Original Article

Prorenin Receptor Homologue VHA-20 is Critical for Intestinal pH Regulation, Ion and Water Management and Larval Development in *C. elegans*

(prorenin receptor / gut / gene expression / *Caenorhabditis elegans* / pH / development)

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Abstract. The prorenin receptor (ATP6AP2) is a multifunctional transmembrane protein; it is a constituent of proton-translocating V-ATPase, a non-proteolytic activator of renin and an adaptor in the Wnt/β-catenin pathway. Here, we studied *vha-20*, one of the two prorenin receptor homologues that are identified by sequence similarity in the *C. elegans* genome. We show that *vha-20* (R03E1.2) is prominently expressed in the intestine, in the excretory cell and in amphid neurons, tissues critical for regulation of ion and water management. The expression of *vha-20* in the intestine is dependent on NHR-31, a nuclear receptor related to HNF4. VHA-20 is indispensable for normal larval development, acidification of the intestine, and is required for nutrient uptake. Inhibition of *vha-20* by RNAi leads to complex deterioration of water and pH gradients at the level of the whole organism including distention of pseudocoelome cavity. This suggests new roles of prorenin receptor in the regulation of body ion and water management and in acidification of intestinal lumen in nematodes.

Introduction

Prorenin receptor (PRR), a protein member of the renin-angiotensin aldosterone system (RAAS), was first cloned and characterized as a molecule that binds and non-proteolytically activates prorenin more than a decade ago (Nguyen et al., 2002). RAAS activation promotes constriction of arterioles and the release of aldosterone, making RAAS one of the key mechanisms for regulation of blood pressure and electrolyte balance in the human body. RAAS is regulated at several steps. Renin plays a critical role in RAAS by catalysing the rate-limiting step of the entire cascade (Skeggs et al., 1957; Fisher and Hollenberg, 2001). Renin and its receptor are thus promising therapeutic targets (Atlas, 2007).

In the human body, PRR is expressed in all cell types. The highest levels of PRR mRNA are found in the heart, brain, placenta, and relatively lower levels in the kidney and liver (Nguyen et al., 2002). The physiological importance of prorenin receptor was confirmed by its overexpression in transgenic rats causing increase in blood pressure, heart rate, glomerulosclerosis index and urinary protein excretion independently of renin function (Burckle et al., 2006; Kaneshiro et al., 2007). While most components of RAAS evolved in animal species higher than cartilaginous fish, prorenin receptor and an-

giotensin I converting enzyme 1 and 2 homologues were found in *Ecdysozoa*, *Drosophila* and *C. elegans* (Fournier et al., 2012).

The PRR importance goes far beyond the cardiovascular field. PRR plays a significant role in development by participating in Wnt signalling and it is also a cofactor of the multi-subunit proton pump, vacuolar H⁺-ATPase (V-ATPase) (Ludwig et al., 1998; Buechling et al., 2010; Cruciat et al., 2010). V-ATPase is important for the maintenance of cellular and vesicular pH, processing of proteins, membrane sorting, activation of lysosomal enzymes and endocytosis (Nishi and Forgac, 2002). This includes endocytosis of the Wnt/Frizzled complex that was shown to be important for transmission of the extracellular Wnt signal into the cytoplasm (Blitzer and Nusse, 2006).

In this work, we functionally studied *vha-20*, one of the two genes that are identified by sequence similarity as the potential homologues of vertebrate prorenin receptors in *C. elegans* (Nguyen and Muller, 2010), WormBase (http://www.wormbase.org) and this work. We show that *vha-20* is expressed in all *C. elegans* developmental stages and is indispensable for *C. elegans* larval development. Surprisingly, *vha-20* is not expressed ubiquitously as is the case of the mammalian prorenin receptor, but predominantly on or close to the apical membrane of enterocytes, in four amphid neurons and on the plasma membrane of the excretory cell, three tissues critical for the maintenance of water/ion balance and body volume at the level of the whole organism. In keeping with its localization, *vha-20* knock-down results in defects of intestine function and in disturbed systemic water metabolism. This suggests that the prorenin receptor plays a role in the maintenance of systemic water/ion homeostasis in nematodes.

Material and Methods

C. elegans strains and maintenance

The *Caenorhabditis elegans* Bristol N2 strain was used in this work and was maintained as described by Sydney Brenner (Brenner, 1974). *E. coli* strain OP 50 was used to feed the nematodes.

Preparation of vha-20::gfp reporters using intrinsic promoters

GFP-reporter constructs 7270, 7271 and 7272 were prepared by PCR amplification of the *vha-20* promoter and coding sequence (without the terminal codon) by three different 5' primers 7112, 7113, 7114 and one universal 3' primer 7115 (Fig. 1). The DNA fragments were cloned into vector pPD95.75 (a gift from Dr. A. Fire, Stanford University, Stanford, CA) using restriction sites for *Sac*I and *Bam*HI. Obtained constructs were co-injected into distal gonads of young adult *C. elegans* hermaphrodites with marker plasmid pRF4. Primers are listed in Table 1.
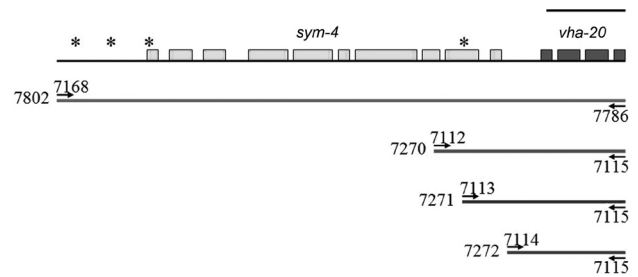

Fig. 1. Genomic regions used as promoter sequences for translation-fusion VHA-20::GFP expression. The arrows represent primers used for PCR amplification of a given gDNA fragment.
* – Symbols represent the sequence AGTTCA, a putative binding site for the regulator of *vha-20* transcription, NHR-31 (Hahn-Windgassen and Van Gilst, 2009). Scale bar 1 kb.

Preparation of vha-20::gfp reporters using operon promoter

To further investigate the *vha-20* expression pattern *in vivo* we used GFP-reporter construct 7802 created by the fusion PCR-based approach (Hobert, 2002). Primers 7168 and 7786 were used for PCR amplification of the 7.6 kb long genomic sequence containing the *vha-20* coding sequence (without the terminal codon), upstream gene *sym-4* and additional 1091 bp 5' sequence from the *sym-4* promoter (Fig. 1). The sequence encoding GFP was amplified from pPD95.75 vector with primers 6232 and 6233. The two sequences were fused together in an additional PCR step using primers 7169 and 6234. The fusion construct was co-injected into the gonad of young adult *C. elegans* hermaphrodites with marker plasmid pRF4.

