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Pbx homeodomain proteins direct Myod activity to promote fast-muscle differentiation

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The basic helix-loop-helix (bHLH) transcription factor Myod directly regulates gene expression throughout the program of skeletal muscle differentiation. It is not known how a Myod-driven myogenic program is modulated to achieve muscle fiber-type-specific gene expression. Pbx homeodomain proteins mark promoters of a subset of Myod target genes, including myogenin (*Myog*); thus, Pbx proteins might modulate the program of myogenesis driven by Myod. By inhibiting Pbx function in zebrafish embryos, we show that Pbx proteins are required in order for Myod to induce the expression of a subset of muscle genes in the somites. In the absence of Pbx function, expression of *myog* and of fast-muscle genes is inhibited, whereas slow-muscle gene expression appears normal. By knocking down Pbx or Myod function in combination with another bHLH myogenic factor, Myf5, we show that Pbx is required for Myod to regulate fast-muscle, but not slow-muscle, development. Furthermore, we show that Sonic hedgehog requires Myod in order to induce both fast- and slow-muscle markers but requires Pbx only to induce fast-muscle markers. Our results reveal that Pbx proteins modulate Myod activity to drive fast-muscle gene expression, thus showing that homeodomain proteins can direct bHLH proteins to establish a specific cell-type identity.

KEY WORDS: Myod, Pbx, Muscle, Zebrafish

INTRODUCTION

Basic helix-loop-helix (bHLH) transcription factors are crucial regulators, in some instances referred to as 'master regulators', of cell-type identities. Among the bHLH proteins, the Myod family drives skeletal myogenesis, similar to the regulation of neurogenesis by Neurod proteins and lymphocyte development by E proteins (Tapscott, 2005; Lee, 1997; Quong et al., 2002). Homeodomain transcription factors have also been termed 'master regulators', but typically of organ or positional identities. For example, the Hox proteins control segmental identities, and Pax6 controls eye development (Pearson et al., 2005; Gehring, 1996). How are positional-identity programs linked with cell-type-identity programs, so that neurons in the eye acquire a different phenotype to neurons in the brain, or that neurons in one hindbrain segment differ from those in another segment? Although the mechanism is not known, one possibility is that positional-identity factors modulate the activity of cell-type-identity factors. As suggested by Westerman et al. (Westerman et al., 2003), homeodomain proteins might act in their region of expression to directly modulate the activity of bHLH proteins and thus confer specific characteristics on a cell-type identity. Although synergistic interactions of bHLH and homeodomain proteins have been described at some promoters (Westerman et al., 2003), the model in which homeodomain proteins can instruct the bHLH proteins to modulate a cell-type program to generate, for example, a particular type of neuron or muscle cell has not yet been demonstrated.

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Previous demonstrations of molecular interactions between the bHLH Myod protein and the homeodomain proteins Pbx and Meis suggest that skeletal myogenesis might be a good system to directly test the role of homeodomain proteins in modulating bHLH-driven cell-type programs (Knoepfler et al., 1999; Berkes et al., 2004). Furthermore, myogenesis is a model system in which to study the acquisition of cellular phenotype diversity; for example, the generation of different fiber types. Skeletal myogenesis in vertebrate embryos is coordinated by the bHLH transcription factors Myod, Myf5, Myog and Mrf4 (Buckingham, 2001). Myod is sufficient to convert fibroblasts and other non-muscle cells into skeletal muscle (Weintraub et al., 1989). Myod directly activates the expression of multiple additional transcription factors, including Myog, and acts in a feed-forward mechanism in cooperation with those factors to directly activate muscle genes expressed later in the differentiation program (Penn et al., 2004; Cao et al., 2006). At a portion of these later target genes, Myod appears necessary to initiate chromatin remodeling before other transcription factors, such as Myog, can bind to the promoters (Cao et al., 2006). Therefore, Myod has a crucial function of identifying and remodeling the target genes that will subsequently be available for activation by other factors. Because Myod directly regulates a broad suite of muscle differentiation genes, one question that arises is how does Myod correctly regulate promoters used in different skeletal muscle types, such as fast- or slow-twitch muscle?

Mutations of the histidine- and cysteine-rich domain or the C-terminal helix III domain of Myod prevent full activation of a subset of Myod target genes and also prevent cooperative DNA binding with Pbx and Meis proteins on specific Myod target promoters, including that of *Myog* (Berkes et al., 2004). Because Pbx and Meis proteins are present on some of these promoters prior to Myod expression, we suggested that a Pbx-Meis complex might mark a subset of genes for Myod activation (Berkes et al., 2004; de la Serna et al., 2005). However, mutations in the Myod protein might alter interactions with additional factors, and a requirement for Pbx-Meis in skeletal myogenesis has not yet been demonstrated. Thus, it

remains to be determined whether a Pbx-Meis complex is necessary for Myod activity at *Myog* or other specific genes in vivo and whether this subset of genes regulates a specific aspect of muscle development.

Zebrafish embryos provide an attractive biological system in which to test the role of Pbx proteins in skeletal myogenesis. Pbx (and Meis) proteins are TALE (three amino acid loop extension)-class homeodomain proteins that are best characterized as cofactors for Hox proteins. In mice, overlapping expression and functional redundancy among the Pbx genes complicates the analysis of their requirements, and skeletal muscle defects have not been reported for knock-outs of individual Pbx genes (Moens and Selleri, 2006). Zebrafish have five Pbx genes, but only *pbx2* and *pbx4* are expressed during the period of early myogenesis (Pöpperl et al., 2000; Waskiewicz et al., 2002). Knock-down of both *pbx2* and *pbx4* in zebrafish results in severe hindbrain-patterning defects that reflect the role of Pbx proteins as Hox cofactors (Waskiewicz et al., 2002), but skeletal muscle development was not assessed. The Pbx-Meis binding site in the mouse *Myog* promoter is conserved in the zebrafish *myog* gene (Berkes et al., 2004), providing an opportunity to test the developmental role of Pbx in myogenesis. Here, we demonstrate that Pbx proteins are necessary for the timely activation of *myog* by Myod, demonstrating that Pbx has a necessary role in marking the *myog* gene for efficient activation during development. Furthermore, we demonstrate that Pbx proteins facilitate Myod activity to drive the expression of a subset of genes necessary for fast-muscle differentiation, thus showing that homeodomain proteins can direct bHLH proteins to establish a specific cell-type identity.

MATERIALS AND METHODS

Zebrafish stocks

Zebrafish (*Danio rerio*) were raised and staged as previously described (Westerfield, 2000). Time (hpf) refers to hours post-fertilization at 28.5°C. In some cases, embryos were raised for periods at room temperature, at approximately 25°C. The wild-type stock used was an AB/WIK hybrid. For microarray analyses, the AB stock was used. The *pbx4* mutant line has been described (Pöpperl et al., 2000).

Morpholino and mRNA injections

Morpholino injections were performed, and working concentrations were determined, as previously described (Maves et al., 2002). Morpholinos (shown 5′ to 3′) were used at the following working concentrations: *pbx2*-MO2 (Waskiewicz et al., 2002), CCGTTGCCTGTGATGGGCTGCTGCG, 0.25 mg/ml; *pbx2*-MO3, GCTGCAACATCCTGAGCACTACATT, 0.5 mg/ml; *pbx2*-MO3 five-base mismatch control (mismatched bases shown in lowercase), GCTcCAAgATCCTcAcCAgTACATT, 0.5 mg/ml; *pbx4*-MO1, AATACTTTTGAGCCGAATCTCTCCG, 0.5 mg/ml; *pbx4*-MO2, CGCCGCAAACCAATGAAAGCGTGTT, 0.5 mg/ml; *myod*-splMO E1I1, AATAAGTTTCTCACAATGCCATCAG, 5 mg/ml; *myod*-splMO E2I2, TTTCGAGCAAACTTACCATTTGGTG, 2.5 mg/ml; *myod*-MO1, GCAAGAAATGTACTTGAATGTTTCC, 0.5 mg/ml; *myod*-MO2, GGAATAGTAAGACAAAGTCCTTCAG, 5 mg/ml; *myf5*-MO1, GATTGGTTTGGTGTTGAAGGTTTCT, 0.25 mg/ml; *myf5*-MO2, GATCTGGGATGTGGAGAATACGTCC, 0.25 mg/ml. *pbx2*-MO2, *pbx2*-MO3, *pbx4*-MO1 and *pbx4*-MO2 were combined, *myod*-MO1 and *myod*-MO2 were combined, and *myf5*-MO1 and *myf5*-MO2 were combined in order to knock down their respective gene products. Embryos that are knocked-down for *pbx2* and *pbx4* function show a developmental delay of approximately two somites during somitogenesis stages, comparable to maternal-zygotic *pbx4*$^{-/-}$*; pbx2*-MO embryos (see Fig. S2 in the supplementary material; C.B.M., unpublished observations). We somite-stage-matched sibling control and MO-treated embryos when collecting embryos for staining or for RNA or protein analyses.

