Basic Helix-Loop-Helix Transcription Factor Twist1 Inhibits Transactivator Function of Master Chondrogenic Regulator Sox9

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Background: Twist1 inhibits chondrogenesis through an undefined mechanism.

Results: Twist1 binds directly to Sox9 and inhibits both Sox9-dependent gene activation and Sox9 binding to target gene enhancer DNA in chondrogenic cells.

Conclusion: Twist1 inhibits Sox9 the transactivator function by impeding its sequence-specific DNA-binding activity.

Significance: These findings provide a mechanistic basis for the antichondrogenic activity of Twist1.

Canonical Wnt signaling strongly inhibits chondrogenesis. Previously, we identified Twist1 as a critical downstream mediator of Wnt in repression of chondrocyte differentiation. However, the mechanistic basis for the antichondrogenic activity of Twist1 has not heretofore been established. Here, we show that Twist1 suppresses cartilage development by directly inhibiting the transcriptional activity of Sox9, the master regulator of chondrogenesis. Twist1, through its carboxyl-terminal Twist-box, binds to the Sox9 high mobility group DNA-binding domain, inhibiting Sox9 transactivation potential. In chondrocyte precursor cells, Twist1, in a Twist-box-dependent manner, inhibits Sox9-dependent activation of chondrocyte marker gene expression by blocking Sox9-enhancer DNA association. These findings identify Twist1 as an inhibitor of Sox9 and further suggest that the balance between Twist1 and Sox9 may determine the earliest steps of chondrogenesis.

Cartilage is a highly specialized and fundamentally essential tissue in vertebrates. It is the basic foundation for the majority of bone tissue produced during development and a critical component of adult joint and airway construction, where it facilitates bone articulation, ameliorates stress impact, and lessens load (1–3). During skeletogenesis, cartilage anlagen appearing at the location of future skeletal elements collectively constitute the primary template upon which bone is subsequently laid in a process termed endochondral ossification (3). Construction of the cartilage anlagen (chondrogenesis) involves the initial aggregation of bipotent mesenchymal cells (osteochondroprogenitors) that subsequently differentiate into proliferating chondrocytes that express abundant cartilage extracellular matrix, including collagen-2 (Col2a1) and aggrecan (Agc1) (4, 5). As skeletogenesis progresses, proliferating chondrocytes progressively exit the cell cycle and undergo hypertrophy prior to maturation into terminal chondrocytes that mineralize their surrounding extracellular matrix and eventually apoptose to be replaced by bone-forming cells, including osteoclasts, osteoblasts, and bone marrow constituents (3).

The initial specification and subsequent differentiation of chondrocytes from osteochondroprogenitors is governed by the coordinate action of morphogenetic signals and responsive transcriptional regulators that drive the expression of genes important for chondrocyte determination, identity, and function. Among the transcription factors linked to control of chondrocyte development, the sex determining region Y (SRY)-related high mobility group (HMG)3 box transcription factor Sox9 has emerged as a master regulator of chondrogenesis. Initially identified as the locus targeted for mutational inactivation in campomelic dysplasia, a severe human chondrodysplasia, Sox9 was subsequently implicated through both molecular cell biology- and mouse genetics-based approaches as a master chondrogenic gene (6, 7). In this regard, Sox9 is expressed in osteochondroprogenitors and prehypertrophic chondrocytes, where it activates chondrocytic marker genes, including Col2a1 and Agc1 (8–13), and its genetic ablation or ectopic expression is sufficient to block or promote chondrocyte differentiation, respectively (14, 15). Sox9 transactivator function during chondrogenesis is further potentiated by two redundantly acting Sox proteins, 1–Sox5/Sox6, that act to promote the binding of Sox9 to chondrocyte marker gene enhancers, possibly by chromatin remodeling (16, 17).

