

**The role of *HyBMP5-8b*, a BMP5-8 orthologue, during
axial patterning and tentacle formation in *Hydra***

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1. Introduction

1.1. Signaling pathways in the development of Bilateria

Cell–cell interactions through signal-transduction pathways are crucial in the coordination of all aspects of embryonic development, not just in vertebrates, but in all bilaterian animals. Embryonic induction was one of the first general principles of development discovered by early developmental biologists (Spemann and Mangold, 1924). Spemann and Mangold observed that two-headed salamanders could be generated, by transplanting the dorsal lip of the blastopore, from one early gastrula into the opposite side of another embryo of the same age. The transplanted tissue, later termed the organizer, was able to induce the fate of neighbouring cells in the host embryo, indicating that cells might communicate with each other through secreted signals. Questions immediately arose about the level of information provided by the organizer. However, it proved impossible in the period from 1930–1980 to learn about the inductive signaling molecules themselves or to discern at a molecular level the responses of cells to these signals. However, research in the past two decades has yielded important advances towards the identification of the molecules that are involved in signaling processes (Cadigan and Nusse, 1997; Gerhart, 1999; Hombria and Brown, 2002; Ingham and McMahon, 2001; Massague and Chen, 2000; McKenna and O'Malley, 2002; Moon et al., 2002; Mumm and Kopan, 2000).

Extensive studies in bilaterian animals revealed that signaling is involved in every event of development from, the earliest steps of axis specification through the diverse kinds of morphogenesis, organogenesis and cell differentiation in the embryo, then through the growth and sexual maturation of the juvenile, and finally the adult's ongoing physiology (Gerhart, 1999).

In Bilateria, at least 17 kinds of signal transduction pathways operate, each distinguished by its transduction intermediates (Gerhart, 1999). Typically, signaling pathways are activated by the binding of a ligand to a transmembrane receptor, which in turn leads to the modification of cytoplasmic transducers. Subsequently, these transducers activate transcription factors that ultimately alter gene expression.

One of the most surprising findings about signaling processes is that only a few pathways are involved in the initiation and maintenance of a wide variety of embryonic developmental processes in bilaterians (Pires-daSilva and Sommer, 2003). The transforming growth factor- β (TGF- β), wingless related (Wnt), Hedgehog (Hh), Notch, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), receptor tyrosine kinase (RTK) and nuclear hormone pathways are used repeatedly throughout the development of individuals.

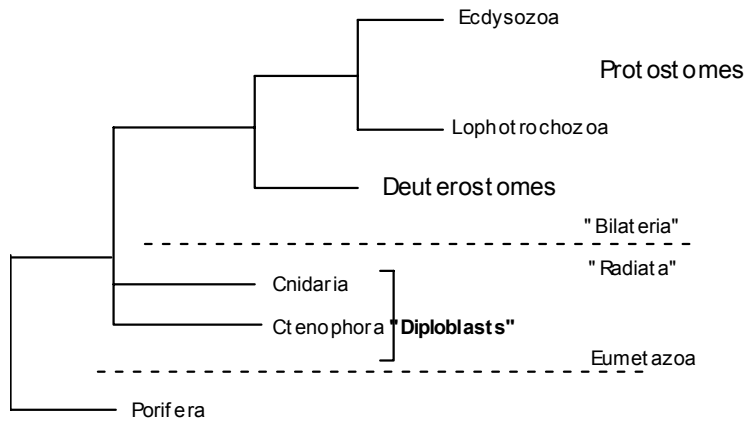


Fig.1.1. Phylogenetic relationships of the major metazoan superphyla (after Martindale et al., 2002).

Most of the studies on signaling pathways have been carried out in a few model organisms (especially those with tractable genetics) and all of them are bilaterians (Fig.1.1.). Looking at how these pathways evolved within the bilaterians might provide insights into how a few signaling pathways can generate so much cellular and morphological diversity during the development of individual organisms and the evolution of animal body plans within the bilaterians. However, a considerable amount of metazoan evolution took place before the bilaterians diverged. Of interest is whether the same signaling pathways are existent in non-bilaterian animals and what role do they play during the development of those animals. By examining non-bilaterian phyla, which arose during the initial phase of metazoan evolution, it should be possible to determine what the ancestral role of each of those pathways might have been and how the earliest animals were assembled.

Of special interest thereby is the role of signaling pathways during axis formation in early metazoans to gain insight into the origin of the bilaterian body axes.

1.2. Differences in the body plan of Bilateria and Radiata

Non-bilaterian metazoans include the Cnidaria (corals, sea anemones, hydras, and jellyfishes), the Ctenophora (comb jellies), the Porifera (sponges) and the Placozoa (*Trichoplax*). Several molecular phylogenetic studies, support that these groups diverged prior to the appearance of the bilaterians (Adoutte et al., 2000; Collins, 1998; Kim et al., 1999; Medina et al., 2001; Wainright et al., 1993).

Cnidarians and ctenophores, collectively known as the Radiata, have been traditionally distinguished from the Bilateria on the basis of three major morphological criteria: body axes, symmetry, and germ layers (Hyman, 1940). Bilaterians are bilaterally symmetrical and have three germ layers, the endoderm, the ectoderm, and the mesoderm. Therefore, they are called triploblastic animals. They have two main axes of polarity, the anterior–posterior and the dorsal–ventral axes. Cnidarians and ctenophores are generally regarded as radial symmetrical and diploblastic consisting of endoderm and ectoderm. They possess a single major axis of polarity, the oral–aboral axis.

With respect to body axes and germ layers, bilaterians are commonly thought to possess the derived condition. Biaxial polarity is thought to have evolved from having a single axis of polarity, e.g., oral–aboral (Nielsen, 1985). However, there is no consensus on the evolution of symmetry (Willmer, 1990). Some authors hypothesize that the ancestral eumetazoan was radial symmetrical, perhaps resembling a cnidarian planula larva (Hyman, 1940; von Salvini-Plawen, 1978) or of the "Gastraea-type" (Häckel, 1874; Nielsen, 1998). Many authors have suggested that some cnidarians either are bilateral, or probably were bilateral at one stage in their evolution (Groger and Schmid, 2001; Martindale et al., 2002), thus raising the possibility that the radial symmetrical character of the phylum is derived. Furthermore, bilateral symmetry may have evolved once, or it may have evolved in multiple lineages (Ax, 1996). One way of gaining insight into the evolution of symmetry is to determine the molecular features, e.g. the signaling pathways, involved in setting up the cnidarian body axis.

1.3. *Hydra* as a model to study the evolution of body axes

1.3.1. Systematics and morphology of *Hydra*

Hydra belongs to the class Hydrozoa (Fig.1.2). Among the cnidarians, *Hydra* is the most extensively studied member due to its long history as an experimental organism and the ease with which it can be cultured and manipulated in the laboratory (Bode and Bode, 1984).

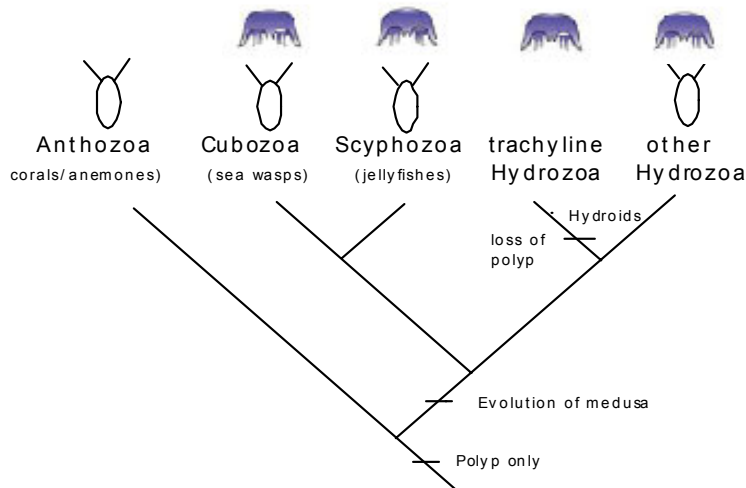


Fig.1.2. Phylogenetic relationship of the phylum Cnidaria (from Martindale et al., 2002). Anthozoans, with their simple life history (egg-embryo-adult) are the basal group of the Cnidaria.

It is important to realize, however, that *Hydra* is highly derived (Collins, 2000; Harrison and Westfall, 1991). *Hydra* is a freshwater polyp, while nearly all other cnidarians are marine. It lacks the medusa stage of the live cycle, which is found in many hydrozoan taxa. It lacks a free-living larva, which is present in all other members of the class Hydrozoa. As so many other model organisms *Hydra* is a readily to cultivate laboratory animal, but is not one of the most representative of its phylum. In general, the developmental processes are much better understood in *Hydra* than in any other cnidarian. Therefore, *Hydra* is still a useful model system to answer questions about evolutionary solutions to developmental problems in an animal phylogenetically distant from bilaterians.

One fact about research in *Hydra* should also be made clear at this point. Developmental phenomena are studied in the adult, although *Hydra* has a normal embryonic development (Martin et al., 1997). But because of the developmental dynamics of the adult polyp and the variety of possibilities to manipulate it experimentally, almost all the efforts to understand *Hydra* development have been directed at the adult. This leads to the comparison of the development in the adult *Hydra* polyp to processes, occurring in the embryos of other animals. *Hydra* embryos are not easily accessible. However, because of the tissue dynamics of *Hydra* (described in chapter 1.3.2), developmental and differentiation processes have to be continuously active. Therefore, it has been possible to use the adult *Hydra* to learn more about the developmental processes in cnidarians.

The body plan of *Hydra* is quite simple. As shown in Figure 1.3, the animal has a single axis with radial symmetry consisting of a head, a body column and a foot along the axis. At the two ends of the single axis are, respectively, the hypostome with a mouth opening, surrounded by a swirl of tentacles (which together constitute the head), and a basal disc of cells (the foot).

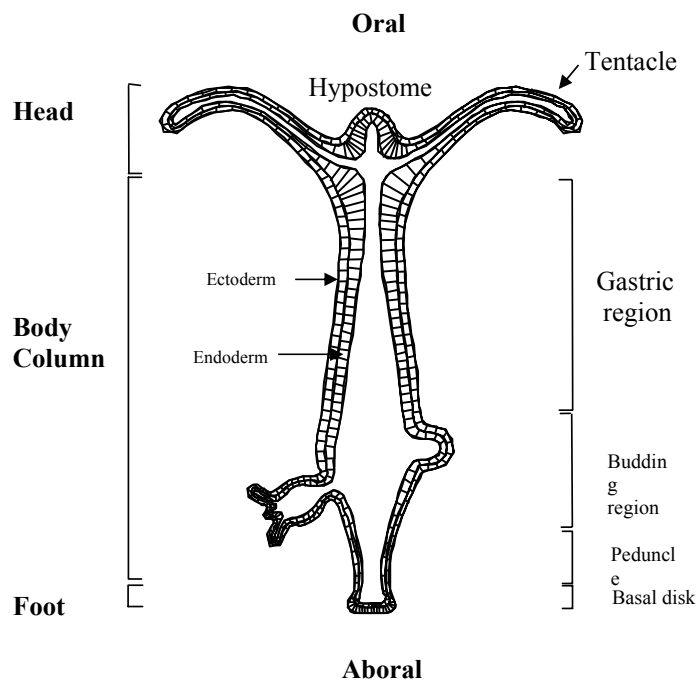


Fig. 1.3. Morphology of a *Hydra* polyp (from Bode, 2003). The regions and the two tissue layers are indicated. The protrusions from the body column are early and later stages of bud development.

Bud formation, *Hydra's* form of asexual reproduction, takes place in the budding zone, which is 60-70% of the distance down the body column from the head. As mentioned above, *Hydra* lacks a medusoid generation, which usually bears the function of sexual reproduction. Gonads occasionally differentiate in *Hydra* in the ectoderm. The basal phylogenetic position of *Hydra* among other metazoa is reflected in its histological organization. As characteristic for diploblastic animals, a *Hydra* polyp is essentially a tube with the wall made up of two epithelial layers, the ectoderm and endoderm. Ectodermal and endodermal epithelial cells build single-layered epithelia, sealed together by narrow bands of septate junctions. Each layer is a single cell thick, which extends throughout the animal. Between these two layers is a basement membrane termed mesoglea that has been shown to play a crucial role in morphogenesis and cell differentiation (Leontovich et al., 2000; Sarras et al., 1991; Sarras et al., 1994; Sarras et al., 1993; Zhang et al., 1994).

There are about 20-25 cell types each with a distinct morphology and regional distribution (Bode et al., 1973). All cell types belong to one of three lineages (Bode, 1996). The epithelial cells of each of the two layers constitute a cell lineage consisting of stem cells in the body column and differentiated cells in the extremities. All of the remaining cells belong to a third cell lineage, the interstitial cell lineage. Cells of this latter lineage are single cells that are scattered among the epithelial cells of both layers, and consist of multipotent stem cells that give rise to four sets of differentiation products: neurons, secretory cells, gametes, and nematocytes, the stinging cells that are typical of cnidarians.

Epithelial cells of both layers have two muscular protrusions mediating body contraction and dilation (Müller, 1950) (Fig.1.4). Endodermal epithelial cells are involved mainly in digestion, whereas the ectodermal epithelial cells function in the protective manner of skin cells.

Both epithelia contain nerve cells. They form a nervous system that appears as a diffuse plexus of nerve cells spread over the whole polyp (Schneider, 1890). A higher density of the neuronal network, however, can be observed in the hypostome and the basal disk. Sensory, ganglion and motor neurons can be identified (Davis et al., 1968).

Cnidaria specific cell types, nematocytes or stinging cells, are located in the ectoderm. *Hydra* has four distinct types of nematocytes. Nematocytes mediate the penetration and capture of prey. The highest concentration of nematocytes can be found in the tentacle-specific battery cells (Campbell, 1967b).

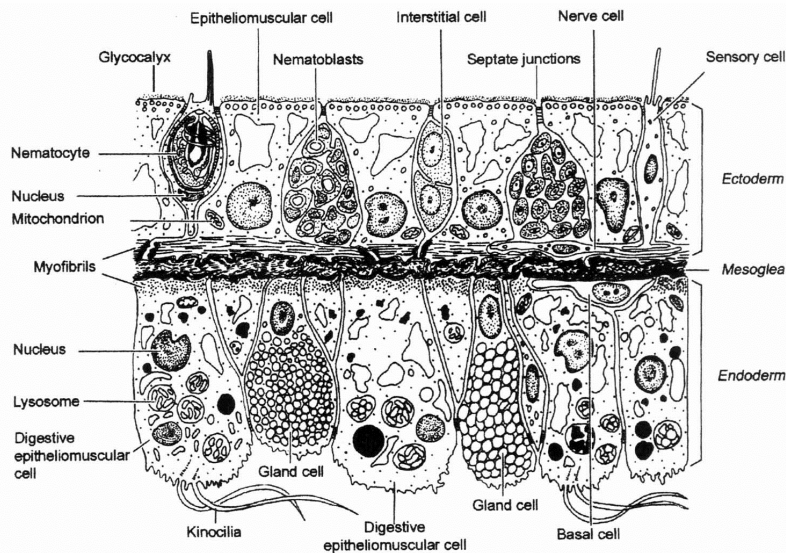


Fig.1.4. Histology of *Hydra* (Tardent, 1978)

1.3.2. Tissue dynamics of the adult *Hydra*

All of the epithelial cells of the body column (the part of the animal exclusive of the head and foot) are mitotically active (Holstein et al., 1991). Only the cells in the tentacles and the foot are arrested in the G2 phase of the cell cycle (Dubel et al., 1987). However, the animal does not increase in size. It maintains its fixed adult size by the shunting of cells into buds, which eventually detach from the adult, and by loss of cells through sloughing from the distal ends of the tentacles and from the foot (Campbell, 1967a; Otto and Campbell, 1977) (Fig.1.5). Hence, the tissues of an adult *Hydra* are in a steady state of production and loss. This creates the earlier mentioned unusual developmental situation in an adult *Hydra*. Because of this tissue dynamics, an epithelial, mitotically active cell in the body column, will during the course of its life, eventually cross either in the head or the foot, where it arrests in the cell cycle and differentiate into a new type of cell, either a tentacle battery cell or a foot basal disk cell. Thus, it seems obvious, that *Hydra* cells must have signaling pathways which allow them to sense their location in the animal. Furthermore, the sensing pathways must be coupled to pathways, which regulate developmental fate decisions.

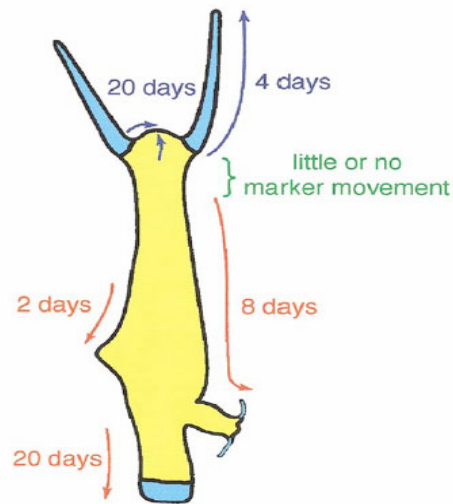


Fig.1.5. Tissue dynamics in the adult *Hydra* (from Steele, 2002). Arrows indicate directions in which cells are displaced after a certain number of days. The area of the animal colored yellow is the region in which the epithelial cells are dividing. The turquoise colored region (tentacles, basal disk) indicates the area in which the cells are arrested in G2 of the cell cycle (Dubel et al., 1987).

1.3.3. Patterning processes in an adult *Hydra* and the head organizer

Because an adult *Hydra* is in a steady state of production and loss of tissue, it is obvious that the processes governing axial patterning need to be constantly active to maintain the morphology of the animal. These same processes are also involved in head regeneration (chapter 1.3.4.). A large body of transplantation experiments has demonstrated that the central element of the axial patterning process is a morphogenetic gradient that has a maximum value in the head. This gradient has been referred to as a gradient of positional value (Wolpert, 1971), the head activation gradient (MacWilliams, 1983b), or the source density gradient (Gierer and Meinhardt, 1972). More recently, it has become clear that the gradient consists of two components. One is an organizer located in the hypostome, whereas the other is the positional value gradient, which is confined to the body column.

A defining characteristic of an organizer region is its ability to induce the formation of a secondary axis when transplanted to another region of an embryo. Among vertebrates, this is true for the dorsal lip of frog embryos, Hensen's node in chick embryos, the embryonic shield of zebrafish embryos and the node region of the

mouse embryo (Smith and Schoenwolf, 1998). The hypostome, or more specifically the head organizer in the hypostome, has the typical characteristic of an organizer (Broun and Bode, 2002; MacWilliams, 1983b). The induced second axis consists of a head and body column but no foot.

The head organizer transmits two signals to the body column. One, which is graded down the body axis, sets up the positional value gradient in the body column (Broun and Bode, 2002; Wilby and Webster, 1970a). The other is a graded distribution of a signal, termed head inhibition, which prevents body column tissue from undergoing head formation (MacWilliams, 1983a; Wilby and Webster, 1970b).

In the context of the tissue dynamics of an adult *Hydra*, these elements probably act in the following manner to maintain the steady state morphology of the adult (Bode, 2003). As the tissue of the hypostome is continuously displaced from its base to its tip and sloughed, the head organizer is continuously undergoing self-renewal. The proteins possibly involved in this self-renewal of the hypostome organizer will be discussed in chapter 1.3.6. Additionally, the organizer continuously produces and transmits signals for the head activation and head inhibition to the body column to maintain the two gradients in a steady state condition. As tissue is displaced up the body column, it is exposed to a higher concentration of the head activation signal, which is translated into a higher level of positional value. When it reaches the apical end of the body column, the level of head activation surpasses that of head inhibition and the tissue is committed to head formation. Similarly, as tissue is displaced down the body column a point is reached where head activation is lower than head inhibition and head formation is initiated that allows the formation of buds.

1.3.4. Regeneration in *Hydra*

Hydra is famous for its regenerative capacity. Just like the multiheaded monster in the Greek mythology that grew two new heads for every one cut off, a cnidarian polyp can regenerate a new head after decapitation (Bode, 2003). When a *Hydra* is bisected in the upper third of the body column, the upper part will regenerate a foot at its basal end, whereas the lower part will regenerate a head at its apical end. Similarly, when a piece of the column is isolated, it will invariably regenerate a head at the apical end (Fig. 1.6), and a basal disk, or foot, at the basal end. Cells located in the middle of the animal

suddenly find themselves at the ends and must adopt the appropriate developmental fates to allow them to regenerate the missing structures (head and foot).

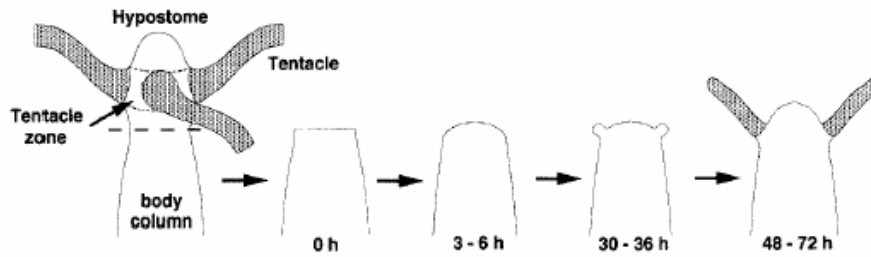


Fig.1.6. Head regeneration (from Bode, 2003).

Furthermore, *Hydra* polyps can be dissociated into a single cell suspension, that reagggregated, regenerates into an intact animal within a few days (Gierer et al., 1972). Cnidarian regeneration occurs by morphallaxis, a process of reorganization of the existing tissue without the necessity of cell proliferation (Hicklin and Wolpert, 1973).

Given the clear necessity that cells in the adult *Hydra* polyp receive extracellular signaling inputs in order to adopt (during regeneration and aggregation) and maintain (in the adult, because of the tissue dynamics) the appropriate developmental fates, it is of interest if or which signaling pathways, respectively, are providing the needed information.

1.3.5. Approaches to identify signaling molecules in *Hydra*

Initially, molecules involved in developmental processes in *Hydra* were identified using biological assays. For example, substances were purified and tested for activities that accelerated regeneration of the head or the foot. Several peptides with biological activity could be identified that way (Hoffmeister, 1996; Schaller, 1973).

A different approach takes advantage of compounds which are known to perturb signaling pathways in other system, such as diacylglycerol (DAG) (Müller, 1989) or lithium chloride (Hassel et al., 1993; Hassel and Berking, 1990). Both substances have dramatic effects on patterning processes in the *Hydra* polyp and help to identify molecules playing a role in patterning. The effect of LiCl will be discussed in detail in the Results section. Furthermore, as discussed earlier, *Hydra* is amenable to

a number of manipulations that alter the axial patterning processes, such as head and foot regeneration and the formation of aggregates that help to identify genes involved in axial patterning.

Differential Display PCR has been successfully applied to identify genes whose expression levels change as a response to changes of pattern formation in *Hydra*. Detailed studies on one gene encoding a peptide termed HEADY, identified using this approach, have revealed a function of the molecule in head formation (Lohmann and Bosch, 2000).

However, of high interest is the role, played by *Hydra* orthologues of bilaterian genes known to be involved in developmental signaling in higher metazoans. A number of genes have been cloned and they also appear to have roles in the patterning processes of *Hydra* (Galliot and Schmid, 2002). This shows that these signaling pathways were present in the common ancestor of *Hydra* and bilaterians and implies that they function in conserved developmental processes of both *Hydra* and higher metazoans.

1.3.6. What is known to date about signaling pathways in *Hydra* ?

It has been shown that *Hydra* contains a number of conserved genes involved in signaling in bilaterians. They are members of the in chapter 1.1 mentioned six basic developmental signaling pathways (Steele, 2002). These include genes of the hedgehog pathway, a hedgehog ligand and a Ci/Gli family transcription factor have been isolated from *Hydra* (Kaloulis, 2000), as well as orthologues of the Notch signaling pathway such as *Notch*, *Suppressor of Hairless*, *Numb* and *hairy/enhancer of split* (P.Towb and J.Posakony, personal communication). At the moment, nothing is known about the function of those pathways in *Hydra*. The genes are still in the process of being characterized. However, their presence in a diploblast implies the presence of a conserved Notch signaling pathway, which originated prior to the divergence of cnidarians and bilaterians.

Several RTK genes have been cloned from *Hydra* and have been shown to be involved in various aspects during development. For example HTK7, a member of the insulin receptor family seems to be involved in cell differentiation and stimulates cell division in an adult *Hydra* (Steele et al., 1996). While *Lemon*, homologous to the human CCK-4 gene, plays a role during budding and gametogenesis, (Miller and

Steele, 2000). *Shin guard*, a receptor protein-tyrosine kinase, with no orthologues in bilaterians, seems to be involved in the commitment of peduncle tissue to foot formation (Bridge et al., 2000).

A single member of the nuclear receptor superfamily has been isolated so far from *Hydra* (Escriva et al., 1997). Phylogenetic analysis revealed that the isolated gene is more closely related to the COUP family of the nuclear receptor superfamily. COUP receptors are involved in the development of a variety of tissues in bilaterians, as for instance the nervous and the circulatory system. Nothing is known about the role of the gene in *Hydra*.

A major finding has been that all the members of the canonical Wnt pathway exist in *Hydra* and have been cloned, and shown to be expressed in adult animals (Hobmayer et al., 2000; Minobe et al., 2000). *In situ* hybridization revealed, that Wnt-signaling is involved in patterning of the oral-aboral axis in *Hydra* as it is transcriptionally upregulated when head organizers are established early during bud formation and head regeneration (Hobmayer et al., 2000).

Only one member of the TGF- β signaling pathway, a receptor activated Smad gene, has been isolated so far from *Hydra* (Hobmayer et al., 2001). The R-Smad gene seems to be involved in nematocyte differentiation and its expression is upregulated in the oocyte precursor cells of polyps undergoing oogenesis. Probably the whole TGF- β pathway is present in *Hydra* as several members of the pathway have been isolated from other cnidarians. These include a type I TGF- β family receptor, two Smad family transcription factors and a gene encoding a member of the bone morphogenetic family, *Am-BMP2/4*, from the coral *Acropora* (Hayward et al., 2002; Samuel et al., 2001). *In situ* hybridization data suggest, the *Acropora* BMP2/4 orthologue, *Am-bmp2/4*, might be involved in axial patterning in the early coral embryo (Hayward et al., 2002). In addition, several TGF- β receptors have been isolated from the sponge *Ephydatia* (Suga et al., 1999), a member of the parazoan phylum Porifera, implying that they originated prior to the divergence between diploblasts and Porifera at the very earliest stages of multicellular life.

Thus, most of the common set of pathways appears to have arisen at least as early as the diploblasts during metazoan evolution, and this invokes the question: 'what were their functions in the ancestor of diploblasts and bilaterians?' Insights into this question can be gained by study of conserved signaling pathways involved in

patterning of the *Hydra* polyp. One critical question concerns the function of the BMP signaling pathway in cnidarians, and whether its function in axis formation of bilaterians is a conserved feature of diploblasts and bilaterians.

1.3.7. Signaling pathways and axis formation in *Hydra*

One pathway that plays a critical role in axial patterning in bilaterians is the Wnt pathway. It is involved in establishing the organizer and subsequently the dorsal-ventral axis in frog and zebrafish embryos (Niehrs, 1999; Schier, 2001) as well as the animal-vegetal axis in sea urchins (Emily-Fenouil et al., 1998; Huang et al., 2000; Vonica et al., 2000). It also provides positional information that determines the polarity of specific regions during early stages of embryonic development such as the polarity of the developing segments in a *Drosophila* embryo (Moline et al., 1999; Pfeiffer et al., 2000; Pfeiffer et al., 2002).

Wnt glycoproteins bind to seven-transmembrane proteins of the frizzled type (Bhanot et al., 1996). Two classes of Wnt proteins can be distinguished, class I acting via β -catenin (e.g. Wnt-1 and Wnt-8) and class II transducing via phosphatidylinositol (e.g. Wnt-5a) (Brown and Moon, 1998). During early *Xenopus* embryogenesis, the class I Wnt pathway is involved in dorsal-ventral axial patterning at two stages, before and after midblastula transition (MBT) (Moon et al., 1997), or the onset of zygotic transcription. Early activation of the pathway mediates the ligand-independent dorsalizing function of the Nieuwkoop centre, which sets up the dorsal-ventral polarity of the embryo and induces the organizer (Harland and Gerhart, 1997; Miller and Moon, 1996; Moon et al., 1997). Ectopic activation of the early Wnt pathway induces complete secondary embryonic axes in *Xenopus* (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991).

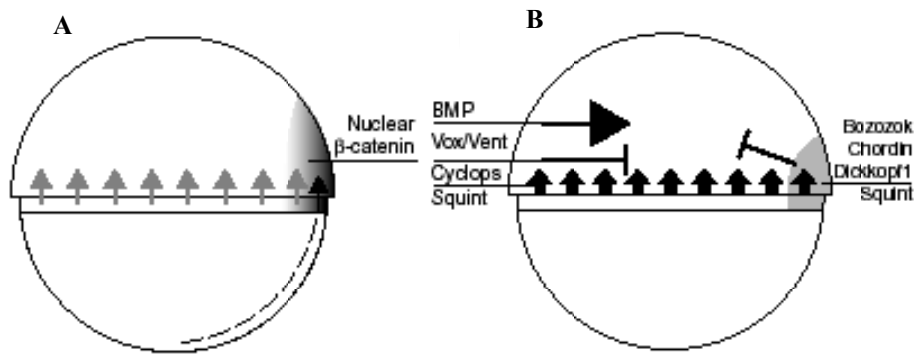


Fig. 1.7. Schematic diagrams illustrating the antagonism of BMP and Wnt signaling during mesoderm patterning in early zebrafish embryos (from Schier, 2001). (A) At the 1-cell stage of the zebrafish embryo the vegetal region of the yolk cell contains a dorsal determinant that is translocated to the future dorsal side, leading to the stabilization and nuclear accumulation of β -catenin (B) Dorsal–ventral and mesendodermal patterning of the blastula embryo. Antagonists such as *Boz*, *Chordin*, and *Dkk1* inhibit ventralizing BMP signals. Bozozok, Chordin, Dickkopf, and Squint are downstream effectors of β -Catenin.

In *Hydra* the pathway also plays a role in axial patterning. *HyWnt*, the *Hydra* orthologue of Wnt-1 is expressed in the hypostome, the upper half of the head, where the head organizer is located (Hobmayer et al., 2000). It is also expressed early on in the developing head organizer as the axis of a developing bud is being set up. Two other members of the pathway, *Hy β -cat* and *HyTcf*, the *Hydra* orthologues of β -catenin and *Tcf/Lef*, are also strongly expressed in the head as well as during bud formation (Hobmayer et al., 2000). More direct evidence for the role of the pathway was obtained by blocking the activity of GSK-3 β , thereby mimicking active Wnt signaling, which led to ectopic head organizer formation throughout the whole body column (M.Broun and H.Bode, personal communication).

The fact that BMP molecules, members of the TGF- β family of signaling molecules, play roles in dorsal-ventral patterning in both *Xenopus*/zebrafish (BMP2,-4,-7) and *Drosophila* (*dpp*, *screw*) embryos suggests that these genes may have such roles in their evolutionary precursors, the diploblasts. In addition, the interaction between the Wnt signaling and the BMP signaling in *Xenopus* and zebrafish in dorsal-ventral patterning (Harland, 2000; Schier, 2001) (Fig.1.7), as well as in patterning the imaginal discs and abdomen in *Drosophila* (Dubois et al., 2001; Kopp et al., 1999), raises the

interesting question if BMP signaling and Wnt signaling also interact with each other in defining polarity during axial patterning in *Hydra*.

1.4. Bone morphogenetic proteins

1.4.1. Bone morphogenetic Proteins - overview

Bone morphogenetic proteins (BMPs) are secreted growth factors, which, based on amino acid homology of a highly conserved seven-cysteine domain in the carboxy-terminal region of the proteins, form a subgroup of the transforming growth factor- β (TGF- β) superfamily (Kingsley, 1994). BMPs have been classified into several subgroups according to their structural similarities. Individually, the members of this TGF- β subfamily are termed BMPs, osteogenic proteins (OPs), cartilage-derived morphogenetic proteins (CDMPs), and growth and differentiation factors (GDFs) (Ducy and Karsenty, 2000).

BMPs were originally isolated by their ability to induce ectopic bone and cartilage formation *in vivo* (Urist, 1965), but it became rapidly evident that BMPs also act as multifunctional regulators in morphogenesis during development in vertebrates and invertebrates (Ebendal et al., 1998; Graff, 1997; Hogan, 1996a; Hogan, 1996b; Wozney, 1998).

BMPs are conserved broadly across the animal kingdom, including vertebrates, arthropods, nematodes and cnidarians. In *Drosophila*, the BMP ligands Decapentaplegic (dpp), and 60A (gbb) have been shown to participate in developmental events as diverse as oogenesis, the development of the basal discs, and as mentioned above the regulation of dorsal-ventral patterning in the early embryo (Raftery and Sutherland, 1999). In vertebrates, BMPs also play roles in dorsal-ventral patterning of the early embryonic mesoderm (Fig. 1.7) and specification of epidermis. In *Xenopus* for example, BMP2, -4 and -7 ventralize early mesoderm and act as negative regulators of neuralization (Harland, 1994; Hemmati-Brivanlou and Melton, 1997). Additionally, vertebrate BMPs play roles in limb development, generation of primordial germ cells, tooth development, and the regulation of apoptosis, to mention a few (Hogan, 1996a).