Injections of *exd* or *shh* (*shha* – ZFIN) mRNA were performed as previously described (Pöpperl et al., 2000; Barresi et al., 2000).

RNA in situ hybridization and immunocytochemistry

RNA in situ hybridizations were performed as previously described (Maves et al., 2002). The following cDNA probes were used: *myod* (Weinberg et al., 1996); *krox-20* [*egr2b* – Zebrafish Information Network (Oxtoby and Jowett, 1993)]; *myog* (Weinberg et al., 1996); *myhc4* (EST fb27a08); *aldh1a2* (Begemann et al., 2001); *mylz2* (Xu et al., 2000); *chrna1* (Sepich et al., 1998); *tmem161a* (IMAGE:7149790); *ttnl* (EST eu247); *atp2a1* (EST fc22f07); *srl* (EST fb94b10); *vmhc* (Yelon et al., 1999); *smyhc1* (Bryson-Richardson et al., 2005); and *cxcr4a* (EST cb824, Zebrafish International Resource Center). We subcloned the *desmin* EST cb290 (Zebrafish International Resource Center) into pCRII-TOPO, which was linearized with *Bam*HI and transcribed with T7 to make the antisense *desmin* probe.

Whole-embryo immunostaining was performed with the following primary antibodies: anti-pan zebrafish Pbx, 1:500 (Pöpperl et al., 2000); anti-Myf5, 1:100 [Santa Cruz, C-20, sc-302 (Hammond et al., 2007)]; anti-myosin heavy chain F59, 1:10 [supernatant (Devoto et al., 1996)]; anti-myosin heavy chain MF20, 1:10 [supernatant (Bader et al., 1982)]; and anti-fast myosin light chain F310, 1:10 [supernatant (Hamade et al., 2006)]. F59, F310 and MF20 antibodies were developed by F. E. Stockdale and D. A. Fischman and were obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa. Stainings were performed as previously described (Feng et al., 2006) with the following modifications: for anti-Pbx staining, embryos were fixed in 4% PFA at 4°C for 4 hours and methanol dehydration was omitted; for anti-Myf5 (Myod) staining, embryos were fixed in 4% PFA for 1 hour at room temperature, methanol dehydration was omitted, and washes and incubations were done in PBS+1% Triton-X; for F59, F310 and MF20 staining, secondaries (Southern Biotech) used were goat anti-mouse IgG1-FITC (1:100, F59 and F310) and goat anti-mouse IgG2b-TRITC (1:100, MF20); for SYTOX Green staining, embryos were rinsed in PBS following antibody staining, and then were incubated in a 1:10,000 dilution of SYTOX Green (Invitrogen) overnight at 4°C.

Embryos were photographed using a Zeiss Axioplan2 microscope, a SPOT RT digital camera (Diagnostic Instruments) and MetaMorph software or imaged using a Zeiss Pascal confocal microscope. Images were assembled using Adobe Photoshop.

Western analysis

Western analysis was performed as previously described (Waskiewicz et al., 2001). Samples were run on NuPage 4-12% BIS-TRIS gels (Invitrogen). The equivalent of approximately one embryo was loaded per lane. Antibodies were used at the following dilutions: anti-pan zebrafish Pbx (Pöpperl et al., 2000), 1:2000; and anti-Histone H4 (Upstate Biotechnology), 1:1000.

Quantitative real-time RT-PCR

Dechorionated embryos were homogenized in TRIzol using a 1 cc insulin syringe, and RNA was isolated following the TRIzol protocol (Invitrogen). 1 μg of RNA plus random hexamers were used in a reverse-transcriptase (RT) reaction with SuperScriptII Reverse Transcriptase (Invitrogen). Real-time PCRs were performed using an Applied Biosystems 7900HT System according to the manufacturer's instructions. The relative expression levels were normalized to those of *ornithine decarboxylase 1* (*odc1*) (Draper et al., 2001). We used the following probe and primer sets (sequences given are 5′ to 3′): myogL1 forward, CTGGGGTGTCGTCCTCTAGT; myogR1 reverse, TCGTCGTTCAGCAGATCCT; myogP1 probe, TGGAGCAGC-GCGTCTGATCA; myodL2 forward, TCAGACGAGAAGACGGAACA; myodR2 reverse, CACGATGCTGGACAGACAAT; myodP2 probe, CAC-CAAATGCTGACGCACGG; odcL2 forward, GACTTTGACTTCGCC-TTCCT; odcR2 reverse, GAGGTGCTTCTTCAGGACATC; odcP1 probe; CGCGCGATATCGTGGAGCAG; desminL1 forward, GAGGCCGAG-GACTGGTATAA; desminR1 reverse, GGTGTAGGACTGGAGCTGGT; desminP1 probe, AGCCAAGCAGGAGACCATGCAA.

cDNA microarray analysis

pbx2-MO*; pbx4*-MO embryos as well as their control siblings (*pbx2*-MO3 mismatch control) were collected at the 10 somite (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

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(using *myod*) as well as the loss of *krox-20* expression and downregulation of *myog* expression in *pbx2*-MO*; pbx4*-MO embryos (see Fig. 1M,N for example). Total RNA was harvested using TRIzol followed by Qiagen RNeasy clean-up according to the Affymetrix recommendations. cRNA labeling and hybridization to Affymetrix Zebrafish Genome arrays were performed using the manufacturer's protocols. The perfect match (PM) probe intensities were corrected by robust multi-array average, normalized by quantile normalization and summarized by medianpolish using the Affymetrix package of Bioconductor. The gene expression profiles of control versus *pbx2*-MO*; pbx4*-MO were compared using the LIMMA package of Bioconductor. Array data are available at NCBI GEO (www.ncbi.nlm.nih.gov/geo/), accession GSE8428.

EMSA

Electrophoretic mobility shift assays (EMSAs) were performed as previously described (Berkes et al., 2004). Pbx4 and Meis3 pCS2 expression constructs were as previously described (Pöpperl et al., 2000; Vlachakis et al., 2000).

RESULTS

Pbx and Myod are required for activation of *myog* expression

To address the role of Pbx in myogenesis, we used antisense morpholino oligos (MOs) to knock down the maternal and zygotic expression of Pbx2 and Pbx4 (Waskiewicz et al., 2002). In control embryos, a pan-zebrafish-Pbx antibody (Pöpperl et al., 2000) identified ubiquitous nuclear Pbx expression in early paraxial mesoderm, and ubiquitous Pbx expression was maintained during somite development and early muscle differentiation (see Fig. S1A-C in the supplementary material), consistent with prior reports of broad *pbx* mRNA expression during early zebrafish development (Pöpperl et al., 2000; Vlachakis et al., 2000; Waskiewicz et al., 2002), and confirming that Pbx proteins are present at the right time and place to potentially regulate early muscle development. By contrast, Pbx protein expression was essentially absent in *pbx2*-MO*; pbx4*-MO embryos (see Fig. S1D in the supplementary material). Western analysis confirmed the loss of Pbx2 and Pbx4 expression (see Fig. S1E in the supplementary material), and the loss of hindbrain expression of *krox-20*, a Pbx-dependent gene (Waskiewicz et al., 2002), confirmed the abrogation of Pbx function in *pbx2*-MO*; pbx4*-MO embryos (see Fig. 1).

We first wanted to test whether Pbx is required for the activation of *myog* expression. During somitogenesis stages in control embryos, *myod* was expressed adjacent to the notochord in adaxial cells (presumptive slow-muscle cells) as well as in more-lateral cells in the posterior of each somite (presumptive fast-muscle cells). *myog* showed a similar pattern of expression, which was delayed relative to *myod* (Weinberg et al., 1996; Groves et al., 2005) (Fig. 1A,D,G,J). In comparison to control embryos, *myod* expression was slightly increased in *pbx2*-MO*; pbx4*-MO embryos, based on in situ hybridization (Fig. 1A,B,G-H,M,N), and this slight increase was confirmed by quantitative real-time reverse transcriptase (RT)-PCR (qRT-PCR; Fig. 1S). Despite the presence of increased levels of *myod* in *pbx2*-MO*; pbx4*-MO embryos, expression of *myog* was severely reduced at the 6-10 somite (s) stages (Fig. 1D,E). As somitogenesis proceeded, *pbx2*-MO*; pbx4*-MO embryos exhibited a delayed pattern of *myog* expression (Fig. 1J,K,M,N). The reduction and delay of *myog* expression were confirmed by qRT-PCR (Fig. 1T). By approximately 24 hours post fertilization (hpf), *myog* expression approached its normal expression pattern but remained at somewhat reduced levels (Fig. 1T and data not shown). These results show that Pbx function is required for the efficient initiation of *myog* expression.

