Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

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born in:	Darmstadt, Germany
Oral-examination:	3rd December, 2013

Molecular Factors of Nematocyst Morphogenesis and Discharge in the Freshwater Polyp Hydra

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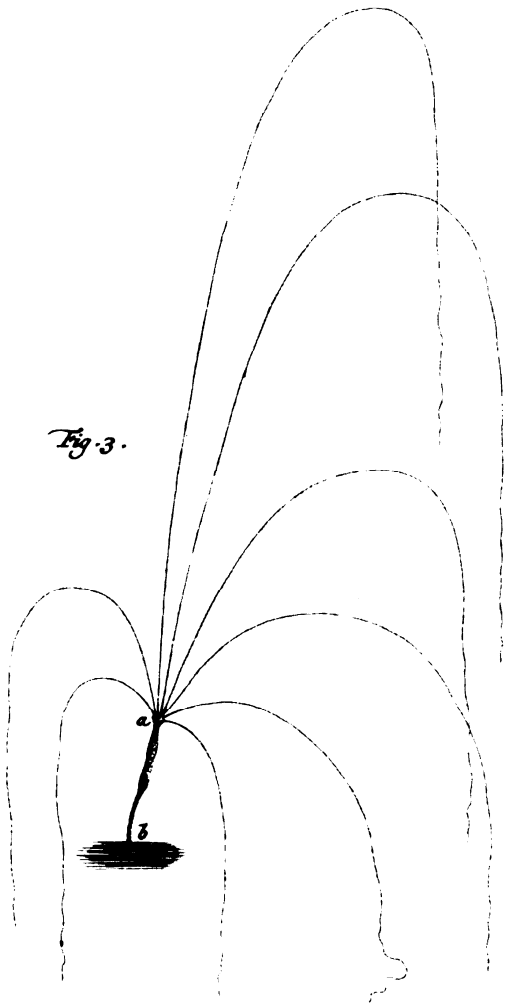
Comme il m'a paru, dès que j'ai commencé à observer les Polypes, que la connaissance des propriétés remarquables, qui se trouvent dans ces Animaux, pouvoit faire plaisir aux Curieux & contribuer en quelque chose aux progrès de l'Histoire Naturelle.

It seemed to me from the start of my observations that knowledge of the remarkable properties of the polyps could bring pleasure to the inquisitive and contribute something to the progress of natural history.

Abraham Trembley, 1744



A. Trembley (1744)
Mémoires, pour servir à l'histoire d'un genre de polypes d'eau douce, à bras en forme de cornes.

PARTS OF THIS THESIS HAVE BEEN COMMUNICATED OR PUBLISHED:

Poster presentations:

Beckmann, A., Balasubramanian, P.G. and Özbek, S. CNIDOIN – A NOVEL ELASTIC PROTEIN FROM HYDRA WITH HOMOLOGY TO SPIDER SILK. COS Labday, Heidelberg (2010).

Beckmann, A. and Özbek, S. CNIDOIN – A NOVEL ELASTIC PROTEIN FROM HYDRA EXPRESSED IN NEMATOCYTES. International COS Heidelberg Symposium (2012).

Beckmann, A., Ziegler, B., Hess, M. and Özbek, S. THE FUNCTION OF TRP CHANNELS AND MECHANORECEPTION IN HYDRA. International COS Heidelberg Symposium (2013).

Beckmann, A., Ziegler, B., Hess, M. and Özbek, S. THE FUNCTION OF TRP CHANNELS AND MECHANORECEPTION IN HYDRA. International Workshop "Unravelling the Developmental Regulatory Network in Early Animals", Tutzing (2013).

Talks:

CNIDOIN – A NOVEL ELASTIC PROTEIN FROM HYDRA EXPRESSED IN NEMATOCYTES. International Workshop Tutzing (2011).

CNIDOIN – A NOVEL ELASTIC PROTEIN FROM HYDRA EXPRESSED IN NEMATOCYTES. Systematic Approaches in Organismal Biology, Heidelberg (2012).

TRP CHANNELS AND MECHANOSENSATION IN HYDRA. Physiologisches Kolloquium, RWTH Aachen (2013).

Publications:

Beckmann, A. and Özbek, S. (2012) THE NEMATOCYST: A MOLECULAR MAP OF THE CNIDARIAN STINGING ORGANELLE. International Journal of Developmental Biology *56* 577-582.

Balasubramanian, P.G., Beckmann, A., Warnken, U., Schnölzer, M., Schüler, A., Bornberg-Bauer, E., Holstein, T.W. and Özbek, S. (2012) PROTEOME OF HYDRA NEMATOCYST. Journal of Biological Chemistry *287* (13), 9672-9681.

Beckmann, A., Xiao, S., Nüchter, T., Kröger, N., Langhojer, F. Petrich, W., Gräter, F., Holstein, T. and Özbek, S. A FAST RECOILING SILK-LIKE ELASTOMER FACILITATES NANOSECOND NEMATOCYST DISCHARGE. Submitted to BMC Biology. In revision.

ABSTRACT

Stinging cells or nematocytes are specialized cells that are unique to Cnidarians. They contain a highly sophisticated organelle, the nematocyst, used for locomotion, defence and capture of prey. The proteome of the nematocyst has provided unique insights into its molecular organisation. The goal of my thesis was to investigate novel molecular factors involved in aspects of nematocyst morphogenesis, structure and function.

A critical part of nematocyst morphogenesis is the initiation of tubule formation by a constriction of the Golgi vesicle membrane. Nematomyosin, a newly identified non-muscle myosin type II, in the present thesis is shown to localize to a collar around the outgrowing tubule indicating an essential role in this process. Blocking of myosin II activity by Blebbistatin leads to malformed nematocyst vesicles. Tubule size control is probably facilitated by a PKD2 channel, shown to be active at the point of maximal tubule outgrowth.

The nematocyst structure has been characterized by stiff and tear-resistant minicollagens, although the discharge process is accompanied by extreme volume changes of the capsule. Here, I have characterized the novel elastic protein Cnidoin that shares structural homology with the spider silk protein Spidroin-2. Cnidoin is expressed in developing nematocytes and locates to wall and tubule structures. Recombinant Cnidoin showed a high tendency to aggregate and to form linear fibres. Cnidoin thus behaves as a typical elastic protein. Being an integral part of the mature nematocyst it could provide the molecular basis for the energy stored that is released in the ultrafast discharge process.

The discharge of nematocysts is triggered by chemical and mechanical stimuli that are detected by the cnidocil at the apical end of the nematocyte. The cnidocil is surrounded by a set of stereocilia, providing a similar arrangement as vertebrate hair cells. A newly identified calcium channel of the TrpA family is shown to locate to stereocilia of the Hydra cnidocil apparatus. The protein can be visualized by immunostainings during developmental stages as well as in mature capsules and thus represents a candidate for mechanosensation during discharge. Nematocalcin, a penta-EF-hand protein, was also located to the stereocilia, but at a more basal position than TrpA, where it might act as a modulatory factor associated with the mechanosensory apparatus.

ZUSAMMENFASSUNG

Nesselzellen oder Nematocyten sind spezialisierte Zellen, die nur bei den Cnidariern vorkommen. Sie besitzen eine hochentwickelte Organelle, die Nematocyste, die zur Fortbewegung, zur Abwehr und zum Beutefang eingesetzt wird. Das Proteom der Nematocyste lieferte einzigartige Einblicke in deren molekulare Organisation. Das Ziel meiner Doktorarbeit war es neue molekulare Faktoren, die an Prozessen der Nematocysten Morphogenese, Struktur und Funktion beteiligt sind, zu untersuchen.

Ein wichtiger Punkt der Nematocysten Morphogenese ist die Ausbildung des Schlauches durch ein Zusammenziehen der Golgivesikel-Membran. Nematomyosin, ein in dieser Arbeit neu identifiziertes Nicht-Muskel Myosin der Klasse II, ist in einem Kragen um den auswachsenden Schlauch herum angeordnet. Dies deutet auf eine essentielle Rolle von Nematomyosin bei diesem Prozess hin. Die Hemmung der Myosin II Aktivität durch Blebbistatin führt zu deformierten Kapselvesikeln. Die Kontrolle der Schlauchlänge wird wahrscheinlich durch einen PKD2 Kanal vermittelt, der zum Zeitpunkt der maximalen Schlauchausdehnung aktiv ist.

Charakteristisch für die Struktur der Nematocysten sind die starren und reißfesten Minikollagen, der Entladungsprozess wird jedoch von ausgeprägten Volumenänderungen der Kapsel begleitet. In der vorliegenden Arbeit habe ich das neue elastische Protein Cnidoin untersucht, das strukturelle Ähnlichkeit zu dem Spinnenseidenprotein Spidroin-2 besitzt. Cnidoin wird während der Entwicklung der Nematocysten exprimiert, das Protein befindet sich in der Kapselwand und im Schlauch. Rekombinantes Cnidoin zeigt eine hohe Tendenz sich zusammenzulagern und Fäden auszubilden. Cnidoin verhält sich wie ein typisch elastisches Protein. Da es ein wesentlicher Bestandteil von reifen Nematocysten ist, könnte es die molekulare Grundlage für mechanisch gespeicherte Energie bilden, die im Entladungsprozess sehr schnell freigesetzt wird.

Die Entladung von Nematocysten wird durch chemische und mechanische Reize ausgelöst, die durch das Cnidocil am apikalen Ende der Nematocyte detektiert werden. Das Cnidocil wird von mehreren Stereocilien umgeben, ähnlich der Struktur von Haarzellen bei Wirbeltieren. Ein neu identifizierter Calciumkanal der TrpA-Familie konnte in den Stereocilien des Cnidocilapparates nachgewiesen werden. Das Protein konnte mit Hilfe von Antikörperfärbungen sowohl während der Entwicklungsphasen als auch in reifen Nesselzellen nachgewiesen werden. Daher ist es wahrscheinlich,

dass TrpA als Mechanosensor eine essentielle Rolle für die Kapselentladung spielt. Das Penta-EF-Hand Protein Nematocalcin ist ebenfalls in den Stereocilien zu finden, allerdings an einer basaleren Position als TrpA. Es könnte als modulierender Faktor mit dem mechanosensorischen Apparat assoziiert sein.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

RESULTS

DISCUSSION

LIST OF TABLES

1. INTRODUCTION

1.1 The phylum Cnidaria

Cnidarians are basal metazoans that form the sister group to all bilaterian animals (Figure 1.1). Corals, jellyfish and sea anemones belong to this phylum, however, not only seawater also freshwater organisms are found in the cnidarian clade. Among the four different classes of Cnidarians the Anthozoans are the most basal one. Corals and sea anemones, including the model organism *Nematostella vectensis*, belong to this class. *Hydra* groups into the Hydrozoans.

Cnidarians were the first animals to develop a nervous system, they possess a nervenet which is a simple nervous system that spans their whole body (Hadzi, 1909; Sakaguchi *et al.*, 1996). In *Hydra*, the mouth region called hypostome is surrounded by a ring of nerve cells and is the strongest innervated tissue. Thirty percent of all nerve cells are in the hypostome, the foot region is also rich in neurons (Bode *et al.*, 1973).

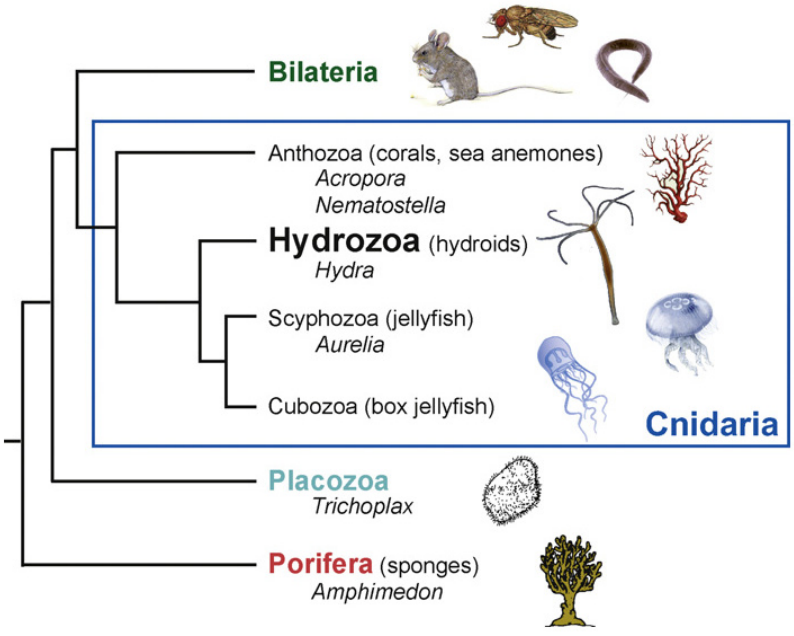


Figure 1.1: Phylogeny of metazoans with focus on Cnidarians (Augustin *et al.*, 2010).

The presence of stinging cells (Cnidocytes) is the unifying and name giving feature of Cnidarians. Those cells contain a highly specialized organelle, the cnida or cnidocyst, which can be classified into three types: nematocysts, spirocysts and ptychocysts. While nematocysts are found in all Cnidarians, the presence of spirocysts and ptychocysts is restricted to the anthozoan subclass Hexacorallia and the

hexacorallian order Ceriantharia respectively (Fautin, 2009). Spirocysts are used to mechanically immobilize prey and by the use of ptychocysts cerianthid anemones build the tube in which they live. Nematocysts are used for defence, locomotion and capture of prey. Among Cnidarians, a large variety of nematocyst types exists. They can be classified into at least 25 types (Weill, 1934; Östman, 2000; Kass-Simon & Scappaticci, 2002) and can be used as a phylogenetic marker (Reft & Daly, 2011).

1.2 *Hydra* as a model system

The freshwater polyp *Hydra* (Figure 1.2 A) has been extensively studied since the mid of the 18th century. Experiments investigating the life cycle, organization and regeneration of *Hydra* were initially performed by Abraham Trembley (Trembley, 1744). The body can be separated into the head with tentacles and mouth (hypostome), the body column and the foot that attaches the animal to the ground. Hydra has a simple diploblastic body plan and is radially symmetric (Figure 1.2 B).

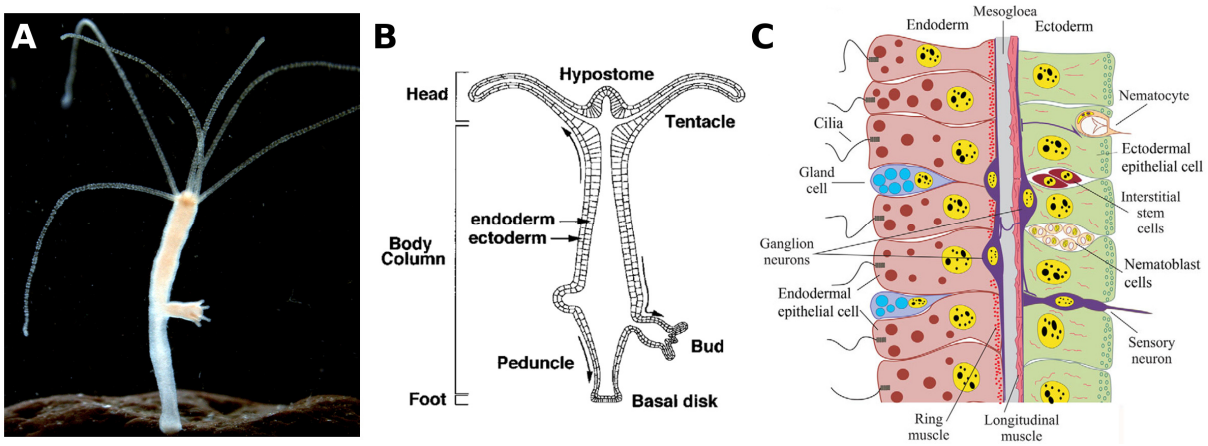


Figure 1.2: Anatomy of *Hydra*. A Photograph of *Hydra magnipapillata* (image by Dr. Melanie Mikosch and Prof. Dr. Thomas Holstein). B schematic representation of Hydra's body plan (Bode, 2001). C different cell types in *Hydra* (Technau & Steele, 2011).

The tissue consists of two cell-layers: the ecto- and the endoderm (Figure 1.2 C). The endoderm harbours secretory gland cells that release digestion enzymes into the gastric cavity. In the ectoderm components of the nervous system such as the sensory neurons and the nematocytes can be found. The two tissue layers are separated by a gel-like extracellular matrix, the mesogloea. The tissue of *Hydra* is highly dynamic. As indicated by arrows in Figure 1.2 B the tissue is in constant movement. It migrates from a region beneath the ring of tentacles by entering the tentacles or by being replaced down the body column (Campbell, 1967). While some

tissue is lost at the tips of the tentacles and the basal disk, the biggest portion is incorporated into newly forming buds. The rates of growth and tissue loss control the body size of *Hydra*. Hydra possesses three stem cell lineages. Endo- and ectodermal epitheliomuscular stem cells divide constantly to produce new epithelial tissue. The interstitial stem cell lineage (i-cells) gives rise to more specialized cell types like neurons, nematocytes, gland cells and germline cells (David & Murphy, 1977).

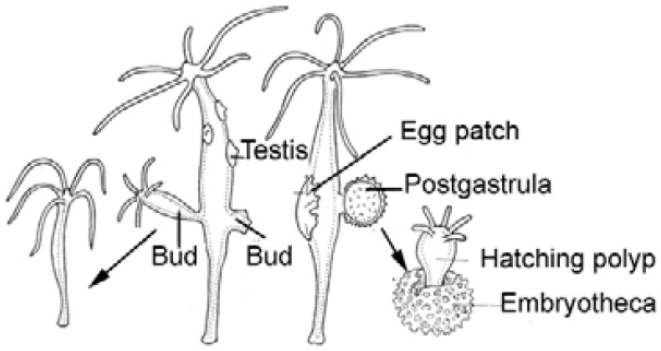


Figure 1.3: Life cycle of *Hydra* (Technau & Steele, 2011).

Hydra can, depending on environmental conditions, either reproduce by budding or sexually (Figure 1.3). Under unfavourable environmental conditions they form gametes on their body column. The sperm are released into the water while the eggs remain attached to the mother animal, even after fertilization. The embryo covers itself with a solid embryotheca that protects it from the environment. In this state, the animals can survive for a long time. Under favourable conditions, the animal hatches and grows out into an adult polyp. If environmental conditions are good, the polyps can reproduce quickly by budding. Tissue from the adult animal is in constant movement and is replaced to form a bud. After the bud developed mouth and tentacles, it separates from the mother animal, settles and grows.

The genomes of *Hydra* and *Nematostella* have recently been sequenced (Chapman *et al.*, 2010, Putnam *et al.*, 2007). With help of these two Cnidarian genomes, the basis for understanding general mechanisms in genome evolution is provided (Steele, 2012). The *Hydra* and *Nematostella* genomes were the first ones to be sequenced among Cnidarians. Their last common ancestor was the stem Cnidarian from which all Cnidarians originate. Both organisms are notably different although belonging to the same phylum (Table 1). The genome of *Hydra* is more than twice as large as the genome of *Nematostella* even though both organisms have a set of 30

diploid chromosomes and their genomes encode about 20 000 proteins. The *Hydra* genome is extremely rich in adenine and thymine whereas in Nematostella the bases occur in more equal amounts. Since the occurrence of the stem *Hydra*, a burst of transposable elements has been observed. Transposable elements might originate in horizontal gene transfer. Different life cycles, forms and environmental conditions lead to the loss as well as to the gain of specific genes. About 11 % of Hydra's and Nematostella's genes are thought to be lineage specific (Forêt *et al.*, 2010). Not only is the content of the genomes but also the regulation of gene expression an important issue. Apart from alternative splicing (occurs in *Hydra* as well as in *Nematostella*, Steele, 2012) and trans-splicing (not found in Anthozoans but in *Hydra*, Douris *et al.*, 2010) about 50 miRNAs are predicted from both genomes, in humans more than 600 are found and regulate the gene expression by binding to transcripts and mediating degradation.

Table 1: Comparison between *Hydra* (Chapman *et al.*, 2010) and *Nematostella* (Putnam *et al.*, 2007) genomic data. Table adapted from Steele, 2012. * 17 of the identified 51 non-tRNA non-rRNA RNAs were identified as miRNAs, this is probably an underestimation.

	Nematostella vectensis	***Hydra magnipapillata***
Genome size (Mbp)	357	900
Chromosome number	15 (1N)	15 (1N)
GC content	49 %	29 %
Protein-coding genes	~ 18 000	~ 20 000
Transposable elements (% genome)	26 %	57 %
miRNA	40	17 (51)*

The genome data offers a powerful tool for comparative developmental and evolutionary analyses at the molecular level.

1.3 Nematocytes in *Hydra*

An adult, well-fed *Hydra* contains 122 000 cells, 56 700 (46%) of which are nematocytes and nematoblasts, their developmental stages (Bode *et al.*, 1973). The tentacles of *Hydra* are densely packed with nematocytes (Figure 1.4 A), 91% of all cells in the tentacles are nematocytes (corresponds to 26 000, Bode *et al.*, 1973). *Hydra* possesses four different types of nematocysts (Figure 1.4 B). The largest ones are the stenoteles. They belong to the penetrant type of nematocysts and are used for prey capture. The stylets at the base of the tubule perforate the prey's integument and the tubule injects toxins into the prey organism. The holo- and the atrichous

isorhiza are glutinant capsules mainly used for locomotion. The smallest nematocytes are the volvents, the desmonemes. Their discharged tubule tightly coils around the prey and prevents it from escaping.

Although structurally very diverse, all nematocysts share the same general build: they consist all of a capsule body and an attached tubule, which is coiled tightly inside the cyst (Figure 1.4 C). In mature nematocytes the nematocyst capsule fills most of the cell.

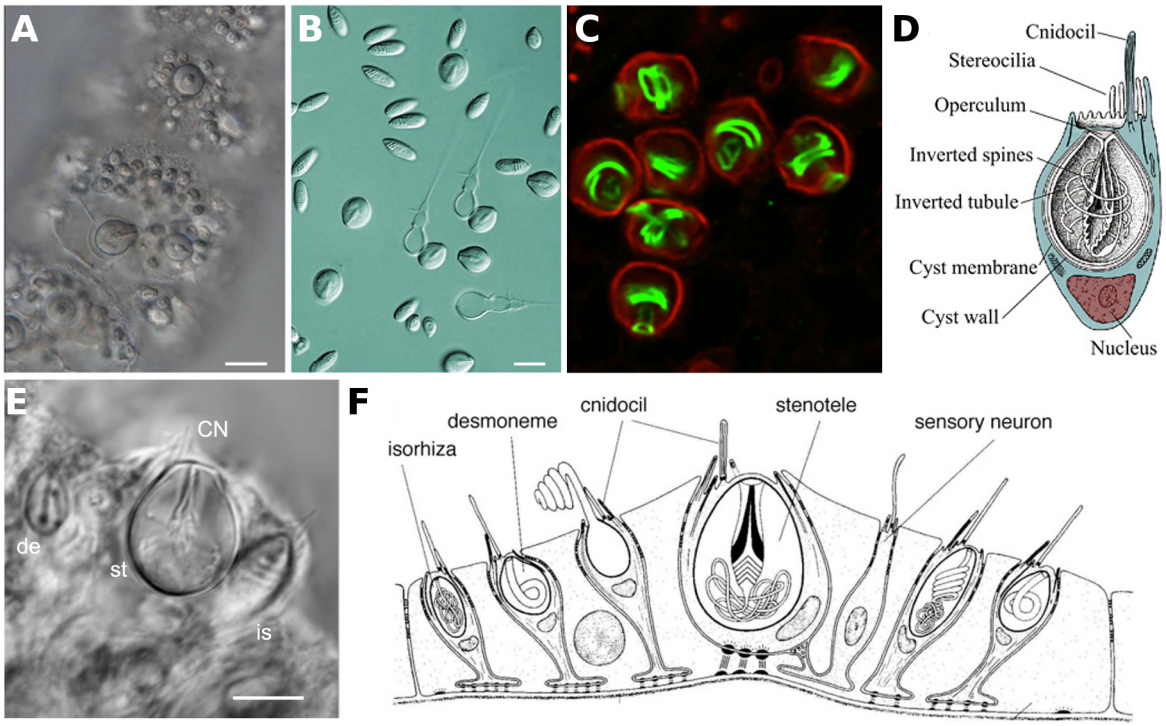


Figure 1.4: Nematocytes in *Hydra*. A view of a tentacle of *Hydra*, densely packed with nematocytes (Balasubramanian *et al.*, 2012). B isolated nematocysts. Different types are visible: the large stenoteles, the oval atrichous and holotrichous isorhiza and the small desmonemes (Balasubramanian *et al.*, 2012). C the general built of a nematocyst can be considered as a capsule body (red) and a coiled tubule inside (green). D schematic representation of a nematocyte (Anderson & Bouchard, 2009). E picture of a battery cell (Beckmann & Özbek, 2012). F schematic drawing of a battery cell (Holstein, 2012).

At the apical end of the nematocyte the cnidocil, a modified cilium, is located (Figure 1.4 D). Together with the surrounding stereocilia it serves as a mechanosensor. In the tentacles of *Hydra,* the nematocytes are arranged within so-called battery cells (Figure 1.4 E&F). The battery cells are modified epitheliomuscular cells, each containing about 20 nematocytes. They harbour one or two central stenoteles, surrounded by some isorhizas and ring of desmonemes as well as a sensory nerve cell (Hufnagel *et al.*, 1985). The nematocytes of one battery cell are connected by neurons and up to five different battery cells are interconnected by neurons (Yu *et al.*,

1985). The discharge of nematocytes is regulated by chemical and mechanical stimulation (Pantin, 1942; Hobmayer *et al.*, 1990; Oliver *et al.*, 2008).

1.4 The cnidocil apparatus

The mechanosensory cnidocil apparatus consists of different cytoskeletal elements that can be revealed upon detergent treatment (Figure 1.5 A). The cnidocil is build by a circular arrangement of 9 pairs of microtubules around a core with increasing amounts of microtubules along the length (Slautterback, 1967). It is encircled by stereocilia (also called outer microvilli, Hausmann & Holstein, 1985). The stereocilia are formed by parallel actin filaments and arise from the apical surface of the nematocyte in a semicircle. At their endings they converge and form a tubule around the central cnidocil (Figure 1.5 B, Hausmann & Holstein, 1985). A ring of small microvilli encircles the nematocyst and the cnidocil (Hausmann & Holstein, 1985), it extends to rods in the cytoplasm that belong to the microtubular basket. These microvilli are thought to anchor the stereocilia and the microtubular basket (Golz, 1994). The basket of microtubules surrounds the capsule and stabilises it inside the cell (Stidwill & Honegger, 1989). At the apical part of the capsule microtubular rods lead over to finer microtubular structures, the microtubules are located directly adjacent to the nematocyst membrane (Stidwill & Honegger, 1989).

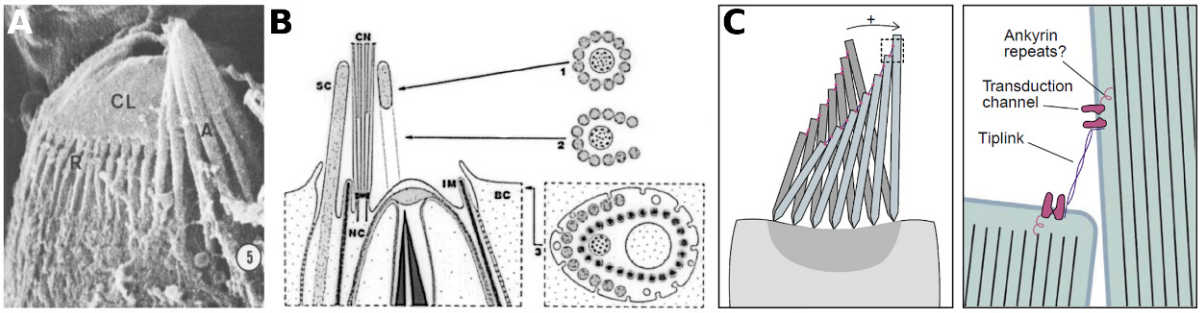


Figure 1.5: The Cnidocil apparatus. A scanning electron micrograph of a *Hydra* nematocyte after detergent extraction that reveals the cytoskeleton (Stidwill & Honegger, 1989) R= rods (microtubular basket that surrounds the capsule), A= actin filament bundles, CL= cuticular layer (electron dense material that covers the operculum). B schematic representation of the stereocilia arrangement in *Craspedacusta* (Hausmann & Holstein, 1985). SC= outer stereocilia, CN= Cnidocil, IM= inner microvilli and supportive rods, NC= nematocyte, BC = battery cell. C drawing of a hair cell. The magnification shows tip-links and the transduction channel (Lin & Corey, 2005).

The arrangement of the stereocilia is highly reminiscent of vertebrate hair cells, as they can be found in the inner ear or the lateral line for example. Deflection of these stereocilia into positive direction leads to stretching of tip-links, by which adjacent stereocilia are connected (Figure 1.5 C). The tip-links in turn are connected to

transduction channels. Upon deflection of the stereocilia, the mechanosensitive transduction channel opens. The resulting inward current depolarizes the cell and leads, in the case of inner ear hair cells, to an action potential. The stereocilia of the cnidocil apparatus have been shown to be connected to the cnidocil and to each other in the contact region (Golz & Thurm, 1991) so a similar mode of signal transduction as in hair cells can be assumed. The molecular nature of the links is unknown, in vertebrate hair cells cadherin23 (Siemens *et al.*, 2004) and protocadherin15 (Ahmed *et al.*, 2006; Kazmierczak *et al.*, 2007) form the tip links.

1.5 Nematocyst discharge

The tissue surface is covered by a mucous cuticle with the cnidocil membrane being the only one with direct contact to the surrounding medium (Golz, 1994). Deflection of the cnidocil leads to a depolarization of the cell and the opening of calcium channels. Calcium is essential for the start of the discharge process (Gitter *et al.*, 1994). The influx of water induces swelling of the capsule and increases the osmotic pressure, the stylets and tubule are expelled with an extreme acceleration of up to 5.410.000 g (Holstein & Tardent, 1984; Nüchter *et al.*, 2006), which is higher than the acceleration of gun bullets and one of the fastest biological processes known. The stylet reaches an average velocity of 9.3–18.6 m/s.

The actual discharge can be considered as an exocytotic process (Özbek *et al.*, 2009) as the membrane that surrounds the nematocyst capsule has to fuse with the nematocyte membrane. The capsules are loaded during maturation with poly-γ-glutamate (Szczepanek *et al.*, 2002). The negative charges inside the capsules are complexed by cations, it could be shown that most nematocysts are extraordinarily rich in calcium (Lubbock *et al.*, 1981). Upon contact of the membrane with the surrounding medium the calcium is lost from the capsule, this enhances the osmotic pressure further (Lubbock *et al.*, 1981). The increased osmotic pressure leads to the influx of water and the tubule everts explosively. First, the stylets eject and perforate the prey's shell. Afterwards, the spines fold back and the tubule is expelled. The hydrostatic pressure thus leads to the discharge of the capsule; in addition it facilitates the injection of toxins through the hollow tubule into the prey organism (Figure 1.6).

However, internal calcium is not alone responsible for the mechanism of discharge. It has been demonstrated that the speed of the discharge process itself depends on extracellular calcium (Watson & Hessinger, 1994; Nüchter *et al.*, 2006), it can even

be inhibited by inferior concentrations of calcium as well as by blockers of mechanosensitive ion channels like the lanthanide Gadolinium (Salleo *et al.*, 1994, Gitter *et al.*, 1994). Although the osmotic pressure is without a doubt one of the driving forces of the slower phases of discharge, it can not provide all the energy needed for the extremely fast initiation (Holstein & Tardent, 1984), therefore mechanically stored energy is assumed to be present in the capsule.
After the discharge, the volume of the nematocyst is reduced to 50% (Holstein & Tardent, 1984). The whole discharge process is completed in less than 3 milliseconds (Holstein & Tardent, 1984), the initial ejection of the spines takes as little as 700 nanoseconds, while the following evagination of the tubule follows a slightly slower kinetic (Nüchter *et al.*, 2006).

