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Transcriptional control of chondrocyte specification and differentiation

Chia-Feng Liu[1,*], **William E. Samsa**[2], **Guang Zhou**[2,3,4], and **Véronique Lefebvre**[1,*]

[1]Department of Cellular and Molecular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH, 44195, USA

[2]Department of Orthopaedics, Case Western Reserve University, Cleveland, OH, USA

[3]Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, OH, USA

[4]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Abstract

A milestone in the evolutionary emergence of vertebrates was the invention of cartilage, a tissue that has key roles in modeling, protecting and complementing the bony skeleton. Cartilage is elaborated and maintained by chondrocytes. These cells derive from multipotent skeletal progenitors and they perform highly specialized functions as they proceed through sequential lineage commitment and differentiation steps. They form cartilage primordia, the primary skeleton of the embryo. They then transform these primordia either into cartilage growth plates, temporary drivers of skeletal elongation and endochondral ossification, or into permanent tissues, namely articular cartilage. Chondrocyte fate decisions and differentiated activities are controlled by numerous extrinsic and intrinsic cues, and they are implemented at the gene expression level by transcription factors. The latter are the focus of this review. Meritorious efforts from many research groups have led over the last two decades to the identification of dozens of key chondrogenic transcription factors. These regulators belong to all types of transcription factor families. Some have master roles at one or several differentiation steps. They include SOX9 and RUNX2/3. Others decisively assist or antagonize the activities of these masters. They include TWIST1, SOX5/6, and MEF2C/D. Many more have tissue-patterning roles and regulate cell survival, proliferation and the pace of cell differentiation. They include, but are not limited to, homeodomain-containing proteins and growth factor signaling mediators. We here review current knowledge of all these factors, one superclass, class, and family at a time. We then compile all knowledge into transcriptional networks. We also identify remaining gaps in knowledge and directions for future research to fill these gaps and thereby provide novel insights into cartilage disease mechanisms and treatment options.

*Corresponding authors: Chia-Feng Liu (liuc2@ccf.org), Véronique Lefebvre (lefebvv@ccf.org), Department of Cellular & Molecular Medicine, Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, NC-10, Cleveland, OH, 44195, USA.

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Keywords

Cartilage; cell lineage; differentiation; master regulator; specification; transcription

1. Introduction

Chondrogenesis is a critical process in the development and healthy maintenance of the vertebrate skeleton [1]. It consists in generating chondrocytes and in directing these cells through successive steps of commitment and differentiation in order to build, renew or remodel cartilage tissues in a tightly controlled spatial and temporal manner. Deciphering the cellular and molecular mechanisms that drive and regulate chondrogenesis is a prerequisite to decoding the causes of the various types of cartilage diseases that affect humans and to developing highly needed treatments for these diseases. Among these diseases, chondrodysplasias are fairly rare, but exist in hundreds of forms [2]. They vary from mild malformations of discrete skeletal elements to generalized skeletal malformations that can be lethal in the perinatal period or result in dwarfism. Osteoarthritis, in contrast, is a highly prevalent, adult-onset disease, primarily characterized by progressive, irreversible degeneration of articular cartilage [3].

Chondrocytes arise in development from multipotent skeletogenic progenitor/stem cells (SSCs), which themselves derive from embryonic cranial neural crest and from paraxial and lateral plate mesoderm. SSCs give rise to two main types of chondrocytes. They become growth plate chondrocytes (GPCs) by proceeding through an intermediate, bipotent osteochondroprogenitor (OCPs) stage (with osteoblast [OB] and chondrocyte potential), and become articular chondrocytes (ACCs) via a multipotent joint progenitor (JP) stage (with chondrocyte, synovial and other joint cell type potential). Once committed to either fate, prechondrocytes (PCs) coalesce into precartilaginous condensations. They next differentiate into early chondrocytes (ECs) and build cartilage primordia by proliferating and producing copious amounts of cartilage extracellular matrix (namely expressing the genes for collagen type II [*COL2A1*] and aggrecan [*ACAN*]). GPCs then follow multiple steps of maturation. They first continue to dynamically proliferate and produce cartilage matrix while aligning themselves in longitudinal columns. These activities allow fast and linear growth of skeletal elements and provide an orderly arranged extracellular matrix template upon which trabecular bone will later be deposited. The so-called columnar chondrocytes (CCs) undergo cell cycle arrest as they transition to prehypertrophy. While still expressing early cartilage matrix genes at maximal levels, prehypertrophic chondrocytes (PHCs) also express stage-specific markers (e.g., *IHH,* Indian hedgehog gene) and initiate expression of hypertrophic markers (e.g., *COL10A1,* collagen type 10 gene). They have a central regulatory role in skeleton development. They emit signals, namely IHH and non-canonical WNTs (including WNT5A), that stimulate CC proliferation and alignment, respectively. IHH also regulates the pace of GPC maturation and induces cortical bone formation in adjacent perichondrium. Next, hypertrophic chondrocytes (HCs) massively expand their cytoplasmic volume and fully switch to a new genetic program. They are as important as CCs to elongate skeletal elements. In addition, they contribute to endochondral ossification (EO) by modifying the cartilage matrix and emitting such signals as the vascular endothelial growth factor VEGFA.

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At the end of the GPC journey, terminal chondrocytes (TCs) express an osteoblast-like phenotype, remodeling and mineralizing the cartilage matrix (e.g., expressing the genes for the bone sialoprotein [*IBSP*] and matrix metalloproteinase 13 [*MMP13*]). They eventually die or fully adopt the OB fate in nascent endochondral bone. Compared to the highly dynamic and transient GPC program, the ACC pathway appears as a conservative one. ACCs proliferate and productively assemble extracellular matrix as they develop their tissue. Once articular cartilage (AC) acquires its adult size, ACCs hardly ever proliferate and they limit their activity to turning over a subset of extracellular matrix components, including aggrecan, but not collagens. Their tissue features layers that are reminiscent of growth plate (GP) zones in terms of differential extracellular matrix composition and cell morphology, but it remains unclear how ACCs form these layers. One school of thought is that the tissue grows in an appositional manner, chondrocytes differentiating from JPs located near the tissue surface and arresting at progressively more mature stages as they reach the subchondral bone [1]. Another view is that JPs are initially distributed uniformly across the tissue and that ACCs directly acquire spatial specialization as they differentiate [4]. It is also unclear when and how distinctions are made between the ACC and GPC programs. As indicated earlier, SSCs appear to adopt either fate as they develop into OCPs or JPs, even though articular and growth plate ECs share many phenotypic features. ACCs are distinguished from GPCs notably by not proceeding to prehypertrophy. As a result, they are unable to elongate their tissue in a fast and considerable manner and to induce its EO. Even if the ACC and GPC programs are distinct, they are not locked. ACCs can indeed switch to the GPC fate in osteoarthritis and mature GPCs can switch to the OB fate. Chondrocyte fate and differentiation decisions are governed and influenced by many types of regulatory factors. We here focus on current knowledge and gaps in knowledge of chondrogenic transcription factors (TFs).

Chondrocytes and all other cell types co-express at any single time thousands of ubiquitous genes and hundreds of lineage-specific and differentiation stage-specific genes. An important and fascinating research enterprise is to decipher the molecular basis of the transcriptional programs that govern the specific identity and activities of each cell type. A pioneer discovery was made 30 years ago when forced expression of the transcription factor MYOD was shown to be sufficient to engage mesenchymal cells into myoblast differentiation [5]. This finding sparked the hypothesis that each cell type is ruled by a master TF, i.e., a TF necessary and sufficient for cell type specification and differentiation. Within a decade, SOX9 was cloned and postulated to be the EC master [6, 7] and RUNX2/3 the GPC masters [8–10]. It then progressively became clear that no cell type is governed by a single master TF and that, instead, cell types might be determined by cores of master TFs. These cores likely contain pioneer and classical TFs. Pioneers bind to condensed chromatin and recruit histone- and DNA-modifying enzymes that write or erase epigenetic modifications. [11]. These modifications are necessary to poise, activate or repress enhancers and promoters, and can have immediate as well as long-lasting consequences on cell lineage and subtype specification, differentiation and other activities. Once chromatin is open, classical TFs bind to enhancers and promoters, and effect gene activation or repression. SOX2, OCT3/4, KLF4 and c-MYC were discovered 10 years ago by the Yamanaka group to be necessary and sufficient to specify and maintain embryonic stem cells

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and to induce pluripotent stem cells from differentiated cell types [12]. While forming a master core, these four TFs nevertheless help themselves with many other factors. As discussed in this review, SOX9 and RUNX2/3 also rely on many functional partners to achieve and fine-tune their chondrogenic master functions.

