

**MOLECULAR ANALYSIS OF THE  
INNATE IMMUNE RESPONSE  
IN *HYDRA***

**Dissertation**

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vorgelegt von  
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“Toward understanding the evolution of disease-associated genes: NOD-like receptors in *Hydra*”

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## ABBREVIATIONS

A	adenine or Ampère
aa	amino acids
Amp	ampicillin
AMP	antimicrobial peptide
AP	alkaline phosphatase
<i>avr</i>	avirulence
b	bases
B	basic region leucine zipper
BCIP	5-bromo-4-chlor-3-indolylphosphate
bHLH-Zip	basic helix-loop-helix leucine zipper
BIR	baculovirus inhibitor of apoptosis protein repeat
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumine
C	cytosine
°C	degree Celsius
CARD	caspase recruitment domain
cDNA	complementary DNA
CH	CHORD
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate
CLR	C-type lectin receptors
cm	centimetre
cm <sup>2</sup>	square centimetre
CMV	<i>Cytomegalovirus</i>
Da	Dalton
DABCO	1,4-diazabicyclo[2.2.2]octane
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DD	DEATH domain
ddNTP	dideoxyribonucleotide triphosphate
DED	DEATH effector domain
DEPC	diethylpyrocarbonate
DFD	DEATH fold domain
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	desoxyribonucleotide triphosphate
dsRNA	double-stranded RNA
DSS	dextran sodium sulphate
DTT	dithiothreitol
ECCO	European Cancer Organisation
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
F	Farad or forward
FISH	fluorescence <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
FKBP	FK506 binding protein

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g	gravity or gram
G	guanine
GFP	green fluorescent protein
h	hours
HA	hemagglutinin
HCl	hydrochloric acid
HMMER	hidden Markov model software package
HR	hypersensitive response
HyNLR	<i>Hydra</i> NOD-like receptor
i.e.	this is
ICE	interleukin 1 converting enzyme
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMD	immune deficiency
<i>in situ</i>	natural location
<i>in vitro</i>	within the glass
<i>in vivo</i>	in the living organism
IP	immunoprecipitation
IPT	Ig like, plexins, transcription factors
J	Joule
kb	kilo base pairs
kDa	kilo Dalton
kV	kilo Volt
LPS	lipopolysaccharide
LRR	leucine-rich repeat
m	metre or milli
$\mu$	micro
$\mu$ F	microfarad
$\mu$ g	microgram
$\mu$ l	microlitre
$\mu$ M	micromolar
M	molar
mA	milliampère
MAB	maleic acid buffer
MAB-B	maleic acid buffer with BSA
MALT	mucosa-associated lymphoid tissue
MAMP	microbe-associated molecular pattern
MAP	mitogen-activated protein
MIC	minimum inhibitory concentration
min	minutes
ml	millilitre
MHC	major histocompatibility complex
mm	millimetre
mol	Mol
mRNA	messenger RNA
NACHT	domain present in NAIP, CIITA, HET-E and TEP1
NaOH	sodium hydroxide
NB-ARC	nucleotide binding adaptor shared by APAF1, <i>R</i> genes and CED-4
NBD	nucleotide-binding domain
NBT	nitroblue tetrasolium

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NCBI	National Center for Biotechnology Information
ng	nanogram
NLR	NOD-like receptor
nm	nanometre
NMR	nuclear magnetic resonance
NO	nitric oxide
NOD	nucleotide binding and oligomerization domain
$\Omega$	Ohm
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBT	PBS containing Tween20
PCR	polymerase chain reaction
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time PCR
R	reverse or resistance
RACE	rapid amplification of cDNA ends
RFLP	restriction fragment length polymorphism
RHD	Rel homology domain
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	double-stranded RNA-mediated interference
RNase	ribonuclease
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature or reverse transcription
RT-PCR	reverse transcription PCR
S <sub>TKc</sub>	serine/threonine protein kinase
SDS	sodium dodecyl sulphate
sec	seconds
S-L	SGT1-like domain
SL	spliced leader
SMART	Simple Modular Architecture Research Tool
SNP	single nucleotide polymorphism
SPR	surface plasmon resonance
ss	single-stranded
T cell	thymus cells
T	Thymine
TAE	TRIS-acetat-EDTA-buffer
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	TRIS-borate-EDTA-buffer
TE	TRIS/HCl-EDTA-buffer
TEMED	N,N,N',N'-Tetraethyldiamine
TES	N-(TRIS(hydroxymethyl)methyl)-2-aminoethanesulfonic acid
TIR	Toll/ interleukin 1 receptor homology
TLR	Toll-like receptor
T <sub>m</sub>	melting temperature
TPR	tetratricopeptide repeat

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TRIS	<i>tris</i> (hydroxymethyl)aminomethane
tRNA	transfer RNA
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
U	units or uracil
UTP	uridinetriphosphat
UTR	untranslated region
UV	ultraviolet
V	Volt
v/v	volume per volume
var	variant
VLR	variable lymphocyte receptor
w/v	weight per volume
WB	Western blot
WGS	whole genome shotgun

# 1 INTRODUCTION

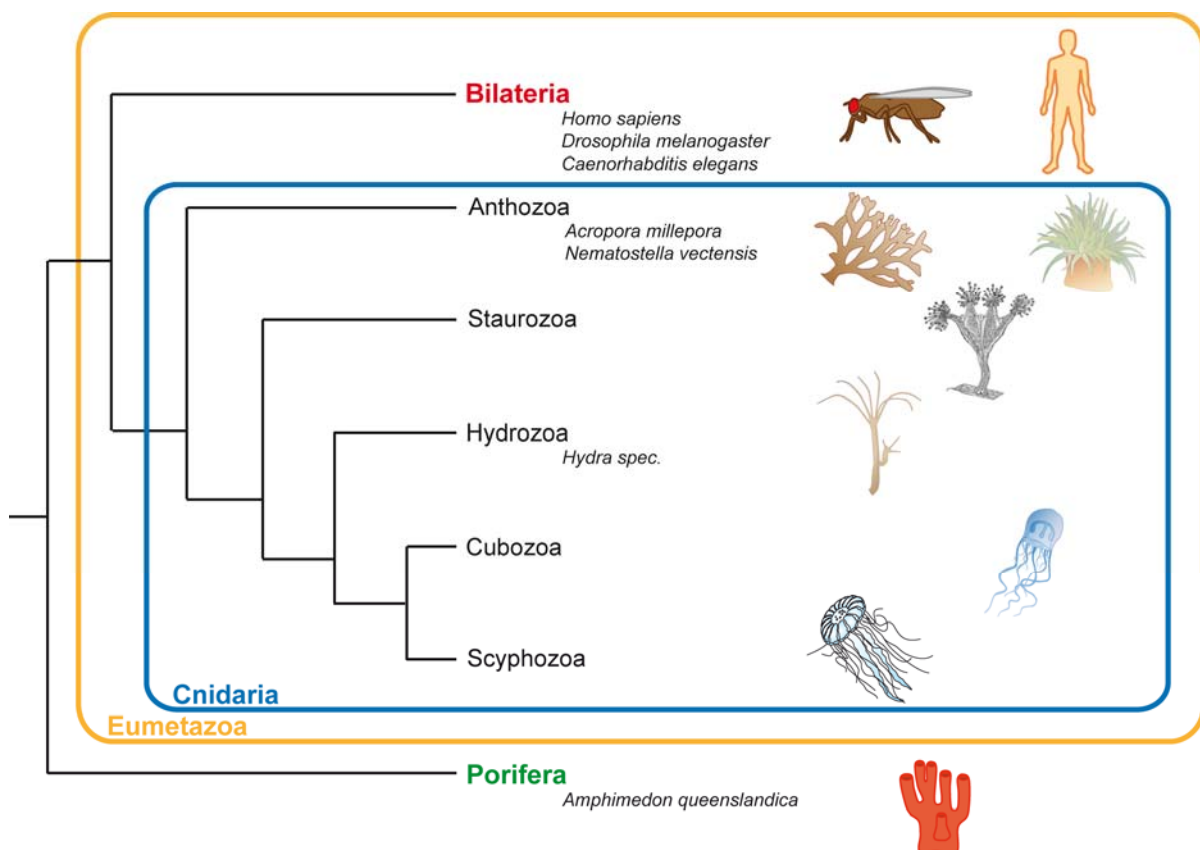
## 1.1 The freshwater polyp *Hydra*

In Greek mythology Hydra was described as a nine-headed monster with eight mortal heads and one immortal. When Heracles cut away one head, two heads were regenerated.

The freshwater polyp of the genus *Hydra* was first described by van Leeuwenhoek in 1702 (van Leeuwenhoek, 1702), but shares no similarities with this mystical beast except for its high regeneration capacity (Bosch, 2007).

The sessile *Hydra* polyps vary from 0.5 to 3cm in size and are found ubiquitarily in freshwater ponds and lakes, where they feed on insect larvae and small crustaceans, such as *Daphnia*.

Representatives of the genus *Hydra* belong to the phylum of Cnidaria which is proposed to be a sister group of the bilateria (Collins, 2002; Martindale *et al.*, 2002; Philippe *et al.*, 2005; Putnam *et al.*, 2007) (see Figure 1.1).



**Figure 1.1: A schematic phylogenetic tree of the animal kingdom.**

The Porifera are a sister group to all Eumetazoans. The Cnidaria represent a sister group to all Bilateria. Within the Cnidaria, five classes are defined: the basal class of Anthozoa; the class of Staurozoa; the Cubozoa; the Scyphozoa; and the Hydrozoa. Included are some representatives of the various taxa. (Schematic drawing of staurozoan representative taken from Collins *et al.*, 2006).

In contrast to Porifera, Cnidaria, as one of the most basal representatives of eumetazoans, possess true tissues connected by tight junctions, sensory, nerve and muscle cells, a gastric cavity and a blastoporus (Nielsen *et al.*, 1996). In contrast to the triploblastic bilateria that are composed of endodermal, ectodermal and mesodermal germ layers the diploblastic Cnidaria lack the mesoderm resulting in one oral-aboral axis and a radial symmetrical body.

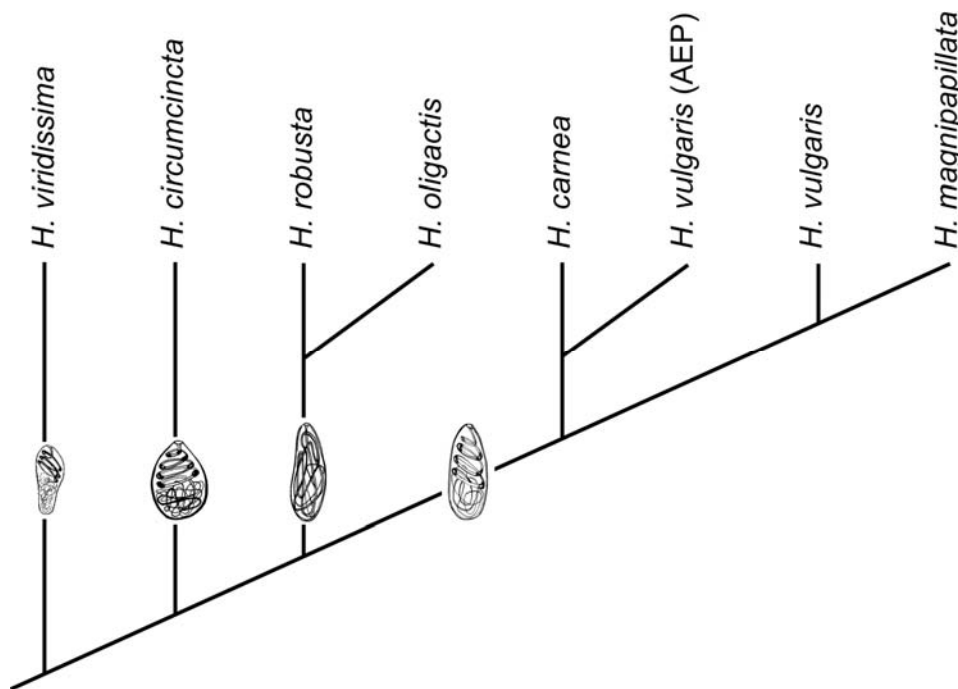
Within the Cnidaria, two alternating forms of differentiation can occur; the polyp being attached to the substratum and the free-floating medusa.

All Cnidaria are characterized by their phylotypic feature; the presence of the cnidocyte (nematocyte) for capturing prey (Tardent, 1995).

The Cnidaria are subdivided into five classes: the most basal class of Anthozoa, including the corals and the sea anemones, followed by the classes of Staurozoa, Scyphozoa, Cubozoa and Hydrozoa, to which *Hydra spec.* belongs (Collins *et al.*, 2006).

Because of the complexity of their cnidocytes, their nervous system, their developmental lifecycles, and their ectodermal gonads the Hydrozoans are referred to as the most derived but secondary reduced class (Collins *et al.*, 2006; Steele, 2002).

Using two nuclear and two mitochondrial marker genes as well as morphological criteria, e.g. the appearance of different holotrichous isorhizas, a special type of cnidocyte, the phylogeny of several *Hydra* species commonly used as model organisms was determined by Hemmrich and colleagues. (Hemmrich *et al.*, 2007a) (see Figure 1.2).



**Figure 1.2: Phylogenetic relations within the genus *Hydra*.**

The tree includes molecular and morphological data with a schematic drawing of holotrichous isorhizas of the different groups. (Taken from Hemmrich *et al.*, 2007).

The species *Hydra viridissima* forms a symbiotic association with unicellular algae of the genus *Chlorella spec.* (Habetha *et al.*, 2003; Oschman, 1967) and is located at the base of this phylogenetic tree followed by *Hydra circumcincta*, the stalk-possessing group of *Hydra robusta* and *Hydra oligactis*, the group consisting of *Hydra carnea* and the laboratory strain of *Hydra vulgaris* (AEP) (Martin *et al.*, 1997) and the most derived group of *Hydra vulgaris* and *Hydra magnipapillata*.

Because of the already mentioned basal placement in the animal tree of life, its simple body structure, its high regeneration capacity, the easy cultivation and the short reproduction times leading to clonal cultures, *Hydra* has been a classical study object for a variety of biological questions, like axial patterning (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1974; Meinhardt and Gierer, 2000), stem cell behaviour (Bosch *et al.*, 2010; Bosch and David, 1987) and regeneration (Bosch, 2007).

The stable transfection of the strain *Hydra vulgaris* (AEP) that frequently undergoes sexual reproduction is now possible *via* microinjection into the developing embryo (Wittlieb *et al.*, 2006). This provides new possibilities for the functional characterization of ancient defensome genes.

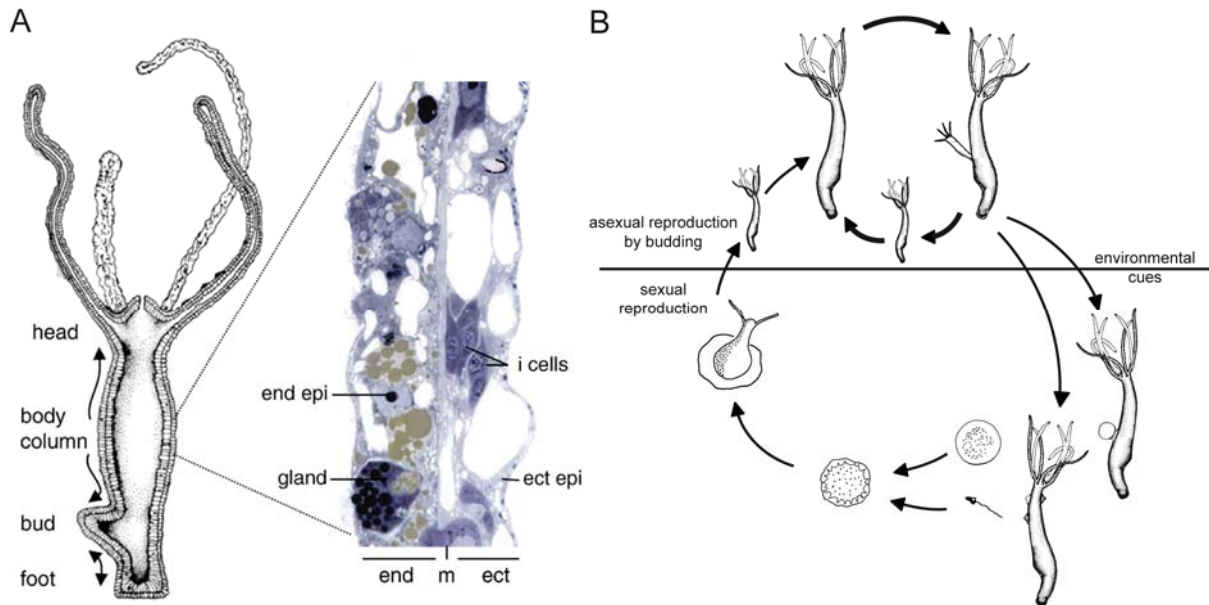
In 2010 the genome of *Hydra magnipapillata* was published (Chapman *et al.*, 2010) and as well as an EST-library of *Hydra magnipapillata* is available, 454 second generation technology was used to generate transcriptome datasets of *Hydra oligactis*, *Hydra viridis* and *Hydra vulgaris* (AEP) (AG Bosch, unpublished data). These datasets provide new important tools for comparative research on *Hydra*. Not only *Hydra* was a subject for extensive genome and transcriptome sequencing, EST databases that are available for many additional cnidarian species and the sequencing of the genome of the anthozoan *Nematostella vectensis* provide new possibilities for comparative research on these ancestral animals (Putnam *et al.*, 2007).

### 1.1.1 *Hydra's* morphology

*Hydra's* body plan is simple and can be subdivided into three parts along the oral-aboral axis: (i) the head region made up of four to twelve tentacles used to capture prey surrounding the hypostome with the mouth; (ii) the tube-like body column on which gonads and buds are formed; and (iii) the foot region that constitutes a basal disc for the attachment to the substrate, depending on the species that may or may not possess a stalk (see Figure 1.3).

*Hydra's* actinomorph body is made up of two monolayered epithelia; the ectoderm at the outside that secretes a glycocalyx as an outer surface and the endoderm that surrounds the gastric cavity. The glycocalyx is supposed to contribute to the protection of the animal against its environment as a physico-chemical barrier (R. Augustin, pers. communication).





**Figure 1.3: Morphology and life cycle of *Hydra*.**

(A) Schematic drawing of *Hydra* including a detailed view on a histological longitudinal section. Abbreviations: end epi (endodermal epithelial cell); gland (gland cell); i cell (interstitial cell); ect epi (ectodermal epithelial cell); m (mesogloea); ect (ectoderm); end (endoderm). (Taken from Bosch, 2007). (B) Life cycle of *Hydra*. The freshwater polyp can either reproduce asexually by budding or sexually by forming gametes. Budding is the prevalent form. (Modified from Bosch, 2009b).

Within the gastric cavity the extracellular digestion of food is performed *via* peristalsis. In addition to prey, potential pathogenic microorganisms can be ingested as well. Both epithelial layers secrete the mesogloea, a thin extracellular matrix separating the two tissue layers and providing stability and elasticity to the polyp. *Hydra* possesses three independent stem cell lines that contribute to these tissues: the ectodermal epithelial cells, the endodermal epithelial cells and the interstitial cells (Bosch, 2009b; Bosch *et al.*, 2010).

The body shape is predetermined by the epithelio-muscle cells of both tissue layers. The ectodermal epithelial cells of the basal disc can secrete mucus for the attachment to the substrate. The endodermal epithelial cells are ciliated and phagocytise the pre-digested food particles and distribute the nutrients to the entire animal. The interstitial stem cell lineage gives rise to several derivatives: (i) the nerve cells forming a diffuse net within the entire body (Bode *et al.*, 1973); (ii) the endodermally located gland cells secreting mucus and digestive enzymes to the gastric cavity; (iii) the different types of nematocytes that are formed by proliferating and differentiating nests of nematoblasts and are taken up in the tentacles by a specially differentiated type of ectodermal epithelial cell, the battery cell, harbouring 8 to 24 cnidocytes (Campbell, 1987; Slautterback, 1967; Wood and Novak, 1982); and (iv) the ectodermally located germ cells.

Asexual budding is the primary mode of reproduction in *Hydra*. The *Hydra* tissue is subjected to a continuous displacement of cells from the centre of the body to the tentacles and the foot

(Campbell, 1967). The amount of new cells is tightly linked to the feeding conditions (Bosch and David, 1984). The polyp's cells are permanently in a steady state equilibrium and the excess of tissue is displaced into the bud that is formed in the lower part of the body column. Under optimal feeding conditions the polyps can form one bud per three days.

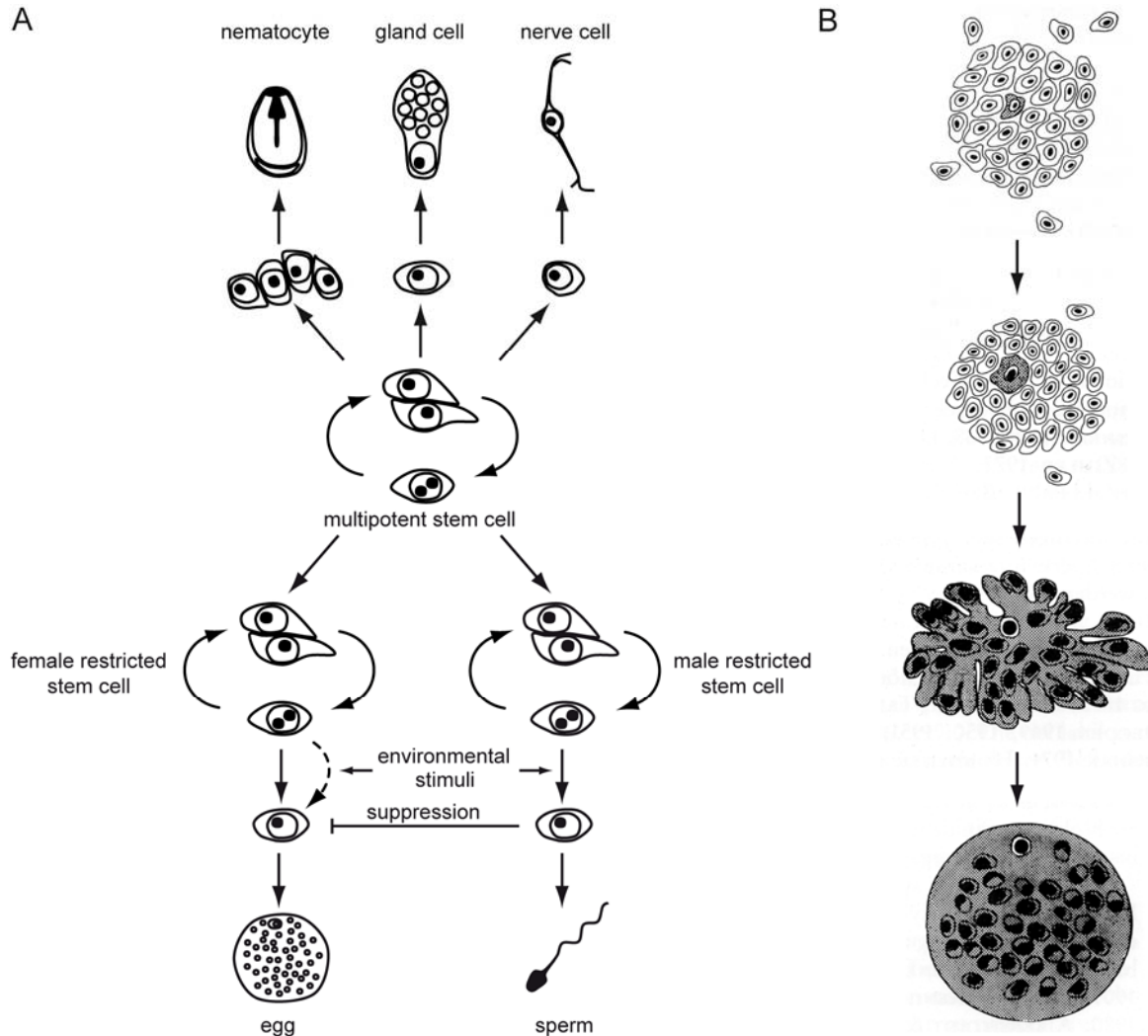
As well as by budding *Hydra* can also reproduce sexually through the formation of ectodermally-located testis and oocytes (Bosch and David, 1987; Littlefield, 1985; Littlefield, 1991). Different species can be hermaphroditic or dioecious (Hemrich *et al.*, 2007a). The formation of gametes can be induced by environmental changes, e.g. the reduction of the temperature in *Hydra oligactis* (Littlefield, 1991) or withholding of food in *Hydra vulgaris* (AEP) (Bosch and David, 1987; Sugiyama and Fujisawa, 1977).

In *Hydra* there is experimental evidence that no separated germ line is present (Bosch and David, 1987). The multipotent interstitial stem cells that give rise to the somatic derivatives also give rise to a germline restricted population of stem cells (Bosch and David, 1987; Littlefield, 1985; Littlefield, 1991). This population is also maintained in sexually undifferentiated polyps (Holstein and David, 1990) (see Figure 1.4).

Spermatogenesis starts with a local proliferation of the interstitial cells at the area of the future testis leading to a swelling of the ectodermal tissue and a subsequent lifting of the covering epithelial cells that will form the outer wall of the testis (Tardent, 1974). The clusters of accumulated interstitial cells synchronously undergo meiotic divisions. The subsequent differentiation processes result in cell layers of differentiation stages within the intercellular space between the mesogloea and the epithelial cells (Munck and David, 1985).

Different *Hydra* species can form one or several oocytes simultaneously. During oogenesis, the egg-restricted interstitial cells aggregate and proliferate beneath the ectodermal epithelial cells giving rise to a cluster of several thousand cells (Honegger *et al.*, 1989). Transplantation experiments have shown that many of these cells have the potency to differentiate into the oocyte (Miller *et al.*, 2000), but only one centrally located cell is selected by an unknown mechanism to become the oocyte. After the determination of the oocyte the surrounding interstitial cells differentiate into nurse cells. These nurse cells accumulate nutrients, like lipids and glycogen, and undergo an apoptotic program that is arrested during embryogenesis, characterized by condensed chromatin and DNA fragmentation into large pieces of 8-15 kb in length, completed after hatching of the offspring (Honegger *et al.*, 1989; Technau *et al.*, 2003). This apoptotic program starts during phagocytosis of the nurse cells by the developing oocyte that dramatically increases in size resulting in a large amoeboid symplastic egg. The nurse cells are incorporated into the cytoplasm and persist in the developing embryo up to several months until the new polyp hatches (Technau *et al.*, 2003). Although the nurse cells undergo an apoptotic program, they are still transcriptional active (Fröblius *et al.*, 2003). The

mature egg breaks through the covering ectodermal epithelial cells and forms a sphere at the outer surface of the mother polyp where it is fertilized and embryogenesis is committed. The embryo develops within the egg without any larval state.



**Figure 1.4: The interstitial cell lineage of *Hydra*.**

(A) Model of the multipotent interstitial stem cell line; the stem cells give rise to nematocytes, gland cells, nerve cells and germline restricted unipotent stem cells. Environmental stimuli can induce gamete formation. Male differentiation can suppress female differentiation. (Modified after Fraune, 2008; Littlefield, 1991) (B) Oogenesis in *Hydra* starting with the accumulation of germline restricted female interstitial cells and the determination of the future oocyte. The oocyte (grey) grows and phagocytoses the surrounding cells that differentiate into nurse cells. (Modified from Holstein and Emschermann, 1995).

As a limnic organism *Hydra* is in permanent contact with its surrounding potential pathogenic microorganisms like protists, fungi, oomycetes, bacteria and viruses. To inhibit an overgrowth with these organisms *Hydra* must have acquired an effective warfare system for its defence.

## 1.2 An introduction into immunity

For each living organism it is of tremendous importance to distinguish self from nonself and to recognize pathogens or degenerated cells to initiate various defence strategies for the protection of its survival and reproduction (Khalturin and Bosch, 2007; Rosenstiel *et al.*, 2009).

In *Homo sapiens* and other jawed vertebrates the adaptive and the innate arm of the immune system can be distinguished (Khalturin and Bosch, 2007; Rosenstiel *et al.*, 2007). The defence mechanisms of the adaptive immune response are relatively slow, but highly specific and mediate an immune memory that protects the organism against attacks of the same pathogen in the future (Cooper and Alder, 2006). In contrast to that, the innate immune response is activated fast and therefore the first line of defence confronting the pathogen. The repertoire of proteins contributing to innate immunity is relatively limited but directed against a broad spectre of pathogens (Beutler, 2004). The innate immune response does not lead to an immune memory and relies on germline-encoded receptors (Takeuchi and Akira, 2010). Many components of the innate immune defence are conserved among all eukaryotes (Hemmrich *et al.*, 2007b; Hibino *et al.*, 2006; Rosenstiel *et al.*, 2009).

The first line of defence is represented by the epithelia that separate an organism from its environment (Schreiber *et al.*, 2005). The epithelial cells are tightly interconnected by cellular junctions and act as a physical barrier. In addition, migrating phagocytes take up invading pathogens and infected cells. Both cells use receptors that can recognize components that are conserved in many potentially pathogenic but also commensal microbes and are therefore called “microbe associated molecular patterns” or MAMPs (Takeuchi and Akira, 2010). These include for example lipopolysaccharide (LPS), flagellin, CpG-DNA or bacterial cell wall components like muramyl dipeptide (MDP). Furthermore, these “pattern recognition receptors” (PRRs) are also capable of recognizing endogenous molecules released from damaged cells termed damage associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). The PRRs mediate immune responses through signal transduction cascades leading to the induction of defence mechanisms such as the onset of inflammation to recruit further immune cells through the production and release of proinflammatory cytokines, type I interferons (IFNs) and chemokines or the production and secretion of effector proteins with an inhibitory or killing activity against microbes, the antimicrobial peptides (AMPs) (Takeuchi and Akira, 2010).

A persistent inflammatory response may also be the cause for tumour formation and the development of cancer as a last consequence *via* an accumulation of gain-of-function and loss-of-function mutations in proto oncogenes like *Kirsten rat sarcoma viral oncogene*

*homolog* (*KRAS*) or *myelocytomatosis viral oncogene homolog* (*MYC*) or in tumour suppressor genes like *adenomatous polyposis coli* (*APC*) or *tumor protein p53* (*TP53*) through the production of radical oxygen species (ROS) as a result of inflammation (Grivennikov *et al.*, 2010).

In addition to the cellular mediated innate immune reactions a humoral innate immunity is represented by the plasma proteins of the complement system (Beutler, 2004).

### 1.2.1 The innate pattern recognition receptors

In mammals four families of PRRs have been identified that can be divided into two groups: the (i) transmembrane proteins of the C-type lectin receptors (CLRs) and the Toll-like receptors (TLRs); the (ii) intracellular located RIG-I-like receptors (RLRs) and the NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

The C-type lectin receptors are characterized by the presence of a carbohydrate-binding domain. With the help of that domain they recognize carbohydrates on microorganisms, such as viruses, bacteria and fungi, and stimulate the production of proinflammatory cytokines or inhibit TLR-mediated immune complexes (Geijtenbeek and Gringhuis, 2009).

The Retinoic acid-inducible gene (RIG)-I-like receptors are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain. Activation by e.g. RNA viruses leads to onset of the expression of type I IFN genes and to activation of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- $\kappa$ B) via TRADD (Tumor necrosis factor receptor type 1-associated via DEATH domain), FADD (Fas-associated via DEATH domain) and caspase 8 or 10 (Kawai and Akira, 2006; Takeuchi and Akira, 2009).

The well-characterized Toll-like receptors sense invading pathogens outside of the cell or in intracellular endosomes and lysosomes (Akira *et al.*, 2006). They are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane domain followed by a cytoplasmic Toll/interleukin 1 receptor (IL1R) homology (TIR) domain. Ten TLRs have been identified in humans; each TLR can recognize particular MAMPs or DAMPs leading to transcriptional upregulation of distinct genes depending on the TLRs and cell types involved. Depending on the usage of distinct adaptor molecules TLR signalling can be divided into two pathways; the MyD88-dependent and the TRIF-dependent signalling pathway (Takeuchi and Akira, 2010).

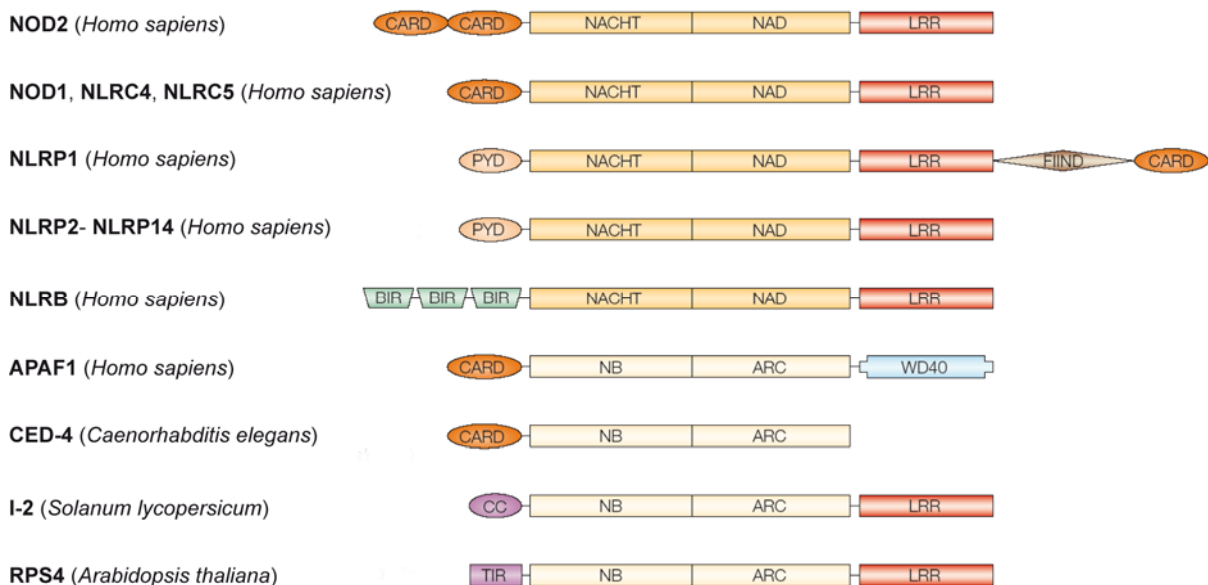
MyD88 (myeloid differentiation primary response gene 88) is composed of a DEATH domain (DD) and a TIR domain and interacts with the DD of the serine/threonine kinase interleukin-1 receptor-associated kinase (IRAK) 4 subsequently leading to the activation of mitogen-

activated protein (MAP) kinase signalling or the activation of NF- $\kappa$ B *via* TAK1, the IKK complex and I $\kappa$ B inducing the expression of cytokine genes and AMPs (Froy, 2005).

TLR3 and TLR4 can use the adaptor protein TRIF (TIR domain-containing adaptor inducing IFN- $\beta$ ) to activate NF- $\kappa$ B and interferon regulatory factor (IRF) controlling the expression of proinflammatory cytokines and type-I IFN genes (Takeuchi and Akira, 2010). *Via* the cooperative binding of RIPK (receptor interacting serine-threonine protein kinase) 1 (Hsu *et al.*, 1996; Stanger *et al.*, 1995) and FADD (Chinnaiyan *et al.*, 1995) and the activation of caspase 8, TRIF can induce apoptosis as well. In addition to the initiation of apoptosis caspase 8 can also contribute to the TLR-induced NF- $\kappa$ B activation (Maelfait and Beyaert, 2008).

### 1.2.2 The NOD-like receptors and their signal transduction cascades

The family of NLRs comprises cytoplasmic PRRs (Rosenstiel *et al.*, 2008). NLRs belong to the family of signal transduction ATPases with numerous domains (STAND) within the group of P-loop NTPases (Leipe *et al.*, 2004). Two subgroups of NBD domain containing proteins are recognized; (i) the AP (apoptotic)-ATPases with e.g. APAF1 (apoptotic protease activating factor-1) (Zou *et al.*, 1997), which harbour the NB-ARC domain (van der Biezen and Jones, 1998) and (ii) the NACHT-NTPases that are mostly represented by the *bona fide* NLRs, which contain a NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) class II transactivator (CIITA), HET-E and telomerase associated protein 1 (TEP1)) domain (see Figure 1.5).



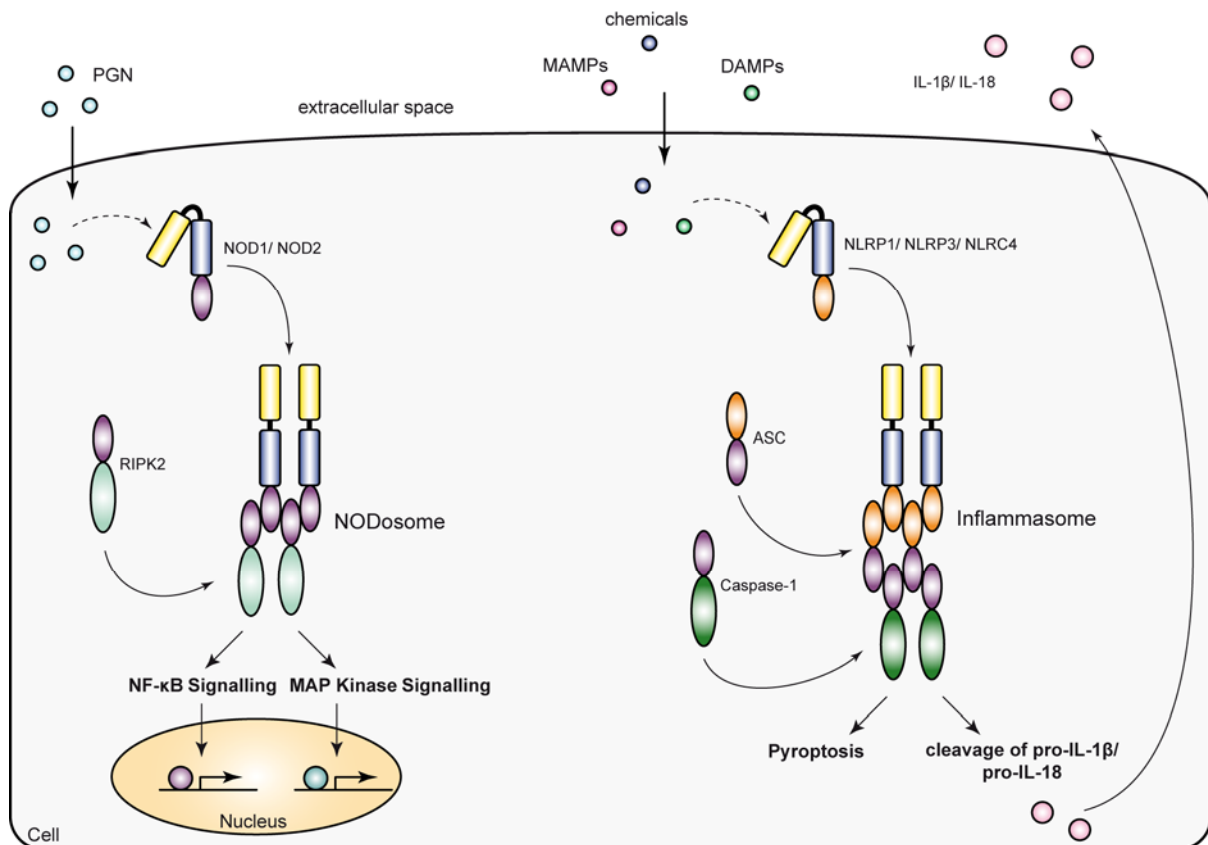
**Figure 1.5: The structure of a variety of NBD domain containing proteins.**

All NLRs have a tripartite domain architecture comprised of an N-terminal effector domain, a central NACHT domain and a C-terminal series of LRRs. They share structural similarities with the APAF1 homologues and plant R proteins. (Modified from Schreiber *et al.*, 2005).

NLRs are a complex family of tripartite receptors that have in common the eponymous central nucleotide-binding domain and oligomerization domain (NOD), synonymously called NACHT or NBD, and C-terminal LRRs (Schreiber *et al.*, 2005). The mammalian NLRs are subdivided into five subfamilies based on the type of their N-terminal effector domain (Chen *et al.*, 2009): NLRA and NLRB each with one human member, the first has a CARD domain with an acidic domain and the latter has three BIR (baculovirus inhibitor of apoptosis protein repeat) domains. The third group of NLRC contains five human members, each of them contains N-terminal CARD domains. To this group belong also the best characterized family members NOD1 (Bertin *et al.*, 1999; Inohara *et al.*, 1999) and NOD2 (Ogura *et al.*, 2001b). The 14 members of the fourth and largest group are called NLRPs. All have in common an N-terminal Pyrin domain. The well characterized NLRP1 and NLRP3 belong to this family as well. The fifth group called NLRX contains one member with a not yet characterized N-terminal part.

For some NLRs the minimal elicitors are known. NOD1 and NOD2 recognize peptidoglycan moieties secreted by bacteria (Chamaillard *et al.*, 2003; Inohara *et al.*, 2003). NLRP3 can recognize a broad spectrum of ligands including MAMPs, like LPS or MDP, but also chemicals, like silica or asbestos, and endogenous DAMPs, like uric acid crystals, suggesting that the agonists might not bind directly to the receptor, but need a co-receptor for recognition (Chen *et al.*, 2009).

Upon recognition of the elicitor by the LRRs, these proteins form large complexes by a conformational change of the LRRs that usually block the oligomerization of the receptors by masking the NACHT domain (Schreiber *et al.*, 2005). The proteins self-oligomerize by the interactions of their NACHT domains (Inohara *et al.*, 2000; Schroder and Tschopp, 2010). This brings the effector domains of these proteins in close proximity to each other, activating the protein complex to recruit downstream proteins *via* homophilic interactions of the effector domains, a mechanism that is termed “induced proximity” (Inohara *et al.*, 2000). According to the identity of the activated NLR different complexes are formed leading to the activation of different signalling cascades. NOD1 and NOD2 form the NODosome with the CARD-containing receptor-interacting serine-threonine kinase 2 (RIPK2) (Inohara *et al.*, 2000; McCarthy *et al.*, 1998; Ogura *et al.*, 2001b). Similar to TLR signalling NF- $\kappa$ B is activated leading to the transcription of inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)- $\alpha$ , IL6 and IL8 and antimicrobial peptides (Chen *et al.*, 2009; Voss *et al.*, 2006). Furthermore, the NODosome can also activate MAP kinase signalling including the p38, extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways (Chen *et al.*, 2009) (see Figure 1.6, left panel).



**Figure 1.6: The signal transduction cascades performed by human NLRs.**

NOD1 or NOD2 sense peptidoglycan (PNG) subunits. Upon activation the proteins form a complex *via* homotypic binding of CARD domains with RIPK2 leading to the activation of NF- $\kappa$ B or MAP kinase signalling. NLRP1, NLRP3 and NLRC4 can sense a variety of agonists (DAMPs, MAMPs and chemicals) leading to the formation of the inflammasome with ASC and caspase 1. The activation of caspase 1 can lead to pyroptosis and the cleavage of proinflammatory cytokines. Colour code of protein domains: yellow (LRR), dark blue (NACHT), violet (CARD), orange (Pyrin), green (caspase), turquoise (serine/threonine kinase).

Upon activation NLRP1, NLRP3 and NLRC4 form an oligomeric complex termed the inflammasome (Chen *et al.*, 2009) (Figure 1.6, right panel). The Pyrin domains interact with the Pyrin domain of the adaptor protein ASC (adaptor protein apoptosis speck protein with caspase recruitment) (Srinivasula *et al.*, 2002) enabling the recruitment of procaspase 1, synonymously called ICE (IL1 converting enzyme). The homotypic interaction between the CARD domains of ASC and procaspase 1 leads to the autoactivation of caspase 1 that subsequently cleaves pro-IL1 $\beta$  and pro-IL18 resulting in their release (Martinon *et al.*, 2002; Schroder and Tschopp, 2010).

The structure of the inflammasome shares similarity with the apoptosome formed by APAF1 upon cytochrome *c* release by damaged mitochondria leading to the recruitment of procaspase 9 *via* a CARD-CARD interaction resulting in induction of apoptosis (Zou *et al.*, 1997; Zou *et al.*, 1999). Indeed, activation of the inflammasome can also lead to a newly described form of programmed cell death: the pyroptosis, which is defined by the rapid



formation of plasma membrane pores, cellular lysis and the release of IL1 $\beta$  and IL18 (Bortoluci and Medzhitov, 2010).

Not only in NLR signalling pathways, but also in the other PRR signalling pathways described above, the homotypic interactions of effector domains made up of the DEATH fold family, like the CARD, DEATH, DED (DEATH effector domain) and Pyrin domains, play key roles in the transduction of the signal. Although they share few sequence similarities, they all have in common the six antiparallel  $\alpha$ -helical bundle structure (Liang and Fesik, 1997; Liu *et al.*, 2003). These domains have been identified in a variety of proteins involved in apoptosis and immunity (Aravind *et al.*, 1999; Chinnaiyan *et al.*, 1995; Medema *et al.*, 1997; Schreiber *et al.*, 2005; Zou *et al.*, 1997).

Dysregulated PRR pathways have been implicated in a variety of severe autoinflammatory diseases (Chen *et al.*, 2009; Rosenstiel *et al.*, 2007; Schreiber *et al.*, 2005). For example, *NOD2* was the first susceptibility gene described for Crohn's disease, a chronic inflammatory bowel disease (Hampe *et al.*, 2001; Ogura *et al.*, 2001a). Different single nucleotide polymorphisms (SNPs) in the part of the *NOD2* gene coding for the LRRs, like the L1007finsC variant leading to a truncated LRR, significantly increase the risk for disease development (Annese *et al.*, 2005).

### 1.2.3 Evolution of NLRs

It is known that the TLRs are a group of phylogenetically very ancient receptors (Hibino *et al.*, 2006; Miller *et al.*, 2007; Wiens *et al.*, 2007). They were originally discovered in *Drosophila melanogaster* (Anderson *et al.*, 1985a; Anderson *et al.*, 1985b). The Toll protein is involved in dorso-ventral patterning and immunity (Lemaitre and Hoffmann, 2007; Lemaitre *et al.*, 1995). The TLRs and conserved signal transduction components can also be found in the sponge and in cnidarians at the base of metazoan evolution (Bosch *et al.*, 2009; Hemmrich *et al.*, 2007b; Miller *et al.*, 2007; Wiens *et al.*, 2007). In contrast, little is known about the evolution of the NLRs.

Although the described mammalian NLRs share high structural similarities with the resistance (*R*) genes of plants it was the general opinion for a long time that these molecules were acquired independently (Ting and Davis, 2005) (see Figure 1.5). The *R* proteins are comprised of a central NB-ARC domain linked to C-terminal LRRs and an N-terminal TIR or coiled coil domain (Meyers *et al.*, 2003; van der Biezen and Jones, 1998). Unlike vertebrate NLRs, these proteins do not detect MAMPs, but specifically recognize pathogen-encoded virulence factors by the avirulence (*avr*) genes (Meyers *et al.*, 2003). This gene-for-gene interaction resembles the function of the adaptive immune system of the jawed vertebrates

and explains the large variety of R proteins. The activation of R proteins leads to the death of the infected tissue *via* hypersensitive response (HR) (Ma and Berkowitz, 2007).

