Molecular analysis of self/non-self recognition in the hermaphroditic urochordate *Ciona intestinalis*

Dissertation

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…at an extremely remote period a group of animals existed resembling in many respects the larvae of our present ascidians, which diverged into two great branches - the one retrograding in development and producing the present class of ascidians, the other rising to the crown and summit of the animal kingdom by giving birth to the vertebrata.

Charles Darwin, The Descent of Man (1871)

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Abbreviations

A	adenine
aa	amino acid
ABC	ammoniumbicarbonat
ACN	acetonitrile
Amp	ampicillin
AP	alkaline phosphatase
BAC	bacterial artificial chromosome
bp	basepair
BCIP	5-brome-4-chlor-3-indolylphosphate
Blast	basic local alignment search tool
Blastn	BLAST-search nucleotide against nucleotide
BSA	bovine serum albumin
C	cytosine
ca.	circa
CCP	complement control protein
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate
cm	centimetre
cm^2	square centimetre
d	day
Da	Dalton
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
dCTP	desoxycytosine triphosphate
DIG	Digoxygenin
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotide triphosphate
ddNTP	didesoxyribonucleotide triphosphate
dscDNA	double-stranded cDNA
dsRNA	double-stranded RNA
DTT	Dithiothreitol
e.g.	for example
EB/EBH	native/denaturing elution buffer
EDTA	Ethylene diamine tetraacetic acid
EF1α	Elongation factor 1α
EST(s)	expressed sequence tag(s)

EtOH	Ethanol
Fig(s).	figure(s)
FSW	filtered sterile seawater
G	guanine
g	gram or gravity
gDNA	genomic DNA
h	hour
HCl	hydrochloric acid
IAA	iodacetamid
in situ	natural location
in vivo	in the living organism
IPTG	isopropyl-β-D-thiogalactopyranosid
JGI	Joint Genome Institute
kb	kilobase
kV	kilovolt
LB-amp	LB-medium with 50µg/ml Ampicillin
l	litre
LEW/LEWH	native/denaturing lysis, equilibration and wash buffer
M	molar
mA	milliampère
MBq	megabequerel
MAB	maleic acid buffer
MAB-B	maleic acid buffer with 1% (w/v) BSA
max.	maximum of
µF	microfarad
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
min	minute(s)
Mio	million
mRNA	messenger-RNA
ms	millisecond(s)
MWCO	molecular weight cut off
NaOH	sodium hydroxide
NBT	nitroblue tetrasolium
n	nano
NCBI	National Centre for Biotechnology Information

OD_{600}	optical density at 600nm
Ω	Ohm
PAA	poly-acrylamid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature or reverse transcription
SDS	*s*odium dodecyl sulfate
s or sec	second(s)
SEM	scanning electron microscopy
ss	single stranded
SSC	Sodium salino cytrate
SSH	Suppression Subtractive Hybridization
T	thymine
Tab.	table
TAE	Tris-Acetat-EDTA-buffer
Taq	*Thermophilus aquaticus*
TBE	Tris-Borat-EDTA-buffer
Tblastn	BLAST-search protein against translated nucleotide
TE	Tris/HCl-EDTA-buffer
TEM	transmission electron microscopy
TEMED	N,N,N´,N´-Tetraethyldiamine
TLR	Toll-like receptor
T_M	Primer-annealing temperature
Tris	*tris*(hydroxymethyl)aminomethane
tRNA	transfer-RNA
U	unit(s)
UTR	untranslated region
UV	ultraviolet
V	Volt
vCRL	variable complement receptor like
vWA	Von Willebrand factor
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-4-indolyl-β-galactopyranoside

1 Introduction

1.1 Self/non-self recognition

Self/non-self recognition is the ability of an individual organism to distinguish its own tissues from those of another individual belonging to the same or another species (Boehm, 2006). The ability to discriminate self from non-self is the fundamental feature which is shared by all multicellular organisms (Khalturin and Bosch, 2007). Self/non-self recognition is necessary for three biological processes (Fig. 1-1):

(i) Every individual has to be able to recognize potential pathogens in order to activate protective mechanisms (pathogen defense).

(ii) In colonial animals, allogeneic cells have to be recognized and eliminated to prevent their integration into the germline which would otherwise endanger transmission of the own genetic information from generation to generation (histocompatibility).

(iii) In hermaphroditic organisms, gametes should recognize each other, but at the same time self-fertilization has to be prohibited to avoid genetic degradation (self-sterility).

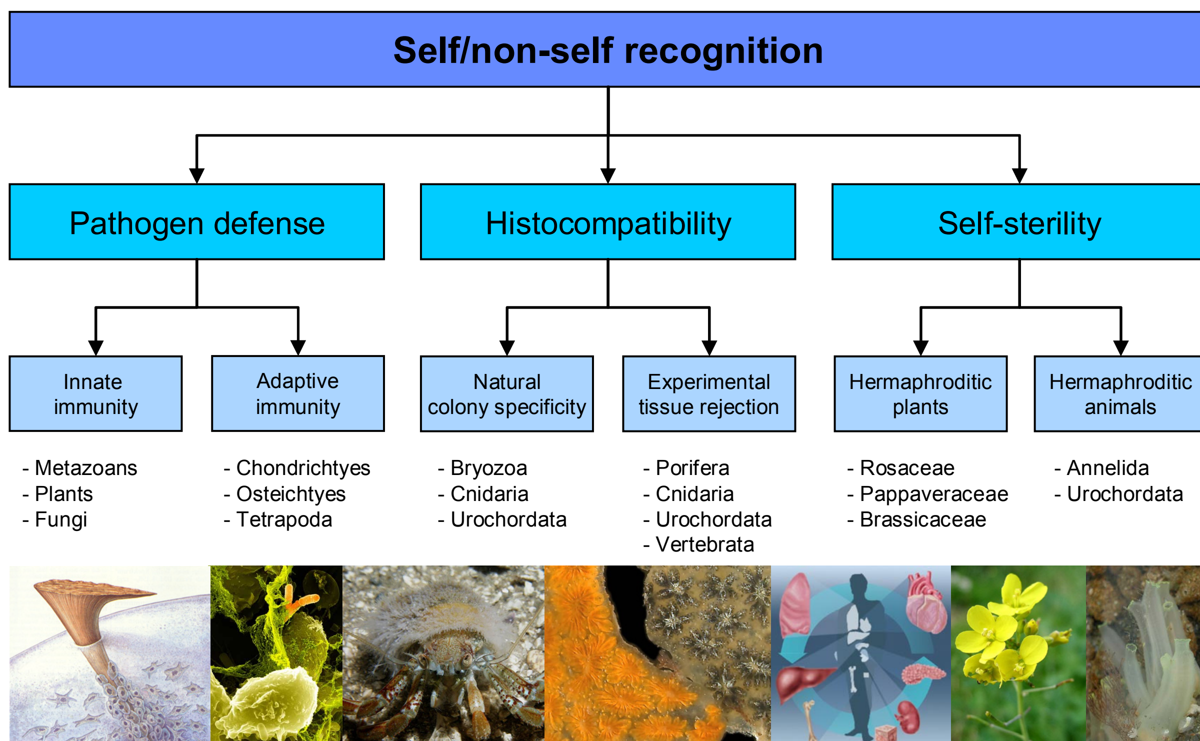

Fig. 1-1: Overview of self/non-self recognition in metazoans and plants.

Self/non-self discrimination can be based on two different mechanisms: recognition of self or recognition of non-self.

Recognition of non-self requires that corresponding receptors should be able to identify an unlimited diversity of foreign cells or molecules. As default state, all defense reactions (e.g. cellular lysis) should be inactive. They are initiated only after detection of an "alien" antigen (Pancer and Cooper, 2006). From the conceptional point of view this system is very effective, but complicated to realize at the molecular level. It implies that receptor has to recognize all possible variants of antigens except those expressed by self tissue. Nevertheless, this type of recognition is employed in the adaptive immune system of higher vertebrates (Cooper and Alder, 2006) and agnathans (Pancer et al., 2004).

Molecular requirements to recognize self are much less complex (Boehm, 2006). Own cells can be marked as self and upon recognition a default reaction (e.g. cellular lysis or fertilization) is inhibited. Such recognition system can be based on pair of receptor and ligand which specifically interact with each other and initiate an inhibitory reaction. Genomic linkage of both receptor and ligand genes would be sufficient for stable transmission over generations. At the same time high polymorphisms of each haplotype within the population are possible (De Tomaso et al., 2005). Therefore, recognizing self instead of non-self is much easier to realize. This mechanism, for example, is typical for mammalian NK-receptors and is utilized in the alternative activation pathway of the complement system (Khalturin et al., 2004).

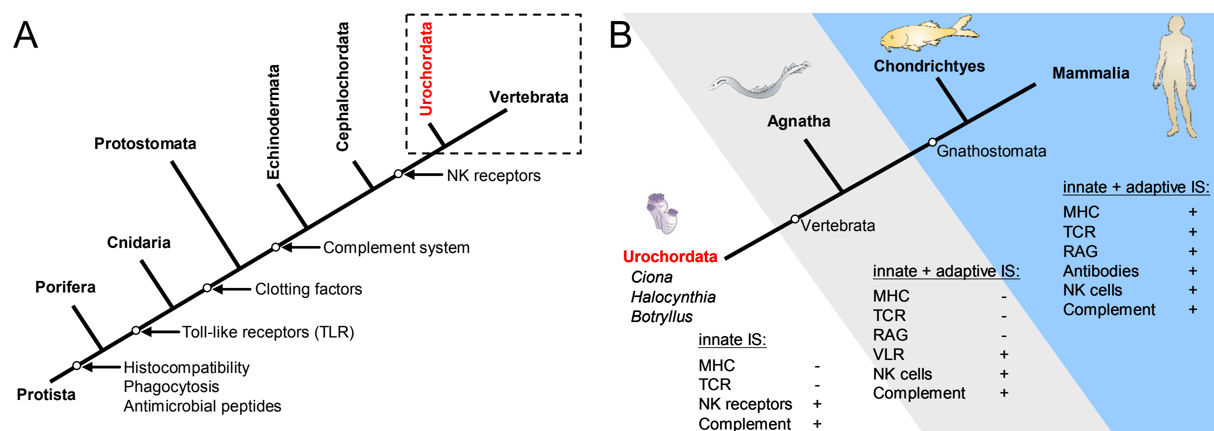

Fig. 1-2: Evolution of pathogen defense. (A) Major phases during development of the innate immune system. (B) Evolution of vertebrate adaptive immune system. Modified from Khalturin, 2002.

It is a well known fact that the molecular mechanisms used to defend against pathogens within the innate immune system are evolutionary conserved (Fig. 1-2 and

Miller et al., 2007). For example, complement components are present in all metazoans. The adaptive immune system, in contrary, is only present in higher vertebrates (Cooper and Alder, 2006).

While molecular mechanisms underlying pathogen defense have been well studied (Janeway et al., 2002) only little data is available on histocompatibility and self-sterility. Did these two self/non-self recognition processes also evolve gradually and are the corresponding receptors conserved among different taxa? Do immunity and histocompatibility share common evolutionary roots? To address these questions model organisms representing different branches of the phylogenetic tree must be used. Especially informative are basal metazoans (sponges and cnidarians) and animals which are closely related to higher vertebrates such as urochordates.

Being sister group of the vertebrates, urochordates occupy a peculiar position within metazoan phylogeny and especially among chordates (Delsuc et al., 2006). Tunicates display two types of self/non-self recognition: histocompatibility and self-sterility (Khalturin et al., 2004). Thus, these animals represent an instructive model system to gain insides into the molecular mechanisms and evolution of self/non-self recognition mechanisms. This study, therefore, focuses on the molecular basis of self/non-self recognition in the tunicate *Ciona intestinalis*.

1.2 The model organism *Ciona intestinalis*

1.2.1 Phylogeny of *Ciona intestinalis*

Deuterostomes can be subdivided into three different lineages of monophyletic origin: the echinoderms, hemichordates and chordates. Within the chordate phylum tunicates (or urochordates) represent the third subphylum apart from vertebrates and cephalochordates (see Fig. 1-3 A). According to recent phylogenetic studies urochordates are the closed living relatives of vertebrates (Delsuc et al., 2006; Delsuc et al., 2008; Singh et al., 2009). This phylogenetic position as basal chordates makes it possible to gain insides into the evolution of the vertebrates by studying urochordates. The urochordate subphylum can be further divided into four classes (Perez-Portela et al., 2009): Ascidicea, Thaliacea, Sorberacea and Larvacea (Appendicularia).

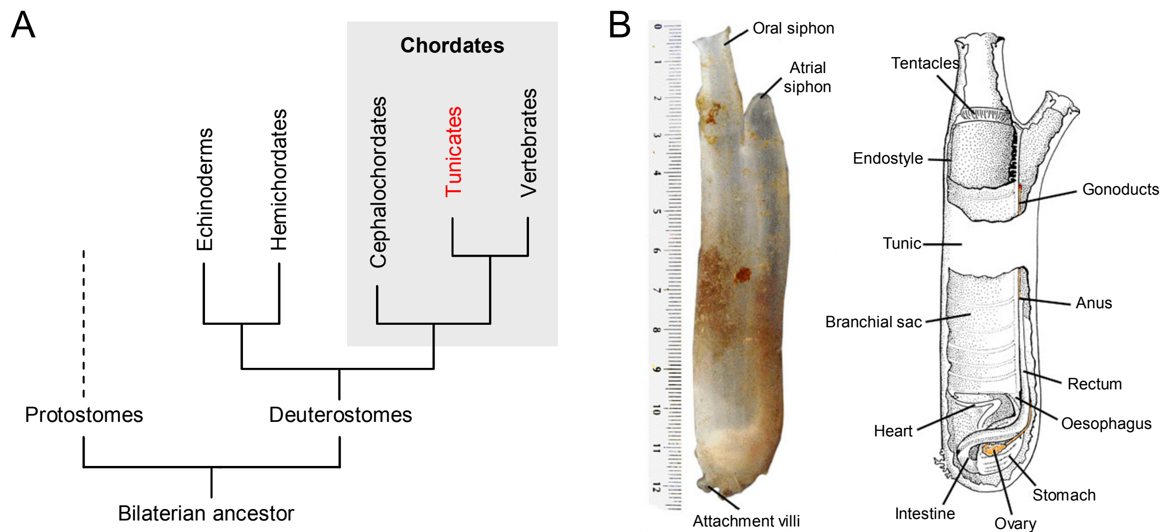

Fig. 1-3: Tunicate phylogeny and body plan of *Ciona intestinalis.* (A) Phylogenetic tree of metazoa, modified from Singh et al., 2009. (B) Photograph and schematic drawing of adult *Ciona intestinalis* specimen, modified from Cirino et al., 2002.

The chorda dorsalis (notochord), neural tube, pharyngeal basket and presence of a post-anal tail are characteristic features of a chordate body plan. These structures can be identified in the larvae of all urochordates. However, in most adult urochordates only the pharyngeal basket remains functioning as a filtering and respiratory organ. The body of all urochordates is covered by the tunic which is the synapomorphic feature of this taxon. The tunic is produced by epidermal cells and consists of proteins and carbohydrates especially tunicin which is analogous to cellulose. The genes responsible for synthesis of tunicin were presumably transferred horizontally from bacteria 500 million years ago (Satoh and Levine, 2005) .

Ciona intestinalis belongs to the class Ascidicea representing the largest phylum among the urochordates comprising of more than 2000 species which are either solitary or colonial. *Ciona* is a solitary ascidian. Adult animals are marine, sessile, semi-transparent and grow up to 15 cm in size (Fig. 1-3 B). Animals can be found around the globe growing on hard substrates like rocks or algae (Dybern, 1963). Food particles are transported through the oral siphon by water flow and stick to mucus produced by endostyle glands. The mixture of mucus and food particles is further transported to the oesophagus and the digestive tract by movement of cilia present on the surface of the pharyngeal basket (Westheide and Rieger, 2006). Like in all urochordates the vascular system in *Ciona* is not closed. Thus, lymph and body fluid are mixed (hemolymph). The heart consists of only one chamber and pumps the hemolymph rhythmically either into the pharyngeal slits or into the body. Within the hemolymph nine morphologically different types of hemocytes can be identified using

electron microscopy. Some of these types express specific marker genes (reviewed in Arizza and Parrinello, 2009) but the lineage and their relationship is unknown as well as the location of hematopoietic tissue.

1.2.2 Development of *Ciona*

Ciona intestinalis is a hermaphrodite. Adult animals produce sperm and oocytes in testis and ovary respectively. Both types of gametes accumulate in gonoducts and are simultaneously released through the atrial siphon into the surrounding sea water. Spawning is induced by light. Despite of simultaneous spawning animals are self-sterile (see section 1.4).

Within 18h after fertilization the embryo develops into a free-swimming tadpole larva (Fig. 1-4 A-C). The larva is the only mobile phase within the life cycle of *Ciona* and has a typical chordate body plan (see section 1.2.1).

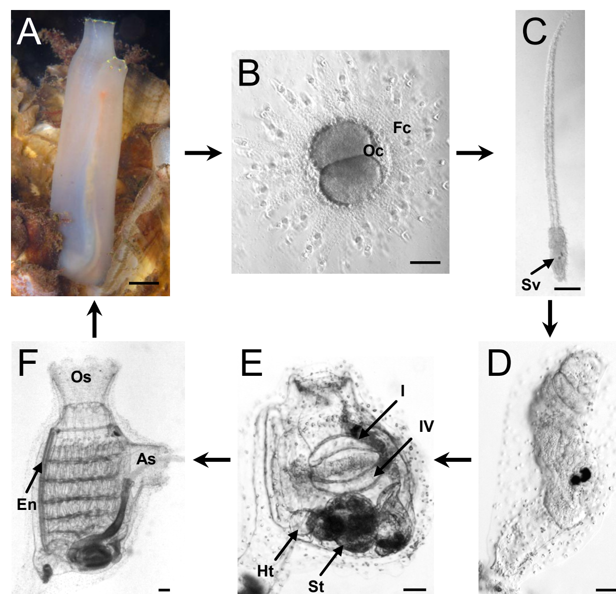

Fig. 1-4: Life-cycle of *Ciona intestinalis.* (A) Adult *Ciona* individuals spawn gametes into the surrounding sea water. (B) Developing oocyte covered with follicle cells. Oc - oocyte, Fc - follicle cell. (C) Free-swimming tadpole larva developed from fertilized embryo. Sv - sensory vesicle. (D-F) After larval settlement the adult body plan is established by rotation of the body axis (D) and metamorphosis into FAS (E) and SAS (F) juvenile stage (first and second ascidian stage, five and 14 days after fertilization). Ht - heart, St - stomach, I/IV - protostigmata, Os - oral siphon, As - atrial siphon, En - endostyle. Bars represent 1cm in (A) and 50µm in (B-F). Scheme modified from Chiba et al., 2004.

The Larva consists of only 2500 cells and two compartments - trunk and tail. Larvae settle on a suitable substrate with their anterior part and undergo complete metamorphosis. Larval structures like the chorda, neural tube and tail are resorbed and the body is turned 90 degrees along its axis (Fig. 1-4 D). After that rotation the

anterior part of the larva is transformed into the oral siphon (Willey, 1893). Within two weeks the adult body plan is established by development of pharyngeal slits, endostyle, heart and hemocytes by the first and second ascidian stage juveniles (Fig. 1-4 E-F). Testis and ovary are developed within two to three months after fertilization and the life-cycle is then completed (Berrill, 1947; Cirino et al., 2002).

1.2.3 *Ciona intestinalis* as model organism

Ciona intestinalis has been studied intensively since the beginning of the last century. Its transparent body enables direct visualization of biological processes in the living animal and the embryonic development is fast and mosaic (Satoh, 2001). These features allow to trace the fates for every single blastomere and the establishment of lineage-maps and, therefore, presented the basis for *Ciona* as a model organism for embryology (Conklin, 1905; Nishida and Satoh, 1983, 1985; Nishida, 1987; Imai et al., 2006). Since the ascidian tadpole larva represents the prototypical chordate body plan despite its cellular simplicity (Corbo et al., 2001) and due to the informative phylogenetic position as sister group of the vertebrates within the chordate lineage, *Ciona* allows to gain insights into chordate evolution.

The *Ciona* genome has been sequenced in 2002 (Dehal et al., 2002). Being 160Mbp, it is much smaller compared to vertebrate genomes such as those of human, mouse or puffer fish (Lander et al., 2001; Venter et al., 2001; Aparicio et al., 2002; Waterston et al., 2002), but is comparable in size to those of other invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* (C. elegans Sequencing Consortium, 1998; Adams et al., 2000). In most of the cases only one homologue of a vertebrate gene family can be found in the *Ciona* genome presumably representing the ancestral state before duplications occurred in the vertebrate lineage (Lemaire, 2006). That feature of the *Ciona* genome is very valuable as it may simplify the analysis of gene regulatory networks due to the missing genetic redundancy (Dehal and Boore, 2005; Holland et al., 2008). Many cDNA libraries from different developmental stages and tissues were sequenced (Satou et al., 2003). To date more than 1.2 million ESTs (expressed sequence tags) and 6893 complete transcript sequences representing almost 50 percent of all 15500 protein coding genes (Simmen et al., 1998) are publicly available at NCBI.

Large-scale expression analysis using whole mount *in situ* hybridization have been performed (*Ciona intestinalis* adult *in situ* hybridization database “CiAID”,

http://bioinfo.s.chiba-u.jp/ciaid/) and genomic scaffolds have been mapped onto chromosomes using FISH (fluorescence *in situ* hybridization) with BAC clones (Shoguchi et al., 2005; Shoguchi et al., 2006).

Generation of transgenic animals is possible which is the fundamental requirement for a model organism in the modern molecular biology. By means of microinjection or electroporation nucleic acids can be introduced simultaneously into single or even hundreds of embryos (Sasakura et al., 2003; Sasakura et al., 2007). These techniques can be used for enhancer-trap experiments (Awazu et al., 2007) or insertional mutagenesis (Sasakura, 2007) as well as for the functional analysis of genes by gain or loss of function experiments either by overexpression or knock-down of gene expression (Davidson et al., 2006; Nishiyama and Fujiwara, 2008).

1.2.4 Presence of two cryptic *Ciona intestinalis* species

Ciona intestinalis is referred to as a cosmopolitan species inhabiting temperate waters around the globe (Hoshino and Nishikawa, 1985; Kobayashi et al., 2002). Animal morphology varies little within individuals of a population. Only slight differences can be observed according to coloration of the tunic and eggs but in total, different populations do not display much morphological variation. Nevertheless, recent studies have shown that British and Japanese animals require other temperatures for optimal growth and display genomic differences (Caputi et al., 2007). These differences are mostly located in intronic regions and populations can be characterized by presence or absence of the transposable element *Cigr*-1 (Suzuki et al., 2005). Using molecular markers which exploit these genetic differences several *Ciona intestinalis* populations around the globe were assigned to either one of the two types (see Fig. 1-5 and Suzuki et al., 2005; Caputi et al., 2007). Type A populates the coast of Japan, Australia, South America, the Mediterranean Sea and the west coast of North America. Type B animals inhabit the east coast of North America and the coast of England, Scotland and Sweden (Suzuki et al., 2005; Caputi et al., 2007).

By comparison of mainly mitochondrial sequence information, separation of the two types was estimated to have occurred more than 20 million years ago (Iannelli et al., 2007; Nydam and Harrison, 2007). Most importantly, crossings of both types do not result in viable and fertile offspring (Suzuki et al., 2005; Caputi et al., 2007). Therefore, all the criteria for the classical reproductive isolation are fulfilled and the

two types can be regarded as different species which have diverged very recently or are maybe still on the way of genetic separation. Hence, in the following sections animals will be referred to as *Ciona intestinalis* species A or B.

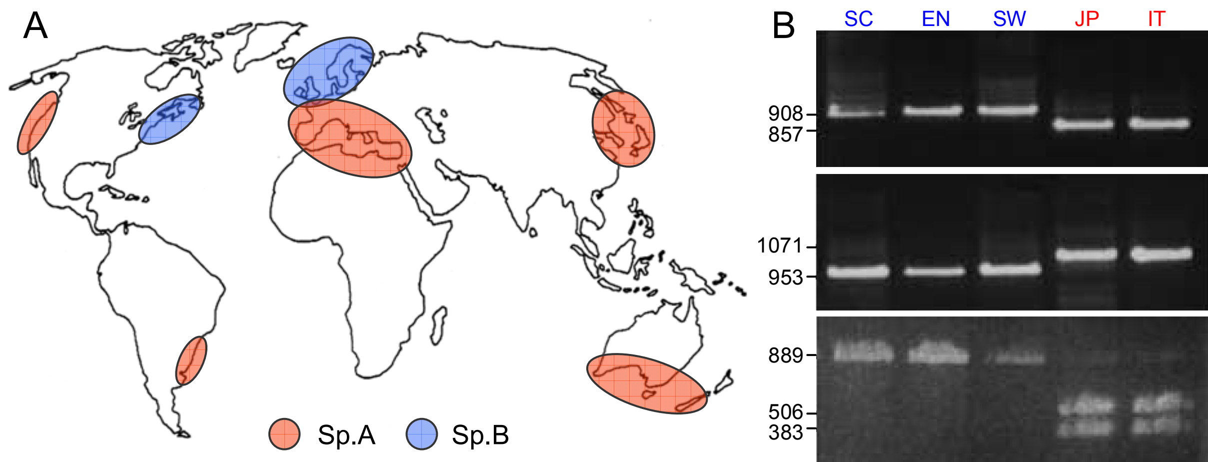

Fig. 1-5: Presence of two cryptic *Ciona intestinalis* species. (A) Distribution of both cryptic *Ciona intestinalis* species A and B around the globe (modified from Caputi et al., 2007). (B) Although morphologically indistinguishable *C. intestinalis* species A and B can be discriminated by use of molecular markers (modified from Suzuki et al., 2005). *Ciona intestinalis* species A and B are colored red and blue respectively. SC - Scotland, EN - England, SW - Sweden, JP - Japan, IT - Italy.

1.3 Histocompatibility

Allogeneic tissue transplantation in vertebrates leads to the rejection of the transplant. Recognition is based on MHC (major histocompatibility complex) receptors representing polymorphic self markers. MHC haplotypes have to be shared in donor and recipient for tolerance of the transplanted tissue. Since vertebrates do not undergo natural transplantation reactions this mechanism is a consequence of the ability of the adaptive immune system to recognize any type of non-self (Janeway et al., 2002).

In contrast to vertebrates, colonial urochordates like *Botryllus schlosseri* undergo naturally occurring transplantation reactions. Already at the beginning of the last century it has been shown that parts of the same colony fuse whereas parts of genetically different colonies initiate a rejection reaction if they come in contact (Bancroft, 1903). This self/non-self recognition is encoded genetically by one genomic locus termed Fu/HC (fusion/histocompatibility locus). This locus is highly polymorphic and, thus, many alleles are present within a population (Oka and Watanabe, 1957; Scofield et al., 1982; Weissman et al., 1990). Therefore, most animals are heterozygotes in Fu/HC. Colonies only fuse if at least one allele is shared (De Tomaso and Weissman, 2003). The *Botryllus* Fu/HC-locus has been

sequenced and two contained polymorphic genes have been characterized termed cFuHc and fester (De Tomaso et al., 2005; Nyholm et al., 2006). Alleles of the cFuHC gene correlate with the ability to fuse or reject but further functional experiments have not been conducted (De Tomaso et al., 2005). Knock-down of fester gene expression diminished sensing capability towards allogeneic colonies resulting in inhibition of rejection. Moreover, a monoclonal α-fester antibody was able to convert rejection into fusion (Nyholm et al., 2006). This data indicates an involvement of both candidate genes in the process of colony specificity in *Botryllus schlosseri* but the definite mechanism still remains unknown.

Solitary urochordates do not undergo natural transplantation reactions like colonial tunicates. Nevertheless, experimental tissue rejection can be observed in several solitary ascidian species. For example tunic transplants of allogeneic animals are rejected between different individuals of *Ciona intestinalis* or *Styela plicata* (Reddy et al., 1975; Raftos and Briscoe, 1990). It has been even proposed that these animals possess an immunological memory since secondary transplants were rejected in a shorter period of time than primary (Raftos et al., 1987). For another solitary ascidian *Halocynthia roretzi* a different kind of histocompatibility reaction is described. Hemocytes isolated from allogeneic individuals lyze each other in a fast cytotoxic reaction termed ACR (allogeneic contact reaction, Arai et al., 2001) which is controlled by one or two polymorphic genomic loci (Fuke and Nakamura, 1985). Interestingly, antibodies raised against N-glycosylated proteins influence both the ACR as well as self-sterility (Fuke, 1990). Thus, the molecular mechanisms underlying these aspects of self/non-recognition seem to be similar or even the same in *Halocynthia*.

Both naturally occurring colony specificity as well as experimental tissue rejection can be observed in many metazoan phyla including bryozoans, sponges, cnidarians and urochordates indicating an early development of this biological feature (Bancroft, 1903; Chadwick-Furman and Rinkevich, 1994; Humphreys, 1994; Hughes et al., 2004). Progenitors of the vertebrate histocompatibility mechanism have been assumed to be present in basal chordates because of the monophyletic origin of chordates (Burnet, 1971; Saito et al., 1994). However, neither MHC-like molecules were found in urochordates and other basal chordates nor a syntenic genomic locus could be identified in the sequenced genomes of *Ciona intestinalis* and *Ciona savignyi* (Dehal et al., 2002; Small et al., 2007). This indicates that although self/non-

self recognition processes are present in many phyla they seem to use different molecular mechanisms to achieve the same function of protecting the genetic individuality of an organism (Buss, 1987; Khalturin and Bosch, 2007).

