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Review

Vacuolar (H^+)-ATPases in *Caenorhabditis elegans*: What can we learn about giant H^+ pumps from tiny worms?

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ABSTRACT

Vacuolar (H^+)-ATPases, also called V-ATPases, are ATP-driven proton pumps that are highly phylogenetically conserved. Early biochemical and cell biological studies have revealed many details of the molecular mechanism of proton pumping and of the structure of the multi-subunit membrane complex, including the stoichiometry of subunit composition. In addition, yeast and mouse genetics have broadened our understanding of the physiological consequences of defective vacuolar acidification and its related disease etiologies. Recently, phenotypic investigation of V-ATPase mutants in *Caenorhabditis elegans* has revealed unexpected new roles of V-ATPases in both cellular function and early development. In this review, we discuss the functions of the V-ATPases discovered in *C. elegans*.

The acidification of intracellular organelles is essential for various cellular functions, including protein degradation and vesicular trafficking. H^+ transport across membranes is mediated primarily by ATP-dependent proton pumps known as vacuolar H^+-ATPases (V-ATPases) [1–3]. These pumps are composed of two discrete domains. The V1 domain is a 650-kDa peripheral macromolecular complex that hydrolyzes ATP to generate the energy required for pumping protons. The Vo domain is a 260-kDa integral membrane protein complex that transports H^+ across the lipid bilayer.

The V1 domain is composed of subunits A to H, whereas the Vo contains the a, c, c″, d, e, and e′ subunits. Several subunits have different isoforms or alternatively spliced variants that are expressed in a tissue-specific manner and that appear to be specific for the function of V-ATPases in those tissues. The activity of V-ATPases is highly regulated by several mechanisms, including the reversible dissociation of the Vo and V1 domains. In addition to the acidification of intracellular compartments, V-ATPases are present in the plasma membranes of some specialized cells, where they perform coupled transport of molecules to carry out cell-specific functions, such as renal acidification, bone absorption, regulation of cytoplasmic pH, and spermatogenesis [1]. In addition, several lines of evidence from genetic studies and cellular analyses have suggested that the Vo domain is involved in membrane fusion, independent of the V1 domain. In this study, we review the main characteristics of the structure and functions of V-ATPases and discuss how several studies that used *Caenorhabditis elegans* as a model system contributed to our understanding of the various functions of V-ATPases.

1. Structure and functions of V-ATPases

V-ATPases are multi-subunit complexes organized into two functional domains that are operated by a rotary mechanism energized by ATP hydrolysis (Fig. 1, reviewed in [1–3]). In the V1 domain, there are eight different subunits, designated A to H. Three copies each of the A and B subunits are organized in an alternating fashion to form a hexamer. ATP hydrolysis occurs at the interface between the A and B subunits. The other V1 subunits constitute peripheral and central stalks that have distinct functions in the rotary mechanism by which the V-ATPases couple ATP hydrolysis to proton pumping. One copy each of the C and H subunits and three copies each of the G and E subunits form peripheral stalks that act as stators [4–8]. One D and one F subunit form a central stalk that serves as a rotor to couple the energy generated by ATP hydrolysis to the actual rotation of the proteolipid ring in Vo to transport protons.

The Vo domain is composed of six different subunits: a, c, c′, c″, d, and e. Higher organisms lack c′, which is universal only in fungi, but contain a type I transmembrane accessory subunit, Ac45 [9,10]. A single Vo domain comprises four or five copies of the c subunit and one copy of the "a" and "d" subunits, whereas the stoichiometry of the 9-kDa

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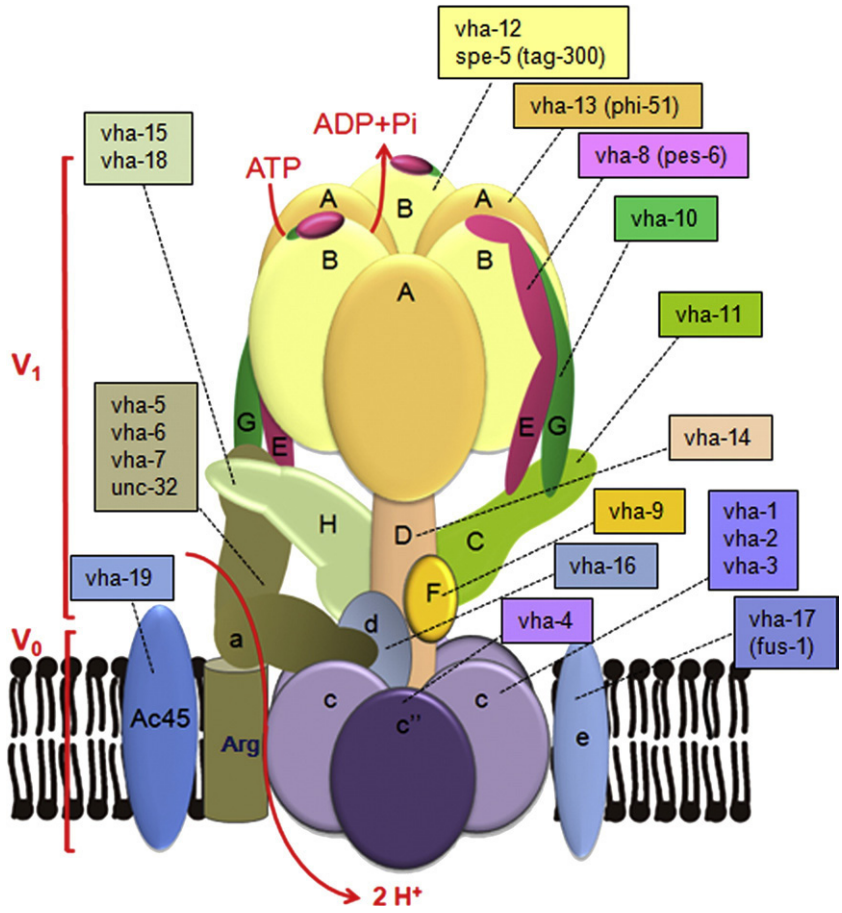

