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Cdx1 Interacts Physically with a Subset of Hox Proteins

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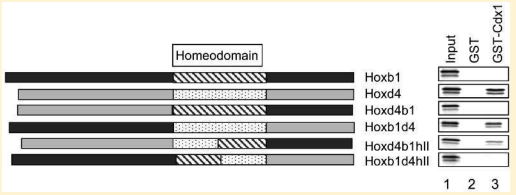
ABSTRACT: *Cdx* and *Hox* gene families encode homeodomain-containing transcription factors involved in anterior−posterior vertebral patterning. Although Cdx proteins are direct transcriptional regulators of *Hox* gene expression, both Hox and Cdx proteins are known to interact with other homeodomain transcription factors, leading us to speculate that Cdx and Hox proteins may also interact physically. In testing this, we found that that Cdx1 is indeed capable of associating with a subset of Hox proteins. This interaction is localized to the homeodomain region of both classes of proteins, is reliant on specific arginine residues in helix I of the Hox homeodomain, and is further modulated by N-terminal Hox sequences. More promiscuous interactions were seen with Hox proteins expressed in vivo, suggestive of bridging factors or post-translational modifications. Finally, we demonstrate that this interaction modulates Cdx−Hox transcriptional activity on a *Hox*-responsive element. This study is the first example of Cdx−Hox protein interactions and suggests that such complexes may modulate Hox and/or Cdx function.



In vertebrates, unsegmented paraxial mesoderm undergoes somitogenesis to generate epithelial somites. These subsequently differentiate into dermamyotome and sclerotome, the latter being the anlage of the vertebrae. Many vertebrae exhibit unique morphological characteristics related to their antero-posterior (AP) position along the major body axis and must therefore be subject to patterning at some stage in their ontogenesis; the *Hox* gene products are central to this patterning.

The 39 murine *Hox* genes are believed to have evolved by a series of duplication events from an ancestral complex related to the *HOM-C* genes of *Drosophila* and are distributed in four clusters denoted *Hoxa*−*Hoxd*.[1−4] In the mouse embryo, *Hox* transcripts are initially detected at late gastrulation around embryonic day (E) 7.5 in the primitive streak region. Transcript distribution then expands to reach a rostral limit in the neurectoderm and paraxial mesoderm.[5,6] Both the time of onset and rostral limit of expression are related to the chromosomal location of a given *Hox* gene within a cluster, with 3′ members being typically expressed earlier and reaching a more anterior limit than 5′ members. Hox-dependent ingression of epiblast cells through the primitive streak is thought to contribute to the establishment of these domains.[7] This results in staggered domains of *Hox* expression along the AP axis, which is believed to reflect a "Hox code" for vertebral patterning.[8,9]

A number of signaling pathways and transcription factors are known to impact *Hox* expression, among which are the vertebrate Cdx (caudal) family of homeodomain transcription factors, Cdx1, Cdx2, and Cdx4.[10−13] A critical role for Cdx upstream of *Hox* expression was initially suggested by the finding of vertebral homeoses and posterior shifts in the expression of a number of *Hox* genes in Cdx1 null mutants and Cdx2 heterozygotes.[14−17] This is likely a direct relationship, as Cdx binding sites have been identified in numerous *Hox* promoters, some of which are capable of conveying *Hox*-like spatiotemporal expression patterns in transgenic models.[18,19]

As with many other transcription factors, Cdx1 and Cdx2 have been shown to physically interact with other effectors of transcription. For example, sequences within the Cdx2 homeodomain are involved in interactions with Brn-4 on the *proglucagon* promoter[20] as well as with HNF1α on the *lactase* promoter.[21] Cdx2 can also enhance the physical interaction between PAX6 and coactivator p300, although the precise interface involved in this relationship has not yet been characterized.[22] In the intestine, Cdx2 has been shown to colocalize with TCF4[23] and has also been proposed to coregulate target genes in concert with GATA6 and HNF4α.[24] Pbx1 has been implicated as a Cdx2 cofactor on the *proglucagon* promoter, an interaction that is mediated through the pentapeptide motif of Cdx2, reminiscent of Pbx−Hox association.[25−27] Finally, interaction between Cdx1 homeodomain sequences and residues within the B-box of LEF1 has been suggested to underlie Cdx1 autoregulation.[28]

As with Cdx proteins, Hox family members also interact physically with several other transcription factors. In particular, Hox DNA binding affinity and specificity are impacted via interaction with members of the three-amino acid loop extension (TALE) class of homeodomain proteins, comprised of Pbx and Meis members. Pbx proteins interact with Hox proteins through the hexapeptide motif found N-terminal to the homeodomain in *Hox* paralog groups 1−10,[25,27] although other sequences have also been implicated.[29,30] Meis proteins can interact directly with Pbx within the DNA-bound