To demonstrate the specificity of the pbx MOs, we injected mRNA for the *Drosophila* pbx gene ortholog, *extradenticle* (*exd*), into *pbx2*-MO*; pbx4*-MO embryos. *exd* mRNA previously was shown to rescue *krox-20* expression in *pbx4*$^{-/-}$ embryos (Pöpperl et al., 2000). We found that *exd* mRNA rescued *krox-20* expression and rescued the reduced and delayed *myog* expression in *pbx2*-MO*; pbx4*-MO embryos (see Fig. S2 in the supplementary material). Additionally, knock-down of either *pbx2* or *pbx4* had little or no effect on *myog* expression, and a five-base mismatch control MO for *pbx2* caused no defects in *myog* expression (data not shown). Therefore, the delay and reduction in *myog* expression in *pbx2*-MO*; pbx4*-MO embryos is specific and dependent upon the loss of function of both pbx genes.

We then addressed whether the requirements for Myod are similar to those for Pbx in the activation of *myog* expression. We used two types of MOs to knock down Myod: translation-blocking MOs (*myod*-MO) to knock down all Myod activity, and splice-blocking MOs (*myod*-splMO) to knock down the helix III-containing exon 3 of zebrafish *myod*, because the helix III domain of Myod is necessary for cooperative binding with Pbx in vitro (Berkes et al., 2004). *myod*-splMOs induced aberrantly spliced cDNAs that are predicted to generate truncated proteins lacking the helix III domain but retaining the activation and bHLH domains of Myod, whereas the *myod*-MOs caused the loss of Myod protein (see Fig. S3 in the supplementary material). If the delay in *myog* expression in *pbx2*-MO*; pbx4*-MO embryos was due to requirements for an interaction between Pbx and Myod, then we expect that knock-down of Myod would also result in a delay in *myog* expression. Similar to *pbx2*-MO*; pbx4*-MO embryos, *myog* expression was reduced and delayed with knock-down of Myod helix III (Fig. 1F,L,T). Knock-down of Myod protein caused a similar delay in *myog* activation and, at later stages, *myod*-MO embryos maintained a slightly more severe reduction of *myog* expression compared with *pbx2*-MO*; pbx4*-MO embryos (Fig. 1O; see also Fig. 4). These results show that Myod is indeed required for the proper expression of *myog* in zebrafish and, in particular, that it has similar requirements as Pbx for the timely initiation of *myog* expression. These results thus support our hypothesis that interaction with Pbx is necessary for Myod to properly initiate *myog* expression.

To test whether Pbx or Myod knock-down cause a delay in all muscle gene expression, we analyzed the expression of *desmin* (*desm*), one of the earliest-expressed muscle-specific genes in zebrafish (Xu et al., 2000) and a known direct transcriptional target of Myod that does not depend on Myod helix III in cultured mouse cells (Bergstrom et al., 2002; Berkes et al., 2004). In control embryos, *desm* is expressed subsequent to *myod*, mainly in adaxial cells (Xu et al., 2000) (Fig. 1P). *desm* expression appeared largely unaffected in *pbx2*-MO*; pbx4*-MO, *myod*-splMO or *myod*-MO embryos, either by RNA in situ hybridizations (Fig. 1P-R; see also Figs 2 and 4) or by qRT-PCR (Fig. 1U). We demonstrate below (see Fig. 4) that zebrafish *desm* is indeed Myod-regulated. These results show that Pbx is not required for the proper initiation of all muscle gene expression.

Microarray analysis identifies Pbx-dependent somite and muscle gene expression

To identify the subset of muscle genes that require Pbx for normal developmental expression, we used Affymetrix expression microarrays to compare gene expression in embryos lacking Pbx function (*pbx2*-MO*; pbx4*-MO embryos) to control MO-treated embryos at the 10s and 18s stages. We chose the 10s stage because that was when *myog* expression showed a strong reduction in *pbx2*-

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MO; *pbx4*-MO embryos (Fig. 1) and when early muscle-specific genes are beginning to be expressed (Xu et al., 2000). We chose the 18s stage because many muscle differentiation genes are not expressed until mid- or late-stage somitogenesis in zebrafish (Xu et al., 2000). Using the cut-offs of >1.5-fold change and a false discovery rate of <0.05, our array analysis identified 188 Pbx-dependent genes at 10s and 258 genes at 18s, including all of the previously known Pbx-dependent genes expressed in the hindbrain, such as *krox-20* (Waskiewicz et al., 2002) (see Tables S1 and S2 in the supplementary material). From these groups, we selected the genes that are known to be expressed in wild-type somites and early skeletal muscle (Thisse et al., 2001; ZFIN, 2006) (Table 1). *myog* is

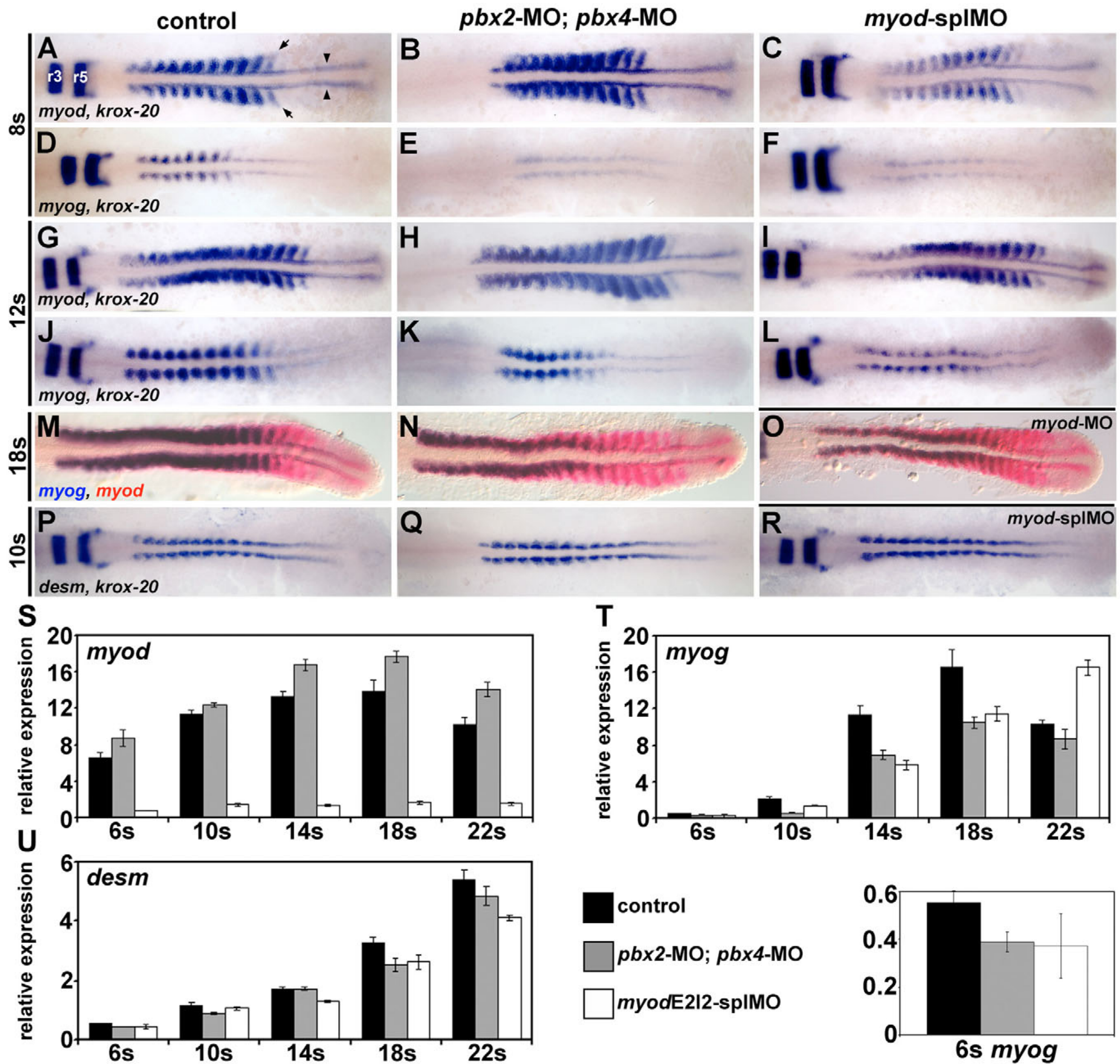

Fig. 1. Pbx and Myod are required for the proper initiation of *myog* expression. (**A-R**) RNA in situ expression of (A-C,G-I) *myod* and *krox-20*, (D-F,J-L) *myog* and *krox-20*, (M-O) *myog* (blue) and *myod* (red), or (P-R) *desm* and *krox-20* in (A,D,G,J,M,P) wild-type control, (B,E,H,K,N,Q) *pbx2*-MO; *pbx4*-MO, (C,F,I,L,R) *myod*-splMO, or (O) *myod*-MO embryos. Similar phenotypes were observed for both *myod* splice-blocking MOs (*myod*-splMOs). Somite (s) staging was confirmed by counting somites using Nomarski optics. All embryos are shown in dorsal view, anterior towards the left. (A) Arrowheads point to adaxial cells; arrows point to lateral somite cells; and r3 and r5 indicate *krox-20* expression in hindbrain rhombomeres. (**S-U**) Graphs of real-time reverse-transcriptase (RT)-PCR quantities for (S) *myod*, (T) *myog* and (U) *desm*. (T, bottom) Enlarged view of 6s stage *myog* expression and the key for S-U. *x*-axes are developmental stage and *y*-axes are relative mRNA expression level. Quantities were normalized to *odc1* expression. Error bars represent standard deviation. *myod*E2I2-splMO targets the exon 2-intron 2 boundary (see Fig. S3 in the supplementary material). The *myod* real-time primers measure only mRNA with properly spliced intron 2. Quantitative (q)RT-PCR shows that less than about 10-15% of normally spliced *myod* transcripts remain in *myod*-splMO embryos (Fig. 1S and see Fig. S3 in the supplementary material; and data not shown). Similar quantities for *myog* and *desmin* were observed for both *myod*-splMOs.

