# *Caenorhabditis elegans* TEN-1 is essential for basement membrane function

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Dekan

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## I. Summary

Teneurins are large transmembrane proteins playing fundamental roles in development. They are highly expressed in the developing and adult nervous system in distinct layers, often in interconnected regions of the brain, where they were proposed to have an important function during target recognition and synapse formation. Beside the nervous system, teneurins are found at places of cell migration, at morphogentically active zones in developing limbs or at muscle attachment sites. The extracellular domains of teneurins interact in a homophilic manner and this interaction may trigger release of the intracellular domain from the membrane. The soluble intracellular domain can translocate to the nucleus and influence gene expression.

To elucidate teneurin function, we studied the role of the single teneurin orthologue *ten-1* in *C. elegans* development. We characterized mutants in the *ten-1* gene and found that TEN-1 is important for gonad development, vulva formation, distal tip cell migration and axonal guidance. Despite of such pleiotropic phenotypes, we initially concentrated on the gonadal defects. We found that *ten-1* does not control germline proliferation but is essential for the maintenance of the gonadal basement membrane. The basement membrane defect in the *ten-1* mutant was very local and most of the basement membranes showed generally wild-type ultrastructure as analyzed by electron microscopy. Similar disorganization of early gonads has been reported for integrin *ina-1*, dystroglycan *dgn-1* and laminin *epi-1* mutant worms. Therefore, we took a candidate gene approach and tested the genetic interactions between *ten-1* and genes encoding various basement membrane proteins and receptors. This analysis revealed that teneurin acts redundantly with integrin and dystroglycan. Moreover, mutation in *ten-1* sensitized the worms to loss of nidogen and led to defects in pharyngeal morphogenesis. Genetic studies also indicated that laminin could be a ligand for TEN-1 but initial data from vertebrate *in vitro* studies have not confirmed this hypothesis. Reporter constructs showed TEN-1 localization in the cytoplasm and membrane of certain head neurons, pharynx and several gonadal cells but no signs of nuclear translocation of the teneurin intracellular domain could be detected.

Our data provide the first evidence for a novel role of teneurin in basement membrane biology and its redundant function with integrin and dystroglycan receptors.

# II. Introduction

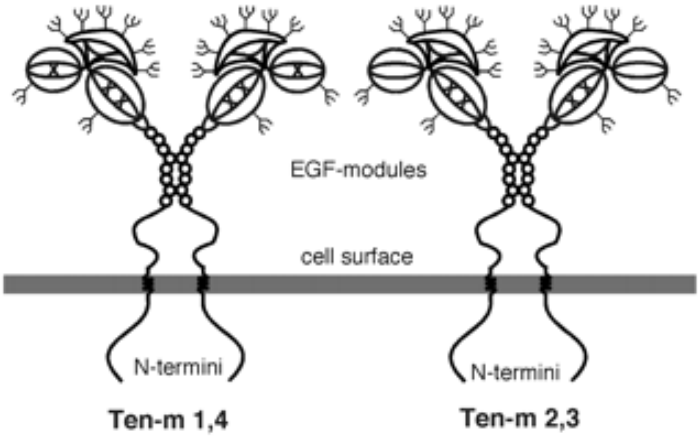
## II.1. Teneurins

Teneurins are large transmembrane proteins with fundamental functions during development in regulating cell-cell interactions and cell adhesion (Tucker and Chiquet-Ehrismann, 2006; Tucker et al., 2007). They are phylogenetically conserved from *Caenorhabditis elegans* to man and were described in several species, including *ten-m*/*odz* and *ten-a* in *Drosophila* (Baumgartner et al., 1994; Fascetti and Baumgartner, 2002; Levine et al., 1994; Rakovitsky et al., 2007), *ten-1* in *C. elegans* (Drabikowski et al., 2005), zebrafish (Mieda et al., 1999), chicken (Minet et al., 1999; Rubin et al., 2002; Tucker et al., 2001; Tucker et al., 2000), rat (Otaki and Firestein, 1999), mouse (Ben-Zur et al., 2000; Oohashi et al., 1999; Zhou et al., 2003), and man (Minet and Chiquet-Ehrismann, 2000). In vertebrates, four teneurin paralogs exist and they were named teneurin-1 to -4, ten-m1 to -m4 or odz-1 to -4.

### II.1.1. Protein domains and structure

Teneurins are type II transmembrane proteins with an approximate molecular mass of 300 kDa. They have an N-terminal cytoplasmic domain and a large extracellular part. The extracellular domain of all teneurins is highly conserved and contains eight tenascin-type EGF-like repeats, a region of conserved cysteines and YD repeats. The second and fifth EGF-like repeat have an odd number of cysteines and it was proposed that the unpaired cysteines may form disulfide bridges with adjacent teneurin molecule leading to homo- or heterodimer formation (Fig. II.1A) (Feng et al., 2002; Oohashi et al., 1999). The EGF-like repeats are followed by a region containing 17 cysteines that are conserved throughout family members in all species and may be required for correct protein folding. Finally, the C-terminal half of the extracellular domain contains 26 YD repeats. This motif is only found in some bacterial proteins (e.g. rearrangement hot spot elements in *E. coli*) and it is predicted to be highly glycosylated (Feng et al., 2002; Minet and Chiquet-Ehrismann, 2000).

A

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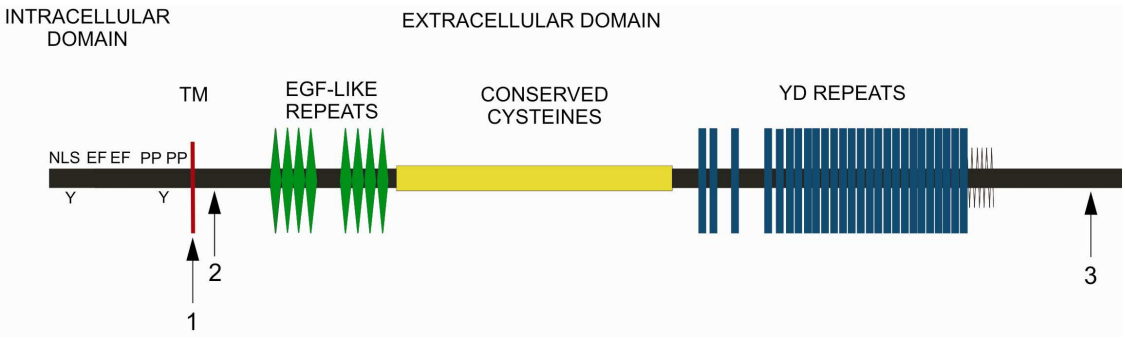


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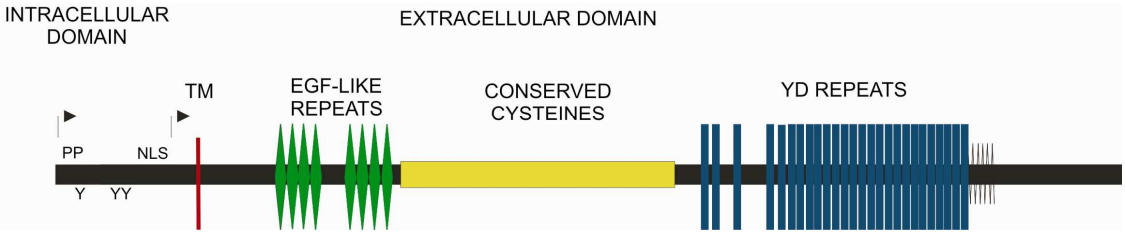


**Figure II.1.** (A) Teneurins are type II transmemebrane proteins connected through covalent bonds in the second and fifth EGF-like repeat. Large globular domains are present in the C-terminal part of the molecule. (B) Domain organization of vertebrate teneurins. The intracellular domain contains nuclear localization signal (NLS), EF-hand like motifs (EF), proline-rich stretches (PP) and putative tyrosine phosphorylation sites (Y). The single transmembrane domain is followed by a large extracellular part consisting of eight tenascin-type EGF-like repeats, a region with conserved cysteines and YD repeats. Three proteolytic cleavage sites are indicated by arrows. (C) Domain organization of *C. elegans* TEN-1. TEN-1L contains proline-rich stretch, putative tyrosine phosphorylation sites and nuclear localization signal. Arrows indicate two N-termini of TEN-1 protein variants. Extracellular domain of TEN-1 shows similar domain organization to vertebrate teneurins. Picture A is taken from (Feng et al., 2002).

Intracellular domains of teneurins show little conservation between the phyla and cannot be aligned in a linear manner. However, most of them contain proline-rich stretches, putative tyrosine phosphorylation sites and nuclear localization signals. The domain organization and predicted structures of teneurins are shown in Fig. II.1A,B.

In *Caenorhabditis elegans* there is a single teneurin ortholog, named *ten-1*. This gene is under control of alternative promoters giving rise to two protein variants, differing in the length of their intracellular domain (Fig. II.1C). The overall domain organization of TEN-1 is highly similar to vertebrate teneurins and the TEN-1 long variant contains a proline-rich stretch and a putative bipartite nuclear localization signal in its cytoplasmic part (Drabikowski et al., 2005).

### II.1.2. Teneurin expression patterns

The main site of teneurin expression is the developing and adult nervous system (Ben-Zur et al., 2000; Mieda et al., 1999; Oohashi et al., 1999; Otaki and Firestein, 1999; Rubin et al., 2002; Tucker et al., 2000; Zhou et al., 2003). Teneurin paralogs are often found in subpopulations of neurons in the developing brain and their expression patterns are largely non-overlapping (Rubin et al., 2002; Rubin et al., 1999; Zhou et al., 2003). For instance, teneurin-1 and -2 are mainly localized in interconnected regions of specific visual pathways, i.e. teneurin-1 is expressed in the tectofugal pathway, while teneuirn-2 is primarily found in the thalamofugal pathway. All four teneurins are also expressed in distinctive, complementary gradients in the developing mouse cortex as well as thalamic nuclei that are connected with appropriate cortical regions. Teneurins show reduced expression in the neocortex of *Emx2-/-* mice and it was proposed that they function in cortical arealization (Li et al., 2006). Moreover, *in vitro* and *in vivo* studies showed that teneurins promote neurite outgrowth implying their important role in axon guidance, target recognition and establishing neuronal connectivity (Leamey et al., 2007a; Minet et al., 1999; Rubin et al., 1999).

Similarly to vertebrate teneurins, *ten* genes in *Drosophila* are expressed in subsets of neurons (Baumgartner et al., 1994; Fascetti and Baumgartner, 2002; Levine et al., 1994) and *ten-m* is found in the developing optic system of the fly, including eye disc and optic

lobes (Minet et al., 1999). Furthermore, expression of both *C. elegans* isoforms is found in a subpopulation of neurons (Drabikowski et al., 2005).

Besides the nervous system, teneurins are often expressed at sites of pattern formation or cell migration. Some avian teneurins were shown to be expressed at morphogenetically active sites of developing limbs, pharyngeal arches, somites and notochord (Tucker et al., 2001; Tucker et al., 2000). *Drosophila ten-m* is found in alternating stripes of the developing embryo, tracheal system, muscle attachment sites and cardiac cells (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner et al., 1994).

In *C. elegans*, expression of *ten-1* from the upstream promoter is mainly detected in the cells of mesodermal origin, like somatic gonad cells (including distal tip cells), pharynx, some muscle and hypodermal cells. The downstream promoter is mainly active in the ectoderm, e.g. dorsal hypodermal cells and leader cells during morphogenesis, arcade cells and excretory duct during postembryonic development (Drabikowski et al., 2005).

### II.1.3. Teneurin function – knockout studies

Fundamental roles of teneurins in development have been demonstrated by genetic studies, mostly in invertebrates. *Drosophila ten-m* mutants are embryonic lethal due to the fusion of adjacent denticle belts (Baumgartner et al., 1994; Levine et al., 1994). Moreover, late *ten-m* mutants show defects in ventral nerve cord development, cardiac cells and eye patterning (Kinel-Tahan et al., 2007; Levine et al., 1994). Similar defects in cuticle formation and eye development have been described for the second *Drosophila* gene, *ten-a* (Rakovitsky et al., 2007). In *Caenorhabiditis elegans*, the single *ten-1* gene is required for several aspects of cell migration and morphogenesis. Mutations in the *ten-1* gene (or its knock down by RNAi) result in a pleiotropic phenotype, including ectopic germline formation, gonad disorganization, distal tip cell migration and axonal guidance defects as well as nerve cord defasciculation (Drabikowski et al., 2005).

Recently, the first vertebrate knockout has been described (Leamey et al., 2007b). Mutation in the mouse teneurin-3 gene leads to defects in eye-specific patterning in the visual system and impairs binocular vision. The visual defects may be suppressed by silencing the inputs from one eye through monocular lesion. Such a mild and specific

phenotype of teneurin-3 knockout mice may be the result of compensation by other family members.

### II.1.4. Teneurin processing

Several reports postulate that teneurins undergo proteolytic processing. A putative furin cleavage site is located between the transmembrane domain and the EGF-like repeats (number 1 on Fig. II.1B). This site is present in all mouse teneurins, both *Drosophila ten-m* and *ten-a,* as well as *C. elegans ten-1* (Drabikowski et al., 2005; Oohashi et al., 1999). Processing at this cleavage site leads to the release of the extracellular domain from the cell surface. Immunostaining with the antibody against the extracellular domain of teneurin-2 co-localizes with the laminin staining in certain chicken basement membranes suggesting that the shed extracellular part may bind to the surrounding extracellular matrix (Tucker et al., 2001). It was also shown that teneurin-2 can be cleaved *in vitro* at the furin site and soluble *ten-m* can be found in the conditioned medium of *Drosophila* Schneider S2 cells (Baumgartner et al., 1994; Rubin et al., 1999).

Several lines of evidence indicate that teneurins undergo regulated intramembrane proteolysis and may be cleaved near or in the transmembrane domain (number 2 on Fig. II.B). As a result, the intracellular domain is released from the membrane and can translocate to the nucleus. For teneurins, neither the exact cleavage site nor the protease(s) have been identified so far.

However, it was shown that there is a functional interaction between teneurin-2 and the zic-1 transcription factor (Bagutti et al., 2003). The intracellular domain of teneurin-2 could be detected in the nuclei of HT1080 cells in discrete spots that often co-localize with endogenous PML (promyelocytic leukemia protein). The translocation of the intracellular domain into the nucleus was confirmed *in vivo* for *C. elegans* TEN-1 (Drabikowski et al., 2005). An antibody against the intracellular domain stains both the membrane and nuclei of developing embryos, in contrast to the antibody against the C-terminal part of TEN-1 that labels membranes exclusively.

Finally, there are some reports indicating that teneurins may be processed at a furin site close to the C-terminus of the protein (number 3 on Fig. II.B). The teneurin C-terminal

associated peptide (TCAP) shows homology to corticotrophin releasing factor family and may modify neurite outgrowth in immortalized hypothalamic cells (Al Chawaf et al., 2007; Wang et al., 2005).

### II.1.5. Teneurin interacting proteins

To uncover the biological function of teneurins, several efforts have been made to identify teneurin interacting proteins. Two such proteins have been found in a yeast two-hybrid screen using part of the teneurin-1 intracellular domain as bait (Nunes et al., 2005). One of them is CAP/ponsin, a cytoskeleton adapter protein playing an important role in cell adhesion (Zhang et al., 2006). Another one, MBD1 (a methyl CpG binding protein) is a known transcriptional repressor (Wade, 2001). The interaction between teneurin-1 and these two proteins was confirmed by immunoprecipitation and co-localization studies. The biological function of teneurin-1 binding to CAP/ponsin or MBD1 is unclear but it may be required for a connection to the actin cytoskeleton or the transcriptional regulation.

Teneurins are also thought to interact in a homophilic manner in their extracellular domains (Leamey et al., 2007a; Rubin et al., 2002) and most likely with other cell-surface or extracellular ligands but till to date, none of them has been identified. Our working model predicts that homophilic interaction or ligand binding initiates cytoskeletal changes and/or proteolytic release of the teneurin intracellular domain and its translocation to the nucleus (Fig. II.2).

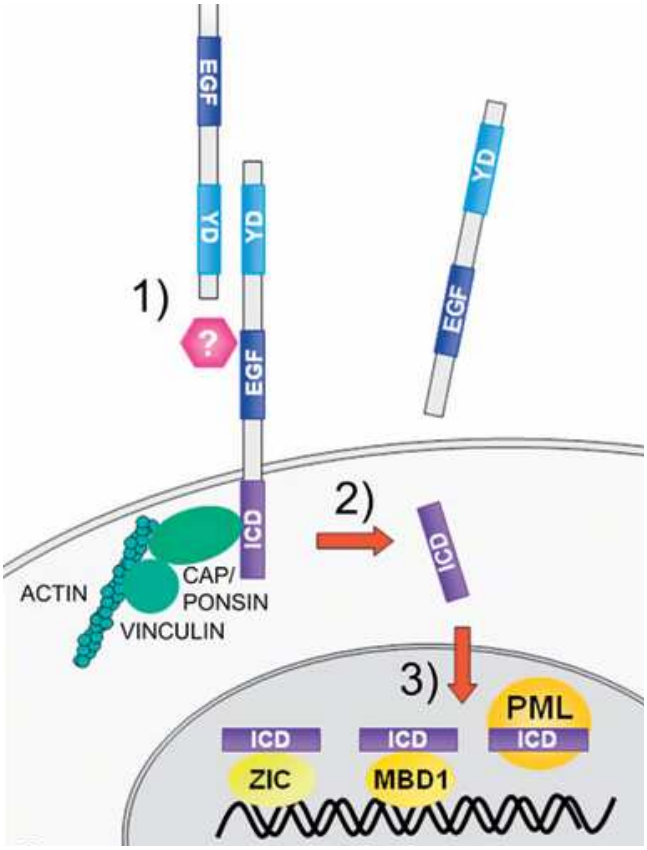


**Figure II.2.** Model of teneurin signaling. Ligand binding or homophilic interaction between teneurin extracellular domains triggers cytoskeletal remodeling or proteolytic release of the intracellular domain. The soluble intracellular domain can translocate to the nucleus and associate with PML bodies, or bind to nuclear proteins (e.g. Zic or MBD1) and regulate gene expression. Picture is taken from (Kenzelmann et al., 2007).

## II.2. *Caenorhabditis elegans* as a model system

In this study, we took advantage of *C. elegans* as our model system. This soil nematode has been widely used in research because of its simple anatomy, short life cycle, invariant cell lineage, powerful genetics and simplicity of culturing conditions. Worms provide an excellent *in vivo* model to study a variety of processes, e.g. cell migration (distal tip cells), cell invasion (anchor cell) or mechanisms of morphogenesis (epidermis). Forward genetic screens allow identifying the *C. elegans* mutations that produce certain phenotypes and have led to the discovery of key proteins required for fundamental developmental events.

### II.2.1. Basement membranes and their receptors

Basement membranes (BMs) are thin, specialized sheets of extracellular matrix proteins that separate tissues and organs, and are required for cell adhesion, migration and differentiation during development (Schwarzbauer, 1999; Yurchenco et al., 2004). Many basement membrane proteins and receptors found in vertebrates are conserved in *C. elegans* (Cox et al., 2004; Hutter et al., 2000) but there are less genes and isoforms in each family. The major BM molecules and receptors described in worms are shown in Figure II.3.

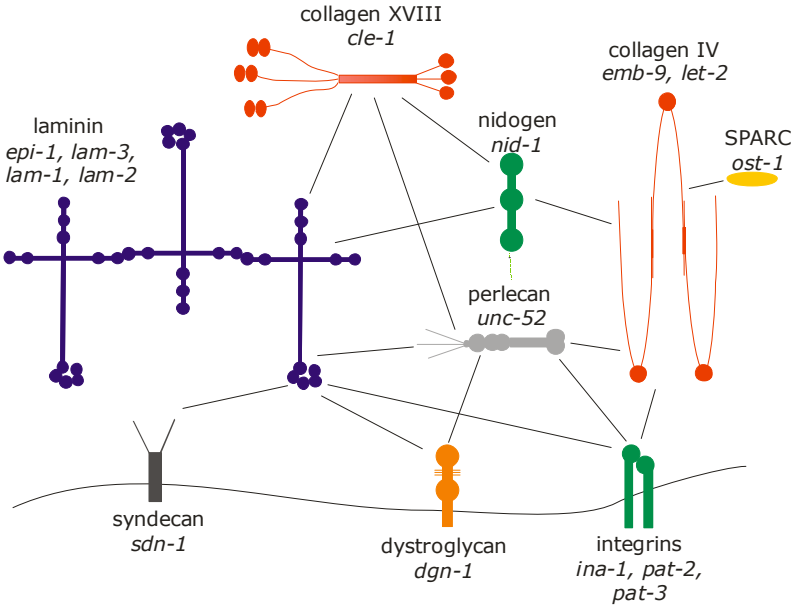


**Figure II.3.** Basement membrane proteins and receptors. Gene names are in italic. Adapted from (Kramer, 2005; Yurchenco et al., 2004).

The composition of basement membranes differs between tissues and developmental stages, e.g. collagen IV EMB-9/LET-2 and nidogen NID-1 are widely present in worm basement membranes, while perlecan UNC-52 is restricted to basement membranes surrounding muscle cells and a LET-2 (exon 9) splice variant predominates during embryogenesis (Graham et al., 1997; Kang and Kramer, 2000; Mullen et al., 1999; Sibley et al., 1993). Basement membranes in worms have an asymmetric appearance between different tissues and range in thickness from 20 nm on epidermis and gonad (Fig. II.4A,F) to 50-100 nm on pharynx and body wall muscles as measured on transmission

electron micrographs (Fig. II.4E,F) (Huang et al., 2003). All basement membranes in *C. elegans* seem to consist of a single sheet although the epidermal basement membranes have often a “lollypop” appearance (dark dots sticking out of BM), which may represent an additional layer.

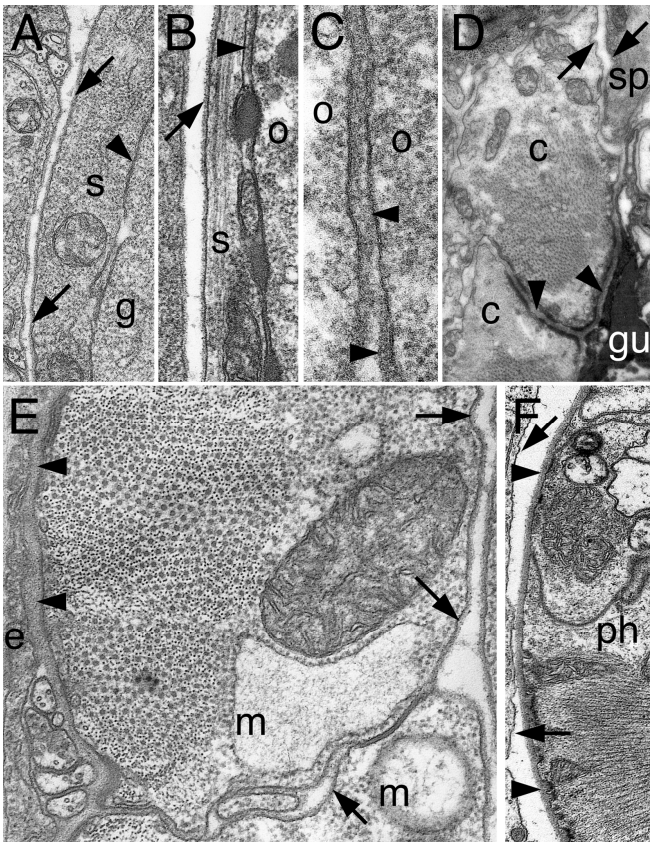


**Figure II.4.** Basement membrane ultrastructure in *C. elegans*. (A) A thin basement membrane (arrows) is present on the gonadal sheath (s), while there is no BM (arrowheads) separating sheath cell and germ cells (g). (B) A basement membrane (arrowhead) starts to form around the developing oocyte (o). (C) Two mature oocytes (o) are ensheathed by BMs (arrowheads). (D) Male tail cross section. A thick BM (arrowhead) covers cloacal muscles (c) on the cuticle side. (E) Body wall muscles are covered by a thick BM (arrowheads) on the side of epidermis and a thin BM (arrows) on the pseudocoelomic side. (F) The pharynx (ph) is ensheathed by a thick BM (arrowheads) (reproduced from Huang et al., 2003).

**Laminins**

Laminins are secreted heterotrimeric molecules that are able to self-polymerize and form networks (Miner and Yurchenco, 2004). The *C. elegans* genome encodes two laminin α chains: LAM-3 and EPI-1, a single β chain LAM-1 and a single γ chain LAM-2. LAM-3 αA and EPI-1 αB chains show high similarity to vertebrate α1/α2 and α3/α4/α5 chains, respectively (Hutter et al., 2000). Both laminin isoforms are broadly distributed among worm basement membranes but EPI-1 is associated with epidermal and gonadal BMs exclusively, and LAM-3 is unique for the nervous system (Huang et al., 2003).

*C. elegans* laminins are required for cell polarity, differentiation, migration and tissue separation. Mutations in the *lam-3* gene causes complete developmental arrest during embryogenesis or at the L1 stage due to pharynx deformation. In *lam-3(n2561)* arrested larvae, pharyngeal cells adhere to their surrounding tissues, while most of the other tissues and organs seem to be normal (Huang et al., 2003). The majority of *epi-1* null mutants arrest as embryos or early larvae, however 27% of worms develop to adulthood. Adult *epi-1* mutants show disruption of basement membranes, muscle polarization defects, axon misguidance and germ cell invasion into adjacent tissue due to gonad epithelialization failure (Huang et al., 2003).

Reduction of *lam-1* or *lam-2* (or both *lam-3* and *epi-1*) function by RNAi result in high embryonic lethality (80-85%) due to cell detachment and severe disorganization of developing embryos. Furthermore, partial loss-of-function mutants in the *lam-1* gene show similar phenotypes to viable laminin α mutants, implying that both α and β subunits are required for basement membrane assembly and integrity (Kao et al., 2006).

**Nidogen**

The single *C. elegans* nidogen NID-1 is broadly distributed among BM but is particularly concentrated around the developing gonad and nervous system. There are three *nid-1* splice variants, which show differential expression during development (Kang and Kramer, 2000). The *cg119* null mutant is viable and fertile but shows defects in synapse organization and function (Ackley et al., 2005; Ackley et al., 2003). However, this mutation does not influence collagen IV localization indicating that nidogen is not essential for BM assembly or stability (Kang and Kramer, 2000). Interestingly, the *ur41*

loss-of-function mutation causes defects in dorso-ventral positioning of specific axons indicating that nidogen plays an important role in axon sorting along the midline (Kim and Wadsworth, 2000).

**Perlecan**

Perlecan is a major heparan sulphate proteoglycan of basement membranes and in worms it is encoded by a single *unc-52* gene. Three main isoforms are expressed from the *unc-52* locus: short (S), medium (M) and large (L) (Mullen et al., 1999). Additionally, several alternative splicing sites exist, therefore as many as 50 perlecan isoforms may be present in *C. elegans* (Rogalski et al., 2001). UNC-52 localization is limited to muscle cells. In body wall muscles, perlecan is found in the basement membrane between muscles and epidermis and is concentrated at the dense bodies and M-lines (Mullen et al., 1999).

Several mutant alleles of the *unc-52* gene have been isolated and their analysis revealed that the M isoform is essential for myofilament assembly. Null mutants of the *unc-52* gene arrest at the two-fold stage because of myofilament lattice disorganization, while mutations eliminating only the L isoform show essentially wild-type phenotype (Mullen et al., 1999). Loss-of-function mutation, *e444*, leads to progressive paralysis and gonad disorganization in adults indicating that it affects only some perlecan isoforms specific for adult worms (Gilchrist and Moerman, 1992).

**Collagen XVIII**

The *C. elegans* gene *cle-1* is a single ortholog of vertebrate collagens type XV/XVIII. Expression from three different promoters gives rise to three protein isoforms (A-C), which share a common endostatin domain. CLE-1 is broadly distributed in BMs but is concentrated in the nervous system. Loss-of-function mutation in *cle-1* results in neuron and distal tip cell migration defects, male tail defects and low penetrance larval arrest (Ackley et al., 2001). It was also shown that CLE-1 is required for synapse organization and function (Ackley et al., 2003). Moreover, it may stabilize the basement membranes as *cle-1* overexpression rescues gonad fragility defects found in fibulin *fbl-1* mutants (Muriel et al., 2006).

**Integrins**

Integrins are heterodimeric ECM receptors consisting of one α and one β subunit. The *C. elegans* genome encodes two α chains: INA-1 and PAT-2, and a single β chain, PAT-3. INA-1/PAT-3 is mostly similar to laminin-binding integrins, while PAT-2/PAT-3 shows high similarity to RGD-binding integrins (Bokel and Brown, 2002).

INA-1 is broadly expressed in developing embryos. However, in L1 larvae it becomes restricted to migrating cells (including distal tip cells) and neurons, as well as organs undergoing morphogenesis (e.g. vulva and uterus). Null mutation of the *ina-1* gene leads to developmental arrest at the L1 stage due to pharyngeal malformation. Weak loss-of-function *ina-1* mutants are viable but show defects in the anterior hypoderm (notched head phenotype), axon defasciculation, and disorganization of the developing gonad (Baum and Garriga, 1997).

Both PAT-2 and PAT-3 integrin chains are strongly expressed in muscle cells (Gettner et al., 1995; Williams and Waterston, 1994). Mutations in *pat-2* or *pat-3* genes cause the Pat (paralyzed at two-fold) phenotype – mutant embryos fail to complete morphogenesis and arrest at two-fold stage due to sarcomere disorganization (Williams and Waterston, 1994). This phenotype is similar to defects observed in lethal *unc-52* mutants (Rogalski et al., 1993) implicating that PAT-2/PAT-3 integrin may be an essential receptor required for perlecan binding in muscle cells.

The role of PAT-3 integrin in larval and adult tissues was investigated by a dominant negative approach. Expression of a HA-βtail transgene (HA-tagged transmembrane and cytoplasmic domain) in gonad, body wall and sex muscles leads to uncoordinated, egg-laying phenotype and gonad migration defects (Lee et al., 2001).

**Dystroglycan**

In vertebrates a single dystroglycan exists, while the *C. elegans* genome contains three dystroglycan related genes: *dgn-1*, *dgn-2* and *dgn-3*. *C. elegans dgn-1* shows the highest similarity to vertebrate and *Drosophila* dystroglycans, although the protein is not processed into α and β subunits. DGN-1 is highly expressed in epithelial cells (including pharyngeal epithelium and somatic gonad cells) and neurons. *C. elegans* dystroglycan is required for gonad epithelialisation, neuronal and distal tip cell migration, and vulva

development (Johnson et al., 2006). In vertebrates, dystroglycan hypoglycosylation in skeletal muscles leads to muscular dystrophies (Haliloglu and Topaloglu, 2004). In contrast, worm DGN-1 is not expressed in muscles and it does not interact genetically with genes encoding components of the dystrophin complex (Johnson et al., 2006). Mutations in *dgn-2* or *dgn-3* do not cause any obvious defects and their expression patterns have not been described in detail, therefore their function remains unknown (James M. Kramer, unpublished).

