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Pbx acts with Hand2 in early myocardial differentiation

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Abstract

Transcription factors of the basic helix-loop-helix (bHLH) family are critical regulators of muscle cell differentiation. For example, Myod drives skeletal muscle differentiation, and Hand2 potentiates cardiac muscle differentiation. Understanding how these bHLH factors regulate distinct transcriptional targets in a temporally and spatially controlled manner is critical for understanding their activity in cellular differentiation. We previously showed that Pbx homeodomain proteins modulate the activity of Myod to promote the differentiation of fast-twitch skeletal muscle. Here, we test the hypothesis that Pbx proteins are also necessary for cardiac muscle differentiation through interacting with Hand2. We show that Pbx proteins are required for the activation of cardiac muscle differentiation in zebrafish embryos. Loss of Pbx activity leads to delay of myocardial differentiation and subsequent defective cardiac morphogenesis, similar to reduced Hand2 activity. Genetic interaction experiments support the hypothesis that Pbx proteins modulate the activity of Hand2 in myocardial differentiation. Furthermore, we show that Pbx proteins directly bind the promoter of the myocardial differentiation gene *myl7* in vitro, supporting a direct role for Pbx proteins in promoting cardiac muscle differentiation. Our findings demonstrate new roles for Pbx proteins in vertebrate cardiac development and also provide new insight into connections between the transcriptional regulation of skeletal and cardiac muscle differentiation programs.

Keywords

pbx; *hand2*; zebrafish; heart development; muscle differentiation

Introduction

Development of the heart involves the complex orchestration of cellular specification, differentiation, and morphogenesis. Even slight disruptions of embryonic cardiac development can lead to congenital heart disease (CHD) in humans. Insight into the genetic basis of CHD has come from the identification of human mutations associated with CHD and also from model organism studies of cardiac development (Clark et al., 2006; Olson, 2006; Pierpont et al.,

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Contributions

AT and LM carried out the experiments. SJT and LM conceived the experiments. CBM provided the zebrafish facility. LM analyzed the data and wrote the paper. All authors have approved the final version of this manuscript.

2007). Several genes encoding transcription factors comprise a conserved regulatory network with major roles in heart development, including the factors Nkx2–5, MEF2, GATA, Tbx and Hand1/2 (Clark et al., 2006; Olson, 2006). These factors control cardiac cell fates and morphogenesis by regulating expression of downstream genes such as those encoding contractile proteins. Although mutations in these transcription factor genes have been associated with CHD in humans, there is often phenotypic heterogeneity associated with single-gene defects, as seen for *NKX2–5* and *TBX5* (Srivastava and Olson, 2000; Clark et al., 2006). This heterogeneity points to potential roles for modifier loci in affecting the phenotypic severity of CHD. A challenge now is to understand how additional factors might modify the transcriptional programs controlled by the major heart factors.

Skeletal muscle has served as a model for understanding general principles guiding the transcriptional control of cellular differentiation. Vertebrate skeletal muscle development is coordinated by a family of four basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs): Myod, Myf5, MRF4 and Myogenin (Pownall et al., 2002; Tapscott, 2005). These factors, in particular Myod, are sufficient to convert non-muscle cells into skeletal muscle (Weintraub et al., 1991; Tapscott, 2005). Myod directly activates the expression of additional transcription factors, including *Myogenin*, and acts in a feed-forward mechanism in cooperation with those factors to directly activate a skeletal muscle differentiation gene expression program (Tapscott, 2005; Cao et al., 2006). Recent studies have shown that Myod and another bHLH protein, Hand, are both critical for striated bodywall muscle formation in *C. elegans* (Fukushige et al., 2006), whereas Hand proteins regulate development of cardiac muscle, but not skeletal muscle, in flies and chordates (Olson, 2006). The conserved roles for bHLH proteins in regulating muscle development suggest that there are fundamental core mechanisms for generating contractile cell types (Fukushige et al., 2006).

Because the MRFs directly regulate the expression of many genes necessary for skeletal muscle differentiation, understanding how these bHLH proteins regulate distinct transcriptional targets in a temporally and spatially controlled manner has been critical for understanding their activity. One mechanism recently proposed is the use of “molecular beacons”, or cofactors that bind the promoter of specific transcriptional targets and act as signals to attract a MRF to that promoter (Berkes et al., 2004). Pbx homeodomain proteins appear to function as such factors. Pbx proteins are TALE (Three Amino acid Loop Extension)-class homeodomain proteins that are best characterized as transcriptional cofactors for Hox proteins (Moens and Selleri, 2006). However, Pbx proteins interact with factors other than Hox proteins and have been shown to cooperatively bind DNA with MRFs, including Myod (Knoepfler et al., 1999; Berkes et al., 2004). Pbx proteins bind directly to promoters of certain Myod transcriptional targets, such as *Myogenin*, and they bind the *Myogenin* promoter prior to Myod in mammalian myoblast cell lines (Berkes et al., 2004). Thus, Pbx appears to mark a subset of Myod target genes for transcriptional activation by Myod.

We recently tested the requirements for Pbx in interacting with Myod in zebrafish skeletal muscle development (Maves et al., 2007). Zebrafish have five *pbx* genes, but only *pbx2* and *pbx4* are expressed during early developmental stages, and both can be readily knocked down to generate Pbx-null embryos (Waskiewicz et al., 2002; Maves et al., 2007). We found that Pbx proteins are indeed required for Myod to activate *myogenin* expression and are necessary for Myod to activate expression of fast-twitch skeletal muscle differentiation genes (Maves et al., 2007). Pbx and Myod also exhibit genetic interactions in the regulation of *myogenin* expression and fast-muscle differentiation (Maves et al., 2007).

Here, we wanted to further test the hypothesis that Pbx proteins regulate cellular differentiation by modulating bHLH factor activity, in particular by testing whether Pbx proteins interact with Hand2 in cardiac muscle differentiation. Although Hand proteins are critical regulators of heart

development in zebrafish and mice, the mechanisms of Hand function in the heart are not well understood (Srivastava et al., 1997; Firulli et al., 1998; Riley et al., 1998; Yelon et al., 2000; Firulli, 2003). *Pbx* gene deficiencies in mice were recently shown to cause cardiac defects that resemble human CHDs (Chang et al., 2008; Stankunas et al., 2008), but early roles of *Pbx* genes in myocardial differentiation have not yet been addressed. Here we show that Pbx proteins are indeed required for cardiac muscle differentiation in zebrafish. Our results suggest that Pbx proteins regulate myocardial differentiation and morphogenesis by interacting with Hand2. These connections between Pbx and bHLH factors support the idea of a core developmental program regulating the transcriptional control of contractile muscle cell differentiation.

Materials and methods

Zebrafish Stocks

Zebrafish (*Danio rerio*) were raised and staged as previously described (Westerfield, 1995). Time (hpf) refers to hours post-fertilization at 28.5°C. In some cases, embryos were raised for periods at room temperature, about 25°C. The wild-type stock used was AB. *hand2*^{s6} embryos were generously provided by Zayra Garavito-Aguilar and Debbie Yelon, and this allele has been previously described (Yelon et al., 2000)

Morpholino and mRNA injections

pbx2; pbx4 morpholino cocktails were previously described (Maves et al., 2007). *hand2* translation-blocking morpholino, generously provided by Debbie Yelon, was injected at 9 ng per embryo at the 1-cell stage (sequence: 5′-CCTCCAACTAAACTCATGGCGACAG-3′). We somite-stage-matched sibling control and MO-treated embryos when collecting embryos for staining or for RNA harvesting. For the experiments of Fig. 5, control MO (*pbx2*-MO3 mismatch control; Maves et al., 2007), was used to balance the total amounts of MO injected.

pCS2-*hand2* was made by using RT-PCR to amplify full-length zebrafish *hand2*, which was cloned into the XhoI site of pCS2. mRNA was synthesized using the mMessage mMachine kit (Ambion). About 200–300 pg of *hand2* mRNA was injected per embryo at the 1-cell stage.

