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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*.

Keywords

hindbrain; zebrafish; transcription; fgf; hox; microarray

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

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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; Gavalas et al., 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; McClintock et al., 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.

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* (166pg each) or *meis3+βgal* (166pg each) were microinjected into 1-2 cell stage zebrafish embryos and raised to 14 hours post fertilization. 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 750μl of Trizol Reagent (Invitrogen) and stored at -80°C. For morpholino (MO) injections, 4ng of MO targeting the translational start site of *ppp1r14al* was microinjected into 1-2 cell stage of embryos. For double morpholino injections, 2ng of *MOfgf8* was injected solely or in combination with 4ng of *MOppp1r14al*. Rescue experiments were performed using 4ng *MOppp1r14al*+300pg *ppp1r14al* mRNA compared to 4ng *MOppp1r14al*+300pg *GFP* mRNA. The sequences of MO are as follows: *MOppp1r14al* 5′-CACCCGATTCGCAGCCATCTCCAGA-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 ⌠L nuclease-free water (Ambion). 1 ⌠g total RNA per sample was shipped on dry ice for microarray analysis. For RT-PCR, cDNA was first synthesized using 1 ⌠g total RNA, 200 units of Superscript III reverse transcriptase (Invitrogen), and 2.5 ⌠M oligo dT primer in a 20 ⌠L reaction for 2 hours at 37°C. Quantitative PCR was performed using QuantiFast SYBR Green PCR kit (Qiagen) using 500ng of cDNA and gene specific primers in a 50 ⌠L 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′-AAACGCAGGAGATGGCCTGA-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.

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 minutes 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′-GGACAGTTGCAGGAGGGCTTTCTTTGTGTGAT-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 anterior zebrafish embryo (Alexandre et al., 1996; McClintock et al., 2001; Vlachakis et al., 2001). In particular, *hoxb1b*, together with its *meis* and *pbx* cofactors, induces ectopic expression of hindbrain genes such as *hoxb1a, hoxb2a, krox20* and *valentino* in the anterior CNS of zebrafish embryos (Fig. 1C;(Choe et al., 2002; Vlachakis et al., 2001)). In fact, the forebrain region undergoes a wholesale transformation to a hindbrain fate in these embryos - including differentiation of ectopic hindbrain Mauthner neurons (Vlachakis et al., 2001). This transformation is accompanied by morphological changes that shorten and thicken the anterior neural region into a characteristic rounded shape, as well as by the lack of eye formation. In contrast, misexpression of *hoxb1b* alone, or either cofactor alone, does not induce ectopic gene expression in the anterior CNS (Fig. 1B) and has no effect on morphology. Hence, we reasoned that misexpression could form the basis of a simple system to isolate novel genes acting downstream of *hoxb1b* in the hindbrain.

hoxb1b-mediated induction of ectopic hindbrain gene expression is readily detected by in situ hybridization (Fig. 1C; (Vlachakis et al., 2001)). To quantify this induction, we used quantitative RT-PCR and assayed expression of three hindbrain genes (*krox20, valentino* and *hoxa3*; (Amores et al., 1998; Moens et al., 1998; Oxtoby and Jowett, 1993)), one

forebrain/midbrain gene (*otx2*; (Li et al., 1994)) and one "house-keeping" gene (*tubulin*). We microinjected *hoxb1b* and *meis3* mRNA – we have previously found that it is not necessary to co-inject *pbx* mRNA (Vlachakis et al., 2001) since *pbx2* and *pbx4* are highly expressed throughout the embryo (Vlachakis et al., 2000; Waskiewicz et al., 2002) - and raised embryos to 14hpf. Successfully injected embryos were identified based on the loss of eyes and the characteristic rounding of the head. The anteriormost region of *hoxb1b/meis3*-injected embryos was dissected at the line indicated in Fig. 1C. As control, we used the anterior-most region of embryos injected with *meis3* alone (dissected as outlined in Fig. 1B). We find that expression of hindbrain genes is robustly induced such that *krox20* transcripts are increased ~10-fold (Fig. 1E) and *valentino* and *hoxa3* are induced ~2-4 fold (not shown). The forebrain/midbrain gene *otx2* is repressed ~2-fold (not shown), as expected based on the loss of forebrain fates and in accordance with our previously reported in situ hybridization data (Vlachakis et al., 2001), while expression of *tubulin* is unchanged (Fig. 1D). We conclude that hindbrain genes are induced 2- to 10-fold in the anterior of *hoxb1b/meis3*-injected embryos at the expense of forebrain/midbrain genes and that this induction is sufficiently pronounced to be detected by quantitative RT-PCR.

Expression profiling efficiently identifies *hoxb1b*-induced genes

To identify novel hindbrain genes acting in the *hoxb1b* genetic cascade, anterior tissue was dissected from embryos misexpressing *hoxb1b/meis3* or *meis3* alone at 14hpf, a stage when cell fate specification is ongoing in the hindbrain, as shown in Fig. 1. Probes were prepared from each tissue and hybridized to microarrays containing 65-mer oligonucleotides (Compugen/Sigma-Genosys oligo set XEBLIB96), representing ~12,500 unique zebrafish genes, by the Kimmel Cancer Center Microarray Core Facility at Thomas Jefferson University. The experiment was carried out in triplicate where each replicate array was hybridized to probe prepared from a separate injection/dissection. Array results were analyzed as outlined in Materials and Methods and genes were considered *hoxb1b* targets if they were up-regulated more than 2-fold in *hoxb1b/meis3*-injected relative to *meis3*-injected controls with a p-value below 0.05 for the three experiments. Using these criteria, we find that 100 genes are up-regulated by *hoxb1b* at 14hpf (Table S1).

Strikingly, we find that six of the ten most highly induced genes – *krox20, hoxa3a, valentino, hoxb3a, hoxa2b, hoxb2a* - have been previously found to be expressed in the hindbrain and four of these (*krox20, valentino, hoxa2a, hoxb2a*) have already been shown to be regulated by *hoxb1b/meis3* (Choe et al., 2002; Vlachakis et al., 2001; Waskiewicz et al., 2001). Given the efficient identification of known *hoxb1b*-regulated hindbrain genes by our assay, we reasoned that the uncharacterized genes in Table S1 might also represent novel *hoxb1b*-regulated genes expressed in the hindbrain. To test this directly, we selected 54 genes for further analysis. Since some known hindbrain genes showed relatively modest induction (e.g. *ephA4a*; 2.07-fold induction), we selected a set of genes to represent a cross-section of high and low induced genes, as well as genes with a range of p-values (including some genes that did not make the cut for Table S1 due to low fold-induction or high p-value). The genes selected for further analysis were re-annotated and are listed in Table 1. cDNA clones containing the longest insert available for each gene were purchased and initial sequencing revealed that 49 of the purchased clones corresponded to the correct gene on the microarray.

In situ hybridization analysis of candidate genes up-regulated by Hoxb1b

We successfully generated in situ probes from 45 clones and detectable expression patterns were observed for 31 of these, with the remaining 14 showing either no signal or high levels of background staining. We cannot distinguish if the failed in situ hybridizations are due to poor probes, or to low expression levels of the targeted genes, but all failed probes were re-

synthesized at least twice. Fig. 2 shows all expression patterns obtained, with the exception of Dr.132502 (*hoxb3a*), for which the expression pattern is known. We note that at least 21 of the 30 genes in Fig. 2 show expression in the hindbrain (sometimes accompanied by expression elsewhere). Twenty of these genes show expression that is restricted to one, or several, rhombomeres, while one is broadly expressed in the hindbrain (Table 1; Fig. 2P). Of the remaining nine genes, at least seven are expressed next to the neural tube at the level of the hindbrain in structures that may correspond to neural crest or sensory placodes (e.g. Fig. 2S). Hence, since *hoxb1b* is normally expressed throughout the caudal region of the developing CNS, 28 of the 30 genes for which we have expression data are expressed in a pattern that is compatible with direct or indirect induction by *hoxb1b*. Only two genes in Fig. 2, Dr.73730 (Fig. 2V) and Dr.105556 (Fig. 2Y), have expression patterns that appear incompatible with either direct or indirect induction by *hoxb1b*, although we note that both genes are expressed near the hindbrain. We also note that of the 28 hindbrain associated genes shown in Fig. 2, only three (*ngf1a/nab, irx5a* and *ephA4*; (Mechta-Grigoriou et al., 2000; Wang et al., 2001; Xu et al., 1994)) have been previously identified as being expressed in the hindbrain, the remaining 25 genes are either novel genes whose expression has not been reported in the published literature, or known genes not previously reported to have hindbrain associated expression. Thus, approximately one half (25/54) of the genes we picked turned out to represent genes not previously known to be hindbrain associated.

