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The transcription factor Lc-Maf participates in *Col27a1* regulation during chondrocyte maturation

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Abstract

The transcription factor Lc-Maf, which is a splice variant of c-Maf, is expressed in cartilage undergoing endochondral ossification and participates in the regulation of type II collagen through a cartilage-specific *Col2a1* enhancer element. Type XXVII and type XI collagens are also expressed in cartilage during endochondral ossification, and so enhancer/reporter assays were used to determine whether Lc-Maf could regulate cartilage-specific enhancers from the *Col27a1* and *Col11a2* genes. The *Col27a1* enhancer was upregulated over 4-fold by Lc-Maf, while the *Col11a2* enhancer was downregulated slightly. To confirm the results of these reporter assays, rat chondrosarcoma (RCS) cells were transiently transfected with an Lc-Maf expression plasmid, and quantitative RT-PCR was performed to measure the expression of endogenous *Col27a1* and *Col11a2* genes. Endogenous *Col27a1* was upregulated 6-fold by Lc-Maf overexpression, while endogenous *Col11a2* was unchanged. Finally, *in situ* hybridization and immunohistochemistry were performed in the radius and ulna of embryonic day 17 mouse forelimbs undergoing endochondral ossification. Results demonstrated that *Lc-Maf* and *Col27a1* mRNAs are coexpressed in proliferating and prehypertrophic regions, as would be predicted if Lc-Maf regulates *Col27a1* expression. Type XXVII collagen protein was also most abundant in prehypertrophic and proliferating chondrocytes. Others have shown that mice that are null for Lc-Maf and c-Maf have expanded hypertrophic regions with reduced ossification and delayed vascularization. Separate studies have indicated that *Col27a1* may serve as a scaffold for ossification and vascularization. The work presented here suggests that Lc-Maf may affect the process of endochondral ossification by participating in the regulation of *Col27a1* expression.

Keywords

Lc-Maf; *Col11a2*; *Col27a1*; skeletal development; endochondral ossification; collagen

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Introduction

Maf family proteins belong to the basic leucine zipper (bZIP) superfamily of transcription factors; they contain a leucine zipper dimerization interface adjacent to a basic DNA binding region [1]. However, the basic regions of the Maf family of proteins contain non-conservative amino acid substitutions at positions that are highly conserved in other bZIP proteins. Maf proteins also contain an additional DNA-binding region on the N-terminal side of the bZIP domain that is highly conserved and required for specific DNA recognition [2]. These differences result in a different tertiary structure and DNA contact interface than the canonical bZIP proteins [3]. Maf proteins often recognize longer DNA sequence elements (13-14 bp) that extend past the typical bZIP recognition element [2-4].

Individual Maf proteins can be widely expressed in diverse cell types and yet are involved in the expression of a variety of tissue-specific gene products both during and after development [5-9]. This characteristic appears to be due, in part, to the ability of bZIP proteins to form heterodimers with a wide variety of other transcriptional regulatory proteins. Due to the nature of dimer formation among bZIP proteins, in which both proteins independently recognize one half of the DNA recognition sequence, Maf recognition elements frequently differ from each other [2]. It has been suggested that Maf proteins acquire their ability to regulate expression of such a diversity of genes through interacting with different transcriptional regulatory proteins in the developing tissues and thereby recognizing a variety of target-specific DNA elements [1, 3, 10-12].

The long form of c-Maf (Lc-Maf) was identified in a yeast two-hybrid screen by its specific interaction with SOX9, a known transcriptional activator of a number of cartilage-specific genes, including those for the type II, IX, XI, and XXVII collagens [7, 13-16]. Lc-Maf was shown to synergize with SOX9 to increase the expression of the cartilage-specific *Col2a1* gene in 10T1/2 cells and MC615 chondrocytes by binding within a *Col2a1* enhancer element to the short, 7-bp recognition element GGCTCTG [7]. Lc-Maf is a splice variant of c-Maf, it has a different 3′UTR region and the protein has an additional 10 amino acids at the carboxyl terminus [7, 17]. Using a probe from the 3′UTR of Lc-Maf, northern hybridizations revealed RNA expression in various mouse tissues, including cartilage [7, 17]. It has been demonstrated that the presence of Lc-Maf and c-Maf in cartilage plays a vital role for correct skeletal development: mice lacking these proteins exhibited decreased fetal bone length and had improper hypertrophic chondrocyte differentiation [18].

