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Chromatin-associated HMG-17 is a major regulator of homeodomain transcription factor activity modulated by Wnt/β-catenin signaling

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ABSTRACT

Homeodomain (HD) transcriptional activities are tightly regulated during embryogenesis and require protein interactions for their spatial and temporal activation. The chromatin-associated high mobility group protein (HMG-17) is associated with transcriptionally active chromatin, however its role in regulating gene expression is unclear. This report reveals a unique strategy in which, HMG-17 acts as a molecular switch regulating HD transcriptional activity. The switch utilizes the Wnt/β-catenin signaling pathway and adds to the diverse functions of β-catenin. A high-affinity HMG-17 interaction with the PITX2 HD protein inhibits PITX2 DNA-binding activity. The HMG-17/PITX2 inactive complex is concentrated to specific nuclear regions primed for active transcription. β-Catenin forms a ternary complex with PITX2/HMG-17 to switch it from a repressor to an activator complex. Without β-catenin, HMG-17 can physically remove PITX2 from DNA to inhibit its transcriptional activity. The PITX2/HMG-17 regulatory complex acts independently of promoter targets and is a general mechanism for the control of HD transcriptional activity. *HMG-17* is developmentally regulated and its unique role during embryogenesis is revealed by the early embryonic lethality of HMG-17 homozygous mice. This mechanism provides a new role for canonical Wnt/β-catenin signaling in regulating HD transcriptional activity during development using HMG-17 as a molecular switch.

INTRODUCTION

The chromatin-associated high mobility group protein (HMG-17) is a member of the HMGN family including HMG-14 that bind to the nucleosome core particle, without specificity for a DNA sequence (1). HMGN proteins are expressed in the nucleus and cytoplasm (2,3) and they regulate chromatin structure (4), histone modifications (5) and the rates of transcription (6). These factors are non-histone proteins that may act to modify chromatin structure to generate a conformation that facilitates and enhances transcription, replication and repair (4). In the nucleus, HMGN proteins appear to associate and dissociate regularly among nucleosomes and reduces the compaction of chromatin fiber (3,7). Thus, HMG molecules bind DNA transiently and continuously move to other binding sites within the chromatin. However, their interaction with chromatin is likely mediated by binding other factors in a multiprotein complex (1,8).

HMG-17 is expressed during early mouse embryogenesis throughout the entire embryo but is down regulated as development proceeds. However, in some actively differentiating cell types or in kidney cells undergoing a mesenchymal to epithelial transition, *HMG-17* expression is not decreased (9). Thus, HMG-17 may be required in tissues or cells undergoing proliferation and differentiation during organogenesis (10).

PITX2 is a 'paired' type homeodomain (HD) transcriptional activator and its activity can be modulated through protein interactions and phosphorylation (11–14). The analyses of *Pitx2*$^{-/-}$ homozygous knockout mice revealed that *Pitx2* is required for heart morphogenesis, development of the mandibular and maxillary facial prominences, tooth and pituitary development (15–18). For PITX2, the C-terminal and HD regions of the protein have been identified as sites for protein–protein interactions (14,19–22). The canonical Wnt signaling pathway is one mechanism where β-catenin and Lef-1 can independently interact with PITX2 to increase its transcriptional activity (22,23). Thus, it is becoming clear that differential mechanisms for β-catenin regulated transcription occur

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through its interaction with other factors and represents a major developmental event. Identifying these alternative pathways is of major interest to elucidate new developmental programs.

The regulated transcriptional activity of PITX2 through its interaction with HMG-17, modulated by β-catenin represents a new alternative Wnt/β-catenin signaling pathway. We demonstrate a novel molecular mechanism for the control of PITX2 HD transcription factor activation through its interaction with HMG-17. HMG-17 inhibits PITX2 DNA binding through an interaction with the PITX2 HD. HMG-17 forms an inhibitory complex with PITX2, which can be activated by canonical Wnt/β-catenin signaling. HMG-17 and PITX2 co-localize to chromatin structures in the nucleus. β-Catenin interacts with PITX2 in the nucleus and de-represses the PITX2/HMG-17 complex. This type of mechanism would allow for the tight spatial and temporal expression of PITX2 target genes during embryogenesis.

MATERIALS AND METHODS

Yeast two-hybrid system

PITX2 was used as bait with a cDNA library to identify interacting factors. PITX2A was cloned in the Gal4 DNA-binding domain vector (pBD-Gal4 vector, Stratagene). PITX2A, PITX2A C173, PITX2A C39 and PITX2A HD were PCR amplified using primers with Sal1 sites and inserted into the vector digested with Sal1. The library contains cDNA prepared from one-day postnatal mouse teeth (molars and incisors). The cDNAs are fused to the Gal4 transactivation domain in the Stratagene two-hybrid vector. This plasmid library contains 10^7 individual cDNAs. The library was amplified in yeast. The recombinant library plasmids and the bait plasmid were co-transformed into a reporter yeast strain (YRG-2). This strain has the *his3* and *lacZ* (β-galactosidase) genes under control of a Gal4 responsive element. Yeast transformed with the library (initially 10^6 transformants) were selected for growth on auxotrophic medium lacking histidine, in the presence of 15 mM 3-AT. The yeast were screened for expression of β-galactosidase (blue/white screening with X-gal) to confirm the interaction. The filter lift assay was done according to the manufacturer's directions (Stratagene). Plasmids were isolated from the yeast and used to retransform the YRG-2 yeast strain. This eliminated the possibility of background genomic mutations. Clones were further screened on tryptophan and leucine minus plates. Strong positive clones were identified and the cDNA sequence analyzed using GenBank to determine the clone identity.

Expression and purification of GST fusion proteins

The human *PITX2A* and *C* constructs were PCR amplified from cDNA clones as described (12,24). The *PITX2*, β-*catenin* and *HMG-17* PCR products were cloned into the pGex6P-2 GST vector (Amersham Pharmacia Biotech) as previously described (12,24). All pGex6P-2 GST plasmids were confirmed by DNA sequencing. The plasmids were transformed into BL21 cells. Proteins were isolated as described (19,24). All proteins were cleaved from the GST moiety by PreScission protease (GE HealthCare). Protein concentration was quantitated with Bradford Reagent (BioRad Laboratories, Hercules, CA). Proteins were examined by electrophoresis on denaturing SDS–polyacrylamide gels, followed by Coomassie blue staining (50% methanol, 10% acetic acid and 0.5% Coomassie brilliant blue stain).

GST–PITX2 pull-down assays

Immobilized GST–HMG-17, GST–β-catenin and GST–PITX2 fusion proteins were prepared as described above and suspended 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 of ethidium bromide). Bacterially over-expressed and purified PITX2, β-catenin or HMG-17 protein (75–400 ng) were added to 5 μg's immobilized GST–HMG-17, GST–β-catenin, GST–PITX2, GST–PITX2 truncated fusion proteins or GST, respectively, in a total volume of 50μl, and incubated for 30 min at 4°C. The beads were pelleted and washed three times with 100 μl binding buffer. The bound proteins were eluted by boiling in SDS-sample buffer and separated on either 10% or 12.5% SDS–polyacrylamide gel or 15% tricine gels. The experiment to analyze co-binding of β-catenin and HMG-17 to PITX2 was performed by adding purified β-catenin and HMG-17 to the appropriate GST–PITX2 constructs. After incubation and extensive washing the beads were divided into equal aliquots, run on an SDS gel and probed with either β-catenin or HMG-17 antibodies. Thus, both HMG-17 and β-catenin were present in the binding reactions. Purified PITX2, β-catenin or HMG-17 proteins were analyzed in the western blot. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using the PITX2, β-catenin or HMG-17 antibody (Upstate) and ECL reagents from GE HealthCare.

Western blot assays

Expression of endogenous or transiently expressed PITX2, β-catenin and HMG-17 proteins was demonstrated using the PITX2 P2R10 antibody (25), HMG-17 and β-catenin antibodies (Upstate). Approximately 10–40 μg of transfected cell lysates were analyzed in western blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using specific antibodies and ECL reagents from GE HealthCare.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides containing a PITX2-binding site within the *Dlx2* promoter with flanking partial BamHI ends were annealed and filled with Klenow polymerase to generate ^{32}P-labeled probes for EMSAs as described (21). The sequence of the sense oligonucleotide for probe *Dlx* TAATCC was 5′-gatccGCTCATGCCTGTAATCCCAGCACTCAGGg-3′ and antisense 5′-gatccCCTGAGTGCTGGGATTACAGGCATGAGCg-3′ leaving the four base overhangs (lowercase letters)

which were end filled and labeled. The PITX2-binding site is underlined. Standard binding assays were performed as previously described (12). A titration of the bacteria expressed and purified HMG-17 protein was used in the assays. The samples were electrophoresed, visualized and quantitated as described previously, except quantitation of dried gels was performed on the Molecular Dynamics STORM PhosphoImager (21).

Expression and reporter constructs

Expression plasmids containing the cytomegalovirus (CMV) promoter linked to the PITX2 cDNA were constructed in pcDNA 3.1 MycHisC (Invitrogen) (12,19,24). Lef-1 and β-catenin S37A expression plasmids have been previously described (26). HMG-17 and deletion constructs were prepared by PCR amplification of the full-length *HMG-17* cDNA and cloned into the pcDNA3.1 MycHisC vector. The *Dlx2* promoter cloned into the luciferase vector has been previously described (21). All constructs were confirmed by DNA sequencing. SV-40 or CMV β-galactosidase reporter plasmids were co-transfected in all experiments as a control for transfection efficiency. All plasmids were double-banded CsCl purified.

