

Towards an understanding of molecular mechanisms controlling gland cell behaviour in *Hydra*

Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität
zu Kiel

vorgelegt von
Stefan Siebert



Kiel, den 12.12.2007

Referent: Prof. Dr. Dr. h.c. Thomas C.G. Bosch

Koreferent: Prof. Dr. Thomas Roeder

Tag der mündlichen Prüfung: 24.01.2008

Meinen Eltern

„Von allen Leistungen des vorigen Jahrhunderts auf dem Gebiete der niederen Thiere ist wol keine von so eingreifendem Einfluss für die Entwicklung der Wissenschaft gewesen, wie die Entdeckung der wunderbaren Lebenseigenschaften der Süßwasserpolypen.“

Nicolaus Kleinenberg: *Hydra* - Eine Anatomisch - Entwicklungsgeschichtliche Untersuchung, S.1, 1872

Teile der vorliegenden Arbeit wurden bereits veröffentlicht

Augustin, R., Franke, A., Khalturin, K., Kiko, R., Siebert, S. Hemmrich, G., Bosch, T. C. G. (2006). "Dickkopf related genes are components of the positional value gradient in *Hydra*." Dev Biol **296**(1): 62-70.

Siebert, S., Anton-Erxleben, F., Bosch, T.C.G. (2007). „Cell type complexity in the basal metazoan *Hydra* is maintained by both stem cell based mechanisms and transdifferentiation”. Dev Biol, in press.

Table of content

	Abbreviations	X
1	Introduction	12
1.1	Regeneration and cellular plasticity.....	12
1.2	The Cnidarian <i>Hydra</i> in the tree of life	12
1.2.1	Phylogeny of <i>Hydra</i>	12
1.2.2	Morphology and histology of <i>Hydra</i>	14
1.2.3	Biology of <i>Hydra</i>	17
1.3	<i>Hydra</i> tissue dynamics	17
1.4	<i>Hydra</i> - An old model in new shape	21
1.5	Concepts and players in <i>Hydra</i> patterning	21
1.6	Dickkopf related proteins in <i>Hydra</i>	23
1.7	Aims of the study.....	25
2	Results	26
2.1	„Homologue hunting” reveals <i>HyDkk1/2/4 C</i> sequence conservation in different <i>Hydra</i> species	26
2.2	<i>HyDkk1/2/4 A</i> and <i>C</i> are clustered within the genomes of different <i>Hydra</i> species.....	27
2.3	Interspecific comparison of <i>HyDkk1/2/4 C</i> 5’ flanking region indicates functional promoter elements	30
2.4	Functional analysis of the <i>HyDkk1/2/4 C</i> promoter using transgenic <i>Hydra</i>	37
2.5	The 5’-flanking region of <i>HyDkk1/2/4 C</i> directs reporter gene expression in zymogen gland cells in a pattern that recapitulates the endogenous expression pattern	38
2.6	The <i>HyDkk1/2/4 C</i> signal peptide directs the reporter protein into secretory vesicles	40
2.7	Transgenic zymogen gland cells change their phenotype when they change their position along the body axis	40
2.8	Zymogen gland cells change their gene expression after elevation of positional value via interference with the canonical Wnt pathway ..	42
2.9	Zymogen gland cells change their gene expression when they change position in the course of regeneration	43

2.10	Histological studies verify that gland cells in different position have different morphologies and that intermediate gland cell morphologies can be found in regenerating tissue	45
2.11	Identification of interstitial cells which give rise to granular mucous gland cells	47
3	Discussion	50
3.1	Functional analysis of the the <i>HyDkk1/2/4 C</i> promoter	50
3.2	<i>HyDkk1/2/4</i> and <i>Hydra</i> patterning	53
3.3	Gland cell differentiation in <i>Hydra</i>	54
3.4	The nature of <i>HyDkk1/2/4 C</i> transcriptional regulation.....	56
3.5	Stability of the cell differentiation state	59
4	Summary	61
5	Zusammenfassung.....	62
6	Materials	63
6.1	Organisms	63
6.2	Media.....	63
6.3	Buffers and solutions	63
6.3.1	General purpose solutions	63
6.3.2	Solutions for <i>in situ</i> hybridisation	65
6.3.3	Kits.....	66
6.3.4	Enzymes	66
6.4	Chemicals	66
6.5	Vectors.....	68
6.6	Radioactive substances	68
6.7	Antibodies	68
6.8	Primer (MWG Biotech).....	68
6.9	Devices and machines.....	69
6.9.1	PCR machines (Thermocycler).....	69
6.9.2	Power supplies	70
6.9.3	Gel electrophoresis chambers	70
6.9.4	Incubators/shakers.....	70
6.9.5	UV-transilluminators.....	70
6.9.6	Electroporation devices.....	70
6.9.7	Centrifuges	70

6.9.8	Microscopy.....	70
6.9.9	Other devices.....	71
6.9.10	Other materials	71
6.10	URLs und Software.....	71
7	Methods.....	73
7.1	Culturing <i>Hydra</i>.....	73
7.2	Incubation experiments.....	73
7.3	Nuclear acid extractions.....	73
7.3.1	Isolation of total RNA	73
7.3.2	Isolation of genomic DNA	73
7.3.3	Isolation of Plasmid-DNA	74
7.3.4	Quantification of nuclear acids.....	74
7.3.5	cDNA synthesis	74
7.4	Polymerase chain reaction (PCR).....	74
7.4.1	Standard-PCR	74
7.4.2	Splinkerette-PCR	75
7.4.3	Insert check PCR.....	76
7.5	Agarose gel-electrophoresis.....	76
7.6	Purification and extraction of PCR products.....	77
7.7	Competent cells	77
7.8	Cloning technique.....	77
7.9	Bacterial stock cultures.....	77
7.10	DNA sequencing	78
7.11	Screening of a <i>Hydra oligactis</i> full length cDNA library for <i>HyDkk1/2/4</i> genes	78
7.12	Regeneration experiments	79
7.13	Expression analysis via <i>in situ</i> hybridisation.....	79
7.13.1	Preparation of Biotin/Dig-labelled RNA probes	79
7.13.2	Protocol <i>in situ</i> hybridisation	79
7.14	Generation of transgenic <i>Hydra</i>	81
7.14.1	Preparation of a HyDkk1/2/4C::EGFP promoter construct.....	81
7.14.2	Transfection of <i>Hydra</i>	82
7.15	Imaging	82
7.16	Histological methods.....	82

7.16.1	Maceration of <i>Hydra</i> tissue	82
7.16.2	Fixation of <i>Hydra</i> for confocal microscopy	83
7.16.3	Fixation of <i>Hydra</i> for transmission electron microscopy	83
7.16.4	Sectioning and staining: semi-thin sections	83
7.16.5	Sectioning and contrasting: ultra-thin sections	84
7.17	Computational methods	84
7.17.1	Multiple alignments	84
7.17.2	mVISTA	84
7.17.3	GATA	84
7.17.4	Footprinter 3.0	84
8	References	85
9	Appendices	92
9.1	<i>HyDkk1/2/4 C</i> cds	92
9.2	<i>HyDkk1/2/4 A</i> cds	92
9.3	<i>HyDkk1/2/4 C</i> - genomic sequence	93
9.4	<i>HyDkk1/2/4 A</i> - genomic sequence	95
10	Acknowledgements	96
11	Erklärung	97

Abbreviations

aa	Amino acid
AB	Antibody
AEP	<i>Hydra vulgaris</i> (AEP)
Amp	Ampicillin
AP	Alkaline Phosphatase
app.	appendix
APS	Ammonium persulfate
bp	Base pair
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
cds	coding sequence
cDNA	complementary DNA
ch.	chapter
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propansulfonate
COI	cytochrome oxidase subunit I
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
dCTP	Deoxycytosine triphosphate
DIG	Digoxygenin
Dkk	Dickkopf
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ECM	extracellular matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EST(s)	Expressed Sequence Tag(s)
EtOH	Ethanol
Fig.	Figure
g	gram
i-cell	interstitial cell
kb	kilobase
kV	kilovolt
LB-amp ⁺	Luria broth
HM	<i>Hydra</i> medium
l	liter
M	molar
MAB	Maleic acid buffer
MAB-B	Maleic acid buffer containing 1 % BSA
mag	<i>Hydra magnipapillata</i>

MetOH	Methanol
μ F	microfarad
min	minute
μ	micro
mg	Milligram
ml	Millil
mM	millimolar
mRNA	Messenger-RNA
NBT	nitroblue tetrasolium
n	nano
NCBI	National Center for Biotechnology Information
OD ₆₀₀	Optical Density at 600 nm
oli	<i>Hydra oligactis</i>
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	double-stranded RNA-mediated interference
RNase	Ribonuclease
rpm	rounds per minute
rRNA	ribosomal RNA
RT	Room temperatur of Reverse Transcription
SDS	Sodium dodecylsulfate
SSC	Sodium salino cytrate
Tab.	table
T _A	Annealing temperature primer
TAE	Tris-Acetate-EDTA-Buffer
<i>Taq</i>	<i>Thermophilus aquaticus</i>
TBE	Tris-Borate-EDTA-Puffer
TEMED	N,N,N',N'- tetraethyl methylenediamine
T _m	melting temperature
tRNA	transfer RNA
U	Unit
UTR	untranslated region
UV	ultraviolette
V	Volt
vir	<i>Hydra viridis</i>
w/v	weigth per volume

1 Introduction

1.1 Regeneration and cellular plasticity

It has long intrigued researchers why some animals possess remarkable powers of self-regeneration and others do not. A newt can regenerate a complete limb and an entire plant might grow even from a single cell or a group of cells. In most organisms, cellular commitment and the differentiated state are very stably controlled and cell type identity seems to be irreversibly fixed. For renewal of differentiated tissue to occur, animals and plants rely on multipotent and/or unipotent stem cells. In the absence of stem cells, and under special circumstances (e.g., regeneration), new tissue can also be formed from differentiated cells by transdifferentiation, a process by which cells are able to dedifferentiate and subsequently redifferentiate. The term transdifferentiation was coined to distinguish the switching of a terminally differentiated cell type into another during insect development (Selman and Kafatos, 1974). For a cellular behaviour to be interpreted as transdifferentiation two conditions must be fulfilled: first, the differentiation states must be clearly defined and distinguishable; and second, a direct ancestor-descendant relationship and common developmental history between the cells must be clearly demonstrated (Eguchi and Kodama, 1993).

The plasticity of the differentiated cell state essentially contributes to the profound difference between animals with high and low regeneration capacity (Bosch, 2007a). One animal in which cellular plasticity plays a major role during normal development is the Cnidarian *Hydra* which has a long history in developmental biology. Already in 1744 Trembley described the remarkable capability of *Hydra* to regenerate missing structures (Trembley, 1744). Since then, *Hydra* has been used as a model system to address multifaceted questions concerning development, stem cells, regeneration and cellular plasticity.

1.2 The Cnidarian *Hydra* in the tree of life

1.2.1 Phylogeny of *Hydra*

The Cnidaria are a basal animal group, which is discussed as the sistergroup to the Bilateria (Collins, 2002; Martindale et al., 2002; Philippe et al., 2005; Putnam et al., 2007, see Fig. 1.1 A). Members of this taxon are characterised by a phylotypic cell type, the stinging cell or cnidocyte, one of the most complex cell types in the animal kingdom

(Tardent, 1995). The level of organisation of Cnidaria is diploblastic, a feature which they share with the Ctenophores. Their body is built from two germ layers - endoderm and ectoderm. The mesoderm, a third germ layer typical for bilaterian animals, is not developed at this level of organisation.

The cnidarian bauplan is described as radial symmetric and it is thought to have evolved around 600 million years ago (Xiao and Knoll, 1999; Chen et al., 2002). Its emergence coincides with (i) the occurrence of true epithelia, characterised by tight junctions and (ii) the emergence of the first nervous systems in the animal kingdom (Nielsen et al., 1996). Within the phylum two forms of body organisation developed – the polyp, characterised by a cylindrical body, and the mostly freefloating medusa. In many cnidarian life cycles both forms alternate. The Cnidaria are currently subdivided into the Anthozoa, the Cubozoa, the Scyphozoa and the Hydrozoa; with *Hydra* belonging to the latter (Fig. 1.1 B).

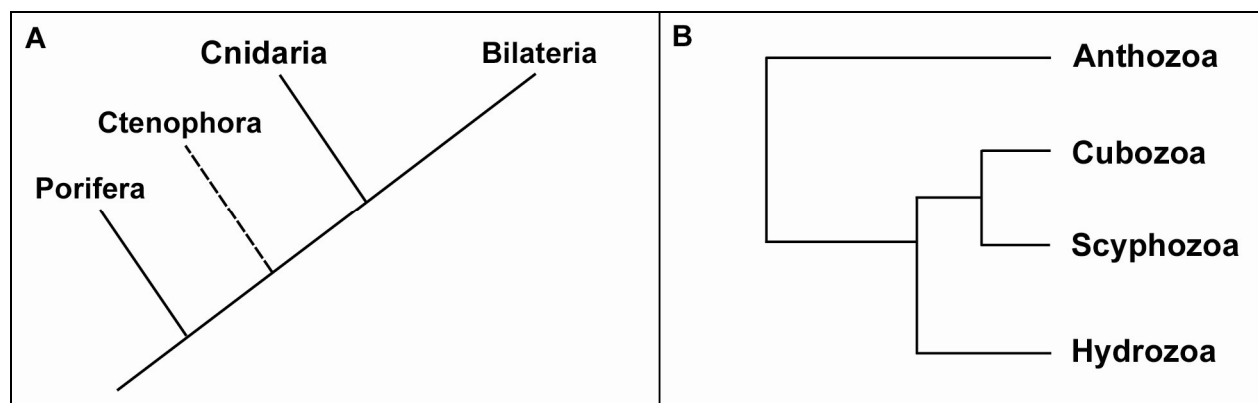


Fig. 1.1: Phylogenetic position of the Cnidaria. (A) Suggested relationships at the base of the animal kingdom (Phillipe, 2005). (B) Within the Cnidaria Anthozoa are regarded as basal (after Collins et al., 2006).

The Anthozoa are regarded as basal within the Cnidaria (Medina et al., 2001; Collins et al., 2006). In contrast to the other cnidarian groups, they have a circular mitochondrial genome like bilaterians do and lack the otherwise phylotypic medusa stage in their life cycles (Bridge et al., 1992; Bridge et al., 1995). The Hydrozoans are seen as the most derived group based on their outstanding cnidocyte type complexity, body plan diversity and molecular evidence (Steele, 2002; Collins et al., 2006, see Fig. 1.1 B). Cross-species analyses need an understanding of the phylogenetic relationships of the species to be analysed. Using two nuclear and two mitochondrial markers as well as morphological criteria, Hemmrich et al. recently resolved the relationship of several species of the genus *Hydra*, which are frequently held in the laboratory (Hemmrich et

al., 2007). This analysis identified the symbiotic *Hydra viridis* as the most basal species (Fig. 1.2).

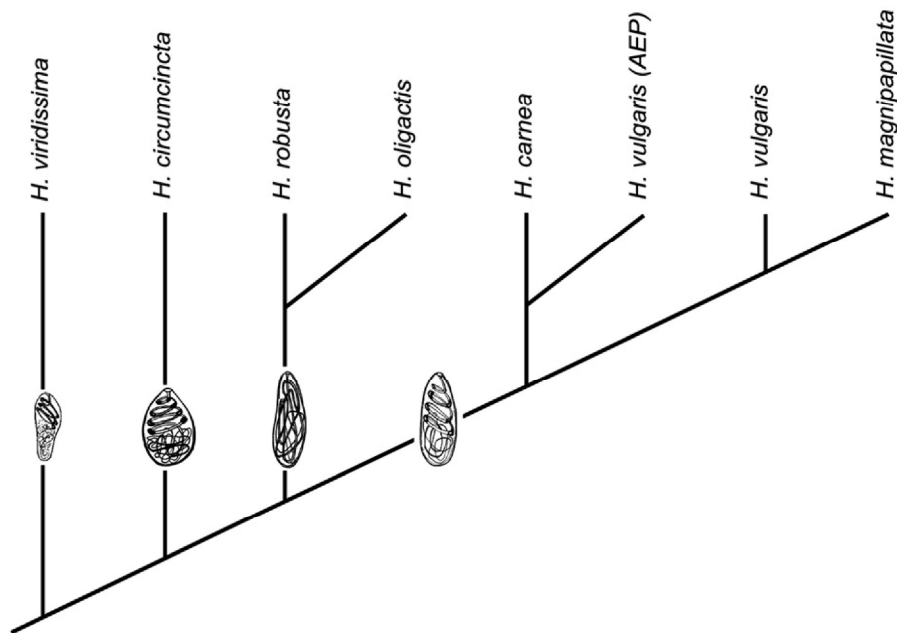


Fig. 1.2: Phylogeny of *Hydra*. Relationship of different *Hydra* species as indicated by a phylogenetic analysis using two nuclear (18S, 28S rRNA) and two mitochondrial (16S rRNA, COI) genes. Also depicted are the holotrichous isorhizas, one of four cnidocyte types, of the different groups (taken from Hemmrich et al., 2007).

1.2.2 Morphology and histology of *Hydra*

The body of *Hydra* is simple, consisting of a hollow tube with a basal disk for adhesion to substrate and an apical structure comprising a hypostome with a mouth opening which is surrounded by a ring of tentacles (Fig. 1.3 A). There are two epithelial cell layers in *Hydra*: the endoderm, aka gastrodermis, and the ectoderm, aka epidermis. They are derived from separate lineages of epithelio-muscle cells (Fig. 1.3 B). Cells of the epidermis comprise (I) the epithelial cells of the gastric region, capable of mitotic division, as well as (II) battery cells of the tentacles, which contain cnidocytes, and (III) basal disk cells. The latter have a secretory character and serve the attachment of the animal. Both epithelial layers are separated by an acellular layer, the mesoglea (Fig. 1.3 B). Transgenic cell labelling experiments have shown that a complete ectoderm can be formed by a very small founder population of epithelial cells from the gastric region (Wittlieb, pers. com.).

Predominantly within the ectoderm resides a population of interstitial cells (i-cells). A self-maintaining multipotent subpopulation of these cells gives rise to a variety of

specialized somatic cells (nerve cells, nematocytes) and germ cells in a position dependent manner (Bode, 1996; Bosch, 2007b).

These stem cells give also rise to gland cells. Interstitial cells in *Hydra* are abundant throughout the gastric region. In the head and foot region they are present in very low numbers (see below and Fig. 1.3 C, David and Plotnick, 1980). In *Hydra* the different cell types are lineage restricted. Neither interconversion between cells from the interstitial lineage and the epithelial cell lineages, nor between the ectodermal and endodermal epithelial cells have been observed (Sugiyama and Fujisawa, 1978).

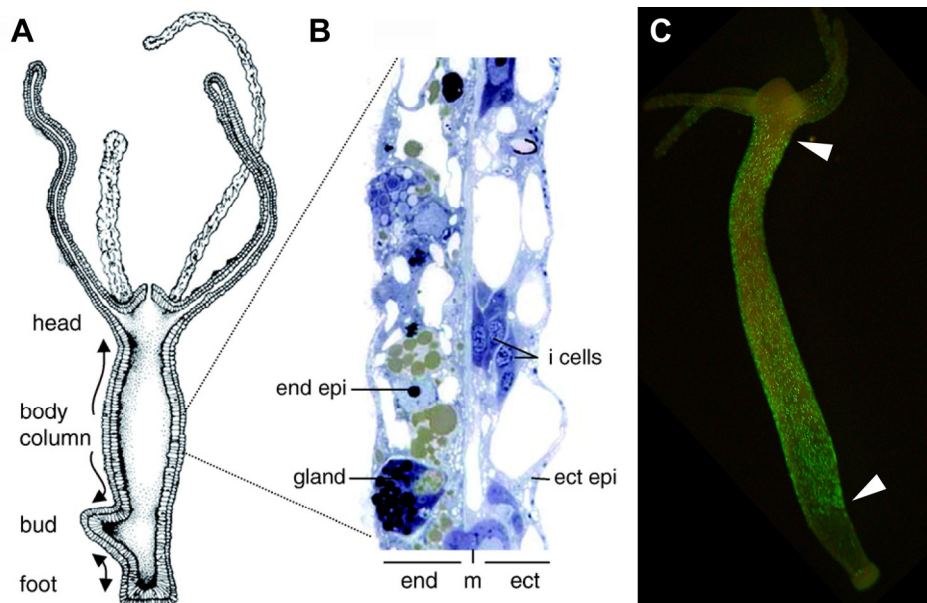


Fig. 1.3: Morphology and histology of *Hydra*. (A) Schematic representation of a *Hydra* polyp. (B) Cellular composition of *Hydra* tissue within the gastric region. end epi: endodermal epithelial cell, gland: zymogen gland cell, ect epi: ectodermal epithelial cell, i-cells: interstitial cells, end: endoderm, ect: ectoderm, m: mesoglea (A and B modified from Bosch, 2007). (C) I-cells are abundant along the body column, but show only very low concentrations in head and foot as indicated by an i-cell specific antibody, mAB C41. Arrows mark boundaries of interstitial cell distribution (modified from Khalturin et al., 2007).

The nervous system of *Hydra* is simple and has a netlike structure with high densities of neurons in the head and the foot (Bode et al., 1973). It consists of individual nerve cells with sensory or ganglionic character residing between epithelial cells of both layers. Subsets of nerve cells have been shown to express specific neurotransmitters or antigens, recognized by specific antibodies. This indicates a molecular diversity depending on the position of the cell (Koizumi and Bode, 1986; Technau and Holstein, 1996).

Within the gastrodermis four types of secretory cells can be found. The head comprises two secretory cell types whose vesicles show intense reactivity to histochemical tests

for mucus (Rose and Burnett, 1968). These mucous gland cells (MGCs), were, based on the morphology of their secretory vesicles, classified as granular MGCs (gMGC) and spumous MGCs (sMGC) (Fig. 1.4 A, Semal-van Gansen, 1954).

Zymogen gland cells (ZMG) are the dominant secretory cell type in the gastric region and are presumed to secrete proteolytic enzymes into the gastric cavity for extracellular digestion (Haynes and Burnett, 1963, Fig. 1.4 B). There is a second secretory cell type (mucG, Fig. 1.4 B) within the gastric region in very low abundancies, which is often neglected in the literature (Gauthier, 1963). The secretory vesicles of this cell type have a spumous occurrence comparable to the one in the head and the staining properties indicate that they secrete mucus (Gauthier, 1963). The identification of the expression pattern of a growing number of genes demonstrates a unique genetic signature for each of the different secretory cells types (Hwang et al., 2007).

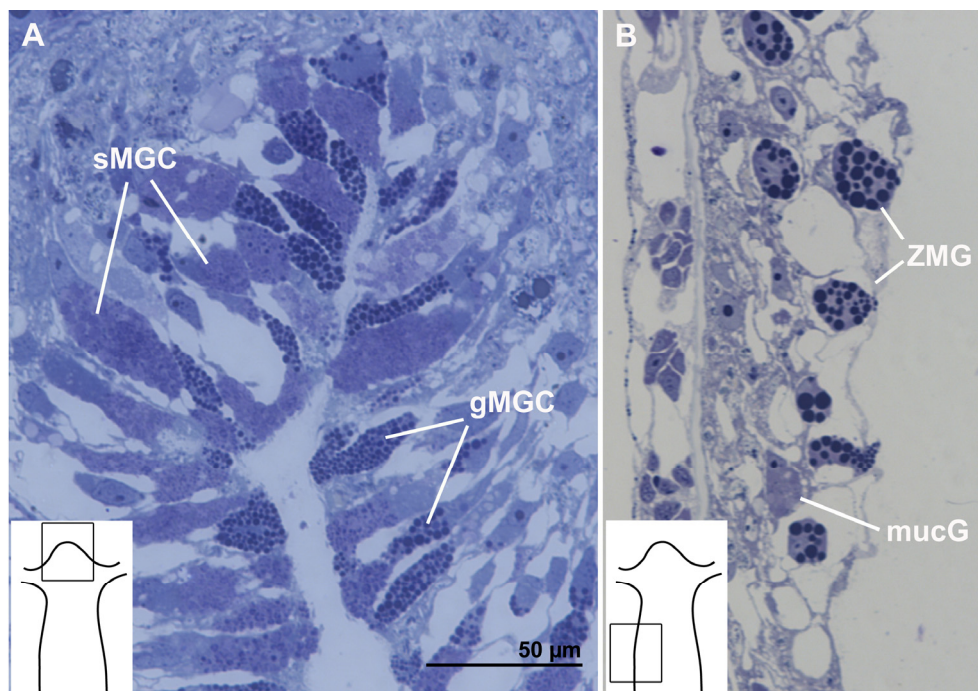


Fig. 1.4: Secretory cells within the *Hydra* gastrodermis. (A) The hypostome comprises spumous (sMGC) and granular mucous gland cells (gMGC). **(B)** In the gastric region zymogen gland cells (ZMG) are the dominant secretory cell type, whereas a second type is present in low abundancies (mucG). (modified from Siebert et al., 2007).

Labelling experiments and measurement of nuclear DNA content showed that the ZMG population contains transiently proliferating cells (Schmidt and David, 1986; Bode et al., 1987). Whereas the origin of ZMGs previously could be demonstrated unambiguously to lie in the interstitial cell population (Schmidt and David, 1986; Bode et al., 1987), the origin of the MGCs in the head, an almost interstitial cell free region, remained elusive.

Rose and Burnett (1968, 1970) addressed this question and performed regeneration experiments with *Hydra viridis* focusing on the origin of secretory cells within the newly formed hypostome. The presented data indicated zymogen gland cells as one source for both mucous gland cell types after hypostomal regeneration (Rose and Burnett, 1968b). On the other hand, in the course of mid-gastric regeneration, gastrodermal basophilic cells, which are supposed interstitial cell derivatives residing within the endoderm, were proposed as the primary source for these cells (Rose and Burnett, 1970). Dübel (1989) indicated that hypostomal MGCs may be derivatives of head-specific interstitial stem cells but did not provide quantitative experimental data supporting this notion. As he did not distinguish the different types of mucous cells in the head and as he proposed an undefined mucous cell of the gastric region to be an additional source of the secretory cells of the hypostome, the relationship of the secretory cells in *Hydra* remains confusing. Furthermore it is unclear to what extent the proposed mechanisms, if present, contribute to the pool of secretory cells in the *Hydra* hypostome.

1.2.3 Biology of *Hydra*

Hydra lives, unlike most hydrozoans, in freshwater ponds or lakes. The animals feed on small crustaceans or larvae which are captured by using the tentacles and cnidocytes. Reproduction in *Hydra* takes place either vegetatively by budding (Clarkson and Wolpert, 1967), facilitating laboratory culture maintenance and propagation, or sexually by the formation of eggs and sperm. In the latter case male polyps develop testis, female polyps develop externally a single egg, which will get fertilized and develop while attached to the mother polyp (Martin et al., 1997). Some *Hydra* species are hermaphroditic and develop testis and eggs simultaneously. The *Hydra* development is direct, its life cycle lacks a medusal stage (Martin et al., 1997).

1.3 *Hydra* tissue dynamics

One of the remarkable features of *Hydra* is its capacity to regenerate missing structures and the ability to reaggregate and rebuild vital polyps even after dissociation into single cells (Gierer et al., 1972). In steady state polyps a complete animal will also regenerate from any 2 % of the tissue mass of the gastric region located between head and foot (Shimizu et al., 1993). Interestingly, the replacement of missing structures does not involve mitotic divisions and DNA synthesis (Park et al., 1970; Hicklin and Wolpert, 1973; Cummings and Bode, 1984). Therefore the mode of regeneration was described

to be morphallactic (Hicklin and Wolpert, 1973) i.e. new structures are build from existing cells which change phenotype and function in a position dependend process (see Morgan, 1901).

Beside these artificial translocations of cells in regeneration experiments, e.g. original gastric cells become head cells in the course of head regeneration, translocation of cells occurs constantly and naturally. Continuous proliferative activity of ectodermal and endodermal cells leads to (i) translocation of cells towards the apical and basal regions and (ii) to the incorporation of body column tissue into forming buds (see Fig. 1.5, Campbell, 1967a; Campbell, 1967b; Selman and Kafatos, 1974). The compensation of this continuous tissue growth happens mostly via budding and secondly via cell loss at both ends of the body and at the tips of the tentacles (Campbell, 1967b).

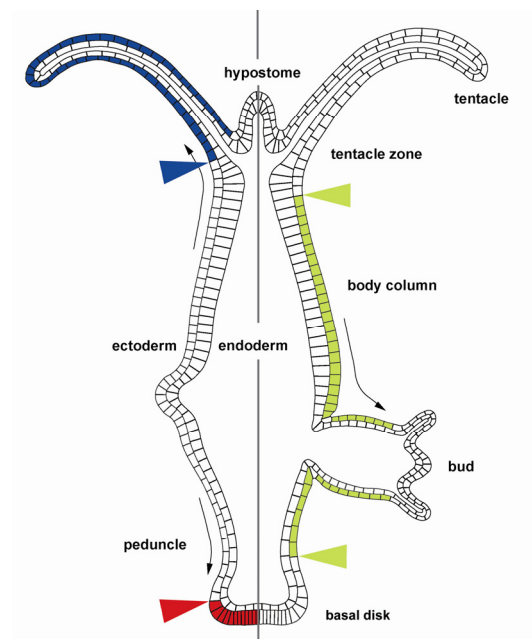


Fig. 1.5: Differentiation boundaries within *Hydra* tissue. (left) Epithelial cells change, when translocated towards the tentacle (blue) or towards the basal disk (red). Blue arrow: transition gastric - tentacle. Red arrow: transition gastric - basal disk. **(right)** Coloured ectoderm marks the area of predominant interstitial cell distribution. Yellow arrows: upper and lower boundary of interstitial cell maximum. Fine arrows mark tissue flux (schematic drawing taken and modified from Bode, 1996).

Despite this dynamic situation and continuous displacement of cells, in each polyp the overall axial distribution of the cell systems remains constant. The continual tissue flux necessitates the permanent activity of patterning processes, sensing of positional information and according changes in differentiation state. An epithelial cell forced in this process towards the basal disk gets a glandular character and will start to produce mucus which serves attachment to the substrate (see Fig. 1.5 left, red arrow, Davis,

1973). Ectodermal cells which become translocated towards the tentacles will take up cnidocytes and turn into battery cells at the tentacle basis (Fig. 1.5, blue arrow). At these two boundaries the cells differentiate finally and become arrested in the G2 phase (4n) of the cell cycle (Fig. 1.5 left, Dübel et al., 1987). The state of the dividing epithelial cells of the gastric region was therefore described to be metastable (Bode et al., 1986). Strikingly, ectodermal tissue in the apical gastric region will be displaced into the tentacles and will not get translocated towards the hypostome, the latter being maintained from a pool of head specific proliferating cells (Dübel et al., 1987).

It could be shown that the cells of the gastrodermis behave different and that both layers move as independent, coherent tissue sheets with different speeds (Campbell, 1967c). Labelling experiments showed, that unlike in the ectoderm, epithelial cells of the gastrodermis contribute not only to the tentacles, but also to the hypostome. Along with the change of position goes a decrease in vacuole sizes. The cells stay mitotically active except for the region around the mouth opening (Dübel, 1989).