Cartilage is an important tissue that serves a vital role as the template for many bones in the developing skeleton. During the process of endochondral ossification, long bones of the body develop from a cartilage intermediate that is progressively replaced by bone. During the first phase, the mesenchyme cells differentiate into chondrocytes. Starting at the center of the cartilaginous template and progressing towards the epiphyses, these cells mature from reserve to proliferating and on to hypertrophic chondrocytes, at which point mineralization begins. At each stage of chondrocyte differentiation, the expression of extracellular matrix proteins are temporally and spatially coordinated due to a tight regulation via transcription factors, signaling molecules, hormones and local growth factors [7, 19]. Precise coordination of the expression of extracellular matrix proteins, such as the cartilage-specific collagens, is essential for correct skeletal development.

Because Lc-Maf was shown to increase *Col2a1* expression, we investigated the role it might play in regulating other cartilage specific collagen genes that are also involved with skeletal development. Specifically, we focused on the D/E enhancer element from *Col11a2* and the 27F/G enhancer element from *COL27A1*. Like the *Col2a1* enhancer, these elements are both

responsive to SOX9 [13-15, 20, 21]. Our findings indicate that the transcription factor Lc-Maf does indeed help activate expression of *Col27a1* but not *Col11a2*.

Hjorten et al. have suggested on the basis of protein localization studies that *Col27a1* plays a role in the later stages of endochondral ossification [22]. In a separate study, MacLean et al. demonstrated that absence of both c-maf and Lc-maf causes abnormal endochondral ossification [18]. The present study suggests that Lc-Maf may affect the process of endochondral ossification by participating in the regulation of *Col27a1* expression.

Materials and Methods

Plasmids

Each of the reporter constructs used in transient transfections contained four tandem copies of an enhancer element, cloned upstream of the 95-bp *Col2a1* minimal promoter and a luciferase reporter gene. Enhancer elements tested in plasmids 4×(D/Em)p95Luc and 4×(27F/Gm)p95Luc were synthesized as complementary oligonucleotides purchased from Invitrogen (Carlsbad, CA). Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before annealing. The plasmid p89Col2a1Bs was used to multimerize the enhancer elements to four tandem copies before transferring them, along with the *Col2a1* minimal promoter, into the luciferase reporter vector pLuc4 as previously described [13, 23]. The other luciferase reporter plasmids were made previously [13-15]. The p89Col2a1Bs plasmid was a gift from Dr. Veronique Lefebvre of the Cleveland Clinic in Cleveland, Ohio. The Lc-Maf expression vector pcDNA3.1-Lc-Maf was a gift from Dr. Wendong Huang of the Baylor College of Medicine in Houston, Texas.

Transient Transfections

Rat chondrosarcoma (RCS) cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), L-glutamine (2mM) and 10% bovine growth serum. Every 3-4 days the cells were passaged using a 0.25% trypsin-1mM EDTA solution.

Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's protocol. A total of 2 μg of DNA was used to transfect each 10-cm^2 well. Each reaction included 1.0 μg of the luciferase reporter plasmid, 0.5 μg of pSV-β-galactosidase as an internal control for transfection efficiency, and 0.5 μg of either pcDNA3.1-Lc-Maf or the empty pcDNA3.1 vector. β -galactosidase levels were measured using the Galacto-Light/Plus system (Tropix, Bedford, MA) following the manufacturer's protocol. Luciferase levels were measured using the Luciferase Assay Reagent (Promega. Madison, WI) following the manufacturer's protocol. Transfection data is reported as Relative Luciferase Units (luciferase units per β-galactosidase unit) ± standard error, normalized to the activity of the appropriate enhancer/reporter gene construct within each experiment. Each graph represents at least three independent experiments, each performed in triplicate. Results were analyzed for statistical significance using Student's t-test.

In Vitro Transcription/Translation

Lc-Maf protein was synthesized using the Single Tube Protein System 3, T7 kit (Novagen, San Diego, CA) according to manufacturer's instruction using the Lc-Maf expression plasmid, pcDNA3.1-Lc-Maf. To verify that transcription and translation occurred as expected, [^{35}S]methionine-labeled Lc-Maf was visualized on SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA)