1.4.2. Bone morphogenetic Proteins - Structure

BMPs are dimeric proteins with a single interchain disulfide bond, and this dimeric conformation is an absolute requirement for the biological action of BMPs (Eimon and Harland, 1999). They are synthesized as large precursor proteins with an amino-terminal signal sequence and a prodomain that is followed by the mature ligand region, the C-terminal sequence containing seven conserved cysteine residues characteristic for BMPs (Fig.1.8). Upon dimerization, the precursor proteins are proteolytically cleaved in the Golgi-Apparatus at the consensus sequence (Arg-X-X-Arg or Arg-X-Lys/Arg-Arg) to release the biologically active carboxy-terminal dimers from the N-terminal proregion (Kingsley, 1994). Candidate proteases responsible for BMP processing before secretion comprise members of the subtilisin-like proprotein convertase (SPC) family. Several of these proteases have been shown to specifically hydrolyze peptide bonds preceded by the sequence RXXR or RXK/R (Creemers et al., 1993; Molloy et al., 1992). It has been recently shown that the downstream sequence adjacent to the cleavage site determines the cleavage efficiency, while the N-terminal region controls the stability of the processed mature protein (Constam and Robertson, 1999).

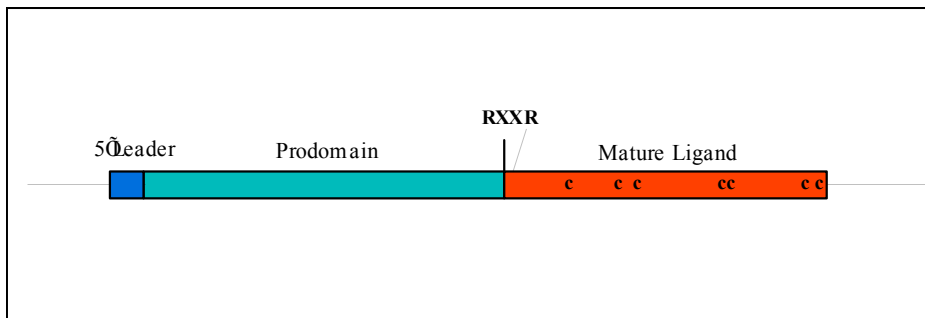


Fig.1.8. Structure of a BMP precursor protein. RXXR: Consensus sequence

Analysis of the crystal structure of two BMP family members, BMP7 and BMP2 (Griffith et al., 1996; Scheufler et al., 1999) has revealed that the core of the monomer is a cysteine knot involving six cysteine residues that are invariably spaced in the C-terminal region of all family members. In general, BMPs have one more conserved cysteine that is involved in intermolecular disulfide bonding. Other

characteristic conserved features of the C-terminal region are the two separated antiparallel beta-sheets, the second of which adopts a twisted crossover conformation, and a four-turn α -helix approximately perpendicular to the strands (Fig. 1.9). The overall folding topology has been described as a hand (Daopin et al., 1992) with the helix mimicking the wrist, the cysteine-knot core the palm, and the beta-sheets the fingers.

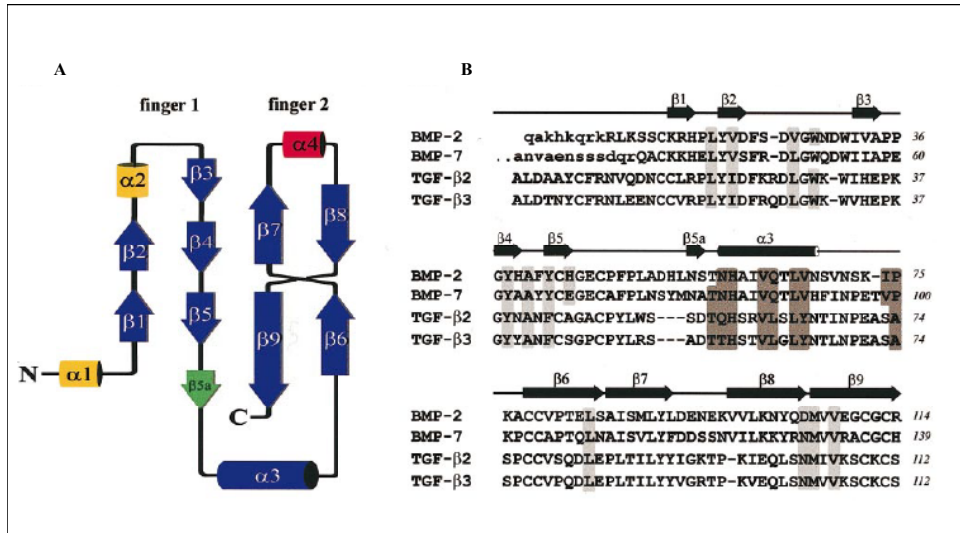


Fig.1.9. Topology diagram of BMP2 (A) and structure-based sequence alignment of TGF- β family proteins. (from Scheufler et al., 1999). (A) Helices are indicated as cylinders, β -strands as arrows. Secondary structure elements, which are part of the TGF- β /BMP basic-scaffold are colored blue. Orange ones ($\alpha 1$ and $\alpha 2$) are uniquely found in TGF- β s, red ones are unique to BMP-7 ($\alpha 4$), and green ones are unique to BMP-2 (β -5a). The secondary structure assignment has been carried out with PROCHECK (Laskowski et al., 1992). (B) The secondary structure assignment refers to the X-ray structure of BMP-2. Residues that are not visible in the models are indicated as lowercase characters. Residues involved in dimer contacts are conserved (contact cutoff-distance 3.7 Å). Those located in the Aenger-region of the protein are shaded in light gray, dark gray indicates residues located in helix $\alpha 3$.

1.4.2. TGF- β signaling pathway

Figure 1.10 shows a scheme of the TGF- β /Smad signal transduction pathway. Upon dimerization of the TGF- β proteins, the molecules are proteolytically cleaved within the cell to yield carboxy-terminal mature proteins. Once secreted, the dimers initiate signaling by binding cooperatively to both type I and type II serine/threonine kinase receptors (Ducy and Karsenty, 2000; Miyazono et al., 2000). The type II receptors are constitutively active kinases, which transphosphorylate serine and threonine residues within the intracellular GS (glycine-serine-rich) domain of the type I receptor subunit (Wrana et al., 1994) upon ligand binding. The activated type I receptor kinase, in turn, phosphorylates particular members of the Smad family of proteins, called receptor-regulated Smads (R-Smads), at serines in a conserved C-terminal SSXS motif, to elicit cellular responses.

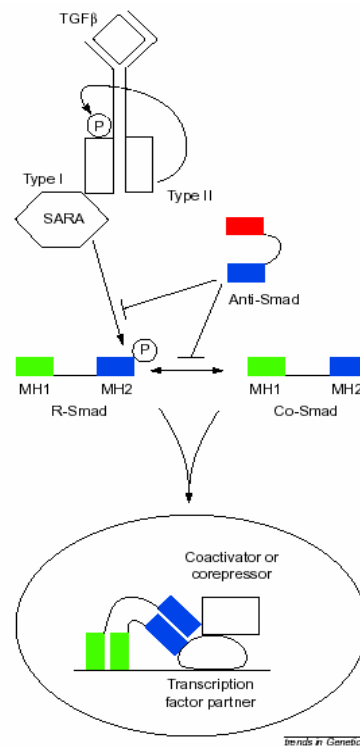


Fig. 1.10. Model for TGF- β signal transduction. (from Patterson and Padgett, 2000). Because the Smads bind DNA weakly, their activity in the nucleus is altered by other transcription partners and other coactivators or corepressors. In most TGF- β pathways, an Anti-Smad is transcribed in response to signaling, which attenuates TGF- β signaling by binding to the receptors and/or Smads.

Genetic studies using *C. elegans* and *Drosophila* have resulted in the identification of these downstream signaling mediators of the receptors for BMP-like ligands in these organisms, which were called, respectively, *sma* (Savage et al., 1996) and *Mothers against dpp* (*Mad*) (Sekelsky et al., 1995). An increasing number of the vertebrate members belonging to this protein family have subsequently been identified and are denoted Smad, a fusion of *sma* and *Mad* (Derynck et al., 1998; Kawabata et al., 1999; Raftery and Sutherland, 1999). Several Smads are known in vertebrates and invertebrates and are classified into three groups based on their structures and functions. Receptor-regulated Smads (R-Smads) transiently associate with type I serine/threonine kinase receptors and undergo direct phosphorylation. R-Smad1, -5, and -8 are activated by BMP specific receptors II (Hoodless et al., 1996; Kawai et al., 2000; Nishimura et al., 1998), whereas R-Smad2 and -3 mediate TGF- β signaling and are activated by activin/nodal specific receptors II (Macias-Silva et al., 1996; Zhang et al., 1996). Phosphorylation leads to association of the R-Smads with the common partner Smad (Co-Smad or Smad4), and results in nuclear translocation of these heteromeric complexes, to permit the assembly of Smad/transcription factor complexes on the promoter of target genes (Baker and Harland, 1996; Lagna et al., 1996; Zhang et al., 1996). Finally, members of the third group of Smads, Smad6 and Smad7, interfere with R-Smads and Co-Smads and are called inhibitory Smads (I-Smads) (Casellas and Brivanlou, 1998; Imamura et al., 1997).

While Smads alone can bind to specific DNA sequences, their binding affinity is considered to be too weak to serve as effective and highly specific DNA binding proteins *in vivo* (Shi et al., 1998). Thus, additional DNA binding partners are thought to be required for efficient DNA binding.

1.4.3. Modulation of BMP signaling

Finely tuned signals between cells are necessary to coordinate all aspects of development. For this reason, BMP signaling is subjected to regulation at multiple levels: intracellularly, at the membrane site, and extracellularly. The first level of regulation resides within the cell cytoplasm, where inhibitory Smads and Smurfs act as modulators (von Bubnoff and Cho, 2001). Finally, in the extracellular space, a control

system exists, whereby high affinity antagonists complex selectively with BMPs to inhibit their biological activities.

The exact concentration of active BMPs seems to be important for rendering a particular biological effect. It was found in *Xenopus* and *Drosophila* that an activity gradient of the members of the BMP family of growth factors is formed during embryogenesis, leading to the generation of different cell types, depending on the location (Dale and Jones, 1999; Dale and Wardle, 1999; De Robertis et al., 2000; Wharton et al., 1993).

Extracellular modulators regulate the extracellular concentration of active BMPs (Barth et al., 1999; Hama and Weinstein, 2001; Liem et al., 2000). An increasing number of these natural antagonists has been identified in vertebrates and invertebrates. In vertebrates, the list of BMP antagonists already comprises more than seven proteins, including noggin, chordin, *chordinlike*, follistatin, FSRP, the DAN/Cerberus protein family, and sclerostin. The *Drosophila* short gastrulation (*sog*) is the orthologue of chordin, and affects dorsal–ventral patterning by antagonizing *dpp* and *screw*, the homologues of, respectively, vertebrate BMP-2/-4 and BMP5-8 (Holley et al., 1995; Neul and Ferguson, 1998). cDNA clones encoding a chordin orthologue have also been isolated from *Hydra* (B.Hobmayer and T.Holstein, personal communication).

In both vertebrates and invertebrates, other regulating factors are involved in the BMP/*dpp*-*screw* pathway. Secreted zinc metalloproteinases antagonize the activity of chordin/*sog*, including *Drosophila* Tolloid (Tld), *Xenopus* Xolloid (Xol), and human BMP-1 (Marques et al., 1997). Another extracellular factor, twisted gastrulation (*tsg*), which is conserved among vertebrate and invertebrate species, influences this proteinase cleavage and modifies the interactions between chordin/*sog* and BMP/*dpp*-*screw* by binding to the complex. The formation of this ternary complex leads to a more efficient inhibition of BMP/*dpp* activity (Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001).

1.5. BMP signaling and axis formation in Cnidaria?

As mentioned earlier, the number of axes in cnidarians is contentious. A second, highly controversial, question concerns the relationship between the major longitudinal polarized axis of cnidarians, the oral-aboral axis, and the anterior-posterior axis of the

Bilateria. Homology between these axes is often assumed. Recent molecular data indicate that conserved patterning systems exist for both the anterior-posterior axis and the dorsal-ventral axis in bilaterian animals. The anterior-posterior axis is characterized by the ordered regional distribution of Hox gene expression domains (Hirth and Reichert, 1999; McGinnis and Krumlauf, 1992). Such a deployment of Hox genes has been proposed to be a universal and definitive metazoan character, the zootype and (Holley and Ferguson, 1997). Whether the zootype hypothesis is valid for the diploblastic phyla is still controversial (Ferrier and Holland, 2001; Schierwater and Desalle, 2001), but it is generally thought to be true for the Bilateria. The dorsal-ventral axis is defined by the actions of genes of the dpp/BMP2/4 class and their antagonists (Holley and Ferguson, 1997). In *Drosophila*, dpp is expressed dorsally and acts to specify dorsal fates whereas its antagonist sog is expressed ventrally. In *Xenopus*, the situation is inverted, with BMP4 being expressed ventrally and chordin acting dorsally. These observations indicate that there is a conserved system for defining the polarity of the dorsal-ventral axis, although an axis inversion with respect to the substratum has occurred in the course of evolution between arthropods and vertebrates (Arendt and Nubler-Jung, 1994; Arendt and Nubler-Jung, 1999; De Robertis and Sasai, 1996). Data from the expression patterns of cnidarian homeobox genes related to those having conserved roles in anterior-posterior axis patterning in arthropods and chordates are inconclusive. Whereas, expression data for members of the Wnt signaling pathway indicated that this pathway has a function in defining the organizer region at the oral end of the *Hydra* axis (Hobmayer et al., 2000).

However, the question of whether the oral-aboral axis of cnidarians and the dorsal-ventral axis of bilaterians are patterned by the same conserved mechanism has not been well studied. In order to address this question, I have investigated BMP signaling in *Hydra* through cloning and characterization of members of this pathway and by analysis of activated components.

2. Material

2.1 Animals

Hydra vulgaris, strain Zürich

Hydra vulgaris, strain Basel

Hydra vulgaris, strain Japan (Zürich) L2

Hydra magnipapillata, strain 105

Hydra magnipapillata, strain reg 16 mutant

2.2. Media

Artemia Medium

Hydra Medium + 36.8 g Seasalt

Dissociation Medium (DM-Medium)

3.6 mM KCl, 6 mM CaCl₂, 1.2 mM MgSO₄, 6 mM Sodium Citrate, 6 mM Sodium Pyruvate, 6 mM Glucose, 12.5 mM TES in H₂O, 50 mg/l Rifampicin, 50 mg/l Kanamycin, 100 mg/l Streptomycin in H₂O; adjust pH to 6.9 before adding antibiotics, stir over night and store at -20°C

Hydra-Medium

1 mM CaCl₂, 0.08 mM MgSO₄, 0.5 mM NaHCO₃, 0.1 mM MgCl₂,
0.03 mM KNO₃ in H₂O

LB-Medium

10 g NaCl, 10 g Bacto-tryptone, 5 g Bacto-Yeast Extract, add deionized H₂O to a final Volume of 1 l, autoclave

LB-Medium + ampicillin

1 liter LB-Medium, autoclave, add 1 ml ampicillin (100mg/ml)

LB-Medium + tetracycline

1 liter LB-Medium, autoclave, add 1 ml tetracycline (12.5 mg/ml).

LB-Agar

1 liter LB-Medium plus 15 g Bacto-Agar

LB-plates with ampicillin

Take autoclaved LB-Agar, let it cool to 50°C, add ampicillin to a final concentration of 100 µg/ml, pour 30-35 ml of medium into 85 mm petri dishes, store at 4°C for up to one month

LB-plates for blue/white selection

Make LB-plates with ampicillin; then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates

2% maltose solution

Dissolve 1g of maltose in 100 ml H₂O, sterilize using syringe filters (0.2µm).

Modified Barth's solution-high salt (MBS)

110 mM NaCl; 1 mM KCl; 2.4 mM NaHCO₃; 0.82 mM MgSO₄·7H₂O; 0.33 mM Ca(NO₃)₂·4H₂O ; 0.41 mM CaCl₂·2H₂O; 10 mM Hepes; adjust to pH 7.6 with NaOH

SM (λ phage medium)

100 mM NaCl; 10 mM MgSO₄; 50 mM Tris-HCl, pH 7.5 in H₂O
autoclave the solution and add filter sterilized gelatin solution to a final concentration of 0.01 %

SOB-Medium

20 g Bacto-Tryptone, 5 g Bacto-Yeast Extract, 0.5 g NaCl in H₂O

SOC-Medium

SOB-Medium plus 20 mM Glucose
(add 2 ml of 1M filter sterilized [0.22 μ m] Glucose to 100 ml autoclaved SOB-Medium)

Top-Agar

1 liter LB-Medium plus 7g Agarose

2.3. Buffers and Solutions

Alkaline phosphatase buffer (NTMT)	100 mM NaCl; 100 mM Tris-HCl, pH 9.5, 50 mM MgCl ₂ ; 0.1 % Tween 20 (prepare fresh from sterile stock solutions in H ₂ O)
Ampicillin Stock	50 mg/ml (in H ₂ O)
Bromophenol Blue solution	add solid bromophenol blue to DEPC- H ₂ O, mix and continue to add more powder until no more will dissolve, allow powder to settle, carefully pipet the saturated supernatant

Denhardt's	1% polyvinylpyrrolidone, 1% Ficoll, 1% BSA fraction V in H ₂ O, filter sterilize
DEPC-H ₂ O	1 ml DEPC/ 1 l deionized H ₂ O, stir o/n with stirring bar, autoclave
Freeze-Thaw Buffer	10 mM Tris-HCl, pH 7.4; 250 mM Sucrose; 10 mM EDTA, pH 8.0
Gel loading buffer - DNA (6x)	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF 30% (v/v) glycerol in H ₂ O
Gel loading buffer- RNA	360 µl deionized formamide 80 µl 10x MOPS buffer 130 µl 37% formaldehyde 44.5 µl 90% glycerol (DEPC-H ₂ O) 95.5 µl DEPC-H ₂ O 40 µl saturated bromophenol blue solution
Gel loading buffer-protein (5x)	0.5% SDS; 200 mM Tris-HCl, pH 6.8; 5% Glycerol; 0.05% Bromophenol Blue solution
Glycine stock solution	40 mg/ml glycine in sterile deionized H ₂ O
Glycine working solution	1:10 dilution of stock in PBT
Hybridization-buffer-A (primary library screen)	1 M NaCl; 100 mM Tris-HCl, pH 8.0; NaPPi; 0.1 mM EDTA, pH8.0; 0.1% SDS, 50 µg/ml Heparin; 300 µg/ml salmon sperm DNA

Hybridization-buffer-B (secondary library screen)	0.5 M Na-phosphate pH 6.4; 0.1 mM EDTA pH 8.0; 7% SDS; 5% Dextran Sulfate
Hybridization solution (<i>in situ</i>)	50% Formamide, 5xSSC, 200 µg tRNA, 0.1% Tween 20, 0.1% CHAPS, 1xDenhardt's, 100 µg/ml heparin in DEPC-H ₂ O
IPTG Stock Solution	200 mg/ml in H ₂ O
MAB	100 mM Maleic Acid, 150 mM NaCl, pH 7.5 in H ₂ O, autoclave
MAB-B	MAB+1% BSA fraction V
Merlin I (Cell resuspension buffer)	50 mM Tris-HCl, pH7.5; 10 mM EDTA, pH 8.0; 100 µg/ml RNase A (DNase free) in H ₂ O
Merlin II (Cell lysis buffer)	0.2 M NaOH, 1% SDS in H ₂ O
Merlin III (Neutralization solution)	61.35 g potassium acetate, 35.7 ml glacial acetic acid in 500 ml H ₂ O
Merlin IV (Celite resin)	dissolve 66.84 g guanidine hydrochloride in 33.3 ml Merlin III by stirring at gentle heat and adjusting pH to 5.5, add H ₂ O to make volume up to 100 ml, continue stirring at gentle heat until fully dissolved, filter the guanidine hydrochloride solution through a whatman filter paper 1 into a

	bottle containing 1.5 g of diatomaceous earth
Merlin V (Column wash solution)	200 mM NaCl; 20 mM Tris-HCl, pH 7.5; 5 mM EDTA; 50% EtOH
MOPS (10x)	0.4 M MOPS free acid; 0.1 M Sodium Acetate; 5 mM EDTA, pH 8.0 in DEPC-H ₂ O (store at RT in dark), adjust pH to 7.0, wrap with foil and store in dark at RT
4% Paraformaldehyde fixative	2 g paraformaldehyde in 50 ml hydra medium
PBS	0.15 M NaCl, 0.08 M Na ₂ HPO ₄ , 0.021 M NaH ₂ PO ₄ , adjust pH to 7.34 in DEPC-H ₂ O
PBT	PBS + 0.1% Tween 20
QuikHyb [®] Hybridization Solution	Stratagene
Rgt buffer	1x Ringer's buffer, 0.1% Tween 20
Ringer's buffer (10x)	1.3 M NaCl, 50 mM KCl, 20 mM CaCl ₂ , 100 mM Hepes
SDS-PAGE running buffer (10x)	250 mM Tris-base; 1.92 M Glycine, 1% SDS
SSC (20x)	3 M NaCl, 0.3 M Na-citrate in H ₂ O
TBE (10x)	890 mM Tris-HCl, 890 mM Boric Acid, 20 mM EDTA pH 8.0 in H ₂ O

TE	10 mM Tris-HCl pH 7.4, 7.5 or 8.0, 1 mM EDTA pH 8.0 in H ₂ O
TMAC wash buffer	3 M TMAC; 50 mM Tris, pH 8.0; 0.2 mM EDTA, pH 8.0 in H ₂ O
TNE (1.2x) NaCl;	62.2 mM Tris-HCl, pH 8.0; 187 mM NaCl; 3.1 mM EDTA, pH 8.0; in H ₂ O
TNET	1x TNE; 0.5% Triton X-100; 1x Complete Protease Inhibitor Mix (Roche); 50 mM NaF; 1 mM NaVO ₄ in H ₂ O
Triethanolamine stock solution (10x)	1 M triethanolamine, pH 7.8
X-Gal	20 mg/ml in DMFA, store at -20°C

2.4. Enzymes

Alkaline Phosphatase, Calf Intestinal (CIAP)	Fisher Scientific
Exonuclease III	Stratagene
M-MLV Reverse Transcriptase	Invitrogen
Proteinase K	Sigma
Platinum <i>Pfx</i> DNA-Polymerase	Invitrogen
RNAse A	Sigma
Restriction endonucleases	New England Biolabs
SP6-, T7-, T3 RNA-Polymerase	Roche
T4 DNA-Ligase	Promega
Taq DNA-Polymerase Biolase	Bioline

2.5. Kits

ABI Prism [®] Big Dye Terminator Cycle	PE Applied Biosystems
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Sequencing Kit

ECL Plus Western Blotting Detection System Amersham Biosciences

ExAssist[®] Interference-Resistant
Helper Phage with XL0LR Strain Stratagene

Tropix Galacton-Plus Assay System ABL120RP

mMESSAGE mMACHINE SP6, T7, T3
in vitro Transcription Kit Ambion

Oligotex[®] mRNA Midi Kit Qiagen

Perfectprep[®] Plasmid Maxi Eppendorf

pGEM[®]-T Easy Vector System I Promega

Prime-It[®] II Random Primer Labeling
Kit Stratagene

QIAEX II Gel Extraction Kit Qiagen

Subcloning Efficiency[™]DH5 α [™]
Chemically competent E. coli Invitrogen

2.6. Chemicals

[α - ³² P]dATP	NEN [®] Life Science Products, Inc.
Acrylamide:Bis 37.5:1 40% w/v solution	EM science, Merck
Agarose	Invitrogen
Alsterpaullone	A.G. Scientific, Inc.
Ampicillin	Sigma
APS	USB
Bacto-Agar	BectonDickinson
Bacto-Tryptone	BectonDickinson
Bacto-Yeast Extract	BectonDickinson
BM-Purple AP Substrate	Roche
Boric Acid	Fisher Scientific
Bromophenol Blue	Fisher Scientific
Bovine Serum Albumin (BSA) Fraction V	Sigma
CaCl ₂	Fisher Scientific
Chaps	Sigma
Chloroform	Fisher Scientific
CuSO ₄	Sigma
DEPC	Sigma
Dextran Sulfate	PharmaciaBiotech
Diatomaceous earth (acid-washed)	Sigma
Dig RNA labeling mix, 10xconc.	Roche
DMFA	Fisher Scientific
DMSO minimum 99.5%	Sigma
DNA-molecular size marker (1 Kb Plus DNA ladder)	Invitrogen
dNTP 100mM each	Promega
DTT	Invitrogen
EDTA	Sigma
Ethanol 200 Proof	Gold Shield Chemical, Co
Ethanol 190 Proof	Gold Shield Chemical, Co
Ethidium Bromide	Fisher Scientific
Euparal	ASCO-Laboratories

Formaldehyde (stabilized 37% solution)	Fisher Scientific
Formamide	Fisher Scientific
Glacial acetic acid	Fisher Scientific
Glucose	Sigma
Glycerol	Fisher Scientific
Glycine	Sigma
Guanidine Hydrochloride	Sigma
HCl	Fisher Scientific
Hemocyanin, Keyhole Limpet (KLH)	CALBIOCHEM [®]
Heparin	Sigma
IPTG	Invitrogen
Kanamycin	Sigma
KCl	Sigma
Levamisole	Sigma
LiCl	Fisher Scientific
Maleic Acid	Fisher Scientific
Maltose	Fisher Scientific
MgCl ₂	Sigma
MgSO ₄	Fisher Scientific
Methylene Blue	Sigma
MOPS	Sigma
NaF	Sigma
NaVO ₄	Sigma
NBT/BCIP AP-substrate	Roche
Paraformaldehyde	Sigma
Penicillin G	Sigma
Potassium acetate	Fisher Scientific
Rifampicin	Sigma
RNA molecular size marker (0.24-9.5 Kb RNA Ladder)	Invitrogen
RNasin [®] Ribonuclease Inhibitor	Promega
Salmon Sperm DNA	Sigma
SDS	Bio-Rad
Sodium Acetate	Fisher Scientific

Sodium Citrate	Fisher Scientific
Sodium Chloride	Sigma
Sodium Hydroxide	Fisher Scientific
Sodium Phosphate, monobasic and dibasic	Sigma
Sodium Pyruvate	Sigma
Sodium Pyrophosphate	Sigma-Aldrich
Streptomycin	Sigma
TES	Sigma
Temed	Sigma
TMAC	Fisher Scientific
Triethanolamine	Sigma
Tris Base	Fisher Scientific
tRNA from yeast	Boehringer, Mannheim
Tween 20	Sigma
Urethane	Sigma
X-Gal	Invitrogen

2.7. Immunochemicals

Anti-Digoxigenin Fab-Fragments, AP conjugate	Roche
Anti-phospho-Smad 1	gift of Peter ten Dijke
Anti-Rabbit IgG (Fc) AP conjugate	Promega
Anti-Rabbit IgG (FC) HRP conjugate	Promega
Fetal Bovine Serum	Life Technologies
Newborn bovine serum	Irvine Scientific™
Sheep Serum	Sigma

2.8. Vector Plasmids

pGEM-T Easy	Promega
pBS SKI ⁻	Stratagene
pCS2 ⁺	Constructed by David Turner and Ralph Rupp, Fred Hutchinson Cancer Center on date of 1/93 (Turner and Weintraub, 1994)
pCS2 ⁺ /BM40-Xtwg	Blitz et al., in preparation
pCS2 ⁺ /dnXBMP1	Blitz et al., 2000
pRMHa M ⁺	gift of Kavita Arora

2.9. Bacteria

E. coli XL-1 Blue MRF ['] strain	Stratagene
Subcloning efficiency TM DH5 α TM	Invitrogen
chemically competent e.coli	

2.10. cDNA Library

Uni-Zap TM XR Custom cDNA library	Stratagene (gift from Dr. Mike Sarras)
Host strain	XL1-Blue MRF [']
Primer	Oligo dT
Cloning Site	EcoRI and XhoI

2.11 Oligonucleotides, Peptide

SP6	5'-ATTTAGGTGACACTATAGAATA-3'
T7-pGEM-T	5'-TAATACGACTCACTATAGGGCG-3'
T7-pBS	5'-GTAATACGACTCACTATAGGGC-3'
T3-pBS	5'-AATTAACCCTCACTAAAGGG-3'
3'pCS2 ⁺	5'-CACTGCATTCTAGTTGTGGTTTGTCC-3'
dpp fl	5'-TAYGTIGAYTTYWSIGAYGT-3'
60Afl	5'-ATGAAAYGCNACNAAYCAYGC3'

Splnktop	5'-CGAATCGTAACCGTTCGTACGAGA ATTCGTACGAGAATCGCTGTCTCT CCACGAGCCAAGA-3'
Splnkbottomhind	5'AGCTTCTTGGCTCGTTTTTTTTTGCAAAA-3'
Splnkbottomxba	5'-CTAGTCTTGGCTCGTTTTTTTTTGCAAAA-3'
SplnkbottomEco	5'-AATTTCTTGGCTCGTTTTTTTTTGCAAAA-3'
Splko	5'-CGAATCGTAACCGTTCGTACGAGAA-3'
Splki	5'-TCGTACGAGAATCGCTGTCTCTCC-3'
HBMPr4	5'-CTGGCATCCACATTGATGAACAATC-3'
HBMPr3	5'-AACAATCATGTTTGGTATTTCTGC-3'
Bmprev3	5'-TCAGCCAGAGTAGCAGCTGC-3'
bmprev2	5'-ACCACGCGTAAACCGTTTTGG-3'
raceR ₀	5'-AAGGATCCGTCGACATCG-3'
raceR ₁	5'-GACATCGATAATACGACT-3'
screwout (GSP1)	5'-ATCTTACAAGCACTAGTGCATAC-3'
screwnest (GSP2)	5'-TTCCAAAACCTTGTTGTGCTCC-3'
BMPforw2ligand	5'-TGTGGGAAACATCTTTTATATGTG-3'
BMPprevligand	5'-CTGGCATCCACATTGATGAAC-3'
BMP7BamHIF	5'-AAAGCAGAGTTACCGGATCCTATTAGATTGCCATT AC-3'
3'seqscreen1	5'-ATTAGGTCTTGGTACATATGCATTG-3'
Prodom1	5'-TCTCCAACGAGCCAAGAAGC-3'
PolyArevbmp6	5'-TTTATTAACGGCACATAAAAACAG-3'
exopcs25-	5'-TTTGCAGGATCCAATGAAAGTAACGTTATTAAGC-3'
exopcs23-	5'-GGCCTTGAATTCCTATTGGCATCCACAGTG-3'
exoligandA5'	5'-CCGTAAGAGAGGCATAGAAGATGATGATTT-3'
exoligandA3'	5'-CTAGAGGCTCGAGTTACTGGCATCCACATTG-3'
exoligandB5'	5'-CCGTAAGAGAGGCAGTATGCAAGATAGTAT-3'
exoligandB3'	5'-CTAGAGGCTCGAGCTATTGGCATCCACAGTG-3'
NheI5'hybmp7b	5'-GGGGCTAGCAATTCCAATAAAGAGCGAAATATC- ATACAAAATAC-3'
XhoI3'hybmp7b	5'-GGGCTCGAGCTATTGGCATCCACAGTGTGGACTA- TC-3'
BluntendHya5'	5'-ATAGAAGATGATGATTTTTTAAAAGCAGCT-3'

Hya3'	5'-GGGTCTAGACTGGCATCCACATTGATGAA-3'
BluntendHyb5'	5'-AGTATGCAAGATAGTATTTTTATAGAAGCT-3'
Hyb3'	5'-CTCGAGCTATTGGCATCCACAGTGTGGACTATC-3'
5E α 1a	5'-GGAAAAGGATCATTTAAATAT-3'
3E α 1a	5'-AACAAATCATTGTGTTAACACC-3'
bamH1hysmadfw	5'-GGGGGATCCATGGCATCAATGGCATCTC-3'
sallhysmadrev	5'-GGGGTTCGACCACTAGTGATTTCGACCGTAAGCTTC-3'
BM40-peptide	5'-MRAWIFFLLCLAGRALAAPL-3

2.12 Electrophoresis

HORIZON [®] 58 Horizontal-Agarose	GibcoBRL
Elektrophoresis Chamber	Life Technologies
PowerPac 300 Power Supply	BioRad
Mini-PROTEAN 3 Electrophoresis Cell	BioRad

2.13 Thermocyclers

Mastercycler [®] Gradient	Eppendorf
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2.14 Centrifuges

Micro centrifuge 5415 D	Eppendorf
Micro centrifuge TX-160 (refrigerated)	TomyTech USA Inc.
Sorvall [®] RT 6000 B (refrigerated)	DuPont
Beckman TJ-6	Beckman
Beckman J2-21	Beckman

2.15 Incubators

Dry-Bath Incubator (heat block)	Fisher Scientific
Dual Chamber Water bath 188	Precision [®] Scientific
Gyrotory [®] Water Bath Shaker G76	New Brunswick Scientific
Bench Hybridization Incubator	New Brunswick Scientific
Isotemp Standard Incubator (Thelco)	Precision [®] Scientific
AMBI-HI-LO CHAMBER (18 °C)	Lab-line Instruments, Inc.

2.16 Freezers

-80 °C ultra low freezer	SO-LOW
-20 °C freezer	Lab-line Instruments, Inc.

