in 5% CO_2 at 37°C for 1 h. After 1 h, 1 ml of culture medium containing 50 µg/ml of ascorbic acid was added to the wells. Two hours later, the cells were treated with transfection reagent with or without siRNAs and medium was replaced every other day thereafter. Cell differentiation was assessed by 0.1% alcian blue staining and immunostaining with anti-type II collagen antibody (Cosmo Bio, #LB-1297) and detected with PicTure Kit (Zymed, CA, USA). For analysis of gene expression level, the cultured cells were collected and total RNA was prepared using RNeasy mini kit (Qiagen, Germany). cDNA was prepared using RNA-PCR kit (Takara, Japan) and PCR reaction using SYBR Green Realtime PCR Master Mix (Toyobo, Japan) was monitored by LightCycler (Roche, Germany). Primer sequences used: For *aggrecen*: 5′-gaggagagaactg gagaag-3′ and 5′-gccgatagtggaatacaac-3′; for *Col2a1*: 5′-cgagtggaagagcggagactac-3′ and 5′-ccagtttttccgagggac agt-3′; for *GAPDH*: 5′-caatgacccccttcattgacc-3′ and 5′-gac aagcttcccgttctcag-3′.

Immunohistochemical analysis

Mouse embryo front limbs (E15.5) were fixed in 4% formaldehyde in PBS for 16 h. After dehydration, tissues were embedded in paraffin and sectioned. Immunohistochemical staining was done with the PicTure Kit (Zymed) for anti-Sox9 rabbit IgG, and anti-Tip60 rabbit IgG (Santa Cruz).

Chromatin immunoprecipitation (ChIP) asssays

The ChIP assayswere performed in HCS-2/8 cells and synovial cells. Cross-linked and fragmentated lysates were immunoprecipitated with control antibody, with anti-Sox9, anti-Tip60 (goat, Santa Cruz) or anti-Sox5 antibodies. PCR was performed to detect the coprecipitated *Col2a1* enhancer region with primer pairs: 5′-cctcctctc catctttccacgagtttgag-3′ and 5′-taccgcagggccgtataagtgattct ttg-3′ (for human), and, 5′-cctcctctccatctttccacgagtttg-3′ and 5′-ggaactgttttgcttcgtcgccgctgt-3′ (for mouse). For control PCR reaction, β-actin gene was used. The sequences were: 5′-gatgacctggccgtcaggcagctcata-3′ and 5′-acgtagccatccaggctgtgctgtccc-3′.

In vivo Sox9 acetylation assays

In vivo Sox9 acetylation assay was done basically according to the previous report (43). Briefly, COS7 cells were transfected with a vector (pcDNA3.1) for expression of Flag- and His-tagged wild-type and K61/253/398A Sox9 with or without GFP-Tip60 18 h before subsequent treatments. The cells were treated with 2 µM tricostatin A (TSA) and 5 mM nicotinamide (NA) for 6 h before collection. The cells were lyzed in lysis buffer [10 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 0.1% Nonidet P-40, 10% glycerol] supplemented with a complete protease inhibitor cocktail (Roche Diagnostics), TSA and NA. The whole-cell lysates were diluted with an equal volume of incubation buffer [25 mM Tris–HCl (pH 7.9), 0.5% Triton X-100, 0.05% SDS] and incubated with Ni-agarose (Qiagen) for 1 h at 4°C with gentle mixing. After the incubation, the resin was washed three times with wash buffer [20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$ and 0.2% Nonidet P-40]. After the final wash, the resin was boiled in SDS-sample buffer and the samples were resolved by SDS–PAGE with subsequent western blotting using an antibody to acrtyl-Lys (Upstate, NY, USA).

RESULTS

Identification of Tip60 as a Sox9 interactive molecule

Using a yeast two-hybrid screen to search for proteins that directly interact with Sox9, we obtained several clones from an HCS-2/8 human chondrosarcoma cDNA library which encoded for Tip60. Tip60 exists in three isoforms: isoform 1 (accession number: NP_874369) encodes additional 33 amino acids at N-terminus of isoform 2 (accession number: NP_006379), while isoform 3 (accession number: NP_874398) excludes 52 amino acids in the N-terminal part. The Tip60 which we obtained included amino acids 18–514 of isoform 2 and was either isoform 1 or 2. Tip60 is expressed relatively strongly in testis, heart, brain, and weakly in kidney, liver and lung (44). To confirm direct interactions between Sox9 and Tip60, we used several methods. First, to identify the Tip60 interaction sites in Sox9, full-length, N-terminal, C-terminal and internal fragments of Sox9 were expressed in AH109 yeast cells as GAL4-DNA-binding domain-fusion proteins, whereas Tip60 was coexpressed as a GAL4-transactivation domain-fusion protein (Figure 1A). Interaction strength between Tip60 and Sox9 fragments was estimated by measuring β-galactosidase activity reflecting the activity of the reporter containing GAL4-binding sites (Figure 1B). To measure the interaction strength between Tip60 and either full length or truncated forms of Sox9, β-galactosidase activity of the reporter gene was measured, standardized to protein concentration and corrected for the β-galactosidase activity obtained after cotransformation of the pGADT7 empty vector with the same series of Sox9 polypeptides. Binding to Tip60 was achieved by an almost full-length Sox9 fragment that included residues 26–509, while the shorter fragments that included amino acid residues between 409 and 509 such as 182–509, 298–509 or 409–509 also showed similar or even higher efficiency. Other Sox9 truncated fragments lacking residues 409–509 bound less efficiently, indicating that Tip60 mainly interacts with the C-terminal transactivation domain of Sox9, however, other Sox9 fragments such as 26–189, which used for two-hybrid screening, or 26–304 also showed some binding activity to Tip60 (Figure 1B). *In vitro* coprecipitation assays using full-length and truncated Sox9 fragments, which were synthesized by *in vitro* transcription/translation and recombinant GST-Tip60, which was immobilized on a

