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Review

Ancient signals: peptides and the interpretation of positional information in ancestral metazoans[☆]

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Abstract

Understanding the ‘tool kit’ that builds the most fundamental aspects of animal complexity requires data from the basal animals. Among the earliest diverging animal phyla are the Cnidaria which are the first in having a defined body plan including an axis, a nervous system and a tissue layer construction. Here I revise our understanding of patterning mechanism in cnidarians with special emphasis on the nature of positional signals in *Hydra* as perhaps the best studied model organism within this phylum. I show that (i) peptides play a major role as positional signals and in cell–cell communication; (ii) that intracellular signalling pathways in *Hydra* leading to activation of target genes are shared with all multicellular animals; (iii) that homeobox genes translate the positional signals; and (iv) that the signals are integrated by a complex genetic regulatory machinery that includes both novel *cis* regulatory elements as well as taxon specific target genes. On the basis of these results I present a model for the regulatory interactions required for axis formation in *Hydra*.

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1. Positional information and the need to have comparative data from ancestral metazoans

The first step in the development of a complex organism is the establishment of a pattern of cells that can differentiate along different pathways. This patterning requires some minimal signals between cells, and that was proposed (Bonner, 2001) to be the origin of multicellular develop-

ment. What are these signals and where did they come from? Central to the development of multicellular body plans is positional information. That is, cells possess positional identity, which is specified by their location on a developmental axis. After they have acquired their positional values, the cells interpret this information by differentiating according to their genetic program. Evolution has developed a variety of different mechanisms for the generation of positional signals. Positional signals in higher organisms can be roughly grouped in two classes: freely diffusible, membrane permeable signals and molecules that bind to specific receptors at the cell surface (Wolpert, 1996). Studies in insects, worms and vertebrates have revealed that many gene families and genetic pathways are involved in encoding posi-

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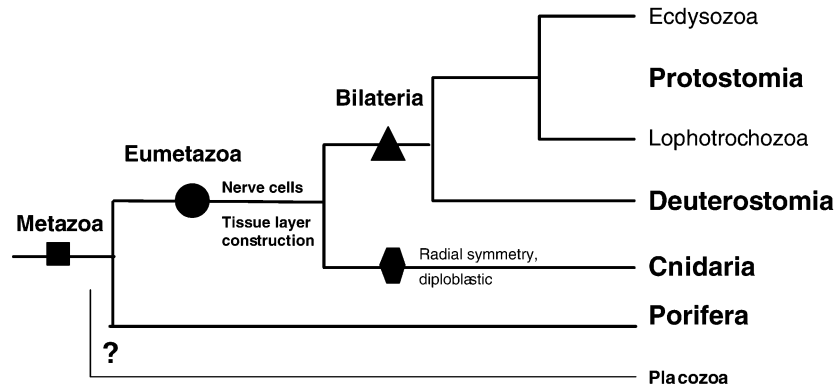


Fig. 1. Metazoan phylogeny showing the phylum Cnidaria as sister group of the Bilateria.

tional identity and that similar pathways control different types of morphogenesis. This similarity in developmental processes between disparate animal phyla has provided convincing evidence for the monophyletic origin of metazoa. Insects, worms and vertebrates all derive from the 'triploblast' or 'Bilateria' clade of metazoans (Fig. 1). Since several animal phyla diverged, however, before the origin of this clade, the discovery of shared molecules tells us little about their origin and original roles, until we have comparative data from more basal animals. The aim of this paper is to review the experimental evidence for positional information in ancestral metazoa. For the purposes of the discussion, ancestral metazoa will be restricted to Placozoa, Porifera and Cnidaria.

2. At the origin of metazoan evolution: placozoa and porifera

It is likely that the signalling beginnings were simple and were only subsequently increased in complexity. Since the 19th century *Trichoplax adhaerens* has been considered the simplest metazoan and proposed to be a model organism for the transition from single cellular protists to multicellular metazoa (e.g. Metschnikoff, 1883; Collins, 1998; Grell and Ruthmann, 1991). *Trichoplax* is composed of a ciliated epithelium that is differentiated dorsally and ventrally and contains just four distinct cell types. In this respect, *Trichoplax* is simpler than most larvae of poriferans, cnidarians and ctenophorans. Its particular morphology, characterized by an extreme form of simplicity has justified the creation of an own phylum, the Placozoa with *Trichoplax adhaerens* as the only