### II.2.2. Epithelial morphogenesis

In *C. elegans*, epithelial cells play a crucial role in the process of embryonic morphogenesis as they determine the shape of the embryo. Epidermal morphogenesis requires changes in the position and shape of epidermal cells as well as their interaction with underlying neuronal cells and body wall muscles. Once the epidermal cells are specified, three major steps of morphogenesis take place: movements of ventral neuroblasts, ventral enclosure and embryo elongation (developmental stages and timing are shown in Fig. II.5).

In the first step of epidermal morphogenesis, **movements of ventral neuroblasts** are required for closure of the gastrulation cleft. Several signaling pathways were shown to be essential for this process, including ephrin (VAB-1, EFN-1 to -4) and semaphorin-2A (MAB-20) pathway components, and the LAR receptor protein tyrosin phosphatase (PTP-3) (Chin-Sang et al., 1999; Chin-Sang et al., 2002; Harrington et al., 2002; Roy et al., 2000; Wang et al., 1999). Mutants in these receptors or ligands show an enlarged and persistent ventral cleft. Disorganization of ventral neuroblasts, which serve as a substrate for epidermal cells, affects the subsequent step of morphogenesis, epidermal enclosure.

Before the ventral enclosure begins, dorsal epidermal cells rearrange to form a single row of cells (process known as dorsal intercalation). Afterward in the process of **ventral enclosure**, epidermal cells from the dorsal side of the embryo migrate ventrally to close up at the ventral midline (Williams-Masson et al., 1997). In the first step, two pairs of leading cells extend their long protrusions towards the ventral midline and rapidly form

junctions between their counterparts. Subsequently, posterior ventral cells fill the ventral pocket and enclose by a purse-string mechanism.

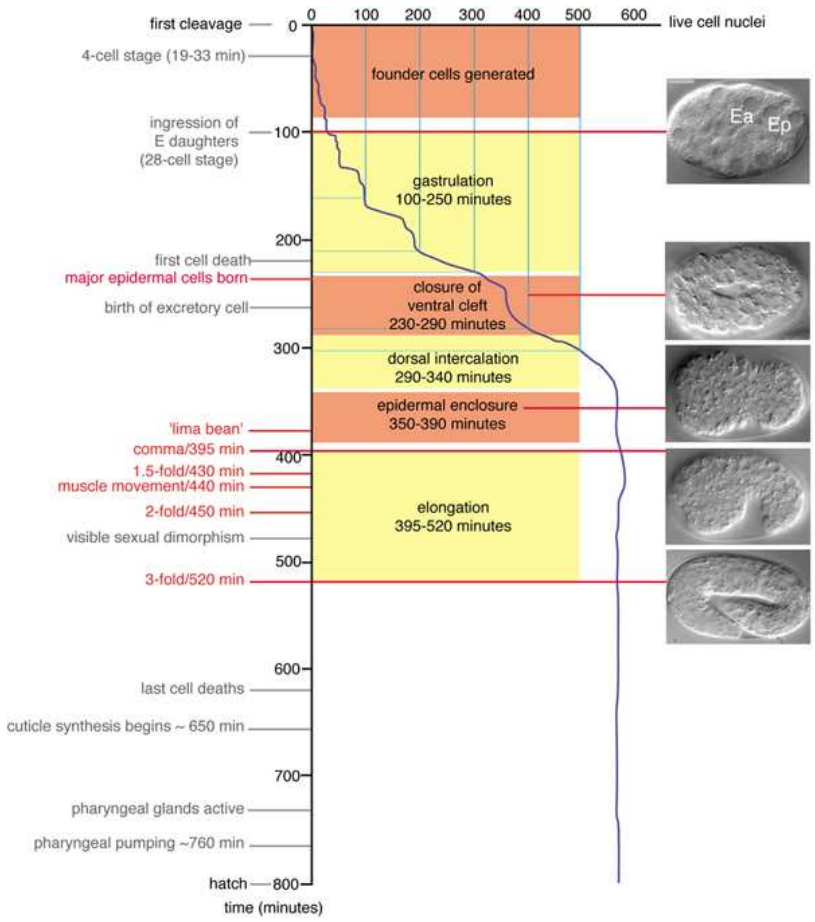


**Figure II.5.** Epidermal morphogenesis timing and developmental stages. Times are indicated for embryonic development at 20°C. Nomarski pictures show worm development at respective stages. Picture is taken from (Chisholm and Hardin, 2005).

Modulation of the actin cytoskeleton is essential for the process of ventral enclosure. Mutants in Rac GTPase, *ced-10*, and its interacting proteins, *gex-2* and *gex-3*, as well as the Arp2/3 complex required for microfilament nucleation, show disorganized epidermis (Severson et al., 2002; Soto et al., 2002). Moreover, components of the cadherin/catenin complex, i.e. HMR-1/cadherin, HMP-1/α-catenin and HMP-2/β-catenin, are required for ventral enclosure as mutations in core proteins of this complex lead to failure of junction formation between leading cells (Raich et al., 1999).

In the last step of morphogenesis, **elongation**, the embryo reduces its diameter and epidermal cells elongate along the anterior-posterior axis. Similarly to ventral enclosure, both reorganization of the actin cytoskeleton and the cadherin/catenin complex are required for the early steps of the elongation process (Costa et al., 1998; Wissmann et al., 1997).

Interaction between epidermal cells and underlying body wall muscles appears to be critical for later stages of elongation as mutations completely eliminating the muscle function cause developmental arrest at the two-fold stage (Williams and Waterston, 1994). Several basement membrane components playing an important role in muscle development are essential for the elongation process. Perlecan/UNC-52 is required for myofilament lattice assembly and collagen IV EMB-9/LET-2 - to maintain the muscle-epidermis attachment during muscle contraction (Gupta et al., 1997; Hresko et al., 1994). Mutants in all these genes arrest during elongation.

In addition, components of fibrous organelles, which transmit the forces of muscle contraction to epidermis and cuticle, are required for the elongation process. They include myotactin/LET-805, spectraplakin/VAB-10 and intermediate filament proteins IFA-3, IFB-1 (Bosher et al., 2003; Hresko et al., 1999; Woo et al., 2004).

### II.2.3. Pharynx development

The pharynx is a linear tube with two bulbs and is ensheathed by a thick basement membrane. It can be divided into six parts: the buccal cavity, procorpus, anterior bulb (metacorpus), isthmus, terminal bulb and pharyngeal-intestinal valve (Fig. II.6). There are seven cell types that form the pharynx: arcade cells, muscles, epithelia, neurons, glands, mariginal cells and valves (Fig. II.6) (Albertson and Thomson, 1976).

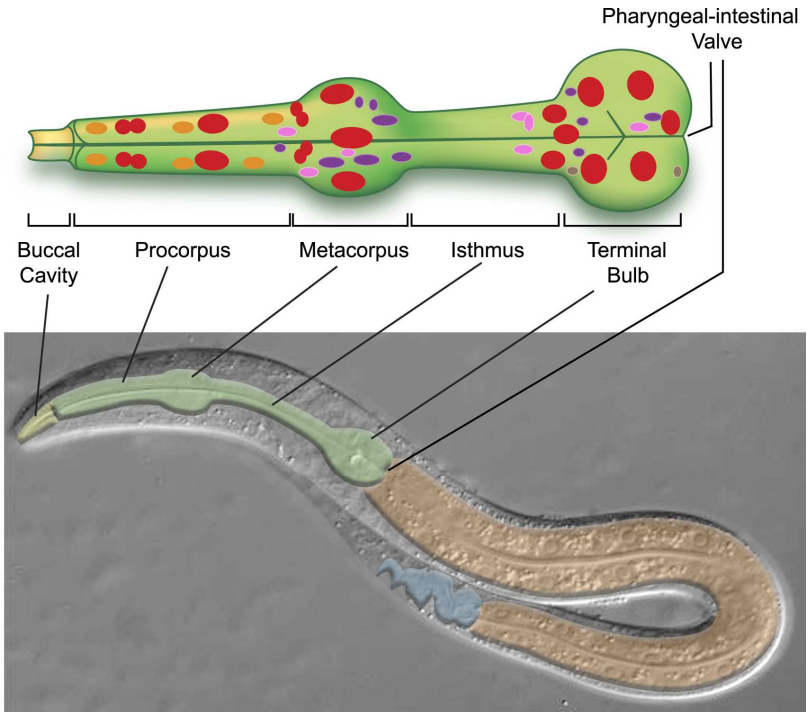


**Figure II.6.** Pharynx organization. Nuclei of different cell types are labeled as follows: red - muscles, purple – neurons, orange – epithelia, pink – mariginal cells, brown – glands. Picture is taken from (Mango, 2007).

The pharyngeal cells are derived from ABa and MS descendants (Sulston et al., 1983) and pharynx development is predominantly regulated by the transcription factor PHA-4 (Gaudet and Mango, 2002; Mango et al., 1994). At the end of gastrulation, the pharyngeal primordium forms an epithelialized ball of cells connected by adherens junctions (Portereiko and Mango, 2001). Subsequently, the foregut connects to the buccal cavity in the morphogenetic process called pharyngeal extension, which can be divided into three steps: (I) rotation of pharyngeal cells, (II) epithelialization of arcade cells and (III) contraction (Fig. II.7) (Portereiko and Mango, 2001).

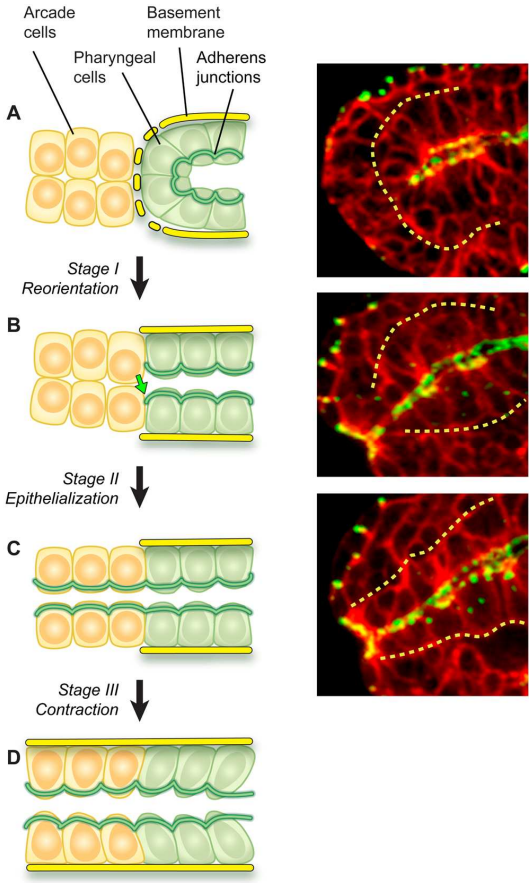


**Figure II.7.** Three steps of pharyngeal extension. Cell membranes are labeled in red with anti-β-spectrin/UNC-70 antibody and adherens junctions are green. Basement membranes are represented by dotted yellow line. Picture is taken from (Mango, 2007).

In the first stage, pharyngeal cells reorient their apical and basolateral polarity relative to the embryonic axes and align with the arcade cells. The basement membrane at the anterior tip of the primordium is removed and pharyngeal cells at this position lose cell contacts. Subsequently, arcade cells (mesenchymal) are converted into epithelial cells and form adherens junctions connecting them to the pharyngeal epidermis. Finally, pharyngeal and arcade cells undergo local contraction that pulls them tightly together.

Mutations in several genes affect pharyngeal morphogenesis causing a Pun (pharynx unattached) phenotype. However, it is unclear, whether they are required for correct cell fate determination, cell differentiation or morphogenesis. Proper pharyngeal attachment requires several transcription factors, e.g. *ast-1* (Schmid et al., 2006), *die-1* (Heid et al., 2001), *elt-5 (Koh and Rothman, 2001)*, transcriptional repressor *lin-35* and ubiquitin-

conjugating enzyme *ubc-18* (Fay et al., 2003) or ubiquitin-ligase *ari-1* (Qiu and Fay, 2006).

Interestingly, many proteins required for the formation or maintenance of epithelia, like cadherins, catenins or discs-large, are not essential for pharyngeal morphogenesis (Costa et al., 1998; Firestein and Rongo, 2001; Pettitt et al., 1996). However, mutation in *zen-4* or *cyk-4* cause a Pun phenotype since the kinesin-like protein ZEN-4 and RhoGAP CYK-4 were shown to be important for arcade cell polarization (Portereiko et al., 2004).

### II.2.4. Gonad development

The gonad primordium consists of four cells: Z1 and Z4 are somatic gonad precursor cells, whereas Z2 and Z3 are germline founder cells. Z1 and Z4 come from the MS lineage, are born late during embryogenesis and migrate to associate with germline precursor cells. Z2 and Z3 are exclusive descendants of $P_4$ and during embryogenesis they are attached to the intestine (Sulston et al., 1983). At hatching, these four cells form a compact primordium which is completely ensheathed by a basement membrane. Germline and somatic gonad precursor cells start to proliferate at the L1 stage. In late L2, somatic gonad cells reorganize and form the somatic gonad primordium of hermaphrodite (SPh) which separates proliferating germ cells into a posterior and an anterior population. Distal tip cells remain at the tips of gonad arms controlling gonad migration and promoting germline mitosis. During L3 stage, proximal germ cells enter meiosis, while distal germline nuclei continue to divide mitotically. Gametogenesis starts at the proximal end of the gonad at the L4 stage and continues throughout adulthood (Hubbard and Greenstein, 2000). Gonad development is summarized in Fig. II.8.

Z1 and Z4 cells give rise to all somatic structures in the gonad, including distal tip cells, sheath cells, spermatheca and uterus (Kimble and Hirsh, 1979). Until late L2, 12 somatic cells are formed from Z1 and Z4 precursors: two distal tip cells, four sheath/spermatheca precursors, two dorsal and three ventral uterine cells, and a single anchor cell (McCarter et al., 1997). Already at these early steps of gonad development, descendants of Z1/Z4 cells closely wrap proliferating germ cells and they are located between the germline and the gonadal basement membrane (Hall et al., 1999; Pepper et al., 2003).

Sheath cells play an important role in the maintenance of gonadal integrity and gametogenesis since they provide nutritional and structural support, and control meiotic progression (Hall et al., 1999; McCarter et al., 1997). The sheath/spermathecal (SS) precursor cells and their descendants are required for germline proliferation (Killian and Hubbard, 2005; McCarter et al., 1997). Moreover, proximal sheath cells and spermatheca play an important role in ovulation (McCarter et al., 1999).

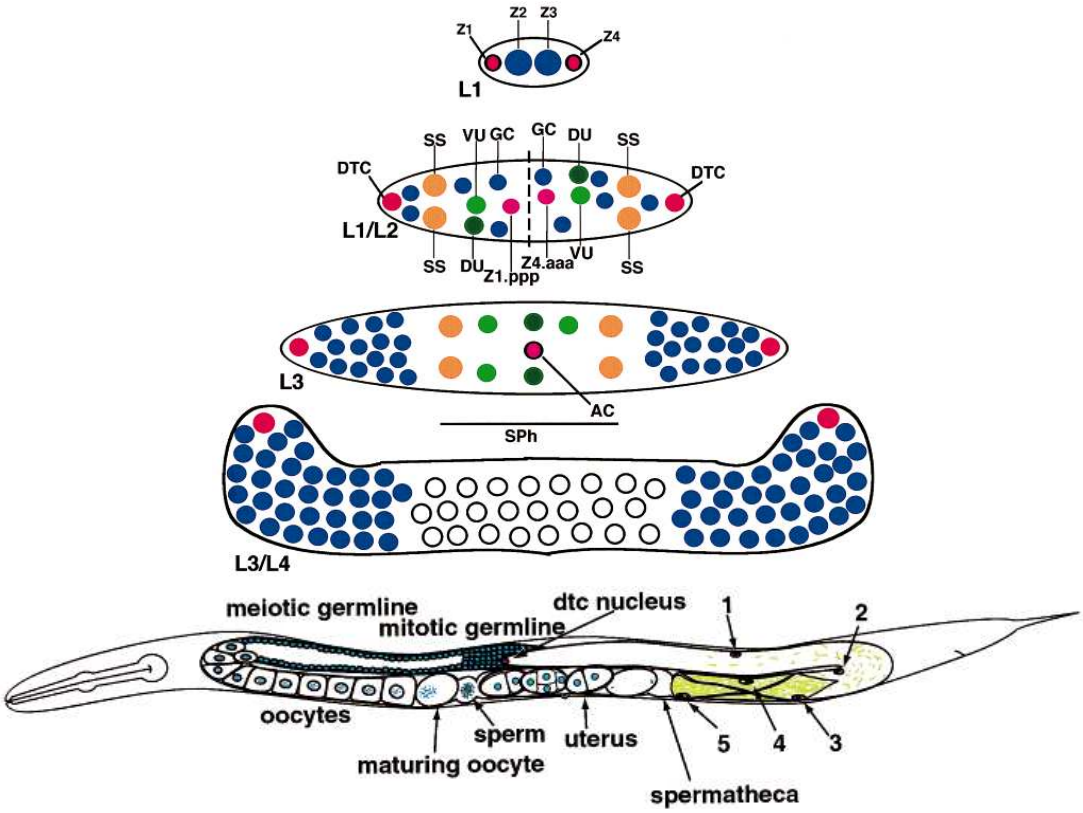


**Figure II.8.** Gonad development in *C. elegans*. The developing gonad is shown at the L1 stage, L1/L2 molt, L3 stage, L3/L4 molt and adult stage. Germ cells (GC) are shown in blue, distal tip cells (DTC) are red, sheath/spermatheca precursors (SS) are yellow, uterine cells are green (ventral uterine - VU, dorsal uterine – DU), and anchor cell (AC) is pink. Cells forming somatic gonad primordium (SPh) are underlined. In adult worms, both germline and somatic structures (including five pairs of sheath cells) are shown. Picture is taken from (Hubbard and Greenstein, 2000).

One of the somatic cells, the anchor cell (AC), plays a crucial role in vulva induction and the formation of the connection between uterus and vulva. During the L3 stage, the AC attaches to the ventral side of the gonadal basement membrane, removes the gonadal and epidermal BMs precisely at its basolateral side and invades the underlying tissue (Fig. II.5). The anchor cell invasion is studied as an *in vivo* model of regulated invasion. The transcription factor FOS-1 is a key regulator of this process and it affects the transcrption of three targets: ZMP-1/matrix metalloprotease, CDH-3/protocadherin and ECM protein hemicentin (Sherwood, 2006).

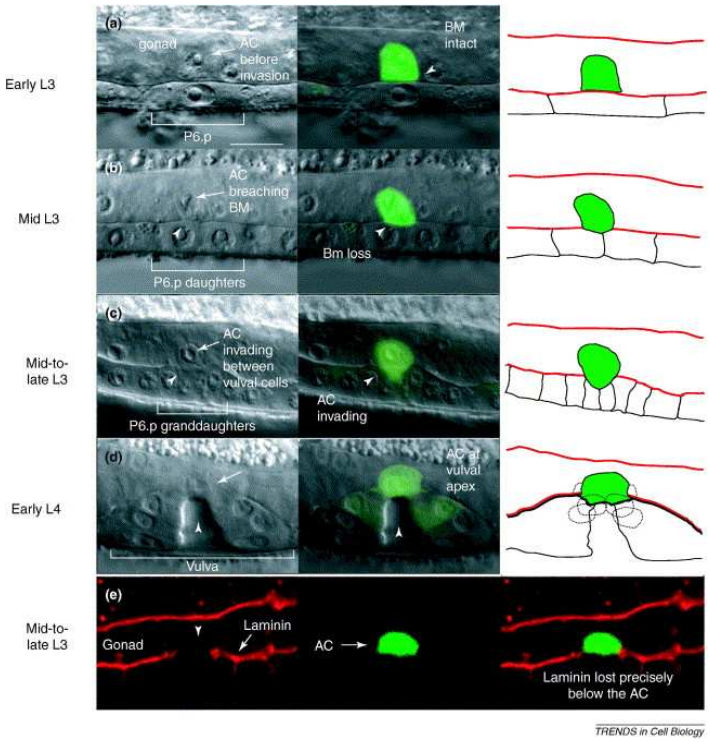


**Figure II.5.** Anchor cell (AC) invasion through the basement membranes. Anchor cell is labeled by GFP expressed under *cdh-3* promoter. In the early L3 stage (a), AC attaches to the ventral side of the gonadal basement membrane, just above P6.p vulva precursor cell. Gonadal and epidermal BMs are removed during the mid L3 stage (b). During mid-to-late L3 stage (d), AC is invading between vulval cells. By the early L4 stage (d), AC invasion is complete. Immunostaining with laminin antibody reveals loss of BMs precisely under the AC (e). Reproduced from (Sherwood, 2006).

## II.3. Aim of the work

Since little is known about teneurin function *in vivo*, we took advantage of the *C. elegans* model organism to elucidate the role of the single teneurin gene *ten-1* during worm development. As *ten-1* mutant worms showed a pleiotropic phenotype with many tissues affected, we initially concentrated on gonadal defects. We discovered that TEN-1 is essential for the maintenance of the gonadal BM during development by analyzing BM organization with GFP markers and at high resolution by transmission electron microscopy. In addition, we used a candidate gene approach to identify receptors and pathways acting redundantly to *ten-1* and found several synergistic genetic interactions between *ten-1* and mutants in BM components and receptors. To shed light on the mechanism of teneurin action in preserving basement membrane integrity, we characterized the defects found in synthetic lethal double mutants and investigated TEN-1 localization.

# III. Results

## III.1. Results – published

### III.1.1. Teneurins – proteins with fundamental roles in development

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Molecules in focus

# Teneurins: Transmembrane proteins with fundamental roles in development

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**Abstract**

Teneurins are a novel family of transmembrane proteins expressed during pattern formation and morphogenesis. Originally discovered as ten-m and ten-a in *Drosophila*, four vertebrate teneurins as well as a *Caenorhabditis elegans* homologue were identified. The conserved domain architecture of teneurins includes an intracellular domain containing polyproline motifs. The long extracellular domain consists of eight EGF-like repeats, a region of conserved cysteines and unique YD-repeats. Vertebrate teneurins are most prominently expressed in the developing central nervous system, but are also expressed in developing limbs. In *C. elegans*, RNAi experiments and studies of mutants reveal that teneurins are required during fundamental developmental processes like cell migration and axon pathfinding. Cell culture experiments suggest that the intracellular domain of teneurins translocates to the nucleus following release from the membrane by proteolytic processing. Interestingly, the human teneurin-1 gene is located on the X-chromosome in a region where several families with X-linked mental retardation are mapped.


*Keywords:* Ten-1; Odz; Odd oz

## 1. Introduction

The teneurins are a family of phylogenetically conserved transmembrane glycoproteins expressed during pattern formation and morphogenesis. They were discovered independently in the early 1990s by two laboratories studying *Drosophila*. Baumgartner and colleagues (Baumgartner & Chiquet-Ehrismann, 1993; Baumgartner, Martin, Hagios, & Chiquet-Ehrismann, 1994) screened a *Drosophila* library with a probe corresponding to the EGF-like repeats of chicken tenascin-C in an effort to identify the arthropod homolog of that extracellular matrix protein. Their screen revealed two novel proteins that they called ten-m and ten-a for 'tenascin-like protein major' and 'tenascin-like protein accessory'. Meanwhile, Levine and colleagues (Levine et al., 1994) reported the results of a screen with novel antibodies to *Drosophila* phosphotyrosine containing proteins that independently identified ten-m. They called the gene encoding their protein *odd Oz* (*odz*) since mutant embryos exhibited an 'oddless' pair-rule phenotype. Both names persisted when the four genes encoding the vertebrate homologues were

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found; the literature contains numerous references to ten-m1-4 as well as Odz1-4 (see Tucker & Chiquet-Ehrismann, 2006, for review). Fortunately, the numbering schemes used by the different groups usually coincided. In 1999, a new name for the protein family was proposed: teneurins (Minet, Rubin, Tucker, Baumgartner, & Chiquet-Ehrismann, 1999). The name refers both to the original name 'ten-a' as well as the nervous system, which is one of the primary sites of teneurin expression both in vertebrates and invertebrates. The name 'teneurin' is now widely used (Table 1), but care should be taken when searching the literature for references to odz, ten-m, tenm, ten-a and ten-1. The latter is the teneurin homologue in *Caenorhabditis elegans* (Drabikowski, Trzebiatowska, & Chiquet-Ehrismann, 2005).

Table 1
Summary of teneurin nomenclature and expression during development (includes only proteins with known expression patterns)

| Species | Name | Synonyms | Expression pattern |
|---|---|---|---|
| *Caenorhabditis elegans* | Ten-1L[a] | | Somatic gonad, vulva, subset of neurons, gut, some hypodermal and muscle cells[a] |
| | Ten-1S[a] | | Subset of neurons, some hypodermal cells[a] |
| *Drosophila melanogaster* | Ten-m[b,c] | Odz[d,e] | Odd-numbered parasegments[b,d], subset of neurons[b,d], tracheal system[b], cardiac cells[b], lymph glands[b], muscle attachment sites[b], morphogenetic furrow[c,e], wing pouch[e], leg and antennal discs[e] |
| | Ten-a[f,g] | | CNS[f,g], eye[f], muscle attachment sites[f,g] |
| *Danio rerio* | Teneurin-3 | Ten-m3[h] | Developing brain, somites, notochord, pharyngeal arches[h] |
| | Teneurin-4 | Ten-m4[h] | Developing brain, spinal cord[h] |
| *Gallus gallus* | Teneurin-1[c,i] | | Developing CNS and eye[c,i] |
| | Teneurin-2[i,j] | | Developing brain and eye[i], AER of limb buds[j], tendon primordia[j], pharyngeal arches[j], heart[j], somites[j], neural tube[j], craniofacial mesenchyme[j] |
| | Teneurin-4[k] | | Developing CNS, ZPA of limb buds, pharyngeal arches[k] |
| *Rattus rattus* | Teneurin-2 | Neurestin[l] | Developing and adult CNS, somites[l] |
| *Mus musculus* | Teneurin-1 | Ten-m1[m], odz1[n,o], ten-m/odz1[p] | Developing and adult CNS[m,o,p], eye[m], smooth muscle cells in lungs[m], kidney glomeruli[m], adult testes[m] |
| | Teneurin-2 | Ten-m2[m], odz1[r], odz2[n,o], ten-m/odz2[p] | Developing and adult CNS[p] |
| | Teneurin-3 | Ten-m3[m], odz3[n,o], ten-m/odz3[p] | Developing and adult brain[n,o,p,t], developing eye[n], spinal cord[n,p], notochord[p], craniofacial mesenchyme[n], tongue[n], dermis[n], saccule[n], developing limb[n], periosteum[n] |
| | Teneurin-4 | Ten-m4[m], odz4[n,o,r,s], ten-m/odz4[p], DOC4[n,r,t] | Developing and adult brain[n,o,p,s,t], developing eye[n], somites[p], spinal cord[n], trachea[n], nasal epithelium[n], saccule[n], joints[n], adipose tissue[n], tail bud and limbs[s] |

[a] Drabikowski et al. (2005).
[b] Baumgartner et al. (1994).
[c] Minet et al. (1999).
[d] Levine et al. (1994).
[e] Levine et al. (1997).
[f] Baumgartner and Chiquet-Ehrismann (1993).
[g] Fascetti and Baumgartner (2002).
[h] Mieda, Kikuchi, Hirate, Aoki, and Okamoto (1999).
[i] Rubin et al. (1999).
[j] Tucker et al. (2001).
[k] Tucker et al. (2000).
[l] Otaki and Firestein (1999).
[m] Oohashi et al. (1999).
[n] Ben-Zur, Feige, Motro, and Wides (2000).
[o] Li et al. (2006).
[p] Zhou et al. (2003).
[r] Ben-Zur and Wides (1999).
[s] Lossie et al. (2005).
[t] Wang et al. (1998).

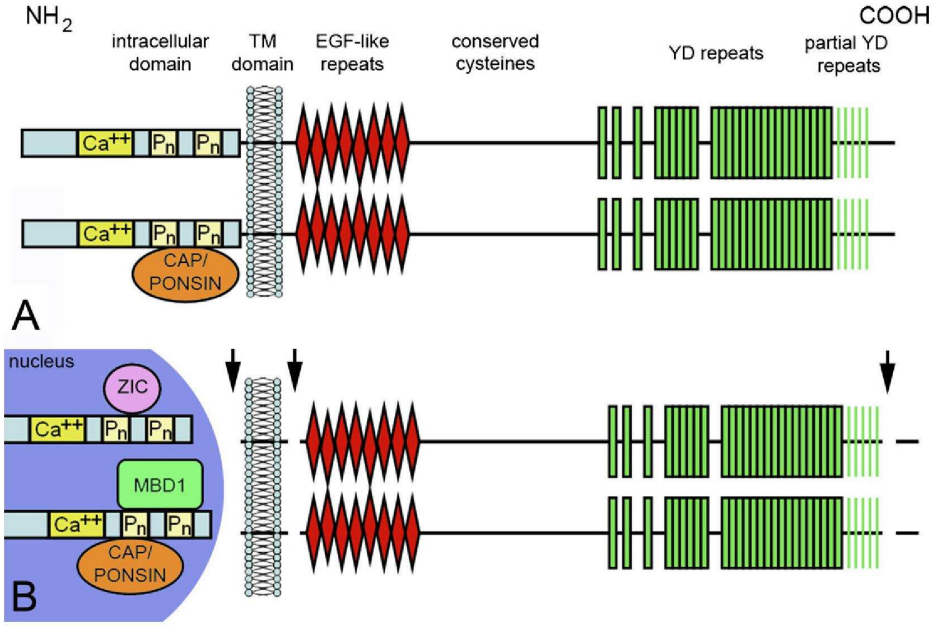


Fig. 1. (A) A stick diagram illustrating the domain organization of a typical vertebrate teneurin. The intracellular domain contains putative $Ca^{2+}$-binding motifs and polyprolines ($P_n$) that bind to CAP/ponsin and potentially link the intracellular domain to the actin-based cytoskeleton. Extracellularly are eight EGF-like repeats, a stretch of conserved cysteine residues, 26 YD-repeats and a series of partial YD-repeats. Teneurins can dimerize via interactions between the second and fifth EGF-like repeats. (B) Some, if not all, teneurins can be processed at multiple sites (arrows). The intracellular domain can be cleaved and transported to the nucleus, where it can interact with the DNA-binding protein MBD-1 and possibly the transcription factor zic. The extracellular domain can be released from the cell surface, and a C-terminal peptide derived from teneurins has been shown to have neuropharmacological properties.