2.17 UV-devices

312 nm Transilluminator FBTI 614	Fisher Scientific
Alpha Imager 2200 v5.5	Alpha Innotech Corporation
UV-Stratalinker 1800	Stratagene

2.18 Blotting Material

PROTRAN [™] pure nitrocellulose transfer and immobilization membran BA85 pore size 0.45 µm , 82 mm pore size 0.45 µm , 137 mm	Schleicher & Schuell GmbH
GeneScreen [™] hybridization transfer Membrane	DuPont NEN [®] Research products
Gel Blot Paper 3 mm	Schleicher & Schuell
Immobilon [™] -P, PVDF membrane	Millipore
Western blotting apparatus	BioRad

2.19. Plasmid Purification (Miniprep)

3cc syringe barrel	Becton Dickinson
Vacuum Manifold Vac-Man [®]	Promega
Wizard [®] Miniprep Column	Promega

2.20 Autoradiography

Autoradiography Cassette FBXC 1417	FisherBiotech
X-OMAT [™] AR	Kodak

2.21 Miscellaneous

ABI Prism [™] 310 Genetic Analyzer	PE Applied Biosystems
Cover Glass	Fisher Scientific
“Falcon” tubes (polypropylene tubes), 15ml/50 ml	Greiner
Glass dishes for hydra culturing	Pyrex [®]
Great Salt Lake Artemia Cysts	Sanders [®] Brine Shrimp Company
Hot Plate-stirrer PC-351	Corning
Micro Centrifuge tubes (1.5 ml; 0.5ml)	USA Scientific
Microcentrifuge tubes (0.4 ml)	Fisher Scientific
Microscope slides	Fisher Scientific
pH Meter 240	Corning
UV/VIS Spectrophotometer DU [®] 530	Beckman
Vortex Genie 2	Fisher Scientific
Watchmaker forceps #5 (0.05 mm x 0.02 mm)	Dumont

2.22 Sequence comparisons and molecular phylogenetic analysis

DNAstar for macintosh	Lasergene
Database searches	http://www.ncbi.nlm.nih.gov/
Sequence alignment	<i>ClustalX</i> version 1.81
<i>Puzzle</i>	http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html
<i>PAUP*</i>	version 4.0b10 for Macintosh (PPC)
Signal peptide prediction	http://www.cbs.dtu.dk/services/SignalP/

3. Methods

3.1. Handling animals

3.1.1. Animals and Culture conditions

Animals were maintained at 18°C in hydra medium with a 12 hour light cycle according to the standard method (Lenhoff and Brown, 1970) and fed every two days three times a week with freshly hatched *Artemia salina* nauplii. Animals were starved for at least 24 hours prior to all experimental manipulations. Unless otherwise stated, the experiments were carried out with animals of the L2 (Japan) strain of *Hydra vulgaris*. The 105 strain (*Hydra magnipapillata*) was used for head regeneration experiments as well as the reg-16 strain a regeneration deficient strain of *H.magnipapillata*.

3.1.2. Preparation of aggregates

50 animals provided about 10 aggregates. Prior to aggregation the head and the foot of each animal was removed. The body columns were collected in a 12 ml tube sitting in an ice bucket. The body columns were washed three times in dissociation medium (DM) on ice. After the last wash the body columns were sitting in 3-5 ml DM on ice for 15-30 minutes, following which they were dissociated by gently pipetting up and down with a Pasteur pipette for about 1 minute. After settling of the sediment for one minute on ice the supernatant was collected in a 50 ml tube. 5 ml of fresh DM medium was added to the remaining body columns and the dissociation by pipetting was repeated three more times. The cell suspension was centrifuged for 5 minutes at 4°C at 100xg in a swinging bucket rotor. The cell pellet was resuspended smoothly in about 5ml DM and further filled up to an appropriate cell density (6000-8000 epithelial cells per aggregate). The resuspended cells were aliquoted into the aggregation tubes (0.4 ml Fisherbrand microcentrifuge tubes). The tubes were centrifuged 5 minutes at 100g at 4°C in a swinging bucket rotor. The centrifuge tubes were incubated upside down in petri dishes filled with DM until the formed aggregates fell down into the dish. After 6 hours they were transferred into hydra medium (HM):DM 1:1, 12 hours later they were

transferred to HM:DM 1:3 and after 36 hours of preparation they were cultured in 100% HM.

3.1.3. Tissue manipulations

For head regeneration experiments, *H.magnipapillata* polyps and polyps of the regeneration-deficient strain of *H.magnipapillata*, reg-16, were bisected either directly beneath the tentacle zone or half way down the body column. After bisection, the lower halves were incubated at 18°C. To examine foot regeneration the upper halves of animals of the L2 strain of *H.vulgaris* were bisected in the middle of the body column or in the middle of the peduncle region and were allowed to regenerate.

To lower the head activation gradient along the *Hydra* body column, animals were treated with LiCl. The polyps were incubated continuously in 0.5 mM LiCl in hydra medium. The animals were fed as usual and washed daily with LiCl/hydra medium solution. To raise the head activation gradient along the body column and to induce ectopic tentacle formation, animals of the *H. vulgaris* L2 strain were treated with either 5 µM alsterpaullone or 2 mM LiCl in hydra medium for two days (Hassel et al., 1993). After that they were transferred back to hydra medium, and examined daily for ectopic tentacle formation. The animals were not fed during the 2 mM LiCl treatment and during the alsterpaullone treatment.

3.2. Molecular techniques

3.2.1. Universally applied techniques

3.2.1.1. Isolation of *Hydra* DNA and RNA

Animals of the L2 strain of *Hydra vulgaris* were starved for at least 3 days. Genomic DNA was isolated essentially as described by Davis et al. (Davis et al., 1980). Total RNA was extracted from *H. vulgaris* L2 polyps according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).

3.2.1.2. Purification of Plasmid DNA

To isolate plasmids from bacteria a procedure based on “Magic Minipreps” of the Promega Corporation was used. Diatomaceous earth (or CELITE) suspended in 7 M guanidinehydrochloride/1M potassium acetate, pH5.5 (Merlin IV), serves as a resin that binds DNA at a high efficiency. Plasmid purification was carried out from 5 ml overnight bacterial cultures. Briefly, bacterial cells were lysed under alkaline conditions, and the lysate was subsequently neutralized and adjusted to high-salt binding conditions. The lysate was centrifuged at 16000 rpm for 10 minutes and the clear supernatant was transferred to a barrel of a Wizard[®] Miniprep Column/ syringe assembly attached to a vacuum manifold containing 1 ml of CELITE suspension. A vacuum of at least 15 inches of Hg was applied to pull the resin/lysate mix into the minicolumn. That accomplished, the vacuum was stopped at the source and 3 ml of Merlin V column wash solution was added. To wash the resin vacuum was applied again. Then, the minicolumn was centrifuged in a microcentrifuge tube at 16000 rpm for 2 minutes to remove any residual Merlin V solution. Finally, the DNA was eluted with 50 µl nuclease free water. The plasmid DNA recovered using this procedure is pure enough for standard applications as restriction enzyme digests and sequencing reactions.

3.2.1.3. Agarose gel-electrophoresis

DNA was separated in 1-1.5% agarose gels in TBE buffer containing ethidium bromide and size evaluated using a 1 kb DNA ladder (Invitrogen).

3.2.1.4. DNA sequencing

DNA sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems). This technique is based on the Sanger dideoxi-mediated chain-termination method (Sanger et al., 1977). The rhodamine acceptor dyes enable band detection in the automated sequencer ABI Prism[™] 310 Genetic Analyzer. Reactions were set up according to the manufacturer protocol. The sequencing data were analyzed using the manufacturer software or the seqMan program of the DNASTar.f (Lasergene) software.

3.2.1.5. Cloning of PCR products

3.2.1.5.1. DNA purification

PCR products were separated in 1% TBE-agarose gels and subsequently extracted and purified using the QIAEX II Gel Extraction Kit (Qiagen) following the manufacturers protocol. DNA that was digested with restriction enzymes prior to the ligation into a recipient vector was phenol/chloroform extracted and ethanol precipitated according to standard protocols (Sambrook et al., 1989).

3.2.1.5.2. Ligation of PCR products into pGEM-T vector

T/A-cloning is based on the property of a Taq-polymerase to add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of amplified PCR products. The pGEM-T Easy Vector Systems take advantage of this fact and add after cutting their vectors a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recirculization of the vector and providing a compatible overhang. Ligation into pGEM-T Easy vector (Promega) was carried out according to the manufacturer protocol.

3.2.1.5.3. Subcloning by Exonuclease III digestion

Conventional cloning requires a DNA insert and a recipient vector to possess either blunt or compatible cohesive ends by restriction digestion. Using exonuclease III, an incompletely processive exonuclease, it is possible to expose complementary DNA between an insert and a vector such that annealing becomes independent of restriction site compatibility (Li and Evans, 1997). Exonuclease III catalyzes the stepwise removal of 5'-mononucleotides from the 3'-hydroxyl termini of double stranded DNA and circular DNAs containing nicks or gaps (Weiss, 1976). I applied the following protocol: The DNA insert was prepared by PCR using a proofreading polymerase (Pfx). Designing oligonucleotides against the DNA of interest, I made sure that they contained 12 bp on their 5' end complementary to the vector ends, in addition to the

gene specific sequence. The PCR product was purified by gel electrophoresis, using the QIAEX II Gel Extraction Kit (Qiagen).

The recipient vector was cut with two different restriction endonucleases to ensure complete digest. The linearized vector was purified via phenol/chloroform extraction, followed by ethanol precipitation. 0.1 µg vector was mixed with 0.5 µg insert in 1x Exonuclease III buffer and 20 U of Exonuclease III in a volume of 20 µl on ice. The reaction was then incubated at 14°C for exactly 1 minute in a water bath with the tube tops open. This allowed the exonuclease III to remove approximately 15 nucleotides at each existing 3' end. 80 µl of 50 mM EDTA pH 8.0 was added to stop the reaction. This was followed by phenol/chloroform extraction and ethanol precipitation. The washed pellet was resuspended in 10 µl of 1x universal digestion buffer. The digested ends were melted at 65°C for 5 minutes. The annealing reaction took place at 37°C for 5 minutes. All 10 µl were used to transform competent *E.coli* cells (Subcloning efficiency™DH5α™).

3.2.1.5.4. Transformation

Chemically competent *E.coli* cells (Subcloning efficiency™DH5α™) were used for transformation. The reaction was carried out according to the manufacturer protocol. Briefly, the ligation reaction was centrifuged and placed on ice. A 50 µl aliquot of DH5α™ cells in a microcentrifuge tube was thawed on ice. 5-10 µl of the ligation reaction was pipetted directly into the competent cells and mixed by gently tapping the tube. The vial was incubated for 30 minutes on ice. The cells were heat shocked for 20 seconds in a 37°C waterbath. The vial was then placed on ice for 2 minutes. 900 µl of pre-warmed SOC medium were added to the vial. The vial was incubated at 37°C for 45 minutes at 250 rpm in a shaking incubator. 20 to 200 µl of the transformation vial were plated out onto LB agar plates (supplemented with the appropriate antibiotic and with IPTG [100 mM] and X-gal [50 mg/ml] for blue/white selection). The plates were inverted and incubated at 37°C overnight. To prove whether white colonies contained an insert, plasmids were digested with restriction endonucleases that flanked either site of the multiple cloning region and checked on an agarose gel.

3.2.1.6. Labeling of a DNA probe with [³²P]dATP

Probes for the screening of cDNA libraries, Northern Analysis and Southern Analysis were labeled with [³²P]dATP using the random primer method (Feinberg and Vogelstein, 1984) and the Prime-It[®] II Random Primer Labeling Kit from Stratagene. The procedure relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The primer-template complexes formed represent a substrate for the Klenow fragment of DNA polymerase I. The enzyme synthesizes new DNA by incorporating nucleotide monophosphates at the free 3'-OH group provided by the primer. The newly synthesized DNA was made radioactive by substituting radiolabeled [³²P]-dATP for the nonradioactive dATP in the reaction mixture. 25 ng of DNA was mixed with 10 µl random hexamers and denatured in a boiling water bath for 5 minutes. The reaction was briefly centrifuged and the following components were added:

10 µl 5x buffer

5 µl labeled [³²P]dATP (3000Ci/mmol)

1 µl Exo(-)Klenow (5 U/µl)

The reaction was incubated for 10 minutes at 37°C and was stopped by adding 2 µl of stop mix. 50 µl of TE-buffer was added before the sample was applied to a G-50 Sephadex column to separate the radioactively labeled DNA from unincorporated dNTPs. The column chromatography was carried out following standard protocols (Sambrook et al., 1989). In all three applications the probe was used only used its specific activity was no less than 10⁸ cpm/µg.

3.2.2. Isolation of *Hydra* BMP orthologues

3.2.2.1. Primer design for degenerate PCR

Designing degenerate oligonucleotides to conserved regions of genes can allow cloning of divergent/ related genes. I have used degenerate oligonucleotides to amplify genes of the BMP family of signalling molecules out of *Hydra*. First I selected stretches of conserved amino acids in the ligand domain of the BMP family. I avoided amino acids with several alternative codons. For the third codon position I chose the nucleotide that is represented at a minimal frequency of 60%. For codons where no single nucleotide

reached this frequency, I used two nucleotides A and T, since *Hydra* has an A/T-rich genome and prefers A and T at the third position of the codon. When I had to represent all four nucleotides I introduced Inosine (I), in order to keep the degeneracy low. Inosine occurs naturally in the wobble position of the anticodon of some transfer RNAs and is known to form base pairs with A, C, and U in the translation process. While not truly universal, Inosine is less destabilizing than mismatches involving the 4 standard bases. Degenerate primers are standardly applied as pairs (sense and antisense oligonucleotide) in a RT-PCR reaction. In order to decrease the degeneracy in the PCR reaction even further I used the splinkerette PCR method, in which I only had to use one degenerate oligonucleotide per PCR reaction.

3.2.2.2. Splinkerette PCR

The splinkerette PCR is an adapter ligation-mediated PCR method frequently used for obtaining genomic sequence upstream or downstream of a known sequence. The method has been first published by R.S. Devon (Devon et al., 1995). I have adapted the method to amplify unknown sequence by using degenerate oligonucleotides designed as described in 3.2.2.1. The procedure of the splinkerette PCR method (shown in figure 3.1) begins with the digestion of genomic DNA with a restriction enzyme to generate a 5' overhang (1) and is followed by a ligation of a linker termed splinkerette adapter (2) to the digested ends of the genomic DNA (3). The flanking sequences are then amplified using a gene specific primer of the gene of interest, in my case a degenerate oligonucleotide, and the universal splinkerette-specific primer (4).

The splinkerette linker (2) is designed to build a hairpin structure on the bottom strand to avoid end-repair priming and thereby amplifying unwanted nonspecific sequence fragments. In the original paper the authors cleaved genomic DNA with the endonuclease BamHI and used an adapter sequence that recognizes the BamHI overhang. Since BamHI cuts too infrequently in *Hydra's* A/T-rich genome, we have changed the oligonucleotides used to build the splinkerette linker to take advantage of more A/T-rich sites (HindIII, EcoRI, XbaI). The splinkerette adapters were generated by annealing the splinkerette oligonucleotides *splinktop* and *splinkbottom* (HindIII, XbaI or EcoRI) with each other. 150 µg/ml of *splinktop* oligonucleotide and 150 µg/ml of *splinkbottom* oligonucleotide were mixed in 20 µl annealing buffer (Tris, pH 7.4, 5 mM MgCl₂). The mixture was heated to 90°C and then cooled to room temperature on

the benchtop for 10-20 minutes. 3 μg of *Hydra* genomic DNA was digested with 12 U of Hind III, XbaI or EcoRI, respectively, in a 40 μl reaction at 37°C for 2 hours, followed by phenol/chloroform extraction and ethanol precipitation. The DNA sample was ligated to the splinkerette linker using 5 U T4 DNA ligase at 4°C overnight:

6 μl splinkerette linker (prepared as described above); 2 μl Hind III digested DNA reaction (~3 μg), XbaI or EcoRI, respectively; 10 μl 2X ligase buffer; 1 μl water; 1 μl T4 DNA ligase (5U).

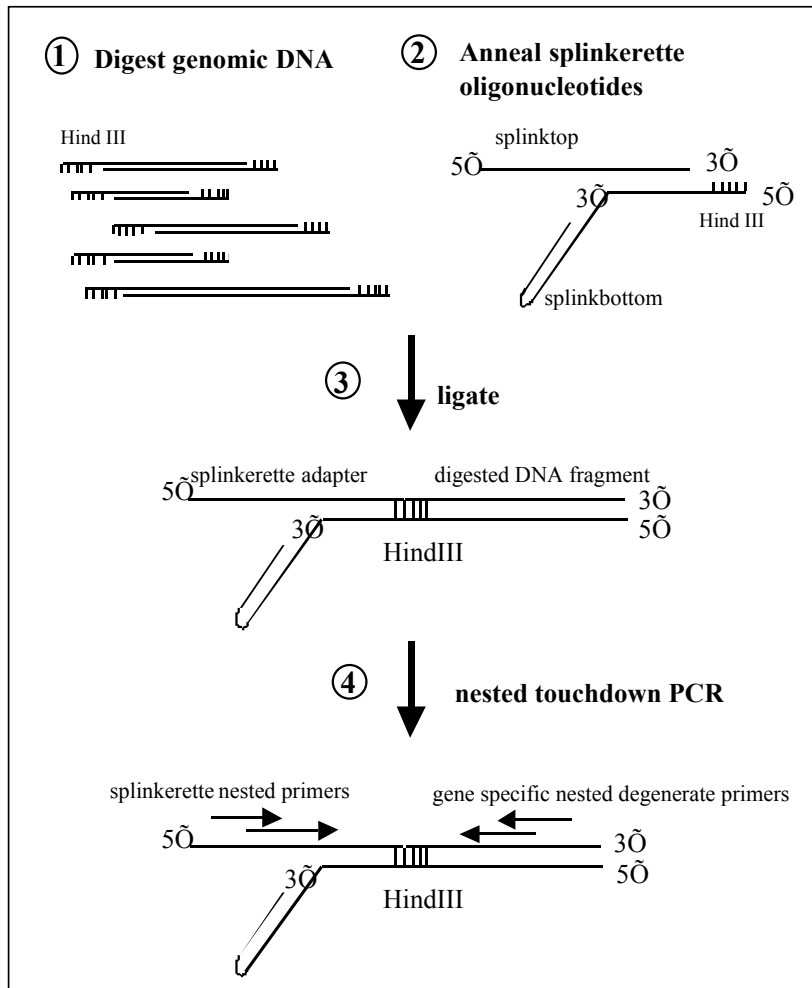


Fig. 3.1. Schematic diagram of the splinkerette PCR procedure using the restriction enzyme HindIII.

Nested touchdown PCR (Don et al., 1991) was performed upon 1% of the ligated material. Touchdown PCR involves decreasing the annealing temperature by 1°C or 2°C every second cycle to a 'touchdown' annealing temperature which is then used for 10 or more cycles. It was originally intended to bypass more complicated optimization processes for determining optimal annealing temperatures. The idea is that any differences in the melting temperature (T_m) of the used oligonucleotides between correct and incorrect annealing gives a two-fold difference in product amount per cycle (four-fold per 1°C). This method therefore enriches for the correct product over any incorrect products. This procedure is especially helpful when using degenerate oligonucleotides. I performed the touchdown PCR traversing 53°C to 43°C. I decreased the annealing temperature by 2°C every second cycle to a annealing temperature of 43°C, which was then used for 20 more cycles. First, primary splinkerette primer, *splko* was employed together with the primary degenerate primer. The following components were assembled:

Component	Volume (μ l)
ligated material (diluted ligation reaction 1:100)	2
buffer (10x) Biolase	5
MgCl ₂ (50 mM).	5
dNTP (10 mM)	2
splko (250 ng/ μ l)	1
dppf1 (400 ng/ μ l)	1
Taq Polymerase (Biolase)	2
ddH ₂ O	36.3

PCR conditions:

1) 1 cycle

Denaturation 30 sec 94°C

Annealing 1 min 53°C

Elongation 2 min 72°C

2) 10 cycles

Denaturation 15 sec 94°C
Annealing 1 min 51°C (every second cycle decrease of temperature by
Elongation 2 min 72°C

3) 10 cycles

Denaturation 15 sec 94°C
Annealing 1 min 43°C
Elongation 4 min 72°C

4) 10 cycles

Denaturation 15 sec 94°C
Annealing 1 min 43°C
Elongation 6 min 72°C

For the second round of amplification 1 µl of the first PCR reaction using the secondary splinkerette primer, *splki*, and the secondary degenerate primer were amplified using the same conditions as in the first round. The PCR products obtained were purified by gel electrophoresis, and cloned into pGEM-T Easy and sequenced. Various combinations of degenerate oligonucleotides in splinkerette PCR reactions were used for amplifying BMP genes with the optimal combination being *dppf1* and 60Af1 (for sequence information see Materials), which were designed against the following amino acid stretches of the conserved ligand domain of BMP genes: YV[D/S]F[S/R]and [L/M]N[S/A]TNHAI (see also Fig.X 4.1.).

To obtain further genomic sequence upstream of the BMP-gene-fragment isolated, specific primers designed against the 5' end of the sequence were employed in splinkerette PCR reactions. Primers used were: *HBMPPr3*, *HBMPPr4*, *BMPPrev2*, *BMPPrev3*.

3.2.2.3. 3'RACE

In order to obtain 3'UTR sequence of a gene of interest, 3'RACE was performed according to Frohman (Frohman, 1993). Primers used were:

(dT)₁₇-R_i-R_o for reverse transcription

raceR_o and raceR_i : outer and inner adapter primer

screwout (example for *HyBMP5-8a*): outer gene specific primer (GSP1)

screwne (example for *HyBMP5-8a*): inner gene specific primer (GSP2)

3.2.2.3.1. Reverse transcription reaction

Using the M-MLV reverse transcriptase, I performed a reverse transcription reaction according to Frohman's recommendations, using little amounts of total RNA (2 µg) and only 50 ng of (dT)₁₇-R_i-R_o primer, following the manufacturer protocol. The obtained first strand product was diluted to 500 µl with TE buffer.

3.2.2.3.2. Amplification protocol

For the PCR reaction the following components were assembled:

Component	Volume (µl)
10x PCR buffer (Biolase)	5
template cDNA	5
MgCl ₂ (50mM)	2.5
dNTPs (10mM)	2
Forward primer (25pmol/µl) GSP1	1
Reverse primer (25pmol/µl) R _o	1
(dT) ₁₇ -R _i -R _o (2pmol/µl)	1
DMSO	5
ddH ₂ O	28.5

To denature the first strand products this cocktail was heated to 95°C for 7 minutes and cooled down to 75°C. 2.5 U of Biolase Taq Polymerase was added to the reaction. The primers were allowed to anneal at 44°C for 2 minutes followed by an extension reaction at 72°C for 40 minutes. After that 30 cycles for the first round of amplification were carried out in the following manner:

1) 30 cycles

Denaturation 15 sec 94°C

Annealing 25 sec 46°C

Elongation 3 min 72°C

2) Elongation 15 min 72°C

The amplification products from the first round were diluted 1:20 in TE buffer. 1 µl of the diluted material using the primers GSP2 (*screwnest*) and R₁ were amplified using the 30 cycle conditions from the first round without the initial 2-minute annealing and 40-minute extension period.

3.2.2.4. Library screening

Isolation of a cDNA containing the entire coding region of a second *Hydra* BMP gene, *HyBMP5-8b*, was accomplished by screening a *Hydra vulgaris* Uni-Zap™XR Custom library oligo (dT) primed, derived from regenerating *Hydra* heads (Deutzmann et al., 2000)(kind gift of Mike Sarras).

3.2.2.4.1. Primary library screen

The library was screened with a cDNA fragment of 306 bp obtained by PCR amplification, using the oligonucleotides *BMPforw2ligand* and *BMPprevligand* that are spanning the mature ligand region of *HyBMP5-8a* starting with the first conserved cysteine residue. The fragment was labeled with [³²P]-dATP as described in 3.2.5.

In the primary round a total of 5x10⁵ clones were screened. Briefly, the titer of the amplified cDNA library was first determined to seed a number of 5x10⁴ plaques/15 centimeter LB-plate. Ten such plates were prepared in the following manner. The *E.coli* strain XL1-Blue MRF⁷ was grown overnight in LB supplemented with 0.2% maltose and 10 mM MgSO₄ (maltose induces expression of maltose receptor, which also acts as a λ-phage docking protein) by shaking at 250 rpm at 30°C. The grown bacteria were spun down for 5 minutes at 2000 rpm in a TJ-6 centrifuge and resuspended in chilled 10 mM MgSO₄. 0.1 ml of the appropriate phage solution in SM-buffer and 0.2 ml of the bacteria suspension were combined in a glass culture tube and

incubated for 15 minutes at 37°C. 3.5 ml of liquid top agar (50°C) was added to the culture tube and poured onto a LB 15-centimeter plate. The plates were incubated overnight at 37°C, dried and chilled at 4°C. Labeled nitrocellulose filters were placed on the chilled plates with blunt forceps in order to transfer the phage colonies onto the membrane. The filters were prepared in duplicates. To electrostatically bind the phage DNA to the nitrocellulose membrane, the filters were first placed for 40 seconds into the denaturation solution (0.5 M NaOH, 1.5 M NaCl), then transferred to the neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0) for 3 minutes and lastly rinsed in 2xSSC to decrease the salt concentration. The dried filters were then baked at 80°C for 1 hour to covalently bind the DNA to the nitrocellulose.

The filters were prehybridized for 2 hours at 41°C in glass bottles and 100 ml hybridization-solution-A. The complete random primed reaction was added to 10 ml of fresh hybridization-solution-A and applied to the filters overnight. The membranes were washed to a final stringency of 0.2xSSC, 0.1%SDS at 41°C. Hybridization was detected by exposure of the filters to X-Ray film (Kodak X-OMAT) with intensifying screens at -80°C overnight. Double positive spots visible on the film were aligned with the original LB-phage plate in order to pick the positive phage colony. The obtained agar plug was dispensed in 1 ml of SM buffer containing one drop of chloroform. The suspension was vortexed and kept at 4°C overnight.

3.2.2.4.2. Secondary library screen

A secondary library screen to purify the positive plaques obtained in the primary round, using 10 centimeter LB-plates, was performed following essentially the same protocol. However, the hybridization conditions applied differed from the primary screen to ensure a higher specificity. Hybridization was carried out in hybridization-solution-B at 60°C. Positive plaques were picked as described earlier and stored at 4°C.

3.2.2.5. *In vivo* excision of the pBS II SK⁻ phagemid from the Zap[®] Express vector

The inserts of plaque-purified positive phage were recovered in the pBluescript SK⁻ plasmid by *in vivo* excision using the Stratagene kit: ExAssist[®] Interference-Resistant Helper Phage with XL0LR Strain. Briefly, separate overnight cultures of XL1-Blue

MRF⁺ cells supplemented with 0.2 % Maltose and 10 mM MgSO₄ and XLOLR cells in LB medium were grown at 30°C. The cells were being gently spun down at 1000xg and resuspended at an OD of 1 in 10 mM MgSO₄. In a 50 ml Falcon tube 200 µl of XL1-Blue MRF⁺ cells, 250 µl of phage stock (containing >1x10⁵ phage particles) and 1 µl of the ExAssist helper phage (>1x10⁶ pfu/µl) were combined and grown at 37°C for 15 minutes. Then, after adding 3 ml of LB medium the tube was incubated at 37°C for 2.5-3 hours with shaking.

The Falcon tube was heated at 65°C for 20 minutes and then spun down at 1000xg for 15 minutes. The supernatant, containing the excised pBluescript phagemid packaged as filamentous phage particles, was decanted into a new sterile Falcon tube. The excised phagemid was grown by adding 200 µl of XLOLR cells to 100 µl phagemid suspension either undiluted or at a dilution of 1:3 and 1:30, incubating the mixtures at 37°C for 15 minutes and plating them on LB-ampicillin plates. Only colonies appeared on the plates, that were containing pBS II SK⁻ double-stranded phagemid with the cloned DNA insert. The helper phage did not grow, since it contains amber stop codons, which XLOLR cells do not suppress. λ-phage also did not propagate in the XLOLR cells because these cells are resistant to lambda infection.

3.2.3. Characterization of *Hydra* BMP orthologues

3.2.3.1. Southern Blot Analysis

Genomic DNA of whole *Hydra* polyps were digested with 4 different restriction endonucleases: EcoRI, EcoRV, XbaI, HindIII (10 µg per digest). The digested DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in 30 µl of water. The DNA aliquots were fractionated in a 0.8% agarose gel together with a 1kb DNA size marker and two positive controls. The agarose gel was photographed with a ruler laid alongside the gel so that band positions could later be identified. Then, the gel was rinsed twice with water, soaked for 10 min in 0.25 HCl solution, rinsed with water and soaked two times for 15 minutes each in denaturation solution (1.5 M NaCl, 0.5 M NaOH). The gel was rinsed with water and soaked two times for 20 min in neutralization solution (1.5M NaCl, 0.5 M Tris pH 8). The transfer of the electrophoretically separated DNA from the gel to a nylon-based membrane was

achieved using the upward capillary transfer over night as described in Sambrook et al., (1989). After the transfer the membrane was rinsed twice in 2xSSC to remove residual agarose and to leach out excess salt. The DNA was then UV crosslinked to the membrane using an UV-stratalinker 1800 (Stratagene). Hybridization with a [³²P]-dATP-labeled 416 bp long cDNA fragment, corresponding to the mature ligand domain of the *HyBMP5-8b* gene, was carried out using QuickHyb hybridization solution (Stratagene) at 68°C for 18 hours rotating in a hybridization oven.

The oligonucleotides used to amplify the cDNA fragment were *exoligandA5'* and *exoligandA3'*. The membrane was prehybridized for 15 min in prewarmed QuickHyb solution in a 50 ml Falcon tube. The probe was applied at a concentration of 2.5x10⁶ total counts/2ml hybridization solution. The membrane was washed to a final stringency of 0.2xSSC/0.2% SDS at 68°C. Hybridization was detected by exposure of the filters to X-Ray film (Kodak X-OMAT) with intensifying screens at -80°C for one week. The hybridized radioactive probe was removed by treating the membrane with several hundred milliliters of boiling 0.1%SDS. The stripped membrane was then hybridized using the same conditions with a second probe, corresponding to the mature ligand domain of *HyBMP5-8a*, also 407 bp in length. The oligonucleotides used to amplify this cDNA fragment were *exoligandB5'* and *exoligandB3'*.

3.2.3.2. Northern Blot Analysis

Total RNA was extracted from whole *H. vulgaris* polyps. Poly(A)⁺ RNA was isolated using oligo(dT) coupled to polystyrene latex beads (Qiagen) as described by the manufacturer. 2 µg of poly(A)⁺ RNA (for *HyBMP5-8b*) or 8 µg of poly(A)⁺ (for *HyBMP5-8a*), respectively, was transferred to a Genescreen (Dupont) nylon-based membran following electrophoresis in a formaldehyde-gel.

3.2.3.2.1. Formaldehyde-Agarose (FA) Gel Electrophoresis

Electrophoresis tanks and casting equipment were cleaned with 0.2% SDS, rinsed with water, dried with ethanol, and then filled with a solution of 3% H₂O₂. After 10 minutes at room temperature, the electrophoresis tanks were rinsed thoroughly with DEPC-H₂O. Glass ware and stirring bar used for preparing the gel were soaked for 20 minutes

in 0.2 N NaOH and then rinsed thoroughly in DEPC-H₂O. 0.25 g agarose were melted in 21 ml DEPC-H₂O. The agarose solution containing a stirring bar was placed on a magnetic stirrer and 2.5 ml of 10xMOPS solution, preheated to 40 °C, and 1.5 ml of 37% formaldehyde were carefully added to the solution by pipetting. The solution was mixed thoroughly and poured onto the gel support. The gel was equilibrated in 1xMOPS buffer for 40 minutes.

Prior to loading the RNA samples the gel was prerun for 5 minutes at 45 V. The mRNA sample was prepared in the following way. After addition of 10 µl loading dye to the RNA sample was incubated at 65°C for 15 minutes and chilled on ice for 5 minutes. Before loading onto the FA gel 2 µl of 0.2mg/ml Ethidiumbromide solution was added to the samples. 5 µg of RNA molecular size marker (Invitrogen) were run on each gel. The gel was run at 45 V for 2 hours. After completion, a picture was taken to insure the RNA was still intact (RNA of the size marker served as a control) and photographed with a ruler so that band positions could later be identified on the membrane.

3.2.3.2.2. Hybridization with the radioactive labeled DNA probe

The gel was soaked 3 times for 20 minutes each time in distilled water and soaked for a short time in 20xSSC before blotting onto the membrane. The transfer of the electrophoretically separated RNA from the gel to the nylon-based membrane was achieved using the upward capillary transfer as described in Sambrook et al., 1989. After the transfer was accomplished, the membrane was rinsed briefly in 2xSSC to remove residual agarose and to leach out excess salt. The RNA was then crosslinked to the membrane using an UV-stratalinker 1800 (Stratagene).

Hybridization with a [³²P]-dATP-labeled cDNA fragment 418 bp in length, corresponding to the mature ligand domain of the *HyBMP5-8b* cDNA, was carried out using QuickHyb hybridization solution (Stratagene) at 68°C for 18 hours rotating in a hybridization oven. The membrane was washed to a final stringency of 0.2xSSC/0.2% SDS at 50°C. Hybridization was detected by exposure of the filter to X-Ray film (Kodak X-OMAT) with intensifying screens at -80°C over night. The same northern analysis procedure was carried out for the detection of *HyBMP5-8a* using a 418 bp cDNA fragment corresponding to the mature ligand domain of *HyBMP5-8a* and 8 µg of poly(A)⁺ RNA.