species (Grell, 1971). This simplicity has been used to argue that Placozoa are basal to Cnidaria and Bilateria (Grell and Ruthmann, 1991; Schierwater and Kuhn, 1998). While the simple organisational status of *Trichoplax* has never been questioned, there is, however, considerable dispute about its basal position. Morphological reduction with losses of organs and tissues took place many times in early evolution of Metazoa and Bilateria, not only in parasitic species. Molecular sequence studies strongly support the view of a derived position of *Trichoplax* suggesting that placozoans are secondarily simplified (Bridge et al., 1995; Collins, 1998). It is not clear, whether this simplification took place during the common history of Bilateria and Placozoa or after Placozoa diverged from Bilateria (Collins, 1998). With regard to signalling, nothing is known about the interactions controlling dorsal–ventral differentiation of the epithelium in this simple animal. Only one transcription factor of the *Antp* superclass gene, *Trox-2*, has been isolated from *Trichoplax* so far (Schierwater and Kuhn, 1998), leaving the molecular machinery controlling *Trichoplax* development sitting in the dark. Thus, as long as the phylogenetic position of *Trichoplax* is a subject of intensive debate, and as long as only few genes are known and no methods are available for their functional analysis, this organism—despite its striking simplicity—offers little insight in the tool kit necessary for multicellularity.

In contrast to placozoans, the phylogenetic position of Porifera is well accepted (Soest et al., 1999). Porifera (sponges) most likely evolved from simple unicellular flagellates, the choanoflagellates. Hexactinellid sponges are often consid-

ered to be the most ancient metazoans (Reiswig and Mackie, 1983; Thiel et al., 2002). Porifera have six to ten different cell types including ciliated choanocytes that drive water through canals and chambers. Collagens was shown to be deposited between cells and contributes to the extracellular matrix as do glycoproteins, which are also present in the extracellular matrix of higher vertebrates (Gundacker et al., 2001). There is a great deal of cell mobility and reversal of cell differentiation (Ruppert and Barnes, 1994) and many sponges have remarkable powers of regeneration. The classic experiment demonstrating the regenerative ability of sponges involves forcing living sponge tissue through a silk mesh. The separated cells quickly reorganize by progressive association of similar cells due to cell adhesion molecules (Müller, 2001; Müller et al., 2001).

Given the absence of a body axis and of organs, will elucidation of signalling pathways in sponges shed light on the basic functional network at the beginning of metazoan evolution? Sponges have signal transduction pathways including receptor tyrosine kinases and protein kinase C that are the basis for physiological processes in higher metazoa (Müller, 2001). This and other observations indicate that sponges share a common ancestor with all other metazoa and that multicellularity is associated with the presence of an extracellular matrix, cell adhesion molecules and membranes associated receptors. Nothing is known, however, about the signals and interactions required for attainment of the definitive sponge form and for differentiation of sponge-specific features such as the choanocytes. Thus, besides providing clear evidence supporting the monophyletic origin of the Metazoa, sponges appear to offer little insight into the molecular machinery needed to make these features, which are inseparably linked to Eumetazoa.

3. Cnidaria are the first in evolution with a defined body plan

Among the basal metazoa, Cnidaria, the sister group of the Bilateria (Fig. 1), are the first in evolution that have a defined body plan including an axis, a nervous system and a tissue layer construction. Cnidarians are radially symmetric. In Hydrozoa, the single body axis is composed of a head, a body column and a foot (Fig. 2). The animals are diploblastic consisting of two epithelia, the ectoderm and the endoderm surrounding a

gastric cavity. In the freshwater hydrozoan *Hydra*, there are approximately 20 cell types distributed among 3 cell lineages (for review see Bosch, 1998). Each of the epithelial layers is made up of a cell lineage, while the remaining cells are part of the interstitial cell lineage, which reside among the epithelial cells of both layers. Multipotent interstitial stem cells give rise to neurons, secretory cells and gametes in a position dependent manner (Bosch and David, 1987). These stem cells also give rise to cnidocytes, which are unique to and characteristic of all cnidarians. Since epithelial cells as well as interstitial cells in adult *Hydra* continuously proliferate, there is continuous tissue displacement towards the extremities in adult polyps. Cells in *Hydra*, therefore, continuously change their position along the body axis and differentiate according to their position into head or foot specific cells. The oral–aboral body axis, which extends from the tip of the hypostome to the basal disc of the foot, must be generated from scratch every time a parent polyp makes a bud (Fig. 2b). Since axis formation and cell differentiation processes are continuously active in adult polyps, *Hydra* generally is regarded as ‘embryonic model system’ in which processes active in bilaterian animals only during certain embryonic stages can be studied in mass cultured adults (Bosch, 1998). The underlying assumption is that the machinery governing bud formation and regeneration mimicks the mechanisms, which are active during early embryogenesis. However, whether the molecular mechanisms underlying the various developmental stages (Fig. 2c) are identical to the mechanisms controlling axis formation during budding and regeneration remains to be shown. Interestingly, preliminary observations indicate that some of the patterning genes well documented in adult polyps are not expressed at all during embryonic stages (Fröblius, A., Bosch, T.C.G., unpubl. observation).