A large expansion of NLR orthologues has also been discovered in teleost fishes. 201 NLRs were detected in the zebrafish and 70 were found in the pufferfish (Stein *et al.*, 2007).

Since except of the structurally related APAF1 orthologues CED4 and DARK of *Caenorhabditis elegans* (Yuan and Horvitz, 1990) and *Drosophila melanogaster* (Rodriguez *et al.*, 1999) no proteins belonging to the NLR family *sensu stricto* were present in these ecdysozoan model organisms, it was assumed that NLRs were vertebrate-specific (Ting and Davis, 2005).

The first hint for invertebrate NLRs was given by the workgroup of Jonathan Rast in 2006 showing that the sea urchin *Strongylocentrotus purpuratus* possesses more than 200 NLR orthologues (Hibino *et al.*, 2006; Rast *et al.*, 2006). Remarkably, the vast majority of these NLRs have DEATH domains instead of CARD or Pyrin domains. The Pyrin domain still seems to be vertebrate specific.

In 2008, a large amount of NLR orthologues was also detected in the chordate *Branchiostoma floridae* (Huang *et al.*, 2008). The amphioxus genome encodes for more than 100 NLRs.

Although these recent studies have begun to elucidate the origins of NLR signalling, it is still unclear how early in evolution these receptors have evolved.

### **1.3 *Hydra* and other cnidarians provide insight into evolution of innate immunity**

As mentioned above, human chronic inflammatory diseases of epithelial barrier organs are frequently associated with loss-of-function variations in PRRs (Rosenstiel *et al.*, 2007). But not only genetic variations, also a change of living conditions within the past century accompanied by a modified microbial community with new metabolic patterns have been implicated to contribute to the development of chronic inflammatory diseases (Rosenstiel *et al.*, 2009; Wen *et al.*, 2008). Basal eumetazoan organisms such as cnidarians can help to understand the processes leading to the development of these diseases. It was shown that many genes that have been lost in ecdysozoan model organisms can be found in cnidarians suggesting the last common ancestor of cnidarians and bilaterians already possessed these genes (Miller *et al.*, 2005; Technau *et al.*, 2005). Therefore, cnidarians represent a good model system to understand, how selective pressures contribute to diversity in conserved immune genes in order to survive a variety of immune challenges (Rosenstiel *et al.*, 2009).

Eugene Rosenberg proposed in his “coral probiotic hypothesis” that corals can adapt to their changing environment quickly by changing their bacterial symbionts and therefore gaining resistance to diseases (Reshef *et al.*, 2006). This “holobiont”-hypothesis gives cues for understanding the role of a symbiotic microflora of humans in the development of barrier disorders.

Many facets of immunity are a subject of recent studies in cnidarians. Work on intraspecies competitions in Anthozoans and the discovery of the allorecognition locus in the colonial Hydrozoan *Hydractinia echinata* show that these animals are capable of distinguishing self from nonself (Cadavid, 2004; Nicotra *et al.*, 2009). Another major field of interest is the symbiotic association of Cnidarians with photosynthetically active unicellular algae, as in the extensively studied field of the Anthozoa-Zooxanthellae mutualism and the effect of coral bleaching due to environmental stressors (Mydlarz *et al.*, 2010; Rosenberg *et al.*, 2009; Weis, 2008). The presence of mannose-binding lectins potentially involved in the recognition of pathogens and symbionts was reported for the coral *Acropora millepora* (Kvennefors *et al.*, 2008). In sea fan corals, it was shown that the attack of a pathogenic fungus leads to tissue melanisation by the usage of the prophenoloxidase known to be involved in the immune response of Arthropods (Mydlarz *et al.*, 2008). Furthermore, orthologues of proteins of the complement system appear to be present in anthozoans and hydrozoans, but so far without any proven function in immunity (Kimura *et al.*, 2009; Miller *et al.*, 2007).

As mentioned above, *Hydra* has a very simple body plan. Except for the thin glycocalyx it does not have any protective layers to separate it from its environment (Bosch *et al.*, 2009). The freshwater polyp does not possess any migratory immune cells, so it has to rely exclusively on an epithelial defence making it a useful model system to study epithelial defence mechanisms (Augustin *et al.*, 2010).

It was demonstrated more than 25 years ago by Rahat and Dimentman that sterile cultivated polyps have a reduced budding rate indicating a beneficial effect of the microbial community on their host (Rahat and Dimentman, 1982). In 2003, Kasahara and Bosch showed that extracts from polyps depleted in interstitial cells have an increased growth inhibition effect against the Gram-positive bacterium *Bacillus subtilis* and Gram-negative bacterium *E. coli* (Kasahara and Bosch, 2003). Fraune and colleagues could show that these interstitial cell depleted animals have a different microbial community indicating an effect of the interstitial cells in shaping the composition of the microbial symbionts (Fraune *et al.*, 2009).

Via 16S rDNA analysis it was detected that different *Hydra* species support particular bacterial guilds and that this association of the host with its symbionts is stable over years (Fraune and Bosch, 2007).

Effector molecules that might take part in this shaping of *Hydra*'s microbial community have also been discovered: *Hydra* expresses a variety of antimicrobial peptides in its endodermal layer. Hydramacin-1 and the arminin protein family are expressed by the endodermal epithelial cells and the expression of the hydramacin-1 protein is inducible *via* treatment with LPS (Augustin *et al.*, 2009a; Bosch *et al.*, 2009; Jung *et al.*, 2009). Another peptide, which shows antimicrobial activity, is kazal2 being strongly expressed by the endodermal gland cells (Augustin *et al.*, 2009b). Other good candidates for being antimicrobial peptides are the members of the *periculin* gene family that are predominantly expressed within the developing egg and the embryo (Fraune, 2008). One reason for the mainly endodermal expression of these peptides might be the uptake of food and accompanying putative pathogenic bacteria by the endodermal epithelial cells (Bosch *et al.*, 2009). Interestingly, many of these peptides do not show any homology with other peptides and are therefore taxonomically restricted, which as been observed for many immune effector molecules (Khalturin *et al.*, 2009).

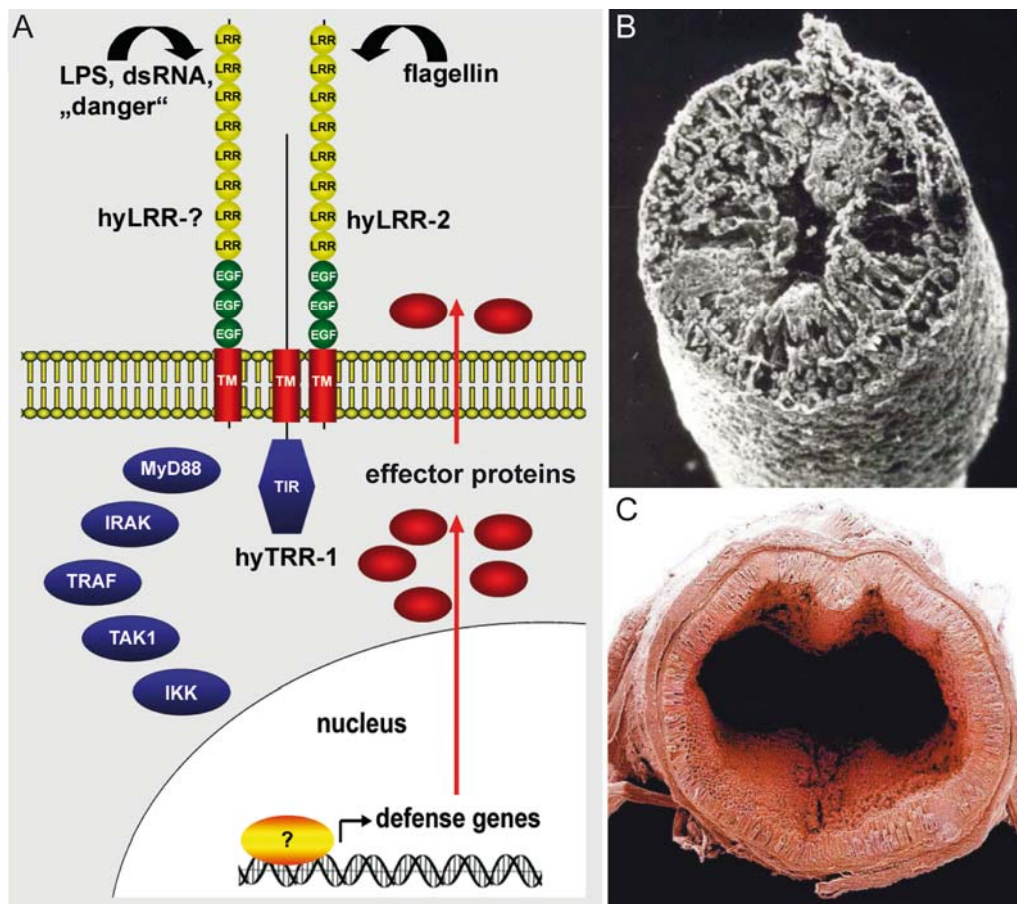
The expression of these taxonomically restricted AMPs may be controlled by conserved signal transduction pathways (Bosch *et al.*, 2009). The presence of a TLR and the conserved components of its signalling pathway as well as components of MAP kinase signalling in *Nematostella* shows their phylogenetically old roots (Hemrich *et al.*, 2007b; Miller *et al.*, 2007). A truncated orthologue for NF- $\kappa$ B was detected in *Nematostella* as well (Sullivan *et al.*, 2007; Sullivan *et al.*, 2009).

*Hydra* does not possess a *sensu stricto* TLR, but it was shown using a HEK293 cell based *in vitro* assay that a TIR-transmembrane domain protein, HyTRR1, can form a functional subunit with its co-receptor HyLRR2 upon stimulation with flagellin (Bosch *et al.*, 2009) (see Figure 1.7).

In addition to this unusual TLR, *Hydra* possesses conserved components of the TLR signalling cascade, like MyD88, TRAF6, TAK1 and IKK and components of MAP kinase signalling, like JNK and AP1 (Hemrich *et al.*, 2007b; Miller *et al.*, 2007; Philipp *et al.*, 2005). In contrast to previous findings the new transcriptome datasets gave first evidence for a putative NF- $\kappa$ B orthologue (G. Hemrich, pers. communication).

Some orthologues of proteins involved in apoptosis were detected in *Hydra*, like HyBcl-2, HyBak, HyBax and ten caspases, most of them resembling the human caspase 3 (Böttger and Alexandrova, 2007; Cikala *et al.*, 1999).

To conclude, *Hydra* is an important tool for studying a variety of immunological questions such as the evolution of epithelial defence mechanisms and the development of barrier disorders.



**Figure 1.7: The epithelial defence in *Hydra*.** (A) Model of the TLR signalling cascade in *Hydra*. Upon activation with flagellin HyTIR-1 and HyLRR-2 interact. Conserved TLR signalling cascade members are present in *Hydra*. The activation of TLR signalling may lead to the secretion of effector proteins. (Modified from Bosch *et al.*, 2009). (B) A cross-section of the gastric region of *Hydra*; (C) A cross-section of the human small intestine. (Taken from Bosch, 2009a).

## 1.4 Aims of the study

NLRs are a family of intracellular innate immune receptors sensing MAMPs and endogenous danger signals (Rosenstiel *et al.*, 2008). Upon activation these receptors form multimeric complexes with downstream signalling components resulting in an immune response including the release of AMPs and pro-inflammatory cytokines (Chen *et al.*, 2009; Voss *et al.*, 2006). Genetic variants of NLRs have been associated with the manifestation of chronic inflammatory diseases of epithelial barrier organs in *Homo sapiens* (Rosenstiel *et al.*, 2007).

Although these receptors are key players in epithelial defence, the information about their evolution within the animal kingdom is very limited (Hibino *et al.*, 2006; Huang *et al.*, 2008; Ting and Davis, 2005). In order to broaden the understanding of NLR evolution, one goal of the study was to screen the genomes of several animal species with a focus on basal metazoans and especially on *Hydra* accompanied by a phylogenetic analysis.

The freshwater polyp *Hydra* is continuously surrounded by potential pathogenic microbes and must have acquired effective epithelial defence mechanisms accompanying its adaption to its ecological niche (Augustin *et al.*, 2010; Rosenstiel *et al.*, 2009). Using *Hydra* as model organism for epithelial defence, the expression of *Hydra* NLRs should be analysed in more detail.

Furthermore, putative interaction partners for these receptors should have been identified and characterized. In order to elucidate the interaction of *Hydra* NLRs with these proteins and the result of these interactions, first functional analyses should be performed.

A disturbed function of immunity may lead to a disturbed microflora (Fraune *et al.*, 2009; Salzman *et al.*, 2010; Zaki *et al.*, 2010). This disturbance has been discovered in a strain of *Hydra oligactis*, which accidentally formed tumour-like structures upon isolation from nature and cultivation under laboratory conditions. The second goal of the study was to characterize this tumour bearing strain using histological and molecular biological methods in order to get insight in tumour formation within a basal invertebrate animal.

## 2 RESULTS

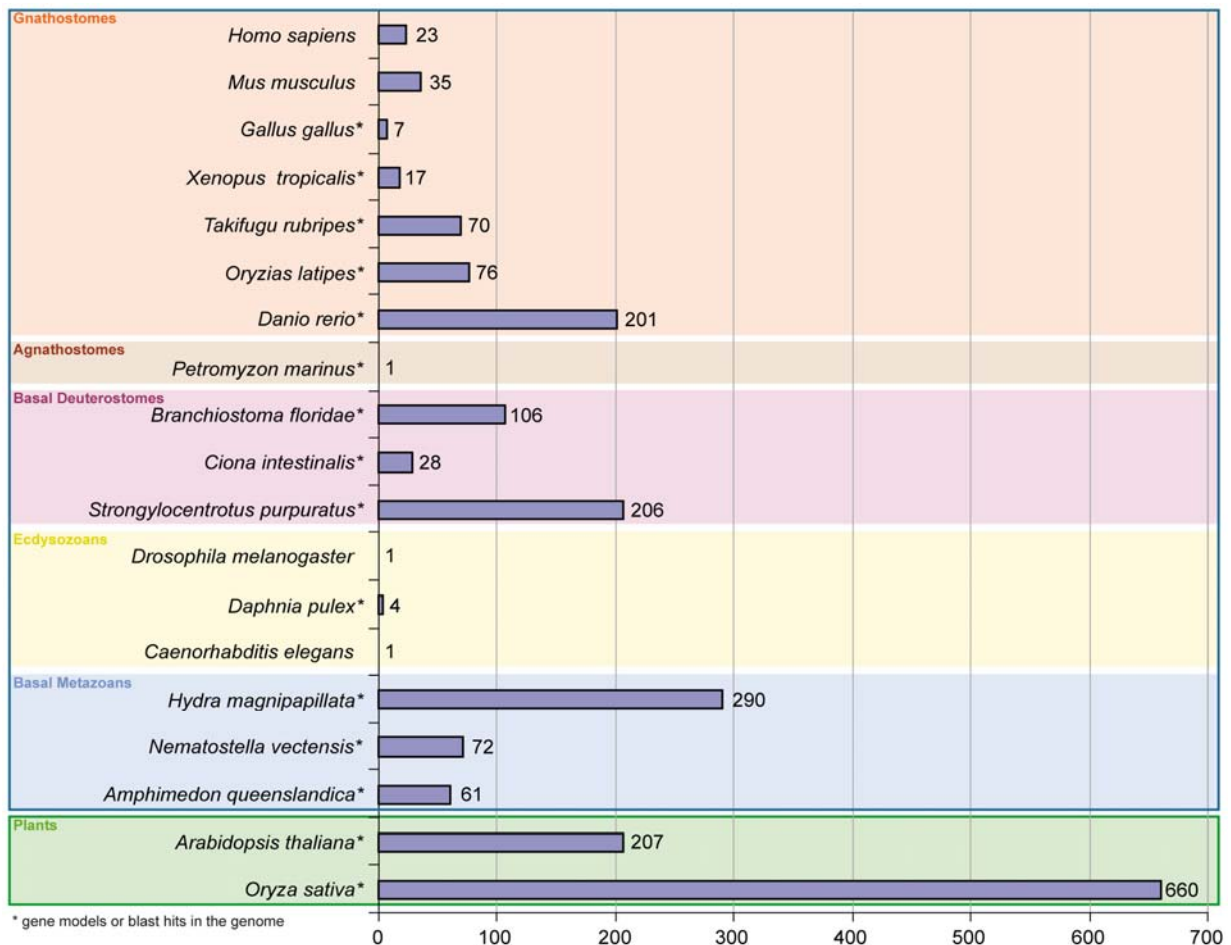
### 2.1 The family of NBD domain containing proteins is highly complex in basal metazoans

Proteins containing NBD domains like NACHT and NB-ARC domains fulfil various functions in immunity or apoptosis (Rosenstiel *et al.*, 2008; Zou *et al.*, 1997). While a host of data has been presented on the biological roles of selected members of the family (e.g. NOD2 and NALP3) in *Homo sapiens* and other vertebrates (Chen *et al.*, 2009; Schroder *et al.*, 2010), hardly anything is known about the evolution of these gene families. To broaden this knowledge and ask the question whether NLRs were already present at the base of animal evolution, a screening for orthologues of NBD domain containing proteins was performed in the genomes and transcriptomes of various animal species *via* BLAST (basic local alignment search tool) (Altschul *et al.*, 1990) searches with the domains of vertebrate NLRs or sequence analysis using hidden Markov model algorithms (HMMER) (Eddy, 1996). All hits were subsequently verified by protein domain predictions. As well as basal species such as the sponge *Amphimedon queenslandica*, the cnidarian anthozoan species *Acropora millepora*, *Nematostella vectensis* and the hydrozoan species *Hydra magnipapillata* were investigated, but also the genomes of one arthropod species, *Daphnia pulex*, the agnathostome *Petromyzon marinus* and three gnathostome species: *Oryzias latipes*; *Xenopus tropicalis*; and *Gallus gallus*. These organisms were included to elucidate the appearance of NBD-domain containing proteins at different diverging nodules of animal evolution.

In Figure 2.1 the absolute numbers of NBD-containing proteins of various multicellular organisms with a sequenced genome are compared. Figure 2.2 shows a detailed view of the domain compositions as well as the numbers of these proteins of selected screened animal species in comparison to published NBD domain containing proteins of other species (Bai *et al.*, 2002; Cecconi *et al.*, 1998; Hibino *et al.*, 2006; Huang *et al.*, 2008; Kanneganti *et al.*, 2007; Meyers *et al.*, 2003; Rast *et al.*, 2006; Rodriguez *et al.*, 1999; Schreiber *et al.*, 2005; Sodergren *et al.*, 2006; Stein *et al.*, 2007; Yuan and Horvitz, 1990; Zmasek *et al.*, 2007; Zou *et al.*, 1997). A detailed list of all hits obtained in this screening with the predicted domain compositions is present in the appendix.

It is known that *Homo sapiens* and *Mus musculus* have with 23 and 35 genes a relatively low number of NBD-coding genes (Bergelson *et al.*, 2001; Kanneganti *et al.*, 2007; Schreiber *et al.*, 2005; Zou *et al.*, 1997), compared to 70 and 201 genes of two teleost fish species, *Takifugu rubriceps* and *Danio rerio* (Stein *et al.*, 2007). To broaden the information about

these genes in vertebrates, the genomes of a bird (*Gallus gallus*), an amphibian (*Xenopus tropicalis*), and another teleost fish (*Oryzias latipes*) were screened.



**Figure 2.1: Number of NBDs in selected multicellular organisms.**

The NACHT and NB-ARC domains were counted in *Gallus gallus*, *Xenopus tropicalis*, *Oryzias latipes*, *Petromyzon marinus*, *Daphnia pulex*, *Hydra magnipapillata*, *Nematostella vectensis* and *Amphimedon queenslandica* at the basis of homology searches via BLAST, HMMER or gene models. The total numbers of NBDs in *Amphimedon queenslandica* cannot be clearly estimated due to the fact that single genome reads were screened. For *Homo sapiens* (Schreiber *et al.*, 2005; Zou *et al.*, 1997), *Mus musculus* (Cecconi *et al.*, 1998; Kanneganti *et al.*, 2007), *Takifugu rubripes* (Stein *et al.*, 2007), *Danio rerio* (Stein *et al.*, 2007), *Branchiostoma floridae* (Huang *et al.*, 2008; Zmasek *et al.*, 2007), *Ciona intestinalis* (Sodergren *et al.*, 2006), *Strongylocentrotus purpuratus* (Hibino *et al.*, 2006; Rast *et al.*, 2006; Zmasek *et al.*, 2007), *Drosophila melanogaster* (Rodriguez *et al.*, 1999), *Caenorhabditis elegans* (Yuan and Horvitz, 1990), *Arabidopsis thaliana* (Meyers *et al.*, 2003) and *Oryza sativa* (Bai *et al.*, 2002) previous publications were taken into consideration.

In the chicken genome, six orthologous gene models for NOD-like receptors and one for APAF1, including the models published by Hughes *et al.* (Hughes, 2006), were detected (see Figures 2.1 and 2.2).

A total of 17 NBD containing gene models were discovered in the genome of *Xenopus tropicalis* (see Figure 2.1). Within these gene models a NAIP and an APAF1 orthologue are present. Most of the gene models encode a NACHT-LRR structure (see Table 9.1 in the appendix).



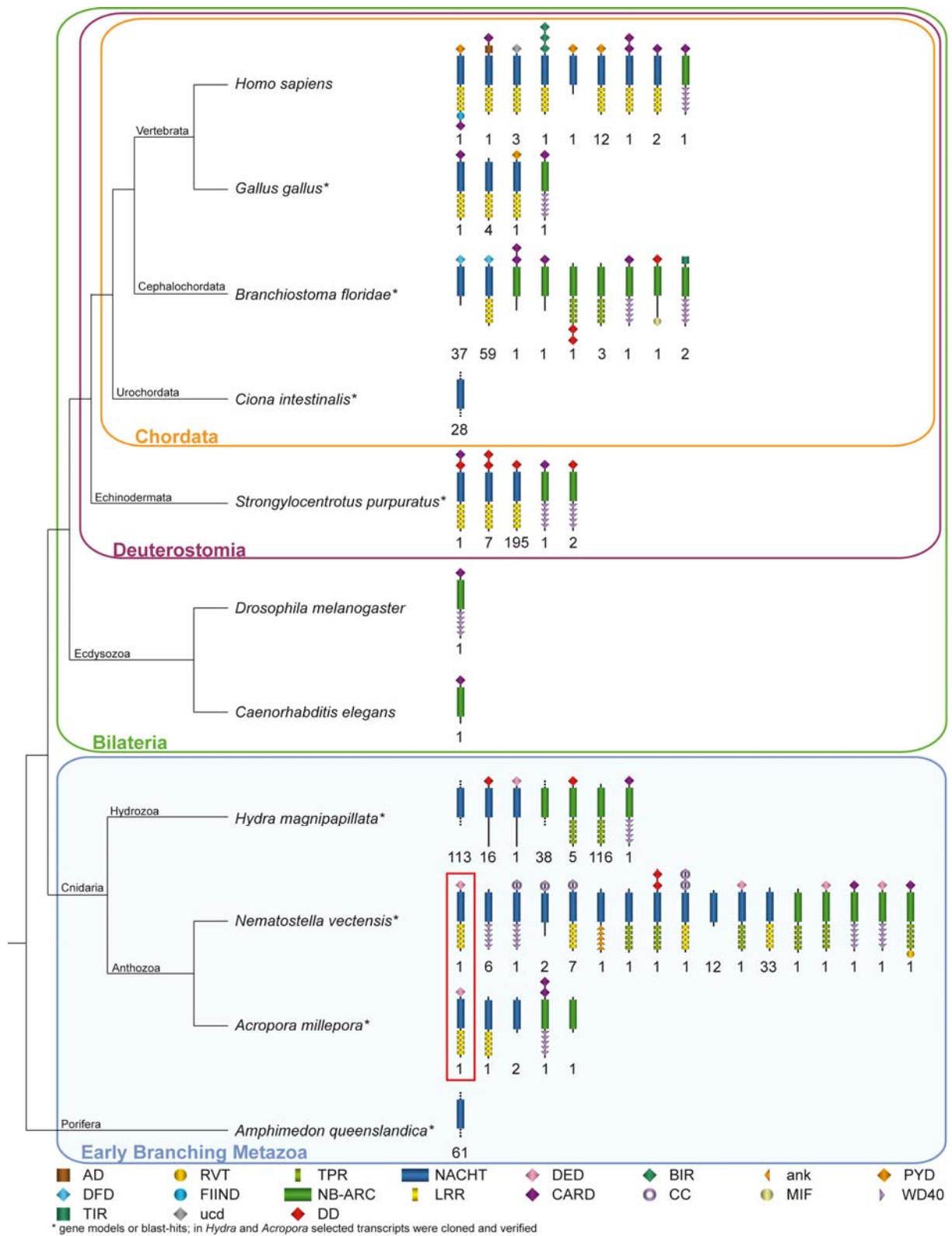


Figure 2.2: Overview of structures of NBD containing proteins in selected animals. (Description: next page)

**Figure 2.2 (continued): Overview of structures of NBD containing proteins in selected animals.**

For *H. sapiens* (Schreiber *et al.*, 2005; Zou *et al.*, 1997), *B. floridae* (Huang *et al.*, 2008; Zmasek *et al.*, 2007), *C. intestinalis* (Sodergren *et al.*, 2006), *S. purpuratus* (Hibino *et al.*, 2006; Rast *et al.*, 2006; Zmasek *et al.*, 2007), *D. melanogaster* (Rodriguez *et al.*, 1999), *C. elegans* (Yuan and Horvitz, 1990), *N. vectensis* (2 NB-ARC domain containing models) (Zmasek *et al.*, 2007) previous publications were taken into consideration. Below the predicted protein structure the numbers of proteins are mentioned. Red box indicates the *bona fide* NLR orthologues in *Nematostella* and *Acropora*; Abbreviations: AD (acidic domain), DFD (DEATH-fold domain), TIR (toll-interleukin-1 receptor), TPR (tetratricopeptide repeat), NB-ARC (nucleotide binding adaptor shared by APAF1, *R* gene products and CED-4), DD (DEATH domain), NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA), HET-E and TP1), LRR (Leucine rich repeat), DED (DEATH effector domain), CARD (Caspase recruitment domain), PYD (Pyrin domain), BIR (baculovirus inhibitor of apoptosis repeat), CC (coiled coil), ank (ankyrin repeat), RVT (reverse transcriptase), FIIND (domain with function to find), ucd (uncharacterized domain) The lengths of proteins and domains are not to scale. The number of repetitions of a repetitive domain (WD40, LRR, TPR, ank) does not reflect the exact number of repeats.

In the genome of the teleost fish *Oryzias latipes* 78 loci for NBDs were detected (see Figure 2.1). About one half of the hits are represented by a gene model, some are supported by ESTs. Some of these NBDs appear to be clustered on different chromosomes, e.g. chromosome 2 (21 NBDs), chromosome 18 (9 NBDs) and ultracontig 205 (6 NBDs) (see Table 9.1 in the appendix).

It is known that the number of NBD-coding genes in two basal chordate species varies a lot (Huang *et al.*, 2008; Sodergren *et al.*, 2006). Whereas *Branchiostoma floridae* NBD-coding genes underwent a broad expansion, leading to 106 representatives (Huang *et al.*, 2008), the urochordate *Ciona intestinalis* does not seem to contain any genes coding for NBDs connected with other domains (Sodergren *et al.*, 2006). To elucidate the situation in a basal vertebrate species, the genome of the agnathostome *Petromyzon marinus* was investigated. Interestingly, no orthologue for NOD-like receptors was detected here, except of an APAF1 orthologue (see Figure 2.1), represented by four short genome contigs with a size range from 4.6 to 17.9 kb. Due to the short contig length and to the fact that the contigs cover different parts of the APAF1 gene, these hits are assumed to belong to one locus in the genome.

Because *D. melanogaster* and *C. elegans* do not have any NLR orthologues except APAF1 (Rodriguez *et al.*, 1999; Yuan and Horvitz, 1990), it was assumed that this gene family is absent in all ecdysozoan species (Ting and Davis, 2005). In order to question this assumption, the genome of the arthropod species *Daphnia pulex* was investigated. In the genome of the freshwater crustacean two gene models coding for proteins with a NACHT domain followed by LRRs were discovered in addition to an APAF1 homologue (see Figure 2.1 and Table 9.1 in the appendix). One of these NLRs gives best BLAST hits to two predicted proteins of the same domain composition of two mosquito species, *Aedes aegypti* and *Culex quinquefasciatus* (XP\_001658101, XP\_00184881). Furthermore, a gene model coding for a NACHT domain and WD40 repeats was detected (see Table 9.1 in the appendix).

Since nothing was known about the presence of NBD coding genes in basal metazoan species, it was previously thought that NLRs evolved at the state of the deuterostomes (Ting and Davis, 2005). The genomes of a demosponge, *Amphimedon queenslandica*, of two cnidarian species, the anthozoan *Nematostella vectensis*, and the hydrozoan *Hydra magnipapillata*, and the EST databases of the anthozoan *Acropora millepora* were investigated to determine whether these basal animals could use NLRs for their defence against pathogens as well.

It is not yet possible to survey the complete NBD gene repertoire of the sponge *Amphimedon queenslandica* because no genome assembly is available. A total of 349 NACHT domains were identified in the trace archive genomic sequence data, which were assembled to 61 genome contigs (see Figures 2.1 and 2.2). This implied presence of a large number of NACHT proteins suggests a relatively complex NBD repertoire in early diverging Metazoa.

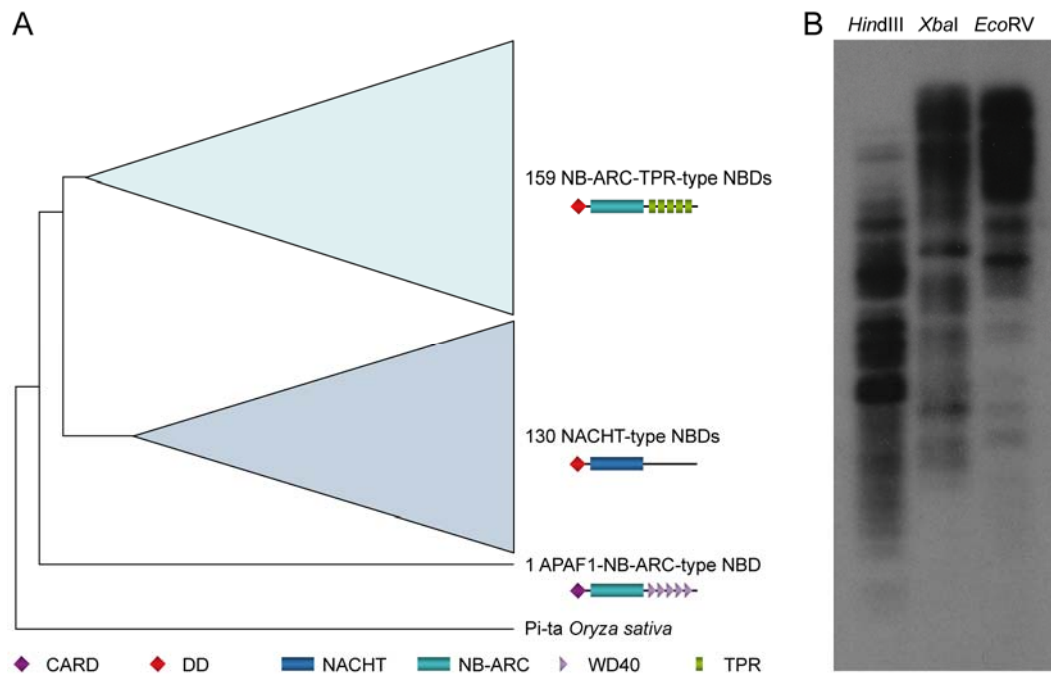
In collaboration with Yvonne Weiss from the workgroup of David J. Miller the EST databases of the anthozoan cnidarian species *Acropora millepora* were screened. One *bona fide* NOD-like receptor orthologue consisting of a DED, a NACHT domain and LRRs was detected and verified by cloning of the entire open reading frame (red box in Figure 2.2). In addition, another NACHT and LRR containing EST, two NACHT and one NB-ARC containing ESTs were detected. An orthologue for APAF1 coding for two CARD domains instead of one was cloned (see Figure 2.2).

In addition to two published NB-ARC domain containing proteins (Zmasek *et al.*, 2007), 70 loci coding for NBD domains were detected in a second anthozoan species, *Nematostella vectensis*. These include an orthologue of the *Acropora millepora* NLR (red box in Figure 2.2), more than 30 NACHT domains linked to LRRs and many other proteins showing a large variety of domain compositions, e.g. NACHT domains that are connected to WD40 repeats or ankyrin repeats and eleven NACHT domains connected to coiled coils. Another group of six proteins link an NB-ARC domain with TPRs. A single *bona fide* APAF1 homologue is present as well (see Figure 2.2).

In the genome of *Hydra magnipapillata* two large groups of in total 290 NBD domain containing proteins were detected (see Figures 2.1, 2.3).

The first group includes 130 NACHT domain coding loci. Using HMMER analysis in 16 of them a DEATH domain was found upstream of the NACHT domain on the genomic contig, in one of them a DED domain (see Figure 2.2). No LRRs appear to be present in *Hydra* orthologues for NOD-like receptors, but ESTs coding for LRR containing proteins were identified (data not shown). Neither by computational analysis methods, nor by attempts to physically link NACHT-coding with LRR-coding contigs *via* PCR, could an NLR with a NACHT-LRR structure be identified. HyNLRs share a high degree of conservation on the

amino acid as well as on the nucleotide level. With a set of primers 170 transcript fragments were amplified and cloned from cDNA that could be assembled to 19 contigs and 30 singletons, indicating that at least 49 genes are definitely transcribed (see also appendix 9.4.1). The database screening for NACHT containing genes is supported by a Southern blot hybridisation resulting in a complex band pattern (see Figure 2.3B).



**Figure 2.3: Analysis of NBDs in *Hydra magnipapillata*.**

(A) The schematic tree was built on the basis of an amino acid alignment of non-fragmented NBDs according to HMMER prediction. The drawing of the protein structure shows a representative protein of this group. Abbreviations: TPR (tetratricopeptide repeat), NB-ARC (nucleotide binding adaptor shared by APAF1, *R* gene products and CED-4), DD (DEATH domain), NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA), HET-E and TP1), CARD (Caspase recruitment domain). (B) Southern blot hybridisation with a probe coding for a NACHT domain from *Hydra magnipapillata*. The genomic DNA from *Hydra magnipapillata* was digested with *Hind*III, *Xba*I and *Eco*RV. The probe used for the hybridisation reflects the NACHT domain, the most conserved part of the HyNLR gene sequences.

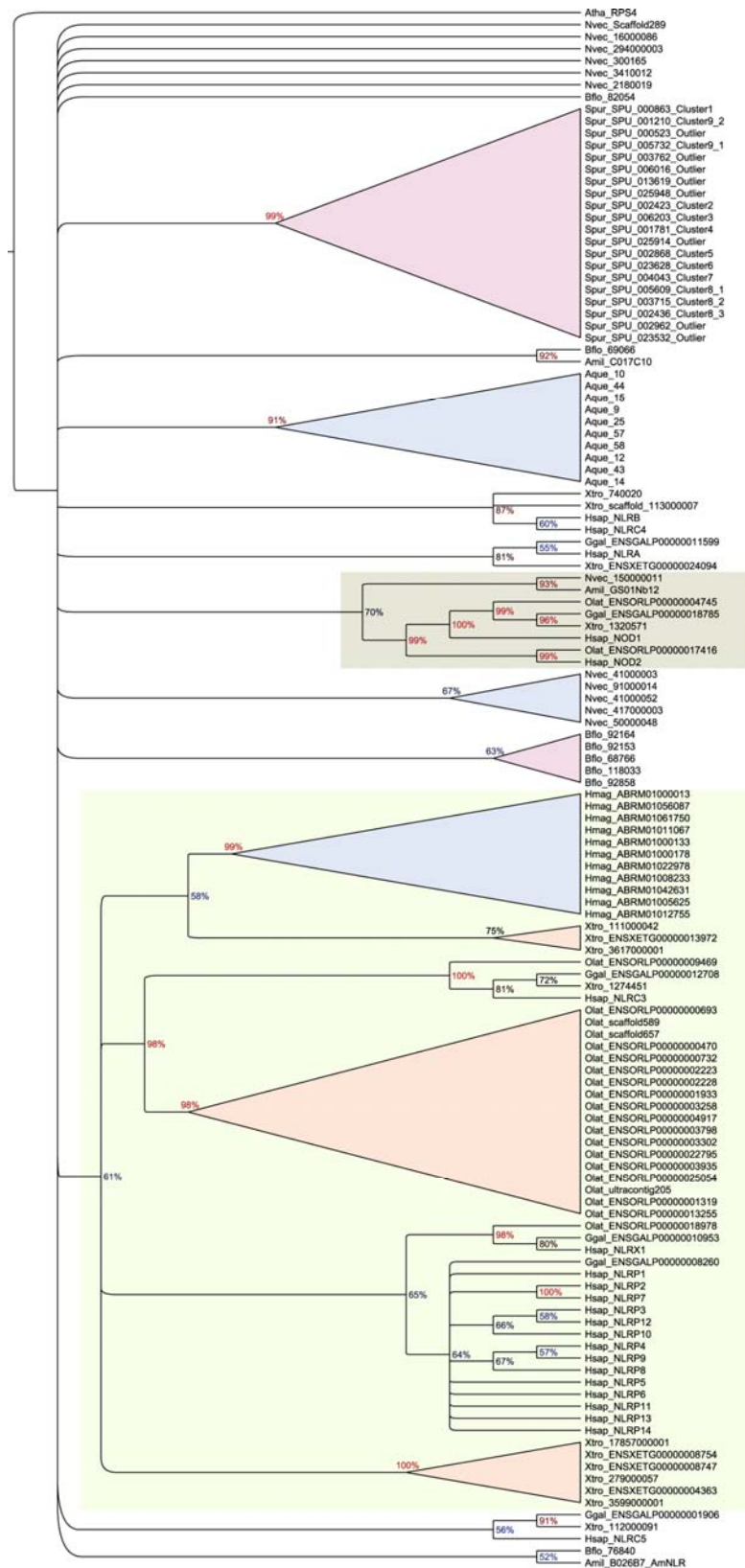
The second group of NBD proteins in *Hydra* is represented by the 160 NB-ARC domain containing proteins (see Figures 2.2, 2.3). In the genome one APAF1 homologue coding for a CARD domain, a NB-ARC domain and WD40 repeats was identified (see Figures 2.2, 2.3). Furthermore, a large group of NB-ARC domains connected with TPRs was found. In 159 NB-ARC domain coding genome contigs 116 were linked to a TPR repeat (see Figure 2.2). Additional five contained not only TPRs but also an N-terminal DEATH domain. This unusual architecture was experimentally verified for several of the *Hydra* gene predictions. Twelve transcript variants were cloned and sequenced from cDNA with primers for conserved regions illustrating that these genes are indeed transcribed (see also appendix 9.4.2).

Except from *Hydra* and *Nematostella* this new uncharacterized protein family has so far only been detected in *Branchiostoma floridae* (Zmasek *et al.*, 2007). Remarkably, these proteins give best BLAST hits to bacterial proteins also containing NB-ARC domains connected to TPRs. RT-PCR performed on mRNA isolated from separated ectodermal and endodermal tissue layers showed that this gene family is exclusively expressed in the ectodermal epithelium of *Hydra* (data not shown).

A phylogenetic analysis using Bayesian interference with selected NACHT-domain representatives (Figure 2.4) resulted in a polytomy and revealed that throughout animal evolution vast species-specific NLR gene expansions frequently occurred.

These expansions can be observed for *A. queenslandica*, *H. magnipapillata*, *S. purpuratus* and partially for *N. vectensis*, *B. floridae*, *X. tropicalis* and for the human NLRP genes. Whereas almost all human NLR genes and their vertebrate orthologues appear to be vertebrate-specific, NOD1 and NOD2 form a cluster not only with vertebrates, but also with two cnidarian representatives indicating a putative phylogenetically old origin for NOD1 and NOD2, at least for their NACHT domains. Remarkably, all *Hydra* NACHT domains are located within one group of exclusive vertebrate sequences, indicating a relatively high sequence similarity between these groups.

An alignment with some representative NACHT domains that were used to build the phylogenetic tree (see appendix 9.2) shows that the various NACHT-domain motifs, like the P-loop and the Mg<sup>2+</sup>-binding site (Koonin and Aravind, 2000), are conserved throughout evolution, although the different NACHT sequences are very heterogeneous.

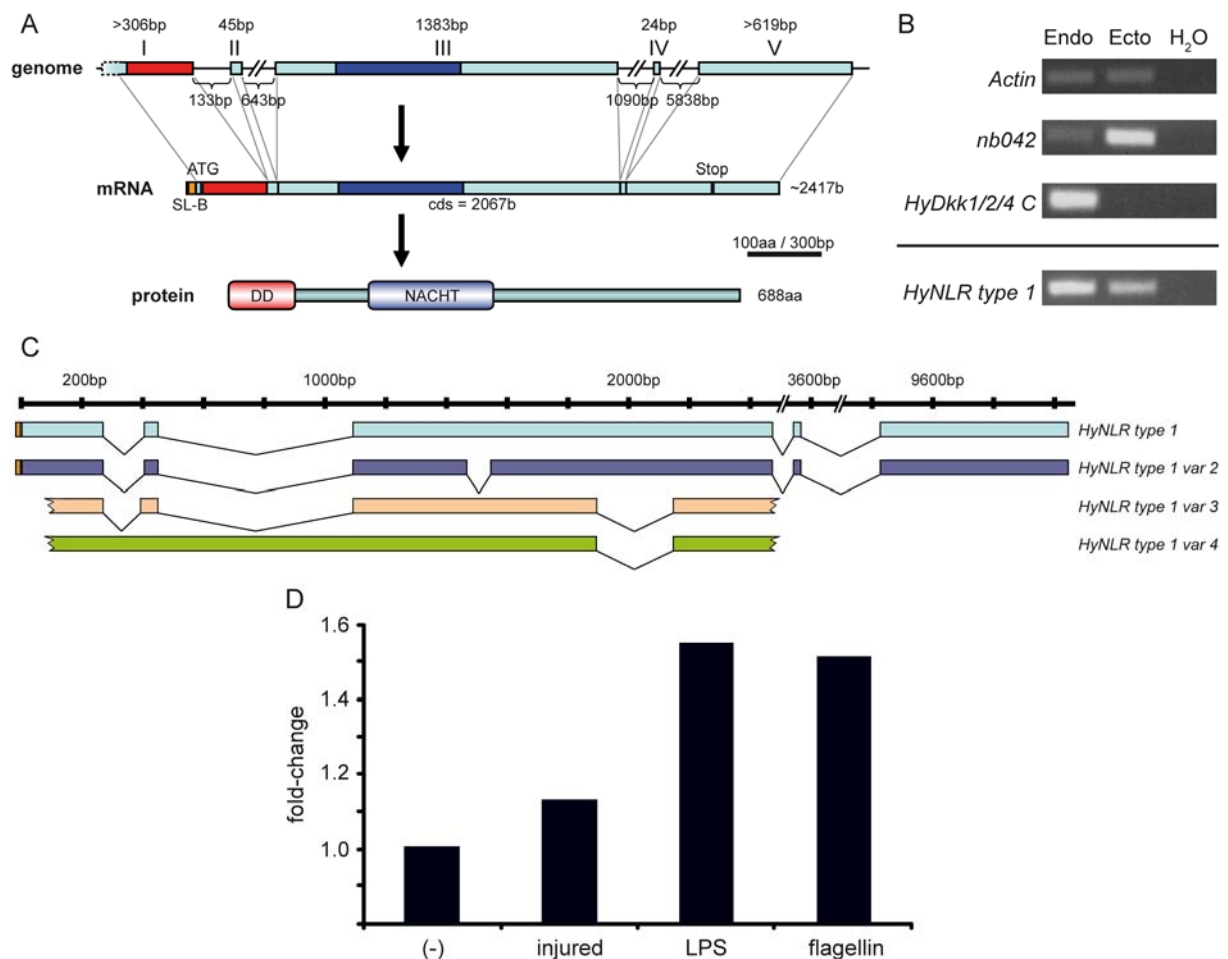


**Figure 2.4: Phylogenetic analysis of representative NACHT-domains across the animal kingdom.**

The tree was built by Bayesian interference of phylogeny. Percentages indicate posterior probabilities. Posterior probabilities below 50 % are not shown; bright yellow box: *Hydra* NACHT domains are located within one group with vertebrate sequences; grey box: indicates a cluster of vertebrate NOD1 and NOD2 orthologues with similar cnidarian NACHT domains; as outgroup the NB-ARC domain of RPS4 (*A. thaliana*) was used. Abbreviations: Amil (*A. millepora*), Atha (*A. thaliana*), Nvec (*N. vectensis*), Spur (*S. purpuratus*), Bflo (*B. floridae*), Aque (*A. queenslandica*), Xtro (*X. tropicalis*), Olat (*O. latipes*), Hmag (*H. magnipapillata*), Hsap (*H. sapiens*), Ggal (*G. gallus*).

## 2.2 The expression profile of *HyNLR type 1*, a *Hydra*-NLR representative gene

In contrast to anthozoans, *Hydra* lacks a classical NLR with a NACHT-LRR structure, but contains several proteins with an N-terminal DEATH domain followed by a NACHT domain. To provide insight into the function of the *Hydra* NLR-like proteins, the transcript architecture and expression pattern of one of *Hydra* NLR-type genes, *HyNLR type 1*, was analyzed in detail (Figure 2.5).



**Figure 2.5: Expression profiling of *HyNLR type 1*.**

(A) The gene structure of *HyNLR type 1*. The gene consists of five exons of various sizes and encodes a transcript of about 2,417 bases. At the 5' end the *trans*-spliced leader B is added. The 688 aa long protein product contains a predicted DEATH and a NACHT domain but lacks LRRs. (B) Semiquantitative reverse transcriptase PCR performed on RNA extracted from the ectodermal and the endodermal cell layers of *Hydra* after procaine treatment showing a predominant expression of *HyNLR type 1* in the endodermal layer. As control genes the housekeeping gene *actin*, the ectodermally expressed nematoblast gene *nb042* (Milde *et al.*, 2009) and the endodermally expressed gland cell gene *HyDkk1/2/4 C* (Augustin *et al.*, 2006) were used. (C) Splice isoforms of *HyNLR type 1*. In addition to the most abundant *HyNLR type 1* transcript (light blue) three other transcript variants (dark blue, orange and green) that show an alternative splice pattern were cloned and sequenced. *HyNLR type 1 var 3* and *4* were cloned partially. (D) Quantitative real time PCR showing a light up-regulation of *HyNLR type 1* after stimulation with LPS and flagellin. The fold-change of the expression of *HyNLR type 1* is compared with the expression levels of the negative control. Abbreviations: endo (endoderm), ecto (ectoderm).

