Structural basis for homeodomain recognition by the cell-cycle regulator Geminin

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Homeodomain-containing transcription factors play a fundamental role in the regulation of numerous developmental and cellular processes. Their multiple regulatory functions are accomplished through context-dependent inputs of target DNA sequences and collaborating protein partners. Previous studies have well established the sequence-specific DNA binding to homeodomains; however, little is known about how protein partners regulate their functions through targeting homeodomains. Here we report the solution structure of the Hox homeodomain in complex with the cell-cycle regulator, Geminin, which inhibits Hox transcriptional activity and enrolls Hox in cell proliferative control. Side-chain carboxylates of glutamates and aspartates in the C terminus of Geminin generate an overall charge pattern resembling the DNA phosphate backbone. These residues provide electrostatic interactions with homeodomain, which combine with the van der Waals contacts to form the stereospecific complex. We further showed that the interaction with Geminin is homeodomain subclass-selective and Hox paralog-specific, which relies on the stapling role of residues R43 and M54 in helix III and the basic amino acid cluster in the N terminus. Interestingly, we found that the C-terminal residue Ser184 of Geminin could be phosphorylated by Casein kinase II, resulting in the enhanced binding to Hox and more potent inhibitory effect on Hox transcriptional activity, indicating an additional layer of regulation. This structure provides insight into the molecular mechanism underlying homeodomain-protein recognition and may serve as a paradigm for interactions between homeodomains and DNA-competitive peptide inhibitors.

The homeodomain transcription factors play fundamental roles in genetic control of development, including body plan specification, pattern formation, and cell fate determination (1–4). This protein family includes Hox, extended Hox, NK, LIM, POU, paired, and atypical subclasses based on evolutionary classifications (5). These proteins are all characterized by a 60-aa DNA binding domain, the homeodomain, which is encoded by a 180-bp DNA sequence known as the homeobox. The homeodomain folds into three α-helices and the latter two form a helix-turn-helix motif typical for DNA binding (6). The molecular mechanisms of how homeodomains recognize target DNA sequences have been extensively studied. For Hox proteins, their homeodomains contain identical DNA base-contacting residues and have very similar DNA-sequence specificity. Cooperative DNA binding with other cofactors is thought to enhance the transcriptional specificity of Hox proteins (7).

In addition to the role in DNA recognition, homeodomains have been found to serve as protein interaction targets to regulate the functions of homeodomain transcription factors or other proteins. For example, the Hox homeodomains are involved in the interactions with proteins, such as Smad1 (8), Smad4 (9), CBP (10), HMG1 (11), and Geminin (12). These protein interactions are mostly described to influence regulatory activities that define the level of target-gene activation or repression, and also link homeodomain proteins function to a variety of developmental pathways, such as chromatin remodeling, cell signaling, and cell cycle regulation (13). Despite the accumulating knowledge on the functional importance of the homeodomain–protein interactions, the molecular mechanism and sequence signatures of homeodomains in modulating DNA binding or regulatory activities through protein–protein interactions remain largely unexplored.

The mutual regulation between Geminin and Hox or Six3 homeodomain proteins have been identified to coordinate cell proliferation and differentiation processes (12, 14). Geminin was initially identified as a cell-cycle regulator critical for maintaining genome stability and euploidy (15, 16). During G1 phase, Cdc6 and Cdt1 are recruited by origin recognition complex to the replication origins and in turn required for the loading of mini-chromosome maintenance complex onto DNA to form the pre-replication complexes (pre-RC) (17, 18). Geminin physically interacts with Cdt1, and sequesters Cdt1 from its role in the pre-RC assembly, thus preventing reinitiation of DNA replication (19, 20). The interaction of Hox with Geminin could promote cell proliferation and mediate Hox-induced enhancement of hematopoietic stem cell activity (21). Meanwhile, this binding also characterizes Geminin as an inhibitor of the transcriptional activity of Hox proteins. This mutual regulation was suggested to result from the competitive binding between Hox and Cdt1 to the coiled-coil domain of Geminin (12).

In the present study, we first delineated the specific homeodomain binding region (HBR) on Geminin through pull-down assay, isothermal titration calorimetric (ITC) measurement, and NMR titrations. Surprisingly, the HBR is located at the C terminus of Geminin and separated from the Cdt1-binding coiled-coil domain. Using solution NMR techniques, we solved the structure of Gem-HBR in complex with the homeodomain of Hoxc9 (Hoxc9-HD). The defined molecular mechanism for the regulation between Hox and Geminin was verified by mutagenesis, and biochemical and cellular assays. We also found the C-terminal residue Serine 184 of Geminin at the complex interface could be phosphorylated by Casein kinase II (CK2), which increased the binding affinity to Hox, and the mutant that mimics S184 phosphorylation showed enhanced inhibitory effect on Hox transcriptional activity. Furthermore, we showed key residues determining the selectivity of Geminin to various homeodomain subclasses and Hox paralogs.

Results

Delineation of Homeodomain-Binding Site on Geminin. We first performed an extensive search for the Hox-binding domain on Geminin by pull-down assay using Hoxc9-HD and deletion fragments of Geminin based on the report that Geminin interacts with Hox homeodomains (12). Our results suggest that direct physical interaction occurs between Hoxc9-HD and the C terminus of Geminin, spanning residues 138–209 (Fig. 1*A*). To narrow down the binding region, we used smaller fragments of Geminin and found that Geminin(151–170) weakly interacts with

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Data deposition: The NMR, atomic coordinates, chemical shifts, and restraints have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2LP0).

