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Characterization of the Role of SOX9 in Cartilage-Specific Gene Regulation

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CHARACTERIZATION OF THE ROLE OF SOX9 IN CARTILAGE-SPECIFIC GENE REGULATION

by

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ABSTRACT

CHARACTERIZATION OF THE ROLE OF SOX9 IN

CARTILAGE-SPECIFIC GENE REGULATION

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Although advances have been made toward understanding the complex mechanisms that regulate the process of DNA transcription, the specific mechanisms of activation for many individual genes remain unknown. In this study, we focus on the role the transcription factor SOX9 plays in activating cartilage-specific genes, specifically *Col9a1* and *Cartilage Link Protein (CRTL1*). Previously, enhancers of these genes containing single SOX9 binding sites were shown to be activated through SOX9 binding. However, the hypothesis was made that in cartilage-specific genes dimeric SOX9, as opposed to monomeric SOX9, is necessary for activation. We identified a putative binding site adjacent to each of the known single SOX9 binding sites in the *Col9a1* D and E enhancers and in the *CRTL1* enhancer. Electrophoretic Mobility Shift Assays (EMSAs) were performed to determine whether SOX9 bound to these putative sites.

Transient transfections were then performed using wild-type and mutant enhancer-reporter plasmids to determine whether these putative SOX9 binding sites were important for activation *in vivo*. Although dimeric SOX9 bound to each of the enhancers *in vitro*, several different effects were seen *in vivo*. In the presence of the wild-type *Col9a1* D enhancer, no activation was seen. However, when the enhancer was extended to include an additional pair of newly found SOX9 binding sites, expression was increased 10-fold. When any of the four SOX9 binding sites within this enhancer were mutated, expression was completely eliminated, suggesting that interdependent dimers or a tetramer of SOX9 is necessary for the activation of transcription. The weaker *Col9a1* enhancer E was found to increase gene expression minimally through binding of either dimeric or monomeric SOX9. However, dimeric SOX9 was required for the activation of gene expression by the *CTRL1* enhancer. Through this study we validate the importance of not just monomeric but of dimeric and possibly tetremeric SOX9 as an activator of cartilage-specific gene expression.

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INTRODUCTION

Gene Expression

Great advances have been made toward understanding the complex mechanisms that regulate the process of DNA transcription. However, the specific mechanisms of activation for many individual genes are unknown. The following research focuses on the mechanism of activation pertaining to a small subset of cartilage genes and suggests a new model for gene activation. Before addressing the current research, three main parts of general transcriptional activation are reviewed including DNA unwinding, formation of transcriptional initiation complex, and the role of transcription factors.

In the first step necessary to begin transcription, eukaryotic DNA is released from its inactive chromatin structure, where it is tightly coiled around protein, to a more open state thus allowing regulatory DNA binding proteins access to the DNA. Genes that are about to be transcribed must undergo these loosening structural changes, which are usually brought about by chromatin remodeling, histone modification, or a combination of these methods (9). Chromatin remodeling factors complex with DNA, hydrolyze ATP, and use the released energy to disrupt the packaging structure. Histone modification reduces the positive charge on histones lessening the binding force with DNA.

Once the eukaryotic DNA is loosened from the chromatin structure, several proteins essential for transcription, including RNA polymerase II (Pol II), are uninhibited from binding to the core promoter. The core promoter is a DNA region where the complex of general transcription factors, co-activators and Pol II are positioned in order to initiate transcription at the start site (4). In most eukaryotes this begins with the protein TFIID binding to the TATA box sequence within the core promoter which results in

recruitment of remaining general transcription factors and the initiation of transcription (4). Participating in the recruitment of these key proteins, which initiate transcription, are gene or tissue specific transcription factors that also play a part in initiation of transcription.

Transcription factors, which act as either activators or repressors, bind to DNA and interact with the promoter to further regulate levels of gene expression. Specifically, activators bind to enhancers and increase gene expression. Enhancer elements can be in close proximity or far away from the promoter, located upstream, downstream, or within a transcriptional unit. When the DNA is in the loosened state needed for transcription, these enhancer regions become available for activators to bind. After recognizing specific DNA structures or sequences, these activators or transcription factors bind to enhancers and induce conformational changes. The conformational changes facilitate interactions between transcription factors and other co-activators or the Pol II complex thus resulting in regulated transcription. Our current study focuses on the interaction of the transcription factor SOX9 with enhancers and on the gene regulation that results.

SOX Transcription Factors

The transcription factor SOX9 belongs to the HMG box superfamily. The proteins within this superfamily contain a high mobility group (HMG) domain which enables them to bind DNA. A subgroup of the HMG superfamily is the SRY-type HMG box (or SOX) protein family named after the testis-determining factor, SRY. Each SOX protein contains an HMG domain composed of three α-helices forming a twisted L-shape maintained by a hydrophobic core. The SOX protein HMG domain binds the DNA upon specific sequence recognition. The consensus sequence for SOX binding has been

specified as the heptameric sequence (A/T)(A/T)CAA(A/T)G (14). Upon recognition of this sequence, SOX proteins bind in the minor groove and the DNA undergoes a conformational change resulting in a 70-85° bend and unwinding (14).

Along with including an HMG domain, the proteins belonging to group E of the SOX gene family (10) (SOX8, SOX9 and SOX10) contain at least one transactivation domain as well as a dimerization domain (14).When the PQS transactivation domain of SOX8, SOX9, and SOX10 is removed the proteins are no longer able to activate transcription (6). SOX9 contains an additional transactivation domain, PQA, which is not present in SOX8 or SOX10. The dimerization domains found in these three proteins allow for cooperative binding between proteins, making possible the formation of DNA-dependent heterodimers and homodimers (1). Dimerization made possible by the presence of this domain has been shown to be key in the regulatory mechanisms of SOX9 in particular.

Dimerized SOX9 activates several genes that are critical for chondrogenesis. The enhancers of these cartilage-specific genes often contain multiple SOX9 binding sites, and these multiple sites are often paired in opposite orientation with a 3-4 base pair separation. The paired sites allow for the DNA-dependent dimerization of SOX9. In these enhancers, it is often seen that upon mutation of one SOX9 binding site in a pair, the other site is rendered inactive as well. The cartilage-specific genes that are activated by SOX9 include *Col2a1, Col11a2, Col9a2, Col27A,* and *CD-RAP,* all of which contain multiple SOX9 binding sites.

Background Studies

Collagens expressed in cartilage that have been shown to be regulated by SOX9 include types II, IX, XI, and XXVII. *Col2a1* was the first collagen gene to be identified as being activated by SOX9. SOX9 binds to three sites in the enhancer region to activate transcription. If any of these sites are mutated, SOX9 is no longer capable of activating transcription (17). *Col11a2* was found to have three paired SOX9 binding regions, one of which is intronic. Dimerization of SOX9 at each of these sites is essential for full transcriptional activation to occur (2, 3). In *Col9a2*, paired SOX9 binding sites, which bind dimerized SOX9, were found to be important in activating gene expression (1). *COL27A1* was also found to have two paired SOX9 binding sites that, when bound by SOX9, activate transcription (7). Taken together, this work has shown that dimerized SOX9 plays an important role in collagen gene activation.

Other work has shown SOX9 to function in the regulation of non-collagen cartilage genes expressing proteins such as cartilage-derived retinoic acid-sensitive protein (CD-RAP) and aggrecan. SOX9 bound to paired binding sites found in a enhancer region for *CD-RAP* are necessary for full gene expression (5, 13, 15). SOX9 has also been found to transactivate aggrecan expression in some cell lines, however, binding sites have not been identified (11). Thus, SOX9 not only acts as a transcription factor important for collagen gene expression but also for the expression of other essential cartilage proteins as well.

In addition to activating cartilage-specific genes, SOX9 plays a critical role in sex determination. Both the anti-Mullerian hormone (AMH) gene, which leads to regression of the female reproductive tract and Steroidogenic Factor 1 (SF1) gene, which is critical

for gonad development, are shown to be regulated by SOX9 (1). In humans, the mutation of SOX9 results in Campomelic dysplasia (CD), a disease that is not only characterized by skeletal abnormalities but also by XY sex reversal in about two-thirds of patients (13). This illustrates the importance of SOX9 regulation in both cartilage gene expression and sex determination.

It was proposed by Bernard *et al.* in 2003 that dimerization of SOX9 is required for activation of genes involved in chondrogenesis but not for sex determination (1). The missense mutation A76E in the dimerization domain of SOX9 disrupts the capability of dimerization and thus the role of dimerized SOX9 was able to be tested. In the experiments conducted, mutant A76E SOX9 protein was not effective in activating either the *Col11a2* or *Col9a1* enhancers but the activation of SF1 enhancer was not affected, supporting this hypothesis (1).

Three recently identified cartilage-specific gene enhancers have been shown by others to be activated by SOX9 (8, 16). However, the role of dimerized SOX9 on these enhancers was not considered. *Col9a1* was shown to have two enhancers, D and E, each containing a single SOX9 binding site essential for activation (16). The gene for cartilage link protein (*CRTL1*), a key component of cartilage extracellular matrix, was also shown to have an enhancer containing a single SOX9 binding site critical for activation (8). We hypothesized that these enhancers may also be subject to regulation by dimerized SOX9 and upon sequence examination we found potential SOX9 binding sites adjacent to each of the sites previously identified. These potential binding sites are each arranged in opposite orientation to the primary site, and the two sites in each pair are separated by 3-4 base pairs as are all functional SOX9 binding site pairs previously identified.

The goal of my work was to test all three of the enhancers to see if the adjacent sites were in fact functional SOX9 binding sites and whether they played a role in the transcriptional activity of each enhancer. We found that SOX9 dimerization was required for full transcriptional activity of the *CRTL1* enhancer but not the *Col9a1* E enhancer. Most surprisingly, we found that the *Col9a1* D enhancer actually contains four SOX9 binding sites arranged in two pairs. The individual mutation of any one of the four sites completely inactivated the enhancer, suggesting that SOX9 interacts with the enhancer not as two separate dimers but rather as two interdependent dimers or even as a tetramer. This is the first time SOX9 has been found to activate transcription in this manner.

Enhancer Synthesis and Purification

Complementary primers were designed for each enhancer region containing either wild-type paired SOX9 binding sites or substitution mutations in either the upstream or downstream site of each pair. These primers were designed for *CRTL1* enhancer, *Col9a1* enhancer D, and *Col9a1* enhancer E, and mutations were designed to prevent SOX9 binding by creating a total of four mismatches with the consensus SOX9 binding sequence at each site (Table 1). Each enhancer had a 5' GATC overhang on each end.

Complementary single-strand DNA oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and annealed. Briefly, oligonucleotides (5-25 nmols in 25 µl TE), formamide (25 µl), and bromophenol blue (1 µl of 1% solution) were heated at 90° C for three minutes before being separated on an 18% polyacrylamide urea gel (30 ml 29% acrylamide 1% bis-acrylamide, 31.5 urea, 7.5 10X TBE, 13.5 ml H_2O, 0.5 ml 10% ammonium persulfate, 35 µl TEMED) for 2-3 hours at 400 volts. The gel was stained in 5% ethidium bromide for 5 minutes with agitation. The oligonucleotides were excised from the gel, and the excised slices were crushed and incubated in 5 ml of TE overnight at 37° C with agitation. The solution was syringe filtered and concentrated by sec-butanol extraction. The DNA was precipitated with 3 M NaCl (33 µl), 1 M $MgCl_2$ (5 µl), and 1.3 ml ethanol at -80° C for 15 minutes and centrifuged for 15 minutes. The DNA pellet was washed in 80% ethanol, dried, and resuspended in 30 µl TE. TNE (500 µl 1 M Tris pH 8, 100 µl 0.5 M EDTA, 1.67 ml 3M NaCl, up to 50 ml H_2O) was added to obtain 1 µg/µl single-stranded oligonucleotide concentration. Equal volumes of complementary strands were combined, boiled for 10 minutes, and cooled to room

temperature to allow for annealing. Concentrations were determined by spectrophotometry.