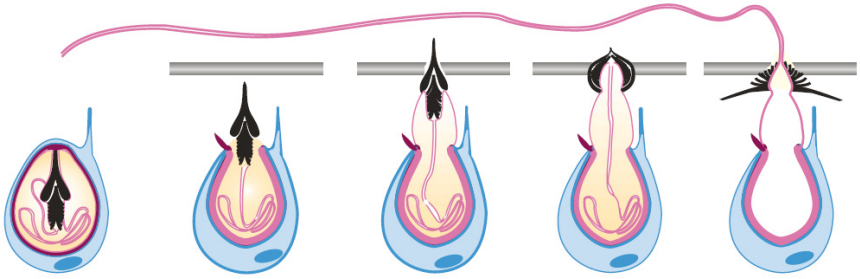


Figure 1.6: Schematic illustration of the discharge of a stenotele (Nüchter *et al.*, 2006).

The discharge is highly regulated, as discharged capsules cannot be reused but have to be replaced and the costs for their synthesis are very high. The animal is able to distinguish between organisms that are not attacked and prey organisms as each organism produces a characteristic frequency of mechanical stimuli by its swimming behaviour. Other cnidarians like anemones have been shown to distinguish between their own and foreign tissue (self non-self recognition; Hidaka *et al.*, 1997; Lubbock & Shelton, 1981). Chemical stimuli, derived from prey organisms, enhance the rate of discharge (Watson & Hessinger, 1989). Optimal discharge is achieved after a combination of chemical and mechanical stimuli (Pantin, 1942).

1.6 Nematocyst development

Nematocytes in *Hydra* arise from the i-cell lineage (Figure 1.7 A), a stem cell population located in the interstitial space of ectodermal epithelial cells. Those are exclusively located in the body column, so that the nematocyte development is restricted to the body column as well. During their development nematocytes migrate through the body column of *Hydra*.

After division of the i-cells, nests of 2-32 nematocytes develop (Slautterback & Fawcett, 1959; Holstein, 1981). The nematocytes are still connected by cytoplasmic bridges (Slautterback & Fawcett, 1959) and all cells of one nest become nematocytes of one type. The nematocyst is built as a self-organized secretion product in a giant post-Golgi vesicle (for details see Beckmann & Özbek, 2012).

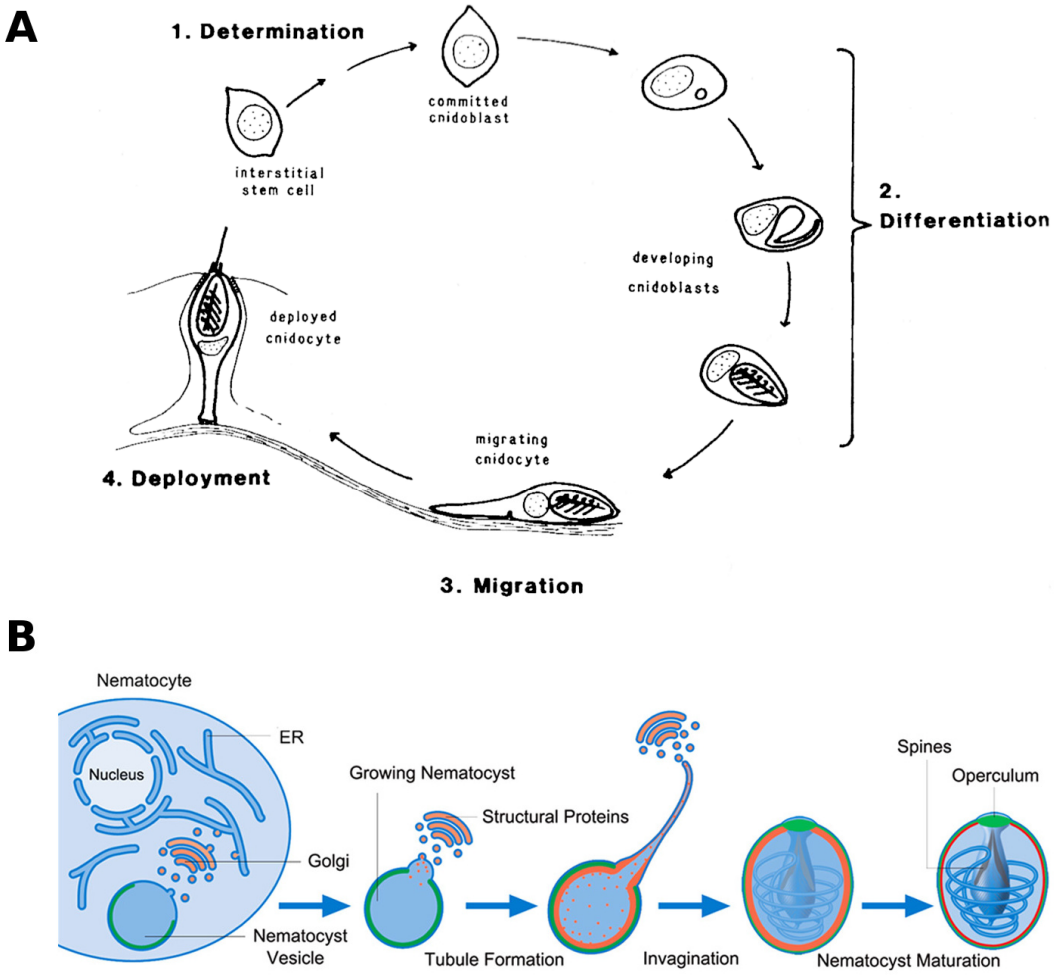


Figure 1.7: Nematocyst development A The life of a nematocyte from the determined i-cell to the mature nematocyte (Campbell, 1988). B Nematocyst development inside a single nematocyte (Beckmann & Özbek, 2012).

Initially the capsule wall is produced, it consists of an outer electron dense (the sklera) and an inner electron lucent layer (the propria, Holstein, 1981). The tubule starts to grow on the outside of the capsule primordium (Figure 1.7 B). After reaching its final length, it is invaginated and coiled up in the capsule matrix (Holstein, 1981). After the invagination process, the formation of spines takes place (Koch *et al.*, 1998) and the wall thins and hardens as a result of disulfide bond cross-linking (Watson & Mariscal, 1984b, Engel *et al.*, 2001). Poly-γ-glutamate synthesis starts after

invagination of the tubule. Afterwards the nest breaks apart and the capsules migrate into the tentacles. Due to ongoing glutamate synthesis, the nematocysts swell as the osmotic pressure increases (Szczepanek *et al.*, 2002). In mature capsules a pressure of 150 bar is achieved (Weber, 1989). Mature nematocytes are finally deployed into the battery cells in the tentacles.

1.7 Aim of my work

The elucidation of the capsule proteome (Balasubramanian *et al.*, 2012) revealed not only interesting general features of the nematocyst, like its structural composition and venom content, but also offered the possibility to investigate its biochemical and physiological properties in more detail by focusing on molecular factors that exhibit specific functions. My work concentrated in detail on three aspects of nematocyst biology: the molecular basis of (1) its elastic properties, (2) the discharge control and (3) the regulation of the nematocyst development.

2. RESULTS

2.1 Cnidoin – the elastic component of the nematocyst

The nematocyst contains many proteins with ECM motifs (Balasubramanian *et al.*, 2012), and can therefore be considered as "a miniature extracellular matrix within a secretory vesicle" (Özbek, 2010). Collagenous components such as the minicollagens form the stiff and tear-resistant structure of the capsule wall and tubule (Figure 2.1 A). During maturation as well as during the discharge process, the nematocyst undergoes severe volume changes (Figure 2.1 B) (Szczepanek *et al.*, 2002; Holstein & Tardent, 1984; Nüchter *et al.*, 2006) and the mature capsule is charged with an extreme pressure of 150 bar (Weber, 1989). The discharge process is one of the fastest biological processes and is completed in milliseconds (Holstein & Tardent, 1984; Nüchter *et al.*, 2006). A purely rigid capsule structure would not support these biomechanical requirements. It was found that all capsule types react in a similar way to osmotic conditions by swelling, shrinking or discharge (Weber, 1989), so the underlying molecular principles are supposed to be the same for all capsule types.

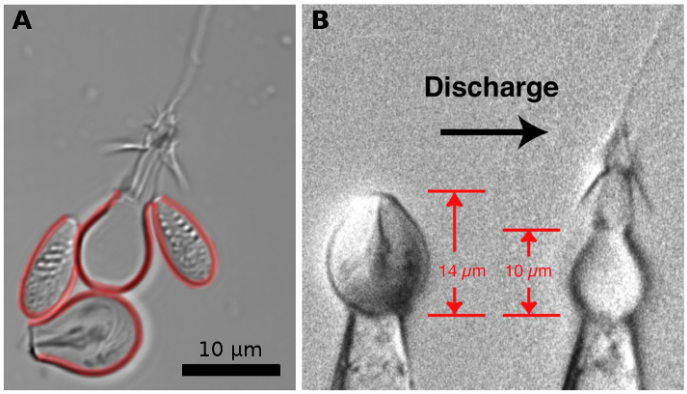


Figure 2.1: Structure and dynamics of a nematocyst. A isolated nematocysts with highlighted capsule wall. B Decrease in volume during discharge (modified after Beckmann *et al.*, in revision).

During the analysis of the capsule proteome, a candidate protein was found that might fulfil the required elastic function due to its possession of an extended glycine-rich repeat domain. It was named Cnidoin because of its similarity to the spider silk protein Spidroin-2 (Hinman & Lewis, 1992). The corresponding gene has been cloned from cDNA (sequences are provided in the attachments). The sequence reveals the presence of a signalpeptide as well as a predicted propeptide. Propeptides in

nematocyte-specific proteins are usually cleaved after a conserved di-basic lysine-arginine motif (Anderluh *et al.*, 2000). The predicted propeptide sequence for Cnidoin is unusually long, comprising 90 amino acid residues. The mature protein contains three minicollagen cysteine rich domains (CRDs, Figure 2.2 A), one at the N-terminus and two as tandem on the C-terminus. CRDs can interlink structural proteins by disulfide bonds. The primary sequence of the mature protein contains 403 amino acids and has a calculated molecular mass of 41.44 kDa. The largest part of Cnidoin is formed by a central repetitive sequence that is similar to the spider silk protein Spidroin-2 (Hinman & Lewis, 1992). Both proteins do even share the same repetitive motif (GxGQQ, Figure 2.2 B). The repetitive sequence in total is extremely rich in glycine, glutamine and methionine (Figure 2.2 C).

Spider silk is tear-resistant and elastic at the same time. This is achieved by the combination of crystalline poly-alanine domains and the elastic glycine- and glutamine-rich sequence (Römer & Scheibel, 2008). Extended stretches of alanine are missing in Cnidoin. Tear resistance could thus be provided by crosslinking the protein to other structural components of the nematocyst like minicollagens.

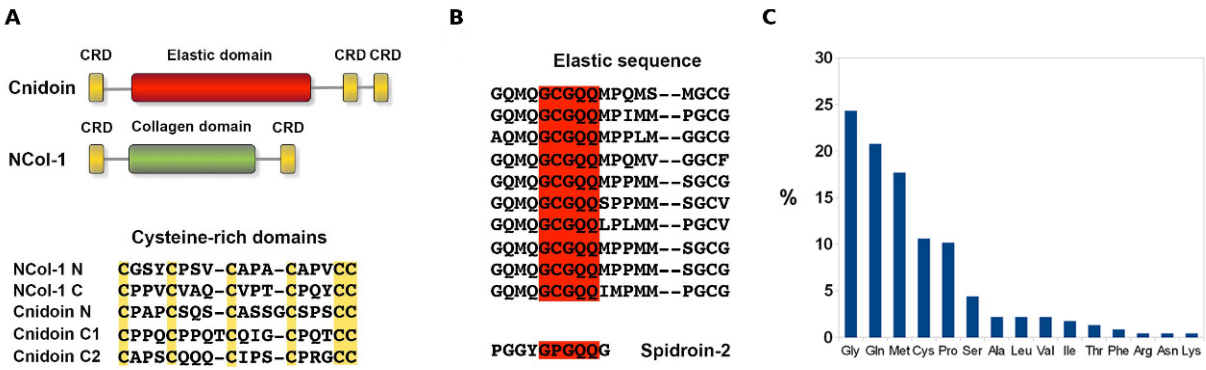


Figure 2.2: Sequence features of Cnidoin. A schematic representation of Cnidoin and Minicollagen-1 and alignment of the CRD domains of both proteins. B alignment of the repetitive domain of Cnidoin and Spidroin-2. C Amino acid composition of the elastic domain of Cnidoin. Modified after Beckmann *et al.*, in revision.

The expression pattern of Cnidoin was addressed by in situ hybridization experiments. Cnidoin was found to be expressed in the body column of *Hydra*, while the foot and head region as well as the tentacles were free of any signal (Figure 2.3 A). The expression is restricted to nests of developing nematocytes (Figure 2.3 B), which could be confirmed by double in situ hybridization using a Minicollagen-1 probe (Figure 2.3 C). Minicollagen-1 is a well-defined nematocyst wall protein (Engel *et al.*, 2001). The expression pattern of Cnidoin and Minicollagen-1 overlap (Figure 2.3 D).

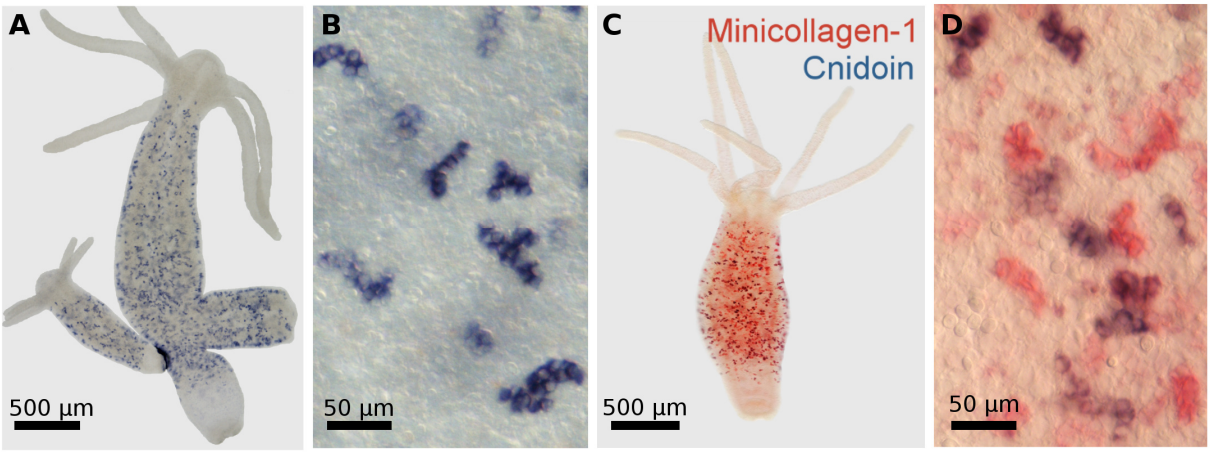


Figure 2.3: Expression pattern of Cnidoin. A the in situ hybridization shows expression of Cnidoin in the body column. B magnification of A, nests of developing nematocytes are stained. C double in situ for Minicollagen-1 (red) and Cnidoin (blue). D magnification of C, the signals for both transcripts overlap. Modified after Beckmann *et al.*, in revision.

To localize Cnidoin on the cellular level, a polyclonal antibody against a CRD epitope was raised. Cnidoin could be detected in nests of developing nematocytes in the body column. The head, foot and the tentacle regions were free of signal (Figure 2.4 A). This pattern is very reminiscent of other structural capsule proteins as Minicollagen-1 or -15 (Engel *et al.*, 2001; Adamczyk *et al.*, 2008) and confirmed the findings by in situ hybridization. The proteins can only be visualized by immunostainings in developmental stages. Later on, the capsules undergo massive wall compaction during maturation and individual structural components cannot be detected any longer. Depending on the fixation method either the wall or the tubule of developing nematocytes was stained (Figure 2.4 B - F). The detected structures could be co-stained by the corresponding structural minicollagens NCol-1 and NCol-15.

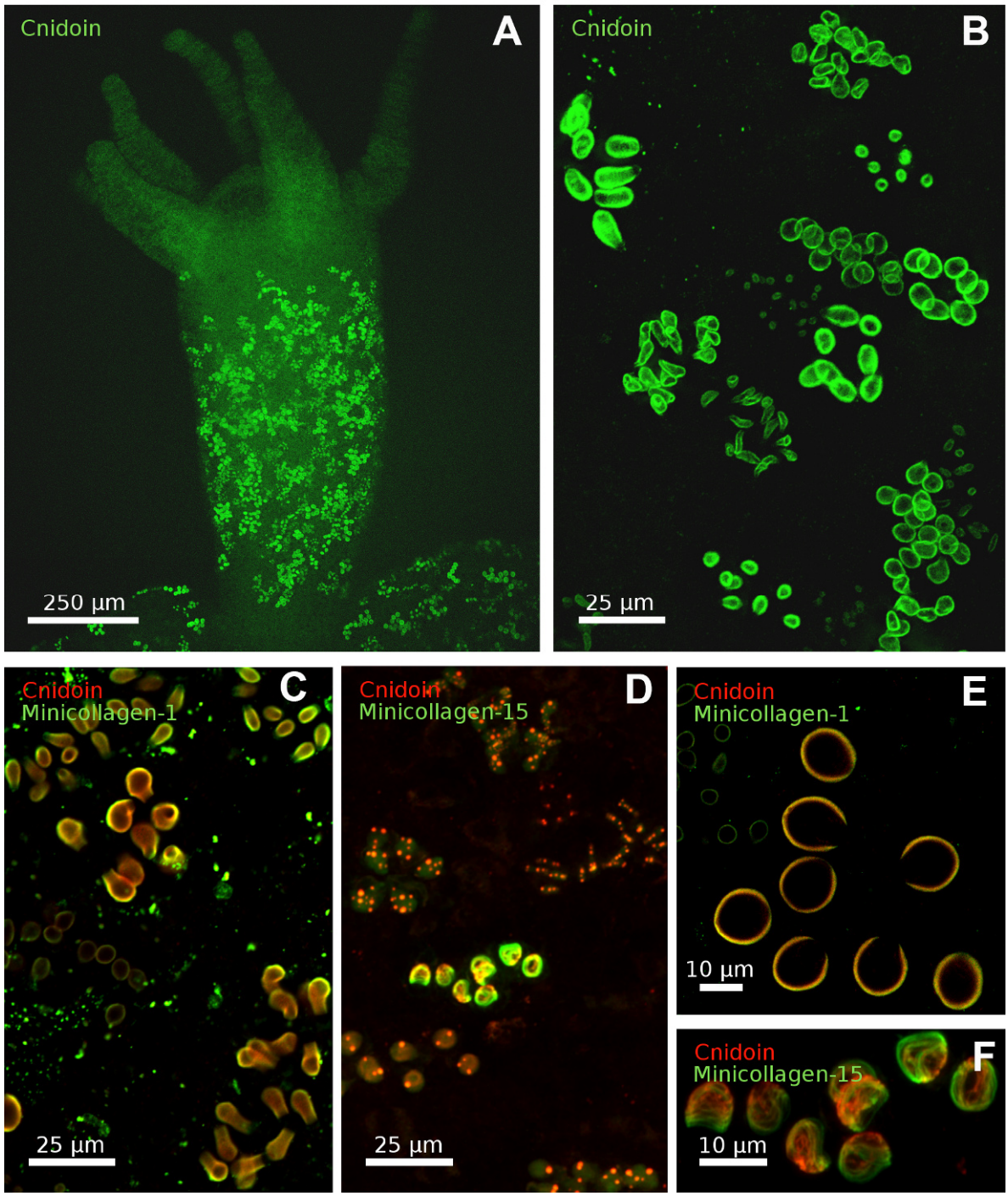


Figure 2.4: Immunostaining of Cnidoin. A overview, B magnification, C co-staining with NCol-1, D co-staining with NCol-15, E close-up of a nest with NCol-1 co-staining, F co-staining of a nest with NCol-15 co-staining. A – C, E PFA-fixed. D, F Lavdovsky-fixed. Modified after Beckmann *et al.*, in revision.

In *Nematostella*, the antibody gave also a distinct signal in nematocytes in the tentacles (Figure 2.5). In anthozoans the pattern of nematocyst development differs from the one described for *Hydra*, the development takes place in the tentacles directly. Spirocysts were detected primarily (Figure 2.5 B). Despite this finding, no homolog protein could be detected by BLAST searches indicating high diversity at

the sequence level.

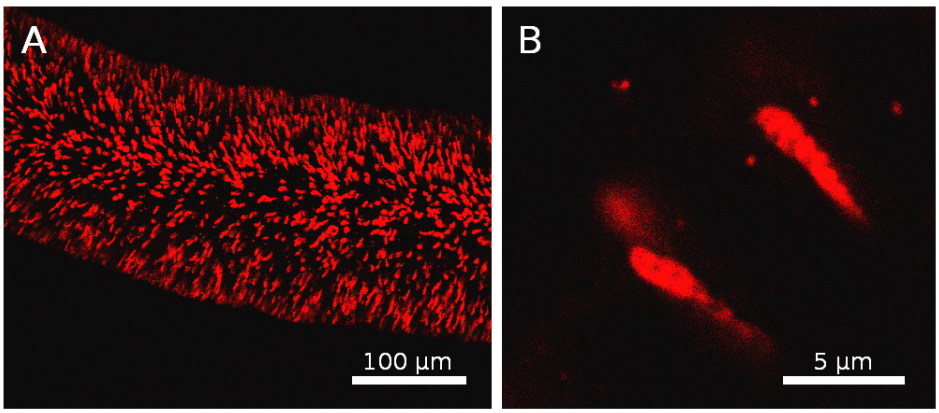


Figure 2.5: Cnidoin in *Nematostella*. A overview of a tentacle stained for Cnidoin. B magnification, showing labelled spirocysts.

In Western Blot analysis Cnidoin could be detected in whole Hydra lysate, as well as in isolated nematocysts. Even after extensive washing with SDS, Cnidoin was still present in the insoluble fraction, designated as nematocyst ghosts (Figure 2.6 A). Cnidoin could only be detected under reducing conditions indicating incorporation into the capsule polymer by disulfide bonds.

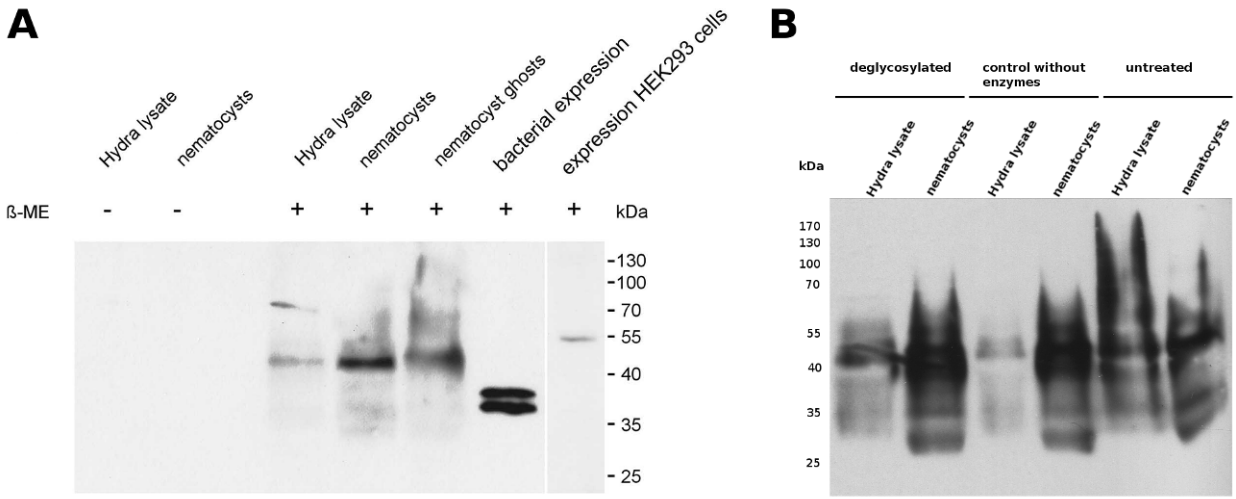


Figure 2.6: Western Blot analysis of Cnidoin. A Detection of Cnidoin in Hydra and nematocyst lysate as well as recombinant protein. B Deglycosylationassay. Modified after Beckmann *et al.*, in revision.

Recombinant protein was produced in HEK293 cells as well as in bacteria. Both proteins were His-tagged and purified using Ni-NTA. The protein produced in HEK293 cells included the putative propeptide and had a calculated molecular weight of 53 kDa. This corresponded well with the apparent molecular weight in SDS-PAGE analysis indicating that a cleavage of the propeptide does not occur in HEK293 cells.

The detected molecular weight in *Hydra* is lower and corresponds with the calculated molecular weight lacking the propeptide. Although unusually long, the propeptide seems to be normally cleaved in *Hydra*. The bacterially produced protein showed indications of unspecific proteolysis probably caused by the denaturing purification procedure.

Cnidoin contains no N-glycosylation but seven O-glycosylation sites. To test whether Cnidoin is posttranslationally modified, Hydra samples and nematocyst lysate were treated with a deglycosylation enzyme mix that is supposed to remove N- as well as O-glycosylations. No shift in molecular weight could be observed after treatment (Figure 2.6 B), excluding post-translational modifications by sugars.

The presence of CRD domains in both termini of Cnidoin could indicate a linkage of the protein to the disulfide network of the capsule wall and tubule. This hypothesis is supported by the fact, that Cnidoin can only be detected by Western Blot analysis under reducing conditions. To investigate whether polymerization is an intrinsic feature of Cnidoin, recombinant protein has been treated with reduced glutathione. In the presence of reduced glutathione, recombinant bacterial Cnidoin monomers showed a strong tendency to polymerize (Figure 2.7 A).

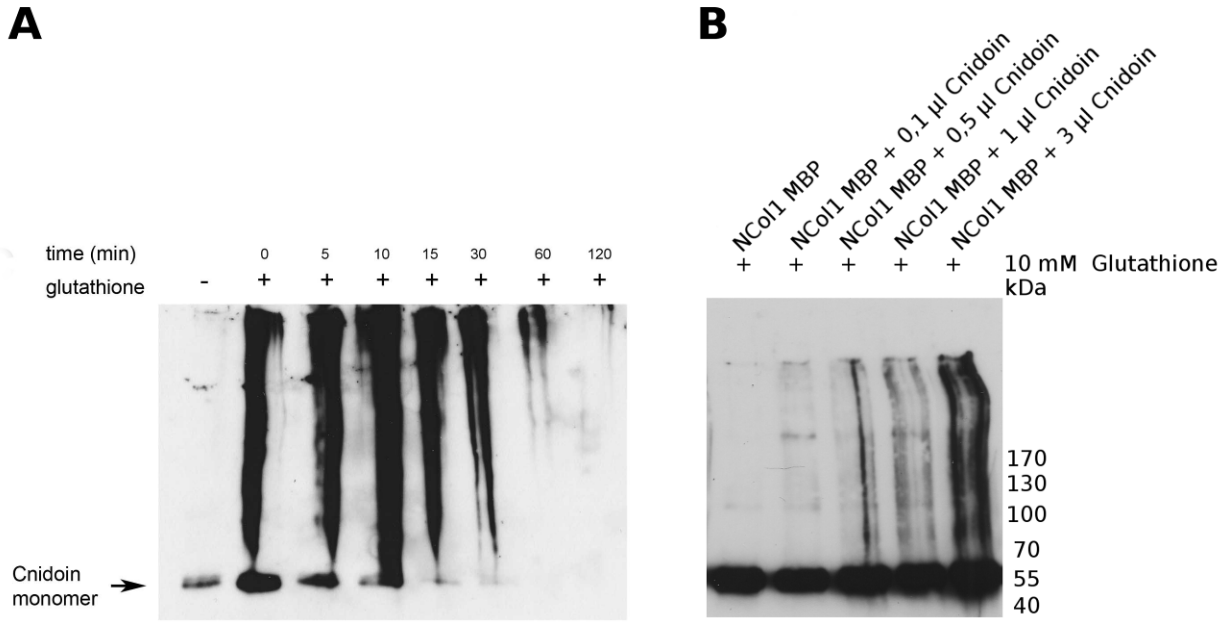


Figure 2.7: Polymerization of Cnidoin in the presence of reduced glutathione. A time dependent polymerization of Cnidoin. B increasing amounts of Cnidoin enhance the polymerization in a Cnidoin – NCol-1 mixture. Modified after Beckmann *et al.*, in revision.

The rate of recombinant NCol-1 MBP polymerization could be enhanced by the addition of increasing amounts of Cnidoin. This provides an indirect evidence for the

interaction of both proteins and the formation of a co-polymer by disulfide bonds via the CRDs (Figure 2.7 B).

The bacterial recombinant protein could only be purified under denaturing conditions with 8M urea, otherwise it immediately aggregated to sheet-like structures. Even in 8M Urea it was not stable. Frozen samples that were brought back to room temperature aggregated quickly and formed prominent macroscopic fibres (Figure 2.8). This coacervation process is also reported for other elastic proteins like elastin (Bellingham *et al.*, 2001). The fibres were unstructured and did not consist of regular unit fibres, even at high magnifications in the electron microscope (Figure 2.8 D).

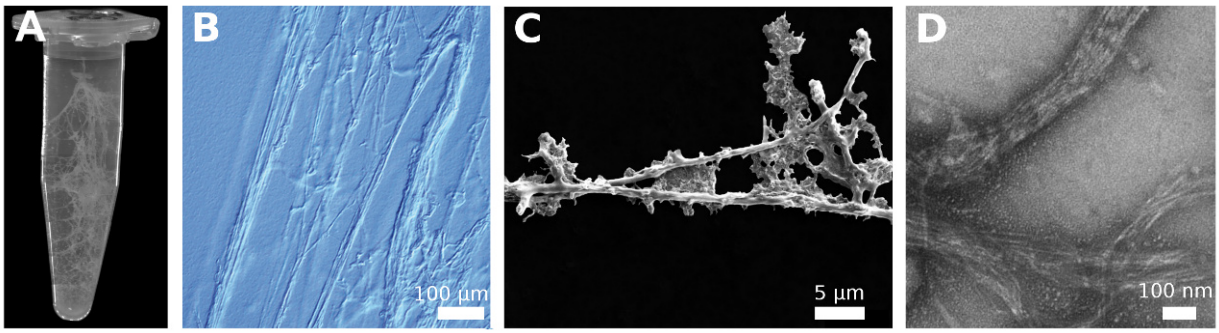


Figure 2.8: recombinant Cnidoin. A reaction tube after coacervation. B lighmicroscopic picture of fibres (modified after Balasubramanian *et al.*, 2012). C scanning electron micrograph of fibres. D negative stained fibres in transmission electron micrograph (modified after Beckmann *et al.*, in revision).

The properties of recombinant Cnidoin are similar to the ones reported for other elastic proteins. To determine the elasticity of Cnidoin molecular dynamics (MD) simulations have been carried out at the Heidelberg Institute for Theoretical Studies (Beckmann *et al.*, in revision). It has been shown (Cheng *et al.*, 2010), that these simulations produce valuable predictions of the proteins elastic properties.

By DisEMBL, Cnidoin is predicted to be highly disordered in its repetitive, putative elastic domain (Figure 2.9 A) while at least the N-terminal part of the protein appears to be structured. By applying a virtual pulling force on a fixed Cnidoin peptide (Figure 2.9 B), the behaviour of the protein upon extension can be simulated.

