Bmpr1bb IS A NOVEL GENE INVOLVED IN RETINOIC ACID INDUCED PATTERNING OF THE ZEBRAFISH HINDBRAIN

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Abstract

Retinoic acid signalling plays a critical role during zebrafish development. The teratogenic effects of retinoic acid have been demonstrated by embryonic deformation resulting from insufficient or excessive levels of this vitamin A derivative. During embryogenesis, bone morphogenetic proteins are closely linked to the physiological interpretation of RA gradients, particularly in the hindbrain. We describe an uncharacterized gene, *Bmpr1bb*, as being significantly downregulated in response to retinoic acid treatment. *In situ* expression demonstrates that *Bmpr1bb* is expressed ubiquitously at 10hpf, and is slowly downregulated until 48hpf where the expression is concentrated in the hindbrain. We propose that *Bmpr1bb* is a downstream target of RA signalling, strongly downregulated during embryogenesis and specified to a specific region of the hindbrain.

Introduction

Retinoic acid (RA) plays an important part in the development of pattern in the zebrafish hindbrain, a fact that has been appreciated since early studies of its teratogenic effects *in vivo*. RA also plays an important role in developing the anterior-posterior axis, inducing a combinatorial expression of *Hox* genes in the hindbrain (Kessel, Gruss 1991). Evidence for a RA gradient comes from studies demonstrating that excess of retinoic acid during embryogenesis disrupts development of the anterior hindbrain as a potent dorsalizing signal (Wilson et al. 2007), (Durston et al. 1989). RA deficiency also has teratogenic effects, most clearly demonstrated by vitamin A deficient animal models (White et al. 2000). Despite the importance of RA signalling in vertebrates, only a handful of genes are known to be involved in RA signalling (Duester 2008). To this end, we aimed to identify and characterize novel downstream targets of RA signalling in the vertebrate hindbrain. Based on RA/RA-antagonist microarray data, we hypothesize that we can identify and characterize a candidate gene that is part of a network of downstream targets responsive to RA levels.

Within the cell, RA levels are tightly controlled, being either degraded by CYP26 in non-target tissues, or bound to retinoic acid receptor (RAR) – retinoid X receptor (RXR) heterodimers and functioning as nuclear receptors (Fig 1**)** (Mark, Ghyselinck & Chambon 2009, Abu-Abed et al. 2001, Sakai et al. 2001). RA signalling in the developing hindbrain is intimately related to its effects on *Hox* gene function. Several Hox genes are direct targets of RA, and contain retinoic acid response elements (RAREs) in their promoter regions (Simeone et al. 1990) (also see (Langston, Gudas 1994) and references therein). Only a few direct targets of RA signalling have been described: *Hox1*, a small number of transcription factors (*HNF-3α*, *Cdx1*), and a number of genes directly involved in retinoid metabolism (*CRABP1*, *CRABP2*) (Balmer, Blomhoff 2002).

Patterning of the embryonic dorsoventral axis of vertebrates requires signalling through bone morphogenetic proteins (BMPs). BMP ligands BMP2 and BMP7 function as heterodimers, catalyzing the assembly of a quadripartite transmembrane serine-threonine kinase receptor complex consisting of two type I and two type II receptors. Once the receptors are complexed, a phosphorylation cascade activates BMP-responsive Smads1/5 which act as transcription factors eliciting the downstream response (Feng, Derynck 2005). BMP signalling in vertebrates is tightly linked to RA levels. Indeed, exogenous RA has been shown to directly downregulate BMPs (Thompson et al. 2003). Bone morphogenetic protein receptors (BMPRs) are also ideal gene candidates for RA signalling, based on evidence for RA-induced

