

Molecular analysis of *Hydra* embryonic development
by means of subtractive hybridization

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Abbreviations

'	minute
"	second
aa	Amino acid
AB	Antibody
BCIP	5-bromo-4-chloro-3-indolylphosphate
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
cm	Centimetre
cpm	Counts per minute
DABCO	Triethylendiamin
DAG	di-acyl glycerol
DAPI	4,6-diamidino-2-phenylindole
dATP	Deoxyriboadenosine triphosphate
dCTP	Deoxyribocytidine triphosphate
ddH ₂ O	Bi-distilled water
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyriboguanidine triphosphate
dH ₂ O	Distilled water
Dig	digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ds	Double stranded
dTTP	Deoxyribothymidine triphosphate
dUTP	Deoxyribouridine triphosphate
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetate, sodium salt
EST	expressed sequence tag
et al.	and others

EtOH	ethanol
Fig.	figure
FITC	Fluorescein isothiocyanate
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HKMTase	Histone lysine-specific methyltransferase
HS	Hybridization solution
i.e.	id est = that is
IPTG	Isopropyl-1-thio- β -D-galactoside
kb	kilobase
kV	Kilovolt
l	Liter
LB	Luria broth
M	Molar
MAB	Maleic acid buffer
MetOH	methanol
μ F	microfarad
mg	Milligram
μ g	Microgram
min	minute
ml	Milliliter
μ l	Microliter
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
NBT	Nitroblue tetrasolium
NCBI	National center for biotechnology information
ng	Nanogram
nm	nanometer
OD600	Optical density at 600 nm
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
pmol	Picomol
PRC	Polycomb Repressive Complex
RACE	Rapid amplification of cDNA ends
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Rounds per minute
RT	Reverse transcription
SDS	Sodium dodecylsulfate
ss	Single stranded
SSC	Sodium salino-cytrate
SSH	Suppression subtractive hybridization
SSH	Suppression subtractive hybridization
Ta	Annealing temperature of the primer
TdT	Terminal deoxynucleotidiltransferase
TEMED	N, N, N', N'- tetraethyl methyldiamine
Tm	Melting temperature of the primer
tRNA	Transport RNA
U	Unit
UTR	Untranslated region
UTR	Untranslated region
UV	Ultraviolet
UV	Ultraviolet
V	volt
Ω	Ohm
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. Introduction

1.1. Features of oogenesis and embryonic development.

The program of development of a multicellular animal includes formation of the most complex cell of an organism, the egg cell, its fertilization and rapid but errorless formation of the enormously complex body. A very obvious and at the same time very important feature of oogenesis and embryonic development is that it happens to every living individual only once. In the course of oogenesis or early embryonic development the body axes are defined, then body regions get specified, then cells proliferate and differentiate to form body parts. It is accepted that axis specification, pattern formation, morphogenetic movements, position-dependent cell fate specification, extensive cell proliferation, differentiation and apoptosis are trademarks of oogenesis and embryonic development. All these events require strict control over gene expression, which is possible only in a properly organized environment of structural proteins, yolk, membrane components and localized maternal mRNAs. After patterning is done, and the body parts and organs have developed, the task of the organism changes to maintenance of the homeostasis, renewing cell populations, fighting pathogens and, finally, reproduction. This change of the task is reflected in the presence of a number of structural and regulatory genes, both maternal and zygotic, necessary for building up an organism, but not for its maintenance. These genes are transiently expressed during oogenesis (if they are maternal) and embryogenesis. For example, maternal *bicoid* mRNA is stored at the anterior end of *Drosophila* egg, its protein forms a gradient, and after patterning the anterior-posterior axis of the fly *bicoid* is not expressed until it is time to make a new egg (Berleth et al., 1988; FlyBase <http://flybase.bio.indiana.edu/>). *Hunchback* is expressed in the ovary of *Drosophila* and its mRNA is present throughout the embryo, but in the anterior part of it, where bicoid binds to *hunchback* enhancers in zygotic nuclei, more *hunchback* is transcribed. It functions, together with bicoid, to pattern the anterior end of the embryo, and it is not expressed in adult fly before it starts with oogenesis (FlyBase <http://flybase.bio.indiana.edu/>). Zygotic gene *serendipity- α* encodes a cytoskeleton component involved in cellularization. It starts to be expressed during the 12-th cell cycle, reaches its peak at the early cycle 14 and disappears by the end of this cycle (for review see Mazumdar and Mazumdar, 2002). These data reflect

the fact that there are processes specific for embryonic development, and that these processes require certain genetic context.

At the same time there are animals, in which pattern formation, cell proliferation and differentiation occurs constantly or happens periodically in adult asexual state. For their unusual nature, these processes were even termed “somatic embryogenesis” (Tokin, 1969). Somatic embryogenesis is typical for the animals occupying both sides of the evolutionary border of Eumetazoa: sponges from below, and cnidarians from above.

1.2. Hydra – a model of constant pattern formation and morphogenesis in adult state

Freshwater *Hydra* is a typical, though simple, metazoan organism with true tissues, primitive nervous system and stable cell types. Since the XVIII-th century *Hydra* has attracted scholars thanks to its enormous regeneration capacity (Trembley, 1744). It is able to restore the rest of its body from a small piece and even from the aggregates of dissociated cells obtained by centrifugation (Gierer et al., 1972; Gierer, 1974; Bosch, 2003). Cell proliferation, differentiation, position-dependant cell fate specification and morphogenetic movements occur permanently in adult polyps as the cells divide in the gastric region and get displaced towards the extremities and into the buds (Bode et al., 1986). Therefore, processes, which are typical for embryogenesis of higher metazoans, can be studied in budding or regenerating *Hydra* polyps. All this, together with the simplicity of organization of the polyp, especially its single body axis, and phylogenetic position of Cnidaria - an ancient sister group (Fig. 1.1) to the bilaterally symmetric Metazoa – stipulated the choice of *Hydra* as a developmental biology and evolutionary model. The interest is so high that a large EST project and now also a genome project are being performed.

In terms of evolution of developmental pathways *Hydra* provides valuable data on the basic tool kit of genes necessary for a multicellular organism to be such. Putative ancestral functions of the genes become elucidated as conserved transcription factors are being found. Often their function in Bilateria is very different from their function in *Hydra*. For example *Hydra* has two homologues of the mesoderm-specific gene *brachyury* (Technau and Bode, 1999), but lacks mesoderm; *Hydra* and other cnidarians have homologues of several Hox and ParaHox genes for example, homologues of

labial, *Abdominal-B*, *Gsx* and *caudal* (Gauchat et al., 2000; Ferrier and Holland, 2001). In bilaterians these genes are involved in positional cell fate specification along the anterior-posterior axis, but cnidarians lack a clear anterior-posterior axis, as the apical-basal axis cannot be unambiguously called its homologue.

1.2.1. Phylogenetic position, anatomy and histology of *Hydra*

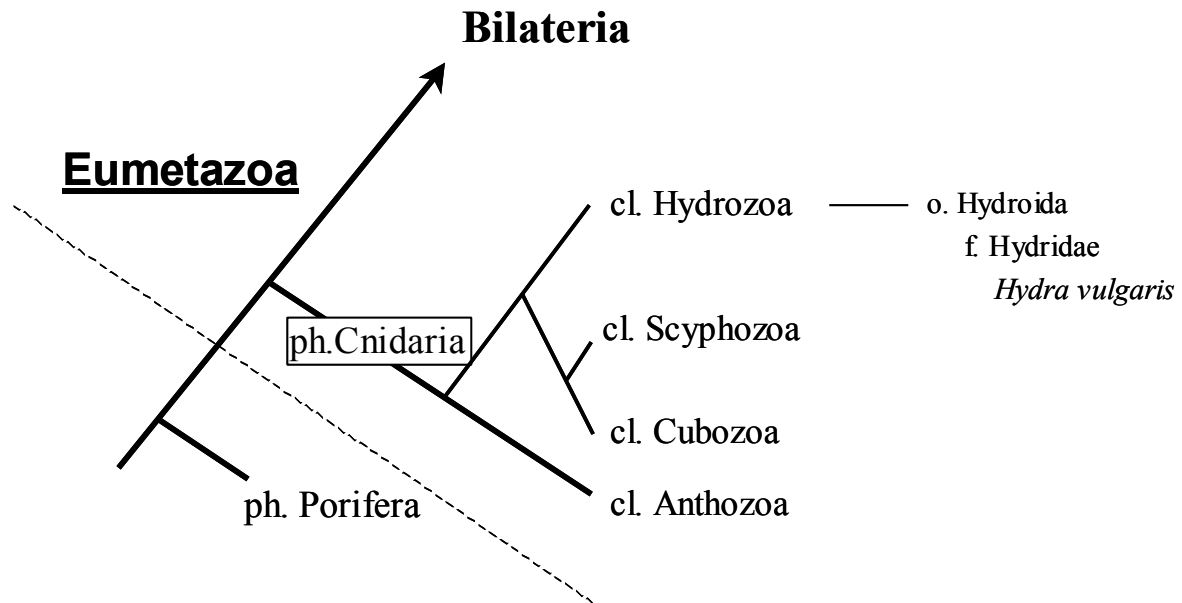


Fig. 1.1 Phylogenetic position of *Hydra* according to Medina et al. (2001) and the ITIS database (<http://www.itis.usda.gov/>)

Hydra belongs to the phylum Cnidaria encompassing radially symmetric diploblastic animals, distinct fossils of which were dated as more than 500 million years old (Xiao et al., 2000). Cnidarians were named this way, thanks to the presence of stinging cells (called cnidocytes or nematocytes) in their body wall. The class Hydrozoa is built up of seven orders, and is typically represented by marine colonial animals with a change of asexual polyp generation and sexual medusa generation. *Hydra*, however, completely lacks medusae and develops simple gonads on the body walls of the male and female polyps. In favorable conditions *Hydra* propagates asexually by budding, but in autumn or in case of depression it converts to sexual process, which will be described below. Anatomy of a polyp is simple. It is a bi-layered tube with a ring of tentacles surrounding the hypostome with a mouth on the apical end (head) and a peduncle with

a basal disc on the basal end (foot). Under good feeding conditions buds develop regularly on the border of the lower two thirds of the length of the animal (Fig. 1.2).

The body wall consists of two cell layers – ectoderm and endoderm. There are three stable cell lineages: ectodermal epitheliomuscular cells, endodermal

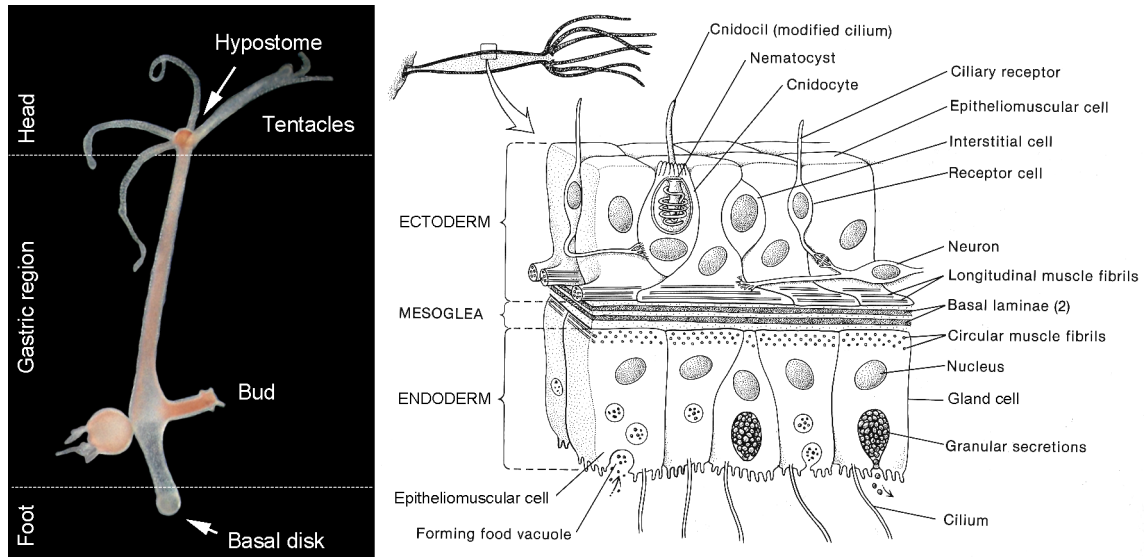


Fig. 1.2 Anatomy of *Hydra* and scheme of its body wall (after Ruppert and Barnes, 1994)

epitheliomuscular cells and interstitial stem cell lineage (David and Campbell, 1972; Campbell and David, 1974). Epitheliomuscular cells are the main cell type, responsible for maintenance of the shape of the animal and for pattern formation in normal and experimental situations. They are usually called simply epithelial cells.

Interstitial stem cells inhabit the space between the basal parts of the ectodermal epithelial cells. As shown on Fig. 1.3, interstitial stem cells can proliferate (~60%) and differentiate (~40%) into somatic cells, such as nerve cells, gland cells and nematocytes, and into gametes (Bosch and David, 1987; Bosch et al., 1991).

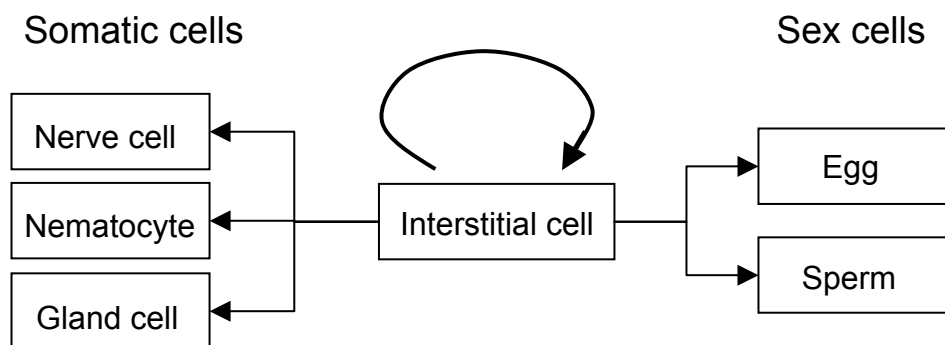


Fig. 1.3 Differentiation pathways of the interstitial cells

1.2.2. Gametogenesis in *Hydra*

Unlike many other animal groups, cnidarians do not have a stable germ line (for review see Extavour and Akam, 2003). Both, spermatogonia and oogonia originate from a subpopulation of interstitial stem cells, which gets committed to a germ fate. At the same time interstitial stem cells, both in sexual and asexual polyps, permanently express germ line marker genes such as *vasa* homologues *Cnvas1* and *Cnvas2* (Mochizuki et al., 2001) and *nanos* homologue *Cnnos1* (Mochizuki et al., 2000). Cnidarians have true gonads – specific regions, which get populated by germ cells migrating into them. *Hydra* gonads are extremely simple. They are just distinct places where germ cells accumulate between the mesoglea and ectodermal epithelial sheet.

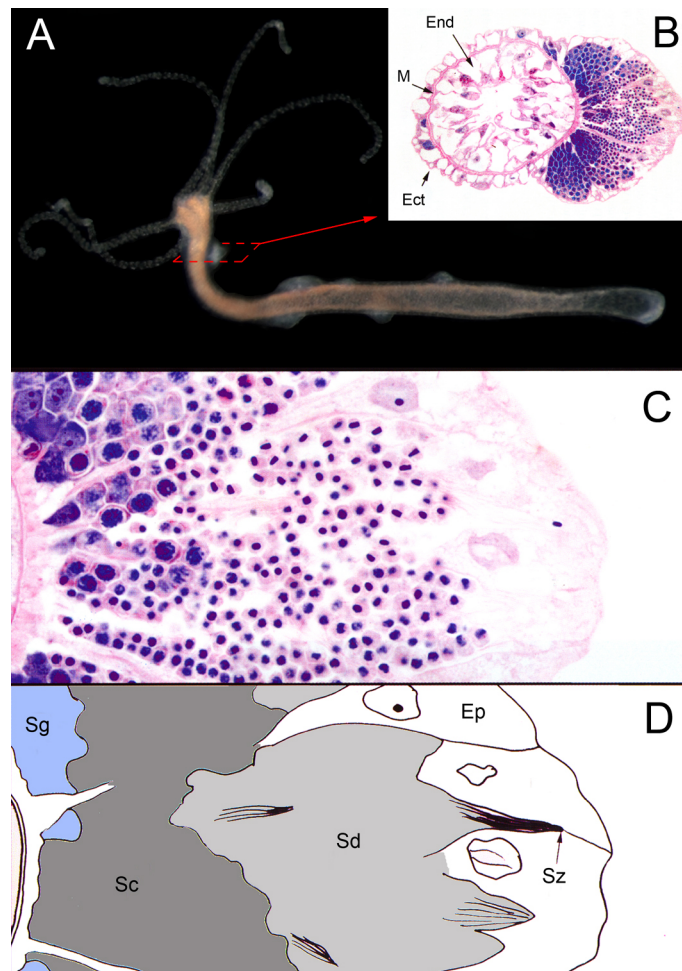


Fig. 1.4 Anatomy and histology of the hydra testis. A) Male polyp. B) Cross-section of the body and testis Ect – ectoderm, End – endoderm, M – mesoglea. C) histology of the testis D) scheme of the section shown on “C”. Sg – spermatogonia, Sc – spermatocytes, Sd – spermatids, Sz – spermatozoa. Ep – epithelial cell. Characteristic location of cells at different stages and changes in nucleus sizes are visible. (After Kuznetsov et al., 2001 with modifications)

While spermatogenesis (Fig. 1.4) goes in a quite conventional way, with cells at earlier stages of sperm development more proximal, and spermatids and mature spermatozoa more distal from the mesoglea (Brien, 1966; Kuznetsov et al., 2001), *Hydra* oogenesis is very peculiar.

Those interstitial cells that will become oogonia start to divide and form clusters, connected by cytoplasmic bridges. The process of conversion of an interstitial cell into oogonium involves significant changes in its intracellular structures. The amount of cytoplasm increases; massive rough endoplasmatic reticulum develops; lipid droplets

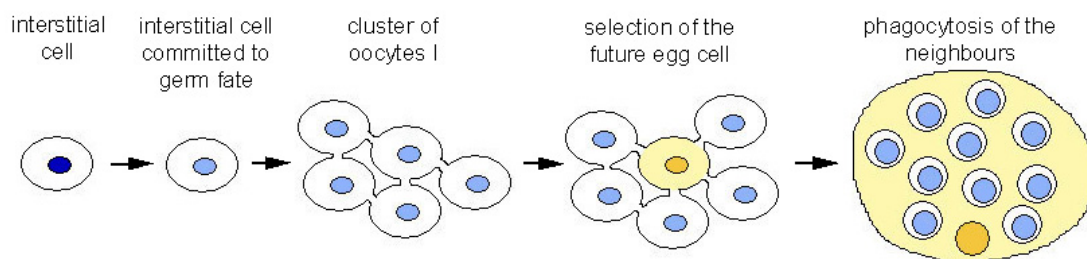


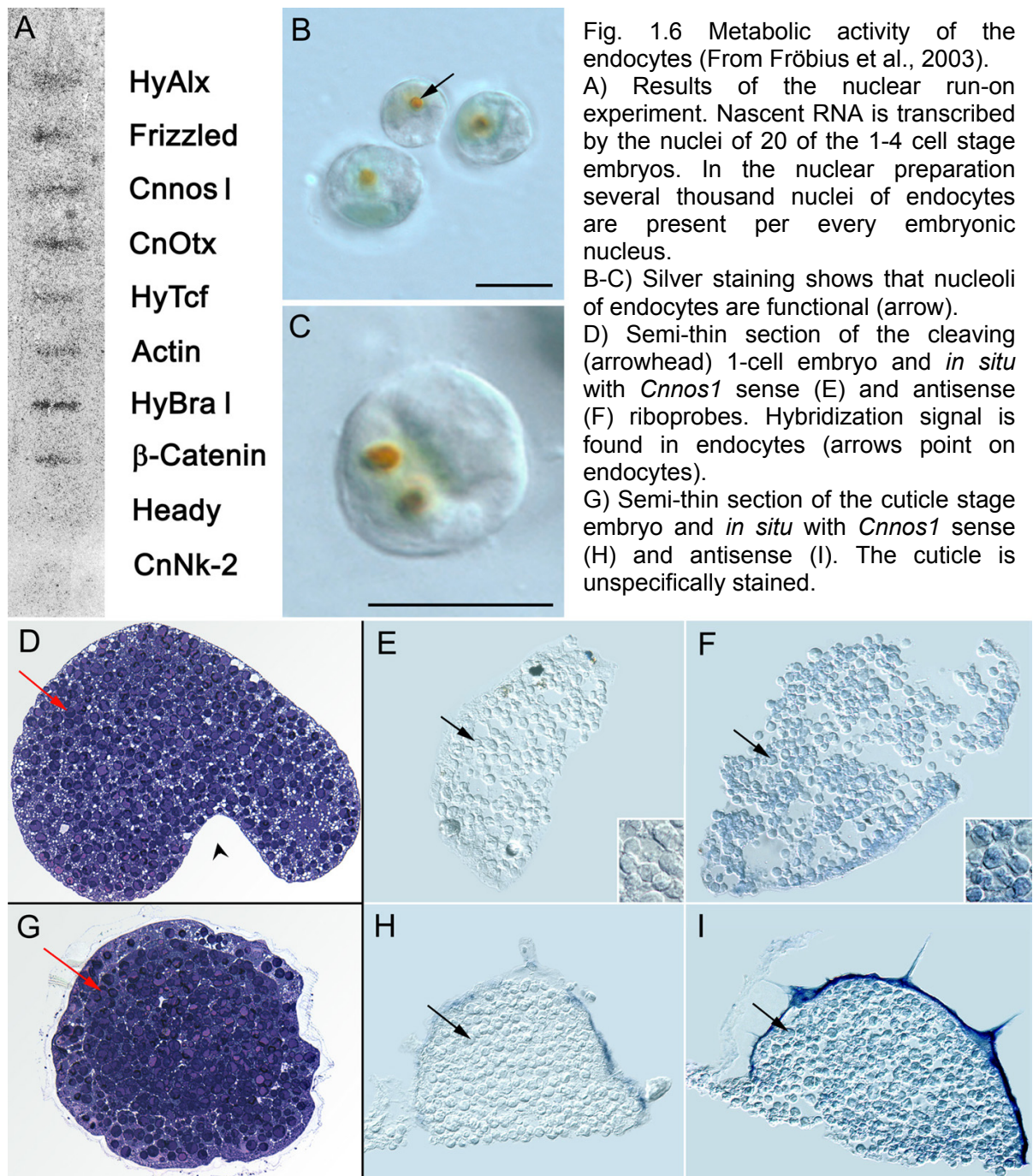
Fig. 1.5 A scheme of oogenesis in *Hydra*

and glycogen inclusions get accumulated. Multiple Golgi complexes form from smooth vesicles; number of mitochondria increases; intensive emission of ribonucleoprotein material from the nucleus into the cytoplasm takes place. Smooth vesicles and membranous tubes integrate into the cell membrane, significantly increasing its surface (Aizenshtadt, 1974). At this time all the cells of the cluster undergo first meiotic division, which can be traced by cytophotometric measurement of the DNA content in cells of the cluster, and become oocytes I (Aizenshtadt and Marshak, 1975). Then one of them gets specified to develop into an egg cell, while the others will become nurse cells of the egg (Fig. 1.5). The mechanism of this induction is unknown. Contact to the mesoglea might be important, because the growing egg was shown always to form cell contacts with the endodermal epitheliomuscular cells, reaching them through the mesoglea with cellular processes (Aizenshtadt, 1978). There are data that homeobox gene *CnOtx* is likely to be involved in defining the future egg (Miller et al., 2000).

The growing oocyte then forms interdigitations with the surrounding oocytes and starts to “bite of” and phagocytose pieces of their cytoplasm. The membranes surrounding these pieces then get disassembled into small vesicles and the cytoplasm becomes incorporated into the cytoplasm of the rapidly growing oocyte. At a certain point the growing oocyte starts to engulf the surrounding oocytes, and though few of

them get lysed and digested, the majority of these *endocytes* or *nurse cells* (2000-9000 cells) remain stored in vesicular bodies within the egg throughout the embryonic development (Aizenshtadt, 1978, Martin et al., 1997). When the oocyte reaches its final size, the ectodermal sheet ruptures, and the oocyte gets exposed to the environment.

Phagocytotic type of oogenesis is not unique for *Hydra*. Oocytes of sponges also uptake surrounding cells, though in case of haplosclerid sponges somatic cells are being engulfed and not oocytes (Ereskovskii, 1999). The most interesting fact is that both in sponges and cnidarians the endocytes do not undergo immediate cytolysis when



phagocytosed by the growing oocyte. On the contrary (Fig. 1.6), in *Hydra* eggs and embryos they remain transcriptionally and, possibly, translationally active (Fröbius et al., 2003). We could demonstrate by means of nuclear run-on experiments with nuclei from twenty 1 to 4 cell stage embryos (such preparations contain several thousand endocyte nuclei per every nucleus of the blastomere) that nascent RNA of several regulatory and structural genes is being transcribed at these early stages (Fig. 1.6A) Moreover, nucleoli of the endocytes can be stained with AgNO₃, which is characteristic of metabolically active nucleoli producing ribosomal RNA (Fig. 1.6 B-C). Finally, *Cnnos1* RNA could be detected in the nurse cells by means of *in situ* hybridization on tissue sections (Fig. 1.6 D-I).

Endocytes were first termed apoptotic cells by Honneger et al. (1989), but detailed study did not appear before last year (Technau et al., 2003), when it was demonstrated that endocytes remain in a state of arrested apoptosis. It was shown that caspase activity was permissive for the onset of oogenesis. Caspase inhibition on stage 1-2 (stage definition according to Miller et al., 2000) caused aborted oogenesis and failure to engulf endocytes, but caspase repression at later stages did not affect oogenesis. So apoptosis induction is necessary for the start but unwanted in the course of oogenesis. Nurse cells are TUNEL-negative and remain TUNEL-negative throughout oogenesis and embryonic development. Chromatin of the endocytes is in some way protected against Dnase I. Treatment with Dnase I causes rapid increase in the number of TUNEL-positive epithelial cells but does not affect nurse cells. The arrest in apoptosis is probably due to increased peroxidase activity in the embryo. Peroxidases and catalases inhibit apoptosis by preventing oxidative stress to mitochondria (Zhang et al., 1997). In *hydra*, endocytes possess elevated level of peroxidase activity from earliest stages of oogenesis till hatching of a young polyp, in which endocytes are still present in the endoderm. Within 1-3 days after hatching, peroxidase activity decreases, starting from the apical end and expanding to the basal regions, and it correlates with the degradation and disappearance of the endocytes. Phagosomes, in which endocytes are kept, were shown not to fuse with the acidic secondary lysosomes until shortly after hatching. All the endocytes in lysosomes were highly degraded and TUNEL-positive (Technau et al., 2003).

1.2.3. Embryogenesis in *Hydra*

Hydra embryogenesis was described in detail by Martin et al. (1997). External fertilization takes place after the oocyte has completed its second maturation division (Tardent, 1985). The egg starts to cleave radially (Fig. 1.7 B-D), with animal blastomeres slightly bigger than vegetative, and at 8 hours post fertilization develops into coeloblastula, consisting of approximately 76 polygonal cells surrounding a large cavity (Fig. 1.7 E). Gastrulation occurs in form of immigration, spreading in a wave from the animal (distal from the body of the mother polyp) to the vegetal pole. The cells get

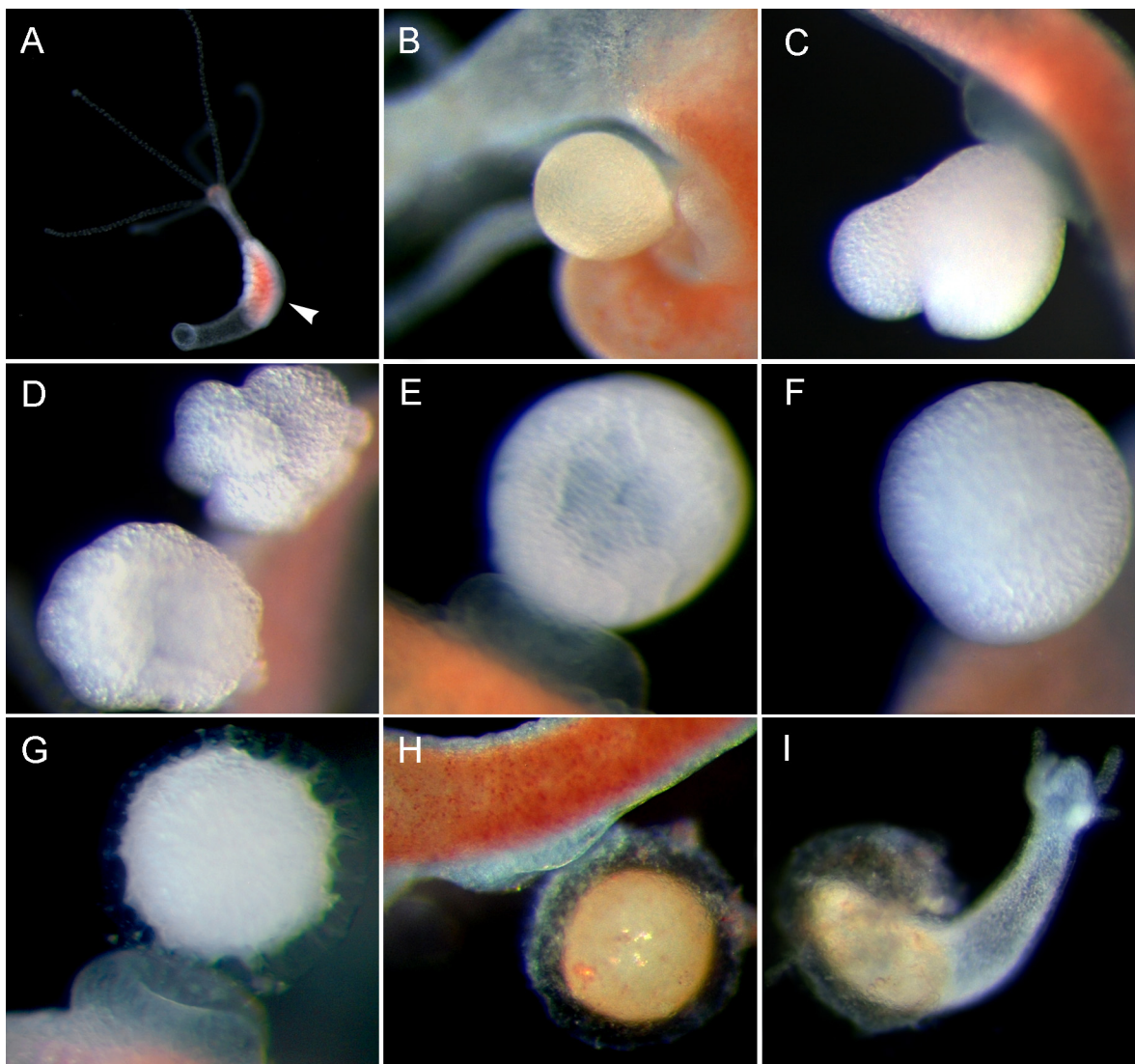


Fig. 1.7 *Hydra* oogenesis and embryogenesis. A) Female *Hydra* polyp. The arrowhead indicates the egg fleck – the accumulation of interstitial cells converting into oogonia. B) Hydra egg. C) First cleavage furrow. D) Cleaving embryos of different stages. E) Coeloblastula. F) Gastrula. G) Spike stage. H) Cuticle stage embryo, still attached to the mother polyp. I) Hatching polyp.

displaced one by one into the blastocoel and fill it completely. By 12 hrs. post fertilization gastrulation is over (Fig. 1.7 F). The result of immigration is a parenchymula-like embryo with an ectodermal layer surrounding the unorganized inner cell mass. Interestingly, the bulk of endocytes is incorporated into immigrating cells, while ectodermal blastomeres retain few endocytes. After approximately twelve more hours without significant changes to be observed, the ectodermal cells start to extend filopodia and secrete a firm multi-layer chitinous cuticle. This “spike” stage (Fig. 1.7 G) lasts for approximately 24 hours. As soon as the cuticle is constructed, the filopodia get drawn in and the embryo reaches the dormant overwintering stage, which can last up to 24 weeks (Fig.1.7 H). Further on I will refer to it as a “cuticle stage”. After some time the embryo detaches from the mother. Prior to hatching the unorganized interior cell mass organizes into endodermal layer; gastric cavity, mesoglea, hypostome, tentacles and basal disk form. After these morphogenetic events are complete, the young *Hydra* starts to contract and expand and finally hatches through a crack in the cuticle (Fig. 1.7 I). Young polyps possess all the derivatives of interstitial stem cells and are able to catch, uptake and digest food. They also can regenerate as good as adult polyps. No physical or chemical agents were shown to date to effectively induce hatching.

1.2.4. Pattern formation in adult animals: generating positional signal

As it was already mentioned, pattern formation is a permanent process in *hydra*. The flow of cells into the buds and into the extremities requires constant guidance by a stable positional information system. This system was studied in detail thanks to polyp’s capacity to regenerate body parts by a morphallactic process. The results of multiple regeneration and grafting experiments allowed Alfred Gierer and Hans Meinhardt to apply Alan Turing’s reaction-diffusion model for description of morphogenetic events in *Hydra* (Gierer and Meinhardt, 1972). Head and foot are postulated to be the sources of pairs of morphogenetic gradients: the short-range gradient of the activator and the long-range gradient of the inhibitor of the head and foot specification. An activator-producing cell induces its closest neighbors to produce activator, and, at the same, time the inhibitor. The activator does not diffuse far from its source and forms a local high concentration patch, while the inhibitor diffuses more easily and represses activator production everywhere but in the pre-existing area of activation. Further development of

the model by Meinhardt (1993) postulated the existence of a gradient of “source density”, describing the ability of the tissue to produce activator. The presence of source density gradient explains why a polyp with amputated head and foot still regenerates the head at the former apical end, and the foot at the former basal end.

The quantity of the activator and inhibitor at every particular place along the body column of a *Hydra* is interpreted by cells as “positional value”, with the head having the highest, and the foot – the lowest positional value. This change in positional value (or source density in Meinhardt’s model) is clearly demonstrated by the change in head regeneration potential. The closer to the former head was the apicalmost tissue of the decapitated stump, the faster it regenerates the head and the earlier it starts to express head-specific genes. The cells react to changes in positional value by changing their differentiation state. The head and the foot act as primary organizing centers and actually establish the system of positional values, which can be observed when transplanted heads or feet induce re-specification of the tissue of the recipient in grafting experiments (Berking, 1981; MacWilliams, 1982). Both cell layers of the body wall contribute to the system of positional coordinates. It was shown (Smid and Tardent, 1982) in a following elegant experiment. Cylindrical piece of hydra gastric region was treated with procaine and endoderm was separated from the ectoderm. The endodermal tube was then inverted and re-inserted into the ectodermal tube. Out of 33 stumps used for the experiment 8 formed heads at the apical side of the ectodermal fragment, 3 formed heads in agreement with the orientation of the endodermal fragment and 22 formed a new axis, perpendicular to the original, so that the head formed at the site where the summary positional value of both ectoderm and endoderm was the highest.

