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Coordinate regulation of retinoic acid synthesis by *pbx* genes and fibroblast growth factor signaling by *hoxb1b* is required for hindbrain patterning and development

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ABSTRACT

The vertebrate hindbrain is composed of a series of lineage-restricted segments termed rhombomeres. Segment-specific gene expression drives unique programs of neuronal differentiation. Two critical embryonic signaling pathways, Fibroblast Growth Factor (FGF) and Retinoic Acid (RA), regulate early embryonic rhombomere patterning. The earliest expressed *hox* genes, *hoxb1b* and *hoxb1a* in zebrafish, are logical candidates for establishing signaling networks that specify segmental identity. We sought to determine the mechanism by which *hox* genes regulate hindbrain patterning in zebrafish. We demonstrate that *hoxb1a* regulates r4-specific patterning, while *hoxb1b* regulates rhombomere segmentation and size. *Hoxb1a* and *hoxb1b* redundantly regulate *vhnf1* expression. Loss of *hoxb1b* together with *pbx4* reverts the hindbrain to a groundstate identity, demonstrating the importance of *hox* genes in patterning nearly the entire hindbrain, and a key requirement for Pbx in this process. Additionally, we provide evidence that while *pbx* genes regulate RA signaling, *hoxb1b* regulates hindbrain identity through complex regulation of FGF signaling.

1. Introduction

The vertebrate hindbrain is transiently divided into a series of lineage-restricted segments, known as rhombomeres. Segmental gene expression generates distinct populations of neurons (Moens and Prince, 2002) including both reticulospinal interneurons (Kimmel et al., 1982) and cranial branchiomotor neurons (BMN) (Chandrasekhar et al., 1997). Two key signaling pathways – Fibroblast Growth Factor (FGF) and Retinoic Acid (RA) – regulate early gene expression within the vertebrate hindbrain. Global loss of either FGF or RA signaling results in profound changes to reticulospinal and BMN differentiation (Alexandre et al., 1996; Holder and Hill, 1991; Maden and Holder, 1991; Maves et al., 2002; Maves and Kimmel, 2005; Papalopulu et al., 1991; van der Wees et al., 1998). Yet, the regulation of hindbrain FGF and RA signaling remains incompletely understood.

Hox (*homeobox transcription factor*) genes are evolutionarily conserved transcription factors that regulate anterior-posterior (A-P) patterning of hindbrain rhombomeres (McGinnis and Krumlauf, 1992). Hox factor binding and subsequent transcription of target genes is dependent on interactions with cofactors such as Meis (Myeloid ecotropic integration site) and Pbx (Pre-B cell leukemia homeobox)(Mann, 1995). Paralog Group 1 (PG1) *hox* genes are expressed early in hindbrain specification and development, with *hoxb1b* expression beginning at 50% epiboly in the presumptive hindbrain and *hoxb1a* expression beginning at tailbud, restricted to rhombomere 4 (r4), the first compartment of the hindbrain to form a distinct segment (Alexandre et al., 1996). Due to their early expression in the hindbrain, and their role as transcriptional regulators, PG1 Hox proteins are hypothesized to play an important role in establishing pattering and segmentation in the hindbrain. Loss of Hox cofactors Pbx2/4 in zebrafish abrogates expression of *hoxa2b*, *hoxb2a*, *hoxb1a*, *hoxb3a*, and *hoxa3* (defining a loss of segmental identity that spans r2-r6). Taken together with an expansion of r1 markers (*epha4a* and *fgfr1*), this implies that Pbx is necessary to define the identity of r2-r6 (Waskiewicz et al., 2002). The expression of *hoxb1b*, however, is normal in Pbx depleted embryos, and Pbx is known to act as a cofactor for all anterior Hox proteins tested (Cooper et al., 2003; Pöpperl et al., 2000; Waskiewicz et al., 2002). As such, researchers proposed a model in which Pbx-Hox-1 complexes lie at the top of a hierarchy to regulate and initiate hindbrain patterning and segmental gene expression. Consistent with this model, knockdown of *hoxa1/b1/d1* in *Xenopus laevis* causes a loss of segmental identity similar to that observed in Pbx-depleted zebrafish embryos (McNulty et al., 2005). However, murine *Hoxa1*;*Hoxb1* compound mutants have milder defects in hindbrain development, exhibiting a loss of r4 accompanied by alterations to the surrounding r3-6 regions (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). Similar phenotypes are observed in zebrafish

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mutants for *hoxb1b* and *hoxb1a*. *Hoxb1a* regulates r4 specific gene expression and associated neurons (Weicksel et al., 2014) whereas *Hoxb1b* is required for segmentation and rhombomere size, with additional phenotypes observed in neural crest, ear, and r4 associated neurons (Weicksel et al., 2014; Zigman et al., 2014). Compound loss of both *hoxb1a* and *hoxb1b* results in hindbrain segmentation defects similar to those observed in *hoxb1b* mutants (Weicksel et al., 2014; Zigman et al., 2014).