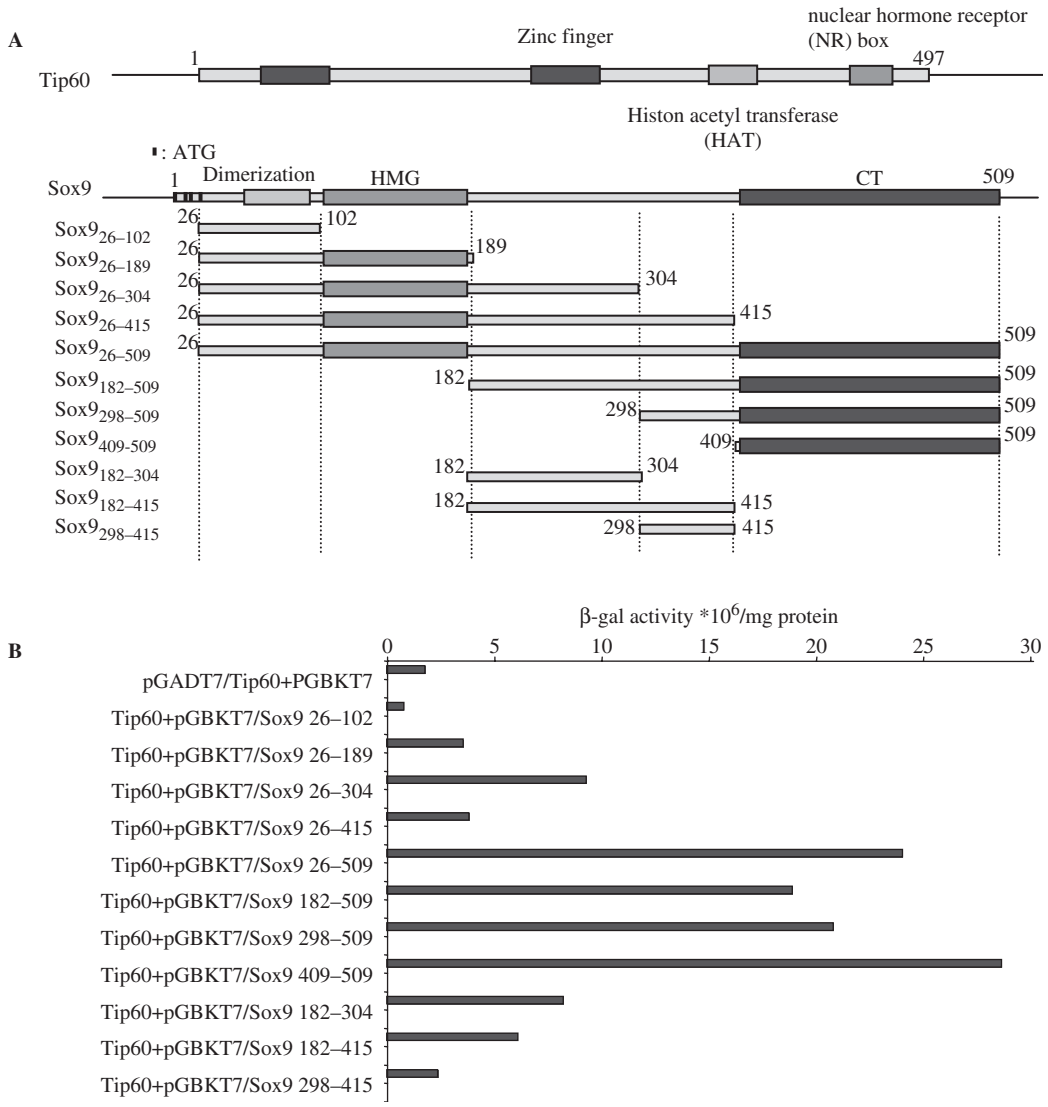

Figure 1. Tip60 associates with Sox9 in yeast cells. (**A**) Schematic diagram of Tip60 and Sox9 constructs. Tip60 contains a putative zinc finger domain, a HAT domain and a nuclear hormone receptor (NR) box. Sox9 contains a dimerization domain, a high-mobility group DNA-binding domain (HMG Box), a C-terminal transactivation domain (CT) and 4 ATG codons at the N-terminus portion in frame; the fourth ATG is sufficient for transactivation of C*ol2a1* reporter *in vivo* and *in vitro*. (**B**) The ability of N-or C-terminally truncated Sox9 to associate with Tip60 in yeast was quantified. All Sox9 fragments containing the CT domain associate with Tip60, while the N-terminal part including the HMG box binds only weakly.

glutathione–Sepharose resin, largely confirmed the yeast two-hybrid assay results although they showed some quantitative differences (Figure 2A). For instance, the Sox9$_{409–509}$ fragment did not show strong interactions with Tip60 in the pull-down assay with GST-Tip60 beads, whereas the yeast two-hybrid assay showed strong interactions (Figure 2A). Overall, the results of the pull-down assays suggested that the C-terminal transactivation domain (Sox9$_{409–509}$) of Sox9 is important for interaction with Tip60 and that additional segments of Sox9 might also contribute to binding (see Figure 1A). We also examined the interactions between Sox9 and Tip60 by coimmunoprecipitation of the proteins in transfected COS7 cells. Figure 2B shows that Tip60 was immunoprecipitated with Sox9 antibody.

Sox9 contains a HMG-box domain that binds directly to a specific sequence in the minor groove of DNA. In EMSA experiments using a *Col2a1* enhancer (48 bp) probe that binds Sox9, the addition of recombinant Tip60 to recombinant Sox9 produced a slower-migrating DNA–protein complex and increased the total amount of complex formed (Figure 3, lane 4, asterisk). The presence of both Sox9 and Tip60-His in the protein–DNA complexes was verified by the addition of anti-Sox9 or anti-Tip60 antibodies. Anti-Sox9 antibody (Figure 3, lane 5) and anti-Tip60 antibody (Figure 3, lane 6) gave rise to supershifts.

Tip60 enhances Sox9-dependent transactivation of a reporter containing multimerized enhancer sequences of Col2a1 dose dependently

In cotransfection experiments of COS7 cells, Tip60 increased the activity of a Sox9-dependent *Col2a1* reporter construct 2.5 to 3 times (Figure 4A). The increase was

