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Range of HOX/TALE superclass associations and protein domain requirements for HOXA13:MEIS interaction

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Abstract

AbdB-like HOX proteins form DNA-binding complexes with the TALE superclass proteins MEIS1A and MEIS1B, and trimeric complexes have been identified in nuclear extracts that include a second TALE protein, PBX. Thus, soluble DNA-independent protein–protein complexes exist in mammals. The extent of HOX/TALE superclass interactions, protein structural requirements, and sites of in vivo cooperative interaction have not been fully explored. We show that *Hoxa13* and *Hoxd13* expression does not overlap with that of *Meis1–3* in the developing limb; however, coexpression occurs in the developing male and female reproductive tracts (FRTs). We demonstrate that both HOXA13 and HOXD13 associate with MEIS1B in mammalian and yeast cells, and that HOXA13 can interact with all MEIS proteins but not more diverged TALE superclass members. In addition, the C-terminal domains (CTDs) of MEIS1A (18 amino acids) and MEIS1B (93 amino acids) are necessary for HOXA13 interaction; for MEIS1B, this domain was also sufficient. We also show by yeast two-hybrid assay that MEIS proteins can interact with anterior HOX proteins, but for some, additional N-terminal MEIS sequences are required for interaction. Using deletion mutants of HOXA13 and HOXD13, we provide evidence for multiple HOX peptide domains interacting with MEIS proteins. These data suggest that HOX:MEIS interactions may extend to non-AbdB-like HOX proteins in solution and that differences may exist in the MEIS peptide domains utilized by different HOX groups. Finally, the capability of multiple HOX domains to interact with MEIS C-terminal sequences implies greater complexity of the HOX:MEIS protein–protein interactions and a larger role for variation of HOX amino-terminal sequences in specificity of function.

Keywords: Hox; HOXA13; Homeodomain; AbdB-like; MEIS; TALE superclass; Limb bud; Müllerian duct; Cofactor

Introduction

Hox genes encode a conserved set of transcription factors that specify regional identity along the developing anteroposterior axis of developing embryos. A common feature of this gene family is that each protein has a 60-amino-acid DNA-binding domain (DBD) referred to as the homeodomain (HD) (Gehring et al., 1994). The mammalian genome contains 39 Hox genes that reside in four separate linkage groups, designated A to D, on four separate chromosomes. Hox genes within each cluster can be categorized into 13 paralog groups based upon their position and homology to the *Drosophila* Hox genes (Krumlauf, 1994).

HOX specificity is acquired, in part, through cofactor interaction (Mann and Affolter, 1998). The most studied HOX cofactors are the vertebrate PBX and MEIS proteins, two members of the TALE (*T*hree *A*mino acid *L*oop *E*xtension) superclass of atypical homeodomain proteins (Burglin, 1997). HOX proteins from paralog groups 1–10 form cooperative DNA-binding complexes with PBX proteins, while those of groups 9–13, referred to as AbdB-like, cooperatively bind DNA in vitro with MEIS1 proteins (Chang et al., 1996; Shen et al., 1997a,b). In addition to heterodimeric interactions on DNA with these TALE superclass proteins, some HOX proteins have been shown

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to form soluble triple complexes including both PBX and MEIS or PREP1 proteins in the absence of DNA (Berthelsen et al., 1998; Jacobs et al., 1999; Shanmugam et al., 1999; Shen et al., 1999).

Cooperative binding between anterior mammalian or *Drosophila* HOX and PBX/Exd proteins, respectively, has been shown to depend upon a highly conserved HOX hexapeptide that lies N-terminal to the homeodomain (Chan et al., 1994; Chang et al., 1995; Johnson et al., 1995; Ryoo et al., 1999). The AbdB-like HOX proteins from paralog groups 9 and 10 have a conserved tryptophan that mediates PBX interaction (Chang et al., 1996; Shen et al., 1997b). Importantly, HOX proteins from groups 11, 12, and 13 lack a similarly positioned, conserved tryptophan, suggesting that cofactor interaction may require other residues. Cooperative interaction between MEIS1 and these AbdB-like HOX proteins on DNA has been reported, but the protein motifs mediating these interactions have not been elucidated. The potential for interaction between MEIS and more anterior HOX proteins without DNA binding has not been tested.

There are a few reports describing coexpression between MEIS and posterior HOX proteins; these have been limited to the adult mouse epididymis, mouse ovary, the developing limb bud, and myeloid cells (Bomgardner et al., 2003; Lawrence et al., 1999; Mercader et al., 1999, 2000; Villaescusa et al., 2004). Complexes of HOX and MEIS proteins have been recovered from myeloid cells showing association of these proteins in an in vivo context (Shen et al., 1999); however, the full extent of HOX:MEIS associations and their importance in development is not understood.

In the developing limb, the expression of Hox genes from paralog group 13, *Hoxa13* and *Hoxd13*, is restricted to the distal autopod (Dolle et al., 1989; Haack and Gruss, 1993; Mortlock et al., 1996; Post and Innis, 1999). However, *Meis1* and *Meis2* are restricted to proximal regions of the developing limb (Mercader et al., 1999, 2000). Therefore in the distal limb bud, HOXA13 and HOXD13 apparently function in the absence of MEIS1 and MEIS2 proteins as potential cofactors. The expression domain of *Meis3* in the limb bud has not been reported. This could imply that in the limb, HOX group 13 proteins work as monomers (Galant et al., 2002) and/or that they interact with other cofactors.

The present study was initiated to explore HOX:MEIS interactions in more detail. We examined expression of *Meis* and *Hox* paralog group 13 genes and encoded protein products in the reproductive tract as one potential in vivo site for HOX:MEIS interaction. Additionally, using a yeast two-hybrid assay, we tested the range of AbdB and non-AbdB-like HOX protein interactions with MEIS and the capabilities of other TALE superclass proteins to interact with HOXA13, and we probed the location of paralog group 13 and MEIS peptide domains necessary for interaction.

Materials and methods

Whole-mount in situ hybridization

Embryos were collected from matings of C57Bl/6J mice at E10.5 and E11.5. Embryos were staged by assigning noon of the day of vaginal plug discovery as E0.5. Antisense mRNA probes were transcribed as previously described for *Hoxa13* and *Hoxd13* (Post and Innis, 1999). A *Meis3* antisense probe was created using a 531-bp template consisting of contiguous coding sequence downstream of the homeodomain and 3′ UTR. Following linearization by *Aat*II, an RNA probe was synthesized using Sp6 RNA polymerase. *Meis2* sense RNA probe was created by *Not*I template linearization and transcription using Sp6 RNA polymerase. Whole-mount in situ hybridization with a single digoxygenin-labeled RNA probe was performed as previously described (Bober et al., 1994), except BM purple (Roche) was used as the substrate for alkaline phosphatase.

RT–PCR

Genital tracts were dissected from male and female P1 (n = 5), and 10-week old female mice (n = 2). For each age, RNA was prepared separately from the ovaries, uterus, and cervix/vagina for the females and testis, epididymis, and prostate of males, using Trizol (Invitrogen). All RT–PCRs were performed using the One-Step RT–PCR kit (Invitrogen) with 150 ng of total cellular RNA per 50-μl reaction. The RT step was for 30 min at 50°C; the PCR used an annealing temperature of 56°C and an extension time of 45 s at 72°C for 40 cycles. PCR primer pairs used, from 5′ to 3′, for *Meis1* (a and b), *Meis2* (c and d), and *Meis3* (e and f) were (a) CGAGCAGTCAGCCAAGGGAC, (b) TCAGTCACCATTGTAGACAACG, (c), GTGCAGCCCATGATTGACCAG, (d), TACTATTGGGCATGAATGTCC, (e) TTGAC-CAGTCTAATCGCACAG, and (f) AGGGAGGTAGGCGTTGAATGG. Each primer pair amplifies products spanning multiple exons, distinguishing spliced mRNA from genomic DNA contamination. Gel image analysis was done using the Quantity One software with a Gel Doc 2000 (BioRad).

Immunohistochemistry

Antibodies to murine MEIS1 and HOXD13 were created using the multiple antigenic peptide method (Posnett and Tam, 1989), using amino acids 107–132 for MEIS1 and 176–198 for HOXD13. Anti-HOXA13 antibody, Ab490, was previously described (Post et al., 2000). For section immunohistochemistry, N-terminal MEIS1 and MEIS2 antibodies were obtained from Dr. A. Buchberg (Jefferson Medical College, Philadelphia, PA). These two antibodies were previously demonstrated to be specific for each protein (Swift et al., 1998; Zhang et al., 2002). An antibody specific for MEIS3 was not available at the time of this study.