HMG-17 siRNA constructs

Two HMG-17 short hairpin RNA (shRNA) constructs were prepared using sequences downstream of the ATG in the HMG-17 cDNA. HMG-17 shRNA1 was made by annealing the sense oligo, 5′-GATCCTCTGCGAGGTTGTCTGCTA**TTCAAGAGA**TAGCAGACAACCTCGCAGATCA-3′ with the antisense oligo, 5′-AGCTTGATCTGCGAGGTTGTCTGCTA**TCTCTTGAA**TAGCAGACAACCTCGCAGAG -3′. HMG-17 shRNA2 was made using the sense oligo, 5′-GATCCAAATGGAGATGCCAAAACA**TTCAAGAGA**TGTTTTGGCATCTCCATTTTCA-3′ and the antisense oligo, 5′-AGCTTGAAAATGGAGATGCCAAAACA**TCTCTTGAA**TGTTTTGGCATCTCCATTTG-3′. The 5′ BamH1 and 3′ HindIII sites are underlined and the hairpin loop is in bold. The annealed oligos were cloned into the pSilencer 4.1-CMV neo siRNA expression vector (Ambion) digested with BamH1 and HindIII per the manufacturer's directions. The HMG-17 sequence is 70 bp downstream of the ATG in clone HMG-17 shRNA1 and 210 bp downstream of the ATG in HMG-17 shRNA2. This vector uses the CMV promoter to drive expression of the transcript. All constructs were confirmed by DNA sequencing. All plasmids were double-banded CsCl purified.

Immunocytochemistry

CHO cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin seeded on 18 mm × 18 mm microscope glass cover slips in 60 mm dishes after transfection by electroporation. CHO cells were mixed with 10 μg of expression plasmids pCMV–PITX2A and MycHisC–HMG-17. CHO cells were electroporated at 380 V and 950 microfarads (μF) (Gene Pulsar XL, Bio-Rad). CHO cells were fed 24 h prior to transfection. Transfected cells were incubated for 24 h, washed with PBS, fixed in ice-cold acetone for 10 min at 4°C and air-dried.

Immunofluorescent double staining was performed as described (27). Fixed CHO cells were washed with PBS for 2 × 5 min, incubated in 10% normal goat serum-PBS 30 min at room temperature (RT), then incubated cells with rabbit anti-PITX2 at 4°C overnight. After overnight incubation, cells were rinsed by washing in PBS for 3 × 5 min and cells were incubated with Alexa Fluor 555 goat anti-rabbit IgG 30 min at 37°C. Cells were washed with PBS for 3 × 5 min. To block binding of the rabbit anti-PITX2 IgG of the secondary staining to the goat anti-rabbit IgGs used in the first round staining, the CHO cells were incubated with rabbit IgG at 1:25 for 2 h at RT. Subsequently, the CHO cells were incubated with 1:300 rabbit anti-HMG17 (CHEMICON International, Inc.) at 4°C overnight, washed in PBS 3 × 5 min and cells were incubated with Alexa Fluor® 488 goat anti-rabbit IgG(H + L) (Molecular Probes, Invitrogen detection technologies) for 30 min at 37°C. The cells were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA) and examined with a Zeiss Axiophot Fluorescent Microscope using Axion4.3 Version software.

Confocal microscope analyses of endogenous Pitx2 and HMG-17 protein expression in LS-8 cells was performed as above with the following modifications. Cold acetone fixed LS-8 cells were washed with PBS for 2 × 5 min, incubated in 10% normal goat serum-PBS for 30 min at RT, incubated with mouse anti-Pitx2 IgG (Novus Biologicals) at 1:100 dilution and rabbit anti-HMG-17 IgG (Chemicon, International, Inc.) at 1:300 dilution at 4°C overnight. The cells were washed in PBS 3 × 5 min and incubated with Alexa Fluor 555 donkey anti-mouse IgG (H&L) and Alexa Fluor 488 goat anti-rabbit IgG (H&L) (Molecular Probes, Invitrogen detection technologies) for 30 min at 37°C. The cells were mounted with Vectashield mounting medium (Vector Laboratories, Inc.). Images were acquired using a Zeiss LSM510 confocal microscope.

Cell culture, transient transfections, luciferase and β-galactosidase assays

CHO, C3H10T1/2 and LS-8 cells were cultured in D-MEM supplemented with 5% or 10% FBS and penicillin/streptomycin and transfected by electroporation. Cultures were fed 24 h prior to transfection, resuspended in PBS and mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid and 0.5 μg of CMV or SV-40 β-galactosidase plasmid. LiCl was added to the appropriate cells after electroporation at a final concentration of 10 mM. Electroporation of CHO cells was performed at 380 V and 950 μF (Gene Pulser XL, Bio-Rad), and C3H10T1/2 cells at 360 V and 950 μF. LS-8 cells were transfected by electroporation as previously described (21). Transfected cells were incubated for 24 h in 60 mm culture dishes and fed with 5% FBS and D-MEM and then lyzed 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 Inc.). All luciferase activities were normalized to β-galactosidase activity.

Immunoprecipitation assay

Approximately 24 h after cell transfection with PITX2 and HMG-17, CHO cells were rinsed with 1 ml of PBS, then incubated with 1 ml ice-cold RIPA buffer for 15 min at 4°C. Cells were harvested and disrupted by repeated aspiration through a 25-gauge needle attached to a 1 ml syringe. The lysates were then incubated on ice for 30 min. 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. Supernatant was transferred to a fresh 1.5 ml microfuge tube on ice and precleared using the mouse ExactaCruz F IP matrix (ExactaCruz F, Santa Cruz Biotechnology) for 30 min at 4°C. Matrix was removed by brief centrifugation and supernatant transferred to a new tube. An IP antibody–IP matrix complex was prepared as per manufacturer's instructions using primary anti-HMG-17 antibody (UPSTATE). The IP antibody–IP matrix complex was incubated with the precleared cell lysate at 4°C for 12 h. After incubation, the lysate was centrifuged to pellet the IP matrix. The matrix was washed two times with PBS and resuspended in 15 μl of ddH2O and 3 μl 6 × SDS loading dye. Samples were boiled for 5 min and resolved on a 10% polyacylamide gel. Western blotting was used with anti-PITX2 antibody and HRP-conjugated rabbit ExactaCruz F reagent to detect immunoprecipitated proteins.

Fluorescence polarization measurements of DNA binding and protein interactions

Fluorescence polarization experiments were done with a PanVera Beacon fluorescence polarization system (PanVera Corp.). 5′-Fluoresceinated oligonucleotides corresponding to the PITX2-binding site (bicoid element), (Oligos Etc.), (5′-F-TTAATCCCCCCCCGGGATTAA-3′), were self-annealed in 10 mM sodium cacodylate, pH 6.5, by heating to 90°C followed by flash cooling to form a stem-loop structure with the bicoid element (underlined). Oligonucleotide concentrations were calculated and binding was assayed in a 100 μl volume at 25°C. The binding experiments contained the following: 65 nM fluoresceinated DNA (IT = 230), and 1.0 μg/ml poly(d[I-C]) in HEPES-binding buffer (20 mM HEPES, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). Poly(d[I-C]) (Sigma) was included as a control for nonspecific DNA binding. After each addition of protein, samples were incubated in the Beacon instrument at 25°C for 30 s before a measurement was taken. The 15 s incubation allowed equilibrium to be reached as previously described (28). Samples were excited at 490 nm, and emission was measured at 530 nm.

The data of each binding isotherm were analyzed by curve fitting using KaleidaGraph software (Synergy Software). Protein additions were stopped at ~10-fold over the calculated dissociation constants to ensure that maximum binding was achieved. The following general curve fit equation was used to define the dissociation constant (29):

$$mP = \frac{mP_i + \Delta mP^*[P]}{(K_d + [P])}$$

where mP_i is the initial mP (millipolarization), ΔmP the overall change in mP and $[P]$ is the protein concentration.

The following general curve fit equation was used to determine the K_i of HMG-17 (30):

$$a = \frac{(K_d + [S])}{(K_d{}^*(1 + [I]/K_i) + [S])}$$

where a is the residual activity ($mP - mP/\Delta mP$), K_d is the previously determined K_d of PITX2, $[S]$ is the concentration of PITX2 that was present for the K_i measurement and $[I]$ is the concentration of HMG-17.

Analysis of HMG-17–PITX2 HD interaction by surface plasmon resonance (SPR)

SPR experiments were performed at 25°C on a BIAcore 3000 (BIAcore AB). Thirteen thousand resonance units (RU) of anti-GST antibody (BIAcore AB) were immobilized simultaneously on two flow cells on a CM5 sensor chip using anti-GST antibody immobilization protocol V1.0 provided by the manufacturer. GST–PITX2 HD and GST (BIAcore AB) at the same concentration (10 μg/ml) were injected separately over the anti-GST surfaces in running buffer (10 mM potassium phosphate, pH 7.4, 150 mM NaCl, 0.5 mg/ml BSA and 0.005% surfactant P-20). The amount of GST–PITX2 HD captured on the sample flow cell was 1950 RU. The other flow cell with GST captured (1600 RU) serves as a reference surface. Twofold linear dilution series (8–0.5 nM) of HMG-17 in the running buffer were injected over sample and reference flow cells at the same time for 8 min at a flow rate of 30 μl/min. After dissociation with running buffer for 4 min, the bound HMG-17 was removed with 30 s pulse of regeneration buffer (10 mM potassium phosphate, pH 7.4, 500 mM NaCl). All measurements were baseline corrected by subtracting the sensorgram obtained with reference surface and buffer blank (double referencing). Data were globally fitted to a 1:1 Langmuir-binding model using BIAevaluation software (Version 4.1) to obtain the association and dissociation rate constants (k_{on} and k_{off}, respectively). The dissociation constant (K_D) was calculated from the kinetic parameters as $K_D = k_{off}/k_{on}$.

Chromatin immunoprecipitation analysis

The triple sequential ChIP assays were performed using the ChIP Assay kit (Upstate) and as previously described (31) except for the following modifications. LS-8 cells were used and lysates were incubated first with β-catenin polyclonal rabbit IgG (Upstate) overnight at 4°C. The following day the samples were washed and eluted using 0.1% SDS, 30 mM DTT and 100 mM NaCl and incubated at 37°C for 30 min. The samples were diluted 1:50 with ChIP Dilution Buffer and incubated overnight with HMG-17 monoclonal rabbit IgG (Upstate) at 4°C. The following day the samples were washed and eluted as

before and then incubated with PITX2 P2R10 antibody (25) overnight at 4°C. Two primers for amplifying the β-catenin/HMG-17/Pitx2-binding site in the *Dlx2* promoter are as follows: sense starting at –716 to –696, 5′-GGAGGGAACCTCAGAATCAG-3′ and antisense at –325 to –347, 5′-ACATCTCTTGTCCAACTTCGCC-3′. PCR conditions include 58°C annealing and 35 cycles, input control contained 5% of the initial chromatin DNA. All the PCR products were evaluated on a 2% agarose gel in 1× TBE for appropriate size and confirmed by sequencing.