The MD simulations correspond in principle to theoretic atomic force microscopy measurements. The peptides are stretched and different parameters as the applied force, secondary structures and the solvent accessible surface area (ΔSASA) are calculated. The force-extension profile of the two Cnidoin peptides is shown in Figure 2.9 C. Cnidoin was stretched and the conformations were sampled at various distances (dZ). The average resisting force against stretching was calculated. A slight

plateau can be observed, overall Cnidoin can be easily extended. The forces were fitted with a worm-like chain model and the persistence length was calculated to be 0.72±0.02 nm. The shorter the persistence length is, the higher is the tendency of the protein to coil and its elasticity. From the simulations it can be concluded that Cnidoin indeed behaves like an elastic protein, comparable to spider silk that has, under the same conditions, a persistence length of 0.74 nm (Cheng *et al.*, 2010). The force needed to extend the peptides results from interactions within the peptide. Cnidoin shows a plateau in the force-extension curve, this shows an internal willingness to recoil and to work against the extension. At low extensions ΔSASA increases (Figure 2.9 D). This indicates the burial of hydrophobic areas in the un- or little stretched configuration of the peptide. The burial of hydrophobic residues is the driving force for the collapse of a stretched Cnidoin molecule.

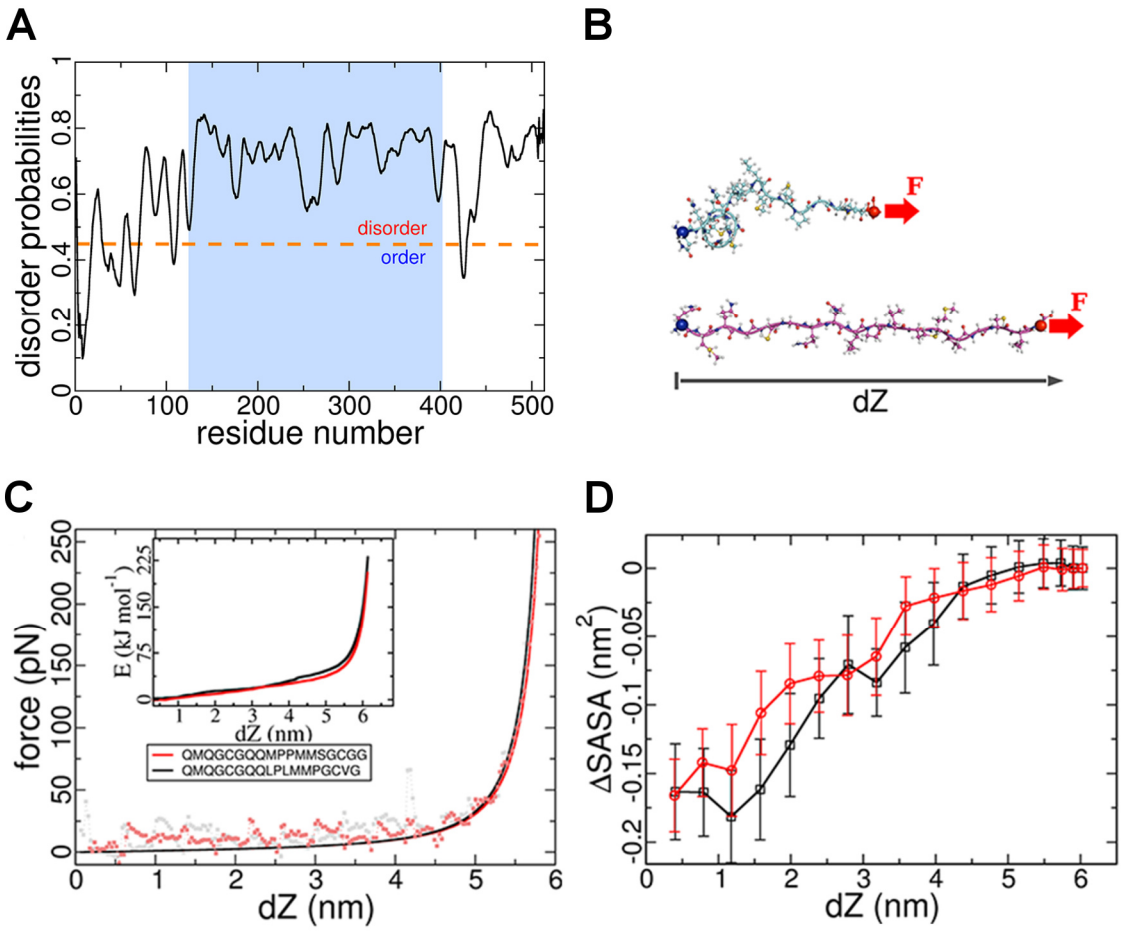


Figure 2.9: MD simulations of Cnidoin. A DisEMBL prediction of the state of order of Cnidoin. The light blue box indicates the repetitive sequence. B exemplary conformations of Cnidoins. A pulling force (red arrow) was applied and extension and behaviour of the protein was simulated. C Umbrella sampling on stretched Cnidoin peptides at varying distances showing the resulting average resisting forces against extension. The resulting free energy is shown in the inset. D hydrophobic surface burial of two Cnidoin peptides measured by disappearance of solvent accessible surface area (ΔSASA**).** Graphics by Dr. Senbo Xiao and Dr. Frauke Gräter (modified after Beckmann *et al.*, in revision).

While the high abundance of glutamine and glycine in the elastic domain of Cnidoin can be explained by its elastic behaviour the role of methionine is still elusive (Figure 2.2 C). The costs for synthesising methionine are high and as the third abundant amino acid within the elastic domain it probably will have a defined role. Whether this role consists in the crosslinking of different elastic domains or whether methionine has an impact on the elasticity of Cnidoin was addressed by additional MD simulations. In these calculations, all methionines in the peptides were replaced by alanines and the peptides were subjected to the same force-quench MD simulations as wildtype peptides. The mutated peptides showed the same behaviour as wildtype peptides, thus methionine has no impact on the elasticity and does not facilitate the nematocyst discharge process.

Elastic protein sequences are unstructured and lack secondary structures like α-helices, β-sheets and coiled coil domains. Infrared spectroscopy can be used to determine the amount of individual secondary structure elements in a protein sample. A construct of four repeated elastic domains was investigated by FTIR in cooperation with the Kirchhoff Institute for Physics (Beckmann *et al.*, in revision; Figure 2.10 A).

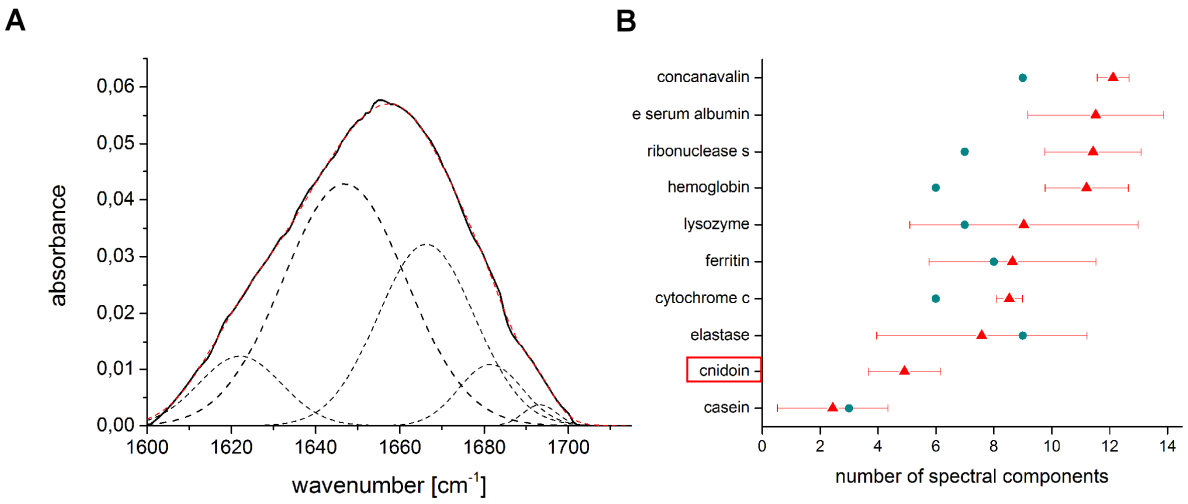


Figure 2.10: FTIR measurements of Cnidoin. A exemplary measurement of Cnidoin showing individual spectral components that account for the measured signal. B Number of spectral components observed in Cnidoin and other reference proteins. The red triangles represent the average number of components derived from the experiments with their standard deviation. Blue dots are the results obtained from Byler & Susi, 1986. Graphics by Prof. Dr. Wolfgang Petrich (modified after Beckmann *et al.*, in revision).

As the infrared spectra of proteins depend on various environmental and experimental conditions, nine additional proteins were used. These proteins were previously investigated by FTIR (Byler & Susi, 1986). From these experiments it can

be concluded that a low number of spectral components represents a low abundance of ordered secondary structures in the protein. Thus these proteins can provide a reference for the obtained spectra of Cnidoin. The elastic domain is expected to be unstructured.

FTIR on dried films of proteins of Cnidoin provided evidence that the elastic domain is lacking prominent secondary structures as it shows only a limited number of spectral components, comparable to casein, a protein known to lack secondary structures.

2.2 Molecular components of the mechanosensory cnidocil apparatus

2.2.1 The ion channel TrpA

Transient receptor potential (Trp) channels play an important role in many cellular processes. The family of Trp ion channels consists of 7 subgroups (Li *et al.*, 2011), but all share a common topology with six transmembrane domains and intracellular C- and N-termini. The pore region is located between the fifth and sixth transmembrane domain and a functional channel is composed of four subunits. Several Trp channel play a role in mechanoreception especially TrpN and TrpA group members (Lin & Corey, 2005). A mouse TrpA homolog has been proposed as a candidate for the hair cell mechanoreceptor (Corey *et al.*, 2004). Recently it was shown that TrpA is located in the sensory structures in the tentacles of the sea anemones *Nematostella* and *Haliplanella* (Mahoney *et al.*, 2011). Nematocyst discharge in these anemones was affected by TrpA selective drugs. The discharge of nematocysts in *Hydra* is dependent on extracellular calcium (Nüchter *et al.*, 2006) but so far, no calcium sensitive molecular components have been identified.

On this basis the *Hydra* homolog of the *Nematostella vectensis* TrpA was cloned from cDNA (sequences are provided in the attachments). The protein consists of 1255 amino acids and has a calculated molecular weight of 141.5 kDa. The N- as well as the C-terminus is predicted to point towards the cytosol, while 6 transmembrane helices span the membrane (Figure 2.11). The N-terminus contains 16 Ankyrin repeats. Ankyrin repeat domains are found in multiple protein families and serve for protein-protein interactions. Due to their folding they can be considered as molecular springs (Li *et al.*, 2006; Gaudet, 2008).

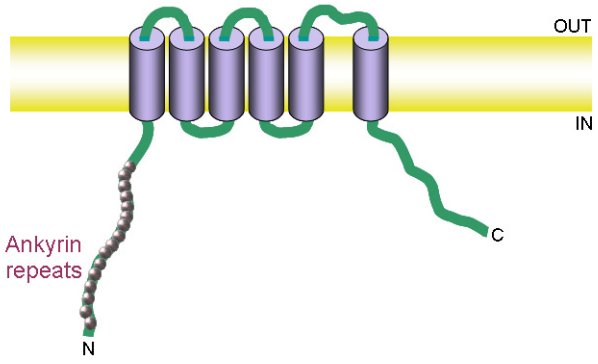


Figure 2.11: Schematic representation of the *Hydra* TrpA.

The in situ hybridization of TrpA revealed the expression of the calcium channel in

nests of developing nematocytes in the body column. The tentacles as well as the foot region did not show any TrpA expression (Figure 2.12 A).

In Western Blot analysis, the antibody detected a clear and single band at the expected molecular weight of 140 kDa (Figure 2.12 B). TrpA could be detected in whole Hydra lysate as well as in head and body column lysate. The preimmuneserum control was free of signal. While the expression of the protein is restricted to developing nematocytes in the body column of *Hydra*, the protein itself can be detected in the whole animal by Western Blot analysis.

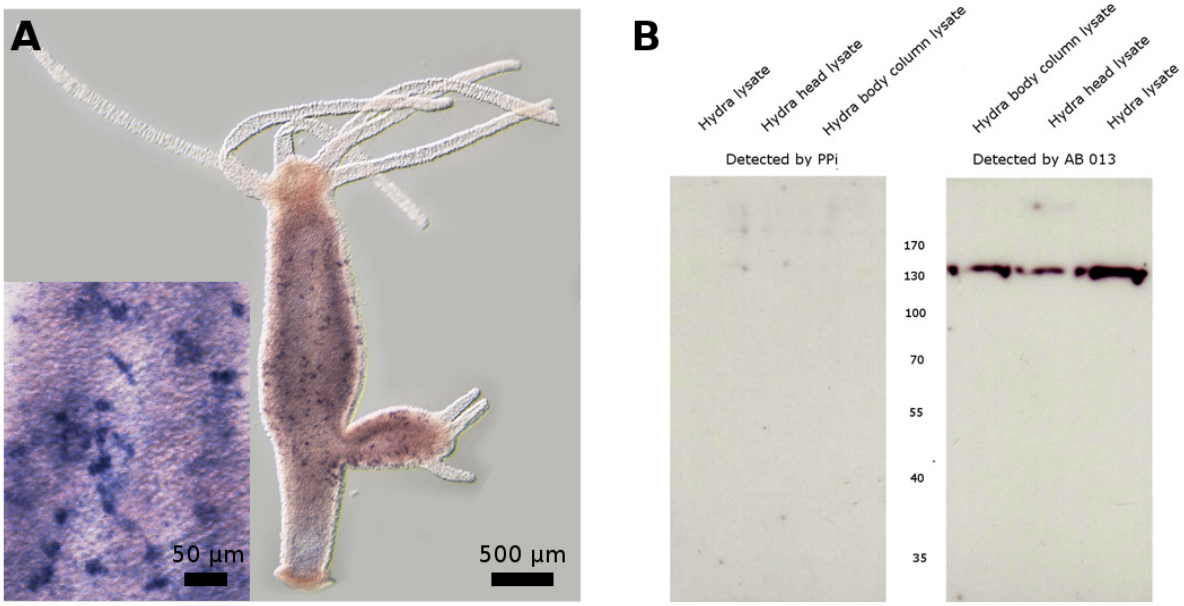


Figure 2.12: In situ hybridization and Western Blot analysis of TrpA. A the in situ shows expression in nests of developing nematocytes in the body column B the protein can be detected by Western Blot analysis in the body column as well as in the head of *Hydra*, indicating that the protein migrates with the maturating nematocytes into the tentacles after expression in the body column (Bachelor thesis from Bérénice Ziegler).

The two localizations of the TrpA protein can also be observed in the immunostainings (Figure 2.13). In the body column of *Hydra*, TrpA is detected in nests of developing nematocytes, while in the tentacles, mature nematocytes are stained. By co-staining with the NOWA –CTLD antibody the nests that are positive for TrpA can be characterized in more detail. NOWA-CTLD stains the tubule in developing nematocysts. TrpA is mainly present in late stages of capsule development. The maturating nematocytes incorporate TrpA and migrate at their final step of development into the tentacles. In mature nematocytes TrpA can be detected in the stereocilia of the cnidocil apparatus that surround the cnidocils that are built by actin filaments. The sensory cnidocil originates in the middle of the TrpA positive stereocilia as visualized by co-staining with α-tubulin.

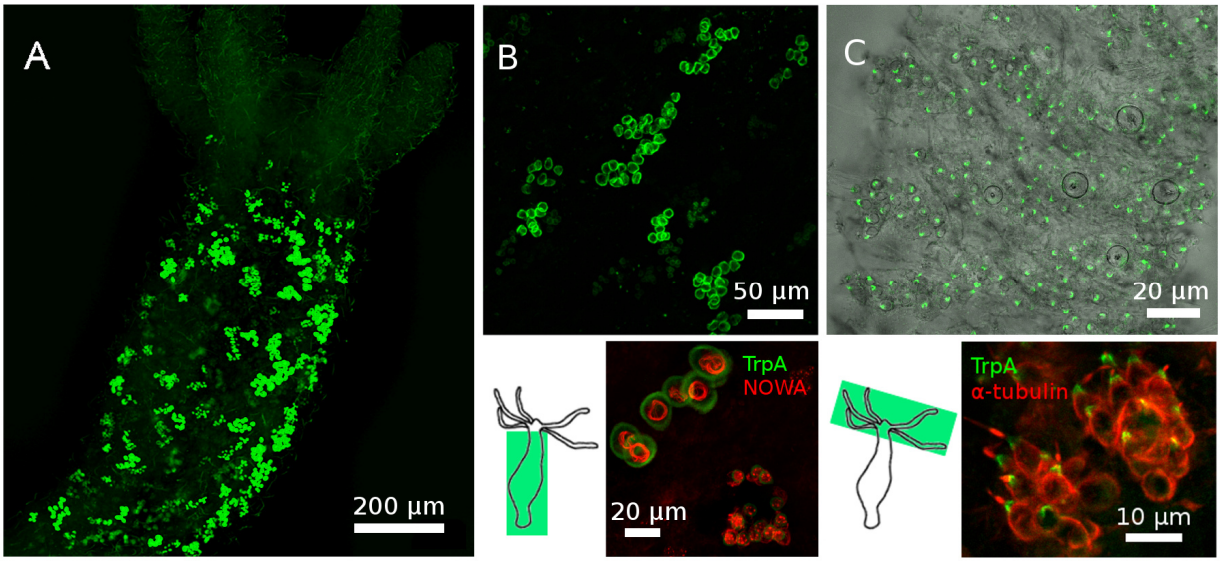


Figure 2.13: Immunostaining of TrpA. A overview of a bud. B nests of developing nematocytes in the body column. Co-staining with NOWA CTLD reveals that only late stages of development are positive for TrpA. C TrpA in the tentacles of *Hydra*. The calcium channel locates to the stereocilia of the cnidocil apparatus. The microtubular cnidocil originates in the middle of the stereocilia.

Upon Hydroxyurea treatment, that causes the loss of interstitial stem cell and their derivatives, the signal is lost. Treatment with the calcium chelator EGTA or decnidocilation did not affect the presence or localisation of the protein in the stereocilia.

A first hint on the function of TrpA during nematocyst discharge is obtained upon treatment of the animals with the styrol dye FM1-43. The dye is known to enter cells by the sensitive channel itself and blocks the channel then from the inside (Drew & Wood, 2007). Sensitivity for FM1-43 could also be shown for the hair cell mechanotransduction channel (Gale *et al.*, 2001). Animals placed into medium containing the dye immediately show labelled nematocytes. The labelling is too fast to be caused by endocytosis, the dye has to enter the cell directly (Figure 2.14).

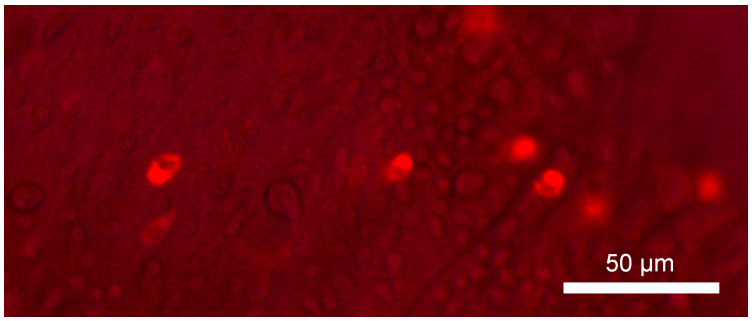


Figure 2.14: Tentacle of a living Hydra that was incubated shortly in 5 µM FM1-43. The overall light red colour is due to the filter of the microscope as the transmitted light and fluorescence image were captured at the same time. Sequential images of both channels were not possible due to movements of the animals. No paralyzing agent was used to not induce artefacts.

Hydra that are incubated in FM1-43 containing Hydra medium are not able to catch *Artemia* anymore. The dye is not toxic to the animals, even long treatments do not cause any defects. Though it can be assumed that the effect of an abolished feeding behaviour is caused by inhibition of an essential factor of nematocyst discharge. Specific inhibitors and activators (Cinnamaldehyde, Polygodial, Zinc chloride, HC 030031, A 967079) of TrpA have been tested at various concentrations, but so far none of them could be used as the needed concentrations were not supported by the animals, the chemicals were toxic to the Hydra.

2.2.2 The penta-EF-hand protein Nematocalcin

The analysis of the nematocyst proteome revealed the presence of a member of the penta-EF-hand protein family in the nematocyte. The protein was called Nematocalcin and the coding sequence was cloned from cDNA (sequence provided in the attachments). The protein consists of 201 amino acids and has a molecular weight of 22.62 kDa. It contains an N-terminal collagen-like (glycine and glutamine-rich) domain and shows high sequence similarity to other calcium-binding proteins like Grancalcin or Sorcin (Figure 2.15). Those proteins have been studied intensively, providing some hints that could also apply to Nematocalcin. Penta-EF-hand proteins fall into two subgroups, with Sorcin and Grancalcin both being members of group II that probably evolved from group I (Maki *et al.*, 2002). Penta-EF-hand proteins contain five EF-hand motifs for binding calcium, but not all motifs are functional, as some lack essential residues for the coordination of calcium. For Nematocalcin two overlapping EF-hand motifs have been predicted by sequence analysis. They correspond to EF2 and EF3 as derived from alignments.

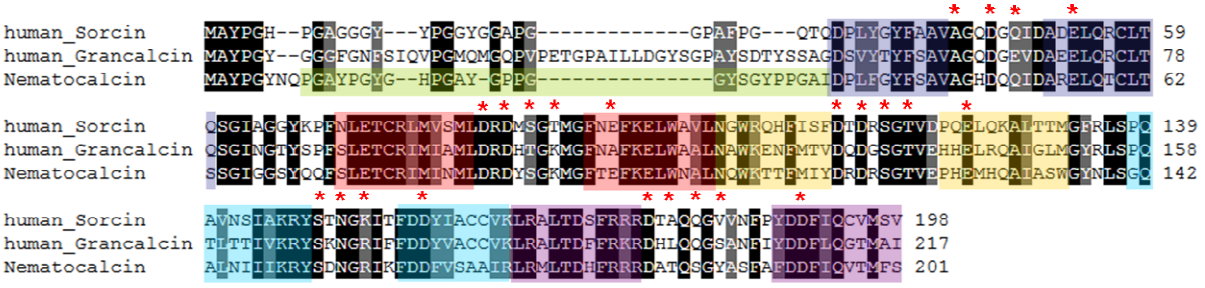


Figure 2.15: Alignment of Nematocalcin with two other penta-EF-hand proteins (human Sorcin [P30626] and Grancalcin [P28676]). Conserved amino acids are indicated by black and grey boxes, amino acids are grouped following their properties: GAVLI, FYW, CM, ST, KRH, DENQ, P. Highlighted is the collagen-like domain of Nematocalcin (green) as well as the helices of the EF-hand motifs. Residues important for calcium coordination are marked by asterisks (after Xie *et al.*, 2001).

The crystal structure of both calcium-free (Jia *et al.*, 2000) and calcium-bound (Jia *et*

al., 2001) Grancalcin as well as calcium-free (Xie *et al.*, 2001; Nastopoulos *et al.*, 2001) Sorcin have been resolved, providing a good evidence for the structure of Nematocalcin, as both proteins are highly conserved. The proteins are mainly alpha-helical.

For investigating the role of Nematocalcin in nematocytes, an antibody was raised. In Western Blot analysis, a signal in *Hydra* lysate as well as in head and body column lysate could be detected at the calculated molecular weight of 22.62 kDa (Figure 2.16). In isolated nematocysts, the signal was not as strong as expected for a capsule protein, indicating that the nematocyst is not the major localisation of the protein although it has been isolated with the nematocysts during the proteome analysis. A signal for Nematocalcin could be detected in reducing as well as in non-reducing probes, indicating that the protein is not crosslinked by disulfide bonds.

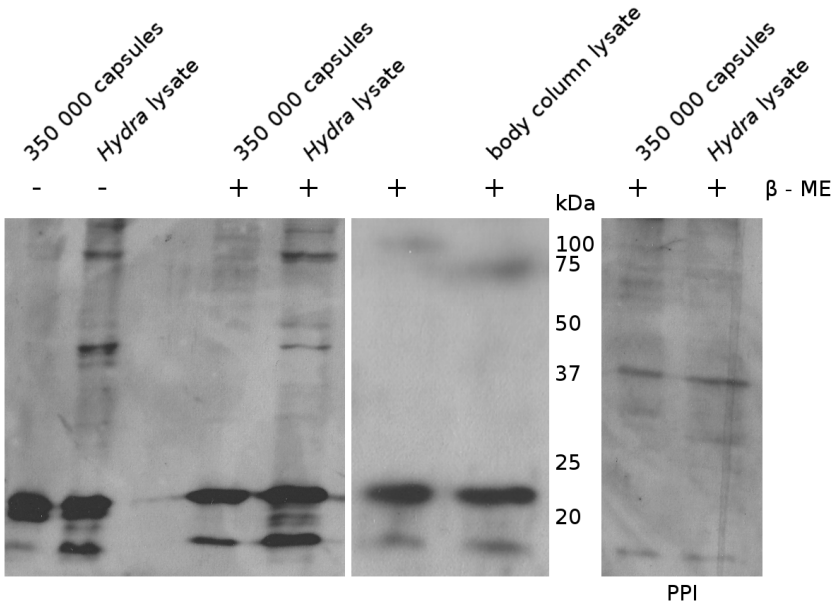


Figure 2.16: Western Blot analysis of Nematocalcin. The protein can be detected in reducing (with ß-Mercaptoethanol) as well as in non-reducing (without ß-ME) samples. Nematocalcin is present in the whole animal. The Blot detected with the preimmunesera (PPI) shows some unspecific binding of the antibody.

Immunostainings with Nematocalcin revealed that the protein is indeed correlated to nematocytes, but not with the capsule itself. Nematocalcin locates to the stereocilia of the cnidocil apparatus (Figure 2.17). The detection in capsules by Western Blot analysis and the presence in the capsule proteome indicate that it binds to the nematocyst in a specific or unspecific manner; the protein does not contain a signal peptide for the localization in capsules. In immunostainings, Nematocalcin could only be detected in mature capsules, no developing stages were visible. All types of

nematocytes showed a signal for Nematocalcin.

To characterize the localization of Nematocalcin in relation to other components of the nematocyte, different co-stainings were performed (Figure 2.17). An antibody for *Nematostella* Minicollagen-1 was used to visualize mature capsules in the tentacles of *Hydra*. While Minicollagen-1 is a major structural component of the capsule wall, Nematocalcin is superposed to the capsule itself. It is located in the stereocilia that surround the cnidocil (Figure 2.17 D). β-tubulin was used to visualize all microtubules, including the subsets of acetylated and tyrosinated tubulin. Acetylated tubulin forms the stable microtubules. They build the cnidocil. Tyrosinated tubulin builds dynamic microtubules. It is found in the microtubular basket surrounding the nematocyst as well as in sensory neurons encircling the mouth. Nematocalcin does not co-localize with microtubules in the cnidocil. In some nematocytes a co-localization in the microtubular basket surrounding the nematocyst can be observed, but the most dominant localization of Nematocalcin is in the stereocilia of the cnidocil apparatus. Phalloidin was used to stain the actin cytoskeleton (Figure 2.17 D+F). Actin is one of the major components of the cnidocil apparatus stereocilia, but it does only partly overlap with Nematocalcin. While Nematocalcin is located at the basal part of the stereocilia, actin extends to more distal parts.

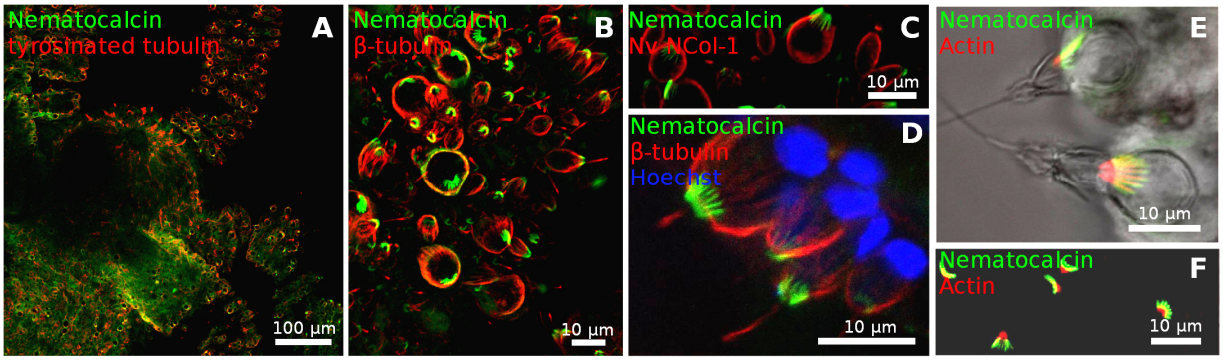


Figure 2.17: Immunostaining of Nematocalcin. A co-staining with tyrosinated tubulin to visualize dynamic microtubule. Nematocalcin can only be detected in mature nematocytes, no developmental stages are visible. B tentacle of *Hydra magnipapillata*. Microtubules form the cnidocil and surround the nematocyst inside the cell to stabilize it. Nematocalcin localizes to the stereocilia that surround the cnidocil. C Nv NCol-1 marks the capsule wall, Nematocalcin is superposed to the actual capsule. D the cnidocil originates in the middle of the Nematocalcin containing stereocilia. E+F actin and Nematocalcin localize both to the stereocilia. While Nematocalcin is located at the basis, actin extends to more distal parts. Actin is visualized by Phalloidin.

The localisation of Nematocalcin to the stereocilia of the cnidocil apparatus could be confirmed by immunogold labelled cryo-TEM (Figure 2.18).

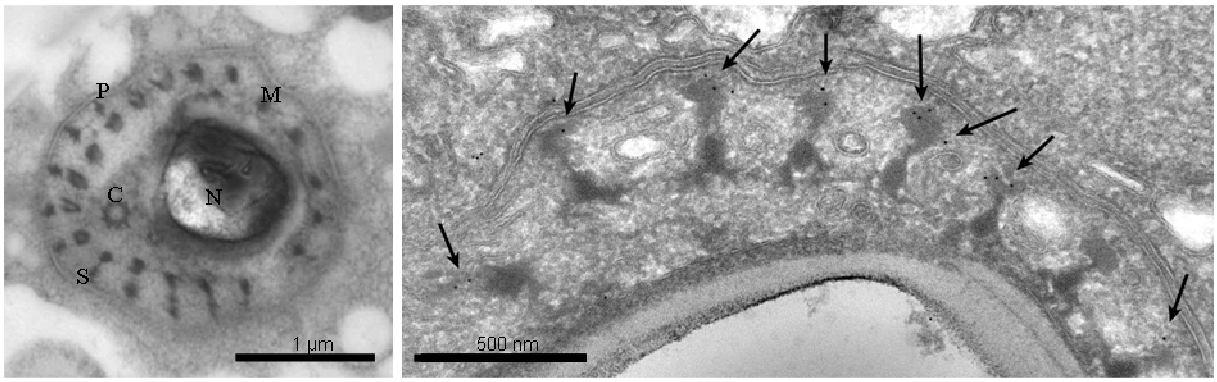


Figure 2.18: Immunogold-labelled cryo TEM (images by Michael W. Hess, PhD, Associate Professor of Cell Biology, Innsbruck Medical University). A cross section of a nematocyte, the nematocyst (N), cnidocil (C), microvilli (M) and the stereocilia (S) as well as the surrounding plasma membrane (P) are visible. B Magnification of a nematocyte cross section. Immunogold particles are visible as little black dots (arrows) and localize to the stereocilia.

Removal of the cnidocils by rinsing the animals with ethylenglycol (Golz & Thurm, 1990) did not alter the Nematocalcin signal. The stereocilia are not tightly linked to the cnidocil and not removed upon decnidocilation.