Fig. 1. Structure of the V-ATPase. A schematic showing the expression of V-ATPase in *C. elegans* is presented, and all known corresponding worm subunits are indicated. V-ATPase consists of a peripheral V1 domain that hydrolyzes ATP and an integral Vo domain that translocates protons across the membrane. The A and B subunits, which are the core subunits of the V1 domain, participate to form ATP binding and hydrolysis pockets. In the Vo domain, the a and e subunits are adjacent to a proteolipid ring, which is composed of the c, c′, and c″ subunits. The V1 and Vo domains are connected by a central stalk composed of the D, F, and d subunits and surrounded by a peripheral stalk composed of the C, E, F, G, and H subunits of the V1 domain. The *C. elegans* genome encodes orthologs of all subunits except c′, which is present in yeast but is absent in mammals. The proteolipid ring in the worm therefore is likely composed of only c and c″ subunits, and may be accessorized by Ac45, which has been reported to interact with the a, c, and d subunits in mammals [77]; however, the stoichiometry of the subunits is not known. Protons are translocated from the cytoplasm to the lumen through two hemi-channels in the "a" subunit by repetitive protonation and deprotonation of arginine, which is buried between the hemi-channels. Glutamates in the proteolipid ring of the V1 domain rotate in the lipid bilayer with a force driven by ATP hydrolysis.

polypeptide e subunit remains unknown [11,12]. The c, c′, and c″ subunits are highly hydrophobic and form a proteolipid ring in the membrane that rotates around the central stalk as protons are pumped. Each proteolipid subunit contains a single buried glutamic acid (Glu) in transmembrane 4 (TM4) of subunits c and c′ and in TM3 of subunit c″. The carboxyl groups of the buried glutamic acids undergo reversible protonation during proton transport [13]. The 100-kDa subunit "a" contains an N-terminal domain facing the cytoplasm, and transmembrane helices of the C-terminal domains are embedded in the lipid bilayer. Located in those TMs are two hemi-channels, one oriented towards the cytoplasm, and the other towards the lumen, through which protons bind and dissociate from the buried carboxyl groups in the proteolipid ring [2]. The essential residue for proton transport is an arginine (R735 in VPH1 of *Saccharomyces cerevisiae*) located in TM7 that appears to be identical across species. This arginine forms a luminal facing hemi-channel and promotes deprotonation of the buried glutamate in each of the proteolipid ring subunits [14]. Recent cryogenic EM studies have reported that the d subunit appears to form the top of the proteolipid ring and contact the "a" subunit [8,15]. Whether it serves as a rotating shaft itself is not known, although mutation of the "d" subunit affects the coupling efficiency [16].

V-ATPases acidify the lumen of various intracellular organelles, including endosomes, lysosomes, and secretory vesicles. The resulting high concentration of protons in these organelles is key to the biological functions of each organelle. First, the acidic pH generated in the lumen by V-ATPases facilitates the dissociation of protein complexes. In this mode, ligand–receptor complexes, such as the low density lipoprotein (LDL) receptor and the insulin receptor that have been internalized into endosomes, are dissociated by the low pH in the endosomes, and the released receptors can then be recycled back to the plasma membrane [17]. Another example is the newly synthesized lysosomal enzymes associated with the mannose-6 phosphate receptors in Golgi-derived vesicles [18]. When these vesicles fuse with a lysosome, those enzymes dissociate from the receptors because of the low pH in the lysosome [19]. In a further step, the low luminal pH activates those unloaded enzymes to catalyze biochemical reactions. In secretory vesicles, protease activity is required for conversion of the precursor forms of peptide hormones and growth factors, such as insulin and EGF, into mature forms [19,20]. The acidic environment of endosomes also facilitates invasion of enveloped viruses, such as influenza virus and vesicular stomatitis virus, as well as toxins, such as anthrax toxin and diphtheria toxin, into host cells by triggering the formation of membrane pores [21]. In certain cases, the low luminal pH of intracellular organelles is a prerequisite for proper vesicular trafficking. The budding of endosomal carrier vesicles containing released ligands from early to late endosomes is dependent upon luminal acidification by V-ATPases. In this process, V-ATPase itself may serve as a binding scaffold for

proteins involved in budding, such as ARF-6, a small G-protein, and ARNO, a guanine-nucleotide exchange factor for ARF-6 [22]. The proton potential across the membrane generated by V-ATPases provides a driving force to transport small molecules and ions into the lumen. At presynaptic terminals, neurotransmitter packaging to synaptic vesicles requires the proton gradient generated by V-ATPases [3].

V-ATPases are also present in the plasma membrane, where they function both in normal physiology and in disease processes. In the mammalian kidney, V-ATPases in the apical membrane of renal α-intercalated cells in the distal tubule and collecting duct secrete protons into the urine, thus regulating plasma pH [23]. Mutations in the human a4 or B1 subunit isoforms of the V-ATPase result in defective acid secretion, leading to the onset of renal tubular acidosis with low plasma pH and excessive urinary loss of K^+ and Ca^{2+} [24,25]. Osteoclasts place V-ATPases on their ruffled membranes attached to bone, reserving some extracellular space in-between the bone and the membrane in which the acidity facilitates degradation of the bone for bone resorption [26]. A genetic defect in the human a3 isoform responsible for plasma membrane targeting of V-ATPases in osteoclasts leads to the failure of bone degradation, a condition called osteopetrosis [27]. This finding suggests that V-ATPases could be a drug target to treat osteoporosis, which is characterized by excessive bone loss. V-ATPases are also present in the apical membrane of epididymal clear cells, which are required for sperm maturation and viability [28]. The plasma membrane localization of V-ATPases may be associated with the invasiveness of highly motile migrating cells, such as vascular endothelial cells and highly metastatic tumor cells [29,30]. In vascular endothelial cells, V-ATPases are localized at the leading edge of the cells, where they seem to play a role in both invasion and migration. The invasiveness of tumor cells is correlated with the activity of the plasma membrane V-ATPase and is successfully inhibited by treatment with bafilomycin A, a specific inhibitor of V-ATPases. These results suggest that inhibition of V-ATPases might be a way to treat tumor metastasis and the excessive vascularization associated with diabetes.