1.4 Self-sterility

Generation of new genotypic variations by recombination of the parental genetic material is the main benefit of sexual reproduction allowing an adaptation to changing environmental conditions. However, sexual reproduction raises new problems. Since many plant and animal species are hermaphrodites, specific mechanisms are required to avoid self-fertilization which would result in loss of genetic diversity and might increase the chances of offspring being affected by recessive or deleterious traits (inbreeding depression). How is this type of self/non-self recognition achieved?

Self-incompatibility was intensively studied in fungi, urochordates and plants. At present, comprehensive understanding of the molecular and mechanistic processes controlling self-sterility is only available in plants. Flowers of many plant species have both male and female reproductive structures or a single plant possesses both types of unisexual flowers. But despite this hermaphroditic state most plant species are self-incompatible (De Nettancourt, 2001). Cells of the pistil are able to discriminate between auto- and heterologous pollen and inhibit development and growth of self pollen tube. In *Brassicaceae* this recognition is controlled by one highly polymorphic genomic locus termed S-locus. In grasses a second Z-locus acts cooperatively together with the S-locus (Hunter, 2009). The S-locus contains two tightly linked genes encoding the male and the female determinant (Takayama et al., 2000; Kachroo et al., 2001). The interaction of stigma SRK (S-locus receptor kinase) and pollen SCR/SP-11 (S-locus cysteine-rich) proteins determine self-incompatibility. If SRK and SCR belong to one haplotype pollination is inhibited. After an interaction of both determinants the ubiquitin ligase ARC1 (Armadillo-repeat-containing 1) is activated via phosphorylation and initiates proteolysis of Exo70A1 which is required for growth of the pollen tube (Zhang et al., 2009). Therefore, fertilization is inhibited. Mechanisms preventing self-pollination differ among plant families. In *Rosaceae* development of autologous pollen is inhibited by RNase secreted from cells of the stigma whereas pollen decay in *Papaveraceae* is the result of apoptosis (Thomas et al., 2003; McClure, 2004). Recently, both the female and male determinants were isolated and characterized from *Papaver rhoeas* (Wheeler et al., 2009).

In urochordates self-sterility has been the subject of intense studies for almost a century (Morgan, 1923). Most solitary urochordates are hermaphrodites with individual animals having both testis and ovary. Ascidian oocytes are covered by a cellular layer consisting of follicle and test cells as well as an acellular vitelline envelope. Spawning of both types of gametes into the surrounding sea water is regulated by changing light conditions and, therefore, occurrs almost synchronously among animals in a population (Cirino et al., 2002). However, despite of external fertilization and simultaneous spawning most ascidians are self-sterile (Lambert, 2005). Thomas Hunt Morgan was the first who performed crosses and analyzed the incidence of cross-sterility among siblings (Morgan, 1923, 1938a, b, 1939a, b, 1942, 1944). He concluded that self-sterility is determined by two to five genomic loci, but the involved loci and encoded molecules have not been isolated and characterized further (Morgan, 1942; Murabe and Hoshi, 2002).

Interestingly, oocytes are not self-sterile from the very beginning. They have to undergo a maturation process to acquire self recognition capability (Lambert, 2005). Completion of this maturation is visualized by breakdown of the germinal vesicle (Fig. 1-6).

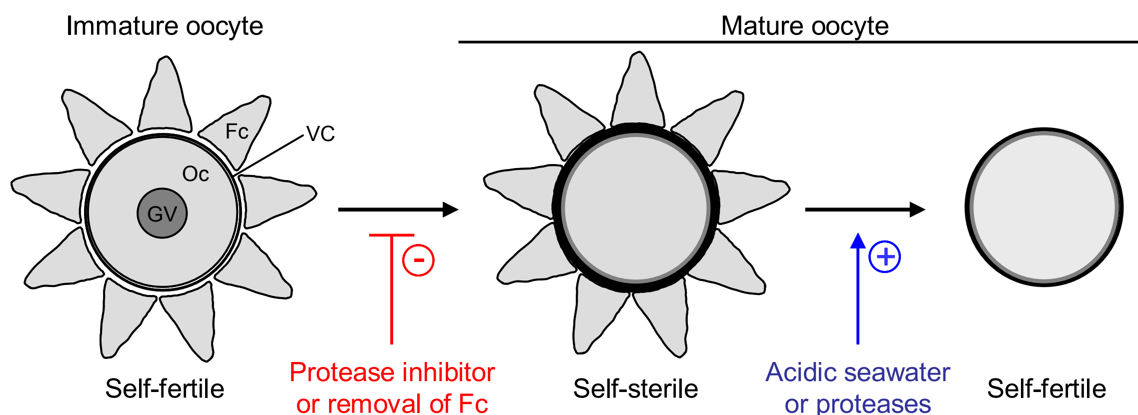

Fig. 1-6: Oocyte maturation in *Ciona intestinalis*. Immature oocytes acquire self-sterility after maturation inside the ovary. Breakdown of the germinal vesicle is a marker for maturation. Acquisition of self-sterility can be inhibited by treatment with protease inhibitors or by removal of follicle cells. Self-sterile mature oocytes become self-fertile if incubated in acid sea water or with proteases. Note that during this treatment follicle cells detach from the oocyte surface.

In *Ciona intestinalis* oocyte maturation can be inhibited by applying protease inhibitors or by removal of follicle cells (De Santis and Pinto, 1991). Interestingly, co-culturing of separated follicle cells is sufficient to establish self-sterility in an individual-specific manner (Pinto et al., 1995). This indicates that follicle cells are essential for a successful maturation process since they either secrete a factor which is the female determinant itself or which proteolytically modifies the self-sterility

factor. This factor is proposed to be located at the vitelline envelope because heterologous sperm are able to bind more firmly to this layer than autologous sperm (Rosati and de Santis, 1978). Moreover, by transmission electron microscopy studies it was shown that the vitelline envelope thickens during oocyte maturation and its thickness decreases by an incubation of oocytes in acidic sea water which removes the self/non-self recognition potential (Pinto et al., 1995). However, during this treatment follicle cells are separated as well. Thus, it cannot be excluded that the loss of self-sterility is connected with the loss of follicle cells. It is also known that follicle cells play a role in several other aspects of fertilization.

For example, in *Halocynthia roretzi* follicle cells are absolutely required for fertilization. Removal of these cells completely abolishes fertilization capacity (Fuke, 1983; Kawamura et al., 1988). In *Ciona* follicle cells (i) produce and secrete a protease which induces germinal vesicle breakdown during oocyte maturation, (ii) mediate the primary block to polyspermy by secreting a glycosidase, (iii) protect the oocyte nucleus from damage caused by UV-light since they contain UV absorbing mycosporine-like amino acids and (iv) they influence egg floatation by their shape (Lambert, 2009). Interestingly and most important, follicle cell shapes vary among ascidians and even between different species of the same genus. These modifications of follicle cell architecture might indicate that different species use other systems to confer self-sterility (Fig. 1-7).

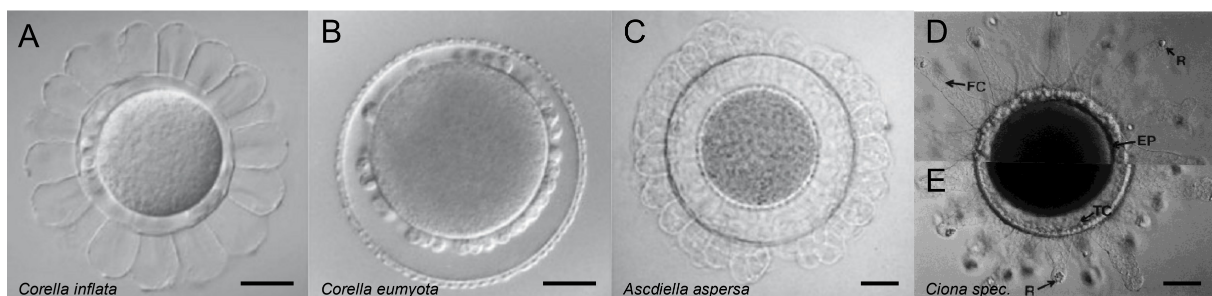

Fig. 1-7: Follicle cell morphology varies in different ascidian species. Microscopic pictures of spawned oocytes from (A) *Corella inflata*, (B) *Corella eumyota*, (C) *Ascidiella aspersa*, (D) *Ciona intestinalis* and (E) *Ciona savignyi*. Note even species of the same genus possess completely different architecture of follicle cells. EP - egg plasma membrane, R - refringent body, TC - test cell, FC - follicle cell. Bars represent 50µm. Modified from Byrd and Lambert, 2000 and Lambert, 2009.

Taken together, much is known about the cellular processes during self/non-self recognition controlling self-sterility in urochordates but the underlying molecular mechanisms for most tunicate species remain completely unclear.

A candidate molecule controlling self-sterility has been described for two *Halocynthia* species *H. roretzi* and *H. aurantium* (Sawada et al., 2002; Sawada et al., 2004; Ban

et al., 2005). These proteins termed HrVC70 and HaVC80 are composed of an N-terminal signal peptide, 12 and 13 EGF domains respectively and are proteolytically processed from a precursor protein containing additionally a transmembrane domain. HrVC70 and HaVC80 display several properties of a potential self/non-self recognition molecule: (i) both proteins are polymorphic and thus presumably individual-specific, (ii) they are present on the vitelline envelope and (iii) can be extracted by acidic sea water. (iv) Extracted HrVC70 is preferentially bound by heterologous sperm and (v) fertilization is inhibited if sperm is pre-incubated with extracted HrVC70 protein. Notably, the inhibitory effect of heterologous HrVC70 is stronger than that caused by HrVC70 extracted from the same individual. Several putative interaction partners for HrVC70 have been identified but no functional interaction was demonstrated (Harada and Sawada, 2007). Only a CRISP-like protein expressed by sperm has been shown to physically interact with the HrVC70 protein, but binding is not individual-specific (Urayama et al., 2008). This data suggests an involvement of oocyte HrVC70/HrVC80 and sperm CRISP-like proteins in the control of *Halocynthia* self-sterility but the exact mechanisms remain unclear.

Apart from *Halocynthia* candidate self-sterility molecules have only been isolated and characterized from *Ciona intestinalis*. By comparing ovarian transcriptomes of three individuals by SSH to screen for individual-specific variable genes expressed in the female gonad, a highly polymorphic gene was isolated which displays structural similarities to the human complement receptor CD46 (Fig. 1-8 A, Kürn et al., 2007). Thus, this gene was termed vCRL1 (variable complement receptor like 1). The vCRL1 protein is composed of a N-terminal signal peptide, a transmembrane domain and contains a variable number of CCP domains as result from alternative splicing (Fig. 1-8 B). As shown in Fig. 1-8 C and D, vCRL1 gene is expressed in hemocytes, siphon and ovary tissue. Within the female gonad expression is restricted to follicle cells surrounding the oocyte. In hemocytes vCRL1 is alternatively spliced which produces several isoforms. Moreover, vCRL1 protein is highly polymorphic and individual-specific (Fig. 1-8 E). Thus, vCRL1 represents a candidate self-sterility receptor in *Ciona intestinalis* species B. However, functional involvement of vCRL1 in allorecognition remains to be proven.

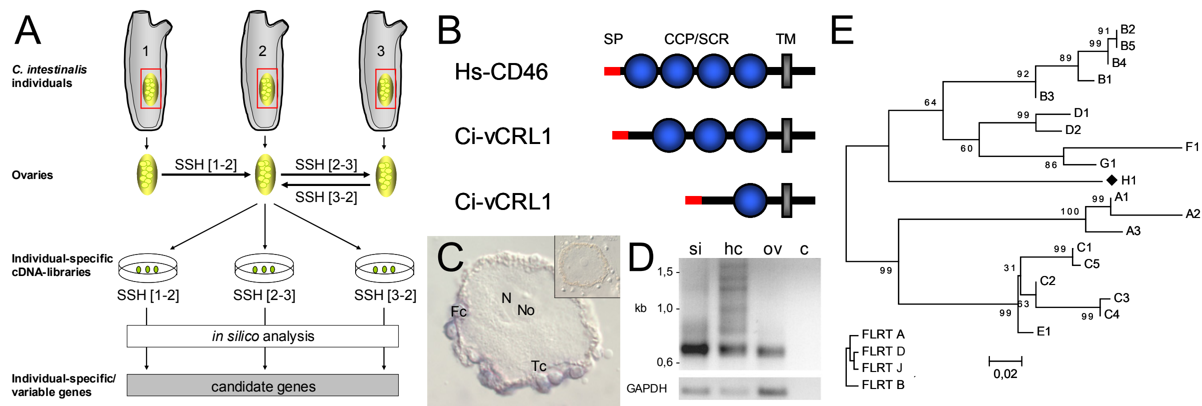

Fig. 1-8: Identification and initial characterization of vCRL1 - an individual-specific variable gene expressed in follicle cells. (A) Isolation of vCRL1 by unbiased SSH screening for individual-specific variable genes expressed in *Ciona* ovary. (B) Domain organization of vCRL1 protein variants compared to human CD46. (C) Expression analysis for vCRL1 gene by *in situ* hybridization with sectioned ovary. Sense control is depicted in upper right corner. (D) vCRL1 RT-PCR expression analysis compared to GAPDH. Si - siphon, hc - hemocytes, ov - ovary, c - no template. (E) Neigbor-Joining phylogenetic tree of vCRL1 proteins from different animals showing high polymorphism compared Ci-FLRT. Modified from Kürn et al., 2007.

Recently two additional candidate self/non-self recognition loci have been isolated from Japanese *Ciona intestinalis* (species A, Harada et al., 2008). The two loci were restricted to 170kb and 1Mb. Both loci lack an obvious sequence homology but they contain syntenic gene pairs encoding a putative receptor and ligand. These genes were termed v- and s-Themis since the encoded proteins are present at the vitelline envelope and sperm respectively. Alleles of Themis genes correlate with the observed cross-fertilization phenotype in almost all cases indicating that self-sterility is controlled by the interaction of v- and s-Themis proteins encoded by both loci. Involvement of Themis in self-sterility in *Ciona intestinalis* species B, however, has not been demonstrated.

1.5 Aims of the study

Tunicates represent an informative system to analyze the molecular mechanisms of self/non-self recognition because they display both self-sterility and histocompatibility reactions. Several variable receptors were isolated from *Botryllus*, *Halocynthia* and *Ciona* (Sawada et al., 2004; Ban et al., 2005; De Tomaso et al., 2005; Nyholm et al., 2006; Kürn et al., 2007; Harada et al., 2008). Some of these genes have been shown to be functionally involved in self/non-self recognition.

In Northern European *Ciona intestinalis* (species B) we have previously isolated the candidate self/non-self recognition receptor vCRL1 which is expressed in ovary (Kürn et al., 2007). Additionally, two genomic loci termed Themis have been isolated in Pacific animals by Harada and colleagues using segregational analysis. Themis loci were shown to confer self-sterility in *Ciona intestinalis* species A (Harada et al., 2008). However, it is not yet clear, if these two Themis loci are sufficient or if an additional locus is involved as it has been proposed by Thomas Morgan (Morgan, 1942; Harada and Sawada, 2008). Furthermore, it has not been shown, whether Themis and vCRL1 are involved in the control of self-sterility in *Ciona intestinalis* species B as different species might use different receptors for self-sterility.

The goal of this study was the detailed characterization of the vCRL1 gene and its role in self/non-self discrimination processes in *Ciona intestinalis* species B.

Specific aims of my work were:

- production of recombinant vCRL1 protein and generation of α-vCRL1 antibodies;
- clarification of vCRL1 protein localization;
- analysis of the genomic organization of the vCRL1 locus which might assist in the identification of potential ligands of vCRL1 protein;
- functional analysis of vCRL1 by antibody interference, segregational analysis and knock-down using stable transgenesis.

2 Results

2.1 Northern European *Ciona intestinalis* belong to species B

In recent publications it has been shown that two morphologically indistinguishable but genetically different *Ciona intestinalis* populations inhabit different locations around the globe (see Fig. 1-5 and Caputi et al., 2007). Since crosses of animals belonging to these two populations do not result in viable and fertile offspring, they can be regarded as separate species according to the classical species definition.

We work with animals from Bergen (Norway) and Helgoland (Germany). Therefore, it was important to examine which species these animals belong to. Three PCR-based markers (Suzuki et al., 2005) were tested that exploit the genetic differences between both *Ciona intestinalis* species (Fig. 2-1). Animals sampled around Bergen (Norway) and Helgoland (Germany) show the same characteristic pattern of *C. intestinalis* species B and are different from Japanese *Ciona* individuals which belong to species A. This experimental evidence is very important since transgenic animals were generated using individuals collected in Norway and constructs have been made based on promoter and terminator sequences from German animals (see section 2.8).

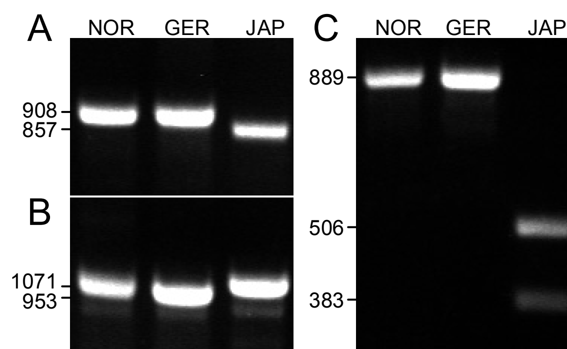

Fig. 2-1: Analysis of cryptic *Ciona intestinalis* species. PCR-based markers 1 (A), 2 (B) and 4 (C) published by Suzuki et al., 2005 were used for amplification from genomic DNA of randomly sampled Norwegian (NOR), German (GER) or Japanese (JAP) *Ciona intestinalis* individuals. The numbers indicate the size in basepairs of amplified fragments.

2.2 Mature *Ciona* oocytes are completely covered by follicle cells

In *Ciona intestinalis* only heterologous sperm are capable to accomplish fertilization (Lambert, 2005). Self/non-self discrimination event between oocyte and sperm is likely to occur at the earliest time point. Therefore, proteins responsible for this mechanism are proposed to be located on the outermost surfaces of both gametes.

Mature *Ciona intestinalis* oocytes are covered by two layers: (i) an acellular layer - the vitelline envelope and (ii) a cellular layer, consisting of test and follicle cells (Honegger and Koyanagi, 2008). In several publications using glycerinated fixed oocytes deprived of follicle cells, it has been proposed that sperm recognition occurs at the acellular layer because heterologous sperm bind more firmly to the vitelline envelope than autologous sperm (Rosati and de Santis, 1978; De Santis et al., 1979; De Santis and Pinto, 1991). However, these experiments do not represent the natural conditions during the fertilization process. Thus, it cannot be excluded that follicle cells are responsible for self/non-self recognition since they represent at least a mechanical barrier for sperm penetration and are the outermost layer of a mature oocyte. To analyze whether follicle cells cover the oocyte completely or if spermatozoa are able to pass through, we conducted a surface analysis of spawned mature oocytes using electron microscopy (Fig. 2-2).

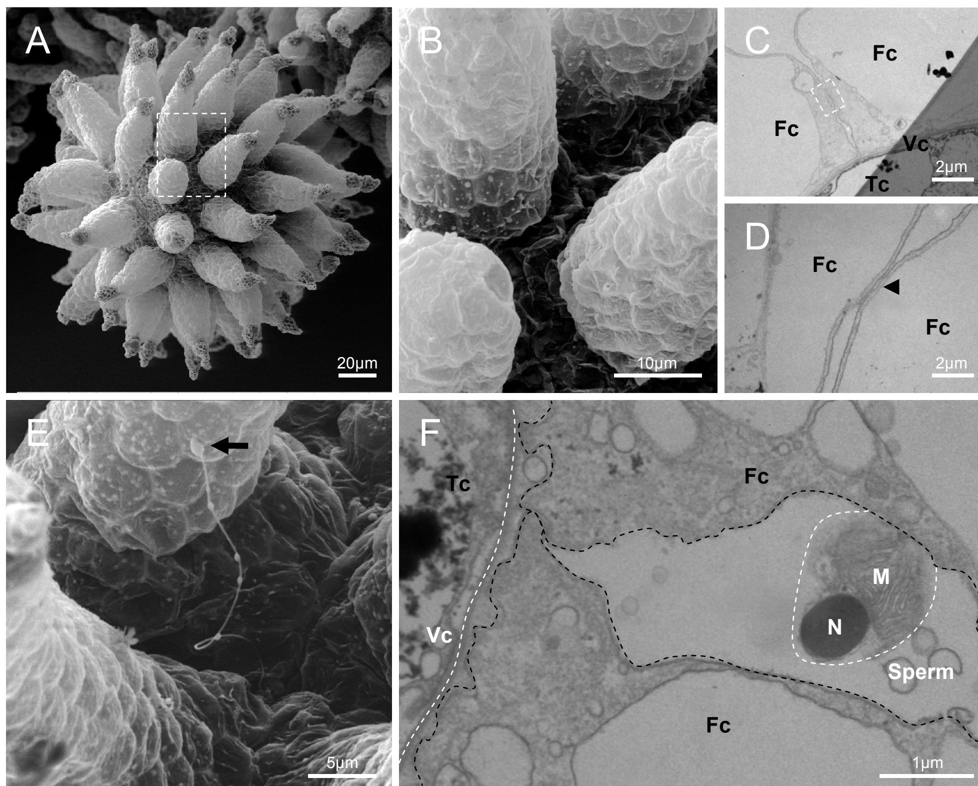

Fig. 2-2: Surface analysis of spawned mature oocytes using electron microscopy. (A-B) SEM micrograph of spawned mature oocyte. (B) Magnification of indicated area in (A). (C-D) TEM pictures showing two adjacent follicle cells located on top of the acellular vitelline envelope and a layer of test cells. (D) Magnification of indicated area in (C). The arrowhead indicates a junction between two follicle cells. (E) SEM and (F) TEM pictures of mature oocytes incubated with heterologous sperm. Note the diameter of the sperm head (arrow) is 1-2µm and therefore larger than the ridge between two follicle cells. The follicle cell surface as well as the sperm head have been highlighted using dashed lines for better visualization. Fc - follicle cell; Tc - test cell; Vc - vitelline envelope, N - nucleus; M - mitochondrion. Modified from Sommer et al., 2010.

As shown in Fig. 2-2, follicle cells are situated very close to each other so that

spermatozoa are incapable to cross this cellular layer. Furthermore, it seems to be the case that follicle cells are interconnected (see Fig. 2-2 C, D). Taken together, evidence from this morphological study and other previous experiments (De Santis and Pinto, 1991) in which immature oocytes deprived of their follicle cells did not acquire self-sterility, suggests that follicle cells are important for self/non-self recognition during fertilization in *Ciona intestinalis*. Moreover, the first contact between of both gametes takes place between sperm and follicle cells of the mature oocyte.

2.3 Analysis of the vCRL gene

2.3.1 Ovarian vCRL variants of different *Ciona* individuals are highly variable

Genes involved in the control of self-sterility in *Ciona intestinalis* need to fulfill several criteria: (i) they must be individual-specific, meaning that they should be highly polymorphic. Therefore, every animal should have its own unique variant to distinguish itself from other individuals. (ii) The candidate gene is very likely to be expressed at the site of recognition since the protein has to be present at the interacting surface. In the case of an interaction between sperm and oocyte these sites are presumably the sperm head surface and the vitelline coat or the follicle cells of the mature oocyte (see section 1.4).

We have previously isolated and initially characterized the individual-specific gene vCRL (variable complement receptor like, formerly termed vCRL1, Kürn et al., 2007) by comparing the ovarian transcriptomes of three *Ciona* individuals using SSH (suppression subtractive hybridization). This gene is highly polymorphic and expressed in follicle cells. Thus, it is a promising candidate as self-sterility receptor in *Ciona intestinalis*. In the previous analysis vCRL transcript sequences were determined using cDNAs isolated from whole animals. Moreover, due to technical reasons no full-length transcripts have been obtained. Hence, no definite conclusion about the vCRL polymorphism in follicle cells could be made using previous data. In my PhD thesis, I analyzed the vCRL polymorphism restricted to ovarian tissue using 5’RACE.

All vCRL sequences were obtained from animals sampled at Bergen (Norway) or Helgoland (Germany) and compared to those obtained from assembled ESTs available at NCBI originating from Japanese animals (animal J, marked with ◊).

Therefore, the alignment not only comprises of vCRL protein sequences of animals from different geographical locations or belonging to different populations but also includes a comparison of both *Ciona intestinalis* species A and B.

Comparison of ovarian full-length vCRL protein variants containing one CCP domain demonstrates the high polymorphism of the vCRL gene in this tissue (Fig. 2-3). Sequences of different individuals show as little as 37% identity. Even vCRL haplotypes obtained from a single individual display only 37-80% or 54-88% similarity on transcript and protein level respectively. The observed great variability is comparable to differences between separate genes but it is not caused by the presence of a gene family as vCRL is a single-copy gene according to southern blot analysis (Kürn et al., 2007 and unpublished data). Surprisingly, in addition to the extracellular CCP domain also the intracellular domain and the signal peptide of the vCRL proteins vary between different animals or even between alleles of a single individual, e.g. sequences A-1 and A-2 (Fig. 2-3 A).

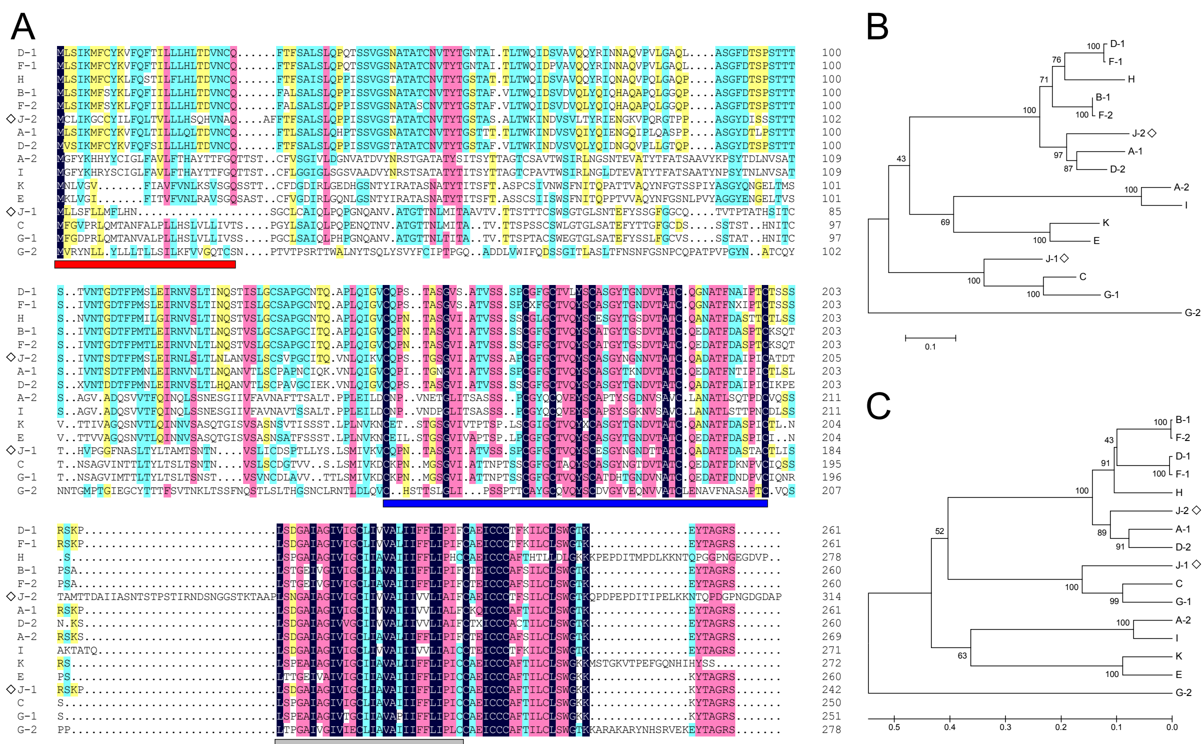

Fig. 2-3: Ovarian vCRL proteins are highly polymorphic. (A) Multiple alignment of 16 vCRL protein variants obtained from 11 *Ciona intestinalis* individuals. All sequences originate from *Ciona intestinalis* species B animals exept those of databank sequences of Japanese animal J (species A, marked with a diamond). Bars underneath the sequence alignment represent domains of the vCRL proteins. Red - signal peptide, blue - CCP domain, grey - transmembrane domain. (B) Neighbor-Joining phylogenetic and (C) UPGMA homology tree drawn using alignment shown in (A). Note that sequences from Japanese animal J do not form a distinct outgroup despite of the geographical separation and representing a different species, indicating that these differences are masked by the extreme polymorphism of the vCRL protein.

Interestingly, sequence similarity among vCRL proteins from different individuals

does not correlate with the geographical distribution of different populations or *Ciona intestinalis* species. As shown in Fig. 2-3 B and C, vCRL protein sequences of animal J (*C. intestinalis* species A) do not form a distinct outgroup in both the phylogenetic as well as the homology tree when compared to sequences of species B individuals. This indicates that vCRL proteins are selected for variability and that this high polymorphism is masking population or even species differences.

2.3.2 Identification of a potential self/non-self recognition locus

In the JGI *Ciona intestinalis* genome project v2.0 the vCRL gene is located in scaffold 269 (Fig. 2-4 A) which is estimated to be 110kb in size. This scaffold is mis-assembled and fragmented by large stretches of missing sequence data due to low coverage by end-sequenced BAC clones and shotgun reads. Thus, currently available sequence information does not allow to clarify the structure of the vCRL gene (see section 2.3.3) and to identify genes which are located in the direct vicinity of vCRL. To overcome this problem a BAC library constructed from genomic DNA of a single Japanese *Ciona intestinalis* individual (Kobayashi et al., 2002) was screened using vCRL probe. Four BAC clones carrying fragments of the vCRL genomic locus were identified using this approach. Furthermore, two BACs of a second library generated from genomic DNA of several individuals were isolated using their end-sequences (Dehal et al., 2002). In total, six BAC clones representing four haplotypes derived from three different animals were isolated, sequenced by shotgun approach and assembled (Fig. 2-4 A). For complete BAC sequences see attached compact disc.