Wild-type and mutant C*OL27A1* 27F/G probes were prepared by annealing complementary oligonucleotides synthesized by Invitrogen. The probes were labeled by end-filling with α-[^{32}P]dGTP using the Klenow fragment and purified using Amersham Biosciences NICK columns according to manufacturer's protocol. Labeled probes were adjusted with unlabeled probe to achieve equivalent specific activities. Lc-Maf protein was pre-incubated for 15 minutes in 20 μl buffer containing 20 mM HEPES, 100mM KCl, 10mM $MgCl_2$, 5% glycerol, 1 mM EDTA, 5 mM DTT, 0.1% NP-40, 0.5 mg ml^{-1} BSA, and 1 μg poly(dGdC) ·poly(dGdC) (Amersham Biosciences, Piscataway, NJ), with or without antibody. Since Lc-Maf differs from c-Maf by only an additional 10 amino acids at the carboxyl terminus, an antibody directed towards the amino terminus of c-Maf was used (Santa Cruz Biotechnology, Santa Cruz, CA). Radiolabeled probe was added to the binding reaction and incubated at room temperature for an additional 15 minutes. The samples were then fractionated by electrophoresis through a non-denaturing 4% polyacrylamide gel in 0.5× TBE at 140 V and visualized by autoradiography.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RCS cells were transiently transfected with either pcDNA3.1-Lc-Maf or the empty pcDNA3.1 vector as described above and were harvested 48 hours after transfection. Percent of transfected cells (transfection efficiency) was determined by including a parallel transfection reaction with a GFP expression plasmid in each experiment. The percent of cells expressing GFP was measured using a BD FACS Canto flow cytometer. RNA was isolated using the PerfectPure RNA Cultured Cell Kit (5 PRIME, Gaithersburg, MD) and reverse transcribed using the ImProm II Reverse Transcription System (Promega, Madison, WI). Rat-specific primers for *Col27a1* and *Col11a2* were designed to span the last intron using Primer 3 Input 0.4.0 software. The primer sequences were as follows: 5'GGAACGGACAGGTCTTTGAA3' (*Col27a1* forward), 5'GGGTCCGGAAGGTGAATAGT 3' (*Col27a1* reverse), 5'GATGAGCTGAGCCCTGAGAC3' (*Col11a2* forward), 5'CCAGGTCTGAGAAGGAAGCA 3' (*Col11a2* reverse). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPD) served as an internal reference. Relative quantification was performed using a Roche 480 LightCycler and SYBR Green Master Mix (Roche, Basel, Switzerland), and results were analyzed using the Roche LightCycler 480 version 1.2 Relative Quantification software package. Results were normalized to adjust for the transfection efficiency of each independent experiment.

In situ hybridizations and Immunohistochemistry

Mouse embryos were collected at embryonic day 17 (E17). Forelimbs were removed, fixed overnight at 4°C in 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS), embedded in paraffin, and sectioned (6-8 μm) before placing onto Superfrost/Plus microscope slides (Fisher).

In situ hybridization probes for the mouse *Col10a1*, *Lc-Maf*, and *Col27a1* genes were kind gifts from the following individuals: *Col10a1* from B. de Crumbrugghe, *Lc-Maf* from W. Huang, and *Col27a1* from J. Pace. Single-strand RNA antisense probes were labeled with digoxigenin-11-UTP using the DIG RNA labeling kit (Roche) according to manufacturer's protocol.

In situ hybridization was performed following the protocol of Murtaugh [24] with the following modifications: after incubation with 1 μg/mL proteinase K (Fisher) for 30 minutes at 37°C and washing with PBT (phosphate-buffered saline plus 0.1% Tween-20), sections were postfixed in 4% PFA/PBS and incubated 4 hours in a humid chamber with

prehybridization buffer. Hybridization to digoxigenin-11-UTP-labeled probes for the *Col10a1*, *Col27a1*, and *Lc-Maf* genes was carried out in a humid chamber overnight in the same hybridization mixture. Following hybridization, sections were treated with RNAse A (Fisher) in TNE (10 mM Tris pH 7.5, 500mM NaCl, 1 mM EDTA) and washed in SSC (15 mM sodium citrate, 150 mM NaCl, pH 7.0). The sections were blocked using 10% serum/MABT (100 mM Maleic Acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5) before visualizing the probes by staining with anti-DIG-alkaline phosphatase-conjugated antibody (Roche) and treating the slides with BM Purple (Fisher).

Immunohistochemistry was performed as described previously [25] using primary antibody directed against mature type XXVII collagen. This antibody was designed, generated, and generously provide by J. Pace [22].

Results

Lc-Maf activated the chondrocyte-specific *COL27A1* 27F/G enhancer element in reporter assays

The intronic *COL27A1* enhancer element 27F/G was previously shown to regulate gene expression in a cartilage-specific manner in response to SOX9 [15].

