Hierarchical Interactions of Homeodomain and Forkhead Transcription Factors in Regulating Odontogenic Gene Expression*

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Shankar R. Venugopalan1, Xiao Li1, Melanie A. Amen, Sergio Florez, Diana Gutierrez, Huojun Cao, Jianbo Wang, and Brad A. Amendt2

From the Texas A&M University Health Science Center, Institute of Biosciences and Technology, Houston, Texas 77030

FoxJ1 is a forkhead transcription factor expressed in multiple tissues during development and a major regulator of cilia development. FoxJ1 heterozygous mice present with defects in odontogenesis, and we correlate these defects to hierarchical interactions between homeodomain factors Pitx2 and Dlx2 with FoxJ1 in regulating their expression through direct physical interactions. Chromatin immunoprecipitation assays reveal endogenous Pitx2 and Dlx2 binding to the Dlx2 promoter and Dlx2 binding to the FoxJ1 promoter as well as Dlx2 and FoxJ1 binding to the amelogenin promoter. PITX2 activation of the Dlx2 promoter is attenuated by a direct Dlx2 physical interaction with PITX2. Dlx2 autoregulates its promoter, and Dlx2 transcriptionally activates the downstream gene FoxJ1. Dlx2 and FoxJ1 physically interact and synergistically regulate both Dlx2 and FoxJ1 promoters. Dlx2 and FoxJ1 also activate the amelogenin promoter, and amelogenin is required for enamel formation and late stage tooth development. FoxJ1 heterozygous mice maxillary and mandibular incisors are reduced in length and width and have reduced amelogenin expression. FoxJ1 mutants show a reduced and defective ameloblast layer, revealing a biological effect of these transcription factor hierarchies during tooth morphogenesis. These transcriptional mechanisms may contribute to other developmental processes such as neuronal, pituitary, and heart development.

Tooth development provides an excellent model system to delineate the molecular mechanisms regulating organogenesis. This process involves reciprocal interaction between the dental epithelium and the neural crest mesenchyme starting with the epithelial condensation and subsequent invagination into the mesenchyme (1). Tooth morphogenesis is a complex process under a tight genetic control. The stages of tooth development and several epithelial-expressed transcription factors are shown in Fig. 1. A time line of selected epithelial transcription factors is also shown as a reference for the hierarchy of their expression. We previously demonstrated that FoxJ1 was expressed during tooth development at E14.5, 18.5, and postnatal day (P) 1 in both ameloblasts and odontoblasts (2). FoxJ1 is a nuclear transcription factor, and it belongs to the winged-helix/fork-head family of transcription factors. It plays a fundamental role in embryonic development and differentiation (3). In addition to the developing tooth germ, FoxJ1 is also expressed in the oral epithelium, salivary gland, airway epithelium, oviduct, spermatids, and choroid plexus (4–6). During ciliogenesis, FoxJ1 regulates programs promoting basal body docking and axoneme formation in cells that are previously committed to the ciliated cell phenotype (7). FoxJ1 regulates T and B cell activities in the immune system (8, 9) and is required for determination of left-right asymmetry of the internal organs in mice (10).

Pitx2 is specifically expressed in the stomatodeal ectoderm as early as day E8.5 and is the earliest transcription marker of tooth development (11–13). Pitx2 belongs to the family of bicoid/paired-related homeobox genes that have a fundamental role in human development, disease, and evolution (14–17). During embryonic development they play a major role in pattern formation and cell fate determination. With the expression of Pitx2, the stomatodeal ectoderm acquires the capacity to induce odontogenic properties in cranial neural crest. Pitx2 is specifically expressed in the dental epithelium throughout tooth development. Because Pitx2 is the earliest marker of tooth development, we hypothesize that Pitx2 regulates early signaling molecules and transcription factors required to regulate early and late stages of tooth development.

Dlx2 is an orthologue of the Drosophila distal-less gene, which play a central role in patterning of jaws (18–20). During tooth development Dlx2 is expressed in the dental epithelial cells starting from the initiation, bud, cap, bell, and differentiation stages of tooth morphogenesis (20, 21). Homozygous mutants of Dlx2 exhibit abnormalities in the craniofacial and neuronal development (22). Dlx1/Dlx2 mutant exhibit arrested maxillary molar development demonstrating their significance in tooth development (19). Dlx2 expression occurs after Pitx2 and is a Pitx2 target gene. During late stages of tooth morphogenesis, the inner enamel epithelium-derived ameloblasts secrete the key enamel protein amelogenin (23). Amelogenin lays down the enamel matrix, which later gets mineralized to form enamel. The promoter region of the amelogenin contains several binding sites for FoxJ1 and Dlx2. This led to our hypothesis that these two factors together regulate amelogenin gene expression.

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: TAMU Health Science Center, Institute of Biosciences and Technology, 2121 West Holcombe Blvd., Houston, TX 77030. Tel.: 713-677-7402; Fax: 713-677-7784; E-mail: bamendt@ibt.tamhsc.edu.

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**EXPERIMENTAL PROCEDURES**

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP assays were performed as previously described using the ChIP assay kit (Upstate Biotechnology) with the following modifications (24, 25). LS-8 cells were cultured for 24 h, harvested, and plated in 60-mm dishes. Cells were cross-linked with 1% formaldehyde for 10 min at 37 °C the next day. All PCR reactions were done under an annealing temperature of 58 °C. The sense primer (5′-GGAGGGGACCTCAGAATCAG-3′) and antisense primer (5′-GAGACCAAGAGACTGAAGAGTTTG-3′) and antisense primer (5′-GGATCTCCCTCAACTGTGGACT-3′) and antisense primer (5′-GAATCTGGCATTGGTATGGTC-3′) were used without chromatin; normal goat IgG was used replacing the Dlx2 and FoxJ1 antibodies to reveal nonspecific immunoprecipitation of the chromatin. Primers upstream of the factor binding sites or to another gene promoter were used as controls.

