Molecular cloning, expression and characterization of the zebrafish *bram1* gene, a BMP receptor-associated molecule

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Summary

We have identified a cDNA clone encoding BMP receptor-associated molecule 1 (BRAM1) from the zebrafish expressed sequence tag (EST) database. The 2606 bp full-length *bram1* cDNA was cloned, and further confirmed by nucleotide sequencing. The zebrafish sequence encodes a protein of 195 amino acids with an evolutionarily conserved MYND domain, which displays ~98% homology with human and mouse BRAM1, and ~64% homology with *C. elegans* BRA-1 and BRA-2. The *bram1* gene, composed of five exons and four introns, spans ~14 kb on linkage group 14 of the zebrafish genome. RT-PCR and whole mount *in situ* hybridization analyses disclosed that zebrafish BRAM1 is a maternal factor. The protein interacts directly with zebrafish BMP Receptor type IA, as observed from GST-pull down and co-immunoprecipitation assays. Furthermore, cotransfection of zebrafish BRAM1 with the corresponding BMP receptor resulted in down-regulation of BMP-mediated signaling. Our results collectively indicate that BRAM1 plays a biological role during zebrafish development.

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)- β superfamily. These proteins contribute to the embryonic development of animals, and are essential for patterning of the embryo along all the body axes [1, 2]. The BMP signaling pathway, such as the members involving BMP signaling pathway, is highly conserved among different species, including human, mouse and zebrafish (*Danio rerio*). The pathway is activated through binding of BMP to type I and type II serine–threonine kinase receptors [3], consequently triggering phosphorylation

of the type I receptor. This step leads to recruitment of the receptor-regulated Smads (R-Smads), specifically, Smad1, 5 or 8, to the receptor complex [4], and subsequent phosphorylation at the SSXS motif in the C-termini of all R-Smad proteins [5, 6]. Next, R-Smads are released from type I receptors and assemble with Co-Smad, Smad4, into heteromeric complexes [7–9]. The complexes translocate to the nucleus and participate directly in the modulation of gene expression via specific interactions with other co-activators or co-repressors [4, 10, 11]. In addition to the Smad family, several other proteins are involved in the BMP signaling pathway. These include TGF- β activated kinase (TAK1) [12], which also participates in the mitogen-activated protein kinase (MAPK) cascade, and TAK1 binding protein 1 (TAB1) [13].

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BMP signaling is suppressed by inhibitory Smads or I-Smads. Smad6 and Smad7 inhibit the BMP signaling pathway by competing with R-Smad proteins for interactions with the activated type I receptor [14]. Moreover, Smad6 competes with Smad4 to block complex formation [15]. Smad7 forms a complex with the HECT domain ubiquitin ligase, Smurf, and triggers the degradation of receptors or R-Smads via proteasomal and lysosomal pathways [16, 17]. The BMP receptor IA (ALK3) interacts with a cytosolic protein, BRAM1 (BMP receptor-associated molecule 1) [18]. This association is specific, since neither ALK2 nor TGF- β type I receptor (T β R-I) bind the protein [18]. BRAM1 contains a conserved MYND domain, which mediates protein-protein interactions, including binding to Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) [19]. This association leads to inhibition of LMP1-mediated NF- κ B activation via targeting of the I κ B α molecule, suggesting that BRAM1 functions as a negative regulator of LMP1-mediated biological function [19]. To date, two homologs of BRAM1 in *C. elegans* (BRA-1 and BRA-2) have been identified [20]. Genetic analysis of a BRA-1 null mutant with previously known *daf-7* (a TGF- β ligand homolog mutant) reveals that BRA-1 is epistatic to *daf-7* and *daf-1* (a type I receptor homolog) [21, 22] and hypostatic to *daf-14* (a Smad homolog) [23]. Moreover, BRA-1 negatively regulates TGF- β signaling by blocking physical interactions between the type I receptor and Smads [21]. However, the physiological role of zebrafish BRAM 1 has yet to be elucidated.

In this study, we isolate zebrafish *bram1*, and report the identification, expression and functional characterization of the corresponding protein that may be involved in early zebrafish development and organogenesis. Further experiments disclose that BRAM1 directly interacts with the BMP receptor, both *in vitro* and *in vivo*. A luciferase reporter assay further shows that BRAM1 downregulates BMP signaling in a dose-dependent manner.