A reporter construct containing this enhancer [4×(27F/G)p95Luc] was cotransfected with an Lc-Maf expression vector [pcDNA3.1-Lc-Maf] into rat chondrosarcoma (RCS) cells to determine whether Lc-Maf exerts any regulatory action on this enhancer element. Cotransfection led to a 4.5-fold increase in reporter gene expression ($p= 0.0002$) (Fig. 1A).

Neither an Lc-Maf consensus binding sequence nor any complete Maf-family consensus binding sequence could be identified within the *COL27A1* 27F/G enhancer element, but there appeared to be a partial sequence (AAA**GACT**TGA) at the 5′ end of the enhancer. A 2-bp mutation was introduced in this area (AAAGA**A**TT**A**A) (Fig. 1D). The activity of the mutant enhancer 27F/Gm, in which the 2-bp mutation was introduced, was tested in transient transfections in RCS cells. This mutant enhancer was no longer activated by Lc-Maf, suggesting that the mutation in 27F/Gm disrupts a site that is necessary for transcriptional activation by Lc-Maf (Fig. 1B). Comparison of the activities of the 27F/Gwt and the 27F/Gm enhancers without Lc-Maf induction showed that the mutant enhancer was only 20% as active as the wild-type in RCS cells ($p=0.0004$), consistent with the loss of a positive regulatory element (Fig. 1C).

Electrophoretic mobility shift assays (EMSA) were performed to determine whether Lc-Maf interacts directly with the 27F/Gwt enhancer sequence. Lc-Maf was synthesized by *in vitro* transcription/translation and incubated with radiolabeled 27F/Gwt or 27F/Gm probe. A shifted DNA-protein complex was observed with the wild-type enhancer probe, and pre-incubation of Lc-Maf with an anti-c-Maf antibody inhibited formation of this DNA-protein complex (Fig. 1E, arrow). Incubation with the 27F/Gm probe demonstrated that the same 2-bp mutation that eliminated Lc-Maf responsiveness in reporter assays also reduced Lc-Maf binding to the enhancer in EMSA.

Lc-Maf weakly inhibited the *Col11a2* D/E enhancer element in reporter assays

As with the *COL27A1* enhancer 27F/G, the *Col11a2* D/E enhancer element was previously shown to regulate gene expression in a cartilage-specific manner in response to SOX9 [13]. A reporter construct containing this enhancer [4×(D/E)p95Luc] was cotransfected with an Lc-Maf expression vector [pcDNA3.1-Lc-Maf] into RCS cells to determine whether Lc-Maf exerts any regulatory action on the *Col11a2* D/E enhancer element. Cotransfection led to a

small decrease in reporter gene expression, to 65% of the original D/E value (p=0.0002) (Fig. 2A).

Substitution mutations were introduced into the *Col11a2* D/E enhancer element at a putative Lc-Maf binding site (Fig. 2D). These mutations made the enhancer non-responsive to Lc-Maf (Fig. 2B), suggesting that Lc-Maf exerts its small effect on transcription through the region mutated in D/Em. Comparison of the activities of the uninduced D/Ewt and the D/Em enhancers in RCS cells showed that D/Em was slightly more active (p=0.0018), consistent with the loss of a negative regulatory element (Fig. 2C). These effects, however, were so weak as to raise questions about whether Lc-Maf would affect *Col11a2* D/E enhancer activity *in vivo*.

Ectopic expression of Lc-Maf increases endogenous *Col27a1* expression in cultured cells

To help determine whether the results observed in reporter assays accurately reflected regulatory mechanisms in growing cells, RCS cells were transiently transfected with either the Lc-Maf expression plasmid [pcDNA3.1-Lc-Maf] or the empty vector control [pcDNA3.1], and RNA was isolated for analysis by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Fig. 3). Quantitative amplification of *Col27a1* transcripts showed that *Col27a1* expression levels were increased 6.0 ± 1.1-fold in response to ectopic expression of Lc-Maf (p=0.011). In contrast, *Col11a2* transcript levels were slightly and non-significantly decreased to 0.6 ± 1.5-fold (p=0.805), indicating that the inhibitory effect Lc-Maf exhibited on the *Col11a2* D/E enhancer in reporter assays was probably not biologically relevant.

Lc-Maf binds to the *Col27a1* F/G enhancer element in vitro.