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complex[31−33] and can also affect the nuclear localization and stability of Pbx proteins themselves.[34−36]

From late gastrulation through tail bud stages, *Cdx* members exhibit expression patterns that overlap with a number of *Hox* genes in the caudal embryo, consistent with direct regulation of *Hox* by Cdx members. In addition, as Cdx proteins can associate with other homeodomain proteins, we postulated that Cdx and Hox proteins may physically interact. Indeed, we found that Cdx1 associated with a subset of Hox proteins in a manner that involved specific residues in the first helix of the Hox homeodomain. This interaction was also dependent on permissive Hox N-terminal sequences. A larger group of Hox proteins interacted with Cdx in a cellular environment, suggesting the presence of bridging factors or post-translational modifications that influenced association. Finally, Cdx and Hox proteins co-occupied a known target sequence in a manner that required Hox−Cdx interaction, and this co-occupation impacted Hox-dependent transcription from these sequences. These findings represent the first description of Cdx−Hox protein interactions and suggest that these interactions impact the function of these factors in vivo.

MATERIALS AND METHODS

Expression Vectors. Open reading frames encoding Hox homeodomain sequences were generated from genomic DNA by polymerase chain reaction (PCR), while full-length Hox cDNAs were derived by reverse-transcription PCR (RT-PCR) using total RNA isolated from embryonic day 8.5−13.5 mouse embryos. PCR products were subcloned into pCR2.1 according to the manufacturer's instructions (Invitrogen). To introduce a FLAG epitope, inserts were subsequently excised using restriction sites introduced into the PCR primers and cloned into a modified pCEP4flag vector (Invitrogen) and subsequently into pSG5 (Stratagene) for in vitro and in vivo expression.

Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). Fusion proteins between Hox members were created by amplifying relevant cDNA sequences in a two-step PCR process. All constructs were verified by sequencing prior to use. Primers used for PCR amplification or mutagenesis are available upon request.

GST Fusion Protein Purification. BL-21 bacteria were transformed with either empty GST expression plasmid or previously described GST-Cdx1 constructs.[25] Cultures were grown for 3 h, treated with 0.5 mM IPTG (Bioshop), and cultured for a further 3 h. Cells were then pelleted, resuspended in PBS containing 1 mM DTT and protease inhibitors [1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 mM PMSF (Sigma)], and lysed by sonication using a Branson Sonifier 450. Triton X-100 (1%) was then added and the lysate cleared by centrifugation at 10000*g*. The supernatant was then incubated with glutathione-agarose beads (BD Biosciences) as per the manufacturer's instructions. The beads were subsequently washed three times and resuspended in PBS with protease inhibitors for use in binding assays.

Tissue Culture and Transfection. COS7 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). P19 embryocarcinoma cells were grown in α-MEM supplemented with 2.5% FBS and 7.5% heat-inactivated donor calf serum.

COS7 cells were transfected with 5 μg of plasmid DNA per 10 cm plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24 h post-transfection and disrupted on ice in 300 μL of lysis buffer [20 mM Tris (pH 8.0), 25 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, and protease inhibitors] for 30 min.

P19 cells were seeded into six-well plates at a density of 400000 cells/well for luciferase-based reporter assays. Cells were transfected using the calcium phosphate precipitation method with 1 μg of reporter vector, varying amounts of expression vector, and 200 ng of β-gal expression vector to a total of 2 μg of DNA/well. Cells were harvested 48 h post-transfection and the lysates processed and analyzed using the Promega Luciferase Assay System according to the manufacturer's instructions. β-Galactosidase activity was assessed using the chlorophenol red-β-D-galactopyranoside assay system (Calbiochem) and values used to correct for transfection efficiency.

Protein−Protein Interaction Assays. In vitro protein−protein interaction assays were conducted as previously described.[28] Briefly, [^{35}S]methionine-labeled Hox proteins were generated in vitro using the TNT Quick Coupled Transcription/Translation system (Promega). Five microliters of ^{35}S-labeled protein was then incubated with 5 μg of GST fusion protein (affixed to glutathione-agarose beads) in 1 mL of TNENi (50 mM Tris, 100 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM DTT, and protease inhibitors) overnight at 4 °C. The beads were then washed three times with TNENi; proteins were resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and Hox protein was revealed by autoradiography and compared to a lane containing a 5% input of radiolabeled protein.

To monitor interaction in a cellular environment, co-immunoprecipitation assays were performed as previously described.[28] Briefly, FLAG-tagged Hox expression vectors were cotransfected with or without Cdx expression vectors as described above. Cell lysates were then prepared and precipitated using anti-FLAG antibody (Sigma), and protein−protein interactions were detected in Western blots using the anti-Cdx1 antibody.[37] Association was compared to a 5% total lysate input lane, and Hox immunoprecipitation was confirmed by Western blotting using the anti-FLAG antibody.