Table 1. Microarray-identified Pbx-dependent genes expressed in somites and early muscle

Gene symbol	Affymetrix ID number	Fold change at 10s (controlMO: *pbx2*-MO; *pbx4*-MO)	Expression pattern at 10s	RNA in situ expression in *pbx2*-MO; *pbx4*-MO at 10s	RNA in situ expression in *myod*-MO at 10s
si:dkey-119m7.4	Dr26487.1.S1_at	3.85	Segmental plate	ND	ND
angptl7	Dr10141.1.A1_at	2.62	Broad through lateral somites	Very reduced	Upregulated
hoxa3a	Dr24774.1.S2_at	2.25	Anterior cells of anterior somites; central nervous system (CNS)	All domains reduced	Not affected
myog	Dr2499.1.S1_at	2.04	Adaxial cells and lateral somites	Very reduced	Very reduced
egfl6	Dr1807.1.A1_at	1.95	Segmental plate; CNS	All domains reduced	Not affected
bhik	DrAffx.1.2.S1_at	1.92	Somites; lateral plate mesoderm; tail bud; polster	ND	ND
fabp3	Dr6814.1.S1_at	1.74	Posterior cells in anterior somites; CNS; eye	All domains reduced	Somite expression reduced
pwp2h	Dr3590.1.S1_at	1.61	Segmental plate; CNS	All domains reduced	Not affected
ppp1r3b	Dr4453.1.S1_at	1.59	Ubiquitous	ND	ND
acta1	AFFX-Dr-acta1-5_at	1.59	Basal ubiquitous expression; stronger in adaxial cells	Reduced	Reduced
meis3	Dr1680.1.S1_at	1.56	Anterior somites; head mesenchyme; CNS	All domains reduced	Not affected
ttn	Dr4681.1.A1_at	1.53	Adaxial cells; lateral somites; heart primordium	Heart domain reduced	Not affected
chrna1	Dr123.1.S1_at	1.52	Adaxial cells	Reduced	Reduced
gstm	Dr1754.1.S1_at	1.52	Diffuse in somites, CNS and eye	All domains reduced	Somite expression reduced
ttnl	Dr1662.1.S1_at	1.52	Adaxial cells; lateral somites; segmental plate	Reduced	Very reduced
Gene symbol	**Affymetrix ID number**	**Fold change at 18s (controlMO: *pbx2*-MO;*pbx4*-MO)**	**Expression pattern at 18s**	**RNA in situ expression in *pbx2*-MO; *pbx4*-MO at 18s**	**RNA in situ expression in *myod*-MO at 18s**
vmhc	Dr10607.1.A1_at	3.20	Adaxial cells; heart	Heart domain reduced	Not affected
myhz1	Dr23067.1.S1_at	2.99	Adaxial cells and lateral somites	ND	ND
myhc4	Dr4812.1.S1_s_at	2.51	Adaxial and lateral somites	Reduced	Very reduced
eng2a	Dr496.1.S1_at	2.30	Muscle pioneers; CNS	CNS expression reduced	Not affected
aldh1a2	Dr5206.1.S1_at	2.26	Somites; lateral plate mesoderm; eye	All domains reduced, but increased expression in tail bud	Not affected
mylz2	Dr2914.1.S1_at	2.08	Adaxial cells and lateral somites	Very reduced	Very reduced
chrna1	Dr123.1.S1_at	2.06	Adaxial cells and lateral somites	Very reduced	Very reduced
ppp1r3b	Dr4453.1.S1_at	1.97	Ubiquitous	ND	ND
angptl7	Dr10141.1.A1_at	1.96	Lateral somites	Reduced	Upregulated
ttn	Dr4681.1.A1_at	1.88	Adaxial cells; lateral somites; heart	Reduced	Reduced
bhik	DrAffx.1.2.S1_at	1.84	Anterior somites; tail bud	ND	ND
tmem161a	Dr5047.1.A1_at	1.80	Adaxial cells and lateral somites	Reduced	Very reduced
ttnl	Dr1662.1.S1_at	1.78	Adaxial cells and lateral somites	Reduced	Very reduced
dhrs3	Dr2644.1.A1_at	1.76	Anterior cells of somites; ventral mesoderm; spinal cord	All domains reduced	Somite expression expanded
atp2a1	Dr20010.7.S1_at	1.76	Adaxial cells and lateral somites	Reduced	Very reduced
acta1	AFFX-Dr-acta1-5_at	1.75	Basal ubiquitous expression, stronger in adaxial cells and somites	Reduced	Reduced
srl	Dr21216.1.A1_at	1.72	Adaxial cells and lateral somites	Reduced	Very reduced
si:dkey-119m7.4	Dr26487.1.S1_at	1.66	Posterior segmental plate	ND	ND
myog	Dr2499.1.S1_at	1.63	Adaxial cells and lateral somites	Reduced	Reduced
fabp3	Dr6814.1.S1_at	1.54	Posterior-ventral somite expression; CNS; eye	Upregulated in somites; reduced in CNS	Upregulated in somites
atp1a2a	Dr10438.1.S1_at	1.52	Lateral somites	Reduced	Reduced
cmyc	Dr1.1.S1_at	1.51	Lateral somites; CNS; eye	CNS and eye reduced	Not affected

10s/18s, 10/18 somites; ND, not determined.

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one of these somite-expressed genes and provides an initial validation of our array analysis (Table 1). Because some of these genes were expressed in domains in addition to the somites, we used RNA in situ hybridization to confirm that most of these genes indeed show reduced somite expression in *pbx2*-MO*; pbx4*-MO embryos (Table 1 and Fig. 2A-P). However, there were some exceptions. For example, *vmhc* was expressed both in adaxial cells and the heart primordium, but only the heart expression was reduced in *pbx2*-MO*; pbx4*-MO embryos (Fig. 2Q,R). Furthermore, *desm* was not significantly changed on the arrays and showed normal expression in *pbx2*-MO*; pbx4*-MO embryos at 18s (Fig. 2S,T). These results indicate that Pbx is indeed required for a subset of skeletal muscle gene expression.

We tested whether these microarray-identified Pbx-dependent genes were also Myod-dependent and found that, of the 20 genes whose somite/skeletal muscle expression was Pbx-dependent, 13 also showed reduced somite/muscle expression in *myod*-MO embryos (Table 1). At the 18s stage, these genes generally exhibited a delayed pattern of expression in *pbx2*-MO*; pbx4*-MO embryos, which was slightly more reduced in *myod*-MO embryos (Table 1), similar to that observed for *myog*. We noted that many of the Pbx- and Myod-dependent genes identified in our 18s arrays are predicted to play roles in fast-muscle differentiation, in particular *myhz1*, *myhc4*, *mylz2*, *atp2a1*, *srl* and *atp1a2a* (Feng et al., 2006; Groves et al., 2005; Bárány, 1967; Reiser et al., 1985; Ohlendieck et al., 1999). Previous work showed that knock-down of Myod in zebrafish causes reduced *mylz2* expression and, therefore, a defect in fast-muscle development (Hammond et al., 2007). We examined *mylz2* expression at early stages and found that, like other 'fast-muscle' genes in zebrafish (Bryson-Richardson et al., 2005; Xu et al., 2000) (data not shown), *mylz2* was initially expressed mainly in adaxial cells, but also in lateral somite cells, and that both of these domains were reduced and delayed in *pbx2*-MO*; pbx4*-MO and *myod*-MO embryos (Fig. 2U-W). Similar results were observed for *myhc4*, *atp2a1*, *ttnl* and *srl* (data not shown). Thus, we have identified a set of genes that are both Pbx- and Myod-dependent. Based on the predicted functions of these genes and their expression defects, our data suggest that Pbx might facilitate Myod-activation of a set of genes that function in fast-muscle differentiation.