**Cell Culture, Transient Transfection, Luciferase, and β-Galactosidase Assays**—Chinese hamster ovary (CHO) cells or LS-8 (oral epithelial cells (26)) were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin and transfected by electroporation. Cultures were fed 24 h before transfection, resuspended in PBS, and mixed with 2.5 µg of expression plasmids, 5 µg of reporter plasmid, and 0.5 µg of SV-40 β-galactosidase plasmid. Electroporation of CHO cells were performed at 380 V and 950 microfarads (Gene Pulser XL, Bio-Rad). Electroporation of LS-8 cells has been previously described (25). Transfected cells were incubated for 24 h in 60-mm culture dishes and fed with 5% FBS and DMEM and then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix).
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Inc.). All luciferase activities were normalized to β-galactosidase activity.

**GST Pulldown Assays**—Immmobilized GST-PITX2, GST-PITX2 ΔN38, GST-PITX2 ΔC39, and GST-PITX2 C39 fusion proteins were expressed in binding buffer (20 mM HEPES, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM DTT with or without 1% milk and 400 μg/ml ethidium bromide). Purified bacteria expressed Dlx2 proteins (50–500 ng) were added to 10–30 μg of immobilized GST-PITX2 fusion proteins or GST in a total volume of 100 μl and incubated for 30 min at 4°C. The beads were pelleted and washed 5 times with 200 μl of binding buffer. The bound proteins were eluted by boiling in SDS-sample buffer and separated on a 10% SDS-polyacrylamide gel. Approximately, 100 ng of purified Dlx2 proteins were analyzed in separate Western blots. After SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted, and detected using PITX2 antibody (Capra Science) and ECL reagents from GE Healthcare.

**Immunoprecipitation Assay**—Approximately 24 h after cell transfection with Dlx2 and FoxJ1, CHO cells were rinsed with 1 ml of PBS and then incubated with 1 ml of ice-cold radiimmunoprecipitation assay buffer for 15 min at 4°C. LS-8 oral epithelial cells were used to demonstrate endogenous Dlx2 and Pitx2 interaction. Cells were harvested and disrupted by repeated aspiration through a 25-gauge needle attached to a 1-ml syringe. Cellular debris was pelleted by centrifugation at 10,000 × g for 10 min at 4°C. An aliquot of lysate was saved for analysis as input control. The supernatant was transferred to a fresh 1.5-ml microcentrifuge tube on ice and precleared using the mouse IgG. Precleared lysate was incubated with protein A/G-agarose beads for 1–2 h at 4°C. After a brief centrifugation, supernatant was transferred to a new tube, and immunoprecipitation was performed with anti-Dlx2 antibody (Santa Cruz Biotechnology). The supernatant was incubated with protein A/G-agarose beads at 4°C for overnight. Immunoprecipitates were collected by brief centrifugation and washed 2 times with PBS and resuspended in 15 μl of double-distilled H2O and 3 μl of 6× SDS loading dye. Samples were boiled for 5 min and resolved on a 10% polyacrylamide gel. Western blotting was performed with anti-FoxJ1 antibody and HRP-conjugated reagent to detect immunoprecipitated proteins.

**Histology and Immunohistochemistry**—Steve Brody (Washington University, St. Louis, MO) kindly provided the FoxJ1−/− mice. All animals were housed in the Program of Animal Resources of the Institute of Biosciences and Technology and were handled in accordance with the principles and procedure of the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Texas A&M Health Science Center, Institutional Animal Care and Use Committee. Mice carrying lacZ-PGKNeo cassette in FoxJ1 exon-1 were mated to generate null alleles. Embryos were collected at various time points considering the day of observation of a vaginal plug to be embryonic day (E) 0.5. The primers used for genotyping were HGT 6F (5′-GCAAGATGGAGAGGT-GGAG-3′), HGT 7R (5′-TGGCGGTGGAATGGGAAAT-3′), and HGT LacZ3 (5′-GACAGATCGCCCTCAGGAA-3′). PCR, allele-specific amplification of genomic DNA isolated from mouse-tail, would generate a 470-bp wild-type (WT) or 524-bp mutant knock-out DNA product (27).

Embryos were treated with 4% paraformaldehyde and dehydrated with sequential concentration of alcohol and finally with xylene. The embryos were embedded in paraffin, and sections were made at 7-μm thickness. To examine tissue morphology, standard hematoxylin and eosin were used. For immunohistochemistry, sections were deparaffinized and treated with 0.1 M sodium citrate buffer for 7.5 min at 100% power in a revolving 800-watt microwave and an additional 2 cycles of 5 min at 50% power. Subsequently, sections were treated with 4% hydrogen peroxide to block the endogenous peroxidase activity. The slides were incubated with 5% goat serum for 30 min followed by overnight incubation with anti-amelogenin antibody at 1:500 dilution (Santa Cruz). After antibody incubation, the slides were treated with biotinylated anti-rabbit secondary antibody (Vector Laboratories) at a concentration of 1:200 for 30 min. Avidin biotin complex and DAB substrate (Vector Laboratories) were used as our reporting system.

