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Multiple enhancers associated with *ACAN* suggest highly redundant transcriptional regulation in cartilage

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Abstract

The chondroitin sulfate proteoglycan core protein aggrecan is the major protein constituent of cartilage aside from collagen, and is largely responsible for its distinctive mechanical properties. Aggrecan is required both for proper cartilage formation in development and maintenance of mature cartilage. Prominent *Acan* transcription is a conserved feature of vertebrate cartilage, although little is known about its specific transcriptional regulation. We examined the genomic interval containing human *ACAN* for transcriptional enhancers directing expression to cartilage, using a functional assay in transgenic zebrafish. We tested 24 conserved non-coding sequences, representing ~6% of the total sequence in the interval, and identified eleven independently capable of regulating reporter gene expression in cartilage. These enhancers were widely spaced, from >100 kb upstream of the gene to within the first intron. While the majority displayed broad cartilage expression in zebrafish larvae, several were restricted to a subset of cartilage cells in the craniofacial skeleton. In older fish, the enhancers displayed differential activity; some maintained expression, either in all cartilage or preferentially in articular cartilage at the joints, while others were not active. This remarkable degree of overlapping regulatory control has been highly conserved; we identified clear orthologues of six enhancers at the chicken *ACAN* locus, arranged in the same order relative to the gene. These were also functional in directing expression to cartilage in transgenic zebrafish. Several enhancers contain potential binding sites for Sox9, consistent with its described role as an upstream regulator of *Acan* expression. However, others lacked Sox9 consensus binding sites, implicating additional pathways and transcription factors as regulators of *Acan* expression in cartilage, either in development or adult tissue. Our identification of these enhancer sequences is the necessary first step in detailed examination of the upstream regulators of *Acan* expression.

Keywords

Aggrecan; Cartilage; Cis-regulatory elements; Zebrafish

1. Introduction

Acan encodes aggrecan, the major chondroitin sulfate proteoglycan (CSPG) core protein of cartilage, a highly abundant component of the cartilage extracellular matrix. Although not

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exclusive to cartilage, aggrecan is found most prominently in cartilage and its deposition is considered the hallmark of chondrogenesis. Together, glycosylated aggrecan, the associated link protein, and hyaluronan lend cartilage its important mechanical properties of resistance to compression and viscosity. Not only is aggrecan essential for cartilage formation during development, but its maintained expression throughout life is likely to be essential to cartilage integrity.

The importance of aggrecan in development of cartilage is evident from the phenotypes of chicken and mouse mutants. The *cartilage matrix deficiency* (*cmd*) mouse mutant (Rittenhouse et al., 1978) is a naturally occurring mutation in *Acan* that is predicted to produce a truncated protein (Watanabe et al., 1994), although no aggrecan is detected in the cartilage matrix of homozygous mutant mice. Mutants display dwarfism, short snout, and cleft palate, and die shortly after birth. The chicken *nanomelia* mutant (Landauer, 1965) is also caused by a mutation in *ACAN* (Li et al., 1993; Primorac et al., 1994), and similarly results in a homozygous lethal chondrodysplasia. In humans, a recessive mutation in *ACAN* causes severe chondrodysplasia, but is not lethal; the mutant protein displays altered interaction with the extracellular matrix protein Tenascin, and does not seem to represent a null mutation (Tompson et al., 2009).

Aggrecan is also likely to play a central role in osteoarthritis and other degenerative processes affecting cartilage and intervertebral discs. Mice heterozygous for the *cmd* mutation appear normal at birth. However, they develop mild dwarfism, apparent at one month, and by one year develop severe curvature and misalignment of the spine, eventually leading to neurological impairment (Watanabe et al., 1997). A heterozygous mutation in *ACAN* has similarly been associated with spondyloepiphyseal dysplasia type Kimberley (SDK), which is clinically characterized by short stature and severe premature osteoarthritis (Gleghorn et al., 2005). Several studies have linked polymorphisms in the *ACAN* coding sequence with osteoarthritis (Horton et al., 1998; Kamarainen et al., 2006; Kirk et al., 2003) and lumbar disk degeneration (Mashayekhi et al., 2010), suggesting that even subtle differences in aggrecan function or levels could contribute to these common, multi-factorial diseases.

The expression of aggrecan in cartilage is highly conserved in evolution, and has been described in *Xenopus* and zebrafish as well as chicken, mouse, and other mammals. While presumably its similar expression in such disparate species involves common regulatory mechanisms, little is known about the regulation of *Acan* transcription. It is a target for positive regulation by the related factors Sox9, Sox5, and Sox6, acting through an upstream enhancer element (Han and Lefebvre, 2008). The homeobox transcription factor SHOX also regulates *Acan* expression, acting cooperatively with Sox5 and Sox6 at the same enhancer (Aza-Carmona et al., 2011). Other genes and signaling pathways are proposed to be upstream (Li et al., 2003; Pei et al., 2009; Pfander et al., 2003; Sen et al., 2004; Tim Yoon et al., 2003; Tsai et al., 2003), but no direct evidence has been shown for interactions with specific cis-regulatory sequences. Nonetheless, it is likely that variations in expression levels of the gene are linked to important pathogenic processes. Therefore, we undertook a comprehensive survey of the human *ACAN* locus to identify additional cis-regulatory elements capable of recapitulating, wholly or in part, the endogenous expression of *ACAN* in cartilage *in vivo*. Using moderate evolutionary sequence conservation as a guide to potential enhancer function, we tested 24 conserved sequences, from an interval of 220 kb containing *ACAN*. We assayed the sequences for their ability to direct reporter gene expression to cartilage in transgenic zebrafish. We uncovered a remarkable degree of overlap in regulatory control at the locus; in all, we identified eleven sequences independently capable of regulating expression in cartilage. Six of these are conserved at the chicken locus, in the same positions relative to the gene, indicating deep conservation of the

regulatory machinery. Five of the six chicken enhancers also display conserved function, directing marker gene expression to cartilage in transgenic zebrafish.

Our identification of multiple enhancers associated with *ACAN* is the first necessary step in the investigation of specific upstream genes and signaling pathways for their effect on gene transcription. The sequences are also candidates for non-coding polymorphisms or mutations associated with changes in expression levels and human disease.