Different substances influence the positional value. Phorbol esters, di-acyl glycerol (DAG) and arachidonic acid, for example, were shown to increase positional value of the tissue, which resulted in formation of ectopic heads or single tentacles in treated animals (Müller, 1989; Müller, 1990; Weinziger et al., 1994). Lithium chloride, on the contrary, lowers the positional value, resulting in development of ectopic feet (Hassel et al., 1993). Similar action was demonstrated for peptides pedibin and Hym 346 (Grens et al., 1996)

The positional value system determines the morphology of hydra, as different regulatory and realizator genes are switched on and off in a position-dependent manner. There are 5 distinct regions of the hydra body, based on the genes expressed:

the hypostome, the tentacle formation zone and tentacle bases, the gastric region, the peduncle and the basal disk. What could be the physical carrier of the positional information remains largely an unanswered question. Changes in the composition of the extracellular matrix (ECM) could play a role in providing cells with a kind of molecular address. Interestingly, there are genes coding for the astacin family matrix metalloproteases, which are expressed in a position dependent manner and might play a role in location-specific ECM modification. For example, *HMP-1* is expressed in a head-to-foot gradient by the endodermal gland cells, and its protein is forming a gradient mirroring the RNA distribution. The expression of this gene is upregulated within 2 hours after decapitation (Yan et al., 1995; Yan et al., 2000a). In contrast to *HMP-1*, *HMP-2* is expressed in the foot-to-head gradient (Yan et al., 2000b), but its protein seems to be equally distributed along the whole gastric region as far as tentacle bases. Finally *Farm1* was found to be expressed everywhere but head and foot, and was shown to be sensitive to lowering of the positional value by LiCl or peptide pedibin (Kumpfmüller et al., 1999).

1.2.5. Pattern formation in adult animals: regional cell fate specification

There are many conserved transcription factors involved in regulating the patterning events. As *Hydra* belongs to the sister group of Bilateria, it is always tempting to compare the regions of expression of orthologous genes in Cnidaria and Bilateria. The most discussed question is, of course, the correspondence of the apico-basal axis of a cnidarian to one of the body axes of a triploblast animal, usually the anterior-posterior axis (Meinhardt, 2004; Finnerty et al., 2004). While this remains largely a speculative issue, especially in positioning the head of *Hydra* at the anterior or posterior end of its putative anterior-posterior axis, based on expression of different genes, there are cases of unquestioned homology. One of such cases is the hypostome. As it was already mentioned, this region has organizer features, the main of which is the ability to induce formation of a secondary axis, when transplanted into ectopic location (Broun and Bode, 2002). Based on embryological data from other cnidarians, which gastrulate by invagination, for example the soft coral *Nematostella vectensis*, it is clear that *Hydra* hypostome corresponds to the region around the blastopore (Scholz and Technau, 2003). And it was dorsal lip of the blastopore, which

was first shown by Spemann and Mangold to be the organizer in the newt (Spemann and Mangold, 1924, cited from Gilbert, 2003). There are genes, expression of which is characteristic for the organizer tissue in other models (mostly vertebrate, for example, *Xenopus laevis*, *Danio rerio*, *Gallus gallus* etc.), and which were also shown to be expressed in *Hydra* hypostome. These are *HyBra1*, the homologue of *brachyury*, *Cngsc*, the homologue of *gooseoid*, *Budhead*, the homologue of *Fork head/HNF3 β* and the members of the Wnt signaling cascade.

Brachyury is coding for a T-box transcription factor, which is involved in vertebrates in early mesoderm specification and, later during gastrulation, in axial and posterior mesoderm formation. Brachyury-BMP4 interplay was recently shown to establish the early pattern of Hox genes expression in *Xenopus* (Wacker et al., 2004). The expression in posterior mesoderm next or around the blastopore is deemed to be the ancestral one, as it is observed both in primitive deuterostomes and protostomes (Technau, 2001; Arendt et al., 2001; Technau and Scholz, 2003). *Hydra brachyury 1*, is continuously expressed in the endodermal cells of the hypostome, the region homologous to the circumblastoporal zone. *HyBra1* is also expressed at the site, where the bud will form, before the onset of any morphogenetic movements. The original expression is confined to a small group of cells, which will eventually become the tip of the hypostome. The area of expression enlarges, as the bud starts to grow, but always remains confined to the apical tip of the bud. During head regeneration it is expressed as early as after 3 hours post-decapitation, and reaches the maximum expression level by 4-6 hours. Tentacle buds emerge always at the lower margin of the expression domain. Notably, *HyBra1* is a marker and regulator of the hypostome formation, but not tentacle formation. Its expression was always observed in the hypostomes of the ectopic heads after DAG treatment, but never in the individual ectopic tentacles. It was also shown that *HyBra1* expression is essential for the formation of the head, but it does not commit the tissue to the head fate, as it can be reversed by transplantation of the *HyBra1*-expressing regenerating tip into a location in a recipient polyp, which is not conducive for head formation.

Gooseoid is a gene coding for a homeodomain transcription factor. It is expressed in the organizer region of vertebrate embryos and plays also a role in formation of the anterior/dorsal structures of the embryo. In frog it is synergistically controlled by *Siamois*, the key homeobox gene responding to β -catenin induction to

form a Nieuwcoop center prior to gastrulation (Fan and Sokol, 1997), and TGF β paracrine factors (Engleka and Kessler, 2001). Both, *Siamois* and *gooseoid* can induce an ectopic axis if their mRNA is injected into ventral vegetal blastomeres. Moreover, mRNA of *Cngsc*, a *Hydra* homologue of *gooseoid*, is also potent to induce an ectopic axis in *Xenopus* embryo (Broun et al., 1999). In *Hydra* *Cngsc* is expressed in three different spatial domains. First, there is a dotted expression in a subset of cells in the ectoderm of the hypostome, which are supposedly nerve cells, as this expression domain does not exist in hydroxyurea-treated hydras (such polyps have strongly reduced population of interstitial cells and their derivatives). Second, there is a ring of *Cngsc* expression in the endodermal epithelial cells at the border between the tentacle zone and the hypostome. And third, there is weak expression of the gene in the endodermal epithelial cells in the body column. During budding, the region of the endoderm underlying the bud placode becomes devoid of *Cngsc* message, and this repression spreads over the whole field of the bud. As the bud starts to evaginate, the ring-like endodermal expression reappears at the apical end, but not in the very tip of the bud. From this stage on, it remains confined to the future tentacle zone. Speckled ectodermal expression is reestablished during the middle stages of bud formation, followed by reappearance of the third domain in the endoderm of the body column. During head regeneration *Cngsc* is first removed from the regenerating tip (endodermal ring), then it reappears after 24 hours as a spot or a narrow ring which gets to its final position a bit away from the tip. Subsequently, tentacle bumps appear right at the lower margin of the ring. The function of the gene is not completely clear. Tentacle formation does not require *Cngsc* expression, as shown on DAG-treated polyps forming ectopic tentacles. It also seems not to be involved in initial stages of head patterning, as it is expressed too late during head regeneration. At the same time, *Cngsc* might function as a separator of two regions of the head: the hypostome and the tentacle region. This would require mutual negative regulation of *Cngsc* and *HyBra1*, which is known for their *Xenopus* orthologues (Artinger et al., 1997). Interestingly, just like in *Hydra* buds and regenerating heads, the *Xbra* and *gsc* expression domains overlap in early Spemann organizer, but later during gastrulation the coexpression is lost as the proteins start to directly repress the transcription of each other.

Budhead is a *Hydra* homologue of *Fork head* (Martinez et al., 1997). In *Drosophila*, as well as in vertebrates *Fork head/HNF-3 β* plays a role in specification of the foregut and hindgut, and is expressed in the organizer prior to and during

gastrulation. Vertebrate *fork head* genes are also required for the dorsal mesoderm development and neural tube patterning (Ruiz i Altaba et al., 1993). In *Hydra budhead* is expressed in the endodermal epithelial cells. The expression is strongest in the tentacle zone (but not in the tentacles) and right above it, but upper portion of the hypostome stays devoid of *budhead* message. The expression is also observed in the endodermal epithelial cells in a head-to-foot gradient, fading away approximately on the level of budding zone (Martinez et al. 1997; Siebert, personal communication). During budding the gene is upregulated in the endoderm underlying the bud placode. Later *budhead* expression encompasses the whole presumptive bud tissue. As the bud evaginates, the *budhead*-free apical area appears and the expression forms a gradient as in adult polyp. During head regeneration *budhead* is first detected 8 hours post-decapitation and follows with minor differences its mode of expression during budding. As well as in case of *HyBra1* and *Cngsc*, in DAG- or arachidonic acid-treated polyps *budhead* expression was observed only when hypostomes but not individual ectopic tentacles formed. Synergistic effect of *HNF-3* and *brachyury* was demonstrated in ascidian *Ciona intestinalis* (Shimauchi et al., 2001). Partial overlap of the expression domains of *HyBra1* and *budhead* in adult polyps and complete overlap during early phases of budding and regeneration might mean that some kind of joint function is conserved in *Hydra* as well.

In vertebrates the organizer is formed as a result of the interplay of the Wnt and TGF β signaling. Members of the Wnt cascade are known from *Hydra*. It is noteworthy that the cloned *Hydra* homologue of *frizzled* (Minobe et al., 2000) appears not to be relevant to the particular Wnt signaling described (Hobmayer et al., 2000). Its mRNA is localized in the endoderm, while all the other isolated genes are ectodermally expressed. While *Hydra* homologues of *dishevelled* and *GSK3* are expressed uniformly, *β -catenin*, *Tcf* and *Wnt* demonstrate dynamic expression patterns. *HyTcf* is expressed in the entire hypostome, and fades in a gradient manner in the upper third of the body. It is also expressed in buds from the earliest stages on, first overall, later in the same pattern as in adults. *β -catenin* is as well upregulated during budding in the domain overlapping with *HyTcf* expression in addition to the low level expression all over the body. But most interestingly, *HyWnt* is expressed in a domain of few epithelial cells at the tip of the hypostome. It is also expressed in several cells, which will eventually become the tip of the hypostome of the bud, and then expands to the neighboring cells,

until it reaches the size of the adult hypostome expression domain. During head regeneration *HyWnt*, *HyTcf* and *Hyβ-Cat* are upregulated within 1 hour after decapitation, which is, probably, a record value for the onset of gene expression in regenerating *Hydra* tissue. It is proposed that Wnt pathway is an early and key element in the setup of *Hydra* head organizer.

In contrast to the head system, few conserved regulators are known to be expressed in the foot. The cells, which get displaced into the foot become re-specified as they move down the peduncle against the gradient of *CnNK-2*, an *NK2* class homeobox gene, expressed in the endoderm of the peduncle and in a foot-to-head gradient in the lower half of the body (Grens et al., 1996). The point of no return in the differentiation into a foot cell is reached, as the cell crosses the peduncle/basal disk border, characterized by the most intense expression of *CnNK-2* in the endoderm, and a ring of expression of paired-like homeobox gene *manacle* in the ectoderm (Bridge et al., 2000). It is important to mention that the decline of positional value results in evident derepression of foot genes, for example *CnNK-2*. It was shown that treatment with LiCl or peptides pedibin and Hym346 caused expansion of the *CnNK-2* expression domain (Grens et al., 1999). This might mean that there is possibly a long range antagonism between the foot and head systems. Indirect evidence of this might be the complementary pattern of budhead and *CnNK-2* expression at the basis of the bud. Transcription of *CnNK-2*, otherwise strong in the budding zone, is completely abolished in the bud field exactly at the site of *budhead* expression (Siebert, personal communication).

Not only conserved, but also novel genes are known to play crucial roles in pattern formation in hydra polyps. Morphogenetically active novel peptides were shown to be required for head (Lohmann and Bosch, 2000) and foot specification (Hoffmeister, 1996; Takahashi et al., 1997; Grens et al., 1999; Harafuji et al., 2001), as well as for differentiation processes of different cell types, especially neurons (Bosch and Fujisawa, 2001). Recently first evidence of direct physical interplay of the conserved (*CnNK-2*) and novel (pedibin) members of the foot patterning machinery was obtained (Thomsen et al., 2004).

1.2.6. What is known about the *Hydra* embryo in molecular terms and what has to be understood?

Hydra is constantly undergoing embryonic processes in adult state and is traditionally regarded by researchers as “eternal embryo”. At the same time its system of positional coordinates remains permanently stable and provides dynamic tissue with unambiguous positional information (MacWilliams, 1983a; MacWilliams 1983b). Experimental interference or destruction of this system shows its ability to reliably reestablish itself. The only process, when positional information has to be generated *de novo* starting from a single cell is embryonic development. Currently the understanding of how similar or different are the processes of embryonic and adult patterning is low. Two different approaches can be applied to elucidate this: i) one may compare the spatial and temporal pattern of expression of genes known to regulate asexual patterning processes and ii) one may search for genes, which are functioning predominantly or exclusively in the embryo. A great help in the latter approach comes from the ever-growing *Hydra* EST project, which counts now (October, 2004) 103091 EST sequences coming from different developmental situations, but not from embryonic tissue.

The results of the first approach demonstrated that there is a big difference in the timing of expression of known developmental genes in embryos and adult polyps (Fröblius et al., 2003). Most of the transcription factors, known to be early response genes during pattern formation in a polyp, such as *gooseoid*, *brachyury*, *Fork head* and *NK2* homologues, start to be expressed as late as at the cuticle stage. Cytoplasmic members of the Wnt cascade are, on the contrary, maternal, but *HyWnt* itself starts to be expressed only at the cuticle stage. This would resemble the situation in *Xenopus*, where *Frizzled*, *disheveled*, *GSK-3* and *β -catenin* are maternal, and *Wnt8* does not appear before gastrulation. At the same time extremely important things are happening in the embryo before the cuticle stage, when “adult” genes are switched on, is reached. The egg cleaves, the body axis is specified, cell layers and cell types are generated. This implies that there must be some other players necessary for early embryonic development. Cnidarian egg has to be characterized in terms of mRNAs and proteins present, which allow the egg to make it to the point when adult patterning system is taking over the process of generating and maintenance of adult bauplan.

1.3. The goals of this study

To get insight in the molecular machinery of the early embryogenesis, I used the second before-mentioned approach and looked for molecular differences between asexual adults and embryos. Genes, which were upregulated during early embryonic development, were isolated in a subtractive hybridization screen. This unbiased strategy allows to isolate fragments of both known and novel genes, which are likely to play a role in a complex process of building a simple animal from scratch, and to estimate, how different is the genetic context during embryonic development from one in asexual hydra .

2. Results

2.1. Generation and analysis of the subtractive cDNA library from early embryos

2.1.1. Generation of the subtracted embryonic library

Poly A⁺ mRNA from embryonic stages ranging from fertilized egg till gastrula and from asexual budding polyps was isolated, and ds cDNA was synthesized. Embryonic ds cDNA was used as a tester, and cDNA from asexual budding polyps - as a driver in a suppression subtractive hybridization. An aliquot of the resulting subtracted cDNA was subsequently checked by 2% agarose gel electrophoresis (Fig. 2.2), while the rest was ligated into pGEM-T vector and cloned into *E. coli* bacteria. The gel analysis showed that, in contrast to unsubtracted cDNA, which formed a smear on the gel, the cDNA sample after subtraction consisted of multiple bands of different size and intensity, providing a hint that subtraction procedure worked.

Electroporated bacteria were plated on LB-ampicillin with X-gal/IPTG and individual white colonies were picked with the use of the Q-Pix robot. The collected

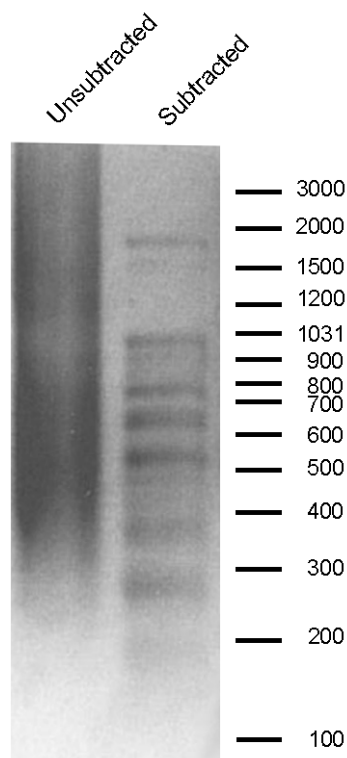


Fig. 2.2 Gel electrophoresis to check the subtraction procedure

clones were propagated in seven 384-well plates in freezing medium and stored at -80° . Replicates of these were sent to the Sequencing Center of the Washington University in St. Louis and sequenced in frames of the *Hydra* EST project. The sequences from this library are annotated in the GenBank as Kiel 3.

2.1.2. Sequence analysis

Sequencing of the Kiel 3 subtracted embryonic library yielded 2851 sequences. The sequences were cleaned of vector and adaptor motifs and clustered with the use of TIGR Indices Clustering Tools with 80% stringency. Sequences shorter than 100 bp were discarded (311 sequences). Remaining sequences were assembled into 76 clusters, consisting of more than 2 sequences, and 12 singletons. The average length of the insert was 379 base pairs. Clusters were named according to the animal name, cDNA library name and cluster number and submitted to GenBank. The clusters were numbered according to the number of ESTs comprising them, with Cluster 001 being the biggest. For example, HVULK3CL007 stands for *Hydra vulgaris* Kiel 3 Cluster 007. To identify putative homologues of known genes, cluster and singleton sequences were subjected to translated BLAST searches against the protein database with the threshold E-value of 10^{-6} . Sequences, which did not give a BLAST hit, were subjected to translated BLAST search against the translated nucleotide database with the same threshold E-value to check for possible hits to untranslated regions of the known transcripts. Also nucleotide-nucleotide BLAST search against all non-embryonic *Hydra* ESTs with the threshold E-value of 10^{-50} was performed to obtain information whether each cluster and singleton was also transcribed in asexual polyps. Only one cluster which gave a BLAST hit, was likely to be a result of bacterial RNA contamination and was excluded from further analysis. Cluster 074 (HVULK3CL074) had strong similarity to bacterial transposase (IS10 transposase from *Shigella flexneri*; E-value of $2e-80$), but as it might have been a transcribed sequence incorporated into *Hydra* genome, it was not discarded. Cluster 024 (HVULK3CL024) had some homology to the chloroplast gene from *Oenothera odorata* (E-value of $3e-13$), but represented most probably a *Hydra* gene, as it was AT-rich (63.8%) and had hits in the *Hydra* EST database from other cDNA libraries. All the other clusters with known homologues gave hits to metazoan genes. Cluster 002, which gave a match ($2e-27$) to the 3' UTR of the *Hydra*

Table 2.1 General information about clusters and singletons of the Kiel 3 SSH library

Cluster ID	Seq length (bp)	Number of Seqs	BLASTX / TBLASTX to NCBI Genbank NR (threshold: 1e-6)	E-value	BLASTN; non-embryonic ESTs	E-value
Metabolism						
HVULK3CL001	181	505	NP_110477.1 betaine-homocysteine methyltransferase [<i>Rattus norvegicus</i>]	8e-11	BP514759.1	6e-77
HVULK3CL008	221	68	AAH05139.1 Ubiquitin specific protease 5 protein [<i>Homo sapiens</i>]	5e-18	CF778956.1	2e-28
HVULK3CL010	701	64	AAK51137.1 nucleoside diphosphate kinase [<i>Hydra vulgaris</i>]	5e-54	CF599726.1	0
HVULK3CL013	444	51	AAS48105.1 cytochrome c [<i>Pectinaria gouldii</i>]	1e-46	BP512961.1	0
HVULK3CL015	399	46	NP_114021.1 nucleoside diphosphate kinase [<i>Rattus norvegicus</i>]	6e-11	CN568053.1	2e-50
HVULK3CL023	292	30	AAH08273.1 ATP synthase, delta subunit [<i>Mus musculus</i>]	6e-13	BP510557.1	1e-136
HVULK3CL025	710	30	AAF64308.1 class μ glutathione S-transferase [<i>Bos taurus</i>]	2e-59	-	-
HVULK3CL039	332	16	AAA36963.1 ornithine decarboxylase [<i>Cricetus cricetus</i>]	2e-28	CN564180.1	0
HVULK3CL044	258	15	XP_215455.2 similar to beta-hexosaminidase [<i>Rattus norvegicus</i>]	5e-26	CN563958.1	1e-145
HVULK3CL046	263	14	CAA59420.1 elongation factor-1 delta [<i>Xenopus laevis</i>]	1e-26	CN563426.1	1e-133
HVULK3CL047	557	13	AAG53660.1 peroxiredoxin 4 [<i>Bos taurus</i>]	9e-89	CD266293.1	0
HVULK3CL062	205	5	NP_776770.1 heat shock 70 kDa protein 8 [<i>Bos taurus</i>]	3e-11	CB888419.1	1e-104
CN569710.1	572	1	AAB03269.1 Serine/threonine-protein kinase PRP4m [<i>Mus musculus</i>]	8e-40	CN633797.1	0
CN621714.1	242	1	XP_215827.1 Similar to N-acetylgalactosamine kinase [<i>Rattus norvegicus</i>]	8e-14	CN570408.1	1e-105
CN622064.1	232	1	AAT06143.1 ATP synthase beta subunit [<i>Obelia sp.</i>]	1e-35	BP505414.1	1e-127
Nuclear proteins						
HVULK3CL003	234	108	XM_314037.1 putative nucleolar protein family A member 3 like [<i>Anopheles gambiae</i>]	5e-9	BP513400.1	2e-74
Development						
HVULK3CL006	384	79	XLA421945 Embryonic ectoderm development [<i>Xenopus laevis</i>]	4e-34	-	-
HVULK3CL056	586	8	AAG33621.1 Mesoderm development candidate 2 [<i>Mus musculus</i>]	1e-29	-	-
Ribosomal proteins						
HVULK3CL009	586	68	CAD29995.1 ribosomal P0 protein [<i>Bombyx mori</i>]	8e-70	CD681265.1	0
HVULK3CL020	640	35	AAT35583.1 ribosomal protein L26 [<i>Pectinaria gouldii</i>]	6e-45	CN565138.1	0
HVULK3CL031	647	24	AAH54266.1 Rps8-prov protein [<i>Xenopus laevis</i>]	3e-71	CN562875.1	0
HVULK3CL034	290	22	XP_393671.1 similar to CG1263-PA [<i>Apis mellifera</i>]	7e-43	CN551117.1	1e-161
HVULK3CL040	274	16	AAK95213.1 40S ribosomal protein S28 [<i>Ictalurus punctatus</i>]	1e-23	CK721565.1	1e-151
HVULK3CL048	826	13	CAA75444.1 ribosomal protein L7a [<i>Takifugu rubripes</i>]	1e-103	CF599814.1	0
HVULK3CL051	438	11	A53221 acidic ribosomal protein P1 [<i>Polyorchis penicillatus</i>]	2e-29	CK720763.1	1e-165
HVULK3CL059	373	6	XP_413758.1 similar to 40S ribosomal protein S27 [<i>Gallus gallus</i>]	4e-37	CN558332.1	0
HVULK3CL069	155	4	XP_394541.1 similar to ribosomal protein S11 [<i>Apis mellifera</i>]	2e-8	CK593212.1	7e-70
HVULK3CL070	314	4	AAO43049.1 40S ribosomal protein [<i>Perinereis aibuhitensis</i>]	2e-32	CK721100.1	1e-126
Defence factors						
HVULK3CL011	703	55	P61914 Equinatoxin II precursor [<i>Actinia equina</i>]	4e-8	BP515372.1	1e-130
CN561433.1	602	1	AY649787 Hydraporin 1 [<i>Hydra vulgaris</i>]	3e-74	-	-
Cytoskeleton						
HVULK3CL017	516	42	BAC16745.1 calponin [<i>Branchiostoma belcheri</i>]	4e-24	CN566751.1	0
HVULK3CL021	863	35	AAR39410.1 beta tubulin [<i>Chlamys farreri</i>]	1e-144	CN563047.1	0
Protease inhibitors						
HVULK3CL019	518	37	AAN28679.1 cystatin B [<i>Theromyzomys tessulatum</i>]	5e-21	CK721551.1	0
HVULK3CL055	289	9	AAP92780.1 hepatopancreas kazal-type proteinase inhibitor [<i>Penaeus monodon</i>]	3e-16	CK721643.1	1e-163
Adhesion molecules						
HVULK3CL043	724	15	NP_957400.1 similar to bystin-like [<i>Danio rerio</i>]	3e-46	CN622683.1	0
Signal transduction						
HVULK3CL065	482	5	BAA81696.1 G protein a subunit 4 [<i>Hydra magnipapillata</i>]	2e-15	CN777118.1	0
HVULK3CL067	590	4	BAA81696.1 G protein a subunit 4 [<i>Hydra magnipapillata</i>]	1e-82	CN777322.1	1e-116
Mobile elements						
HVULK3CL074	578	3	AAD50250.1 IS10-right transposase [<i>Shigella flexneri</i>]	2e-80	CN560406.1	0
Proteins with unknown function						
HVULK3CL016	532	44	XP_371343.1 similar to KIAA0663 gene product [<i>Homo sapiens</i>]	6e-12	CN772160.1	0
HVULK3CL018	401	39	CAG09100.1 unnamed protein product [<i>Tetraodon nigroviridis</i>]	2e-16	BP507524.1	1e-180
HVULK3CL022	622	34	XP_415866.1 similar to RIKEN cDNA 3100002B05 [<i>Gallus gallus</i>]	6e-74	CA302685.1	0
HVULK3CL024	801	30	CAA45898.1 hypothetical protein [<i>Oenothera odorata</i>]	3e-13	CN565799.1	0
HVULK3CL026	448	30	XP_421133.1 similar to hypothetical protein MGC4504 [<i>Gallus gallus</i>]	8e-13	CN558754.1	0
HVULK3CL028	239	27	AAH65662.1 Hypothetical protein zgc:77390 [<i>Danio rerio</i>]	2e-7	-	-
HVULK3CL049	763	12	CAE69232.1 Hypothetical protein CBG15274 [<i>Caenorhabditis briggsae</i>]	9e-13	CN622837.1	0
HVULK3CL063	490	5	CAF91296.1 unnamed protein product [<i>Tetraodon nigroviridis</i>]	9e-8	CN561028.1	1e-171
CN561395.1	135	1	P38978 ks1 protein precursor head-specific protein [<i>Hydra vulgaris</i>]	7e-16	CN551935.1	2e-69
Fragments without BLAST match, but with ESTs from asexual Hydra						
HVULK3CL004	307	96	No blast match	-	CK594059.1	1e-164
HVULK3CL005	597	81	No blast match	-	CN554189.1	0
HVULK3CL007	235	79	No blast match	-	CF601483.1	4e-69

Table 2.1 (continued)

Cluster ID	Seq length (bp)	Number of Seqs	BLASTX / TBLASTX to NCBI Genbank NR (threshold: 1e-6)	E-value	BLASTN; non-embryonic ESTs	E-value
HVULK3CL029	149	26	No blast match *	-	CN554437.1	7e-73
HVULK3CL032	195 / No ORF	23	No blast match *	-	CN768241.1	4e-32
HVULK3CL033	435	22	No blast match	-	CF599573.1	1e-165
HVULK3CL035	389	21	No blast match *	-	CA302704.1	1e-169
HVULK3CL036	211	20	No blast match *	-	CN630233.1	1e-105
HVULK3CL041	152 / No ORF	16	No blast match	-	CK593132.1	2e-79
HVULK3CL042	261	15	No blast match	-	CN554160.1	1e-130
HVULK3CL045	177	14	No blast match	-	CF778920.1	1e-71
HVULK3CL052	501	10	No blast match	-	BP508522.1	1e-104
HVULK3CL053	118	10	No blast match *	-	CK594011.1	1e-61
HVULK3CL058	128	7	No blast match	-	CN570816.1	1e-67
HVULK3CL060	165	5	No blast match	-	CN629163.1	5e-65
HVULK3CL061	102	5	No blast match *	-	CN624078.1	3e-46
HVULK3CL064	340	5	No blast match	-	CN554189.1	1e-64
HVULK3CL066	310	5	No blast match	-	CN565538.1	1e-168
HVULK3CL071	130	4	No blast match *	-	CN553512.1	5e-64
HVULK3CL072	326	4	No blast match *	-	CN554807.1	1e-166
HVULK3CL075	127	3	No blast match *	-	CN567891.1	6e-33
HVULK3CL076	299	2	No blast match	-	CN551154.1	1e-157
CN569861.1	140	1	No blast match	-	CK721220.1	3e-69
CN621864.1	120 / No ORF	1	No blast match	-	CV514407.1	1e-33
Fragments without BLAST match and without ESTs from asexual <i>Hydra</i>						
HVULK3CL002	478	110	AJ005934.1 lamin 3'-UTR [<i>Hydra attenuata</i>] – see text for details	2e-27	-	-
HVULK3CL012	351	52	No blast match	-	-	-
HVULK3CL014	685 / No ORF	48	No blast match	-	-	-
HVULK3CL027	309	27	No blast match	-	-	-
HVULK3CL030	119	26	No blast match	-	-	-
HVULK3CL037	228 / No ORF	20	No blast match	-	-	-
HVULK3CL038	292	19	No blast match	-	-	-
HVULK3CL050	135 / No ORF	11	No blast match	-	-	-
HVULK3CL054	207 / No ORF	10	No blast match	-	-	-
HVULK3CL057	796	8	No blast match	-	-	-
HVULK3CL068	640	4	No blast match	-	-	-
HVULK3CL073	100 / No ORF	3	No blast match	-	-	-
CN562093.1	319	1	No blast match	-	-	-
CN562313.1	387	1	No blast match	-	-	-
CN562427.1	209 / No ORF	1	No blast match	-	-	-
CN568480.1	413	1	No blast match	-	-	-
CN568924.1	102	1	No blast match	-	-	-

lamin gene was annotated as a novel gene, because it was shown to be a strong embryonic differential (Fig. 2.5), possessing a complete open reading frame of 126 amino acids preceded by a splice leader sequence (Stover and Steele, 2001), implying that it was an independent RNA.

The outcome of the BLAST searches allowed us to subdivide the sequences into the following groups: i) genes with known homologues from other metazoan animals and with ESTs from asexual *Hydra*, ii) genes with known NR homologues in other metazoan animals but without ESTs from asexual *Hydra*, iii) genes with no identifiable homology but matching the ESTs from asexual *Hydra*, and iv) genes with no identifiable homologues and no entries in the EST dataset from asexual *Hydra*. As summarized in Table 2.1, a total of 46 (~52%) sequences shared homology with proteins from other organisms. Based on the function of the homologous proteins found by BLAST search, these clusters were assigned putative functions (Fig. 2.3). Largest functional groups

represented by known genes were “Metabolism”, “Ribosomal proteins” and, interestingly, “Proteins with unknown function”, but still larger were the groups containing possible novel genes: “Fragments without BLAST match, but with ESTs from

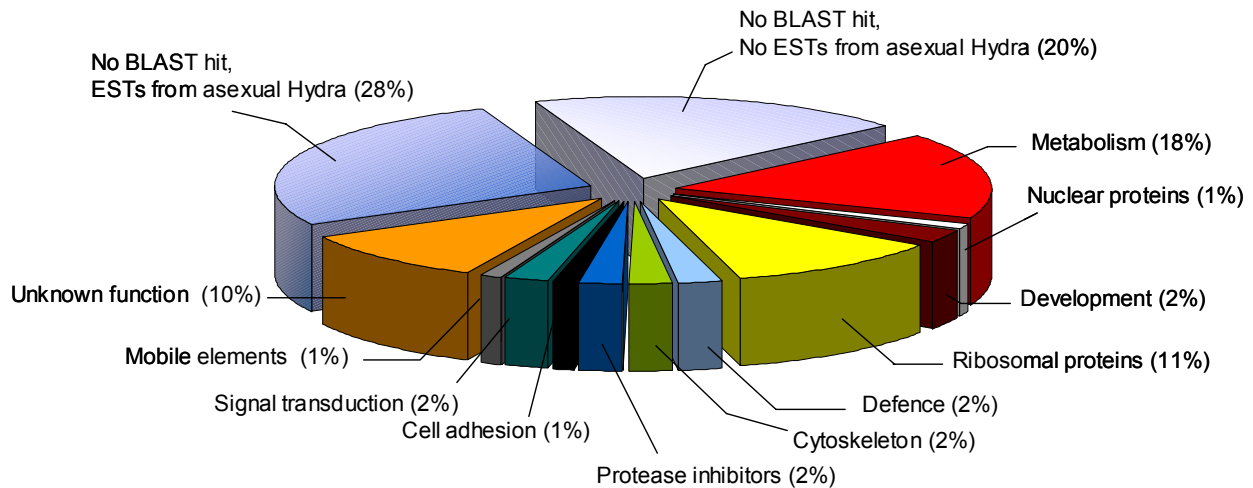


Fig. 2.3 Distribution of the clusters and singletons according to their putative function

asexual *Hydra*” and “Fragments without BLAST match and without ESTs from asexual *Hydra*”

41 (~48%) of clusters and singletons of the library did not generate a significant BLAST match. Keeping in mind that SSH procedure often yields not full length cDNA sequences, but cDNA restriction fragments, it was necessary to avoid ungrounded annotation of Kiel 3 EST clusters and singletons as “No BLAST hit”. For that *Hydra* ESTs from other cDNA libraries, matching Kiel 3 SSH library clusters and singletons,

Table 2.2 Information about putative function of Kiel 3 clusters based on the EST analysis

Cluster ID	BLASTN; non-embryonic ESTs	E-value	BLASTX of the corresponding EST (threshold: 1e-6)	E-value
Metabolism				
HVULK3CL029	CN554437	7e-73	AAF24308 Signal recognition particle 68 [Homo sapiens]	2e-36
HVULK3CL036	CN630233	1e-105	NP_080371 Small nuclear ribonucleoprotein D3 [Mus musculus]	1e-29
HVULK3CL071	CN553512	5e-64	AAH63946 Heat shock 70kDa protein 5 (glucose-regulated protein) [Danio rerio]	1e-21
HVULK3CL072	CN554807	1e-166	BAB21109 Elongation factor 1 delta [Bombyx mori]	6e-23
HVULK3CL075	CN567891	6e-33	AAT39418 Rhamnose-binding lectin precursor [Branchiostoma belcheri tsingtaunese]	6e-09
CN569861.1	CK721220	3e-69	Q962X7 Protein transport protein SEC61 gamma subunit [Branchiostoma belcheri]	1e-16
CN621864.1	CV514407	1e-33	NP_498235.1 ADP-Ribosylation Factor related (20.5 kD) (arf-1) [Caenorhabditis elegans]	3e-41
Ribosomal				
HVULK3CL035	CA302704	1e-169	XP_415408 Similar to similar to 26S proteasome non-ATPase regulatory subunit [Gallus gallus]	2e-7
HVULK3CL053	CK594011	1e-61	AAK95155 Ribosomal protein L28 [Ictalurus punctatus]	6e-22
HVULK3CL061	CN624078	3e-46	CAD27733 S6 ribosomal protein [Paracentrotus lividus]	2e-33
Signal transduction				
HVULK3CL032	CN768241	4e-32	XP_413717 Thyroid hormone receptor interacting protein 4; activating signal cointegrator 1 [Gallus gallus]	6e-49

were also subjected to BLASTX analysis with the same threshold e-value. By this procedure, 11 of 24 Kiel 3 clusters and singletons, which matched ESTs from other *Hydra* cDNA libraries but did not retrieve BLAST hits, were assigned putative functions (Table 2.2). This significantly changed the outlook of the diagram (Fig. 2.4). “Metabolism” became the largest cluster followed by “Fragments without BLAST match and without ESTs from asexual *Hydra*”.