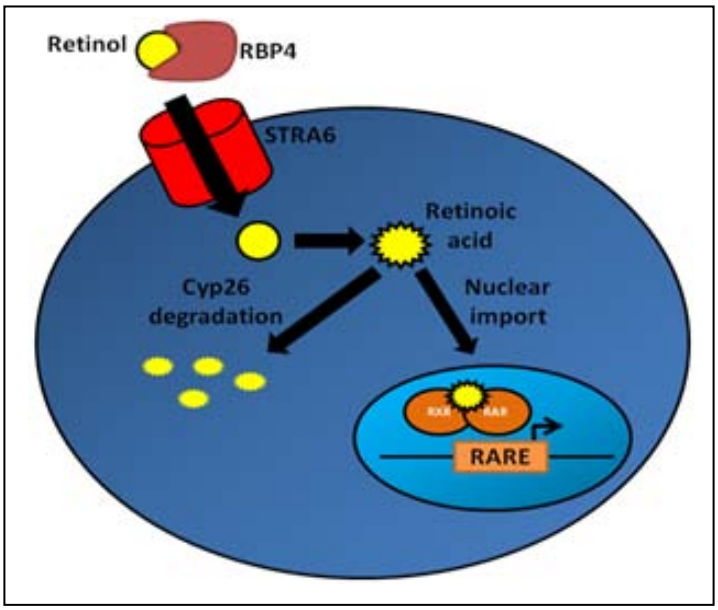

Figure 1. Schematic diagram of retinoic acid synthesis and signalling. Carried from the liver, retinol is bound by RBP4, and imported into the cell by the RA receptor STRA6. Inside the cell, RA is either A) degraded in non-target tissues by CYP26, or B) transported into the nucleus and bound to RAR-RXR heterodimers. RXR-RAR proteins bind retinoic acid response elements, and either transcriptional activate or inhibit gene transcription through co-repressors or co-activators. Based off of Duester, 2008**.**

BMPR signalling (Wan et al. 2006), and regulatory interpretation of RA gradients (Norlin et al. 2001). In order to elucidate RA target genes, Feng et al used a microarray-based approach to identify genes whose expression differed under RA treatment and RA inhibition (Feng et al. 2009). They describe *Dhrs3a* as a regulator of RA biosynthesis but the vast majority were uncharacterized. From this data set we selected *Bmpr1bb* as a candidate gene for interpretation of RA signalling. We hypothesize that *Bmpr1bb* is a candidate gene for direct response to the RA level in the developing zebrafish hindbrain. The developmental expression of *Bmpr1bb* was analyzed using Northern blotting and quantitative PCR (qPCR), and found to be significantly upregulated from the 50% epiboly to the 12hpf stage in addition to being strongly downregulated by RA. *In situ* expression analysis confirmed these results, demonstrating a ubiquitous expression of *Bmpr1bb* at the 10hpf stage, and a dynamic regulation specifying it to the hindbrain at 48hpf. These results suggest that *Bmpr1bb* is a good candidate for interpreting the RA-gradients present during zebrafish development.

Results

Gene selection

A microarray of zebrafish cDNA identified several hundred genes whose expression was significantly altered by RA, a RA antagonist, or both(Feng et al. 2009). From this data set, we chose *Bmpr1bb* (probe set ID: Dr.8289.1.S1_at) as a candidate gene involved in the RA response in the zebrafish hindbrain; *Bmpr1bb* is highly downregulated upon RA treatment, and upregulated upon treatment with a RA antagonist (control:1.0, RA fold change:0.5, RA-antagonist:1.3). Additional BMPs were identified by the microarray (*Bmp2b*, *Bmper*, *Bmp7a*), providing further evidence that RA may play an important role in the regulation of BMPs.

Validation of the *Bmpr1bb* transcript

The reported cloning of *Bmpr1bb* has recently been described, but with little other information reported (Little, Mullins 2009). *Bmpr1bb* is a 1994bp mRNA transcript, but the full transcript is likely longer than this because the 3' UTR is unknown (NCBI Gene ID: 100149664). Given the incomplete nature of this gene, it was necessary to validate the transcript. The duplicate co-ortholog *Bmpr1b* shares 81% homology with *Bmpr1bb*, and given that *Bmpr1bb* has been predicted from a single contig we treated the sequence with scepticism. Only 634bp have been mapped to a contig, with a significant portion of the 5' UTR not present (Fig 2). This 5' fragment of the gene maps to the distal tip of chromosome 10 whereas *Bmpr1b* is on chromosome 5, supporting the distinction of these two transcripts. There are