An important task in deciphering whether TFs directly impact gene expression is to delineate the DNA sequences (cis-acting elements) through which genes are activated and repressed. Sequences closely associated with transcription start sites (within ~50bp) mediate the assembly of the basal transcriptional machinery, i.e., a complex of ubiquitous factors that recruit RNA polymerase. Proximal enhancers (typically within ~1kb of transcription start sites) bind ubiquitous and/or cell type-specific TFs capable of interacting with the basal transcription machinery to activate or repress transcription. Remote enhancers have long been known to exist, but they were initially thought to be rare. The recent advent of whole-genome approaches, including chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), has revealed that they are actually very abundant and have primary roles in cell type-specific transcriptional regulation. It was first shown that the Yamanaka factors specify pluripotent stem cell identity and activities by binding primarily, not to promoters, but to enhancers located up to several hundreds of kb around promoters [13]. These remote enhancers are often found in clusters, and are therefore named super-enhancers. Since then, many cell types have been shown to feature master factor-bound super-enhancers. Namely, non-hypertrophic GPCs possess several hundreds of super-enhancers associated with cartilage-specific genes and bound by SOX9 [14, 15]. These super-enhancers feature DNA motifs for multiple TF types in addition to SOX9 binding sites, and thus support the notion that SOX9 does not work alone.

To date, the prevailing model is thus that chondrocytes have their specific activities governed at the genome level by dynamic cores of master factors binding to super-enhancers and assisted by many modulatory factors binding at the level of super-enhancers, proximal enhancers and promoter regions. The human genome encodes more than 3000 TFs, categorized into superclasses, classes and families, according to the features of their DNA-binding and other functional domains [16]. We review current knowledge of chondrogenic TFs based on this classification (Table I). We end with an integrative view of their interactions during chondrocyte differentiation and with suggestions for future research.

2. Beta-scaffold transcription factors with minor groove contacts

We start with a superclass of TFs that feature a β-scaffold in their DNA-binding domain and that, unlike all other types of TFs, contact DNA primarily in the minor groove. This class happens to contain all currently known chondrogenic master TFs.

2.1. High-mobility-group (HMG)-domain factors

A major class within this first TF superclass is comprised of proteins with a characteristic, high-mobility-group (HMG)-type DNA-binding domain. This domain is comprised of 3 α-helices that fold into an L-shape and that strongly bend the DNA helix upon intercalating into its minor groove. The HMG-domain class includes the SOX and TCF/LEF families.

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2.1.1 SOX transcription factors—SOX proteins are critically involved in cell type specification and differentiation in many lineages [17]. They bind DNA to motifs matching or resembling the C[A/T]TTG[A/T][A/T] sequence, and typically increase their otherwise low DNA-binding affinity and specificity through homodimerization or cooperation with specific partners.

SOX9: A first milestone in the search for chondrogenic TFs was the discovery that heterozygous mutations within and around *SOX9* cause campomelic dysplasia, a severe cartilage malformation syndrome [18, 19]. Since then, many studies have revealed that SOX9 has major functions in chondrogenesis. *SOX9* is expressed in this process from as early as the SSC stage, but its importance at this stage remains unknown. No defects have indeed been reported in *Sox9*-null mouse limb buds (*Sox9*$^{fl/fl}$*Prx1Cre*) until SSCs are due to differentiate into PCs [20]. The SOX9 protein may thus be kept inactive in SSCs. In mouse chimeric embryos derived from a mixture of *Sox9*$^{+/+}$ and *Sox9*$^{-/-}$ pluripotent stem cells, *Sox9*$^{+/+}$ SSCs formed precartilaginous condensations, whereas *Sox9*$^{-/-}$ SSCs either died or were displaced to the outskirts of condensations [20, 21]. The target genes of SOX9 in PCs remain unknown. In contrast, SOX9 is well proven to govern cartilage primordia and GP formation by transactivating cartilage-specific genes. ChIP-seq assays have recently widened the known spectrum of SOX9 targets in non-hypertrophic GPCs, to virtually all genes for cartilage extracellular matrix components and to many genes for cartilage-specific regulatory factors [14, 15]. SOX9 binds DNA by forming homodimers on SOX motifs oriented in opposite direction and separated by 3–4 nucleotides. It binds primarily to enhancers and associates occasionally with repressed regions and promoters. SOX9 was once thought to prevent GPC maturation. This notion was mainly based on the fact that *SOX9* expression is turned off at the PHC to HC transition. Recent studies have confirmed that SOX9 is necessary to prevent premature growth arrest of CCs and activation of PHC markers, but have also revealed that the SOX9 protein remains present through the early HC stage and is necessary to allow HCs to undergo overt differentiation before dying or converting into OBs [22, 23]. SOX9 was proposed to repress *Col10a1* in CCs by binding as a monomer in association with GLI2/3 to an upstream enhancer [24]. It was also shown to be necessary in PHCs/HCs for *Col10a1* expression and was proposed to directly activate the gene by cooperating as a homodimer with MEF2C on a proximal enhancer [23]. Besides being essential in cartilage primordia and GPs, SOX9 is also necessary to maintain a high level of aggrecan in adult AC [25]. Together, these studies and more have provided strong evidence that SOX9 is a master classical TF in PCs, ECs, GPCs and ACCs, and that it might have distinct actions at different stages of chondrocyte differentiation. The possibility that it also acts as a pioneer factor remains untested.

SOX5 and SOX6: *Sox5* and *Sox6* are co-expressed with *Sox9* in chondrocytes. Belonging to the SOXD group, SOX5 and SOX6 are structurally and functionally very similar, but share with SOX9 (a SOXE protein) only 50% identity in the HMG domain and, unlike SOX9, do not feature a transactivation domain. Their molecular actions thus differ from those of SOX9. *Sox5* and *Sox6* are largely redundant in chondrogenesis [26, 27]. Whereas single knockout mice are born with mild defects, *Sox5/6* double-null fetuses die in utero with severe chondrodysplasia. They weakly express cartilage-specific genes, despite normal

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expression of *Sox9*, and hence fail to overtly develop cartilage primordia, GPs and AC. Molecular assays have demonstrated that SOX5/6 homo/heterodimers bind in close proximity of SOX9 on cartilage-specific super-enhancers. They preferentially bind to pairs of SOX motifs oriented in the same direction and potentiate transactivation by strengthening SOX9 binding to target sites [14]. Forced expression of SOX5/6 and SOX9 is sufficient to convert SSCs into chondrocytes [28]. The three SOX proteins are therefore often referred to as the master chondrogenic SOX trio. *SOX5* and *SOX6* mutations have not been linked to major skeletal diseases in humans, but heterozygous deletions affecting *SOX5* cause intellectual disability, short stature and mild dysmorphism [29]. The latter traits advocate that optimal chondrogenesis requires full expression of *SOX5* in humans.

Other SOX proteins: SOX8, a SOXE protein closely related to SOX9, is expressed in SSCs and human adult ACCs [30, 31]. *Sox8*-null mice have osteopenia due to premature OB differentiation, but were not found to have cartilage malformations [32, 33]. This leaves open the question of a role for SOX8 in chondrocytes. SOX4, SOX11, and SOX12 (SOXC proteins) are closely related to one another, but not to SOX8/9 and SOX5/6. They are expressed strongly in SSCs and PCs, and weakly in chondrocytes [34]. They act redundantly to keep SSCs alive and they critically help delineate cartilage primordia by securing the non-chondrocytic fate of perichondrium cells and JPs [34, 35]. They are also pivotal to initiate GP formation through actions both in perichondrium cells and in GPCs [36]. Thus, SOX4/11/12 have cell- and non-cell-autonomous roles in the chondrocyte lineage, complementing and even opposing those of the SOX5/6/9 trio.

2.1.2. TCF/LEF proteins—The family of T-cell factor/lymphoid enhancer factors comprises four members: TCF7 (formerly called TCF1), TCF7L1 (TCF3), TCF7L2 (TCF4) and LEF1 [37]. In the absence of canonical WNT signals, these HMG-type TFs repress gene expression. Upon canonical WNT signaling, the transcriptional co-activator β-catenin translocates to the nucleus, binds to TCF/LEF factors, and converts them into transcriptional activators. TCF/LEF factors bind to CTTTG[A/T][A/T] DNA motifs, which match or resemble those of SOX proteins. TCF7L2 is specifically expressed in HCs, in which it may act together with RUNX2 [38, 39]. TCF7L2 was also shown to be expressed in primary osteoarthritic ACCs and to potentiate the pro-catabolic and apoptotic actions of NFκB [40]. These findings match the ability of canonical WNT signaling to promote GP maturation and AC degeneration [41].

