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EXPLORING THE MECHANISM OF CLEAVAGE AND SECRETION OF THE MAJOR SPERM PROTEIN (MSP) DOMAIN OF THE VAPB/VPR-1 PROTEIN

by

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A DISSERTATION

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2020

EXPLORING THE MECHANISM OF CLEAVAGE AND SECRETION OF THE MAJOR SPERM PROTEIN (MSP) DOMAIN OF THE VAPB/VPR-1 PROTEIN

HALA ZEIN-SABATTO

GRADUATE BIOMEDICAL SCIENCES

ABSTRACT

VAPB is one of three mammalian VAP proteins. It is a type-II ER transmembrane protein whose N-terminal major sperm protein domain (MSPd) is cleaved and secreted. Since the MSPd faces the cytosol, rather than the ER lumen, how it is cleaved and secreted is not yet known. In humans, P56S is a substitution mutation within the VAPB protein that segregates with cases of familial Amyotrophic Lateral Sclerosis (ALS) and prevents the secretion of VAPB MSPd. The work described in this thesis uses *C. elegans* to study how the N-terminal MSPd of VAPB is proteolytically processed, secreted, and regulated.

C. elegans VPR-1 is the nematode homolog of VAPB. In Chapter 2 of this thesis, overexpression of VPR-1 with an N-terminal FLAG tag revealed that the N-terminal VPR-1 peptide is secreted from intestinal cells to bind the distal gonad. Genome-editing techniques were also used to tag the termini of endogenous *vpr-1*. Immunofluorescent imaging of endogenously tagged VPR-1 revealed a polar localization of VPR-1 termini in intestinal cells. In addition, western blots of these endogenously incorporated epitope tags revealed two stable VPR-1 products. Mass spectrometry determined that the smaller of the two products is the cleaved N-terminal peptide of VPR-1 that spans the MSPd and ends at a conserved leucine at the 156th amino acid position. This indicates that VPR-1 and other VAP homologs may be cleaved at L156 to release the MSPd.

Chapter 3 describes an RNAi screen using *C. elegans* to identify genes required for MSPd cleavage or secretion. *C. elegans* null for *vpr-1* are sterile and can be rescued by *vpr-1* expression in the neurons, germline, or intestine. The brood size of *vpr-1* null worms expressing *vpr-1* in the intestine after RNAi knockdown was assessed for 422 genes. This screen identified a v-SNARE and several proteasome components as potential mediators of VPR-1 MSPd proteolysis and trafficking. Further analysis is needed in order to verify these candidates. In all, results from these two chapters advanced our understanding of MSPd cleavage and secretion and may pave the way to understanding ALS pathology.

Keywords: VAPB, VPR-1, major sperm protein, MSP, *Caenorhabditis elegans*, Amyotrophic Lateral Sclerosis,

DEDICATION

This dissertation is dedicated to the memory of Dr. Michael Miller, my Ph.D. mentor. By leaving us, Mike taught me that while doing well designed research is important for a career in academia, being a great teacher and mentor is how we keep our research alive.

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All thanks and praises are due to God for allowing me to complete this dissertation.

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INTRODUCTION

1.1 Overview

VAPs (VAMP-associated proteins) are a family of conserved endoplasmic reticulum (ER) integral protein that are ubiquitously expressed [1, 2]. VAPs were first identified in *Aplysia californica* through a yeast two-hybrid screen aiming to identify vesicle-associated membrane proteins (VAMP, also known as synaptobrevin) -associated proteins that play a role in neuronal exocytosis of neurotransmitters [3]. VAP proteins play a role in ER functions such as lipid transport and metabolism, membrane trafficking, and the unfolded protein response [1, 2]. They can also function as an extracellular signaling molecule via the cleavage and secretion of their N-terminal peptide in a cell type-specific manner [4, 5]. How intracellular and extracellular functions of VAP proteins are balanced is not yet understood. Cell-specificity and multiple isoforms may be contributing factors that underlie these diverse roles [1].

Several mutations within a human VAP gene, VAPB, have been linked to the neurodegenerative disease Amyotrophic Lateral Sclerosis (ALS) [6-8]. Among these mutations, the P56S point mutation prevents the cleavage and secretion of the VAPB N-terminal peptide and may contribute to the pathology of ALS. However, how the N-terminal peptide is cleaved and secreted is not fully understood [9]. In the work included in this thesis, I used the unsegmented worm, *C. elegans*, as an in vivo model to investigate this issue. My studies identified the cleavage site within the *C. elegans*

homolog of VAPB and discovered several genes that potentially mediate the cleavage of VAPB or the secretion of the cleaved peptide.