Hydra has a long history as a model system in developmental biology because of its remarkable capacity to regenerate missing body parts. Most spectacularly, hydra polyps when dissociated into a suspension of single cells and pelleted into aggregates by centrifugation will organize themselves into complete polyps within a few days (Gierer et al., 1972; Fig. 2e). This ability for self-organization is at least partially due to the continuous production of cells and signal factors in adult tissue. It represents a beautiful experimental system for the study of de novo pattern formation

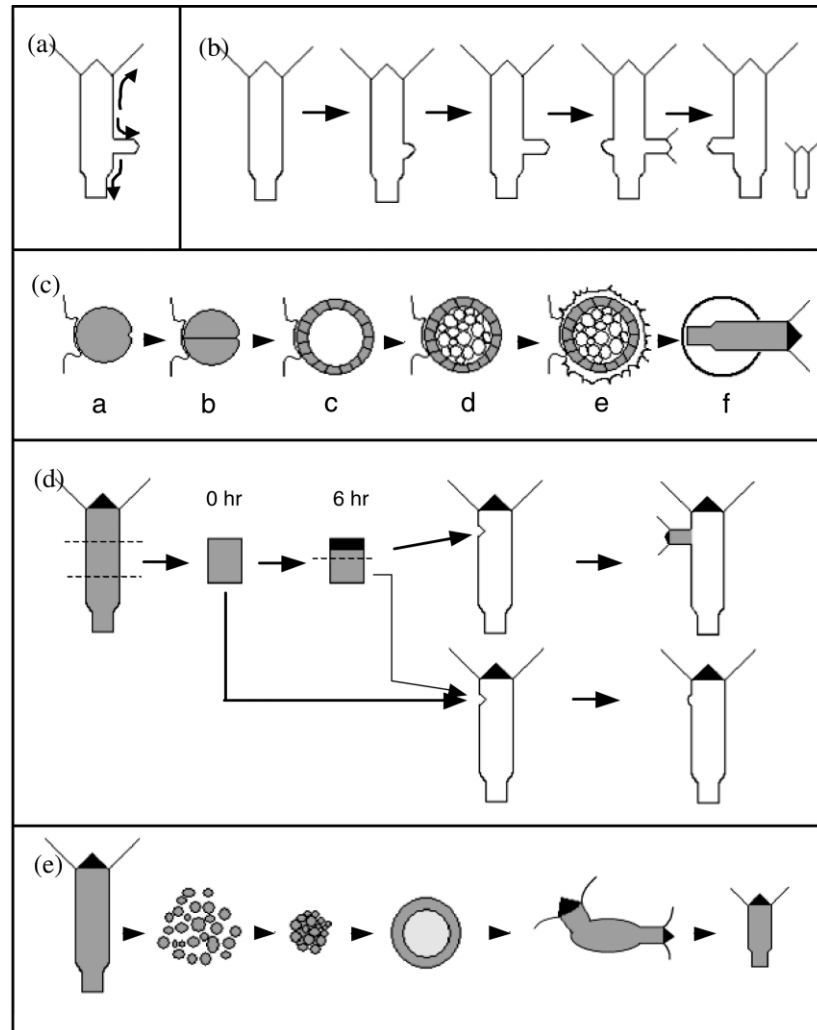


Fig. 2. Axis formation in *Hydra*. (a) Schematic drawing of a *Hydra* polyp. Arrows indicate tissue movement as a result of continuous cell proliferation of epithelial cells in the body column. (b) Axis formation by budding. (c) Patterning during embryogenesis: (a) uncleaved egg showing the sperm entry pit at the future oral end of the adult polyp; (b) two-cell stage; (c) blastula; (d) late cleavage stage; (e) early cuticle stage. The cells of the outer layer extend tiny filopodia upon which cuticle material is deposited; and (f) Emerging hatchling. As the cuticle breaks open, the hatchling exits head-end first. (d) Regeneration of tissue sections and experimental demonstration of the generation of a prepattern which then induces the formation of the visible pattern. (e) Self-organization and shaping of the body plan from aggregates of individual cells.

and points to an important process of patterning in multicellular organisms: visible patterns are preceded by prepatterns or morphogenetic fields. Regenerating tissue pieces cut from the gastric regions of *hydra* show a directional property called polarity. Such pieces regenerate a head in the part of the sections, which is closest to the original head. The decision of where to form a head is made few hours after cutting, long before any head-like structure is visible. If the area that will

become the head of the regenerated animal is cut out and transplanted to the gastric region of another *hydra* (Fig. 2d), it will induce the formation of a second head in the host tissue (MacWilliams, 1983a,b). Which molecules are involved in the generation of this prepattern, which distinguishes a future head area from other parts of the regenerate? How is the pattern established during regeneration and budding? Molecular studies have provided a first glimpse on the genetic components

required. Here I will focus on a number of recent discoveries, which took our understanding a step further.