In Vitro Transcription/Translation

SOX9 was generated by *in vitro* transcription/translation of a SOX9 expression plasmid using the TNT Coupled Wheat Germ Extract System (Promega). Briefly, Wheat Germ Extract (25 µl), reaction buffer (2 µl), T7 RNA polymerase (1 µl), amino acid mixture (1 nmol), ribonuclease inhibitor (40 U), and SOX9 DNA template (1 µg) were combined in a single tube and incubated at 30° C for 60-90 minutes. In negative control, DNA template was omitted from the reaction. Control reactions were prepared as described with the substitution of ^{35}S-methionine for methionine with either DNA template for SOX9, control template, or no DNA template. Products from reactions containing ^{35}S-methionine were separated by SDS-PAGE (10%) and visualized using autoradiography in order to verify correct protein product size (Figure 1).

Electrophoretic Mobility Shift Assay (EMSA)

To determine whether SOX9 binds to the identified potential binding sites, EMSAs were performed using each wild-type and mutant enhancer for *Col9a1* D1/D2, *Col9a1* M1/M2/D1/D2, and *Col9a1* E1/E2, and *CRTL1* C1/C2 as DNA probes. To prepare DNA probes for EMSAs, 5' GATC overhangs of each double-stranded oligonucleotide were ^{32}P radiolabeled by end filling. DNA Polymerase I Klenow fragment (5 U), α^{32}P-dGTP (500 uCi), dNTPs not including dGTP (250 nmol each), and double-stranded enhancer probe (2 pmol) were incubated at 37° C for 30-45 minutes. Probes were purified from unincorporated α^{32}P-dGTP by Sephadex G-50 filtration using nick-columns (Amersham Biosciences). Probe radioactivity was determined by

scintillation counting and different probes were diluted to the same specific activity with non-radiolabeled probe.

DNA-protein binding reactions were performed with radiolabeled probes (2 µl) and SOX9 (1 µl) generated by *in vitro* transcription/translation. Reactions were carried out at room temperature for 45 minutes in a DNA binding buffer containing 20 mM Hepes (pH 7.9), 10% glycerol, 50 mM KCl, 0.05% Nonidet NP-40, 0.5 mM EDTA, 0.5 mM DTT, and 1 mM PMSF. dGdC (0.5-2 µg) was added as a non-specific competitor. In negative controls, blank samples made in transcription/translation reaction were substituted for SOX9. To supershift SOX9 in one lane of each duplicate, anti-SOX9 antibody (480 ng) was pre-incubated with SOX9 for 20-30 minutes before the addition of probes. Samples were separated using a 4% polyacrylamide gel, dried to filter paper, and visualized by autoradiography.

Plasmid Construction

4x Enhancer-Reporter Plasmids

Purified enhancers were used to construct plasmids containing four tandem copies of an enhancer upstream of a minimal promoter and a luciferase reporter gene. Enhancers (5 µg) were phosphorylated using T4 kinase (10 U), kinase 10x buffer (4 µl), ATP (4 µl 10 mM) in a 40 µl reaction incubated at 37° C for 30 minutes. Phenol chloroform extraction and ethanol precipitation were then performed to purify the enhancers. The double-stranded enhancers with sticky ends resembling ends digested with *Bam*HI and *Bgl*II were ligated upstream of the *Col2a1* minimal promoter in previously digested and dephosphorylated Bluescript vector containing an ampicillin resistance gene. Competent cells were transformed with the ligated plasmid and plated on ampicillin agar plates. The

next day, transformed colonies were grown in LB broth (5 ml) containing ampicillin at 37° C with shaking for 12-16 hours. Plasmid DNA was extracted from overnight cultures using the Miniprep plasmid prep kit (Qiagen). To multermerized the enhancer to four copies in tandem, Bluescript plasmid containing one copy of the enhancer and the minimal promoter was double digest with *Bam*HI/*Hind*III and *Bgl*II/*Hind*III resulting in a DNA segment containing the enhancer and the promoter and another segment containing one copy of the enhancer and the vector without the promoter. The digestion products were separated on a 1% agarose gel and bands of the correct size were excised from the gel. Qiaex II DNA extraction (Qiagen) products were used to extract the DNA from the gel. By ligating the two segments together using T4 ligase, a plasmid containing two copies of the enhancer and the minimal promoter was formed. After transforming cells with ligated DNA, plating cell, picking colonies and growing overnight cultures, and performing plasmid prep on the overnight culture, another double digest was performed on this plasmid. This produced a DNA segment that contained two copies of the enhancer region and the minimal promoter and another segment that contained two copies of the enhancer and the vector without the promoter. After separating the digested plasmid on a gel, cutting out the appropriate bands, and extracting the DNA from the gel, these two DNA segments were ligated together resulting in a plasmid that contained four copies of the enhancer and the minimal promoter in the Bluescript vector. This new plasmid was again used to transform cells, which were plated, colonies picked, cultures grown, and plasmid preps performed on the cultures. This plasmid was then digested to remove the four copies of the enhancer and the minimal promoter. After being separated on a gel, excising the appropriate bands, and extracting the DNA from the gel, this segment was

ligated into the luciferase reporter plasmid p95Luc immediately upstream of the reporter plasmid. The plasmids were sequenced by the BYU DNA Sequencing Center to verify correct construction.

Col9a1 M1/M2/D1/D2 Enhancer-Reporter Plasmid

To create the *Col9a1* M1/M2/D1/D2 enhancer plasmid, PCR was used to amplify the 96-basepair D enhancer region described by Bernard *et al*. (1), which was then ligated into Bluescript vector containing the minimal promoter. The single copy of the enhancer and promoter were then removed and ligated into the luciferase reporter plasmid. Briefly, PCR was performed using primers complimentary to the ends of the enhancer region of interest (Forward TTAAGGATCCAGTGGGCACATTTTTAC, Reverse GGCGAGATCTATC TGCTATAGGAGTAC). DNA template was extracted from human white blood cells using the DNeasy Tissue Kit (Qiagen). PCR reaction contained Platinum Taq DNA polymerase (1 U) (Invitrogen), 10x PCR buffer without $MgCl_2$ (5 µl), dNTP mix (10 nmols), $MgCl_2$ (75 nmols), forward and reverse primers (5 pmols each), DNA template (0.25-1.0 µg), and water (up to 50 µl). The reaction started at 94° C for 2 minutes followed by 35 cycles of PCR (94° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds). PCR product was purified using the QIAquick PCR Purification kit (Qiagen). The purified PCR product was then digested with *Bam*HI/*Bgl*II (15 U each) (Fisher) with 10x Buffer B at 37° C for 3.5 hours. Enzymes and salts were removed using QIAquick PCR Purification kit (Qiagen). The PCR product was ligated into linear Bluescript vector (20-50 ng) upstream of the *Col2a1* minimal promoter with T4 Ligase (12 U) (Fisher) and 10x Ligase Buffer (4 µl) at 16° C overnight. Competent cells were transformed with the ligated plasmid, plated, colonies picked, and cultures grown.

Plasmid DNA was extracted from overnight cultures using Miniprep plasmid prep kit (Qiagen).

The M1/M2/D1/D2 enhancer and the promoter were removed from Bluescript using *Hind*III and *Spe*I. Due to the presence of a *Hind*III cut site within the enhancer, the plasmid was first digested with SpeI (70 U) for 4 hours followed by an incomplete digestion with *Hind*III (5 U) for 10 minutes. The digest products were separated on a 2% agarose gel and the segment containing the complete enhancer and promoter was extracted from the gel and ligated into the luciferase reported plasmid, p95Luc. The plasmid was sequenced by the BYU DNA Sequencing Center to verify correct construction.

Mutagenesis

To prevent SOX9 binding, mutations were made to each of the four putative SOX9 binding sites located within the *Col9a1* M1/M2/D1/D2 enhancer – M1*/M2/D1/D2, M1/M2*/D1/D2, M1/M2/D1*/D2, M1/M2/D1/D2*. Mutations to *Col9a1* M1/M2/D1/D2 in p95Luc were made using QuikChange II Site-Directed Mutagenesis (Stratagene). Briefly, complementary primers which contained the mutations were designed and purified as described in *Enhancer Synthesis and Purification*. Primers were M1*/M2/D1/D2 forward AACCTCAGCCCTC TGGCAGCTTCCACTGTATT, M1*/M2/D1/D2 reverse AATACAGAGGAAGCTGCC AGAGGGCTGAGGTT, M1/M2*/D1/D2 forward TCTGAAAGCTTCCACCTGATTC CTATAGCAGTT, M1/M2*/D1/D2 reverse AACTGCTATAGGAATCAGGTGGAA GCTTTCAGA, M1/M2/D1*/D2 forward TGTATTCCTATAGCAGTTCTGGCAGCTG CCATTGTACTCCTATA, M1/M2/D1*/D2 reverse TATAGGAGTACAATGGCAGCT

GCCAGAACTGCTATAGGAATACA, M1/M2/D1/D2*-A forward AGCAGTTCTGA AAGCTGCCGGCGTACTCCTATAGCAGATAGATC, M1/M2/D1/D2*-A reverse GA TCTATCTGCTATAGGAGTACGCCGGCAGCTTTCAGAACTGCT, M1/M2/D1/D2* forward AGCAGTTCTGAAAGCTGCCGGCGTGCTCCTATAGCAGATAGATC, M1/M2/D1/D2* reverse GATCTATCTGCTATAGGAGCACGCCGGCAGCTTTCAG AACTGCT. These primers (125 ng each) were extended in a reaction containing 10x reaction buffer (5 µl), M1/M2/D1/D2 plasmid template (20-50 ng), dNTP mix (1 µl), P*fuUltra* HF DNA polymerase (2.5 U), and water (up to 50 µl). The reaction was started at 95° C for 30 seconds and then 16-18 cycles were performed (95° C for 30 seconds, 55° C for l minute, 68° C for 7 minutes) followed by 2 minutes at 4°C.

*Dpn*I (10 U) was added to each amplification reaction to digest the parental methylated template and incubated at 37° C for 1 hour. After incubating XL1-Blue Supercompetent cells (50 µl) on ice for 30 minutes, the cells were transformed with the digested product (1-2 µl) by heat shocking for 45 seconds at 42°C. The samples were incubated on ice for 2 minutes before adding 0.5 ml NZY+ broth preheated to 42° C and incubating for 1 hour at 37° C with shaking. The transformed cells were plated on LB ampicillin plates and incubated at 37° C for 16 hours. Colonies were picked, overnight cultures grown, and plasmid prep performed as previously described. The plasmids were sequenced by the BYU DNA Sequencing Center to identify those containing the correct mutations.

Due to the number and spacing of mutations required in M1/M2/D1/D2*, the mutagenesis was performed in two steps. The M1/M2/D1/D2*-A primers introduced

three mutations to the wild-type plasmid and the M1/M2/D1/D2* primer added the fourth.

Tissue Culture

Rat chondrosarcoma (RCS) cells and 10T1/2 fibroblast cells were a gift from Dr. Benoit de Crombrugghe of the University of Texas M.D. Anderson Cancer Center. Cells were cultured at 37° C with 5% CO_2 in supplemented Dulbecco's modified Eagle medium (D-MEM) (Gibco). Supplemented media contained 50 U/ml Penicillin (Cellgro), 50 μg/ml Streptomycin (Cellgro), 2 mM L-glutamine (Cellgro) and 10% fetal bovine serum (HyClone).

Transient Transfection

RCS and 10T1/2 cells were transiently transfected in triplicate using enhancer reporter plasmids, pSV-β-galactosidase expression plasmid as an internal control for transfection efficiency, and empty p95Luc vector for negative control. Additionally, SOX9 pcDNA3.1 expression vector was used in 10T1/2 transfections with empty pcDNA3.1 vector as a negative control. Each transfection was repeated at least 3 times. Lipofectamine Reagent (Invitrogen) was used to transfect the cells following manufacturer's instructions. Briefly, each 38.5 cm^2 well was seeded with 2 x 10^5 cells and incubated 18-24 hours with supplemented Dulbecco's modified Eagle medium (see Tissue Culture). The medium was removed and total plasmid DNA (2 μg) and 0.5% Lipofectamine reagent in Opti-MEM (Gibco) (1 ml) were added to each well and incubated 5-24 hours. One ml of 20% FBS in D-MEM was then added to produce a final concentration of 10% FBS and cells were incubated 13-24 hours. This was followed by a

second incubation in fresh 10% FBS in D-MEM (with or without supplementary L-glutamine and antibiotics) for 18-24 hours.