RNA in situ hybridization

RNA in situ hybridizations were performed as previously described (Maves et al., 2007). The following cDNA probes were used: *tnnt2* (Sehnert et al., 2002); *ttna* (EST wu:fb96b11); *cmlc1* (Reiter et al., 1999); *vmhc* (Yelon et al., 1999); *aldh1a2* (Begemann et al., 2001); *myl7* (Yelon et al., 1999); *lft1* (EST cb73, Thisse et al., 2001); *hand2* (Yelon et al., 2000); *nkx2.5* (Lee et al., 1996); *scl* (Schoenebeck et al., 2007); *gata4* (Reiter et al., 1999); *myod* (Weinberg et al., 1996); *krox-20* (*egr2b*-Zebrafish Information Network; Oxtoby and Jowett, 1993); *amhc* (Berdougo et al., 2003); *nppa* (Berdougo et al., 2003).

Embryos were photographed using a Zeiss Axioplan2 microscope and Zeiss AxioCam MRc digital camera or a Zeiss Stemi SV6 and Nikon CoolPix 4500 digital camera. Images were assembled using Adobe Photoshop.

cDNA microarray analysis

pbx2-MO; *pbx4*-MO embryos as well as their control siblings (*pbx2*-MO3 mismatch control) were collected at the 10s or 18s stage from three independent sets of injections. A subset of embryos from each set of injections were used for in situ hybridization to confirm somite staging (using *myod*) and loss of *krox-20* expression in *pbx2*-MO; *pbx4*-MO embryos (see Figures 2 and 3 for examples). Total RNA harvesting, cRNA labeling and hybridization to Affymetrix Zebrafish Genome arrays, and statistical analyses of the gene expression profiles

of control versus *pbx2*-MO; *pbx4*-MO were performed as previously described (Maves et al., 2007). We used the cut-offs of >1.5-fold change and a False Discovery Rate of <0.05 to identify genes whose expression is significantly dependent on Pbx function. Array data are available at NCBI GEO (www.ncbi.nlm.nih.gov/geo/), accession GSE8428.

EMSA

Electrophoretic mobility shift assays were performed as previously described (Berkes et al., 2004). *pbx4* and *meis3* pCS2 expression constructs were previously described (Pöpperl et al., 2000; Vlachakis et al., 2000).

Results

Pbx2 and Pbx4 are required for normal heart function

To address the role of Pbx proteins in zebrafish heart development, we used anti-sense morpholino oligos (MOs), injected at the 1-cell stage, to knock down the maternal and zygotic expression of both Pbx2 and Pbx4 (Waskiewicz et al., 2002). We previously demonstrated that these combined *pbx2* and *pbx4* morpholinos specifically and effectively knock down all Pbx protein at least up to 24 hours post fertilization (hpf) (Maves et al., 2007). In wild-type zebrafish embryos, the two heart chambers, the atrium and ventricle, are distinct and functioning by 48 hpf (Glickman and Yelon, 2002; Fig. 1A). In *pbx2*-MO; *pbx4*-MO embryos, a weakly contractile heart forms and appears morphologically abnormal, accompanied by mild pericardial edema (30/30 embryos exhibited a similar phenotype). Heart function appears disrupted, with blood pooling by the atrium (Fig. 1B). We confirmed the defect in heart function by assessing the heartbeat at 48 hpf. Wild-type control embryos had 156±18 beats/min (n=10) and *pbx2*-MO; *pbx4*-MO embryos had 86±16 beats/min (n=9). Although it is not clear from these live observations whether abnormal heart size or shape causes the functional defects, our finding that Pbx proteins are required for normal heart function is consistent with previous studies in zebrafish (Pöpperl et al., 2000) and mice (Chang et al., 2008; Stankunas et al., 2008).

Microarray analysis identifies Pbx-dependent heart gene expression

To investigate the underlying requirements for Pbx proteins in heart development, we used Affymetrix expression microarrays to compare gene expression in *pbx2*-MO; *pbx4*-MO embryos with control siblings (injected with *pbx2*-MO mismatch control), as previously described (Maves et al., 2007). Two stages were examined: 10 somites/14 hpf, when early heart specification occurs, and 18 somites/18 hpf, when early heart differentiation occurs (Yelon et al., 1999; Glickman and Yelon, 2002). Microarray experiments on total RNA from whole embryos identified Pbx-dependent genes in all tissues (Maves et al., 2007). From the gene lists generated from the arrays, we identified the genes that are expressed in wild-type heart precursors, according to the publicly available RNA in situ database (Thisse and Thisse, 2005), or that are known to regulate vertebrate heart development (Table 1). For each of these heart genes, we performed RNA in situ hybridization or quantitative RT-PCR to confirm that these genes indeed show altered expression in *pbx2*-MO; *pbx4*-MO embryos (Table 1; Fig. 2). Embryos were co-labeled with *krox-20*, a Pbx-dependent hindbrain gene, to confirm full Pbx knock-down (Waskiewicz et al., 2002; Maves et al., 2007; Fig. 2). In *pbx2*-MO; *pbx4*-MO embryos, *tnnt2*, *ttna*, *cmlc1*, *vmhc*, *myl7*, and *lft1* all show reduced heart primordium expression, whereas *bon*, *sox32*, and *hand2* show increased expression, consistent with our array results (Table 1; Fig. 2). *aldh1a2* shows reduced somite and eye expression in *pbx2*-MO; *pbx4*-MO embryos, consistent with our array results (Table 1; Figs. 2I–J; Maves et al., 2007), but shows expanded lateral plate mesoderm expression (Figs. 2I–J). These array analyses and validations thus define the genomic expression requirements for Pbx proteins at early and

intermediate stages in heart development and support the hypothesis that Pbx proteins are required for proper heart development.

Of the Pbx-dependent heart genes (Table 1), most (*tnnt2*, *ttna*, *cmlc1*, *vmhc*, and *myl7*) are involved in cardiac contractile function and can be categorized as myocardial differentiation genes. In particular, *cardiac troponin T2* (*tnnt2*), *titin* (*ttna*), *cardiac myosin light chain-1* (*cmlc1*), *ventricular myosin heavy chain* (*vmhc*), and *myosin light polypeptide 7* (*myl7*) have all been shown to be necessary for heart muscle differentiation and function in zebrafish (Sehnert et al., 2002;Xu et al., 2002;Auman et al., 2007;Chen et al., 2008). Therefore, the disrupted heart function in *pbx2*-MO; *pbx4*-MO embryos is likely due to defective myocardial differentiation.