Methods

Fish and embryo maintenance

Adult zebrafish (*Danio rerio*) were maintained at 28 °C under a 14 h light/10 h dark cycle. Wild-

type zebrafish embryos were obtained from a natural spawning AB strain, and developmental stages were determined from time after fertilization (hours post-fertilization; hpf) at 28 °C, as well as morphological criteria.

Cloning of zebrafish bram1 cDNA

A zebrafish cDNA clone (GenBank accession no. *AW174019*) containing part of the *bram1* open reading frame (ORF) was identified by BLAST analysis of the NCBI EST database. Full-length *bram1* cDNA was determined by 5'-RACE studies on a zebrafish cDNA library with the 5'-RACE system Version 2.0 (GibcoBRL). The primers used for the outer and inner 5'-RACE amplification reactions were: GSP1 (5'-TTGGCAGTTGCCTTCATTTAC-3') and GSP2 (5'-TGTCTGTGTGCCTTTGTGGATCA-3'). Amplification was performed for a total of 30 cycles under the following condition: 30s at 96 °C, 30 s at 65 °C, and 2 min at 72 °C. The amplified product was cloned into the pUC18 vector (BAYOU BIOL-ABS). Multiple alignments were performed with CLUSTALW version 1.8.

GST pull-down assay

GST-BMPRIA C-terminal protein (from a.a. 201 to the C-terminal end cloned into pGEX-3X expression vector) was produced in *E.coli* BL21. Briefly, the protein was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h at 16 °C. GST fusion proteins were prepared as described by the manufacturer (Pharmacia), and purified with glutathione-agarose beads (Pharmacia). The purity and quantity of recombinant proteins were determined by SDS-PAGE, followed by staining with Coomassie blue.

In vitro transcribed and translated [³⁵S]Met-labeled BRAM1 was generated using pCDNA3.1-BRAM1 in a 50 μ l reaction containing [³⁵S]Met (Pharmacia) with the TNT coupled transcription-translation system, according to the manufacturer's instructions (Promega).

The GST pull-down experiment was performed using 10 μ g GST or GST fusion protein bound to glutathione beads, and 10 μ l ³⁵S-labeled protein synthesized *in vitro* in a total volume of 500 μ l binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) at

4 °C overnight. Beads were extensively washed with binding buffer, resuspended in SDS sample buffer, and separated by SDS-PAGE. Radiolabeled proteins were visualized by autoradiography.

Coimmunoprecipitation

COS-7 cells were transfected with pCDNA3.1-BMPRIA-HA and/or pFlag-BRAM1. Cell extracts prepared using RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, and 0.5% NP-40) supplemented with a complete protease inhibitor cocktail (Roche) were incubated with mouse anti-FLAG M2 affinity gel (Sigma) at 4 °C overnight with rotation. Next, beads were washed three times with RIPA buffer, and boiled in SDS sample buffer for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE, and electroblotted onto PVDF membranes that were subsequently incubated with mouse anti-FLAG monoclonal antibody M2 (Sigma) or rabbit anti-HA polyclonal antibody (Santa Cruz) with 1:1000 or 1:500 dilution, respectively, for 1 h in 3% skimmed milk. Tagged proteins were detected using HRP-conjugated secondary antibodies and chemiluminescence (ECL, Pierce).

Luciferase reporter assay

3T3 cells (5×10^6) were transfected with 1 μ g p(SBE)₄-luc, 1 μ g BMPRIA-HA and different doses of Flag-BRAM1 by electroporation (0.24 kv, 960 μ F). Following transfection, cells were treated with BMP2 (80 ng/ml) for 16 h in DMEM containing 0.1% FBS. A pSV40- β -galactosidase vector (50 ng) was co-transfected for the monitoring of transfection efficiency. For harvesting, lysis buffer (200 μ l) was added to each dish and 50 μ g of the protein-containing supernatant was used for luciferase determination with a luminometer. Additionally, 50 μ g proteins were assayed for β -galactosidase activity. Reporter activity is expressed as a ratio of relative light units/ β -galactosidase activity. Data are presented as means $\pm$ SEM of experiments performed in triplicate.