Plus reagent (Invitrogen) was used to enhance transfection efficiency in RCS transfections. Plasmids (2 μg) were diluted in D-MEM (100 μl) and then incubated with Plus reagent (15-16 μl) for 15 minutes before adding 5% Lipofectamine reagent in D-MEM (100 μl) and incubating 15 minutes. The solution was added to cells in 800 μl fresh D-MEM and incubated for 3 hours. One ml of 20% FSB in D-MEM was added to produce a final concentration of 10% FBS and cells were incubated 21 hours followed by a second incubation in fresh complete media for 18-24 hours.

Cells were rinsed (3x) with cold PBS and placed on ice. Lysis solution (Tropix) with 0.5 mM DTT was added to each well, cells were harvested by scraping, and cell debris pelleted by 5 minute centrifugation at 14,000 rpm.

β-galactosidase production was measured using the Galacto-Light Plus system (Tropix) according to manufacture's instruction. Briefly, 100 μl Reaction Buffer (substrate plus diluent) was added to cell extract (2 μl). After 30 minutes of incubation, Accelerator (100 μl) was added to terminate the enzyme activity and trigger light emission measured by the luminometer (TD-20/20 Turner Designs).

Luciferase production in transfected cells was measured using the Luciferase Assay System (Promega). Luciferase assay reagent (100 μl) containing luciferin substrate was added to cell extract (20 μl) and light produced from the luciferase reaction was measured by the luminometer (TD-20/20 Turner Designs).

Statistical Analysis

Analysis of variance (ANOVA) model was fit using transfection data to determine the statistical significance between treatments. The basic model fit was $y=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$ where μ is the overall mean of enhancer activity, α_i is the effect of SOX9 for the *i*th level, where *i*th levels are the presence or absence of SOX9, and β_j is the effect of the enhancer for the *j*th level, where the *j*th levels are p95Luc, wild-type, and mutant enhancers. The interaction $(\alpha\beta)_{ij}$ is used to show whether the effects of the presence or absence of SOX9 differs between enhancers. Random error is accounted for with the term $\varepsilon_{ijk.}$ Effects were found to be significant using a confidence interval of 95% ($\alpha = 0.05$). We looked at the a) marginal mean of enhancer activity with each wild-type and mutant as compared to p95Luc, b) marginal mean of enhancer activity in the presence or absence of SOX9, and c) marginal mean of enhancer activity between two enhancers with and without SOX9. Bonferroni post hoc tests were used to adjust for multiple comparisons to obtain a family-wise error rate of 0.05.

Co-Immunoprecipitation

RCS cells were cultured in T75 culture flasks until 70-100% confluent. Cells were lysed by incubation for 30 minutes with 1% NP-40 buffer (1.3-1.6 ml per 100 cm^2 cell culture) containing 150 mM NaCl, 50 mM Tris (pH 8) and protease inhibitors including Leupeptin (1 µg/mg), Pepstatin (1 µg/ml), and PMSF (50 µg/ml). This and all subsequent steps were carried out on ice or at 4°C. Lysed cells were harvested by scraping, centrifuged at 10,000 rpm, and supernatant collected. Supernatant was incubated with 0.05-0.01 volumes of preimmune rabbit serum for one hour. To clear the lysate, A/G Plus agarose beads (20 µl of 25% suspension) (Santa Cruz Biotechnology) were incubated

with the lysate for 30-60 minutes with rotation, centrifuged at 2,500 rpm for 30 seconds and then supernatant collected. The lysate clearing step was repeated one to three times. A/G beads with crosslinked SOX9 antibody were added to cell lysate and incubated for 4 hours with rotation. The beads were pelleted by centrifuging at 2,500 rpm for one minute and supernatant discarded. Beads were washed (2-4x) with 1 ml NP-40 buffer or PBS. Pellet was resuspended in 40 µl Laemmli buffer with 5% β-mercaptoethanol, boiled for 3-5 minutes, and separated on a 10% polyacrylamide gel. The gel was rinsed for 30 minutes in water, stained overnight with SYPRO Ruby protein gel stain (Bio-Rad), and destained for one hour in water. Unique bands were excised from the gel and stored at -20ºC.

A challenge with heavy background resulted from preimmune antibody added to preclear nonspecific binding proteins from the cell lysate. Excess preimmune antibody that was not removed form the cell lysate bound to A/G beads and because it was not crosslinked to the beads it would be seen among the immunoprecipitated proteins separated on the gel. To reduce this background, several incubations with A/G beads in the cell lysate were performed to remove the preimmune antibody. The proteins bound with each incubation were separated on a polyacrylamide gel so the level of background could be determined.

Crosslinking

Special consideration needed to be taken due to the similar size of SOX9 protein (56 kDa) and IgG heavy chain. Anti-SOX9 antibody was crosslinked to A/G beads to eliminate heavy chain protein seen on the polyacrylamide gel after protein separation so that the presence of SOX9 could be seen.

Anti-SOX9 polyclonal antibody (Chemicon International) was crosslinked to A/G agarose beads using dimethylpimelimidate (DMP) (Pierce Chemical). Briefly, A/G agarose beads (500 µl of 25% suspension) were washed with PBST (PBS containing 0.1% Tween 20 detergent). Beads were resuspended in PBST and anti-SOX9 antibody (100 µg) was added and incubated at 4° C with rotation overnight to allow for binding of antibody to the A/G agarose beads. Beads with bound antibody were then washed (3x) and resuspended in 0.2 M sodium borate buffer (pH 9). DMP crosslinker (100 mg) was dissolved in 0.2 M sodium borate buffer and incubated with the beads at room temperature for one hour to allow for the crosslinking reaction to occur. The beads were washed (3x) with 0.2 M triethanolamine (pH 8) and incubated in 0.2 M triethanolamine for two hours at room temperature with rotation. This was followed by three washes with 0.1 M glycine buffer (pH 2.5) and then three washes with PBS buffer. The crosslinked beads were stored in PBS at 4°C.

Immunoblot

After protein samples were separated on a 10% SDS polyacrylamide gel, proteins were transferred to Westran Clear Signal membrane (Schleicher and Schuell Bioscience) at 4° C in buffer containing 0.025 M Tris, 0.19 M glycine, 0.025% SDS, and 20% methanol.

After transfer, the blot was rinsed in TBS-T (50 mM Tris pH 8, 15 mM NaCl, and 0.05% Tween-20) and blocked with 5% non-fat dry milk (Biorad) for one hour at room temperature with agitation. Following blocking, the blot was rinsed in TBS-T. Anti-SOX9 antibody (0.48 mg/ml) was diluted 1:5000 in blocking solution and incubated with blot for 2.5 hours at room temperature with agitation. The blot was then rinsed with TBS-

T once for 15 minutes and then three times for 5 minutes, all with agitation. The blot was then incubated in blocking buffer containing 1:5000 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody (0.8 mg/ml) for 1.5 hours with agitation. The blot was rinsed again with TBS-T once for 15 minutes and then three times for 5 minutes, all with agitation. ECL Western blotting detection reagents (Amersham Bioscience) were using to visualize secondary antibody by chemiluminescent reaction. After adding detection reagent to the blot, the blot was exposed to autoradiography film.

Tryptic Digestion

An in-gel tryptic digest was performed on gel slices to prepare proteins for peptide analysis as previously reported (12). Briefly, acetonitrile solution (50% acetonitrile and 50 mM ammonium bicarbonate) (1.5 ml) was added to the gel slice and incubated 30 minutes with shaking. The solution was then aspirated and reduction buffer (100 mM ammonium bicarbonate, 5 mM DTT) (150 µl) was added to the gel slice and incubated at 60ºC for 30 minutes. The buffer was aspirated and alkylation buffer (100 mM ammonium bicarbonate, 20 mM iodoacetamide) (150 µl) was added to the gel slice and incubated in the dark for 30 minutes. Following the incubation, the solution was aspirated and 100 mM ammonium bicarbonate (1 ml) was added to the gel slice and incubated for 30 minutes with shaking. The ammonium bicarbonate was aspirated and the gel slice was sectioned to increase surface area before being incubated in acetonitrile solution (500 µl) for 30 minutes with shaking. The solution was aspirated and the gel pieces dried by vacuum centrifugation. Gel pieces were then hydrated in trypsin solution (trypsin 20 ng/µl, 25mM ammonium bicarbonate) and excess solution was removed. Ammonium bicarbonate was added until all gel pieces were submerged. After overnight

incubation at 37ºC with motion, formic acid (88%) (1 µl) was added to the gel pieces which were then sonicated for 20 minutes.

Mass Spectrophotometry

Protein samples were sent to the Brigham Young University Proteomics and Biological Mass Spectrophotometry Facility or the Columbia University Protein Core Facility for identification using oMALDI and MS/MS mass spectrophotometry. Columbia's facility performed tryptic digestion in house.

Col9a1 D Enhancer

After the putative SOX9 binding site, D1, was identified adjacent to the known site, D2, EMSAs were performed to determine whether SOX9 binds to D1 *in vitro.* Probes used included wild-type, D1/D2, as well as mutants D1*/D2 and D1/D2* (Table 1A). The EMSAs showed that SOX9 binds as a dimer and a monomer to the wild-type Col9a1 D1/D2 enhancer (Figure 2). Inclusions of anti-SOX9 antibody supershifted both complexes confirming that they both contain SOX9. Mutations of the D1 site (D1*/D2) allowed for monomeric but prevented dimeric SOX9 binding. Mutation of the D2 site (D1/D2*) prevented both dimeric and monomeric binding, even though the D1 site was still intact.

To determine whether the binding of SOX9 to *Col9a1* D1/D2 and D1*/D2 *in vitro* correlated with transcriptional activity *in vivo*, transient transfections were done in rat chondrosarcoma cells (RCS) where SOX9 is readily expressed (Figure 3). To our surprise, the presence of *Col9a1* D1/D2 or D1*/D2 enhancers did not result in an increase in transcriptional activity compared with the no-enhancer control, p95Luc. Although they did not contain the full length enhancer previously identified by Bernard *et al*. (1), these enhancers contained the same SOX9 binding site, D2, which had been identified as the critical site for activation. We speculated that another critical sequence must exist in the upstream region contained in Bernard's enhancer but omitted from our D1/D2 enhancer. To test this hypothesis, the same enhancer region as described by Bernard *et al.* (Table 1) was used to construct a single-copy enhancer-reporter plasmid, which was tested in transient transfections in RCS cells (Figure 4). The presence of this full enhancer region

resulted in high activation as compared to the D1/D2 enhancer, indicating that the upstream region does indeed contain critical elements. In searching for possible transcription factor binding sites in this newly included upstream region, two new putative SOX9 binding sites were identified (Table 1B). Substitution mutations were made within these newly identified putative SOX9 binding regions, M1 and M2, so that the importance of these sites could be assessed. The D1 and D2 sites were also mutated in the context of this longer enhancer. Surprisingly, introducing a mutation to any one of the four putative SOX9 binding sites within the M1/M2/D1/D2 enhancer resulted in a complete elimination of activity (Figure 5), indicating that the two pairs do not work independent from one another. Rather, these results suggest that the four binding sites give rise to the formation of a SOX9 tetramer and that protein binding at all four sites is required for enhancer activity.

To verify that the activity of the M1/M2/D1/D2 enhancer was a result of the presence of SOX9, transient transfections were performed in 10T1/2 fibroblast cells. Because SOX9 is not expressed in this cell line, comparisons were able to be made between activity with the enhancer in the presence and absence of SOX9. Co-transfection with a SOX9 expression plasmid increased the activity of the M1/M2/D1/D2 enhancer 6 fold (Figure 6A). Mutation of any one of the four SOX9 binding sites markedly decreased enhancer responsiveness to SOX9, suggesting that SOX9 does, in fact, bind to each of the four sites and that this binding is required for transcriptional activity (Figure 6B).

EMSAs were performed to confirm the ability of SOX9 to bind to the M1/M2/D1/D2 enhancer and also to see whether SOX9 could bind when only one of the

four SOX9 binding sites was mutated (Figure 7). From the supershifts resulting upon the addition of anti-SOX9 antibody, we see that SOX9 is capable of binding to M1/M2/D1/D2. All mutants except M1/M2/D1/D2* are also able to bind SOX9 to some degree. Whether this is monomer, dimer, trimer, or tetramer SOX9, however, is not clear. These results do suggest, however, that although SOX9 binding may still be possible if one of the four sites is mutated, such binding does not promote transcriptional activation.