Pbx requirements resemble those for Hand2 in early myocardial differentiation

The reduced *vmhc* and *myl7* expression in *pbx2*-MO; *pbx4*-MO embryos resembles that seen in zebrafish *hand2* mutants, and Hand2 is required for myocardial differentiation in zebrafish (Yelon et al., 2000; Schoenebeck et al., 2007). We therefore asked whether the Pbx-dependent genes identified by our array analyses are also Hand2-dependent. We used a morpholino against *hand2* to knock down *hand2* function. We confirmed that the morpholino knock-down caused reduced *myl7* and *vmhc* expression, similar to *hand2*−/− embryos (Table 1; Yelon et al., 2000), and we also confirmed that *myl7* expression defects in *hand2*-MO embryos were rescued by *hand2* mRNA (Supplemental Fig. 1). We find that, indeed, most of the Pbx-dependent genes, in particular the myocardial differentiation genes, are also Hand2-dependent (Table 1). In addition, *hand2* expression itself is regulated by both Pbx and Hand2, as *hand2* expression increases in *pbx2*-MO; *pbx4*-MO embryos and in *hand2*-MO embryos (Table 1; Figs. 2Q-R). Zebrafish *hand2*−/− embryos show reduced *hand2* expression because the mutant alleles directly affect *hand2* transcription (Yelon et al., 2000), whereas the *hand2* MO targets Hand2 translation. Although we do not know the levels of Hand2 protein expression in *hand2*-MO or *pbx2*-MO; *pbx4*-MO embryos, we interpret the increased *hand2* expression in *hand2*-MO and in *pbx2*-MO; *pbx4*-MO embryos as indicative of decreased Hand2 activity. Thus, Pbx-deficient embryos resemble Hand2-deficient embryos in two respects: they both have similar requirements for myocardial differentiation gene expression, and they both regulate *hand2* expression.

We observe, however, two notable differences between Pbx and Hand2 regulation of the microarray-identified genes. First, *aldh1a2* expression in the lateral plate mesoderm increases in *pbx2*-MO; *pbx4*-MO embryos but is not affected in *hand2*-MO embryos (Table 1; Figs. 2I–J). Second, the endodermal genes *bon* and *sox32* show increased expression in *pbx2*-MO; *pbx4*-MO embryos but are not affected in *hand2*-MO embryos (Table 1). We address these differences in the Discussion, and, nevertheless, we conclude that our microarray analysis and validations reveal similar requirements for Pbx and Hand2 in early myocardial differentiation.

Early myocardial specification appears normal in Pbx-deficient embryos

In order to address the origins of the myocardial differentiation defects in Pbx-deficient embryos, we investigated early myocardial specification in *pbx2*-MO; *pbx4*-MO embryos. An early fate map of zebrafish cardiac progenitors at the 6–9 somite stage (Schoenebeck et al., 2007) has defined markers of anterior lateral plate mesoderm (ALPM; *gata4*, *gata5*, *tbx20*), rostral ALPM (presumptive blood and vasculature; *scl*), caudal ALPM (*hand2*), and medial-caudal ALPM (*nkx2.5*). These markers exhibit normal expression in *hand2*−/− embryos, showing that *hand2* is not needed for early cardiac specification (Schoenebeck et al., 2007). We analyzed expression of these fate-map markers in *pbx2*-MO; *pbx4*-MO embryos and *hand2*-MO embryos at 7–9 somites (Fig. 3). The *hand2* expression domain is expanded in both *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos at 7 somites (Figs. 3J–3L), similar to the

hand2 upregulation at 18 somites (Figs. 2Q–R) and further supporting the correspondence between loss of Pbx and Hand2 activities. Expression of the other early cardiac fate-map markers appears largely normal in *pbx2*-MO; *pbx4*-MO embryos or in *hand2*-MO embryos (Fig. 3 and data not shown), although *scl* expression may be subtly reduced in *pbx2*-MO; *pbx4*-MO embryos (Fig. 3G–3H). Thus, early cardiac progenitors and myocardial specification appear intact in the absence of Pbx. Furthermore, aside from *hand2*, expression of these early cardiac fate-map markers was not identified as altered in our *pbx2*-MO; *pbx4*-MO microarray experiment. This analysis shows that Pbx, like Hand2, is not required for establishing early domains of specific heart precursors but rather for subsequent myocardial differentiation.

Pbx-deficient and hand2-MO embryos exhibit similar cardiac differentiation and morphogenesis defects

We next wanted to investigate the extent of the myocardial differentiation defects in *pbx2*-MO; *pbx4*-MO embryos compared to *hand2*-MO embryos. Subsequent to the activation of myocardial differentiation genes at around the 13–18 somite stage (15.5–18 hours post fertilization, hpf; Yelon et al., 1999), the bilateral myocardial precursors migrate to the midline, merge to form the cardiac cone, and then, by 26 hpf, elongate to form the heart tube (Yelon and Glickman, 2002). Myocardial differentiation appears to be required for myocardial morphogenesis because zebrafish embryos with defective myocardial differentiation have defects in myocardial morphogenesis (Yelon et al., 1999; Reiter et al., 1999; Yelon et al., 2000). However, zebrafish loci that are required for myocardial differentiation can exhibit very different requirements for myocardial morphogenesis. For example, zebrafish *hand2* mutants show reduced myocardial precursors with defective midline migration and subsequent cardia bifida, whereas *nkx*-deficient embryos have normal midline migration but then fail to properly elongate the heart tube (Yelon et al., 2000; Trinh et al., 2005; Targoff et al., 2008). Because zebrafish *pbx2*-MO; *pbx4*-MO embryos have early myocardial differentiation defects, we wanted to determine whether they also have a characteristic myocardial morphogenesis defect. We therefore examined the expression of *myl7*, *vmhc* and *amhc* at three time points of myocardial morphogenesis: the 21 somite-stage (21s), when the myocardial precursors have merged to form the cardiac cone (Fig. 4A), 25 hpf, when the heart tube has elongated (Fig. 4D), and 42 hpf, when the heart tube has looped such that the atrium lies to the right of the ventricle (Fig. 4G). In control embryos, *myl7* labels all myocardial precursors, *vmhc* labels ventricular precursors, and *amhc* labels atrial precursors (Berdougo et al., 2003). We find that *myl7*- and *vmhc*-expressing heart precursors continue to be reduced and show delayed midline migration at 21s in both *pbx2*-MO; *pbx4*-MO embryos and *hand2*-MO embryos, while *amhc* expression is absent (Fig. 4A-C). At 25 hpf, myocardial precursors in *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos have merged abnormally at the midline and have failed to elongate into the heart tube (Fig. 4D–F). At 42 hpf, cardiac morphogenesis appears similarly abnormal in both *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos: there appear to be separate *vmhc*-expressing and *amhc*-expressing chambers, however their shapes are abnormal, appear somewhat smaller than controls, and have not looped properly (Fig. 4G–I). These analyses reveal that the *hand2*-MO phenotype does not fully correspond to the *hand2* null mutant phenotype at 25 hpf and beyond, as most *hand2*-MO embryos show delayed midline fusion of heart precursors rather than cardia bifida, or separate lateral hearts, at 25 hpf and 42 hpf (Yelon et al., 2000; also see Fig. 5). However, this suggests that the heart morphogenesis defects in fully Pbx-deficient embryos correspond to reduced Hand2 function. We also analyzed differentiated hearts of *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos compared to control embryos, using dual labeling of the atrium-specific antibody S46 with the whole heart marker MF20. We see similar results as the in situ analysis at 42 hpf, in that both chambers are reduced and similar morphogenesis defects occur in *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos (data not shown). Taken together, our analyses show that *pbx2*-MO; *pbx4*-MO embryos have both

myocardial differentiation and morphogenesis defects that closely resemble those seen in embryos with reduced Hand2 function.

To further investigate and compare the myocardial differentiation defects in *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos, we examined expression of *natriuretic peptide precursor A* (*nppa*), a myocardial differentiation gene that is regulated by Hand2 in mice (Thattaliyath et al., 2002). *nppa* expression in the zebrafish heart initiates around 24 hpf (data not shown). By 28 hpf, *nppa* fails to be activated in either *pbx2*-MO; *pbx4*-MO or *hand2*-MO embryos (Fig. 4J). Even by 42 hpf, *nppa* expression remains significantly downregulated in *pbx2*-MO; *pbx4*-MO or *hand2*-MO embryos (data not shown). These findings thus further corroborate the correspondence between the requirements for Pbx and Hand2 in myocardial differentiation.

