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SOX9 in cartilage development and disease

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Abstract

SOX9 is a pivotal transcription factor in chondrocytes, a lineage essential in skeletogenesis. Its mandatory role in transactivating many cartilage-specific genes is well established, whereas a pioneer role in lineage specification, which along with transactivation defines master transcription factors, remains elusive. Abundant, but yet incomplete evidence exists that intricate molecular networks control SOX9 activity during the multi-step chondrogenesis pathway. They include a highly modular genetic regulation, posttranscriptional and posttranslational modifications, and varying sets of functional partners. Fully uncovering SOX9 actions and regulation is fundamental to explain mechanisms underlying many diseases that directly or indirectly affect SOX9 activity and to design effective disease treatments. We here review current knowledge, highlight recent discoveries, and propose new research directions to answer remaining questions.

Introduction

Cartilage, a distinctive and essential tissue in vertebrates, is built and maintained by a unique cell type, the chondrocyte. Chondrocytes derive in embryogenesis from multipotent skeletal progenitor cells, as do osteoblasts and a few other cell types. Upon lineage specification, chondrogenic cells condense and undergo early differentiation to establish cartilage primordia that constitute the primary skeleton of the embryo [1]. They then remodel the tissues into growth plates or permanent cartilage. Growth plates are temporary structures that ensure skeletal elongation and the progressive replacement of cartilage by bone. They are made of layers of chondrocytes at sequential stages of differentiation: early-stage cells proliferating in columns, and terminally maturing prehypertrophic and hypertrophic cells. The latter eventually die or participate in endochondral ossification through osteoblastic transdifferentiation. In joints and in the respiratory and auditory systems, chondrocytes mature into quiescent, lowly anabolic cells that ensure permanent cartilage homeostasis throughout life. Elaborate molecular networks control chondrocyte specification and differentiation [1–3]. They mediate their effects at the genetic level through specific sets of transcription factors, of which SOX9 is central [4]. SOX9 belongs to a family of twenty

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Conflict of interest statement

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SRY-related HMG box-containing (SOX) proteins, most of which contribute to cell type specification and differentiation in discrete lineages [5]. The first clue that SOX9 is essential in development, including chondrogenesis, came with the discovery that heterozygous mutations occurring within and around *SOX9* cause Campomelic Dysplasia (CMPD), a severe skeleton malformation syndrome often associated with XY sex reversal, and milder skeletal dysplasias, namely acampomelic campomelic dysplasia (ACMPD) and Pierre Robin Sequence (PRS) [6–9]. Studies in animal models and molecular studies have uncovered many aspects of SOX9's specific roles and modes of actions and regulation in development and diseases, but many questions remain. It is thus a good time to review current knowledge, underline recent discoveries, and set priorities for future studies.

Roles and partners of SOX9

Lineage-tracing approaches using mouse $Sox9^{Cre}$ and $Sox9^{CreER}$ knockin alleles have shown that many progenitor cell types express *Sox9* and give rise to more cell types than those currently known to be SOX9-dependent [10,11]. In the skeletal system, these cells include osteoblasts, tenocytes and synovial fibroblasts, but solid evidence is still lacking that SOX9 is needed in skeletal progenitors and in other progeny than chondrocytes. Whole transcriptome profiling assays (RNA-seq) detected few changes between $Sox9^{wild\text{-}type}$ and $Sox9^{null}$ mouse embryo limb bud skeletogenic cells, and whole epigenome and targetome assays (ChIP-seq) showed that SOX9 helps, but is not required to, remove transrepression marks and deposit active promoter and enhancer marks at chondrocyte-specific genes [12]. These findings are consistent with a contributing rather than mandatory pioneer role of SOX9 in setting a chondrogenic chromatin landscape, but more work is needed to identify the chondrogenic pioneers that act before or with SOX9 and thereby answer the important question of whether SOX9 is a master chondrogenic factor, i.e., a transcription factor involved in both the epigenetic and the transcriptional governance of the chondrocyte lineage. FOX transcription factors, including FOXC, FOXF, and FOXP proteins, are chondrogenic pioneer candidates: they are expressed before and at the onset of chondrogenesis; they are necessary for proper chondrogenesis; FOX-binding sites are enriched near SOX9-binding sites on chondrocyte enhancers; and several family members are pioneers in non-chondrogenic processes [13–17].