We next examined the detailed expression pattern of genes expressed within the hindbrain. The expression patterns for 18 of the 21 hindbrain genes is shown in Fig. 3 -two known hindbrain genes (*iro5*/Dr.83684 and *ephA4*/Dr.47585), as well as one gene whose in situ signal was too weak to permit analysis by double in situ hybridization (Dr.150323) were not analyzed. Of these 18 genes, we find that four are expressed primarily in r5 (Dr. 80253, Dr. 15418, Dr.76397, Dr.122018), five primarily in r3/r5 (Dr.80366, Dr.85688, Dr.83971, Dr. 150422, Dr.81187), two primarily in r4 (Dr.82617, Dr.84702), two primarily in r2/4 (Dr. 106214, Dr.79266) and one primarily in r2/r4/r6 (Dr.79031). The remaining four genes are expressed in multiple adjacent rhombomeres (Dr.74192, Dr.23693, Dr.75543) or throughout the hindbrain (Dr.12108). In summary, at least 1/3 (20/54) of the *hoxb1b*-induced genes selected for analysis are expressed in rhombomere-restricted patterns during the stage when hindbrain formation takes place.

Many *hoxb1b*-induced r5/r6 genes act downstream of *vhnf1* and *valentino*

We next made use of existing mutant lines to determine where the newly identified genes act in the genetic hierarchy controlling hindbrain development. Previous analyses have indicated that *hoxb1b* is required for expression of *vhnf1* in r5/r6 (Choe and Sagerstrom, 2004) and that *vhnf1* is in turn required for r5/r6 expression of *val* (Hernandez et al., 2004; Sun and Hopkins, 2001; Wiellette and Sive, 2003). We therefore examined the expression of several of the newly identified genes in *vhnf1* and *val* mutant embryos. We find that three genes expressed in r5 (Dr.80253, Dr.15418, Dr.76397; Fig. 4A-L) and two genes expressed in r3/r5 (Dr. 80366, Dr. 85688; Fig 4M-T) are not expressed in r5 of *vhnf1* and *val* mutants. This finding indicates that these five genes act downstream of *vhnf1* and *val*.

We also examined the expression of Dr.79031 (that is expressed in r2/r4/r6) and find a contiguous domain of Dr.79031 expression caudal to r3 in *vhnf1* and *val* mutant embryos (Fig. 4U-W). This domain appears larger than just r4, suggesting that r6 expression of Dr. 79031 persists in *vhnf1* and *val* mutants. Similarly, expression of Dr.79266 (that is expressed in r2/r4) is unaffected in r2/r4 in *vhnf1* and *val* mutants, but expression appears to expand caudally in both mutants (Fig. 4Y-BB).

Lastly, we confirmed that the newly identified genes are regulated by *hoxb1b*. We injected *hoxb1b/meis3* mRNA and detect ectopic expression in the anterior embryo of all genes

tested (Fig. 4D, H, L, P, T, X, BB). In addition, disrupting the activity of Hox cofactors from the Pbx and Meis families prevents expression of the newly identified hindbrain genes (Fig. S1B, D, F, H). Taken together, these results indicate that the genes identified in our screen are indeed *hoxb1b* regulated.

The *ppp1r14al* gene regulates formation of the caudal hindbrain

We next set out to determine if the novel hindbrain genes identified in our expressionprofiling screen are required for hindbrain development. We selected the r4-restricted Dr.82617 gene, since its expression in a single rhombomere simplifies functional analysis and since a full-length clone was readily available. Analysis of the Dr.82617 sequence revealed similarity to Protein phosphatase 1 regulatory subunit 14a. Protein phosphatase 1 (PPP1) is one of the main serine/threonine phosphatases in eukaryotic cells (reviewed in (Cohen, 2002)). While there are four PPP1 isoforms in most mammals, these are broadly expressed and show high sequence identity, suggesting that the various PPP1 proteins display similar substrate specificities. Accordingly, all five Ppp1 genes identified in zebrafish to date (*ppp1caa*, *ppp1cab*, *ppp1cb*, *ppp1cbl* and *ppp1cc*) are broadly expressed (*ppp1* expression data were retrieved from the Zebrafish Information Network (ZFIN), University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org/; 5/17/10). Instead, substrate specificity is provided when a single PPP1 catalytic subunit (PPP1c) associates with one of a large number of available regulatory (PPP1r) subunits (reviewed in (Cohen, 2002)). Ppp1r14a is one such regulatory subunit that was initially identified as CPI-17 with the ability to inhibit PPP1c activity in smooth muscle. Notably, the inhibitory activity of Ppp1r14a is further enhanced by PKC-mediated phosphorylation of Ppp1r14a itself (Eto et al., 1995; Eto et al., 1997).

Although our analysis identified Dr.82617 as Ppp1r14a, a distinct zebrafish gene (Unigene Dr.14203) had been previously designated Ppp1r14a. Phylogenetic analysis indicates that the two genes cluster together with Ppp1r14a proteins from other species (Fig. 5A), suggesting that they may have resulted from a previously reported duplication of the zebrafish genome. As a result, we have designated Dr.82617 as *ppp1r14a-like* (*ppp1r14al*).

Our initial analysis of genes from the expression screen identified *ppp1r14al* as being expressed in r4 (Fig. 2, 3). A more detailed analysis confirmed this finding at early somitogenesis stages (12-14hpf; Fig. 5B, C) and also revealed expression in ventral mesoderm (arrowheads in Fig. 5C). Thereafter, *ppp1r14al* expression is no longer r4-specific, but persists in the lateral neural tube (arrowheads in Fig. 5D, E) and also becomes detectable in cranial ganglia (arrows in Fig. 5D, E). In contrast, *ppp1r14a* (Dr.14203) does not show restricted gene expression at early stages of development and is not expressed in the hindbrain (*ppp1r14a* expression data were retrieved from the Zebrafish Information Network (ZFIN), University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org/; 5/17/10), but is expressed in intestinal smooth muscle by 3dpf (Georgijevic et al., 2007).

To examine the role of *ppp1r14al* in zebrafish development, we made use of MOs targeting the *ppp1r14al* translation initiation site. We find that disrupting *ppp1r14al* function leads to reduced *fgf3* expression in r4 at the end of gastrulation (10.5hpf; Fig. 6A, B). We also observe modest reductions in the r4 expression of *dual specificity phosphatase 2* (*DUSP2*; Fig. 6C, D) and *hoxb1a* (Fig 6E, F), but not of *ephrinB2*, *cyp26b* or *irx7* (Fig. S2A-F). Expression levels of *krox20* in r3 and r5 are not affected, although the r3 and r5 expression domains may be somewhat closer together in MO-injected embryos (Fig. 6G-J). Since *fgf3* appears to be the earliest gene affected upon interfering with *ppp1r14al* function, we examined expression of *pea3*, a known Fgf-target (Raible and Brand, 2001). We find that the size of the *pea3* expression domain is reduced somewhat (Fig. 6K, L), consistent with

impaired Fgf-signaling in MO-injected embryos. We also observe a slight reduction in the size of the *val* domain in r5/r6 (Fig. 6M, N), consistent with previous reports that *val* expression is Fgf-dependent (Hernandez et al., 2004; Wiellette and Sive, 2003). Lastly, we co-injected MOs with *ppp1r14al* mRNA mutated to be refractory to the MOs. We find that *ppp1r14al* mRNA efficiently rescues the MO phenotype at 10.5hpf (Fig. 6O-R), confirming the specificity of the MOs. In particular, less than 10% of MO-injected embryos show normal *fgf3* expression and over 50% are severely affected (Fig. 6P, R). This situation is reversed when *ppp1r14al* mRNA is co-injected with the MO, leading to no embryos with a severe phenotype and more than 70% with strong *fgf3* expression (Fig. 6Q, R).