Pbx and Hand2 genetically interact in cardiac morphogenesis

In two respects, our results strongly suggest that Pbx interacts with Hand2 in myocardial differentiation. First, most of the Pbx-regulated heart genes identified in our microarray analysis are also Hand2-regulated (Table 1), and second, the myocardial differentiation and morphogenesis phenotype of *pbx2*-MO; *pbx4*-MO embryos closely resembles that of zebrafish *hand2* knock-down embryos (Fig. 4). We therefore wanted to determine whether Pbx and Hand2 exhibit genetic interactions in myocardial differentiation by simultaneously knocking down the functions of both factors using combinations of *pbx2; pbx4* and *hand2* morpholinos. We envisioned three possible results for *pbx2*-MO; *pbx4*-MO; *hand2*-MO embryos: 1. They may show a more severe phenotype than *hand2* null embryos. This result may occur if Pbx and Hand2 act additively and independently to regulate myocardial differentiation. 2. They may show a less severe phenotype than *pbx2*-MO; *pbx4*-MO embryos. Because *hand2* expression is upregulated in *pbx2*-MO; *pbx4*-MO embryos (Fig. 2), blocking *hand2* function in *pbx2*-MO; *pbx4*-MO embryos may act to rescue the *pbx2*-MO; *pbx4*-MO phenotype. 3. They may resemble *hand2* null embryos, which exhibit cardia bifida at 25 hpf and 42 hpf and are more severe than *pbx2*-MO; *pbx4*-MO embryos or *hand2*-MO embryos (Yelon et al., 2000; Fig. 5E). This result may occur if Pbx provides no further function in heart development than facilitating Hand2 function. Whereas knock down of either Pbx or Hand2 function led to delayed and abnormal heart morphogenesis (Figs. 5B–C, 5F–G), as described above for Figure 4, when we knocked down both Pbx and Hand2 function, we observed that, at both 24 hpf and 42 hpf, most *pbx2*-MO; *pbx4*-MO; *hand2*-MO embryos exhibit cardia bifida (Figs. 5D, 5F–G). With simultaneous Pbx and Hand2 knock-down, we did not observe any evidence for a more severe heart phenotype or loss of *myl7* or *vmhc* expression than that observed in *hand2* null embryos, nor did we observe any evidence that Hand2 knock-down rescued the Pbx knock-down (Fig. 5 and data not shown). The simultaneous Pbx and Hand2 knock-down thus corresponds to the *hand2* null phenotype, supporting an interaction between Pbx and Hand2 in promoting myocardial differentiation and morphogenesis.

Pbx proteins bind to the myl7 promoter in vitro

We next wanted to determine whether Pbx directly regulates expression of cardiac muscle differentiation genes. Because Pbx recruits Myod to the *Myogenin* promoter (Berkes et al., 2004), Pbx may also recruit Hand2 to myocardial differentiation gene promoters. The zebrafish *myl7* promoter has been partially characterized through *gfp* reporter analyses in transgenic embryos (Huang et al., 2003). A fragment containing 870bp of 5′ upstream regulatory sequence mimics endogenous *myl7* expression (Huang et al., 2003). Using the binding-site prediction program Match1.0, we identified a potential Pbx binding site at −778bp that appears to more closely resemble the Pbx/Meis consensus binding site than the conserved Pbx/Meis site in the *myogenin* promoter (Fig. 6A; Berkes et al., 2004). We tested whether this site is capable of binding Pbx4, together with cofactor Meis3, through gel-shift assays (EMSA), similar to how we have assayed Pbx/Meis binding in previous studies (Berkes et al., 2004; Maves et al.,

2007). We find that Pbx4/Meis3 binding to the −778/−752bp oligo is inhibited by unlabeled wild-type oligo but not by an oligo containing a mutated Pbx binding site (Fig. 6B), showing that Pbx proteins can bind directly to the *myl7* promoter in vitro. As a control, we observe similar results with an oligo containing the *myogenin* Pbx/Meis site (Fig. 6B). Because there is a non-canonical E-box sequence adjacent to the Pbx binding site in the *myl7* promoter, similar to the non-canonical E-box adjacent to the Pbx site in the *myogenin* promoter (Fig. 6A; Berkes et al., 2004), we wanted to address whether Hand2 protein interacts with Pbx at the *myl7* promoter. We performed additional gel shift assays but were unable to observe any binding of Hand2 protein or Hand2 combined with E12 protein to the −778/−752 oligo, nor were we able to observe any cooperative binding of Hand2/E12 with Pbx/Meis to the −778/−752 oligo (data not shown). Thus, the mechanism by which Pbx and Hand2 proteins interact at myocardial differentiation gene promoters remains to be determined. Although we do not yet know whether Pbx proteins bind *myl7* or other myocardial promoters in vivo, our EMSA results strongly support a direct role for Pbx regulation of myocardial differentiation gene expression.

Discussion

In this work, we test the hypothesis that Pbx proteins regulate cellular differentiation by modulating bHLH factor activity, in particular by testing whether Pbx proteins interact with Hand2 in cardiac muscle differentiation. Our data reveal that Pbx proteins in zebrafish are necessary for proper heart development and function by regulating gene expression required for myocardial differentiation. Our results show that Pbx proteins have similar requirements to Hand2 in myocardial differentiation and morphogenesis and, furthermore, demonstrate a genetic interaction between Pbx and Hand2. Thus, our findings underscore the critical roles of Pbx proteins in promoting muscle cell differentiation and suggest that Pbx proteins may modulate bHLH factor activity more broadly than previously appreciated.

Our results demonstrate that Pbx and Hand2 are required for the expression of similar cardiac muscle differentiation genes and show similar requirements for cardiac morphogenesis. However, we observed two notable differences between Pbx and Hand2 regulation of the microarray-identified Pbx-dependent genes. First, *aldh1a2* expression in the lateral plate mesoderm increases in *pbx2*-MO; *pbx4*-MO embryos but is not affected in *hand2*-MO embryos (Table 1; Fig. 2). *aldh1a2* encodes an enzyme responsible for the synthesis of retinoic acid. Because retinoic acid normally restricts the number of cardiac progenitor cells (Keegan et al., 2005), increased *aldh1a2* expression in *pbx2*-MO; *pbx4*-MO embryos could lead to reduced myocardial differentiation. However, the increased *aldh1a2* expression in *pbx2*-MO; *pbx4*-MO embryos occurs during somitogenesis, while retinoic acid acts on cardiac progenitors during gastrulation (Keegan et al., 2005). Second, the endodermal genes *bon* and *sox32* show increased expression in *pbx2*-MO; *pbx4*-MO embryos but are not affected in *hand2*-MO embryos (Table 1). In zebrafish, loss of *bon*, which encodes a Mix homeodomain factor, or *sox32* function leads to delayed cardiac morphogenesis and cardia bifida (Kikuchi et al., 2000;Alexander et al., 1999). Thus, the connection between increased endodermal marker expression in *pbx2*-MO; *pbx4*-MO embryos and cardiac differentiation and morphogenesis is not immediately clear. In spite of these differences between Pbx and Hand2 gene regulation, we conclude that our microarray analysis and validations reveal similar requirements for Pbx and Hand2 in early myocardial differentiation and suggest that they act together in a common pathway.