Independent of its role in acidification, the membrane-integral Vo domain of the V-ATPase has been proposed to have a role in membrane fusion [31]. Genetic studies using yeast have indicated that Vo domains from donor and acceptor membranes form complexes that occur downstream from trans-SNARE pairing; formation of these complexes depends on the presence of both Rab-GTPase and calmodulin. The extreme hydrophobicity of the Vo domain has been suggested to facilitate the mixing of the lipid bilayer after tethering of vesicles. This new membrane fusion function of the Vo domain has been shown to be physiologically important, because mutation of the Vo domain results in obvious defective exocytic events in multicellular organisms such as the fruit fly and mouse. A *Drosophila* mutant that lacks *vha-100* encoding the "a" subunit accumulates synaptic vesicles at the presynaptic terminal [32]. This mutant has severe defects in neural transmission caused by impairment of synaptic vesicular fusion. In addition, islets isolated from mice with a deletion of the a3 subunit gene secrete insulin inefficiently, although insulin production and packaging to the secretory vesicles are not affected [20,32]. This compelling evidence strongly implies that the V-ATPase Vo domain participates in vesicular membrane fusion that is critical for both exocytic and endocytic pathways and important for various cellular activities, including neurotransmitter release and hormone secretion, and further suggests that the Vo domain may play a role in other physiological phenomena involving membrane–membrane fusion.

2. *C. elegans* as a model system to study V-ATPases

V-ATPases are ubiquitous among eukaryotes and are highly conserved over evolutionary time. A simple, unicellular yeast mutant system has made large contributions to the V-ATPase research field; it has resulted in the discovery of new subunits, allowed structure–function analyses to be performed, and resulted in the elucidation of the biochemical properties of the giant ATP hydrolysis enzyme. However, the functions and structure of V-ATPases are more complex in an organotypical context. V-ATPases are present both in the endomembranes of intracellular organelles and in the plasma membranes of specific tissues. The multi-subunit membrane complex pumps protons across the lipid bilayer using ATP hydrolysis to acidify or transport molecules, and it can assist in membrane fusion. How do V-ATPases exert these multi-functions in the context of different tissues, and how are these functions regulated?

To answer these questions, it is essential to use a multicellular model system with differentiated tissues in which reverse genetic and transgenic approaches are easily feasible to track down the expression patterns of particular genes in particular tissues. Although a variety of studies using a mouse genetic system, one type of model system, have allowed some of these questions to be addressed, many studies using other model systems have revealed unexpected functions and regulation of V-ATPases. For example, in a molecular genetic study using *Xenopus* embryos, it was reported that the asymmetry of H^+-flux across the plasma membrane resulting from an uneven distribution of V-ATPases in early developmental stages was crucial for correct left–right body axis determination [33]. In the plant model system *Arabidopsis*, it was demonstrated that reduced Golgi-localized V-ATPase activity was required for hormone-dependent hypocotyl growth inhibition [34]. In plant systems, it is possible to investigate responses to environmental cues such as salt adaptation [35,36]. Because the first animal model of a V-ATPase knock-out was made in *Drosophila*, the Malpighian tubule of the fly, which is analogous to the vertebrate kidney tubule, has been used as a platform to analyze the phenotypes of V-ATPase subunit mutants [37–39]. In addition, genetic screening for synaptic malfunction in *Drosophila* has revealed that a mutation in subunit "a" results in a synaptic vesicular fusion defect, supporting the proposed novel role of the Vo domain as a component of the membrane fusion machinery [32]. Another insect system has provided evidence to support a novel and unique role of V-ATPases in the goblet cells of the midgut [40]. In the tobacco hornworm *Manduca sexta*, V-ATPases in the apical membrane of goblet cells lining the midgut help to create an electrical membrane potential by generating a proton gradient across the membrane. This gradient is then used by membrane potential-dependent $K^+/2H^+$ antiporters to secrete K^+ ions into the lumen. This transport of K^+ ions out of the goblet cells is important for ion homeostasis because the tobacco hornworm has a diet high in K^+. These previous contributions of various model systems to the field of V-ATPases highlight the importance of using a broad spectrum of model systems to further investigate critical, as yet unknown functions of V-ATPases.

C. elegans, a free-living soil nematode, has proven to be a powerful genetic model system to study evolutionarily conserved genes. Although the worm is a simple organism, it has been shown to share many of the essential biological pathways present in higher organisms, including development, neuronal activities, and aging process. In addition, approximately 40% of the genes of the worm are predicted to have mammalian orthologs, and many of these gene products have conserved, essential biological functions and reflect the functions of their counterparts in higher organisms. Finally, a short life cycle (3 days) and life span (2–3 weeks) along with a simple anatomy (959 somatic cells in hermaphrodites) and a compact genomic organization (98 Mbp in 6 chromosomes) make this simple nematode amenable to genetic analyses to confirm the biological functions of known conserved genes, or to find new genes in conserved biological pathways. Several recent studies of V-ATPases in *C. elegans* have shown that this simple worm is a versatile model system, and can be used not only to further investigate the known functions of the multi-functional enzyme but also to discover new functional and regulatory characteristics of V-ATPases.

3. Genomic structure of V-ATPases in *C. elegans*

The *C. elegans* genome harbors 21 orthologs of 13 V-ATPase subunits that share high similarity with those of yeast and mice (Table 1). The ortholog of the yeast c′ subunit is not present in the genome of *C. elegans*. Excluding *spe-5* and *unc-32*, which were isolated based on their developmental and behavioral phenotypic defects, the subunits are denoted by *vha*, an abbreviation of *v*acuolar H^+-ATPase. These genes are distributed throughout the entire genome, including autosomes I to V and the X chromosome.