Whole-mount immunostaining was performed as done previously (Yokouchi et al., 1995) on dissected P1 female genital tracts. Primary antibodies were used at dilutions of 1:10,000 for anti-MEIS1 and anti-HOXD13, and 1:5000 for anti-HOXA13 and rabbit preimmune serum. The secondary antibody, donkey anti-rabbit HRP conjugate (Amersham), was used at a dilution of 1:500. For section immunohistochemistry, reproductive tracts were dissected out of P1 mice, fixed with 4% paraformaldehyde for 1 h at room temperature, and paraffin-embedded. Sections of 5μm were used for analysis. Sections were dewaxed, rehydrated through a series of graded ethanol concentrations, and blocked overnight in PBST with 5% Carnation milk. Samples were incubated at room temperature for 3 h with primary antibodies at dilution of 1:1000 for anti-MEIS1, anti-MEIS2, anti-HOXD13, rabbit preimmune serum, and 1:50 for affinity-purified anti-HOXA13. Sections were incubated for 2 h with biotin-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch) at a dilution of 1:500. Vector Elite reagent was used according to manufacturer's protocol (ABC, Vector Labs) to boost the signal. Samples were developed with either DAB (Brown; Figs. 3J and P) or Vector VIP (Purple; Vector Labs; Figs. 3E–I and K–O) for 5–15 min to achieve desired color intensity.

Co-immunoprecipitation

NIH 3T3 cells were transfected with expression vectors as indicated in Fig. 4. In brief, 550,000 cells were seeded in 6-cm dishes (three per condition) 18 h prior to transfection. Following the transfection protocol for Lipofectamine Plus (Invitrogen), each plate received 4 μg of total DNA (2 μg per construct) with 16 μl of Plus reagent and 24 μl of Lipofectamine. Forty-eight hours posttransfection, cell lysates were prepared by collecting cells from three 6-cm dishes by treatment with trypsin-EDTA. For each condition, cells were pelleted by centrifugation, supernatant removed, followed by addition of 1 ml cold lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA), and mechanically disrupted with a 26-gauge needle. Immunoprecipitation was performed using anti-FLAG M2 affinity gel following manufacturer's protocol (Sigma). Eluted proteins were separated by electrophoresis on 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose for Western analysis using primary antibodies at 1:10,000 (anti-HOXA13 and anti-MEIS1 107–132) followed by donkey anti-rabbit HRP secondary antibody (Amersham) at 1:15,000, each in PBST with 5% Carnation nonfat dry milk. Protein was visualized using Supersignal chemilluminescent substrate (Pierce).

Yeast two-hybrid

All yeast two-hybrid preys were created by cloning coding sequences into the vector pJG4-5 on the 3′ end of the B42 transcriptional activation domain (AD). Bait constructs were made by cloning coding sequences into the vectors pEG202 or pEG202NLS (includes nuclear localization signal) on the 3′ end of the LexA DNA-binding domain (DBD). All HOX and TALE superclass constructs were derived from murine cDNAs. Control baits used were pRFHMI (Bicoid HD) and pBait (TGFβRI). The bait plasmid and *lacZ* reporter vector (pSH17-4) were transformed into haploid yeast strain RFY206 (MATa), and preys were transformed into the strain EGY48 (MATα) as described previously (Gietz et al., 1992). Testing for bait and prey interactions was accomplished by interaction mating (Finley and Brent, 1994). After selecting for diploid yeast, strains were transferred to media containing X-gal with either glucose or galactose and grown overnight at 30°C. Interaction readings were visually scored 24 h after growth on X-gal-containing media.

Sequence analysis

Comparison of protein sequences was done using the MEGALIGN program (DNASTAR) with the CLUSTAL W alignment algorithm (Thompson et al., 1994). A neighbor-joining tree was generated in MEGALIGN, from the CLUSTAL W sequence alignments. Accession numbers for the *Mus musculus* protein sequences: HOXA13 (NP_032290), HOXB13 (NP_032293), HOXC13 (NP_034594), HOXD13 (NP_032301), MEIS1A (Q60954), MEIS1B (NP_034919), MEIS2A (CAA04138), MEIS2B (CAA04139), MEIS2C (CAA04140), MEIS2D (CAA04141), MEIS3 (AAC52949), PREP1 (AAH52701), PREP2 (AAM18702), TGIF (NP_033398), and PBX1A (NP_899198). The accession number for *Gallus gallus* HOXA13 proteins sequence is NP_989470.

Results

Coexpression of HOXA13 and HOXD13 with MEIS proteins in the female reproductive tract (FRT)

Meis1 and *Meis2* are expressed in a separate proximal limb region (data not shown) from that of *Hoxa13* and *Hoxd13* (Figs. 1A and D) at early stages precluding interaction (Mercader et al., 1999, 2000). By whole-mount in situ hybridization on developing mouse embryos, we analyzed the limb bud expression of *Meis3* at E10.5 and E11.5 (Figs. 1B and E). Similar to that of *Meis1* and *Meis2*, *Meis3* is not expressed in the distal limb bud. Thus, lack of *Meis3* expression in the distal limb bud at these developmental time points excludes the possibility for a functional interaction to occur between paralog group 13 HOX and MEIS proteins.

Previous work demonstrated *Meis2* expression in the adult female reproductive tract henceforth referred to as FRT (Oulad-Abdelghani et al., 1997); however, the precise cell types were not delineated, and earlier developmental

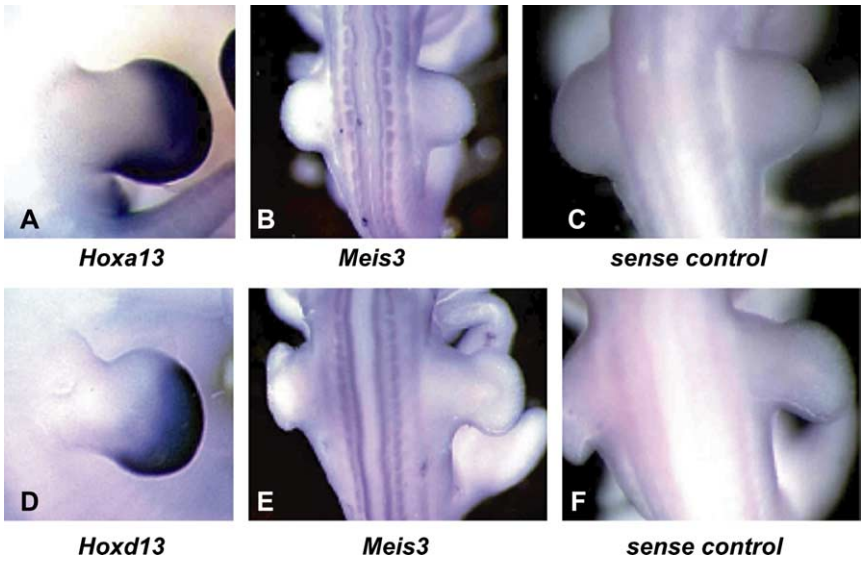

Fig. 1. Expression patterns preclude a limb bud interaction between MEIS3 and either HOXA13 or HOXD13. Whole-mount in situ hybridization on E10.5 (B and C), and E11.5 (A, and D–F) mouse embryos using antisense and sense RNA probes. Distal limb bud expression of (A) *Hoxa13* and (D) *Hoxd13*. *Meis3* is expressed (B and E) in the somitic mesoderm, but not in the limb bud at these stages. Probe specificity is shown by the absence of any signal from embryos incubated with a sense RNA probe (C and F).

stages were not examined. Thus, the onset of *Meis2* expression in the FRT is unknown. *Hoxa13* is known to be expressed in the mesenchymal region of the cervix/vagina of both the developing and mature FRT (Ma et al., 1998; Post and Innis, 1999; Taylor et al., 1997; Warot et al., 1997). We define the developing FRT as the prepubertal female reproductive tract immediately after birth (P1), a time during peak morphological differentiation of the mouse reproductive system (Podlasek et al., 2002; Taylor et al., 1997). To determine whether *Meis1*, *Meis2*, and *Meis3* are also expressed in the developing and mature FRT, we performed RT–PCR using RNA collected from the ovary, uterus, and cervix/vagina regions of P1 and 10-week-old (10 W) females. Consistent with previous results (Taylor et al., 1997), *Hoxa13* expression was detected only in RNA derived from the cervix/vagina tissue of the mature FRT, whereas expression was also detected in the ovary and uterus in addition to the cervix/vagina in the developing FRT, albeit at lower levels (Fig. 2). Each *Meis2* splice variant (*Meis2a–d*) was expressed in the ovary, uterus, and cervix/vagina RNA at nearly equivalent levels. The two *Meis1* splice variants (*Meis1a* and *Meis1b*) are expressed in

