Dissertation

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presented by

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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
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).


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 MECHANOESENSATION IN HYDRA. Physiologisches Kolloquium, RWTH Aachen (2013).

Publications:


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.


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.
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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).

![Image of Hydra magnipapillata](image by Dr. Melanie Mikosch and Prof. Dr. Thomas Holstein)

![Figure 1.2: Anatomy of Hydra. A Photograph of Hydra magnipapillata. B schematic representation of Hydra’s body plan. C different cell types in Hydra.](image by 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 (Forét *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.

<table>
<thead>
<tr>
<th></th>
<th>Nematostella vectensis</th>
<th>Hydra magnipapillata</th>
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<tbody>
<tr>
<td>Genome size (Mbp)</td>
<td>357</td>
<td>900</td>
</tr>
<tr>
<td>Chromosome number</td>
<td>15 (1N)</td>
<td>15 (1N)</td>
</tr>
<tr>
<td>GC content</td>
<td>49 %</td>
<td>29 %</td>
</tr>
<tr>
<td>Protein-coding genes</td>
<td>~ 18 000</td>
<td>~ 20 000</td>
</tr>
<tr>
<td>Transposable elements (% genome)</td>
<td>26 %</td>
<td>57 %</td>
</tr>
<tr>
<td>miRNA</td>
<td>40</td>
<td>17 (51)*</td>
</tr>
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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).](image)

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., 2013).
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 (Slatterback, 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).

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 (Slatterback & Fawcett, 1959; Holstein, 1981). The nematocytes are still connected by cytoplasmic bridges (Slatterback & 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.
2.1 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.

During the analysis of the capsule proteome, a candidate protein was found that might fulfill 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 signal peptide 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.

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 2.2: 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.
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.
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 Deglycosylation assay. 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.](image)

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).

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 ($\Delta$SASA) 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 $\Delta$SASA 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 ($\Delta$SASA). 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.](image)

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.](image)

The in situ hybridization of TrpA revealed the expression of the calcium channel in
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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).](image)

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.
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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, HC030031, A967079) 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).](image-url)

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

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
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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.](image)

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 characterisation 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).

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).](image)

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 preimmuniserum (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 loose 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.
RESULTS

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.
RESULTS

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
GdCl₃ 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
Results

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.](image)

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.

3.1 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](image)

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 maturing 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 heterologous 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*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number</th>
<th>Query coverage</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>peflin-like</td>
<td>XP_002168493.2</td>
<td>90%</td>
<td>36%</td>
</tr>
<tr>
<td>programmed cell death protein 6-like</td>
<td>XP_002169270.2</td>
<td>83%</td>
<td>35%</td>
</tr>
<tr>
<td>calpain-B-like</td>
<td>XP_002167620.1</td>
<td>80%</td>
<td>37%</td>
</tr>
<tr>
<td>grancalcin-like</td>
<td>XP_004212885.1</td>
<td>61%</td>
<td>34%</td>
</tr>
</tbody>
</table>

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 (Slatterback & Fawcett, 1959; Holstein, 1981). The capsule primordium is surrounded by an arrangement of microtubules (Slatterback, 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 (Slatterback, 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 CaCl₂, 0.1 mM MgCl₂, 0.1 mM KCl, 1 mM NaH₂CO₃, 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$_2$O 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$_2$O 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.**

<table>
<thead>
<tr>
<th>primer sequence (5’- 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnidoin forward</td>
<td>ATGTCTCGATTACTACTCTTC</td>
</tr>
<tr>
<td>Cnidoin reverse</td>
<td>TTATCTCTTTTTACCAAAAGCTCC</td>
</tr>
<tr>
<td>TrpA forward</td>
<td>ATGGATAGCAGACTCAGATGACTGG</td>
</tr>
<tr>
<td>TrpA reverse</td>
<td>TCAATCAACTTTTGTGTGAACGAGAACTTGG</td>
</tr>
<tr>
<td>Nematocalcin forward</td>
<td>ATGGCATATTCAGGATACAACTC</td>
</tr>
<tr>
<td>Nematocalcin reverse</td>
<td>CTAGCTGGGAAACAACCAG *</td>
</tr>
<tr>
<td>Nematomyosin forward</td>
<td>ATGGCAAGAAGAAAGATGTTTA</td>
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<tr>
<td>Nematomyosin reverse</td>
<td>TCAATCGGTGCGTCTCCTATC</td>
</tr>
<tr>
<td>PKD2 forward</td>
<td>ATGAGTGCTACAAAAAGAATTAGAAGTACAAAAG</td>
</tr>
<tr>
<td>PKD2 reverse</td>
<td>GTAATAATTTTCACACTCCTCGCTG</td>
</tr>
</tbody>
</table>