Precartilaginous condensation is the first morphologically recognizable step in chondrogenesis. It is absolutely dependent upon SOX9 [18] (Figure 1), but its effector and other regulatory mechanisms remain largely elusive. Recent effort using *Sox9* mutant mouse limb buds have indicated that SOX9 upregulates genes for specific autocrine and paracrine regulatory factors and for cytoskeleton and actin network components [12]. These candidate genes for the implementation of precartilaginous condensation warrant full validation.

Overt chondrogenesis occurs as condensed prechondrocytes differentiate into early-stage chondrocytes, cells whose main activity is to produce cartilage extracellular matrix. This step requires SOX9 and SOX5/SOX6 [18,19]. SOX5 and SOX6 are structurally and functionally very similar to one another, but not to SOX9, and act redundantly [19–22]. They cooperatively bind with SOX9 on active enhancers and super-enhancers associated with hundreds of cartilage-specific genes, and thereby potentiate SOX9's ability to transactivate

[14,15]. Occasional binding to the promoters of ubiquitously expressed genes and to transcriptionally repressed gene loci suggest that the trio may also upregulate and inhibit more genes than those specifically expressed in early chondrocytes [15,23]. The RUNT-domain factor RUNX1 also contributes to overt chondrogenesis, and was recently shown to physically interact with the SOX trio and enhance activation of chondrocyte-specific genes [24]. This finding is in line with the abundance of RUNX binding sites near SOX trio motifs in chondrocyte enhancers [14,15]. ChIP-seq and transactivation (reporter) assays in chondrocytes should be considered to fully validate this RUNX1/SOX partnership. Of note, this role of RUNX1 is distinct from that of RUNX2 and RUNX3, which drive growth plate chondrocyte maturation [1].

The SOX trio remains required to maintain chondrocyte at the early differentiation stage as they proliferate in columns in growth plates and maintain permanent cartilage homeostasis. This activity may involve cooperativity between SOX9 and the Hedgehog pathway-dependent GLI transcription factors [25]. Despite being still highly expressed in prehypertrophic chondrocytes, the SOX trio delays progression to this stage and inhibits rather than activates prehypertrophic marker genes. The three SOX RNAs abruptly vanish and the SOX9 protein (SOX5/6 have not been tested) slowly decays as chondrocytes undergo overt hypertrophy [26]. SOX9 is required at this stage to prevent cell apoptosis, to express to at least some of the early hypertrophic markers, and to prevent chondrocytes from converting to the osteoblast lineage [19,20,26–28]. These activities namely involve keeping RUNX2 and canonical WNT signaling levels in check. Besides SOX5/SOX6, co-factors of SOX9 likely include the JUN and FOSL2 AP1 factors [29]. Cooperativity between GLI, JUN/FOSL2 and SOX9 is based on frequent binding sites for these factors near those of SOX9 in chondrocyte enhancers [14,15] and in vivo evidence of the expression and importance of the factors in chondrogenesis [25,29]. In contrast, SOX9 may compete with FOXA2 to delay terminal chondrocyte maturation [25]. Complementary studies are warranted to fully validate these findings and possibly identify additional factors.