We next examined the effect of *ppp1r14al* overexpression and observe elevated *fgf3* expression in r4 (Fig. 7A, B), further supporting a role for *ppp1r14al* in the regulation of *fgf3* expression. Notably, *ppp1r14al* overexpression also affects formation of the otic vesicle, detected as an increased number of otoliths (Fig. 7C, D), consistent with the known role for *fgf3* in otic vesicle formation (Kwak et al., 2002; Maroon et al., 2002; Phillips et al., 2001).

Since *ppp1r14al* is expressed in lateral cells in several rhombomeres (arrowheads in Fig. 5D, E), as well as in cranial ganglia (arrows in Fig. 5D, E), at later stages, we also examined gene expression in those tissues in later-stage MO-injected embryos. We find that expression of *ascl1a (zash1a)*, which is regulated by *fgf3* in the forebrain (Walshe and Mason, 2003), is down regulated in MO-injected embryos already by mid-somitogenesis (13hpf; Fig. S3A, B). Similarly, *ngn1, fgf20* and *pax2a* (Krauss et al., 1991; Liao et al., 1999; Whitehead et al., 2005), three genes expressed laterally within the neural tube, are down regulated in MO-injected embryos by late somitogenesis (22-24hpf; Fig. S3C-H). This effect appears to be specific to the hindbrain, since *ngn1* and *pax2* expression at the MHB is largely unaffected (asterisks in Fig. S3C, D, G, H). Notably, *dlx2* expression in the cranial ganglia (arrows in Fig. S3I, J) is down regulated only modestly. While it is possible that this role for *ppp1r14al* is independent of its role in r4, a recent lineage tracing analysis in the mouse (Makki and Capecchi, 2010) revealed that numerous structures outside the hindbrain (including the cranial ganglia) derive from cells that initially express *hoxa1* (the murine *hoxb1b* homolog). Hence, *hoxb1b* may regulate *ppp1r14al* expression at most of the embryonic sites that we observe in this analysis.

When observing MO-injected embryos at later stages of development, we also noticed increased cell death in the neural tube (Fig. S4). Acridine orange staining confirmed increased cell death in the hindbrain and anterior neural tube of MO-injected embryos at 12-13hpf (Fig. S4B). Co-injection of MOs targeted to p53 does not rescue this cell-death (not shown), demonstrating that it is not due to p53-mediated MO-toxicity. In contrast, using the same rescue strategy as in Fig. 6, we find that *ppp1r14al* mRNA rescues the cell death (Fig. S4C). Notably, acridine orange staining of 9hpf MO-injected embryos was indistinguishable from that of control embryos (not shown), indicating that the reduction in *fgf3* expression precedes the increased cell death and suggesting that cell death is a later consequence of loss of *ppp1r14al* and/or *fgf3* activity.

Simultaneous disruption of *ppp1r14al* and *fgf8* interferes with r5/r6 formation

The experiments in Fig. 6 reveal a robust effect of *ppp1r14al* on *fgf3* expression, but modest effects on the expression of other hindbrain genes. This observation suggests that reduced *fgf3* expression does not have profound effects on overall hindbrain development. Indeed, it has been previously reported that *fgf3* acts redundantly with *fgf8* in a r4 signaling center to regulate formation of r5/r6 (Maves et al., 2002; Walshe et al., 2002). In particular, individual disruption of *fgf8* or *fgf3* function (using MOs and/or the *acerebellar* line that carries a *fgf8* mutation) produces modest phenotypes, while simultaneous disruption of *fgf8* and *fgf3*

function leads to significant loss of *krox20* expression in r5 and *valentino* expression in r5/r6 (Maves et al., 2002; Walshe et al., 2002).

Since *fgf3* expression is reduced (Fig. 6), but *fgf8* expression is unaffected (not shows) in embryos injected with *ppp1r14l* MO, we reasoned that simultaneous disruption of *ppp1r14al* and *fgf8* might produce a phenotype similar to simultaneous disruption of *fgf3* and *fgf8*. As reported (Maves et al., 2002; Walshe et al., 2002), we find that *fgf8* disruption has a modest effect on *pea3, krox20* and *val* expression (Fig. 8B, F, J). This effect is similar to that observed upon disruption of *ppp1r14al* (Fig. 6; Fig. 8C, G, K). In contrast, co-injection of *ppp1r14al* and *fgf8* MOs causes pronounced reduction in the expression of *pea3, krox20* and *val* (Fig. 8D, H, L). This latter phenotype is similar to that reported for simultaneous loss of *fgf3* and *fgf8* (Maves et al., 2002; Walshe et al., 2002), supporting the notion that *ppp1r14al* acts by regulating *fgf3* expression.

ppp1r14al expression is regulated by Hoxb1b

We next determined if *ppp1r14al* expression requires Hoxb1b activity. As discussed, Hoxb1b functions in a complex that also includes Meis and Pbx cofactors and we have found that such complexes cannot form in the absence of Meis proteins. Hence, we used a dominant negative Meis construct (Choe et al., 2002) to disrupt Meis/Pbx/Hoxb1b complexes during zebrafish embryogenesis and find that *ppp1r14al* expression is lost (Fig. 9B). We also find that injection of *meis3, pbx4* and *hoxb1b* mRNA induces ectopic expression of *ppp1r14al* in the anterior embryo (Fig. 9C), as expected based on the design of our screen. Lastly, we identified a Meis/Pbx/Hox binding element in the first intron of the *ppp1r14al* gene and, using ChIP analysis, we find that endogenous Meis, Pbx and Hoxb1 proteins occupy this element in wild type embryos (Fig. 9D).

Discussion

In this report we use ectopic expression of *hoxb1b/meis3* followed by dissection and microarray analysis to isolate novel *hoxb1b*-regulated hindbrain genes. A total of 100 genes fulfilled our criteria of at least a 2-fold induction with a p-value under 0.05 over three independent experiments. 54 genes were characterized further and we find that 25 of these represent novel genes or known genes not previously reported as expressed in the hindbrain. This fraction of novel hindbrain genes (25/54) may in fact be an underestimate since only 31 of the 54 genes gave detectable expression patterns by in situ hybridization. Specifically, while some of the genes that failed to show an expression pattern are likely to represent true false positives (i.e. genes identified as up-regulated by *hoxb1b* when they are really not), other genes may have failed to show an expression pattern for technical reasons (such as poor probe synthesis) and may in fact be regulated by *hoxb1b*. We also note that there is little correlation between the fold induction of a gene by *hoxb1b* in our assay and the likelihood that the gene will be expressed in the hindbrain. This is clear from Table 1, which shows that 12 of the genes induced by more than 3-fold display hindbrain specific expression and 10 of the genes induced by only 2-3 fold are expressed in the hindbrain. This lack of correlation is also illustrated by the fact that *ephA4a*, a known gene expressed in r1, r3 and r5, was induced only 2.071-fold by ectopic *hoxb1b/meis3* expression. Lastly, since the 54 genes we analyzed were not biased to the most highly induced ones, but were selected to represent the full range of observed fold induction and p-values, it is likely that genes in Table S1 that were not analyzed will harbor a similar fraction (~50%) of hindbrain expressed genes.