Pbx function is required for efficient fast-muscle differentiation in zebrafish embryos

To further address the requirements for Pbx and Myod in the development of fast and slow muscle in zebrafish, we examined additional markers that label fast- or slow-muscle precursors. *smyhc1*, which encodes a slow myosin heavy chain and is expressed specifically in adaxial cells/slow-muscle precursors (Bryson-Richardson et al., 2005), was expressed normally in *pbx2*-MO*; pbx4*-MO and in *myod*-MO embryos (Fig. 2X-Z). Engrailed staining labels muscle pioneers – an early-differentiating subset of the slow-

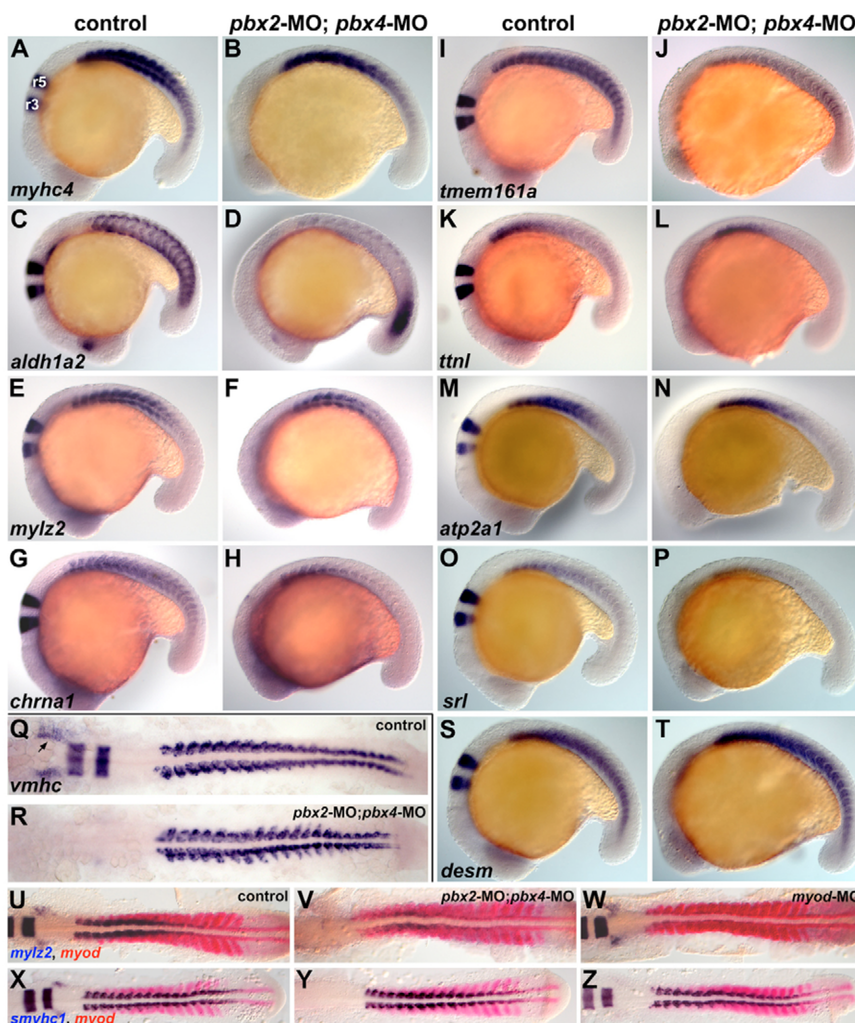

Fig. 2. Validation of microarray-identified Pbx-dependent genes. (**A-Z**) RNA in situ expression of genes from Table 1 in (A,C,E,G,I,K,M,O,Q,S,U,X) wild-type control, (B,D,F,H,J,L,N,P,R,T,V,Y) *pbx2*-MO*; pbx4*-MO, or (W,Z) *myod*-MO embryos. (S,T) Control gene, *desm*; (X-Z) slow-muscle gene, *smyhc1*. Although *pbx2*-MO*; pbx4*-MO embryos appear delayed relative to controls, all embryos are stage-matched at (A-T) 18-somite stage (18s), (U-W) 16s or (X-Z) 14s. *krox-20* was included in all in situs to control for the *pbx* MOs. (A) r3 and r5 indicate *krox-20* expression in hindbrain rhombomeres. (Q) Arrow marks *vmhc* expression in the heart primordium. Embryos are shown in (A-P,S-T) left-side view or (Q-R,U-Z) dorsal view, anterior towards the left.

muscle cell population (Devoto et al., 1996; Hatta et al., 1991) – which appeared to develop normally in *pbx2*-MO*; pbx4*-MO embryos (data not shown). The F59 anti-myosin heavy chain antibody, which specifically labels slow-muscle precursors during somitogenesis stages (Devoto et al., 1996), was expressed normally in *pbx2*-MO*; pbx4*-MO, in *myod*-splMO and in *myod*-MO embryos (Fig. 3A,B,E). The F310 anti-myosin light chain antibody labels fast-muscle myosins in zebrafish (Hamade et al., 2006). In contrast to F59 staining, F310 staining was delayed and reduced in *pbx2*-MO*; pbx4*-MO, in *myod*-splMO and in *myod*-MO embryos (Fig. 3C,D,F and see Fig. S4 in the supplementary material). *myod*-MO embryos showed slightly more reduced fast-muscle development than *pbx2*-MO*; pbx4*-MO or *myod*-splMO embryos (Fig. 3F and see Fig. S4 in the supplementary material). Expression of a pan-muscle myosin marker MF20 (Bader et al., 1982) initiated on time, and its slow-muscle expression appeared normal in *pbx2*-MO*; pbx4*-MO, in *myod*-splMO and in *myod*-MO embryos (Fig. 3A-F and see Fig. S4 in the supplementary material). Thus, upon loss of Pbx or Myod function, we observed delayed and reduced fast-muscle differentiation, using multiple markers, and we observed no change, including no delay, reduction, or increase, in slow-muscle-differentiation marker expression. These results, combined with our microarray findings, show that Pbx function is needed to promote efficient fast-muscle differentiation, as is Myod.

Multiple populations of fast-muscle fibers have been described in zebrafish. Primary fast muscle comprises medial fast fibers, which express low levels of Engrailed in response to Hedgehog signaling (Wolff et al., 2003), and lateral fast fibers, which are dependent on Fgf signaling (Groves et al., 2005). A secondary population of fast-muscle precursors, initially marked by *pax3* and *pax7* expression, contributes fast-muscle fibers to the growing myotomes, beginning at around 24 hpf (Hollway et al., 2007; Stellabotte et al., 2007). Because Engrailed expression appeared normal in *pbx2*-MO*; pbx4*-MO embryos (data not shown) and because fast-muscle differentiation in *pbx2*-MO*; pbx4*-MO embryos appeared to recover by about 24 hpf (Fig. 3), we conclude that Pbx is necessary for the efficient differentiation of primary lateral fast fibers.

We next looked for evidence that lateral somite cells are maintaining a prolonged undifferentiated state in the absence of Pbx. Decreased fast-muscle differentiation and loss of Myod function are associated with increased and prolonged *pax3* and *pax7* expression, markers of undifferentiated myogenic precursors (Groves et al., 2005; Hammond et al., 2007). We could not identify a significant upregulation of *pax3* or *pax7* in *pbx2*-MO*; pbx4*-MO embryos (data not shown). However, our microarray analysis identified the upregulation of *cxcr4a* in *pbx2*-MO*; pbx4*-MO embryos (see Table S2 in the supplementary material). *cxcr4a*, the expression of which in the anterior-lateral cells of each somite is initially very similar to *pax7*, is normally downregulated in all but the most posterior somites at the 18s stage (Hollway et al., 2007) (see Fig. S5 in the supplementary material). We observed prolonged maintenance of *cxcr4a* in trunk somites in *pbx2*-MO*; pbx4*-MO embryos as well as in *myod*-MO embryos (see Fig. S5 in the supplementary material). These results suggest that Pbx, as well as Myod, are important for the efficient differentiation of lateral somite cells.