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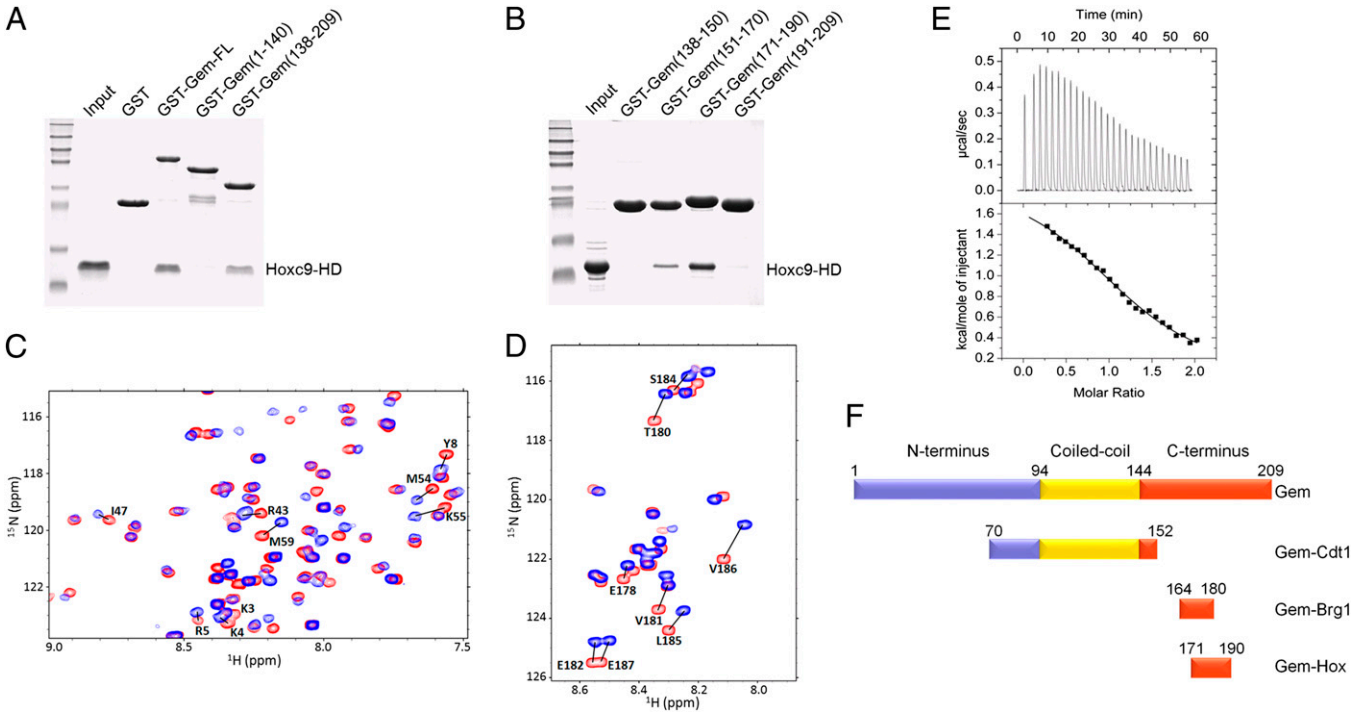

Fig. 1. C terminus of Geminin specifically interacts with Hoxc9. (*A*) Hoxc9-HD was pulled down by GST-tagged Gem-FL and its deletion mutants, and visualized by Coomassie blue staining. (*B*) The defined region of Geminin required for Hoxc9-HD interaction. Hoxc9-HD was pulled down by GST-tagged fragments of Geminin C terminus and visualized by Coomassie blue staining. (*C*) Part of the ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Hoxc9-HD in free form (red) overlaid with that titrated with nonlabeled Gem-HBR at a molar ratio of 1:2 (blue). (*D*) Part of the ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Gem-HBR in free form (red) overlaid with that titrated with nonlabeled Hoxc9-HD at a molar ratio of 1:2 (blue). (*E*) ITC measurement of Hoxc9-HD binding to Gem-HBR. (*F*) Schematic illustration of reported and currently identified protein binding motifs on Geminin.

Hoxc9-HD and Geminin(171–190) (Gem-HBR) strongly interacts with Hoxc9-HD (Fig. 1*B*).

The specificity of the interaction between Hoxc9-HD and Gem-HBR was further validated through NMR titration experiments. In heteronuclear single quantum coherence (HSQC) spectra of ^{15}N-labeled Hoxc9-HD titrated with Gem-HBR and vice versa, the chemical-shift perturbations of amide proton and nitrogen resonances of a defined set of residues indicate a specific binding (Fig. 1 *C* and *D* and Fig. S1). In contrast, titration of Hoxc9-HD with Geminin(151–170) showed no obvious changes in chemical shift, suggesting that the weak binding observed in the GST pull-down assay was nonspecific, or the interaction was too weak to be detected by NMR (Fig. S2*A*). Titration of ^{15}N-labeled Hoxc9-HD with Geminin(151–190) and vice versa showed similar chemical-shift pattern as the titration with Gem-HBR, except that the peaks broadened more rapidly, indicating a higher affinity (Fig. S2 *B* and *C*). These results demonstrate that Gem-HBR in the longer fragment plays the major role in the interaction with Hoxc9-HD, and the preceding region may contribute to strengthen the binding through nonspecific contacts. We also did NMR titration of ^{15}N-labeled Hoxc9-HD with Geminin(1–160) (Fig. S2*D*). No obvious chemical-shift changes were observed in the helix bundle region of the homeodomain, suggesting that the N-terminal segment and central coiled-coil domain of Geminin have no specific binding to Hoxc9-HD, which agrees with the pull-down result.