**Real Time PCR**—FoxJ1 shRNA was commercially purchased from S. A. BiosciencesA (SureSilencing shRNA Plasmid for FOXJ1, catalog #KH02286). The shRNA sequence was 5′-GCTACTGACAGCCTGGACTTT-3′. Endogenous Dlx2 activation was measured using sense (5′-GTAGCCCTATTCTTGTATTCTCTT-3′) and antisense (5′-ACTGTCAAAGACTCCAGTCATCTCT-3′) primers. RNA extraction was performed using RNasy Mini kit from Qiagen. RT-PCRs were performed using iScript Select cDNA synthesis kit from BioRad. Real-time PCRs were performed using iQ SYBR Green Supermix kit, and all Ct values were normalized by β-actin level. All PCR products were examined by melt curves and sequenced.

**RESULTS**

**PITX2 Endogenously Binds to the Dlx2 Promoter**—We have previously shown that PITX2 activates the Dlx2 promoter (28). However, we now demonstrate endogenous Pitx2 binding to the Dlx2 chromatin. ChIP were performed in LS-8 cells (an oral epithelial cell line (26)) as they endogenously express both Pitx2 and Dlx2. Pitx2 antibody was used to immunoprecipitate the Pitx2/Dlx2 chromatin complex, and primers specifically flanking the Pitx2 binding site were used to amplify the chromatin (Fig. 2A). Normal rabbit IgG and nonspecific Msx2 promoter primers (–702 to –429) were used as negative controls in the Pitx2 immunoprecipitated chromatin complex. Dlx2 primers specific for Pitx2-binding site amplified the Pitx2-immunoprecipitated chromatin complex, demonstrating that Pitx2 binds to the Dlx2 promoter (Fig. 2B, lane 2). No amplification was detected in the Dlx2 primer-only and nonspecific control primers showing the specificity of the experiment (Fig. 2B, lanes 3 and 6). Also, the normal rabbit IgG immunoprecipitated chromatin did not yield a PCR product (Fig. 2B, lane 5). Chromatin input collected before the immunoprecipitation with Pitx2 antibody was amplified with Dlx2 primers and produced a PCR product showing the presence of chromatin in the starting material (Fig. 2B, lane 4).

Preliminary data indicated that Dlx2 potentially autoregulates its promoter. Bioinformatic screens revealed several Dlx2
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binding elements in the Dlx2 promoter, and a ChIP assay was performed using a Dlx2 antibody (Fig. 2, A and C). The Dlx2 antibody immunoprecipitated chromatin containing the Dlx2 binding site in the Dlx2 promoter (Fig. 2C, lane 2). Multiple controls were performed as in Fig. 2B and demonstrate the specificity of the ChIP assay. All PCR products were sequenced to confirm their identity. These results confirm that endogenous Pitx2 and Dlx2 bind to the Dlx2 promoter.

**PITX2 and Dlx2 Activate the Dlx2 Promoter and Dlx2 Reciprocally Attenuates PITX2 Transcriptional Activation of the Dlx2 Promoter**—Pitx2 and Dlx2 are homeodomain transcription factors and show an overlapping expression during craniofacial and brain development. To test the significance of Pitx2 and Dlx2 binding to the Dlx2 promoter, transient transfection assays were performed in CHO cells. PITX2A is a full-length construct, and PITX2A ΔN38 is a N-terminal deletion construct, whereas the PITX2A ΔT1261 is an Axenfeld-Rieger syndrome mutation and has a stop codon in the C-terminal OAR domain (29) (Fig. 3A). PITX2A activated the Dlx2 promoter at ~30-fold, and the PITX2A ΔN38 activated at ~25-fold (Fig. 3B). Interestingly the PITX2A ΔT1261 activated the Dlx2 promoter at only ~5-fold (Fig. 3B). Thus, the PITX2A N terminus had no effect on activation of the Dlx2 promoter; however, deletion of the PITX2 C-terminal tail OAR domain in the PITX2A ΔT1261 mutant had reduced activation. We have previously shown that the OAR domain is essential for PITX2 transcriptional activity and as a protein interaction domain (30).

Interestingly, Dlx2 activates the Dlx2 promoter at 20-fold, demonstrating that Dlx2 can autoregulate its promoter (Fig. 3B). We asked if PITX2 and Dlx2 could work together to synergistically activate the Dlx2 promoter. Surprisingly, Dlx2 attenuated PITX2A activation from ~30 to ~14-fold (Fig. 3B). Dlx2 also reduced the activity of PITX2A AN38 from ~25- to ~10-fold (Fig. 3B). However, Dlx2 and PITX2A ΔT1261 activated the promoter at ~30-fold, revealing no effect of Dlx2 attenuation with this construct (Fig. 3B). These experiments suggested that Dlx2 physically interacted with the PITX2A OAR domain to regulate transcription. To demonstrate endogenous activation of Dlx2 expression, CHO cells were transfected with PITX2 or Dlx2, and endogenous Dlx2 expression was measured by real time PCR. PITX2 activated Dlx2 expression ~10-fold, and Dlx2 activated Dlx2 at ~6-fold (Fig. 3C). To further demonstrate the PITX2 Dlx2 interaction, co-transfections were performed with the TK-Bic luciferase promoter (contains four PITX2 binding sites upstream of the TK3 minimal promoter (30)) and PITX2 and/or Dlx2. PITX2 activated this promoter at 14-fold, whereas Dlx2 did not activate the promoter (Fig. 3D). However, Dlx2 attenuated PITX2 activation of this minimal promoter, demonstrating a direct repression of PITX2 transcriptional activity by Dlx2 (Fig. 3D).