Lc-Maf and Col27a1 transcripts showed similar expression patterns

To observe the expression patterns of *Lc-Maf* and *Col27a1* during endochondral ossification of the developing limbs, we performed *in situ* hybridizations on the forelimbs of mouse embryos at embryonic day 17 (E17), which is after the beginning of ossification in this tissue. A probe directed against *Col10a1*, a marker for prehypertrophic and hypertrophic chondrocytes, was included as a control. *Lc-Maf* staining was weak relative to the two collagen genes, but it was most prominent in the prehypertrophic and proliferating chondrocytes with lower levels of expression in the reserve zone (Fig. 4). Similarly, *Col27a1* staining was most prominent in prehypertrophic and proliferating chondrocytes, with decreased staining in reserve chondrocytes.

Immunostaining for type XXVII collagen was strongest in prehypertrophic chondrocytes

Previous immunohistochemistry experiments by others, which examined *Col27a1* expression at different stages of endochondral ossification and in different tissues than those used in the present study (human phalanges and femoral growth plates) [22] suggested that type XXVII collagen would be most abundant in hypertrophic chondrocytes. It was surprising, therefore, that our *in situ* hybridizations showed more *Col27a1* transcripts in prehypertrophic than in hypertrophic chondrocytes.

To examine type XXVII collagen protein localization patterns in mouse forelimbs at E17, we performed immunohistochemical analyses of mouse radius and ulna sections. We observed that the type XXVII collagen protein was most abundant in prehypertrophic chondrocytes, consistent with our *in situ* hybridization results (Fig. 5).

Discussion

In 2003, MacLean et al. reported on the phenotype of mice bearing a targeted inactivation of *c-Maf* [18]. Because the Lc-Maf protein is translated from a splice variant of *c-Maf*, both the c-Maf and Lc-Maf proteins were absent in these null mice [17]. Analysis of tibiae at various stages of embryonic and post-natal development showed that at E15.5, the number of hypertrophic chondrocytes was decreased. By 16.5, however, the *c-Maf/Lc-Maf*-null tibia contained an expanded domain of hypertrophic chondrocytes, associated with delayed vascular invasion and reduced ossification at the center of the tibia. This same abnormality was still detectable at 4 weeks after birth [18]. This phenotype suggested that c-Maf and/or Lc-Maf play(s) a role in regulating the timing of initial terminal differentiation of chondrocytes prior to vascular invasion, and also in the disappearance of hypertrophic chondrocytes at the time of chondrocyte apoptosis and vascular invasion.

Lc-Maf has been shown to cooperate with SOX9 to increase expression of the type II collagen gene, *Col2a1* [7]. Inactivation of Lc-Maf, therefore, might be expected to alter chondrocyte differentiation by decreasing *Col2a1* expression. Examination of mice with decreased *Col2a1* expression, however, reveals some differences with the *c-Maf/Lc-Maf*-null phenotype. *Dmm* mice contain a mutation in the C-propeptide coding region of *Col2a1*, which results in a form of type II collagen that cannot be secreted into the cartilage extracellular matrix [26]. *Dmm* mice show disorganization of the normal columnar array of chondrocytes in the proliferative zone, but no expansion of the hypertrophic zone as in *c-Maf/Lc-Maf*-null mice [27, 28]. Similarly, mice that are heterozygous for a targeted inactivation of *Col2a1* show disorganization of chondrocytes in the proliferative zone, but no changes in the hypertrophic zone were reported [29]. It is unlikely, therefore, that the *c-Maf/Lc-Maf*-null phenotype of hypertrophic zone expansion with delayed vascular invasion and reduced ossification is due solely to a reduction in *Col2a1* expression.

Type XXVII collagen was discovered in 2003 and was shown to be expressed in cartilaginous tissues [20, 30]. More recent work by Hjorten et al. examining *Col27a1* expression in the growth plate demonstrated, as we have also seen, that the *Col27a1* gene is most actively transcribed in proliferating chondrocytes [22]. At the timepoint and in the tissues examined in our study, type XXVII collagen protein was also most abundant in proliferating as well as prehypertrophic chondrocytes. Interestingly, Hjorten et al. observed that even though *Col27a1* transcription was most active in proliferating chondrocytes, type XXVII collagen appeared to accumulate progressively over the life of the chondrocyte, so that eventually it became most abundant in the pericellular region around hypertrophic chondrocytes. It was also found surrounding what appeared to be lacunae that once had held hypertrophic chondrocytes before they underwent apoptosis [22]. On the basis of this protein localization, Hjorten et al. suggested that type XXVII collagen plays a role in the later stages of cartilage remodeling during endochondral ossification. Indeed, they consider type XXVII collagen a “good candidate for a scaffold of mineralization in cartilage and as the supporting environment for invading blood vessels” [22]. If this is indeed the case, one might predict that a mouse with decreased expression of *Col27a1* would show delayed vascular invasion and reduced ossification.