Beside this epithelial movement in a sheet, Campbell classified two further modes of cellular motility, namely (i) active and (ii) passive movements. Cnidoblasts have been shown to actively migrate within the epithelial layers towards the tentacles whereas nerve, interstitial or gland cells are passively translocated embedded into the epithelial matrix (Campbell, 1967c). Recently, with focus on cnidoblasts and interstitial cells this movement could be visualized *in vivo* using transgenic animals whose interstitial cells and their derivatives were partly marked with EGFP (Khalturin et al., 2007).

Previous observations have revealed two strategies that underlie the maintenance of tissue organisation: differentiation from multipotent stem cells (Bosch and David, 1987) and phenotype conversion (Bode, 1992). The latter appears to be particularly evident in the plasticity of the differentiated state of the nerve net as immunocytochemical studies have revealed that neurons displaced from region to region appear to undergo changes in phenotype (Bode, 1992; Technau and Holstein, 1996). Within the tentacles and in the hypostome exists a population of neurons which is defined by an antiserum against the neuropeptide FMRFamide, termed FLI⁺ neurons (Koizumi and Bode, 1986). Even though the tissue is moving constantly and the neurons get translocated to the distal ends of the tentacles and the hypostome, where they get eventually sloughed off, the pattern and the basal border of the FLI⁺ cells stays the same indicating a continuous addition of new neurons at the base of the tentacles. Bode et al. reasoned that these cells originated (i) from translocated neurons, which acquired the new phenotype, and (ii) in newly differentiating neurons, as they could demonstrate lower densities of these

neurons in regenerating heads after removal of interstitial cells (Koizumi and Bode, 1986).

As the translocation of neurons and subsequent conversion of the phenotype can not sufficiently explain nerve net maintenance within the hypostome, two hypotheses have been proposed concerning the origin of neurons in an environment with low numbers of interstitial cells: (i) local commitment of pluripotent stem cells which thereby creates the apical (and basal) boundary of the interstitial cell distribution (see Fig. 1.3 C, Fig. 1.5 right, yellow arrows, Yaross and Bode, 1978) and (ii) migration of committed precursors which eventually differentiate within the hypostome (Teragawa and Bode, 1990; Teragawa and Bode, 1991; Technau and Holstein, 1996).

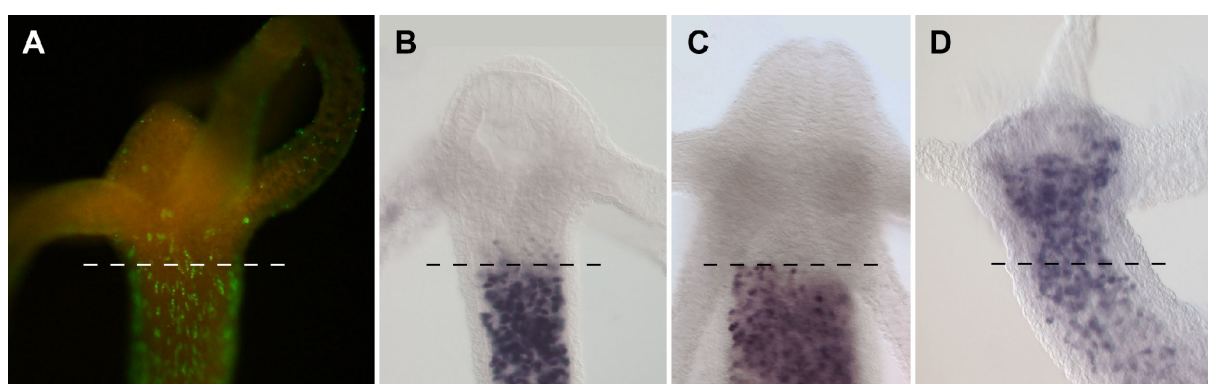


Fig. 1.6: I-cell localisation and transcriptional boundaries of genes from the i-cell lineage. (A) Position-dependent localisation of interstitial cells defined by mAB C41 (picture courtesy of K. Khalturin). Interstitial cells are located predominantly in the body column. (B,C) In situ hybridisation with zymogen gland cell specific genes *HyDkk1/2/4 C* and *A* (see below) and (D) *HySCP* (*HySCP* in situ hybridisation by K. Khalturin).

Fundamental for the understanding of cell type stability and positional value is the identification of the factors involved in the formation of boundaries, which define morphogenetic fields along the *Hydra* body column. Strikingly, the head boundary of the interstitial cell distribution seems to correspond to boundaries in the transcription pattern of genes like the zymogen gland cell genes *HyDkk1/2/4 A* and *C* (Fig.1.6 A - C). This boundary is not based on the absence of the respective cell type in more apical regions, as gland cell gene *HySCP* continues to be transcribed in head tissue (Fig 1.6 D). As transcriptional clues responsible for this pattern must be hardwired into the regulatory units, the analysis of the promoter regions of these genes might reveal factors involved in boundary maintenance and cell type changes (transdifferentiation).

1.4 *Hydra* - An old model in new shape

In the last years more and more sequence data could be acquired at tremendous speed from organisms at the base of animal evolution shedding new light on the ancestral gene repertoire (Miller et al., 2005; Miller et al., 2007; Putnam et al., 2007). Comparative data from different taxa revealed that Cnidarians share many genes with the bilateria. Furthermore these data indicate that many ancestral genes were present and have been retained in Cnidaria but got lost in the protostomian lineage (Miller et al., 2005; Technau et al., 2005). Recently, a National Human Genome Research Institute - funded *Hydra magnipapillata* genome project at the J. Craig Venter Institute resulted in a first assembled draft genome data set with 6 X coverage (<http://hydrazome.metazome.net>). Accompanied by a still growing set of at present 174.000 expressed sequence tags (ESTs) and the development of critical molecular techniques like stable transgenesis (Wittlieb et al., 2006), this marks a new era in *Hydra* research. Especially transgenesis offers new insights into patterning and differentiation processes as well as the establishment of direct lineage relationships using transgenes driven by gene specific promoter constructs. The structural organisation of *Hydra* makes it possible to trace individual labelled cells *in vivo* and to analyse the reconstitution of the cell type repertoire and homeostasis in the context of regeneration (Wittlieb et al., 2006; Khalturin et al., 2007). First phylogenetic comparisons to characterise interspecifically conserved transcription factor binding motifs within putative regulatory regions have been successfully conducted (Siebert et al., 2005) and will offer, together with functional testing of promoters, new insights into *Hydra* gene regulatory networks. Furthermore RNA interference techniques have been successfully applied in *Hydra* (Lohmann et al., 1999; Galliot et al., 2007; Miljkovic-Licina et al., 2007). Taken together, recent developments meant an extensive advance towards a complete molecularisation of the model organism *Hydra*.

1.5 Concepts and players in *Hydra* patterning

How does the framework for stable patterning and homeostasis in a constantly renewing animal look like? Early at the beginning of the last century as an undergraduate student Ethel Brown detected organiser capacities of the *Hydra* hypostome (Brown, 1909). Fragments from the apical tip were able to induce a second axis when transplanted into the gastric region of *Hydra*. The factors involved in this process were unknown for a long time. Recently it could be demonstrated that members

of the canonical Wnt pathway, including *HyWnt3a*, β -catenin, *Dishevelled* and *Tcf/Lef*, are involved in setting up the organiser (Hobmayer et al., 2000; Broun et al., 2005; Lee et al., 2006; Guder et al., 2006b). Generally, the vertebrate Wnt/ β -catenin pathway is activated via binding of Wnt to its receptor Frizzled/LRP which leads, transduced via cytoplasmatic Dishevelled, to the inactivation of GSK-3 β activity. The inactivation, which is essentially a destruction of a protein complex consisting of GSK-3 β with the co-regulatory proteins Axin and APC, leads to the stabilisation, accumulation and nuclear translocation of β -catenin. Here β -catenin, after it has formed complexes with Tcf/Lef DNA-binding factors, activates Wnt target genes (see Lee et al., 2006). A homologue of the Wnt-receptor frizzled is present in *Hydra* (Minobe et al., 2000).

Within the Cnidaria the presence of 11 of the 12 Wnt subfamilies could be shown in the Anthozoan *Nematostella* and most of them are also present in *Hydra* (Kusserow et al., 2005; Guder et al., 2006b). In *Nematostella* these genes are expressed along the oral-aboral axis in partially overlapping domains suggesting a role in axial patterning (Kusserow et al., 2005; Lee et al., 2006).

Interestingly, genes of the Wnt system expressed in the *Hydra* hypostome demonstrate features predicted by theoretical models of axial patterning in which organizer regions, generated by mechanisms that involve local self-enhancement coupled to antagonistic reactions, play pivotal roles (Gierer and Meinhardt, 1972; Meinhardt, 2007). Shortly these reaction-diffusion systems (see also Turing, 1952) are based on a non linear autocatalysis of an activator substance. This activator promotes also a rapidly diffusing antagonist which acts on a longer range and also slows down the activator production (Gierer and Meinhardt, 1972). In nature, the signals involved and the propagation of these signals might comprise various factors and transducers (Meinhardt, 2007). These systems allow the formation of an organizer and explain inhibition of further organizers in the periphery of an existing one.

In the model the *Hydra* foot acts as a second organising structure (Meinhardt, 2007). To explain the formation of a stable axis which requires maintenance of a single organizer on a larger scale, a further variable was introduced – the source density, which is defined as the competence to perform the autocatalytic production of the activator (Gierer and Meinhardt, 1972). This competence is positively influenced by the activator in the head and shows in *Hydra* a graded distribution along the body column with high values in the apical region which are decreasing down to the foot. The model allows to explain polar regeneration of any piece of the body column and the regeneration of different structures from originally neighbouring tissue (Meinhardt, 2007).

A graded signal within the *Hydra* body column which might mediate a positional value has been discussed and proposed for long, as it could explain many of the observable patterning processes (Tardent, 1954; Wolpert et al., 1972; Wolpert et al., 1974; MacWilliams, 1983). Associated with the concept of positional value is the morphogen concept (Gurdon and Bourillot, 2001). Morphogens are secreted factors which take part in patterning process mediating signals in a concentration dependend manner. Generally the formation of gradients is a fundamental problem in biology (Ashe and Briscoe, 2006; Grieneisen et al., 2007; Meinhardt, 2007) and the elucidation of the factors involved in *Hydra* axis formation could contribute to a further understanding of these mechanisms.

In addition to various Wnt pathway genes, many other pathways and conserved molecules playing a role in the development of higher animals were identified in *Hydra* like the TGF β /BMP pathway (Hobmayer et al., 2001), Notch signalling (Käsbauer et al., 2007; Khalturin et al., 2007) or receptor tyrosine signalling (Steele et al., 1996; for summary see Bosch, 2007a). Interestingly also „mesodermal“ genes can be found despite a diploblastic level of organisation (Technau und Bode, 1999).

1.6 Dickkopf related proteins in *Hydra*

In vertebrates, proteins of the Dickkopf (Dkk) family are known to interfere with Wnt signalling in a mostly antagonistic manner (Krupnik et al., 1999; Niehrs, 2006). Dkk proteins contain a signal peptide and are characterised by two cystein-rich domains separated by a linker region (Glinka et al., 1998, see Fig. 1.7 A). Each of these domains contains ten cysteins, which are arranged in a characteristic pattern, and additional conserved residues. Whereas the N-terminal domain is unique to Dickkopf proteins, the C-terminal domain shows conserved cysteins and predicted disulfide–bonding pattern typical for a colipase fold (Aravind and Koonin, 1998; Niehrs, 2006). Colipases are small protein co-factors needed e.g. by pancreatic lipase for efficient dietary lipid hydrolysis (van Tilbeurgh et al., 1999). The colipase fold is also present in a variety of proteins like toxins or protease inhibitors and is generally involved in specific protein–protein interactions (Aravind and Koonin, 1998; Boisbouvier et al., 1998; Hubbard et al., 1999; Kaser et al., 2003). It was shown that the second domain is sufficient for antagonistic interference with Wnt signalling (Brott and Sokol, 2002; Mao and Niehrs, 2003) which happens via modulation of the Wnt co-receptors of the LRP5/6 class. If the latter is present in Cnidaria remains to be clarified. Four Dkks have been characterised in

vertebrates. Whereas Dkk1, Dkk 2 and Dkk 4 modulate Wnt signalling, Dkk3 seems to serve different function. Dkk proteins could not be found in the protostome lineage of the bilaterians (Niehrs, 2006).

HyDkk3, one bona fide Dickkopf, has been indentified in *Hydra* (Fig. 1.7 A, Fedders et al., 2004). It shows a diagnostic 12 aa linker region of Dkk3 proteins between the two cystein rich domains. It is expressed in differentiating cnidoblasts (Fedders et al., 2004). Additionally two further short proteins with similarity to the second cystein rich domain of Dkk proteins were identified (Fig. 1.7 A, B, Augustin et al., 2006; Guder et al., 2006a). They were termed *HyDkk1/2/4* as they grouped with vertebrate Dkk1, Dkk2 and Dkk4 in a phylogenetic analysis (Guder et al., 2006a).

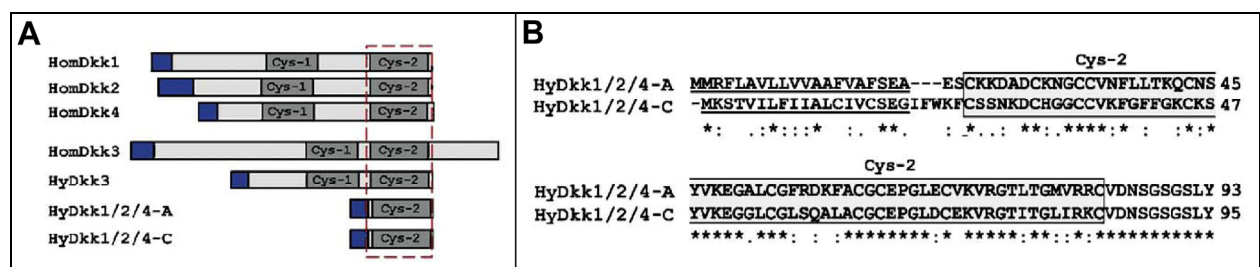


Fig. 1.7: Dickkopf proteins in *Hydra*. (A) Like human *Dkks* (*HomDkk1-4*), *HyDkk3* is composed of two cystein rich domains, whereas *HyDkk1/2/4s* only share the second cystein rich domain. Blue: signal peptide. (B) Amino-acid sequence alignment of *HyDkk1/2/4 A* and *C*. *, same aa, :, conserved substitutions, ., semi-conserved substitutions. The signal peptide is underlined and the cystein rich domain is shaded (A, B taken from Augustin et al., 2006).

Both proteins, *HyDkk1/2/4 A* and *HyDkk1/2/4 C*, have a predicted N-terminal signal sequence, consist out of 93/95 aa and show a sequence identity of around 64% on nucleotide level. Whereas *HyDkk1/2/4 A* is expressed in zymogen gland cells down to the penduncle, *HyDkk1/2/4 C* transcription is activated in the same cells in a graded manner (Fig. 1.8 A, B, Augustin et al., 2006; Guder et al., 2006a). The transcription patterns of both genes show a strikingly sharp boundary below the tentacle formation zone (see above). Overexpression experiments of *HyDkk1/2/4 A* in *Xenopus* embryos resulted in comparable phenotypes as after injection of *Xenopus Dkk1*, which lead to the proposal of an ancient Wnt-Dickkopf antagonism (Guder et al., 2006a). Expression data of both genes in the course of tissue manipulations also allowed to suggest modulation of Wnt signalling by these proteins (Augustin et al., 2006). In the Anthozoan *Nematostella* two of three identified *Dkk* genes group within the *Dkk3* subfamily (Lee et al., 2006). The third, *NvDkk1/2/4*, has both cystein rich domains and is complementary

expressed to the *NvWnt* domain during embryogenesis and in the early polyp (Lee et al., 2006). Interference of this protein with Wnt signalling remains to be clarified.



Fig. 1.8: Dickkopf related proteins *HyDkk1/2/4 A* and *HyDkk1/2/4 C* are expressed in zymogen gland cells (ZMG) of the gastric region. (A) *HyDkk1/2/4 A* is expressed homogenously in zymogen gland cells down to the peduncle, whereas *HyDkk1/2/4 C* (B) is activated in the same cells in a graded manner (B taken from Augustin et al., 2006).

1.7 Aims of the study

The formation and maintenance of transcriptional boundaries in dynamic tissues is not well understood. As *HyDkk1/2/4 C* shows a striking position dependent expression pattern, unraveling the regulatory inputs on its promoter could help to understand (i) the biochemical nature of the boundary below the head and (ii) how a graded expression is achieved.

To get insights into this regulation, phylogenetic footprinting approaches using orthologous sequences from related *Hydra* species followed by functional experiments using *Hydra* transgenesis will be applied in this study. As transgenic techniques allow unique possibilities of *in vivo* tracking, they will be used to elucidate gland cell dynamics.

The sequence conservation on the nucleotide level varied from 95,6 % (*H. magnipapillata* / *H. vulgaris* (AEP)) to 90,0 % between *H. magnipapillata* and *H. oligactis* and was with 67,8 % the lowest in the comparison of *H. magnipapillata* and *H. viridis*. In accordance with the molecular phylogeny of the genus *Hydra* (Hemrich et al., 2007), *H. viridis* has the most distant sequence as can be also seen in the phylogenetic representation based on a full length amino acid alignment (Fig. 2.1 B). The highest sequence similarity was observed between *H. magnipapillata* and *H. vulgaris* (AEP).

When analysing the expression pattern by means of *in situ* hybridisation, the graded activation in zymogen gland cells (Fig. 2.2 A - D) and the sharp boundary of expression below the head (Fig. 2.2 E) could be observed in all species demonstrating a conserved transcriptional regulation.



Fig. 2.2: *HyDkk1/2/4 C* *in situ* hybridisation shows conserved expression pattern in different *Hydra* species. (A) *H. magnipapillata*, (B) *H. vulgaris* (AEP), (C) *H. oligactis* and (D) *H. viridis* (picture courtesy of C. Lange), (E) Conserved boundary of expression below the tentacle formation zone (*H. magnipapillata*).

2.2 *HyDkk1/2/4 A* and *C* are clustered within the genomes of different *Hydra* species

To get first insights into the putative regulatory region of *HyDkk1/2/4 C* I analysed the available genomic information of *H. magnipapillata* using the trace archive (NCBI) and the available *H. magnipapillata* genome browser (<http://hydrazome.metazome.net>). Screening the assembled genome data with the full length sequence information of *HyDkk1/2/4 C* identified a single contig, Contig 39193, which comprised around 300 kb (Fig. 2.3 A). Interestingly, when analysing the periphery of the *HyDkk1/2/4 C* locus, *HyDkk1/2/4 A* could be identified ranging from – 4,993 bp (ATG) to – 3,909 bp (Stop codon) relative to the translation initiation site of *HyDkk1/2/4 C* suggesting a gene duplication event (Fig. 2.3 A, B). To exclude genomic missassembly, the physical

linkage of both genes was checked using mate pair information of the trace archive. The two traces of template 1061024507168 align to *HyDkk1/2/4 C* and *HyKK1/2/4 A*, respectively, and therefore support the cluster.

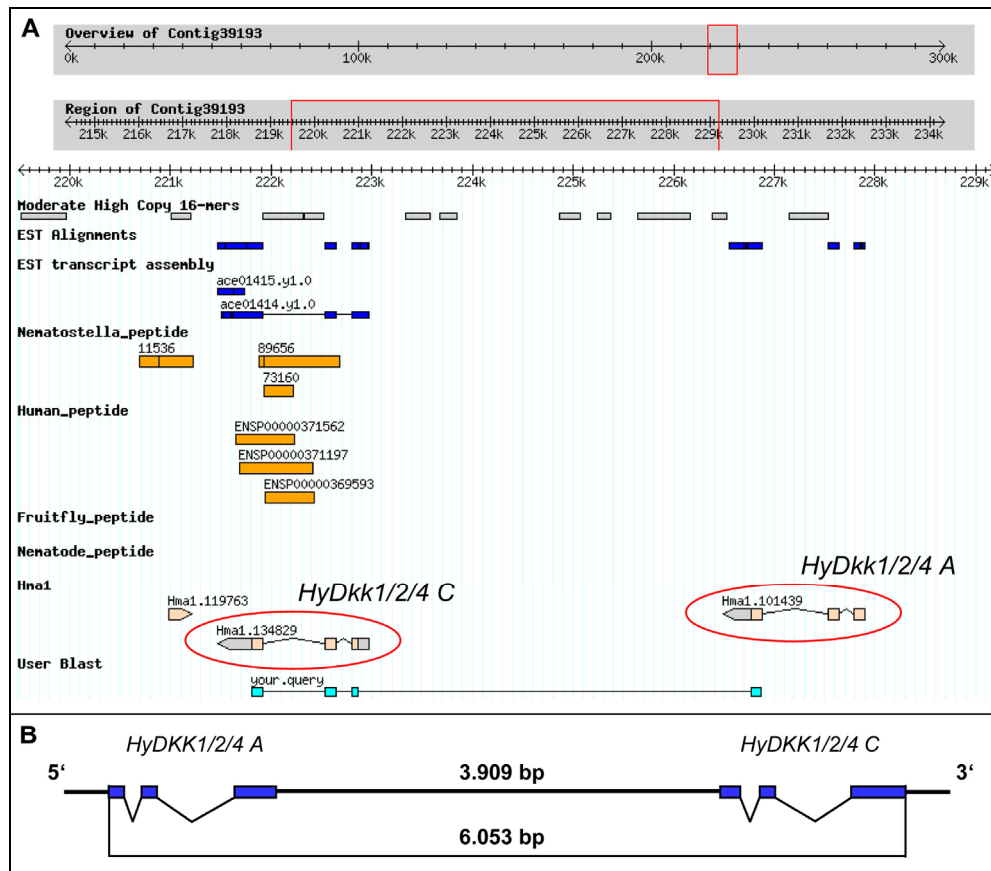


Fig. 2.3: Clustered localisation of *HyDkk1/2/4 A* and *HyDkk1/2/4 C* on genomic contig 39193. (A) Screenshot from the genome browser output after BLAST of full length *HyDkk1/2/4 C*. Both genes are localised on the minus strand. **(B)** *HyDkk1/2/4 A* lies upstream of *HyDkk1/2/4 C*. Given distances are relative to translation start and end.

Genomic analysis revealed that both *HyDkk1/2/4 A* and *C* are single copy genes and both consist out of three exons (Fig. 2.3, 2.4, app. 9.3, 9.4). The clustered localisation of the two genes was used to obtain 5'-flanking region of *HyDkk1/2/4 C* from *H. vulgaris* (AEP). Sequence specific primers directed (I) against exon 3 of *HyDkk1/2/4 A* pointing towards *HyDkk1/2/4 C* and (II) against exon 1 of *HyDkk1/2/4 C* pointing towards *HyDkk1/2/4 A* resulted in a approximately 4 kb long PCR fragment (see FIG. 2.4 A) demonstrating a conserved genomic distance in *H. vulgaris* (AEP) compared to the situation in *H. magnipapillata*. The intergenic region was partially sequenced yielding 1,470 bp of putative regulatory region upstream ATG of *HyDkk1/2/4 C*. To broaden the basis for a phylogenetic comparison of this region I used a PCR strategy, involving digestion of genomic DNA, adapter ligation and nested PCR (Splinkerette PCR, see ch.

7.4.2), to get sequence information of the orthologous regions from *H. oligactis* and *H. viridis*. By this approach I was able to amplify 1,090 bp *HyDkk1/2/4 C* 5'-flanking region from *Hydra oligactis* and 852 bp from *Hydra viridis*, respectively. In both cases sequence information was obtained also for intron one. The size of this intron varies between 130 bp (*H. oligactis*) and 393 bp (*H. viridis*) (Fig. 2.4 B). Clustering of the two genes could be shown also for *Hydra oligactis* using primers against exon 3 of *HyDkk1/2/4 A* and a primer against the 5'-flanking region of *HyDkk1/2/4 C* (primer position -1.041 to -984 bp). The resulting single PCR fragment (Fig. 2.4 A) indicated a closer genomic distance between the two genes of around 2.9 kb in this species.

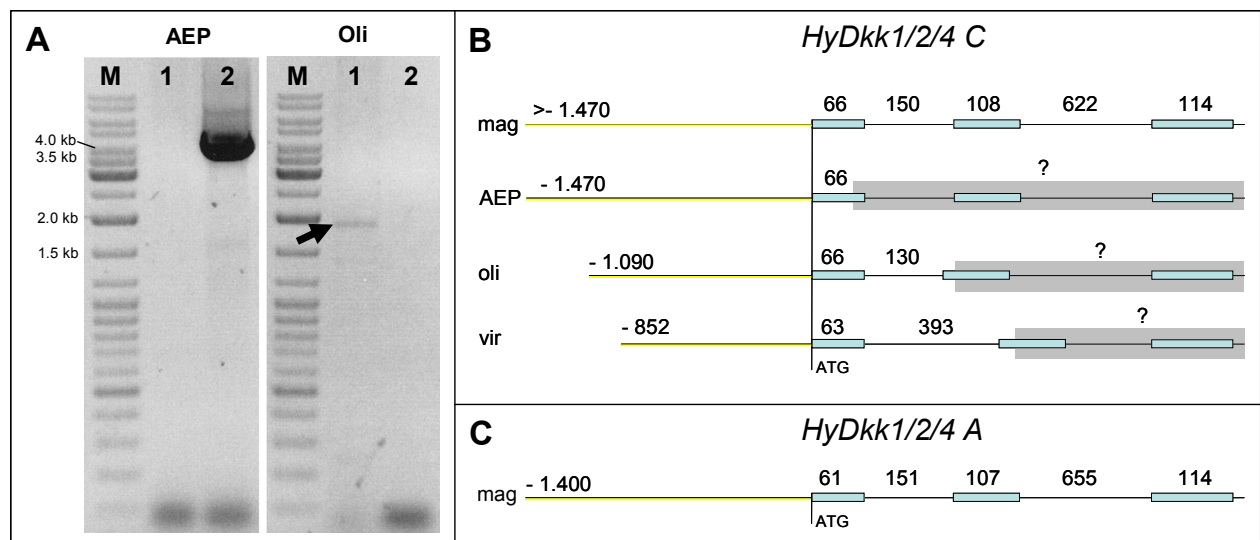


Fig. 2.4: Available data on genomic organization of *HyDkk1/2/4* genes in different *Hydra* species. **(A)** PCR on genomic DNA revealed clustering of the *HyDkk1/2/4* genes in *Hydra vulgaris* (AEP) and *Hydra oligactis*. PCR reactions using primer combinations directed against *HyDkk1/2/4 A* and *C* sequence pointing towards each other resulted in specific fragments indicating a genomic distance of around 4 kb in *H. vulgaris* (AEP) and 2.9 kb in *H. oligactis* (see also text). M: Marker, Lane AEP (1): control without DNA template, Lane AEP (2): PCR on genomic DNA (AEP) template. Lane Oli (1): PCR on genomic DNA (oli) template, arrow marks PCR product. Lane Oli (2): control without DNA template. **(B)** Available genomic sequence information for *HyDkk1/2/4 C* of *H. magnipapillata* (mag), *Hydra vulgaris* (AEP), *H. oligactis* (oli) and *Hydra viridis* (vir). Blue boxes indicate exons, grey boxes indicate unknown genomic organization. Figures indicate numbers of nucleotides, yellow: known 5' sequence information. **(C)** Genomic organisation of *HyDkk1/2/4 A* in *H. magnipapillata*. Figures indicate numbers of nucleotides.

Due to a lack of sequence information for *HyDkk1/2/4 A* in *H. viridis*, the genomic situation in this species remains to be elucidated. The available genomic sequence information on *HyDkk1/2/4 C* from the different species is summarised in Fig. 2.4 B (app. 9.2, 9.3). For comparative studies also 5'-flanking sequence information of *HyDkk1/2/4 A* was obtained from the *H. magnipapillata* genome data. Here, sequence

information was given for the first 754 bp upstream ATG whereas in more 5' direction a stretch of 318 unassigned nucleotides (N's) followed. Comparison with rough data from the trace archive (NCBI) including the matepair information allowed to elongate the 5'-flanking region *in silico* to approx 1.4 kb (Fig. 2.4 C). All obtained 5'-flanking regions were used in a phylogenetic comparison.

2.3 Interspecific comparison of *HyDkk1/2/4 C* 5' flanking region indicates functional promoter elements

A first conservation analysis of the *HyDkk1/2/4 C* 5'-flanking regions from the different species using the mVista online resource (<http://genome.lbl.gov/vista/index.shtml>) revealed high sequence conservation within the first 850 bp between the closely related species *Hydra magnipapillata* and *Hydra vulgaris* (AEP) with a decreasing conservation in regions upstream -1 kb relative to ATG, where conservation did not exceed values of 50 % (Fig. 2.5 A).

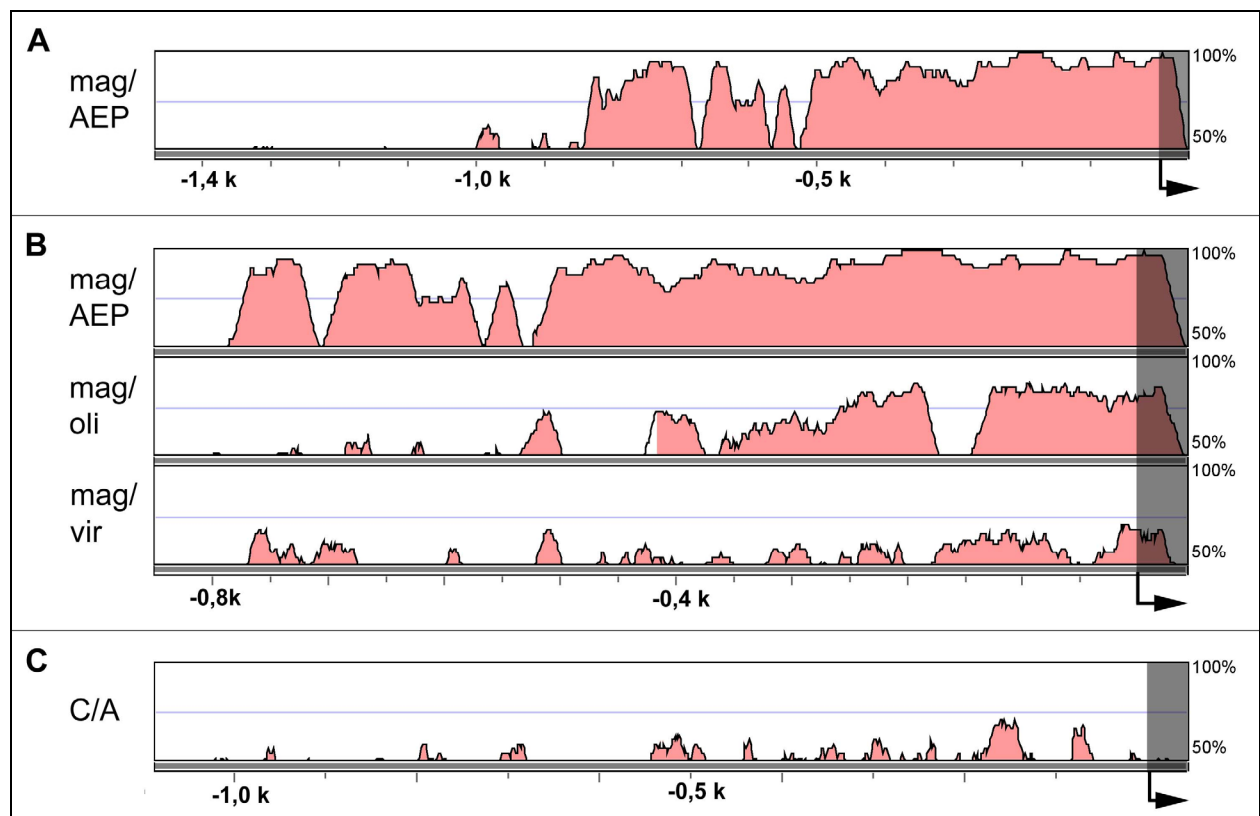


Fig. 2.5: mVista conservation profiles for the *HyDkk1/2/4* 5'-flanking regions from different *Hydra* species. (A) Profile of the comparison of 1,470 bp upstream *HyDkk1/2/4 C* from *H. mag* versus *H. AEP*. (B) Profiles of the comparisons of 852 bp upstream *HyDkk1/2/4 C* from *H. mag* versus sequences from *H. AEP*, *H. oli* and *H. vir*. (C) Comparison of 1,090 bp 5'-flanking region of *HyDkk1/2/4 C* with the corresponding region of *HyDkk1/2/4 A* (sequences from *H. mag*). mVista settings: Calc. window: 40 bp, Cons width: 40 bp, Cons. Identity: 50%, grey: coding sequence. AVID program was used for alignment.