## 2. Structure

The structure of a typical teneurin is summarized in Fig. 1A. All teneurins described to date are type 2 transmembrane proteins (i.e., the amino terminus is inside the cell). The proteins are large, with a typical molecular weight of just over 300 kDa. In vertebrates, teneurins have a highly conserved and unique intracellular domain with proline-rich stretches, which are characteristic of SH3-binding sites, and two EF-hand-like putative $Ca^{2+}$ binding sites. The former can bind CAP/ponsin in a yeast two-hybrid screen, which potentially links the intracellular domain of teneurins to the actin-based cytoskeleton through CAP/ponsin's interactions with vinculin (Nunes et al., 2005). There are also highly conserved putative phosphorylation sites in the intracellular domain; the roles of these domains are unknown. The phylogenetically conserved extracellular domain of teneurins contains eight tenascin-type EGF-like repeats. The free cysteines in the second and fifth EGF-like repeats can form cross links with their counterparts on an adjacent molecule, resulting in the formation of teneurin dimers (Feng et al., 2002). The central part of the extracellular domain of teneurins contains 17 highly conserved cysteine residues that probably play a role in the proper folding of this portion of the molecule. The C-terminal half of the extracellular domain contains 26 YD repeats (consensus sequence $GX_{3\text{-}9}YXYDX_2GR$[L,I or V]$X_{3\text{–}10}G$) and a series of partial YD repeats. Before being found in teneurins, YD repeats had only been described in the cell wall proteins of a few prokaryotes (Minet & Chiquet-Ehrismann, 2000). Ten-1, the *C. elegans* teneurin, is unique among the teneurins described to date in that it has two variants generated by two promoters (Drabikowski et al., 2005). The longer variant, designated ten-1L, has a proline-rich stretch and a nuclear localization sequence, both of which are missing from the short variant (ten-1S). The extracellular domains of ten-1 are remarkably similar to those found in vertebrates: they can be aligned with the vertebrate teneurins along the entire length and exhibit the same domain structure.

## 3. Expression and processing

In *Drosophila*, ten-a is found in the larval nervous system and muscle attachment points (Fascetti & Baumgartner, 2002), whereas ten-m/odz is expressed first in odd-numbered parasegments and later in the nervous system, muscle attachment points and tracheal precursor cells (Baumgartner et al., 1994; Levine et al., 1994). Ten-m/odz is also prominently expressed in the morphogenetic furrow of imaginal discs (Levine, Weiss, & Wides, 1997; Minet et al., 1999). Both ten-1L and ten-1S are expressed by subsets of neurons in adult *C. elegans* (Drabikowski et al., 2005). Ten-1L is also expressed in the muscle cells of the vulva, some hypodermal cells as well as in the somatic gonad and gut. In embryos, ten-1S is expressed in hypodermal cells and ten-1L is expressed in the precursor cells of the gonad, gut and pharynx. Teneurin expression in vertebrates is best studied in the chicken embryo and in the mouse. In the chicken embryo, teneurin-1 and teneurin-2 are both highly expressed in the developing visual system, with teneurin-1 being primarily expressed in the tectofugal visual pathway, and teneurin-2 in a subset of the tectofugal pathway as well as the thalamofugal visual pathway (Fig. 2A; Rubin, Tucker, Brown-Luedi, Martin, & Chiquet-Ehrismann, 2002). The proteins are found both in puncta near cell bodies as well as in axonal tracts. Teneurin-2 and teneurin-4 are also expressed in developing limbs, with teneurin-2 found in the apical ectodermal ridge (Tucker et al., 2001) and teneurin-4 in the zone of polarizing activity (Tucker, Martin, Kos, & Chiquet-Ehrismann, 2000). In the mouse, the expression of each teneurin in the developing CNS has been described in detail. For example, in the murine cerebellum teneurin-1 is primarily expressed in the granular layer, teneurin-2 is expressed in the granular layer, Purkinje cells and the molecular layer, and teneurin-3 and teneurin-4 are primarily expressed by Purkinje cells, though teneurin-4 mRNA is also found in cerebellar white matter (Zhou et al., 2003). Recently, the expression of the teneurin-4 gene was shown to be reduced in the cortex of *emx2*$^{-/-}$ mice (Li, Bishop, & O'Leary, 2006). When examined by in situ hybridization, the same authors demonstrated that teneurins-1 through 4 (which they call Odz1-4) are each expressed in distinctive rostro-caudal gradients within the cortical plate, and that each teneurin is also expressed in brain nuclei that are connected with the appropriate teneurin-positive parts of the cortex. As in the chicken embryo, teneurin-4 has been shown to be expressed in the limbs of mouse embryos, where its expression is regulated by HoxD (Cobb & Duboule, 2005). A summary of the expression patterns can be found in Table 1.

Considerable progress has been made in recent years regarding teneurin processing and binding partners (Fig. 1B). Most notably the intracellular domain can be cleaved near (or possibly in) the transmembrane domain and transported to the nucleus, giving teneurins the potential to act as transcription factors (Bagutti, Forro, Ferralli, Rubin, & Chiquet-Ehrismann, 2003). Nuclear transport has been demonstrated experimentally by measuring luciferase activity from a reporter construct following transfection of an expression construct with the corresponding transcriptional activator TF fused to the N-terminus of teneurin-2. Interestingly, the activ-

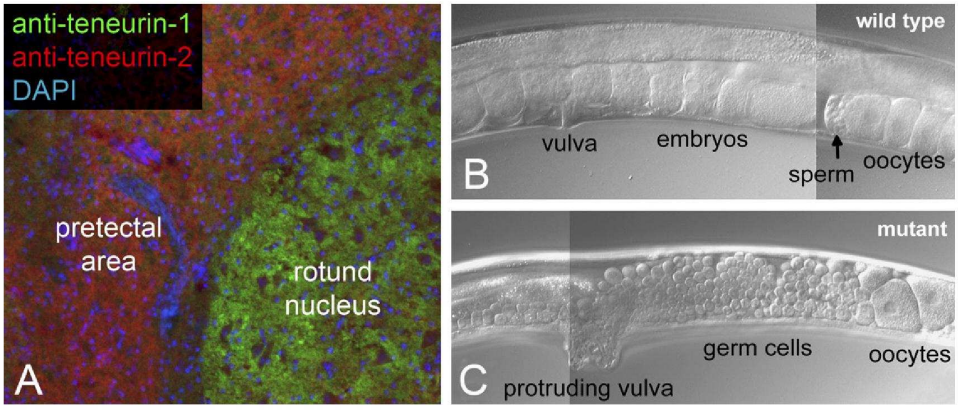


Fig. 2. (A) In vertebrate embryos different teneurins have distinct and often mutually exclusive patterns of expression. For example, in the developing avian brain teneurin-1 is concentrated in the rotund nucleus, whereas teneurin-2 is found in the surrounding pretectal area. (B and C) *C. elegans* mutants lacking ten-1 exhibit a number of defects, including an abnormal nervous system, a protruding vulva and the disintegration of the gonad, which results in the release of germ cells into the pseudocoelom.

ity of the TF fusion construct was increased 6.5-fold upon transfection of a stable cell line that overexpressed the extracellular domain of teneurin-2, suggesting that teneurin–teneurin interactions may control teneurin-mediated transcriptional regulation. Nuclear localization of the intracellular domain was also confirmed by immunocytochemistry following the expression of this region alone in HT1080 cells. In addition, antibodies raised against the intracellular domain of teneurin-1 stain puncta in the nuclei of chicken embryo fibroblasts (Nunes et al., 2005), and antibodies against the N-terminus of ten-1 stain nuclei in *C. elegans* embryos, whereas antibodies against the C-terminus give plasma membrane-related staining (Drabikowski et al., 2005). In addition to CAP/ponsin, the DNA-binding protein MBD-1 has been shown to associate with the intracellular domain in a yeast-two hybrid screen and to co-localize with the intracellular domain of teneurin-1 in discrete puncta in the nucleus (Nunes et al., 2005). There is also a functional interaction between the intracellular domain of teneurin-2 and the transcription factor zic. When zic and the intracellular domain of teneurin-2 are co-transfected into COS-7 cells, there is a marked down-regulation of transcription from a luciferase reporter containing a zic-responsive promoter and a reduction of the teneurin-2 intracellular domain found in the nucleus (Bagutti et al., 2003). Taken together, these data explain how a large transmembrane protein can act as a regulator of morphogenesis.

In addition to the processing of the intracellular domain, teneurin-2 has a furin cleavage site outside the plasma membrane. This site is cleaved in vitro and may account for the labeling of extracellular matrix sometimes seen with antibodies to the extracellular domain of teneurin-2 (Rubin, Tucker, Martin, & Chiquet-Ehrismann, 1999; Tucker et al., 2001). Finally, all teneurins have a potential dibasic cleavage motif near the C-terminus that would generate a short peptide. Interestingly, teneurin C-terminus associated peptides (TCAP) share structural and physiological properties with the corticotropin-releasing factor family of peptides and may play important roles in mediating animal behavior (Lovejoy, Chawaf, & Cadinouche, 2006).

Alternative splicing has been demonstrated for avian teneurin-2 by RT-PCR; in this case, the two major variants, one full length and the other lacking the part of the extracellular domain carboxy to the EGF-like repeats, have similar developmental regulation and patterns of expression (Tucker et al., 2001). Others have proposed that numerous splice variants of teneurin-4 exist, but if and when they are expressed needs to be verified (Lossie, Nakamura, Thomas, & Justice, 2005).

## 4. Biological function

Evidence of the fundamental importance of teneurins in basic developmental events comes from the study of mutant embryos in *Drosophila* and mouse, as well as RNAi knockdown and mutants in *C. elegans*. Ten-m/Odz mutants in *Drosophila* created by P element insertion are embryonic lethal (Baumgartner et al., 1994; Levine et al., 1994). Embryonic phenotypes range from mild to severe fusion of ventral denticle belts that result from the absence of odd-numbered segments. In some older *Drosophila* ten-m/Odz mutant embryos the ladder-like arrangement of the ventral nerve cord is clearly disrupted. In *C. elegans*, ten-1 has been knocked down with RNAi and a deletion mutant has been characterized (Drabikowski et al., 2005). Interference of ten-1 expression with RNAi results in a broad range of phenotypes that include abnormal pathfinding and fasciculation of neuronal processes as well as abnormal migration of the distal tip cells of the gonad. In a *ten-1* deletion mutant (Fig. 2B and C) there is a severe phenotype that includes gonadal disintegration and the release of the germ cells into the pseudocoelom and protrusion of the vulva. These worms also have an abnormal nervous system. To date, teneurin knockout mice have not been described. However, delays in gastrulation as well as neural tube defects prior to embryonic lethality were observed in mice homozygous for a point mutation near the C-terminus of teneurin-4 (Lossie et al., 2005). Each of these studies point to fundamental roles for teneurins in embryonic morphogenesis and the development of the nervous system.

## 5. Clinical relevance

In humans, the teneurin-1 gene is found on the X chromosome at q25, a region where several forms of X-linked mental retardation have been mapped. Given the high levels of teneurin-1 expression in the developing avian visual system and the importance of teneurins in neuronal morphogenesis in both vertebrates and invertebrates, it is intriguing that some forms of X-linked mental retardation feature poor vision and seizures (e.g., Gustavson et al., 1993).

There are no reports of teneurins being expressed in tumors in the literature, but searches of on-line microarray databases reveal that some teneurins are upregulated in cancer. Since teneurins have many characteristics of key transcriptional regulators during embryonic pattern formation, growth and cell migration, studies of teneurins during tumorigenesis should prove interesting and may have diagnostic or therapeutic value.

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## III.1.2. *ten-1*, an essential gene for germ cell development, epidermal morphogenesis, gonad migration, and neuronal pathfinding in *Caenorhabditis elegans*

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My contribution: I characterized *ten-1(ok641)* worms and analyzed the phenotypes in the mutant and rescued worms.

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# *ten-1*, an essential gene for germ cell development, epidermal morphogenesis, gonad migration, and neuronal pathfinding in *Caenorhabditis elegans*

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**Abstract**

*ten-m* (*odz*) is the only pair-rule gene discovered in *Drosophila* that encodes a transmembrane protein and not a transcription factor. The vertebrate Ten-m orthologues have been implicated in pattern formation and neuronal development. To investigate the role of this protein in development, we characterize here the structure and function of the *Caenorhabditis elegans* orthologue *ten-1*. We found that two promoters control the expression of two different *ten-1* transcripts. This results in the expression of type II transmembrane protein variants differing in their intracellular domains. Both *ten-1* transcripts show complex, but distinct, expression patterns during development and in the adult. Interference with Ten-1 expression by RNAi experiments leads to multiple phenotypes resulting in defects in hypodermal cell migration, neuronal migration, pathfinding and fasciculation, distal tip cell migration, the establishment of the somatic gonad, and gametogenesis. The RNAi phenotypes were confirmed by the analysis of a deletion mutant which revealed that Ten-1 is essential for somatic gonad formation. The intracellular domain of the long form was detected at the cell membrane and in the nucleus. We propose that Ten-1 acts as a receptor for morphogenetic cue(s) and directly signals to the nucleus by translocation of its intracellular domain to the nucleus following its proteolytic release from the cell membrane.


*Keywords:* *C. elegans*; Morphogenesis; Embryogenesis; Germ line; Nuclear translocation; Ten-m; Odz; Teneurin

## Introduction

Cell–cell interactions are crucial determinants regulating the development of multicellular organisms. Despite of the great number of cell types and different organs in animals, only few signaling pathways are involved in their generation. They include Hedgehog, Wnt, TGF-β, receptor tyrosine kinase, nuclear receptor, Jak/STAT, and Notch pathways (Barolo and Posakony, 2002). The same signaling pathways are used repeatedly throughout the development of an organism. For example, the Notch receptor originally discovered in *Drosophila* is involved in cell specification in the central and peripheral nervous system, oogenesis, spermatogenesis, myogenesis, heart formation, and imaginal disc formation (for reviews, see Artavanis-Tsakonas et al., 1999; Greenwald, 1998). In *C. elegans*, the Notch orthologues Lin-12 and Glp-1 are involved in induction of germ line mitosis, blastomere specification in early embryos, AC/VU cell specification in the somatic gonad, vulva precursor cells, and uterine cell specification (for reviews, see Greenwald, 1998; Kimble and Simpson, 1997). The basic mechanism of signaling by Notch is conserved from *Drosophila* to man and disturbances in Notch-mediated signaling processes are involved in cancer as well as neurological diseases (Joutel and Tournier-Lasserve, 1998). The invertebrate animal models *Drosophila* and *C. elegans* have been used successfully to identify molecular mechanisms underlying cell–cell interactions and many important

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proteins involved in cell–cell signaling have been discovered thanks to these model organisms (Baumeister and Ge, 2002; Lambie, 2002; Lehmann, 2001; Livingston and Wilt, 1993).

The *Drosophila* gene ten-a was originally identified based on the presence of tenascin-type EGF-like repeats (Baumgartner and Chiquet-Ehrismann, 1993). The *ten-a* expression pattern suggests an important function during development. A peak of *ten-a* mRNA accumulation is seen during embryogenesis and the expression level declines during larval and pupal stages, though transcripts remain detectable in the eye and brain of pupae (Baumgartner and Chiquet-Ehrismann, 1993). By immunostaining, the major site of *ten-a* expression was found to be at muscle attachment sites and in the developing central nervous system (Fascetti and Baumgartner, 2002). Originally, a partial cDNA for Ten-a was reported (Baumgartner and Chiquet-Ehrismann, 1993), but by analysis of the genomic region harboring the ten-a gene a complete transcript could be predicted (Minet and Chiquet-Ehrismann, 2000). The expression of this long form of Ten-a was confirmed by Fascetti and Baumgartner (2002). The Ten-a protein is highly similar to a second Drosophila protein named Ten-m (Baumgartner et al., 1994) which was independently described also as Odd oz or odz (Levine et al., 1994).

Interestingly, *ten-m/odz* is a pair-rule gene (Baumgartner et al., 1994; Levine et al., 1994). Pair-rule genes are required for the regulation of the segmental pattern formation in *Drosophila* embryogenesis. They encode transcription factors regulating the expression of the segment polarity genes. Thus, it is intriguing to find a cell surface protein that belongs to the class of pair-rule genes. Since *ten-m/odz* mutants of *Drosophila* are embryonic lethal, a potential function of *ten-m* in later development cannot easily be addressed. However, the expression patterns in the developing embryos and the adult fly suggest further important activities of this protein in later developmental processes as well. Its presence often coincides with locations of morphogenetic cell movements, e.g., during gastrulation, the development of the tracheal system, on pioneering axons, and in the developing eye (Baumgartner et al., 1994; Levine et al., 1994, 1997).

Vertebrate genomes contain four *ten-a/ten-m* homologues, termed ten-m1–4 (Oohashi et al., 1999), odz1–4 (Ben-Zur et al., 2000), or teneurin 1–4 (Minet and Chiquet-Ehrismann, 2000). Since this protein family is conserved in metazoans and in the chicken is expressed at locations known to deliver morphogenetic cues (Tucker et al., 2000, 2001), it is reasonable to predict that it fulfils fundamentally important functions conserved throughout metazoan development. We therefore decided to characterize the *ten-a/ten-m* orthologue of *C. elegans*, which we named *ten-1*, and to analyze its function by interfering with Ten-1 expression by gene deletion and by RNA interference experiments.

## Materials and methods

### *General methods and strains*

*C. elegans* strains were cultured at 20°C as described in Brenner (1974). Wild type refers to *C. elegans* variety Bristol, strain N2. VC518 [*ten-1* (ok641)] was obtained from the C. elegans Knockout Consortium and backcrossed 10 times before further analysis. Flanking positions of the deletion are R13F6 coordinates 4938/7069 resulting in the sequence read at the break position of GAATCTTGTGGTTCAAGACT/TACAATTGCATCAGCAAAA. Furthermore, the following strains were used: NL2099 [*rrf-3*(pk1426)], NL2098 [*rrf-1*(pk1417)], SU93 [(jcIs1 IV) *jam-1*::GFP], RU7 [*ten-1a*::GFP], RU9 [*ten-1b*::GFP], RU85 [SU93 *jam-1*::GFP, *rrf-3*], RU86 [*ten-1a*::GFP, *rrf-3*], RU87 [*ten-1b*::GFP, *rrf-3*], RU91 [*ten-1a*::GFP *ten-1* (ok641)], RU92 [*ten-1* (ok641), *ten-1b*::GFP], RU93 [*ten-1* (ok641) lim-7::GFP + rol-6(su1006)], RU95 [*ten-1* (ok641), F36A3 (III)].

### *cDNA analysis and DNA constructions*

Molecular cloning procedures were performed according to standard methods (Sambrook et al., 1989). cDNA fragments were prepared by RT-PCR using Superscript II RNaseH(−) reverse transcriptase (Life Technologies) from mRNA isolated from mixed stage worms. The entire *ten-1* cDNA was amplified in overlapping pieces with Expand HiFi polymerase (Roche) using the following primers: CATACGTCTGGAGGAGCACCG/TTGAATTGGCAATGACTCGAAG; and TCTGATTCTTCTCGAATTGTCG/ATTTTCATCAGGTGTTGTCGAC; ACAATTCAAACCGTCTTACTG/CTATTCAGATTTTCGGAACTTCC. The 5′ cDNA ends were amplified using SL1 as a 5′ primer and the gene-specific primer AGCACGTGTCGCTATCGTCG. The PCR products were cloned, sequenced, and assembled into the full-length ten-1 coding sequences including the 5′ UTRs of 8120 bp encoding Ten-1L and 7626 bp encoding Ten-1S. These sequences were submitted to the DDB/EMBL/GenBank databases under accession numbers AB206835 and AB206836, respectively. Conserved motifs and protein domain were searched for using the program Motif Scan at http://myhits.isb-sib.ch.

4 kb of the upstream promoter was amplified with the primers CATTGGTCAATTGGCGCGCCCATTCGCAGACG and ATTAGGCGGTGGGGGTACCGCATTCG and cloned into the *Asc*I/*Kpn*I sites of pPD117.01 (gift of A. Fire). 3 kb of the downstream promoter was amplified with the primers GAATTCGCATGCAAATGTGAAGCATG and CCACCAGGTACCGGATCACCATTGTTC and cloned into the *Sph*I/*Kpn*I sites of pPD117.28 (gift of A. Fire).

DNA encoding the long intracellular domain was amplified with the primers CAGAGTGCGGCCGCCCGTGCGTTTCG and GGCTAGGAATTCATTCCATTTG-

GATGG. DNA encoding the short intracellular domain was amplified with the primers TTACAATTTTTCAGGCGG-CCGCAAGTTGGC and GGCTAGGAATTCATTCCATTT-GGATGG. Both fragments were cloned into the *Sph*I/*Kpn*I sites of pPD118.28 (from A. Fire) to create translational fusions with GFP under the heat shock promoter hsp16.2.

*Transgenic animals*

Transgenic animals were generated by microinjection of DNA into the distal arms of gonads as described (Mello et al., 1991). GFP-promoter constructs were injected at a concentration of 1 ng/μl along with 50 ng/μl PvuII-digested worm genomic DNA. Heat shock promoter-driven constructs were injected at a concentration of 10 ng/μl together with 90 ng/μl pRF4. The heat shock was induced by placing worms at 33°C for 20 min in M9 buffer. VC518 (ok641) was rescued by injecting the cosmid F36A3 at a concentration of 5 ng/μl together with 10 ng/μl of pPD and 90 ng/μl of pBluescript (Stratagene).

*RNAi*

A cDNA fragment corresponding to the upstream promoter-specific transcript was amplified with the primers ACCGTTACCTCATCCATCAG and CGTTGTCCAGAT-GCTGGTGAG. Three fragments of cDNAs corresponding to the common transcripts were amplified with the primers ACCGTTACTAAGCCTGCACG and TTACATGATC-CATCCGATGC and all DNA fragments were cloned into pBluescript II (Stratagene). RNA was synthesized and purified using the MEGAscript kit (Ambion) according to the manufacturer's protocol. After transcription, single-stranded RNA molecules were annealed and prepared for microinjection. Ten-1 RNAi was injected at a concentration of 1 μg/μl. Injected worms were transferred to new plates once a day and the brood size was counted. Embryos were considered dead if they did not hatch within 12 h after removal of the mother.

*Antibody production, immunofluorescence, and microscopy*

Anti-Ten-1 antibodies were raised against the Ten-1-specific peptides from the N-terminus of the long variant (MFQHRTTNAQGPPPNRPMPR) and the common C-terminus (PAHQSGLLASVHSWKFRKSE). The peptides were synthesized and the rabbits immunized at Neosystem (Strasbourg, France). All sera were affinity purified using the respective peptide-coupled columns. Peptides were coupled to CH-activated Sepharose (Amersham Biosciences) according to the manufacturer's protocol. Purified antibodies recognize on Western blots a single band of overexpressed protein in worm extracts. We were, however, not able to detect the endogenous protein on Western blots. In contrast, by immunofluorescence, we were able to detect endogenous Ten-1 protein and this staining was abolished by the inclusion of the respective peptides used for immunization. For immunofluorescence, embryos were fixed with 3% formaldehyde in PBS for 30 min. Fluorescent and Nomarski images were obtained with a Zeiss axioscope microscope. Confocal images were acquired with an Olympus Fluoview FV500 microscope.

## Results

*A single C. elegans ten-1 gene with two promoters*

The *C. elegans* genome contains only one gene homologous to the *ten-a* and *ten-m* genes of *Drosophila*. It is located on the left arm of chromosome III and was mapped to the cosmid R16F6. The entire cDNA encoding Ten-1 was cloned and sequenced using mRNA isolated from mixed stage worms (as described in Materials and methods). We found a few small discrepancies to the entry in Wormbase due to errors in the prediction of exon/intron junctions and a major discrepancy at the 5′ end (described below). We determined the transcription start by a 5′ RACE analysis using splice leader 1 (SL1) as the 5′ primer and a *ten-1*-specific oligonucleotide as the 3′ primer. The RT-PCR reaction using mixed stage mRNA as template resulted in two products differing in size. By sequencing these bands, two different cDNA species could be identified. One of them corresponded with a minor difference at the 5′ end to the transcription start of the predicted open reading frame R16F6.4 and the other one contained in addition the predicted open reading frame F28F5.1 together with a newly discovered exon as depicted in Fig. 1A. Thus, the *ten-1* gene is under the control of alternative promoters, ten-1a and ten-1b, resulting in two different transcripts that encode two Ten-1 proteins (Fig. 1B). The long form (Ten-1L; accession number XXXXX) contains N-terminal to a predicted transmembrane sequence an intracellular domain of 218 aa, whereas the short form (Ten-1S; Accession number XXXXX) starts with the membrane-proximal 36 aa that is common between both variants. The intracellular sequences do not show homology to any domains found in other proteins but the long form harbors a proline-rich stretch at the N-terminus and a potential bipartite nuclear localization signal. *C. elegans* Ten-1 has a predicted transmembrane domain. This suggests that Ten-1 is a type II transmembrane protein which has been experimentally confirmed for chicken teneurin-2 (Rubin et al., 1999). The extracellular part is highly homologous to all vertebrate and Drosophila teneurins and harbors 8 EGF-like repeats. They are followed by a region with conserved cysteines. The C-terminal part of Ten-1 is composed of YD repeats. These short repeats are highly similar to repeats found in the *rhs* elements of *E. coli* as noticed in previous work (Minet and Chiquet-Ehrismann, 2000; Minet et al., 1999). Two potential furin cleavage sites could be located at a distance

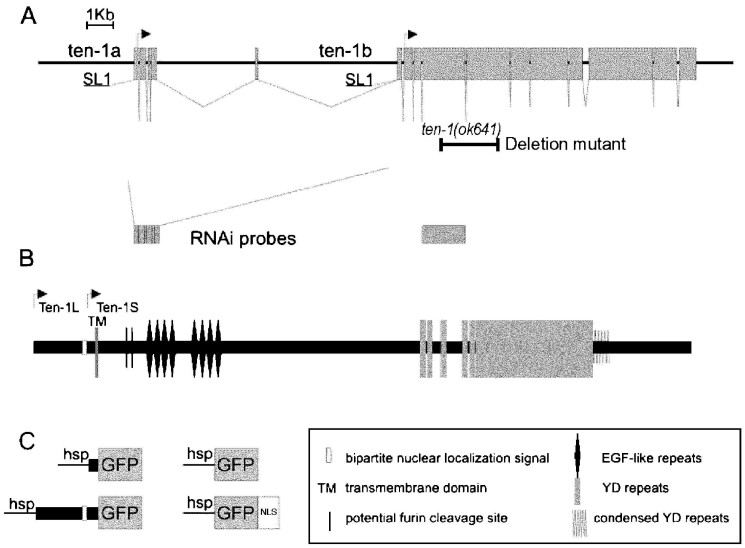


Fig. 1. Gene and protein structure of ten-1. (A) Exon–intron organization of the ten-1 gene and indication of the two transcription and translation initiation sites from the upstream (ten-1a) and downstream (ten1b) promoters, respectively. The positions of the deletion in the ten-1 (ok641) mutant and RNAi probes are indicated. (B) Model of the Ten-1 proteins. The two alternative N-termini are represented by arrows indicating the beginning of the protein Ten1l and Ten1s, respectively. Protein domains are explained in the legend below the model (boxed). (C) Models of the Ten-1 intracellular domain-GFP constructs expressed under the heat shock promoter.

of 100 and 120 aa from the transmembrane domain (Fig. 1B), near the site of an experimentally confirmed furin cleavage site in vertebrate teneurin-2 (Rubin et al., 1999).

*The two forms of Ten-1 have distinct expression patterns*

We first analyzed the expression pattern of the *ten-1* gene by promoter-GFP translational fusions (Fig. 2). In the embryo, the upstream promoter (ten-1a) is most active in the descendants of the C and EMS blastomers (Figs. 2A and B). During postembryonic development, GFP expression was detected in the pharynx, gut, coelomocytes, posterior body wall muscles, vulva muscles in hermaphrodites, and diagonal muscles in males (Figs. 2C–F). The ten-1a promoter is also active in some hypodermal cells including the hyp-11 cell, hypodermal seam cells, and rectal hypodermis. In the somatic gonad, it is active throughout its development starting with z1 and z4 cells in the embryo (Figs. 2A and C). During gonad development, it is expressed in the distal tip cells and the linker cell in males, in gonad and spermatheca sheath cells, and the utse cells of the uterus. In males, ten-1a is active in the vas deferens and spicule socket cells. Furthermore, GFP expression in DVB neurons and a few ring interneurons could be detected.

In the embryo, the downstream promoter (ten-1b) is most active in the descendants of the ABp cell and in the hypodermis (Figs. 2G and H). The dorsal hypodermal cells and the ventral leader cells were most prominently labeled (Figs. 2G and H). During postembryonic development, GFP fluorescence was visible in specialized epithelial cells including the arcade cells of the anterior end and the excretory duct. Ten-1b is also active in a subset of neurons including CAN and HSN neurons as well as neurons of the lumbar and retro-vesicular ganglion and some nerve ring interneurons (Figs. 2I–K). In males, GFP fluorescence is also visible in R8 and R9 ray neurons (Fig. 2K).

In summary, the ten-1a promotor resulting in the expression of the Ten-1L protein is mainly active in mesoderm whereas the ten-1b promoter that regulates the transcript encoding the short Ten-1S variant is more highly expressed in ectoderm.