Total RNA isolation and reverse transcription

Fifty µl of cultured nematodes were frozen in -80 °C and dissolved in 300 µl of resuspension buffer (10 mM Tris-HCl; 10 mM EDTA, 5% 2- mercaptoethanol; 0.5% SDS; pH 7.5). After adding 8 µl of proteinase K the sample was mixed vigorously and incubated 1 h at 55 °C, with following phenol-chloroform extraction and ethanol precipitation. DNase I treatment was carried out with the use of RNeasy Spin column system (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was prepared with the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Kyoto, Japan) using random hexamers.

Quantitative PCR and droplet digital PCR

cDNA obtained as described above was used as a template for quantitative PCR (qPCR) and droplet digital PCR (ddPCR). qPCR was performed in the Light Cycler 2.1 from Roche (F. Hoffmann-La Roche, Basel, Switzerland) and evaluated using LightCycler® Software 4.1. Droplet digital PCR was performed in the QX200™ Droplet Digital™ PCR System (Bio-Rad, Hercules, CA) according to the manufacturer's protocol using ddPCR™ Supermix for Probes. Evaluation was done using

Table 1. Primers used in the study

No.	Sequence
4778	GCT CTA GAG ATT GGT CAC GAA AAC TTC CTG TC
4779	CGG GGT ACC TGA CTG AAG AAC TCT TCA CAA CC
6632	AGC TTG CAT GCC TGC AGG TCG ACT
6633	AAG GGC CCG TAC GGC CGA CTA GTA GG
6634	GGA AAC AGT TAT GTT TGG TAT ATT GGG
7109	ATG AAG TTT GTT CTT CTC GTC TCC
7111	TCT CAA CAC CGT CGG ATT TGG TTG
7112	CAT GCA TGC ATA TCA CAC TGC TGT GGA TGT ACG
7113	CAT GCA TGC TCG CGC CAA GAC CTC AAG TGT TCT TC
7114	CAT GCA TGC TTT TCG ATC CTC CAC TCT GCT TAC C
7115	GCG GAT CCT CT TCA TTC TTG TGG TGG TCA TAC
7168	CAT GAT GAG ACG TTA TGG ATT ACT G
7169	GAT GAG ACG TTA TGG ATT ACT GTT TTC
7190	AAG AAG GCT TGT AAG AGA GGA ATG
7191	GAT TTC GAA TTT CTT GAC TTT TCC
7196	CAA ACG GCC GTC TTC TCA
7197	GGT GTG GTT GCC TTG TAT GA
7497	CAA TGC TGA TGT TTT CCG TGT
7498	AAC GTC TGC GAC AGC TTC TT
7721	AGT CGG TGT GCG AAG GAG
7722	GAT AGC GAC CGC ATC CAC
7786	AGT CGA CCT GCA GGC ATG CAA GCT GTC TTT CTT CAT TCT TGT GGT GGT CAT

QuantaSoft 1.3.2.0 software. The values were normalized to *ama-1* in both methods and expressed as arbitrary units. Primers and probes used are listed in Table 2**.**

RNA interference

Inhibition of the VHA-20 function was achieved by RNA-mediated interference (RNAi) performed by microinjecting the dsRNA produced *in vitro* from the *vha-20* sequence into the ovarial syncytium of young adult *C. elegans* hermaphrodites. The region that spans exons 1 and 2 was cloned into pCRII-TOPO cloning vector (Invitrogen, Waltham, MA) using primers 7109 and 7111. dsRNA was prepared by using SP6 and T7 RNA polymerases (Fermentas, Thermo Fischer Scientific, Waltham, MA). dsRNA was purified by phenol/chloroform extraction and ethanol precipitation and injected into gonads of young adult *C. elegans* hermaphrodites at a concentration of 1 µg/ µl. The progeny of injected worms was kept at 16 °C and 24 °C and used for subsequent

Table 2. The list of primers and probes used for RT-qPCR and ddPCR

Gene	Primer 1	Primer 2	Probe
ama-1	7721	7722	133
vha-20	7497	7498	113
acs-2	7190	7191	136
fat-7	7196	7197	54

experiments. Control animals were injected with non-specific RNA as described (Simeckova et al., 2007).

Fluorescence microscopy

Fluorescence microscopy was performed using the Olympus BX60 microscope equipped with an UV source model U-ULH and DP30BW camera (Olympus, Tokyo, Japan).

Acridine orange staining

Acridine orange staining was performed as described (Oka and Futai, 2000; Ji et al., 2006) with modifications. Animals subjected to *vha-20* knockdown and control animals in L1 stage were collected in staining solution (0.05% acridine orange in phosphate buffer) (Sigma-Aldrich, St. Louis, MO) and incubated for 25 min at room temperature. After washing in phosphate buffer, animals were mounted, and Nomarski and fluorescence images were obtained. Loss of acidic compartmental pH was detected by *in vivo* staining with acridine orange done similarly as described by Chauhan et al. (2013) and recorded as a shift of excitation/emission properties characteristic of acidic pH detected by Olympus mirror unit U-MWG (maximum excitation 510-550 nm, maximum emission 590 nm) and U-N41014 (maximum excitation 450 nm, maximum emission 510 nm) for the excitation/emission characteristic of neutral pH. Fluorescence was recorded by monochromal camera DP30BW at the same setting for control and experimental sam-

ples. Composite images were prepared using the ImageJ program available at http://imagej.nih.gov/ij/ (Schneider et al., 2012) with red channel used for recordings of red fluorescence and green channel for green fluorescence. This setting allowed detection of acidic pH as an orange/red colour and neutral pH as a bright green colour in composite images.

Results

Bioinformatic analysis of C. elegans prorenin receptor

A Blast search in UniProt and NCBI databases using the protein sequence of human prorenin receptor (accession number O75787) as query identified putative homologues from various metazoan species with significant value of $E < 10^{-04}$ over 95–100 % of the sequence (Fig. 2). UniProt lists most of them as prorenin receptors according to the annotation in the protein family database Pfam. In *Caenorhabditis elegans*, the hit ($E = 2 \times 10^{-07}$) is coded by a gene with the cosmid name R03E1.2 and gene name *vha-20* and is localized on chromosome X. It has only one known form of mRNA listed in Wormbase (http://www.wormbase.org/species/c_elegans/gene/WBGene00010993; WS 243, accessed August 5, 2014 and January 7, 2015). Although the Pfam profile uses only the most conserved receptor segment for the sequence per annotation, the Blast results and the profile sequence alignment shows that the homology spans the entire sequence and includes all features identified in the human sequence (Fig. 2).