The complete transcript of *HyNLR type 1* was amplified using 3'RACE (rapid amplification of cDNA ends)-PCR technique and sequenced subsequently from cDNA. The gene is represented on the *Hydra magnipapillata* genome scaffold contig35932 (<http://hydrazome.metazome.net>) and is transcribed from five exons of various sizes to an mRNA with a length of about 2,417 bases with a coding sequence of 2,067 bases (Figure 2.5A). The 5' untranslated region (5' UTR) contains the *Hydra*-specific *trans*-spliced leader B sequence (Stover and Steele, 2001). Thus, a clear transcription start cannot be estimated easily. Sequencing of different PCR products revealed that the *trans*-spliced leader can be added on two different splice acceptor sites resulting in two different 5' UTRs varying in their length (see also appendix 9.4.1.1). The transcript codes for a protein of 688 amino acid (aa) residues with a predicted DEATH domain and a subsequent NACHT domain. BLAST searches with the complete protein against the mammalian protein databases result in NLRPs as best hits, resulting from a similarity in DEATH and NACHT domains. The C-terminal part that follows the NACHT domain does not contain LRRs and gave no significant matches with the NCBI non redundant (NR) database, implying that this part of the protein is unique for *Hydra*.

Since *in situ* hybridisation did not lead to interpretable results, the gene's expression profile was assessed using semiquantitative reverse transcription (RT)-PCR following a procaine treatment to separate the two tissue layers of *Hydra* (Figure 2.5B). The *HyNLR type 1* gene is predominantly expressed in the endodermal tissue layer and to a decreased amount in the ectodermal layer.

In addition to the most abundant *HyNLR type 1* transcript three additional transcript variants that are transcribed from both alleles were amplified from cDNA as a result of alternative splicing events (Figure 2.5C, see also appendix 9.4.1.1). The complete transcript of *HyNLR type 1 var 2* was cloned and subsequent sequencing showed that the transcript lacks 80 nucleotides in the third exon. The variants 3 and 4 were partially amplified from cDNA. In the *HyNLR type 1 var 3* the second exon contains twelve additional nucleotides at the 5' end and another deletion of 253 bases in the third exon. *HyNLR type 1 var 4* contains the same deletion and in addition the exons I, II and III are not spliced at all. In all cases the canonical splice sites GU-AG were used.

In order to analyse whether the expression of the *HyNLR type 1* transcript is inducible upon stimulation with bacteria or MAMPs, incubation experiments were performed. In the first experiment polyps were incubated either with a suspension of the freshwater bacterium *Pseudomonas fluorescens* or *Escherichia coli*, or with a supernatant of the human pathogenic bacterium *Pseudomonas aeruginosa* or *P. aeruginosa* surface components. After the



incubation, RT-PCRs were performed. The transcription of the *HyNLR type 1* gene was not induced upon treatment with these stimuli (data not shown).

A possible explanation for no observed induction of the *HyNLR type 1* transcript might be the poor accessibility of the endodermal cell layer for the bacterial stimuli. Under normal conditions, the mouth of *Hydra* is tightly closed and separates the gastric cavity from the environment. In order to overcome this problem a second incubation experiment was performed. Now the MAMPs, LPS and flagellin, were directly injected into the gastric cavity of *Hydra* in addition to the incubation of the polyps within these MAMPs. To exclude an effect due to tissue injury, *Hydra* medium alone was injected into control animals. A quantitative real-time (qRT)-PCR was conducted using the extracted mRNA as template. The fold-changes of the *HyNLR type 1* expression were calculated in comparison to the expression levels of the non treated animals. Upon stimulation with LPS, an upregulation of 1.55-fold was detected, after challenging the polyps with flagellin a 1.5-fold induction was observed.

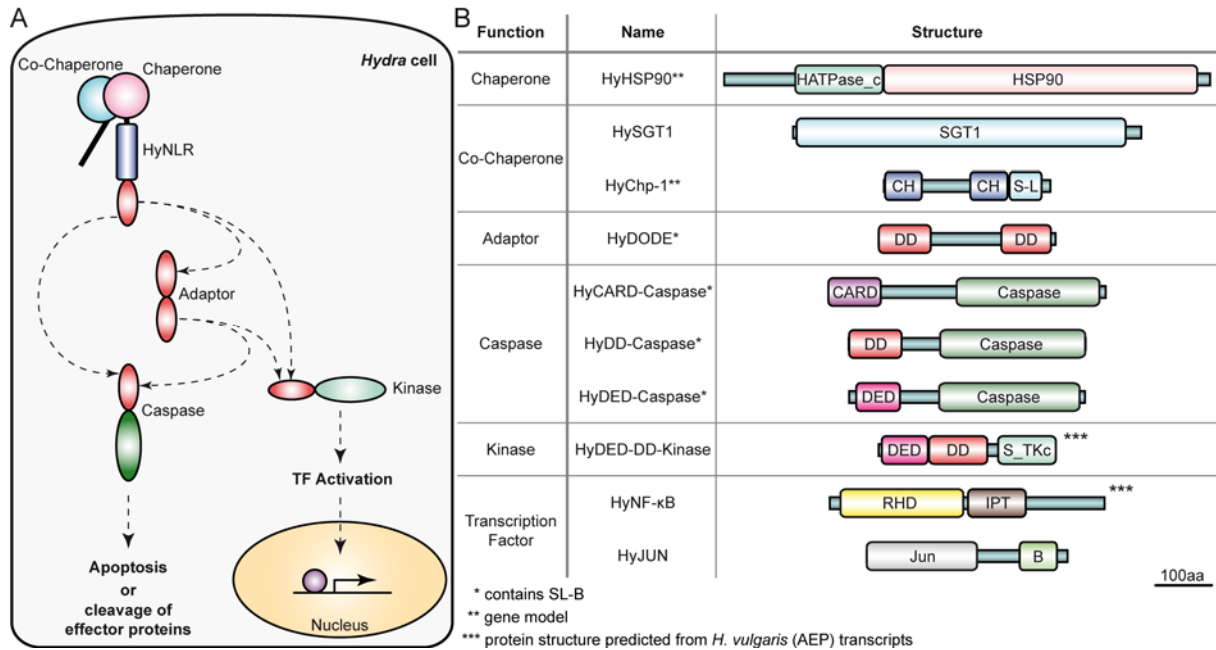
### 2.3 The putative interactome of HyNLR type 1 and other HyNLRs

Upon activation NLRs recruit several supporting proteins that are necessary to form large molecular complexes, e.g. the inflammasome comprised of NLRP1 or NLRP3 monomers (Schroder and Tschopp 2010), ASC (apoptosis-associated speck-like protein containing a CARD) (Masumoto *et al.*, 1999) and caspase 1 (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992) or the NODosome, formed by NOD1 or NOD2 in association with the serine-threonine kinase RIPK2 (Inohara *et al.*, 2000; Ogura *et al.*, 2001b). Additionally, chaperones and co-chaperones are required to foster and modulate the functional state of the complexes (Hahn, 2005; Mayor *et al.*, 2007). To elucidate the conservation of the NLR-associated molecular pathways, the *Hydra magnipapillata* genome was screened for genes whose protein products may interact with the HyNLR type 1 protein in an ancient NLR signalling cascade (Figure 2.5). To further investigate their roles in a HyNLR signalling cascade, selected candidate genes were subsequently cloned from cDNA using RACE PCR techniques in order to obtain their entire coding sequence.

Three orthologues of the NLR-chaperone HSP90 (Mayor *et al.*, 2007), and orthologues of the NLR co-chaperones Chp-1 (Hahn, 2005) and SGT1 (Mayor *et al.*, 2007) were identified (Figure 2.5B) and the full open reading frame (ORF) of one of the co-chaperones, *HySGT1*, consisting of 1,979 bases, was cloned and sequenced.

To identify the initiators of downstream signals elicited by NLR activation, the EST and genome databases were screened for genes whose predicted protein products contain an effector domain of the DEATH-fold domain family for homotypic interactions with HyNLRs.

A gene encoding a protein consisting of two DEATH domains was identified and the entire ORF of 915 bases was cloned (Figure 2.6B). This Double-DEATH protein HyDODE could function as an adaptor protein like ASC in the human NLRP1 and NLRP3 inflammasomes and could serve as a bridge for the interaction of *Hydra* NLRs with further effector proteins like caspases or kinases.



**Figure 2.6: Screening for the putative interactome of HyNLRs.**

(A) A putative model how the signal transduction of an NLR in *H. magnipapillata* could be performed. The NLR needs a chaperone and a co-chaperone to foster and modulate its functional state. Upon activation it could either activate a caspase or a kinase directly or indirectly *via* an adaptor protein. The caspase could induce apoptosis or cleave substrate proteins and the kinase could activate transcription factors that may regulate the expression of e.g. antimicrobial peptides. (B) Table of the putative interactome of HyNLRs. The complete ORFs for *HySGT1*, *HyDODE*, *HyCARD-Caspase*, *HyDD-Caspase*, *HyDED-Caspase* and *HyJUN* were cloned. Abbreviations: DD (DEATH domain), DED (DEATH effector domain), CARD (Caspase recruitment domain), CH (CHORD), S-L (SGT1-like domain), S\_TKc (serine/threonine protein kinase), RHD (Rel homology domain), IPT (Ig like, plexins, transcription factors), B (basic region leucine zipper); The *HyDD-Caspase*, the *HyDED-Caspase* and *HyDODE* transcripts contain the *trans*-spliced leader B, the protein structure of HyNF-κB and HyDED-DD-Kinase are derived from transcripts from *H. vulgaris* (AEP).

Furthermore, the complete transcript sequence consisting of 1,431 bases coding for a caspase containing a CARD domain at the N-terminus was identified and cloned (Figure 2.6B).

The *Hydra magnipapillata* orthologues of two suggested transcripts encoding *Hydra* caspases that contain an N-terminal DED (HyDED-Caspase, 1,215 bp) or a DEATH domain (HyDD-Caspase) (Böttger and Alexandrova, 2007) were cloned and the ORF of the *HyDD-Caspase* gene was completed from 1,182 to 1,224 bases using *trans*-spliced leader PCR. The HyDD-Caspase is unique for *Hydra*. Thus, HyNLRs could form an ancient inflammasome with one of these caspases directly or indirectly *via* homotypic interaction of DEATH-like domains leading to cell death or the processing of other target proteins.

Another possibility could be a signal transduction through a kinase that activates subsequent NF- $\kappa$ B or MAP kinase signalling pathways. A group of kinase coding genes was identified in the transcriptome database of *H. vulgaris* (AEP) (3) and in the genome of *Hydra magnipapillata* (3) that have a unique protein domain composition consisting of an N-terminal DED and a DEATH domain followed by a kinase domain not orthologous to RIPK1 or RIPK2. The DEATH-kinase structure resembles the domain composition of the interleukin-1 receptor-associated kinase 1 (IRAK1), but these proteins give best BLAST hits to MAP kinases. A BLAST search without the kinase domain resulted in the apoptosis signalling adaptor protein FADD (Fas-associated *via* death domain) as best hit. The unusual domain composition of these genes was verified by partially amplifying one *H. vulgaris* (AEP) orthologue in *H. magnipapillata* cDNA (Figure 2.6B).

This protein kinase could activate the *H. magnipapillata* orthologues for NF- $\kappa$ B or JUN. The *Hydra* NF- $\kappa$ B orthologue, partially cloned from cDNA, contains a Rel homology domain necessary for DNA binding and an Ig-like domain, but seems to lack the C-terminal part of other NF- $\kappa$ B orthologues containing ankyrin repeats and a DEATH domain used for transactivation (see Figure 2.6B). An orthologue for JUN, HyJUN is present in *Hydra* as well. Remarkably, the transcripts for *HyDODE*, the *HyCARD-Caspase*, the *HyDD-Caspase* and the *HyDED-Caspase* contain the same *trans*-spliced leader as *HyNLR type 1*.

A Southern blot hybridisation performed for *HySGT1*, *HyDODE*, the *HyDD-Caspase*, the *HyDED-Caspase* and the *HyCARD-Caspase* showed that, in contrast to the *HyNLRs*, these genes are single or low copy genes (data not shown).

A prerequisite for an interaction of protein products is the co-expression of the coding genes. Since the expression for *HySGT1*, *HyDODE*, the *HyCARD-Caspase* and the *HyDED-Caspase* could not be determined *via in situ* hybridisation, the same RT-PCR on procaine treated tissue samples, mentioned in section 2.2, was performed for all candidate genes and in addition, a primer combination was used to amplify a couple of *HyNLRs* (pan *HyNLR*) (Figure 2.7A).

The *HyNLR* group, *HyDODE*, the *HyCARD-Caspase* and the *HyDD-Caspase* showed a predominant expression in the endodermal layer and a weaker expression in the ectodermal tissue, whereas *HySGT1*, *HyDED-Caspase* and the *HyDED-DD-Kinase* are equally expressed in both layers. *HyNF- $\kappa$ B* and *HyJUN* showed an endodermal expression as well, but a stronger expression level in the ectodermal tissue. The RT-PCR result for the *HyDD-Caspase* is supported by the *in situ* hybridisation, which shows the predominant endodermal expression (Figure 2.7B). Thus, all genes are co-expressed in the endodermal tissue of *Hydra* and most of them are expressed in the ectoderm as well.



**Figure 2.7: Analysis of the gene expression patterns of the putative HyNLR interactome.**

(A) Semiquantitative RT-PCR performed on RNA extracted from the ectodermal and the endodermal cell layers of *Hydra* after procaine treatment. As control genes the housekeeping gene *actin*, the ectodermally expressed nematoblast gene *nb042* and the endodermally expressed gland cell gene *HyDkk1/2/4 C* are used. The *HyDD-Caspase*, *HyDODE* and the *HyCARD-Caspase* are predominantly expressed in the endodermal layer, *HySGT1*, the *HyDED-Caspase* and the *HyDED-DD-Kinase* are equally expressed, *HyNF-κB* and *HyJUN* are predominantly expressed in the ectodermal layer. (B) *In situ* hybridisation performed for the *HyDD-Caspase* showing an endodermal expression in the whole body column. Abbreviations: ec/ecto (ectoderm), en/endo (endoderm).

The *HyNLR* group, *HyDODE*, the *HyCARD-Caspase* and the *HyDD-Caspase* showed a predominant expression in the endodermal layer and a weaker expression in the ectodermal tissue, whereas *HySGT1*, *HyDED-Caspase* and the *HyDED-DD-Kinase* are equally expressed in both layers. *HyNF-κB* and *HyJUN* showed an endodermal expression as well, but a stronger expression level in the ectodermal tissue. The RT-PCR result for the *HyDD-Caspase* is supported by the *in situ* hybridisation, which shows the predominant endodermal expression (Figure 2.7B). Thus, all genes are co-expressed in the endodermal tissue of *Hydra* and most of them are expressed in the ectoderm as well.

To investigate the inducibility of the expression of *HySGT1*, *HyDODE*, the *HyCARD-Caspase*, the *HyDD-Caspase*, the *HyDED-Caspase* and *HyCARD* upon bacteria or MAMP stimulation the mRNA obtained from the incubation experiment mentioned in section 2.2 served as RT-PCR template for these candidate genes as well. For none of the candidate genes an alteration of transcription levels was observed (data not shown).

## 2.4 The exons of HyNLRs and their putative interaction partners are in phase

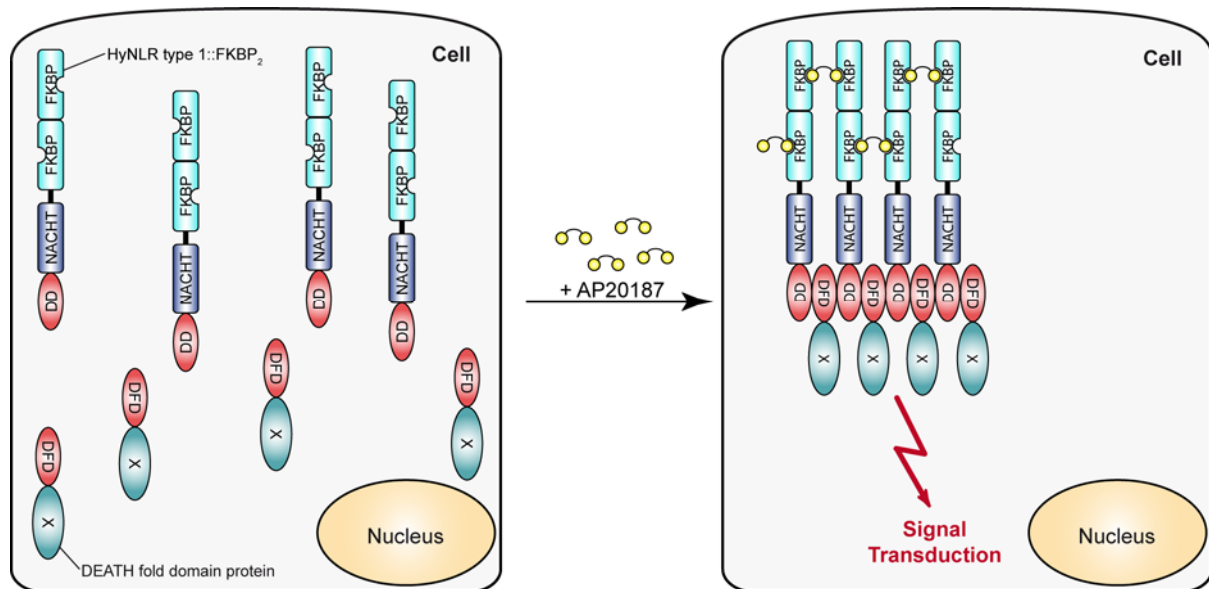
The broad variety of NLR orthologues in *Hydra* gave rise to speculations about their origins. They may have arisen from few precursors by gene duplication and exon shuffling events. Another striking observation was that many putative interacting proteins have a protein domain composition which is so far unique for *Hydra*, e.g. HyDODE, the HyDD-Caspase or the HyDED-DD-Kinase. A prerequisite for successful exon shuffling is that all exons start and end in the same phase of a base triplet and that an entire domain is encoded by one exon (Liu and Grigoriev, 2004; Liu *et al.*, 2005). By this it can be assured that the exons do not destroy the open reading frame upon rearrangement and that the whole domain can be transferred into other proteins. In most cases across the animal kingdom these exons start and end in phase 1 of the base triplet (Liu and Grigoriev, 2004). To investigate whether the HyNLRs and their interaction partners may also have been a target for exon shuffling, the exon-intron structures of two *HyNLR* representatives (*HyNLR type 1* and the *HyNLR* coding for an N-terminal DED-domain), *HyDODE* as well as the *HyDD-Caspase* were analysed. Remarkably, all exons of all investigated genes were in the same phase, i.e. phase 1, of the coding base triplet indicating that these genes may indeed have arisen by exon shuffling events (see appendix 9.4.1.1, 9.4.1.2, 9.5.4, 9.5.6).

## 2.5 Functional characterisation of HyNLR type 1 and its putative interaction partners

Numerous putative interaction partners were detected in the *H. magnipapillata* genome. They seemed to be co-expressed in both epithelial layers of *Hydra* and therefore could interact in an ancient NLR signalling cascade. To demonstrate their interactions two different approaches were performed. In an *in vitro* assay the direct interaction of HyNLR type 1 and two caspases was investigated. An unbiased *in vivo* assay was performed to analyse the effect of the activation of HyNLR type 1. For both assays the principle described in Figure 2.7 was used.

Since the endogenous ligands for HyNLRs are not known, the C-terminal part of HyNLR type 1 was replaced with two FKBP subunits that can be artificially linked by the addition of a small lipophilic ligand (AP20187). This oligomerisation should mimic the endogenous activation of the HyNLR (Figure 2.8) as it was shown for NOD2 by Ogura *et al.* (Ogura *et al.*, 2001b).

Upon oligomerisation the DEATH domains of the HyNLR type 1::FKBP<sub>2</sub> chimera should recruit the downstream signalling protein through homophilic interaction with its DEATH fold domain.



**Figure 2.8: Principle of activation of the HyNLR chimeric protein.**

The C-terminal part of the HyNLR type 1 protein was replaced with two FKBP subunits. Upon induced oligomerisation using the small lipophilic ligand AP20187 the DEATH domains of the HyNLR should recruit the downstream proteins *via* homophilic interactions with the DEATH fold domain. Abbreviations: DD (DEATH domain), DFD (DEATH fold domain), NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA), HET-E and TP1), FKBP (FK506 binding protein), X (unknown domain).

### 2.5.1 HyNLR type 1 and two *Hydra* caspases interact *in vitro*

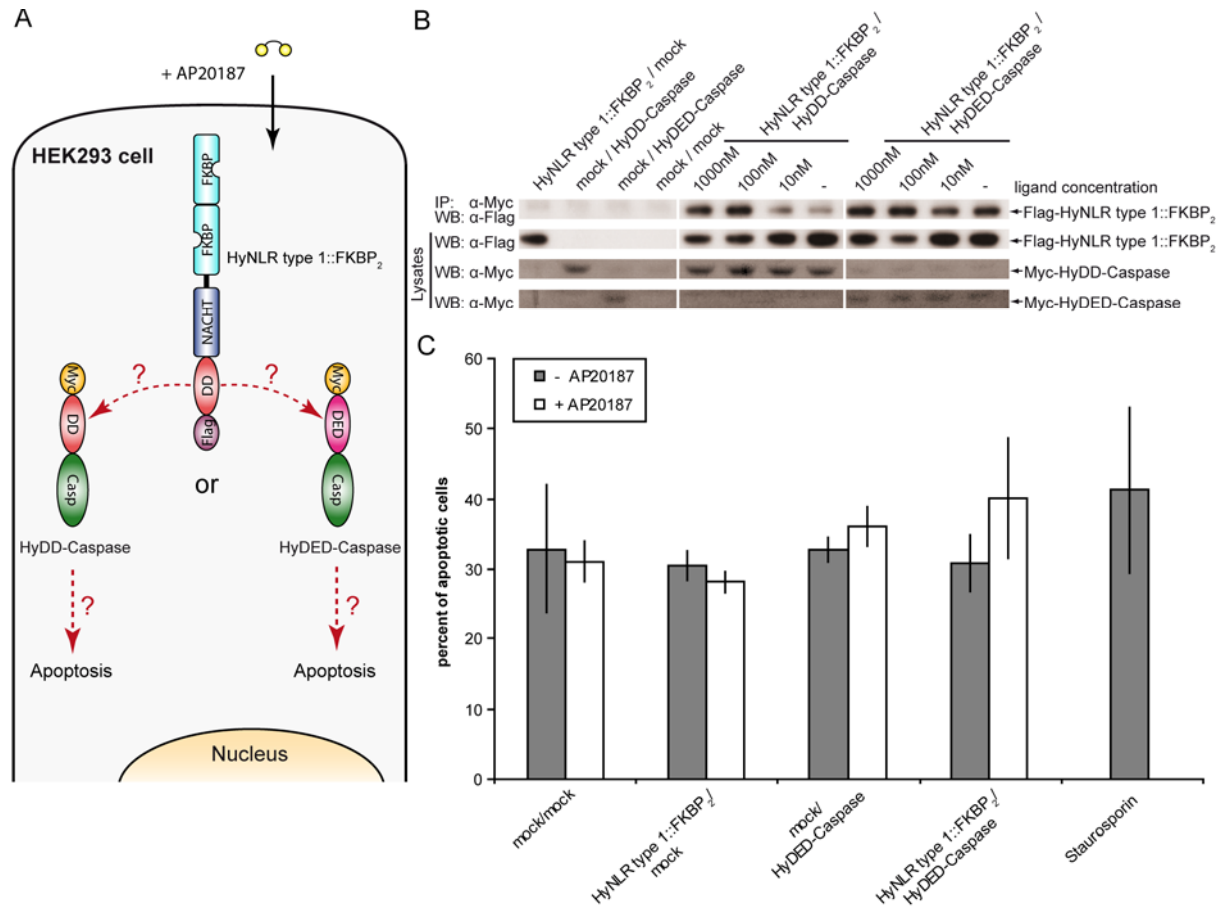
The interaction of the HyNLR type 1::FKBP<sub>2</sub> protein with the HyDD-Caspase and the HyDED-Caspase was investigated *in vitro* using HEK293 cells as heterologous expression system. The cells were co-transfected with expression-constructs for the Flag-tagged chimeric HyNLR type 1::FKBP<sub>2</sub> and the Myc-tagged HyDD-Caspase or HyDED-Caspase (Figure 2.9A, see also appendix 9.7.1).

The immunoblotting analysis showed that all transfected constructs were similarly expressed (Figure 2.9B).

Remarkably, the HyNLR type 1::FKBP<sub>2</sub> protein co-precipitated with the HyDD-Caspase upon forced oligomerisation in a ligand concentration-dependent manner. Addition of 100 nM AP20187 led to a robust co-precipitation of both overexpressed proteins, whereas in the absence of the ligand a weak interaction was detectable.

Also with the HyDED-Caspase the HyNLR type 1::FKBP<sub>2</sub> was co-precipitated. In this case the interaction was rather constitutive and not depending on the ligand concentration.

These results give first hints that the HyDD-Caspase, as well as the HyDED-Caspase, may be direct endogenous interaction partners for the HyNLR type 1 protein and may be involved in HyNLR-mediated cytoplasmic defence responses.



**Figure 2.9: Co-immunoprecipitation experiments of the HyNLR type 1::FKBP<sub>2</sub> chimera and two *Hydra* caspases expressed in HEK293 cells.**

(A) HEK293 cells were co-transfected with an expression construct coding for the Flag-tagged HyNLR type 1::FKBP<sub>2</sub> protein under the control of a CMV promoter, and either an expression construct coding for the Myc-tagged HyDD-Caspase or the Myc-tagged HyDED-Caspase in order to check their interactions and to measure apoptosis. (B) The co-immunoprecipitation experiment performed on the co-expressed HyNLR type 1::FKBP<sub>2</sub> protein and the HyDD-Caspase or the HyDED-Caspase with increasing amounts of AP20187. Line 1: Immunoprecipitation was performed using an α-Myc antibody to catch the HyDD-Caspase or the HyDED-Caspase. Western blot was performed using an α-Flag antibody to detect the co-precipitated HyNLR type 1::FKBP<sub>2</sub> fusion protein. The amount of the precipitated HyNLR type 1::FKBP<sub>2</sub> chimera increases with the ligand concentration in the case of the HyDD-Caspase, whereas the amount of the chimeric protein does not increase in the case of the expression of the HyDED-Caspase. Line 2: Expression control for the Flag-tagged HyNLR type 1::FKBP<sub>2</sub> chimeric protein in HEK293 cells. Line 3: Expression control for the Myc-tagged HyDD-Caspase. Line 4: Expression control for the Myc-tagged HyDED-Caspase. (C) Percentage of FITC Annexin V positive HEK293 cells expressing HyNLR type 1::FKBP<sub>2</sub> protein and the HyDED-Caspase measured by flow cytometry, (mean ± standard deviation;  $n = 4$ ; t test was performed for the HyNLR type 1::FKBP<sub>2</sub>/HyDED-Caspase expressing cells,  $P = 0,06$ ). Abbreviations: WB (Western blot), IP (immunoprecipitation), DED (DEATH effector domain), DD (DEATH domain), NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA), HET-E and TP1), FKBP (FK506 binding protein), Casp (Caspase domain).

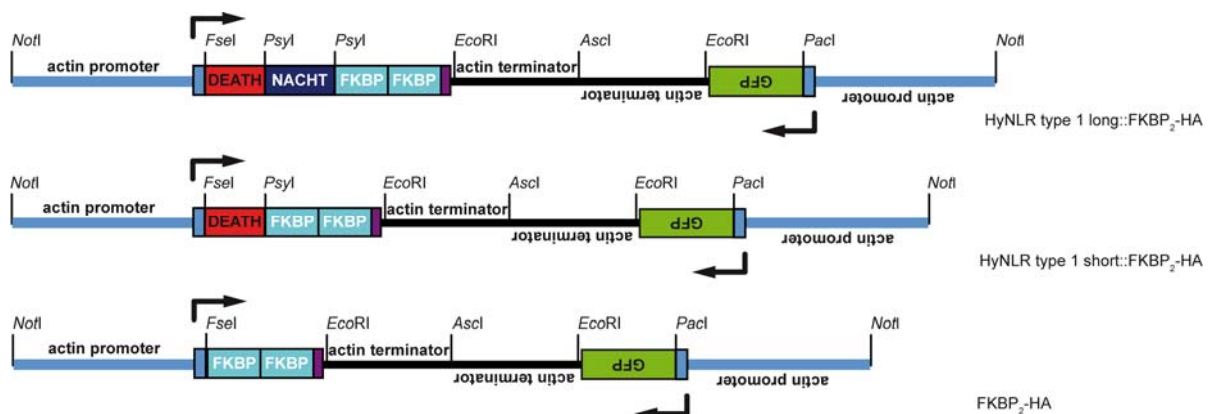
In order to analyse whether the interaction of the HyNLR type 1 chimera and one of the caspases leads to apoptosis, the HEK293 cells co-expressing these constructs were subjected to

a FITC-Annexin V staining and subsequent counting by flow cytometry after incubation with and without the ligand (Figure 2.9C). The cells co-expressing HyNLR type 1::FKBP<sub>2</sub> and the HyDD-Caspase did not undergo apoptosis upon ligand addition (data not shown). In contrast to that, the HyDED-Caspase showed the ability to induce apoptosis upon forced oligomerization of the HyNLR type 1 resulting in a similar amount of apoptotic cells like in the staurosporin-treated positive control. This was not the case in the absence of the ligand.

These results indicate that the HyNLR type 1 and the HyDED-Caspase can indeed interact *in vitro* and that the activated HyNLR on his part can activate the HyDED-Caspase to induce apoptosis. This can be seen as a first hint that apoptosis may be used as an innate immune defence mechanism in *Hydra*. To prove these results the function of the HyNLR type 1 protein needs to be characterized *in vivo*.

### 2.5.2 Characterization of HyNLR type 1 *in vivo*

To confirm the *in vitro* findings three different constructs were generated for overexpression experiments in transfected *Hydra* (Figure 2.10).



**Figure 2.10: Generation of overexpression constructs to elucidate the function of HyNLRs *in vivo*.**

Schematic drawing of the three constructs that have been generated. All constructs were double expression constructs using GFP under the expression control of the *actin* promoter and terminator in an opposite reading frame orientation. HyNLR type 1 long::FKBP<sub>2</sub>-HA contains the DEATH and NACHT domain of HyNLR type 1 fused to the two FKBP subunits with a C-terminal HA tag under the control of an *actin* promoter and terminator. The HyNLR type 1 short::FKBP<sub>2</sub>-HA construct contains the DEATH domain of HyNLR type 1 fused to the FKBP domains. A construct containing only the FKBP subunits (FKBP<sub>2</sub>-HA) was generated to serve as a negative control.

All expression constructs contained a GFP coding sequence fused to the first nucleotides of *actin* to trace the transgenic cells (see also appendix 9.7.2). In the opposite reading frame direction three different ORFs containing a codon optimized FKBP<sub>2</sub> fused to a C-terminal HA-tag were inserted, that were fused to some *actin* coding bases as well. The HyNLR type 1 long::FKBP<sub>2</sub>-HA construct contained both the DEATH and the NACHT domain of the HyNLR, whereas the HyNLR type 1 short::FKBP<sub>2</sub>-HA chimera contained only

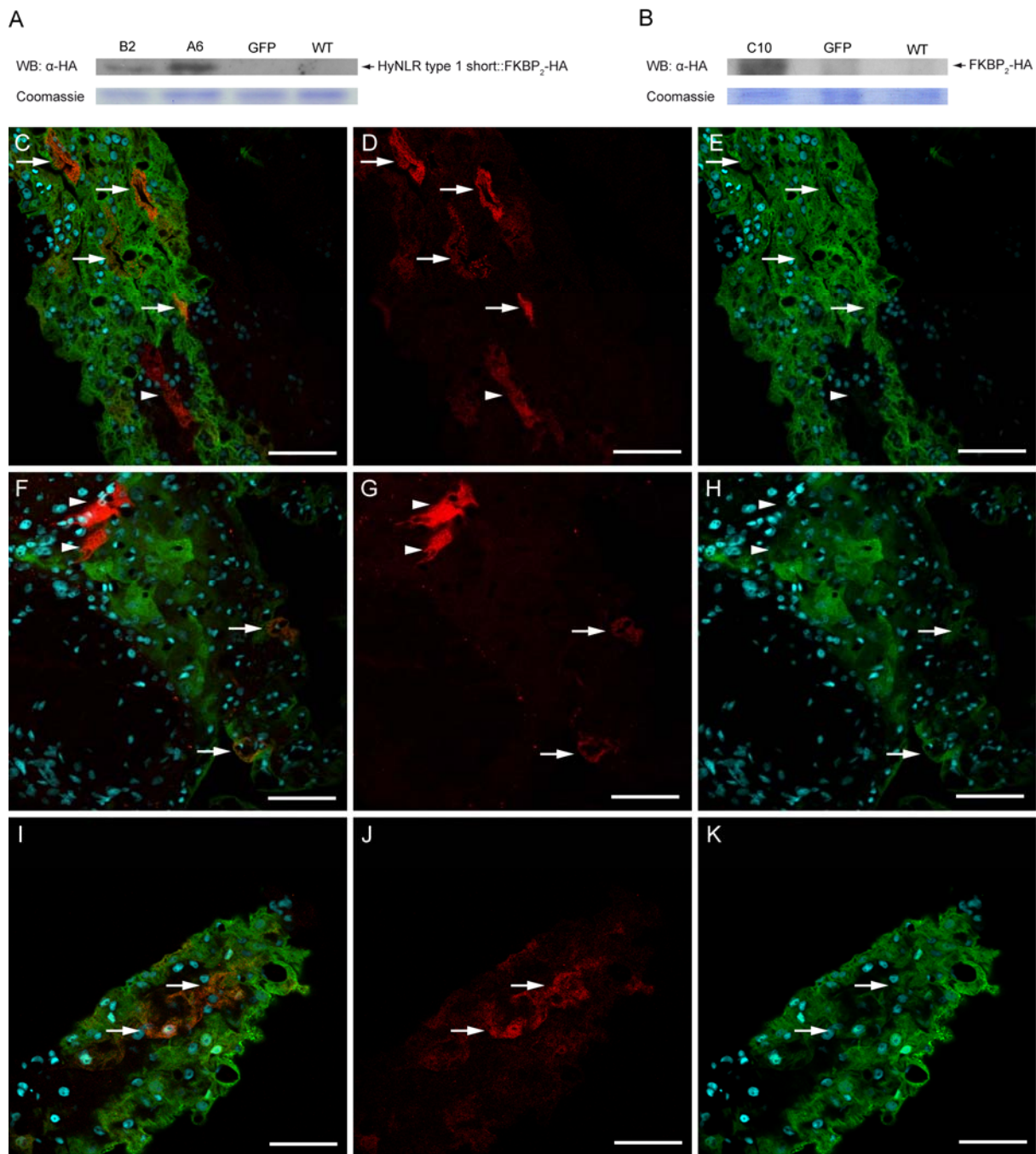


the DEATH domain in case that the long version is constitutively active due to the oligomerisation of the NACHT domains. The third construct contained no sequence parts of HyNLR type 1 and was designed as a negative control to exclude unspecific cell signalling effects due to the oligomerisation of the FKBP domains alone.

In total, six transgenic *Hydra* lines expressing GFP were obtained by microinjection of the expression constructs into developing embryos. For none of the three lines transfected with the long HyNLR construct could an expression of the HA-fused chimera be detected, neither by western blotting, nor by immunohistochemical approaches (data not shown).

The two lines A6 and B2 showed an expression of the short fusion protein of the expected size of about 48 kDa in the immunoblot (Figure 2.11A).

The line C10, being transfected with the negative control, also expressed the protein of the estimated size (26 kDa) (Figure 2.11B). The immunoblot results are supported by the immunofluorescence staining performed on all three lines (Figure 2.11C-K). Unfortunately, only a small amount of GFP positive cells additionally expressed the HA-tagged protein (arrows) in all tested lines. Some HA-positive cells did not express the GFP (arrowheads). The expression of GFP and the fusion proteins seems to be uncoupled in all tested lines and GFP cannot be used as a tracer to select for HA-positive cells. Although the expression of these fusion proteins was successful, living polyps cannot be used for apoptosis assays or other experiments because a too small amount of GFP-positive cells expresses the chimeric protein. A possible solution for further experiments might be the enrichment of the GFP and HyNLR type 1::FKBP<sub>2</sub> expressing cells *via* cell sorting for *in vitro* assays, e.g. Annexin V staining, to detect apoptosis. The animals might also be used for co-precipitation experiments to find the endogenous downstream interacting protein. For future studies new constructs should be generated, in which the expression of the chimeric HyNLR protein is directly coupled with a tracer to enrich these cells *in vivo*.



**Figure 2.11: Detection of the fusion proteins in *Hydra*.**

(A) Western blot performed to confirm the expression of HyNLR type 1 short::FKBP<sub>2</sub>-HA using an  $\alpha$ -HA antibody; the detected proteins in the tested lines B2 and A6 had the expected size of 48 kDa; (B) Western blot performed to confirm the expression of FKBP<sub>2</sub>-HA using an  $\alpha$ -HA antibody; the detected protein in the tested line C10 had the expected size of 26 kDa; in (A) and (B) the coomassie-stained gels are shown as loading control; as a negative control, GFP expressing (GFP) and wildtype animals (WT) were used. (D), (E), (F) immunofluorescence staining performed on the line B2 using an  $\alpha$ -HA antibody; (G), (H), (I) immunofluorescence staining performed on the line A6 using an  $\alpha$ -HA antibody; (J), (K), (L) immunofluorescence staining performed on the line C10 using an  $\alpha$ -HA antibody; (C), (F), (I) overlay of all three detected wavelengths, indicating that only a small amount of cells expresses the HA-tagged protein; (D), (G), (J) detection of the HA-tagged protein; (E), (H), (K) detection of the DAPI-stained nuclei and the GFP-positive cells; red ( $\alpha$ -HA); blue (DAPI); green (GFP); arrows (cells coexpressing GFP and the HA-tagged protein), arrowheads (cells expressing only the HA-tagged protein), scalebars (50  $\mu$ m).

## 2.6 Tumour bearing polyps provide an example for a disturbed microbial community

It is known from experiments conducted in mammals that pattern recognition receptors and their signalling pathways are crucially involved not only in host-commensal mutualism, but also in intestinal tissue homeostasis and tumorigenesis (Rakoff-Nahoum and Medzhitov, 2007; Rakoff-Nahoum *et al.*, 2004). In *Hydra*, a tumour bearing strain of *Hydra oligactis* showed an imbalanced homeostasis of the associated microbiota (Figure 2.12). This wildlife strain was isolated from the environment of St. Petersburg by Dr. Boris Anokhin.

In *H. oligactis*, endosymbiotic bacteria belonging to the group of  $\alpha$ -Proteobacteria have been detected in the endodermal as well as in the ectodermal epithelial cells in an equal distribution (Figure 2.12A, B) (Fraune and Bosch, 2007). In contrast to that, the fluorescence *in situ* hybridisation (FISH) assay performed on the tumour bearing polyps led to a dramatically different result (Figure 2.12C, D).

Remarkably, as shown in Figure 2.11C, the ectodermal tissue was free of endosymbiotic bacteria, whereas the endodermal layer contained large clumps of bacteria not belonging to the same species as the normal endosymbiont of *H. oligactis* (Figure 2.12C, D). The transmission electron microscopy picture shows that these bacteria reside within cells. They have a rod-like shape and a size of about 1  $\mu\text{m}$  in length. Their cytoplasm looks very heterogeneous with one to two electron-dense areas per cell of different sizes (Figure 2.12E). A 16S rDNA analysis demonstrated that these bacteria belong to the class of  $\beta$ -Proteobacteria and with a probability of 56 % to the order of Neisseriales (Figure 2.12F). This group was never detected in any other *H. oligactis* microbial community (S. Fraune, personal communication) indicating that the tumour bearing polyps have a drastic change in their microbiota.

After isolation of this strain from the environment, it spontaneously developed tumour-like structures upon cultivation under laboratory conditions for approximately one month.

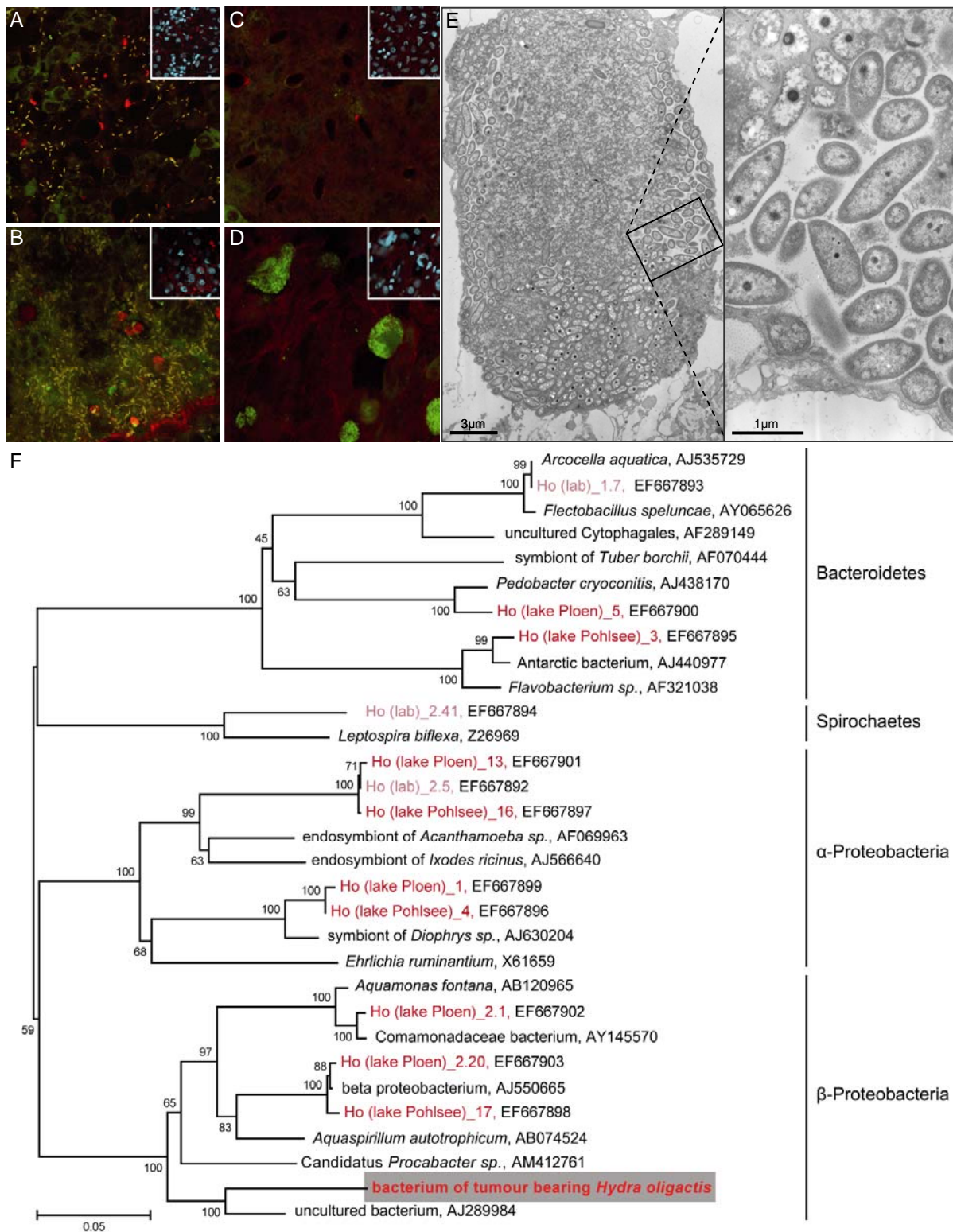
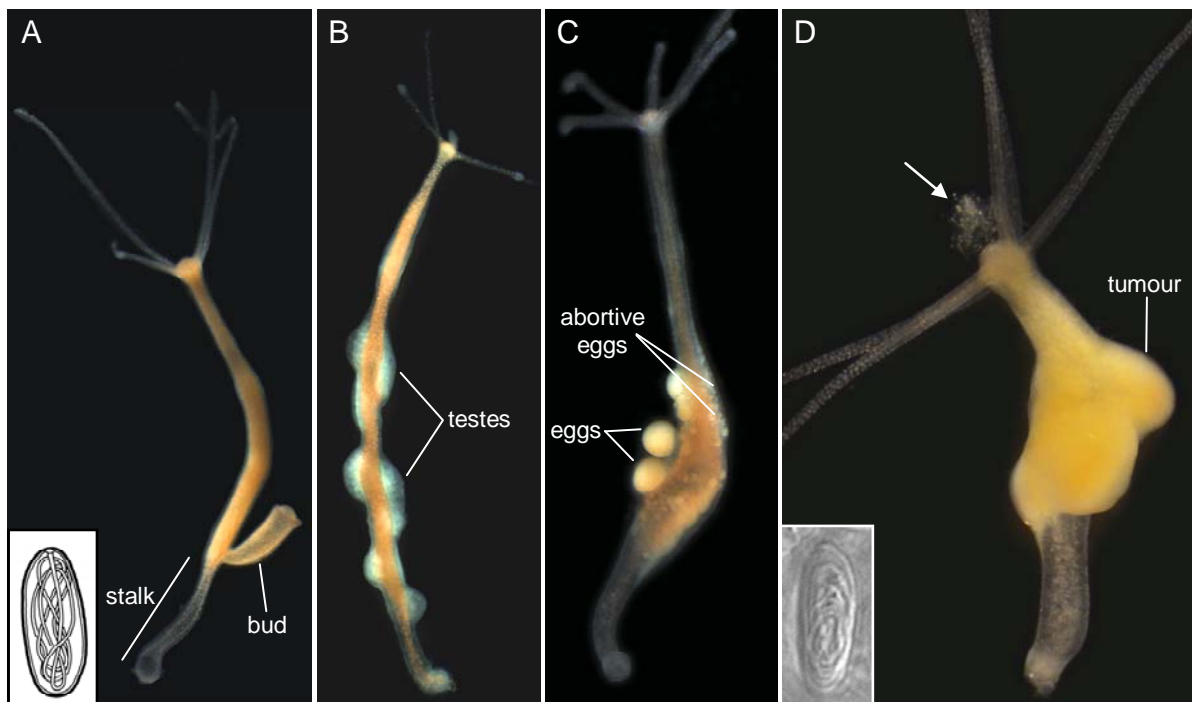


Figure 2.12: Analysis of microbiota of the tumour bearing *Hydra*. (Description: next page)

**Figure 2.12 (continued): Analysis of microbiota of the tumour bearing *Hydra*.**

(A, B) FISH performed on control *H. oligactis* ectodermal (A) and endodermal (B) tissue, showing an equal distribution of bacterial endosymbionts. (C, D) FISH performed on tumour bearing *H. oligactis* ectodermal (C) and endodermal (D) tissue showing that the endogenous endosymbiotic strain is not present and that clumps of bacteria are located within the endoderm. Green (eubacterial probe), blue (DAPI), red (phalloidin), yellow (probe specific for the endosymbiont of *H. oligactis*, results in an overlay of the red specific probe and the green eubacterial probe). (E) TEM image showing a clump of bacteria with a heterogenous cytoplasm surrounded by a membrane. (F) Phylogenetic positions (16S rDNA sequences, neighbor-joining tree) of identified bacterial phylotypes in the tumour bearing polyps and in control *H. oligactis* polyps. Bootstrap values are shown at the corresponding nodes ( $n = 1000$ ). The branch length indicator displays 0.05 substitutions per site, the gray shadowed phylotype represents the dominant clone isolated from the tumour bearing samples. Light red (phylotypes isolated from the *H. oligactis* laboratory culture), dark red (phylotypes isolated from *H. oligactis* samples from the lake Ploen or lake Pohlsee).