SOX9 structure/function

Reaching deep understanding of the molecular actions of SOX9 and how diseases may disrupt them requires detailed knowledge of the protein’s structure/function attributes. Such information is still being gathered. Like all twenty SOX proteins, SOX9 features an SRY-related high-mobility-group (HMG) domain (Figure 2A) [5]. This domain penetrates the DNA minor groove and binds motifs matching or resembling C[A/T]TTG[A/T][A/T]. This property combines with DNA bending and protein interaction abilities to establish transcriptional complexes. This domain also harbors the nuclear import and export signals of the protein. Within the SOX family, SOX9 forms the SOXE group with SOX8 and SOX10 because the three proteins share a higher degree of identity with one another in the HMG domain than with other SOX proteins and also feature group-specific domains. One of these domains (DIM) confers preferential binding t targets at sites containing inverted SOX motifs separated by 3–4 nucleotides (Figure 2B). Data were recently provided that DIM may promote protein homodimerization, not through DIM:DIM interactions, as long thought, but through DIM:HMG interactions [30]. Consistent with tight structures and essential functions

of these domains, all CMPD-causing *SOX9* missense mutations occur in the HMG and DIM domains (Angelozzi and Lefebvre, in press).

The C-terminus of SOX9 has been known to contain a potent transactivation domain, called TAC (transactivation domain at the C-terminus), ever since nonsense mutations located before or within TAC were found to cause CMPD [31] (Figure 2A). By definition, transactivation domains contact the promoter-based basal transcriptional machinery directly or via transcriptional co-activators. Accordingly, TAC can interact with the transcriptional co-activators CBP and P300 [15,32] and MED25 (Mediator Complex Subunit 25) [33]. In contrast, its interaction with β-catenin inhibits transactivation by both proteins and induces their degradation [34]. TAC is about 100-residue-long, but the residues critical for transactivation and other protein interactions remain undeciphered. Very recently, another transactivation domain was identified in the middle of SOX9 (TAM) [35]. Both TAM and TAC have intrinsic transactivation capability and the two domains synergize to activate chondrocyte-specific genes and reporters in vitro. The minimal sequence required for TAM activity is 40-residue long. It is predicted to form a protein-binding pocket, but its preys remain unknown. These data, along with demonstration that SOX10 TAM is essential in mouse development [36], strongly suggest that SOX9 decisively involves TAM in its chondrogenic actions. Finally, vertebrates have evolved a unique domain in SOX9 [35,37]. This domain of up to 45 residues is called PQA because it contains only prolines, glutamines and alanines. It helps stabilize SOX9 and facilitates transactivation in vitro, but lacks intrinsic transactivation capability. Only two missense mutations in TAC and one microdeletion in PQA have been reported to be pathogenic so far, and all resulted in disorders of sex development (DSD) with ACMPD or no skeletal defects [38,39]. The mildness of the diseases may be due to the mutation types or to the fact that, unlike HMG and DIM, TAM, TAC and PQA are less structured and can therefore better tolerate point variants. This finding should not undermine the likelihood that each domain may be critical for SOX9's chondrogenic and other activities and thus deserves further investigations.

SOX9 post-translational modifications

The chondrogenic activities of SOX9 depend not only on functional partners' availability and protein integrity, but also on post-translational modifications (Figure 2A). Several have been detected and linked to cartilage development and diseases, but their functional impact in vivo remains unvalidated [40]. Among them, phosphorylation of S64 and S181 has received much attention. It was shown to increase SOX9 transcriptional activity in vitro and proposed to help delay chondrocyte maturation downstream of PTHrP signaling in a PKA (cAMP-dependent protein kinase A)-dependent manner [41,42]. S64 and S181 phosphorylation may also be achieved by other kinases downstream of other pathways. For instance, it was proposed to occur and promote SOX9 SUMOylation downstream of BMP and canonical WNT signaling during neural crest delamination [43]. Further, SHP2, a Src homology domain-containing tyrosine phosphatase and key player in RAS/MAPK signaling, was recently found to be critical for proper skeletal patterning and growth [44]. It was shown to block SOX9 phosphorylation by PKA and hence subsequent SUMOylation. Of note, gain-of-function mutations in *PTPN11* (encoding SHP2) cause Noonan and LEOPARD syndromes, which include skeletal dysplasia, whereas loss-of-function mutations cause