Sequence comparison of *H. magnipapillata* with *H. oligactis* showed a decline in conservation at around -400 bp (Fig. 2.5 B). Only low conservation values were detectable in the comparison with the most distantly related *Hydra viridis*. However the common origin of the sequences was still visible (Fig. 2.5 B).

Even though clustering of *HyDkk1/2/4 A* and *HyDkk1/2/4 C* genes implied a common origin and a gene duplication event, the 5'-flanking regions showed no strong homology or conserved regions (Fig. 2.5 C). Considering that the genes are transcribed within the same cell type, this was unexpected.

As sequence inversions or shuffled features are frequent characteristics of noncoding sequences, I used the GATA alignment tool to get deeper insight into the sequence relatedness. The GATA tool uses the NCBI-BLAST program to identify all small subalignments between two input sequences that score above a chosen cut off and visualises them. The alignments (Fig. 2.6) are graphed as two shaded boxes which are connected by a line indicating the orientation of the alignment. Black stands for the same orientation whereas red indicates a +/- orientation of the aligned sequence. Solid colours of boxes indicate high scores, light colours indicate low scores of the particular alignment.

The comparison (Fig. 2.6 A) of 1,470 bp 5'-flanking region from *H. magnipapillata* and *H. vulgaris* (AEP) showed a colinear arrangement of the sequences within the first 900bp with a few stretches of nucleotides present in the *H. vulgaris* (AEP) sequence, presented as white areas, which are absent in *H. magnipapillata*. Furthermore, eight subalignments were displayed, where conserved sequences of *H. magnipapillata* were detected upstream 0.9 kb in AEP in an inverted orientation.

When sequence information of *H. oligactis* was included into the analysis and compared to *H. magnipapillata* and *H. vulgaris* (AEP), the program identified sequence conservation adjacent to the translation start and highlighted a conserved stretch around -400 bp which was present in all three species (Fig. 2.6 A, B, red arrow, Fig. 2.7 red box).

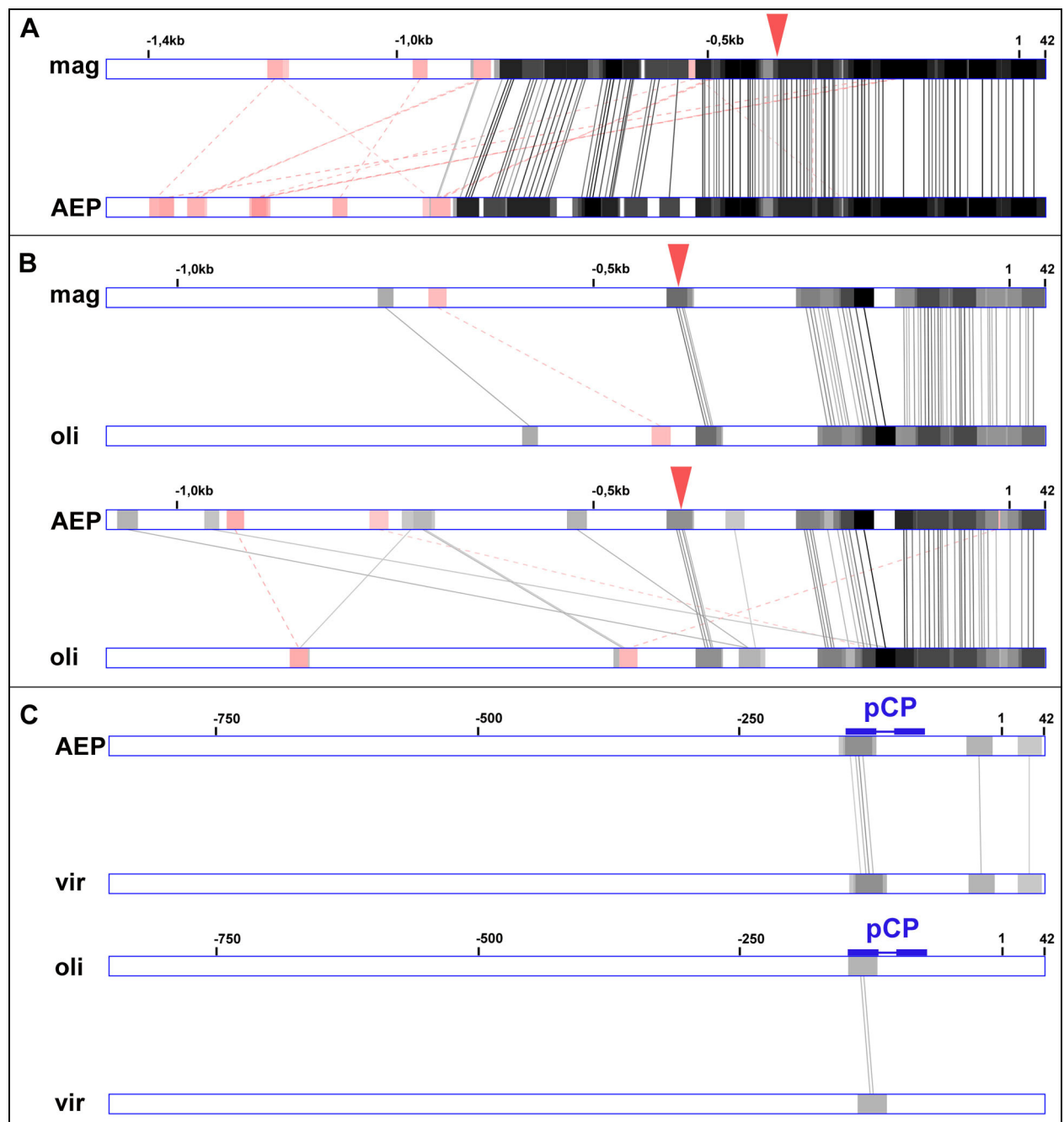


Fig. 2.6: Comparative sequence analysis of *HyDkk1/2/4* C flanking region using the GATA alignment tool. (A) Comparison of 1,470 bp 5' flanking region from *H. mag* and *AEP*. **(B)** Comparisons between *H. mag* and *H. oli* as well as *H. AEP* and *H. oli* highlighted a region around -400 bp (red arrow) which is shown to be conserved in all three species. Default alignment parameters were applied. **(C)** Comparisons of *H. AEP* and *H. oli* to *H. vir* reveal a conserved element starting 42 bp in front of the transcription start site and is therefore taken as part of the putative *HyDkk1/2/4* C core promoter (pCP, blue bar). Default alignment parameters were applied.

In more 5'-direction, no sequence conservation across more than two species was detected (Fig. 2.6 B). The identified alignments were therefore taken only as weak hints for functional relevance. When having a look directly at the nucleotide alignment of all three species, the overall divergence of the *H. oligactis* sequence upstream the

indicated conserved region (-415 to -383 bp, red bar) became evident (Fig. 2.7). Taken together, these results pointed to important elements within the first 440 bp upstream of ATG.

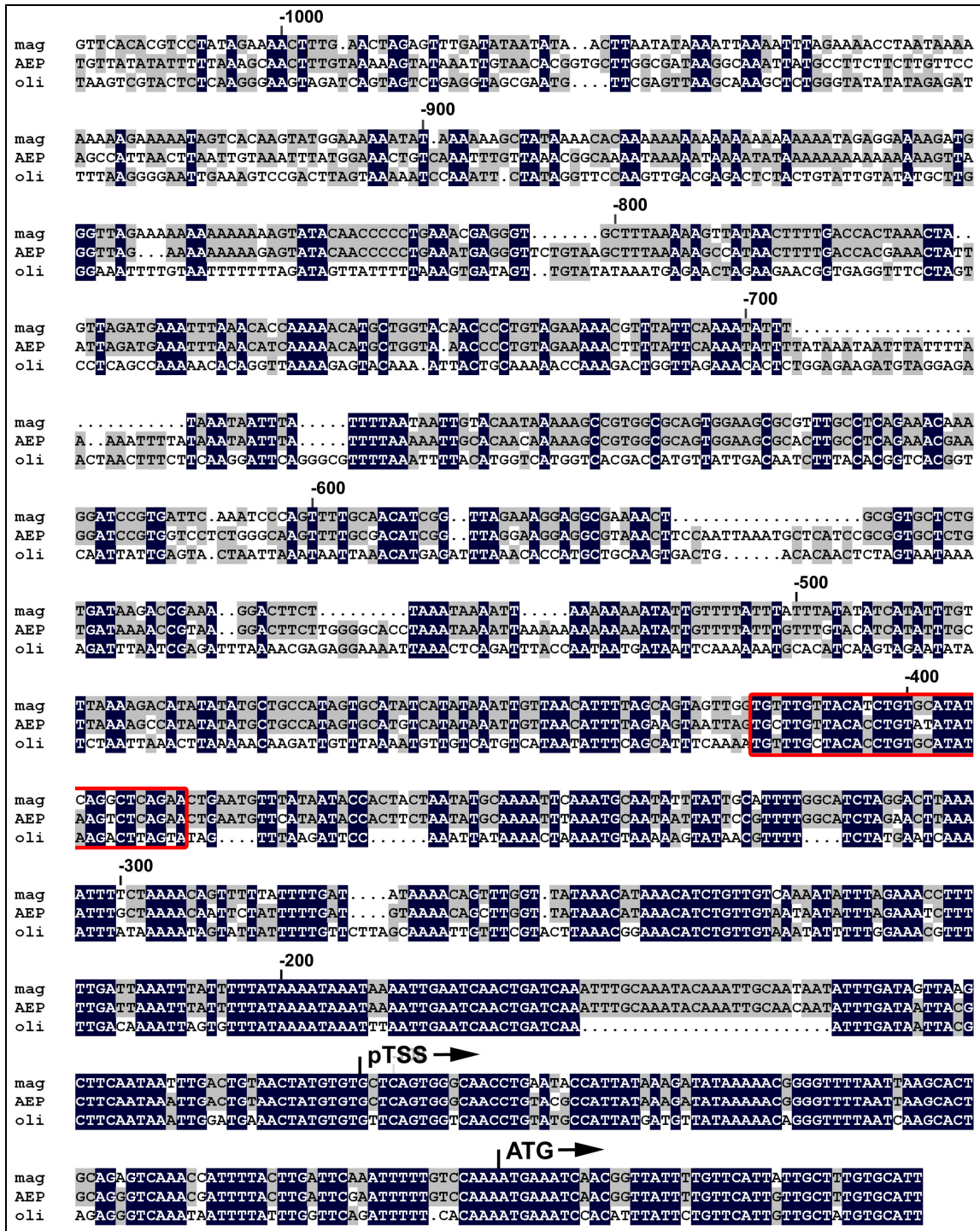


Fig. 2.7: Multiple alignment of 1,090 bp of *HyDkk1/2/4* C' 5' flanking region from *H. mag*, *H. AEP* and *H. oli* indicates high sequence identities within the first 440 bp upstream ATG. Red box: conserved region indicated by the GATA software, pTSS: putative transcription start site, ATG translation start.

To get insights into the length of the 5' untranslated region of *HyDkk1/2/4 C* I used the EST data to deduce the transcription start site (TSS). The ESTs with the longest 5' information (ESTs: CN559611, CN559480, gene model: Hma1.134829) indicated a transcription start at – 103 bp upstream ATG (see Fig. 2.7).

As the phylogenetic analysis showed *H. viridis* as the most distantly related species I wondered if inclusion of this species into the analysis would result in a more detailed picture of putative functional elements within 500 bp proximal to ATG and compared in a second GATA comparison the *H. viridis* sequence to the other three species. When comparing *H. viridis* to *H. magnipapillata* only two TA-rich alignments were indicated under the chosen standard conditions which were not detected in the comparisons with *H. vulgaris* (AEP) and *H. oligactis* (data not shown), which marked them as weak positive elements. Interestingly a sequence of 26 bp was shown to be conserved within *H. vulgaris* (AEP), *H. oligactis* and *H. viridis* (Fig. 2.6 C). As this conserved sequence started 42 bp in front of the putative transcription start I interpreted this region as part of the *HyDkk1/2/4 C* core promoter (putative Core Promoter: pCP, see Fig. 2.6 C). The particular sequence within *H. magnipapillata* showed two further nucleotide exchanges suggesting a core promoter consensus start of ATTTxXTAxTTAxXCTxCAATAxTT (see Fig. 2.8 A). A bona fide TATA signal could not be detected in this region. The core promoter might include the transcription start site and 19 additional nucleotides further downstream (Fig. 2.8 A, blue bar).

The multiple nucleotide alignment of all four species demonstrated that only few conserved islets are still detectable, which might represent evolutionary conserved functional sequences and thereby putative proximal promoter elements (Fig. 2.8 A).

To account for possible elements which were not detectable in the multiple alignment due to shuffling or to misalignment caused by diverging sequences I performed a further analysis using the Footprinter 3.0 resource (<http://genome.cs.mcgill.ca/cgi-bin/FooterPrinter3.0/FooterPrinterInput2.pl>), which uses the phylogenetic relationship among homologous sequences to identify the best conserved motifs. When using a motif size of eight nucleotides and a maximum number of allowed mutations of one, it reported ten motives from which elements 1 - 7 showed a collinear arrangement (represented as colored boxes in the multiple sequence alignment, Fig. 2.8 A, B). Interestingly the analysis suggested elements 6 and 7 are localised in more 5' direction in the three other species compared to *H. viridis* (Fig. 2.8 A, B). In particular element 5 (red Box) displays a remarkable conservation (Fig 2.8 A, B).

The previously addressed conserved element within the other three species (see Fig. 2.7) was not supported by the *H. viridis* sequence information as could be seen in Fig. 2.8 A (grey bar).

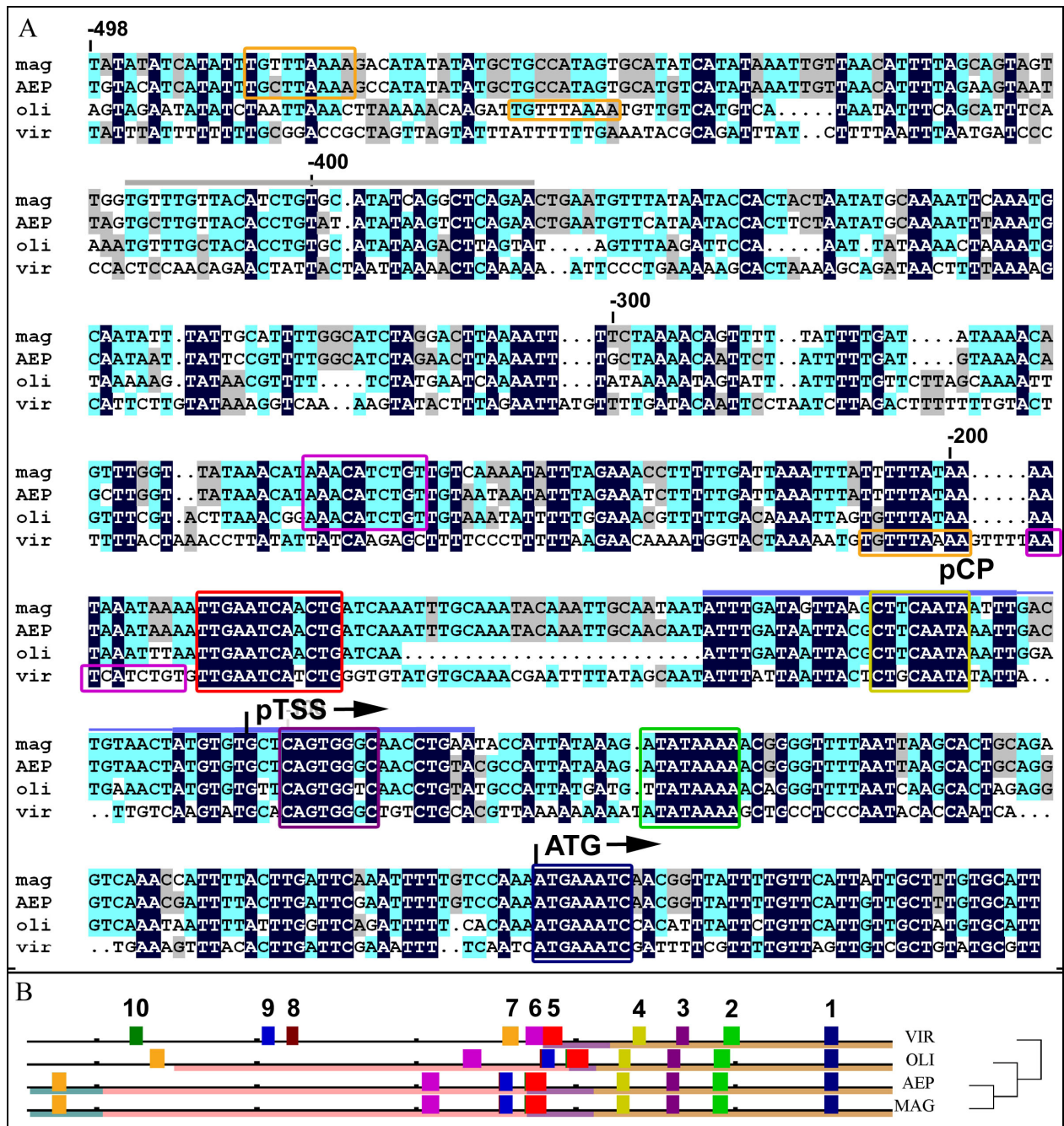


Fig. 2.8: Analysis of 500 bp of *HyDkk1/2/4* C 5' flanking region from *H. mag*, *H. AEP*, *H. oli* and *H. vir* indicates core promoter (pCP) and putative cis-regulatory elements. (A) Multiple alignment of sequence information from -500 to 42 bp of the *HyDkk1/2/4* C gene from the four species. grey bar: previously addressed element (Fig. 2.7), pCP (blue bar): putative core promoter, pTSS: putative Transcription start site, ATG translation start, various coloured boxes indicate conserved elements 1 – 7 from the Footprinter 3.0 analysis shown in B. (B) Phylogenetic footprinting analysis using Footprinter 3.0 indicates conserved octamers of sequence.

Taken together, the phylogenetic comparison of the *HyDkk1/2/4 C* 5'-flanking region including four different *Hydra* species shed light on conserved elements and pointed to the importance of the proximal region of the 5'-flanking region up to -500 bp. Especially the inclusion of sequence information from *Hydra viridis* allowed further refinement of the conservation profile. Nevertheless, as could be seen in figures 2.5 A and 2.6 A, sequence conservation between the closely related species *H. magnipapillata* and *H. vulgaris* (AEP) decreases around - 1 kb. The most conservative interpretation of these results pointed to important regulatory elements within 1 kb upstream ATG of the *HyDkk1/2/4 C* gene. To functionally characterize this putative regulatory sequence *in vivo*, I generated transgenic polyps which express EGFP under the control of 1,027 bp *HyDkk1/2/4 C* 5' -flanking sequence from *Hydra magnipapillata*.

2.4 Functional analysis of the *HyDkk1/2/4 C* promoter using transgenic *Hydra*

Stable transgenesis via microinjection in eggs of *Hydra vulgaris* (AEP) had been successfully established using a circular transfection construct in which an EGFP reporter was driven by 5'-flanking region of the actin gene (Wittlieb et al., 2006). Depending on the cell lineage in which the integration took place, EGFP expression could be observed within the ectodermal, the endodermal or the interstitial cell lineage of the hatchling. In the case of multiple integrations within different cells of the developing embryo, which frequently happened (Wittlieb et al., 2006), more than one cell lineage was affected. Depending on the time point of integration, the ratio between transgenic to non transgenic cells of the hatchling varied. As gland cells in *Hydra* are derivatives of the interstitial cell line (Schmidt and David, 1986; Bode et al., 1987), the integration of the *HyDkk1/2/4* transfection construct had to take place within a cell of the interstitial cell lineage capable of differentiation into a zymogen gland cell. In the case of a reporter which is driven by a functional gland cell specific regulatory region these transfected interstitial cells should not be identifiable. Only in case of differentiation towards a zymogen gland cell, which may include precursor states, within the correct spatial domain the cell should start to produce the reporter protein (see Fig. 2.9 A).

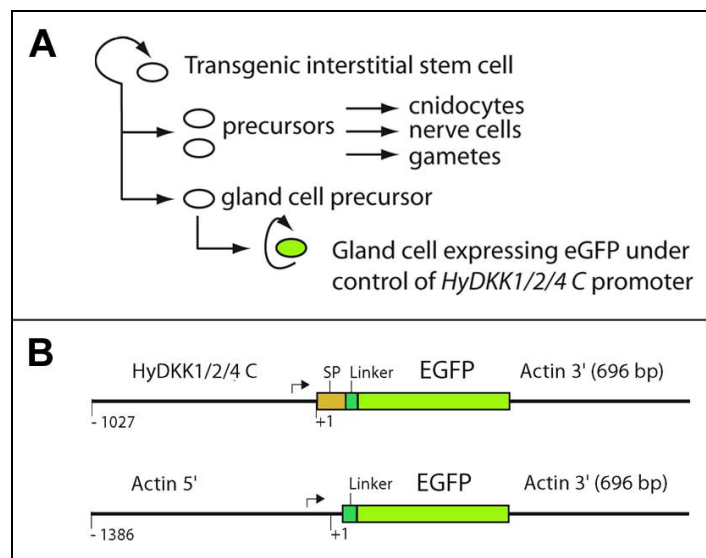


Fig. 2.9: Generation of transgenic polyps expressing EGFP under control of the *HyDkk1/2/4 C* promoter. (A) Interstitial cell differentiation in *Hydra*: Integration of the transgene in the interstitial cell lineage should result in gland cell specific reporter activation. (B) Expression constructs for generation of transgenic *Hydra*. Top: Construct containing 1,027 bp *HyDkk1/2/4 C* 5' flanking region and 66 bp coding sequence comprising the *HyDkk1/2/4 C* signalpeptide and three additional residues. Below: Control construct with EGFP driven by 1,386 bp Actin 5'-flanking region. Modified from Siebert et al., 2007.

As mentioned before, the *HyDkk1/2/4* genes contain predicted signal peptides. To address whether the signal peptide influences the reporter protein localisation it got incorporated into the transfection construct. The transformation construct was therefore made by placing the 1,027 bp *HyDkk1/2/4* C 5'-flanking region and 66 bp coding sequence in front of the reporter gene EGFP (Fig. 2.9 B top, see ch. 7.14.1). The coding sequence comprised information for the predicted *HyDkk1/2/4* C signal peptide and three subsequent residues, to allow proper cleavage. Animals expressing EGFP in the interstitial cell lineage under the control of the actin promoter (Fig. 2.9 B bottom) were used as controls (Khalturin et al., 2007).

2.5 The 5'-flanking region of *HyDkk1/2/4* C directs reporter gene expression in zymogen gland cells in a pattern that recapitulates the endogenous expression pattern

None of the embryos showed an EGFP signal before hatching, which usually occurred two weeks after injection. Out of 66 injected embryos, 21 (32%) hatched from which two lines contained EGFP positive gland cells and no EGFP expression in any other cell type. Whereas one of these lines developed an epithelial phenotype and stopped feeding, the founder population of the second line could further expand into a mass culture by clonal propagation via budding. Since founder polyps had only very few positive gland cells (Fig. 2.10 A), the integration event most likely took place late in the precursor population.

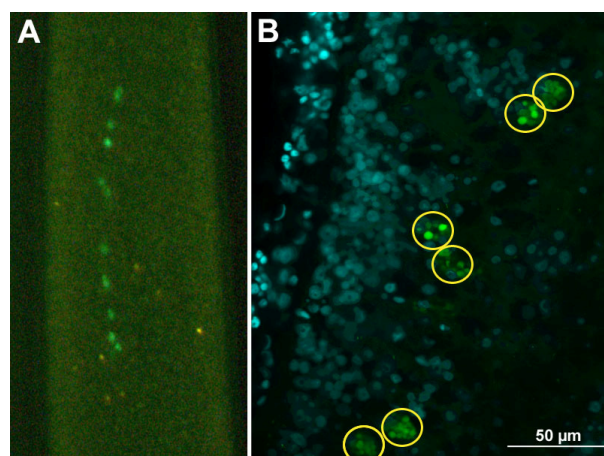


Fig. 2.10: Transgenic gland cells in *Hydra*. (A) Stereo microscopy identifies rows of transgenic zymogen gland cells (ZMG). (B) Confocal analysis of a polyp containing EGFP expressing cells reveals that transgenic gland cells occur in pairs indicating mitotic activity. Green: EGFP expressing cells. Blue, HOECHST stained nuclei. Modified from Siebert et al., 2007.

Stereo- and confocal microscopy (Fig. A and B) showed pairs of gland cells lined up in rows along the body column. This is consistent with the view (Schmidt and David, 1986; Bode et al., 1987) that differentiated gland cells undergo cell divisions. Therefore these animals provide first *in vivo* evidence for characteristics previously described for gland cells. In order to get a larger fraction of gland cells carrying the transgene I started mass-culturing and selected for animals with high numbers of positive gland cells. Fig. 2.11 shows an example of such a polyp and demonstrates that the 1,027 bp 5'-flanking region of the *HyDkk1/2/4 C* gene is able to direct the expression of EGFP in gland cells in a pattern that recapitulates precisely the endogenous expression pattern of the *HyDkk1/2/4 C* gene (Fig. 2.11 see insert for comparison). These results identify the 1.027 bp as sufficient for *HyDkk1/2/4 C* expression *in vivo*.

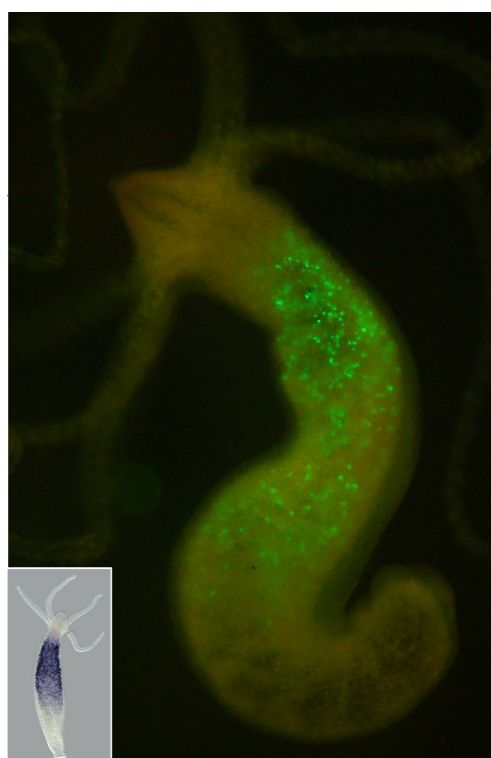


Fig. 2.11: Polyps carrying the *HyDkk1/2/4 C* driven transgene show authentic reporter localisation. Polyp in which many but not all ZMGs are expressing *EGFP*. Note that the EGFP distribution recapitulates the *HyDkk1/2/4 C* expression shown in inser. Modified from Siebert et al., 2007.

Since cells in *Hydra* are continuously proliferating and continuously displaced towards the apical respectively basal end of the body axis and since EGFP appears to be a relatively stable reporter gene (see below), passive translocation of the reporter protein towards the terminal regions was expected. Surprisingly, transgenic animals showed a relatively sharp border of zymogen gland cells with EGFP+ vesicles (Fig. 2.11) indicating spatially controlled loss of the EGFP-reporter protein.

2.6 The HyDkk1/2/4 C signal peptide directs the reporter protein into secretory vesicles

Confocal microscopy was used to analyse the reporter localisation within the transgenic cells. The incorporated signal peptide apparently drives the reporter protein into secretory vesicles within the zymogen gland cells (Fig. 2.12 A). In control transgenic *Hydra* expressing EGFP in ZMGs driven by the *Hydra* actin promoter without signal peptide, the reporter protein is localised in the cytoplasm (Fig. 2.12 B).

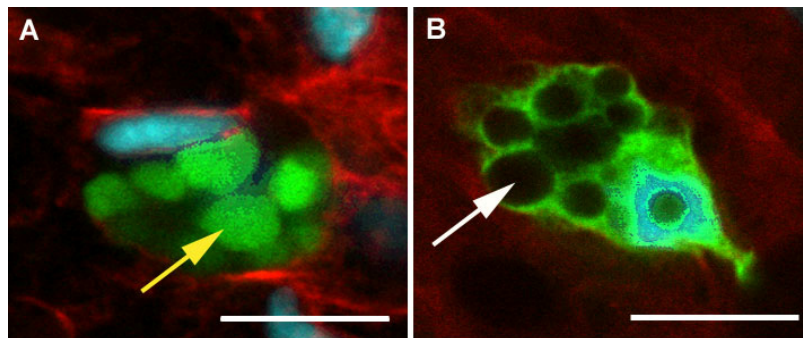


Fig. 2.12: EGFP localisation in transgenic zymogen gland cells. (A) Confocal analysis of transgenic ZMG in the gastric region showing EGFP protein localized in secretory vesicles (yellow arrow). Green, EGFP protein; blue, HOECHST stained nucleus; red, actin filaments, scale bar 10 μ m. **(B)** Control transgenic polyp with EGFP expression under control of actin promoter. Note that EGFP is localized within the cytoplasm and secretory vesicles are EGFP negative (white arrow). Green, EGFP protein; blue, HOECHST stained nucleus; red, actin filaments, scale bar 10 μ m. Modified from Siebert et al., 2007.

2.7 Transgenic zymogen gland cells change their phenotype when they change their position along the body axis

Transgenic polyps with ZMGs marked by the expression of EGFP were used to follow the fate of individual gland cells and to determine whether individual ZMGs would change their phenotype upon changes of their position along the body axis. Confocal microscopy was used to examine labelled ZMGs located in the subtentacle region (Fig. 2.13 A) at the boundary of *HyDkk1/2/4 C* expression. In contrast to transgenic ZMGs within the gastric region which showed large secretory granules packed with EGFP (Fig. 2.12 A), transgenic ZMGs in the subtentacle region were found which have only few granules filled with EGFP (Fig. 2.13 B - D) indicating transcriptional inactivation of the transgene and gradual release of EGFP+ vesicles.