In the Hoxc9-HD/Gem-HBR titration spectra, amide proton and nitrogen resonances changed as a function of the added binding partner, indicating that the complex existed in fast exchange on the NMR time scale. Chemical-shift perturbation maps suggest that the N-terminal arm and C-terminal helix of Hoxc9-HD, primarily responsible for DNA recognition, are involved in the interaction with Gem-HBR. Through NMR and ITC measurements, we determined the dissociation constant of the complex to be 22.2 ± 4.5 μM (Fig. 1*E* and Table S1). This modest binding affinity is in agreement with the fast exchange state of the complex formation demonstrated by NMR titrations. We also determined the binding affinity between Hoxc9-HD and Geminin full-length protein (Gem-FL), which was calculated to be 3.3 ± 0.3 μM based on ITC measurement (Fig. S2*E*), indicating that although Gem-HBR provides the specific binding site for Hoxc9-HD, amino acids in other regions of Geminin could further enhance the binding affinity through nonspecific contacts.

Our results, obtained by multiple approaches, consistently demonstrate that the specific Hox-binding region of Geminin is situated at the C terminus, encompassing residues 171–190, which is different from the Cdt1-binding domain (22, 23) and partially overlaps with the Brg1-binding region (24) (Fig. 1*F*).

Structure Determination of Hoxc9-HD/Gem-HBR Complex by NMR. Using the solution NMR techniques, we determined the 3D structure of the Hoxc9-HD/Gem-HBR complex. The complex structure, calculated with intermolecular NOE constraints, was well-defined with good backbone geometry and no significant restraint violation (Fig. S3 *A*–*C* and Table S2). To independently confirm the observed orientation of Gem-HBR bound to Hoxc9-HD, the paramagnetic relaxation enhancement experiment was performed with a Hoxc9-HD mutant (C6S/M59C) that allowed covalent attachment of a paramagnetic proxyl group to the introduced cysteine. Binding of the spin-labeled Hoxc9-HD mutant to Gem-HBR resulted in line broadening of Gem-HBR residues 181–190, which further supports that Gem-HBR interacts with Hoxc9-HD in a head-to-head manner (Fig. S3 *D* and *E*). Hoxc9-HD adopts the well-characterized homeodomain-fold comprising three helices arranged in a bundle (Fig. 2*A*). Helices II and III of Hoxc9-HD form a helix-turn-helix motif, which is typical for DNA binding. The N-terminal arm preceding helix I of Hoxc9-HD and Gem-HBR are highly flexible in free form but become more rigid in the complex, as demonstrated by the ^{1}H-^{15}N heteronuclear NOEs (Fig. S3 *F* and *G*). Gem-HBR in the

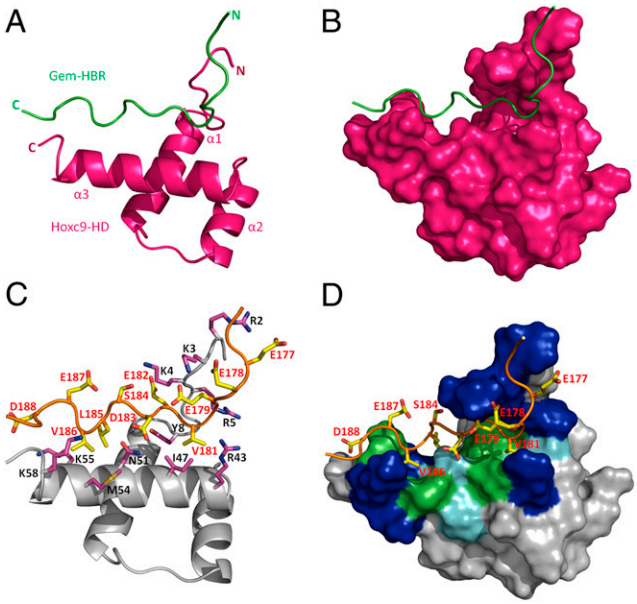

Fig. 2. Three-dimensional structure of Hoxc9-HD/Gem-HBR complex. (*A*) Ribbon diagram of a representative structure from the ensemble. Hoxc9-HD is colored in magenta, and Gem-HBR is depicted in green. (*B*) The complex shown with Gem-HBR in ribbon and Hoxc9-HD in surface representation. (*C*) Expanded view of the complex binding interface. Geminin residues are labeled in red and Hox residues in black. (*D*) The complex is illustrated by surface representation for Hoxc9-HD and by stick model for Gem-HBR (blue: basic residues; cyan: polar residues; green: hydrophobic residues; gray: noninteracting residues).

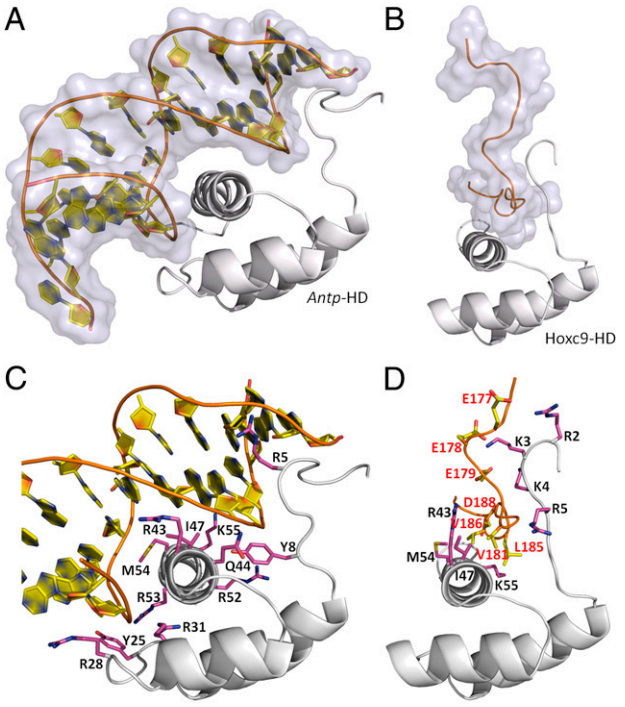

Fig. 3. Structural comparison of *Antp*-HD/DNA and Hoxc9-HD/Gem-HBR complexes. Structural alignment of homeodomains in complexes of *Antp*-HD/DNA (*A*, PDB: 1AHD) and Hoxc9-HD/Gem-HBR (*B*). Comparison of detailed structural features of homeodomain binding interfaces between DNA (*C*) and Gem-HBR (*D*). Protein residues are labeled as described in Fig. 2*C*.

complex is well ordered on its C terminus and inserts itself into the cavity formed by helix III and N-terminal arm of Hoxc9-HD (Fig. 2 *A* and *B* and Fig. S3*A*).