2.2. RUNX transcription factors

RUNX proteins (also called core-binding factors, CBFs) have crucial roles in chondrogenesis and other processes [42]. They are composed of a α-subunit, encoded in humans by *RUNX1/CBFA2*, *RUNX2/CBFA1*, and *RUNX3/CBFA3*, and a β-subunit, encoded by *CBFB.* The α-subunit binds DNA to [A/G]ACC[A/G]CA motifs through its RUNT domain, and its affinity for DNA is increased by the β-subunit. Heterozygous mutations causing *RUNX2* haploinsufficiency cause cleidocranial dysplasia (CCD), a bone disease largely explained by master roles of RUNX2 in OBs [43].

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RUNX2 and RUNX3—Another milestone in the search for chondrogenic master TFs was the discovery that *Runx2*-null mice die at birth lacking PHCs/HCs in several locations, besides also lacking all intramembranous and endochondral bones [8, 44]. Adding support to the notion of a master role for RUNX2 in GPCs, Forced expression of RUNX2 in CCs was shown to be sufficient to induce chondrocyte hypertrophy [9]. Later, *Runx2* and *Runx3* were both found to be highly expressed in PHCs/HCs, and redundant master actions were demonstrated by showing that *Runx3*-null mice have virtually normal GPs, whereas *Runx2/3*-null fetuses fully lack maturing chondrocytes [10]. Proving cell-autonomous roles for the RUNX proteins in chondrocytes, mice inactivating *Runx2* specifically in chondrocytes (*Col2Cre*) were shown to develop the same cartilage phenotype as *Runx2*-null mice [45]. RUNX2/3 are transcriptional activators and their targets in chondrocytes likely include prehypertrophic (*Ihh*), hypertrophic (*Col10a1*) and terminal (*Mmp13*) markers, but the whole targetome of RUNX2/3 remains unknown. Besides genes co-expressed in chondrocytes and OBs, such as *Mmp13* [46], it will be interesting to determine if chondrocyte-only markers, such as *Col10a1* and *Ihh*, are truly direct targets of RUNX2/3, as suggested in vitro [10, 47], and to identify how RUNX2 targets distinct genes in chondrocytes and in OBs. RUNT-domain motifs are frequent near the SOX5/6/9 binding sites in chondrocyte enhancers [14, 15], suggesting interplay between RUNX and SOX proteins.

RUNX1—Because *Runx1*-null mouse embryos die from hematopoietic failure, the significance of *Runx1* expression in skeletogenesis was initially studied by transgenically rescuing *Runx1* expression in these embryos in endothelial/hematopoietic progenitors [48]. All cartilage and bone elements develop normally in these mice, except the sternum and occipital bone primordia, which show a chondrocyte maturation delay. These defects match those of *Runx2/3* mutants in other primordia, and can be explained by the relative expression levels of the 3 genes in skeletal elements. Specific inactivation of *Runx1/2* in limb bud and sternum SSCs (*Prx1Cre*) or ECs (*Col2Cre*) confirmed that the two genes are partially redundant in sternum chondrogenesis [49]. In a recent study, *Runx1* expression was found upregulated in superficial adult ACCs upon mechanical loading and in osteoarthritis, suggesting roles for *RUNX1* in adult joints [50].

Taken together, studies on RUNX subunits and complementary studies on CBFB [51, 52] have pointed to key roles for RUNX TFs in ECs, GPCs, and ACCs.

2.3. MADS-domain transcription factors

MADS-domain proteins participate in eukaryotic processes varying from homeosis in flowering plants to cell differentiation in animals. The MADS domain features α-helices, which wrap as a coiled coil around CC[A/T]$_6$GG DNA motifs, and β-sheets, which provide a homodimerization interface. MEF2 proteins are transcriptional activators named after their myogenic roles, but they also contribute to other processes, including chondrogenesis [53]. **MEF2C** is essential in neural crest cells for craniofacial development [54]. It is co-expressed with **MEF2D** in GPCs, and the two proteins were shown to work together with RUNX2 and SOX9 to enhance *Ihh* and *Col10a1* expression in PHCs/HCs [23, 55]. They

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were also proposed to target a number of other genes, but their full targetome remains to be defined.

2.4. REL-homology-domain (RHD) transcription factors

RHD factors act downstream of many signaling pathways. Their RHD domain contacts DNA in the major and minor grooves, and their C-terminus mediates homo/heterodimerization. The main families, NFkB and NFATC, comprise several chondrocyte regulatory factors.

NFκB proteins—Five nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) proteins are recognized: NFκB1, NFκB2, RELA, RELB and REL [56]. They bind DNA to GGG[A/G]N[T/C][T/C][T/C]CC motifs. Their activation, through nuclear translocation, is induced by stress signals and inflammatory cytokines. This results in chondrocyte catabolic actions and *Sox9* downregulation [57]. In contrast, **RELA/p65**, which is expressed in healthy SSCs and GPCs, was shown in vitro to promote cell survival and proliferation in response to growth hormone [58, 59]. This finding will merit in vivo validation in future studies.

NFATC proteins—Nuclear factors of activated T-cells (NFATC1 to 5) exist in vertebrates only, and they control many processes [60]. Their nuclear translocation requires dephosphorylation by the calcium-dependent calcineurin phosphatase. It occurs downstream of many signaling pathways. NFATC proteins bind DNA efficiently in partnership with such factors as AP1, GATA4, FOXP and MEF2. NFATC3 was first suggested to be involved in chondrogenesis downstream of calcineurin in a limb bud mesenchymal cell assay in vitro [61]. In vivo, NFATC1 was detected in superficial ACCs and pre/hypertrophic GPCs, and NFATC2 in adult, but not fetal ACCs [62, 63]. Interestingly, while mice lacking NFATC1 and/or NFATC3 in chondrocytes do not show increased susceptibility to osteoarthritis, mice lacking NFATC2 do, and mice lacking NFATC1 and NFATC2 are even more susceptible. Chondrocytes in the latter mice show imbalance between anabolism and catabolism, including ectopic hypertrophy. NFATCs are thus required for AC homeostasis and might be involved in specifying the ACC versus GPC fate.

2.5. STAT proteins

The family of signal transducers and activators of transcription (STATs) comprises 7 members that have key roles in biological processes by regulating cell proliferation, survival, and differentiation. STAT proteins are present in a latent form in the cytoplasm, and their phosphorylation downstream of a variety of growth factors, hormones and cytokines results in homo/heterodimerization and in translocation to the nucleus. STAT proteins preferentially bind to DNA motifs containing the canonical TTC[N2–4]GAA sequence. Important roles have been proposed in chondrocytes. For instance, both **STAT1** and **STAT5** inhibit CC proliferation downstream of FGFR3 signaling [64, 65]. STAT5 was also implicated in mediating effects of growth hormone on GPCs [59]. **STAT3** activation by pro- and anti-inflammatory cytokines was shown to either negatively or positively regulate chondrocyte differentiation in vitro [66, 67].

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These studies and many more have started to reveal that β-scaffold transcription factors with minor groove contacts have prominent, diverse actions in chondrocyte fate determination, differentiation and activities, and that full understanding of their extent and regulation should be the focus of many new research projects.

3. Zinc-coordinating transcription factors

Zinc-coordinating transcription factors form the largest superclass of all protein types. They account for about 50% of all human TFs and are distributed into 3 main classes [16, 68]. C2H2 proteins use 2 cysteines and 2 histidines to hold a zinc ion, while C4-zinc-finger-of-nuclear-receptor-type and diverse-C4-zinc-finger proteins hold the ion with 4 cysteines. Zinc coordination stabilizes a α-helix, forming a zinc-finger that binds 3–4 nucleotides in the DNA major groove. The recognized nucleotides vary among α-helices. Most proteins feature at least 2 zinc-fingers and thus bind motifs of 6 or more nucleotides. This unique modular organization of diverse zinc-fingers provides this protein class with a large, almost infinite, spectrum of DNA-binding recognition opportunities. As such, it was ingeniously embraced by molecular biologists to custom-design zinc-finger nucleases (ZFN) for precise genomic editing [69]. Many zinc-finger proteins are expressed in the chondrocyte lineage and critically impact chondrogenesis.

3.1. C2H2 zinc-finger transcription factors

SP/Krüppel-like (KLF) proteins form a main family in the C2H2 class. They typically feature 3 zinc-fingers and work as transcriptional activators or repressors. **SP1** (specificity protein 1) is ubiquitously expressed and transactivates many housekeeping, cell cycle-regulated and cell type-specific genes. It was also shown in chondrocytes to bind and upregulate the promoters of *Col2a1*, *Sox9,* and other genes, and to be repressed by cytokines that powerfully affect chondrocytes, namely transforming growth factor-β and interleukin-1 [70–72]. **SP7/OSX** is specifically expressed in PHCs and OBs. The most striking phenotype of *Sp7*-null mice is the lack of bones, explained by the fact that SP7 is a master transcriptional activator in OBs [73]. While GPs do not appear affected in these mice, mice carrying one *Sp7* null allele and one allele inactivated in chondrocytes (*Col2Cre*) delay TC maturation, suggesting that SP7 controls this process cell-autonomously [74]. **ZNF521/ZFP521** has 30 Krüppel-like zinc-fingers. Like SP7, it is expressed in PHCs and OBs, but unlike SP7, it functions as a transcriptional repressor. A target of PTHrP (parathyroid hormone-like protein) signaling, it inhibits RUNX2 activity in both cell types [75]. Its inactivation prompts chondrocyte hypertrophy, as seen in *Pthlh*-null mice (lacking PTHrP), and it rescues the phenotype of mice with a constitutively active PTHrP receptor (PTH1R), a model for Jansen metaphyseal chondrodysplasia [76] ZFP521 thus contributes to controlling the pace of GPC maturation.