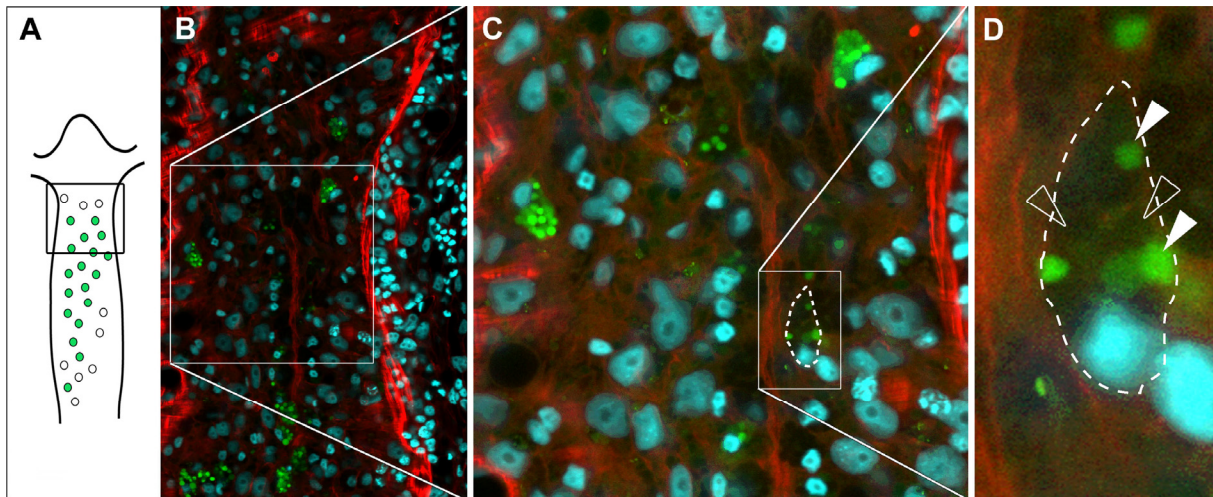


Fig. 2.13: Monitoring individual EGFP expressing gland cells at the boundary of *HyDkk1/2/4 C* expression. (A) Scheme of region of interest. (B) Confocal microscopy. Green, EGFP protein; blue, HOECHST stained nucleus; red, actin filaments. (C) Magnification of area shown in B. (D) Magnification of gland cell indicating that only part of the secretory vesicles are filled with EGFP protein. Figure taken from Siebert et al. 2007.

I next considered the behaviour of labelled ZMG gland cells during head regeneration. As shown in Fig. 2.14 A, transgenic polyps were cut within the maximum of EGFP positive cells shortly below the boundary of expression (Fig 2.14 A) and were allowed to regenerate a head. Sharply contrasting the transgenic animal shown in Fig. 2.11, regeneration resulted in animals (n=100) which all contained numerous EGFP+ ZMGs in the head region (Fig. 2.14 B, C) indicating that these cells took part in a morphallactic regeneration process. In the absence of feeding, such EGFP+ gland cells can be detected in the regenerating head for more than 4 weeks post-regeneration (data not shown). Thus, changing the position along the body axis does not cause a ZMG to immediately release the EGFP reporter from the vesicles.

Confocal microscopy indicated (Fig. 2.14 D, E) that not all gland cells present in the regenerating head tissue were EGFP+ and that unlabelled gland cells were interspersed between the labelled gland cells. This may be due to the fact that the transgenic animals used were mosaics comprising EGFP+ gland cells as well as EGFP- gland cells. Alternatively, it may indicate the presence of gland cells which lack a common developmental history with transgenic ZMGs (see below). Interestingly about one week post regeneration (Fig. 2.14 C) the most apical tip of the regenerated head in all animals (n=40) appeared to be at least partially free of EGFP+ cells (see below). As the described head regeneration means essentially a translocation of the gland cell to an ectopic position I next tried to manipulate the environment of the transgenic cells via changing the positional value within the body column using alsterpaullone.

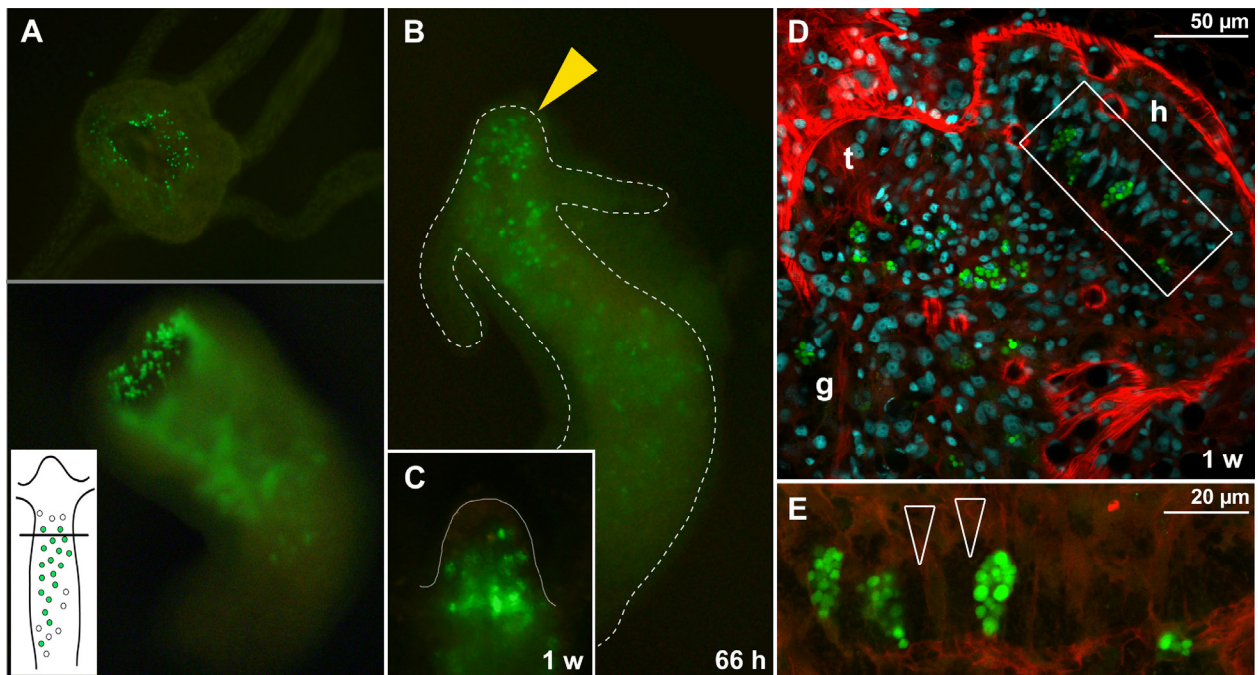


Fig. 2.14: During head regeneration, EGFP labelled zymogen gland cells get integrated in the newly formed hypostomal region. (A) Polyp immediately after cutting. Insert shows cutting level in the upper gastric region. **(B)** Transgenic polyp undergoing head regeneration for 66 hrs. Yellow arrowhead points to the tip of the newly formed head containing EGFP positive cells. **(C)** Insert shows polyp undergoing regeneration for 7 days indicating that the tip of the hypostome is partially free of transgenic ZMGs (n=40). **(D)** Confocal analysis of polyp 7 days after onset of head regeneration demonstrates presence of EGFP positive cells within the newly formed hypostome. t, tentacle; h, hypostome; g, gastric region. **(E)** Magnification of area shown in Fig 3 C shows rows of cells where non labelled cells are interspersed (empty arrow head) between EGFP positive cells. Green, EGFP protein; blue, HOECHST stained nucleus; red, actin filaments. Figure taken from Siebert et al. 2007.

2.8 Zymogen gland cells change their gene expression after elevation of positional value via interference with the canonical Wnt pathway

By inhibiting GSK 3 β , alsterpaullone leads to the stabilisation of beta-catenin which in turn translocates into the nucleus and activates effector genes of the Wnt-pathway. As a consequence head specific genes are activated all over the body column, ectopic tentacles and eventually ectopic heads form. I monitored the conversion using the head specific gene *ks1*. At d9 after the start of treatment animals could be observed, which had developed numerous ectopic tentacles and which showed *ks1* expression all over and thereby indicating a conversion to head tissue. In the course of the experiment *HyDkk1/2/4 C* transcription was progressively ceased (data not shown, see Augustin et al., 2006) and animals free of *HyDkk1/2/4 C* transcript could be observed on day 9 as

shown in Fig. 2.15 B. Interestingly in transgenic animals (n=30) the silencing of the reporter transcription did not result in the disappearance of the EGFP signals in ZMGs (Fig. 2.15 C, animal on d9 after start of treatment) indicating not only the persistence but also a switch of the transcriptional signature of these cells.

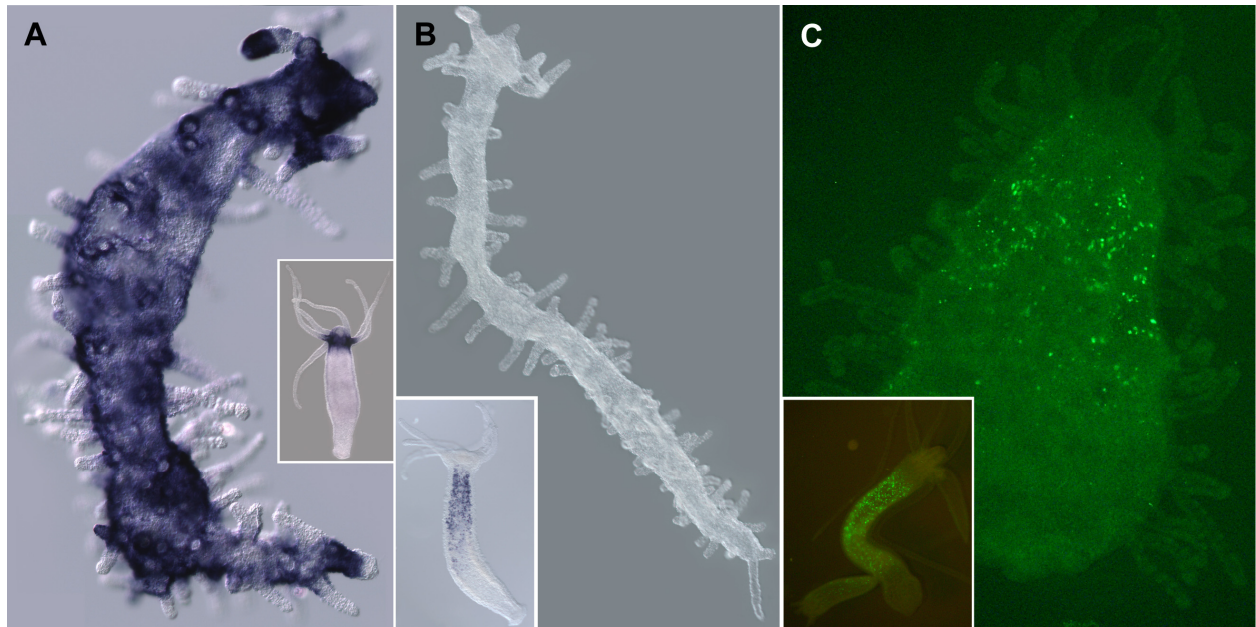


Fig. 2.15. Canonical Wnt activation via alsterpaullone treatment leads to transcriptional inactivation of *HyDkk1/2/4 C* expression. Animals shown 9 days after beginning of the treatment. **(A)** Headspecific *ks1* expression is activated all over the body column in the course of the treatment. **(B)** Changing of the cellular environment leads to successive inactivation of *HyDkk1/2/4 C* transcription and results in *HyDkk1/2/4 C* transcript free animals. **(C)** Animals carrying transgenic zymogen cells keep the EGFP signal even after ceasing *HyDkk1/2/4 C* driven EGFP transcription in the course of the treatment. Inserts show untreated control animals.

2.9 Zymogen gland cells change their gene expression when they change position in the course of regeneration

To get deeper insights into the observed transcriptional inactivation of *HyDkk1/2/4/EGFP* expression and to determine whether the gradual disappearance of EGFP+ secretory granules indicated in Fig. 2.13 (B to D) is a result of an inactivation of the EGFP reporter gene, I performed *in situ* hybridisation with both a *HyDkk1/2/4 C* and an *EGFP* specific probe with head regenerating polyps. Both probes gave identical results. As shown in Fig. 2.16 (A-C) for the *EGFP* probe, regeneration is accompanied by gradual disappearance of EGFP transcripts in cells of the regenerating tip whereas EGFP protein was persisting in gland cell vesicles within head regenerating tissue (Fig. 2.16 D). EGFP transcripts were detectable up to 48 h after cutting (Fig. 2.16 B). Since

the regenerating animals shown in Fig. 2.14 (B, C) and Fig. 2.16 D were not fed during the experimental period, I next tested whether feeding would influence the stability of the EGFP filled vesicles. Fig. 2.16 (E and F) shows that feeding of the regenerated animals results in successive release of the secretory granules and re-establishment of the *HyDkk1/2/4 C* specific spatial distribution pattern.

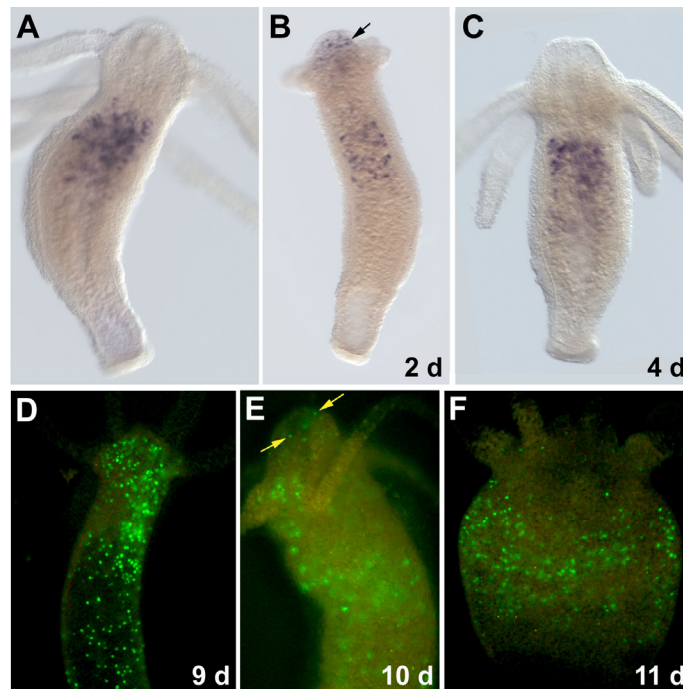


Fig. 2.16: Disappearance of EGFP and *HyDkk1/2/4 C* transcripts in the course of head regeneration (A-C) in contrast to the persistence of EGFP protein in the absence of feeding (D). (A) *In situ* hybridisation using a EGFP probe in control polyps. (B) *In situ* hybridisation using an EGFP probe in a polyp undergoing head regeneration for 2 days (n=30). Arrowhead indicates localization of transcript in cells of the regenerating tip. (C) 4 days after head regeneration no EGFP transcript can be detected anymore in cells within the head region (n=30). (D) In a polyp 9 days following decapitation EGFP protein is present in gland cell vesicles in the tip of the regenerated head. (E) Feeding of polyp in D results in rapid release of EGFP from vesicles. Polyp on day 10 after decapitation. (F) Feeding of polyp in E results in release of remaining EGFP positive vesicles and re-establishment of EGFP distribution pattern seen in steady state polyps (see Fig 1 H). Polyp on day 11 after decapitation. Figure taken from Siebert et al. 2007.

Since feeding appears to trigger the release of secretory vesicles, this may also explain the fact that ZMGs in normal polyps in an axial position where EGFP is transcriptionally inactivated contain both EGFP positive and EGFP negative vesicles (Fig. 2.13 D). Following one more feeding event, the remaining EGFP signal in the cell shown in Fig. 2.13 D will disappear and the cell will become EGFP negative. Taken together, EGFP labelling allowed not only to track translocation of transgenic gland cells during

regeneration and chemical manipulation of the tissue, but also to conclude that ZMGs originating from the gastric region are ending up in head tissue. These observations also indicated that the transition of a ZMG from gastric to head tissue involves turning off their zymogen gland cell specific gene expression (Fig. 2.16, A –C) and resetting the gene expression programme.

2.10 Histological studies verify that gland cells in different position have different morphologies and that intermediate gland cell morphologies can be found in regenerating tissue

Monitoring individual EGFP labeled ZMGs during regeneration has shown (Fig. 2.14 A, B and 2.16 D) that these cells are incorporated in head tissue. Despite the drastic change in spatial location, morphological features such as the size and shape of EGFP positive vesicles appeared to remain constant - as long as polyps were not fed (Fig. 2.14 D, E). So I tried to follow the transition also by using histological means.

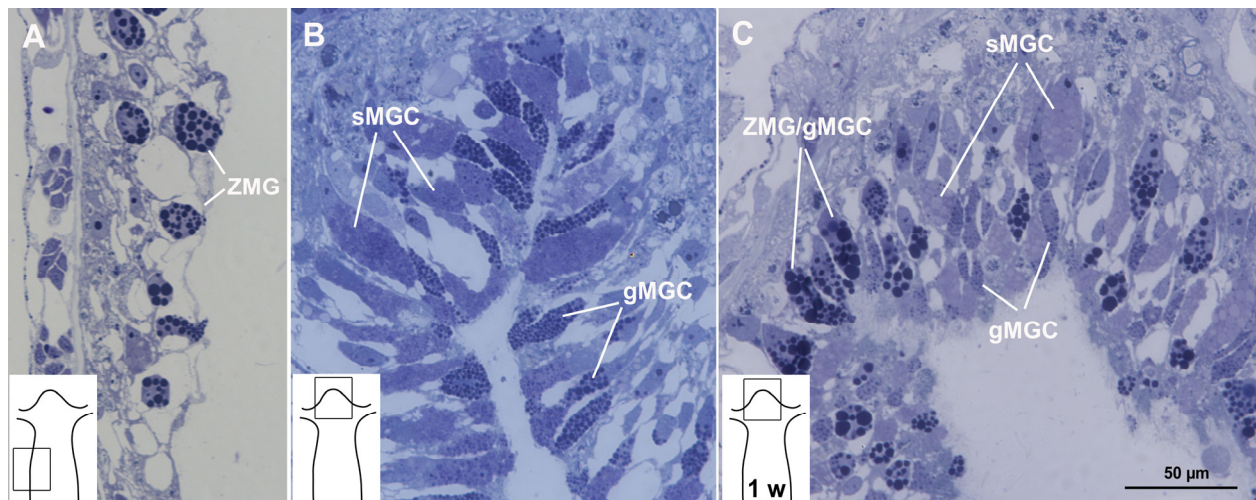


Fig. 2.17: Histology of normal and head regenerating polyps. (A) Histology of gastric region. ZMG, zymogen gland cell; **(B)** Histology of head region. sMGC, spumous mucous gland cell; gMGC, granular mucous gland cell. **(C)** Histology of regenerating head. sMGC, spumous mucous gland cell; gMGC, granular mucous gland cell. ZMG/gMGC, a cell originating from the former gastric region displaying morphological features typical for both ZMG and gMGC. Figure taken from Siebert et al. 2007.

Fig. 2.17 shows that in histological sections ZMGs in gastric region (Fig. 2.17 A) could be distinguished from the two types of MGCs in the head (Fig. 2.17 B) due to the differences in cell shape and size of the secretory vesicles.

While gMGCs contained numerous small granules in high density (Fig 2.17 B), sMGCs are larger in size and contained less conspicuous vesicles. As shown in Fig. 2.17 C, in

animals undergoing head regeneration in the absence of feeding, ZMGs can be clearly identified in the regenerating tip. This suggests that these ZMGs are in the process of transdifferentiation into head specific MGCs. Fig. 2.17 C also shows, that in addition to ZMGc the regenerated hypostome contains gMGCs and sMGCs indicating that these cells which are normally absent in the gastric region, represent newly differentiated MGCs (see below).

Ultrastructural analysis (Fig. 2.18) confirmed this view and showed in regenerating head tissue the presence of gland cells with vesicles of both the ZMG and the gMGC type (Fig. 2.18 E and F).

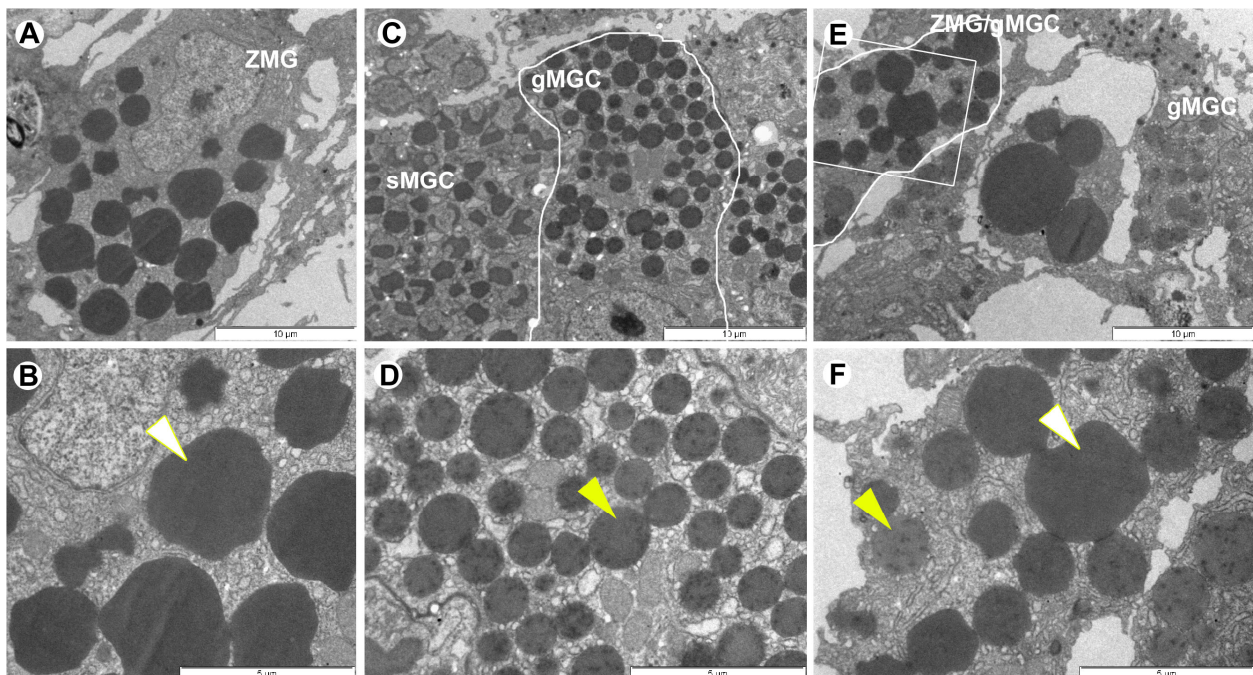


Fig. 2.18: Ultrastructure of gland cells in normal and head regenerating polyps. (A, B) Zymogen gland cell (ZMG) of the gastric region. (B) Magnification of area in A shows large vesicles which occur homogenous (white arrow). (C) Spumous mucous gland cell (sMGC) and a granular mucous gland cell (gMGC) in a non regenerated hypostome. (D) Magnification of area in C shows gMGC characterised by small vesicles which occur spotty (yellow arrow). (E) Cell in transition in a regenerated hypostome (1 week). ZMG/gMGC: cell with features of both kind of cells, gMGC: granular mucous gland cell. (F) Magnification of area in E. Cell contains both homogenous, large vesicles characteristic for ZMGs (white arrow) as well as small, spotty vesicles characteristic for gMGCs (yellow arrow). Figure taken from Siebert et al. 2007.

In figures 2.18 A and B, ZMGs of the gastric region are shown which are characterised by large secretory granules of homogeneous occurrence. In contrast, hypostomal gMGCs (Fig. 2.18 C and D) have much smaller granules which contain conspicuous electron dense spots.

An ultrastructural analysis of tissue which undergoes head regeneration (Fig. 2.18 E and F) identifies gland cells with both types of granules. The presence of such gland cells in regenerating tissue is consistent with the view that gastric ZMG can convert to a mucous granular cell type. Taken together with the observations shown above (Fig. 2.16 to 2.17) these results provide compelling evidence that gMGCs in the regenerated hypostome originate from ZMGs in the gastric region by a transdifferentiation process. Cells which contained ZMG vesicles and vesicles of the sMGC type could not be observed in regenerated hypostomes.

2.11 Identification of interstitial cells which give rise to granular mucous gland cells

Previous observations (Rose and Burnett, 1970; Dübel, 1989) indicated that hypostomal mucous cells may originate from interstitial cells. Interstitial cells, however, were reported (David and Plotnick, 1980) to be present predominantly in the gastric region and only present in very low numbers in head and foot regions. Fig. 2.19 shows a pair of spindle shaped large interstitial cells from a mazerate of *Hydra* hypostomes.

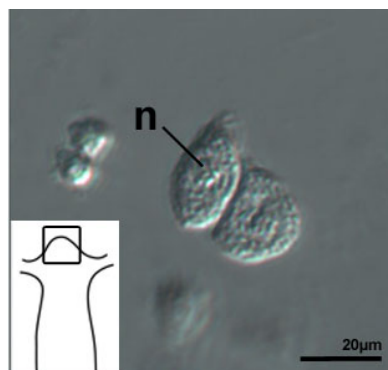


Fig. 2.19: Interstitial stem cell pair from mazerated hypostomes: n: nucleus

To assess the contribution of stem cell-based mechanisms in hypostomal mucous cell differentiation, I used *in situ* hybridisation and a molecular probe, *HyTSR1*, known (C. Guder, pers. com., Miljkovic-Licina et al., 2004) to be expressed exclusively in gMGCs of the hypostome. To identify both the gastric ZMGs and the hypostomal gMGCs populations I performed double *in situ* hybridisation using *HyTSR1*-specific and *HyDkk1/2/4 C* specific probes. As shown in Fig. 2.20 (A), in tissue undergoing head regeneration for 36 h both gland cell populations can clearly be distinguished. *HyTSR1* expressing cells are restricted to the regenerating tip while *HyDkk1/2/4 C* expressing

ZMGs can be found along the body column and also in the regenerating tip between the *HyTSR1* cells.

Strikingly, when examining the morphology of *HyTSR1* expressing cells in *Hydra* regenerates in more detail at higher magnification (Fig. 2.20 B) it could be shown that they vary both in cell shape and intracellular localization of the *HyTSR1* transcripts. Some *HyTSR1* expressing cells did not contain any vesicles and had an overall morphology of large interstitial cells (Fig. 2.20 C, Fig. 2.19). Other *HyTSR1* expressing cells (Fig. 2.20 D and E) had morphological features typical for hypostomal granular mucous gland cells with small vesicles located at one end of the cell and an *in situ* signal in the cytoplasmic region around the nucleus. To examine these cells in greater detail an ultrastructural analysis of *Hydra* tissue undergoing head regeneration for 48 and 96 h was performed. As shown in Fig. 2.20 F, within the gastrodermal epithelium near the mesoglea interstitial cell-like cells with large nuclei could be observed. Interestingly, within the cytoplasm of these cells a well developed endoplasmatic reticulum (ER) could be identified (Fig. 2.20 F). This is in contrast to interstitial cells in the gastric region which do not contain an elaborated ER. In 96 h regenerates non-epithelial cells of spindle-like shape (Fig. 2.20 G) were present, which in addition to a well developed endoplasmatic reticulum had secretory granules with the described (compare Fig. 2.18 D) spotty character of mucous granular cells. The observations suggested that the *HyTSR1* expressing cells shown in Fig. 2.20 (C to E) represent lineage-restricted interstitial cells which in the head differentiate into mature gMGCs. This showed that stem cell-based differentiation of gMGCs is an integral part of the gland cell differentiation pathway in the course of regeneration. The absence of EGFP+ gland cells in the most apical tip of the polyps examined one week after onset of regeneration (Fig. 2.14 C) supported that notion.

Most interestingly, when performing *in situ* hybridisation with 96 hrs regenerates using *HyTSR1* probe not only mature gMGCs expressing *HyTSR1* could be identified (Fig. 2.20 H) but also *HyTSR1* expressing gland cells which clearly showed morphological features typical for ZMGs of the gastric region. Fig. 2.20 I shows, that some of them not only expressed the gMGC-specific gene *HyTSR1* but even contained a mixture of small and large vesicles. This suggested that these “chimeric” cells were caught in transdifferentiation from a gastric ZMG towards a head-specific gMGC.

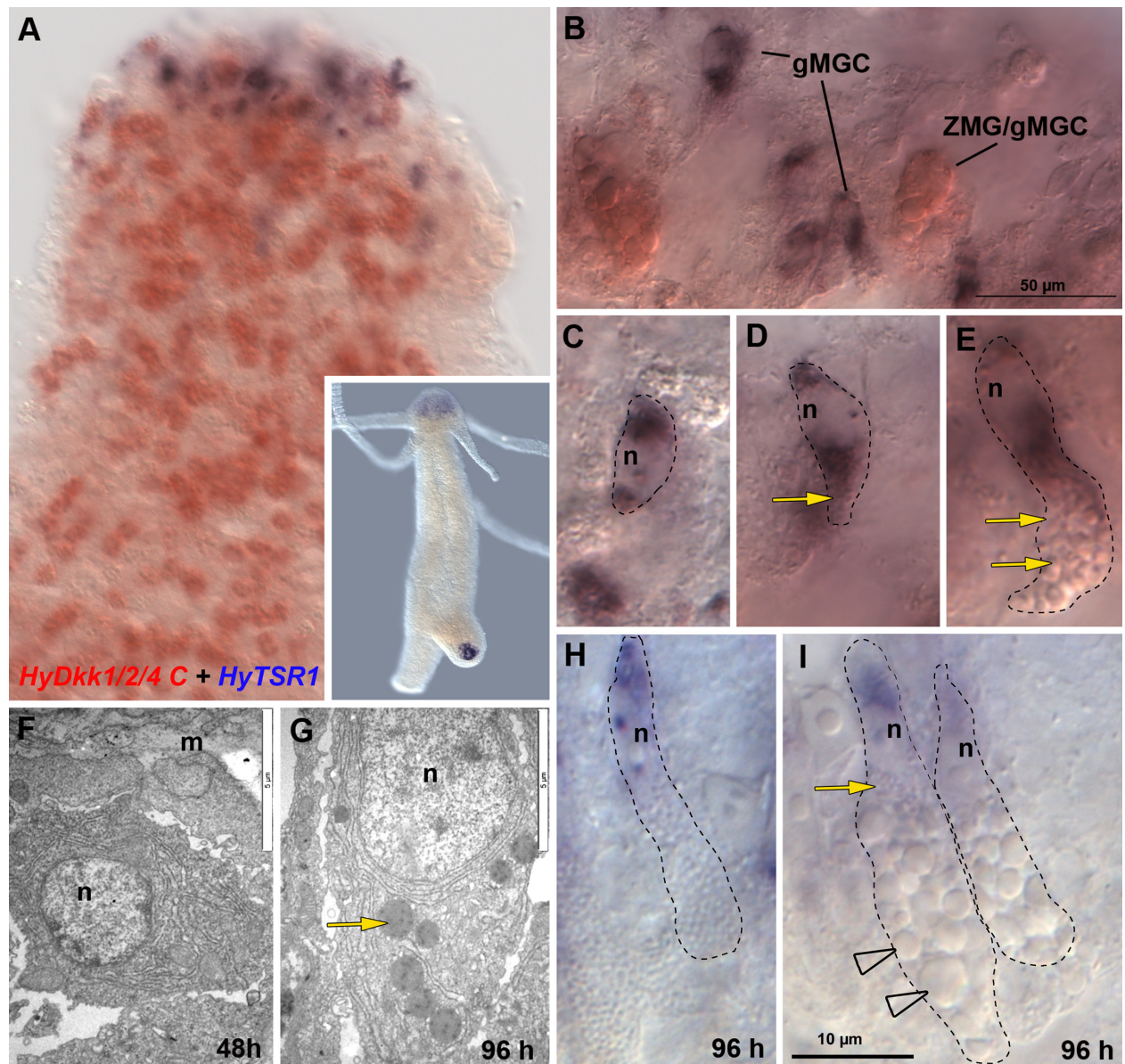


Fig. 2.20: Molecular evidence for two sources of hypostomal gMGCs: new differentiation from a subpopulation of undifferentiated interstitial cells and transdifferentiation from zymogen cells. (A) Expression profile of *HyDkk1/2/4 C* and *HyTSR1* in the regenerating tip 36 h after decapitation. Insert shows the *HyTSR1* expression pattern in budding polyp. (B) Larger view of area in regenerating tip shows ZMGs and new evolving gMGCs from spindle shaped cells. (C-E) Different morphologies of *HyTSR1* expressing cells in 36 h regenerate: (C) early developmental stage showing large nucleus and distinct transcript localisation at the two poles of the cell, (D) advanced stage showing first secretory vesicles at one pole of the cell, (E) later stage with a transcript-free secretory part of cell filled with vesicles (yellow arrows). (F) Ultrastructural analysis reveals undifferentiated cells with large nuclei and distinct endoplasmic reticulum within the endoderm (48 h regeneration, m: mesoglea) without secretory vesicles and (G) with secretory vesicles (yellow arrow) showing electron dense spots characteristic for gMGCs (96 h regeneration). (H, I) Expression of *HyTSR1* in a regenerated tip 96h after decapitation in a mature gMGC (H) and in a gland cell showing ZMG morphology (I) indicating a transdifferentiation event. Arrow: small gMGC-like vesicles, Arrowhead: large vesicles typical for ZMGs. Figure taken from Siebert et al. 2007.