In addition to Hand2, other transcription factors could potentially interact with Pbx proteins in heart development. GATA and Nkx family factors are expressed in myocardial precursors and play critical roles in early myocardial differentiation and morphogenesis (Olson, 2006; Schoenebeck and Yelon, 2007). However, zebrafish *gata5* mutants have early defects in *gata4* and *nkx2.5* expression and thus have early myocardial specification defects that are not

observed in Pbx-null embryos (Reiter et al., 1999; Fig. 3). Zebrafish Nkx-deficient embryos have heart tube extension defects but do not exhibit early myocardial differentiation defects (Targoff et al., 2008). Pbx proteins are well-known cofactors for Hox proteins (Moens and Selleri, 2006), so Pbx proteins may regulate heart development through these factors. The full roles for Hox proteins in cardiac development are not known, but, in zebrafish, Hoxb5b negatively regulates cardiomyocyte numbers (Waxman et al., 2008). If Pbx acts with Hoxb5b in this role, the *hoxb5b* knock-down phenotype (increased *amhc*-expressing cardiomyocytes; Waxman et al., 2008) is not consistent with the reduced *amhc* expression in Pbx-null embryos (Fig. 4). Thus, although Pbx may function with additional factors in myocardial differentiation, the requirements for Pbx most closely correspond with those for Hand2, underscoring the interaction between these two factors.

The advantage of using zebrafish in these studies is that we characterized the Pbx-null embryonic heart phenotype by knocking down both Pbx2 and Pbx4. In zebrafish, *pbx4* likely plays a major role relative to *pbx2* during early heart development. Zebrafish zygotic *pbx4* mutant embryos have been reported to have impaired circulation and pericardial edema at 5 days post fertilization (Pöpperl et al., 2000), and we find that *pbx4*−/− embryos have reduced *myl7* and *vmhc* expression and cardiac morphogenesis defects (L.M., unpublished data), although not as severe as *pbx2*-MO; *pbx4*-MO embryos. *Pbx* gene deficiencies in mice were recently shown to cause cardiac defects that resemble human CHDs (Chang et al., 2008; Stankunas et al., 2008). In particular, loss of specific *Pbx* genes led to distinct cardiac outflow tract defects, with *Pbx1* playing a major role (Stankunas et al., 2008). The role of Pbx in cardiac outflow tract development likely reflects Pbx function within cardiac neural crest cells (Chang et al., 2008). These studies did not address whether early myocardial differentiation is affected in *Pbx*-deficient mice, thus, it is not yet clear whether Pbx proteins have conserved roles in zebrafish and mammalian heart development. In order to gain full insight into the potential roles of Pbx in CHD in humans, it will be necessary to characterize the requirements for *Pbx* genes in early mouse heart development, in particular to test whether *Pbx* genes are required for early myocardial differentiation. The ability to generate mouse embryos deficient for multiple alleles of *Pbx1*, *Pbx2*, and *Pbx3* (Capellini et al., 2006; Stankunas et al., 2008) will greatly contribute to analyzing Pbx requirements in early heart development.

Our zebrafish studies suggest that *Pbx* genes may interact with *Hand1* or *Hand2* in mammalian heart development, but this is not yet explored. *Pbx* genes and *Hand2* both function in limb development in zebrafish (Pöpperl et al., 2000; Yelon et al., 2000) and in mice (Charite et al., 2000; Capellini et al., 2006). However, whereas Pbx and Hand2 act together in the zebrafish heart, in the mouse limb they appear to function in parallel pathways (Capellini et al., 2006), and *Hand2* expression is not dependent on Pbx in the mouse limb (Capellini et al., 2006) as it is in the zebrafish heart (this work). Future examinations of Pbx and Hand2 functions in the heart or limb would benefit from complementary approaches in zebrafish and mouse.

Our studies reveal several similarities between the roles of Pbx in cardiac muscle development and in skeletal muscle development (this work and Maves et al., 2007). In both muscle systems: 1. Pbx is needed for muscle differentiation and contractile gene expression, 2. Pbx is needed for proper initiation of muscle gene activation, because Pbx-dependent genes eventually turn on, 3. Pbx genetically interacts with a bHLH factor, Myod or Hand2, 4. Expression of the bHLH genes *myod* or *hand2* increases (while their activity decreases) in Pbx-null embryos, and 5. Pbx-null embryos have similar, but not as severe, differentiation phenotypes compared to complete loss of Myod or Hand2. These similarities strongly suggest that Pbx may have comparable interactions with Hand2 in cardiac development as Pbx does with Myod in skeletal muscle.

These connections between Pbx and bHLH factors support the idea of a core developmental program regulating the transcriptional control of contractile muscle cell differentiation, in which Pbx proteins facilitate the ability of bHLH factors to recognize and activate a set of myosins and other genes needed for differentiation. We speculate that Pbx/bHLH interactions could function even more broadly in cellular differentiation, in contexts such as neuronal differentiation and pancreas development, where Pbx and the NeuroD family of bHLH factors both play important roles (Dutta et al., 2001; Waskiewicz et al., 2002; Westerman et al., 2003).

In skeletal muscle, Pbx and Myod exhibit both genetic as well as biochemical interactions and cooperatively bind in a complex together on the *Myogenin* promoter, which contains Pbx and Myod binding sites (Berkes et al., 2004; Maves et al., 2007). By binding to Myod target gene promoters, Pbx is thought to act as a signal for Myod to recognize and activate those promoters (Berkes et al., 2004). While we were able to demonstrate that Pbx proteins can bind the myocardial *myl7* promoter in vitro (Fig. 6), we were unable to demonstrate that Hand2 can bind the *myl7* promoter, either in the absence or presence of Pbx. Several studies have revealed that Hand proteins may activate promoters independently of DNA binding by physically interacting with transcriptional complexes (Dai et al., 2002; McFadden et al., 2002; Thattaliyath et al., 2002; Morin et al., 2005). Also, Liu et al. (Liu et al., 2009) have recently shown that mice lacking the DNA-binding domain of Hand2 have normal early heart development. Whether Pbx/Myod and Pbx/Hand2 interactions share common biochemical mechanisms remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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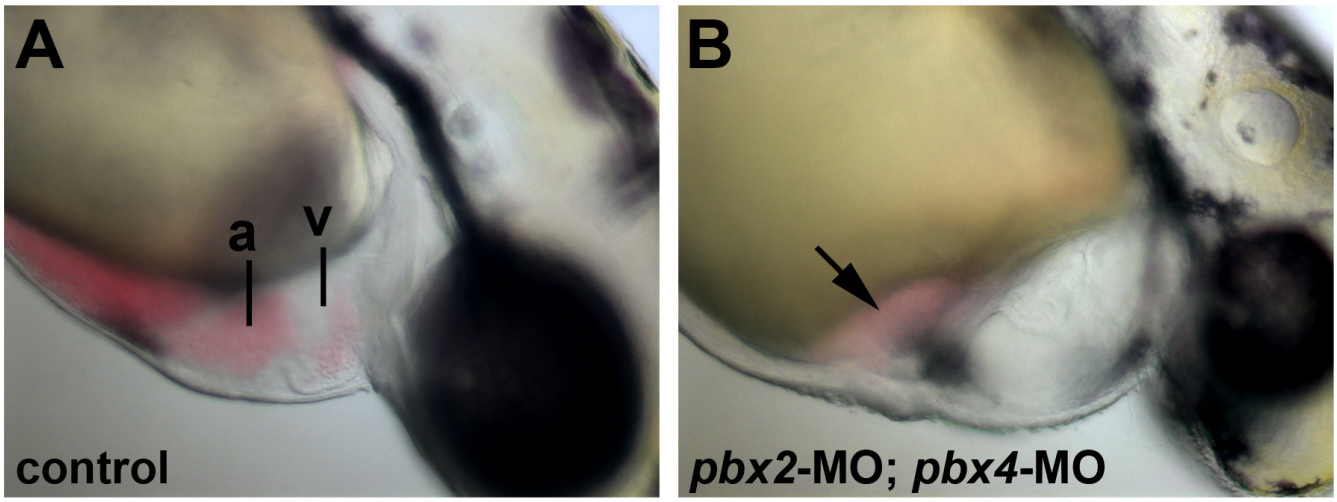

Fig. 1.