RT-PCR and Real-time RT-PCR

Stage-specific total RNA from at least 100 normal embryos was isolated using the Trizol A reagent (Gibco/BRL). Total RNA (1 μ g) was employed for

reverse transcription. The following primers were used: *bram1*, 3'-TGCCACGACAAATACTC CA AGATCTTCAG-5' (sense) and 3'-GTTTGCAT TTCTCTCCATCTCGGCCT-5' (antisense); *bmpr1a*, 3'-ATCTACGGCACGCAGGG AAA A5' (sense) and 3'-AGCGCCTGGTTCC CATACTG-5' (antisense); *bmp4*, 3'-AGCAGCA TCCCAGAGG ACGAA-5' (sense) and 3'-GTCC TTCCCGTG GCGCCTT-5' (antisense); and *ef-1 α* , 3'-GAGGG GCTTGTCTCGGTGGGA-5' (sense) and 3'-GTG TTGCCTTCGTCCCAATTTCAG-5' (antisense). RT-PCR was performed under the following conditions: 45 s at 96 °C, 30 s at 60 °C, and 30 s at 72 °C for 30 cycles. Quantitative RT-PCR was performed in accordance with the manufacturer's instructions, using a LightCycler (Roche Diagnostics) and the FastStart DNA Master SYBR Green I system (Roche Diagnostics) in which fluorescence binds to double-stranded DNA. PCR products were separated on a 2% agarose gel, and results normalized against data from control mRNA, *ef-1 α* .

Whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde. *In situ* hybridization was performed using a digoxigenin-labeled antisense RNA probe [24]. NBT/BCIP was employed as a substrate for alkaline phosphatase. To prepare the antisense riboprobe, the *EcoRI-XbaI* fragment of *bram1* cDNA was subcloned into pBluescriptKS+ (Stratagene), linearized by *EcoRI* digestion, and transcribed with T7 RNA polymerase.

Results and discussion

Isolation of the zebrafish bram1 cDNA sequence

BRAM1 is conserved in human, mouse, and *C. elegans* [6, 7, 9]. To ascertain the presence of BRAM1 in zebrafish, we employed the mouse sequence to search the NCBI EST database, using the tBLAST program. As a result, an EST clone (Accession No. *AW174019*) containing an insert of ~2.5 kb displaying a partial open reading frame, translation stop codon, and 3' untranslated region containing the polyadenylation signal sequence (AATAAA) was identified. Based on the EST sequence, we designed a pair of primers for

5'RACE analysis. The full-length cDNA obtained was subjected to DNA sequence analysis. Consequently, we identified an additional 387 nucleotides. The nucleotide and deduced amino acid sequences of this gene are shown in Figure 1. Analysis of the 2606 bp cDNA sequence revealed that zebrafish *bram1* contains a 289 bp 5'uncoding region, a 1729 bp 3'uncoding region, poly A-tail, and an ORF from nucleotides 290–877 that encodes a putative protein of 195 amino acids with a 44 residue MYND domain from amino acid 152 to the C-terminus (Figure 1a). The two crucial positions for translational initiation are the purine at –3 and guanine at +4. Both these nucleotides are conserved in zebrafish cDNA. Additionally, the *bram1* gene is mapped to linkage group 14, based on the Sanger zebrafish genomic sequence project database (http://www.sanger.ac.uk/Projects/D_rerio/blast-server.shtml). The zebrafish *bram1* genomic sequence spans 14,394 bp, encompassing five exons and four introns (Figure 1b). The translation initial codon, ATG, is located on the third exon.

Analysis of zebrafish BRAM1 homologs

The deduced amino acid sequences of BRAM1 homologs of human, mouse, *C. elegans*, and *Drosophila* from the EST library of NCBI are shown in Figure 2a. BRAM1 of human, mouse and zebrafish are highly homologous within the entire sequence, and almost identical within the C-terminal MYND domain (amino acids 151–195) (Figure 2a). The amino acid sequence of zBRAM1 (zebrafish BRAM1), displays 77%, 75%, 31%, and 24–25% identity to human (hBRAM1), mouse (mBRAM1), *C. elegans* (BRA-1 & BRA-2), and *Drosophila* (CG8569 & CG1815), respectively, as determined from the CLUSTAL W multiple sequence alignment program (Figure 2a). All homologs contain the highly conserved MYND domain. Specifically, the zebrafish MYND domain displays 98% identity to hBRAM1 and mBRAM1, 64% with *C. elegans*, and 40% and 55% homology with the *Drosophila* homologs, CG8569 and CG1815, respectively (Figure 2b). Moreover, zebrafish BRAM1 displays significant homology (>60%) with hBRAM1 and mBRAM1 outside the MYND domain, and a lower degree of similarity with *C. elegans* and *Drosophila* homologs, suggesting that these proteins play similar biological roles.