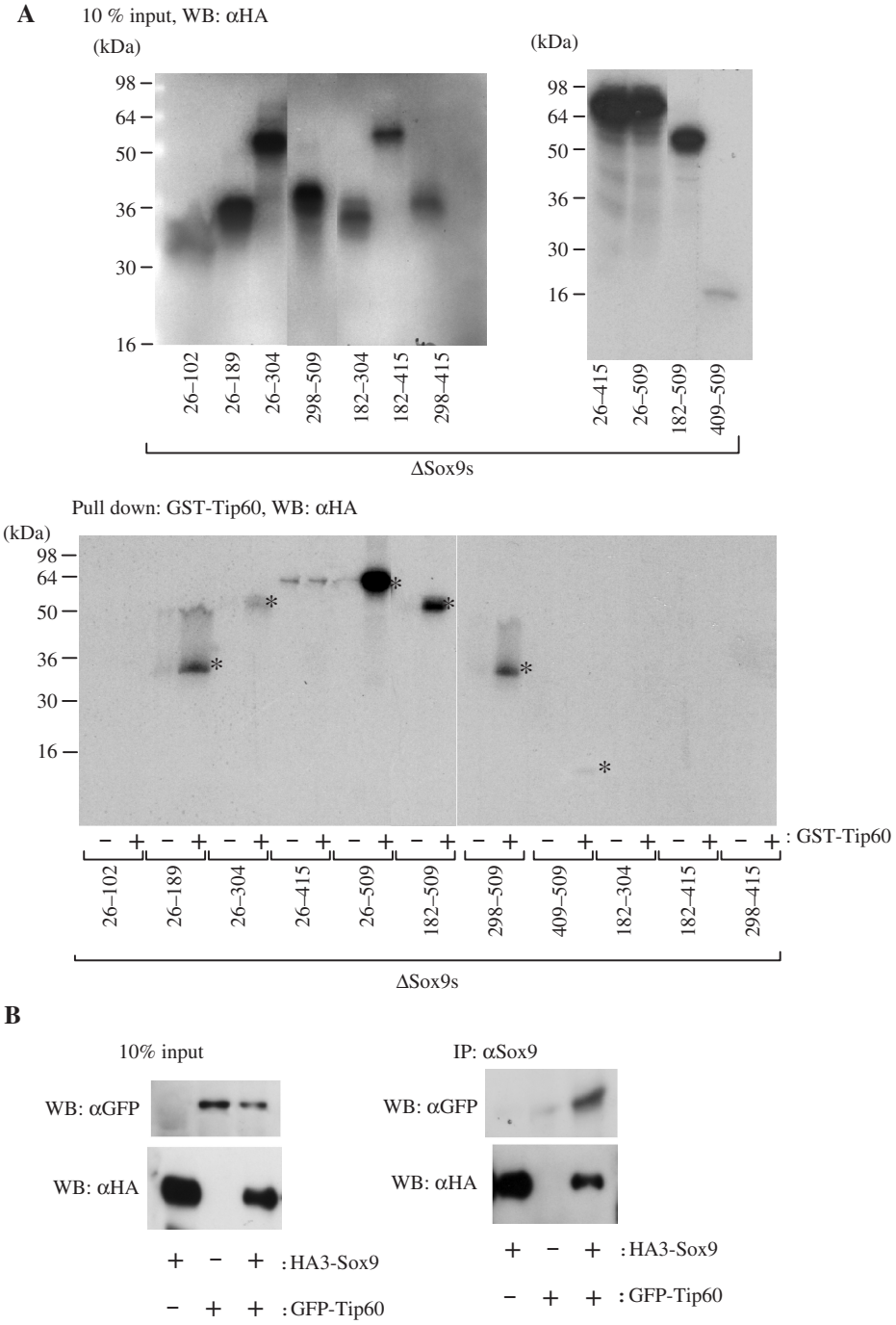

Figure 2. (**A**) *In vitro* association of Sox9 and Tip60. N-terminal or C-terminal truncated HA-Sox9 proteins were generated by *in vitro* transcription–translation, loaded onto SDS–PAGE, and detected by western blot using an HA antibody. Ten percent of the input Sox9 proteins (upper panel). Numbers indicate the molecular mass of protein markers in kiloDalton. Pull-down assay with GST-Tip60 (lower panel). Significant pull down of Sox9 fragment (*) was seen with Sox9 (26–509, 182–509, 298–509,), which contains the CT domain. Sox9 fragments (26–189), (26–415) and (409–509), showed weak binding activity in Figure 1. (**B**) *In vivo* association of Sox9 with Tip60. Full-length HA3-Sox9 or GFR Tip60 or HA3-Sox9 and GFP-Tip60 were transiently overexpressed in COS7 cells. Equal amounts of cellular proteins were immunoprecipitated with anti-Sox9 antibody. Coimmunopreciptated proteins were detected by either anti-GFP antibody for Tip60 or anti-HA antibody for Sox9. (Left): Ten percent input of expressed proteins were determined by anti-GFP antibody and anti-HA antibody. (Right): Detection of Tip60 after coimmunoprecipitation with Sox9.

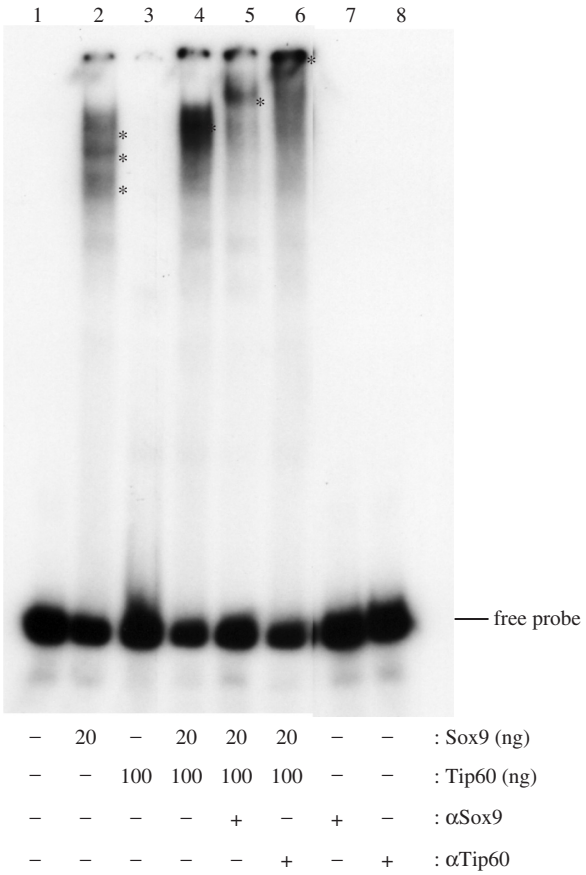

Figure 3. Tip60 binds to HMG sites *of Col2a1* in the presence of Sox9 and stabilizes Sox9–DNA complex. An oligonucleotide probe covering a 48-bp Sox9-dependent enhancer DNA segment of *Col2a1* intron1 was used for Sox9 binding by EMSA. His-tagged recombinant Sox9 and Tip60 were purified from Sf9 cells infected with baculoviruses expressing Sox9 and Tip60, respectively, using Ni–agarose. In the presence of Tip60, the intensity of the Sox9–DNA complex increased and migrated more slowly (lane 4). The existence of Sox9 and/or Tip60 in this slow-migrating complex was verified by adding specific antibodies which caused a supershift (lanes 5 and 6). Asterisks indicate the mobility of the major complexes in each lane.

dependent of Sox9 binding to DNA, since a mutant *Col2a1* reporter, (MA6)4x48p89/Luc construct, which lacked Sox9 binding (45), did not show any transactivation in the presence of Sox9 and Tip60 (Figure 4A). The Tip60-mediated increase in transactivation by Sox9 of the *Col2a1* reporter was also dose dependent (Figure 4B).

Subnuclear localization of Tip60 changes after binding Sox9

Because Tip60 is reported to act as a HAT, we evaluated potential effects of Tip60 on the subnuclear localization of Sox9. In the chondrocyte cell line HCS-2/8 expressing both endogenous Tip60 and Sox9, both proteins showed colocalization in the nucleus in a diffuse pattern (Figure 5A). Cos7 cells in which HA-tagged Sox9 was overexpressed revealed a granular subnuclear localization, whereas Cos7 cells expressing a green fluorescent protein (GFP)-Tip60 fusion polypeptide showed a punctated subnuclear localization when each of these was overexpressed separately (Figure 5B). When HA-Sox9 and GFP-Tip60 were co-overexpressed in COS7 cells, Sox9 showed a slightly more diffuse localization, whereas the Tip60 distribution changed from a punctuated to a diffuse localization, largely overlapping with Sox9. A proportion of Sox9 and Tip60 staining remained separate (Figure 5B).