Figure 2.13 shows a morphological comparison of a tumour bearing polyp with a normal undifferentiated *H. oligactis* polyp, as well as with a male and a female differentiated animal.

**Figure 2.13: Different phenotypes of *Hydra oligactis*.**

(A) A sexually undifferentiated budding polyp with a small insertion of a schematic drawing of a *H. oligactis* specific holotrichous isorhiza (taken from Holstein and Emschermann, 1995). (B) A male differentiated polyp with shortened tentacles and numerous testes. (C) A female differentiated polyp producing several eggs and abortive eggs simultaneously. (D) A polyp from the tumour bearing *H. oligactis* strain releasing cells through its mouth (arrow). Insertion (holotrichous isorhiza from the tumour bearing strain indicating that this strain belongs to the species of *H. oligactis*).

Below the budding zone *H. oligactis* possesses a stalk region (Hemmrich *et al.*, 2007a). The colour of *H. oligactis* is brown to orange due to the inclusion of carotenoids. Its synapomorphic feature is a special type of holotrichous isorhiza with an unordered arrangement of the external tubes (Figure 2.13A, see also Figure 1.2) (Hemmrich *et al.*, 2007a). *H. oligactis* is strictly dioecious and this species hardly ever switches between the two sexes (Littlefield, 1991). Under laboratory conditions sexual reproduction can be artificially

induced by cultivating the animals at 10 °C for approximately two weeks (Littlefield, 1991). Gametogenesis is accompanied by shortening of the tentacles (Brien, 1965) due to the consumption of interstitial cells. This leads to the death of the animal if gametogenesis is proceeded for a long time, a phenomenon called “crise gamétogénique” (Brien and Reniers-Decoen, 1949). The male polyp contains numerous spirally arranged testes throughout the entire body column (Figure 2.13B). The female differentiated polyp contains synchronously a couple of eggs in the distal gastric region and in addition numerous “abortive eggs” (Figure 2.13C) (Brien and Reniers-Decoen, 1949). The tumour bearing polyps have an abnormal body shape with bulges of tissue in the distal gastric region (Figure 2.13D). By their unordered arrangement of the external tube within the holotrichous isorhiza they could be identified as *H. oligactis* species. The yellowish polyps hardly ever bud and for reproduction of the culture the polyps had to be cut longitudinally. Remarkably, the animals frequently release cells through their mouth (arrow).

In the following chapters these tumour bearing polyps are characterised under histological and biomolecular aspects.

### 2.6.1 Histological characterisation of the tumour bearing polyps

In order to gain a deeper understanding of the cellular composition of the tumour-like structures, the animals were cross-sectioned and subjected to light microscopy (Figure 2.14A-L). The tissue sections show that the tissue layers of the tumour bearing *Hydra* are thickened in comparison to the control tissue (Figure 2.14A-H).

The tumour-like structure consists of numerous interstitial cells (Figure 2.14C, D). Interstitial cells with two nucleoli were detected as well (Figure 2.14G, arrow), indicating a high synthesis activity of these cells. In addition, numerous rounded cells with a morphology resembling apoptotic cells were present in the ectodermal tissue layer (arrowheads).

Remarkably, these cells resemble nurse cells in shape and staining characteristics (Figure 2.14F) (see also Fröbius *et al.*, 2003).

Nurse cells are differentiated interstitial cells arrested in apoptosis, which are taken up by the developing egg during oogenesis (Honegger *et al.*, 1989). The cells lose their nucleolus and contain condensed chromatin (as shown in Figure 2.15A) (see also Technau *et al.*, 2003).

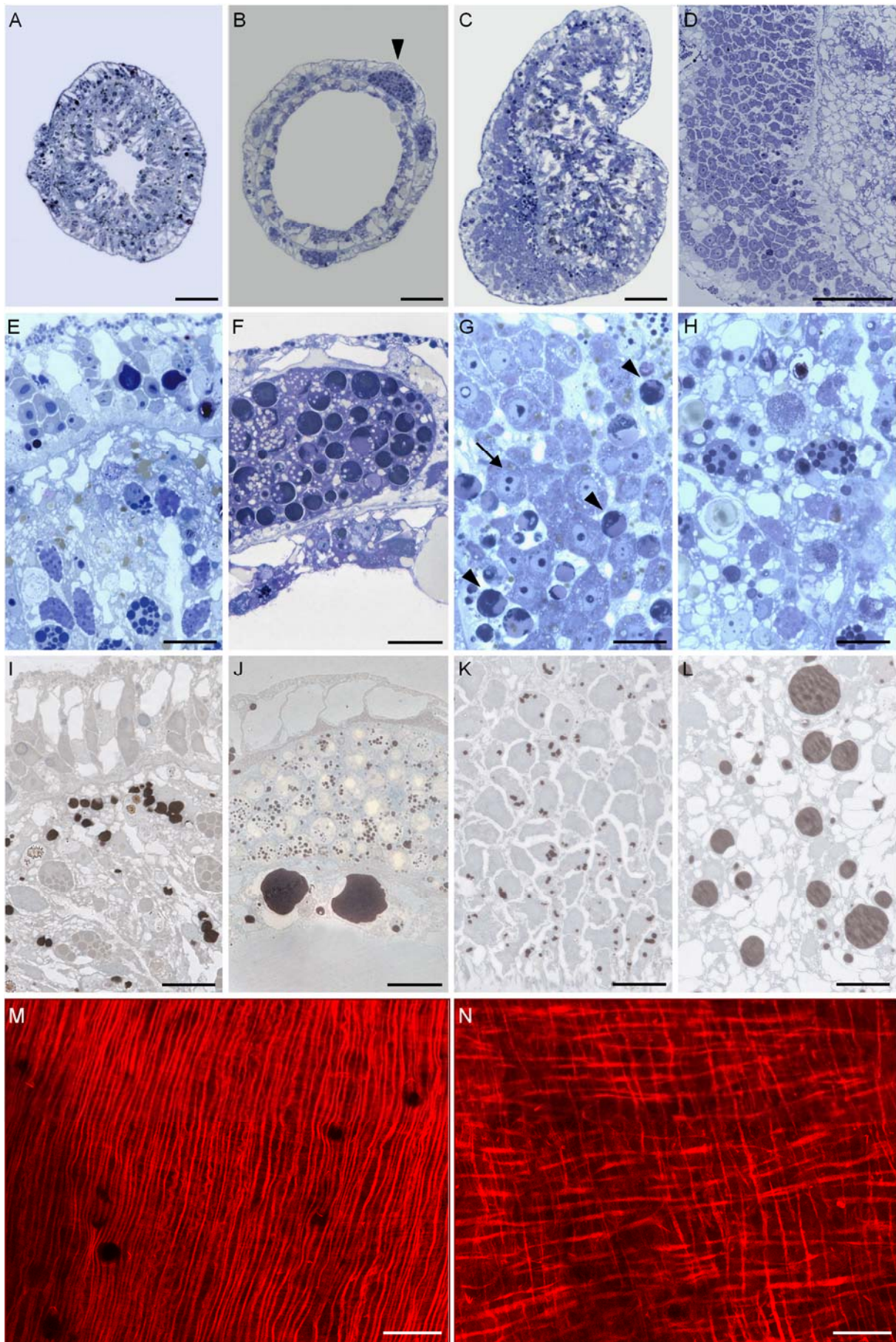
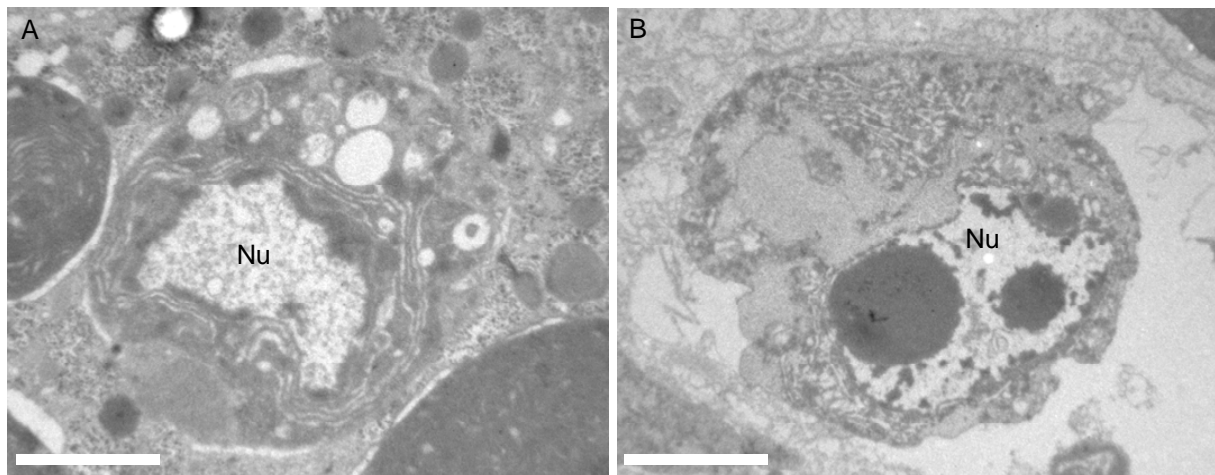


Figure 2.14: Histological characterisation of the tumour bearing polyps. (Description: next page)

**Figure 2.14 (continued): Histological characterisation of the tumour bearing polyps.**

(A-L) Cross-sections of a control polyp (A, E, I), a female polyp (B, C, D) and a tumour bearing polyp (C, D, G, H, K, L) stained with Richardson (A-H) or Sudan Black (I-L). (A) Overview of a cross-section in a control polyp. (B) Cross-section overview in a female differentiated polyp with a developing oocyte (arrowhead). (C) Cross-section overview of a tumour bearing polyp. (D) Enlargement of the cross-section showing that the tumour cells are interstitial cells. (E) Enlargement of the ectodermal and endodermal tissue of the control polyp. (F) Enlargement of the developing oocyte with ingested nurse cells in a female polyp. (G) Enlargement of the ectodermal layer of the tumour bearing polyp showing a cell with two nucleoli (arrow) and rounded cells (arrowheads). (H) Enlargement of the endodermal layer of the tumour bearing polyp. (I) Cross-section of a control polyp showing the endodermally located lipid droplets. (J) Cross-section of a developing oocyte with ingested nurse cells. (K) Cross-section of the ectodermal tissue of a tumour bearing polyp showing small lipid droplets located in the interstitial cells. (L) Cross-section of the endodermal tissue of the tumour bearing polyp showing large lipid droplets. (M) Phalloidin staining of actin filaments in a control polyp and (N) in a tumour bearing polyp indicating that the arrangement of the actin filaments is looser than in the control tissue. Scalebars: A-D (100  $\mu\text{m}$ ), E-L (20  $\mu\text{m}$ ).

In the case of the tumour bearing polyps, rounded cells containing condensed chromatin can be found in the ectodermal layer, but these cells are not phagocytosed (Figure 2.15B).

**Figure 2.15: The rounded cells of the tumour bearing polyps resemble nurse cells.**

TEM pictures of (A) a nurse cell in a female differentiated *H. oligactis* polyp. The cell is located within the developing oocyte. (B) A rounded cell of the tumour bearing polyp, that is not phagocytosed by a developing oocyte. Both cells contain nuclei with condensed chromatin. Scalebars (5  $\mu\text{m}$ ); abbreviation: Nu (nucleus).

A terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay to detect apoptotic cells within the tumour bearing polyps resulted in no dramatically induced apoptosis throughout the whole body column in comparison to the control animal, although apoptotic cells could be detected within the tumour (Figure 2.16B).

The Sudan Black staining performed on the tumour bearing polyps shows a different size and distribution of lipid droplets in comparison to the sexually undifferentiated control tissue, e.g. the unusual presence of numerous small lipid droplets in the ectodermal interstitial cells (Figure 2.14K) and the dramatically enlarged lipid droplets on the endodermal tissue layer (Figure 2.14L). This feature can also be observed within the developing oocyte (Figure



2.14J). The nurse cells contain small lipid droplets and large lipid droplets can also be found in the endoderm.



**Figure 2.16: Detection of apoptosis in a tumour bearing polyp in comparison to a control animal.**

TUNEL staining was performed in a control polyp (A) and in a tumour bearing polyp (B). There is no enlarged apoptosis detectable in the body column of the tumour bearing polyp although there are TUNEL positive cells within the tumour (box).

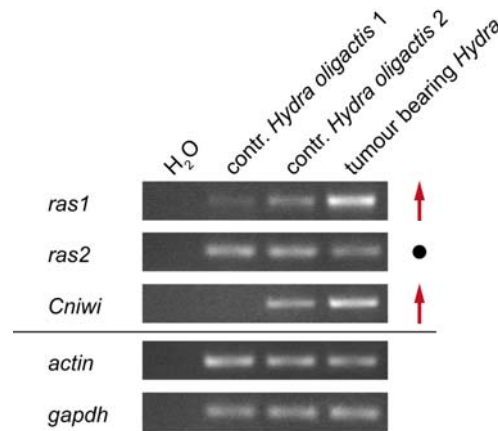
A phalloidin staining of the actin filaments shows that in comparison to the control animals the actin filaments of the tumour bearing polyps have a much looser arrangement than the filaments of the control polyps (Figure 2.14M, N). This may be a result of the large number of the interstitial cells that intercalate between the epithelial cells.

## 2.6.2 Gene expression analysis in the tumour bearing polyps

For many examples of cancerogenesis in vertebrates it is known that the oncogene KRAS, that is involved in the regulation of the cell cycle, can be dysregulated.

Two orthologues genes of the small GTPases KRAS (Der *et al.*, 1982) and RRAS (Lowe *et al.*, 1987), *ras1* and *ras2*, have been identified in a previous study in *H. magnipapillata* (Bosch *et al.*, 1995). In order to analyse a putative involvement of the RAS signalling pathway in the tumour bearing *Hydra*, the orthologues were partially amplified from *H. oligactis* and their expression levels were determined *via* RT-PCR (Figure 2.17).

The expression of the RRAS orthologue *ras2* was not altered in the tumour bearing polyps, whereas the expression level of the KRAS orthologue *ras1* was induced in comparison to the control *H. oligactis*.



**Figure 2.17: *Ras1* and *Cniwi* transcripts are upregulated within the tumour bearing polyps.**

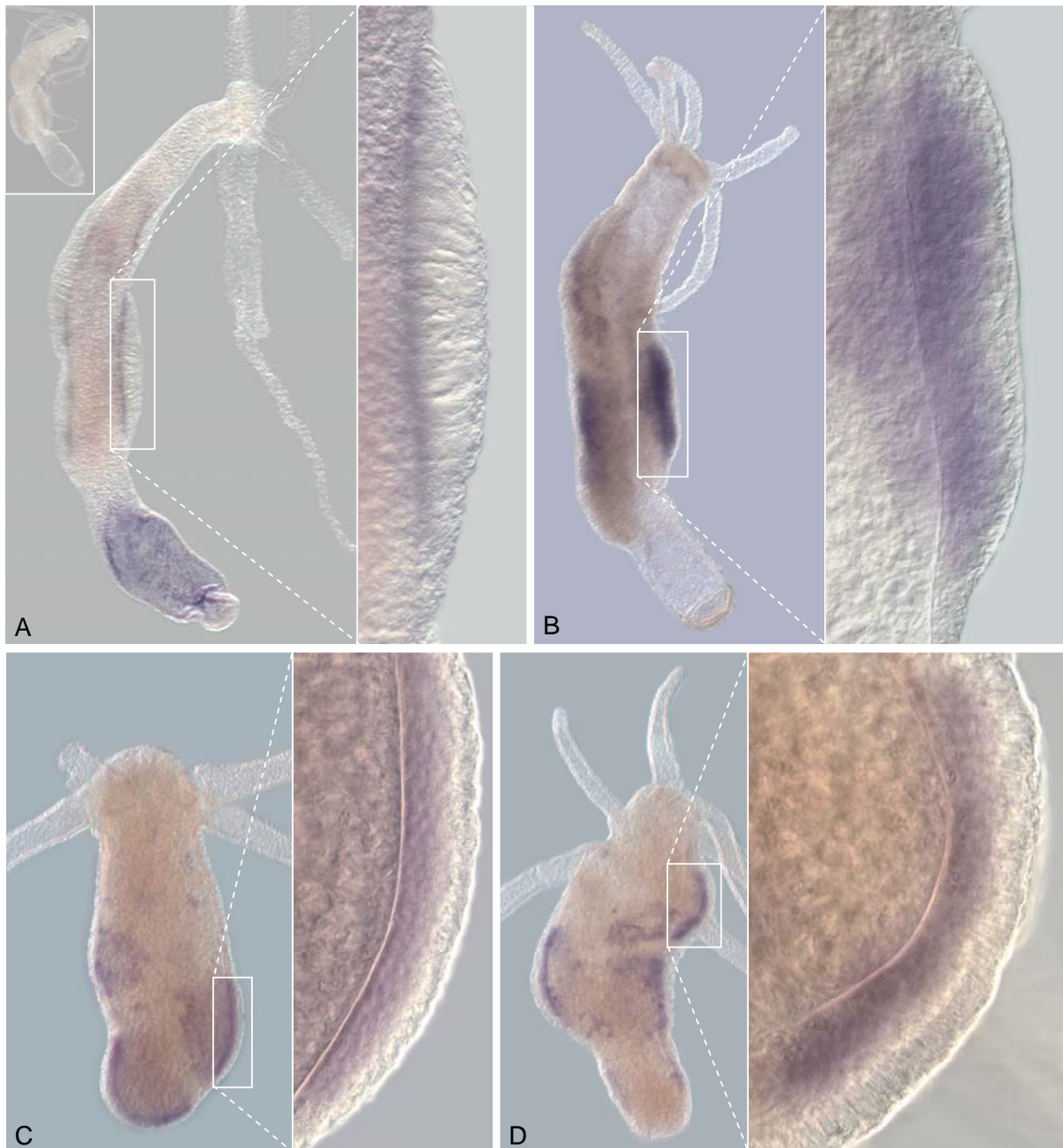
The RT-PCR was performed on mRNA isolated from tumour bearing polyps and two isolates from control polyps for the transcripts of *ras1*, *ras2* and *Cniwi*; the housekeeping genes *actin* and *gapdh* served as equilibration controls.

Since the tumour-like structures contain large amounts of undifferentiated interstitial cells and some cells that resemble the morphology of nurse cells, one could hypothesise that the interstitial cells in the tumours predominantly differentiate into gamete cells. Therefore, the expression level of *Cniwi*, a gene being primarily expressed within the interstitial cells determined to become germline cells (Puchert, 2008; Seipel *et al.*, 2004), was analysed as well. Indeed, the transcription of this gene was induced in the tumour bearing polyps.

In order to confirm these results, one of the two NANOS1 (Jaruzelska *et al.*, 2003) orthologues, *Cnnos1*, that have been identified in *H. magnipapillata* (Mochizuki *et al.*, 2000), served as marker for interstitial cells restricted to gametogenesis in an *in situ* hybridisation (Figure 2.18).

In sexually undifferentiated polyps of the species *H. magnipapillata*, this gene is expressed in few groups of interstitial cells along the body column (Mochizuki *et al.*, 2000). This was also the case for sexually undifferentiated *H. oligactis* polyps (data not shown). In male as well as in female differentiated animals, this gene is expressed on the bases of the testis (Figure 2.18A) and within the developing eggs (Figure 2.18B). In the tumour bearing polyps, the *Cnnos1* gene is expressed in large patches within the tumours, in cells that are located between the ectodermal epithelial cells and the mesogloea (Figure 2.18C, D).

The induced expression of *Cniwi* and the large tissue patches of the tumour bearing polyps expressing *Cnnos1*, indicate that the dramatically increased interstitial cells forming the tumour are indeed determined to become germline cells.

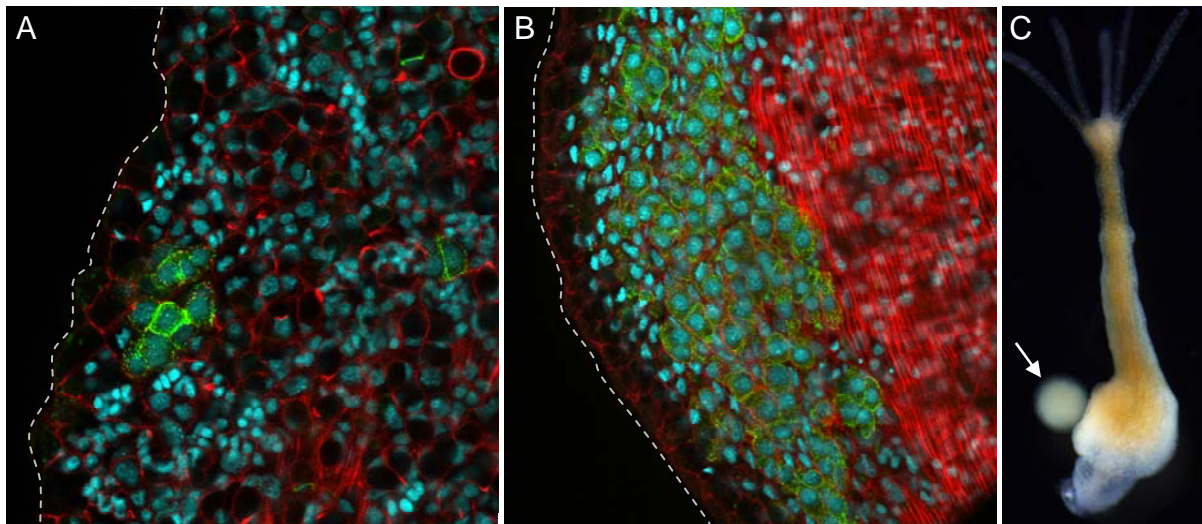


**Figure 2.18: *Cnmos1* is expressed within the interstitial cells that are located in the tumour.**

*In situ* hybridisation with *Hydra oligactis* was performed on a male differentiated polyp (A), the small insertion showing the sense control, a female differentiated polyp (B) and two tumour bearing polyps (C,D).

### 2.6.3 The female germline marker periculin 1a is expressed within the tumours

Since the interstitial cells within the tumour-like structure appeared to be cells restricted to the female germline, an immunofluorescence staining using an antiserum against the cationic part of the periculin 1a protein (Bosch *et al.*, 2009; Fraune, 2008), which is exclusively expressed within female germline cells, was performed to confirm the previous results (Figure 2.19).



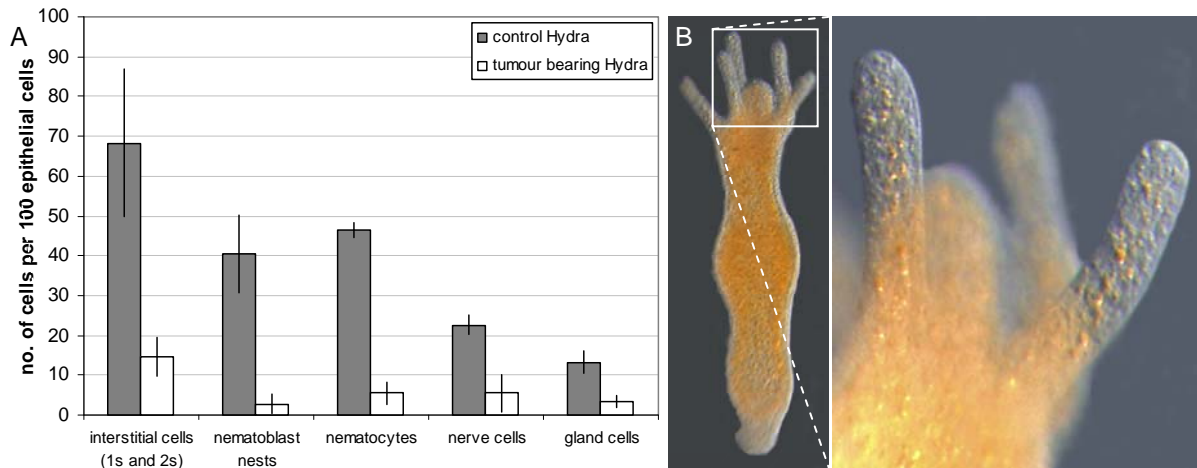
**Figure 2.19: Immunofluorescence staining of periculin 1a.**

Immunofluorescence staining using an antiserum generated against the cationic part of the periculin 1a protein with female control *H. oligactis* (A) and tumour bearing *H. oligactis* (B). Red (phalloidin), blue (DAPI), green ( $\alpha$ -periculin 1a antiserum). (C) Induction of gametogenesis in a tumour bearing *Hydra*. Arrow (egg).

In control tissue the antibody stains small nests of interstitial cells (Figure 2.19A). The periculin 1a protein is located within vesicles. In the tissue of the tumour bearing polyp, large amounts of periculin 1a-expressing cells were detected (Figure 2.19B), indicating that the interstitial cells that build up the tumour are female restricted interstitial cells. Like in the control population of *H. oligactis*, oogenesis is never performed by the tumour bearing *Hydra* under normal culturing conditions. Oogenesis can be induced in these polyps by cultivating them at 10 °C for about 14 days (Figure 2.19C). Remarkably, the differentiated polyps looked different than the female control polyps (as shown in Figure 2.19C). They do not contain abortive eggs and they produce only one egg. This may indicate a disturbance of the differentiation pathway of the interstitial stem cells or an onset of a loss of interstitial cells.

#### 2.6.4 Dying tumour bearing polyps have lost a large amount of interstitial cells

Regularly tumour bearing polyps died. That led to a limited number of animals and therefore to a limited number of experiments that could be performed. Two of these dying animals have been macerated and the amount of interstitial cells and their derivatives has been counted in relation to the number of epithelial cells in comparison to two macerated control polyps (Figure 2.20A).



**Figure 2.20: Analysis of the interstitial cell lines in dying tumour bearing polyps.**

(A) Counting of interstitial cells and their derivatives in relation to 100 epithelial cells. Two female control polyps and two dying tumour bearing polyps were macerated and cells of two slides per animal were counted. The mean value of the two animals was built. (B) A dying tumour bearing polyp showing a decreased length of tentacles and a smooth surface of the tentacles.

The numbers of interstitial stem cells and all their derivatives, including nematoblasts, nematocytes, nerve cells and gland cells, were dramatically decreased in the dying tumour bearing polyps in comparison to the control animals. This finding is additionally supported by the observation that the dying animals had shortened tentacles with a smooth appearance indicating that hardly any nematocytes were present in the tentacles of these animals (Figure 2.20B). This indicates a misbalance in the tissue homeostasis in dying tumour bearing polyps. This misbalance may be due to the enhanced differentiation of interstitial cells into germline-restricted stem cells. This may lead to a subsequent lack of multipotent stem cells giving rise to other differentiation products of the interstitial stem cell lineage with the death of the polyp as a last consequence.

## 3 DISCUSSION

### 3.1 Screening for NBD-containing proteins at the base of the animal kingdom

In the screening for NBD-containing proteins performed as part of this thesis an extraordinary large number of NACHT and NB-ARC domain containing proteins was identified at the base of animal evolution (see section 2.1).

The presence of 349 NACHT domains in the raw genome data of *Amphimedon queenslandica* that led to 61 assembled contigs was the first hint that this sponge may use a large repertoire of NLRs for immune defence (see section 2.1). A subsequent screening of the assembled genome will help to confirm these preliminary results. Little is known about the immune system of the sponge (Böhm *et al.*, 2001; Wiens *et al.*, 2005). At the molecular level, components of the TLR signalling pathway, orthologues for one TLR, MyD88, NF- $\kappa$ B and IRAK-4 have been discovered in the demosponge *Suberites domuncula* (Wiens *et al.*, 2005; Wiens *et al.*, 2007). Although these filter-feeders do not possess true tissue, the TLR orthologue is expressed at the epithelial surfaces that separate it from its external environment indicating an ancient form of epithelial defence. However, an effective defence cannot be achieved using only one TLR. It is likely that the sponge uses NLR orthologues for pattern recognition as well.

Two anthozoan cnidarian species, *A. millepora* and *N. vectensis* have one *bona fide* NLR with a DED at its N-terminus, a NACHT domain and LRRs (see section 2.1). The expression of this receptor was confirmed by PCR in *Acropora*. Unfortunately, the total number of *Acropora* NLRs cannot truly be determined due to the lack of a sequenced genome.

In the genome of *Nematostella* a total of 72 NBD-containing proteins were identified (see section 2.1). Interestingly, many genome scaffolds in *Nematostella* contain several NLR genes indicating a putative transcriptional co-regulation of these genes. Moreover, several of them contain various C-terminal repetitive domains, including ankyrin repeats, WD40 domains, LRRs and TPRs. Remarkably, 11 gene models were detected displaying structural similarities to plant R proteins by the presence of an N-terminal coiled coil domain. The appearance of this domain must be proven by analysing the coding sequence, but it could be seen as another hint for a last common ancestor of plants and animals that might have used NBD containing proteins for defence. However, these coiled coil domains could also have been acquired in an analogous manner to the plant R genes.

It seems that in *H. magnipapillata* as a representative of the Hydrozoa that branched from the Anthozoa at least 540 million years ago (Chapman *et al.*, 2010) a vast expansion of the NBD-

containing proteins occurred. 130 NACHT and 160 NB-ARC containing genes were found in the genome of *H. magnipapillata*.

Being cnidarians, the body plans of *Nematostella* and *Hydra* are relatively simple. Both are built up of two monolayered epithelia; the ectoderm and the endoderm that are separated by the mesogloea. Although migratory cells with a putative immune function have been reported for anthozoans (Ellner *et al.*, 2007; Mydlarz *et al.*, 2008), both animals have to rely on an effective epithelial defence mechanism (Bosch, 2008; Bosch *et al.*, 2009). Together with ingested prey, numerous putatively pathogenic microorganisms can be taken up and pass into the gastric cavities of the animals (Bosch *et al.*, 2009). The phagocytic endodermal epithelial cells cannot distinguish between food particles and pathogens and as a result, *Hydra* individuals could be easily infected. However, in *Hydra* it was shown that they also live in close contact to symbiotic bacteria that may also benefit to their fitness (Fraune and Bosch, 2007). In the case of *H. oligactis* these symbiotic bacteria are located inside the epithelial cells surrounded by a membrane (Fraune and Bosch, 2007). Therefore it is important to differentiate between pathogenic and non-pathogenic organisms. An immune defence reaction against extracellularly-located pathogens would be detrimental to *Hydra*, because antimicrobial peptides that are secreted into the gastric cavity will be released from the cavity through the mouth. A more effective scenario would be the direct defence against phagocytosed cytoinvasive bacteria by the release of antimicrobial peptides into the phagosome or an intracellular defence mechanism performed by the NLRs. This could be a possible explanation for the high number of intracellular NLRs in *Nematostella* and in *Hydra* compared to a low number of membrane-spanning TLRs (one in *Nematostella* and two truncated versions in *Hydra*) (Bosch *et al.*, 2009; Hemmrich *et al.*, 2007b; Miller *et al.*, 2007). It is highly likely that the TLRs and the NLRs in these organisms function together in a complex network leading to apoptosis or the production of AMPs to control the microbial community that surrounds *Hydra* and *Nematostella*.

Finding classical NLRs in anthozoan cnidarians directly contradicts the assumption that NLRs evolved at the level of the teleost fishes (Ting and Davis, 2005). This led to a reinvestigation of their apparent absence from ecdysozoans.

It was reported that the ecdysozoans *D. melanogaster* and *C. elegans* do not contain any NLRs, only the APAF1 orthologues DARK and CED-4 are present (Rodriguez *et al.*, 1999; Yuan and Horvitz, 1990). It was shown by Miller, Tenchau and colleagues that these species are rapidly evolving and divergent from other animal species (Miller *et al.*, 2005; Miller *et al.*, 2007; Technau *et al.*, 2005). Several gene losses were reported for this group. Nevertheless in the genome of the arthropod *D. pulex* four NBD containing genes were identified, with two of them having orthologs in dipteran insects consisting of a NACHT domain connected with

LRRs. In contrast to previous statements (Ting and Davis, 2005), NLR-resembling proteins are present in arthropods, but they are very limited in number.

Therefore it is likely that arthropods rely on other proteins for the recognition of pathogens. In addition to a relatively small number of peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) that can activate the Toll or the immune deficiency (IMD) pathway in *Drosophila* (Lemaitre and Hoffmann, 2007; Pal and Wu, 2009), arthropods use another highly variable system for pattern recognition. They can express thousands of isoforms of the immunoglobulin (Ig)-superfamily receptor Down syndrome cell adhesion molecule (Dscam) that are generated through a mechanism of alternative splicing (Watson *et al.*, 2005).

In the sea urchin *S. purpuratus* as well as in the chordate *B. floridae*, a vast expansion of NLRs and TLRs has occurred (Hibino *et al.*, 2006; Huang *et al.*, 2008; Rast *et al.*, 2006). In contrast, the urochordate *C. intestinalis* does not seem to have any NLRs as only single NACHT domains were detectable (Sodergren *et al.*, 2006). In the jawless vertebrate *P. marinus* no NLRs were detected by the performed screening (see section 2.1). Interestingly, this species has in addition only two TLR orthologs (Ishii *et al.*, 2007). However, the lamprey uses a highly variable receptor system; the variable lymphocyte receptors (VLRs) composed of highly diverse leucine-rich repeats (LRR) that are somatically rearranged for the specific recognition of pathogens expressed by migratory immune cells (Pancer *et al.*, 2004).

In order to validate the relatively small amount of NBD containing proteins in jawed vertebrates the analysis was extended with a screening of a teleost fish species, *O. latipes*, an amphibian, *X. tropicalis*, and *G. gallus* as a representative of birds. Although all jawed vertebrates use the somatic recombination of Ig-domain coding gene families within migratory immune cells to generate highly specific antibodies directed against thousands of epitopes (Cooper and Alder, 2006), the total numbers of NBD containing genes vary greatly within this group. In general, the gene family seems to be expanded within the teleost fish, with more than 200 representatives in the zebrafish *D. rerio* (Stein *et al.*, 2007), but the number of NBD coding genes of land living vertebrates is relatively low with 17 orthologs in the frog, 7 genes in the chicken, 35 representatives in the mouse (Cecconi *et al.*, 1998; Kanneganti *et al.*, 2007) and 23 in man (Schreiber *et al.*, 2005; Zou *et al.*, 1997).

The phylogenetic analysis shows that in *Hydra* as well as in many other species throughout the entire animal kingdom like in *A. queenslandica* and *S. purpuratus*, and partially in *N. vectensis*, *B. floridae*, *O. latipes*, *X. tropicalis* and also *H. sapiens* species-specific NLR gene expansions occurred. The same was reported for two additional bony fish species: *D. rerio*



and *T. rubripes* (Stein *et al.*, 2007). With regard to a possible function in immunity, this may indicate species-specific adaptations in a variety of ecological niches.

Each organism must defend itself effectively against putative pathogens that persist in its external environment. A prerequisite for an effective defence is a highly specific recognition of pathogens by the use of a highly variable repertoire of receptor proteins. It is very likely that different defence strategies have evolved dependent on an organism's adaptation to its habitat and corresponding to its phenotype. In addition to species-specific recognition strategies, conserved pathways such as the TLR signalling pathway including MAP kinase and NF- $\kappa$ B signalling are highly conserved throughout animal evolution (Hemmerich *et al.*, 2007b; Hibino *et al.*, 2006; Miller *et al.*, 2007; Wiens *et al.*, 2007).

A good example for a well-adapted immune answer can be found in plants. They are sessile but have a very high self-regeneration capacity. They use a large repertoire of intracellular R proteins for the specific recognition of pathogens by their avirulence (avr) factors (Chisholm *et al.*, 2006; Lukasik and Takken, 2009; Mansfield, 2009). These R proteins share structural similarities with the NLRs of the animal kingdom (Głowacki *et al.*, 2010; van der Biezen and Jones, 1998). They contain an N-terminal coiled coil or a TIR domain, a central NB-ARC domain and C-terminal LRRs. An even higher specificity is achieved through alternative splicing and a very fast evolution of these R genes (Bergelson *et al.*, 2001; Dinesh-Kumar and Baker, 2000). The tissue that is affected by pathogen attack dies by a mechanism called hypersensitive response (HR) to prevent a spreading of the pathogen (Ma and Berkowitz, 2007). Before this study it was assumed that plants and vertebrates have invented the tripartite NBD-containing receptors independently because no NLRs have been discovered in *D. melanogaster* and *C. elegans* (Ting and Davis, 2005), but the current thesis gives the first evidence that a scenario is very likely where the last common ancestor of plants and animals already had at least the domains that are necessary to built up this kind of receptors. Indeed, NACHT and NB-ARC domains are already present in some eubacterial species, where they perform other functions (Koonin and Aravind, 2000; Leipe *et al.*, 2004). However, these domains might have been acquired through horizontal gene transfer. In addition, the NBD domains can be connected to repetitive domains like TPRs or WD40 repeats or to TIR domains. In contrast, it seems that the 6-helical DEATH fold which the DEATH, the DED, the CARD and the Pyrin domain have in common is unique to the animal kingdom (see also Aravind *et al.*, 1999). It is already present in the sponge (Müller *et al.*, 2001) and it was proposed that it evolved from ankyrin repeats (Boldin *et al.*, 1995).

One can conclude that the numbers of NBD-coding genes of various species does not follow any rule, but that each organism needs an effective and highly specific immune response against invading pathogens. NLRs as well as TLRs are two ancient immune receptor families

and each organism has developed its own pathogen recognition strategy to be best adapted to its ecological niche and its physiological predispositions.

### 3.1.1 *Hydra* uses a complex repertoire of NBD-coding genes

In the genome of *H. magnipapillata* 130 NACHT and 160 NB-ARC-containing genes have been identified (see section 2.1). Even on the nucleotide level the NBD coding genes of *Hydra* are very similar to each other making it difficult to work specifically on one particular representative gene. As supported by phylogenetic analysis, it is very likely that this variety was achieved by gene duplication events and exon shuffling. The *Hydra* genome contains a high number of transposable elements (Chapman *et al.*, 2010). In this screening many NLR like genes were found in close proximity to transposases (data not shown). A prerequisite for a successful exon shuffling is the location of domains on single exons and that all exons are in the same phase of the coding base triplets so that the open reading frame is not destroyed as a result of shuffling (Liu and Grigoriev, 2004; Liu *et al.*, 2005). In the gene *HyNLR type 1* and another screened representative of *HyNLRs* this is indeed the case (see section 2.4 and appendix 9.4.1.1, 9.4.1.2, 9.5.4, 9.5.6). All exons are phase 1 exons, so the domain compositions can be changed easily. Transcripts of other *HyNLRs* were amplified containing the second exon two times or containing NACHT domain coding exons sharing a 100 % identity at the nucleotide level, but having completely different effector domain coding exons (data not shown). In addition, the gene *HyNLR type 1* having one distinct representative in the genome of *H. magnipapillata* appears to be a small gene family of 4 slightly differently expressed genes in the transcriptome of *H. vulgaris* (AEP), a closely related *Hydra* species (data not shown). This can be seen as another hint for the rapid evolution of the *HyNLR* genes. These observations lead to the conclusion that in the case of *HyNLRs* gene duplications and exon shuffling events seem to be used as a relatively fast and effective mechanism for genes that need to evolve fast due to their putative role in immune defence. Although many of these genes were partially amplified from cDNA proofing that they are indeed transcribed, some of them may also represent pseudogenes. Interestingly, the phylogenetic analysis showed that *Hydra* NACHT domains are located within a cluster of exclusively vertebrate NACHT domains (see Figure 2.4). This may indicate a similar role in immunity and makes *Hydra* a good model organism to study NLR function, e.g. the function of human NLRPs.

In contrast to the NLR orthologues detected in *Acropora* and *Nematostella*, the surprisingly large portion of NACHT domain coding genes in *Hydra* lack C-terminal LRRs (see Figure 2.2). Three different scenarios could be possible: (i) there are NLRs present in the *Hydra* genome that contain LRRs, but due to the relatively short average genome scaffold lengths of 63.4 kb in the CA assembly and 92.5 kb in the RP assembly (Chapman *et al.*, 2010), it was not possible to detect the LRRs. Therefore the presence of LRRs in *HyNLRs* cannot be

excluded, but if there were NACHT domains connected to LRRs it would be very unlikely that never a NACHT domain and an LRR are located at the same scaffold accidentally; (ii) there may be a *Hydra*-specific domain for the detection of a ligand present at the C-terminal part of the HyNLRs, this is supported by the finding that the C-terminal part of the HyNLR type 1 did not have any homologous sequences in other organisms, this uncharacterized part may represent a taxonomically restricted ligand detection motif. It would be useful to clarify the tertiary structure of this part of HyNLR type 1; potentially one could find a repetitive folding element similar to e.g. the LRRs of Toll-like receptors (Bell *et al.*, 2003). In addition, one could test the ligand recognition ability of the C-terminal part by generating fusion proteins with the N-terminal part of e.g. the human NOD2 protein in a HEK293 cell-based assay. An activation of the chimera would lead to an activation of NF- $\kappa$ B that could be detected using e.g. luciferase assays; (iii) the HyNLRs might need a second protein encoding LRRs as a co-receptor. In a recent publication it was shown that the *Hydra* Toll like receptor resembling protein (HyTRR)-1 lacking the external LRRs of *bona fide* TLRs interacts with HyLRR-2 in HEK293 cells upon stimulation with flagellin (Bosch *et al.*, 2009). This could also be the case for HyNLRs and might be supported by the finding of 26 ESTs encoding LRRs that are not connected to other domains.

Besides an orthologue for APAF1, a large family of proteins with a tripartite domain composition composed of an N-terminal DEATH domain, a NB-ARC domain and C-terminal TPRs has been discovered in *Hydra* (see section 2.1, Figure 2.2). Predicted proteins of similar domain compositions have so far only been reported for *B. floridae* and *N. vectensis* without any proposed function (Zmasek *et al.*, 2007). As mentioned before, in bacteria and fungi proteins with a NB-ARC domain connected to TPRs are present as well (Leipe *et al.*, 2004). And the amplified transcripts from *H. magnipapillata* give best BLAST hits to bacterial proteins. But these transcripts could be amplified from *H. magnipapillata* cDNA, they show an exon-intron structure on the genome and contain the *trans*-spliced leader B sequence (data not shown). Therefore, a contamination from bacteria or fungi is excluded. RT-PCR performed on mRNA isolated from ectodermal and endodermal tissue samples showed that at least a group of these genes is exclusively expressed within the ectodermal layer of *Hydra*. The presence of an NB-ARC domain is a first hint for a possible function of these proteins in immunity or cell death in *Hydra*. To support this idea, e.g. knockout or overexpression constructs similar to the HyNLR type 1::FKBP<sub>2</sub> fusion protein that can be artificially activated may be used to generate transgenic *Hydra* in future studies.

### 3.2 HyNLR type 1, an ancient NOD-like receptor that could build up an ancient inflammasome

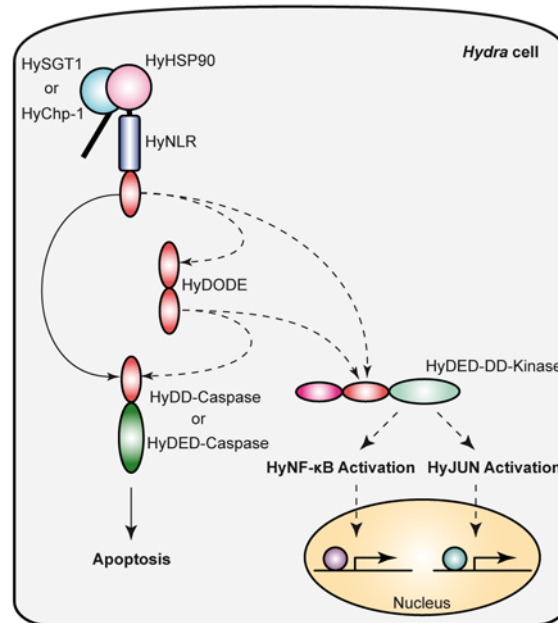
One representative of the discovered *Hydra* NOD-like receptor gene family, termed *HyNLR type 1*, was analysed in detail (see section 2.2). The gene coding for a C-terminal DEATH domain followed by a NACHT domain and a long part without any domain predictions is predominantly expressed in the endodermal epithelium and shows a weak expression in the ectoderm as well (see Figure 2.5). The endoderm is surrounding the gastric cavity of *Hydra*, the place where the digestion of food is performed. The endodermal epithelial cells that phagocyte pre-digested food particles come into close contact to co-ingested pathogens. This makes the effective protection of the endoderm of great importance as mentioned before.

Not only one *HyNLR type 1* transcript is generated, but 3 additional variants result from alternative splicing (see Figure 2.5). With a specific antibody targeted against the DEATH domain of HyNLR type 1 one could detect the resulting shorter proteins *via* immunoblotting to check if the alternatively spliced transcripts are indeed translated to regulate NLR signalling in *Hydra*. Remarkably, this putative immune-modulatory function of splicing might be conserved throughout evolution, as a short splice isoform of the *NOD2* transcript is found in *H. sapiens* that negatively regulates NOD2 signalling (Rosenstiel *et al.*, 2006). Also in plants, alternative splicing plays an important role in R protein signalling (Dinesh-Kumar and Baker, 2000; Ferrier-Cana *et al.*, 2005; Gassmann, 2008).

The transcription of *HyNLR type 1* can be weakly induced by the injection of flagellin and LPS into the gastric cavity of *Hydra* (see Figure 2.5). This induction of gene expression dependent on MAMP treatment can be seen as a hint for the involvement of this gene in immunity. A reason for this relatively weak induction could be the fact that this receptor must be constitutively expressed. A strong expression regulation is not very likely in contrast to the expression of putative effector genes that might be up-regulated upon pathogen recognition. On the other hand, *Hydra* is continuously surrounded by bacteria and their cell components. This could lead to a constitutive induction of the gene involved in immunity. The development of a stable gnotobiotic culture could lead to more drastic changes of gene expression levels upon stimulation with MAMPs.