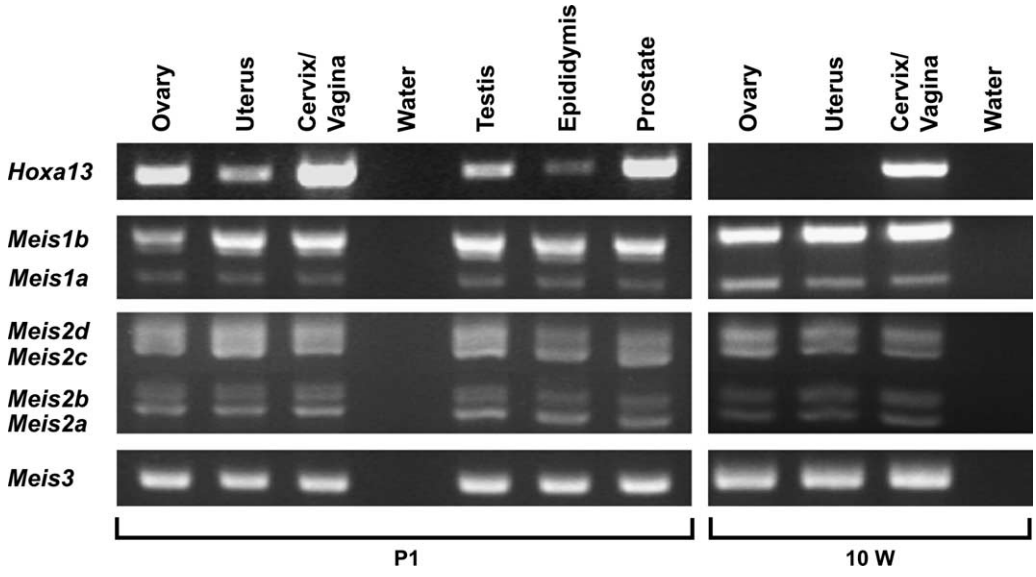

Fig. 2. Expression of *Hoxa13*, *Meis1*, *Meis2*, and *Meis3* in both the developing male and female and the mature female reproductive tracts. Total cellular RNA was prepared from the testis, epididymis, and prostate of newborn (P1) male mice and ovary, uterus, and cervix/vagina of both newborn and 10-week-old (10 W) female mice, and used for RT–PCR. Primers distinguish the splice variants of *Meis1* (*Meis1a* and *Meis1b*) and *Meis2* (*Meis2a–d*) based upon size differences.

each of the three RNA populations tested; however, *Meis1b* expression consistently appeared greater than that of *Meis1a* (similar trend seen in the developing male reproductive tract). As seen for *Meis1* and *Meis2*, *Meis3* is also expressed in each of the RNA populations. Unlike *Hoxa13* where expression differences exist between the developing and mature FRT, no differences were observed for *Meis1–3*.

Hoxa13, *Hoxd13*, and *Hoxb13* are expressed in the male reproductive tract (Economides and Capecchi, 2003; Podlasek et al., 2002). To determine whether *Meis1-3* is also expressed within the same tissues as *Hoxa13*, RT–PCR was performed using RNA derived from the testis, epididymis, and prostate of P1 male mice. Similar to the expression in the FRT, *Meis1*, *Meis2*, and *Meis3* are expressed in each of the tested RNA populations (Fig. 2). P1 expression of *Hoxa13* is detected in the prostate as well as in both the epididymis and testis. Expression of *Hoxd13* was also detected in prostate RNA and weakly in that of the epididymis and testis (data not shown). These experiments broadly define the male and female reproductive tracts as tissues where *Hoxa13* and *Meis1-3* are coexpressed.

To determine whether HOXA13 and HOXD13 are coexpressed within the same regions and cells as MEIS proteins, we performed immunostaining on developing FRTs (Fig. 3). Consistent with the RT–PCR data, by whole-mount immunostaining, HOXA13 expression appears highest in the caudal regions of the developing FRT (Fig. 3A), which includes the cervix and vagina. This restricted expression pattern was also observed for HOXD13 (Fig. 3B). Using an antibody that recognizes both MEIS1A and MEIS1B, expression was found to extend throughout the entire FRT (Fig. 3C). To further demonstrate which cell types within the developing cervix and vagina are expressing HOXA13, HOXD13, MEIS1, and MEIS2 and whether expression occurs in the same or separate cell populations, immunohistochemistry was performed on histological sections from P1 females (Figs. 3E–P). The available antibodies were all raised in the same species making double-labeling experiments to demonstrate cellular coexpression impractical. HOXA13 expression was shown to extend throughout the mesenchyme (MES) of the cervix and vagina (Fig. 3F) but not that of the stratified squamous epithelium (SSE, Fig. 3L).

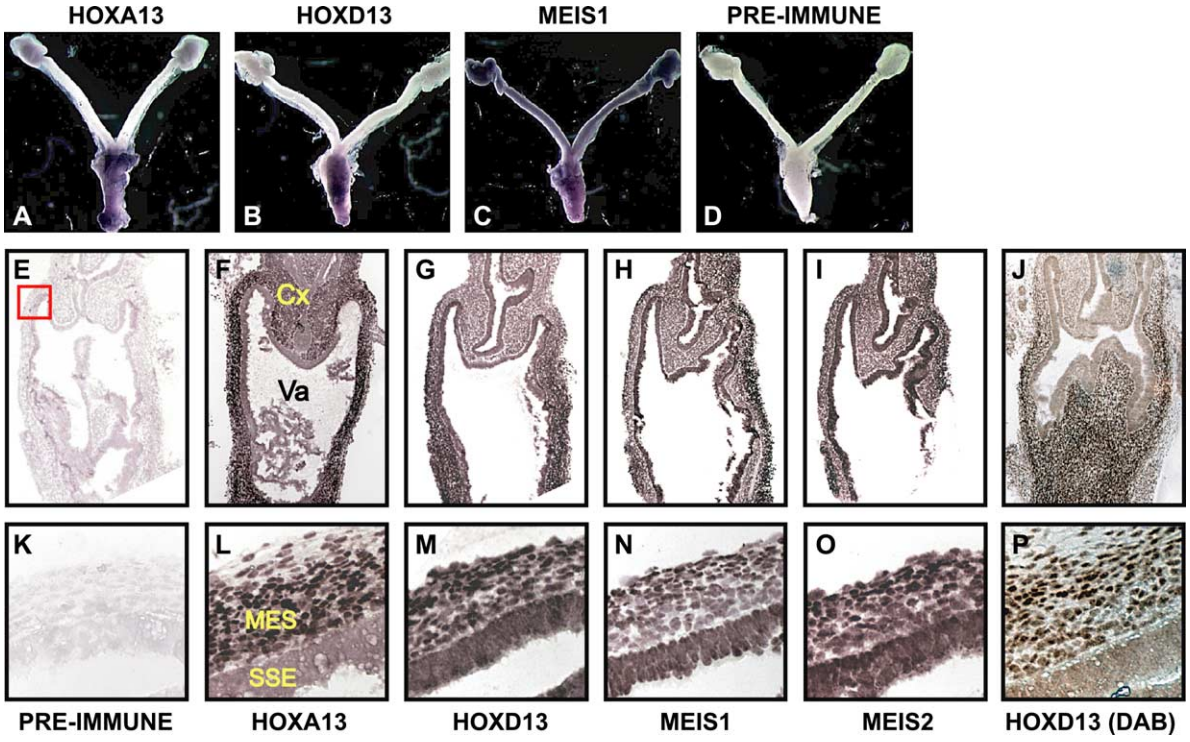

Fig. 3. Expression of HOXA13, HOXD13, MEIS1, and MEIS2 in the developing female reproductive tract. Whole-mount immunostaining of newborn FRTs shows strong HOXA13 (A), and HOXD13 (B) expression detected in the caudal cervix and vagina region, while expression is faintly observed in the more rostral structures of the uterus and ovaries. MEIS1 expression (C) is observed throughout the ovary, uterus, and cervix/vagina region. Section immunohistochemistry (E–J at 100×; K–P at 400×) shows expression of HOXA13 (F and L), HOXD13 (G, J, M, and P), MEIS1 (H and N), and MEIS2 (I and O) in cell types within the developing cervix and vagina. Preimmune control is shown to demonstrate antibody specificity (E and K). Nuclear HOXA13 expression (F) is detected in both the cervix (Cx) and vagina (Va). Cell type-specific expression, area indicated by red box (E), can be seen at a higher magnification (K–P). For HOXA13, expression is limited to the mesenchymal cells (MES), and not cells in the stratified squamous epithelial (SSE) layer (L). The expression pattern of HOXD13 is similar to that of HOXA13, with expression in both the cervix and vagina (G and J), and limited to the mesenchymal cells (M and P). The mesenchyme-restricted expression of HOXD13 is better visualized using DAB as the substrate (P). MEIS1 and MEIS2 are expressed throughout both the cervix and vagina (H and I), and expression is nuclear in both MES and SSE cells (N and O).