RESULTS

HMG-17 interacts with the PITX2 HD

HMG-17 was identified as a PITX2 interacting partner using the yeast two-hybrid assay. Over 500 000 clones were screened using an oral epithelial cell (LS-8 cells) library as prey and the PITX2A full-length, HD and C-terminal tail were used as the bait. A stringent screening process identified 40 clones that grew on histidine/tryptophan/leucine minus plates. After sequencing 17 were confirmed to be HMG-17. HMG-17 interacted with the PITX2A full-length protein, PITX2 HD and the PITX2 C-terminal tail. Transformants containing the HMG-17 clone were re-streaked on an SD agar plate without leucine, trptophan and histidine. LacZ expression of the cotransformants was determined by the filter lift assay (yeast two-hybrid data not shown).

To map the PITX2 interaction region, GST-pull-down experiments were performed using purified bacteria expressed proteins. A schematic of the immobilized GST-PITX2A wild type and truncated proteins are shown in Figure 1A. The structure of the HMG-17 protein and Coomassie blue stained gel of the purified HMG-17 protein used in the assays are shown in Figure 1B. Immobilized PITX2 HD (GST-PITX2 HD) bound the HMG-17 protein (Figure 1C). GST-PITX2 ΔC173, which has the complete C-terminal tail deleted bound HMG-17 (Figure 1C). HMG-17 bound to the PITX2°C-terminal tail (GST-PITX2 C173) and specifically to the last C-terminal 39 residues (GST-PITX2 C39) (Figure 1C). As a control, GST beads or the PITX2 N terminus did not bind the HMG-17 protein (Figure 1C, data not shown). We have designated these two binding sites in the PITX2 protein as HMG-17-binding domain (BD) #1 and #2 (Figure 1A).

Because HMG-17 interacts with the PITX2 HD, which confers DNA-binding activity, we asked if HMG-17 affected the DNA-binding properties of PITX2. PITX2 specifically binds to the *Dlx2* promoter and this element (TAATCC) was used as the probe in the EMSA. HMG-17 is a small peptide (89 residues) and does not cause a large shift in the probe band and only a small amount of HMG-17/DNA complex is observed immediately above the free probe (Figure 1D). HMG-17–DNA binding is transient and may not be stable in these experiments (4). However, HMG-17 inhibited PITX2A and PITX2C isoform (80 ng) DNA-binding activity (Figure 1D). PITX2A and PITX2C are the major isoforms involved in embryogenesis and have identical HDs and C-terminal tails (19,32). HMG-17 was titrated at 20, 40 and 60 ng to 80 ng of PITX2 isoforms and the higher HMG-17 concentration inhibited over 90% of PITX2A and PITX2C binding to the *Dlx2* promoter probe (Figure 1D).

β-catenin de-represses HMG-17 inhibition of PITX2 transcriptional activity

CHO cells were co-transfected with PITX2A, HMG-17 and the full-length Dlx2 3276 luc promoter to determine if HMG-17 regulated the transcriptional activity of PITX2A. CHO cells do not endogenously express Pitx2 and only low levels of β-catenin (22). Therefore, CHO cells were used in the assay without interference from endogenous Pitx2 activity. The pluripotent C3H10T1/2 cells, CHO cells and LS-8 cells endogenously express HMG-17 demonstrating the ubiquitous expression pattern of this factor (Figure 2B) (4). PITX2A activated the *Dlx2* promoter by ~30-fold (Figure 2A). Co-expression of PITX2A and HMG-17 revealed a 3-fold decrease in PITX2A activation from 30-fold to 10-fold (Figure 2A). We have previously shown that β-catenin interacts with PITX2 to synergistically activate gene expression (22). Furthermore, β-catenin directly interacts with the PITX2 HD to regulate PITX2 transcriptional activation (33). We asked if β-catenin S37A (constitutively active form) would regulate the HMG-17 repression of PITX2. Co-transfection of PITX2A and β-catenin S37A resulted in a 43-fold synergistic activation of the *Dlx2* promoter (Figure 2A). Co-transfection of PITX2A, HMG-17 and β-catenin S37A activated the *Dlx2* promoter by 65-fold compared to 10-fold activation for PITX2 and HMG-17 co-transfection (Figure 2A). The activity of the PITX2/HMG-17 complex is switched from a repressor to an activator in the presence of β-catenin (65-fold, Figure 2A). The action of β-catenin was not cell specific as β-catenin de-repressed the HMG-17 inhibition of PITX2 in transfected C3H10T1/2 and LS-8 cells (data not shown). A western blot demonstrates equal expression of transfected PITX2, β-catenin and HMG-17 (Figure 2C).

Consistent with increased PITX2A transcriptional activation by transfected β-catenin, cells treated with LiCl increased PITX2C activation of the *Dlx2* promoter (Figure 2D). HMG-17 repression is not PITX2 isoform dependent as would be expected since both isoforms contain identical HDs and C-terminal tails (19). LiCl is a potent GSK-3 inhibitor and inhibits β-catenin phosphorylation and stabilizes the pool of cellular β-catenin similar to Wnt signaling (34). HMG-17 did not activate the *Dlx2* promoter after LiCl treatment of CHO cells however, PITX2C activation of the *Dlx2* promoter in the presence of HMG-17, increased from 10- to 37-fold after treatment with LiCl (Figure 2D). The increase in PITX2C activation with LiCl treatment and HMG-17 was less than the activation by transfected β-catenin S37A with PITX2A and HMG-17 observed in Figure 2A. This is presumably due to the increased pool of β-catenin S37A from the transfected plasmid compared to the endogenous β-catenin pool stabilized by LiCl treatment. Furthermore, LiCl

