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# The Hox cofactors Meis1 and Pbx act upstream of *gata1* to regulate primitive hematopoiesis

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ABSTRACT

During vertebrate development, the initial wave of hematopoiesis produces cells that help to shape the developing circulatory system and oxygenate the early embryo. The differentiation of primitive erythroid and myeloid cells occurs within a short transitory period, and is subject to precise molecular regulation by a hierarchical cascade of transcription factors. The TALE-class homeodomain transcription factors Meis and Pbx function to regulate embryonic hematopoiesis, but it is not known where Meis and Pbx proteins participate in the hematopoietic transcription factor cascade. To address these questions, we have ablated Meis1 and Pbx proteins in zebrafish, and characterized their molecular effects on known markers of primitive hematopoiesis. Embryos lacking Meis1 and Pbx exhibit a severe reduction in the expression of *gata1*, the earliest marker of erythroid cell fate, and fail to produce visible circulating blood cells. Concomitant with a loss of *gata1*, Meis1- and Pbx-depleted embryos exhibit downregulated embryonic hemoglobin (*hbae3*) expression, and possess increased numbers of *pu.1*-positive myeloid cells. *gata1*-overexpression rescues *hbae3* expression in Pbx-depleted; *meis1*-morphant embryos, placing Pbx and Meis1 upstream of *gata1* in the erythropoietic transcription factor hierarchy. Our study conclusively demonstrates that Meis1 and Pbx act to specify the erythropoietic cell lineage and inhibit myelopoiesis.



## Introduction

Primitive hematopoiesis influences morphology of the developing embryonic circulatory system (Baumann and Dragon, 2005; Hove et al., 2003) and produces circulating erythrocytes that facilitate tissue oxygenation during periods of rapid embryonic growth (Orkin and Zon, 2008). Analyses in vertebrate models have identified a cascade of transcription factors that are critical for the specification of primitive erythrocytes. However, the upstream mechanisms by which these factors are regulated remain largely unclear. Previous research has shown that overexpressing posteriorly-expressed *hox* genes partially rescues erythropoietic gene expression in mutants with defects in primitive blood cell differentiation (Davidson et al., 2003; Davidson and Zon, 2006). These data support a model whereby Hox transcription factors serve to regulate primitive hematopoiesis. The Hox cofactors Meis1 and Pbx have also been implicated in hematopoiesis; *Pbx1*-knockout and *Meis1*-deficient mice exhibit profound embryonic anemia (Azcoitia et al., 2005; DiMartino et al., 2001; Hisa et al., 2004). Notably, the precise molecular function of Meis1 and Pbx in regulating primitive hematopoiesis remains to be elucidated, and it is not yet known where Meis1 and Pbx participate in the hematopoietic transcription factor hierarchy. In the present work, we analyze the

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function of Meis1 and Pbx by ablating these proteins and characterizing their molecular effects on known regulators of zebrafish primitive hematopoiesis.

Several lines of evidence suggest that Hox transcription factors act as master regulators of hematopoietic cell fate decisions (Abramovich and Humphries, 2005). Mice bearing deletions in *Hoxb3*, *Hoxb4*, *Hoxb6*, *Hoxa7*, *Hoxc8*, and/or *Hoxa9* possess defects in the development of multiple hematopoietic lineages (Brun et al., 2004; Izon et al., 1998; Kappen, 2000; Ko et al., 2007; Lawrence et al., 1997; Magnusson et al., 2007a; Shimamoto et al., 1999; So et al., 2004). In zebrafish, *hoxb6b*, *hoxb7a*, and *hoxa9a* regulate primitive erythropoiesis and contribute to hematopoietic stem cell formation (Davidson et al., 2003; Davidson and Zon, 2006). *Hox* genes are also implicated as proto-oncogenes in hematological malignancies (Kroon et al., 2001; Nakamura et al., 1996; Pineault et al., 2003; Slape and Aplan, 2004). The specificity of Hox proteins is achieved through their interaction with other DNA-binding cofactors (Mann, 1995; Mann and Affolter, 1998; Mann and Chan, 1996). Such cofactors include the Three Amino acid Loop Extension (TALE)-class homeodomain transcription factors Meis (Myeloid Ecotropic Integration Site), Pbx (Pre-B-Cell Leukemia Homeobox), and Prep/PKnox (Pbx Knotted Homeobox). Meis/Prep and Pbx coordinately bind DNA with Hox proteins, increasing their DNA-binding affinity as well as specificity (Berthelsen et al., 1998a; Chan and Mann, 1996; Chan et al., 1996; Chang et al., 1997; Ebner et al., 2005; Knoepfler et al., 1996; LaRonde-LeBlanc and Wolberger, 2003; Mann, 1995; Mann and Chan, 1996). Embryos lacking Meis and Pbx display phenotypes that are consistent with a total lack of Hox function. For example, loss of both Pbx2 and Pbx4 in the zebrafish hindbrain generates an anteriorizing homeotic transformation of the neural tube, in which rhombomeres 2–6 take on the molecular and neuronal identity of rhombomere 1 (Popperl et al., 2000; Waskiewicz et al., 2002). A nearly identical phenotype results from the knockdown of *Hoxa1*, *Hoxb1*, and *Hoxd1* gene products in *Xenopus* (McNulty et al., 2005). In zebrafish, overexpressing *meis3* in combination with *pbx4* and *hoxb1b* posteriorizes the neural tube, transforming the presumptive forebrain and midbrain regions into a hindbrain fate (Vlachakis et al., 2001). Combined, these data illustrate the significant role that TALE-class proteins play as Hox cofactors *in vivo*. It should be noted that Pbx and Meis/Prep1 proteins also form stable heterodimeric complexes in the absence of Hox proteins (Berthelsen et al., 1998b; Chang et al., 1997; Rieckhof et al., 1997), and are regulated both pre- and post-transcriptionally. For example, Meis1 is normally sequestered in the cytoplasm, but Pbx–Meis complexes are actively transported into the nucleus (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Jaw et al., 2000; Mercader et al., 1999; Rieckhof et al., 1997; Vlachakis et al., 2001). Meis and Pbx proteins also bidirectionally stabilize each other. This stabilization is dependent upon domains that mediate Meis–Pbx complex formation (Jaw et al., 2000; Longobardi and Blasi, 2003; Waskiewicz et al., 2001).