Reciprocal Blast using the *C. elegans* sequence R03E1.2 as query hits again the Pfam annotated prorenin receptors including the human one (UniProt:O75787; NCBI:NP_005756.2, $E = 2 \times 10^{-07}$). However, it identifies an additional putative prorenin receptor, protein T14B4.3 ($E = 7 \times 10^{-17}$) not recognized with the human sequence query. Both sequences are readily identified in the second iteration of PSI-BLAST with the human sequence as query ($E = 10^{-54}$ and $E = 7 \times 10^{-10}$ for R03E1.2 and T14B4.3, respectively, over the entire sequence). In agreement, this additional sequence is also annotated as renin receptor in UniProt and WormBase. All available nematode proteomes contain two distinct prorenin receptor paralogues. We did not observe this duplication in *Chordata* or *Drosophila*. It appears that the renin receptor diversified into two paralogues in nematodes. In the present paper, we concentrate on R03E1.2, *vha-20*. T14B4.3, which diverged from its metazoan homologues much further, is being studied separately. Contrary to R03E1.2, which is recognized by WormBase as well as by published searches for orthologues as prorenin receptor (Shaye and Greenwald, 2011), T14B4.3 was not detected as a prorenin receptor homologue but shows relation to R03E1.2.

For confirmation of the genomic organization of R03E1.2 listed in WormBase, we prepared cDNA from *C. elegans* mixed population cultures and searched for transcripts using PCR with primers derived from known
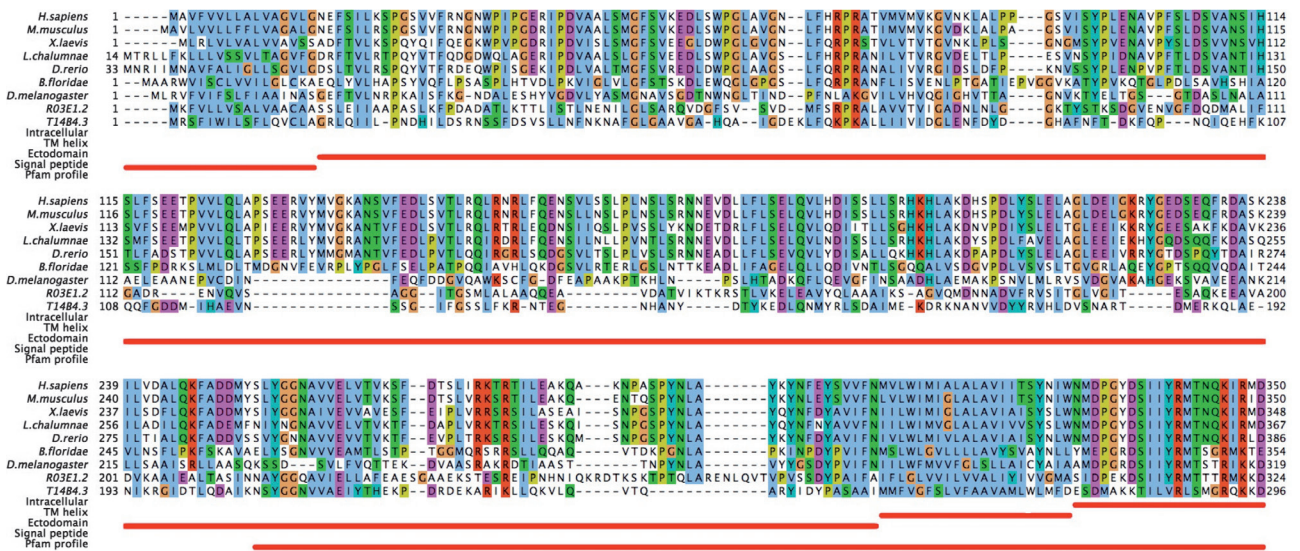

Fig. 2. Alignment of prorenin receptor amino acid sequences
Sequences selected from Blast and PSI-BLAST (Altschul et al., 1997) results with R03E1.2 (Q9XTY9) as query (species, UniProt identifier): *Homo sapiens* (O75787), *Mus musculus* (Q9CYN9), *Xenopus laevis* (Q7T0S3), *Latimeria chalumnae* (H3AI82), *Danio rerio* (F1QIH1), *Branchiostoma floridae* (C3YJH1), *Drosophila melanogaster* (Q9VHG4) and two *Caenorhabditis elegans* paralogues labelled by their cosmid names R03E1.2 (Q9XTY9) and T14B4.3 (Q22482).
Red bars indicate putative positions of the excretion signal peptide, extracellular ectodomain, transmembrane domain, intracellular cytoplasmic domain and the region identified by the *Renin_r* (PF07850) profile from the Pfam database (Punta et al., 2012).
Aligned with PROMALS (Pei et al., 2007), displayed with Jalview (Waterhouse et al., 2009), amino acid types coloured according to clustal scheme (jalview.org/help/html/colourSchemes/clustal.html).

splice leaders. We confirmed that *vha-20* is trans-spliced with the SL2 splice leader sequence and has a short 5'-UTR consisting of only three nucleotides (AGG) and the splice leader sequence (data not shown). This supports the *vha-20* genomic organization in an operon with the upstream gene, *sym-4*. The intergenic region is only 549 bp long. We did not detect splicing with SL1.

vha-20 expression profile during development

The *vha-20* expression pattern during *C. elegans* development was established by RT-qPCR from total RNA prepared from synchronized *C. elegans* populations in individual developmental stages (embryos, larval stages L1 to L4, and adults). This showed that the *vha-20* expression increases during larval development. The maximum is in the L2 stage, when the expression increases almost 5-fold compared to the expression in adult worms (Fig. 3). We also investigated whether starvation influences the *vha-20* expression but did not find any measurable effect (data not shown).

Transgenic VHA-20::GFP reveals a tissue-specific expression pattern and specific VHA-20 intracellular localization

In order to visualize VHA-20 tissue-specific expression and its intracellular localization, we created multiple independent transgenic *C. elegans* lines carrying the complete amino acid coding region of VHA-20 fused with the *gfp* gene with four different 5'genomic regions including predicted promoters (Fig. 1).