Expression of the zebrafish bram1 gene

The temporal expression profile of the *bram1* gene was examined by RT-PCR analysis of mRNA in embryos at different development stages (0, 4, 8, 10, 12, 16, 24, 72, 144 hpf, and adult fish). The *bram1* transcript was readily detected in embryos upon fertilization (day 0), and levels declined significantly after 4 hpf (Figure 3a). However, expression was slightly enhanced at 72 hpf. Interestingly, *bram1* mRNA is present during the early stages of development before the onset of zygotic transcription, indicating that these transcripts are maternally supplied. BRAM1 molecules from human and mouse interact with BMPR-1A, and are therefore possibly involved in BMP-mediated signaling. Accordingly, we hypothesize that these three molecules are expressed in a cooperative manner. To test this hypothesis, we examined the expression profiles of zebrafish *bmp receptor type 1a* (*bmpr1a*) and *bmp4*. The *bmpr1a* message is maternally provided, similar to *bram1*, but expression was elevated during the gastrulation stages, followed by a decline until the somitogenesis stages (16 hpf). Expression of *bmp4* was absent prior to gastrulation, but showed a regular increase during the gastrulation stages, and decreased during somitogenesis. However, zebrafish *bram1*, *bmpr1a*, and *bmp4* mRNA levels were elevated again at the end of somitogenesis, with a peak between 24 and 72 hpf, followed by a decrease until the adult stage. Zebrafish *ef-1a* appears to be present at a constant level during all the developmental stages examined. To further confirm the RT-PCR data, we performed real-time RT-PCR to compare the amounts of the three transcripts described above during the developmental stages (0, 4, 8, 10, 16, 24, 30, 36, 48, 72 and 144 hpf). Assuming the level at 24 hpf was 1, we observed that expression of *bram1* was significant before gastrulation and declined during the gastrulation and somitogenesis stages (Figure 3b). Northern blot analysis of adult zebrafish tissues revealed high levels of the *bram1* transcript in the ovary (data not shown). The *bmpr1a* level generally increased during cleavage and blastula stages, with a peak at 8 hpf. Expression of *bmp4* was initiated at 8 hpf, and generally decreased until the pharyngula period. Levels of *bram1*, *bmpr1a*, and *bmp4* started to increase after 24 hpf, and displayed minor peaks at 36 and 48 hpf. Our results suggest that zebrafish *bram1*, *bmpr1a*, and

Figure 1. Nucleotide, deduced protein, and genomic organization of *bram1*. Panel (a) shows the nucleotide and deduced amino acid sequences of BRAM1. The open reading frame is presented in uppercase font. Start and stop codons are underlined. The MYND domain is specified within a solid box. Panel (b) shows gene structure of *bram1*. Five exons are separated by four introns 80, 2879, 144, and 8685 bp in length.

Figure 2. Comparison of zebrafish BRAM1 and its homologs. Panel (a) shows alignment of BRAM1 proteins from zebrafish (z), human (h), mouse (m), *C. elegans* (BRA-1 and BRA-2), and *Drosophila* (CG8569 and CG1815). The asterisk (*) indicates identical amino acids; (:) represents a strong similarity; (.) implies weaker similarity; and hyphens (-) indicate gaps in the alignment. The MYND domain is underlined. Panel (b) shows similarities and identities of zebrafish BRAM1 with its homologs. The MYND domain is highlighted within a gray box. The percentage identities and similarities between whole sequences and zebrafish BRAM1 are shown in bold. The percentage identity of the N-terminal and C-terminal regions is indicated below the structure maps. The numbers of the predicted amino acid sequence are specified on the right.

Figure 3. Gene expression analysis of *bram1* during different zebrafish embryonic developmental stages. Panel (a) shows RT-PCR analysis of *bram1*, *bmp1a*, *bmp4* at ten developmental stages. Amplified DNA products were separated by electrophoresis on 2% agarose gels. Control PCR products were generated using primers specific for the *ef-1α* gene. Panel (b) shows real-time RT-PCR analysis of *bram1* transcripts at twelve different developmental stages. Total RNA from different stages was isolated, and gene expression determined by Faststart PCR, using reverse-transcribed cDNA. Relative expression of *bram1*, *bmp1a*, and *bmp4* was normalized in comparison to that of internal control, *ef-1α*.

bmp4 are expressed simultaneously, supporting the potential cooperation of these three proteins in the BMP signaling pathway. Thus, high *bram1* expres-

sion during the early developmental stage may contribute to cell fate and influence organogenesis during later development.

