



## Review

# Why polyps regenerate and we don't: Towards a cellular and molecular framework for *Hydra* regeneration

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**Abstract**

The basis for *Hydra*'s enormous regeneration capacity is the "stem cellness" of its epithelium which continuously undergoes self-renewing mitotic divisions and also has the option to follow differentiation pathways. Now, emerging molecular tools have shed light on the molecular processes controlling these pathways. In this review I discuss how the modular tissue architecture may allow continuous replacement of cells in *Hydra*. I also describe the discovery and regulation of factors controlling the transition from self-renewing epithelial stem cells to differentiated cells.

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If we have had or will have a finger cut off, we cannot restore it. If, however, we dissociate an intact *Hydra* into single cells, a perfect polyp will reconstitute itself from the pellet of centrifuged cells within the next few days. What is the difference between "us" and "them"? Why possess some animals remarkable powers of self-regeneration and others not? *Hydra* is the superstar of regeneration since more than 200 years. In the 1740s, the Swiss scientist Abraham Trembley (1744) discovered that freshwater polyps could regenerate their heads and feet and – if cut into a few pieces – all of them would regenerate to form new individuals (Lenhoff and Lenhoff, 1988). Scientists have long wondered how *Hydra* regenerates so well. *Hydra*'s regeneration capacity and the underlying mechanism responsible for specification of positional information has inspired (and is still inspiring) computational biologists to demonstrate that mathematical equations can be applied to explain morphogenetic events in animals (for review see Meinhardt and Gierer, 2000; Meinhardt, 2002, 2004a,b; Crampin et al., 2002; Marciniak-Czochra, 2006). *Hydra* also presents excellent opportunities for understanding how gradi-

ents of morphogens could be set up and maintained to control local developmental processes (Wolpert et al., 1972, 1974). By application of quantitative cellular techniques much has been learned about *Hydra*'s cell populations, and the mechanisms controlling pluripotency, lineage commitment, and position dependent cell differentiation (for reviews see Bode, 1996; Bosch, 2006). But precisely how in *Hydra* the regenerating tissue is reorganized, how positional information is encoded at the molecular level, and how cells respond to diffusible positional signals (or "morphogens") remained largely mysterious. An impressive accumulation of gene sequences, novel tools and the development of genomic resources over the past few years has brought a new perspective on *Hydra*'s regeneration capacity. A National Science Foundation-funded large-scale *Hydra* EST Project ([www.hydrabase.org](http://www.hydrabase.org)) resulted in 170,000 ESTs. A National Human Genome Research Institute-funded *Hydra* genome project at the J. Craig Venter Institute currently provides 6x coverage of the *Hydra magnipapillata* genome with an assembled draft genome sequence appearing later this year. *Hydra* became also amenable to reverse genetics through RNAi experiments, further expanding the capabilities of this model organism (Lohmann et al., 1999; Takahashi et al., 2005; Cardenas and Salgado, 2003; Chera et al., 2006; Amimoto et al., 2006). Finally, transgenic *Hydra* (Wittlieb et

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al., 2006; Steele, 2006) now pave the way for many important scientific and technological applications making resources and methods available to fully explore the biological opportunities that the polyp provides. *Hydra*'s unique advantages as model for morphological and molecular studies of regeneration include (i) the optical transparency of the two tissue layers allowing the direct visualization of individual cells by means of GFP fluorescence and facilitating *in vivo* tracking of cells within the intact organism; (ii) the rapid growth rate with a population doubling time of 3.5 days; and (iii) the mass-culturing of clonally derived animals. Since in *Hydra* the epithelial cells are key players in regeneration, I will focus here on epithelial stem cells. We will first follow them at the site of regeneration and then discuss the mechanisms by which they are thought to become morphologically and molecularly distinct from their neighbours in the head and foot region.

### Regeneration in *Hydra* occurs by morphallaxis

*Hydra* is made up of two cell layers – the ectoderm and endoderm – separated by a thin extracellular matrix (ECM) called the mesoglea (Fig. 1). The polar body plan has a head and tentacles on one end and a foot on the opposite end of a hollow column (Fig. 1A). The cells either belong to the ectodermal or endodermal epithelial cell lineage, or to the interstitial cell lineage. Epithelial cells are epitheliomuscular cells covering the outside of the animal or lining the gastric cavity. Interstitial cells are mostly localized in the interstitial space between ectodermal epithelial cells and differentiate into nerve cells, cnidocytes, gland cells, and – during sexual differentiation – into gametes

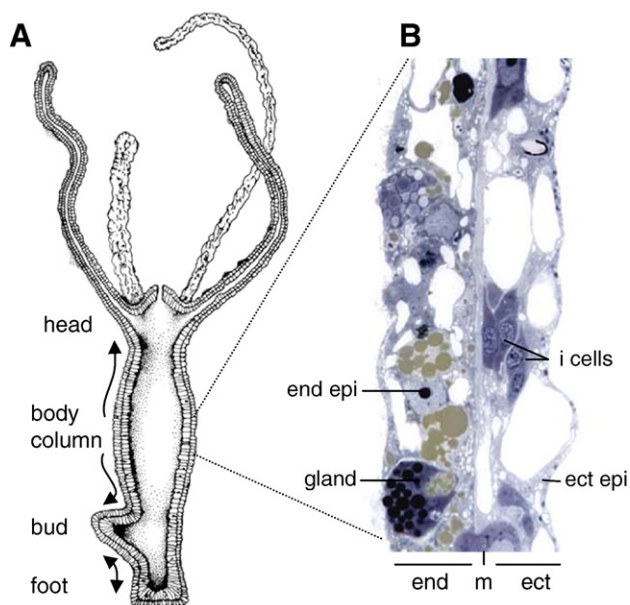


Fig. 1. The freshwater polyp *Hydra*. (A) Schematic longitudinal cross section indicating the simple epithelial organization. Arrows indicate the direction of tissue displacement. (B) Photograph of a section of part of the epithelial lining of the body column, showing the diploblastic organization. Note how interstitial cells and gland cells are interspersed between ectodermal and endodermal epithelial cells, respectively. End, endoderm; ect, ectoderm; m, mesoglea; Photograph courtesy of Dr. Friederike Anton-Erxleben (Kiel).