Approximately 15% of *C. elegans* genes are organized in closely spaced gene clusters called operons [92]. The genes in a single *C. elegans* operon are often co-regulated at the transcriptional level because they are under the control of a single promoter, and some of them are functionally related. V-ATPase genes appear to be an obvious example of the coordinated regulation of genes whose translational products function together. More than 50% of V-ATPase genes (11 out of 20 genes) are polycistronically transcribed because they are clustered in operons (Table 2). Genes *vha-1* and *vha-2*, both of which encode the c subunit of Vo, share the operon CEOP3620, whereas *vha-3*, which encodes another c subunit and *vha-11*, which encodes the A subunit of V1, share another operon, CEOP4638 [41,42]. Because protein function is sometimes hypothesized based on the function of other genes clustered in the same operon, there is a possibility that some of the genes clustered with V-ATPases might have a role related to the functional and structural features of V-ATPases. Interestingly, a couple of genes implicated in the mitochondrial unfolded protein response pathway (UPR(mt)) are encoded by V-ATPase operons [43]. Formation of a complex consisting of the homeodomain-containing transcription factor DVE-1 and the small ubiquitin-like protein UBL-5 is correlated with UPR(mt), and both genes are required for the UPR(mt). DVE-1 is recruited to the nucleus upon the activation of UPR(mt), where it then binds to the regulatory regions of mitochondrial chaperone genes. These events are mitigated when the activity of *clpp-1*, which encodes a mitochondrial Clp1-like protease, is reduced. It is noteworthy that *clpp-1* shares an operon with *vha-9*, whereas *ubl-5* shares one with *vha-10*. The relationship between V-ATPases and the UPR (mt) remains to be explored.

4. Putative assembly machinery for V-ATPases in *C. elegans*

The V1 and Vo domains of V-ATPases are able to assemble independently, and they associate later [44]. In yeast, the assembly of the Vo domain requires dedicated chaperones for correct assembly in the endoplasmic reticulum (ER). These proteins include the integral membrane proteins VMA12 (vacuolar membrane ATPase activity-12), VMA21, and the peripheral membrane protein VMA22 [45,46]. VMA12 and VMA22 appear to form a transient complex with Vph1p, an “a” subunit localized in vacuoles, whereas VMA21 interacts with proteolipid subunits and promotes the assembly of the ring structure. Pkr-1 appears to play a role in the efficient assembly and export of the Vo domain, but the molecular mechanism of the most recently identified assembly factor is not

Table 1
V-ATPase subunits in *Caenorhabditis elegans* [1,63,78].

Subunit	Function	Yeast gene	Mouse gene (tissue/cell)	*C. elegans* gene (chromosome; expression)	RNAi or mutant phenotype and other information	References
V_1						
A	ATP hydrolysis, regulation via non-homologous domain, stator subunit	VMA1	A	vha-13 (V; also known phi-51, intestine, excretory cell)	Embryonic lethal, decreased expression in calcineurin mutants	[79]
B	ATP hydrolysis, binds actin and aldolase, stator subunit	VMA2	B1 (renal, epididymis, lung) B2 (ubiquitous)	vha-12 (X; pharynx, intestine); spe-5 (I; also known as tag-300)	Embryonic lethal; spermatogenesis-defect	[1,50,80,81]
C	Regulatory, stator subunit, binds actin	VMA5	C1 (ubiquitous); C2a,b (lung, renal, epididymis)	vha-11 (IV; excretory cell, intestine)	Embryonic lethal	[1,52]
D	Rotary subunit	VMA8	D	vha-14 (III)	Embryonic lethal	[82]
E	Stator subunit, binds RAVE and aldolase	VMA4	E1 (testis); E2 (ubiquitous)	vha-8 (IV; also known as pes-6; excretory cell, hypodermis)	Embryonic lethal, larval arrest	[1,53,59]
F	Rotary subunit	VMA7	F	vha-9 (II)	Embryonic lethal	[83]
G	Stator subunit, binds RAVE	VMA10	G1 (ubiquitous); G2 (neural); G3 (renal, epididymis)	vha-10 (I)	Embryonic lethal	[1,57]
H	Regulatory, stator subunit	VMA13	Two alternatively spliced variants	vha-15 (X; also known as phi-52; muscle, intestine, neurons); vha-18 (V)	Embryonic lethal; embryonic lethal	[1,80]
V_0						
a	H^+ transport, targeting, binds aldolase, stator subunit	VPH1 (vacuole) STV1 (Golgi)	a1 (neural); a2 (endothelial); a3 (osteoclasts); a4 (renal, epididymis)	vha-5 (IV; also known as rdy-1; pharynx, excretory cell, epidermal cell); vha-6 (II; intestine); vha-7 (IV; hypodermis, uterus, spermatheca); unc-32 (III; nerve-ring, ventral-nerve-cord)	Larval arrest; larval arrest; wild-type; reverse ventral coiler	[1,49,54,71,73]
c	H^+ transport, rotary subunit	VMA3	c	vha-1 (III) and vha-2 (III; excretory cell, rectum); vha-3 (IV; excretory cell, gastrointestinal, hypodermis)	Embryonic lethal; larval arrest; larval arrest	[41,42] [84]
c′	H^+ transport, binds Vma21, rotary subunit	VMA11	No mammalian homolog	No *C. elegans* gene		[10]
c″	H^+ transport, rotary subunit	VMA16	c″	vha-4 (II; excretory cell, rectum)	Embryonic lethal	[41,85]
d	Coupling, rotary subunit	VMA6	d1 (ubiquitous); d2 (renal, epididymis, osteoclast)	vha-16 (I; excretory cell, hypodermis, intestine, ventral- and dorsal-nerve-cord)	Larval arrest	[1,64]
e		VMA9		vha-17 (IV; also known as fus-1, excretory cell, gut cells)	Epidermal cell fusion defect	[63]
Ac45	Accessory protein	No yeast gene	Atp6ap1	vha-19 (IV)	Larval arrest	[9,77]

Table 2
V-ATPase subunits in operons of *C. elegans* [74].