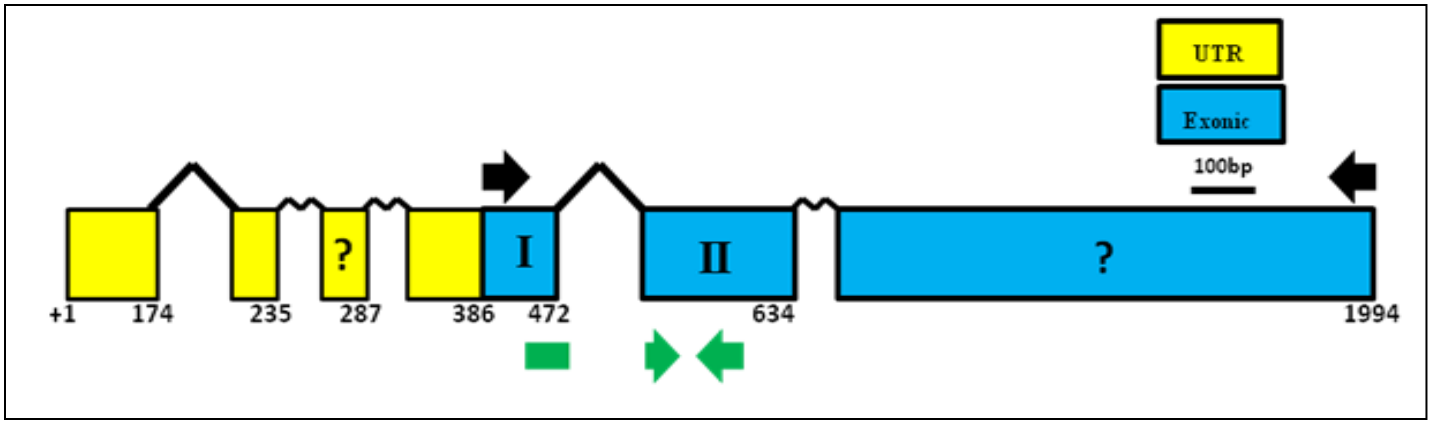

Figure 2. The *Bmpr1bb* gene has largely been constructed from a single contig (NW001882198.1) that is not annotated on the zebrafish genome. A blast analysis of the contig maps to the distal tip of chromosome 10. Prediction for the gene is from conserved homologues in other species. The UTR region from 236-287bp is not mapped and has likely been lost in zebrafish. Similarly, the majority of exonic sequence is not mapped in the genome, as it lies outside the contig. The 3'UTR is unknown, and the *Bmpr1bb* mRNA transcript is expected to be longer than 1994bp. Primer sites used to amplify the open reading frame are shown in black, and for the qPCR analysis in green.

two known splice sites in *Bmpr1bb*, joining exons 1 and 2 in the 5'UTR, and exons 2 and 3 in the ORF. The gene is not annotated to a genomic location, and there are no known splice junctions after the third exon. Much of the gene prediction came from conserved homologous sequences, only some of which map to the single contig. There are no known expressed sequence tags for this gene, which is likely due to the incomplete nature of the transcript and high degree of homology with *Bmpr1b*, confusing the large-scale bioinformatics approach. The coding DNA sequence of *Bmpr1bb* specifies a 535AA protein with a membrane targeting signal, and a single predicted transmembrane domain (Fig 3). Through functional domain prediction, we support that *Bmpr1bb* is a type I BMP-receptor, because of the conserved QS motif (Fig 3). The Bmpr1bb protein is highly conserved, and contains a number of key functional domains that support its designation as a type I BMPR (Fig 4). Surprisingly, the membrane targeting sequence is not present in the human homolog, and the activin receptor domain is divergent. *Bmpr1bb* was successfully cloned using full length ORF primers (see Fig 2), and sequencing verified that *Bmpr1bb* is indeed a real transcript distinct from *Bmpr1b*.