In the present study, we have demonstrated that Lc-Maf can increase the transcriptional activity of a cartilage-specific, SOX9-responsive enhancer element from the *Col27a1* gene [15]. Furthermore, we have demonstrated that ectopic expression of Lc-Maf causes a 6-fold increase in endogenous *Col27a1* gene expression in cultured chondrocytic cells. Finally, we have shown that *Lc-Maf* and *Col27a1* transcripts are co-localized in growth plates undergoing endochondral ossification in E17 mice, with maximal expression in the proliferating and prehypertrophic regions. The expression patterns of c-Maf and Lc-Maf are

very similar, and the experiments presented here have not ruled out c-Maf as an additional regulator of *Col27a1* expression. These results have, however, clearly demonstrated that Lc-Maf activates expression of *Col27a1*. Taken together, the results presented here suggest that Lc-Maf participates in the regulation of *Col27a1* expression during endochondral ossification. By extension, these results suggest a potential explanation for the delayed vascular invasion and reduced ossification in growth plates of *c-Maf/Lc-Maf*-null mice.

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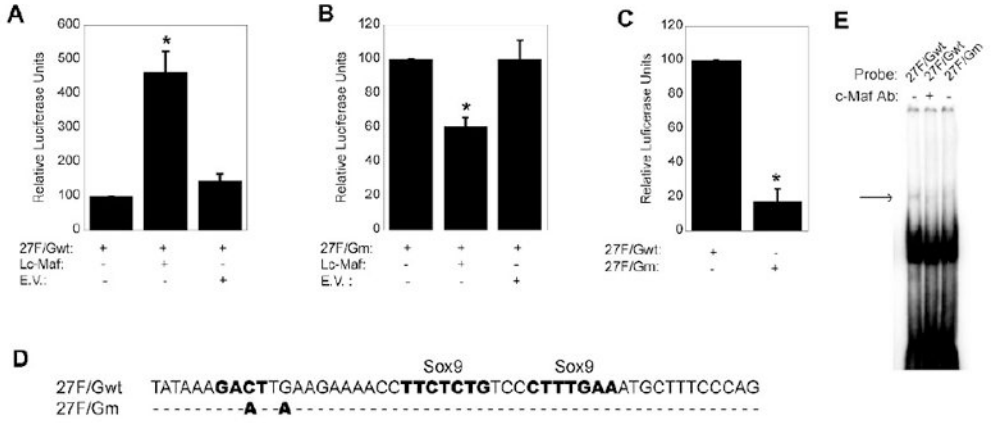

Figure 1. Lc-Maf activates the *COL27A1* 27F/G enhancer element

(A-C) RCS cells were transiently transfected with wild-type or mutant enhancer/reporter plasmids plus pcDNA3.1-Lc-Maf (Lc-Maf) or the empty pcDNA3.1 vector (E.V.) as indicated. Enhancer activities that are significantly different from the corresponding wild-type (A and C) or mutant (B) enhancer activities are indicated with an asterisk ($p \leq 0.0004$). (D) The wild-type *COL27A1* 27F/G sequence is shown with the Sox9 binding regions in bold and the best-available Lc-Maf binding region also bold. The nucleotide substitutions for the mutant enhancer are indicated below the wild-type sequence. (E) Electrophoretic mobility shift assays were performed with the *COL27A1* 27F/Gwt and 27F/Gm enhancer elements as DNA probes with *in vitro* transcribed/translated Lc-Maf protein and c-Maf antibody as indicated. The arrow indicates the DNA-protein complex formed between the 27F/Gwt probe and *in vitro*-synthesized Lc-Maf.