Although Sox9 is expressed in osteochondroprogenitors, these cells nonetheless retain bipotent differentiation properties, and signaling constraints on its chondrogenic activity must therefore be enforced to maintain an uncommitted fate. In this regard, we and others have shown previously that signaling through the canonical Wnt/β-catenin pathway strongly inhibits chondrogenesis and that Wnt signaling has been proposed

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3 The abbreviations used are: HMG, high mobility group; bHLH, basic helix-loop-helix; Luc, luciferase.
to function as a molecular switch between chondrocytic and osteoblastic fates during mesenchymal progenitor cell differentiation (18–20). The mechanism by which Wnt/β-catenin signaling represses chondrocyte differentiation remains to be fully clarified. Although it may involve, at least in part, repression of Sox9 gene expression, this has not been established conclusively, and additional mechanisms are possibly implicated. In this regard, we identified previously the transcriptional regulator Twist1 as a downstream mediator of Wnt signaling in repression of chondrocyte differentiation (18). In chondrogenic cells, we found that canonical Wnt signaling induces Twist1 expression, which is conversely diminished in parallel with expression of certain Wnt family members (18). Furthermore, in chondrocyte precursors, ectopic expression of Twist1 repressed, whereas Twist1 depletion enhanced expression of chondrocyte marker genes, including Col2a1 and Agc1 (18).

Twist1 is an essential and multifunctional protein implicated in a diverse range of normal and pathological processes spanning development and differentiation to congenital malformation syndromes and cancer. First identified in Drosophila as a critical determinant of gastrulation and mesoderm formation, Twist1 is phylogenetically conserved (21, 22). In mammals, Twist1 is developmentally expressed in mesoderm-derived embryonic tissues and postnatally in adult mesoderm-derived mesenchymal stem cells, where it functions as a major regulator of mesenchymal cell differentiation (23, 24). Consistent with its critical role in mesoderm development, inactivating mutations in the Twist1 gene elicit the autosomal dominant inherited disorder Saethre-Chotzen syndrome, characterized by a broad range of congenital anomalies, including short stature, craniosynostoses, high forehead, ptosis, small ears, and maxillary hypoplasia with high and narrow palate (25, 26). More recently, Twist1 has also been shown to function as an inducer of the epithelial-to-mesenchymal transition and a driver of cancer metastasis (27–30).

The pleiotropic activities of Twist1 derive from its role as a bipotent transcriptional regulator capable of activating or repressing target gene expression through direct or indirect mechanisms. As a member of the basic helix-loop-helix (bHLH) family of DNA-binding transcription factors, Twist1 is characterized by a domain encompassing a region of basic amino acids adjacent to two α-helices separated by an interhelical loop (31). Homodimerization or heterodimerization between Twist1 and other bHLH family members leads to juxtaposition of the basic domains, generating a composite interface through which Twist1 dimers specifically bind to DNA regulatory elements (E boxes) present in the promoters of Twist1 target genes, many of which contribute to the specification of mesenchymal cell phenotypes (32, 33). Activation or repression of target gene transcription by promoter-bound Twist1 is determined by its differential recruitment of stimulatory or inhibitory coregulators that function to modify chromatin and/or regulate RNA polymerase II transcription complex formation (33). Beyond its ability to directly regulate target gene transcription through interaction with E box elements, Twist1 can also function to indirectly modulate transcription through physical interactions with DNA-binding transcription factors (32, 34). In this regard, Twist1 is known to bind to and inhibit MyoD/MEF2 and Runx2, master transcriptional regulators of myogenesis and osteogenesis, respectively, blocking the differentiation of mesenchymal progenitor cells down either of these lineages (32, 34). Thus, Twist1 appears to play a critical role in restricting cell differentiation by forming inhibitory interactions with master transcriptional regulators of mesenchymal cell fate. Our prior findings that Twist1 acts downstream of canonical Wnt signaling to negatively regulate chondrogenesis extends the inhibitory role of Twist1 to the chondrocyte lineage (18). However, the mechanistic basis by which Twist1 inhibits chondrocyte gene expression and differentiation has heretofore remained obscure. Here we provide evidence that Twist1 negatively regulates chondrocyte gene expression by direct inhibition of Sox9, the master regulator of chondrogenesis. We show that Twist1, through its carboxyl-terminal Twist-box, binds directly to the Sox9 DNA-binding domain. This interaction inhibits Sox9-dependent activation of chondrocyte marker gene expression by impeding Sox9-enhancer DNA association. These results thus identify a mechanistic basis for Twist1-mediated inhibition of chondrogenesis and reveal a new pathway for regulation of early cartilage formation.

EXPERIMENTAL PROCEDURES

Cell Culture—C3H10T1/2, ATDC5 (RIKEN cell bank), HEK293, mouse limb bud (MLB), and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO2 incubator. Normal human primary chondrocytes (NHAC) were purchased from Lonza (Walkersville, MD) and maintained in chondrocyte growth medium (CGM) (Lonza).