Since we detected the SL2 splice leader sequence in expressed *vha-20* in agreement with its predicted organization in an operon, we created transgenic lines 7802 (A, B) carrying a 1091 bp region of the predicted promoter of the first gene of the predicted operon, *sym-4*, its complete gene, and the entire genomic sequence of *vha-20* up to the stop codon, exchanged for GFP coding gene using the pPD95.75 vector. Transgenic lines ex-
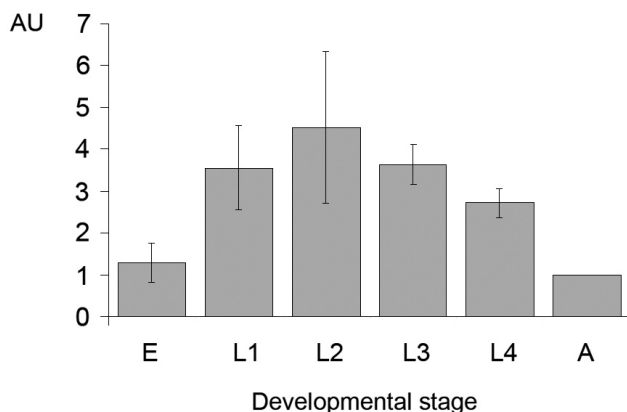
pressed VHA-20::GFP in the apical membrane of enterocytes, in the plasma membrane of the excretory cell, all arms of the excretory cell and weakly in two pairs of putative chemosensory amphid neurons localized posteriorly to the nerve ring (Fig. 4 A, C). Our conclusion that these cells are indeed amphid neurons is based on the localization of the cells and the fact that they send dendrites to the tip of the head, which is a typical feature of amphid neurons in *C. elegans*. The expression in the putative amphid neurons and in the enterocytes started during embryogenesis in the two-fold and three-fold embryonic stages, respectively, and lasted until adulthood. The expression of VHA-20::GFP in the excretory cell started in the L1 stage and lasted until adulthood.

Since Wormbase lists the SL1 leader sequence in *vha-20* transcripts, we tested whether *vha-20* is also expressed independently of the operon. We created three types of transgenic lines (7270, 7271, 7272) of the complete *vha-20* protein-coding sequence fused with the gene for GFP derived from pPD95.75 and with the sequence preceding the *vha-20* coding region made in three different lengths (1491 bp, 991 bp and 567 bp). All three lines had the same VHA-20::GFP expression pattern, revealing that *vha-20* is expressed also directly under the regulation of its own promoter independently of *sym-4* from all three putative promoters, and identified the short promoter as likely the minimal promoter of *vha-20*. The intrinsic *vha-20* promoter was sufficient to strongly promote the VHA-20::GFP expression in the apical membrane of enterocytes and in two pairs of amphid neurons, but failed to activate expression in the excretory system (Fig. 4 E, G). The VHA-20 expression in all three lines was first detected during embryogenesis in the two-fold stage in the four amphid neurons, and the expression in the apical membrane of intestine started when the embryo reached the three-fold stage. The expression of VHA-20::GFP in all of these cells remained detectable throughout the whole life cycle. In some transgenic animals, we observed weak expression of VHA-20::GFP in additional pairs of amphid neurons (Fig. 4 G).

To test which of these two expression patterns represents the functional expression of *vha-20*, we inhibited the *vha-20* expression by RNAi on both of the VHA-20::GFP backgrounds. We observed reduction of the GFP signal in both lines, suggesting that *vha-20* can utilize both promoters (not shown).

VHA-20 is essential for embryonic and larval development

The inhibition of *vha-20* by injecting dsRNA into N2 adults led to impaired development. Embryonic lethality was observed in 9 % of progeny at room temperature and was slightly higher at 16 °C (11 %). Most larvae were arrested during L1 stage (69 %) and L2 stage (17 %). The most prominent feature of affected larvae was formation of large vacuoles with fluid in the position of the pseudocoelome. These vacuoles finally fused
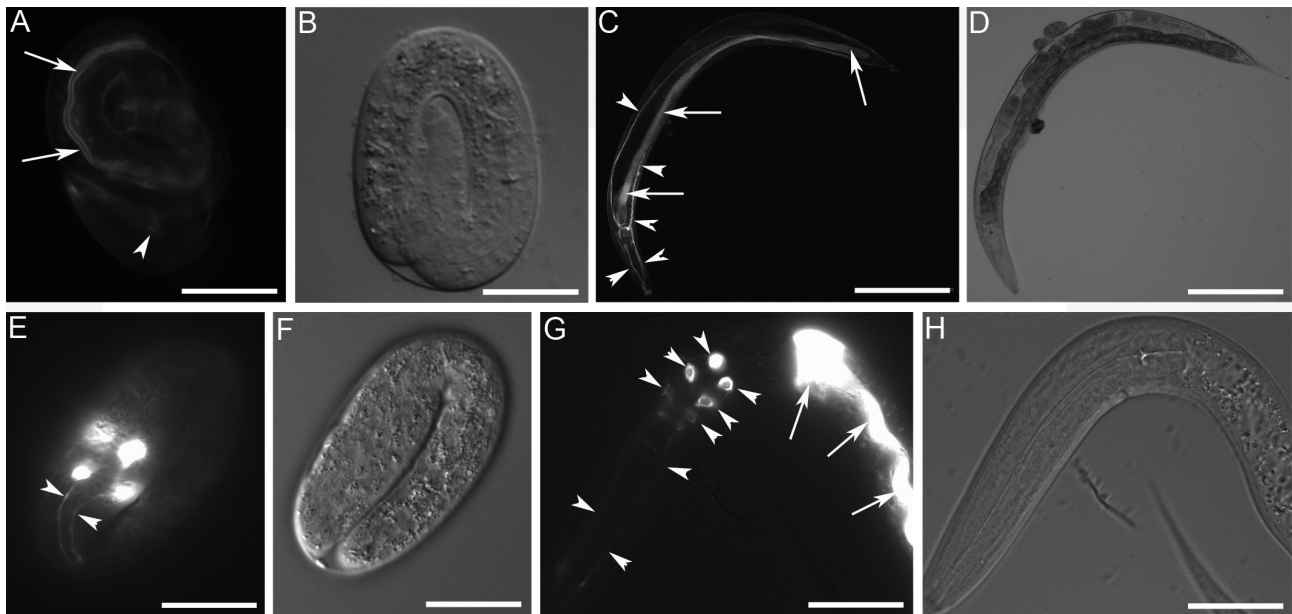

Fig. 3. Expression of *vha-20* in *C. elegans* developmental stages

E – embryos, **L1–L4** – larval stages, **A** – young adult hermaphrodites. Arbitrary units indicate fold of expression relative to the expression found in young adults.