17 (20%) of subtracted clusters and singletons had no match to the ESTs from asexual polyps. Within these two groups of sequences not providing a BLAST hit, 9 sequences were found, which did not have good open reading frames, and were therefore potential untranslated regions or non-coding RNAs.

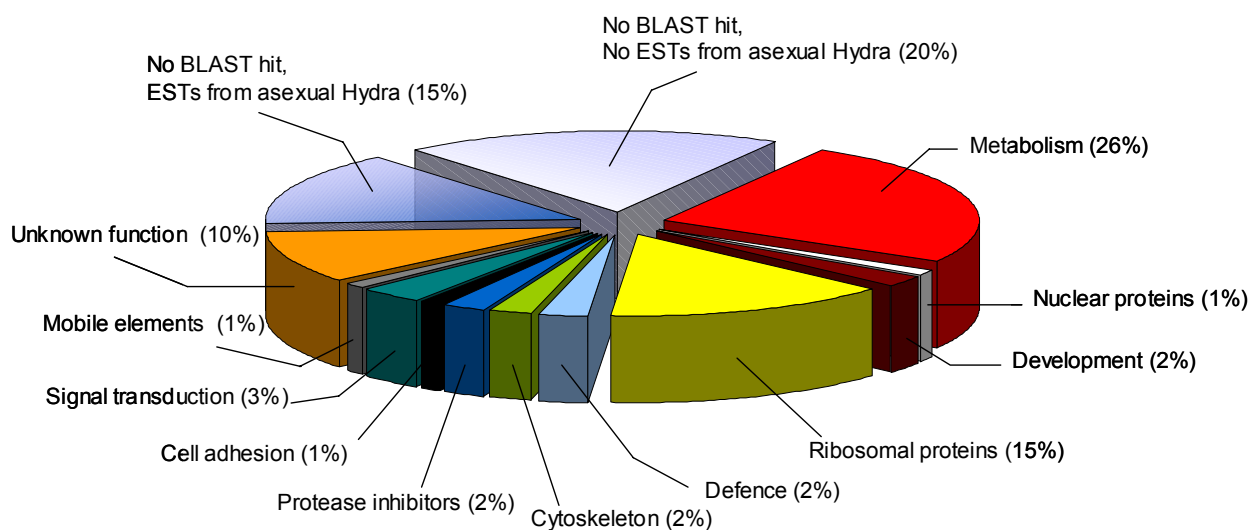


Fig. 2.4 Distribution of the clusters and singletons according to their putative function based on integrated data from Kiel 3 and other *Hydra* cDNA libraries

2.1.3. Verification of the quality of the subtracted library by RT PCR

In the process of subtractive hybridization false positive clones may be obtained. Subtraction efficiency may vary from as high as 95% (Diatchenko et al., 1996) to as low as 5% (Diatchenko et al., 1998). To verify the quality of the subtraction, the expression of 15 of 88 clusters and singletons (17%), was assessed by RT PCR. Single stranded cDNA samples were prepared from independent mRNA preparations (not the ones used for SSH library) from asexual budding polyps and from early embryos from egg till

gastrula stage. The concentration of cDNA samples was equilibrated with primers against *β-actin* (18 cycles) and *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (26 cycles). Nematocyte lineage-specific gene *HyDkk3* was used as a control adult gene (Fedders et al., 2003). The results of RT PCR are presented on Fig. 2.5A and B. None of the clusters checked was expressed stronger in asexual budding adult polyps than in embryos, though expression of some clusters turned out to be very weakly upregulated (for example, Cluster 001, Cluster 029 or Cluster 061). This shows that subtraction worked efficiently, and the number of false positives is low. At the same time

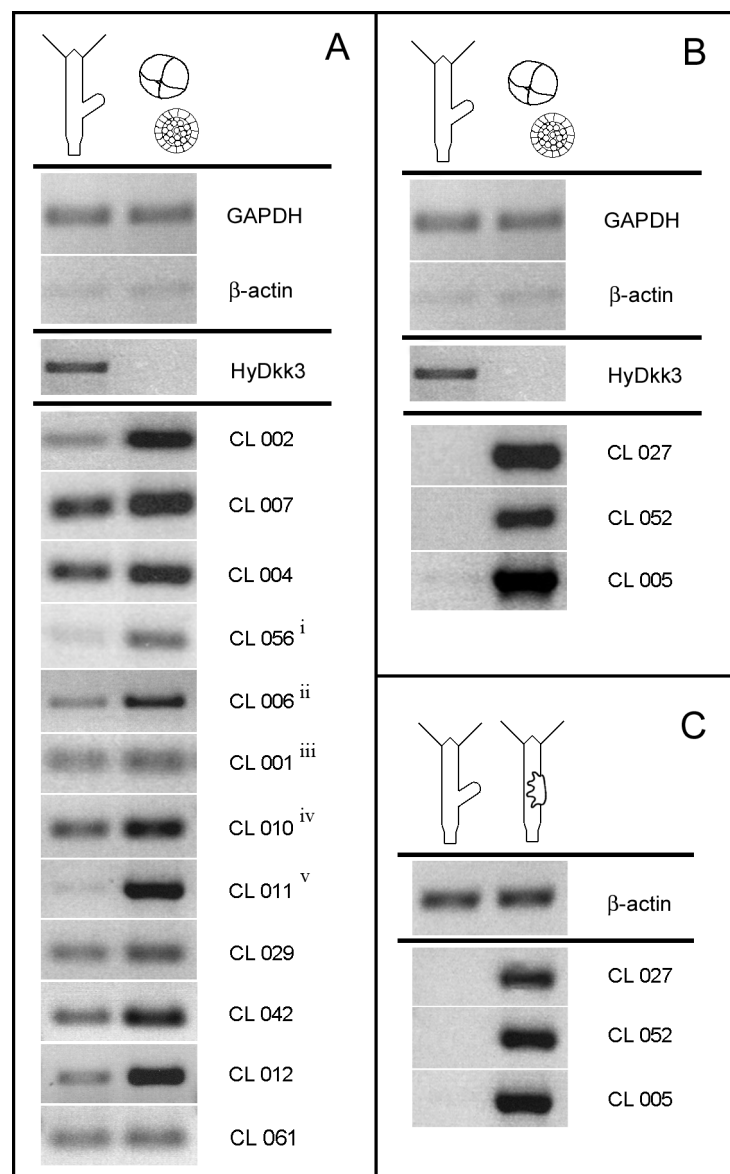


Fig. 2.5 Results of RT PCR analysis of the selected clusters. A) clusters expressed in adults, but upregulated in embryos; B) clusters expressed exclusively or nearly exclusively in embryos; C) check for maternal expression of the clusters on (B). Female polyps with egg flecks used.

it became clear that based on the strength of expression the tested clusters could be divided into two groups: i) clusters, expression of which is upregulated in embryos, but is also strong in asexual budding adults (Fig. 2.5A), and ii) clusters, expression of which appears to be exclusively embryonic (Fig. 2.5B). These latter clusters were additionally checked to get information if their expression was maternal or zygotic. For that the ss cDNA samples from asexual budding adults and female polyps with egg flecks were tested for expression of these clusters. All three of them turned out to be maternally expressed (Fig. 2.5C).

2.1.4. Examples of genes with known homologues

Among the clusters of the Kiel 3 library there was a homologue of mouse *bystin*

```

1      TTTTTAGTCCCTGTGTAATAAGTAAAAAAAAAGTCATGGGCAAAATAAAAACTGAAAAAAAA
1      M G K I K T E K K

61     GGAAAGACGAGGGCTGAGTGATGATATTTTTAGCTGTTGATTCAGAAGTCAAACAAAAAAAA
10     E R R G L S D D I L A V D S E V K Q K N

121    TCGTAAAGCAAAAAGAAGCATCTCTAAGAAGACGAGATGAAGACGATAAGTTTGTGGCGA
30     R K A K E A S L R R R D E D D K F V G D

181    TAAACTTTCCAAAAAATTTCTTTTGAAGCTAGAGCCCAGCAAGAAGAATTACAAGAAGA
50     K L S K K I L L Q A R A Q Q E E L Q E E

241    GTATCCACCTTCAGAATATGTTACAAAAAAAAGTTACCTTGGTAATAAGAAGGATGAATC
70     Y P P S E Y V T K K S Y L G N K K D E S

301    TTCAGATGAAAATGAGTTTGAAGATGATGATCATTTTTATGAAAATATTCAAATTGATGA
90     S D E N E F E D D D H F Y E N I Q I D E

361    AGAAGATGAGAAAGCAATGAAAAACTTTATGTCAGATACACCTGCAACACAAAAAAGTTT
110    E D E K A M K N F M S D T P A T Q K T L

421    AGCTGATATTATTATGGAAAAAATTAACGAAAAAAAAGTTGAGATAAGAAGTCAGATGTC
130    A D I I M E K I N E K K T E I R S Q M S

481    AGAAACATCTGAAAAACCAGTAATGGATGAAAGAATAGTATCTGTTTTTAAAAGTGTGG
150    E T S E K P V M D E R I V S V F K S V G

541    GGAAATACTATCAAAGTATAGATCTGAAAAATTTCTAAAGCATTAAATCATAACCAAC
170    E I L S K Y R S G K F P K A F K I I P T

601    ATTGCAAAATTTGGGAAGAAGTACTCTATTGCACAGATCCAGATAGTTGGACACCAGCTGC
190    L Q N W E E V L Y C T D P D S W T P A A

661    TATGTATCAAGCATCACGAATATTTATTTTCATGTTTTAATTCAAACATGGCACAAAGATT
210    M Y Q A S R I F I S C F N S N M A Q R F

721    TATT
230    I

```

Fig. 2.6 Sequence of the 5' of *HyBystin*. Bystin domain is highlighted grey.

(Cluster 043, e-value 3e-46). Cluster 043 corresponds to the 5' region of the transcript. It contains a start codon preceded by a stop codon, and, according to SMART prediction, a bystin domain (Fig. 2.6). The open reading frame goes further 3'. The reason why *Hydra* homologue of *bystin* might be of interest is because in mice *bystin* is one of the molecules, which, together with trophinin, tastin and cytokeatin facilitates adhesion of the trophoblast cells of the blastocyst and endometrial epithelial cells (Suzuki et al., 1998; Suzuki et al., 1999). Finding a mammalian embryonic adhesion molecule homologue in *Hydra* embryos might be not a mere coincidence.

Another interesting gene with a putative developmental function is the homologue of the *mesoderm development candidate 2* (*mesdc2*) from mouse (Cluster 056, e-value 1e-29). Like in case of *bystin*, SSH cluster also contains the 5' part of the coding sequence (Fig. 2.7) Like its mouse counterpart, *Hydra* homologue of *mesoderm*

```

1      AAAATATACAAAAAATGACGCGATATAATCATTTACAATTTCTTTTTAAATTGTTTTTGC
1      M T R Y N H L Q F L F K L F L

61     TGTTTAATATTTTAATAGTAGCTTCTTCTAAAGATAGTAAACAAAAAGTTAATAAAATTG
16     L F N I L I V A S S K D S K Q K V N K I

121    GAAAAAATGTAGCTGATTACAACGATGTTGATGTTGAAAACTTCTCGATCAGTGGAATG
36     G K N V A D Y N D V D V E K L L D Q W N

181    AAAATGATGAGGATGACGAAGATGAAGATACAGATGATCCTCGCTTAAAACCAAGGCCAA
56     E N D E D D E D E D T D D P R L K P R P

241    AAATCAATATGGAAGAGATTTTTTAAAGCTGGCGGTAGTCAAGAAGAAATTTTAAAAGCAT
76     K I N M E E I F K A G G S Q E E I L K A

301    CGAAAAAAGGACAACCATTAATGATTTTTGCTAATGTTGCTGGAAATCCTTCACGTAAGG
96     S K K G Q P L M I F A N V A G N P S R K

361    AGACAGAAACTGTTTCAGCTTTGTGGCAAAGTAAAGAAATAATCAAATTCAGTTC
116    E T E T V S A L W Q T S L R N N Q I Q V

421    AAAGATACGTCATATCAGACAACCGTGTTTTGTTTCAGCTTGAAGATGGTAGCCAAGCAT
136    Q R Y V I S D N R V L F Q L E D G S Q A

481    TTGAAATAAAAGATTACCTAACATCATTAGATACTTGTGAGAGCGTGCATTTGAAAATT
156    F E I K D Y L T S L D T C E S V S F E N

541    TGAATTTTCCCGGAAAAGGAGCCGGCAAACAAAACCTCGAGCTGTG
176    L N F P G K G A G K T K L E L

```

Fig. 2.7 Sequence of the 5' of *Hydra* homologue of *Mesdc2*. Signal peptide domain is highlighted grey.

development candidate 2 contains a signal peptide and no other conserved domains. SignalP analysis predicts a signal peptide with cleavage site after residue 25 (Fig. 2.8).

In mouse *mesdc1* and *mesdc2* genes were found to be located in the region of the chromosome 7, deletion of which, as determined by BAC transgenic rescue, turned to be critical for differentiation of the mesoderm (Wines et al., 2001). How exactly *mesdc2* protein functions remains unknown. It is certainly also not known how its homologue from *Hydra* acts. But here again we meet an example of a gene existing earlier than its function, because *Hydra* has a gene likely to be necessary for mesoderm

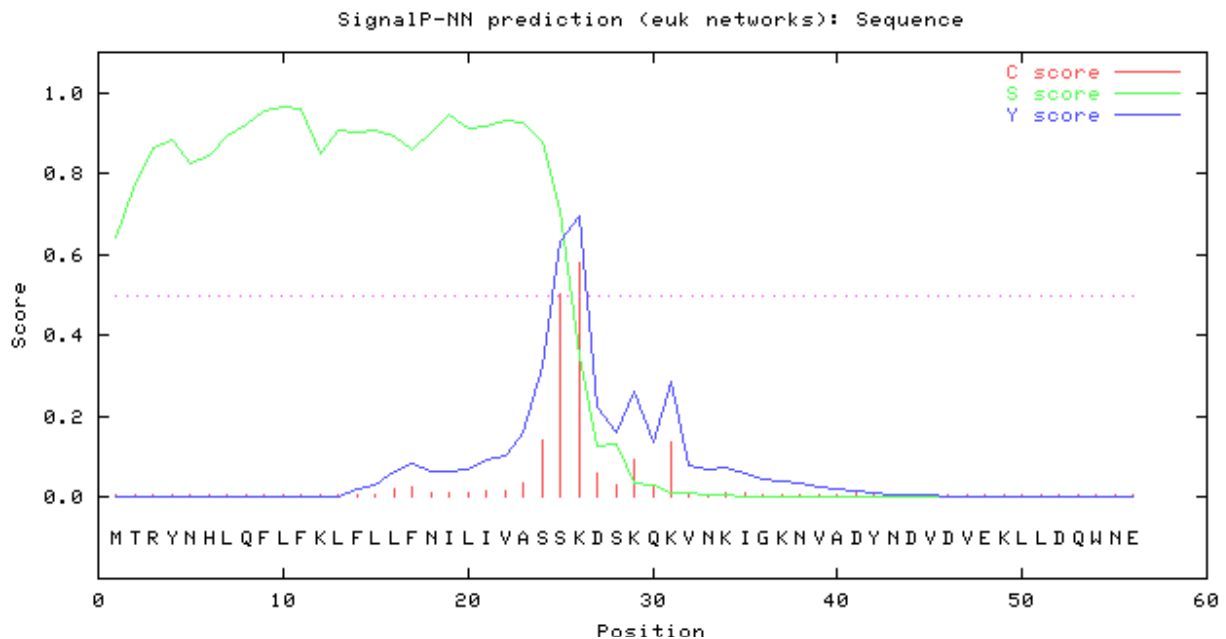


Fig. 2.8 SignalP analysis of the N-terminus of the *Hydra* homologue of *mesoderm development candidate 2*. Green curve shows the probability that the sequence is a signal peptide, highest peaks on the blue curve and longest red bars point on the putative cleavage site.

formation but lacks mesoderm.

The third interesting and strongly differentially expressed gene is the homologue of *equinatoxin II* from *Actinia equina* (Cluster 011, $4e-8$), *Hydra Toxin 1* (*HyTox-1*). The SSH cluster contains a full length coding sequence with an open reading frame of 186 amino acids, a splice leader sequence (Stover and Steele, 2001) in the 5'-UTR, and a 3'-UTR (Fig. 2.9 A). Different domain prediction engines present alternative variants of the protein structure. *HyTox-1* possesses an C-terminal cytotoxic domain (residues 45 - 182 according to BLAST, or 18 to 186 according to SMART) and a transmembrane domain (residues 5 - 27 according to SMART or 1 - 19 according to TMpred) or a signal peptide (residues 1 - 18 according to SignalP). This leads to two possible structures of the protein, as shown on Fig. 2.9 B-D. It can either be a secreted molecule, like equinatoxin II, which is a potent pore forming protein (Kristan et al., 2004), or, according to TMpred prediction, a transmembrane protein with the cytotoxic domain on the

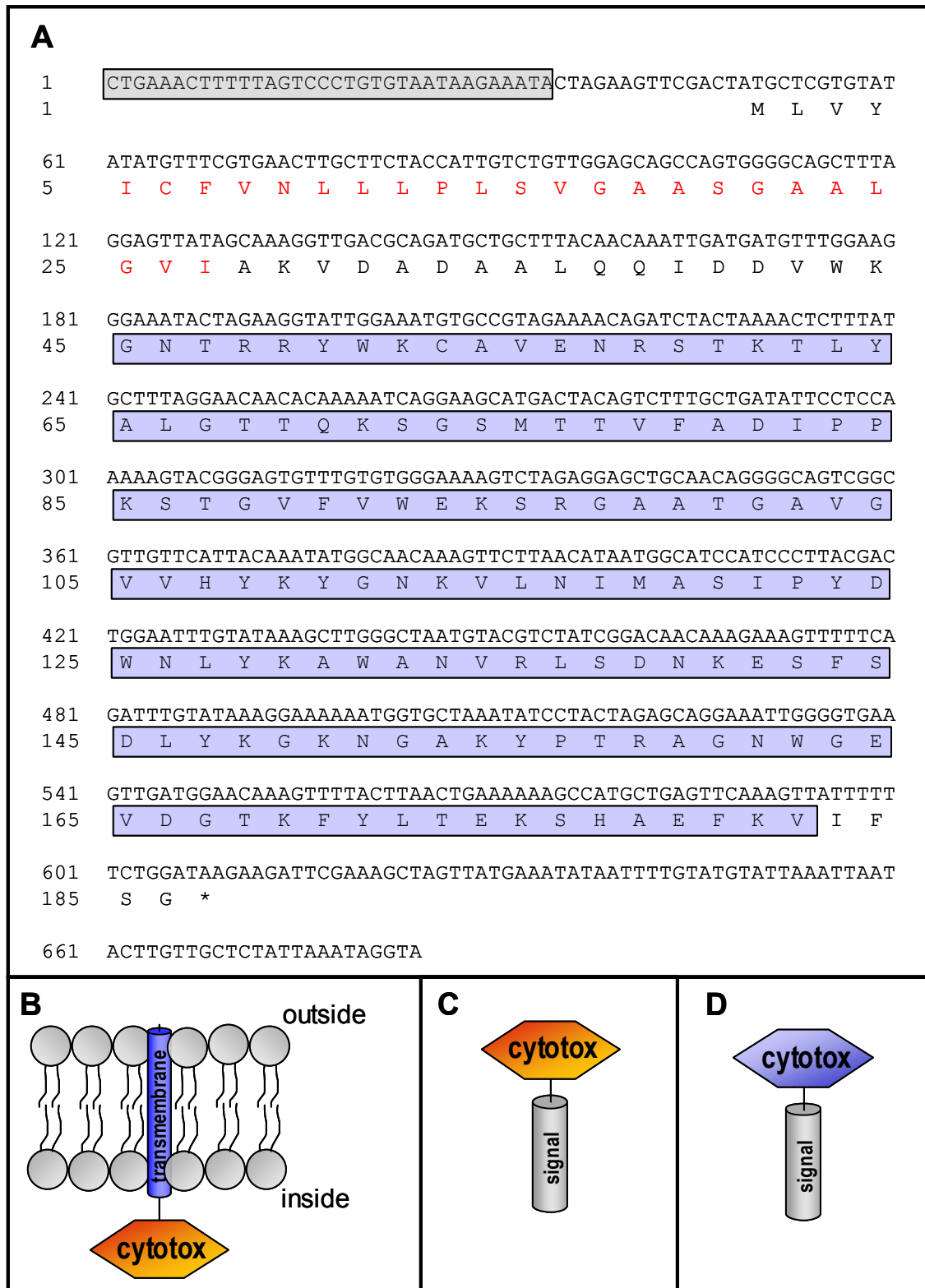


Fig. 2.9 Sequence of the *HyTox-1* gene (A) and comparison of possible domain structure of *HyTox-1* (B and C) and equinatoxin II (D). Splice leader is highlighted grey, transmembrane domain is printed in red, cytotoxic domain is boxed in blue.

cytoplasmic side. In both cases it might play a role in defence mechanisms of the otherwise poorly protected embryo, making it poisonous for a predator.

HyTox-1 is not the only transcript coding for a protein with a putative defence function. *Hydraporin 1* was also found in the Kiel 3 SSH library, but this was only a singleton (CN561433). *Hydraporin 1* is a novel gene from *Hydra*, which was found in another SSH assay targeted to obtain genes induced by *Pseudomonas aeruginosa* supernatant. The putative Hydraporin-1 is a peptide with the fold similar to one of the anti-fungal protein of *Aspergillus giganteus* (Zill, personal communication).

2.2. HyEED-1 – a conserved regulatory gene involved in embryogenesis

2.2.1. Isolation of *HyEED-1*

47% of the EST clusters in the library represent genes with known homologues. As an example of a highly conserved gene upregulated in embryos, a cluster coding for polycomb group gene (cluster 006, Fig. 2.5; Table 1) was chosen for a detailed analysis. It showed strong similarity to vertebrate *embryonic ectoderm development* or *EED*, and was named *Hydra Embryonic Ectoderm Development-1 (HyEED-1)*. In bilaterian animals, *EED* (or *Extra Sex Combs* in *Drosophila*) is known to be involved in regulation of gastrulation, long-term silencing of *Hox* genes, maintenance of the stem cell qualities and also in inactivation of X-chromosome in mammals (Ng et al., 2000; Wang et al., 2002; Plath et al., 2003; Valk-Lingbeek et al., 2004). *EED* forms a complex with another polycomb protein, Enhancer of Zeste (*EED-E(Z)* complex). The inhibitory effect of the complex on target genes is reached by specific methylation of histone H3 at the Lys27 residue (Cao et al., 2002; Plath et al., 2003; Okamoto et al., 2004; Kirmizis et al., 2004).

By means of PCR with degenerate primer against a conserved stretch in the 5' region from the received SSH fragment followed by 5' RACE splinkerette PCR and 3' RACE PCR, a 1561 bp long full-length cDNA sequence of *HyEED-1* was obtained (GenBank accession number: AY347258). It contained a 5' UTR, an open reading frame coding for 420 amino acids, and a 3' UTR ending with poly-A tail. Interestingly, this was not

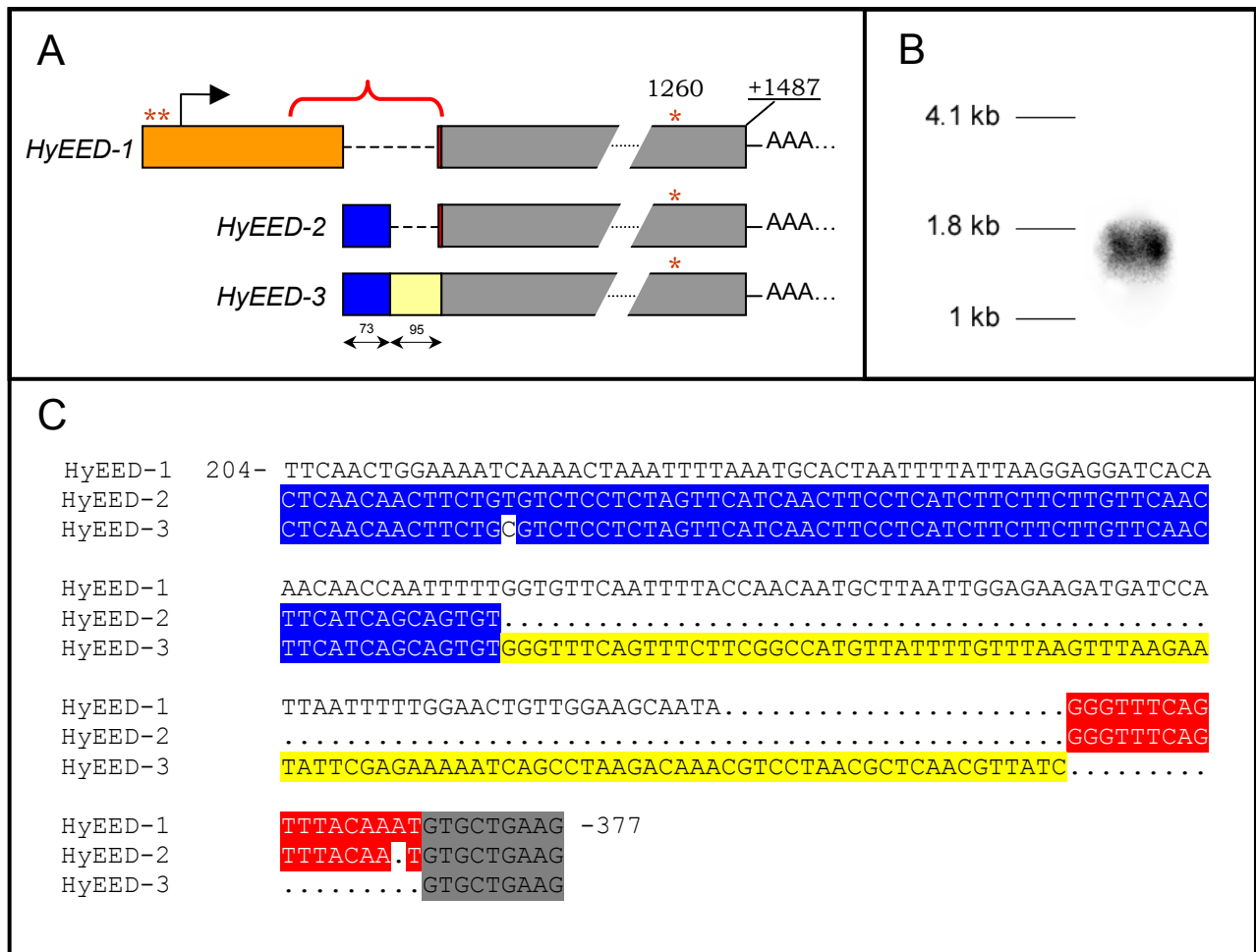


Fig. 2.10 Analysis of *HyEED* structure. A) cDNA variants of *HyEED*. Red bracket marks the part of *HyEED-1* shown on (C). B) Northern blot hybridization. C) Alignment of the variable region of *HyEED*. Position of the presented fragment within the submitted sequence of *HyEED-1* is shown. Stretches of sequence are highlighted in the same color as in (A). Unhighlighted *HyEED-1* sequence corresponds to the orange box on (A).

the only transcript present. Two other transcripts, carrying insertions and deletions of different size were also cloned. These transcripts, named *HyEED-2* and *HyEED-3*, were isolated in the course of PCR with the degenerate primer. 5' RACE PCR did not yield any more sequence. On their 5' they possess a 73 bp motif, which is not present in *HyEED-1*. Additionally, *HyEED-3* has a stretch of 95 nucleotides, not present in *HyEED-1* and *2*. Instead, *HyEED-1* and *-2* have 17 nucleotides not present in *HyEED-3* (Fig. 2.10 A and C). Though several cDNA variants of *HyEED* were found, the Northern blot hybridization with a probe corresponding to bases 887-1200 of the sequence submitted to GenBank (which corresponds to the conserved region of the gene) did not reveal the presence of transcripts of visibly different length (Fig. 2.10 B). The hybridization signal

gave a size of approximately 1.6 kb, which nearly perfectly matched the size of the isolated *HyEED-1* (1561 bp). Southern blot hybridization with an intronless probe constructed against specific region of *HyEED-1* transcript (positions 56-319 of the submitted sequence) yielded 2 bands (Fig. 2.11A). Interestingly, Southern blot hybridization with the same probe against conserved region of all three transcripts, as was used for the Northern blot hybridization, also resulted in a two bands pattern in case of *Hind III* - digested genomic DNA, and a thick band at the size of more than 10 kb in case of *Pst I* digestion (Fig. 2.11B). The latter band could be in fact also two

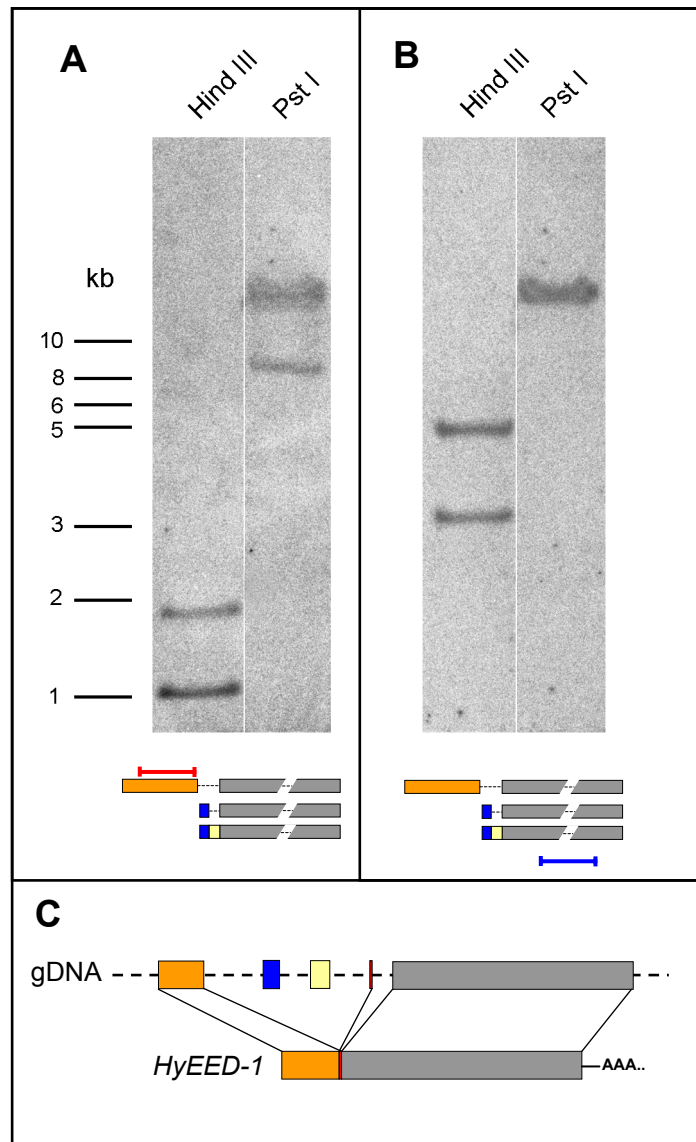


Fig. 2.11 Southern blot hybridization with *HyEED* probes. A) Result of the hybridization with a *HyEED-1* transcript-specific intronless probe. The position of the probe within the *HyEED-1* transcript is marked with a red bar on the scheme under the hybridization image. B) Result of the hybridization of the same membrane with an intronless probe, recognizing the conserved region of the transcripts. The position of the probe within the *HyEED* transcripts is marked with a blue bar on the scheme under the hybridization image. B) Possible genomic organization of *HyEED* and an example of formation of *HyEED-1* splice variant.

bands, which are not separated on the gel far enough from each other. This is possible, considering the size of the band (more than 10 kb).

Obtaining same number of bands both with the *HyEED-1* transcript-specific probe and a probe against the conserved region of all three transcripts makes me think that all transcripts are products of alternative splicing from the same gene, which is present in the genome in two copies (Fig. 2.11C). The fact that bands obtained with these two probes are not co-localized is most probably due to the presence of restriction sites within the intron, which is shown to be located on the genomic level between the regions used for the preparation of the probes. According to RT PCR with primers recognizing different cDNA variants, *HyEED-1*, *HyEED-2* and *HyEED-3* are all upregulated in the embryos in comparison to asexual budding adults (Fig. 2.12). Alternatively spliced products of the same gene are also likely to be spatially co-expressed.

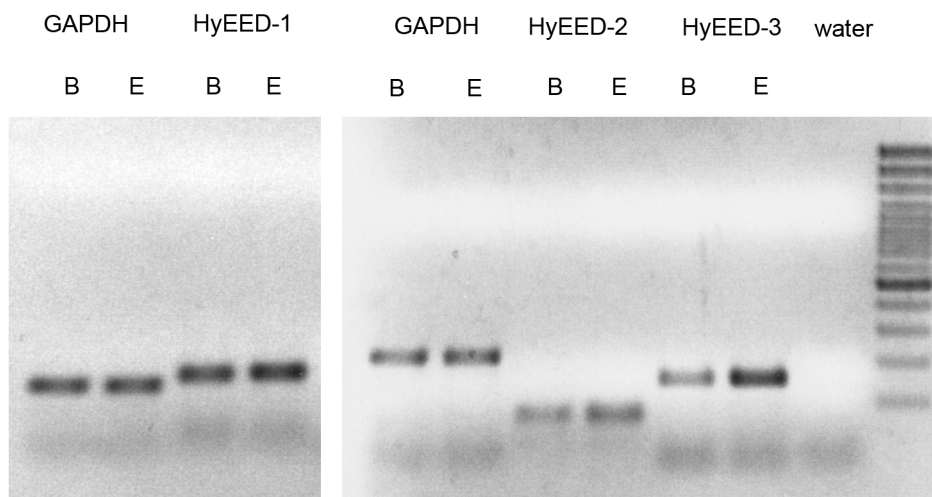


Fig. 2.12 RT PCR with transcript-specific primers, recognizing different variants of HyEED. B - cDNA from asexual budding polyps, E - cDNA from embryos.