The contact between Hoxc9-HD and Gem-HBR is mainly through a combination of electrostatic and hydrophobic interactions. The Geminin-binding site of Hoxc9-HD exhibits two positively charged surfaces separated by one hydrophobic region (Fig. 2 *C* and *D*). The first positively charged surface is formed by residues R2, K3, K4, and R5, and is in close contact with E177, E178, E179, and E182 of Gem-HBR. The second surface is located on the C terminus, consisting of K55 and K58, which engages E187 and D188 of the C-terminal Gem-HBR. Residues R5, R43, I47, and M54 make hydrophobic contacts with V181 and V186 on Gem-HBR.

Comparison of Hoxc9-HD/Gem-HBR and *Antp*-HD/DNA Complex Structures. We compared our complex structure with the NMR structure of a *Drosophila* Hox homeodomain (*Antp*-HD) bound to DNA. The Geminin-binding site of Hox is located in the same region as that occupied by DNA in the *Antp*-HD/DNA complex (25) (Fig. 3 *A* and *B*). We aligned the solution structures and compared the intermolecular contacts of the two complexes (Fig. 3 *C* and *D*). Helix III of the homeodomain, the DNA recognition helix involved in the major groove binding, also plays a major role in the interaction with Gem-HBR. Residues R43, I47, and M54 provide the majority of the hydrophobic contact and K55 makes the electrostatic contact to both DNA and Gem-HBR. Residues R43, R52, and R53 connecting phosphate groups of DNA through salt bridges are not engaged in electrostatic interactions with Gem-HBR. The flexible N-terminal arm of the homeodomain, which binds to the minor groove of DNA, also interacts with the N terminus of Gem-HBR mainly through electrostatic forces. Additionally, residue R5 hydrophobically interacts with both DNA and Gem-HBR. Contacts reported for the *Antp*-HD/DNA complex include salt bridges involving R28 and R31 at the start of helix II and hydrophobic contact by Y25 in the loop preceding helix II. Although chemical-shift changes in this loop region were observed in the Hoxc9-HD/Gem-HBR complex, no direct contact was identified. In addition, hydrogen bonds formed by residues Y8, Y25, and Q44 with DNA were observed in the *Antp*-HD/DNA complex, but no explicit hydrogen bond was found in the Hoxc9-HD/Gem-HBR complex. Despite these differences in intermolecular contacts, Hox-HD binds DNA and Gem-HBR in a similar mode mainly by using the conserved residues of helix III and the N-terminal arm to clamp DNA or Gem-HBR. Therefore, Gem-HBR acts as a competitive inhibitor of DNA for binding the homeodomain of Hox proteins.

Mutational Analysis of Hoxc9-HD and Gem-HBR Interface. To validate the protein contacts identified in the complex structure, we made a series of mutations on residues at the intermolecular interface. The binding affinity of the mutants to the wild-type partner was determined by ITC measurement and NMR titrations. These two approaches yielded consistent conclusions on the importance of a number of charged and hydrophobic residues (Table S1). Specifically, we made three triple-mutants of Gem-HBR by replacing residues EEE(177–179), VED(181–183), and VED (186–188) separately (E to Q, V to A, and D to N). These substitutions resulted in obviously attenuated affinity of the complex, which was five- to sevenfold lower than that of the wild-type. For the mutant of Hoxc9-HD, in which N-terminal basic residues RKKR (2–5) were all substituted by alanine, the affinity was fourfold weaker compared with that of the wild-type. When six acidic residues conserved in human and mouse Geminin were replaced by neutrally charged amino acids (Gem-HBRmt6), the binding to Hoxc9-HD was entirely abolished (not detectable by either ITC or NMR), indicating that these residues are essential and collaboratively contribute to the binding (Fig. S4). The importance of electrostatic complementarities is also supported by a pull-down assay showing the disruption of Gem-HBR and Hoxc9-HD interaction at high salt concentrations (Fig. S5*A*).

Gem-HBR Inhibits Hox Binding to Target DNA. To investigate whether Gem-HBR itself could block DNA binding to Hox, we performed an EMSA, applying the recombinant Hoxc9 full-length protein (Hoxc9-FL) and a double-stranded oligonucleotide encompassing the consensus binding sequence for Hoxc9 (Fig. 4*A*). Hoxc9 induced significant shift of the oligonucleotide band during electrophoresis. Preincubation of Gem-HBR with Hoxc9 resulted in prominent, dose-dependent reduction of the shifted band. This result demonstrates that the 20-aa-long Gem-HBR could compete against DNA for binding to Hoxc9, supporting its role as the specific site of Geminin recognized by Hox proteins. Furthermore, the mutant Gem-HBRmt6 did not attenuate the Hoxc9-DNA band. This finding is in agreement with the abrogated binding of the mutant Gem-HBRmt6 determined by ITC and NMR analysis, and further validates the identified intermolecular contacts.