**Dlx2 Physically Interacts with PITX2A**—A GST pulldown assay was performed to determine whether Dlx2 directly interacted with PITX2A. Dlx2 (300 ng) was incubated with immobilized GST-PITX2, GST-PITX2 ΔN38 (N-terminal deletion of PITX2), GST-PITX2 ΔC39 (C-terminal 39 amino acids deletion), and GST-PITX2 C39 (C-terminal 39 amino acids) in one reaction (Fig. 4A). After incubation, each aliquot was extensively washed and resolved separately on a polyacrylamide gel probing for Dlx2 (Fig. 4B). Dlx2 bound to PITX2, PITX2 ΔN38, and PITX2 C39, but it did not interact with PITX2 ΔC39 (Fig. 4B). Dlx2 interacts specifically with the last 39 amino acids of the PITX2 C-terminal tail containing the OAR domain. Co-immunoprecipitation assays using a Dlx2 antibody to immunoprecipitate the Dlx2-PITX2A complex in transfected CHO cell lysates confirmed the interaction of Dlx2 and PITX2 (Fig. 4C). Endogenous Dlx2 and Pitx2 interactions are shown using LS-8 cells without transfected factors (Fig. 4D).

**Dlx2 Binds to and Activates the FoxJ1 Promoter in Concert with FoxJ1**—We have previously identified FoxJ1 as a new factor in craniofacial and tooth development (2); however, the factors regulating FoxJ1 expression during the late stages of tooth development are not known. Sequence analysis revealed a Dlx2 consensus binding site (CAATT)A in the FoxJ1 promoter (Fig. 5A). ChIP assays were performed in LS-8 cells, as both factors are endogenously expressed in these cells. Dlx2 antibody was used to immunoprecipitate the Dlx2-FoxJ1 chromatin complex. FoxJ1 primers designed specifically for the region flanking the Dlx2 binding site located at −1779 and −1774 bp were used to amplify the chromatin (Fig. 5A). Dlx2 immunoprecipitated the FoxJ1 chromatin complex, and the primers yielded a PCR

**The abbreviations used are:** TK, thymidine kinase; Amelx, amelogenin; Ab, ameloblast; OAR, otp, aristale, and Rx.
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FIGURE 3. PITX2 and Dlx2 activate the Dlx2 promoter, and Dlx2 attenuates PITX2 transcriptional activity. A, is shown a schematic of PITX2A full-length construct, the PITX2A ΔN38 construct with the N-terminal deletion, and a PITX2A ΔT1261 construct with a stop codon in the OAR domain that truncates the C-terminal tail. B, CHO cells were transfected with 2.5 μg of Dlx2 and/or PITX2A, PITX2A ΔN38, and PITX2A ΔT1261 expression plasmids, and 5 μg of Dlx2 promoter plasmid. All DNAs were double CsCl-banded for purity, and cells were transfected by electroporation. To control for transfection efficiency, all transfections included the SV-40 promoter plasmid. All DNAs were double CsCl-banded for purity, and all transfections included the SV-40 promoter in the reporter vectors. CHO cells were transfected with 5 μg of empty expression vector, PITX2A, and Dlx2 DNAs. All Ct values were normalized to β-actin levels. All reactions have three replicates each, and the experiments were repeated three times. All PCR products were examined by melting curves and sequenced to ensure specificity. D, Dlx2 represses PITX2 activity independent of Dlx2 binding to DNA. Bicoid motifs (Bic) were cloned in flanking the TK minimal promoter at expected, Dlx2 and FoxJ1 synergistically activated the Dlx2 promoter 40-fold, confirming their synergistic activity with β-galactosidase activity. C, real-time PCR results indicated the endogenous Dlx2 mRNA level was both up-regulated by the overexpressed PITX2 gene and Dlx2 gene compared with the empty vector. CHO cells were transfected with 5 μg of empty expression vector, PITX2A, and Dlx2 DNAs. All Ct values were normalized to β-actin levels. All reactions have three replicates each, and the experiments were repeated three times. All PCR products were examined by melting curves and sequenced to ensure specificity. D, Dlx2 represses PITX2 activity independent of Dlx2 binding to DNA. Bicoid motifs (Bic) were cloned in flanking the TK minimal promoter in the reporter vectors. CHO cells were transfected with 5 μg of bicoid motif reporter plasmid, 2.5 μg of empty, PITX2A, and Dlx2 expression plasmids. To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter (0.5 μg). The activities are shown as mean fold-activation compared with the reporter plasmid with empty expression plasmids and normalized to β-galactosidase activity (± S.E.) from four independent experiments. HD, homeodomain.

product of 262 bp (Fig. 5B, lane 3). However, the FoxJ1 primers only and chromatin-immunoprecipitated with goat immunoglobulin (IgG control) did not yield a PCR product (Fig. 5B, lanes 1 and 2). All PCR products were sequenced to confirm their identity.

Because the ChIP assays demonstrated that endogenous Dlx2 bound to the FoxJ1 promoter, we asked if Dlx2 could transcriptionally regulate the promoter. We co-transfected the FoxJ1 2.6-kb promoter with Dlx2 and/or FoxJ1 plasmids into CHO cells. Dlx2 by itself activated the FoxJ1 promoter at ~12-fold, and FoxJ1 activated its promoter at low levels (Fig. 5C). However, co-expression of FoxJ1 with Dlx2 synergistically activated the FoxJ1 promoter at ~45-fold (Fig. 5C). Lef-1 transfection was used as a control. To demonstrate that the Dlx2 synergistic interaction with FoxJ1 was due to protein interactions and not independent DNA binding activities, transfection experiments were repeated using a TK-Luc reporter with three Fox binding sites cloned upstream of the TK minimal promoter. FoxJ1 activated the TK-Fox promoter at ~13-fold, whereas Dlx2 did not activate the reporter (Fig. 5D). Co-transfection of FoxJ1 with Dlx2 increased FoxJ1 activation of TK-Fox to ~25-fold (Fig. 5D). As controls, the Fox binding sites were mutated in the TK-Fox reporter (TK-MutFox), which abolished activation by FoxJ1 (Fig. 5D).