Defective heart development in *pbx2*-MO; *pbx4*-MO embryos. (A–B) Lateral views of live (A) wild-type and (B) *pbx2*-MO; *pbx4*-MO embryos at 48 hpf. *pbx2*-MO; *pbx4*-MO embryos show mild pericardial edema and blood pooled near the atrium (arrow). Anterior is to the right. a, atrium. v, ventricle.

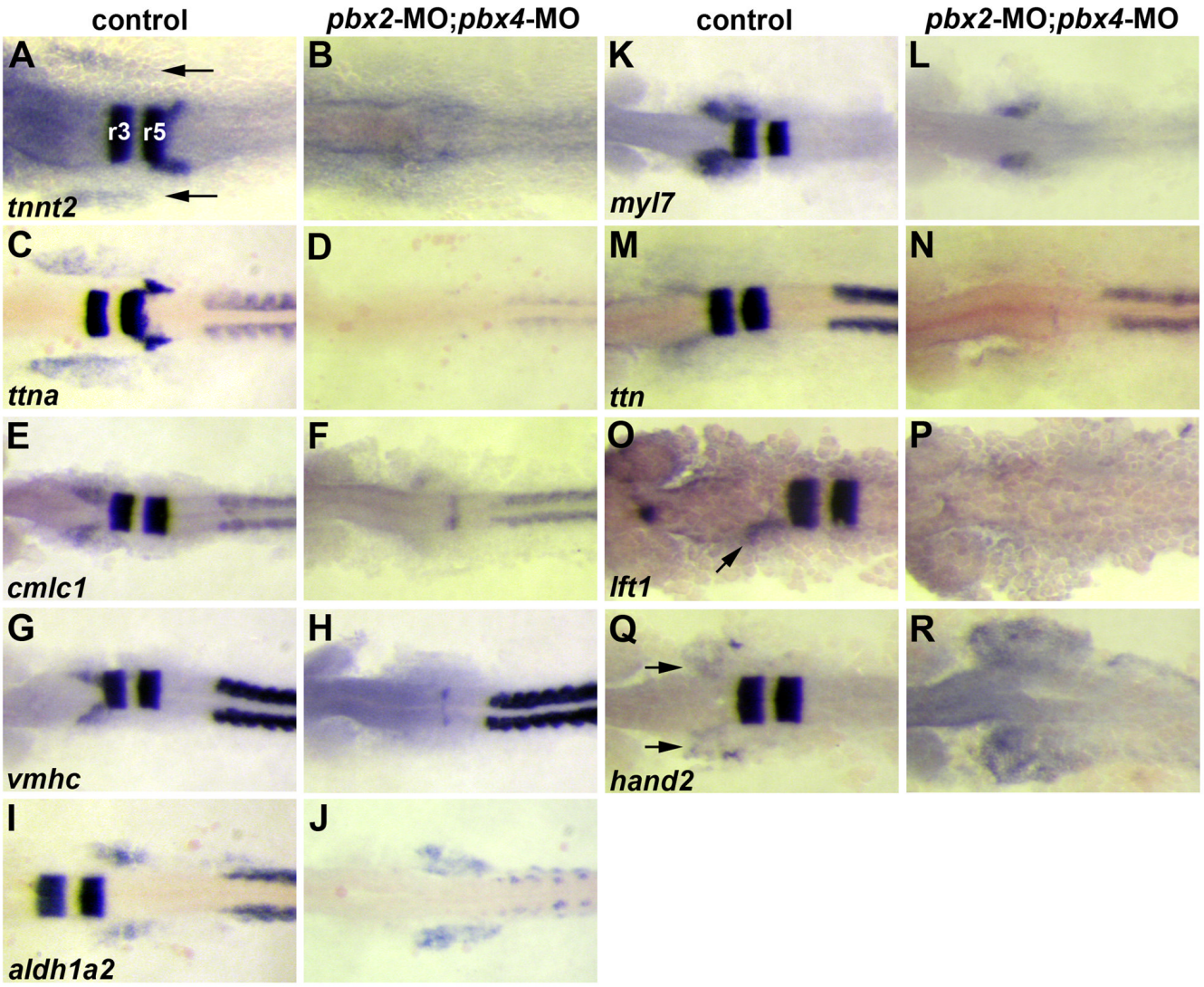

Fig. 2.

Validation of microarray-identified Pbx-dependent heart genes. (A–R) RNA in situ expression of genes from Table 1 in (A,C,E,G,I,K,M,O,Q) wild-type control or (B,D,F,H,J,L,N,P,R) *pbx2*-MO; *pbx4*-MO embryos. (A–D) are at 10 somite stage (10s). (E–N, Q–R) are at 18s. (O–P) are at 20s to achieve more robust expression of *lft1* in controls. *krox-20*, labeled in hindbrain rhombomeres r3 and r5 in (A), is included in all in situs to control for loss of Pbx. The wild-type heart primordium expression domains of *tnnt2*, *ttna*, *cmlc1*, *vmhc*, *myl7*, *lft1* and *hand2* are just anterior-lateral to r3 *krox-20* expression (arrows in A,O,Q). n≥10 for each marker in control or *pbx2*-MO; *pbx4*-MO. Embryos are shown in dorsal view, anterior towards the left.

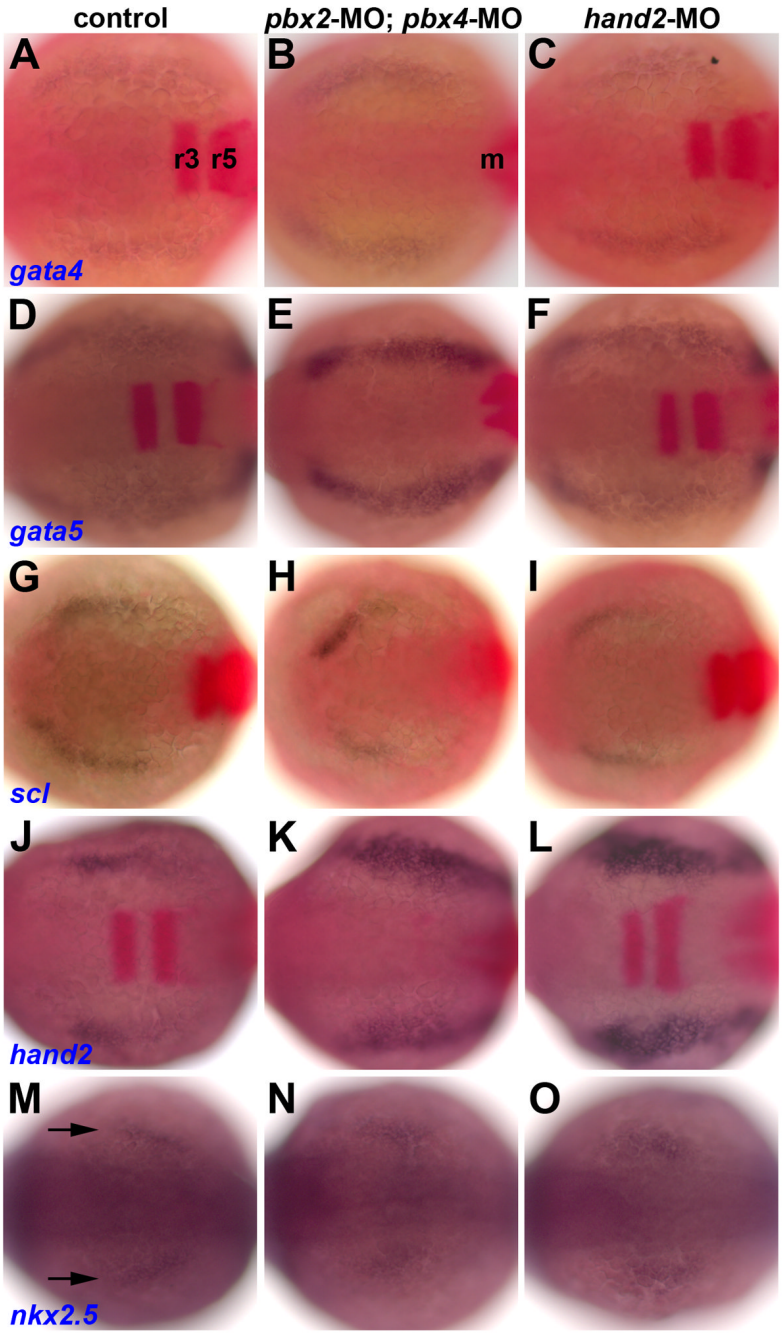

Fig. 3.