3 Discussion

3.1 Functional analysis of the the *HyDkk1/2/4 C* promoter

Phylogenetic comparisons of the orthologous 5'-flanking regions from four different *Hydra* species lead to insights into the conservation of regulatory regions of the *HyDkk1/2/4 C* gene and the identification of conserved elements with potential functional relevance. Comparison of *Hydra magnipapillata* and *Hydra vulgaris* (AEP) sequences suggested that all relevant elements needed for temporal and spatial expression reside within the first 1,000 bp upstream ATG (Fig. 2.5 A). By using 1,027 bp of 5'-flanking region of the *HyDkk1/2/4 C* gene to control EGFP expression I generated transgenic polyps which indeed faithfully recapitulated *HyDkk1/2/4 C* expression which characterises this region as sufficient for authentic expression (Fig. 2.11).

Cis-elements conserved in multiple and distantly related species have a higher probability to be functional than non-functional (Bergman et al., 2002). Hence, comparisons including the species *Hydra oligactis* and *Hydra viridis*, the latter being the most basal *Hydra* species with respect to the genus' phylogeny (Fig. 1.2), promised to enhance our understanding of the essential elements in *HyDkk1/2/4 C* regulation. As sequence conservation between *H. magnipapillata* and *H. oligactis* declines at about -430 bp (Fig. 2.7), I reasoned that this region might be sufficient for driving the reporter in the expected manner. Especially the inclusion of the most distantly related species *Hydra viridis* in the study resulted in a higher resolution of potential regulatory element identification and suggests that this species might aid functional annotation of cis-regulatory sequences in further analyses of this kind (Fig. 2.8).

Sequence similarity and genomic localisation of *HyDkk1/2/4* genes pointed to a gene duplication event (Fig. 2.3). Furthermore *HyDkk1/2/4 C* is expressed in a subpopulation of *HyDkk1/2/4 A* positive cells (Augustin et al., 2006). However no obvious sequence similarity was detected when comparing 5'-flanking regions from *HyDkk1/2/4 A* and *C* in *H. magnipapillata* (Fig. 2.5 C). This suggests that the cell type specific transcriptional activation is still retained but 5'-sequence divergence after the gene duplication altered transcription patterns (see below). As duplication events remove selective pressures and give room for diversification of cis-regulatory regions and coding sequence, they are seen as an important mechanism in the generation of diversity (Krakauer and Nowak, 1999). Gene duplications have tremendously contributed to the genomic

complexity in Cnidarians (Chourrout et al., 2006; Thomsen and Bosch, 2006; Bielen et al., 2007).

Two strategies will be crucial in the further characterisation of the *HyDkk1/2/4 C* promoter: (i) generation of transgenic animals carrying truncated versions of the functional promoter to assess the *in vivo* relevance of potential regulatory elements revealed by my analysis, and (ii) *in vitro* testing of the *in silico* predicted elements in DNase I footprinting and electrophoretic mobility shift assays.

For the further functional dissection of cis-acting elements, the development of a reliable integration reporter is critical. This is especially needed in projects comparable to the one described here, where the transgene expression is only activated in an interstitial cell derivative. Ideally, an integration reporter would indicate transfection of the precursor, the interstitial cell, and the reporter of interest would be activated in the course of a differentiation event. To test if a truncated version of the promoter still supports normal *HyDkk1/2/4 C* transcription, first injections of *Hydra* embryos with a modified transformation vector (originally designed by K. Khalturin, unpublished) carrying a cassette of 474 bp *HyDkk1/2/4 C* promoter driving EGFP and Actin::dsRed as an integration reporter have been performed. This work is still in progress.

DNase I protection assays are a useful tool in the determination of DNA-Protein interaction and have been successfully applied in *Hydra* (Endl et al., 1999). Labelled DNA to be analysed is incubated with either nuclear extract or recombinant protein and is partially protected by bound proteins in a subsequent digestion with DNase I. This leaves a “footprint” when the DNA fragments are separated via gel electrophoresis. As the *HyDkk1/2/4 C* transcription is repressed in the head, which could be demonstrated in various experiments (see Fig. 2.15, 2.16 B, C), a comparison of nuclear protein from head and gastric could reveal differences in the binding pattern on the *HyDkk1/2/4 C* flanking region, thus revealing sequence elements involved in the mediation of the transcriptional boundary. To obtain larger fractions of tissue for the isolation of nuclear protein, alsterpaullone treated animals, in which canonical Wnt signalling is activated, could be used instead of head tissue. As it was shown, *HyDkk1/2/4 C* is transcriptionally silenced in the course of the conversion from gastric tissue towards head tissue (see Fig. 2.15).

In contrast to a previous study, dealing with an ectodermal gene (Endl et al., 1999), here, the gene of interest is expressed in a subpopulation of zymogen cells. Hence in a nuclear extraction from gastric region most of the nuclear protein will come from non secretory cells. Here, a new methodical approach using fluorescence-activated cell

sorting (FACS) might solve this problem and is currently under development with encouraging first result (G. Hemmrich, pers. com.). Using transgenic *Hydra* expressing a reporter in a certain cell type, like here in zymogen cells, an enrichment of these cells after dissociation of the polyps should be feasible. Direct extraction of nuclear protein after cell sorting would provide an enriched cell type specific transcription factor set which could be used in DNase I footprinting or electrophoretic mobility shift assays (EMSA) (Galliot et al., 1995; Siebert et al., 2005). EMSAs are used to verify binding of transcription factors to a DNA sequence element via analysis of migration of the DNA during gel electrophoresis. A fragment of DNA migrates slower when bound by a protein.

However, as *HyDkk1/2/4 C* seems not to be an effector gene of an evolutionary conserved signal transduction cascade, there are no obvious suspects which possibly take part in regulation and which could facilitate *in silico* prediction approaches. Growing collections of transcription factor binding sites, like Transfac (Matys et al., 2006), are therefore of limited help as generally a variety of hits are reported even on selected regions after phylogenetic footprinting. Further problems result from the AT- richness of the *Hydra* genome as many AT rich binding profiles from higher animals generally tend to be over represented in prediction studies. As in performed analyses no informative elements were detected, an *in silico* prediction of potential binding factors is not presented in this study.

With the progress of proteomics and mass spectrometry new approaches of protein identification have been established (Nordhoff et al., 1999; Woo et al., 2002; Yaneva and Tempst, 2003; Nordhoff and Lehrach, 2007). Combinations of two-dimensional electrophoresis and mass spectrometry could be shown to be a powerful tool for the identification of DNA-binding proteins observed in EMSA (Woo et al., 2002). The *Hydra* peptide collection currently available (19.845 peptides, www.compagen.org), should allow peptide mass fingerprinting, even though it does not comprise full length sequences. Taken together, mass spectrometry promises to be the method of choice for the identification of DNA binding proteins in *Hydra*. DNA sequence elements shown to be bound by proteins from nuclear extract could be used for affinity purification and enrichment of these factors prior to two-dimensional gel electrophoresis and mass spectrometric analysis.

3.2 *HyDkk1/2/4* and *Hydra* patterning

Previously, our working group and others (Augustin et al., 2006, Guder et al., 2006) have speculated that the *HyDkk1/2/4* genes are components of a conserved Wnt signalling cascade. The present study demonstrates that the inclusion of the N-terminal *HyDkk1/2/4 C* signal peptide in the EGFP expression vector led to the translocation of the EGFP reporter protein towards secretory vesicles of zymogen gland cells (Fig. 2.12 A). Furtheron, I could show that all secretory vesicles of gland cells within the *HyDkk1/2/4 C* expression domain seem to be EGFP positive (2.12 A). These vesicles or part of the secreting cells are released in the course of feeding as EGFP signals are partially reduced from feeding event to feeding event (Fig. 2.16). This indicates, in the most parsimonious interpretation, a regulated secretion of EGFP into the gastric cavity. On the assumption that EGFP reporter protein localisation reflects the localisation of native *HyDkk1/2/4 C* protein, this indicates involvement of *HyDkk1/2/4 C* in *Hydra* axial patterning as rather unlikely.

Only the N-terminal signal peptide was integrated in the *HyDkk1/2/4 C* reporter construct (Fig. 2.9 B) which obviously achieved the EGFP translocation into the rough endoplasmatic reticulum. Subsequently EGFP was translocated towards the prominent vesicle type of these cells. This can be interpreted as a default secretion mechanism as no further signal sequences are present in the construct. If a different behaviour of the native *HyDkk1/2/4 C* protein would be postulated, this would mean further processing signals immanent in the *HyDkk1/2/4 C* sequence and also the presence of a second type of secretory vesicles which behaves differently compared to the ones described above. As almost all cells show at least some constitutive secretion, it remains to be shown, if native *HyDkk1/2/4 C* gets released constitutively into the extracellular space. Only this would allow a concentration depend effect or cellular signalling per se.

Therefore to further assess *HyDkk1/2/4 C* function, two approaches are essential: (i) the localisation of *HyDkk1/2/4 C* protein *in vivo* and (ii) functional studies including gain/loss of function experiments. I tried to address both questions in the course of my PhD studies. In detail, for the generation of *HyDkk1/2/4 A* and *C* specific antibodies both full length proteins (without signal peptide) were overexpressed in *E. coli*. For each protein three mice were immunized (data not shown, see also Olbrich, 2005). However, none of the antisera did show any reactivity neither when used on western blots nor in immunohistochemistry.

Furthermore, I tried to re-establish RNAi in the lab. Successful RNAi approaches have been performed in *Hydra* using electroporation strategies for delivery of dsRNA or by feeding bacteria expressing dsRNA of interest (Lohmann et al., 1999; Galliot et al., 2007; Miljkovic-Licina et al., 2007). Both approaches and additional dsRNA incubation experiments were performed without significant depletion effects, indicating a gene dependent efficiency of the methods (data not shown). One variable which was not stressed to an end and leaves room for optimisation, was the number of dsRNA deliveries in an experiment, as for some genes several application are needed for efficient silencing (B. Galliot, pers. com., Miljkovic-Licina et al., 2007).

Overexpression experiments using transgenic *Hydra* represent another possibility to assess protein function. A phenotype for a functional Wnt antagonist could be for instance a diminished potential for head regeneration. Based on this reasoning, I generated transgenic *Hydra* overexpressing a HyDkk1/2/4 C – EGFP fusion protein driven by the *Hydra* actin promoter (data not shown). So far, in different lines of transgenic animals expression could be observed only in the ectodermal as well as in the endodermal cell lineage. The fusion protein showed a cytoplasmatic localisation. Despite of incorporation of the signal peptide no hints for secretion could be observed in these cell lineages, which would be crucial for Dickkopf function. These animals show normal patterning. The effect of this construct when expressed within the interstitial cell lineage remains to be elucidated.

3.3 Gland cell differentiation in *Hydra*

Whereas in the past much attention was brought to epithelial cells and nerve cells in the course of *Hydra* patterning, less focus had been set on secretory cells. Previous studies on the nervous system in *Hydra* using antibodies and transplantation experiments indicated that many neurons change phenotype as they are displaced from one region to another (Koizumi and Bode, 1986; Koizumi et al., 1988; reviewed in Bode, 1992). The epithelial cells undergo changes when displaced into tentacle or foot regions as they differentiate into battery cells and adhesive cells of the basal disk, respectively (Fig. 1.5 left, Bode et al., 1986; Wittlieb et al., 2006). Thus, changing the local environment causes changes in the regulatory input received by the cells. This leads to an alteration in their differentiation state.

Recently, the identification of Dkk related proteins (Augustin et al., 2006; Guder et al., 2006a) and other proteins, like e.g. the protease inhibitor Kazal (Chera et al., 2006,

Augustin, pers. com.), suggested gland cells as a source of signalling molecules and other proteins with non digestive function.

The *Hydra* gastric cavity comprises two functionally but not spatially separated compartments (i) the region of the hypostome down towards the tentacle region and (ii) the gastric region (see Figs. 1.3, 1.4). Both areas are characterised by distinct groups of secretory cells with clearly distinguishable morphological features and physiologically distinct functions (Fig. 1.4). Secretory cells of the hypostome produce mucus to facilitate the passage of food whereas cells of the gastric region produce proteolytic enzymes for digestion purposes (Haynes and Burnett, 1963; Rose and Burnett, 1968). In this study, animals expressing EGFP driven by the gland cell-specific *HyDkk1/2/4 C* promoter were used to monitor gland cell differentiation in *Hydra* in real-time and in the context of the native environment (Fig. 2.10, 2.13, 2.14).

It could be shown that zymogen cells have remarkable phenotypic plasticity in response to positional signals and that both stem cell-based mechanisms as well as transdifferentiation events appear to play a role in maintaining secretory cell complexity within the *Hydra* hypostome (Fig. 2.20). Although further study is required to determine the specific molecular and cellular cues that permit this cellular plasticity, the data allow to present a simple conceptual model that characterises several steps in gland cell differentiation (Fig. 3.1). Gland cell progenitors in *Hydra* are interstitial stem cells with extensive, probably unlimited, self-renewal capacity and which are predominantly located in the gastric region (Schmidt and David, 1986; Bode et al., 1987; Bosch and David, 1987). Upon commitment, these cells give rise to transiently amplifying gland cells (Schmidt and David, 1986; Bode et al., 1987). How the fate decision towards actual gland cell differentiation is made is still unclear. Using the marker gene EGFP, morphological criteria and molecular data, it could be shown that differentiated ZMGs can transdifferentiate into gMGCs (Fig. 2.14, 2.18, 2.20). Upon entering the head tissue, the ZMGs stop the expression of *HyDkk1/2/4 C* and start to express molecular marker *HyTSR1*. In steady state polyps this will happen with a time offset as there is a region where none of both genes is expressed (compare Fig. 2.2 E, Fig. 2.20 A (insert)). In addition, using *HyTSR1*, evidence is provided that gMGC can also originate from interstitial cell-like precursors (Fig. 2.20, B -E).

Due to the lack of appropriate molecular markers this model does not give insight in the origin of sMGC, the second type of MGCs in the head. In the histological studies no hints for transdifferentiation of zymogen into spumous cells (sMGC) were found.

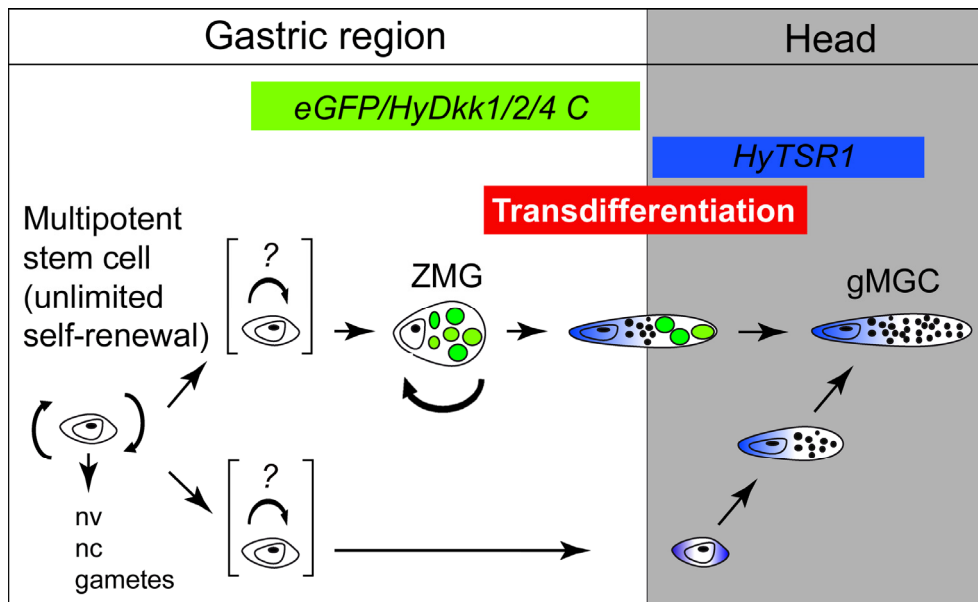


Fig. 3.1: Conceptual model for the origin of granular mucous gland cells in the *Hydra* hypostome.

Multipotent stem cells capable of unlimited self renewal give rise to gland cell precursors which eventually differentiate to zymogen gland cells (ZMG) in the gastric region or to granular mucous gland cells (gMGC) in the head. Zymogen gland cells are capable of direct transdifferentiation into gMGC. This is accompanied by transcriptional inactivation of *EGFP/HyDkk1/2/4 C* and activation of *HyTSR1* expression (blue). Green: EGFP protein. Taken from Siebert et al. 2007.

Taken together, the data presented here indicate, that in steady state polyps zymogen gland cell are an important source for granular mucous cells and therefore contribute to cell-type homeostasis in the hypostome of *Hydra*. Differentiation from low abundant interstitial cells present in the hypostome, might serve maintenance purposes, which once differentiated to MGC further proliferate. If long range immigration of precursors determined to become MGCs into the hypostome play a role in steady state polyps remains to be clarified.

3.4 The nature of *HyDkk1/2/4 C* transcriptional regulation

Even though we are still on the way towards understanding of the regulatory inputs on the *HyDkk1/2/4 C* promoter, it is worthwhile to think about the theoretical signals that have to be integrated to achieve the transcriptional pattern observed (Fig. 2.11). *HyDkk1/2/4* transcription shows a sharp boundary below the head. A similar boundary is also visible in various other genes expressed in zymogen gland cells (e.g. Hma.4041, Hma.9449, Hma.3371, Hma.4697, Hma.1694, see online web resource: <http://hydra.lab.nig.ac.jp/hydra/> and Hwang et al., 2007) indicating that the zymogen cell state of the gastric compartment has a well defined upper boundary.

Assuming that a morphogen gradient is involved in formation of this boundary, this could imply a factor emanating from the head region that inhibits the activation of a ZMG specific transcription factor set within the head (Fig. 3.2). Only below a permissive threshold the zymogen fate would be established. This factor might be seen as coupled to or as a part of an activator – inhibitor system in the sense of Gierer and Meinhardt (Gierer and Meinhardt, 1972).

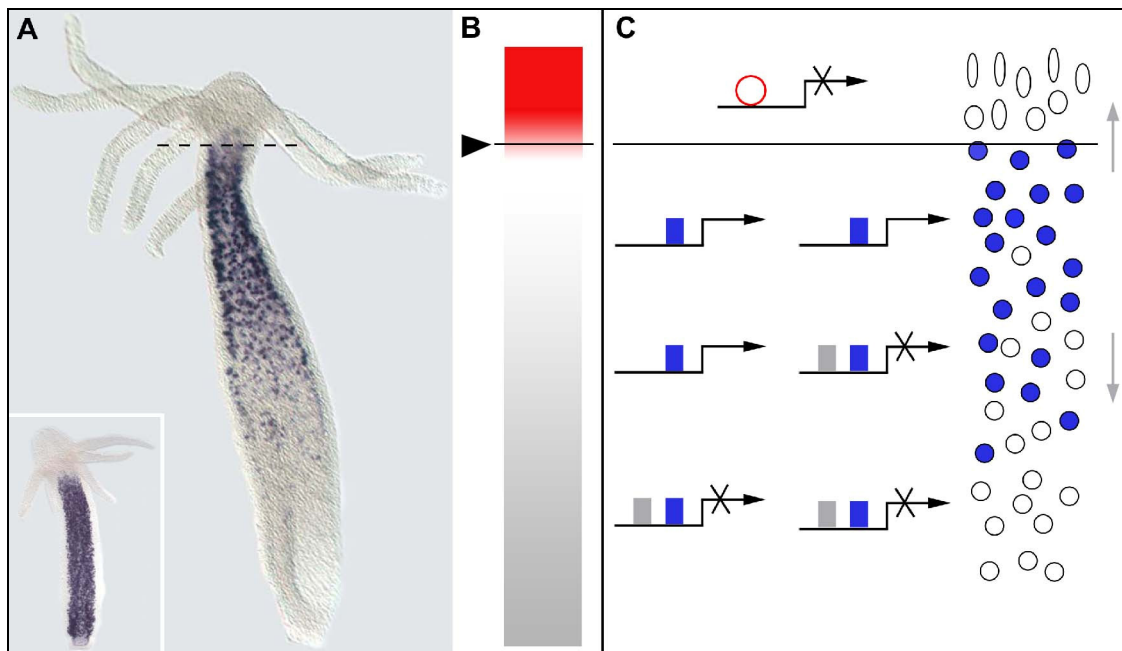


Fig. 3.2: Model for transcriptional activation of *HyDkk1/2/4 C*. (A) In contrast to *HyDkk1/2/4 A* (insert), *HyDkk1/2/4 C* is activated in zymogen gland cells in a graded manner (polyp taken from Augustin et al., 2006). (B) An inhibitory factor emanating from the head creates an upper boundary for the establishment of the ZMG cell fate (black arrow marks permissive boundary). A long ranging signal originating from the foot mediates transcriptional inactivation of *HyDkk1/2/4 C*. (C) ZMG cells crossing the boundary in apical direction inactivate *HyDkk1/2/4 C* transcription, loose zymogen character and transdifferentiate. Within the gastric region *HyDkk1/2/4 C* is generally activated in zymogen gland cells. In basal parts transcription is inactivated via binding of a repressing signal resulting in the characteristic transcription pattern. blue box: transcription activating complex, grey box: repressive factor(s) emanating from the basal part of *Hydra*, arrows indicate tissue flux.

Various models have been proposed for the generation of sharp boundaries based on morphogen gradients. Assuming that the morphogen is a secreted peptide which undergoes binding to cell surface receptors, degradation and chemical modifications, one strategy to steepen the gradient, which would allow sharp differences in gene expression, can be the integration of positive feedback loops at different levels of the signalling cascade. This can be achieved, for instance, by allowing the morphogen to inhibit degradation of components of its own signalling cascade (for review see Lander,

2007). If the morphogen is allowed to upregulate the production of its own receptor, this is also positive feedback and has spatial effects. Signalling is in this case maximal in cells with higher number of receptors and, on the assumption that receptor binding leads to degradation of the morphogen, this would suppress signalling in the periphery and sharpen the boundary of the signal (Lander, 2007).

Interestingly, activation of Wnt signalling via alsterpaullone leads to the activation of the head specific gene machinery which might involve promoting the theoretical repressing factor(s). As a consequence, this leads to downregulation of *HyDkk1/2/4* genes (see Fig. 2.15, Augustin et al., 2006; Guder et al., 2006a). When cells cross the inhibitory threshold in apical direction, a hypostomal secretory cell fate might be triggered and the cells change state and transdifferentiate (Fig. 3.2 C). Promoter studies as described above might elucidate signalling cascades involved in this regulation (see ch. 3.1).

It has been shown that *HyDkk1/2/4 C* is activated in a subpopulation of *HyDkk1/2/4 A* positive cells in a graded manner (Augustin et al., 2006). Coding sequence similarity, genomic clustering and expression in the same cell type imply that *HyDkk1/2/4* genes are a result of a gene duplication event (Fig. 2.3, Augustin et al., 2006). As the expression pattern of many zymogen genes resemble the homogenous distribution of *HyDkk1/2/4 A*, this pattern might be interpreted as basal (see online web resource: <http://hydra.lab.nig.ac.jp/hydra/> and Hwang et al., 2007). Both *HyDkk1/2/4* genes are co-expressed in the apical part of the gastric region. However in the course of cis-regulatory diversification *HyDkk1/2/4 C* might have come under control of a regulatory module which leads to repression of its transcription in the basal parts of *Hydra* (Fig. 3.2).

This is supported by transplantation experiments followed by *in situ* hybridisations suggesting inhibitory effects emanating from an ectopically attached foot on the expression of *HyDkk1/2/4 C* (Augustin et al. 2006). Simplified, in the terms of modelling, a long ranging signal from the foot is likely to inhibit activation in a concentration dependent manner. As the *HyDkk1/2/4 C* expression pattern is actually a read out of a graded signal, the characterisation of the 5'-flanking region, shown to be sufficient for the expression, offers a new level of analysis which may elucidate factors or signalling cascades mediating a gradient.

3.5 Stability of the cell differentiation state

Transdifferentiation events have been reported for both vertebrates and invertebrates. Vertebrate embryogenesis is a highly dynamic process in which cells differentiate within changing frameworks of transcriptional boundaries and signalling gradients from a totipotent egg towards highly specialised somatic cells, germ cells and pools of multipotent stem cells. Generally the differentiated cell type is regarded to be stable. In some cases conversions occur and cell types change their phenotyp. A well known example is the regeneration of the lens of various species of urodeles (Tsonis and Del Rio-Tsonis, 2004; Yoshii et al., 2007). In mammals, examples of transdifferentiation are seen in the development of esophageal smooth muscle (Patapoutian et al., 1995), the liver (Taub, 2004), and in the Schwann cells of the peripheral nervous system (Harrisingh et al., 2004). Recently, mouse auditory epithelial support cells were shown to transdifferentiate into sensory hair cells (White et al., 2006). Well-documented is also the transdifferentiation of pancreatic exocrine cells towards hepatocytes in response to experimental treatments (Tosh and Slack, 2002).

In invertebrates, experimentally induced transdifferentiation has been documented in several cases (Schmid and Alder, 1984; Bode et al., 1986; Kawamura and Fujiwara, 1994; Kawamura and Fujiwara, 1995; Reber-Muller et al., 2006). In the Cnidarian *Podocoryne*, terminally differentiated mononucleated striated muscle cells were found to dedifferentiate in response to mechanical disruption of the muscle and exposure to signals from the extracellular matrix (Schmid and Reber-Muller, 1995). The cells then transdifferentiate into smooth muscle cells and resume cell division; finally, these smooth muscle cells can differentiate into nerve cells (Schmid and Reber-Muller, 1995). Although our current knowledge on the molecular mechanisms involved in regulating transdifferentiation is very limited, a number of studies point to an important role of ECM remodelling (Streuli, 1999; Leontovich et al., 2000; Echeverri et al., 2001; Straube and Tanaka, 2006). In *Drosophila*, studies on transdetermination, a transformation process between different imaginal discs, have shown that fate switches take place in response to the inductive signal of the c-Jun N-terminal kinase (JNK) signalling pathway, which down-regulates Polycomb group (PcG) proteins (Klebes et al., 2005; Lee et al., 2005; McClure and Schubiger, 2007). Thus, understanding the molecular mechanisms enabling cells to transdifferentiate may also require unravelling the changes that occur on chromatin.

Slack and Tosh proposed a simple model for the occurrence of transdifferentiation (Slack and Tosh, 2001; Tosh and Slack, 2002). During embryogenesis two tissues get specified via expression of a single transcription factor, which is activated within a morphogen gradient. Assumed that this factor distinguishes the two tissues postnatally, a change in the microenvironment or a somatic mutation could alter its expression and this would induce change of the cell type (Slack and Tosh, 2001; Tosh and Slack, 2002). In many cases changes in the cellular phenotyp occurs during regeneration or experimentally manipulations, meaning under conditions where the cellular environments and also the factors influencing the cell are artificially changed.

In *Hydra*, however, constant mitotic activity in the epithelia and resulting tissue dynamics lead to permanent and life long changes in the microenvironment of the cells (see Fig. 1.5). Transdifferentiation processes are therefore triggered continuously. In higher animals “final” differentiation of cells might be in many cases connected to a loss of sensitivity for several external factors. This could be reached by histone modifications or DNA methylation. However, in some cases the cells might keep responsiveness to external stimuli, but the respective factors, which would cause changes, are simply missing. Therefore many cells might be more plastic than directly accessible, maintaining a metastable state (Bode et al., 1986). Understanding of cellular behaviour at boundaries in *Hydra* might help to elucidate the factors involved in maintaining cell identity or in mediating cell fate switches in higher animals.

4 Summary

To characterise critical regulatory events that control cell behaviour and cell plasticity in a basal organism, I have generated a transgenic *Hydra* line which expresses EGFP specifically in zymogen gland cells. These cells are derivatives of the interstitial stem cell lineage and have previously been found to express two Dickkopf related genes, *HyDkk1/2/4 A* and *HyDkk1/2/4 C* (Augustin et al., 2006). To get insights into the transcriptional regulation of *HyDkk1/2/4 C*, I isolated the orthologous 5' flanking regions from four different *Hydra* species. Interspecific sequence comparisons revealed conserved potential functional elements and suggested that the first 1000 bp upstream of the translation initiation site might be sufficient for authentic transcription. When functionally tested, transgenic *Hydra* recapitulated faithfully the previously described graded activation of *HyDkk1/2/4 C* expression along the body column as well as a sharp boundary of expression below the head, indicating that the promoter contains all elements essential for spatial and temporal *HyDkk1/2/4 C* expression control.

Using these transgenic animals I investigated gland cell differentiation. Tracing individually labelled zymogen gland cells allowed me (i) to show that changes in position along the single body axis are accompanied by changes in gene expression and cell morphology; and (ii) to define a distinct ancestor-descendant relationship between zymogen gland cells in the gastric region and granular mucous cells in the head using additionally histological and molecular data. In addition it could be demonstrated that during head regeneration (iii) a part of the mucous gland cell population in the head is directly derived from interstitial cells.

The observations support the view that in *Hydra* both stem cell-based mechanisms and transdifferentiation are required for maintaining the different types of cells found along the body axis. The study describes the first transgenic *Hydra* line expressing a reporter gene under the control of a cell-type specific promoter.