2. Results

2.1 Non-coding sequence conservation at the *ACAN* locus

We took an unbiased approach to identify cis-regulatory sequences associated with *ACAN*, in addition to the proximal upstream enhancer that was previously described (Han and Lefebvre, 2008). We interrogated a 222 kb window of genomic sequence containing the human *ACAN* gene, extending to the genes on either side, using the PhastCons track on the UCSC genome browser. Through comparison of 28 vertebrate genomes, we identified 24 conserved non-coding sequences, representing the most conserved ~6% of the interval excluding coding sequence, for functional analysis (Table 1). These included sequences encompassing the 5'UTR (*-1ACAN*) and the 3'UTR (*+71ACAN*) of *ACAN* itself. The sequences are named according to their position in kilobases relative to the transcription start site of *ACAN*, and are broadly distributed across the interval, including seven from introns of the *ACAN* gene itself (Fig. 1).

2.2 Functional evaluation of potential enhancers

We cloned the 24 potential regulatory sequences into a reporter plasmid containing *egfp* coding sequence in conjunction with the heterologous *cFos* minimal promoter, based on the Tol2 transposable element (Hu et al., 2011). We injected each of these constructs, together with RNA encoding the Tol2 transposase, into zebrafish embryos at the 1-cell stage. Because of the highly efficient mosaic expression of Tol2 vectors, we could examine the injected embryos directly for EGFP expression in skeletal structures from 1-6 days post fertilization (dpf).

Thirteen of the tested sequences did not regulate specific expression consistent with endogenous *Acan* expression, and were not further analyzed. For the remaining eleven constructs, the injected fish were raised to adulthood, and EGFP expression in their progeny was examined in detail after germline transmission.

2.3 Multiple enhancers regulate broad expression in developing cartilage

Strikingly, we identified a large number of enhancers directing expression to cartilage. Eight of these regulate broad expression in craniofacial cartilages (Fig. 2; Fig. 3I, J). These vary in strength and exact timing of expression, with some active very early, at 2 dpf, while others are not detected until 3 dpf. Six were expressed robustly (Fig. 2), while two, *-45ACAN* and *-55ACAN*, showed significantly lower levels of expression (Fig. 3I, J).

Transgenics for *-11ACAN*, in addition to broad cartilage expression, showed strong expression in skeletal muscle, inconsistent with endogenous gene expression (Fig. 3A, B). This enhancer is homologous to the known regulatory element from the mouse gene, which does not display similar ectopic expression in transgenic mice (Han and Lefebvre, 2008). The sequence we initially analyzed was larger than the mouse enhancer. Therefore, we cloned the smaller equivalent human sequence for analysis. In transient transgenics with the shorter enhancer, we still observed prominent expression in skeletal muscle (data not

shown), indicating that the ectopic expression was not due to the inadvertent cloning of an inappropriate activating sequence outside the native enhancer.

The remaining two enhancers were active only in a subset of cartilage cells. The $-64ACAN$ enhancer was active at 2 dpf in the earliest forming cartilage, but by 3 dpf was active mosaically in only some cells (Fig. 3C-E). The expression was observed in multiple independent lines, so was not a result of mosaic inactivation of a particular transgene insertion. At 6 dpf, the expression was largely localized to medial cartilage, but was also still seen in scattered cells throughout the cartilage. The $-107ACAN$ enhancer was broadly active in cartilage at 2 and 3 dpf. However, by 6 dpf, EGFP was largely confined to cells in the midline ethmoid plate and the medial portions of the ceratobranchial cartilages (Fig. 3G), similar to the expression regulated by $-64ACAN$. In addition, EGFP was also seen in bone, including the membranous bones opercle and cleithrum (Fig. 3H), and in perichondral cells surrounding the craniofacial cartilages.

2.4 Regulation of early expression in neural crest and notochord

Our initial screen was for enhancer activity in cartilage, but many of the identified enhancers had additional activity before the formation of morphologically apparent cartilage. The craniofacial cartilages, which strongly express *Acan* later in development, are derived from cranial neural crest cells. Interestingly, one enhancer regulated early and broad expression in neural crest, including in the trunk, while several additional ones regulated expression in the branchial arches consistent with neural crest-derived mesenchyme (Fig. 4A-E). The notochord has many similarities with cartilage in gene expression and composition of extracellular matrix, including abundant aggrecan. Consistent with this, we found three enhancers that regulate expression of EGFP in the notochord at 1 dpf (Fig. 4F-H). Two enhancers that regulated robust expression in branchial arches at 1 dpf ($-55ACAN$ and $-45ACAN$) were weakly active in differentiated cartilage, suggesting that their primary activity is in early cartilage precursors.

2.5 Maintained enhancer activity in juveniles

By one month, perichondral ossification has replaced the bulk of most cartilage elements in the craniofacial skeleton with bone, surrounding the remaining cartilage core. There has also been substantial joint maturation and formation of articular cartilages. Both types of cartilage can be visualized with a universal cartilage reporter transgenic line $-1.4coll1a1:egfp$ (Fig. 5A). A gene trap into the *trps1* gene (Talbot et al., 2010), an early patterning gene for joints, expresses EGFP preferentially in joints (Fig. 5B). Several *ACAN* enhancers were broadly active in cartilage of the juvenile fish, and regulated expression patterns similar to $-1.4coll1a1:egfp$ (Fig. 5E, H), while $-108ACAN$ regulated expression more strongly in joints, in a pattern similar to *trps1* (Fig. D, G).

2.6 Conservation of regulatory architecture

The first exon of human *ACAN* is non-coding, and because of limited conservation is not annotated in many other species. Thus enhancers lying within the first intron in human are seemingly located upstream of the gene in many other species. Conversely, an additional upstream non-coding exon has been annotated for the mouse gene (Walcz et al., 1994), making the homologues of the $-11ACAN$ and $-34ACAN$ enhancers fall within the first intron. Interestingly, the $+30ACAN$ enhancer overlaps with an expressed sequence tag (EST) from a chondrosarcoma line, and the orthologous chicken sequence overlaps with two ESTs isolated from cartilage, suggesting that this conserved enhancer sequence is normally transcribed in cartilage.