Early heart specification is normal in *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos. (A–O) RNA in situ expression at the 7–9 somite stage of (A–C) *gata4*, (D–F) *gata5*, (G–I) *scl*, (J–L) *hand2*, and (M–O) nkx2.5, all in blue, in (A,D,G,J,M) control, (B,E,H,K,N) *pbx2*-MO; *pbx4*-MO, or (C,F,I,L,O) *hand2*-MO embryos. Also included in (A–L), in red, are *krox-20*, labeled in (A) in r3 and r5 in the hindbrain, and *myod*, labeled m in (B) and used to confirm somite staging, but is not visible here in all embryos. Arrows in (M) mark *nkx2.5* expression. n≥13 for each marker in control, *pbx2*-MO; *pbx4*-MO, or *hand2*-MO. Embryos are shown in dorsal view, anterior towards the left.

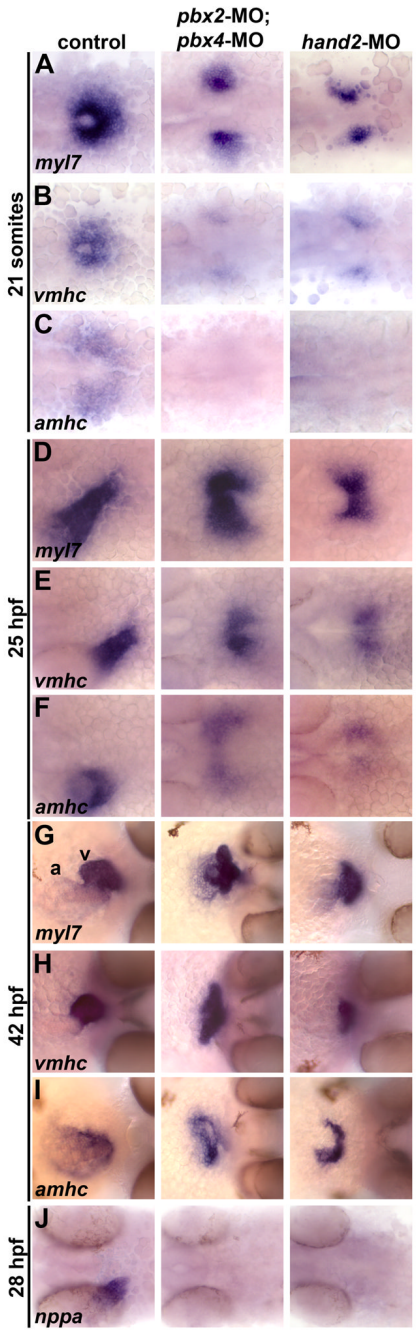

Fig. 4.

Pbx-deficient and *hand2*-MO embryos exhibit similar cardiac differentiation and morphogenesis defects. (A–J) RNA in situ expression of (A,D,G) *myl7*, (B,E,H) *vmhc*, (C,F,I) *amhc*, and (J) *nppa* at (A–C) 21 somites, (D–F) 25 hours post fertilization (hpf), (G–I) 42 hpf, and (J) 28 hpf. In control embryos shown, *myl7* labels all myocardial precursors, *vmhc* labels ventricular precursors, and *amhc* labels atrial precursors (Berdougo et al., 2003). Ventricle (v) and atrium (a) are labeled in (G). n≥20 for each marker in control, *pbx2*-MO; *pbx4*-MO, or *hand2*-MO. (A–F) Embryos are shown in dorsal view, anterior towards the left. (G-I) Embryos are shown in frontal views, dorsal towards the right.

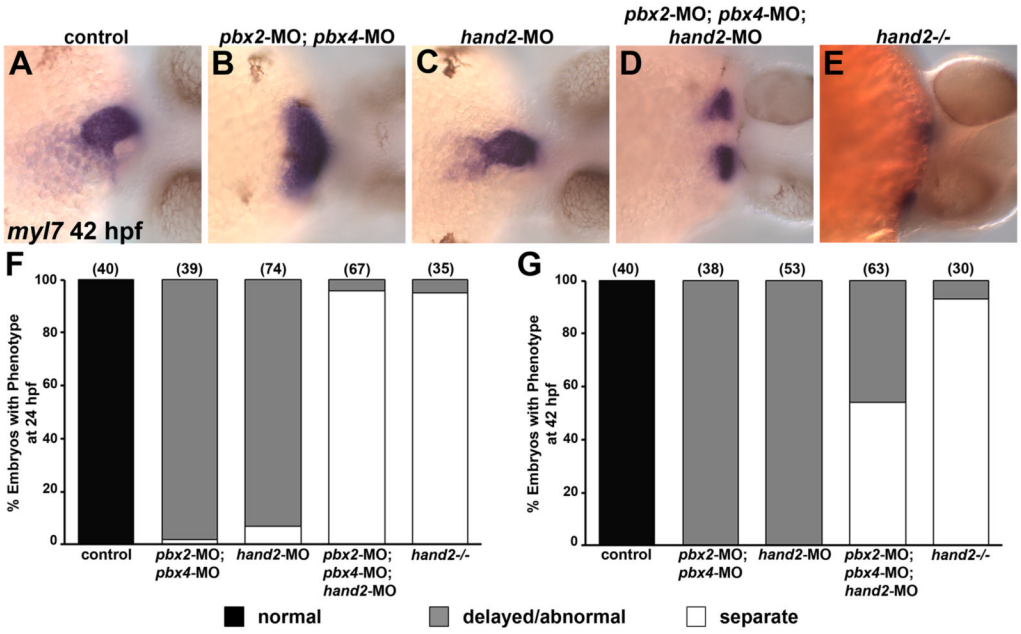

Fig. 5.

Pbx and Hand2 genetically interact in cardiac morphogenesis. (A–E) RNA in situ expression of *myl7* at 42 hpf in (A) control, (B) *pbx2*-MO; *pbx4*-MO, (C) *hand2*-MO, (D) *pbx2*-MO; *pbx4*-MO; *hand2*-MO, or (E) *hand2*−/− embryos. Embryos are shown in frontal views, dorsal towards the right. (F–G) Quantification of phenotypes observed using *myl7* expression at (F) 24 hpf or (G) 42 hpf. Normal phenotypic classes (black) correspond to tube (24 hpf) or looped (42 hpf) patterns depicted in Fig. 4D and Fig. 5A, respectively. Delayed or abnormal phenotypic classes (gray) correspond to images shown for *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos in Fig. 4D (24 hpf) or Fig. 5B–C (42 hpf). The separate, or cardia bifida, phenotypic classes (white) correspond to two *myl7* domains and resemble those described for *pbx2*-MO; *pbx4*-MO and *hand2*-MO embryos in Fig. 4A or for *pbx2*-MO; *pbx4*-MO; *hand2*-MO and *hand2*−/− embryos in Fig. 5D–E. Numbers in parentheses denote numbers of embryos. *hand2*−/−embryos were derived from clutches that contained about 25% mutant embryos; their *hand2*+/+ and +/− siblings showed normal *myl7* expression.