(Bosch and David, 1987; Bosch, 2006). Any isolated fragment of the *Hydra* body which is larger than a few hundred epithelial cells can regenerate into a miniature version of the animal (Fig. 2A). Even aggregates of dissociated cells (Fig. 2B) will regenerate into viable polyps (Noda, 1971; Gierer et al., 1972; Technau et al., 2000). This ability for self-organization is due to the continuous production of cells and signal factors in the adult tissue. Regenerating tissue pieces cut from the gastric regions show a directional property called polarity (Fig. 2A). Such pieces regenerate a head in the apical end of the isolated fragment. A foot is always regenerated at the basal end of such a piece. Polarity is thought to be based on gradients of molecules whose concentration provides positional information (Wolpert et al., 1974; MacWilliams, 1983a,b). The commitment, for example, of the apical tissue to undergo head formation is made a few hours after cutting, long before any head-like structure is visible (MacWilliams, 1983a,b). Thus, regeneration in *Hydra* represents a beautiful experimental system for the study of *de novo* pattern formation and points to an important process of patterning in multicellular organisms: visible patterns are preceded by prepatterns or morphogenetic fields.

In the early 20th century, Thomas Hunt Morgan coined the terms morphallaxis and epimorphosis to describe the two major types of regeneration which can be observed in various animal groups (Morgan, 1901). Morphallaxis refers to the type of regeneration that occurs in the absence of cellular proliferation and involves the transformation of existing body parts or tissues into newly organized structures. Epimorphosis refers to regeneration that requires active cellular proliferation. In planarians as in some vertebrates such as salamanders, both the generation of new tissue at the wound site via cell proliferation (blastema formation) and morphallaxis are needed for complete regeneration (Brockes et al., 2001; Agata, 2003; Reddien and Sánchez Alvarado, 2004; Sanchez Alvarado, 2006). There, cells near the site of the injury lose their specialized properties and revert to a primordial state in a process called de-differentiation. It is thought that those stem cells then multiply rapidly and redifferentiate to form the tissue needed to rebuild the limb or organ (Brockes and Kumar, 2005; Slack, 2006).

In the marine hydrozoan *Podocoryne*, some cells under certain conditions can de-differentiate or trans-differentiate (Schmid and Reber-Muller, 1995; Reber-Muller et al., 2006). Early regenerative processes in *Hydra*, however, always occur in the absence of DNA synthesis as a morphallactic process in which cells from the gastric region differentiate into head or foot specific cells (Cummings and Bode, 1984). Pulse labeling experiments have demonstrated that the number of labeled cells in regenerating tissue declines sharply at the site of cutting during the first 12 h (Holstein et al., 1991) pointing to the release of factor(s) which inhibit mitosis. My lab has reinvestigated the issue of cell proliferation at the injury site by using transgenic polyps and *in vivo* tracking of GFP expressing endodermal epithelial cells in regenerating tissue (Wittlieb et al., 2006). We have shown (Fig. 2C; Wittlieb et al., 2006) that at the tip of the regenerating tissue there is no localized cell proliferation of endodermal epithelial cells. These

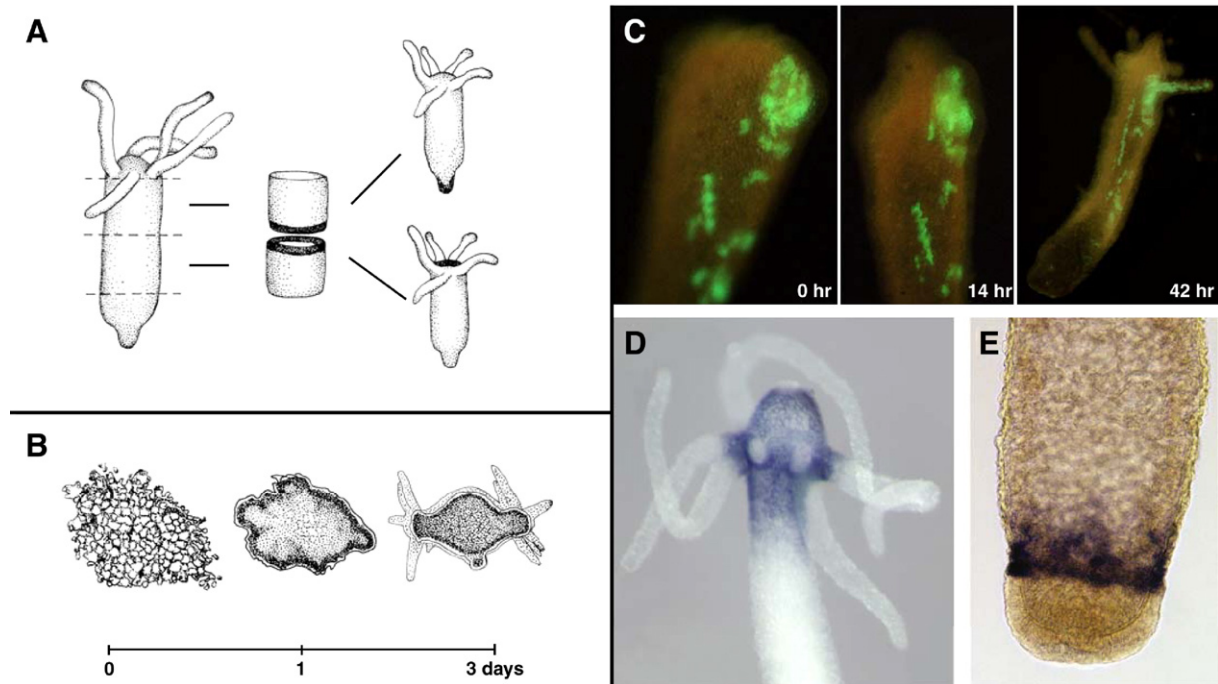


Fig. 2. Regeneration in *Hydra*. (A) Classical experiment demonstrating that a head or a foot can regenerate at the same axial location. (B) Regeneration of an intact polyp from a clump of isolated cells. Schematic drawing of pictures shown in Holstein et al. (2003). (C) In vivo tracking of EGFP expressing endodermal epithelial cells reveals that head regeneration occurs via morphallaxis in the absence of local cell proliferation. (D) Expression of peptide encoding gene Hym301 in the head region; in situ photograph courtesy of Dr. Konstantin Khalturin (Kiel). (E) Position dependent expression of *anklet* near the foot; in situ photograph courtesy of Dr. Yoshitaka Kobayakawa (Fukuoka).

*in vivo* observations, therefore, strongly support the previous findings and clearly demonstrate that regeneration in *Hydra* occurs almost exclusively by morphallaxis. Regeneration in *Hydra* has also been described at the ultrastructural level as a rapid wound healing process initiated by the endoderm (Bibb and Campbell, 1973). Elegant *in vitro* studies (Takaku et al., 2005) with isolated ectodermal and endodermal epithelial cells led to the conclusion that in reorganizing the epithelial layers the endodermal epithelial cells display unexpected motility. Cells at the regenerating tip get activated within 2–3 h after amputation (Technau and Bode, 1999) and undergo phenotypic alterations of cellular, biochemical, and functional properties, leading to the expression of new cell surface antigens (e.g. Bode et al., 1988) and genes (e.g. Takahashi et al., 2005; Amimoto et al., 2006; see Figs. 2D and E).