* The Nematocalcin reverse primer comprises 3’UTR. Further subcloning was necessary to obtain a pure Nematocalcin cDNA clone.
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% H2O) 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°C. 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 Denaturing 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) MgCl₂ 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$_2$PO$_4$, 8 mM Na$_2$PO$_4$] 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 \[ e^{-\frac{AIC_{i\text{max}} - AIC_{i}}{2}} \]
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 QMQCGGQQMPMPMMSGCGG and QMQCGGQQQLPLMMPGCQVG, 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·mol⁻¹·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·mol⁻¹·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 GdCl₃ (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$_2$PO$_4$ 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 an 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.


Bode, H., Berking, S., David, C.N., Gierer, A., Schaller, H. and Trenkner, E. (1973) QUANTITATIVE ANALYSIS OF CELL TYPES DURING GROWTH AND MORPHOGENESIS IN HYDRA. Development Genes and
REFERENCES


David, C.N. and Murphy, S. (1977) CHARACTERIZATION OF INTERSTITIAL STEM CELLS IN HYDRA BY CLONING. Developmental Biology(2), 372-383.


Eismann, B., Beckmann, A. and Özbek, S. **MANUSCRIPT ON NEMATOMYOSIN IN PREPARATION.**


DOMINANT POLYCYSTIC KIDNEY DISEASE (ADPKD), IS A Ca2+-PERMEABLE NONSELECTIVE CATION CHANNEL. Proceedings of the National Academy of Sciences 98 (3), 1182-1187.


REFERENCES

VESICLE. Protoplasma 248 (4), 635-640.


Reft, A.J. and Daly, M. (2011) MORPHOLOGY, DISTRIBUTION, AND EVOLUTION OF APICAL STRUCTURE OF NEMATOCYSTS IN HEXACORALLIA. J Morphol.


Trembley, A. (1744) MEMOIRES, POUR SERVIR A L'HISTOIRE D'UN GENRE DE POLYPES D'EAU DOUCE, A BRAS EN FORME DE CORNES. Jean and Herman Verbeek, Leiden.


Weill, R. (1934) CONTRIBUTION A L'ETUDE DES CNIDAIRES ET DE LEURS NEMATOCYSTES. Travaux de la Station Zoologique de Wimereux 10, 11.


MONOCLONAL ANTIBODY IN NEURONS IN SITU IN THE HEAD REGION OF HYDRA. Journal of Morphology 184 (2), 183-193.
## 6.1 Antibodies

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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Eurogentec No</th>
<th>Antigenic peptide</th>
<th>Fixative</th>
<th>Duration of fixation</th>
<th>Dilution</th>
<th>Blocking for Western Blot</th>
<th>Species</th>
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<td>Cnidoin</td>
<td>DE10035</td>
<td>GCAIPQSCQICPSGRCGCGA</td>
<td></td>
<td></td>
<td>1:200</td>
<td>milk</td>
<td>Guinea pig</td>
</tr>
</tbody>
</table>
Table 5: Compilation of primary antibodies used for co-stainings.