The expression of *Acan* in cartilage is highly conserved among vertebrates, suggesting that the regulatory sequences might also be highly conserved. As expected, all eleven enhancers were highly conserved among placental mammals, but for a subset of these, the conservation extended to other vertebrates, including marsupials and birds. The six enhancers with clear homologues at the chicken locus are arranged in the same order and orientation relative to the coding sequence (Fig. 6A), demonstrating deep conservation of the regulatory architecture at the *Acan* locus.

2.7 Conserved function of chicken ACAN enhancers

To test the significance of the sequence conservation observed at six of the enhancers, we cloned the homologous chicken sequences (Table 2) into our enhancer analysis vector. In transient transgenic zebrafish, five displayed conserved transcriptional activity in cartilage (Fig. 6B-F). The enhancers homologous to *+4ACAN* and *-11ACAN* regulated strong and specific expression in craniofacial cartilage in many (~80% and 30%, respectively) of the injected embryos at 3 dpf (not shown), and the expression was maintained at 6 dpf (Fig. 6D, E). While the enhancer homologous to *-113ACAN* also regulated prominent cartilage expression at 3 dpf in ~30% of injected embryos (Fig. 6B), expression had been largely down-regulated by 6 dpf (not shown). The homologue of *+30ACAN* also regulated expression in cartilage, visible by 6 dpf (Fig. 6F). However, the expression was not as strong or widespread, and present in a smaller percentage of embryos. The homologue of *-107ACAN* regulated expression prominently in heart at 3 dpf (~65% of injected embryos), and also less strongly in the cartilage in a subset of embryos (Fig. 6C). By 6 dpf, expression was also evident in perichondral cells associated with cartilage (data not shown), similar to the expression regulated by the homologous human enhancer.

2.8 Conserved consensus sequences suggest complex regulation of Acan transcription

We hypothesized that important regulatory inputs into *Acan* transcription will be conserved, and therefore took advantage of multi-species alignments to define potential transcription factor binding sites (TFBSs). For the six most highly conserved enhancers, we aligned sequences from human, mouse, opossum, and chicken, and simultaneously searched for predicted TFBSs in aligned regions. One known upstream regulator of *ACAN* is the transcription factor Sox9. In addition to the previously described Sox9 binding site in the *-11ACAN* enhancer, we found predicted sites in the *-113ACAN* and *+30ACAN* enhancer sequences (Fig. 7A, E). Two of the remaining three sequences contained no conserved predicted binding sites for Sox9 or related factors, but instead showed evidence for regulation by a variety of other transcription factors (Fig. 7B, D). One highly conserved sequence, *-34ACAN*, has multiple regions of alignment across species, and also regulates broad cartilage expression in transgenic embryos, but we found no predicted TFBSs in aligned regions.

3. Discussion

3.1 Enhancers associated with human ACAN regulate cartilage expression in zebrafish

Enhancers are frequently not associated closely with their corresponding genes, and can be located over a megabase away either upstream or downstream. In order to comprehensively survey a large locus for enhancers, an efficient functional assay is critical. A small subset of enhancers are highly conserved, and these are likely to be associated with developmentally important genes with tissue-specific expression (Pennacchio et al., 2006; Visel et al., 2008; Woolfe et al., 2005). Not surprisingly, these highly conserved sequences are often equivalently functional across divergent species. However, for most enhancers, there is no readily detectable sequence conservation extending beyond mammals. Despite this apparent lack of sequence conservation, we and others have previously shown that enhancer function

can be deeply conserved, with mammalian enhancers regulating gene appropriate expression in transgenic zebrafish (Fisher et al., 2006a; Rada-Iglesias et al., 2011). Here we have exploited this convenient assay system to identify multiple enhancers associated with the *ACAN* locus. Importantly, the fact that these enhancers function across species strongly suggests that the important trans factors acting upon the enhancers are conserved, and also likely their binding sites.

Interestingly, we found little correlation between physical and functional characteristics among the tested sequences. For example, proximity to the gene, or to each other, did not correlate in any obvious way with activity. LOD score was also a poor predictor of enhancer activity; none of the positive enhancers had as high a score as the $-64ACAN$ sequence, which had no activity in our assay. $-64ACAN$ and similar sequences may have other important functions subjecting them to negative selection, that would not be apparent in an assay for positive transcriptional regulation. The negative results may also reflect limitations of our experimental system. We based our sequence selection for analysis on the most conserved core for each element, and could have failed to include important functional sequence in the less conserved flanks. A negative result could also reflect a real functional difference between human and zebrafish. The necessary activating signals for an individual enhancer may not all be present in zebrafish, or the human enhancer may simply have diverged sufficiently to no longer be recognized by zebrafish transcription factors. Therefore, a positive result in our assay is likely to represent deeply conserved enhancer function, while a negative result should be interpreted more cautiously.

Previously, one upstream enhancer had been identified for mouse *Acan*, and shown to bind several likely upstream regulators (Han and Lefebvre, 2008). We found the homologous human sequence to regulate, in addition to the expected cartilage expression, robust ectopic expression in skeletal muscle of transgenic zebrafish, not a normal site of endogenous *Acan* expression. Given the high homology between the human and mouse sequences, it is likely that this difference is due to the environment (mouse vs. zebrafish) rather than an intrinsic difference in the human sequence. The ectopic activity is consistent with the expression in zebrafish either of an activating trans factor in skeletal muscle that is not present in mouse at equivalent stages, or the absence in zebrafish of a negative regulator normally present in skeletal muscle of mouse. However, despite the ectopic expression, the enhancer also regulates appropriate cartilage expression.