#### 3.2.1 Characterisation of the HyNLR interactome

An *in silico* screen for potential interaction partners of *Hydra* NLRs resulted in several interesting candidate genes leading to the model shown in Figure 3.1 (see also section 2.3).



**Figure 3.1: A model for the HyNLR signalling cascade.**

Upon activation the HyNLR might recruit the HyDD-Caspase or the HyDED-Caspase resulting in apoptosis or the HyDED-DD-Kinase resulting in the activation of transcription factors like HyNF- $\kappa$ B or HyJUN. This interaction might be performed directly or *via* the adaptor protein HyDODE. Arrows: supported by experimental evidence; dashed arrows: possible interaction.

Three orthologs for HSP90 and one for SGT1 were identified in the *Hydra* genome. The HSP90-SGT1 complex is conserved in plants, fungi and vertebrates and one task is keeping the NBD containing receptors in a functional state (da Silva Correia *et al.*, 2007; Mayor *et al.*, 2007). It is known that the SGT1 protein binds the NLRs in their LRR region (da Silva Correia *et al.*, 2007). Since *Hydra* NLRs do not contain LRRs, co-immunoprecipitation experiments could lead to the discovery of an alternative binding site. An orthologue for the co-chaperone Chp-1 is also present in the *Hydra* genome. Chp-1 is orthologous to the plant co-chaperone RAR1 that is implicated in R protein-mediated disease resistance (Hahn, 2005). In humans the Chp-1 protein is known to form a complex with HSP90 and NOD1 (Hahn, 2005; Wu *et al.*, 2005). The function of this co-chaperone in innate immune signalling seems to be highly conserved. The detection of these proteins also in *Hydra* strongly supports the idea of an ancient phylogenetic origin of the NLR signalling.

Interestingly, the HyNLR type 1 protein shows the highest similarity with proteins of the NLRP family that form the inflammasome in *H. sapiens*. Several components of an ancient inflammasome are present in the *Hydra* genome. An adaptor protein with two DEATH domains was identified that could function in analogy to the ASC protein that mediates the binding of caspase 1 to the human inflammasome complex (Chen *et al.*, 2009; Srinivasula *et al.*, 2002). Activation of this caspase leads to the cleavage of the cytokine precursor proIL1 $\beta$  with the subsequent release of IL1 $\beta$  (Cerretti *et al.*, 1992). Three caspases coding for an N-terminal DEATH like fold are present in the *Hydra* genome. In contrast to previous studies

there is a caspase with an N-terminal CARD domain present in *Hydra*. This caspase may interact with the *Hydra* orthologue for APAF1 and form the apoptosome. Two additional caspases, one coding for an N-terminal DEATH domain, the other coding for a DED domain, that were originally identified by Böttger and Alexandrova in *Hydra vulgaris* (Böttger and Alexandrova, 2007) are present in the *Hydra magnipapillata* genome as well. Interestingly, the HyDD-Caspase appears to be specific for the genus *Hydra*. One or both of these caspases could form an ancient inflammasome with HyNLRs that may activate cleavage of a substrate protein or induce apoptosis.

Moreover, all exons of HyDODE and the HyDD-Caspase genes code for entire domains and are in phase 1 which is a hint for exon shuffling events to obtain these *Hydra* specific genes as discussed before (see appendix 9.5.4, 9.5.6).

Upon activation the human NOD1 or NOD2 proteins form the NODosome complex with the RIPK2 to induce NF- $\kappa$ B, JUN or p38 signalling (Chen *et al.*, 2009; Inohara *et al.*, 1999; Ogura *et al.*, 2001b). No direct orthologue for RIPK2 was detected in the *Hydra* genome, but a new *Hydra*-specific kinase containing all domains necessary for this signal transduction, the HyDED-DD-Kinase. This might function in analogy to RIPK2 and activate NF- $\kappa$ B or JUN signalling in *Hydra*.

NF- $\kappa$ B proteins are a family of Rel-homology domain containing proteins that serve as transcription factors in a variety of signal transduction cascades (Vallabhapurapu and Karin, 2009). In humans it consists of 5 family members: RelA; c-Rel; RelB; NF- $\kappa$ B1 (p50 and its precursor p105); and NF- $\kappa$ B2 (p52 and its precursor p100). The Rel proteins code for the Rel homology domain, an Ig-like domain and a transcription activation domain (TAD), whereas p100 and p105 do not code for a transcription activation domain, but instead for ankyrin repeats followed by a DEATH domain. These proteins need the dimerisation with Rel proteins to activate transcription. Upon activation of these proteins, the ankyrin repeats are cleaved from the protein resulting in the active protein that can translocate to the nucleus (Vallabhapurapu and Karin, 2009).

Like the NF- $\kappa$ B orthologue from *Nematostella* (Sullivan *et al.*, 2007; Sullivan *et al.*, 2009), the HyNF- $\kappa$ B that is comprised of the Rel homology domain and an Ig-like domain seems to lack the C-terminal ankyrin repeats as well as the DEATH domain and homology searches result in the p100/p105 group as best hit as well.

In contrast to this, a NF- $\kappa$ B orthologue was detected in the phylogenetically older demosponge *A. queenslandica* coding for all domains found in p100 and p105 (Gauthier and Degnan, 2008), suggesting that this form is more ancient and that the cnidarian NF- $\kappa$ B orthologues are secondarily shortened. Since p100 or p105 cannot activate the transcription

alone, they need a Rel protein containing a transcription activation domain to form a heterodimer. If it does not form a heterodimer, it might serve as a repressor of transcription. On the other hand, the protein may also contain a not yet identified TAD domain like as in the case for the Rel proteins and activate transcription without any co-activator. Future sequence analysis will help to elucidate the protein domain structure.

Furthermore, it may need a protein like I $\kappa$ B containing ankyrin repeats to repress its autoactive state. An I $\kappa$ B-like protein is present in *Nematostella* (Sullivan *et al.*, 2007), but was so far not identified in *Hydra*. An important task for future work will be to screen for additional Rel-homology domain containing proteins coding for a transcription activation domain and for I $\kappa$ B orthologues to elucidate the function of this transcription factor in *Hydra*. In addition, it will be of great interest to find target genes of HyNF- $\kappa$ B and to perform HyNF- $\kappa$ B activation assays using reporter gene constructs.

All investigated putative interaction partners of HyNLRs are co-expressed within both epithelia of *Hydra*, most of them predominantly in the endodermal layer, so a pre-requisite for an interaction is fulfilled (see Figure 2.7). As mentioned above, the endoderm surrounds the gastric cavity and is exposed to ingested material putatively containing pathogenic microorganisms. The ectodermal layer must also be protected as it is permanently exposed to the surrounding environment. The ectodermal epithelial cells secrete the glycocalyx surrounding the entire animal whose function might be to build up a physical barrier against pathogens. This glycocalyx may also contain antimicrobial active components and serve as a chemical barrier as well (R. Augustin, pers. communication). In contrast, the endodermal epithelium is not protected by an extracellular layer and may therefore require an increased level of protection leading to an increased expression level of the investigated transcripts.

It seems that the DEATH and DED domains are the folds of the NLR orthologues at the base of animal evolution. This must also be carried on by their interaction partners: HyDODE is comprised of two DEATH domains instead of ASC with its Pyrin and CARD domain. The HyDD-Caspase and the HyDED-Caspase contain DEATH and DED domains instead of the CARD-domain of the human caspase 1 and the HyDED-DD-Kinase contains a DED and a DEATH domain instead of the CARD domain in the human RIPK2. It seems that in higher vertebrates the DEATH domains were replaced by CARD domains (Hibino *et al.*, 2006). The first CARD domain in a NLR is present in one NLR orthologue in *S. purpuratus*, but also here the majority of the NLR gene models contain DEATH domains (Hibino *et al.*, 2006). As no Pyrin domains were detected neither in *Hydra* nor in any other invertebrate species by the performed screenings, these results support the view that the Pyrin domain is vertebrate-specific (Rosenstiel *et al.*, 2008).

One can conclude that the general mechanisms, such as the activation of a caspase or a kinase by an NLR and the usage of adaptor proteins as well as alternative splicing in NLRs are conserved throughout evolution, whereas the domain compositions and sequences of the proteins that are involved in the signalling cascade are not conserved and are at least in the case of the HyDD-Caspase and the HyDED-DD-Kinase unique to *Hydra* and therefore taxonomically restricted.

### **3.2.2 *Trans*-spliced leader additions indicate a putative translational co-regulation of the HyNLR interactome**

Remarkably, the transcripts for *HyDODE*, the *HyCARD-Caspase*, the *HyDD-Caspase* and the *HyDED-Caspase* contain the same *trans*-spliced leader as the HyNLRs. The *Hydra*-specific *trans*-spliced leader B is added to all these transcripts (Stover and Steele, 2001). It is supposed that spliced leaders (SL) are added to at least one third of all *Hydra* genes (Chapman *et al.*, 2010). During spliced leader addition the original 5'UTR is replaced by a short RNA leader sequence commonly transcribed from the spaces in between the 5S rRNA gene clusters (Drouin and de Sá, 1995) using splice donor and acceptor sites of the leader sequence and the transcribed gene (Hastings, 2005; Van der Ploeg, 1986). SL *trans*-splicing was observed in various animal groups including trypanosomes, dinoflagellates, cnidarians, rotifers, nematodes, flatworms and urochordates, but not in fungi, plants and vertebrates (Derelle *et al.*, 2010; Lall *et al.*, 2004; Matsumoto *et al.*, 2010; Van der Ploeg, 1986; Wallace *et al.*, 2010). In *Hydra* 10 SL sequences were identified (Chapman *et al.* 2010). A common ancient origin of SL addition with independent losses in many lineages and a repeated evolution of the mechanism are a current subject of discussion (Douris *et al.*, 2010; Hastings, 2005).

The function of SL *trans*-splicing is not fully understood. It was shown to be involved in translational regulation in trypanosomes by providing the 5' cap structure (Lee and Van der Ploeg, 1997). In flatworms it provides the start codon for the initiation of translation (Cheng *et al.*, 2006), it can improve translational efficiency (Lall *et al.*, 2004; Maroney *et al.*, 1995; Zeiner *et al.*, 2003) or it can help to resolve multicistronic transcripts into capped monocistronic mRNAs (Blumenthal, 2005; Marlétaz *et al.*, 2008; Satou *et al.*, 2006). In *H. magnipapillata* 32 putative operons were detected by genome screenings indicating that these SL additions might play a role in resolving polycistronic mRNAs to functional mRNA subunits (Chapman *et al.*, 2010). It is tempting to speculate that putative immune relevant genes might be transcribed simultaneously within one operon and that the SL-B is added to all these transcripts for a translational coregulation to ensure that all pathway members are expressed simultaneously within the same cell. Until now the operon structure of these genes



cannot be supported, because no genome scaffold with clustered NLR signalling genes could be identified in the *Hydra* genome. In contrast to that, in *N. vectensis* some NBD-coding genes were indeed located on the same scaffolds which could be a hint at transcriptional co-regulation. However, in contrast to *Hydra*, no *trans*-spliced leader sequences are present in *Nematostella* (Chapman *et al.*, 2010).

### 3.3 Programmed cell death as an ancient immune answer?

*Hydra* does not possess migratory immune cells and has an enormous self-renewing capacity (Bosch, 2008; Bosch, 2007). A mechanism could be conceived in which pathogen-infected cells could undergo apoptosis and not be ingested by neighbouring cells, because this might lead to the spreading of the pathogen, and simply be released into the gastric cavity and to the environment. The damaged tissue could easily regenerate.

To prove this hypothesis it is important to find a *Hydra* pathogen that can be used in incubation experiments. It was observed that *Hydra* can release rounded cells through the mouth aperture and first incubation experiments with the spores of the oomycete *Saprolegnia sp.* have shown that *Hydra* can lose large amounts of ectodermal and endodermal cells upon spore attack (R. Augustin, pers. communication).

The experiments conducted in this thesis in a HEK293 cell-based assay have shown that HyNLR type 1 is able to recruit two *Hydra* caspases; the *Hydra* specific HyDD-Caspase and the HyDED-Caspase as an ortholog of caspase 8 *in vitro*. This recruitment leads to elevated apoptosis of HEK293 cells with the HyDED-Caspase. The interaction of these proteins is most probably performed *via* the homo- or heterotypic interactions of the DEATH fold domains of the caspases and the NLR. An interaction of a DEATH domain with a DED domain is unusual, but cannot be excluded, because both domains belong to the same fold family. In addition, these domains are only the result of a prediction and future work must be conducted to elucidate the true domain foldings and their binding capacities, using e.g. nuclear magnetic resonance (NMR) spectroscopy or surface plasmon resonance (SPR) technology.

These results can be seen as first hints that apoptosis could be indeed an ancient form of immune defence in *Hydra*. Further experiments need to be performed to prove this hypothesis. The interaction of the proteins must be supported independently by assays such as yeast two hybrid screens or co-localization experiments. The induction of apoptosis could be studied in addition by PARP-cleavage detection assays or DNA laddering experiments. Of course, *in vivo* studies need to be performed. Transgenic *Hydra* expressing HyNLR type 1::FKBP<sub>2</sub> fusion constructs should have been used for apoptosis assays. Upon

addition of the ligand AP20187 the green glowing GFP-expressing cells should undergo apoptosis and disappear. With the transgenic *Hydra* lines that were created in this thesis it was not possible to perform reliable apoptosis assays, because the amount of GFP and HyNLR type 1::FKBP<sub>2</sub> co-expressing cells was too low. New constructs must be generated where the successful expression of the HyNLR can be directly tagged visually in living animals. For the construction of the present expression vectors the NLR was voluntarily not linked to a fluorescent protein to inhibit its self-assembly. However, with these constructs co-immunoprecipitation experiments can be conducted *via* the HA-tag of the HyNLR fusion protein to identify the endogenous interaction partner. Subsequent assays to detect caspase activity could be performed in future work.

Nevertheless, it is important to strictly regulate the putative apoptotic immune reaction in *Hydra* because it is not feasible to induce apoptosis only upon stimulation with a MAMP. *Hydra* is permanently surrounded by bacteria that are not harmful to it and apoptosis can only be permitted if a real danger threatens the animal. NLRs are good candidates for this activation of apoptosis because they are located intracellularly and might induce the host reaction to pathogens only if the pathogen is cytoinvasive similar to the function of the *R* genes in plants. A requirement for simultaneous activation of several HyNLRs to induce downstream signalling can not be excluded as well.

The programmed death of an infected cell is known to play a role in an effective pathogen defence in many examples. Plants, sessile organisms without migrating immune cells, use a mechanism of programmed cell death, the HR, leading to apoptosis at the site of infection to limit the spread of pathogens within the whole plant (Ma and Berkowitz, 2007). HR is accompanied by the generation of ROS and NO. It is induced by gene-for gene interactions of the plant *R* gene with its pathogen encoded avirulence (*avr*) gene and provides host resistance to the pathogen (Rafiqi *et al.*, 2009). But not only in plants generation of ROS is an important protective mechanism. Recent studies using human cell lines showed an increased ROS generation upon NOD2 stimulation (Lipinski *et al.*, 2009). Furthermore, ROS appear to be involved in the activation of the NALP3 inflammasome *via* the thioredoxin-interacting protein (TXNIP) (Zhou *et al.*, 2010).

In mammals, cytotoxic T-cells recognize virus-infected cells by the presentation of a foreign antigen and induce their apoptotic program by the release of the pro-apoptotic cytotoxic proteins granzyme and granulysin that activate apoptosis by the cleavage of the procaspase 3 and BH3 interacting domain death agonist (BID) (Lieberman, 2003).

In invertebrates, caspases are involved in host immune defence as well. In *D. melanogaster* it was shown that loss-of-function mutants of *dredd*, the ortholog of caspase 8, are highly susceptible to Gram-negative bacteria and are defective in the production of the antimicrobial

peptide drosomycin (Kuranaga and Miura, 2007). Furthermore the caspase inhibitor protein DIAP2 (*Drosophila* inhibitor of apoptosis 2) has been reported to be required for the IMD pathway regulating host defence against Gram-negative bacteria (Leulier *et al.*, 2006).

In *C. elegans*, an infection of the intestine with *Salmonella typhimurium* causes an increase in the gonadal cell death. A loss-of-function mutant of the caspase ced-3 with suppressed cell death activation is highly susceptible of being killed by *Salmonella* infections (Aballay and Ausubel, 2001).

In the demosponge *Suberites domuncula* the expression of a caspase is highly inducible by the addition of the synthetic triacyl lipopeptide Pam(3)Cys-Ser-(Lys)(4), a ligand for the mammalian TLR1 and TLR2 (Wiens *et al.*, 2007).

In mammals, the inflammasome-dependent activation of caspase 1, primarily leading to the modification of endogenous signalling molecules, may also result in the induction of pyroptosis, a kind of programmed cell death that is accompanied by the release of the pro-inflammatory cytokines IL1 $\beta$ , IL18 and IL33 to recruit immune cells (Bortoluci and Medzhitov, 2010).

Autophagy can also be involved in the control of infections by directing intracellular or ingested pathogens to lysosomes leading to their destruction and can also lead to apoptotic death of the cell as a consequence (Bortoluci and Medzhitov, 2010).

Therefore programmed cell death seems to be a conserved mechanism of innate immune defence.

### 3.3.1 A putative immune modulatory function of HyNLRs

The HyDD-Caspase that can also interact with the HyNLR type 1 did not show the ability to induce apoptosis *in vitro* (see Figure 2.9). This could have many reasons. Firstly, this caspase is *Hydra* specific and therefore its substrate proteins might not be present in human cell lines. The replacement of the caspase domain of the HyDD-Caspase and the HyDED-Caspase with a human caspase domain could help to compare their activation. Secondly, if the activation of the caspase may not lead to apoptosis in *Hydra* the caspase might have another immune modulatory role. The human caspase 1 e.g. that is activated by the inflammasome converts pro-inflammatory cytokines into their active released forms (Schroder and Tschopp, 2010). It is known that caspases in vertebrates and invertebrates have many roles in developmental processes, such as cell proliferation, cell migration, cell differentiation and cell shaping depending on the cleavage of different substrate proteins (Kuranaga and Miura, 2007).

Caspases are also reported to modulate immune responses, like caspase 12 that has an inhibitory role in the antimicrobial response by interacting with RICK (LeBlanc *et al.*, 2008).

Furthermore, caspase 1 is able to cleave the TLR adaptor Mal and can interfere with TLR signalling (Ulrichs *et al.*, 2010). In a recent study it was shown that mice deficient for NLRP3 or ASC and caspase 1 were highly susceptible to dextran sodium sulphate (DSS) induced colitis. This was accompanied by a loss of epithelial integrity and a commensal overgrowth and bacteremia (Zaki *et al.*, 2010). Thus, the activation of caspase 1 is involved in the maintenance of intestinal homeostasis and protection against ulcerative colitis. As discussed above invertebrate caspases also have immune regulatory roles.

The HyDD-Caspase might also be involved in the cleavage of substrate proteins that can directly mediate defence against pathogens, such as proteins that interfere with immune signalling or even effector molecules. A genetic knockout of the two caspases in *Hydra* can help to elucidate their roles in immunity.

If modulations of an immune answer rather than apoptosis are the result of the activation of HyNLR type 1, the transgenic polyps expressing the HyNLR type 1::FKBP<sub>2</sub> fusion protein could also have been used for minimum inhibitory concentration (MIC) assays in order to analyse the activation of antimicrobial peptides as putative effector genes of HyNLR signalling. This could have been supported by comparative gene expression analyses such as qRT-PCR or transcriptome comparisons in order to discover putative target genes of HyNLR signalling that could either be conducted using the caspases or the HyDED-DD-Kinase. Furthermore, a comparative 16S rRNA gene analysis of the bacterial community might help to elucidate the role of NLR signalling in *Hydra*. As the amount of cells expressing the fusion protein is too low for a reliable conduction of these experiments, new transgenic lines must be established in the future.

### 3.4 The tumour bearing polyps have an altered microbial community

Pathogens cannot only cause apoptosis, but they can also induce tumour formation and cancer (Grivennikov *et al.*, 2010; Hussain and Harris, 2007; Rakoff-Nahoum, 2006). In the tissue of the tumour bearing *Hydra* large clumps of intracellularly-located bacteria belonging to the order of Neisseriales have been detected within the endodermal layer (see section 2.6). This group cannot be detected in undifferentiated control polyps of the same species. Representatives of this group, such as *Neisseria gonorrhoeae* and *Neisseria meningitidis*, cause the human diseases gonorrhoea and meningitis (Virji, 2009). This may be a hint that the bacteria of the tumour bearing *Hydra* may also have the potential to infect an animal species.

Remarkably, a changed microbial community with a shift to *Vibrio spp.* has also been discovered in the tumour harbouring tissue of the coral *Porites compressa* (Breitbart *et al.*, 2005).

It is unclear whether the bacteria of the tumour bearing polyps are the cause for or a secondary effect of the tumour. Due to the low number of animals curing experiments with antibiotics could not be performed. If this bacterial strain is cultivatable, inoculation experiments with healthy polyps could be performed to check whether they can induce tumour growth in *Hydra*.

An example of a bacterial pathogen that can cause cancer in the human stomach is *Helicobacter pylori*. It causes long-term infections of the gastric mucosa accompanied with chronic inflammation that can lead to the ulceration of the stomach wall and to gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Grivennikov *et al.*, 2010; Hussain and Harris, 2007). It may be possible that the bacteria of the investigated *Hydra* strain also cause the tumour formation.

In general, chronic inflammation as a result of infection with viruses or bacteria, because of inherited diseases such as Crohn's disease or ulcerative colitis, or as a result of exposure to chemicals such as asbestos can be linked to tumorigenesis by providing a pro-tumorigenic microenvironment delivering reactive oxygen species (ROS) or reactive nitrogen intermediates (RNI) being capable of inducing DNA damage and genomic instability and delivering cytokines that may promote tumour growth (Hussain and Harris, 2007). Furthermore, epigenetic changes as a result of inflammation can favour tumour initiation (Grivennikov *et al.*, 2010; Hussain and Harris, 2007).

The production of ROS to inhibit pathogen growth is an ancient mechanism in immune defence. In addition to vertebrate immunity it was described in plant defence against insects (Liu *et al.*, 2010; Moloi and van der Westhuizen, 2006) in *C. elegans* (Chávez *et al.* 2009) and insect immunity (Molina-Cruz *et al.*, 2008). Until now it is not known, whether *Hydra* produces ROS upon immune stimulation. Answering this question could form an interesting part of future work in *Hydra* immunity.

It was shown by Fraune *et al.* that the amount of interstitial cells in the tissue of *Hydra* has a direct effect on the composition of its microbial community (Fraune *et al.*, 2009). A loss of the interstitial cell lineage and all its derivatives in the *H. magnipapillata* strain sf1 resulted in a shift from the dominating  $\beta$ -Proteobacteria phylotype to a new dominating phylotype of the Bacteroidetes group. If a loss of a cell lineage can change the microbial community than an excess of this cell lineage may change the microbial composition as well. The tissue homeostasis in the tumour bearing polyps is altered as well and because the tumour is made up of many interstitial cells these may cause the change in the microflora by their expressed gene products, e.g. antimicrobial peptides. A good candidate gene for shaping the microbial community expressed by the interstitial cells is *periculin 1a*. The expression of *periculin 1* is inducible upon several stress stimuli, e.g. monosodium urate, LPS, flagellin or dsRNA (Bosch

*et al.*, 2009) and the protein is located within vesicles. It is built up of an anionic and a cationic part and it shows antimicrobial activity. The expression of *periculin 1a* is restricted to the female germ cells. The tumour consists of female germ cells that express the periculin 1a protein (see Figure 2.19). Since there is enlarged periculin 1a expression this may cause the change of the microbial composition.

### 3.5 The tissue homeostasis is disturbed in the tumour bearing polyps

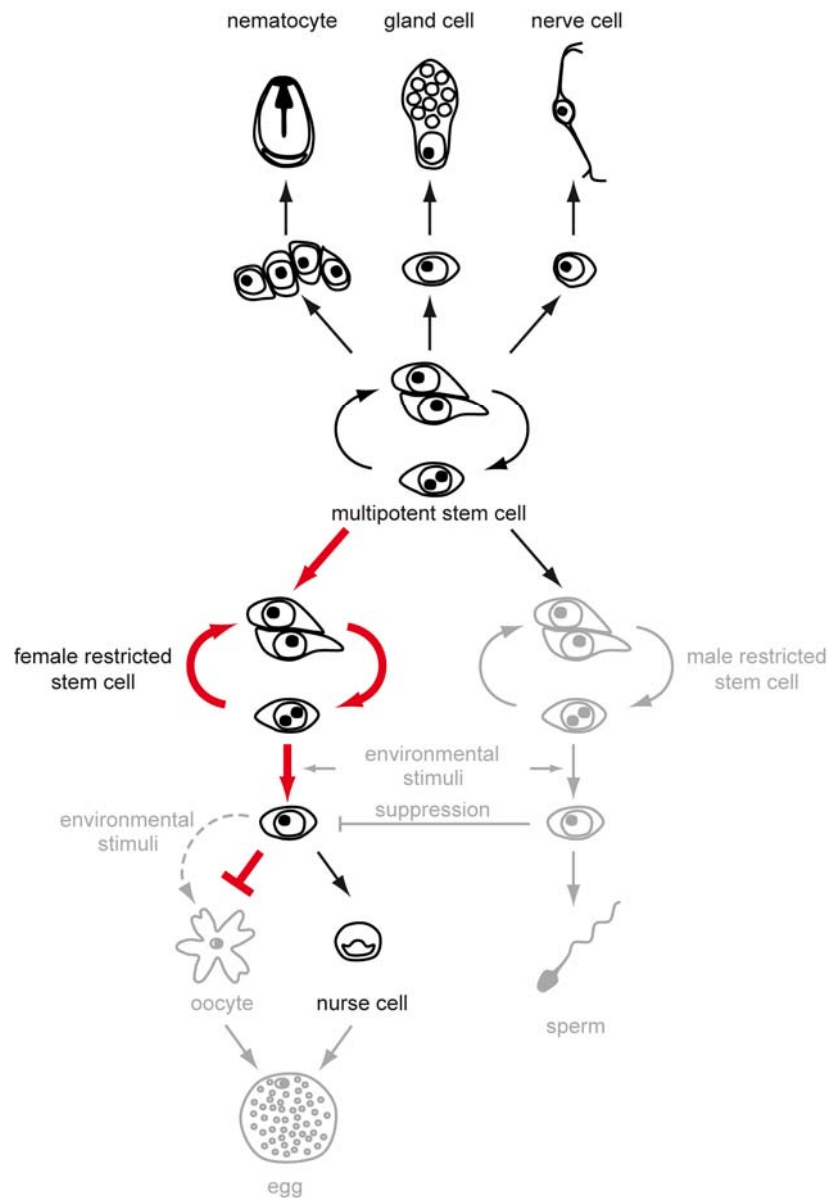
Tumour formation and cancer, the formation of malign tumours or neoplasia, are a tremendous problem in the health care system. Statistically, one third of European population develops cancer (ECCO, 2009). And it is a cause of death for many people. Therefore cancer research is a main focus in the medical research worldwide (Christofori, 2006; Grivennikov *et al.*, 2010; Stratton *et al.*, 2009). Mouse models are used to study tumourigenesis but only few cases of tumours in various invertebrate phyla including annelids, arthropods, molluscs and ascidians resulting from hereditary or environmental factors including pathogen attack have been described (Scharrer and Lochhead, 1950).

Uncontrolled growth of hemocytes as a classical neoplasia, also called “clam leukemia”, has been discovered in a variety of bivalve molluscs (Barber, 2004; Delaporte *et al.*, 2008; Farley, 1969a; Farley, 1969b). Few cases of tumour growth have been reported for decapod crustaceans (Vogt, 2008). Planarians and *Drosophila* have been used as invertebrate model organisms to study tumour formation (Jang *et al.*, 2007; Oviedo and Beane, 2009; Rodahl *et al.*, 2009). Examples for tumour formation have been detected in many coral species from more than ten families (Breitbart *et al.*, 2005; Peters *et al.*, 1986; Squires, 1965; Yamashiro *et al.*, 2000). In the case of corals abnormal growth that lead to enlarged skeletal elements are referred to as tumours (Breitbart *et al.*, 2005).

In this study the first case of a tumour within a hydrozoan species is described. The tumour is made up of numerous interstitial cells including cells that resemble nurse cells. The cells express *Cnnos1*, a marker for germline cells in *Hydra* (Mochizuki *et al.*, 2000), and *periculin 1a* (Fraune, 2008), which validates the assumption that these cells are restricted to the female germ line. Figure 3.2 shows a model that can be used to understand the abnormal proliferation of the interstitial stem cells giving rise to the tumour tissue.

It was shown by Littlefield, Bosch and David that *Hydra* possesses three interstitial stem cell lines that have the capacity to self-renew (Bosch and David, 1987; Littlefield, 1991); the multipotent stem cells that give rise to all derivatives of the interstitial cell lineage, nematocytes, gland cells, nerve cells and gametes. The male- and female-restricted stem cell lines have the ability to self-renew and give rise to spermatozoa, oocytes and nurse cells.

During oogenesis the interstitial cells start to proliferate and give rise to a large group of cells (Honegger *et al.*, 1989). One cell that is located in the middle of this group differentiates into the oocyte and starts to phagocytose the surrounding interstitial cells that differentiate into nurse cells. Nurse cell differentiation is indicated by the appearance of lipid vesicles in interstitial cells. *H. oligactis* is strictly dioecious and gametogenesis can be induced by environmental stimuli, such as temperature decrease (Littlefield, 1991).



**Figure 3.2: Model of the interstitial cell lineage in the tumour bearing *Hydra*.**

The multipotent stem cell lineage in *Hydra* has a self-renewing capacity and gives rise to its derivatives nematocytes, gland cells, nerve cells and germline cells. *H. oligactis* is strictly dioecious and either male or female gametes are produced upon induction *via* environmental stimuli. There are sub-populations of gametogenesis restricted stem cell lines that also have the possibility to self-renew. In the case of the tumour bearing polyps, there is an over-proliferation of the female restricted stem cell lines with a blocking of the terminal differentiation of the oocyte, but sometimes nurse cells appear that are not phagocytosed by the oocyte. (Modified after Fraune, 2008; Littlefield, 1991).

The tumour bearing polyps show an over-proliferation of the female restricted germline cells without the terminal differentiation into the oocyte. Nurse cell resembling cells occur, which are not phagocytosed by a developing oocyte. In histological sections one can clearly see the lipid droplets that are stored by the interstitial cells indicating the onset of the differentiation into nurse cells as shown in Figure 2.13J. Many rounded cells can be seen within the tumour, but only few apoptotic cells can be found, which fits to the observation that TUNEL positive nurse cells are only found when the new polyp hatches (Technau *et al.*, 2003). The general opinion in the literature is that nurse cells start to differentiate after the oocyte is selected (Honegger *et al.*, 1989; Miller *et al.*, 2000; Technau *et al.*, 2003). In the tumour bearing polyps there is no sign of oocyte differentiation. Either there is no oocyte selected, or if there is one, this may have lost the ability to phagocytose. An *in situ* hybridisation with a marker gene that is exclusively expressed within the developing oocyte could help to clarify the situation.

The tumour bearing polyps still have the ability to produce eggs, as it could be shown by incubating a tumour bearing polyp under decreased temperature conditions. However, only one egg is produced in contrast to the normal oogenesis in *H. oligactis*, that is defined by the production of many eggs simultaneously and the appearance of small abortive eggs (Brien and Reniers-Decoen, 1949). This supports the findings of the disturbed oogenesis and could be seen as a hint for a lack of interstitial cells that could differentiate into oocytes.

The lack of interstitial cells fits to the observation that numerous tumour bearing polyps died due to a decreased amount of interstitial cells that therefore could not give rise to nematocytes, gland cells and nerve cells as shown in Figure 2.19. This might be due to the fact that all multipotent stem cells undergo augmented differentiation into the female restricted stem cells and as a consequence, a too small amount of multipotent stem cells remains. The frequent longitudinal cutting of the animals for culturing purposes may have further promoted this situation.

*Drosophila* and *Hydra* oogenesis share striking similarities (Alexandrova *et al.*, 2005; Miller *et al.*, 2000; Technau *et al.*, 2003). In both species, the oocyte and nurse cells arise from germ-line cells. And in both species apoptosis of germline-derived nurse cells is involved in oocyte differentiation. Nurse cells enter the apoptotic program, but are prevented from complete degradation for a defined time frame (Miller *et al.*, 2000). The species orthologues for caspase 3 are thought to be involved in this different apoptotic processes in *Hydra* and *Drosophila* (Technau *et al.*, 2003). Although the *Hydra* nurse cells reside in apoptosis, they are still transcriptionally active and express a large number of genes involved in development (Fröblius *et al.*, 2003).



Ovarian tumours have also been described for *D. melanogaster* (Bai *et al.*, 2002; King, 1969; Smith and King, 1966). Here, mutations in the genes *ovarian tumor (otu)*, *Sex-lethal (Sxl)* and *sans fille (snf)* result in the formation of grossly abnormal egg cysts consisting of hundreds to thousands of germ line cells surrounded by an unaffected somatic follicle cell layer (King, 1969; King and Riley, 1982). The tumorous germ cells are small and fail to differentiate into nurse cells and oocytes.

Also, in hard- and soft-shell clams ovarian tumours have been reported (Barry and Yevich, 1972; Darriba *et al.*, 2006; Hesselman *et al.*, 1988; Van Beneden *et al.*, 1993; Yevich and Berry, 1969). Three types of gonadal neoplasm histotypes have been defined in bivalves: (i) germinoma (follicular proliferation of immature germ cells that can expand into and beyond interstitial tissues); (ii) gonadal stromal neoplasms (composed of spindle cells); and (iii) gonadoblastoma (an intermixture of primitive follicular and stromal elements) (Darriba *et al.*, 2006). However, these tumours have not been characterized at the molecular level.

### **3.5.1 Does an increased expression of oncogenes lead to tumour formation in *Hydra*?**

The tumour bearing polyps show an enlarged expression of the KRAS orthologue *ras1* (see section 2.6.3). The oncogene KRAS encodes for a small GTPase involved in many signal transduction pathways as well as in the control of the cell cycle and is known to play a role in various cases of cancerogenesis in humans (Friday and Adjei, 2005). In most cases, point mutations lead to an expression of a constitutively active form of KRAS, but also gene amplifications or overexpression of KRAS have been reported to be implicated in tumourigenicity and cancer (Coleman *et al.*, 1994; Galiana *et al.*, 1995). Sequencing of the *ras1* coding sequence of the tumour bearing polyps with a subsequent single nucleotide polymorphism (SNP) analysis in comparison to the *ras1* gene of the control population would be useful to rule out whether this gene might be a cause for the tumour formation in these polyps. A generation of overexpression constructs coding for a constitutively active form of *ras1* in *Hydra* could help to understand tumourigenesis in these animals.

An enlarged expression of *ras1* might be a cause for the tumour, but can also be seen as a result of the increased proliferation of the interstitial cells. One cannot exclude that the detected increase of the *ras1* expression is due to a greater number of *ras1* expressing interstitial cells in the cDNA sample as it is likely the case for the increased expression of *cnivi*. To rule out this possibility it will be necessary to use a non-inducible gene exclusively expressed in interstitial cells as equilibration control.

An orthologue of the human *c-myc* protooncogene coding for the transcription factor Myc (Bister and Jansen, 1986) and for its dimerization partner Max (Eisenman, 2001) have been

identified and characterized in *Hydra* (Hartl *et al.*, 2010). Both are bHLH-Zip DNA binding proteins controlling the expression of up to 15 % of all human genes and regulate cell growth, proliferation differentiation metabolism and apoptosis (Eilers and Eisenman, 2008; Eisenman, 2001). A deregulation of *c-myc* leading to elevated Myc levels leads to tumourigenesis and characterizes about 30 % of all human cancers (Dang *et al.*, 2008; Nesbit *et al.*, 1999). The Myc and Max orthologues *myc1* and *max* from *Hydra* are expressed in fast proliferating stem cell populations of the interstitial cell lineage in *Hydra*, such as the multipotent stem cells, nematoblasts and gland cells, and Myc is able to induce cell transformation in avian fibroblast assays (Hartl *et al.*, 2010). It would be interesting to analyse the expression levels of *myc* and *max* in the tumour bearing polyps. If these were elevated this could be another reason for the induction of the tumour.

Another reason for tumour formation in these polyps could be environmental changes. These *Hydra* polyps have been collected from the environment and were subsequently cultivated under laboratory conditions. This represents an extreme change of the environment. Maybe this was sufficient to incompletely induce oogenesis. Nevertheless, this culture was stable for years, so it seems unlikely that just one change in the culturing conditions may have caused this extreme change in tissue homeostasis.

### 3.6 Tumourigenesis in an immortal animal?

As mentioned above, neoplasia is a burden on healthcare systems worldwide, but relatively few cases have been described in invertebrate species. A putative reason for this might be the relatively short lifespan of many invertebrates. *Hydra* is often considered to be immortal (Brien, 1951; Tardent, 1978) and can proliferate by budding. All three cell lines of *Hydra*, the ectodermal and the endodermal epithelial cells as well as the interstitial cells, are multipotent stem cells and have the ability to self-renew for a very long period of time or even perpetually. Therefore they show characteristics which cells of vertebrate species only have in embryogenesis (Bosch *et al.*, 2010). Aspects of ageing have not been clearly shown for *Hydra* and it is proposed that it is immortal (Martínez, 1998), except for the “crise gamétogénique” that leads to the death of the female *H. oligactis* polyp after sexual reproduction because of the consumption of the interstitial cells (Brien and Reniers-Decoen, 1949; Yoshida *et al.*, 2006). Because *Hydra* is immortal, it is hard to define tumourigenesis or cancer as most cells have the ability to self-renew and it was shown that *Hydra* cells also have the ability to transdifferentiate (Siebert *et al.*, 2008). In the case of the tumour bearing polyps, the interstitial cells divide uncontrolled leading to the formation of a tumour and a loss of tissue homeostasis.

A classification of these tumours from a “classic” medical point-of-view is hard because of *Hydra*’s simple body plan. One can clearly say that these tumours are not malignant or cancerous, because they do not seem to be tissue-invasive, as no clumps of interstitial cells were detected in the endodermal layer. Furthermore, the histological sections show that the borders of the neoplasia appear to be well defined. First transplantation experiments show, that the tumour cannot grow on the tissue recipients (data not shown) but this may also be due to the fact that the acceptor animal was male. It is known that the male germ line has a suppressive effect on the female germ line in *Hydra* (Littlefield, 1991). Therefore it may be correct to refer to these tumours, in a neutral way speaking, as a result of uncontrolled cell proliferation without implications of true cancer.

Interestingly, the polyps that carry a tumour cannot produce buds and so the tumour is not transferred to the next generation under natural conditions. To obtain a clonal culture the polyps were cut longitudinally which would never occur in nature. Although the tumours were stable for years, it was not possible to enlarge the culture over a critical number of animals. Frequently, animals died due to a lack of interstitial cells, so these tumours cannot affect future generations of *H. oligactis*.

An immortal animal must have evolved effective mechanisms to protect itself from a variety of diseases, either caused by pathogens or somatic mutations. These animals need an effective innate immune defence machinery that is so far characterized by the usage of Toll-like receptors, a large complexity of NOD-like receptors, conserved and taxonomically restricted putative signalling pathway components, a variety of antimicrobial peptides, and a conserved apoptosis machinery. In addition they need an efficient DNA repair mechanisms to protect themselves from the acquisition of dangerous mutations. The identification of new components of innate immune defence and the protection of self and the characterization of their networks in *Hydra* will be a task of future work.

### **3.7 *Hydra*, a valuable model organism to analyse innate immunity and tissue homeostasis**

Taken together, the results of this thesis show that NLRs are conserved throughout animal evolution and therefore phylogenetically older than previously thought.

The repertoire of *Hydra* NLRs appears to be very complex. Some mechanisms of NLR signalling seem to be conserved throughout animal evolution, such as the signal transduction through DEATH-fold effector domains between the NLR and downstream signalling components, e.g. adaptor proteins (HyDODE) or caspases (HyDD-Caspase, HyDED-Caspase), whereas other aspects have been solved in a species-specific way. Examples for the

latter are the species-specific expansions of NLR genes, the unique domain compositions of NLR signalling components (HyDODE, HyDD-Caspase, HyDED-DD-Kinase), but also effector proteins, such as AMPs (Augustin *et al.*, 2009a; Jung *et al.*, 2009; Khalturin *et al.*, 2009).

Furthermore, the analysis of the tumour bearing *Hydra* strain shows a disturbance in tissue homeostasis. Through an unknown trigger female germline-restricted interstitial stem cells over-proliferate and form tumour-like structures. These polyps cannot proliferate by budding indicating severe defects. These polyps are a valuable tool for further studies of the control of cell proliferation and apoptosis.

Although *Hydra*, as a representative of early branching metazoan animals, has a very simple body plan, it already possesses many mechanisms of innate immunity and maintenance of tissue homeostasis. All these mechanisms need to be tightly controlled and are interwoven in a complex regulatory network. Because of its simplicity, *Hydra* is a useful model organism to gain insights into these complex networks.

### 3.8 Future prospects

NLRs are sensors of MAMPs and endogenous “danger” signals (Chen *et al.*, 2009). They are key regulators of the maintenance of epithelial barrier integrity in mammals (Rosenstiel *et al.*, 2007). However, these receptors are not only present in vertebrates (Hibino *et al.*, 2006; Huang *et al.*, 2008). The repertoire of NLR proteins throughout the animal kingdom is very diverse and especially in early branching metazoans very complex. This rich repertoire was most likely acquired through species-specific gene amplifications in adaptation to their habitat and its concomitant pathogens. Not only receptors, but also basic principles of their signal transduction pathways could be conserved throughout animal evolution. However, species-specific pathway components may have evolved as well. In *Hydra*, the activation of NLR signalling may lead to apoptosis as an ancient form of immune response.

The results of this study may lead to new and interesting questions that may form part of future investigations:

- When in the evolution of Metazoa did the first NLRs appear?
  - Did these NLRs already have a role in immunity?
  - What is the mechanism of action of the ancestral NLR-signalling cascade?
  - How is it regulated?
- Does the activation of NLR-signalling in *Hydra* lead to apoptosis *in vivo*?
- How are the different immune responses integrated in *Hydra*?

- What are the target genes of HyNLRs?
- What is the result of their activation?

Furthermore, the analysis of a tumour bearing strain of *H. oligactis* showed an altered microbial community. The cells that contribute to the tumour are female germline-restricted cells and the tumour formation resembles *Hydra* oogenesis in several aspects.

Questions that arise as a result of these observations include:

- What causes tumourigenesis?
  - Are bacteria or mutated oncogenes the reason for the tumour-formation?
  - Are environmental changes the trigger for the tumour growth?
- Which genes are affected by tumour-formation with regard to their expression?
- If tumour formation represents an incomplete misguided oogenesis, is there a cell that differentiates into an oocyte simultaneously with nurse cells?
  - Why can oogenesis not be completed?

## 4 SUMMARY

The initial sensing of invading potential pathogenic microorganisms by pattern recognition receptors (PRRs) of the innate immune system is pivotal for the survival of the host. Nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) are a family of cytoplasmic PRRs that recognize conserved microbe-associated molecular patterns (MAMPs) or endogenous danger signals. Upon activation, these proteins trigger the release of pro-inflammatory cytokines and antimicrobial peptides (AMPs) via a variety of signal transduction cascades in mammals. All pathways have in common the initial formation of a multimeric complex of the activated NLR and downstream signalling proteins through homophilic interactions of effector domains. Many genetic variants of NLRs have been linked to chronic inflammatory disorders of the human epithelial barrier organs.

Although these receptors are of fundamental importance for understanding the maintenance of the human barrier integrity not much is known about their evolutionary roots.

This thesis involved screening the genomes of a variety of animal species with a focus on basal Metazoa and particularly on the freshwater polyp *Hydra* for NLR orthologues. It was subsequently demonstrated that the family of NLRs is evolutionary conserved and that frequently throughout animal evolution species-specific gene-amplifications occurred.

*Hydra* is continually surrounded by potential pathogenic microorganisms and must rely on an effective epithelial defence machinery. Using *Hydra* as a model organism for epithelial defence, the expression profile of one of the identified *Hydra* NLRs, *HyNLR type 1* was analysed in detail. This gene is predominantly expressed in the endodermal epithelium and is subject of alternative splicing. A co-expressed putative HyNLR-interactome has been identified as well. First functional *in vitro* studies using an artificially activatable chimeric protein including the N-terminal part of HyNLR type 1 could show that *Hydra* caspases may link NLR signalling to apoptosis as an ancient mechanism of innate immune defence.

As well as a genetic background, environmental changes and an altered composition of the host's microbiota are proposed to contribute to the development of barrier diseases. A tumour bearing *Hydra* strain with an altered microbial composition was analysed using histological and molecular biological techniques. The tumour was shown to be composed of female germline-determined interstitial stem cells. The tumour-like structure shows numerous parallels to oogenesis in *Hydra*, including lipid storage, the appearance of nurse cells and the expression of germline-specific genes. This first detailed description of tumour formation in a basal eumetazoan animal provides new possibilities for the analysis of an imbalanced tissue homeostasis.

## 5 ZUSAMMENFASSUNG

Das initiale Erkennen eindringender potenziell pathogener Mikroorganismen durch Muster-Erkennungsrezeptoren (*pattern recognition receptors* (PRRs)) des angeborenen Immunsystems ist entscheidend für das Überleben des Wirts. *Nucleotide binding and oligomerization domain* (NOD)-like Rezeptoren (NLRs) stellen eine Familie von zytoplasmatischen PRRs dar, die konservierte Mikroben-assoziierte molekulare Muster (*microbe-associated molecular patterns* (MAMPs)) oder endogene Gefahrensignale erkennen. Nach ihrer Aktivierung leiten diese Proteine in Säugetieren die Freisetzung proinflammatorischer Zytokine und antimikrobieller Peptide (*antimicrobial peptides* (AMPs)) über eine Vielfalt von Signaltransduktionskaskaden ein. Allen Signalwegen gemein ist die initiale Bildung eines multimeren Komplexes aus dem aktivierten NLR und nachgeschalteten Signalproteinen durch homophile Interaktionen ihrer Effektor-domänen. Viele genetische Varianten der NLRs sind mit chronisch entzündlichen Erkrankungen der menschlichen Barriereorgane in Verbindung gebracht worden.

Obwohl diese Rezeptoren von fundamentaler Bedeutung für das Verständnis der Aufrechterhaltung der humanen Barriere Integrität sind, ist nicht viel über ihre evolutiven Wurzeln bekannt.

Diese Arbeit umfasste das Überprüfen der Genome zahlreicher Tierarten mit einem besonderen Fokus auf basalen Metazoen und insbesondere auf dem Süßwasserpolyphen *Hydra* auf Orthologe für NLRs. Es konnte weiterhin gezeigt werden, dass die Familie der NLRs evolutionär konserviert ist und dass häufig während der Evolution der Tiere artspezifische Gen-Amplifikationen aufgetreten sind.