In the dependence of calcium penta-EF-hand proteins change their localisation. Such has been shown for example for Grancalcin (Teahan *et al.*, 1992), Sorcin (Meyers *et al.*, 1995) and Peflin (Kitaura *et al.*, 2001). In the presence of calcium, they bind to membranes. Treatment of *Hydra* with EGTA could lead to a redistribution of Nematocalcin. In several experiments it was found to localize to the cnidocil or the microtubule basket surrounding the capsule after EGTA treatment. However, the same pattern could be observed in some non-treated animals. Thus the effect of an EGTA-mediated reduction in external calcium needs to be clarified in the future.

Nematocalcin is located at the stereocilia of the cnidocil apparatus, as well as the calcium channel TrpA. To test whether both proteins localize to the same structure, co-stainings were performed (Figure 2.19), each together with another protein of the sensory cnidocil apparatus structure. None of the proteins co-localizes with microtubules. The Cnidocil originates in the middle of the stereocilia, where TrpA co-localizes with actin, while Nematocalcin overlaps only a little and is mainly located at the basal part (Figure 2.19). As revealed by ultrastructure investigations (Golz & Thurm, 1991) only the upper part of the actin filaments in the stereocilia is membrane-attached. The basis reaches as a rootlet into the cytoplasm providing evidence that the sensory part is at the tip. On this basis, TrpA localizes to the sensory region, while Nematocalcin at the base might be part of the signal transduction machinery.

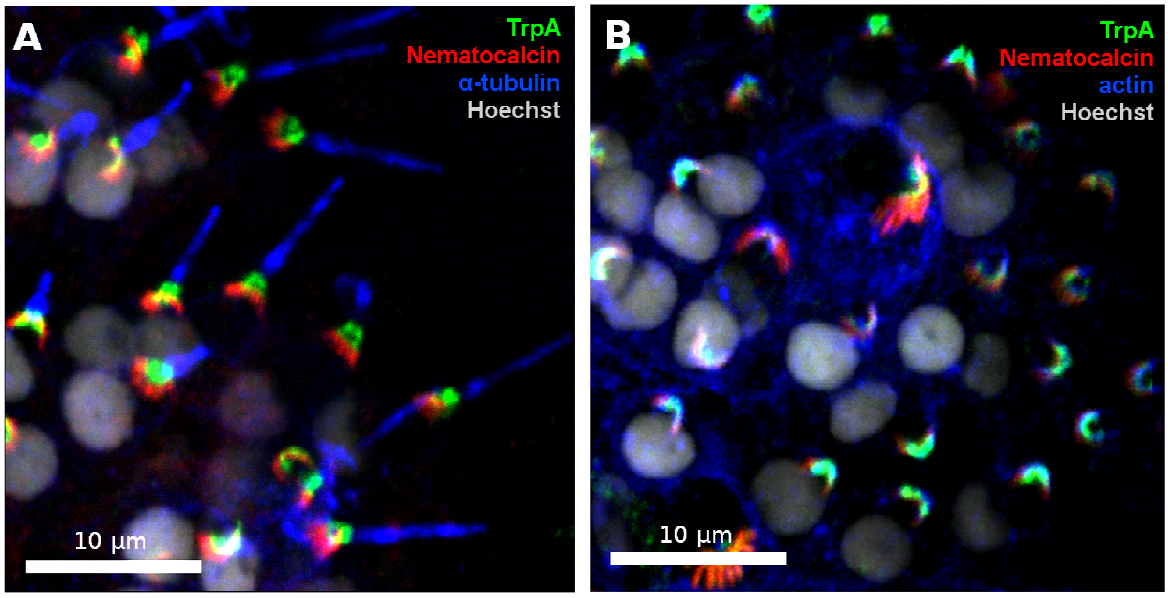


Figure 2.19: TrpA and Nematocalcin localize both to the stereocilia in the cnidocil apparatus. A co-staining of TrpA, Nematocalcin and α-tubulin. B co-staining of TrpA, Nematocalcin and actin. Actin is visualized by an actin antibody.

Transgenic animals expressing Nematocalcin eGFP under control of the nematocyte specific NOWA promotor were created by microinjection into embryos (according to Wittlieb *et al.*, 2006). The animals show GFP expression in developing nests of nematocytes as well as in mature capsules (Figure 2.20).

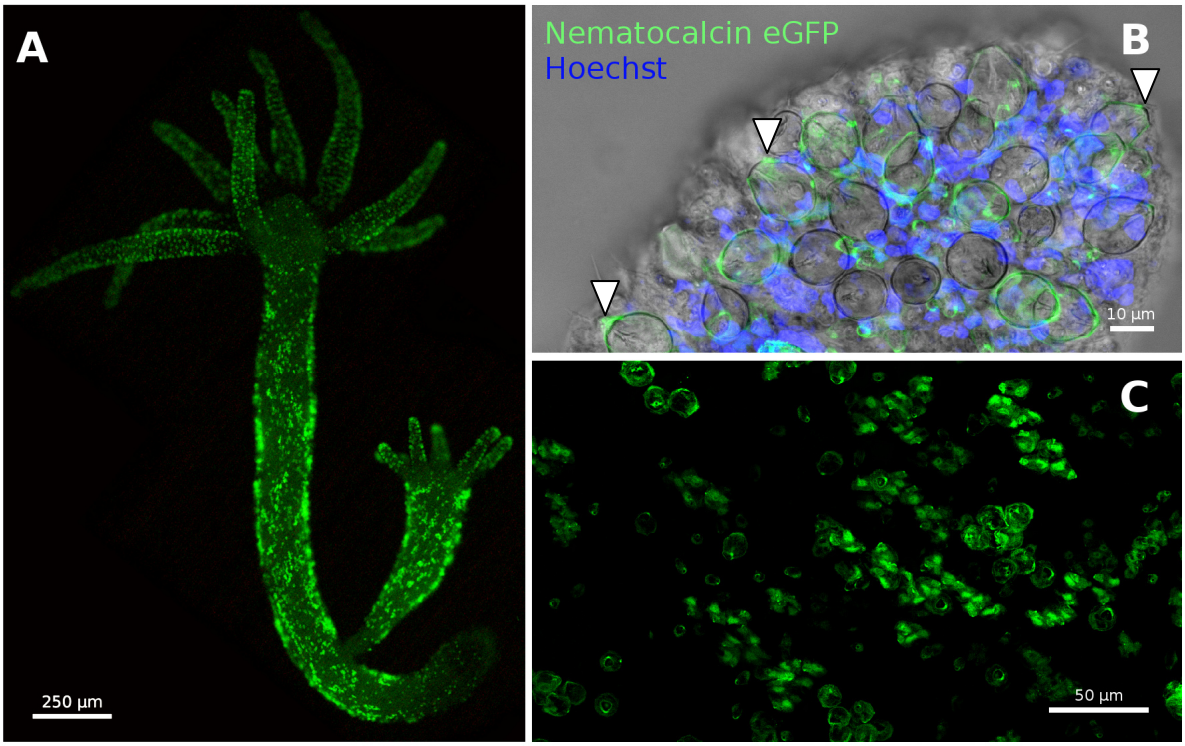


Figure 2.20: Transgenic animals expressing Nematocalcin eGFP under the control of the NOWA Promotor. A overview of a living animal, paralysed by 3 mM Heptanol in Hydra medium. B confocal stack of a tentacle tip shortly fixed with PFA and stained with Hoechst to visualize the nuclei. The prominent cnidocil apparatus of some capsules is marked by arrowheads. C confocal stack of the body column of a shortly PFA fixed Nematocalcin transgenic animal. Due to expression under control of the NOWA promotor nests of developing nematocytes are visible.

In the immunostainings, no expression of Nematocalcin could be observed during nematocyte development. The presence of GFP-expressing nests in the transgenic animals thus results from the NOWA promotor, which might be active earlier than the native Nematocalcin promotor or drive stronger expression levels. In mature capsules, Nematocalcin GFP can be detected in the nematocytes as well as in the cnidocil apparatus. Thus the transgenic animals confirm the immunostainings for Nematocalcin and provide a powerful tool for further studies on this protein. The intracellular presence of Nematocalcin might be due to accumulation caused by overexpression.

The putative active EF-hands EF2 and EF3 have been mutated by replacing the calcium coordinating aspartic acid and glutamic acid residues through alanine. The mutation of all residues avoids an expression of the whole protein in vivo, probably due to a massive misfolding, though a functional characterization of Nematocalcin could not be accessed.

The in situ hybridization of Nematocalcin did not exclusively show the expected expression in nematocytes. The sense control did not show any signal, while the antisense probe gave an intense signal in the whole animal. Both endo- and ectodermal tissue showed expression (Figure 2.21).

In the tentacles, some almost mature nematocytes had a strong signal. This might indicate the actual expression pattern of Nematocalcin, while the broad expression pattern might result from other, similar penta EF-hand proteins.

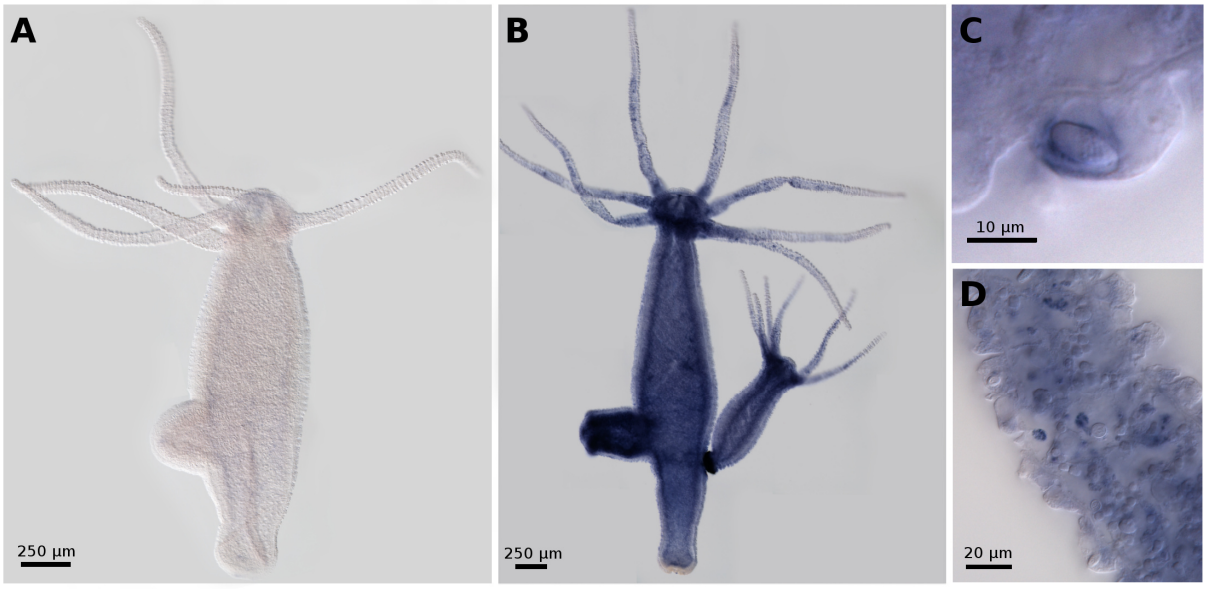


Figure 2.21: In situ hybridization for Nematocalcin. A sense control. B antisense signal in the whole animal. C Nematocyte showing expression by staining with antisense probe. D expression in a tentacle by staining with antisense probe. Ecto- and endodermal tissue shows expression, only rarely a correlation to nematocytes can be observed.

2.3 Novel molecular factors in tubule morphogenesis

2.3.1 Nematomyosin – shaping the nematocyst vesicle

A non muscle myosin type II was identified in the nematocyst proteome analysis (Balasubramanian *et al.*, 2012). To elucidate the function of a myosin in the context of the nematocyst the corresponding gene was isolated from *Hydra magnipapillata* cDNA, cloned and sequenced (sequence attached). It corresponds to the gene models Hma2.213345 and XP_002167357.1 respectively. The encoded protein is a typical type II myosin with a head and a tail domain. The head has ATP binding properties and the tail is thought to interact with other myosins by coiled coil interactions. As the protein was found in the nematocyst proteome and is therefore believed to be associated with the nematocysts of *Hydra*, it was called Nematomyosin, although it does not contain a signal peptide for the localisation in capsules. Nematomyosin consists of 1946 amino acids and has a theoretical molecular weight of 224.57 kDa. For functional characterization of the protein an antibody against the head region was created.

With immunostainings Nematomyosin could be detected in the body column of *Hydra*, where it associates with nests of developing nematocytes (Figure 2.22).

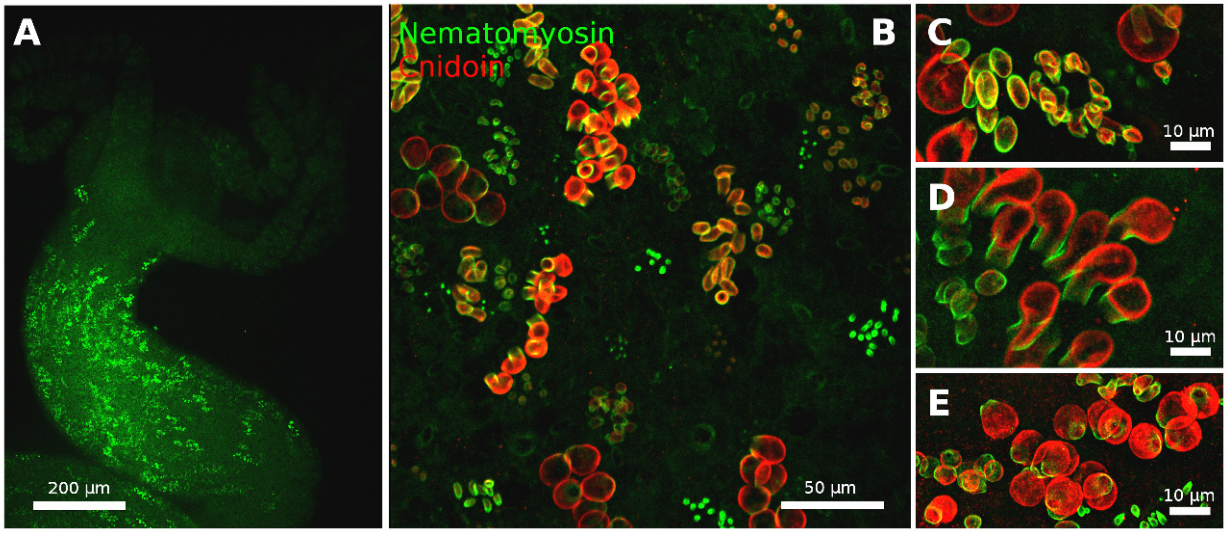


Figure 2.22: Immunostaining of Nematomyosin. A overview of a bud. B the co-staining with Cnidoin reveals the developmental stages. C-E Magnifications of the co-staining with Cnidoin. Different developmental stages are shown.

Through co-staining with Cnidoin the developing capsule wall was made visible, so that it was possible to distinguish between different capsule types and developmental stages. In early developmental stages Nematomyosin surrounds the forming capsule wall (Figure 2.22 C). When the tubule grows out, Nematomyosin is restricted to a

collar around the tubule basis (Figure 2.22 D). In almost mature capsules with coiled tubule, some residual protein is left at the apical part of the nematocyst vesicle (Figure 2.22 E). The preimmunesera did not give any signal.

Based on the immunostainings the assumption can be made that Nematomyosin plays an important role in the development of nematocytes. It is associated with the outgrowing tubule and the capsule wall. As a motile protein it could create the force to induce a constriction in the capsule vesicle and thus have an important influence on the shape of the developing nematocyst. Inhibition of Nematomyosin by the specific Myosin type II inhibitor Blebbistatin (Kovács *et al.*, 2004) leads to malformed nematocysts (Eismann *et al.*, manuscript in preparation; Figure 2.23).

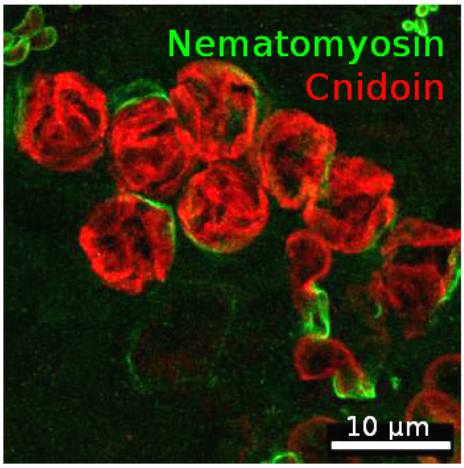


Figure 2.23: Nest of stenoteles after Blebbistatin treatment (1 µM). Severe deformations of the nematocyst vesicle are visible. (modified from the Bachelor thesis of Björn Eismann)

By the hydrolysis of ATP, bound to the head region, myosins convert chemical energy into movement along actin fibres. So far, no interaction or co-localisation to actin could be shown in nematocytes.

2.3.2 PKD2 – a polycystin-2 like channel in *Hydra* nematocyte development

Nematocysts within cnidarians and even within the four different types being present in *Hydra* differ in shape and size, particularly in the tubules. They can have dramatically different sizes and a variable amount of spines and attachments (Weill, 1934). As the development of all nematocysts is considered to follow similar principal mechanisms, the formation has to be carefully regulated.

Hydra possesses multiple Trp channels one of which is the putative mechanosensitive PKD2 channel. PKD2 is a well-characterized ion channel and mutations in either PKD1 or PKD2 lead in humans to polycystic kidney disease, one of the most abundant inherited diseases (see Köttgen, 2007 for review). PKD2 is thought to act as a mechanosensor and to detect liquid flow. The channel has been found to play important roles not only in the human kidney, but also in many other organisms such as in the cell wall repair of yeast (Aydar & Palmer, 2009), the Drosophila sperm or sea urchin acrosome reaction (Kierszenbaum, 2004) for example.

PKD2 was identified as a non selective calcium channel, which means it gates calcium, but also other, smaller, cations (González-Perrett *et al.*, 2001). HyPKD2 is a transmembrane ion channel with 839 residues and a molecular weight of 96.02 kDa. The protein contains six transmembrane domains and two calcium-binding EF-hand motifs in the C-terminal tail (Figure 2.24 A). Both, the C- as well as the N-terminal tail are predicted to point towards the cytoplasm. PKD2 does not contain a signal peptide for specific localization in the nematocyst vesicle. The corresponding gene has been cloned from *Hydra magnipapillata* cDNA (sequences are attached).

The *Nematostella* homolog of PKD2 is expressed in nematocytes (Fabian Rentzsch, personal communication). The *Hydra* homolog of PKD2 could not be detected by in situ hybridizations. The sense and the antisense probe gave the same unspecific staining pattern, although different probes and conditions were tested.

Nevertheless an antibody that was raised against a peptide sequence in the C-terminal part of the *Hydra* PKD2 channel gave a clear signal in Western Blot analysis (Figure 2.24 B). The signal could be detected in whole Hydra lysate and was present in both head and body column lysate. The antibody against the *Hydra* protein also detected a signal in *Nematostella* lysate at ~100 kDa which matches the calculated theoretical molecular weight of 103.16 kDa). The preimmunesera did not show any prominent signals.

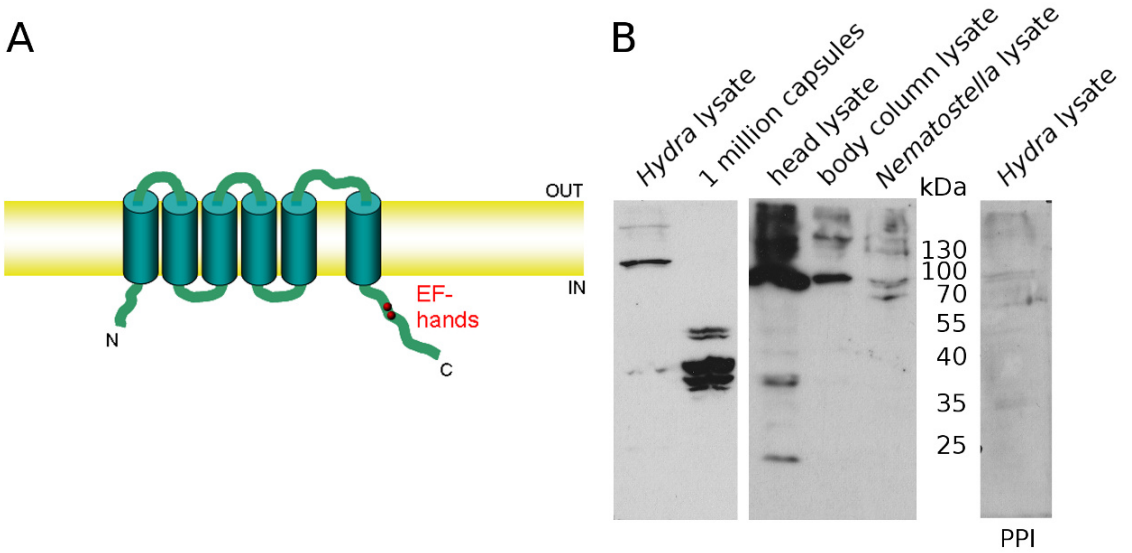


Figure 2.24: Domain organisation of PKD2 and Western Blot analysis. A schematic representation of *Hydra* PKD2 channel. B PKD2 can be detected in Hydra lysate and is present in the head as well as in the body column lysate. The antibody against the *Hydra* PKD2 detects also a signal of the corresponding molecular weight in Nematostella. In capsules, no signal for PKD2, but a prominent smaller protein is detected. The preimmuneserum (PPI) does not show any prominent signal.

Surprisingly, although expressed at least in nematocytes in *Nematostella*, the antibody did not detect the protein in lysate of isolated *Hydra* capsules in Western Blot analysis. Although the capsules were applied in excess, no band at 96 kDa was detectable, but signals with lower molecular weight were visible. This might be due to degradation of the channel inside the capsules or unspecific binding of the antibody.

The detected signals differ in intensity (Figure 2.25). PKD2 is first present at the onset of tubule outgrowth. The brightest signal is obtained in nests of nematocytes with an outgrown tubule. The signal covers the complete nematocyst vesicle. This might indicate a massive expression and presence of the PKD2 protein during tubulation. The protein is correlated to the outgrowth of the tubule; other localisations might result from mass-flow due to the strong expression. When the tubule is invaginated, some remaining signal of PKD2 can still be detected associated to the tubule.

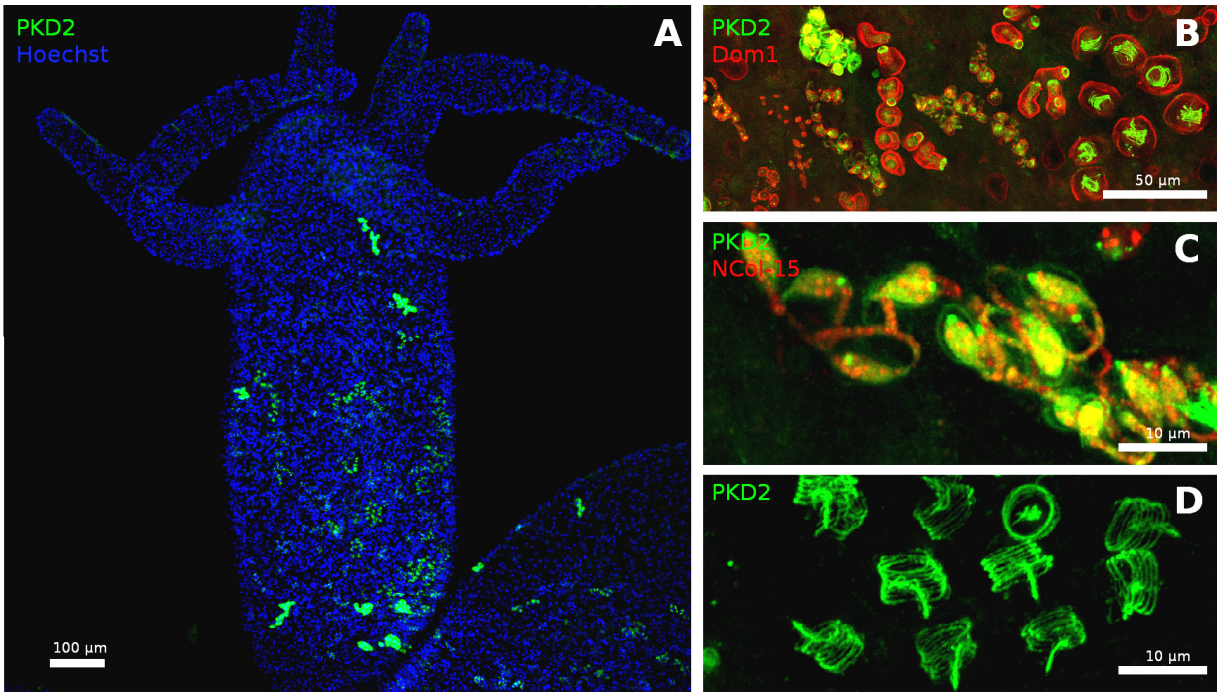


Figure 2.25: Immunostainings of PKD2 in *Hydra magnipapillata*. A overview of the staining pattern. PKD2 can be detected in nests of developing nematocytes in the body column. B Co-staining of PKD2 and anti-Dom1, a capsule wall marker. The detected signals differ in intensity during nematocyte development. PKD2 appears first at the onset of tubulation. At developmental stages with a maximal length of the non-invaginated tubule, PKD2 is most abundant in the nematocyte (see C). After Invagination the signal looses intensity. C Co-staining of PKD2 and Minicollagen-15 at outward tubule stage. D After invagination, PKD2 remains associated with the tubule.

By triple staining of PKD2, Minicollagen-15 and Cnidoin or Dom1 respectively, the development of nematocytes and the different phases of tubulation can be visualized (Figure 2.26). While the tubular proteins Minicollagen-15 and Cnidoin mainly colocalize, PKD2 labels the onset of tubulogenesis and later on the whole nematocyte. By co-staining of the capsule outer wall protein Dom1 with Minicollagen-15 and PKD2 all major components of the developing capsule (wall and tubule) can be detected.

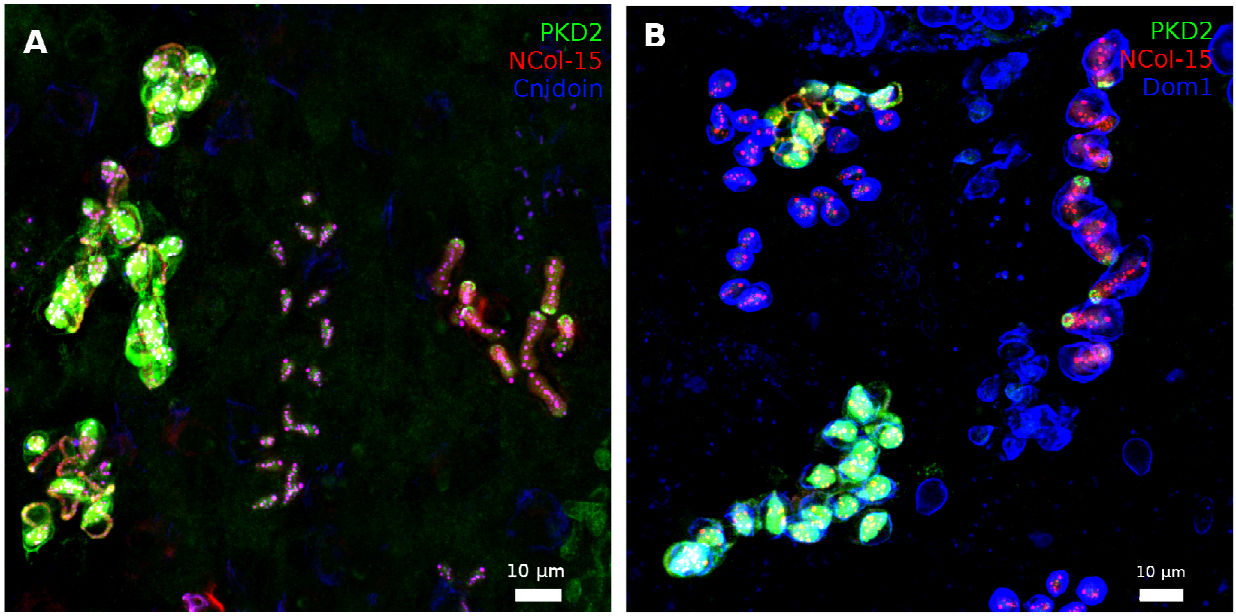


Figure 2.26: fine-localisation of PKD2 by triple-immunostainings. A co-staining of the tubular proteins NCol-15 and Cnidoin with PKD2. B co-staining of the nematocyst wall antigen Dom1, the tubule protein NCol-15 and PKD2.

PKD2 can also be detected by immunostainings in *Nematostella vectensis* where it is present in a subset of nematocytes in the tentacles (Figure 2.27). Some non capsule related, possibly unspecific staining signals were present in the mesenteries. Co-staining with Minicollagen-1 reveals that those nematocysts showing a PKD2 signal are not positive for Minicollagen-1. They might represent different developmental stages.

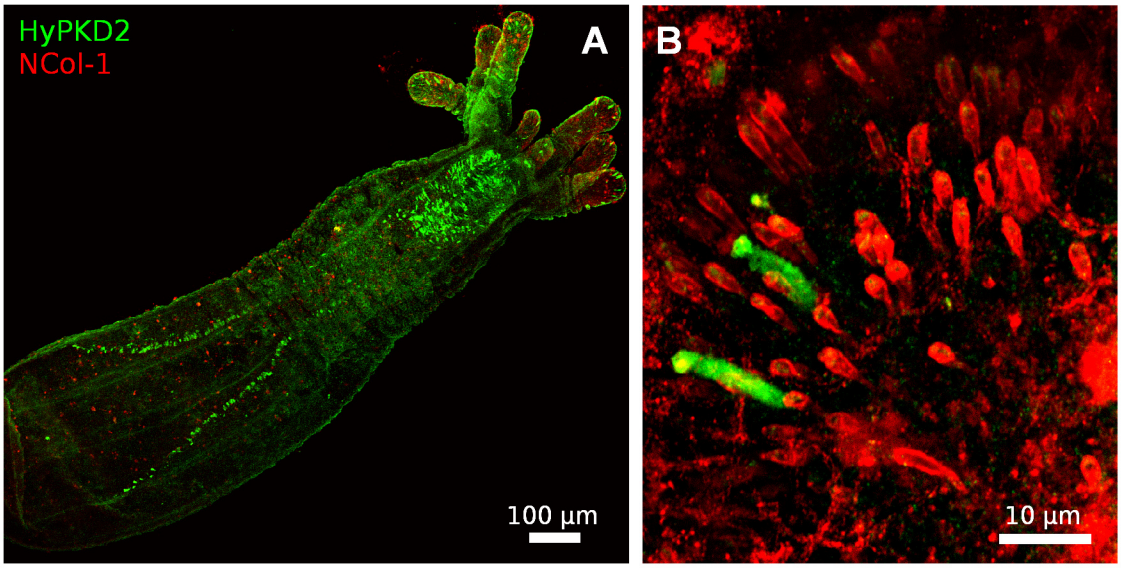


Figure 2.27: Staining of *Nematostella* with HyPKD2 antibody. A overview of a whole primary polyp co-stained for HyPKD2 and NCol-1. B magnification of a tentacle.

HyPKD2 could be involved in determining the tubule length by sensing the tension in the vesicle membrane, it has been proposed to control the growth of renal tubules as well (Lubarsky & Krasnow, 2003). An inhibition of the channel should then lead to malformed capsules. Gadoliniumchloride is known to block mechanosensitive ion channels (Ermakov *et al.*, 2010), even PKD2 mediated currents could be inhibited by $GdCl_3$ application (González-Perrett *et al.*, 2001). Treatment with 100 µM Gadoliniumchloride leads to a dramatic reduction of the PKD2 signal, higher concentrations are not tolerated by the animals (Literature values for PKD2: half block 206 µM, full inhibition 400 µM; Anyatonwu & Ehrlich, 2005).