<table>
<thead>
<tr>
<th></th>
<th>Fixation</th>
<th>Dilution</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minicollagen-1</strong></td>
<td>Lavdovsky is best,</td>
<td>1:500</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>PFA for Cnidoin co-staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Minicollagen-15</strong></td>
<td>Lavdovsky</td>
<td>1:1000</td>
<td>rabbit</td>
</tr>
<tr>
<td><strong>NOWA CTLD</strong></td>
<td>ice-cold Methanol</td>
<td>1:400</td>
<td>rabbit</td>
</tr>
<tr>
<td><strong>α-tubulin</strong></td>
<td>ice-cold Methanol</td>
<td>1:1000</td>
<td>mouse</td>
</tr>
<tr>
<td><strong>β-tubulin</strong></td>
<td>PFA</td>
<td>1:1000</td>
<td>mouse</td>
</tr>
<tr>
<td><strong>tyrosinated tubulin</strong></td>
<td>PFA</td>
<td>1:1000</td>
<td>rat</td>
</tr>
<tr>
<td><strong>Nematostella Minicollagen-1</strong></td>
<td>Lavdovsky is best,</td>
<td>1:500</td>
<td>guinea pig</td>
</tr>
<tr>
<td></td>
<td>PFA works also</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phalloidin Alexa568</strong></td>
<td>PFA</td>
<td>1:200</td>
<td>-</td>
</tr>
<tr>
<td><strong>actin</strong></td>
<td>ice-cold Methanol</td>
<td>1:90</td>
<td>mouse</td>
</tr>
<tr>
<td><strong>Domain 1</strong></td>
<td>Lavdovsky</td>
<td>1:500</td>
<td>guinea pig</td>
</tr>
</tbody>
</table>
## 6.2 ISH probes

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

<table>
<thead>
<tr>
<th>Clone (No in vector library)</th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnidoin full-length</td>
<td>Bp in full-length</td>
<td>1542</td>
</tr>
<tr>
<td>Cnidoin confirmed</td>
<td></td>
<td>561</td>
</tr>
<tr>
<td>Nematocalcin confirmed</td>
<td></td>
<td>513</td>
</tr>
<tr>
<td>Nematocalcin full-length</td>
<td></td>
<td>679</td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td>749</td>
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<td>T7</td>
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<td>1023</td>
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<tr>
<td>T7</td>
<td></td>
<td>1028</td>
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<tr>
<td>Bp in full-length</td>
<td></td>
<td>1542</td>
</tr>
<tr>
<td>Cnidoin confirmed</td>
<td></td>
<td>561</td>
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<tr>
<td>Nematocalcin confirmed</td>
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<td>Nematocalcin full-length</td>
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<td>1023</td>
</tr>
<tr>
<td>T7</td>
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<td>1028</td>
</tr>
</tbody>
</table>