*Spatiotemporal expression pattern of the zebrafish *bram1* gene*

We analysed the *bram1* expression pattern during early embryonic development by whole-mount *in situ* hybridization with zebrafish embryos from the 2-cell stage to 72 hpf, using a digoxigenin-labeled riboprobe. As shown in Figure 4, *bram1* transcripts were uniformly expressed throughout the embryo in cleavage and blastula stages, and strong staining for *bram1* was detected. These

findings indicate that maternally supplied *bram1* transcripts are ubiquitously and uniformly distributed in all blastomeres (Figures 4a, b). During gastrulation, lower level of *bram1* transcripts are present (Figure 4c), consistent with data obtained from real-time RT-PCR experiments. At the tail-bud stage, *bram1* was expressed throughout the embryos. During the somitogenesis stages, expression was restricted to the ventral region of the trunk and anlagen of the eyes (Figure 4d). At 24 hpf, faint uniform expression of *bram1* was

Figure 4. Expression pattern of *bram1* during embryonic development. Embryos from two-cell (a), high (b), shield (c), 10-somites (d), 24 hpf (e), 36 hpf (f), 48 hpf (g), 72 hpf (h) stages were analyzed using whole-mount *in situ* hybridization by probing with digoxigenin-labeled *bram1*-specific antisense probes.

Figure 5. BRAM1 interacts with the BMP receptor to downregulate the BMP signaling pathway. Panel (a) shows the result of co-immunoprecipitation of zebrafish BRAM1 and BMPRIA. COS-7 cells were transiently transfected with zebrafish BMPRIA-HA and Flag-zBRAM1(1-195), Flag-zBRAM1(67-195), Flag-zBRAM1(1-128) or Flag-zBRAM1(129-195) deletion constructs, followed by immunoprecipitation with anti-Flag, and western blot analysis with anti-HA antibodies. Panel (b) shows interactions between BRAM1 and BMPR-IA, as determined by GST-pull-down assays. [35 S]Met-labeled BRAM1 was generated by *in vitro* transcription and translation. BRAM1 was incubated with affinity-purified GST-BMPR C terminal fusion protein, and recovered using glutathione-Sepharose beads. Panel (c) shows BRAM1 inhibits BMP signaling pathways. Data from experiments involving the co-transfection of zebrafish BMPRIA-HA and Flag-BRAM1-expressing constructs (0.5, 1, & 3 μ g) with a BMP-responsive reporter gene construct, p(SBE) $_4$ -luc show that zebrafish BRAM1 interferes with BMP signaling in 3T3 cells.

observed in the brain and eyes (Figure 4e). At 36 hpf, *bram1* transcripts increased in the retina, cerebellum, and buds (Figure 4f). At 48 and 72 hpf, decreased *bram1* expression was evident throughout the brain (Figure 4g, h). It worth noting that the expression pattern of *bmp receptor* is overlapping with the expression pattern of zebrafish *bram1* [25–30].

Zebrafish BRAM1 specifically interacts with zebrafish BMPR-IA and downregulates BMP signaling in mammalian cells

Analogous to human and mouse BRAM1, zebrafish BRAM1 interacts with its BMP type I receptor. Thus, we investigated whether this association affects the BMP signaling pathway. COS-7

cells were transiently transfected with Flag-zBRAM1 and zBMPRIA-HA, followed by immunoprecipitation with anti-Flag antibody. Flag-tagged BRAM1 proteins were precipitated with anti-Flag antibody, and immunoblot analysis revealed co-precipitation of BMPRIA-HA (Figure 5a). Reprobing with the anti-Flag antibody confirmed that an equivalent level of BRAM1 protein was precipitated. Our data show that BRAM1 interacts specifically with the cytoplasmic domain of BMPR-IA in mammalian cells. To analyze the BMP receptor binding domain of BRAM1, deletion mutants, Flag-BRAM1(67–195), Flag-BRAM1(1–128), and Flag-BRAM1(129–195) (Figure 5a) were transiently expressed, along with BMPR-IA-HA.