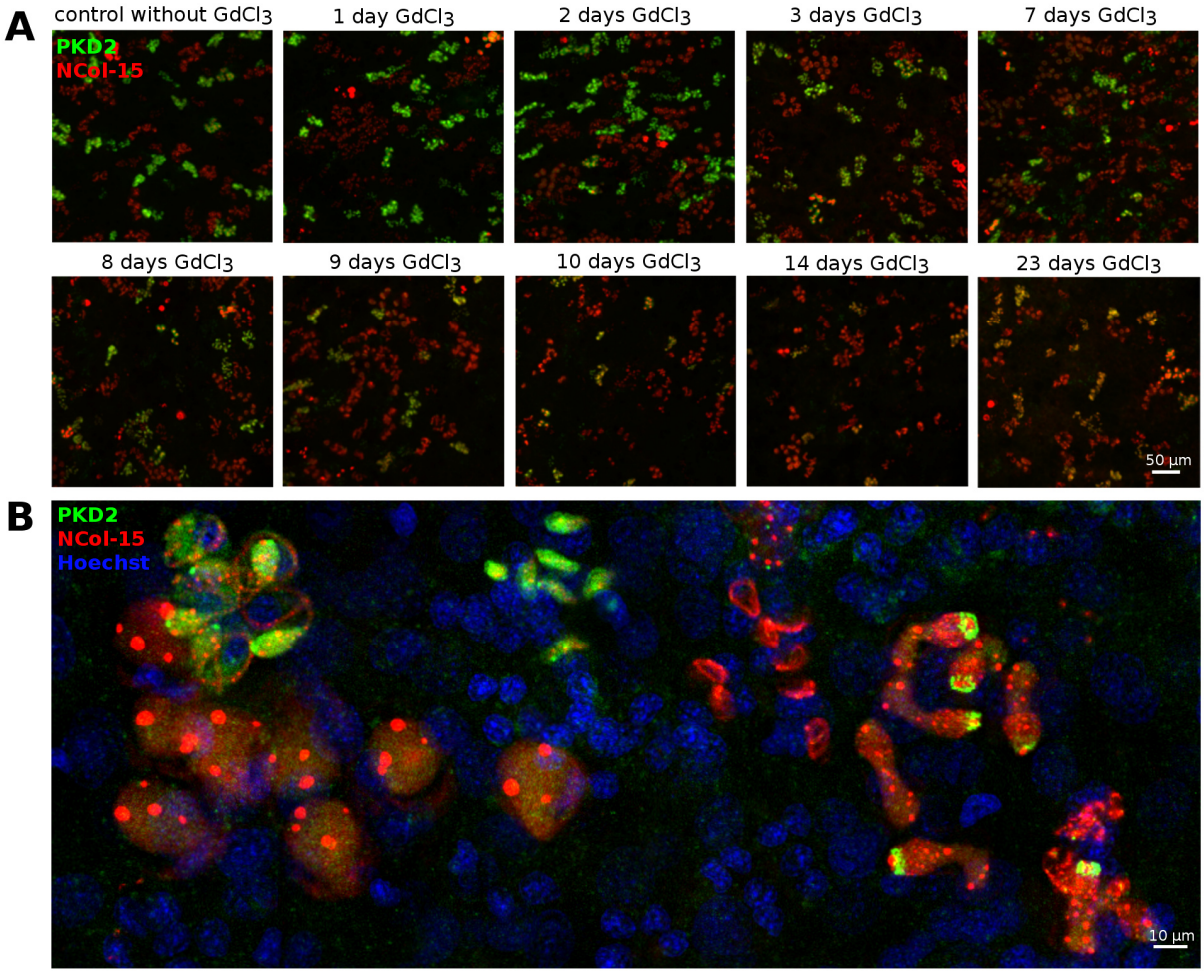


Figure 2.28: Gadoliniumchloride treatment and nematocyte morphology after treatment. A overview of the effect of Gadoliniumchloride on the presence of PKD2 as well as the overall presence of developing nematocytes visualized by Minicollagen-15. All images are acquired upon the same conditions. B close-up of developing nests after 14 days of Gadoliniumchloride treatment (23 days incubation with Gadoliniumchloride gave the same results) The staining pattern is the same as in untreated animals, but the intensity is reduced.

The general number of developing nematocytes in the body column of *Hydra* seems not to be affected even after 23 days of treatment, as visualized by co-staining with

Minicollagen-15 (Figure 2.28 A). Although the amount of PKD2 seems to be reduced upon Gadoliniumchloride treatment, malformed capsules could not be observed. The morphology of the capsules remained unaffected and the remaining localisation of PKD2 was the same as for untreated animals (Figure 2.28 B).

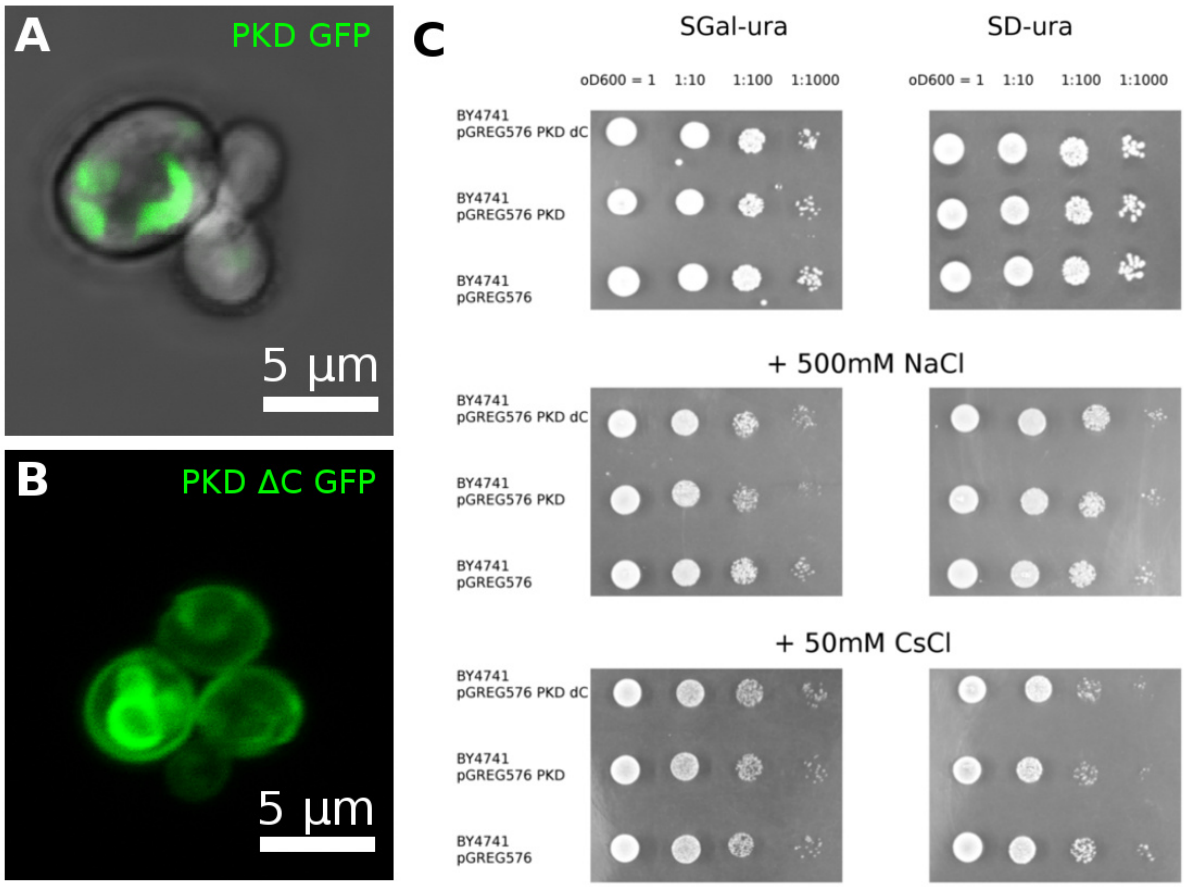


Figure 2.29: Expression of GFP-tagged PKD2 in yeast. A confocal section of yeast expressing wild-type PKD2, the channel is retained inside the cell. B Expression of a C-terminal truncated mutant of PKD2. The channel is transported to the plasmamembrane. C growth tests on different media with yeast expressing wildtype and mutant PKD2 as well as the empty vector.

To address functional properties of the HyPKD2 channel I performed experiments in yeast (*Saccharomyces cerevisiae* BY4741). The localisation of PKD2 within the cell seems to be regulated by the C-terminal cytoplasmic tail of the channel. In yeast, C-terminal truncation of the protein alters the trafficking inside the cell. While the wild type channel is retained inside the cell in the ER, a mutant with a C-terminal truncation was transported to the plasma membrane (Figure 2.29). PKD2 is supposed to be a non-selective calcium channel. To test the activity of the ion channel, different growth tests were performed. Yeast expressing HyPKD2 wildtype, PKD2 C-terminal truncation and empty vector were compared regarding their growth on different media. On SD media the channel is not expressed, only in the galactose-

containing SGal-media the expression of the transgenic construct is activated. The addition of sodium chloride or caesium chloride to the SGal-medium would result in an influx of caesium and sodium if the channel was active in the plasma membrane and toxicate the cells. Therefore a reduced growth was expected if the channel was functional. In the performed growth tests, no difference could be observed between yeasts expressing PKD2, PKD2 ΔC or empty vector.

3. SUMMARY, DISCUSSION AND PERSPECTIVES

3.1 Cnidoin – elastic component of the nematocyst

Different elastic proteins are used among the animal kingdom facilitating various functions. Examples can be found in mussels (byssal threads; Benedict & Waite, 1986), in insects (spider silk, reviewed in Römer & Scheibel, 2008; jumping cicadas [resilin], Burrows *et al.*, 2008) as well as in vertebrates (Elastin in blood vessels; Sage, 1982).

The elastic protein Cnidoin from *Hydra* represents a basal member of elastomeric proteins. Cnidoin possesses a remarkable repetitive sequence, being rich in glycine, glutamine and methionine. While glycine and glutamine are well known components of elastic sequences, the role of methionine in this context is still elusive. The repetitive sequence is highly similar to the elastic sequence in Spidroin-2 (Hinman & Lewis, 1992) with the repetitive motif being GxGQQ. FTIR measurements confirmed the lack of secondary structures in the large elastic domain. Cnidoin contains a signal peptide for the incorporation in the nematocyst and a propeptide. The protein is uniquely expressed in nematocytes of all types and incorporated in the wall and tubule of the nematocyst. The fact that the different localizations of Cnidoin can only be revealed by applying different fixatives might indicate an altered molecular arrangement or different interaction partners of Cnidoin in the capsule wall and tubule.

Cnidoin possesses terminal CRD domains and recombinant protein shows a strong tendency to aggregate and to form fibres even under harsh conditions like 8 M urea. In the presence of reduced glutathione it polymerizes with itself very quickly. This shows that Cnidoin has features to also act with other proteins containing CRD domains, like minicollagens or NOWA (Engel *et al.*, 2001; Engel *et al.*, 2002; Adamczyk *et al.*, 2008) although a covalent interaction remains to be demonstrated. All these proteins are structural components of the capsule wall or tubule. Therefore the linkage of structural proteins by disulfide bonds can be considered as one of the major features of nematocysts. Disulfide bonds are very stable and the dense network provides the molecular basis for the extraordinary properties of the capsule. While the minicollagens form a stiff and tear resistant meshwork, the incorporation of Cnidoin renders the nematocyst structure elastic. The nematocyst is loaded with an extreme pressure of 150 bar by the synthesis of poly-γ-glutamate (Weber, 1989;

Szczepanek *et al.*, 2002). The mature capsule has a stretched configuration (Figure 3.1). The release of this mechanically stored energy enables the extreme acceleration by which the spines and the tubule are released during discharge. As shown by MD simulations, Cnidoin acts like a typical elastic protein. The driving force for the collapse of Cnidoin is the burial of hydrophobic residues inside the protein. With Cnidoin, the long-time postulated elastic component of the nematocyst could be identified.

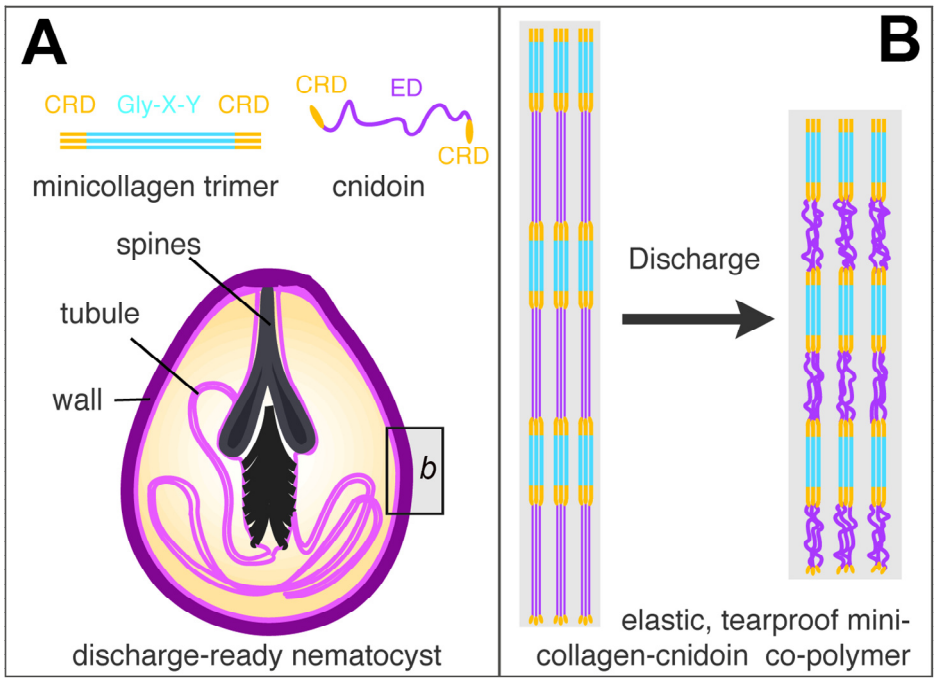


Figure 3.1: Schematic representation of a nematocyst with the contributions of minicollagens and Cnidoin. In the discharge-ready nematocyst, the minicollagen-Cnidoin co-polymer is stretched. Upon discharge, the elastic Cnidoin relaxes, the stored energy is released.

The elastic properties might not only play a role in the storage of mechanical energy to facilitate the discharge. They might also be used to stabilize the capsule and to prevent a rupture of the rigid collagenous network during discharge. The tubule is attached to the capsule wall and when it is expelled it must not rupture. Otherwise an adhesion on the prey or substrate would not be mediated. The prey could escape, nematocyst-mediated locomotion would be impossible. The rupture of the nematocyst would also risk damage of the surrounding tentacle tissue. This could be avoided by rendering the capsule structure elastic, so that it can withstand the biomechanical stress.

The molecular properties make Cnidoin an interesting target for biotechnological applications. The elastic polymers could be used as biological plastic, the combination with minicollagens induces a stiffness and tear-resistance to these

polymers comparable to spider silk that have already been subjected to biotechnical applications (Omenetto & Kaplan, 2010). By the proportion of each component, the properties could be fine-tuned.

3.2 The control of nematocyst discharge: TrpA & Nematocalcin

The apical surface of nematocytes carries the cnidocil apparatus. A sensory structure by which the animal can detect prey-derived mechanical and chemical stimuli. The cnidocil apparatus consists of the central cnidocil and a horseshoe-like arrangement of stereocilia. The molecular components involved in triggering discharge were unknown so far. The cnidocil apparatus provides structural similarity to the hair cells in the inner ear of vertebrates, therefore a similar mechanotransduction machinery can be expected. Deflection of the cnidocil and the stereocilia by a passing prey could lead to a calcium influx, depolarisation of the cell and finally to nematocyst discharge. For hair cells in mice, TrpA has been proposed to be the mechanotransduction channel (Corey *et al.*, 2004) but it was shown not to be essential for hearing in the mouse model (Kwan *et al.*, 2006). Nevertheless, TrpA is clearly involved in mechanosensation in the nematode *Caenorhabditis elegans* (Kindt *et al.*, 2007) and in the sea anemone *Nematostella vectensis* (Mahoney *et al.*, 2011) were it triggers nematocyst discharge.

The Hydra homologue of the TrpA ion channel could be identified and cloned. Its sequence comprises six transmembrane domains and 16 ankyrin repeats in the cytosolic N-terminus.

The expression of HyTrpA1 was restricted to nematocytes and was shown to take place in nests of developing nematocytes. In Western Blot analysis, the protein can be detected in the whole animal, even in the tentacles. This means after expression in developing nematocytes, TrpA is incorporated into its final localization within the cell and migrates with the maturating nematocyte to the tentacles. In immunostainings the protein can be detected in nests of developing nematocytes as well as in the stereocilia of the cnidocil apparatus of mature nematocytes.

The discharge process is known to depend on extracellular calcium. Therefore TrpA provides a good candidate for a mechanosensitive transduction channel. The N-terminal ankyrin-repeats might bind to the actin cytoskeleton of the stereocilia. Due to the protein binding properties of the ankyrin-repeats and its spring-like behaviour (Gaudet, 2008), this linkage could correspond to the gating spring. A similar

arrangement has been proposed for the vertebrate hair cell, but could so far not been proven without doubt.

In addition, the channel could be associated to tip-links that bind to adjacent stereocilia. While in vertebrate hair cells cadherin23 and protocadherin15 are known components of the tip-links (Siemens *et al.*, 2004; Ahmed *et al.*, 2006), the molecular nature of these links in cnidarians is still elusive, although the presence of inter-stereocilia-connections has been shown by electron microscopy (Golz & Thurm, 1991). So far, no cadherin23 or protocadherin 15 homologues have been identified in *Hydra*.

Penta-EF-hand proteins have been shown to bind directly to ion channels. The penta-EF-hand protein Peflin binds to a TrpN channel from *Xenopus* in yeast two-hybrid screens, GST-pulldowns and a cell model (Wiemuth *et al.*, 2012), Sorcin has been shown to interact with the cardiac calcium release channel (ryanodine receptor, RyR) and to modify the open-probability of the channel (Lokuta *et al.*, 1997). In *Hydra* the penta-EF-hand protein Nematocalcin could be detected in immunostainings in stereocilia as well as TrpA. But while actin and TrpA encompass a more distal part of the structure, Nematocalcin is located at the basal part. Indeed, only the upper part of the stereocilia is associated with membranes, the rods anchor the structure in the cytoplasm (Golz & Thurm, 1991). The tip of the stereocilia is therefore suspected to be the localisation of the mechanotransduction machinery. Whether Nematocalcin and TrpA interact on the molecular level has yet to be shown. So far, an interaction of both proteins could not be proven on the biochemical level. TrpA could interact with Nematocalcin or other calcium-sensitive proteins by its ankyrin-repeats to be regulated by calcium. Moreover calcium itself has been shown to influence the gating of TrpA directly (Doerner *et al.*, 2007). The regulation and gating behaviour of TrpA will be subject of further studies, including the characterization in HEK293 cells by patch-clamp measurements. In this system a mechanical pulse can be applied by a pointed liquid flow to test the mechanosensitivity of the channel. A functional characterization of the channel in a hetereologous system seems to be essential, as functional studies in *Hydra* appear to be very hard to address. The inhibition of Hydra's feeding behaviour by incubation with FM1-43 provides a first hint. But FM1-43 is not selective for TrpA. It could also act on other Trp channels. Different specific chemical inhibitors and activators of TrpA have been applied (Cinnamaldehyde, Polygodial, Zinc chloride, HC 030031, A 967079), but none of them could provide

functional data as they were not tolerated by the animals. In *Nematostella*, a modulation of the TrpA ion channel by TrpA activators (URB-596 and Polygodial) and inhibitors (Ruthenium Red and streptomycin) has been shown to have an impact on the discharge rate (Mahoney *et al.*, 2011).

The cnidocil is mechanosenory without any doubt and the discharge process itself has been shown to depend on external calcium (Nüchter *et al.*, 2006) but the calcium signals during discharge could not be visualized so far. Experiments I performed with the calcium-sensitive dye Fura-2 were not able to detect a calcium signal in *Hydra* nematocytes during discharge induced by electrical stimuli. It might be worthwhile to try a more sensitive dye but the problem may be that of the uptake of the dye as *Hydra* is covered by a mucous glycocalyx that might prevent internalisation of the dye. Furthermore all Hydra cells will take up the dye and it might be difficult to distinguish the nematocyte derived signal from the tissue background. Nevertheless it would be very interesting to visualize the calcium signals during discharge, as this could clarify the site of calcium entry from the surrounding medium and the progression of the signal inside the cell. It is still unclear whether other signalling molecules or proteins transport the calcium signal further.

The identified penta-EF-hand protein Nematocalcin could play a role in calcium signalling prior to or during discharge. The protein is highly conserved when compared to other well-characterized penta-EF-hand proteins like Sorcin or Grancalcin. It has been shown that penta-EF-hand proteins like Grancalcin (Jia *et al.*, 2000) form dimers by the interaction of their fifth EF-hand motifs. Whether the same is true for Nematocalcin has to be clarified in the future. Nematocalcin can be detected in immunostainings in the stereocilia surrounding the cnidocil at a more basal position than TrpA. This localisation of Nematocalcin was also confirmed by immunogold TEM. Grancalcin has been shown to be relocated from the cytoplasm to the plasma membrane upon binding of calcium (Boyhan *et al.*, 1992). Calcium signalling is thought to involve the N-terminal glycine and proline rich domain (Jia *et al.*, 2000), binding of calcium results in structural changes and hydrophobic residues are exposed to the aqueous environment (Lollike *et al.*, 2001) which facilitates localization to hydrophobic structures like the membranes. The localization of Nematocalcin in nematocytes in the absence of calcium remains controversial. In some preparations it could be detected in a more cytosolic distribution, maybe associated with the microtubular basket that surrounds the capsule. Whether this

behaviour is random, depends on received stimuli or on the presence of calcium will have to be clarified in future. As Nematocalcin was detected during the proteome analysis of isolated nematocytes, it can be speculated that it indeed is associated tightly with the nematocyst, at least a fraction of it.

Grancalcin interacts with L-Plastin, a protein known to have actin bundling activity (Lollike *et al.*, 2001). As actin is a major structural component of the stereocilia of the cnidocil apparatus in *Hydra*, this might provide a hint for the function of Nematocalcin. It might play a role in regulating the actin bundling of the stereocilia and therefore in adaption of the sensory structure to a given stimulation. For hair cells adaptation has been proposed to be mediated by a myosin, attached to tip-links and moving along actin filaments as well as calcium dependent closure of the transduction channel (see Hudspeth, 2005 for review).

Although the immunostainings show a clear localization of Nematocalcin in the stereocilia of the cnidocil apparatus that has been confirmed by TEM and the transgenic animals, the in situ showed a broader expression. This could be due to very similar penta-EF-hand proteins in *Hydra*, which are expressed more ubiquitously. NCBI BLAST analysis of Nematocalcin against *Hydra magnipapillata* revealed the presence of four highly similar predicted proteins (Table 2).

Table 2: Compilation of Nematocalcin-similar proteins in Hydra.

Name	Accession number	Query coverage	Identity
peflin-like	XP_002168493.2	90%	36%
programmed cell death protein 6-like	XP_002169270.2	83%	35%
calpain-B-like	XP_002167620.1	80%	37%
grancalcin-like	XP_004212885.1	61%	34%

The observed expression pattern in the in situ hybridization might therefore be due to binding of the probe to similar transcripts or be caused by unspecific background staining. The gene is too short to select another probe of appropriate size. Nevertheless, some stained nematocytes could be observed, indicating expression of Nematocalcin in almost mature nematocytes.

The transgenic animals expressing Nematocalcin eGFP under the control of the NOWA promotor confirm the immunostainings and the presence of Nematocalcin in the cnidocil apparatus. Furthermore they provide a powerful tool for further research.
Unfortunately the mutation of calcium-coordinating residues abolished expression of the corresponding transgenic construct. It might be worthwhile to check the expression of different intermediate variants or to change the amino acids to glutamine and asparagine instead of alanine in order to perturb the protein structure less and to produce a functional protein.

3.3 Regulation of nematocyst morphogenesis: Nematomyosin & PKD2

The formation of nematocysts in Cnidarians is a complex process that has to be carefully regulated. The different types of capsules require distinct morphologies of their tubules. Tubules of various capsules differ in shape, length and diameter as well as in the possession of spines (Weill, 1934). In Hydra, nematocytes differentiate from i-cells. The nematocyst is formed as a giant post-Golgi vesicle (Slautterback & Fawcett, 1959; Holstein, 1981). The capsule primordium is surrounded by an arrangement of microtubules (Slautterback, 1963) that originate in a pair of centrioles near the Golgi (Westfall, 1966). The microtubules might guide the arriving vesicles to the site of fusion (Slautterback, 1963) or change the size of the capsule primordium to shape the growing nematocyst (Holstein, 1981). Wall proteins form a thin layer along the vesicle membrane covered with microtubules that thickens by the addition of further structural proteins. The microtubules seem to be required for a straight capsule wall. If the wall extends above the microtubules it appears wrinkled (Watson & Mariscal, 1984b).
The identified nematocyte-specific protein Nematomyosin belongs to the non-muscular type II myosins. They are characterized by a head-domain, which hydrolyses ATP to convert chemical energy into movements, and a tail-domain, that can interact with other proteins by coiled-coil interactions. These myosins are responsible for movements of organelles and vesicles inside the cell along actin filaments. Non-muscle type II myosins have also been described to locate to Golgi membranes and on Golgi derived vesicles (Ikonen *et al.*, 1997). The interaction of myosin II with the Golgi membrane is enhanced by G-protein activation. Myosin type II is involved in budding and coating of a subpopulation of non-clathrin coated vesicles (Narula & Stow, 1995) that derive from the trans-Golgi-network (TGN).

Nematomyosin has been shown to be associated with the nematocyst by its detection during the proteome analysis. Immunostainings revealed the presence of the protein in nests of developing nematocytes. The abundance of Nematomyosin alters during the developmental stages. While it surrounds the whole capsule at early stages, it is restricted to a collar around the basis of the outgrowing tubule. In almost mature capsules, only some protein is left at the apical part of the capsule. Due to its localization, a function of Nematomyosin during the outgrowth of the tubule can be assumed. Taking into account that myosins can create movements and forces, it could shape the nematocyst vesicle. By extending the vesicle in early phases of development it allows the capsules to assemble inside. When the tubule starts forming, a constriction of the nematocyst vesicle is needed. After the tubule has invaginated, the structure of the capsule is determined and the myosin is not necessary for the development of the capsule any more. It is crucial for the further understanding of Nematomyosin and the capsule morphogenesis itself to reveal interaction partners of Nematomyosin. Typically myosin interacts with actin, while forces along microtubules are created by dynein and kinesin. So far, no actin cytoskeleton appears to be involved with the capsule development. As described in the beginning of this section the nematocyst primordium is covered by a basket of microtubules. It might therefore important to consider a direct or indirect interaction of Nematomyosin with microtubules. Such interactions could be elucidated by binding assays and pulldown experiments. It has been proposed that myosin can bind to microtubules or microtubule associated proteins in vitro (Shimo-Oka *et al.*, 1980) but the cross-play of myosins with actin and microtubuli is still elusive. It has been shown that myosins can intercalate directly in the membrane (Schewkunow *et al.*, 2008) by inserting the tail region between the phospholipids of the membrane. Therefore Nematomyosin could be either inserted into the nematocyst vesicle membrane or interact with the proteinous capsule itself and shape the nematocyst primordium and growing tubule by tearing and constriction. Which possibility accounts for Nematomyosin remains to be shown. The fact that Nematomyosin is crucial for the development of intact capsules is provided by experiments where Nematomyosin is inhibited by Blebbistatin. This leads to malformed capsules.

The scaffold of the outgrowing tubule is formed by the proteoglycan Chondroitin (Adamczyk et al., 2010) that is associated to Nematogalectin (Hwang et al., 2010). Nematogalectin in turn might recruit Minicollagens to the tubule by binding their N-

glycosylation sugar residues (Engel *et al.*, 2001). As soon as the everted tubule is formed outside of the capsule wall, the Golgi regresses and degrades (Slautterback & Fawcett, 1959). The tubule is inverted and coiled inside the matrix of the nematocyst. During the step of invagination fibres have been described (Watson & Mariscal, 1984a) to be located at the inner wall of the external tubule and the outer wall of the inverting tubule. These fibres interconnect pleats of the tubule and appear to bind calcium (Watson, 1988). The discrete function of these fibres is unclear. They might facilitate the invagination process. The invagination has been proposed to be accompanied by increased osmotic stress and therefore a loss of fluid from the capsule (Watson & Mariscal, 1984a).

In immunostainings PKD2 can be detected in nests of developing nematocytes. The strongest signal can be detected at stages with maximally outgrown tubule. The signal appears first at the onset of tubulation, at the site were the tubule grows out. At these early stages of tubulogenesis PKD2 shows a similar distribution as Nematogalectin B (Hwang *et al.*, 2010). Interestingly a Galectin-3 acts as a natural brake on cystogenesis (Chiu *et al.*, 2006) in the polycystic kidney disease and Nematogalectin is important for the tubulation of *Hydra* nematocytes (Hwang *et al.*, 2010). Thus PKD2 and Nematogalectin might interact and control the outgrowth of the nematocyst's tubule. Co-staining both proteins might provide further hints towards the mechanics and regulation of tubulation.

At stages of outgrown tubule, the signal is very bright and located inside the whole nematocyte. This is probably due to massive expression and therefore large amounts of the protein in the ER. Nevertheless the protein is still thought to be in the membrane of the nematocyst vesicle. It could be involved in sensing the tension created by the outgrowing tubule. As different capsule types require different tubule length a regulation of the tubule growth is absolutely needed. A distinct signal, which could be mediated by PKD2, has to be given when the tubule is ready for invagination.

Apart from pure calcium signalling, Polycystins have been shown or suspected to act on multiple signalling pathways: mTOR, NF-κB, Hippo (canonical Wnt is modulated by Hippo) (Boletta, 2009, Banzi *et al.*, 2006, Happé *et al.*, 2011, Varelas *et al.*, 2010). Which ones are linked to nematocyst morphogenesis has to be shown. Interestingly it has been shown that NF-κB is essential for the nematocyte development in *Nematostella* (Wolenski *et al.*, 2012). PKD2 could not only sense the size of the

outgrown tubule but also have an impact on the production and crosslinking of structural components as it was shown to regulate the secretion and assembly of ECM components (Mangos *et al.*, 2010). In addition, PKD2 has been identified as an important factor of the growth of the *Schizosaccharomyces pombe* cell wall as well as in vesicle trafficking (Aydar & Palmer, 2009). In polycystic kidney disease, caused by mutations in either PKD1 or PKD2, defects in the ECM can be observed (Ramasubbu *et al.*, 1998). This is of particular interest, as a significant portion of nematocyst proteins contain ECM motifs (Balasubramanian *et al.*, 2012).

As shown by the immunostainings, PKD2 seems to enter the nematocyst passively by tubule invagination. There, it is not thought to be active anymore. This fact is further proven by Western Blot analysis, in which the full-length protein could not be detected in isolated nematocysts. In Western Blot analysis, the protein was detected in head as well as in body column lysate. The presence in the head is unexpected if the protein plays a role in capsule development, as in the tentacles only mature capsules are present. The signal might result from contaminant body column tissue in the head lysate sample. In Immunostainings, no signal in the tentacles can be observed.

A regulation of the localization of PKD2 is crucial in nematocytes to ensure its proper function. The experiments in yeast showed that the C-terminal domain of PKD2 is involved in protein trafficking. Only the C-terminal truncated mutant was transported to the plasmamembrane while the wildtype was retained in the ER when expressed in yeast. The C-terminal part of the protein contains clusters of acidic amino acids. Those might be recognized by the phosphofurin acidic cluster machinery (PACS), as it was already shown for the human PKD2 channel (Köttgen *et al.*, 2005). Although located at the plasmamembrane in yeast, the channel did not show any activity in growth tests. This might indicate that it needs to be activated or the C-terminal truncation affects its function. PKD2 has been shown to be inhibited by low pH (González-Perrett *et al.*, 2001) although no values are given, the growth conditions of yeast were maybe not optimal for the channel.