*Hydra* ist stets von potenziell pathogenen Mikroorganismen umgeben und auf effektive epitheliale Abwehrmechanismen angewiesen.

*Hydra* wurde als Modellorganismus für epitheliale Abwehr benutzt und das Expressionsprofil eines der identifizierten *Hydra* NLRs, *HyNLR type 1*, detailliert untersucht. Dieses Gen wird vor allem im endodermalen Epithel exprimiert und unterliegt alternativem Spleißen. Ein koexprimiertes putatives Interaktom für HyNLRs konnte ebenfalls identifiziert werden. Erste funktionelle *in vitro* Studien mit einem künstlich aktivierbaren chimären Protein, das den N-terminalen Bereich von HyNLR type 1 enthält, konnten zeigen, dass *Hydra* Caspasen die NLR Signalgebung mit Apoptose als einer alten Form der angeborenen Immunantwort verknüpfen können.

Es wird vermutet, dass nicht nur ein genetischer Hintergrund, sondern auch Veränderungen der Lebensumstände und eine veränderte Komposition der Wirt-Mikrobiota an der Entstehung

von Barriere-Erkrankungen beteiligt sind. Ein spontan aufgetretener Tumor in einer *Hydra*-Linie, die eine veränderte mikrobielle Zusammensetzung aufwies, wurde histologisch und molekularbiologisch untersucht. Es konnte gezeigt werden, dass der Tumor von interstitiellen Stammzellen, die auf die weibliche Keimbahn festgelegt sind, gebildet wird. Die Tumorartige Struktur zeigt viele Parallelen zur *Hydra*-Oogenese auf. Dies beinhaltet die Speicherung von Lipiden, das Auftreten von Nährzellen und die Expression von Keimbahn-spezifischen Genen. Diese erste detaillierte Beschreibung einer Tumorbildung in einem basalen Eumetazoen bietet neue Möglichkeiten für die Analyse einer gestörten Gewebekomöostase.



## 6 MATERIALS

### 6.1 Organisms and cell lines

<i>Hydra</i> strains:	<i>H. magnipapillata</i> (105)	
	<i>H. vulgaris</i> (AEP)	
	<i>H. oligactis</i>	
	Tumour bearing <i>H. oligactis</i>	Dr. Boris Anokhin
	<i>H. oligactis</i> St. Petersburg	Dr. Boris Anokhin
Feeding strain:	<i>Artemia salina</i>	Silver Star
Cell line:	HEK293 (ACC305)	German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany)
Bacterial strains:	<i>E. coli</i> ElectroMAX™ DH5 $\alpha$ -E™	invitrogen
	<i>Pseudomonas fluorescens</i>	Department of Dermatology, Kiel
	<i>P. aeruginosa</i> (ATCC33348)	Department of Dermatology, Kiel
	<i>P. aeruginosa</i> (ATCC33354)	Department of Dermatology, Kiel

### 6.2 Media

<i>Artemia</i> medium	31.8 g sea salt/l Millipore water
DMEM medium (Ready Mix)	PAA Laboratories
LB medium	10 g peptone, 5 g yeast extract, 5 g NaCl, water up to 1 l, autoclaved
LB medium with ampicillin	LB medium, add 50 $\mu$ g/ml ampicillin, water up to 1 l, autoclaved
LB agar	LB medium, add 15 g agar agar, water up to 1 l, autoclaved
TSB medium	3.0 g Tryptic Soy Broth, 17.0 g peptone, 2.5 g glucose, 5.0 g NaCl, 2.5 g K <sub>2</sub> HPO <sub>4</sub> , water up to 1l
MG medium	40 mM K <sub>2</sub> HPO <sub>4</sub> , 30 mM glucose, 22 mM KH <sub>2</sub> PO <sub>4</sub> , 7 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.5 mM MgSO <sub>4</sub>
<i>Hydra</i> medium	1 mM CaCl <sub>2</sub> , 1 mM NaCl, 0.1 mM MgSO <sub>4</sub> , 0.1 mM KCl, 1 mM TRIS-HCl (pH 7,8)
Volvic	Danone Waters
SOB medium	20 g peptone, 5 g yeast extract, 0.55 g NaCl, 1.86 g KCl, water up to 1l, autoclaved, add 10 ml 1M MgSO <sub>4</sub> und 10 ml 1M MgCl <sub>2</sub> (both sterile)
SOC medium	SOB medium, add 0.02 M glucose (sterile)

## 6.3 Buffers and solutions

### 6.3.1 General

APS stock solution	10 % in Millipore water
Denhardt's (50x)	1 % Polyvinylpyrrolidone, 1 % Ficoll, 1 % BSA fraction V in Millipore water (sterile)
DNA loading buffer	50 % glycerol, 10 mM EDTA (pH 8.0), 0.1 % SDS, 0.1 % Bromophenol blue, 0.1 % Xylene cyanol
EDTA solution	0.5M EDTA (pH 8.0)
Hoechst stock solution	1mg/ml in Millipore water
Mowiol	5 g Mowiol 4-88 in 20 ml 100 mM TRIS (pH 8.0), stir for 16h at room temperature, then add 10 ml glycerol, stir for 16h 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.
Mowiol/DABCO	2 ml Mowiol, add some crystals of DABCO
NTM buffer	100 mM NaCl, 100 mM TRIS-HCl, 50 mM MgCl <sub>2</sub> , pH 9.5
NTMT buffer	NTM, add 0.1 % Tween20
Paraformaldehyde stock solution	8 % paraformaldehyde in Millipore water, heat in water bath at 50 °C, add droplets of NaOH until the powder lyses, pH 7.4
PBS buffer	150 mM NaCl, 80 mM K <sub>2</sub> HPO <sub>4</sub> , pH 7.34
PBS/ glycerol	9:1 of PBS and glycerol
PBT buffer	PBS, add 0.1 % Tween20
Phalloidin stock solution	0.1mg/ml in Millipore water
SDS stock solution	10 % in Millipore water
Sheep serum	inactivation at 56 °C for 30 min
SSC buffer (20x)	3M NaCl, 0.3M sodium citrate (pH 7.0)
TAE buffer (50x)	242 g TRIS base, 57.1 ml 100 % acetic acid, 100 ml 0.5M EDTA, Millipore water up to 1l
TBE (10 x)	0.89 M TRIS base, 0.89 M boric acid, 0.02 M EDTA
TBS buffer (10x)	200 mM TRIS, pH 7.6, 1.37 M NaCl
TBST buffer	TBS, add 0.1 % Tween20
TE buffer	10 mM TRIS-HCl (pH 7.5), 1 mM EDTA

Urethane relaxing solution 2 % urethane in *Hydra* medium

### 6.3.2 *In situ* hybridisation

Blocking Solution 80 % MAB-B, 20 % heat-inactivated sheep serum

Glycine working solution 4 mg glycine per ml PBT

Hybridisation Solution 50 % formamide, 5x SSC, 0.1 % Tween20, 0.1 % CHAPS, 1x Denhardt's, 100 µg/ml Heparin in Millipore water

MAB 100 mM maleic acid, 150 mM NaCl, pH 7.5

MAB-B MAB plus 1 % BSA fraction V

Triethanolamine 100 mM triethanolamine (pH 7.8)

### 6.3.3 Southern blot

Sephadex G-50 suspension 5 g Sephadex G-50 in 100 ml TE buffer, autoclaved

Washing solution 1 2x SSC, 0.1 % SDS

Washing solution 2 0.2x (0.5x) SSC, 0.1 % SDS

Denaturing solution 1.5M NaCl, 0.5M NaOH

Neutralisation solution 1M TRIS, pH 7.4, 1.5M NaCl

Hybridisation solution 50 % formamide, 4.8x SSC, 10 mM TRIS-HCl, pH 7.5, 1 % SDS, 1x Denhardt's, 10 % dextran sulfate

### 6.3.4 Procaine

Dissociation medium 3.6 mM KCl, 6 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 6 mM sodium citrate, 6 mM sodium pyruvate, 12.5 mM TES, pH 6.9, sterile

Solution A 1:1:1 dilution of 1 % Procaine-HCl in Millipore water, Dissociation medium, *Hydra* medium, pH 4.5

Solution B 1:1:1 dilution of 1 % Procaine-HCl in Millipore water, Dissociation medium, *Hydra* medium, pH 2.5

### 6.3.5 TUNEL

Blocking solution 20 % heat-inactivated sheep serum in PBS

TdT staining solution 5 µM DIG-dUTP, 150U/ml TdT in TdT buffer

**6.3.6 Western blot**

Blocking solution	TBST, add 5 % milk powder
Coomassie solution	0.05 % Coomassie in decolourising solution
Decolourising solution	50:1:40 of methanol, acetic acid, Millipore water
Gel buffer 1	0.5M TRIS-HCl (pH 6.8)
Gel buffer 2	1.5M TRIS-HCl (pH 8.8)
Loading buffer (2x)	135 mM TRIS-HCl (pH 6.8), 20 % glycerol, 4 % SDS, 2 % $\beta$ -mercaptoethanol, 0.001 % bromophenol blue
Running buffer	125 mM TRIS, 0.96 M glycine, 0.5 % SDS
Separating gel (12 %, 90 ml)	36 ml 30 % acrylamide/0.8 % bisacrylamide, 22.5 ml gel buffer 2, 900 $\mu$ l 10 % SDS, 45 $\mu$ l TEMED, 450 $\mu$ l 10 % APS, 30.1 ml water
Stacking gel (45 ml)	5.85 ml 30 % acrylamide/0.8 % bisacrylamide, 11.25 ml gel buffer 1 450 $\mu$ l 10 % SDS, 45 $\mu$ l TEMED, 225 $\mu$ l 10 % APS, 27.45 ml water
Transfer buffer	25 mM TRIS, 192 mM glycine, 10 % methanol

**6.3.7 Coimmunoprecipitation**

Anode buffer I	30 mM TRIS, 20 % methanol
Anode buffer II	300 mM TRIS, 20 % methanol
Cathode buffer	25 mM TRIS, 20 % methanol, 40 mM 6-aminocaproic acid
RIPA buffer	50 mM TRIS-HCl (pH 7.4), 1 % NP40, 0.25 % sodium deoxycholate, 150 mM NaCl

**6.3.8 FISH**

Hybridisation solution (4 ml)	360 $\mu$ l 5M NaCl, 40 $\mu$ l 1M TRIS-HCl (pH 7.4), 200 $\mu$ l formamide, 1398 $\mu$ l Millipore water, 2 $\mu$ l 10 % SDS
Washing buffer (50 ml)	4.5 ml 5M NaCl, 1 ml 1M TRIS-HCl, 44.45 ml Millipore water, 50 $\mu$ l 10 % SDS

**6.3.9 Maceration**

Maceration solution 1:1:13 of acetic acid, glycerol, water

**6.3.10 DNA extraction**

Lysis Buffer 100 mM TRIS-HCl, pH 8.0, 100 mM EDTA, 1 % SDS

**6.3.11 Histology**

Lead citrate solution 1.33 g  $\text{Pb}(\text{NO}_3)_2$ , 1.76 g sodium citrate in Millipore water, mix for 30 min, add 8 ml 1M NaOH and fill up to 50 ml with Millipore water

Richardson 0.5 % Methylene blue, 0.5 % borax, 0.5 % Azur II in  $\text{ddH}_2\text{O}$

Sudan Black B Solution 1 g Sudan Black B in 100 ml 100 % ethanol, heat to cooking using a water bath, cool down and filtrate

**6.3.12 DNA sequencing**

Sequencing gel for LI-COR (41 cm) 10.5 g urea, 14 ml Millipore water, 3.75 ml Rotiphorese® Gel 40, 2.5 ml 10x TBE, 38  $\mu\text{l}$  TEMED, 175  $\mu\text{l}$  10 % APS

Sequencing gel for LI-COR (66 cm) 21 g urea, 28 ml Millipore water, 5.55 ml Rotiphorese® Gel 40, 5 ml 10x TBE, 76  $\mu\text{l}$  TEMED, 350  $\mu\text{l}$  10 % APS

**6.4 Chemicals**

1,2-Propylene oxide	Roth
6-Aminocaproic acid	Sigma
Acetic acid	Roth
Acetic anhydride	Roth
Agar 100 resin	Agar Scientific
Agar agar	Roth
Agarose	Roth
Ammonium persulfate (APS)	Merck
Ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ )	Roth
Ampicillin	Merck
AP20187	Ariad
Azur II	Merck
Boraxcarmin solution	Chroma-Gesellschaft
Boric acid	Roth
Bovine serum albumine (BSA) fraction V	Roth
Bromophenol blue	Sigma
Calcium chloride ( $\text{CaCl}_2$ )	Roth

CHAPS	Roth
Chloroform	Roth
Coomassie Brilliant Blue R-250	Serva
DABCO	Sigma
dCTP (100 mM)	Fermentas
DEPC	Roth
Developer	Agfa
Dextran sulfate	Roth
dGTP (100 mM)	Fermentas
DIG-dUTP	Roche
Dipotassium phosphate ( $K_2HPO_4$ )	Merck
dNTP mix (10 mM)	Fermentas
dTTP (100 mM)	Fermentas
EDTA	Roth
Ethanol	Roth
Ethidium bromide	Roth
Euparal	Roth
Ficoll	Sigma
Fixer	Tetenal
Flagellin	Alexis
Formamide	Roth
FuGENE® 6 Transfection Reagent	Roche
Gelatine	Sigma
Glass wool	Serva
Glucose	Roth
Glutaraldehyde	Sigma
Glycerol	Roth
Glycine	Roth
Heparin	Roth
Hoechst	Calbiochem
Hydrochloric acid (HCl)	Roth
Isopropanol	Roth
Lead (II) nitrate ( $Pb(NO_3)_2$ )	Merck
Lead acetate	Merck
Levamisole	Roth
Lipopolysaccharide (LPS)	Borstel
Magnesium chloride ( $MgCl_2$ )	Merck
Magnesium sulfate ( $MgSO_4$ )	Merck
Maleic acid	Roth
Methanol	Roth
Methylene blue	Merck
Milk powder	Roth
Monopotassium phosphate ( $KH_2PO_4$ )	Merck
Mowiol 4-88	Calbiochem
TES	Sigma
NBT/BCIP	Roche
Nitrogen ( $N_2$ )	Westfalia
Nonidet P40 (NP40)	Sigma
Osmium tetroxide ( $OsO_4$ )	Roth
Paraformaldehyde	Sigma
Peptone	Roth

Phalloidin-Tetramethylrhodamine B isothiocyanate	Sigma
Phenol	Roth
Phosphatase inhibitor cocktail 2	Sigma
Pioloform	Plano
Polyvinylpyrrolidone	Sigma
Potassium chloride (KCl)	Roth
Procaine hydrochloride	Sigma
Protease inhibitor tablets	Roche
Protein A/G PLUS-Agarose	Santa Cruz Biotechnology
Rhodamine B Isothiocyanate-Dextran R9379	Sigma
Rotiphorese® Gel 30	Roth
Rotiphorese® Gel 40	Roth
Salmon sperm DNA	Invitrogen
Sea salt (Reef crystals™)	Aquarium Systems
Sephadex G-50	Pharmacia
Sheep serum	Sigma
Sodium acetate	Roth
Sodium cacodylate	Sigma
Sodium chloride (NaCl)	Roth
Sodium citrate (Na <sub>3</sub> (C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ))	Roth
Sodium deoxycholate	Sigma
Sodium dodecyl sulfate (SDS)	Roth
Sodium hydroxide (NaOH)	Roth
Sodium pyruvate	Sigma
Sudan Black B	Merck
Tetramethylethylenediamine (TEMED)	Serva
Triethanolamine	Sigma
TRIS base	Roth
TRIS-HCl	Roth
TritonX100	Merck
TRIzol®	Invitrogen
tRNA yeast	Sigma
Tryptic Soy Broth	Sigma
Tween20	Roth
Uranyl acetate	Merck
Urea	Roth
Urethane	Sigma
Volvic water	Danone waters
Xylene cyanol	Sigma
Yeast extract	Roth
β-Mercaptoethanol	Sigma

## 6.5 Kits

Amersham ECL Plus™ Western Blotting Reagent Pack	GE Healthcare
ARGENT™ Regulated Homodimerization Kit	Ariad
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied biosystems
DIG RNA Labeling Kit (SP6/T7)	Roche
DNeasy Blood & Tissue Kit	Qiagen
First Strand cDNA Synthesis Kit	Fermentas

FITC Annexin V Apoptosis Detection Kit I	BD Pharmigen™
NucleoSpin® Extract II	Macherey Nagel
NucleoSpin® Plasmid QuickPure	Macherey Nagel
pGEM®-T Kit	promega
QIAfilter™ Plasmid Maxi Kit	Qiagen
QIAfilter™ Plasmid Midi Kit	Qiagen
RNeasy® Mini-Kit	Qiagen
SequiTherm EXCEL™ II DNA Sequencing Kit	Epicentre
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific

## 6.6 Enzymes

DNase I	Fermentas
Gateway® BP Clonase® II enzyme mix	Invitrogen
Gateway® LR Clonase® II enzyme mix	Invitrogen
Klenow fragment	Fermentas
Platinum® Taq Polymerase High Fidelity	Invitrogen
Proteinase K	Sigma
RNase A	Fermentas
T4 ligase	Fermentas
<i>Taq</i> DNA polymerase	GE Healthcare
Terminal Deoxynucleotidyl Transferase (TdT)	Fermentas

### 6.6.1 Restriction enzymes

<i>Bsu</i> RI	Fermentas
<i>Eco</i> RI	Fermentas
<i>Eco</i> RV	New England Biolabs
<i>Fse</i> I	New England Biolabs
<i>Hin</i> 6I	Fermentas
<i>Hind</i> III	Fermentas
<i>Psy</i> I	Fermentas
<i>Xba</i> I	Fermentas

## 6.7 Vectors

pGEM®-T	Promega
pCEV-GwA	provided by G. Jacobs (see appendix)
pC <sub>4</sub> M-F <sub>v</sub> 2E	Ariad
pCMV-Myc	Clontech
pDONR®221	Invitrogen
hydeath-fkbp	synthesised by GENEART (see appendix)
ligR1	provided by K. Khalturin (see appendix)

## 6.8 Oligonucleotides

A list of the used oligonucleotides can be found in the appendix.



## 6.9 Radioactive nucleotides

$\alpha$ -[<sup>32</sup>P]-dATP

GE Healthcare

## 6.10 Antibodies

Alexa Fluor® 488 donkey anti-mouse IgG (H+L)

Anti HA High Affinity Rat mAB (clone 3F10)

Anti-Digoxigenin-alkaline phosphatase linked Fab fragments

Anti-FLAG® M2 Antibody

Anti-Periculin antiserum N31

Cy3 AffiniPure Donkey Anti-Mouse IgG

ECL™ Mouse IgG Horseradish Peroxidase-linked Whole Ab

ECL™ Rabbit IgG Horseradish Peroxidase-linked Whole Ab

HA-Tag (6E2) mouse monoclonal antibody

Myc-Tag (71D10) Rabbit mAb

Peroxidase AffiniPure Goat Anti-Rat IgG

Invitrogen

Roche

Roche

Sigma-Aldrich

V. Klimovich

Jackson Immunoresearch

GE Healthcare

GE Healthcare

Cell Signaling Technology

Cell Signaling Technology

Jackson Immunoresearch

## 6.11 DNA and protein size standards

GeneRuler™ DNA Ladder Mix

Precision Plus Protein Dual Color Standard

Prestained Protein Marker, Broad Range

Fermentas

Bio-Rad

New England Biolabs

## 6.12 Lab equipment and other materials

### 6.12.1 PCR machines

Cyclone gradient

Primus 96 *plus*

Primus 25

7900HT Fast Real Time PCR-System

Peqlab

MWG-Biotech

MWG-Biotech

Applied Biosystems

### 6.12.2 Power supplies

E835

PP3000

EPS-500/400

EPS-3500

Consort

Biometra

Pharmacia Biotech

Pharmacia Biotech

### 6.12.3 Gel electrophoresis chambers

MINI SUB-CELL®

Novex Mini-Cell

SUB-CELL® GT

SE250 PAGE gel chamber

PerfectBlue™ Twin L

Bio-Rad

Invitrogen

Bio-Rad

Hoefer Scientific Instr.

Peqlab

**6.12.4 Incubators/ shakers**

CB150 (Cell culture incubator)	Binder
Certomat® H	B. Braun
Certomat® R	B. Braun
HIS25	Grant BOEKEL
KS10 (rotation shaker)	Edmund Bühler
Thermoincubator	Heraeus Instruments
Thermomixer Compact	Eppendorf
ThermoStat plus	Eppendorf
Oven/ incubator	Memmert
C/R5 (waterbath)	Julabo
Rotation incubator RM5	Assistant

**6.12.5 UV devices**

UV lamp Chroma 43	Vetter GmbH
ImaGo Compact Imaging System	B&L Systems
UV-Stratalinker® 1800	Stratagene

**6.12.6 Electroporation devices**

Gene Pulser II	Bio-Rad
Pulse Controller II	Bio-Rad

**6.12.7 Centrifuges**

Centrifuge 5415 D	Eppendorf
Centrifuge 5417 R	Eppendorf
miniSpin	Eppendorf
Multifuge 3 S-R	Heraeus
Speed Vac® Plus	Savant
Universal Vacuum System Plus	Savant

**6.12.8 Microscopy**

CLSM TCS SP/UV	Leica
DC300F (digital camera)	Leica
Transmission electron microscope EM 208 S	Philips
Axiovert 100	Zeiss
Axioskop 2	Zeiss
AxioCam (digital camera)	Zeiss
SZX16	Olympus
DP71 (digital camera)	Olympus
Wild M3C binocular	Heerbrugg
KL1500 LCD	Schott

**6.12.9 Photometer**

Nanodrop ND1000 photometer	Thermo Scientific
BioPhotometer	Eppendorf



Amersham Hyperfilm ECL	GE Healthcare
Cover slips	Roth
Membrane filter disc (0.025 $\mu\text{m}$ )	Millipore
Extra thick filterpaper	Invitrogen
Amersham Hybond <sup>TM</sup> N+ membrane	GE Healthcare
UVette®	Eppendorf
Cuvettes	Sarstedt
Electroporation cuvettes (1mm)	Peqlab
NuPAGE® 4-12 % Bis-TRIS Gel	Invitrogen
Pasteur pipettes (150mm, 230mm)	Eydam
Petri dishes (35x10mm, 60x15mm, 92x16mm)	Sarstedt
Plastic dishes	Westmark
Roti®-PVDF membrane	Roth
Grid Box	Plano
Grid G2500	Plano
Scalpel blades	Merck
Pipette tips (2.5 $\mu\text{l}$ , 200 $\mu\text{l}$ , 1000 $\mu\text{l}$ , 5 ml)	Sarstedt
Gel Blotting Paper GB002	Schleicher & Schuell
BioMax MS (x-ray film)	Kodak
Latex gloves	Roth
Nitril gloves	Roth
Microscope Slides	Walter-CMP
Flat embedding mould G3690 (rubber)	Plano
Filtropur S (0.2 $\mu\text{m}$ , 0.45 $\mu\text{m}$ )	Sarstedt
Plastic foil	Aldi
Micro mortar	Fisher
Parafilm	Pechiney Plastic Packaging

### 6.12.13 Other materials

Sieves	Nuova
Glass bottles	Schott
Pipettes (2-20 $\mu\text{l}$ , 20-200 $\mu\text{l}$ , 0.1-1 ml, 0.5-5 ml)	Gilson
Pipette (0.1-2.5 $\mu\text{l}$ )	Eppendorf
Easypet	Eppendorf
250 ml tubes	Sorval
Micro cannulation needle	Hamilton Bonaduz AG
Beaker glasses	Schott
BioMax® MS Intensifying Screen	Kodak
X-ray cassettes	Kodak
Erlenmeyer flasks	Schott
Graduate zylinder	Schott

### 6.13 URLs

AG Bosch internal BLAST server	<a href="http://134.245.171.51/blast/">http://134.245.171.51/blast/</a>
BLAST searches	<a href="http://blast.ncbi.nlm.nih.gov/Blast.cgi">http://blast.ncbi.nlm.nih.gov/Blast.cgi</a>
Compagen	<a href="http://compagen.zoologie.uni-kiel.de/">http://compagen.zoologie.uni-kiel.de/</a>
Compagen_NG	<a href="http://134.245.171.51/compagen_ng/blast/blast_cs.html">http://134.245.171.51/compagen_ng/blast/blast_cs.html</a>
Genome Browser	<a href="http://genome.ucsc.edu">http://genome.ucsc.edu</a>
<i>Gallus</i>	
<i>Oryzias</i>	
<i>Xenopus</i>	
<i>Petromyzon</i>	
Genome Browser <i>Daphnia</i>	<a href="http://genome.jgi-psf.org/Dappu1/Dappu1.home.html">http://genome.jgi-psf.org/Dappu1/Dappu1.home.html</a>
Genome Browser <i>Hydra</i>	<a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a> <a href="http://hydrazome.metazome.net">http://hydrazome.metazome.net</a>
Genome browser <i>Nematostella</i>	<a href="http://genome.jgi-psf.org/Nemve1/Nemve1.home.html">http://genome.jgi-psf.org/Nemve1/Nemve1.home.html</a>
Ribosomal database project	<a href="http://rdp.cme.msu.edu/">http://rdp.cme.msu.edu/</a>
SMART	<a href="http://smart.embl-heidelberg.de/smart/">http://smart.embl-heidelberg.de/smart/</a>
Stellabase	<a href="http://evodevo.bu.edu/stellabase/">http://evodevo.bu.edu/stellabase/</a>

### 6.14 Software

Axio Vision 3.1	Zeiss
BioEdit	<a href="http://www.mbio.ncsu.edu/BioEdit/bioedit.html">http://www.mbio.ncsu.edu/BioEdit/bioedit.html</a>
Cell^A Imaging software	Olympus
DNAMAN4.15	Lynnon Coprporation
e-Seq 3.0	LI-COR
FigTree1.3.1	<a href="http://tree.bio.ed.ac.uk/software/figtree/">http://tree.bio.ed.ac.uk/software/figtree/</a>
HMMER	<a href="http://hmmer.janelia.org/">http://hmmer.janelia.org/</a>
IM 50 4.0	Leica
InterProScan	<a href="http://www.ebi.ac.uk/Tools/InterProScan/">http://www.ebi.ac.uk/Tools/InterProScan/</a>
Jalview	<a href="http://www.jalview.org/">http://www.jalview.org/</a>
MEGA 3.0	<a href="http://www.megasoftware.net/index.html">http://www.megasoftware.net/index.html</a>
MEGA 4.0	<a href="http://www.megasoftware.net/index.html">http://www.megasoftware.net/index.html</a>
MINIMUS	<a href="http://sourceforge.net/apps/mediawiki/amos/index.php?title=AMOS">http://sourceforge.net/apps/mediawiki/amos/index.php?title=AMOS</a>
MrBayes 3.1.2	<a href="http://mrbayes.csit.fsu.edu/">http://mrbayes.csit.fsu.edu/</a>
MUSCLE	<a href="http://www.ebi.ac.uk/Tools/muscle/index.html">http://www.ebi.ac.uk/Tools/muscle/index.html</a>
PhosphoImager AIDA Image Analyser	Raytest
Photoshop CS4	Adobe
Pintail	<a href="http://www.bioinformatics-toolkit.org/Pintail/">http://www.bioinformatics-toolkit.org/Pintail/</a>
Wallac 1414 WinSpectral v1.30	PerkinElmer

## 7 METHODS

### 7.1 Cultivation and incubation of *Hydra* and HEK293 cells

#### 7.1.1 Cultivation of *Artemia salina*

*Artemia salina* served as food for all used *Hydra* species. For hatching, eggs were incubated overnight at 32 °C in *Artemia* medium under permanent air supply. Afterwards, nauplii were kept at room temperature for several days. For feeding the *Hydra* polyps, nauplii were collected and, by using a close meshed sieve, washed with water to remove salts. Afterwards, *Artemia* were resuspended in *Hydra* medium.

#### 7.1.2 Cultivation of *Hydra*

*Hydra* polyps were kept in plastic dishes at 18 °C in *Hydra* medium with a 12 h day-night cycle according to standard methods (Lenhoff and Brown, 1970). All animals were fed two to four times per week. For comparative incubation experiments all animals were kept under the same feeding conditions. All experiments for the NOD-like receptor study were performed on *H. magnipapillata*. The transgenic polyps were obtained using *H. vulgaris* (AEP). The tumour bearing polyps were kept in a petri dish that was replaced after each feeding. Only polyps with more than five tentacles were cut longitudinally to expand the culture. A laboratory male strain of *H. oligactis* and a female strain of *H. oligactis* isolated by Dr. B. Anokhin from the surrounding of St. Petersburg served as control strains. In *H. oligactis* gametogenesis was induced by incubating polyps at 10 °C for 14 days.

#### 7.1.3 HEK293 cell culture

HEK293 cells were cultivated in 6-well plates containing DMEM medium at 37 °C and 5 % CO<sub>2</sub>.

#### 7.1.4 Tissue separation with procaine

A modified protocol from Epp *et al.* and Bode *et al.* was used to separate the ectodermal and endodermal tissue of *Hydra magnipapillata* (Bode *et al.*, 1987; Epp *et al.*, 1979).

Head and foot were removed; animals were incubated in solution A for 5 min and in solution B for 1.5 min at 4 °C. Polyps were transferred to dissociation medium at 18 °C. The ectodermal and endodermal tissues formed a ring- and a rod-like structure respectively and were separated using needles. The separated tissues were subjected to RNA extraction.

### 7.1.5 Preparation of *Pseudomonas aeruginosa* MAMP solutions

*P. aeruginosa* (ATCC33354) was cultivated in TSB medium over night at 37 °C. The culture was centrifuged at 6000 rpm for 10 min. The pellet was resuspended in water and heat-inactivated at 65 °C for 15 min. The sample was centrifuged and the supernatant was sterile-filtered (0.45 µm).

*P.s aeruginosa* (ATCC33348) was cultivated in MG medium without shaking. The supernatant was sterile-filtered.

### 7.1.6 Incubation of *Hydra* polyps in bacteria and MAMPs

*E. coli* DH5α and *Pseudomonas fluorescens* were cultured in 30 ml LB medium at 37 °C and room temperature respectively until they reached an OD<sub>600</sub> of 5. The cells were centrifuged for 15 min at 2,500 g, washed three times with sterile Millipore water and resuspended in *Hydra* medium to an OD<sub>600</sub> of 10. Furthermore, *Pseudomonas aeruginosa* (ATCC33348) supernatant in MG medium (cultivated without shaking) and *Pseudomonas aeruginosa* (ATCC33354) cell membrane components in Millipore water (bacteria were heat shocked and sterile filtered) were used as stimuli. *Hydra* medium and MG medium served as negative controls. Animals were incubated for 4 h in the different stimuli prior to extraction of RNA using TRIzol®.

### 7.1.7 Injection of MAMPs into *Hydra*

The animals starved for 48 h and were placed in fresh Volvic water over night prior to injection. LPS (200 ng/ml in Volvic water) or flagellin (2.5 µg/ml in Volvic water) were injected through the hypostome into the gastric cavity of 20 polyps without buds or gonads using a 1 ml syringe and a micro cannulation needle (Hamilton Bonaduz AG). Untreated animals and animals injected with pure Volvic water (wounding control) served as negative controls. MAMPs were injected and surrounded the animals in the same concentration. Polyps were incubated for 4 h prior to RNA isolation using TRIzol®.

## 7.2 General molecular biologic methods

### 7.2.1 Isolation of RNA from *Hydra*

Before the isolation of RNA, polyps were starved for at least two days. At least 20 polyps per approach were collected within a 1.5 ml reaction tube on ice and washed several times with ice-cold *Hydra* medium. The supernatant was removed completely and 10 µl TRIzol® reagent (Invitrogen) per mg tissue were added. The sample was vortexed immediately until the tissue was completely homogenised. After incubating the samples for 5 min, they were

either stored at  $-20\text{ }^{\circ}\text{C}$  or the extraction protocol was proceeded. Only RNase-free material and solutions were used from this point of time on. Per ml TRIzol® 0.2 ml Chloroform were added, followed by a vigorous shaking for 15 sec. After 3 min of incubation at RT the samples were centrifuged for 15 min at  $4\text{ }^{\circ}\text{C}$  and 12,000 g to separate the aqueous and the organic phase. The upper aqueous phase was collected and transferred into a fresh tube. Per ml TRIzol® 0.5 ml Isopropanol were added at room temperature and the tube was shaken softly for several times followed by incubation at room temperature for 10 min. Afterwards, the samples were centrifuged for 10 min at  $4\text{ }^{\circ}\text{C}$  and 12,000 g. The supernatant was removed and the pellet was washed in 1 ml 75 % ethanol per ml TRIzol® and centrifuged for 5 min at  $4\text{ }^{\circ}\text{C}$  at 7,500 g. After removing the entire supernatant the pellet was air-dried on ice. The pellet was resuspended within 80  $\mu\text{l}$  Millipore water on ice.

To remove contaminations with genomic DNA the samples were treated with DNase I according to the pipetting scheme shown in Table 7.1.

**Table 7.1: Pipetting scheme for the treatment with DNase I.**

<b>Component</b>	<b>Volume [<math>\mu\text{l}</math>]</b>
10x Buffer	10
DNase I (1 U/ $\mu\text{l}$ )	8
RNaseOUT Ribonuclease Inhibitor	2
RNA	80
<b>Final volume</b>	<b>100</b>

The samples were incubated for 1 h at  $37\text{ }^{\circ}\text{C}$ . To stop the reaction 2  $\mu\text{l}$  EDTA (25 mM) were added and the samples were heated to  $65\text{ }^{\circ}\text{C}$  for 10 min.

Afterwards the RNA was purified using the “RNeasy® Mini-Kit” from QIAGEN according to the manufacturer’s “RNA Cleanup” protocol. The RNA was eluted by adding 20  $\mu\text{l}$  Millipore water to the silica column.

### **7.2.2 Quantification of nuclear acids**

The quantity and quality of isolated nuclear acids were determined at the absorbance at 260 nm using the Nanodrop spectrophotometer.

### **7.2.3 First strand cDNA synthesis**

The synthesis of cDNA was performed using the “First Strand cDNA Synthesis Kit” (Fermentas) according to the manufacturer’s protocol. For subsequent comparative analyses of gene expression levels *via* PCR, equal amounts of RNA were added to the reactions.



For synthesis either the included Oligo(dT)18 primer or sequence specific primers were used. For subsequent 3'RACE PCRs an adapter primer was used according to the manufacturer's instructions. Afterwards the cDNA was diluted with 130  $\mu$ l Millipore water.

## 7.2.4 Polymerase chain reaction (PCR)

### 7.2.4.1 Standard PCR

A standard PCR reaction was set up in a final volume of 20  $\mu$ l. Table 7.2 contains the pipetting scheme for the reaction.

**Table 7.2: Pipetting scheme for standard PCRs.**

Component	Volume [ $\mu$ l]
DNA + H <sub>2</sub> O	11.8
Buffer containing MgCl <sub>2</sub> (10x)	2
Forward primer (10 $\mu$ M)	2
Reverse primer (10 $\mu$ M)	2
dNTP mix (1 mM)	2
<i>Taq</i> polymerase (5 U/ $\mu$ l)	0.2
<b>Final volume</b>	<b>20</b>

The used PCR program is shown in Table 7.3.

**Table 7.3: Standard PCR program.**

Step	Temperature	Duration
Initial Denaturation	94 °C	3 min
Denaturation	94 °C	40 sec
Hybridisation } 30-40x	*	40 sec
Elongation }	72 °C	**
Terminal Elongation	72 °C	3 min

\* Hybridisation temperature was depending on the melting temperature of the used primer pairs.

\*\* Elongation time was depending on the length of the amplified PCR fragment. A synthesis rate of the *Taq* polymerase of 1,000 bp per minute was used to estimate elongation time.

The PCR products were analysed *via* agarose gel electrophoresis.

### 7.2.4.2 Colony check PCR

A colony check PCR was performed to analyse plasmids for ligated inserts. A standard PCR reaction of a final volume of 10  $\mu$ l was set up without template DNA. Single bacterial colonies carrying plasmids served as template. These were picked from agar plates and transferred directly to the reaction mix. The PCR was performed using the plasmid-specific SP6 and T7 primers at a hybridisation temperature of 52 °C for 40 cycles.

### 7.2.4.3 High Fidelity PCR

To analyse open reading frames of genes and for the amplification of DNA fragments for expression constructs, a DNA polymerase with proof reading ability (Platinum® *Taq* Polymerase High Fidelity, invitrogen) was used. The pipetting scheme for the reaction is shown in Table 7.4.

**Table 7.4: Pipetting scheme for high fidelity PCRs.**

Component	Volume [ $\mu$ l]
DNA + H <sub>2</sub> O	11
Buffer (10x)	2
MgSO <sub>4</sub> (50 mM)	0.8
Forward primer (10 $\mu$ M)	2
Reverse primer (10 $\mu$ M)	2
dNTP mix (1 mM)	2
Platinum® <i>Taq</i> Polymerase High Fidelity (5 U/ $\mu$ l)	0.2
<b>Final volume</b>	<b>20</b>

The used PCR program is shown in Table 7.5.

**Table 7.5: High fidelity PCR program.**

Step	Temperature	Duration
Initial Denaturation	94 °C	2 min
Denaturation	94 °C	40 sec
Hybridisation	*	40 sec
Elongation	68 °C	**
Terminal Elongation	68 °C	**

\* Hybridisation temperature was depending on the melting temperature of the used primer pairs.

\*\* Elongation time was depending on the length of the amplified PCR fragment. A synthesis rate of the *Taq* polymerase of 1,000 bp per minute was used to estimate elongation time.

### 7.2.4.4 3' Rapid Amplification of cDNA Ends (RACE) PCR

To elongate the sequence information of transcripts, RACE PCRs were performed. To obtain more sequence information in the 3' direction of transcripts, the cDNA synthesis described before was conducted with an adapter primer that contains a specific sequence 5' of the Oligo(dT) sequence. The 3' RACE PCRs were performed using high fidelity *Taq* polymerase in two steps. In the first step a transcript-specific forward primer was used together with a reverse primer hybridising to the adaptor sequence. In addition, two reactions were prepared that contained only one of both primers to exclude unspecific amplifications. The products were separated using agarose gel electrophoresis and specific fragments were excised, purified and served as templates for the second PCR step. In this semi-nested PCR a transcript specific primer located in 3' direction of the first specific primer is used together with the adaptor primer. The reactions containing one of both primers were prepared as well. The

products were analysed *via* agarose gel electrophoresis and the specific fragments were excised, purified, cloned and sequenced.

#### **7.2.4.5 5' RACE PCR using *trans*-spliced leader primers**

For the amplification of unknown 5' ends of partially sequenced transcripts, forward primers hybridising in the *trans*-spliced leader sequence were used together with transcript-specific reverse primers in a high fidelity PCR. As described before, two steps with two strand-specific primers were performed. The products were analysed *via* agarose gel electrophoresis and the specific fragments were excised, purified, cloned and sequenced.

#### **7.2.4.6 Semiquantitative reverse transcriptase PCR**

In order to compare expression levels of specific genes within different cDNA samples, like cDNA from MAMP treated animals, tumour bearing polyps or procaine-separated tissue, semiquantitative reverse transcriptase PCRs were performed using the standard PCR protocol. Before the first comparison of target gene transcript levels, the amounts of template cDNAs were equilibrated using primers for housekeeping genes, like *actin* (M32364) (Fisher and Bode, 1989) or *glyceraldehydes 3-phosphate dehydrogenase (gapdh)* (AF307863). Each time the housekeeping gene reactions were carried out together with the target gene reactions. For the tissue separation experiment the genes *nb042* (FJ232916, expressed in ectodermal nematoblasts) (Milde *et al.*, 2009) and *HyDkk1/2/4 C* (DQ127904, expressed in endodermal gland cells) (Augustin *et al.*, 2006) served as separation controls. Template master mixes were prepared to ensure an accurate mixture of all samples. The PCR cycles were adjusted to the different expression levels of the genes taking into account that they represent the exponential amplification phase. All reactions were conducted twice. The products were analysed using agarose gel electrophoresis.

#### **7.2.4.7 Quantitative real-time PCR (qRT-PCR)**

In order to analyse the expression levels of *HyNLR type 1* after MAMP injection, a quantitative real-time PCR (TaqMan) was performed according to the manufacturer's guidelines (Applied Biosystems Inc, USA) using a 7900HT Fast Real Time PCR-System. Expression levels of *HyNLR type 1* were compared to *actin*. Expression levels were calculated relative to *actin* using the standard-curve method (Livak and Schmittgen, 2001) and performed in duplicate.

### 7.2.5 Electrophoretic separation of DNA samples

DNA samples were separated using horizontal 1-1.5 % agarose gel electrophoresis in 1x TAE buffer. The gels contained 0.01 % ethidiumbromide. As size marker the “GeneRuler™ DNA Ladder Mix” (Fermentas) was used.

### 7.2.6 Purification or extraction of DNA fragments from agarose gel fragments

The desired PCR products or other DNA fragments were cut out on a UV illumination table and transferred into 1.5 ml reaction tubes. Afterwards DNA was extracted using the “NucleoSpin® Extrakt II” Kit by Macherey Nagel. The same kit was used for direct purification of PCR products. Both were performed according to the manufacturer’s protocol. DNA was eluted by adding 20 µl NE buffer to the silica column.

### 7.2.7 Ligation of PCR products into the pGEM®-T vector

For sequencing or probe generation for *in situ* hybridisation, PCR products were ligated into the pGEM®-T vector by promega *via* TA cloning. The *Taq*-polymerase generates adenosin (A) overhangs on the synthesised DNA strands. These “sticky ends” are used for the ligation into the vector carrying thymidin (T) overhangs. Reactions were set up as follows:

**Table 7.6: Pipetting scheme for the ligation of DNA fragments into the pGEM®-T vector.**

Component	Volume [µl]
pGEM®-T (50 ng/µl)	0.25
2x Buffer	5
T4-Ligase (3 U/µl)	1
DNA (4-70 ng)	3.75
<b>Final volume</b>	<b>10</b>

Ligations were carried out over night at 4 °C. Afterwards the samples were diluted 1:2 with Millipore water prior to a dialysis performed on a semi-permeable membrane filter disc on ice-cold water for 1 h.

### 7.2.8 Generation of electrocompetent *E. coli* cells

*E. coli* ElectroMAX DH5α-E cells (invitrogen) were stored at -80 °C and used as stock culture for the generation of electrocompetent cells. 1 µl of the stock culture was added to 20 ml SOB medium and cells were cultured overnight at 220 rpm and 37 °C. 6 ml of this culture were added to 450 ml SOB medium twice in parallel. The culture was grown at 220 rpm and 37 °C until the logarithmic growth phase was reached at OD<sub>600</sub> = 0.6. The cells were harvested by centrifugation for 15 min at 3,000 g and 4 °C. The pellet was washed in

ice-cold Millipore water and centrifuged at 3,000 g for 10 min. This step was repeated. During resuspension the cells were merged successively. The pellet was re-suspended within 36 ml of 15 % ice-cold glycerol. After a centrifugation step at 3,000 g for 10 min the pellet was dissolved in 3,600  $\mu$ l 15 % ice-cold glycerol and aliquots of 40  $\mu$ l were immediately frozen in liquid nitrogen prior to storage at -80 °C.

### **7.2.9 Transformation of *E. coli***

The cells containing 7  $\mu$ l of the dialysed ligation sample were electroporated with the “Gene Pulser II” (Bio-Rad) using the following conditions: 1.8 kV, 25  $\mu$ F. Immediately after electroporation the cells were rinsed with 1 ml pre-warmed SOC medium and incubated at 37 °C and 220 rpm for 1 h. 100 to 1000  $\mu$ l were plated on ampicillin-containing LB-agar. Only cells containing the plasmid providing resistance against the antibiotics were able to grow over night at 37 °C.

### **7.2.10 Preparation of plasmids**

#### **7.2.10.1 Mini-preparation**

For sequencing and probe generation, Mini-preparations were performed using the “NucleoSpin® Plasmid QuickPure” Kit (Macherey Nagel) according to the manufacturer’s instructions.

#### **7.2.10.2 Midi-preparation**

For subsequent injection of expression constructs into *Hydra* embryos a Midi-preparation was performed using the “QIAfilter™ Plasmid Midi Kit” (Qiagen) according to the manufacturer’s instructions. Afterwards an additional precipitation step was performed by the addition of 1/10 volume 3 M Sodium Acetate and 2 volumes of Ethanol. The tubes were inverted and centrifuged for 15 min at 20,000g at 4 °C. The pellet was washed in 300  $\mu$ l 75 % ethanol, the centrifugation step was repeated and the pellet was dried on air and dissolved in 75  $\mu$ l Millipore water over night at 4 °C.

#### **7.2.10.3 Maxi-preparation**

For the transfection of Hek293 cells expression constructs were isolated by a Maxi-preparation using the “QIAfilter™ Plasmid Maxi Kit” (Qiagen) according to the manufacturer’s instructions.

### 7.2.11 Sanger DNA sequencing

Two different protocols were used for the method originally published by Sanger *et al.* (Sanger *et al.*, 1977). 300 ng of Mini-prepped plasmid DNA served as template for all reactions.

#### 7.2.11.1 Sequencing using the LI-COR system

For the reaction the “SequiTherm EXCEL™ II DNA Sequencing Kit” (Epicentre) was used with IRD700® and IRD800® labelled 5' ends of T7 and SP6 primers as shown in Table 7.7:

**Table 7.7: Pipetting scheme for the LI-COR sequencing reaction.**

Component	Volume [ $\mu$ l]
DNA (300 ng) + H <sub>2</sub> O	8
Buffer (3.5x)	7.2
SequiTherm EXCEL II DNA Polymerase (5 U/ $\mu$ l)	0.8
IRD labelled primer (5 $\mu$ M)	1
<b>Final volume</b>	<b>17</b>

A total of 4  $\mu$ l of this mixture were added to 2  $\mu$ l of nucleotide mix containing ddATP, ddCTP, ddGTP or ddTTP respectively. The samples were subjected to the following PCR program:

**Table 7.8: PCR program for the LI-COR sequencing reaction.**

Step	Temperature	Duration
Initial Denaturation	95 °C	5 min
Denaturation	95 °C	30sec
Hybridisation	50 °C	15sec
Elongation	70 °C	1 min

The reaction was stopped by the addition of 3 $\mu$ l loading buffer. Prior to loading 0.7 $\mu$ l of the samples on a 41 or 66 cm sequencing gel in the “4300 DNA Analyzer” (LI-COR Biosciences) they were denatured for 10 min at 95 °C. The results were analysed using the e-Seq 3.0 program.