Flag-tagged BRAM1 proteins were precipitated with anti-Flag antibody, and immunoblot analysis revealed co-precipitation of BMPRIA-HA. Immunoprecipitation data showed that only Flag-BRAM1(1–128) did not co-precipitate with BMPRIA-HA. In contrast, the other deletion mutants precipitated with BMPR-IA. Our data thus strongly imply that the C-terminal region of BRAM1 interacts with BMPR-IA, particularly through the MYND domain of BRAM1.

Zebrafish BRAM1 interacts with BMPR-IA through the MYND domain in mammalian cells. We additionally performed a GST pull-down assay to determine whether the binding of zebrafish BRAM1 to BMPR-IA is direct. Specifically, the GST-BMPRC' fusion protein was expressed in BL21 bacterial cells and affinity column-purified (Figure 5b). Zebrafish BRAM1 was labeled with [³⁵S] using *in vitro* transcription and translation, and incubated with GST-BMPRC' using glutathione-Sepharose beads overnight at 4 °C to pull down the protein complexes. *In vitro*-translated BRAM1 interacted with GST-BMPRC' (Figure 5b), indicating that the homolog is directly associated with BMPR-IA in zebrafish.

To establish the function of the BRAM1–BMPRIA complex in BMP signaling, cells were co-transfected overnight with the specified BRAM1-expressing vector, BMPR-IA expressing vector, and BMP-responsive promoter–reporter plasmids ((SBE)₄-Luc), followed by serum starvation 12 h, and treated with BMP2 or left untreated for 16 h. BRAM1 inhibited the transcriptional activity induced by BMP2 in a dose-dependent manner (Figure 5c). The data suggest that the

BRAM1 inhibits BMP signaling, possibly through its C-terminal MYND domain.

Thus, in this study, we have reported the isolation of the zebrafish *bram1* gene, and have additionally examined the developmental profile of this gene in zebrafish, which is coordinated with the expression of BMP and BMP type I receptor. The direct interactions between zebrafish BRAM1 and BMP receptor type IA, and inhibition of BMP-mediated reporter gene expression, support a potential role of zebrafish BRAM1 in BMP-mediated signaling. Further analyses of the interactions between BRAM1 and mediator molecules in BMP signaling pathways are required to elucidate the mechanism by which BRAM1 inhibits BMP signaling. It would additionally be feasible to investigate the physiological role of BRAM1 in zebrafish development by morpholino knock-down of BRAM1 expression, followed by *in vivo* analysis of fish development.

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References

1. Nohe A., Keating E., Knaus P. and Petersen N.O., Signal transduction of bone morphogenetic protein receptors. *Cell Signal* 16: 291–299, 2004.
2. ten Dijke P., Korchynskyi O., Valdimarsdottir G. and Goumans M.J., Controlling cell fate by bone morphogenetic protein receptors. *Mol. Cell. Endocrinol.* 211: 105–113, 2003.
3. Wrana J.L., Attisano L., Wieser R., Ventura F. and Massague J., Mechanism of activation of the TGF- β receptor. *Nature* 370: 341–347, 1994.
4. Heldin C.H., Miyazono K. and ten Dijke P., TGF- β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 390: 465–471, 1997.
5. Kretschmar M., Liu F., Hata A., Doody J. and Massague J., The TGF- β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev.* 11: 984–995, 1997.
6. Macias-Silva M., Abdollah S., Hoodless P.A., Pirone R., Attisano L. and Wrana J.L., MADR2 is a substrate of the TGF- β receptor and its phosphorylation is required for