Chromatin Immunoprecipitation (ChIP). ChIP was performed essentially as previously described.[38] Briefly, P19 cells were transfected as described above using 5 μg of expression plasmids and 1 μg of *Hoxb8* regulatory element reporter construct.[28] Twenty-four hours following transfection, cells were fixed in 1% formaldehyde for 10 min at room temperature and then quenched using 0.125 M glycine. Cells were washed using PBS, lysed using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM DTT, and protease inhibitors), and sonicated at 30% output for 2 min using a Branson Sonifier 450. Lysates were cleared and immunoprecipitated with 5 μg of the anti-FLAG antibody (Sigma) and eluted with 50 μL of elution buffer (1% SDS and 0.1 M $NaHCO_3$). An aliquot of the eluant was subsequently immunoprecipitated using the anti-Cdx1 antibody.[37] DNA was then isolated and assessed for interaction with the Hoxb8 template by quantitative PCR (qPCR). The relative level of enrichment was sequentially corrected for input and αFLAG pull-down efficiency and compared to that of cells transfected with FLAG-Hoxd4 alone as previously described.[39,40] The reverse experiment was conducted in a similar manner, first immunoprecipitating for Cdx1 and subsequently

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for Hox and using cells transfected with Cdx1 alone as a negative control. Data were obtained from qPCR from four independent transfections. Specific primer sequences are available upon request.

RESULTS

On the basis of previous findings, we speculated that Cdx1 may physically associate with Hox proteins. To test this, we first assessed the ability of several full-length Hox members to interact with Cdx1 in vitro using a GST pull-down assay. Using this approach, we found that full-length Hoxd4 interacted with Cdx1 while Hoxb1, Hoxa6, Hoxc8, and Hoxa9 proteins did not exhibit detectable association (Figure 1A). These data are consistent with a direct interaction and suggest specificity with respect to the ability of Hox proteins to associate with Cdx1.

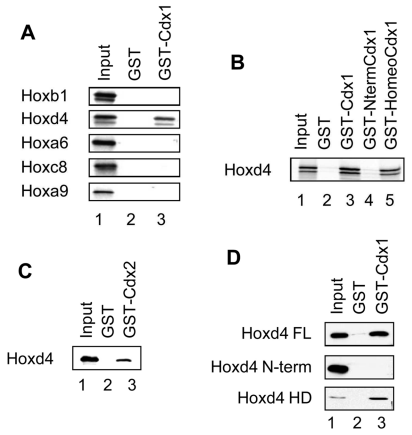

Figure 1. Cdx interacts with a subset of Hox proteins. Hox cDNAs were transcribed and translated in vitro in the presence of [^{35}S]methionine and the products used in GST pull-down assays. Interacting proteins were resolved by SDS–PAGE and revealed by autoradiography. (A) Interaction of full-length Hox proteins with GST-Cdx1. (B) Full-length Hoxd4 associated with full-length GST-Cdx1 and the GST-Cdx1 homeodomain, but not GST-Cdx1 N-terminal sequences. (C) Full-length Hoxd4 interacted with full-length GST-Cdx2. (D) Full-length GST-Cdx1 interacted with full-length Hoxd4 (FL) and the Hoxd4 homeodomain (HD), but not Hoxd4 N-terminal (N-term) sequences. Inputs shown represent 5% of the protein used in the pull-down assay.

To identify the region(s) of Cdx1 necessary for Hoxd4 interaction, proteins containing either the Cdx1 N-terminus (residues 1–160) or the homeodomain and C-terminus (residues 163–214) were generated and assessed for binding. Hoxd4 was found to interact with the C-terminal Cdx1 fragment, but not with N-terminal sequences (Figure 1B). As the regions C-terminal to the homeodomain are poorly conserved between Cdx members and both Cdx1 and Cdx2 were able to bind Hoxd4 (Figure 1C), this suggests that the Cdx homeodomain appeared to be necessary for interaction with Hoxd4, although additional, divergent, C-terminal sequences cannot be formally excluded.

Two regions of Hoxd4 were initially assessed for interaction with Cdx1: the N-terminal region including the hexapeptide motif (residues 1–151) and the homeodomain-containing C-terminal sequences (amino acids 152–250). Only the C-terminal Hoxd4 fragment interacted with Cdx1 (Figure 1D). Together with the observations described above, these findings suggested that the homeodomain regions of each transcription factor were involved in these interactions.