Blastn searches using full-length vCRL sequences revealed that all six isolated BACs contain parts of the genomic locus neighboring the vCRL gene. Comparison of 40kb sequence downstream of the vCRL gene illustrates high inter- as well as intra-individual (allelic) polymorphism in this genomic locus. As shown in Fig. 2-4 B, differences between haplotypes of the same animal are similar to those between haplotypes of unrelated individuals.

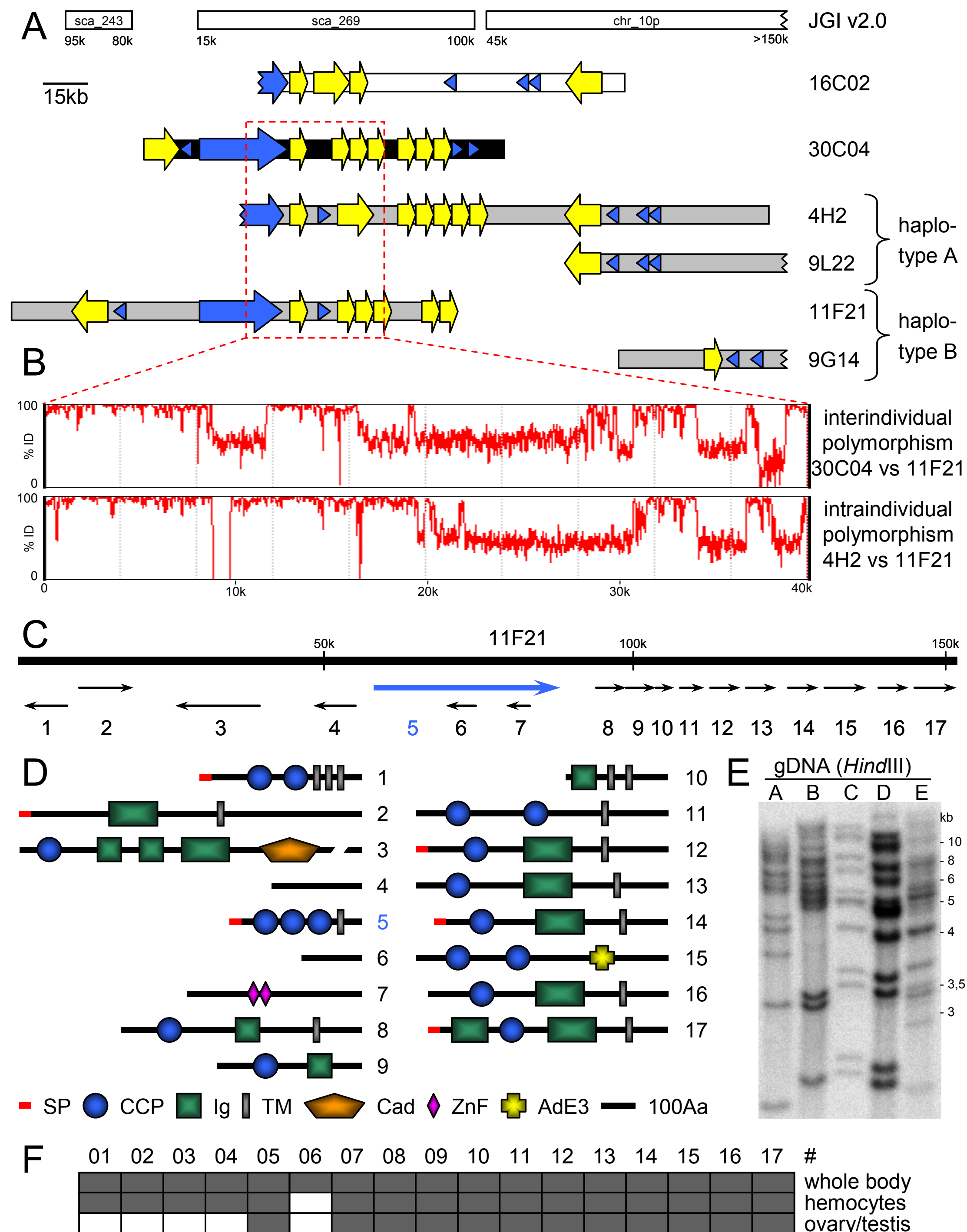

Fig. 2-4: Isolation of the vCRL genomic locus. (A) Schematic overview of six sequenced BAC clones representing both haplotypes of a single individual (grey) as well as two haplotypes from different animals (black and white) compared to their approximate localization in the JGI *Ciona intestinalis* genome v2.0. Blue arrow - vCRL gene, blue triangle - repetitive vCRL 5' sequence, yellow arrow - Ig/CCP domain containing genes. (B) Identity plot of indicated 40kb sequence marked by dashed red line in (A) comparing either clone 30C04 or 4H2 with 11F21. (C) Putative gene map of BAC 11F21 by gene prediction. Arrows represent genes and their location on + or - strand. vCRL gene (#5) is colored in blue. (D) Domain structures of predicted proteins encoded by 11F21. SP - signal peptide, CCP - complement control protein domain, Ig - immunoglobulin-like domain, TM - transmembrane domain, Cad - cadherin domain, ZnF - zinc finger domain, AdE3 - adenovirus E3 domain; (E) Southern blot analysis using genomic DNA of five unrelated *Ciona* individuals digested with *Hind*III and probe directed against sequence between CCP and Ig domains encoded by predicted gene #8. Note that all animals show different hybridization patterns as well as varying numbers of signals indicating presence of a gene cluster and high interindividual polymorphism of this genomic locus. (F) Expression analysis for predicted genes of BAC 11F21. Grey/white boxes indicate presence/absence of ESTs respectively. Modified from Sommer et al., 2010.

The vCRL gene is flanked by several genes encoding receptors with Ig and CCP domains. These genes might code for ligands of the vCRL protein because of their close location to the vCRL gene (Fig. 2-4 A, C) and their expression in the same tissues - hemocytes or gonads - according to EST analysis (Fig. 2-4 F). Number of genes in the cluster and their position differ among individuals and even between haplotypes of the same individual (Fig. 2-4 A) because parts of this cluster are shuffled and re-arranged. Thus, composition of this gene cluster is individual specific. Polymorphism of this genomic locus was analyzed by Southern blot. Genomic DNA of five unrelated *Ciona* individuals was digested with *Hind*III and hybridized with a probe directed against the intervening sequence between the CCP and Ig domains encoded by predicted gene #8 (BAC clone 11F21, Fig. 2-4 E). Southern blot analysis suggests that at least 13 Ig/CCP encoding genes are present in the *Ciona intestinalis* genome. Potentially these genes can be ligands for vCRL receptor.

2.3.3 Structure of the vCRL gene

Since scaffold 269 of the *Ciona* genome project v2.0 cannot be used to reliably determine the structure of the vCRL gene, sequence information from BAC clones (see section 2.3.2) was used to reconstruct the structure of vCRL locus. As shown in Figs. 2-4 and 2-5, the complete vCRL gene is located on BAC clone 11F21 spanning from nucleotide positions 57993 to 86822. Exon/intron boundaries were determined using high stringent blastn comparisons with full-length cDNAs and canonical GT/AG splice donor/acceptor sites respectively.

By applying these criteria two alternatively spliced transcripts could be assembled encoding vCRL proteins containing one and three CCP domains (Fig. 2-5). For sequences of both transcripts see attached CD. A schematic overview of the orientation of both transcripts compared with BAC clone 11F21 is depicted in Fig. 2-5. The first three introns between exons encoding the signal peptide and the spacer sequence N-terminal of the first CCP domain are 8691, 10768 and 4883bp long being much larger than all other introns. Interestingly, all exons start and end in the same open reading frame. This feature might facilitate alternative splicing events, since the overall topology of the protein domains is not affected by insertion or deletion of exons.

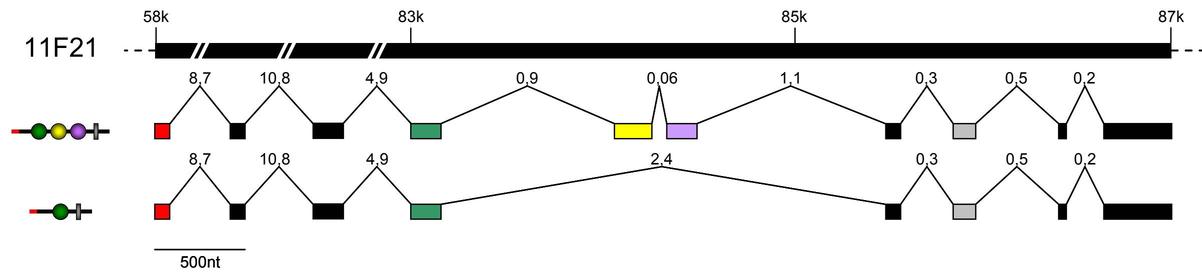

Fig. 2-5: Schematic overview of vCRL gene structure. Exon/intron structure of two alternatively spliced transcripts coding for vCRL proteins containing one or three CCP domains in comparison to BAC clone 11F21. Both vCRL variants encoded by BAC 11F21 were assigned *in silico* using blast analysis with full-length vCRL transcripts of different individuals to determine exon/intron boundaries in combination with canonical splicing sites. Numbers indicate intron sizes in kilobases.

2.4 Production of recombinant vCRL proteins and α-vCRL sera

The major aim of this study was to determine whether vCRL gene is involved in self/non-self recognition, for example in the control of self-sterility. To approach this task recombinant protein of different vCRL variants had to be produced and purified to be used for the generation of specific polyclonal antisera. Several expression systems can be utilized for the production of recombinant proteins having different technical advantages or disadvantages. Three different expression systems - cnidarian *Hydra vulgaris* AEP, yeast *Pichia pastoris* and *Escherichia coli,* were tested for the production of vCRL protein. *Hydra* and *Pichia* are capable of protein modifications such as glycosylation or establishment of disulfide bridges in contrast to *E. coli*, which in return is supposed to grow faster and produce higher amounts of protein. Results obtained from each of the expression systems are discussed in the following sections.

2.4.1 Production of recombinant vCRL protein using *Hydra vulgaris* AEP

Freshwater polyp *Hydra vulgaris* AEP was used for heterologous gene expression because the recombinant protein purified from this animal is more likely to resemble the native *Ciona* vCRL protein in terms of glycosylation pattern and folding. The vCRL1 hemocyte variant encoding three CCP domains was expressed as his-tagged protein fused to EGFP (Fig. 2-6 A-C). Transgene was introduced by microinjection of embryos (Wittlieb et al., 2006). The expression construct was integrated into the genome of several embryos giving rise to three different lines expressing the transgene in either ectoderm, endoderm or both tissues. EGFP fluorescence intensity varies among vCRL transgenic lines and is much weaker compared to GFP endo

polyps, which express only EGFP (Fig. 2-6 D). This indicates that different amounts of vCRL-EGFP fusion protein are produced by different lines most likely due to varying number of integrated copies of the expression construct. Amounts of recombinant protein produced by transgenic lines were analyzed by Western blot with monoclonal α-GFP or α-his-tag antibody. As shown in Fig. 2-6 E, a signal of approximately 66kDa is detectable in all three vCRL-GFP expressing lines using both antibodies which is consistent with the predicted size. However, signal intensity detectable for these lines is much lower compared to the 28kDa signal of GFP endo line using α-GFP antibody. Therefore, the produced amount of recombinant protein is much higher in GFP endo line compared to vCRL expressing lines. Interestingly a faint signal of 28kDa which resembles the size of the GFP protein can also be detected in non-transgenic AEP wildtype polyps using α-GFP antibody. This could indicate that they possess a derived GFP-like protein which is not functional since no fluorescence can be observed in these animals.

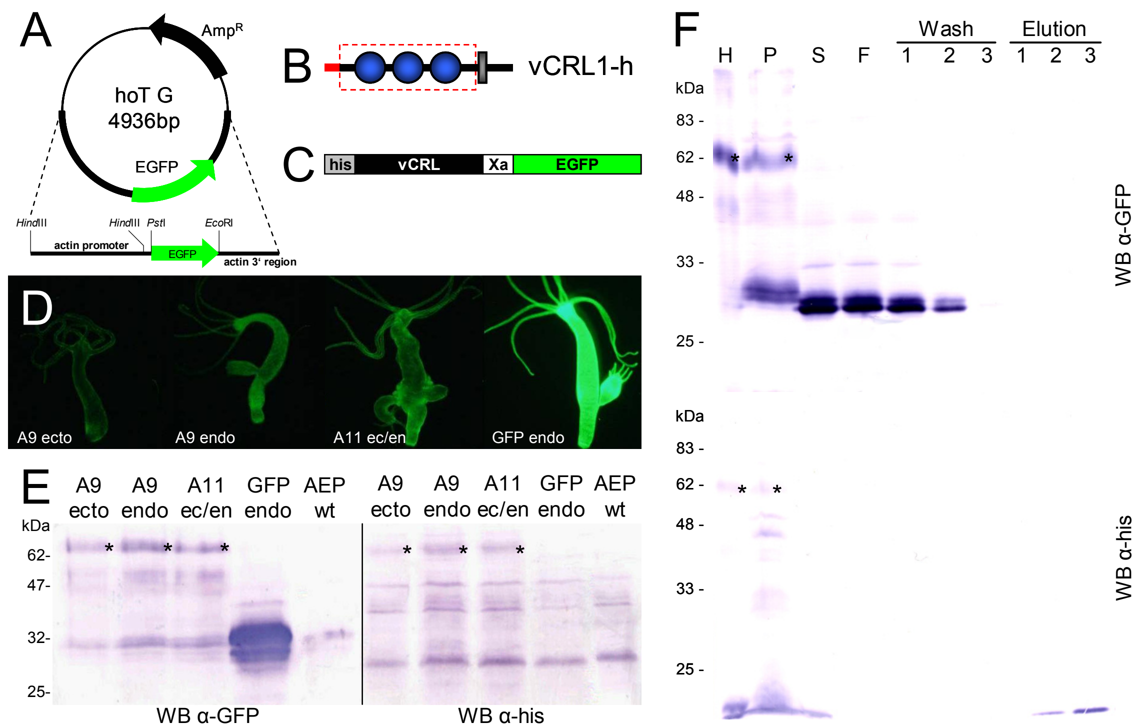

Fig. 2-6: Production of recombinant vCRL protein in *Hydra vulgaris* AEP. (A) Schematic drawing of the vector used to generate the expression construct (modified from Wittlieb et al., 2006). (B) Domain structure of the expressed vCRL variant. Only the extracellular portion including three CCP domains was used (dashed red line). (C) Graphical representation of the expressed fusion protein. His - hexameric Histidin, Xa - Factor Xa cleavage site. (D) Fluorescence images of three transgenic *Hydra* polyps representing different genetic lines according to integration of the vCRL-EGFP fusion construct compared to polyp expressing GFP in endodermal tissue. (E) Western blot analysis using whole polyps and monoclonal α-GFP or α-his-tag antibody. The 66kDa vCRL-EGFP fusion protein is marked with asterisk. (F) Western blot analysis of Ni-NTA affinity purification of recombinant vCRL protein under native conditions using α-GFP or α-his-tag antibody. H - lysate of whole polyps, P - pelleted protein, S - supernatant, F - flow through. The vCRL-EGFP fusion protein is marked by asterisk.

66kDa recombinant vCRL-EGFP fusion protein carried a his-tag and was purified by Ni-NTA affinity chromatography under native conditions. Purification was monitored by Western blot analysis. As shown in Fig. 2-6 F, the intact fusion protein was detectable in whole lysate of transgenic polyps and additionally only in the insoluble pelleted fraction by α-his-tag and α-GFP antibodies. No fusion protein could be detected in supernatant, flow through or elution fractions. A 28kDa signal was detectable in the supernatant and in the washing fractions using α-GFP antibody and a signal of less than 10kDa was detected in the supernatant as well as in the second and third elution fractions using α-his-tag antibody (Fig. 2-6 F). This indicates that the fusion protein was degraded during the purification process despite the presence of protease inhibitors. The degradation is probably due to strong proteolytic activity of *Hydra* cell lysate. As the amount of detected fusion protein in whole lysate as well as in the pelleted fraction was higher using α-GFP compared to α-his-tag antibody, degradation occurred between his-tag and vCRL protein resulting in loss of the his-tag and purification capability using Ni-NTA affinity chromatography.

2.4.2 Production of recombinant vCRL protein using *Pichia pastoris*

Recombinant proteins expressed by *Pichia pastoris* can be purified directly from culture medium without cellular lysis. Moreover, *Pichia* is able to perform protein modifications like glycosylation and establishing disulfide bridges. Hence, this system is suitable for stable large scale expression of already solubilized proteins.

Yeast cells were transformed with expression vector encoding his-tagged vCRL protein (Fig. 2-7 A-C) and selected according to copy number of integrated constructs. Resistance to Zeocin is not absolute but proportional to number of integrated copies. Therefore, the fastest growing transformant on Zeocin containing agar plates was selected for establishment of an expression culture (Fig. 2-7 D). After reaching appropriate optical density protein expression of the culture was induced by addition of methanol for two days. Harvested culture supernatant was subjected to native Ni-NTA affinity chromatography and purification of recombinant vCRL protein tested by SDS-PAGE and α-his-tag Western blot (Fig. 2-7 E and F). The expected size of the expressed recombinant vCRL protein is 24kDa but a larger protein, 34kDa in size, was purified in large quantities from the culture medium. This band was detected very weakly by α-his-tag antibody compared to recombinant vCRL protein isolated from *Escherichia coli* but could be verified as vCRL using

MALDI-TOF. Therefore, the size difference and weak reactivity against α-his-tag antibody indicates a strong glycosylation of the expressed vCRL protein in *Pichia pastoris*. However, not all vCRL protein variants were expressed well in *Pichia* (data not shown). Therefore, this expression system was not developed further since several vCRL proteins could be purified in large quantities from *Escherichia coli* (see section 2.4.3).

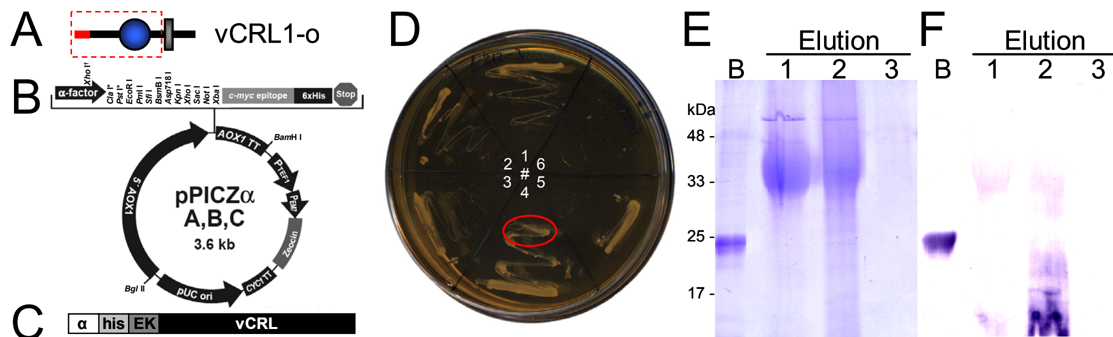

Fig. 2-7: Production of recombinant vCRL protein in *Pichia pastoris*. (A) Domain structure of the expressed vCRL variant. Only the extracellular portion including the CCP domain (dashed red line) was used. (B) Schematic overview of pPICZαA expression vector taken from user manual (Invitrogen). (C) Graphical representation of the expressed his-tagged vCRL protein. α - α-factor signal sequence, his - hexameric Histidin, EK - enterokinase cleavage site. (D) Zeocin selection of six transformants on agar plates. Note the differential growth of streaked colonies indicating different copy numbers of integrated expression construct. (E) Analysis of Ni-NTA affinity chromatography purified culture supernatant by SDS-PAGE and (F) α-his-tag Western blot. B - recombinant vCRL protein purified from *E. coli*.

2.4.3 Production of recombinant vCRL protein using *Escherichia coli*

Escherichia coli represents the standard system for heterologous gene expression because of its fast growth, ease of culture and large amounts of protein. To account for the high polymorphism of the vCRL protein, the extracellular portion of three different vCRL variants was expressed and purified. vCRL1-h encodes a protein with three CCP domains isolated from hemocytes and vCRL1-o as well as vCRL2-o code for proteins containing one CCP domain isolated from ovary tissue (Fig. 2-8 A). The expressed proteins are termed vCRL1 and vCRL2 because they represent two different allele classes of the vCRL gene. Using denaturing Ni-NTA affinity chromatography his-tagged vCRL proteins were purified from lysates of IPTG-induced transformants and subjected to SDS-PAGE (Fig. 2-8 B). Finally, purified vCRL proteins were re-natured by dialysis against phosphate buffer. As shown in Fig. 2-8 C, several bands could be detected for vCRL1-h and a second protein band of 27kDa was observed for vCRL1-o as well as vCRL2-o indicating contamination with other proteins. However, the major protein bands showed sizes of 36, 24 and 28kDa

for each of the respective constructs correlating with predicted sizes of recombinant proteins. As control, proteins were isolated from *Escherichia coli* not transformed with any expression construct. MALDI-TOF was used to verify all protein preparations and identified the contaminating 27kDa protein to be an FKBP-type peptidyl-prolyl cis-trans isomerase from *E. coli* (NCBI accession number NP_289896). Concentration for all four preparations was measured. For each of the constructs 1-2mg recombinant protein were obtained per litre expression culture.

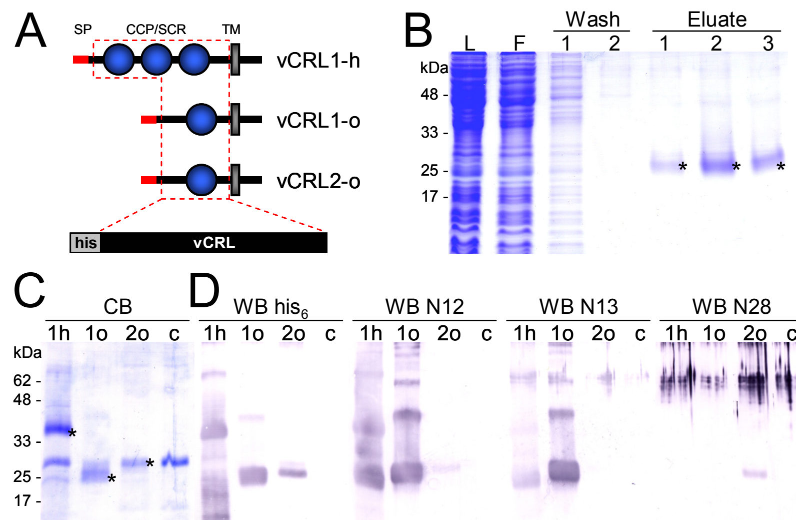

Fig. 2-8: Production of recombinant vCRL protein in *Escherichia coli*. (A) Domain structures of the expressed vCRL variants and graphical representation of the expressed his-tagged vCRL proteins. Only the extracellular portion (dashed red line) was used. His - His-tag. (B) Coomassie stained SDS-PAGE of Ni-NTA chromatography fraction for construct vCRL1-o. L - whole bacterial lysate, F - flow through. Eluted vCRL1-o protein bands are marked by asterisk. (C) Purified recombinant vCRL protein variants. CB - Coomassie stained SDS-PAGE. (D) Analysis of polyclonal α-vCRL mouse sera. WB - Western blot using either monoclonal α-his-tag antibody or polyclonal α-vCRL sera N12, N13 or N28. N12, N13 and N28 were generated using purified vCRL1-h, vCRL1-o and vCRL2-o as antigen respectively. Note the different specificity of α-vCRL sera depending on the used antigen. vCRL protein bands are marked by asterisk. 1h, 1o and 2o - protein variants vCRL1-h, vCRL1-o or vCRL2-o; c - proteins purified under same conditions from *E. coli* cells not transfected with expression construct. Taken from Sommer et al., 2010.

Purified vCRL proteins were used to generate polyclonal α-vCRL sera in mice. Acquired sera were tested against their respective antigens by Western blot analysis. Monoclonal his-tag antibody was used as positive control (Fig. 2-8 D). Polyclonal α-vCRL sera N12, N13 and N28 were produced using different vCRL variants - vCRL1-h, vCRL1-o and vCRL2-o respectively, for immunization of mice. Therefore, they display different specificities. N12 recognizes all three different recombinant proteins, whereas N13 and N28 specifically recognize either vCRL1 or vCRL2 protein variants (Fig. 2-8 D). Taken together, these results show that three different variants of vCRL recombinant protein were produced in *Escherichia coli* and three different α-vCRL sera were generated which specifically recognize either vCRL1, vCRL2 or both vCRL protein variants.

2.5 vCRL protein is present on oocyte surface and hemocytes

Proteins responsible for self/non-self recognition have to be located on the surface of interacting effector cells. Therefore, any receptor responsible for self-sterility must be present at the site where initial contact between gametes takes place. In *Ciona intestinalis*, the surface of a mature oocyte is completely covered by follicle cells and any direct contact of sperm with the vitelline coat is thereby mechanically prevented (see Fig. 2-2). Since initial contact takes place between follicle cells and sperm, it has been proposed that the individual-specific vCRL gene, expressed in follicle cells, might be involved in self-sterility (Kürn et al., 2007). Exact localization of the protein, however, has not been demonstrated. Polyclonal α-vCRL sera (see Fig. 2-8) were used to address this issue. As shown in Fig. 2-9, vCRL protein is localized in the cytoplasm as well as on the surface of follicle cells and on the vitelline coat both in developing (Fig. 2-9 B and C) and mature oocytes (Fig. 2-9 E and F).

In addition to ovary tissue, vCRL gene is strongly expressed in hemocytes. Moreover, alternative splicing of vCRL transcript which generates an additional intra-individual variability is restricted to these cells (see Fig. 1-8). As exemplified by Fig. 2-9 H, large amounts of vCRL protein are present on the surface and in the cytoplasm of all types of hemocytes. It has been proposed that hemocytes are the major immune effector cells in *Ciona* which could mediate self/non-self recognition processes in pathogen recognition or in histocompatibility reactions (Arizza and Parrinello, 2009).

Taken together, the vCRL protein is present at all interfaces where self/non-self recognition may take place. Thus, protein localization supports the idea that vCRL is involved in self-sterility or histocompatibility in *Ciona intestinalis* species B.

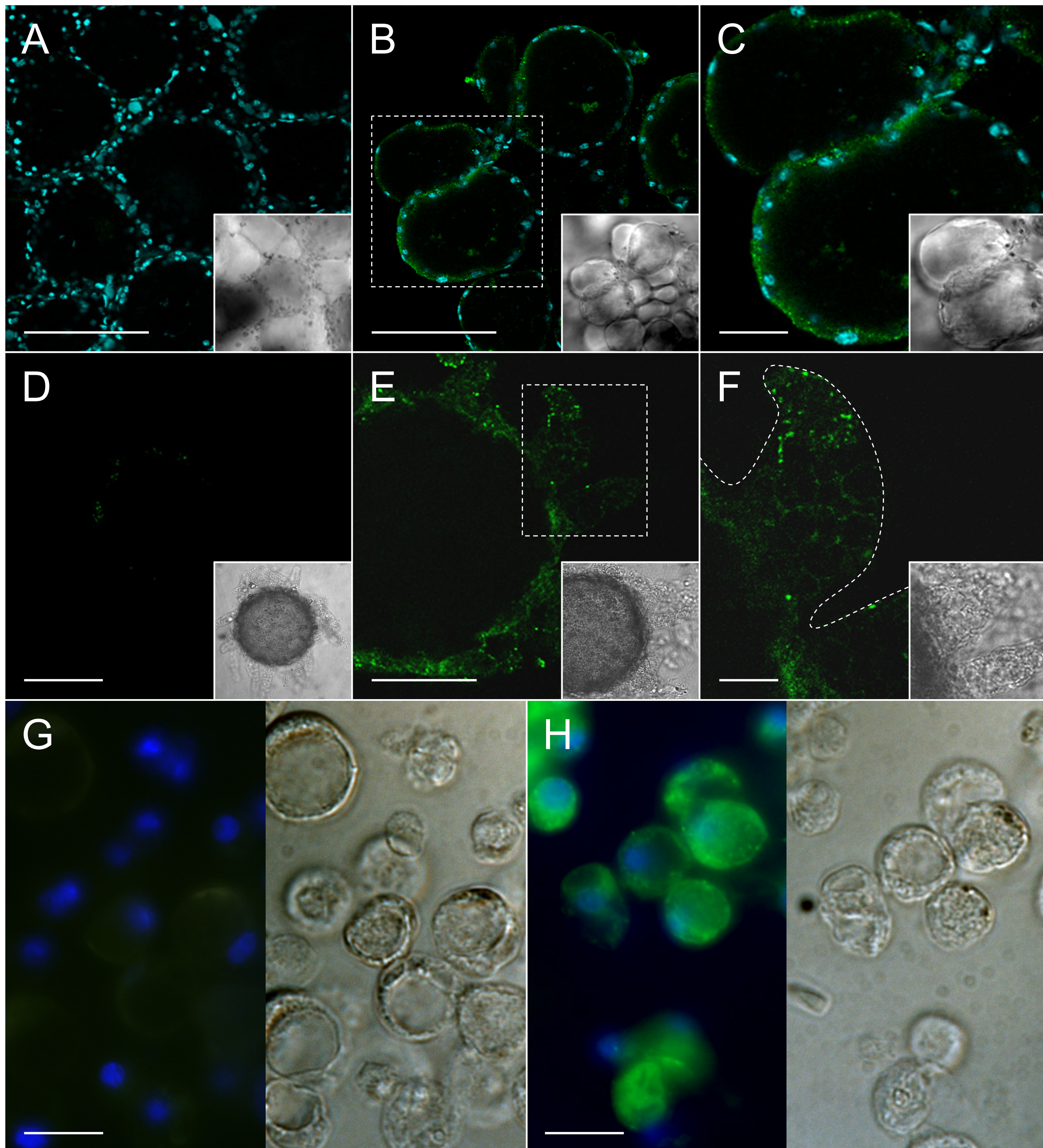

Fig. 2-9: vCRL protein is located on the surface of oocytes and hemocytes. Localization of vCRL protein by immunohistological staining using ovary sections (A-C), spawned mature oocytes (D-F) and hemocytes (G-H). Tissues were incubated with control (A,D,G) or α-vCRL serum (B,C,E,F,H) to detect vCRL protein. Dark field photographs are shown in the lower right corner (A-F) or on the right side of the corresponding fluorescent image. Pictures C and F are magnifications of B and E, respectively. Blue - HOECHST, green - control or α-vCRL serum. Bars represent 50µm (A-F) and 5µm (G,H). Modified from Sommer et al., 2010.