*RNA interference affects morphogenesis of the hypodermis, somatic gonad formation, and neuronal migration and pathfinding*

To analyze the phenotype of Ten-1-deficient worms, we performed RNA interference experiments (Fire et al., 1998). Worms were injected with double-stranded RNA directed against the ten-1a transcript or with a probe common to both transcripts (cf. Fig. 1). Injection of either probe into wild-type N2 worms resulted in low and variable penetrance phenotypes. Injecting the RNAi hypersensitive *rrf-3* strain (Simmer et al., 2002) resulted in the same phenotypes at greater but still variable penetrance. Interference with the ten-1a-specific transcript caused defects in germ line development and somatic gonad formation while interference with both transcripts

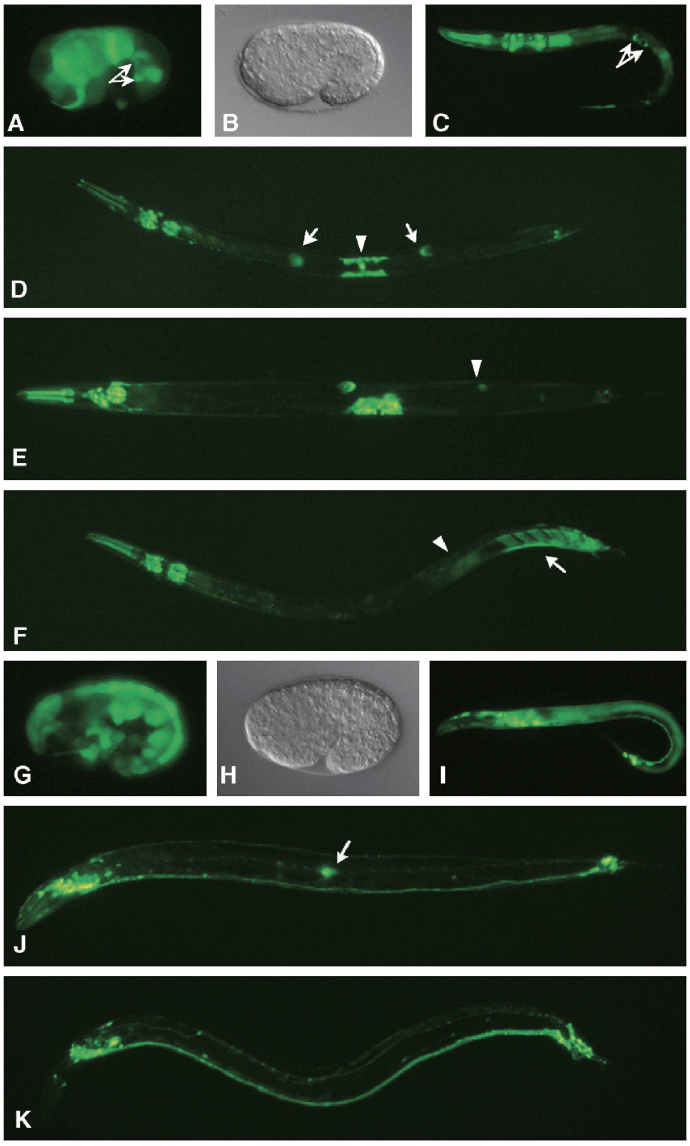


Fig. 2. Ten-1 expression patterns. The ten-1a promoter-GFP translational fusion protein is predominately expressed in the mesoderm (A–F). At the 1.5-fold stage, pharynx, gut, and somatic gonad precursor cells are labeled and the z1 and z4 cells are indicated by a double arrow (A). The interference contrast picture of the same embryo is shown in B. In the L1 larvae, the pharynx, some gut cells, hyp11, and precursors of the somatic gonad show fluorescence and the z1 and z4 cells are indicated by a double arrow (C); in a ventral view of an L4 larvae, some neurons in the nerve ring are labeled and the anchor cell flanked by the vulva muscle precursors is pointed out by an arrowhead and the distal tip cells by arrows (D). In the adult hermaphrodite, fluorescence is seen in the pharynx, some nerve ring neurons, the distal tip cells, the vulva muscles, and a coelomocyte is indicated by an arrowhead (E). In the adult male, in addition to the same expression pattern in the head region as the hermaphrodite, the vas deferens (arrowhead), diagonal muscles (arrow), and spicule sheath cells are labeled (F). The ten-1a promoter-GFP translational fusion protein is predominately expressed in the ectoderm (G–K). At the 1.5-fold stage, hypodermal cells are strongly labeled (G). The interference contrast picture of the same embryo is shown in H. In the L1 larvae, neurons in the nerve ring and tail are strongly stained (I); in the adult hermaphrodite, predominant staining is seen in neurons of the nerve ring and the tail connected by the ventral nerve cord (J). In the adult male, a similar pattern as in the hermaphrodite is seen with the addition of some male-specific neurons in the tail (K).

caused additional hypodermal and neuronal defects as described below.

*rrf-3* worms injected with RNAi directed against both transcripts had significantly less progeny than control animals, and this decrease in F1 progeny could be rescued by mating with *rrf-3* males. The progeny of *rrf-3* worms injected with the ten-1a-specific RNAi showed 15 ± 5% embryonic lethality whereas the lethality increased to 58 ± 8% after injection of RNAi directed against both transcripts. In control experiments with RNAi against a GFP transcript, no increased lethality in the offspring of injected worms was observed. The data of this analysis are presented in Table 1. To determine whether Ten-1 acts cell autonomously or non-autonomously in sperm development, we injected *rrf-1* worms known to be defective in somatic RNAi (Sijen et al., 2001). There was no significant difference in the number of progeny of injected worms using ten-1 or GFP RNAi (Table 1). This is, however, no proof of non-autonomous action of Ten-1 in sperm development because *rrf-1* mutant worms are not hypersensitive in the germ line and are expected to react as wild-type worms that show a mild phenotype. Furthermore, the somatic gonad is already developed at the time of injection and *ten-1* is expressed in developing sperm.

The embryonic lethality of worms injected with RNAi against both ten-1 transcripts most likely resulted from abnormal embryonic elongation due to hypodermal cell migration defects as can be seen in Fig. 3A. Surviving embryos hatched as variably abnormal larvae (Fig. 3B). To visualize better the effect of ten-1 RNAi on hypodermal cell migration, we injected dsRNA into su93 worms expressing a Jam-1-GFP fusion (Mohler et al., 1998) in a *rrf-3* background. The hypodermal cells failed to migrate correctly and acquired disorganized and aberrant shapes (Figs. 3E–G). The posterior part of the body was more strongly affected (Figs. 3B and G) and also the morphology of the rays in the male tail was aberrant (Fig. 3C). Furthermore, the distal tip cell sometimes meandered resulting in severely disturbed gonad migration (Fig. 3H). Often worms had few or no sperm (Fig. 3J) or existing sperm was pushed to the uterus by ovulating oocytes (not shown). Endomitotic oocytes developed in the uterus and gonad probably due to malfunction of the sheath cells and lack of sperm (Fig. 3I). Due to the mispositioning and/or malfunction of the gonad and spermatheca sheath cells, oocytes ruptured during ovulation (Fig. 3J). In addition, we noticed that some worms were constipated and that the gut was often translucent suggesting abnormalities in gut function (not shown).

To visualize better the effect of ten-1 RNAi on somatic gonad development, we crossed *rrf-3* worms with worms expressing the ten-1a promoter GFP translational fusion construct and injected them with RNAi. In more severe cases, somatic gonad cells did not envelop the gonad and often remained as an isolated group of cells (Figs. 3M and N). As a consequence, germ cells spread throughout the body cavity and no mature oocytes and sperm developed as can be seen in the corresponding interference contrast picture (Fig. 4N). In addition, vulva muscles were often mispositioned or they did not attach to the body wall or to the vulva (Figs. 3M and N). Worms often burst through the vulva. This is likely a consequence of aberrant vulva-uterine connection and/or malformation of the vulva muscles since the Ten-1a promoter is active both in utse and vulva muscle cells.

The effect of ten-1 RNAi on neuronal development was addressed by injecting *rrf-3* worms carrying the ten-1b-GFP promoter construct. As a consequence, the migration of certain neurons was aberrant (Figs. 4A and F). Furthermore, axonal pathfinding of some of the neurons was disturbed and they extended axons in the wrong direction and sometimes made loops and early turns (Figs. 4C, G, and H). Abnormalities of the ventral cord became obvious and sometimes the integrity of the ventral cord was lost (Fig. 4D) or the axons of the ventral cord were defasciculated (Figs. 4I–K).

### *The ten-1 (ok641) deletion mutant shows abnormal somatic gonad development and a tumorous germline*

To confirm the phenotypes of *ten-1* knock-down by RNAi, we analyzed the phenotype of a *ten-1* deletion mutant. The strain obtained from the C. elegans Knock-out Consortium, VC518 (ok641), carries an in-frame deletion of 2130 bp/675 aa removing 4 EGF-like repeats and a part of the cysteine-rich region. This is not a null mutation. The mRNA is expressed at a similar level as the wild-type transcript (data not shown). The mutant protein seems to have a dominant-negative effect, since more than 30% of the heterozygous worms show one or more of the phenotypes mentioned below (Table 2). Homozygous *ten-1* deletion worms show a variable phenotype including embryos arresting during elongation (Fig. 5A). 26% of worms die during larval development. 39% of worms were sterile and 15% burst through the vulva ($n$ = 175). The most prominent phenotype was observed in the development of the somatic gonad. The

Table 1
RNA interference in rff-3 and rrf-1 worms

| RNAi injected | $F_0$ progeny | Embryonic lethality | $F_1$ progeny | rrf-1 early L4 | rrf-1 early L4 mated with wt males |
|---|---|---|---|---|---|
| Ten-1a specific | 115 ± 35, $n$ = 12 | 15 ± 5%, $n$ = 1379 | 84 ± 33*, $n$ = 15 | | |
| Common probe | 68 ± 21, $n$ = 19 | 58 ± 8%, $n$ = 1227 | 83 ± 38*, $n$ = 19 | 60 ± 12, $n$ = 17 | 372 ± 96, $n$ = 12 |
| GFP | 140 ± 31, $n$ = 17 | 3 ± 2%, $n$ = 2242 | 156 ± 23, $n$ = 19 | | 384 ± 41, $n$ = 11 |

* Only progeny of worms that survived throughout the experiment was taken into account.

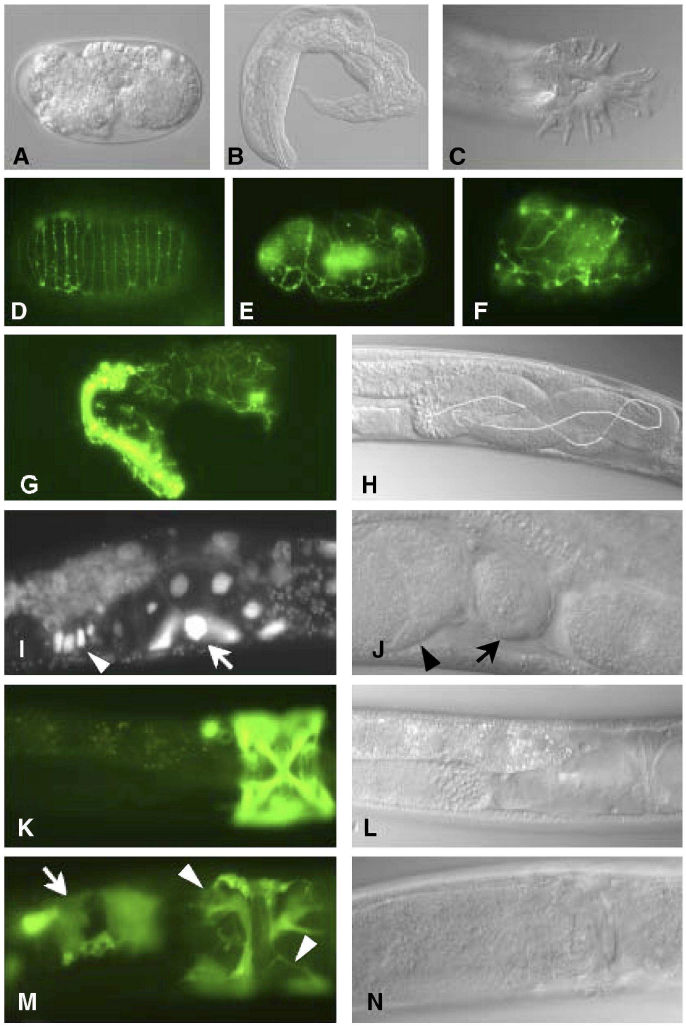


Fig. 3. Depletion of Ten-1 protein by RNAi results in severe morphological defects. The following phenotypes were observed in rrf-3 worms injected with RNAi against both *ten-1* transcripts (A–C and H–J) in rrf-3 worms crossed with the Jam-1-GFP-expressing worms RU85 [SU93 jam-1::GFP, *rrf-3*] (D–G) or in rrf3 worms crossed with the ten1a promoter GFP worms RU86 [ten-1a::GFP, *rrf-3*] (K–N): embryos arrested during elongation (A); variable abnormal larvae (B); malformed rays in the male tail (C); disorganized and aberrant shapes of hypodermal cells (E–G) as compared to the non-injected embryo (D); the distal tip cell with a "confused migration" phenotype and the path is marked by a white line (H); endomitotic oocytes develop in the gonad (arrow) and in the uterus (arrowhead) as can be seen by DAPI staining (I); oocyte rupture during ovulation and a ruptured oocyte in the gonad is indicated by an arrow and in the spermatheca by an arrowhead (J); the sheath cells of the gonad form clumps (arrow in M) and as a result the germline fills the body cavity (N, same field as M); the vulva muscles do not attach to the vulva nor to the body wall (arrowheads in M) in comparison to the non-injected RU86 [ten-1a::GFP, *rrf-3*] worm (K and L).

distal tip cells meander and often fail to turn (Fig. 5C). The worms have little or no sperm. Several ovulation defects have been observed: oocyte rupture during ovulation, endomitotic oocytes form in the gonad arm and in the uterus, and embryos develop in the gonad arm. The gonad loses its integrity and germ cells fill the worm (Fig. 5B). In addition to the two gonad arms, a tumorous germline forms around the vulva (Figs. 5E and H). In 20% of mutant worms, the somatic gonad develops normally and embryos do not burst through the vulva. These worms have a similar brood size as wild-type worms. To better visualize the effect of the *ten-1* deletion, we crossed the *ten-1* (ok641) mutant with worms expressing GFP under the *lim-7* promoter to label somatic gonad sheath cells. This revealed that the sheath cells developed but often formed clumps and did not

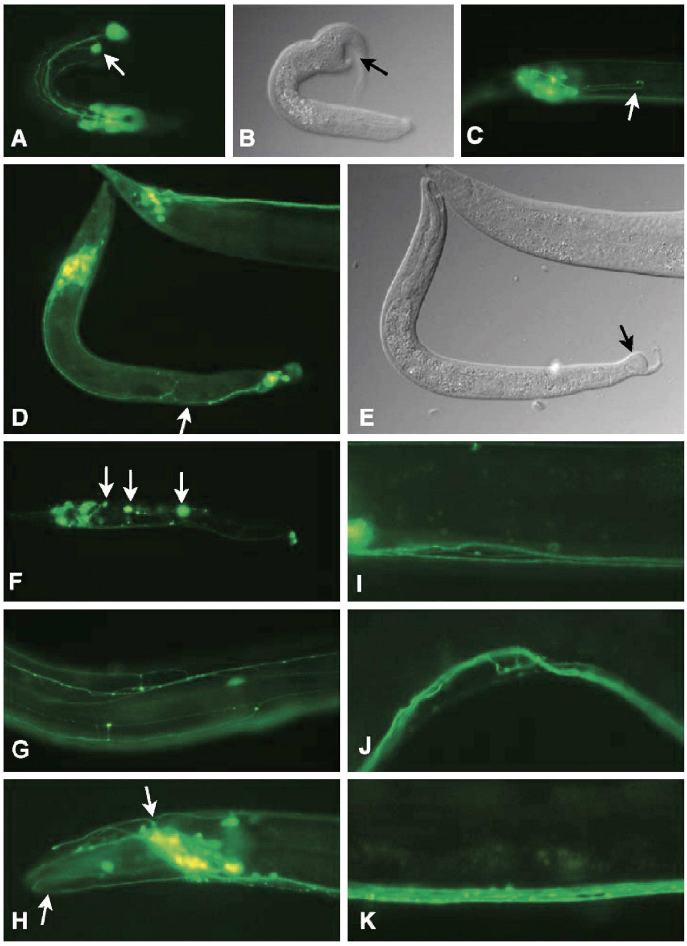


Fig. 4. Depletion of Ten-1 protein results in severe neuronal defects. To visualize neuronal phenotypes, rrf-3 worms were crossed with ten-1b promoter GFP worms (RU 87 [*ten-1b*::GFP, *rrf-3*]) and injected with RNAi against both *ten-1* transcripts (A–K). Neuronal migration defects are visible (arrows in A and F). The integrity of the ventral cord is disrupted (arrow in D) along with defects in the posterior hypodermis of the same animals shown by interference contrast (arrows in B and E). Axonal pathfinding is defective and axons meander randomly (arrows in C, G, and H). The axons in the ventral cord defasciculate (I and J), as can be appreciated when compared to the ventral cord of a non-injected worm (K).

cover the entire germline. In the worms that developed germline tumors, these were partially covered by sheath cells as well (Figs. 5D–G). To visualize a potential effect of the *ten-1* (ok641) deletion on neuronal development, we crossed this mutant with RU9 [*ten-1b*::GFP] worms. The morphology and pathfinding of some neurons were clearly abnormal (Fig. 5H).

We were able to partially rescue the *ten-1* (ok641) phenotype by injecting the cosmid F36A3 carrying the entire genomic region of the *ten-1* gene (A. Coulson, personal communication). Worms carrying the F36A3 cosmid showed reduced larval lethality. None of the rescued worms burst through the vulva nor exhibited a tumorous germline (Table 2).

### *The long cytoplasmic domain of Ten-1 can be detected in nuclei*

We raised antibodies against both the N- (anti-N) and the C-terminal peptides (anti-C) of the mature long form of the Ten-1 protein. The anti-N antiserum is recognizing the long form exclusively whereas the anti-C antibody is expected to recognize both Ten-1 variants. Antibody staining revealed that Ten-1 is expressed ubiquitously in early embryos (Figs. 6A–F). This result was confirmed by in situ hybridization (not shown). We used antibodies against the N-terminus of the long Ten-1L variant (anti-N) and against the common C-terminus of both protein variants (anti-C). Anti-C stained all membranes while

Table 2
Phenotypes and rescue of ten-1 deletion worms

| | N2, $n = 20$ | Ten-1±, $n = 100$ | VC518 (ok641), $n = 175$ | RU95 [(ok641) F36A3], $n = 217$ |
|---|---|---|---|---|
| Embryonic lethality (%) | 0 | 5 | 5 | 5 |
| Larval lethality (%) | 0 | 16 | 25 | 10 |
| Sterile (%) | 0 | 10 | 37 | 3 |
| Burst through the vulva (%) | 0 | 3 | 11 | 0 |
| Fertile, viable (%) | 100 | 66 | 22 | 82 |

anti-N stained in addition to membranes also the nuclei of Ten-1-expressing cells. Within the nucleus, the Ten-1 staining was punctuate (Fig. 6I). The nuclear staining was particularly prevalent in cells lining the gut where anti-C stained the cell membranes and anti-N stained predominantly the nuclei (Figs. 6G and H). These findings led us to speculate that the endogenous Ten-1 might normally be proteolytically processed and that the translocation of the intracellular domain could be a physiological process in Ten-1 signaling. The nuclear translocation of the long intracellular domain of Ten-1 could be confirmed by overexpression of both intracellular domains as GFP fusion proteins under a heat shock promoter. Transient expression of these constructs revealed no difference between a cytoplasmic GFP and a fusion between the short intracellular domain and GFP. Both constructs resulted in the same cytoplasmic fluorescence (Figs. 6J and K). However, the fusion construct between the long intracellular domain and the same GFP accumulated in the nuclei of the cells (Fig. 6L). Also in this case, the nuclear staining was punctuate, in contrast to that seen following the expression of a GFP containing a standard nuclear localization signal resulting in homogeneous nuclear staining (Fig. 6M).

## Discussion

### *The ten-1 gene encodes two variants of Ten-1 proteins*

In this work, we have characterized the gene structure, the expression pattern, and the function of *ten-1* in *C. elegans*. *ten-1* is the single *C. elegans* orthologue of the two *Drosophila* genes *ten-a* (Baumgartner and Chiquet-Ehrismann, 1993; Fascetti and Baumgartner, 2002; Minet and Chiquet-Ehrismann, 2000) and *ten-m/odz* (Baumgartner et al., 1994). The overall structure of the Ten-1 protein is conserved throughout phyla along the entire length of the protein. The most highly conserved region contains EGF-like repeats. Proline-rich sequences are present in the intracellular domains of *C. elegans*, *Drosophila*, and vertebrate *ten-1* homologues; however, they are located at

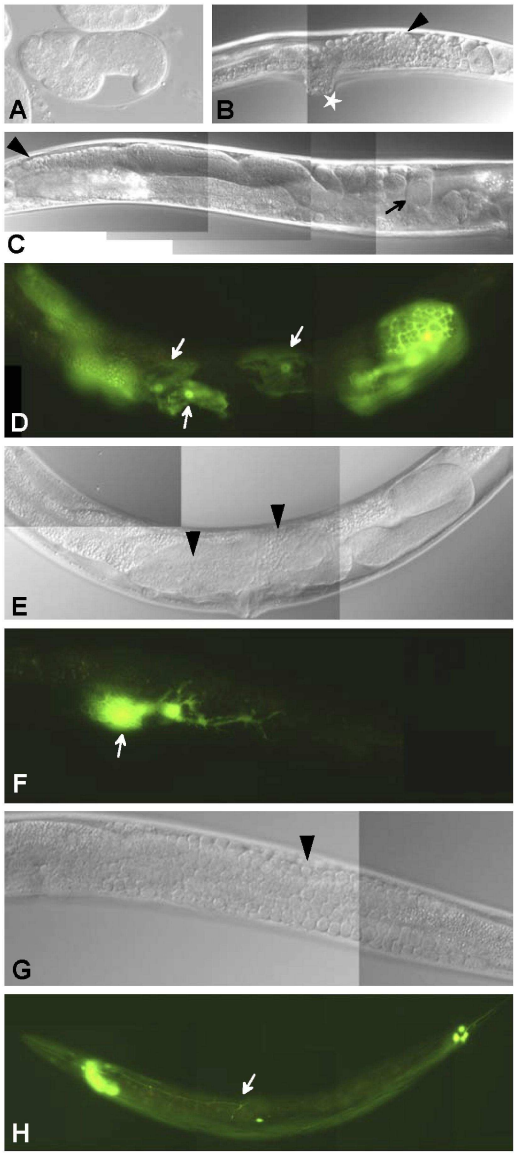


Fig. 5. VC518 (ok641) *ten-1* deletion mutants show severe morphological defects. Embryos arrest during the elongation stage (A). The gonad looses its integrity, the germline (arrowhead) fills the body cavity, tumorous germline forms, and the worms have a protruding vulva indicated by a star (B). The gonad fails to turn and extends to the head (arrowhead) and a ruptured oocyte (arrow) is seen in the spermatheca (C). To visualize the gonad sheath cells, ok641 worms were crossed with lim-7::GFP-expressing worms (D–G). Tumorous germline (arrowheads) forms in the proximity of the vulva (E and G). These additional germ cells are partially covered by gonadal sheath cells seen by GFP fluorescence in the same worms (arrows in D and F). To visualize neuronal phenotypes, ok641 worms were crossed with ten-1b promoter GFP worms (RU 87 [ten-1b::GFP]). Axonal migration defects are indicated by an arrow (H).

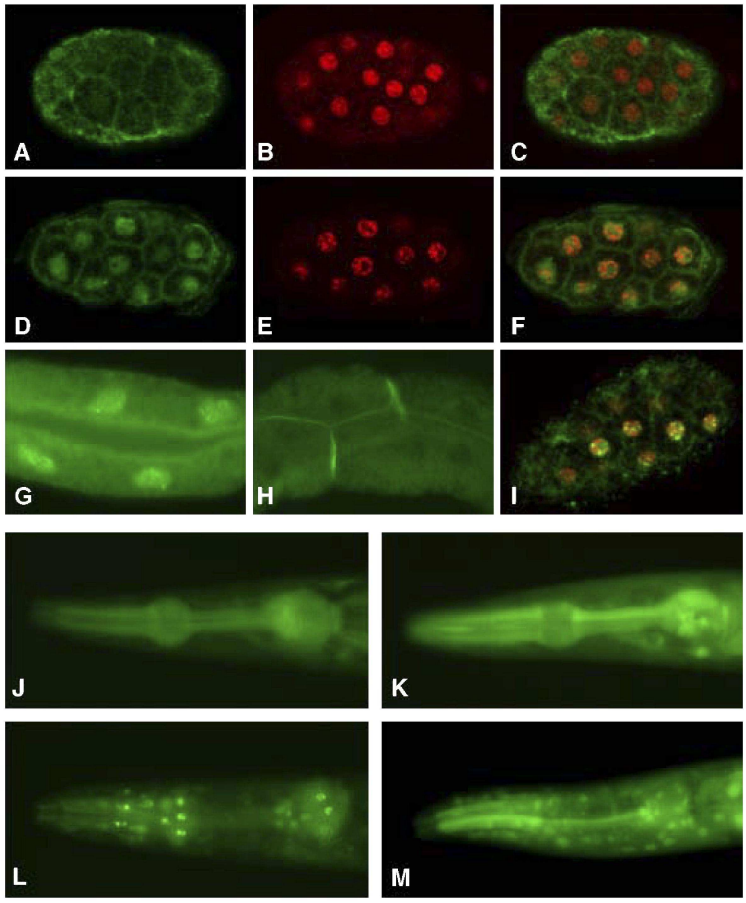


Fig. 6. The long form of the intracellular domain localizes to the nucleus. Confocal images of wt embryos stained with anti-N (A) and anti-C (D) antibodies. PI nuclear staining (B and E) and merged images (C and F). The anti-C antibody stains cell membranes and the anti-N antibody stains both membranes and nuclei. In I, a merged image of PI nuclear staining in red and anti-N staining in green clearly reveals the staining of sub-nuclear structures by the anti-N antibody. Gut staining with anti-N antibodies reveals a nuclear staining (G) and anti-C antibodies stain the membranes (H). The expression patterns of heat shock-induced expression of a short intracellular domain GFP fusion construct reveal the same cytoplasmic staining (J) as cytoplasmic GFP alone (K) whereas the long intracellular domain GFP fusion construct results in a punctuate nuclear staining (L) distinct from the NLS-GFP construct which shows homogeneous nuclear staining (M).

variable positions. The nuclear localization signal may also be conserved since arginine and lysine stretches are present at similar positions in the *C. elegans*, *Drosophila*, and vertebrate proteins. Indeed, overexpression of the chicken teneurin-2 intracellular domain in cultured cells results in its nuclear localization (Bagutti et al., 2003). Furin recognition sequences are present at a distance of approximately 140 aa from the transmembrane domain in all species.

In contrast to *Drosophila*, where two genes encode two differentially expressed proteins (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner et al., 1994; Fascetti and Baumgartner, 2002), we found that in *C. elegans* two different gene products are derived from a single gene due to the presence of two promoters. They give rise to two different transcripts that encode large type II transmembrane proteins differing in their N-terminal sequences. Thus, despite the existence of a single *ten-1* gene, *C. elegans* expresses two Ten-1 proteins with distinctive spatial and temporal expression patterns and thus differential functions in *C. elegans* development.

### *Ten-1 function in cell–cell recognition*

The two protein variants differ in their N-terminal intracellular domains that are either 36 or 218 aa in length. The extracellular domains with a length of 2419 aa are shared between the two variants. The expression of the shorter form of Ten-1 regulated by the downstream

promotor ten-1b shows the most prominent expression in the dorsal hypodermis and the anterior leading cell of the ventral hypodermis during embryonic development and later is found in a subset of neurons. Its main function may be the promotion of cell–cell recognition between hypodermal cells as well as between axons in the ventral nerve cord, since interference with protein expression can lead to abnormal hypodermal cell positioning resulting in an altered body shape, bursting of embryos during elongation and hatching, and at later stages to the defasciculation of the axons in the ventral nerve cord. Furthermore, the pathfinding of certain axons is severely disturbed. Since there is early Ten-1a expression in descendants of ABp cells, this might be a direct axon guidance defect caused by interference with Ten-1 expression; however, an indirect effect on pathfinding caused by morphological disturbances cannot be excluded. A function in pathfinding is supported by studies on vertebrate orthologues. All four vertebrate Ten-1 orthologues called teneurin 1–4 (Minet and Chiquet-Ehrismann, 2000; Rubin et al., 1999), ten-m1–4 (Oohashi et al., 1999), or odz-1–4 (Ben-Zur et al., 2000), respectively, show high expression in brain. Furthermore, the rat teneurin-2 orthologue neurestin has been shown to be upregulated in olfactory neurons during regeneration and synaptogenesis (Otaki and Firestein, 1999) and recombinantly expressed teneurin-1 can affect neurite outgrowth in vitro (Minet et al., 1999; Rubin et al., 1999).

*Ten-1 function in somatic gonad development*

The long form of Ten-1 is encoded by transcripts from the upstream promotor ten-1a and its expression pattern is highly interesting. It is already expressed in the germ line and in all cells during early embryogenesis. Later in development, it is found among several other locations in the distal tip cell, a cell of crucial function in regulating the morphogenesis of the gonad (Austin and Kimble, 1987; Seydoux and Schedl, 2001). This focal expression of Ten-1 in organizing centers such as Ten-1L in the distal tip cell and Ten-1S in the anterior leading cell of the ventral hypodermis is reminiscent of similar observations in vertebrates where, e.g., teneurin-2 was found to be expressed in the cells of the apical ectodermal ridge, the organizing center of morphogenesis of limbs (Tucker et al., 2001). In accordance with its expression in the distal tip and sheath cells, we observed multiple phenotypes including defects in gonad migration and somatic gonad development in our experiments of interfering with Ten-1 function by RNAi and gene deletion. Defects in the germline and the development of germline tumors appear to be a result of aberrant signaling from the somatic gonad since the *ten-1* (ok641) mutant can be rescued by injection of a non-complex transgenic array not expected to be expressed in the germline.

*Ten-1 signaling by release of the intracellular domain and translocation to the nucleus*

The most interesting finding was the discovery that the intracellular domain of the long form of Ten-1 can be detected in cell nuclei. This implies that Ten-1 signaling functions through proteolytic cleavage of the intracellular domain and that this domain can subsequently translocate to the nucleus. Such a mechanism is well known for other signaling molecules regulating development, the most prominent example being Lin-12/Notch (Greenwald, 1998). The intracellular domain of Lin-12/Notch and the intracellular domain of another plasma membrane receptor SREBP serve as transcription regulators, and ATF6 is a bona fide transcription factor regulated by intramembranous proteolysis (for review, see Hoppe et al., 2001). We, therefore, envisage a similar mechanism for Ten-1L and propose that Ten-1L constitutes a novel member of the family of membrane-anchored transcription modulators. Such a mechanism of action is supported by studies with recombinantly expressed teneurin-2 in cell culture experiments (Bagutti et al., 2003). In the nucleus, the intracellular domain of Ten-1L seems to localize to certain subnuclear structures. This is reminiscent of the immunostaining of the vertebrate teneurin-2 intracellular domain which also showed a punctuate staining pattern that colocalized with PML bodies (Bagutti et al., 2003). PML bodies are subnuclear structures believed to function in transcription, DNA repair, replication, and posttranscriptional regulation of gene expression, and may represent a storage compartment of proteins involved in transcription control (Borden, 2002). No PML gene has been identified in the *C. elegans* genome, but many other proteins known to be localized in PML bodies, such as Sp100, p53, or CBP, exist in the nematode. It should thus be considered that similar subnuclear structures might exist in *C. elegans* as well.

In summary, we have characterized Ten-1 as a novel transmembrane protein of *C. elegans* and have demonstrated its requirement for gametogenesis, early embryogenesis, and hypodermal cell migration. In later stages of development, it is involved in neuronal migration and pathfinding, distal tip cell migration, and the establishment of the somatic gonad. Furthermore, it is required for pharynx and gut development as well as for proper defecation. We propose that Ten-1 acts as a receptor for morphogenetic cues and that it directly signals to the nucleus by proteolytic release of its intracellular domain from the cell membrane and by translocation to the nucleus.

## Acknowledgments

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## III.2. Results – submitted

### III.2.1. *C. elegans* teneurin, *ten-1*, is required for gonadal and pharyngeal basement membrane integrity and acts redundantly with integrin *ina-1* and dystroglycan *dgn-1*

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Short title: TEN-1 function in basement membranes

Abbreviations: BM, basement membrane; DIC, differential interference contrast; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage; Pun, Pharynx Unattached; SGP, somatic gonad precursor cells.