Northern blotting of *Bmpr1bb* expression

In order to determine the presence of alternative transcripts, the length of the *Bmpr1bb* 3' UTR, and the developmental expression of the gene, we first performed a Northern blot on various stages of zebrafish development. As seen in Fig 5, *Bmpr1bb* was detected at ~2700bp, suggesting that this gene might have a long 3'UTR (~700bp). *Bmpr1bb* was detected at 50% epiboly, 10hpf, 24hpf, and at the adult developmental stage. No *Bmpr1bb* expression was detected at 10hpf with RA treatment, consistent with the microarray data suggesting its downregulation in the presence of RA. The Northern blot did not reveal the presence of alternative splicing, but did demonstrate a possible dynamic developmental regulation of *Bmpr1bb*. To investigate this further with a finer measure of expression, we therefore performed a qPCR analysis.

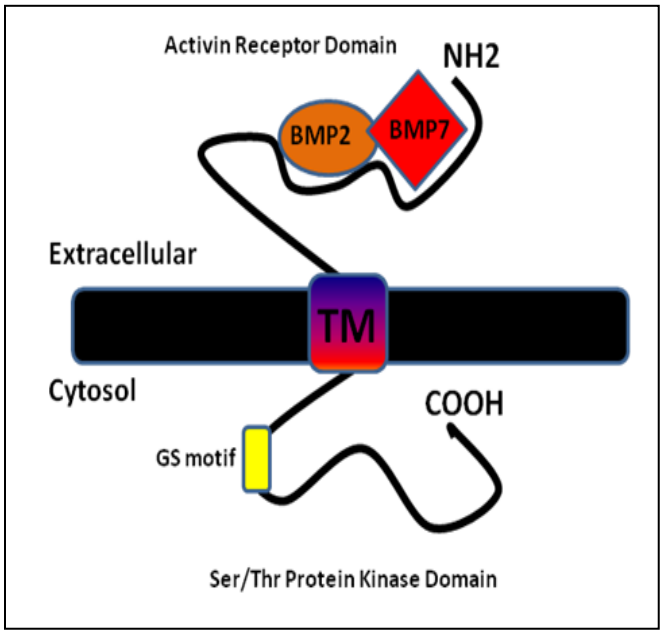

Figure 3. Proposed model for the Bmpr1bb protein. Transmembrane topology prediction supports a model with a membrane targeting sequence (AA 13-32) and a single transmembrane domain. This model places the conserved activin receptor domain extracellular, and the Ser/Thr kinase domain intracellular. A GS motif is the phosphorylation target for the BMPR type II receptor, specifying an active state to the protein. A BMP2-BMP7 heterodimer binds to Bmpr1bb, activating the intracellular phosphorylation cascade. Domain prediction was made with InterProScan, and membrane topology prediction with TMHMMv2.0.

Quantitative PCR

To complement the Northern blot results, we next analyzed the expression of *Bmpr1bb* over several developmental stages to establish how expression of this gene might be regulated during embryogenesis. As shown in Fig 6a, *Bmpr1bb* has a low expression at the 50% epiboly stage, increasing to a maximum at 12hpf. The expression slowly decreases throughout the remainder of the developmental stages analyzed (24hpf, 48hpf). We next compared *Bmpr1bb* with and without the addition of RA (Fig 6B). *Bmpr1bb* expression is significantly reduced upon RA treatment, which is consistent with the Northern blot results and the microarray data. These three pieces of evidence strongly suggest that *Bmpr1bb* is downregulated in response to RA. To verify the results *in vivo*, we next analyzed *Bmpr1bb* expression with an *in situ* hybridization.

***Bmpr1bb* is expressed ubiquitously and then specified to the hindbrain**

The results reported thus far have established that *Bmpr1bb* is expressed maximally at 10hpf, and is further downregulated as development continues (18hpf, 24hpf, 48hpf). The control *in situ* probe, *Vhnf1*, is expressed at the rhombomere 4/5 boundary, and is expanded along the AP axis upon RA treatment (Hernandez et al. 2004). We confirmed these results, affirming that our RA treatment was successful (Fig 7A, B). We have here within described the RA-induced downregulation of *Bmpr1bb* at the 12hpf stage, and consistent with the results from the microarray analysis, Northern blot and qPCR, *Bmpr1bb* is downregulated upon RA treatment (Fig 7C, D). Consistent with the developmental expression profile established by the qPCR data, *Bmpr1bb* expression decreases as the zebrafish develops: from a maximum expression at 10hpf to a minimal, restricted level at 48hpf (Fig 8A-D). *Bmpr1bb* maintains ubiquitous expression at 18hpf, becoming restricted to the hindbrain region at 24hpf, and finally a distinct boundary in the hindbrain at 48hpf. These results suggest a dynamic regulation of *Bmpr1bb* during development, leading to a distinct expression in the hindbrain where *Bmpr1bb* signalling may be integral to pattern development.