Hox N-Terminal Sequences Influence Association with Cdx1. To determine if Cdx could associate with diverse Hox proteins, members representative of a range of paralog groups were assayed for their capacity to interact with Cdx1. As the domain of association was confined to the C-terminus of Hoxd4, homeobox sequences from members of *Hox* paralog groups 1, 4–6, 8–10, 12, and 13 were used as templates for in vitro translation. While the majority of Hox homeodomains interacted with Cdx1, Hoxa1 sequences bound comparatively weakly in these assays, while Hoxb1 and Hoxc8 homeodomains did not interact detectably (Figure 2A). Thus, robust

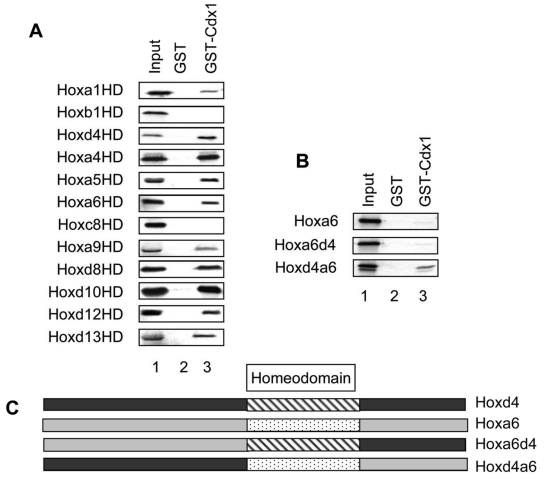

Figure 2. Hox N-terminal sequences influence Cdx1 binding. GST pull-down assays performed with ^{35}S-labeled Hox proteins and full-length GST-Cdx1. (A) Hox homeodomain sequences assessed for interaction with full-length GST-Cdx1. (B) Interaction between chimeric Hoxa6–Hoxd4 proteins and GST-Cdx1. (C) Schematic representation of the chimeric constructs used in panel B. Inputs shown represent 5% of the protein used in the pull-down assay.

association with Cdx1 in vitro is a characteristic that is restricted to a subset of Hox homeodomains. Further, these data demonstrate that the Hoxa6 and Hoxa9 homeodomains in isolation were capable of interacting with Cdx1, which contrasts with observations using the full-length protein, suggesting that sequences present in full-length Hox proteins could negatively influence Cdx binding in some cases.

To investigate further the influence of N-terminal Hox sequences on association with Cdx1, chimeric proteins between Hoxd4 (which interacted with Cdx1 as both a homeodomain fragment and a full-length protein) and Hoxa6 (which interacted only as a homeodomain protein) were constructed. Initial fusion proteins were generated between Hoxd4 N-terminal residues 1–151 and Hoxa6 C-terminal residues 154–232 (i.e., from the beginning of the Hoxa6 homeodomain to the end of the protein); the resultant chimera is denoted as Hoxd4a6. The converse fusion protein was also derived and is denoted Hoxa6d4.

Interaction assays revealed that Hoxd4a6 associated with Cdx1 while Hoxa6d4 did not (Figure 2B). As the Hoxa6 and Hoxd4 homeodomains are both able to interact with Cdx1 in isolation, and Hoxd4 (but not Hoxa6) also interacts as a full-length protein, it would appear that the N-terminal region of Hoxa6 (and potentially other Hox proteins such as Hoxa9)

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confers a dominant inhibition on the ability of Hox homeodomains to interact with Cdx1.

Discrete Hox Homeodomain Sequences Are Necessary for Cdx Interaction. The observations described above indicated that interaction between full-length Hox proteins and Cdx1 necessitates both permissive Hox N-terminal sequences and homeodomain residues capable of productive interaction. Both full-length Hoxd4 and its homeodomain in isolation interacted with Cdx1, while Hoxb1 did not interact in either form. Therefore, to better define the residues necessary to confer interaction, fusion proteins juxtaposing Hoxd4 and Hoxb1 homeodomain sequences were generated. The Hoxd4b1 construct was comprised of Hoxd4 N-terminal residues 1−151 fused to the Hoxb1 homeodomain amino acids 199−297, while the converse is termed Hoxb1d4 (Figure 3). Interaction assays revealed that the Hoxb1d4 chimeric

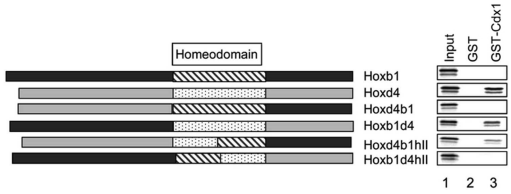

Figure 3. Sequences within helix I of the Hoxd4 homeodomain confer interaction with Cdx1. The left panel shows a schematic representation of the swapping strategy used to identify regions of the Hoxd4 homeodomain that conferred interaction with Cdx1. In the right panel, Hox proteins schematized on the left were translated in vitro in the presence of [^{35}S]methionine and assessed for interaction with full-length GST-Cdx1 as revealed by autoradiography. Inputs shown represent 5% of the protein used in the pull-down assay.

protein interacted with Cdx1, while the Hoxd4b1 fusion protein did not (Figure 3). This is in agreement with the finding that the C-terminus of Hoxd4, but not that of Hoxb1, can interact with Cdx1 and also illustrates that the N-terminus of Hoxb1 does not have an inhibitory effect on Cdx1 association.