Col9a1 E Enhancer

EMSAs were performing using enhancers E1/E2, E1*/E2, and E1/E2* (Table 2A) to determine whether SOX9 binds to the recently identified E1 site (Figure 8). Here again we see both dimerized and monomeric SOX9 bound to the wild-type enhancer. When the E1 site is mutated, SOX9 still binds as a monomer to the E2 site, but when the E2 site is mutated, no SOX9 binding occurs.

To determine whether the SOX9 binding seen *in vitro* leads to activation *in vivo*, RCS cells were transiently transfected with E1/E2, E1*/E2, and E1/E2* enhancer reporter plasmids (Figure 9). Although E1/E2 did show some transcriptional activity, activation was increased by less than 2 fold compared to the no-enhancer control, p95Luc. In the E1*/E2 enhancer, this low level of transcriptional activation persisted, suggesting that monomeric SOX9 was able to induce activation as much as the dimer in this instance. The E1/E2* enhancer was much more transcriptionally active than expected, considering that the EMSA showed no SOX9 bound to that enhancer. It is likely that the introduction of the E2* mutation inadvertently created a new binding site for a different transcriptional activator, making it impossible to detect the effect of the loss of SOX9 binding.

To determine whether transcriptional activation by these enhancers in RCS cells was a result of SOX9 binding, transient transfections were performed in 10T1/2 fibroblast cells with and without the addition of SOX9 (Figure 10). Consistent with the RCS transfection and EMSA results, the E1*/E2 mutant enhancer did remain partially responsive to monomeric SOX9. The results observed with the E1/E2* enhancer, however, were not expected. This enhancer was more strongly activated by SOX9 than was the wild-type, even though no SOX9 bound this enhancer in EMSAs. It is possible that the over-expression of SOX9 in 10T1/2 cells secondarily increased the expression of another activating protein, which in turn bound and activated the mutant enhancer.

CRTL1 Enhancer

EMSAs were performing using enhancers C1/C2, C1*/C2, and C1/C2* (Table 2B) to determine whether SOX9 binds to the newly identified C2 site (Figure 11). Both dimeric and monomeric SOX9 bound to the wild-type C1/C2 enhancer. Mutation of the C2 site prevented dimeric but allowed monomeric SOX9 binding, but mutation of the C1 site eliminated all SOX9 binding.

To determine whether the SOX9 binding seen *in vitro* corresponds to transcriptional activation *in vivo*, RCS cells were transiently transfected with C1/C2, C1*/C2, and C1/C2* enhancer reporter plasmids (Figure 12). The wild-type C1/C2 enhancer was weak in RCS cells but did show statistically significant enhancer activity. Mutation of either the C1 site (C1*/C2) or the C2 site (C1/C2*) abolished enhancer activity, demonstrating that monomeric SOX9 binding cannot activate this enhancer. Instead, dimeric SOX9 binding is required for activation by C1/C2 enhancer.

Transient transfections of the C1/C2 enhancer-reporter plasmid were also performed in 10T1/2 cells (Figure 13A). These experiments showed that in the presence of SOX9, enhancer activity of C1/C2 is increased about 10 fold. Mutation of either the C1 or C2 site reduces SOX9 responsiveness drastically (Figure 13B). Thus we confirm that SOX9 is causing the increase in expression.

Co-Immunprecipitation

The presence of the dimerization domain in SOX9 allows for homodimer formation but SOX9 is also thought to form heterodimers with a variety of other proteins. Using co-immunoprecipitaion on RCS cells, we attempted to identify proteins that hetrodimerize with SOX9, because these could be possible co-activators of importance in transcription.

To verify the presence of SOX9 in the mix of extracted proteins immunoprecipitated for the cell lysate, western blots were performed (Figure 14). These blots showed SOX9 was affectively being immunoprecipitated from the cells. From the co-immunoprecipitations, unique bands were identified compared to negative controls. Unique bands were seen around 30 and 50 kDa and have been submitted for identification by mass spectrometry.

Dimeric SOX9

From the experiments performed, we have gained insight into how SOX9 binds and regulates the enhancers for the *Col9a1* and *CRTL1* genes. Evidence is presented here that within each pair of SOX9 binding sites, a primary and a secondary binding site exist. EMSAs for *Col9a1* D1/D2 and E1/E2, and *CRTL1* C1/C2 all showed dimeric SOX9 binding to wild-type enhancer, monomeric SOX9 binding upon mutation of one site, and no SOX9 binding upon mutation of the other site. When mutations were introduced to the secondary SOX9 binding site of the pair, SOX9 was still able to bind to the other site as a monomer. However, when the primary site in the pair was mutated, no SOX9 was able to bind. The presence of primary and secondary SOX9 binding sites may indicate that the formation of a SOX9 dimer occurs in a specific order for each pair. First, one monomer of SOX9 binds to the primary site and once bound, the second SOX9 protein is able to bind, allowing for dimer formation.

Binding of SOX9 to an enhancer *in vitro,* however, does not necessarily lead to gene activation *in vivo*. Although monomeric SOX9 was able to bind and activate the *Col9a1* E1*/E2 enhancer in RCS cells, monomeric SOX9 also bound to enhancers *Col9a1* D1*/D2 and *CRTL1* C1/C2* in EMSAs but no activation occurred in RCS cells. SOX9 was also able to bind the *Col9a1* M1/M2/D1/D2 mutants M1*/M2/D1/D2, M1/M2*/D1/D2, and M1/M2/D1*/D2 in EMSAs. Even though SOX9 bound to these mutant enhancers in EMSAs, they had no transcriptional activity in RCS cells. Furthermore, we showed that the short D1/D2 enhancer bound dimeric SOX9 in EMSA but had absolutely no enhancer activity in RCS cells.

Interestingly, bound dimeric SOX9 results in completely different activation for each enhancer considered in this study. For the *Col9a1* M1/M2/D1/D2 enhancer, the binding of dimeric SOX9 was not sufficient to induce activation. All four SOX9 binding sites were required for activation to occur. The *Col9a1* E1/E2 enhancer was only activated slightly by dimeric SOX9, and the same level of activation occured upon binding of monomeric SOX9. The *CRTL1* C1/C2 enhancer was activated significantly by dimeric SOX9 and not at all by monomeric SOX9.

Based upon the different activation levels with dimeric SOX9 bound to the enhancers, it is quite possible that other proteins besides SOX9 are influencing the regulation of one or more of these enhancers. Unique bands obtained from co-immunoprecipitation of SOX9 may contain proteins that alter the activity of SOX9. Upon identification of these proteins, follow-up experiments would determine each protein's role as a co-regulator with SOX9.

Tetrameric SOX9

Upon first look at the arrangement of SOX9 binding sites in the *Col9a1* M1/M2/D1/D2 enhancer, the hypothesis was made that SOX9 dimerization would occur at each binding site pair and that binding of dimerized SOX9 at each pair would result in some level of gene activation. We had anticipated when one of the SOX9 binding pairs was mutated, an intermediate level of expression would occur. The fact that upon mutation of any of the four sites no activation is seen indicates that the two pairs do not work independent from one another. This implies that the four binding sites give rise to the formation of a SOX9 tetramer or two interacting dimers. Thus, here we see a novel

model for SOX9 binding where gene activation is dependent on the formation of a SOX9 tetramer instead of simply dimers.

The spacing between SOX9 binding sites within pairs has been shown to be critical for function. We suggest that the spacing between the two pairs of SOX9 binding sites within the *Col9a1* M1/M2/D1/D2 enhancer may also be structurally critical to allow for the formation and binding of a SOX9 tetramer. To determine the importance of this spacing, follow-up experiments will test the activation of wild-type binding pairs with 4 base pairs added between the two pairs of sites.

Although the structure allowing for tetrametric SOX9 seems probable, the possibility of other proteins making up part of the activating complex also exists. Without defined bands each being shifted upon addition of anti-SOX9 antibody in EMSAs, it remains uncertain exactly which proteins are included in the complex.

Despite the uncertainty about which proteins are a part of the activator, the tetrameric complex apparently causes extraordinary strength of activation compared to monomeric and dimeric SOX9 activation. Only 1x M1/M2/D1/D2 reporter plasmids were used in transient transfections as opposed to 4x enhancer for the other reporter-plasmids, which results in an unequal comparison of the strength of the enhancers. In order to make a direct comparison between the strength of the activation for the *Col9a1* enhancers, M1/M2/D1/D2 and E1/E2, transient transfections need to be performed using a reporter plasmid containing 4x M1/M2/D1/D2. Because a simple 1x copy M1/M2/D1/D2 reporter-plasmid activated the expression of the reporter gene ten-fold, whereas the 4x copy of E1/E2 activated the reporter gene only two fold, it is likely that M1/M2/D1/D2 is the predominant enhancer of *Col9a1*.

In conclusion, the experiments performed in this study further elucidate the role of SOX9 in the regulation of the *Col9a1* and *CRTL1* genes. Activation of the *Col9a1* gene is largely due to tetrameric SOX9 binding at the M1/M2/D1/D2 enhancer and minimally activated by dimeric or monomeric SOX9 binding at the E1/E2 enhancer. Despite the weakness of the enhancer, dimeric SOX9 is necessary for the activation of the *CRTL1* C1/C2 enhancer. Through this study we validate the importance of not just monomeric but of dimeric and perhaps tetrameric SOX9 as an activator of cartilage gene expression.

TABLES

A

		Nucleotide Sequence
Col9a1	D1/D2	D1 D2 TATAGCAGTTCTGAAAGCTGCCATTGTACTCCTATAGCAGATCTAAAAGC
Col9a1	D1*/D2	D1* D2 TATAGCAGTTCTG**GC**AGCTGCCATTGTACTCCTATAGCAGATCTAAAAGC
Col9a1	D1/D2*	D1 D2* TATAGCAGTTCTGAAAGCTGCC**GGC**GT**G**CTCCTATAGCAGATCTAAAAGC

B

		Nucleotide Sequence
Col9a1	M1/M2/D1/D2	M1 M2 AGTGGGCACATTTTTACTGGAACCTCAGCCCTCTGAAAGCTTCCACTGTA D1 D2 TTCCTATAGCAGTTCTGAAAGCTGCCATTGTACTCCTATAGCAGAT
Col9a1	M1*/M2/D1/D2	M1* M2 AGTGGGCACATTTTTACTGGAACCTCAGCCCTCTG**GC**AGCTTCCACTGTA D1 D2 TTCCTATAGCAGTTCTGAAAGCTGCCATTGTACTCCTATAGCAGAT
Col9a1	M1/M2*/D1/D2	M1 M2* AGTGGGCACATTTTTACTGGAACCTCAGCCCTCTGAAAGCTTCCAC**CTG**A D1 D2 TTCCTATAGCAGTTCTGAAAGCTGCCATTGTACTCCTATAGCAGAT
Col9a1	M1/M2/D1*/D2	M1 M2 AGTGGGCACATTTTTACTGGAACCTCAGCCCTCTGAAAGCTTCCACTGTA D1* D2 TTCCTATAGCAGTTCTG**GC**AGCTGCCATTGTACTCCTATAGCAGAT
Col9a1	M1/M2/D1/D2*	M1 M2 AGTGGGCACATTTTTACTGGAACCTCAGCCCTCTGAAAGCTTCCACTGTA D1 D2* TTCCTATAGCAGTTCTGAAAGCTGCC**GGC**GT**G**CTCCTATAGCAGAT

Table 1 *Col9a1* D wild-type and mutant enhancers contain putative SOX9 binding sites. Previously identified SOX9 binding site (underlined in black) as well as putative SOX9 binding sites are shown in red. Bases that are SOX9 binding consensus sequence mismatches are underlined in red. SOX9 binding sites containing substitution mutations (shown in bold) have names with asterisks. A) The 50-bp region containing the previously identified SOX9 binding site, D2, and the putative SOX9 binding site, D1. B) The 96-bp region including D1 and D2 as well as upstream putative SOX9 binding sites M1 and M2. The sequences bracketed by green dashed lines were used in EMSAs.