Fig. 4. VHA-20::GFP expression in transgenic *C. elegans*
Panels **A** to **D** show transgenic line 7802 carrying the transgene organized in the operon with *sym-4*. **A** – An embryo in three-fold stage expressing the transgene labelled with GFP in the apical membrane of enterocytes (arrows) and weakly in amphid neurons in the head (arrowhead). **B** shows the same live embryo in Nomarski optics. **C** – An adult hermaphrodite expressing the transgene in the apical membrane of enterocytes (arrows) and on the plasma membrane of all four arms of the excretory cell (arrowheads). **D** shows the same animal as in panel C in Nomarski optics. Panels **E** to **H** show expression of the transgene from the *vha-20* own promoter (line 7270). **E** – Expression of the transgene in four amphid neurons in the head of a two-fold embryo. Arrowheads mark axons. **F** – The embryo shown in panel E in Nomarski optics. **G** – An L4 larva showing expression of the transgene on the plasma membrane of the cell bodies and axons of four apmphid neurons (arrowheads). Strong expression of the transgene is seen in the apical membrane of enterocytes delimiting the intestinal lumen (arrows). **H** – The animal shown in G in Nomarski optics. Scale bars: A, B, E, F, G, H 20 µm, C and D 200 µm.

and revealed a distended pseudocoelome in an otherwise live and moving larva (Fig. 5 A, B). The remaining 14 % of arrested larvae surviving for 7 days at L1 stage were able to escape arrest and develop into adults and laid eggs (Table 3).

VHA-20 is essential for pH maintenance in the intestine and normal intestinal function

Since the prominent localization of VHA-20::GFP at the apical membrane of enterocytes is in keeping with the known intestinal function of *C. elegans* V-ATPase a-subunit VHA-6 that is important for the maintenance of pH gradient across the apical membrane (Allman et al., 2009), we investigated whether also VHA-20 might be required for pH homeostasis in the *C. elegans* intestine.

The pH in the pharynx and intestine has been shown to form a gradient from approximately pH 6 in the anterior pharynx to pH 3.6 in the distal intestine (Chauhan et al., 2013). In order to see the dependence of the intestinal pH on functional VHA-20, we used *in vivo* staining with acridine orange in control animals and in the progeny of parents inhibited for *vha-20* by RNAi. At neutral pH, acridine orange is present prevalently in monomers, while at acidic pH acridine orange aggregates, which

changes its solubility and its excitation/emission properties (Ahmed and Pelster, 2008; Han and Burgess, 2010). In contrast to control animals that show stronger fluorescence in red spectra than in green spectra, yielding orange colour in composite view (Fig. 5 C), animals with inhibited *vha-20* yield green colour at the identical settings. This indicates a loss of acidic pH in the intestinal lumen of the *vha-20* inhibited animals. Since precise pH pulsing has been shown to be critical for absorption of digested food constituents (Allman et al., 2009), we tested whether impaired food absorption might participate in the *vha-20* loss-of-function phenotype. We determined the expression of *acs-2* and *fat-7*, two genes involved in fatty-acid metabolism whose expression is known to be regulated by food deprivation (Van Gilst et al., 2005; Vohanka et al., 2010). In keeping with defective food uptake in *vha-20* inhibited animals, the expression profile of *acs-2* increased and *fat-7* decreased as expected in fasting (Fig. 6).

vha-20 intestinal expression is regulated by HNF4 homologue, NHR-31

NHR-31 is the *C. elegans* homologue of HNF4 and is important for the development of the excretory channel by regulating transcription of several V-ATPase subu-
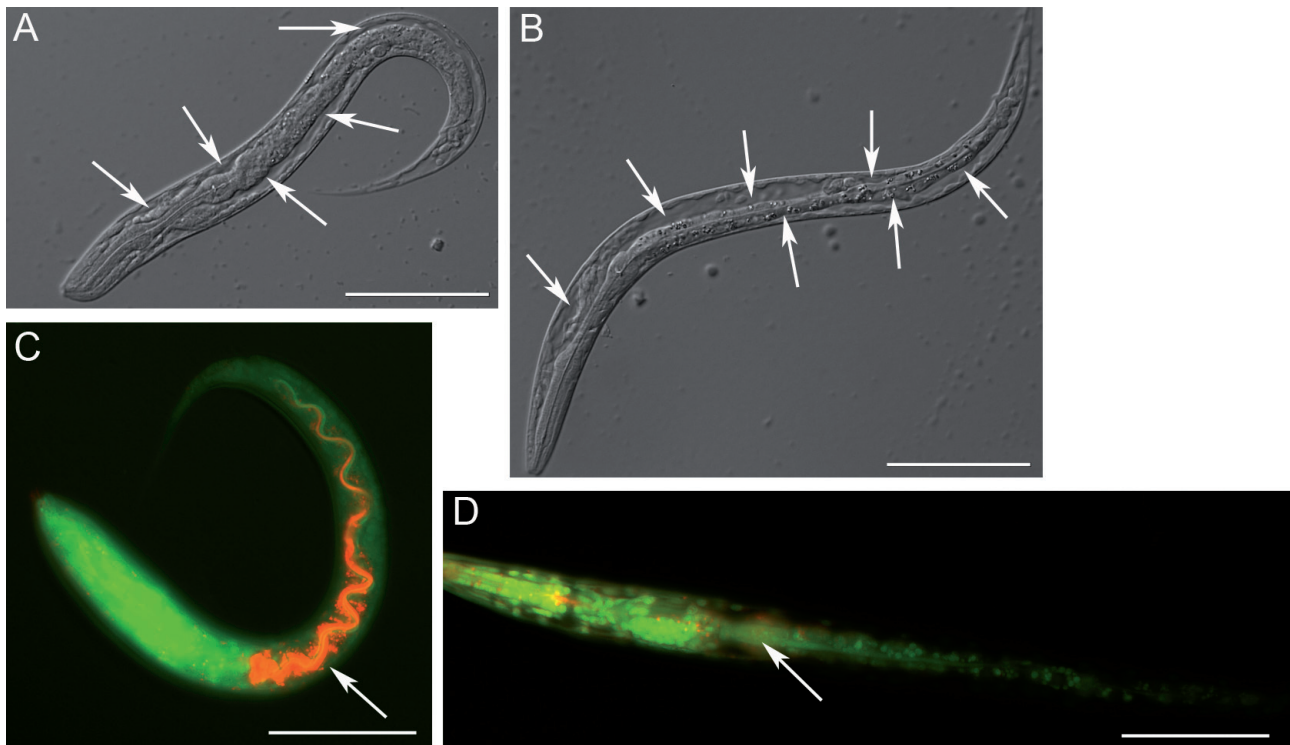
Fig. 5. The effect of inhibition of *vha-20* by RNAi on larval development and intestinal pH detected by altered fluorescence of acridine orange. **A** and **B** show progressive formation of vacuoles (arrows) in pseudocoelome of L1 (A) and L2 (B) larvae in Nomarski optics. **C** and **D** show fluorescence of acridine orange detected in a control L1 larva and in a larva inhibited for *vha-20* (D). Wild-type larva shows higher fluorescence in red channel than in green channel, which results in the orange-red fluorescence in the gut lumen (arrow) indicating acidic pH (C). In contrast, the L1 larva with inhibited *vha-20* shows neutral intestinal pH detected by predominant green fluorescence (arrow) in a composite picture taken at the same settings (D). Scale bar: 50 μm.