The events during regeneration resemble normal morphogenesis and involve the interplay of several cell types, signaling pathways, extracellular matrix components, and soluble factors. There are, however, distinct differences between regeneration and normal morphogenesis. Regeneration always starts with a wound; and wound healing may require some special action not essential during normal morphogenesis. In a recent study, Chera et al. (2006) provide an example for such a special action. They explored the function of the evolutionarily conserved *Kazal1* gene which is expressed in endodermal gland cells and upregulated during regeneration. *Kazal1* silencing by RNA interference resulted in dramatic tissue disorganization followed by a massive death of gland cells and the accumulation of autophagosomes within the cytoplasm of digestive cells. Chera

et al. (2006) conclude that in intact *Hydra* *Kazal1* serine-protease-inhibitor activity is required to prevent excessive autophagy and to exert a cytoprotective function to survive the wounding stress (see also Galliot et al., 2006, for review).

#### Regeneration requires epithelial cells, intact ECM, and a critical size

Regeneration in *Hydra* can be carried out by epithelial cells only. Evidence for this comes from experiments in which cell types were selectively eliminated from *Hydra* and the developmental capabilities of the resulting animals studied. When the interstitial cell lineage is removed, the resulting epithelial polyps can perform all morphogenetic processes including head and foot regeneration and budding (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). Moreover, chimeric *Hydra* containing epithelial cells from normal hydra and interstitial cells from mutant *reg16* reveal that the defect responsible for the low head regeneration potential in this mutant resides in the epithelial cells (Nishimiya et al., 1986). Thus, all factors and genes necessary for regeneration must be activated in epithelial cells. There are, however, two additional requirements for head regeneration to occur normally. First, an intact ECM (mesoglea) separating the two cell layers is necessary. The mature mesoglea contains macromolecules such as laminins, collagens, heparan sulfate proteoglycans and fibronectin-like molecules (reviewed in Sarras and Deutzmann, 2001). Regeneration starts with the immediate retraction of the mesoglea which subsequently has to be rebuilt (Shimizu et al.,

2002). Head regeneration is blocked in a reversible manner by drugs affecting collagen processing or secondary collagen and proteoglycan structure (Sarras et al., 1991). Regeneration of cell aggregates into polyps is also blocked by polyclonal and monoclonal antibodies raised to isolated ECM (Sarras et al., 1993). Shimizu et al. (2002) extended these studies and demonstrated by antisense experiments in which translation of matrix-associated components was blocked, the fundamental importance of cell–ECM interactions during epithelial morphogenesis. The key role of the mesoglea in *Hydra* epithelial homeostasis is also underlined by the discovery (Kuznetsov et al., 2002) that the survival of *Hydra* epithelial cells depends on their anchorage to extracellular matrix molecules. Key regulators for degrading or remodelling the ECM are metalloproteases (Deutzmann et al., 2000; Fowler et al., 2000; Yan et al., 2000a, b; Leontovich et al., 2000; Shimizu et al., 2002; Sarras et al., 2002). In a SSH screening project aimed to identify genes that are differentially expressed during regeneration and budding, my lab recently obtained strong support for this view by identifying numerous metalloproteases in a cDNA library enriched for genes which are upregulated or downregulated during regeneration and budding (Hemmrich, Augustin and Bosch, unpubl.). Within the 3634 sequences analyzed we detected not only the previously known protease encoding genes but also not yet described proteases including a *Hydra* homolog to sea urchin metalloprotease SpAN, a *Hydra* gene related to *C. elegans* metalloproteinase ADAM, and a cDNA clone encoding a cysteine protease Cathepsin L homolog. Thus, proteases appear to play a key role in *Hydra* tissue remodelling. Older studies showed that the second requirement for successful regeneration is a critical minimum tissue size (Shimizu et al., 1993). The smallest tissue that can regenerate must have about 300 epithelial cells (Shimizu et al., 1993). Smaller tissue pieces always disintegrate. Technau et al. (2000) more recently could show that within a regenerating tissue piece it needs a cluster of

5–15 epithelial cells for the de novo formation of activation centers. Thus, the mystery of *Hydra*'s regeneration capacity can be confined to a clump of a few hundred epithelial cells. It is in this tiny group of epithelial cells that the patterning process starts.

#### All epithelial cells in the gastric region are epithelial stem cells with continuous self-renewing capacity and remarkable phenotypic plasticity

Stem cells are defined as cells that have the ability both to proliferate indefinitely and to differentiate into specific cells. Both the epithelial cells as well as the interstitial cells in the *Hydra* body column continuously undergo self-renewing mitotic divisions (Dübel et al., 1987). Epithelial cells are proliferating with a doubling time of about 3.5 days (Bosch and David, 1984). To prove that *Hydra* epithelial cells indeed have stem cell properties, we have made use of transgenic polyps and transplanted a single GFP-expressing endodermal epithelial cell into a nontransgenic polyp (Fig. 3). By doing so we have generated (Wittlieb et al., 2006) polyps in which the entire ectodermal or endodermal epithelium contains the transgene (Fig. 3). Thus, *Hydra* epithelial cells are capable, by successive divisions, both of indefinite self-renewal and of producing different types of specialised cells such as tentacle or foot specific epithelial cells. Since there is no evidence for subpopulations of epithelial cells which cannot repopulate the host tissue, all *Hydra* epithelial cells in the gastric region, therefore, must be considered as stem cells. It is this feature which makes adult *Hydra* tissue different from tissue of other invertebrates and vertebrates: cells in *Hydra* by continuously proliferating and responding to positional signals retain features which most cells in other animals only have during the short period of embryogenesis. Supporting earlier observations, our *in vivo* tracking of GFP labelled epithelial cells also showed