A

		Nucleotide Sequence
CRTL1	C1/C2	C1 C2 TCCAGCGCCACAAAGAGACATTCTGCACACACACT
CRTL1	C1*/C2	C1* C2 TCCAGCGCCGCCGAGAGACATTCTGCACACACACT
CRTL1	C1/C2*	C1 C2* TCCAGCGCCACAAAGAGACGTGCTGCACACACACT

B

		Nucleotide Sequence
Col9a1	E1/E2	E1 E2 AAATCCCTCCTTCAGTATAACCTTGTTAGCTTTGAGGACACACCCCTAGG
Col9a1	E1*/E2	E1* E2 AAATCCCTCCGTCCGTATAACCTTGTTAGCTTTGAGGACACACCCCTAGG
Col9a1	E1/E2*	E1 E2* AAATCCCTCCTTCAGTATAACCGCGTCAGCTTTGAGGACACACCCCTAGG

Table 2 *Col9a1* E and CRTL1 wild-type and mutant enhancers contain putative SOX9 binding sites. Previously identified SOX9 binding site (underlined in black) as well as putative SOX9 binding sites are shown in red. Bases that are SOX9 binding consensus sequence mismatches are underlined in red. SOX9 binding sites containing substitution mutations (shown in bold) have names with asterisks. A) Col9a1 E enhancer containing the previously identified SOX9 binding site, E2, and the putative SOX9 binding site, E1. B) *CRTL1* enhancer containing the previously identified SOX9 binding site, C2, and the putative SOX9 binding site, C1.

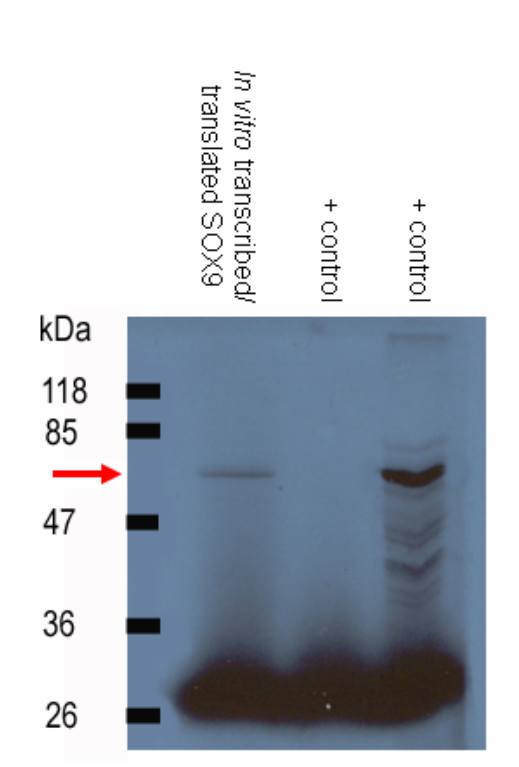

Figure 1 SOX9 made using *in vitro* transcription/translation is seen at 56 kDa (red arrow). In the positive control lane, bands contain proteins from *in vitro* transcription/translation control expression vector. In the negative control, no bands are seen.

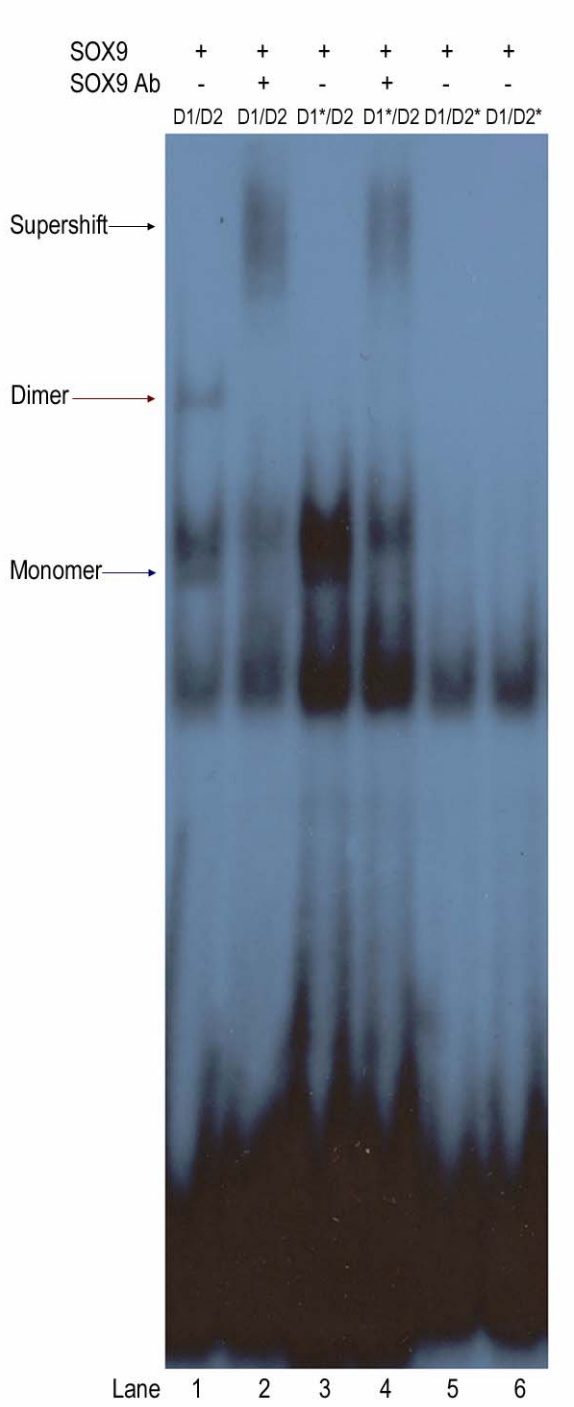

Figure 2 Dimeric and monomeric SOX9 bind to *Col9a1* D1/D2 enhancer and monomeric SOX9 binds to mutant D1*/D2. Electrophorectic Mobility Shift Assays were performed using *in vitro* made SOX9 and wild-type or mutant enhancers. Dimeric SOX9 is seen in lane 1, monomeric SOX9 is seen in lanes 1 and 3, and anti-SOX9 antibody induced supershifts are seen in lanes 2 and 4.

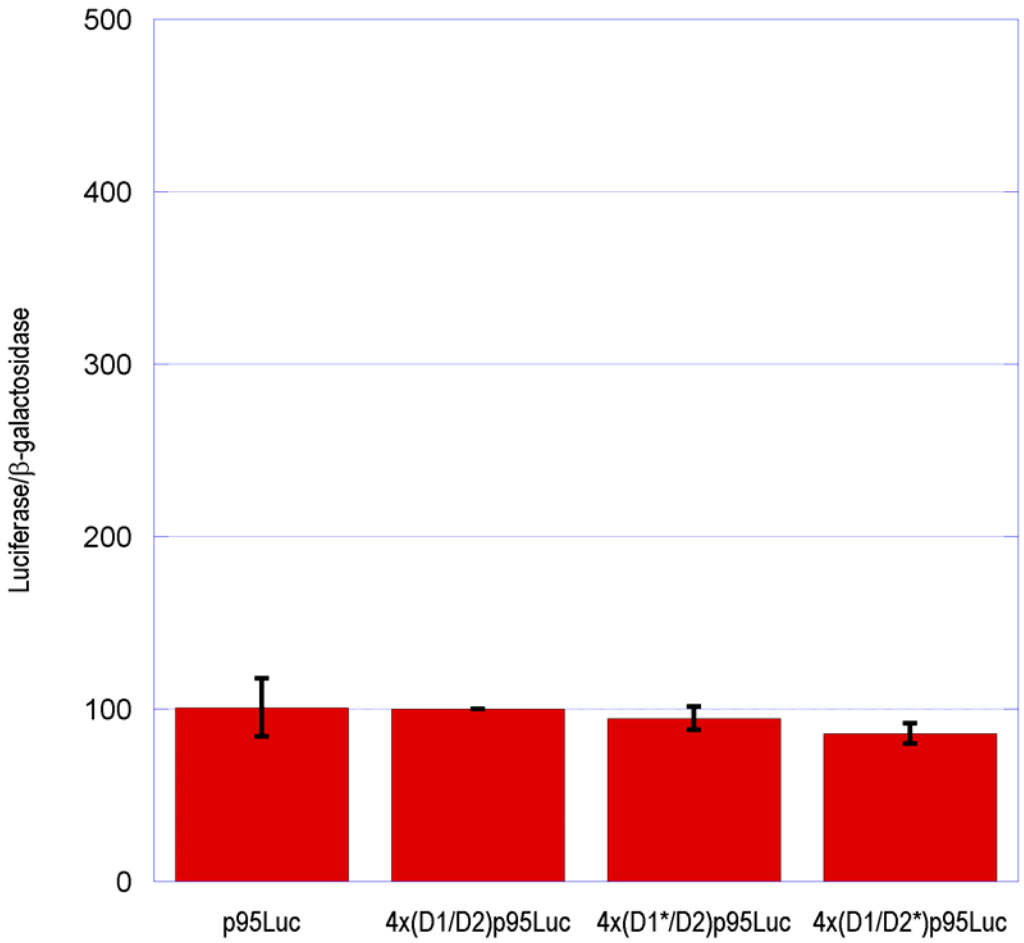

Figure 3 The *Col9a1* D1/D2 enhancers do not activate gene expression. Transient transfections were performed in RCS cells using the wild-type (D1/D2) or mutant (D1*/D2, D1/D2*) enhancers-reporter plasmids. The no-enhancer reporter plasmid, p95Luc, functioned as the control. None of the enhancers induced statistically significant levels of activation compared to the control.

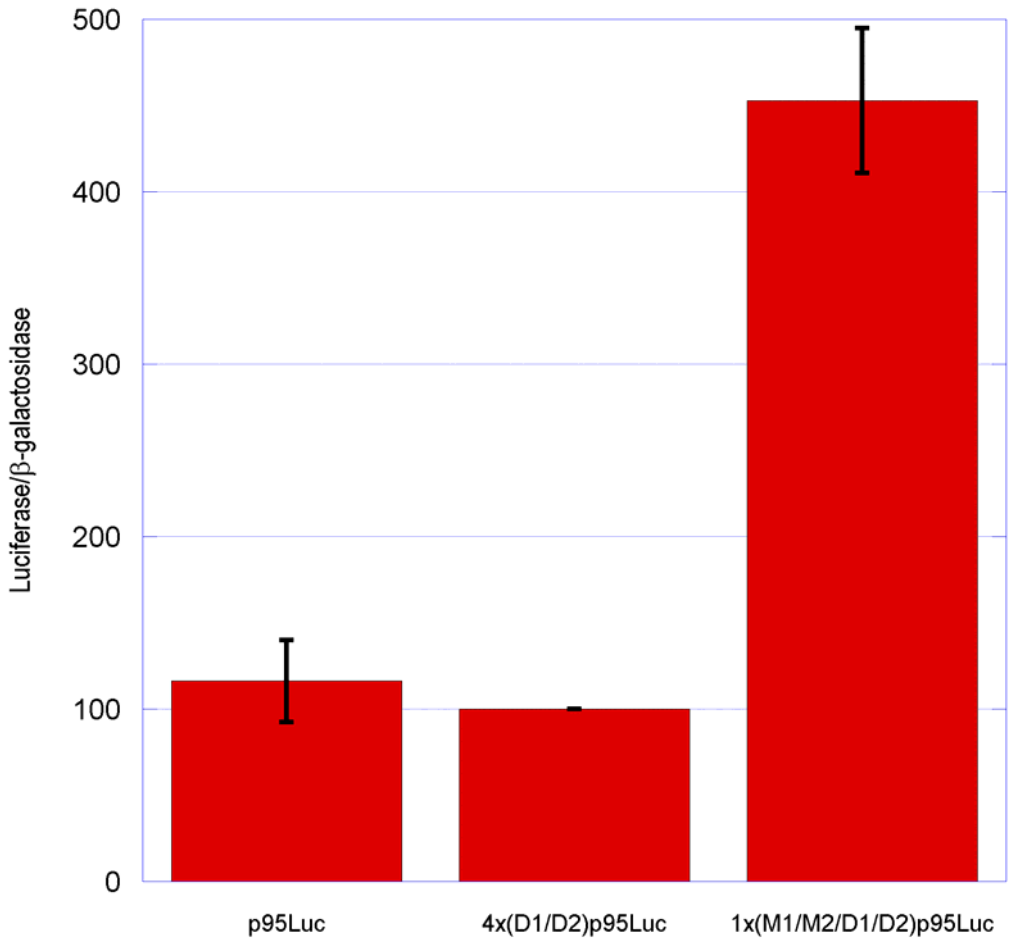

Figure 4 The *Col9a1* M1/M2/D1/D2 enhancer activates gene expression whereas D1/D2 does not. Transient transfections were performed in RCS cells using reporter plasmids containing the short D1/D2 enhancer or longer M1/M2/D1/D2 enhancer. The no-enhancer reporter plasmid, p95Luc, functioned as the control. Only M1/M2/D1/D2 induced statistically significant levels of activation compared to the control.