To delineate further Hox homeodomain sequences necessary for interaction with Cdx1, a second set of chimeric Hox sequences were generated. As the third helix is the site of Hox−DNA binding and is highly conserved, we reasoned that it is unlikely that the differential Cdx1 interaction among Hox proteins is due to variance in this domain. We therefore generated chimeras with the junction between the first and second helices of Hox homeodomains as depicted in Figure 3. Hoxd4b1hII is the N-terminus of Hoxd4 including the first helix (residues 1−180) fused to Hoxb1 sequences commencing at the second helix (amino acids 228−297). The converse Hoxb1d4hII construct is Hoxb1 (amino acids 1−227) fused to Hoxd4 (residues 181−250). Interaction analysis using these chimeric proteins revealed that Hoxd4b1hII, but not Hoxb1d4hII, interacted with Cdx1 (Figure 3), suggesting that sequences within helix I dictate association, at least in the case of Hoxd4.

Identification of Specific Hox Residues Implicated in Interaction with Cdx1. A comparison of helix I sequences between interacting and noninteracting Hox members revealed several residues potentially involved in dictating association with Cdx1. In particular, the arginines at positions 3, 24, and 29 of the Hoxd4 homeodomain (residues 201, 222, and 227 of the full-length protein) are conserved in other interacting Hox members. These residues are, however, absent in the noninteracting Hoxb1 and Hoxc8 homeodomains (Figure 4A and data not shown). To assess the relevance of these residues

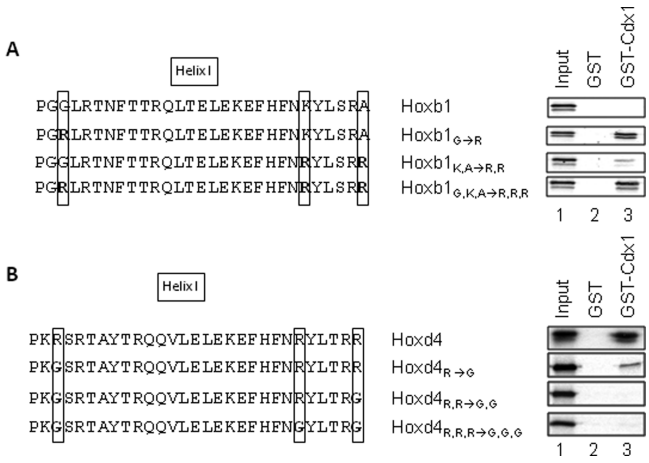

Figure 4. Identification of specific residues involved in Hox−Cdx interaction. The left halves of panels A and B show schematic representations of the residues modified by site-directed mutagenesis to create Hoxb1 and Hoxd4 mutants, respectively. Autoradiographic results of GST-Cdx1 pull-down assays assessing the interaction with Hoxb1 and Hoxd4 mutants are illustrated in the right halves of panels A and B, respectively. Inputs shown represent 5% of the protein used in the pull-down assay.

in conferring Cdx1 association, the noninteracting protein Hoxb1 was sequentially mutated to alter one or more of the residues in these positions to arginine. The single mutant Hoxb1$_{G\rightarrow R}$ changed the residue at homeodomain position 3 from glycine to arginine, while the mutant Hoxb1$_{K,A\rightarrow R,R}$ has mutations at positions 24 (lysine to arginine) and 29 (alanine to arginine) of the homeodomain. Finally, the triple mutant Hoxb1$_{G,K,A\rightarrow R,R,R}$ is a combination of the single and double mutants. Pull-down assays revealed that, in marked contrast to the wild-type protein, Hoxb1$_{G\rightarrow R}$ interacted with Cdx1 in a manner comparable to that of Hoxd4, while the double mutant Hoxb1$_{K,A\rightarrow R,R}$ also bound, albeit comparatively weakly. The triple mutant Hoxb1$_{G,K,A\rightarrow R,R,R}$ also interacted with Cdx1 (Figure 4A).