ZBTB20/ZNF288 belongs to a family of proteins that bind DNA via both a Krüppel-type and a BTB/POZ domain. The gene for this transcriptional repressor was recently found to be strongly expressed in HCs and its conditional inactivation with *Col2Cre* to cause short stature [77]. GPC proliferation is reduced and hypertrophic zones are elongated. *Sox9* expression is prolonged and *Col10a1* expression reduced. ZBTB20 is thus a newly identified

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major promoter of HC differentiation. Heterozygous mutation in the *ZBTB20* gene cause Primrose syndrome, which is characterized by recognizable facial features, macrocephaly, enlarged and calcified external ears, in addition to other non-skeletal defects {Primrose, 1982 #181}.

SNAI1/SNAIL and SNAI2/SLUG are SNAIL-family transcriptional repressors. They have 4 and 5 zinc-fingers, respectively. They regulate many processes, including mesoderm formation and epithelial-to-mesenchymal transition [78]. *Snai2* is widely expressed in the chondrocyte lineage, but *Snai1* is mostly found in PCs and PHCs/HCs [79]. Inactivation of either gene with *Prx1Cre* has no obvious effect, but co-inactivation has uncovered redundant roles in GPCs. Specifically, chondrocyte proliferation is impaired, possibly due to *Cdkn1a* upregulation, and PHC/HC maturation is delayed, resulting in dwarfism. SNAI1 is a target and mediator of FGFR3 signaling [80]. SNAI2 was also found to inhibit mesenchymal stem cell (MSC)-derived chondrogenesis in vitro and to be upregulated in osteoarthritic cartilage [81].

Last but not least, the **GLI family** of C2H2 zing-finger TFs is composed of three members in mammals (GLI1-3) that are central mediators of Hedgehog signaling [82]. *Gli2*-null mice show, like *Ihh*-null mice, an elongated hypertrophic zone and reduced bone formation, whereas *Gli3* inactivation rescues the defects of *Ihh*-null mice. These findings indicate that GLI2 is a main mediator and GLI3 a negative regulator of the Hedgehog pathway in GPCs. AS described earlier, GLI factors cooperate with SOX9, RUNX2 and other TFs in chondrocyte transcriptional regulation.

Altogether, many studies agree that C2H2 TFs have important and varied roles at all chondrocyte differentiation steps. Research must be pursued to reveal their entire targetomes and their specific positions in chondrogenic regulatory networks.

3.2. Transcription factors with a nuclear receptor-type C4 zinc-finger

Nuclear receptors are unique to metazoans. They are encoded by 49 genes in humans and have key roles in development and adulthood. Some class members are translocated to the nucleus upon ligand binding. Others are already nuclear in the absence of ligand, and are converted from transcriptional repressors to transcriptional activators upon ligand binding. Nuclear receptors recognize their targets at hormone response elements (HREs). These elements are composed of two repeats of 6 nucleotides, whose relative orientation (direct or inverted) and proximity are specific to each type of nuclear receptor.

The **glucocorticoid receptor** (*NR3C1*) is expressed ubiquitously and controls many physiological and pathological processes. It is dispensable in GPCs and ACCs in mice under physiological conditions [83, 84], but its over-activation in children treated with glucocorticoids likely underlies the deleterious effects observed on GPs [85]. In contrast, the **thyroid hormone receptors α and β** (*NR1A1* and *A2*) are critical for normal skeletal growth [86]. Both genes are expressed in GPCs. Interestingly, while *Nr1a1*-null mice exhibit features of hypothyroidism, including impaired GPC maturation and growth retardation, *Nr1a2*-null mice show signs of hyperthyroidism, including accelerated GPC maturation and short stature. The **retinoic acid receptors α, β and γ** (*NR1B1* to *B3*) are expressed in all

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GPCs, except HCs [87]. Their inactivation using *Col2Cre* has revealed redundancy in promoting chondrocyte proliferation and cartilage matrix production postnatally. Interestingly, since the GP is largely devoid of retinoid ligand, the receptors most likely work as ligand-free transcriptional repressors under physiological conditions. Their targets were proposed to include *Sox9*. The **peroxisome proliferator-activated receptors** α, β/δ **and** γ (*PPARA, D,* and *G*, respectively) have key roles in lipid and glucose metabolism in many cells, and PPARγ is an adipogenic master. Inactivation of *Pparg* in chondrocytes during development leads to dwarfism, with reduced expression of *Sox9*, *Col2a1*, *Acan*, *Ihh* and *Col10a1* [88], and inactivation in adulthood exacerbates the development of post-traumatic osteoarthritis, at least in part by increasing cartilage catabolism [89]. In contrast, inactivation of PPARδ has no obvious effect on cartilage development and protects mice from developing post-traumatic osteoarthritis [90]. The importance of PPARα in chondrocytes in vivo remains unknown.

Nuclear hormone receptors are thus important regulators of chondrocyte activities. Their direct and indirect actions in the cell type-specific transcriptional networks remain underestimated and deserve further studies.

3.3. Diverse C4-type zinc-finger transcription factors

GATA1-6 are transcriptional activators that use two zinc-fingers to bind to [A/T]GATA[A/G] DNA motifs [91]. Only **GATA6** is expressed during chondrogenesis. Mainly found in SSCs and PCs, it prevents ectopic expression of *Shh* (Sonic Hedgehog) and hence polydactyly [92, 93]. Heterozygous mutations in the gene for one of its relatives, **TRPS1**, cause Tricho-Rhino-Phalangeal syndrome, characterized by short stature and craniofacial abnormalities. *Trps1* is expressed in PCs, perichondrium cells, JPs, and PHCs [94]). TRPS1 is a transcriptional repressor that facilitates the PHC to HC transition. It may downregulate *Ihh* (Indian hedgehog) and *Runx2* expression by interacting with RUNX2 [94] and GLI3 [95]. Future studies will likely uncover additional targets and functional partners.

4. Helix-turn-helix transcription factors

Helix-turn-helix (HTH) proteins form the second largest superclass of TFs [16]. They arose in prokaryotes and represent 25% of all human TFs. The HTH domain is composed of two α-helices linked by a short “turn” sequence. One helix recognizes a specific DNA sequence and the other stabilizes DNA binding. Several classes of HTH proteins contain members importantly involved in chondrogenesis.

4.1. Homeodomain transcription factors

The homeodomain preferentially binds DNA to ATTA-containing motifs and is found in homeotic factors (hence its name) and many types of non-homeotic TFs [96].

The fly was the first animal found to rely on a cluster of homeobox genes (**HOX**) to specify the formation of antennas, legs and other body structures. Mammals were later found to have 39 HOX genes, distributed in 4 paralogous clusters. As in flies, the mammalian HOX genes have decisive, overlapping roles in body plan formation along the anterior-posterior axis. This includes specification of positional identity in the axial and appendicular skeleton [97].

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Most HOX genes are expressed in SSCs and perichondrium cells, and not in differentiated chondrocytes [98]. Their inactivation causes agenesis or homeosis of specific structures. For instance, mouse *Hox13* inactivation prevents autopod skeletogenesis because SSCs fail to form digital precartilaginous condensation [99], and mouse *Hox11* inactivation transforms the sacrum into lumbar vertebrae [100]. Mutations in human HOX genes cause several types of skeletal malformations [101].