#### 7.2.11.2 Sequencing using the capillary system

For the reaction the “BigDye® Terminator v1.1 Cycle Sequencing Kit” (Applied Biosystems) was used as shown in Table 7.9:

**Table 7.9: Pipetting scheme for the BigDye® sequencing reaction.**

Component	Volume [µl]
DNA (300 ng) + H <sub>2</sub> O	6
SB buffer (5x)	1
BigDye	2
Primer (5 µM)	1
<b>Final volume</b>	<b>10</b>

The PCR was performed using the following program:

**Table 7.10: PCR program for the BigDye® sequencing reaction.**

Step	Temperature	Duration
Initial Denaturation	96 °C	1 min
Denaturation	96 °C	10 sec
Hybridisation	*	5 sec
Elongation	60 °C	4 min

\* depending on melting temperatures of used primers

For visualisation of sequencing results the “3730 DNA Analyzer” (Applied Biosystems) was used according to the manufacturer’s instructions.

## 7.2.12 Southern blotting

### 7.2.12.1 Isolation of genomic DNA from *Hydra*

For the isolation about 200 polyps were collected. After removing the *Hydra* medium 500µl Lysis Buffer were added and the animals were homogenised by using a plastic pistil. 50 µl of a 1 mg/ml Proteinase K solution were added and the homogenate was incubated at 50 °C for 2 h. An equal volume of phenol was added; the tube was inverted several times and centrifuged for 15 min at 11,000 g at room temperature. The supernatant was transferred into a fresh tube and the phenol step was repeated. The supernatant was transferred into a fresh tube and an equal volume of chloroform was added. The tube was inverted carefully and centrifuged at 11,000 g for 10 min. This step was repeated as well. The supernatant was transferred into a fresh tube and sodium chloride was added to a final concentration of 0.2 M prior to the precipitation of the DNA with two volumes of 96 % ethanol. The tube was inverted carefully until the DNA formed a white clot. This clot was transferred into a fresh tube and washed in 70 % ethanol. The ethanol was removed completely and the pellet was air dried. The DNA was dissolved over night at 4 °C in Millipore water. RNA contaminations were removed by a digestion with RNase A. 10 mg/ml were added prior to an incubation of 1 h at 37 °C. The quality of the DNA was determined *via* gel electrophoresis.

### 7.2.12.2 Restriction digestion of genomic DNA

A total of 20  $\mu\text{g}$  of the isolated DNA were digested using three different restriction enzymes independently. The pipetting scheme for the reaction is shown in Table 7.11.

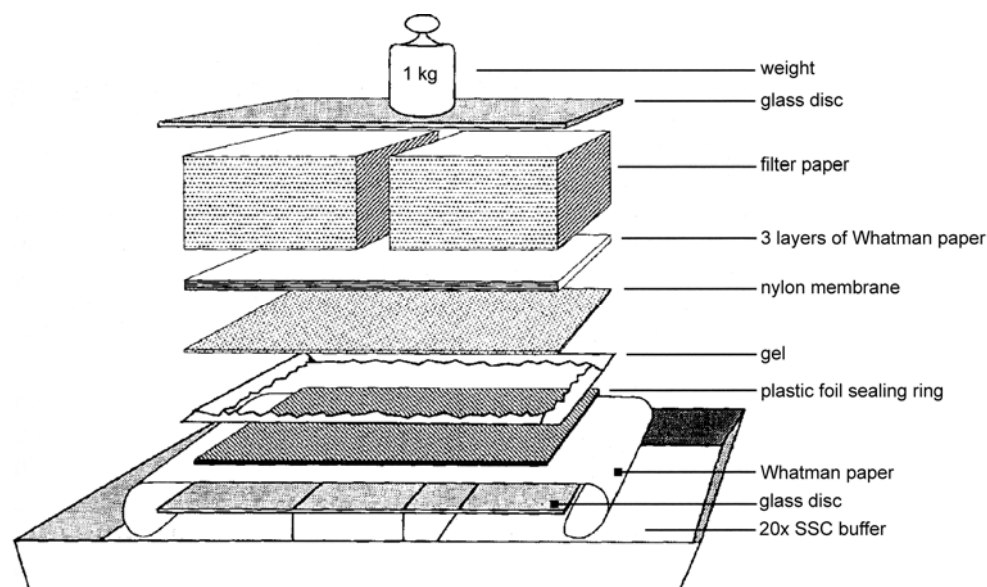
**Table 7.11: Pipetting scheme for the digestion of genomic DNA.**

Component	Volume [ $\mu\text{l}$ ]
Enzyme (HindIII/ XbaI/ EcoRV) (10 U/ $\mu\text{l}$ )	5
10x Buffer	5
DNA (20 $\mu\text{g}$ ) + H <sub>2</sub> O	40
<b>Final volume</b>	<b>50</b>

The digestion was performed at 37 °C for 2 h.

### 7.2.12.3 Southern blotting

The digested DNA was separated on a 0.6 % agarose gel using a low voltage. The gel was photographed and depurinated for 10 min with 0.2 M hydrochloric acid. The gel was briefly rinsed with water and the DNA was denatured by incubating the gel twice in denaturing solution for 20 min. Then the DNA was neutralised by two gel incubations in neutralisation solution for 20 min each. Afterwards the gel was rinsed with 20x SSC for 10 min. The DNA was transferred by capillary forces to a Hybond N+ membrane (GE Healthcare) using the construction shown in Figure 7.1 according to the method published by Southern (Sanger *et al.*, 1977).



**Figure 7.1: Schematic drawing of the Southern blot capillary transfer.** (Mikosch, 1999)



The DNA was blotted for 18 h. The filter was equilibrated for 5 min in 6x SSC and dried on air. The DNA was UV-cross-linked to the membrane using 130 mJ in the stratalinker. Afterwards the membrane was incubated at 80 °C for 45 min.

#### 7.2.12.4 Radioactive labelling of DNA probes

PCRs with sequence-specific primers were performed with cDNA or plasmids containing the desired insert to obtain the 300 to 400 bp templates for the labelling reactions. Purified PCR products served as template. Table 7.12 shows the pipetting scheme for the labelling of DNA with  $\alpha$ -<sup>32</sup>P-dATP.

**Table 7.12: Pipetting scheme for the generation of radioactive DNA probes.**

<b>Component</b>	<b>Volume [<math>\mu</math>l]</b>
DNA (100/150 ng) + H <sub>2</sub> O	72/62
10x buffer	10
Random hexamer primer (10 mM)	10
or	
Sequence specific primers (F+ R) (100 $\mu$ M)	10 each
dCTP/ dGTP/ dTTP mix (0.5 mM)	4
Klenow fragment (10 U/ $\mu$ l)	2
$\alpha$ - <sup>32</sup> P-dATP	2
<b>Final volume</b>	<b>100</b>

A total of 100 ng DNA (150 ng for the NACHT-probe) were mixed with random hexamer primers (or sequence specific primers for the NACHT-probe) and denatured at 95 °C for 5 min and cooled down slowly to room temperature. Afterwards the buffer, the dCTP/ dGTP/ dTTP mix, the Klenow fragment and the  $\alpha$ -<sup>32</sup>P-dATP were added. The synthesis of DNA strands was carried out at 37 °C for 3 h.

#### 7.2.12.5 Purification of radioactive probes

To remove non-integrated  $\alpha$ -<sup>32</sup>P-dATP and primers the probes were purified using Sephadex G-50 columns. A 1 ml syringe was filled with a plug of glass wool and Sephadex suspended in 1x TE buffer, after a centrifugation at 194 g for 1 min. After addition of the probe, the column was centrifuged at 1,700 g for 1 min. For the determination of the integration rate, 1  $\mu$ l of the probe was measured using a scintillation counter using the Cerenkov-method (Haberer, 1966) before and after the purification.

#### **7.2.12.6 Hybridisation of Southern blots**

The membranes were transferred into 50 ml tubes. Per cm<sup>2</sup> of membrane 0.2 ml of pre-heated hybridisation solution were added, containing denatured salmon sperm DNA (final concentration 100 µg/ml). The membranes were pre-hybridised for 1 h at 42 °C.

Per ml hybridisation solution one million counts of probe were added to the tube. Prior to the addition the probe was denatured at 95 °C for 5 min. The hybridisation was performed over night at 42 °C. Afterwards the excessive probe was removed by washing the membrane twice in washing solution 1 for 10 min at room temperature and once with washing solution 2 for 30 min at 65 °C. The membrane was shrink-wrapped and exposed to a Phosphoimager-screen for various incubation times prior to the detection in the “Phosphoimager FLA-5000”. After the detection of specific bands the membrane was exposed to an X-ray film at -80 °C. After several time points the films were developed.

#### **7.2.13 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)**

Polyps were relaxed for 2 min using freshly prepared urethane relaxing solution and fixed over night at 4 °C by adding an 8 % paraformaldehyde solution. Samples were transferred into 12-well plates and dehydrated for 2 min in 100 % ethanol and rehydrated with 50 % ethanol for 2 min. Afterwards they were washed twice in PBS for 5 min, twice in PBT for 15 min and twice in PBS for 10 min. Next, the samples were equilibrated in Terminal Deoxynucleotidyl Transferase (TdT) buffer (Fermentas) for 30 min prior to the labelling reaction using 5 µM DIG-dUTP and 150 U/ml TdT in TdT buffer for 2 h at 37 °C. The samples were washed in TBS for 1 min and incubated in 0.5 M EDTA (pH 8.0) to stop the labelling. Afterwards all samples were washed twice with PBS containing 1 mM EDTA for 1 min and heated to 65 °C for 30 min in PBS/EDTA to inhibit endogenous alkaline phosphatases. Animals were washed four times in PBS for 10 min each and once with PBT for 15 min prior to the incubation with blocking solution for 30 min. The binding of the anti-Digoxigenin-alkaline phosphatase linked Fab fragments (Roche, 1:4,000 in blocking solution) was performed over night at 4 °C. Afterwards animals were washed four times in PBT for 15 min to remove excess antibodies. The samples were equilibrated three times in NTMT for 5 min. The colour reaction was performed with NBT/BCIP (1:100 in NTMT) and stopped by exchanging the solution with water. Animals were dehydrated with increasing ethanol concentrations and embedded in Euparal.

### 7.3 *In situ* hybridisation

#### 7.3.1 Generation of a Digoxigenin-labelled RNA probe

The desired transcript fragments 300 to 400 bp length were cloned from cDNA into the pGEM®-T vector and sequenced afterwards. Depending on the direction of integration a PCR was performed with one of the vector-specific primers (T7/SP6) and one gene-specific primer.

A total of 0.5 µg purified PCR product were used as template for the “DIG RNA Labeling Kit (SP6/T7)” (Roche) according to the manufacturer’s protocol.

#### 7.3.2 Dot blot with the RNA probes

In order to analyse the accurate labelling of all RNA probes a dot blot was performed. The probe templates were diluted 1:10, 1:10<sup>2</sup>, 1:10<sup>3</sup>, 1:10<sup>4</sup>, 1:10<sup>5</sup> and 1:10<sup>6</sup> and 1 µl was spotted onto a Hybond N+ membrane. The membrane was dried and the DNA was UV-cross-linked to the membrane using 120 mJ in the stratalinker. The following steps were carried out at hybridisation temperature. The prehybridisation was conducted for 30 min with hybridisation solution prior to the hybridisation with a probe dilution of 1:10,000 for 4 h. Afterwards, the membrane was washed in 50 % hybridisation solution/50 % 2x SSC for 10 min and twice in 2x SSC containing 0.1 % CHAPS. The following steps were conducted at room temperature. The membrane was washed twice with MAB for 5 min and once for 10 min with MAB-B prior to the incubation with anti-Digoxigenin-alkaline phosphatase linked Fab fragments (dilution 1:2,000 in blocking solution) for 1 h. The excess antibody was removed by washing the membrane in MAB for 10 min and twice in PBT for 10 min. Afterwards the membrane was equilibrated in NTMT for 5 min prior to the staining reaction with 10 µl NBT/ BCIP per ml NTMT. The reaction was stopped in water.

#### 7.3.3 Whole-mount *in situ* hybridisation with *Hydra*

The protocol was adapted from previous works (Grens *et al.*, 1996; Philipp *et al.*, 2005).

##### Day 1

Only polyps that were starved for at least two days were used. Animals were relaxed in freshly prepared urethane relaxing solution for 2 min followed by a quick addition of a fresh 8 % paraformaldehyde in *Hydra* medium (pH 7.4). The solutions were replaced by a new 4 % paraformaldehyde solution prior to the overnight fixation at 4 °C.

### Day 2

Only RNase-free materials were used. The following steps were performed at room temperature. The animals were treated in 99 % ethanol for at least 10 min until the animals were completely decolourised. The samples were rehydrated by a treatment with 75 % ethanol, followed by a treatment with 50 % ethanol and a treatment with 25 % ethanol in PBT. All steps were carried out for 5 min. Afterwards, the samples were washed three times with PBT for 10 min followed by a treatment with Proteinase K in PBT (end concentration 10 µg/ml) for 20 min. The reaction was stopped by the addition of glycine working solution and the immediate exchange of this solution with new glycine working solution followed by 10 min of incubation. The samples were washed three times with PBT for 10 min and afterwards treated twice with triethanolamine for 10 min. The triethanolamine was exchanged and 2.5 µl/ml acetic anhydride were added. After 5 min additional 2.5 µl/ml acetic anhydride were added. After three washing steps with PBT for 5 min the animals were refixed with 4 % paraformaldehyde in PBT for 1 h. The solution was removed by washing the samples three times with PBT for 7 min. Afterwards the animals were washed twice in 2x SSC for 7 min followed by a heating step in 2x SSC for 15 min at 70 °C. The following steps were performed at hybridisation temperature, usually 56 °C. The animals were washed in 50 % 2x SSC/50 % hybridisation solution for 10 min, followed by a washing step in hybridisation solution for 10 min. Afterwards the samples were pre-hybridised for at least 2 h in hybridisation solution containing 20 µl/ml tRNA. Different pre-dilutions of the probes were used ranging from 1:1 to 1:10. 1 µl probe per ml hybridisation solution was denatured in 50 µl hybridisation solution at 70 °C for 10 min. The hybridisation solutions of the samples were replaced with new hybridisation solution with tRNA and the probe was added to a final concentration of 1:1000 to 1:10,000 and the samples were incubated for 36 h. A sense and an antisense probe were always used in parallel.

### Day 3

The probe was removed by the following 10 min washing steps: 100 % hybridisation solution, 75 % hybridisation solution/25 % 2x SSC, 50 % hybridisation solution/50 % 2x SSC and 25 % hybridisation solution/75 % 2x SSC. Afterwards the animals were incubated twice in 2x SSC containing 0.1 % CHAPS for 30 min.

The following steps were performed at room temperature. The samples were washed twice in MAB for 10 min followed by a 1 h treatment in MAB-B. The samples were incubated in blocking solution for 2 h at 4 °C. The binding of the anti-Digoxigenin-alkaline phosphatase linked Fab fragments (Roche, dilution 1:2,000) was performed overnight at 4 °C.

#### Day 4

The samples were washed with blocking solution for 20 min, followed by a washing step with MAB-B for 30 min. After six additional washing steps with MAB, the samples were rinsed with NTMT for 5 min and treated with NTMT containing 1 mM Levamisole for 5 min. The solution was replaced by the staining solution containing 10 µl NBT/BCIP per ml NTMT with 1 mM Levamisole. The samples were incubated in the dark until a clear staining was detectable. The reaction was stopped by exchanging the staining solution with Millipore water twice. The animals were dehydrated with increasing amounts of Ethanol prior to embedding in Euparal.

### **7.4 Analysis of *Hydra*-associated bacteria**

#### **7.4.1 Extraction of DNA from *Hydra*-associated bacteria**

Genomic DNA was extracted from 10 *Hydra* polyps using the “DNeasy Blood & Tissue” Kit (Qiagen). Prior to DNA extraction, the animals were washed three times with sterile filtered *Hydra* medium. The animals were lysed in 180 µl of ATL buffer containing 20 µl Proteinase K for 3 to 6h at 56 °C. After the addition of 200 µl of AL buffer the samples were incubated for 10 min at 70 °C. 200 µl of 100 % ethanol were added and the lysates were purified using DNeasy columns and eluted with 100 µl AE buffer.

#### **7.4.2 Phylogenetic 16S rDNA analysis**

Bacterial 16S rRNA gene fragments were amplified *via* standard PCR (30 cycles) using the Eub\_27F and Eub\_1492R primers (Weisburg *et al.*, 1991). The products were cloned into pGEM®-T vector and transformed into *E. coli* DH-5α as described before. The inserts were checked by performing a colony-check PCR. The PCR products were precipitated over night using sodium acetate and two volumes of ethanol. The samples were centrifuged at 4,000 g for 30 min and 4 °C. The pellet was dried on air and resuspended to subject them to restriction fragment length polymorphism (RFLP) analysis using the restriction enzymes *Bsu*RI and *Hin*6I. The products were separated on a 1.2 % agarose gel. Representative plasmids were sequenced as described before. The sequences were subjected to the chimera check program Pintail to estimate chimeric sequences (Ashelford *et al.*, 2005). Closely related sequences were identified by using the “Sequence match” of the ribosomal database project. An alignment was produced using Bioedit (Hall, 1999). The alignment was used to create a neighbor-joining tree (Saitou and Nei, 1987) by using MEGA4.0 (Kumar *et al.*, 2008; Tamura *et al.*, 2007) with a bootstrap re-sampling of 1,000 replicates.

### 7.4.3 Fluorescence *in situ* hybridisation (FISH)

Macerates were produced as described before. Hybridisations of fixed *Hydra* cells were done according to the protocol used by Manz *et al.* with fluorescent-labelled rRNA oligonucleotide probes (Manz *et al.*, 1992): a universal eubacterial probe (EUB338, 5' labelled with fluorescein) and a probe specific for the *Hydra oligactis* endosymbiont (HoSym1030 *E. coli* positions 1030-1048, 5' labelled with Cy3) (Fraune and Bosch, 2007). Hybridisations were performed at 46 °C for 90 min and washed at 48 °C for 15 min. The formamide concentration in the hybridisation buffer was varied between 0 and 30 %, while the sodium chloride concentration in the post-hybridisation buffer was adjusted accordingly. The fluorescence signal by probe HoSym1030 to target cells was stable; the intensity of this signal was stable between 0 and 20 % formamide and decreased slightly at 30 % formamide. With non-target cells, there was no signal even under low-stringency conditions (no formamide). Therefore, 10 % formamide was used routinely for single hybridisations and for double hybridisations with EUB338. Afterwards the samples were stained with Hoechst and mounted with DABCO/Mowiol. Samples were analysed using the confocal laser scanning microscope.

## 7.5 Overexpression of HyNLR type 1 in *Hydra*

### 7.5.1 Generation of expression constructs for *Hydra vulgaris* (AEP)

Three expression constructs were generated for *Hydra*. The first construct (HyNLR type 1 short::FKBP<sub>2</sub>-HA) contained the DEATH domain coding part of *HyNLR type 1* fused to two FKBP coding subunits fused to a hemagglutinin (HA)-tag coding sequence of the pC<sub>4</sub>M-F<sub>v</sub>2E of the “ARGENT™ Regulated Homodimerization Kit” (Ariad). The sequences were codon-optimized for the expression in *Hydra* by GENEART (hydeath-fkbp, vector backbone pGA4) and flanked by an *FseI* and *EcoRI* cutting site for the site-directed mutagenesis into the ligR1 expression vector provided by Dr. K. Khalturin having the pGEM®-T vector as backbone. The DEATH and FKBP sequence were separated by a *PsyI* cutting site for the insertion of the NACHT-coding part of *HyNLR type 1* in order to obtain the HyNLR type 1 long::FKBP<sub>2</sub>-HA. The NACHT coding part was amplified from cDNA with primers containing the *PsyI* cutting sites. The third construct (FKBP<sub>2</sub>-HA) contained the two FKBP subunits amplified with *FseI* and *EcoRI* cutting site containing primers from the GENEART vector. The NACHT and FKBP sequences were sub-cloned into the pGEM®-T vector and electroporated into *E. coli*. Afterwards, Mini-preparations were performed. The same was done with the GENEART vector. The NACHT sequence containing plasmid was cut with *PsyI* and the NACHT sequence was inserted into the GENEART vector using T4 ligase (Fermentas). The three different vectors were cut with *FseI* and *EcoRI* and the inserts were ligated into the ligR1 vector thereby exchanging the DsRed coding sequence. Now all coding sequences were under

the control of a *Hydra actin* promoter followed by the first 10 amino acids of actin and an *actin* terminator. All expression vectors were proofed by sequencing. Prior to the injection into *Hydra* embryos Midi-preparations of all plasmids were performed as described before.

### **7.5.2 Generation of transgenic *Hydra* polyps**

Transgenic polyps were generated using the method published by Wittlieb *et al.* (Wittlieb *et al.*, 2006). A plasmid concentration of 2 µg/µl was used and 1 % Rhodamine B Isothiocyanate-Dextran R9379 (Sigma) were added as tracer. The solution was injected into *Hydra* embryos within the 1 to 2 cell developmental stage.

### **7.5.3 Western blotting with *Hydra* protein extract**

#### **7.5.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

A total of 10 *Hydra* polyps of each tested line were lysed in 100 µl 2x loading buffer containing 20 µl/ml β-mercaptoethanol by denaturing them for 10 min at 99 °C and subsequent vortexing. Prior to loading them on the 12 % TRIS-glycine gel together with the broad range “Prestained Protein Marker” (New England Biolabs), the probes were centrifuged at 10,000 g. 20 µl of each probe were loaded following the method published by Laemmli (Laemmli, 1970). The run was started at 40 mA, when the bands reached the border between the stacking and the separating gel the amperage was raised to 100 mA. Two gels were run in parallel and the first gel was stained in Coomassie solution directly afterwards, whereas the second gel was used for Western blotting.

#### **7.5.3.2 Western blotting**

The gel was placed in transfer buffer for 15 min without shaking. A PVDF-membrane was rinsed with methanol for 1 min and afterwards placed into the transfer buffer together with the Whatman papers. Proteins were blotted using a semidry electro blotting device with amperage of 3 mA/cm<sup>2</sup> for 80 min. After blotting the gel was stained with Coomassie to determine the amount of transferred protein. The membrane was dried. After a treatment with methanol for 1 min and 5 min with transfer buffer the membrane was placed in TBST buffer for 5 min. The blocking was carried out in TBST containing 4 % milk powder at 4 °C over night. Afterwards the membrane was washed four times in TBST for 5 min. The membranes were incubated with the primary antibody (“Anti HA High Affinity” antibody (Roche) diluted 1:1,500 in blocking solution) for 1 h. By several washing steps (twice for 10 sec, once for 10 min and three times for 5 min) with TBST the excess first antibody was removed. Afterwards, the membrane was exposed to the horseradish peroxidase-conjugated secondary antibody (“Peroxidase AffiniPure Goat Anti-Rat IgG” (Jackson ImmunoResearch) dilution 1:10,000 in

TBST) for 1 h. This antibody was removed using the same washing steps as before. The proteins were detected using the “SuperSignal West Pico Chemiluminescent Substrate” (Thermo Scientific) according to the manufacturer’s instructions. The signal was detected by exposing the membrane to the “Amersham Hyperfilm ECL” (GE Healthcare) for several time points ranking from 1 min to 1 h.

## 7.6 Heterologous expression of *Hydra* genes in HEK293 cells

### 7.6.1 Generation of the HEK293 cell expression constructs

For heterologous expression in HEK293 cells, three different expression constructs were generated. The first expression construct contained the long variant of the *HyNLR type 1* fragment of the *Hydra* expression construct fused to two FKBP subunits (see section 7.5.1). *Via* Gateway® recombination cloning the insert should be transferred into the pCEV-GwA vector. Therefore the insert was amplified with gateway primers and sub-cloned into the pDONR™221 vector (Invitrogen) using the “Gateway® BP Clonase® II enzyme mix” (Invitrogen) according to the manufacturer’s protocol. Afterwards the insert was transferred into the pCEV-GwA expression vector using the “Gateway® LR Clonase® II enzyme mix” (Invitrogen) according to the manufacturer’s guidelines and thereby fused to an N-terminal Flag-tag.

The *HyDD-Caspase* and the *HyDED-Caspase* were amplified from plasmids containing the entire open reading frame with restriction site containing primers. The open reading frames were ligated into the pCMV-Myc vector (clontech) using T4 ligase and thereby fused to N-terminal Myc-tags using the *EcoRI* and *XhoI* cutting sites. A Maxi-preparation was conducted prior to transfection of the HEK293 cells.

### 7.6.2 Transfection of HEK293 cells

HEK293 cells were grown for 24 h prior to transfection *via* lipofection. 3 µg of plasmid DNA (Flag-*HyNLR type 1::FKBP<sub>2</sub>*, Myc-*HyDD-Caspase*, Myc-*HyDED-Caspase*) were mixed with 12 µl FuGENE®6-reagent and 100 µl of FCS-free DMEM-medium were added. The solutions were incubated for 30 min and added to the HEK293 cells after a replacement of the culture medium. The cells were incubated for 24 h at 37 °C and 5 % CO<sub>2</sub>.



### **7.6.3 Coimmunoprecipitation**

#### **7.6.3.1 Addition of AP20187 to the HEK293 cells**

Various amounts of AP20187 (final concentrations: 1  $\mu$ M, 0.1  $\mu$ M, 10 nM and 1 nM) were added to oligomerize and activate the FKBP-linked proteins. The cells were incubated at 37 °C and 5 % CO<sub>2</sub> for 24 h.

#### **7.6.3.2 Coimmunoprecipitation**

Transfected HEK293 cells were washed with PBS and rinsed with 500  $\mu$ l RIPA buffer containing phosphatase- and protease-inhibitors (phosphatase-inhibitor: 1:100, protease inhibitor: 1 tablet per 10 ml RIPA buffer). Cells were shaken for 15 min at 4 °C using a plate shaker. The free-floating cells were transferred into a tube and vortexed three times for 45 sec. The samples were centrifuged at 18,500 g and 4 °C for 15 min. The supernatant was transferred into two fresh tubes; the first sample was used for immunoprecipitation whereas the other one served as control extract. A preclearance step was performed with one sample by the addition of 10  $\mu$ l “Protein A/G PLUS-Agarose” (Santa Cruz Biotechnology) and subsequent rotation of the probe on a roll-incubator for 90 min at 4 °C to remove the proteins that bind unspecifically to agarose beads. The samples were centrifuged for 5 min at 425 g and 4 °C and the supernatant was transferred into a fresh tube. Afterwards the precipitation antibody (“Myc-Tag Rabbit mAb”, Cell signalling technology, dilution 1:1,000) was added and the samples were rotated for 1 h at 4 °C. 20  $\mu$ l Protein A/G PLUS-Agarose were added and the samples were rotated over night at 4 °C. After a centrifugation step for 5 min at 425 g the supernatant was discarded and the pellet was washed five times with 500  $\mu$ l RIPA buffer for 20 min at 4 °C.

#### **7.6.3.3 SDS-PAGE**

The agarose beads were centrifuged for 5 min at 425 g and 4 °C and resuspended in 20  $\mu$ l loading buffer, then heated to 100 °C for 10 min. Afterwards they were centrifuged at 425 g for 1 min and 12  $\mu$ l supernatant were loaded on the “NuPAGE® 4-12 % Bis-Tris Gel” (Invitrogen) together with control extracts and the “Precision Plus Protein Dual Color Standard” (Bio-Rad). The gel electrophoresis was conducted at 50 mA for 90 min.

#### **7.6.3.4 Western blotting**

The “Amersham Hybond-P” membrane (GE Healthcare) was rinsed for 10 sec in methanol and placed in Millipore water for 5 min. Three pieces of “Extra thick filterpaper” (Invitrogen) were placed in cathode buffer, anode buffer I and anode buffer II respectively. The anode

buffer II filter was placed at the base of the semidry blotting aperture, followed by the anode buffer II filter, the membrane, the separating gel and the cathode buffer filter. Blotting was conducted with  $0.8 \text{ mA/cm}^2$  and 300 V for 2 h.

After blotting the membrane was placed into a 50 ml-tube for blocking with TBST containing 5 % milk powder for 1 h prior to the incubation with the primary antibodies (“Myc-Tag Rabbit mAb” (Cell Signalling Technology), “Anti-FLAG® M2 Antibody” (Sigma-Aldrich)) in a dilution of 1:1,000 in TBST containing 5 % milk powder. One short and three 20 min washing steps with TBST removed excess antibodies. The incubation with the secondary antibody (“ECL™ Rabbit IgG Horseradish Peroxidase-linked Whole Ab” or “ECL™ Mouse IgG Horseradish Peroxidase-linked Whole Ab” (GE Healthcare), dilution 1:2,000) was conducted for 2 h at room temperature. After the same washing procedure as described before, the detection was performed using the “Amersham ECL Plus™ Western Blotting Reagent Pack” and the “Amersham Hyperfilm ECL” (both GE Healthcare) according to the manufacturer’s instructions for various exposure times.

#### **7.6.4 Annexin V staining and quantification of HEK293 cells**

HEK293 cells were co-transfected with the HyDD- or HyDED-Caspase and HyNLR type 1::FKBP<sub>2</sub> expression constructs. Four samples were conducted in parallel within a 24-well plate. Cells were pre-treated with or without 100 nM AP20187 as described before. For the detection of apoptosis, the “FITC Annexin V Apoptosis Detection Kit I” (BD Pharmingen™) was used. Cells incubated in 1  $\mu\text{M}$  Staurosporin for 2-4 h were used as positive control. Cells were washed in 1 ml PBS and were detached by pipetting up and down and transferred into a 15 ml-tube. 5 ml PBS were added for washing. Cells were centrifuged for 5-10 min at 300 g. The supernatant was replaced with the staining solution containing 200  $\mu\text{l}$  Annexin V binding buffer, 5  $\mu\text{l}$  FITC-Annexin V and 10  $\mu\text{l}$  propidium iodide staining solution. The samples were transferred into FACS tubes and incubated for 10 min in the dark. Afterwards 300  $\mu\text{l}$  PBS were added. The amount of Annexin V positive cells was determined using the “FACSCalibur” flow cytometer (BD Biosciences). First the forward scatter and the side scatter of the cells were used to gate the main cell population. The total amount of apoptotic cells per 10,000 showing a FITC-Annexin V and a low propidium iodide staining was determined. The mean values and standard deviations were calculated. A student’s t test was performed for the HyNLR type 1::FKBP<sub>2</sub>/ HyDED-Caspase expressing cells.

## 7.7 Histological methods

### 7.7.1 Maceration of *Hydra* cells

Single dying tumour bearing (2) and control polyps (2) were placed in a tube and the medium was replaced by 50  $\mu$ l maceration solution as described previously (David, 1973). They were incubated in a water bath at 32 °C for 30 min. The cells were dissociated by flipping the tube. The cells were fixed by the addition of an equal volume of an 8 % paraformaldehyde solution. Aliquots of the suspension were placed onto gelatine-covered glass-slides with a droplet of a 1 % Tween20 solution. Slides were air-dried over night and embedded with PBS/glycerol for microscopic analysis. Per animal the amount of interstitial cells per 100 epithelial cells was counted twice.

### 7.7.2 Preparation of *Hydra* tissue for transmission electron microscopy

Polyps were relaxed in freshly prepared urethane relaxing solution and fixed in 3.5 % glutaraldehyde in 0.05 M cacodylate buffer containing 10 mM MgCl<sub>2</sub> (pH 7.4) for 18 h at 4 °C. After a washing step using 0.075 M cacodylate buffer for 30 min the animals were post-fixed with 1 % OsO<sub>4</sub> in 0.075 M cacodylate buffer for 2 h at 4 °C. The animals were washed for 30 min prior to the 15 min dehydration steps using 30, 50, 60, 70, 80, 90, 100 and 100 % ethanol. After incubating the samples twice in 1,2-propylene oxide for 20 min they were infiltrated with agar 100 resin. The components were mixed according to the manufacturer's descriptions for the "hard" version. Several 30 min steps of infiltration were performed using 1,2-propylenoxide and agar 100 resin in mix ratios of 1:2, 1:1 and 2:1 followed by an over night incubation with pure agar resin at room temperature. Afterwards the samples were placed into rubber forms containing fresh resin and polymerized at 60 °C.

#### 7.7.2.1 Semi-thin sections and staining

Tissue blocks were trimmed using a razor blade prior to cutting with the Ultracut S ultratome (~ 0.5  $\mu$ m thickness). The sections were placed on a glass slide and stained as described by Richardson *et al.* (Richardson *et al.*, 1960) at 60 °C for 1 to 2 min. Other sections were stained with Sudan Black B according to Lison for 5 to 10 min and washed with water (Lison, 1934).

#### 7.7.2.2 Ultra-thin sections and contrasting

Ultra-thin sections (thickness 60 to 70 nm) were cut with the Ultracut S ultratome and placed on round grids pre-treated with 1.2 % pioloform dissolved in chloroform. The sections were contrasted using 2.5 % uranylacetate for 5 min, rinsed with water and treated with freshly

prepared lead citrate solution for 3 min (Reynolds, 1963) and analysed using the transmission electron microscope EM 208 S.

### 7.7.3 Immunohistochemical staining of proteins in *Hydra*

Animals were relaxed in freshly prepared urethane relaxing solution for 2 min and fixed by the addition of 8 % paraformaldehyde in *Hydra* medium for 1 h. During fixation some animals expressing the HA-tagged protein were cut longitudinally. Afterwards, animals were washed four times in PBT for 15 min followed by a washing step in PBS with 0.5 % TritonX100 for 30 min. A blocking was performed in PBT with 1 % bovine serum albumin (BSA) for 1 h. The incubation with the primary antibody was performed over night at 4 °C. The following dilutions were used: For the periculin 1a labelling: 1:500 dilution of the “antiserum N31” (Klimovich); for the detection of the HA-tagged protein: 1:200 dilution of the “HA-Tag (6E2) mouse monoclonal antibody” (Cell Signaling Technology®). The samples were washed four times in PBT containing 1 % BSA for 15 min. Afterwards the samples were treated with the fluorescence-labelled secondary antibody (for the  $\alpha$ -periculin 1a antiserum: Alexa Fluor® 488 donkey anti-mouse IgG: 1:1,000; for the  $\alpha$ -HA antibody: Cy3 AffiniPure Donkey Anti-Mouse IgG, dilution 1:1,000) in PBT with 1 % BSA for 2 h. The samples were washed four times with PBS containing 0.5 % Tween20 and 1 % BSA for 15 min. In the case of the periculin 1a staining the animals were treated with phalloidin (1:1,000 in PBS containing 1 % BSA and 0.5 % TritonX100 and Tween20) for 1 h. Afterwards, these samples were treated three times with PBS containing 1 % BSA and 0.5 % Tween20 for 10 min. All samples were treated with Hoechst (dilution 1:500 in PBS containing 1 % BSA and 0.5 % Tween20) and were directly embedded in DABCO/Mowiol and stored at 4 °C prior to microscopical analysis using the confocal laser scanning microscope.

## 7.8 Computational analyses

### 7.8.1 Analysis of DNA sequences

The program DNAMAN 4.15 was used for general small scale sequence analysis like assemblies, alignments or the analysis of open reading frames.

### 7.8.2 Screening for NLR orthologues within annotated genomes

The genomes of *Xenopus tropicalis*, *Oryzias latipes*, *Gallus gallus*, *Petromyzon marinus*, *Nematostella vectensis* and *Daphnia pulex* were screened by hand via TBLASTN searches (Altschul *et al.*, 1990) with mammalian NOD-like receptors and APAF1 and their NBD domains, with the NLR orthologue of *Acropora millepora*, and by text searches containing

the keywords “NOD”, “NLRC”, “NLRP” or “NLRX”. All hits were controlled manually by checking their ORFs, domain compositions and best BLAST hits using DNAMAN 4.15, SMART (Letunic *et al.*, 2006) and BLASTP. In *Nematostella* the annotated gene models from Stellabase (Sullivan *et al.*, 2006) were screened for NACHT domains, LRRs and effector domains in addition. The hits were matched to the genome screening afterwards.

### **7.8.3 Screening for NACHT domains in the whole genome shotgun (WGS) reads of *Amphimedon queenslandica***

Using a NACHT domain consensus sequence from *Nematostella vectensis* a TBLASTN search was performed on a local installed WGS database of *Amphimedon queenslandica* using the compagen server. The hits with sequence identity of more than 94 % were assembled using the MINIMUS assembler included in the AMOS package (Sommer *et al.*, 2007). Only contigs consisting of two or more reads were taken into consideration for further analysis. The ORFs were determined manually using DNAMAN and NACHT domains were predicted using SMART.

### **7.8.4 Screening of *Acropora millepora* ESTs**

A local installation of *Acropora millepora* ESTs on the compagen server and ESTs of the workgroup of D. J. Miller were screened for NLR orthologues *via* TBLASTN and HMMER (Eddy, 1996) searches with the single domains of mammalian NLRs. All hits were screened for ORFs and the predicted domains. Best BLAST hits were obtained manually using SMART and BLASTP.

### **7.8.5 Screening of the *Hydra magnipapillata* databases for NBD coding genes**

Two approaches were performed to detect *Hydra* NLR orthologues. To obtain a first overview, the *Hydra magnipapillata* EST were screened for effector, NACHT and NB-ARC domains and LRRs *via* TBLASTN searches with vertebrate NLR and APAF1 domains and *via* HMMER analysis. Second, the *Hydra magnipapillata* hydrazome genome browser was screened using the identified ESTs for further NBD-domain coding genes. To estimate the entire NACHT and NB-ARC coding gene repertoire, a TBLASTN screening was performed on the non-assembled whole genome shotgun reads using the *Nematostella* NACHT consensus sequence and one NB-ARC sequence (connected with a TPR) as query using compagen. All hits were assembled using MINIMUS to obtain haplotype representatives. The hits were matched to the *Hydra* genome “Celera Assembler” assembly (Chapman *et al.*, 2010) *via* BLAST-searches and all scaffolds were screened for protein domains *via* InterProScan (Zdobnov and Apweiler, 2001). All effector domains with the right orientation (5' of the NBD

domain and encoded by the same strand) and TPRs (3' of the NB-ARC domain and encoded by the same strand) were taken into consideration.

Extensive searches for LRRs connected to NACHT domains were performed with different approaches: (i) the NACHT-coding genome scaffolds were screened for LRRs *via* InterProScan, (ii) TBLAST searches on the *Hydra* genome were performed using all published *Strongylocentrotus purpuratus* NLRs clearly connected to LRRs (Hibino *et al.*, 2006) as query sequence, (iii) PCRs were performed using primers hybridising in weakly predicted LRR regions in 3' orientation to NACHT-coding genome sequences and (iv) PCRs were performed to connect NACHT-encoding ESTs with LRR-encoding EST.

#### **7.8.6 Screening of the *Hydra magnipapillata* databases for NLR interaction partners**

All ESTs coding for effector domains not connected to a NACHT domain were used to find putative interaction candidates for *Hydra* NLRs. They were matched against the hydrazome genome browser to identify putative further domains. Screenings for RIPK1 or 2 orthologs were performed *via* TBLASTN searches with the human protein sequences and *via* screening all *Hydra* genome scaffolds for protein kinase and effector domains using InterProScan. No true orthologs were detected. Similar searches were performed for caspase genes coding for effector domains. Using the assembled second generation *Hydra* transcriptome assembly of various species (Compagen-NG), transcripts coding for effector domains connected with kinase domains were identified *via* TBLASTN searches with RIPK1 and 2. The sequences of the *HyDD-Caspase* and the *HyDED-Caspase* were provided by Prof. A. Böttger. All other interaction partners were detected by TBLASTN analysis using the hydrazome genome browser and human genes as query sequence.

#### **7.8.7 Calculation of phylogenetic trees**

The *Hydra* NBD-domain tree was calculated using MEGA 3.0 (Kumar *et al.*, 2008) using the neighbor-joining algorithm (Saitou and Nei, 1987) and the amino acid alignments of completely predicted NBD-domains. These alignments were produced using MUSCLE and Jalview. Afterwards the branches were replaced manually by triangles to obtain a graphic overview of the different NBD-containing protein types in *Hydra*.

For the calculation of the NACHT-domain tree SMART-predictions of rather complete NACHT-domains were used. For organisms with a large amount of NLR proteins (*Hydra magnipapillata*, *Amphimedon queenslandica*, *Nematostella vectensis*) previous trees were calculated on amino acid alignments using the ClustalW Multiple Alignment Tool (Thompson *et al.*, 1994) of BioEdit (Gap open: 10; Gap extend: 2) (Hall, 1999) and the neighbor-joining

algorithm of MEGA 4.0 (Tamura *et al.*, 2007) (Bootstrap:1,000; Poisson correction model; complete deletion). Representatives of all groups supported with a bootstrap value > 50 % were selected. For already published NLRs of *Strongylocentrotus purpuratus* and *Branchiostoma floridae* representatives were selected on the base of the published trees (Hibino *et al.*, 2006; Huang *et al.*, 2008). These prototype amino acid sequences were aligned by hand on the basis of the NACHT domain alignment published by Koonin and Aravind (Koonin and Aravind, 2000). As out-group the NB-ARC domain of an R protein (RPS4) of *Arabidopsis thaliana* was selected. The NACHT domains of *Daphnia pulex* were left out for the final tree because they appeared to produce too much noise. For the calculation of the tree a local install of MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) was used. 2,600,000 generations were calculated using the GTR model and four chains with a burnin of 25 % and the invgamma rate variation. Only posterior probabilities > 50 % are shown. The tree was visualised using FigTree1.3.1 (Rambaut, 2006).