Like HOXA13, HOXD13 is expressed throughout the MES of the vagina (Va) and cervix (Cx). However, HOXD13 expression does not extend as far rostral into the cervix (Figs. 3G and J). HOXD13 expression is restricted to the MES (Figs. 3M and P). Expression of both MEIS1 and MEIS2 extends throughout the cervix and vagina (Figs. 3H and I) in both the mesenchyme and stratified squamous epithelial layers (Figs. 3N and O). HOXA13, HOXD13, MEIS1, and MEIS2 are predominantly nuclearly localized; this was confirmed by sections stained with the nuclear tropic dye, hematoxylin (data not shown). This is the earliest and most thorough demonstration of MEIS expression in the FRT. These results demonstrate that within the cervix and vagina of the developing FRT, paralog group 13 HOX proteins are coexpressed in the nuclei of mesenchymal cells with MEIS proteins, presenting an opportunity for functional interactions to occur.

MEIS1B interacts with HOXA13 in mammalian cells

MEIS1A and MEIS1B have been shown to interact with HOX proteins in triple complexes as non-DNA-binding partners (Shanmugam et al., 1999; Shen et al., 1999), and MEIS1A/HOXA9 complexes can form in the absence of DNA (Shen et al., 1997b). To test whether HOXA13 and MEIS1B could be recovered as a complex from cervix/vagina cells, co-immunoprecipitation was attempted using cellular extracts from P1 FRT preparations. Unfortunately, all available antibodies were created in rabbits. This precluded the clear visualization of proteins of interest by Western analysis (data not shown).

We thus elected to perform co-immunoprecipitation using lysates generated from NIH 3T3 cells transfected with C-terminal FLAG-tagged full-length HOXA13 and N-terminal Myc-tagged full-length MEIS1B (Fig. 4). Using an anti-FLAG agarose conjugate, HOXA13-FLAG was immunoprecipitated from lysates. MEIS1B was recovered after immunoprecipitating HOXA13-FLAG (Fig. 4, lane 4), but not from lysates without HOXA13-FLAG (Fig. 4, lane 5). Similar results were obtained using HOXD13-FLAG (data not shown). These results indicate that group 13 HOX proteins can be recovered from mammalian cells in a soluble complex with MEIS1B.

MEIS1A and MEIS1B interact specifically with HOXA13

To further characterize direct HOXA13-MEIS1 peptide interactions, we used a yeast two-hybrid system (Gyuris et al., 1993). The full-length coding sequences for *Meis1a* and *Meis1b* (amino acids 1–390 and 1–465, respectively) were cloned in-frame on the 3′ end of the B42 AD (Activation Domain), placing MEIS1 peptide sequences at the carboxy-terminus of expressed prey molecules. Baits were created that divided the complete HOXA13 coding sequence into three overlapping segments (Figs. 5A and B5–7), each cloned on the 3′ end of the LexA DBD (DNA-binding domain). An interaction with MEIS1B was observed with the bait containing the coding sequence for amino acids 150–313 of HOXA13, but not with those for HOXA13 amino acids 1–165 or 283–386 (Fig. 5C, baits 5–7). Identical results were observed with MEIS1A (data not shown). Since the prey protein is only expressed on plates containing galactose, by the *GAL1* promoter, the *lacZ* expression can be concluded to result from the interaction between either MEIS1A or MEIS1B and the 150–313 HOXA13 bait and not from autoactivation of the reporter by the bait protein alone, as seen for the LexA DBD-Gal4 AD-positive control (Fig. 5C, bait 2). The specificity of this interaction was verified by demonstrating that MEIS1A and MEIS1B preys do not interact with either the LexA DBD bait alone, the LexA DBD fused with the Bicoid HD, or the LexA DBD fused with the TGFβRI (Fig. 5C, baits 1, 3, and 4). In addition, the bait containing amino acids 150–313 of HOXA13 did not interact with the B42 AD only, confirming that the interaction is between HOXA13 and MEIS1B and not the adjacent heterologous activation domain. As a final

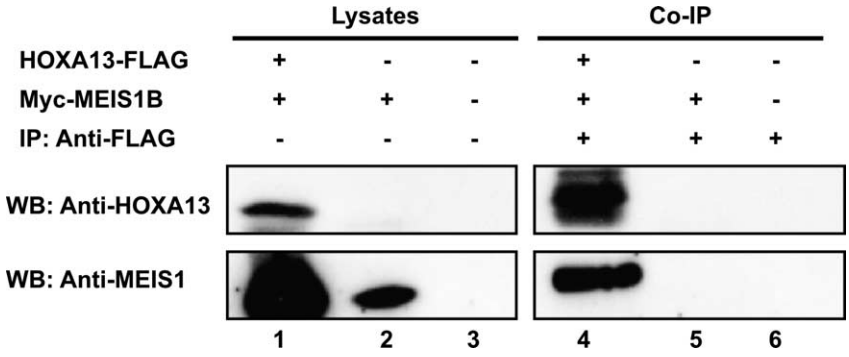

Fig. 4. HOXA13 and MEIS1B interact in mammalian cells. Co-immunoprecipitation demonstrates an in vivo interaction between MEIS1B and HOXA13. Myc-MEIS1B was expressed in NIH 3T3 cells with or without HOXA13-FLAG. Whole cell lysates were prepared and subjected to immunoprecipitation using an anti-FLAG agarose conjugate. Cell lysates (lanes 1–3) and proteins eluted from the agarose conjugate (lanes 4–6) were resolved by SDS–PAGE and subjected to Western blotting separately with anti-HOXA13 and anti-MEIS1 antibodies. Lanes 1 and 4 are from cells transfected with HOXA13-FLAG and Myc-MEIS1B expression constructs, lanes 2 and 5 from cells transfected with Myc-MEIS1B and empty expression vector, and lanes 3 and 6 received only the empty vector.