Discussion

Approximately 600 genes have been shown to be RA-responsive at the 2-4 somite stage in zebrafish (Feng et al. 2009), and the vast majority of these are yet to be characterized. A number of these genes are regulated directly by RA-bound receptors, a classic example being the *Hox* genes (Langston, Gudas 1994). Both RA excess and deficiency can cause malformation in the embryo, so it is important to understand the regulation of RA on both upregulated and downregulated RA-responsive

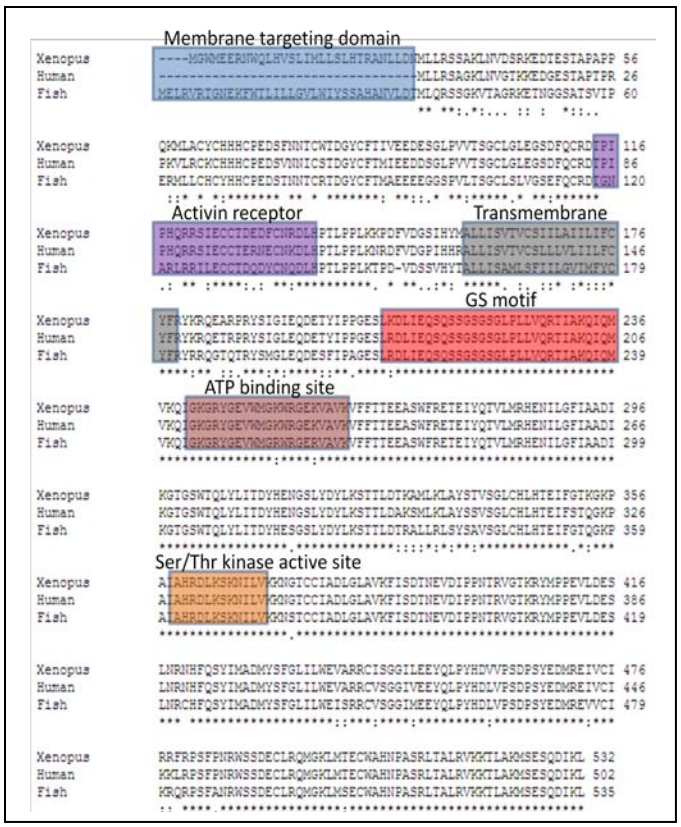

Figure 4. Sequence comparison of related Bmpr1b proteins demonstrates a high degree of conservation. A divergent N terminus does not appear to be in a functional domain based on protein topology prediction, and may be the membrane target sequence. "*" denotes an invariant AA,":" denotes conservative substitution "." denotes semiconservative substitution. Sequences were aligned with ClustalW2, and domains were predicted as described above. GeneID: Xenopus 780089, Human 658, Fish 100149664.

genes. The results described here suggest that *Bmpr1bb* is strongly downregulated by RA during early embryogenesis, and the dynamic expression observed over the remainder of developmental stages observed could be characteristic of a RA-induced downregulation. Further studies should include analyzing the expression of this gene at these developmental stages with the addition of excess RA and a RA-inhibitor.

Bmpr1bb shows a dynamic expression pattern during embryogenesis, yet the mechanism of this downregulation remains unresolved. Further characterization is needed at the molecular level to determine how RA has such a potent affect on several different BMPs, and how this regulation is variable in different tissues. RA induced downregulation of *Bmpr1bb* may be accomplished in a number of ways, the obvious way being transcriptional repression (RAR-RXR heterodimers with a co-repressor). Yet, RA is known to have unconventional affects on gene expression. A recent study demonstrated that RA bound RARα is capable of direct translational repression (Poon, Chen 2008). Finally, the wide range of gene regulation induced by RA might occur through transrepression of other nuclear receptors (Gupta et al. 2008). Future studies would involve a close inspection of the promoter region for RARE. In addition to the transcriptional control and expression pattern of RA on *Bmpr1bb*, it may have an effect on the Bmpr1bb protein as well. RAR-RXR heterodimers can alter protein kinase activity (Alsayed et al. 2001), and RAR proteins have recently been reported in the cytosol and membranes of some cells, suggesting a new paradigm by which RARs could integrate cytoplasmic events (Masia et al. 2007). The molecular function of the Bmpr1bb protein remains undetermined. Bmpr1bb's high degree of homology with other type-I BMPRs suggests it is an effector kinase that activates SMADs 1/5, and antibody immuno-stain for these activated kinases with and without RA is certainly a key question of interest.