Pbx functions with Myod to promote fast-muscle, but not slow-muscle, differentiation

Previous work has shown that *myod* functions redundantly with *myf5* in slow-muscle development, because loss of *myod* together with *myf5* causes loss of slow-muscle myosin heavy chain expression (Hammond et al., 2007). Redundancy with Myf5 might thus mask requirements for Pbx acting with Myod. In particular, if Pbx is necessary for Myod to activate slow-muscle gene expression, then

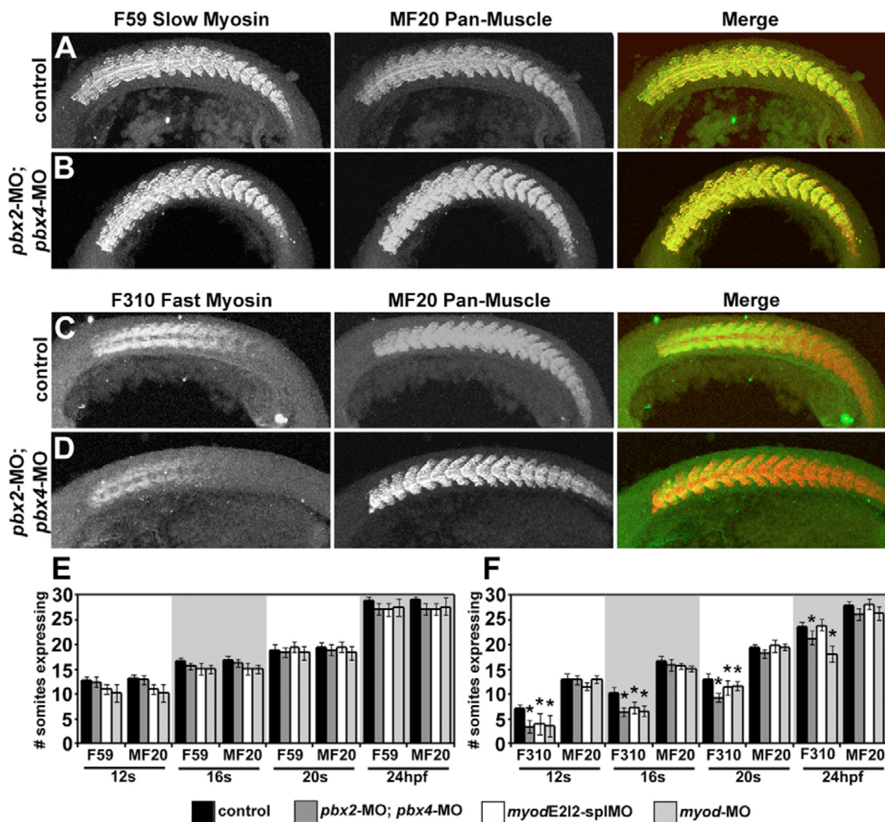

Fig. 3. Pbx is required for the proper initiation of fast-muscle differentiation. (**A-D**) Antibody staining of slow muscle with F59 antibody (green; A,B), or fast muscle with F310 antibody (green; C,D), in (A,C) wild-type control or (B,D) *pbx2*-MO*; pbx4*-MO embryos. Antibody staining of muscle with MF20 antibody (red; A-D) is included to control for somite staging. Embryos are shown in left-side view, anterior towards the left. (**E**,**F**) Quantification of (E) F59/slow- and (F) F310/fast-muscle marker expression. MF20 expression is included to control for somite (s) staging. Graphs show number of somites expressing a muscle marker at different developmental stages. Similar phenotypes were observed for both *myod* splice-blocking MOs (*myod*-splMOs). Error bars represent standard deviation. *P* values (Mann-Whitney test; normalized to somite number): **P*<0.0001 compared to same-stage control; except for *pbx2*-MO*; pbx4*-MO F310 compared to control at 24 hpf, *P*<0.03.

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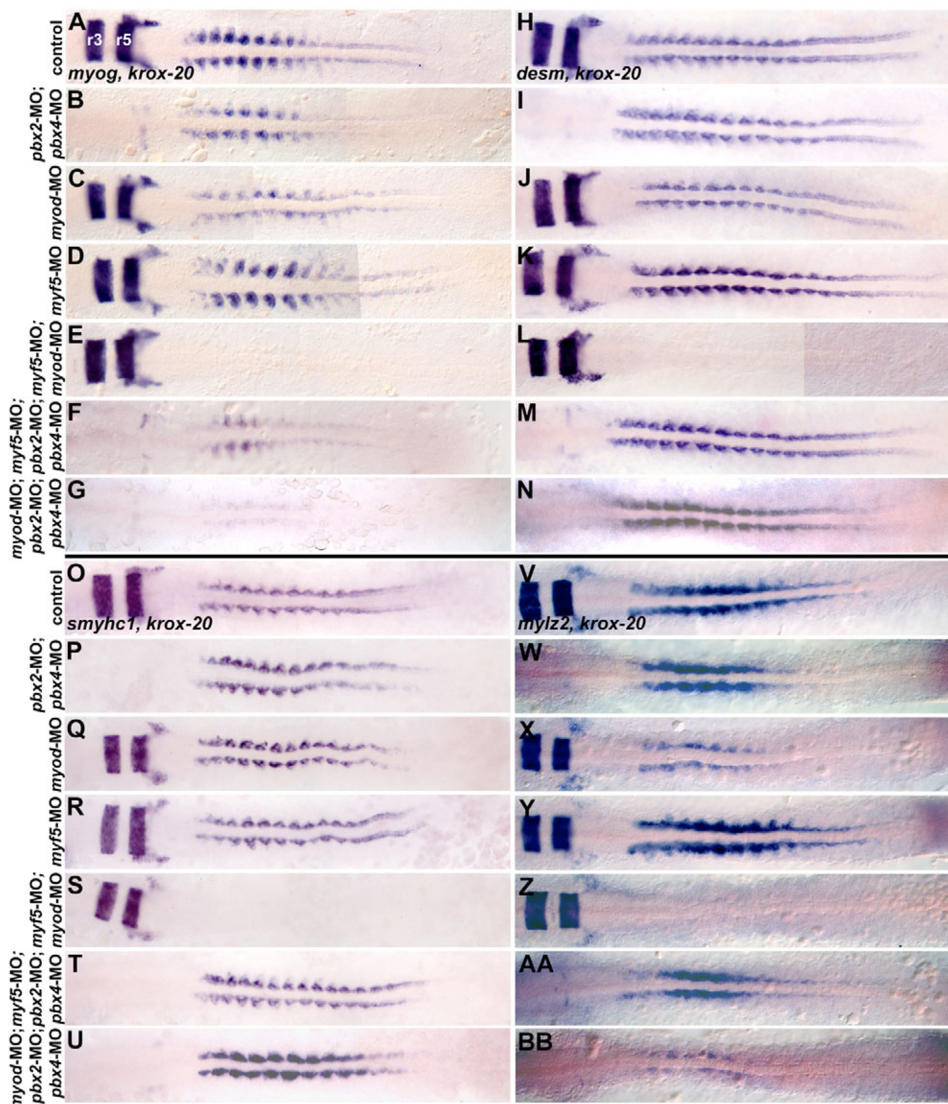

Fig. 4. Pbx functions with Myod in fast-muscle, but not slow-muscle, differentiation. RNA in situ expression at the 10-somite (10s) stage of (**A-G**) *myog* and *krox-20*, (**H-N**) *desm* and *krox-20* or (**O-U**) *smyhc1* and *krox-20*, or at 16s of (**V-BB**) *mylz2* and *krox-20* in (A,H,O,V) wild-type control, (B,I,P,W) *pbx2*-MO*; pbx4*-MO, (C,J,Q,X) *myod*-MO, (D,K,R,Y) *myf5*-MO, (E,L,S,Z) *myf5*-MO*; myod*-MO, (F,M,T,AA) *myf5*-MO*; pbx2*-MO*; pbx4*-MO, or (G,N,U,BB) *myod*-MO*; pbx2*-MO*; pbx4*-MO embryos. Embryos are shown in dorsal view, anterior towards the left. Somite staging was confirmed by counting somites using Nomarski optics. (A) r3 and r5 indicate *krox-20* expression in hindbrain rhombomeres.

knock-down of Myf5 together with Pbx should cause slow-muscle defects. Alternatively, because the helix III Pbx-interacting domain of Myod is conserved in Myf5 (Bergstrom and Tapscott, 2001), knock-down of Myod together with Pbx might cause slow-muscle defects. We found that *myf5*-MO embryos showed no defects in *myog* or *desm* expression (Fig. 4D,K), whereas *myf5*-MO*; myod*-MO embryos showed complete loss of *myog* and *desm* expression (Fig. 4E,L), confirming that our *myf5* and *myod* MOs were functioning. *myf5*-MO*; pbx2*-MO*; pbx4*-MO embryos resembled *pbx2*-MO*; pbx4*-MO embryos, in that *myog* expression was delayed in lateral somite cells and *desm* expression was unaffected (Fig. 4F,M). *myod*-MO*; pbx2*-MO*; pbx4*-MO embryos, however, showed almost complete loss of *myog* expression at 10s (Fig. 4G), whereas *desm* expression was unaffected (Fig. 4N). By 18s, in *myod*-MO*; pbx2*-MO*; pbx4*-MO embryos, *myog* expression was still much reduced compared with *myod*-MO embryos (data not shown). These results suggest that Pbx proteins are needed for both Myod and Myf5 to activate *myog* expression.

