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Meis Cofactors Control HDAC and CBP Accessibility at Hox-Regulated Promoters during Zebrafish Embryogenesis

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SUMMARY

Hox proteins form complexes with Pbx and Meis cofactors to control gene expression, but the role of Meis is unclear. We demonstrate that Hoxb1-regulated promoters are highly acetylated on histone H4 (AcH4) and occupied by Hoxb1, Pbx, and Meis in zebrafish tissues where these promoters are active. Inhibition of Meis blocks gene expression and reduces AcH4 levels at these promoters, suggesting a role for Meis in maintaining AcH4. Within Hox transcription complexes, Meis binds directly to Pbx and we find that this binding displaces histone deacetylases (HDACs) from Hoxb1-regulated promoters in zebrafish embryos. Accordingly, Pbx mutants that cannot bind Meis act as repressors by recruiting HDACs and reducing AcH4 levels, while Pbx mutants that bind neither HDAC nor Meis are constitutively active and recruit CBP to increase AcH4 levels. We conclude that Meis acts, at least in part, by controlling access of HDAC and CBP to Hox-regulated promoters.

INTRODUCTION

Hox proteins are transcription factors that control anteroposterior body axis formation in animal embryos by regulating gene expression in discrete domains (reviewed in McGinnis and Krumlauf, 1992). In many situations, Hox proteins form complexes with Pbx and/or Meis cofactors—TALE class transcription factors that are broadly expressed in most tissues and at most stages of embryogenesis—to control transcription of target genes (reviewed in Mann and Affolter, 1998). There is great variability among Hox-regulated promoters both in their constellation of Meis, Pbx, and Hox binding sites and in the transcriptional outcome (activation versus suppression; Mann and Affolter, 1998). No general rules have emerged for how particular combinations of cofactors control transcriptional outcome, but it appears that the presence of Meis cofactors correlates with active transcription of Hox-regulated genes in many instances (Ferretti et al., 2000, 2005; Jacobs et al., 1999; Ryoo et al., 1999), although there are exceptions (Gebelein et al., 2002). In *Drosphila*, Meis proteins may act in part by facilitating nuclear translocation of Pbx proteins (Rieckhof et al., 1997), but some Meis activities require its DNA binding domain (Noro et al., 2006) and Meis proteins may not control Pbx nuclear translocation in other organisms (e.g., zebrafish; Choe et al., 2002; Vlachakis et al., 2001), suggesting that Meis cofactors have additional functions in activating transcription.

An evolutionarily conserved Hox-regulated cascade is required for activation of paralog group 1 (PG1) and PG2 *hox* gene expression in rhombomere 4 (r4) of the vertebrate hindbrain, and this cascade requires Meis function (Choe et al., 2002; Ferretti et al., 2000, 2005; Jacobs et al., 1999; McClintock et al., 2002; Pöpperl et al., 1995, 2000; Waskiewicz et al., 2001). In zebrafish (Figure 1A), this cascade is initiated by Hoxb1b, which activates *hoxb1a* expression. Once Hoxb1a is expressed, it can maintain its own expression in r4. In addition, Hoxb1b and Hoxb1a activate *hoxb2a* expression in r4. Importantly, Meis and Pbx cofactors are required for Hoxb1b and Hoxb1a to drive PG1 and PG2 gene expression in zebrafish r4 (Choe et al., 2002; Choe and Sagerström, 2005; Pöpperl et al., 2000; Vlachakis et al., 2001; Waskiewicz et al., 2001, 2002). We have used this simple Hox-regulated cascade to examine the function of Meis proteins and we find that Meis cofactors control accessibility of HDAC and CBP histone modification enzymes to Hox-regulated promoters during zebrafish development.

RESULTS AND DISCUSSION

Meis Proteins Are Required for Histone H4 Acetylation at the *hoxb1a* and *hoxb2a* Promoters

We initially dissected 12–14 hpf embryos (Figure 1B) into posterior pieces (that express *hoxb1a*) and anterior pieces (that do not express *hoxb1a*). ChIP analysis using antibodies raised to endogenous proteins revealed that Pbx, Meis, and Hoxb1a/b occupy the *hoxb1a* promoter in tissues expressing *hoxb1a*, but not in nonexpressing tissues (Figure 1C). The *hoxb2a* promoter is similarly occupied by Pbx, Meis, and Hoxb1a/b in posterior, but not anterior, tissues (Figure S1, see the Supplemental Data available online). We next examined acetylation of histone H4 (AcH4), a marker of transcriptionally active chromatin, at the *hoxb1a* and *hoxb2a* promoters. As expected, ChIP analysis revealed a higher level of AcH4 at the *hoxb1a* and *hoxb2a* promoters in posterior tissues (where both genes are expressed) than in anterior (nonexpressing) tissues (Figure 1D). In contrast,