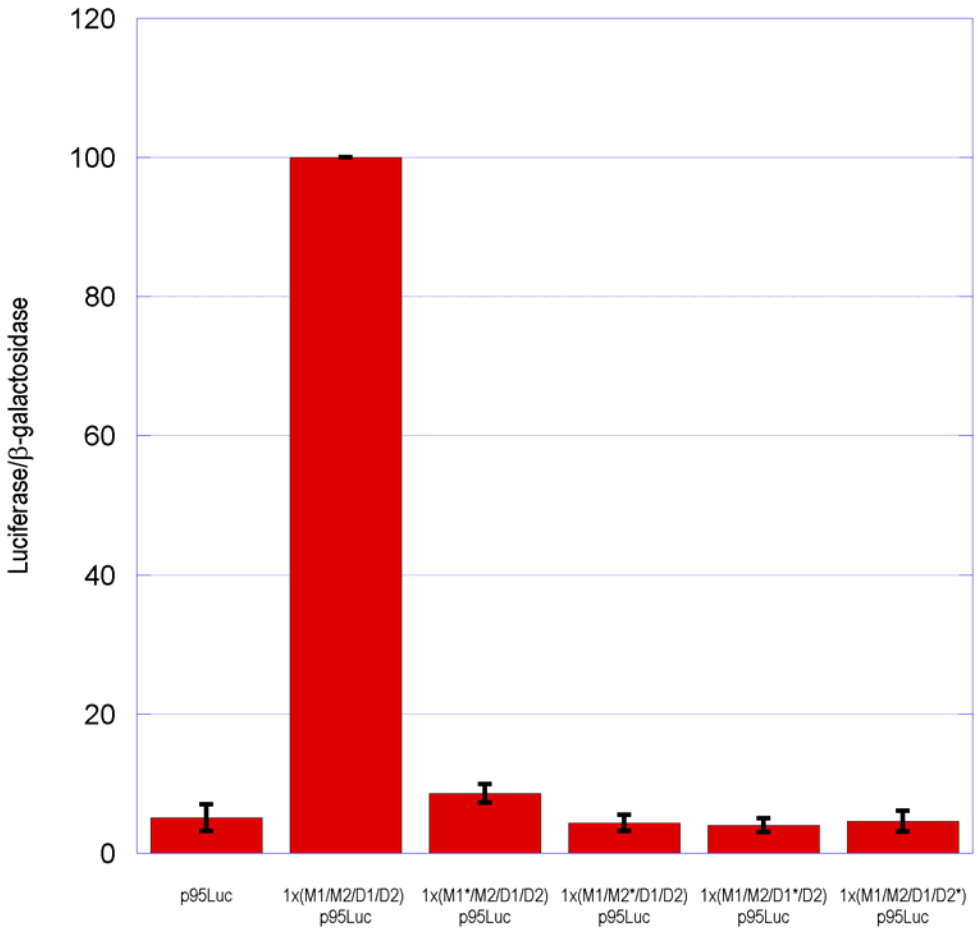

Figure 5 The *Col9a1* M1/M2/D1/D2 enhancer is unable to activate gene expression when any of the four binding sites are mutated. Transient transfections were performed in RCS cells using reporter plasmids containing the wild-type enhancer (M1/M2/D1/D2) or mutant enhancers (M1*/M2/D1/D2, M1/M2*/D1/D2, M1/M2/D1*/D2, M1/M2/D1/D2*. The no-enhancer reporter plasmid, p95Luc, functioned as the control. None of the mutants resulted in statistically significant levels of activation compared to the control.

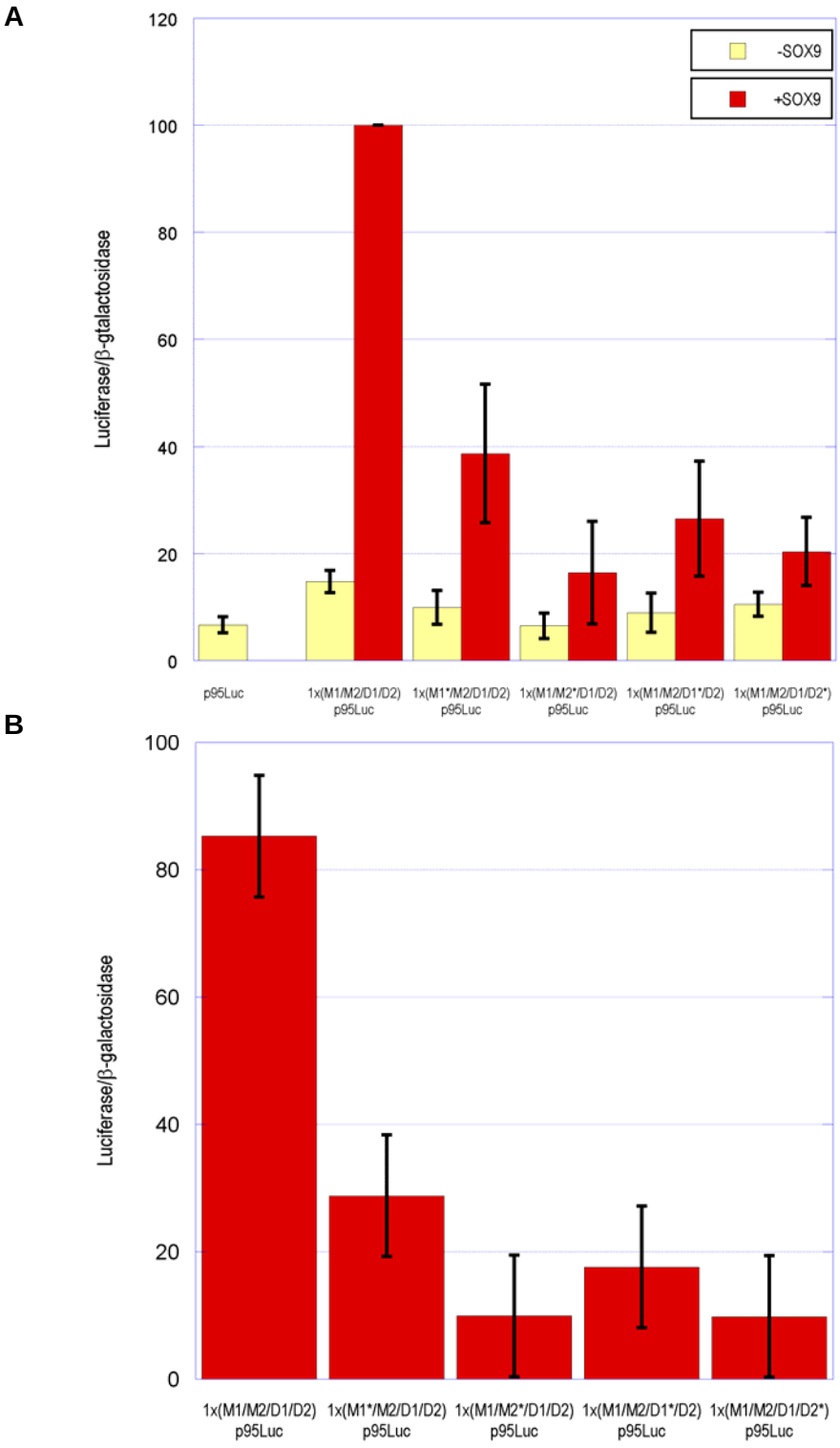

Figure 6 The *Col9a1* M1/M2/D1/D2 enhancer activates gene expression in fibroblast cells when SOX9 is present. Transient transfections were performed in 10T1/2 fibroblast cells. A) Cells were co-transfected with SOX9 expression vector (red), or empty expression vector (yellow). B) Activation differences for each enhancer with and without SOX9 are plotted. The activation with M1/M2*/D1/D2, M1/M2/D1*/D2, and M1/M2/D1/D2* is not statistically significant.

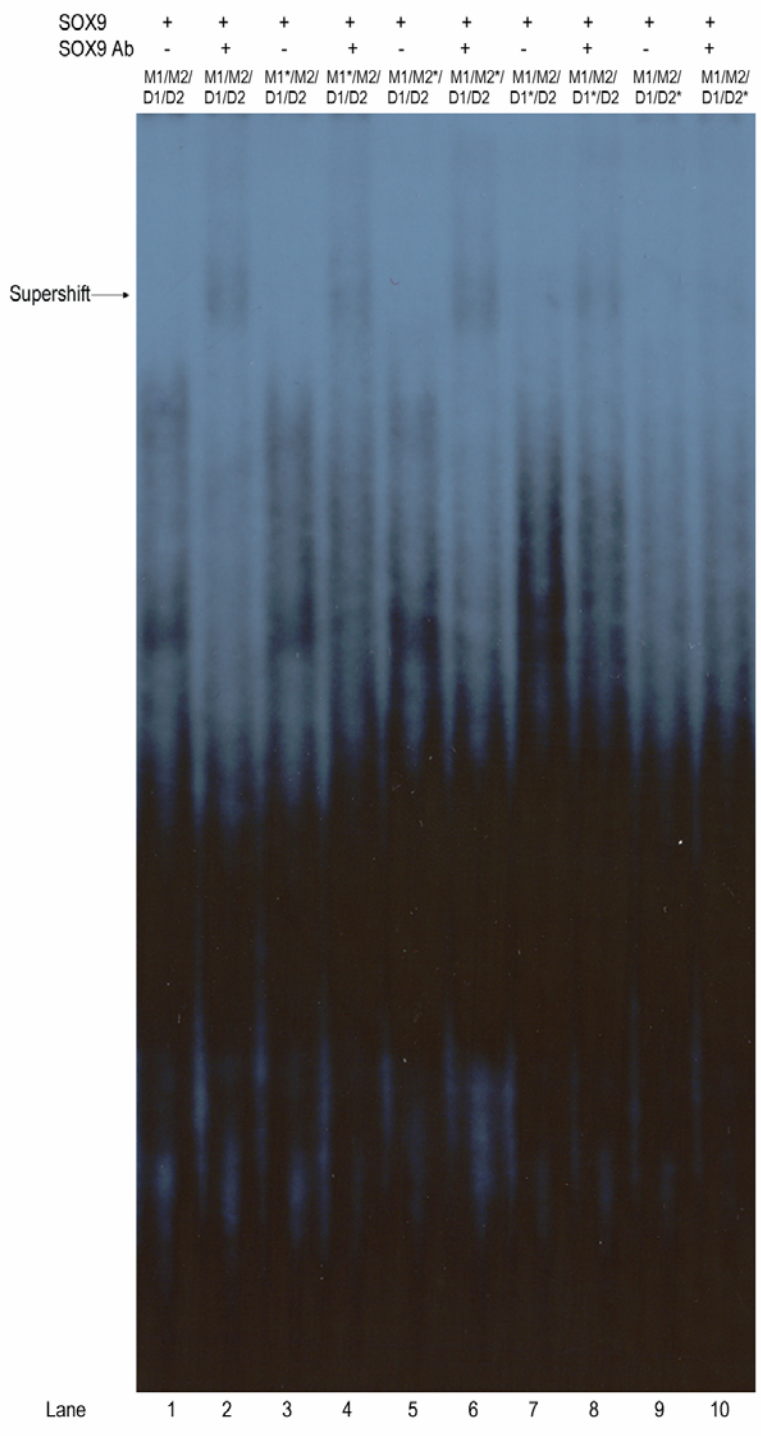

Figure 7 SOX9 binds to *Col9a1* M1/M2D1/D2 enhancer and mutants M1*/M2/D2/D2, M1/M2*/D2/D2, and M1/M2/D2*/D2. Electrophorectic Mobility Shift Assays were performed using *in vitro* made SOX9 and wild-type or mutant enhancers. Anti-SOX9 antibody induced supershifts are seen in lanes 2, 4, 6 and 8.