Transient Transfection and Luciferase Assay—C3H10T1/2, ATDC5, MLB, and HeLa cells were seeded into 12-well plates 24 h prior to transfection with the internal control pact-β-galactosidase expression plasmid and a specified luciferase reporter plasmid without or with either pcDNA-V5-Sox9 or pcDNA-Myc-Twist1. Transient transfections were performed using FuGENE6 (Roche) following the instructions of the manufacturer. Luciferase reporter plasmids correspond to the following: Sox9BS3-Luc contains three tandem 18-base pair Sox9 binding sites (35). Col2-Luc contains the rat Col2a1 promoter and enhancer (36). The luciferase assay was performed using a luciferase assay kit (Promega Corp., Madison, WI).

Coimmunoprecipitation—HeLa cells were cotransfected individually or together with expression plasmids pcDNA-myc-Twist1 and pcDNA-V5-Sox9. Cells were lysed 48 h posttransfection with 50 mM Tris 8.0, 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100, 1% deoxycholate, 50 μg/ml PMSF, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Whole cell lysates were precleared with protein G-Sepharose (GE Healthcare) and immunoprecipitated by incubation with anti-V5 antibody (Invitrogen) and protein G-Sepharose (GE Healthcare). Immune complexes were washed three times with 50 mM Tris 8.0, 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100, 1% deoxycholate, 50 μg/ml PMSF, and protease inhibitor mix at 4 °C. Immunoprecipitated proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and processed by Western
Twist1 Inhibits Sox9

Pull-down Assays—GST and GST-Twist1 derivatives were expressed in *Escherichia coli* strain BL21 (Stratagene, La Jolla, CA). Soluble lysates were prepared in resuspension buffer (1 × PBS, 2 μM MgCl₂, 0.5% Triton X-100, 5 μM DTT, 50 μg/ml PMSF, and protease inhibitor mixture). Resuspended cells were treated with 1 mg/ml lysozyme (Sigma-Aldrich) for 5 min at 4 °C, followed by sonication and clarification by centrifugation at 14,000 rpm for 30 min at 4 °C. Supernatants were collected and incubated with pre-cleaned glutathione-Septarose beads (GE Healthcare) for 1 h at 4 °C. WT and mutant Sox9 derivatives were expressed and radiolabeled with [35S]methionine using a TNT® quick-coupled transcription/translation system (Promega Corp.) followed by incubation with glutathione-Septarose-immobilized GST or GST-Twist1 derivatives in resuspension buffer for 4 h at 4 °C. Immobilized proteins were washed three times in resuspension buffer, and bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE gel, and detected by PhosphorImager analysis. For MBP pull-down experiments, MBP-Sox9 derivatives were purified from crude *E. coli* lysates on amylene resin (New England Biolabs, Beverly, MA). 35S-labeled Twist1 was prepared as described above and used for the pull-down assays.

Nucleofection and Real-time Quantitative PCR—Nucleofections were performed according to the instructions of the manufacturer using a Nucleofector® II instrument and Nucleofector® kit T (Amaxa Biosystems, Köln, Germany) RNA was isolated after 72 h following nucleofection using an RNAesy® mini kit (Qiagen, Germantown, MD). Quantitative PCR was performed using a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA) and Absolute SYBR Green ROX mix (ABgene Epsom, UK). Expression levels determined by quantitative PCR were normalized to an unaffected control, 18s. The primers used for real-time PCR are as follows: *Agc1*, 5′-GGAATCTCCTAGCTTCTCG-3′ and 5′-ACTG-CAGCGATGACCCCTC-3′ and 18s, 5′-CATGTGTTGTTTGTAG-GAAAAGCA-3′ and 5′-GTCGTGGGTTCTGCTAGT-3′.

Chromatin Immunoprecipitation—ChIP assays were performed as described previously (37). *Agc1* A1 enhancer primers were as follows: 5′-ATGTGACCTGGGTCAGATAAGGAA-3′ and 5′-GAATATTCTTCTTCCCTGAAAGCCGTC-3′, as described previously (17). The unrelated control primers used were β-actin 5′-GATCATATTGCTGCCTCTGCCG-3′ and 5′-GAGAGCTTAAAAACGCAG-3′.