As the full length coding sequence of *HyEED-1* was obtained, and attempts to get more sequence information about the other two *HyEED* variants were unsuccessful, the focus was given to *HyEED-1* (Fig. 2.13). The HyEED-1 protein possesses characteristic multiple WD-40 repeats, which are necessary for stable or reversible protein-protein interaction. Comparison of HyEED-1 with other proteins (Fig. 2.14 A)

1 CAAATTAAGCTCTGAGATAATATTGCTAGATAATACATGCTAATAGGAAGTTTCTGATGGATACTGTTAATGATTT
1 M D T V N D F

76 TATCGACGATAGTTATCGTGATAATGATGATGATACTGACAGTAGTCAAACATATTCAACTCCAGTAAAACAAA
8 I D D S Y R D N D D D T D S S Q T Y S T S S K T K

151 AGGAAAAAAGGTATGCAAAAAAATGGAAAAAGCAAAATAAACATGATGAGAATTCAACTGGAAAAATCAAACT
33 G K K V C K K K W K K Q N K H D E N S T G K S K L

226 AAATTTTAAATGCACATAATTTTATTAAGGAGGATCACAAACAACCAATTTTGGTGTTCATTTTACCAACAATG
58 N F K C T N F I K E D H K Q P I F G V Q F Y Q Q C

301 CTTAATTGGAGAAGATGATCCATTAATTTTGGAACTGTTGGAAGCAATAGGGTTTCAGTTTACAAATGTGCTGA
83 L I G E D D P L I F G T V G S N R V S V Y K C A E

376 AGATTCTGGACAAATTTTATTGCTTCAAAGTTATGCTGATAGTGATCCAGAAGAAAGTTTCTATGCATGTTTCATG
108 D S G Q I L L L Q S Y A D S D P E E S F Y A C S W

451 GACATATGATCCAGATAACAGAAATCCTTTGTTTTGTTTTGCTGGAGCTAAAGGAATCATTCATATACTTAATCC
133 T Y D P D N R N P L F C F A G A K G I I H I L N P

526 ATGTATACGTCAAGTTGCTACTTATCTTCAAGGACATGGCAGTGCATCAATGAGTTAAAGACGCACCCCATGTA
158 C I R Q V A T Y L Q G H G S A I N E L K T H P I E

601 ACCATTGATAATTTCTCAGTGAAGTAAAGATCACACTATTAGAATGTGGAACATTAAGACAGAAGTTTGCCTGTC
183 P L I I L S A S K D H T I R M W N I K T E V C V A

676 TATTTTGGTGGTGTAGATGGTCACAGAGATGAAGTCTGGGTATTGATTTTGATGTACTTGGTACAAAAATAGT
208 I F G G V D G H R D E V L G I D F D V L G T K I V

751 TTCTTGTGGAATGGATCATTTCTTTAAAATTTTGGTCACTTGAGACAGAGAAATGCAAAAAAGTAATCAATGATTC
233 S C G M D H S L K F W S L E T E K C K K V I N D S

826 GCATGCTCACTCAAGTACAGAAAGGATCTTTCATACATTAAATGTTTATTATCCTGAGTATACTACTCGAGAAGT
258 H A H S S T E R I F H T L N V H Y P E Y T T R E V

901 TCATCGTAATTACGTTGATTTGTTGTTTGGTTAGGTGATCTTGTATATCAAATCTTGTGACAATCAAGTTGT
283 H R N Y V D C C V W L G D L V I S K S C D N Q V V

976 ATGTTGGAAAACAAGCAACCAATAGAAACAGTTATGAAAAGGAAAAATAACTCTGATGTTGGTGTGTTTGTGCT
308 C W K T K Q P I E T V M K R K N N S D V G V F V L

1051 ACACAAGTTTGATATTGATTTGTGCGACATCTGGTTCATAAGATTGTCAGTAGATCTGAATCAAACGATTTTAGC
333 H K F D I D L C D I W F I R F A V D L N Q T I L A

1126 CCTTGGAAACCAATTTGGTAAAGTTTATCTCTATGATTTAGAAGGAGAGCATCCTGCCCATGCCAAACCAACGAT
358 L G N Q I G K V Y L Y D L E G E H P A H A K P T I

1201 ACTTTTTCATTTCAAGTGTACAACGGTTGTTTCGCCAAATTTTCGTTCAATAGTTTCAGCCAAAGTACTAATTGCTGT
383 L F H S K C T T V V R Q I S F N S S A K V L I A V

1276 TTGTGATGATGGCTCTGTATGGAGATGGGACAAGCAGACATAATGAAAAATCTCGTGAAATAGTAAAAATGAA
408 C D D G S V W R W D K Q T * *

1351 GCTATAATGAAGAAGCTGTGATGACACTTTGAAAGTAGCAATATTTCCATAAGAGAATAACAAACAATTGATTTG
1426 TAATATGAAAAATAATTAATGCTAAAATGTTCAAAGGAATAAAATCAGTTTTTAATAAATACTGTATAAGATAAG
1501 AAAAAATGTACATTTGTTAGTAAATAGAATAAAGTTCTGTTTTAAAAA

Fig. 2.13 Nucleotide and amino acid sequence of *HyEED-1* (GenBank AY347258). The fragment originally obtained as SSH cluster 006 is shown in red; the 264 bp long *HyEED-1*-specific fragment used for the Southern blot hybridization is highlighted yellow. The conserved 313 bp long intronless probe used for Southern and Northern hybridization is highlighted grey; fragment used for preparation of the probe for *in situ* hybridization is shown in bold and underlined.

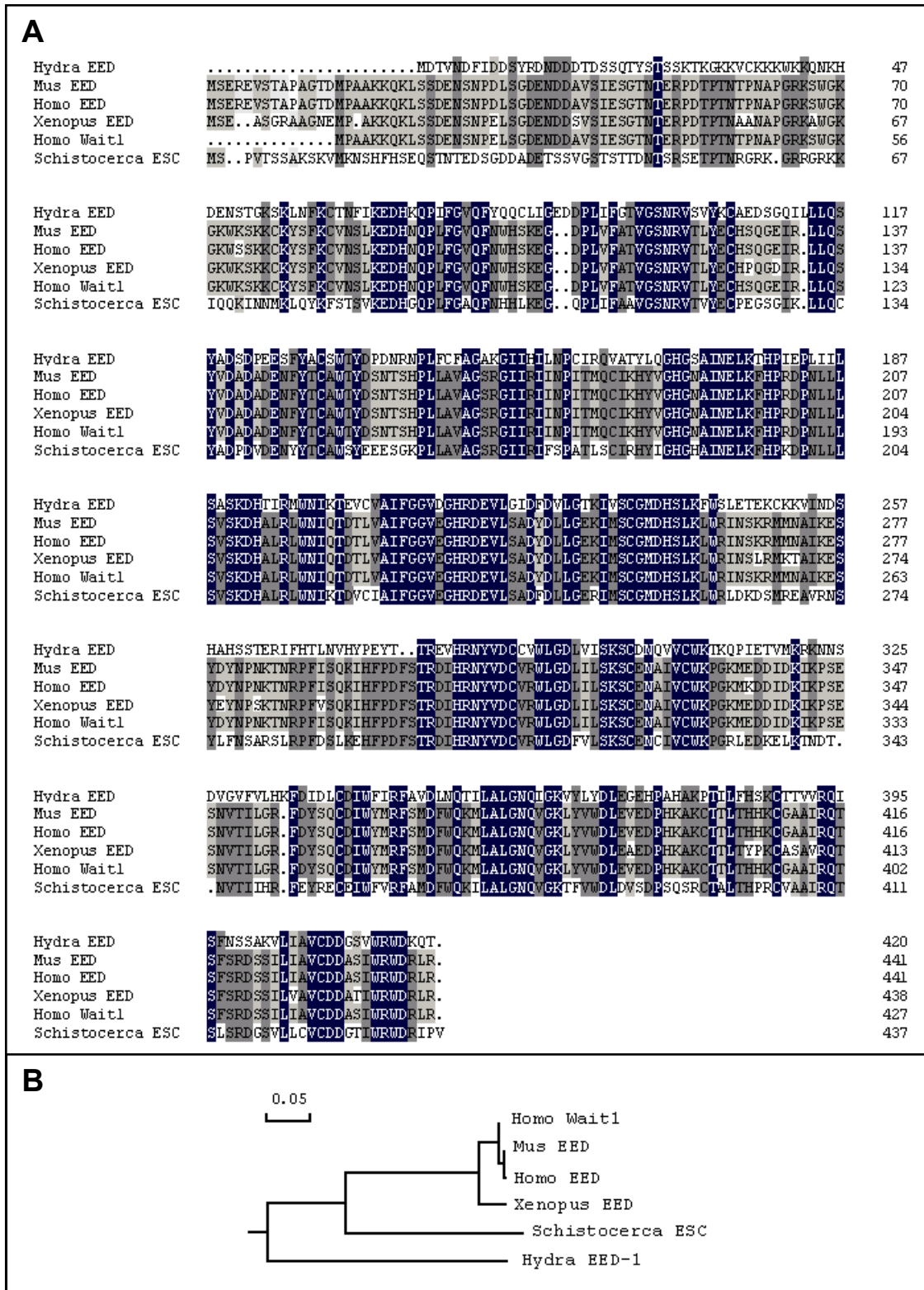


Fig. 2.14 A) Alignment of HyEED-1 with related proteins from other animals. B) Phylogenetic comparison of HyEED and other metazoan EED/ESC proteins. Scale bar indicates an evolutionary distance of 0.05 aa substitution per position in the sequence.

reveals significant conservation on the amino acid level (43% identities); at the same time phylogenetic tree based on the multiple alignment analysis unambiguously places HyEED at the basis of the metazoan EED/ESC tree (Fig. 2.14 B).

2.2.2. Analysis of *HyEED* expression

To get some insight about the dynamics of *HyEED* expression, ss cDNA samples from different embryonic stages were compared to ss cDNA samples from adult polyps undergoing different pattern formation processes, such as budding, head and foot regeneration (Fig. 2.15). It became clear, that *HyEED* is constantly expressed in adult polyp, but its transcription level is not affected by adult patterning processes. At the same time it gets upregulated in embryos.

Whole mount *in situ* hybridization with digoxigenin-labeled RNA probe against the conserved region of the *HyEED* transcripts revealed that during early stages (egg to gastrula) *HyEED* is expressed throughout the embryo (Fig. 2.16). A drastic change in expression happens at the cuticle stage. *In situ* hybridization on 16 µm-thick frozen sections of cuticle stage embryos demonstrated that *HyEED* message appears to be gone from all the cells of the embryo except for a subpopulation of round or spindle-shaped cells, lying individually or in couples (Fig. 2.17). These cells morphologically strongly resembled interstitial cells of adult polyps. *HyEED*-positive cells were positioned both at the outer wall of the embryo, most probably in the thin ectodermal

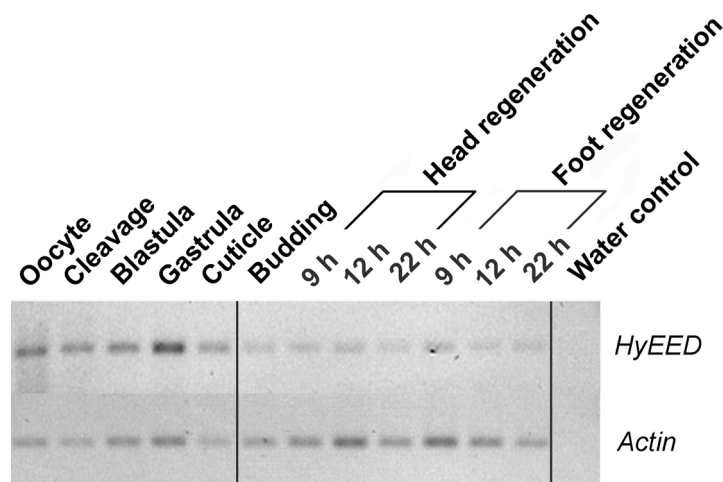


Fig. 2.15 Results of RT PCR during different embryonic stages and developmental events in adult polyps.

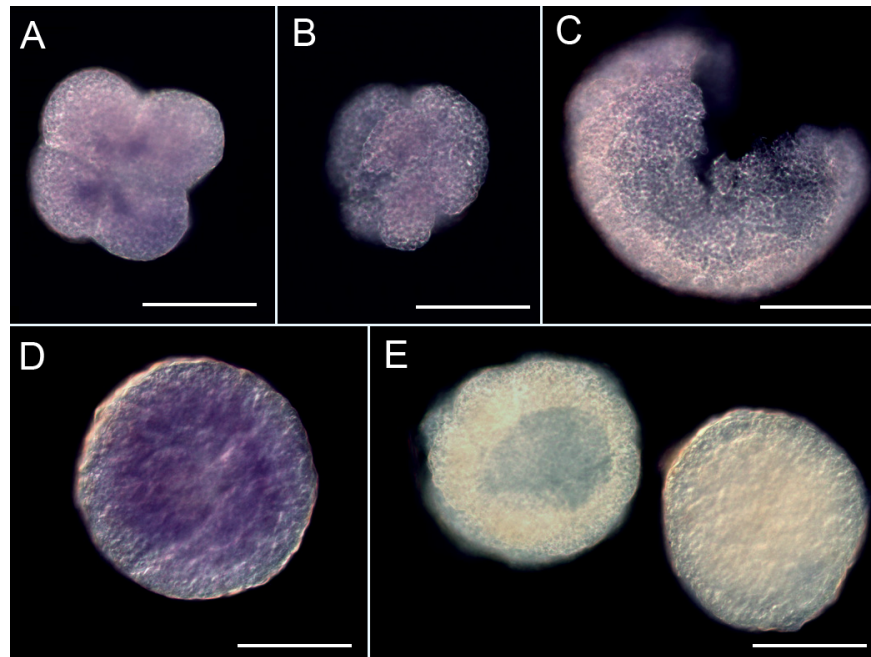


Fig. 2.16 Expression of *HyEED* during early stages of embryogenesis. A) 4 cells stage B) 8 cells stage C) Blastula D) gastrula E) blastula and gastrula hybridized to the sense probe of *HyEED*. Bar 0.5 mm

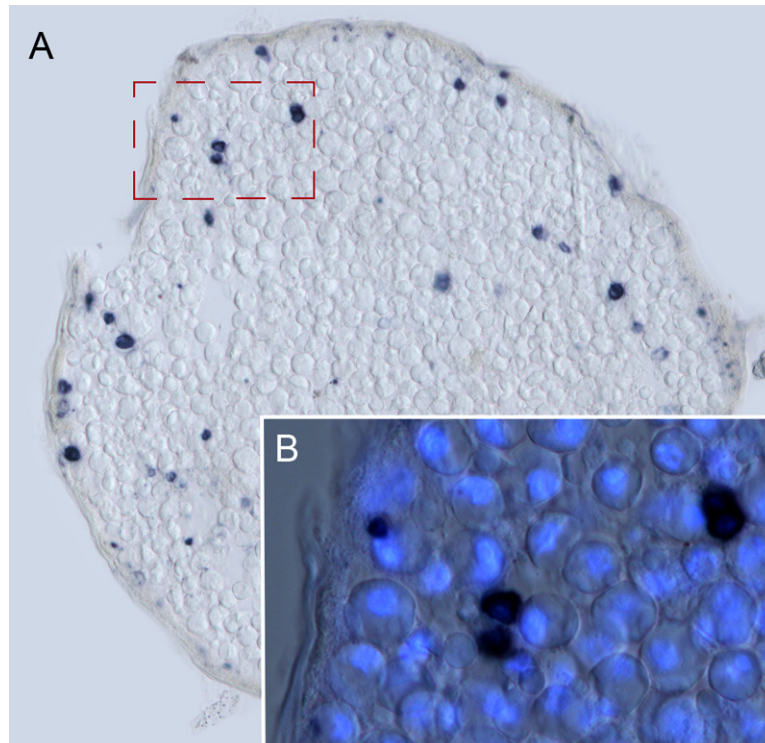


Fig. 2.17 Expression of *HyEED* during the cuticle stage. A) Nascent interstitial cells are located on the outside as well as deep within the embryo. B) Close-up of the area boxed in (A). Cell nuclei on (B) are counterstained with DAPI.

layer, which is not visible on the sections after *in situ* procedure, and also in the depth the embryo among the prospective endodermal cells. To clarify if the cells expressing the gene are really interstitial cells, *in situ* hybridization on adult polyps was carried out, and *HyEED* expression was indeed found to be restricted to the interstitial cells, and in sexy polyps also to germ line cells. Asexual polyps (Fig. 2.18A) expressed *HyEED* in all interstitial cells of the body, and expression was visibly not affected by budding (as already shown by RT PCR). In polyps in the process of gametogenesis interstitial cells start to proliferate and form clusters (Fig. 2.18B). In these cells the expression is more

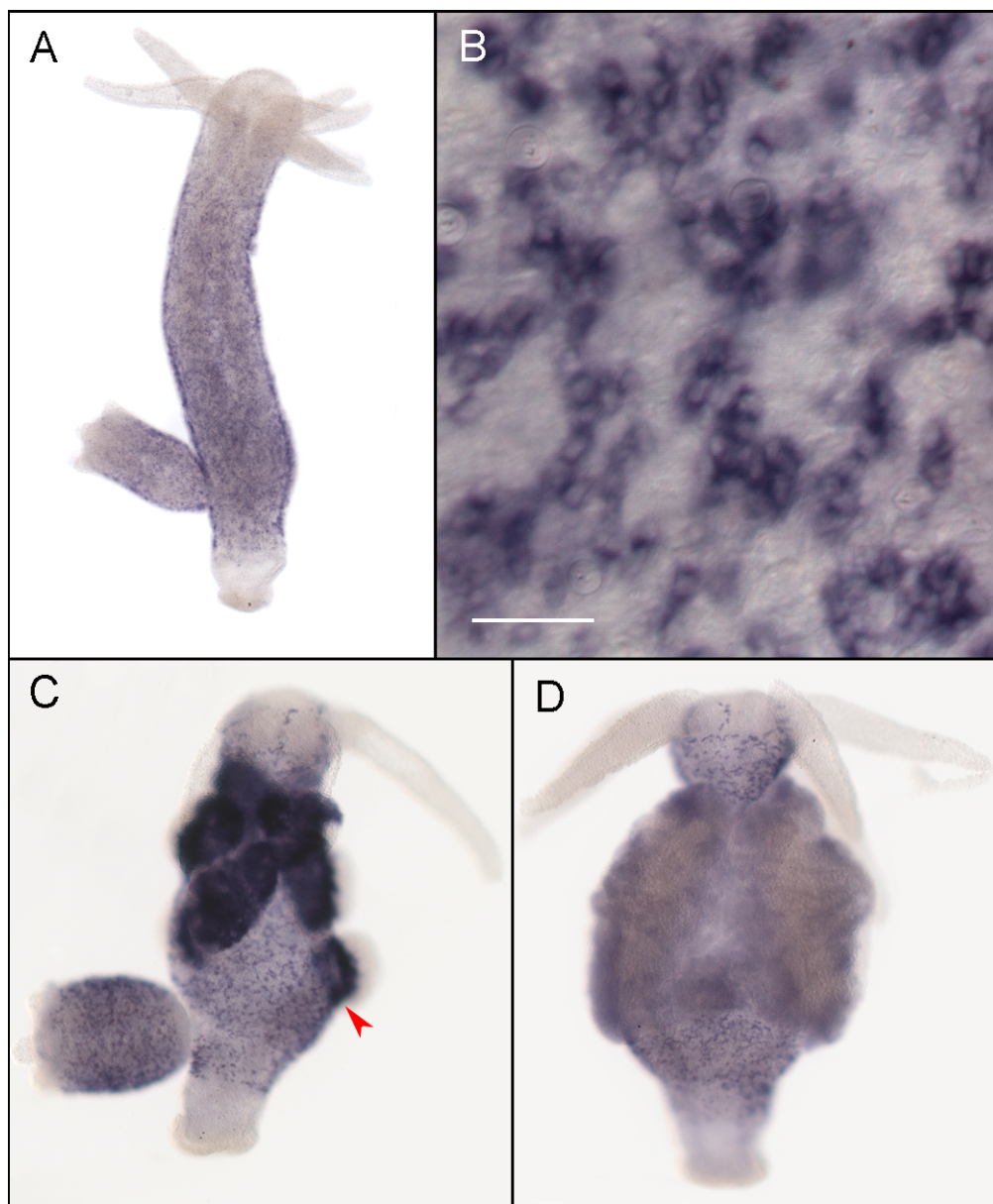


Fig. 2.18 Expression of *HyEED* in adult polyps. A) Asexual polyp B) Interstitial cells in a female polyp (bar 30 μ m) C) Male polyp, arrowhead points on the testis D) Female polyp with two egg flecks

intensive than in the interstitial cells of the asexual polyp. In addition to expression in the interstitial cells, *HyEED* is strongly expressed in the bases of the testes (Fig. 2.18C) and weakly within the egg fleck (Fig. 2.18D).

One of the ways to specifically localize interstitial cells in adult polyp is to stain them with C-41 monoclonal antibody (Fig. 2.19 A, B). To check, whether *HyEED* expressing cells could be stained with an interstitial-cell specific antibody, 16 μm -thick frozen sections of cuticle stage embryos of the same developmental age as taken for *in situ* hybridization, and of adult polyps were cut and stained with C-41. Cuticle stage embryos, in contrast to control adult polyps on the same slide, did not show any staining. This implies, that *HyEED* starts to be expressed earlier in the nascent interstitial cells than C-41 epitope appears.

As it was mentioned before, the function of EED proteins in all bilaterian animals is chromatin remodulation in complex with E(Z) by means of specific methylation of

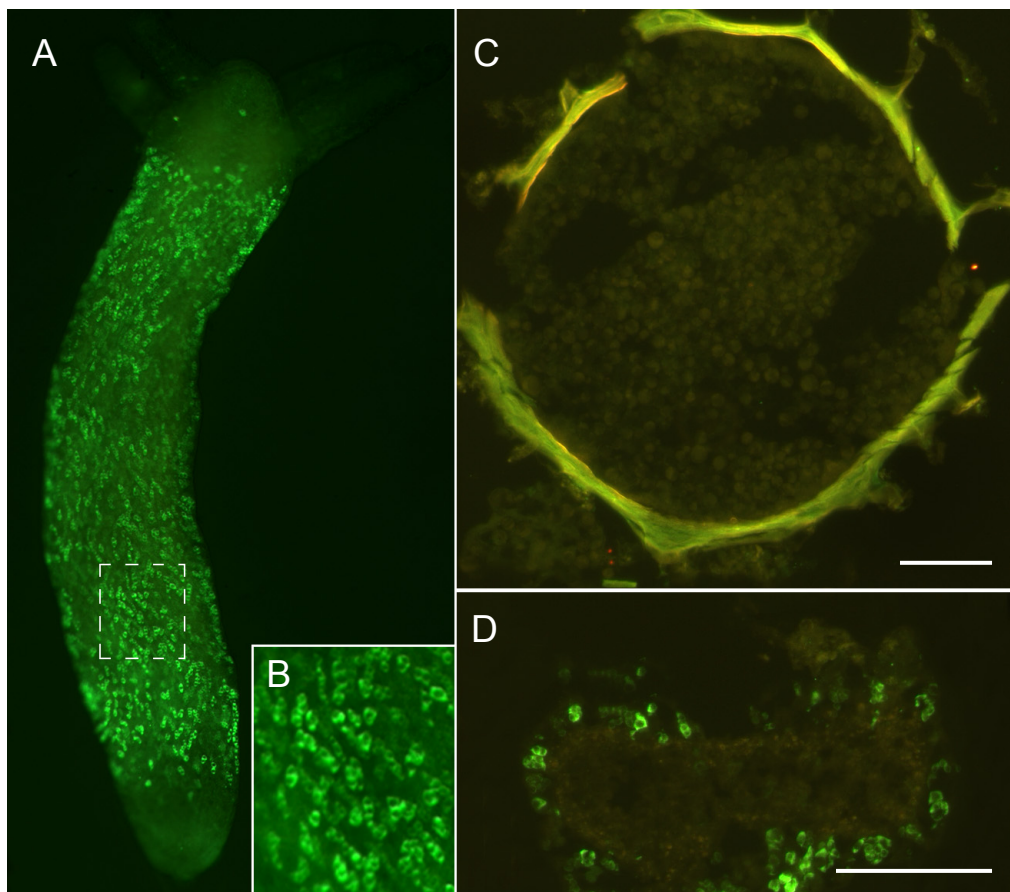


Fig. 2.19 C-41 staining of adult polyp and cuticle stage embryo. A) C-41 staining of an asexual Hydra B) close-up of the area marked with dashed line on (A). Interstitial cells visible. C) Cuticle stage embryo is C-41-negative. D) Control cross-section of the polyp. Interstitial cells stained. Bars 0.2 mm.

histones, namely methylation of Lys 27 in the histone H3. A fragment of *Hydra* histone H3 is known (AAQ96279), and it is 100% identical to the mouse histone, including the EED-E(Z) complex recognition site (KAARK₂₇SAP). To get some indirect evidence on the possible function of HyEED protein, 14 μm thick frozen sections of *Hydra* testes were stained with the monoclonal anti-H3di/tri-methyl K27 antibody (a kind gift from Dr. D. Reinberg, Howard Hughes Medical Institute, NJ; for antibody details see Okamoto et al., 2004). Specific methylation was observed in the large nuclei at the bases of the testes and in distinct nuclei within the body wall (Fig. 2.20). From the size and location of the antibody-positive nuclei it was clear that they were ones of the spermatogonia and spermatids - exactly the cells, in which *HyEED* was found to be expressed. Individual nuclei in the body wall corresponded most probably to the interstitial cells, which expressed *HyEED*.

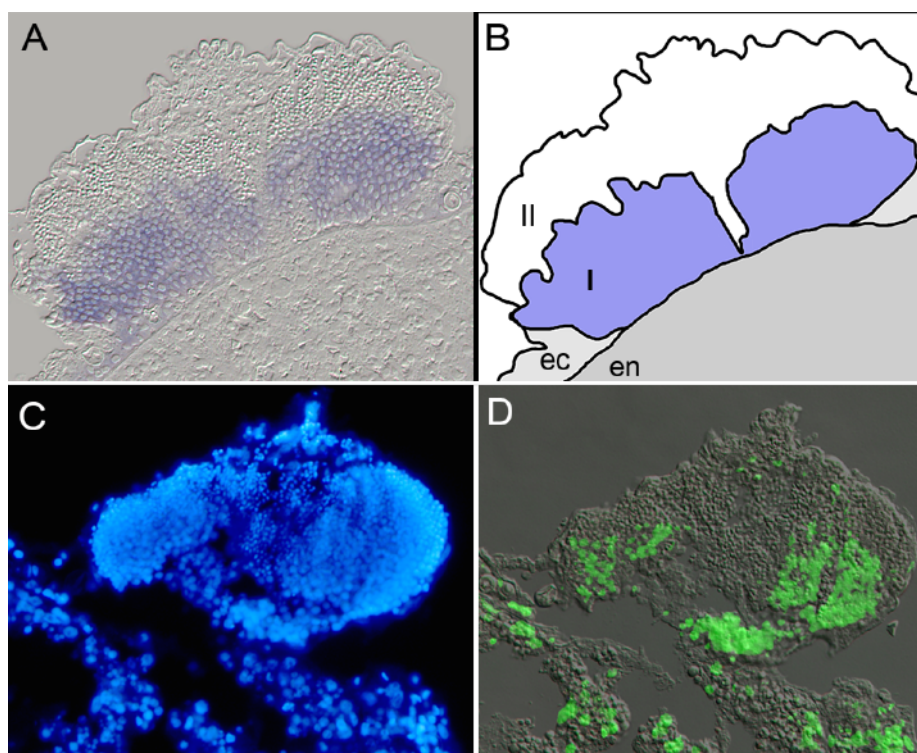


Fig. 2.20 Colocalization of HyEED mRNA and specific type of histone methylation.

A) Semi-thin section (2 μm) of a whole mount *in situ* of a male polyp. Testis is shown with Nomarsky optics. Difference in the size of the nuclei in different strata of the testis is evident. B) A scheme of the testis shown on (A) ec - ectoderm, en-endoderm, I - the zone of spermatogonia and spermatocytes showing *in situ* signal, II - the *in situ* signal-free area of spermatids and mature sperm. C) DAPI staining of the nuclei in the testis and a fragment of the body wall (frozen section). Note two areas of large nuclei in the basal area of the testis. D) An overlay of the Nomarsky optics image of the testis shown on (C) and indirect immunofluorescence visualization of staining with anti-H3di/tri-methyl K27 antibody. The staining is confined to the large nuclei at the basis of the testis and to few presumable interstitial cells nuclei in the body wall.

2.3. HyEMB-1 – a novel gene involved in embryogenesis

2.3.1. Cloning and analysis of HyEMB-1

Cluster 027 was chosen as an example of an embryo-specific gene. In adult polyps its transcription could be detected only during oogenesis (Fig. 2.5 C). Neither RT PCR nor the Northern blot hybridization revealed signs of the presence of its transcript in asexual polyp (Fig. 2.21 A and B). Southern blot hybridization showed that it was a single copy gene. According to the Northern blot signal, the size of the transcript is slightly bigger than 1.0 kb.

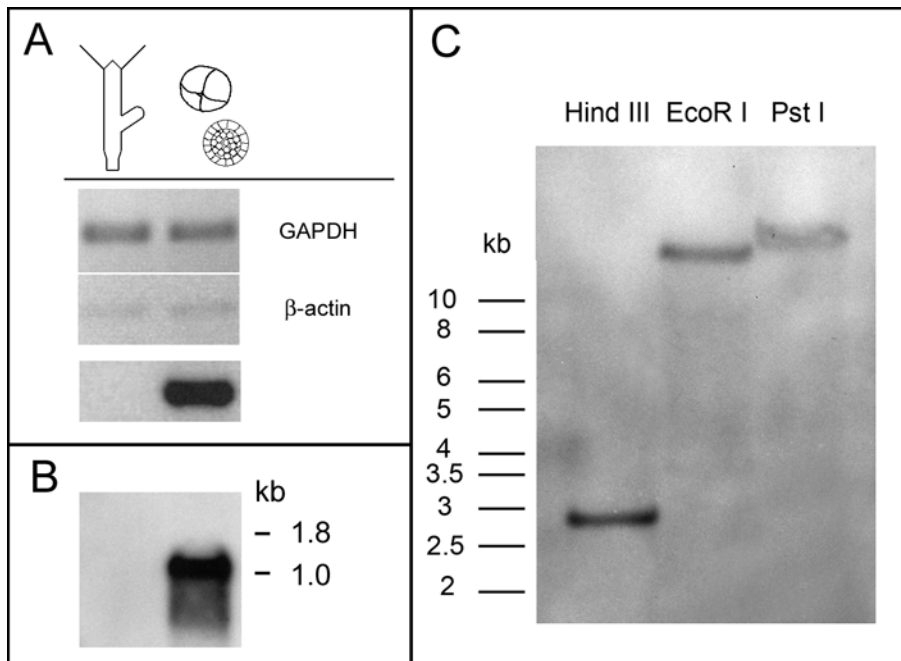


Fig. 2.21 Analysis of HyEMB-1 A) RT PCR analysis and B) Northern blot hybridization show that HyEMB-1 is present in embryos and absent in adults. C) Southern blot hybridization reveals existence of a single copy of HyEMB-1

By means of 5'- and 3' RACE PCR the full length coding sequence was obtained. The 1158 transcript has a 68 bp long 5' UTR, an open reading frame coding for 342 amino acids and a 3' UTR ending with a poly-A tail (Fig. 2.22). The gene was called *Hydra Embryonic- I (HyEMB-1)*.

Though there is no stop codon between the putative first methionine and the 5' end of the sequence, it is very likely that the translation start is at this position. Two

1 GAACAAGTAATTTTAAATCTCTCAGTAATTTAAAGTCAACTATCAGTAACAATAGTAAAG

61 GTCTTAGAATGTTGAAGACATTTGTTTTATTGCTTTGTGTTGGGATATCCTCGGCTGAAT
1 M L K T F V L L L C V G I S S A E

121 TTGTAAGTACTACTAAATGTGGTTTTAAAGTCAGAAAGCCGGAATTTTTACGCTATTGCA
18 F V T A T K C G F K V R K P E F L R Y C

181 TATCGCCAGGAAGCAGAATGTACATTGGAGATTTTGATGGAGACAAACACGATGATGTAA
38 I S P G S R M Y I G D F D G D K H D D V

241 TGTGTATTGATTTAGTTTCTGGTGACATGAGCATAATACTTTCAAAGGTTTCAACTATTC
58 M C I D L V S G D M S I I L S K V S T I

301 GTAAAAAAGTTTTTTATAACATGGAAGCTTGATAGGTGCAAATATGTTCTTCTTGGTG
78 R K K V F Y N M E A C I G A K Y V L L G

361 ATTTTAACGGTGATCATCGCACTGATATTATCTGCCAGTTACATAATGGTGAAAAATACA
98 D F N G D H R T D I I C Q L H N G E K Y

421 TTTACCTGGCCACTCTAAATGGATCTTTCTCAACTTACACTATGTTAAATGCTGTACCGT
118 I Y L A T L N G S F S T Y T M L N A V T

481 ATAGTACGCCAAGTGTAACAACTTCTGTACACAAAGTAATTACAGACCTGTGATAGGTG
138 Y S T P S V K T F C T Q S N Y R P V I G

541 **ATTTTGATGGAGATAAATTTGATGACTATATGTGTCATGAACTACTACTGGTCTGAATGT**
158 D F D G D K F D D Y M C H E T T T G R M

601 **CAATTATTTACGGTCAAAAAAATGTTGCTGATTTATTTCAGAGAAGAATCTATGCTAAAAG**
178 S I I Y G Q K N V A D L F R E E S M L K

661 **ATACTTTTATGTGCCGAGGAAAGATTTGACTGGTTTTATTTAATGGAGACAAAATGTGTG**
198 D T F M C R G K I L T G L F N G D K M C

721 **ATATTATGTGCCATGACAAAACATACGGGACTATTGTTATTGCTTCTGTTAAAGACGATC**
218 D I M C H D K T Y G T I V I A S V K D D

781 **ATGTGAATATTATGTACAACCTCAACTTGGAAATGCATTGAAAGAAGCTCTTCTGTCTATGT**
238 H V N I M Y N S T W K C I E R S S S V M

841 TTGCTGACGTAGACGGGGACAGCTATGACGATTTGCTATGCAAACAACTGGAAAAGTGT
258 F A D V D G D S Y D D L L C K Q T G K V

901 TACAGATTTTAAGAAATACTCGCAATAAAATGTTTTACGGACCAGTAGAAGCTGAGTTTT
278 L Q I L R N T R N K M F Y G P V E A E F

961 ATCCTGAAGCCAACCCAAACAAAATCTATTATACTGTTGAGACCGGAGATTTTTAATGGCG
298 Y P E A N P N K I Y Y T V E T G D F N G

1021 ATGGCAAAGACGATTTATTAGGCCATGGTTTTGACGGATCACTTCAGATTGCTGAAGCAC
318 D G K D D L L G H G F D G S L Q I A E A

1081 TTTGTTTAAACTGTAATATTTTTATGAAGTTCAGTTCCTTGATAAAATAAAAAATACTTA
338 L C L K L *

1141 TAAAAAAAAAAAAAAAAA

Fig. 2.22 Nucleotide and amino acid sequence of *HyEMB-1*. Signal peptide is highlighted dark grey and shown in white letters, arrowhead marks the putative cleavage site, FG-GAP domains predicted by SMART are highlighted light grey. FG-GAP domains with lower than threshold e-value are shown with red letters. Fragment used for hybridization probe preparation is printed in bold.