While the studies of Pbx-depleted embryos (Waskiewicz et al., 2002) and Hox-1 *Xenopus* morphants (McNulty et al., 2005) both show a loss of segmental identity, the loss of PG1 *Hox* genes in zebrafish and murine model have a much more subtle phenotype (Weicksel et al., 2014; Zigman et al., 2014). This paradox likely reflects a more complex role for Pbx in regulating hindbrain patterning than originally proposed, and this argues that other Pbx-dependent factors are required for hindbrain patterning. Therefore we hypothesize that hindbrain segmentation requires not only Hox-Pbx complexes, but also Pbx complexes with other cofactors than Hox, and the loss of both of these may be sufficient to prevent hindbrain segmentation.

The effectors of Pbx and Hox PG1 genes remain incompletely characterized. Given their broad effects on segmental patterning, it is logical to assume they have roles in regulating core signaling pathways, such as RA and FGF. RA synthesis is catalyzed by the rate-limiting Aldh1a2 (Aldehyde dehydrogenase 1 family, member A2; and also known as Retinaldehyde dehydrogenase or Raldh2) enzyme, which provides a source of RA posterior to the hindbrain (Niederreither et al., 1999; Niederreither et al., 2000). Cyp26 (Cytochrome P450 family 26) enzymes act to inhibit RA signaling by hydroxylating RA (Abu-Abed et al., 2001; Niederreither et al., 2002) in the forebrain, midbrain, and anterior hindbrain (de Roos et al., 1999; Hernandez et al., 2004; Sakai et al., 2001; Sirbu et al., 2005; Swindell and Eichele, 1999). This creates a gradient of RA across the A-P axis of the hindbrain where higher concentrations of RA activate progressively more posterior genes (Gavalas, 2002). Reduced RA levels result in the loss of posterior hindbrain identity (Begemann et al., 2001; Dupe and Lumsden, 2001; Maden et al., 1996) while increased levels of RA disrupt the development of the anterior hindbrain (Gould et al., 1998; Hill et al., 1995; Morrison et al., 1997), with such alterations commonly ascribed to changes in *hox* gene expression (Ferretti et al., 2000; Frasch et al., 1995; Gould et al., 1998; Morrison et al., 1997; Papalopulu et al., 1991; Yan et al., 1998). Additionally, there is evidence that *hox* and *pbx* genes act upstream of retinoic acid signaling. In mouse, loss of *Hoxa1/Pbx1* or *Pbx1/2* causes a reduction in *Aldh1a2* expression (Vitobello et al., 2011), and *pbx2/4* depleted zebrafish embryos also exhibit reduced *aldh1a2* expression in the trunk and retina. (French et al., 2007; Maves et al., 2007).

FGF signaling is frequently associated with the development of the midbrain, cerebellum, and the midbrain-hindbrain boundary (Meyers et al., 1998; Reifers et al., 1998; Sleptsova-Friedrich et al., 2001), however it is also required for inter-rhombomere signaling within the hindbrain. The FGF ligands, *fgf3* and *fgf8 (fgf8a)*, are expressed in the r4 signaling center and loss of these genes, or pharmacological inhibition thereof, disrupts the development of the surrounding rhombomeres, in particular r5/6 (Marin and Charnay, 2000; Maves et al., 2002; Walshe et al., 2002; Wiellette and Sive, 2004). *Hox* genes have been linked to regulation of the expression of FGFs in the hindbrain. The loss of *pbx2/4* results in a failure of *fgf3* and *fgf8* to be expressed in r4, and *hoxb1b* has been shown to indirectly regulate *fgf3* through the protein phosphatase *ppp1r14al* (Choe et al., 2011).

In this manuscript, we investigate the phenotypes of *hoxb1a*, *hoxb1b*, and *hoxb1a;hoxb1b* mutants to determine the respective and combinatorial roles of PG1 *hox* genes in regulating hindbrain RA and FGF signaling. Our work illustrates that *hoxb1b* is required for appropriate FGF signaling in the hindbrain, while PG1 *hox* genes do not appear to overtly regulate RA signaling. We uncover a novel role for *Hoxb1a/b* in regulating *vhnf1*. The loss of both *hoxb1b* and *pbx4* is required to revert the hindbrain to the r1 ground state, and this phenotype is plausibly explained by changes to RA signaling in combination with the *hoxb1b* driven changes to FGF signaling. Taken together, our results do not support a simple linear pathway in which PG1 Hox proteins function alone at the top of a hierarchy of hindbrain pattern formation. Instead, PG1 *hox* genes function in concert with *pbx* and other transcription factors to regulate FGF and RA signaling to establish hindbrain identity.