RESULTS

Twist1 Inhibits Sox9 Transactivation Activity—Our prior studies revealed that Twist1 strongly suppresses the chondrocyte lineage (18), and we speculated that this repression is exerted through a transcriptional pathway that is centrally important for chondrocyte differentiation. Among the chondrogenic transcription factors that we considered as possible targets of Twist1, Sox9 is a master regulator of chondrogenesis that exhibits a developmental expression pattern partially overlapping that of Twist1 (38, 39). This observation, coupled with the fact that Twist1 targets other lineage-specific transcriptional regulators for inhibition, prompted us to ask whether Twist1 could also inhibit Sox9 transactivator function. To this end, we initially examined the ability of Twist1 to suppress transcription from a Sox9-dependent reporter plasmid following ectopic expression of both proteins in murine C3H10T1/2 multipotent mesenchymal stem cells. As expected, ectopic expression of Sox9 resulted in robust (~16-fold) activation of a Sox9-dependent reporter template bearing three multimimerized Sox9 binding sites upstream of a luciferase (Luc) gene (Sox9BS₃-Luc) (Fig. 1A). Notably, Sox9-dependent activation of this reporter plasmid was strongly inhibited by Twist1 in a dose-dependent manner (Fig. 1A). Twist1 also inhibited Sox9-dependent activation of a luciferase reporter template driven by the native Col2a1 enhancer (Fig. 1B).

Twist1-mediated inhibition of Sox9 transactivation function was specific because Twist1 had no influence on transcription driven by the *Herpes simplex* virus VP16 transactivation domain (Fig. 1C). Finally, Twist1-mediated inhibition of Sox9 was observed in a broad range of cell types other than C3H10T1/2 cells, including murine ATDC5 chondrocyte progenitor cells, murine MLB limb bud mesenchymal cells, and human HeLa cervical carcinoma cells (Fig. 1, D–F). Together, these findings reveal that Twist1 can specifically inhibit the transactivation function of Sox9.

The Twist1 C Terminus Is Required for Inhibition of Sox9 Transactivation Activity—To identify the domain(s) in Twist1 responsible for inhibition of Sox9, we comparatively assessed a panel of Twist1 truncation and missense mutants (Fig. 2A) for their respective abilities to inhibit Sox9-dependent transactivation of the Sox9BS₃-Luc reporter in C3H10T1/2 cells. This analysis revealed that deletion of the C terminus, but not the N terminus, of Twist1 significantly impaired its ability to inhibit Sox9 transactivation activity (Fig. 2B). Interestingly, deletion of the Twist1 N terminus produced a derivative (Twist1 ΔN) possessed of both its bHLH dimerization interface and C terminus that was a more potent Sox9 inhibitor than wild-type Twist1 (Fig. 2B). The Twist1 bHLH dimerization interface, however, was dispensable for Sox9 inhibition because a Twist1 derivative (H2pro) bearing a single amino acid substitution sufficient to disrupt this interface nonetheless still inhibited Sox9 transactivation activity (Fig. 2B). The deleterious impact of the H2pro mutation on Twist1 dimerization properties was confirmed by analysis in a MyoD-responsive reporter system. Compared with Twist1 WT, Twist1 H2pro was significantly compromised in its ability to inhibit MyoD-dependent transactivation (Fig. 2C), a function known to be dependent upon Twist1/MyoD heterodimer formation (34). Differences among Twist1 mutants in their respective abilities to inhibit Sox9 could not be attributed to differences in their relative expression levels because all mutants were expressed comparably in C3H10T1/2 cells (Fig. 2D). Taken together, these results implicate the Twist1 C terminus in inhibition of Sox9 transactivation function.

The Twist1 C Terminus Bounds Directly to the Sox9 DNA Binding Domain—Twist1 could inhibit the transactivation function of Sox9 through direct physical interaction or indirectly through alternative mechanisms. Accordingly, we sought to determine whether, and through what domains, Twist1 and Sox9 bind directly to each other. For this purpose, we compar-
atively examined GST-Twist1 WT, as well as GST-Twist1 N- and C-terminal truncation derivatives (Fig. 2A), for their respective abilities to bind to a panel of radiolabeled Sox9 truncation derivatives (Fig. 3A) produced by transcription and translation in vitro. GST-Twist1 WT bound to WT Sox9, indicating that the two full-length proteins interact in vitro (Fig. 3B), a finding confirmed by the ability of purified recombinant MBP-Sox9 (WT) to bind to WT Twist1 produced by transcription and translation in vitro (Fig. 3C). Confirmation that the two proteins interact directly was rendered by the observation that purified E. coli-expressed GST-Twist1 WT and MBP-Sox9 WT bound to one another in vitro (Fig. 3D). Notably, the Sox9-interaction profiles observed for Twist1 truncation mutants directly correlated with their respective Sox9 inhibitory potentials. Thus, Twist1 ΔN, a more potent inhibitor of Sox9 than Twist1 WT, also bound Sox9 WT more strongly than the latter (Fig. 3B). Conversely, Twist1 ΔC, a much weaker inhibitor of Sox9 than Twist1 WT, was severely compromised for Sox9 binding activity (Fig. 3B). The strict concordance between the Sox9-binding and inhibitory potentials of Twist1 and its truncation mutants strongly suggests that Twist1 inhibits Sox9 through direct physical interaction.