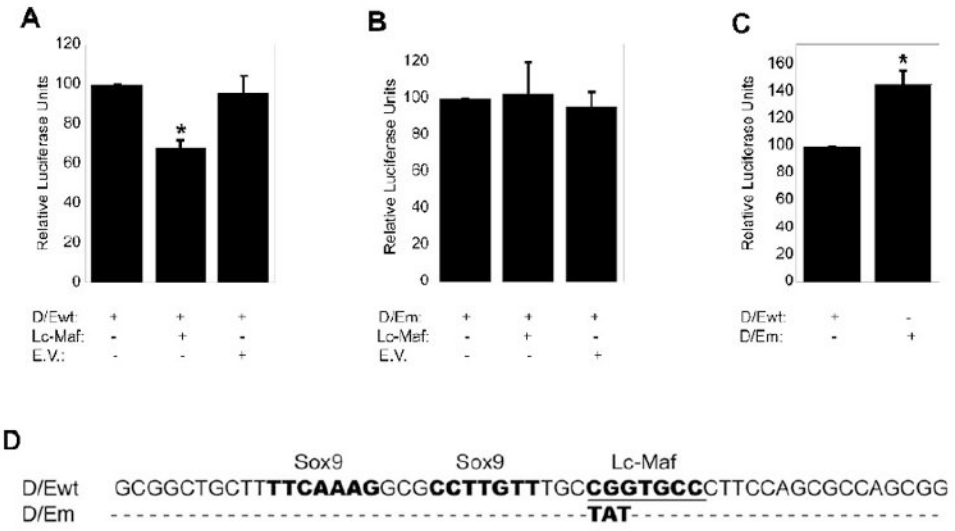

Figure 2. Lc-Maf interacts with the *Col11a2* D/E enhancer element to inhibit enhancer activity

(A-C) RCS cells were transiently transfected with wild-type or mutant enhancer/reporter plasmids plus pcDNA3.1Lc-Maf (Lc-Maf) or the empty pcDNA3.1 vector (E.V.) as indicated. Enhancer activities that are significantly different from the corresponding wild-type (A and C) or mutant (B) enhancer activities are indicated with an asterisk ($p \leq 0.0018$). (D) The wild-type *Col11a2* D/E sequence is shown with the Sox9 binding regions in bold and the putative Lc-Maf binding regions in bold and underlined. The nucleotide substitutions for the mutant enhancers are indicated below the wild-type sequences.

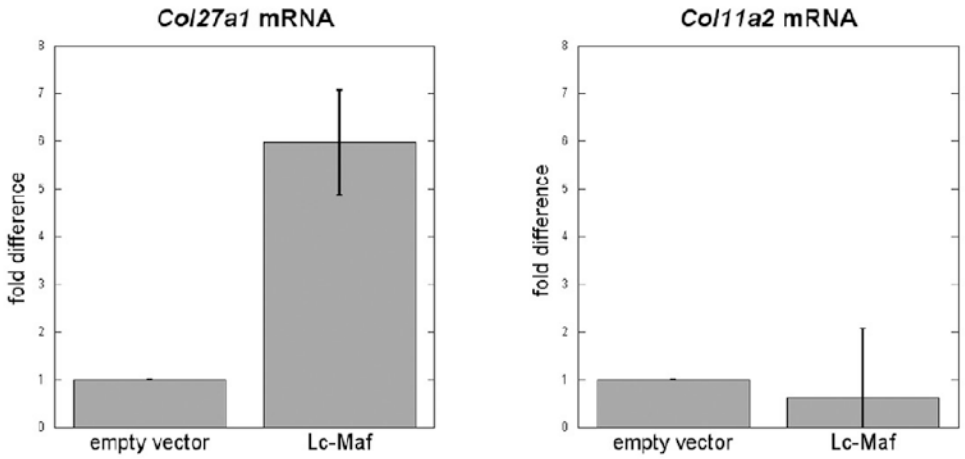

Figure 3. Ectopic expression of Lc-Maf increases endogenous *Col27a1* expression

RCS cells were transfected with the pcDNA3.1Lc-Maf (Lc-Maf) or the empty pcDNA3.1 vector. RNA was isolated, and q-RT-PCR was performed to measure expression levels of endogenous *Col27a1* and *Col11a2*. Data reported is the average of three independent experiments, each performed in triplicate, ± standard error. Relative quantification analysis revealed that Lc-Maf cause a 6-fold increase in the expression of endogenous *Col27a1*, while the expression of endogenous *Col11a2* was not changed.