Two recent studies used microarray analysis to isolate PG1 *hox*-regulated genes. Rohrschneider et al compared gene expression between wild type and *hoxb1a*-depleted rhombomere 4 (Rohrschneider et al., 2007) and successfully identified 12 genes regulated

by *hoxb1a* in r4. There are several differences between our study and the Rohrschneider study. First, they focused on a different PG1 *hox* gene (*hoxb1a* versus *hoxb1b*). Second, our study used gain of function (ectopic *hoxb1b* expression), while Rohrschneider used loss of function (*hoxb1a* depletion by morpholino injection). Third, our study would identify any gene activated by *hoxb1b* in the caudal hindbrain (r4-r7), while Rohrschneider focused on *hoxb1a*-regulated genes in r4. In spite of these differences, our screen identified two of the 12 genes from the Rohrschneider screen (*collagen VIIa* and *calretinin*). We conclude that, taken together, these two screens provide a comprehensive analysis of PG1 target genes in the hindbrain. In a distinct study, van den Akker et al used ectopic *hoxb1b* expression to identify target genes (van den Akker et al., 2010). However, this study analyzed embryos prior to gastrulation (40% epiboly) - well before the onset of neural development - and did not co-express *hox* cofactors. As a result, none of the highest-scoring *hoxb1b*-regulated genes identified by van den Akker et al appear in our dataset.

In order to determine if the genes identified in our screen are important for hindbrain development, we analyzed the *ppp1r14al* gene in detail. *ppp1r14al* encodes a regulatory subunit that associates with protein phosphatase 1 (PPP1) to modulate its activity, but *ppp1r14a* was originally identified as inhibiting PPP1 activity in smooth muscle (Eto et al., 1995) and has not been previously implicated in hindbrain development. Given the ubiquitous expression and broad substrate specificity of PPP1 proteins, it is difficult to identify a likely substrate for the PPP1/Ppp1r14al complex in the hindbrain and we instead examined a panel of r4 genes to determine if they were affected by loss of *ppp1r14al*. Since *ppp1r14al* is predicted to act downstream of PG1 *hox* genes, it is not surprising that we see only modest effects on the expression of *hoxb1a* or the size of r4 in *ppp1r14al*-depleted embryos. In contrast, we observe robust reduction of *fgf3* expression in r4 of *pp1r14al*-depleted embryos. This is in agreement with previous reports indicating that *fgf3* acts downstream of PG1 *hox* genes in r4 (Waskiewicz et al., 2002). We note that, while *fgf* signaling is known to regulate formation of r5 and r6 (Maves et al., 2002; Walshe et al., 2002), we observe only minor effects on *valentino* expression in r5 of *ppp1r14al*-depleted embryos. This is likely due to the fact that *fgf8*, which acts together with *fgf3* to regulate r5/r6, is unaffected in *ppp1r14al*-depleted embryos. Indeed, apart from the robust loss of *fgf3* expression, the *ppp1r14al* loss of function phenotype closely resembles the subtle effect of loss of *fgf3* reported by Walshe et al (Walshe et al., 2002). By simultaneously disrupting both *fgf8* and *ppp1r14al*, we observe a more pronounced phenotype with clear loss of *krox20*, *pea3* and *val* expression – similar to the phenotype reported for simultaneous disruption of *fgf3* and *fgf8* (Maves et al., 2002; Walshe et al., 2002). We also observe increased cell death at later stages in *ppp1r14al* MO-injected embryos. Since the cell death occurs subsequent to the loss of *fgf3* expression and since co-injection of *ppp1r14al* mRNA (which restores *fgf3* expression) rescues the cell death, our data are consistent with the cell death being due to loss of *fgf3*, although we can not formally rule out the possibility that increased r4 expression of survival factors other than *fgf3* may also be induced in the rescue experiments. Taken together, our data suggest a role for *ppp1r14al* upstream of *fgf3* in the establishment of the key r4 signaling center during early hindbrain development. Notably, while we demonstrate that *ppp1r14al* is likely directly regulated by Hoxb1, Meis and Pbx, we do not know how *ppp1r14al* regulates *fgf3* expression. Intriguingly, *fgf3* expression is regulated by GATA4 in other systems (Murakami et al., 1999) and GATA4 activity is modulated by phosphorylation on Ser-105 (Kitta et al., 2003), making GATA4 a potential substrate for Ppp1r14al.

Lastly, we note that our screen does not select specifically for genes that are directly regulated by Hoxb1b. Accordingly, r5 expression of a number of genes identified in the screen is lost in *vhnf1* and *val* mutant embryos. Since *vhnf1* and *val* both act downstream of PG1 *hox* genes (Choe and Sagerstrom, 2004; Choe et al., 2002; Waskiewicz et al., 2001;

Waskiewicz et al., 2002), this finding suggests that such genes are indirectly regulated by Hoxb1b, although it remains possible that they receive combinatorial input from Hoxb1b and vHnf1 and/or Val.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

> *hox* genes are required for hindbrain formation, but *hox* targets are largely unknown > We identify 100 genes up-regulated by zebrafish *hoxb1b* > Of 54 genes analyzed further, 25 represent novel hindbrain genes > Expression of the *ppp1r14al* gene is directly regulated by *hoxb1b* in rhombomere 4 > *ppp1r14al* encodes a protein phosphatase subunit and regulates *fgf3* expression

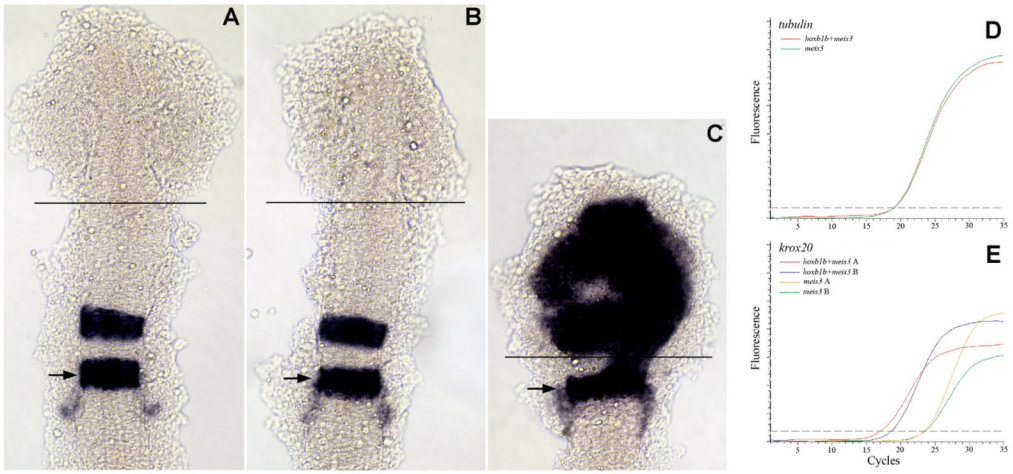


Figure 1.

An ectopic expression/dissection strategy to identify *hoxb1b*-regulated genes. (A-C) Uninjected (A), *meis3+βgal* mRNA-injected (B) or *meis3+hoxb1b* mRNA-injected (C) embryos were analyzed by in situ hybridization for expression of *krox20*. The position of r5 is indicated by arrows and the site where the anterior embryo was dissected is indicated by black lines in A-C. (D-E) Quantitative RT-PCR was used to assay expression of *tubulin* (D) and *krox20* (E) in *meis3*-injected and *meis3+hoxb1b*-injected embryos. Two independent injections are shown in E. Embryos in A-C were flat mounted at 14hpf and are shown in dorsal view with anterior to the top.