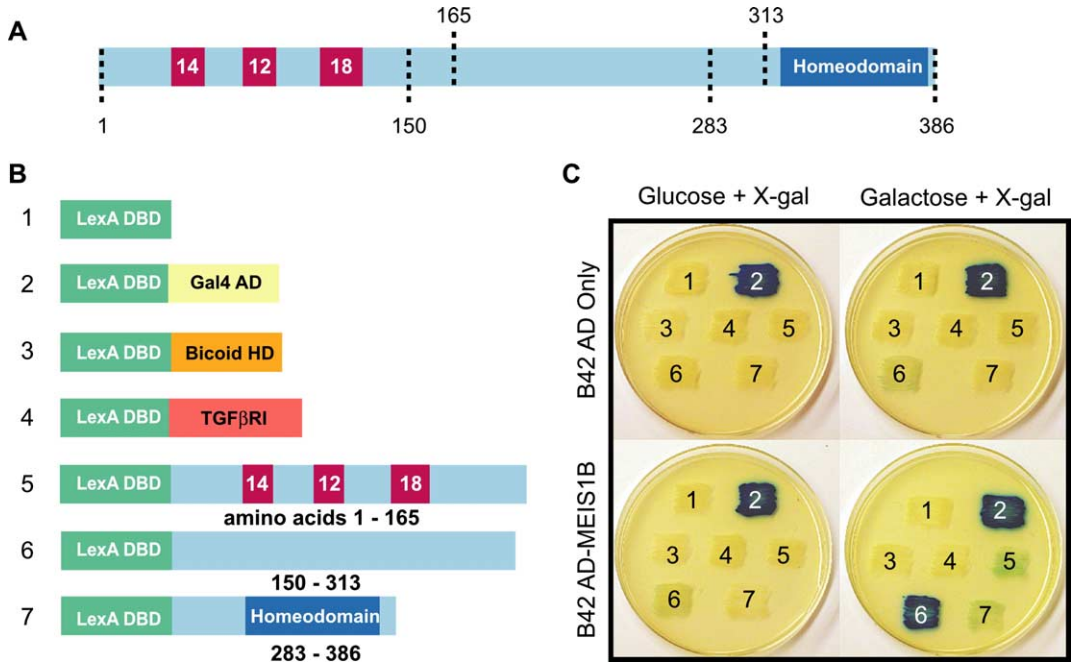

Fig. 5. Amino acids 150–313 of HOXA13 specifically interacts with MEIS1B in yeast. (A) Diagram of full-length HOXA13 (1–386) with three polyalanine tracts (red) and C-terminal homeodomain (dark blue). HOXA13 was subdivided into three overlapping yeast two-hybrid baits; amino acids 1–165, 150–313, and 283–386. (B) Baits tested for interaction with MEIS1B; (1) LexA DNA-binding domain (DBD) only, (2) LexA DBD + Gal4 activation domain (AD), (3) LexA DBD + Bicoid homeodomain (HD), (4) LexA DBD + TGFβRI, (5) LexA DBD + HOXA13 1–165, (6) LexA DBD + HOXA13 150–313, and (7) LexA DBD + HOXA13 283–386. (C) Results for the interaction assay for each bait with the B4 AD only prey or B42 AD with full-length MEIS1B. The prey protein is only expressed on media containing galactose.

test of specificity, we performed a bait/prey swap, making MEIS1A and MEIS1B the bait and HOXA13 150–313 the prey. Here, a strong interaction between HOXA13 and both MEIS1A and MEIS1B was observed (data not shown).

MEIS1A and MEIS1B interact specifically with other HOX proteins

To determine whether all paralog group 13 Hox proteins could interact with MEIS1A and MEIS1B, the protein sequences encoded by the entire first exons of *Hoxb13*, *Hoxc13*, and *Hoxd13* were cloned into the pJG4-5 prey vector, and the expressed preys were tested for interaction with both full-length MEIS1A and MEIS1B baits. A specific interaction of comparable intensity to that of HOXA13 was seen for each paralog group 13 member (Table 1). First exon encoded protein sequences of HOXA13 were also sufficient for interaction (see Fig. 7A). Of interest, full-length HOX proteins (HOXD13, HOXA11, HOXA9) failed to interact with either MEIS1A or MEIS1B (data not shown). However, deletion of 27 amino acids from the carboxy-terminus of full-length HOXD13 (using only aa 1–312) or conversion of the homeodomain residues $I^{47}Q^{50}N^{51}$ to alanines (Caronia et al., 2003), which severely reduces DNA binding, allowed interaction.

We then tested whether other HOX proteins could interact with MEIS1 proteins. The entire first exon encoded proteins for the *AbdB-like* genes *Hoxd12*, *Hoxa11*, *Hoxa10*, *Hoxa9*, and the non-*AbdB-like* genes *Hoxb8*, *Hoxa5*, *Hoxb4*, and *Hoxa2* were cloned into the pJG4-5 prey vector. None of these contained their homeodomain. A reproducible interaction was demonstrated between each of the expressed HOX preys and both full-length MEIS1A and MEIS1B baits (Table 1). The interactions were shown to be specific, in that no interaction was observed between any of the HOX preys and the control baits including the LexA DBD alone, or LexA DBD with either the Bicoid HD or TGFβRI. The results demonstrate that in the context of eukaryotic yeast nuclei, MEIS1A and MEIS1B interact with representative members of anterior, non-AbdB-like HOX proteins (paralog groups 2, 4, 5, and 8) as well as posterior, AbdB-like HOX proteins (paralog groups 9–13). Although previous studies demonstrated the inability for cooperative DNA-binding interactions to occur between MEIS1 proteins and the anterior HOX proteins (Shen et al., 1997a), those findings do not preclude an interaction in solution, which could affect the concentration of free and bound HOX and MEIS transcription factors in cells.

Paralog group 13 HOX proteins do not interact with other TALE superclass members

MEIS1 belongs to the TALE superclass of homeodomain proteins (Burglin, 1997). We sought to determine whether other members of this group could interact with HOXA13. We amplified the full-length coding sequences for *Meis2b*,

Table 1
Full-length MEIS1A and 1B interact with HOX proteins of most paralog classes

Preys	LexA DNA-binding domain baits				
	Empty	Gal4AD[a]	Bicoid HD	TGFβR1	MEIS1A/1B
Empty[b]	0	+++	0	0	0
HOXA13 (150–313)	0	+++	0	0	+++
HOXB13 (1–200)	0	+++	0	0	+++
HOXC13 (1–243)	0	+++	0	0	+++
HOXD13 (1–256)	0	+++	0	0	+++
HOXD12 (1–200)	0	+++	0	0	+++
HOXA11 (1–236)	0	+++	0	0	++
HOXA10 (1–308)	0	+++	0	0	+
HOXA9 (1–192)	0	+++	0	0	+++
HOXB8 (1–142)	0	+++	0	0	+++
HOXA5 (1–187)	0	+++	0	0	+++
HOXB4 (1–151)	0	+++	0	0	+++
HOXA2 (1–126)	0	+++	0	0	++

0, no interaction detected by *lac*Z activity.
+++, strong interaction detected by *lac*Z activity.
++, medium interaction detected by *lac*Z activity.
+, minor interaction detected by *lac*Z activity.
[a] Gal4AD is a positive control for *lac*Z activity.
[b] Prey protein is the B42 AD alone.

Meis3, *Prep1*, *Prep2*, and *Tgif* by RT–PCR. These products were cloned 3′ of the LexA DBD and tested for interaction in yeast with the HOXA13 prey (amino acids 150–313). MEIS2B interacted with the HOXA13 prey, while those of PREP1, PREP2, and TGIF did not (Table 2A). As the bait, MEIS3 was a strong transcriptional activator in the absence of an expressed prey protein. To circumvent this autoactivation, *Meis3* was cloned into the prey vector, and the expressed prey was shown to interact with the HOXA13 150–313 bait (Table 2B). Preys containing the C-terminal 127 amino acids of MEIS2C and MEIS2D, which do not include any portion of their homeodomains, also interacted with the HOXA13 150–313 bait (data not shown). To test for additional N-terminal or C-terminal HOX residues required for TALE superclass interactions, we tested preys that contained the protein sequences for HOXD13 1–212 and HOXA13 150–360 (data not shown). No interaction occurred with either PREP1 or PREP2; however, we cannot rule out that other domains of HOXA13 or HOXD13 as full-length molecules interact with more diverged TALE members. These results demonstrate that MEIS2 and MEIS3 proteins, in addition to MEIS1, have the capacity to interact with HOXA13. Interaction of TALE superclass members with group 13 HOX proteins appears to be restricted to the MEIS proteins and not with closely related PREP1 and PREP2 proteins or the more distantly related TGIF (Fig. 6A).

Table 2B
MEIS3, in prey configuration, interacts specifically with HOXA13

Prey	LexA DNA-binding domain baits				
	Empty	GAl4 AD[a]	Bicoid HD	TGFβRI	HOXA13
Empty	0	+++	0	0	0
MEIS3	0	+++	0	0	+++

0, no interaction detected by *lac*Z activity.
+++, strong interaction detected by *lac*Z activity.
−, not tested for interaction.
[a] Gal4AD is a positive control for *lac*Z activity.

The C-termini of MEIS1A and MEIS1B mediate interaction with HOXA13

To localize the HOXA13-interacting peptide domains of MEIS1A and MEIS1B, we created various yeast two-hybrid preys by subdividing these proteins and determining their ability to interact in yeast (Fig. 6B). Interaction with the HOXA13 150–360 bait was observed with full-length MEIS1B (1–465) as well as N-terminal deleted MEIS1B preys comprising amino acids 214–465 or 358–465. A MEIS1B prey composed of amino acids 369–465 was also sufficient for interaction. C-terminal MEIS1-deleted preys comprising amino acids 1–329 or 214–346 failed to interact with the HOXA13 bait. In-frame deletion of the region between the homeodomain and the C-terminal region of

Table 2A
HOXA13 peptide (150–313) interacts with MEIS proteins

Preys	LexA DNA-binding domain baits						
	MEIS1A	MEIS1B	MEIS2B	MEIS3	PREP1	PREP2	TGIF
Empty[a]	0	0	0	+++	0	0	0
HOXA13 (1–165)	0	0	0	+++	–	–	–
HOXA13 (150–313)	+++	+++	+++	+++	0	0	0
HOXA13 (283–386)	0	0	0	+++	–	–	–

0, no interaction detected by *lac*Z activity.
+++, strong interaction detected by *lac*Z activity.
−, not tested for interaction.
[a] Prey protein is the B42 AD alone.