We describe the identification of sequences independently capable of regulating expression in cartilage, consistent with *Acan* expression. Based on their proximity to the *ACAN* coding sequence, we presume that they are normally involved in its transcriptional regulation. One of the neighboring genes, *ISG20*, encodes a ribonuclease strongly induced in lymphatic cells by interferon (Gongora et al., 1997; Gongora et al., 2000). The other flanking gene, *HAPLN3*, is physically closely associated with *ACAN*, and it has been speculated that its ancestral gene contributed part of the coding sequence of the ancestral CSPG gene when it arose through domain shuffling (Kawashima et al., 2009). *Hapln3* is expressed broadly, including prominently in the cardiovascular system, but is not apparently expressed in cartilage (Ogawa et al., 2004). Therefore, it is unlikely that the enhancers we identified function in the transcriptional regulation of these flanking genes. A rigorous demonstration of their function in *ACAN* transcription will depend on mutating them in the context of the intact locus. Such experiments would also directly address the question of how functionally redundant the individual enhancers are.

3.2 Significance of multiply redundant gene regulation

Apparently redundant transcriptional regulation has been noted previously, in particular for several developmentally important genes in *Drosophila* (Hobert, 2010). It has been proposed

that one important benefit to the organism of such seemingly unnecessary redundancy is that it lends robustness to gene expression in stressful situations (Frankel et al., 2010; Perry et al., 2010). In the case of *Acan*, mutations in mouse and human demonstrate the importance not just for its expression, but for maintaining its expression level within narrow parameters. Its conserved expression in the cartilage of all extant vertebrates further supports its critical importance. Therefore, we speculate that the large number of independently regulated *Acan* enhancers has evolved as a mechanism to ensure consistent, high levels of gene expression throughout life.

3.3 Conservation of regulatory architecture and evolution of *Acan*

The *Acan* gene evolved by domain shuffling, and arose in the vertebrate lineage (Kawashima et al., 2009). Its appearance was likely a key event in the evolution of cellular cartilage in the vertebrate lineage. CSPG (as assayed by Alcian blue staining) is a prominent feature of cartilage in all extant vertebrates, and is presumed to correspond to *Acan* expression. Therefore, we speculated that the cis-regulatory mechanisms governing *Acan* might also be evolutionarily ancient. Through comparison of multiple vertebrate sequences, we identified conservation of six enhancers at the chicken *Acan* locus, in the same order relative to the gene. Several of these were also conserved at the locus in *Xenopus tropicalis* (data not shown).

In teleost fish with sequenced genomes, including zebrafish, there are two annotated *acan* orthologues. We directly compared the sequences upstream of the two zebrafish genes with other vertebrates. Using relaxed alignment parameters and an algorithm designed to detect alignments despite small scale rearrangements, we nonetheless only identified one possible homologous enhancer, a sequence aligned with *+30ACAN* at the *acanb* locus. It has been proposed that following the whole genome duplication in the teleost lineage, retained pairs of duplicates have evolved to have different expression patterns through differential retention of regulatory sequences (Cresko et al., 2003; Kleinjan et al., 2008; Kluver et al., 2005). However, if the *Acan* locus in the last common ancestor before the genome duplication had a similarly complex and redundant regulatory apparatus, we might predict that both teleost duplicates would still be expressed in cartilage, given the large number of independent enhancers regulating that expression. In zebrafish, *acana* on chromosome 7 is expressed prominently in cartilage (Kang et al., 2004), as is *acanb* on chromosome 25 (our unpubl. obs.), consistent with this prediction.

3.4 Potential upstream regulators of *Acan* expression

To define potential upstream regulators of the identified enhancers, we analyzed them with one of many available algorithms used to predict transcription factor binding sites. We imposed the additional restriction that the binding sites be conserved from mammals to avians. There will certainly be functional binding sites, less well conserved, that are not predicted by our analysis. In addition, alternative algorithms can utilize quite different starting data sets and criteria for matches to consensus sequences, frequently resulting in somewhat different sets of predicted binding sites. Overall, our analysis for potential TFBSs points to a number of possible upstream regulators of *ACAN* transcription in cartilage, and further suggests that enhancers mediating very similar expression patterns are subject to quite different regulation. However, as with any computational prediction of TFBSs, our analysis should serve as a guide for future functional validation.

Some of the enhancers we identified regulate broad and sustained expression in cartilage, although even these did not all regulate identical expression patterns. In addition, some of the sequences displayed strikingly different activity, for example regulating expression in only a small subset of cartilage cells, such as *-107ACAN* and *-64ACAN*. We would predict

that these differences in expression patterns are mediated by different upstream regulators for each enhancer, and in fact we found quite different patterns of predicted TFBSs among the enhancer sequences. Previous characterization of one enhancer, equivalent to the human *-11ACAN* sequence, showed that it is bound by Sox9 (Han and Lefebvre, 2008). Consistent with this, there is a predicted binding site for an SRY-related protein, conserved to chicken. We also find similar conserved sites in two other enhancers, *-113ACAN* and *+30ACAN*; both of these regulate broad and specific cartilage expression, although at 1 dpf *-113ACAN* is active in the notochord while *+30ACAN* is active in the branchial arches. A sequence overlapping with one of these, *+30ACAN*, was also tested for potential enhancer activity by Han and Lefebvre (2008). They detected no activity; however, the sequence they tested was significantly shorter (320 bp) than the one we analyzed, and did not contain the complete predicted Sox9 binding site.

There is evidence for several cartilage-expressed genes that Sox9 regulates their transcription through binding as a dimer, to an inverted repeat of two binding sites separate by 3 or 4 bases (Bernard et al., 2003; Bridgewater et al., 2003; Coustry et al., 2010; Genzer and Bridgewater, 2007; Jenkins et al., 2005; Sock et al., 2003). Our approach to TFBS prediction, requiring both a high match to the consensus sequence and a high degree of conservation, revealed an inverted repeat sequence meeting these criteria in *-11ACAN*. We also examined all enhancers using relaxed criteria to define individual Sox9 binding sites and looking manually for inverted repeats with the correct spacing. Through this approach, we identified two additional potential sites for Sox9 regulation, in the *-113ACAN* and *-107ACAN* enhancers (data not shown). The first of these was not conserved in chicken, and did not overlap with the Sox9 binding site predicted by our other approach. An important complement to these TFBS predictions will be experimental validation through direct demonstration of binding, especially in the case of Sox9, which may have additional binding requirements for cartilage genes.