Tip60 modulates chondrogenesis during endochondral ossification

During endochondral ossification, Sox9 is expressed specifically in resting and proliferative cartilage but not in hypertrophic cartilage, whereas Tip60 is expressed throughout the cartilage including hypertrophic cartilage (Figure 6A). To examine the possible role of Tip60 in chondrogenesis, we synthesized a siRNA specific for Tip60 to downregulate Tip60 mRNA levels (see Materials and methods section) and an inactive control siRNA. The Tip60-siRNA partially downregulated Tip60 protein expression at a concentration of 20 nM and 100 nM. Control siRNA did not affect Tip60 expression (Figure 6B). The ability of Tip60 to interfere with chondrogenic differentiation was then investigated in micromass cultures of mesenchymal cells derived from 11.5-day embryonic mouse limbs (46–48). The mesenchymal cells started to develop alcian blue positive cartilaginous nodules after 2 days of culture (Figure 6C). The siRNA of Tip60 significantly reduced the accumulation of proteoglycans around cartilaginous nodules after 2 or 4 days in culture, whereas control siRNAs did not decrease alcian blue staining. Accumulation of type II collagen was also inhibited in Tip60 siRNA-treated cultures after 4 days culture as shown by immunostaining (Figure 6D). Gene expression of aggrecan and Col2a1 was also downregulated in micromass cultures by Tip60 siRNA as measured by quantitative RT–PCR (Figure 6E).

Detection of Tip60–Sox9 interaction on Col2a1 enhancer by ChIP

To further explore whether Tip60 associated with the same chromatin segment of the *Col2a1* enhancer element of intron 1 which is the target of Sox9, we performed ChIP experiments using HCS-2/8 cells which express both Sox9 and Tip60, As shown in Figure 7, anti-Tip60 precipitated the same *Col2a1* chromatin fragment in the *Col2a1* intron 1 enhancer that was precipitated with the anti-Sox9 antibody; the coprecipitated *Col2a1* fragment was identified by PCR (Figure 7A, upper photo). Interestingly, Sox5, which is known to bind to the same *Col2a1* enhancer in EMSA assays, also interacted with this segment in the ChIP assay (Figure 7A). In human synovial cells, which express Tip60 but not Sox9, ChIP with anti Tip60 did not indicate any precipitation of the *Col2a1* enhancer, suggesting that recruitment of Tip60 to the *Col2a1* promoter requires Sox9. To confirm the association of Sox9, Tip60 and Sox5 with the Col2a1 enhancer, ChIP analysis was also performed with primary mouse chondrocytes. As in HCS-2/8cells, all three components were found to associate with the *Col2a1* enhancer, while the *β-actin* gene which does not contain Sox9-binding

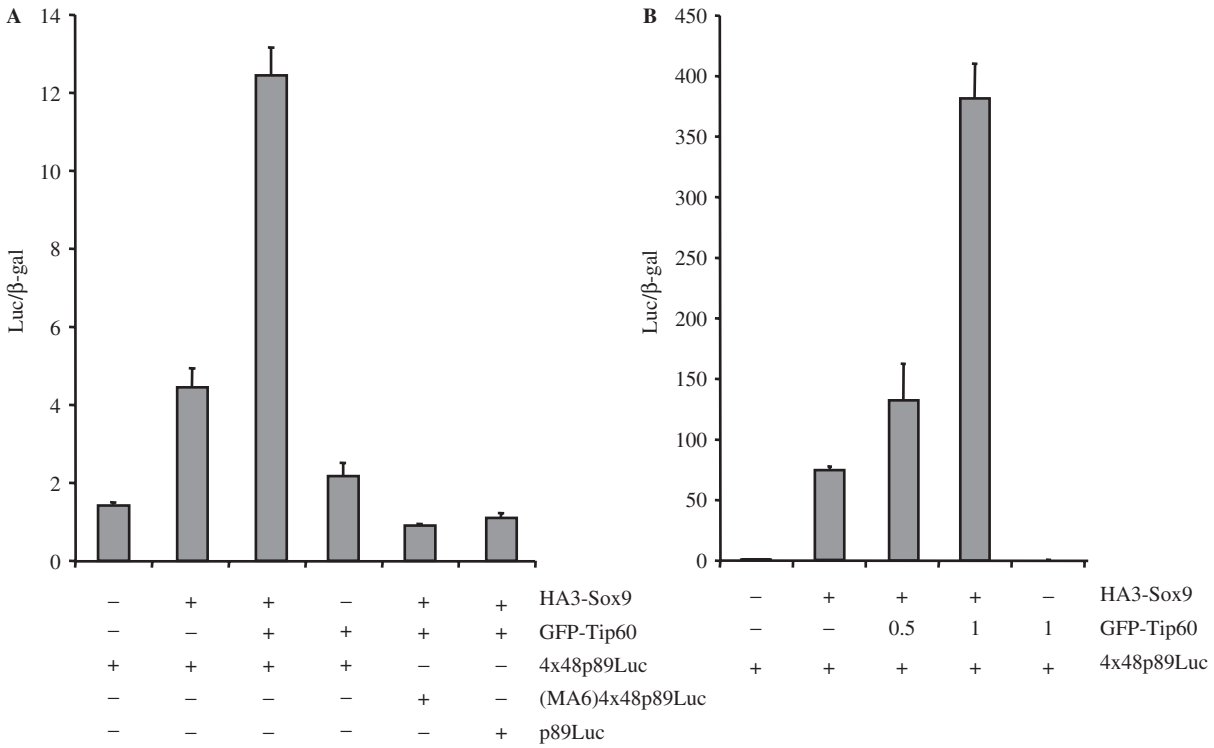

Figure 4. Tip60 enhances Sox9 transcriptional activity. (**A**) Tip60 enhances Sox9 transactivation activity using a reporter with a multimerized *Col2a1* enhancer (4 × 48 *Col2a1* p89). COS7 cells were cotransfected with HA3-Sox9 (3 ng) and/or GFP-Tip60 (1 μg) expression vectors with 4 × 48 *Col2a1* p89 (0.77 μg), or the inactive mutant mA6 of 4 × 48 *Col2a1* p89 (0.77 μg), or with p89 (0.77 μg) luciferase reporter constructs and SV40/β-galactosidase (3 ng) reporter construct as indicated. (**B**) Tip60 enhances Sox9 transactivation activity of a reporter with a multimerized *Col2a1* enhancer (4 × 48 *Col2a1* p89) dose dependently. Cos7 cells were cotransfected with HA3-Sox9 (100 ng) and/or GFP-Tip60 (0.5 or 1 μg) expression vectors together with 4 × 48 *Col2a1* p89 (0.77 μg) luciferase reporter construct and SV40/β-galactosidase (3 ng) reporter construct as indicated.

elements [searched by TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html)] did not reveal any ChIP signals (Figure 7B).