4. Axis formation in *Hydra*: an ancient animal in molecular terms

The traditional view of position dependent differentiation along the *Hydra* body axis - controlled by head activating and inhibiting diffusible substances - has been re-cast into modern molecular terminology. More than 100 genes have been identified and characterized from *Hydra* (reviewed in Bosch and Khalturin, 2002). Numerous *Hydra* genes governing axial patterning and cell differentiation are orthologs of genes found in bilaterians indicating that important receptor and signaling molecules originated early during metazoan evolution. Most recently, several EST (expressed sequence tags) analyses have been initiated using budding hydra as the source of mRNA (Bode and Steele, pers. communication; Fujisawa, pers. communication). This promises to generate quantitative information on gene expression during *Hydra* axis formation within the next few years. There is also a solution to another problem encountered when approaching ancestral metazoans at the molecular level: the lack of genetic tools. Recent studies in *Hydra* suggest that gene transfection techniques can be applied to these animals. *Hydra* genes have been silenced by dsRNAi (Lohmann et al., 1999; Lohmann and Bosch, 2000; Smith et al., 2000) or modified antisense oligonucleotides (Leontovich et al., 2000; Yan et al., 2000; Zhang et al., 2001). Moreover, gene regulation can be studied by introduction of reporter gene constructs into *Hydra* using electroporation (Brennecke et al., 1998; Miljkovic et al., 2002 reviewed in Bosch et al., 2002) or the particle gun (Böttger et al., 2002).

4.1. The generation of positional information in *Hydra* depends on organizer tissue and includes novel peptides

Axis formation in *Hydra* is controlled by an head organizer located in the hypostome, which is the apical part between the tentacle ring and the mouth (Broun and Bode, 2002). Several genes have been discovered and expressed in tissue whenever a head is formed. One of them is the *Brachyury* homolog *HyBra1*, which is expressed in the hypostome. Its transcriptional activation

correlates with the changes of the head activation level (Technau and Bode, 1999) and, therefore, may be an essential part of the organizer activity. Other genes early expressed during head formation include genes of the WNT signalling cascade *HyWnt*, *Hy-cat*, *HyTcf* (Hobmayer et al., 2000), homeobox gene *prdl-1* (Gauchat et al., 1998) and the novel peptide *Heady* (Lohmann and Bosch, 2000). When *Hydra* cells in single cell suspension are allowed to organize themselves into complete polyps, Technau et al. (2000) have shown that the de novo formation of organizer centers where head activation takes place requires a minimum of approximately 15 epithelial cells. *HyBra1* and *HyWnt* are expressed in such aggregates in locally restricted areas corresponding to such clusters of epithelial cells (Technau et al., 2000). Current efforts in several labs are directed towards finding out whether these genes are activated independently or in a hierarchical sequence and to identify the downstream effectors that execute the *Hydra* organizer blueprint.

To serve as molecules involved in organizer formation, the corresponding genes must not only be expressed at the proper time in the proper cells. The proteins encoded by these genes must be able to induce neighbouring cells to change their differentiation pathway. A classical assay for the presence of such 'morphogenetic' substances in *Hydra* is lateral transplantation (MacWilliams, 1983a; Fig. 2d). Using this assay system, four peptides have been shown to be part of the positional information system along the apical-basal body axis capable to induce head or foot specific differentiation: HEADY, pedibin/Hym-346 and Hym-323 (Bosch and Fujisawa, 2001). The 12-amino-acid peptide HEADY is a potent inducer of apical fate and also sufficient for head induction (Lohmann and Bosch, 2000). *Heady* represents a novel gene that is absent in the genomes of other animals and may be a gene essential for the cnidarian way of head determination (Bosch and Khalturin, 2002). At the opposite end of the body axis, peptide pedibin/Hym-346 plays a role in the positional value gradient acting as morphogenetic signal for foot differentiation (Hoffmeister, 1996). Pedibin/Hym-346 is synthesized as a precursor of 49 amino acids and lowers the positional value gradient along the body column and favours foot formation. During the course of a systematic screening of peptide signaling molecules, another novel peptide,

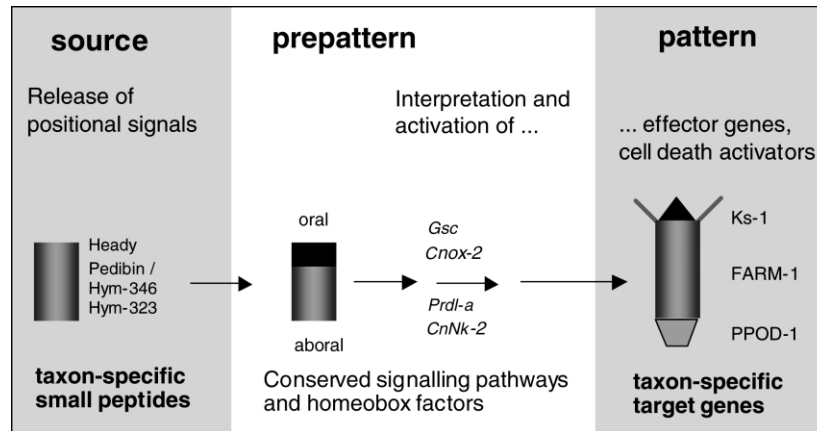


Fig. 3. Principles and participants in the formation of the *Hydra* body plan. Positional signals including taxon specific short peptides provide positional information and lead to a prepattern that has oral-aboral information. Homeobox transcription factors interpret this positional information most likely in a combinatorial manner. To generate taxon (Cnidaria) specific structures such as battery cells containing cnidocytes in tentacles, these conserved signalling pathways regulate the expression of taxon specific target genes such as ks-1. To shape the end of the body axis, homeobox factors might also activate cell death activators.