2.6 *In vitro* antibody interference

vCRL protein meets all criteria expected for a candidate gene controlling self-sterility in *Ciona intestinalis*. It is individual-specific and located at the surface of mature oocytes (follicle cells and vitelline envelope). Thus, it is present at the interaction site between oocyte and sperm. To functionally test if the vCRL protein is involved in the

recognition between gametes, α-vCRL sera were used to interfere with auto- and heterologous fertilizations.

Addition of α-vCRL serum should interfere with the fertilization process if the vCRL protein is the determining factor controlling self-sterility. The expected outcome would be that autologous fertilization is promoted if the inhibitory vCRL protein is blocked by the antibody (Fig. 2-10 A). Heterologous fertilization should only be affected if the antiserum activates the vCRL receptor (Fig. 2-10 B). In that case fertilization would be inhibited since binding of α-vCRL serum would mimic autologous sperm and trigger the arrest of oocytes.

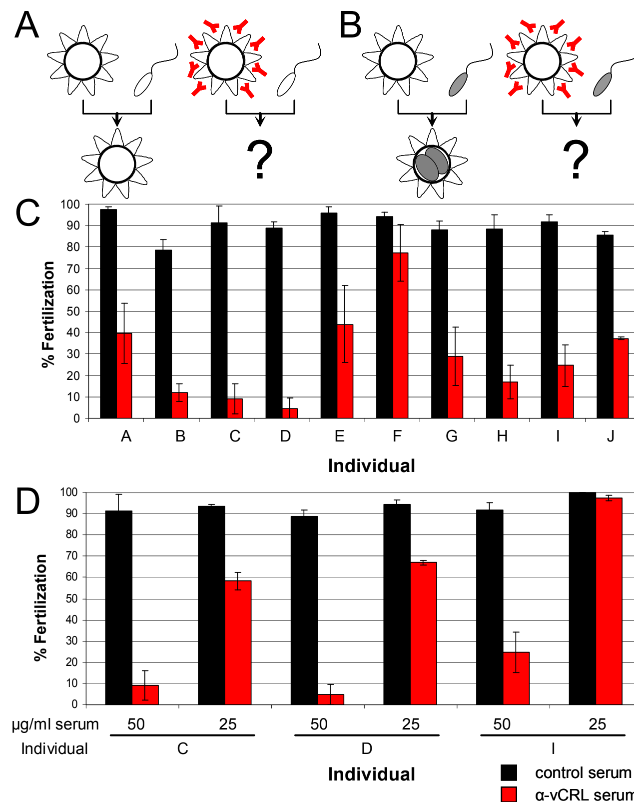

Fig. 2-10: Polyclonal α-vCRL serum inhibits heterologous fertilization in *Ciona*. Experimental setup for antibody interference using (A) autologous and (B) heterologous fertilization. (C) Fertilization rate of heterologous inseminations of oocytes for ten different unrelated *Ciona* animals in the presence of either control or α-vCRL serum. Addition of α-vCRL serum drastically decreases fertilization rate compared to controls. Note the differential response among the ten animals tested indicating variable antibody specificity resulting from high interindividual polymorphism of vCRL protein. (D) The inhibitory effect of α-vCRL serum is concentration dependent. Black - control serum, red - α-vCRL serum. The data represent the mean of three replicates carried out for each experiment ± standard deviation. Results for normal heterologous fertilization were the same as for control serum and thus were not included. Figure taken from Sommer et al., 2010.

Surprisingly, addition of α-vCRL serum did not influence autologous fertilization, but it drastically decreased the fertilization rate of heterologous inseminations compared to controls (Fig. 2-10 C). In most of the animals the fertilization rate is decreased to less than 40% but inhibitory effects vary between the ten animals tested. This variation is

most likely due to the differences in antibody specificities resulting from the high polymorphism of the vCRL variants expressed by each individual. The inhibitory effects were still detectable using as little as 25µg/ml α-vCRL serum and they directly correlate to the used concentration of α-vCRL serum (Fig. 2-10 D). This indicates that the observed block of heterologous fertilization caused by α-vCRL serum is due to a specific interaction. Although this data suggests that the vCRL protein is involved in the process of fertilization, the mode of action was unexpected as the antibody did not promote self-fertilization.

2.7 Segregational analysis

vCRL antisera blocked heterologous, but did not promote autologous fertilization. Thus, it was not clear whether vCRL protein is a self-sterility receptor in *Ciona intestinalis.* To address this question *in vivo*, segregational analysis was performed. The basis for this analysis is the haploid sperm hypothesis proposed by Thomas Morgan (Morgan, 1944). This theory is based on the following statements:

(i) self-sterility is defined by several genomic loci;

(ii) all self-sterility loci are polymorphic leading to many alleles within the population;

(iii) all alleles of all loci have to be identical between parents to prevent fertilization;

(iv) sperm express only one allele of each locus;

(v) oocytes express both alleles of every locus.

Block of self-fertilization is stable, very strict and prevents mating only of those individuals which are genetically absolutely identical in self-sterility loci. This enables the examination of factors involved in this process by segregational analysis. Assuming only one genomic locus is controlling self-sterility and both parents are heterozygous in this locus having different alleles, four different genetic combinations can occur within the F1 offspring (Fig. 2-11 A). If these siblings would be crossed to other individuals of this cross, 16 different combinations would be possible of which four - 25% of siblings - would be cross-sterile (Fig. 2-11 B). By increasing the number of genomic loci the percentage of cross-sterile combinations within the F1 generation will drastically decrease. The expected probability of cross-sterility can be calculated using the formula shown in Fig. 2-11 C. This formula incorporates allelic state (homo-/heterozygous) of the parental self-sterility loci. For example, if both parents are

heterozygous in all loci, percentages of cross-sterility within F1 generation of 6.25%, 1.6% and 0.4% would be expected assuming two, three and four loci respectively.

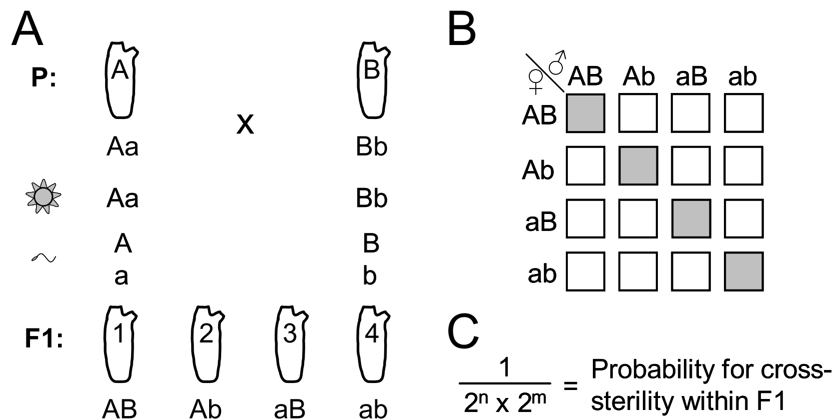

Fig. 2-11: Principle of performed segregational analysis. (A) Basic outline of segregational analysis assuming haploid sperm hypothesis and one locus system. (B) Graphical representation of crossing of F1 siblings from (A). Cross-sterile and cross-fertile combinations are represented by grey and white boxes respectively. (C) Formula to calculate probability for cross-sterile combinations depending on parental genotypes. n/m - number of heterozygous loci of the individual parents A/B. Modified from Sommer et al., 2010.

Several crosses were set up and analyzed for cross-sterility among siblings. Animals were reared in the laboratory and smaller than normal (<5cm). Such animals had fewer gametes and, therefore, the experimental setup was limited to a group of six animals that could be analyzed simultaneously by pairwise crossings. In total, 407 heterologous crosses were performed (see Tab. 2-1).

Tab. 2-1: Statistics of performed crosses. Comb. - total crossed combinations minus autologous controls, Sterile - number of cross-sterile combinations. Data for all performed crossings is attached in appendix (Fig. 9-1). Table modified from Sommer et al., 2010.

Cross	Experiments	Comb.	Sterile	%	Theoretical explanation
AxB	6*6+1	31	2	6.5	two heterozygous loci per parent
	6*6	30	2	6.7	het/het x het/het
		61	**4**	**6.6**	→ 6.25%
BxG	6*6	30	4	13.3	one locus of one parent homozygous
	4*4+5	17	3	17.6	hom/het x het/het
	6*6+2	32	0	0	→ 12.5%
	6*6+2+4+2	38	4	10.5	
	1*8	7	1	14.3	
		124	**12**	**9.7**	
CxD	6*6	30	2	6.7	one locus of one parent homozygous
	6*6	30	4	13.3	hom/het x het/het
		60	**6**	**10.0**	→ 12.5%
CxF	6*6+3*2	36	8	22.3	one locus of one parent homozygous
	6*6	30	2	6.7	hom/het x het/het
	6*6	30	0	0	→ 12.5%
	6*6	30	2	6.7	
		126	**12**	**9.6**	
ExF	6*6	30	8	26.7	two of four loci homozygous
	3*3	6	2	33.3	e.g. hom/het x hom/het
		36	**10**	**27.8**	→ 25%
∑		**407**	**44**		**Two locus system**

Among all crossings no frequencies of cross-sterility below six percent have been observed (Tab. 2-1). This indicates that self-sterility in *Ciona intestinalis* species B is governed by not more than two genomic loci. The observed frequencies of cross-sterile animals of about 10% and 28% percent can be explained by a two locus system. The most straightforward explanation in that case is that one or both parents are homozygous in one of the two self-sterility loci respectively. Therefore, the crossing experiments predict that parental animals A, B and C were heterozygous for both self-sterility loci whereas individuals D, E, F and G are homozygous in one of the two loci.

As shown in Fig. 2-11 A, only four different genotypes among F1 siblings are possible if one locus is assumed whereas potentially 16 different offspring genotypes can occur if a two locus system is controlling self-sterility in *Ciona* and both parents are heterozygous in these loci. Therefore, segregational analysis of a two locus system is more complex as depicted in Fig. 2-12.

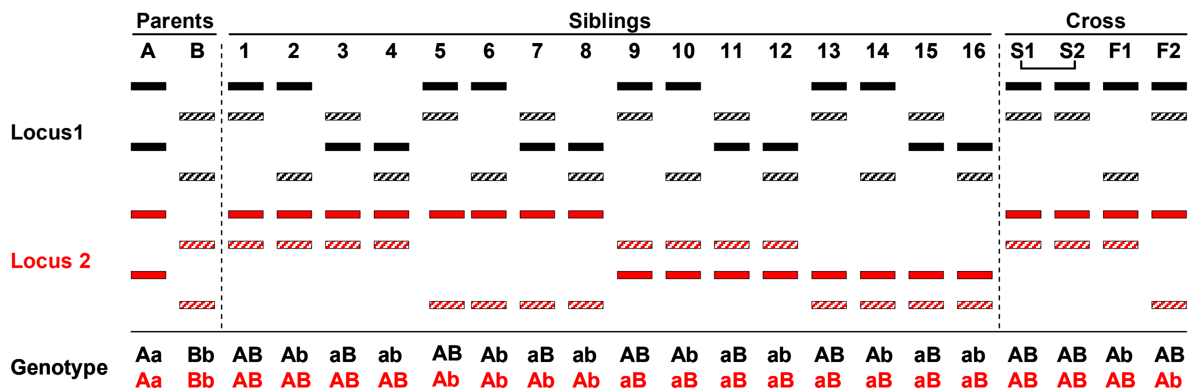

Fig. 2-12: Hypothetical segregational analysis assuming a two locus system. Parents A and B are both heterozygous in both genomic loci resulting in 16 different genotypes among F1 siblings. Alleles of animals A and B are filled and shaded respectively and alleles of one locus are colored accordingly. S1/S2 - cross-sterile animals (connected with bars), F1/F2 - cross-fertile animals, A/a and B/b - alleles of parental animals A and B respectively. Note that cross-sterile animals must always have the same alleles whereas cross-fertile animals can have different or the same alleles if only one locus is analyzed.

To track individual parental alleles it is necessary to use markers, which are able to amplify alleles of only one certain locus but from both parents. In the best case these amplicons should have different sizes (amplified length polymorphism, AFLP). That diminishes the need for sequencing. Therefore, design of specific markers is crucial for this experiment. To generate markers for the segregational analysis of the vCRL gene full-length transcript sequences were determined for all parental animals by 5'RACE. Based on this information primers were designed against a constant region located within the sequence coding for the CCP domain and in the 3'UTR. The whole

genomic region encoding this sequence was amplified and sequenced for each parent. By comparison of these genomic sequences primers were designed which amplify fragments shorter than 2kb of this locus which finally could be used for segregational analysis of the vCRL gene by AFLP.

If vCRL receptor is responsible for self-sterility, than in all cross-sterile F1 siblings both alleles of vCRL gene must be identical. Segregational analysis with crosses AxB, CxD and ExF (Fig. 2-13 A, B and C) revealed that vCRL alleles do not correlate with the fertilization outcome. Although AFLP patterns for cross-sterile animals 20/20 and 26/27 of AxB cross as well as 90/91 of ExF cross are identical, all other cross-sterile combinations (7 out of 10) do not show the same banding pattern. Thus, vCRL is not involved in self-sterility.

In *Ciona intestinalis* species A (from Pacific ocean) two self-sterility loci - Themis-A and Themis-B - have been identified recently (Harada et al., 2008), but the involvement of these loci in self-sterility in species B has not yet been tested. Moreover, crossing data indicate presence of a two locus system (Tab. 2-1). Thus, segregation of Themis-A and B alleles was also analyzed.

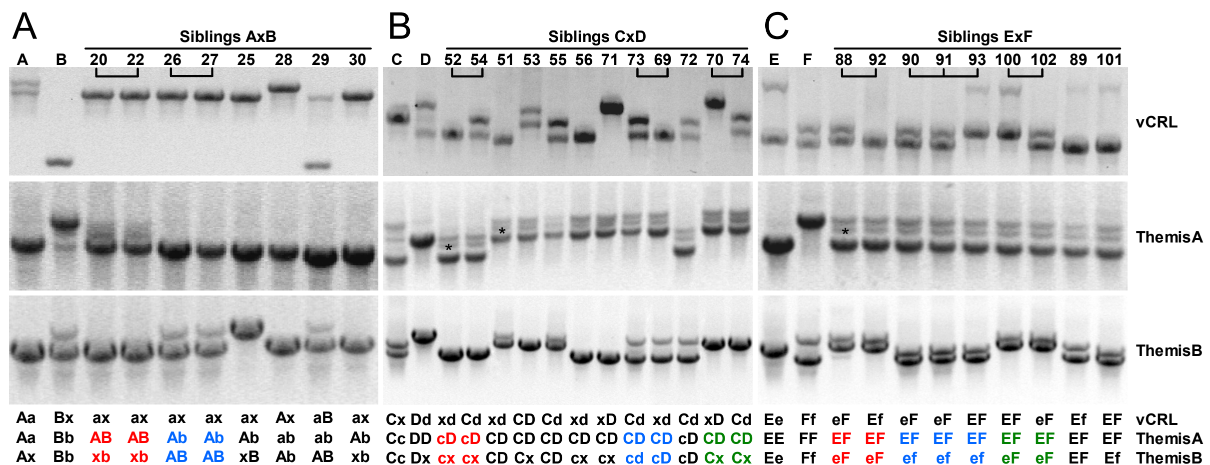

Fig. 2-13: Themis-A and B but not vCRL alleles segregate with fertilization phenotype. Segregational analysis for alleles of vCRL, Themis-A and B using crosses AxB (A), CxD (B) and ExF (C). Cross-sterile animals are connected with bars. Individual genotypes are given below the gel photo using letters according to the parents. Capital and small letters represent alleles of a heterozygous locus. If a parental allele could not be detected it was designated as “x”. Themis-A and B alleles of cross-sterile animals are colored. Unspecific Themis-A amplicons are marked with asterisks. Note, animals CxD #56 and #71 show same Themis AFLP patterns and therefore should be cross-sterile but crossing data for this combination is not available (see Fig. 9-1). Modified from Sommer et al., 2010.

As shown by Fig. 2-13, Themis alleles perfectly correlate with the fertilization outcome of all analyzed siblings. Moreover, the observed hetero- and homozygotic states of Themis genes meet the expectations for parental genotypes in self-sterility loci predicted based on the crossing data (see above and Tab. 2-1). For example,

one locus of parental animal F was supposed to be homozygous and the other locus heterozygous according to the percentage of cross-sterile animals. Indeed, this animal is homozygous for Themis-A and heterozygous for Themis-B. Taken together, data from the segregational analysis indicate that not the vCRL gene but both Themis loci cooperatively control self-sterility in North Atlantic *Ciona intestinalis*. Thus, both *C. intestinalis* species A (Atlantic) and B (Pacific) use the same components for self-incompatibility. This might therefore implicate an additional yet not characterized molecular mechanism which prevents inter-species fertilization because of their reproductive isolation.

2.8 Functional analysis of vCRL by knock-down of expression

vCRL is not responsible for self-sterility in *Ciona intestinalis* species B. However, the vCRL gene is highly polymorphic and individual-specific. Thus, it might have a different role in self/non-self recognition. But what function does the vCRL protein exhibit and how can it be revealed?

The only way to find out the role of vCRL is to pursue functional experiments. They can be realized either by ectopic overexpression of the vCRL gene or by knock-down of its expression. In case of vCRL ectopic overexpression is not very likely to obtain an informative phenotype. Furthermore, the vCRL gene is individual-specific and thus only overexpression of the endogenous vCRL variant seems to be suitable which complicates the analysis. Therefore, knock-down of vCRL gene expression is a more promising approach to elucidate its function. Knock-down can be achieved by different methods. The use of morpholinos is technically limited because one morpholino is only able to interfere with one mRNA molecule. In contrast to that, RNA interference (RNAi) includes an amplification step. Thus, a small amount of interfering RNA is sufficient to knock-down expression in even highly expressed genes.

However, RNAi method is not established in *Ciona intestinalis*. Initial experiments injecting adult animals with dsRNA and siRNA did not result in reduced vCRL expression although the molecular machinery required for RNAi is present in the *Ciona* genome (data not shown). Therefore, I developed a novel hairpin-based vector system which enables the functional analysis of the vCRL gene by stable transgenesis. This technique has several advantages: (i) The method can be applied

for every gene. (ii) The construct is integrated into the genome. Thus, the resulting effects are stable and reproducible. Furthermore, multiple knock-down lines can be generated by crossing transgenic animals which carry different constructs.

Constructs were based on the pMiLRneo vector (Fig. 2-14 A) and contained 2kb of the 5' flanking sequences of the *Ciona intestinalis* EF1α or vWA-like genes (Fig. 2-14 B). EF1α gene is strongly expressed in all adult tissues (see Aniseed database http://crfb.univ-mrs.fr/aniseed). In contrast to that, expression of vWA-like gene is restricted to hemocytes (Wakoh et al., 2004). Expression was monitored by EGFP reporter and transcription was terminated by 1,5kb 3' flanking region of the EF1α gene (Fig. 2-14 B).

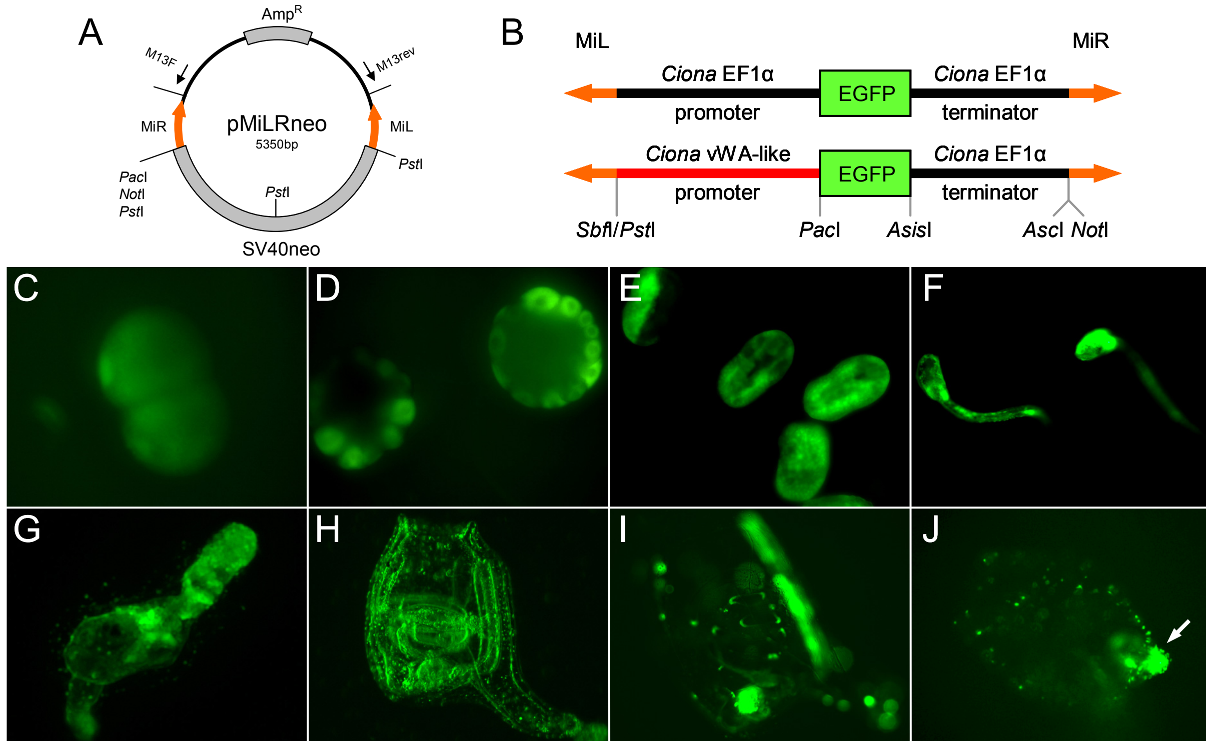

Fig. 2-14: Transgenic *Ciona intestinalis*. (A) pMiLRneo vector and (B) constructs used for test transfection. (C-I) Fluorescence pictures of developmental stages of embryos transfected with EF1α promoter::EGFP constructs. (C) 2-cell, (D) gastrula, (E) neurula, (F) larvae, (G) rotation, (H) FAS, (I) SAS. (J) FAS stage juvenile of vWA-like promoter::EGFP construct. EGFP fluorescence was detected in all tissues using EF1α whereas only few cells are stained by the vWA-like promoter construct. Note, accumulated EGFP-positive cells might indicate presence of a restricted hematopoetic tissue (arrow). Modified from Sommer et al., 2010.

For EF1a promoter constructs EGFP fluorescence was detected starting from the 2-cell stage and in all adult tissues depending on the time point of integration (Fig. 2-14 C-I). On the contrary, only EGFP positive hemocytes and some tunic cells were identified using vWA-like promoter construct only from first ascidian stage on (Fig. 2-14 J). This confirms that the used EF1α and vWA-like promoters are strong and ubiquitous or hemocyte-specific respectively since hemocytes appear at the

transition from rotation phase to first ascidian stage (Chiba et al., 2004). Thus, both promoter constructs faithfully recapitulate normal expression pattern. Moreover, restricted hematopoietic tissues seem to be present in *Ciona intestinalis* since many accumulated EGFP positive hemocytes can be observed in the basal part of juvenile animals electroporated with vWA-like promoter constructs (Fig. 2-14 J).

Electroporation of EF1α promoter driven hairpin constructs did not affect early embryonic development. However, there was a strong effect on metamorphosis. Most of the embryos transfected with vCRL hairpin vectors did not complete metamorphosis whereas development was not impaired by the control *Hydra* MyD88 hairpin (Fig. 2-15).

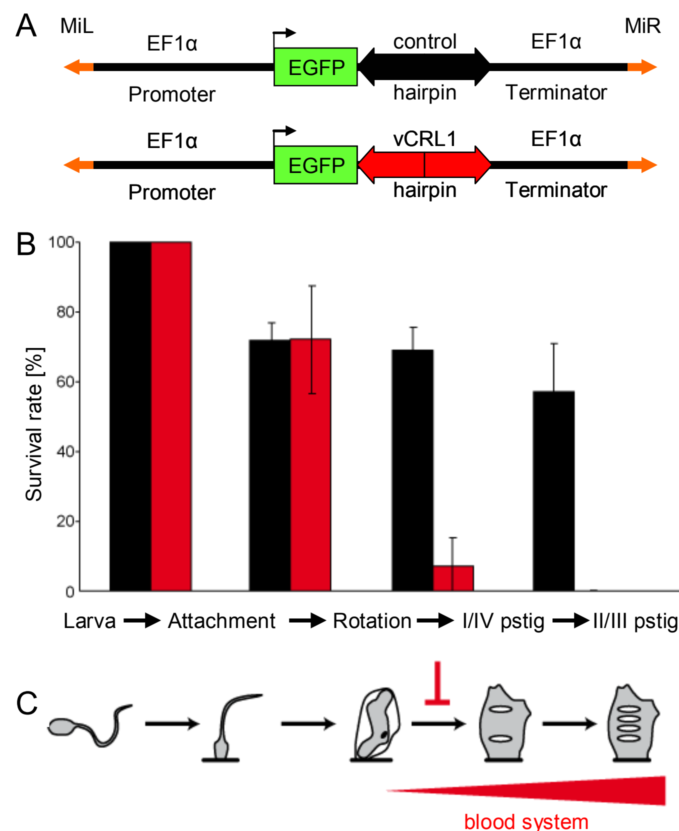

Fig. 2-15: Knock-down of vCRL expression effects metamorphosis. (A) Hairpin constructs used for stable RNAi. (B) Percentage of embryos transfected with vCRL or control hairpin constructs which successfully developed through the indicated stage. Data represents the mean of three independent experiments. Notably, knock-down of vCRL expression results in an arrest at rotation stage. Pstig - protostigmata. Both I/IV and II/III protostigmata stages represent FAS juveniles. (C) Summary of developmental defect caused by silencing of vCRL expression which correlates with the establishment of the blood system. Taken from Sommer et al., 2010.

As shown in Fig. 2-15, development of animals electroporated with vCRL hairpin constructs arrested mostly at the transition from rotation to first ascidian stage. Interestingly, hemocytes can first be observed at this point of development (Chiba et al., 2004). Furthermore, the same phenotype was caused by vWA-like promoter

driven expression of the vCRL hairpin but no statistical data is available for the effects caused by these constructs. Taken together, the data indicates that knock-down of vCRL gene expression in hemocytes causes severe problems in the formation of a functioning blood system and thus developmental arrest.

3 Discussion

3.1 Presence of two cryptic *Ciona intestinalis* species

Ciona intestinalis has been referred to as single cosmopolitan species (Hoshino and Nishikawa, 1985; Kobayashi et al., 2002). However, is it really one species everywhere in temperate waters around the globe? Recently, several research groups identified molecular discrepancies between animals used for their studies and available databank sequences. *Ciona intestinalis* animals could be divided into two subtypes and it was shown that both are reproductively isolated (Suzuki et al., 2005; Caputi et al., 2007; Iannelli et al., 2007; Nydam and Harrison, 2007). Thus, at least two cryptic morphologically indistinguishable *Ciona intestinalis* species exist.

However, to exploit *Ciona* as a model organism, it is required that animals used for research purposes by different laboratories belong to the same species and in the best case have a common genetic background. This is of special importance when genetic experiments are conducted. Furthermore, only in that case the sequenced *Ciona* genome and other molecular resources can be utilized effectively. For example, presence of two cryptic *Ciona intestinalis* species complicates the identification of proteins isolated from species B by MALDI-TOF which relies on genomic databases derived from species A (Fedders, 2008). Moreover, as both cryptic species are genomically different and reproductively isolated, different self/non-self recognition mechanisms may be present in *Ciona intestinalis* species A and B.

Therefore, it was important to analyze which species the animals belong to that were used in this study. *Ciona intestinalis* animals sampled around Helgoland (Germany) and Bergen (Norway) belong to species B based on three molecular markers (see section 2.1). Among several individuals tested no species A animals were found (data not shown) demonstrating that populations are geographically more restricted than previously thought. This questions the status of *Ciona intestinalis* as a cosmopolitan species. Since *Ciona* animals sampled at Norway, Sweden, England and Germany belong to the same species and are different from Italian animals it is likely that individuals in whole Northern Europe represent species B (see figure 1-5 A). Thus, it is feasible to compare experiments conducted with animals from these locations and more important to analyze gene functions by generating transgenic animals using sequences obtained from any of the Northern European populations.

This information is of importance as transgenic *Ciona intestinalis* were generated to analyze the function of vCRL receptor by stable RNAi (see section 2.8) using individuals collected in Norway and constructs based on promoter and terminator sequences from German animals. In that case all individuals belong to the same species (genetic background) and, therefore, cross-species effects can be excluded.