- nuclear accumulation and signaling. *Cell* 87: 1215–1224, 1996.
7. Kawabata M., Inoue H., Hanyu A., Imamura T. and Miyazono K., Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO J.* 17: 4056–4065, 1998.
 8. Lagna G., Hata A., Hemmati-Brivanlou A. and Massague J., Partnership between DPC4 and SMAD proteins in TGF- β signaling pathways. *Nature* 383: 832–836, 1996.
 9. Wu R.Y., Zhang Y., Feng X.H. and Derynck R., Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. *Mol. Cell. Biol.* 17: 2521–2558, 1997.
 10. Derynck R. and Zhang Y.E., Smad-dependent and Smad-independent pathways in TGF- β family signaling. *Nature* 425: 577–584, 2003.
 11. ten Dijke P. and Hill C.S., New insights into TGF- β -Smad signaling. *Trends Biochem. Sci.* 29: 265–273, 2004.
 12. Yamaguchi K., Shirakabe K., Shibuya H., Irie K., Oishi I., Ueno N., Taniguchi T., Nishida E. and Matsumoto K., Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* 270: 2008–2011, 1995.
 13. Shibuya H., Yamaguchi K., Shirakabe K., Tonegawa A., Gotoh Y., Ueno N., Irie K., Nishida E. and Matsumoto K., TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* 272: 1179–1182, 1996.
 14. Nakao A., Afrakhte M., Moren A., Nakayama T., Christian J.L., Henchel R., Itoh S., Kawabata M., Heldin N.E., Heldin C.H. and ten Dijke P., Identification of Smad7, a TGF- β -inducible antagonist of TGF- β signaling. *Nature* 389: 631–635, 1997.
 15. Hata A., Lagna G., Massague J. and Hemmati-Brivanlou A., Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* 12: 186–197, 1998.
 16. Zwijsen A., Verschuere K. and Huylebroeck D., New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS Lett.* 546: 133–139, 2003.
 17. Murakami G., Watabe T., Takaoka K., Miyazono K. and Imamura T., Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol. Biol. Cell* 14: 2809–2817, 2003.
 18. Kurozumi K., Nishita M., Yamaguchi K., Fujita T., Ueno N. and Shibuya H., BRAM1, a BMP receptor-associated molecule involved in BMP signaling. *Genes Cells* 3: 257–264, 1998.
 19. Chung P.J., Chang Y.S., Liang C.L. and Meng C.L., Negative regulation of Epstein-Barr virus latent membrane protein 1-mediated functions by the bone morphogenetic protein receptor 1A-binding protein, BRAM1. *J. Biol. Chem.* 277: 39850–39857, 2002.
 20. Georgi L.L., Albert P.S. and Riddle D.L., daf-1, a *C. elegans* gene controlling dauer larva development, encodes a novel receptor protein kinase. *Cell* 61: 635–645, 1990.
 21. Morita K., Shimizu M., Shibuya H. and Ueno N., A DAF-1-binding protein BRA-1 is a negative regulator of DAF-7 TGF-beta signaling. *Proc. Natl. Acad. Sci. USA* 98: 6284–6288, 2001.
 22. Estevez M., Attisano L., Wrana J.L., Albert P.S., Massague J. and Riddle D.L., The daf-4 gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* 365: 644–649, 1993.
 23. Inoue T. and Thomas J.H., Targets of TGF-beta signaling in *Caenorhabditis elegans* dauer formation. *Dev. Biol.* 217: 192–204, 2000.
 24. Tsang M., Kim R., de Caestecker M.P., Kudoh T., Roberts A.B. and Dawid I.B., Zebrafish nma is involved in TGFbeta family signaling. *Genesis* 28: 47–57, 2000.
 25. Chen J.N., van Eeden F.J., Warren K.S., Chin A., Nusslein-Volhard C., Hafler P. and Fishman M.C., Left-right pattern of cardiac BMP4 may drive asymmetry of the heart in zebrafish. *Development* 124: 4373–4382, 1997.
 26. Yelick P.C., Abduljabbar T.S. and Stashenko P., zALK-8, a novel type I serine/threonine kinase receptor, is expressed throughout early zebrafish development. *Dev. Dyn.* 211: 352–361, 1998.
 27. Yan Y.T., Gritsman K., Ding J., Burdine R.D., Corrales J.D., Price S.M., Talbot W.S., Schier A.F. and Shen M.M., Conserved requirement for EGF-CFC genes in vertebrate left-right axis formation. *Genes Dev.* 13: 2527–2537, 1999.
 28. Bisgrove B.W., Essner J.J. and Yost H.J., Multiple pathways in the midline regulate concordant brain, heart and gut left-right asymmetry. *Development* 127: 3567–3579, 2000.
 29. Chen J.N. and Fishman M.C., Genetics of heart development. *Trends Genet.* 16: 383–388, 2000.
 30. de Caestecker M.P., Bottomley M., Bhattacharyya S., Payne T.L., Roberts A.B. and Yelick P.C., The novel type I serine-threonine kinase receptor Alk8 binds TGF-beta in the presence of TGF-betaRII. *Biochem. Biophys. Res. Commun.* 293: 1556–1565, 2002.