3.2.3.3. *In situ* hybridization

Since this method involves labeling RNA with digoxigenin-labeled nucleotides, minimization of the introduction of RNase is critical. To do so, gloves, RNase-free pipette tips and tubes were used. All aqueous solutions used in the first half of the hybridization procedure including the actual hybridization step were treated with DEPC, and then autoclaved to remove DEPC. Fixation, processing, hybridization and visualization of the riboprobes in whole-mount preparations were conducted as previously described (Grens et al., 1996; Grens et al., 1999; Martinez et al., 1997)

3.2.3.3.1. Synthesis of anti-sense and sense RNA probes

HyBMP5-8a and *HyBMP5-8b* sense and antisense digoxigenin-labeled RNA probes were made using an RNA *in vitro* transcription kit (Roche). One probe was generated against the to date identified open reading frame (ORF) of *HyBMP5-8a*. This fragment was amplified from cDNA by PCR using a proofreading polymerase (Pfx) and the oligonucleotides *prodom1* and *polyArevbmp6*. The PCR fragment was cloned into the pGEM-T Easy vector (Fig. 3.2)

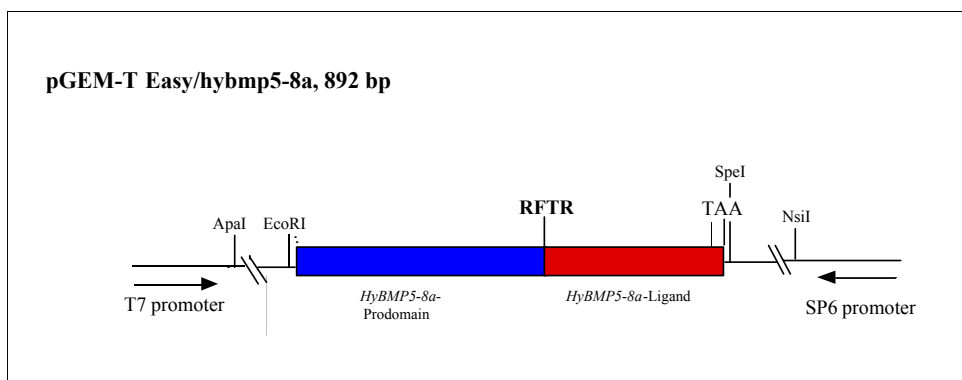


Fig. 3.2. Structure of the *HyBMP5-8a* clone used for preparing digoxigenin labeled RNA probes. The figure shows the location of the cDNA sequence in the multiple cloning site (MCS) of pGEM-T Easy. RFTR: tetrabasic cleavage site of *HyBMP5-8a*.

Two probes were generated against the sequence of *HyBMP5-8b*, one against the full length clone obtained in the library screen (Fig. 3.3A) and the other only to 62 bp 5'UTR followed by 335 bp of the prodomain of *HyBMP5-8b* (Fig. 3.3B). This 5'-fragment of *HyBMP5-8b* was amplified by PCR from the original clone using a proofreading polymerase (Pfx) and the oligonucleotides *BMP7BamHIF* and *3'seqscreen1*. This PCR fragment was cloned into the pGEM-T Easy vector.

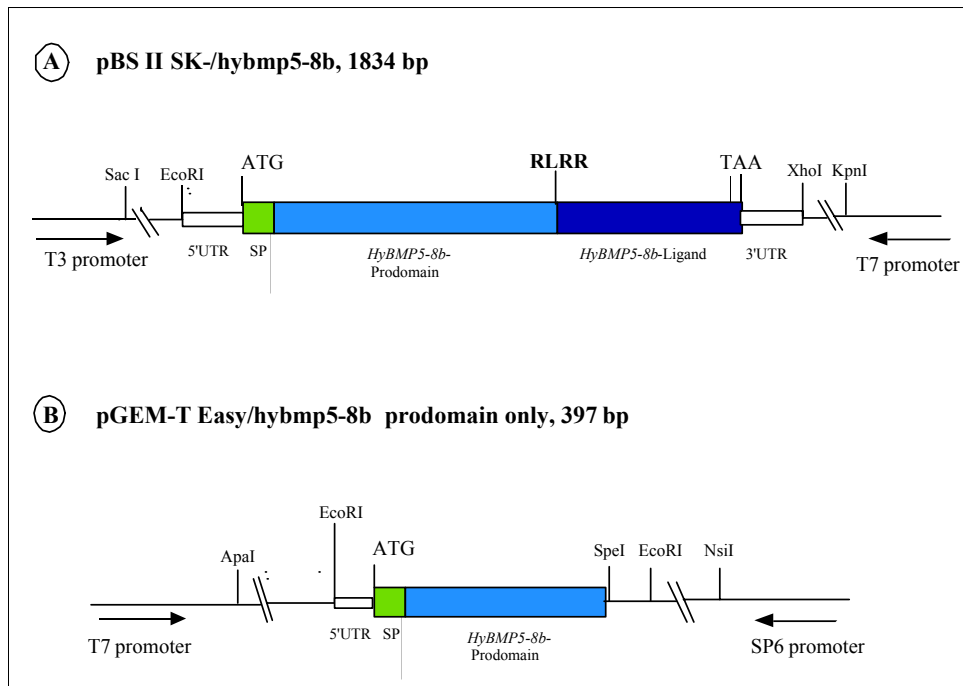


Fig. 3.3. Structure of the *HyBMP5-8b* clones used for preparing digoxigenin labeled RNA probes. The figure also shows the location of the clones in the MCS of their respective vectors. (A). Full length clone of *HyBMP5-8b* obtained in the library screen in pBS II SK⁻. RLLR: tetrabasic cleavage site of *HyBMP5-8b*, SP: signal peptide (B). 397 bp of the prodomain region of *HyBMP5-8b* cloned into the MCS of pGEM-T Easy. SP: signal peptide

Plasmids were linearized with the appropriate restriction enzyme 5' or 3' of the cDNA region of interest, to prepare sense or antisense RNA probes, respectively. The digested template DNA was examined on a gel to confirm that the cleavage was complete, phenol/chloroform extracted, precipitated with 1/10 3M Na-acetate pH 5.2 and 2 Vol 100% Ethanol and resuspended in DEPC-H₂O. The *in vitro* digoxigenin labeling of RNA probes was performed according to the Roche protocol in a 20 μ l

reaction. After the *in vitro* transcription reaction, the digoxigenin labeled RNA was precipitated by adding 30 μ l DEPC-water and 25 μ l LiCl solution (7.5 M LiCl, 50 mM EDTA in DEPC-water). Sense RNA probes were used in the initial *in situ* Hybridizations to confirm that the staining patterning obtained with the antisense RNA probes was specific.

3.2.3.3.2. Hybridization procedure on whole mounts

All *Hydra* polyps were starved at least one day prior to fixation. Animals were relaxed in 2% urethane for 60-120 seconds in HM and fixed overnight at 4°C in 4% paraformaldehyde in HM. Samples were subsequently treated with ethanol and proteinase K to facilitate diffusion of the probes into the epithelial bilayer. The proteinase K digest was followed by a treatment with acetic anhydride to neutralize the positively charged proteins located on the tissue surface. This prevents unspecific binding of the riboprobes to NH_2^+ groups of tissue surface proteins that were exposed by the proteinase K treatment. To stabilize digested tissues, samples were then re-fixed with 4% paraformaldehyde. The fixative was removed by several 5 minute washes with PBT, after which the samples were heated to 80°C for 20 min to inactivate endogenous alkaline phosphatases.

Samples were then prehybridized in hybridization solution at 55°C to block nonspecific hybridization sites. This was followed by a 1.5 day hybridization at 55°C with the digoxigenin-labeled RNA probe, used at a concentration of approximately 0.4 ng/ μ l, and a subsequent wash in hybridization solution and 2xSSC at 55°C. Samples were then washed in MAB and pre-blocked in MAB-B for 2 hours. Alkaline phosphatase-conjugated anti-digoxigenin Fab fragments were diluted 1:400 in MAB-B and preabsorbed for at least 2 hours against fixed *Hydra* polyps before being used. The preabsorbed Fab fragments were diluted to a final concentration of 1:2000 and applied to the samples over night at 4°C. Animals were then washed eight times for one hour in MAB at room temperature and kept overnight in MAB. Then, the specimens were washed three times briefly in alkaline phosphatase buffer (NTMT). Samples were put in BM Purple AP substrate solution (Roche) for generation of a color reaction at 37°C. Specimens were lastly dehydrated with ethanol and mounted in Euparal.

3.2.4. *Xenopus* injection experiments

3.2.4.1. Cloning of different injection constructs

pCS2⁺ is a multipurpose expression vector. Although originally designed for expressing proteins in *Xenopus* embryos from either injected RNA or DNA, pCS2⁺ is also useful for high-level transient expression in a wide variety of mammalian and avian cells. pCS2⁺ contains a strong enhancer/promoter (simian CMV IE94) followed by a polylinker and the SV40 late polyadenylation site. An SP6 promoter is present in the 5' untranslated region of the mRNA from the sCMV promoter, allowing *in vitro* RNA synthesis of sequences cloned into the polylinker. The vector backbone is from pBluescript II KS⁺ and includes the ampicillin resistance gene and a fl origin for producing single stranded DNA. A map of the pCS2⁺-vector including the restriction sites of polylinker 1 and polylinker 2 is shown in Figure 3.4. All *HyBMP5-8a* and *HyBMP5-8b* constructs used for microinjection into *Xenopus* embryos were cloned into the polylinker 1 of pCS2⁺.

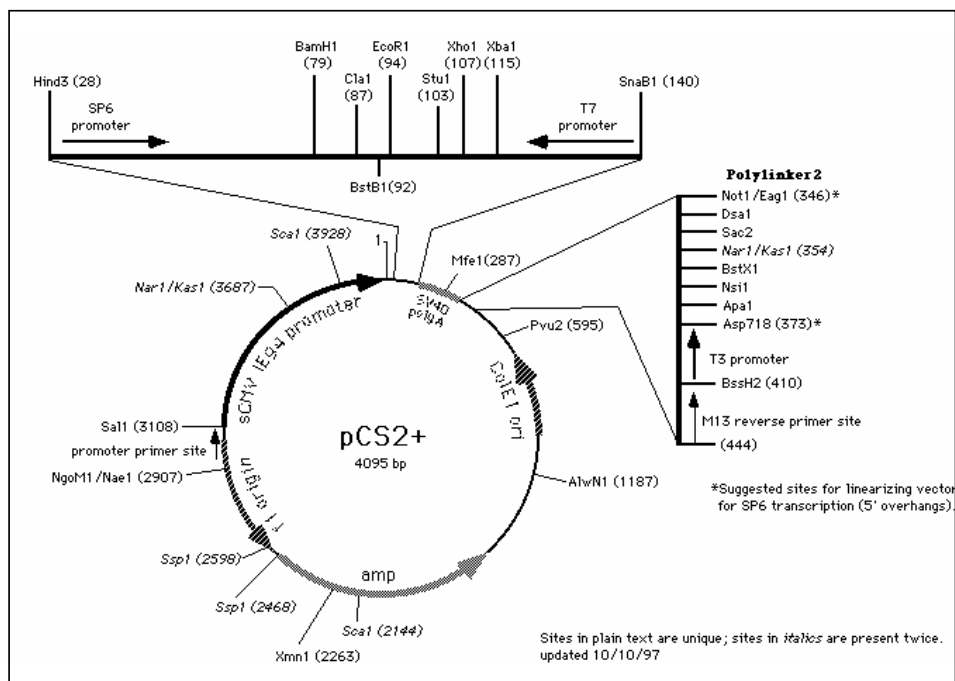


Fig. 3.4. pCS2⁺ vector map (<http://sitemaker.med.umich.edu/dlturner.vectors>), polylinker 1 contains the SP6 and T7 promoter

A) Full length *HyBMP5-8b* in pCS2⁺ (pCS2⁺/hybmp5-8bFL)

The entire ORF of *HyBMP5-8b* was amplified by PCR using the oligonucleotides *exopcs25* and *exopcs23*. The PCR product was subcloned into the restriction sites EcoRI and XhoI of polylinker 1 of pCS2⁺ by exonuclease III digestion (see 3.2.1.5.3. and Li and Evans, 1997) and sequenced. The structure of the construct is shown in Figure 3.5.

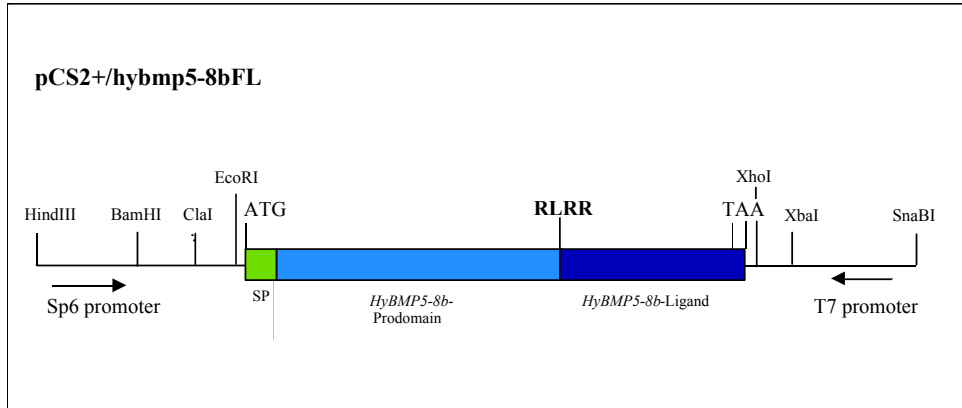


Fig. 3.5. Structure of the construct hybmp5-8bFL and location in polylinker 1 of pCS2⁺. SP: signal peptide of *HyBMP5-8b*; RLRR: tetrabasic cleavage site of *HyBMP5-8b*.

B) Full length *HyBMP5-8b* minus signal peptide in pCS2⁺ (pCS2⁺/BM40-hybmp5-8b)

In order to maximize secretion of the *HyBMP5-8b* protein in *Xenopus* embryos, the *HyBMP5-8b* coding sequence minus its signal peptide was inserted in frame into pCS2⁺/BM40-Xtwg (Scott et al., 2001). This pCS2⁺ vector construct carries the *Xenopus* twisted gastrulation sequence coupled to the 20 amino acid signal peptide sequence region of human BM-40, an extracellular matrix protein BM-40/osteonectin/SPARC (Kohfeldt et al., 1997). The signal peptide sequence consisting of the first 22 bp of *HyBMP5-8b* was predicted by using the search program at <http://www.cbs.dtu.dk/services/SignalP/>, (Nielsen et al., 1997). The pCS2⁺/BM40Xtwg construct was linearized with the restriction endonucleases NheI and XhoI to eliminate all *Xtwg* sequence, followed by phenol/chloroform extraction and ethanol precipitation. The entire ORF of *HyBMP5-8b* minus the 22 amino acids signal peptide sequence was amplified using the oligonucleotides *NheI5'hybmp7b* and *XhoI3'hybmp7b*. The PCR-

product was purified, digested with the restriction endonucleases *NheI* and *XhoI*, purified again using Qiaex II (Qiagen) and ligated using T4 DNA-Ligase in frame into the linearized pCS2⁺ vector containing the BM40 signal peptide sequence. The structure of pCS2⁺/BM40-hybmp5-8b is shown in Figure 3.6.

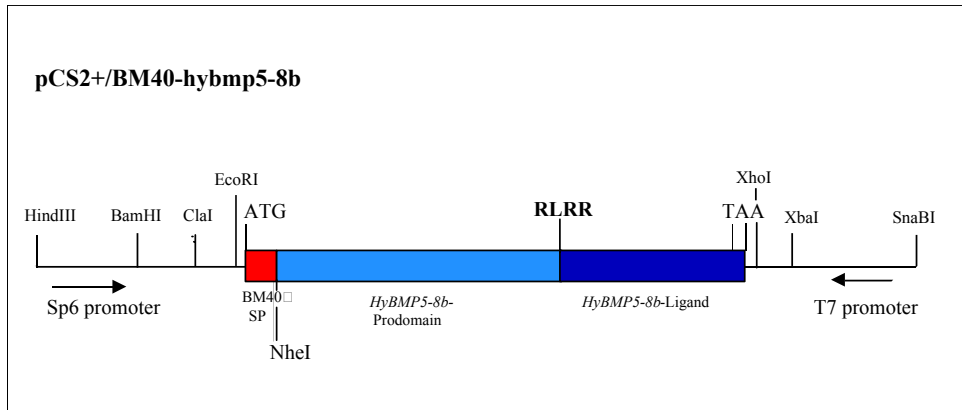


Fig. 3.6. Structure of the construct BM40-hybmp5-8b and its location in polylinker 1 of pCS2⁺. BM40-SP: signal peptide of human BM40; RLRR: tetrabasic cleavage site of *HyBMP5-8b*

C) Xactivin-hybmp5-8a and Xactivin-hybmp5-8b fusion proteins in pCS2⁺

In order to construct activin/*HyBMP* fusion genes, the mature ligand region, starting with the first amino acid after the cleavage site of *HyBMP5-8a* and *HyBMP5-8b* was amplified by PCR using two different sets of oligonucleotides and a proofreading DNA Polymerase (Pfx). Oligonucleotides used for *HyBMP5-8a* were *exoligandaA5'* and *exoligandaA3'*. Oligonucleotides used for *HyBMP5-8b* were *exoligandB5'* and *exoligandB3'*. The purified PCR products were inserted into with *StuI* and *XhoI* linearized pCS2⁺-Xactivin/FLAG-dnXBMP1 cDNA (Blitz et al., 2000). This pCS2⁺ construct consists of the signal peptide, prodomain and tetrabasic cleavage site of *Xenopus* activin followed by a FLAG-tag and the protease region of *Xenopus* BMP1 and EGF repeats of *Xenopus* BMP1.

The 3' end of pCS2⁺/FLAG-dnXBMP1 encompassing the protease region of BMP1 and its EGF repeats was eliminated by digesting the vector with the restriction enzymes *StuI* and *XhoI*. The PCR products encompassing the mature ligand region of *HyBMP5-8a* and *HyBMP5-8b*, were ligated in frame into the linearized pCS2⁺-

dnXBMP1 by exonuclease III digestion (see 3.2.1.5.3. and Li and Evans, 1997). The activin-HyBMP5-8a and activin-HyBMP5-8b chimeras contain the *Xenopus* activin signal peptide, the *Xenopus* activin prodomain and the *Xenopus* activin tretrabasic cleavage site. This segment is followed by mature peptides of *HyBMP5-8a* or *HyBMP5-8b* starting from the 36th and 33rd amino acids before the first cysteine residue, respectively. The structure of those two chimera constructs is shown in Figure 3. 7.

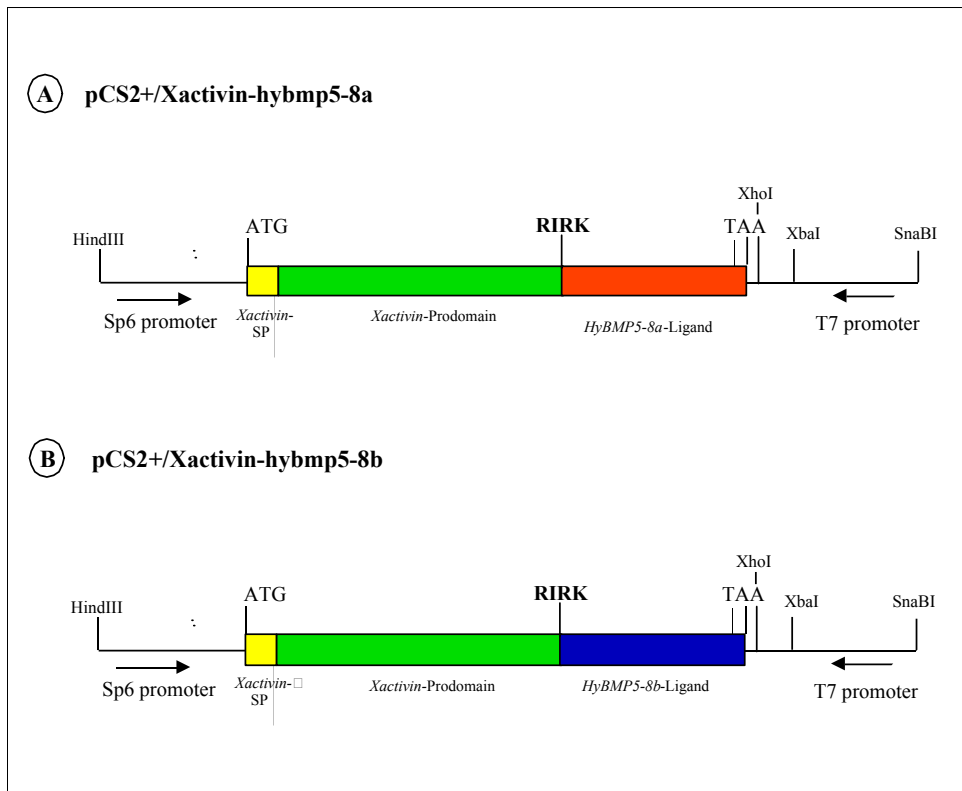


Fig. 3.7. Structure of Xactivin-HyBMP5-8a (A) and Xactivin-HyBMP5-8b (B) and location in polylinker 1 of pCS2⁺, note: several restriction enzyme sites at the 5' end of the polylinker have been eliminated. RIRK: tetrabasic cleavage site of *Xenopus* activin. SP: signal peptide of *Xenopus* activin.

3.2.4.2. *Xenopus* injection assays

Xenopus laevis females were injected with 600 units of human chorionic gonadotropin. *Xenopus* eggs laid in high salt modified Barth solution (MBS) were fertilized *in vitro*.

They were dejellied using 2% cysteine (pH 7.8), washed, and transferred to 0.1xMBS (Gurdon, 1976) in a petri dish with 1% agarose bottom.

Embryonic stages were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Capped polyadenylated synthetic *HyBMP5-8a* and *HyBMP5-8b* mRNAs were prepared by linearizing pCS2⁺ constructs (3.2.3.4.1. A,B or C) with NotI and using the mMessage mMachine kit (Ambion) for *in vitro* transcription. It has been observed that capped SP6 mRNAs from pCS2⁺ which include the SV40 polyA site at the 3' end (DNA templates linearized at polylinker II) are more stable than conventional capped RNAs when injected into *Xenopus* embryos. In addition, these mRNAs produce substantially more protein than conventional mRNAs (10X or more in many cases, R. Rupp, L. Snider, and D. Turner, unpublished). These effects appear to reflect polyadenylation of the injected pCS2⁺ mRNA.

RNA concentration and RNA quality was analyzed by spectrophotometry and electrophoresis. Embryos were microinjected in 1xMBS and thirty minutes after microinjection transferred and maintained in 0.1xMBS. Two-cell to four-cell embryos were microinjected with *in vitro* synthesized mRNA as described (Wright et al., 1989). Animal caps were dissected in 1xMBS using watchmaker's forceps at blastula stages 8 to early 9 and maintained in 0.3xMBS until sibling control embryos reached tailbud stages.

3.3. Immunohistochemical procedures

3.3.1. Whole mount staining with anti-P-Smad1 antibody

Activation of BMP signaling in adult *Hydra* polyps was detected by whole mount immunocytochemistry using the anti-phospho-Smad 1 antibody (PS1) (Faure et al., 2000) (kindly provided by Peter ten Dijke) following a procedure described by L.Javois (Javois et al., 1988), modified as below. All *Hydra* polyps were starved at least one day prior to fixation. Animals were relaxed in 2% urethane in HM for 60-120 seconds and fixed overnight at 4°C in 4% paraformaldehyde in HM. Samples were subsequently treated with methanol and PBS to facilitate diffusion of the antibody in the tissue. Following heating in an 80°C waterbath for 30 minutes (to destroy endogenous alkaline phosphatase activity), the samples were washed with PBS and unspecific protein

sticking was blocked by incubating the samples for 1 hour at room temperature in PBS containing 10% neonatal calf serum (blocking solution).

The samples were then incubated at 4°C over night with the primary antibody, PS1 (1:5000 in blocking solution, containing 100 µg/ml KLH). Following which they were washed four times in PBS and incubated for 1 hour in anti-rabbit IgG (Fc)-conjugated alkaline phosphatase diluted 5000-fold in blocking solution. Following four washing steps in PBS, the samples were rinsed several times in alkaline phosphatase buffer (NTMT). To visualize PS1 labeling the samples were incubated at room temperature in the alkaline phosphatase substrate solution (200 µl of NBT/BCIP stock solution in 10 ml NTMT). After the color reaction had developed (~30 minutes), the reaction was terminated, by transferring the samples through three changes of stop solution (Rgt-buffer). The samples were lastly dehydrated with ethanol and mounted in Euparal.

3.4. Database Searches and Phylogenetic Analysis

3.4.1. Database Searches

Database searches were carried out by using the BLAST server at the National Center of Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>)

3.4.2. Phylogenetic Analysis

Amino acid sequences of the TGF-β superfamily gene products were aligned using the multiple alignment program ClustalX (Thompson et al., 1997). A diverse sampling of TGF-β genes was used to provide a thorough search for potentially closely related genes. 161 confidently aligned sites of the C-terminus of the selected genes were analyzed. Phylogenetic analysis was performed using the program *Puzzle* (Strimmer and von Haeseler, 1996), a maximum likelihood based method applying quartet puzzling. The Dayhoff (Dayhoff et al., 1978) method was used as a substitution model and, because of blocks of high conservation within the sequence, I assumed a rate heterogeneity (mixed + 8 gamma rates) along the sequence. The required parameter alpha of the gamma distribution was estimated by the program from the data set and

found to be 1.08. 1000 replicate analyses were performed. Branch lengths correspond to the number of changes. Numbers at each node correspond to the percentage of replicates in which the node was supported, a measure of statistical support (Strimmer and von Haeseler, 1996).

An analysis using the maximum parsimony program *PAUP 4.0b10 2* (Swofford, 1998) yielded basically the same tree topology. The heuristic search option of *PAUP 4.0b10* was used, with 500 random sequence taxon addition replicates along with tree bisection and reconnection branch swapping (TBR). 4 different trees were obtained. The robustness of the branches of the obtained trees was assessed for the consensus tree by applying the bootstrap/jackknife analysis in PAUP. 100 bootstrap replicates were performed using random taxon addition with tree bisection and reconnection branch swapping (TBR) in the heuristic search option.

4. Results

4.1. Isolation of two BMP orthologues from *H. vulgaris*

4.1.1. Isolation of *HyBMP5-8a*

A *Hydra* orthologue of BMP genes was amplified using degenerate oligonucleotides designed against the second β -strand (*dppf1*) and the beginning of the α -helix (60Afl) of vertebrate BMP genes (Fig. 4.3). A single PCR product was recovered by using these degenerate oligonucleotides in a splinkerette PCR (Devon et al., 1995) on *Hydra* genomic DNA (step one, Fig. 4.1). Sequence analysis of the cloned PCR product showed sequence similarity to bilaterian BMP genes. Based on the sequence of the predicted protein, the fragment was designated *HyBMP5-8a*. Additional sequence was obtained using the splinkerette method (step 2 and 3, Fig. 4.1) and 3'RACE-PCR (step 4, Fig. 4.1).

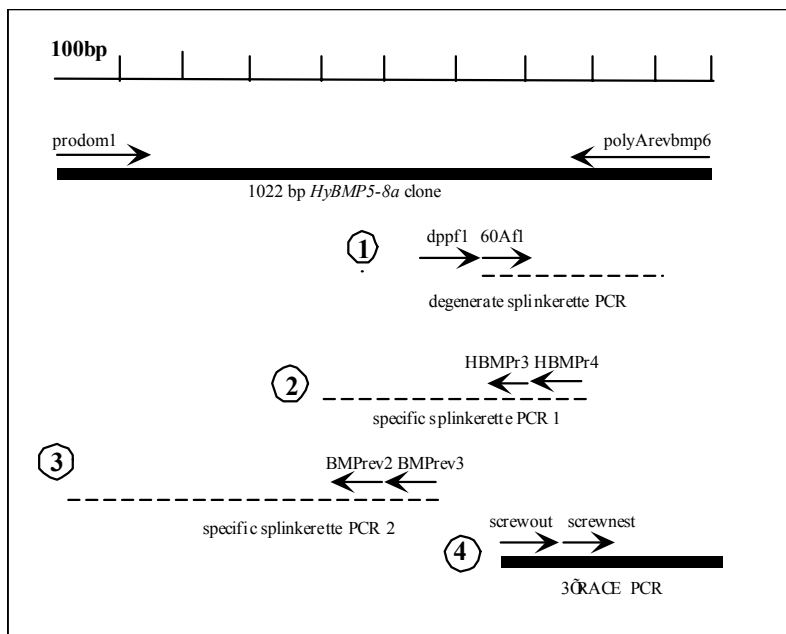


Fig. 4.1. Schematic representation of the strategy to characterize the *HyBMP5-8a* cDNA fragment. (1) Initial degenerate splinkerette PCR. (2)/(3) Splinkerette PCR using specific primers to identify further 5' sequence. (4) 3' RACE. Arrows: primers used. Full lines: cDNA. Dotted lines: genomic DNA.

To confirm the putative sequence of *HyBMP5-8a*, primers designed to the extreme ends of the sequence were used with a proof-reading polymerase (Pfx) to amplify the gene from cDNA. The *HyBMP5-8a* cDNA sequence comprises 1022 bp including a single open reading frame of 888 bp, but lacks a start methionine suggesting the presence of an intron.

4.1.2. Isolation of *HyBMP5-8b*

In order to identify additional BMP genes a cDNA library derived from regenerating *Hydra* heads (Deutzmann et al., 2000), was screened using a cDNA fragment spanning the *HyBMP5-8a* mature ligand (Fig. 4.3). Following the secondary screen, several positive clones were sequenced and shown to be derived from the same gene. The predicted open reading frame (ORF) for this gene was 404 amino acids in length and had sequence similarity to BMP genes. However, the gene displayed only 80% similarity to the initially isolated *Hydra* BMP gene. Therefore, I designated the full-length clone as *HyBMP5-8b*.

4.1.3. Sequence analysis of *HyBMP5-8a* and *HyBMP5-8b*

Using splinkerette PCR and 3'RACE resulted in 1022 bp sequence for *HyBMP5-8a* with a 5' truncated single open reading frame (ORF). This sequence included a C-terminal ligand region of 102 amino acids typical of a BMP protein, a conserved maturation BMP cleavage site (RFTR) and most of the prodomain (Fig. 4.2).

The *HyBMP5-8b* cDNA contains a single ORF of 1215 nucleotides that encodes a protein of 404 amino acids. *HyBMP5-8b* displays all conserved features of BMP family genes (Fig. 4.2) with a 22-amino-acid signal peptide for secretion at the N-terminus, predicted by using the search program at <http://www.cbs.dtu.dk/services/SignalP/> (Nielsen et al., 1997). Following the signal peptide is a 243-amino-acid prodomain that is flanked by a conserved BMP maturation cleavage site (RLRR). The 102-amino-acid region in the -terminus is typical for BMP ligands (Fig. 4.2).

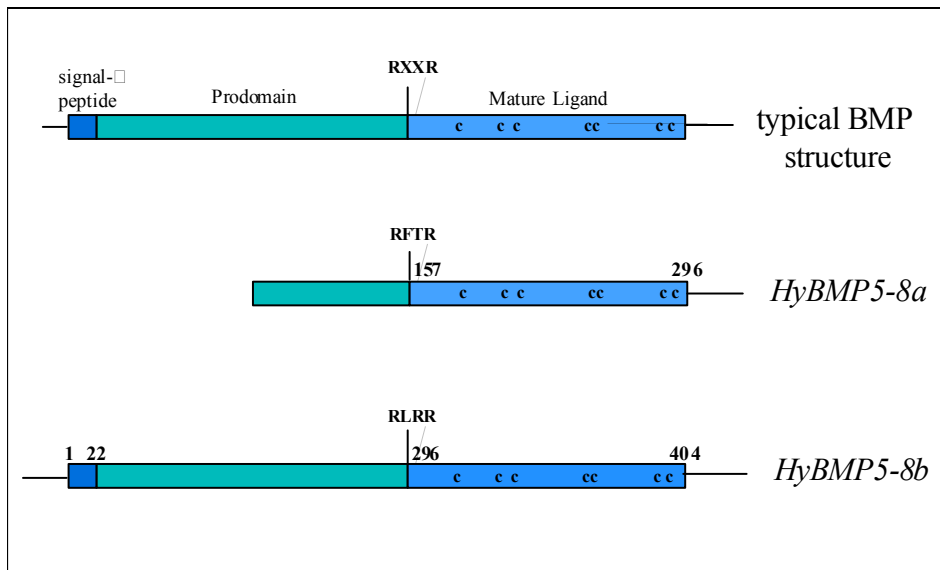


Fig. 4.2. Schematic drawing of the predicted *HyBMP5-8a* and *HyBMP5-8b* proteins. The three regions correspond to the signal peptide, the prodomain, the mature ligand region and the location of the tetrabasic cleavage sites RLRR and RFTR, respectively, which conform to the consensus RXXR cleavage signal for proteolytic processing of BMP signaling proteins. Numbers specify amino acid positions.

The positively charged residues RFTR at position 154-157 of the *HyBMP5-8a* amino acid sequence and RLRR at position 266-269 of the *HyBMP5-8b* amino acid sequence conform to the bilaterian BMP ligand consensus sequence RXXR, which acts as a cleavage signal for proteolytic processing by subtilisin like proteases (Creemers et al., 1993; Molloy et al., 1992). Furthermore, the location of the tetrabasic sequences 36 or 33 amino acids further N-terminal of the first conserved cysteine residue, makes them very likely cleavage sites for protein maturation (Fig. 4.3). This shows BMP processing to form active signaling molecules is basal to diploblasts and triploblasts.

```

CLUSTAL X (1.81) multiple sequence alignment

HyBMP58b      MKVTLLSIIRLIVYVFNVFNVIPTKERNIIQNTLLDLFGKHNRPRPDVFSHLGRNETSA
HyBMP58a      -----

HyBMP58b      KKYMLDLYEYSVNTERQGMNFTTNSQNISINIVDDADTVVSFLNNAYVVRPNTTDESGEM
HyBMP58a      -----

HyBMP58b      FFDVSSNYVVEKVLATALQVYLDITYKTVIAEKLIISVYKIVVPKKKYVLLASKLINASV
HyBMP58a      -----

HyBMP58b      SQWHEFNVLEASLSWIEFSETNNGILLVCQNLQKVNIPIESCGIVDFKGREEFRPFLVSF
HyBMP58a      -QWHEFNVLEAMLSWVKYSETNNGILIVCKSLQQEAILVENCVVGFKGSKDYQPFLVSF
*****      ***::*****:*.**: * :*.**:*.*** :::*****

HyBMP58b      YQSGKEEEFPAKQIRNTEST-----LKLQERLRRSMQDSIFIEAAE
HyBMP58a      YQSSKNEEFFALKAPDFDSTSRLAQHQYFLDNKFKTSKSARLPKRFTRGIEDDDFLKAAA
**.*:*** * : ::** * :*: *.:*.* **:*

HyBMP58b      QLS----FVRNKTRNSGENNLGSRCDKHPLYIGFKDLGWSDWIIAPDGYRANCYGGDCS
HyBMP58a      TLAEKNNFVQSKLISKRFKNNNISDCGKHLLYVSFKDIGWSDWIIAPDGYITSYCGGDCS
*:      **:* * : ::** * *.* **:*.*:*****:***** :.*****

HyBMP58b      FPLDNNANATNHAIQTLVHMYPEIIPKPCCAPNKLNTLQVFLDERNNVVMKRYSNMI
HyBMP58a      YPLESNLNATNHAILRALVHTIFPKKIPKPCCAPNKLNAMSILFFDDRNNVVMQEI PNMI
:*. * *****:*** :*: *****:..**:*:*****:..**

HyBMP58b      VQHCQCQ
HyBMP58a      VHQCQCQ
*.:****

```

Fig. 4.3. Alignment of the predicted amino acid sequences of the two *Hydra* BMP5-8 genes with each other. The residues within them that are conserved between HyBMP5-8a and HyBMP5-8b are marked by asterisks.