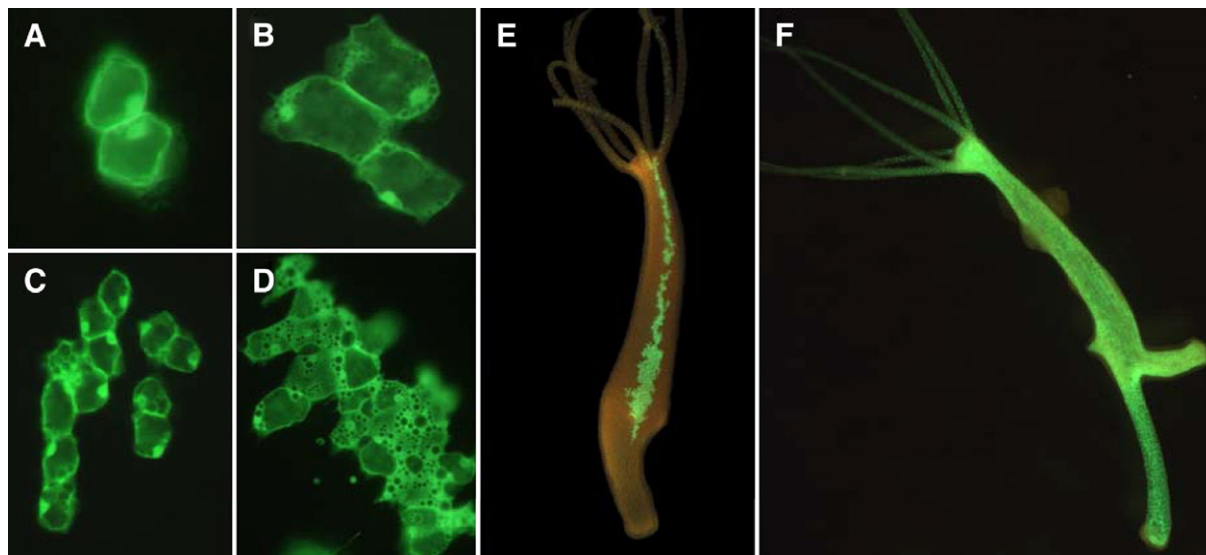


Fig. 3. *Hydra* epithelial cells have stem cell properties and are capable, by successive divisions, both of indefinite self-renewal and of producing different types of specialised cells such as tentacle or foot specific epithelial cells. The picture shows proliferation and differentiation of a pair of EGFP expressing endodermal epithelial cells in a nontransgenic host (A) leading successively (B–E) to a transgenic polyp expressing EGFP in all (F) of its endodermal epithelial cells.

that there is continuous tissue displacement towards the extremities (see Fig. 1A). Tissue moves from the gastric column into the head region at the base of the tentacles and finally into the tentacles themselves. Displacement of ectodermal epithelial cells into the tentacles results in differentiation of battery cells which contain cnidocytes. Displacement of epithelial cells towards the lower body regions results in differentiation of epithelial cells into basal disk cells which begin to secrete mucus. This remarkable plasticity of epithelial cells in response to positional signals allows *Hydra* to build complex structures such as the tentacles with only a limited number of different cell types.

### Factors that enable the transition from self-renewing epithelial stem cells to differentiated epithelial cells

How do the cells in regenerating tissue “know” that their localization in the gastric region has changed to a position at the apical or basal end, respectively? One of the central dogmas of developmental biology is that the behavior of a cell is determined by its position in the embryo (Wolpert, 1996). With the elucidation of the chemical nature of signalling molecules which affect the differentiation of epithelial stem cells in *Hydra* (Fujisawa, 2003), the unravelling of signal transduction pathways in *Hydra* (Bosch, 2003) and *Nematostella* (Darling et al., 2005), and the transcriptome and genome projects underway in a number of cnidarian species (Miller et al., 2005; Technau et al., 2005), it becomes clear that regeneration in *Hydra* requires a complex signalling machinery.

#### *Secreted peptides and their impact on head and foot regeneration*

Using a combination of unbiased biochemical approaches (Takahashi et al., 1997) and classical assay systems for the presence of “morphogenetic” substances (MacWilliams, 1983a), several peptides could be linked with epithelial differentiation along the apical–basal body axis capable to induce head or foot specific differentiation. The properties of such epitheliopeptides acting as positional signals were reviewed recently (Bosch and Fujisawa, 2001; Fujisawa, 2003). Briefly, the 12-amino-acid peptide HEADY (Lohmann and Bosch, 2000), a novel gene that is absent in the genomes of other animals (Bosch and Khalturin, 2002), is a potent inducer of apical fate and also sufficient for head induction since disruption of HEADY function by dsRNA mediated interference (RNAi) resulted in severe defects in head formation (Lohmann and Bosch, 2000). Another novel peptide, Hym-301 (Takahashi et al., 2005), was initially discovered as part of a project aimed at isolating novel peptides from *Hydra* (Takahashi et al., 1997). In an adult, the gene is expressed in the ectoderm of the tentacle zone and hypostome, but not in the tentacles (see Fig. 2D). Treatment of regenerating heads with synthetic Hym-301 peptide causes an increase in the number of tentacles formed, while treatment with Hym-301 dsRNA leads to a reduction of tentacles formed during bud formation or head regeneration (Takahashi et al., 2005). Treatment of epithelial

animals indicates that the gene directly affects the epithelial cells that form the tentacles. The expression pattern plus these manipulations indicate that the gene has a role in tentacle formation. Konstantin Khalturin in my lab recently has produced transgenic *Hydra* expressing Hym-301 under the control of the *Hydra* actin promoter in all their epithelial cells (Khalturin and Bosch, unpubl.). Preliminary data indicate that during regeneration those animals show striking abnormalities in tentacle development. Thus, peptide Hym-301 appears to be causally involved in the regulation of tentacle formations. At the opposite end of the body axis, two peptides, pedin and pedibin, stimulate foot-regeneration (Hoffmeister, 1996). Both peptides are also among the peptides isolated by the *Hydra* peptide project (Takahashi et al., 1997). Hym-346, the *H. magnipapillata* homologue to pedibin, appears not only to accelerate foot regeneration but also to increase foot activation potential in gastric tissue (Fujisawa and Shimizu, pers. communication). Another novel peptide, Hym-323, (Harafuji et al., 2001) 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 indicate (Harafuji et al., 2001) that 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, 2000, 2003; Darmer et al., 1998) demonstrate that peptides are abundant within the phylum Cnidaria and that they play multiple roles in cell communication, cell differentiation and regeneration in *Hydra*.

### To regenerate is to communicate: signal transduction pathways in *Hydra* regeneration

Cell–cell communication and the exchange of information between cells and cell layers is a must in regeneration. Without cell cell communication, regeneration would not happen. Studies in insects and worms have shown that only a few signalling pathways generate much cellular and morphological diversity during the development of individual organisms (Pires-daSilva and Sommer, 2003). Helpful in identifying the signalling network’s biochemical components which in *Hydra* regeneration communicate exogenous signals to the transcriptional machinery 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, that signaling in *Hydra* regenerating tissue shows remarkable conservation to the signaling pathways used by the developing vertebrate embryo.