Gem-HBR Is Essential for Inhibition of Hox-Mediated Luciferase Gene Activation. To explore the importance of Gem-HBR on Hox-mediated gene activation in a cellular environment, a reporter assay was performed with a well-defined luciferase reporter plasmid, pTHCR, containing a 90-bp Hox cross-talk region, and Hoxd9, which bears a homeodomain nearly the same as Hoxc9-HD (26). Overexpression of Hoxd9 resulted in an approximate fourfold increase in luciferase activity in HeLa cells (Fig. 4*B*). Cotransfection with increasing amounts of Gem-FL suppressed the Hoxd9-induced reporter activity. In contrast, no repression was observed when the wild-type Gem-FL was replaced by the Gem-FLmt6 mutant, which removed the six negatively charged groups by E-to-Q mutations. This observation suggests that the C-terminal acidic residues of Geminin are critical to antagonize the role of Hox proteins in transcriptional activations, and strongly supports the structural mechanism we proposed.

CK2 Phosphorylation at S184 of Geminin Could Enhance Its Inhibitory Effect on Hoxc9 Transcriptional Activity. A previous report has found that the C terminus of Geminin, not the coiled-coil domain, could be phosphorylated by serine/threonine protein kinase CK2 (27). To investigate whether residues T180 and S184 of Geminin located at the complex interface are targets of CK2, we performed an in vitro phosphorylation assay followed by MS analysis. The results demonstrate that S184, but not T180, could be phosphorylated by CK2 (Fig. S6 *A–D*). NMR titrations showed that the mutant S184E, which mimics phosphorylation of S184, bound to Hoxc9-HD with ~fivefold higher affinity than the wild-type (Fig. S6 *E* and *F*). The higher affinity may result from the negatively charged group introduced at residue 184 that enhanced the electrostatic interactions with the positively charged residues in the N-terminal arm of Hoxc9-HD (Fig. 2 *C* and *D*). In the luciferase assay, the Gem-FL S184E mutant showed stronger inhibition of the Hoxc9-induced reporter gene expression compared with the wild-type Gem-FL (Fig. 4*C*), which is consistent with the enhanced binding of Gem-HBR to Hoxc9-HD upon the phosphorylation-mimicking mutation. Collectively, these results indicate that the inhibitory effect of Geminin on Hox transcriptional activity could be regulated by CK2 phosphorylation.

Gem-HBR Has Differential Affinity to Homeodomain Subclasses and Hox Paralogs. More than 100 homeodomain proteins are present in the cellular environment and their homeodomains share a conserved structural fold. An interesting question is whether the inhibitory effect of Geminin is specific to Hox or common to homeodomain proteins of various subclasses. To address this issue, we used NMR titrations to further determine the binding constant between Gem-HBR and homeodomains of six subclasses: Msx1 (extended Hox class), Pax6 (paired class), ISL1 (LIM class), Oct4 (POU class), Six3 (Atypical class), and Hox paralogs Hoxb1 and Hoxd10 (Hox class). These homeodomains could also serve as natural mutants of Hoxc9-HD to verify the determined binding mechanism.

As shown in Fig. S7 *A–H*, Hoxd10 has the highest binding affinity comparable to that of Hoxc9 ($K_d \sim 10^{-5}$ M). Hoxb1, Msx1, ISL1, and Oct4 showed modest affinity ($K_d \sim 10^{-4}$ M), and Pax6 and Six3 exhibited the weakest binding ($K_d > 10^{-4}$ M). Based on the Hoxc9-HD/Gem-HBR structure and the sequence alignment of the eight homeodomains (Fig. 5*A*), residues at positions 43 and 54 contribute to the observed binding affinity difference. The bulky side chains of R43 and M54 in Hoxc9 and Hoxd10 are in favor of hydrophobic interactions with Gem-HBR. In other homeodomains, one or both of these two amino acids are changed to residues with shorter side chains as Thr