Dlx2 Directly Interacts with FoxJ1—Because Dlx2 and FoxJ1 synergistically activated the FoxJ1 promoter, we asked if these proteins would also synergistically regulate the Dlx2 promoter in a similar fashion. We co-transfected the Dlx2 promoter with Dlx2 and/or the FoxJ1 plasmids into CHO cells (Fig. 6A). Dlx2 by itself activated the Dlx2 promoter at ~20-fold, whereas FoxJ1 activated the promoter minimally at ~5-fold (Fig. 6A). As expected, Dlx2 and FoxJ1 synergistically activated the Dlx2 promoter at ~40-fold, confirming their synergistic activity with another promoter construct (Fig. 6A). These data reveal that Dlx2 not only autoregulates its activity; it can also synergize with FoxJ1 to increase activation of the Dlx2 and FoxJ1 promoters. Lef-1 transfection was used as a control.

Co-immunoprecipitation was performed to determine whether Dlx2 and FoxJ1 physically interact. CHO cells were transfected with an empty vector, Dlx2, and/or FoxJ1 plasmids. Dlx2 antibody did not immunoprecipitate the Dlx2-FoxJ1 complex in the transfected empty vector (mock), Dlx2 plasmid only, or the FoxJ1 plasmid only cell lysates (Fig. 6B, lanes 2–4). However, when Dlx2 and FoxJ1 were co-transfected, Dlx2 formed a
complex with FoxJ1 and was immunoprecipitated by Dlx2 antibody (Fig. 6B, lane 5). To demonstrate the expression of FoxJ1, the cell lysates were directly analyzed by Western blot (Fig. 6B, lanes 6 and 7, Input). 100 ng of FoxJ1 pure protein was used as a Western blot control (Fig. 6B, lane 1). This co-immunoprecipitation data corroborates the transfection data, demonstrating that these two proteins can physically interact to form a complex and regulate gene expression.

**FoxJ1 and Dlx2 Endogenously Bind to the amelogenin (Amelx) Promoter**—It has been previously reported that Dlx2 may regulate amelogenin and control enamel formation during tooth development (31). We asked if FoxJ1 and Dlx2 directly regulated the Amelx promoter. ChIP assays were performed in LS-8 cells and demonstrated that both endogenous FoxJ1 and Dlx2 bind the Amelx promoter at −189 to −196 bp (Fig. 7, A and B). The FoxJ1 antibody immunoprecipitated chromatin corresponding to the Amelx promoter and FoxJ1 binding site (Fig. 7B, left panel). Controls included the IgG control and primers to an upstream region of the Amelx promoter that does not contain a Dlx2 DNA binding site, −6452 to −6714 (Fig. 7D, right panel). The FoxJ1 antibody immunoprecipitated chromatin containing the Dlx2 binding site, demonstrating that FoxJ1 interacts with Dlx2 bound to chromatin (Fig. 7D, middle panel). Thus, both endogenous FoxJ1 and Dlx2 bind to the Amelx promoter in LS-8 cells independently and in a co-complex.

**Dlx2 and FoxJ1 Co-activate the Amelx Promoter**—To investigate the role of Dlx2 and FoxJ1 proteins in regulating amelogenin gene expression, we cloned the Amelx promoter into a luciferase vector. LS-8 cells were used for transfection, and FoxJ1 was transfected at an increasing concentration of 0.5, 1.25, 2.5, and 5 μg with the Amelx promoter (Fig. 8A). FoxJ1 activated the Amelx promoter in a dose-dependent fashion from −3-, −8-, −16-, and −28-fold (Fig. 8A). FoxJ1 alone is sufficient to activate the Amelx promoter. Subsequently, we co-transfected Dlx2 and/or FoxJ1 with the Amelx promoter into LS-8 cells. Dlx2 activated the Amelx promoter at ~27-fold (Fig. 8A). However, when FoxJ1 and Dlx2 were co-transfected, they activated the Amelx promoter at ~55-fold (Fig. 8). These experiments demonstrate that Dlx2 and FoxJ1 independently activate the Amelx promoter and together they may have a role in amelogenesis and enamel formation.

FoxJ1 shRNA was used to knock down endogenous FoxJ1 expression in LS-8 cells to test if this would affect endogenous amelogenin expression. FoxJ1 shRNA (5 μg) and negative control shRNA were transfected into LS-8 cells, and total mRNA was extracted 48 h after transfection. Real-time PCR assays...
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FIGURE 5. Endogenous Dlx2 binds to and activates the FoxJ1 promoter in concert with FoxJ1. A, shown is a schematic representation of FoxJ1 2.6-kb promoter with one Dlx2 binding site, noted by the + symbol. The locations of the sense primer (S) and the antisense primer (AS) are shown in the distal promoter to amplify the immunoprecipitated chromatin. B, a ChIP assay was performed in LS-5 cells. Lane 1 contains the FoxJ1 primer-only control, and lane 2 is the immunoprecipitation using normal goat immunoglobulin G and FoxJ1 primers. Lane 3 is the Dlx2-immunoprecipitated chromatin amplified using the specific FoxJ1 promoter primers. Lane 4 is the chromatin input amplified using the FoxJ1 primers. Lane 5 is the PCR marker. All PCR products were sequenced to confirm their identity. C, CHO cells were transfected with 2.5 μg of Dlx2 and/or FoxJ1 and Lef-1 (control) expression plasmids and 5 μg of FoxJ1 2.6-kb promoter plasmid. Transfections were done as in Fig. 3 (mean ± S.E.) from at least three independent experiments). D, Dlx2 interacts with DNA-bound FoxJ1. FoxJ1 binding sites (4) and mutated FoxJ1 binding elements (MutFox) were cloned in front of the minimal TK promoter in the reporter vector. CHO cells were transfected with 5 μg of reporter plasmids and/or 2.5 μg of empty, FoxJ1, and Dlx2 expression plasmids. Transfections were performed as described in Fig. 3 (± S.E.) from four independent experiments.