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**Abstract**

The *C. elegans* teneurin ortholog, *ten-1*, plays an important role in gonad and pharynx development. *ten-1* null mutants are sterile due to local basement membrane deficiency leading to early gonad disruption. They also arrest as L1 larvae with malformed pharynges and disorganized pharyngeal basement membranes. The pleiotropic phenotype of *ten-1* mutant worms is similar to defects found in basement membrane receptor mutants *ina-1* and *dgn-1* as well as in the mutants of the extracellular matrix component laminin, *epi-1*. We show that the *ten-1* mutation is synthetic lethal with *ina-1* and *dgn-1* indicating that TEN-1 could be a receptor acting redundantly with integrin INA-1 and dystroglycan DGN-1. The morphological defects found in *epi-1* deficient worms are enhanced by lack of *ten-1* suggesting that laminin EPI-1 is a potential extracellular ligand for TEN-1. Moreover, *ten-1* deletion sensitizes worms to loss of nidogen *nid-1* causing a pharynx unattached phenotype in *ten-1*;*nid-1* double mutants. TEN-1 appears to be an important receptor required for basement membrane maintenance and/or adhesion in particular organs and cells.

## Introduction

Teneurins are large transmembrane proteins that play important roles in cell signaling and cell adhesion (Tucker and Chiquet-Ehrismann, 2006; Tucker *et al.*, 2007). Teneurins are phylogenetically conserved among metazoans and they were described in several species, including *ten-1* in *Caenorhabditis elegans* (Drabikowski *et al.*, 2005), *ten-m*/*odz* and *ten-a* in *Drosophila* (Baumgartner et al., 1994; Fascetti and Baumgartner, 2002; Levine et al., 1994; Rakovitsky et al., 2007), zebrafish (Mieda *et al.*, 1999), chicken (Minet et al., 1999; Rubin et al., 2002; Tucker et al., 2001; Tucker et al., 2000) and mouse (Ben-Zur et al., 2000; Oohashi et al., 1999; Zhou et al., 2003). In vertebrates, four teneurin paralogs exist and they were named teneurin-1 to -4, ten-m1 to -m4 or odz-1 to -4.

The extracellular domain of all teneurins is composed of eight tenascin-type EGF-like repeats, a region of conserved cysteines, and YD repeats which are found in a few bacterial proteins (Minet and Chiquet-Ehrismann, 2000). The intracellular domain contains proline-rich stretches and putative tyrosine phosphorylation sites but is less conserved than the extracellular part and cannot be aligned in a linear way between the phyla. Teneurins are thought to interact in a homophilic manner (Bagutti et al., 2003; Leamey et al., 2007a; Oohashi et al., 1999; Rubin et al., 2002) and to date, no other ligand has been identified.

The name “teneurins” refers to their high expression in the developing and adult nervous system (Ben-Zur et al., 2000; Mieda et al., 1999; Oohashi et al., 1999; Otaki and Firestein, 1999; Rubin et al., 2002; Tucker et al., 2000; Zhou et al., 2003). In the developing mouse cortex, all teneurins are expressed in distinctive gradients and may be required for neocortical patterning (Li *et al.*, 2006). Several reports point out their role in the development of visual pathways. Leamey and co-workers (2007a) have found that teneurins are upregulated in visual versus somatosensory areas of the neocortex. Moreover, expression of different teneurins is largely non-overlapping and can be found in interconnected regions of the developing visual system (Leamey et al., 2007a; Rubin et al., 2002; Rubin et al., 1999). For instance, teneurin-1 staining is found in the tectofugal pathway and teneurin-2 is primarily expressed in the thalamofugal pathway. In addition, teneurins were shown to promote neurite outgrowth *in vitro* (Minet *et al.*, 1999; Rubin *et al.*, 1999) and *in vivo* (Leamey *et al.*, 2007a), suggesting an important function for

teneurins in axon guidance and target recognition. Recently, the first vertebrate teneurin knockout was described (Leamey *et al.*, 2007b). Teneurin-3 regulates eye-specific patterning in the visual system and the knockout mice show impaired binocular vision. Such a mild phenotype may be a result of functional redundancy and compensation by other teneurins.

Beside prominent expression in the nervous system, teneurins are also found in non-neuronal tissues. They are expressed in alternating stripes of the fly embryo, cardiac cells, muscle attachment sites, tracheal system in *Drosophila* (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner *et al.*, 1994), limb buds, branchial archs, somites in chicken (Tucker et al., 2001; Tucker et al., 2000) or gonadal somatic cells, pharynx and muscles in *Caenorhabditis elegans* (Drabikowski *et al.*, 2005). Teneurin expression in these tissues is often associated with pattern formation and cell migration.

The *in vivo* function of teneurins is mainly inferred from studies of *Caenorhabdities elegans* and *Drosophila* mutants. Mutation in the fly *ten-m* gene causes embryonic lethality due to the fusion of adjacent denticle belts (Baumgartner *et al.*, 1994; Levine *et al.*, 1994). Moreover, defects in the ventral nerve cord, cardiac cells and eye patterning are found in late *ten-m* mutant embryos (Kinel-Tahan et al., 2007; Levine et al., 1994). Similar defects in cuticle and eye development have been observed for the second *Drosophila* teneurin gene, *ten-a* (Rakovitsky *et al.*, 2007). In *Caenorhabditis elegans*, deletion in the *ten-1* gene causes a pleiotropic phenotype, including ectopic germline formation, nerve cord defasciculation, defects in distal tip cell migration and axonal pathfinding (Drabikowski *et al.*, 2005).

The single teneurin ortholog in *Caenorhabditis elegans*, *ten-1*, is under control of alternative promoters giving rise to two protein variants. The isoforms differ only in their intracellular domains and their expression patterns are complex but mostly non-overlapping: TEN-1 long (TEN-1L) is found mainly in the mesoderm, including pharynx, somatic gonad, various muscles and neurons, and TEN-1 short (TEN-1S) is predominantly expressed in some hypodermal cells and in a subset of neurons (Drabikowski *et al.*, 2005).

We report here the role of TEN-1 in gonadal basement membrane maintenance and pharynx development. Mutation in the *ten-1* gene leads to gonad rupture and sterility.

Similar disorganization of the early gonads has been reported for basement membrane mutants, i.e. integrin α *ina-1*, dystroglycan *dgn-1* and laminin αB *epi-1* (Baum and Garriga, 1997; Huang *et al.*, 2003; Johnson *et al.*, 2006). Furthermore, the genetic interactions between *ten-1*, *ina-1*, *dgn-1*, *epi-1* and *nid-1* suggest that teneurin, integrin and dystroglycan have related and partly redundant functions in *Caenorhabditis elegans* development. We hypothesize that TEN-1 is a novel basement membrane receptor or regulator acting together with INA-1 and DGN-1.

## Materials and Methods

### General methods and *C. elegans* strains

*C. elegans* strains were maintained at 20˚C as described (Brenner, 1974). The following strains were used in this study: wild-type N2, variety Bristol, CH120: *cle-1(cg120) I*, CB444: *unc-52(e444) II*, VC518: *ten-1(ok641) III*; TM0651: *ten-1(tm651) III*; NG39: *ina-1(gm39) III*; NG144: *ina-1(gm144) III*; CB189: *unc-32(e189) III*; CX2914: nDf16/*dpy-17(e164) unc-32(e189) III*; CH119: *nid-1(cg119) V*; CH121: *dgn-1(cg121)/dpy-6(e14) unc-115(mn481) X*. The *tm651* deletion removes nucleotides R13F6: 3661-4550 of the *ten-1* coding sequence.

The following GFP marker strains were used: RU7: *kdEx7* [*ten-1a::gfp*]; RU97: *ten-1(ok641) kdEx45* [*F36A3, III*]; SS0747: *bnIs1* [*pie-1::GFP::PGL-1*] (gift of Susan Strome); IM253: *urEx131* [*lam-1::gfp*] (gift of William Wadsworth), CH1878: *dgn-2(ok209) dgn-3(tm1092) dgn-1(cg121)*; *cgEx308* [*DGN-1::GFP*] (gift of James Kramer).

Double mutant worms were maintained as [*ten-1(ok641)*;*ina-1(gm144)*; *kdEx45*], [*ten-1(ok641/+)*;*nid-1(cg119)*], [*ten-1(ok641)*;*dgn-1(cg121/+)*; *kdEx45*] or [*ten-1(ok641/+)*;*dgn-1(cg121)*; *cgEx308*] strains and genotyped by PCR for the phenotypic analysis.

### Constructs and plasmids

The translational *Pten-1a::GFP::TEN-1L* minigene reporter construct was generated by cloning SpeI-HindIII cDNA fragment and HindIII-XhoI genomic fragment of TEN-1 long variant into p123T vector (Mo Bi Tec). The following restriction sites were introduced into the primers: SpeI and XhoI flanking the *ten-1* coding sequence, SacII at the 5’ end of the *ten-1a* promoter and ApaI downstream of the 3’ UTR.

Long intracellular domain, transmembrane domain and a short fragment of the extracellular part were amplified using 5’-AACAGTCTACCGAATCCCAACC-3’ and 5’-ATAACTAGTATGTTCCAGCACAGGTAAACTACCACG-3’ primers and cDNA from mixed stage N2 worms as a template. For the extracellular domain of *ten-1* we used 5’-GCTGAAATACCCACTCGCCAGC-3’ and 5’-ATCTCGAGCTATTCAGATTTTCGGAACTTCC-3’ primers and R06H12 cosmid as a template. Sequence encoding GFP was amplified from pPD117.01 vector and its NcoI

site was mutated to CCTTGG. GFP was fused by PCR to the N-terminus of *ten-1* cDNA fragment, which was cloned into SpeI-NcoI sites of *ten-1* minigene. HA tag was added at the C-terminus of *ten-1* coding sequence by PCR and cloned into HpaI-XhoI sites. The *Pten-1a::GFP::TEN-1L* construct contained 4235 bp of the *ten-1a* promoter and 512 bp sequence downstream of the stop codon. PCR fragments were generated with Pfu Turbo DNA polymerase (Stratagene).

**Transgenic animals**

Transgenic lines were generated as previously described (Mello *et al.*, 1991). The *Pten-1a::GFP::TEN-1L* plasmid was injected into *ten-1(ok641)* mutant worms. Injections of *GFP::TEN-1* minigene at low concentration (5 ng/µl) resulted in a very weak GFP fluorescence, mainly in the nervous system. Therefore, we injected the worms with high concentrations of the transgene (40 ng/µl) and obtained several lines giving stronger GFP fluorescence. We used pRF4 [*rol-6*] as a co-injection marker.

**RNA interference**

RNA-mediated interference (RNAi) was performed as described (Kamath and Ahringer, 2003). The K08C7.3 RNAi clone was obtained from the Ahringer feeding library. Wild type and *ten-1(ok641)* synchronized L4 hermaphrodites were placed on RNAi plates and grown at 15˚C for 72 hours. Single adult worms were placed on fresh RNAi plates and allowed to lay eggs for 24 hours. These plates were examined for 3 days to determine embryonic lethality and postembryonic phenotypes.

**Phenotypic analysis**

Young adult hermaphrodites were placed on separate plates and allowed to lay eggs for 24 hours. The progeny was analyzed for embryonic and postembryonic phenotypes: lethality, larval arrest, sterility and bursting at the vulva.

**Time course of germline development**

Gravid hermaphrodites were synchronized by bleaching. Eggs were allowed to develop overnight in M9 liquid culture and starved L1 larvae were placed on bacteria plates. We

scored the number of germ cells in 20 worms for each genotype at following time points: 0, 8, 12, 16, 20 hours.

**Microscopy**

Animals were mounted on 2% agarose pads in a drop of M9 buffer containing 25 mM sodium azide. DIC and fluorescence images were acquired with Z1 microscope (Zeiss) and AxioCam Mrm camera (Zeiss) using 63x/1.4 NA Plan-APOCHROMAT objective (Zeiss) and AxioVision software.

## Results

### Both *ten-1(ok641)* and *ten-1(tm651)* are functional null alleles

In our previous study we described the *ten-1* mutation, *ok641*, that carries an in-frame 2130 bp deletion removing four EGF-like repeats and a large part of the conserved cysteines region (Drabikowski *et al.*, 2005). We now obtained another allele, *tm651*, lacking 890 bp and introducing a frameshift into the *ten-1* coding sequence (Fig. 1). This deletion results in a loss of the transmembrane domain and the entire extracellular part. Therefore, *tm651* is most likely a null allele. Since phenotypes of both *ten-1* mutants show similar penetrance (Table 1), we assume that *ok641* represents a functional null allele as well.

To confirm this hypothesis, we created heterozygous worms carrying nDf16 deficiency in trans to *tm651* or *ok641* and investigated whether the mutant phenotypes became aggravated after complete removal of one copy of the *ten-1* gene. The *ok641*/nDf16 and *tm651*/nDf16 worms displayed a similar range of defects to *ok641* and *tm651* homozygous animals and the values observed were very close to those calculated under the assumption of *ten-1* mutants being null alleles (Table 2).

These data and the fact that *ok641* and *tm651* deletions affected protein regions that are common to both TEN-1 isoforms, suggested that there was no functional TEN-1 present in any of the *ten-1* mutants.

### Gonads of *ten-1* mutant worms burst early in development

Previous studies demonstrated that TEN-1 plays an important role in gonad development and function (Drabikowski *et al.*, 2005). Homozygous *ten-1(ok641)* worms are viable but 15-20% of them are sterile or burst through the vulva due to ectopic germline forming in the midbody region. Occasionally, gonads disintegrate completely and germ cells float in the pseudocoelom.

To determine the basis and the developmental stage of ectopic germline formation, we performed a time course experiment of germ cell proliferation in the early gonads of *ten-1(ok641)* mutants. We used worms carrying a P-granule GFP marker to distinguish between germ cells and somatic gonad precursor cells. Interestingly, we found that germ cells were released from the gonads of *ten-1* mutant already at the early L3 stage (Fig.

2B). At the same time point, there were no germ cells present around the developing somatic gonad primordium in the wild type worms (Fig. 2A). A sharp DIC boundary surrounding the gonad was visible in the wild-type as well as a large part of *ten-1(ok641)* gonad (Fig. 2A,B) but absent on the dorsal side of the mutant gonad, where the germ cells leaked out into the pseudocoelom. Gonad bursting was not the result of germline overproliferation causing increased pressure on the gonadal basement membrane, since we did not find any difference in the number of germ cells between wild type and *ten-1* mutants at this stage (Fig. 2C).

**Gonadal basement membrane is not maintained in the *ten-1* mutant**

Bursting of the early gonads in the *ten-1* mutant suggested that mutant worms have defects in the basement membrane formation or maintenance. Therefore, we examined the organization of the basement membrane in the *ten-1(ok641)* worms using a laminin-β *LAM-1::GFP* marker that labels all basement membranes in worms.

At hatching, both wild-type and *ten-1* mutant gonad primordia were compact and completely surrounded by laminin (unpublished data). As the gonadal precursor cells divided, a discontinuity appeared in the *ten-1(ok641)* gonadal basement membrane. The laminin layer surrounding the developing gonad appeared to get thinner or was not as stable as in wild type (Fig. 3B) but germ cells did not loose contacts yet and gonads kept their tubular shape, similarly to wild-type (Fig. 3A). At the L3 stage, there was no laminin-GFP detectable in the center of the mutant gonad and the germ cells were released. Gonad disruption appeared always on the dorsal side (Fig. 3D). During gonad isolation in the adult worms we observed that gut and gonad were often joined together in *ten-1* mutants suggesting that the regional deficiency in part of the gonadal basement membrane led to germ cell adhesion to the gut.

Local removal of gonadal basement membrane is known to take place during anchor cell invasion. In wild type worms this occurs on the ventral side of the gonad (Fig. 3E), while the break in the *ten-1* mutant appeared always on the dorsal side. Since *ten-1* is highly expressed in the anchor cell, we considered the possibility that the protein was required for anchor cell formation, guidance or correct spatial attachment and that the anchor cell position could be affected in the *ten-1* mutant animals. We, therefore, analyzed the

localization of the anchor cell in *ten-1* mutant worms expressing GFP under the *ten-1a* promoter. We did not find any defects in the position of the anchor cell in the mutant worms and it attached normally to the ventral side of the gonadal basement membrane at the L3 stage (Fig. 3F).

We also analyzed the gonadal basement membrane ultrastructure by electron microscopy and did not find any obvious general defects in its organization (unpublished data). The basement membrane was absent in the region of the break but appeared normal in the distal parts of the gonad. Moreover, we did not find any whorls or clumps of extracellular material typical for some other basement membrane mutants such as *epi-1*, *lam-1* or *dig-1* (Benard et al., 2006; Huang et al., 2003; Kao et al., 2006).

In summary, the gonadal basement membrane in the *ten-1(ok641)* hermaphrodites was properly assembled at hatching but was not maintained later in development. The localized basement membrane deficiency was not the result of impaired anchor cell invasion but must be due to another cause resulting in defects in the basement membrane assembly, stability or protein expression. Basement membranes beyond the gonad (and pharynx) did not show any major changes in the *ten-1* mutant worms as examined with *LAM-1::GFP* marker (unpublished data).

**Gonadal defects of *ten-1* mutants are similar to those found in the dystroglycan *dgn-1*, integrin *ina-1* and laminin *epi-1* mutants**

Gonadal epithelialization defects were reported for the dystroglycan *dgn-1(cg121)* mutant (Johnson *et al.*, 2006), several integrin α chain mutants *ina-1* (Baum and Garriga, 1997) and laminin α chain mutants *epi-1* (Huang *et al.*, 2003). Dystroglycan and integrins are cell surface receptors that interact with laminin and are required for basement membrane assembly, adhesion and signal transduction (Bokel and Brown, 2002; Higginson and Winder, 2005). EPI-1 is one of two laminin α chains found in *C. elegans* genome. Laminins are secreted proteins that play fundamental roles in basement membrane formation and function (Miner and Yurchenco, 2004; Previtali et al., 2003). Both *C. elegans* laminin isoforms are broadly distributed among the basement membranes but the gonadal basement membrane contains the EPI-1 isoform only (Huang *et al.*, 2003). Gonads of *dgn-1* and *epi-1* mutants were variably misshapen (Fig. 4C,E), burst during

development and led to worm sterility. Early gonads of *ina-1(gm39)* worms hardly ever burst (Fig. 4F) and rather seemed to be swollen in the center. However, at the L4 stage *ina-1* mutant gonads were clearly ruptured and the germ cells clustered around the developing vulva (Fig. 4G), similarly to *ten-1(ok641)* gonads (Fig. 4H).

We analyzed the organization of the laminin network surrounding the developing gonad in *dgn-1* mutants using *LAM-1::GFP* marker. Although the DIC pictures of *ten-1* and *dgn-1* mutants appeared similar, *dgn-1(cg121)* hermaphrodite gonads did not have any localized break such as the *ten-1(ok641)* gonads. In contrast, the *dgn-1* mutant gonads were generally disorganized and *LAM-1::GFP* seemed to be more diffuse throughout the gonadal surface in comparison to *ten-1* mutant gonads (Fig. 4D). Gonads in the adult *dgn-1(cg121)* as well as *epi-1(RNAi)* worms were also more disorganized than in the *ten-1(ok641)* mutant (unpublished data).

Nevertheless, gonadal defects described for the above three mutants, i.e. *dgn-1*, *ina-1* and *epi-1*, resembled the defects that we observed in the *ten-1(ok641)* worms (Fig. 4B) suggesting that TEN-1 could be an additional receptor required for gonadal basement membrane maintenance.

TEN-1 was found to be expressed in early gonads, including Z1 and Z4 cells, somatic gonad precursor cells during L2 stage (Fig. 4I,J), and anchor cell in L3 stage (Fig. 3E). TEN-1 expression in these gonadal somatic cells suggested that they could play an important role in the basement membrane maintenance. However, it is unclear whether TEN-1 functions in these cells to control correct basement membrane assembly, somatic cell position in the gonad or cell adhesion.

### *ten-1* is synthetic lethal with *dgn-1, ina-1, epi-1* and *nid-1*

The similar gonadal phenotypes of *ten-1*, *dgn-1*, *ina-1* and *epi-1* mutants suggested that TEN-1 could be a basement membrane receptor with similar and partly redundant function to dystroglycan and/or integrin receptors. To asses the interaction between *ten-1* and genes encoding various basement membrane components, we constructed double mutant combinations. In the crosses we used *ten-1(ok641)* and *dgn-1(cg121)* null alleles, the *ina-1(gm144)* loss-of-function mutant, and an RNAi approach in the case of *epi-1*.

To further analyze the genetic interaction network of *ten-1*, we included additional genes encoding basement membrane proteins, namely nidogen *nid-1*, perlecan *unc-52* and collagen XVIII *cle-1*. *C. elegans* nidogen deletion does not affect basement membrane assembly (Kang and Kramer, 2000) but *nid-1* mutants show defects in neuromuscular junction organization (Ackley *et al.*, 2003) and axonal tracts positions (Kim and Wadsworth, 2000). Interestingly, the *nid-1(cg119)* null mutant was found to be synthetic lethal with *dgn-1* as a result of pharyngeal defects (J. M. Kramer, personal communication). Mutation *e444* in the perlecan *unc-52* gene causes progressive paralysis in worms as well as gonad disorganization and germ cell release into the body cavity (Gilchrist and Moerman, 1992). Loss-of-function mutation in the collagen *cle-1* gene leads to cell migration and axon guidance defects. Some larvae of *cle-1(cg120)* mutant are unable to pump and arrest at the L1 stage with misshapen pharynges (Ackley *et al.*, 2001).

Interestingly, we observed more severe phenotypes in several double mutants than in any single mutant alone (Table 3). Synthetic lethality was found in *ten-1*;*dgn-1, ten-1*;*ina-1, ten-1*;*nid-1* and *ten-1*;*epi-1* double mutants. Lack of dystroglycan or nidogen in the *ten-1* mutant background led to developmental arrest during late embryogenesis or L1 larval stage in almost 100% of worms. Double mutant larvae were translucent suggesting a feeding defect. Morphological defects found in *epi-1* deficient worms (Fig. 5C) were enhanced by *ten-1* deletion. More than 90% of *ten-1*;*epi-1* double mutants arrested during embryogenesis or as early larvae and showed dramatic disorganization of developing tissues (Fig. 5E). Moreover, *ten-1;ina-1* mutants showed severe morphological defects, not found in any single mutant alone (Fig. 5B, D), and nearly 100% of double mutant worms arrested as disorganized embryos or L1 larvae (Fig. 5F).

In contrast, mutations in *unc-52* or *cle-1* did not cause synthetic lethality in the *ten-1* mutant background. These two mutations did not enhance embryonic lethality, larval arrest or sterility of the *ten-1(ok641)* worms. However, we cannot exclude that *unc-52* and *cle-1* interact genetically with *ten-1* in other processes, like axon guidance or distal tip cell migration.

**TEN-1 functions with NID-1 and DGN-1 in pharynx development**

Since larval arrest was significantly increased in several double mutants, we decided to investigate the phenotypes of the starved L1 larvae of *ten-1*;*dgn-1* and *ten-1*;*nid-1* double mutants suspecting that these three proteins could have an important role in pharyngeal morphogenesis. This hypothesis was supported by the fact that the long TEN-1 isoform was expressed in the developing pharynx (Fig. 6A, B) and was detectable in the pharyngeal basement membrane till adulthood (Fig. 6C, D). As 30% of *ten-1* single mutant worms arrest as L1 translucent larvae, we examined their pharyngeal defects. The wild type foregut is a short tube with two bulbs, surrounded by a thick basement membrane (Fig. 7A, B). As viewed by DIC microscopy, *ten-1* arrested larvae had variably misshapen pharynges and the outline of the pharynx was often barely visible (Fig. 7C). In addition, we examined the pharyngeal basement membrane organization with the *LAM-1::GFP* marker and found that it seemed to be disordered and missing in some parts of the pharynx (Fig. 7D).

In contrast to *ten-1* mutant worms, only a low percentage of *dgn-1* and *nid-1* single mutants arrested during larval stages. Pharynges of *dgn-1* larvae showed mostly wild type appearance (Fig. 7E), while the few *nid-1* arrested larvae had a bent anteriormost part of their foregut (Fig. 7F). Another phenotype found at low penetrance in the *nid-1* single mutant was Pharynx Unattached (Pun), where the pharyngeal epithelium did not connect to the arcade cells of the hypoderm.

Removal of *dgn-1* in the *ten-1* mutant background enhanced the defects found in the *ten-1* single mutant and double mutants of *ten-1*;*dgn-1* arrested as larvae with their pharynges variably misshapen (Fig. 7G). Interestingly, *ten-1*;*nid-1* double mutants arrested as larvae which were unable to feed because their pharynges were not attached to the lips (Fig. 7H).

In summary, our data suggest that *ten-1* and *dgn-1* act redundantly in pharyngeal morphogenesis and/or function. Moreover, both *ten-1* and *dgn-1* caused synthetic lethality in the *nid-1* mutant background implying an important role for these two receptors in the process of pharyngeal attachment.

## Discussion

Teneurins are conserved molecules with fundamental functions during development (Tucker and Chiquet-Ehrismann, 2006; Tucker *et al.*, 2007). In *C.elegans*, the major phenotypes observed in *ten-1* mutants are larval lethality and sterility (Drabikowski *et al.*, 2005). In the present work, we have analyzed the physiological and molecular mechanisms underlying these developmental defects. Our data provide the first indication of a link between TEN-1 and basement membranes and show its important function in gonad, pharynx and hypoderm development, most likely as a receptor signaling redundantly to INA-1 and DGN-1.

### Important function of TEN-1 in somatic gonad precursor cells

We found that TEN-1 is essential for the maintenance of the basement membrane early in development of the gonads in *C. elegans*. The basement membrane surrounding the gonad was formed properly at hatching but during larval development ruptured at a very specific location on the dorsal side in the middle of the gonad. The upstream promoter of *ten-1* is active in the somatic gonad precursor cells (SGPs) of L1-L2 larvae and RNAi specific for the TEN-1 long variant is known to cause gonadal disorganization (Drabikowski *et al.*, 2005). Therefore, SGPs may play an important role in extracellular matrix production and/or assembly of the gonadal basement membrane. The source of the basement membrane proteins and the process of their incorporation into the basement membrane are poorly understood. SGPs could function in basement membrane assembly by expression of specific receptors on their surface that bind and organize extracellular matrix proteins provided by adjacent tissue. Such a situation was described for fibulin-1, which is secreted by the intestine and is deposited on the gonadal surface (Muriel *et al.*, 2005). Also in the case of laminin isoforms it was suggested that their differential distribution is at least partly based on differential assembly mediated by cell surface receptors (Huang *et al.*, 2003). TEN-1 is a good candidate to function as a receptor promoting basement membrane assembly in the gonad. Another possibility is that TEN-1 is essential for SGPs polarization, adhesion or migration. It was shown that alterations in SGPs position in the early gonads of *dgn-1* and *epi-1* mutants result in severe epithelialization defects and gonad disorganization (Johnson *et al.*, 2006).

Apparently, lack of TEN-1 in the immature somatic epithelium or distal tip cells does not influence larval germ cell proliferation.

**TEN-1 as a basement membrane receptor acting redundantly with laminin-binding integrin and dystroglycan**

Mutants in the *ten-1*, *ina-1* and *dgn-1* genes share several phenotypic features, including gonad disorganization, protruding vulva, defasciculation of the ventral nerve cord, distal tip cell migration and axonal guidance defects (Baum and Garriga, 1997; Drabikowski *et al.*, 2005; Johnson *et al.*, 2006; Meighan and Schwarzbauer, 2007). Moreover, *ten-1* and *ina-1* mutants show malformation of their pharynges. This strongly suggests that these three receptors may have related functions in several tissues and processes.

Disorganization of early gonads in *ten-1* mutant worms resembled the defects found in *ina-1* and *dgn-1* mutant worms, however, penetrance of gonad disruption and sterility was different. Only 10-20% of the *ten-1* null mutant and *ina-1* loss-of-function mutant showed gonad bursting and sterility. Gonadal defects could not be assessed in the *ina-1* null background as *ina-1(gm86)* worms arrest at the L1 larval stage (Baum and Garriga, 1997). In contrast, in *dgn-1* null mutants almost 100% of adult worms were sterile. Therefore, DGN-1 may represent the major receptor required for gonad epithelialization and basement membrane assembly, while INA-1 and TEN-1 may compensate for each other or have more subtle functions in gonad development. Moreover, INA-1 and TEN-1 could act together in pharyngeal morphogenesis and function, since single mutants in these genes arrested as L1 larvae with malformed pharynges (~30% in the *ten-1* and almost 100% in the *ina-1* null mutants (Baum and Garriga, 1997)).

Double mutants between *ten-1*, *ina-1* and *dgn-1* showed synergistic genetic interaction implying that these three genes act in similar developmental processes and have at least partly redundant function. Related roles of these receptors in gonad development could not be directly assessed because of functional redundancy in other developmental processes, i.e. pharyngeal or hypodermal morphogenesis. Double mutants of *ten-1*;*dgn-1* and *ten-1*;*ina-1* were synthetic lethal and arrested during embryogenesis or early larval stages.

Arrested larvae of *ten-1*;*dgn-1* worms were translucent with misshapen pharynges. Although *DGN-1::GFP* is expressed in the developing pharynx, *dgn-1* mutant worms do not arrest as L1 larvae and do not show any obvious pharyngeal defects. However, lack of *dgn-1* in the *ten-1* mutant background strongly enhanced the defects found in the *ten-1* single mutant suggesting that there is compensation between *ten-1* and *dgn-1* in pharynx development and function. Interestingly, lack of the *ten-1* gene in *nid-1* mutant worms had the same effect as the removal of *dgn-1* in the *nid-1* mutant background (J. M. Kramer, personal communication) and both double mutants show a Pun phenotype. Therefore, loss of teneurin or dystroglycan sensitizes the worms strongly to loss of nidogen, which confirms the functional redundancy between TEN-1 and DGN-1 receptors.

Furthermore, *ten-1*;*ina-1* double mutants were synthetic lethal and arrested as embryos or early larvae, frequently with severe morphological defects. Integrin loss of function mutants, *ina-1(gm39),* show malformation of the anterior hypoderm manifesting as a notched head phenotype (Baum and Garriga, 1997), while *ten-1* null mutants have low penetrance morphological defects in the posterior body (Drabikowski *et al.*, 2005). Combination of mutations in both genes resulted in worms arrested as L1 larvae with the entire body deformed. Mosaic analysis revealed that INA-1 is important in hypodermis (Baum and Garriga, 1997) and the short variant of TEN-1 is known to be expressed in hypodermal cells of the developing embryo (Drabikowski *et al.*, 2005). Therefore, mild defects found in single mutants may be due to compensation by the presence of an alternative receptor. This strongly suggests that *ina-1* and *ten-1* could act together in several developmental processes, including hypodermal morphogenesis.