3.2 Follicle cells contribute to self-sterility in Northern European *Ciona intestinalis* (species B)

Almost all published fertilization experiments intended to analyze the site of self-/non-self recognition between sperm and oocyte were performed using fixed oocytes or those mechanically stripped of their follicle cells (Rosati and de Santis, 1978; De Santis and Pinto, 1991; Pinto et al., 1995). Under these experimental conditions an influence of follicle cells on gamete interaction cannot be assessed and thus was neglected. Moreover, both methods do not represent natural physiological situation. It is impossible to detach all follicle cells from oocytes completely. Thus, there is no guarantee that all cell fragments are removed. Hence, an influence of follicle cells cannot be completely excluded.

However, follicle cells are clearly involved in self-sterility of *Ciona intestinalis* because they are required for successful oocyte maturation (De Santis and Pinto, 1991; Pinto et al., 1995). Moreover, they represent a mechanical barrier for sperm penetration since they are the outermost layer of a mature oocyte and cover it completely leaving no space for sperm to pass through (Fig. 2-2). Even if this mechanical barrier might not be absolute, the close proximity of follicle cells limits the sperm binding area available on the vitelline envelope and oocyte surface. Thus, only few sperm are able to establish a physical contact to these layers which reduces the probability of self-fertilization since low local sperm concentration does not overcharge the regulatory molecular mechanisms.

Moreover, it has been shown that only autologous follicle cells are able to induce an acquisition of self-sterility during oocyte maturation (Pinto et al., 1995). This indicates that they secrete a factor which is either representing the female determinant itself or which is essentially modifying the self-sterility factor proteolytically that is already present on the vitelline membrane or oocyte surface. Thus, it is tempting to

hypothesize that follicle cells might express genes which are responsible for the control of gamete self/non-self recognition.

3.3 Molecular mechanisms controlling self-sterility in Northern European *Ciona intestinalis* (species B)

Self-sterility of *Ciona intestinalis* has been studied already since the middle of the last century. Thomas Morgan analyzed the basis of gamete self-incompatibility and identified most of the basic cellular as well as some genetic characteristics (Morgan, 1923, 1938a, b, 1939a, b, 1942, 1944). Based on his research he formulated the haploid sperm hypothesis which is still valid and represents the fundament for the recent molecular studies.

Briefly, this theory consists of the following features. Self-sterility is defined genetically by three to five genomic loci which are highly polymorphic and thus many alleles are present within the population. Fertilization is inhibited only if all alleles of all loci are identical between interacting gametes. Sperm express only one allele of each locus whereas oocytes carry both alleles of every locus.

Assuming this hypothesis would be true, self-sterility of *Ciona intestinalis* would be stable and very strict because only animals identical in all alleles of the self-sterility loci would be cross-sterile. Morgan's experiments have been confirmed by more recent studies in Japan but the incidence of cross-sterility was higher. Thus, the number of involved genomic loci was estimated to be only two to three in Japanese *Ciona intestinalis* (Murabe and Hoshi, 2002; Harada and Sawada, 2008; Harada et al., 2008).

Two of these candidate self/non-self recognition loci have been isolated and delineated to 170kb and 1Mbp (Harada et al., 2008). Despite lacking obvious sequence similarity both loci contain a syntenic gene pair which encodes a receptor and ligand (v- and s-Themis). The former encodes a protein located at the vitelline envelope and the latter is expressed in testis. Most important, alleles of these genes correlate with the outcomes of fertilization experiments in almost all the cases. Thus, gamete self-incompatibility in *Ciona intestinalis* species A seems to be controlled by the interaction of v- and s-Themis proteins encoded by both loci (Harada et al., 2008). Since not all experimental crossing phenotypes can be explained with these

two loci it remains to be answered if additional loci are involved which have not been identified yet (Harada and Sawada, 2008).

As mentioned above both cryptic *Ciona intestinalis* species do not only display genomic differences, but are also not able to cross-fertilize and generate fertile offspring. Thus, it is an interesting question whether both *Ciona* species use the same or different molecular mechanisms to prevent self-fertilization. And if they employ different mechanisms what are the self-sterility receptors in Northern European animals?

Several years ago an unbiased search for variable genes expressed in *Ciona* ovary was conducted (Kürn et al., 2007). This led to the identification of the highly polymorphic gene vCRL which was proposed to function as self/non-self recognition receptor. This hypothesis is based on the following indications: vCRL protein is individual-specific as well as present on the surface of follicle cells and hemocytes (Fig. 2-9). Moreover, it is structurally analogous to the human complement receptors CD46 and CD55 which in higher vertebrates protect the own tissue from lysis by the complement system (see Russell, 2004 and section 3.5). Interestingly, besides this protective function CD46 is also involved in fertilization. In human, CD46 is present at the inner surface of the acrosome which is exposed after sperm capacitation (Anderson et al., 1993). CD46 antibodies are able to inhibit fertilization (Riley-Vargas et al., 2004). Most important, mutations in CD46 gene have been associated with male infertility (Kitamura et al., 1997; Nomura et al., 2001). In rodents, CD46 is expressed exclusively in the male gonad and its complement control function in other tissues is carried out by Crry (Riley-Vargas and Atkinson, 2003). However, in contrast to vCRL mammalian complement receptors do not vary among individuals (Natsuume-Sakai et al., 1980). Thus, could the individual-specific vCRL protein be involved in the prevention of self-fertilization in Northern European *Ciona intestinalis*?

If the vCRL protein would be the self-factor controlling self-sterility an incubation of oocytes with α-vCRL antibodies might promote self-fertilization since vCRL protein which normally inhibits autologous sperm is blocked by the serum. Heterologous fertilization should not be affected. However, addition of α-vCRL sera inhibited heterologous fertilization in a concentration dependent manner and with high specificity (Fig. 2-10 C and D), whereas autologous fertilization was not promoted. This finding was unexpected. The most probable explanation would be that addition of α-vCRL serum causes a steric effect rather than triggering a specific blocking

response. The vCRL protein is present at the oocyte surface and α-vCRL antibodies bind to it. Therefore, no space might be left for sperm to enter the interfollicular ridge and interact with the oocyte surface. This mode of interference cannot be excluded because sera of non-immunized mice were used as control and not an antibody against a follicle cell surface protein. Such an antibody might also cause spatial problems and, therefore, interfere with sperm passage. Unfortunately, such control antibody is not available at present. However, to my mind conformational effect is unlikely to cause the observed strong inhibition of heterologous fertilization. The size of an antibody molecule is small compared to the interfollicular ridge and very little amounts of serum were used to achieve a reduction in fertilization. Thus, it is probable that sperm were not physically hampered from entry or passage by addition of the sera. As another possibility, α-vCRL sera could have activated the inhibitory vCRL protein. vCRL protein on the oocyte surface might have been bound by α-vCRL serum which mimics an interaction with autologous sperm. Therefore, α-vCRL serum might have triggered an arrest in oocytes and inhibited heterologous fertilization, but did not affect autologous fertilization.

Interference experiments with purified recombinant vCRL proteins were also conducted. Theoretically, recombinant protein might bind autologous sperm and mask them as non-self. As a result, autologous fertilization might be promoted. However, in our experiments, addition of purified recombinant vCRL proteins did not show any effect on fertilization. This might be caused by varying affinities of the used recombinant and the endogenous vCRL protein to its sperm ligand. vCRL protein is highly variable and individual-specific. Therefore, autologous vCRL protein binds to its receptor on the sperm surface with a much higher affinity than vCRL protein variants from other individuals. Another possible explanation could be that the recombinant vCRL proteins produced in *Escherichia coli* were not glycosylated or folded correctly. Thus, recombinant vCRL proteins might have been non-functional.

As the α-vCRL serum did not promote self-fertilization in antibody interference assay, it remained an open question whether vCRL protein is controlling self-sterility in Northern European *Ciona intestinalis*.

This issue was further clarified using crossing experiments combined with the analysis of vCRL segregation. First, it was demonstrated that not more than two loci are conferring gamete self-incompatibility in *Ciona intestinalis* species B because the frequency of cross-sterility among F1 siblings was never lower than approximately six

percent (see Tab. 2-1). Therefore, the number of involved loci is the same as estimated for Japanese *Ciona* (species A, see above). Segregational analysis for the vCRL gene revealed that it did not correlate with the observed fertilization outcome. Thus, vCRL protein does not represent a self-sterility factor.

In contrast to vCRL, alleles of both Themis loci displayed a complete correlation with the fertilization phenotype (Fig. 2-13). Therefore, both Themis loci control self-sterility not only in Japanese animals (species A, Harada et al., 2008) but also in Northern European *Ciona intestinalis* (species B). That is a very interesting finding because both *Ciona intestinalis* species are genomically as well as physiologically different and reproductively isolated. The use of the same components for self-incompatibility implicates an additional, not yet characterized molecular mechanism which prevents inter-species fertilization.

3.4 Variable vCRL receptor is required for metamorphosis

Since vCRL was shown not to be responsible for the control of self-sterility what other functions does this highly polymorphic individual-specific gene exert?

There are several ways to study genes functionally including injection of proteins (Shoyele and Slowey, 2006), injection of ds- and siRNA (Mello and Conte, 2004; Grosshans and Filipowicz, 2008) or to use transgenic animals (Sasakura et al., 2007).

An injection of protein acts only locally and due to the extreme polymorphism of the individual-specific vCRL protein, the endogenous vCRL protein will have the highest affinity to its receptor. Thus, it is likely that injection of recombinant vCRL protein will not cause any effects.

An injection of ds- and siRNAs also acts only locally but it might cause informative effects despite of the high variability of the vCRL gene. It would be sufficient that only a short stretch of the dsRNA is identical to the endogenous vCRL transcripts of the injected individual. Thus, a functional siRNA could be generated and vCRL expression might be diminished which would allow analysis of the gene function. Therefore, I injected both ds- and siRNAs into *Ciona individuals* but knock-down was not achieved.

Transgenic animals allow analysis of a gene function in the whole body or a certain tissue depending on the promoter used. This technique therefore represents the best

way to functionally analyze the vCRL gene. However, ectopic overexpression of the vCRL gene is less probable to cause an informative phenotype compared to knock-down of vCRL expression. The vCRL gene is individual-specific and, thus, only overexpression of the endogenous vCRL variant would be suitable. Therefore, knock-down of vCRL gene expression represents the most promising approach to elucidate its function.

Hence, we attempted to knock-down vCRL gene expression by stable transgenesis using hairpin constructs. Interestingly, development of embryos electroporated with vCRL hairpin constructs arrested at the transition from late rotation to first juvenile stage, whereas control hairpin constructs did not cause abnormalities (Fig. 2-15). Diminished vCRL gene expression causes developmental arrest at exactly the phase at which hemocytes originate since the first circulating hemocytes are detected at first ascidian stage (Chiba et al., 2004). Both the ubiquous EF1α as well as the hemocyte-specific vWA-like promoter caused the same developmental problems, indicating that the arrest during metamorphosis is due to knock-down of vCRL gene expression specifically in hemocytes. Hemocytes are circulatory cells and the main immune effector cells in urochordates (Rowley et al., 1984; Arizza and Parrinello, 2009). They infiltrate infected tissues and phagocytize invading pathogens or kill them using for example the prophenoloxidase system and complement factors (Alberts et al., 2004).

Taken together, vCRL might be a self-marker on the surface of developing *Ciona* blood cells. It is individual-specific and may function as inhibitory receptor which prevents destruction of own cells. Therefore, this system might be functionally similar to the mode of action of natural killer (NK) receptors and protection from the inappropriate activation of the complement system in mammals.

3.5 Self/non-self recognition systems in urochordates

Little is known about how self/non-self recognition systems in the animal kingdom function. Complete molecular machinery of allorecognition is known only for the representatives of jawed vertebrates. Besides that, there are just five invertebrate species (cnidarian *Hydractinia symbiocarpus* and four tunicate species - *Botryllus schlosseri*, *Halocyntia roretzii*, *H. aurantium* and *Ciona intestinalis*) where at least some components - mostly receptors and their ligands - have been identified

(Sawada et al., 2004; Ban et al., 2005; De Tomaso et al., 2005; Nyholm et al., 2006; Kürn et al., 2007; Harada et al., 2008; Nicotra et al., 2009). Currently available data on self/non-self recognition suffers greatly from a very patchy phylogenetic distribution of model systems and, therefore, does not answer two fundamental questions: Do allorecognition systems in different animal groups share common evolutionary roots or did they originate independently? Are there any evolutionary connections between allorecognition and components of the immune system in invertebrate animals?

Urochordates represent an ideal model system to gain insides into the evolution of self/non-self recognition systems since they occupy a key phylogenetic position being the closest living relatives of the vertebrates (Delsuc et al., 2006; Delsuc et al., 2008; Singh et al., 2009). Even more important they display both histocompatibility reactions as well as self-sterility besides pathogen defense (Bancroft, 1903; Morgan, 1923). Several polymorphic receptors have been isolated from *Botryllus*, *Halocynthia* and *Ciona* (Sawada et al., 2004; Ban et al., 2005; De Tomaso et al., 2005; Nyholm et al., 2006; Kürn et al., 2007; Harada et al., 2008). Some of these genes were shown to be functionally involved in self/non-self discrimination processes. As shown in Fig. 3-1, self/non-self recognition receptors differ significantly among tunicate genera. These recognition molecules vary on sequence as well as on a structural level with only one exception. Analysis of the vCRL genomic locus revealed that a cluster of genes encoding Ig and CCP domain containing proteins (PVL, potential vCRL ligand) is located within 10-70kb from the vCRL gene (see figure 2-4). These genes might code for a ligand of the vCRL protein as receptors and their ligands which mediate self/non-self recognition are likely to be closely located to one another in the genome to ensure co-segregation as one functional unit (De Tomaso and Weissman, 2003; Hunter, 2009; Nicotra et al., 2009). Moreover, PVL gene cluster is variable and individual-specific. This genomic situation, therefore, structurally resembles the organization of the *Botryllus schlosseri* Fu/HC locus containing cFuHC and fester genes which are involved in the control of colony specificity (Fig.3-1, De Tomaso et al., 2005; Nyholm et al., 2006). However, the *Ciona* vCRL and the *Botryllus* Fu/HC loci do not share sequence homology or synteny. That may indicate that the molecular mechanisms controlling histocompatibility in *Ciona intestinalis* and *Botryllus schlosseri* have evolved independently but are based on structurally similar components. Taken together, the data obtained from this study indicate that different species employ different molecular mechanisms to carry out the same function of

self/non-self recognition, however, the underlying molecules can be structurally similar. Thus, there is no common evolutionary origin of self/non-self recognition machinery among tunicates.

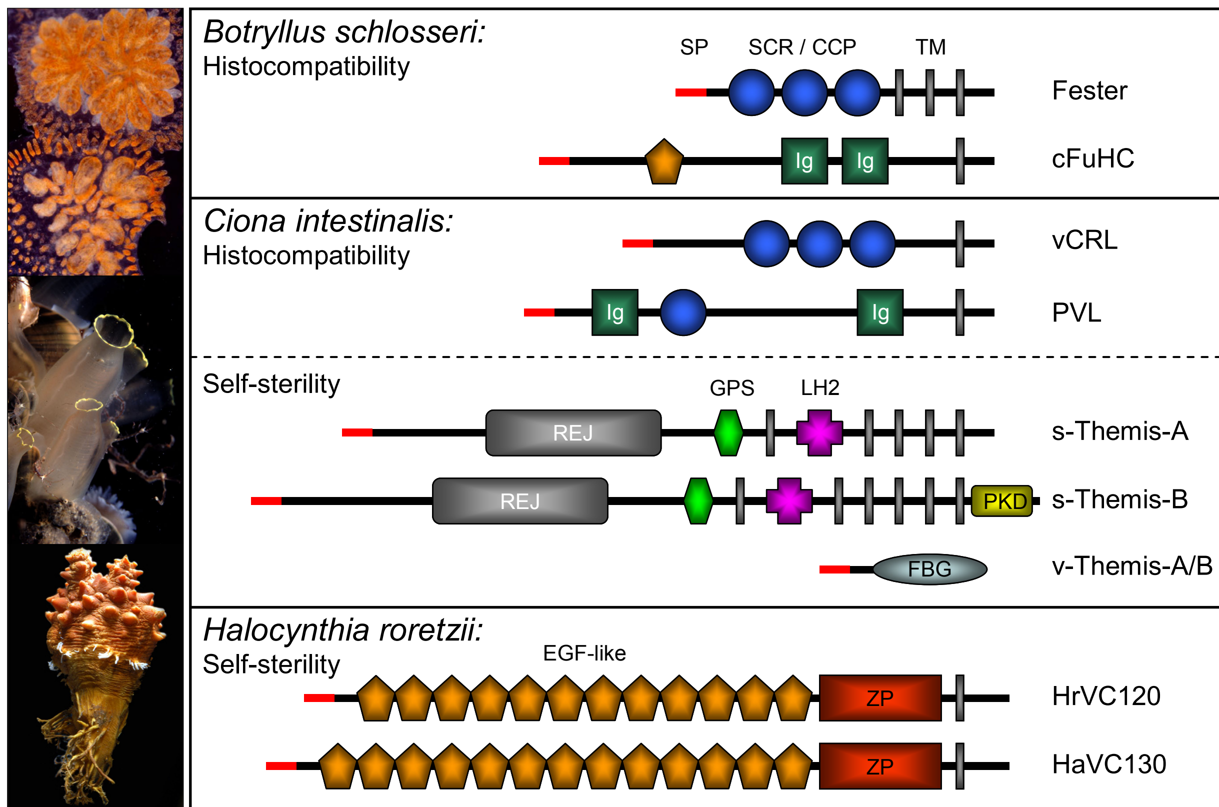

Fig. 3-1: Urochordate self/non-self recognition molecules. Candidate and proven recognition molecules of the colonial tunicate *Botryllus schlosseri* and the solitary ascidians *Ciona intestinalis* and *Halocynthia roretzii*. PVL - potential vCRL ligand (Ig/CCP containing protein), SP - signal peptide, SCR/CCP - complement control protein domain, TM - transmembrane domain, Ig - immunoglobulin-like domain, REJ - receptor for egg jelly domain, GPS - G protein-coupled receptor proteolytic site, LH2 - lipoxygenase homology domain 2, FBG - fibrinogen C-terminal β/γ chain gamma chain domain, EGF-like - epidermal growth factor-like domain, ZP - Zona pellucida domain. Modified from Sommer et al., 2010.

This study aimed to elucidate the molecular mechanisms of self/non-self recognition in *Ciona intestinalis*. This hermaphroditic urochordate is self-sterile although gametes are spawned into the surrounding seawater simultaneously because oocytes are able to discriminate between auto- and heterologous sperm (Lambert, 2005). How are these features encoded on a molecular level?

For Japanese *Ciona intestinalis* (species A) alleles of the two genomic Themis loci were shown very recently to correlate with the fertilization outcome (Harada et al., 2008). Here in this study, cross-sterility and the ability to cross-fertilize were shown to be completely dependent on the alleles of two Themis loci also in Northern European *Ciona intestinalis* (species B) and independent of the vCRL locus. Thus, two different reproductively isolated species use the same molecular mechanisms for the control

of self-sterility. Recognition is facilitated by polymorphic self-marker present on oocytes and sperm. Interaction between both protein pairs inhibits fertilization. Due to their polymorphism binding and the successive inhibition can only be triggered if the involved male and female alleles are identical (Fig. 3-2 A).

Although *Ciona intestinalis* is not a colonial urochordate individuals display histocompatibility reactions by rejection of tunic transplants from allogeneic animals (Reddy et al., 1975). Moreover, foreign potentially pathogenic microorganisms have to be recognized and eliminated, whereas reactivity to own cells has to be avoided. How are these antagonistic functions achieved in the absence of MHC which is the mediating self-marker of higher vertebrates?

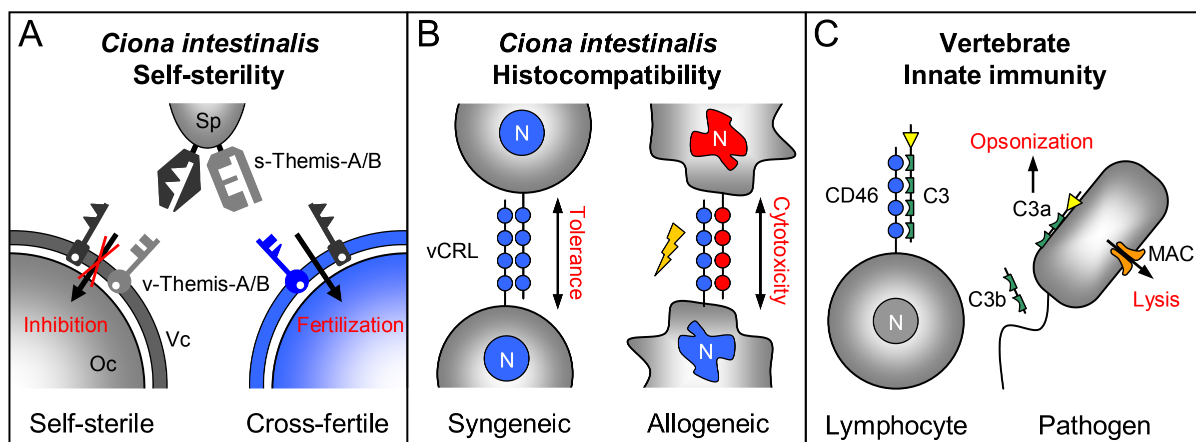

Fig. 3-2: Molecular mechanisms of self/non-self recognition in *Ciona intestinalis*. (A) Two genomic loci encoding receptor and ligand pairs termed Themis control self-sterility in *Ciona intestinalis*. v-Themis is present on the surface of oocytes and s-Themis on the sperm surface. An interaction between both protein pairs inhibits fertilization. Note, due to sequence polymorphism only animals carrying the same alleles in both loci are cross-sterile. (B) Histocompatibility in *Ciona intestinalis* is mediated by the individual-specific vCRL protein. Hemocytes express the vCRL gene which marks the cell as self and inhibits binding of soluble complement components or other immune factors. Foreign cells do not carry the self-marker vCRL and thus are lyzed. Furthermore, knock-down of vCRL gene expression leads to developmental arrest at the transition from rotation to first ascidian stage because hemocytes become auto-reactive or are lyzed since they miss the appropriate self-marker. (C) “Missing-self” concept of innate immunity exemplified by the mammalian complement system. By default soluble complement factors attach to the plasma membrane of cells which do not carry complement receptors. These proteins inhibit binding and processing of complement components and, thus, the formation of pores. Modified from Sommer et al., 2010.

The vCRL protein is individual-specific (Fig. 2-3) and present on the surface of hemocytes (Fig. 2-9) which are the main immune effector cells in these animals (Arizza and Parrinello, 2009). Knock-down of vCRL gene expression exclusively in hemocytes resulted in developmental arrest at the stage where hemocytes emerge (Fig. 2-15). Furthermore, vCRL protein shows structural similarities to human complement receptors like CD46 (see Kürn et al., 2007 and Fig. 1-8). Cells carrying these receptors are protected against the lytic activity of soluble complement factors

by binding them and preventing pore formation (Zipfel and Skerka, 2009). Taken together, vCRL might be an individual-specific inhibitory receptor which protects cells of the own tissue by marking them as self and, thus, prevents lysis by the complement system or other immune components (Fig. 3-1 B). If cells are not carrying the self-marker vCRL either because they are of an allogeneic origin (non-self) or due to knock-down by RNAi they are not recognized as self and tolerance is not established. Instead, these cells are attacked by immune components and lyzed. Consequently, knock-down of vCRL gene expression leads to developmental arrest since in the course of metamorphosis hemocytes are either not developed or destroyed. Taken together, the vCRL protein might be a self-marker mediating histocompatibility in *Ciona intestinalis*. The data therefore indicates that molecular mechanisms of histocompatibility in *Ciona intestinalis* are functionally equivalent to the mode of action of NK receptors in mammals and protection from the complement system (Fig. 3-2 C). Complement system in *Ciona intestinalis* seems to be an important factor as more genes encoding complement components have been identified in the *Ciona* genome than they are present in humans (Azumi et al., 2003). In general other genes or gene families do not display the redundancy or complexity observed in higher vertebrates (Corbo et al., 2001). Thus, the complement system seems to be selectively expanded in urochordates despite their overall genomic simplicity. In summary, this indicates that components of the complement system might have an evolutionary ancient function not only in the immune system, but also in histocompatibility.

4 Summary

The ability to distinguish self from non-self is a fundamental feature of all metazoans allowing them to recognize potential pathogens, keep their genetic integrity and assure successful reproduction. The molecular mechanism of self/non-self recognition is completely known only for vertebrates. But how do other animals distinguish self from non-self?

Urochordates are the closest living relatives of vertebrates which display naturally occurring histocompatibility reactions and in addition are self-sterile hermaphrodites. These animals, therefore, require two self/non-self recognition systems. In *Ciona intestinalis* several variable receptors have been isolated from two cryptic species inhabiting the Pacific Ocean and the Northern Atlantic (A and B): Themis has been shown to control self-sterility in species A while in species B the highly polymorphic vCRL gene has been identified by our research group. vCRL was proposed to be a self/non-self recognition receptor, but its exact function remained unknown.

In my thesis I analyzed the function of the individual-specific vCRL gene in *Ciona intestinalis* species B. Anti-vCRL antibodies, segregational analysis and transgenic knockdown animals were used to elucidate an involvement of vCRL in self/non-self discrimination processes. *In vitro* antibody interference assays showed that vCRL is involved in gamete interaction. However, segregational analysis demonstrated that the vCRL locus is not controlling self-sterility. In contrast to that, fertilization outcomes could be solely explained by segregation of two Themis loci. Thus, gamete self-incompatibility in both species of *Ciona intestinalis* is controlled cooperatively by two Themis loci.

Interestingly, knock-down of vCRL gene expression resulted in a drastic developmental arrest during metamorphosis which is caused by an impaired formation of the blood system. The data, therefore, demonstrate that vCRL might serve as self marker on the surface of developing blood cells. Thus, in the absence of MHC variable complement component might be utilized as an individuality marker in *Ciona*. My findings demonstrate a possible evolutionary link between invertebrate histocompatibility and innate immunity. Moreover, components of the complement system might have an evolutionary ancient function not only in the immune system, but also in histocompatibility.

5 Zusammenfassung

Die Fähigkeit der Selbst-/Nichtselbsterkennung ist eine fundamentale Eigenschaft aller vielzelligen Tiere, um potentielle Pathogene zu erkennen, die eigene genetische Integrität zu wahren und eine erfolgreiche Fortpflanzung hermaphroditer Organismen zu gewährleisten. Die molekularen Mechanismen dieser Erkennungsreaktionen sind bisher nur bei Vertebraten komplett verstanden. Wie aber unterscheiden andere Tiergruppen Selbst von Nichtselbst?

Urochordaten sind die nächstlebenden Verwandten der Wirbeltiere, die über natürlich auftretende Histokompatibilitätsreaktionen verfügen und selbststerile Hermaphroditen sind. Folglich benötigen sie zwei unterschiedliche Systeme zur Selbst-/Nichtselbst-erkennung. Für *Ciona intestinalis* wurden variable Rezeptoren aus beiden kryptischen Spezies (A und B) isoliert, welche im Pazifischen Ozean und dem Nordatlantik vorkommen: Themis kontrolliert die Selbststerilität in Spezies A, wohingegen aus Spezies B das hochpolymorphe Gen vCRL durch unsere Arbeitsgruppe isoliert wurde. Es wurde vorgeschlagen, dass vCRL möglicherweise Erkennungsmechanismen vermittelt, Funktionelle Untersuchungen fehlten jedoch.

Diese Arbeit beschäftigte sich darum mit der funktionellen Analyse von vCRL in *Ciona intestinalis* Spezies B. Anti-vCRL Seren, Segregationsanalyse und transgene Tiere wurden verwendet, um den Einfluss von vCRL auf Selbst-/Nichtselbst-erkennungsprozesse zu studieren. *In vitro* Interferenz-Versuche erwiesen, dass vCRL funktionell an der Interaktion der Gameten beteiligt ist. Jedoch zeigte eine Segregationsanalyse, dass nicht vCRL die Selbststerilität kontrolliert sondern zwei Themis loci, deren Allele mit dem Befruchtungsausgang korrelierten.

Interessanterweise resultierte das Abschalten der vCRL Genexpression in einer drastischen Arretierung der Entwicklung während der Metamorphose, da kein funktionelles Blutsystem gebildet werden konnte. Folglich könnte vCRL als Selbstmarker auf sich entwickelnden Blutzellen dienen. Somit würden in *Ciona* variable Komponenten des Komplementsystems als Individualitätsmarker verwendet werden. Dies deutet darauf hin, dass die Histokompatibilität und das angeborene Immunsystem der Invertebraten evolutionär miteinander verbunden sind. Außerdem könnten Komponenten des Komplementsystems eine evolutionär ursprüngliche Funktion nicht nur im Immunsystem sondern auch in der Histokompatibilität haben.