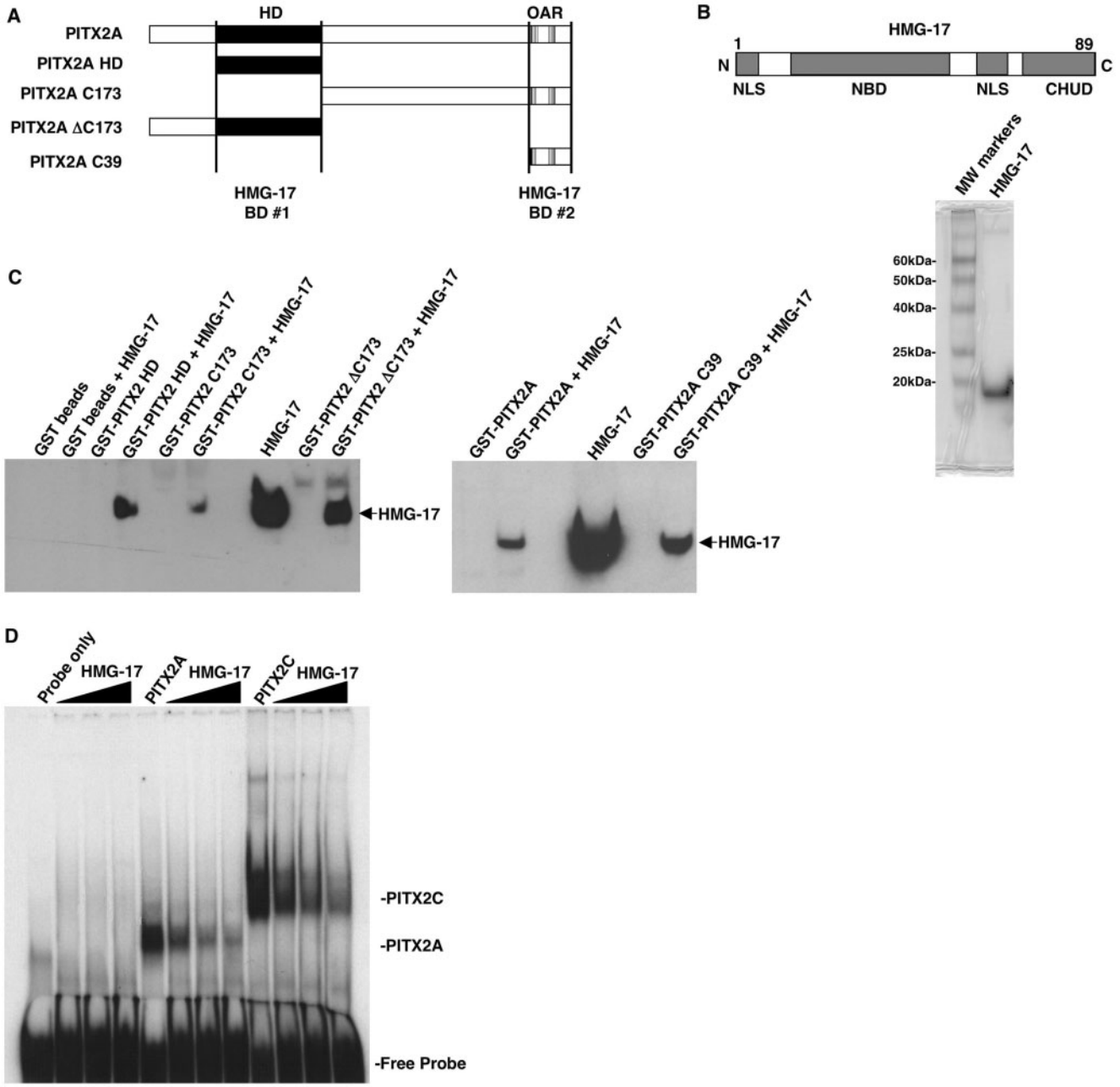

Figure 1. HMG-17 interacts with two regions of the PITX2 protein. (**A**) Schematic of the PITX2A protein and the N-terminal and C-terminal truncated proteins. The location of the two HMG-17-binding domains (BD) in the PITX2 protein are shown. (**B**) Schematic of the HMG-17 protein; NLS, nuclear localization signal; NBD, nucleosomal-binding domain; CHUD, chromatin-unfolding domain (4). The purified HMG-17 protein used in the various assays was visualized by Coomassie blue staining. (**C**) GST–PITX2A protein pull-down assay with bacterial expressed and purified HMG-17 protein (100 ng). To demonstrate HMG-17 binding to PITX2A, PITX2A protein was incubated with purified HMG-17 protein. HMG-17 binds to the PITX2 homeodomain (HD) and this region is termed HMG-17 BD #1. HMG-17 binds to a region containing the C-terminal OAR domain (conserved 14 residue motif), which is termed HMG-17 BD #2. As a control GST-beads were incubated with purified HMG-17 to demonstrate the specificity of HMG-17 binding to the GST–PITX2A fusion proteins. (**D**) HMG-17 protein (20, 40 and 60 ng) was incubated with the Dlx2 promoter sequence containing a PITX2-binding element (TAATCC) as the radioactive probe in an electrophoretic mobility shift assay (EMSA). HMG-17 did not bind in this assay to DNA however, PITX2A and PITX2C proteins (80 ng) bound to the DNA. HMG-17 titration (20, 40 and 60 ng) with 80 ng of either PITX2A or PITX2C revealed that HMG-17 inhibited PITX2 binding in a dose-responsive manner. The EMSA experiments were analyzed in 8% native polyacrylamide gels. The free and bound forms of DNA were quantitated using the Molecular Dynamics STORM PhosphoImager. The free probe and bound DNA are indicated.

treatment of PITX2C and HMG-17 transfected cells did not activate the *Dlx2* promoter at the levels of PITX2C expression alone with LiCl due to the limited endogenous β-catenin pool and exogenous HMG-17 (Figure 2D). However, both methods of increasing β-catenin activity reveal a role for β-catenin in de-repressing HMG-17 inhibition of PITX2. Similar results were observed in LS-8 transfected cells (Figure 2E).

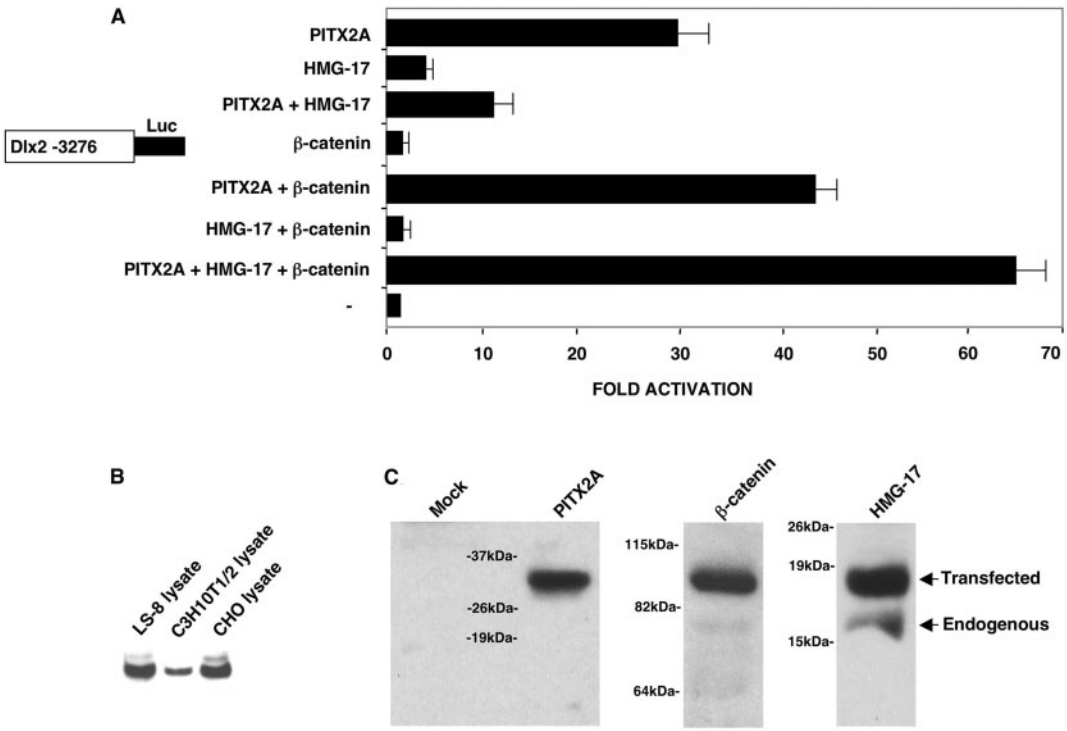

Figure 2. The repression of PITX2 activity by HMG-17 is modulated by β-catenin. (**A**) CHO cells were transfected with the Dlx2 3.2 kb luciferase reporter gene (5 μg) and co-transfected with CMV-PITX2, CMV-β-catenin S37A, CMV-HMG-17, or the CMV plasmid without PITX2, HMG-17 or β-catenin (–) (2.5 μg). To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter (0.5 μg). Cells were incubated for 24 h, and then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared to the Dlx2 promoter plasmids without PITX2, β-catenin or HMG-17 expression and normalized to β-galactosidase activity (±SEM from five independent experiments). (**B**) HMG-17 is endogenously expressed in LS-8, C3H10T1/2 and CHO cells. (**C**) Western blot of transfected cell lysates (20 μg); PITX2A was detected using the PITX2 Ab (25) as previously described; β-catenin was detected using the antibody from Santa Cruz Biotech; and HMG-17 was detected using an antibody from Chemicon International. The proteins were visualized using ECL reagents from Amersham. Molecular weight markers are shown on the left of each blot. (**D**) Endogenous β-catenin de-repressed the PITX2/HMG-17 complex. CHO cells were transfected as described above and transfected cells were treated without (–) and with (+) LiCl at a final concentration of 10 mM. The activities are shown as mean fold activation compared to the *Dlx2* promoter plasmid without PITX2 or HMG-17 expression and normalized to β-galactosidase activity (±SEM from four independent experiments). (**E**) LS-8 oral epithelial cells were transfected as in panel D to determine if the activation was cell dependent. The activities are shown as mean fold activation compared to the *Dlx2* promoter plasmids without PITX2 or HMG-17 expression and normalized to β-galactosidase activity (±SEM from eight independent experiments).

Knockdown of endogenous HMG-17 increased PITX2 transcriptional activity

We next asked if endogenous HMG-17 was repressing PITX2 activation. Inhibition of endogenous HMG-17 expression by HMG-17 siRNA (siHMG-17) increased PITX2C activation of the *Dlx2* promoter from 33- to 45-fold (Figure 3A). A negative control (siNEG supplied by Ambion), and siGADPH did not affect the activity of PITX2C in CHO cells (Figure 3A).

Western blot analyses were performed to demonstrate reduced endogenous HMG-17 protein by HMG-17 siRNA. Endogenous HMG-17 protein is detected in mock and PITX2 transfected lysates (Figure 3B, lanes 2 and 3). Transfected HMG-17 migrates slightly slower than endogenous HMG-17 in the gel due to a myc/his tag on the transfected protein (Figure 3B, lanes 4 and 5). Interestingly, PITX2 stabilized both endogenous and transfected HMG-17 (Figure 3B, compare lanes 4 and 5). Endogenous HMG-17 was completely inhibited by siHMG-17 expression (Figure 3B, lane 6). The siNEG control had no effect on endogenous HMG-17 expression (Figure 3B, lane 7). As a loading control the western blot was stripped and re-probed with the GAPDH antibody. Similar levels of GAPDH were observed in all lanes demonstrating that equal protein amounts were assayed (Figure 3C). As another control a western blot demonstrates that PITX2 and HMG-17 expression did not affect GAPDH expression. More importantly siNEG and siHMG-17 did not affect GAPDH expression demonstrating the specificity of these siRNAs (Figure 3D). siGADPH inhibited endogenous GAPDH expression ~50% after only 24 h (Figure 3D). It should be noted that all siRNA experiments were assayed after 24 h, and the complete loss of HMG-17 protein after 24 h suggests that it is a labile protein. A western blot of transfected PITX2C demonstrates PITX2C expression (Figure 3E).

PITX2 and HMG-17 co-localize in the cell nucleus

HMG-17 protein was visualized using FITC in the CHO cell nucleus associated with chromatin structures and in foci (Figure 4A), (35,36). There are two adjacent nuclei on the left side of the panels. PITX2 was visualized with