Hym-323 has been discovered in *Hydra* (Harafuji et al., 2001). The peptide is 16 amino acids long, shares no structural similarity to Hym-346, and is encoded in the precursor protein as a single copy. Northern blot analysis, in situ hybridization analysis and immunohistochemistry showed that it is expressed in both ectodermal and endodermal epithelial cells throughout the body, except for the basal disk and the head region. Transplantation and regeneration experiments indicated that Hym-323 is enhancing the foot-patterning process in *Hydra* by acting on epithelial cells. Upon initiation of foot formation, the stored Hym-323 peptide is released from the epithelial cells and induces differentiation of basal disk cells of the foot. These and other studies (e.g. Takahashi et al., 1997; Koizumi, 2002; Grimmelikhuijzen et al., 2002) have convincingly demonstrated that peptides are abundant within the phylum Cnidaria and that they play multiple roles in cell communication, cell differentiation and patterning in *Hydra*.

Since peptides are so widely used in so many different biological processes, the question arises whether *Hydra* also needs all the classical growth factors and cytokines, which in higher organisms control cell communication and differentiation. So far, there is at least indirect evidence for the presence of one classical signal factor in *hydra*. This is due to the cloning of a receptor protein tyrosine kinase, HTK7, which is closely related to the vertebrate insulin receptor (Steele et al., 1996).

Since exogenously added insulin affects cell division in *Hydra*, it seems that insulin-like signal factors are present at this early stage of metazoan evolution. The HTK7 receptor appears to cause production of a signal, which stimulates cell division in the body column. However, this conclusion has to be taken *cum grane salis* as long as no causal link has been demonstrated between the HTK7 receptor and its putative endogenous ligand and as long as no insulin encoding gene has been detected in *Hydra*. The EST database offers unique opportunities to identify such genes. In sum, despite their basal position in phylogeny and in contrast to the view (Bonner, 2001) that the signalling beginnings were simple, Cnidaria appear to use a surprisingly large spectrum of molecules for transmission of developmental signals.

4.2. Intracellular signalling pathways in *Hydra* are shared by all multicellular animals

Studies in insects and worms have shown that cell–cell interactions during embryonic development involve the *Hedgehog*, *Wnt*, *transforming growth factor- β* , receptor tyrosine kinase, *Notch*, JAK/STAT and nuclear hormone pathways (Pires-DaSilva and Sommer, 2003). Apparently, only a few signalling pathways generate much cellular and morphological diversity during the development of individual organisms and the evolution of animal body plans. How did these pathways

evolve? Which signalling pathways are involved in pattern formation and cell differentiation in *Hydra*?

In *Hydra*, the detailed properties of the signalling networks biochemical components just become apparent. A major breakthrough in identifying individual components was the introduction of pharmacological tools. These treatments disrupt distinct molecular processes and thus demonstrate their contribution to signal transduction. It turns out, not too surprisingly anymore, that the same few classes of signalling pathways are also controlling patterning and position dependent gene expression in *Hydra*. The mitogen-activated protein kinase (MAPK) and the PI/protein kinase C (PKC) systems are two such cascades, often coupled to a network that communicates exogenous signals to the transcriptional machinery and other cellular effectors. Several genes have been identified in *Hydra* encoding receptor and non-receptor protein tyrosine-kinases (Bosch et al., 1989; Steele et al., 1996; Reidling et al., 2000). Recently, pharmacological inhibition revealed that the *Hydra src* type receptor tyrosin kinase *STK* (Bosch et al., 1989) is involved in head formation, as inhibition of its activity prevents head, but not foot, regeneration (Cardenas et al., 2000). Interestingly, choanoflagellates, which have long been suspected to be the closest unicellular relatives of animals, have a receptor tyrosine kinase that includes multiple extracellular ligand-binding domains and resembles that of RTKs in sponges and humans (King and Carroll, 2001). This provides support for the idea that the origin of the Metazoa is linked to the ability to receive and transduce signals and that the evolution and diversification of receptor protein-tyrosine kinases was a fundamental step in the development of metazoans (King and Carroll, 2001; Steele, 2002).