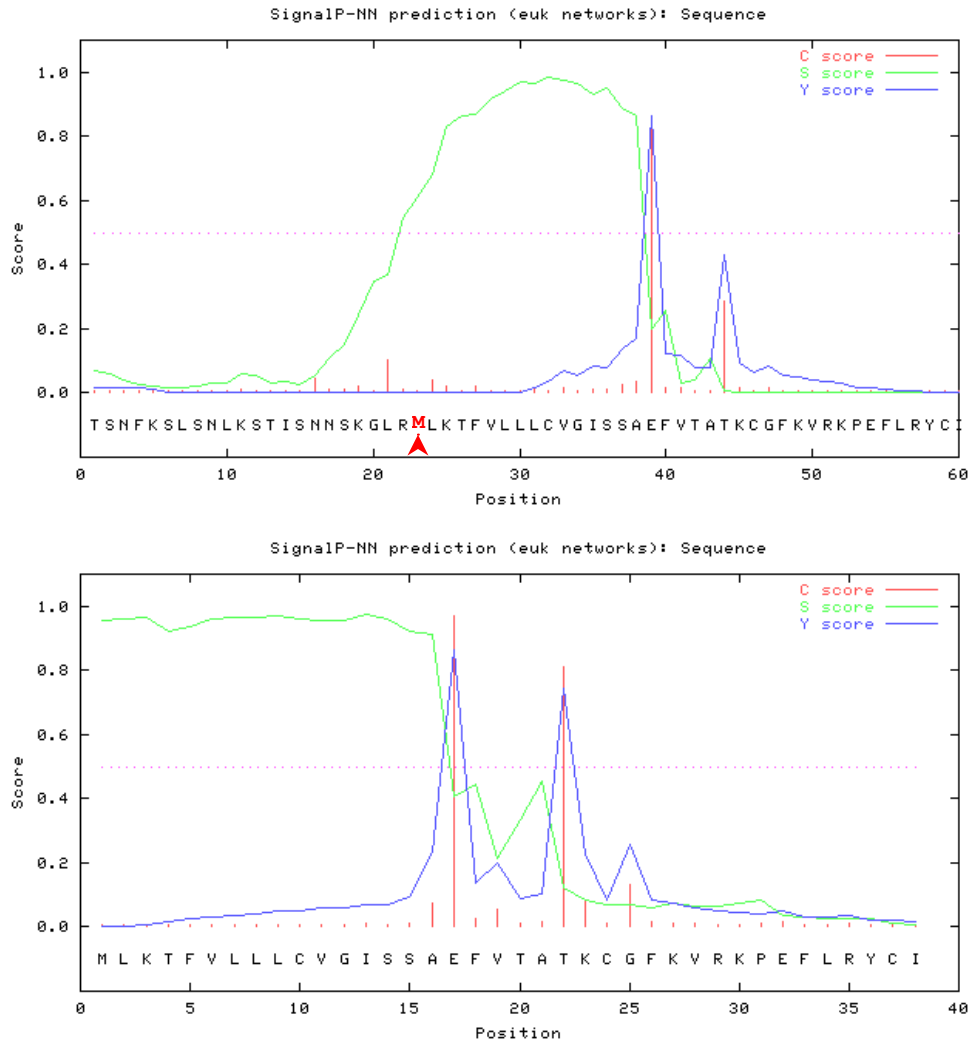


Fig. 2.23 SignalP comparison of signal peptide prediction charts of the two possible variants of HyEMB-1. On the upper panel the complete region 5' from the putative first ATG is translated and analysed. Putative first methionine is marked with an arrowhead. On the lower panel the protein sequence starts from the putative first methionine.

facts speak in favor of this: the length of the sequence corresponds to the Northern blot hybridization signal, and SignalP protein analysis program predicts a signal peptide starting exactly from this methionine (Fig. 2.23). The green curve represents the S-score, which reflects the probability that the sequence of interest stands for the signal peptide. The 50% probability is marked with the dotted horizontal line. The C-score (red bars) shows possible signal peptide cleavage sites. The Y-score (blue curve) is a derivative of the first two scores, and together with the C-score gets better prediction of the cleavage site. From the comparison of the graphs it seems very likely that the methionine at position 69 of the DNA sequence is the first amino acid of the protein. There is an abrupt loss of signal peptide qualities by the more N-terminal amino acids.

At the same time, the probability of the first 16 amino acids, starting from this methionine, to be a signal peptide is nearly 1 (100%). Both, C-score and Y-score predict a putative cleavage site between the residues 16 and 17.

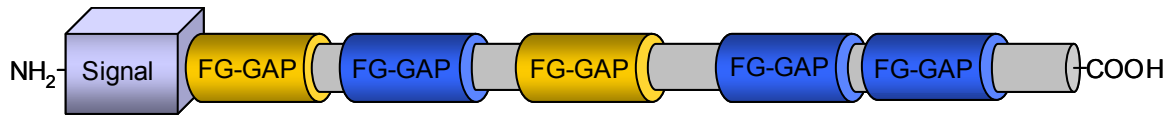


Fig. 2.24 Domain structure of HyEMB-1. Orange FG-GAP domains are predicted with lower than threshold e-value.

Within the amino acid sequence SMART search predicts five FG-GAP domains, two of which are predicted with lower than threshold probability (Fig. 2.24). FG-GAP domains form β -propeller structures and are related to integrin domains on the α -chain of the integrin receptor, where they play a role in ligand binding in the ECM. Taken together, the amino acid sequence implies that HyEMB-1 is a secreted protein with possible role in extracellular matrix.

2.3.2 Analysis of *HyEMB-1* expression by *in situ* hybridization

To get information about the *HyEMB-1* expression localization, *in situ* hybridization on 16 μm frozen sections of embryos was performed. At the early stages of embryogenesis the transcript is localized to the cytoplasm of the egg and, later, of the blastomeres, but is not detectable in endocytes (Fig. 2.25 A-D). This situation lasts all the way through cleavage and gastrulation. When post-gastrula embryos start to form spikes, a change in the expression occurs: the overall signal becomes significantly weaker, while a sub-population of small strongly stained cells arises. At this time point the cells have DAPI-positive nuclei (Fig. 2.25 E-F). Interestingly, however, during the following 24 hours as the cuticle is being built, these cells first become DAPI-negative, and finally, by the cuticle stage, appear to be some kind of cell debris (Fig. 2.25 G-J). Late cuticle stage embryos lack nearly all the remains of these cells.

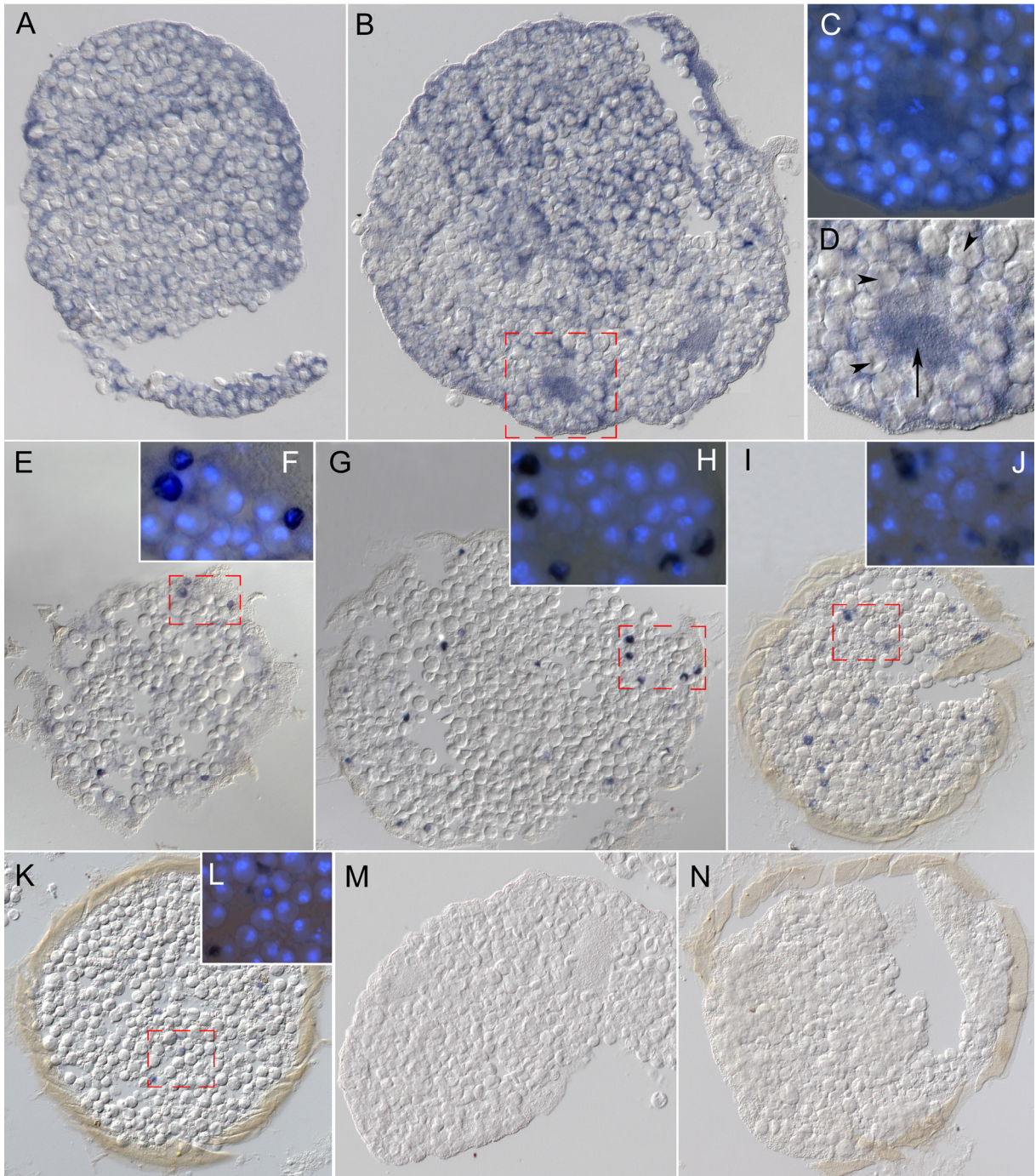


Fig. 2.25 Expression of *HyEMB-1* in hydra embryos. A) Fertilized egg. B) Gastrula. C) DAPI staining of the area boxed on (B). Endocyte nuclei visible, in the center the blastomere chromosomes are stained. D) Same fragment as in (C), viewed with Nomarsky optics. Endocytes (arrowheads) are free of *HyEMB-1* message, while the blastomere (arrow) is intensively stained. E) Early spike stage embryo. Overall blue staining gets weaker, intensively stained cells appear. F) DAPI staining of the area boxed on (E). Nuclei of the *in situ*-positive cells are stained. G) Later spike stage embryo. Even less overall staining, strong *in situ* signal is seen in several cells, most of which appear DAPI negative (H). I) Cuticle stage embryo with the stained DAPI-negative (J) cell fragments visible. K) The remains of the cell disappear during in the later cuticle stage embryos. L) DAPI staining of the area boxed on (K). M) Cleaving egg and N) cuticle stage embryo hybridized with the sense probe.

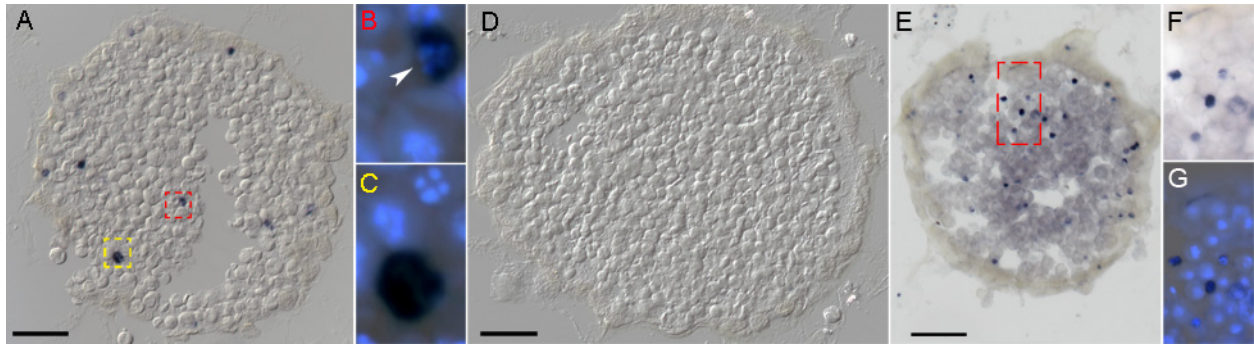


Fig. 2.26 *HyEMB-1* expression correlates with apoptosis.

A) Spike stage embryo hybridized with the *HyEMB-1* antisense probe. B) An area corresponding to the red box in (A). *In situ* - positive cell has a DAPI-positive nucleus with blocks of condensed chromatin (arrowhead). C) An area corresponding to the yellow box in (A). *In situ* - positive cell does not have a DAPI-positive nucleus. D) Spike stage embryo hybridized with the *HyEMB-1* sense probe. E) TUNEL-positive nuclei in the spike stage embryo. F) A close-up of the area boxed in (E). G) DAPI fluorescence of the same area. TUNEL and DAPI signal co-localize, indicating that TUNEL staining was specific. Bar 80 μ m

This process looked like elimination of the subpopulation of cells. As long as many of them had clumped chromatin, it was likely that they were apoptotic. At the same time it is known that early embryos are TUNEL-negative, and endocytes remain TUNEL negative till shortly after hatching (Technau et al., 2003). To check if the presence of the seemingly dying cells correlated with appearance of TUNEL-positive cells, the 14 μ m frozen sections of the spike and cuticle stage embryos were subjected to TUNEL assay. It became clear that late embryos at the spike and cuticle stage normally have a certain number of TUNEL positive nuclei (Fig. 2.26).

It has to be mentioned that *HyEMB-1* has a close relative in the Kiel 3 SHH library. This is Cluster 005. Cluster 005 is a very strong differential, which is also faintly expressed in adult polyps and is impressively upregulated during oogenesis (See Fig. 2.5 B and C). The overlapping piece of sequence is 80.74% identical on the nucleotide level and 75.14% identical on the amino acid level (Fig. 2.27). According to RT PCR with primers specifically constructed to recognize either *HyEMB-1* or Cluster 005, it is very likely that these are really two independent transcripts. At the same time, such a strong similarity most probably causes cross-hybridization on the *in situ* preparations and on the Northern and Southern blots. The fact that the Northern blot hybridization results in a single band (See Fig. 2.21) means that two transcripts are most probably of the same length. This is not surprising, as they are very similar, and the stop codon is also in the same position (Fig. 2.27 B). Single band on the Southern blot is harder to

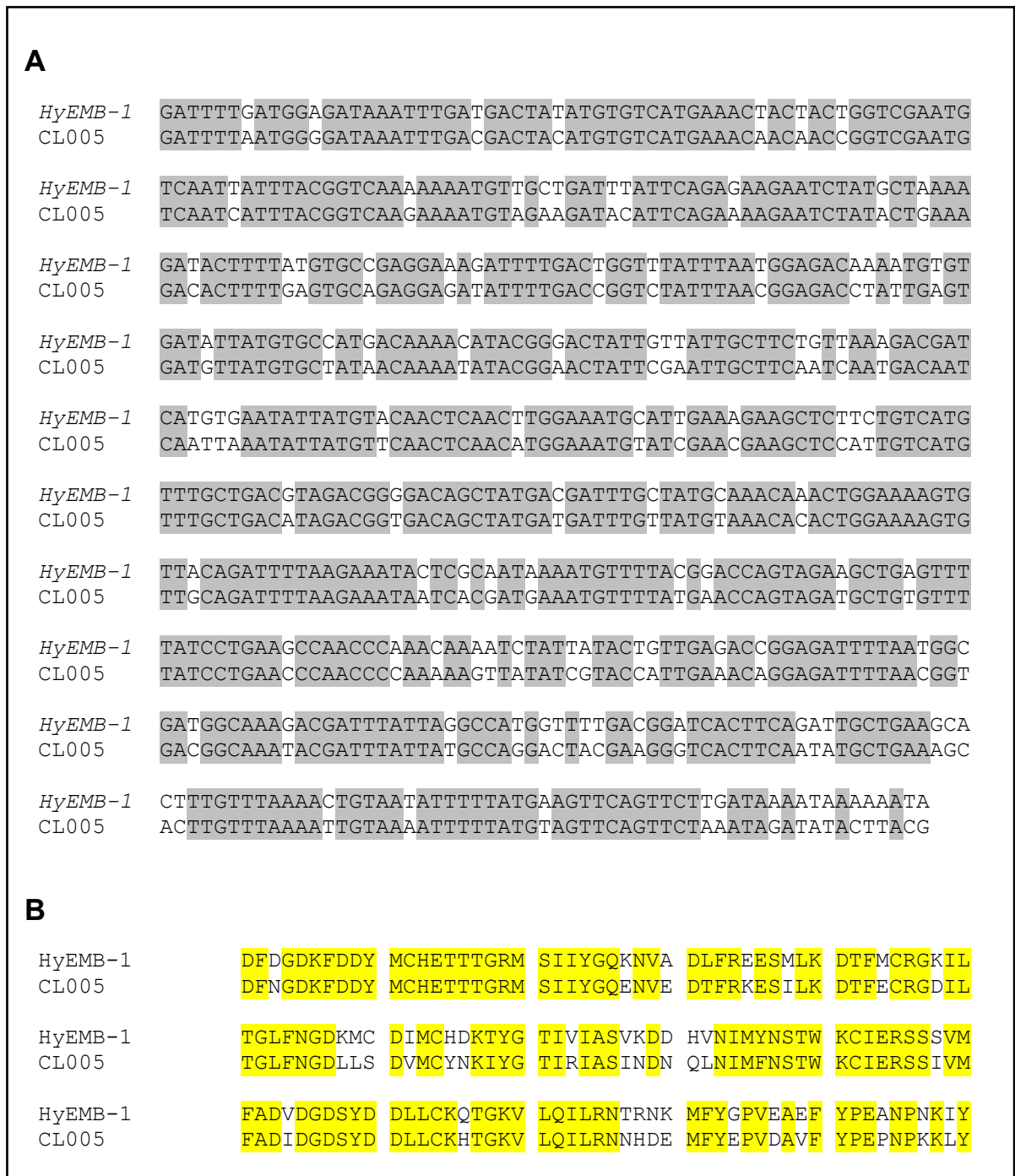


Fig. 2.27 Nucleotide (A) and amino acid (B) alignment of the overlapping part of Cluster 005 and *HyEMB-1*.

explain, but this might mean that *HyEMB-1* and Cluster 005 could be two allelic variants of the same gene.

3. Discussion

3.1. Embryonic development: what is going on in molecular terms?

In 1986 Lewis Wolpert wrote: “*It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life.*” (cited from Wolpert, 1991). Gastrulation is, definitely, very important, but in genetic terms there are two points, when an embryo crosses not less critical thresholds. These are i) activation of zygotic genome and ii) conversion of labile inducible expression state of an embryonic primordium, characterized by transient expression of regulatory genes and definition of gene expression territories, into stable self-maintaining expression in a specified spatial domain.

Embryonic development is a unique process, when starting from a single cell a complex program is unfolded to form a complex body. In essence, the developing egg is a self-building and self-organizing machine. In mechanistic terms, development proceeds as a progression of states of spatially defined gene expression (Davidson et al., 2002). Through this progression specification occurs, means in each region of the developing body cells start to express some strictly limited subset of genes.

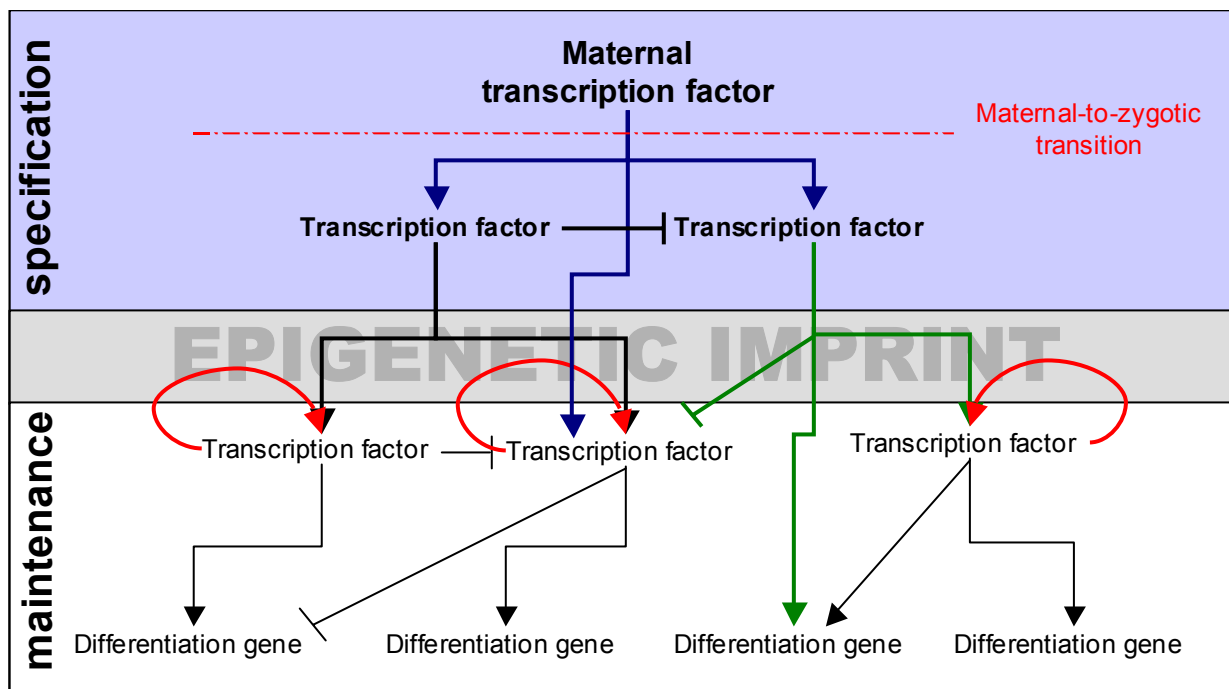


Fig. 3.1 A scheme of a gene regulatory network in development

Specification is guided by signaling ligands from other cells in consequence to their own prior states of specification, combined with maternal factors spatially distributed within the ooplasm and, later, within the blastomeres. Specification state of a cell reflects, actually, which transcription factors are present in the nucleus of this cell at a given moment. The input coming from the transcription factors is interpreted by cis-regulatory regions of the target genes, and a new spatial pattern arises. At a certain time point the state is reached, when everything is specified, and the developmental task is not to create patterns, but to maintain patterns. This is the time, when necessary expression of regulatory genes has to become autocatalytic. This is largely done by imposing an epigenetic imprint. The genes, which have to be inactive, are repressed by means of chromatin modification (Fig. 3.1). Before the autocatalytic state is achieved, literally all the patterning is done: axis specification, morphogenetic territories definition, cell fate specification are over. Proliferation and differentiation remain the key processes to be controlled.

Maternal-to-zygotic transition fulfills three major tasks: i) to destroy oocyte-specific transcripts, that should not be subsequently expressed (histone H1oo, MSY2), ii) to replenish maternal transcripts of genes, which have to be expressed constitutively (actin, for example), and iii) to promote the dramatic change in gene expression, when genes, which were not expressed in the oocyte start to be expressed (Zeng and Schultz, 2003). To illustrate the third point, one may remember that with the use of quantitative, high-resolution, two-dimensional gel protein database it was shown that during this transition in mice one third of the proteins increase by an average of fivefold, another third decrease by an average of sevenfold, and 10% undergo transient changes in rates of synthesis (Latham et al., 1991).

A number of maternal RNAs both of transcription factors and structural proteins are accumulated in the oocyte, but are never seen after some certain point in development. For example, maternal *bicoid* mRNA is stored at the anterior end of the *Drosophila* egg, its protein forms a gradient, and after patterning the anterior-posterior axis of the fly *bicoid* is not expressed until it is time to make a new egg (Berleth et al., 1988). *Hunchback* is expressed in the ovary of *Drosophila* and its mRNA is present throughout the embryo, but in the anterior part of it, where bicoid binds to *hunchback* enhancers in zygotic nuclei, more *hunchback* is transcribed. It functions, together with bicoid, to pattern the anterior of the embryo, and it is not expressed in adult fly before it starts with oogenesis (FlyBase <http://flybase.org/>). In mammals maternal genes are also

of importance. *MATER*, along with other potential markers of germ cells or oocytes: *ZAR1* (zygotic arrest 1), *GDF9* (growth and differentiation factor 9), *BMP15* (bone morphogenetic protein 15), *VASA*, *Histone H100* and other essential proteins are found only in oocytes and early embryos (Tanaka et al., 2001; Pennetier et al., 2004). The presence of maternally encoded complete set of histones was reported in sea urchin (Mandl et al, 1997). These are the only histones existing in the early cleaving embryo, and they are completely replaced by the midblastula stage. Not only histones are replaced. The same is true for cytoskeletal proteins. Lamin LIII is the only lamin present in *Xenopus* oocyte nuclei. It serves as a lamin pool for the formation of pronuclei and early cleavage nuclei. It is present in embryos up to the tail bud stages, and is completely gone afterwards (Stick and Hausen, 1985). There is an embryo-specific actin pSpec4 in sea urchin (Garcia et al., 1984). There are also typical embryo-specific transcription factors, for example ENK - an embryo-specific NK-class homeobox transcription factor, expressed in the inner cell mass of the blastocyst, and expression of which is no longer observed after day-9.5 stage of mouse gestation (Wang et al., 2003).

Less is known about zygotic embryo-specific transcripts. One of such genes is *serendipity- α* , which was already mentioned before. It codes for a cytoskeleton component involved in cellularization of *Drosophila* periblastulae. Expression starts during the 12-th cell cycle, reaches its peak at the early cycle 14 and disappears by the end of this cycle (Mazumdar and Mazumdar, 2002).

Taken together, these data reflect the fact that there are processes specific for embryonic development, and these processes require certain genetic context.

3.2. Advantages and disadvantages of SSH

Suppression subtractive hybridization (SSH) is a method of choice to search for differences in the transcript pool at two selected stages. We used it to find genes, which were upregulated in the early embryo in comparison to asexual budding adult. The most serious and decisive advantage of the SSH is that it allows to select genes on an unbiased basis. This leads to significant representation of novel genes in the resulting library - a thing not possible in homologue hunting. At the same time, the SSH procedure has several inborn drawbacks, the main of which originates from the

necessity to enzymatically digest the ds cDNA of the tester prior to adaptor ligation. As a result, subtracted library is composed not of genes, but of fragments of genes,

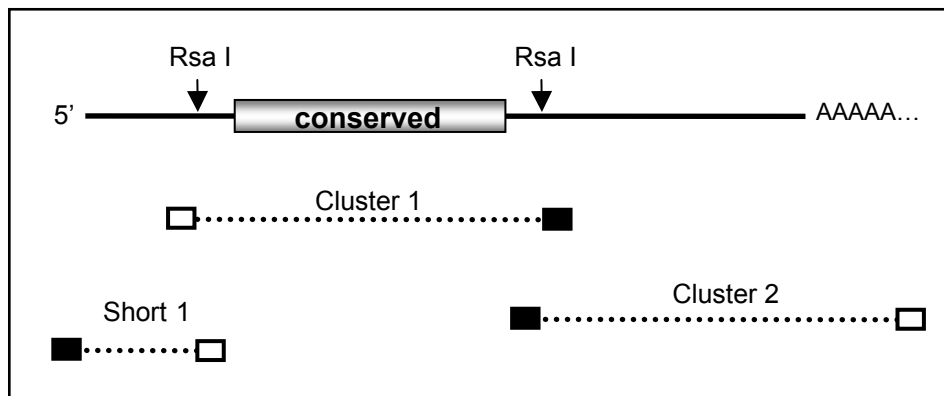


Fig. 3.2 SSH fragments yielded by an imaginary mRNA. Arrows indicate restriction endonuclease digestion sites (for example, *Rsa I*), white and black boxes stand for SSH adaptors.

upregulated at a certain developmental situation. Moreover, the interpretation of the sequence information is also not unambiguous, especially for the clusters without significant homology to some known gene. Situations leading to possible misinterpretations are illustrated on Fig. 3.2. Imaginary differentially expressed mRNA coding for a protein with a conserved domain and possessing two restriction sites for the enzyme used in ds cDNA digestion provides three SSH fragments: Cluster 1, Cluster 2, and a short fragment (Short 1). Short 1 will be discarded because of the insufficient length, so there will be only Cluster 1 and Cluster 2 left. First of all, they will not be recognized as belonging to the same gene, because they do not overlap. There is an example of this in Kiel 3 library: clusters 065 and 067 stand for different regions of the same G-protein gene. Second, if the gene has a conserved region (for example, coding for a DNA-binding domain of the protein), Cluster 1 will provide a BLAST match to a known gene, and will be annotated as its homologue, but Cluster 2, if the two proteins have diverged strong enough, will retrieve no homologues, and will be annotated as novel. This means that no BLAST match for an SSH fragment does not mean that it necessarily stands for a novel gene - such conclusion can be drawn only when a full length coding sequence is known.

Another technical problem is the size of the SSH fragment. First, long templates without cutting sites for the restriction enzyme used will not be effectively amplified by PCR, and eventually will get lost from the library. Second, as there is no size selection step in the SSH procedure, the size solely depends on the restriction enzyme used. In

mammals Rsa I yields fragments with the average size of approximately 600 bp (Diatchenko et al., 1996), but Kiel 3 library has an average cluster sequence length of 379 bp, and the smaller is the cluster sequence length, the more difficult is subsequent analysis. In perspective, it appears reasonable to test different blunt end producing restriction enzymes, to select an endonuclease, which yields fragments with the average size between 0.6 - 1 kb, and to include a size selection step prior to ligation of adaptors and hybridization with the driver cDNA.

3.3. Verification of the library: necessary or not?

As it was mentioned before, SSH may yield falsely differential cDNAs, which were by some reason not subtracted and then amplified by PCR. The efficiency of subtraction can vary from 95% of differentially expressed sequences to 5% (Diatchenko et al., 1996; Diatchenko et al., 1998). This makes it necessary to test the outcome of the subtractive hybridization. The most reasonable approach for testing the library and finding clones bearing differentially expressed inserts is macroarray analysis. The embryonic SSH library clones from six 384-well plates (2304 clones) were gridded on nylon membranes, lysed and hybridized first with the radioactive ss cDNA probe from asexual budding adult polyps, and then, after stripping the probe, rehybridized with the radioactive ss cDNA probe from embryos. 110 differentially hybridized clones, and 42 clones not providing any hybridization signal neither with embryonic, nor with "adult" probe, were picked, propagated and sequenced in a pilot experiment. Though sequencing of the complete library made the results of this preliminary in-house sequencing redundant, there are several points to be discussed. The sequences of the picked differentially hybridizing clones were clustered, and some of the clusters were checked by RT PCR. All of the clusters checked proved to be true differentials (stronger expressed in embryos than in asexual adult polyps). At the same time 3 of 78 clones found on the filter, which gave stronger hybridization signal with "adult" probe, were sequenced, and none them proved to be indeed false positive, when checked by RT PCR. These clones corresponded to clusters 002, 042 and 061 (See Fig. 2.5). Hence, macroarray gives valuable hints of what is differential, and also provides expression data, as the strength of the hybridization signal must be proportional to the representation of the ss cDNA in the probe, but macroarray preparation and

hybridization in the case of Kiel 3 library turned to be more prone to errors than subtractive hybridization itself, primarily because of the unspecific hybridization of the complex cDNA probe to some clones on the macroarray.

Analysis of RT PCR and sequencing data lead to the following conclusions:

1. Not a single false positive was detected among 15 of 78 clusters, implying that the overall number of false positives is low.
2. SSH procedure yielded two types of differentially expressed fragments. The first type are fragments, which are upregulated in embryos, but are still strongly expressed in adults (Fig. 2.5A). The second type differentials are expressed exclusively or nearly exclusively in embryos (Fig. 2.5B).
3. All of the three second type differentials were shown to be maternal transcripts, probably accumulated in the oocyte during oogenesis (Fig. 2.5C).
4. Though SSH proved to be a very reliable method for obtaining genes upregulated at a certain developmental stage, the number of ESTs comprising an SSH cluster does not reflect neither the strength of expression of the gene nor the extent of differentiability of its expression. For example, cluster 001, standing for betaine-homocysteine methyltransferase comprises 505 ESTs. Macroarray and RT PCR data indicate that this is neither a strongly expressed gene, nor a strong differential. Cluster 052 is, on the contrary, very strongly differential, though it consists of only 10 ESTs.
5. A gene may give BLAST hits to the ESTs from asexual polyp, but still be very strongly differential, like clusters 005, 011 and 052.

3.4. Sequence analysis of the Kiel 3 clusters and singletons

3.4.1. General information

In silico analysis of 2851 SSH inserts sequenced in frames of the *Hydra* EST project yielded four classes of EST clusters and singletons: i) the ones, which had homologues in other metazoans and also gave a hit in the EST database from asexual *Hydra* cDNA libraries, ii) the ones, which had homologues in other metazoans, but did not have ESTs from *Hydra*, iii) clusters without significant homology to any known gene but with ESTs from adult asexual animals and iv) clusters without known homologues

and without ESTs from asexual *Hydra* (Fig. 3.4). The clusters and singletons with BLAST hits were additionally subdivided into functional groups (Table 2.1, Fig. 2.3). The biggest groups, representatives of which had homologues in other organisms were “metabolism”, “Ribosomal proteins” and “Proteins with unknown function”. Still bigger were the groups represented by clusters and singletons, which did not give a BLAST hit.

As it was mentioned before, SSH procedure yields not full length cDNA sequences, but cDNA restriction fragments. Not to falsely annotate Kiel 3 EST clusters

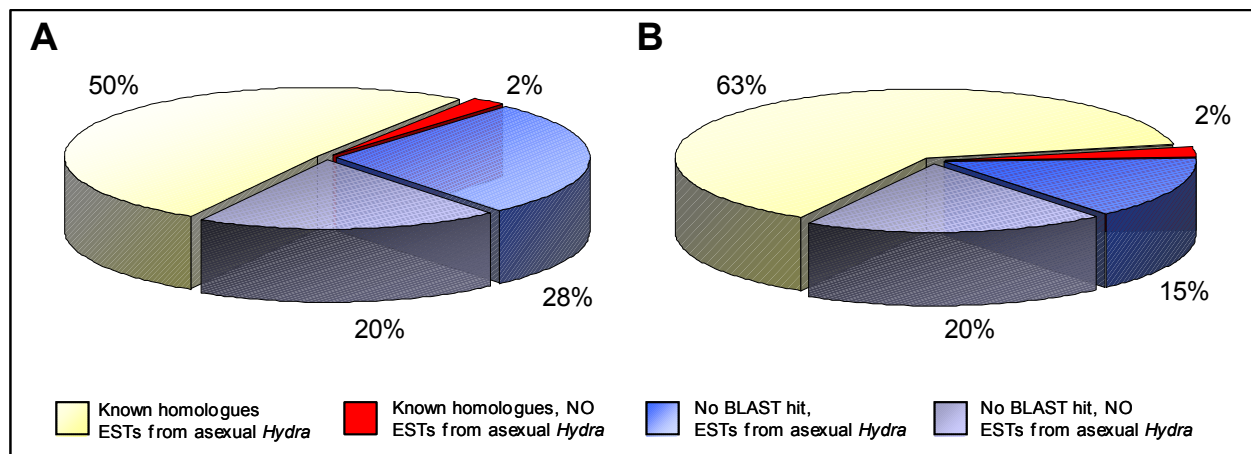


Fig. 3.4 Distribution of Kiel 3 clusters and singletons according to the presence or absence of homologous sequences.