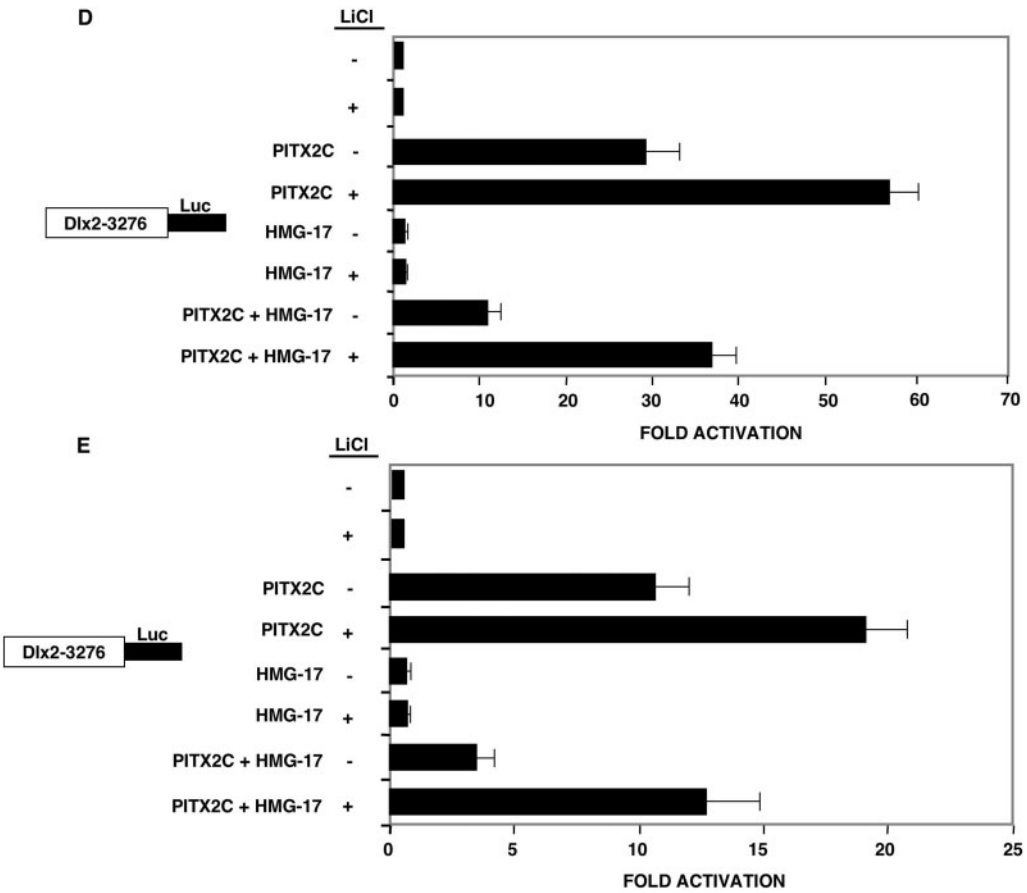

Figure 2. Continued.

Texas red in distinct regions of the nucleus and associated with chromatin structures (Figure 4B). Merging the two fluorescent proteins reveals a strong overlap in their nuclear localization (Figure 4C). DAPI staining reveals the nuclear/chromatin structure (Figure 4D), and merging of the DAPI stain with HMG-17 and PITX2 expression correlates with these factors recruitment to nuclear chromatin structures (Figure 4E).

Endogenous Pitx2 and HMG-17 expression was observed in LS-8 nuclei (Figure 4H and I). Endogenous Pitx2 expression appears diffusely throughout the nucleus (Figure 4H), whereas HMG-17 expression is more localized to small foci (Figure 4I). Merging the two images reveals that Pitx2 is co-localized to nuclear regions where HMG-17 resides (Figure 4J). While all HMG-17 appears to co-localize with Pitx2 not all Pitx2 staining co-localizes with HMG-17.

Furthermore, HMG-17 expression occurs in the dental and oral epithelial cells of an E14.5 molar tooth bud (data not shown). The tooth epithelial specific expression of HMG-17 directly overlaps that of PITX2 (37).

Specificity of DNA binding and the PITX2/HMG-17 interaction

The specificity of PITX2 and HMG-17 for DNA were determined by binding to 5′- fluoresceinated oligonucleotides corresponding to the PITX2-binding site (bicoid element). Fluorescence polarization experiments determined the binding curves for PITX2 and HMG-17 (Figure 5A and B). The calculated equilibrium dissociation constant, K_d is 129 ± 17 nM for PITX2 binding to the bicoid element (Figure 5A). The calculated equilibrium dissociation constant, K_d is 1.3 ± 0.40 μM for HMG-17 binding to the bicoid element (Figure 5B). Thus, HMG-17 has a low affinity for DNA and correlates with HMG-17 binding to DNA non-specifically.

HMG-17 binding to PITX2 was determined after PITX2 DNA binding reached equilibrium. A specific PITX2/HMG-17 complex was identified by titrating HMG-17 protein to DNA bound PITX2. Surprisingly, HMG-17 addition to the PITX2/DNA complex resulted in decreased polarization, demonstrating that HMG-17 was removing PITX2 from the DNA (Figure 5C). These data correlate with the EMSA data demonstrating that the PITX2/HMG-17 complex cannot bind DNA. The inhibitory constant (binding constant) of HMG-17 for PITX2 is, K_i 105 ± 5 nM (Figure 5C). Thus, HMG-17 has 10-fold higher affinity for PITX2 than for DNA and HMG-17 has a higher binding affinity for PITX2 than PITX2 has for DNA. HMG-17 physically interacts with the PITX2 HD and this interaction releases PITX2 from the DNA.

The interaction between HMG-17 and PITX2 HD (HD only) was examined by real-time SPR-binding assay.

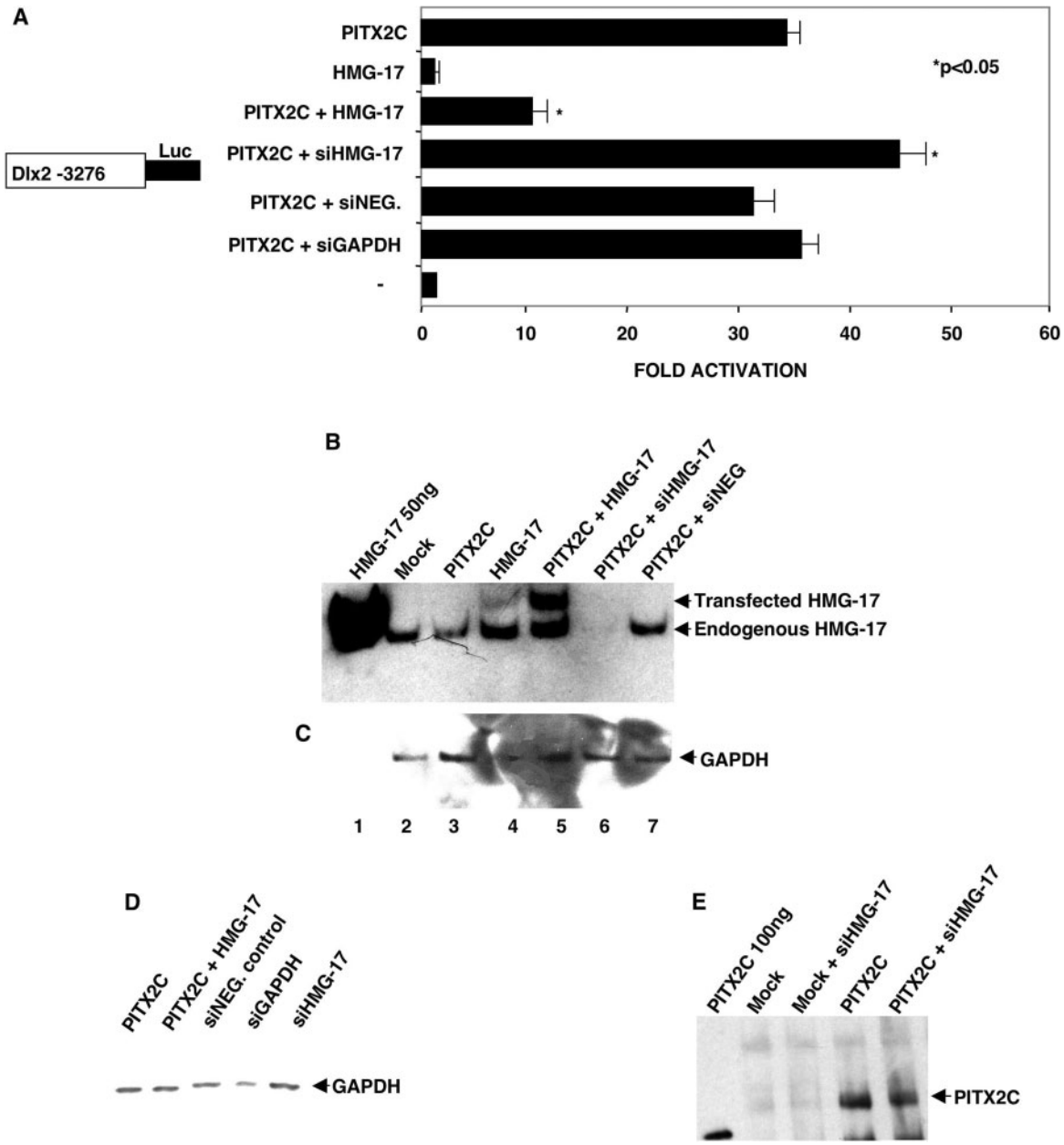

Figure 3. Reduced endogenous HMG-17 protein increased PITX2 transcriptional activation. (**A**) CHO cells were transfected as in Figure 2 with the Dlx2 3.2 kb luciferase reporter (5 μg). The cells were co-transfected with expression vectors, shRNA expression vectors or shRNA vectors alone (–) (2.5 μg). The activities are shown as mean fold activation compared to the *Dlx2* promoter plasmids without PITX2, siRNA or HMG-17 expression and normalized to β-galactosidase activity (±SEM from five independent experiments). (**B**) Western blots of endogenous and transfected HMG-17 in CHO cells. Lane 1 is purified HMG-17 protein, lane 2 is endogenous HMG-17, lane 3 is endogenous HMG-17 after PITX2C transfection, lane 4 is both endogenous and transfected HMG-17. The transfected HMG-17 migrates slightly slower in the gel due to a myc/his tag on the C-terminal tail of the protein. Lane 5 is endogenous and transfected HMG-17 with transfected PITX2C, lane 6 demonstrates a complete inhibition of endogenous HMG-17 expression by transfected HMG-17 siRNA. Lane 7 is endogenous HMG-17 with the transfected siNegative control and PITX2C. (**C**) The western blot in panel B was stripped and re-probed with GAPDH antibody to demonstrate equal loading of the cell lysates. (**D**) Western blot of cell lysates probed with the GAPDH antibody. (**E**) Western blot of transfected PITX2C with and without HMG-17 siRNA. The transfected PITX2C protein migrates slightly slower in the gel due to a myc/his tag on the C-terminal tail of the protein.