**Result**

- Nests with antisense
- Nests with antisense
- Ubiquitous signal with antisense
- Ubiquitous signal with antisense
- No difference between sense & antisense
- No difference between sense & antisense
- A cocktail of all 4 antisense probes showed nests (alone no difference between sense & antisense)
- No difference between sense & antisense
- Ubiquitous signal with antisense
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**

```
1   ATG TCT CGA TTA CTA CTT CTA TTA CTG GTT TGC TTG ATA TTG TAG 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
61  TCA AAG TCC GAA AAA AAA GAT CAT AAA AGC AAA GAG CATCAA AAA GAA AAA CAC GAA GAA
121  S   K   S   E   K   K   D   H   K   T   K   E   H   Q   K   K   H   K
211  AAA AAG AAA ACT CCT AAA TCA ACT GAA ACT GGA GAT GAA TCA GAA GGT
361  S   G   T   Q   R   S   E   T   L   E    D   T   D   H   T   T   S
511  ACA GAC ACT GAG AAG ACG ACA ATT GCA AAA AGA GC T GCA CTT CCG GGA GGA GCA ACA TTC
661  S   G   C   S   P   S   C   A
811  TCC GGA ACT CAA CGA TCA GAA AGC CTT GAT GCA AGA CAT AGA TAT GAG GTT GAG
961  G   G   C   G
1211 GGG GGT TGC GGT GGA CAA ATG CAA GGT TGT GGA CAA CAA ATG CCT CAA ATG CCA GCT TCT CTA
1411 S   G   G   C   G   S   P   S   C   M   N   S   M   P   Q   M   P   A   S   L
1611 TCA TCT GGA TGT TCT CCT AGC TGC ATG AGT CAA GAA GAT AGT CCT CAA ATG CCA GCT TCT CTA
1811 S   S   G   C   S   P   S   C
2011 GGT TGC GGT GGA CAA ATG ATG GGT TGT GGA CAA CAA ATG CCT CAA ATG TCA TGA GGG
2411 G   G   C   G   Q   G   G   Q   S   E   T   L   G   B   Q   M   P   Q   M   S   M   G
2811 GCA CAA ATG CAA GGG TGT GGA CAA CAA ATG CGG CCA TTA ATG GGT GTC GCA TG CCAA GCT CGT
3211 A   Q   M   Q   G   C   G   Q   Q   M   P   L   G   M   G   C   G
3611 GGT TGT GGG CAA CAA TCG CCA CCA ATG ATG TCA GG A TGT GTC GGA CAA ATG CAA GGT TGT GGA CAA
4011 G   G   Q   Q   L   R   A   P   M   V   S   M   T   P   G   C   G   G   Q
4411 ATG CAA GGT TGT GGA CAA CAA ATG CCG CAA ATG GTC GTG GGA TGT TTG GCA CAA ATG GTG
4811 M   Q   G   C   G   Q   Q   M   P   Q   M   V   G   C   G   F   Q   M   V
5211 GGT TGC GGC ACA CAA ACA TCT TAA AAA GCC TGC CTC GCA CCT AAC TCA ATT
5611 G   G   C   G   T   Q   T   Q   T   F   Q   S   S   L   K   A   P   C   A   P   N   S
6011 GGT TGT GAG CAG CAG CTT GCT GCT CAA ATG GTA CAA ATG ACG CCA GGT TGT GGT GGA CAA
6411 G   G   C   G   C   G   Q   Q   M   P   Q   M   V   S   M   T   P   G   C   G   G   Q
6811 ATG CAA GGT TGT GGA CAA CAA ATG CCG CAA ATG GTC GTG GGA TGT TTG GCA CAA ATG GTG
7211 M   Q   G   C   G   Q   Q   M   P   Q   M   V   G   C   G   F   Q   M   V
7611 GGT TGC GGC ACA CAA ACA TCT TAA AAA GCC TGC CTC GCA CCT AAC TCA ATT
8011 G   G   C   G   T   Q   T   Q   T   F   Q   S   S   L   K   A   P   C   A   P   N   S
8411 GGT TGT GAG CAG CAG CTT GCT GCT CAA ATG GTA CAA ATG ACG CCA GGT TGT GGT GGA CAA
8811 G   G   C   G   C   G   Q   Q   M   P   Q   M   V   S   M   T   P   G   C   G   G   Q
9211 ATG CAA GGT TGT GGA CAA CAA ATG CCG CAA ATG GTC GTG GGA TGT TTG GCA CAA ATG GTG
9611 M   Q   G   C   G   Q   Q   M   P   Q   M   V   G   C   G   F   Q   M   V
10011 GGT TGT GAG CAG CAG CTT GCT GCT CAA ATG GTA CAA ATG ACG CCA GGT TGT GGT GGA CAA
10411 G   G   C   G   C   G   Q   Q   M   P   Q   M   V   S   M   T   P   G   C   G   G   Q
10811 CAA ATG CCT CCA ATG ATG TCT GGG TGT GGT GGT CAA ATG CAA GGT TGT GGA CAA CAA ATG
11211 Q   M   P   P   M   M   S   G   C   G   G   Q   Q   M   Q   G   C   G   Q   Q   M
```
6.3.2 TrpA