A) Based only on embryonic SSH sequences; B) Based on embryonic SSH sequence and on sequence of homologous ESTs from asexual *Hydra*.

and singletons as “No BLAST hit”, *Hydra* ESTs from other cDNA libraries matching Kiel 3 SSH library clusters and singletons, were also subjected to BLASTX analysis with the same threshold e-value. This significantly changed the outlook of the diagram (Table 2.2, Fig. 3.4). In terms of putative functions of the clusters, this change made “Metabolism” the largest functional group followed by “Fragments without BLAST match and without ESTs from asexual *Hydra*”.

Large representation of metabolic and ribosomal cDNAs fragments is not surprising. There are obviously oocyte- and embryo-specific features of metabolism. Notably, some of the Kiel 3 clusters belonging to the “Metabolism” functional group, namely *betaine-homocysteine methyltransferase* (Cluster 001), *glutathione S-transferase* (Cluster 025), and *ornithine decarboxilase* (Cluster 039), match with the oocyte- and 8-cell specific SSH clusters from mouse (Zeng and Schulz, 2003).

3.4.2. Novel genes in the Kiel 3 SSH library

It is most probable that at the level of transcription factors *Hydra* uses conserved patterning machinery both for adult and embryonic patterning. Key metazoan transcription factors, such as *brachyury*, *gooseoid*, *Otx*, *NK2*, *forkhead* and many others were found in *Hydra* (Technau and Bode, 1999; Brown et al., 1999; Smith et al., 1999; Grens et al., 1996; Martinez et al., 1997). They are expressed in position-dependent manner, and structurally closely resemble their bilaterian orthologues. In several cases they were even shown to save mutant phenotypes in *Drosophila*, like *CnAsh*, an *achaete-scute* homologue (Grens et al., 1995), induce ectopic axis in frog embryos, like *Hydra* β -*catenin* (Hobmayer et al., 2000) or ventralize *Xenopus* animal caps, like *HyBra1* (Marcellini et al., 2003). At the same time, *Hydra* EST project shows that *Hydra* possesses a huge number of novel genes. The knowledge of the function of these genes remains extremely obscure, as long as there are no good genetic tools developed for this model at the moment. Some of the novel genes prove, though, to be of great importance, for example, donor hydra tissue treated with HEADY peptide (Lohmann and Bosch, 2000) induces ectopic axis in the recipient polyp, and novel peptide pedibin (Grens et al., 1999) significantly modulates positional information, resulting in the shift in the expression domain of the homeobox gene *CnNK-2*. The direct physical link between this conserved transcriptional regulator and pedibin was recently demonstrated by Thomsen and colleagues (Thomsen et al., 2004), who showed that CnNK2 directly regulates transcription of pedibin. Apart from patterning events, novel genes, coding for neuropeptides are involved in development and differentiation of the nerve cells (Plickert et al., 2003).

That is why it was interesting that 35% of the clusters received were fragments of genes with no homology to any known gene. To be accurate, they cannot be all called “novel genes”, because the experimental procedure for subtractive hybridization included enzymatic digestion of the tester ds cDNA, which might have resulted, first, a loss in some cases of the conserved part of the gene sequence, leading to no good BLAST hit, and, second, in receiving two or more non-overlapping clusters from a single original cDNA (see Fig. 3.2). In any case, the ratio of the fragments not giving a BLAST hit was significant in the Kiel 3 library, and at least some of these clusters, like *HyEMB-1*, really are novel genes. Moreover, 20% of the clusters were not only providing no BLAST match, but also had no ESTs from asexual polyps, so they might be possibly not

only *Hydra*- or Cnidaria-specific, but *Hydra* embryo-specific. Another thing to be noted is that of 32 clusters and singletons, which did not give a good BLAST match, 9 did not have a reasonable open reading frame. This might mean either that these are 3'- or 5'- untranslated regions of novel or highly derived genes or non-coding RNAs, possible regulatory function of which is now widely discussed (Sado et al., 2001; Volpe et al., 2002).

3.5. HyEMB-1 - a novel embryonic gene

One of the embryo-specific novel genes was analyzed in detail. *HyEMB-1* encodes a 342 amino acids protein with several FG-GAP domains. These domains resemble ligand binding domains of α -integrins, but in integrins they are positioned in the N-terminal part of the protein, prior to the transmembrane domain, while domain structure of *HyEMB-1* implies that it might be a secreted protein with a signal peptide at the N-terminus followed by FG-GAP domains. By RT PCR it was demonstrated that *HyEMB-1* is expressed already by the female polyp in the process of oogenesis. *In situ* hybridization experiments revealed that its mRNA is present in every blastomere of the early embryo, but not in the endocytes engulfed by the growing oocyte. Expression of *HyEMB-1* is dynamic during embryogenesis. Till the late gastrula stage its RNA is abundant in all the blastomeres, but it is not detectable in the endocytes. Then within the next 24 hours, as the embryo is rearranging its cytoskeleton, building spikes and secreting the cuticle, *HyEMB-1* expression significantly goes down. While overall expression in the blastomeres is decreasing, some small cells remain *HyEMB-1* - positive. At the early spike stage these cells have DAPI-positive nuclei, while at the later spike stage the nuclei show clumped chromatin and finally completely loose DAPI staining. Cuticle stage embryos contain sometimes *in situ* - positive material looking like not cells, but remains of cells. But majority of late cuticle stage embryos are *HyEMB-1* negative. To correlate this process of disintegration of a certain kind of cells with the programmed cell death process in the embryo, TUNEL staining was performed. Certain amount of TUNEL-positive nuclei is present in the embryo at the stage when *HyEMB-1*-positive cells become DAPI-negative and degenerate.

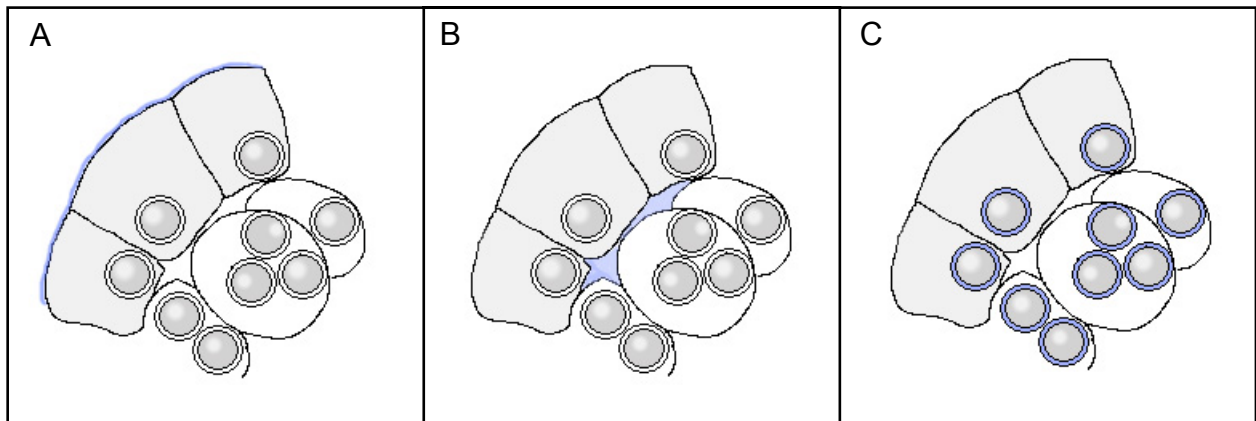


Fig. 3.5 Possible localization of HyEMB-1 protein in a fragment of *Hydra* gastrula. A) In the sheet of ECM surrounding the embryo on the outside B) In the intercellular space between the blastomeres C) In the space surrounding the endocyte in a phagosome. Ectoderm is shown light grey, inner blastomeres - white, endocytes in phagosomes grey, HyEMB-1 - blue.

It is hard to hypothesize about the possible function of the protein, but assuming from the presence of the FG-GAP domains that it is really an ECM protein, one might think of three possible locations in the embryo, where it could be functioning. First, this could be in the thin layer of matrix surrounding the embryo on the outer surface (Fig. 3.5A); second, it could be in the intercellular space of the embryo or in the newly forming basal membrane after gastrulation is over (Fig. 3.5B), and, finally, it could be within the phagosomes containing endocytes (Fig. 3.5C). The latter possible location is the most interesting. The need for keeping the endocytes within the embryonic cells throughout the whole embryonic process remains uncomprehended. Technau et al. (2003) suppose that endocytes are not communicating with the blastomeres, but our results documented transcription of the nascent RNA of transcription factors by the 1-4 cell stage embryos, and endocytes being *in situ* - positive for the *Cnnos1* riboprobe (Fröbius et al., 2003), and it is difficult to imagine the possible function of all these regulatory genes and proteins in metabolically inactive and dying cells. Antibody localization of the protein would, certainly, clarify this issue.

3.6. Hydra Embryonic Ectoderm Development. Epigenetics and the origin of the interstitial cells.

3.6.1. Functions of the Polycomb proteins

The role of epigenetic code in pattern formation and cell differentiation was long underestimated. However, increasing amount of data are accumulating, which show that chromatin modification is indeed crucial for determination of what genes are expressed in a cell at a given moment. There are several ways to modify chromatin, main of which are DNA methylation, histone modification and RNA interference (Zhang and Reinberg, 2001; Sado et al., 2001; Di Croce et al., 2002; Yelin et al., 2003; Orlando, 2003). These three mechanisms share the tasks. Interplay of RNAi and histone modification mechanisms was recently demonstrated by Volpe et al. (2002).

N-terminal tails of histones can be methylated, phosphorylated and acetylated. Polycomb group repressor proteins and Trithorax group activator proteins are the key players in forming a genetic imprint by means of histone methylation. They coexist, creating a dynamic situation, in which genes not only can be imprinted once and for all and maintained in one terminal state, but also switched on and off. Epigenetic control of pattern formation and cell specification is completely unstudied in *Hydra*. At the same time, *Hydra* is constantly in a process of patterning and cell differentiation, so conserved gene subjected to more detailed analysis was *Hydra* homologue of the polycomb gene *embryonic ectoderm development (EED)*.

As it was already mentioned, polycomb group proteins in *Drosophila* were shown to play essential role in the long-term silencing of the *Hox* genes, after their expression domains are established early in development by transiently expressed gap and pair-rule genes (Reinitz and Levine, 1990; Qian et al., 1991; Simon, 1995). Mammals use polycombs for the same purpose. Changes in expression of the polycomb group genes result in shifts of the expression domains of the *Hox* genes. Mouse EED, for example, targets *Hox a4*, *Hox a7*, *Hox b6* and *Hox c8* (Wang et al., 2002). Another important function of the EED-Ezh2 complex is its role in X-chromosome inactivation (Plath et al., 2003; Okamoto et al., 2004). Recently a strong link between stem cell qualities of the cells and polycomb group genes expression was uncovered. Polycombs were proposed to maintain the “stemness” of the stem cell (see review Valk-Lingbeek et al., 2004).

There are several Polycomb Repressive Complexes (PRCs). PRC1 - is a stable repression complex, which maintains a long term repression of the gene. PRC-2, consisting of Enhancer of Zeste, Embryonic Ectoderm Development, Suppressor of Zeste12, RbAp46 and RbAp48, in contrast to PRC1, acts early in embryogenesis and prepares the ground for PRC1-dependent long-term epigenetic memory. Histone lysine-specific methyltransferase (HKMTase) activity of the PRC2 is dependent on the SET domain of the Enhancer of Zeste protein (Orlando, 2003; Kuzmichev et al., 2004). EED proteins do not have methyltransferase activity themselves, but target PRC to the site, which has to be methylated. Recently it was shown that in human there are 4 different isoforms of EED, translated from different start codons of the same mRNA, which differentially target the complex either to lysine 27 of the nucleosomal histone H3 or to lysine 26 of the linker histone H1 (Kuzmichev et al., 2004). *EED* is present in mammalian genome in several copies. Different EED proteins can be synthesized not only from different start codons of the same RNA, but also from alternatively spliced RNAs. In different tissues different types of RNA prevail (Rietzler et al., 1998).

It is necessary to stress that, in contrast to members of Polycomb repressive complex 1, *EED* can be expressed transiently. For example, in the imaginal wing disc, ESC-E(Z) complex is necessary only for the establishment of the *Hox* genes expression boundaries but not for the maintenance of their repression state like PRC1 (Beuchle et al., 2001). Another feature of EED is its involvement in differentiation of particular cell types. In mammals it was shown that EED causes defects in gastrulation and extraembryonic tissue development resulting in early lethality (Faust et al., 1998). One of the effects is the lack of giant trophoblast cells differentiation (Wang et al., 2002). EED is supposed to be negatively regulating the gene of the bHLH transcription factor *Mash2* (homologous to *Drosophila achaete-scute*, and to *Hydra CnAsh*), which, if not repressed, switches off giant trophoblast cells differentiation from trophoblast stem cells by inhibiting *Hand 1* expression.

To summarize, EED proteins in other models are parts of a complex, which represses transcription of genes in a target-specific manner by means of histone methylation. They can be expressed transiently and act in pattern formation, cell type differentiation and maintenance of the stem qualities of a cell. In mammals EED genes were shown to be present in the genome in several copies and be alternatively spliced. Different protein isoforms can also be synthesized from different start codons of the same mRNA.

3.6.2. *Hydra EED* - the earliest known genetic marker of the interstitial cells

Polycomb group proteins are known from all kingdoms of multicellular organisms. They were found in plants (Grossniklaus et al., 1998), fungi (Stankunas et al., 1998) and, certainly, animals. Within Cnidaria, *Podocoryne carnea* homologue of *Polycomb* was reported two years ago (Lichtneckert et al., 2002). It is expressed at all stages of the life cycle in all tissues, but with different intensity. *E(Z)* homologue is cloned from a coral *Acropora millepora* in the search for genes expressed during its embryonic and larval development (Miller, personal communication). A fragment of *Hydra* homologue of *EED* was cloned in an SHH experiment presented in this thesis. The full length transcript coding for 420 amino acids was obtained by 5' and 3' RACE PCR. Interestingly, as in case of mammalian *EEDs*, this was not the only variant of cDNA. Two other shorter cDNA fragments were also found. All three variants, which were named *HyEED-1*, *HyEED-2*, and *HyEED-3* possess different insertions, but the general size of their cDNAs must not vary strongly, as long as the Northern blot hybridization with a probe, which recognizes all of them, yields a single band. According to RT PCR recognizing different cDNA variants, *HyEED-1*, *HyEED-2* and *HyEED-3* are all upregulated in the embryos in comparison to asexual budding adults. Southern blot analysis yielded, however, the same amount of bands with transcript-specific and conserved probes, which implies, that all transcribed variants might be products of the same gene, which is present in two copies in the genome.

Additional work is necessary to understand fully the need for and specificity of different transcriptional variants of *HyEEDs*. *In situ* hybridization with a probe recognizing the whole family of transcripts demonstrated that *HyEED* RNA is present in the oocyte and later in the developing embryo. At the later stages of the embryogenesis *HyEED* is expressed in a subset of cells in the ectoderm and, notably, endoderm of the embryo. These *EED*-expressing cells are morphologically resembling interstitial cells of the adult polyp, though they are still C41-negative. Interestingly, in adult polyps *HyEED* is also expressed in the interstitial cells. Thus it is very likely that these *in situ* - positive cells in the embryo are indeed interstitial cells, and *HyEED* expression is an earlier marker of the interstitial cells than C41 epitope.

This finding revives the discussion about the origin of the interstitial cells. Scholars, who investigated the embryogenesis of marine hydroids on the histological level, first found interstitial cells in the ectodermal layer of *Tubularia*

mesembryanthemum (Brauer, 1891) and *Clava squamata* (Harm, 1903). Recently Martin et al. (1997) used CP4 monoclonal antibody against an uncharacterized epitope in the interstitial cells of *Hydra*, and also could stain them first in the ectoderm of the late cuticle stage embryo. In argument to the ectodermal hypothesis, there are strong histological and electron microscopy data supporting endodermal origin of the interstitial cells. It was documented in different species of both athecate hydroids, *i.e.* closest marine relatives of *Hydra* such as *Tubularia*, *Hydractinia*, *Clava*, *Coryne* and others (Fennhoff, 1980; Van de Vyver, 1980), and thecate hydroids of the genus *Obelia* (Polteva and Aizenshtadt, 1980) that interstitial cell arise as a result of asymmetric division of the endodermal blastomeres and migrate into the ectoderm. Results presented in this study support the endodermal hypothesis of interstitial cells origination, showing that however derived freshwater *Hydra* is, it still follows general rules of hydroid embryogenesis.

HyEED expression data are in accordance with the knowledge about how EED proteins function in other models. *HyEED* expression is transient and differentiation-dependent. All the cells, which express *HyEED* are multi- or totipotent. It is expressed in

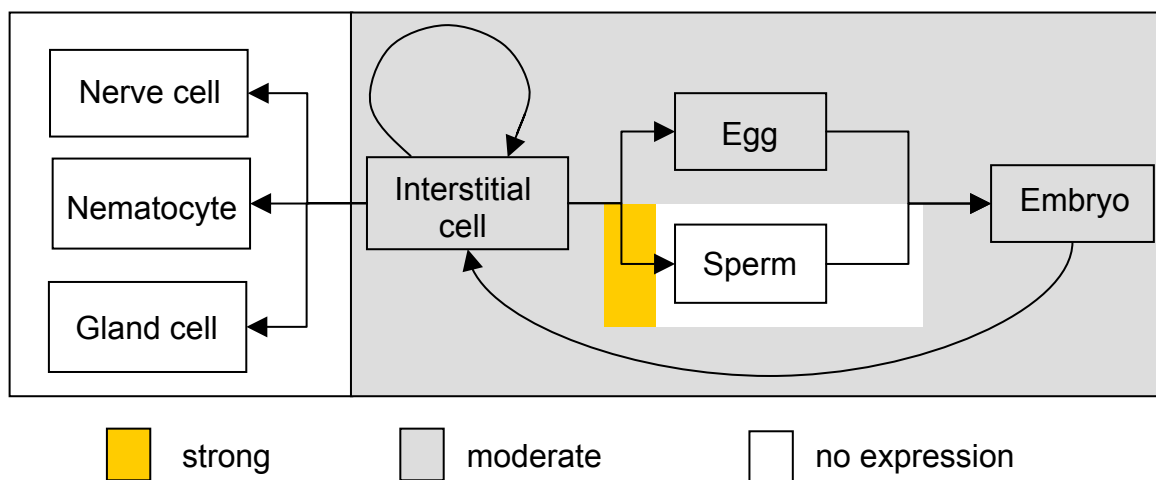


Fig. 3.14. *HyEED* expression correlates with “stemness” of the cell

early embryos, where every blastomere has most wide differentiation capacities; it is expressed in the early interstitial cells when ectoderm is specified to be ectoderm, and prospective endoderm is determined, and it is expressed in the interstitial cells of adult polyps, which are also multipotent cells and are, in fact, germ line cells constantly expressing such germ line-specific markers like *vasa* and *nanos*. Expression is turned down as soon as the cell is committed to any kind of terminal differentiation (Fig. 3.14).

One may speculate that expression of *HyEED* is restricted to multipotent cells, which are “waiting for the imprint”. As soon as stable imprint is set by Polycomb Repressive Complex I, the cell becomes a differentiated derivative of the stem cell and loses *HyEED* expression.

The mechanism of EED action appears to be conserved from the most basal metazoans, like *Hydra*, to mice and flies. In case of *Hydra*, it was demonstrated that nuclei with specific pattern of histone methylation, typical for EED-E(Z) complex, belong to the cells, which strongly express *HyEED*. As long as E(Z) is the methyltransferase, which actually accomplishes the function of the PRC2, it would be very interesting to isolate its *Hydra* homologue in order to compare gene silencing mechanisms in very primitive and most advanced animal groups. In every model studied, EED turned to be very specific and conservative in its targets. These are, primarily, transcription factors. Targets of *Hydra* EED are not known. RNAi knock-down experiments might provide some answers. It would be interesting to analyze expression of *CnAsh*, the *achaete-scute* homologue (Grens et al., 1995) expressed in a subset of interstitial cells differentiating into nematocytes, in an RNAi experiment, because it is known to be negatively regulated by EED in mice, and at early stages it is co-expressed with *HyEED*.

3.7. Concluding remarks

Embryonic development of *Hydra* is not too well characterized in genetic terms. Subtractive hybridization of embryonic and asexual adult cDNA pools yielded a fraction of gene fragments, which were upregulated in embryos compared to asexual adults. The genes encoded by these fragments were primarily metabolic, ribosomal and, strikingly, ones without significant homology to any other sequence. No obvious transcriptional regulators were found in this screening. Though the size and character of the library is not sufficient to draw a general conclusion about what genes are expressed, and what are not expressed in the embryo, and the library preparation procedure also does not allow the use of the number of ESTs in SSH clusters as an indicator of the expression level of the corresponding genes, it is possible to state that genetic context of the egg and embryo is strongly different from adult. Both, known and

putative novel genes are differentially expressed. Some genes, like *HyEMB-1*, are not expressed in asexual polyps at all.

Epigenetic mechanisms of regulation of gene expression by means of chromatin remodulation, appear to be important for proper embryonic development and for cell differentiation in *Hydra*. One of the molecules upregulated during embryonic development and in the stem cell lineage was a polycomb group regulatory gene *HyEED*. Epigenetic factors are also known to play critical role in the maintenance of cell identity. The way they act in *Hydra* - an animal, in which stable positional information is preserved, but cells are constantly changing their differentiation state - remains unknown and challenging.

Axis specification, cell layer formation, early cell lineage differentiation in *Hydra* happen before conventional “adult” transcription factors start to be expressed. The questions, what transcription factors are functioning before the time point, when adult transcription factors are turned on, how important are maternal transcription factors and in general maternal spatial determinants, still have to be answered. Extra effort has to be given to examination of *Hydra* gastrulation. While cnidarians gastrulating by invagination, like *Nematostella*, express a conventional set of gastrulation genes (Scholz and Technau, 2003; Martindale et al., 2004), *HyBra1* appears to be silent during gastrulation in *Hydra* (Technau and Bode, 1999; Fröbius et al., 2003). This might reflect the fact that multipolar immigration is a unique kind of gastrulation, which, from the first look, lacks an organizer. Is there really no organizer in *Hydra* before adult morphology is formed prior to hatching? To-date knowledge about upregulation of *HyBra1* expression at the time of hatching (Technau and Bode, 1999) speaks in favor of that, though it could be also not true, because low level of *HyBra1* transcript is detectable by RT PCR throughout embryogenesis (Fröbius et al., 2003). In *Podocoryne*, *brachyury* homologue is very weakly upregulated at the site of ingression (Spring et al., 2002). Detailed analysis of other conventional regulator molecules, especially intracellular maternal members of the Wnt pathway is also necessary to get some insight about early axis formation.

The question of what key regulators are guiding the early stages of *Hydra* embryonic development remains unanswered. Most probably these are conserved molecules. At the same time, according to the data presented above, they clearly have to act in a genetic context, largely from novel proteins, which differs greatly from the situation in adult polyp, and is permissive for various developmental processes.

Creation of a large scale normalized embryonic library and probing it with the SSH cDNA pool of cDNAs upregulated in embryos would be helpful to get more detailed information about what genes are involved in creating the embryonic microenvironment and, especially, regulation of the early development.

4. Summary

Freshwater *Hydra* undergoes typical embryonic processes, such as pattern formation and cell differentiation, in adult state, as it proliferates by budding and the cells change their position within the animal. At the same time, *Hydra* can propagate sexually and then conventional embryogenesis occurs. Till recently there was completely no understanding how similar or different were the processes of adult and embryonic patterning in genetic terms. Earlier we could demonstrate the difference in timing of expression of regulatory genes in adult and embryonic states (Fröblius et al., 2003). Here the results of suppression subtractive hybridization - an unbiased way to get fragments genes upregulated in embryos in comparison to adults - are presented. Sequencing of the embryonic SSH library yielded 2851 sequences, which were assembled into 76 clusters and 12 singletons. The clusters were largely represented by metabolism-related genes, but 35% of clusters and singletons generated no BLAST match to any known sequence from another organism. Moreover, 20% of clusters did not match ESTs from asexual *Hydra* polyps. Though probably not all of these clusters stand for novel genes, the amount of *Hydra*-specific molecules involved in embryonic development appears to be significant.

Two expression strategies were uncovered for the differentially expressed genes. Some were upregulated in embryos but still strongly expressed in asexual adults, while the others were exclusively embryonic. All three embryonic clusters tested turned to be maternally expressed in female polyps undergoing oogenesis. One of these clusters, corresponding to a novel gene *HyEMB-1* was further analyzed. It was shown to be expressed in the blastomeres but not in the endocytes of the early embryos, and its expression was turned down by the cuticle stage. In the course of this study a homologue of the polycomb group gene *embryonic ectoderm development (EED)* was found as upregulated during embryogenesis. Like in other models, *HyEED* expression is characteristic of cells, which are multipotent in their differentiation capacities. In *Hydra* these were embryonic cells and interstitial cells. It is also the earliest known to-date genetic marker of interstitial cells in embryogenesis, proving their endodermal origin.

Most probably *Hydra* uses conserved transcription factors to regulate embryonic development, but it became evident that genetic context differs significantly between adult polyp and embryo.

4. Zusammenfassung

Der Süßwasserpolymp *Hydra* vollzieht als adultes Tier typische embryonale Prozesse wie Musterbildung und Zelldifferenzierung, zum Beispiel während der Knospung. Zusätzlich pflanzt *Hydra* sich auch sexuell fort, wobei eine klassische Embryogenese stattfindet. Bis vor kurzem gab es keinerlei Erkenntnisse über Unterschiede oder Übereinstimmungen von genetischen Steuerungsprozessen bei adulter und embryonaler Musterbildung.

Wir konnten bereits zeigen, dass sich der Zeitpunkt der Expression regulatorischer Gene in adulten und embryonalen Stadien unterscheidet (Fröbuis et al. 2003). In der vorliegenden Arbeit sind die Ergebnisse einer subtraktiven Hybridisierungsmethode, SSH, dargestellt, mit der erwartungsgemäß Fragmente von Genen gefunden werden konnten, die im Embryo, aber nicht im adulten Tier hochreguliert sind. Die Sequenzierung der erzeugten Bibliothek von cDNA-Fragmenten ergab 2851 Sequenzen, die zu 76 Clustern und 12 Singletons zusammengefaßt werden konnten. Die Cluster repräsentieren zu einem großen Teil Stoffwechselfgene. 35% der Cluster und Singletons ergaben keinerlei Übereinstimmungen mit bekannten Sequenzen anderer Organismen (BLAST-Analyse). Zu 20% der Cluster lassen sich keine korrespondierenden Sequenzen in EST-Datenbanken aus asexuellen Hydren finden. Obwohl möglicherweise nicht jedes dieser Cluster für ein neues Gen steht, scheinen *Hydra*-spezifische, in die in Embryogenese involvierte Gene darunter zu sein.

Die durch SSH identifizierten Gene unterteilten sich in zwei Klassen. Die Expression von Genen der ersten Klasse ist im Embryo hochreguliert, jedoch auch im asexuellen, adulten Tier signifikant vorhanden. Gene der zweiten Klasse sind ausschließlich embryonal exprimiert. Drei der exklusiv embryonalen Cluster wurden getestet: alle zeigten eine maternale Expression in weiblichen Polypen während der Oogenese. Eines dieser Cluster repräsentiert das bisher unbekannte Gen *HyEMB-1*. Seine Expression wurde in Blastomeren, nicht aber in den Endozyten früher Embryonen nachgewiesen. Im Kutikulastadium wird sie herunterreguliert. Des Weiteren wurde ein homologes Gen aus der Polycomb-Genngruppe, *embryonic ectoderm development (EED)*, gefunden, dessen Expression während der Embryogenese hochreguliert wird. Wie in anderen Modellen ist die *HyEED*-Expression charakteristisch für Zellen, die eine multipotente Differenzierungskapazität besitzen. In *Hydra* sind dies embryonale und

interstitielle Zellen. Mit *HyEED* ist der erste bisher bekannte genetische Marker für interstitielle Zellen in der Embryogenese gefunden, der ihren entodermalen Ursprung beweist.

Höchstwahrscheinlich benutzt *Hydra* konservierte Transkriptionsfaktoren, um die Embryonalentwicklung zu steuern. Die große Anzahl der im Zuge der Embryogenese differentiell exprimierter Gene macht jedoch deutlich, dass dieses in einem grundsätzlich anderen genetischen Kontext als im adulten Tier geschieht.

5. Materials

5.1. Organisms used

<i>Hydra</i> strain:	AEP
Feeding:	<i>Artemia salina</i> (Crustacea, Branchiopoda)
Bacterial strains:	<i>Escherichia coli</i> , strains XL-1 Blue and DH5- α

5.2. Media

<i>Artemia</i> medium	31,8 g sea salt per 1 l millipore water
<i>Hydra</i> medium (HM)	1 mM CaCl ₂ , 1 mM NaCl, 0,1 mM MgSO ₄ , 0,1 mM KCl, 1 mM Tris-HCl (pH 7,8)
Freezing medium	10 g Tryptone; 5 g Yeast extract; 20 g NaCl; 36 mM K ₂ HPO ₄ ; 13.2 mM KH ₂ PO ₄ ; 1.7 mM Na-citrate; 0.4 mM MgSO ₄ ; 6.8 mM (NH ₄) ₂ SO ₄ ; 4.4% Glycerol; millipore water up to 1 liter; 50 mg/ml ampicillin after autoclaving and cooling down 20 g of LB broth base can be used instead of tryptone, yeast extract and NaCl.
LB-amp medium	10 g Tryptone; 5 g Yeast extract; 5 g NaCl millipore water up to 1 liter; 50 mg/ml ampicillin after autoclaving and cooling down
LB -agar + ampicillin	10 g Tryptone; 5 g Yeast extract; 5 g NaCl; 15 g agar-agar, millipore water up to 1 liter; 50 mg/ml ampicillin after autoclaving and cooling down

SOB - medium	20 g Bacto-Trypton, 5 g Yeast extract, 0,58 g NaCl, 0,19 g KCl in 1 l Millipore water
SOC-medium	10 ml SOB-Medium, 100 µl 2 M Glucose (sterile-filtered), 100 µl sterile-filtered 2M Mg ²⁺ solution (20.33 g MgCl ₂ ·6H ₂ O; 24.65 g MgSO ₄ ·7H ₂ O/100 ml Millipore water)
Tissue freezing medium	Jung

5.3. Buffers and solutions

5.3.1. General purpose solutions

Ampicillin stock solution	50 mg/ml, stored at –20°C in 1ml aliquots
APS	10 % (w/v) in Millipore water
Denaturation solution	1.5 M NaCl, 0.4 M NaOH
Denhardt's (50 x)	1 % Polyvinylpyrrolidon, 1 % Ficoll, 1 % BSA Fraction V, sterile-filtered, stored at –20°C.
Depurination solution	0.1 M HCl
DNA gel loading buffer	50 % Glycerol, 10 mM EDTA pH 8, 0,1 % SDS, 0,025 % Bromphenol blue, 0,025 % Xylene cyanol
EDTA	0.5 M in Millipore water
Glycogen solution	35 mg/ml in Millipore water
Heparin	10 mg/ml in Millipore H ₂ O, stored at –20°C
Hybridization solution (Northern, Southern, Macroarrays)	50 % Formamide (v/v), 4,8 x SSC, 10 mM Tris-HCl pH 7,5, 1% SDS, 1 x Denhardt's, 10 % Dextran sulfate, 20 µg/ml sonicated salmon sperm DNA
Hybridization solution (Southern)	6xSSC, 0.1% SDS, 1 x Denhardt's, 5% Dextran sulfate, 20 µg/ml sonicated salmon sperm DNA

IPTG stock solution	200 mg/ml in Millipore water, stored at -20°C in 200µl aliquots
Lysis buffer (for gDNA isolation)	10 mM Tris-HCl pH 8, 150 mM EDTA pH 8, 0.5% SDS, after homogenization Proteinase K added to the final concentration of 100 µg/ml
Lysis buffer (for bacteria)	1.5 M NaCl, 0.4 M NaOH
Membrane stripping solution	0,5 % SDS (boiling)
Neutralization solution (Macroarrays, Southern blots)	1,5 M NaCl, 1 M Tris-base pH 7,5
NTMT	100 mM NaCl, 100 mM Tris pH 9,5, 50 mM MgCl ₂ , 0.1% Tween 20
RNA loading buffer	50% Glycerol, 1 mM EDTA pH 8,0, 0,25% Bromphenol blue, 0,25% Xylencyanol,
RNA gel running buffer	0.1 M MOPS pH 7, 40 mM Na-acetate, 5 mM EDTA pH 8
RNaseOut	Invitrogen
SDS stock solution	10 % (w/v) in Millipore water
Sephadex G - 50	5 g Sephadex G-50 in 100 ml 1xTE, autoclaved
SSC (20 x)	3 M NaCl, 0,3 M Na citrate, pH 7,0
TAE (50 x)	242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA, pH 8.0, water up to 1 liter
TBE (5 x)	54 g Tris base, 27,5 g boric acid, 20 ml 0,5 M EDTA, pH 8,0 water up to 1 liter
TBE sequencing buffer (10 x Long Run Buffer)	1,34 M Tris-Base, 450 mM boric acid, 25 mM EDTA, at 50 °C pH must be 8,3 - 8,7.
TE	10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0
Tris-HCl stock solutions	1 M Trish-HCl, pH 7.2; 7,5; 8.0; 9.5.
Washing solution 1 (for membranes)	2 x SSC, 0,1 % SDS
Washing solution 2 (for membranes)	0,2 x SSC, 0,1 % SDS
X – Gal stock solution	20 mg/ml X-Gal in Di-methyl-formamide, stored at -20°C in 500 µl aliquots