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## 9 APPENDICES

### 9.1 NLR orthologues of the screened animal species

#### 9.1.1 Genome hits for NLR orthologues

Table 9.1: List of all NBD coding sequences in the screened genomes.

see CD-ROM

#### 9.1.2 NLR orthologues in *Acropora millepora*

see CD-ROM

#### 9.1.3 NACHT domains in *Amphimedon queenslandica*

see CD-ROM

#### 9.1.4 NACHT consensus sequence from *Nematostella vectensis*

```
RSVLLLEGDSGAGKTTTLTKKLASDWAKGVLSGSSTFPEVELLLVLKCEMKEGNEG GGI FQAIQEQLLP EEITVEE  
RLVIFQYIKHNQ GKVMVILDGLDEIP SQANSVKESLNKVISRNALALS YILVTSRPDKTLYRHFVGGN IILQIKGL  
CNVDDYISNFFPSDPDY
```

### 9.2 Bayesian inference analysis

#### 9.2.1 NACHT domains used for the alignments

see CD-ROM

#### 9.2.2 Alignment used for the phylogenetic tree

see CD-ROM

#### 9.2.3 Representative alignment for the phylogenetic tree





## 9.4 *Hydra* NBD containing transcripts

### 9.4.1 *Hydra* NLR transcripts

#### 9.4.1.1 HyNLR type 1

##### Coding sequence

see CD-ROM

##### Genomic Sequence

see CD-ROM

##### Translation

688 AA, MW=81221

```

1      AGTTAAAATGGATGAAAACATTCTTCAAAATCAACAGAATAGGTATCTATTTAGTCGAAG
1      M D E N I L Q N Q Q N R Y L F S R S

61     TGTCGGAATAGATTGAAAAGACCTTGGACGATGTTTACAAATTTTAGATAGCTATTTAGA
19     V G I D W K D L G R C L Q I L D S Y L D

121    TGTGATAGATATTGAAATCTAAAAATTGTTGTGATAAAGCATATCAAATGTTGACCAAATG
39     V I D I E S K N C C D K A Y Q M L T K W

181    GACAGAAAAGGAAAATCCATCATTAAATGAACTAAAAATGCTTTGCAAATATGGAGAA
59     T E K E N P S L N E L K N A L Q T M E K

241    AAACGATTTGATGAAAACATTGGAAGATCTTCAAAATACGTTTCATCAAAAAGAAATTGAAA
79     N D L M K T L E D L Q I R S S K E I E K

301    AAGTAATTTAGTCAAGACAGATTTCTTGAGTGATGCGCTGAAAAATTTTATCTCGAAAC
99     S N L V K T D F L S D A L K N F Y L E T

361    TTATAAAACAATTGAGGAAATTCACCACAATTAACAACCTTATCAAGTTAACTTTTTT
119    Y K T I E E I Q P Q L N K P Y Q V N F L

421    AGATAAATTTGTTGATCTTTATGTTGTTGATGAAGTTGAAGTCCAAAAAGATGCAATTA
139    D K F V D L Y V V D E V E V Q K D A I N

481    TATTGCAGAGCTAGATCAATTTTTAAAGAAACAAATGAGTTATACATCAATTCTATTTGA
159    I A E L D Q F L K K Q M S Y T S I L F E

541    AAAGCATTTAAAAGATCTGTCTTTATTTCTAATATCTGGTATTGCTGCTATTGGTAAAA
179    K H L K D L S L F L I S G I A A I G K T

601    ATGGTTGCTTAGAAAAATTTTACTAGATTGGTCAAATGGTTTTATTTGGAAAAATATTGA
199    W L L R K F L L D W S N G F I W K N I D

661    TTTAGTGTTTTACTTGAGAGTGAAACAACCTTAATTTGTATGAAAATATTTCTAATATTAA
219    L V F Y L E C K Q L N L Y E N I S N I N

721    TGAATTAAGTATGTTTCTACAAAGATATTTTAAAAGGTTATAATATTTCTTTAGATT
239    E L L D V F Y K D I L K G Y N I S L D F

781    CATGCAATCTAAACCATCAATTATGTTTATAATTGATGGCTTAAACGAATTTAAATACTT
259    M Q S K P S I M F I I D G L N E F K Y F

841    TGACCAATTAATAAGCAATACACACTGTAGTTCACAAGAAATTCCTATCTTAATGTCTT
279    D Q L I S N T H C S S Q E I P I L N V F

901    TACAGAAATTTATAAATACAAAGCTGTGATATCAGGAAGAGTAAATACAATATCACAATA
299    T E I Y K Y K A V I S G R V N T I S Q Y

961    TGAAAAATGTGGTCACGCGTTATAAAGATAAGTTAACCATTAGAGTTATGGGTTATAATGA
319    E N V V T R Y K D K L T I R V M G Y N E

```

1021 AAATGGAATAAAATATTATTTGAGGAATAATTTAATAAAAAAACAAAACGCAGAGTGAA  
339 N G I K Y Y L R N N L I K K T K R R V K

1081 AACTATGTTAAAAGAGTCTTCGATTGCAAAAACCATGGCAAAAGTACCCTTTTTTTTATC  
359 T M L K E S S I A K T M A K V P F F L S

1141 ATGTATGTGTACAATTGTTGCTGATTTAAAAGTAAAATATAACTTTATAAATCTCACTAT  
379 C M C T I V A D L K V K Y N F I N L T M

1201 GACAGATTTGTATGCATATAATTTTTTATACTTTTTTCAAAGACACATTTTAAATGAAA  
399 T D L Y A Y N F L Y F F Q R H I F N E N

1261 TCATGAACCAATTTTCAAATAATGGAAAATAATCTAAACAAGCAATGTATTTTAAACAT  
419 H E P I F K I M E N N L N K Q C I L N I

1321 ATGTAAAATGGCATATATTTTTTTTATTGAAAATAAACTACTTTTTCCGATATAGAAAT  
439 C K M A Y I F F I E N K T T F S D I E I

1381 CCAAATTTTATCAGTGGTTTTGATCAAATTGAAAGTGGGCTCCTTGAAAAGGTGTTTTT  
459 Q T F I S G F D Q I E S G L L E K V F L

1441 AATGAAAAGTGGTTTTTATTGAAAAGATTGAAACAAATTTTGGTTATCATTATCGGTTTTG  
479 M K S G F I E K I E T N F G Y H Y R F C

1501 TCAGACAGCAATGATAGAGCTATGTGTCTCTGTTTCATGCATACTATAGTTATAGTGGGAA  
499 Q T A M I E L C V S V H A Y Y S Y S G K

1561 AAAGATTATGAAAATCAAAGTTAAAAGACTGTTTTTCAATTATATATGGTTTATTAA  
519 K I M E N Q K L K D C F S I I Y G L L N

1621 TACAAATGAAAATAGTTTTATCAAGTACTTAGCTAACTTGACAAATTCAAATACTGAAA  
539 T N E N S F I K Y L A N L T N S N T E K

1681 GATATCTTTGAATCATGTGATTGATGTGATTCAAAAGAAATTAACGTGATCGTGAGTA  
559 I S L N H V I D V I P K E I N C D R E Y

1741 TCAAGATAAACAAAAGTTGTTTATGCAATGTTTTTTGAATCGCAGGCAACTATTACAGA  
579 Q D K Q K L F M Q C F F E S Q A T I T D

1801 CGATATTAAGCATCAATTGATGAAAGAAAGTGAAGATTTTGATAAATGGTACAAAAAC  
599 D I K A S I D E R K W K I L I N G T K T

1861 ATCTTATGAAGTTTTATGTGAAAAATATTTTGTAACCATCTCGTTAACTCAGGAGGGAA  
619 S Y E V L C E K Y F V N H L V N S G G K

1921 GTTGACATCTTTAGAAAATTGACAAAAATTTATTAAGTATAAAGAAAAAGATCTTATTAA  
639 L T S L E I D K N L L T D K E K D L I K

1981 GCAATGTTCAAGGAATGTAAGATAATCCGTATAAAGCGGAGTTATCGTTTTTTTATTGA  
659 Q C S R N V K D I R I K R S Y R F F I D

2041 TCAATTCACGAAGCGATGGATAAGAAGTGAATAAAGTGTTTTTTATTGGAGGGTATTAAT  
679 Q F T K R W I R S E \*

**Background Colour:** Exon structure

**Red:** DEATH domain

**Blue:** NACHT domain

**Bold:** Start and Stop codon

**Splice variants**

see CD-ROM

### 9.4.1.2 HyNLR containing a DED domain (partial)

see CD-ROM

#### Translation

517 AA, MW=60097

```

1      CCCACGCGTCCGAAAAAAGAAAAGTATTTTGTGGAAATGGATAATGAAGAACATAAAGA
1      P R V R K K E K Y F V E M D N E E H K D

61     TATATGCTAAAGATTGCAAATAATCTGCTGGAAAAAGATGTTAAAGCAATTAAGTTTAA
21     I L L K I A N N L L E K D V K A I K F Y

121    TTATGCATCAAAAATTGGCTATGCTCATCTTGAAAAGATCAAAACTTCTATTGAATTAAT
41     Y A S K I G Y A H L E K I K T S I E L I

181    TCAAACTTTGAACATCGTATGGTTCTTGGGGTTGATCACTATGATGCTTTTGTGAAGT
61     Q T L E H R M V L G V D H Y D A F V E V

241    ACTTAACAAAATTGGAAGGAATGACCTGGCAAAACAATTTTCTCAAACTTTGAGTTCACC
81     L N K I G R N D L A K Q F S Q T L S S P

301    AGAAAGTTCTACCAGAACTTCTTCTAAGAAAGATATATCAGAAATATGCACAGCACTCAA
101    E S S T R T S S K K D I S E I C T A L K

361    AAGTTTCTATCTTACAAAATTATGGGAACATAAATGAAGTTCAAGTACCACTAAGATTGCC
121    S F Y L T N Y G N I N E V Q V P L R L P

421    TGCTAATGTTGATTTAATGCATAAATTTGTTGATCTATGTATTGTTGATGCAGGTAATAC
141    A N V D L M H K F V D L C I V D A G N T

481    TCAAGTAGATGCTGTTTTAGTGTGAGCGAGAGGAGTTTCTAAAAAAGCAATTGCGTTA
161    Q V D A V F S V E R E E F L K K Q L R Y

541    TACACCAATTCATATAATGAAATTTTATGAAAGAAAAATCTGTAACTCTAATATCTGG
181    T P I P Y N E I F M K E K S V T L I S G

601    AATAGCTGGTATTGGGAAAACATGGTTGCTTAGAAAATGTTTGCTTGATTGGTCAAATGG
201    I A G I G K T W L L R K C L L D W S N G

661    TCTAATTTGAAAAATGTTAAACTTGTGTTTTATTTGGAATGCAGAAGGCTTAATCGATA
221    L I W K N V K L V F Y L E C R R L N R Y

721    TCAAAATATTGGTAGTATTAACGAATTAATAAATTTTCTACAGAGATATTGTAATGA
241    Q N I G S I N E L I N F F Y R D I V N D

781    TTTTGATATTAGTATTATTCTGCGCTGTTTATAATTGATGGATTAGATGAGTTTAAATA
261    F D I S I Y S A L F I I D G L D E F K Y

841    TTTAAATGAATTAATAAATTGCAGTTCGACTAGTAACATCCAATTGTTAATGTTTTAAC
281    L N E L I N C S S T S N Y P I V N V L T

901    AGAAATTCGAAATAATAAATATGTAGTTGCTGGTAGAGTTTATGCAATTGATCAGTATCA
301    E I R N N K Y V V A G R V Y A I D Q Y Q

961    AAGTATATCTACTGAGGATTGTGATAAGATAAACATTCAAATTATGGGATTTAATGAAGA
321    S I S T E D C D K I N I Q I M G F N E D

1021   TGGAATAAATAAATTATATAGAAAATAATGTTTTAGAGGAAAAAAGATGTTGTGAAAGC
341    G I N N Y I E N N V L E E K K D V V K A

1081   AACTTTGAAGGAATCTGCAGTTGCAAAATCAATGTCACTCTGTTCCATTTTTTTATCTTT
361    T L K E S A V A K S M S S V P F F L S F

1141   CATGTGTAAGATTATTAGTTGTTCAAATAATTTATACAGAAATTCTTTTTTAAACAATGAC
381    M C K I I S C S N N L Y R N S F L T M T

```



1201 **AGACCTGTATGCAAATATTTTTTTTACACTTTTTTGCAAAAACACATTATCAAAAACAATGA**  
 401 D L Y A N I F L H F L Q K H I I K N N E

1261 **ATCAATTTCTCAATTTATGGAAAACAATCCAATAAAAAATATGTTCTAAACATTTGTAA**  
 421 S I S Q F M E N N S N K K Y V L N I C K

1321 **AATGCGTACCAATTGTTTGTGAAAATAAAGTAATTTTTTTCAAAGAAGAAATTCAAAC**  
 441 I A Y Q L F V E N K V I F F K E E I Q T

1381 **TTTTGTCAATGAGTTTGATAAAAAATGAAGAACTTTTTTTGGATTTATTGAGAGGATTGA**  
 461 F V N E F D K N E E T F F G F I E R I E

1441 **AACAAGTCTAGGTGAATATATTTTATCAATTTGCACATTTGACAATAATGGAGTTTTGTGC**  
 481 T S L G E Y I Y Q F A H L T I M E F C A

1501 **ATCTATATATGCATATAATTGTTTAAAGTAGTGAAGAGATTATGACCAATGAAAA**  
 501 S I Y A Y N C L S S E E I M T N E

**Background Colour:** Exon structure

**Red:** DEATH domain

**Blue:** NACHT domain

**Bold:** Start codon

### 9.4.1.3 Other HyNLRs

see CD-ROM

### 9.4.1.4 Probe for Southern blot hybridisation

TGGTATTGGGAAAACATGGTTGCTTAAAAAATGTTTGTCTTGATTGGTCAAATGATATAATTTGGaAAAAATGTTGA  
 ACTTGTTTTTTTATTTTGGGAATGCAGAAGGATTAATCAATATGAAAATATTTCAAATATTAACGATTTACTAAGCGT  
 TTTCTACAAAGACATTATAAGCAACTTTAATATTAGTAATCATACTGTACTGTTTATAATTGATGGGTTAGATGA  
 GTTTAAATATTTCAATGAATTATCAAACCCAAGATTAAGTGTAcTATCCCTATTGTTAATGTTTTAGCAGAAAT  
 TCAAAGATATAAACATTTAGTTGCTGGTAGAGTTTATGCAATTGATC

### 9.4.2 *Hydra* TPR-NLR transcripts

see CD-ROM

## 9.5 Putative interactome of HyNLRs

### 9.5.1 HSP90 (gene models)

see CD-ROM

### 9.5.2 HySGT1

see CD-ROM

### 9.5.3 HyChp-1

See CD-ROM

## 9.5.4 HyDODE

### Coding sequence

see CD-ROM

### Translation

304 AA, MW=35528

```

1      CCCACGCGTCCGCACATACTGAAACTTTTTAGTCCCTGTGTAATAAGATGACTCAAATA
1                                          M T Q N

61     TATCTAGTTTGACAAAGTCAAAACTTGACAATGTCTCGCTGAAATCTGGAAAGAGTTTG
5     I S S L T K S K L A Q C L A E I W K E F

121    CAGAAGAAATCCCTTTACCAAAATATAAGATAGCTTCTATTGAACAAGAACAAAACTGT
25    A E E I P L P K Y K I A S I E Q E Q K L

181    TAGTAGAAAGAGCTTATCGTGTACTTACTTTTTGGTACCAATCTGATCCTGATGCATTTA
45    L V E R A Y R V L T F W Y Q S D P D A F

241    ATGAAAACAACATAAAAATGAAGTTAAAAAGTTTAAAGCGGAATGATATAATTAGAGCTG
65    N E N N I K M K L K S L K R N D I I R A

301    TGTTTGGAGAAGAAGATGATCAACCTGCAAAACCCAAAGAAACAAACGTCTCTAAAGTTG
85    V F G E E D D Q P A K P K E T N V S K V

361    AGGACCTTCTTATCGTCAGATTCGTGTGCTTTCAGACTTCTTAGATCCTGACTATGGGT
105   E D L S Y R Q I R V L S D F L D P D Y G

421    GGGAAGAATTTGCCCATCACCTTTTTGAACATGACAATGAAATGATAAATGAAATACAAA
125   W E E F A H H L F E H D N E M I N E I Q

481    AGCTTCGTCATGAGTATGCTGGAGGTGGTAACCCTGCATACCTGTTCTTAAAAACAATTGC
145   K L R H E Y A G G G N P A Y L F L K Q L

541    TTCAAAGAAGGCCAAACATACTTTTTGAATGAATTTTTGGATGCTTGTAAGGAAGTAAAGA
165   L Q R R P N I L L N E F L D A C K E V K

601    GAGCAGATATCATACTATGCAAAATGAAAATTTTAGTGAATTAGTTATATCAGCGAAT
185   R A D I I H Y A N E N F S G I S Y I S E

661    TAGATAAAAACCAACTAAGAGATTTCCGCCGATAAGGTACGTGGAATATAGTTCCAAGTA
205   L D K N Q L R D F A D K V R G N I V P S

721    ATTGGGCTGAAATCGCAAGCGAGTTTGTGCGAGTTTTCTTCTGAAGATATAAAAAGAATAG
225   N W A E I A S E F V E F S S E D I K R I

781    AACTTGAAAGGCTGGCTCCAAATAGTTATAGTCCGACGAAAAAATTGTTGAAAAGCTTA
245   E L E R L A P N S Y S P T K K L F E K L

841    AACAAATTCATCCAAATATGGAGTTAGATAATTTAATAAATGTATGTGAAAAAATCAGGA
265   K Q I H P N M E L D N L I N V C E K I R

901    GGAAAGATGTTGCTAATAAACTTCGTGAATTTGCAATAAATAATAGCAGCATAGCGCAAT
285   R K D V A N K L R E F A I N N S S I A Q

961    AGTTATTTTTTTAATGTTTTCAAGTTTTTTTATTAATTTTTTGTATTATTTAATGATT
320   *

1021   TTATTTTTGAGTTTATTAGGTTTTTATCGGTAGTTGATAATATCTATTCTTTAATTTAA
340

1081   TGTTTAGAATGC
360

```

**Background Colour:** Exon structure

**Red:** DEATH domain

**Bold:** Start and Stop codon

### 9.5.5 HyCARD-Caspase

See CD-ROM

### 9.5.6 HyDD-Caspase

#### Coding Sequence

See CD-ROM

#### Translation

407 AA, MW=47096

```

1      TGTAATAAGAAACAACAAAATGGCAGAAGACATTGTTTCTAATGAATCATTCATTTTAA
1      M A E D I V S N E S F I F K

61     AATGAGCTCTAGTATTGGAACATATTGGGTAACTTGGACGTGCTCTAGGAATCAAAGA
15     M S S S I G T Y W V T L G R A L G I K D

121    CAGTGTTTTAGAAATGCTTGATAAGGAAAATCCCAAAGTTATAGATAAAGCATATGCTAT
35     S V L E M L D K E N P K V I D K A Y A M

181    GTTAAACATTGGACTGCAAAGAACACAAATGCAACTATTGACGAGTTGAAAATTGTTTT
55     L K H W T A K N T N A T I D E L K I V L

241    AATGGAATTAGAAAGAAATGATTTAAAAAAGAAGTAGAAAAAATTTCAAGTACTTTAAT
75     M E L E R N D L K K E V E K I S S T L I

301    TTCAAAATGACTATTGCTGACAAGAAAGTAAATAGCCAAAATCTATCATTAGGCAATGA
95     S K M T I A D K K V N S Q N L S L G N E

361    ACAGGATTTAAATGTTGAGGAGAAGAAATCTATTCAAGAAACCCTAACCTCAAGTAAACA
115    Q D L N V E E K K S I Q E T L T S S K Q

421    ATCTTTGTTCATCAGAACATGAATCAACAGTAACAGATGAAAAACCTAATCAAGCTCAATG
135    S L S S E H E S T V T D E K P N Q A Q C

481    CTATCCTATGACACGTAAAAAACTTGGCAAACCTTTTCATTTTAAACAATATGCACAATGA
155    Y P M T R K K L G K L F I F N N M H N D

541    TGAAAAGACTACTCATAGACTTGCAGCAAAATATGATGTCAAAAAAATTAAGCAACCTT
175    E K T T H R L A A K Y D V K K I K A T F

601    CAGCAAAATAGAATTTGAAGTCGTAGTTTATAAACCGAATAGCTTTGAAGAGATGAAAA
195    S K I E F E V V V Y K P N S F E E M K K

661    AAATTTAGAAGAAGTTTAAAGGAACCATATGGCGAGTACAATATTTTCAATATTTT
215    N L E E V L K E P Y G E Y N I F S I F F

721    CATTGCTCATGGACAGGATGGTAAACTATTTGGACCAAATGAACTAATAACAAAAATC
235    I A H G Q D G K L F G P N E T N N K K S

781    ATTTTCAATACTTGAATTGAAAAAATGCTTGAGGATAACTCTTGGCTGAAAAATATTC
255    F S I L E L K K M L E D N S W L K N I P

841    CAAACCTGGTCTTTTTCGAAAAGCTGTTCGGCTTCGATAAAGATTTTCAACAAATGACCTT
275    K L V F C E S C R G F D K D F Q Q M T L

901    AACTAATTTTGAAGCTAAATCTAATGAAAAGAATGTAATACGGCAAAATTTGCTGACTT
295    T N F E A K S N E K N V N T A K F A D L

961    ATTTATGGTTTTTCTACTGCTGAGTCTTTTGTATCAATATCTATGCCTTCAGATAATGA
315    F I G F S T A E S F V S I S M P S D N D

```

1021 TGTTCAAATGAAAACTTTTACAGCCCGTGGGTCATTGAACTTTGCAAACAAATTCAGGA  
 335 V Q M K N F Y S P W V I E L C K Q I Q E

1081 AAAATACATGACAACTGACTTTCTACAGATTTACCAGGACCTCCAGCGATACATGAGTAG  
 355 K Y M T T D F L Q I Y Q D L Q R Y M S S

1141 CACACCTTTATTGTCTAATGGCGAAGAAGTTTATTTCCAGATGCCAGAACTTCAATCTAC  
 375 T P L L S N G E E V Y F Q M P E L Q S T

1201 TTTAAGATATAAACTTTATCTTAATATGGAGAGAAAACTTAAAAAAAAATAATGTTAAAT  
 395 L R Y K L Y L N M E R K T \*

**Background Colour:** Exon structure

**Red:** DEATH domain

**Green:** Caspase domain

**Bold:** Start and Stop codon

### 9.5.9 HyDED-Caspase

see CD-ROM

### 9.5.8 HyDED-DD-Kinases

see CD-ROM

### 9.5.9 HyJUN

see CD-ROM

### 9.5.10 HyNF- $\kappa$ B

see CD-ROM

## 9.6 Vectors

### 9.6.1 pCEV-GwA (constructed and provided by Dr. G. Jakobs)

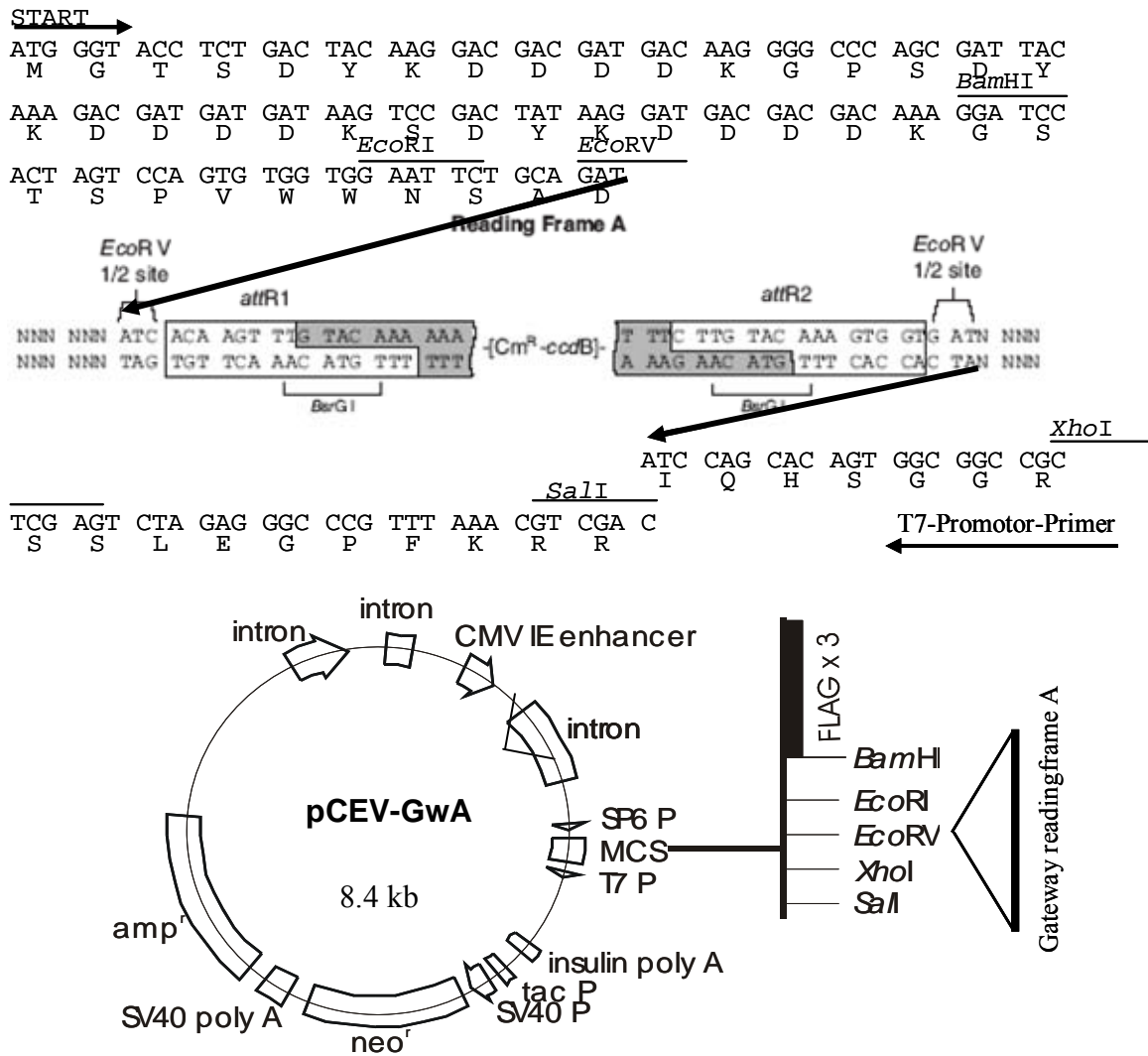


Figure 9.2: Map of the pCEV-GwA vector including multiple cloning site.

**Partial sequence:**

see CD-ROM

### 9.6.2 pGA4 including hydeath-fkbp (GENEART)

Plasmid Map:

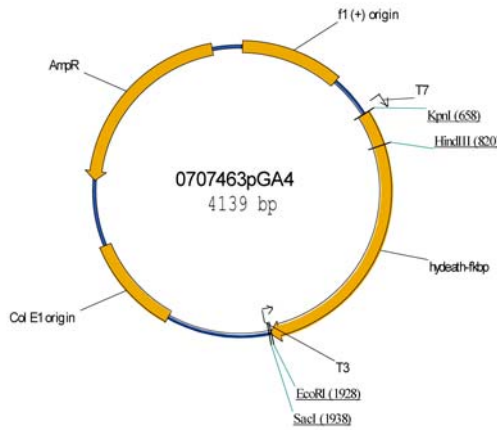


Figure 9.3: Map of the pGA4 vector including hydeath-fkbp.

Vector sequence:

see CD-ROM

### 9.6.3 ligR1 (constructed and provided by Dr. K. Khalturin)

ligR1

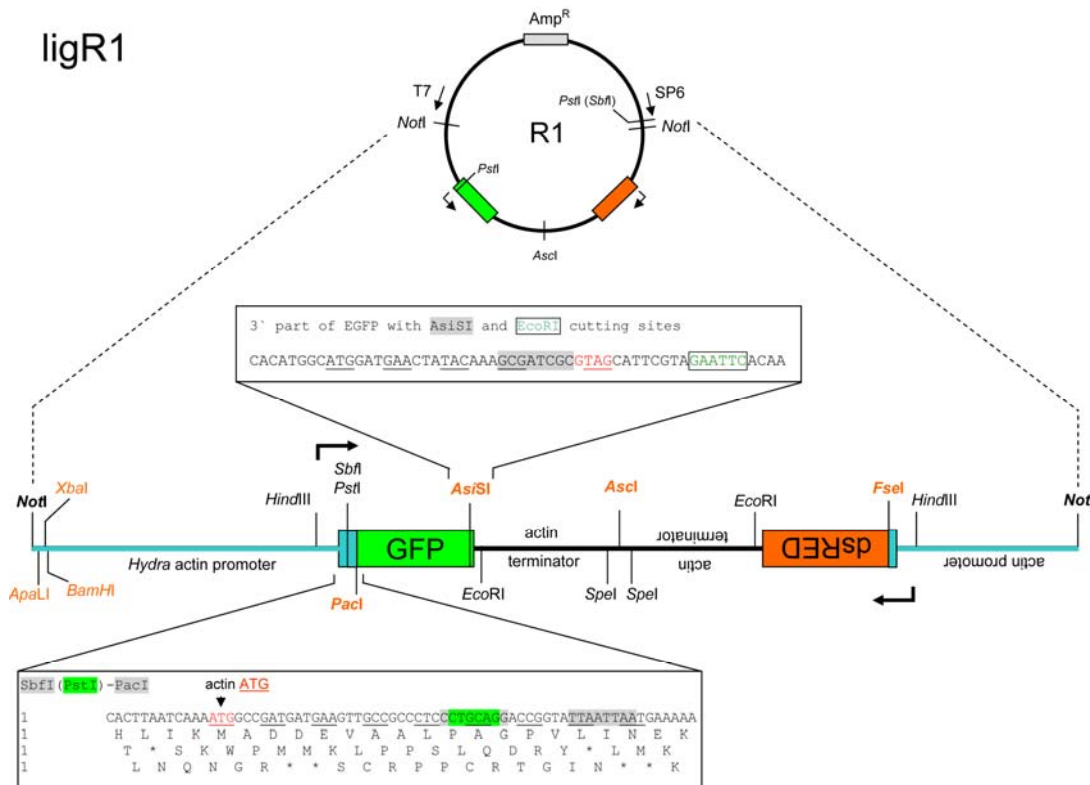


Figure 9.4: Map of the ligR1 vector (vector backbone pGEM-T).

ligR1 expression cassette sequence:

see CD-ROM

## 9.7 Expression constructs

### 9.7.1 Expression constructs for HEK293 cell-based assays

#### 9.7.1.1 Flag-HyNLR-FKBP

**ATGGGTACCTCTGACTACAAGGACGACGATGACAAGGGGCCAGCGATTACAAAGACGATGATGATAAGTCCGAC**  
**TATAAGGATGACGACGACAAAGGATCCACTAGTCCAGTGTGGTGAATTCTGCAGATATC** **ACAAGTTTGTACAAA**  
**AAAGCAGGCTTCGACGAAAATATTCTTCAAATCAACAAAATAGATATCTTTTTTCAAGATCAGTTGGAATTGAT**  
**TGGAAAGATCTTGAAGATGTTTACAAATTCTTGATTCATATCTTGATGTTATTGATATTGAATCAAAAAATTGAT**  
**TGTGATAAAGCTTATCAAATGTTAACAAAATGGACAGAAAAAGAAAATCCATCATTAAATGAACTTAAAAATGCT**  
**TTACAAACAATGGAAAAAATGATCTTATGAAAACATTAGAAGATCTTCAAATTAGATCATCAAAAGAAAATTGAA**  
**AAATCAAATCTTGTTAAAACAGATTTTCTTTCAGATGCTTTAAAAAACTTTTATCTTGAAACATATAAAAACAATT**  
**GAAGAAATTCACCACAACCTTAATAAACCATATCAAGTTAATTTTCTTGATAAAATTTGTTGATCTTTATGTTGTT**  
**GATGAAGTTGAAGTTCAAAAAGATGCTATTAATATTGCTGAATTAGATCAATTTCTTAAAAAACAAATGTCATAT**  
**ACATCAATTTCTTTTGAAAAACATCTTAAAGATCTTG** **GACCAGGTC** **TTTCTTTATTTCTAATATCTGGTATTGCT**  
**GCTATTGGTAAAACATGGTTGCTTAGAAAATTTTACTAGATTGGTCAAATGGTTTTTATTTGGAAAAATATTGAT**  
**TTAGTGTTTTACTTGGAGTGTAACAACCTTAATTTGTATGAAAATATTTCTAATATTAATGAATTACTTGATGTT**  
**TTCTACAAGATATTTTAAAAGGTTATAATATTTCTTTAGATTTTCATGCAATCTAAACCATCAATTTATGTTTATA**  
**ATTGATGGCTTAAACGAATTTAAATACTTTGACCAATTAATAAGCAATACACACTGTAGTTCACAAGAAAATTCCT**  
**ATTTCTTAATGTCTTTACAGAAATTTATAAATACAAAGCTGTGATATCAGGAAGAGTAAATACAATATCACAAATAT**  
**AAAAATGTGGTCACGCGTTATAAAGATAAGTTAACCATTAGAGTTATGGGTTATAATGAAAAATGGAATAAAAATAT**  
**TATTTGAGGAATAATTTAATAAAAAAACAAAATGCAGAGTGAAAATG** **GACCAGGTC** **CCGGTGTTCAGTTGAA**  
**ACAATTTACCCCGGTGATGGAAGAACATTTCCAAAAAGAGGACAAAATTTGCGTTGTTTCATTATACTGGAATGTTA**  
**GAAGATGGTAAAAAGTTGATTCATCAAGAGATAGAAAACAACCTTTTAAATTTATGTTAGGAAAAACAAGAGTT**  
**ATTAGAGGTTGGGAAGAAGGTGTTGCTCAAATGTCAGTTGGACAAAGAGCTAAATTAACAATTTCTCCAGATTAT**  
**GCTTATGGTGCTACAGGACATCCCGGTATTATTCCACCACATGCTACATTAGTTTTTTGATGTTGAATTACTTAAA**  
**TTAGAAACAAGAGGTGTACAAGTAGAACTATTAGTCCCGGTGATGGTAGAACTTTTCTTAAAAGAGGTCAAACCT**  
**TGCGTAGTACATTATACTGGAATGCTTGAAGATGGAAAAAAGTAGATAGTAGCAGAGATAGAAACAACCTTTT**  
**AAATTTATGCTTGGTAAACAAGAAGTAATAAGAGGTTGGGAAGAAGGTGTAGCACAAATGAGTGTAGGTCAAAGA**  
**GCAAACTTACAATTAGCCCTGATTATGCATATGGTGCAACTGGTCATCCCGGAATTATACCACCTCATGCAACT**  
**CTTGTATTTGATGTAGAATTGTTAAAACCTTGAAACATCATATCCATATGATGTTCCAGATTATGCT** **TAA** **GACCCA**  
**GC** **TTTCTTGTACAAAGTGGT**

Blue: Flag tag

Pink: *attR1* site

Green: *attR2* site

Grey: *PsyI* cutting site

**Bold:** Start and Stop codon

Red: *HyNLR type 1* DEATH domain-coding part

Blue: *HyNLR type 1* NACHT domain-coding part

Light blue: FKBP<sub>2</sub> part

### 9.7.1.2 Myc-HyDD-Caspase

**ATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTG**CTTATGGCCATGGAGGCCCGAATTC**TGGCAGAAGAC**  
 ATTGTTTCTAATGAATCATTCAATTTTTAAAATGAGCTCTAGTATTGGAACATATTGGGTAAACACTTGGACGTGCT  
 CTAGGAATCAAAGACAGTGTTTTAGAAATGCTTGATAAGGAAAATCCCAAAGTTATAGATAAAGCATATGCTATG  
 TAAAAACATTGGACTGCAAAGAACACAAATGCAACTATTGACGAGTTGAAAATTGTTTTAATGGAATTAGAAAAGA  
 AATGATTTAAAAAAGAAGTAGAAAAAATTTCAAGTACTTTAATTTCAAAAATGACTATTGCTGACAAGAAAAGTA  
 AATAGCCAAAATCTATCATTAGGCAATGAACAGGATTTAAATGTTGAGGAGAAGAAAATCTATTCAAGAAACCCTA  
 ACCTCAAGTAACAATCTTTGTGCATCAGAACATGAATCAACAGTAACAGATGAAAAACCTAATCAAGCTCAATGC  
 TATCCTATGACACGTAAAAAACTTGGCAAACCTTTTCATTTTTAACAAATATGCACAATGATGAAAAGACTACTCAT  
 AGACTTGCAGCAAAATATGATGTCAAAAAAATTAAGCAACCTTCAGCAAAAATAGAATTTGAAGTCGTAGTTTAT  
 AAACCGAATAGCTTTGAAGAGATGAAAAAATTTAGAAAGAGTTTTAAAGGAACCATATGGCGAGTACAATATT  
 TTTTCAATATTTTTCAATTGCTCATGGACAGGATGGTAAACTATTTGGACCAAATGAAAATAATAACAAAAAATCA  
 TTTTCAATACTTTGAATTGAAAAAATGCTTGAGGATAACTCTTGGCTGAAAAATATTTCCCAAACCTGGTCTTTTGC  
 GAAAGCTGTCGGGCTTCGATAAGGATTTTTCAACAAATGACCCTAACTAATTTTGAAGCTAAATCTAATGAAAAG  
 AATGTAAATACGGCAAAATTTGCTGACTTATTTATTGGTTTTTCTACTGCTGAGTCTTTTGTATCAATATCTATG  
 CCTTCAGATAATGATGTTCAAATGAAAACTTTTACAGCCCGTGGGTCAATTGAACTTTGCAAACAAATTCAGGAA  
 AAATACATGACAACCTGACTTTCTACAGATTTACCAGGACCTCCAGCGATACATGAGTAGCACACCTTTATTGTCT  
 AATGGCGAAGAAGTTTTATTTCCAGATGCCAGAACCTTCAATCTACTTTAAGATATAAACTTTATCTTAATATGGAG  
 AGAAAAACT**TAACTCGAG**

**Blue:** Myc tag

**Pink:** *EcoRI* cutting site

**Blue:** *XhoI* cutting site

**Bold:** Start and Stop codon

### 9.7.1.3 Myc-HyDED-Caspase

**ATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTG**CTTATGGCCATGGAGGCCCGAATTC**TGAATCCTCAT**  
 ATCAGTAGTTGGAATCAATTTTTATGTTTCATTGGCTGATGAATTATTTAAAAGATAATATAAAAAGAAAATCAAGTTT  
 ATTTTGAAGACTACATACCTGCTAGTGTTCGTGAGACATTAGATGATGGTCATACACTCTTGAATGAATTGGAG  
 AAGCGGAATATAATCTCATGTAATGACCTGTCTAAACTAGGCCAGATTCTTGCTGATATTGGAAGGAAAAGACCTT  
 TTATCTAAAATAAAATCTTATATGGAAGAAAAAGCAAATTAACGAGTGTGAATGAGATATCCCAAAAAAGTGGT  
 TCAACATTCAAGTTCAACATTCAATTCAACATTCAATTCAAGCTCAAACCTTCTGCTTCAGTTTCTGCTTCAACTTCT  
 GTTTTAAATTTGGACTCAATTAAGAAAGAATATTTTGTAGAAAATACTTCAATTGAGTTGGGAATATACAGCATG  
 CACAATGTTTCTAGTGAATATGTATTATATTACGCAACTACTTTGATAAAGAAGTAATTATAACTGATGAAAAT  
 AAAAAGCTAGAACAGCGCTTAGGAAATGGAGCAGACGAAAGTCAACTAAAGGAGACATTTACTTGGCTGAACTTT  
 GATGTTTTGGTATACAAAAATAAAAGTTCTAAAGATATAGTGCAATTATTATATAGTAATCTCAAAAAATAGTTCCG  
 ATACAAGATTGTTTTGTATGTTGTATTTTGTACATGGATATAGAAATGGAATATATGGCAGTGATGGATCCAGG  
 CTATCGTTTTGAAGAATTGTGGACCACTGTTGAGGACGCATCTTCTGAATCACTTCAGGGTAAACCTAAGTTGTTTT  
 TTCATTCAAGCTTGTCAAACCGAAGTAAATGTTAAGGAAATGTCTTTATCAGATATTGGAATAAAAAGTTGATTTT  
 GAAGATGTTTTGACATCTTTAGCAACATTACCAGGTAAAGTTGCGTTTTCGTGATGTAAGTACTAGAGGCTCATGGTAT  
 ATTCAAACACTTTGTGAGACTTTGAAAAATCGTGCTTATGATTCTTCACTTACTTGAATTTTTGACGGAACTTAAC  
 TCTAATATGGCGTCGAAAGTAGATTACTACAAAGTATGTTCTGAATATAAAAATCGCTACCCAAATGTCTTCAATT  
 CAAAGTTGCACCTTATCAAAAAAATTTTTTTCAAACCATATGACGTAAGTCTCTTTCGCGGTTACGTATTTCTT  
**TAACTCGAG**

**Blue:** Myc tag

**Pink:** *EcoRI* cutting site

**Blue:** *XhoI* cutting site

**Bold:** Start and Stop codon



## 9.7.2 Expression constructs for transgenic *Hydra*

### 9.7.2.1 HyNLR type 1 long::FKBP<sub>2</sub>-HA

**GGCCGGCC**AGGAATGGACGAAAATATTCTTCAAATCAACAAAATAGATATCTTTTTTCAAGATCAGTTGGAATT  
 GATTGGAAAGATCTTGAAGATGTTTACAAATCTTGATTCATATCTTGATGTTATTGATATTGAATCAAAAAAT  
 TGTGTGATAAAGCTTATCAAATGTTAACAAAATGGACAGAAAAAGAAAATCCATCATTAAATGAACTTAAAAAT  
 GCTTTACAAACAATGGAAAAAATGATCTTATGAAAAATTAGAAAGATCTTCAAATTAGATCATCAAAAAGAAAT  
 GAAAAATCAAATCTTGTTAAACAGATTTTTCTTTAGATGCTTTAAAAAACTTTTATCTTGAAACATATAAAAA  
 ATTGAAGAAATTCACCACAACCTTAATAAACCATATCAAGTTAATTTTTCTTGATAAAATTTGTTGATCTTTATGTT  
 GTTGATGAAGTTGAAGTTCAAAAAGATGCTATTAATATTGCTGAATTAGATCAATTTCTTAAAAAACAAATGTCA  
 TATACATCAATTCTTTTTGAAAAACATCTTAAAGATCTTG**GACCAGGTC**TTCTTTATTTCTAATATCTGGTATT  
 GCTGCTATTGGTAAAACATGGTTGCTTAGAAAATTTTTACTAGATTGGTCAAATGGTTTTATTTGGAAAAATATT  
 GATTTAGTGTTTTACTTGGAGTGTAACAACCTTAATTTGTATGAAAATATTTCTAATATTAATGAATTACTTGAT  
 GTTTTCTACAAAGATATTTTAAAGGTTATAATATTTCTTTAGATTTTCATGCAATCTAAACCATCAATTAATGTTT  
 ATAATTGATGGCTTAAACGAATTTAAATACTTTGACCAATTAATAAGCAATACACACTGTAGTTCACAAGAAAT  
 CCTATTCTTAATGTCTTTACAGAAATTTATAAATACAAAGCTGTGATATCAGGAAGAGTAAATACAATATCACAA  
 TATGAAAATGTGGTCACGCGTTATAAAGATAAGTTAACCATTAGAGTTATGGGTTATAATGAAAATGGAATAAAA  
 TATTATTTGAGGAATAATTTAATAAAAAAACAACATGCAGAGTGAAAACCTG**GACCAGGTC**CCGGTGTTCAGTT  
 GAAACAATTTACCCGGTGATGGAAGAACATTTCCAAAAAGAGGACAACTTGCGTTGTTTATTACTGGAATG  
 TTAGAAGATGGTAAAAAGTTGATTCATCAAGAGATAGAAAACAACCTTTTAAATTTATGTTAGGAAAAACAAGAA  
 GTTATTAGAGGTTGGGAAGAAGGTGTTGCTCAAATGTCAGTTGGACAAAGAGCTAAATTAACAATTTCTCCAGAT  
 TATGCTTATGGTGTACAGGACATCCCGGTTATTATTTCCACCACATGCTACATTAGTTTTTGTGATTTGAATTACTT  
 AAATTAGAAACAAGAGGTGTACAAGTAGAACTATTAGTCCCGGTGATGGTAGAACTTTTCTAAAAGAGGTCAA  
 ACTTGCGTAGTACATTATACTGGAATGCTTGAAGATGGAAAAAAGTAGATAGTAGCAGAGATAGAAAACAACCT  
 TTTAAATTTATGCTTGGTAAACAAGAAGTAATAAGAGGTTGGGAAGAAGGTGTAGCACAAATGAGTGTAGGTCAA  
 AGAGCAAACTTACAATTAGCCCTGATTATGCATATGGTGCAACTGGTTCATCCCGGAATTATACCACCTCATGCA  
 ACTCTTGTATTTGATGTAGAATTGTTAAACTTGAAACATCATATCCATATGATGTTCCAGATTATGCT**TAA**CAT  
 TCGTAGAATTC

**Pink:** *FseI* cutting site

**Blue:** *EcoRI* cutting site

**Grey:** *PsyI* cutting site

**Red:** *HyNLR type 1* DEATH domain-coding part

**Blue:** *HyNLR type 1* NACHT domain-coding part

**Light blue:** FKBP<sub>2</sub> part

**Bold:** Stop codon

### 9.7.2.2 HyNLR type 1 short::FKBP<sub>2</sub>-HA

**GGCCGGCC**AGGAATGGACGAAAATATTCTTCAAATCAACAAAATAGATATCTTTTTTCAAGATCAGTTGGAATT  
 GATTGGAAAGATCTTGAAGATGTTTACAAATCTTGATTCATATCTTGATGTTATTGATATTGAATCAAAAAAT  
 TGTGTGATAAAGCTTATCAAATGTTAACAAAATGGACAGAAAAAGAAAATCCATCATTAAATGAACTTAAAAAT  
 GCTTTACAAACAATGGAAAAAATGATCTTATGAAAAATTAGAAAGATCTTCAAATTAGATCATCAAAAAGAAAT  
 GAAAAATCAAATCTTGTTAAACAGATTTTTCTTTAGATGCTTTAAAAAACTTTTATCTTGAAACATATAAAAA  
 ATTGAAGAAATTCACCACAACCTTAATAAACCATATCAAGTTAATTTTTCTTGATAAAATTTGTTGATCTTTATGTT  
 GTTGATGAAGTTGAAGTTCAAAAAGATGCTATTAATATTGCTGAATTAGATCAATTTCTTAAAAAACAAATGTCA  
 TATACATCAATTCTTTTTGAAAAACATCTTAAAGATCTTG**GACCAGGTC**CCGGTGTTCAGTTGAAACAATTTCA  
 CCCGGTGATGGAAGAACATTTCCAAAAAGAGGACAACTTGCGTTGTTTATTACTGGAATGTTAGAAGATGGT  
 AAAAAAGTTGATTCATCAAGAGATAGAAAACAACCTTTTAAATTTATGTTAGGAAAAACAAGAAGTTATTAGAGGT  
 TGGGAAGAAGGTGTTGCTCAAATGTCAGTTGGACAAAGAGCTAAATTAACAATTTCTCCAGATTATGCTTATGGT  
 GCTACAGGACATCCCGGTTATTATTTCCACCACATGCTACATTAGTTTTTGTGATGTTGAATTACTTAAATTAGAAA  
 AGAGGTGTACAAGTAGAACTATTAGTCCCGGTGATGGTAGAACTTTTCTAAAAGAGGTCAAACCTTGCGTAGTA  
 CATTATACTGGAATGCTTGAAGATGGAAAAAAGTAGATAGTAGCAGAGATAGAAAACAACCTTTTAAATTTATG  
 CTTGGTAAACAAGAAGTAATAAGAGGTTGGGAAGAAGGTGTAGCACAAATGAGTGTAGGTCAAAGAGCAAAA  
 ACAATTAGCCCTGATTATGCATATGGTGCAACTGGTTCATCCCGGAATTATACCACCTCATGCAACTCTTGTATT  
 GATGTAGAATTGTTAAACTTGAAACATCATATCCATATGATGTTCCAGATTATGCT**TAA**CATTCGTA**GAATTC**

**Pink:** *FseI* cutting site

**Blue:** *EcoRI* cutting site

**Grey:** *PsyI* cutting site

**Red:** *HyNLR type 1* DEATH domain-coding part

**Light blue:** FKBP<sub>2</sub> part

**Bold:** Stop codon

### 9.7.2.3 FKBP<sub>2</sub>-HA

**GGCCGGCC**AGGTGTTCAAGTTGAAACAATTTACCCGGTGATGGAAGAACATTTCCAAAAAGAGGACAAACTTGC  
 GTTGTTTCATTATACTGGAATGTTAGAAGATGGTAAAAAAGTTGATTCATCAAGAGATAGAAACAAACCTTTTAAA  
 TTTATGTTAGGAAAACAAGAAGTTATTAGAGGTTGGGAAGAAGGTGTTGCTCAAATGTCAGTTGGACAAAGAGCT  
 AAATTAACAATTTCTCCAGATTATGCTTATGGTGCTACAGGACATCCCGGTATTATTCCACCACATGCTACATTA  
 GTTTTTGATGTTGAATTACTTAAATTAGAAAACAAGAGGTGTACAAGTAGAAACTATTAGTCCCGGTGATGGTAGA  
 ACTTTTCCTAAAAGAGGTCAAACCTTGCGTAGTACATTATACTGGAATGCTTGAAGATGGAAAAAAGTAGATAGT  
 AGCAGAGATAGAAACAAACCTTTTAAATTTATGCTTGGTAAACAAGAAGTAATAAGAGGTTGGGAAGAAGGTGTA  
 GCACAAATGAGTGTAGGTCAAAGAGCAAACTTACAATTAGCCCTGATTATGCATATGGTGCAACTGGTCATCCC  
 GGAATTATACCACCTCATGCAACTCTTGTATTTGATGTAGAATTGTTAAAACCTTGAAACATCATATCCATATGAT  
 GTTCCAGATTATGCT**TAA**CATTCGTA**GAATTC**

**Pink:** *FseI* cutting site

**Blue:** *EcoRI* cutting site

**Light blue:** FKBP<sub>2</sub> part

**Bold:** Stop codon

## 9.8 Bacteria of the tumour bearing *Hydra*

### 9.8.1 Bacterial 16S rDNA sequence

see CD-ROM

### 9.8.2 Alignment of the bacterial 16S rDNA sequences

see CD-ROM

## 9.9 *Hydra oligactis* sequences for Ras orthologues

### 9.9.1 *ras1* (partial)

GGTGTGGAAAAAGTGCTCTAACAATACAACCTTATACAGAACCATTTTGTGGAAGATTATGACCCTACTATTGAG  
 GATTCGTATATTAAGCAGGTTGTTGTAGATGGCGCAATATGCATTCTGGATATTCTTGATACAGCAGGTCAAGAA  
 GAGTACAGTGCTATGCGAGAACAATATATGCGTACTGGAGAAGGTTTTTTGTGCATGTTTGTCTGTGACAAGTCTT  
 AAATCATTTCAAGAAATTGATAACTTTAGAACAACAAGCCCTAAGGGTAAAAAGATGCTGACAAAAGTA

### 9.9.2 *ras2* (partial)

AAAAAGTGCACCTAACCATACAATTCATTCAATCGCATTTTGTCCAAGACTATGATCCAACGATTGAAGATTCTTA  
 CAGGAAACAGTGTGTAATTGATGATAAAGTTGCACATTTAGATATATTGGATACAGCTGGGCAAGAGGAGTTTAG  
 TGCTATGCGAGAGCAATACATGAGAACAGGGGAGGGTTTTCTTCT

## 9.10 Oligonucleotides

**Table 9.2:** List of the used oligonucleotides.

see CD-ROM

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## **11 ERKLÄRUNG**

Hiermit erkläre ich, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis eingeständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater Prof. Dr. Dr. h.c. Thomas C. G. Bosch in Anspruch genommen. Des Weiteren erkläre ich, dass ich noch keinen Promotionsversuch unternommen habe.

Teile dieser Arbeit wurden bereits zur Publikation eingereicht.

Kiel, den 10.08.2010

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