Furthermore, cell-adhesion molecules, once believed to function primarily in tethering cells to extracellular ligands, are now recognized as having broader functions in cellular signalling cascades. Examples include the β -catenin/plakoglobin/armadillo gene family that plays an important role in cadherin-mediated cell adhesion as well as in developmental signaling. Cell interactions in *Hydra* depend on cadherins (Hobmayer, personal commun.) and β -catenin homologues (Hobmayer et al., 1996). There is indirect evidence that a β -catenin-dependent signaling mechanism controls transcription of target genes in the organizer region

because a *Wnt* gene is differentially upregulated in cells of the organizer region (Technau et al., 2000; Hobmayer et al., 2000). Wnt proteins are signaling molecules that act through a receptor called Frizzled and control transcription through a β -catenin-dependent signaling mechanism. Thus, already at an early stage of evolution of multicellularity, there is the dual role of β -catenin in cadherin-mediated cell adhesion as well as in developmental signaling. A direct link between cell adhesion and morphogenetic signaling has been observed in dissociation/re-aggregation experiments: *Hydra* cell clusters formed by homotypic adhesion acquire a new cellular affinity and a capacity to act as head organizers only when they have reached a certain size (Technau et al., 2000). The recently identified *Sweet Tooth* receptor protein tyrosine kinase (Reidling et al., 2000) adds another aspect to the role of potential cell adhesion molecules in *Hydra*. This novel receptor protein tyrosine kinase with C-type lectin extracellular domains may participate in signal-transduction processes not by establishing specific transmembrane complexes, but by acting as a pattern recognition receptor for foreign cells (Reidling et al., 2000).

Receptor tyrosine kinases activate effectors such as phospholipase C- γ (PLC- γ), leading to the activation of PKC. *Hydra* has several PKC genes (Hassel, 1998; Hassel et al., 1998) and head regeneration is associated with an increase in PKC activity (Müller, 1985; Hassel et al., 1998). In addition, members of serine/threonine protein kinases have been cloned from *Hydra* (Herold et al., 2002). One of them, belonging to the PKB/Akt family is upregulated during head regeneration (Herold et al., 2002). Thus, not only tyrosine kinases and PKC, but also other serine/threonine kinases can be traced back in evolution to basal metazoans. Moreover, cloning of *HySmad1*, a highly conserved orthologue of receptor-activated Smads represents evidence of the presence of TGF- β signalling pathway in Cnidaria (Hobmayer et al., 2001). The TGF- β superfamily is a group of multifunctional cytokines that includes bone morphogenetic proteins (BMPs), TGF- β s and others (Massagué, 1998). These secreted signaling molecules regulate many aspects of cellular responses, including cell proliferation, differentiation, migration and apoptosis. BMP subfamily members have a critical role in embryonic patterning and in maintaining tissue homeostasis in adult life, where-

as TGF- β subfamily members mainly modulate the immune response. In *Hydra*, BMP related genes are differentially expressed at early stages of head formation and inhibition of BMP signaling leads to ectopic head structures along the body axis (Bode, personal commun.). Thus, although there is no evidence for the presence of TGF- β subfamily members in *Hydra* nor in any other invertebrate despite serious efforts devoted to cloning them, the TGF- β signaling pathway through activation of specific Smad proteins seems to be shared by all multicellular animals.

On the one hand, *Hydra* appears to share the few classes of signaling pathways with all other multicellular animals. Since on the other hand, only a few signalling molecules have been identified in unicellular eukaryotes, the evolution of signalling pathways and the availability of a wide spectrum of kinases might have been a prerequisite for the occurrence of multicellularity.

4.3. Positional signals are integrated by homeobox genes

There is good evidence that patterning in *Hydra* is governed by a positional value gradient that is maximal in the head and decreases down the body column towards the foot (MacWilliams, 1983a,b). A major challenge when analysing position dependent cell differentiation is to elucidate how this positional information is translated into the precise spatial and temporal expression of key regulatory genes. Differentiation of cells at the basal end of the axis in *Hydra* into stalk and foot specific cells depends on two important signal factors, pedibin and pedin (Hoffmeister, 1996). Homeodomain factor *CnNK-2* is sensitive to these peptides and involved in translating the positional value gradient into changes in cell behavior and foot specific differentiation (Grens et al., 1996). *CnNK-2* and *pedibin* are coexpressed in entodermal epithelial cells located at the basal end of the body column, which has a low positional value. Expression of both genes attenuates in a gradient-like manner from the foot towards the gastric region. In polyps treated with pedibin, the *CnNK-2* expression is greatly extended towards the gastric region (Grens et al., 1999). Thus, the peptide appears to cause a decrease in positional value of gastric tissue, leading to an increased spatial domain of expression of homeobox gene *CnNK-2*.