metachondromatosis. Ubiquitination, acetylation and deacetylation of SOX9 were also suggested to be decisive. Recently, *DDRGK1* mutations were shown to cause Shohat-type spondyloepimetaphyseal dysplasia [45]. DDRGK1, a component of the UFM1 conjugation pathway, was found to inhibit SOX9 ubiquitination and subsequent proteasomal degradation. Its SOX9 residue target remains uncharacterized, but be K398 or other residues previously shown to be ubiquitinated [46]. Sirtuins are NAD^+-dependent lysine deacetylases involved in many processes, including chondrogenesis. *Sirt1*$^{+/-}$ mice are growth-delayed and develop osteoarthritis precociously, and *Sirt1*$^{-/-}$ pups neonatally with severe skeletal malformations [47], whereas *Sirt7*$^{-/-}$ mice are resistant to osteoarthritis [48]. SIRT1 may promote chondrogenesis by increasing SOX9 nuclear localization [49]. It can deacetylate SOX9, but the affected residues remain unknown. In contrast, SIRT7 suppresses SOX9 activity in vitro, but appears unable to deacetylate SOX9, leaving open its mechanism of action [48]. Altogether, these studies point to posttranslational mechanisms that could be paramount in chondrogenesis and thus call for more investigations to fully validate them and likely to uncover many more.

SOX9 gene regulation

The specific spatiotemporal pattern of the *SOX9* RNA implies that *SOX9* regulation starts at the gene and RNA levels. Many pathways have been shown to control *SOX9* expression in chondrogenesis: Hedgehog, PTHrP, BMP, TGFβ, FGF, non-canonical WNT and hypoxia signaling induce or upregulate it, whereas canonical WNT, NOTCH, retinoic acid and inflammatory pathways decrease it [1,2]. Their importance is illustrated by the fact that mutations in various components of these pathways cause skeletal dysplasias. An example is achondroplasia, due to activating mutations in *FGFR3* [50]. Focusing on the *SOX9* proximal region to explain changes in *SOX9* expression, these studies have provided in vitro evidence that HIF-1α (hypoxia) [51], SMAD (BMP) [52], RBPjk/NICD (NOTCH) [53], STAT3 (many cytokine and growth factor pathways) [54], and YAP1/TEAD (Hippo) [55] bind specific motifs in this region (Figure 3). While proximal regulatory elements may indeed critically modulate *SOX9* expression, it is worth noting that the *SOX9* promoter is insufficient to drive reporter gene expression in chondrocytes and other SOX9-expressing cells in transgenic mice [56,57]. Thus, distant elements may also mediate the effects of signaling pathways.

The discovery that translocations and microdeletions occurring far around *SOX9* cause CMPD, ACM PD, PRS or DSDs provided the first clue years ago that *SOX9* cis-acting elements are numerous and spread over large distances (Figure 3). Delineation of the *SOX9* topologically associated domain to the 2-Mb gene desert around *SOX9* has confirmed this notion [58]. To date, a dozen enhancers have been delineated throughout this domain and have been shown to be active in multiple or discrete tissues, chondrocyte-specific stages, or anatomical sites [8,56,57,59–61]. Moreover, a recent GWAS study linked nose shape traits in humans to four loci located between 91 and 974 kb upstream of *SOX9* and containing active promoter/enhancer marks [62]. Together, current knowledge supports the notion that SOX9 is modularly controlled, likely by more enhancers than currently known, and that variants in enhancers may contribute to skeletal pattern diversity among humans as well as among vertebrate species.

Identifying the transcription factors that control *SOX9* enhancers is a difficult task still in its infancy. SOX9 can positively regulate several of its gene enhancers [57,59], leading to the important concept that pathways affecting SOX9 protein stability may secondarily affect *SOX9* gene expression through this feedback loop. There is also evidence that SOX5/SOX6 contribute to the activity of several *SOX9* enhancers [59], that STAT3 targets a rib-specific enhancer [61], and that MSX1, a homeodomain transcription factor involved in limb and craniofacial development, controls a mandible-specific SOX9 enhancer [8]. Further, very recent ChIP-seq assays for PITX1, a bicoid-class homeodomain transcription factor required for hindlimb development, have uncovered a dozen of PITX1-bound limb enhancers upstream and downstream of *SOX9*, consistent with *Sox9* downregulation in $Pitx1^{-/-}$ mouse embryos [63].