In addition to their role in hindbrain patterning, there is evidence that TALE-class proteins also play an important role in the regulation of embryonic hematopoiesis. *Pbx1*-knockout mice display a lethal reduction in definitive multipotent blood progenitors, leading to reduced hematocrit and severe embryonic anemia (DiMartino et al., 2001). *Meis1*-deficient mice display a severe reduction in myeloerythroid progenitors (Azcoitia et al., 2005; Hisa et al., 2004), and *Prep1*-deficient mice exhibit profound anemia (Di Rosa et al., 2007; Ferretti et al., 2006; Penkov et al., 2005). Although these studies demonstrate a strong correlation between Hox cofactor function and hematopoiesis, they fail to elucidate the precise molecular function of Meis and Pbx during blood cell differentiation.

In the present study, we provide evidence that Meis and Pbx proteins are essential regulators of zebrafish primitive hematopoiesis. We demonstrate that inhibiting zebrafish Meis1 and Pbx protein synthesis cripples the production of circulating erythrocytes, and generates defects in erythropoietic gene expression. We also demonstrate that Meis–Pbx complexes are required for proper expression of *gata1*, but are not required to initiate *scl* expression. This phenotype is strikingly different from that of a *cdx4*/*cdx1*-depleted zebrafish embryo, which completely lacks early *scl* expression. We propose a model placing Meis1 and Hox downstream of Cdx, and upstream of *gata1* in the molecular hierarchy of primitive hematopoiesis.

## Materials and methods

### *Zebrafish strains, genotyping, and morpholinos*

The b557 allele of *pbx4* (also known as *lazarus* or *lzr*) was originally identified through the altered hindbrain expression pattern of *egr2b* (*krox20*), as previously described (Popperl et al., 2000). Pbx-depleted embryos were generated by injecting one-cell stage embryos from a heterozygous mutant *lzr*$^{+/-}$ (*pbx4*$^{+/-}$) incross with a combination of the following four previously described Pbx translation-blocking morpholinos:

*pbx2*-MO1, CCGTTGCCTGTGATGGGCTGCTGCG (1 ng);
*pbx2*-MO2, GCTGCAACATCCTGAGCACTACATT (2 ng);
*pbx4*-MO1, AATACTTTTGAGCCGAATCTCTCCG (3 ng);
*pbx4*-MO2, CGCCGCAAACCAATGAAAGCGTGTT (3 ng) (Erickson et al., 2007).

This method yields 75% Pbx-depleted embryos (*lzr*$^{+/-}$ and *lzr*$^{-/-}$) and 25% partially-depleted embryos (*lzr*$^{+/+}$). The effectiveness of this approach at removing >95% of total Pbx protein has been documented using a pan-Pbx antibody (Maves et al., 2007; Waskiewicz et al., 2002). Pbx-depleted embryos are phenotypically indistinguishable from maternally and zygotically mutant *lzr* embryos injected with *pbx2* morpholinos, and were identified through *in situ* hybridization assays for the downregulation of *eng2a* (Erickson et al., 2007) and abrogation of *egr2b* (*krox20*) expression (Popperl et al., 2000; Waskiewicz et al., 2002).

Meis1-deficient embryos were generated by injecting one-cell AB embryos with 4 ng of translation-blocking *meis1* morpholino; GTATATCTTCGTACCTCTGCGCCAT, as previously described (French et al., 2007). The specificity of this morpholino was assessed through the observation of expected hindbrain phenotypes (French et al., 2007), mRNA rescue experiments (French et al., 2007), and immunohistochemical analysis of Meis1 protein levels using the P2A6 monoclonal antibody (Fig. 3).

Gata1-deficient embryos were generated by injecting one-cell AB embryos with 5 ng of translation-blocking *gata1* morpholino; CTGCAAGTGTAGTATTGAAGATGTC, as previously described (Galloway et al., 2005).

Cdx-depleted embryos were generated by injecting one-cell *cdx4* $^{-/-}$ (*kgg*$^{tv205}$) embryos with 5 ng of translation-blocking *cdx1a* morpholino; CAGCAGATAGCTCACGGACATTTTC, as previously described (Davidson and Zon, 2006).

### *Whole-mount in situ hybridization and histochemical staining*

Examination of gene expression by whole-mount *in situ* hybridization was performed essentially as previously described (French et al., 2009; Gongal and Waskiewicz, 2008). Proteinase K treatment (10 μg/ml) was performed for 30 s (12 to 16 hpf embryos) and 3 min (24 hpf embryos). Two-color *in situ* and histochemical staining of hemoglobin by o-dianisidine (Sigma) were preformed as previously described (Erickson et al., 2007; Lieschke et al., 2001).

Embryos were photographed using a Zeiss AxioImager Z1 compound microscope with an Axiocam HR digital camera under a 10× objective, using an Olympus stereoscope with a QImaging micropublisher camera, or using a Leica stereoscope with a Leica