FIGURE 6. Dlx2 and FoxJ1 synergistically activate the Dlx2 promoter through direct physical interactions. A, CHO cells were transfected with 2.5 μg of Dlx2 and/or FoxJ1 expression plasmids and 5 μg of Dlx2 3.2-kb promoter plasmid. Transfections were done as in Fig. 3 (mean ± S.E.) from at least three independent experiments). B, a co-immunoprecipitation assay is shown. FoxJ1 and/or Dlx2 expression plasmids (5 μg) were transfected in CHO cells and incubated for 24 h. Cells were harvested and lysed, and the FoxJ1-Dlx2 protein complex was immunoprecipitated (IP) using the Dlx2 antibody. The immunoprecipitated complex was resolved on a 10% polyacrylamide gel and transferred to a polyvinylidene fluoride filter, and Western blotting (WB) was done using FoxJ1 antibody. Lanes 2–5 are the immunoprecipitated proteins and controls; lanes 6–9 are the input samples.

with FoxJ1 primers and amelogenin primers revealed a 50% reduction in FoxJ1 transcripts that resulted in an ~30% reduction in amelogenin transcripts (Fig. 8, B and C). These data further confirmed FoxJ1 activation of the Amelx promoter. However, it is essential to demonstrate an in vivo role for FoxJ1 in late stage tooth development and the regulation of amelogenesis. We next examined the FoxJ1−/− mice for craniofacial and tooth defects.

Defective Ameloblast Cell Layer Thickness in FoxJ1 Null Mice—Immunohistochemical staining previously identified FoxJ1 expression in the dental epithelium at E14.5 and 18.5 (2). FoxJ1 was expressed both in the ameloblasts and odontoblasts at P1, suggesting a role for FoxJ1 in late stage tooth development and enamel formation. Germ line-targeted disruption of FoxJ1 was used in this study to characterize the function of FoxJ1 during tooth morphogenesis. The FoxJ1−/− mice were previously reported to have cilia and heart defects, but the craniofacial/tooth defects were not analyzed (27). We examined FoxJ1−/− embryonic days E14.5 and E18.5 and post-natal days P1 and P7 and 32-week-old mouse incisors and molars. FoxJ1−/− embryos were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to study the tissue morphology.

In E14.5 FoxJ1−/− mice, both incisor and molar tooth germs developed, suggesting that the early patterning and inductive events of tooth formation are not affected in FoxJ1−/− mice (Fig. 9, A–D). However, the tooth germs (outlined with a dotted line) at this stage were smaller in size and hypoplastic compared with the wild type (Fig. 9, A–D). The dental epithelium differentiates into enamel-secreting ameloblasts, and the mesenchyme differentiates into the dentin-secreting odontoblasts. At E18.5, defects in incisor ameloblast and odontoblast differenti-
A pattern were identified as loosely packed cells that are not as well organized in FoxJ1/H11002/H11002 mice compared with wild type littermates (Fig. 9, E–H). We investigated ameloblast and odonto-blast differentiation in FoxJ1/H11002/H11002 mice at P1. At this stage the FoxJ1/H11002/H11002 mice revealed defective ameloblasts compared with wild type mice (Fig. 9J). The thickness and density of the ameloblast layer was reduced in the FoxJ1/H11002/H11002 incisor (Fig. 9J). Stratum intermedium is a layer of cells that abuts the ameloblasts layer and plays a role in proper enamel formation in concert with ameloblasts. At this stage the stratum intermedium (Si) layer was widely spaced as compared with well compacted and tightly packed stratum intermedium in the wild type (Fig. 9, I and J). Of the 49 litters examined and 25 null embryos sectioned, all presented with this unique dental phenotype (Fig. 9K).

**FIGURE 7. Endogenous Dlx2 and FoxJ1 bind to the amelogenin promoter.** A, shown is a schematic of the FoxJ1 binding site in the amelogenin promoter and primers flanking the site. B, a ChIP assay is shown. The left panel shows the PCR products from the immunoprecipitated chromatin using primers flanking the FoxJ1 binding site in the Amelx promoter. FoxJ1 Ab immunoprecipitated the Amelx promoter chromatin, and IgG control Ab did not immunoprecipitate the promoter. The right panel shows the controls, demonstrating that the FoxJ1 Ab did not immunoprecipitate chromatin using upstream Amelx primers not flanking a FoxJ1 binding site. As a control, the primers did amplify the chromatin from the input sample before immunoprecipitation. The middle panel is a ChIP assay using the Dlx2 antibody to immunoprecipitate the FoxJ1-Dlx2 complex binding to the FoxJ1 DNA binding site. C, shown is a schematic of the Dlx2 binding site and primers flanking the site in the Amelx promoter. D, shown is a ChIP assay. The left panel demonstrates Dlx2 Ab immunoprecipitation of the Amelx promoter and the chromatin input-positive control and IgG Ab negative control. The right panel shows controls, as in the right panel in B. The middle panel is a ChIP assay using FoxJ1 antibody to immunoprecipitate the Dlx2-FoxJ1 complex binding to the Dlx2 DNA element.