Many non-HOX homeodomain genes also participate in skeletogenesis. An example is ***SHOX*** (short stature homeobox). Its heterozygosity causes Leri-Weill dyschondrosteosis {Ross, 2005 #182}, featuring short stature and skeletal dysplasia, and its nullizygosity causes Langer mesomelic dysplasia [102]. These diseases involve accelerated maturation of GPCs and premature closure of GPs. Mice lack a *SHOX* ortholog, but possess ***Shox2***, a paralog of human *SHOX*. Deletion of *Shox2* using *Prx1Cre* causes severe rhizomelia, whereas deletion using *Col2Cre* causes a much milder phenotype [103]. These findings indicate that *Shox2* controls chondrocytes mostly at the SSC stage or non-cell-autonomously. Another example is ***PRRX1/MHOX*** **(**its regulatory elements drive *Prx1Cre* expression), whose inactivation causes agnathia-otocephaly in humans [104] and multiple skeletal malformations in mice [105]. *PRRX1* too controls chondrocytes at the SSC stage or non-cell-autonomously. ***DLX5*** and ***DLX6*** sit tail-to-tail in the genome and are expressed in SSCs, perichondrium cells, PHCs/HCs, and OBs. Mutations in human *DLX5* and *DLX5/6* regulatory elements cause split-hand/foot malformation (SHFM) syndrome [106], and *Dlx5/6*-null mice exhibit lower-to-upper-jaw homeosis, skull agenesis, delayed axial skeleton growth and ossification, and SHFM-like appendicular defects [107, 108]. Findings from transgenic overexpression of *Dlx5* in chondrocytes in wild-type and *Dlx5/6*-null mice suggest cell-autonomous roles for DLX5/6 in chondrocyte maturation [109]. Since, unlike RUNX2, DLX5/6 are not sufficient to initiate chondrocyte pre/hypertrophy, DLX5/6 likely act downstream of RUNX2. DLX5 and SP7 were recently suggested to pair off in OBs [110]. They might thus act similarly in chondrocytes. **NKX3.2/BAPX1** is an important transcriptional repressor in chondrogenesis [111]. Its nullizygosity in humans causes spondylo-megaepiphyseal-metaphyseal dysplasia, i.e., short trunk and GP and ossification defects in the axial and appendicular skeleton [112]. Induced by SHH and maintained by bone morphogenetic protein (BMP) signaling in SSCs, NKX3.2 promotes chondrogenesis by inducing *Sox9* upregulation and repressing *Runx2* [113]. Later, it may delay GPC maturation by repressing *Runx2* and *Mef2c* in CCs, and may maintain cells alive by binding to NFκB [58, 114]. *NKX3.2* expression in normal and osteoarthritic human ACCs correlates negatively with expression of markers for HCs, catabolic behavior and inflammation, suggesting important roles in maintaining AC healthy [115]. NKX3.2 might act in redundancy with relatives, namely NKX3.1, such that the contribution of NKX factors to chondrogenesis may be currently underestimated.

Overall, homeodomain-containing proteins have commanding, often overlapping or redundant roles in skeleton patterning and chondrogenesis. Most are expressed primarily in SSCs and perichondrium cells, and may thus affect chondrocytes through lasting roles (e.g., epigenetic modifications in SSCs) or through non-cell-autonomous actions (e.g., by

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activating signals in the perichondrium that impact adjacent chondrocytes). Their target genes remain scarcely known.

4.2. Paired-domain transcription factors

Mammals contain nine paired-domain proteins (PAX1 to 9) and all have important roles in development [116]. The paired domain preferentially binds GTCACGGT motifs and owes its name to featuring two HTH regions. Several PAX proteins additively possess a full or partial homeodomain, and the two domain types synergize to bind DNA. This is the case of PAX3 and PAX7, which act redundantly in neural crest formation and other processes [117], but not of **PAX1 and PAX9**, which are expressed in subsets of SSCs and are necessary for cell survival [118, 119]. PAX proteins work as transcriptional repressors or activators by recruiting distinct sets of co-factors. Their full targetome remains unknown, but PAX1/9 were proposed to directly activate *NKX3.2* in the sclerotome [120]. PAX7 was recently shown to have both pioneer and classical TF functions [116]. More studies are thus needed to define the molecular actions of PAX proteins in SSCs.

4.3. Forkhead-domain transcription factors

Humans express 40 proteins that use a forkhead/winged-helix domain (FOX) to bind [A/C]AA[C/T] sequences in the major and minor groove of DNA. These proteins are categorized into 19 groups (FOXA to FOXS) and several are known to achieve pivotal pioneer, transactivation and transrepression functions in major processes, including skeletogenesis [121, 122].

FOXC1 and C2 were the first FOX proteins to be linked to skeletogenesis. They are expressed from mesodermal to PC stages [123, 124]. *Foxc1*-null and *Foxc2*-null mice die upon birth with craniofacial and axial skeleton malformations and multiple other abnormalities [123, 125]. Skeletal defects include early patterning and chondrocyte and OB differentiation problems. Single heterozygotes are normal, but double heterozygotes exhibit many of the single-null mouse defects, indicating functional overlap between the two factors [126]. FOXC1 was proposed to interact with GLI2 in chondrocytes to stimulate the expression of IHH target genes, including *Pthlh*, *Ptc1*, and *Gli1*, and may also directly upregulate *Col10a1* [127], but further studies are required to fully uncover the cell- and non-cell-autonomous actions of FOXC proteins in chondrogenesis.

Deletion of **FOXA2 and A3** are expressed in EC and GPC chondrocytes and their inactivation (*Col2Cre*) was shown to specifically impair HC differentiation [128]. Intriguingly, FOXA2/3, like FOXC1/2, were proposed to help activate *Col10a1* through a FOX recognition site, raising the possibility that several FOX groups act in redundancy or through complementary actions to promote chondrocyte hypertrophy.

Adding further complexity to the actions of FOX proteins in chondrocytes, **FOXP1, P2 and P4** are transcriptional repressors expressed strongly in PCs and perichondrium cells and weakly in CCs [77]. Their single and compound inactivation (*Col2Cre*) and their overexpression in transgenic mice have revealed cooperative actions in delaying GP

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maturation and EO, possibly through inhibiting RUNX2 transcriptional activity. They may thus antagonize the actions of FOXC and FOXA proteins in skeletogenesis.

FOXO1, FOXO3, and FOXO4 are ubiquitously expressed and control oxidative stress and cell metabolism, proliferation and apoptosis in many lineages. Their inactivation using *Col2Cre* was shown to cause elongation of HC zones, a postnatal increase in body length, and progressive hyperkyphosis in adulthood [129]. Resembling those of mice lacking PTEN (inhibitor of AKT activation), these defects are consistent with the ability of AKT to phosphorylate and degrade FOXO proteins. *FOXO1* and *FOXO3* are highly phosphorylated in osteoarthritic ACCs, likely in response to inflammatory cytokines, and their knockdown was shown to sensitize primary chondrocytes to oxidative stress [130, 131].

Thus, at least four FOX groups have key functions in developing and adult cartilage. These findings and the frequent presence of FOX recognition motifs in chondrocyte-specific super-enhancers [14, 15]. call for further studies to fully decipher their specific molecular actions in chondrocytes and progenitors.

4.4. Tryptophan cluster transcription factors

A specific class of TFs is made of proteins that feature multiple highly conserved and closely spaced tryptophan residues in their HTH DNA-binding domain. Within this class, the ETS family is highly implicated in cancer, but contains at least one member, **ERG**, also involved in skeletogenesis. ERG is strongly expressed in PCs and JPs [132]. Its inactivation in JPs (*Gdf5Cre*) does not affect joint formation [133], but its transgenic overexpression (*Col2a1*) prevents GPC maturation and induces expression of the ACC marker tenascin-C. Interestingly, mice inactivating ERG in JPs develop osteoarthritis as they age and are more sensitive to surgically induced osteoarthritis than wild-type mice. Molecular studies have suggested that the genes for PTHrP and lubricin (encoded by *Prg4*, a superficial ACC marker) might be part of the ERG targetome. Together, these findings suggest that ERG may help specify the ACC fate of JPs, but may act in redundancy with relatives. Further studies are needed to fully understand the contribution and regulation of ERG and relatives in chondrogenesis.

5. Basic-domain transcription factors

The basic-domain protein superclass accounts for less than 10% of all human TFs, but is nevertheless critical in many lineages. Basic domains are rich in positively charged amino acids that participate in formation of α-helices and facilitate interaction with DNA. Basic domains are often associated with a dimerization domain that helps stabilize binding to DNA. This superclass is divided into basic-helix-loop-helix (bHLH), basic-leucine zipper (bZIP), and basic-helix-span-helix (bHSH) classes.

5.1. Basic-helix-loop-helix (bHLH) transcription factors

The bHLH motif has two α-helices joined by a loop, and it generally binds DNA to the CANNTG motif, called E box [134]. Several class members are master regulators in a discrete lineage, e.g., MYOD (myogenic), MYC (cell proliferation/oncogenic), SCX (tenogenic) and NEUROD (neurogenic). Several bHLH proteins intervene in

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chondrogenesis, but none has yet been found to meet the criteria of a master chondrogenic regulator. Several TF families comprise the bHLH class.