3765 base pairs
1255 amino acids
141.53 kDa

Ankyrin-repeats
transmembrane domain
antigenic peptide
| 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 K N N Y G T T |
| 841 | GCA TGG CAC TAT GCA TGT AGG CTT GGT AAT AAA AAA CTT TGG CTT AAG ATT CTG ACT GAC ATT |
| 281 | A L H Y A C 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 CCT 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 GTG TGC ATA AGG GTA GTA AGT CTT ATA AAT TAT GGA TCA AAT GTA TTT GCT |
| 321 | N G G C I R S V T L I N Y G S N V F A |
| 1021 | ATA 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 AAT AAT AAA ACT AAA |
| 361 | R D L N K G D V F P V L E E I S N K F K |
| 1141 | GCT TGG CAA CAT GGT CCT CAT ATT GTT GAA GAT CTT ATT GAG TTA CTC ATT ATC AAA GGA |
| 381 | A L Q H V S S I V D L E I L L I K G |
| 1201 | GCT CTC AAA AAT GTT CCC GAA ATT AAA CAT GAA CA A CAG AGA AAC GCA TTT GTT AAC AGT |
| 401 | A L K N V P E D K H E Q R N A F V N S |
| 1261 | AAA ACC AAA GAG GAT CAC ACA CCT TGT CAT ATT GCA GCA TGT GGT 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 GGG GAT GTC AAC GCT CAA ACA GAT TCT GCC 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 GAT GAA GAG GTA GAA AAT TTT TTA ATA ATG TAT |
| 461 | F L H F A M S G H E R V V N P L I M Y |
| 1441 | GAT CTT ATT GAA CTG AGG GAT GGT CAA ATT AAT TCA ACT GAT GTT AAT AAC AAT GAT CCT |
| 481 | D A N I Q A V D N D L M T P L H R A C L |
| 1501 | TCC GGG CGA TTA TCA GTG ATT AAA TTA GAT GAG AAA GGA GCT TTA CTT GAA GAT 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 AAT TTT ACT CCT GCT ATT GTA TGT CCT GCT GGT TGT AAG GGT CAT GAT GAA GGT AT A |
| 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 GTT CAA ATT AAT TCA ACT GAT GTT AAT AAC AAT GAT CCT |
| 541 | T Y L I A R G V Q I N S T D V N N K K N A |
| 1681 | CTT CAT GTT GCA GTT AAA GAG AAC CAA TTA GAA ACT TTA AAG TTT TTA TTT GAT AAT CAT |
| 561 | L H V A V K E N D E T L K F L L D N H |
| 1741 | CAA TTT AAA AAG ATG AAT CAT TCA GAT AAA GAC AAT AGG GCA CCT GTG CAT TAT GCA GCA |
| 581 | Q F K K F M N D S D K D N R A P V H Y A A |
| 1801 | GCT GAT GGA AAT CCT CAG GCA TGG 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 | GCT GAT AAT CAA GAA AGG ACT CCT TCT CAT TTG GCA TCT GAG AAG GGT GCT CAA CCT TCA TCT GTG |
| 621 | G D N Q E R T P L H L A S E K G H L S C |
| 1921 | G TA A AA CT CTC ATT TCT ACA TGT CCT GGT GAA GAT ATT AAC TCT ACT GAT GCC CAT GGA ATG |
| 641 | V K L L I S A G E I N S T D A H G M |
| 1981 | ACT CGG CTT CAT TTA GCT GCA TCA AAT GAT CAT AGA AAA GTA GTG AAT CCT CTC 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 TTG 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 AGT CAG ACT CAT CAA ATT TTA GCT GAA AAT GGT TCT ATT 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 GAA AAA GAT GGT TAT ACA CCT CTG CAC CAT GCA GCC CTT GCA GAA CAT GTT GAA TGC |
| 721 | C D K N G Y T P L H L 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 AAC GAG AGA AAA AAT |
| 741 | I V A L L D Q G A N I Q L L T T K E R K N |
| 2281 | TGT TTA TAT CTT GCA GTT GAC AAC TCA GAA AGA GAA GCT GGC ATG GGG 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 |
A