We then assessed slow- and fast-muscle differentiation in these MO combinations using *smyhc1*, F59, *mylz2* and F310. As previously reported, all slow-muscle differentiation was lost in *myf5*-MO*; myod*-MO embryos (Hammond et al., 2007) (see Fig. 4S). All fast-muscle differentiation was also lost in *myf5*-MO*; myod*-MO embryos (Fig. 4Z and data not shown), revealing that *myf5* acts with *myod* in fast muscle. *myf5*-MO*; pbx2*-MO*; pbx4*-MO embryos resembled *pbx2*-MO*; pbx4*-MO embryos (Fig. 4T,AA and data not shown). Importantly, slow-muscle differentiation was not delayed in *myf5*-MO*; pbx2*-MO*; pbx4*-MO embryos (Fig. 4T and data not shown). These results show that Pbx is not necessary for Myod to activate slow-muscle gene expression. We also observed that slow-muscle differentiation was not delayed in *myod*-MO*; pbx2*-MO*; pbx4*-MO embryos (Fig. 4U and data not shown). However, similar to *myog* expression, *myod*-MO*; pbx2*-MO*; pbx4*-MO embryos showed severely reduced activation of *mylz2* (Fig. 4BB) and very reduced F310 staining even at 24 hpf (data not shown). Taken together, these results show that Pbx homeodomain proteins are necessary to modulate the activity of Myod and, probably, Myf5, in driving fast-muscle-specific differentiation.

Pbx is required downstream of Shh signaling for Myod to induce fast-muscle, but not slow-muscle, gene expression

To further demonstrate that Pbx directs Myod to drive fast-muscle, but not slow-muscle, development, we tested whether Pbx and Myod are required for the ability of Sonic hedgehog (Shh) signaling to

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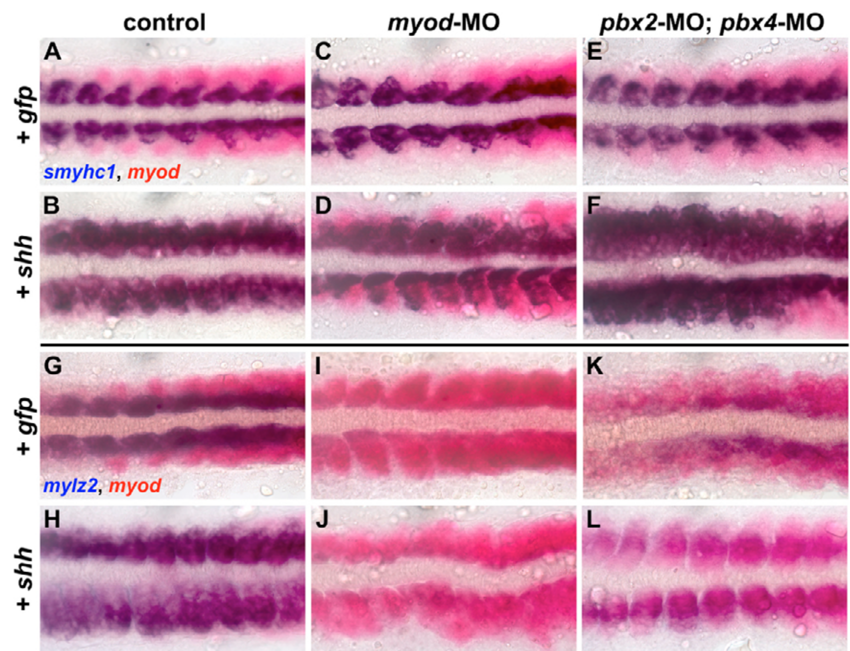

Fig. 5. Pbx is required downstream of Shh signaling to induce fast-muscle, but not slow-muscle, gene expression. (**A-L**) RNA in situ expression of (A-F) *smyhc1* (blue) and *myod* (red) at the 14-somite (14s) stage or (G-L) *mylz2* (blue) and *myod* (red) at 16s in embryos injected with (A,G) *pbx2*-MO mismatch control and *gfp* mRNA, (B,H) *pbx2*-MO mismatch control and *shh* mRNA, (C,I) *myod*-MO and *gfp* mRNA, (D,J) *myod*-MO and *shh* mRNA, (E,K) *pbx2*-MO*; pbx4*-MO and *gfp* mRNA, or (F,L) *pbx2*-MO*; pbx4*-MO and *shh* mRNA. The most-anterior 8-10 somites of each embryo are shown, in dorsal view, anterior towards the left. Control embryos injected with *shh* mRNA (control+*shh*) show increased expression of *smyhc1* (B, 106/135 embryos; 29/135 show normal *smyhc1* expression) or increased expression of *mylz2* (H, 88/121; 33/121 show normal *mylz2* expression). *myod*-MO+*shh* embryos show less-frequent and less-extensive induction of *smyhc1* compared with control+*shh* (46/90 embryos resemble D; 44/90 show normal *smyhc1* expression) and fail to upregulate *mylz2* expression beyond that seen in *myod*-MO+*gfp* embryos (84/88 embryos resemble J; 4/88 show slightly increased *mylz2* expression). *pbx2*-MO*; pbx4*-MO+*shh* embryos show strongly increased expression of *smyhc1* (77/79 embryos resemble F; 2/79 have normal *smyhc1* expression). Approximately half (43/102; L) of *pbx2*-MO*; pbx4*-MO+*shh* embryos show expansion of weak levels of *mylz2* across the somites; the other half (59/102) resemble *pbx2*-MO*; pbx4*-MO+*gfp* embryos. Similar results were observed in multiple experiments.

drive slow- or fast-muscle gene expression. Previous studies have shown that injecting *shh* mRNA into wild-type embryos induces the upregulation of *myod* and slow-muscle gene expression, such as *smyhc1*, causing expanded slow-muscle differentiation across the lateral somites (Currie and Ingham, 1996; Blagden et al., 1997; Du et al., 1997) (Fig. 5B). We tested whether this slow-muscle expansion requires Myod and found that *shh*-induced upregulation of *smyhc1* was partially inhibited in *myod*-MO embryos (Fig. 5D). However, *shh* mRNA readily induced increased *myod* and *smyhc1* expression in *pbx2*-MO*; pbx4*-MO embryos (Fig. 5F), showing that Pbx is not needed for Shh/Myod-induced slow-muscle development. Whereas *shh* induces repression of fast-muscle markers at late stages (30 hpf) (Blagden et al., 1997; Ju et al., 2003) (data not shown), we found that, at earlier stages, *shh* can induce the expansion of fast-muscle markers, such as *mylz2*, in control embryos (Fig. 5H and data not shown). Shh-induced upregulation of fast-muscle markers was inhibited in *myod*-MO embryos, even though *myod* expression was upregulated (Fig. 5J and data not shown). In contrast to *smyhc1*, Shh/Myod induction of fast-muscle markers was inhibited in *pbx2*-MO*; pbx4*-MO embryos (Fig. 5L and data not shown). Thus, our experiments here show that the slow- or fast-muscle-inducing effects of Shh are at least in part mediated by Myod, but that Pbx is required only for the induction of fast-muscle markers. Taken together, these experiments further demonstrate that Pbx functions specifically in regulating Myod activation of fast-muscle gene expression.

Pbx cooperates with Myod to regulate fast-muscle gene expression independent of Myog or retinoic acid and might directly regulate *mylz2*

We next wanted to address how directly Pbx and Myod function in the activation of fast-muscle-specific genes. There are several reasons why Myog could be the main direct target of Pbx-Myod. First, the *myog* promoter contains the best-characterized binding site for a Pbx-Myod complex (Berkes et al., 2004). Second, *myog* expression is affected strongly and early in *pbx2*-MO*; pbx4*-MO embryos and in *myod*-MO embryos (Fig. 1). Also, work in mammalian cell culture has demonstrated that Myog is a key downstream effector of Myod (Cao et al., 2006). If Myog is the main effector of Pbx-Myod in zebrafish, then knocking down Myog function should cause similar defects as those seen in *pbx2*-MO*; pbx4*-MO embryos and *myod*-MO embryos. We confirmed that Myog protein is almost completely abolished in *myog*-MO embryos (data not shown). However, we observed no delays or defects in fast-muscle gene expression in *myog*-MO embryos (data not shown). We also analyzed additional Pbx-dependent genes and found that *acta1*, *ttn*, *chrna1* and *ttnl* expression all appeared to be normal in *myog*-MO embryos (data not shown). These results reveal that Pbx-Myod must have direct targets, in addition to *myog*, to mediate the defects in fast-muscle gene expression.