6 Material

6.1 Organisms

Ciona intestinalis	ordered from Biologische Anstalt Helgoland (Alfred-Wegener-Institut für Polar- und Meeresforschung) or from SARS International Centre for Marine Molecular Biology (Bergen, Norway)
Hydra vulgaris	strain AEP
Artemia salina	Silver Star
Escherichia coli	strain ElectroMAX^{TM}DH5α^{TM} (Invitrogen)
	strain ElectroMAX^{TM}DH10b^{TM} (Invitrogen)
	strain Rosetta 2(DE3)pLysS (Novagen)

6.2 Chemicals

Acetonitrile	Roth
Agar Agar	Roth
Agarose	Roth
Ammonium acetate	Roth
Ammonium bicarbonate	Roth
Ammonium sulfate	Sigma
Ampicillin	Merck
APS (ammonium persulfate)	Merck
β-Mercaptoethanol	Merck
Boric acid	Roth
Bromphenol blue	Serva
BSA fraction V	Roth
$CaCl_2$	Roth
$CaCl_2$ x H_2O	Roth
CHAPS	Sigma
Chloroform	Fluka
DABCO	Sigma
dNTPs (10mM)	Fermentas
Dextran sulfate	Roth
Dithiothreitol	MP Biomedicals

DMRIE-C	Invitrogen
Gene Ruler™ DNA Ladder Mix	Fermentas
EDTA	Sigma
Developer G150	Hugo Rost & Co GmbH
Acetic acid	Roth
Acetic anhydride	Sigma
Ethanol	Roth
Ethidium bromide (50 mg/ml)	Roth
Euparal 3C 239	Chroma
$FeCl_2$ x 4 H_2O	Merck
$FeCl_3$ x 6 H_2O	Merck
FITC dextran WD40	Sigma
Formamide	Roth
Glucose	Merck
Glutaraldehyde	Fluka
Glycine	Merck
Glycerine	Roth
HCl	Roth
HOECHST	Calbiochem
Heparin	Sigma
Iodacetamide	BioChemica
IPTG	Sigma
Isoamylalcohol	Roth
Isopropanol (2-Propanol)	Roth
KCl	Merck
K_2HPO_4 x 2 H_2O	Roth
K_2HPO_4	Roth
KH_2PO_4	Roth
Lead acetate	Merck
Lithium chloride	Roth
Maleic acid	Roth
Seawater salt, Ocean Zac plus	Zoo Zajak, Duisburg
Methanol	Roth
$MgCl_2$	Merck
$MgCl_2$ x 6 H_2O	Merck

$MgSO_4 \times 7 H_2O$	Merck
Mowiol 4-88	Calbiochem
Sodium acetate	Roth
$Na_2EDTA \times 2 H_2O$	Roth
Na_2SO_4	Roth
NaCl	Roth
$NaH_2PO_4 \times H_2O$	Roth
Na_2HPO_4	Roth
$NaNO_3$	Merck
NaOH	Roth
Sodium citrate	Roth
NBT/BCIP	Roche
NH_4Cl	Merck
Ni-NTA	Novagen
Osmium tetroxide	Roth
Paraformaldehyde	Agar Scientific Ltd.
Phenol	Roth
Pioloform	Plano
Polyvinylpyrrolidon	Sigma
Propylenoxide	Fluka
Rotiphorese Gel 40	Roth
Sheep serum	Sigma
Sodium dodecyl sulfate	Roth
Sephadex G50	Pharmacia
Sodium cacodylate	Fluka
Nitrogen (liquid)	Messer Griesheim
Substrate solution (NBT/BCIP)	Roche
Superfix	Tetenal Photowork GmbH & Co
TEMED	Serva
Triethanolamine	Merck
Tris	Roth
TRIzol® Reagent	Invitrogen
tRNA	Sigma
Tryptone	Difco Laboratories
Tween-20	Roth

Uranyl acetate	Merck
Urea	Roth
X-Gal	Sigma
Xylene	Roth
Yeast extract	Gibco BRL® Life Technologies

6.3 Media

Artemia medium	31.8g seawater salt per litre Millipore H_2O
Hydra medium	1mM CaCl2, 1mM NaCl, 0.1mM MgSO4, 0.1mM KCl, 1mM Tris-HCl (pH 7.8)
LB medium	1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, autoclaved
LB-Amp medium	LB medium with 50µg/ml Ampicillin (added after autoclaving)
LB Agar	LB medium with 1.5% (w/v) Agar Agar, autoclaved
SOB medium	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 25mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, autoclaved
SOC medium	SOB medium with 20mM glucose (sterile filtered, added after autoclaving)
Freezing medium	36mM K_2HPO_4, 13.2mM KH_2PO4, 0.35M NaCl, 1.7mM NaCitrate, 0.4mM $MgSO_4$, 6.8mM $(NH_4)_2SO_4$, 4.4% (v/v) glycerol, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, autoclaved

6.4 Buffer and solutions

6.4.1 General purpose solutions

Blocking solution (IHC)	1% (w/v) BSA in PBT
Denaturing solution	1.5M NaCl, 0.5M NaOH
Denaturing elution buffer (EPH)	8M urea, 50mM NaH_2PO_4, 300mM NaCl, 1M imidazole, pH 8.0
Denhardt's (50x)	1% polyvinylpyrrolidon, 1% (w/v) ficoll, 1% (w/v) BSA fraction V, filter sterilized, stored at -20°C
DNA loading buffer (6x)	Fermentas

DNA lysis buffer	10mM Tris-HCl, 150mM EDTA, 0.2% (w/v) SDS, pH 8.5
Elution buffer, native (EB)	50mM NaH_2PO_4, 300mM NaCl, 1M imidazole, pH 8.0
Elution buffer, denat. (EBH)	EB including 8M urea
Heparin	10mg/ml in Millipore H_2O, stored at -20°C
Lead citrate solution	1.33 g $Pb(NO_3)_2$, 1.76g $Na3(C_6H_5O_7)$ x 2 H_2O, 8 ml 1 M NaOH, filled up to 50ml with Millipore water
LEW	50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0
LEWH	50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, 8M urea, pH 8.0
Ligase buffer (2x)	Promega
Mowiol	15% (w/v) mowiol 4-88, 66mM Tris, 30% (v/v) glycerol, pH 8.0, stored at -20°C
NTM	0.1M NaCl, 0.1M Tris, 50mM $MgCl_2$, pH 9.5
NTMT	NTM containing 0.1% (v/v) Tween20
Paraformaldehyde	8% (w/v) in PBT or FSW, pH 7.5
PBS	0.15M NaCl, 80mM Na_2HPO_4, 20mM NaH_2PO_4, pH 7.3
PBS*	0.5M NaCl, 80mM Na_2HPO_4, 20mM NaH_2PO_4, pH 7.3
PBT	PBS with 0.1% (v/v) Tween20
PCR buffer (10 x)	Amersham
RNaseOut	Invitrogen
Sephadex G50	5% (w/v) Sephadex G50 in TE, autoclaved
SSC (20x)	3M NaCl, 0.3M sodium citrate, pH 7.0
TAE (50x)	2M Tris acetate, 50mM EDTA, pH 8.0
TBE (10x)	1.3M Tris, 0.45M boric acid, 25mM EDTA, pH 8.5
TE	10mM Tris, 1mM EDTA, pH 7.5
X-Gal	20mg/ml in dimethylformamide

6.4.2 Blots

Denaturing solution	1.5M NaCl, 0.5M NaOH
Depuring solution	0.2M HCl

Developer G150	0.02% (v/v) G150 in aqua dest.
Fixing solution	20% (v/v) Superfix in Millipore H_2O
Hybridization solution (Southern)	6x (v/v) SSC, 0.5% (w/v) SDS, 5x Denhardt's
Hybridization solution (Macroarray)	50% (v/v) formamide, 4.8x (v/v) SSC, 10mM Tris, 1% (w/v) SDS, 1x Denhardt's, 10% (w/v) Dextran-sulfate, pH 7.5
Neutralizing solution	1M Tris, 1.5M NaCl, pH 7.4
Salmon sperm DNA	10mg/ml in aqua dest.
Wash solution 1	2x SSC, 0.1% (w/v) SDS
Wash solution 2	0.2x SSC, 0.1% (w/v) SDS

6.4.3 SDS polyacrylamide gel electrophoresis and Western blot

Loading buffer (2x)	135mM Tris, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.001% (w/v) bromphenol blue, pH 6.8
Running buffer (5x)	125mM Tris, 1M glycine, 0.5% (w/v) SDS
Discoloration solution	Methanol:acetic acid:H_2O (50:10:40)
Coomassie staining solution	0.05% (w/v) Coomassie in discoloration solution
Gel buffer pH 6,8	0.5M Tris-HCl, pH 6.8
Gel buffer pH 8,8	1.5M Tris-HCl, pH 8.8
Transfer buffer	25mM Tris, 192mM glycine, 10% (v/v) methanol

6.5 Kits

Agar 100 resin	Agar Scientific, Ltd., Essex
DIG RNA Labelling Kit (SP6/T7)	Roche
DNeasy Blood&Tissue Kit	Qiagen
First strand cDNA Synthesis Kit	Amersham Biosciences
Megaprime DNA Labelling Kit	Amersham
Megascript RNAi kit	Ambion
Micro BCA protein assay Kit	Pierce
NucleoSpin® Plasmid Quick Pure Kit	Macherey-Nagel
NucleoSpin® Extract II Kit	Macherey-Nagel
PCR-Select cDNA Subtraction Kit	BD Bioscience
pGEM-T Easy Vector System	Promega
Qiafilter Plasmid Midi Kit	Qiagen

QuickPrep Micro mRNA Purification Kit	Amersham Biosciences
SequiTherm EXCEL II LC	Epicentre
Superscript II Double Strand cDNA Synthesis	Invitrogen
TRIzol Reagent	Invitrogen

6.6 Enzymes

Unless stated otherwise, all restriction endonucleases were obtained from NEB.

*Bgl*I	Fermentas
NotI	Fermentas
*Pst*I	Fermentas
Platinum *Taq* DNA polymerase	Invitrogen
Proteinase K	Sigma
Shrimp alkaline phosphatase	Fermentas
SP6 RNA Polymerase	Roche
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase	Fermentas
T7 RNA polymerase	Roche
Taq DNA polymerase	Fermentas
Trypsin (sequencing grade)	Promega

6.7 Oligonucleotides (Primer)

Oligonucleotides not included in Kits were synthesized by MWG. A list of all used primers is attached in appendix (section 9.2).

6.8 Radioactive substances

Redivue™ α-[^{32}P]-dCTP	Amersham Biosciences

6.9 Vectors

hoT G	Wittlieb et al., 2006
pGEM-T	Promega
pET28a	Novagen
pPICZαA	Invitrogen
LigAC-6	K. Khalturin, unpublished

pMiLRneo	Klinakis et al., 2000

For detailed vector descriptions see appendix 9.3 and figures 2-6 and 2-7.

6.10 Commercial antibodies

Mouse-anti-his$_6$	Novagen
Sheep-anti-mouse alkaline phosphatase	Chemicon
Donkey-anti-mouse Alexa 488	Invitrogen

6.11 Electric devices and other material

6.11.1 PCR machines (Thermocycler)

Primus 96 *plus*	MWG-Biotech
PTC-240 Tetrad 2	MJ Research

6.11.2 Electrophoresis devices and power supplies

B1A, B2, D3 (gel chamber)	Owl Separation Systems Inc.
SE250 (PAGE gel chamber)	Hoefer Scientific Instruments
SEDECM (electroblotter)	Peqlab
EV-231 (power supply)	Consort
EPS-3500 (power supply)	Pharmacia Biotech

6.11.3 Incubators

HIS25	Grant Boekel
KS10 (rotary shaker)	Edmund Bühler
Thermo incubator	Heraeus Instruments
Thermomixer compact	Eppendorf
Thermomixer Certomat H	B. Braun Biotech

6.11.4 UV devices

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

6.11.5 Electroporation

Gene Pulser II	Bio-Rad

Pulse Controller II	Bio-Rad
GenePulser XcellTM	Bio-Rad

6.11.6 Centrifuges

Centrifuge 5417 R	Eppendorf
MSC-6000	Kisker Biotech
Multifuge 3 S-R	Heraeus Instruments
Sorvall RC-5B	DuPont Instruments
SpeedVac Plus SC110A	Savant

6.11.7 Microscopy

SZX16	Olympus
DP71 (digital camera)	Olympus
EM 208 S (electron microscope)	Phillips
S420 (scanning electron microscope)	Leo
CPD030 (critical point drying machine)	BAL-TEC
SCD050 (sputter-coater)	BAL-TEC
AxioCam	Zeiss
Axioskop 2	Zeiss
CLSM TCS SP/UV	Leica
DC300F (digital camera)	Leica

6.11.8 Other devices

4700 Proteomics Analyzer Workstation	Applied Biosystems
AF-10 (ice machine)	Scotsman
BioPhotometer	Eppendorf
CellTram Air pump	Eppendorf
CellTram vario pump	Eppendorf
DNA Analyzer 4300L	LI-COR
Freezer –20°C	Siemens
Freezer –80°C	Forma Scientific
Refridgerator +4°C	Liebherr
Weighing machine	Sartorius
LaminAir HB2448 (model Firefly)	Heraeus Instruments
Microwave	Moulinex

Milli-Q Academic System	Millipore
Nanodrop ND 1000 photometer	Thermo Scientific
pH-Meter pH 211	Hanna Instruments
Phosphoimager FLA-5000	FUJI
QFill2	Genetix
QPix	Genetix
Sterile hood	Heraeus
Szintillation counter	Siemens
Ultrasonic bath Sonorex Super RK106	Bandelin Sonorex
Ultratome Ultracut S	Leica
Thoma chamber	Saaringia
VARIOKLAV 400 EV	H+P Labortechnik GmbH
Wallac WinSpectral	Perkin Elmer
Vortex Genie 2	Scientific Industries

6.12 Other materials

12-and 48-well plate	Roth
96- and 384-well plate	Genetix
Beakers	Roth
Cellophane	Roth
Chromatography paper	Whatman
Cover slips	Roth
Cuvettes (Uvette®)	Eppendorf
Dialysis tube, MWCO 12-14kDA	Medicell International Ltd.
Electroporation cuvettes	peQLab
Glass slides	Walter
Hybond N^+ Nylon membrane	Amersham Biosciences
Injection needles (0,4x20mm)	Braun
Latex and Nitril gloves	Roth
Pasteur pipettes (150mm, 230mm)	Sarstedt
Petri dishes	Sarstedt
Pipette tips (10, 20, 200, 1000, 5000µl)	Sarstedt
Pipettes (2,5 to 5000µl)	Eppendorf
Plastic tubes (15ml, 50ml)	Sarstedt
Phosphoimaging plates	FUJI

PVDF-membrane	Roth
Q-Tray	Genetix
Reaction tubes (0,2ml; 0,5ml; 1,5ml)	Sarstedt
Radiographic film (BioMax MS)	KODAK
Sieves	Nuova
Scalpell blades	Merck
Spirulina	HS-Products
Syringe (different volumes)	Braun
Sterile filters (0,20 µm, 0,45 µm pore size)	Sarstedt
UV lamp Chroma 43	Vetter
Vivaspin columns (different sizes)	Sartorius Stedim Biotech

6.13 Computer programs and data banks

6.13.1 Data banks (DB)

Blast databank	http://www.ncbi.nlm.nih.gov/blast/
Ciona genome, JGI v2.0	http://genome.jgi-psf.org/Cioin2/Cioin2.home.html
Ciona genome, ANISEED	http://crfb.univ-mrs.fr/aniseed/

6.13.2 Sequence analysis software

ClustlW2	http://www.ebi.ac.uk/Tools/clustalw2/index.html
ConSite	http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite
DDD-PCR	http://www.ncbi.nlm.nih.gov/UniGene/ddd.cgi
GeneMark.hmm	http://exon.biology.gatech.edu/eukhmm.cgi
SMART	http://smart.embl-heidelberg.de
eSeq v3.0	LICOR
DNAMAN v4.15	Lynnon Coprporation
MEGA v4.0.2	http://www.megasoftware.net
SEQtools v8.3	http://www.seqtools.dk

6.13.3 Other software

Image processing	Photoshop 8.0.1 (Adobe)
Microscopy	Axio Vision 3.1 (Zeiss)
	IM 50 4.0 (Leica)
Scintillation counter	Wallac 1414 WinSpectral v 1.30 (Perkin Elmer)
PhosphoImager	AIDA Image Analyser (Raytest)

7 Methods

7.1 Animal culture

7.1.1 *Ciona intestinalis*

Living *Ciona intestinalis* individuals were ordered from “Biologische Anstalt Helgoland” (Alfred Wegener Institute) and put directly after arrival into an aquarium containing water from North Sea at 15°C with constant aeration. Water was cleaned in a closed system including biofilter and protein separator. Animals were fed twice a week using *Spirulina*. During feeding filters were stopped to allow continuous uptake. Under these conditions animals survived one to four weeks. Animals were also sampled at Bergen seaside and cultured at SARS International Centre for Marine Molecular Biology (Bergen, Norway) in the laboratory of Dr. Di Jiang.

7.1.2 *Hydra vulgaris*, strain AEP

Animals kept in a climate chamber at 18°C in *Hydra* medium (Lenhoff and Brown, 1970) were fed three times a week using *Artemia salina* larvae. Culture dishes were cleaned 6-8h after feeding.

7.1.3 *Artemia salina*

Hatching of *Artemia salina* eggs was induced by incubation in 30°C saltwater for 48h. Before feeding *Hydra* polyps with the larvae they were washed with freshwater and resuspended in *Hydra* medium.

7.2 Isolation of nucleic acids

7.2.1 Isolation of genomic DNA

Ciona intestinalis genomic DNA was either isolated using whole animals or separated tissues of a single individual. Either separated tissues were homogenized directly in DNA lysis buffer or whole animals were first grinded in liquid nitrogen and the resulting powder than mixed with DNA lysis buffer. 50µg/ml Proteinase K was added and the solution incubated at 55°C for 3h. Proteins and other cell components were separated by centrifugation at 14000g with phenol and chloroform. After pelleting the

DNA using 96% cold Ethanol and washing with 70% EtOH, pellet was air dried and solved in 150µl TE. If gDNA had to be extracted from several small samples DNeasy Blood&Tissue Kit (Quiagen) was used according to the manufacturer's protocol.

7.2.2 Isolation of plasmid DNA

4ml LB medium containing appropriate antibiotic was inoculated with a single bacterial colony and grown for 16h at 37°C and 220rpm. Plasmids were isolated from these cultures using NucleoSpin Plasmid Quick Pure Kit (Macherey-Nagel). If plasmid DNA of high quality or in large amounts was needed, isolation was performed from 250ml cultures using Qiafilter Plasmid Midi Kit (Qiagen). In both cases extractions were performed according to the manufacturer's protocol.

7.2.3 Isolation of mRNA

Extraction of mRNA from *Ciona intestinalis* was performed using QuickPrep micro mRNA Purification Kit (Amersham Bioscience) according to manufacturer's protocol.

7.2.4 Quantification of nucleic acids

Amount and purity of solved nucleic acids was assessed measuring extinction at 230, 260 and 280nm using BioPhotometer (Eppendorf) or NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

7.3 cDNA synthesis

Single- as well as double-stranded cDNA (ss- or dscDNA respectively) was synthesized by reverse transcription using Superscript II Double Strand cDNA Synthesis Kit (Invitrogen). Synthesis was performed with NotI-d$(T)_{18}$ primer and the resulting dscDNA resuspended in 20µl H_2O per 1µg applied mRNA.

7.4 Polymerase chain reaction (PCR)

Nucleic acid sequences can be detected and amplified by PCR, which is based on three aspects: (i) denaturation of a DNA template, (ii) specific binding to a

complementary template sequence of two primers (annealing) pointing in opposite directions and (iii) elongation of these primers by a thermostable DNA polymerase (Saiki et al., 1985; Mullis et al., 1986). By repeating the cycle of denaturation, annealing and elongation a DNA fragment located between the two primers can be amplified exponentially.

7.4.1 Standard PCR

Standard PCRs were performed using Illustra *Taq* DNA polymerase (GE Healthcare). Reactions were assembled as shown in Tab. 7-1. In case large or non abundant templates had to be amplified, PCR was performed utilizing more processive Platinum-*Taq*-Polymerase (Invitrogen). 0.6µl 50mM $MgCl_2$ and accordingly less water were included in the reaction. Amplifications with both DNA polymerases were performed with the following PCR program: 95°C 5min, up to 40x (95°C 30s, Tm 30s, 72°C 1min/1kb), 72°C 5min. Tm equals the primer annealing temperature estimated by manufacturer MWG.

Tab. 7-1: Components of a standard PCR

Component	Volume [µl]
PCR buffer (10x)	2
Primer A (10µM)	1
Primer B (10µM)	1
DNA template (~ 60ng)	1
dNTPs (10mM)	0.2
Taq DNA polymerase (5U/µl)	0.2
Aqua dest.	14.6
total volume	**20**

7.4.2 Splinkerette PCR

Full-length cDNA sequences of *Ciona* individuals had to be identified by splinkerette PCR due to high polymorphism in 5'part of vCRL transcripts. Splinkerette was ligated to dscDNA 5'ends and PCR performed using primers hybridizing to the splinkerette as well as to the non-polymorphic vCRL 3'UTR. Amplification was carried out in two successive PCRs. Products of the first reaction served as templates for the second PCR nested PCR. To increase specificity a Touch-down PCR was used. The annealing temperature was lowered 0.5°C each of the first ten cycles beginning 3°C above and ending 2°C below Tm. Remaining cycles were performed with constant Tm. Sequences of primers as well as of the splinkerette are listed in appendix 9.2.

7.4.3 Semi-quantitative RT-PCR (Reverse transcription-PCR)

Semi-quantitative RT-PCR was carried out to compare gene expression in different tissues. mRNA was isolated in parallel from different tissues and converted to cDNA using reverse transcriptase. This cDNA was used as template in the following standard PCR. Amplification of a housekeeping gene (β-Actin) was carried out additionally for equilibration and the number of cycles limited to stop the reaction in exponential phase to be able to compare intensities of PCR products.

7.5 Gel electrophoresis

7.5.1 Agarose gel electrophoresis

Nucleic acids were separated in 0.7-2% (w/v) agarose in 1xTAE using horizontal gel electrophoresis and stained with ethidium bromide. Fragment sizes were estimated by comparison to DNA or RNA ladders (Fermentas).

7.5.2 Purification of nucleic acids from agarose gels

Purification of bands excised from agarose gels was performed utilizing NucleoSpin Extract II kit (Macherey-Nagel) according to manufacturer's protocol.

7.5.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (Laemmli, 1970). Samples were diluted in 2x loading buffer, boiled for 5min and loaded onto 10-18% polyacrylamide gels (10x10cm^2 with 1mm width, see Tab. 7-2). Electrophoresis was performed at constantly 30mA and prestained protein marker (New England Biosciences) was used to estimate protein sizes. Proteins were visualized by staining with Coomassie.

Tab. 7-2: Components for stacking and separating gel of SDS-PAGE

Component	Stacking gel	Separating gel
40% Acrylamid/ 0.8% Bisacrylamid	0.9ml	5.0ml
Gelpuffer pH 6,8	1.25ml	-
Gelpuffer pH 8,8	-	2.5ml
Aqua dest.	3.3ml	2.4ml
10% SDS	50µl	100µl
TEMED	5µl	5µl
10% APS	25µl	50µl

7.6 Precipitation of nucleic acids

If concentrations were insufficient or nucleic acids suspended in an unsuitable solvent, they were precipitated by centrifugation after addition of 0.1x volume 3M sodium acetate and 3x volume ethanol. Pellets were washed with 70% EtOH, air dried and suspended in an appropriate amount of solvent. Apart from using precipitation nucleic acids were also concentrated by vacuum centrifugation.

7.7 Cloning technique

7.7.1 T/A-ligation

All PCR products to be sequenced were ligated in pGEM-T vector (pGEM-T vector system, Promega) according to manufacturer's protocol.

7.7.2 Construction of expression vectors

DNA fragments that had to be cloned into an expression vector as well as the target plasmid were digested with restriction endonucleases, separated by gel electrophoresis and purified (see 7.5.1 and 7.5.2). Both DNA fragments were ligated using T4 DNA ligase (New England Biosciences). Digestions as well as ligations were performed according to manufacturer's instructions.

7.7.3 Electrocompetent bacteria

450ml SOB medium was inoculated twice with 6ml overnight culture of *Escherichia coli* DH5α cells (Invitrogen) grown in SOB. Cells were pelleted at OD_{600} of 0.4-0.6, washed three times with ice-cold water and resuspended in 3.6ml 15% (v/v) glycerol. 40µl aliquots were frozen in liquid nitrogen and stored at -80°C.

7.7.4 Transformation of electrocompetent *E. coli* DH5α

40µl electrocompetent *E. coli* DH5α cells were mixed with ligation reactions or plasmids in electroporation cuvettes of 1mm gap and transformed using Gene Pulser II (Bio-Rad, 1,8kV, 200Ω, 25 µF). Cells were suspended in 960µl SOC medium immediately after electroporation and incubated at 37°C and 220rpm for one hour. 50-500µl bacteria suspension was plated on LB-agar containing appropriate

antibiotics. If blue/white screening was performed plates were supplemented with 80µg/ml X-Gal as well as 400µM IPTG.

7.7.5 Colony-check PCR

Sizes of inserted DNA-fragments of grown transformants were analyzed by colony-check PCR. Primers were flanking the cloning site on the vector or hybridizing with the inserted DNA fragment in this standard PCR. Colonies were transferred into the reaction tube and the contained plasmids served as template. PCR was performed with 35 cycles using standard program.

7.7.6 Bacterial stock cultures

500µl of an overnight culture was mixed with an equal volume of 50% (v/v) glycerol and stored at -80°C.

7.8 DNA sequencing

7.8.1 Sequencing

DNA sequencing was performed by chain terminator method (Sanger et al., 1977) using plate sequencer DNA Analyser 4300L (LI-COR) and 5'-IRD700 or IRD800 labeled primers in combination with SequiTherm EXCEL II DNA sequencing kit LC (Epicentre) according to manufacturer's protocols. Products were separated on 6% polyacrylamide gel containing 21g Urea, 28ml H_2O, 5.55ml Rotiphorese Gel 40, 5ml 10x TBE, 76µl TEMED and 350µl 10% (w/v) APS. Electrophoresis was conducted using standard conditions and 0.8x TBE. Basecalling and qualitity assement were performed using eSeq v3.0 program.

7.8.2 Sequence analysis

General sequence analysis like conversion into complementary and reverse sequences, searching for open reading frames or cutting sites of restriction endonucleases, translation as well as comparison of several sequences by multiple alignments was conducted using DNAMAN v4.15 (Lynnon Corporation). Alignments were imported to MEGA v4.0.2 (Tamura et al., 2007) and served as basis for

construction of homology trees according to UPGMA protocol (Unweighted Pairwise Grouping Method using Arithmetic means", Sneath and Sokal, 1973) or generation of phylogenetic trees by Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic trees were drawn using a bootstrap of 500.

The vCRL gene and its organization on sequenced BACs (see 3.10.2) were analyzed by blastn searches using vCRL transcript sequences as query. The putative vCRL transcript of BAC 11F21 was generated manually using this information. BAC sequences were compared by pairwise alignments using Clustlw2 (standard options). Graphical representation of these alignments was achieved using ConSite program. Open reading frames on all BACs were predicted using "*C.intestinalis* ES-3.0" database of Eukaryotic GeneMark.hmm program. Predicted peptides of less than 150aa or that cannot be assigned to *C. intestinalis* ESTs using tblastn search (est_others database, restricted to *Ciona intestinalis*, no gene models) were excluded from further analysis. Domains were predicted for all peptides using SMART.

7.9 Heterologous gene expression

Production of recombinant vCRL protein was performed employing three different organisms. Three vCRL variants encoding for the extracellular domain were expressed: (i) one vCRL1 variant isolated from hemocyte cDNA encoding three CCP domains, (ii) a vCRL1 and (iii) a vCRL2 variant originating both from ovary cDNA and coding for one CCP domain (Fig. 2-8 A). Sequences of the modules used for construction of expression vectors are included in appendix (section 9.3.2). For final plasmid sequences see attached CD.

7.9.1 Production of recombinant protein in *E. coli* Rosetta 2(DE3)pLysS

Transformation of thermocompetent *Escherichia coli* Rosetta 2(DE3)pLysS cells (Novagen) with vCRL expression vectors was carried out according to manufacturer's protocol. Vectors were based on pET28a plasmid in which vCRL sequences were inserted by *Xho*I as well as *Nde*I digestion. Transformants were grown in 5ml cultures and expression was induced at OD_{600} of 1.4-1.6 by addition of 1mM IPTG for 4h. Expression was analyzed by SDS-PAGE and his-tag Western blot comparing samples taken before and after induction. Recombinant vCRL protein was extracted from 500ml induced culture. Cells were pelleted and lyzed by sonication,

which was carried out ten times using 10% power and 70% cycle for 15s on Sonopuls HD2200 with VS70T sonotrode (Bandelin). His-tagged vCRL proteins were purified by denaturing Ni-NTA affinity chromatography, renaturated during dialysis against 50mM HEPES pH 8.0 and concentrated (see 7.9.4 to 7.9.6).

7.9.2 Production of recombinant protein in *Pichia pastoris*

Preparation of competent *Pichia pastoris* cells as well as transformation was carried out according to manufacturer's protocol. Expression constructs were based on pPICZαA vector in which vCRL sequences were inserted by *Xho*I as well as *Nde*I digestion. pPICZαA additionally contains the Zeocin gene allowing dosage dependent selection of clones according to the number of integrated copies of the construct. Recombinant vCRL protein was purified by Ni-NTA affinity chromatography under native conditions from 250ml expression culture which was inoculated with the clone being able to grow at the highest Zeocin concentration and grown according to manufacturer's instructions.

7.9.3 Production of recombinant protein in *Hydra vulgaris*, strain AEP

Embryonic stages (two- to four-cell stages) of the freshwater polyp *Hydra* AEP were transfected by microinjection. Embryos were fixed using CellTram vario pump (Eppendorf) and 0.1µl vector solution (0.6µg/µl) was injected with a glass needle. Expression vector was based on hoT G (Wittlieb et al., 2006). vCRL sequence was inserted in frame between *Hydra* actin promoter and EGFP by *Pst*I digestion. Microinjection as well as cultivation of animals was carried out in *Hydra* medium. Polyps hatched 15-45 days after fertilization at 18°C and construct integration was monitored by EGFP fluorescence. Transgenic lines were generated by clonal selection of transformants. 15 animals of each line were homogenized in 2x loading buffer and vCRL protein production was assessed by Western blot.