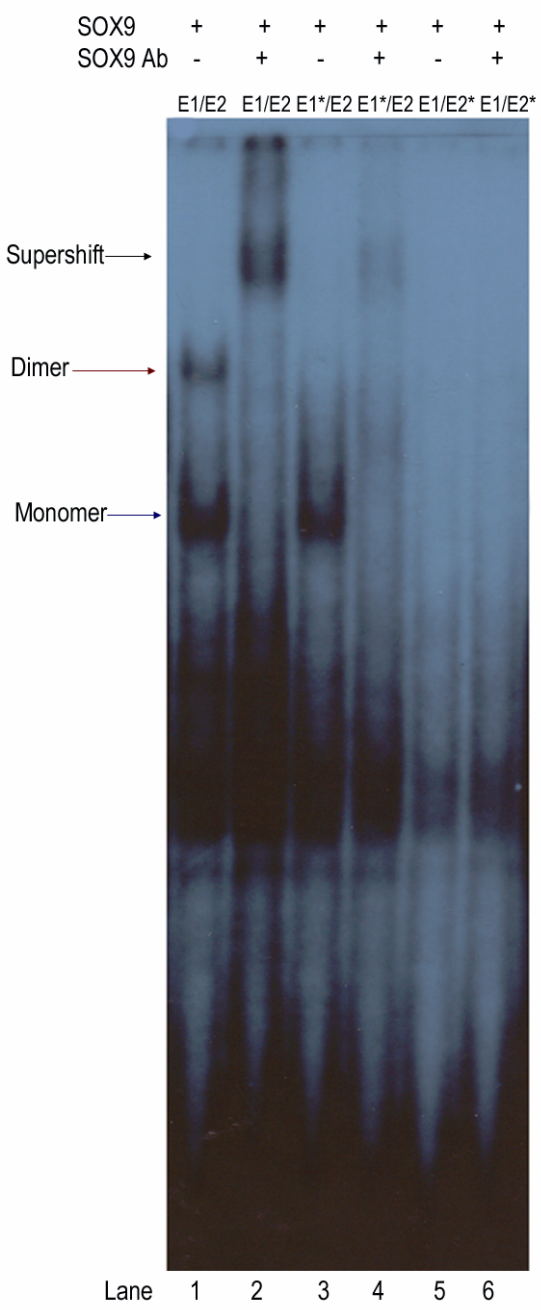

Figure 8 Dimeric and monomeric SOX9 bind to *Col9a1* E1/E2 enhancer and monomeric SOX9 binds to mutant E1*/E2. Electrophorectic Mobility Shift Assays were performed using *in vitro* made SOX9 and wild-type or mutant enhancers. Dimeric SOX9 is seen in lane 1, monomeric SOX9 is seen in lanes 1 and 3, and anti-SOX9 antibody induced supershifts are seen in lanes 2 and 4.

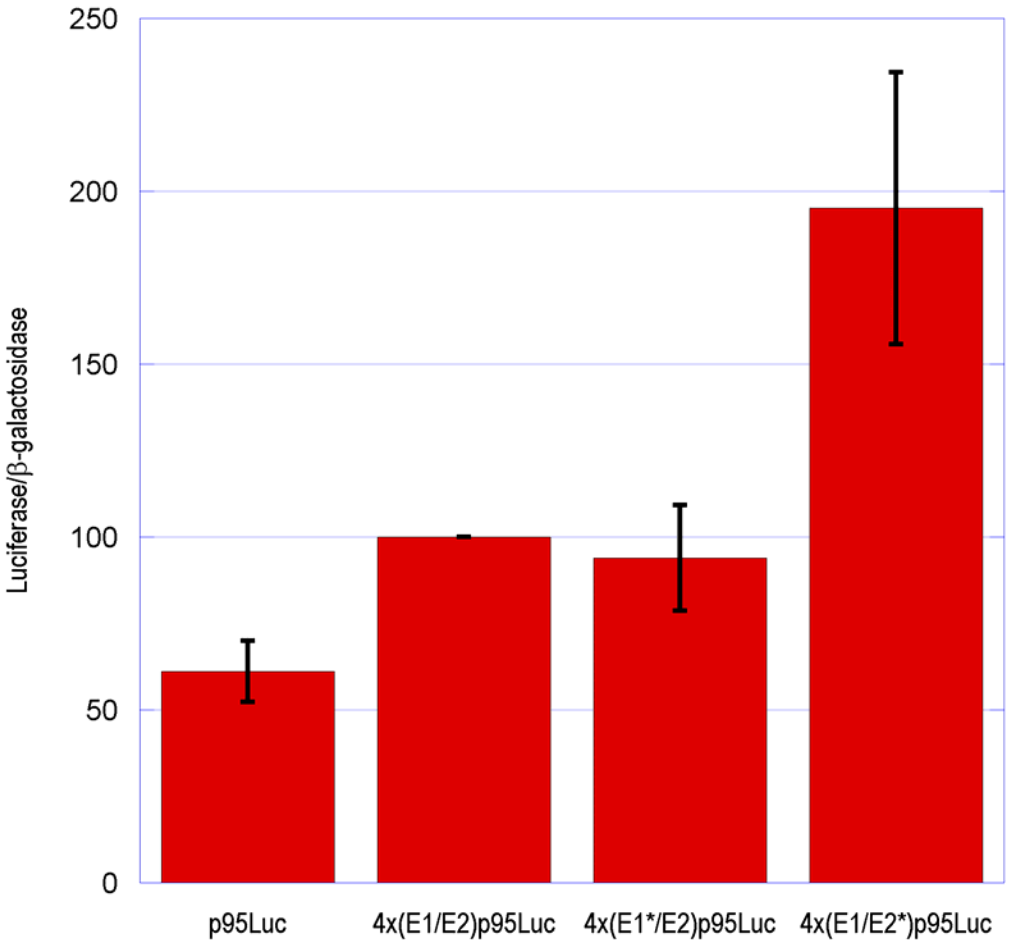

Figure 9 The *Col9a1* enhancers E1/E2 and E1/E2* activate gene expression equally. Transient transfections were performed in RCS cells using reporter plasmids containing wild-type (E1/E2) or mutant (E1*/E2, E1/E2*) enhancers. The no-enhancer reporter plasmid, p95Luc, functioned as the control. All enhancers induced statistically significant levels of activation compared to the control. The difference in levels of activation induced by E1/E2 and by E1*/E2 were not statistically significant.

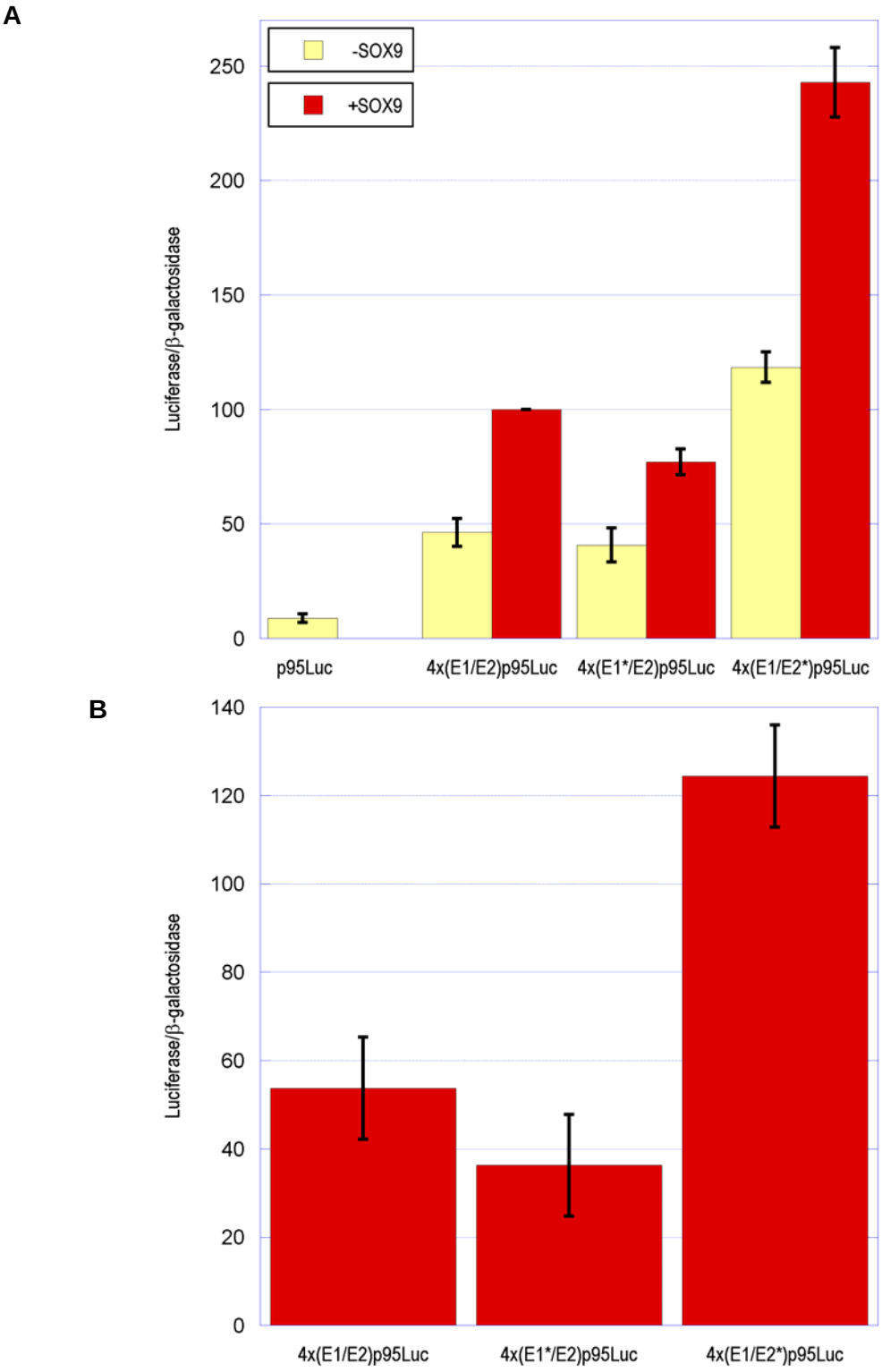

Figure 10 All *Col9a1* E1/E2 enhancers activate gene expression in fibroblast cells when SOX9 is present. Transient transfections were performed in 10T1/2 fibroblast cells. A) Cells were co-transfected with SOX9 expression vector (red), or empty expression vector (yellow). B) Activation differences for each enhancer with and without SOX9 are plotted.

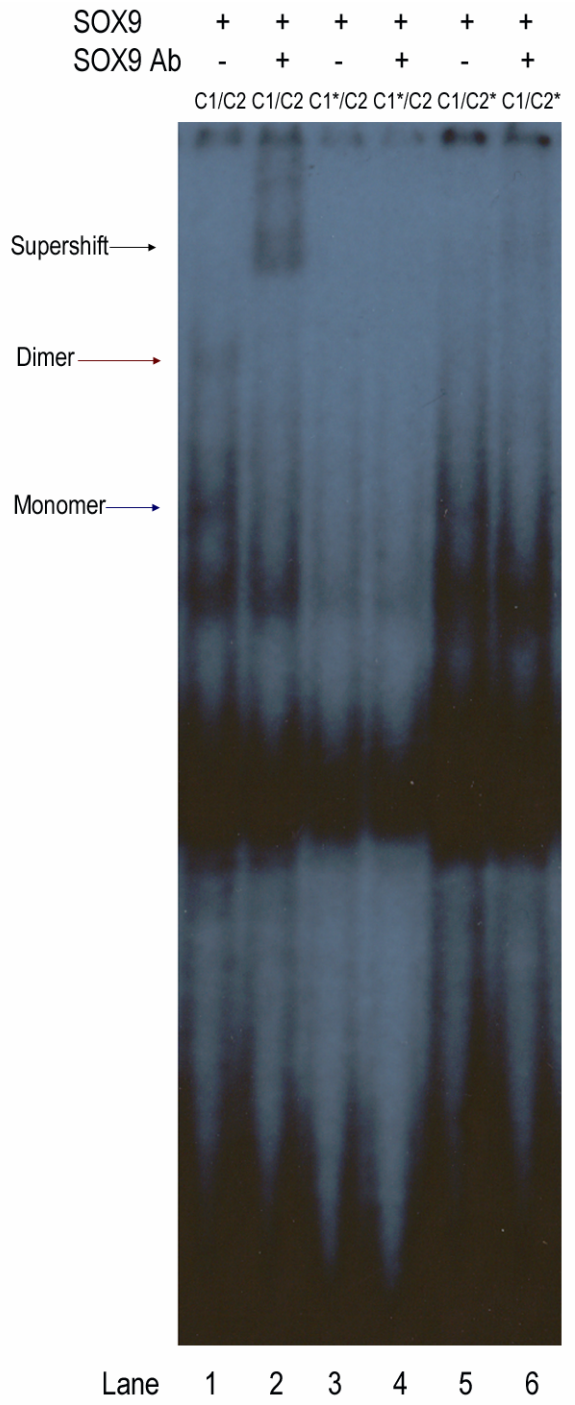

Figure 11 Dimeric and monomeric SOX9 bind to *CRTL1* C1/C2 enhancer and monomeric SOX9 binds to mutant C1/C2*. Electrophorectic Mobility Shift Assays were performed using *in vitro* made SOX9 and wild-type or mutant enhancers. Dimeric SOX9 is seen in lane 1, monomeric SOX9 is seen in lanes 1 and 5, and anti-SOX9 antibody induced supershifts are seen in lanes 2 and 6.