In a converse experiment, we mutated one or more of these arginines in the context of full-length Hoxd4 and assessed the ability of the mutants to interact with Cdx1. Mutation of the arginine at position 3 of the first helix significantly weakened the association with Cdx1, while mutation of the arginine at position 30 together with position 3 eliminated detectable binding (Figure 4B), as did mutation of all three arginine residues (Figure 4B). These findings suggest that, in the context of the full-length protein, these residues are necessary and sufficient to confer interaction between Hox and Cdx proteins in vitro.

Cdx−Hox Interactions in Vivo. To begin to assess the effects of a cellular environment on Hox−Cdx association, we conducted co-immunoprecipitation assays using proteins expressed in COS7 cells. Using this approach, we found that full-length Hoxd4, Hoxa6, and Hoxa9 interacted with Cdx1, while Hoxb1 and Hoxc8 did not (Figure 5A). Consistent with the in vitro assays, Hoxb1$_{G,K,A\rightarrow R,R,R}$ also interacted with Cdx1, while Hoxd4$_{R,R,R\rightarrow G,G,G}$ had a decreased level of association compared to that of the wild-type protein (Figure 5A). These results lend

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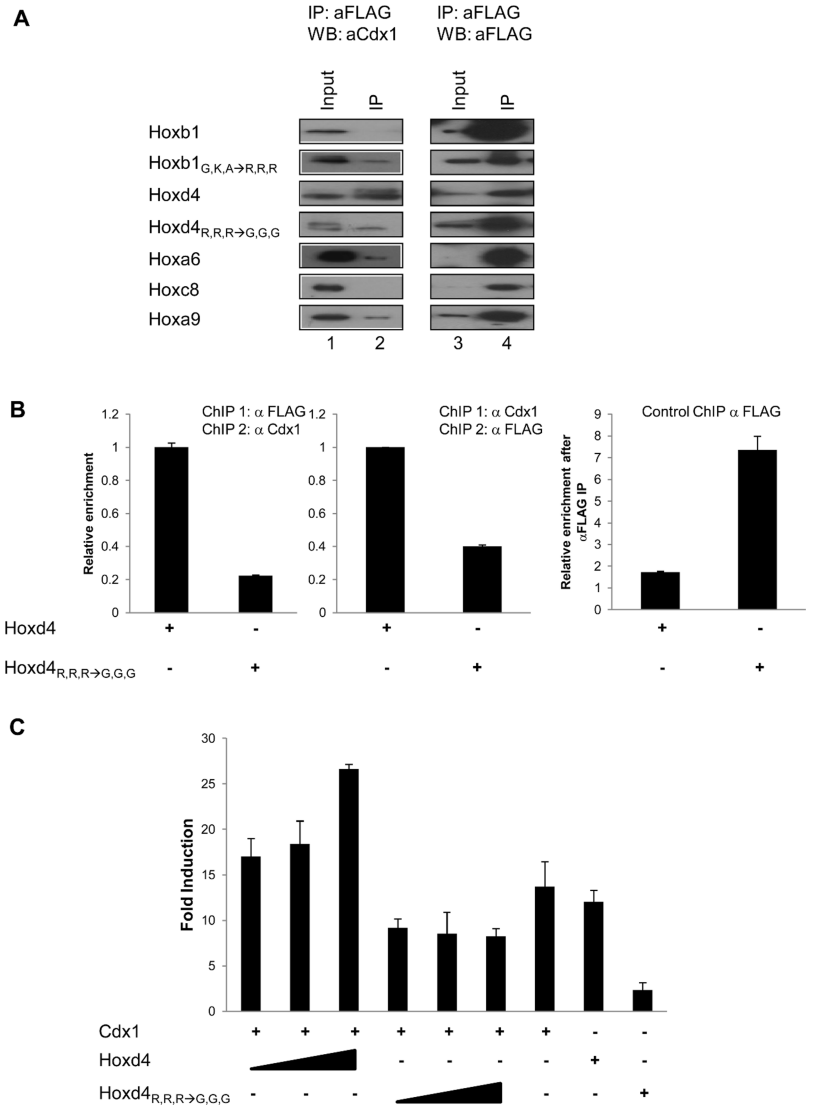

Figure 5. Hox–Cdx interactions in vivo. (A) Denoted FLAG-tagged Hox proteins were assessed for interaction with full-length Cdx1 in COS7 cells as revealed by immunoblotting for αFLAG or αCdx1. (B) ChIP–re-ChIP for P19 cells transfected with Cdx1 and Hoxd4 or Hoxd4$_{K,R,R\rightarrow G,G,G}$. Chromatin was immunoprecipitated first with anti-FLAG and subsequently with anti-Cdx1 antibodies, and analysis was conducted on the *Hoxb8* regulatory element in quadruplicate (left). The experiment was subsequently repeated, first immunoprecipitating with anti-Cdx1 and subsequently with anti-FLAG (middle) antibodies. qPCR data reflect enrichment relative to wild-type Hoxd4, corrected for relative inputs and first immunoprecipitation. Of note, mutation of Hoxd4 does not inhibit its binding to DNA (right). (C) Effect of 100 ng of Hoxd4 or Hoxd4$_{K,R,R\rightarrow G,G,G}$ and 50 ng of Cdx1 together on the activity of the *Hoxb8* regulatory element assessed by reporter analysis. β-Gal activity was used to correct for transfection efficiency. Error bars represent the standard deviation of triplicate transfections. Inputs shown represent 5% of the protein used in the pull-down assay.

further support to the importance of these arginine residues in mediating Cdx–Hox interactions in vivo.