7.9.4 Ni-NTA affinity chromatography

All vCRL expression constructs encoded his_6-tag fusion proteins which allowed an enrichment of the labelled protein out of a complex protein mixture. This compound was incubated over night with 5% (v/v) Ni-NTA resin in LEW or LEWH under native

or denaturing conditions respectively and loaded onto a 5ml syringe stacked with glass wool. The column was washed three times with 3ml LEW or LEWH and the recombinant protein eluted with EB or EBH in five fractions of 1.5ml volume.

7.9.5 Dialysis

Proteins purified by Ni-NTA affinity chromatography under denaturing conditions were re-natured by slowly reducing urea concentration. The protein/urea mixture was transferred to a dialysis tube (MWCO 12-14kDA, Medicell International Ltd.) and dialyzed twice against 100x volume of a suitable solvent buffer for 24h at 4°C.

7.9.6 Protein quantification

Total protein amount was measured photometrically utilizing micro BCA protein assay kit (Pierce) according to manufacturer’s protocol.

7.9.7 Concentration of proteins

If proteins were highly diluted the solution was concentrated by size exclusion chromatography using Vivaspin columns (Sartorius Stedim Biotech) according to manufacturer’s instructions. Additionally Vivaspin columns were used to quickly exchange buffer of protein solutions.

7.9.8 Matrix-assisted laser desorption/ionization Time-of-Flight (MALDI-TOF)

Matrix-assisted laser desorption/ionization Time-of-Flight method allows identification of proteins by laser mediated ionisation and migration of the ionized fragments in an electric field. A matrix is used to protect the protein from destruction via direct laser beam and facilitates ionization as well as vaporization.

Proteins were reduced in 2x loading buffer supplemented with 50mM DTT and afterwards free cystein thiol-groups alkylated by addition of 150mM IAA. Separation was performed by SDS-PAGE. A single excised protein band was incubated in ACN/25mM ABC and dehydrated in ACN. Proteins were digested in-gel with Trypsin (Promega, sequencing grade) and peptides solved in ACN. This solution was concentrated by vacuum centrifugation to 1µl volume and mixed with 9µl 0.1% (v/v)

TFA in ACN. Desalting, spotting and further analysis was carried out by Dr. Christoph Gelhaus (zoophysiology, CAU Kiel) using 4700 Proteomics Analyzer Workstation (Applied Biosystems). Proteins were identified by searching protein and nucleotide data bases available at MASCOT (http://www.matrixscience.com) or MS-Fit (http://prospector.ucsf.edu/mshome.htm).

7.9.9 Generation of vCRL antiserum

Generation of mice sera was performed in collaboration with Dr. Vladimir Klimovich (Research Center of Radiology and Surgical Technology, St. Petersburg). Briefly, mice were injected with bands of purified recombinant vCRL proteins excised from SDS-PAGE gels. Specificity of α-vCRL sera was elevated by boosting of mice with solubilized purified proteins.

7.10 Blots

7.10.1 Southern blot

20-50µg isolated genomic DNA was digested with 50-100U restriction endonucleases separated by agarose gel electrophoresis, depurinated in 250mM HCl and incubated in denaturing solution for 30min. The gel was equilibrated in neutralizing solution for 25min and DNA transferred to Hybond N^+ Nylon membranes (Amersham Biosciences) by capillary blotting. Hybridizations were carried out over night in hybridization solution at 55°C, followed by washing twice in 0.2x SSC/0.1% SDS at 55°C and 60°C depending on signal/background ratio. Autoradiographed imaging-plates were analyzed using FLA-5000 phosphoimager (FUJI). DNA-probes were radioactively labelled with P-[32P]-dCTP using Megaprime DNA labelling System (Amersham Biosciences, see section 7.10.3).

7.10.2 Macroarray

To identify BACs containing parts of the vCRL genomic locus, two libraries constructed from DNA of a single animal as well as multiple *Ciona intestinalis* individuals (Kobayashi et al., 2002) were screened using vCRL probe. Clones were grown on Performa high-density membrane (Genetix) covered with LB medium, lyzed and DNA fixed to the membrane by UV cross-linking. Membranes were hybridized

following conventional Southern blotting protocol at 42°C. BACs of positive clones were isolated using Qiafilter Plasmid Midi Kit (Qiagen). 3µg DNA was digested with *Hind*III and submitted to fingerprinting by southern blot analysis. Four BAC clones of the single individual library and additionally two BACs originating from different *Ciona intestinalis* individuals of the second library were sequenced by shotgun approach (Genome sequencing centre, St. Louis, USA).

7.10.3 Radioactive labelling of DNA-fragments

DNA-probes were labelled radioactively with α-[^{32}P]-dCTP utilizing Megaprime DNA labelling System (Amersham Biosciences) by random primed labelling. Reactions were set up as shown in Tab. 7-3. Not incorporated α-[^{32}P]-dCTP was removed by size-exclusion chromatography using Sephadex G50 columns. Incorporation rate was determined using scintillation counter (Cerenkov protocol).

Tab. 7-3: Components for radioactive labelling of DNA-probes.
Primers A and B were used to generate DNA template.

Component	Amount [µl]
PCR product, 150ng in aqua dest.	70
Random hexamer primer, 100µM	8
Primer A, 10µM	1
Primer B, 10µM	1
denature for 5min at 95°C	
10x Klenow buffer	10
AGT dNTP mix, 0.5mM	4
Klenow fragment (1U/µl)	2
α-[^{32}P]-dCTP (370MBq/ml)	3
incubate at 37°C for 2h	

7.10.4 Western blot

Proteins separated by SDS-PAGE were transferred to PVDF membranes by semi-dry electroblotting with 3mA/cm² for 2h. Dried membranes were re-activated in methanol, equilibrated in transfer buffer and blocked with PBT supplemented with 3% (w/v) BSA. After over-night incubation with primary antibody membranes were washed with PBT and incubated with secondary antibody (sheep-anti-mouse coupled to alkaline phosphatase, 1:2000, Chemicon). Antibodies were diluted in 1.5% (w/v) BSA in PBT. Unbound antibodies were washed away with PBT and membranes stained in NBT/BCIP 1:200 in NTMT.

7.11 Immunohistochemistry (IHC)

Ciona intestinalis tissue was fixed with 4% (w/v) paraformaldehyde in seawater at 4°C for 16h and transferred to methanol. After washing with PBT permeabilization was performed in PBS with 0.5% TritonX100. Blocking was carried out in 1% (w/v) BSA in PBT and tissue incubated with primary antibody diluted 1:100 to 1:500 in blocking solution. After washing with 1% (w/v) BSA in PBT, donkey-anti-mouse Alexa 488 secondary antibody (Invitrogen, 1:1000 in blocking solution) was added. Unbound antibody was washed away with PBT and DNA staining of cell nuclei was performed using 1µg/ml HOECHST dye. Tissue was embedded in DABCO/Moviol. Unused fixed tissue was stored in methanol at -80°C.

7.12 Fertilization experiments

Mature *Ciona* animals were cultured under continuous illumination for at least 48h to inhibit spawning. Accumulated gametes were extracted directly from gonoducts after dissection. Dry sperm was diluted in FSW to OD_{600} of 1.0 just before insemination. Approximately 500 oocytes in 250µl FSW were mixed with 10µl of sperm solution from the same (autologous) or of another individual (heterologous). After 75min incubation at RT cells were fixed by addition of one volume 2M sulphuric acid and fertilization was assessed by cleavage status. All fertilization experiments were carried out in 48-well plates with flat bottom.

7.13 Interference experiments

7.13.1 Transient RNA interference (RNAi)

PCR fragments carrying T7 RNA polymerase promoter sequences served as templates for synthesis of dsRNA using Megascript RNAi kit (Ambion) according to manufacturer’s protocol. siRNAs designed according to manufacturer’s guidelines against vCRL 3’UTR sequence as well as a control siRNA showing no blast hit to *Ciona intestinalis* EST project (see appendix 9.4) were purchased from Ambion. 1µg/ml siRNA or 30µg dsRNA was injected three times every second day into the body cavity of five *Ciona intestinalis* specimen using 1ml syringe with 0.4x20mm needle. RNAs were injected together with 3% (v/v) DMRIE-C transfection reagent and 1% (w/v) FITC dextran WD40. Ovaries of single individuals were dissected and

hemolymphe was pooled for the five animals injected with the same RNA. After extraction of mRNA vCRL expression was assessed by RT-PCR.

7.13.2 Stable RNA interference (RNAi)

To achieve a stable knock-down of vCRL gene expression constructs carrying a vCRL hairpin sequence were introduced into *Ciona intestinalis* embryos by electroporation (Sasakura et al., 2007; Sasakura et al., 2008). Electroporations were performed by Dr. Satoko Awazu and Dr. Di Jiang (SARS International Research Centre for Marine Molecular Biology, Norway) using GenePulser XcellTM (Bio-Rad) at parameters 15ms, 50V and 60µg of construct DNA.

The hairpin was designed according to the following guidelines: (i) vCRL transcript sequence coding for transmembrane domain as well as 3'UTR was used as template because it shows the least polymorphism between individuals, (ii) sense and antisense sequences were separated by a spacer of at least 300nt. This spacer is essential for replication of the constructs in bacteria.

Cloning was performed using LigAC-6 (generated by Konstantin Khalturin, modified pGEM-T vector) vector as backbone and the assembled cassettes were transferred to pMiLRneo expression plasmid to create the final constructs.

A hairpin targeting *Hydra vulgaris* AEP MyD88 (generated by Sören Franzenburg) was used as control for specific knock-down of vCRL gene expression as this sequence does not show any significant hit in the genome database (JGI v2.0) and therefore should not interfere with gene expression in *Ciona intestinalis*.

Integration of the constructs was monitored by EGFP reporter gene expression driven by two different promoters. The *Ciona* EF1α and hemocyte-specific vWA-like promoter (Wakoh et al., 2004) were chosen because the former is a very strong promoter driving expression to all tissues and the vWA-like promoter was chosen to analyze the vCRL function only in hemocytes. The hemocyte-specific vWA-like promoter was isolated using primers against sequence covering 2kb upstream of the vWA-like gene (Cioin2:293819, JGI genome browser v2.0) which was confirmed to be specifically expressed in hemocytes by comparing hemocyte and embryo EST libraries available at NCBI using DDD-PCR. The EF1α promoter was isolated using primers designed for a 2kb section upstream of the transcript model ci0100150109 (ANISEED genome browser). In all constructs 1.5kb sequence downstream of

ci0100150109 was used as a terminator. Sequences and an overview of all used constructs for stable knock-down of vCRL gene expression are listed in appendix 9.3.3 and figures 2-14 and 2-15, respectively.

For knock-down experiments only those electroporated animals were chosen which showed more than 75% transgenic cells in the whole embryo and normal tadpole development. Tadpoles were allowed to settle in 2ml antibiotic seawater in 24well culture dishes with one tadpole per well. Water was changed every two days. Progress of metamorphosis was scored by monitoring if development of an individual failed and arrested at attachment, rotation or first ascidian stage at the end of 12 days since planting in the well.

7.13.3 Antibody interference

To analyze the role of vCRL during fertilization in-vitro, oocytes were pre-incubated in FSW containing different concentrations of mouse-anti-vCRL serum or as control pre-immune serum for 60min at RT and crossing experiments were carried out as described above (see section 7.12).

7.14 Segregational analysis

Ciona intestinalis animals were crossed in different combinations and dry sperm, ovary as well as somatic tissue were frozen separately of these parental animals. Embryos were cultured in closed system at SARS International Centre for Marine Molecular Biology (Bergen, Norway) until sexual maturity. Siblings were checked for cross-fertilization ability against other individuals of the same cross as described above (see 7.12) and whole animals were preserved in 70% ethanol for preparation of genomic DNA. Synthesis of dscDNA was performed using ovary mRNAs isolated from parental animals. The complete full-length vCRL transcript sequences were determined by splinkerette PCR (7.4.2). Primers were designed against conserved transcript sequences coding for the CCP domain and the 3'UTR. Using these primers fragments of the vCRL genomic loci from parental animals were amplified and sequenced by primer walk. All vCRL alleles of the crosses were finally analyzed using primers designed against parental genomic vCRL sequences.

To analyze Themis-A and Themis-B alleles in the crosses primers s-Themis-A_for/rev or 151120_for/rev (Harada et al., 2008) were used.

7.15 Imaging

7.15.1 Light microscopy

Confocal imaging was performed using Leica CLSM TCS SP/UV and Leica DC300F digital camera. Light microscopic analyses were carried out using Zeiss Axioskop 2 and Zeiss AxioCam digital camera as well as Olympus SZX16 and OlympusDP71 digital camera.

7.15.2 Scanning electron microscopy

Tissue was fixed in 2.5% glutaraldehyde in FSW for 16h at 4°C. After washing with 75mM cacodylate buffer for 30min, post-fixation was carried out with 1% OsO_4 in 75mM cacodylate buffer for 2h at 4°C. After washing with PBS*, tissue was dehydrated in rising Ethanol series (25%, 50%, 75%, 100%) for 10min twice each step. Oocytes were critical point dried in an ethanol-carbon dioxide mixture (BAL-TEC CPD030), sputter-coated (BAL-TEC SCD050) and viewed at 10kV using S420 SEM (Leo).

7.15.3 Transmission electron microscopy

Samples were fixed as described for scanning electron microscopy (see section 7.15.2) and infiltrated with Agar 100 resin after incubation in 1,2-propylenoxide twice for 15min. Components of the embedding resin were mixed according to the manufacturer's protocol for the "hard" version. Infiltration occurred in several steps for 30min each with 1:2, 1:1 and 2:1 propylenoxide and Agar 100 resin respectively. Afterwards tissue was incubated in Agar resin overnight at RT and placed into rubber forms (Flacheinbettung/Plano) with fresh resin. Polymerization occurred at 60°C. From these blocks ultra-thin sections of 60-70nm thickness were prepared using Ultracut S ultratome. Sections were captured on round grids pretreated with 1.2% (w/v) pioloform in chloroform and contrasted with 2.5% uranyl acetate for 5min. After rinsing with water, grinds were treated with lead citrate solution for 3min (Reynolds, 1963) and analyzed using a transmission electron microscope EM 208 S.

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9 Appendices

9.1 Crossings among F1 siblings

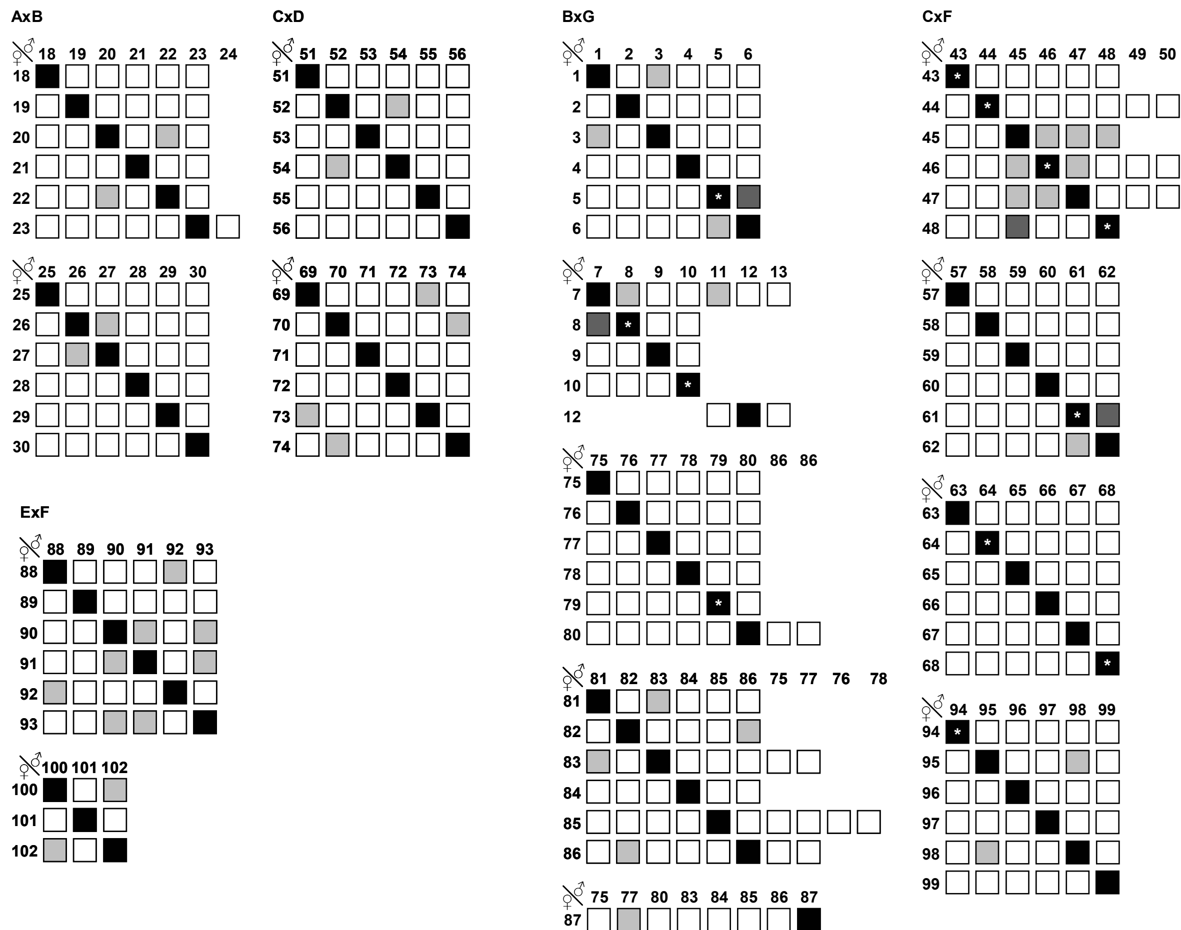

Fig. 9-1: Cross-sterility among F1 siblings. Offspring of a single cross were tested for pairwise cross-sterility by *in vitro* fertilization. Numbers indicate a single offspring individual and letters represent parental animals. Black - autologous controls (sterile), white - successful fertilization, grey - sterile combination showing no fertilization. Note, that some oocyte preparations were contaminated with heterologous sperm (indicated by asterisk). Thus, a small percentage of eggs were fertilized.

9.2 Oligonucleotides (primers)

Primers used for: Vector - flanking vector primers to test insert integration. Species subtyping - differentiation of both *Ciona intestinalis* species A and B. Rec. protein *Pichia*/*E.coli* - production of recombinant protein in *Pichia pastoris* and *Escherichia coli*. Rec. protein *Hydra* - production of recombinant protein in *Hydra* AEP. vCRL - amplification of vCRL alleles. Transgenic *Ciona* - generation of hairpin constructs to knock-down vCRL gene expression using transgenic *Ciona intestinalis*. 5'/3 RACE - Splinkerette and primers for 5'/3'RACE. Ig/CCP Southern - probe generation for Southern blot analysis of Ig/CCP genes neighboring vCRL. Segregational analysis - RFLP primers to analyze segregation of vCRL, Themis-A and Themis-B loci.

Name	Sequence 5'-3'	Tm [°C]	Comment
SP6	CGATTTAGGTGACACTATAG	53,2	Vector
T7	TAATACGACTCACTATAGGG	53,2	Vector
M13_F	GTAAAACGACGGCCAGT	52,8	Vector
M13_R	GGAAACAGCTATGACCATG	54,5	Vector
pET28a_R	GGTTATGCTAGTTATTGCTC	53,2	Vector
pET28a_R_700	GGTTATGCTAGTTATTGCTC	53,2	Vector
pPICZaA_for	CGGTTACTCAGATTTAGAAG	53,2	Vector
AOX_pPicholi_rev	AACTTGAACTGAGGAACAG	52,4	Vector
m1_for	TGTATTTACTATTTTCAACG	47,1	Species subtyping
m1_rev	CTCCACTGCTAGCAACTGC	58,8	Species subtyping
m2_for	GTGTCTTAGAAGAGGAGAAT	53,2	Species subtyping
m2_rev	CTTTGTCCACTGAGAGCACT	57,3	Species subtyping
m4_for	GCTTTGCTATGGAGGACCAG	59,4	Species subtyping
m4_rev	TCAAGAAGCGCTCCTAGCTC	59,4	Species subtyping
vCRL_Nde_E_f	AAAACATATGTCCGCAATTCAATTGCCACAAC	64,4	Rec. protein *Pichia*/*E.coli*
vCRL1b_Xho_E_r	TTTTCTCGAGTTATCCCGGAGACAATGACGAAC	68,2	Rec. protein *Pichia*/*E.coli*
vCRL1o_Xho_E_r	TTTTCTCGAGTTATCCATCAGATAGTGCTTTTGCTG	67,2	Rec. protein *Pichia*/*E.coli*
vCRL2o_Nde_E_f	AAAACATATGGCCCAAGCTCCATTTACATTC	64,2	Rec. protein *Pichia*/*E.coli*
vCRL2o_Xho_E_r	TTTTCTCGAGTTATGCTCCTGTACTTAATGTTGATGG	67,2	Rec. protein *Pichia*/*E.coli*
vCRL_F_(Pst_6H)	TTGCCTGCAGTACATCACCACCATCACCATTCCGCAATTCAATTGCCACAA	>75	Rec. protein *Hydra*
vCRL_R_Xa_PST)	TTTTCTGCAGCTCTTCCTTCAATTCCCGGAGACAATGACGAACCGCC	>75	Rec. protein *Hydra*
vCRL_3.6	CATGCTTKACTATTGKTGGTGTTTC	59,7	vCRL
vCRLutr18_R	TAAAGATAGGCGTGCAAGGTACAG	61,0	vCRL
vCRLutr18_F	GAATATTCTTTGCATGTTCCAGG	57,1	vCRL
vCRL_5.1	TAGGCAGAGCTGTTTGCTAGC	59,8	vCRL
vCRL_5.2	TTTCGTCATCCGAGGCTTCAAATAA	59,7	vCRL
vCRL_5.3	CATTGGTGTTGCTCATAGTATCTAGT	60,1	vCRL
EF1a_P_f_Sbf	AAAACCTGCAGGGAATGTAACTTGCTTTACCATCGCG	70,6	Transgenic *Ciona*
EF1a_P_r_PacI	TTTTTTAATTAAAGTCTTGTCTTTCATTTTGGAAGGTTG	63,1	Transgenic *Ciona*
EF1a_T_f_Asis	AAAAGCGATCGCGTGACTGCAGCCAACGACAC	72,1	Transgenic *Ciona*
EF1a_T_r_Asc	TTTTGGCGCGCCCAATCTAACTGATAAGCAGAGTCATG	71,6	Transgenic *Ciona*
bProm_for	AAAACCTGCAGGCTTTGGTTATAGGCTATTGTTTGAGG	69,5	Transgenic *Ciona*
bProm_rev	TTTTTTAATTAATAGTAGCAGCTTCATATTTAATAACTTCAC	62,6	Transgenic *Ciona*
Asis_up_Adapter	CGCAAGAATTCAACGTACGAAACCGGTAAGCGAT	69,5	Transgenic *Ciona*
Asis_downAdapter	CGCTTACCGGTTTCGTACGTTGAATTCTTGCGAT	69,5	Transgenic *Ciona*
vCRL_hp_for_Spl	CACACGTACGGTTCAATATTCATGTGCAACTGATCATAC	69,5	Transgenic *Ciona*
vCRL_hp_rev_Eco	CACAGAATTCCATTTATAAAGATAGGCGTGAAAGGTAC	67,3	Transgenic *Ciona*
vCRL_hp_space1	TTTTCGTACGGGTAACGCCAGGGTTTTCCC	69,5	Transgenic *Ciona*
vCRL_hp_rev_Age	CACAACCGGTCATTTATAAAGATAGGCGTGAAAGGTAC	69,5	Transgenic *Ciona*
MyD_Ad_up	CGCGTAAGACGAATTCTACAT	55,9	Transgenic *Ciona*
MyD_Ad_down	GTAGAATTCGTCTTACGCGAT	55,9	Transgenic *Ciona*
SF_MyD88as_F_Asi	TACAAAGCGATCGCGTTTTAAAATTCTG	60,7	Transgenic *Ciona*
SF_MyD88s_F_SplI	TTAATCGTACGAAACCAATGGATTGCATTAAT	61,8	Transgenic *Ciona*
SplBotBlunt5'-P	CCTTGGCTCGTTTTTTTTTGCAAAAA	58,5	5' RACE
KK_SplTop	CGAATCGTAACCGTTCGTACGAGAATTCGTACGAGAATCGCTGTCCTCTC CAACGAGCCAAGG	>75	5' RACE
SplOut_Pri2	GAATCGTAACCGTTCGTACGAG	60,3	5' RACE
KK_SplOut	CGTAACCGTTCGTACGAGAATTCG	62,7	5' RACE
SplinnPri2	CGTACGAGAATCGCTGTCCTC	61,8	5' RACE
NotI	CTGGAAGAATTCGCGGCCGCAGG	67,8	3' RACE
NotId(T)18	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTT	68,5	3' RACE
PVL1(s)_5.1	AGCTGGATATTTGCCCATAATTC	57,1	Ig/CCP Southern
PVL1(s)_3.1	GTAAACAACAGCAAAACCACAGC	58,9	Ig/CCP Southern
CCP_for8	GCAACMTGTCAGGAGRATGC	59,4	Segregational analysis
vCRL_CCP2_r	CATCATTACCACATGTAGCAGAC	58,9	Segregational analysis
Geno_rev1	GAACCAGTTTGAATAAAACAATACCG	58,5	Segregational analysis
s_Themis-A_f	TCTTACCTTGGGTTGTGGTTTTGG	61,0	Segregational analysis
s_Themis-A_r	TCCGGAATGATGACGTTTTTCAC	58,9	Segregational analysis
151120_for	AACACGCTGCAGTCAACTTTGGA	60,6	Segregational analysis
151120_rev	TCGATCAGGAAACTCTCCCAGGTA	62,7	Segregational analysis

9.3 Vector descriptions

9.3.1 Commercial vectors

9.3.1.1 pGEM-T

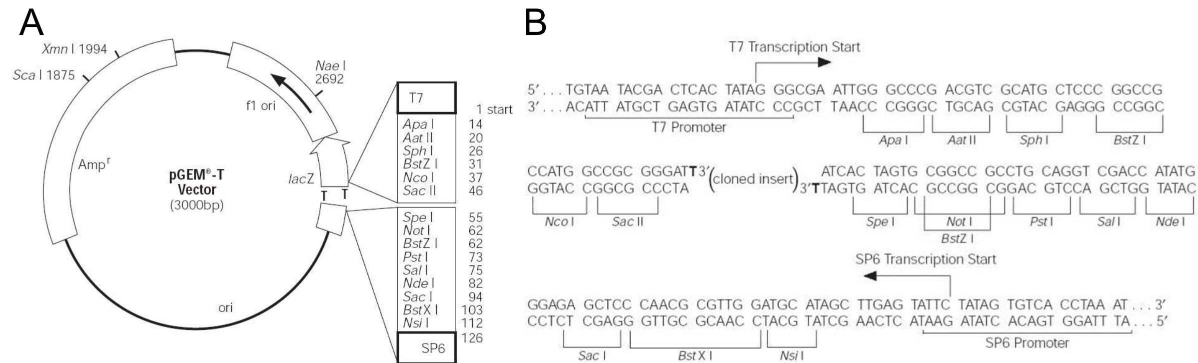

Fig. 9-2: pGEM-T vector. (A) Vector card and (B) multiple cloning site of pGEM-T vector; modified from manufacturer's manual.

9.3.1.2 pET28a

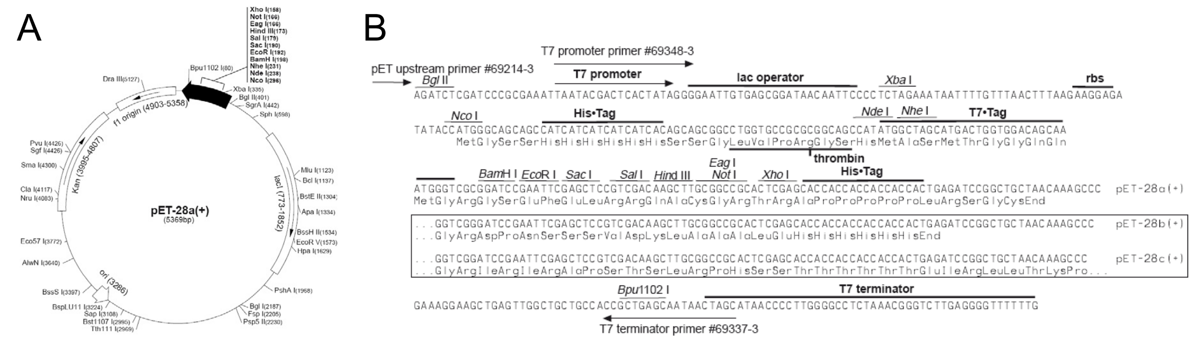

Fig. 9-2: pET28a vector. (A) Vector card and (B) multiple cloning site of pET28a vector; modified from manufacturer's manual.