Table 3. The effect of vha-20 inhibition on the number of progeny

| Breeding temperature	Injected hermaphrodites	Progeny scored	Embryonic lethality	Larval lethality		
				Total	**L1**	**L2**
16 °C	15	1446	148 (11 %)	1243 (86 %)	1003 (69 %)	240 (17 %)
24 °C	13	1458	122 (9 %)	1064 (73 %)	847 (58 %)	217 (15 %)

nits required for proper functioning of the excretory system in *C. elegans* (Hahn-Windgassen and Van Gilst, 2009). It also regulates the *vha-20* expression (Hahn-Windgassen and Van Gilst, 2009). We investigated whether the intestinal expression of *vha-20* is also dependent on NHR-31. Inhibition of *nhr-31* by microinjection of dsRNA induced developmental defects of the excretory channel as described (Hahn-Windgassen and Van Gilst, 2009). In addition to the published data, we also observed intestinal defects similar to the defects observed in *vha-20* knockdown animals including dilatation of the intestine, formation of vacuoles and larval arrest (Fig. 7). *nhr-31* RNAi caused larval lethality in 31 % of the larvae (695 progeny scored).

To visualize the NHR-31 effect on *vha-20* intestinal expression, we used transgenic lines expressing VHA-20::GFP. Compared to controls (Fig. 4 A, C), transgenic larvae inhibited for *nhr-31* had decreased expression of the transgene in the intestine as well as in the excretory

system (Fig. 7 A, C, D). Approximately 30 % lethality of larvae was observed in the progeny of *nhr-31* inhibited parents and corresponded to the easily observable decrease of VHA-20::GFP signal in the gut. VHA-20::GFP expressed from the operon with *sym-4* (line 7802) was markedly decreased in the progeny of parents with inhibited *nhr-31*. Interestingly, larvae inhibited for *nhr-31*, similarly as larvae inhibited for *vha-20*, had severely malformed appearance but were viable, with preserved motoric functions and pharyngeal pumping.

Discussion

In this work we show that VHA-20, one of the two prorenin receptor homologues that are identified by sequence similarity in *C. elegans,* functions in tissues that are critical for the ion balance maintenance: in amphid neurons, in the gut, especially on the apical part of the enterocytes, and in the membrane of the excretory cell.
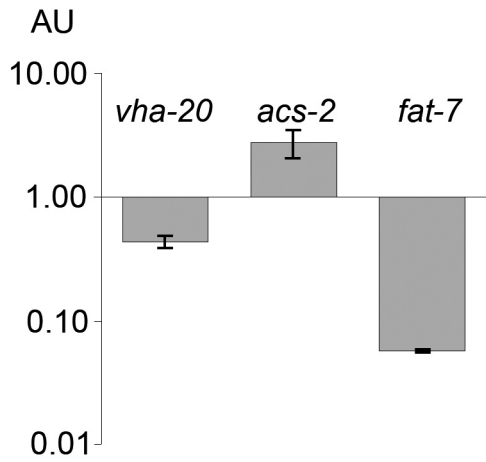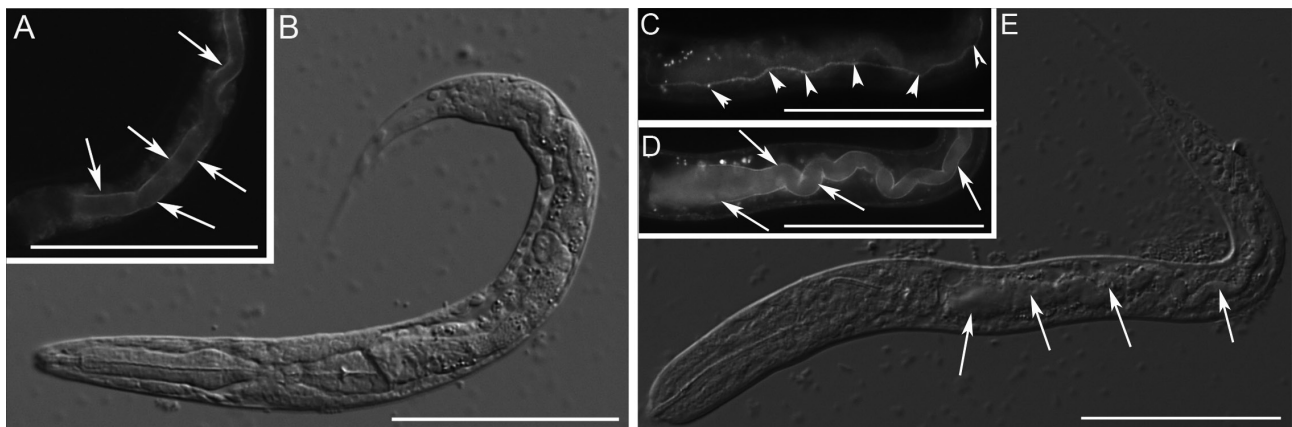
Fig. 6. The effect of *vha-20* inhibition by RNAi on the expression of *acs-2* and *fat-7* at the mRNA level detected by reverse transcription and droplet digital PCR. Expression of genes in arbitrary units given as ratio with controls, normalized to *ama-1* and shown in the logarithmic scale. *vha-20* inhibition, which is documented by ddPCR targeted at a region outside of the region used for preparation of dsRNA, results in an expressional shift of *acs-2* and *fat-7* consistent with starvation. The results shown are from a representative experiment made in triplicates.

We show that VHA-20 is important for acidification of the lumen of enterocytes. We also show that VHA-20 is critical for larval development, most obvious in the L1 and L2 stages. The developmental expression pattern shows that the highest abundance of *vha-20* is in L2 stage; however, our knockout experiments show that larval arrest occurs mostly in the L1 stage. This fact is probably due to the necessity of VHA-20 for proper larval development from its very beginning, in which the relative abundance of the *vha-20* transcript does not play a major role. The VHA-20 inhibition also leads to defects consistent with its critical role in systemic ion and water management visualized as a dramatic distention of the pseudocoelome cavity.