To determine if Hox and Cdx members interacted on common target promoters, we used a ChIP–re-ChIP approach to assess the occupancy of the *Hoxb8* regulatory element, which is Cdx target.[18,28] To this end, P19 cells were transfected with Cdx1 in combination with Hoxd4 or Hoxd4$_{R,R,R\rightarrow G,G,G}$ and the response element. As expected, Cdx1, Hoxd4, and Hoxd4$_{R,R,R\rightarrow G,G,G}$ were all enriched at this locus (data not shown), and mutation of Hoxd4 did not abrogate its ability to bind to DNA (Figure 5B, right panel). Furthermore, ChIP–re-ChIP for Hoxd4 showed robust enrichment with Cdx1, while the Hoxd4$_{R,R,R\rightarrow G,G,G}$ mutant did not exhibit binding above background (Figure 5B, left and middle panels). These results indicated that Hoxd4 and Cdx1 can co-occupy a given promoter and that this interaction depends on the aforementioned Hoxd4 residues.

To determine if the interaction between Cdx1 and Hox proteins has potential biological impact, we assessed the effect of coexpression of Cdx1 and Hoxd4 on transcription directed by the *Hoxb8* promoter region, which contains four consensus Cdx binding sites that can be driven by Cdx1.[18] Transfection analyses in P19 embryocarcinoma cells revealed that Cdx1 and Hoxd4, but not Cdx1 and Hoxd4$_{R,R,R\rightarrow G,G,G}$, activated this reporter (Figure 5C). These data are consistent with the physical interaction between Cdx and Hox having an impact on transcription of at least some common target genes.

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DISCUSSION

Physical interaction between transcription factors can have a profound impact on their function through, for example, altering binding specificity and/or avidity, affecting subcellular localization, or altering interaction with other cofactors. Both Cdx and Hox family members are involved in vertebral patterning along the AP axis, and both are known to physically interact with a number of other transcription factors. For example, Cdx members have been shown to interact with additional homeodomain proteins, including Pbx1, Brn-4, TCF4, GATA6, HNF4A, and HNF1α.[20,24,26,41] Similarly, Hox proteins interact with homeodomain partners such as Pbx and Meis family members.[42] These findings led us to hypothesize that Cdx and Hox proteins may interact physically. Indeed, we found that both Cdx1 and Cdx2 are capable of direct interaction, and these interactions require specific arginine residues within Hox proteins. Moreover, this interaction appears to be functionally important on at least some target promoters. These data represent the first finding of a physical interaction between Hox and Cdx family members and suggest that this interaction may modulate target gene expression in vivo.

Specificity of Hox−Cdx Interactions. Initial experiments suggested that the Cdx1 and Hoxd4 homeodomains are necessary for their interaction, and there is ample precedence for the homeodomain serving such a role.[43−45] Hox homeodomains are highly conserved, particularly within a given paralog group. To determine the extent of Hox proteins capable of interacting with Cdx, homeodomains representative of different paralog groups were assessed for their ability to interact. These studies demonstrated that most of the Hox C-terminal sequences assessed were capable of interaction with Cdx1, suggesting that conserved residues within the homeodomain may dictate this interaction.

When comparing the ability of Hox proteins to associate with Cdx1, we found that certain Hox proteins that bound Cdx1 as C-terminal fragments could no longer bind as full-length proteins. Subsequent analysis led to the demonstration that sequences N-terminal to the Hox homeodomain may, in some instances, negatively influence interaction with Cdx1, suggesting additional means by which Hox−Cdx interactions could be modulated.