Finally, several miRNAs and IncRNAs have been shown to affect SOX9 RNA level or translation during chondrogenesis in vitro [40]. Direct roles for miRNAs are supported by conserved binding sites in the *SOX9* 3' untranslated region. Recently, the IncRNAs referred to as *ROCR* (regulator of chondrogenesis RNA) were located 94 kb upstream of *SOX9* and found critical for *SOX9* expression and successful chondrogenesis of human mesenchymal stem cells [64]. In all cases, in vivo validation remains to be provided.

Summary and outlook

Ever since *SOX9* mutations were linked to campomelic dysplasia, substantial research efforts have been devoted towards uncovering the ins and outs of the gene in the chondrocyte lineage. To date, the roles of SOX9 at the multiple steps of chondrocyte differentiation are fairly well understood, although more work should still be done to assess the impact of SOX9 binding to many genes with known or yet-unknown roles in cartilage. Less understood, despite a potential for breakthroughs, is the impact of *SOX9* expression on progenitor cells. Further, key mechanisms involved in SOX9 regulation at the gene, RNA and protein level have been uncovered, but have also led us to humbly acknowledge that the SOX molecular network is likely far more sophisticated than currently appreciated. Important goals for the future are to continue to dissect the many nuts and bolts of SOX9 actions and regulation. Definitive knowledge will come with the use of cutting-edge approaches in vitro and in vivo, including the Crispr/Cas technology to genetically modify transcribed and cis-acting regions of SOX9, its targets, co-factors and regulators. New findings are promised to be insightful for many areas of fundamental science, for better grasping the mechanisms underlying a large number of cartilage diseases and for designing better strategies to treat these diseases.

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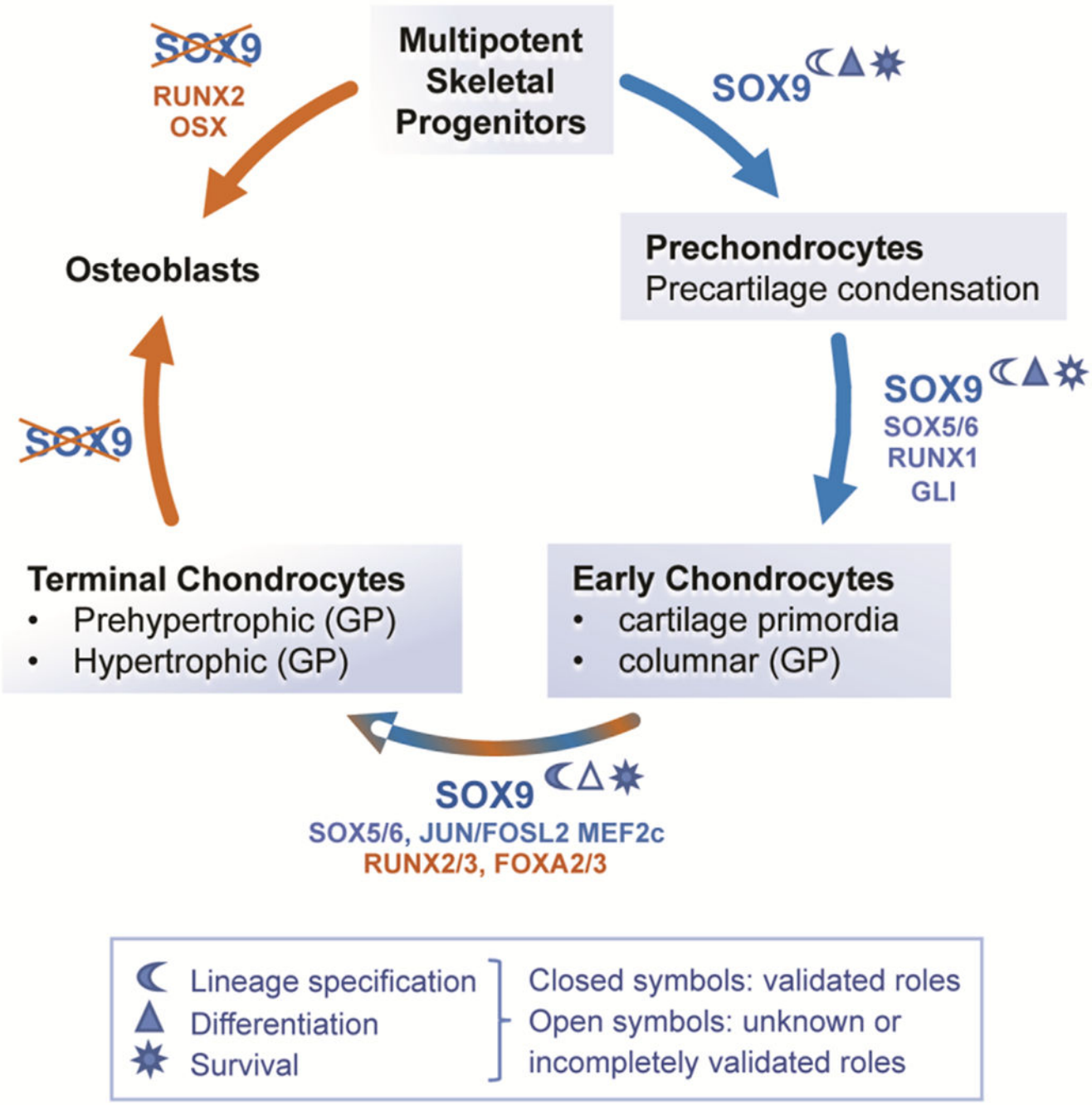