The remaining highly conserved enhancers do not contain predicted SRY/Sox binding sites, and presumably these are regulated independent of Sox9. We do find predicted binding sites for other transcription factor families, whose members include potential regulators such as Hox proteins, Gli transcription factors, and Ets-related transcription factors. Importantly, each sequence functions independently to regulate almost identical expression in developing cartilage, despite the fact that they are likely regulated by quite different factors. We would argue that this system of regulatory control, with multiple enhancers having different input but equivalent output, insures a maintained high level of *Acan* expression in a variety of environmental conditions.

4. Experimental Procedures

4.1 Identification of conserved non-coding sequences

A region encompassing *ACAN* was analyzed, starting from the upstream gene *ISG20* and ending at the downstream gene *HAPLN3*, corresponding to genome coordinates chr15:86,993,001-87,225,000 on the NCBI 36/hg18 assembly from March, 2006. Conserved non-coding sequences (CNSs) were selected using the PhastCons algorithm (Siepel et al., 2005) as implemented on the UCSC genome browser, incorporating 28 vertebrate genome sequences.

4.2 Transgenesis and EGFP expression analyses

Twenty-four sequences were chosen for analysis, representing ~6% of the non-coding sequence in the interval. Primers for PCR amplification were designed using Primer 3 software, based on each conserved sequence plus 200 base pairs on either side. The genome

coordinates of the sequences cloned for analysis are given in Table 1. All CNSs were amplified from genomic DNA with high fidelity DNA polymerase, cloned into the Tol2 transposon based vector pGWcfosEGFP (Fisher et al., 2006a; Fisher et al., 2006b) which was altered with the addition of recombination sites for phiC31 as described (Hu et al., 2011), and analyzed for expression in transient transgenics.

Fish were cared for following standard protocols (Westerfield, 2007), and in accordance with animal care regulations at the University of Pennsylvania. Each construct was injected in at least two separate experiments, and mosaic GFP expression analyzed in a minimum of 200 embryos. Embryos were screened from 1 to 6 dpf using an Olympus MVX10 epifluorescence microscope. For those constructs regulating a consistent expression pattern, embryos were raised to adulthood and their progeny examined for expression after germline transmission. Unless otherwise noted, all constructs were examined in at least three independent transgenic lines, and among these each showed consistent expression.

4.3 Sequence analysis and alignments

Homologous sequences in other genomes were defined using the MultiZ alignment on the UCSC browser. In several cases for the chicken enhancer sequences, there was no apparent alignment with the human sequence as the base genome, but alignment was detected when another species (mouse or opossum) was used as the base. The genome coordinates of all sequences used for alignments and TFBS predictions are listed in Table S1.

For the six most conserved enhancers, the sequences from human, mouse, opossum, and chicken were aligned using DiAlign on the Genomatix Software Suite, and analyzed for potential TFBSs in the aligned regions by MatInspector using the program defaults, comparing with the vertebrate subsection from the latest matrix library (Release 8.3, Oct. 2010). We performed additional analyses with the entire upstream intervals (to the closest gene) from the above species, compared separately to the two zebrafish genes, with the global alignment program Shuffle-LAGAN implemented through the VISTA web server. For pairwise alignments other than human to mouse, the parameters were relaxed to 50% identity over 50 bases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aza-Carmona M, Shears DJ, Yuste-Checa P, Barca-Tierno V, Hisado-Oliva A, Belinchon A, Benito-Sanz S, Rodriguez JJ, Argente J, Campos-Barros A, Scambler PJ, Heath KE. SHOX interacts with the chondrogenic transcription factors SOX5 and SOX6 to activate the aggrecan enhancer. *Human molecular genetics*. 2011; 20:1547–1559. [PubMed: 21262861]
- Bernard P, Tang P, Liu S, Dewing P, Harley VR, Vilain E. Dimerization of SOX9 is required for chondrogenesis, but not for sex determination. *Human molecular genetics*. 2003; 12:1755–1765. [PubMed: 12837698]
- Bridgewater LC, Walker MD, Miller GC, Ellison TA, Holsinger LD, Potter JL, Jackson TL, Chen RK, Winkel VL, Zhang Z, McKinney S, de Crombrughe B. Adjacent DNA sequences modulate Sox9 transcriptional activation at paired Sox sites in three chondrocyte-specific enhancer elements. *Nucleic acids research*. 2003; 31:1541–1553. [PubMed: 12595563]