C. elegans maintains its body shape using a combination of three major mechanisms: semi-rigid exoskeleton, internal body fluid (water and ion) content and internal hydrostatic pressure generated by the precisely regulated tonus of longitudinal body wall muscles (Kage-Nakadai et al., 2011; Petzold et al., 2011). Ion and water management in intestinal lumen and intestinal cells is critical for the absorption of nutrients (Allman et al., 2009), as well as prevention of ion and water loss during defecation. Immediately prior to defecation and the rectal valve opening, the ions are pumped outside of the intestinal lumen (Pfeiffer et al., 2008; Allman et al., 2009; Chauhan et al., 2013). The maintenance of water and ion balance in the body cavities and tissues is critical for normal nematode development and life and is tightly regulated in response to environmental conditions by sensing organs, the amphid neurons, by resorption in the gut and excretion by the excretory cell (reviewed in Choe, 2013). The prominent expression of VHA-20 in tissues important for the body ion/water balance suggests its primary function in this mechanism. Moreover, the *vha-20* knockdown leads to phenotypical changes almost identical to inhibition of genes required for osmoregulation, as reported by Liégeois et al. (2007).

A characteristic feature of larvae with inhibited VHA-20 is an atrophy of enterocytes accompanied by enlargement of the intestinal lumen in keeping with impaired absorption of nutrients. This was confirmed by the expression pattern of *acs-2* and *fat-7*, two genes whose protein products were previously shown to be involved

Fig. 7. The effect of inhibition of *nhr-31* by RNAi on the development of L1 larvae expressing VHA-20::GFP from the short promoter (7270) (panels A and B) and from the operon with *sym-4* (7802) (panels C, D, E). **A** – Inhibition of *nhr-31* decreased expression of VHA-20::GFP in the intestine and malformation of the intestine (arrows) (dilatation of the intestinal lumen) similarly as in animals with inhibited *vha-20*. **B** shows the same animal as in A in Nomarski optics. Panels **C** and **D** show decreased expression of the transgene organized in the operon in the excretory cell and intestine. Both panels show the same animal in two different focus planes. Arrowheads mark one channel of the excretory cell with defective expression of VHA-20::GFP in panel C. Inflated intestine with decreased expression of VHA-20::GFP is marked by arrows in panel D. Panel **E** shows the same animal as in panels C and D in Nomarski optics. Arrows mark malformed intestine. Scale bar: 50 μm.

in fatty acid metabolism and that respond at the mRNA level to food availability (Van Gilst et al., 2005).

The dependence of *vha-20* (denominated as cosmid R03E1.2) expression on NHR-31 was identified in a study focused on the NHR-31 role in the regulation of expression of V-ATPase-related genes in the excretory cell. This dependence was observed at the level of total RNA from the whole organism (Hahn-Windgassen and Van Gilst, 2009). Our experiments with GFP-tagged transgene confirmed that *vha-20* is regulated by NHR-31 and showed that *vha-20* is regulated by NHR-31 both in the intestine and in the excretory cell.

NHR-31 is a homologue of HNF4, NR2A nuclear receptor which is one of the founders of the NR gene family. NR2A has multiplied in *Rhabditidae* to a large number of paralogues. These multiplied paralogues show variable sequence conservation with HNF4. Recent functional studies also identified functional similarity to HNF4 in a growing number of multiplied receptors, indicating functional evolutionary pressures for HNF4 multiplication (Kostrouchova and Kostrouch, 2014). Interestingly, HNF4 is similarly important for the development of kidneys in vertebrates (Grigo et al., 2008) as NHR-31 for the proper development and function of the excretory cell (Hahn-Windgassen and Van Gilst, 2009).

Evolution of new protein functions includes the mechanism of gene sharing. In this concept, proteins can acquire two or more unrelated functions in different cells and tissues. The evolutionary pressures for the selection of particular proteins may be as diverse as the protein solubility (and thus achievable high optical density in the eye lens) and their regulation at the level of promoters allowing expression of large quantities in some cells and very precisely regulated small quantities in other cell types (Piatigorsky et al., 1988; Piatigorsky and Wistow, 1989; Piatigorsky, 2007).

Our work shows that VHA-20 acts as a regulator of systemic ion and water management in nematodes. This is reminiscent of prorenin receptor utilization in RAAS in mammals. The utilization of prorenin receptor at two levels of the same regulatory cascade suggests an evolutionary pressure for selection of proteins already employed in the cascade. We have encountered a similar situation in the case of hedgehog-related proteins identified in the regulatory cascade of nuclear receptors (Kouns et al., 2011).

In the case of prorenin receptor, this pressure may reflect a potency of prorenin receptor to bind and sterically alter proteins or to act as an adaptor, as it is in the assembly of V-ATPase, a step that is likely to induce conformational changes of interacting proteins (constituents of V-ATPase) as well. The additional selection pressure may have been its regulation at the level of the promoter, similarly as in several cases of gene sharing (Piatigorsky, 1992).

References

Ahmed, K. H., Pelster, B. (2008) Ionic determinants of pH of acidic compartments under hypertonic conditions in trout hepatocytes. *J. Exp. Biol*, **211**, 3306-3314.

Allman, E., Johnson, D., Nehrke, K. (2009) Loss of the apical V-ATPase a-subunit VHA-6 prevents acidification of the intestinal lumen during a rhythmic behavior in C. elegans. *Am. J. Physiol. Cell Physiol*. **297**, C1071-1081.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. **25**, 3389-3402.

Atlas, S. A. (2007) The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J. Manag. Care Pharm.* **13**, 9-20.

Blitzer, J. T., Nusse, R. (2006) A critical role for endocytosis in Wnt signaling. *BMC Cell Biol.* **7**, 28.

Brenner, S. (1974) The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Buechling, T., Bartscherer, K., Ohkawara, B., Chaudhary, V., Spirohn, K., Niehrs, C., Boutros, M. (2010) Wnt/Frizzled signaling requires dPRR, the Drosophila homolog of the prorenin receptor. *Curr. Biol.* **20**, 1263-1268.

Burckle, C. A., Jan Danser, A. H., Muller, D. N., Garrelds, I. M., Gasc, J. M., Popova, E., Plehm, R., Peters, J., Bader, M., Nguyen, G. (2006) Elevated blood pressure and heart rate in human renin receptor transgenic rats. *Hypertension* **47**, 552-556.

Chauhan, V. M., Orsi, G., Brown, A., Pritchard, D. I., Aylott, J. W. (2013) Mapping the pharyngeal and intestinal pH of Caenorhabditis elegans and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS Nano* **7**, 5577-5587.

Choe, K. P. (2013) Physiological and molecular mechanisms of salt and water homeostasis in the nematode Caenorhabditis elegans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **305**, R175-186.

Cruciat, C. M., Ohkawara, B., Acebron, S. P., Karaulanov, E., Reinhard, C., Ingelfinger, D., Boutros, M., Niehrs, C. (2010) Requirement of prorenin receptor and vacuolar H+-ATPase-mediated acidification for Wnt signaling. *Science* **327**, 459-463.

Fisher, N. D., Hollenberg, N. K. (2001) Is there a future for renin inhibitors? *Expert Opin. Investig. Drugs* **10**, 417-426.