5 Zusammenfassung

Zur Charakterisierung von wesentlichen regulatorischen Einflüssen, die Zellverhalten und zelluläre Plastizität in einem basalen Organismus kontrollieren, erzeugte ich transgene Hydren, die EGFP spezifisch in zymogenen Drüsenzellen exprimieren. Diese sind Derivate der interstitiellen Stammzelllinie und es konnte zuvor gezeigt werden, dass diese Zellen zwei Dickkopf ähnliche Proteine exprimieren, *HyDkk1/2/4 A* and *HyDkk1/2/4 C* (Augustin et al., 2006). Um Einsichten in die transkriptionelle Regulation von *HyDkk1/2/4 C* zu erlangen, isolierte ich orthologe 5' flankierende Regionen aus vier verschiedenen Hydraarten. Interspezifische Sequenzvergleiche führten zur Identifikation von potentiell funktionalen Elementen und deuteten an, dass 1000 bp 5' des Translationsstarts für eine korrekte Expression ausreichend sind. Der funktionale Test ergab, dass transgene Hydren das von *HyDkk1/2/4 C* bekannte gradierte Expressionsmuster einschließlich der deutlichen transkriptionellen Grenze unterhalb der Kopfregeion zeigen. Der verwendete Promoter enthält somit alle essentiellen Elemente zur räumlichen und zeitlichen Kontrolle der *HyDkk1/2/4 C* Expression.

Transgene Tiere wurden anschliessend zur Analyse von Drüsenzeldifferenzierung verwendet. Die Dokumentation von markierten Zellen entlang der Körpersäule ergab, dass Positionsänderungen mit Veränderungen der Genexpression und Zellmorphologie einhergehen. Weiterhin konnte mithilfe der EGFP markierter Zellen sowie anhand von histologischen und molekularbiologischen Daten gezeigt werden, dass gastrale zymogene Drüsenzellen Vorläufer von granulären mukösen sekretorischen Zellen des Kopfes sind und weiterhin, dass sich während der Kopfregeneration ein Teil der mukösen sekretorischen Zellen des Kopfes direkt aus interstitiellen Zellen ableitet.

Diese Beobachtungen unterstützen die Sicht, dass in *Hydra* stammzellbasierte Differenzierung und Transdifferenzierung bei der Entstehung und Beibehaltung der Zellentypen entlang der Körpersäule eine Rolle spielen. Diese Studie beschreibt die erste transgene Hydralinie, in der die Expression eines Reporters unter der Kontrolle eines zelltypspezifischen Promoters steht.

6 Materials

6.1 Organisms

Hydra strains:	<i>Hydra magnipapillata</i> , <i>Hydra oligactis</i> , <i>Hydra vulgaris</i> (AEP), <i>Hydra viridis</i>
Feeding organism:	<i>Artemia salina</i>
Bacterial strains:	<i>Escherichia coli</i> (strain DH5 α) <i>Escherichia coli</i> (strain XL1-blue)

6.2 Media

<i>Artemia</i> medium	31,8 g sea salt per 1 l Millipore water
Hydra-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)
LB-amp medium	10 g Tryptone; 5 g Yeast extract; 5 g NaCl millipore water up to 1 l; 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 l; 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
SOC-Medium	10 ml SOB-Medium, 100 μ l 2 M Glucose (sterile filtered), 100 μ l sterile-filtered 2M Mg ²⁺ solution (20.33 g MgCl ₂ ·6 H ₂ O; 24.65 g MgSO ₄ ·7H ₂ O/100 ml Millipore water)

6.3 Buffers and solutions

6.3.1 General purpose solutions

Ampicillin stock solution	50 mg/ml, stored at -20°C in 1ml aliquots
APS	10 % (w/v) in Millipore water
Denhardt's (50 x)	1 % Polyvinylpyrrolidon, 1 % Ficoll, 1 % BSA Fraction V, sterile-filtered, stored at -20°C.

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
Hoechst stock solution	1 mg/ml in Millipore
Hybridization solution (HS) (macroarray)	100 ml Denhardt's (50x), 250 ml 20x SSPE, 20 ml 10% SDS-Solution, water up to 1 l, stored at -20°C 100 µg/ml(HS) salmon sperm (denatured at 95° for 5' before use)
IPTG stock solution	200 mg/ml in Millipore water, stored at -20°C in 200µl aliquots
Lead citrate solution	1.33 g Pb(NO ₃) ₂ , 1,76 g Na ₃ (C ₆ H ₅ O ₇)• 2H ₂ O in Millipore, mix for 30 minutes, add 8 ml 1 M NaOH and fill up to 50 ml with Millipore water
Lysis buffer (gDNA Isolation)	10 mM Tris-HCL pH 8,5, 150 mM EDTA ph 8,0, 0,2% SDS, 100 µg/ml Proteinase K
Maceration solution	acetic acid, glycerol, water (1:1:13)
NTMT	100 mM NaCl, 100 mM Tris pH 9,5, 50 mM MgCl ₂ , 0.1% Tween 20
Phalloidin stock solution	0,1 mg/ml in Millipore
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
SSPE (20 x)	175,3 g NaCl, 27,6 NaH ₂ PO ₄ •H ₂ O, 7,4 g EDTA, water up to 1 l, adjust pH
TAE (50 x)	242 g Tris base, 57.1 ml glacial acetic acid,100 ml 0.5 M EDTA, pH 8.0, water up to 1l
TBE sequencing buffer (10X)	162 g Tris base, 27,5 g boric acid, 50 ml 0,5 EDTA water up to 1 l
TE	10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0
Tris-HCl stock solutions	1 M Tris-HCl, pH 7.2; 7,5; 8.0; 9.5.
Washing sol.1 (macroarray)	2 x SSC, 0,1 % SDS
Washing sol. 2 (macorarray)	0,2 x SSC, 0,1 % SDS

6.3.2 Solutions for *in situ* hybridisation

Blocking solution	80 % MAB-B, 20 % heat-inactivated sheepserum (inactivation at 56°C for 30')
Glycine working solution	4 mg/ml in Millipore H ₂ O
Hybridisation solution	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
Hybridisation (washing steps)	50 % formamide, 5 x SSC, 0,1 % Tween 20, 0,1% CHAPS, 1 x Denhardt's, 100 µg/ml heparin
MAB	100 mM maleic acid, 150 mM NaCl, pH 7,5
MAB-B	MAB /1 % BSA fraction V, stored at -20° C
MAB-T	MAB/0.1% Tween 20
Mowiol	5 g Mowiol 4-88 in 20 ml 100mM Tris pH 8,0 ml, stir for 16 h at room temperature, then add 10 ml Glycerol, stir for 16 h at room temperature. Clarification of the solution by centrifugation (5000 rpm, 20 min). Stored at -20°C in aliquots (500 µl). Prewarm 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 HM. To dissolve the powder 100 µl/10 ml of 10 M NaOH was added and the solution was heated to 55°C. 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°C 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 (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

6.3.3 Kits

Agar 100 resin	Agar Scientific, Ltd., Essex
DIG RNA Labelling Kit (SP6/T7)	Roche
Expand longue Template PCR System	Roche
First strand cDNA synthesis kit	Amersham Biosciences/GE Healthcare
NucleoSpin Extract II	Machery-Nagel
NucleoSpin Plasmid/Plasmid Quick Pure	Machery-Nagel
pGEM [®] -T Easy Vector System	Promega
Qiafilter plasmid Midi Kit	Qiagen
SequiTherm EXCEL [™] II DNA Sequencing Kit	Epicentre

6.3.4 Enzymes

DNAseI	Amersham
Klenow-Fragment	Fermentas
NotI	Fermentas
SequiTherm EXCEL II DNA Polymerase	Epicentre
Shrimp alkaline Phosphatase	Fermentas
Taq DNA-Polymerase	Fermentas
T4 DNA-Ligase	Promega
Proteinase K	Sigma
Platinum High Fidelity polymerase	Invitrogen
Platinum Taq DNA Polymerase	Invitrogen
RNase A	Sigma
XbaI	Fermentas

6.4 Chemicals

Acidic acid	Roth
Acetic Anhydride	Sigma
Agar-Agar	Roth
Agarose	Roth
Ammonium-acetat	Roth
Ampicillin	Merck
APS	Roth
Azur II	Merck
Biotin-RNA labeling Mix, 10x conc.	Roche
Boric acid	Roth
Bromphenol blue	Fluka
Bovine serum albumin Fraction V	Merck

CHAPS	Sigma
Chloroform	Roth
dATP (100 mM)	Fermentas
DABCO	Sigma
Dextran-Sulfat	Roth
dNTPs (100mM)	Fermentas
EDTA	Sigma
Ethanol	Roth
Ethidium bromide (50 mg/ml)	Roth
Euparal	Roth
Fast Red Tablets	Roche
Formaldehyde (37%)	Merck
Formamide	Roth
Gelatine	Sigma
Glutaraldehyde	Fluka
Glycerol	Roth
HEPES	Sigma
HOECHST	Calbiochem
Hydrochloric acid	Roth
IPTG (0,1M)	Sigma
Isopropanol	Roth
Isoamyl alcohol	Merck
LB Broth Base	Life Technology
Lead acetate	Merck
Levamisol	Roth
Magnesium chloride	Merck
Magnesium sulfate	Merck
Maleic acid	Sigma
Methanol	Roth
Methylene Blue	Merck
Mowiol 4-88	Calbiochem
NBT/BCIP	Roche
Nonidet P40 (NP 40)	Sigma
Osmium tetroxid	Roth
Paraformaldehyde	Merck
Phalloidin	Fluka
Phenol	Roth
Pioloform	Plano
Potassium chloride	Roth
Propylenoxide	Fluka
RapidGel™-XL-40% Concentrate	USB
Rhodamine B Isothiocyanate-Dextran	Sigma
RNaseOut	Invitrogen
Salmon sperm DNA 10 mg/ml (sonicated)	Stratagene
Sheep serum	Sigma
Sea salt (Ocean Zac plus)	Zoo Zajac, Duisburg
SEPHADEX G50 medium	Pharmacia
Sodium acetate	Roth
Sodium azide	Merck
Sodium cacodylate	Fluka
Sodium chloride	Roth
Sodium citrate	Roth

Sodium-di-hydrogenphosphate	Roth
Rotiphorese [®] Gel 30	Roth
TEMED (N,N,N',N'-Tetraethylmethyldiamin)	Merck
Triethanolamine	Sigma
Tris-base	Roth
Tris HCl	Roth
TRizol [®] Reagent	Invitrogen
tRNA yeast (10mg/ml)	Sigma
Tryptic Soy Broth	Sigma
Trypton	Roth
Tween20	Merck
Uranylacetate	Merck
Urea	Roth
Urethan	Sigma
X-Gal	Sigma
Yeast extract	Roth

6.5 Vectors

pGEM [®] -T	Promega
hotG	Originally designed by J. Lohmann

6.6 Radioactive substances

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

6.7 Antibodies

Anti-Dig-AP	Roche
Anti Bio-AP	Roche

6.8 Primer (MWG Biotech)

Name	5'- Sequence - 3'	T _m	Comment
AEP Pr R (IRD 700)	CAGGGGTTTACC AGCATG	56,0	AEP promoter
GFP R(75) (IRD 700)	GTGCCCATTA ACATCACCAT C	57,9	hoT G construct
SP 6 (IRD 700)	ATTTAGGTGACACTATAGAATAC	53,5	
SP 7 (IRD 800)	TAATACGACTCACTATAGGG	53,2	
M13 F (IRD 700)	CCCAGTCACGACGTTGTAAAACG	62,4	
M13 R (IRD 800)	AGCGGATAACAATTTACACAGG	58,6	
St SlpOut Pr2	GAATCGTAACCGTTCGTACGAG	60,3	Splinkerette vir
StSploutPri3	GAATCGTAACCGTTCGTACG	57,3	Splinkerette oli

SplinnPri2	CGTACGAGAATCGCTGTCCTC	61,8	Splinkerette vir
St_SplInnPri_3	TACGAGAATCGCTGTCCTC	56,7	Splinkerette oli
AEP gen D2 Fw	CGTTGACCATTTCAGGAAGTGG	59,8	Intergenic aep
AEP gen D1 rev	CGCTGCAAACAATGCACAAAGC	60,3	Intergenic aep
Dlp2 oli ex3 fw	GTTGACAATTTCAGGCAGTGG	57,3	Intergenic oli
Dlp1 oli prom rw	CCAGAGCTTTGCTTAACTCG	57,3	Intergenic oli
Dlp oli spl out1	CAACACCCGCCATTACAATC	57,3	Splinkerette oli
DO_DLPoli_in_1	CAATGCACATAGCAACAATGAAC	57,1	Splinkerette oli
Dlp vir in	CAAATCCGAAATTCAAACAGCATCC	59,7	Splinkerette vir
DLP vir out	ATGCCGCATAGTCCACCTTC	59,4	Splinkerette vir
GFP_F(29)	GAGTTGTCCCAATTCTTGTTG	55,9	in situ GFP
GFP_(R(711))	GTATAGTTCATCCATGCCATG	55,9	in situ GFP
R DLP1 F	CGTACACGTTTCACACGTCCT	59,4	<i>HyDkk1/2/4 C</i> promoter
P DLP1 R	CCTCCATGGCAATCTTTGTTTGA	58,9	<i>HyDkk1/2/4 C</i> promoter
DLP1 pr Xbal	ACTCTAGACGTACACGTTACACGTCC TATAG	68,2	Transfection construct
DLP1 pr Not1	CAGCGGCCGCCAGAATATTCCTTCGC TGCAAAC	74,3	Transfection construct
Dlp1 AEP f	TCCAAAATGAAATCAACGGTTATTT	54,8	FL AEP <i>HyDkk1/2/4 C</i>
RA_dlp3_F(2)	CGG ATG CAGAATGCGAAAATGGTTGC	64,8	FL AEP <i>HyDkk1/2/4 A</i>
RA_dlp3_R(2)	GGTCAACGCATTTTCTGATCATCCCG	64,8	FL AEP <i>HyDkk1/2/4 A</i>
<i>HyDkk1/2/4 C</i> F	CCACTTCCGGAGTTGTCAAC	59,4	in situ <i>HyDkk1/2/4 C</i> FL AEP <i>HyDkk1/2/4 C</i>
<i>HyDkk1/2/4 C</i> R	CAGTGGGCAACCTGAATACC	59,4	in situ <i>HyDkk1/2/4 C</i>
HyTSR1(22) FW	CTGTTTGCCTCAATGCTGAC	57,3	in situ <i>HyTSR</i>
HyTSR1(949) R	TACCACCAAATGCAGGTTTGG	57,9	in situ <i>HyTSR</i>

Primer used for: **FL**: Full length amplification, **in situ**: *in situ* probe generation, **Splinkerette**: used in splinkerette PCR, **promoter**: amplification of promoter from genomic DNA, **intergenic**: amplification of intergenic region on genomic DNA.

6.9 Devices and machines

6.9.1 PCR machines (Thermocycler)

Cyclone gradient	peqLab
Primus 96 <i>plus</i>	MWG-Biotech
Primus 25	MWG-Biotech
Robocycler Gradient 96	Stratagene
Tetrad2	MJ Research/Biorad

6.9.2 Power supplies

EPS 3500	Amersham Pharmacia Biotech
LNG 350-06	Heinzinger

6.9.3 Gel electrophoresis chambers

EasyCast minigel system B1A	Owl Separation Systems
EasyCast minigel system B2	Owl Separation Systems
Multiphor 2	Amersham Pharmacia Biotech
EPS 3500	Amersham Biosciences
LNG 350-06	Heinzinger
PerfectBlue™ Twin L	Peqlab

6.9.4 Incubators/shakers

HIS25	Grant Boekel
Thermo-Incubator	Heraeus Instruments
KS10 (Shaker)	Edmund Bühler
Mini 10	Thermo Hybaid
Thermomixer compact	Eppendorf
Thermomixer Certomat H	B. Braun Biotech
Ultratemp 2000	Sternkopf

6.9.5 UV-transilluminators

Imaging-system	Biorad
ImaGo compact imaging system	B+L Systems
UV-Stratalinker 1800	Stratagene

6.9.6 Electroporation devices

Gene Pulser II	Biorad
Pulse Controller II	Biorad

6.9.7 Centrifuges

Centrifuge 5415 D	Eppendorf
Centrifuge 5417 R (Kühlzentrifuge)	Eppendorf
Minifuge RF	Heraeus Instruments
Labofuge 1	Heraeus Instruments
Multifuge 3 S-R	Heraeus Instruments

6.9.8 Microscopy

AxioCam	Zeiss
---------	-------

Axioskop 2	Zeiss
SZX16	Olympus
DP71 (digital camera)	Olympus
CLSM TCS SP/UV	Leica
DC300F	Leica
EM 208 S (electron microscope)	Philips
Axiovert 100	Zeiss

6.9.9 Other devices

Eraser (erasing phosphoimager screens)	Raytest
CellTram Air pump	Eppendorf
CellTram vario pump	Eppendorf
Dialysis membrane	Millipore
LICOR DNA Analyser Gene Read IR 4300	MWG Biotech
Milli-Q Academic System	Millipore
LaminAir HB2448 (model Firefly)	Heraeus Instruments
Phosphoimager FLA-5000	Fuji
Pipette Puller 700 C	Kopf Instruments, Tujunga, CA
Ultratome Ultracut S	Leica
Vortex Genie 2	Scientific Industries
VARIOKLAV Sterilizer Typ 400 EV	H+P Labortechnik GmbH
Wallac WinSpectral	Perkin Elmer
Milli-Q Academic System	Millipore
pH-Meter pH 211	Hanna Instruments
Nanodrop ND 1000	Nanodrop Technologies

6.9.10 Other materials

12-well microtiter plates	griner bio one
Chromatography paper 3MM	Whatman
Diamantmesser	C. Schmied Labortechnik
Elektroporationsküvetten	Peqlab
Flacheinbettung G3690 (rubber)	Plano
Gene Ruler DNA ladder mix	Fermentas
Grid Box	Plano
Grid G2500	Plano
Microcon YM 50 columns	Microcon
X-Ray film (BioMax MS)	KODAK

6.10 URLs und Software

Blast database queries -	http://www.ncbi.nlm.nih.gov/blast/
NCBI Trace archive	http://www.ncbi.nlm.nih.gov/Traces/trace.cgi?
<i>Hydra</i> ESTs	http://www.compagen.org
<i>Hydra</i> genome browser	http://hydrasome.metazome.net
Vista	http://genome.lbl.gov/vista/index.shtml

Footprinter	http://genome.cs.mcgill.ca/cgi-bin/FootPrinter3.0/FootPrinterInput2.pl
GATA	http://gata.sourceforge.net/
SignalP 3.0	http://www.cbs.dtu.dk/services/SignalP/
Mega 4	http://www.megasoftware.net/index.html
BioEdit 7.0.5.3	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
SignalP	http://www.cbs.dtu.dk/services/SignalP/
Cell^A Imaging software	Olympus
eSeq v.3 Sequencing software	LI-COR Inc., Michigan Technology University
Wallac 1414 WinSpectral v1.30	PerkinElmer
Cerenkov protocol	
J2SE 1.4.2	Sun microsystems

7 Methods

7.1 Culturing *Hydra*

Animals of the strains *Hydra magnipapillata* (strain 105), *Hydra vulgaris* (AEP), *Hydra viridis* and *Hydra oligactis* were cultured in *Hydra* medium (HM) at 18°C with 12 hour light cycle according to the standard method (Lenhoff and Brown, 1970). *Hydra* mass cultures were held in 20 X 20 cm X 6 cm plastic dishes. Polyps were fed 3 - 4 days a week with newly hatched and freshwater rinsed nauplii of brine shrimp *Artemia salina*. 24 h starved animals were used for all experiments.

7.2 Incubation experiments

Treatment with alsterpaullone was performed as described (Broun et al., 2005). Alsterpaullone was dissolved in DMSO and diluted with *Hydra* medium (HM) to 5µM. Animals were kept in 5µM alsterpaullone for 48h and then transferred to HM. Animals were fixed at different timepoints after beginning of treatment for further analysis.

7.3 Nuclear acid extractions

7.3.1 Isolation of total RNA

Total RNA of *Hydra* was isolated with TRIzol[®] Reagent (Invitrogen) according to the manufacturer's protocol.

7.3.2 Isolation of genomic DNA

250 *Hydra* polyps were placed into an Eppendorf tube and the excessive *Hydra* medium was removed. 0.5 ml of lysis buffer was added and the animals were homogenized using a pestle and incubation for 2 h at 55°C. To inactivate proteinase K and to extract proteins, 500 µl phenol was added. The sample was shaken for several seconds and centrifuged for 15' at 12,000 g at RT. The supernatant was removed and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The sample was again shaken and centrifuged for 15' and supernatant was removed. Equal volume of chloroform/isoamyl alcohol (24:1) was added and protein was extracted for a second time. Afterwards the upper phase was removed. NaCl was added to the final

concentration of 0.5 M and DNA was precipitated with a double volume of ice-cold 99% ethanol (EtOH). Precipitated DNA was transferred to a fresh tube. After washing in 70% EtOH and air-drying, the DNA was dissolved in 100 μ l Millipore water.

7.3.3 Isolation of Plasmid-DNA

Bacterial 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 using „NucleoSpin Plasmid QuickPure“ (Macherey-Nagel). For *Hydra* transfection experiments the “Qiafilter Plasmid Midi Kit” (Qiagen) was used for plasmid isolation. A 3 ml starter culture was used to inoculate 100 ml LB medium, which was grown at 37°C overnight. Both extractions were performed according to the manufacturer’s protocols.

7.3.4 Quantification of nuclear acids

Concentration and purity of isolated DNA was determined by measuring the optical density at 260/280/230 nm using a NanoDrop ND 1000 spectrophotometer.

7.3.5 cDNA synthesis

First strand cDNA was synthesised using the “First strand cDNA synthesis Kit” (Amersham Biosciences/GE Healthcare) according to the manufacturer’s protocol. Not I (dt)₁₈ Primer were used for reverse transcription.

7.4 Polymerase chain reaction (PCR)

7.4.1 Standard-PCR

For a standard PCR the following components were assembled:

Component	Volume [μ l]	Final concentration
Millipore	12,8	-
10x PCR-Buffer	2,0	1x
dNTPs [1mM]	2,0	100 μ M
Forward-Primer [10 μ M]	1,0	500 nM
Reverse-Primer [10 μ M]	1,0	500 nM
Template cDNA	1,0	-
Taq-Polymerase [5u/ μ l]	0,2	0,05 u/ μ l
Total	20,0	

Standard PCRs (Taq polymerase) were performed under following conditions using an annealing temperature $T_A = T_m$ (*MWG Biotech*) minus one degree Celsius.

Standard-PCR-program: 2 min 94°C, up to 40 X (30 s 94° C, 30 s T_A ($T_m-1^\circ\text{C}$),
1 min/1 kb 72°C), 5 min 72 °C

For the amplification of larger genomic fragments the Expand Long Template PCR System (Roche) was used according to the manufacturer's instructions. Platinum Taq DNA polymerase (Invitrogen) was used for Hot start PCR to increase specificity and yield.

7.4.2 Splinkerette-PCR

The splinkerette-PCR is an adapter ligation-mediated PCR method frequently used for obtaining genomic sequence upstream or downstream of a known sequence. The 5'-flanking region of *HyDkk1/2/4 C* was amplified using genomic templates of *Hydra oligactis* and *Hydra viridis* which were prepared by Stefan Thomsen in a previous study (Thomsen et al., 2004). Briefly genomic DNA template had been digested with 18 different restriction enzymes (producing GATC-, CTAG- or CG- overhangs or blunt ends) and the DNA fragments had been subsequently ligated to "splinkerettes", a special type of vectorette (Devon et al., 1995). A top-strand (5'- CGAATCGTAA CCGTTCGTAC GAGAATTCGT ACGAGAATCG CTGTCCTCTC CAACGAGCCA AGG-3') and different bottom strands according to the overhangs produced by the restriction enzymes (5'- GATCCCTTGG CTCGTTTTTT TTTGCAAAAA-3' for GATC-, 5'- CTAGCCTTGG CTCGTTTTTT TTTGCAAAAA-3' for CTAG-, 5'-CGCCTTGGCT CGTTTTTTTT TGCAAAAA-3' for CG-, 5'-CCTTGGCTCG TTTTTTTTTG CAAAAA-3' for blunt ends) had been designed and annealed. The 18 different DNA preparations were used in nested PCR approaches combining sequences specific primers pointing towards the unknown 5' region and splinkerette primers against the attached splinkerette pointing towards the already known part of the sequence. In each approach two PCRs were performed. The PCR product of an outer primer combination was diluted (1:30) and used in a second PCR with an inner primer combination. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen). As the splinkerette is highly represented in the DNA template, a Touch-Down-PCR approach was used in the

first PCR to increase the specificity of amplification. T_A was lowered by 1°C every next cycle in the first amplifications (Fig. 7.1).

1. PCR		
Touch-Down-PCR:		2 min 94°C
	3 x 7 cycles	30 s 94°C First: 30 s $T_A (T_m - 1^\circ\text{C}) + 6^\circ$ Last: 30 s $T_A (T_m - 1^\circ\text{C})$ 1min/1kb 72°C
	1 x 20 cycles	30 s 94°C 30 s $T_A (T_m - 1^\circ\text{C})$ 3 min 72°C 5 min 72°C
2. PCR		
Standard-PCR-program:		2 min 94°C ,
	up to 40 x	30 s 94°C , 30 s $T_A (T_m - 1^\circ\text{C})$ 1 min/1 kb 72°C
		5 min 72°C

Fig. 7.1: Programs used for Splinkerette PCRs

Specificity of the product was checked using each primer alone in a control PCR. Specific PCR-fragments were ligated into vector pGEM-T subsequently sequenced.

7.4.3 Insert check PCR

Standard PCR conditions were used in insert check PCRs which serve the identification of bacterial colonies carrying a plasmid of interest. A pipette tip was used to inoculate a $10\mu\text{l}$ standard PCR reaction with few cells of the colony. Inserts size were determined via performing PCR using vector specific primers (SP6/T7, M13F/M13R) followed by a gel electrophoretic analysis of the PCR product. The insert size gives a hint on the insert identity.

7.5 Agarose gel-electrophoresis

DNA was separated in 1-2% agarose gels containing ethidium bromide in 1x TAE buffer using horizontal gel electrophoresis devices. Depending on the expected fragment sizes, different DNA-size standards were used in parallel.

7.6 Purification and extraction of PCR products

To purify or extract PCR products from agarose gels, the “NucleoSpin Extract 2 Kit” from Macherey Nagel was used. For dialysis of DNA or to change buffers, “YM-50” columns from Millipore were used.

7.7 Competent cells

For preparation of competent cells, a single colony of *E. coli* was grown overnight in 3 ml SOB medium containing 12 µg/ml tetracyclin at 37°C. This culture was used to inoculate 50 ml of SOB, which was incubated for several more hours until the logarithmic growth phase was reached ($OD_{600}=0.5-0.6$). Bacteria were then harvested by centrifugation for 10' at 3000 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 cells were resuspended in 200 µl 10% glycerol and frozen at -80° C in 40 µl aliquots.

7.8 Cloning technique

Purified PCR products were ligated into the pGEM-T vector system (Promega) as described by the manufacturer (10 µl reactions). Ligation mixtures were dialyzed against water using nitrocellulose filter membranes (0,025 µm) from Millipore to increase the transformation efficiency. Electro competent cells of *E. coli* strains XL-1 blue or DH-10B were transformed (BIORAD electroporation device, 1 mm cuvette). The cells were thawed on ice. 5 - 10 µl of the dialysed ligation reaction was mixed with the bacteria and electric pulse was applied (25 µF, 200 Ω, 1.8 kV). Electroporated cells were mixed with prewarmed 960 µl of SOC medium (37°C) and allowed to recover for 1 h at 37°. 100 µl of the liquid bacterial culture was plated onto LB-agar plates (50 µg/ml ampicillin) and incubated overnight. An insert check PCR allowed identification of colonies carrying the plasmid of interest.

7.9 Bacterial stock cultures

500 µl of bacterial culture were mixed with 500 µl sterile 50 % glycerol and stored at -80°C.

7.10 DNA sequencing

Sequencing of DNA was performed on a LI-COR 4300 plate sequencer. Sequencing reactions for the LI-COR system were carried out using the SequiTherm EXCEL II DNA sequencing kit LC (Epicentre) according to the manufacturer's protocol. The 6 % polyacrylamide gel contained 14 ml H₂O, 3.75 ml 40 % Rapid Gel XL solution, 2.5 ml 10 x TBE long run buffer, 10.5 g urea, 38 µl TEMED and 175 µl ammonium persulfate (APS). 5'-IRD700 or IRD800 labeled sequencing primers were used for standard sequencing runs. Basecalling and quality assessment was conducted using e- Seq software package provided with the sequencer.

7.11 Screening of a *Hydra oligactis* full length cDNA library for *HyDkk1/2/4* genes

For the identification of *HyDkk1/2/4* genes within *Hydra oligactis*, I screened an existing full length cDNA library (Spudy, 2006). Five filters were available comprising around 40,000 spotted clones. For the synthesis of the radioactively labelled probe, a full length DNA fragment of *HyDkk1/2/4 C* from *Hydra magnipapillata* was used. The gene AK17 from *Ciona intestinalis* served on the filters as a guide spot and a labelling reaction for AK17 was prepared in parallel. The templates were denatured and cooled down to room temperature. During the cooling process, random hexamers were added. The probes were prepared as follows:

Component	Volume [µl]
Denatured cDNA probe (100ng)	74
Random hexamer primer (10mM)	10
10x Klenow fragment buffer (10x)	10
D A-G-T-Mix (1mM)	2
Klenow fragment (10 U/µl)	2
[αP32]-dCTP (250µCi/µl)	2
Total	100

The assembly of the labelling reactions was followed by a 2,5 h incubation at 37°C. Unincorporated ³²P-dCTP was removed by size-exclusion chromatography using Sephadex G50 columns. Incorporation rates were determined using a scintillation counter (Cerenkov protocol) measuring counts per minute (cpm) of 1 µl of the labelling reaction before and after the usage of the columns. The filters were prehybridised in

100 ml hybridisation solution (HS) for 3 h. The hybridisation solution was exchanged and hybridisation occurred overnight at 55 °C at a final probe concentration of 3×10^5 cpm/ml. The removal of HS was followed by two washing steps with 2 x SSC/0,1 % SDS (20') and one washing step with 0,2 x SSC/0,1 % SDS (20'). Afterwards filters were sealed and exposed to X-ray films. After development of the films 42 signals could be identified. Eight positive clones were picked from the library and the inserts were subsequently sequenced.

7.12 Regeneration experiments

In all regeneration experiments, animals were bisected within the maximum of EGFP/HyDkk1/2/4 C expressing cells shortly below the boundary of expression. Regenerating animals were not fed unless otherwise stated and fixed to different time points for further analysis.

7.13 Expression analysis via *in situ* hybridisation

7.13.1 Preparation of Biotin/Dig-labelled RNA probes

Gel-purified PCR products were used for probe preparation. Biotin/Dig-labeled probes were prepared using “Biotin RNA labelling kit” or “Dig RNA labeling kit (SP6/T7)” (Roche) according to the manufacturer’s protocol.

7.13.2 Protocol *in situ* hybridisation

In situ hybridisations were adapted from previous works (Grens et al., 1996; Philipp et al., 2005).