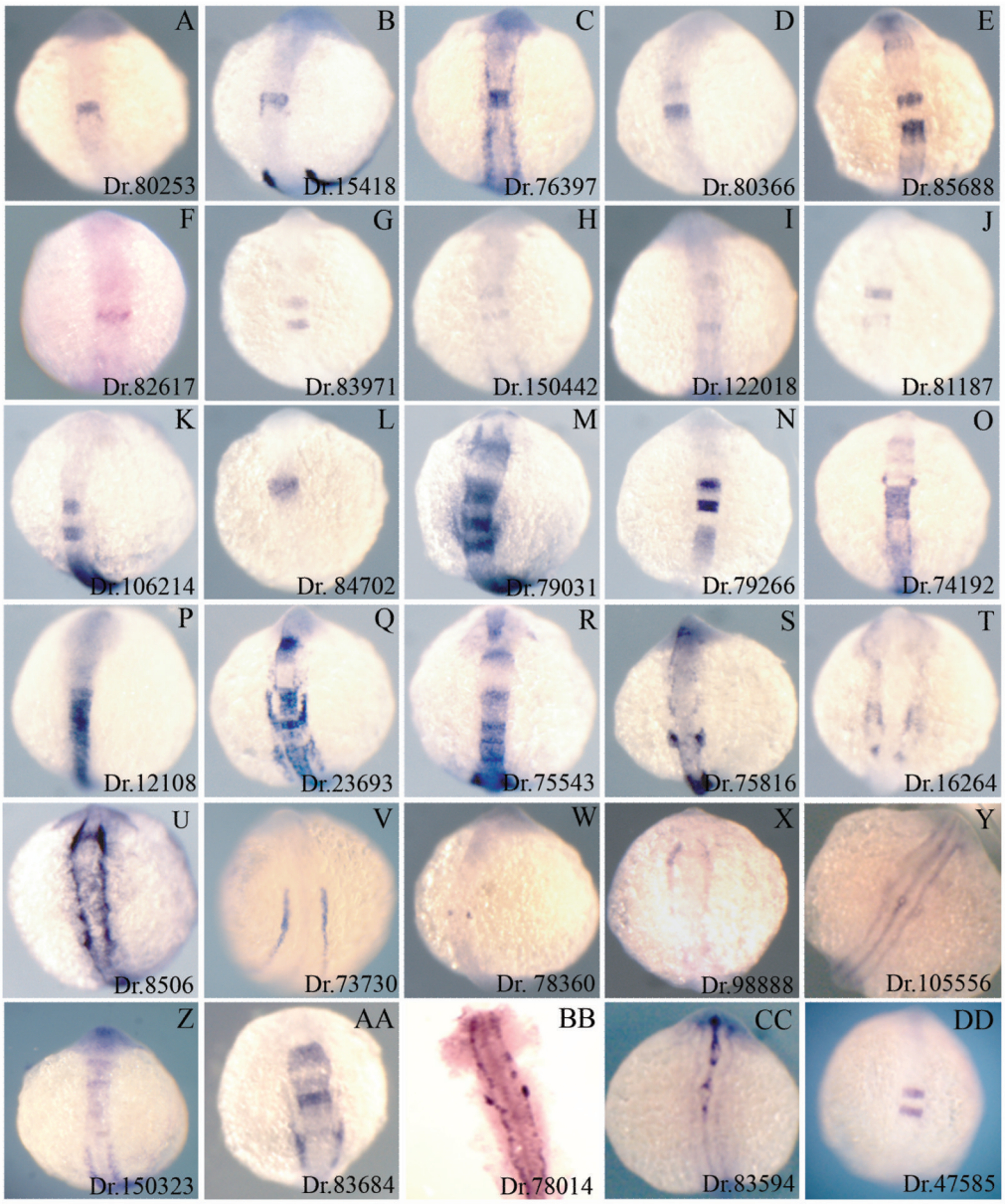


Figure 2.

Expression patterns of *hoxb1b*-regulated genes. 30 genes identified as up-regulated by *hoxb1b* (see Table 1 and Table S1) were assayed by in situ hybridization to determine their expression patterns. All embryos are at 14hpf and are shown as whole mounts in dorsal view with anterior to the top. The Unigene ID for each gene is given in the lower right of each panel and corresponds to the Unigene IDs in Table 1 and Table S1.

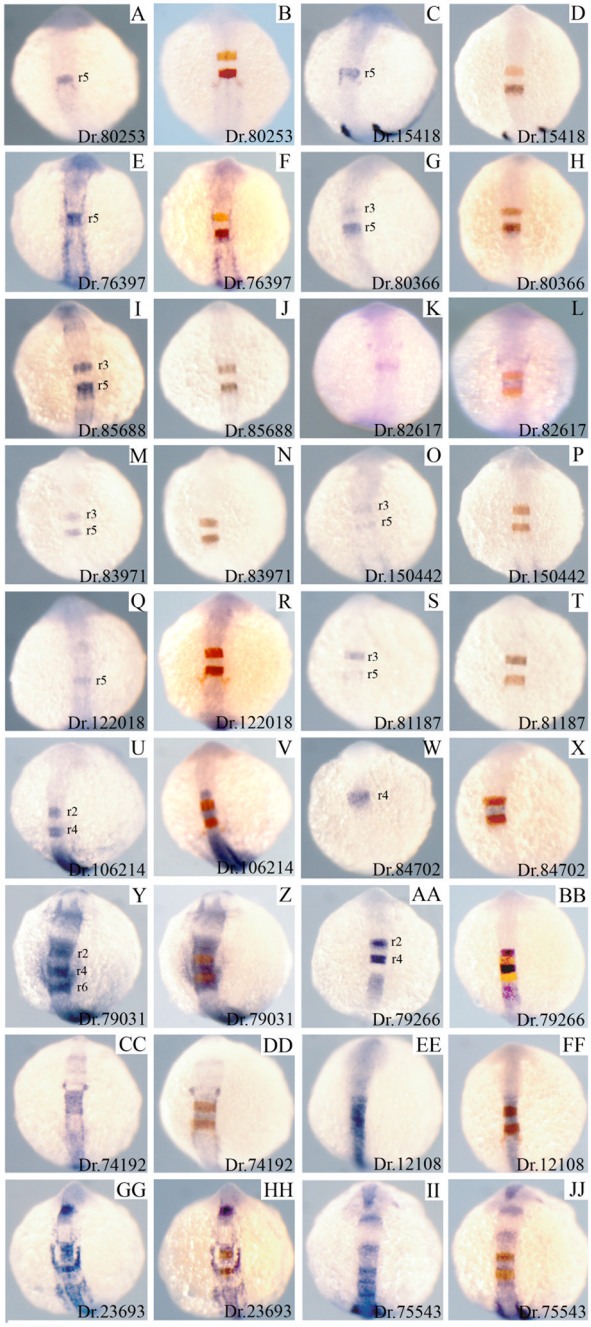


Figure 3.

Hindbrain expression patterns of *hoxb1b*-regulated genes. 18 *hoxb1b*-regulated genes identified as expressed in the hindbrain were assayed by themselves (1st and 3rd column) or as double in situ hybridization together with *krox20* (2nd and 4th column; *krox20* is expressed in r3 and r5 and is detected in red). Based on the double in situ hybridizations, it was determined which rhombomeres express each of the novel genes and this is indicated in each panel in columns 1 and 3. All embryos are at 14hpf and are shown as whole mounts in dorsal view with anterior to the top. In situ panels in columns 1 and 3 are duplicated from Fig. 2 for ease of comparison.