A recent study showed that *Bmpr1bb* is involved in establishing the dorsoventral pattern in zebrafish, redundant with its duplicate co-orthologue *Bmpr1b*. The authors established that the knockdown of *Bmpr1b/Bmpr1bb*, along with *Bmpr1a*, caused increasingly dorsalized phenotypes. (Little, Mullins 2009). Despite the functional redundancy proposed for zebrafish, slight differences in knockout phenotypes in mouse suggests that these genes might have evolved independent functions which are yet to be

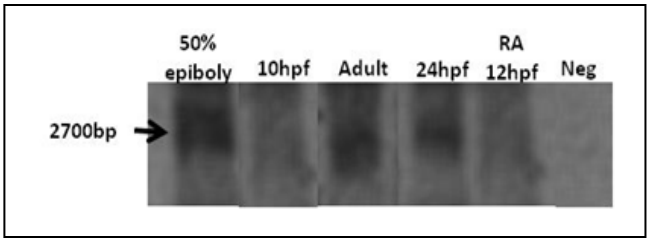

Figure 5. Northern blot of *Bmpr1bb* from zebrafish developmental timeframes demonstrates a dynamic regulation of *Bmpr1bb* expression, and a large downregulation upon treatment with RA. Hybridization to the blot was performed overnight at 65ºC, and washed in 1%SSC, 0.1%SDS for 20 minutes (three times), and again with 0.3%SSC, 0.1%SDS for 20minutes (two times). The Kodak film was developed after 72hours exposure at -80ºC.