In summary, TEN-1, INA-1 and DGN-1 are not required for basement membrane formation and maintenance in general, but they are crucial in particular tissues and organs such as the gonad and pharynx. The lack of a phenotype in all basement membranes could also reflect redundancy between these three receptors, where deletion of a single gene can be compensated for by the presence of other receptors.

**Possible ligands of the TEN-1 receptor**

Our data indicate a novel role for TEN-1 as a basement membrane receptor and raise the question of its ligand(s). Since the *ten-1* mutant phenotype resembled in many aspects the phenotypes of *epi-1* as well as the laminin binding receptors *ina-1* and *dgn-1*, suggested that laminin EPI-1 could be a ligand for TEN-1 as well. Laminin *epi-1* mutants are generally sick and show cell polarization defects, tissue disorganization and physical disruption of basement membranes (Huang *et al.*, 2003). Similar phenotypes have been described for laminin β loss-of-function mutants, *lam-1(rh219)* (Kao *et al.*, 2006). Thus, mutations in the laminin genes cause more severe defects than *dgn-1* or *ina-1* single mutants suggesting that these two receptors might be functionally redundant or additional laminin receptors exist. Mutation in *ten-1* gene strongly enhanced the effects of *epi-1* depletion by RNAi, leading to almost complete lethality of *ten-1*;*epi-1* worms. This result supports the hypothesis of EPI-1 being a ligand for TEN-1, however, direct interaction between TEN-1 and EPI-1 needs confirmation by further biochemical studies.

Nidogen NID-1 could be another ligand interacting with TEN-1. *C. elegans* nidogen is found in most basement membranes but predominates around the nerve ring and in the developing gonad (Kang and Kramer, 2000). Nevertheless, loss of *nid-1* alone causes very mild defects, mainly in the nervous system (Ackley et al., 2003; Kim and Wadsworth, 2000). The defects are, however, dramatically enhanced, if a *nid-1* deletion is combined with a mutation in basement membrane receptors *ten-1* or *dgn-1* or axon guidance molecules such as the *sax-3* Robo receptor or the *unc-40* netrin receptor (J. M. Kramer, personal communication). In such sensitized backgrounds, lack of *nid-1* causes a highly penetrant Pun phenotype. It appears that correct attachment of pharyngeal epithelium to hypodermal arcade cells requires several basement membrane receptors and guidance molecules as well as nidogen. Currently, NID-1 is considered more as a regulatory molecule changing the conformation of basement membranes or ligand availability (Hobert and Bulow, 2003; Kim and Wadsworth, 2000) rather than being a purely structural component linking laminin and collagen networks (Fox *et al.*, 1991).

**Conservation in other organisms**

Our data provide the first indication of TEN-1 connection to basement membrane function. There is little evidence from previous studies in vertebrates suggesting that teneurins could be basement membrane receptors. However, in retrospect, the finding that induction of filopodia formation in neuroblastoma cells by teneurin-2 depends on the substrate and is more prominent on laminin than on poly-L-lysine (Rubin *et al.*, 1999) may reflect a direct interaction between theses proteins. Furthermore, chicken teneurin-2 was found to co-localize with laminin in particular basement membranes of the optic cup and the heart endocardium (Tucker *et al.*, 2001).

Teneurins in vertebrates have been most intensively studied in the nervous system. Teneurin-3 knockout mice show defects in the positioning of specific visual circuits leading to impaired binocular vision (Leamey *et al.*, 2007b). Such a mild phenotype in the single mutant might be due to functional redundancy with other teneurins or, in the context of our present study, with other basement membrane receptors. Teneurins may have redundant functions to integrins and dystroglycan in higher organisms as well and they may be implicated in related aspects of cellular behavior. It is known from human diseases that dystroglycan hypoglycosylation causes not only muscular dystrophies but also central nervous system abnormalities such as mental retardation, disorganization of brain lamination and ocular defects as found in patients suffering from Walker–Warburg Syndrome, Muscle–Eye–Brain disease and Fukuyama-Congenital Muscular Dystrophy (Montanaro and Carbonetto, 2003; Moore et al., 2002; Ross and Walsh, 2001). In the case of integrins, mutations in the $\alpha_6$ chain lead to epidermolysis bullosa, a skin disease caused by abnormal hemidesmosome attachment to the basement membrane and fragility of the dermal-epidermal junction (Pulkkinen *et al.*, 1997). Furthermore, congenital myopathy was observed in patients carrying mutations in the integrin $\alpha_7$ chain (Hayashi *et al.*, 1998).

Therefore, our studies in *C. elegans* could be instructive for further analyses of teneurins, integrins or dystroglycan function in vertebrates, since they point out high redundancy not only between several receptors of the same family but also between structurally distinct basement membrane receptors.

**Acknowledgments**

We would like to thank: the Shohei Mitani's lab and the Japanese National BioResource Project for providing the *ten-1(tm651)* strain and the *C. elegans* Gene Knockout Consortium for *ten-1(ok641)* strain; James M. Kramer, William Wadsworth and Susan Strome for GFP marker strains; Andrew Fire for GFP expression vectors. We acknowledge Julie Ahringer, Cambridge University Technical Services Limited and MRC geneservice for the RNAi clone. Some strains used in this study were provided by the *Caenorhabditis* Genetics Center (CGC). This work was supported by the Novartis Research Foundation.

**Figure legend**

**Figure 1. Genomic organization of *ten-1* gene and location of *tm651* and *ok641* deletions.** Exons are depicted as boxes and introns are shown as lines. Expression of *ten-1* is regulated by alternative promoters: *ten-1a* and *ten-1b*, resulting in two type II transmembrane protein variants differing in the length of their intracellular domain. Fragments of exons encoding different protein domains are labeled as follows: red - single transmembrane domain, green – EGF-like repeats in two groups, yellow – region of conserved cysteines, and blue – stretch of YD repeats. Black horizontal lines show the regions deleted in two *ten-1* mutants: *tm651* and *ok641*.

**Figure 2. Germ cells are released from the early gonad of *ten-1(ok641)* mutant through the central break.** Germ cell number and localization were evaluated using the P-granule marker *pie-1::GFP::PGL-1*. (A) Wild-type L3 gonad. The somatic gonadal primordium forms in the middle of the gonad and germ cells fill the two gonad arms (only one arm is shown). (B) Ruptured gonadal primordium of a *ten-1(ok641)* L3 larva. Germ cells are released into the body cavity and localize in the vicinity of the developing somatic gonad primordium. (C) Time course of germline development in wild type animals and *ten-1(ok641)* mutants. There is no germline overproliferation in the early gonads of the *ten-1(ok641)* mutant. Scale bar: 20 μm.

**Figure 3. The basement membrane breaks on the dorsal side of the *ten-1(ok641)* gonads.** Basement membranes were visualized by the *LAM-1::GFP* marker. (A) The wild-type L2 gonad is uniformly covered by laminin. (B) In the *ten-1(ok641)* mutant, the gonadal basement membrane becomes thinner or fails to assemble correctly (arrowhead) at the L2 stage. (C) The wild-type L3 gonad is covered by laminin. (D) There is no laminin present in the center of the *ten-1(ok641)* L3 gonad. The basement membrane is absent completely and germ cells are released. (E) Wild-type L3 larva expressing GFP under *ten-1a* promoter. GFP-labeled anchor cell is attached to the ventral side of the gonad. (F) Disrupted L3 gonad of *ten-1(ok641)* worms. Anchor cell formed normally and attached to the basement membrane on the ventral side of the gonad. Scale bar: 20 μm.

**Figure 4. Misshapen gonadal primordia are found in several basement membrane mutants, i.e. dystroglycan *dgn-1*, integrin *ina-1* and laminin *epi-1*.** DIC pictures of early gonads in wild type (A), *ten-1(ok641)* (B), *dgn-1(cg121)* (C) and the corresponding *LAM-1::GFP* pattern (D), *epi-1(RNAi)* (E), *ina-1(gm39)* L2 larva (F), *ina-1(gm39)* L4 larva (G) and *ten-1(ok641)* L4 larva (H). Mutant gonads do not form a tube-like structure but grow into a disorganized mass. Expression from the upstream promoter of *ten-1* is found in the L2 gonad of wild type worms (I, J). Scale bar: 20 μm.

**Figure 5. Morphological defects found in *epi-1(RNAi)* worms, *ten-1; epi-1(RNAi)* and *ten-1; ina-1* double mutants.** Wild-type (A) and *ten-1(ok641)* L1 larvae (B). *epi-1* depleted worms are often misshapen but defects are relatively mild (C). Arrested larva of *ina-1(gm144)* mutant (D). Morphological defects of *epi-1(RNAi)* worms were enhanced by *ten-1(ok641)* deletion and caused deformation of the entire body in the arrested larvae (E). Similar defects were found in *ten-1(ok641);ina-1(gm144)* double mutants (F). Severity and penetrance of the defects were greatly enhanced in the double mutants compared to single mutants. Scale bar: 20 μm.

**Figure 6. The long TEN-1 isoform is expressed in the developing and adult pharynx.** GFP::TEN-1 transgene (*kdEx121*) is expressed in the developing pharynx of the early embryo (A, B) and the adult pharyngeal basal membrane (C, D). Expression of the *kdEx121* is also found in some head neurons (D). Scale bar: 20 μm.

**Figure 7. Pharyngeal defects in *ten-1*, *nid-1*, *dgn-1* single and double mutants.** Pharynx morphology of L1 larva is shown. *LAM-1::GFP* marker labels the pharyngeal basement membrane. Wild-type pharynx is outlined by a sharp DIC boundary visible by DIC microscopy (A). Basement membrane organization in the wild-type larva visualized by *LAM-1::GFP* (B). Arrested larvae of *ten-1(ok641)* mutant have misshapen pharynges and the pharyngeal outline is invisible on DIC pictures (C). In the *ten-1* mutant, the basement membrane around the pharynx is disorganized or missing in some parts (arrows) (D). The pharynx of the *dgn-1* mutant worms shows no obvious defects (E). Arrested larvae of *nid-1* mutants have sometimes bent pharynges (arrow) (F) or their

pharynges do not attach to the hypoderm (similar to the double mutant shown in H). Variably misshapen pharynges were found in the *ten-1;dgn-1* double mutants (G). An unattached pharynx (Pun) phenotype observed in *ten-1;nid-1* double mutants (H). White arrowheads mark the anterior and black arrowheads posterior ends of the pharynges. Scale bar: 20 μm.

**Tables**

**Table 1. Phenotypes of *ten-1* deletion mutants.**

| Genotype | Embryonic lethality (%) | Larval arrest (%) | Sterile and/or vulva defects (%) | Fertile adults (%) |
|---|---|---|---|---|
| wild type | 0.9 | 0 | 0 | 99.1 |
| *ten-1(tm651)* | 6.4 | 32.1 | 16.7 | 44.8 |
| *ten-1(ok641)* | 5.8 | 31.7 | 17.4 | 45.2 |

**Table 2. Embryonic lethality and larval arrest phenotypes appearing in the progeny of nDf16/*ten-1 unc-32* transheterozygotes.**

| Genotype | Embryonic lethality (%) | Larval arrest (%) | Adults – total (%) | % Unc in adult worms | n |
|---|---|---|---|---|---|
| nDf16/*ten-1(tm651) unc-32(e189)* | 29.7 | 27.2 | 43.1 | 32.1 | 492 |
| nDf16/*ten-1(ok641) unc-32(e189)* | 33.5 | 22.6 | 43.9 | 30.1 | 310 |
| Expected value for nDf16/*ten-1*[a] | 29.5 | 24.0 | 46.5 | 33.3 | |

[a] calculated ratio of phenotypes expected if the *ten-1* mutants are null mutants

**Table 3. *ten-1* is synthetic lethal with *dgn-1*, *ina-1*, *epi-1* and *nid-1*.**

| Genotype | Embryonic lethality | Larval arrest | Sterile and/or vulva defects[a] | Fertile adults | n |
|---|---|---|---|---|---|
| wild type | 0.9 | 0 | 0 | 99.1 | >300 |
| *ten-1(ok641)* | 5.8 | 31.7 | 17.4 | 45.2 | >300 |
| *dgn-1(cg121)* | 5.4 | 2.2 | 92.4 | 0 | 92 |
| *ten-1(ok641);dgn-1(cg121)* | 14.0 | 84.2 | 1.8 | 0 | 57 |
| *ina-1(gm144)* | 10.0 | 30.6 | 23.5 | 35.9 | 170 |
| *ten-1(ok641);ina-1(gm144)* | 12.9 | 85.7 | 1.4 | 0 | 70 |
| *nid-1(cg119)* | 4.2 | 7.3 | 0.3 | 88.2 | >300 |
| *ten-1(ok641);nid-1(cg119)* | 34.7 | 65.2 | 0 | 0 | 88 |
| *epi-1(RNAi)* | 17.1 | 29.5 | 53.4 | 0 | >300 |
| *ten-1(ok641);epi-1 (RNAi)* | 48.5 | 44.2 | 7.3 | 0 | 293 |
| *cle-1(cg120)* | 0.7 | 0.7 | 1.1 | 97.5 | 283 |
| *ten-1(ok641);cle-1(cg120)* | 3.4 | 24.5 | 23.1 | 49.0 | 147 |
| *unc-52(e444)* | 3.5 | 1.5 | 5.6 | 89.4 | 198 |
| *ten-1(ok641);unc-52(e444)* | 4.8 | 21.5 | 36.5 | 37.2 | 293 |

Percentage of wild-type and mutant worms (single and double mutants) showing the following phenotypes: embryonic lethality, larval arrest, sterility or vulval defects, and wild-type fertile adults.

[a] "Vulva defects" category includes protruding vulva and bursting at the vulva phenotypes.

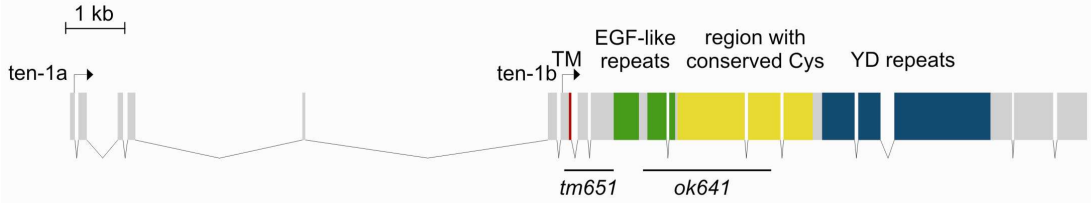



Figure 1.

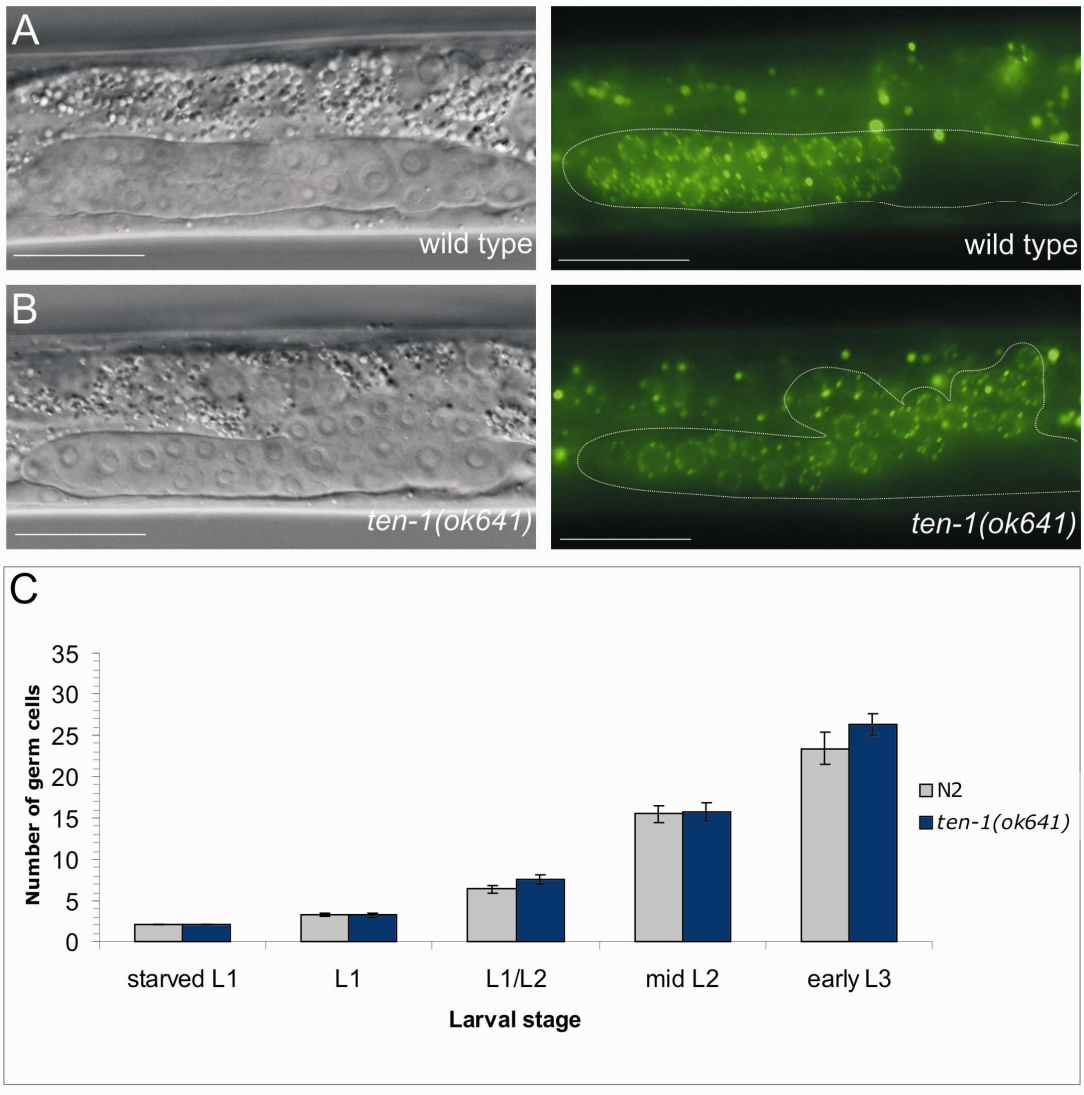



Figure 2.

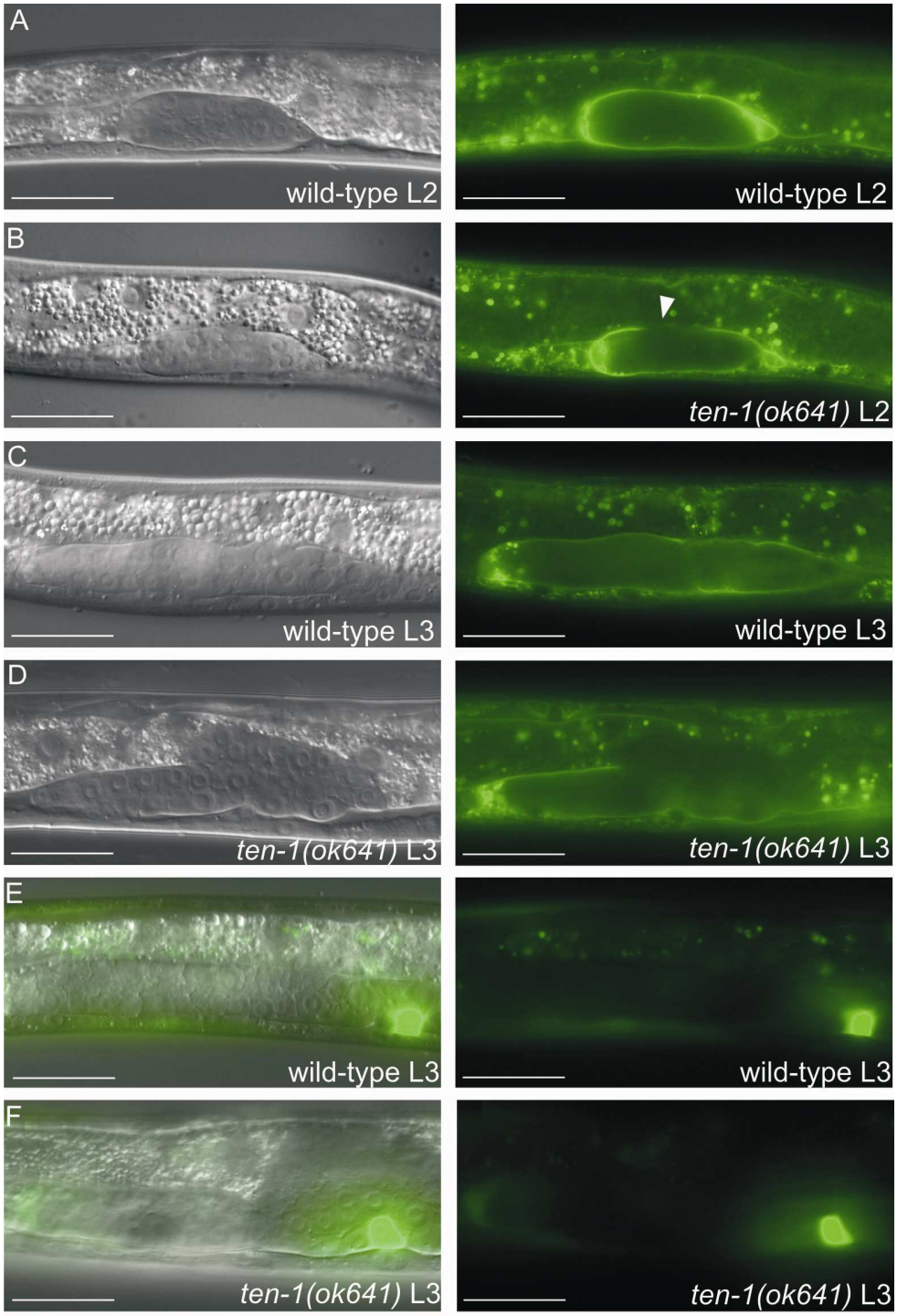

A
wild-type L2
wild-type L2
B
ten-1(ok641) L2
C
wild-type L3
wild-type L3
D
ten-1(ok641) L3
ten-1(ok641) L3
E
wild-type L3
wild-type L3
F
ten-1(ok641) L3
ten-1(ok641) L3

Figure 3.

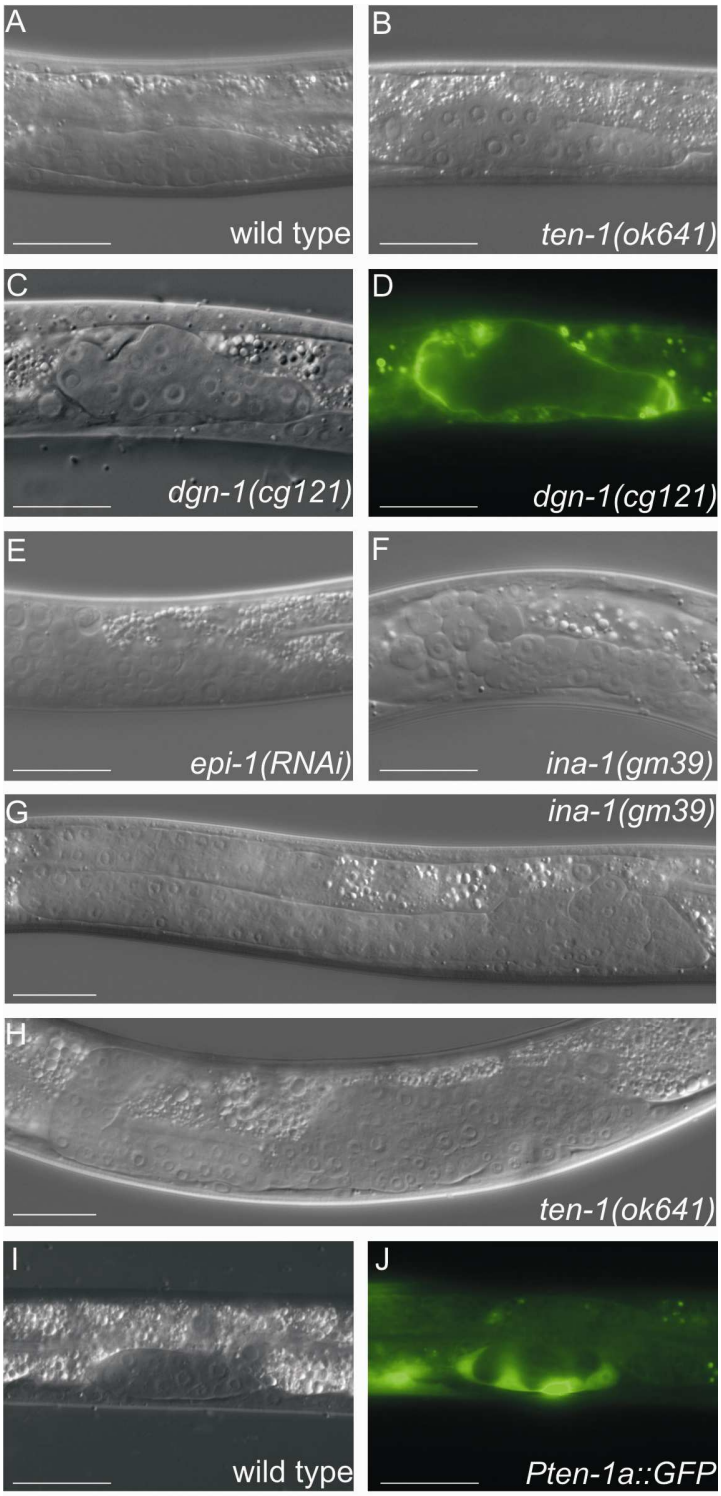

A
wild type
B
ten-1(ok641)
C
dgn-1(cg121)
D
dgn-1(cg121)
E
epi-1(RNAi)
F
ina-1(gm39)
G
ina-1(gm39)
H
ten-1(ok641)
I
wild type
J
Pten-1a::GFP

Figure 4.

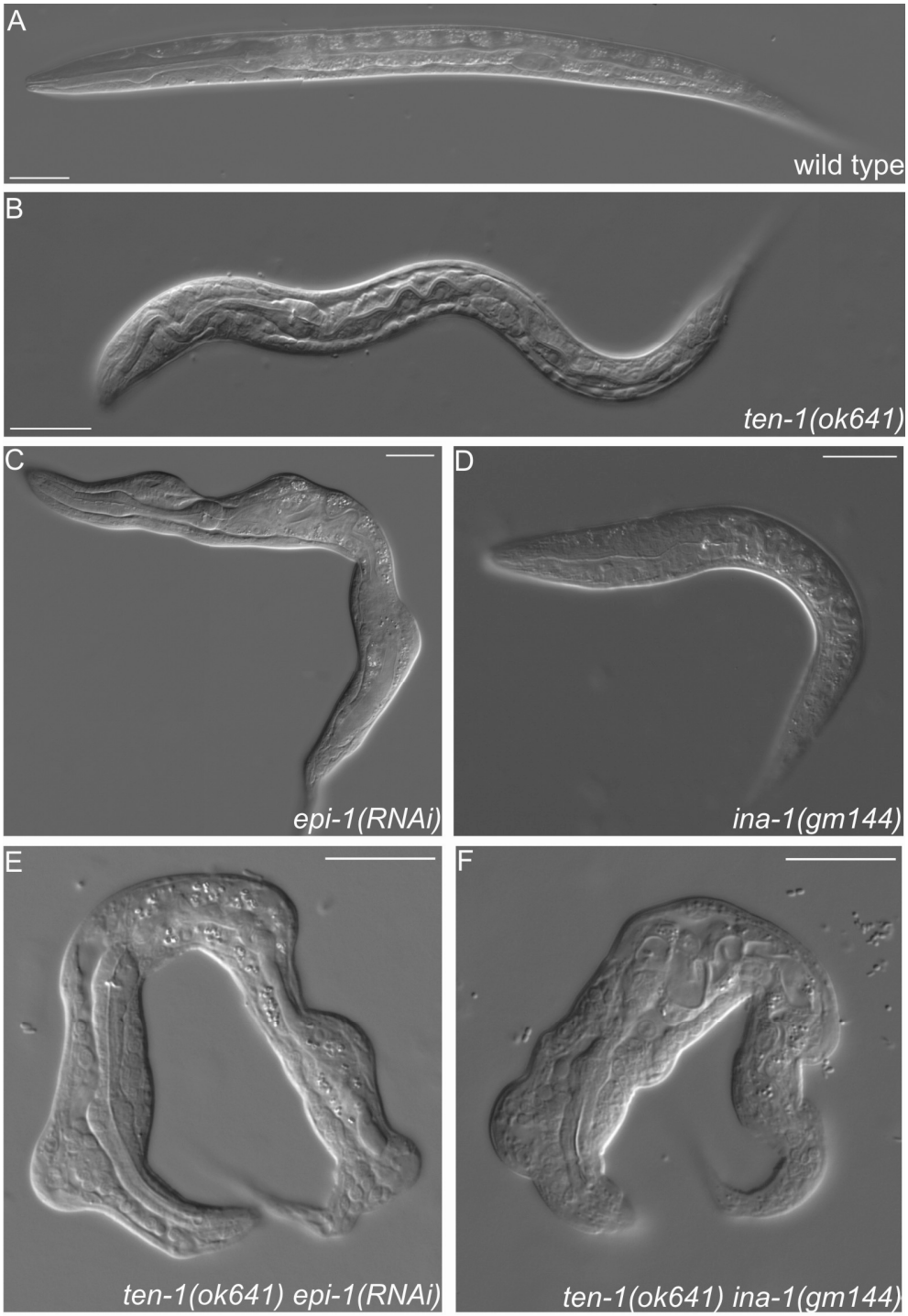

A
wild type
B
ten-1(ok641)
C
epi-1(RNAi)
D
ina-1(gm144)
E
ten-1(ok641) epi-1(RNAi)
F
ten-1(ok641) ina-1(gm144)

Figure 5.

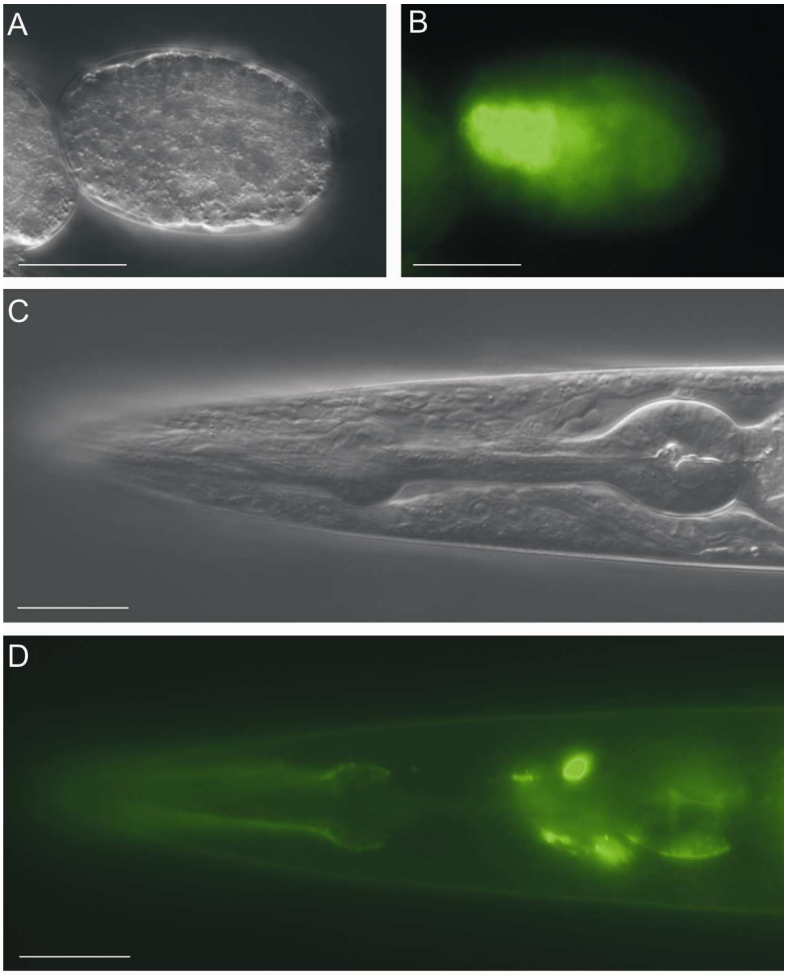



Figure 6.