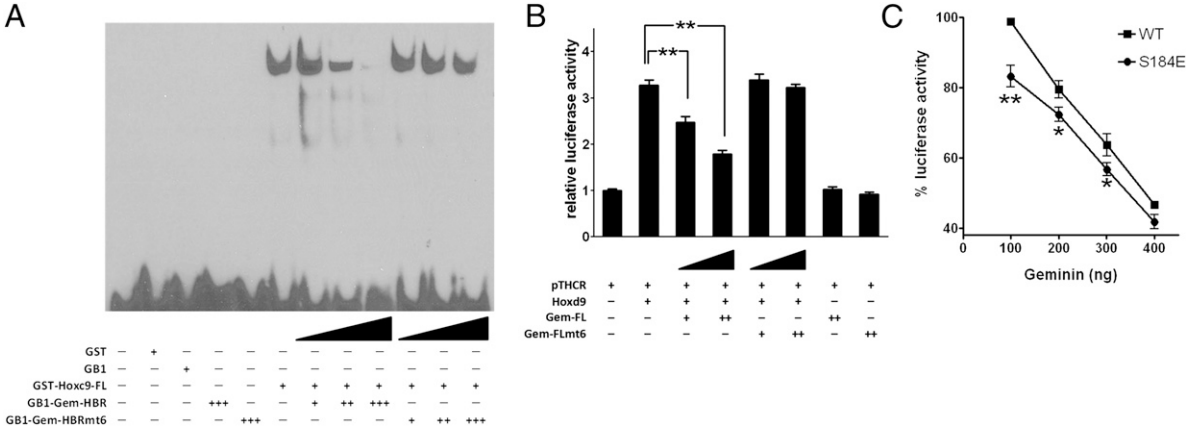

Fig. 4. Effects of wild-type and mutated Geminin on Hox-DNA binding and Hox-induced transcription of the reporter gene. (*A*) Inhibition of Hoxc9-DNA binding. EMSA was performed using GST-tagged Hoxc9-FL (2 μg) and increasing amount (10, 25, and 60 μg) of GB1-tagged Gem-HBR or Gem-HBRmt6. The SDS/PAGE Coomassie staining gel of the input proteins is shown in Fig. S5*B*. (*B*) Inhibition of Hoxd9-mediated transcriptional activation. Gem-FL or Gem-FLmt6 alone transfected with pTHCR did not alter the reporter activity (bars 7 and 8). Gem-FL cotransfected with Hoxd9 suppressed the luciferase activity induced by Hoxd9 (bars 3 and 4), whereas no repression was observed when Gem-FLmt6 was cotransfected with Hoxd9 (bars 5 and 6). Results are presented as mean ± SEM and obtained from three independent experiments performed in triplicates. Data were analyzed by one-way ANOVA followed with Newman–Keuls multiple comparison test. $^{**}P < 0.01$. (*C*) Comparison of Gem-FL wild-type (WT) and S184E mutant in inhibition of Hoxd9-mediated transcriptional activation. Increasing amount of Gem-FL and Gem-FL S184E were cotransfected with Hoxd9. Percent luciferase activity is the relative light units detected from the luciferase enzyme assay in cotransfected cells relative to control cells transfected with Hoxd9 alone. Results are presented as mean ± SEM and obtained from four independent experiments each performed in duplicates. Data were analyzed by Student *t* test. $^{*}P < 0.05$. $^{**}P < 0.01$ significantly different between wild-type and mutant.

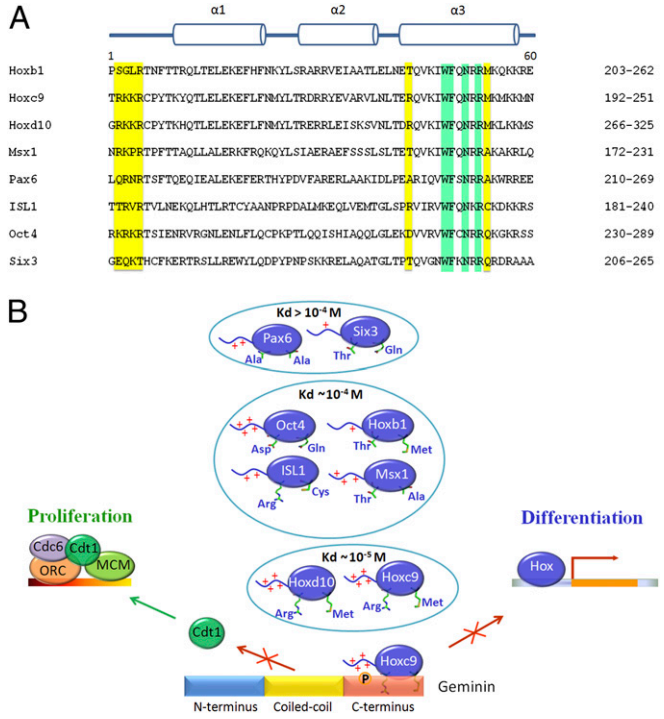

Fig. 5. Differential binding affinity of homeodomain subclasses and Hox paralogs to Geminin. (*A*) Sequence alignment of homeodomains of Hox paralogs and various subclasses. Residues Trp48, Phe49, Asn51, and Arg53, which are conserved across all homeodomains with major implications for DNA binding and overall stability of the homeodomain structure, are highlighted in green. Residues mainly contributing to the differential binding affinity with Geminin are highlighted in yellow. Residue numbering within the homeodomain is shown above and in the full-length protein is shown on the right. (*B*) A schematic illustration for the coordination of cell differentiation and proliferation through mutual regulation of posterior Hox proteins and Geminin. The C terminus of Geminin interacts with the homeodomain to inhibit Hox-induced gene transcription and cell differentiation; Hox prevents Geminin from binding to Cdt1, possibly through steric hindrance, and subsequently facilitates DNA synthesis and cell proliferation. CK2 could phosphorylate S184 at the C terminus of Geminin and enhance its binding with Hox. Furthermore, homeodomains of six subclasses (Pax6, Six3, Oct4, ISL1, Msx1, Hox) and three Hox paralogs (Hoxb1, Hoxc9, Hoxd10) have different binding affinity to Gem-HBR, in which posterior Hox proteins (Hoxc9, Hoxd10) showed the strongest binding. Residues that mainly account for the binding constant difference are the N-terminal basic amino acids at positions 2–5 (each represented by a "+") and amino acids at positions 43 and 54 in helix III (shown with side-chain cartoon).

(Hoxb1, Msx1, and Six3), Ala (Pax6), or Asp (Oct4) at position 43, and Ala (Msx1, Pax6), Cys (ISL1), or Gln (Oct4, Six3) at position 54, which could lead to the reduced affinity to Geminin. To validate the importance of these two residues, we made a pair of swap mutants for Hoxc9 and Pax6 by exchange of the respective residues at positions 43 and 54. The K_d between Hoxc9-HD (R43A/M54A) and Gem-HBR is 93.4 ± 14.8 μM, which is around fourfold higher than that between the wild-type Hoxc9-HD and Gem-HBR (22 μM) (Fig. S7*I*). The K_d between Pax6-HD (A43R/A54M) and Gem-HBR is 127.7 ± 18.2 μM, which demonstrates greatly increased affinity compared with that between the wild-type Pax6-HD and Gem-HBR (>1,000 μM) (Fig. S7*J*). These results further support that the differential affinity of Gem-HBR to homeodomain subclasses is largely attributed to residues at these two positions. In addition, as revealed by the complex structure and the mutagenesis study, the N-terminal basic residue cluster at positions 2–5 provides positive charges and, consequently, favorable electrostatic interactions between Hox and Geminin. The less basic amino acids in this region of most other homeodomains could also result in the weaker binding (Fig. 5*B*). Taken together, the differential binding affinity of various homeodomains is in support of our complex structure and suggests that the strength of electrostatic and hydrophobic forces determines the selectivity of Geminin toward specific Hox homeodomains.