In vertebrates, all phases of wound healing are either directly or indirectly controlled by cytokines which may act in an autocrine or paracrine manner. Surprisingly, by now there is remarkably little evidence that cytokines and large secreted proteins such as growth factors are involved in regeneration and cellular differentiation in *Hydra*. There are two exceptions so

far. One is HyBMP5-8b (Reinhardt et al., 2004), a BMP5-8 orthologue and member of one of the most complex groups of cytokine superfamilies, consisting of various TGF- $\beta$  isoforms and other family members such as Activin A. Expression patterns of HyBMP5-8b in normal animals and in manipulated tissues under conditions that alter the positional value gradient indicate that HyBMP5-8b is active in tentacle formation and in patterning the lower end of the body axis (Reinhardt et al., 2004). There is evidence that the BMP antagonist gremlin, a cystein knot protein belonging to the CAN family that in vertebrates antagonizes preferentially BMP2 and BMP4 (Michos et al., 2004), is one of the factors involved in head regeneration (Holstein, pers. communication; see Fujisawa, 2006). Evidence for a second putative cytokine in *Hydra* was obtained in an *in silico* search for genes encoding insulin-related proteins which led to the identification of three candidate genes for insulin (Steele and Fujisawa, pers. communication; see Fujisawa, 2006). One of them is expressed in neurons while the other two are expressed in the ectoderm (Steele and Fujisawa, pers. communication). This together with the identification and characterization of the *Hydra* insulin receptor gene (HTK-7) (Steele et al., 1996) almost certainly will stimulate now efforts to understand the role of insulin signalling in *Hydra*.

*Anklet, a protein with a perforin and an EGF domain mediates foot specific differentiation*

An interesting but not yet completely understood protein functionally involved in the transition of the ectodermal epithelial stem cells into foot specific “basal disk” epithelial cells is the protein “anklet” (Amimoto et al., 2006) in *H. oligactis*. Amimoto et al. (2006) screened *H. oligactis* by DD-PCR for genes expressed early during foot regeneration. The screening identified anklet which has a signal sequence in its N-terminus, and one MAC/PF (Membrane attack complex/Perforin) domain, as well as one EGF domain. In foot-regenerating animals, *anklet* is first expressed in the newly differentiated basal disk cells at the regenerating basal end, and then expression becomes restricted in the lowest region of the peduncle (see Fig. 2E). Since this spatially specific expression pattern pointed to a role of anklet in basal disk formation, Amimoto et al. (2006) included in their study a functional analysis by suppressing the transcription level of anklet using RNA-mediated interference (RNAi). Suppression of the level of expression of the *anklet* gene led to a smaller foot and significant decrease in basal disk size, and during foot regeneration to a delay in basal disk regeneration. This shows that *anklet* is involved in the formation and maintenance of the basal disk in *Hydra*. With regard to the interesting structural features of anklet with both a perforin and a EGF domain, Amimoto et al. (2006) speculate that anklet may not serve as cytokine or growth factor but that the perforin domain may provide anklet with a cytotoxic function and may promote the drastic phenotypic changes which can be observed in cells approaching the boundary between the peduncle and the basal disk. In future experiments it will be interesting to see whether and how in

transgenic *Hydra* which are overexpressing anklet (or any other of the identified differentiation factors) the regeneration and differentiation processes are accelerated or modified.

*The ancient pathway: RTKs, Ras, and PI(3)K*

By now, several genes encoding receptor and non-receptor protein tyrosine-kinases have been identified in *Hydra* (Bosch et al., 1989; Steele et al., 1996; Reidling et al., 2000; Kroiher et al., 2000; Steele, 2002). Receptor tyrosine kinases (RTKs) activate Ras GTPase and the PLC $\gamma$ -PKC pathway leading to the activation of the phosphatidylinositol-3-OH kinase (PI(3)K)-Akt cell survival pathway (Schlessinger, 2000). A large family of RTK ligands with various functions in development are the fibroblast growth factors (FGFs). A *Hydra* FGFR-like gene, *kringelchen* (Sudhop et al., 2004) is activated during axis formation and bud detachment. The use of both synthetic inhibitors as well as antisense oligonucleotides against *kringelchen* specifically inhibits bud detachment. While this suggests a role of the *kringelchen* FGF receptor in this process, final elucidation of the function awaits the identification of the corresponding ligand and insight in the signalling cascade downstream of *kringelchen*. Since the *Hydra* genome appears to lack conserved FGF molecules (Bosch, pers. observation), the molecular analysis of *kringelchen* receptor activation might uncover unexpected surprises.

Cardenas et al. (2000) explored the role of RTK signalling in *Hydra* by specifically focussing on the role of the *Hydra* src-type receptor tyrosine kinase, STK (Bosch et al., 1989). Pharmacological inhibition revealed that STK is a key component of the signal transduction system involved in head formation (Cardenas et al., 2000). STK activity is strongly increased 6 h after decapitation, and the inhibition of its activity prevents head but not foot regeneration (Cardenas et al., 2000). Thus, this part of the analysis provided a striking correlation—high levels of STK are associated with head but not foot regeneration. To go beyond correlation, in a more recent study Cardenas and Salgado (2003) used the RNAi approach to block STK activity. When they depleted the STK mRNA level in this way, they created polyps which were unable to regenerate normal head structures. Thus, STK may play a major role in the initial commitment of cells to develop head structures.

Another central component of the mitogen activated protein kinase (MAPK) pathway which was identified in *Hydra* already more than one decade ago and shown to be differentially expressed during head regeneration is the small GTPase Ras2 (Bosch et al., 1995). Upon decapitation the transcript level of *ras2* (but not of the related gene *ras1*) decreases rapidly in the upper gastric region which is adjacent to the former head (Bosch et al., 1995). The disappearance of *ras2* mRNA can be prevented completely by direct stimulation of PKC. While the nature of the factor required to maintain *ras2* expression is not known yet, there are three observations which underline the importance of this signal transduction pathway in *Hydra* head formation: (i) head regeneration is associated with an increase in PKC activity (Müller, 1989; Hassel et al., 1998), (ii) *Hydra* has several PKC genes (Hassel, 1998; Hassel et al., 1998) with

some of them expressed in endodermal cells closed to wounded sites, and (iii) conversion of the gastric region into head tissue by activation of PKC has drastic and immediate consequences for the expression of a number of position dependent expressed *Hydra* genes. In addition to tyrosine protein kinases, a serine/threonine protein kinase belonging to the PKB/Akt family is upregulated during head regeneration (Herold et al., 2002). Since Manuel et al. (2006) recently presented evidence for the participation of another pathway, the PI(3)K–PKB pathway, involved in head regeneration in *Hydra*, the transduction pathways mediated by PKC, STK and PI(3)K may include the participation of ERK 1–2 as a point of convergence.