9.3.1.3 LigAC-6 vector sequence

```
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CCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGAC
CACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAAGCGATCGCG
TAGAATTCACAATTCGATTATATTTATACTGGACTATTTTTACATCTGTTCGGTTATTTTCACATTTATTTTTCT
ATATATATCTTATAAACGTTTTAAAACCCATGTAATTTTTGTTAAGCTGTAATATAAAAGACGTCCTAACAAACT
TCTTTTATTACTGAATTTCCTTTAATTATAATAAATAACAAGTTTTAAAATAAATTCAGGCAATTAAGGCGCTCC
TGAGGTACTAAAATTAATGTAAACATTTAAAATTAACTTGGATGGTCTTAAGTACTGTACTCGTGATTTTGTTAT
ACTTTATTATTAGAAAAGTCGTCTATTAACTTTTTGTTCCTTAATTTACTTGATTAAATTGTCGCTTAATTTATC
AAATCAGGTTTTGCGCGTTATTTTAGAGAAAAACTTATTAGAAAAATGAATAAGCAAAGTTTAGGCTAACATGTT
TTTTTATTATTTTAAATAGTTCAAGTCAATGACGTATAAAATGCATTTGCAAAAAATTTTAAGTAACCCTATAAA
CTTAGCAATAGTAGATACTGGATGCAAGCATTCAGTAGCAGCATTGCATATCTGCTGTCTTTACGTACAAATAAC
AGCAAAAATGGACCTTTATTGGCTTCACATCGTCGTAAAACATGTGTTATTGGACTTGTCACAAATGTGTTAAGT
ATACAGAGCTTAGCTCTTGATGTTGATCACTAGTCGGCGCGCCATCAGTTTAAACGAATGCGGCCGCACTTGTGT
ATAAGAGACAGCTGTCGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTT
GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGG
AAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGC
TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT
TGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAA
AAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA
AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCC
CTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCG
CTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTA
TCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAG
TGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA
AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAG
ATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAA
AACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGA
AGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCT
ATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAG
GGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATA
AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGT
TGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTG
GTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCC
ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCA
CTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAG
TACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATC
TTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACC
AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGA
ATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTT
GAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGATGCGGTGTGA
AATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAA
TAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTC
AAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCG
AGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAAC
GTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGC
GTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAA
GTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTAT
A

Cutting sites of used restriction endonucleases are highlighted in magenta.

9.3.2 vCRL cDNA sequences used to generate expression plasmids

>vCRL1-h (3x CCP domain)
CTTGGTGTTGCTCATAGTATCTAGTCCTGGATGTTTGTCCGCAATTCAATTGCCACAACCTGGAAACCAAGCAAA
TGTTGCAACTGGAACCACAAACCTCACGATCACTGCAACAGTAACAACTAGTCCGTCTTCATGTTCGTGGTCAGG
AACAGGACTAACTGCTATACAATTTTACTCCTCTCTGTTTGGTTGTGCTCCAAGCAGCACTTCCTCACATAGCAT
TACATGCACAAATTCAGCAGGAGTCATTAATACCACTCTTACATATCTTACTGCAATGACAAGCAACAGCACAGT
TTCCGTAAATTGTGATTCATCTGTTGTTACCTCATTGTCAATGGTAGTAAAAGCATGCCAACCAAACACTTCAAG
TGGTGTTAGTGCAACCGTTAGTTCTTCACCATGTGGATTTGGATGCACAGTTCAATATTCGTGTGCATCTGGTTA
TAATGGGAACGATGTTACTGCAACATGTCAGGAGGATGCTACATTTGATGGCATCGACCCTGCCTGTATTCAAAT
CACATGTGCAAATTCTGATCATCTGTGCTCCAATCTTCAGTATTGTTTTAATCAAACTGGTTCAACCAATGCTGG
CACAACTGTTACAATTCCATGCAATACTGGTTATAGTGGTTCTGTGTCTGCTACATGTGGTAATGATGGTAGTTG
GACAATAACACCTCAACCCCCATGTCCAGCTTTGTGCCCTGTGTCAGATATAACATCCACACCAGGAAGGTGTAC
AACTGCTACTGGTGAAATATTACAAGGCCAAAGCGTAACTGTCACTTGTTGCATTGGTTATGAAGGAGTGAGCAC
AACGGCTATCTGTACTTCAGATAGAAGTTGGTCTAATCTCCCAACATGTACAGCTGATACAACAACAACGACCCG
TGAAGGAAGTGTAGCAAGCACCACAAGCACTATTCAAAACAGTCCAACTGGCGGTTCGTCATTGTCTCCGGGAGC
TATAGCGGGCATTGTTATTGGATGCTTAATTGCTGTTGCAATCATCATATTTTTCCTGATTCCTATCTGTTGTGC
GGAGATTTGCTGTTGTGCTTTTACAATACTTTGCTTGTCTTGGGGAAAAAAAAAGCCATATCCAGAGCCAGATAT
AACCATTCCAGACTTGAAAAAGAATACACAGCCGGACGGTCCTAATGGCGAAGGTGTTGTACCTTGAACATTATA
CAAGAAATTTGTTTACTTTCAAATTCTTAGCAGTTTCTTTGGTTCATAATTTTGTTTTGAATATTCTTTGCATGT
TCCAGGTTTTAATTTTATCTGTTTGTTCGTGTTTTTGAATTGTTTGTATAAATTGAGTCTTCAAGACAACCATTT
TAATTTAATTAAAGTTTGAGAAACGTTTCCAATATTCTGTGTAGCTCAGTTAACTTCCCATGTACATATGGTTTA
TTGGTTTATTAAACAATGTAATTGTATTATTACTAATTTTGTATTTGTTCCACGTAGATGTATGCATGATTTTAT
TGTACTAGAAACACCAACAATAGTAAAGCATG

>vCRL1-o (1x CCP domain)
CATTGGTGTTGCTCATAGTATCTAGTCCTGGTTATTTGTCCGCAATTCAATTGCCACAACCTGAAAACCAAACAA
ATGTTGCAACTGGAACCACAAACCTCATGATCACTGCAACAGTAACAACTAGTCCATCTTCATGTTCGTGGTTGG
GAACAGGACTAAGTGCAACCGAATTTTATACCACTGGTTTTGGTTGTGATTCAAGCAGCACTTCCACACACAATA
TTACATGCACAAATTCAGCAGGAGTCATTAATACCACTCTTACATACCTTACTTCATTGACAAGCAACACCAACG
TTTCACTAAGCTGTGATGGTACTGTTGTTTCATTGTCAATGATAGTAAAAGCATGCCAACCAAACACTGCAAGTG
GCGTTATCGCAACCGTCAGTTCTTCACCATGTGGATTTGGATGCACAGTTCAATATTCGTGTGCAACTGGTTATA
CTGGGACTGATTTTACTGCAACATGTCACGAGGATGCTACATTTGATGGCATCGACCCTGTCTGTGATCAAAGCT
CGACAACAACAGCAAAAGCACTATCTGATGGAACTATAGCGGGCATTGTTATTGGATGCATAATTGTTGTTGCAA
TCATCATAGTTGTCCTGATTGCTATCTTTTGTGCGGAGATTTGCTGCCGCACTCTTTCAATACTTTGCTTGTCTT
GGGGAACAAAAGAGCCAGATGACCCCACGCAAAAAGGGAAAAAGAATACACAGCCGGACGGTCCTAATGGCGAAG
GTGTTGTACCTTGAACATTATACAAGAAATTTGTTTAGTTTCAAATTCTTAGCAGTTTATTTGGTTCATAATTTT
GTTTTGAATATTCTTTGCATGTTCCAGGTTTTAATTTTATCTGTTTGTTCGTGTTTTAAATTGTTTGTATAAATT
GAGTCTTCAAGACAACCATTTTATTTTAATTAAAGTTTGAGAAAAGTTTTCAATATTCTGTGTAGCTCAGTTAAC
TTCTTATGTACATATGGTTTATTGGTTTATTAAACAATGTAATTGTATTATTACTAATTTTGTATTTGTTCCACG
TAGATGTATGCATGATTTTATTGTACTAGAAACACCACCAATAGTAAAGCATGTTATACATTTAAATTAATATAA
ACCCCCCACATAAATATATTGATTTTTACTGTACCTTGCACGCCTATCTTTA

>vCRL2-o (1x CCP domain)
ATGTGTTTAATAAAGGGGTGCTGTTATATACTATTTCAATTCACAGTTTTGTTACTACACTCACAATATGTAAAT
GCCCAAGCTCCATTTACATTCTCTGCGATATCCCTACAACCCCCAATATCATCAGTTGGTTCAACTACCACTGCT
ACTTGTAATGTGACCTATACTGGAAGTACAGCGTTTCTATTAACATGGAAAATAAGCTCTGTTTCTGTTTTGACA
TACTTGATTGAAAATGGAAACGTTCCACAACGTGGTACTCCACCTGCTTCTGGATACAATGTATCTTCAAGCACA
ACAACATCCATTGTTAACACTGGTGACACTTTTCCCATGAACCTTGAAATCAGGAATTTAAGTTTGGCCCTCAAT
CAATCAACAGTTTCTCTCGGTTGTTCGGAACCCGGTTGTATCACTCCAGTTCAGTTACAAATTGGAGTATGCCAA
CCAAGCACGGCAAGTGGTGTTATCGCAACCGTCAGTTCTTCATCATGTGGATTTGGATGTACAGTTCAATATTCG
TGTGCATCTGGTTATACTGGGAACGATGTTACTGCAACATGTCAGGCGGATGCTACATTTGATGCCATCCCAATA
TGTATCAGTCCGACACCATCAACATTAAGTACAGGAGCAATAATTGGCATTGTTATTGGATGCATAACTGCCGCT
GCACTCATCACATTTGTCCTGATTGCTATCTTTTGTAAGCAGTTTTGCTGCTGCACTTTTTCAATACTTTGTTTG
TCTTGGGGAGAAAAAAAATACACAGCCGGACGGTCCTAATGGCGAAGGTGATGTACCTTGAAAATTATACAAGAA
AATTATTTAGTTTCAAATTCGTAGCAGTTTCTTTGGTTCATAATTTTGTTTTGAATATTCTTTGCATGTTCCAGG
TTTTAATTTTATCTGTTTGTTTGTTCGTGTTTTTGAATTGTTTGTATAAATTGAGTCTTCAAGACAACCATTTTA
TTTTAATTAAAGTTTGAGAAACAAATTCAATATTCTGTGTAGCTCAGTTAACTTATTATGTACATATGGTTTATT
GGTTTATTAAACAATGTAATTGTATTATTACTAATTTTGTATTTGTTCCACGTAGATGTATGCATGATTTTATTG
TACTAGAAACACCACCAATAGTAAAGCATGTTATATATTTAAATTAATATAAACCCCCCACATAAATATATTAAT
TTTTACTGTACCTTGCACGCCTATCTTTA

Specific hybridization sites of primers used to generate expression constructs are highlighted in red (forward) and green (reverse) and the complete sequences of the assembled constructs are contained on attached CD.

9.3.3 Sequences of hairpin vector components

In the following sections sequences of the modules which were used to generate the different constructs are listed. Sequences contained in primers used to generate the fragments are highlighted in different colours: magenta - cutting site of restriction endonuclease, cyan - specific primer hybridization sequence, yellow - start/stop codon and grey - intervening spacer sequence. For complete sequences of the assembled constructs see attached CD.

9.3.3.1 EF1α promoter

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CCTGCAGGGAATGTAACTTGCTTTACCATCGCGTGGTGGGAAAACGACAGTCGTTATAGCACGAGTGTTCATACA
CCTCGTGCCAGCTTACGAGCTACCATATATGTTGTGGGCGAATAAAGGTTTTATAAATATAACAGGTTTTATAAA
TATAACAACGCCATTTTAAAGTCGGTTACATAATTCTGTAACTAGTTCAAATTGAACGGTAAACGTAAATAAAAA
CCTTGACCGTCTTACCCAATTATATAAAAACACTTTGAACGCTTTTTAGGATGGAAGGGTATGGCCATGCTAGAT
AATTCTGTGGACCATCTCAACCCAACCTATTACAGAACGGTCGTAATAATGAAAATGGATACCATTTTTAGGCAT
ATAGACTGATTCCTCTACTTTCTAGAAACGTAAGCAGTATACACAGAAAAAATGAAGTGTGATTCTGTGCAATTA
AACCGTTCTAAATTCATAGCCGACTGAATTTCTAATTAAGTGAATGTCTGACCTAGATTTATTGTTAAGTTTAGC
ACCAAATCTGAGCCAGCGATAAGCAGTCTAATTAAATTGGCTGCTGGCGATAAAATAGGTCATCCTGAAAAATCG
TTTGCGCCTTTATTTAAAATATAGTAGAGTGGGGAAAGACGGACATCTTATCGTTCTATTTTCTCGTCCCATTT
CGTAGTAAACAAAGAACATTCAAAAAATATAAAACCATAACTTCAAAACTTCAATAGACCGTTGTCAACTGTTTA
AAACACAGTAAGAGAATTTGGATATTATGTGCTAAAGGTGTCCCATCTCCCCCCACCCTACTATATCTGTTTATA
GTTCTGTGGGGTAAGATGAGATACCGTTAACACCTAAACATTTTTACTTTAAACAATCAACCACGTTTTTTATGG
TCGTGATGGGCATGAGGTTACATAATTCTGAAAATATTTTTTGCCCCCGACCAAAAGACGCGAAGAGTAAAAACA
TGTCTCAGCTTATATTCCCCACATAAATATATTTTTGTACTGTTTGGTGAATTTATAAACTTATATTACCATGCA
TATACGTTATGTTACTGGTATTTTCTCAGTAGGCAAATTCATTTGTCCACGTTTTATAGGTTTTTATTTATGATT
TTTAAAATGCTAAAAATGTGGGAGGGGGTTGAAAGTACAATACAAACACACAAAACAACTCAAACTAAAGATTTA
TAGTTATGCTAATTCACCTACACAATATAACAAGATGTGTAATGCAACCATGTGTTTATGATGAGCGCTAACATA
TTTTGTAACCACTCAAATTCCCCGCCACACGAGGATAATGAATAGGTGACTCTGTAGTCTGTACATCTTAGACTG
AAATAAAGATTATAAATCTACGAAATAAAATAATTTCTGCTCACTGATTATACTTCTGTTTTATAGATTAGAAAC
CGTTTCTAATAAATGACCTAATTCGCTATACACACACGCTGTGCGCGAGATAATCATTCTCGCACCCCGTTTATT
GTGTTAAAATTGCCGCCTAGATTCACAAAGCGTGACGGCTAGAGCCAGCAACGTGTCGCCTTCAATTACGCAACA
TCCGGGTTGCGCAATTCTGGATATAAAAGAACTAACAAAGATGACGTAGCTACCTTTTTCAGTTCAGACTTACGA
AAGACTCACGTGTCGGCGGTCTACTTGTCCTTTTCGAGCTGTGGCAATTTGGTGAGTGGTTCTATCTTATATCTG
AGTACATCTCTAAGGAATTATAGTTTGATTAGTTAAGTTTTTATTGTTAGGAAAGATGAAATCATTAGGTTTTAC
TTAGTTTAAGTATGTTAGTACTGGTTAGGCGTTTGAATTATTGAAAAACTCAGTTCGTTAACTGTAGTAGTTCTG
GTAGCTTAGCAAGTATACCCTGTATACGCCTTTTGGCTTTTTAACAATAACTTAAACTTATTTTACAGCAAATTT
CTGTGCATTCGGTTAACCCCAACCTTCCAAAATGAAAGACAAGACTTTAATTAA
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9.3.3.2 vWA-like promoter

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CCTGCAGGCTTTGGTTATAGGCTATTGTTTGAGGATCCGAGGGGCAACTGTTTATTAATTTTGATATTTAACATT
TTATATGCCATGTGTGTTTCTGCAAGCCATCTTGAAGTATAATCGAGCACGGTATTAGTTTTGCGTATAAAGATA
TAACCAGCGGGGCTGATCGCATATATATAGCTATAGCTTACGCACGAATAATATGGCGATGTCTGACTGACGATC
ATTAAAAGCATGTATACAACCAACTACAGGTTATGGGAAATATAGTAGGGTGGGGGAAGATGTGACACCTTTAGC
ACATAATATCCAAATATCATGATAGTGTTTTAAAGAATTAACAACGGTCAATGGAAGTCGTGAGGATACGGTTTA
ATAATTCTATGAATGTTTTTGGTTACTAACAAATGAGACGATAAAATAGAATGTAATAGTGTCTCATCTTTCCCC
AACCCACTATAAATACAGCAAATTGTAAGTTATGGGAAAAAATACAACAGTGAAAGTATAACAGTTTTTGTTTCT
AACGCTATAATAGCAACAGCAGAATAAACAGGTAGTACGTATGCATGCACTGATTCAAGACTGAAATCAAAAGAA
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AACATTGTTGTTGTTTTGGTTCGATACTTAACTCATGTTGTTTAAGTTTTTAATCGTGTTTTGAGGAAGTTCCGC
TGCTTTGCGCGAGCGGCGGAACTCTGGTTCCGAGAATGTTTGATGTCACGTTTCCTTTAGCGAATCTTTAAAGAA
ATATCAAAGTTTTTGTTTAACCTCTTATTCGCGAGGACACAATTATGATTCGTTTAATGAGTCTGTTAGTTGTGC
GGGACATTACTGGGTAAAATTAATAGAAAAATGCCAAAAAAAGCTGAACGATTAAATTTTGAGCTTTCTTAGAAA
AATGAGTAAAAATGGCAAATGTAGGAAGTGCTATTATGACGCATGTTGTAGATGATCGAAAGCGAGTAATGCGTA
ATATGTCTGGCACAGCTGCAATGTTAATACATCACAATTTAATTACTATACAAGAGCAATAGTTCATTTAATATG
CCACAAAGTACGTTGCCGGTGCGCAGTGAAAAATATCACAGACAATACACGTTCTTCCGTTGACGGACTTGAAAT
ACGTGTAGCTGATAAGTGAAGGAACCTGAGTTGACAAACGAATTCTTCCGGCAAACAGAGACCGCATTTTCACGA
TTTTGGTTTACCGTAGCATAAATACGTAATATTTTGTTTTGGTTTTATACACCGACAGCCGGTGTATTTTGCAAG
GATTACCTGCTTCCTATTTGACAACAAACATTGCAAATGATGGAGAATAAACAAATAATAAATCCGACCGAAGCA
AAAATATTTTGCAAGCAATCTCTTTTATATGTGGGTGAGGTGCCATTGGGTGGCACGCCATGGGTTCTAGGATTT
AAGCCAGAATTAGTGGCTATAGGTACATTAATAACTGTTCTGACATTTCATTGTATTTAATGTAATGAACTATAC
AAAAGGCACATATATTTAAATCCGCCACCTTACAAAAGTAAAAAATCCGATTTCTATATTAGCATTAATGGCTAT
GAAATAGTTTCAGTTCTTAACGTCAGGTAATTCCAAAGTATTTTATTTGATTTAATTAGAGGATTTATTCCTTGC
CTCACAGTGAAACTTATTAAGCAGCTTAAACTTTTGCGGTTCGTGCACGAATTTCGTGGTATGACGTCATTATAT
CACAATGTAGTGGAAAAGTTAACGTTACATTAAAAAAAAAACGATTAATTTGTAAAACGTAATAAGGTTATGCAG
CTTGAGTTTGACGAAAGCAGGGCTCAGTTATACGTCATGATACGAACGTATATGTCTACGAACATTGGCGCGCCG
GAATTCGCTAAGTAGCAATGTGTATGAGTAATTAGGCATCATTACTCAGAGCCATATTAGGGGCATCGATAGTGC
GCAGTGAAAATGACGGAAAAACGCAACGGCGGGTATATAAGGCCTGCGTGGATGAGGTATATATGACACGTTCAT
CGAACGAAGTTAAAATATTATTTTGTGAAGTTATTAAATATGAAGCTGCTACTATTAATTAA

9.3.3.3 EGFP

TTAATTAATGAAAAAATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT
GATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAA
TTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTGTTATGGTGTTCAATGC
TTTTCAAGATACCCAGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAA
AGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTT
AATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTAT
AACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAAC
ATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTA
CCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT
CTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAAGCGATCGC

9.3.3.4 EF1α terminator

GCGATCGCGTGACTGCAGCCAACGACACCGCCCACCATCACTTCTGCAGTTTTACTCCACCAGCTTGCTCACCCT
TCTAACCATTTCAGCCTAGTTAAACCCCCAGTTCAATTTGTCAGCCTTTTAAATTGCGTCATGACATTTCAGTTG
TTACTGTGTGCCGCTCTGTCCACATGGTGTAGTTATGTTTTTCATTACCCCCACCGAAAACACTTTCGTAGGAAA
AAACCCTCCGTTTCGTGCCAACTTAAAAGTGTTATTTTTGTTTCTAGCGCTGCTTAACTTATTTAAAATGACAAG
ACTATCTATGATTTTAAACCATGGCTTTGAATGGCGTACATGTATTATTGTGTTTATATTCAGTTAAACCGTACT
ATTACTGCTTGGAAAATTCAGTTCATTCCTTCACCGAAGGCTTTAACGTGGTTGGTTGCGGTGTTGTCAATTTGC
TGCTGGAAATAAAGCTTCTGTTAAGTGTATACTGTCTCTTGGTTTGCAAGATTATTATGGATCCAGATGTTGTAT
ATACAAGAGTGAAGATTTAGCGTTTTTATGTGGTAAAACGCCCATGGTGATCTATTTACAGTTTTTAAGTTGAGC
TGATCAATTTTCACATGATGATCCTGAACGACCAGGGGCACAAAGGCATTTGCAGATTGTCCCATCTCTCACTAC
GACCGATCTACCACTATTTTCACAACGGCCAGTACATTTCGAAGTCTGGTATTTAGCGAAGTAGTGTTCAATTGC
CAAAGCCATGTTTCTCTGCTTCGCTCTGTCTGGGATGACCGATGATATGGGTCGCAGCTGTAAAATTACAAATAG
TCAGTTTTTCTGACGAAAGTGAATGTTGATTACATTCAAATGAAAGTCGTTGATGGCTAATGCCATCGTTCATAC
GCTTTAATTAGCAGATTATGTGGTTATATTAGATATCTTTACCAGTAATATTGAGAACGCCAAGGTAAAATCTAG
TTTTTTGATACTTAATGAACAGCCATATTAAAAGCCATACATGAAAAAAAAATTAAAAAAAACATACATAAACTC
TCATAAGCTGACACAGTAGGTCGTGGCTGACACGAGGTGTATGAAACAAAACGCTCGTGTTTTAACGACTGTTGT
TGCACCGTCACGCGAGAATAAAGTAACTTCCATTCATACATGATACAGTTCTTACCTTGAAATCGATGACTGCAG
GGCTAAGTTTAACTGATGCTGTCCAGTTTTCGTAAGCATTTGTTGTAAGTCGGTTTCTAGCAAAGAAACTGAGAG
CACTAGCTGTATGAGACTGCCCACCAATCACATTCGATATAACTTCCTTAGCAGCCATTGTAAAACTTCCTAAAT
ATTAGTAAATAACGAATAATAATGTGTACATGACTCTGCTTATCAGTTAGATTGGGCGCGCCATCAGTTTAAACG
AATGCGGCCGC

9.3.3.5 vCRL hairpin

GCGATCGCTTACCGGTCATTTATAAAGATAGGCGTGAAAGGTACAGTAAAAATCAATATATTTATGTGGGGGTT
TATATTAATTTAAATATATAACATGCTTTACTATTGGTGGTGTTTCTAGTACAATAAAATCATGCATACATCTAC
GTGGAACAAATACAAAATTAGTAATAATACAATTACGTTGTTTAATAAACCATATGTACATAAGAAGAAACTGAG

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TTACACAGAATATTGAATACGTTTCTCAAACTTTAATTAAAATAAAATGGTTGTCTTGAAGACTCAATTTATACA
AACAATTTTAAACACGAACAAACAGATAAAATTAAAACCTGGGACATGCAAAGAATATTCAAAACAAAATTATGA
ACCAAAGAAACTGCTAAGAATTTGAAAGTAAACAAATTTCTTGTATAATGTTCAAGGTACAACACCTTCGCCATT
AGGACCGTCCGGCTGTGTATTCTTTTTCAAGTCTGGAATGGTTATATCTGGCTCTGGATCTTTTGTTCCCCAAGA
CAAGCAAAGTATTGAAAAAGCACAACAGCAAATCTCCGTACAAAAGATAGGAATCAGGAAGAATATGATGAGTGC
AACAACAATTATGCATCCAATAACAATGCCCGCTATAGCTCCATCAGATAGTGATTTACTTCTCGAACTTTGAAT
ACAGACAGGGTCACTGTCAAATGTAGCATCCTCCTGGCATGTTGCATTAACATCGTTCCCAGTATGATCAGTTGC
ACATGAATATTGAACTTTAATTATCATGGACAATGTGGTAACAACAGCTAAATCACAATTTACGGTAACTGTGCT
GTTGCTTGTCAATGAAGTAAGATATGTAAGAGTGGTCATAATGACTCCTGTTAGTCCTGTTCCTTCCCACGAACA
TGCAGTTGGGCTAGTTGATACTGTTGCAGTGATCATCAGGTTTGTGGAATCCCGCGGCCATGGCGGCCGGGAGCA
TGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGAC
TGGGAAAACCCTGGCGTTACCCGTACGGTTCAATATTCATGTGCAACTGATCATACTGGGAACGATGTTAATGCA
ACATGCCAGGAGGATGCTACATTTGACAGTGACCCTGTCTGTATTCAAAGTTCGAGAAGTAAATCACTATCTGAT
GGAGCTATAGCGGGCATTGTTATTGGATGCATAATTGTTGTTGCACTCATCATATTCTTCCTGATTCCTATCTTT
TGTACGGAGATTTGCTGTTGTGCTTTTTCAATACTTTGCTTGTCTTGGGGAACAAAAGATCCAGAGCCAGATATA
ACCATTCCAGACTTGAAAAAGAATACACAGCCGGACGGTCCTAATGGCGAAGGTGTTGTACCTTGAACATTATAC
AAGAAATTTGTTTACTTTCAAATTCTTAGCAGTTTCTTTGGTTCATAATTTTGTTTTGAATATTCTTTGCATGTT
CCAGGTTTTAATTTTATCTGTTTGTTCGTGTTTAAAATTGTTTGTATAAATTGAGTCTTCAAGACAACCATTTTA
TTTTAATTAAAGCTTGAGAAACGTATTCAATATTCTGTGTAACTCAGTTTCTTCTTATGTACATATGGTTTATTA
AACAACGTAATTGTATTATTACTAATTTTGTATTTGTTCCACGTAGATGTATGCATGATTTTATTGTACTAGAAA
CACCACCAATAGTAAAGCATGTTATATATTTAAATTAATATAAACCCCCCACATAAATATATTGATTTTTACTGT
ACCTTTCACGCCTATCTTTATAAATGGAATTCTTGCGATCGC
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9.3.3.6 Control *Hydra* MyD88 hairpin

```
GCGATCGCGTTTTAAAATTCTGGGCATTTCACACTTTTTATACAATATTGGTATCACTTTGTTCTTATTAAATTC
AAATGATAAAGATAATGCAATTTTAGCTTGTGTTCGACAGTTATTTGATTTGTTGAAATTAGGAGATAAAATAAC
TATAACTTTCCTGCATTTTTCTTCAATAACACTGCATAACTGCTCAAAGGGATTGTACCCAGGCAAAAAATCACA
ATAATCAATACAAACTTTATACCCATATTCATTCTCTAAACGATTCTTAATTAACTTAGCAAAATTTATATCATC
TTTTGCATATGATATAAAGACGTCATAATTATCATTGAGTGTAAAATTACTAACATGAGTTATGGAGCATTGCAA
ATCTTGATTGTCATTTTCAATAAAATTGTTATCATTAATCGTACGAAACCAATGGATTGCATTAATAAAGATCAA
TTACTTTATCAGTCTCTGTCATATCCTATAATGAGTCAAGTTTCATTTTTACTTAATCCTATTTGTGATAATAAA
GATTGGAGAGCCTTAGCTGGTGTATTTGATTTTACGATGGAAGAAGTGAATGGTTTGTTATCTGCTTGTGACCCA
ACAATGGAGTTATTTAAATATTATGAGTTGAAATATGGCAAAAAAGTTACATTATCATATGTTATAGAATCATTA
AATAATATTGGAAGAAAAGATGTTGTACAAGATATTCTTAAATACATTAATGATAACAATTTTATTGAAAATGAC
AATCAAGATTTGCAATGCTCCATAACTCATGTTAGTAATTTTACACTCAATGATAATTATGACGTCTTTATATCA
TATGCAAAAGATGATATAAATTTTGCTAAGTTAATTAAGAATCGTTTAGAGAATGAATATGGGTATAAAGTTTGT
ATTGATTATTGTGATTTTTTGCCTGGGTACAATCCCTTTGAGCAGTTATGCAGTGTTATTGAAGAAAAATGCAGG
AAAGTTATAGTTATTTTATCTCCTAATTTCAACAAATCAAATAACTGTCGAACACAAGCTAAAATTGCATTATCT
TTATCATTTGAATTTAATAAGAACAAAGTGATACCAATATTGTATAAAAAGTGTGAAATGCCCAGAATTTTAAAA
CGAATTC
```

9.3.3.7 Adapter for integration of control *Hydra* MyD88 hairpin

```
up:      CGCGTAAGACGAATTCTACAT
down: TAGCGCATTCTGCTTAAGATG
```

The adapter is integrated by ligation in *Asis*I digested vector. As result only one *Asis*I cutting site remains intact and an *Eco*RI site is introduced facilitating directional cloning.

9.4 si-RNAs

vCRL-A

```
Target:                 5' AAATTGAGTCTTCAAGACAAC   3'
Sense siRNA strand:     5'   AUUGAGUCUUCAAGACAACUU 3'
Antisense siRNA strand: 5' UUUAACUCAGAAGUUCUGUUG   3'
```

```
vCRL-B
Target:                  5′ AAACACCACCAATAGTAAAGC   3′
Sense siRNA strand:      5′   ACACCACCAAUAGUAAAGCUU 3′
Antisense siRNA strand:  5′ UUUGUGGUGGUUAUCAUUUCG   3′

control
Target:                  5′ AAATCGCTCTAATAGATGTCC   3′
Sense siRNA strand:      5′   AUCGCUCUAAUAGAUGUCCUU 3′
Antisense siRNA strand:  5′ UUUAGCGAGAUUAUCUACAGG   3′
```

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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.

Kiel, den 22.02.2010

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Felix Sommer