5.3.2. Solutions for *in situ* hybridization, antibody and TUNEL staining

Blocking solution	80 % MAB-B, 20 % heat-inactivated sheep serum (inactivation at 56 ⁰ for 30')
Glycine working solution	4 mg/ml in Millipore H ₂ O
Hybridization solution (prehybridization and hybridization)	50 % formamide, 5 x SSC, 0,1 % Tween 20, 0,1% CHAPS, 1 x Denhardt's, 100 µg/ml heparin, 100 µg/ml yeast tRNA
Hybridization solution for washes	50 % formamide, 5 x SSC, 0,1 % Tween 20, 0,1% CHAPS, 1 x Denhardt's, 100 µg/ml heparin
Lavdovsky's fixative	50% EtOH, 3,7%PFA, 4% acetic acid
MAB	100 mM maleic acid, 150 mM NaCl, pH 7,5
MAB-B	MAB /1 % BSA fraction V, stored frozen at -20 ⁰
MAB-T	MAB/0.1% Tween 20
Mowiol	6 g Glycerol, 2,4 g Mowiol 4-88, 6 ml Millipore H ₂ O leave for 2 hours at room temperature, then add 12 ml 0,2 M Tris pH 8,5 and incubate at 53°C until Mowiol is dissolved. Clarification of the solution by centrifugation (5000 rpm, 20 min). Stored at -20°C. Preheated to room temperature before use.
NTMT	100 mM NaCl, 100 mM Tris pH 9,5, 50 mM MgCl ₂ , 0,1 % Tween20
4% Paraformaldehyde (PFA)/HM	5 ml frozen aliquots of 4% PFA in hydra medium. To dissolve the powder 100 µl/10 ml of 10 M NaOH was added and the solution was heated to 55 ⁰ . After complete dissolving, the pH is adjusted to 7.2-7.4
4% Paraformaldehyde (PFA)/PBS	5 ml frozen aliquots of 4% PFA in PBS. To dissolve the powder 100 µl/10 ml of 10 M NaOH was added and the solution was heated to 55 ⁰ . After complete dissolving the pH is adjusted to 7.2-7.4

PBS (1x)	0,15 M NaCl, 0,08 M Na ₂ HPO ₄ , 0,021 M NaH ₂ PO ₄ , pH 7,4
PBT	PBS + 0,1 % Tween20
Proteinase K stock solution (100 x)	1 mg/ml Proteinase K in Millipore H ₂ O, stored in 50 µl aliquots at -70°C
Triethanolamine	0,1 M Triethanolamine, pH 0,1
Urethane stock solution	20 % (w/v) urethane in Millipore water

5.4. Kits

BD Advantage™ 2 PCR Enzyme System	BD Biosciences
Clontech PCR-Select™ cDNA Subtraction Kit	BD Biosciences
DIG RNA Labelling Kit (SP6/T7)	Roche
DYEnamic ET Dye Termination Cycle Sequencing Kit	Amersham Biosciences
Expand Long Template polymerase mix	Roche
First – Strand cDNA Synthesis Kit	Amersham Biosciences
GFX Micro Plasmid Prep Kit	Amersham Biosciences
High Pure Plasmid Isolation Kit	Roche
Megaprime™ DNA labelling Kit	Amersham Biosciences
NucleoSpin® Plasmid	Macherey-Nagel
Nucleo Trap® mRNA Mini Kit	Macherey-Nagel
pGEM®-T Vector System	Promega
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
QuickPrep micro mRNA Purification Kit	Amersham Biosciences
Ready-To-Go DNA Labelling Beads (-dCTP)	Amersham Biosciences
SequiTherm EXCEL™ II DNA Sequencing Kit	Epicentre
Superscript™ Double Strand cDNA Synthesis Kit	Invitrogen
TRizol® Reagent	Invitrogen

5.5. Enzymes (not from kits)

DNase 1	Amersham Pharmacia
Klenow fragment	Fermentas
Platinum Taq Polymerase	Invitrogen
Proteinase K	Sigma
RNase A	Sigma
Restriction endonucleases	Fermentas
SP6 RNA Polymerase	Roche
T7 RNA Polymerase	Roche
Taq DNA-Polymerase	Amersham
Terminal deoxinucleotidiltransferase	Amersham

5.6. Chemicals

Acetic acid (glacial)	Roth
Acetic anhydride	Sigma
Agarose	Sigma
Agar-agar	Roth
Ammonium acetate	Roth
Ammonium persulfate	Merck
Ampicillin	Merck
BM-Purple	Roche
Boric acid	Roth
Bromphenol blue	Fluka
Bovine serum albumin Fraction V	Roth
CHAPS	Roth
Chloroform	Fluka
DEPC (Diethyl pyrocarbonate)	Roth
Dextran sulfate	Roth
Dimethylformamide	Merck
DNA size standards (50 bp/100 bp/1kb ladders)	Fermentas
dATP (100mM)	Fermentas

dCTP (100mM)	Fermentas
dGTP (100mM)	Fermentas
dTTP (100mM)	Fermentas
Dig-dUTP	Amersham
EDTA	Roth
Ethanol	Roth
Ethidium bromide (50 mg/ml)	Roth
Euparal	Roth
Formaldehyde (37%)	Roth
Formamide	Roth
Glucose	Merck
Glycerol	Roth
HEPES	Sigma
Hydrochloric acid	Roth
IPTG	Sigma
Isopropanol	Roth
LB Broth Base	Life Technology
Levamisol	Roth
Lithium chloride	Roth
Magnesium chloride	Merck
Magnesium sulfate	Merck
Maleic acid	Sigma
Methanol	Roth
Mowiol 4-88	Calbiochem
MOPS	Roth
Paraformaldehyde	Sigma
Phenol	Roth
Poly-L-lysine	Sigma
Potassium chloride	Roth
RapidGel™-XL-40 % Concentrate	USB
Salmon sperm DNA 10 mg/ml (sonicated)	Stratagene
Sheep serum	Sigma
SDS (Sodium dodecylsulfate)	Roth
See salt (Ocean Zac plus)	Zoo Zajac, Duisburg

SEPHADEX G50 medium	Pharmacia
Sodium acetate	Roth
Sodium azide	Merck
Sodium chloride	Roth
Sodium citrate	Roth
di-Sodiumhydrogenphosphate	Roth
Sodium-di-hydrogenphosphate	Roth
Sodium hydroxide	Roth
Substrate solution for alkaline phosphatase (NBT/BCIP)	Roche
TEMED (N,N,N',N'-Tetraethylmethyldiamine)	Merck
Tetracyclin	Sigma
Triethanolamine	Sigma
Tris-base (Trihydroxymethylamonimethane)	Roth
tRNA from yeast (10mg/ml)	Sigma
Tryptic Soy Broth	Sigma
Trypton	Roth
Tween20	Merck
Urea	Roth
Urethan	Sigma
X-Gal	Sigma
Xylene cyanol	Fluka
Yeast extract	Roth

5.7. Radioactive substances

α -[³² P]-dATP	Amersham
α -[³² P]-dCTP	Amersham

5.8. Vectors

pGEM [®] -T	Promega
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5.9. Antibodies

Anti-DIG-AP Fab Fragment	Roche
Anti-H3di/tri-methyl K27	Dr. D. Reinberg
C 41	Prof. Dr. C.N. David
FITC-conjugated antimouse Ig G	Sigma

5.10. Primers and oligos

All the primers were ordered at the MWG Biotech if not mentioned otherwise

Standard primers:

AK Actin34	5' AAG CTC TTC CCT CGA GAA ATC 3'
AK Actin35	5' CCA AAA TAG ATC CTC CGA TCC 3'
GAPDH-F	5' GCCTTATGACAACCATTCAT 3'
GAPDH-R	5' TCAACAACAGAAACATCTGC 3'
SP6	5' ATT TAG GTG ACA CTA TAG AAT AC 3'
T7	5' TAA TAC GAC TCA CTA TAG GG 3'
T3	5' AAT TAA CCC TCA CTA AAG GG 3'
M13F	5' CCC AGT CAC GAC GTT GTA AAA CG 3'
M13R	5' AGC GGA TAA CAA TTT CAC ACA GG 3'

Sequencing primers:

SP6(IRD800)	5' ATT TAG GTG ACA CTA TAG AAT AC 3'
T7(IRD800)	5' TAA TAC GAC TCA CTA TAG GG 3'
M13R(IRD800)	5' AGC GGA TAA CAA TTT CAC ACA GG 3'
M13F(IRD800)	

SSH - Primer:

SSH_PCR primer 1	5' CTA ATA CGA CTC ACT ATA GGG C 3'
SSH_Nested PCR p.1	5' TCG AGC GGC CGC CCG GGC AGG T 3'
SSH_Nested PCR p.2	5' AGC GTG GTC GCG GCC GAG GT 3'

SSH_cDNA synthesis p. 5' TTT TGT ACA AGC TT₃₀N₁ N 3'

3' und 5' RACE PCR:

Splinkerette (Top strand) 5' CGAATCGTAACCGTTCGTACGAGAATTCGTA-
CGAGAATCGCTGTCCTCTCCAACGAGCCAAGG 3'

Splinkerette (Bottom blunt) 5' CCTTGGCTCGTTTTTTTTTGCAAAAA 3'

cb_SplOutPri_2 5' GAATCGTAACCGTTCGTACGAG 3'

cb_SplInnPri_2 5' CGTACGAGAATCGCTGTCCTC 3'

st_SplOutPri_3 5' GAA TCG TAA CCG TTC GTA CG 3'

st_SplInnPri_3 5' TAC GAG AAT CGC TGT CCT C 3'

Not1 5' AAC TGG AAG AAT TCG CGG CCG CAG GAA T18 3'

3'RACE 5' GAC TCG AGT CGA CAT CGA T₁₇ 3'

Adapter 5' GAC TCG AGT CGA CAT CGA 3'

Specific primers

EEDstopR 5' ATTATGTCTGCTTGTCCCATCTCCAT 3'

EEDstartF 5' ATGGATACTGTTAATGATTTTATCGACG 3'

UK-EED5'Rin 5' ACCAGATGTCGCACAAATCAATATCA 3'

UK-EED3'Rin 5' ATCTTGTGACAATCAAGTTGTATGTTGG 3'

UK-EED3'Out 5' AGGTCTCGAGAAGTTCATCGTAATTA 3'

UK-EED5'Out 5' ATCGTTGGTTTGGCATGGGCA 3'

EED-F(306) 5' ACAAATGTGCTGAAGATTCTGGAC 3'

EEDgapR1 (Sigma ARK) 5' AGCAACTTGACGTATACATGG 3'

EEDgapR2 (Sigma ARK) 5' ATCTGGATCATATGTCCATGA 3'

UK-DegEED1 5' AGATCGAAGGA(AT)(CT)G(AG)GCC(AGCT)(AGCT) 3'

EEDFULLR2(35) 5' ATTCCGGCCGCGAATTCTTCCCT 3'

EEDFULLR1(95) 5' ACTTCCTATTAGCATGTATTATCTAGC 3'

HYEED-1R(319) 5' ATCATCTTCTCCAATTAAGCATTGTTGG 3'

HyEED2-3F 5' GTCTCCTCTAGTTCATCAACTTCC 3'

HyEED3R 5' GATAACGTTGAGCGTTAGGACGT 3'

HyEED-2-R 5' TTGTAAACTGAAACCCACACTGCT 3'

2E4R-2(63) 5' GACCTTTACTATTGTTACTGATAGTTGAC 3'

2E4-F(44) 5' ATCCTCGGCTGAATTTGTAAGT 3'

2E4-R(71)	5' AGTAGCAGTTACAAATTCAGCCGA 3'
2E4-R(136)	5' ATTCTGCTTCCTGGCGATATGC 3'
2E4-R(182)	5' ACACATTACATCATCGTGTTTGTCTC 3'
2E4-5'2	5' CAGAAGCAATAACAATAGTCCCG 3'
2E4-5'3	5' CCGTAAATAATTGACATTCGACCAG 3'
CI12F	5' CTTCAATTGATCCTCCGACAT 3'
CI12R	5' AGTGTTTGCTTGCAAGTCCAC 3'
CI14F	5' AGTATAGCCGAATTACCAACTAAT 3'
CI14R	5' GGTTTTGTATCGTATCGACGT 3'
CI27F	5' TAGGTAAACCAGCTGGTGGA 3'
CI27R	5' CGTTTTGAAGATGAAACAGTGG 3'
GGCI3con1_F1	5' AATGCAATCATCAATGTCTCCACCG 3'
GGCI3con2_R	5' ATCGATTGGATGTAGGGGATTAACTATG 3'
GGCI41F	5' GAAGATGTAACTCCGATGATAAATAAC 3'
GGCI41R	5' ACAGATTTTATTGCTCATATAGGAATCC 3'
GGCI60F	5' GGAATAAGCATTGACATGTATTC 3'
GGCI60R	5' AGACATTCAGAGCACATGAAGA 3
GGCI6con1-2_5'1	5' AGCATATTGAAGTGACCCTTCGTAG 3'
GGCI6con3-5'1	5' AGCAATCTGAAGTGATCCGTCAAAC 3'
2e4_5'RACE1	5' ATGAAACTACTACTGGTCTGAATGTC 3'
2e4_3'RACE1	5' ACCAGTCAAATCTTTCCTCGGC 3'
2E4F	5' ACATAATATTCACATGATCGTCTTTA 3'
2E4R	5'ACATTCTGTACACAAAGTAATTACA 3'
6A8-F	5' ATTTGACGACTACATGTGTCATG 3'
6A8-R	5' AAGTGACCCTTCGTAGTCCT 3'
1M16-F	5' ACGACTACATGTGTCATGAAAC 3'
1M16-R	5' ACAAGTGCTTTCAGCATATTGAAG 3'
6C15-R	5' ATCAAGAAGCTTGAAGCTTTCAGT 3'
6C15-F	5' ACGACTTTGCTATCAAACAAACCT 3'
2D3-F	5' ATCGTAAACGGAAAGTACGTTTTG 3'
2D3-R	5' TAGGTAAACCAGCTGGTGGA 3'
2O1-F	5' AGATGCTCATGCCCTCAGTAT 3'
2O1-R	5' GGGAAACGAATGAGATTTGTTTTC 3'
3C1-F	5' AGTTCATAGCCTAATCTAATTTGATC 3'

3C1-R	5' ATTTCTTACCCAAAATCGGAACG 3'
Equin-F	5' GAAACTTTTTAGTCCCTGTGTAAT 3'
Equin-R	5' ATTTCTGCTCTAGTAGGATATTTA 3'
1E16F	5' AGTGTTTGCTTGCAGTCCACCA 3'
1E16R	5' ATCCCCTCCATAATAATCAACCC 3'
2K21F	5' AATTTGAATAATTTTTGAAAAATGACTTAC 3'
2K21R	5' ACTATTTGTATCGAGCTATACTAA 3'
4P15F	5' AGTATAGCCGAATTACCAACTAAT 3'
4P15R	5' ATGTCTCTGATAAAACATATTCTGC 3'
2M1F	5' ACATAATATTCACATGATCGTCTTTAA 3'
2M1R	5' ACACAAAGTAATTACAGACCTGTG 3'
6F2R	5' ATACAATTAGGTAAATCCCCTCC 3'
6F2F	5' AGTGTTTGCTTGCAGTCCAC 3'
4d24-R1	5' TCTTCTTGACTACCGCCAGCT 3'
4D24-F	5' ATAGTAGCTTCTTCTAAAGATAGTAAAC 3'
3O16F	5' AGTACTGCTACATAAATTTGTGAATAC 3'
3O16-R	5' AGCGTGACAGCACTCTTTG 3'
4C24-F	5' ACTATGCTACTTCTTCAAAAATGTGC 3'
1G2-F	5' ACTCTATAAGCAGGAGATCCAG 3'
1G2-R	5' ACGGTCTTGTCACAGAGCAC 3'
1G5-F	5' ACGCTTGCAATTCTTATATTCTATC 3'
1G5-R	5' ATCGTAGTAGAAACGTCCCTA 3'
bet-hom-metR	5' CCTGCTTTAACGTAACCACG 3'
bet-hom-metF	5' GTCCACTTTTTATTCTCGTGCA 3'
4C24-R	5' AGTCCTGTTCACTGATAGTAGTG 3'

5.11. Devices and machines

PCR machines

Cyclone gradient	peqLab
i-Cycler 96	Biorad
Primus 96 plus	MWG-Biotech

Primus 25
PTC-255 Peltier Thermal Cycler

MWG-Biotech
MJ Research

Power supplies

EPS 3500
LNG 350-06
PS 250

Amersham Pharmacia Biotech
Heinzinger
HYBAID

Gel electrophoresis chambers

EasyCast minigel system B1A
EasyCast minigel system B2
Multiphor 2

Owl Separation Systems
Owl Separation Systems
Amersham Pharmacia Biotech

Incubators and shakers

Thermoshaker Certomat H
HIS25
Thermoincubator
KS10 (Rotary shaker)
Mini 10
Thermomixer Compact

B.BraunBiotech
Grant BOEKEL
Heraeus Instruments
Edmund Bühler
Thermo Hybaid
Eppendorf

UV transilluminators

ImaGo
UV-Stratalinker 1800
UV-Kontaktlampe Chroma 43

B+L Systems
Stratagene
Vetter GMBH

Electroporation devices

Gene Pulser II
Pulse Controller II

Biorad
Biorad

Centrifuges

Biofuge 13
Centrifuge 5415 D
Centrifuge 5417 R

Heraeus
Eppendorf
Eppendorf

Minifuge RF	Heraeus
Labofuge 1	Heraeus
Multifuge 3 S-R	Heraeus

Microscopy

AxioCam	Zeiss
Axioskop 2	Zeiss

Other machines

BioPhotometer	Eppendorf
Eraser (erasing phosphoimager screens)	Raytest
Gene Quant Photometer	Pharmacia Biotech
Kryotome	Leica
LaminAir HB2448 (model Firefly)	Heraeus Instruments
LI-COR DNA Gene Analyzer Gene Read IR 4200	MWG Biotech
MegaBACE 500	Amersham Biosciences
Milli-Q Academic System	Millipore
NEN Global IR ² DNA Analyzer	LI-COR
Phosphoimager FLA-5000	FUJI
pH-meter pH 211	Hanna Instruments
QFill 2	Genetix
QPix	Genetix
Ultramicrotome	LKB
VARIOKLAV sterilizer Type 400 E	H+P Labortechnik GmbH
Vortex Genie 2	Scientific Industries
Wallac WinSpectral	PerkinElmer

5.12. Other materials

96 well microtitre plates	Genetix
384 well microtitre plates	Genetix
Chromatography paper 3MM	Whatman
Hybond N Nylon membrane	Amersham Biosciences
Hybond N ⁺ Nylon membrane	Amersham Biosciences

Microcon YM 50 columns	Microcon
Phosphoimaging plates	Fuji
Plasticware	Sarstedt
QTrays 20 cm x 20 cm	Genetix
X-Ray film (BioMax MS)	KODAK

5.13. Computer programs

ESTs clustering and sequence cleaning

TGICL EST clustering package	TIGR (http://www.seqtools.dk)
SEQ CLEAN	TIGR (http://www.seqtools.dk)

cDNA Macroarray analysis software:

AIDA Image Analyzer v3.2	(http://www.raytest.de)
AIDA Array Compare v3.2	(http://www.raytest.de)

Text editing

MS Word 2000

Image editing

Adobe Photoshop 7.0

Quantitative data analysis

MS Excel 2000

Software for scintillation counter

Wallac 1414 WinSpectral v1,30 (PerkinElmer) Cerenkov protocol

Sequence analysis software

DNASIS v2.5
ESeq LICOR v2.0
MEGABACE Sequence Analyzer software (Molecular Dynamics)
SEQtools v8.0xxx (http://www.seqtools.dk)

Sequence analysis in Internet

EST and Genome projects:

Hydra magnipapillata:

Database of Japan: <http://www.ddbj.nig.ac.jp/>

EMBL: <http://www1.embl-heidelberg.de/>

Genome Sequencing Center St. Louis: <http://www.genome.wustl.edu/EST/index.php>

Sputnik (MIPS): <http://mips.gsf.de/proj/sputnik/hydra/>

Caenorhabditis elegans:

Sanger Center: http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml

Worm base: <http://www.wormbase.org/db/searches/blat>

NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/Genome/NematodeBlast.html>

Drosophila melanogaster:

Fly Base: <http://www.fruitfly.org/>

NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/Genome/FlyBlast.html>

Strongylocentrotus purpuratus:

<http://sugp.caltech.edu/sequence/blast.psp>

Ciona intestinalis:

<http://ghost.zool.kyoto-u.ac.jp/indexr1.html>

<http://genome.jgi-psf.org/ciona4/ciona4.home.html>

Fugu rubripes:

<http://fugu.hgmp.mrc.ac.uk/>

<http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html>

Danio rerio:

<http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html>

Gallus gallus:

<http://www.chick.umist.ac.uk/>

Mus musculus:

<http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html>

BLAST searches

NCBI: <http://www.ncbi.nlm.nih.gov/blast/>

Protein domain search

SMART: <http://smart.embl-heidelberg.de/>

SignalP V2.0: <http://www.cbs.dtu.dk/services/SignalP-2.0/>

TMpred: http://www.ch.embnet.org/software/TMPRED_form.html

6. Methods

6.1. Animals and culture conditions.

All the experiments were performed with *Hydra* polyps of the AEP strain belonging to the *vulgaris* group. The strain was obtained as a result of the crosses of several American wild strains. CA7 was collected in 1980 in the basin in the vicinity of Lake Tahoe (California, USA), PA1 was collected on the same year in Philadelphia and, finally PA2 was collected in 1994 also in Philadelphia. Then the following crosses were made:

1.
P) PA1 x CA7
F1) Polyps named A, E and G

2.
P) A4 x E2
F2) Polyps named AE

3.
P) AE20 x PA2
F3) AEP

AEP animals are identical to *H. vulgaris* in general morphology and structure of nematocytes. At the same time they are not identical to *H. vulgaris* on molecular level. RAPD analysis and also hybridization analysis with probes against *PPOD* family peroxidases demonstrate significant differences (Kuznetsov, 2002; Thomsen unpublished data).

Asexual animals were kept in artificial hydra medium (HM) at 18⁰ and fed daily with freshly hatched brine shrimp *Artemia salina* nauplii, which were obtained by overnight incubation of two tee spoons of *Artemia* eggs in 500 ml aerated bottles with salt water. Prior to feeding, the nauplii were washed with tap water to avoid changing the salt concentration in the *Hydra* culture dishes.

Oogenesis was induced by starving daily fed polyps for five days and subsequent feeding twice a week.

Prior to DNA or RNA isolation the animals were starved for 48 hours, to avoid contamination of the preparations with brine shrimp nucleic acids.

6.2. Genomic DNA extraction

200 *Hydra* polyps were placed into the eppendorf tube and homogenized in 0.5 ml of lysis buffer (10 mM Tris-HCl pH 8, 150 mM EDTA pH 8, 0.5% SDS) then proteinase K was added to the homogenate to the final of 100 µg/ml and digestion was allowed to proceed for 1 hour at 50⁰. Equal volume of phenol was then added, the sample was shaken for several seconds and centrifuged for 15' at 12000 g. The supernatant was removed and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to it. The sample was again shaken and centrifuged for 15' and supernatant was removed. Equal volume of chloroform/isoamyl alcohol (24:1) was added and one more round of protein extraction was performed. If after the third centrifugation the interphase was still visible, the last extraction step was repeated. After the final centrifugation, the upper phase was removed, NaCl was added to it to the final concentration of 0.5 M and DNA was precipitated with a double volume of ice-cold 99% ethanol (EtOH). Thread-like high molecular weight DNA was removed with a pipette tip, washed in 70% EtOH, air-dried and dissolved in 100 mM Tris-HCl pH 8. RNaseA was then added to the final concentration of 50 µg/ml and RNA digestion was carried out for 30' at 37⁰. Afterwards proteinase K was added to the final of 50 µg/ml and the sample was incubated at 37⁰ for 15' more. Then protein extraction was performed as described above with centrifugation times of 10' instead of 15'. Finally, pure high molecular weight DNA was precipitated with ethanol, washed in 70⁰ ethanol, air-dried and redissolved in 10 mM Tris-HCl pH 8. The quality of DNA was analyzed on 1% agarose gel and DNA concentration and purity were determined photometrically.

6.3. Total RNA extraction and mRNA extraction.

Total RNA of Hydra was isolated with TRIzol[®] Reagent according to the manufacturer's protocol. 200 polyps were taken, normally, for one preparation. PolyA⁺ mRNA was extracted from total RNA or directly from tissue using QuickPrep[™]Micro mRNA Purification Kit (Amersham) or Nucleo Trap[®] mRNA Mini Kit (Macherey-Nagel) according to the provided protocols.

6.4. cDNA synthesis by reverse transcription

First strand cDNA synthesis was performed with the use of the First Strand cDNA Synthesis Kit (Amersham) with oligo-dT primer according to the manufacturers protocol. First strand cDNA was used for 3' RACE PCR and for RT PCR. For 5' RACE PCR double stranded cDNA was produced as follows:

Component	Amount
First strand cDNA reaction product	20 μ l
DEPC-treated water	91 μ l
10 mM dNTP mix	3 μ l
5x Second strand buffer	30 μ l
<i>E. coli</i> DNA ligase (10 U/ μ l)	1 μ l
<i>E. coli</i> DNA polymerase I (10 U/ μ l)	4 μ l
<i>E. coli</i> RNAse H (2 U/ μ l)	1 μ l

The components were gently mixed and incubated at 16⁰ for 2 hours. Then 2 μ l of 5 U/ μ l T4 DNA polymerase were added to blunt the ends of the ds cDNA and the incubation was continued for 5 more minutes. The tube was then placed on ice and 10 μ l of 0.5M EDTA pH 8 was added to stop the reaction. Afterwards 160 μ l of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tube, the content was vortexed thoroughly and centrifuged at room temperature for 5' at 14000 g to separate the phases. 140 μ l of the aqueous phase was removed, 70 μ l of 7.5 M ammonium

acetate was added, followed by 0.5 ml ice-cold absolute ethanol. Precipitated ds cDNA was centrifuged for 20' at room temperature at 14000 g. The pellet was then washed with 70% EtOH, air-dried and dissolved in 50 µl of water.

6.5. Ligation of splinkerettes

If ds cDNA was used for 5' RACE PCR, splinkerette adaptors (Devon et al., 1995) were ligated to it (Fig. 6.1). Splinkerette is a ds DNA molecule consisting of the top strand and a bottom strand, which, in its 3' region, is complementary to the top strand, and in its 5' region is forming a hairpin structure. Such an adaptor increases the specificity of the 5' RACE PCR, because splinkerette outer nested primer (blue triangle on Fig. 6.1 B) does not have an annealing site before the complementary strand for the top strand is built in the first PCR cycle starting from the reverse specific outer nested primer (black triangle on Fig. 6.1 B).

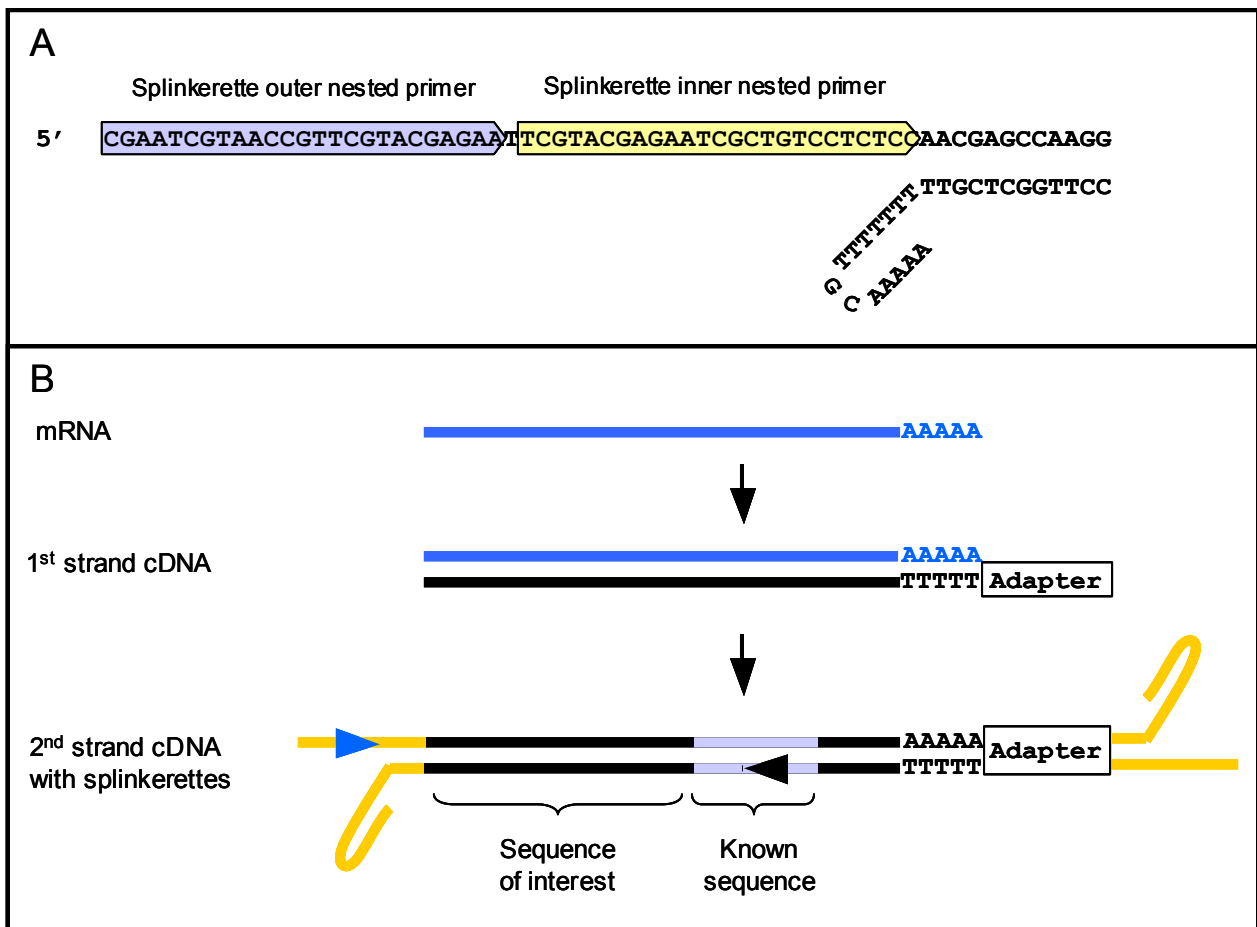


Fig. 6.1 Schematic representation of the splinkerette (A) and of the process of cDNA synthesis and splinkerette ligation (B)

Top and bottom splinkerette strands were mixed together in equimolar quantities, heated in the PCR machine to 94⁰ for 3 minutes, then incubated at 50⁰ for 5 minutes and, finally, allowed to slowly cool down to room temperature together with the PCR block. Then ligation reaction was set up as follows:

Component	Amount
ds cDNA	34 μ l
10x ligation buffer	5 μ l
PEG 4000 50% w/v	5 μ l
T4 ligase (2 U/ μ l)	3 μ l
Annealed splinkerette (50 pmol/ μ l)	3 μ l

Ligation was carried out for 36 hours at 16⁰. Afterwards the ligase was inactivated by heating for 10' at 65⁰ and unligated splinkerettes were removed by filtration through the Microcon YM 50 column as described in the manual.

6.6. Polymerase chain reaction

PCR (Saiki et al., 1985) was used in multiple applications in the course of this study. In every individual case the method was modified according to the goal of the experiment.

6.6.1. Fragment amplification

For the amplification of the fragments of known genes the pipetting scheme was as shown below:

Component	Amount
DNA solution	1 μ l
10x PCR buffer with MgCl ₂	2 μ l
dNTP (10 mM)	0.3 μ l

<i>Taq</i> DNA polymerase (5 U/ μ l)	0.1 μ l
Forward primer (10 pmol/ μ l)	1 μ l
Reverse primer (10 pmol/ μ l)	1 μ l
Water	14.6 μ l

The PCR program was the following:

Initial denaturation	95 ⁰	3'	
Denaturation	94 ⁰	30''	40 cycles
Primer annealing	Ta=Tm-1 ⁰	30''	
Polymerization*	72 ⁰	60''	
Final extension	72 ⁰	3'	
Final hold	4 ⁰		

* Polymerization time was chosen depending on the size of the template DNA with a general assumption that the polymerase works at 1 kb/min rate.

6.6.2. RT PCR

This semi-quantitative method was used to evaluate expression level of genes on different developmental stages. First, the concentrations of two or more ss cDNA samples were equilibrated with primers against *β -actin* (19 PCR cycles) and GAPDH (27 PCR cycles). Actin- and GAPDH-equilibrated cDNAs were then used for PCR with the primers against the gene of interest. Water control (no template cDNA) and equilibration control (GAPDH or actin primers) were included in every case. The results of the PCR were analyzed on the 1-2% agarose gel.

6.6.3. 3' RACE PCR

To obtain parts of the unknown sequence lying downstream from the known piece of the gene, 3' RACE PCR was used. In this case two nested forward primers

were generated against the known sequence. Adaptor primer, representing the 5' part of the oligo-dT primer used for reverse transcription (Fig. 6.1) was taken as a reverse primer. Then a semi-nested PCR was performed. In the first reaction the outer specific primer and the adaptor primer were used, then the PCR product was diluted 1:100 and 1 μ l of the dilution was subjected to the second PCR, in which the inner specific primer and adaptor primer were used. To increase the specificity, hot start Platinum *Taq* polymerase was used for the first reaction. The pipetting scheme for the first reaction is presented below:

Component	Amount
DNA solution	1 μ l
10x PCR buffer	2 μ l
MgCl ₂ (50 mM)	0.6 μ l
DNTP (10 mM)	0.4 μ l
Platinum <i>Taq</i> DNA polymerase (5 U/ μ l)	0.1 μ l
Forward outer specific primer (10 pmol/ μ l)	1 μ l
Adapter primer (10 pmol/ μ l)	1 μ l
Water	13.9 μ l

The PCR program for the first reaction was the following:

Initial denaturation	95 ⁰	3'	
Denaturation	94 ⁰	30''	2x10 cycles
Primer annealing*	T _a , $\Delta T = -0.5^0$	30''	
Polymerization	72 ⁰	60''	
Denaturation	94 ⁰	30''	20 cycles
Primer annealing	T _a	30''	
Polymerization	72 ⁰	60''	
Final extension	72 ⁰	3'	
Final hold	4 ⁰		

* Here the “touch-down” principle was applied. The T_a of the first cycle was selected as T_m+4° , then in every following cycle T_a was decreased for the value of 0.5° , covering the span of 5° in 10 cycles. This allows to select the most stringent primer annealing temperature and to create statistic preference of the specific PCR products over the unspecific ones. The second part of the program was the same as standard fragment amplification PCR.

In the second reaction diluted PCR product of the first PCR was subjected to the fragment amplification program with 40 amplification cycles.

6.6.4. 5' RACE PCR

To obtain fragments of the sequence located upstream from the known sequence, 5' RACE PCR was used. Double stranded cDNA with splinkerettes was taken as a template, and a nested hot start touch-down PCR reaction was performed with splinkerette outer primer and specific reverse outer primer. The pipetting scheme and PCR program were essentially the same as for the first reaction of the 3' RACE PCR, except for the use of Expand Long Template polymerase mix (Roche) in several cases, when no distinct PCR fragment could be received otherwise. The product of the first reaction was diluted 1:100 and $1\ \mu\text{l}$ was used for the second PCR with splinkerette inner primer and specific reverse inner primer. As in the case of 3' RACE, the second PCR is a simple fragment amplification.

6.6.5. Degenerate primer PCR

To obtain a fragment of cDNA between the known part of the *HyEED* gene, which was cloned in the course of subtractive hybridization, and the phylogenetically conserved part upstream to it, degenerate primer was synthesized based on the sequence of mouse and human homologues of *HyEED*. Amplification was performed with the use of touch-down program, same as for the first reaction of the RACE PCR.