### *The canonical Wnt pathway and regeneration*

A well known signaling cascade that has been implicated in multiple biological processes in development is the Wnt pathway (Sancho et al., 2004; Bejsovec, 2005; Cadigan and Liu, 2006). Wnt proteins are secreted factors that act through a receptor called Frizzled, require antagonists such as Dickkopf proteins and control transcription through a  $\beta$ -catenin-dependent signaling mechanism (Cadigan and Liu, 2006). *Hydra* has several components of the Wnt cascade (Hobmayer et al., 2000; Broun et al., 2005; see Lee et al., 2006, for review). During head regeneration, HyWnt is found as a tight spot at the terminus of the regenerating body axis.  $\beta$ -Catenin and TCF are also upregulated, but over a wider region of the head (Hobmayer et al., 2000). Treatment of *Hydra* with alsterpaullone (Broun et al., 2005), which specifically blocks the activity of GSK-3 $\beta$  elevates the level of  $\beta$ -catenin in the nuclei of body column cells, confers characteristics of the head organizer on the body column, and induces the expression of genes of the Wnt pathway in the body column (Broun et al., 2005). These results provide direct evidence for a role of the canonical Wnt pathway in the formation and maintenance of the head organizer in hydra.

The mechanisms underlying the regulation of Wnt signalling in *Hydra* are not yet understood. Well known antagonists of Wnt signalling are Dickkopf proteins 1, 2 and 4 (Bejsovec, 2005). In a yeast signal peptide secretion screen directed towards isolation of regeneration specific genes in *Hydra*, Holstein's team (Guder et al., 2006) isolated a small 95 amino acid containing protein with high sequence similarity to the conserved cysteine pattern in Dickkopf proteins. Guder et al. (2006), therefore, termed the gene *Hydra* *hydck1/2/4*. In contrast to conventional members of the Dickkopf family which have two cysteine rich domains, *hydck1/2/4* contains only a single Dickkopf-like cysteine rich domain 2. The gene is expressed in endodermal gland cells and is also an early regeneration responsive gene both in foot and head regeneration. Guder et al. (2006) found a rapid and dramatic increase of *hydck1/2/4* message at the side of injury within 30 min after head removal. This early up-regulation was clearly related to the injury stimulus, since it also occurred by simply cutting the animal at any side in the body column. Guder et al. (2006) presume, therefore, that the early release of Dickkopf proteins at the side of cutting is an essential trigger for head regeneration. Is

HyDkk1/2/4 acting as a Wnt antagonist? When HyDkk1/2/4 mRNA was injected in *Xenopus* embryos, HyDkk1/2/4 has similar Wnt-antagonism activity as XDkk1 in *Xenopus* embryos. Moreover, in *Hydra* *hydck1/2/4* expression is complementary to that of *hywnt3a*, brachyury and other head-specific genes (Hobmayer et al., 2000; Technau and Bode, 1999). Finally, experimental activation of the Wnt/ $\beta$ -Catenin signalling leads to complete downregulation of *hydck1/2/4* transcripts. Although these observations may point to a role in Wnt signalling, this may not be the whole story for a number of reasons. First, early during regeneration *hydck1/2/4* and *hywnt3a* are co-expressed making a direct role of HyDkk1/2/4 as Wnt antagonist more complicated. Second, preliminary data from H. Shimizu (pers. communication) and our own lab show that in *H. magnipapillata* strain A10 in the complete absence of all *hydck1/2/4* expressing cells, the animals display normal morphogenesis indicating that at least in this strain *hydck1/2/4* plays no essential role in morphogenesis. Third, my lab recently has shown (Augustin et al., 2006) that there is a closely related gene to *hydck1/2/4* (in the Augustin et al. study termed HyDkk1/2/4-A), *hydck1/2/4-C*, which is not responsive to regeneration signals at all. The co-expression of both genes could be functionally significant because preliminary evidence based on yeast-two-hybrid system suggests that HyDkk1/2/4-A and HyDkk1/2/4-C interact with the same putative receptor (R. Kiko and T.C.G. Bosch, unpublished). Further experimental data are required, but my view at present is that these observations make a direct role of small Dickkopf-related molecules as regulators of Wnt signaling in *Hydra* less likely.

One important class of proteins shown to be involved in cell fate and terminal differentiation processes in many vertebrates and invertebrates are Notch proteins. Therefore, to complete our understanding of cell communication during regeneration in *Hydra*, questions concerning Notch signalling and cross talk between Notch and other pathways need to be addressed in future efforts. Interestingly, in *Hydra* expressing a Notch–GFP fusion protein, nuclear localization of Notch can be prevented by treating the animals with the synthetic presenilin inhibitor DAPT (A. Böttger, pers. communication; see Fujisawa, 2006). Since such DAPT treated polyps have defects in the interstitial cell differentiation pathway, Notch signalling appears to be involved in differentiation processes in *Hydra* as in bilaterian animals.

### **Control of transcription at the site of regeneration**

How is the positional information provided by peptides and other factors translated into the precise spatial and temporal expression of key regulatory genes? Several studies in hydra have provided compelling evidence that transcription factors classified as homeobox (Broun et al., 1999; Gauchat et al., 2000), paired box (Gauchat et al., 1998), fork head/HNF-3 motif (Martinez et al., 1997), and T-box (Technau and Bode, 1999) containing proteins are of particular importance in *Hydra* regeneration and development. Early studies have also shown that at least some of those transcription factors have retained their binding specificities during the course of

evolution. *Hydra* nuclear proteins, for example, change their binding activity to cAMP responsive elements (CREs) during regeneration (Galliot et al., 1995). The effects appear to be a general response to regeneration and not specific for head or foot formation. A *Hydra* protein related to the cAMP response element binding protein (CREB) was shown to participate in the CRE binding complex (Galliot et al., 1995) indicating a role for CRE-binding proteins during regeneration. More recently, Kaloulis et al. (2004) further explored the role of the cAMP-response element-binding protein pathway using an antibody against *Hydra* CREB which specifically detects phosphoSer133-CREB positive nuclei. Kaloulis et al. (2004) observed a dramatic increase in the number of phospho-CREB-positive nuclei in head-regenerating tips early during regeneration. Since a p80 CREB-binding kinase belonging to the ribosomal protein S6 kinase family showed an enhanced activity and a hyperphosphorylated status during head but not foot regeneration (Kaloulis et al., 2004), the mitogen-activated protein kinase/ribosomal protein S6 kinase/CREB pathway is involved in *Hydra* regeneration.

