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A screen for *hoxb1*-regulated genes identifies *ppp1r14al* as a regulator of the rhombomere 4 Fgf-signaling center

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ABSTRACT

Segmentation of the vertebrate hindbrain into multiple rhombomeres is essential for proper formation of the cerebellum, cranial nerves and cranial neural crest. Paralog group 1 (PG1) *hox* genes are expressed early in the caudal hindbrain and are required for rhombomere formation. Accordingly, loss of PG1 *hox* function disrupts development of caudal rhombomeres in model organisms and causes brainstem defects, associated with cognitive impairment, in humans. In spite of this important role for PG1 *hox* genes, transcriptional targets of PG1 proteins are not well characterized. Here we use ectopic expression together with embryonic dissection to identify novel targets of the zebrafish PG1 gene *hoxb1b*. Of 100 genes up-regulated by *hoxb1b*, 54 were examined and 25 were found to represent novel *hoxb1b* regulated hindbrain genes. The *ppp1r14al* gene was analyzed in greater detail and our results indicate that Hoxb1b is likely to directly regulate *ppp1r14al* expression in rhombomere 4. Furthermore, *ppp1r14al* is essential for establishment of the earliest hindbrain signaling-center in rhombomere 4 by regulating expression of *fgf3*.

Introduction

hox genes were first identified in *Drosophila* genetic screens as important regulators of embryonic development (reviewed in (Lewis, 1994)). In particular, mutations in *hox* genes give rise to homeotic phenotypes where one body structure is transformed, more or less completely, into a different structure. Subsequently, *hox* genes were shown to carry out analogous functions in vertebrates (reviewed in (Krumlauf, 1994)). In vertebrates, genome duplications have produced four *hox* clusters, except in teleost fish that contain seven clusters as the result of an additional duplication event (Amores et al., 1998; Kuraku and Meyer, 2009). *hox* genes that occupy the same position in each cluster are referred to as paralogous genes (reviewed in (Alexander et al., 2009)) and their expression is co-linear with their position in the *hox* clusters such that 3′ genes are expressed earlier and further anteriorly than 5′ genes. Accordingly, the earliest expressed vertebrate *hox* genes belong to paralog group 1 (PG1).

PG1 *hox* genes act together with *hox* genes from PG2, 3 and 4 to regulate formation of the caudal hindbrain. In particular, PG1–4 *hox* genes act to impart distinct identities on rhombomeres 4–7. In the mouse, *Hoxa1*, which is expressed in the caudal hindbrain, is the earliest-acting *hox* gene and it is required for appropriate patterning of rhombomere (r) 4, 5 and 6 (Carpenter et al., 1993; Mark et al., 1993). *Hoxa1* is also required to activate *Hoxb1*, which is expressed exclusively in r4. Accordingly, in *Hoxb1* mutants, r4 is misidentified and takes on r2-like characteristics (Goddard et al., 1996; Studer et al., 1996). In addition, *Hoxa1*/*Hoxb1* double mutants show a more severe phenotype than the single mutants (Gavalas et al., 1998, 2001; Rossel and Capecchi, 1999; Studer et al., 1998), indicating that these *hox* genes may regulate non-overlapping sets of genes. Notably, the additional genome duplication in teleosts has led to a re-shuffling of responsibilities among *hox* genes in zebrafish. In particular, the only zebrafish *hoxa1* gene (*hoxa1a*) is not expressed in the hindbrain. Instead, a second *hoxb1* copy (*hoxb1b*) has taken on the role performed by *Hoxa1* in the mouse, while zebrafish *hoxb1a* plays the same role as murine *Hoxb1* (McClintock et al., 2001, 2002). Hindbrain patterning and PG1 *hox* genes have been implicated in developmental defects during human development. In particular, mutations in *Hoxa1* have been linked to defects of the brainstem (which derives partly from the embryonic hindbrain) that are associated with some cases of autism (Bosley et al., 2007; Tischfield et al., 2005).