All the steps were carried out at room temperature under agitation if not mentioned otherwise. 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 7.4. Fixed polyps were then washed twice with 100% methanol and could be stored at -20°C. In some cases for better penetration of the probe, antibody and substrate animals were cut longitudinally after fixation. Prior to hybridisation the animals were rehydrated stepwise (100% EtOH, 75% EtOH, 50%EtOH/50% PBT, 25% EtOH/75% PBT, PBT (5' each step)) and washed 3x10' in PBT. Afterwards the polyps were treated for 20' at RT with 10 µg/ml proteinase K. The digestion was stopped via rinsing with 4 mg/ml glycine/PBT solution and an additional 10' wash. Then, after 3x5'

washes in PBT the animals were treated 2x5' with 0.1M triethanolamine pH 7.8, followed by two washes: once with 2.5 μ l/ml acetic anhydride/triethanolamine and once with 5 μ l/ml acetic anhydride/triethanolamine for 5 min each, to cover 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 70°C in prewarmed 2XSSC to inactivate endogenous alkaline phosphatases. Then the polyps were washed once for 10' with 50% hybridization solution/50% 2xSSC, once with pure hybridisation solution (HS; 50% formamide, 5xSSC, 1mg/ml CHAPS, 1x Denhardt's, 100 μ g/ml heparin, 100 μ g/ml tRNA, 0.1% Tween 20), and prehybridized in HS at hybridization temperature for 2 h. Digoxigenin-labeled riboprobe was then added to the samples and hybridization was carried out for 18-60 hours at 57°C. After hybridisation the samples were subjected to a series of 10' washes at hybridisation temperature with 100% HS, 75%HS/25% 2xSSC, 50%HS/50% 2xSSC, 25%HS/75% 2xSSC, then the animals were washed 2x30' with 0,1% CHAPS/2xSSC at hybridisation temperature. Afterwards the animals were washed two times with MAB at RT and blocked first for 1 hour in MAB/1%BSA, then for 2 hours in a solution containing 80% MAB/1%BSA and 20% of heat-inactivated sheep serum at 4°C. Antidigoxigenin antibody (alkaline phosphatase coupled) were added to the blocking solution in final dilution of 1:2000 and polyps were incubated with the antibody overnight at 4°C. Unbound antibody was removed by 8x40' washes with MAB-T (or MAB), then the animals were washed twice for 5' in NTMT/Levamisole (1mM), and 10 μ l/ml of NBT/BCIP mixture in NTMT was applied. Staining reaction took place at RT in the dark and was stopped by brief washes in PBS, followed by dehydration in methanol or ethanol dilution series. Afterwards the polyps were embedded in Euparal.

Double in situ hybridisations were performed using DIG- and Biotin-labelled RNA probes simultaneously. After the standard protocol and incubation with AntiBiotin AP antibody, FAST RED was used as the first substrate. The staining reaction was stopped by a 10' wash in 100mM glycine-HCL, pH 2.2/0.1% Tween. Four short washes with MAB were followed by 2X10' MAB and subsequent preblocking in MAB/1% BSA for 1h. Afterwards 2 h incubation in blocking solution (80% MAB/1%BSA and 20% of heat-inactivated sheep serum) at 4°C took place with subsequent antiDIG AP antibody incubation over night at 4°C. Unbound antibody was again removed by 8x40' washes with MAB-T (or MAB), then the animals were washed twice for 5' in NTMT, a 10 μ l/ml of NBT/BCIP mixture in NTMT and the animals were processed as described above.

7.14 Generation of transgenic *Hydra*

7.14.1 Preparation of a *HyDkk1/2/4C::EGFP* promoter construct

The transfection construct used was hoT G, originally designed by Jan U. Lohmann. In HoT G the plasmid pUC19 was used as a backbone. In the original construct 1,386 bp of the *H. vulgaris* actin 5'-flanking region, the transcription start site, the native initiator codon, and the first 10 amino acids of *H. vulgaris* actin were put in front of a GFP reporter gene which was followed by 3' genomic region of the *H. vulgaris* actin gene including the termination polyadenylation signal (Wittlieb et al., 2006). Subsequently, GFP was replaced by EGFP by site-directed mutagenesis and two additional restriction sites were introduced (Fig. 7.2, Böttger et al., 2002). hoT G was further modified by K. Khalturin who inserted an additional Not I restriction site at an existing PstI cutting site between promoter and reporter (Fig. 7.2).

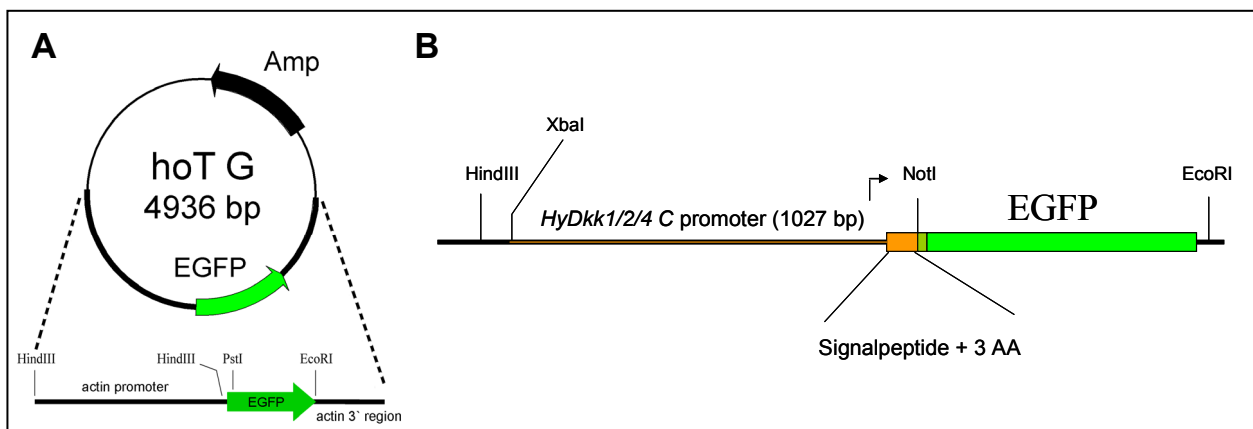


Fig. 7.2: Transfection constructs used for generation of the transgenic lines which were used in this study. (A) Control animals expressed EGFP within their interstitial cell lineage driven by the *Hydra* actin promoter (Khalturin et al., 2007). The construct used for this line was hoT G (taken from Wittlieb et al., 2006). **(B)** The actin promoter of hoT G was exchanged by 1.027 bp *HyDkk1/2/4 C* 5'flanking region and coding sequence for signal peptide and three additional aa using XbaI and NotI cutting sites.

To test the putative promoter region of *HyDkk1/2/4 C* a fragment of 1,093 bp, comprising 1027 bp *HyDkk1/2/4 C* 5'flanking region and 66 bp coding for the predicted *HyDkk1/2/4 C* signal peptide and three subsequent residues, was amplified from *H. magnipapillata* gDNA using Platinum High Fidelity polymerase and primers R DLP1 F/P DLP1 R. By reamplification, two cutting sites were attached to the fragment using primers Dlp_pr XbaI and Dlp_pr Not I. The fragment was cloned into the modification of hoT G EGFP expression vector using XbaI and a newly inserted Not I cutting site.

Microcon YM-50 columns were used for buffer exchange between the successive digestions of fragment and vector. The resulting transfection constructs were sequenced and positive clones were grown for plasmid preparations. The plasmid DNA was purified using Qiagen MidiPrep Kit.

7.14.2 Transfection of *Hydra*

For transfection, *Hydra* embryos were removed from *H. vulgaris* (AEP) females and microinjected with the expression construct at the two- to eight-cell stage. Microinjection was performed using an inverted microscope and two micromanipulators. Embryos were held by a micropipette by using a CellTram Air pump. The construct was injected by using a CellTram vario pump. Glass needles for microinjection were produced by using Vertical Pipette Puller 700 C. The construct was injected into the embryos in a final plasmid concentration of 1 µg/µl. The injection solution contained 2% RITC to visualise the injection success. If possible, several blastomeres were injected. During the injection procedure, embryos were kept in HM at RT. Microinjected embryos were transferred to 12-well microtiter plates and incubated at 18°C in HM.

7.15 Imaging

In vivo observations were made and documented using Olympus SZX16 and Olympus DP71 digital camera. To slow down movements of adult polyps, animals were put in between two layers of transparent foil.

Laser-scanning confocal data were acquired using Leica CLSM TCS SP/UV and Leica DC300F digital camera. Light microscopic analyses were performed using Zeiss Axioskop 2 and Zeiss AxioCam digital camera.

7.16 Histological methods

7.16.1 Maceration of *Hydra* tissue

Maceration preparations were performed as described previously (David, 1972). Hypostomes of 20 polyps were removed using a scalpel and were transferred into a 1.5 ml Eppendorf tube, excessive hydramedium was removed and 20 µl of maceration solution were added. The tube was incubated at 32°C for 15' with occasional gentle shaking. In the course of this incubation tissues disintegrated to single cells. The cells

were then fixed with equal volume of 8% formaldehyde. The resultant cell suspension was distributed over a square space on a glass slide pre-treated with 1% gelatine solution. To reduce surface tension of the cell suspension a small drop of 1% Tween-20 detergent was placed on a glass slide prior to the cell suspension. The preparations were dried overnight at room temperature and analysed using phase contrast microscopy.

7.16.2 Fixation of *Hydra* for confocal microscopy

Polyps were relaxed in 2% urethane prior to fixation in 4% paraformaldehyde. Animals were washed six times for 15' and overnight in PBT. Following washing samples were stained with phalloidin (1:1000) and then rinsed three times for 10' in PBT. Prior to embedding in Mowiol/DABCO animals were incubated in HOECHST (1:500).

7.16.3 Fixation of *Hydra* for transmission electron microscopy

Polyps were relaxed in 2% urethane prior to fixation in 3.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 18 h at 4°C. After washing with 0.075 M cacodylate buffer for 30', animals were postfixed with 1% OsO₄ in 0.075 M cacodylate buffer for 2 h at 4°C. After additional washing for 30' the tissue were dehydrated in 30%, 50%, 60%, 70%, 80%, 90%, 100%, 100% EtOH (each step 15'). After incubation of 2 x 10' in 1,2-propylenoxide, it was proceeded with infiltration of Agar 100 resin. Components of the embedding resin were mixed according to the manufacturer's protocol for the "hard" version. Infiltration occurred in several steps each of which took 30' with the following mixtures of propylenoxide and Agar 100 resin: 1:2, 1:1 and 2:1. This was followed by incubation in the pure Agar resin overnight at RT and under agitation. Finally samples were placed into rubber forms (Flacheinbettung/Plano) in a new portion of the resin and were let polymerise at 60° C.

7.16.4 Sectioning and staining: semi-thin sections

Blocks with embedded tissues were trimmed with a razor blade. Semi-thin sections were cut at the Ultracut S ultratome (~0,5 µm thickness). Semi-thin sections were captured on a glass slide and were stained according to Richardson et al. (1960) with a solution containing 0.5% Methylene Blue, 0.5% borax, and 0.5% Azur II in ddH₂O at 60°C for 1–2' (Richardson et al., 1960).

7.16.5 Sectioning and contrasting: ultra-thin sections

Ultra-thin sections were prepared using also the Ultracut S ultratome. Sections (60-70 nm thickness) were captured on round grids which were pretreated with 1.2% pioloform dissolved in chloroform. Ultra-thin sections were contrasted with 2.5% uranylacetate for 5', rinsed with ddH₂O, then treated with lead citrate solution (prepared freshly from lead acetate and sodium citrate) for 3' (Reynolds, 1963) and analysed using a transmission electron microscope EM 208 S.

7.17 Computational methods

7.17.1 Multiple alignments

Sequence alignments were generated using ClustalW (Thompson et al., 1994) included into the BioEdit v.7.053 sequence analysis software package. To infer phylogenetic relationships neighbour-joining (NJ) was used. Tree was drawn using MEGA4.

7.17.2 mVISTA

Conservation profiles were inferred by using the mVista server. The AVID alignment algorithm was used (Bray et al., 2003).

7.17.3 GATA

Standard GATA (Nix and Eisen, 2005) alignment settings were used, with a window size of 24 and a lower cut off score of 70 (raw)/219.0 (bit). BLASTN parameters were: Nt: Match, 5, Mismatch, -4, Gap: Creation: -10, Extension -4.

7.17.4 Footprinter 3.0

Footprinter analysis (Blanchette and Tompa, 2003) was performed using a motif size of eight. Allowed maximum of mutations and maximum mutations per branch were chosen as one. The used phylogenetic tree was (vir,(oli, (AEP, mag))).

8 References

- Aravind, L. and E. V. Koonin (1998). "A colipase fold in the carboxy-terminal domain of the Wnt antagonists--the Dickkopfs." Curr Biol **8**(14): R477-8.
- Ashe, H. L. and J. Briscoe (2006). "The interpretation of morphogen gradients." Development **133**(3): 385-94.
- Augustin, R., A. Franke, et al. (2006). "Dickkopf related genes are components of the positional value gradient in Hydra." Dev Biol **296**(1): 62-70.
- Bergman, C. M., B. D. Pfeiffer, et al. (2002). "Assessing the impact of comparative genomic sequence data on the functional annotation of the Drosophila genome." Genome Biol **3**(12): RESEARCH0086.
- Bielen, H., S. Oberleitner, et al. (2007). "Divergent functions of two ancient Hydra Brachyury paralogues suggest specific roles for their C-terminal domains in tissue fate induction." Development **134**(23): 4187-97.
- Blanchette, M. and M. Tompa (2003). "FootPrinter: A program designed for phylogenetic footprinting." Nucleic Acids Res **31**(13): 3840-2.
- Bode, H., S. Berking, et al. (1973). "Quantitative analysis of cell types during growth and morphogenesis in Hydra." Wilhelm Roux' Arch. Dev. Biol. **139**: 231-243.
- Bode, H., J. Dunne, et al. (1986). "Transdifferentiation occurs continuously in adult hydra." Curr Top Dev Biol **20**: 257-80.
- Bode, H. R. (1992). "Continuous conversion of neuron phenotype in hydra." Trends Genet **8**(8): 279-84.
- Bode, H. R. (1996). "The interstitial cell lineage of hydra: a stem cell system that arose early in evolution." J Cell Sci **109 (Pt 6)**: 1155-64.
- Bode, H. R., S. Heimfeld, et al. (1987). "Gland cells arise by differentiation from interstitial cells in Hydra attenuata." Dev Biol **122**(2): 577-85.
- Boisbouvier, J., J. P. Albrand, et al. (1998). "A structural homologue of colipase in black mamba venom revealed by NMR floating disulphide bridge analysis." J Mol Biol **283**(1): 205-19.
- Bosch, T. C. (2007a). "Symmetry breaking in stem cells of the basal metazoan Hydra." Prog Mol Subcell Biol **45**: 61-78.
- Bosch, T. C. (2007b). "Why polyps regenerate and we don't: towards a cellular and molecular framework for Hydra regeneration." Dev Biol **303**(2): 421-33.
- Bosch, T. C. and C. N. David (1987). "Stem cells of Hydra magnipapillata can differentiate into somatic cells and germ cells." Dev Biol **121**: 182-192.
- Böttger, A., O. Alexandrova, et al. (2002). "GFP expression in Hydra: lessons from the particle gun." Dev Genes Evol **212**(6): 302-5.
- Bray, N., I. Dubchak, et al. (2003). "AVID: A global alignment program." Genome Res **13**(1): 97-102.
- Bridge, D., C. W. Cunningham, et al. (1995). "Class-level relationships in the phylum Cnidaria: molecular and morphological evidence." Mol Biol Evol **12**(4): 679-89.
- Bridge, D., C. W. Cunningham, et al. (1992). "Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure." Proc Natl Acad Sci U S A **89**(18): 8750-3.
- Brott, B. K. and S. Y. Sokol (2002). "Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins." Mol Cell Biol **22**(17): 6100-10.
- Broun, M., L. Gee, et al. (2005). "Formation of the head organizer in hydra involves the canonical Wnt pathway." Development **132**(12): 2907-16.
- Brown, E. N. (1909). "The production of new hydrants in Hydra by insertion of small grafts." J. Exp. Zool.(7): 1-23.

- Campbell, R. D. (1967a). "Tissue dynamics of steady state growth in *Hydra littoralis*. I. Patterns of cell division." Dev Biol **15**(5): 487-502.
- Campbell, R. D. (1967b). "Tissue dynamics of steady state growth in *Hydra littoralis*. II. Patterns of tissue movement." J Morphol **121**(1): 19-28.
- Campbell, R. D. (1967c). "Tissue dynamics of steady state growth in *Hydra littoralis* III. Behaviour of specific cell types during tissue movements." J. Exp. Zool.(164): 379-392.
- Chen, J. Y., P. Oliveri, et al. (2002). "Precambrian animal life: probable developmental and adult cnidarian forms from Southwest China." Dev Biol **248**(1): 182-96.
- Chera, S., R. de Rosa, et al. (2006). "Silencing of the hydra serine protease inhibitor Kazal1 gene mimics the human SPINK1 pancreatic phenotype." J Cell Sci **119**(Pt 5): 846-57.
- Chourrout, D., F. Delsuc, et al. (2006). "Minimal ProtoHox cluster inferred from bilaterian and cnidarian Hox complements." Nature **442**(7103): 684-7.
- Clarkson, S. G. and L. Wolpert (1967). "Bud morphogenesis in hydra." Nature **214**(5090): 780-3.
- Collins, A. G. (2002). "Phylogeny of Medusozoa and the evolution of cnidarian life cycles." J. Evol. Biol. **15**: 418-432.
- Collins, A. G., P. Schuchert, et al. (2006). "Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models." Syst Biol **55**(1): 97-115.
- Cummings, S. G. and H. Bode (1984). "Head regeneration and polarity reversal in *Hydra attenuata* can occur in the absence of DNA synthesis." Roux's Arch Dev Biol **194**: 79-86.
- David, C. N. (1972). "Quantitative method for maceration of *Hydra* tissue." Roux's Arch Dev Biol **171**: 259-268.
- David, C. N. and I. Plotnick (1980). "Distribution of interstitial stem cells in *Hydra*." Dev Biol **76**(1): 175-84.
- Davis, L. E. (1973). "Histological and ultrastructural studies of the basal disk of *Hydra*. I. The glandulomuscular cell." Z Zellforsch Mikrosk Anat **139**(1): 1-27.
- Devon, R. S., D. J. Porteous, et al. (1995). "Splinkerettes--improved vectorettes for greater efficiency in PCR walking." Nucleic Acids Res **23**(9): 1644-5.
- Dübel, S. (1989). "Cell differentiation in the head of *Hydra*." Differentiation **41**: 99-109.
- Dübel, S., S. A. Hoffmeister, et al. (1987). "Differentiation pathways of ectodermal epithelial cells in *hydra*." Differentiation **35**(3): 181-9.
- Echeverri, K., J. D. Clarke, et al. (2001). "In vivo imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema." Dev Biol **236**(1): 151-64.
- Eguchi, G. and R. Kodama (1993). "Transdifferentiation." Curr Opin Cell Biol **5**(6): 1023-8.
- Endl, I., J. U. Lohmann, et al. (1999). "Head-specific gene expression in *Hydra*: complexity of DNA- protein interactions at the promoter of *ks1* is inversely correlated to the head activation potential." Proc Natl Acad Sci U S A **96**(4): 1445-50.
- Fedders, H., R. Augustin, et al. (2004). "A Dickkopf- 3-related gene is expressed in differentiating nematocytes in the basal metazoan *Hydra*." Dev Genes Evol **214**(2): 72-80.
- Galliot, B., M. Miljkovic-Licina, et al. (2007). "RNAi gene silencing affects cell and developmental plasticity in *hydra*." C R Biol **330**(6-7): 491-7.
- Galliot, B., M. Welschof, et al. (1995). "The cAMP response element binding protein is involved in *hydra* regeneration." Development **121**(4): 1205-16.

- Gauthier, G. F. (1963). "Cytological studies on the gastroderm of Hydra." J Exp Biol **152**: 13-39.
- Gierer, A., S. Berking, et al. (1972). "Regeneration of hydra from reaggregated cells." Nat New Biol **239**(91): 98-101.
- Gierer, A. and H. Meinhardt (1972). "A theory of biological pattern formation." Kybernetik **12**(1): 30-9.
- Glinka, A., W. Wu, et al. (1998). "Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction." Nature **391**(6665): 357-62.
- Grens, A., L. Gee, et al. (1996). "CnNK-2, an NK-2 homeobox gene, has a role in patterning the basal end of the axis in hydra." Dev Biol **180**(2): 473-88.
- Grieneisen, V. A., J. Xu, et al. (2007). "Auxin transport is sufficient to generate a maximum and gradient guiding root growth." Nature **449**(7165): 1008-13.
- Guder, C., I. Philipp, et al. (2006b). "The Wnt code: cnidarians signal the way." Oncogene **25**(57): 7450-60.
- Guder, C., S. Pinho, et al. (2006a). "An ancient Wnt-Dickkopf antagonism in Hydra." Development **133**(5): 901-11.
- Gurdon, J. B. and P. Y. Bourillot (2001). "Morphogen gradient interpretation." Nature **413**(6858): 797-803.
- Harrisingh, M. C., E. Perez-Nadales, et al. (2004). "The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation." Embo J **23**(15): 3061-71.
- Haynes, J. and A. L. Burnett (1963). "Dedifferentiation and Redifferentiation of Cells in Hydra Viridis." Science **142**: 1481-3.
- Hemrich, G., B. Anokhin, et al. (2007). "Molecular phylogenetics in Hydra, a classical model in evolutionary developmental biology." Mol Phylogenet Evol **44**(1): 281-90.
- Hicklin, J. and L. Wolpert (1973). "Positional information and pattern regulation in hydra: the effect of gamma-radiation." J Embryol Exp Morphol **30**(3): 741-52.
- Hobmayer, B., F. Rentzsch, et al. (2001). "Identification and expression of HySmad1, a member of the R-Smad family of TGFbeta signal transducers, in the diploblastic metazoan Hydra." Dev Genes Evol **211**(12): 597-602.
- Hobmayer, B., F. Rentzsch, et al. (2000). "WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra." Nature **407**(6801): 186-9.
- Hubbard, T. J., B. Ailey, et al. (1999). "SCOP: a Structural Classification of Proteins database." Nucleic Acids Res **27**(1): 254-6.
- Hwang, J. S., H. Ohyanagi, et al. (2007). "The evolutionary emergence of cell type-specific genes inferred from the gene expression analysis of Hydra." Proc Natl Acad Sci U S A **104**(37): 14735-40.
- Käsbauer, T., P. Towb, et al. (2007). "The Notch signaling pathway in the cnidarian Hydra." Dev Biol **303**(1): 376-90.
- Kaser, A., M. Winklmayr, et al. (2003). "The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions." EMBO Rep **4**(5): 469-73.
- Kawamura, K. and S. Fujiwara (1994). "Transdifferentiation of pigmented multipotent epithelium during morphallactic development of budding tunicates." Int J Dev Biol **38**(2): 369-77.
- Kawamura, K. and S. Fujiwara (1995). "Cellular and molecular characterization of transdifferentiation in the process of morphallaxis of budding tunicates." Semin Cell Biol **6**(3): 117-26.
- Khalturin, K., F. Anton-Erxleben, et al. (2007). "Transgenic stem cells in Hydra reveal an early evolutionary origin for key elements controlling self-renewal and differentiation." Dev Biol **309**(1): 32-44.

- Klebes, A., A. Sustar, et al. (2005). "Regulation of cellular plasticity in *Drosophila* imaginal disc cells by the Polycomb group, trithorax group and lama genes." Development **132**(16): 3753-65.
- Koizumi, O. and H. R. Bode (1986). "Plasticity in the nervous system of adult hydra. I. The position-dependent expression of FMRFamide-like immunoreactivity." Dev Biol **116**(2): 407-21.
- Koizumi, O., S. Heimfeld, et al. (1988). "Plasticity in the nervous system of adult hydra. II. Conversion of ganglion cells of the body column into epidermal sensory cells of the hypostome." Dev Biol **129**(2): 358-71.
- Krakauer, D. C. and M. A. Nowak (1999). "Evolutionary preservation of redundant duplicated genes." Semin Cell Dev Biol **10**(5): 555-9.
- Krupnik, V. E., J. D. Sharp, et al. (1999). "Functional and structural diversity of the human Dickkopf gene family." Gene **238**(2): 301-13.
- Kusserow, A., K. Pang, et al. (2005). "Unexpected complexity of the Wnt gene family in a sea anemone." Nature **433**(7022): 156-60.
- Lander, A. D. (2007). "Morpheus unbound: reimagining the morphogen gradient." Cell **128**(2): 245-56.
- Lee, N., C. Maurange, et al. (2005). "Suppression of Polycomb group proteins by JNK signalling induces transdetermination in *Drosophila* imaginal discs." Nature **438**(7065): 234-7.
- Lee, P. N., K. Pang, et al. (2006). "A WNT of things to come: evolution of Wnt signaling and polarity in cnidarians." Semin Cell Dev Biol **17**(2): 157-67.
- Lenhoff, H. M. and R. D. Brown (1970). "Mass culture of hydra: an improved method and its application to other aquatic invertebrates." Lab Anim **4**(1): 139-54.
- Leontovich, A. A., J. Zhang, et al. (2000). "A novel hydra matrix metalloproteinase (HMMP) functions in extracellular matrix degradation, morphogenesis and the maintenance of differentiated cells in the foot process." Development **127**(4): 907-20.
- Lohmann, J. U., I. Endl, et al. (1999). "Silencing of developmental genes in Hydra." Dev Biol **214**(1): 211-4.
- MacWilliams, H. K. (1983). "Hydra transplantation phenomena and the mechanism of Hydra head regeneration. II. Properties of the head activation." Dev Biol **96**(1): 239-57.
- Mao, B. and C. Niehrs (2003). "Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling." Gene **302**(1-2): 179-83.
- Martin, V. J., C. L. Littlefield, et al. (1997). "Embryogenesis in hydra." Biol Bull **192**(3): 345-63.
- Martindale, M. Q., J. R. Finnerty, et al. (2002). "The Radiata and the evolutionary origins of the bilaterian body plan." Mol Phylogenet Evol **24**(3): 358-65.
- Matys, V., O. V. Kel-Margoulis, et al. (2006). "TRANSFAC and its module TRANSCOMP: transcriptional gene regulation in eukaryotes." Nucleic Acids Res **34**(Database issue): D108-10.
- McClure, K. D. and G. Schubiger (2007). "Transdetermination: *Drosophila* imaginal disc cells exhibit stem cell-like potency." Int J Biochem Cell Biol **39**(6): 1105-18.
- Medina, M., A. G. Collins, et al. (2001). "Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA." Proc Natl Acad Sci U S A **98**(17): 9707-12.
- Meinhardt, H. (2007). "1 models of biological pattern formation: from elementary steps to the organization of embryonic axes." Curr Top Dev Biol **81**: 1-63.
- Miljkovic-Licina, M., S. Chera, et al. (2007). "Head regeneration in wild-type hydra requires de novo neurogenesis." Development **134**(6): 1191-201.

- Miljkovic-Licina, M., D. Gauchat, et al. (2004). "Neuronal evolution: analysis of regulatory genes in a first-evolved nervous system, the hydra nervous system." Biosystems **76**(1-3): 75-87.
- Miller, D. J., E. E. Ball, et al. (2005). "Cnidarians and ancestral genetic complexity in the animal kingdom." Trends Genet **21**(10): 536-9.
- Miller, D. J., G. Hemmrich, et al. (2007). "The innate immune repertoire in cnidaria--ancestral complexity and stochastic gene loss." Genome Biol **8**(4): R59.
- Minobe, S., K. Fei, et al. (2000). "Identification and characterization of the epithelial polarity receptor "Frizzled" in *Hydra vulgaris*." Dev Genes Evol **210**(5): 258-62.
- Morgan, T. H. (1901). "Regeneration in the egg, embryo and adult." Am Natur **35**: 949-973.
- Niehrs, C. (2006). "Function and biological roles of the Dickkopf family of Wnt modulators." Oncogene **25**(57): 7469-81.
- Nielsen, C., N. Scharff, et al. (1996). "Cladistic analyses of the animal kingdom." Biol J Linn Soc **57**: 385-410.
- Nix, D. A. and M. B. Eisen (2005). "GATA: a graphic alignment tool for comparative sequence analysis." BMC Bioinformatics **6**: 9.
- Nordhoff, E., A. M. Krogsdam, et al. (1999). "Rapid identification of DNA-binding proteins by mass spectrometry." Nat Biotechnol **17**(9): 884-8.
- Nordhoff, E. and H. Lehrach (2007). "Identification and characterization of DNA-binding proteins by mass spectrometry." Adv Biochem Eng Biotechnol **104**: 111-95.
- Olbrich, D. (2005). "Molekularbiologische Untersuchungen zur Musterbildung von *Hydra*." Diploma thesis.
- Park, H. D., A. B. Ortmeier, et al. (1970). "Cell division during regeneration in *Hydra*." Nature **227**(5258): 617-9.
- Patapoutian, A., B. J. Wold, et al. (1995). "Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle." Science **270**(5243): 1818-21.
- Philipp, I., T. W. Holstein, et al. (2005). "HvJNK, a *Hydra* member of the c-Jun NH2-terminal kinase gene family, is expressed during nematocyte differentiation." Gene Expr Patterns **5**(3): 397-402.
- Philippe, H., N. Lartillot, et al. (2005). "Multigene analyses of bilaterian animals corroborate the monophyly of Ecdysozoa, Lophotrochozoa, and Protostomia." Mol Biol Evol **22**(5): 1246-53.
- Putnam, N. H., M. Srivastava, et al. (2007). "Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization." Science **317**(5834): 86-94.
- Reber-Muller, S., R. Streitwolf-Engel, et al. (2006). "BMP2/4 and BMP5-8 in jellyfish development and transdifferentiation." Int J Dev Biol **50**(4): 377-84.
- Reynolds, A. S. (1963). "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy." J Cell Biol **17**: 208-212.
- Richardson, K. C., L. Jarett, et al. (1960). "Embedding in epoxy resins for ultrathin sectioning in electron microscopy." Stain Technol **35**: 313-23.
- Rose, P. G. and A. L. Burnett (1968). "An electron microscopic and histochemical study of the secretory cells in *Hydra viridis*." Wilhelm Roux' Arch. **161**: 281-297.
- Rose, P. G. and A. L. Burnett (1968b). "An electron microscopic and radioautographic study of hypostomal regeneration in *Hydra viridis*." Wilhelm Roux' Arch. **161**: 298-318.
- Rose, P. G. and A. L. Burnett (1970). "The origin of mucous cells in *Hydra viridis* " Wilhelm Roux' Arch. **165**: 177-191.
- Schmid, V. and H. Alder (1984). "Isolated, mononucleated, striated muscle can undergo pluripotent transdifferentiation and form a complex regenerate." Cell **38**(3): 801-9.