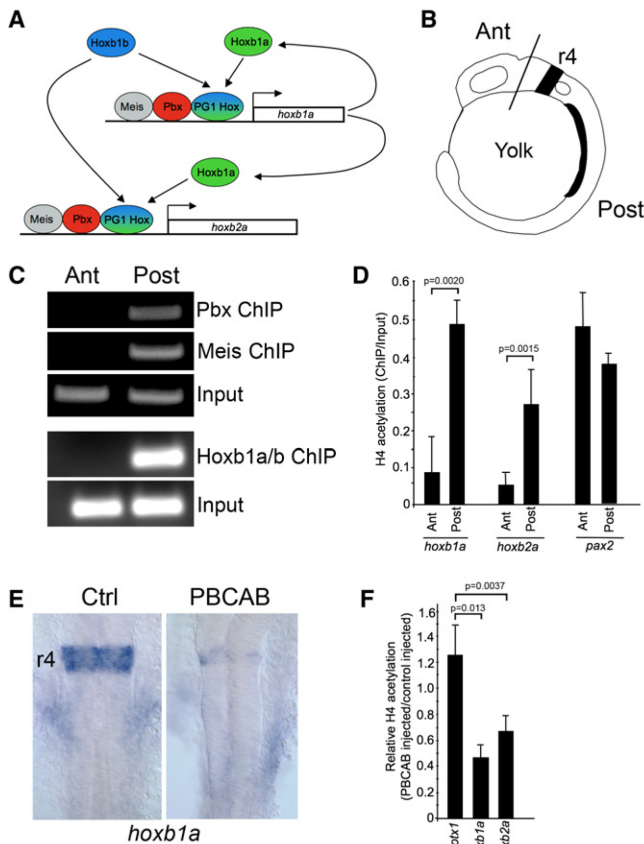

Figure 1. Meis Proteins Are Required for Histone H4 Acetylation at Hox-Regulated Promoters

(A) Diagram of *hoxb1a* and *hoxb2a* regulation by Hox, Pbx, and Meis factors in zebrafish rhombomere 4 (PG1, paralog group 1).

(B) Dissection of 14 hpf zebrafish embryos (black line indicates position of cut) produces a posterior piece (Post) expressing *hoxb1a* (black shading) and an anterior piece (Ant) not expressing *hoxb1a*.

(C) ChIP analysis reveals that Pbx, Meis, and Hoxb1a/b occupy the *hoxb1a* promoter in posterior (Post), but not anterior (Ant), tissues at 14 hpf.

(D) ChIP analysis of zebrafish embryos reveals that AcH4 is higher in posterior than anterior tissues for the *hoxb1a* and *hoxb2a* promoters, but not for the *pax2* promoter.

(E) Expression of a Meis dominant-negative construct (PBCAB; right panel) blocks *hoxb1a* expression (dark blue stain) in zebrafish r4 (compare to control embryo; left panel). Panels show close-ups of the hindbrain with anterior to the top.

(F) ChIP analysis reveals that using a dominant-negative construct (PBCAB) to interfere with Meis function reduces AcH4 at the *hoxb1a* and *hoxb2a* promoters, but not at the *otx1* promoter, in zebrafish embryos. Data are expressed as the ratio of PBCAB-injected/control-injected embryos.

Error bars indicate standard deviation calculated from a minimum of three experiments.

the *pax2* promoter, which is expressed in both anterior and posterior tissues independently of Hox proteins, shows similar levels of AcH4 in both tissues (Figure 1D).

Various dominant-negative constructs have been used to interfere with Meis function in zebrafish and *Xenopus* embryos (Choe et al., 2002; Dibner et al., 2001; Waskiewicz et al., 2001). In particular, the PBCAB dominant-negative construct blocks the function of all known zebrafish Meis/Prep proteins by preventing their nuclear translocation (without affecting the nuclear localization of Pbx or Hox proteins; Choe et al., 2002; Choe and Sagerström, 2004) and interferes with transcription of *hoxb1a* and *hoxb2a* in r4 (Choe et al., 2002) (Figure 1E and Figure S1C). Using the PBCAB construct, we find that embryos with reduced Meis function have reduced AcH4 levels at both the *hoxb1a* and the *hoxb2a* promoter (Figure 1F), while total H4 levels are unaffected at both promoters (Figure S1D). As expected, Meis proteins are not detected at the promoter in embryos expressing the PBCAB dominant-negative construct (Figure S1E). We note that Pbx, and perhaps Hoxb1a/b, also are not detected, likely due to chromatin compaction resulting from the loss of AcH4. These results indicate that Meis proteins may be required for transcription of Hox-regulated genes by acting at, or upstream of, the histone H4 acetylation step.