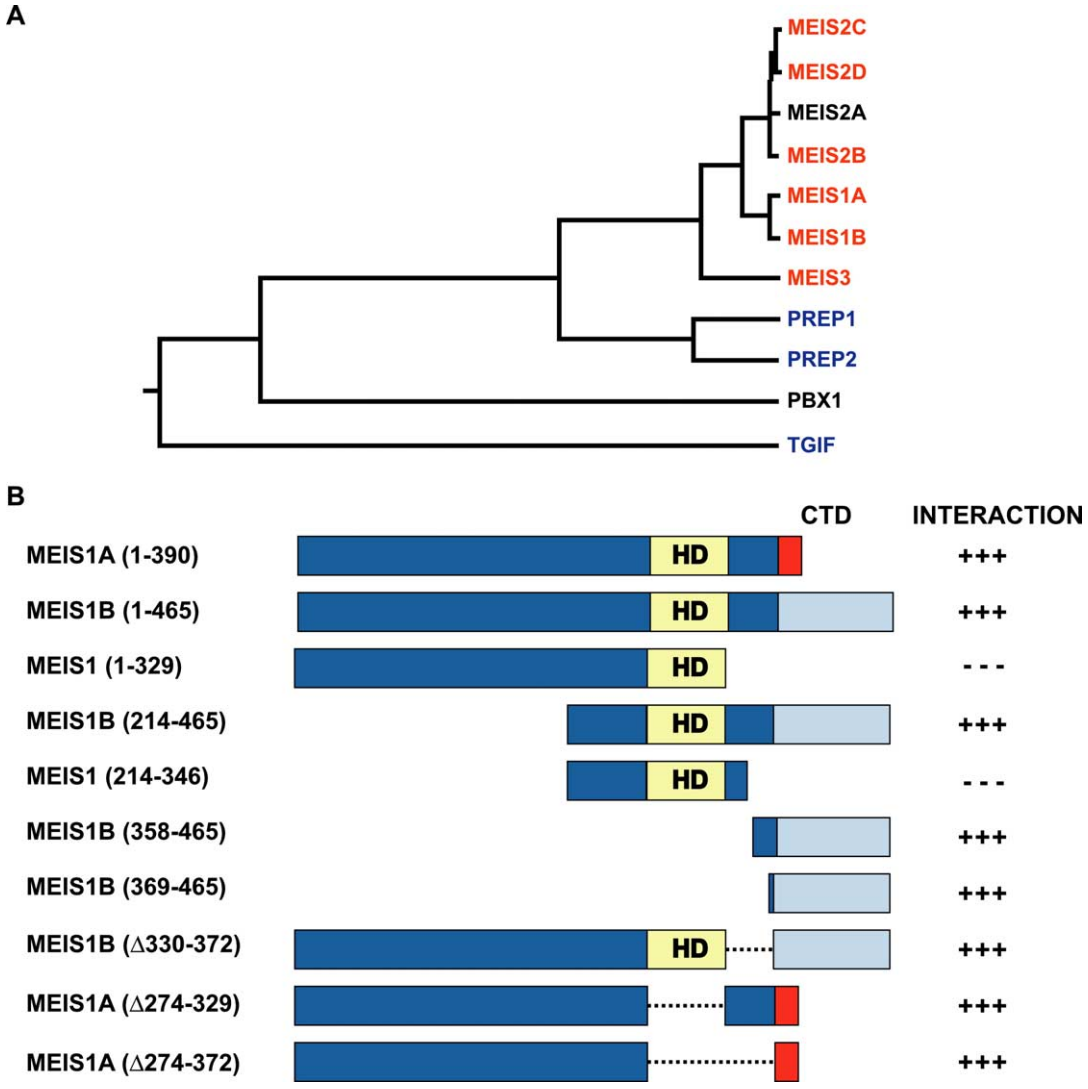

Fig. 6. MEIS proteins interact with HOXA13 through their C-terminal domain. (A) Neighbor joining tree based on amino acid sequence identity for selected TALE superclass members. TALE proteins which where demonstrated to interact with HOXA13 amino acids 150–313 are shown in red, those that did not interact in blue, and not tested in black. (B) Diagrammatic representation of MEIS1 preys tested for interaction with the HOXA13 150–360 bait, and their interaction status. The MEIS homeodomain (HD) is in yellow, and unique C-terminal domains (CTD) of MEIS1A and MEIS1B are colored in red and light blue, respectively. Dashed line represents an in-frame deletion of protein sequence.

MEIS1B (MEIS1B Δ330–372), which is identical in MEIS1A, also strongly interacted with the HOXA13 bait. Together, these data demonstrate that within the unique C-terminal domain (CTD) of MEIS1B resides the motif(s) responsible for interaction with HOXA13. It was thus surprising that full-length MEIS1A (1–390) also interacts with the HOXA13 150–360 bait. The 18-amino-acid CTD of MEIS1A (373–390) alone failed to interact with the HOXA13 bait (data not shown), perhaps due to the small size of the prey molecule. However, the necessity of the C-terminal 18 amino acids was demonstrated using MEIS1A preys with in-frame deletions of amino acids 274–329 (removes the homeodomain) and 274–372 (removes the homeodomain and adjacent C-terminal identical sequences). These preys both were able to interact with the HOXA13 bait. Collectively, these results demonstrate that the C-terminal peptide domains (CTD) of MEIS1A and MEIS1B are necessary for interaction with HOXA13. Moreover, the CTD of MEIS1B was shown here to be sufficient for interaction.

Differences in MEIS1B peptide sufficiency for HOX interaction

To determine the MEIS1B peptide sufficiency for additional HOX interactions, MEIS1B baits consisting of amino acids 1–465 (full length) and 340–465 (C-terminal 126 amino acids) were tested for interaction with various HOX preys (Table 3). Two different patterns of interaction were observed. First exon encoded peptide sequences of HOXB8,

Table 3
Differences in MEIS1 sufficiency for HOX interaction in yeast

Preys	LexA DNA-binding domain baits					
	Empty	Gal4AD[a]	Bicoid HD	TGFβR1	MEIS1 (1–465)	MEIS1B (340–465)
Empty[b]	0	+++	0	0	0	0
HOXA13 (150–313)	+/–	+++	+/–	0	+++	+++
HOXB13 (1–200)	0	+++	0	0	+++	+++
HOXC13 (1–243)	+/–	+++	+/–	0	+++	+++
HOXD13 (1–256)	0	+++	0	0	+++	+++
HOXD12 (1–200)	0	+++	0	0	+++	+++
HOXA11 (1–236)	0	+++	0	0	++	0
HOXA10 (1–308)	0	+++	0	0	+	0
HOXA9 (1–192)	0	+++	0	0	+++	+++
HOXB8 (1–142)	+/–	+++	+/–	0	+++	+++
HOXA5 (1–187)	0	+++	0	0	+++	0
HOXB4 (1–151)	0	+++	0	0	+++	0
HOXA2 (1–126)	0	+++	0	0	++	0

0, no interaction detected by *lac*Z activity.
+++, strong interaction detected by *lac*Z activity.
++, medium interaction detected by *lac*Z activity.
+, minor interaction detected by *lac*Z activity.
+/–, varies between no *lac*Z activity to minor *lac*Z activity in replication.
[a] Gal4AD is a positive control for *lac*Z activity.
[b] Prey protein is the B42 AD alone.

HOXA9, HOXD12, and all paralog group 13 HOX proteins interacted with both MEIS1B baits. First exon encoded peptide sequences of HOXA2, HOXB4, HOXA5, HOXA10, and HOXA11 could interact with the full-length MEIS1B, but not with the C-terminal 126 amino acids. The HOXA10 and HOXA11 preys interacted with a MEIS1B bait including amino acids 214–465 (albeit attenuated, data not shown). Thus, within the AbdB-like group of HOX proteins, HOXA10 and HOXA11 appear to require additional MEIS1B sequence for interaction. These experiments demonstrate differences in MEIS peptide sufficiency for the broad repertoire of possible HOX:MEIS associations.