- Schmid, V. and S. Reber-Muller (1995). "Transdifferentiation of isolated striated muscle of jellyfish in vitro: the initiation process." Semin Cell Biol **6**(3): 109-16.
- Schmidt, T. and C. N. David (1986). "Gland cells in Hydra: cell cycle kinetics and development." J Cell Sci **85**: 197-215.
- Selman, K. and F. C. Kafatos (1974). "Transdifferentiation in the labial gland of silk moths: is DNA required for cellular metamorphosis?" Cell Differ **3**(2): 81-94.
- Semal-van Gansen, P. (1954). "L'histologie de l'endoderme de l'hydre d'eau douce." Ann. Soc. Zool. Malac. Bel. **85**: 217-278.
- Shimizu, H., Y. Sawada, et al. (1993). "Minimum tissue size required for hydra regeneration." Dev Biol **155**(2): 287-96.
- Siebert, S., F. Anton-Erxleben, et al. (2007). "Cell type complexity in the basal metazoan Hydra is maintained by both stem cell based mechanisms and transdifferentiation." Dev Biol **1**(313): In Press.
- Siebert, S., S. Thomsen, et al. (2005). "Control of foot differentiation in Hydra: phylogenetic footprinting indicates interaction of head, bud and foot patterning systems." Mech Dev **122**(9): 998-1007.
- Slack, J. M. and D. Tosh (2001). "Transdifferentiation and metaplasia--switching cell types." Curr Opin Genet Dev **11**(5): 581-6.
- Spudy, B. (2006). "Molekulare Analyse der angeborenen Immunantwort bei *Hydra*." Diploma thesis.
- Steele, R. E. (2002). "Developmental signaling in Hydra: what does it take to build a "simple" animal?" Dev Biol **248**(2): 199-219.
- Steele, R. E., P. Lieu, et al. (1996). "Response to insulin and the expression pattern of a gene encoding an insulin receptor homologue suggests a role for an insulin-like molecule in regulating growth and patterning in Hydra." Dev. Gen. Evol. **206**: 247-259.
- Straube, W. L. and E. M. Tanaka (2006). "Reversibility of the differentiated state: regeneration in amphibians." Artif Organs **30**(10): 743-55.
- Streuli, C. (1999). "Extracellular matrix remodelling and cellular differentiation." Curr Opin Cell Biol **11**(5): 634-40.
- Sugiyama, T. and T. Fujisawa (1978). "Genetic analysis of developmental mechanisms in Hydra. II. Isolation and characterization of an interstitial cell-deficient strain." J Cell Sci **29**: 35-52.
- Tardent, P. (1954). "Axiale Verteilungsgradienten der interstitiellen Zellen bei Hydra und Tubularia und ihre Bedeutung für die Regeneration." Wilhelm Roux' Arch. Entwicklungsmech. Organismen **146**: 593-649.
- Tardent, P. (1995). "The Cnidarian cnidocyte, a hightech cellular weaponry." Bioessays **17**: 351-362.
- Taub, R. (2004). "Liver regeneration: from myth to mechanism." Nat Rev Mol Cell Biol **5**(10): 836-47.
- Technau, U. and T. W. Holstein (1996). "Phenotypic maturation of neurons and continuous precursor migration in the formation of the peduncle nerve net in Hydra." Dev Biol **177**(2): 599-615.
- Technau, U., S. Rudd, et al. (2005). "Maintenance of ancestral complexity and non-metazoan genes in two basal cnidarians." Trends Genet **21**(12): 633-9.
- Teragawa, C. K. and H. R. Bode (1990). "Spatial and temporal patterns of interstitial cell migration in *Hydra vulgaris*." Dev Biol **138**(1): 63-81.
- Teragawa, C. K. and H. R. Bode (1991). "A head signal influences apical migration of interstitial cells in *Hydra vulgaris*." Dev Biol **147**(2): 293-302.
- Thompson, J. D., D. G. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-

- specific gap penalties and weight matrix choice." Nucleic Acids Res **22**(22): 4673-80.
- Thomsen, S. and T. C. Bosch (2006). "Foot differentiation and genomic plasticity in Hydra: lessons from the PPOD gene family." Dev Genes Evol **216**(2): 57-68.
- Thomsen, S., A. Till, et al. (2004). "Control of foot differentiation in Hydra: in vitro evidence that the NK-2 homeobox factor CnNK-2 autoregulates its own expression and uses pedibin as target gene." Mech Dev **121**(2): 195-204.
- Tosh, D. and J. M. Slack (2002). "How cells change their phenotype." Nat Rev Mol Cell Biol **3**(3): 187-94.
- Trembley, A., Ed. (1744). *Memoires pour servir a l'histoire d'un genre de polyps d'eau douce*. Leiden, J.u.H.Verbeek.
- Tsonis, P. A. and K. Del Rio-Tsonis (2004). "Lens and retina regeneration: transdifferentiation, stem cells and clinical applications." Exp Eye Res **78**(2): 161-72.
- Turing, A. (1952). "The chemical basis of morphogenesis." Philos. Trans. B. **641**: 37-72.
- van Tilbeurgh, H., S. Bezzine, et al. (1999). "Colipase: structure and interaction with pancreatic lipase." Biochim Biophys Acta **1441**: 173-184.
- White, P. M., A. Doetzlhofer, et al. (2006). "Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells." Nature **441**(7096): 984-7.
- Wittlieb, J., K. Khalturin, et al. (2006). "Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis." Proc Natl Acad Sci U S A **103**(16): 6208-11.
- Wolpert, L., M. R. Clarke, et al. (1972). "Positional signalling along Hydra." Nature New Biology **239**: 101-103.
- Wolpert, L., A. Hornbruch, et al. (1974). "Positional information and positional signalling in Hydra." Amer. Zool. **14**: 647-663.
- Woo, A. J., J. S. Dods, et al. (2002). "A proteomics approach for the identification of DNA binding activities observed in the electrophoretic mobility shift assay." Mol Cell Proteomics **1**(6): 472-8.
- Xiao, S. and A. H. Knoll (1999). "Fossil preservation in the Neoproterozoic Doushantuo phosphorite Lagerstätte, South China." Lethaia **32**(3): 219-40.
- Yaneva, M. and P. Tempst (2003). "Affinity capture of specific DNA-binding proteins for mass spectrometric identification." Anal Chem **75**(23): 6437-48.
- Yaross, M. S. and H. R. Bode (1978). "Regulation of interstitial cell differentiation in Hydra attenuata. IV. Nerve cell commitment in head regeneration is position-dependent." J Cell Sci **34**: 27-38.
- Yoshii, C., Y. Ueda, et al. (2007). "Neural retinal regeneration in the anuran amphibian *Xenopus laevis* post-metamorphosis: transdifferentiation of retinal pigmented epithelium regenerates the neural retina." Dev Biol **303**(1): 45-56.

9 Appendices

9.1 *HyDkk1/2/4 C cds*

>mag

```
ATGAAATCAACGGTTATTTTTGTTTCATTATTGCTTTGTGCATTGTTTGCAGCGAAGGAATATTCTGGAAATTTTGCTC
ATCAAACAAAGATTGCCATGGAGGCTGTTGTGTAAATTCGGGTTTTTTGGAAAATGCAAATCTTACGTAAAAGAAG
GTGGACTCTGCGGTCTTAGCCAAGCACTTGCCTGTGGTTGCGAACCAGGACTTGACTGCGAGAAAAGTAAGAGGAACT
ATTACAGGATTGATTAGGAAATGTGTTGACAACCTCCGGAAGTGGTTCTTTGTATTAA
```

>oli

```
ATGAAATCCACATTTATTCTGTTTCATTGTTGCTATGTGCATTGTTTGCAGCGAAGGAATATTCTGGAAACTTTGCTC
ATCAAACAAAGATTGTAATGGCGGGTGTGTGTTAAGTTTGGGTTTTATTGGAAAATGCAAATCTTATGTAAAAGAAG
GTGGACTCTGTGGGCTTAGCCAAACACTCGCATGTGGTTGTGAACCAGGGCTCGACTGCGAAAAAGTAAGAGGAACT
ATTACAGGATTGATTAGAAAAGTGTGTTGACAACCTCTGGAAGTGGATCTTTGTATTAA
```

>AEP

```
ATGAAATCAACGGTTATTTTTGTTTCATTGTTGCTTTGTGCATTGTTTGCAGCGAAGGAATATTCTGGAAAATGTGCAA
ATCAAACAAAGATTGCGATGGAGGCTGTTGTGTAAATTAGGGTTTTTAGGAAAATGCAAATCTTACGTAAAAGAAG
GTGGACTCTGCGGCCTTAGCCAAACATTTGCGTGTGGTTGCGAACCAGGACTCGACTGCGAGAAAAGTAAGAGGAACT
ATTACAGGATTGATTAGAAAATGTGTTGACAACCTCCGGAAGTGGTTCTTTGTATTAA
```

>vir

```
ATGAAATCGATTTTTCGTTTTGTTAGTTGTGCTGTATGCGTTGTTTCTTTGGAAGCAAGTTGGCTTTGTCGATCTAA
CAATGATTGCAAAAATGGATGCTGTTTGAATTTCCGATTTGTTAAGAAGTGTCTGCCATACGTTAAAGAAGGTGGAC
TATGCGGCATCCGTGAAAAAATTTTCATGTGGCTGTGAACCAGGTCTCGAATGTGAAAAAGTAAGGGGAACCATCACA
GGCTTGATTA AAAAATGCGTTAACCAATGATAAAGGAAGTGGATCATTGTATTAA
```

9.2 *HyDkk1/2/4 A cds*

>mag

```
ATGATGAGATTTTTAGCAGTCCTTTTAGTTGTAGCTGCATTTCGTTGCATTTAGTGAAGCCGAGTCTTGCAAAAAGGA
TGCAGACTGCAAAAATGGTTGTTGTGTGAATTTCTTACTTACTAAGCAATGTAATAGCTATGTTAAAGAAGGTGCAC
TCTGCGGTTTTTCGTGACAAATTTGCATGCGGTTGTGAACCAGGACTCGAATGCGTAAAAGTACGAGGAACTTTAACT
GGGATGGTTAGAAGATGCGTTGACAATTCAGGAAGTGGATCTTTGTATTAA
```

>oli

```
ATGAGATCATTAGCAGTCCTTCTAATTGTAGCTGTATTTGCTGCTTTCAGCGAAGCCCAGTTTTGCAAAAAGTGATCG
AGATTGCAAAGATGGTTGCTGCGTAAACTTTTTGGTTACTAAGAGATGTAATAGTTATGTTAAAGAAGGTGGACTAT
GCGGTATCCGTAACAAACTTTTCGTGCGGTTGTGAACCAGGACTCGACTGCGTAAAAGTACGAGGAACTTTAACC GGG
GTTATCAGCAAATGCGTTGACAATTCAGGCAGTGGATCTTTGTATTAA
```

>AEP

```
ATGAAATTTTTAGCAGTCCTTTAATTGTAGCTGCATTTCGTTGCATTTAGTGAAGCCAAGTCTTGCAAAAACGGATGC
AGAATGCGAAAATGGTTGCTGTGTAAATTTCTTACTTACTAAGCAATGCAATAGCTATGTTAAAGAAGGTGCACTAT
GTGGTTTTTCGTGACAGATTTACATGCGGTTGTGAACCAGGACTCGAATGTGTA AAAAGTACGAGGAACTTTAACC GGG
ATGATCAGAAAATGCGTTGACCATTCAGGAAGTGGATCTTTGTATTAA
```

9.3 *HyDkk1/2/4 C* - genomic sequence

>mag (*cds*)

```
TGGTTAGCCGGTGGTAGAATAAGTGGTAGTCGGTGGAGGTTCCAACGCCTCCACCGGCTACCACATGGTAAATACAG
TAGTGTTAAAGCTGTTCTCATAAGATTAGATTACGATGAGTAGTATTATTCTGTGAAAATTTTGAACCTGCATATT
ATTAAGAAAAAAGCTGTGCTAAAGCCCTCTTACATGAACGGCACTTTAACCATACCTTATAGACTGCTATGATTTA
AGTGCACATACTTCTAATATTCTGAGCAAACCTTTTTTTCATCTATGTTTTTCCGTTTTAAGAACAGTTACAATAATA
AGATACATGAATAAATATTTACATAGACAGGAGAAAAATAAGACAATGTTAGTCTTACCGCTAAGTGCTTACCGCAT
TAATTTAAAGCTGCTCAATTCAAAATCAACCGCTCTTTTTGATAACATTACCTCTTACCGTACACGTTACACGTC
TATAGAAAACCTTTGAACTAGAGTTTGATATAATACTTAATATAAAAATTTAAATTTAGAAAACCTAATAAAAAAA
AAGAAAAATAGTCACAAGTATGGAAAAAATATAAAAAAGCTATAAAACACAAAAAATAAAAAAATAGAGG
AAAAGATGGGTTAGAAAAAATAAAAAAGTATAACAACCCCTGAAACGAGGGTGCTTTAAAAAGTTATACTTTTGA
CCACTAACTAGTTAGATGAAATTTAAACACCAAAAACATGCTGGTACAACCCCTGTAGAAAAACGTTTATTCAAAA
TATTTTAAATAATTTATTTTAAATAATTGTACAATAAAAAAGCCGTGGCGCAGTGGAAGCGCGTTTGCTCAGAAACAA
AGGATCCGTGATTCAAATCCCAGTTTTGCAACATCGGTTAGAAAAGGAGGCGAAAACCTGCGGTGCTCTGTGATAAGAC
CGAAAGGACTTCTTAAATAAAATTTAAAAAATATTTGTTTTATTTATTTATATATCATATTTGTTTTAAAAGACATAT
ATATGCTGCCATAGTGCATATCATATAAATTGTTAACATTTTAGCAGTAGTTGGTGTGTTGTTACATCTGTGCATATC
AGGCTCAGAACTGAATGTTTATAATACCACTACTAATATGCAAAATTCAAATGCAATATTTATTGCATTTTGGCATC
TAGGACTTAAAATTTTCTAAAACAGTTTTTATTTTGATATAAAAACAGTTTGGTTATAAACATAAACATCTGTTGTCA
AAATATTTAGAAAACCTTTTTGATTAATTTATTTTATAAAAATAAATAAAATTTGAATCAACTGATCAAATTTGCAA
TACAAATTTGCAATAAATATTTGATAGTTAAGCTTCAATAAATTTGACTGTAACATATGTGTGCTCAGTGGGCAACCTGAA
TACCATTATAAAGATATAAAAACGGGGTTTTAATTAAGCACTGCAGAGTCAAACCATTTACTTGATTCAAATTTTT
GTCCAAAATGAAATCAACGGTTATTTTGTTCATTATTGCTTTGTGCATTGTTTGCAGCGAAGGAATATTTCTGGTAA
GAACTTGATTTATTTGTATTTGTATGATTGATTTTAGAAAAGTTAGCTGATAACTAACTGGTAACTAAGTTGCTCTT
TCCAATTAATAGTTAAATAGTGAGTTTTAAATATTTTTTATATTTTAAACTTTTTCTTTTTTATTTAGAAATTTTG
CTCATCAAACAAAGATTGCCATGGAGGCTGTTGTGTTAAATTCGGGTTTTTTGGAAAATGCAAACTTTACGTAAAAG
AAGGTGGACTCTGCGGTCTTAGCGTAAGTTATTCCAACCTGTTTTCTTTTTTAAATTTAAATGATTTCAAATTAAC
TGTTAATTTCTTTAATTTTAAATTTATTTTATATATTTTATATATCAATTTTTTAAATAGAGGTATATATATATATA
TATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
ATATANNNNNNNNNTATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
TATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
ATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
TATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
ATATACATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
TATATATATTTATTTATTTATATATCTAGCAAGCACTTGCATGTGGTTGCGAACCAGGACTTGACTGCGAGAAAAGTA
AGAGGAACTATTACAGGATTGATTAGGAAATGTGTTGACAACCTCCGGAAGTGGTTCTTTGTATTTAATTAAATGTACC
TTTTTCAAAAAATATATACATAATTTATTTTATTCAAATTTGTTTGTATATTATTGTATTGACTTTTTACTTTGCAT
TTATTTTGTATGTGTAATATGGTAGATTATTTGCCTCCAAAAGATTCAATTTGAGATTTTATGGTATAGAGTAAAA
TAGATCAAATGAGAGAGAGAGAGGATAGAATAAAATAGAGGATAGACTAAGATAATTTTATCAATGTTTATTCTAAT
GCTCCTAGTTGTTTCATTGAATAAATATTTATATAAAAAATTTAAAACAAAGTAAATAATTTTGACTGTAAATGATCAAA
```

>AEP (*cds*)

```
AATAGATCAGCTATATAATTTAAAAACTGAAAAACAAGTTAATTATTCGTATTTTCGTAATTTATTCGAAAGAAAAAA
AAAAAAAAAAAAAAGGTTAAAATTTTTTGACAATAATCATTATCAAAAATCACATTTTCTCGCTTTTTATTTTATTTT
TTGTGCGTATTTATATATATAAAATTTATATATATTCACCTTTACTTTTACCTAATCTAGTCATGATATTTTACTTT
ATCTTTTATATATTTTACTTAAAATATATATAAAGTTTTATATATTAACCTTACTTTTACGCAATCTATTTATGAT
ATTATGTATTAACGATATTTTACTTTTATTTATTTATATATCTTTTGTAAACATTCATTTAATTTTTCTTTTATGTTA
TATATTTTTAAAGCAACTTTGTAAAAGTATAAATTTGTAACACGGTGCTTGGCGATAAGGCAAATPATGCCTTCTTC
TTGTTCCAGCCATTAACCTAATTGTAAATTTATGGAACTGTCAAATTTGTTAAACGGCAAAAATAAAAAATAAATAT
AAAAAAAAAAAAAAGTTAGGTTAGAAAAAAGAGTATAACAACCCCTGAAATGAGGGTCTGTAAAGCTTTAAAA
AGCCATAACTTTTGACCACGAAACTATTATTAGATGAAATTTAAACATCAAAAACATGCTGGTAAACCCCTGTAGAA
AACTTTTATTCAAATATTTTATAAATAATTTATTTTAAAAATTTTATAAATAATTTATTTTAAAAATTTGCACAAC
AAAAGCCGTGGCGCAGTGGAAGCGCACTTGCCTCAGAAACGAAGGATCCGTGGTCTCTGGGCAAGTTTTGCGACA
TCGGTTAGGAAGGAGGCGTAAACTTCCAATTAATGCTCATCCGCGGTGCTCTGTGATAAAACCGTAAGGACTTCTT
GGGACACCTAAATAAAATTTAAAAAATAAATATTTGTTTTATTTGTTTGTACATCATATTTGCTTAAAAGCCATAT
ATATGCTGCCATAGTGCATGTCAATAAATTTGTTAAATTTAGAAAGTAATTTAGTGTCTGTTACACCTGTATATATA
AGTCTCAGAAGTGAATGTTTCATAATACCCTTCAATATGCAAAATTTAAATGCAATAATTTCCGTTTTGGCATC
TAGAACTTAAAATTTGCTAAAACAATTTCTATTTTGTATGTAACAGCTTGGTTATAAACATAAACATCTGTTGTAA
TAATATTTAGAAATCTTTTTGATTAATTTATTTTATAAAAATAAATAAAATTTGAATCAACTGATCAAATTTGCAA
TACAAATTTGCAACAATATTTGATAATTACGCTTCAATAAATTTGACTGTAACATATGTGTGCTCAGTGGGCAACCTGTA
CGCCATTATAAAGATATAAAAACGGGGTTTTAATTAAGCACTGCAGGGTCAAACGATTTTACTTGATTCGAATTTTT
GTCCAAAATGAAATCAACGGTTATTTTGTTCATTGTTGCTTTGTGCATT
```

>oli (cds)

AGTAGTCTGAGGTAGCGAATGTTTCGAGTTAAGCAAAGCTCTGGGTATATATAGAGATTTTAAGGGGAATTGAAAAGTC
 CGACTTAGTAAAAATCCAAATTTCTATAGGTTCCAAGTTGACGAGACTCTACTGTATTGTATATGCTTGGGAAATTTT
 GTAATTTTTTTTAGATAGTTATTTTTAAAGTGATAGTTGTATATAAATGAGAAGTACAAGAACGGTGAGGTTTCCTAG
 TCCTCAGCCAAAAACACAGGTTAAAAGAGTACAAAATTTACTGCAAAAACCAAAGACTGGTTAGAAAACACTCTGGAGA
 AGATGTAGGAGAAGTAACTTTCTTCAAGGATTCAGGGCTTTTAAATTTTACATGGTCAATGGTCACGACCATGTTAT
 TGACAATCTTTACACGGTCACGGTCAATTATTGAGTACTAATTAATAAATTAACATGAGATTTAAACACCATGCTG
 CAAGTGACTGACACAACCTCTAGTAATAAAAAGATTTAATCGAGATTTAAAACGAGAGGAAAAATTAACCTCAGATTTAC
 CAATAATGATAATTTCAAAAAATGCACATCAAGTAGAATATATCTAATTAACCTTAAAAACAAGATTGTTTAAAAATGT
 TGTCAATGTCATAATATTTTCAGCATTTCAAAATGTTTGTACACCTGTGCATATAAGACTTAGTATAGTTTAAAGATTC
 CAAATTATAAAACTAAAATGTAAAAAGTATAACGTTTTTCTATGAATCAAAAATTTATAAAAAATAGTATTTATTTTGT
 TCTTAGCAAAATTTGTTTCGTACTTAAACGGAAACATCTGTTGTAAATATTTTTGGAAACGTTTTTGACAAAATTAGT
 GTTTATAAAATAAATTTAATTGAATCAACTGATCAAATTTGATAATTACGCTTCAATAAATTTGGATGAAACTATGTG
 TGTTTCAGTGGTCAACCTGTATGCCATTATGATGTTATAAAAACAGGGTTTTAATCAAGCACTAGAGGGTCAAATAAT
 TTTATTTGGTTTCAGATTTTTTACAAAATGAAATCCACATTTATTCTGTTTCATTGTTGCTATGTCATTGTTTGCAGC
GAAGGAATATTCTGGTAAAAACACTTTCTTTAGACTTTCTTTAACTTTCTTAAGACTTTCTTTTTTTAGACTTAA
AAAACATTTTTTAGTTAAACGATTATTTATTAGACATTTTGAAGTTATAGAAAAATTTAAATTTTTTA
TCATCAAAACAAAGATTGTAATGGCGGGTGTGTGTTAAGTTTGGGTTTATTGGAAAAATGCAAATCTTATGTA
AGGTTGGACTCTGTGGGCTTAGC

>vir (cds)

CGGTATACAGTCGGTTAATGGTTGGCAAGAAATGCCAACAGTTAGCCAACCTGTAGCCAATACTGGCCCGTCAGTTGG
 ACCAACTAAAGCGTGTTTACTGGGTAAACACTAAAAATGCGTTGCAAAGTTTATATGATAGATTATTTTTTATAT
 GATTTTGTATATTTTTAAATAATTACTTAAGTTATGTAAATTTTTGAATTTCTCGAAGTGCTACTTAGCTTTAAAAAA
 GGTTGCACAACAGTTAAAAAATATATTGCTCTTGAATTTTTATCTCGAAAGTTTATGTAAATAAGTGGACAGCTG
 ATTTTAAAAGTACTTTCAAGAGGCACCATTACTTTATTTTTATTTATTTATTTTTTTTGGCGACCGCTAGTTAGTAT
 TTATTTTTTGAATACGCAGATTTATCTTTTAAATTTAATGATCCCCACTCCAACAGAAGTATTACTAATTTAAACT
 CAAAAAATCCCTGAAAAAGCACTAAAAGCAGATAAATTTAAAAGCATTCTTGTATAAAGTCAAAAGTATACTTT
 AGAATTATGTTTTGATACAATTCCTAATCTTAGACTTTTTTTGTAATTTTTTACTAAACCTTATATTATCAAGAGCTT
 TTCCCTTTTTAAGAACAAAATGGTACTAAAAATGTGTTTAAAAGTTTAAATCATCTGTGTTGAATCATCTGGGTGTA
 TGTGCAAACGAATTTTATAGCAATATTTATTAATTAATCTGCAATATATTTATGTCAGTATGCACAGTGGGCTGTC
 TGCACGTTAAAAAATAATATAAAAAGCTGCCTCCCAATACACCAATCATGAAAGTTTACACTTGATTCGAAATTTT
 CAATCATGAAATCGATTTTCGTTTTGTTAGTTGTCGCTGTATGCGTTGTTTCTTTGGAAGCAAGTTGGTAAGCATT
 TTATTTATTTGTAACATTTTTTTGTTATAAAACCACATAAACCAGAGTTATGAAAGGTTTCCAGAAAAACAGTTCCC
 ATTAATAATATCGTCGGTTTTAAAAGTAAAACATAAGTATCAATTATGAAATTCGGAGAAAAACATGTTTTTACACGTAA
 TTAATTTTTGAAATGAAATTTGCTTTTGAATGATCAAGGCATCATAAGCACTTGCTAGTTTTTTCATATTTATGGAAT
 GTCAAAAAATAAATCACTTAGTGACGTTTAACTCAATTCTCATTAAAACCTGACTTAGTGACTTTTACCTCTAAC
 CAACCATATATACTCTAATTTAAAGTATTACACCTTTGATATTTTCTATATCTACGTAATGATTTCTGTGTTTTAGC
TTTTGTCGATCTAACAAATGATTGCAAAAATGGATGCTGTTTGAATTTCCGATTTG

9.4 *HyDkk1/2/4 A* - genomic sequence

>mag (**cds**)

TAATCCTCACACTGCTCGCATTGATCAATGATACCGCAAAATTTTGTGCAAGCTGATCTTCGCAATCCCTCAGCTCG
 ACTTACCCAATTGTTCTGGGGAAGTGGCATTAAAACATAACCGTGGCGTGTTC AATTATATCTGTTACAAAAATTTA
 ATACAGTTGCTACAAGACCCGCCATTAAGCCATTTCTCAACATAATGCAGCTTCTTGCCCGTGTA AAAATCTGGAAT
 GGTATGCAGGTCCCTTTCCCTTTCCCCTAAAAAACGGATGAGTGGAAACTCGCCTTAACACGTATTTTATGAGGTT
 TTAAAAATGCCTAGATTGTGACGAACACGTGTTTATATGGATTTAAATTCGAAAAGTAAAACTAGACGTGGTGGCTG
 GGTTAAGAAGTTACTGGTGCCTTGGAGAGATTATAATTATATAGTTGATAAACTTTTGTCACTCCGTACATATTTT
 GGAACGAGATTATATATTTGCATTACGTTATTATTACGAGAGATATATTATGTGTAAATTTGTCACCAAGGTTATAAA
 GGGCAGTAAAAATAACTTGACTATTTATTAAGAAAAATACGGGATAAAATTACATTTTTTTTATAAAACAATTTTTTC
 TATTA AAAATAATTTTTTTCATAAATTATATTCCAAAAGTAGCCATTTTAACGACGTTACGGAATCAGGGAATCAGTG
 ACAGGGGTGCTTATATTACAATTACAAAATTACTAATCCATAATTAACACAAGTCTAAATTTCAAAAAGAATTGCGA
 TAGTTACCAAGTATAATAATGGTTACCAATAGCAAGTCTTGAAAATTTAGA ACTGAAACCAATTTAATCTATTTT
 CAGTAAAATAAACAGATACCAAAAGCATTGCATCACAAACCAATGCCTCGATTGTGCAATTAATAAAAATCCAAAAA
 ACACCTGCATTTATTCATCTGGATAAAATACTCAAATACATCTTGCATCAACATTTGTTTATTGTTCTTCATTTAAAT
 ATTAGACATGCGCAATCAACACTTAAAAAGAAGTATAAAAAGTAGCTTTTATACAAACGTTCTTTACATACATCTTT
 TCTGATTTATCA**ATGATGAGATTTTAGCAGTCTTTTAGTTGTAGCTGCATTCGTTGCATTTAGTGAAGCCG**AGGT
 ATTTATAAACTTTTAAATAGGATATAAAAATATCTAGATAACTAAATCAATTTATAGACAGTTATACATCAATAAGTA
 TTTTTGACGTATACAAATATGACGCATATATACTTAATACAACAATTGTTATTATCTATTTTTTTTATA**GTCTTG**
CAAAAAGGATGCAGACTGCAAAAATGGTTGTTGTTGTAATTTCTTACTTACTAAGCAATGTAATAGCTATGTTAAAG
AAGGTGCACTCTGCGGTTTTCGTGTAAGTTTTTTAAAAATTTAATAATTTTTGTTCTTTTACTTGTTCCTTCAATTT
 AATTCCTAAAAATCCTTAAGTTTTTTCAGTTCTTTAAAAAATTTTTTTTTTTCATTTATTAATAAACTTAATTTATTAAC
 AATAGAAGACGCATAAAAAATATTCTAAAAAGTAAAATGAAAATTTCTTTCTTTTATTATAATGGAAGAAAATGTTTT
 TCCAATTACAACCTTAAATATTTTTGTGTTTTAAATATTTTATCTATTAATTTTTTTTTTATTCAAGTAGGTTTCTCCTT
 TTCTATTTTTTAATTTTTTATGTTTAAGTTAGAAACAGCAGTTAATAAGTTTTTCTAATATTTATTTCTAATATTTAA
 TTAAATCTAATAATAATTTTTCTAATATTAATAGGTTATTCTCATATTTCTATATGAATTTTTAGGAGCTTTAGAAA
 CTAAAAGTTCTAAATTA AAAATAAAATTTTCATTTTCGTTAAACAGCTTCAATTGGTTTCTTAAGTAGAAAAAAAAAAAA
 AAAA ACTCCTTATACCTCTATAAACTATTGTAAATATTTATACCTCTATAAACTATTGTAAATATAGTAAATATTT
 TAACAAGTATTA AAAAGAACATGTTATTTGCTTTTATTATTATTATATATACTTTTTA**GGACAAATTTGCATGC**
GGTTGTGAACCAGGACTCGAATGCGTAAAAGTACGAGGAACTTTAACTGGGATGGTTAGAAGATGCGTTGACAAATC
AGGAAGTGGATCTTTGTATTAAATTAATACAGAAAAAAAAAAGATAAATTTAAAAAAGAAACAAATATATAATTCTGA
 GTTAAATTATTA AAAAGTTTACATATTTTTCGTTTCAAACCCCTCAAGTTAACGCTCTGCACGCAGCGTATACTATAA
 AATCGCCTAAGTTCGGTACTTTGAA CATTCAATAAAAACAGGCCAAAAATAAACGGTTTTTTCAGTTTTTTTTCCCTA
 GTAATACAGGCGTTTATCTGACAGTTCCTTATATATTCCATTATATCGTTAATTTATATGTAAAGATAAGACTTA
 AAAATTTCTTATCTTAACAATGTAAATTAATAAAAATAAAATTTATTTGAAATGAAACCAGGTAGTTTGACAGA ACTT

10 Acknowledgements

Herrn Prof. Dr. Dr. h.c. Thomas C.G. Bosch danke ich herzlich für die Bereitstellung des Themas, für die motivierende Betreuung und die Unterstützung der Feldforschung.

Mein besonderer Dank gilt auch Frau Dr. Friederike Anton-Erxleben für großartige Unterstützung bei allen „bildgebenden“ Verfahren sowie anregende Diskussionen. Frau Antje Thomas sei gedankt für die Hilfe in histologischen Belangen, Herrn Jörg Wittlieb für die Mühen bei der Transfektion von *Hydra*.

Weiterhin danke ich der gesamten Arbeitsgruppe für ein produktives Klima und jedes gute Gespräch.

Ich danke den Menschen, die in anstrengenden Zeiten da waren, besonders meiner Familie für den steten Rückhalt.

11 Erklärung

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

Kiel, den 12.12.2007