Discussion

Hox-Binding Region Is Separate from the Cdt1-Binding Site on Geminin. In this study we demonstrate that the C terminus of Geminin specifically binds to the Hox homeodomain. It was previously believed that the Hox-binding region of Geminin is overlapped with the Cdt1-binding site in the central coiled-coil domain (28), which stems from the results of peptide array mapping of Hox-binding regions (12). However, the peptides used in the arrays may not accurately represent the behavior of a folded protein. In our pull-down assay, no interaction was observed between Hoxc9-HD and the coiled-coil domain of Geminin, in accordance with a previous report (23). We also applied the more sensitive NMR titrations, which indicate that the coiled-coil domain may nonspecifically bind to the N-terminal flexible region of Hoxc9-HD. Based on our current finding and the previous observation that Hox prevented the formation of Geminin-Cdt1 complex, we propose that Hoxc9 occupies the C terminus of Geminin and inhibits its interaction with Cdt1, possibly through steric hindrance, but not by direct competition of the binding pocket.

Geminin binding to Hox and Cdt1 at separate regions permits different regulatory pathways of its inhibitions of Cdt1-dependent cell proliferation and Hox-mediated cell differentiation (Fig. 5*B*). Particularly, we showed that the interaction of Geminin with Hox and the resulted inhibition of Hox transcriptional activities could be modulated through phosphorylation of residue S184 in the C terminus of Geminin by CK2, which is known to contribute to regulating cell growth and differentiation (29–31). The Brg1-binding region of Geminin is localized at residues 164–180 in the C terminus (24). Therefore, the Hox-binding region is partially overlapped with the Brg1-binding site, implying that Brg1 may be able to compete with Hox for Geminin and consequently regulate their interaction.

Homeodomains Encode Unexplored Information for Protein Recognition. In the past decades, great efforts have been devoted to elucidate specific DNA recognition by homeodomains (6, 7). The determined homeodomain-DNA structures demonstrate a similar mode of DNA binding to various homeodomains. In addition to the monomeric homeodomain-DNA structures, structures of Hox-DNA-Exd/Pbx ternary complexes have been solved, which revealed the involvement of the extended and unstructured region outside the homeodomain that links to a DNA-bound cofactor protein Exd or Pbx (32–35).

More recently, a number of protein interactions engaging the homeodomains have been identified to be involved in various developmental pathways. Some of the protein partners, including Smad1, CBP, HMG1, Geminin, and other Hox proteins, are suggested to bind at regions responsible for DNA recognition (13). These protein partners are described to regulate DNA binding and transcriptional activity of the homeodomain proteins, and may also reciprocally involve homeodomains in other regulatory pathways. Other protein partners bind to the non–DNA-contact region of homeodomains, as exemplified by Pax, which did not interrupt Hox-DNA binding but links Hox to the novel regulatory function on eye development (36).

Here we provide the structural mechanism for Hox–Geminin interaction, which is DNA-independent and engages only the homeodomain core. Our findings highlight that although homeodomains have been broadly viewed and extensively studied as DNA-binding domains, their sequences also encode information for specific protein recognition. It is anticipated that the DNA binding inhibitors of homeodomains may share similar binding features with Geminin, considering that the homeodomain has

extremely conserved structural fold. The N-terminal basic residues and the third helix of the homeodomains form a cavity which provides the docking site for DNA or protein. The side-chain carboxylates of glutamates and aspartates of protein partners, which generate an overall charge pattern resembling the DNA phosphate backbone, attract homeodomain through electrostatic interactions that combine with the van der Waals contacts to form the stereospecific complex.

Geminin Selectively Interacts with Posterior Hox Homeodomains. Comparing homeodomains of Hox paralogs and other subclasses, their binding affinity to Gem-HBR could differ at over 100-fold. The binding strength is tuned by hydrophobicity of residues at positions 43 and 54 in helix III and electrostatic forces contributed by the basic amino acids at positions 2–5 in the N terminus (Fig. 5*B*).

Vertebrate Hox genes are arranged in four clusters (a to d), and grouped into 13 paralog groups. The linear arrangement of genes within each cluster facilitates controlled spatial and temporal expression along the anterior-posterior axis of the body (34). Comparing their sequence signatures for Geminin binding, residues R43 and M54 are nearly conserved in all Hox except group 1 (Fig. S7*K*). Central and anterior Hox proteins have one or three fewer basic amino acids at the N terminus positions 2–5 than posterior Hox. In this regard, the binding affinity to Geminin is expected to increase from anterior to posterior Hox, as also demonstrated by the higher K_d of Hoxb1 compared with Hoxc9 and Hoxd10, which indicates that posterior Hox proteins are preferred for the cross-talk with Geminin for regulations of cell proliferation and differentiation.

Geminin has previously been found to interact with Six3, a homeodomain protein of the atypical subclass, to control the balance between proliferation and differentiation during early vertebrate eye development (14). In our assay, the Six3 homeodomain showed little binding to Gem-HBR, which agrees with the observation that Geminin was not able to inhibit the DNA binding to Six3 (14). Taken together, these results suggest that Geminin may target other regions of Six3 to antagonize its transcriptional activity, with a different mechanism from the repression of Hox protein functions.

Our results indicate that the subtle sequence variation among homeodomains may fine-tune specific homeodomain–protein interactions. Importantly, this finding demonstrates a molecular mechanism for tight control of diverse cellular functions of homeodomain proteins. Moreover, narrowing down residues that may be dedicated to particular molecular functions could provide the basis for targeted therapeutic interventions (37). Hox is well documented as an anticancer target (38); however, specific inhibition of its transcriptional activity has not been realized. The selective Hox-inhibitory peptide and the structural mechanism identified in the present study may be used and further studied for development of novel therapeutic agents.

Materials and Methods

The human Hoxc9-HD and Gem-HBR were cloned and expressed using a modified pET32a vector. Proteins were purified using ion exchange and gel-filtration chromatography. The detailed description of NMR experiments, structure calculation, biochemical and cellular assays is provided in *SI Materials and Methods*.