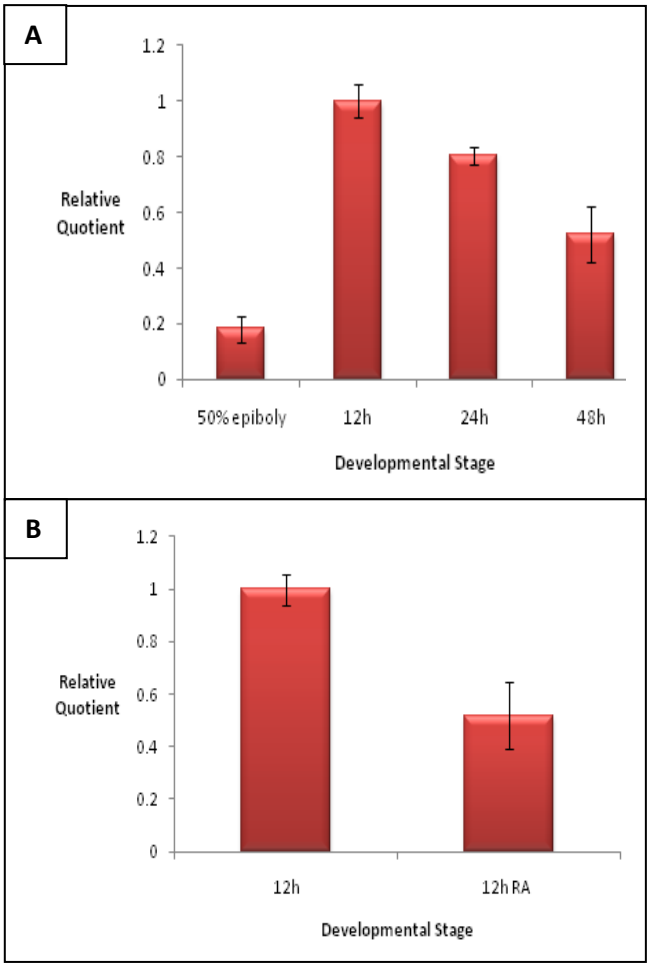

Figure 6A. Quantitative PCR of *bmpr1bb* from a series of zebrafish developmental stages shows a peak expression at 12hpf, slowly decreasing as development continues. **6B**. *Bmpr1bb* expression at 12hpf relative to a retinoic acid treated embryo shows a marked RA-induced downregulation. The qPCR expression data was normalized to the *odc* control, and compared to the 12hpf RQ. The data was analyzed with a relative quotient, where the *Bmppr1bb* amplification was compared to the *odc* control at each timeframe, and then relative to 12hpf. Error bars show standard error of the mean.

these proteins differ in their regulation, and only a close analysis of the *Bmpr1a* expression pattern will confirm this hypothesis. Our characterization of the RA-induced downregulation of Bmpr1bb suggests a possible regulatory divergence that could be investigated in other systems. Of note, a study by Li et al reported that RA induces the expression of Smad1 and Smad5 (Li et al. 2003). They shower that this was a BMPR specific process, but independent of Bmpr1a. These studies, along with the results described here, suggest that *Bmpr1b/Bmpr1bb* might be the BMPR responsive to RA levels in many different tissues.

Materials and Methods

Cloning *Bmpr1bb*

Total RNA was harvested from WT zebrafish at 11hpf using TRIZOL reagent and standard protocols. A first strand cDNA synthesis was performed from 5µg of total RNA, using an oligo dT primer and Superscript III Reverse Transcriptase (Invitrogen, CA) according to the manufacturers specifications. *Bmpr1bb* was amplified with Phusion polymerase (New England Biolabs, MA), using primers specific for the full length ORF (*Bmpr1bb*F: 5' ATGGAGCTCCGGGTACGGAC, *Bmpr1bb*R: 5' TCACAGTTTAATGTCCTGCGACAC) (also see Figure 2). The full length *Bmpr1bb* gene was cloned into the pGEX-GR expression plasmid using a blunt-ended EcoRV ligation, with the NTD fused to GST. PCR and sequencing confirmed the correct orientation of the gene and that it was in frame with the GST tag.

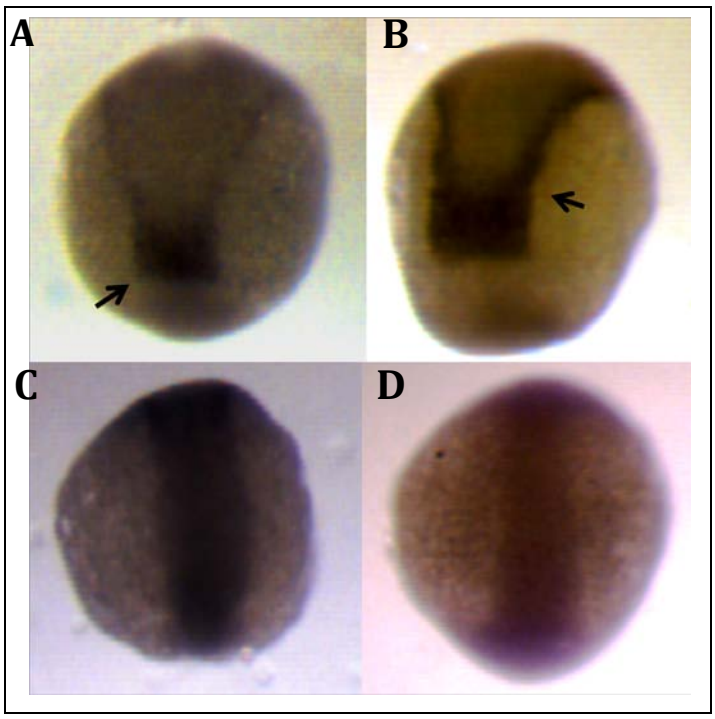

Figure 7. Whole-mount *in situ* hybridization of wild-type embryos at the 1-somite stage, untreated (**A, C**) or treated with RA (**B, D**). **A**. *Vhnf1* is expressed at the r4/5 boundary at 10hpf. **B**. *Vhnf1* expression is expanded along the AP axis upon treatment with RA. **C**. *Bmpr1bb* is expressed maternally and ubiquitously, and is consistently downregulated upon treatment with RA.