- Coustry F, Oh CD, Hattori T, Maity SN, de Crombrughe B, Yasuda H. The dimerization domain of SOX9 is required for transcription activation of a chondrocyte-specific chromatin DNA template. *Nucleic acids research*. 2010; 38:6018–6028. [PubMed: 20484372]
- Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A, Postlethwait JH. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. *Developmental dynamics: an official publication of the American Association of Anatomists*. 2003; 228:480–489. [PubMed: 14579386]
- Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS. Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science*. 2006a; 312:276–279. [PubMed: 16556802]
- Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS. Evaluating the biological relevance of putative enhancers in zebrafish using transposon-mediated transgenesis. *Nature Protocols*. 2006b; 1:1297–1305.
- Frankel N, Davis GK, Vargas D, Wang S, Payre F, Stern DL. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature*. 2010; 466:490–493. [PubMed: 20512118]
- Genzer MA, Bridgewater LC. A Col9a1 enhancer element activated by two interdependent SOX9 dimers. *Nucleic acids research*. 2007; 35:1178–1186. [PubMed: 17264118]
- Gleghorn L, Ramesar R, Beighton P, Wallis G. A mutation in the variable repeat region of the aggrecan gene (AGC1) causes a form of spondyloepiphyseal dysplasia associated with severe, premature osteoarthritis. *American journal of human genetics*. 2005; 77:484–490. [PubMed: 16080123]
- Gongora C, David G, Pintard L, Tissot C, Hua TD, Dejean A, Mechti N. Molecular cloning of a new interferon-induced PML nuclear body-associated protein. *The Journal of biological chemistry*. 1997; 272:19457–19463. [PubMed: 9235947]
- Gongora C, Degols G, Espert L, Hua TD, Mechti N. A unique ISRE, in the TATA-less human Isg20 promoter, confers IRF-1-mediated responsiveness to both interferon type I and type II. *Nucleic acids research*. 2000; 28:2333–2341. [PubMed: 10871365]
- Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. *Molecular and cellular biology*. 2008; 28:4999–5013. [PubMed: 18559420]
- Hobert O. Gene regulation: enhancers stepping out of the shadow. *Current biology: CB*. 2010; 20:R697–699. [PubMed: 20833307]
- Horton WE Jr, Lethbridge-Cejku M, Hochberg MC, Balakir R, Precht P, Plato CC, Tobin JD, Meek L, Doege K. An association between an aggrecan polymorphic allele and bilateral hand osteoarthritis in elderly white men: data from the Baltimore Longitudinal Study of Aging (BLSA). *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 1998; 6:245–251.
- Hu G, Goll MG, Fisher S. PhiC31 integrase mediates efficient cassette exchange in the zebrafish germline. *Developmental dynamics: an official publication of the American Association of Anatomists*. 2011; 240:2101–2107. [PubMed: 21805532]
- Jenkins E, Moss JB, Pace JM, Bridgewater LC. The new collagen gene COL27A1 contains SOX9-responsive enhancer elements. *Matrix biology: journal of the International Society for Matrix Biology*. 2005; 24:177–184. [PubMed: 15922909]
- Kamarainen OP, Solovieva S, Vehmas T, Luoma K, Leino-Arjas P, Riihimäki H, Ala-Kokko L, Mannikko M. Aggrecan core protein of a certain length is protective against hand osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2006; 14:1075–1080.
- Kang JS, Oohashi T, Kawakami Y, Bekku Y, Izpisua Belmonte JC, Ninomiya Y. Characterization of dermacan, a novel zebrafish lectican gene, expressed in dermal bones. *Mechanisms of development*. 2004; 121:301–312. [PubMed: 15003632]
- Kawashima T, Kawashima S, Tanaka C, Murai M, Yoneda M, Putnam NH, Rokhsar DS, Kanehisa M, Satoh N, Wada H. Domain shuffling and the evolution of vertebrates. *Genome research*. 2009; 19:1393–1403. [PubMed: 19443856]
- Kirk KM, Doege KJ, Hecht J, Bellamy N, Martin NG. Osteoarthritis of the hands, hips and knees in an Australian twin sample--evidence of association with the aggrecan VNTR polymorphism. *Twin*

- research: the official journal of the International Society for Twin Studies. 2003; 6:62–66. [PubMed: 12626230]
- Kleijnjan DA, Bancewicz RM, Gautier P, Dahm R, Schonhaler HB, Damante G, Seawright A, Hever AM, Yeyati PL, van Heyningen V, Coutinho P. Subfunctionalization of duplicated zebrafish pax6 genes by cis-regulatory divergence. *PLoS genetics*. 2008; 4:e29. [PubMed: 18282108]
- Kluver N, Kondo M, Herpin A, Mitani H, Scharl M. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. *Development genes and evolution*. 2005; 215:297–305. [PubMed: 15818483]
- Landauer W. Nanomelia, a Lethal Mutation of the Fowl. *The Journal of heredity*. 1965; 56:131–138. [PubMed: 14328419]
- Li H, Schwartz NB, Vertel BM. cDNA cloning of chick cartilage chondroitin sulfate (aggrecan) core protein and identification of a stop codon in the aggrecan gene associated with the chondrodystrophy, nanomelia. *The Journal of biological chemistry*. 1993; 268:23504–23511. [PubMed: 8226878]
- Li J, Kim KS, Park JS, Elmer WA, Hutton WC, Yoon ST. BMP-2 and CDMP-2: stimulation of chondrocyte production of proteoglycan. *Journal of orthopaedic science: official journal of the Japanese Orthopaedic Association*. 2003; 8:829–835. [PubMed: 14648273]
- Mashayekhi F, Shafiee G, Kazemi M, Dolati P. Lumbar disk degeneration disease and aggrecan gene polymorphism in northern Iran. *Biochemical genetics*. 2010; 48:684–689. [PubMed: 20496110]
- Ogawa H, Oohashi T, Sata M, Bekku Y, Hirohata S, Nakamura K, Yonezawa T, Kusachi S, Shiratori Y, Ninomiya Y. Lp3/Hapln3, a novel link protein that co-localizes with versican and is coordinately up-regulated by platelet-derived growth factor in arterial smooth muscle cells. *Matrix biology: journal of the International Society for Matrix Biology*. 2004; 23:287–298. [PubMed: 15464361]
- Pei M, He F, Wei L, Rawson A. Melatonin enhances cartilage matrix synthesis by porcine articular chondrocytes. *Journal of pineal research*. 2009; 46:181–187. [PubMed: 19054299]
- Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, Shoukry M, Minovitsky S, Dubchak I, Holt A, Lewis KD, Plajzer-Frick I, Akiyama J, De Val S, Afzal V, Black BL, Couronne O, Eisen MB, Visel A, Rubin EM. In vivo enhancer analysis of human conserved non-coding sequences. *Nature*. 2006; 444:499–502. [PubMed: 17086198]
- Perry MW, Boettiger AN, Bothma JP, Levine M. Shadow enhancers foster robustness of *Drosophila* gastrulation. *Current biology: CB*. 2010; 20:1562–1567. [PubMed: 20797865]
- Pfander D, Cramer T, Schipani E, Johnson RS. HIF-1 α controls extracellular matrix synthesis by epiphyseal chondrocytes. *Journal of cell science*. 2003; 116:1819–1826. [PubMed: 12665562]
- Primorac D, Stover ML, Clark SH, Rowe DW. Molecular basis of nanomelia, a heritable chondrodystrophy of chicken. *Matrix biology: journal of the International Society for Matrix Biology*. 1994; 14:297–305. [PubMed: 7827752]
- Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature*. 2011; 470:279–283. [PubMed: 21160473]
- Rittenhouse E, Dunn LC, Cookingham J, Calo C, Spiegelman M, Dooher GB, Bennett D. Cartilage matrix deficiency (cmd): a new autosomal recessive lethal mutation in the mouse. *Journal of embryology and experimental morphology*. 1978; 43:71–84. [PubMed: 632744]
- Sen M, Cheng YH, Goldring MB, Lotz MK, Carson DA. WISP3-dependent regulation of type II collagen and aggrecan production in chondrocytes. *Arthritis and rheumatism*. 2004; 50:488–497. [PubMed: 14872491]
- Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, Clawson H, Spieth J, Hillier LW, Richards S, Weinstock GM, Wilson RK, Gibbs RA, Kent WJ, Miller W, Haussler D. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome research*. 2005; 15:1034–1050. [PubMed: 16024819]
- Sock E, Pagon RA, Keymolen K, Lissens W, Wegner M, Scherer G. Loss of DNA-dependent dimerization of the transcription factor SOX9 as a cause for campomelic dysplasia. *Human molecular genetics*. 2003; 12:1439–1447. [PubMed: 12783851]