Figure 1.

Current knowledge and gaps in knowledge regarding SOX9's main roles in skeletal progenitors, chondrocytes and osteoblasts. *SOX9* is expressed from the multipotent skeletal progenitor stage until chondrocyte hypertrophic differentiation (blue shading of boxes) and has been shown to be involved in chondrocyte lineage specification, differentiation, and survival. Repression of *SOX9* is required for osteoblast differentiation from progenitor cells and terminal chondrocytes. Confirmed and candidate co-factors of SOX9 are indicated (blue) as well as other factors involved in specific cell differentiation stages (brown).

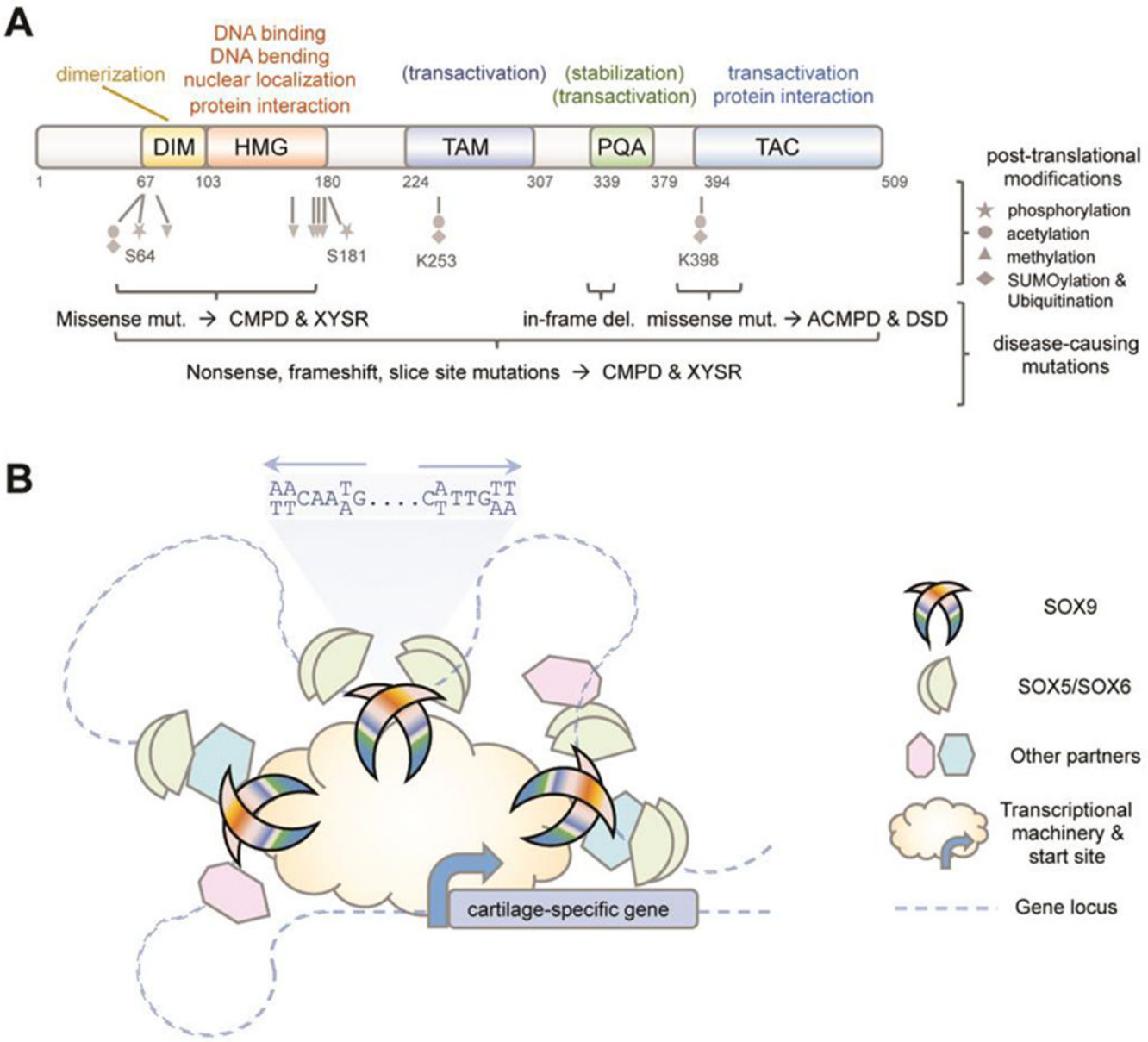

Figure 2.

Current knowledge of SOX9 protein's modes of action and regulation. (A) Domain organization of the protein, with indication of functions validated in vivo (no parentheses), functions identified only in vitro (in parentheses), and post-translational modifications identified only in vitro (grey). The location of mutations (mut.) causing diseases as severe as campomelic dysplasia (CMPD), XY sex reversal (XYSR), acampomelic CMPD (ACMPD) and disorders of sex development (DSD) are indicated (brackets). (B) Schematic of the main mode of action of SOX9 in chondrocytes. SOX9 binds as a homodimer to pairs of inverted SOX recognition sites on multiple enhancers associated with cartilage-specific genes. It functionally interacts with SOX5/SOX6 and other co-factors that bind to nearby sites on the enhancers. SOX9 uses its transactivation domains to contact transcriptional co-activators and basal transcriptional machinery components and thereby induce gene transactivation.