Meis Proteins Overcome HDAC-Mediated Repression of the *hoxb1a* Promoter

While Meis proteins have not been shown to regulate histone acetylation, Pbx proteins reportedly interact with histone deacetylases (HDACs) (Saleh et al., 2000). To explore this further, we identified a zebrafish *hoxb1a* promoter fragment that contains all Meis-, Pbx-, and Hox-binding sites previously identified as necessary for expression of mouse *hoxb1* in r4 (Ferretti et al., 2000, 2005; Jacobs et al., 1999). This fragment is sufficient to recapitulate the *hoxb1a* expression pattern in zebrafish embryos (Figure S2A and S2B) and we used it for reporter assays in HEK293 cells (experiments in HeLa cells yielded similar results; Figure S3). We find that transfecting Meis3 or Pbx4 has only limited effects on expression of the reporter, whereas transfecting Hoxb1b induces expression ~6-fold (Figure 2A, columns 1–4). This Hoxb1b-mediated activation is dependent on endogenous Pbx, since BMHoxb1b (a point mutant that cannot bind Pbx; Vlachakis et al., 2001) does not activate the reporter (Figure 2A, column 5). Cotransfecting Pbx4 with Hoxb1b leads to a dose-dependent reduction in reporter activation (Figure 2A, compare columns 7 and 8 to column 4). This effect has been reported previously as being caused by Pbx recruiting HDACs to repress transcription of Hox-regulated genes (Saleh et al., 2000). Accordingly, we observe that treatment with the HDAC inhibitor Trichostatin A (TSA) restores reporter expression to cells transfected with Pbx4 and Hoxb1b (Figure 2A, compare columns 7 and 8 to column 9). We hypothesized that Meis proteins might act to overcome this HDAC-mediated repression. Indeed, cotransfecting Meis3 together with Pbx4 and Hoxb1b restores expression of the reporter similar to treatment with TSA (Figure 2A, column 11). In contrast, BMMeis3 (a Meis3 mutant that cannot bind Pbx; Vlachakis et al., 2001) does not restore expression (Figure 2A, column 12), indicating that Meis3 must bind Pbx4 to overcome HDAC-mediated repression. Accordingly, blocking endogenous Meis activity by the PBCAB dominant-negative construct blocks reporter activation by Hoxb1b (Figure 2A, compare column 6 to column 4).

We next generated a series of Pbx4 deletion constructs (Figure 2B, top panel) and tested their ability to bind Meis and/or HDAC (Figure 2B, bottom panel). We find that ΔPBCA2, ΔPBCA3, and ΔPBCB5 do not bind Meis3 (Figure 2B, lanes 13–15), but vary in their ability to bind HDAC1 such that ΔPBCA2 binds HDAC1 at wild-type levels (Figure 2B, lane 18), ΔPBCA3 partially binds