Operon	Subunit	Other genes in the operon	Ortholog, functions and characteristics	References
CEOP1216	spe-5	pas-3	Proteasome alpha subunit	
		chp-1	CHORD-containing protein	[86]
CEOP1264	vha-10	F46F11.9	GSG-1/meiosis	[87,88]
		ubl-5	UBL5/ubiquitin-like protein	[89]
		F46F11.6	Unknown	
		F46F11.12	Small nucleolar RNA gene	
CEOP2316	vha-4	T01H3.2	Multiple dysferlin and beta-propeller domain	
CEOP2476	vha-9	clpp-1	Clp-like protease	[43]
		ZK970.t4	tRNA pro	
CEOP3556	unc-32	tpk-1	Thiamine pyrophosphokinase	[90]
		trxr-2	Thioredoxin reductase 2	
CEOP3620	vha-1, vha-2	R10E11.6	AP1 subunit gamma-binding protein 1 (gamma-adaptin)	[41,91]
CEOP3880	vha-14	F55H2.5	Cytochrome B581	
CEOP4638	vha-3, vha-11			[52]
CEOP5412	vha-13	Y49A3A.1	Choline/ethanolamine phosphotransferase choline esterase1	
		cyn-1	Peptidyl-prolyl cis–trans isomerase	
		Y49A3A.3	Unknown conserved protein similar to Barwin-related endoglucanase	

known. Based on BLAST searches, VMA21 is highly conserved over evolutionary time and has orthologs in various eukaryotes, including higher organisms such as humans, whereas orthologs of VMA12 and VMA22 are found only in fungi, and Pkr-1 seems to be yeast-specific. This suggests that eukaryotes may share similar assembly mechanisms for the proteolipid ring, whereas the association of the "a" subunit with the proteolipid ring has evolved dynamically. This association seems to be a necessary step in evolution to support the variety of "a" subunits that provide a critical basis for the various functions of V-ATPases in different tissues and cellular compartments. In *C. elegans*, F09E5.11, R07E5.7, and F02E9.1, identified by BLAST searches, encode predicted orthologs of *S. cerevisiae* VMA12, VMA21, and VMA22, respectively. Whether these proteins are assembly factors for the Vo domain of *C. elegans*, as they are in yeast, remains to be determined.

5. Functions of V-ATPases in *C. elegans*

As a pioneer in this field, the Futai laboratory used molecular genetic tools available in the *C. elegans* model system to study the role of V-ATPases in the development of nematodes. Their early studies investigating V-ATPase subunit genes in *C. elegans* revealed several unique features of V-ATPase genes in this organism [41,42]. First, three genes, *vha-1*, *-2*, and *-3*, encode proteolipid subunit c in *C. elegans*, whereas only one mammalian counterpart has been reported (Table 1, Fig. 2). Among these genes, *vha-2* and *vha-3* produce completely identical c subunits because most of the differences in the DNA sequences between these genes are wobble substitutions; however, these genes are expressed in different tissues. Thus, the tissue-specific expression of proteolipid isoforms may be related to different functions and regulatory mechanisms of V-ATPases in various tissues in *C. elegans*.

The first paper describing the V-ATPases of *C. elegans* is the landmark work on nematode genetics by Sydney Brenner [47,48]. In these papers, Brenner described the *unc-32* mutant, one of the *unc* (uncoordinated) mutants defective in normal patterns of behavior. This mutant has a ventral coiler phenotype during reversing and, later, it was shown that this phenotype was due to mutations in one of the four "a" subunits [49]. The *unc-32* gene is ubiquitously expressed throughout the entire worm. However, the *unc-32* transcripts exist as six different alternatively spliced variants, and a few of these transcripts that contain exon 4b are specifically expressed in the nervous system, including in motor neurons and in most head neurons. The mutation in the allele that causes the *unc* phenotype is located at the consensus splice acceptor site of exon 4b, indicating that these neuron-specific splice variants have a function required for normal neuronal activity. The other embryonic lethal alleles contain mutations that affect all of the other spliced forms, and therefore, certain *unc-32* isoforms are absolutely required for viability.

V-ATPase is required for spermatogenesis in *C. elegans* [50]. Six mutant alleles of *spe-5*, encoding the B subunit in *C. elegans*, exhibit defective spermatogenesis, with excessive vacuolation in the spermatocytoplasm and distension of the fibrous body–membranous organelle (FB–MO) complex that segregates molecular components to spermatids during their maturation [51]. In mammals, V-ATPases in the apical membrane of narrow and clear cells are responsible for the acidic luminal environment in the epididymis and vas deferens that is required for the maturation of sperm and for their storage in a quiescent state [28]. Interestingly, two B subunit orthologs are present in both mammals and *C. elegans*, and one of the mammalian B subunits is expressed only in certain epithelial tissues, including the epididymis (Table 1). This leads to two intriguing questions: 1) Where is *spe-5* expressed in *C. elegans*? and 2) How does V-ATPase activity contribute to normal spermatogenesis in this nematode?