In addition to their role of transcriptional activators there is evidence that in *Hydra* homeobox genes integrate positional signals and allow spatially restricted gene expression by acting as transcriptional repressors. Examples include *Cnox-2*, an ortholog of the ParaHox *Gsx* gene, which prevents body column tissue from forming a head. *Cnox-2* is expressed in the body column, but not in the head region and becomes downregulated on the protein level after head removal (Shenk et al., 1993a,b). By analyzing the protein binding sites of the promoter of *ks-1*, a tentacle specific gene (Weinziger et al., 1994), we showed (Endl et al., 2000) that more proteins including *Cnox-2* bind to the *ks-1* promoter in the body column than in head tissue where the *ks-1* gene is actually expressed. Thus, *Cnox-2* and maybe other repressors may prevent the transcription of *ks-1* and other head specific genes in body column cells. The postulated mechanisms and the number of homologous homeobox genes cloned from Cnidaria suggest that a highly regulated control system appeared at a relatively early stage in animal evolution.

4.4. Differential expression of target genes is based on a complex genomic control apparatus

Signalling between and within cells ultimately leads to gene activation and inactivation. Changes in the regulation of genes may lead to the creation of new patterns of development during evolution. Recently, we started to investigate the regulatory interactions involved in *Hydra* patterning at the promoter level. By in vitro footprinting and gel retardation techniques, we have examined DNA/protein interactions at 1.5 kb *cis* regulatory region of *ks1* (Endl et al., 1999). The *ks1* gene is expressed in ectodermal tentacle epithelial cells and is sensitive to patterning signals along the apical-basal body axis (Weinziger et al., 1994). Since *ks1* is required for head formation (Endl et al., 1999), it may play an essential role in generating a cnidarian specific feature, the battery cell with its cnidocytes. Surprisingly, 47 target sites for nuclear proteins were detected in the *ks1* promoter. Ten of them are binding sites for general DNA binding proteins, the remaining 37 *ks1* promoter elements were binding sites for nuclear proteins from basal tissue. In addition to 26 elements with sequence similarity to known transcription factor binding sites, we could identify a number of *cis*-

regulatory sequence elements in the *ks1* promoter region, which are not related to any known consensus sequence but nevertheless are specific binding sites for nuclear proteins. These 11 *cis* regulatory elements, termed ‘Hyko’ (Hyko; German: hydra head specific) elements, could represent binding sites for *Hydra* specific transcription factors. We suggested elsewhere (Endl et al., 1999; Bosch and Khalturin, 2002) that such ‘uniqueness’ regulatory proteins could code for species or group specific characters and that *Hydra* specific developmental features, such as e.g. tentacle formation might be regulated by nuclear proteins binding to the Hyko sites.

The view that molecular mechanisms that direct spatially restricted gene expression in *Hydra* are complex is supported by analysing the regulation of foot specific genes. As outlined above, in *Hydra* two genes have been found so far participating in development and differentiation of foot specific structures. The homeobox protein CnNK-2 functions as transcriptional regulator (Grens et al., 1996), while the signal peptide pedibin serves as an ‘extrinsic’ positional signal (Grens et al., 1999). One mechanism that provides persistent expression of CnNK-2 is an autoregulatory circuit. By characterizing the regulatory region of CnNK-2 using EMSAs we recently discovered first evidence for such an autoregulatory circuit, mediated by binding of CnNK-2 protein to an upstream NK-2 element in the CnNK-2 locus DNA (Thomsen, S., Khalturin, K., Bosch, T.C.G., in prep.). Analysis of the *cis*-regulatory sequence of peptide encoding gene pedibin revealed that it too contains NK-2 DNA binding domains (Thomsen, S., Khalturin, K., Bosch, T.C.G., in prep.). Since CnNK-2 physically interacts with the *pedibin* NK2 DNA binding domain in foot tissue, CnNK-2 appears to function as a transcriptional regulator of foot specific signals. In support of this view, characterization of the regulatory region of foot specific peroxidases revealed additional NK-2 binding elements (Thomsen, Hoffmeister-Ullerich and Bosch, unpubl.). We suggest that the primary function of the peptides is to induce expression of CnNK-2 (and interacting transcriptional regulators) in a temporally and spatially overlapping pattern. CnNK-2, in turn, controls localized expression of *pedibin* by either repression or activation. In addition, CnNK-2 maintains its own expression by a feedback loop. In this view, CnNK-2 and pedibin together synergistically regulate foot specific dif-

ferentiation. Pedibin is upstream of, and controls, the expression of CnNK-2. The latter, in turn, presumably controls not only pedibin and its own expression, but also genes further downstream whose products directly are involved in foot specific differentiation.

Taken together, regulation of gene expression in *Hydra* is as complex as in any other metazoan. *Hydra* has retained key regulatory genes that would have been found in the last common ancestor of Cnidaria and Bilateria, more than 550 million years ago. Therefore, regulation of gene expression in *Hydra* includes novel *cis* regulatory elements, which may be binding sites for novel *trans* acting factors. This may be due to the fact that Cnidaria had plenty of time to evolve its own, non-bilaterian features, the most distinctive of which are the single body axis and battery cells containing cnidocytes in the tentacles.