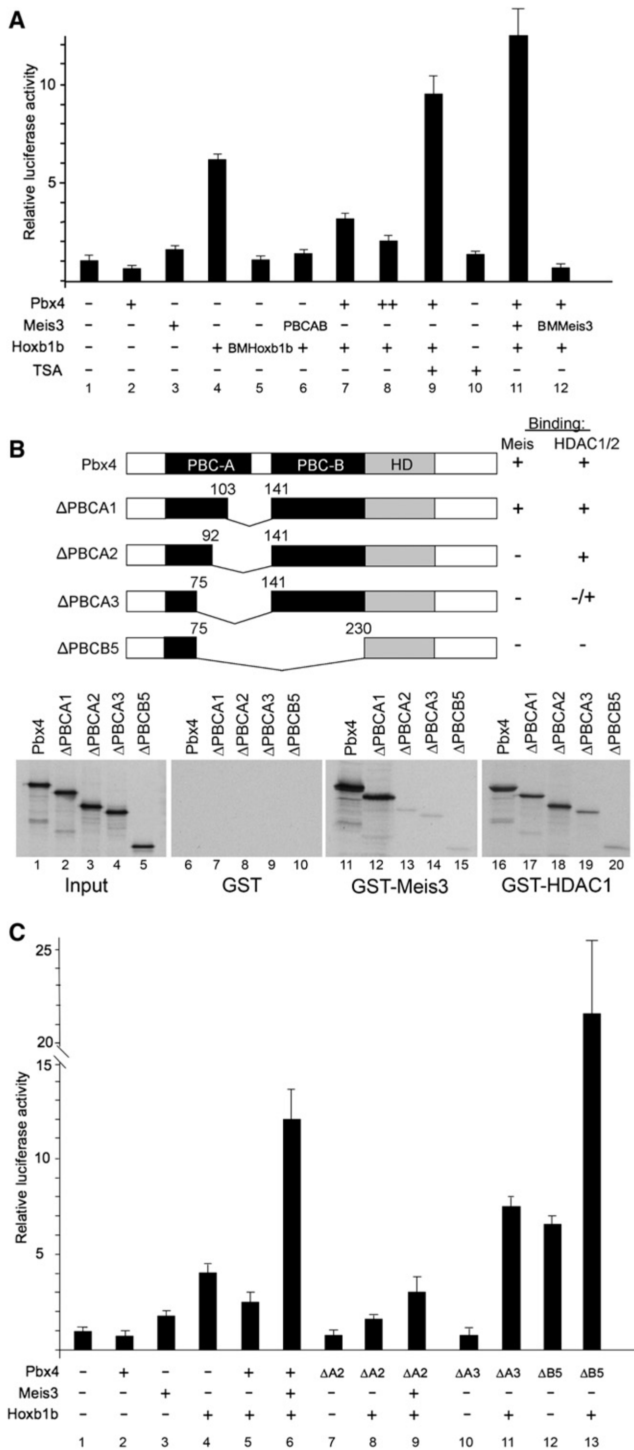

Figure 2. Meis Proteins Counteract HDAC Activity at the *hoxb1a* Promoter

(A) A *hoxb1a:luciferase* reporter was cotransfected into HEK293 cells with expression vectors as indicated below the graph. Data are expressed as fold increase in luciferase activity over transfection with reporter construct alone and are normalized for transfection efficiency by inclusion of a *renilla* luciferase control plasmid. BMHoxb1b, Hoxb1b mutant that does not bind Pbx proteins; PBCAB, Meis dominant negative; BMMeis3, Meis3 mutant that does not bind Pbx proteins; TSA, HDAC inhibitor Trichostatin A.

(B) Deletions of the Pbx4 protein generate constructs that bind HDAC but not Meis (ΔPBCA2), that partially bind HDAC but not Meis (ΔPBCA3), and that bind neither HDAC nor Meis (ΔPBCB5). GST pull-down data (bottom panel) and a summary of the deletion constructs (top panel) are shown.

(C) A *hoxb1a:luciferase* reporter was cotransfected with expression vectors as indicated below the graph. Data are expressed as fold increase in luciferase activity over transfection with reporter construct alone and are normalized for transfection efficiency by inclusion of a *renilla* luciferase control plasmid (ΔA2, ΔPBCA2; ΔA3, ΔPBCA3; ΔB5, ΔPBCB5).

Error bars indicate standard deviation calculated from a minimum of three experiments.

HDAC1 (Figure 2B, lane 19), and ΔPBCB5 binds HDAC1 only very weakly (Figure 2B, lane 20). Testing these deletion constructs in the reporter assay revealed that ΔPBCA2 acts similarly to wild-type Pbx4 in its ability to repress the reporter when cotransfected with Hoxb1b (Figure 2C, compare columns 5 and 8 to column 4), consistent with ΔPBCA2 retaining HDAC1 binding. However, while Meis3 substantially enhances reporter activation in cells cotransfected with Pbx4 and Hoxb1b (Figure 2C, column 6), it has minimal effect in cells transfected with ΔPBCA2 and Hoxb1b (Figure 2C, column 9), consistent with Meis only overcoming HDAC-mediated repression if it can bind Pbx. In contrast, cotransfection of ΔPBCA3 or ΔPBCB5 with Hoxb1b leads to robust activation of the reporter even in the absence of cotransfected Meis3 (Figure 2C, columns 11 and 13), indicating that if Pbx4 cannot bind HDACs, Meis is no longer needed to induce expression. Interestingly, this suggests that various Meis domains, including C-terminal activation domains identified in some studies (e.g., Huang et al., 2005), may not be required for Hox-mediated transcription, at least under our conditions. We also note that ΔPBCB5 is about three times more active than ΔPBCA3, consistent with ΔPBCA3 retaining partial HDAC binding (Figure 2B). Together, these findings indicate that Meis binds Pbx4 to overcome HDAC-mediated repression.

Meis Proteins Counteract HDAC Activity and Increase Histone H4 Acetylation at Hox-Regulated Promoters in Zebrafish Embryos

We next tested if the ΔPBCA2 and ΔPBCB5 constructs have the predicted effect on histone H4 acetylation and expression of Hox-regulated promoters in zebrafish embryos. Specifically, since ΔPBCA2 binds HDAC, but not Meis, we expect it to recruit HDACs, promote histone deacetylation, and repress expression of endogenous Hox-target genes. Indeed, expressing ΔPBCA2 in wild-type embryos reduces AcH4 at the *hoxb1a* and *hoxb2a* promoters (Figure 3B), similar to the effect observed using PBCAB to block endogenous Meis function (Figure 1F), while the *otx1* promoter is unaffected. Analysis of *hoxb1a* expression (Figure 3A) revealed only weak repression by ΔPBCA2 in wild-type embryos. We reasoned that this mild effect might be due to competition from endogenous Pbx for binding to the *hoxb1a* promoter and therefore assayed *lazarus* (*lzr*) mutant embryos that retain maternal Pbx4, but lack zygotic Pbx4 (Pöpperl et al., 2000). We find that ΔPBCA2 leads to near complete repression of *hoxb1a* in *lzr* embryos (Figure 3A). Notably, a mutant form of ΔPBCA2 that cannot bind DNA has no effect on *hoxb1a* expression in *lzr* embryos, demonstrating that ΔPBCA2 must bind DNA to mediate its effect.