In human renal tissue PKD2 interacts closely with PKD1 by coiling of their C- termini (Hanaoka *et al.*, 2000), however both proteins do not completely overlap in localization in human renal tissue, suggesting that they might be active individually as well as in a complex together (Foggensteiner *et al.*, 2000). The C- terminus of PKD1 is thought to be cleaved upon mechanical stimuli and transported in the nucleus,

where it might act on gene expression (Chauvet *et al.*, 2004). Several proteins from *Hydra* have been predicted as PKD1-like. It might be possible that both proteins do also interact in the capsule morphogenesis.

As the functional characterization of PKD2 in *Hydra* is still missing, this gene might be a good candidate for siRNA treatment. By siRNA treatment the effect of PKD2 on developing nematocytes should be clearly visible. If it is required as a tube size sensor or as a regulator of polymerization, knock-down should lead to deformed capsules that show abnormal tubules. The inhibition of PKD by Gadoliniumchloride reduced the PKD2 signal, but no malformed capsules could be observed. The remaining channel activity might be sufficient to maintain normal development and the nematocyst development might follow the all-or-nothing principle.

4. MATERIALS & METHODS

4.1 Animals

Hydra magnipapillata was used for all experiments except the creation of transgenic animals. Animals were cultured in hydra medium (1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1 mM NaH_2CO_3, pH 7.8) at 18 °C and fed two to three times a week with freshly hatched *Artemia salina* nauplii. Animals used for the experiments were starved for 24 h.

For the creation of transgenic animals, *Hydra vulgaris* AEP was used. Animals were fed daily for a month and then starved for a week to induce gametogenesis.

The starlet sea anemone *Nematostella vectensis* was cultured in 1/3 seawater pH 7.8 at 18°C. The animals were fed once a week with *Artemia*. To induce gametogenesis the incubation temperature was shifted from 18°C in the dark to 26°C in the light. An alternate method is to maintain the culture in aquariums. There the animals produce constantly some eggs and sperm that are released into the medium, where the fertilization occurs. The development from the embryo to the planula larvae takes 2 days at 18°C, till a primary polyp is formed 10 days have passed. For immunostainings primary polyps were used.

4.2 Transgenic animals

For Nematocalcin transgenic animals were produced by injection (FemtoJet) of embryos according to Wittlieb *et al.*, 2006. The transgenic construct consists of a pBluescript II SK- derivate with the NOWA promotor driving the gene expression. The protein is visualized in the animals by its C-terminal eGFP tag. Transgenic constructs were checked by gene gun whether they are expressed or not.

To access the functional aspects of Nematocalcin, the putative calcium-coordinating sites (D86, D88, E97 for EF2 and D116, D118, E127 for EF3) were mutated to alanine by site directed mutagenesis by PCR. The mutated construct was not expressed in Hydra as determined by gene gun experiments.

4.3 Nematocyst isolation

Intact nematocysts were isolated from whole hydra tissue after freezing the tissue in 1.5 ml reaction tubes for at least one hour at -80°C. The thawed tissue was homogenized by a syringe. The isolation of nematocysts represents a density

gradient centrifugation. 10% sucrose was added to the solutions in order to prevent an osmotic triggered discharge of the nematocysts. After addition of 50 % (v/v) Percoll in ddH_2O with 10% sucrose and 0.003 % Triton-X 100 the homogenized tissue samples were centrifuged at 7 500 g and 4 °C for 15 minutes. The supernatant was discarded and the pellet was resuspended in 50 % (v/v) Percoll in ddH_2O with 10% sucrose and 0.003 % Triton-X 100 and centrifuged again for 15 minutes. The pellet was resuspended in PBS with 0.003% Triton-X 100, 10% Sucrose. After one more centrifugation for 10 minutes at 7500 g and 4°C, the capsules were resuspended in a little volume PBS, 10% Sucrose. Isolated nematocysts were quantified in a Neubauer counting chamber and used directly for experiments or frozen at -20°C. For counting an aliquot of the capsules was diluted 1:10. Each square of the chamber was counted and the mean was multiplied by 100.

4.4 Preparation of cDNA and molecular cloning

Isolation of whole RNA was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was transcribed by Superscript III Reverse Transcriptase (Invitrogen) into cDNA. Using a pair of appropriate primers (see Table 3) the gene of interest was amplified by PCR.

Table 3: Primer for gene amplification from cDNA.

Tm = melting temperature

* The Nematocalcin reverse primer comprises 3'UTR. Further subcloning was necessary to obtain a pure Nematocalcin cDNA clone.

	primer sequence (5'- 3')	T_m (°C)
Cnidoin forward	ATGTCTCGATTACTACTTC	53.88
Cnidoin reverse	TTATCTCTTTTTACCAAAAGCTCC	59.98
TrpA forward	ATGGATAGCAACTCAGAGTCACTGG	66.74
TrpA reverse	TCAATCAACTTTGTTTTTGAACGAAGAACTTG	66.92
Nematocalcin forward	ATGGCATATCCAGGATACAATC	60.36
Nematocalcin reverse	CTAGCTGGGAAACAACCAG *	60.60
Nematomyosin forward	ATGGACAACGAAGAAGATAGTTTA	60.78
Nematomyosin reverse	TCAATCGGTGCCGTCCTCATC	67.37
PKD2 forward	ATGAGTGCTACAAAAAGAATTAGAAGTACAAAAG	65.62
PKD2 reverse	GTAATAATTTCACACTCACTCGCTG	62.96

The PCR product was purified and ligated at 4°C overnight into the pGEM-T vector (Promega) by TA cloning. As the Precisor polymerase was used in the PCRs to amplify the gene of interest from cDNA, an additional A-tailing with the EuroTaq polymerase had to be performed prior to ligation in pGEM-T. The ligation was precipitated with ethanol and transformed into E. coli by electroporation. Positive clones were selected by colony PCR. The plasmids were extracted by a Miniprep kit (Promega). The sequence was verified by automated sequencing at Eurofins MWG Operon.

4.5 In situ hybridization

The in situ probes were amplified from the pGEM-T clones by PCR using the M13 forward and reverse primers. The purified and quality checked PCR product was transcribed in vitro to DIG labelled RNA by Sp6 and T7 RNA polymerases, purified by precipitation with ammonium acetate and quality checked on a 1% agarose gel (see Table 6 for a compilation of all probes used). The in situ probes were diluted to approximately 5 ng/µl with hybridizing solution and then used in a 1:100 – 1:1000 dilution for the experiment. For TrpA a cocktail of four different probes was used.

Animals were relaxed with 2% urethane in hydra medium and fixed overnight with freshly prepared 4% PFA in hydra medium. The fixed animals were transferred to 100% ethanol and rehydrated in 5 minute steps using 75%, 50%, 25% ethanol in PBS, 0.1% Tween20 (PBT). After three 5 min washing steps with PBT the animals were incubated with 1x Proteinase K in PBT for 7 minutes. The reaction was stopped by adding 4 mg/ml glycine in PBT. Then, the animals were equilibrated in 0,1 M Triethanolamin (TEA) for 2x 5 min and incubated for 5 min each with 0.25% and 0.5% acetanhydride in TEA, followed by 2 washing steps with PBT. Then, a re-fixation with 4% PFA was performed for 20 min at room temperature, followed by five 5 min washing steps with PBT. The animals were incubated with hybridizing solution (50% Formamide, 5x SSC [0.75 M NaCl, 0.075 M Trisodium citrate, pH 7.0], 1x Denhardt's [1% Polyvinylpyrrolidone, 1% Ficoll, 1% BSA], 200 µg/ml yeast RNA, 100 µg/ml Heparin, 0.1% Tween20, 0.1% Chaps, 10% H_2O) for 10 min and then pre-hybridized in hybridizing solution for 2 hours at 55°C. The probes were diluted in hybridizing solution and denatured by heating (75°C, 10 min). The animals were incubated with the probes for 2.5 days at 55°C. Unbound probes were removed by 5 minute washing steps with 100%, 75%, 50%, 25% hybridizing solution in 2x SSC

followed by 2 incubations for 30 min in 2xSSC, 0.1% Chaps. The animals were equilibrated in Maleic acid buffer (MAB: 100 mM Maleic acid, 150 mM NaCl, pH 7.5) for 2x 10 min and blocked in 1% blocking reagent (Roche) in MAB for 2 hours at room temperature. For the detection of the DIG labelled RNA probes an anti-DIG antibody coupled to alkaline phosphatase was used at 1:4000 in blocking solution at 4°C overnight. Unbound antibody was washed out during eight 30-60 min washing steps with MAB, followed by an overnight washing step. To detect the signal the animals were first equilibrated 2x 10 min in NTMT (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 0.1% Tween20) at room temperature and than incubated in NBT/BCIP (Roche, premixed solution) 1:50 in NTMT in the dark at 37°C. In some cases separate NBT and BCIP solutions (Roche) were used. 3.75 µl of each stock solution was added per ml staining solution. When reaching the optimal signal to background ratio, the reaction was stopped by adding 100% ethanol. The animals were rehydrated by incubation for 5 min in 75%, 50% and 25% ethanol in 0.1x PBS. After a final rehydration step in PBS the animals were mounted on microscopic slides in PBS 90% glycerol.

For double *in situ* hybridization with Minicollagen-1 the animals were incubated simultaneously with DIG-labelled Cnidoin and FITC-labelled Minicollagen-1 probes. After incubation with the DIG antibody (1:2000) and MAB washing, the staining with NBT/BCIP was performed. The staining reaction was stopped with ethanol and after rehydration the animals were incubated with the FITC antibody (1:2000) overnight. After MAB washing, the minicollagen-signal was detected with the FastRed substrate (Roche). The reaction was stopped with 100 mM glycine, 0.1% Tween20, pH2.2. Samples were washed in PBS and mounted on microscopic slides with PBS 90% glycerol. In situ images were captured with the Nikon Eclipse 80i using DIC contrast. Image processing was performed with the Nikon software NIS Elements, Adobe Photoshop Elements or Gimp.

4.6 Recombinant protein expression and purification

Recombinant expression in *E. coli* BL21 (DE3) cells was performed from a pET21b vector (Novagen), which introduces a C-terminal polyhistidine tag and is inducible by IPTG. Cnidoin protein was exclusively found in inclusion bodies and purified under denaturing conditions (8M urea) using Ni-NTA beads.

Expression in HEK293 cells was obtained by transfecting the cells with pCEP Pu

vector. The vector induced a His-tag and a BM40 signal peptide for the secretion of the recombinant protein into the medium. Protein purification from the medium was performed using Ni-NTA beads. Minicollagen-1 was expressed in HEK293 cells in fusion with the maltose binding protein (MBP) to prevent it from aggregation (after Engel *et al.*, 2001).

4.7 SDS PAGE & Western Blot

Isolated nematocysts were solubilised by heating (95 °C, 10 min) in sample buffer with or without 2-mercaptoethanol as indicated in the experiments. Hydra lysate was prepared by dissolving an animal in reducing or non-reducing sample buffer by heating and vortexing. Nematocyst ghosts, the insoluble fraction of nematocysts, were obtained by extended SDS washing of the isolated capsules. The ghosts were afterwards solubilised in sample buffer. When Hydra head and body column lysate was analyzed, 5 heads and 1 body column were considered to contain equal amounts of tissue. The heads and body columns were dissolved in sample buffer by heating and vortexing.

The samples were separated by SDS-PAGE, using 12% separating and 4% stacking gels, and transferred to nitrocellulose membranes (GE Healthcare, Amersham Hybond ECL) by wet blotting with 350 mA for one hour or overnight at 20 mA. Blocking was performed for 1 h with 5% skim milk or BSA (depending on the antibody, see Appendix Table 4) in PBS 0.2% Tween20. After 3x 10 min washing with PBS 0.2% Tween20 the membrane was incubated with the primary antibody (1:1000 in 1% milk / BSA in PBS 0.2% Tween20) for 1.5 h or overnight, followed by three 10 min washing steps. The primary antibody was detected using a secondary antibody coupled to horseradish peroxidase (1:10 000 in 1% milk / BSA in PBS 0.2% Tween20) for 1 h. Detection of chemoluminescent signals by ECL was used to visualize the signals on films (GE Healthcare, Amersham Hyperfilm ECL).

For the polymerization assay of Cnidoin reduced glutathione was added to a final concentration of 1 mM to recombinantly expressed Cnidoin. The protein was incubated at 37°C. Samples were taken at indicated time points, mixed with non-reducing sample buffer and boiled for 5 minutes at 95°C. Afterwards the samples were kept on ice, until all time points were covered. The samples were separated by a gradient SDS-PAGE (4-20%, Bio-Rad), blotted on nitrocellulose, blocked with 5% BSA and detected by an anti-penta-his antibody.

The copolymerization of recombinant Minicollagen-1 MBP (maltose binding protein) and Cnidoin was performed in the presence of 10 mM reduced glutathione. Minicollagen-1 MBP was purified by Ni-NTA from HEK293 cell culture supernatant. Cnidoin was obtained by isolating recombinant protein from E. coli. As indicated in the experiment, various amounts of Cnidoin were added to the Minicollagen-1 containing sample in the presence of glutathione. The sample was mixed with non-reducing sample buffer and boiled immediately at 95°. Separation was obtained by using 4-20% gradient SDS-PAGE and detection was performed as described above.
Deglycosylation of Cnidoin was accessed by incubating nematocyst and Hydra samples with deglycosylation mix according to the manufacturer's instructions (New England Biolabs, Protein Deglycosylation Mix). This enzyme mix will remove almost all N-linked and simple O-linked glycans. Incubation was prolonged to 6 hours. 10 mM DTT were added to the 10x Glycoprotein Denaturating Buffer to break the disulfide bonds of the capsules. The control reaction (included in the kit) under the same modified conditions worked fine. Sample separation and detection were performed as described above.

4.8 Immunocytochemistry

Hydra magnipapillata were relaxed in 2% urethane in hydra medium and then fixed in freshly prepared 4% PFA in hydra medium, Lavdovsky fixative (50% ethanol, 10% formaldehyde, 4% acetic acid, 36% water) or ice-cold methanol depending on the antibody (see Appendix Table 4 for the requirements of each antibody). The fixative was removed by three 10 min washing steps with PBS 0.1% Triton X100. The antibody was diluted in PBS 1% BSA and incubated overnight at 4°C. For co-stainings of two or three antibodies (see Appendix Table 5) all antibodies were incubated simultaneously overnight at 4°C. To remove unbound antibodies three 10 min wash steps with PBS 0.1% Triton X100 were performed. The incubation with the second antibodies was performed for 2 hours at room temperature. The secondary antibodies were diluted 1:400 in PBS 1% BSA. In some experiments Hoechst33258 staining of nuclei was performed. Therefore the samples were incubated with Hoechst33258 1:1000 in PBS after the incubation with the secondary antibody for 10 minutes. To remove unbound antibodies the animals were washed 3 times with PBS and then mounted on object slides with PBS 90% glycerol.
Nematostella were relaxed in the dark. Then they were paralyzed by the careful

addition of equal volume of 7.14% (350 mM) $MgCl_2$ in Nematostella medium. Afterwards fixation and immunostaining was performed as described above.
Fluorescence images were captured with the Nikon A1R confocal laser-scanning microscope, in part at the Nikon Imaging Center, Heidelberg. Image analysis and processing was performed with Nikon NIS Elements, Adobe Photoshop Elements and Gimp.

4.9 Immunogold-labelled Transmission Electron Microscopy

Native, untreated Hydra samples were rapidly frozen by high pressure freezing to prevent the formation of ice crystals that could damage the tissue (Holstein *et al.*, 2010). Cryo-fixation preserves the structures of the samples very well and prevents artefacts by chemical fixation. By freeze-substitution the samples were stabilized. The rehydrated and fixed samples were subjected to cryo-sectioning that preserved the fine internal structures very well. Resin-sections have been tried as well, but cryo-sectioning produced better samples. Immunostainings were performed on these sections with the Nematocalcin antibody. The signal was detected with secondary antibodies that were coupled to colloidal gold particles. The electron dense gold is visible in the transmission electron microscope as a black dot.

4.10 Transmission Electron microscopy of recombinant Cnidoin

Supernatants of recombinant Cnidoin samples solubilised in 8M urea were absorbed to freshly glow-discharged thin carbon films supported by thick perforated carbon layers and negatively stained with uranyl formiate following standard procedures (Engel, 1994).

4.11 Scanning Electron microscopy of recombinant Cnidoin

Fibres from purified Cnidoin protein were washed several times with 8M Urea to remove smaller aggregates. The fibres were attached to poly-L-lysine (Sigma-Aldrich) coated glass cover slides and incubated for 10 min in 0.2% glutaraldehyde, 2% formaldehyde in aqua dest. After 3 washes (distilled water) the sample was incubated with 2.5% glutaraldehyde and after a 10 min washing step (distilled water) contrasted with 4% osmium tetroxide for 45 min at room temperature. The sample was dehydrated by washing with increasing concentrations of ethanol before transferring to 100% acetone. After critical point drying, the samples were coated and

imaged.

4.12 Fourier-Transform Infrared-Spectroscopy (FTIR)

The degree of ordering was assessed on the basis of Fourier-transform mid-infrared spectroscopy of dried films of Cnidoin elastic domain. For recombinant expression of the Cnidoin elastic domain a DNA fragment coding for amino acids GGQM-AGCG was amplified by PCR and cloned four times as a tandem repeat into the pet21 vector (Novagen). Protein purification was performed under denaturing conditions via the C-terminal his-tag. For elution, buffer conditions were changed to PBS (PBS [136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2PO_4] including 10 mM DTT, 500 mM NaCl, 250 mM Imidazole, protease inhibitor mix) and the eluted protein was immediately applied for the experiment. For comparison, nine further proteins (hemoglobin, bovine serum albumin, concanavalin, ribonuclease s, lysozyme, ferritin, cytochrome c, elastase, casein) were investigated in the same manner. Thus, 10 different protein solutions at 1 mg/ml each were prepared in the elution buffer for Cnidoin elastic domain (see above). Each solution was pipetted to 10 wells (50 µL per well) of a 96-well silicon sample carrier and left to dry. This procedure was repeated on three further sample carriers and each sample carrier was henceforth investigated separately in order to check for consistency and reproducibility. Details of the technical setup for spectroscopy are described in Rohleder *et al.*, 2005. After vector normalization in the region from 1600 cm^{-1} to 1715 cm^{-1} and background subtraction, the median absorbance of each 9-fold replication on each sample carrier was calculated. In order to investigate the degree of ordering of the proteins we followed the implications of Byler and Susi (Byler & Susi, 1986) in that, both, a low number of spectral components in the overall Amid I band as well as the existence and strength of a peak around 1645cm^{-1} are indicative for a low degree of ordering. The number of spectral components was estimated from fitting one Gaussian curve per spectral component and comparing the fit results on the basis of the Akaike Information Criterion (Akaike, 1974). Median spectra were fitted with up to 13 Gaussian curves and up to 10 random starting conditions for any fixed number of Gaussian curves. An example of a median spectrum of Cnidoin is shown in Figure 2.10 A together with a fit result for 5 Gaussian curves (i.e. five spectral components). All fit results were analyzed by means of the corrected Akaike Information Criterion

(AICc). Weights for the fit with i Gaussian curves were set to be $\frac{e^{-\frac{AICc_{\min}-AICc_i}{2}}}{\sum_i e^{-\frac{AICc_{\min}-AICc_i}{2}}}$ and the weighted arithmetic mean was calculated in order to yield the optimum number of Gaussians as well as its standard deviation.

For the investigation of peak positions and widths, those spectral components, which contributed less than 1% to the total signal (area under the curve) were omitted from further analysis in order to avoid misleading conclusions. The peak around 1645 cm^{-1} in the case of Cnidoin had an average width of 17.3 cm^{-1} and, mathematically, the difference to the widths of the other peaks is significant on the basis of a two-sided t-test.

However, despite the clear indications for the lower degree of ordering in Cnidoin it has to be noted that many assumptions and simplifications enter into this analysis such that the results should be considered as supplementary information supporting the hypothesis of Cnidoin being a mainly unordered protein.

4.13 Molecular dynamics (MD) simulations

The overall organisation of Cnidoin was addressed by DisEMBL predictions (Linding *et al.*, 2003). Two repetitive sequence units, namely *QMQGCGQQMPPMMSGCGG* and *QMQGCGQQLPLMMPGCVG*, were selected for investigation in MD simulations. Both of the units contain the *GCGQQ* motif and also a high content of methionine, two major features of Cnidoin.

All simulations were carried out using MD software package Gromacs 4.5.3 (Hess *et al.*, 2008). The two Cnidoin peptide units with extended initial structures were first solvated in boxes of TIP4P water with an ion concentration (Na^+ and Cl^-) of 0.1 mol/litre (Jorgensen, 1983). A cut-off of 1.0 nm was used for non-bonded interactions, and the Particle-Mesh Ewald method for long-range electrostatic interactions (Darden *et al.*, 1993). Periodic boundary conditions were employed to remove artificial boundary effects. In order to use a time step of 0.2 fs, all covalent bonds were constrained using the LINCS algorithm (Hess *et al.*, 1997). All simulations were performed under a constant temperature of 300 K and a constant pressure of 1 bar, using Nose-Hoover temperature coupling and Parrinello-Rahman pressure coupling methods, with coupling time constants of 0.4 ps and 4 ps respectively (Parrinello, 1981; Hoover, 1985; Nose, 2002). The simulation systems were first energy

minimized by using the steepest descent method. Equilibration of the solvent molecules was carried out for 500 ps, with all heavy atoms in the peptide restrained by a force constant of 1000 $kJ \cdot mol^{-1} \cdot nm^{-2}$. The two Cnidoin units were then fully equilibrated for 500 ns individually. Energy and coordinates of the simulation systems were collected every 1000 time steps.

Umbrella sampling along the end-to-end distance of the two-peptide units was performed to probe their elasticity. Structures of the two units with shortest end-to-end distances in the above-mentioned 500 ns equilibration were chosen as starting points. Two new simulation systems comprising ~55,000 atoms, which were large enough to accommodate fully extended peptides, were set up. The peptides were extended by a pulling force in force-probe MD simulations (Grubmuller *et al.*, 1996). Peptide conformations covering Z-components of end-to-end distances between 0.4 and 7.0 nm were chosen as starting structures for umbrella sampling, with distance intervals of 0.4 nm. A force constant of 500 $kJ \cdot mol^{-1} \cdot nm^{-2}$ was used for the umbrella potential. The sampling times were changed with peptide extensions, with longer simulation times of 150 ns for extensions shorter than 2.0 nm because of higher fluctuations, and shorter times of 50 ns for longer extensions. The potential of mean force was calculated by using the weighted histogram analysis method (Kumar, 1992).

4.14 Gadolinium chloride treatment

100 µM $GdCl_3$ (Sigma Aldrich) were added to the culture medium. Animals were fed 2-3 times a week Samples were taken at indicated timepoints and used for immunocytochemistry.

4.15 Expression of HyPKD2 in yeast

HyPKD2 was amplified by PCR with primers containing sites for homologous recombination (rec-sites). The PCR product and SalI linearized pGREG576 plasmid was co-transformed into *Saccharomyces cerevisiae* BY4741. By homologous recombination yeast creates the vector with the N-terminal GFP tagged PKD2 (Jansen *et al.*, 2005). The expression of the construct is driven by an inducible Gal-promotor. Positive clones can be selected by growth on –ura and no growth on –his SD Plates.

Growth tests on SGal –ura 500 mM NaCl, 50 mM CsCl as well as complementation

assays with the PLY240 strain (possesses no internal Potassium transporter) and growth inhibition zone assays with calcium and potassium were performed. They did not show any functionality of the channel. For the growth tests a fresh overnight culture was washed with water and brought to an OD_{600} of 1. 7 µl of this culture as well as 3 dilutions (1:10, 1:100m 1:1000) were applied on the appropriate agar plates. The wildtype channel was retained in the secretory pathway inside the cells as visualized by the Nikon A1R confocal. Therefore a C-terminal truncation mutant was created lacking amino acids 707 - 839.

SD –ura plates:

Yeast Nitrogen Base	5.9 g/l
KH_2PO_4	1 g/l
Drop out supplements –ura	1.94 g/l
Glucose	2 %

pH 5.6 – 5.8 through addition of arginine

Agar	1.5%

SGal –ura plates contain 4% galactose instead of glucose to drive protein expression through activation of the Gal-promotor.

4.16 Hydroxyurea treatment

Animals were treated with 10 mM Hydroxyurea in Hydra medium and fed daily to remove mature nematocytes. When the animals were not able to catch prey any more, they were used for further experiments.

4.17 EGTA treatment

The animals were incubated in 5 mM EGTA in Hydra medium at 18°C for 30 minutes and then used for immunostainings.

4.18 Decnidocilation

To remove the cnidocils, animals were rinsed with 6 % ethylene glycole in Hydra medium (after Golz & Thurm, 1990). Afterwards the animals were washed with Hydra medium and used for further experiments.

4.19 TrpA inhibitors and activators

The styrol dye FM1-43 (life technologies, molecular probes) was added in 5 µM concentration to the culture medium. Stained nematocytes were immediately visible under the fluorescence microscope. *Artemia* were added but no feeding behaviour could be observed.

The more specific activators (zinc chloride [Sigma Aldrich], polygodial [Tocris bioscience] and cinnamaldehyde [Sigma Aldrich]) and inhibitors (HC-030031 [Sigma Aldrich] and A 967079 [Tocris bioscience]) were added to the Hydra medium in various concentrations and incubated overnight to test toxicity of the substances. None of them was supported in the concentrations needed. Upon overnight incubation in Hydra medium containing these substances, the animals died and dissolved. Therefore even short-time incubation with these substances is not recommended as toxic effects might interfere with the experimental results.

4.20 Fura-2 mediated calcium measurements

To visualize the calcium signalling during nematocyst discharge first experiments with Fura-2 (Invitrogen, molecular probes) were performed. The dye changes its absorption upon binding of calcium from 340 nm to 380 nm. The emission is constantly at 510 nm. Therefore Fura-2 is considered as a ratiometric dye and used to measure intracellular calcium. The ratio of fluorescence upon excitation with 340/380 nm represents intracellular calcium ratios.

Hydra were incubated in 1 µM Fura-2 acetoxymethyl ester (Fura-2 AM) in Hydra medium. The ester mediates membrane permeability of the dye. Once inside the cell, the AM is cleaved by esterases so that the dye is trapped inside the cells. The animals were placed on an object slide were aluminium foil electrodes had been attached in close distance. The discharge of nematocysts was triggered by a electric pulse of 30 µsec and 24 V DC (according to Holstein & Tardent, 1984) given by a Grass SD 5 Stimulator. Not all nematocytes could be brought to discharge by this method. The fluorescence was excited with a Lambda DG-4 (Sutter Instrument) and observed on a Nikon microscope. No change in the 340/380 ratio of fluorescence could be observed. An additional problem was that the animals were still moving, so that individual areas were hard to track. The animals contracted upon the electrical pulse. Sectioning of the animal, 2% Urethane, 3 mM Heptanol and embedding in 5% Gelatine were used to immobilize the animals, but without success.

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6. APPENDIX

6.1 Antibodies

Table 4: Compilation of primary antibodies characterized in this thesis.

	Cnidoin	TrpA	Nematocalcin	Nematomyosin	PKD-2
Eurogentec No	DE10035	DE12142	ED09003	DE11239	DE12089
Antigenic peptide	GCAPSCQQQCIPSCPRGCCGA EP100174	CFKKMNDSDKDNRAPV EP123012 CDYSNGKVEQSPKPEK EP123013	GRIKFDDFVSAAIR EP090113	LSQLYKEQLQGLMNTLC EP113682	CLKKIQSKINMKSGDD EP121672
Fixative	4% PFA (wall) Lavdovsky (tubule)	-20° MeOH	4% PFA	4% PFA	Lavdovsky
Duration of fixation	30 min RT 5 hours RT	Overnight at 4°	3 hours RT	30 min RT	overnight
Dilution	1:250	1:200	1:250	1:100	1:1000
Blocking for Western Blot	milk	milk	milk	BSA	BSA
Species	Guinea pig	Rat	Rabbit	Rat	Rat

Table 5: Compilation of primary antibodies used for co-stainings.

	Fixation	Dilution	Species
Minicollagen-1	Lavdovsky is best, PFA for Cnidoin co-staining	1:500	rabbit
Minicollagen-15	Lavdovsky	1:1000	rabbit
NOWA CTLD	ice-cold Methanol	1:400	rabbit
α-tubulin	ice-cold Methanol	1:1000	mouse
β-tubulin	PFA	1:1000	mouse
tyrosinated tubulin	PFA	1:1000	rat
Nematostella Minicollagen-1	Lavdovsky is best, PFA works also	1:500	guinea pig
Phalloidin Alexa568	PFA	1:200	-
actin	ice-cold Methanol	1:90	mouse
Domain 1	Lavdovsky	1:500	guinea pig

6.2 ISH probes

Table 6: Compilation of all ISH probes used (unless otherwise stated the sense probe did not give a signal).