The C-terminal amino acid region of both *HyBMP5-8a* and *HyBMP5-8b* contains seven invariant cysteine residues characteristic of BMP signaling peptides (Fig. 4.4), necessary for dimerization and formation of the characteristic TGF- β cysteine-knot structure. Sequence comparison with other BMP orthologues shows that both *HyBMP5-8a* and *HyBMP5-8b* are highly conserved over the mature ligand region.

The genes show closer similarity to each other (82% identity) than to deuterostome BMP5-8 genes and the *Drosophila* 60A gene (58-65% identity). Both *HyBMP5-8a* and *HyBMP5-8b* show a lower degree of similarity to BMP2/4 genes, with 47-52% identity, suggesting they are more closely related to BMP5-8 genes.

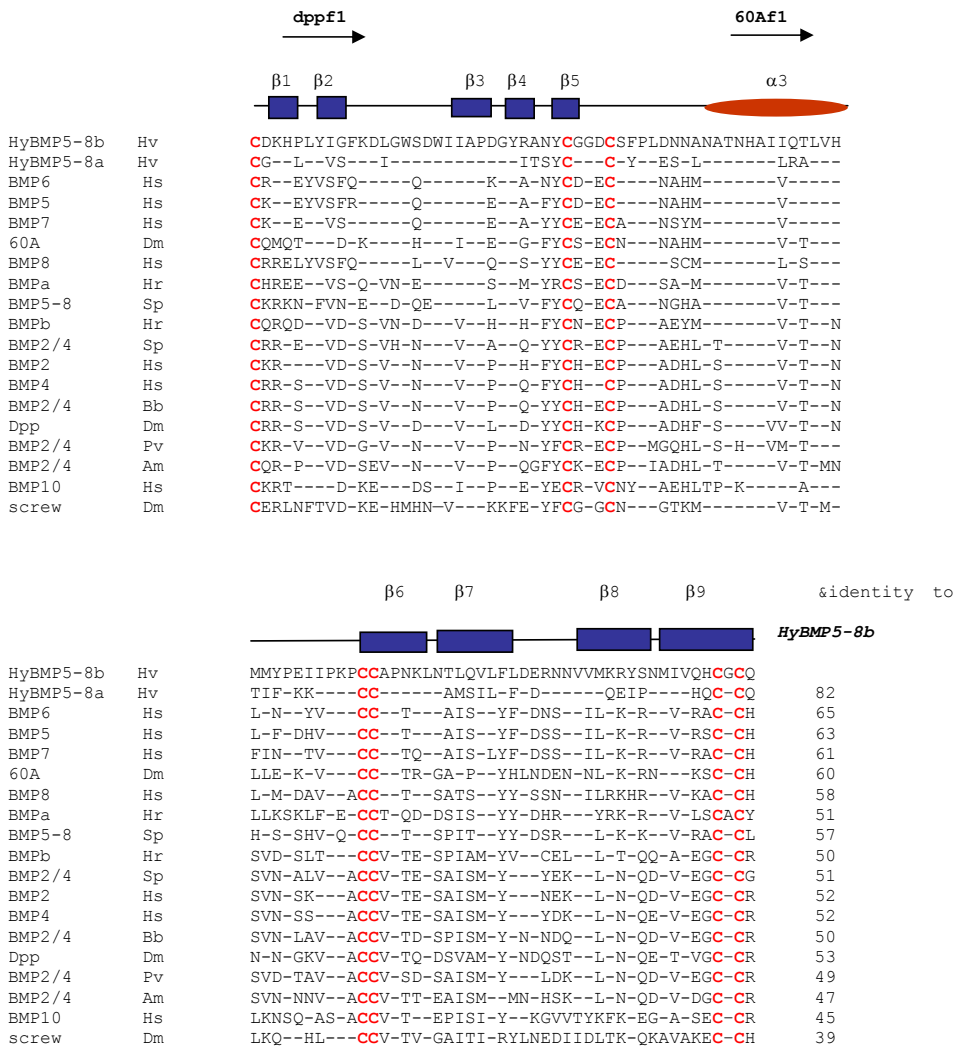


Fig. 4.4. Comparison of the C-terminal region starting with the first conserved cysteine residue of *HyBMP5-8a* and *HyBMP5-8b* with other members of the BMP subfamily of TGF- β signaling molecules. The various sequences are displayed in order of identities shared with *HyBMP5-8b*. Horizontal lines indicate amino acid residues identical to *HyBMP5-8b*. The seven characteristic cysteine residues are indicated in bold red letters.

Comparing the prodomains of *HyBMP5-8a* and *HyBMP5-8b* against the nonredundant database at NCBI using BLAST, showed low conservation over the prodomain. The closest match to the prodomains of *HyBMP5-8a* and *HyBMP5-8b* was

the prodomain of the human BMP7 protein with 26% identity. Proteins of the BMP2/4 group on the other hand displayed approximately 23% identity. This low degree of conservation over the prodomain is a common feature of the BMP family (Scheufler et al., 1999).

The complete nucleotide sequence and predicted amino acid sequences of *HyBMP5-8a* and *HyBMP5-8b* can be found in chapters 8.2. and 8.3. of the Appendix.

4.1.4. Phylogenetic Analysis

To clarify the relationship of *HyBMP5-8a* and *HyBMP5-8b* to other BMP genes, phylogenetic analyses were performed using maximum parsimony and maximum likelihood methods. 102 amino acids of the C-terminal region of the two *Hydra* genes starting from the first conserved cysteine were aligned with related TGF- β proteins chosen by degree of similarity from the Blast search (Fig. 4.4). Both maximum parsimony and maximum likelihood (Fig. 4.5) strongly supported the grouping of *HyBMP5-8a* and *HyBMP5-8b* with the vertebrate BMP5-8 genes and the *Drosophila* 60A gene. But neither analyses resolved the relationship of the *Hydra* BMP genes to the other BMP5-8 genes (Fig. 4.5).

This was as the 102-amino-acid-C-terminal region used in the alignment is highly conserved between all BMP family members and did not provide enough information to resolve the relationship. Therefore, additional stretches of conserved amino acid residues in other regions of the TGF- β proteins were aligned in order to provide more resolution.

The amino acid sequence immediately N-terminal to the conserved ligand region showed enough similarity for a convincing alignment using ClustalX and editing by eye. This generated an alignment consisting of a part of the prodomain, the cleavage site and the complete mature ligand domain (for alignment see chapter 8.4. of the Appendix).

Phylogenetic trees constructed using the extended alignment confirmed that *HyBMP5-8a* and *HyBMP5-8b* are closest to BMP5-8 genes as indicated by the level of statistical support (Fig. 4.6, Fig. 4.7).

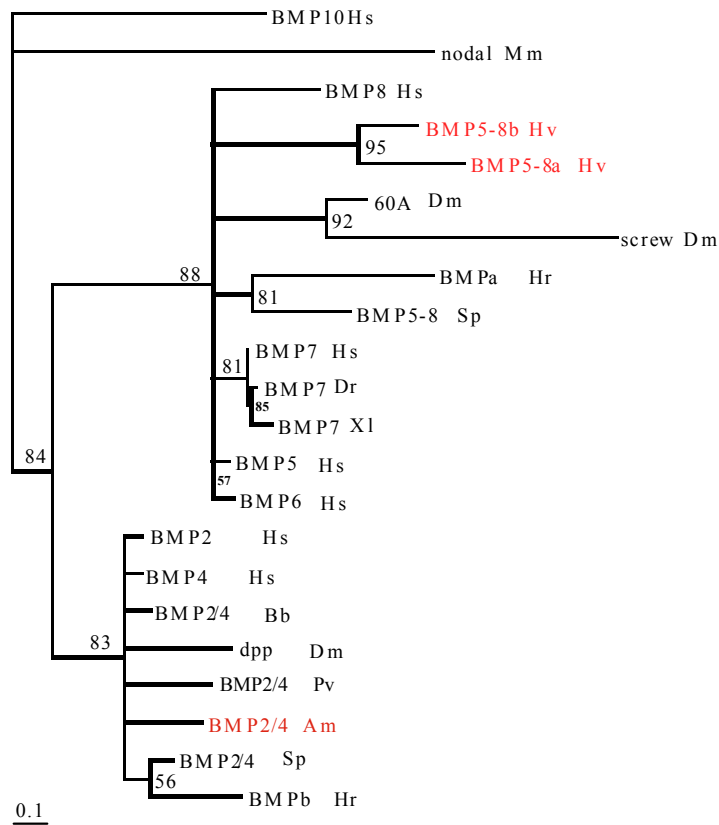


Fig. 4.5. Maximum likelihood tree of the BMP gene family and *nodal* constructed from the amino acid sequences of the ligand region of BMP genes (starting with the first conserved cysteine) using quartet puzzling. The tree includes sequences of *Hydra vulgaris* (Hv), *Acropora millepora* (Am), *Drosophila melanogaster* (Dm), *Strongylocentrotus purpuratus* (Sp), *Halocynthia roretzi* (Hr), *Branchiostoma belcherii* (Bb), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Homo sapiens* (Hs) and *Mus musculus* (Mm). Numbers at the nodes are percentage quartet puzzling support values and the scale indicates maximum likelihood branch lengths. *Nodal* was chosen as the outgroup.

Both the maximum likelihood and maximum parsimony analysis show that the *Hydra* sequences are equally related to all BMP5-8 genes (Fig. 4.6, Fig. 4.7). These trees also show that both genes originated in the *Hydra* lineage following the divergence of cnidarians and bilaterians by a gene duplication event making both these genes equally related to all bilaterian BMP5-8 family genes.

Phylogenetic trees generated from these alignments were unable to resolve the relationship between BMP5-8 and BMP2/4 genes. BMP10 and GDF2 were used as

outgroups to the BMP5-8 genes due to the high degree of similarity shown by sequence alignment. Trees generated were unable to resolve the relationships of these to BMP5-8 and BMP2/4 genes but suggest the gene duplication event giving rise to BMP2/4 and BMP5-8 genes occurred prior to the divergence of Cnidaria and Bilateria.

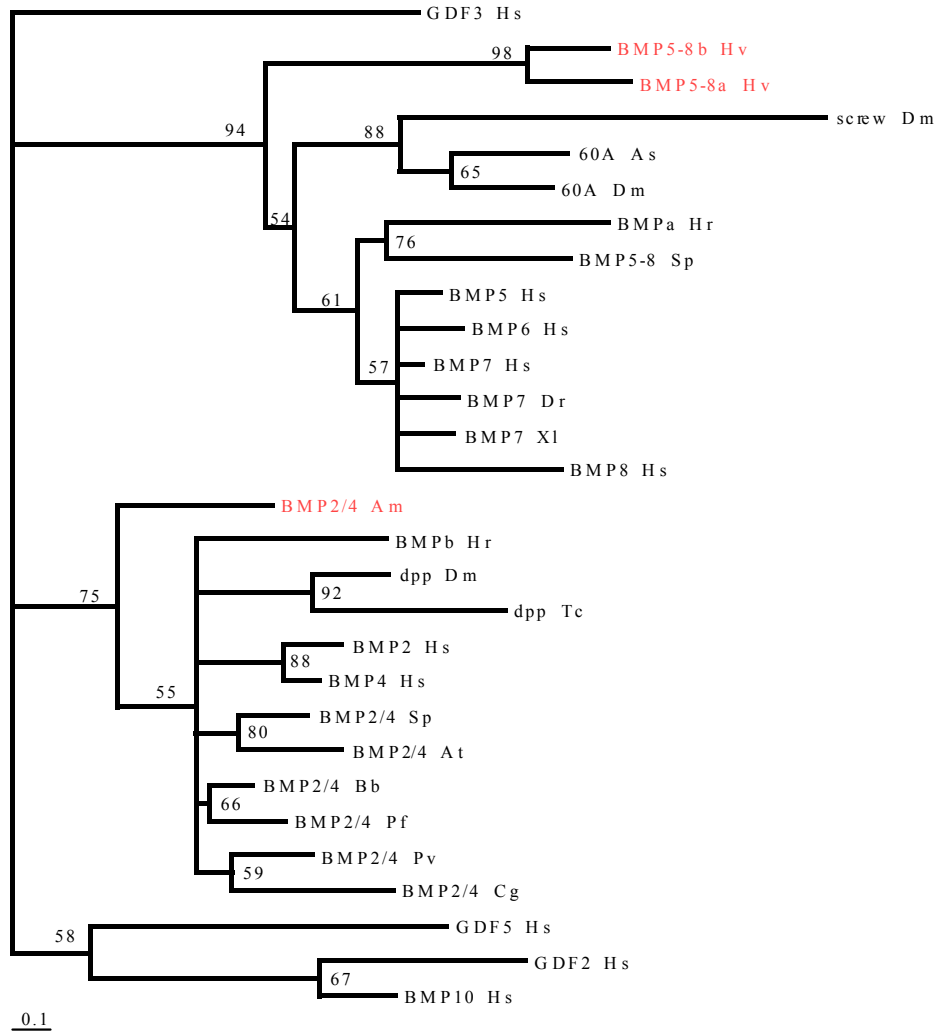


Fig. 4.6. Maximum likelihood tree of BMP genes constructed from an alignment of 163 residues over the C-terminal region by quartet puzzling using GDF3 as outgroup. The tree includes sequences of *Hydra vulgaris* (Hv), *Acropora millepora* (Am), *Drosophila melanogaster* (Dm), *Anopheles stephensi* (As), *Tribolium castaneum* (Tc), *Patella vulgaris* (Pv), *Crassostrea gigas* (Cg), *Strongylocentrotus purpuratus* (Sp), *Archaster typicus* (At), *Halocynthia roretzi* (Hr), *Branchiostoma belcherii* (Bb), *Ptychodera flava* (Pf), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Homo sapiens* (Hs). Numbers at the nodes are percentage quartet puzzling support values and the scale indicates maximum likelihood branch lengths.

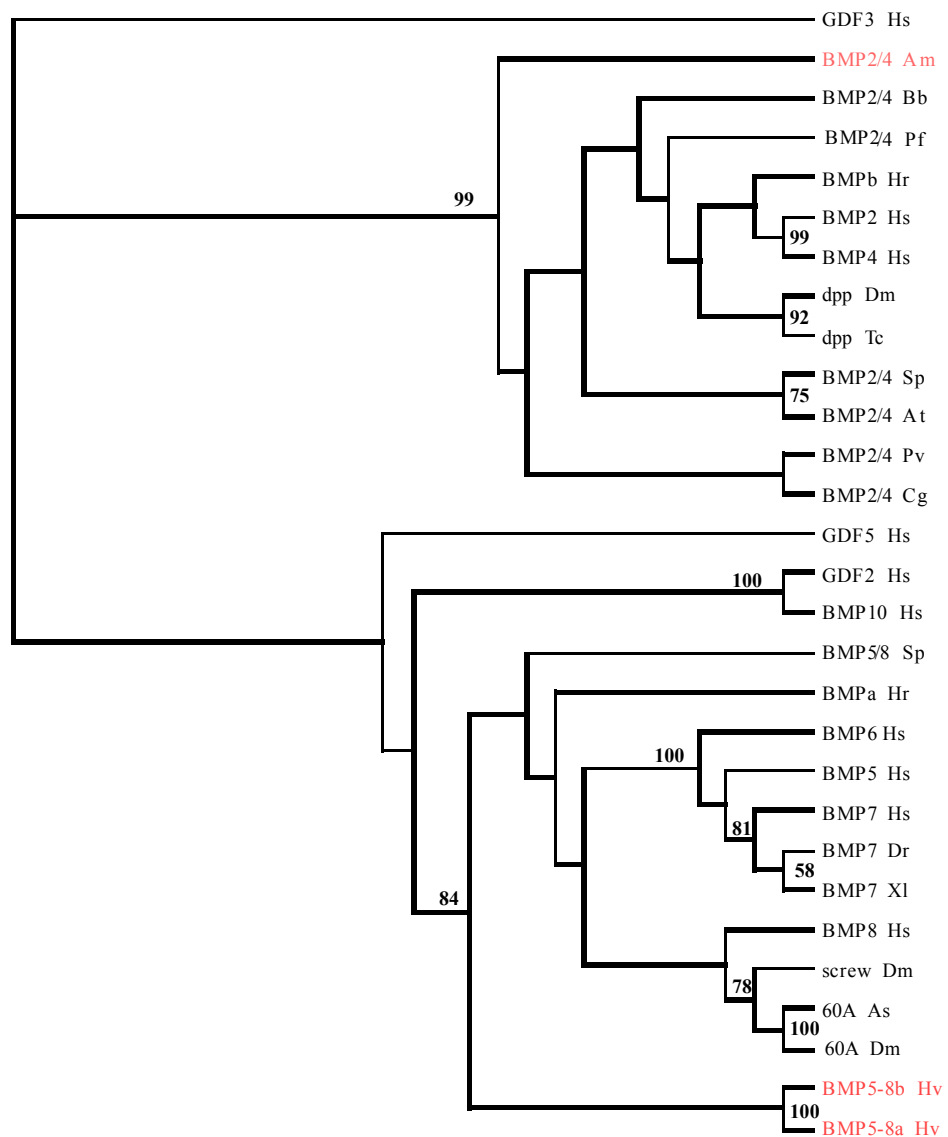


Fig. 4.7. Maximum parsimony tree of BMP genes. An alignment of 163 residues over the C-terminal region was used to generate a tree using the program PAUP 4.2 and GDF3 as outgroup. For species used and gene names see figure 6.

4.2. Southern analysis

In order to determine if there are more copies of BMP related genes in the *Hydra* genome a Southern blot was screened. First it was screened under stringent conditions with a 416 bp cDNA fragment, corresponding to the mature ligand domain of the *HyBMP5-8a* gene, starting from the second amino acid after the tetrabasic cleavage site. The same blot was screened with a 407 bp fragment corresponding to the mature ligand region of *HyBMP5-8b* starting from the first amino acid after the tetrabasic cleavage site. Under the stringent conditions applied only one strong band was detected using the *HyBMP5-8a* probe (Fig. 4.8), indicating that the gene has a single copy in the *Hydra* genome. Using the *HyBMP5-8b* probe, in three of the digests double bands could be detected. This result has two possible explanations: the probe is spanning an intron sequence or there is a second copy of the gene in the *Hydra* genome.

Using less stringent conditions it was possible to detect both genes at once with only one of the probes (data not shown). Additional bands were also visible indicating that there are more distantly related BMP genes in *Hydra*.

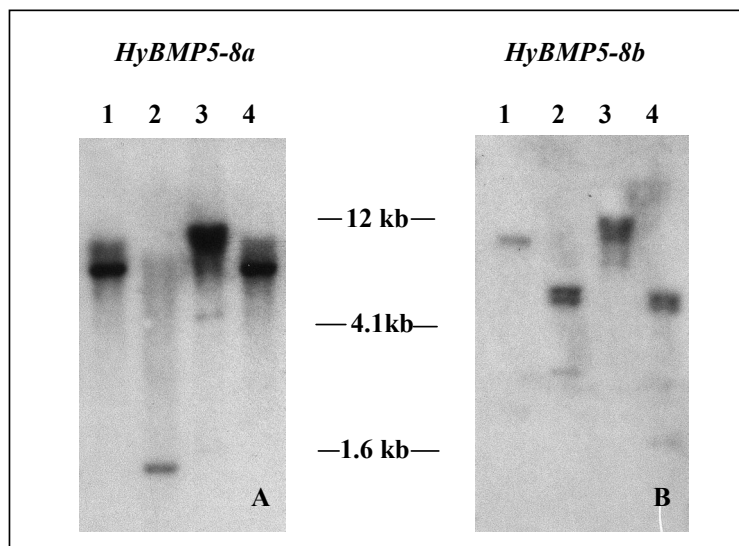


Fig. 4.8. Southern Blot analysis. The same southern blot was probed for *HyBMP5-8a* and *HyBMP5-8b*. 10 μ g of digested genomic DNA was loaded per lane. Restriction enzymes used: (1) EcoRI, (2) EcoRV, (3) XbaI, (4) HindIII.

4.3. Northern analysis

Northern Blot analysis under stringent conditions using the same probes for hybridization, showed that *HyBMP5-8a* and *HyBMP5-8b* each encode a single mRNA species of approximately 2.3 kb (Fig. 4.9). Using 2 µg of poly(A)-RNA preparations, a strong signal was observed applying the *HyBMP5-8b* fragment as a probe. However, under the same conditions the *HyBMP5-8a* gene gave no signal at all. By using an excessive amount of poly(A)-RNA in a second Northern analysis (Fig. 4.9), the *HyBMP5-8a* mRNA was detectable.

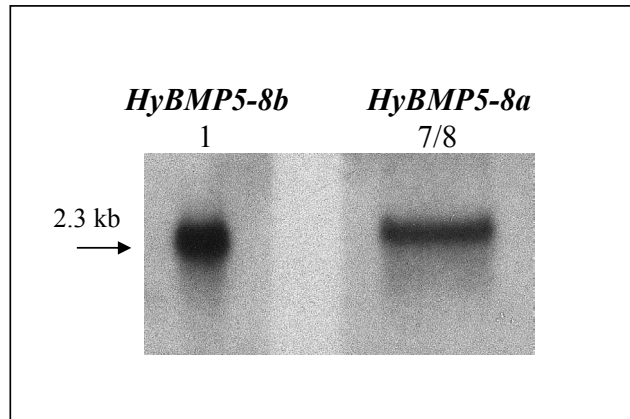


Fig. 4.9. Northern Blot analysis. Lane 1 represents 2 µg of poly(A)-RNA hybridized with the *HyBMP5-8b* fragment, lanes 7 and 8 represent 9 µg of poly(A)RNA hybridized with the *HyBMP5-8a* fragment.

In summary, *HyBMP5-8a* exists as a single copy in *Hydra*. *HyBMP5-8a*, is a less abundant mRNA as *HyBMP5-8b* and this is reflected when performing *in situ* hybridization (see section 4.5.).

4.4. Evolutionary conservation of the function of *HyBMP5-8b*

Several *Hydra* genes introduced into either *Xenopus* or *Drosophila* embryos mimic the activity of the host's orthologue of the gene demonstrating that the function of the gene has been conserved (Broun et al., 1999; Grens et al., 1995; Hobmayer et al., 2000). Similarly, the BMP2/4 orthologue of the coral *Acropora* has dorsalizing activity in *Drosophila* embryos (Hayward et al., 2002) demonstrating that its function as a BMP is

conserved. Since the *HyBMP5-8b* sequence was full length and the structure of the predicted protein strongly resembled that of other BMP5-8 proteins, it was of interest to determine if it was sufficiently conserved to mimic the biological function of its bilaterian homologues.

Xenopus BMP's 2, 4 and 7 induce ventral mesoderm, ventralize dorsal mesoderm and suppress the neuralization of prospective epidermis during early *Xenopus* embryogenesis (Clement et al., 1995; Dale et al., 1992; Jones et al., 1992; Suzuki et al., 1997; Wilson and Hemmati-Brivanlou, 1995). Therefore, we tested whether *HyBMP5-8b* has similar ventralizing activity. Surprisingly, the injection of *HyBMP5-8b* mRNA into *Xenopus* embryos did not promote the development of ventral mesoderm, but led instead to the formation of secondary axes (Fig. 4.10B) as opposed to normally developed control embryos (Fig. 4.10A). Injection of 3 ng of full length *HyBMP5-8b* mRNA resulted in the induction of a secondary axis in 45% of the injected embryos (n=29).

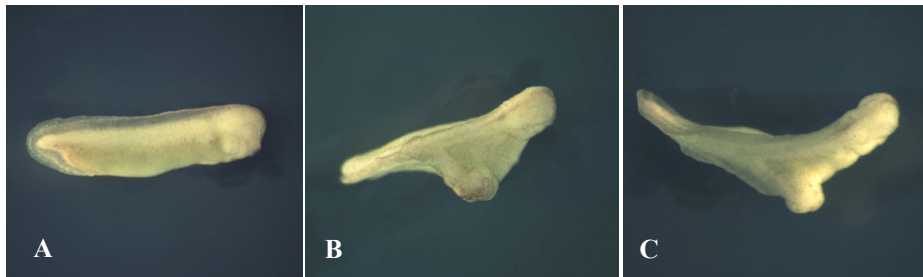


Fig. 4.10. *HyBMP5-8b* mRNA and BM40-*HyBMP5-8b* mRNA induce a secondary axis when injected into wild-type BMP2. Synthetic RNAs were injected into *Xenopus* embryos at the 2-cell stage and embryos developed to tailbud stage. (A) Control. (B) *HyBMP5-8b* (3ng mRNA/injection). (C) BM40-*HyBMP5-8b* (3ng mRNA/injection).

Secondary axes in *Xenopus* embryos can also be induced by ectopically expressing molecules, such as activin or Vg1, that are responsible for the formation of the dorsal mesoderm (Dale et al., 1993; Thomsen et al., 1990; Thomsen and Melton, 1993). To determine whether the formation of the secondary axes induced by *HyBMP5-8b* injection was due to the inhibition of BMP signaling and not due to signaling via an activin/nodal pathway, the behavior of *HyBMP5-8b* was analyzed in dissected ectodermal explants (“animal caps”). Animal caps respond to activin/nodal signaling and undergo elongation movements mimicking mesodermal convergence and

extension (Thomsen et al., 1990), whereas inhibition of BMP signaling in animal caps results in the differentiation of cement glands (an ectodermal adhesive organ) (Hawley et al., 1995). After injection of 3 ng of *HyBMP5-8b* mRNA into the animal pole of four-cell stage embryos, animal caps were isolated at the blastula stage and cultured in saline solution until sibling embryos reached tailbud stages. Uninjected control animal caps differentiated into epidermis (Fig. 4.11A).

Several of the RNA injected cultured animal caps formed ectopic cement glands, as confirmed by the presence of columnarized cells containing pigment granules (30%, n=20; Fig. 4.11B), as well as the secretion of mucus (data not shown). None of them showed signs of elongation movements. This result strongly supports the idea that *HyBMP5-8b* inhibited BMP signaling.

In order to determine if a failure in secretion of the *HyBMP5-8b* protein led to the formation of secondary axes in *Xenopus* embryos by retention of endogenous *Xenopus* BMP precursor proteins, the *HyBMP5-8b* signal peptide sequence was replaced with the sequence of a signal peptide of the human protein BM40. This signal peptide is known to be very efficient for secretion of proteins across different phyla (Mayer et al., 1993; Scott et al., 2001). However, the overexpression of this BM40-*HyBMP5-8b* mRNA led also to the induction of secondary axes (44%, n=25; Fig. 4.10C). Furthermore, the construct seemed to be even more efficient than the wildtype *Hydra* protein in inducing ectopic cement gland formation in dissected ectodermal explants (50%, n=20; data not shown).

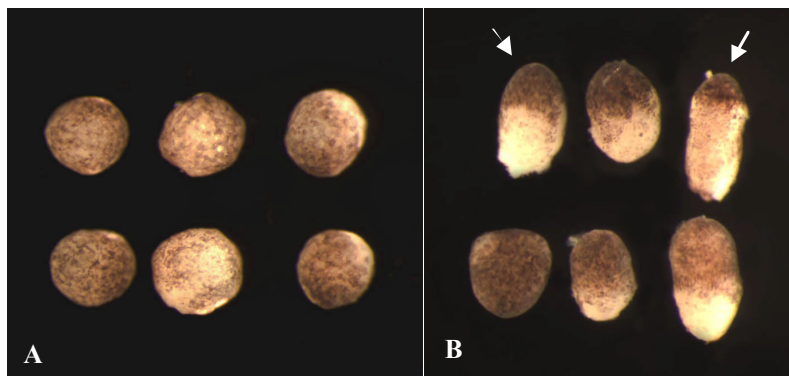


Fig. 4.11. *HyBMP5-8b* induces cement glands in animal cap ectoderm. (A) Control, uninjected control animal caps remain as atypical epidermis. (B) *HyBMP5-8b* (3 ng mRNA/injection), arrows point to cement gland tissue, that is revealed by pigment aggregation. Animal cap explants were excised at blastula stage and cultured until tailbud stage.

The bioactivity of BMP's requires proteolytic cleavage of the pro-BMP precursor following the multibasic motif RXXR to yield the active, carboxy-terminal mature protein dimer (Aono et al., 1995; Constam and Robertson, 1999; Cui et al., 1998). Studies in *Xenopus* show that Vg-1, a critical determinant of dorsal-ventral axis specification, potently induces axial mesoderm in animal cup explants only if fused to a foreign BMP prodomain that allows efficient precursor processing (Dale et al., 1993; Thomsen and Melton, 1993). To attempt to rule out that a failure in processing of *HyBMP5-8b* results in the inhibition of BMP signaling in *Xenopus* embryos we also constructed a chimeric *Xenopus*-activin-*HyBMP5-8b* fusion mRNA. This chimeric protein contains the signal peptide and prodomain of *Xenopus* activin, fused downstream of its RIRK cleavage site to the *HyBMP5-8b* ligand coding sequence. Similar fusions have previously been shown to yield active ligands (Constam and Robertson, 1999; Dale et al., 1993; Suzuki et al., 1997; Thomsen and Melton, 1993). Interestingly, injection of only 1 ng capped mRNA of this activin-*HyBMP5-8b* fusion construct into *Xenopus* embryos had dorsalizing effects leading to the formation of secondary axes in 68% of the injected embryo (n=25, data not shown). This fusion construct also induced the formation of ectopic cement glands in animal caps (50%, n=20; data not shown).

Therefore, as these experiments indicate that the *Hydra* BMP5-8 orthologue had the unexpected effect of inducing dorsal instead of ventral structures, and these results are most consistent with inhibition of endogenous BMP signaling, I suggest that *HyBMP5-8b* is not a functional homologue of the vertebrate BMP7. The gene is equally related to all the members of the BMP 5-8 group and can not substitute for BMP7 function.

A chimeric *Xenopus-HyBMP5-8a* fusion mRNA was constructed in the same way as the *Xenopus*-activin-*HyBMP5-8b* chimera. Injection of 3 ng capped mRNA of this construct into *Xenopus* embryos did not have any effect on the development of the embryos.

4.5. Expression pattern of the *Hydra* BMP5-8 orthologues in whole mounts

4.5.1. Expression of the *Hydra* BMP5-8 orthologues in the adult animal

To determine the expression patterns of the two *Hydra* BMP genes, *in situ* hybridization was carried out on whole mounts of *Hydra*. A clearly defined expression pattern for the *HyBMP5-8a* gene using a probe against the complete cloned sequence could not be obtained. Plausibly, this was due to a low level of expression as indicated by Northern Blot analysis. In contrast, *HyBMP5-8b* is expressed in two locations along the body column in an adult *Hydra*: in the head and in the lower 1/3-1/2 of the body column (Fig. 4.12A-C). This expression was observed in three different strains of *H.vulgaris* (L2, Zürich, Basel) as well as in the 105 strain of *H.magnipapillata*.

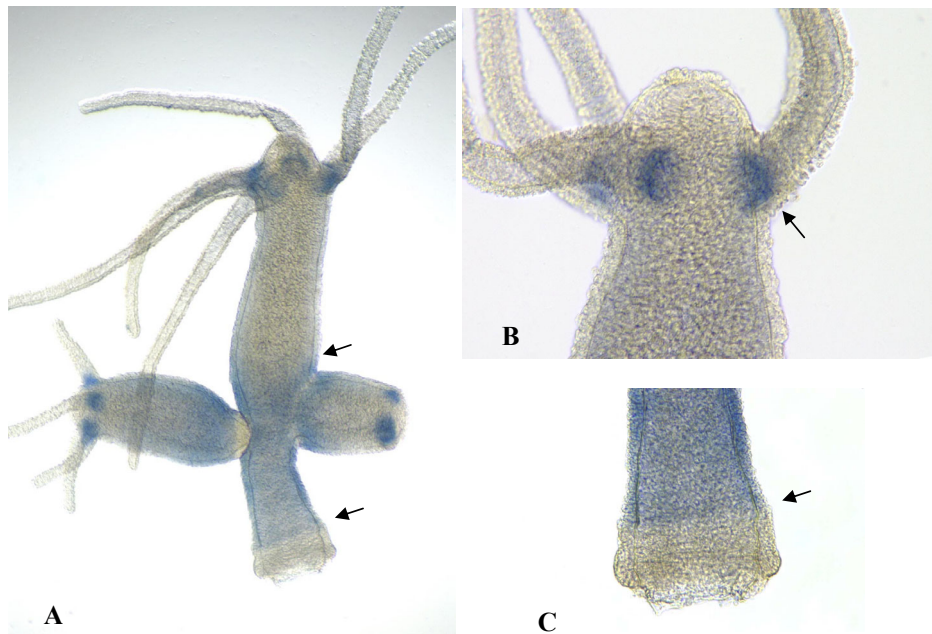


Fig. 4.12. Expression pattern of *HyBMP5-8b* in an adult *H. vulgaris* (Zürich strain) as detected by whole-mount *in situ* hybridization. (A) Whole animal: arrows indicate the apical and basal boundary of the expression in the lower third of the body column. (B) Higher magnification view of the head: arrow indicates the expression at the base of a tentacle. (C) Higher magnification view of the foot: arrow indicates the sharp basal border of expression.