HOXA13 has multiple MEIS interaction domains

To identify HOXA13 residues critical for MEIS interaction, we created a series of nested, smaller HOXA13 preys and tested them for their ability to interact with full-length MEIS1A and MEIS1B baits (Fig. 7A). A HOXA13 prey consisting of amino acids 150–360 interacts with both MEIS1A and MEIS1B. N-terminal deletions of this construct to residue 237 did not disrupt interaction with MEIS1A or MEIS1B, yet a protein truncated to residue 247 was unable to interact with either MEIS1A or MEIS1B. Progressive C-terminal deletions of the HOX 150–360 prey, as far as residue 250, were also able to interact with MEIS1A and MEIS1B. However, further C-terminal deletions to amino acid 246 or to 242 abrogated interaction. The shared sequence in all MEIS-interacting HOXA13 preys is amino acids 237–250 (Fig. 7C, domain II). Comparison of amino acid sequences for all murine paralog group 13 HOX proteins and chicken HOXA13 shows that residues Y, Q, Y (uncharged polar side chains), and D (charged polar side chain) are conserved. To test whether this region is necessary for MEIS interaction, a prey with an in-frame deletion of these amino acids was created. This prey, A13 150–313 (Δ237–250), robustly interacts with both MEIS1A and MEIS1B. Taken together, the data suggested that more than one domain for MEIS interaction exist within amino acids 150–313 of HOXA13. However, one domain appears to be within the conserved region defined by HOXA13 amino acids 237–250.

Multiple MEIS interaction domains in HOXD13

To determine whether HOXD13 also contains multiple domains capable of mediating MEIS interaction, a series of HOXD13 mutant preys were created. A prey containing amino acids 1–312 strongly interacts with MEIS1A and MEIS1B (Fig. 7B). This prey, like HOXA13 150–360, terminates at amino acid 312 just before helix 3/4 of the homeodomain. C-terminal truncations as far as residue 139 of HOXD13 retained interaction with MEIS1A and MEIS1B. Further deletion to residue 118 eliminated interaction. Conversely, an N-terminal truncation to residue 139, construct 139–312, was able to interact with both MEIS1A and MEIS1B, although it shares only one amino acid with the minimal N-terminal interacting prey (1–139). Within the region between amino acids 118 and 139 resides a domain with high sequence identity among all murine group 13 paralogs and chicken HOXA13 (Fig. 7C, domain I). An in-frame deletion of domain I (Δ123–135) in the construct 1–180 eliminates interaction with MEIS1A and MEIS1B [compare D13 1–180 (Δ123–135) to D13 1–180]. Addition of further C-terminal sequences to this clone restored interaction in the absence of domain I [D13 1–256

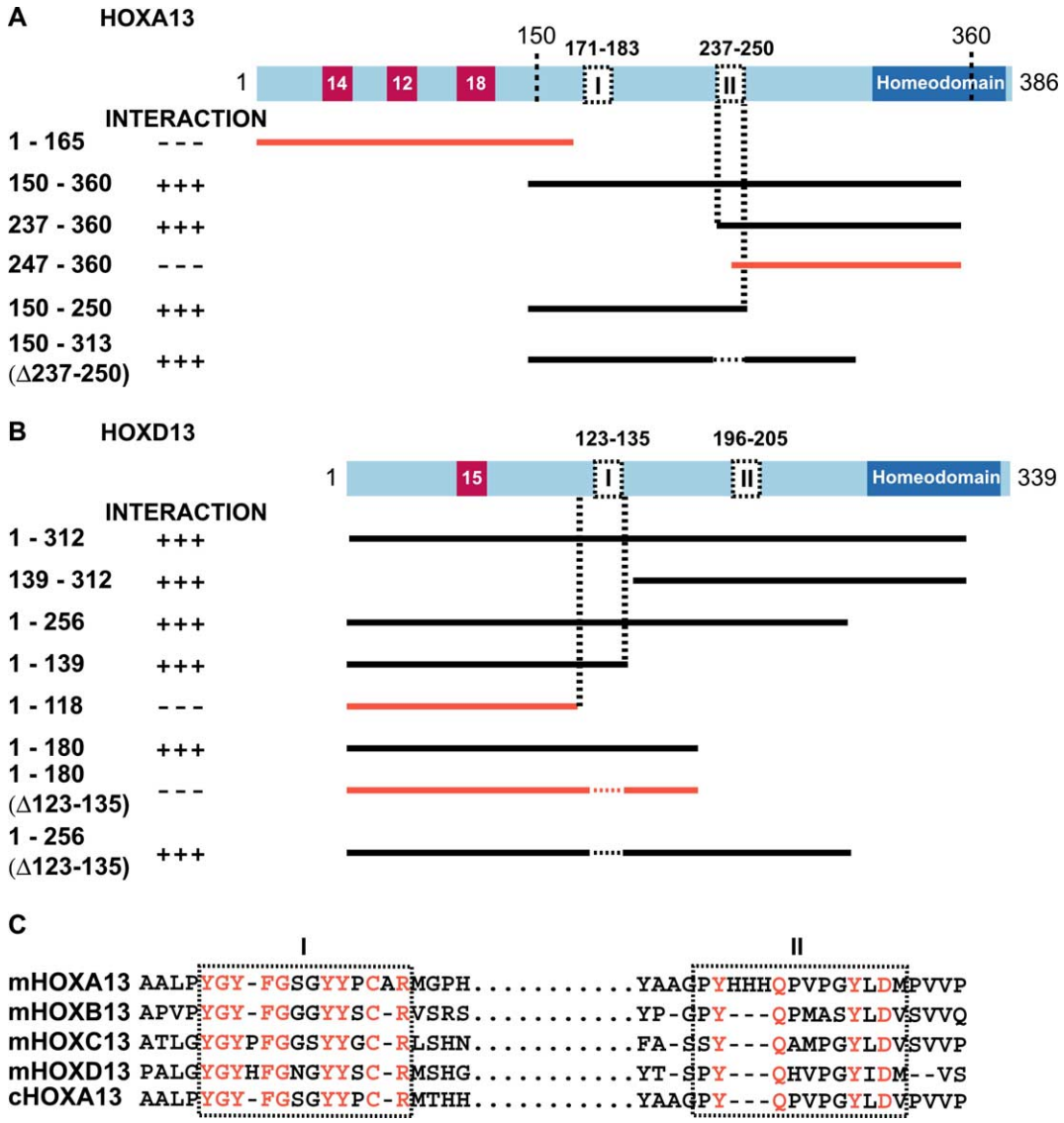

Fig. 7. HOXA13 and HOXD13 have multiple MEIS interaction domains. Diagrammatic representation of various (A) HOXA13 and (B) HOXD13 preys tested for interaction with MEIS1A and MEIS1B. The MEIS-interacting preys are indicated in black and noninteracting preys in red. In-frame deletion of amino acid residues is indicated by a horizontal dashed line. (C) Sequence alignment of mouse HOXD13 amino acids 123–135 (domain I) and HOXA13 amino acids 237–250 (domain II) with mouse paralog group 13 HOX proteins and chicken HOXA13. In red are residues conserved among all compared proteins.

(Δ123–135)]. Thus, HOXD13, like HOXA13, is capable of interacting with MEIS1A and MEIS1B through multiple domains.

Discussion

Paralog group 13 HOX proteins function with or without MEIS protein cofactors

In the developing limb bud, the paralog group 13 HOX proteins, HOXA13 and HOXD13, play an essential role in the proper patterning and development of the distal autopod (Fromental-Ramain et al., 1996). However, during development, the expression of *Meis1* and *Meis2* is restricted to a separate proximal region (Mercader et al., 1999, 2000). Here, we show that like *Meis1* and *Meis2*, *Meis3* is not expressed in the distal limb bud. This suggests that HOXA13 and HOXD13 contribute to early limb development in a MEIS-independent manner and either function as monomers or utilize other cofactors. Whether later stages or selected cells within the developing limb utilize group 13 HOX:MEIS complexes remains to be explored.