TWIST1 is a classical bHLH factor, whose gene is expressed in SSCs and perichondrium cells downstream of canonical WNT and NOTCH signaling. *TWIST1* heterozygous mutations cause Saethre-Chotzen syndrome, characterized by short stature and craniosynostosis among other skeletal anomalies. TWIST1 homo/heterodimers inhibit differentiation of SSCs into downstream cell types, including chondrocytes [51, 135, 136]. They repress genes involved in cell specification either directly or by physically interacting with master regulators, including SOX9, RUNX2, MYOD, and PPARγ [137]. TWIST1 may be involved in keeping SOX9 inactive in SSCs.

The **bHLH-PAS family** includes the hypoxia-inducible factor **HIF1α**, which dimerizes with the HIF1β subunit (also called ARNT) through a PAS (Per-Arnt-Sim) domain. The HIF1α gene is ubiquitously expressed, but its protein product is rapidly degraded, unless oxygen levels drop below 5%. Presumptive joint regions and the center of GPs are hypoxic and rely on HIF1α to ensure cell survival and to promote cell proliferation and differentiation [138]. HIF1α transactivates genes involved in anaerobic metabolism and proline hydroxylation (a key step in collagen synthesis), and might even directly stimulate *Sox9* expression [139]. Besides HIF1α, the bHLH-PAS family also comprises TFs regulating the circadian rhythm, namely CLOCK, BMAL1, PER1/2 and CRY1/2. Several studies have started to reveal important roles for these factors in promoting GP activity and in ensuring adult AC healthy homeostasis [140, 141].

The **bHLH-O family** is made of bHLH proteins with distinctive Orange domain, which helps in heterodimer partner selection. Prominent members are the Hairy/Enhancer of Split-related **HES** and **HEY** transcriptional repressors, whose genes are direct targets of NOTCH signaling. HES1 and HES5 delay the differentiation of SSCs into chondrocytes and facilitate GPC hypertrophy ([142]. As for HIF1α, a key target might be *Sox9*.

The **bHLH-Z family** is made of bHLH proteins that dimerize through a leucine zipper. It includes SREBP1 and 2, proteins essential for cholesterol metabolism. These factors may be critical in GPCs, as suggested by the observation that chondrocyte-specific inactivation of the gene for S1P, a proprotein convertase required to cleave SREBP precursor proteins into mature forms, results in severe chondrodysplasia in the mouse, with significant downregulation of SREBP-dependent pathways [143, 144].

Taken together, these findings have started to reveal that the bHLH class, like other classes of TFs, contributes several members in the regulation the chondrocyte lineage at multiple steps and in various, yet-incompletely-defined ways.

5.2. Basic-leucine-zipper (bZIP) transcription factors

The ZIP domain is an amino acid sequence that is mainly made of heptad repeats, in which hydrophobic residues (often leucine) occupy every first and fourth position and stabilize protein dimers. The basic region of the domain is involved in DNA sequence recognition and the leucine-zipper in dimerization and DNA-binding stabilization. The bZIP class includes

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several closely related families. Members can homodimerize or heterodimerize with relatives in their own family or in another family.

The **ATF/CREB family** (activating transcription factor/cyclic AMP response element binding) is involved in cell survival, growth, and stress responses in various processes. Its members bind DNA to GTGACGT[A/C][A/G] motifs (cAMP-response element, CRE). **ATF4** can function as a transcriptional activator or repressor. It is induced transcriptionally and stabilized translationally by oxidative, hypoxic, and ER stress, and has key roles in allowing cells to adapt to these conditions [145, 146]. First shown to promote collagen synthesis and cell type-specific genes in OBs, it was subsequently found to stimulate CC proliferation and PHC differentiation, namely through upregulating *Ihh* expression [147]. Although earlier studies suggested that **ATF3** could participate in promoting HC differentiation, conditional inactivation in chondrocytes (*Col2Cre*), revealed that ATF3 is dispensable for cartilage development [148]. The factor, however, exacerbates post-traumatic osteoarthritis downstream of inflammatory cytokines. Other studies have suggested that **ATF2 and CREB** could promote GPC cell cycle progression, and that **ATF6** could promote chondrocyte hypertrophy by interacting with RUNX2 [149]. **BBF2H7** (encoded by *CREB3L2*) is a membrane-bound TF most highly expressed in CCs [150]. *Creb3l2*$^{-/-}$ mice exhibit severe chondrodysplasia due to defective expression of genes encoding proteins involved in the export of cartilage extracellular matrix proteins from the endoplasmic reticulum to the Golgi apparatus. These findings call for additional studies to fully uncover the important roles that multiple ATF/CREB TFs play, possibly often in redundancy of each other, in developing and adult cartilage.

The **AP1 family** is primarily comprised of **JUN** (c-JUN, JUNB, JUND) and **FOS** (c-FOS, FOSB, FOSL1/FRA1 and FOSL2/FRA2) proteins [151]. Many of these proteins are expressed in GPCs [152]. AP1 factors are activated in response to various growth factors and inflammatory cytokines, and regulate cell proliferation, differentiation and death. They bind DNA at TPA (TGA[G/C]TCA) or cAMP (TGACGTCA) consensus sequences. Homodimers of c-JUN are potent transcriptional activators, whereas FOS proteins lack a transactivation domain and partner with JUN to transactivate genes. *Fos* inactivation in the mouse results in postnatal growth retardation, partly due to shortening of CC zones at the expense of HC zones [153]. In contrast, global and *Col2Cre*-mediated inactivation of *Fosl2* delays HC differentiation and results in smaller HC zones [154]. FOSL2 also enhances OB differentiation [155] and is thus one more TF promoting both GPC maturation and OB differentiation. *Jun* is expressed in JPs. Its inactivation using *Prx1Cre* was shown to block specification of these cells, resulting in cartilage primordia fusions in place of joints, but it did not otherwise affect skeletal development in utero [156]. JUN is thus necessary to repress the chondrogenic fate of JPs, but is dispensable during EC and GPC differentiation. Nevertheless, a recent study showed that a large majority of SOX9-bound genomic regions coincided with a small fraction of JUN-bound regions in non-hypertrophic GPCs [152]. In vitro assays have suggested that SOX9 and JUN are able to physically interact with one another and to additively activate a *Col10a1* enhancer. More studies are needed to assess the nature and full extent of actions of the various AP1 members in chondrocytes.

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The **MAF family** has two members, **c-MAF and MAF-B,** expressed in PCs, perichondrium cells and HCs [157–159]. Inactivation of *Maf* mildly delays EO in mouse fetuses [158]. Like JUN, c-MAF is able to physically interact with SOX9 in vitro and to cooperate with SOX9 in transactivating a reporter gene, namely one containing a *Col2a1* cartilage-specific enhancer [157]. The in vivo significance of these findings remains unknown, as is the possibility that c-MAF acts in partial redundancy with MAFB and other bZIP proteins. Heterozygous mutations in *MAFB* cause multicentric carpotarsal osteolysis syndrome, mimicking juvenile rheumatoid arthritis, and also associated with minor facial anomalies and non-skeletal defects {Zankl, 2012 #183}.

The **CCAAT-enhancer-binding protein** family has six members: C/EBPα to C/EBPζ. These members bind DNA to CCAAT motifs through a C-terminal bZIP domain, and transactivate genes through N-terminal domains. C/EBPα is a master regulator of adipogenesis, and CEBPβ is critical for macrophage function. C/EBPβ and δ are abundantly expressed at all stages of GPC differentiation [160]. *Cebpb*-null mice are dwarf and show reduced expression of PHC/HC markers [161]. C/EBPβ was proposed to directly target the genes for the cyclin-dependent kinase inhibitor p57Kip2 and IHH, and to interact with RUNX2 to activate *Ihh* [161, 162]. C/EBPβ is also produced by ACCs, especially in osteoarthritic lesions, where it could have deleterious effects [161]. *Cebpb*-null mice are indeed more resistant to post-traumatic osteoarthritis than wild-type mice. In addition to further defining the molecular targets and partners of C/EBPβ, it will also be important to test whether C/EBPβ acts in partial redundancy with close relatives.

5.3. Basic-helix-span-helix (bHSH) transcription factors

The bHSH class is primarily represented by the **AP2 family**, which contains 5 members: AP2α, β, γ, δ, and ε [163]. These proteins bind DNA as homo/heterodimers to palindromic GCC(N3)GGC sequences and use N-terminal domains to activate or repress transcription. AP2α, β, and γ are expressed in neural crest and in facial and limb bud SSCs in the embryo, and direct cell survival and proliferation. **AP2α** is also expressed in GPCs and delays cell differentiation. Its inactivation in the mouse leads to severe craniofacial malformations, among other defects. In contrast, inactivation of either **AP2β** or **AP2γ** does not affect chondrogenesis, possibly due to redundancy with family members. **AP2ε** was recently shown to be expressed in HCs and ACCs, and upregulated in human osteoarthritis [164]. Inactivation of its gene in the mouse does not impact chondrogenesis, but protects mice against post-traumatic osteoarthritis. AP2ε was proposed to directly contribute to *Col2a1* repression in HCs [165]. Further studies are thus warranted to fully explore the actions and regulation of AP2 proteins in chondrocytes.