4.5. Are apoptosis genes in *Hydra* shaping the ends of the body axis?

Apoptosis, also known as programmed cell death, is triggered by the activation of cysteine proteases called caspases (Cryns and Yuan, 1998). Caspases are initially synthesized as inactive zymogens, but become activated by proteolytic processing during apoptosis. In *Caenorhabditis elegans*, CED-3 is the only caspase involved in apoptosis. CED-3 becomes activated by autoprocessing through interaction with the adaptor protein CED-4 in cells fated to die during development (Cryns and Yuan, 1998). Cikala et al. (1999) have shown that *Hydra* has at least two caspases with an overall structure similar to CED-3 and caspase3. *Hydra* cells undergoing apoptosis can be identified by various methods including staining with acridine orange and the TUNEL assay. Apoptosis in *Hydra* appears to have the same morphological features as apoptosis in higher animals and presumably is used to eliminate ‘excess’ cells, e.g. in starving polyps (Bosch and David, 1984; Cikala et al., 1999). Little or no signs of cell death can be detected in asexual polyps in the body column. At the end of the body axis, in foot, head and tentacle tissue, however, there is a slight increase in the number of apoptotic cells (Cikala et al., 1999; Kuznetsov et al., 2001). This is consistent with the fact that hydra cells are continuously displaced towards the extremities and raises the question: what are the regulators, which have

access to the cell death genes in *Hydra* and determine where and when the cells have to die? In developing *Drosophila*, it recently has been shown (Lohmann et al., 2002) that the Hox gene *Deformed (Dfd)* directly activates the expression of the death activator *reaper (rpr)* and that this is both necessary and sufficient to account for *Dfd*'s ability to modulate segmental morphology in the fruitfly head. Direct links between Hox genes and components of the apoptotic orchestra have not been documented yet in *Hydra*. Therefore, it is intriguing to speculate that this Hox-gene dependent induction of cell death is a more general phenomenon and is part of an evolutionary old patterning machinery.

5. From humble beginnings come promising perspectives

Hydra began its scientific career as a model of regeneration in 1744 (Trembley, 1744) and has since become a celebrated example of a developmental system (for review see Wilkins, 2000; Pennisi, 2000; Steele, 2002). The rich diversity of peptides, with functions as diverse as intercellular communicators, neurotransmitters and signals for spatial and temporal control of axis formation and cell differentiation, hints at the wealth of information passed between interacting cells. As numerous developmental control genes are discovered and the wide spectrum of molecular techniques is applied more widely to *Hydra*, a complex system of signalling emerges.

What is the molecular basis for the obligate interaction between the positional signals and the transcription machinery controlling the expression of patterning genes? What are the genetic mechanisms that underlie the development of taxon specific features such as the cnidocyte containing tentacles? In one scenario (Fig. 3), short peptides might provide positional information in developing or regenerating tissue causing a prepattern that has oral-aboral information. Cells in these prepattern then serve as inducers of the visible pattern by using signalling pathways and homeobox transcription factors shared by all multicellular animals to interpret positional information and to activate effector genes. The generation of taxon specific structures could underlie the control of taxon specific signal molecules as well as taxon specific effector genes. Several examples of taxon specific signals and effector genes have been described in

Hydra including the novel peptides *Heady* and *pedibin/Hym-346* as well the tentacle specific gene *ks1* (reviewed in Bosch and Khalturin, 2002). To shape the ends of the body axis, homeobox genes might also activate cell death activators.

There are many aspects of this model that need to be tested. The generation of positional information needs to be defined in molecular terms and co-stimulatory molecules need to be incorporated. Also, it will be interesting to screen for more positional signals and putative morphogens. *Heady* as well as the foot specific molecules *pedibin* and *pedin* indicate that small peptides can serve as substances that vary in spatial distribution and have morphogenetic effects. Whether these peptides are sufficient to generate the prepattern which then induces the formation of the visible pattern remains to be shown. Furthermore, we need to achieve a better understanding of the signals involved in the spatial and temporal control of stem cell proliferation and differentiation. Technically, most urgent are rigorous functional tests of the genes already identified and proposed to be involved in the patterning processes. Armed with specific molecular modulators such as morpholinos or RNAi, inducible transgenes and reporter gene technology, the pseudo-genetic approach to the study of position dependent cell differentiation promises to advance the original goals of *Hydra* developmental biology. It will also be important to establish technologies that allow the quantitative and integrative analysis of multiple signaling pathways and transcription factors in *Hydra* and also other basal metazoa. To determine how promoters integrate the activities of several transcription factors, investigators will need to use data from gene-expression-profiling experiments, together with new bioinformatics approaches. New imaging techniques that allow visualization of signaling pathways and transcription in vivo, coupled with the further development of Cnidarian models that allow subtle and controlled perturbation of gene expression, should help us to refine and expand our understanding of the molecules and signaling pathways required to form a basal metazoan. Understanding how signals are generated and interpreted in basal metazoa is not only a scientific challenge, but also has clear relevance for understanding a major step in evolution, the origin of multicellularity.

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