Reciprocal efforts to map the Twist1-binding domain on Sox9 revealed that neither deletion of the Sox9 N terminus (Sox9 ΔN) nor the Sox9 C terminus (Sox9 ΔC) impaired its ability to bind to GST-Twist1 (WT or ΔN) (Fig. 3, A and B). Significantly, deletion of the Sox9 DNA-binding domain (Sox9 ΔHMG) abrogated the ability of Sox9 to bind to GST-Twist1 (WT or ΔN), thus delimiting the relevant Twist1 interaction surface on Sox9 to its HMG DNA-binding domain (Fig. 3B). Interestingly, the isolated Sox9 HMG domain bound promiscuously to all GST-Twist1 truncation derivatives, including Twist1 ΔC, indicating that sequences outside of the Sox9 HMG domain impart binding specificity on this surface (Fig. 3B). Taken together, these results identify the principal reciprocal interaction surfaces on either protein to encompass the C terminus of Twist1 (amino acids 173–206) and the HMG domain within Sox9 (amino acids 100–187).

To determine whether Twist1 and Sox9 interact in vivo, we examined the ability of the two proteins to coimmunoprecipitate with one another following their ectopic expression in HeLa cells. This analysis revealed specific precipitation of Myc epitope-tagged Twist1 by V5-specific antibodies only in the presence, but not in the absence, of coexpressed V5 epitope-
tagged Sox9 (Fig. 3E). These results confirm that Twist1 and Sox9 associate in mammalian cells.

Inhibition of Sox9 Transactivation Function by Twist1 Requires Its C-terminal Twist Box—Previously, Twist1 was identified as an inhibitor of osteoblast differentiation, and its antiosteogenic activity was mapped to a novel domain within its C terminus, the Twist-box spanning amino acids 186–206, that interacts with the DNA-binding domain of Runx2 to inhibit its function as a transcriptional activator (32). Our observation that Twist1 inhibits Sox9 transactivation function in a manner requiring its C terminus prompted us to examine the possible contribution of the Twist-box to Twist1-mediated Sox9 inhibition. To this end, we comparatively evaluated Twist1 WT and mutant derivatives bearing individual amino acid substitutions within the Twist-box for their respective abilities to inhibit Sox9-dependent reporter gene activation in C3H10T1/2 cells. As expected, ectopic expression of Twist1 WT significantly inhibited Sox9 transactivation activity (Fig. 4, A and B). Individual substitutions at either of two conserved amino acids within the Twist-box (L187G, F191G) slightly but reproducibly impaired the ability of Twist1 to inhibit Sox9 transactivation activity, whereas substitution of a third conserved amino acid (R195G) was without effect (Fig. 4, A and B). However, combinatorial substitution of all three amino acids (LFR3G, L187G, F191G, R195G) significantly impaired the Sox9 inhibitory function of Twist1 (Fig. 4, A and B) and also severely diminished its Sox9-binding activity (C and D). Taken together, these results strongly suggest that direct interaction between the Twist1 Twist-box and Sox9 underlies Twist1-mediated inhibition of Sox9 transactivation activity.