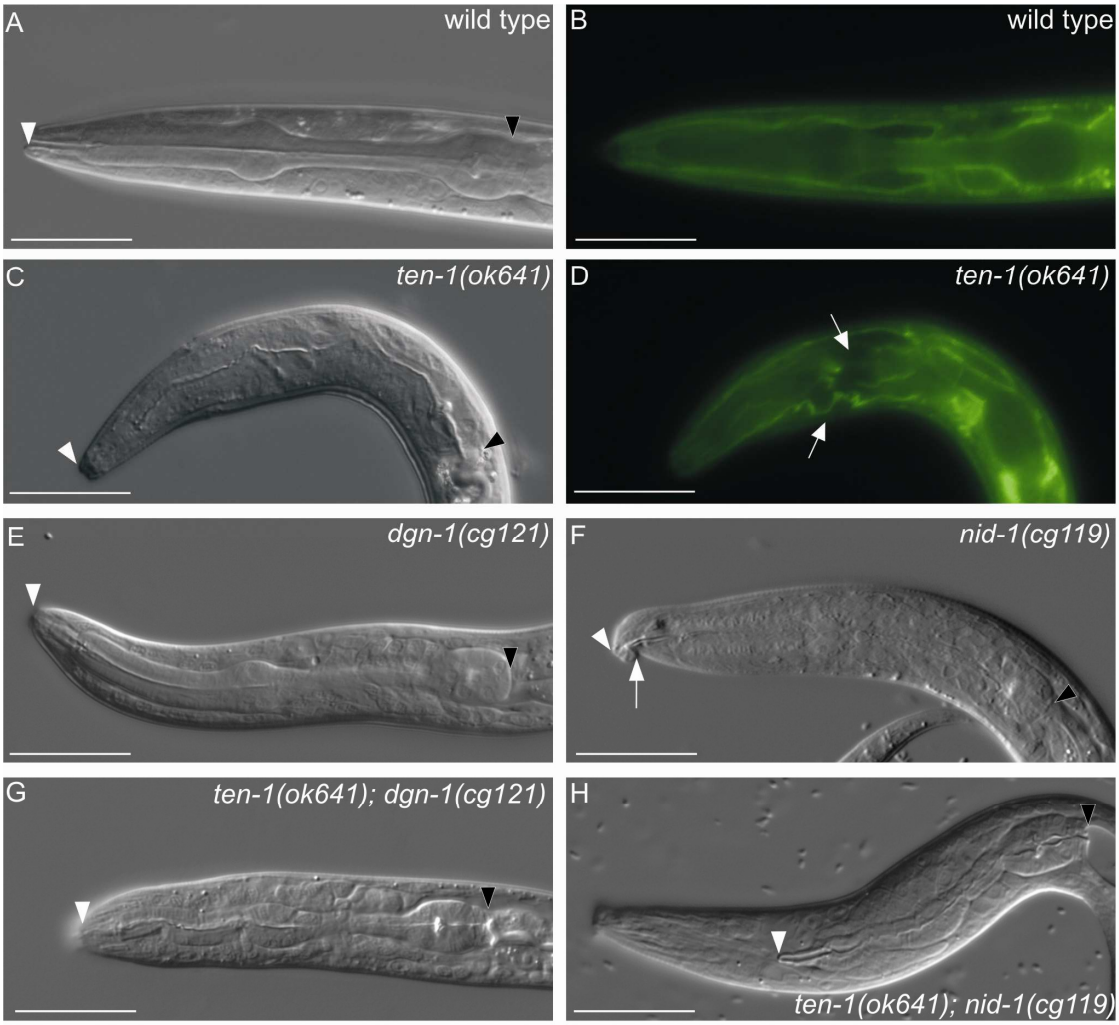

A
wild type
B
wild type
C
ten-1(ok641)
D
ten-1(ok641)
E
dgn-1(cg121)
F
nid-1(cg119)
G
ten-1(ok641); dgn-1(cg121)
H
ten-1(ok641); nid-1(cg119)

Figure 7.

## III.3. Results – unpublished

### III.3.1. Expression of truncated *ten-1* transcripts in *tm651* and *ok641* mutants

In our study, we used two mutants in the *ten-1* gene. Since one of the alleles, *ok641*, carrries an in-frame deletion, it is possible that TEN-1 missing 710 amino acids in its extracellular part is made and that such a truncated protein retains some functionality. The second mutation, *tm651*, deletes the transmembrane domain and introduces a frameshift into the *ten-1* coding sequence. It is expected that such a protein is not produced at all because of the premature stop codon and mRNA degradation by nonsense-mediated mRNA decay. Due to a lack of an antibody recognizing the endogeneous protein on Western blots, we performed a semi-quantitative RT-PCR to determine the *ten-1* transcript levels in the wild type versus mutant worms. As an internal control for the experiment, we used the *ama-1* gene encoding the large subunit of RNA polymerase II. The primers were designed within the first 750 nucleotides of the *ten-1* transcript. We did not find any difference in the transcript level in any of the *ten-1* mutants (Fig. III.1) and concluded that truncated *ten-1* transcripts are made at the same levels as the intact mRNA and they are not degraded.

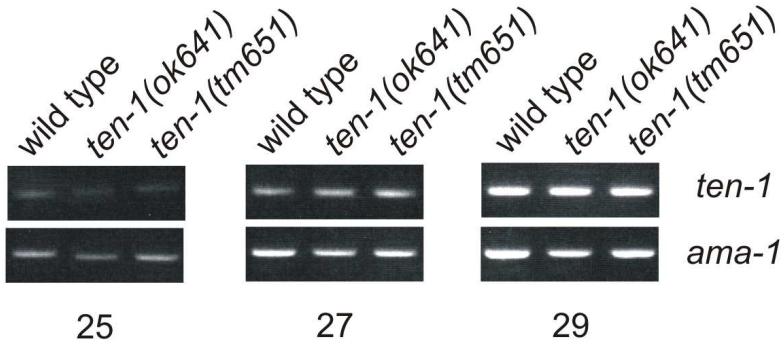



Figure III.1. Semi-quantitative RT-PCR comparing *ten-1* mRNA levels in *tm651* and *ok641* mutant worms. *ama-1* was used as an internal control. The transcript levels in wild type and mutant worms are shown for 25, 27 and 29 PCR cycles.

However, our genetic data (Table 2, submitted manuscript) implicate that TEN-1 function is abolished completely, even if the truncated proteins are produced. It still remains to be determined what the exact molecular features of the *ten-1* transcripts are in the mutants. It is possible that there is an alternatively spliced short TEN-1 isoform that contributes to wild-type transcript levels in RT-PCR, therefore the PCR from the 3' region of the transcript should be performed.

### III.3.2. Somatic gonad disorganization in *ten-1* mutant worms

The gonadal phenotype of *ten-1(ok641)* worms is similar to defects found in several basement membrane mutants. It was reported that mislocalization of SGPs in L1 larvae lead to gonad epithelialization defects and gonad bursting in *dgn-1* and *epi-1* mutant worms (Johnson et al., 2006). Therefore, we analyzed the position of early somatic cells in the gonads of *ten-1* mutants. We crossed *ten-1(ok641)* worms with the JK2049 strain expressing the *Plag-2::GFP* marker labeling Z1 and Z4 cells, and their descendants. We found that in ~20 % of *ten-1* mutants at the L1 stage one or both somatic cells were mispositioned and they interdigitated between Z2 and Z3 germ cell precursors (Fig. III.2). In 5-10% of worms (both mutant and wild type) one of the somatic precursors was missing but this may be due to the marker loss in one of the cells. This preliminary observation needs further investigation and the gonadal somatic cell lineage should be followed up later in development. This would allow us to determine how the initial SGP mislocalization affects organization of the somatic gonad and basement membranes at later larval stages.

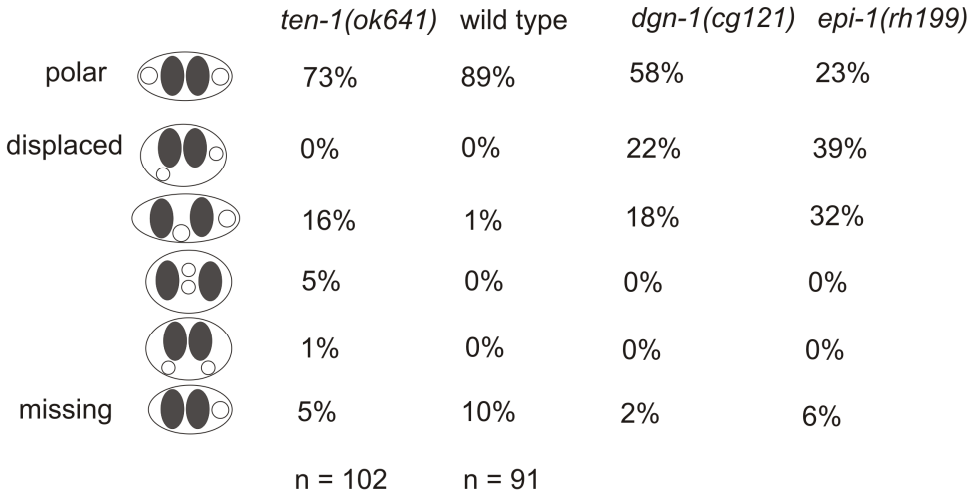



Figure III.2. Mislocalization of somatic gonad precursor cells (Z1 and Z4 – white circles) in *ten-1*, *dgn-1* and *epi-1* mutants. Germ cell precursors are shown as grey ovals. Data for *dgn-1* and *epi-1* mutants are taken from (Johnson et al., 2006).

### III.3.3. Basement membrane ultrastructure in the *ten-1* mutant worms

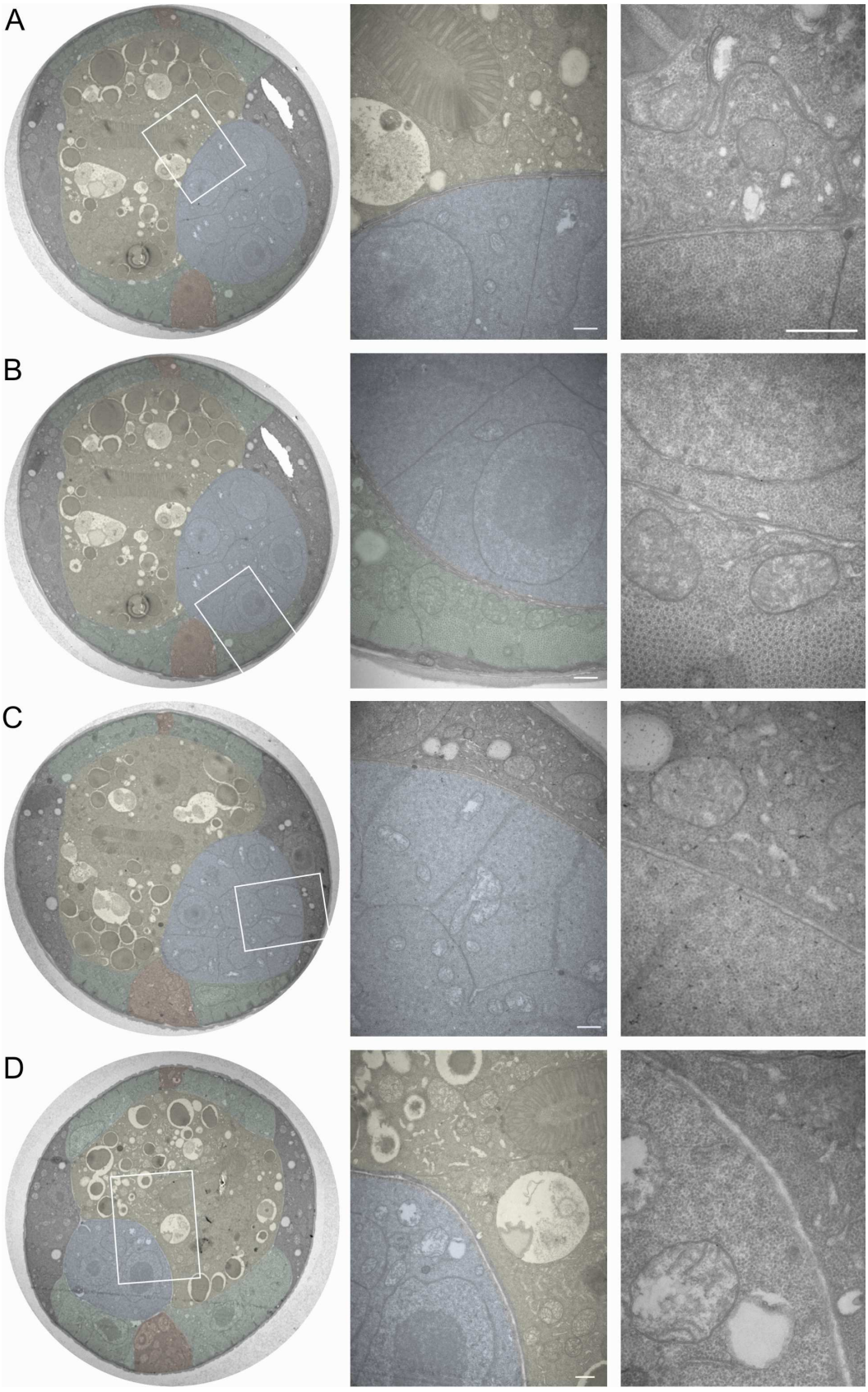
*In collaboration with Ursula Sauder, ZMB Center of Microscopy of the University of Basel, Switzerland.*

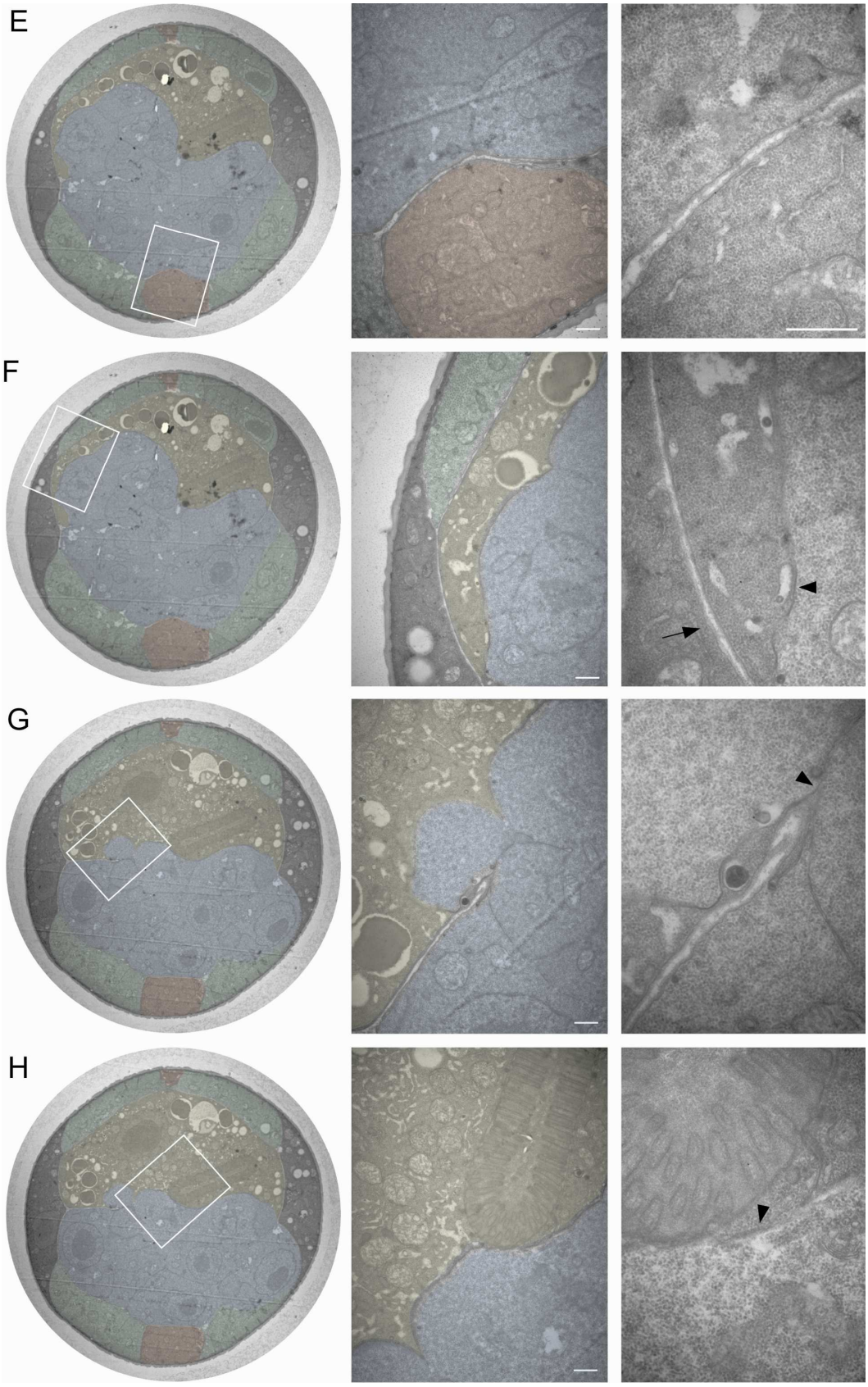
We showed that *ten-1* mutant worms have a defect in the gonadal BM integrity and a localized break appears in the center of their L2/L3 gonad (Figure 3, submitted results). Therefore, we analyzed gonadal basement membrane ultrastructure in the *ten-1* mutant worms by electron microscopy. L3 larvae were prepared by a two-step fixation, embedded in Epon resin and thin sectioned at 2 μm intervals. Wild-type gonads were completely ensheathed by basement membranes, which appeared as thin mesh of extracellular material (Fig. III.3A-C). In *ten-1(ok641)* worms, gonads had round shape and were completely covered by BM in sections localized distally from the break (Fig. III.3D). However, in the midbody region basement membrane was absent on the dorsal side of the broken gonad, germ cells leaked out through the break and invaded the intestine (Fig. III.3F-H). In contrast, basement membranes on the ventral side of the burst gonad showed generally wild-type ultrastructure (Fig. III.3). Moreover, we did not find

any whorls or clumps of extracellular material that could suggest general defect in basement membrane organization. Such a phenotype was described for some basement membrane mutants such as *epi-1*, *lam-1* or *dig-1* (Benard et al., 2006; Huang et al., 2003; Kao et al., 2006).

Figure III.3. Basement membrane morphology in *ten-1(ok641)* mutant worms. Cross-sections through a wild type worm (A-C) and a *ten-1(ok641)* mutant (D-H). The middle panel shows enlarged regions marked on overviews (left) with white rectangles. Tissues are labeled as follows: gonad – blue, intestine – yellow, hypodermal ridges (together with the nerve cords) – red, and muscles – green, epidermis – unlabeled. The right panels show further enlargements of the same region to visualize the basement membranes between different tissues.

Basement membranes at the boundaries between gonad and intestine (A), gonad and muscles (B), and gonad and epidermis (C) are shown in wild-type larva. The gonad of the *ten-1* mutant worm appears wild-type in a section 2 μm distant from the break (D). In the midbody region, the mutant gonad breaks on its dorsal side. BMs on the ventral side of the worm (e.g. between hypodermal ridge and gonad) show a normal structure (E). However, it is evident that no BM covers dorsal germ cells (arrowhead), while the BM between intestine and epidermis or muscles has a normal ultrastructure (arrow) (F). A BM surrounds the gonad only to a certain point (arrowhead), after which germ cells clearly leak out of the gonad (G). There is no BM present between germ cells and intestine (arrowhead) (H). Scale bar is 500 nm.


A
B
C
D


E
F
G
H

### III.3.4. TEN-1L localization

We showed that TEN-1 is required for BM function, therefore one of our aims was the localization of TEN-1 in cells, particularly in the gonad. Due to the lack of an antibody working in immunochemistry in larvae and adult worms, we created a TEN-1L full length construct fused to GFP and expressed it under its own promoter. Since little is known about TEN-1 cleavage mechanisms and their regulation, we placed GFP on the N-terminus of the protein as this could give us a possibility of monitoring intracellular domain translocation to the nucleus.

The expression levels of the GFP:TEN-1L construct injected at 5 ng/µl were low although we used genomic DNA as a carrier, which is supposed to improve transgene expression. We obtained a better GFP signal, when the construct was injected at high concentration (40 ng/µl). However, GFP::TEN-1L could only be detected in cells that were shown to have the highest expression of a promoter::GFP transcriptional fusion. Surprisingly, we did not detect any GFP signal in nuclei of any cells, not even in embryos.

We found that TEN-1L was already expressed in ~12-16 cell stage embryos and later in the group of cells that is probably the developing pharynx (Figure 6, submitted manuscript). At early stages, GFP::TEN-1 did not seem to be localized at the membrane but rather showed a dotted cytoplasmic pattern (Fig, III.4A). At the three-fold stage, TEN-1L expression was detected in the cytoplasm and membrane of pharyngeal cells and some head neurons (Fig. III.4B), and this pattern remained untill adulthood (Fig. 6, submitted manuscript). Moreover, GFP::TEN-1L was found in distal tip cells, the anchor cell and uterus (Fig. III.4C-E). Expression in early somatic cells of the gonad is quite low in worms carrying *Pten-1a::GFP* transgene and we were not able to detect GFP::TEN-1L in the gonadal cells (except for DTCs and the anchor cell).

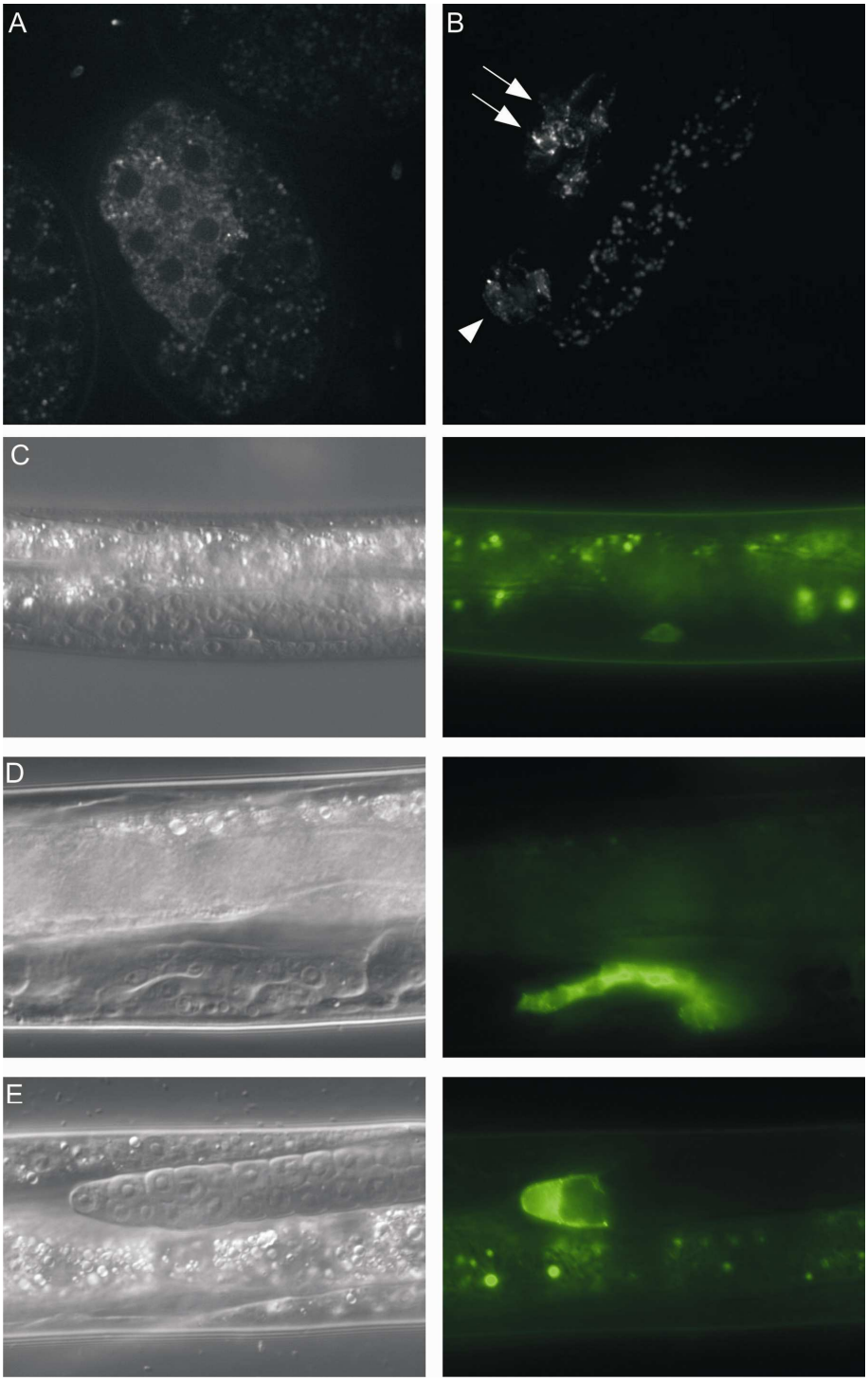



Figure III.4. Localization of GFP::TEN-1L (*kdEx121* transgene). (A) Early embryo, single optical section. (B) Three-fold embryo, overlay of Z-stacks. GFP fluorescence is visible in terminal bulb of the pharynx (arrowhead) and some head neurons (arrows). (C) L3 larva – TEN-1 is expressed in the invading anchor cell. L4 larva – TEN-1 is found in the developing uterus (D) and distal tip cell (E). Pictures were taken on spinning disk confocal microscope (A-B) and Zeiss Z1 miscroscope (C-E).

### III.3.5. Does TEN-1 bind laminin? – adhesion in *C.elegans* cell culture

Our genetic data support the hypothesis that TEN-1 is a novel basement membrane receptor and one of its potential ligands could be laminin. We decided to make use of *C. elegans* cell culture to investigate TEN-1-laminin interaction and its influence on cell adhesion. Since there are no purified worm laminins available, we used two vertebrate proteins: mouse EHS laminin 111, which is similar to LAM-3, and human placenta laminin 511 with homology to EPI-1. For *in vitro* culture, we isolated embryonic cells from two worm strains: RU7 and CH1878. RU7 worms carry the *Pten-1a::GFP* transgene, which labels *ten-1* expressing cells. Green cells from CH1878 strain overexpress worm dystroglycan, which is a well-known laminin receptor, and these cells could serve as a positive control.

Initially, we used cells from the RU7 strain and EHS laminin for coating. Since worm cells are reported to adhere well only to peanut lectin, we coated the plates with peanut lectin first and subsequently with laminin 111. After three days in culture, numerous cells attached and differentiated, mostly into muscles (labeled by phalloidin staining) and neurons (cells with long processes). The majority of GFP positive cells differentiated into neuronal cells. The percentage of green cells in suspension was very low and we did not observe any preference for adhesion of GFP positive cells to laminin (Figure III.5A). One possible explanation is that non-green cells were still able to adhere because of the peanut lectin, while ignoring the laminin coating.

In the next experiment, we used CH1878 cells and coated the wells with peanut lectin (PL), peanut lectin + laminin (PL+LN) or laminin alone (LN). Cells adhered well to PL and PL+LN but they clumped together when plated on laminin alone. Nevertheless, green cells seemed to adhere to LN equally well as GFP negative cells (Fig. III.5B). From this we concluded, that *C. elegans* cells do not recognize vertebrate laminin.

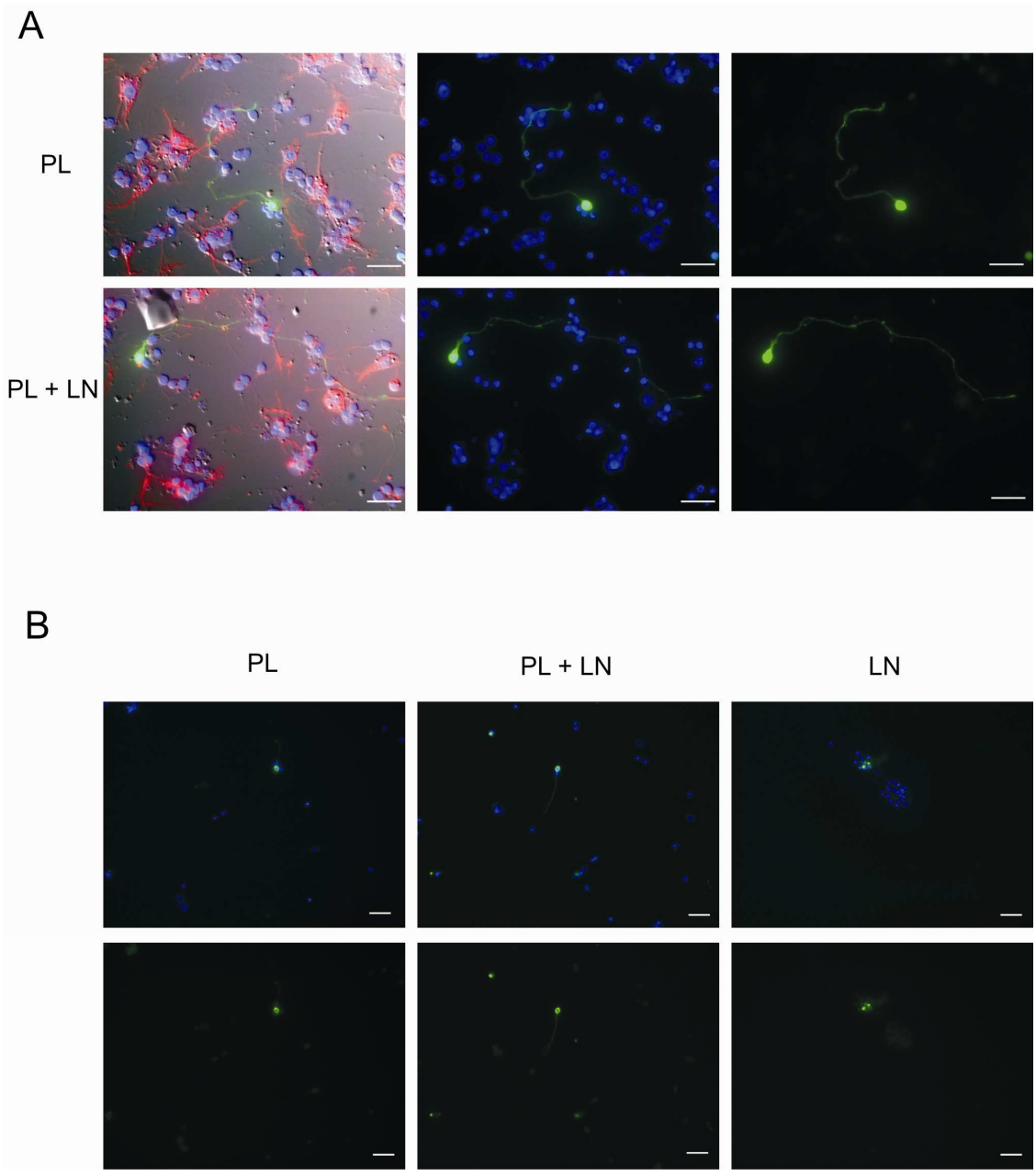



Figure III.5. Cell culture of *C. elegans* embryonic cells plated on peanut lectin (PL), peanut lectin and laminin (PL+LN) or laminin alone (LN). Cells were stained with TRITC-phalloidin (red) and Hoechst (blue). GFP positive cells express *Pten-1a::GFP* (A) or *DGN-1::GFP* (B). Scale bar is 10 μm.