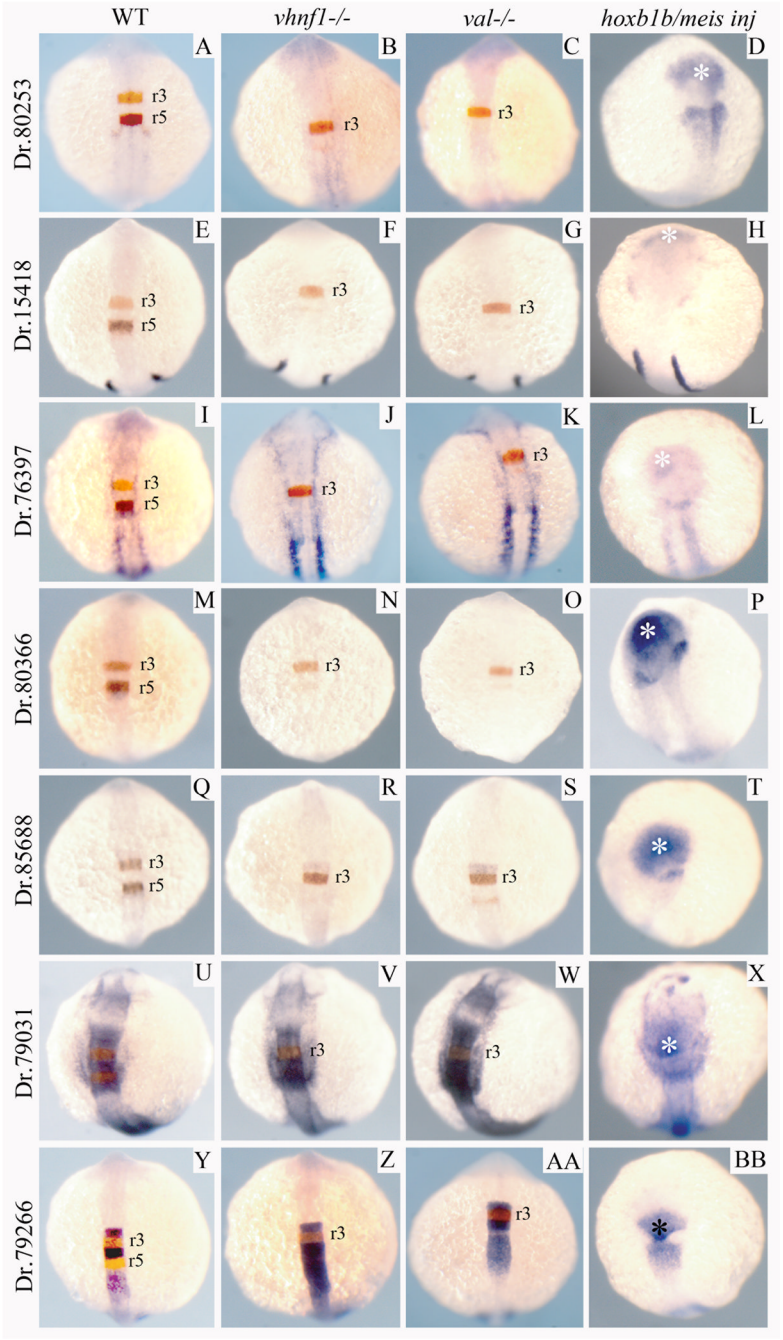


Figure 4.

Expression of *hoxb1b*-regulated genes in *vhnf1* mutant, *valentino* mutant and *hoxb1b/meis3*-injected embryos. Seven *hoxb1b*-regulated genes were analyzed for their expression in wild type (A, E, I, M, Q, U, Y), *vhnf1* mutant (B, F, J, N, R, V, Z), *valentino* mutant (C, G, K, O, S, W, AA) or *hoxb1b+meis3*-injected (D, H, L, P, T, X, BB) embryos. Genes were analyzed as double in situ hybridizations with *krox20* (A-C, E-G, I-K, M-O, Q-S, U-W, Y-AA; *krox20* detected in red) or as single in situ hybridizations (D, H, L, P, T, X, BB). All embryos are at 14hpf and are shown as whole mounts in dorsal view with anterior to the top. In situ panels in the left-hand column are duplicated from Fig. 3 for ease of comparison.

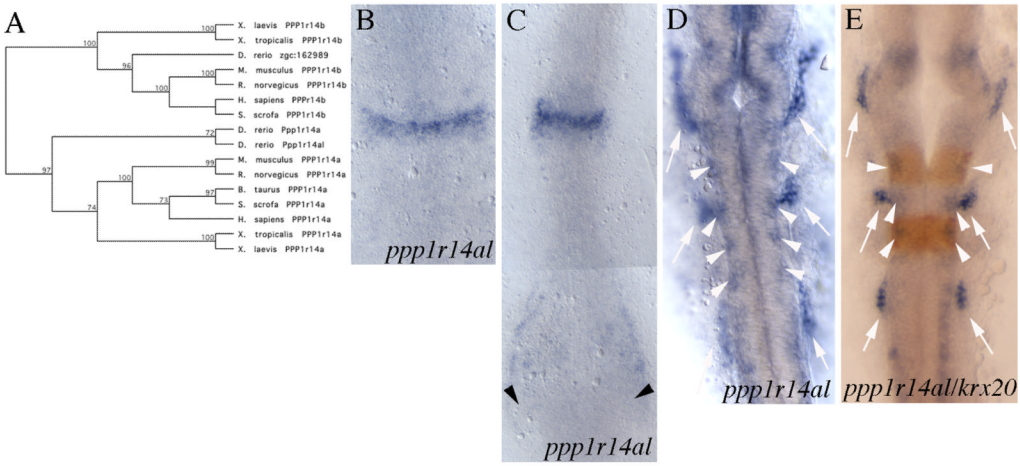
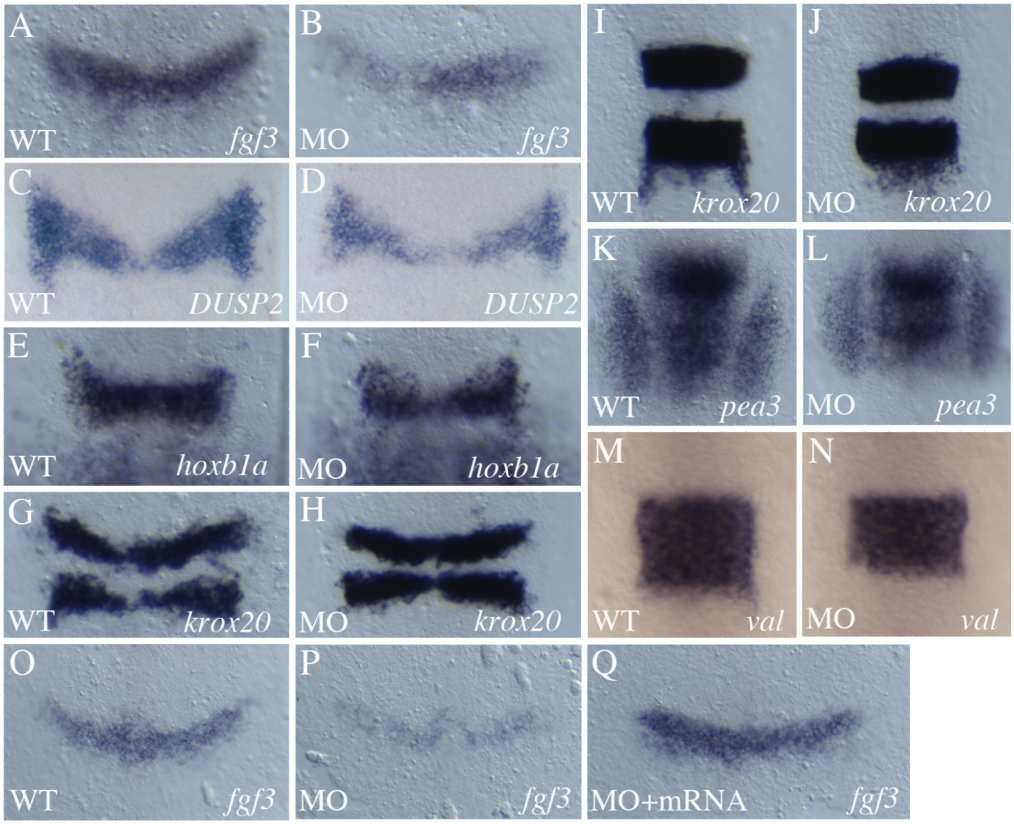


Figure 5.

Sequence and expression analysis of the *ppp1r14al* gene. (A) Phylogenetic tree demonstrating that zebrafish *ppp1r14al* groups with other *ppp1r14a* genes, not with *ppp1r14b* genes. (B-E) *ppp1r14al* expression pattern at 9hpf (B), 11hpf (C) and 20hpf (D, E). Arrows indicate cranial placodes (D, E) and arrowheads indicate staining in ventral mesoderm (C) and in the lateral neural tube (D, E). E is a double in situ hybridization with *krox20* detected in red. Embryos in B-E are shown as flat mounts in dorsal view with anterior to the top.



R

	WT	Mild	Severe
Control injected	100% (54/54)	0% (0/54)	0% (0/54)
MO + *GFP* mRNA	9.3% (5/54)	37.0% (20/54)	53.7% (29/54)
MO + *ppp1r14al* mRNA	71.8% (56/78)	29.2% (22/78)	0% (0/78)

Figure 6.