The sensorgrams fit well to a 1:1 binding model ($Chi^2 = 0.61$) (Figure 5D). The dissociation constant ($K_D = 3.04$ pM) obtained from the analysis indicated the HMG-17–PITX2 HD interaction to be of high affinity. These data indicate a stronger affinity of HMG-17 for PITX2 than the polarization experiments due to binding of the PITX2 HD peptide compared to the full-length protein. Moreover, the kinetic parameters suggested that this strong interaction was due to a fast complex formation with association rate k_{on} of $(4.39 \pm 0.03) \times 10^5\,M^{-1}s^{-1}$, and a very stable complex with dissociation rate k_{off} of $(1.33 \pm 0.09) \times 10^{-6}\,s^{-1}$. However, the stability of the HMG-17/PITX2 HD complex could be easily destroyed by slight increase of salt concentration. This is

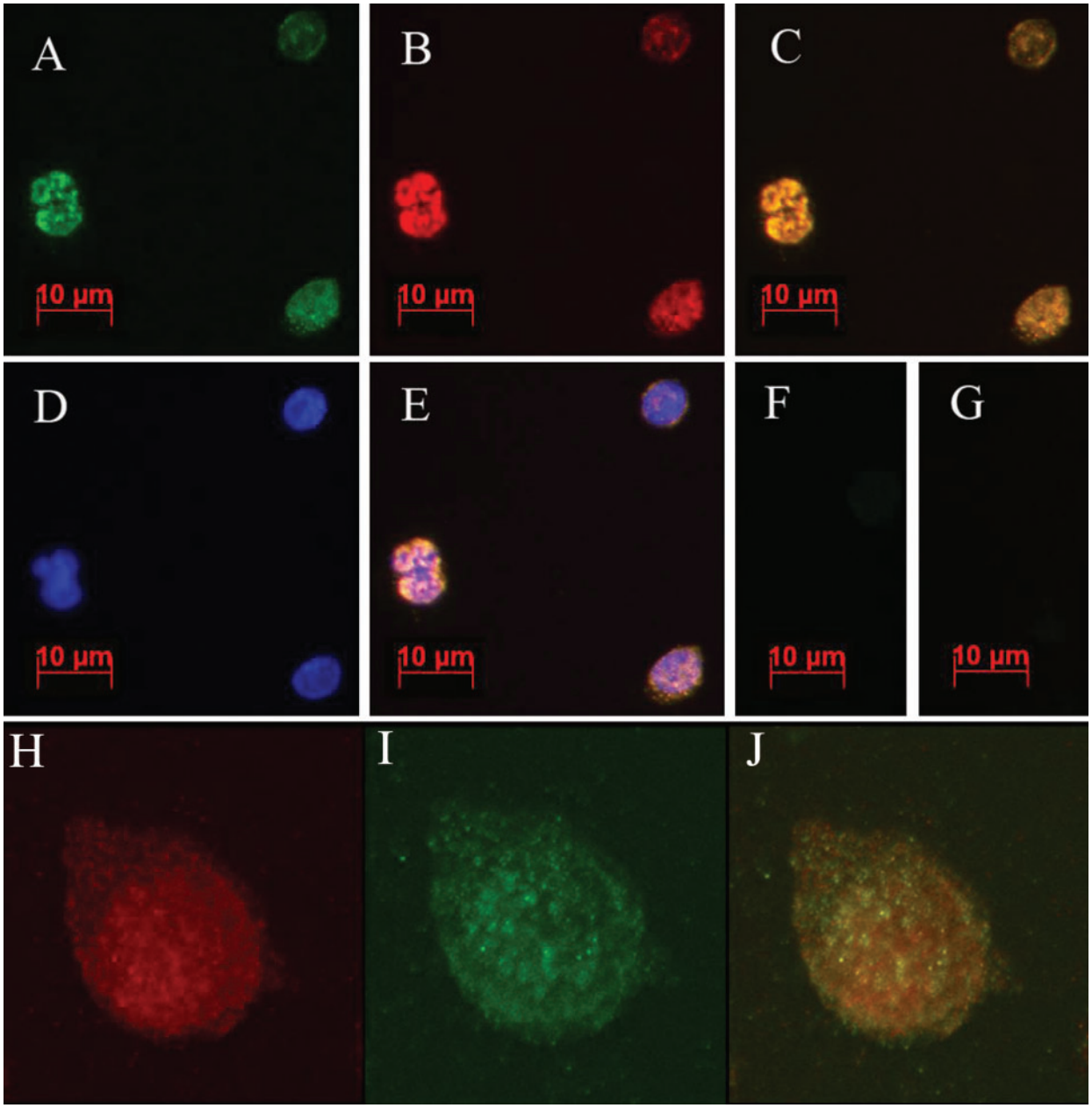

Figure 4. PITX2 and HMG-17 co-localize in the nucleus. (**A**) HMG-17 expression was detected using an HMG-17 antibody and visualized with the Alexa Fluor 555 goat anti-rabbit IgG. There are two adjacent nuclei on the left side of the panels. (**B**) PITX2 expression was detected with the PITX2 antibody (25) and the Alexa Fluor 488 goat anti-rabbit IgG(H + L) (Molecular Probes, Invitrogen detection technologies). (**C**) The corresponding stained nuclei from panel A and B were merged to yield the image in panel C, demonstrating co-localization of HMG-17 and PITX2. (**D**) DAPI staining of the nuclei in panels A–C, demonstrating intact nuclei. (**E**) Merging of panels A, B and C showing the DAPI stain overlaps with HMG-17 and PITX2 staining. (**F**) Negative control or secondary only antibody for Alexa Fluor 555 goat anti-rabbit IgG and (**G**) is the negative control for secondary only antibody using the Alexa Fluor 488 goat anti-rabbit IgG. (**H**) Confocal image (×40) of endogenous Pitx2 expression in LS-8 nuclei and (**I**) endogenous HMG-17 expression. (**J**) Merged images of Pitx2 and HMG-17 expression.

based on the observation that using the regeneration buffer, which contained 200 mM or 500 mM of NaCl, removed bound HMG-17 from the HMG-17/PITX2 HD complex (Figure 5E). This indicated that HMG-17 and PITX2 HD bind to each other through ionic interactions and the interaction can be regulated by salt concentration. Therefore, strong interacting proteins can modify PITX2 and HMG-17 ionic interactions. The HMG-17/PITX2 interaction is measured differently in the two assays, however both measurements demonstrate a high affinity association between the two proteins.

PITX2/HMG-17/β-catenin complex

HMG-17 antibody (Ab) immunoprecipitates PITX2 only in the presence of β-catenin. The previous experiments demonstrated specific PITX2 and HMG-17 protein