Tip60 amplifies Sox9 transactivation enhanced by Sox5 in a natural *Col2a1* promoter

To test whether Tip60 might enhance the activity of *Col2a1* reporters, Tip60 was cotransfected with Sox9 in the presence of Sox5. Tip60 stimulated 4-fold the activity of a multimerized 48-bp *Col2a1* enhancer linked to a minimal *Col2a1* promoter (Figure 8A). With a reporter that contained a 3000 b promoter segment and 3020 b of exon1 and intron1 of *Col2a1*, the Tip60 enhancement was 8-fold, suggesting that Tip60 may act on a transcriptional complex containing both Sox9 and Sox5 also in the entire *Col2a1* gene (Figure 8B).

Since Tip60 slightly enhanced Sox5-induced transcription of the 3-kb–3-kb Col2al promoter–enhancer construct also in the absence of Sox9 (Figure 8B), the question remained whether Tip60 can bind directly to Sox5. To test this option, we carried out *in vivo* binding assay using cell lysates containing ectopically expressed Flag-Sox5, HA3-Sox9 and GFP-Tip60 (Figure 8C). The data show that anti TIP60 did not coprecipitate Sox5 with Tip60 and/or Sox9, suggesting that Tip60 does not bind to Sox5, but recruits Sox5 when binding to Sox9 in association with the DNA in the Col2a1 enhancer.

Tip60 acetylates Sox9 through Lys61/253/398, but enhancement of transcriptional activity of Sox9 by Tip60 is not affected by acetylation

Acetylation of lysine residues of transcription factors is an important mechanism regulating their activity and stability (43,49). Since Tip60 has been originally reported as a HAT, we examined whether acetylation of Sox9 by Tip60 affects its transcriptional activity. For detection of acetylated Sox9, we precipitated expressed Flag-Sox9-His in Cos7 cells by Ni–agarose and subsequently analyzed the acetylated protein by western blot using anti-acetyl-Lys antibody. In the absence of Tip60, treatment with the deacetylase inhibitors TSA and NA resulted in increased amounts of acetylated Sox9 (Figure 9A). In the presence of Tip60, acetylation of Sox9 was further increased and stabilized, and the amount of acetylated Sox9 was independent from the presence or absence of TSA and NA (Figure 9A).

It has been reported that lysine residues which are acetylated are also targets for SUMOylation (43). Since Sox9 is SUMOylated through K61, K253 and K398 (36), we examined whether K61/253/398A mutant is also acetylated by TIP60. Interestingly, the K61/253/398A

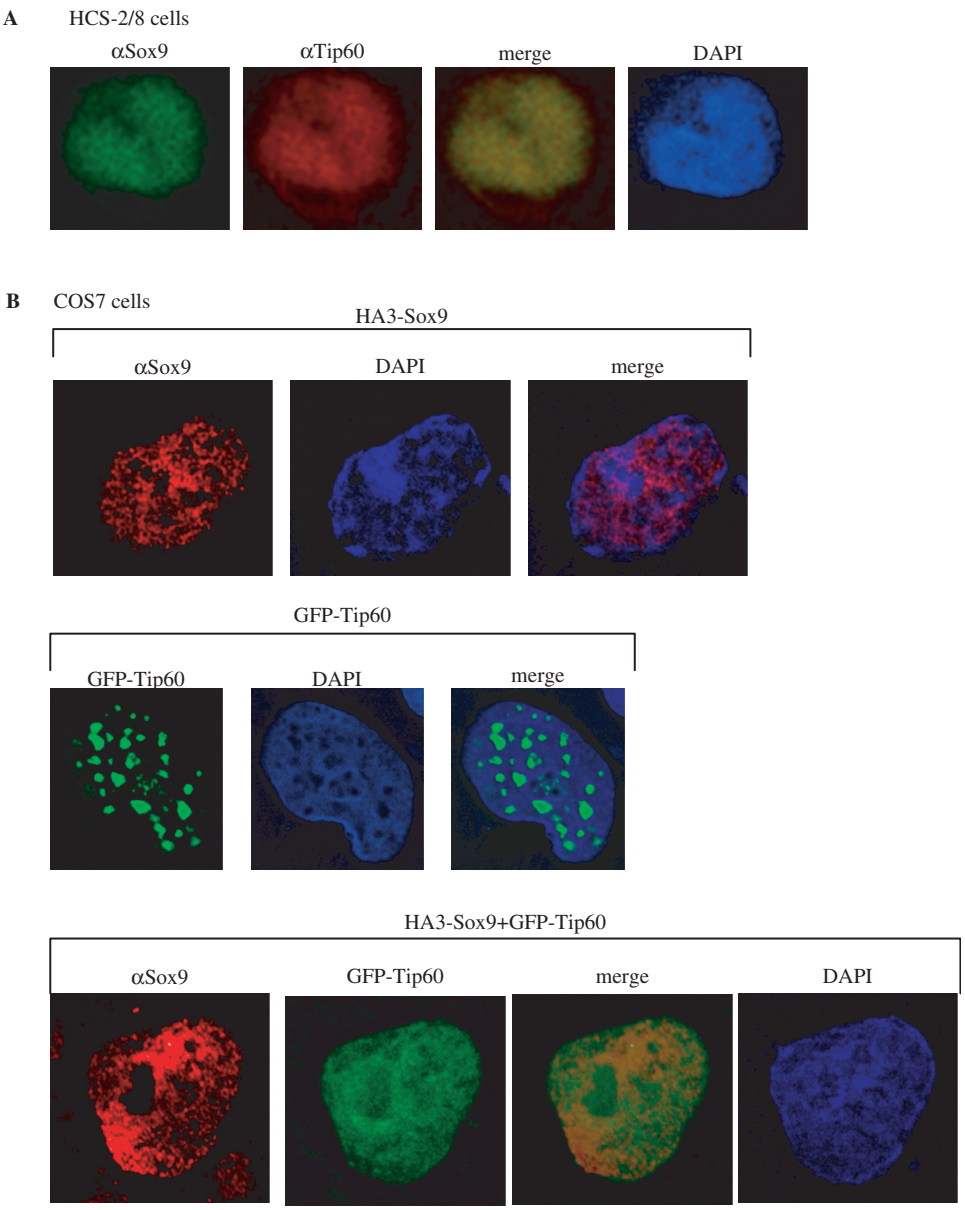

Figure 5. Subnuclear co-localization of Sox9 with Tip60 in the nucleus. (**A**) Sox9 protein and Tip60 protein in HCS-2/8 cells were stained with anti-Sox9 (green) and Tip60 (red) antibodies, respectively. The subnuclear localization of proteins was detected by indirect immunofluorescence and analyzed by deconvolution microscopy. The subnuclear distribution of endogenous Sox9 and Tip60 in HCS-2/8 cells overlapped in a diffuse staining pattern. (**B–D**) COS7 cells were transfected with expression vectors encoding HA3-Sox9 (B), or GFP-Tip60 (C) or both (D). Sox9 was stained with anti-Sox9 antibody and detected by indirect immunofluorescence, and Sox9 and Tip60 were analyzed by deconvolution microscopy. Sox9 alone displayed a punctuated distribution in the nucleus (B), while Tip60 alone showed a somewhat patchy distribution in the nucleus (C), but the Tip60 distribution became more diffused in the presence of Sox9 (D); about 50% of the nuclear area shows codistribution of Sox9 and Tip60.

mutant showed the same basal level of acetylation as wt Sox9, however, Tip60 did not enhance acetylation of the mutant, indicating that TIP60 acetylates the same lysine residues which are SUMOylated. Treatment with TSA and nicotinamide protected deacetylation of Sox9 even in the presence of Tip60, suggesting that Sox9 may contain acetylated lysine residues other than K61, K253 and K398.