Disruption of *ppp1r14al* leads to loss of *fgf3* expression. (A-N) Control (A, C, E, G, I, K, M) and *ppp1r14al* MO-injected (B, D, F, H, J, L, N) embryos were assayed for expression of *fgf3* at 10.5hpf (A, B), *DUSP2* at 10.5 hpf (C, D), *hoxb1a* at 11.5 hpf (E, F) *krox20* at 10.5hpf (G, H) and at 12hpf (I, J), *pea3* at 12hpf (K, L) and *valentino* at 12.5hpf (M, N). (O-R) Analysis of *fgf3* expression in control (O), *ppp1r14al* MO-injected (P), *ppp1r14al* MO + *ppp1r14al* mRNA injected (Q; note that mRNA is mutated to resist MO effect) reveals rescue of the *ppp1r14al* MO phenotype at 10.5hpf. Actual data is given in R. Phenotypes were scored as severe (similar to panel P), or mild (similar to panel B). Embryos in A-Q are shown as flat mounts in dorsal view with anterior to the top.

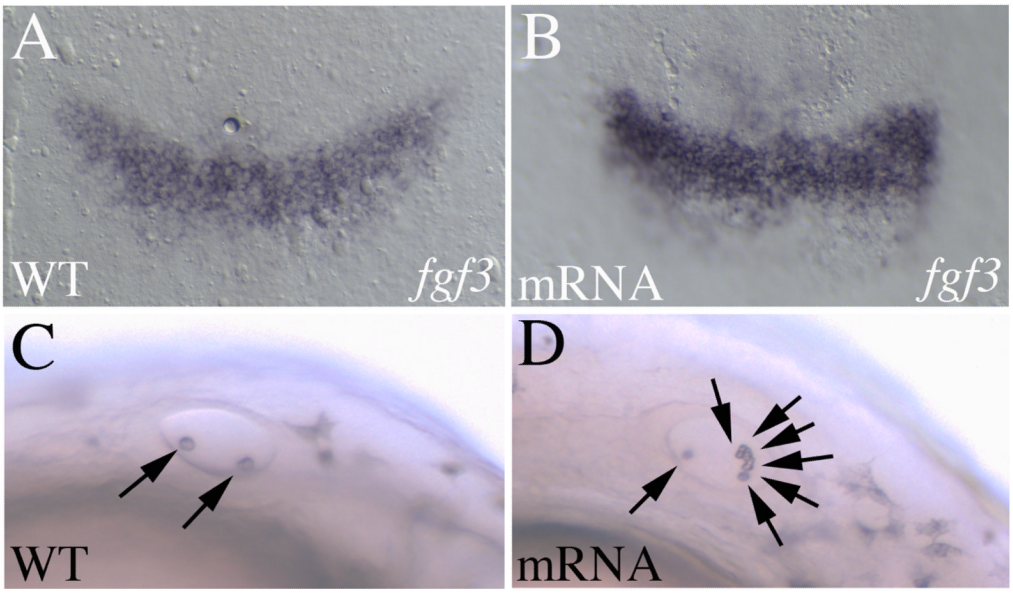


Figure 7.

Overexpression of *ppp1r14al* leads to elevated *fgf3* expression. Control (A, C) or *ppp1r14al* mRNA-injected (B, D) embryos were assayed for *fgf3* expression (A, B), or observed under brightfield for the presence of otoliths (C, D). A, B are shown as flat mounts in dorsal view with anterior to the top. C, D are live embryos in lateral view with anterior to the left. Arrows in C, D indicate otoliths.

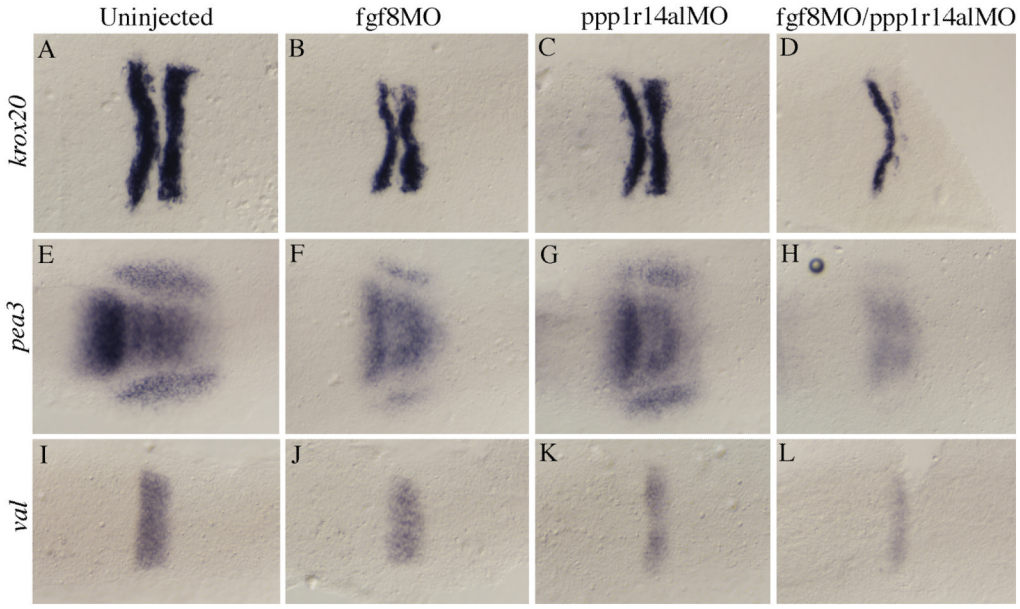


Figure 8.

Combined loss of *ppp1r14al* and *fgf8* function leads to disruption of the r4 signaling center. Uninjected (A, E, I), *fgf8* MO-injected (B, F, J), *ppp1r14al* MO-injected (C, G, K) or *fgf8* + *ppp1r14al* MO-injected embryos (D, H, L) were assayed for expression of *krox20* (A-D), *pea3* (E-H) or *valentino* (I-L) by in situ hybridization. All embryos are dorsal views at 11hpf with anterior to the left.

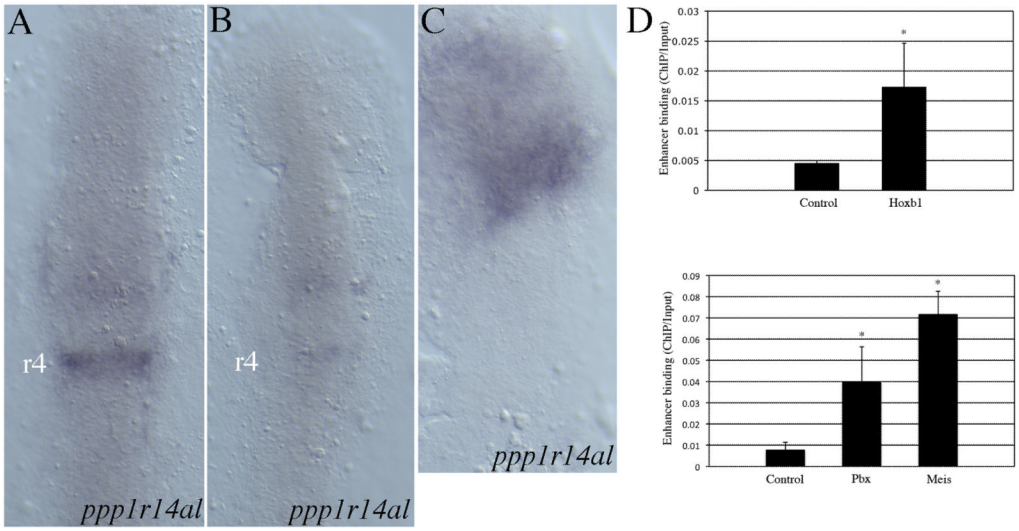


Figure 9.

ppp1r14al is regulated by Hoxb1b together with Pbx and Meis cofactors. (A-C) *ppp1r14al* expression was analyzed in control (A), dominant negative Meis-injected (B) and *hoxb1b/pbx4/meis3*-injected (C) embryos. Embryos in A-C are flat mounts at 11hpf in dorsal view with anterior to the top. (D) Chromatin immunoprecipitation from 11 hpf zebrafish embryos revealed binding of Hoxb1b (top panel), Meis and Pbx (bottom panel) proteins to the regulatory region of *ppp1r14al*. Asterisks indicate values that are statistically different ($p < 0.05$) from the control value based on Student's T-test.