4.5.2. *HyBMP5-8b* plays a role in tentacle formation

The head of a *Hydra* consists of two parts. The apical half, the hypostome, is a dome containing the mouth, while the tentacles emerge from the lower half, the tentacle zone. As shown in figure 4.12B, *HyBMP5-8b* expression in the head is restricted to the tentacle zone. More specifically, expression of the gene is confined to a band of endodermal epithelial cells that bridges the tentacle zone/tentacle border (Fig. 4.12B). As part of the tissue movements in the animal, tissue is displaced in an apical direction from the body column into the tentacle zone. From there it is displaced across the tentacle zone/tentacle border onto the tentacles, and eventually sloughed at the tentacle tips (Campbell, 1967a). Thus, these cells transiently express *HyBMP5-8b* as they traverse the tentacle zone/tentacle border, suggesting the gene plays a role in maintaining the steady state patterning of the tentacles in the adult. This expression of *HyBMP5-8b* in the adult head suggests the gene would also have a role in the development of tentacles. Since tentacle development occurs during bud formation and head regeneration, and can be induced to occur ectopically along the body column, this possibility was examined in these different circumstances.

4.5.2.1. Bud formation

Bud formation, *Hydra*'s form of asexual reproduction, involves *de novo* axis formation. The first sign of a developing bud is the formation of a circular placode in the ectoderm of the budding zone (Otto and Campbell, 1977). The placode and surrounding tissue evaginate and subsequently elongate into a cylindrical protrusion. Thereafter, a head forms at the distal end and a foot at the proximal end of the protrusion, and eventually the young polyp detaches from the parent (Otto and Campbell, 1977).

The expression of *HyBMP5-8b* during tentacle formation in a developing bud begins just before the emergence of the tentacles. By bud stage 5 (bud stages as described (Otto and Campbell, 1977), spots of *HyBMP5-8b* expression appeared in the endodermal epithelial cells just below the protruding tip (Fig. 4.13D). Subsequently, tentacle buds emerge through the *HyBMP5-8b* spots and elongate into tentacles thereby setting up the adult pattern of expression in the head (Fig. 4.13E, 4.13F). This pattern

of expression indicates that the gene has a role in the initiation as well as the maintenance of tentacle formation.

One other point concerns the timing of expression. In a young bud the first two tentacles form on the side of the bud facing the foot of the parent (Otto and Campbell, 1977). Similarly, the first *HyBMP5-8b* spots appeared on this side of the bud (Fig.4.13D). Thus, begin of expression of the gene is tightly coupled with the emergence of the tentacle bud.

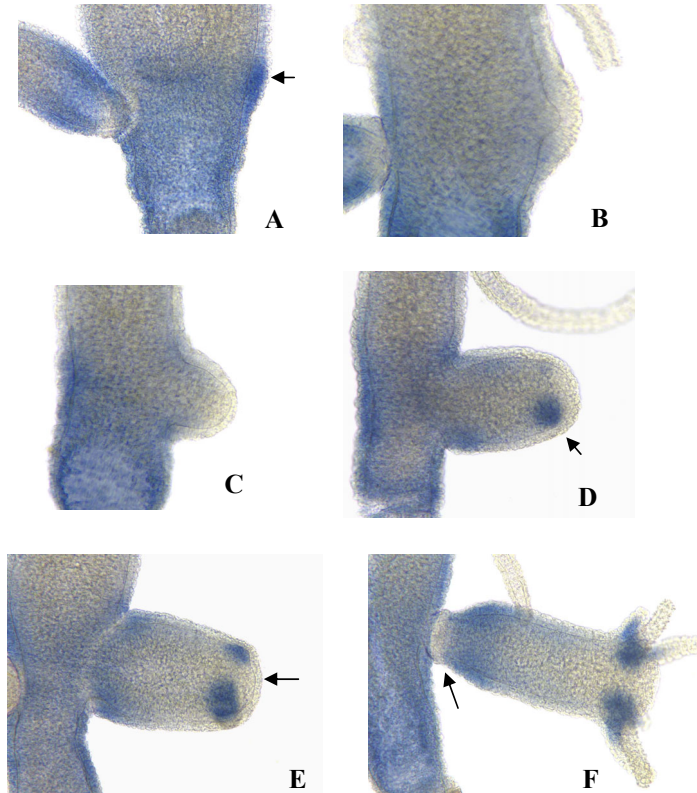


Fig. 4.13. Changes in the *HyBMP5-8b* expression pattern during bud formation in *H. vulgaris* (L2 strain). (A) Stage 1: placode stage. Arrow points to site of emergence of a new bud. (B) Stage 2-3. (C) Stage 4. (D) Stage 5. Arrow indicates the first appearing tentacle spot facing the foot of the parent animal. (E) Stage 6. Arrow points to the tip of a bud, which does not show any *HyBMP5-8b* expression. (F) Stage 9. Arrow indicates the newly formed foot devoid of *HyBMP5-8b* expression.

4.5.2.2. Head regeneration

Removal of the head leads to the morphallactic regeneration of a new head at the distal end of the remaining body column (Bode, 2003). Wound healing occurs within 3 to 5 hours after decapitation, and the head organizer is re-established, e.g. regaining its inducing capacity, within 48-72 hours at the apical end following decapitation (M.Broun, personal communication). Tentacle and hypostomal structures appear after 30-36 hours.

We monitored the reappearance of *HyBMP5-8b* expression during head regeneration in animals of the 105 strain, the wildtype strain of *H. magnipapillata*. The animals were bisected directly below the tentacles. As early as 1 hour after decapitation, expression was visible in a small area at the apex of the animal in the endodermal epithelial cells (Fig. 4.14A). This expression remained unchanged during the following 20 hours, but increased in area and staining intensity by 24 hours (Fig. 4.14B). Thereafter, (28-32 hours) the stain in the cap diminished and spots appeared at the lower edge of the cap. By 36 hours, the cap stain had disappeared completely and only spots remained (Fig. 4.14C). By 40-48 hours, tentacle buds emerged precisely through the spots of *HyBMP5-8b* expression (Fig. 4.14D). Thus, the same relationship between the appearance of the *HyBMP5-8b* spots and tentacle emergence was observed during bud formation and head regeneration.

To obtain further evidence that *HyBMP5-8b* is activated during head formation we analyzed its expression in reg-16, a mutant strain of *H. magnipapillata*, which is defective in head regeneration (Achermann and Sugiyama, 1985). When reg-16 is bisected, most animals do not regenerate a head, while some form a partial head and a few regenerate a complete head. Expression of *HyBMP5-8b* was followed for 6 days in animals bisected beneath the tentacles. As in the wild type animals, expression was detected at the apex of the animal throughout the first 24 hours (Fig. 4.14E, Fig. 4.14F). Unlike the wildtype, the intensity of stain decreased by 24 hours (Fig. 4.14F) and vanished by 36 hours (Fig. 4.14G). In the few regenerates (~5%) that formed a single medial tentacle or two tentacles on opposite sites of the tip, *HyBMP5-8b* was expressed at the base of those tentacles as observed in wild type animals (Fig. 4.14H). The results obtained with reg-16 tighten the correlation of the expression of *HyBMP5-8b* with tentacle formation, as the gene is not expressed when tentacles do not form.

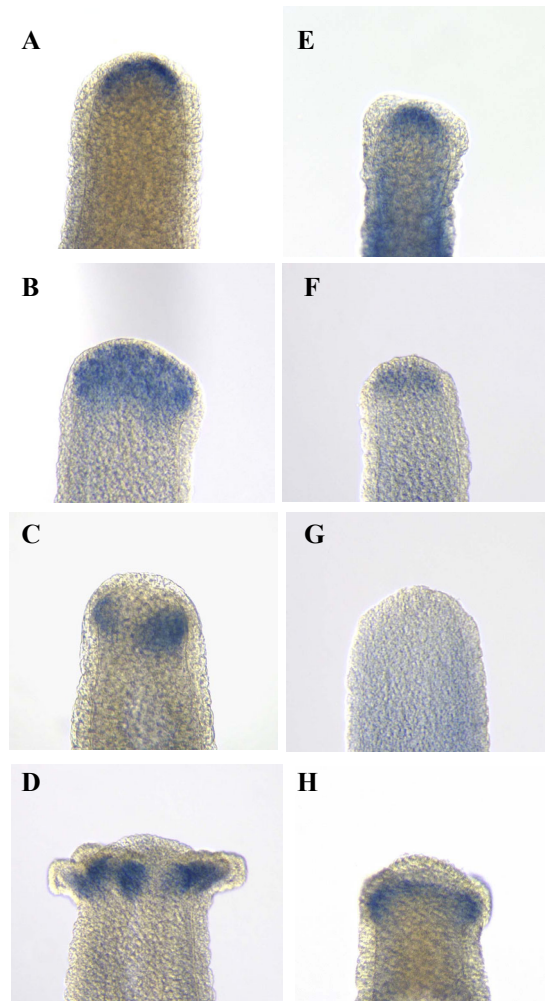


Fig. 4.14. *HyBMP5-8b* expression in the regenerating head following decapitation in *H. magnipapillata* (A-D) and in the regeneration deficient mutant strain (*H. magnipapillata*, reg 16 (E-H)). (A,E) 1 hour. (B,F) 24 hours. (C,G) 36 hours. (D,H) 48 hours. (H) Only 5% of reg 16 regenerates formed a head with one or two tentacles at opposite sites.

4.5.2.3. *HyBMP5-8b* is specifically associated with tentacle formation

To demonstrate that the gene is directly involved in tentacle formation, it would be useful to examine its expression in a situation where tentacles but no other part of the head forms. Treatment of whole animals with either LiCl (Hassel and Bieller, 1996) or alsterpaullone (M. Broun, personal communication) results in the formation of

individual ectopic tentacles on the body column. Hypostome or complete heads, do not form after 2 mM LiCl treatment (Hassel and Bieller, 1996), and form only about a week later after the appearance of ectopic tentacles following alsterpaullone treatment (M.Broun, personal communication).

Animals were treated for 48 hours with either 2 mM LiCl or 5 μ M alsterpaullone, and then transferred back into hydra medium. 2-3 days after end of treatment, ectopic tentacles began to emerge in the upper part of the body column in LiCl-treated animals, and over most of the body column in alsterpaullone-treated animals. The changes in *HyBMP5-8b* expression that occurred during the development of the ectopic tentacles resembled the expression pattern of this gene as tentacles develop during bud formation and head regeneration. *HyBMP5-8b* was first expressed in spots in the upper third of the body column for LiCl-treated animals (Fig. 4.15A), or all over the body column in alsterpaullone-treated animals (Fig. 4.15B). A day later, ectopic tentacles emerged from those spots (Fig. 4.15A, 4.15C). Developing and fully formed tentacles had the characteristic staining pattern in the endodermal epithelial cells at the base of each ectopic tentacle (Fig. 4.15A, 4.15C). Thus, *HyBMP5-8b* expression in the lower part of the head is associated specifically with the formation of tentacles.

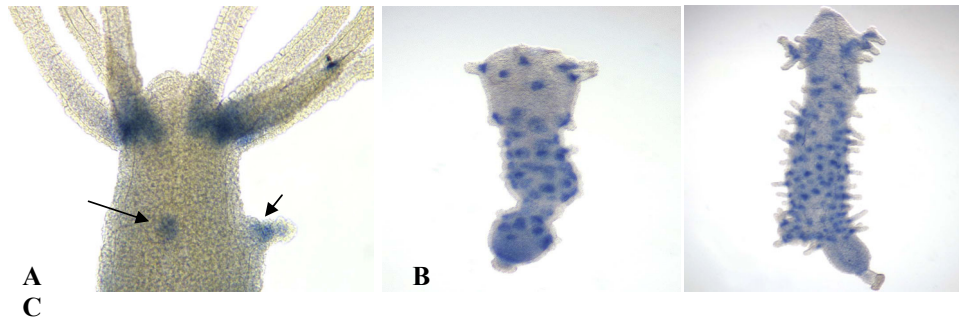


Fig. 4.15. *HyBMP5-8b* expression in 2 mM LiCl (A) or alsterpaullone (B,C) treated animals. (A) Expression in the upper part of an animal 5 days after start of the LiCl treatment. Left arrow indicates a spot of *HyBMP5-8b* expression before ectopic tentacle is visible, while the right arrow indicates an ectopic tentacle with *HyBMP5-8b* expression at the base. (B) Alsterpaullone-treated animal with spots of *HyBMP5-8b* expression all over the body column 5 days after start of treatment. (C) Alsterpaullone-treated animal with clearly visible ectopic tentacles 6 days after start of treatment.

4.5.3. Expression of *HyBMP5-8b* in the lower half of the body column is controlled by the positional value gradient

4.5.3.1. Expression in adults and developing buds

The second domain of *HyBMP5-8b* expression is located in the lower part of the body column. The gene is expressed in the ectodermal epithelial cells of the budding zone and the peduncle, but not in the foot (Fig. 4.12A, 4.12C). The basal border of this expression domain is quite sharp (Fig. 4.12C), while the apical border varies from fairly-to-less sharp. As mentioned earlier, processes governing axial patterning are constantly active due to the tissue dynamics in an adult *Hydra*. In both tissue layers, the epithelial cells of the body column are constantly in the mitotic cycle. This results in the continuous displacement of the tissue of both layers towards the extremities, apically into the head and basally into the foot (Campbell, 1967a). Hence, as ectodermal epithelial cells move into the budding zone they begin to express *HyBMP5-8b* and continue expression as they move through the budding zone and the peduncle. As the tissue enters the foot expression of *HyBMP5-8b* is abruptly turned off.

A similar pattern was observed during bud formation. After elongation into a cylindrical protrusion, the ends of the bud begin to develop. By stage 5, *HyBMP5-8b* is expressed quite strongly at the proximal end (Fig. 4.13E). Then, as the basal ends starts to constrict and the foot develops, expression is shut off (Fig. 4.13F). At this point the expression pattern has become that observed in the adult. The pattern of expression in the adult occurs within the context of the tissue dynamics of an adult *Hydra*. The fact that the pattern is constant while the tissue of the lower part of the body column is continuously moving in a basal direction suggests that the processes governing axial patterning may regulate expression of *HyBMP5-8b* along the body column.

4.5.3.2. *HyBMP5-8b* expression is controlled by the positional value gradient

The axial patterning of an adult *Hydra* polyp is maintained by a positional value gradient (Wolpert, 1971). The gradient is maximal near the head decreasing down the body column to a minimal value near the foot. The head forms at high levels of positional value while the foot forms at low levels. The expression of *HyBMP5-8b* in the lower third of the body column suggested that *HyBMP5-8b* might be activated in response to positional values below a specific threshold, which would correspond to the budding zone and peduncle. Changing the level of the positional value throughout the animal might contract or expand the domain of *HyBMP5-8b* expression. To explore this idea, animals were treated with 0.5 mM LiCl or alsterpaullone, two reagents, which affect the level of the gradient throughout the body column.

Treatment with 0.5 mM LiCl decreases the level of positional value, causing cells of the body column to behave as if they were located closer to the foot (Hassel et al., 1993; Hassel and Berking, 1990). This treatment can also induce the formation of ectopic basal disks along the body column. Two days after begin of treatment the upper boundary of *HyBMP5-8b* expression had been shifted apically (Fig. 4.16B) compared to the control (Fig. 4.16A). Longer treatment led to a continual apical expansion of expression along the body column. During this expansion the apical border of *HyBMP5-8b* expression was less sharp than in the control animals (Fig. 4.16B). By 8 days after the start of treatment, the expression reached its upper limit at the border between the 1- and 2-regions of the body column (Fig. 4.16C). Further treatment with LiCl (as long as 17 days) did not result in any further apical expansion of *HyBMP5-8b* expression. However, ectopic feet formed along the body column and no staining of *HyBMP5-8b* could be found in this ectopic basal disk tissue (Fig. 4.16C).

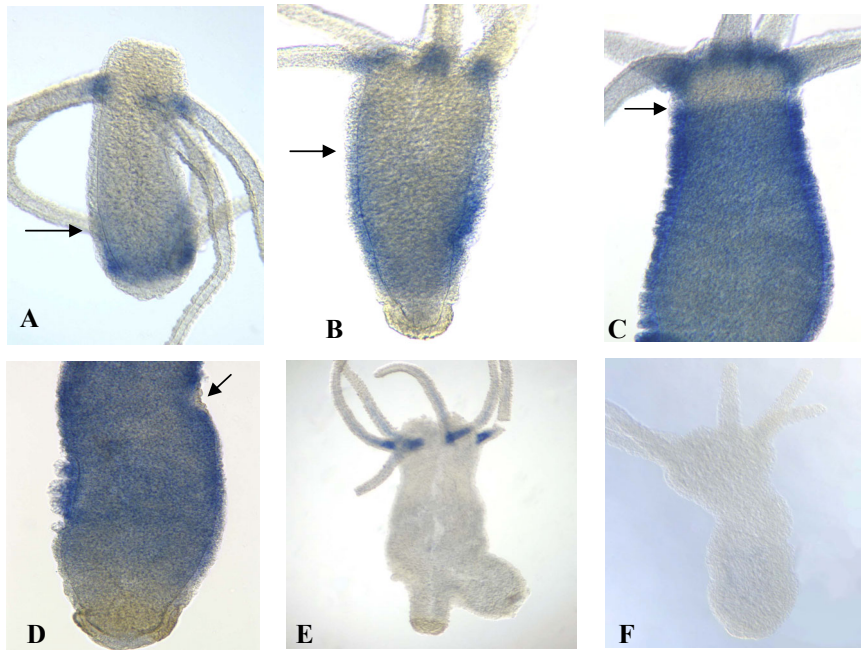


Fig. 4.16. Changes in expression of *HyBMP5-8b* along the body column due to treatment with 0.5 mM LiCl and alsterpaullone. (A) Control animal. Arrow points to the apical border of *HyBMP5-8b* expression in body column. (B) 3 days of 0.5 mM LiCl treatment. Arrow points to the apical border of *HyBMP5-8b* expression in the body column. (C) 15 days of 0.5 mM LiCl treatment. Arrow indicates the boundary of *HyBMP5-8b* expression at the 1 region. (D) 17 days of 0.5 mM LiCl treatment. Arrow indicates newly formed ectopic basal disk tissue that is devoid of *HyBMP5-8b* expression. (E) 1 day after start of alsterpaullone treatment. (F) 2 days after start of alsterpaullone treatment.

Conversely, treatment of *Hydra* polyps with alsterpaullone converts body column tissue into head organizer tissue and raises the level of head activation throughout the body column (M.Broun, personal communication). As described above, such animals generate ectopic tentacles in the body column 4-6 days after treatment and later secondary heads (M.Broun, personal communication). *Hydra* polyps were treated for 2 days with 5 μ M alsterpaullone and then transferred back into hydra medium. The expression of *HyBMP5-8b* in the lower body column vanished completely after 24 hours of alsterpaullone treatment (Fig. 4.16D). By 48 hours, even the expression at the base of the tentacles was no longer observed (Fig. 4.16E). Hence, raising the level of the head activation gradient blocked expression of *HyBMP5-8b* in the body column. Thus, both treatments indicate, that the range of *HyBMP5-8b*

expression along the body column, is affected by the level of positional value. A plausible role for the expression of the gene in the lower part of the body column will be dealt with in the Discussion.

4.5.4. *HyBMP5-8b* expression is associated with organizer formation during bud formation, head regeneration and foot regeneration

The morphogenetic gradients, which control most of the axial patterning in *Hydra* are set up by the head organizer in the hypostome (Broun and Bode, 2002; Cohen and MacWilliams, 1975; MacWilliams, 1983a; MacWilliams, 1983b; Wilby and Webster, 1970a; Wilby and Webster, 1970b). There is also evidence for a foot organizer, which exerts more limited influence (Cohen and MacWilliams, 1975; MacWilliams, 1983b). These organizers are set up during the initiation of axis formation during bud formation as well as during head or foot regeneration. The expression pattern of this BMP signaling molecule during the earliest stages of each of these three different processes suggests that the gene may also have a role in the initiation of axis formation or organizer formation.

As described above, *HyBMP5-8b* expression appears very early in the apical tip of a regenerating head where head organizer formation is taking place. Some time later after organizer formation is complete, expression of the gene in the apical tip ceases. Similar expression patterns also occur during bud initiation and foot regeneration.

4.5.4.1. Initiation of bud formation

In an adult, *HyBMP5-8b* is also expressed transiently in a third location. The first step of bud formation, stage 1, is the enlargement of a small cluster of cells in the ectoderm in the budding zone into a placode (Otto and Campbell, 1977). *HyBMP5-8b* was expressed in those ectodermal cells that will form the circular placode (Fig. 4.13A). The next placode invariably forms on the opposite side of the body column from an existing bud, and forms slightly more apical than the existing bud. This was also invariably the location where the spot of *HyBMP5-8b* occurred. By bud stage 2 however, as the placode and the surrounding tissue start evaginating, the gene was no longer expressed (Fig. 4.13B). Furthermore, the apical tip of the elongating bud

remained free of expression of the gene throughout the whole course of bud formation (Fig. 4.13C-F). Hence, expression occurs very early on as the head organizer is initially being set up, but not thereafter in the developing hypostome of a bud.

4.5.4.2. Foot regeneration

When an adult *Hydra* is bisected at any axial level along the body column, the apical piece will regenerate a foot at its basal end. Animals were bisected either in the middle of the body column or directly above the foot. In both cases, *HyBMP5-8b* was strongly expressed in the regenerating tip at the basal end within an hour after bisection (Fig. 4.17A). The stain in the regenerating tip was found exclusively in endodermal epithelial cells. Furthermore, the level of expression was much higher than observed in the neighboring adult peduncle, and remained elevated during the course of foot regeneration. When the morphological foot began to appear expression of the gene in the regenerating tip vanished (Fig. 4.17B).

Removal of the foot also affected expression in the neighboring peduncle and budding zone in that the level of expression was reduced compared to controls (Fig. 4.17A, 4.17B). Subsequently, when the foot had regenerated, the level of expression rose to the level observed in the adult in about 36 hours (Fig. 4.17C). This reduction and recovery indicates that the foot activates *HyBMP5-8b* expression in the lower body column.

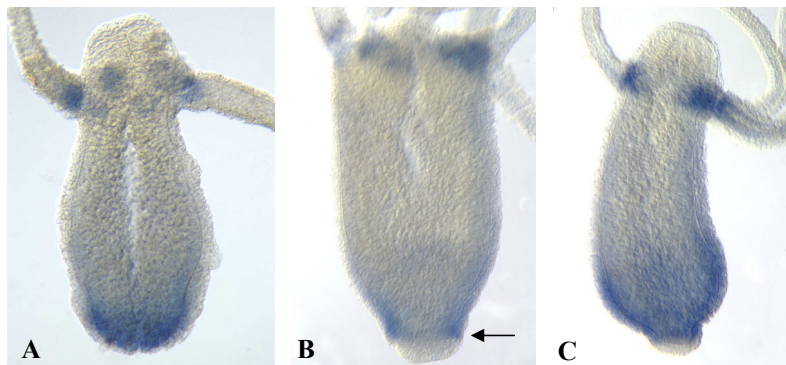


Fig. 4.17. *HyBMP5-8b* expression during foot regeneration following bisection in the lower third of the body column. (A) 1 hour after bisection. (B) 30 hours after bisection. Arrow points to newly formed foot tissue, which does not show *HyBMP5-8b* expression. (C) 36 hours after bisection.

An important point concerns the timing of the appearance of *HyBMP5-8b* expression during head and foot regeneration. The time required for the complete regeneration of either extremity depends on the axial level of sectioning. For head regeneration, the further down the body column the bisection takes place the slower is the rate of regeneration (MacWilliams, 1983a; Webster and Wolpert, 1966). Conversely, for foot regeneration, the further up the body column that bisection occurs, the slower is the rate of regeneration (Mookerjee and Bhattacharjee, 1967). The initiation of *HyBMP5-8b* expression following bisection did not follow this pattern. Regardless of the axial location of bisection, expression of the gene appeared within an hour (data not shown).

Thus, as with bud initiation, the gene is expressed very early during the process. Plausibly, the gene is playing a similar role in all three situations.

4.5.4.3. Aggregate formation

A possible caveat for the instructive role of *HyBMP5-8b* during head regeneration and foot regeneration comes from the fact, that the polyp's body axis and its polarity remain intact. I therefore analyzed aggregates from dissociated cell suspensions, where positional information is completely lost (Gierer et al., 1972). Head and foot organizers form *de novo* at random locations in the aggregates and induce surrounding tissue to develop new body axes, which finally separate into intact polyps (Gierer et al., 1972).

HyBMP5-8b was expressed in epithelial cells of the entire aggregate 2 days after aggregation (Fig. 4.18A). Due to the fragile nature of an aggregate it was not possible to obtain expression data earlier than that. This uniform expression could be observed during the following hours. 4 days after aggregation however, new head protrusions became visible and those did not display any expression of *HyBMP5-8b* (Fig. 4.18B). The intensity of the staining in the remaining tissue faded within the following 24 hours. Shortly before tentacles were emerging, the gene was expressed in the typical spot like pattern in the head protrusions. Newly formed tentacles display *HyBMP5-8b* expression at the base of their tentacles (Fig. 4.18B, 4.18C).

The course of the *HyBMP5-8b* expression pattern during aggregate formation combines characteristics of both head/foot regeneration and bud formation. The gene is upregulated during the early stages of axis formation. Once the organizers are formed

no expression can be found in the newly determined organizer tissue. Shortly before the emergence of tentacles, however spotlike expression can be observed at the future tentacle sites.

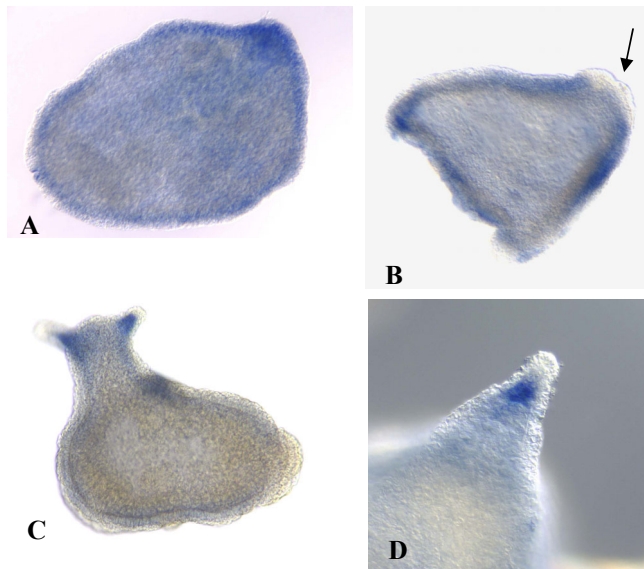


Fig. 4.18. Changes of *HyBMP5-8b* expression during aggregate development. (A) 2 days. (B) 4 days. Arrow indicates a new head protrusion marked by the absence of *HyBMP5-8b* expression. (C) 5 days. (D) Detail of the expression of the gene in the tentacle.

4.6. Preliminary data for anti-Phospho-Smad1 antibody staining in

Hydra

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Whole mount *in situ* hybridization revealed an interesting pattern of expression of *HyBMP5-8b* in the adult *Hydra* polyp and during *de novo* axis formation. However, since BMP signaling molecules are secreted extracellular ligands, expression data do not establish if an activation of BMP signaling is actually taking place in the locations where the protein is being transcribed. Therefore, it was of interest to examine when and where the pathway is active endogenously.

As described earlier, the TGF- β superfamily of growth factors is known to transmit signals to the nucleus mainly through receptor regulated Smads (R-Smads),

intracellular signaling components that are highly conserved in metazoans. Ligand-stimulated C-terminal phosphorylation through Ser/Thr kinase receptors is required for the signaling activity of the R-Smads, and therefore provides a direct means with which to assess the activity of endogenous BMP signaling pathways. Smads 1/5/8 phosphorylation is induced by BMP signaling but not by the activin signaling pathway whereas the converse is true for Smad2 phosphorylation (Baker and Harland, 1996; Hoodless et al., 1996; Macias-Silva et al., 1996).

Recently, a polyclonal antibody has been developed that specifically recognizes the phosphorylated form of BMP-regulated Smads1/5/8. This antibody was raised against *Xenopus* Smad1 protein, but it has been shown to crossreact with its *Drosophila* orthologue *Mad* (Tanimoto et al., 2000).

An orthologue of R-Smads, *HySmad1*, has been isolated from *Hydra* (Hobmayer et al., 2001). Sequence and phylogenetic analyses identified this gene as a *Hydra* orthologue to the R-Smad1/5/8 family (Hobmayer et al., 2001). Amongst other conserved features typical for R-Smad proteins, *HySmad1* exhibits a C-terminal TSVS motif (Fig. 4.19). A similar C-terminal SSXS motif found in R-Smads in higher metazoans, is used for serine/threonine phosphorylation by an activated type I receptor complex. *HySmad1* might act as a potential transducer for the *HyBMP5-8b* BMP ligand. Therefore, the antibody anti-phospho-Smad1 (α -PS1) was tested, to determine if it could recognize a *Hydra* orthologue.

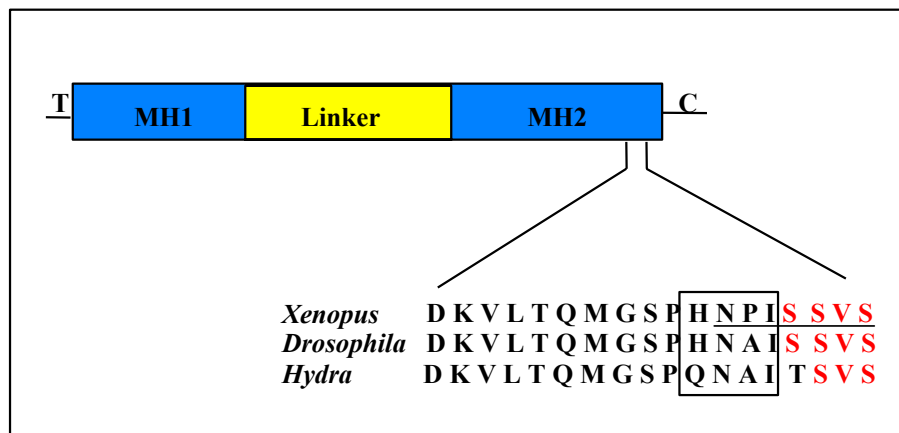


Fig. 4.19. Diagram of antigen sites. Schematic illustration of the highly conserved amino- (MH1) and carboxyl- (MH2) terminal domains of Smad proteins. The underlined amino acid sequence corresponds to the sequence of the synthesized phosphopeptide against which antiphosphoSmad1 antibody was raised. The location of serine residues phosphorylated by TGF β type I receptors are highlighted. Residues that are not conserved are boxed.

First, the antibody staining was examined by whole mount fixation and immunostaining using α -PS1 and a α -Rabbit-AP conjugated secondary AB (Fig. 4.20). The background was very high (Fig. 4.20A, 4.20G). This was partly due to unspecific binding by proteins of the rabbit serum itself, as a background stain can be seen when unimmunized rabbit serum was used in the immunochemistry procedure (Fig. 4.20C). Additionally, it is known that antibodies raised against peptides coupled to keyhole limpet hemocyanin (KLH) cause a background problem when used in *Hydra*. I therefore added 100 μ g/ml KLH protein to the antibody solution to reduce the background staining and could observe a considerably cleaner immunostaining result (Fig. 4.20B, 4.20H).

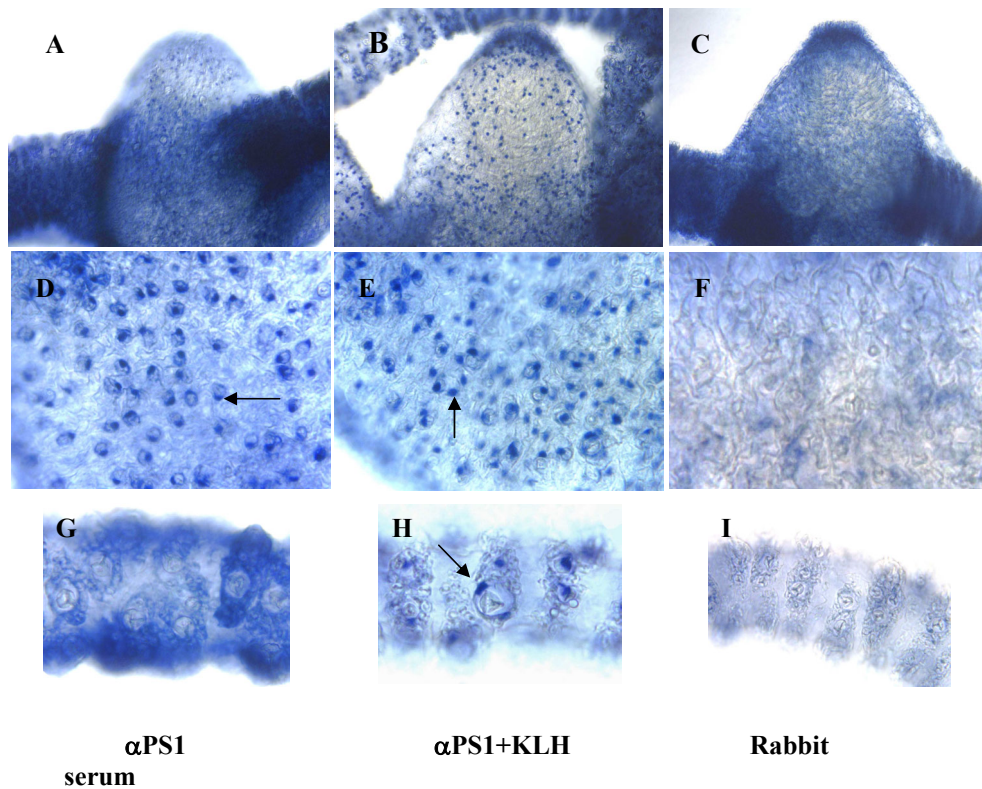


Fig. 4.20. Whole-mount immunohistochemistry in adult *Hydra*. (A,D,G) with α -phospho-Smad1 antibody. (B,E,H) with α -phospho-Smad1 antibody supplemented with 100 μ g/ml KLH. (C,F,I) with unimmunized rabbit serum as a control. (A-C) Head detail. (D-F) Peduncle detail. (G-I) Tentacle detail. Arrows in D,E and H point to stained nematocyte nuclei.