Secreted factors are also required for hindbrain patterning. Indeed, one of the earliest events during hindbrain patterning is the establishment of a signaling center in r4 that secretes Fgf3 and Fgf8 (Maves et al., 2002; Walshe et al., 2002). Fgf3 and Fgf8 are required for proper formation of r5 and r6, apparently by acting together with the *vhnf1* gene to regulate expression of *krox20* in r5 and *valentino* in r5/r6 (Hernandez et al., 2004; Wiellette and Sive, 2003). Nevertheless, it remains unclear how this r4 signaling center is set up and what role *hox* genes may play in this process.

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To fully understand the role of PG1 *hox* genes in hindbrain development, it is necessary to identify genes regulated – directly or indirectly – by PG1 proteins. Some direct PG1 target genes are known, but many such targets are other *hox* genes (e.g. *hoxb1*, *hoxa2*, *hoxb2*; (Maconochie et al., 1997; Popperl et al., 1995; Tumpel et al., 2007)) — although there are also examples of non-*hox* direct targets (e.g. *krox20*; (Wassef et al., 2008)). In terms of indirect target genes, any gene whose expression is lost in PG1 mutants would be a candidate, but in most cases it has not been determined if such genes can actually be induced by PG1 proteins. Here we take advantage of the ease of gene misexpression and dissection in zebrafish to identify genes induced by *hoxb1b*. We identify 100 genes that are up-regulated more than 2-fold by *hoxb1b*. Subsequent expression analysis of 54 genes revealed that 28 are expressed in hindbrain-associated structures. Three of these have been previously reported as expressed in the hindbrain, while the remaining 25 are either novel genes or known genes not previously reported as expressed in the hindbrain. Furthermore, 20 of the 28 hindbrain-associated genes show rhombomere-restricted expression. One r4-restricted gene, the protein phosphatase 1 regulatory subunit *ppp1r14al*, was selected for detailed analysis to determine its role in hindbrain development and to confirm its regulation by Hoxb1b. We find that *ppp1r14al* is required for *fgf3* expression in the r4 signaling center and that loss of *ppp1r14al* leads to defects in hindbrain patterning, as well as disruption of subsequent neurogenesis. Lastly, chromatin immunoprecipitation reveals that Hoxb1, as well as Pbx and Meis cofactors, occupy the *ppp1r14al* promoter in developing zebrafish embryos. Hence, our screening strategy efficiently identified bona fide *hoxb1b* target genes in zebrafish hindbrain development and identified a regulator of the r4-signaling center.

Materials and methods

Zebrafish

Zebrafish and their embryos were handled and staged according to standard protocols (Kimmel et al., 1995).

Microinjections and embryo dissection

All mRNAs for microinjections were synthesized in vitro using the SP6 mMessage mMachine kit (Ambion) as previously described (Vlachakis et al., 2001). For microarray experiments, *hoxb1b* + *meis3* (166 pg each) or *meis3* + *βgal* (166 pg each) were microinjected into 1–2 cell stage zebrafish embryos and raised to 14 hours post fertilization (hpf). Embryos were then manually dechorinated in fish Ringer solution on a 1% agarose-bed 35-mm culture dish. Anterior tissues were dissected and collected using a pair of forceps and were then resuspended in 750 μl of Trizol Reagent (Invitrogen) and stored at −80 °C. For morpholino (MO) injections, 4 ng of MO targeting the translational start site of *ppp1r14al* was microinjected into 1–2 cell stage of embryos. For double morpholino injections, 2 ng of *MOfgf8* was injected solely or in combination with 4 ng of *MOppp1r14al*. Rescue experiments were performed using 4 ng *MOppp1r14al* + 300 pg *ppp1r14al* mRNA compared to 4 ng *MOppp1r14al* + 300 pg *GFP* mRNA. The sequences of MO are as follows: *MOppp1r14al* 5′-CACCCGATTCG-CAGCCATCTCCAGA-3′, *MOfgf8* 5′-TCAACCGTGAAGGTATGAGTCTC-3′ (Maves et al., 2002). For rescue experiments, 6 nucleotides at the 5′ end of the *ppp1r14al* mRNA were changed using the primer 5′-GGAATTCGATGGCCGCCAACAGAGTCGGGAGGCG-3′ to prevent targeting by MOs, while encoding the same amino acids as in wild type *ppp1r14al*. Pbx MOs were reported previously (Waskiewicz et al., 2002).