	Clone (No in vector library)	Bp in full-length	anti-sense	sense	result
Cnidoin full-length	pGEMT Cnidoin fulllength (191)	1542	Sp6	T7	Nests with antisense
Cnidoin confirmed	pGEMT Cnidoin confirmed (190)	561	Sp6	T7	Nests with antisense
Nematocalcin confirmed	pGEMT Nematocalcin confirmed (194)	513	Sp6	T7	Ubiquitous signal with antisense
Nematocalcin full-length	pGEMT Nematocalcin BamHI Xmal (196)	679	T7	Sp6	Ubiquitous signal with antisense
TrpA ISH	pGEMT TrpA ISH (449)	749	Sp6	T7	No difference between sense & antisense
TrpA ISH1	pGEMT easy TrpA ISH 1 (450)	300	Sp6	T7	A cocktail of all 4 antisense probes showed nests (alone no difference between sense & antisense)
TrpA ISH2	pGEMT easy TrpA ISH 2 (451)	539	Sp6	T7	
TrpA ISH3	pGEMT easy TrpA ISH 3 (452)	717	T7	Sp6	
TrpA ISH4	pGEMT TrpA ISH (453)	1009	T7	Sp6	
PKD2 full-length + UTR	pGEMT 76 NCBI (454)	2627	Sp6	T7	No difference between sense & antisense
PKD2 Hma	pGEMT Hma2.231576 (455)	1113	Sp6	T7	
PKD2 ISH2	pGEMT 76 ISH2 (456)	743	Sp6	T7	
Nematomyosin ISH1	pGEMT Nmyo ISH1 (457)	1023	Sp6	T7	Ubiquitous signal with antisense
Nematomyosin ISH2	pGEMT Nmyo ISH2 (458)	1028	Sp6	T7	

6.3 cDNA and protein sequences

6.3.1 Cnidoin

1542 base pairs

513 amino acids in total (without signal- and propeptide 402 amino acids)
54.20 kDa for total transcript (without signal and propeptide 41.44 kDa)

signal peptide
propeptide
CRD
elastic domain
antigenic peptide

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   1      ATG TCT CGA TTA CTA CTT CTA TTA CTG GTT TGC TTG ATA TTG CAT AGA TAT GAG GTT GAG
   1       M   S   R   L   L   L   L   L   L   V   C   L   I   L   H   R   Y   E   V   E

  61      TCA AAG TCC GAA AAA AAA GAT CAT AAA ACG AAA GAG CAT CAA AAA GAA AAA CAC GAA GAA
  21       S   K   S   E   K   K   D   H   K   T   K   E   H   Q   K   E   K   H   E   E

 121      AAA AAA ATA ACT AAA GAT ATT GAA ATT GCA AAA GTG GAG GAT AAA AAA GGG AAC GAA GAA
  41       K   K   I   T   K   D   I   E   I   A   K   V   E   D   K   K   G   N   E   E

 181      AAA AAG AAA ACT CTC AAA TCA ACT GAA GAA TCT ACT GAA ACT GGA GAT GAA TCA GAA GGT
  61       K   K   K   T   L   K   S   T   E   E   S   T   E   T   G   D   E   S   E   G

 241      TCC GGA ACT CAA CGA TCA GAA ACG CTT GAG GCA AGA TCA GAT ACT GAT CAT ACT ACA TCC
  81       S   G   T   Q   R   S   E   T   L   E   A   R   S   D   T   D   H   T   T   S

 301      ACA GAC ACT GAG AAG ACG ACA ATT GCA AAA AGA GCT GCA CTT CCG GGA GGA GCA ACA TTC
 101       T   D   T   E   K   T   T   I   A   K   R   A   A   L   P   G   G   A   T   F

 361      TCT GTA CCA CAA GCA GTT AAG CAA TTG AGA TGC CCA GCA CCA TGT TCT CAA TCA TGT GCA
 121       S   V   P   Q   A   V   K   Q   L   R   C   P   A   P   C   S   Q   S   C   A

 421      TCA TCT GGA TGT TCT CCT AGC TGT TGC ATG AAT AGT ATG CCT CAA ATG CCA GCT TCT CTA
 141       S   S   G   C   S   P   S   C   C   M   N   S   M   P   Q   M   P   A   S   L

 481      TCA CCA ATG ATG GGT GGA TGT GGT AAT CAA ATG CAG GGT TGT GAC CAA CAA TAT ATG ATG
 161       S   P   M   M   G   G   C   G   N   Q   M   Q   G   C   D   Q   Q   Y   M   M

 541      GGG GGT TGC GGT GGA CAA ATG CAA GGT TGT GGA CAA CAA ATG CCT CAA ATG TCA ATG GGG
 181       G   G   C   G   G   Q   M   Q   G   C   G   Q   Q   M   P   Q   M   S   M   G

 601      TGT GGA GGA CAA ATG CAA GGA TGC GGA CAA CAA ATG CCT ATA ATG ATG CCA GGT TGT GGA
 201       C   G   G   Q   M   Q   G   C   G   Q   Q   M   P   I   M   M   P   G   C   G

 661      GCA CAA ATG CAA GGG TGT GGG CAA CAA ATG CCG CCA TTA ATG GGT GGT TGC GGA GGA CAA
 221       A   Q   M   Q   G   C   G   Q   Q   M   P   P   L   M   G   G   C   G   G   Q

 721      ATG CAA GGT TGT GGA CAA CAA ATG CCG CAA ATG GTC GGT GGA TGT TTT GGA CAA ATG GTG
 241       M   Q   G   C   G   Q   Q   M   P   Q   M   V   G   G   C   F   G   Q   M   V

 781      GGT TGC GGC ACA CAA ACA TTT CAA AGT TCA TTA AAA GCG CCC TGC GCA CCT AAC TCA ATT
 261       G   C   G   T   Q   T   F   Q   S   S   L   K   A   P   C   A   P   N   S   I

 841      GGT TGT GGT CAG CAG CTT CGT GCT CCA ATG GTA TCA ATG ACG CCA GGT TGT GGT GGA CAA
 281       G   C   G   Q   Q   L   R   A   P   M   V   S   M   T   P   G   C   G   G   Q

 901      ATG CAA GGT TGT GGA CAG CAA ATG CCA CCA ATG ATG TCT GGG TGT GGT GGA CAA ATG CAA
 301       M   Q   G   C   G   Q   Q   M   P   P   M   M   S   G   C   G   G   Q   M   Q

 961      GGT TGT GGG CAA CAA TCG CCA CCA ATG ATG TCA GGA TGT GTC GGA CAA ATG CAA GGT TGT
 321       G   C   G   Q   Q   S   P   P   M   M   S   G   C   V   G   Q   M   Q   G   C

1021      GGG CAG CAA TTG CCA CTG ATG ATG CCA GGT TGT GTC GGG CAA ATG CAA GGT TGT GGA CAA
 341       G   Q   Q   L   P   L   M   M   P   G   C   V   G   Q   M   Q   G   C   G   Q

1081      CAA ATG CCT CCA ATG ATG TCT GGG TGT GGT GGT CAA ATG CAA GGT TGT GGA CAA CAA ATG
 361       Q   M   P   P   M   M   S   G   C   G   G   Q   M   Q   G   C   G   Q   Q   M
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1141    CCA CCA ATG ATG TCT GGC TGT GGT GGT CAA ATG CAA GGT TGT GGG CAG CAA ATA ATG CCA
 381     P   P   M   M   S   G   C   G   G   Q   M   Q   G   C   G   Q   Q   I   M   P

1201    ATG ATG GCT CCT ATC ATG CCA GGT TGT GGC GGA CAA ATG CAA GCT GGC TGT GGT GGA CAG
 401     M   M   A   P   I   M   P   G   C   G   G   Q   M   Q   A   G   C   G   G   Q

1261    CAA GAA GAA CAA ATG CAG TTT AAA GTA AAA CTC CTT CCA CCT CAA ATT TAC TCT ATA CAA
 421     Q   E   E   Q   M   Q   F   K   V   K   L   L   P   P   Q   I   Y   S   I   Q

1321    CAA CAA CAA CCT CAA CAG CAA TCG CAA TGC CCA CCA CAA TGC CAG CCA CAA ACA TGT CAG
 441     Q   Q   Q   P   Q   Q   Q   S   Q   C   P   P   Q   C   Q   P   Q   T   C   Q

1381    ATT GGA TGT CCA CAA ACA TGT TGT ATG CAA TCT CAA CCT CAA ACT GCT ATG CAA ATG CCA
 461     I   G   C   P   Q   T   C   C   M   Q   S   Q   P   Q   T   A   M   Q   M   P

1441    CAA CCA ATG ATG GTA ATG GGT GGA TGT GCA CCT TCA TGC CAG CAG CAA TGC ATA CCT TCG
 481     Q   P   M   M   V   M   G   G   C   A   P   S   C   Q   Q   Q   C   I   P   S

1501    TGT CCA AGA GGT TGT TGT GGA GCT TTT GGT AAA AAG AGA TAA
 501     C   P   R   G   C   C   G   A   F   G   K   K   R   *
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6.3.2 TrpA

3765 base pairs

1255 amino acids
141.53 kDa

Ankyrin-repeats
transmembrane domain
antigenic peptide

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   1    ATG GAT AGC AAC TCA GAG TCA CTG GAG TTA GAA AGA AGT AGT TAT TTT GAT GAG ACT GAC
   1     M   D   S   N   S   E   S   L   E   L   E   R   S   S   Y   F   D   E   T   D

  61    ATT AAG CTA GCA AAT CTT AGC CAT GAT AAA GAA ATA AAT AAA GAT GAA TTT GCA AAC TTT
  21     I   K   L   A   N   L   S   H   D   K   E   I   N   K   D   E   F   A   N   F

 121    GGC AAT GCT AAT GAA ACA GAT TTG ACT GAA TTA TTG GCA CAC AAA AAT TCA AAG GAA GTA
  41     G   N   A   N   E   T   D   L   T   E   L   L   A   H   K   N   S   K   E   V

 181    AAT TTA GAA AAA ACT GAT AAA AAA AAG TTA ACT ATA TAT GAG CAA CAG TCA TTG TCT AAT
  61     N   L   E   K   T   D   K   K   K   L   T   I   Y   E   Q   Q   S   L   S   N

 241    AAA AAT TCT AAA ACA TCT GTT AAG CTT AGA AAT CTT AAA CCT AGA GTA GAG GAT GAA AGA
  81     K   N   S   K   T   S   V   K   L   R   N   L   K   P   R   V   E   D   E   R

 301    GAA GGT TTA GTG CAC GCT GAA ATA CAA TCC TCT GAT ACT TCT TCT CTT CAA AGT GAA AAT
 101     E   G   L   V   H   A   E   I   Q   S   S   D   T   S   S   L   Q   S   E   N

 361    AAA GAT AAT CAA AAA GAT TCA TTA GAT GGA AAA AAA AGA ATA TCT ATG TTC AAC TTC CCA
 121     K   D   N   Q   K   D   S   L   D   G   K   K   R   I   S   M   F   N   F   P

 421    AAG TGG CTC AAA GAT TAT TCT AAT GGA AAA GTT GAA CAA TCA CCT AAA CCA GAG AAG GAG
 141     K   W   L   K   D   Y   S   N   G   K   V   E   Q   S   P   K   P   E   K   E

 481    ATT GAA GGT GAT TTT tCA AGG TTT tCT CTC CAT CTT GCT GCA AAA GAA GGA ACT ATA GGG
 161     I   E   G   D   F   S   R   F   S   L   H   L   A   A   K   E   G   T   I   G

 541    CGT ATT CAA CAC ATT ATT GAA AAT AAT AAA AAG CAA AAC TCA GTA ACA TCG AAA GAG TTT
 181     R   I   Q   H   I   I   E   N   N   K   K   Q   N   S   V   T   S   K   E   F

 601    ATC AGC ACA AGA GAT GGA TGG AAT AAA AAT GGT TTT AGT GCT TTG CAT TTA GCT GCA CGG
 201     I   S   T   R   D   G   W   N   K   N   G   F   S   A   L   H   L   A   A   R

 661    TAT AAT CAA AAA GAT GTT GTT GCA TAT CTG CTT GAA AAT GGA TCT TTA ATT GAT AGT CCA
 221     Y   N   Q   K   D   V   V   A   Y   L   L   E   N   G   S   L   I   D   S   P

 721    GAT AGA GAT GAT GGT AAT ACA GCT CTC TTA TTG GCA GCA AAA TAT GGA ATG ACA ACA ACT
 241     D   R   D   D   G   N   T   A   L   L   L   A   A   K   Y   G   M   T   T   T
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 781  GCA TCA TTT CTG ATT GAA AAA GGA GCA AAT GTT ATG TTT AAA AAT AAT TAT GGA ACT ACA
 261   A   S   F   L   I   E   K   G   A   N   V   M   F   K   N   N   Y   G   T   T

 841  GCA TTG CAC TAT GCA TGT AGG CGT GGT AAT AAA AAA CTT TTG CTT AAG ATT CTT AGC ATT
 281   A   L   H   Y   A   C   R   R   G   N   K   K   L   L   L   K   I   L   S   I

 901  CCA AAT GTA GAT ATA AAT GTT CAA GAC ATT AAT TTG AAT ACT CCG CTT CAT CTT GCT ATG
 301   P   N   V   D   I   N   V   Q   D   I   N   L   N   T   P   L   H   L   A   M

 961  AAT GGT GGT TGC ATA AGG GTA GTA AGT ACT CTT ATA AAC TAT GGA TCA AAT GTA TTT GCT
 321   N   G   G   C   I   R   V   V   S   T   L   I   N   Y   G   S   N   V   F   A

1021  ATA AAT AAT AAA GGG GAA ATT CCT ATA CAT TAT GCA GCT GCT TCA ACT GTA GAT AAC ATA
 341   I   N   N   K   G   E   I   P   I   H   Y   A   A   A   S   T   V   D   N   I

1081  AGA GAT GAG CTG AAT AAA GGA GAT TAC TTT GTC TTA GAA GAA ATC AGT AAT AAA ACT AAA
 361   R   D   E   L   N   K   G   D   Y   F   V   L   E   E   I   S   N   K   T   K

1141  GCT TTG CAA CAT GTT CCT TCA TCA ATT GTT GAA GAT CTT ATT GAG TTA CTT ATC AAA GGA
 381   A   L   Q   H   V   P   S   S   I   V   E   D   L   I   E   L   L   I   K   G

1201  GCT CTC AAA AAT GTT CCC GAA GAT AAA CAT GAA CAA CAG AGA AAC GCA TTT GTT AAC AGT
 401   A   L   K   N   V   P   E   D   K   H   E   Q   Q   R   N   A   F   V   N   S

1261  AAA ACC AAA GAG AAT CAC ACA CCT TTG CAT ATT GCA GCA TGT TGT GGT AAT GAA AAA TCA
 421   K   T   K   E   N   H   T   P   L   H   I   A   A   C   C   G   N   E   K   S

1321  TTA CAT AAA TTG TTG AGA GTT GGT GGA GAT GTC AAC GCT CAA ACA GAT TCT GGC TTA ACT
 441   L   H   K   L   L   R   V   G   G   D   V   N   A   Q   T   D   S   G   L   T

1381  CCT TTA CAC TTT GCT GCT ATG AGC GGG CAT GAA AGA GTA GTA AAT TTT TTA ATA ATG TAT
 461   P   L   H   F   A   A   M   S   G   H   E   R   V   V   N   F   L   I   M   Y

1441  GAT GCT AAT ATT CAA GCA GTT GAC AAT GAC TTA ATG ACT CCA TTG CAC AGG GCT TGT CTA
 481   D   A   N   I   Q   A   V   D   N   D   L   M   T   P   L   H   R   A   C   L

1501  TTC GGG CGA TTA TCA GTT GTT AAA TTA TTA GAT GAG AAA GGA GCT TTA CTT GAA GTT AAA
 501   F   G   R   L   S   V   V   K   L   L   D   E   K   G   A   L   L   E   V   K

1561  GAT AAA AAC AAC TTT ACT CCT GTT ATA TGT GCT GTG TGT AAG GGT CAT GTT GAA GTT ATA
 521   D   K   N   N   F   T   P   V   I   C   A   V   C   K   G   H   V   E   V   I

1621  ACA TAT CTG ATT GCT AGG GGT GTT CAA ATT AAT TCA ACT GAT GTT AAC AAT AAG AAT GCT
 541   T   Y   L   I   A   R   G   V   Q   I   N   S   T   D   V   N   N   K   N   A

1681  CTT CAT GTT GCA GTT AAA GAG AAC CAA TTA GAA ACT TTA AAG TTT TTA TTG GAT AAT CAT
 561   L   H   V   A   V   K   E   N   Q   L   E   T   L   K   F   L   L   D   N   H

1741  CAA TTT AAA AAG ATG AAT GAT TCA GAT AAA GAC AAT AGG GCA CCT GTG CAT TAT GCA GCA
 581   Q   F   K   K   M   N   D   S   D   K   D   N   R   A   P   V   H   Y   A   A

1801  GCT GAT GGA AAT CTT CAG GCA TTG GAA TTT TTA ATT CAA AAA AAT GCT CCA ATT GAT GTT
 601   A   D   G   N   L   Q   A   L   E   F   L   I   Q   K   N   A   P   I   D   V

1861  GGT GAT AAT CAA GAA AGG ACT CCT CTT CAT TTG GCA TCT GAG AAG GGT CAC TTA TCT TGT
 621   G   D   N   Q   E   R   T   P   L   H   L   A   S   E   K   G   H   L   S   C

1921  GTA AAA CTT CTC ATT TCT ACA TCT GCT GGA GAA ATT AAC TCT ACT GAT GCC CAT GGA ATG
 641   V   K   L   L   I   S   T   S   A   G   E   I   N   S   T   D   A   H   G   M

1981  ACT CCG CTT CAT TTA GCT GCA TCA AAT GAT CAT AGA AAA GTA GTG AAT CTC CTT ATT GAG
 661   T   P   L   H   L   A   A   S   N   D   H   R   K   V   V   N   L   L   I   E

2041  TCC GGT GCT GAT GTT TCT TTA CGT GAT AAT TGT GAC TGG AGC CCT TTA GAT TAT GCT GCA
 681   S   G   A   D   V   S   L   R   D   N   C   D   W   S   P   L   D   Y   A   A

2101  AAA AAT GGT CAT GAA AAG AGT CTG CAA ATT TTA CTT GAA AAT GGT GCT TTT ATT AAT GCT
 701   K   N   G   H   E   K   S   L   Q   I   L   L   E   N   G   A   F   I   N   A

2161  TGT GAC AAA AAT GGT TAT ACA CCT CTG CAC CAT GCA GCA CTT GCA GGA CAT GTT GAA TGC
 721   C   D   K   N   G   Y   T   P   L   H   H   A   A   L   A   G   H   V   E   C

2221  ATA GTT GCA TTA CTA GAT CAG GGT GCT AAT ATT CAA CTA CTA ACT AAG GAG AGA AAA AAT
 741   I   V   A   L   L   D   Q   G   A   N   I   Q   L   L   T   K   E   R   K   N

2281  TGT TTA TAT CTT GCA GTT GAG AAC TCA GAA AGA GAA GCT GGC ATG GCG ATT GTT AAG CAC
 761   C   L   Y   L   A   V   E   N   S   E   R   E   A   G   M   A   I   V   K   H
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2341    AAA AGA TGG CAT GAA GCT CTT CTC AAT ATA GAT AGC AAA AGA GCT CCT GTT ATG GAA AAA
 781     K   R   W   H   E   A   L   L   N   I   D   S   K   R   A   P   V   M   E   K

2401    ATA ATC GAA CTT GCA CCT GAA GTT GCA GAA GTT GCA TTA GAT AAT TGC ATT ACT TAT TCT
 801     I   I   E   L   A   P   E   V   A   E   V   A   L   D   N   C   I   T   Y   S

2461    GAT CTT GAT AAA AAA CAT TCT GAT TAT TCA ATT GAA TAC AAT TTT CAA TTT GTA GAT ACT
 821     D   L   D   K   K   H   S   D   Y   S   I   E   Y   N   F   Q   F   V   D   T

2521    GAC CCC ATA AAC AGT TTA AAT TCT TTT TTT GCA CCT TCT TTG ATT GTT CAA TAC AAA CGT
 841     D   P   I   N   S   L   N   S   F   F   A   P   S   L   I   V   Q   Y   K   R

2581    GGG AAA TTG TTG AGC CAT CCT CTA GTT GTT GAG CTT ATT AAT CAG AGG TGG TCT CGA ATG
 861     G   K   L   L   S   H   P   L   V   V   E   L   I   N   Q   R   W   S   R   M

2641    GGT CGG TGG GTG TAC TTA TCT TCA CTA AGT TTT TAT TTA GTG TTT GTT TCA CTT CTT ACA
 881     G   R   W   V   Y   L   S   S   L   S   F   Y   L   V   F   V   S   L   L   T

2701    GCA CTT GTG GTT ATA GAA AGA ATG AGT TTA AAC AAG CAA CCA ACA AAA ATA TGC TCA GGA
 901     A   L   V   V   I   E   R   M   S   L   N   K   Q   P   T   K   I   C   S   G

2761    AAG AAA TTT ACT GAC ATA ATT ACA TGG TCG ACT CTC GGC ATT GCA TGC ATA CAA ATT TTA
 921     K   K   F   T   D   I   I   T   W   S   T   L   G   I   A   C   I   Q   I   L

2821    TGG AAA TTG ATA TTA ACT ATA TAT ATT GGA ATA AGT TAT ATA AAC AAT CCA GTT AAA ATA
 941     W   K   L   I   L   T   I   Y   I   G   I   S   Y   I   N   N   P   V   K   I

2881    TTA GAA TTT TTT TTA TAC ATT TCA ACA GCA TTG TTT ATG GTT CCT TTT ATT ACT TGC CAA
 961     L   E   F   F   L   Y   I   S   T   A   L   F   M   V   P   F   I   T   C   Q

2941    TTT CAT TTG AAA ATG ACA AAT TTA GAG TCT ATG AAA TGG CAA TCT GGA TCA CTT TCA ATT
 981     F   H   L   K   M   T   N   L   E   S   M   K   W   Q   S   G   S   L   S   I

3001    TTA CTT GCA TGG TCA AAA ATA CTG TTA TAT TTA GAG AAT TTA CCA TTT ATC GGT TTA TAT
1001     L   L   A   W   S   K   I   L   L   Y   L   E   N   L   P   F   I   G   L   Y

3061    ATT GTC ATG TTT ACT GAG GTC CTT TAC ACA TTA CTA AAA GTT TTA TTG GTT TTT GGC ACT
1021     I   V   M   F   T   E   V   L   Y   T   L   L   K   V   L   L   V   F   G   T

3121    CTT CTA ATT GGT TTT GGT TTA TCT TTT TAT GCA CTA CTT GAT CTT CAA TCA GCA TTT AGT
1041     L   L   I   G   F   G   L   S   F   Y   A   L   L   D   L   Q   S   A   F   S

3181    GAT TAT GGG CGC TCT ATT GTC AAA ACA TTT GTC ATG ATG CTA GGT GAA ATA AGC TAC GAC
1061     D   Y   G   R   S   I   V   K   T   F   V   M   M   L   G   E   I   S   Y   D

3241    TCT ATC TTT ACA AAT AAC TAC TCA GAT GAA AAT AGT CAT CTT CTA CCT AAT TTA GAA ATA
1081     S   I   F   T   N   N   Y   S   D   E   N   S   H   L   L   P   N   L   E   I

3301    TCT ATT GTC ATT TTT CTT CTA TTT GCT ATG ATA ATG ATA ATT GTG GTT ATG AAT TTG CTT
1101     S   I   V   I   F   L   L   F   A   M   I   M   I   I   V   V   M   N   L   L

3361    GTT GGT CTT GCA GTT GGT GAT ATT GAA TCA GTT CGC AAC AAT GCA TAT CTA AGA GTT TTA
1121     V   G   L   A   V   G   D   I   E   S   V   R   N   N   A   Y   L   R   V   L

3421    CAG AGA CAA GTT TAT TTT TTA AGT ATT TTA GAT CGA ACC TAT CCT AAA TTT ATC AGA AAA
1141     Q   R   Q   V   Y   F   L   S   I   L   D   R   T   Y   P   K   F   I   R   K

3481    TTT GTG TAT AAA GCT TCT TAT ATA CAA AAA CCT AAT CAA AAG AGT TGG TTT AAA AAG TTT
1161     F   V   Y   K   A   S   Y   I   Q   K   P   N   Q   K   S   W   F   K   K   F

3541    ATC CTA TGG TTG GGT AAT TTT CAA AGG AAA GCA TTG GAA GAA GAA CAA ACA GAT GCA AAA
1181     I   L   W   L   G   N   F   Q   R   K   A   L   E   E   E   Q   T   D   A   K

3601    AAA GAT TTA ATA ATG CTT GAA ATT GCA TCT AAT AGG GAA GCA ATA ATA AAG CAA AAA AAA
1201     K   D   L   I   M   L   E   I   A   S   N   R   E   A   I   I   K   Q   K   K

3661    AAG ACT AAA TCG ATT TTA GAC GAT CTC GAA AAG CAA TTG AAA AGA ACT AAA AAA ATT GCT
1221     K   T   K   S   I   L   D   D   L   E   K   Q   L   K   R   T   K   K   I   A

3721    AAA TTC ATT GCA ACA AGT TCT TCG TTC AAA AAC AAA GTT GAT TGA
1241     K   F   I   A   T   S   S   S   F   K   N   K   V   D   *
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6.3.3 Nematocalcin

606 base pairs

201 amino acids
22.62 kDa

EF-hand motif
antigenic peptide

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   1      ATG GCA TAT CCA GGA TAC AAT CAA CCG GGT GCA TAC CCT GGG TAT GGT CAT CCT GGG GCT
   1       M   A   Y   P   G   Y   N   Q   P   G   A   Y   P   G   Y   G   H   P   G   A

  61      TAC GGT CCT CCT GGT GGT TAT TCT GGA TAT CCT CCT GGT GCA ATT GAC CCC CTT TTT GGA
  21       Y   G   P   P   G   G   Y   S   G   Y   P   P   G   A   I   D   P   L   F   G

 121      TAC TTC TCT GCT GTA GCA GGT CAT GAT CAA CAA ATT GAT GCT CGT GAG CTC CAA ACC TGC
  41       Y   F   S   A   V   A   G   H   D   Q   Q   I   D   A   R   E   L   Q   T   C

 181      TTA ACA TCT TCA GGA ATT GGT GGT TCA TAT CAA CAG TTC AGT TTA GAG ACT TGC CGT ATT
  61       L   T   S   S   G   I   G   G   S   Y   Q   Q   F   S   L   E   T   C   R   I

 241      ATG ATC AAT ATG CTT GAT AGA GAT TAT TCT GGC AAA ATG GGT TTT ACT GAG TTC AAA GAG
  81       M   I   N   M   L   D   R   D   Y   S   G   K   M   G   F   T   E   F   K   E

 301      TTA TGG AAT GCA CTA AAT CAA TGG AAG ACT ACA TTT ATG ATT TAT GAC AGA GAT CGA TCT
 101       L   W   N   A   L   N   Q   W   K   T   T   F   M   I   Y   D   R   D   R   S

 361      GGT ACA GTA GAG CCT CAT GAA ATG CAT CAA GCT ATA GCT TCT TGG GGA TAT AAT TTG AGT
 121       G   T   V   E   P   H   E   M   H   Q   A   I   A   S   W   G   Y   N   L   S

 421      GGT CAA GCG CTT AAT ATC ATT ATA AAA AGA TAT TCT GAT AAT GGT CGA ATT AAG TTC GAT
 141       G   Q   A   L   N   I   I   I   K   R   Y   S   D   N   G   R   I   K   F   D

 481      GAC TTT GTT TCT GCA GCT ATA AGG TTG CGA ATG TTA ACA GAT CAT TTT CGA CGT AGA GAT
 161       D   F   V   S   A   A   I   R   L   R   M   L   T   D   H   F   R   R   R   D

 541      GCC ACT CAA TCA GGA TAT GCT AGT TTT GCT TTT GAT GAT TTC ATT CAA GTG ACA ATG TTT
 181       A   T   Q   S   G   Y   A   S   F   A   F   D   D   F   I   Q   V   T   M   F

 601      TCA TAA
 201       S   *
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6.3.4 Nematomyosin

5838 base pairs

1945 amino acids
224.57 kDa

head domain
tail domain
antigenic peptide

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   1      ATG GAC AAC GAA GAA GAT AGT TTA AAG TAT CTG TCT GTT GAT CGG CGT GCA ATA GCT GAC
   1       M   D   N   E   E   D   S   L   K   Y   L   S   V   D   R   R   A   I   A   D

  61      CCC GTT GCT CAC GCT GCT TGG GCT GCA CAG AAG TTA GTT TGG GTG CCT AGT GAA GAG CAT
  21       P   V   A   H   A   A   W   A   A   Q   K   L   V   W   V   P   S   E   E   H

 121      GGG TTT GTT TCT GCT AGT ATC AAA GAG GAA AAA GGG GAT AAA GTT ATT GCC GAA ATT GAA
  41       G   F   V   S   A   S   I   K   E   E   K   G   D   K   V   I   A   E   I   E

 181      GGA GGG AAA AGA GTG ACA TTT CAT AAA GAT GAT ATT CAA AGG ATG AAT CCA CCC AAA TTT
  61       G   G   K   R   V   T   F   H   K   D   D   I   Q   R   M   N   P   P   K   F

 241      GAC AAG GTG GAA GAC ATG GCT GAT CTG ACT TGT TTA AAT GAA GCT AGT GTG TTG CAT AAT
  81       D   K   V   E   D   M   A   D   L   T   C   L   N   E   A   S   V   L   H   N
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 301  ATT AAA GAT CGA TAT TTT TCA GAT CTC ATT TAT ACT TAC TCT GGT TTG TTT TGT GTT GTT
 101   I   K   D   R   Y   F   S   D   L   I   Y   T   Y   S   G   L   F   C   V   V

 361  GTA AAC CCA TAC AAG AAG ATA CCA ATA TAC AGT GAT AAT GTT GTT AAC TTA TAT CGA GGA
 121   V   N   P   Y   K   K   I   P   I   Y   S   D   N   V   V   N   L   Y   R   G

 421  AAA AAA CGA CAT GAG CTT CCA CCA CAT GTC TAT GCA ATT ACT GAT AAT GCA TAT CGC AGC
 141   K   K   R   H   E   L   P   P   H   V   Y   A   I   T   D   N   A   Y   R   S

 481  ATG TTA CAA GAT CGT GAT AAT CAG TCT ATC CTC TGC ACG GGT GAA TCT GGT GCA GGA AAA
 161   M   L   Q   D   R   D   N   Q   S   I   L   C   T   G   E   S   G   A   G   K

 541  ACT GAA AAC ACA AAA AAG GTT ATA CAG TAC TTG ACT GCT ATA GCT GGT CGT CAT AAA CAA
 181   T   E   N   T   K   K   V   I   Q   Y   L   T   A   I   A   G   R   H   K   Q

 601  GAT GAT AAA TCA GCA CAG GGT CAA CTT GAG GTA CAG TTA CTC CAA GCC AAT CCT ATT CTT
 201   D   D   K   S   A   Q   G   Q   L   E   V   Q   L   L   Q   A   N   P   I   L

 661  GAA GCA TTT GGA AAT GCA AAA ACT GTG AAA AAT GAT AAT TCA TCA CGT TTT GGT AAA TTC
 221   E   A   F   G   N   A   K   T   V   K   N   D   N   S   S   R   F   G   K   F

 721  ATC AGA ATT ATG TTT GAT AAT TCT GGT TTT ATC TCT GGA GCA AAC ATT GAG TCC TAT TTG
 241   I   R   I   M   F   D   N   S   G   F   I   S   G   A   N   I   E   S   Y   L

 781  TTG GAG AAA GGT CGA TTA GTT CGC CAA GCA CCA GAA GAA AGA CTT TTC CAC ATT TTT TAC
 261   L   E   K   G   R   L   V   R   Q   A   P   E   E   R   L   F   H   I   F   Y

 841  CAG TTG CTT TTG GGT GCA AGT CCT GAA GTT AAA AAA CAA TTT CTA CTT CTT GAT CCA AAA
 281   Q   L   L   L   G   A   S   P   E   V   K   K   Q   F   L   L   L   D   P   K

 901  AGT TAT ATT TTT ATG TCA AAT GGA CTT GTT CAG CTT CCT AAC ATG GAT GAC CGT GCA GAA
 301   S   Y   I   F   M   S   N   G   L   V   Q   L   P   N   M   D   D   R   A   E

 961  TTT AAA TTA ACT TTG GAG GCT ATG AGG GAT ATG GGT ATT ACC CAA GAA GAA CTT AAT CCT
 321   F   K   L   T   L   E   A   M   R   D   M   G   I   T   Q   E   E   L   N   P

1021  ATT TTC AAA GTT TTA TCA GCT TGT CTA TTA TTT GGT AAC TTG GAT TTC AAA ATG GAA AGA
 341   I   F   K   V   L   S   A   C   L   L   F   G   N   L   D   F   K   M   E   R

1081  AAA TCA GAT CAA GCT GCG TTG CCC GAT AAT ACT ATT TCA CAG CAA ATT TCT CAT CTT CTT
 361   K   S   D   Q   A   A   L   P   D   N   T   I   S   Q   Q   I   S   H   L   L

1141  GGA ATT GCT GTT ACT GAC TTT ACA AAT GCT TTA TTA AAA CCA AGA GTT AAA GTT GGA CGA
 681   G   I   A   V   T   D   F   T   N   A   L   L   K   P   R   V   K   V   G   R

1201  GAG TTT ACA CAA AAA GCT CAA ACT AAA GCT CAG TGC GAA TTT GCT GTT GAA GCT CTT ACA
 401   E   F   T   Q   K   A   Q   T   K   A   Q   C   E   F   A   V   E   A   L   T

1261  AAA GCT ATG TAT GAG CGT CTT TTC AAA TGG TTA GTC ACT CGC ATT AAC AAG AGT TTA AAT
 421   K   A   M   Y   E   R   L   F   K   W   L   V   T   R   I   N   K   S   L   N

1321  CGA TCA AAA CGT GAA GGT GCC TCT TTC GTT GGA ATC TTG GAT ATT GCT GGT TTT GAA ATT
 441   R   S   K   R   E   G   A   S   F   V   G   I   L   D   I   A   G   F   E   I