**FIGURE 8. FoxJ1 and Dlx2 activate the Amelx promoter.** A, LS-8 cells were transfected with 0.5, 1.25, 2.5, and 3.75 μg of FoxJ1 and/or 2.5 μg of Dlx2 expression plasmids and 5 μg of Amelx promoter plasmid. Transfections were done as in Fig. 3 (mean ± S.E.) from at least three independent experiments). B, FoxJ1 shRNA and negative control (NC) shRNA (5 μg) were transfected into LS-8 cells. Real-time PCR results indicate the FoxJ1 mRNA level was reduced ~50% by the shRNA compared with the negative control group. C, the same mRNA samples revealed an ~30% reduction of endogenous Amelx transcript levels in FoxJ1 shRNA-transfected cells. All Ct values were normalized by β-actin transcripts. All reactions have triplicates, and the experiments were repeated three times. All PCR products were examined by melting curves and sequencing to ensure specificity.
The defective ameloblast layer in FoxJ1−/− tooth germ led us to investigate the P7 FoxJ1−/− mice for enamel defects. At P7 the FoxJ1−/− mice produced both enamel and dentin; however, enamel (En) thickness was reduced ~30%, p < 0.05 (Fig. 10B). The basal lamina layer abutting the ameloblast layer was almost completely absent in the FoxJ1−/− mice, and the ameloblast (Ab) layer was poorly organized and lost the palisaded arrangement (Fig. 10B and D). Also, in the cervical loop region the ameloblast layer is poorly polarized, and the basal lamina (BL) is extremely thin (Fig. 10D).
At 32 weeks the FoxJ1−/− mouse had a full set of erupted teeth. Consistent with the histological phenotype, both the length (~36% shorter) and width of the incisor was reduced in the FoxJ1−/− mouse (Fig. 11, B and C). Also, the enamel deposition lines (perikymata, see arrow) in the WT mouse were fully formed at this stage, whereas the FoxJ1−/− mouse exhibited poorly organized enamel deposition incremental lines in the upper incisors (Fig. 11, A and B). The labial surface of the lower incisor in the WT mouse exhibits yellow pigmentation due to the deposition of iron salt, but the FoxJ1−/− incisor was smooth and chalky white in color (Fig. 11D). Taken together, all these data indicate that FoxJ1 has a role in tooth development especially during amelogenesis.

Reduced Amelogenin Expression in FoxJ1−/− Mice—As we observed poorly organized and reduced ameloblast layer thickness in the FoxJ1 mutant mice and demonstrated FoxJ1 activating the Amelx promoter, we asked whether amelogenin expression levels would be affected in FoxJ1−/− mice. Immunohistochemical staining for amelogenin expression was performed in P4 WT and FoxJ1−/− mouse incisors and molars (Figs. 12, A–D and 13, A–D). At P4 the ameloblasts of WT mice show robust amelogenin expression both in the incisor and molar (Figs. 12C and 13C). Whereas in FoxJ1−/− mice, consistent with the reduced ameloblast layer thickness in P1 and P7, the amelogenin expression levels were also reduced both in the incisors and molars (Figs. 12D and 13D). These data demonstrate reduced amelogenin expression in FoxJ1−/− mice due to FoxJ1 activation of the amelogenin promoter, providing evidence that FoxJ1 has a direct role in amelogenesis. Furthermore, amelogenin expression is not completely lost in these mutant mice due to the transcriptional activity of Dlx2, which is upstream of FoxJ1 expression during tooth development.

As controls, FoxJ1+/− P4 heterozygous mice did not present with ameloblast or odontoblast differentiation defects compared with wild type littermates (Fig. 14). Both incisors and molars were analyzed, and we observed no significant defects in the FoxJ1 heterozygous mice.

**DISCUSSION**

Dlx2 Attenuates PITX2 Transcriptional Activation of the Dlx2 Promoter—Tooth development involves sequential and reciprocal interaction between the dental epithelium and the mesenchyme. Each stage of tooth development is defined by specific set of genes expressed in the dental epithelium and the mesenchyme, dictating cell fate determination. For example, Pitx2 is expressed very early on in the dental epithelium that enables the epithelium to acquire the odontogenic potential, whereas the Dlx2 and FoxJ1 genes are expressed subsequently. The dental epithelium in the late stages differentiates to ameloblasts, which is a unique cell type marked by the expression of amelogenin and formation of enamel. Although mouse genetics studies enabled us to understand the functions of these genes at specific developmental stages, it is not known how these genes are related to one another in a regulatory network. The aim of this study is to unravel the molecular interactions between...
these factors at the level of transcription and identify how they regulate each other and the downstream targets in a hierarchical fashion. Because Pitx2 is the earliest marker of tooth development, it may be possible that Pitx2 regulates signaling molecules and transcription factors that are expressed in the early dental epithelium. Pitx2 binds to the 5'-TAATCC-3' DNA sequence to regulate gene expression. Chromatin immunoprecipitation assays have previously demonstrated that Pitx2 binds to the Lef-1 promoter and synergistically regulates Lef-1 isoform expression in concert with Lef-1 and β-catenin (25, 32). In this report we have identified a new transcriptional mechanism, where the Dlx2 protein has a repressive effect on PITX2 activation of the Dlx2 promoter. Pitx2 expression precedes Dlx2 expression during craniofacial/tooth development and activates Dlx2. Once Dlx2 is expressed, it physically interacts with Pitx2 and attenuates Pitx2 transcriptional activity. Thus, another factor is required to maintain Dlx2 expression during tooth development.