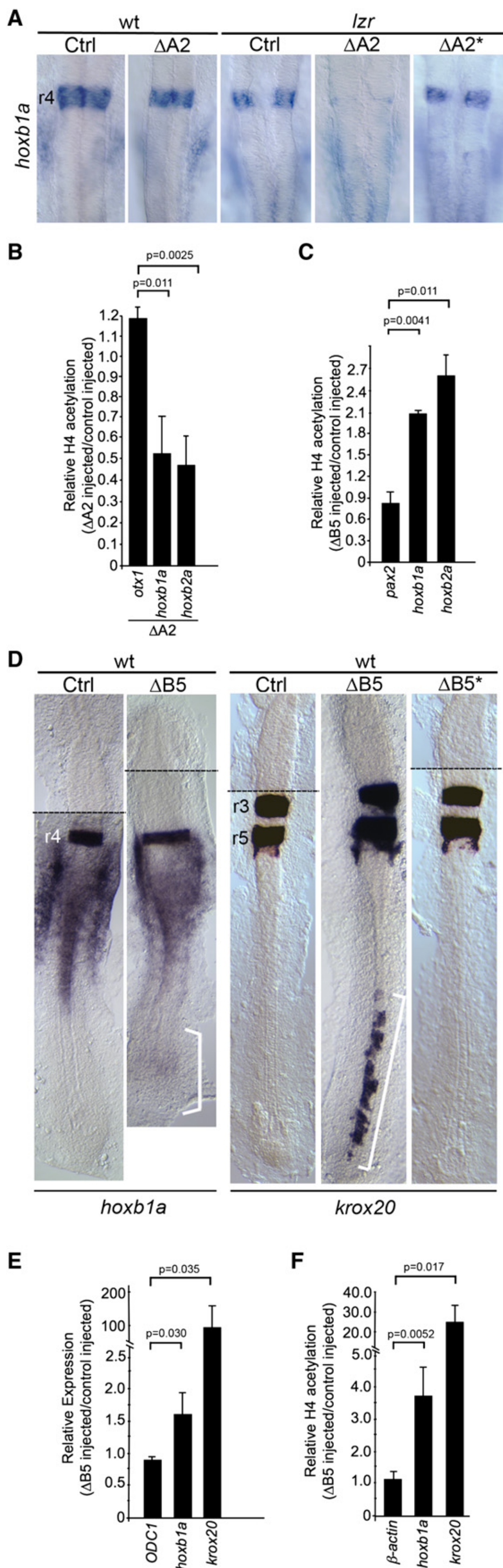

Figure 3. The ΔPBCA2 and ΔPBCB5 Constructs Modulate H4 Acetylation and Expression of Hox-Regulated Promoters in Zebrafish Embryos

(A) Expression of the ΔPBCA2 construct has little effect on *hoxb1a* expression in wild-type embryos, but reduces *hoxb1a* expression (dark blue stain) in r4 of *lazarus* (*pbx4* mutant) embryos. Expression of a ΔPBCA2 mutant that cannot bind DNA (ΔA2*) has no effect. Panels show close-ups of the hindbrain with anterior to the top.

(B) ChIP analysis at 22 hpf reveals reduced AcH4 at the *hoxb1a* and *hoxb2a* promoters, but not at a control promoter, in ΔPBCA2-injected embryos. Data are expressed as the ratio of ΔPBCA2-injected/control-injected embryos.

(C) ChIP analysis at 15 hpf reveals increased AcH4 at the *hoxb1a* and *hoxb2a* promoters, but not at a control promoter, in ΔPBCB5-injected embryos. Data are expressed as the ratio of ΔPBCB5-injected/control-injected embryos.

(D) Expression of the ΔPBCB5 construct induces ectopic expression of *hoxb1a* and *krox20* (white brackets) in zebrafish embryos. A ΔPBCB5 mutant (ΔB5*) that cannot bind DNA has no effect. Panels show flat-mounted embryos with anterior to the top. Dashed lines indicate where two images have been merged.

(E) Quantitative RT-PCR analysis reveals increased expression of *hoxb1a* and *krox20*, but not *ODC1,* in the tail region (white brackets in [D]) of ΔPBCB5-injected embryos. Data are expressed as the ratio of ΔPBCB5-injected/control-injected embryos.