6.7. Southern blot hybridization

For Southern blotting genomic DNA of *Hydra* polyps was digested with different restriction enzymes. In every case appropriate buffer system (Fermentas) was used.

The pipetting scheme for the restriction was the following:

Component	Amount
Genomic DNA	20 μ g
10x Buffer with BSA	5 μ l
Enzyme (10 U/ μ l)	4 μ l
Millipore water	To the final of 50 μ l

Digestion was performed at 37⁰ for 4 hours, afterwards the enzymes were heat-inactivated (if possible), applied onto the 1% agarose gel in 1x TAE buffer and separated in 1x TAE in the 3 V/cm electric field. In every case 20 μ g DNA was used per lane. The gel was then photographed and blotted onto the Hybond N⁺ nylon membrane.

Prior to blotting the gel was treated for 10-15' with depurination solution (0.1N HCl) until the bromphenol blue dye turned completely yellow, then rinsed with distilled water and incubated with moderate agitation in denaturation solution (0.4 N NaOH, 1.5 M NaCl) for 30' until bromphenol blue dye returned to its blue colour. Then the gel was rinsed with water and washed for 30' in neutralization solution (1.5 M NaCl/0.5 M Tris-HCL pH 7.2). After neutralization, the DNA was capillary transferred overnight onto the nylon membrane with 20x SSC as a transfer agent, the membrane was air-dried and DNA was UV-crosslinked to the membrane.

Before hybridization the membrane was rinsed in 2x SSC, then prehybridized at 60⁰ for 1-2 hours in hybridization solution (6x SSC, 0.1% SDS, 5% dextran sulfate, 1x Denhardt's, 20 μ g/ml sonicated salmon sperm DNA). During this time 50 ng of the gel-purified PCR product was labeled with [α P³²]-dCTP.

The pipetting scheme for the labeling was the following:

Component	Amount
Purified and denatured fragment	50 ng
10x Klenow fragment buffer	5 μ l
Klenow fragment (2 U/ μ l)	2 μ l
10 mM mix of dATP, dGTP and dTTP	1.5 μ l
[α P ³²]-dCTP	3 μ l
Random hexamere primer 0.2 μ g/ μ l or specific primers 10 pmol/ μ l	5 μ l 3 μ l each
Millipore water	To the final of 50 μ l

Reaction was allowed to proceed for 30-60', afterwards the product was purified on a Sephadex G-50 medium column, made by filling the 1ml plastic syringe with equilibrated in TE buffer, autoclaved Sephadex G50-medium. Incorporation rate was measured with a liquid scintillation counter according to Cerenkov protocol. The probe was then denatured and added into the hybridization solution to the final concentration of 2×10^6 Cpm/ml.

The blots were hybridized overnight and then washed at a 60^o first shortly with 2xSSC/0.1% SDS and then 2x30' 0.2xSSC/0.1% SDS. Wet membranes were then sealed into plastic bags and exposed either with a PhosphorImager screen at room temperature or with an X-ray film at -80^o.

6.8. Northern blot hybridization

For Northern blot 30 μ g of total RNA or 0.5 - 1 μ g of poly-A⁺ messenger RNA was used per lane. Prior to gel electrophoresis, the gel chamber was treated for 30' with 0.5% SDS, then rinsed with 1M NaOH and washed with Millipore water. Formaldehyde-containing gel was made according to the standard procedure (Sambrook et al., 1989) by mixing 0.5 g of agarose melted in 31.5 ml of water, 9 ml of 37% formaldehyde and 10 ml of 5x Running buffer (0.1 M MOPS pH 7, 40 mM Na-acetate, 5 mM EDTA pH 8).

After polymerization the gel was prerun for 5' in the 1x Running Buffer. Sample RNA was mixed with the following components:

Component	Amount
Sample RNA	30 μ g in 4.5 μ l
5x MOPS Running buffer	2 μ l
37% formaldehyde	3.5 μ l
Formamide	10 μ l

The mixture was heat-denatured for 10' at 70⁰, chilled on ice, mixed with the 10x RNA loading buffer (50% glycerol, 1mM EDTA pH 8, 0.25% xylenecyanol, 0.25% Bromphenol blue) and applied onto the gel. The samples were run at 3 V/cm in 1x RNA gel running buffer. The marker lane was then cut from the gel, stained with 0.5 μ g/ml ethidium bromide in 0.1M ammonium acetate, destained in water and photographed. After that capillary transfer with 20x SSC was carried out. The blotted RNA was UV-crosslinked to the dried membrane, the membrane was soaked in 2x SSC and prehybridized for 1-2 hours in hybridization solution (50% formamide, 4.8x SSC, 10 mM Tris-HCl pH 7.5, 1% SDS, 1x Denhardt's, 10% dextran sulfate, 20 μ g/ml sonicated salmon sperm DNA) at 42⁰. Probe labeling with [α P³²]-dCTP was carried out the same way as described above. Hybridization was performed overnight at 42⁰, then the blots were washed at 60⁰ as described for Southern blot hybridization, and exposed to the PhosphorImager screen or X-ray film. Fragment size determination was carried out based on the position of ribosomal bands or marker bands with the use of calibration curve.

6.9. Cloning and sequencing

6.9.1. AT-ligation

Taq polymerase used in polymerase chain reaction produces A-overhangs on the 3' ends of the PCR fragments. This allows easy ligation into a linearized vector with 3'-T overhangs. Half of the PCR product was analyzed on an agarose gel, then, if there

was a single strong band, the second half of the PCR reaction was cleaned from remaining nucleotides and primers with PCR Purification Kit (Qiagen) as described in the manufacturers protocol. 1 μ l of the eluted DNA was used for ligation. If the PCR resulted in several bands, the products of the correct size were excised and purified from the gel with the use of the Gel Extraction Kit (Qiagen) according to the provided manual. After gel extraction, the sample was incubated for 5' at 72⁰ in 1xPCR buffer with *Taq* polymerase and dNTPs, allowing the enzyme to A-tail the PCR product. This procedure proved to be necessary, as the gel extraction procedure seemed to remove A-overhangs causing very low transformation rates. After A-tailing, the sample was once again cleaned with PCR Purification Kit, and 1 μ l of the eluted DNA was used for ligation. In this study pGEM-T vector system (Promega) was used for AT-cloning. The ligation was carried out as proposed by the manufacturer with minor changes (i.e. the volume of ligation reaction was scaled down by a factor of 2). After ligation, the T4-ligase was heat-inactivated for 10' at 65⁰. This step proved to increase significantly the number of transformants.

6.9.2. Competent cells

XL1-blue and DH5- α *Escherichia coli* cells were used. For preparation of competent cells, a single colony of *E. coli* was grown overnight in 3 ml SOB medium containing 12 μ g/ml tetracyclin at 37⁰. This culture was used to inoculate 50 ml of SOB, which was incubated for several more hours until the logarithmic growth phase was reached (OD₆₀₀=0.5-0.6). Bacteria were then harvested by centrifugation for 10' at 3000 g, the pellet was resuspended in 50 ml of ice-cold sterile ddH₂O, centrifuged again, washed in 25 ml ddH₂O, then in 2 ml 10% glycerol. After the final centrifugation step the cells were resuspended in 200 μ l 10% glycerol and frozen at -80⁰ in 40 μ l aliquots.

6.9.3. Electroporation and checking for the presence of the insert

The *E. coli* cells were thawed on ice, then 1 μ l of the heat-inactivated ligation mix was mixed with the bacteria and electric pulse was applied (25 μ F, 200 Ω , 1.8 kV).

Electroporated cells were mixed with 960 μl of SOC medium and allowed to recover for 1 hour at 37⁰. After that 100 μl of the liquid bacterial culture was plated onto the LB-agar plates with 50 $\mu\text{g/ml}$ ampicillin and incubated overnight. For blue/white selection of the insert-containing clones, 40 μl of 50 mg/ml X-Gal and 4 μl of 200 mg/ml IPTG were plated prior to plating the bacteria. White colonies were then picked with a sterile pipette tips and streaked onto fresh plates. At the same time, the tips were used to inoculate PCR reactions for colony check PCR. Colony check PCR is, in essence, a standard fragment amplification PCR, in which bacterial cells bearing plasmids are introduced into the reaction mix instead of template DNA solution. Normally, primer pairs against vector sequence were used (SP6-T7 or M13forward-M13reverse).

6.9.4. Plasmid purification

The clones carrying plasmids with inserts of correct size were taken for plasmid isolation. 4 ml of the overnight culture of each clone was taken per preparation. Plasmids were isolated with the use of High Pure Plasmid Isolation Kit (Roche) according to the provided protocol.

6.9.5. Sequencing with Li-COR

DNA sequencing with the LiCOR system was carried out using the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies). This sequencing protocol is based on the stochastic termination of the DNA polymerization in a PCR with a single primer upon inclusion of the di-deoxynucleotide instead of deoxynucleotide (Sanger et al., 1977). The sequencing reaction and amplification program were set up according to the manufacturer's protocol. Approximately 1 μg of plasmid DNA was used for the sequencing PCR with 5'-IRD800 labeled primer. Separation of the samples in the 6% polyacrylamide gel (14 ml millipore water, 3.75 ml 40% Rapid Gel XL solution, 2.5 ml 10x TBE long run buffer, 10.5 g urea, 38 μl TEMED, 175 μl ammonium persulfate) and sequencing data collection was performed on LI-COR DNA Gene Analyzer Gene Read

IR 4200 (MWG Biotech). Sequence assembly was performed with the use of E-Seq software provided with the sequencer.

6.9.6. Sequencing with MEGABACE 500

MEGABACE 500 capillary sequencer is a high throughput machine using the same Sanger termination sequencing principle as the LiCOR system. The difference between these two methods is that instead of 4 reactions with different terminators and labeling products by attachment of fluorochrome to the primer, differentially labeled terminator nucleotides are used in capillary sequencing PCR reaction, allowing it to run in one tube. Sequencing reaction and PCR program were performed as described in the protocol for the DYEnamic™ ET Terminator Kit/Cycle Sequencing Kit (Amersham). In average, 200 ng of plasmid was taken for the sequencing PCR. After the PCR, 1 µl of 7.5 M ammonium acetate was added to the 10 µl PCR reaction, the product was precipitated with 30 µl of 99% ethanol and the pellet was washed with 100 µl of 70° ethanol. All centrifugation steps (40' each) were carried out at 4° with the speed of 4500 rpm. After pellet was washed, ethanol was discarded, DNA was air-dried and dissolved in 20 µl of Millipore water. Then the samples were sequenced and data analyzed with MEGABACE Sequence Analyzer software.

6.10. Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization (SSH) is a PCR-based method for selective enrichment of one cDNA pool (tester) with templates not present in another (driver), invented by Diatchenko et al., (1996). Tester and driver double stranded cDNA (ds cDNA) are synthesized from mRNA preparations from two developmental stages to be compared. Tester ds cDNA is digested with a blunt end producing restriction enzyme (in our case Rsa I), subdivided into two portions, and each is ligated with a different dephosphorylated adaptor. This insures that only one strand of adapter is actually covalently bound to the 5' of the DNA. Adaptors have sequence motifs to allow annealing of two nested primers, when recessed termini are filled in.

Two hybridizations are then preformed. In the first, an excess of driver cDNA is added to each sample of the tester. The samples are then heat-denatured and

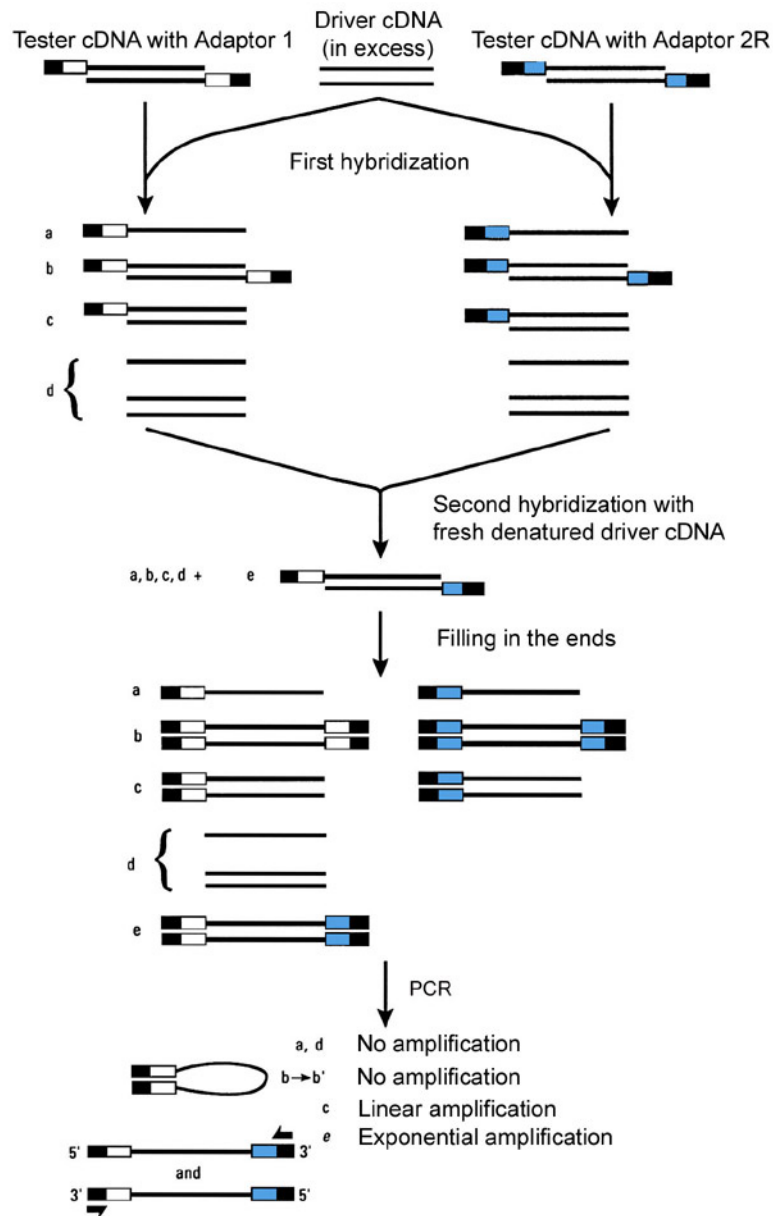


Fig. 6.2 Schematic representation of the SSH

annealed, generating the type **a**, **b**, **c**, and **d** molecules in each sample (Fig. 6.2). Due to second order kinetics of hybridization, abundant molecules form duplexes faster than rare molecules, thus the amount of abundant and rare sequences is partially equalized among type **a** single stranded cDNA (ss cDNA) molecules. At the same time, type **a** molecules are significantly enriched for differentially expressed sequences, because the non-differential tester cDNAs form type **c** molecules with the driver.

During the second hybridization, the two primary hybridization samples are mixed together without denaturing, and more of the denatured driver cDNA is added. During the second hybridization, a new kind of hybrid molecule can form – the type **e** molecule. It is a duplex built of the subtracted and equalized ss cDNAs with different adaptors on the ends. The recessed termini are then filled in, and the entire population of molecules is amplified by polymerase chain reaction (PCR) with primers recognizing different adaptors. While all the types of hybrids either can not be amplified due to the lack of primer annealing sites (**a** and **d** type) or (type **b**) due to suppression PCR effect (Siebert et al., 1995), or can be only amplified linearly (type **c**) because of a single primer annealing site, only type **e** hybrid is amplified exponentially. To further reduce background products and enrich for differentially expressed sequences, the second PCR with nested primers is performed. The resulting product is subsequently ligated into a T/A cloning vector, and can be analyzed in various ways.

In this study messenger RNA of polyps and embryos was isolated with the use of QuickPrep™ Micro mRNA Purification Kit (Amersham). Then ds cDNA was synthesized as described in 6.4. Embryonic ds cDNA was used as a tester and ds cDNA from asexual budding adults as a driver. The subsequent procedure was carried out with the use of PCR-Select™ cDNA Subtraction Kit as described in manufacturers protocol. Both rounds of PCR amplification were carried out with the use of BD Advantage™ 2 PCR Enzyme System, containing a mixture of modified Taq polymerase and a proofreading polymerase and an antibody, blocking their active centers, and thus allowing “hot start”. 20 PCR cycles were used in the first amplification reaction, and 12 cycles in the second PCR. The resulting mixture was analyzed by gel electrophoresis and ligated into pGEM-T vector.

6.11. Library propagation and macroarray preparation

6.11.1. Library propagation, replication and storage

Subtracted library ligated into pGEM-T vector was electroporated in DH5α *E. coli* *bacteria* as described in 6.9.3. The cells were then plated onto the 20x20 cm QTrays with LB-agar plates with 50 µg/ml ampicillin. Prior to that, transformation efficiency was determined by plating a small aliquot of bacteria on a small LB-agar plates. Thus one

could estimate the volume of bacterial suspension to be plated onto the large QTrays to obtain approximately 2000 clones/QTray. This is the density, which is most appropriate for picking with the QPix robot. Individual white clones were then picked and propagated in the Freezing medium (10 g Tryptone; 5 g Yeast extract; 20 g NaCl; 36 mM K₂HPO₄; 13.2 mM KH₂PO₄; 1.7 mM Na-citrate; 0.4 mM MgSO₄; 6.8 mM (NH₄)₂SO₄; 4.4% Glycerol; millipore water up to 1 liter; 50 mg/ml ampicillin after autoclaving and cooling down) in the wells of 384-well plates overnight in humid chamber at 37⁰. When the cultures in the wells were dense enough, plates were frozen and kept at -80⁰. Replicates of the library were made by hand with the use of sterile 384-pin stamp.

6.11.2. Gridding the library on nylon membranes and processing the filters

Hybond-N membrane was used for the arrays. Prior to picking, a sheet of Whatman 3MM paper slightly larger than the size of the membrane was cut, dipped into LB medium, the liquid was allowed to drain, and the sheet was placed onto the filter block of the QPix robot. Then a sheet of nylon membrane was put onto the Whatman 3MM paper, avoiding air bubbles in between, and the clones from the plates were gridded in duplicates in 4x4 pattern, with the GAPDH insert bearing clones used as a guide spot. This means that each pin of the 384-pin gridding head makes 16 spots on a membrane in a 4x4 pattern (Fig. 6.3), corresponding to the clones with the same coordinates but from different plates.

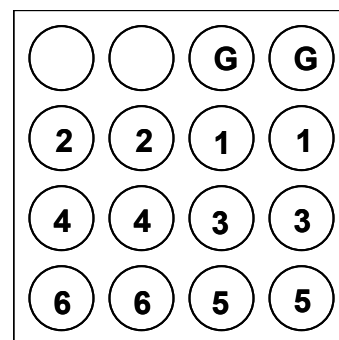


Fig. 6.3 Gridding pattern of a single pin. G - guide spot, numbers correspond to the numbers of the plates

After the gridding was complete, the membranes were placed bacterial side up onto the freshly made QTrays with LB-agar-ampicillin, and the clones were allowed to grow overnight at 37⁰. After the colonies have reached approximately 1.5-2 mm diameter, they were lysed with alkaline for 5' on two sheets of Whatman 3MM paper soaked in 1.5 M NaCl, 0.4 M NaOH, then neutralized 2x5' on two sheets of Whatman 3MM paper soaked in 1.5 M NaCl, 0.5 M Tris pH 7.2, and then equilibrated with 2x SSC 2x5' also on two sheets of Whatman 3MM paper. Finally, the filters were air-dried and baked for 1 hour at 80⁰ and additionally UV-crosslinked, to fix DNA on the membrane. The filters were stored at -20⁰.

6.11.3. Hybridization of the filters.

The filters were hybridized with the radioactive probe and washed according to the Northern blot hybridization protocol (6.8). The probe was prepared as follows:

Component	Amount
Denatured cDNA probe	100 ng
10x Klenow fragment buffer	5 μ l
Klenow fragment (2 U/ μ l)	2 μ l
10 mM mix of dGTP and dTTP	1.5 μ l
[α P ³²]-dATP	3 μ l
[α P ³²]-dCTP	3 μ l
Random hexamere primer 0.2 μ g/ μ l	5 μ l
Millipore water	To the final of 50 μ l

Two radioactive nucleotides were used because the labeling efficiency by Klenow fragment turned to be significantly lower with complex templates like cDNA pools than with uniform templates. Normally the efficiency of 25% was reached in contrast to 70-90% in case of a gel-purified PCR fragment template.

After washing, the filters were exposed with the PhosphorImager screen, and the hybridization picture was processed with Aida software. Subsequently the probe was stripped from the filter with several washes in boiling 0.5% SDS, and the filter was rehybridized with the probe from a different developmental stage.

6.12. In situ hybridization

6.12.1. Preparation of the Digoxigenin-labeled probes

Gel-purified PCR products were used for probe preparation. To minimize the length of the unspecific sequence in the probe, two fragments were synthesized (Fig. 6.4) and labeled separately: the one, containing SP6 promoter - with SP6 RNA

polymerase, the other, containing T7 promoter - with T7 RNA polymerase. Dig-labeled probes were prepared with the use of “Dig RNA labeling kit” according to the manufacturers protocol.

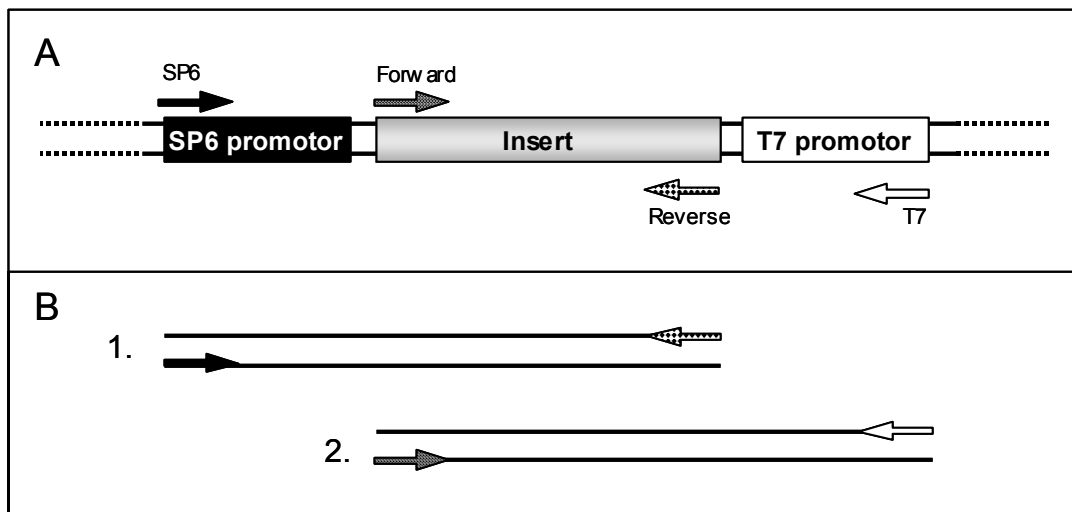


Fig. 6.4 The scheme of fragment amplification for Dig-RNA labeling

- A) The polylinker of the vector with polymerase promoters and primer annealing sites.
 B) Fragments received by PCR with one vector- and on insert-specific primer used for Dig-labeling

6.12.2. *In situ* hybridization on whole mount embryos

All the steps were carried out at room temperature if not mentioned otherwise. Washes were performed with gentle agitation. PBT solution contained 1% of Tween 20, instead of 0.1%, as used in all other applications. This method of *in situ* hybridization was modified from Corbo et al. (1997).

The embryos were fixed overnight in 4% paraformaldehyde/hydra medium, pH 7.2-7.4. Then the samples were washed 8x5' in 100% ethanol, once for 5' in Xylene/Ethanol (1:1), once for 5' in Xylene/Ethanol (3:1), and incubated in Xylene/Ethanol (3:1) for 1 hour. Subsequently, xylene was replaced by ethanol by 5' wash in Xylene/Ethanol (1:1) and 5x5' in 100% ethanol. After that, the embryos were washed once for 5' with 100% methanol, once with Methanol/2% paraformaldehyde on PBT (1:1) and refixed for 25' in 2% paraformaldehyde/PBT. After 5 washes in PBT the embryos were treated with 4 µg/ml proteinase K in PBT for 5'. The enzyme was stopped with a 4 mg/ml glycine/PBT wash followed by 5x5' in PBT. Then the samples were once again post-fixed in 2% paraformaldehyde/PBT and 5x5' washed with PBT. Following

that, the embryos were washed for 5' in hybridization solution/PBT (1:1) and for 5' in pure hybridization solution (50% formamide, 5xSSC, 1mg/ml CHAPS, 1x Denhardt's, 100 µg/ml heparin, 100 µg/ml tRNA, 0.1% Tween 20). Then the samples were prehybridized for 2 hours at 56⁰ and the Dig-labeled sense and antisense probes were added to the different batches of embryos, and hybridization was carried out for 60 hours.

After hybridization the samples were subjected to a series of 10' washes at hybridization temperature with 100% HS, 75%HS/25% 2xSSC, 50%HS/50% 2xSSC, 25%HS/75% 2xSSC, then the embryos were washed 2x30' with 1 mg/ml CHAPS/2xSSC at hybridization temperature. Afterwards the samples were washed once with MAB at room temperature and blocked first for 1 hour in MAB/1%BSA, then for 2 hours in solution containing 80% MAB/1%BSA and 20% of heat-inactivated sheep serum. Antidigoxigenin Fab fragments (Roche) were added to the blocking solution in final dilution of 1:2000 and samples were incubated with the antibody overnight at 4⁰. Unbound antibody was removed by 8x40' washes with MAB-T, then the embryos were washed twice for 5' in NTMT, and 10µl/ml of NBT/BCIP mixture in NTMT was applied. Staining reaction was stopped by a brief wash in MAB, followed by dehydration in methanol or ethanol dilution series. Afterwards the embryos were embedded into Euparal.

6.12.3. *In situ* hybridization on whole mount polyps

Starved animals were relaxed for maximum of 2' in freshly prepared 2% urethane/hydra medium solution and fixed overnight in 4% paraformaldehyde/hydra medium, pH 7.2-7.4. Fixed polyps were then washed twice with 100% methanol and stored at -20⁰. Prior to hybridization the animals were rehydrated stepwise (70% EtOH -> 50%EtOH/50% PBT -> 25% EtOH/75% PBT -> PBT) and washed 3x10' in PBT. Afterwards the polyps were treated for 20' at room temperature with 10 µg/ml proteinase K. Digestion was stopped with 4 mg/ml glycine/PBT solution by means of one short wash followed by a 10' wash. Then, after 3x5' washes in PBT the animals were treated 2x5' with 0.1M triethanolamine pH 7.8, once with 2.5 µl/ml acetic anhydride/triethanolamine and once with 5 µl/ml acetic anhydride/triethanolamine, to

screen all charged residues left after proteinase K digestion. After that, the polyps were washed 3x5' in PBT and post-fixed in 4% paraformaldehyde/PBT for 1 hr. The fixative was removed by 5x5' washes with PBT, then the polyps were washed once in 2xSSC for 10' and heat-treated for 15' at 70⁰ to inactivate endogenous alkaline phosphatases. Then the polyps were washed once for 10' with 50% hybridization solution/50% 2xSSC, once with pure hybridization solution (HS; 50% formamide, 5xSSC, 1mg/ml CHAPS, 1x Denhardt's, 100 µg/ml heparin, 100 µg/ml tRNA, 0.1% Tween 20), and prehybridized in HS at hybridization temperature for 2 hours. Digoxigenin-labeled riboprobe was then added to the samples and hybridization was carried out for 8-60 hours at 55-65⁰.

After hybridization the samples were subjected to a series of 10' washes at hybridization temperature with 100% HS, 75%HS/25% 2xSSC, 50%HS/50% 2xSSC, 25%HS/75% 2xSSC, then the animals were washed 2x30' with 1 mg/ml CHAPS/2xSSC at hybridization temperature. Afterwards the animals were washed once with MAB at room temperature and blocked first for 1 hour in MAB/1%BSA, then for 2 hours in solution containing 80% MAB/1%BSA and 20% of heat-inactivated sheep serum. Antidigoxigenin Fab fragments were added to the blocking solution in final dilution of 1:2000 and polyps were incubated with the antibody overnight at 4⁰. Unbound antibody was removed by 8x40' washes with MAB-T, then the animals were washed twice for 5' in NTMT, and 10µl/ml of NBT/BCIP mixture in NTMT was applied. Staining reaction was stopped by a brief wash in MAB, followed by dehydration in methanol or ethanol dilution series. Afterwards the polyps were embedded into Euparal.

Male polyps used for the semi-thin sectioning were taken after substrate reaction, partially dehydrated by two 20' washes in 70% ethanol, then gradually infiltrated with LR White in several steps: 70% ethanol - LR White 2 / 1 (v/v) for 30', 1 / 1 (v/v) for 30', 1 / 2 (v/v) for 30', LR White for 1 hour, LR White overnight. LR White was then polymerized at 60⁰ in gelatin capsules for 24 hours. 2.5 mm sections were cut with LKB ultramicrotome.

6.12.4. *In situ* hybridization on frozen sections

Embryos were fixed overnight in 4% paraformaldehyde on hydra medium. Cuticles of the cuticle stage embryos were then punctured with a needle and fixation in

this case was allowed to proceed for 2-3 hours more. Afterwards embryos were washed twice in 100% methanol and stored in 100% methanol at -20° . Prior to cutting methanol was replaced stepwise (70% EtOH \rightarrow 50% EtOH/PBT \rightarrow 25%EtOH/PBT \rightarrow PBT) with 1xPBT, then embryos were washed 3x5' in PBT. Embryos were then embedded into Jung Tissue Freezing medium and 16 μ m kryosections were cut. The sections were attached then to 0.1 mg/ml L-Polylysine-coated cover slides. The slides were washed once in 25%EtOH/PBT and 3 times in PBT, then the sections were treated with 10 μ g/ml proteinase K/PBT for 5' at room temperature. Proteinase digestion was stopped by a brief wash with 4mg/ml glycine/PBT followed by 5' wash in glycine/PBT and 3x5' in PBT. Afterwards sections were treated 2x5' with 0.1M triethanolamine, pH 7.8, then once with 2.5 μ l/ml acetic anhydride/0.1M triethanolamine and once with 5 μ l/ml acetic anhydride/0.1M triethanolamine, followed by 3x5' in PBT. The slides were then post-fixed in 4% paraformaldehyde/PBT for 15', washed 5x5' in PBT, then once in 2xSSC, once in 50% hybridization solution/2xSSC and once in hybridization solution (50% formamide, 5xSSC, 1mg/ml CHAPS, 1x Denhardt's, 100 μ g/ml heparin, 100 μ g/ml tRNA, 0.1% Tween 20). Prehybridization was then carried out in hybridization solution (HS) in the humid chamber at hybridization temperature ($55-65^{\circ}$) for 2 hours, then heat-denatured probe was added and sections were hybridized for 8-60 hours in the humid chamber. After hybridization, the sections were washed at hybridization temperature with HS, 75% HS/2xSSC, 50%HS/2xSSC, 25%HS/2xSSC, 10' each step, then 2x30' with 2xSSC/1mg/ml CHAPS.

Afterwards the sections were washed for 10' with MAB, then blocked first for 1 hour in MAB/1%BSA, then for 2 hours in solution containing 80% MAB/1%BSA and 20% of heat-inactivated sheep serum. Antidigoxigenin Fab fragments were added to the blocking solution in final dilution of 1:2000 and sections were incubated with the antibody overnight at 4° . Unbound antibody was removed by 8x30' washes with MAB-T, then the sections were washed twice for 5' in NTMT, and 10 μ l/ml of NBT/BCIP mixture in NTMT was applied to the sections. Staining reaction was stopped by a brief wash in MAB, followed by incubation in 100% methanol. Afterwards the sections were either embedded into Euparal or rehydrated, stained with 100 ng/ml water solution of DAPI and embedded into DABCO/Mowiol.

6.13. Antibody staining

6.13.1. Antibody staining of *Hydra* polyps

This method was modified after Engel et al. (2001). The polyps were relaxed in fresh solution of 2% urethan/Hydra Medium and fixed with Lavdovsky's fixative (50% EtOH/3,7%PFA/4% acetic acid) overnight at RT. Subsequently the samples were washed 5x5' with 1xPBT and permeabilized by a 30' wash with PBS/0.1%Triton X100. Then blocking was performed by means of a wash with MAB/1% BSA for 1 hour. After blocking, the solution was exchanged with MAB/1% BSA containing primary antibody in appropriate dilution, and the samples were stained overnight at +4⁰. One batch of animals was left overnight in blocking solution without adding the primary antibody as a negative control. Unbound primary antibody was removed by 5x15' washes in MAB/0.1% Tween 20. Then the samples were blocked again for 1 hour in MAB/1% BSA, and secondary antibody in appropriate dilution was added. The staining was allowed to proceed for 2-5 hours. If the antibody was FITC-conjugated, then staining and further procedures were performed in the dark. Subsequently, the unbound antibody was removed by 5x15' washes in MAB-T, then the samples were rinsed with water and counterstained for 2' in 0.1 µg/ml DAPI solution in the dark. After staining the samples were embedded in 0.1%DABCO/Mowiol (1 volume of 200 mg/ml DABCO and 2 volumes of Mowiol).

6.13.2. Antibody staining of frozen sections

Animals were fixed overnight in Lavdovsky fixative and 14 µm thick frozen sections were prepared as described in 6.12.4. Antibody staining of frozen sections was performed as described in 6.13.1.

6.14. TUNEL assay

To check if the cells were dying by apoptosis, TUNEL assay was performed. The procedure was modified after Technau et al. (2003).

Embryos were fixed in 4% paraformaldehyde/hydra medium and washed 3x5' in methanol. Methanol was gradually replaced with PBT (75% MetOH/PBT -> 50% MetOH/PBT -> 25%MetOH/PBT -> PBT), then the embryos were embedded into Tissue Freezing Medium and 14 μ m thick frozen sections were prepared as described in 6.12.4. Afterwards the slides were washed twice for 10' in PBS/0.2% Tween 20 and once in PBS. The sections were then equilibrated in terminal deoxynucleotidyltransferase (TdT) buffer for 1 h under a piece of parafilm. End-labeling was carried out overnight at room temperature in 50 μ l TdT buffer containing 0.5 μ M digoxigenin-dUTP and 150 U/ml TdT. Labeling was stopped by addition of PBS/1mM EDTA, incubation at 65°C (2x1 hour), followed by four washes in PBS. Heat inactivation of endogenous alkaline phosphatases is also achieved at this step. Next, the slides were washed in MAB/0.1% Triton X-100/2% BSA for 15' and blocked in 80% MAB/2% BSA ÷ 20% heat inactivated sheep serum for 2 hours. Then the slides were incubated in a 1:2000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase overnight at 4°C, washed 8x15' in MAB/0.1% Tween 20, and 2x5' in NTMT. Then color reaction with BCIP and NBT was performed (10 ml substrate/ 1ml NTMT), the sections were counterstained with DAPI and embedded in DABCO-Mowiol as described in 6.13.1.

7. References

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9. Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation eigenständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe.

Kiel, den 03.11.2004

Grigory Genikhovich