To extend these findings to a more biologically relevant setting, we comparatively evaluated Twist1 WT and C-terminal mutant derivatives for their respective abilities to inhibit Sox9-dependent expression of the chondrocyte marker gene Agc1 in ATDC5 chondrocyte precursor cells. We first confirmed Agc1 to be a target of Sox9 regulation in ATDC5 cells using lentiviral-based shRNA-mediated Sox9 depletion. Relative to control shRNA, Sox9-specific shRNA significantly reduced expression of both Sox9 and Agc1, validating Sox9 as an activator of Agc1 expression in ATDC5 cells (Fig. 5A). Ectopic expression of Twist1 WT in ATDC5
FIGURE 3. The Twist1 C terminus binds to the Sox9 HMG domain. A, schematic diagram of Sox9 truncation/deletion derivatives used for in vitro binding assays. B, top panel, recombinant full-length Sox9 (WT) and its specified truncation/deletion derivatives were expressed and radiolabeled with [35S]methionine by translation in vitro prior to incubation with glutathione-Sepharose-immobilized GST or GST-Twist1 WT/truncation derivatives, as indicated. Bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and visualized by PhosphorImager analysis to detect Sox9 derivatives (top panel) or Coomassie Blue staining to detect GST-Twist1 derivatives (bottom panel). IN, 10% of the in vitro translated protein used for binding reactions. Note that in vitro-translated Sox9 WT migrates as a doublet during SDS-PAGE because of translation initiating at an internal methionine. C, recombinant full-length Twist1 WT was expressed and radiolabeled with [35S]methionine by translation in vitro prior to incubation with amylose-immobilized maltose binding protein (MBP) or MBP-Sox9 WT, as indicated. Bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and visualized by PhosphorImager analysis to detect Twist1 (top panel) or Coomassie Blue staining to detect MBP or MBP-Sox9 (bottom panel). D, purified GST or GST-Twist1 WT was incubated with purified amylose-immobilized MBP or MBP-Sox9 WT as indicated. Bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and processed by WB analysis using GST-specific antibodies to detect GST and GST-Twist1. E, myc-Twist1 and V5-Sox9 were expressed with or without one another in HeLa cells prior to immunoprecipitation (IP) of whole cell lysates using antibodies specific for the V5 epitope, as indicated. Immunoprecipitates were resolved by SDS-PAGE and processed by Western blot (WB) analysis using V5 or myc-specific antibodies as indicated. IN, 10% of the whole cell lysate used for IP reactions.
Twist1 Inhibits Sox9

Twist1 Inhibits the Sequence-specific Association of Sox9 with the agc1 Enhancer A1 in a Manner Requiring Its Twist-box—

Our observation that the Twist-box in Twist1 mediates both direct interaction with the Sox9 DNA-binding domain and inhibition of Sox9 transactivation activity led us to ask whether the two events are mechanistically related. To this end, we used ChIP analysis to comparatively evaluate the impact of Twist1 WT or Twist1 mutant derivatives, including Twist-box mutants, on the binding of Sox9 to the Agc1 A1 enhancer (17) in ATDC5 cells. In the absence of coexpressed Twist1, Sox9 was readily detected on the Agc1 A1 enhancer but not an unrelated sequence from the /H9252-actin gene, confirming sequence-specific association of Sox9 with the Agc1 enhancer in ATDC5 cells (Fig. 6, A and B). This finding is concordant with our observation that Sox9 regulates Agc1 expression in ATDC5 cells (Fig. 5, A). Importantly, coexpression of Twist1 WT, Twist1 ΔN, and Twist1 H2Pro, all of which bind to Sox9, significantly diminished occupancy of the Agc1 enhancer by Sox9 (Fig. 6, A and B). By contrast, Twist1ΔC and Twist1 LFR3G, derivatives compromised for Sox9 interaction because of truncation and mutation, respectively of their Sox9-interacting Twist-box, did not significantly diminish Agc1 enhancer occupancy by Sox9 (Fig. 6, A and B).
These results thus reveal a vital role for the Twist1 Twist-box in inhibition of Sox9 sequence-specific DNA-binding activity.

To corroborate this finding, we asked if Twist1 could inhibit the transactivation activity of Sox9 when the latter was artificially recruited to promoter DNA through a heterologous DNA-binding domain. When fused to the Gal4 DNA-binding domain, Sox9 readily activated transcription from a reporter template bearing multimerized Gal4 DNA-binding sites (Fig. 6D). Significantly, however, Gal4-Sox9-driven reporter gene activation was unaffected by coexpressed Twist1, indicating that Twist1 cannot inhibit the transactivation activity of promoter-bound Sox9. Together, these findings support the notion that Twist1 inhibits the transactivation function of Sox9 by impeding its sequence-specific DNA binding activity.