Martinez et al. (1997) have characterized a *Hydra* homologue of the fork head/HNF-3 class of winged-helix proteins, termed budhead, whose expression patterns suggest a role(s) similar to that found in vertebrates. In the adult *Hydra*, budhead is expressed in the upper part of the head, which has organizer properties. Although the expression pattern is consistent with a role in head formation, the mechanisms of action of budhead in *Hydra* are not yet clear. The functions of two transcriptional regulators belonging to the T-box gene family in *Hydra* regeneration and axis formation are at least superficially

exposed. In vertebrates, T-box transcription factors function in many different signaling pathways, notably bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) pathways. In *Hydra*, the brachyury homologue *HyBra1* is expressed in the endoderm very early during head regeneration and is confined to the region that will form the hypostome (Technau and Bode, 1999). Transplantation experiments indicate that the expression occurs before head determination has occurred, but expression does not irreversibly commit tissue to forming a head (Technau and Bode, 1999). In a new study, using the *Xenopus* animal cap system, Marcellini et al. (2003) investigated the inductive capacity of *HyBra1* and showed that it mimics the action of endogenous *Xenopus* Brachyury by inducing mesoderm but not endoderm. In diploblastic *Hydra* there is no mesoderm. Marcellini et al. (2003), therefore, suggest that the acquisition by Brachyury of properties later in evolution used to generate mesodermal fate predated the emergence of the mesoderm. The second T-box gene discovered in *Hydra* is *Cngsc*, a hydra homologue of the homeobox gene *gooseoid* (Broun et al., 1999). When injected into the ventral side of an early *Xenopus* embryo, *Cngsc* induces a partial secondary axis. The isolation of *Cngsc* and *HyBra* in *Hydra* and the fact that injection of their mRNA in *Xenopus* could mimic many of the properties of *Xenopus* organizer specific genes was an important discovery because it implied right from the outset conserved transcription factors in the execution of organizer activity in *Hydra*. On the basis of comparative expression data, Broun et al. (1999) suggested an evolutionary conservation of gooseoid, brachyury and HNF3 b interaction. Since, however, to date no downstream target genes have been

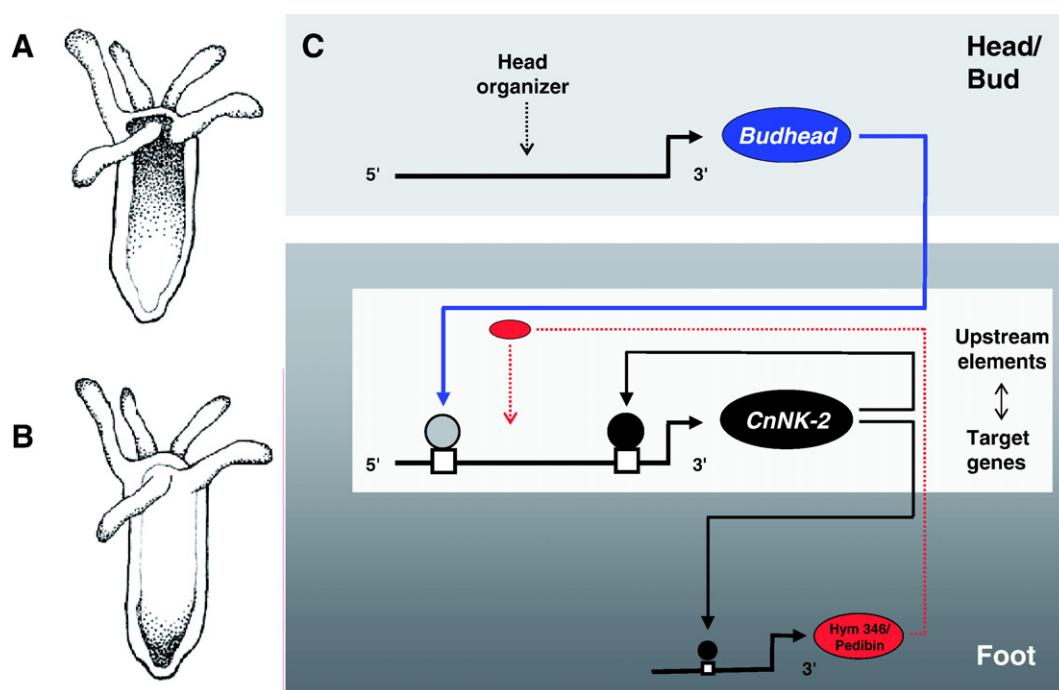


Fig. 4. Complexity of transcriptional regulation in foot epithelial cells. (A) Schematic diagram of the expression pattern of *budhead* in the upper part of the body column. (B) Schematic diagram of the expression pattern of *CnNK-2* in the foot region. (C) Key elements of the genetic regulatory network controlling *CnNK-2* expression. Modified with permission from Siebert et al., 2005.

discovered and little is known about the regulation of *HyBra1* and *gooseoid* genes themselves, further experimental comparisons are required before this conclusion can be drawn.

There is also experimental evidence that spatially restricted gene expression in *Hydra* is controlled by 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 at the protein level after head removal (Shenk et al., 1993a,b). By analyzing the protein binding sites of the promoter of *ks-1*, we showed (Endl et al., 1999) that *Cnox-2* binds to the *ks-1* promoter in the body column but not 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. This may imply that the default state of at least some of the spatially restricted developmental genes is “on” and that locally active transcriptional repressors cause the differential expression patterns.

Gene expression profiles are consequence of transcription factor activities, which, in turn, are controlled by extra-cellular signals. The relationships between all these regulators constitute a genetic regulatory network, which can be used to predict the behavior of the cell in changing environments. While we are far from understanding the genetic regulatory networks for any of *Hydra*'s cell types, emerging genomic technologies and promoter analysis of a foot specific homeobox gene, *CnNK-2*, has inspired us to outline a genetic regulatory network for foot regeneration (Thomsen et al., 2004; Siebert et al., 2005; Thomsen and Bosch, 2006). As stated above, 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, 1999). *CnNK-2* and *pedibin* are coexpressed in endodermal epithelial cells located at the basal end of the body column. 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 an attempt to unravel the transcriptional regulatory network controlling foot specific gene expression, we analyzed the *CnNK-2* 5'-flanking sequence by phylogenetic footprinting (Siebert et al., 2005). Unexpectedly, *budhead*, a nuclear factor involved in head and bud formation (Martinez et al., 1997), was found to bind specifically to the *CnNK-2* regulatory region (Siebert et al., 2005). As *budhead* is expressed opposite to *CnNK-2* in the head region (Fig. 4; Martinez et al., 1997), our results point to a molecular crosstalk between the head, bud and foot patterning systems during axis formation in *Hydra*. As schematically shown in Fig. 4, members of the signaling network controlling foot formation are peptide pedibin which is upstream and controls the expression of *CnNK-2*. *CnNK-2*, in turn, controls localized expression of pedibin and presumably also genes further downstream whose products are directly involved in foot differentiation. In addition, we obtained experimental evidence that *CnNK-2* regulates its own expression by an autocatalytic feedback loop (Thomsen et al., 2004). *Budhead* is proposed (Siebert et al., 2005) to be a transcriptional regulator of *CnNK-2*.