(F) ChIP analysis reveals increased AcH4 at the *hoxb1a* and *krox20* promoters, but not a control promoter, in the tail region (white brackets in [D]) of ΔPBCB5-injected embryos. Data are expressed as the ratio of ΔPBCB5-injected/control-injected embryos.

Error bars indicate standard deviation calculated from a minimum of three experiments.

Based on its activity in the reporter assay, the ΔPBCB5 construct, which does not bind HDACs or Meis, is expected to promote histone acetylation and expression of endogenous Hox-target genes. Indeed, expression of ΔPBCB5 increases AcH4 at the *hoxb1a* and *hoxb2a* promoters 2–2.5-fold (Figure 3C) and induces weak ectopic *hoxb1a* expression posteriorly (bracket in Figure 3D) in wild-type embryos. Notably, *hoxb1b* is expressed in this posterior domain during gastrula and early segmentation stages (when *hoxb1a* expression is initiated), consistent with ΔPBCB5 acting together with endogenous Hoxb1b to drive this ectopic *hoxb1a* expression. Dissections allowed us to focus specifically on this posterior tail domain and, using quantitative RT-PCR, we find that ΔPBCB5 induces *hoxb1a* expression 1.5–2-fold in this domain (Figure 3E). ChIP analysis of dissected tail regions also reveals a 3–4-fold increase in AcH4 at the *hoxb1a* promoter in this posterior domain (Figure 3F). The modest induction of ectopic *hoxb1a* expression in this posterior domain is likely due to the presence of factors that repress *hoxb1a* expression posterior to r4 (e.g., *vhnf1*; Hernandez et al., 2004; Sun and Hopkins, 2001; Wiellette and Sive, 2003). We reasoned that the *hoxb1a(*$\beta-$*globin):eGFP*^{um8} transgenic line (that contains only a 1.0 kb fragment from the *hoxb1a* promoter; Figures S2A and S2B) might be less susceptible to such repression. Indeed, ΔPBCB5 induces robust ectopic expression of the transgenic promoter (Figure S2C). We also examined the effect of ΔPBCB5 on *krox20* expression. *krox20* was recently shown to contain a Meis:Pbx:Hox-regulated element in its enhancer (Wassef et al., 2008) and we confirmed that this element is occupied by Meis, Pbx, and Hox proteins (Figure S4). *krox20* is normally expressed in rhombomeres 3 and 5, but we find that *krox20* expression is strongly upregulated

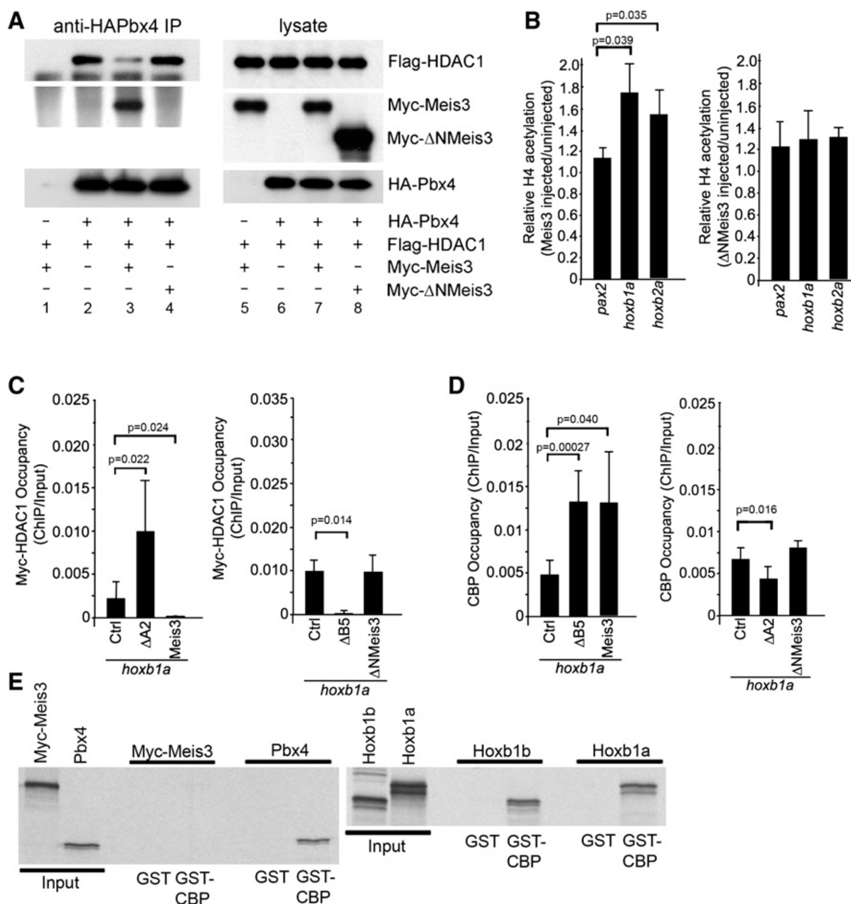

Figure 4. Meis Proteins Compete with HDACs for Binding to Pbx In Vitro and Modulate HDAC and CBP Accessibility to Hox-Regulated Promoters in Zebrafish Embryos

(A) Competitive coimmunoprecipitation experiment. HEK293 cells were transfected as indicated below the blots. After 48 hr, cells were lysed and either immunoprecipitated with anti-HA (to precipitate HA-Pbx4; lanes 1–4) or run as lysate (lanes 5–8), followed by western blotting as indicated to the right. ΔNMeis3, Meis3 mutant that cannot bind Pbx proteins.