DISCUSSION

Twist1 is a critical modulator of mesenchymal stem cell fate during skeletogenesis. Prior studies have clearly established Twist1 as a negative regulator of osteoblast differentiation. During development, Twist1 is expressed in mesodermal-derived osteoblast progenitors, and its expression level decreases during the course of osteogenesis, suggesting that Twist1 functions to maintain an uncommitted cell fate (32). Furthermore, Twist1 overexpression inhibits, whereas its targeted depletion enhances, osteoblast differentiation in vitro and/or in vivo (18,
The mechanisms by which Twist1 inhibits osteoblast differentiation, while complex and not completely elucidated, have nonetheless been deciphered in some detail. These include direct and indirect physical and functional interactions with osteogenic transcription factors, including Runx2, and signaling pathways, including the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling nodes (32, 40–42).

In addition to its role as a negative regulator of osteoblast differentiation, more recent evidence suggests that Twist1 inhibits chondrogenesis. Developmentally, Twist1 is expressed in mesenchymal progenitors and immature chondrocytes, but its expression decreases to undetectable levels in mature chondrocytes (23, 38). Furthermore, we showed previously that Twist1 is induced by canonical Wnt signaling and mediates the restrictive action of this signaling pathway on chondrocyte differentiation from mesenchymal progenitors (18). Moreover, we and others have shown that overexpression of Twist1 inhibits, whereas its targeted depletion enhances, acquisition of the chondrocyte phenotype (18, 43). Thus, abundant evidence supports a critical role for Twist1 as a repressor of chondrocyte differentiation. However, the mechanistic basis for its anti-chondrogenic activity has not heretofore been elucidated. Here, we provide evidence that Twist1 inhibits Sox9, the cardinal transcriptional regulator of chondrogenesis. We show that Twist1, through its C-terminal Twist-box, binds directly to the Sox9 HMG DNA-binding domain, leading to inhibition of Sox9-dependent gene activation. Our conclusion that Twist1 inhibits Sox9 through direct physical interaction is supported by functional analyses that revealed a strict concordance between the ability of individual Twist1 mutant derivatives to both bind to and inhibit the transcriptional activity of Sox9. Functionally, the interaction between Twist1 and Sox9 leads to inhibition of chondrocyte marker gene expression. Mechanistically, this inhibition derives from the ability of Twist1 to impede the sequence-specific association of Sox9 with target gene enhancer DNA. Other direct mechanisms for Twist1-medi-
Twist1 Inhibits Sox9

Twist1 Inhibits Sox9

Molecular and genetic analyses revealed that only Twist1 derivative retaining the Twist-box bound to Runx2 and inhibited its transcriptional activity, whereas a single amino acid substitution in the Twist-box resulted in premature osteoblast differentiation by direct interaction with the Runx2 DNA-binding domain, thus precluding its sequence-specific association of with cognate binding sites in osteogenic target genes. Notably, the relevant Runx2 interaction domain in Twist1 required for its osteoantagonistic function mapped to a novel 20 amino acid domain within its C terminus (amino acids 186–206), the so-called Twist-box (32). Molecular and genetic analyses revealed that only Twist1 derivatives retaining the Twist-box bound to Runx2 and inhibited its transcriptional activity, whereas a single amino acid substitution in the Twist-box resulted in premature osteoblast differentiation in vivo (32). These findings clearly identify the Twist-box as an osteoantagonistic domain. Our findings herein suggest an additional antichondrogenic function for the Twist-box through inhibitory interaction with Sox9. First, we showed that the Twist-box mediates direct interaction between Twist1 and the Sox9 HMG DNA-binding domain. Second, we observed that deletion or mutation of the Twist-box in Twist1 effectively abolishes its ability to bind to Sox9 and inhibit Sox9-dependent activation of chondrocyte marker gene expression. Third, we found that truncation or mutation of the Twist-box abrogates the ability of Twist1 to inhibit the sequence-specific association of Sox9 with its cognate binding sites in the Agc1 enhancer. On the basis of these and prior findings, we therefore speculate that the antiosteogenic and antichondrogenic properties of Twist1 are conferred by a common mechanism (Twist-box-mediated inhibition of DNA-binding activity) targeting the two lineage-specific master regulators of skeletogenesis, Runx2 and Sox9. The bipotent inhibitory activity of the Twist-box may thus contribute prominently to ability of Twist1 to control mesenchymal cell allocation down distinct lineages. In summary, our findings provide a mechanistic basis to explain the antichondrogenic activity of Twist1 through inhibition of Sox9, and thus serve to fill in a missing gap in the pathway leading from canonical Wnt signaling to inhibition of chondrogenesis.

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Twist1 Inhibits Sox9

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