Overall, key elements of the mechanisms that control self-renewal and differentiation of epithelial cells in *Hydra* include

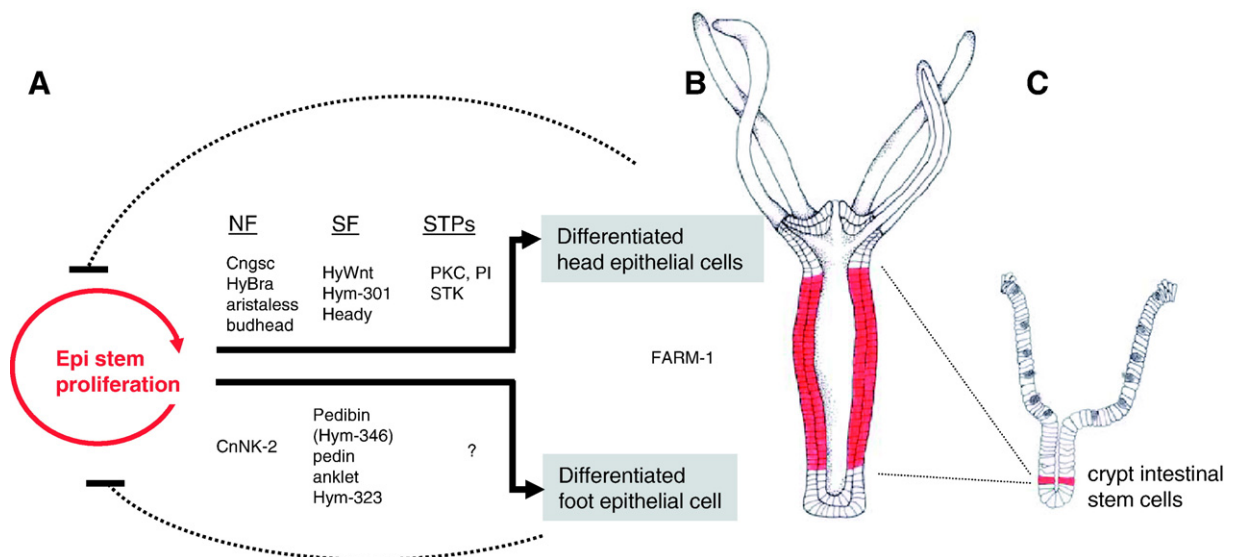


Fig. 5. Regulator and effector genes that affect epithelial cell differentiation in adult *Hydra*. NF, nuclear factors proposed to affect epithelial cell differentiation; SF, secreted factors proposed to affect epithelial cell differentiation; STP, signal transduction pathways proposed to affect epithelial cell differentiation. Dotted lines indicate suggested but not yet proven negative feed back loops. For references, see text. (B) Schematic diagram of a polyp. Red color indicates the localization of epithelial cells with indefinite proliferation capacity. (C) For comparison, schematic diagram of a villus in the mammalian small intestine with one of the crypts that contribute to renewal of its epithelium. Note that the few intestinal stem cells (stained red) are located near the bottom of the crypt (modified with permission from Moore and Lemischka, 2006).

both conserved and novel molecules, paracrine signalling pathways and autoregulatory feed back loops. With regard to the molecules and interactions depicted in Fig. 5, regulation of gene expression in *Hydra* appears to be as complex as in any other metazoan. Two aspects may deserve more detailed investigation in the future: (i) the mechanisms controlling the balance between self-renewal and differentiation of epithelial stem cells in *Hydra*; and (ii) the signals which specify the gastric region. To this end, the only gene expressed in epithelial cells exclusively in the gastric region is Farm-1 (Kumpfmüller et al., 1999), an astacin metalloprotease which is sensitive to positional signals specifying foot differentiation.

### The *Hydra* epithelium—a recipe for successful regeneration

*Hydra* has chosen a life cycle in which proliferation occurs mostly asexual by budding. That requires that each bud obtains the complete cellular repertoire from the mother polyp. By giving all the epithelial cells in the budding region stem cell properties and by filling the interstitial space with multipotent interstitial stem cells with the potential to differentiate not only into somatic cells but also into gametes, buds obtain all what they need. Thus, it is the stem cellness of the tissue which allows *Hydra* its unique life cycle. It seems that this feature alone is sufficient to explain *Hydra*'s unprecedented regeneration capacity. Does this reflect a particularly simple or even “primitive” molecular and cellular tissue architecture? I think no, for two reasons. First, in molecular terms *Hydra* as all other members of the phylum Cnidaria is astonishingly complex. The genomes in different *Hydra* species vary but in general are large with *Hydra vulgaris* having a genome of 1250 Mbp (Zacharias et al., 2004). That is about half size of the human haploid genome. Moreover, Cnidaria not only have about the same number of genes as human and share most of their genes with human (Miller et al., 2005) but their protein sequences, surprisingly, are often more similar to human sequences than to those from fly and worm (Kortschak et al., 2003). Thus, at the level of genomic complexity and gene complement, *Hydra* is much more complex than was previously imagined. Given the morphological simplicity, this complexity is surprising. It indicates, however, that the difference between “them” and “us” in terms of regeneration is unlikely to be based on the available complement of genes. Second, there is also no evidence that *Hydra* cells are fundamentally different from those of zebrafish or human. However, there may be a profound difference in the differentiation potential and plasticity of the cells between *Hydra* and vertebrates. Vertebrates including man depend on specialized cells with limited differentiation potential to perform sophisticated functions. Cells in *Hydra*, in contrast, are capable to produce and receive positional signals continuously even in adult tissue and, therefore, have features which most cells in vertebrates have only during the short period of embryogenesis.

Are regeneration studies in *Hydra* telling us anything relevant with respect to regeneration in man? Analysis of one of the most extensively studied mammalian epithelial stem cell systems, the crypt of the small intestine, has revealed that stem cells in vertebrates are present only in extremely low numbers

(Moore and Lemischka, 2006; see Fig. 5C) and – due to the complexity of the niche microenvironment – difficult to study directly. In contrast, in *Hydra* most of the epithelial cells have high self-renewing capacity and high phenotypic plasticity. As I have tried to outline above, in *Hydra* all raw materials for regeneration come from only three stem cell lineages. With the right instructions, these stem cells have the potential to enter all possible differentiation pathways and – in contrast to vertebrates – can be directly visualized and experimentally manipulated (Figs. 2C and 3). Since fundamental regulatory mechanisms are expected to be conserved in the animal kingdom, and since most vertebrate gene families appear to have deep evolutionary roots (Kortschak et al., 2003; Miller et al., 2005; Technau et al., 2005), these instructions most likely are the same for *Hydra* stem cells as for human cells. Thus, 265 years after Trembley, molecular dissectioning of the components controlling epithelial homeostasis and decision making in *Hydra* offers the hope to reveal fundamental principles that underlie all stem cell systems.

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