Outside of the limb, *Hoxa13* is expressed, among other places, in both the developing male and female reproductive tract as well as the mature female reproductive tract and is essential for their proper development (Ma et al., 1998; Podlasek et al., 2002; Post and Innis, 1999; Taylor et al., 1997; Warot et al., 1997). While *Meis2* is known to be expressed in the adult FRT (Oulad-Abdelghani et al., 1997),

we have shown that it is also expressed in the developing FRT and that HOXA13, HOXD13, MEIS1, and MEIS2 proteins are coexpressed in the nuclei of mesenchymal cells in the cervix and vagina. This work indicates that the developing reproductive tract presents a context where group 13 HOX proteins may have functional interactions with MEIS cofactors. It is notable that proximal limb expression of group 11 and 13 HOX proteins downregulate the expression of MEIS and that distal expression of MEIS downregulates group 13 HOX expression (Capdevila et al., 1999; Mercader et al., 1999). Here, we demonstrate that this mutually exclusive expression is not observed in the reproductive tract. Whether these proteins actually associate in the reproductive tract must now be explored.

Range of HOX:MEIS interactions

In vitro experiments have demonstrated that representative members from each AbdB-like HOX paralog group (9–13) can cooperatively bind DNA with MEIS1. In this same experimental context, MEIS1 was unable to cooperatively bind DNA with more anterior HOX proteins (paralog groups 1–8) (Shen et al., 1997a). It should be pointed out that the yeast two-hybrid system has been used successfully several times to study HOX protein interactions (Chan et al., 1994, 1995; Johnson et al., 1995). Utilizing a yeast two-hybrid system to monitor protein–protein interaction, we showed all paralog group 13 HOX proteins, HOXD12, HOXA11, HOXA10, and HOXA9 can interact with MEIS1A and MEIS1B. Importantly, we also observed interactions between MEIS1A and MEIS1B with the anterior, non-AbdB-like HOX proteins, HOXA2, HOXB4, HOXA5, and HOXB8. Taken together, this suggests that association of these factors can occur not only between DNA-bound AbdB-like HOX proteins and MEIS, but also in solution where neither component or only one may be bound to DNA. These interactions may not have been observed in prior studies using proteins of paralog groups 4, 6, 7, and 8 as the methods were evaluating only DNA-bound complexes, which places a different requirement on the physical association (Shen et al., 1997a). Extensive, thorough work by that group with DNA site selection assays, using HOXB7 and MEIS1B, only identified single HOX or MEIS binding sites. Since we did not test groups 6 and 7 proteins for interaction, we cannot rule out the possibility that MEIS interactions are discontinuous among all HOX proteins. Our assay is potentially more sensitive, yet it was specific.

Selective interactions of HOXA13 and TALE superclass proteins

MEIS proteins belong to a class of atypical homeodomain proteins known as the TALE superclass (Burglin, 1997). Within this group of related proteins, interactions with HOX proteins have been demonstrated for PBX and MEIS proteins, and a third member, PREP1, that interacts with PBX already complexed with a HOX protein (Mann and Affolter, 1998). Here, we demonstrated that TALE superclass proteins that are capable of interacting with HOXA13 include not only MEIS1A and MEIS1B, but also MEIS2A, MEIS2C, MEIS2D, and MEIS3. More diverged family members PREP1, PREP2, and TGIF were unable to interact in this assay. The possible range of interactions demonstrated here would be expected to have broad in vivo relevance, particularly in tissues that express multiple MEIS isoforms as we have demonstrated for the reproductive tract.

Peptide domains contributing to interactions between HOX and MEIS proteins

HOX proteins from paralog groups 1–8 and PBX proteins interact through a HOX hexapeptide, while those of paralog groups 9 and 10 utilize a conserved ANW peptide motif, each N-terminal to the HOX homeodomain (Mann and Chan, 1996; Shen et al., 1997b). Interaction through the hexapeptide motif has been demonstrated to affect in vitro cooperative DNA-binding and in vivo functionality (Chang et al., 1995; Medina-Martinez and Ramirez-Solis, 2003; Shen et al., 1997b). The tryptophan residue (W) of HOXA9 is not necessary for cooperative DNA binding with MEIS1B (Shen et al., 1997b), thus suggesting different sequence requirements for interaction. In our study, we demonstrated for all tested HOX proteins that MEIS-interacting motifs are within the peptide sequences encoded by the first exons, thus excluding homeodomain sequences. We also showed that multiple domains are capable of mediating interaction with MEIS1A and MEIS1B.

Domain I has high amino acid identity among all paralog group 13 HOX proteins and is conserved in the *Drosophila* AbdB protein (Mortlock et al., 2000). Pinpointing the interaction motifs beyond domain I remains to be done and may be challenging as sequence conservation outside of the homeodomain is remote among HOX proteins of different paralog groups. Another domain appears to be represented by HOXA13 amino acids 237–250, here referred to as domain II. Within this domain, four residues (YQYD) are completely conserved between paralog group 13 HOX proteins. Other AbdB-like HOX proteins appear to lack strict conservation of the sequences comparable to domains I and II. However, review of several HOX protein sequences from paralog groups 9–12 reveals the existence of several tyrosine-rich segments. Whether these sequences mediate interaction with MEIS1 would need to be systematically tested to understand the generality of multiple MEIS interaction domains.

Within MEIS proteins, we demonstrate that the HOXA13 interacting region lies within the unique C-termini of MEIS1A and MEIS1B. Remarkably, the C-terminal HOXA13-interacting domain of MEIS1A is only 18 amino acids and has limited sequence identity with the C-terminal

MEIS1B domain. This suggests that few conserved MEIS residues are required for HOXA13 interaction or that altogether unique peptides mediate binding. While the C-terminal domain of MEIS1B is shown here to be sufficient for interaction with all group 13 HOX proteins and with HOXD12, HOXA9, and HOXB8, the C-terminal domain of MEIS1B was not sufficient to interact with HOXA2, HOXB4, HOXA5, HOXA10, and HOXA11; interaction with these proteins requires the contribution of additional N-terminal MEIS peptide sequences. Unique functional outcomes may result from different HOX:MEIS complexes.

Multiple interaction domains and range of HOX:MEIS interactions

Based on previous work with the hexapeptide motif, we anticipated that we would identify a single HOX peptide motif mediating interaction with MEIS proteins. However, recent studies have revealed cooperative in vitro DNA binding between Exd and AbdA or Ubx, even when the HOX hexapeptide is mutated (Galant et al., 2002; Merabet et al., 2003). The AbdA hexapeptide mutant maintains the ability to transform segment identity in vivo in an Exd-dependent manner. Additionally, the first report documenting cooperative interactions between Ubx and Exd was performed using a Ubx variant lacking the hexapeptide motif (Chan et al., 1994). These results cumulatively shed light on our observation, relative to HOX:MEIS interaction, in pointing to additional residues besides the hexapeptide that may promote physical interaction with the Exd cofactor. Of great mechanistic importance, the AbdA hexapeptide mutant retained normal in vivo binding site selection; however, on one target, the mutant protein changed from a repressor to an activator (Merabet et al., 2003). This suggests the possibility that utilization of different cofactor interaction motifs may alter the transregulatory properties of the HOX protein in a target gene-specific manner. Our studies point to the capability of multiple, separate domains of HOXA13 and HOXD13 and possibly for additional HOX proteins for MEIS interaction. Another interpretation of our data is that these proteins are interacting nonspecifically in yeast two-hybrid, which might also explain the data with the anterior HOX proteins. However, we believe that the demonstrated restriction of TALE superclass interactions, variable requirement for N-terminal MEIS peptide sequences for interaction with HOX proteins, and absence of an interaction for numerous baits and preys argue for specificity in our experimental results. Future studies are needed to identify the full complement of interaction motifs and determine their contribution to cooperative DNA binding and in vivo transregulatory function.

It is well established that a major mode of HOX function occurs through the regulation of target genes via direct DNA binding with or without cofactors. However, many studies have revealed functions for homeodomain proteins, including HOX, where the homeodomain is unable to bind DNA as a monomer or is devoid of the DNA-binding domain altogether (Ananthan et al., 1993; Caronia et al., 2003; Chen et al., 2004; Copeland et al., 1996; Fitzpatrick et al., 1992; Suzuki et al., 2003; Zappavigna et al., 1994). Thus, we hypothesize that biologically meaningful HOX:MEIS interactions may not be confined to only those on DNA. Future studies to understand HOX function should include the possibility that HOX proteins exert functional effects through protein–protein interactions where the HOX protein is either a non-DNA-binding partner or is interacting in solution.

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