APPENDIX

761

2341

AAA AGA TGG CAT GAA GCT CTT CTC AAT ATA GAT AG C AAA AGA GCT CCT GTT ATG GAA AAA

781

KRKWH feature ALLNIDSKRKAPVMEK

2401

ATA AGA GCA TTT GCA GAA GCT GAA GCA ATT TCA TAT CAT GAC ATT ACT TAT CCT

841

DPINSLNSFAPSLIVQYKR

2461

GTT CCG TGG GTG TAC TTA TCT TCA CTA AGT TTT TAT TAA GTG TTT GTT TCA CTT CCA AAA

881

ALVIEIRMLNSFLSFAPEVLVPSLLE

2521

GAT CTT GAT AAA AAA CAT TCT GAT TAT TCA ATT GAC AGG TGG GTT CA GAG

921

DKKHDSSFLYSFLVFLVPSLLE

2581

GGG AAA TGG TTG AGC CAT CCT CTA GTC GTT GAG GTT ATT AAT CAG AGG TGG TCT GGA ATG

961

KRLSHPLVLVVELINQRWSMR

2641

ATA ATC GAA CTT GCA CCT GAA GTT GCA GAA GTT GC A TTA GAT AAT TGC ATT ACT TAT TCT

1001

IKLIAWPYSKILTVSLGIA

2701

GCA CTT GTG ATT GAA AGA AGT TTA AAC AAG CCA CCA ACA AAA ATG TCA TCA GAA

1041

ALVEIRMLNSFLSFAPEVLVPSLLE

2761

AAG AAA TTT ACT GAC ATA ATT ACA TGG TCG ACT CT C GGC ATT GCA TGC ATA CAA ATT TTA

1081

KFDTIITWSTLGIAQCIL

2821

TGG AAA TTG ATG ATG ACT ATT ACT GAA GTA ATG ATG ATA AAC AAT CCA GAT AAA AAA

1121

WRKLILTIIYIIGISYINNPVKI

2881

TTA GAA TTT TTT TTA TAC ATT TAC ACA GCA GTA TTT ATG GTT CCT TTT ACT TAC CAA

1161

LEFFLYISTALFMVFPFITCQ

2941

TTAT TGT TCG TAA AAA AGA AGT TAA AAT GAT TTA TTT TTA TTA TTA GAG AAT TTA CCA TTT ATT

1201

KLAWSKILLLYENLPFIGLY

3001

TAA CTT GCA TGG TCA AAA ATA CTG GTA TAT TAA GAT TAT TAA CCA TTT ATC GCT GTA TTA TAT

1241

ATTGTCATGTTACCTCTTACATAAAATTATTTACCTGTTTGGGACT

3061

IVMFTEVLTYTLLKVLLVFGT

3121

CTT CTA ATT GGT TTT TTA TCT TTT TAT CAT CTA GAT CCT CTA AAA TCA GCA TTT AGT

1281

LLIGPGFLSFYALDDLQSAFS

3181

GAT TAT GGG GCC TCT ATT GCA AAA ACA TTT GTC ATG CTA GGT GAA GAA ATG AGC TAC GAC

1321

DYGRSIVKTKFVMMGLGRISYD

3241

TCT ATC TTT ACA AAT AAC TAC TCA GAT GAA AAT AGT CAT CTT CTA CCA AAT GAA GAA ATA

1361

SIPHTNNYDNERSNHHLPNLEI

3301

TCT ATT GTC ATT TTT CTA TTA TCT TTT TAT CAT GTA ATG AGA AAT TTT ACT GCA ATG TTA

1401

SIVFILLFLHAMTIMIIVVMNNL

3361

GGT GGT CTT GCA GTT GGT GAT ATT GAA TCA GCT GCC AAC AAT GCA TAT CTA AGA GCT

1441

VGLAVGDIIESVRSNNAYLRVL

3421

CAG AGA CAA GTT TTT TTA AGT ATT TTA GAT CGA ACC TAT CCT AAA TTT ATC AGA AAA

1481

QRQVLYSVILSDRLDRTYFKPIRK

3481

TTT GTGATT AAA GCT TCT TAT ATA AAA CCT AAT AAG AGT TGG TTT AAA AAG TTT

1521

FYYKASIQKPKNSKWFKKFK

3541

ATC CTA TGG TTG GTG ATAT TTA CAA AGG AAA GCA TGG GTA GAA GAA GAA ACA GAT GCA AAA

1561

ILWLGNPQKRKALEEEQQTDAK

3601

AAA GAT TTA ATG ATT GAT TAA ATT GTA GCAA GCA TAT ATT AAG AGA GCA ATG ATA AAA AAG AAA

1601

KDLIMLEIASNREAIKIQKKK

3661

AAG ACT AAA TCG ATT TTA GAC CAT CTC GAA AAG CAA TTA AGG AAA ACT AAA AAA ATT GCT

1641

KTCKSILDDELKQEKLRTKIKI

3721

AAA TTC ATT GCA ACA AGT TCT TGG TCA AAA AAC AAA GAT GAT

1681

KFIAATSFFKTKVDS*. 