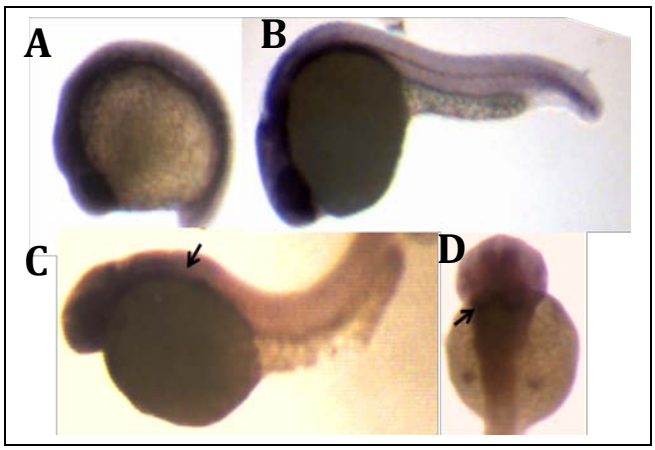

Figure 8. Whole-mount *in situ* hybridization of wild-type embryos, probed for *Bmpr1bb*. Bmpr1bb maintains ubiquitous expression at 18hpf (**A**), and only becomes more restricted at the 24hpf (**B**). At 24hpf, Bmpr1bb shows distinct expression in the eye and hindbrain, but is absent in the forebrain. At 48hpf, Bmpr1bb is further downregulated (**C**), but maintains a sharp expression in a distinct region of the hindbrain (**D**).

Northern blot analysis of *Bmpr1bb*

Fish lines were maintained under standard conditions at 28.5ºC at the University of Alberta Fish Facility. RNA was extracted from WT whole fish embryos at specific developmental stages (50% epiboly, 10hpf, 12hpf, 24hpf, 48hpf, adult, and 12hpf with 0.33µM RA treatment) using TRIZOL reagent standard protocols. 10µg total RNA was loaded for each stage, run on a 3% formaldehyde - 0.8% agarose gel before being transferred to GeneScreen Plus overnight. The RNA was then crosslinked to the membrane with a UV Stratalinker (120mJ, 30 seconds). The probe for *Bmpr1bb* was made from a full length linear fragment, digested out of the pGEX-GR vector with BamHI, and synthesized with α^{32}P-dCTP and Klenow by standard protocol. (Abu-Abed et al. 2001).

Quantitative PCR of *Bmpr1bb*

The qPCR was performed in triplicate on an ABI StepOne Real time cycler (95 ºC 2', 95 ºC 15s, 60 ºC 1', 40X). A standard curve was created using a serial dilution of *Bmpr1bb* and *ornithine decarboxylase* (*odc*) constructs, to assess the PCR efficiency (Bmpr1bb F: 5' CCGCTCACGCGAACGT, Bmpr1bb R: 5' GACAGTGATGGTAACAGTGGCATAATA), also see Fig 2) (Odc F: 5' TGAACCTCCTTGGCTGTCTGA, Odc R: 5' TGCAAAAGAAACAGACGAATGG. Both efficiencies were ~100% (data not shown), and *odc* was deemed an appropriate control. Products were visualized on a 2.0% agarose gel and a melting curve was performed to assure appropriate amplification.

In situ hybridization

Whole mount in situ hybridization was performed essentially as described (Prince et al. 1998) with the following modifications: probes were not hydrolyzed, and various proteinase K (10µg/mL) treatments were used. Proteinase K was used for 3min (18hpf), 5min (24hpf), and 10min (48hpf) and then fixed so the total treatment time was 30min. Probes were made from full length *Vhnf1* and *Bmpr1bb*, and hybridized overnight at 65°C. Anti-DIG antibody was used 1:5000 overnight at 4°C, and then stained for 6 hours. Embryos were photographed with an Olympus stereoscope and a Qimaging micropublisher camera. Embryos were raised at 25°C and staged according to published hallmarks (Kimmel et al. 1995).

Acknowledgements

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