The staining pattern using α -PS1 supplemented with 100 μ g/ml KLH in an adult *Hydra* polyp can be described as follows: A spotted staining pattern could be observed in the hypostome (Fig. 4.20B), the tentacles (Fig. 4.20H) and the peduncle (Fig. 4.20D, 4.20E). Furthermore, higher magnification revealed that the staining in the peduncle and the tentacles resided in the nuclei of nematocyte cells (Fig. 4.20D, 4.20E, 4.20H). To find the antibody stain in cell nuclei is expected since phosphorylated Smad gets translocated together with Smad4 into the cell nucleus and acts there as a transcription factor (Raftery and Sutherland, 1999). The above described staining pattern in the tentacles, the hypostome and the peduncle can not be found in animals exposed to unimmunized rabbit serum (Fig. 4.20C, 4.20F, 4.20I). It was not possible to determine which cells or cell nuclei in the hypostome were stained, respectively. No α -PS1 specific stain could be observed anywhere in the upper part of the body column (data not shown).

How did the α -PS1 staining pattern relate to the *HyBMP5-8b* expression? The antibody stain did overlap in two locations with the expression of *HyBMP5-8b*: in the lower third of the body column and in the tentacles. The hypostome was the only place where α -PS1 staining could be observed but no *HyBMP5-8b* expression was detectable. No expression of the gene and no specific α -PS1 staining could be seen in the upper part of the body column.

Although the antibody staining in *Hydra* whole mounts appeared to be specific, it was necessary to determine if α -PS1 was indeed binding to *HySmad1* and not to other *Hydra* proteins located in nematocyte nuclei. It has been demonstrated that *Mad*, the *Drosophila* orthologue of Smad1, transfected into *Drosophila* S2 cells localizes into the nucleus when activated *tkv*, a *dpp* type I receptor, had been cotransfected (Maduzia and Padgett, 1997). Furthermore, activated *Mad* in S2 cells can be detected by western blot analysis using α -PS1 raised against *Xenopus* Smad1, whereas endogenous activated *Mad* alone is not detectable (L.Parker and K.Arora, personal communication). Cnidarian genes have been proven to be active when introduced into *Drosophila* embryos (Grens et al., 1995; Hayward et al., 2002). I therefore tried to overexpress *HySmad1* in *Drosophila* S2 cells to be able to monitor cross-reactivity of α -PS1 via Western blot analysis. I could not detect a specific band for *HySmad1* (Fig. 4.21).

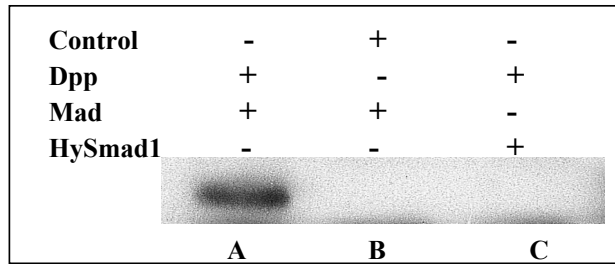


Fig. 4.21. Western Blot analysis. (A) Anti-phosphoSmad1 antibody specifically detects activation of overexpressed Mad in S2 cells. (B) Control. (C) Anti-phosphoSmad1 antibody does not detect activation of overexpressed HySmad1 in S2 cells.

In summary, although preliminary data suggest activation of BMP signaling can be visualized with an anti-phospho-Smad antibody, the ability of the antibody to crossreact with the *Hydra* orthologue of Smad1 has still to be shown.

4.7. *HyBMP5-8b* is not transcriptionally upregulated during oogenesis

It has been shown that BMP signaling acts in different aspects of oogenesis in higher metazoans. In *Drosophila*, the *Dpp* receptors *Punt* and *Thick Veins*, as well as the Smad-family members *Mad* and *Medea*, are required for germ line stem cell proliferation (Xie and Spradling, 1998). In early mouse embryos, BMP ligands and *Smad5* regulate initiation of the mouse germ line and generation of primordial germ cells (Chang and Matzuk, 2001; Lawson et al., 1999). *In situ* hybridization data obtained for *HySmad1* show that the gene is elevated throughout most stages of *Hydra* oocyte development. This indicates that BMP signaling might be involved in various processes of oogenesis in early metazoans as well (Hobmayer et al., 2001).



Fig. 4.22. Expression of *HyBMP5-8b* during oogenesis. (A) Control *in situ* Hybridization using the *H.vulgaris* strain AEP inducible to undergo sexual differentiation. The strain exhibits the same expression pattern as all the other *H.vulgaris* strains in the lab. Arrows point to the expression of the gene at the base of a tentacle and in the lower part of the body column. (B) Stage 2. (C) Stage 4. Arrows indicate the expression at the base of a tentacle and in the lower part of the body column. (D) Stage 6. Oocyte stages are defined as described in Miller et al., 2000.

Development of the oocyte in *Hydra* starts with the rapid proliferation of egg-restricted interstitial stem cells to form large cell clusters of interstitial oocyte precursors (Littlefield, 1991). Precursors in the center of a developing egg field stop cycling and differentiate into nurse cells, while one of these central precursors is selected by an unknown mechanism to become the oocyte (Honegger et al., 1989; Miller and Steele, 2000). The oocyte starts to phagocytose the neighboring nurse cells, becomes spherical, penetrates the ectodermal cell layer and finally completes meiosis (Miller and Steele, 2000).

In order to determine if *HyBMP5-8b* is the ligand involved in activation of *HySmad1* during oogenesis I examined the expression of *HyBMP5-8b* during several stages of oogenesis. I could not detect expression of the gene during the course of the development of an oocyte (Fig. 4.22B-D). This finding indicates that *HyBMP5-8b* is not involved in germ-cell specific differentiation processes and is not responsible for

the upregulation of the expression of *HySmad1*. A possible candidate gene for *HySmad1* activation might be an orthologue of a BMP2/4 protein yet to be isolated from *Hydra*.

4.8. *HyBMP5-8a* and *HyBMP5-8b* are expressed at a low level during embryogenesis

Embryogenesis in *Hydra* is quite simple (Martin et al., 1997). Briefly, the fertilized egg undergoes 6-7 cleavage divisions resulting in a blastula consisting of a single layer of cells. Then, gastrulation occurs by ingression of single cells from all parts of the blastula into the blastocoel thereby forming a solid stereo gastrula. Subsequently, the cells of the outer layer, the presumptive ectoderm, secrete a thick cuticle and the embryo enters a relatively quiescent stage (the cuticle stage). 2-3 weeks (sometimes months) after fertilization, the mass of epithelial cells in the center of the embryo organize into an epithelial layer, the endoderm. Within a day thereafter, the cuticle cracks at the apical end, and the hatchling emerges and elongates from a spherical form into the cylindrical shape of the adult animal forming a head and foot during and shortly after elongation.

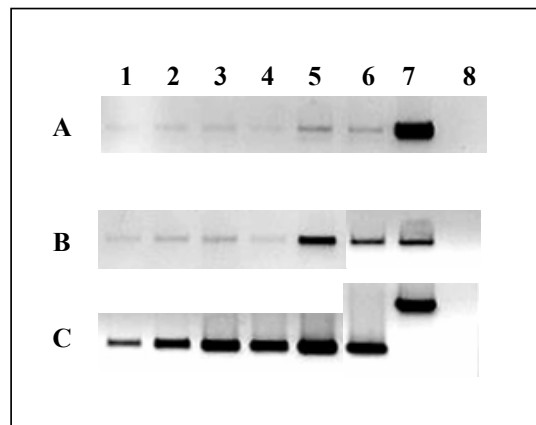


Fig. 4.23. Expression pattern of *HyBMP5-8a* (A) and *HyBMP5-8b* (B) during several stages of embryogenesis as measured with RT-PCR. EF α was used as an internal loading control (C). 1: collection of 2 cell, 4 cell and 8 cell stages, 2: Blastula, 3: Gastrula, 4: Cuticula (3-10 days old), 5: cDNA of *H.vulgaris* strain AEP, 6: cDNA of *H.vulgaris* strain L2, 7: genomic DNA, 8: RT without RNA template. The primers used to amplify an EF α fragment were flanking an intron sequence and thus allowed for the detection of genomic DNA contamination in cDNA samples (lane 7). The EF α cDNA amplification product is 300 bp in length, the EF α amplification product from genomic DNA is 600 bp in length.

An attempt to obtain expression data performing an *in situ* hybridization failed due to scarceness of available embryos and difficulties conducting *in situ* hybridization on available embryos. However, the expression of *HyBMP5-8a* and *HyBMP5-8b* could be examined via RT-PCR. Total RNA was isolated from 50 embryos at several embryonic stages (early cleavage stages, blastula, gastrula, cuticula) reverse transcribed with M-MLV as described in 3.2.2.3.1. and 1 µl of the first-strand cDNA used as a template in a 20 µl PCR reaction. *HyBMP5-8a* (*BluntendHya5'* and *Hya3'*) and *HyBMP5-8b* (*BluntendHyb5'* and *Hyb3'*) specific primers were used. Primers for the EF-1a gene (*5EFα1* and *3EFα1*) were applied as control. PCR conditions were: 3 minutes 94°C, 1 minute 58°C, 1 minute 72°C (30 cycles), followed by 10 minutes 72°C.

Both genes were detectable at a low level throughout the whole course of embryogenesis (Fig. 4.23).

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5. Discussion

5.1. The common ancestor of cnidarians and bilaterians had two different types of BMP ligands

Comparison of the carboxy-terminal regions of members of the TGF-beta superfamily of proteins has led to classifying them into several subfamilies. BMP proteins form the largest group within this family and can be classified into two subgroups, BMP2/4 and BMP5-8 (Kingsley, 1994). Orthologues of each subfamily have been found in both deuterostomes and protostomes and a member of the BMP2/4 group, *bmp2/4-Am*, had been isolated from the coral *Acropora* (Hayward et al., 2002).

Sequence analysis indicates that *HyBMP5-8a* and *HyBMP5-8b* are more closely related to the BMP5-8 group, than to the BMP2/4 group. This view is supported by the phylogenetic analysis, which also suggests that the two *Hydra* BMP5-8 genes are a result of a duplication event in *Hydra*, following the divergence of the cnidarians and the bilaterians. Furthermore, both the maximum likelihood and the parsimony analysis indicate that all three cnidarian BMP genes are equally related to their bilaterian orthologues, strongly supporting the origin of an ancestral BMP gene into a BMP5-8 gene and a BMP2/4 gene prior to the divergence of cnidarians and bilaterians.

5.2. *HyBMP5-8a* and *HyBMP5-8b* are not functionally equivalent to bilaterian BMPs

To show whether a gene possesses a conserved function, derived from an ancestral gene in the last common ancestor, a gene can be introduced into evolutionarily distant animals and tested if it is able to functionally compensate or mimic the endogenous orthologue. This is true for a number of cnidarian genes, which have been shown to have the same biological activity as their bilaterian counterpart. For example, *Cnash*, a *Hydra* *acheate-scute* orthologue will rescue a *Drosophila* *acheate-scute* mutant (Grens et al., 1995). And, introduction of *Cngsc* or *Hyβ-cat*, the *Hydra* *gooseoid* and *β-catenin* orthologues, into the ventral part of a *Xenopus* embryo induced the formation of secondary axes (Broun et al., 1999; Hobmayer et al., 2000). Similarly, the *Acropora*

orthologue of a BMP2/4 gene is functionally conserved as it has dorsalizing activity in *Drosophila* embryos (Hayward et al., 2002).

However, when *HyBMP5-8b* was introduced into *Xenopus* embryos it did not mimic the function of the *Xenopus* homologue, BMP7. Instead of promoting the development of ventral mesoderm as predicted, injection of *HyBMP5-8b* mRNA into *Xenopus* ventral blastomeres led to the formation of secondary axes. Experiments using *Xenopus* dissected ectodermal explants (animal caps) then revealed that this induction of secondary axes was a result of inhibiting *Xenopus* endogenous BMP signaling, rather than a dorsalizing signal per se.

There are several explanations for this behavior of the *HyBMP5-8b* gene. One is that *HyBMP5-8b* dimerizes with endogenous BMP subunits, but the resulting dimers are incapable of maturation and therefore secretion. This could be due to differences for instance in the location or sequence of the tetrabasic cleavage site of the *Hydra* polypeptide. The insufficient cleavage of precursor BMP polypeptides would lead to a lack of active BMP ligands in the extracellular space and thereby to a disruption of BMP signaling. Another possibility is that the *Xenopus* BMP receptors are blocked by binding of *Hydra/Xenopus* BMP heterodimers or of *Hydra* BMP and differences in the secondary structure of the *Hydra* BMP mature ligand then preclude receptor activation. A third possibility is that the *HyBMP5-8b* ligand actively competes with the *Xenopus* BMP ligands for binding to the endogenous receptors, leading to a reduction in BMP signaling.

To rule out the possibility that the inhibition of BMP signaling was caused by specific features of the *HyBMP5-8b* prodomain, e.g. a different position and sequence of the tetrabasic cleavage site, a *Xenopus*-activin-*HyBMP5-8b* fusion protein was constructed. As mentioned above the prodomain of BMP proteins is less conserved than their ligand region. Furthermore, it has been shown that some *Xenopus* TGF- β proteins injected into *Xenopus* embryos are only active when fused to the *Xenopus* activin prodomain (Dale et al., 1993; Thomsen and Melton, 1993). Therefore, we hypothesized it could be possible, that the ligand region by itself will be able to function as its *Xenopus* homologue BMP7. However, injection of the X-activin-*HyBMP5-8b* chimera mRNA led to the same results: induction of secondary axes by inhibiting BMP signaling in early *Xenopus* embryos.

This suggests that *HyBMP5-8b* is not able to function in *Xenopus* as a vertebrate BMP7, but does not preclude it from possessing a signaling capability in

Hydra. Phylogenetic analysis shows that *HyBMP5-8b* is equally related to all the members of the BMP5-8 group, not simply to vertebrate BMP7 genes, therefore it is not implicit that it would have a conserved function in axis formation.

However, preliminary data suggest that *HyBMP5-8b* does act as a BMP peptide in *Hydra*. Staining of whole mounts of *Hydra* with an antibody raised against the phosphorylated version of the receptor activated Smad1 (α PS1) shows overlapping activation of BMP signaling with the *HyBMP5-8b* expression, implying that this BMP ligand binds to BMP receptors and activates thereby downstream signaling. To show, that α PS1 is specifically cross-reacting with the *Hydra* orthologue of Smad1, cell culture was used to show cross-reactivity, but this was inconclusive. This could be due to a variety of reasons: a) *HySmad1* could not be translated by *Drosophila* cells, b) the *Drosophila Thick Veins* receptor is not able to phosphorylate *HySmad1* or c) the antibody is not specifically binding to the *Hydra* Smad1 orthologue. To overcome these difficulties, it would be necessary to develop an antibody specifically to phosphorylated *Hydra* Smad1.

5.3. Roles of *HyBMP5-8b* in axis formation / axial patterning

Several results indicate that *HyBMP5-8b* plays a role in two different aspects of axial patterning in *Hydra*.

5.3.1. A role in organizer formation

A *Hydra* has two organizer regions. One, the head organizer is in the hypostome, while the other, the foot organizer, is in the foot. The head organizer produces and transmits two signals that are involved in axial patterning. One is a signal for setting up the head activation gradient in the body column, and the second is a signal, which inhibits body column tissue from forming a head. The foot organizer has similar properties. Tissue containing the head organizer is clearly defined by its ability to induce body column tissue to form a second axis when transplanted to the body column of a host animal (Broun and Bode, 2002). In contrast, tissue of the body column which is also capable of forming a second axis when transplanted to a host does it not by induction, but by self-organization of the transplanted tissue (Broun and Bode, 2002; Yao, 1945) In

essence, the transplant is undergoing regeneration. Thus, the inductive capacity associated with an organizing region for the head is confined to the hypostome.

As part of the formation of a new head, the head organizer develops. During bud formation this is a central aspect of the initiation of the new axis of the bud. Indirect evidence indicates that it is set up in the evaginating tip during early stages of bud formation (Sanyal, 1966). During head regeneration, formation of the head organizer takes place to re-establish the steady state tissue dynamics based on the head activation and head inhibition gradients. Transplantation experiments that have been carried out provide direct evidence that the head organizer is set up rapidly and early during bud formation being essentially complete in 18 hours by stage 3 (M. Broun, personal communication). In contrast, rebuilding the organizer during head regeneration is a much slower process requiring about 48-72 hours (M. Broun, personal communication).

The expression patterns of *HyBMP5-8b* during bud formation and head regeneration suggest it plays a role in the early stages of head organizer formation. It is initially expressed in the location where the head organizer begins to form, as well as through the early stages of organizer formation. More explicitly, the gene is expressed only during the earliest stage of bud formation as the organizer forms. And, it is expressed during the first 24-36 hours of the 72 hour period of organizer formation during head regeneration. When organizer formation is well underway in each of the two processes, expression of *HyBMP5-8b* is downregulated.

Once the head organizer is formed, it must continually undergo renewal to maintain itself in the context of tissue constantly moving up through the hypostome, which is eventually sloughed at the apex (Campbell, 1967b). Apparently, *HyBMP5-8b* plays no role in the maintenance of the head organizer as it is not expressed in the adult hypostome.

In contrast, two other genes appear to be involved in the maintenance of the head organizer. It has been shown that the canonical Wnt pathway is involved in the formation of the head organizer (M. Broun and H. Bode, personal communication). Two of the genes of this pathway, *HyWnt* and *HyTcf*, the *Hydra* orthologues of *Wnt* and *TCF*, are expressed continuously in the hypostome (Hobmayer et al., 2000). They are also expressed in the placode during stage 1 of bud formation. Unlike *HyBMP5-8b*, the two genes continue to be expressed in the developing apical tip throughout bud development ending up in the adult hypostome (Hobmayer et al., 2000). The two

genes undergo similar patterns of expression during head regeneration appearing early in the apical tip and remaining there throughout the process. Thus, in contrast to the role of *HyBMP5-8b*, it appears as though genes of the Wnt pathway are required continuously from the initiation of organizer formation to the maintenance of this structure in the adult.

HyBMP5-8b may also play a role in the formation of the foot. Following removal of the foot the foot organizer regenerates (Cohen and MacWilliams, 1975), although the timing of this process is not known. Also, following foot removal, *HyBMP5-8b* expression rises sharply and immediately. Expression remains high at the basal end until a foot is visibly forming. Then as in head regeneration, expression of this gene ceases in the foot. Thus, it is plausible that *HyBMP5-8b* plays a general role in the initiation and formation of both the head and foot organizers, but not in their maintenance.

One other piece of evidence supports this view. Transplantation experiments show that head organizer formation begins the earlier the further up the body column bisection occurs (Webster and Wolpert, 1966). The initiation of *HyBral* expression follows this pattern as it begins to be expressed later when the bisection occurs in mid-body column compared to bisection at the head/body column border (Technau and Bode, 1999). Conversely, foot organizer formation begins earlier the further down the body column the head removal occurs (Mookerjee and Bhattacharjee, 1967). In contrast, *HyBMP5-8b* expression starts within an hour regardless of the axial level of bisection. This may reflect that the role of the gene in the building of the organizer begins very early during head or foot regeneration as it does during bud formation.

It would be of interest to determine the activity of BMP signaling during head and foot organizer formation by monitoring the staining pattern of a α -PS1 antibody to generate further support for this hypothesis.

5.3.2. A role in axial patterning

HyBMP5-8b is also expressed in the ectodermal epithelial cells of the budding zone and the peduncle. Expression is fairly uniform in this region. The border is very sharp at the peduncle/foot boundary, and fairly sharp at the budding zone/upper body column border. In the context of the tissue dynamics of the body column, tissue is continuously displaced down the *Hydra* body column through the budding zone and

peduncle onto the foot. Expression of *HyBMP5-8b* is constantly initiated as body column tissue is displaced into the budding zone, and later abruptly turned off as the tissue moves off the peduncle onto the foot. Thus, the gene could play a role in specifying the budding zone and peduncle regions of the oral-aboral axis of the body column. This role for the gene could be similar to that of BMP2/4 orthologues, which define a particular portion of the dorsal-ventral axis in *Xenopus* and *Drosophila* (Hemmati-Brivanlou and Thomsen, 1995; Irish and Gelbart, 1987; Wharton et al., 1993). Additionally, the gene could be involved in the differentiation of nematoblast cells into nematocytes in this region. Preliminary data using the α -PS1 antibody showed a staining of cell nuclei of nematocyte cells in the peduncle, indicating that BMP signaling is active in those cells.

Two factors control the expression of *HyBMP5-8b* in this region. One of them is the positional value gradient, which is a major factor in controlling axial patterning in the body column. Treatments with reagents that change the positional value gradient along the body column support this view. When treated with 0.5 mM LiCl, which lowers the positional value gradient, the range of expression expands up the body column. Conversely, when treated with alsterpaullone, which suddenly and drastically raises the level of positional value throughout the body column (M.Broun and H.Bode, personal communication), the expression of *HyBMP5-8b* in the lower third of the body column vanishes within one day. These responses indicate that the gene is activated when the level of positional value drops below a certain threshold value. The foot also exerts control over the expression of the gene in the lower half of the body column. When the foot is removed, expression in the budding zone and peduncle drops. Once the foot has regenerated, the level of expression of *HyBMP5-8b* eventually regains a normal level.

Hence, the gene probably plays a role in specifying tissue of part of the body column and its expression is controlled by processes, that control axial patterning along the oral-aboral axis.

5.4. *HyBMP5-8b* plays a role in tentacle formation

Because of the tissue dynamics morphogenetic and differentiation processes are continuously active in *Hydra* (Campbell, 1967a). A striking example in which both

types of processes take place involves tentacle formation. The expression patterns of *HyBMP5-8b* related to tentacle formation indicate it plays a role in one or both of these processes.

During bud formation and head regeneration, the gene begins to be expressed in a ring of spots below the apical cap of a bud that has reached the cylindrical protrusion stage. Shortly thereafter tentacle buds emerge from the spots. This suggests that the gene may be required for initiation of tentacle formation. This view is reinforced by the tight correlation between the appearance of spots and tentacle formation. In the head regeneration deficient mutant, *reg-16*, in which tentacles are rarely formed, *HyBMP5-8b* is expressed only when tentacles appear. And, when an ectopic tentacle is induced along the body column, its appearance is always preceded by the expression of the gene as a spot on the body column, from which the ectopic tentacle subsequently emerges.

Once the tentacles have formed, the gene is constantly expressed at the tentacle zone/tentacle border indicating it continues to play a role as tentacle zone tissue is converted into tentacle tissue. Conversion of tentacle zone tissue into tentacle tissue takes place continuously as part of the tissue dynamics involving the displacement of tissue from the body column through the tentacle zone onto the tentacles and eventual loss at the tentacle tips.

5.4.1. Two possible roles for *HyBMP5-8b* during tentacle formation

The first is in the actual morphogenesis of a tentacle. This is initiated by the evagination of small circular region of the tentacle zone tissue. Subsequently, the evaginating epithelial cells of both layers change shape from the cylindrical cells of the tentacle zone to the flat, squamous battery cells of the tentacle (Campbell, 1967b; Dubel et al., 1987). The expression of *HyBMP5-8b* in a narrow band at the border could mean it is involved in this transition in cell shape resulting in the evagination. As tissue continues to be displaced onto the tentacles from the tentacle zone, epithelial cells continue undergo changes in shape. One gene known to play a role in this process is *HyAlx*, a *Hydra aristaless* orthologue, which has a similar expression pattern to that of *HyBMP5-8b* (Smith et al., 2000). Newly developing tentacles evaginate through spots of *HyAlx* during head formation, and the gene is expressed in a narrow band at the tentacle zone/tentacle border in the adult (Smith et al., 2000). The one difference is

that *HyAlx* is expressed in the ectoderm instead of the endoderm. Using RNAi during bud formation results in a significant delay in the appearance of tentacles indicating the gene plays a role in the morphogenesis of a tentacle (Smith et al., 2000). Given the similarity in the expression pattern in the tentacles to *HyAlx*, *HyBMP5-8b* could play a role in morphogenesis.

The second possible role of *HyBMP5-8b* could be related to differentiation. As the epithelial cells of both layers are displaced across the tentacle zone/tentacle border, they undergo dramatic changes in their cellular properties. While in the tentacle zone, the epithelial cells are proliferating, but as they cross the tentacle zone/tentacle border and enter the tentacle, they become permanently arrested in the G2-phase of the cell cycle (Dubel et al., 1987). At the same time, the ectodermal epithelial cells begin to undergo terminal differentiation to form the tentacle-specific battery cells (Campbell, 1967b; Dubel et al., 1987). These changes occur suddenly, as a cell in the tentacle zone expresses different genes than does its immediate neighbor on the tentacle side. For example, *CnOtx* (Smith et al., 1999) and *Cnox3* (Bode, 2001) are expressed in the ectodermal epithelial cells of the tentacle zone. However, the expression of both of these genes stops suddenly at the border. Conversely, as cells cross the border, several genes not expressed in the tentacle zone are expressed at a high level as soon as the cells enter the tentacle. These include an insulin receptor homologue, HTK (Steele et al., 1996), an annexin gene (Schlaepfer et al., 1992), TS19, which is a cell-surface antigen (Bode et al., 1988), and a *Hydra* metalloproteinase, HMP1 (Yan et al., 1995). In this situation, the narrow band of expression of *HyBMP5-8* at the border could facilitate the switch in expression of a number of these genes. Additional support for this hypothesis is the preliminary data using the α -PS1 antibody. The cell nuclei of nematocytes of the tentacle are stained indicating that BMP signaling is active in those cells. *HyBMP5-8b* is not expressed throughout the tentacle but as the cells move into the tentacle they cross the tentacle zone/tentacle border and are under the influence of *HyBMP5-8b*. As BMP genes encode secreted extracellular ligands and the translated proteins are able to act several cell diameters away from their synthesis.

Thus, *HyBMP5-8b* could be involved in the morphogenesis of a tentacle or in the differentiation of the tentacle-specific cells, or it might be involved in both processes. More direct evidence has not yet been obtained since the procedure for introducing dsRNA has been successfully performed so far only with genes expressed

in the ectoderm (Lohmann et al., 1999; Smith et al., 2000), while *HyBMP5-8b* is expressed in the endoderm.

5.5. Differences in *HyBMP5-8b* expression in bud formation and head regeneration can be explained by Meinhardt's model

As mentioned above there is a difference between the expression pattern of *HyBMP5-8b* during head regeneration and budding in the early stages. This difference can be explained with the different dynamics of organizer formation during budding and head regeneration (see chapter 5.3.1.) or with the reaction-diffusion model of Meinhardt, (1993). During head regeneration, *HyBMP5-8b* is initially expressed over the entire presumptive head region, and only later is expression restricted to spots located in the presumptive tentacle zone. In contrast, during bud formation, the gene is never expressed at the tip in the presumptive hypostome, but only in the region below in a spotlike pattern where tentacles will form. Similar differences in expression during bud formation and head regeneration have been observed with other tentacle markers such as *HyAlx* (Smith et al., 2000) and TS19 (Bode et al., 1988).

These results reflect a difference in the patterning processes during head regeneration and bud formation that are consistent with a reaction-diffusion model (Meinhardt, 1993) of the patterning events governing the adult steady state of *Hydra*. The model predicts the sequential formation of hypostome and tentacle zone during head regeneration and bud formation. However, they occur in different orders. During head regeneration apical tissue is initially specified to form tentacles. Subsequently the apical end is specified to form the hypostome which forces tentacle formation into a ring below the apex. Conversely, during bud formation, the apical end is specified to form the hypostome, and the tentacle zone is specified below at a later step. This model nicely explains the difference in the expression patterns of *HyAlx*, the TS-19 antigen and *HyBMP5-8b* during head formation in the two developmental processes. Each marker is initially expressed at the apical end during head regeneration. Subsequently, expression occurs in a ring of spots below the apex (Bode et al., 1988; Smith et al., 2000).

However, during bud formation, none of the markers is initially expressed at the apex. Instead each is only expressed below the apex (Smith et al., 2000; Technau and

Holstein, 1995). *HyAlx* first forms as a continuous ring around the base of the apex, which subsequently breaks up into a ring of spots (Smith et al., 2000). In a slightly different pattern both the TS-19 antigen and *HyBMP5-8b* appear as a ring of spots below the apex.

Thus, the expression pattern of *HyBMP5-8b* associated with tentacle formation adds to the evidence supporting Meinhardt's model that a reaction-diffusion model provides a framework for the mechanisms underlying axial patterning in *Hydra*.

It should be noted that the initial expression of *HyBMP5-8b* in the cap during head regeneration could have two roles. One in tentacle formation as just described, and one in organizer formation as described in the previous section.

5.6. *HyBMP5-8a* and *HyBMP5-8b* in embryogenesis and oogenesis

It has been shown that *HySmad1* is significantly upregulated throughout oogenesis (Hobmayer et al., 2001), therefore, I examined the expression of *HyBMP5-8b* during this phase. However, expression can not be detected at any stage of the development of the oocyte, indicating that *HyBMP5-8b* is not involved in germ-cell specific differentiation processes and is not responsible for the upregulation of the expression of *HySmad1*. A possible candidate gene for *HySmad1* activation might be an orthologue of a BMP2/4 protein.

Bilaterian BMP genes have been implicated in patterning the early bilaterian embryo (Hogan, 1996a; Patterson and Padgett, 2000; Raftery and Sutherland, 1999; Zhao, 2003). Expression data for the *Acropora* homologue of the bilaterian BMP2/4 genes, *bmp2/4-Am*, suggests a role of this gene in axial patterning as well. *Bmp2/4-Am* is expressed during gastrulation of the anthozoan embryo and seems to be specifically associated with the blastopore (Hayward et al., 2002).

Technical difficulties and low availability of embryos precluded detection of *HyBMP5-8a* and *HyBMP5-8b* expression during embryogenesis. However, using RT-PCR analysis, showed that both *HyBMP5-8a* and *HyBMP5-8b* are expressed at a low level during different stages of embryogenesis. It would be of interest, to determine if the role of BMP genes in axial patterning during embryogenesis is conserved. Since *in situ* hybridization on *Hydra* embryos is problematic it would be helpful to explore BMP function in early cnidarian embryos in different members of the family, that do

not impose such difficulties. One example is the aforementioned anthozoan *Acropora*. But more convincing expression data in cnidarian embryos have been generated using the sea anemone *Nematostella* (Scholz and Technau, 2003), making this anthozoan a promising model organism for early cnidarian development.

5.7. Evolution of BMP function

In bilaterians, BMP signaling molecules are involved in a variety of developmental processes ranging from pattern formation and morphogenesis, to growth and differentiation. In some embryos a single BMP gene may play multiple roles during the course of development (Hogan, 1996b; Zhao, 2003).

In bilaterians, BMP genes are expressed in many embryonic organs and tissues, including those in which reciprocal interactions between different cell layers are important for morphogenesis and differentiation (Hogan, 1996a). In the *Drosophila* embryo, for example, *dpp* together with *wg* is required for the initial specification of cardiac mesoderm (Frasch, 1995; Jagla et al., 1997; Wu et al., 1995). An ascidian BMP orthologue induces differentiation of a specific anterior cell type of pigment cells, the otolith (Darras and Nishida, 2001). It has been shown that BMP ligands in *Xenopus* and in *Drosophila* are important for the specification of the embryonic ectoderm (Hemmati-Brivanlou and Melton, 1997; Rusch and Levine, 1996; Sasai and De Robertis, 1997).

With respect to morphogenesis, vertebrate BMPs can trigger cartilage formation when implanted at ectopic sites in adult animals (Hogan, 1996a). More specifically, it has been shown that BMP ligands are important for the initial steps of chondrogenesis: they are involved in the formation of the prechondrogenic condensations and have a role in the transition of condensed chondroprogenitors into chondrocytes (Pizette and Niswander, 2000). BMPs together with Fgfs also mediate the branching morphogenesis of the lung (Weaver et al., 2000; Weaver et al., 1999). In the chick feather bud and the chick limb, BMPs have been shown to modulate outgrowth promoted by a stimulatory Fgf signal (Jung et al., 1999; Niswander and Martin, 1993; Noramly and Morgan, 1998; Pizette and Niswander, 1999).

In an adult *Hydra*, *HyBMP5-8b* appears to have a number of roles, including tentacle morphogenesis and differentiation of cells at the tentacle/tentacle zone border. It is also involved in two patterning processes: initiation of a new axis during bud

formation, and the specification of tissue at the aboral end of the oral-aboral axis. That this gene may have several functions in an adult *Hydra* is not surprising since these developmental processes are continuously active in the context of the tissue dynamics of the animal.