APPENDIX
6.3.3 Nematocalcin

606 base pairs
201 amino acids
22.62 kDa

**EF-hand motif**

**antigenic peptide**

```
1  ATG GCA TAT CCA GGA TAC AAT CAA CCG GGT GCA TAC CCT GGT GG TAT GGT CAT CCA GG CAC
1  M   A   Y   P   G   Y   N   Q   P   G   G   Y   G   Q   A   P   G

61  TAC GGT CCT CCT GGT GAT TCT GGA TAT CCT CCT GGT GCA ATT GAC CCC CTT TTT GGA
61  Y   G   P   P   G   G   Y   S   G   Y   P   P   G   G   Y   P   L

121  TAC TTC TCT GCT GTA GCA GGT GAT AAA AAA TAT CTA GAT GTG TTC CAT CAA CTG CAT
121  Y   F   S   A   V   A   G   H   D   Q   Q   I   D   A   R   E   C

181  TTA ACA TCT CCA GGA ATT GGT TCA TAT CAA CAG TCC AGT TTA GAT GCT AGC CCA ACC TTG
181  L   T   S   G   I   G   G   S   Y   Q   Q   F   S   L   E   T   C   R   I

241  ATG AAC ATG CCT GAT AGA GAT TAT TCT GCC AAA ATG GGT TTT ACT GAG TTC ACT GAG GCA
241  M   I   N   M   L   D   R   D   Y   S   G   K   M   E   A   R   K

301  TTA TGG AAT GCA CTA AAT CAA TGG AAG ACT ACA TTT ATT CAA GCG TAT AAC GAC ACA TAT
301  L   W   N   A   L   N   Q   W   K   T   T   F   M   H   D   R   D   R

361  GGT ACA GTA GAG CCT CAT GAA ATG GTA GCT CTC GGT GAT ATT CTA AAC GAT GAT ACA CTT
361  G   T   V   E   P   H   E   M   H   Q   A   I   A   S   W   G   Y   N   L

421  GGT CAA GCG CTT AAT ATC ATT ATA AAA AGA TAT TCT GGC AAT ATG GGT TTT ACT GAG TTC AGT
421  G   Q   A   L   N   I   I   K   R   V   S   B   D   G   T   E   F

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

541  GCC ACT CAA TCA GGA TAT GCT AGT TTT GCT TTT GAT GAT TCT ATT CAA GTC ACA ATG TTT
541  A   T   Q   S   G   Y   A   S   F   A   F   D   D   E   Q   V   T   M

601  TCA GA
601  S   *  
```

6.3.4 Nematomyosin

5838 base pairs
1945 amino acids
224.57 kDa

**head domain**

**tail domain**

**antigenic peptide**

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

61  CCC GTC GCT CAG GCT TGG GCC CCT ATG AAG TTG TTC TGG GTG CCT AGT GGA GAC CAT
61  F   V   A   H   A   A   W   A   A   Q   K   L   V   W   V   P   S   E   H

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121  G   F   V   S   A   I   K   K   E   E   K   G   D   K   V   I   A   E   I

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181  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 GAT CTG ACT TGT TTA AAT GAA GCT GAT GTG TTG CAT AAT
241  D   K   V   E   D   M   A   D   L   T   C   L   N   E   A   S   V   L   R   N
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APPENDIX

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141 K K R H E L F 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 T GC ACG GGT GAA TCT GGT GCA GGA AAA
161 M L Q D R N Q S I L CT E G S A G K
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G   R   Q   G   F   P   N   R   I   L   F   Q   E   F   R   Q   R   Y   E   I   L

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E   A   L   E   M   D   P   N   L   F   R   I   G    Q   S   K   I   F   F   R

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G   E   A   H   N   D   L   K   S   H   D   K   L   T   D   E   N   K   L

TTC GCT CAA CTT CAA CCT GAA AAT GAT CTC AA TTA GCC AAG AGT AGG ACT CAG

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K   L   Q   Q   R   K   I   E   L   E   E   L   N   D   F   E   I   K   L

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A   E   E   B   E   S   A   K   T   A   E   R   K   K   K   L   Q   Q

CAG TAT CAC GAA GCA CAC AAT GAG CGT CAA CTG AGA TGT AAT GAA AAA CCC ATT GCA CCA

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A   A   R   E   K   V   E   A   K   R   E   L   E   K   D   L   N   K   L

APPENDIX
6.3.5 PKD2

2520 base pairs
839 amino acids
96.02 kDa

transmembrane domain
EF-hand motif
antigenic peptide
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APPENDIX
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