Furthermore, the WT and K61/253/398A mutant of Sox9 showed enhanced transcriptional activity of 4×48 Col2a1 promoter–reporter construct after coexpression of Tip60 (Figure 9B), suggesting that the enhanced transcriptional activation byTip60 is independent from Sox9 acetylation.

DISCUSSION

Sox9 has been shown to be a key factor for the initiation of chondrogenesis and regulation of cartilage-specific gene expression from early stages of chondrogenic differentiation until onset of chondrocyte hypertrophy. To identify Sox9 modulating proteins, we carried out a yeast

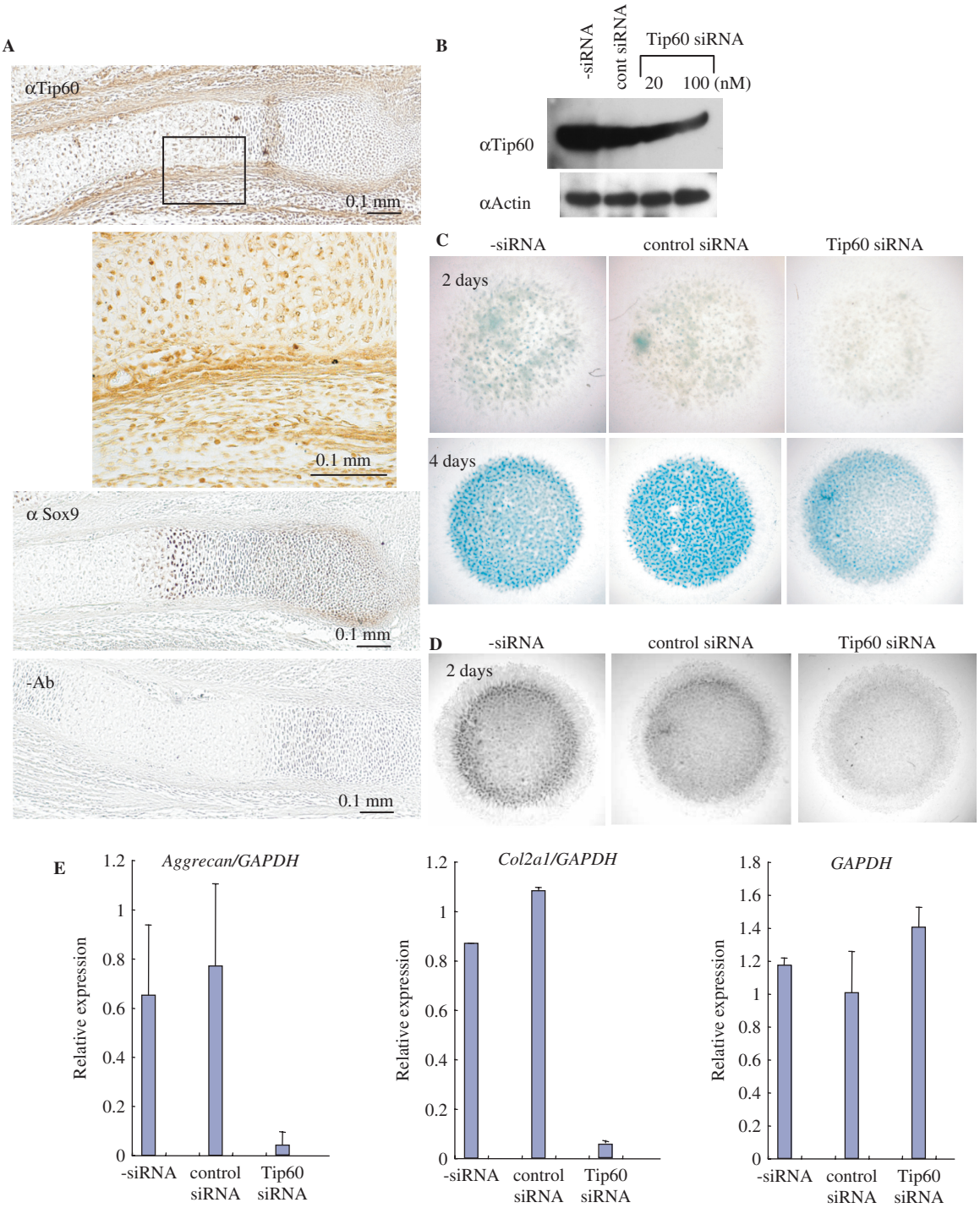

Figure 6. Expression of Tip60 in cartilage and delayed chondrogenesis after inhibition of Tip60 expression. (**A**) Longitudinal sections through a hindlimb of E15.5 embryo were stained with anti-Tip60 and Sox9 antibodies as indicated. Chondrocytes shows intracellular staining with both antibodies. (**B–E**) Knockdown of Tip60 with siRNA in high-density culture of E11.5 mouse limb bud mesenchymal cells. (B) Downregulation of Tip60 levels with siRNA specific for Tip60 compared to a control siRNA. Control siRNA (100 nM) and 20 or 100 nM siRNA were added to the high-density culture, Tip60 and actin were detected by western blot in cell extracts using specific antibodies 4 days after addition of siRNAs. (C) Upper panel: Alcian blue staining after 2 days (above) and 4 days (below) in cultures treated with 20 nM control siRNA and Tip60 siRNA. (D) Type II collagen immunostaining after 2 days in culture. (E) Quantitative RT–PCR analysis for aggrecan and Col2a1 mRNA in 2-day cultures standardized for GAPDH mRNA.