1381  TTC AAA ATA AAT TCA TTT GAA CAG TTA TGT ATA AAT TAT ACT AAT GAA AAA CTG CAA CAA
 461   F   K   I   N   S   F   E   Q   L   C   I   N   Y   T   N   E   K   L   Q   Q

1441  CTG TTC AAC CAC ACT ATG TTT ATT CTT GAA CAA GAA GAA TAC CAG AAA GAA GGA ATT GAC
 481   L   F   N   H   T   M   F   I   L   E   Q   E   E   Y   Q   K   E   G   I   D

1501  TGG AAA TTT ATT GAC TTT GGA CTT GAC TTA CAA CCA ACT ATT GAC TTG ATT GAG AAA CCT
 501   W   K   F   I   D   F   G   L   D   L   Q   P   T   I   D   L   I   E   K   P

1561  ATG GGC ATT ATG GCC TTA CTT GAT GAG GAA TGT TGG TTC CCA AAA GCT ACA GAT AAA AGT
 521   M   G   I   M   A   L   L   D   E   E   C   W   F   P   K   A   T   D   K   S

1621  TTA GTA GAG AAA ATA AAT AAA GCT CAT GCT AAG CAT CCA AAG TAC ATG AAA CCT GAC TTT
 541   L   V   E   K   I   N   K   A   H   A   K   H   P   K   Y   M   K   P   D   F

1681  AGA GCT AAC TCA GAT TTC TGT ATC ATT CAT TAT GCT GGT CGT GTT GAT TAT TCT GCA GCC
 561   R   A   N   S   D   F   C   I   I   H   Y   A   G   R   V   D   Y   S   A   A

1741  CAA TGG CTT ACA AAG AAT ATG GAT CCA CTA AAT GAC AAT GTT GTT GCT TTA TTG GCG GCT
 581   Q   W   L   T   K   N   M   D   P   L   N   D   N   V   V   A   L   L   A   A

1801  TCA AGC GAA CCA TTT GTA GAT GCA TTG TGG AAA GAT ATT GAA AAT GTT GTT AGC ATG AGT
 601   S   S   E   P   F   V   D   A   L   W   K   D   I   E   N   V   V   S   M   S
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1861    GTT ACA GAA GGT ATG GAT ACT GCA TTT GGT GCA GCA AAA ACA AAA AAA GGA ATG TTT AGA
 621     V   T   E   G   M   D   T   A   F   G   A   A   K   T   K   K   G   M   F   R

1921    ACT CTG TCG CAA CTT TAT AAG GAA CAA TTG CAA GGT CTC ATG AAT ACA TTA AAT TGT ACA
 641     T   L   S   Q   L   Y   K   E   Q   L   Q   G   L   M   N   T   L   N   C   T

1981    AAG CCA AAC TTT GTT CGT TGC ATC ATT CCA AAC TAT GAA AAA CGA AGT GGA AAG ATC ACC
 661     K   P   N   F   V   R   C   I   I   P   N   Y   E   K   R   S   G   K   I   T

2041    AAC TTT TTG GTT CTT GAC CAA CTG AGA TGT AAT GGT GTT TTA GAA GGT ATT CGT ATT TGC
 681     N   F   L   V   L   D   Q   L   R   C   N   G   V   L   E   G   I   R   I   C

2101    AGG CAA GGT TTC CCT AAC AGA ATT CTT TTC CAA GAA TTT AGA CAG CGA TAT GAA ATT CTT
 701     R   Q   G   F   P   N   R   I   L   F   Q   E   F   R   Q   R   Y   E   I   L

2161    TGC CCT GGT GTT GTT CCA AAA GGC TTT ATG GAT GGA CGC AAT GCA TCC AAG AAA ATG ATT
 721     C   P   G   V   V   P   K   G   F   M   D   G   R   N   A   S   K   K   M   I

2221    GAG GCT TTG GAA ATG GAT CCA AAT TTG TTC AGA ATC GGT CAA TCA AAA ATA TTT TTC CGT
 741     E   A   L   E   M   D   P   N   L   F   R   I   G   Q   S   K   I   F   F   R

2281    GCT GGT GTG TTA GCC CAT CTT GAA GAA GAA CGT GAT ATT AAA CTT ACT GAA ATA GTA ATT
 761     A   G   V   L   A   H   L   E   E   E   R   D   I   K   L   T   E   I   V   I

2341    CAA TTC CAA GCT TTT TGC AGA GGT AAT ATT GCT CGT AAA AAT TAT AAG AGG CGC ATT CAG
 781     Q   F   Q   A   F   C   R   G   N   I   A   R   K   N   Y   K   R   R   I   Q

2401    CAG TTA TCA GCT ATT AGA GTT ATT CAA CGA AAT GGT CGC AGC TGG ATG AAA TTA AGA AAT
 801     Q   L   S   A   I   R   V   I   Q   R   N   G   R   S   W   M   K   L   R   N

2461    TGG CAA TGG TGG CGC TTG TTC ACA AAA GTA AAA CCT TTG TTG AAT GTT ACA AGA CAC GAT
 821     W   Q   W   W   R   L   F   T   K   V   K   P   L   L   N   V   T   R   H   D

2521    GAA GAG CTT CGC GCC AAA GAA GAG GAG TAT AAA AAA GTT CTT GAT AAA TAC GAA AAA GTT
 841     E   E   L   R   A   K   E   E   E   Y   K   K   V   L   D   K   Y   E   K   V

2581    GAA AGT GCA CAC AAT GAC CTC AAA AAG AGT CAT GAC AAG CTT ACT GAT GAA AAT AAA CTG
 861     E   S   A   H   N   D   L   K   K   S   H   D   K   L   T   D   E   N   K   L

2641    TTA GCT GAG CAA CTT CAA GCT GAA ATA GAG CTG TGT CAA GAA GCA GAA GAG AAT GTT AGT
 881     L   A   E   Q   L   Q   A   E   I   E   L   C   Q   E   A   E   E   N   V   S

2701    CGT CTC CAG CAA CGA AAG ATT GAA TTA GAG GAA TTA TTG AAT GAT TTT GAA ATC AAG TTA
 901     R   L   Q   Q   R   K   I   E   L   E   E   L   L   N   D   F   E   I   K   L

2761    GCT GAG GAG GAA GAG AGA TCA GCC AAG ACT GCT GAG GAA AAA AAA AAG TTA CAA CAA GGA
 921     A   E   E   E   E   R   S   A   K   T   A   E   E   K   K   K   L   Q   Q   G

2821    ATT CAA GAA TTA GAA GAA AGT CTT GAG GAG GAA GAA GCT CAA AGA CAA AAG TTG CAA CTC
 941     I   Q   E   L   E   E   S   L   E   E   E   E   A   Q   R   Q   K   L   Q   L

2881    GAA AAG GTT CAA GTC GAA GCT AAA CTA AAA GCA TTA GAA GAC GAT TTA AGA CTA GTC GAA
 961     E   K   V   Q   V   E   A   K   L   K   A   L   E   D   D   L   R   L   V   E

2941    GAC TCC AAT GCT AAA CTA TCA CAA GAC AAA AAA CAA CTT GAA GAG AGA ATG GAT GAA CTA
 981     D   S   N   A   K   L   S   Q   D   K   K   Q   L   E   E   R   M   D   E   L

3001    AGC ACA AAG TTG TCG GCA GAA GAA GAT AAA TCA AAA GGT TTA ATA AAA TTA AAA GTT AAA
1001     S   T   K   L   S   A   E   E   D   K   S   K   G   L   I   K   L   K   V   K

3061    CAA GAA ACA TTA ATA TCA GAA TTA GAA GAA CGT GTT GCA AAA AGT GAA AAA ACT GCT GTT
1021     Q   E   T   L   I   S   E   L   E   E   R   V   A   K   S   E   K   T   A   V

3121    GAT TAC CAA AAA GAA ATT AGA AAA CTT CAG CAA GAA ATT AGT GAT TTG CGA AAT CAA TTG
1041     D   Y   Q   K   E   I   R   K   L   Q   Q   E   I   S   D   L   R   N   Q   L

3181    GCA GAA GCA CAA CAA CGT ATT GCT GAG TTA GAA GAT GAT CTT GCT CGT CGT GAT AAT GAA
1061     A   E   A   Q   Q   R   I   A   E   L   E   D   D   L   A   R   R   D   N   E

3241    CTG GCT GCA GCT ATC AAG AGA GGA GAT GAG GAA ATG TCA AAA AGA GTT AAT GTC GAA AAA
1081     L   A   A   A   I   K   R   G   D   E   E   M   S   K   R   V   N   V   E   K

3301    GCA AAA CGT GAT GTT GAA GCT CAT CTA GAA GAA GTT AAA GAT GAT TAT GAA CAA GAA AAA
1101     A   K   R   D   V   E   A   H   L   E   E   V   K   D   D   Y   E   Q   E   K

3361    GCA GCT CGT GAA AAA GTT GAA AAA GCT AAA AGA GAA CTA GAG AAG GAT CTT AAT GAA TTG
1121     A   A   R   E   K   V   E   K   A   K   R   E   L   E   K   D   L   N   E   L
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3421    CGT GAA GAA TTA GAA GTC AAC ACA GAT GCA ACT ACA GTG CAG AAA GAG CTT CAG CGT AAA
1141     R   E   E   L   E   V   N   T   D   A   T   T   V   Q   K   E   L   Q   R   K

3481    CGT GAA GAA GAT TTT AAT GAA TTA AAA AAA CAA GTT GAA ATA GAG GCT AGA GAA CAT GAA
1161     R   E   E   D   F   N   E   L   K   K   Q   V   E   I   E   A   R   E   H   E

3541    AAA CAA ATG GAC TCA CTG CGA GGA AAA CAC AAC CAA CTT GTG AAT GAA CTT CAG GAT CAG
1181     K   Q   M   D   S   L   R   G   K   H   N   Q   L   V   N   E   L   Q   D   Q

3601    TTG GAT CAA TTT AAA AAG GGA AAA AAT GCA CTG GAA AAA AAT AAA ACG GCA TTA GAG AAT
1201     L   D   Q   F   K   K   G   K   N   A   L   E   K   N   K   T   A   L   E   N

3661    GAG AAT GCT GAA ATT GCT GCT GAT TTG CAG CGT GTT TCT TTG TTG AAG CAG GAG AGC GAT
1221     E   N   A   E   I   A   A   D   L   Q   R   V   S   L   L   K   Q   E   S   D

3721    AGA AAA GCC AAA AAT TTT GAA GCT CAG TTA AGT GAA GCA AAT GCA ATT CGC ATG GGA CAA
1241     R   K   A   K   N   F   E   A   Q   L   S   E   A   N   A   I   R   M   G   Q

3781    GAG GAA ATG ATT TCA AAA CTT GAT GGA CAA TGT GCT AAG TTA ACT AAA GAA TGT GAT TCA
1261     E   E   M   I   S   K   L   D   G   Q   C   A   K   L   T   K   E   C   D   S

3841    TTA AAT CAA CAA ATT GAT GAA GTG GAA TCA AAA GCA GCC AAT CTC GAA CGA GCT AAA CAA
1281     L   N   Q   Q   I   D   E   V   E   S   K   A   A   N   L   E   R   A   K   Q

3901    GCT GCA GAG ACT TCT CTG TCA GAC CTT CAA GAT GCT TTA CAT GAA GAA ACA CGC CAG AAA
1301     A   A   E   T   S   L   S   D   L   Q   D   A   L   H   E   E   T   R   Q   K

3961    CTT GCT CTT CAA ACA AAA GTT CGC GAG GCT GAA GAT GAA GCC AAT CGT CTT CAA GAA CAA
1321     L   A   L   Q   T   K   V   R   E   A   E   D   E   A   N   R   L   Q   E   Q

4021    CTT GAA GAA GAA GAA GAT GAG AAA AAA GCT GTT CAA AAA ACT CTC ACA CAA GTT CAA TTG
1341     L   E   E   E   E   D   E   K   K   A   V   Q   K   T   L   T   Q   V   Q   L

4081    CAG CTT GAT AGT TGC AAA AAA GAA ATC GAA TTA AAA GTT ACA CTT CTT GAA GAA GCT GAG
1361     Q   L   D   S   C   K   K   E   I   E   L   K   V   T   L   L   E   E   A   E

4141    TCA GCT CGT CAA AAA CAA AAA CGA GAA AAT GAA GAA CTT CGC TCT GAC AAT GAG AGG TTT
1381     S   A   R   Q   K   Q   K   R   E   N   E   E   L   R   S   D   N   E   R   F

4201    CAG TCT GAA ATT TCC AAA CTT GAC AAA GCT CGT AAG AAG TTA CAA GGA GAT TTG GAT GAT
1401     Q   S   E   I   S   K   L   D   K   A   R   K   K   L   Q   G   D   L   D   D

4261    GTT ACT GTG CTC ATG GAA AGA GAA AGA AAT AAT GCA TCC CAA ATG GCT GCA AAA CAA AAG
1421     V   T   V   L   M   E   R   E   R   N   N   A   S   Q   M   A   A   K   Q   K

4321    AAA TTC GAT CAA CTT TTA TCT GAA GAA AAA TCT AGA TCC CAG GCT CTT GCA AGT GAG CGA
1441     K   F   D   Q   L   L   S   E   E   K   S   R   S   Q   A   L   A   S   E   R

4381    GAT AAT GTA GAA AAA GTT GCT CGT GCA AAT GAA ACA AAA ATT CTT TCA TTA CAA AGT GCA
1461     D   N   V   E   K   V   A   R   A   N   E   T   K   I   L   S   L   Q   S   A

4441    AAT GAA GAA TTA GAG GAC AAA TTA GCA GAG TCT GAG CGT GTA CGT AAA AGT CTT ATG GCA
1481     N   E   E   L   E   D   K   L   A   E   S   E   R   V   R   K   S   L   M   A

4501    GAA CTC CAA GAG CTT ATT GAT TCT AAA GAC GGT GCC GGT AAG AAT ATA CAT GAA CTT GAT
1501     E   L   Q   E   L   I   D   S   K   D   G   A   G   K   N   I   H   E   L   D

4561    AAA GCT AAA CGA CTT CTT GAA CAG CAA CTT GCC GAG CAG AAA ACT CAA GTT GAA GAA CTT
1521     K   A   K   R   L   L   E   Q   Q   L   A   E   Q   K   T   Q   V   E   E   L

4621    GAA GAT GAG CTG CAG GCC ACC GAA GAT GCC AAG CTA AGA TTA GAA GTA AAT ATG CAA GCA
1541     E   D   E   L   Q   A   T   E   D   A   K   L   R   L   E   V   N   M   Q   A

4681    CAA AAA GCT CAG TTT GAA AGA GAA CTT GCA GCA AAA GAA GAT TCT ATT GAA GAA GGA AGA
1561     Q   K   A   Q   F   E   R   E   L   A   A   K   E   D   S   I   E   E   G   R

4741    AAA GGA CTT ATC AAA CAG TTA CGT GAA ATG GAA CTC GAA CTT GAA GAG GAA CGC AAA GTA
1581     K   G   L   I   K   Q   L   R   E   M   E   L   E   L   E   E   E   R   K   V

4801    AGC AAA GCT GCT GGT GCA TCT AAA CGA AAG TTA GAG AGT GAT GTA AAG GAA CTT ACT GCT
1601     S   K   A   A   G   A   S   K   R   K   L   E   S   D   V   K   E   L   T   A

4861    CAG TTA GAT CAA GCT AAC AGA CTT AAA GAA GAT AGT CAA AAG CAG CTC AAG AAA TAT CAA
1621     Q   L   D   Q   A   N   R   L   K   E   D   S   Q   K   Q   L   K   K   Y   Q

4921    GTC CAT CTT AAA GAT GTT CAA CGT GAT TTA GAT GAA GCA AAG GCT GCA CGT GAA GAG TTG
1641     V   H   L   K   D   V   Q   R   D   L   D   E   A   K   A   A   R   E   E   L
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4981      TCT GCT CAG GTC AAA GAT AAT GAA AAG AAA TTA AAG GGA TTA GAG TCA GAT TTC GTA CAA
1661       S   A   Q   V   K   D   N   E   K   K   L   K   G   L   E   S   D   F   V   Q

5041      ATG CAA GAA GAT CTC AGC ACA GCT GAG CGT GCT CGT CGT ACT ATT GAA GCA GAA CGC GAT
1681       M   Q   E   D   L   S   T   A   E   R   A   R   R   T   I   E   A   E   R   D

5101      GAA CTT CTT GAA GAG TTA AAT AAC AAC ACT TCT GCA AAG ACA GCT GTA GCT GAA GAA CGC
1701       E   L   L   E   E   L   N   N   N   T   S   A   K   T   A   V   A   E   E   R

5161      AAA CGA TGG GAG GCT CAA ATT GCT GCC CTT GAA GAA GAA TTA GAA GAA GAA CGA ACT CAG
1721       K   R   W   E   A   Q   I   A   A   L   E   E   E   L   E   E   E   R   T   Q

5221      ACC GAG CTT ATG CAA GAT AAG GTT TCA CGA GGT AAT CTA CAA ATA GAC CAA ATG TCT GCT
1741       T   E   L   M   Q   D   K   V   S   R   G   N   L   Q   I   D   Q   M   S   A

5281      GAA TTA GCT GAA GAA AGA GCC GCT TTG CAA TCC CTT GAC AAC TCT CGT ATG ACA TTA GAA
1761       E   L   A   E   E   R   A   A   L   Q   S   L   D   N   S   R   M   T   L   E

5341      CGC GCC AAC AAA GAA TTG CAA AAT AAA CTT GCT GAT TTA GAA TCA AGT TTA CGC TCA CGT
1781       R   A   N   K   E   L   Q   N   K   L   A   D   L   E   S   S   L   R   S   R

5401      ACC AAG AAC ACA GTA GCA ACT CTT GAA GCC AAA ATT AGC AAT CTG GAA GCC CAA CTT GAT
1801       T   K   N   T   V   A   T   L   E   A   K   I   S   N   L   E   A   Q   L   D

5461      CAA GAA GCC AAA GAA AAA CAG AAT ATT ACA AAA TTA CTT AGA AGA ACT GAA AAG CGA GCT
1821       Q   E   A   K   E   K   Q   N   I   T   K   L   L   R   R   T   E   K   R   A

5521      AAA GAA CTT CAA GTG CTT GTT GAT GAG GAG CGT GGT CAC ACT GAA TCG TAC AAA CAA CAA
1841       K   E   L   Q   V   L   V   D   E   E   R   G   H   T   E   S   Y   K   Q   Q

5581      GTT GAG AAA GCT AAT AAC AGG GTA AAA AGC ATA AAA CGA CAA TTG GAT GAA TCC GAG GAA
1861       V   E   K   A   N   N   R   V   K   S   I   K   R   Q   L   D   E   S   E   E

5641      GAA GTT AGT AGA CTA AAC GGA ACC AAG AGA AAG ATG CAA AGA GAT CTT GAT GAA AAT ACC
1881       E   V   S   R   L   N   G   T   K   R   K   M   Q   R   D   L   D   E   N   T

5701      GAA GCG CTT GAA TCA GCA CAA AGA GAA CTT ACA CAG TTA AAA TCA AGG ATG AAG ACT GCC
1901       E   A   L   E   S   A   Q   R   E   L   T   Q   L   K   S   R   M   K   T   A

5761      ACT ACG CCT AGC ACC CGT TCT ACA GGA CGT CGC CGT AAA GAT GAC GAT GAA CAA AAT GAT
1921       T   T   P   S   T   R   S   T   G   R   R   R   K   D   D   D   E   Q   N   D

5821      GAG GAC GGC ACC GAT TGA
1941       E   D   G   T   D   *
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6.3.5 PKD2

2520 base pairs

839 amino acids
96.02 kDa

transmembrane domain
EF-hand motif
antigenic peptide

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   1      ATG AGT GCT ACA AAA AGA ATT AGA AGT ACA AAA GAT TTA CAA CTG AGT CAA CAA CAG GAG
   1       M   S   A   T   K   R   I   R   S   T   K   D   L   Q   L   S   Q   Q   Q   E

  61      TTG TCG GTA CGT AAT GAC TAT CGC GGA TCA AGT GTC TCA TTT GCA TCA TCT AAA AGT TCA
  21       L   S   V   R   N   D   Y   R   G   S   S   V   S   F   A   S   S   K   S   S

 121      CCT TGG CAT GAT GAC ACT AGT TTT ATT TTA AAG CAA AAG GCT TTA TCT TCT AGC GAG AAA
  41       P   W   H   D   D   T   S   F   I   L   K   Q   K   A   L   S   S   S   E   K

 181      GAA TTA AGT AAT TTG CAA AGT ACA ACT TCT ATG AAT CAA TTC AAA AGA CCA AAG CCT ATA
  61       E   L   S   N   L   Q   S   T   T   S   M   N   Q   F   K   R   P   K   P   I

 241      CCT TTT AAG GAA ATG TTC TTG AGA AGC TTA AGA GGT TTA TGG AAA ACA CGA TAC TCT GAT
  81       P   F   K   E   M   F   L   R   S   L   R   G   L   W   K   T   R   Y   S   D
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 301  GAA CTT AAG GAT AAA ACA GAA GCC ATG AAA GTT ACA TTG AAG GAA CTT GCA ATT TAT GTT
 101   E   L   K   D   K   T   E   A   M   K   V   T   L   K   E   L   A   I   Y   V

 361  TTT TTT TTA ATG ATT GCT TGT ATA ATT ACC TTT GGA ATG ACC AGC ACT TCA ATG TTT TAT
 121   F   F   L   M   I   A   C   I   I   T   F   G   M   T   S   T   S   M   F   Y

 421  ATG ACA AAT GCA ATC CAA GGT GTA CTT GCT CCC ACT CCT CCC AAA GAA ATG CCA GGA GAA
 141   M   T   N   A   I   Q   G   V   L   A   P   T   P   P   K   E   M   P   G   E

 481  AGT GGA GAT GTG TTT GAG TAT TTA AAA GAT ACA GTC ATA GGT GGA CTT TAT GTG GAA AAT
 161   S   G   D   V   F   E   Y   L   K   D   T   V   I   G   G   L   Y   V   E   N

 541  TAT TAT GAT GAT TCA CCT GTA AAA ACT TCT GAG TTG GGT TAT ATC TTT TTC GAA AAT AAG
 181   Y   Y   D   D   S   P   V   K   T   S   E   L   G   Y   I   F   F   E   N   K

 601  TTA TTA GGT CGC CCA AGA CTT CGT CAA GTT CGA GTT AGT AAT GAA TCT TGC ATA GTT CAT
 201   L   L   G   R   P   R   L   R   Q   V   R   V   S   N   E   S   C   I   V   H

 661  GAA TAT TTT CGA GAT GAA ATA AAA GAA TGT TAT GCC CCA TAC TCA CCT AGT ACT GAA TTT
 221   E   Y   F   R   D   E   I   K   E   C   Y   A   P   Y   S   P   S   T   E   F

 721  GTT GGG AAA TTT GGT TTG GAA AAT GGA ACT GCC TGG GAG TAT CAA TCT GAA AAA GAG CTA
 241   V   G   K   F   G   L   E   N   G   T   A   W   E   Y   Q   S   E   K   E   L

 781  GAT GGT CAA AGT TTT TCT GGG CCT ATA TCT ACT TAT GGA GGA GGT GGT TAT ACA GTT TTG
 261   D   G   Q   S   F   S   G   P   I   S   T   Y   G   G   G   G   Y   T   V   L

 841  TTT GGA GCA GAT AGC GCA GAG TCT AAT AGC ATT ATT GAT GCT CTA CAG AGT AAT CGA TGG
 281   F   G   A   D   S   A   E   S   N   S   I   I   D   A   L   Q   S   N   R   W

 901  CTT GAT CGT GGA ACA AGA GCT GTA TTT TTC GAT TTT GCT GTT TAC AAT GCC AAC ATT AAT
 301   L   D   R   G   T   R   A   V   F   F   D   F   A   V   Y   N   A   N   I   N

 961  CTG TTT TGT GTT GTT CGC TTG CTA CTT GAG TTT CCA GCT ACT GGT GGA TGC TTC CCG ATA
 321   L   F   C   V   V   R   L   L   L   E   F   P   A   T   G   G   C   F   P   I

1021  TTT TAT TTT CAA ACA TTA AAG CTG TTG CGC TAT GTG ACC TCT ATG GAT TAT TTT GTA ATG
 341   F   Y   F   Q   T   L   K   L   L   R   Y   V   T   S   M   D   Y   F   V   M

1081  GCG TGT GAA GCA ATA TTT ATC CTT CTT CTC ATT TAT TAC TCC ATT GAA GAA GCA ATT GAG
 361   A   C   E   A   I   F   I   L   L   L   I   Y   Y   S   I   E   E   A   I   E

1141  ATA AAA AAA CAT GGT ACT TCA TAT TTC TCA GAT GTA TGG AAT GTC ATG GAT ATT ATA ATA
 381   I   K   K   H   G   T   S   Y   F   S   D   V   W   N   V   M   D   I   I   I

1201  GTG TTA CTG GGT TTT ATT TGT GTG ATT TTT AAT GGT GTT CGT ACG GTA TCA GTT GCT AAT
 401   V   L   L   G   F   I   C   V   I   F   N   G   V   R   T   V   S   V   A   N

1261  AAG TTA CGT ATT ACG TTG GAG GAA CGA GAT AAG TAT GCA AAT TTT GAG ATA TTG GCT GAC
 421   K   L   R   I   T   L   E   E   R   D   K   Y   A   N   F   E   I   L   A   D

1321  ATG CAG ACA AAA TTT AAT GAC CTT GTA GCA GTT TAT ATA TTT CTT ATT TGG ATT AAG TTA
 441   M   Q   T   K   F   N   D   L   V   A   V   Y   I   F   L   I   W   I   K   L

1381  TTC AAG TAT CTC TCA TTC AAC AAA ACA ATG ACA CAG TTA CAA TCA ACT CTC TCT CGT TGT
 461   F   K   Y   L   S   F   N   K   T   M   T   Q   L   Q   S   T   L   S   R   C

1441  GCT AAA GAT ATT GCT GGT TTT TCA GTG ATG TTT TTT ATT GTT TTC TTC GCC TAT GCA TTA
 481   A   K   D   I   A   G   F   S   V   M   F   F   I   V   F   F   A   Y   A   L

1501  TGG GGC TAC CTA CTT TTA GGG CCT CAA TTA GCT GAT TAT TCT ACG TAC TTA AAC AGC ATA
 501   W   G   Y   L   L   L   G   P   Q   L   A   D   Y   S   T   Y   L   N   S   I

1561  TTT GCA TGT TTT CGT ATT ATT CTT GGT GAC TTT GCT TGG TCT GAT ATA AAT GGT GCT GCA
 521   F   A   C   F   R   I   I   L   G   D   F   A   W   S   D   I   N   G   A   A

1621  CCA AGT ATG GGC CCA ATA TTT TTC ATT TCT TAT GTA TTC ATG GTG TTT TTT ATT TTG ATC
 541   P   S   M   G   P   I   F   F   I   S   Y   V   F   M   V   F   F   I   L   I

1681  AAC ATG TTT TTG GCC ATT ATT AAT GAC ACT TAT TCT GAA GTG AAA TCA GAT CTT GCA GAG
 561   N   M   F   L   A   I   I   N   D   T   Y   S   E   V   K   S   D   L   A   E

1741  CAA AAA GAT GAA TTT GAA ATT GGT GAT TAC TTT AAA AAA GGA TAT GAT AAA ATG ATG AGC
 581   Q   K   D   E   F   E   I   G   D   Y   F   K   K   G   Y   D   K   M   M   S

1801  AAT TTA TCT TTT AAG CGA GAA AAA ATT GTT GAC ATT CAA AAA GCT CTT CAA ACT GCT GAT
 601   N   L   S   F   K   R   E   K   I   V   D   I   Q   K   A   L   Q   T   A   D
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1861    GTG AAC CAT GAT AAT ATG TTA GAC TTC GAA GAA TGG AGA GCA CAG TTA AAA TTG CGT GGC
 621     V   N   H   D   N   M   L   D   F   E   E   W   R   A   Q   L   K   L   R   G

1921    CAT GCT GAT TTA GAA ATT GAG GCT GTA TTT ACT CAG TAT GAT TTA GAT GGA GAT CGT GTA
 641     H   A   D   L   E   I   E   A   V   F   T   Q   Y   D   L   D   G   D   R   V

1981    CTC AAT GAA ATT GAA CAA CAA AAA ATG CAT GAT GAT CTT GAT ATA AAA ATG GAA GAG TTA
 661     L   N   E   I   E   Q   Q   K   M   H   D   D   L   D   I   K   M   E   E   L

2041    GAA GAT GAA ATT GAA GAA GTT CGC AAG TCA AAA GGA AAT TTA AAA AAA ATT CAA AGT AAA
 681     E   D   E   I   E   E   V   R   K   S   K   G   N   L   K   K   I   Q   S   K

2101    ATA AAC ATG AAA TCT GGT GAT GAT GAT GAT GAT GAT GAT GAA GAT GAT GAT GAA AAT GAC AGT
 701     I   N   M   K   S   G   D   D   D   D   D   D   E   D   D   D   E   N   D   S

2161    GAA CCA ACA GGA CTT GTA AAA TAT GAA GAG TTC TCA ATC CTG GCT CGA CGC GTT GAT CGT
 721     E   P   T   G   L   V   K   Y   E   E   F   S   I   L   A   R   R   V   D   R

2221    GTT GAA CAA AGT ATT GGC AGT ATG GTG TTG AAG ATT GAT TCC GTT CTT TTA AAG TTG GAA
 741     V   E   Q   S   I   G   S   M   V   L   K   I   D   S   V   L   L   K   L   E

2281    GCT ATG GAT AAA GCT AAA ATA AAA GGA AGA GAG ACA ATG ACA AAG CTT TTA GAC AGT ATT
 761     A   M   D   K   A   K   I   K   G   R   E   T   M   T   K   L   L   D   S   I

2341    GCT GAG CAA GAA TTG AGT GTT TTT AAA GAG TTT GGG AAT AAA TCT AGC ACA AAT ATA TTT
 781     A   E   Q   E   L   S   V   F   K   E   F   G   N   K   S   S   T   N   I   F

2401    CGC CCA ACA TCA ACA ACT TCA AAA CAA TCT AAT GAT AGC GTC ATC TCT ATC AAA GAG GAC
 801     R   P   T   S   T   T   S   K   Q   S   N   D   S   V   I   S   I   K   E   D

2461    CAT GAT GGT GAT GAC GAT GAT GAT GGT GAT AAT GAT GAT GGT GAC GAT CCT GTT AAC TAA
 821     H   D   G   D   D   D   D   D   G   D   N   D   D   G   D   D   P   V   N   *
```

7. ACKNOWLEDGMENTS

I would like to thank several people that contributed to this thesis directly or indirectly:

Suat Özbek

For giving me the opportunity to work on this fascinating project. I am thankful for the many discussions and his door being always open for smaller or larger problems.

Thomas Holstein

For being my second referee and sharing his scientific knowledge and fascination on nematocytes with me.

All current and former lab members

For a nice working atmosphere.

Madeline Bucher, Benjamin Trageser, Björn Eismann and **Bérénice Ziegler**

For working on my projects as bachelor or master students and contributing data to the field of nematocyst research. Might it be included in this thesis or not.

Jutta Tennigkeit

For excellent technical assistance.

My collaborators

Frauke Gräter and **Senbo Xiao** for the MD calculations of Cnidoin.

Wolfgang Petrich, Niels Kröger and **Florian Langhojer** for the FTIR measurements on recombinant Cnidoin.

Rob Steele for the revelation of genomic TrpA sequence.

Frank Kühn for working on the electrophysiological characterisation of TrpA.

Michael Hess for the immunogold TEM of Nematocalcin.

Fabian Rentzsch for introducing PKD2 to us.

Adam Bertl for giving me the opportunity to work in his yeast lab in Darmstadt.

My friends

For being always there.

My parents

For their endless love and support.

Tino Linn

For everything.