### III.3.6. Does teneurin-2 bind laminin?

Due to the difficulties with the *C. elegans* cell culture, we decided to test the interaction between teneurin and laminin in vertebrates since we think that such an interaction might be conserved. However, in vertebrates this analysis has also disadvantages because four teneurin paralogs and even more laminin isoforms exist. In our experiment, we tested for a possible interaction between chicken teneurin-2 and mouse EHS laminin 111.

For the cell adhesion assay, we used HT1080 fibrosarcoma cells stably transfected with a truncated teneurin-2 TE construct (transmembrane domain and EGF-like repeats) or TEY construct encoding the entire extracellular part (Rubin et al., 2002). As a substrate, EHS laminin 111 and fibronectin were used. We found that the stable cell lines expressing TE or TEY of teneurin-2 adhered equally well to laminin and fibronectin as HT1080 cells did (Fig. III.6).

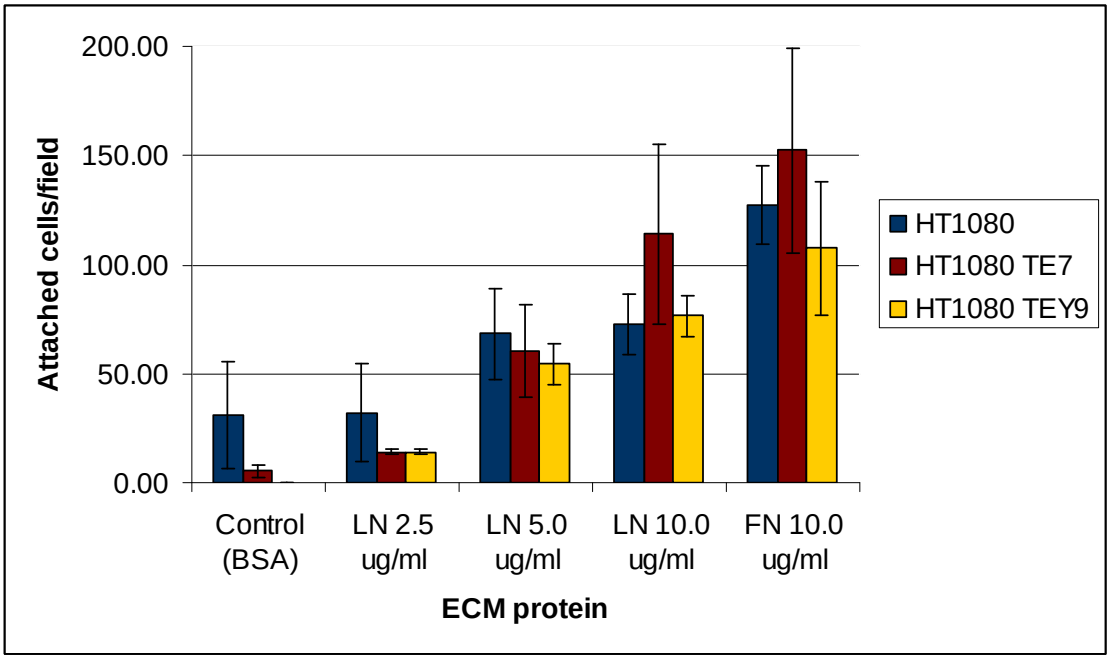



Figure III.6. HT1080 cells expressing TE or TEY of teneurin-2 do not preferentially attach to EHS laminin. Cells were plated for 30 min on the plates with various laminin concentrations.

However, TEY cells seem to have a distinct morphology when plated on laminin (already at the concentration of 2.5 ng/µl) as they spread much more then TE or HT1080 cells (Fig. III.7). These cells appeared slightly bigger and flattened also on fibronectin and even on uncoated cell culture plastic. Therefore, we cannot exclude that this is a clone specific property. It is, however, interesting to note that teneurins may affect the morphology of cells adhering to laminin.

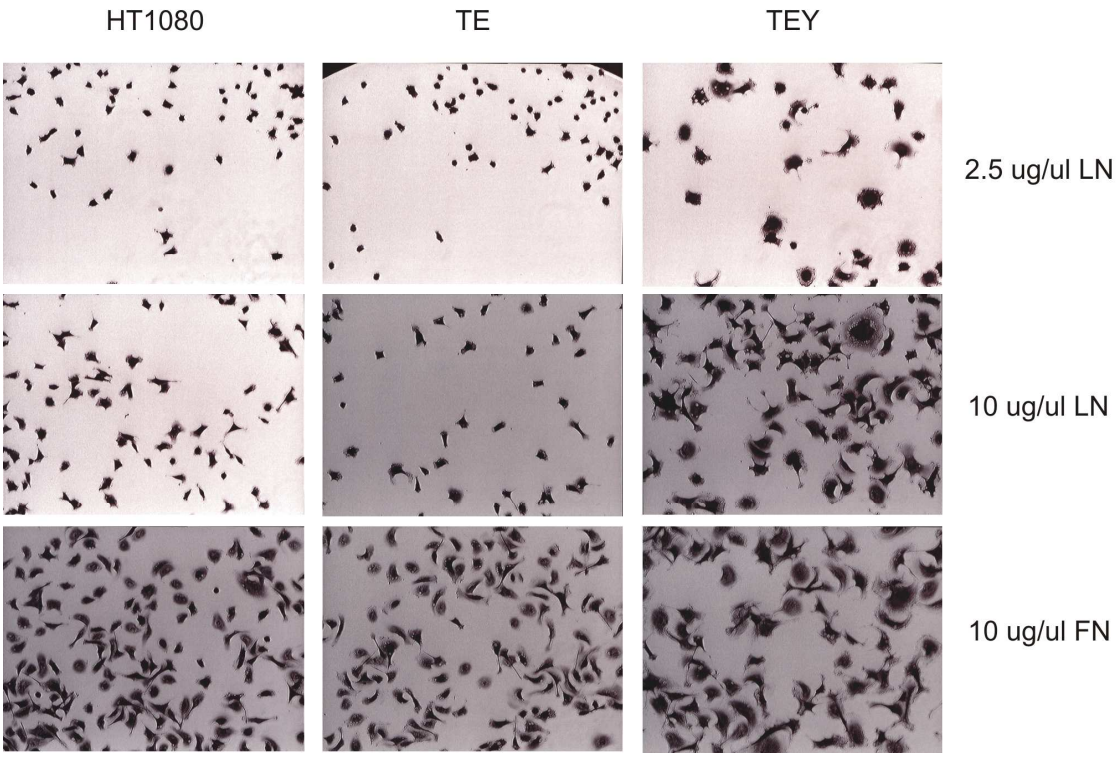



Figure III.7. Morphology of HT1080 fibrosarcoma, TE and TEY cells adhering to EHS laminin and fibronectin. TEY cells spread more on laminin than TE or HT1080 cells, however they are also slightly bigger on fibronectin.

# IV. Discussion

## IV.1. Our aspiration

Teneurins are conserved transmembrane proteins implicated in regulation of cell adhesion and cell signaling (Tucker and Chiquet-Ehrismann, 2006; Tucker et al., 2007). They show very specific and distinct expression during development and are often found at sites of pattern formation or cell migration. Their important role during development was confirmed by genetic studies, mostly in invertebrates. Elucidating teneurin function in vertebrates is challenging due to existence of four paralogs and most likely numerous splice variants. Until now, only a teneurin-3 knockout mouse has been described with a mild defect in visual pathway patterning which may suggest functional redundancy with other teneurins. Several key questions concerning teneurin function still remain open such as the identification of extracellular ligand(s), the role of proteolytic processing or the significance of intracellular domain translocation to the nucleus.

We took advantage of the *C. elegans* model system since in worms a single teneurin gene with two isoforms exists. Thanks to the simplicity of genetics, we could identify *ten-1* partners acting in parallel and partly redundant pathways. We showed for the first time that teneurin is important for basement membrane function and integrity in specific contexts. Therefore, we aimed to elucidate the role of TEN-1 in BMs by analyzing the basal lamina ultrastructure, TEN-1 localization, and adhesion properties.

Our finding of a functional connection between worm teneurin, and BM molecules and receptors, was completely unexpected. Until now, we had almost no evidence from vertebrate or invertebrate studies that teneurin could be a link to extracellular matrix. Our genetic study points a new direction for further investigations on the role of teneurin, particularly in the search of ligands. It will be interesting to study whether the main function of teneurins is BM assembly, adhesion, migration or signal transduction.

## IV.2. *ten-1* acts redundantly with genes encoding basement membrane proteins and receptors

We characterized the gonadal phenotype of *ten-1(ok641)* worms and realized that similar disorganization of gonads appears in integrin *ina-1*, dystroglycan *dgn-1* and laminin *epi-1* mutants. These four mutants share also several other phenotypic features, such as protruding vulva, impaired neuronal guidance and DTCs migration (Baum and Garriga, 1997; Drabikowski et al., 2005; Huang et al., 2003; Johnson et al., 2006; Meighan and Schwarzbauer, 2007).

We tested the genetic interaction between *ten-1* and genes encoding BM components and receptors, and found that 90-100% of *ten-1*;*ina-1*, *ten-1*;*dgn-1*, *ten-1*;*epi-1* and *ten-1*;*nid-1* double mutants arrested during embryogenesis or L1 larval stage. Arrested double mutants showed various phenotypes, i.e. a range of pharyngeal and hypodermal defects. Such synergistic genetic interactions seemed to be specific for laminin and laminin-binding receptors as well as nidogen. In contrast, synthetic lethality did not occur when *ten-1(ok641)* was combined with mutations in *unc-52*, the major heparan sulphate proteoglycan of BMs, or *cle-1*, which encodes collagen XVIII acting together with nidogen at synapses.

We conclude that TEN-1, INA-1 and DGN-1 are not required for BM function in general, but play an important role in particular organs, such as the gonad, hypoderm or pharynx. It is also possible that lack of broader defects in BMs results from functional redundancy and overlapping roles between these three receptors.

## IV.3. TEN-1 is essential for basement membrane maintenance

Studies with the basement membrane marker *LAM-1::GFP* revealed that *ten-1* mutant worms show a specific deficiency in the BM, always appearing on the dorsal side of the developing gonad. High resolution analysis by electron microscopy confirmed that L3 gonads of *ten-1* mutants lacked a BM only locally, on their dorsal side and the distal arms showed normal BMs. BMs surrounding other tissues like muscle, epidermis or gut, showed generally a normal ultrastructure and no BM fragments were found in the body cavity. It is of course possible that a more general function of TEN-1 in BMs is compensated for by the presence of other receptors, i.e. integrin or dystroglycan. Analysis

of the BM ultrastructure in the relatively intact L2 gonads could provide some insight into SGP localization which could be connected with the cause of gonad bursting. Such a study is, however, technically difficult to perform.

We know from the localization studies of *LAM-1::GFP* that also the pharyngeal BM appeared to be disorganized in *ten-1(ok641)* worms causing L1 larval arrest. However, the BM ultrastructure in the head of *ten-1* mutants has not been investigated so far and the cause of defects remains unclear.

### IV.4. Localization of TEN-1

One of our questions was the cellular localization of TEN-1. Teneurin seems to be a novel BM receptor and it is expected that BM receptors are polarized to the basal side of cells. Due to a lack of antibodies, we generated a tagged TEN-1L full length construct but unfortunately its expression level was very low. This may be due to a lack of important regulatory sequences located more distally from the putative promoter or in the omitted introns. GFP::TEN-1 seemed to show cytoplasmic and membrane localization but we could not observe any polarization to the basal side of cells.

At the moment, antibodies against the intracellular and extracellular domains of TEN-1 are being raised in our lab and they will hopefully let us determine the localization of endogenous teneurin in worms. As an alternative, we could create a reporter fusion by insertion of the reporter into a fosmid clone using bacterial recombineering techniques (Dolphin and Hope, 2006). Fosmids contain genomic loci together with all flanking regulatory elements and their chromatin structure is supposed to be closer to physiological situation, and this may support more appropriate expression.

### IV.5. Role of TEN-1 domains and isoforms

Teneurins are multidomain transmembrane proteins and proteolytic processing may play a role in their signaling (Bagutti et al., 2003; Drabikowski et al., 2005). We think it is important to identify the domains that are required for TEN-1 function in BMs. Analysis of rescuing activity in the *ten-1* mutants using constructs lacking certain domains of

TEN-1 could provide important information about teneurin domains that are indispensable in worm development.

We also aimed to identify genes that are required for cleavage and translocation of the intracellular domain to the nucleus by monitoring changes in the localization of GFP::TEN-1L. We were, however, not able to detect any GFP in the nucleus of neuronal, pharyngeal or somatic gonad cells. This may result from fast degradation of the cleaved intracellular domain, or low expression levels of the GFP reporter construct in general. The importance of a possible nuclear translocation of the intracellular domain could be proven by testing the rescuing activity of a TEN-1 construct lacking the N-terminal domain.

TEN-1 exists in two isoforms but their distinct functions have not been extensively investigated. It is known from RNAi experiments that the long isoform plays a crucial role in gonad development, while knockdown of both isoforms results in embryonic lethality and severe hypodermal defects (Drabikowski et al., 2005). The *ok641* and *tm651* mutations affect both TEN-1 isoforms. To clarify the isoform specific function, we have ordered a mutant specific for the long protein variant, which will allow us to identify the defects specific for this isoform. Genetic analysis using such a mutant could answer the question, whether one or both *ten-1* isoforms are required for BM function and whether both act redundantly with dystroglycan and integrin receptors.

### IV.6. TEN-1 function in somatic gonad cells

In my project, we concentrated on the analysis of the role of TEN-1 in gonad development and BM maintenance. Expression of the transcriptional reporter under control of the *ten-1* upstream promoter revealed that the long TEN-1 isoform is found in early SGPs until L3 stage, when it becomes restricted to the AC. Expression of TEN-1 in the early somatic cells, which localize mostly to the ventral side of the gonad, do not answer the question why the break appears on the dorsal side exclusively. SGPs may play a role in BM assembly by expression of specific receptors or they may produce ECM components themselves. They are also known to wrap around the germ cells at the early stages of gonad development and they may require laminin in the gonadal BM for correct

adhesion and positioning. We found that Z1 and Z4 cells were mislocalized in L1 larvae of *ten-1* mutants and this could result in epithelialization defects, local BM deficiency and subsequent leakage of germ cells from the gonad. Analogous defects in the position of somatic precursor cells were reported for *dgn-1* and *epi-1* mutants which are also sterile due to gonad disorganization (Johnson et al., 2006). An essential function of TEN-1 in SGPs could be confirmed by expression of TEN-1L under the control of the *lag-2* promoter specifically expressed in Z1, Z4 cells and their descendants.

Several lines of evidence indicate that teneurins could be proteolytically processed in the region between the EGF-like repeats and the transmembrane domain (Baumgartner et al., 1994; Rubin et al., 1999; Tucker et al., 2001). Therefore, it is possible that TEN-1 acts cell nonautonomously and the cleaved extracellular domain may be incorporated into the gonadal BM, where it could have a structural or signaling function. Significance of proteolytic cleavage at the furin site could be addressed by expression of a secreted TEN-1 extracellular domain under a gut or body wall muscle promoter and the analysis of rescuing activity of such constructs. A reporter tag added to the extracellular domain of TEN-1 would allow to examine its incorporation into gonadal BM.

Besides the expression in SGPs at the L2 stage, TEN-1L is found in the AC during invasion. However, the local BM deficiency in broken gonads of *ten-1(ok641)* worms did not seem to result from mislocalization of the AC and impaired invasion through the BMs. Mutants in the *ten-1* gene show vulva development defects and bursting that could suggest AC invasion defects. Interestingly, it was shown that downregulation of *ina-1* or *pat-3* levels by RNAi results in AC invasion defects. Moreover, polarization of markers (e.g. MIG-2, F-actin or phosphatidylinositol (4,5)$P_2$) is lost in the invading AC, when dominant negative PAT-3 HA-βtail is expressed (Paul Sternberg, unpublished results). Since the AC is a well described system to study cell invasion *in vivo*, it will be interesting to test whether teneurin plays a similar function during AC invasion as integrins.

## IV.7. *ten-1* acts redundantly with *dgn-1*, *nid-1* and *ina-1* in pharyngeal and hypodermal morphogenesis

We found that *ten-1(ok641)* caused synthetic lethality in a *dgn-1* and *nid-1* mutant background and double mutants arrested as L1 starved larvae suggesting a feeding defect. The analysis of pharynx morphology in *ten-1* single mutants showed that their pharynges were misshapen and the BM was partially absent, however the defects were not as clearly localized as in the case of the gonad. Pharyngeal malformations were enhanced when *ten-1(ok641)* was combined with mutation in the dystroglycan *dgn-1* gene. Functionality of pharynges in arrested larvae could be tested by measuring pharynx pumping-rate or efficiency of feeding with fluorescent bacteria. So far, we have not performed such assays and the role of TEN-1 and DGN-1 in pharyngeal BM maintenance and/or pharynx development remains to be investigated.

In the case of *ten-1*;*nid-1* worms, it was apparent that their pharynges were not functional as double mutants showed a Pun phenotype. Unattached pharynges may result from a failure of the anterior pharyngeal cells to initially attach to the arcade cells, or from weak attachment that is broken as a result of pharyngeal muscle contractions. It is also possible that teneurin and nidogen are required for correct alignment of pharyngeal and epidermal cells at the beginning of the pharyngeal extension process. 4D Nomarski recordings of developing *ten-1*;*nid-1* embryos could give us more insight into pharyngeal morphogenesis and help in elucidating TEN-1 function in this process.

Another interesting finding of our study was the genetic interaction between *ten-1* and *ina-1* resulting in embryonic and larval lethality of *ten-1*;*ina-1* worms due to hypodermal defects. Such a phenotype may result from disturbances in neuroblast migration, ventral enclosure or elongation during epidermal morphogensis. To answer the question about the role of teneurin and integrin in hypodermal development, further studies including 4D recordings of worm embryogenesis are required. This could be followed by rescue experiments in *ten-1*;*ina-1* worms by expression of *ten-1* under neuronal, hypodermal or muscle specific promoters as interactions between these tissues are crucial for *C. elegans* morphogenesis. It is likely that interaction with BMs is required for the process of epidermal morphogenesis (e.g. muscle formation and then elongation). Laminins are detected between tissue layers at the completion of gastrulation but BMs assemble only at

the end of epidermal enclosure, at the lima to comma stage (Huang et al., 2003; Johnson et al., 2006). TEN-1 and INA-1 could be essential for BM formation or later during elongation, when BMs are already present.

### IV.8. EPI-1 is a potential basement membrane ligand for TEN-1

We found that *ten-1* mutations severely enhanced the defects caused by knockdown of *epi-1* by RNAi and led to hypodermal disorganization and larval arrest. This suggests that laminin EPI-1 could be a BM ligand for teneurin. Direct interaction between TEN-1 and EPI-1 needs further confirmation *in vitro*. Expression and purification of TEN-1 or its fragments would allow testing the interaction with laminin in worm lysates, as an antibody against EPI-1 exists. Moreover, by such biochemical approaches novel interacting proteins could be identified by mass spectrometry.

We attempted to prove the interaction between teneurin and vertebrate laminin in *C. elegans* primary cell culture. However, cell adhesion assays are also difficult to perform in worms due to a lack of cell lines and appropriate culture conditions. It is possible that vertebrate laminins are too divergent from *C. elegans* orthologs and they may not contain crucial sequences recognized by worm receptors. For instance, it was shown that leech neurons can only sprout on leech laminin but not on mouse protein (Chiquet et al., 1988). Another possibility is that we did not observe any preference for adhesion to laminin of cells expressing TEN-1 or DGN-1 because of functional redundancy and presence of other laminin receptors, e.g. integrins.

A simple alternative for *C. elegans* cell culture could be the *Drosophila* system. Schneider S2 cells express the fly teneurin orthologs *ten-m* and grow primarily in suspension (Baumgartner et al., 1994). Therefore, they could be used to test the interaction of Ten-m with laminin as a substrate. *Drosophila* laminin was shown to be secreted from Kc cells, and S2 cells transfected with laminin-binding PS1 integrin spread, when cultured in conditioned medium from Kc cells (Gotwals et al., 1994). Interaction between laminin and *ten-m* in *Drosophila* would also prove that teneurin binding to laminin is evolutionary conserved.

We demonstrated that fibrosarcoma cells overexpressing the extracellular domain of teneurin-2 did not preferentially adhere to laminin 111 as analyzed by cell adhesion assays. It is possible that these two particular isoforms of teneurin and laminin simply do not interact. On the other hand, teneurins may influence rather the cell morphology and spreading than attachment. For instance, it was shown that teneurin-2 induces formation of filopodia and enlarged growth cones in neuroblastoma cells and these effects are substrate-dependent (Rubin et al., 1999).

### IV.9. TEN-1 signaling

Both, integrins and dystroglycan, are important links between extracellular matrix and the cytoskeleton. They were shown to play an important role in actin remodeling and interact with several proteins (e.g. talin, ILK, dystrophin) that anchor them to the cytoskeleton (D'Abaco and Kaye, 2007; Delon and Brown, 2007; Henry and Campbell, 1999; Higginson and Winder, 2005). Induction of filopodia formation in neuroblastoma cells by teneurin-2 suggests that teneurins are also required for cytoskeleton reorganization. Interestingly, one of the cytoskeleton adaptor proteins, CAP/ponsin, was found to interact *in vitro* with the intracellular domain of teneurin-1. In *C. elegans*, a single CAP ortholog exist (Y45F10D.13), however its expression patterns and mutant phenotype remain unknown. An interaction between teneurin and Y45F10D.13 could be studied by a genetic or biochemical approach. It is, however, possible that this interaction is not conserved since intracellular domains of vertebrate and invertebrate teneurins are relatively divergent.

The success of our genetic studies encouraged us to initiate a high-throughput RNAi screen for suppressors and genes causing synthetic lethality in the *ten-1* mutant background. By this approach, we hope to find more genes acting in parallel to *ten-1* as well as components of the teneurin signaling pathway.

# V. Appendix

## V.1. Experimental procedures

### Semi-quantitative RT-PCR

Mixed stage worms (N2, *ok641* and *tm651*) were washed in M9 buffer, frozen and ground in liquid nitrogen. RNA was extracted with Trizol (Invitrogen) according to manufacturer's protocol and cleaned up on Oligotex mRNA columns (QIAGEN). 500 ng of mRNA was reverse-transcribed by SuperScript$^{TM}$ II Reverse Transcriptase kit (Invitrogen) using random hexamers. Amplification of cDNA was performed using *ten-1*_RTPCR_F5, *ten-1*_RTPCR_R5, *ama-1*_sense and *ama-1*_antisense primers. The following PCR program was used: 95°C for 1 min, followed by 25-29 cycles of 60°C for 30 s, 72°C for 30 s, and 95°C for 20 s, and finished with 72°C for 7 min. Products of PCR reactions were visualized on the agarose gel.

### Two-step fixation for electron microscopy

Worms were washed in M9 and anaesthetized in 8% ethanol in M9 for 5 min. They were placed in a fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.1M sucrose, 10 mM PBS, pH 7.4), cut open with a needle on both anterior and posterior end and fixed for 2 hours. Worms were embedded in 2% agarose, cut into small blocks and washed three times in PBS. Subsequently, worm pieces were fixed with a second solution (1% osmium tetroxide, 1.5% potassium ferrocyanide in PBS) for 2 hours and washed three times in water. Worms were stained with 1% uranyl acetate for 1 hour. Samples were dehydrated in ethanols (10 min in 50% ethanol, 10 min in 70% ethanol, 10 min in 90% ethanol and 10 min in 100% ethanol) and acetone (10 min). Blocks with worms were embedded in Epon resin (Fluka): first in Epon-acetone (1:1) for 1-2 hours and then in pure resin for 2-4 hours. Samples polymerized for 24-48 hours at 60°C and 60 nm sections were prepared with Ultracut E. Sections were stained in uranyl acetate for 60 min and then 2 min in Millonig's lead acetate stain. Pictures were taken on Philipps Morgagni 80 KV miscroscope.

### *C. elegans* cell culture

Synchronized adult worms from one egg plate were bleached. After 5 min lysis solution was diluted two times with the sterile egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 25 mM HEPES, pH = 7.3). Isolated eggs were washed three times with the egg buffer and separated from adult carcasses and debris by filtration through 70 μm filter. Eggs were pelleted at 2000 rpm for 3 min at 4°C. Eggshells were removed by chitinase treatment: 5U/ml of chitinase from *Streptomyces griseus* (Sigma) was added to the eggs and digestion continued for 20-40 min with gentle rocking. When approximately 80% of eggshells were removed, embryos were pelleted and resuspended in 1 ml of L-15 medium (GIBCO® Invitrogen) containing 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Subsequently, embryos were dissociated by gentle pipetting with a drawn out Pasteur pipette. Cell suspension was filtered through a 5 μm filter to remove whole embryos and L1 larvae. An additional 1 ml of cell culture medium was flushed through the filter to remove adherent cells. Cells were pelleted and resuspended in 2 ml of culture medium.

Cells were cultured in 4-well chamber coverglasses (LAB-TEK® Nunc International) that were coated with peanut lectin (0.5 mg/ml in water) and/or laminin (5 μg/ml in PBS with 0.01% Tween) for 10-20 min. Peanut lectin was allowed to dry and laminin-coated wells were washed once with PBS. 0.5 ml of cell suspension was added to each well and cells were maintained at room temperature in a humidity chamber. Culture medium was exchanged after 24 hours and non-adherent cells were removed.

After 3 days in culture, cells were fixed with 750 μl of 4% paraformaldehyde in PBS for 30 min. Then, slides were washed two times with PBS and cells were permeablized in 0.1% Triton-X in PBS for 5 min. After washing in PBS (2x), cells were stained with TRITC-phalloidin (1:500) and Hoechst (1:1000) for 20 min. Cells were washed three times in PBS and mounted in Moviol (Dabco) mounting medium.

### Cell adhesion assay

60-well microtiter plates (Nunc International) were coated for 1 hour at RT with 5 μl of the appropriate ECM protein in PBS containing 0.01% Tween. The coating liquid was

removed and wells were blocked for 1 hour with 10 µl BSA (10 mg/ml in PBS). Wells were washed once with PBS.

Cells were detached from the plates using trypsin/EDTA and resuspended in serum free DMEM medium at the concentration of $1.5x10^5$ cells/ml. 10 µl of cell suspension was plated per well. Cells were incubated at 37°C for 30 min, then washed once in PBS and fixed for 30 min in 10 µl of 4% paraformaldehyde in PBS. After washing in water, cells were stained in 0.1% crystal violet solution for 30 min. Plates were washed extensively with water and air dried. Pictures of the entire well were taken and cells were counted in triplicates.

**List of primers used for genotyping and semi-quantitative RT-PCR**

| Primer name | Sequence (5'-3') |
|---|---|
| VC518_1 | TGACACTGACGGAAGATGCCG |
| VC518_WT | TCAGTTGACCATGAGCTGAGC |
| VC518_3 | CAAACAGTTCCGTCTCCAGCC |
| *tm651*_5'_1 | GCTGAAATACCCACTCGCAGC |
| *tm651*_WT | GCACTCATTAGAAGAACCAGC |
| *tm651*_3'_2 | AGTGTCACATCGTCCCCTTCC |
| New DGN-1A | CTAGTGACAACCGCCATTTCCG |
| New DGN-1B | GAATTCCAAAGGCTCAGAGAGC |
| New DGN-1C | CCATCCAGAAAGTGTTGTTGGC |
| *ina-1(gm144)* seq | TTGCCACTTCGATTTCATCGATGC |
| *ina-1(gm144)* REV | ACGGGATAGGTCGAGAGTCTCC |
| *unc-52(e444)* seq | AAGCACCTTGAACGTCACACCTGG |
| *unc-52(e444)* REV | TCTGACTGGATCCGCGACTCC |
| *nid-1(cg119)*_5' | ATTCCGACCAGCCGTCCTCCC |
| *nid-1(cg119)*_WT | TCTGGAAGCTTCGAGGGAGTCATCAACAG |
| *nid-1(cg119)*_3' | AGCTCTTGCTAAACCCCTCACCTCG |
| cle1 | GGGAGCACCAGCACCACC |
| cle2 | CTCACCCAAAACTCAATGC |
| cle4 | GGCTCTTCTCCATCCATCAC |
| *ten-1*_RTPCR_F5 | ATTGTTGGGGAAGTGGAG |
| *ten-1*_RTPCR_R5 | CACCATTGTTCATAGTGCC |
| *ama-1*_sense | CAGTGGCTCATGTCGAGT |
| *ama-1*_antisense | CGACCTTCTTTCCATCAT |

## V.2. List of abbreviations

AC – anchor cell

BM – basement membrane

CAP – Cbl-associated protein

DMEM – Dulbecco's Modified Eagle's Medium

DIC – differential interference contrast

DTC – distal tip cell

ECM – extracellular matrix

EGF – epidermal growth factor

FN – fibronectin

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

L1 – first larval stage

L2 – second larval stage

L3 – third larval stage

L4 – fourth larval stage

LAR – leukocyte antigen-related

LN – laminin

MBD – methyl CpG binding

Pat – paralyzed at two-fold

PBS – phosphate-buffered saline

PML – promyelocytic leukemia protein

PL – peanut lectin

Pun – pharynx unattached

SGP – somatic gonad precursor

SPh – somatic gonad primordium of hermaphrodite

TCAP – teneurin C-terminal associated peptide

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