6. Transcription factors with a β-hairpin exposed by a α/β-scaffold

The last TF superclass reviewed here is represented by two classes of proteins that have only remotely related DNA-binding domains: NF1 and SMAD proteins. The NFI class comprises four members (NFIA, NFIB, NFIC, and NFIX) and the SMAD class has eight of them (SMAD1 to 8/9) in mammals. All have important roles in various processes [166] **NFI** homo/heterodimers bind DNA at the palindromic TTGGC(N5)GCCAA sequence to

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contribute to transcriptional activation or repression. *NFIX* missense and dominant-negative mutations cause Sotos and Marshall-Smith syndrome, respectively. These disorders include overgrowth and kyphosis, which are also seen in *Nfix*-null mice [167]. While known to be important in skeleton formation and homeostasus, their expression pattern and roles in chondrocytes remain unclear. **SMAD** TFs are categorized into co-SMAD (SMAD4), regulatory-SMADs (SMAD1-3, 5 and 8/9), and inhibitory SMADs (SMAD6 and 7). They mediate canonical signaling pathways initiated by members of the TGFβ superfamily, namely TGFβ and bone morphogenetic proteins (BMPs). They have important, overlapping roles in cooperation with other TFs, including SOX9 and RUNX2, at virtually every step of skeletal patterning and chondrogenesis [168, 169].

7. Integrative view, perspectives and conclusions

This review has highlighted that dozens and even perhaps hundreds of TFs control the lineage specification and differentiation activities of chondrocytes and their progenitors and thereby contribute prominently to the great diversity in size, shape and properties of cartilage tissues.

Chondrogenesis starts in prospective skeletal sites of the embryo with the recruitment, lineage commitment and condensation of SSCs. These steps involve a very large number of TFs whose study is challenging due to extensive functional overlap. Patterning factors control cell survival and proliferation spatially and temporally, and are generally distinguished from masters and associated factors, which control lineage determination and differentiation. Some factors, however, may have both types of activities. HOX proteins, PAX1/9, SHOX2, PRRX1, DLX5/6, SOX4/11/12, FOXC1/2, and AP2α/β/γ likely fall primarily in the first group, and SOX9, NKX3.2, TWIST1, JUN, SNAI2, and HES/HEY proteins in the second group. Several of these early factors remain expressed in perichondrium cells and JPs during cartilage primordia and growth plate development. They may thus continue to determine skeletal patterning and overt development at those stages. Early chondrocyte differentiation in cartilage primordia is governed by the SOX5/6/9 master trio in concert or in parallel with SP1, PPARγ, BBF2H7, and CLOCK proteins. RELA, SNAI1/2, FOXO1/3/4, HIF1α, and ATF2/CREB contribute to this step to promote cell proliferation and survival, and possibly also differentiation. During GPC maturation, chondrocytes still rely on SOX5/6/9 to maintain expression of EC markers at the PHC, to repress PHC markers, and to activate HC markers. They also involve ZNF521, NR1A2, SHOX/2, FOXP1/2/4, FOS, and AP2α/ε to help the SOX trio. In addition, they require the actions of the RUNX1/2/3 masters to initiate activation of pre-, hypertrophic and terminal markers. These factors are helped with MEF2C/D, ZBTB20, SNAI1/2, SP7, NR1A1, PPARγ, TRPS1, DLX5/6, FOXC1/2, FOXA2/3, ATF3/4/6, FOSL2, HES/HEY proteins, c-MAF, and C/EBPβ. Along with the discovery of GPC regulators, great progress has been made to reveal factors controlling healthy ACC specification, differentiation and maintenance. These factors comprise SOX5/6/9, ERG, NFATC1/2, NKX3.2, PPARγ, and CLOCK proteins. Other factors are harmful for these cells. They include ATF3, C/EBPβ, RUNX1/2, NFκB, and PPARδ.

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Although already very long and complex, this list of chondrocyte-determining TFs is still far from being definitive. More TFs than discussed here have been found expressed in the chondrocyte lineage and many additional factors likely remain to be uncovered. In addition, important knowledge gaps remain for all factors already listed. A few of the many outstanding questions that need answers are the following. Among the many TFs known to have key roles in SSCs, which ones, if any, are chondrogenic pioneers? Do SOX9 and RUNX proteins have pioneer factor roles in ECs, maturing GPCs and ACCs in addition to classical TF roles? What are the master factors that decisively distinguish the ACC fate from the GPC fate? What is the full targetome of each chondrogenic factor and how does this targetome change as cells progress through successive differentiation stages? How do chondrogenic TFs functionally interact with one another?

Definitive understanding of chondrogenic transcriptional networks will require more studies in vivo and in vitro. Studies on human samples are invaluable to provide direct insights into physiological and pathological processes, but they are insufficient to fully dissect cellular and molecular actions. Among animal models, the mouse has been most often used and should continue to be used. A major advantage of mice is that loss- and gain-of-function assays can be readily controlled spatially and temporally using conditional approaches. This is critical in chondrogenesis to pinpoint cell- and non-cell-autonomous functions and stage-specific functions of TFs. To decode the molecular actions of TFs, ChIP-seq is a state-of-the-art approach to achieve non-biased characterization of the genome occupancy of TFs. Recent studies have revealed an abundance of chondrocyte-specific super-enhancers bound by the SOX5/6/9 trio and presenting binding motifs for many types of TFs. ChIP-seq should thus ideally be used for every chondrogenic factor and at each stage of chondrocyte differentiation in order to reveal the dynamic changes that likely occur during chondrogenesis in the occupancy of gene regulatory elements by TFs. ChIP-seq studies must be complemented with functional studies, namely RNA-seq in loss- and gain-of-function models, reporter assays with relevant enhancers and promoter segments, and protein-protein interaction assays. Beyond studying the genomic actions of TFs, it is also important to characterize the multiple levels of regulation of these factors. These levels include the epigenetic control of chromatin as well as transcriptional, posttranscriptional and posttranslational regulation. One should also take into consideration that various types of non-coding RNAs are implicated in transcriptional regulation and that many TFs have non-transcriptional roles that can also critically influence cell fate and differentiation.

In conclusion, the last two decades have allowed a wealth of discoveries to be made in the molecular control of chondrogenesis, and this has included the revelation that the pathway is driven and kept in check by dozens of TFs. Key nodes in transcriptional hierarchies and networks have started to be established, and genomic hubs in the form of super-enhancers have started to be located within and around cartilage-specific genes. However, gaps in knowledge remain at multiple levels. Guided by all notable milestones already achieved, prompted by novel ideas, and supported by ever-evolving technological capabilities, researchers of the next decade are well equipped to reach full understanding of chondrogenic transcriptional networks and full understanding of the interactions of these networks with other regulatory pathways, to apply acquired knowledge to fully decipher molecular

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mechanisms disrupted in cartilage malformation and degeneration diseases, and to design efficient approaches to prevent, treat and cure these diseases.

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Abbreviations

AC	articular cartilage
ACC	articular chondrocyte
CC	columnar chondrocyte
EC	early chondrocyte
EO	endochondral ossification
GP	growth plate
GPC	growth plate chondrocyte
HC	hypertrophic chondrocyte
JP	joint progenitor cells
OB	osteoblast
OCP	osteochondroprogenitor
PC	prechondrocyte
PHC	prehypertrophic chondrocyte
SSC	skeletal progenitor/stem cell
TC	terminal chondrocyte
TF	transcription factor