(B) ChIP analysis reveals increased AcH4 at the *hoxb1a* and *hoxb2a* promoters, but not at the *pax2* promoter, in Meis3-injected embryos (left panel). ΔNMeis3 has no effect on AcH4 levels (right panel). Data are expressed as the ratio of injected/uninjected embryos.

(C) ChIP analysis of zebrafish embryos reveals that Myc-HDAC1 (Myc-HDAC1 mRNA was injected to partially label the cellular HDAC1 pool) occupancy at the *hoxb1a* promoter is increased by ΔPBCA2, but decreased by Meis3 and ΔPBCB5.

(D) ChIP analysis reveals that CBP occupancy at the *hoxb1a* promoter in zebrafish embryos is increased by ΔPBCB5 and Meis3, but decreased by ΔPBCA2.

(E) GST pull-down experiments demonstrate that Pbx4, Hoxb1a, and Hoxb1b, but not Myc-Meis3, bind CBP.

Error bars indicate standard deviation calculated from a minimum of three experiments.

posteriorly in ΔPBCB5-injected embryos (Figures 3D and 3E). This upregulation is accompanied by a substantial increase in AcH4 at the *krox20* promoter (Figure 3F), confirming that an ectopic gene expression program is initiated posteriorly in ΔPBCB5-injected embryos. We conclude that ΔPBCA2 (which binds HDAC, but not Meis) promotes histone deacetylation and represses transcription, while ΔPBCB5 (which binds neither HDAC nor Meis) promotes histone acetylation and activates transcription, consistent with our conclusion from Figure 2 that Meis binds Pbx to overcome HDAC-mediated repression of Hox-target genes.

Meis Proteins Control HDAC and CBP Accessibility at the *hoxb1a* Promoter by Competing for Pbx Binding

We note that our observations can be explained if Meis proteins compete with HDACs for binding to Pbx. Indeed, our deletion analyses (Figure 2B) suggest that the sites required for Meis and HDAC binding reside near one another in Pbx4. Consistent with this hypothesis, coimmunoprecipitation revealed that HDAC1 interacts with Pbx4 following cotransfection into HEK293 cells (Figure 4A, lane 2). When Meis3 is cotransfected with Pbx4 and HDAC1, it replaces HDAC1 as the Pbx4 interaction partner (Figure 4A, lane 3), demonstrating that Meis3 and HDAC1 compete for binding to Pbx4. Furthermore, ΔNMeis3 (a Meis3 binding mutant that cannot interact with Pbx proteins; Vlachakis et al., 2001) cannot displace HDAC1 from Pbx4 (Figure 4A, lane 4). This finding suggests that Meis proteins may act to displace HDACs from Pbx proteins bound to Hox-regulated promoters in vivo. To test this possibility directly, we again made use of ChIP analysis in zebrafish embryos. Although AcH4 is already present at the *hoxb1a* and *hoxb2a* promoters in control embryos (Figure 1), we find that injection of Meis3 can further increase AcH4 at the *hoxb1a* and *hoxb2a*, but not the *pax2*, promoter (Figure 4B, left panel), consistent with Meis displacing HDACs also in vivo. In contrast, the ΔNMeis3 construct, which does not bind Pbx, has no effect on AcH4 levels (Figure 4B, right panel). As observed for ΔPBCB5 injection in Figure 3, Meis3 injection does not induce ectopic *hoxb1a* expression (data not shown), but does induce ectopic expression from the *hoxb1a(β−globin):eGFP*^{um8} transgene (Figure S2C). We next assayed HDAC1 occupancy at the *hoxb1a* promoter in zebrafish embryos and found that overexpression of Meis3 or ΔPBCB5 decreases HDAC1 occupancy at the *hoxb1a* promoter (Figure 4C). Furthermore, expression of ΔPBCA2 increases HDAC1 occupancy at the *hoxb1a* promoter, likely because endogenous Meis proteins are unable to displace HDACs from ΔPBCA2 occupying the *hoxb1a* promoter, and the ΔNMeis3 construct has no effect. We conclude that Meis proteins bind Pbx to displace HDACs from the *hoxb1a* promoter in vivo.