Our microarray analysis identified the expression of *aldh1a2*, which encodes an enzyme that is crucial for the synthesis of retinoic acid (RA) (Begemann et al., 2001; Grandel et al., 2002), as Pbx-dependent (Table 1; Fig. 2D). Because RA promotes fast-muscle development in zebrafish (Hamade et al., 2006), *aldh1a2*/RA signaling could be an effector of Pbx. Incubating wild-type embryos in an RA bath induces the upregulation of *myod* and *myog* expression, and precocious fast-muscle differentiation (Hamade et al., 2006). We found that, although RA is still able to strongly increase *myod* expression in *pbx2*-MO*; pbx4*-MO embryos, RA did not rescue *myog* expression nor the defect in fast-muscle development in *pbx2*-MO*; pbx4*-MO embryos (data not shown). Therefore, Pbx is required for the efficient activation of fast-muscle genes by Myod in a manner that is independent of its role in RA synthesis. Taken together, our findings are consistent with the hypothesis that Pbx not only recruits Myod to the *myog* promoter (Berkes et al., 2004) but also to additional fast-muscle gene promoters.

To determine whether Pbx directly regulates fast-muscle gene expression, we have initiated an analysis of fast-muscle gene promoters. We identified a near-consensus Pbx-Meis binding site in

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the *mylz2* promoter and determined that in vitro-translated Pbx-Meis heterodimers bind to this site in electrophoretic mobility shift assays (see Fig. S6 in the supplementary material). Although additional studies are necessary to test the biological relevance of this site, the presence of a Pbx-binding site in the *mylz2* promoter suggests a direct role for Pbx in the regulation of fast-muscle gene expression.

DISCUSSION

Previous studies suggested that Pbx marks a subset of genes for activation by Myod but they did not demonstrate a necessary or biological role for this function (Berkes et al., 2004). We set out to address two specific hypotheses of Pbx function: that it is required for *myog* expression and that it is required for a biologically relevant subset of Myod-regulated gene expression. Our analysis of zebrafish embryos lacking Pbx function demonstrates that Pbx is indeed necessary in order for Myod to accurately regulate the expression of *myog* and that the subset of Myod-regulated genes that also require Pbx confer the fast-skeletal-muscle phenotype in zebrafish embryos. More generally, our results provide the initial support for an instructional role of homeodomain proteins in modulating the activity of bHLH proteins in driving a specific cellular phenotype. Previous work has shown that Myod, Neurod and other bHLH proteins drive a broad program of gene expression to establish a general cell type, such as muscle or neuron, whereas our study demonstrates that homeodomain proteins modulate this broad program to establish the regional identity of that cell type by marking certain subsets of genes for activation.

Our work demonstrates that Pbx is needed for the activity of a non-homeodomain protein. Pbx proteins are most-characterized for their role as Hox cofactors, but Pbx proteins also physically and functionally interact with other homeodomain proteins, including with Pdx1 (Peers et al., 1995; Kim et al., 2002) and Engrailed (Peltenburg and Murre, 1996; Kobayashi et al., 2003; Erickson et al., 2007). Our demonstration that Pbx proteins modulate the set of genes regulated by the bHLH protein Myod suggests that Pbx proteins could have more-widespread interactions than previously appreciated. Indeed, the increasing data on the requirements for Pbx in mice and zebrafish underscore the expanding roles for Pbx beyond that of Hox cofactors (Moens and Selleri, 2006; Erickson et al., 2007) (this work). Although previous studies in worms have shown that Pbx homologs are required for muscle development via their roles in mesoderm development (Liu and Fire, 2000), our study is the first to show that Pbx is required for vertebrate myogenesis.

Although we had hypothesized that Pbx would be required for a subset of Myod activity and therefore for the differentiation of a subset of muscle cell types, it is unexpected that Pbx is needed specifically for fast-muscle differentiation. Pbx proteins are expressed in presumptive slow- (adaxial) and fast- (lateral somite) muscle cells along with Myod. The Pbx- and Myod-dependent genes identified in our study are expressed in both of these cell populations. Adaxial cells thus express both Pbx-dependent and non-Pbx-dependent genes. Therefore, Pbx and Myod appear to be acting at the level of promoter activity and not in specific cell types. If Pbx proteins are ubiquitously expressed, how does Pbx help target Myod to specific promoters? Pbx and Myod directly bind the mouse *Myog* promoter (Berkes et al., 2004), and we found that Pbx can bind the zebrafish *myog* (A.T. and L.M., unpublished observations) and *mylz2* promoters, suggesting a direct role for Pbx in recruiting Myod to fast-muscle gene promoters. An alternative, although not necessarily mutually exclusive, hypothesis is that additional Pbx-dependent intermediates might exist that modulate the activity of Myod on fast-muscle gene regulatory regions. In support of this hypothesis, our microarray analysis identified at least seven genes whose expression was Pbx-dependent but not Myod-dependent (Table 1), although the functions of these genes in muscle development is unknown. Whether acting directly or indirectly, Pbx is necessary for the efficient expression of *myog* and fast-muscle genes, but not for the entire myogenic program. Therefore, our results provide the first evidence that homeodomain proteins directly modulate the activity of a bHLH protein in directing the phenotype of a general differentiation program.

What happens to cells that are delayed in differentiating into fast muscle in the absence of Pbx? We did not find any evidence for an increase in slow-muscle differentiation in the absence of Pbx, and we did not find a severe loss of cells, because *myod* was still expressed strongly in the somites. Thus, these cells might remain in a prolonged undifferentiated state. In the absence of Fgf8 signaling, lateral somite cells fail to properly activate *myod*, *myog* and fast-muscle gene expression but show increased expression of *pax3* and *pax7*, suggesting prolonged maintenance of an undifferentiated state (Groves et al., 2005; Hammond et al., 2007). Loss of Myod and Myf5 function also leads to increased *pax3* and *pax7* expression (Hammond et al., 2007). *pax3* and *pax7* expression does not normally decline in the somites until after *pbx2*-MO*; pbx4*-MO embryos have started to recover fast-muscle differentiation; thus, we have not been able to identify a significant upregulation of *pax3* or *pax7* in *pbx2*-MO*; pbx4*-MO embryos. We did, however, see prolonged maintenance of *cxcr4a* expression in *pbx2*-MO*; pbx4*-MO embryos and in *myod*-MO embryos. Thus, Pbx function might be important for the efficient progression of fast-muscle differentiation. Perhaps the upregulation of *aldh1a2* expression in the tail of *pbx2*-MO*; pbx4*-MO embryos (Fig. 2D) further reflects a defect in differentiation.

The delayed differentiation of a specific muscle type in *pbx2*-MO*; pbx4*-MO embryos is reminiscent of that observed for *Myod* or *Myf5* knock-outs in mice. Whereas loss of *Myod* and *Myf5* together in mice causes a loss of all skeletal muscle development (Rudnicki et al., 1993), loss of *Myod* alone results in delayed hypaxial muscle differentiation, and loss of *Myf5* alone results in delayed epaxial muscle differentiation (Braun et al., 1994; Kablar et al., 1997). Such studies analyzing muscle-differentiation delays have revealed that Myod and Myf5 can play unique roles, and, thus, have had significant impacts on our understanding of muscle development.

Our demonstration that Pbx is needed for the activity of a bHLH protein, in addition to previous examples of homeodomain-bHLH interactions, suggests that homeodomain modulation of bHLH activity could be a widespread mechanism to modulate cell-type diversity. Previous studies have shown interactions between homeodomain proteins and bHLH proteins at specific promoters (Westerman et al., 2003). In particular, the bHLH protein NeuroD1, along with its bHLH heterodimer partner E47, binds with the homeodomain protein Pdx1 to synergistically activate the insulin promoter in B cells within the pancreas (Glick et al., 2000). Mouse *Pbx1*$^{-/-}$ embryos have defects in pancreas differentiation, and Pbx1 interacts with Pdx1 (Kim et al., 2002), but it is not known whether Pbx modulates bHLH activity in the pancreas. In addition to their biochemical interactions, genetic interactions have suggested that bHLH and homeodomain proteins can modulate the activity of each other. Interactions between the homeodomain Nkx2-2 and Olig bHLH proteins in the mouse spinal cord regulate a cell-fate decision between an interneuron fate and a glial fate (Sun et al., 2001). This study suggested that the activity of Olig is dependent on its context with a homeodomain protein. Also, a Hairy-related bHLH protein can inhibit the activity of a Hox protein in regulating epidermal cell

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fates in *Caenorhabditis elegans* (Alper and Kenyon, 2001). However, in these cases, the mechanism by which these modulations occur is not known.

Our results provide a novel demonstration of how interactions between different types of 'master regulatory' factors control cell-type diversity. Homeodomain factors control positional identities, whereas bHLH proteins control cell-type identities. We propose that these identity programs merge together such that homeodomain proteins modulate the set of bHLH-activated genes to achieve region-specific cellular phenotypes. Recent studies are continuing to underscore the role of homeodomain proteins in modulating cellular diversity within a cell type, because Hox proteins are now understood to modulate skin-cell phenotype in different regions of the body (Rinn et al., 2006). We propose that homeodomain proteins, by instructing bHLH proteins to regulate a subset of their target genes, provide competence for a cell to execute a region-specific differentiation program.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/18/3371/DC1

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