Embryonic lethality is one of the most common phenotypes observed in loss-of-function mutants or in RNAi-mediated knockdowns of V-ATPases in *C. elegans*, suggesting that V-ATPase activity is essential for embryogenesis (Table 1). The first phenotypic study of V-ATPase-defective worms was carried out by injection of dsRNA [52]. Injecting worms with *vha-11* dsRNA caused embryonic lethality in the offspring and eventually stopped ovulation in the parent. Interference or knock-out of V-ATPase activity by RNAi in *C. elegans* resulted in impairment of receptor-mediated endocytosis of yolk protein to the embryo, which may be one of the causes of lethality in the embryos [53]. RNAi against *vha-8*, encoding the E subunit of the V1 domain, blocked the uptake of the yolk protein YP170 into developing oocytes, which is critical for normal embryo maturation. Immunocytochemical studies of early embryonic cells revealed that V-ATPases appear as dot-like structures near the nucleus, indicating that they are localized in intracellular organelles [52,54]. Therefore, it is likely that the involvement of V-ATPases in receptor-mediated endocytosis is associated with the functions of the enzymes at the intracellular organelle level rather than at the plasma membrane level in terms of supporting the normal development of embryos. In this regard, mice embryos lacking V-ATPase subunit c are able to develop until no further than the blastocyst stage when implanted in the uterine epithelium. Blastocysts isolated from these embryos show impaired endocytosis as well as organellar acidification [55]. In addition, mammalian V-ATPases can modulate vesicle trafficking by recruiting ARNO and Arf6, essential regulators of the endocytic pathway, to the budding sites of endosomes [22]. Studies in *C. elegans* could provide genetic evidence of the basic molecular mechanisms by which V-ATPases in the intracellular organelles affect receptor-mediated endocytosis.

The GFP-reporter expression patterns of genes in *C. elegans* can provide valuable information regarding the function of the encoded proteins (Fig. 2). Most V-ATPase subunits are primarily expressed in the H-shaped excretory cell, which is believed to function in toxin and metabolic waste excretion as well as osmoregulation (Table 1, Fig. 2) [56]. Mutation of the V-ATPase in worms causes a malfunction of this organ; the *vha-5* mutant dies at the larval stage with its body cavity full of fluid, which is a similar phenotype to that caused by laser ablation of the excretory cell.

Necrotic cell death is implicated in neurodegeneration in neurodegenerative diseases and stroke. Neurodegeneration caused by over-

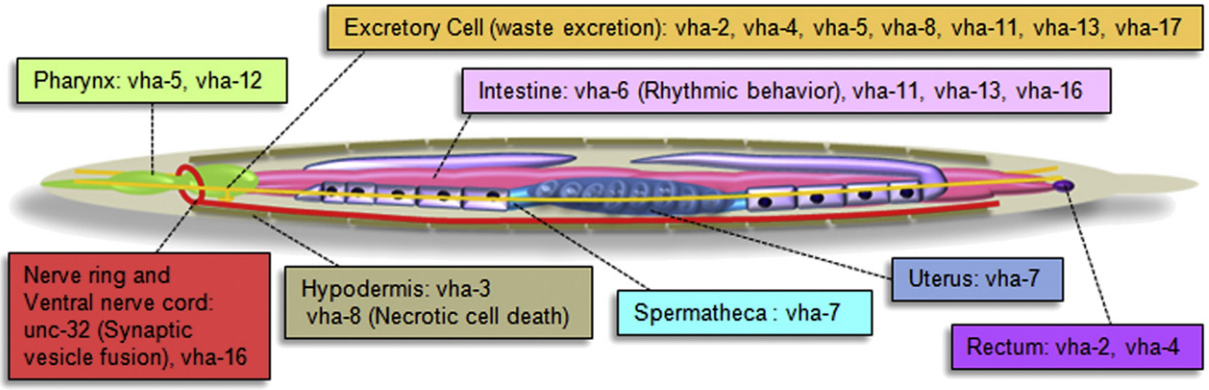

Fig. 2. Tissue-specific expression of V-ATPase subunits in *C. elegans*. GFP expressions driven by promoter of each V-ATPase subunit coding gene are indicated. Some of the subunits are expressed in a tissue-specific manner in *C. elegans*. Most of them are expressed in the excretory cell (yellow), which is responsible for osmotic and ionic regulation and toxic waste elimination, analogous to the renal system of higher animals (Table 1). The gene *unc-32* codes for the neuron (red)-specific "a" subunit in the worm and is essential for synaptic vesicle fusion [49,54]. The gene *vha-6* codes for another "a" subunit, which is almost exclusively expressed in the intestine (pink) and is essential for the acidification of the intestinal lumen that is required for defecation regularity (rhythmic behavior) [76]. The gene *vha-7*, which codes for the other "a" subunit, is expressed in the uterus (dark blue) and spermatheca (teal blue). The gene *vha-8* codes for the E subunit and is highly expressed in the hypodermis (light brown); one of its mutant phenotypes is the death of hypodermal cells due to necrosis [59]. The gene *vha-4* codes for the c" subunit and is highly expressed in the rectum (dark purple) as well as in the excretory cell, although the tissue-specific function of this gene is not known.

expression of *mec-4(d)*, a calcium-conducting ion channel subunit, in mechanosensory neurons is protected in V-ATPase mutants of *C. elegans* [57]. Specific aspartyl and calpain proteases are required for neurodegeneration. These enzymes are usually inert and benign in the cytoplasm, but are activated by an abrupt increase in the intracellular Ca^{2+} concentration that is an initiating event in necrotic cell death [58]. Lysosomes ruptured by those proteases are hypothesized to be an acidic source, leading to a decreased cytosolic pH. Another study of *C. elegans* provided support for the promoting role of V-ATPases in necrotic cell death; V-ATPase activity may underlie larval developmental arrest, which is the most common phenotype of V-ATPase mutants or of worms treated with RNAi against V-ATPases (Table 1). The dead bodies of L1 larvae of *vha-8*, E subunit mutants, also exhibit swollen vacuoles and nuclear fragmentation in the hypodermis, which are hallmarks of necrotic cell death [59]. It has been reported that acidosis accompanying necrotic cell death after stroke in humans activates acid-sensing, calcium-permeable ion channels [60,61]. These findings suggest that the molecular mechanisms underlying necrotic cell death are conserved from nematodes to humans, and further investigation of pH homeostasis in neurodegeneration will likely provide insight into similar pathologies in humans.