The activity of *HyBMP5-8b* during tentacle formation may involve morphogenesis as well as differentiation. The expression pattern in the lower body column suggests *HyBMP5-8b* could be specifying tissue to form the budding zone and eventually to form a bud. The gene could also be involved in the differentiation of nematocyte cells, as the experiments with the α -PS1 antibody indicate. This would be consistent with the results obtained for the expression of the R-Smad gene, *HySmad1*, in a fraction of developing nematocyte nests as well as in individual, differentiated nematocytes in the gastric region. Furthermore, a cell type northern hybridization for *HySmad1* also indicates that BMP signaling might be involved in nematocyte differentiation.

Thus, our results show that BMP signaling in differentiation and morphogenesis of tissues is a conserved feature and might have been present in the earliest multicellular animals.

Among bilaterians, BMPs are also involved in laying out the general positional information in the very early embryo. For example, in *Drosophila*, the BMP ligands *dpp* and *screw*, have been shown to participate in the regulation of dorsal-ventral patterning (Raftery and Sutherland, 1999). Similarly, in vertebrates, BMPs play roles in dorsal-ventral patterning of the early embryonic mesoderm. In *Xenopus*, for example, BMP2, 4, and 7 ventralize early mesoderm (Graff, 1997). In *Hydra*, the expression of *HyBMP5-8b* in the lower third of the adult *Hydra* polyp could be involved in the maintenance of the aboral region of the oral-aboral axis in the steady state adult animal.

More specifically, there is an analogy with axis formation in *Xenopus*. The Wnt pathway, and more specifically, β -catenin is involved in setting up the organizer which defines the dorsal end of the *Xenopus* embryo. BMP4 in conjunction with BMP7 defines the ventral end. In *Hydra*, the head organizer defines the oral end of the axis. *HyWnt* signaling has been shown to be involved in the development and maintenance of the head organizer (Hobmayer et al., 2000). And, as in the *Xenopus* embryo, *HyBMP5-8b* is expressed at the opposite end of the oral-aboral axis. An interaction between Wnt signaling and BMP signaling appears to be conserved in *Hydra*, as shown

by the disappearance of *HyBMP5-8b* expression in the body column following alsterpaullone treatment. Alsterpaullone is a specific GSK-3 β inhibitor whose action leads to a constitutively active wnt signaling along the *Hydra* body column and treatment subsequently converts the body column into tissue with head organizer properties (M.Broun and H.Bode, personal communication). This change is reflected in the expression of *HyWnt* all over the body column instead of only in the hypostome, and in the disappearance of *HyBMP5-8b* expression from the body column.

However, this analogy with the molecular mechanisms for setting up the vertebrate organizer and the dorsal-ventral axis has its limitations. The fact, that *HyBMP5-8b* is also expressed during the initiation of bud formation in the location where the head organizer will be set up indicates that the gene may also play a role in the very early stages of axis formation, or head organizer formation. The strong expression during head and foot regeneration supports this view.

In sum, the roles of *HyBMP5-8b* are in general analogous to roles exhibited by BMP genes in bilaterians. More specifically, the function of *HyBMP5-8b* in patterning events resemble those of bilaterian BMPs, suggesting that the function of these genes arose early in metazoan evolution, prior to the divergence of the Bilateria and diploblasts. The finding of several TGF- β receptors in the sponge *Ephydatia* supports this view (Suga et al., 1999). It would be of interest to determine the function of BMP signaling in those simple metazoans, to investigate if the role of this signal transduction pathway in axial patterning is a common trait of all metazoan animals, or constitutes only eumatazoans.

6. Summary

Developmental gradients play a central role in axial patterning in the diploblastic *Hydra*. As part of the effort towards elucidating the molecular basis of these gradients as well as investigating the evolution of the mechanisms underlying axial patterning in metazoan animals, genes encoding signaling peptides are under investigation.

I report the isolation and characterization of *HyBMP5-8b*, a BMP5-8 orthologue, from *Hydra*, a member of the diploblastic Cnidaria. Processes governing axial patterning are continuously active in adult *Hydra*. Expression patterns in normal animals and during bud formation were examined. These patterns, coupled with changes in patterns of expression in manipulated tissues during head regeneration, foot regeneration as well as under conditions that alter the positional value gradient indicate that the gene is active in three different processes. The gene plays a role in tentacle formation, in patterning the lower end of the body axis, and in early stages of head and foot organizer formation.

During early embryonic development of the Bilateria, a number of secreted proteins and their receptors provide positional information that determines the polarity of body structures. One example of this process is the development of the fly wing, where orthogonal gradients of Wg and dpp, determine the shape of the wing. Another example is the determination of ventral fates in early vertebrate embryos by BMPs, whereas activation of the Wnt-signaling pathway sets up the dorsal part of the embryo and induces the organizer. A central molecular mechanism of induction by the organizer resides in the inhibition of BMP signaling.

The organizer region at the oral end of the body axis in *Hydra*, responsible for setting up developmental gradients in the body column, is defined and maintained by Wnt signaling. Treatment with Alsterpaullone, a GSK-3 β inhibitor, leads to a constitutively active Wnt signaling along the *Hydra* body column and subsequently converts the whole body column into tissue with head organizer properties, with *HyWnt* expression all over the body column. Furthermore, the treatment leads to inhibition of *HyBMP5-8b* expression in the lower part of the body column. Thus, the interaction of Wnt and BMP signaling in setting up polarity during axis formation might be a common feature of eumetazoans.

6. Zusammenfassung

Gradienten spielen eine zentrale Rolle während der Achsenbildung in *Hydra*. Gene, die Signalpeptide kodieren werden charakterisiert, um die molekulare Natur dieser Gradienten zu verstehen. Außerdem tragen diese Untersuchungen dazu bei, die Evolutionsmechanismen zu erkennen, die an der Achsenbildung in Vielzellern beteiligt sind.

In dieser Arbeit präsentiere ich die Isolation und Charakterisierung des zu BMP5-8 Genen orthologen *Hydra* Genes, *HyBMP5-8b*. Die Expression des BMP Genes wurde in normalen adulten und knospenden Tieren untersucht. Außerdem wurde die Expression während der Kopf- und Fußregeneration und in Tieren deren Positionswertgradient verändert wurde, beobachtet. Diese Experimente zeigen, dass *HyBMP5-8b* während dreier verschiedener Prozesse aktiv ist. Es ist wichtig für die Tentakelbildung und für die Musterbildung im basalen Teil der Körperachse. Außerdem scheint es für die frühen Stadien der Neubildung des Organisators wichtig zu sein.

Verschiedenste sekretierte Proteine und deren Rezeptoren sind während der frühen Embryonalentwicklung der Bilaterier für die Bereitstellung von Positionsinformation verantwortlich, die zur Polarität von Körperstrukturen führt. Ein Beispiel dafür ist die Entwicklung des Insektenflügels, in dem orthogonale Wg- und dpp-Gradienten die Form des Flügels bestimmen. Ein weiteres Beispiel ist der ventralisierende Einfluss von BMPs auf Gewebe des frühen Froschembryos. Der Wnt-Signaltransduktionsweg hingegen ist verantwortlich für die Festlegung der dorsalen Seite des Froschembryos und induziert den Organisator. Die induzierende Wirkung des Organisators besteht unter anderem in der Inhibierung von BMP-Liganden.

Der Organisator im Hypostom der *Hydra*, verantwortlich für die Induktion und den Erhalt der Positionswertgradienten in der Körperachse, wird wahrscheinlich durch die Wnt-Signaltransduktionskaskade aufrechterhalten. Behandlung mit Alsterpaullone, einem GSK-3 β Inhibitor, führt dazu, dass die Gewebe der ganzen Körperachse Kopforganisatoreigenschaften besitzen, da die Wnt-Signalkaskade überall aktiv ist. Das äußert sich in der Expression von *HyWnt* entlang der gesamten Körperachse und in der Inhibierung der *HyBMP5-8b* Expression im unteren Teil der Körperachse. Dies deutet darauf hin, dass die Interaktion von Wnt- und BMP-Signaltransduktionskaskaden bei der Festlegung von Polarität während der Achsenbildung eine gemeinsame Eigenschaft der Eumetazoen ist.

7. References

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8. Appendix

8.1. Abbreviations

AP	Alkaline Phosphatase
APS	Ammonium Persulfate
BMP	Bone morphogenetic protein
Chaps	(3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate)
cm	centimeter
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DM	dissociation medium
DMFA	N,N'-dimethyl-formamide
DMSO	dimethyl sulfoxide
Dpp	decapentaplegic
DTT	Dl-Dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
dATP	deoxynucleotide adenosin triphosphate
dNTP	deoxynucleotide triphosphate
EtOH	ethanol
HM	hydra medium
in. Hg	inches of mercury
IPTG	isopropylthio- β -D-galactoside
KLH	hemocyanin, keyhole limpet
LB	Luria-Bertani
min	minutes
MOPS	(3-[N-Morpholino]propane-sulfonic acid)
NBT/BCIP	Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate
ORF	open reading frame
rpm	rotations per minute
RT	reverse transcription
SDS	sodium lauryl-sulfate

TES	(N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid)
TGF- β	transforming growth factor- β
TMAC	tetramethylammonium chloride
Tris	trihydroxymethylamino methan
Tween 20	polyoxyethylene-sorbitan monolaurate
UTP	uridine triphosphate
μg	microgram
UTR	untranslated region
μl	microliter
μM	micromolar
λ -Phage	lambda-phage
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

8.2. Nucleotide and predicted amino acid sequence of *HyBMP5-8a*

Nucleotides and amino acids are numbered on the left. The nucleotide sequence contains a partial ORF, encoding a protein of 296 amino acids. The open rectangle marks positively charged residues that conform to the RXXR cleavage signal for proteolytic processing. Squares denote potential N-glycosylation sites with the sequence N-(X)-[ST]-(X), where (X) is any amino acid but proline. The conserved C-terminal region, containing seven cysteine residues is marked red. The cysteine residues are encircled.

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1  L  Q  R  A  K  K  L  Q  I  F  L  D  V  S  Y  K  T  V  P  L
2  CTCCAACGAGCCAAGAAGCTTCAGATTTTTTTAGATGTTTCTTACAAAAGTGTGCCTCTA

21 N  K  F  R  L  E  V  F  K  I  V  V  P  K  K  K  Y  I  L  L
62 AATAAGTTTAGATTAGAAGTTTCAAGATAGTTGTTCTTAAGAAAAAGTACATACTACTA

41 D  S  K  I  I  M  A  S  V  S  Q  W  H  E  F  N  V  L  E  A
122 GACTCTAAAATCATAATGGCTTCAGTTTCTCAATGGCATGAATTTAATGTATTAGAAGCT

61 M  L  S  W  V  K  Y  S  E  T  N  N  G  I  L  I  V  C  K  S
182 ATGCTGAGTTGGGTTAAATATTCAGAAACAAATAATGGTATTCTGATAGTGTGTAAGT

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81 L Q Q E A I L V E N C G V V G F K G S K
242 CTACAGCAAGAAGCCATTCTTTGTTGAAAACGTGGTGTGGATTAAAGGTAGTAAA

101 D Y Q P F L V S F Y Q S S K N E E F F A
302 GATTATCAGCCATTTCTTGTTCATTTTACCAATCCAGTAAAAATGAGGAGTTTTTTGCT

121 L K A P D F D S T S R L A Q H Q Y F L D
362 TTGAAGGCTCCTGACTTTGACTCAACATCAAGACTTGTCAACACCAATACTTTCTAGAT

141 N K F K T S K S A R L P K R F T R G I E
422 AATAAGTTCAAAACTTCTAAATCTGCAAGACTTCCAAAACGGTTTACGCGTGGTATAGAA

161 D D D F L K A A A T L A E K N N F V Q S
482 GATGATGATTTTTTAAAGCAGCTGCTACTCTGGCTGAAAAAATAACTTCGTCAAAGT

181 K L I S K R F K N N N I S D C G K H L L
542 AAGTTAATTTCAAAAAGATTTAAAAATAACAATATATCTGACTGTGGGAAACATCTTTTA

201 Y V S F K D I G W S D W I I A P D G Y I
602 TATGTGTCATTTAAGGATATTGGGTGGTCCAGACTGGATTATAGCACCCGATGGTTATATA

221 T S Y C G G D C S Y P L E S N L N A T N
662 ACCAGTTACTGTGGAGGTGATTGTAGTTATCCACTGGAGAGTAATTTAAATGCAACTAAC

241 H A I L Q A L V H T I F P K K I P K P C
722 CATGCAATCTTACAAGCACTAGTGCATACAATATTTCCAAAGAAAATTTCCAAAACCTTGT

261 C A P N K L N A M S I L F F D D R N N V
782 TGTGCTCCAAATAAACTCAATGCAATGTCAATTTTATTCTTTGACGATCGTAATAATGTT

281 V M Q E I P N M I V H Q C G C Q
842 GTTATGCAAGAAATACCAAACATGATTGTTTCATCAATGTGGATGCCAGTAACTTAAAAAT
902 ATACAAATGAGGAGTTTATTTTCAAAACGTGGTATGAGCAATTTTGTTTTATGTGCCGT
962 TTTAAATCTTATTTAGTTTAAAAAATAATAATTTAATCATGAAAATCCAAAAA
1022 AAAAAAAAAAAAAA

8.3. Nucleotide and predicted amino acid sequence of *HyBMP5-8b*

Nucleotides and amino acids are numbered on the left. The nucleotide sequence contains a complete ORF, encoding a protein of 404 amino acids. The signal peptide sequence consisting of the first 22 bp of *HyBMP5-8b* was predicted by using the search program at <http://www.cbs.dtu.dk/services/SignalP/>, Nielsen et al., (1997) and is underlined. The open rectangle marks positively charged residues that conform to the RXXR cleavage signal for proteolytic processing. Squares denote potential N-glycosylation sites with the sequence N-(X)-[ST]-(X), where (X) is any amino acid but proline. The conserved C-terminal region, containing seven cysteine residues is marked red. The cysteine residues are encircled.

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1  AGACAAACAGCTTTTATATAACTCATGGTCAATTCATTTTATTTCTCATAAACTTGTTTT
61  ATTATTAATAAAAAGCCGAGTTACCGAGTACTATTAGATTGCCATTACAAGAGAAAAAA

                                     M K V T L L S I I
121  AGTTAAATTTATATAAATGTCAGTGCCACTGAAATGAAAGTAACGTTATTAAGCATTATA

10  R L I V Y V F V N F V N G I P T K E R N
181  AGATTGATTGTTTATGTTTTTGTGAATTTTGTGAATGGGATTCCAACATAAGAGCGAAAT

30  I I Q N T L L D L F G K H N R P R P D V
241  ATCATACAAAATACTTTGCTTGATCTATTTGGAAAACATAACCGTCCAAGACCCGACGTT

50  F S H L G R N E T S A K K Y M L D L Y E
301  TTCTCTCATTTAGGTAGAAATGAAACGTCAGCTAAGAAATACATGCTAGATTTGTATGAG

70  Y S V N T E R Q G M N N F T T N S Q N I
361  TATCCGTAACACAGAACGTCAAGGTATGAATAACTTCACAACCAATTCACAAAATATA

90  S N I V D D A D T V V S F L N N A Y V P
421  TCAAATATTGTTGACGATGCGGATACAGTTGTCAGCTTCTAAACAATGCATATGTACCA

110  R P N T T D E S G E M F F D V S S N Y V
481  AGACCTAATACTACTGACGAGTCGGGTGAAATGTTTTTTGATGTATCTTCGAACTATGTC

130  V E K V L A T A L Q V Y L D I T Y K T V

```


541 GTAGAAAAAGTACTTGCTACTGCTCTTCAAGTATACCTTGATATTACCTATAAAACTGTG
150 I A E K L I I S V Y K I V V P K K K Y V
601 ATCGCAGAAAAGTTAATAATTTCTGTTTATAAGATAGTTGTCCCTAAGAAAAAGTACGTA
170 L L A S K L I N A S V S Q W H E F N V L
661 CTGCTGGCCTCAAACATAATTAACGCATCAGTTTCTCAATGGCATGAGTTTAATGTTTTA
190 E A S L S W I E F S E T N N G I L L V C
721 GAAGCTTCATTAAGCTGGATTGAGTTTTTCAGAACTAATAATGGAATATTATTAGTATGT
210 Q N L Q K V N I P I E S C G I V D F K G
781 CAAAATCTACAAAAGGTAAATATACCTATTGAAAGTTGCGGAATCGTTGATTTTAAAGGT
230 R E E F R P F L V S F Y Q S G K E E E F
841 CGAGAGGAATTCAGACCATTTTTTAGTATCTTTTTACCAATCCGGTAAGGAGGAAGATTT
250 P A K Q I R N T E S T L K L Q E R L R R
901 CCCGCAAAACAAATTCGAAATACCGAATCAACTTTAAACTTCAAGAACGTTTAAGACGT
270 S M Q D S I F I E A A E Q L S F V R N K
961 AGTATGCAAGATAGTATTTTTTATAGAAGCTGCAGAACAGTTGAGTTTCGTTTCGAAATAAA
290 T R S N G S E N N L G S R C D K H P L Y
1021 ACGCGTTCAAATGGCTCGGAAAATAATCTTGGATCTCGTTGTGATAAACATCCATTATAT
310 I G F K D L G W S D W I I A P D G Y R A
1081 ATTGGATTTAAAGATTTAGGGTGGTCGGATTGGATTATAGCACCTGATGGCTATCGTGCA
330 N Y C G G D C S F P L D N N A N A T N H
1141 AATTACTGTGGAGGAGATTGCAGTTTTCCACTTGATAATAACGCAAACGCAACAAATCAT
350 A I I Q T L V H M M Y P E I I P K P C C
1201 GCAATTATACAAACACTTGTTTCATATGATGTATCCAGAGATCATTCCAAAGCCCTGTTGT
370 A P N K L N T L Q V L F L D E R N N V V
1261 GCGCCTAATAAGCTGAACACATTACAGTTTTTATTCTTGATGAACGCAATAATGTTGTT
390 M K R Y S N M I V Q H C G C Q
1321 ATGAAGCGCTATTCAAACATGATAGTCCAACACTGTGGATGCCAATAGGTTGAGTAAGAT
1381 AAGGTTTTCAAATACCGATAAACTGCCAAAAGCTAAGACATTAGTTATTCTGTATTCGT

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1441 AGAAAGCAGTTATTTAAACCTAATTTGAATAAAGTAATACAGCGAAGACATCATTGTGGT
1501 CTTTATTCTATAACGTGAAACAACCTTTTTGAAAGTCTCAGACAACATAAAGTGCGAGGTAA
1561 TATTTAAAAAAGTGTAAAAAAATTCAAACGTGAGAACATAAAAATGTATGGGTCAAGTAAA
1621 TTTAATTGAATCATAAACATATACCAAAAAACTTCAGAAATATATTGATGCATTTATATA
1681 TCATAAAAAATCTTTTACCTGTCTGCAGTTTAGTGGTACCAAAGTTTGTGTGCAGAGTGA
1741 CAGTGGTAACAGATGTTTGTATTGTTTATCTTAAGCAAATACATTTGTAATATACAGTT
1801 GAAAATATAAAATTATAAAAAAAAAAAAAAAAAAAAA

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8.4. Alignment of *Hydra* BMPs with other BMP proteins

CLUSTAL X (1.81) multiple sequence alignment

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BMP2/4 Branchiostoma b.      SWESFDVRSVAVTKWKNSPERNYGLEVEVVS PKRGALSNNHHVRLRRS
BMP2/4 Ptychodera f.        SWESFDIRPAVAKWKASQEENHGVEVELTEVQNSQISPHHVRLRRS
BMP2/4 Strongylocentrotus p. SWESFDVRPAMRVWLEEPKNGHLEIELIDSRGRPSPNHHVVRTRE
BMP2/4 Archaster t.         SWHSFDVRPAIRDWRRSHLANHGIEVEVLDHRGRPIAAHHLRIARS
BMP2 Homo s.                RWESFDVTPAVMRWTAQGSHANHGFFVEVAHLEEKQGVV-HVRISSRS
BMP4 Homo s.                RWETFVSPAVLRWTREKQPNYGLAIEVTHLHQTRTHQ-HVRISSRS
BMPb Halocynthia r.        TWERFDVSAETQKWLKDK--NHGLVVEIVRDGDESTPAPHVRLRRD
BMP2/4 Patella v            SWESFDVHPAVLKWKRKPSYNYGIEVRIVS--DSPSISTHVRLRRS
BMP2/4 Crassostrea g.       SWESFDIHPAVLKWKNPTLNHGLKVRVLSFKNKPSTDSHVRLRRD
dpp Drosophila m.           DTVSLDVQPAVDRWLASPQRNYGLLVEVRTVRSCLKPAPHHVRLRRS
dpp Tribolium c.            TTVSIVDFPVARWMDPKTNHGILIVVYSIGAKKSPPEHLRLRR--
BMP2/4 Acropora m.         --TSFDVKQAVQHWLQNPPEFNGLEVHLMDSANSPVTHVSLSEDGT
GDF2 Homo s.                GWETLEVSSAVKRWVRS DSTKSKNKLEVTVES-HRKGCD-LDISVP
BMP10 Homo s.               EWETFVDVTDAIRRWQKSGS--STHQLEVHIESKHDEAEDRLEIDTS
BMP5 Homo s.                GWLVFDITVTSNHVVINPQNNLG--LQLCAETGDGRSINSAGLVGR
BMP6 Homo s.                GWLEFDITATSNLWVVPQHNMG--LQLSVVTRDGVVHHAAGLVGR
BMP7 Homo s.                GWLVFDITATSNHWVVNPRHNLG--LQLSVETLDGQSINLAGLIGR
BMP7 Danio r.               GWLVFDLTVTSNHVVINPQNLG--LQLLVETSHGARMNRAGLVGS
BMP7 Xenopus l.             GWLTFDITATGNHVVMPHYNLG--LQLSVESMDMQNVNLVGLVGR
BMP8 Homo s.                GWLVLDVTAASDCWLLKRHKDLG--LRLYVETEDGHVSDLAGLLGQ
BMPa Halocynthia r.        GWLVFDITSATRDWVEVQESNLG--IRVAVETREGRSVNKAGIVGK
HyBMP5-8b Hydra v.         QWHEFNVLEASLSWIEFSETNNG--ILLVCQNLQKVNI PSCGIVDF
HyBMP5-8a Hydra v.         QWHEFNVLEAMLSWVKYSETNNG--LIVCKSLQQEAILNCGVVGF
BMP5-8 Strongylocentrotus p. GWLVFDMTSATSTWRSYPGANVG--LQLRVESLQGLNIDDAGVVG
60a Anopheles s.           GWLEINVTGAVNLWLKNRQANHGGLYIGAYFGDRVEREKDIGFVSA
60A Drosophila m.          GWLELNVTEGLHEWLKSKDNHGIYI GAHAVNRPDREVKDIGLIHR
screw Drosophila m.        GWLEFNLTDTLRYWLHNKGLQRRNELRISIG---DSQLSAAGLVTP
gdf3 Homo s.                GAVHFNLLDVAKDWNNDNPRKNFGLFLEILVKEDRDSGVNDTCARLR
GDF5 Homo s.                GWEVFDIWKLFNRNFKNSAQ---LCLELEAWERGRAVDLGLGFDRA

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BMP2/4 Branchiostoma b.	RPLLLTYTDDGKANCRRHSLYVDFSDVGWNDWIVAP
BMP2/4 Ptychodera f.	RPLLLTYTDDGKPNCRRRSRYVDFSDVGWNDWIVAP
BMP2/4 Strongylocentrotus p.	RPQIVTYSDDGKANCRRHLYVDFSDVHWNDWIVAP
BMP2/4 Archaster t.	RPLLVTFDDGKPNCRRHPLYVDFDVGWNSWIVAP
BMP2 Homo s.	RPLLVTFGHGDS-SCRRHPLYVDFSDVGWNDWIVAP
BMP4 Homo s.	RPLLVTFGHGDNKNCRRHSRYVDFSDVGWNDWIVAP
BMPb Halocynthia r.	RPLLLTYTHDGK-SCQRQDLYVDFSDVNWDWIVAP
BMP2/4 Patella v	RPLLVTYTDDGKNECKRHVLYVDFDVGWNDWIVAP
BMP2/4 Crassostrea g.	RPLLVTFDDNNQCRRKELNVDFKAVGWNWIFAP
dpp Drosophila m.	QPLLFYTDGDDTCRRHSRYVDFSDVGWDDWIVAP
dpp Tribolium c.	QPLLFYTDGDKPCRRRQMYVDFSGVWNDWIVAP
BMP2/4 Acropora m.	KPLLLTFSHDR--YQQRHPLYVDFSEVGWNDWIVAP
GDF2 Homo s.	LFFFVFSNDHSGHCQKTSLRVNFEDIGWDSWI IAP
BMP10 Homo s.	NPLLVFSDDQGNVCKRTPLYIDFKELIGWDSWI IAP
BMP5 Homo s.	QPFMVAFFKASKQACKKHELYVSRDLGWQDWI IAP
BMP6 Homo s.	QPFMVAFFKVKSTACRKHLYVSRDLGWQDWI IAP
BMP7 Homo s.	QPFMVAFFKATRQACKKHELYVSRDLGWQDWI IAP
BMP7 Danio r.	QPFMVAFLKASKQCKKHELYVSRDLGWQDWI IAP
BMP7 Xenopus l.	QPFMVAFFKTSKQACKKHELYVSRDLGWQDWI IAP
BMP8 Homo s.	QPFVVTFFRASRQVCRRHLYVSRDLGWLDWV IAP
BMPa Halocynthia r.	QAFLVAFLEAGQACHREELYVSRDQVNWEDWI IAP
HyBMP5-8b Hydra v.	RPFVLSFYQSGGSRCDKHPHYIGFKDLGWSDWI IAP
HyBMP5-8a Hydra v.	QPFVLSFYQSSISDCGKHLLYVSRDLGWSDWI IAP
BMP5-8 Strongylocentrotus p.	EFFMVVFFQ--DWQCKRKNLFVNFEDLDWQEWI IAP
60a Anopheles s.	QPFVLYVANSQHKSCRIQQLYVSRFKDLQWHEWI IAP
60A Drosophila m.	QPFMIGFFRGPTRSCQMOTLYIDFKDLGWHDWI IAP
screw Drosophila m.	EFFIVGYFNGPPQSCERLNFTVDFKELHMHNWV IAP
gdf3 Homo s.	SLLVVTLNPDQKNLCHRHLQFLINFRDLGWHKWI IAP
GDF5 Homo s.	KALFLVFGRTKKARCSRKALHVNFKDMGWDDWI IAP
BMP2/4 Branchiostoma b.	PGYQAYYCHGECPPFLADHLNSTNHAIVQTLVNSVNPLAVP
BMP2/4 Ptychodera f.	PGYNAYFYCDGECPPFLADHLNSTNHAIVQTLVHVSVKASAVP
BMP2/4 Strongylocentrotus p.	AGYQAYYCRGECPPFLAEHLNTNHAIVQTLVNSVNPAVLP
BMP2/4 Archaster t.	AGYQAYYCGGECPPFLVDHLNATNHAIVQTLVNSASPOLAP
BMP2 Homo s.	PGYHAFYCHGECPPFLADHLNSTNHAIVQTLVNSVN-SKIP
BMP4 Homo s.	PGYQAFYCHGDCPPFLADHLNSTNHAIVQTLVNSVN-SSIP
BMPb Halocynthia r.	HGYHAFYCNHGECPFLAEYMNATNHAIVQTLVNSVDPSTLP
BMP2/4 Patella v	PGYNAYFCRGECPFPMGQHLNSTHHAVMQTLVHVSVDPTAVP
BMP2/4 Crassostrea g.	PGYNAYYCDGSCWHPYDDHMNTNHAIVQDLVNSIDPRAAP
dpp Drosophila m.	LGYDAYYCHGKCPFLADHFNSTNHAIVQTLVNNMNPVKVP
dpp Tribolium c.	LGYDAYYCGGECEYPIPDHMNTNHAIVQSLVNSMCKPEVP
BMP2/4 Acropora m.	PGYQGFYCKGECPPFIADHLNTNHAIVQTLMNSVNPNNVP
GDF2 Homo s.	KEYEAYECKGGCFPLADDVTPTKHAIQTLVHLKFPPTKVG
BMP10 Homo s.	PGYEAAYECRGVCNYPLAEHLTPTKHAIQALVHLKNSQKAS
BMP5 Homo s.	EGYAAFYCDGECSPFLNAHMNATNHAIVQTLVHLMFPDHPV
BMP6 Homo s.	KGYAANYCDGECSPFLNAHMNATNHAIVQTLVHLMNPEYVP
BMP7 Homo s.	EGYAAAYCEGECAPFLNSYMNATNHAIVQTLVHFINPETVP
BMP7 Danio r.	EGYAAAYCEGECVFLNSYMNATNHAIVQTLVHFINPETVP
BMP7 Xenopus l.	EGYAAAYCDGECAPFLNSFMNATNHAIVQTLVHFINPETVP
BMP8 Homo s.	QGYSAAYCEGECSPFLDSCMNATNHAIQSLVHLMMPDAVP
BMPa Halocynthia r.	SGYMAAYRCGECDFPLSANMNATNHAIVQTLVHLLKSKLFP
HyBMP5-8b Hydra v.	DGYRANYCGGDCSFLDNNANATNHAIQTLVHMMYPEIIP
HyBMP5-8a Hydra v.	DGYITSYCGGDCSYPLESNLNATNHAILLRALVHTIFPKKIP
BMP5-8 Strongylocentrotus p.	LGYVAFYCGECAPFLNGHANATNHAIVQTLVHHMSPSHVP
60a Anopheles s.	EGYGAAYYCSGECNPLNAHMNATNHAIVQTLVHLNHPKVP
60A Drosophila m.	EGYGAAYYCSGECNPLNAHMNATNHAIVQTLVHLLPKKVP
screw Drosophila m.	KKFEAYFCGGCNPFLGTKMNATNHAIVQTLMHLKQP-HLP
gdf3 Homo s.	KGFMANAYCHGECPSLTISLNSSNYAFMQALMHAVDP-EIP
GDF5 Homo s.	LEYEAFHCEGLCEPFLRSHLEPTNHAIVQTLMNSMDPESTP

BMP2/4 Branchiostoma b. KACCVPTDLSPIISMLYLNNENDQVVLKN-YQDMVVEGCGCR
BMP2/4 Ptychodera f. QACCVPTELSPIISMLYLDEYDKVILKN-YQEMVVEGCGCR
BMP2/4 Strongylocentrotus p. KACCVPTELSAISMLYLDEYKVVILKN-YQDMVVEGCGCG
BMP2/4 Archaster t. KACCVPTDLSAISMLYLDDSDSVILRN-YQDMVVEGCGCR
BMP2 Homo s. KACCVPTELSAISMLYLDENEKVVILKN-YQDMVVEGCGCR
BMP4 Homo s. KACCVPTELSAISMLYLDEYDKVVLKN-YQEMVVEGCGCR
BMPb Halocynthia r. KPCCVPTELSPIAMLYVDECELVVILKT-YQQMAVEGCGCR
BMP2/4 Patella v. KACCVPSDLAISMLYLDELKVVILKN-YQDMVVEGCGCR
BMP2/4 Crassostrea g. KPCCVPTELSSLSLLYDEHGAVVLKV-YQDMVVEGCGCR
dpp Drosophila m. KACCVPTQLDSVAMLYLNDQSTVVLKN-YQEMTVVCGCR
dpp Tribolium c. GPCCVPTQLGQMSMLYLGSDGSVILKN-YKEMVVVCGCR
BMP2/4 Acropora m. PACCVPTTLEAISMLFMNEHSKVVILKN-YQDMVVDGCGCR
GDF2 Homo s. KACCVPTKLSPIISVLYKDDMGVPTLKYHYEGMSVAECGCR
BMP10 Homo s. KACCVPTKLEPISILYLD-KGVVTTYKFKYEGMAVSECGCR
BMP5 Homo s. KPCCAPTCLNAISVLYFDDSSNVILKK-YRNMVVRACGCH
BMP6 Homo s. KPCCAPTCLNAISVLYFDDNSNVILKK-YRNMVVRACGCH
BMP7 Homo s. KPCCAPTQLNAISVLYFDDSSNVILKK-YRNMVVRACGCH
BMP7 Danio r. KPCCAPTQLHGISVLYFDDSSNVILKK-YRNMVVRACGCH
BMP7 Xenopus l. KPCCAPTQLNGISVLYFDDSANVILKK-YKNMVVQACGCH
BMP8 Homo s. KACCAPTCLSATSISVLYYDSSNNVILRK-HRNMVVKACGCH
BMPa Halocynthia r. EPCCTPQDLDSISVLYYDDHRNVVYRK-YRNMVVLSCACY
HyBMP5-8b Hydra v. KPCCAPNKLNTLQVLFDERNNVVMKR-YSNMIVQHCGCQ
HyBMP5-8a Hydra v. KPCCAPNKLNAMSILFFDDRNNVVMQE-IPNMIVHQCGCQ
BMP5-8 Strongylocentrotus p. QPCCAPTCLSPITVLYYDSSRNVVLKK-YKNMVVRACGCL
60a Anopheles s. KPCCAPTCLIPIISVLYHIDEANVNLKK-YKNMVVKSCGCH
60A Drosophila m. KPCCAPTRLGALPVLYHLNDEVNLLKK-YRNMIVKSCGCH
screw Drosophila m. KPCCVPTVLGAIITILRYLNEDIIDLTK-YQKAVAKECGCH
gdf3 Homo s. QAVCIPTKLSPIISMLYQDNNDNVILRH-YEDMVVDECGCG
GDF5 Homo s. PTCCVPTRLSPISILFIDSANNVVYKQ-YEDMVVESCGCR

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Reinhardt, B.; Frank, U.; Gellner, K.; Lohmann, J.U. and Bosch, T.C.G. (1999). High-Resolution, Fluorescence-Based Differential Display on a DNA Sequencer Followed by Band Excision. *BioTechniques* 27: 268-271.

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• Stipendium

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