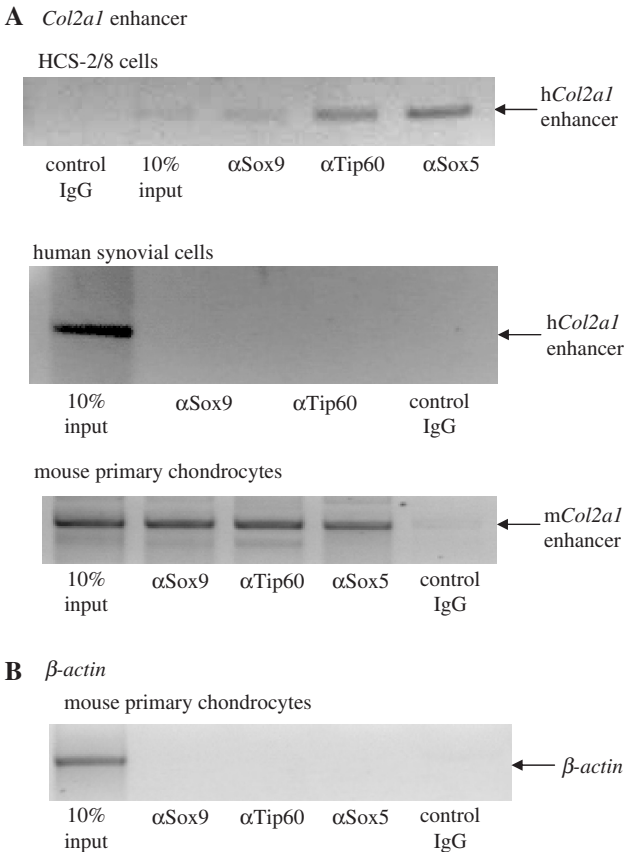

Figure 7. Tip60 and Sox5 associate with a Sox9-target locus in the Col2a1 enhancer. (**A**) ChIP analysis of human chondrocytic cell line HCS-2/8 (upper panel) and of primary mouse chondrocytes (lower panel) with antibodies against Tip60, Sox9, Sox5 demonstrates binding of all three factors to the Sox9 target. In human synovial cells, which do express Tip60 but not Sox9 (data not shown) the *Col2a1* locus is not precipitated by these antibodies (middle panel). The enhancer region of *Col2a1* intron1 was amplified by PCR. (**B**) CHIP of the *β-actin* gene of mouse primary chondrocytes with each antibody as a control confirms the absence of unspecific chromatin immunoprecipitates.

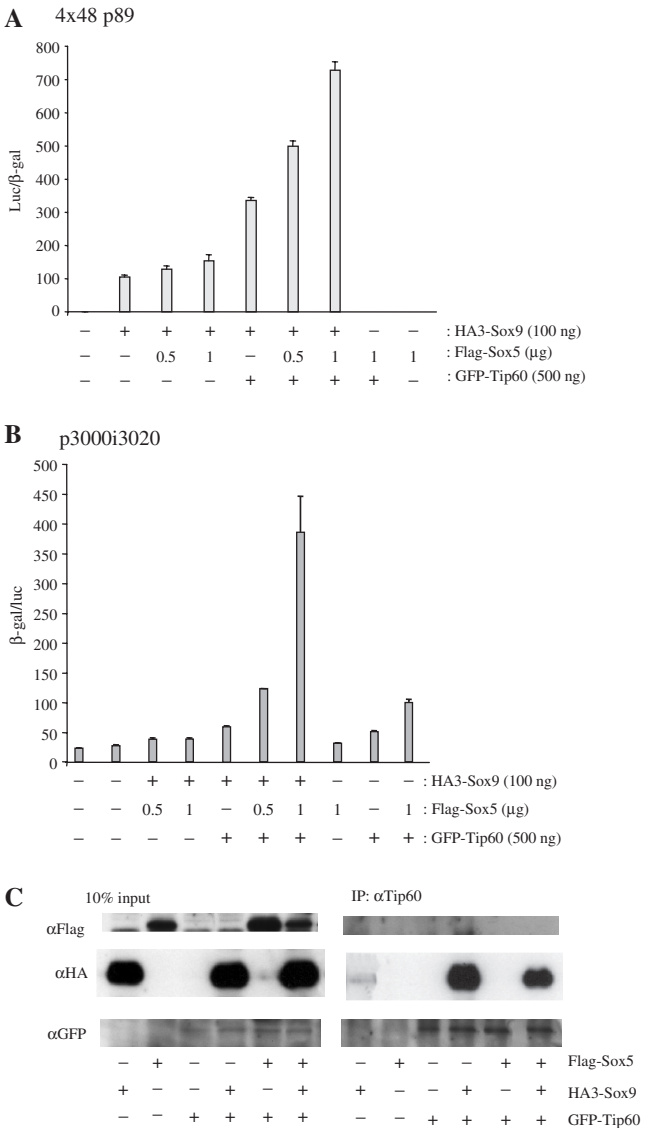

Figure 8. (**A**) Tip60 enhances Sox5-mediated transactivation of Sox9-induced transcription of a luciferase reporter gene containing a multimerized *Col2a1* enhancer (4 × 48 *Col2a1* p89). Cos7 cells were transfected with HA3-Sox9 (100 ng) and/or GFP-Tip60 (0.5 μg) expression vectors with a 4 × 48 *Col2a1* p89 (0.77 μg) luciferase reporter construct and a SV40/β-galactosidase (3 ng) reporter construct as indicated. (**B**) Tip60 further enhances Sox5 transactivation activity of a p3000i3020 *Col2a1* promoter reporter activated by Sox9. Sox5 shows little transactivation of Sox9-dependent activity in the absence of Tip60, but strong activity in the presence of Tip60. (**C**) Sox5 did not show direct interaction with Tip60. COS7 cells were transfected with expression vectors of Flag-Sox5 (1 μg) and/or HA3-Sox9 (1 μg) and/or GFP-Tip60 (1 μg). The cell lysates were immunoprecipitated with anti-Tip60 antibody, and Tip60 and interactive proteins were detected by anti-Flag, anti-HA and anti-GFP antibodies.

two-hybrid screen using a human chondrocyte cDNA library. We previously identified in this screen proteins of the PIAS family which act as SUMO ligases (36). Here, we report on another protein identified in this screen, the HAT Tip60 which interacts with Sox9 and enhances its transcriptional activity.

Specific interactions between Sox9 and Tip60 reported in this study were confirmed by several assays. A yeast two-hybrid assay using Sox9 deletions showed that the C-terminal transcriptional activation domain of Sox9 is the major Tip60-binding segment. The Tip60–Sox9 interaction was further supported by *in vitro* pull-down assays, by coimmunoprecipitation of Sox9 and Tip60 after cotransfection of COS7 cells, by their co-localization in the nucleus of these cells as detected by immunofluorescence, and by EMSA experiments demonstrating the existence of a DNA–protein complex that contains both Sox9 and Tip60. Immunofluorescence analysis of transfected HCS2/8 cells transiently expressing Tip60 showed a punctuated subnuclear localization. When Tip60 was co-overexpressed with Sox9, the distribution of Tip60 dramatically changed to a diffuse pattern. This phenomenon may be explained by a change in the Tip60 functions. The majority of cellular Tip60 exists in a s\nuclear multiprotein complex and is responsible for DNA damage-related responses (28). In the nucleus of Sox9 co-expressing cells, Tip60 may be involved in transactivation of different chromatin structures, thus assuming