Fournier, D., Luft, F. C., Bader, M., Ganten, D., Andrade-Navarro, M. A. (2012) Emergence and evolution of the renin-angiotensin-aldosterone system. *J. Mol. Med. (Berl.)* **90**, 495-508.

Grigo, K., Wirsing, A., Lucas, B., Klein-Hitpass, L., Ryffel, G. U. (2008) HNF4α orchestrates a set of 14 genes to

down-regulate cell proliferation in kidney cells. *Biol. Chem.* **389**, 179-187.

Hahn-Windgassen, A., Van Gilst, M. R. (2009) The Caenorhabditis elegans HNF4α homolog, NHR-31, mediates excretory tube growth and function through coordinate regulation of the vacuolar ATPase. *PLoS Genet.* **5**, e1000553.

Han, J., Burgess, K. (2010) Fluorescent indicators for intracellular pH. *Chem. Rev.* **110**, 2709-2728.

Hobert, O. (2002) PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. *Biotechniques* **32**, 728-730.

Ji, Y. J., Choi, K. Y., Song, H. O., Park, B. J., Yu, J. R., Kagawa, H., Song, W. K., Ahnn, J. (2006) VHA-8, the E subunit of V-ATPase, is essential for pH homeostasis and larval development in C. elegans. *FEBS Lett.* **580**, 3161-3166.

Kage-Nakadai, E., Uehara, T., Mitani, S. (2011) H+/myo-inositol transporter genes, hmit-1.1 and hmit-1.2, have roles in the osmoprotective response in Caenorhabditis elegans. *Biochem. Biophys. Res. Commun.* **410**, 471-477.

Kaneshiro, Y., Ichihara, A., Sakoda, M., Takemitsu, T., Nabi, A. H., Uddin, M. N., Nakagawa, T., Nishiyama, A., Suzuki, F., Inagami, T., Itoh, H. (2007) Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats. *J. Am. Soc. Nephrol.* **18**, 1789-1795.

Kostrouchova, M., Kostrouch, Z. (2014) Nuclear receptors in nematode development: Natural experiments made by a phylum. *Biochim. Biophys. Acta* **1849**, 224-237.

Kouns, N. A., Nakielna, J., Behensky, F., Krause, M. W., Kostrouch, Z., Kostrouchova, M. (2011) NHR-23 dependent collagen and hedgehog-related genes required for molting. *Biochem. Biophys. Res. Commun.* **413**, 515-520.

Liégeois, S., Benedetto, A., Michaux, G., Belliard, G., Labouesse, M. (2007) Genes required for osmoregulation and apical secretion in Caenorhabditis elegans. *Genetics* **175**, 709-724.

Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D. K., Schagger, H. (1998) Identification and characterization of a novel 9.2-kDa membrane sector-associated protein of vacuolar proton-ATPase from chromaffin granules. *J. Biol. Chem.* **273**, 10939-10947.

Nguyen, G., Delarue, F., Burckle, C., Bouzhir, L., Giller, T., Sraer, J. D. (2002) Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J. Clin. Invest.* **109**, 1417-1427.

Nguyen, G., Muller, D. N. (2010) The biology of the (pro)renin receptor. *J. Am. Soc. Nephrol.* **21**, 18-23.

Nishi, T., Forgac, M. (2002) The vacuolar (H+)-ATPases – nature's most versatile proton pumps. *Nat. Rev. Mol. Cell Biol.* **3**, 94-103.

Oka, T., Futai, M. (2000) Requirement of V-ATPase for ovulation and embryogenesis in Caenorhabditis elegans. *J. Biol. Chem.* **275**, 29556-29561.

Pei, J., Kim, B. H., Tang, M., Grishin, N. V. (2007) PROMALS web server for accurate multiple protein sequence alignments. *Nucleic Acids Res*. **35**, W649-652.

Petzold, B. C., Park, S. J., Ponce, P., Roozeboom, C., Powell, C., Goodman, M. B., Pruitt, B. L. (2011) Caenorhabditis elegans body mechanics are regulated by body wall muscle tone. *Biophys. J.* **100**, 1977-1985.

Pfeiffer, J., Johnson, D., Nehrke, K. (2008) Oscillatory transepithelial H(+) flux regulates a rhythmic behavior in C. elegans. *Curr. Biol*, **18**, 297-302.

Piatigorsky, J., O'Brien, W. E., Norman, B. L., Kalumuck, K., Wistow, G. J., Borras, T., Nickerson, J. M., Wawrousek, E. F. (1988) Gene sharing by δ-crystallin and argininosuccinate lyase. *Proc. Natl. Acad. Sci. USA*, **85**, 3479-3483.

Piatigorsky, J., Wistow, G.J. (1989) Enzyme/crystallins: gene sharing as an evolutionary strategy. *Cell* **57**, 197-199.

Piatigorsky, J. (1992) Lens crystallins. Innovation associated with changes in gene regulation. *J. Biol. Chem.* **267**, 4277-4280.

Piatigorsky, J. (2007) *Gene Sharing and Evolution: the Diversity of Protein Functions*. Harvard University Press, Cambridge, MA.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A., Finn, R. D. (2012) The Pfam protein families database. *Nucleic Acids Res.* **40**, D290-301.

Schneider, C. A., Rasband, W. S., Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671-675.

Shaye, D. D., Greenwald, I. (2011) OrthoList: a compendium of C. elegans genes with human orthologs. *PLoS One* **6**, e20085.

Simeckova, K., Brozova, E., Vohanka, J., Pohludka, M., Kostrouch, Z., Krause, M. W., Rall, J. E., Kostrouchova, M. (2007) Supplementary nuclear receptor NHR-60 is required for normal embryonic and early larval development of Caenorhabditis elegans. *Folia Biol. (Praha)*, **53**, 85-96.

Skeggs, L. T., Jr., Kahn, J. R., Lentz, K., Shumway, N. P. (1957) The preparation, purification, and amino acid sequence of a polypeptide renin substrate. *J. Exp. Med.* **106**, 439-453.

Van Gilst, M. R., Hadjivassiliou, H., Yamamoto, K. R. (2005) A Caenorhabditis elegans nutrient response system partially dependent on nuclear receptor NHR-49. *Proc. Natl. Acad. Sci. USA*, **102**, 13496-13501.

Vohanka, J., Simeckova, K., Machalova, E., Behensky, F., Krause, M. W., Kostrouch, Z., Kostrouchova, M. (2010) Diversification of fasting regulated transcription in a cluster of duplicated nuclear hormone receptors in C. elegans. *Gene Expr. Patterns* **10**, 227-236.

Waterhouse, A .M., Procter, J. B., Martin, D. M., Clamp, M., Barton, G. J. (2009) Jalview Version 2 – a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191.