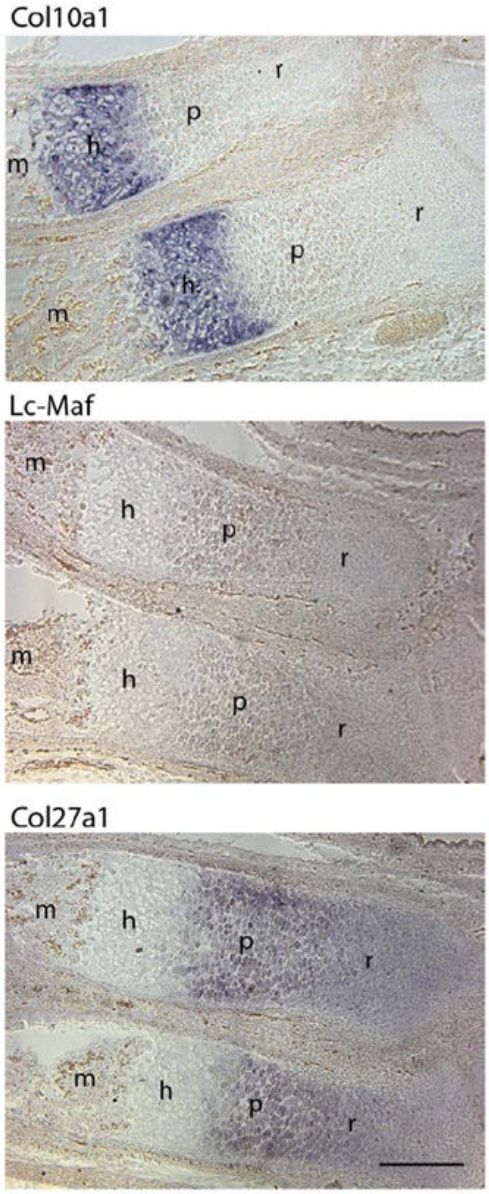

Figure 4. Expression patterns of *Lc-Maf and Col27a1* during endochondral ossification in the murine forelimb

In situ hybridization on longitudinal sections of the radius and ulna of mouse embryos at E17, after the onset of mineralization. Panels are arranged with the diaphysis at the left. Expression of *Col10a1* was observed in hypertrophic and prehypertrophic chondrocytes as expected. *Lc-Maf* mRNA was detected most strongly in the proliferating and prehypertrophic regions. *Col27a1* mRNA was also detected most strongly in the proliferating and prehypertrophic chondrocytes, with less expression in the reserve region. Scale bar, 200 μm. m, mineralized; h, hypertrophic; p, proliferative and prehypertrophic; r, reserve chondrocytes.

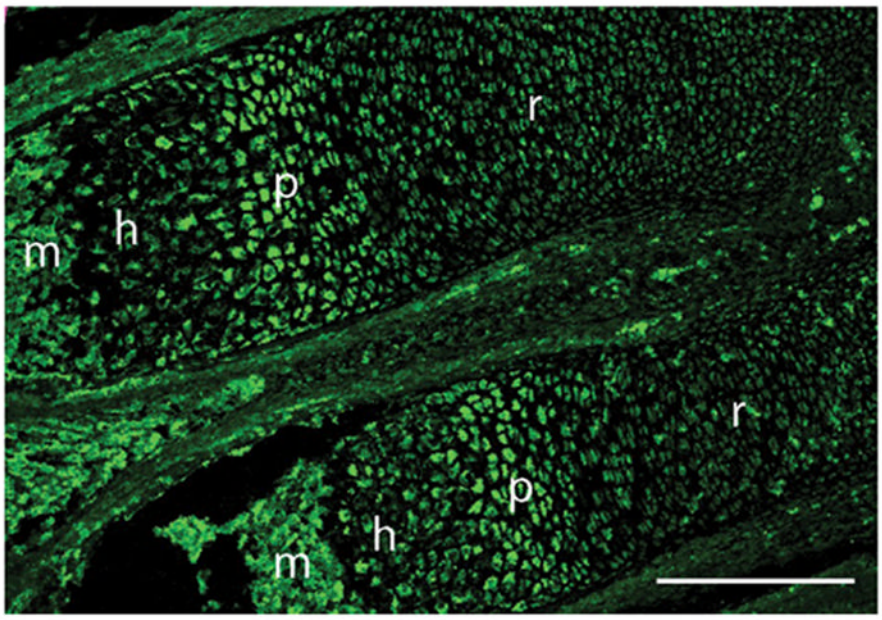

Figure 5. Murine type XXVII collagen protein is most abundant in prehypertrophic chondrocytes in E17 radius and ulna

Longitudinal section of a radius and ulna at E17, showing the mineralized portion of the diaphysis at left. Immunohistochemistry was performed using a primary antibody directed against mature type XXVII collagen and a FITC-labeled secondary antibody. Scale bar, 200 μm. m, mineralized; h, hypertrophic; p, prehypertrophic and proliferative; r, reserve chondrocytes.