Dlx2 and FoxJ1 Synergistically Activate the Dlx2 and FoxJ1 Promoters—Dlx2 plays a central role in the patterning of jaws and is expressed in the distal epithelium of the maxillary jaw and the proximal mesenchyme of both maxillary and mandibular jaw (18–20). During tooth morphogenesis, Dlx2 is expressed in the dental epithelial cells starting from the initiation, bud, cap, bell, and differentiation stages (20, 21). Dlx2 transcription factors bind to DNA to regulate gene expression. For example, Dlx5 and/or Dlx6 expression can be regulated by Dlx2. It was shown that Dlx2 bound to the Dlx5/Dlx6 enhancer region and transcriptionally activated them, suggesting a role for Dlx2 in regulation of other Distal-less family of genes (33). Chromatin immunoprecipitation assays demonstrated that Dlx2 directly bound to the Dlx2 promoter, and in transfection assays Dlx2 repressed PITX2 activity and simultaneously activated its own promoter. Thus, when PITX2 activity is suppressed, Dlx2 alone is sufficient to activate its own promoter providing an auto-regulatory mechanism for Dlx2 expression.

Interestingly the 2.6-kb promoter of FoxJ1 also has a consensus Dlx2 DNA binding site. We have previously demonstrated that FoxJ1 is expressed in the dental epithelium derived ameloblasts (2). Furthermore, Pitx2 regulates FoxJ1 expression and synergistically activates the FoxJ1 promoter in concert with Lef-1 and β-catenin (2). In this report we have identified Dlx2 activating the FoxJ1 promoter, providing a new mechanism for regulating gene expression during late stages of tooth development, which is also independent of PITX2 transcriptional activity.

Apart from regulating its own expression, Dlx2 also bound to and activated the FoxJ1 promoter. Corroborating with the expression patterns of Dlx2 and FoxJ1 in ameloblasts, our data provide an interesting mechanism where Dlx2 functions in regulating both Dlx2 and FoxJ1 gene expression. In addition, FoxJ1 synergistically interacts with Dlx2 to increase the transcription of Dlx2 and FoxJ1 gene expression.

Dlx2 and FoxJ1 Regulate Amelogenin Expression—Interestingly, the Dlx2 LacZ reporter transgenic mice showed expression of Dlx2 in the dental epithelium with an inverse linear relationship between the enamel thickness and Dlx2 promoter activity level (21). Supporting this finding, in the Dlx1/Dlx2 mutants the dental epithelial cells were poorly organized, with the loss of palisade pattern of the ameloblasts. In the transient transfection assays, Dlx2 trans-activated the amelogenin TK-β-galactosidase reporter constructs, suggesting a role for Dlx2 in enamel formation during odontogenesis (31).

In this study we have used targeted deletion of FoxJ1 mice to characterize its function during tooth development. We show that early patterning or initiation of tooth morphogenesis was not affected in FoxJ1−/− mice. However, in late stage tooth development the ameloblast and stratum intermedium cell layers were defective. These structures shown all work together during enamel formation and biomineralization. In the adult mouse the length and the width of the incisors were significantly affected with the disrupted enamel deposition lines in the upper incisor and chalky white, pigment-less lower incisor. The defect in ameloblasts maturation led us to investigate amelogenin expression in FoxJ1−/− mice.

Amelogenin was identified as the main enamel matrix protein comprising 80–90% that of the total enamel protein (23). Crystal pattern organization and regulation of enamel thickness were suggested to be the functions of amelogenin (23, 34). It was shown that the CCAAT/enhancer-binding protein α (C/EBPα) activated amelogenin expression, and Msx2 functions as a transcriptional repressor of amelogenin expression.
Hierarchical interaction of Pitx2, Dlx2, and FoxJ1 in regulating Dlx2, FoxJ1, and AmelX gene expression. Pitx2 activates Dlx2 and FoxJ1. Dlx2 attenuates Pitx2 activation of Dlx2 and FoxJ1. Dlx2 auto-regulates its expression and activates FoxJ1 and amelogenin. FoxJ1 activates amelogenin. Dlx2 acts as a repressor of Pitx2 function, and Dlx2 activates late stage tooth morphogenesis genes, FoxJ1, and amelogenin in concert with FoxJ1.

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FoxJ1 Interactions with Homeodomain Factors

(35). The FoxJ1−/− mouse tooth germ showing significantly reduced amelogenin expression (although not completely abolished) opened up a possibility that FoxJ1 may regulate amelogenin along with Dlx2. Corroborating with previous reports, we demonstrated that Dlx2 activated the AmelX promoter. We further report that FoxJ1 activated the AmelX promoter and that FoxJ1 and Dlx2 directly interact to regulate the AmelX promoter. Because FoxJ1 is highly expressed during late stage tooth development, it must have a role in regulating genes at this stage. Dlx2 expression is maintained by the autoregulatory activation of Dlx2 and, in combination with FoxJ1, provides an enhanced transcriptional complex for amelogenin expression.

In summary, our study has identified novel mechanisms that explain the hierarchical expression of four major genes during tooth development (Fig. 15). Pitx2 activates Dlx2 and Dlx2 represses Pitx2 activity. Interestingly Dlx2 activates its own promoter as well as the FoxJ1 promoter. Dlx2 and FoxJ1 physically interact to synergistically regulate their own promoters as well as additively regulate the amelogenin promoter. Although these data are focused on craniofacial and tooth development, Pitx2, Dlx2, and FoxJ1 are all co-expressed in other tissues, and these transcriptional mechanisms can apply to other developmental processes.