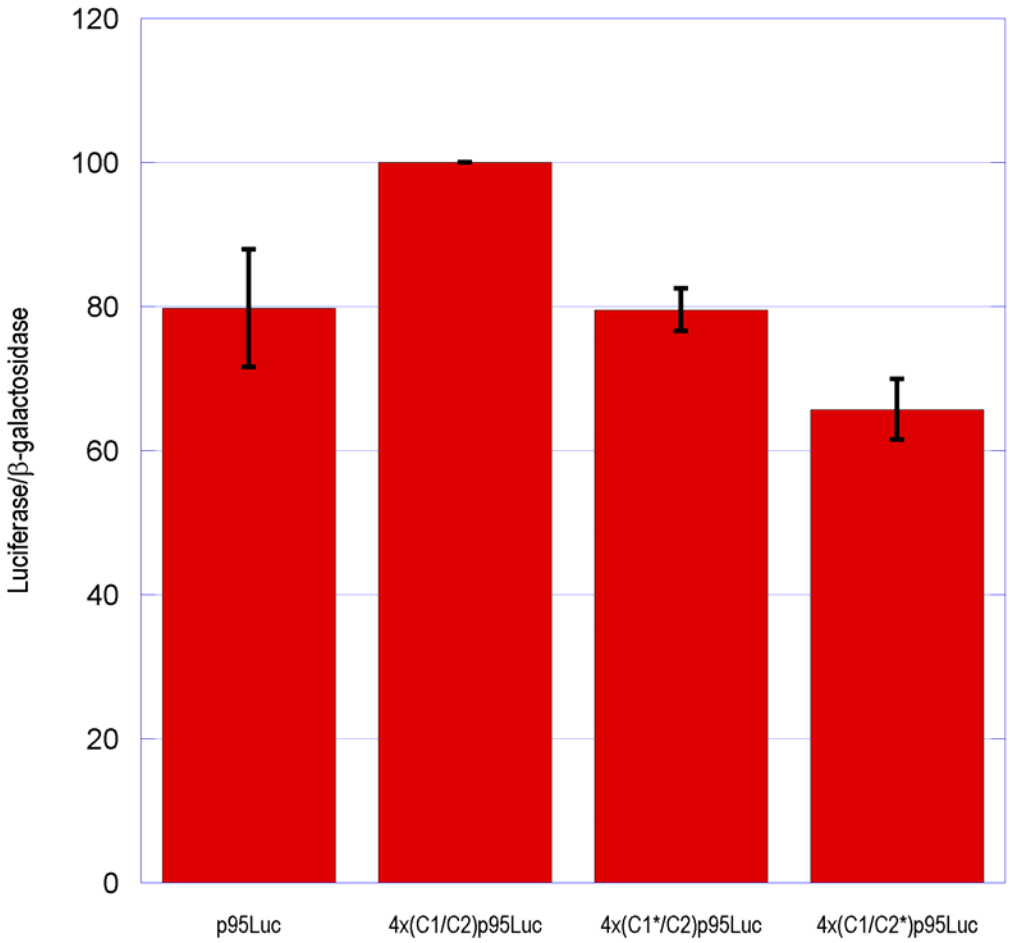

Figure 12 The *CRTL1* C1/C2 enhancer activates gene expression whereas mutant enhancers do not. Transient transfections were performed in RCS cells using reporter plasmids containing wild-type (C1/C2) or mutant (C1*/C2, C1/C2*) enhancers. The no-enhancer reporter plasmid, p95Luc, functioned as the control. Only the wild-type enhancer induced statistically significant levels of activation compared to the control.

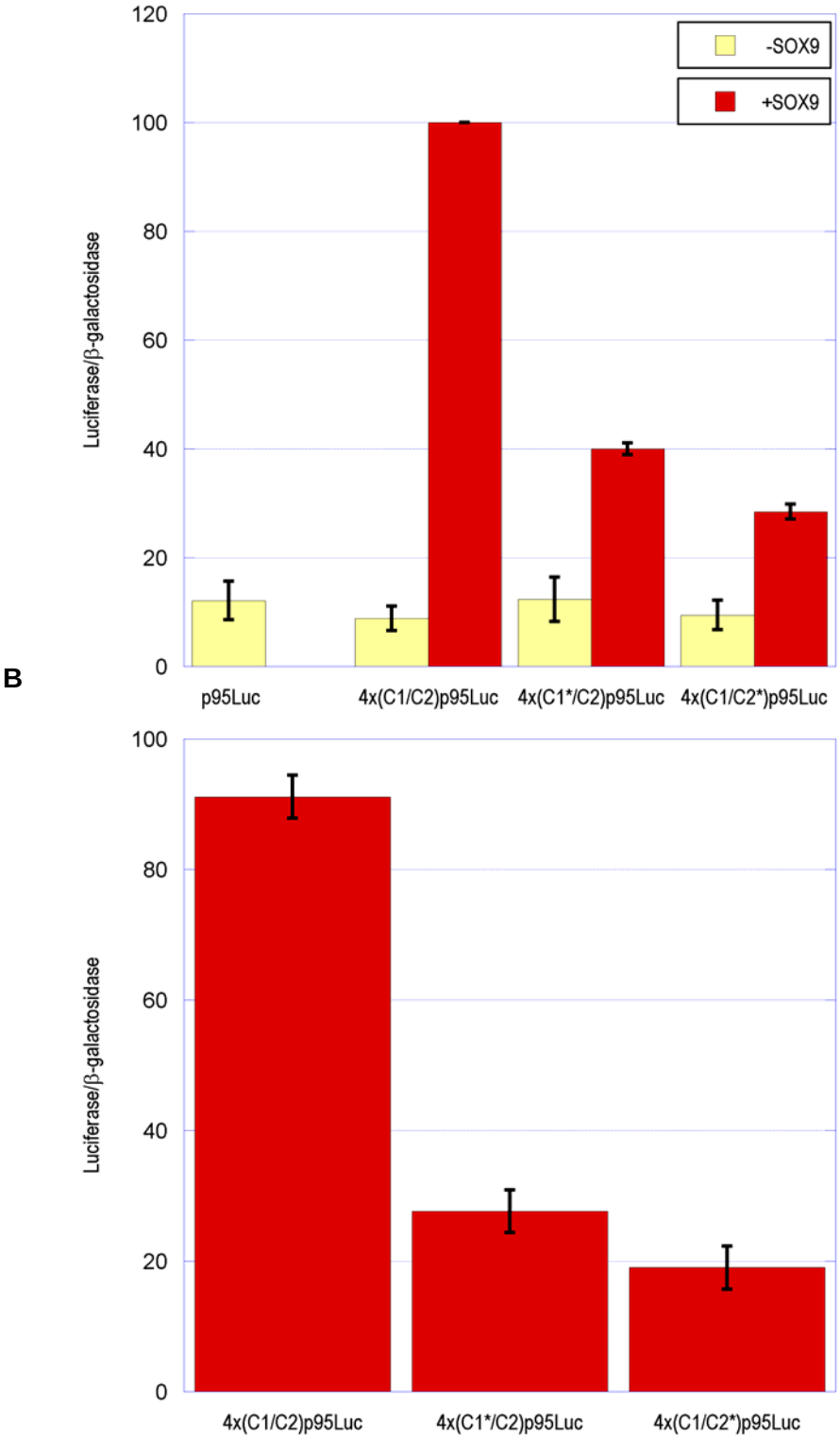

Figure 13 All *CRTL1* C1/C2 enhancers activate gene expression in fibroblast cells when SOX9 is present. Transient transfections were performed in 10T1/2 fibroblast cells. A) Cells were co-transfected with SOX9 expression vector (red), or empty expression vector (yellow). B) Activation differences for each enhancer with and without SOX9 are plotted.

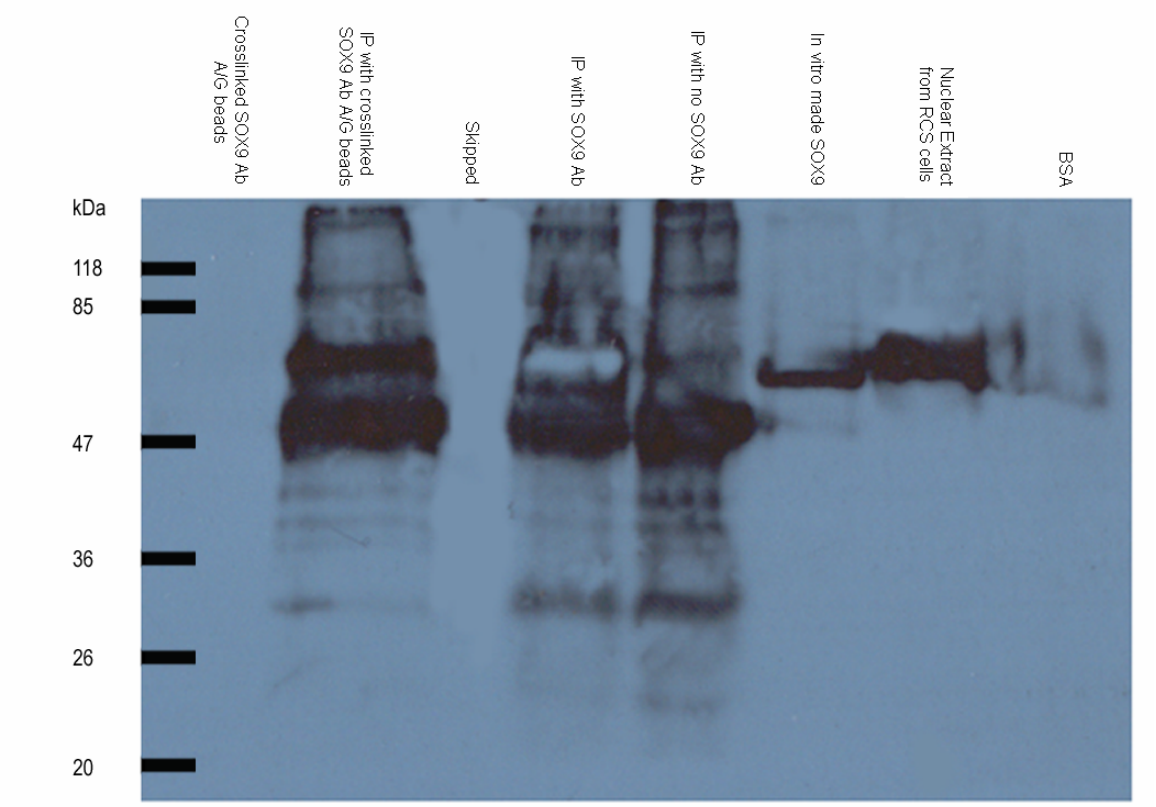

Figure 14 Western blot of immunoprecipitated proteins confirms the presence of SOX9. In lane 1, no bands are seen which indicates the crosslinking procedure was successful. In lanes 2 and 4, bands show the presence of SOX9 (56 kDa) and antibody heavy-chain and light chain (50 kDa and 30kDa respectively). In lane 5, SOX9 is not present. In lanes 6 and 7, SOX9 is seen. In lane 8, no strong bands are present.

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