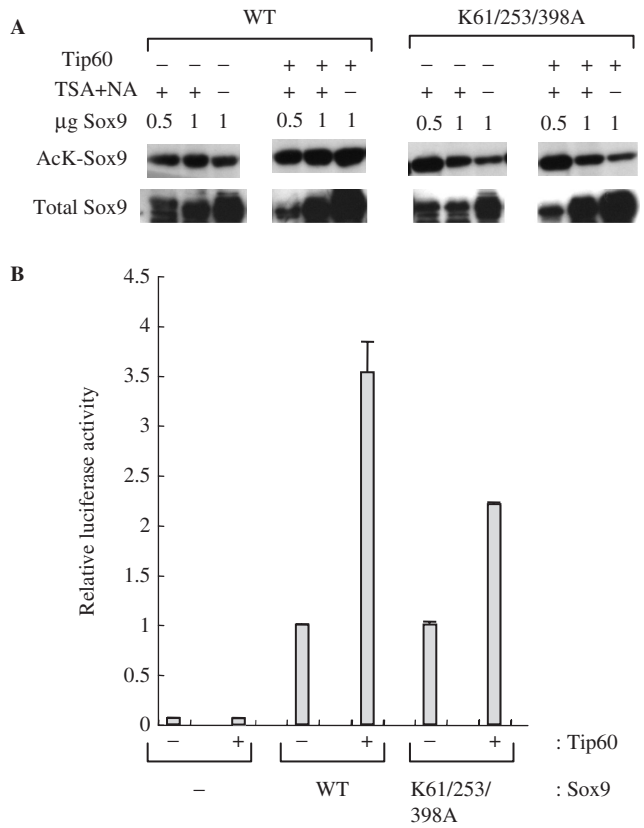

Figure 9. (**A**) Enhanced acetylation of Sox9 but not the K61/253/398A mutant by Tip60. COS7 Cells were transfected with 0.5 and 1 μg wild-type (WT) or K61/253/398A mutant Flag-Sox9-His expression vector. Sox9 from the cell lysates was precipitated with Ni–agarose, and the acetylated Sox9 was analyzed by western blotting using anti-acetyl Lys antibody. Total Sox9 in the same precipitates was detected by anti-Flag antibody. Acetylation of Sox9 was enhanced by the addition of deacetylation inhibitors trichostatin A (TSA) and nicotinamide (NA), which were added to some samples 6 h before collection. Cotransfection with a Tip60 expression vector (1 μg) resulted in enhanced Sox9 acetylation in the presence of TSA and NA; the enhanced level of acetylation was maintained in the absence of TSA and NA. Tip60 did not enhance acetylation of the mutant Sox9 K61/253/398A, and the acetylation level was not maintained in the absence of TSA and NA. (**B**) Not only wt Sox9, also the K61/253/398A mutant exhibited increased transcription activity of a luciferase reporter gene containing a multimerized *Col2a1* enhancer (4×48 *Col2a1* p89) after cotransfection with Tip60. Cos7 cells were transfected with WT or K61/253/398A mutant of Flag-Sox9-His (100 ng) and/or GFP-Tip60 (1 μg) expression vectors with a 4×48 *Col2a1* p89 luciferase reporter construct (0.77 μg) and a SV40/β-galactosidase (3 ng) reporter construct as indicated.

a diffuse subnuclear localization. Also in our previous study, activated Sox9 assumed a diffuse subnuclear localization in the presence of Sox9-binding protein, PIAS proteins and SUMO (36). These observations support the notion that changes in the subnuclear localization following activation of transcriptional factors may be due to changes in the chromatin structure.

A ChIP experiment indicated that Tip60, Sox5 and Sox9 interacted with the same *Col2a1* chromatin DNA fragment in chondrocytes; this segment contains a chondrocyte-specific enhancer. Sox5 and Sox6, which are coexpressed with Sox9 during chondrocyte differentiation, share a high degree of sequence identity. They bind to DNA as homo- or hetero-dimer but do not show a clearly defined transcriptional activation domain. Cotransfection of Sox5 and Sox6 together with Sox9 enhances Sox9 stimulated expression of the endogenous *Col2a1* and *aggrecan* genes in chondrogenic mesenchymal cells, suggesting that the three Sox proteins cooperate with each other in the activation of downstream genes (7,21). Because of their high degree of sequence identity it was hypothesized that Sox5 and Sox6 would have overlapping functions. Indeed, single-null Sox5 or single-null Sox6 mutant mice have a mild skeletal phenotype whereas the double Sox5 and Sox6 mutants show practically no cartilage and no overtly differentiated chondrocytes (23). Because chondrogenic mesenchymal condensations do form in the double mutants, it was concluded that Sox5 and Sox6 were required for overt differentiation of cells in mesenchymal condensations (23).

In reporter gene experiments in cotransfected COS7 cells, Tip60 increased Sox9-dependent activation of a *Col2a1* reporter containing a multimerized short chondrocyte-specific enhancer. This increased transactivation of Sox9 by Tip60 was further enhanced by coexpression of Sox5. Furthermore, the transcriptional activity of a reporter gene which includes a 3000 b promoter, first exon and 3020 b first intron of *Col2a1* was strongly enhanced by Tip60 when transactivated by Sox9 and Sox5. *In vivo* binding experiment of Tip60, Sox9 and Sox5 revealed that Tip60 binds directly to Sox9, but not to Sox5. These results indicate that Tip60 may recruit Sox5 only in association with the Col2a1 enhancer, and promotes the ability of Sox5 to enhance the trancriptional activity of Sox9. In fact, ChIP assays indicated that Sox9, Tip60 and Sox5 interacted with a *Col2a1* intron 1 segment containing a chondrocyte-specific enhancer, suggesting that these interactions are physiologically significant. Synovial cells which do express Tip60, but not Sox9, did not show interaction of Tip60 with the *Col2a1* enhancer, suggesting that the binding of Tip60 to *Col2a1* enhancer is accelerated by the presence of Sox9.

In the embryonic limb, Tip60 was detected in resting, proliferative and maturing chondrocytes. SiRNA knockdown of Tip60 mRNA resulted in delayed chondrogenesis, indicating that Tip60 is required for chondrocyte differentiation through the regulation of Sox9 and Sox5 transcription activity. This is in line with a recent report showing that Tip60 is expressed transiently during early heart development (35), and confirms the notion that specific transactivations of target transcription factors by Tip60 occur in developmental pathways.

Tip60 has been known as a histone acetylase (33). Here, we report for the first time that it may also play a role in regulating transcriptional activity of Sox9. There is ample evidence for transcription regulatory mechanisms by acetylation; for example, acetylation of C-terminal regulatory domain of P53 by both p300/CBP and PCAF (2) has been demonstrated to be critical for its regulation. Thus, acetylation of Sox9 by Tip60 may well be important for its transcriptional activity (50).

Previously, we have shown that in chondrocytes Sox9 lysine residues K61, 253 and 398 are SUMOylated by families of PIAS (36). Here, we provide evidence that

Tip60 enhances acetylation of some or all of these lysine residues since the Sox9 mutant K61/253/398A did not show enhanced acetylation by Tip60. Surprisingly, however, Tip60 also enhanced transcriptional activity of the Sox9 mutant K61/253/398A, suggesting that the observed enhancement of transcription activity of Sox9 may involve an additional mechanism, while the acetylation of Sox9 by Tip60 may not play an exclusive role. Yet, the realization that chondrogenic differentiation is governed by Tip60 HAT in connection with specific target transcription factors suggests possible strategies for therapeutic manipulating in development and disease.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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