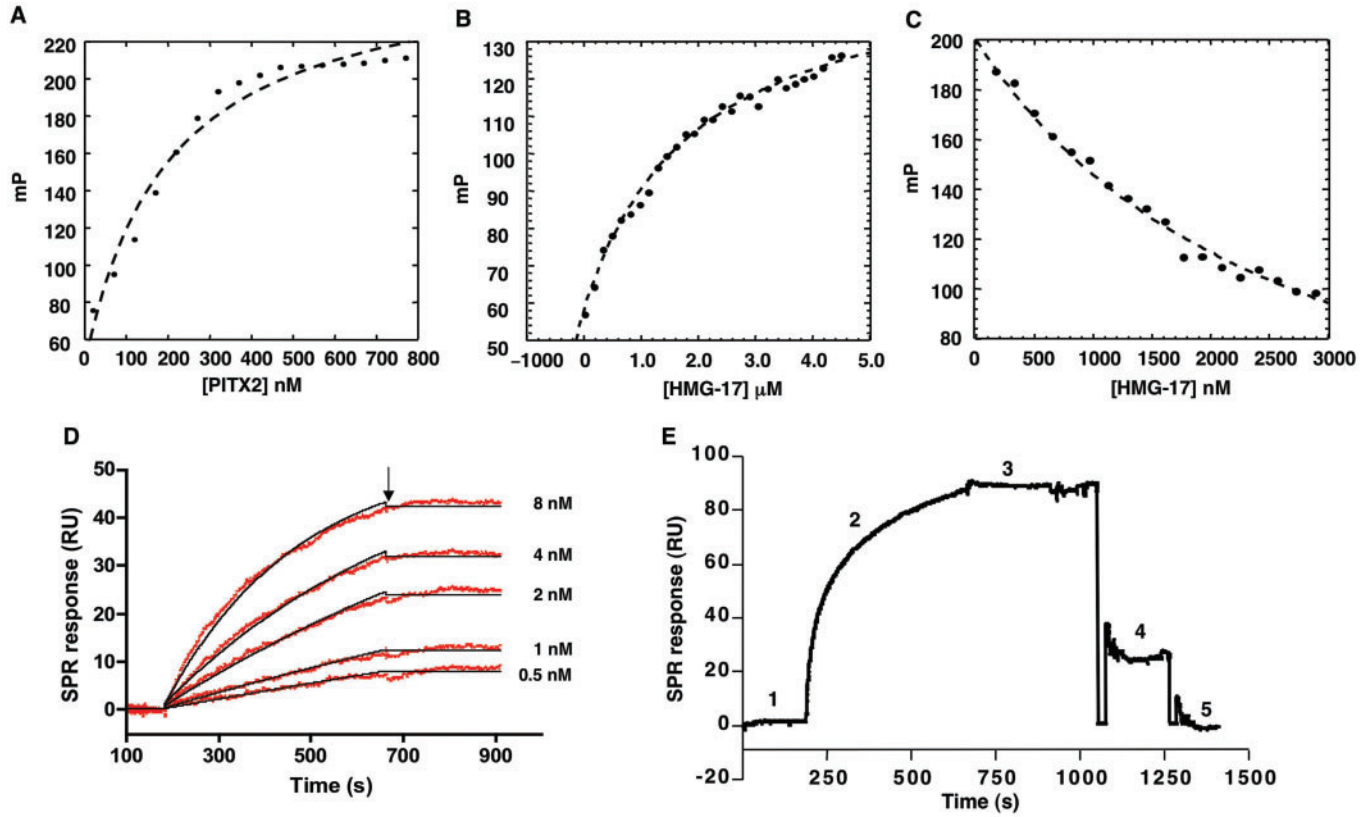

Figure 5. Binding isotherms and surface plasmon resonance (SPR) analysis of HMG-17/PITX2 interaction. Millipolarization (mP) is plotted against the concentration of PITX2 and HMG-17 or both binding to fluoresceinated oligodeoxynucleotides (F-bicoid DNA) in HEPES-binding buffer. (**A**) PITX2 binding to F-bicoid DNA. (**B**) HMG-17 binding to F-bicoid DNA. (**C**) HMG-17 binding to DNA bound PITX2. (**D**) SPR-binding assay. HMG-17 at indicated concentrations was injected over immobilized PITX2 HD (HD, homeodomain) (GST-tagged) at a flow rate of 30 μl/min. After 8 min of injection (indicated by the arrow), the HMG17/PITX2 HD complex was washed with running buffer for 4 min. Shown here are overlay of baseline-adjusted sensorgrams (red jagged lines) and fitted curves (black smooth lines) using 1:1 Langmuir-binding model. Kinetic rate constants $k_{on} = (4.39 \pm 0.03) \times 10^5\,M^{-1}s^{-1}$ and $k_{off} = (1.33 \pm 0.09) \times 10^{-6}\,s^{-1}$ were derived from the fitting, and the dissociation constant K_D of 3.04 pM ($K_D = k_{on}/k_{off}$) was obtained. (**E**) Dissociation of HMG17–PITX2 HD complex by salt. HMG17 (128 nM) was injected over the PITX2 HD surface (i) for 8 min (ii), and followed by 4 min wash with running buffer (iii). After regeneration with two consecutive 30 s pulses of buffers containing 200 mM (iv) and 500 mM of NaCl (v), bound HMG17 was removed from the HMG17/PITX2 HD complex. RU, resonance units. Data are one representative of two experiments.

interactions. Interestingly, the HMG-17 Ab weakly co-immunoprecipitated a PITX2/HMG-17 complex (Figure 6A). A band was detected on longer exposure times indicating that an interaction occurred between PITX2 and HMG-17. This weak interaction could correspond to the HMG-17 interaction with the PITX2 C-terminal tail as revealed in the GST-pull-down experiments in Figure 1 or due to low levels of endogenous β-catenin in the cells. These results are often observed when the Ab cannot recognize an epitope buried in a protein complex. However, when β-catenin was co-expressed with PITX2 and HMG-17 the HMG-17 Ab immunoprecipitated PITX2 suggesting that the complex formed between these three proteins caused a conformational change allowing the Ab to recognize the HMG-17 protein (Figure 6A). As a control the HMG-17 Ab does not recognize β-catenin in a western blot (data not shown). Because β-catenin is a high-molecular weight protein (~89 KDa), its interaction with PITX2 may displace or reposition HMG-17 and allow the HMG-17 Ab to recognize it in the complex.

GST-pull-down assays provide an alternative method to identify a PITX2/HMG-17/β-catenin complex. Immobilized GST–PITX2A, GST–PITX2 HD and GST–PITX2 C173 were incubated with both HMG-17 (75 ng) and β-catenin (400 ng) in one reaction (protein concentrations were adjusted to equal similar amounts of protein molecules) and after incubation and extensive washing the reaction was divided into two aliquots, each aliquot resolved separately on a polyacrylamide gel. One western blot was probed using the HMG-17 antibody (Figure 6B). As controls, GST–PITX2A alone and GST–PITX2A incubated with only β-catenin did not produce an HMG-17 protein band (Figure 6B). However, HMG-17 bound to PITX2A in the presence of β-catenin (Figure 6B). HMG-17 bound to PITX2 HD and PITX2A C173 demonstrating that HMG-17 binds to both regions of PITX2 in the presence of β-catenin. The second western blot revealed β-catenin binding to PITX2A and the PITX2A HD in the presence of HMG-17 (Figure 6C). However, β-catenin did not bind to the PITX2 C-terminal tail and is specific only for the HD.

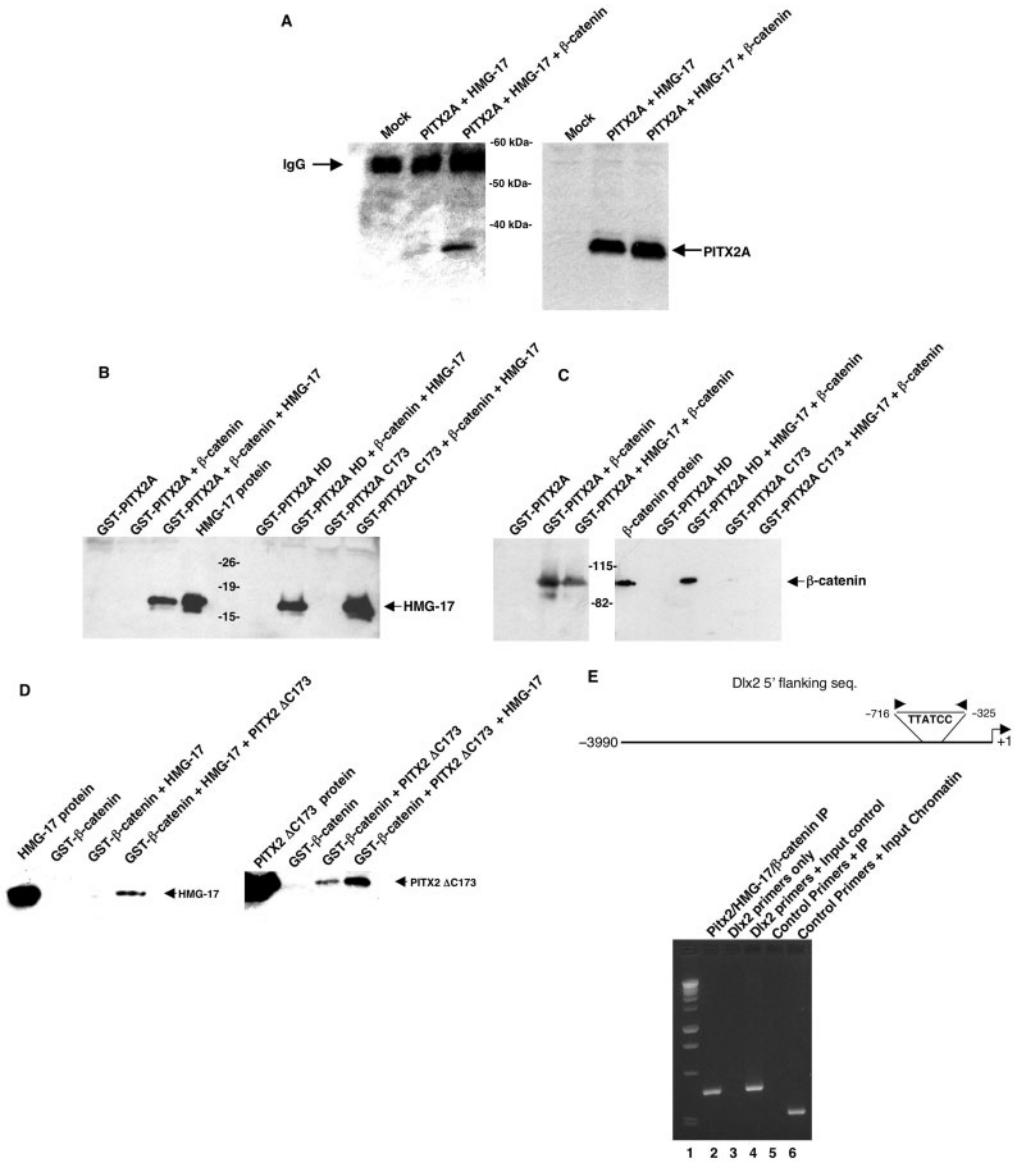

Figure 6. PITX2, HMG-17 and β-catenin form a complex. (**A**) Co-immunoprecipitation (IP) experiments demonstrate a PITX2/HMG-17/β-catenin complex in CHO cells. PITX2A, HMG-17 and/or β-catenin (2.5 μg) were transfected into CHO cells. Cell lysates were incubated with HMG-17 antibody and the IP complex was isolated and resolved on a 10% SDS–polyacrylamide gel and probed for PITX2 using the PITX2 Ab. The HMG-17 antibody was unable to immunoprecipitate the PITX2/HMG-17 complex. Co-expression of β-catenin with PITX2 and HMG-17 allowed the HMG-17 antibody to recognize the PITX2/HMG-17/β-catenin complex and precipitated the complex. HMG-17 Ab does not bind to β-catenin. The proteins were visualized using ECL reagents from Amersham. (**B**) PITX2A, HMG-17 and β-catenin complex formation by purified proteins. GST–PITX2A and truncated PITX2A protein pull-down assay with bacterial expressed and purified HMG-17 (75 ng) and β-catenin proteins (400 ng). Binding reactions contain both HMG-17 and β-catenin and after incubation the reactions were divided into two equal aliquots and resolved on separate 10% SDS–polyacrylamide gels and transferred to PVDF membranes. One aliquot was probed for HMG-17 interactions using the HMG-17 antibody. HMG-17 interacted with the PITX2 HD and C-terminal tail in the presence of β-catenin. (**C**) The duplicate aliquot was probed for β-catenin interactions using the β-catenin antibody. β-catenin interacted with the PITX2 HD but not the C-terminal tail. These results demonstrate that both HMG-17 and β-catenin bind to the PITX2 HD as a complex. As a control GST-beads were incubated with purified HMG-17 and β-catenin to demonstrate the specificity of binding to the GST–PITX2A fusion proteins. (**D**) GST-β-catenin was immobilized on beads and incubated with HMG-17 to demonstrate that these two proteins do not physically interact. Addition of PITX2 ΔC173 protein to GST-β-catenin and HMG-17 demonstrated that HMG-17 interacts with PITX2 bound to β-catenin. Furthermore, PITX2 ΔC173 interacts with β-catenin and addition of HMG-17 increases the PITX2 ΔC173 interaction with β-catenin. The proteins were visualized using ECL reagents from Amersham. Molecular weight markers are noted. (**E**) Triple sequential ChIP using β-catenin Ab first, followed by HMG-17 Ab and PITX2 Ab immunoprecipitations. The Dlx2 primers produced the correct PCR product from the triple antibody IP (lane 2), Dlx2 primer only control (lane 3), Dlx2 primers with chromatin input (lane 4), unrelated control primers with the triple IP chromatin (lane 5) and control primers with input control (lane 6). All bands were sequenced to confirm their identity.