Lastly, we considered that histone acetyl transferase (HAT) enzymes might need to be recruited to Meis:Pbx:Hox complexes in order to maintain high AcH4 levels and active transcription. In particular, several Hox proteins reportedly bind the CBP/p300 HAT enzyme (Chariot et al., 1999; Saleh et al., 2000). We

therefore examined *hoxb1a* promoter occupancy by CBP. We find that expression of Meis3 or ΔPBCB5 increases CBP occupancy at the *hoxb1a* promoter (Figure 4D). ΔPBCA2 reproducibly reduces CBP occupancy to a small extent, consistent with the mild effect of ΔPBCA2 on gene expression in wild-type embryos in Figure 3, while ΔNMeis3 has no effect on CBP occupancy (Figure 4D). While this finding explains how Meis proteins promote histone H4 acetylation at the *hoxb1a* promoter, it also suggests that Meis proteins do not recruit CBP directly, since ΔPBCB5 (that cannot bind Meis) is sufficient to increase CBP occupancy. Accordingly, we find that CBP does not bind Meis3, but binds both Pbx4 and PG1 Hox proteins (Figure 4E). We postulate that Meis proteins promote CBP recruitment indirectly, possibly by displacing HDACs to permit CBP binding. We conclude that Meis proteins are required as Hox cofactors because they modulate HDAC and CBP accessibility at Hox-regulated promoters.

EXPERIMENTAL PROCEDURES

DNA Constructs

All DNA constructs were generated using standard molecular biology techniques. See the Supplemental Experimental Procedures for detailed description.

Cell Culture, Transfection, and Luciferase Assays

HEK293 and HeLa cells were transfected with 0.5 μg of each expression plasmid using FuGENE 6 (Roche) and harvested after 36 hr. Luciferase activity was normalized using cotransfected *Renilla* luciferase. Trichostatin A (TSA) treatments were for 12 hr starting 24 hr after transfection. See the Supplemental Experimental Procedures for detailed description of methods used.

GST Pull-Down and Immunoprecipitation

GST pull-down and immunoprecipitation experiments were performed as described (Runko and Sagerström, 2003; Vlachakis et al., 2001). See the Supplemental Experimental Procedures for detailed description.

Microinjections, In Situ Hybridization, and Quantitative RT-PCR, qRT-PCR

ΔPBCA2 (700 pg), *ΔPBCA2** (700 pg), *ΔPBCB5* (500 pg), *ΔPBCB5** (500 pg), *PBCAB* (500 pg), *meis3* (500 pg), *ΔNMeis3* (500 pg) or *myc-HDAC1* (500 pg) mRNA were microinjected into 1–2-cell stage zebrafish embryos and raised. For qRT-PCR, cDNA was prepared from dissected embryos and subjected to quantitative PCR with gene specific primers (Table S1). In situ hybridizations were carried out as described previously (Choe et al., 2002; Choe and Sagerström, 2004). See Supplemental Experimental Procedures for detailed description of methods used.

Antisera and Chromatin Immunoprecipitation, ChIP

Polyclonal rabbit antisera were raised to full-length Meis3, Pbx4, and Hoxb1b. The Meis3 antiserum does not recognize Prep1, Pbx2, Pbx4, Hoxb1b, or Hoxb1a, but crossreacts weakly with Meis1, 2, and 4 (referred to as "Meis antiserum" above). The Hoxb1b antiserum does not recognize Pbx2, Pbx4, Prep1, Meis1, Meis2, Meis3, or Meis4, but crossreacts weakly with Hoxb1a (referred to as "Hoxb1a/b antiserum" above). The Pbx4 antiserum crossreacts with Pbx2, but does not recognize Prep1, Meis1, Meis2, Meis3, Meis4, Hoxb1b, or Hoxb1a (referred to as "Pbx antiserum" above). ChIPs were performed based on protocols published previously (Salma et al., 2004). Zebrafish embryos were dissociated and crosslinked in 1% formaldehyde. Genomic DNA was sheared to 200–1000 bp DNA fragments by sonication and 1% of sample volume (Input) was set aside for normalization. Samples were incubated with the appropriate antibody overnight, immune complexes were collected and washed, followed by reversal of crosslinks. Quantitative PCR was performed using promoter-specific primers (Table S1). PCR amplification was quantified and normalized to the corresponding input sample (1% of total input). Control amplifications using primers to the *hoxb1a* ORF did not yield signals above background. Control amplification from ChIPs using preimmune serum or no antibody was subtracted (except in Figures 1 and 3 where background was less than 1% of signal). Data are expressed as the average of a minimum of three experiments with error bars indicating standard deviation. Statistical significance was determined using the Student's t test in Microsoft Excel. See Supplemental Experimental Procedures for detailed description of methods used.

SUPPLEMENTAL DATA

Supplemental Data include four figures, one table, and Supplemental Experimental Procedures and can be found at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00344-X.

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