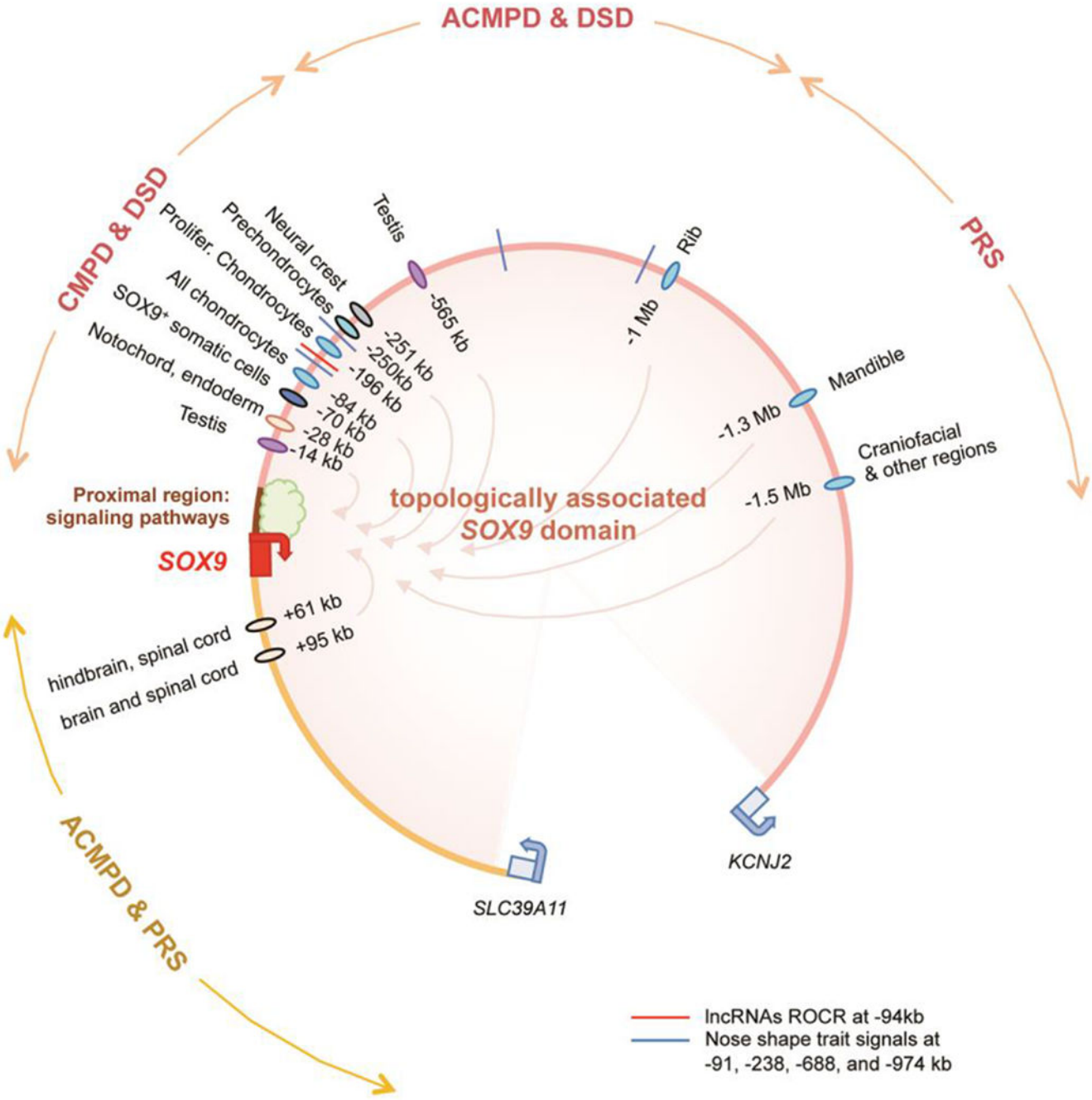

Figure 3.

Current knowledge of the complex modular organization of *SOX9* cis-acting elements. The domain topologically associated with *SOX9* spans 1.9 Mb upstream and 0.5 Mb downstream of the gene. The *SOX9* proximal region is believed to be a hub for signaling pathways. The distal regions house at least a dozen and likely more enhancers driving gene expression in various tissues, chondrocyte differentiation stages, and cartilage anatomical sites. The upstream region also houses *ROCR, SOX9*-regulating lncRNAs. Chromosomal translocations, microdeletions and duplications in the distal regions cause chondrodysplasias

(CMPD, ACMPD, and PRS) and disorders of sex development (DSD) with a severity generally proportional to their proximity to the *SOX9* gene body.