Furthermore, HMG-17 and β-catenin do not interact as β-catenin would be pulled down by HMG-17 binding to the PITX2 C-terminal tail. GST–pull downs and IP assays did not detect a β-catenin/HMG-17 complex (data not shown). While it is possible HMG-17 and β-catenin bind to PITX2 separately in these assays, excess amounts of HMG-17 and β-catenin were added to each reaction with a limited amount of immobilized PITX2. If the proteins were binding separately we would not expect the high levels of binding by each protein to PITX2 when both are present.

GST–β-catenin was immobilized on beads and did not interact with purified HMG-17 protein (Figure 6D). However, when PITX2 protein was added to the binding reaction, HMG-17 bound to PITX2 ΔC173 (does not contain the C-terminal tail), which bound to β-catenin. As expected PITX2 ΔC173 bound to β-catenin and interestingly addition of HMG-17 to the binding reaction increased PITX2 ΔC173 binding to β-catenin (Figure 6D). Thus, HMG-17 appears to facilitate the interaction between PITX2 and β-catenin. These data correlate with the IP data to demonstrate a PITX2/HMG-17/β-catenin complex forms by binding to the PITX2 HD.

A triple sequential ChIP assay demonstrates that the Pitx2/HMG-17/β-catenin ternary complex resides on the *Dlx2* promoter chromatin. LS-8 cells were used in the ChIP assay as these cells endogenously express all three factors. The first ChIP assay used the β-catenin antibody, followed by the HMG-17 antibody and then the PITX2 antibody. After the last IP with the PITX2 antibody, the *Dlx2* promoter chromatin was amplified by PCR using primers specific for the *Dlx2* promoter flanking a PITX2-binding site. The primers amplified a 390 bp product from the triple antibody IP (Figure 6E, lane 2). As a control the Dlx2 primers only did not produce a PCR product, however the primers did produce the correct size band from the input chromatin (Figure 6E, lane 4). Control primers to an unrelated gene did not produce a product from the triple IP chromatin (Figure 6E, lane 5). The control primers did work with the input chromatin (Figure 6E, lane 6). Thus, the endogenous complex of Pitx2/HMG-17/β-catenin binds to the *Dlx2* promoter *in vivo*. These data correlate with the IP data to demonstrate a PITX2/HMG-17/β-catenin complex forms by binding to the PITX2 HD.

DISSCUSSION

HMG-17 is a novel embryonic regulator of HD transcriptional activity modulated by β-catenin

HMG-17 is a member of the HMG genes whose expression is restricted to embryonic tissues and reduced in adult tissues (10). It is expressed in a variety of tissues that correlate with *Pitx2* expression (10,25). HMG-17 is an acetylated non-histone chromosomal protein that acts to unfold the higher order chromatin structure to enhance transcription (38). In the absence of transcription, HMG-17 is released from chromatin and accumulates in interchromatin clusters. Thus, HMG-17 intranuclear distribution may be related to the transcriptional activity of the cell (36). HMG-17 does not bind to consensus chromatin sequences but is associated with other proteins (1). These protein complexes may regulate the dynamic organization of HMG-17 in the nucleus and direct the proteins to specific sites in chromatin. HMG-17 appears to move rapidly and constantly throughout the entire nucleus and is associated with other proteins to form a multi-protein complex (1). Thus, the intracellular trafficking and chromatin targeting of HMG-17 may be regulated by proteins interacting with HMG-17 (1). Our data corroborates these findings and demonstrates that HMG-17 forms a complex with PITX2 at chromatin structures. Clearly, mechanisms must exist that direct HD transcription factors to the sites of active transcription in the nucleus. A simple diffusion strategy will not provide concentrated transcription factors to sites specific for the rapid increase in gene expression required for normal embryo development.

Histone proteins have been well defined as modulators of transcription and play major roles in remodeling chromatin and interactions with HMGN proteins. Chromatin unfolding is facilitated by specific interactions between the C-terminal of HMG-17 and the N-terminal of histone H3 (39). Furthermore, HMGN proteins may compete with histone H1 proteins for binding to the chromatin, which would play a role in regulating the structure and activity of the chromatin fiber (7). HMG proteins alter the local structure of DNA or chromatin by inducing a conformation that facilitates the binding of specific regulatory factors (4,8). Thus, it is possible that HMG-17 associates with the spatial organization of gene expression within eukaryotic nuclei (40).

The HMG domain of Sox proteins can interact with several transcription factors directly through their DNA-binding domains (41). Curiously, detection of the HMG-domain interacting factors by immunoprecipitation was fairly weak. This weak interaction was supported by moderate increases in salt concentrations that produced strong reductions in the amounts of co-precipitated partner protein (41). Furthermore, these interactions were not stable under electrophoretic mobility shift conditions. These data correlate with our data except that we have identified a ternary complex containing β-catenin that appears to stabilize the complex. The contact between the Sox HMG domain and DNA-binding domains of other transcription factors are weak, conversely we have demonstrated a strong interaction between PITX2 and HMG-17. However, we have shown that the PITX2/HMG-17 complex can be easily dissociated by slight increases in salt concentrations. Thus, the HMG-17 interaction with PITX2 is reversible and provides a mechanism to change interaction partners in solution as proposed for the Sox HMG domain (41). This provides a mechanism for changes in HMG-17 binding to PITX2 to functionally change its activity in response to other factors interacting with PITX2.

The inactive PITX2/HMG-17 complex would be concentrated at sites of active transcription as shown for HMG-17. Upon Wnt signaling β-catenin would form a ternary complex with PITX2/HMG-17 and switch this complex to a highly active form. We speculate that β-catenin binding to the PITX2/HMG-17 complex causes

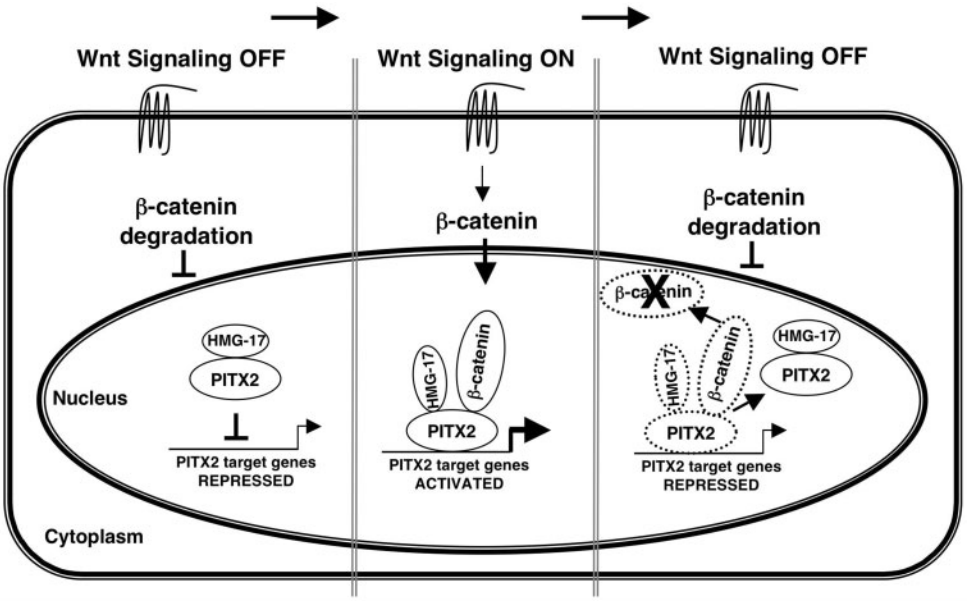

Figure 7. Model for the novel regulation of homeodomain transcriptional activity by HMG-17 modulated by β-catenin. Without Wnt signaling, β-catenin is degraded in the cytoplasm, HMG-17 binds to PITX2 with a high affinity and prevents PITX2 from binding DNA. PITX2 target genes are repressed due to the inability of PITX2 to bind and activate the target gene promoters. In the presence of Wnt signaling, β-catenin is translocated to the nucleus and interacts with the HMG-17/PITX2 complex changing it from a repressor complex to an activator complex. Subsequently when Wnt signaling is turned off and nuclear β-catenin is limited or turned over then HMG-17 forms an inactive complex with PITX2. This mechanism would allow for the tight coordinated control of homeodomain transcriptional activity during development.

a conformational change in HMG-17 that allows for the PITX2 HD to recognize its DNA target sequence and promotes the association of a higher ordered chromatin-associated transcription complex. Previous reports have demonstrated that HMG-17 acts as a transcriptional facilitator or coactivator by unfolding the higher order chromatin structure allowing transcription factors access to the DNA (35,36). These data support our results demonstrating an increase in transcription when the ternary complex of PITX2, HMG-17 and β-catenin are all present, and HMG-17 in this protein complex can then facilitate RNA polymerase II interaction with the DNA and overcome the repressive activity of the chromatin fiber. We have proposed a model for the interaction of PITX2 with HMG-17 and conversion of a repressor complex to an activator dependent on canonical Wnt signaling and β-catenin interaction (Figure 7). Without Wnt signaling, β-catenin is degraded and PITX2 complexes with HMG-17 in an inactive form. Wnt signaling allows β-catenin translocation to the nucleus where it complexes with PITX2 and HMG-17 to yield an active transcriptional complex. When Wnt signaling is off and nuclear β-catenin is reduced, HMG-17 inactivates PITX2 (Figure 7). This interaction would tightly control the transcriptional activity of PITX2. Clearly other chromatin-associated factors may play a role in this multi-protein complex however, the major players in this novel pathway are PITX2, HMG-17 and β-catenin.

HMG-17 appears to be a major regulator of the timing of early embryonic development in the mouse (42). The early embryonic lethality of the HMG-17$^{-/-}$ homozygous mice support the role of HMG-17 as a regulator of early embryonic development.

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Conflict of interest statement. None declared.

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