As opposed to in vitro assays, a wider array of Hox proteins interacted with Cdx1 in COS7 cells, potentially because of the presence of bridging proteins capable of associating with both transcription factors. In this regard, the Hox binding partners Meis1a and Pbx1 are both expressed in COS7 cells, and the latter has also been shown to be a transcriptional coregulator of Cdx2.[26] We have also found that Pbx1 and Meis1a were capable of interacting with Cdx1 and Cdx2 in vitro (our unpublished observations), consistent with such proteins contributing to the more promiscuous Cdx−Hox interactions seen in vivo. It is notable, however, that we were unable to immunoprecipitate Pbx−Hox−Cdx complexes from COS cells. This may be related to the observation that Pbx−Hox and Meis−Hox interactions are likely DNA-dependent.[42,46−48]

The expanded number of interacting proteins in vivo compared to the number in vitro could also suggests post-translational modifications of either Cdx or Hox. Phosphorylation is an important regulator of transcriptional capacity.[49] To this end, Cdx2 can be phosphorylated, and this is thought to be involved in the regulation of its transcriptional potency.[50] Furthermore, phosphorylation of Hox proteins has been shown to have important transcriptional implications. For example, phosphorylation of the protein product of *Hox* gene *Sex combs reduced* (Scr) in *Drosophila* inactivates its transcriptional activity in vivo. Moreover, the *Drosophila* Hox protein Ultrabithorax (Ubx) has been shown to be phosphorylated, with dominant negative forms leading to differential transcriptional repression and homeoses.[51] The bases for these differences in transcriptional activity are currently unknown, but changes in protein−protein interacting partners may underlie these functional changes. In this regard, it is tempting to speculate that altered function via post-translational modification of the N-terminal inhibitory Hox sequences may contribute to the greater promiscuity of Hox−Cdx interactions seen in vivo.

Discrete Hox Residues Are Involved in Cdx Interaction. To identify the specific Hox residues necessary for association with Cdx, we generated fusion proteins between an interactor (Hoxd4) and a noninteractor (Hoxb1). Analysis of these chimeric proteins led to the finding that helix I of the Hoxd4 homeodomain is involved in binding to Cdx1. Closer inspection of this region identified a number of potential residues, notably several arginines, in the homeodomain of Hoxd4 that were conserved in other interactors.

Polar residues, such as arginine, are typically enriched in protein−protein interfaces.[52,53] The essential role of these residues in Hox−Cdx interactions was demonstrated by mutation analysis. Most notable was the apparent role played by the arginine at position 3 of the homeodomain, as mutation of this singular residue to a glycine in the interacting protein Hoxb4 abrogated binding. In this regard, the homeodomains of interacting Hox paralog groups 2−8 typically have an arginine at position 3 while interactors from groups 9−13 harbor a lysine, a semiconserved substitution. Interestingly, paralog group 1 members Hoxa1 and Hoxb1 lack arginine or lysine residues at this position, consistent with their lack of interaction with Cdx1. Notably, however, conversion of glycine to arginine in position 3 of the Hoxb1 homeodomain was sufficient to confer association with Cdx1, underscoring the importance of this singular residue in influencing binding.

Functional Consequence of Cdx−Hox Interaction. Previous studies have shown that the N-terminal arm of the Hox homeodomain is important for interaction with DNA as well as with accessory proteins.[27,54−56] Interestingly, residues at positions 2 and 3 of this region have been shown to influence binding of Hoxd4 to DNA.[57] Moreover, the interaction of the N-terminal arm with DNA is affected by Hox−Pbx interaction, which may be involved in conferring functional specificity among Hox proteins.[27] Finally, it has been shown that N-terminal regions of Hoxd4 inhibit the recruitment of Pbx and Meis,[58] while the N-terminus of Hoxa9 has been shown to have a positive effect on trimer formation,[59] supporting our findings that Hox proteins have a differential impact on partner association and subsequent transcriptional activity. These data underscore the importance of this particular Hox domain, and the arginine at position 3, in both protein−protein and protein−DNA interactions. On the basis of these observations, it is tempting to speculate that interaction with Cdx within this region would also have an impact on Hox function. Consistent with this, we found that Cdx1 and Hoxd4 co-occupied a previously described Cdx1 target region. This binding correlated with transcriptional activation from these sequences, and both co-occupation and transcription were abrogated by mutation of the arginine residues of Hoxd4.

Combinatorial control of promoter regions is thought to be important in conferring functional specificity during development. For example, Cdx2, HNF1α, or GATA4 alone does not strongly induce expression of the *sucrase isomaltase* promoter in intestinal cells. In combination, however, they exhibit a potent synergistic effect, consistent with the proposal that they operate in a common transcriptional complex.[24] Their dynamic and overlapping expression patterns also suggest that they operate together during specific developmental windows.[41] In a similar manner, the graded, overlapping expression domains of Hox proteins are thought to operate in a "*Hox* code" to specify positional identity.[8,9] The complexity of mammalian development implies that other transcriptional mediators are involved in this process. The overlapping expression domains of Cdx members and many Hox members, together with the findings from this study, are consistent with Cdx and Hox acting as coregulators at certain common target genes, although further work will be required to address this paradigm in vivo.

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