- Talbot JC, Johnson SL, Kimmel CB. *hand2* and *Dlx* genes specify dorsal, intermediate and ventral domains within zebrafish pharyngeal arches. *Development*. 2010; 137:2507–2517. [PubMed: 20573696]
- Tim Yoon S, Su Kim K, Li J, Soo Park J, Akamaru T, Elmer WA, Hutton WC. The effect of bone morphogenetic protein-2 on rat intervertebral disc cells in vitro. *Spine*. 2003; 28:1773–1780. [PubMed: 12923462]
- Tompson SW, Merriman B, Funari VA, Fresquet M, Lachman RS, Rimoin DL, Nelson SF, Briggs MD, Cohn DH, Krakow D. A recessive skeletal dysplasia, SEMD aggrecan type, results from a missense mutation affecting the C-type lectin domain of aggrecan. *American journal of human genetics*. 2009; 84:72–79. [PubMed: 19110214]
- Tsai AD, Yeh LC, Lee JC. Effects of osteogenic protein-1 (OP-1, BMP-7) on gene expression in cultured medial collateral ligament cells. *Journal of cellular biochemistry*. 2003; 90:777–791. [PubMed: 14587033]
- Visel A, Prabhakar S, Akiyama JA, Shoukry M, Lewis KD, Holt A, Plajzer-Frick I, Afzal V, Rubin EM, Pennacchio LA. Ultraconservation identifies a small subset of extremely constrained developmental enhancers. *Nature genetics*. 2008; 40:158–160. [PubMed: 18176564]
- Walcz E, Deak F, Erhardt P, Coulter SN, Fulop C, Horvath P, Doege KJ, Glant TT. Complete coding sequence, deduced primary structure, chromosomal localization, and structural analysis of murine aggrecan. *Genomics*. 1994; 22:364–371. [PubMed: 7806222]
- Watanabe H, Kimata K, Line S, Strong D, Gao LY, Kozak CA, Yamada Y. Mouse cartilage matrix deficiency (*cmd*) caused by a 7 bp deletion in the aggrecan gene. *Nature genetics*. 1994; 7:154–157. [PubMed: 7920633]
- Watanabe H, Nakata K, Kimata K, Nakanishi I, Yamada Y. Dwarfism and age-associated spinal degeneration of heterozygote *cmd* mice defective in aggrecan. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:6943–6947. [PubMed: 9192671]
- Westerfield, M. *The Zebrafish Book*. 5 ed. University of Oregon Press; Eugene, OR: 2007.
- Woolfe A, Goodson M, Goode DK, Snell P, McEwen GK, Vavouri T, Smith SF, North P, Callaway H, Kelly K, Walter K, Abnizova I, Gilks W, Edwards YJ, Cooke JE, Elgar G. Highly conserved non-coding sequences are associated with vertebrate development. *PLoS biology*. 2005; 3:e7. [PubMed: 15630479]

Figure 1. Conserved non-coding sequences at *ACAN* locus

A) The UCSC genome browser of the interval containing the human *ACAN* gene is shown, extending from the upstream *ISG20* gene to the *HAPLN3* gene downstream. Above the gene track are depicted the conserved non-coding sequences identified by the PhastCons algorithm (at bottom) as the most highly conserved based on a comparison of 28 vertebrate sequences. In green directly above the gene are the sequences that act as positive enhancers in cartilage, while the negative sequences are at the top in red.

Figure 2. Multiple *ACAN* enhancers regulate broad cartilage expression in transgenic larvae
 For reference, the expression of the cartilage marker transgene *-1.4colla1:egfp* is shown (A), together with a corresponding diagram labeled with cartilage elements of the ventral craniofacial skeleton (B). C-T) For the established *ACAN* transgenic lines, each row is labeled with the enhancer, and each column with the stage (2, 3, or 6 dpf). Several enhancers are already active in the ceratohyal cartilages at 2 dpf (arrows in C, I, L and O), while all are broadly expressed in cartilage at 3 and 6 dpf. Scale bar (in T) represents 200 μm. Abbreviations: bb, basibranchials; bh, basihyal; cb, ceratobranchials; ch, ceratohyal; ep, ethmoid plate; hs, hyosymplectic; mc, Meckel's cartilage; pq, palatoquadrate.

Figure 3. *ACAN* enhancers with unique activities

A, B) The *-11ACAN* enhancer shows prominent ectopic activity in skeletal muscle (bracket in A, arrows in B), as well as gene-appropriate activity in cartilage (arrowhead in B). Two enhancers at *-64* and *-107* showed most prominent activity in a subset of cartilage, close to the midline (C, D, F, G); *-64ACAN* also regulated broader cartilage expression, but mosaically, seen at higher power in the hyosymplectic cartilage (E). In contrast, *-107ACAN* was also active in bone, seen in the cleithrum and opercle at 4 dpf (H). *-55ACAN* is active throughout the cartilage (I), although it regulates expression at a lower level and with higher background than most of the identified enhancers. Similarly, *-45ACAN* is expressed, although not strongly, in cartilage, and expression is also evident in perichondral cells adjacent to the cartilage (J). Scale bar (in J) represents 200 μ m. Abbreviations: cl, cleithrum; op, opercle.