During the development of *C. elegans*, extensive cell–cell fusion events take place to generate an invariant pattern of syncytia in the epidermis [62]. The stereotyped fusion events are highly reproducible, because they are tightly regulated and restricted to only particular cells that fuse with appropriate neighbors. A genetic screen to identify genes essential for normal cell–cell fusion isolated a *fus-1* mutant carrying a defective gene encoding the V-ATPase "e" subunit, an integral membrane component that interacts with the Vo domain [63]. V-ATPases in the worm seem to negatively regulate the programmed cell–cell fusion, because the loss-of-function allele, predicted to produce an inactive V-ATPase, exhibits excessive hyperfusion of epidermal cells. This hyperfusion effect is re-enforced by the fact that RNAi knock-down of either Vo or V1 subunits also results in a similar hyperfusion phenotype. This observation, which is in contrast to the findings for V-ATPase d2 subunit knock-out mice, whose osteoclasts mostly remain mono-nucleated due to the failure of cell–cell fusion, raises the questions of how V-ATPases act in inhibitory or activating modes and whether the underlying mechanisms involve proton pumping activity or membrane fusion events mediated by the Vo domain [64]. Further genetic approaches using *C. elegans*, such as screening to identify suppressors of the *fus-1* hyperfusion phenotype, may help address how V-ATPases function in an anti-fusion mode in the nematode and may provide insight into the underlying mechanisms of cell–cell fusion in higher organisms.

An interesting study using *C. elegans* suggested a possible link between heme homeostasis and the regulation of V-ATPases in endosomal trafficking. Heme is an iron-containing porphyrin that serves as a prosthetic group for proteins that perform various biological processes, including gas exchange and detoxification [65]. *C. elegans* is a heme auxotroph and acquires heme from an environmental supply to incorporate into hemoproteins [66]. Genome-wide microarray screening and bioinformatics approaches identified *hrg-1* and *hrg-4*, two of four orthologs that encode heme-binding transmembrane proteins and that are transcriptionally up-regulated when worms are cultured in low concentrations of heme [67]. HRG-1, which is localized in acidic intracellular organelles, requires an acidic pH to bind to heme, whereas HRG-4, which resides in the plasma membrane, does not. RNAi knock-down of either *hrg-1* or *hrg-4* resulted in abnormal sensing of heme, but only *hrg-1* depletion significantly affected heme uptake, suggesting that HRG-4 is involved in intestinal heme uptake, whereas HRG-1 mediates heme homeostasis, possibly by sensing the concentration of heme in the endosomal compartment. This study may shed light on how endocytosis and the trafficking of nutrients is de-regulated in cancer cells to support transformed phenotypes such as invasiveness and cell survival that are correlated with enhanced V-ATPase activity [68]. HRG-1, the only ortholog of HRG expressed in mammals, resides in the endosomal compartment, and its trafficking to the plasma membrane is highly induced in insulin-like growth factor I receptor (IGF-IR)-over-expressing mouse embryonic fibroblasts. Yeast two-hybrid and immunocytochemical studies have revealed that HRG-1 interacts and co-localizes with the cytosolic V1 domain as well as the membrane-embedded Vo domain. Suppression of HRG-1 expression in mammalian cells results in a decrease in V-ATPase activity that causes an increase in endosomal pH and impaired receptor-mediated endocytosis, which are associated with decreases in both cell migration and survival. In addition, HRG-1 enhances heme transport in a V-ATPase-dependent manner because treatment with bafilomycin A mitigates the effect of over-expression of HRG-1 on increased heme transport. Therefore, it has been proposed that HRG-1-mediated regulation of endosomal pH through interaction between HRG-1 and V-ATPases is essential for the function of the endocytic pathway by which cells acquire nutrients, mediate signaling, and internalize cell surface proteins in response to growth factor receptor activation.

6. Tissue-specific expression of "a" subunits and relevance to function

V-ATPase "a" subunits are present in several isoforms that contain information to target V-ATPases to different membranous locations. Two yeast isoforms, Vph1p and Stv1p, localize V-ATPases in vacuolar and Golgi/endosomal membranes, respectively [69,70]. In mammals, there are four isoforms (a1–a4) that are expressed in a tissue-specific manner. The a4 isoform is expressed in renal intercalated cells and epididymal clear cells where it is localized in the apical membrane, while the a3 isoform is expressed in osteoclasts where it is translocated to the plasma membrane from the lysosome upon activation of osteoclasts [26,28]. In the brain, the a1 isoform is present in both synaptic vesicles and the presynaptic plasma membrane in presynaptic nerve terminals, whereas the a2 isoform localizes only to the apical endosomes of the renal proximal tubule cells. It is also clear that a given cell type can express more than one "a" isoform in the plasma membrane, as has been demonstrated for rat vas deferens and epididymal cells [28]. In *C. elegans*, there are four isoforms of the "a" subunit, which also exhibit tissue-specific expression patterns according to GFP reporters [49,54]. The *vha-5* gene is expressed in excretory cells and hypodermal cells, while *vha-6* is expressed exclusively in the intestine. *vha-7* is expressed in hypodermal cells and the uterus, whereas certain alternatively spliced isoforms of *unc-32* are expressed predominantly in neuronal cells. The introduction of *unc-32*, *vha-5*, or *vha-6* dsRNA into gonads halts embryogenesis and development of F1 progeny at the L1 or L2 larval stages, whereas *vha-7* RNAi does not cause an obvious phenotype.

These genetic variations in the "a" subunit of the V-ATPase have been investigated intensively and have led to the discovery of novel functions of V-ATPases. Studies in yeast and fruit flies have suggested that the Vo domain can play a role in membrane fusion independently of the V1 domain. Vacuoles in the *S. cerevisiae* Vph1p mutant, which lacks the "a" subunit, do not fuse efficiently [31], and synaptic vesicles accumulate in the synaptic terminals of neurons lacking the "a" subunit Vha100 in *Drosophila*, presumably due to impaired synaptic vesicle exocytosis [32]. Inefficient membrane fusion in both cases was independent of proton pumping and required SNARE function.