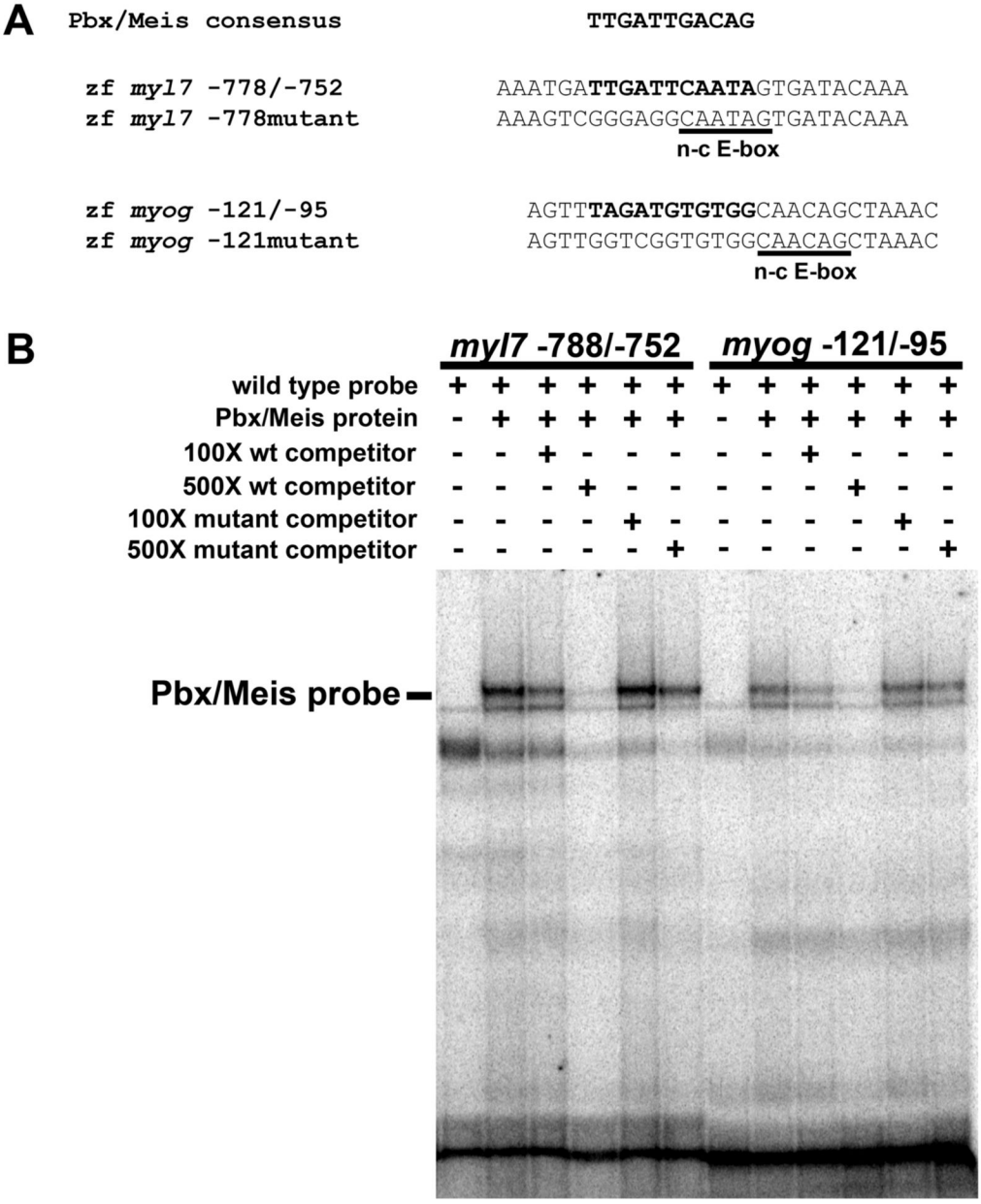

Fig. 6.

Pbx proteins bind the *myl7* promoter in vitro. (A) Pbx/Meis consensus binding site sequence (Berkes et al., 2004) aligned relative to putative Pbx binding site sequences (highlighted in bold) in the zebrafish *myl7* promoter and *myogenin* (*myog*) promoter. The non-canonical E-box (n-c E-box) sites are underlined. Also shown are sequences of mutated Pbx binding sites. Sequences represent oligos used in EMSA. (B) Pbx4 and Meis3 proteins were generated by in vitro translation using a reticulocyte lysate and subjected to EMSA with the specified *myl7* or *myog* probes. Pbx/Meis-bound probe band is indicated. The non-specific bands were observed in multiple gel shifts. Addition of unlabeled *myl7* −778/−752 probe or *myog* −121/−95 probe decreases binding of Pbx/Meis, whereas addition of mutant unlabeled probes does not.

Table 1

Microarray-identified Pbx-dependent heart genes

Gene Symbol	Affymetrix ID number	Fold change at 10s (controlMO: *pbx2*-MO; *pbx4*-MO	Wild-type expression at 10s	RNA in situ expression in *pbx2*-MO; *pbx4*- MO at 10s	RNA in situ expression in *hand2*−/− or *hand2*- MO at 10s
tnnt2	Dr.14260.1.S1_a_at	1.55	Heart primordium	Reduced	Reduced
ttna	Dr.4681.1.A1_at	1.53	Heart primordium; somites	Reduced ([1])	Not affected
bon	Dr.8084.1.S1_at	−1.87	Not expressed	10X increase over control (qRT-PCR)	Similar to control (qRT-PCR)
sox32	Dr.12573.1.S1_at	−1.88	Endoderm	3X increase over control (qRT-PCR)	Similar to control (qRT-PCR)
Gene Symbol	**Affymetrix ID number**	**Fold change at 18s (controlMO: *pbx2*-MO; *pbx4*-MO**	**Wild-type expression at 18s**	**RNA in situ expression in *pbx2*-MO; *pbx4*- MO at 18s**	**RNA in situ expression in *hand2*−/− or *hand2*- MO at 18s**
cmlc1	Dr.8379.1.S1_at	3.62	Heart; somites	Heart domain reduced	Heart domain reduced
vmhc	Dr.10607.1.A1_at	3.20	Heart; somites	Heart domain reduced ([1])	Reduced ([2])
aldh1a2	Dr.5206.1.S1_at	2.26	Lateral plate mesoderm (LPM); somites; eye	LPM domain increased; eye and somite domains reduced; increased in tail bud ([1])	Not affected
myl7	Dr.642.1.S1_at	1.90	Heart	Reduced	Reduced ([2])
ttna	Dr.4681.1.A1_at	1.85	Heart; somites	Reduced ([1])	Heart domain reduced
lft1	Dr.8208.1.S1_at	1.60	Heart; forebrain; notochord	Reduced in heart and forebrain	Reduced in heart
hand2	Dr.8328.1.S1_at	−1.70	Heart; ventral mesoderm	Increased	Reduced in *hand2*−/− ([2]); Increased in *hand2*-MO
sox32	Dr.12573.1.S1_at	−2.05	Not expressed	5X increase over control (qRT-PCR)	Similar to control (qRT-PCR)

[1] Maves et al., 2007;

[2] Yelon et al., 2000