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1. Gehring WJ (1987) Homeo boxes in the study of development. *Science* 236:1245–1252.
2. Gehring WJ, Affolter M, Bürglin T (1994) Homeodomain proteins. *Annu Rev Biochem* 63:487–526.
3. Krumlauf R (1994) Hox genes in vertebrate development. *Cell* 78:191–201.
4. Wellik DM (2009) Hox genes and vertebrate axial pattern. *Curr Top Dev Biol* 88: 257–278.
5. Banerjee-Basu S, Baxevanis AD (2001) Molecular evolution of the homeodomain family of transcription factors. *Nucleic Acids Res* 29:3258–3269.
6. Gehring WJ, et al. (1994) Homeodomain-DNA recognition. *Cell* 78:211–223.
7. Mann RS, Lelli KM, Joshi R (2009) Hox specificity unique roles for cofactors and collaborators. *Curr Top Dev Biol* 88:63–101.
8. Yang X, Ji X, Shi X, Cao X (2000) Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation. *J Biol Chem* 275:1065–1072.
9. Wang N, et al. (2006) TGFbeta/BMP inhibits the bone marrow transformation capability of Hoxa9 by repressing its DNA-binding ability. *EMBO J* 25:1469–1480.
10. Shen WF, Krishnan K, Lawrence HJ, Largman C (2001) The HOX homeodomain proteins block CBP histone acetyltransferase activity. *Mol Cell Biol* 21:7509–7522.
11. Zappavigna V, Falciola L, Helmer-Citterich M, Mavilio F, Bianchi ME (1996) HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J* 15:4981–4991.
12. Luo L, Yang X, Takihara Y, Knoetgen H, Kessel M (2004) The cell-cycle regulator geminin inhibits Hox function through direct and polycomb-mediated interactions. *Nature* 427:749–753.
13. Merabet S, Hudry B, Saadaoui M, Graba Y (2009) Classification of sequence signatures: A guide to Hox protein function. *Bioessays* 31:500–511.
14. Del Bene F, Tessmar-Raible K, Wittbrodt J (2004) Direct interaction of geminin and Six3 in eye development. *Nature* 427:745–749.
15. McGarry TJ, Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* 93:1043–1053.
16. Melixetian M, Helin K (2004) Geminin: A major DNA replication safeguard in higher eukaryotes. *Cell Cycle* 3:1002–1004.
17. Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. *Annu Rev Biochem* 71:333–374.
18. Liu C, et al. (2012) Structural insights into the Cdt1-mediated MCM2-7 chromatin loading. *Nucleic Acids Res* 40:3208–3217.
19. Wohlschlegel JA, et al. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* 290:2309–2312.
20. Tada S, Li A, Maiorano D, Méchali M, Blow JJ (2001) Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat Cell Biol* 3:107–113.
21. Ohno Y, et al. (2010) Hoxb4 transduction down-regulates Geminin protein, providing hematopoietic stem and progenitor cells with proliferation potential. *Proc Natl Acad Sci USA* 107:21529–21534.
22. Lee C, et al. (2004) Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. *Nature* 430:913–917.
23. Saxena S, et al. (2004) A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. *Mol Cell* 15:245–258.
24. Seo S, et al. (2005) Geminin regulates neuronal differentiation by antagonizing Brg1 activity. *Genes Dev* 19:1723–1734.
25. Billeter M, et al. (1993) Determination of the nuclear magnetic resonance solution structure of an Antennapedia homeodomain-DNA complex. *J Mol Biol* 234:1084–1093.
26. Zappavigna V, et al. (1991) HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. *EMBO J* 10:4177–4187.
27. Roukos V, et al. (2007) Geminin cleavage during apoptosis by caspase-3 alters its binding ability to the SWI/SNF subunit Brahma. *J Biol Chem* 282:9346–9357.
28. Kroll KL (2007) Geminin in embryonic development: Coordinating transcription and the cell cycle during differentiation. *Front Biosci* 12:1395–1409.
29. Dominguez I, Sonenshein GE, Seldin DC (2009) Protein kinase CK2 in health and disease: CK2 and its role in Wnt and NF-kappaB signaling: Linking development and cancer. *Cell Mol Life Sci* 66:1850–1857.
30. St-Denis NA, Litchfield DW (2009) Protein kinase CK2 in health and disease: From birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. *Cell Mol Life Sci* 66:1817–1829.
31. Trembley JH, Wang G, Unger G, Slaton J, Ahmed K (2009) Protein kinase CK2 in health and disease: CK2: A key player in cancer biology. *Cell Mol Life Sci* 66:1858–1867.
32. Passner JM, Ryoo HD, Shen L, Mann RS, Aggarwal AK (1999) Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature* 397:714–719.
33. Piper DE, Batchelor AH, Chang CP, Cleary ML, Wolberger C (1999) Structure of a HoxB1-Pbx1 heterodimer bound to DNA: Role of the hexapeptide and a fourth homeodomain helix in complex formation. *Cell* 96:587–597.
34. LaRonde-LeBlanc NA, Wolberger C (2003) Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior. *Genes Dev* 17: 2060–2072.
35. Joshi R, et al. (2007) Functional specificity of a Hox protein mediated by the recognition of minor groove structure. *Cell* 131:530–543.
36. Plaza S, et al. (2008) Cross-regulatory protein-protein interactions between Hox and Pax transcription factors. *Proc Natl Acad Sci USA* 105:13439–13444.
37. Morgan R, et al. (2007) Antagonism of HOX/PBX dimer formation blocks the in vivo proliferation of melanoma. *Cancer Res* 67:5806–5813.
38. Shah N, Sukumar S (2010) The Hox genes and their roles in oncogenesis. *Nat Rev Cancer* 10:361–371.