Table 1

Genes selected for analysis[1]

	Genbank[2]	Fold Change[3]	p-value[4]	Description (gene name)[5]	Unigene[6]	Sequence[7]	Probe[8]	ISH Signal[9]	Expression[10]
1	AI601442	14.32	0.025	si:dkeyp-95d10.1; Similar to DUSP23	Dr.78360	X	X	X	**lateral to hindbrain**
2	Y13944	10.78	0.008	Homeo box B3a (hoxB3a)	Dr.132502	X	X	X	**rhombomere 5/6**
3	AW184433	8.86	0.001	Membrane-spanning 4-domains, subfamily A, member 17A.11	Dr.40434	X	X		
4	AW281753	8.68	0.004	Zwilling (zwi)	Dr.81187	X	X	X	**rhombomere 3/5**
5	AI667023	8.41	0.009	Myelin protein zero	Dr.78005	X	X		
6	BI671376	7.29	0.012	Sb:cb1035	Dr.148642	X	X		
7	AI641757	6.99	0.014	Wu:fc23f06 – similar to HNF1-beta-like or HNF1 gamma	Dr.15418	X	X	X	**rhombomere 5**
8	AI957504	6.83	0.008	Wu:fd06c09	Dr.122018	X	X	X	**rhombomere 5**
9	AF052249	5.82	0.032	Forkhead box D3	Dr.75816	X	X	X	**lateral to hindbrain**
10	BM024206	5.45	0.000	cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1)	Dr.76359	X			
11	AI942951	5.44	0.001	Wu:fc79c11	Dr.106214	X	X	X	**rhombomere 2/4**
12	AI878761	5.41	0.025	EGF-like-domain, multiple 6	Dr.79266	X	X	X	**rhombomere 2/4**
13	AI793592	4.96	0.003	Wu:fc51e09	Dr.150442	X	X	X	**rhombomere 3/5**
14	AI461323	4.86	0.024	PR domain containing 12	Dr.23693	X	X	X	**multiple rhombomeres**
15	AW567515	4.17	0.006	Tissue inhibitor of metalloproteinase 2b	Dr.81512				
16	BI534285	3.81	0.021	Zgc:55558	Dr.83658				
17	AW279672	3.72	0.011	retinol dehydrogenase 12 (all-trans and 9-cis) RDH12	Dr.32031	X			
18	BI430048	3.53	0.001	Zgc:92288	Dr.85849	X	X		
19	AI721660	3.28	0.005	Wu:fc30f10	Dr.150323	X	X	X	**rhombomere boundaries**
20	BI845046	3.27	0.010	Wu:fj78f01 (Si:dkeyp-84a8.1); similar to Ngfi-A binding protein 1	Dr.83971	X	X	X	**rhombomere 3/5**
21	AI965251	3.13	0.020	Wu:fc65f09	Dr.79031	X	X	X	**rhombomere 2/4/6**
22	AI957828	3.10	0.004	Rho family GTPase 3b	Dr.32839	X	X		
23	BI890305	3.03	0.005	Kin of IRRE like 3	Dr.75543	X	X	X	**multiple rhombomeres**
24	BI880801	2.75	0.005	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	Dr.83871	X	X		
25	AY017309	2.73	0.009	Iroquois homeobox protein 5a (irx5a)	Dr.83684	X	X	X	**rhombomere**
26	AI722745	2.72	0.002	Transcription factor AP-2 alpha (tfap2a)	Dr.8506	X	X	X	**lateral to hindbrain**
27	AW115765	2.56	0.008	Zgc:55283 - similar to growth factor-receptor-binding protein 4 (grb4) Nck2	Dr.80366	X	X	X	**rhombomere 3/5**

	Genbank[2]	Fold Change[3]	p-value[4]	Description (gene name)[5]	Unigene[6]	Sequence[7]	Probe[8]	ISH Signal[9]	Expression[10]
28	AA495100	2.50	0.004	fa06f01 ICRFzfls cDNA clone	None				
29	BG304232	2.46	0.008	Zgc:66107 - similar to Acheron (La ribonucleoprotein domain family, member 6)	Dr.6975				
30	BI672091	2.43	0.026	ft34d03; similar to cytoglobin	Dr.29018	X	X		
31	AI416207	2.43	0.026	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived b	Dr.74192	X	X	X	**multiple rhombomeres**
32	AI601770	2.40	0.006	Wu:fc11e07	Dr.78014	X	X	X	**lateral to hindbrain**
33	BG728637	2.37	0.006	Wu:fj66h02		X	X		
34	AF229448	2.37	0.010	Jagged 1a	Dr.83677	X			
35	BM071271	2.31	0.006	Potassium channel tetramerisation domain containing 12.2	Dr.84702	X	X	X	**rhombomere 4**
36	BG891906	2.31	0.000	Actin filament associated protein 1-like 1	Dr.16264	X	X	X	**lateral to hindbrain**
37	BM026691	2.28	0.048	Zgc:65997	Dr.86139	X	X		
38	BM182277	2.20	0.020	Matrix metalloproteinase 2 (mmp2)	Dr.76397	X	X	X	**rhombomere 5**
39	BI710499	2.09	0.003	Cellular retinoic acid binding protein 1 (crabp1a)	Dr.83594	X	X	X	**hindbrain vesicle?**
40	BG727376	2.08	0.014	Wu:fa10d10	Dr.73730	X	X	X	**lateral mesoderm**
41	U89380	2.07	0.014	Ephrin type-A receptor 4a (epha4a)	Dr.47585	X	X	X	**rhombomere 3/5**
42	AF277097	2.05	0.001	SRY-box containing gene 9b	Dr.114501	X			
43	BI897419	2.05	0.043	Limb and neural patterns (lnpa)	Dr.6104	X	X		
44	BG883367	2.02	0.006	Transcribed locus; similar to tox3	Dr.85688	X	X	X	**rhombomere 3/5**
45	BE605613	1.99	0.027	Wu:fj20a04	Dr.80832	X	X		
46	AI794528	1.97	0.012	Zinc finger, CCHC domain containing 24	Dr.76547	X	X		
47	BG985478	1.94	0.007	V-ets erythroblastosis virus E26 oncogene homolog 1a (ets1a)	Dr.98888	X	X	X	**lateral to hindbrain**
48	BI864920	1.92	0.064	Wu:fc17g06; similar to ankyrin 2,3/unc44	Dr.105556	X	X	X	**broad expression**
49	AW420407	1.85	0.005	Transcribed locus	Dr.2211				
50	BM102082	1.81	0.036	Sulfatase FP2a	Dr.12108	X	X	X	**broadly in hindbrain**
51	AW077128	1.71	0.017	Growth arrest-specific 6 (gas6)	Dr.80253	X	X	X	**rhombomere 5**
52	BI980238	1.65	0.179	Transcribed locus - similar to spectrin	Dr.121661	X	X		
53	AA494787	1.34	0.412	Zgc:174862	Dr.75213	X	X		
54	BI890937	1.28	0.062	Zgc:73377; similar to protein phosphatase 1 subunit 14A	Dr.82617	X	X	X	**rhombomere 4**

[1] Each gene selected for analysis is listed in Table 1.

[2] Genbank number for each clone analyzed.

[3] Average fold increase for each gene across three replicates of *meis3* versus *meis3+hoxb1b* injected embryos.

[4] p-value for the fold increase given in column 3.

[5] Gene name from Genbank and/or ZFIN.

[6] Unigene number for each clone analyzed.

[7] Purchased clones whose sequence matched the sequence deposited on the array are indicated by an X. Other clones were not pursued.

[8] Clones for which in situ probes were successfully synthesized are indicated by an X. Other clones were not pursued.

[9] Clones for which detectable expression patterns were identified are indicated by an X.

[10] Brief description of expression pattern for each clone. Detailed expression patterns are shown in figure 2.