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Table 1

Transcription factors involved in chondrocyte fate and differentiation decisions

Transcription factors	Expression											Cell fate and differentiation actions	Trans cr. role	DNA-binding motif	Human skeletal diseases
	SSC	PC	EC	CC	PHC	HC	TC	perIC	JP	ACC	OB				
1. Beta-scaffold transcription factors with minor groove contacts															
1.1. HMG-domain transcription factors															
1.1.1. SOX transcription factors															
SOX9	+	+	+	+	+	+			+	+	−	Master activator of the PC to the HC and ACC stages	TA (TR)	[T/A][T/A]CAA[T/A]G[n3-4]C[A/T]TTG[A/T][A/T]	Campomelic dysplasia
SOX5/SOX6	−	+	+	+	+					+	+	Promote EC, CC, HC, and ACC differentiation	help in TA	ATTGTT[nN]ATTGTT	LAMSHF syndrome (*SOX5*)
SOX8	+	+	+							+	+			(A/T)(A/T)CAA(A/T)G	
SOX4/SOX11/SOX12	+	+	+	+	+	+	+	+	+		+	Promote cell survival, SSC/JP non-chondrogenic fate, and CC alignment	TA	AACAA	Coffin-Siris syndrome (*SOX11*)
1.1.2. TCF/LEF transcription factors															
TCF7L2						+						Promotes HC differentiation	TA	CTTTG[A/T][A/T]	
1.2. RUNX transcription factors															
RUNX1/2/3	+	+		+	+	+	+	+	+		+	Master activators of the PHC to TC stages	TA	[A/G]ACC[A/G]CA	Cleidocranial dysplasia (*RUNX2*)
1.3. MADS-domain transcription factors															
MEF2C/D				+	+	+					+	Activators of the PHC to TC stages	TA	CC[A/T]$_6$GG	
1.4. REL-homology-domain (RHD) transcription factors															
RELA/p65	+		+	+	+	+					+	Promotes SSC and GPC survival and proliferation	TA	GGG[A/G]n[C/T][C/T][C/T]CC	
NFATC1/2/3					+	+				+	+	Promote ACC maintenance	TA	GGAAA	
1.5. STAT transcription factors															
STAT1 and STAT5	+	+	+	+	+	+	+	+	+	+	+	Inhibit CC proliferation	TA	TTC[N2–4]GAA	
2. Zinc-coordinating transcription factors															
2.1. C2H2 zinc-finger transcription factors															
SP1	+	+	+	+	+	+	+	+	+	+	+		TA	GGCGGGG	
SP7					+	+	+				+	Promotes TC differentiation	TA	GGCGGGG	

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Transcription factors	Expression											Cell fate and differentiation actions	Trans cr. role	DNA-binding motif	Human skeletal diseases
	SSC	PC	EC	CC	PHC	HC	TC	periC	JP	ACC	OB				
ZNF521/ZFP521					+	+					+	Slows down CC to PHCs/HC transition	TR		
ZBTB20/ZNF288			+		+	+	+					Inhibits chondrocyte hypertrophy	TR		Primrose syndrome
SNAI1 and SNAI2	+		+	+	+	+	+	+	+	+	+	Promote CC proliferation and PHC/HC differentiation	TR	CAGGTG	
GLI1 to 3	+	+	+	+			+	+	+		+	Mediator/Inhibitor of Hedgehog signaling	TA/TR		
2.2. C4 zinc-finger of nuclear receptor-type															
Glucocorticoid receptor	+	+	+	+	+	+	+	+	+	+	+	Dispensable in normal conditions	TA/TR	AGAACA[n3]TGTTCT	
Thyroid hormone receptors α and β				+	+	+		+			+	Accelerates (NR1A1) and inhibits (NR1A2) GPC maturation	TA/TR	AGGTCA[n4]TGACCT	
Retinoic acid receptors α, β and γ				+	+			+			+	Enhance CC proliferation and matrix production	TA/TR	AGGTCA[n5]AGACCA	
Peroxisome proliferator-activated receptors α, β/δ and γ	+			+	+	+						Enhances GPC gene expression (PPARG)	TA/TR	AGGTCA	
2.3. Diverse C4-type zinc-finger transcription factors															
GATA6	+	+										Inhibits ectopic expression of *Shh* in SSCs	TA	[A/T]GATA[A/G]	
TRPS1	+	+			+			+	+		+	Facilitates PHC to HC transition	TR	[A/T]GATA[A/G]	Tricho-Rhino-Phalangeal syndrome
3. Helix-turn-helix transcription factors															
3.1. Homeodomain transcription factors															
HOX family	+							+				Specify skeletal element identity and early patterning	TA	(C/G)TAATTG	Synpolydactyly; hand-foot-genital syndrome
SHOX and SHOX2	+								+			Delay GPC maturation non-cell-autonomously	TA		Leri-Weill dyschondrosteosis and Langer mesomelic dysplasia
PRRX1	+								+			Contributes to skeletal early patterning	TA	AATTA	Agnathia-otocephaly
DLX5/DLX6	+					+	+		+		+	Promote PHC/HC differentiation	TA	[C/T]AATT[A/T]	Split-hand/foot malformation syndrome
NKX3.2/BAPX1	+	+		+						+		Promotes EC differentiation; represses GPC and ACC maturation	TR	TTAATTG	Spondylo-megaepiphyseal-metaphyseal dysplasia
3.2. Paired-domain transcription factor															
PAX1/PAX9	+								+			Support cell survival	TA/TR	GTCACGGT	
3.3. Forkhead-domain transcription factors															
FOXC1/FOXC2	+		+	+	+	+		+			+	Contribute to skeletal patterning; promote endochondral ossification	TA	[A/C]AA[C/T]	

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Transcription factors	Expression											Cell fate and differentiation actions	Trans cr. role	DNA-binding motif	Human skeletal diseases
	SSC	PC	EC	CC	PHC	HC	TC	periC	JP	ACC	OB				
FOXA2/FOXA3				+	+	+						Promote HC differentiation	TA	[A/C]AA[C/T]	
FOXP1/FOXP2/FOXP4	+	+						+	+			Delay GP maturation and EO	TR	T[G/A]TTTAC[T/A[T/C][T/A]	
FOXO1/FOXO3/FOXO4	+	+	+	+	+	+	+	+	+	+	+	Promote TC maturation	TA	[T/G]TAAACA[A/T]	
3.4. Tryptophan cluster transcription factors															
ERG	+	+							+			Promotes AC homeostasis	TA	ACAGGAA[G/A]	
4. Basic-domain transcription factors															
4.1. Basic helix-loop-helix (bHLH) transcription factors															
TWIST1	+							+				Prevents SSC differentiation into skeletal cells	TR	CANNTG, E-box	Saethre-Chotzen syndrome
HIF1α/2α	+	+	+	+	+	+	+	+	+	+	+	Promote JP and GPC survival and differentiation	TA	CANNTG, E-box	
HES and HEY	+		+	+	+	+		+				Delay EC differentiation; promote HC differentiation	TR	CACGTGC	
SREBP1/2	+	+	+	+	+	+	+	+	+	+	+	Essential for lipid homeostasis	TA	ACTACANNTCCC	
4.2. Basic leucine-zipper (bZIP) transcription factors															
4.2.1. ATF/CREB family													TA/TR	GTGACGT[A/C][A/G]	
ATF4/ATF3	+	+	+	+	+	+	+	+	+	+	+	Promote CC proliferation and PHC differentiation			
ATF2 and CREB	+	+	+	+	+	+	+	+	+	+	+	Promote CC proliferation			
ATF6			+	+	+	+						Promotes HC differentiation			
BBF2H7			+	+	+							Promotes CC proliferation and PHC differentiation			
4.2.2. AP1 family													TA	(TGA[G/C]TCA) or TGACGTCA	
cJUN					+	+	+	+	+		+	Represses the chondrogenic fate in JPs; may promote GPC maturation			
FOS								+			+	Delays HC differentiation			
FOSL2					+	+	+	+			+	Promotes HC differentiation			
4.2.3. MAF family															
c-MAF and MAF-B		+				+	+	+				Promote endochondral ossification		Multicentric carpotarsal osteolysis syndrome (*MAFB*)	
4.2.4. CCAAT-enhancer-binding protein family															
C/EBPβ and C/EBPδ	+	+	+	+	+	+	+	+	+	+	+	Promote PHC/HC differentiation			

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Transcription factors	Expression											Cell fate and differentiation actions	Trans cr. role	DNA-binding motif	Human skeletal diseases
	SSC	PC	EC	CC	PHC	HC	TC	periC	JP	ACC	OB				
4.3. Basic leucine-zipper (bZIP) transcription factors															
4.3.1. AP2 family														GTCGGTG	
AP2α, β, and γ	+		+	+	+			+				SSC survival and proliferation; delay GPC maturation			
AP2ε						+				+	+	Repress *Col2a1* expression in HC			
5. Transcription factors with a β-hairpin exposed by a α/β-scaffold															
NFIC/X	+	+	+	+	+	+	+	+	+	+	+	Promote endochondral ossification	TA/TR	TTGGC(N5)GCCAA	Marshall-Smith and Sotos syndromes
SMAD1 to 9	+	+	+	+	+	+	+	+	+	+	+	Promote chondrogenesis at virtually every step	TA/TR	GC-rich motifs (CTGGAGCC)	

Abbreviations: ACC, articular chondrocyte; CC, columnar chondrocyte; EC, early chondrocyte; EO, endochondral ossification; GP, growth plate; GPC, growth plate chondrocyte; HC, hypertrophic chondrocyte; JP, joint progenitor cells; OB, osteoblast; PeriC, perichondrium cell; PC, prechondrocyte; PHC, prehypertrophic chondrocyte; SSC, skeletal progenitor/stem cell; TC, terminal chondrocyte; TA, trancriptional activator; TR, transcriptional repressor