Liegeois et al. [71] cleverly used *C. elegans* to elucidate the differential roles of Vo and V1 domains in apical secretion and excretory organogenesis. They found that the *vha-5*-encoded subunit "a" is expressed mainly in two distinct tissues. One is the H-shaped excretory cell that extends long processes called excretory canals, where osmoregulation takes place; most of the other V-ATPase subunits are robustly expressed in this cell type. The other tissue type is the main epidermal syncytium [72], which controls body length and apical cuticle secretion. With regard to the function of excretory cells and the epidermis, *vha-5 (mc38)* mutants die at the L1 stage, and they are filled with fluid due to a failure in osmoregulation, a phenotype similar to the phenotype caused by laser ablation of excretory cells [72]. The *vha-5* (mc38) mutant has abnormal alae, a specialized structure of the lateral cuticle that is synthesized by seam cells right underneath the alae and the epidermal syncytium. RNAi knock-downs of several V1 subunits (*vha-8* and *-13*) and Vo subunits (*vha-1*, and *-4*) result in changes in alae formation in L1 larvae. RNAi that targets Vo subunits causes the alae of hatchlings to be severely malformed, whereas RNAi that targets V1 results in normal alae that are indistinguishable from those of wild-type. This result indicates that the Vo domain is essential for apical secretion of cuticle components from the main epidermal syncytium. Furthermore, a study using a plasmid rescue strategy of re-introducing mutant forms of VHA-5 with modified charged or bulky hydrophobic residues to *vha-5* mutant animals clearly demonstrated that mutations in the plausible V1-interacting region cause excretory canal malformation, whereas mutations in the transmembrane domain result in abnormal alae. These results indicate the molecular basis for the differential function of *vha-5* in those two distinct tissues. Finally, the secretion of Hedgehog-related protein, which is required for alae formation through an exosomal pathway involving the multi-vesicular body (MVB), is affected in *vha-5* mutants, whereas collagen secretion independent of the exosome is normal, suggesting that the Vo domain could drive the fusion of MVB with the apical membrane by forming a transmembrane complex, as proposed by previous yeast and *Drosophila* studies [31,32].

The unique association of actin cytoskeleton dynamics with the molting process in *C. elegans* suggests that reorganization of the actin cytoskeleton is involved in the regulation of exocytosis mediated by the Vo domain [73]. These worms go through four molting steps before they become young adults. Molting requires bulk transport of cuticle material to the apical surface of the hypodermis, which is at its peak right before the formation of molts. VHA-5 co-localizes with actin filaments that are organized circumferentially around the worm right before molting; these actin filaments are otherwise randomly distributed. The B and C subunits of V-ATPases have been reported to bind to the actin cytoskeleton that is known to have roles in exocytosis such as the positioning of exocytic vesicles [74,75]. It is probable that the circumferential actin organization that occurs ahead of molting facilitates efficient cuticle secretion by recruiting the Vo domain for exocytic fusion of multi-vesicular bodies containing exosomes.

Well-fed *C. elegans* worms exhibit a rhythmic defecation behavior that requires oscillations in intestinal pH [76]. The resting luminal pH in the worm intestine is about 4; it is rapidly elevated up to near pH 6 at every defecation, and then acidification is reestablished prior to the next cycle. In addition, the proton gradient generated by V-ATPase activity between the intestinal lumen and epithelial cells is crucial for proton-coupled nutrient uptake in mammals. The intestine-specific "a" subunit VHA-6 is localized to the apical membrane of intestinal epithelial cells, and RNAi knock-down of this gene prevents full acidification of the intestinal lumen and prevents the uptake of nutrients such as dipeptides and consequent fat storage in the intestine, resulting in larval arrest due to starvation. These observations indicate that the functional aspects of V-ATPases in the gastrointestinal lumen are conserved from the nematode intestine to the mammalian stomach, thus the *C. elegans* model provides an opportunity to study the role of acid–base transport and acute regulation of V-ATPase activity in rhythmic behavior.

7. Conclusions

Studies that utilize a combination of biochemistry, molecular biology, and cell biology provide a wealth of information that can be used to understand the structure and mechanochemistry of V-ATPases. Whereas yeast genetic studies have revealed physiological aspects of this multi-subunit enzyme at the cellular level, various genetic approaches utilizing multicellular organelles have led to new findings concerning the physiological relevance of the functions of V-ATPases in distinct organotypic contexts. Recent genetic and cellular analyses in *C. elegans* have shown that V-ATPases are critical for embryogenesis and larval development, indicating that this multi-subunit enzyme, which is ubiquitously expressed in various tissues at different developmental stages, has essential roles. In addition, the requirement of V-ATPases for osmoregulation by the *C. elegans* excretory cell, which is analogous to mammalian renal tubules, strengthens the notion that the proton pumping activity of V-ATPases is required for proton-coupled material transport. The Vo domain of V-ATPases may also be a component of the membrane fusion machinery; this novel role is supported by studies that have shown that the Vo domain is critical for cuticular secretion and cell–cell fusion in the epidermis of *C. elegans*. Dynamic rearrangement of the actin cytoskeleton correlated with the unique developmental process of molting in *C. elegans* provides evidence of spatio-temporal

regulation of V-ATPases in the secretory pathway. Screening of this heme-auxotrophic nematode also allowed isolation of the heme-binding protein, HRG-1, which interacts with V-ATPase to regulate nutrient uptake. Finally, V-ATPases are also implicated in necrotic cell death and in intestinal rhythmic acidification, which are processes that are important in pathological progression of human diseases such as stroke, neurodegenerative diseases, and acid indigestion. Evolutionarily conserved modes of V-ATPase function, both in organic physiology and disease pathways, raise the possibility that *C. elegans* can be a useful model not only to elucidate plausible new functions of the multi-functional enzymatic complex in a multicellular environment, but also to screen therapeutic agents that can potentially target V-ATPases.

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