Figure 4. *Acan* enhancers regulate early tissue-specific expression

GFP expression was examined in embryos of established transgenic lines at 1 day post fertilization (dpf), as indicated in the panels. A, B) One upstream enhancer regulates early expression in neural crest cells of the head (A) and trunk (B), and in the brain and spinal cord (A, B). Three additional enhancers (C-E) regulate expression in craniofacial mesenchyme of the branchial arches, also consistent with neural crest expression. F-H) Three enhancers show activity in the notochord, a known site of prominent *Acan* expression. Scale bars in A (for A, C-E) and in B (for B, F-H) represent 200 μ M.

Figure 5. ACAN enhancers mediate sustained gene expression in juvenile fish

For comparison, the expression of the general cartilage marker *-1.4colla1:egfp* (A) and the *trps1* gene trap, which is preferentially expressed in joints (B) are shown in juvenile fish at 1 month. The *ACAN* lines with appreciable GFP expression at a similar stage are shown, as indicated in the panels. Note that some transgenes are expressed broadly in cartilage (C, E, G, H), while *-108ACAN* appears stronger in joints (D), more similar to *trps1*. I)

-107ACAN shows little expression in cartilage, but instead is expressed in areas of bone growth, as seen at the edge of the opercle and in the pectoral fin rays. Abbreviations: fr, fin rays; op, opercle.

Figure 6. Deep conservation of *ACAN* regulatory architecture

A) The UCSC genome browser window of the chicken *ACAN* locus (chr10:14,726,001-14,822,000), showing the location of enhancer elements conserved with mammals (top track in green). For clarity, the elements are named according to the homologous human enhancers, not according to their location relative to the chicken gene. B-F) In transient transgenics, the chicken homologues for five of the human enhancers also regulate EGFP expression in cartilage. Expression is shown at 6 dpf, except for *-113ACAN*, which is shown at 3 dpf.

Figure 7. ACAN enhancers with highly conserved predicted transcription factor binding sites
 A portion of each identified human enhancer sequence is shown aligned with its homologues from mouse, opossum, and chicken; identical bases are indicated with dots below. For $+30ACAN$, the chicken sequence had an internal gap, so zebrafish was used for the alignment instead; see supplemental information for exact intervals used for alignments. For each alignment, only the portion containing high likelihood predicted TFBSs is shown; note also that for two sequences (B, E), internal portions with poor alignment are not shown, indicated by dashed lines at the left. Two overlapping predicted sites are indicated with different shades of grey; for more than two, the entire region is shaded and the predictions indicated above. Abbreviations of binding site matrix families: SORY, Sox/SRY and related HMG box factors; GLIF, GLI zinc finger family; HNF1, Hepatic Nuclear Factor 1; NFAT, Nuclear factor of activated T-cells; FKHD, Fork head domain factors; PAX6, PAX-4/PAX-6 paired domain binding sites; ETSF, Ets1 factors; ABDB, Abdominal-B type homeodomain transcription factors; MEF2, myocyte-specific enhancer binding factor; GATA, GATA binding factors; MOKF, Mouse Krüppel like factor; MYT1, MYT1 C2HC zinc finger protein; MYBL, Myb-like transcriptional regulators.

Table 1

The conserved sequences at the human *ACAN* locus that were analyzed for enhancer function are listed above. The designation for each sequence incorporates the distance in kilobases relative to the annotated transcription start site; negative numbers are upstream, positive downstream. The shaded sequences are those with positive enhancer activity. The coordinates are from the NCBI 36/hg18 assembly from March, 2006; the LOD score (on a scale of 0 to 1000) is that assigned by the PhastCons track, incorporating the 28 vertebrate genome sequences available at the time of the analysis.

Element	Coordinates	LOD	Expression pattern
-144ACAN	87003404-87004562	52	
-122ACAN	87025546-87026605	84	
-113ACAN	87034223-87035213	29	Early in notochord; strong in larval cartilage; sustained in juvenile cartilage and joints
-108ACAN	87039068-87039792	81	Strong in larval cartilage; sustained in juvenile joints
-107ACAN	87040091-87041225	57	Early in neural crest; in medial cartilage elements, perichondral cells and bone; sustained in juvenile bone
-102ACAN	87045053-87045562	65	
-95ACAN	87052291-87053315	101	
-94ACAN	87053785-87054831	66	
-87ACAN	87060803-87062902	72	
-78ACAN	87069667-87070274	33	Early in notochord; strong in larval cartilage; sustained in juvenile cartilage and joints
-64ACAN	87083110-87084133	58	Mosaically in larval cartilage, more prominently in medial elements; sustained in medial cartilages in juvenile
-55ACAN	87092105-87092887	100	Early in branchial arches; weak in larval cartilage
-45ACAN	87104195-87104855	31	Early in branchial arches; weak in larval cartilage
-41ACAN	87106809-87107440	30	
-34ACAN	87113459-87115112	256	Early in notochord; strong in larval cartilage; sustained in juvenile cartilage and joints
-11ACAN	87136508-87137575	121	Ectopic in skeletal muscle; moderate in larval cartilage
-1ACAN	87146979-87148410	92	
+4ACAN	87151581-87152285	99	Strong in larval cartilage
+20ACAN	87167180-87167884	88	
+30ACAN	87177574-87178484	223	Early in branchial arches; strong in larval cartilage; sustained in juvenile cartilage and joints
+34ACAN	87181726-87182184	26	
+63ACAN	87210355-87210922	130	
+64ACAN	87211950-87213336	825	
+71ACAN	87218352-87219650	256	

Table 2

The conserved sequences at the chicken *ACAN* locus that were analyzed for enhancer function are listed above. For simplicity, sequences are designated by the homologous human enhancers rather than their locations relative to the chicken gene. The coordinates are from the WUGSC 2.1/galGal3 assembly from May, 2006.

Element	Genome coordinates
-113 <i>ACAN</i> homologue	chr10:14,810,570-14,811,486
-107 <i>ACAN</i> homologue	chr10:14,807,292-14,808,281
-34 <i>ACAN</i> homologue	chr10:14,786,954-14,787,830
-11 <i>ACAN</i> homologue	chr10:14,783,328-14,784,182
+4 <i>ACAN</i> homologue	chr10:14,775,895-14,776,497
+30 <i>ACAN</i> homologue	chr10:14,764,335-14,765,952