2. Results

2.1. Hoxb1a is required for r4 specification and hoxb1b regulates hindbrain segmentation and rhombomere size

To determine the role of zebrafish PG1 *hox* genes in regulating hindbrain signaling pathways, we created a *hoxb1b*^{ua1006} mutant through TALEN (TAL effector nuclease) mediated mutagenesis (Cermak et al., 2011). An allele containing a 13 bp insertion 63 bp downstream of the start codon was identified (Fig. 1A). This mutation is predicted to generate a 43 amino acid long protein where the first 21 amino acids correspond to the correct wildtype Hoxb1b protein (G22FfsX23). This truncated protein completely lacks the DNA-binding homeodomain and is predicted to be a hypomorph and lack transcriptional activity. We obtained from the Sanger institute (Kettleborough et al., 2013), a *hoxb1a*^{sa1191} mutant containing a G to A transition resulting in a stop codon at amino acid 269 (W269X) (Fig. 1B). This allele is predicted to cause a truncation within the homeodomain and lack amino acids critical for homeodomain DNA binding activity (McClintock et al., 2001; Scott et al., 1989), likely resulting in a hypomorph. To determine if the *hoxb1b*^{ua1006} and the *hoxb1a*^{sa1191} mutations retain biological activity, we assayed for the ability of the mutant *hoxb1b or hoxb1a* mRNA to cause homeotic transformations (Fig. 1C–H). Overexpression of wildtype *hoxb1b* causes an anterior duplication of r4, resulting in ectopic expression of *hoxb1a* transcript in presumptive r2 (McClintock et al., 2001) (Fig. 1D; 66/88 embryos affected). In contrast, overexpression of the mutant *hoxb1b*^{ua1006} mRNA failed to produce an anterior duplication of r4 (Fig. 1E; 0/82 embryos affected) providing evidence that the mutant Hoxb1b protein has strongly reduced biological activity. Similarly, overexpression of wildtype *hoxb1a* causes ectopic expression of *hoxb1a* transcript in presumptive r2 (McClintock et al., 2001) (Fig. 1G; 30/34 embryos affected). Overexpression of the mutant *hoxb1a*^{sa1191} mRNA failed to produce an anterior duplication of r4 (Fig. 1H; 0/38 embryos affected) providing evidence that the mutant Hoxb1a protein also has strongly reduced biological activity.

Previous work has shown that knockdown of paralog group 1 *hox* genes in zebrafish results in mild morphological phenotypes (McClintock et al., 2002). We examined zebrafish homozygous for *hoxb1a*$^{sa1191/sa1191}$ (hereafter referred to as *hoxb1a*$^{-/-}$; embryos genotyped for the *sa1191* allele via dCaps PCR, see Section 4.4 for details) and homozygous for *hoxb1b*$^{ua1006/ua1006}$ (hereafter referred to as *hoxb1b*$^{-/-}$; embryos genotyped for the *ua1006* allele via PCR, see Section 4.4 for details) for morphological defects (Fig. S1A–L). *Hoxb1a*$^{-/-}$ mutants lack any major morphological defects, and *hoxb1b*$^{-/-}$ mutants have a small otic vesicle (Fig. S1A–C, E–G, I–K). *Hoxb1a*$^{-/-}$*;hoxb1b*$^{-/-}$ double mutants display a small otic vesicle accompanied by cardiac edema and die between 3 dpf and 4 dpf (Fig. S1A, D, E, H, I, L). These phenotypes are consistent with a role for PG1 *hox* genes in hindbrain development, as indicated by changes in otic vesicle size, and additionally provide evidence that the concurrent loss of both *hoxb1a* and *hoxb1b* results in more severe defects in embryonic development.

Researchers have previously examined rhombomere boundary specification in zebrafish *hoxb1b*$^{-/-}$ mutants (expression of *sema3gb* (Zigman et al., 2014)). We wished to extend this analysis to *hoxb1a*$^{-/-}$ and embryos doubly homozygous for mutations in *hoxb1a* and *hoxb1b*. We find that embryos lacking *hoxb1a*$^{-/-}$ are overtly normal (*mariposa (forkhead box B1a; foxb1a*; Fig. 2A,B), whereas embryos lacking *hoxb1b* display defects to r2/3, r3/4 and r6/7 boundaries (Fig. 2A,C,