RNA isolation and qRT-PCR

Total RNA from dissected anterior tissues was isolated using standard protocols and dissolved in 20 μL nuclease-free water (Ambion).1 μg total RNA per sample was shipped on dry ice for microarray analysis. For RT-PCR, cDNA was first synthesized using 1 μg total RNA, 200 U of Superscript III reverse transcriptase (Invitrogen), and 2.5 μM oligo dT primer in a 20 μL reaction for 2 h at 37 °C. Quantitative PCR was performed using QuantiFast SYBR Green PCR kit (Qiagen) using 500 ng of cDNA and gene specific primers in a 50 μL reaction and detected in a 7300 realtime PCR system (Applied Biosystems). Sequences of PCR primers were as follows: *tubulin*, 5′-CTGTTGACTACGGAAAGAAGT-3′ and 5′-TATGTGGACGCTCTATGTCTA-3′; *krox20*, 5′-AAACGCAGGA-GATGGCCTGA-3′ and 5′-GGTACTGGGAGTCGATGGAA-3′.

Microarray analysis

Microarray analysis was carried out by the Kimmel Cancer Center microarray facility at Thomas Jefferson University. Biotinylated cRNA probes were synthesized by linear amplification from total mRNA and hybridized to microarray slides containing 16,399 distinct 65-mer oligonucleotides (Compugen/Sigma-Genosys oligo set XEBLIB96), corresponding to approximately 12,500 zebrafish cDNAs. The experiments were performed in triplicate and each replicate array was hybridized with probe prepared from a separate injection and dissection. Background readings were subtracted from experimental readings, followed by normalization where each data point was divided by the 50th percentile of all data points. p-values were derived using Student's T-test. Genes up-regulated by *hoxb1b* + *meis3* were defined as follows: 1) up-regulation by more than 2-fold by *hoxb1b* + *meis3* as compared to *meis3*, and 2) a p-value lower than 0.05. Microarray data has been submitted to GEO under accession number GSE30632.

In situ hybridization

Plasmids containing zebrafish cDNA sequences were purchased from OpenBiosystems (Huntsville, AL). DIG-labeled antisense probes for *hoxb1b* + *meis3* target genes were synthesized using PCR-amplified DNA inserts from the plasmids whose inserts had been verified by sequencing. *hoxb1a*, *krox20*, *valentino*, *pax2*, *dlx2a* and *pea3* were described previously (Akimenko et al., 1994; Brown et al., 1998; Kiefer et al., 1996; Krauss et al., 1991; Moens et al., 1996; Oxtoby and Jowett, 1993; Prince et al., 1998). Plasmids containing *fgf20a* and *ngn1* cDNA was purchased from OpenBiosystems. In situ hybridizations were carried out as described previously (Choe et al., 2002).

Acridine orange staining

Acridine orange staining was performed as described previously (Kwak et al., 2006). Briefly, dechorinated embryos at desired stages were incubated in 0.2% acridine orange (Sigma) in phosphate-buffered saline (PBS) for 30 min at room temperature. Embryos were then washed 5 times with PBS and apoptotic cells were visualized under a UV microscope. Live images were captured using the SPOT software (version 4.6, SPOT imaging solutions).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously using antibodies to Hoxb1b/a, Pbx and Meis (Choe et al., 2009). A Hox/Pbx/Meis binding site located in the first intron of the *ppp1r14al* gene was assayed using primers 5′-GGTGCTAAAAAGTAACAGCCCCCACTGAGG-3′ and 5′-GGA-CAGTTGCAGGAGGGCTTTCTTTGTGTGAT-3′.

Results

An assay for the identification of hoxb1b target genes

Several reports have demonstrated that misexpression of paralog group 1 (PG1) *hox* genes drives ectopic gene expression in the