1.2 Domain structure of VAP and its conservation across species

VAP proteins are a highly conserved family of eukaryotic proteins found in both vertebrate and invertebrate species [2]. VAPA and VAPB are two well-defined mammalian VAP paralogs [10] and share a sequence identity of 63% [1]. The less studied mammalian VAPC is an alternatively spliced isoform of VAPB that lacks the C-terminal sequence [10]. Most invertebrates have only one identified VAP gene. For example, Scs2p is the VAP protein in the budding yeast *S. cerevisiae* [11], and *vpr-1* encodes for the single VAP protein homolog VPR-1 in *C. elegans* [9]. Despite the species analyzed, all VAP proteins share a higher percentage of amino acid identity at the N-terminus than the C-terminus [1, 10-12]. Scs2p and VAP-33 discovered in *Aplysia* share 26.8% identity and 66.3% similarity in the N-terminal region [11]. Scs2p also shares 16 conserved N-terminal amino acids with all mammalian VAP homologs [1, 11]. Human and *Aplysia* VAP share 50% overall amino acid identity but are 65% identical across the first 121 amino acids [12]. This level of conservation in sequence indicates a conserved function of the VAP N-terminal region across species. This N-terminal sequence is defined as the major sperm protein domain (MSPd), which is named after the nematode major sperm protein (MSP) due to the sequence and structural homology (see below).

The MSPd spans the majority of the VAP protein N-terminus (Figure 1). The MSPd folds into an immunoglobin-like, seven-stranded β-sheet sandwich [13]. This stretch of amino acids shares a homology with the nematode MSP, an abundant sperm

protein that polymerizes to form cytoskeletal networks to help nematode sperm crawl.

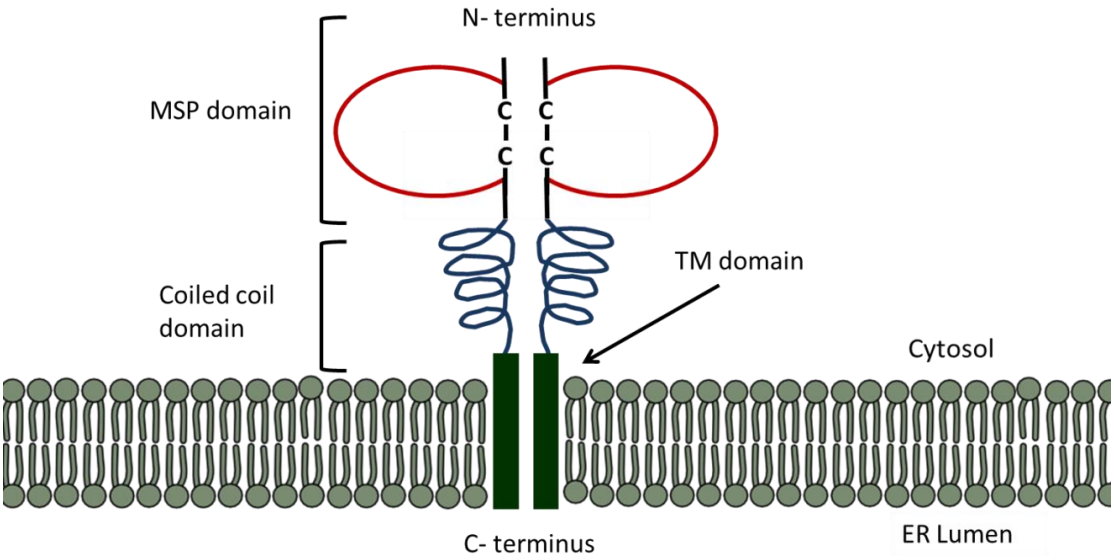

Figure 1: A diagram of VAP protein domains. VAPs have three domains: a transmembrane domain (green), a coiled-coil domain (blue), and the MSP domain (red). Protein here shown as a dimer within the ER membrane.

MSP is also secreted from the sperm to stimulate oocyte maturation and sheath cell contraction [14]. Human VAP MSPd shares 22% sequence identify with nematode sperm MSP [12]. In *C. elegans*, VPR-1 MSPd and sperm MSP are 25% identical and functionally conserved in extracellular signaling [9]. Recombinant VPR-1 MSPd, *Drosophila* VAP, and wild-type human VAP can rescue oocyte maturation and sheath cell contraction in *C. elegans* gonads lacking sperm and MSP [9]. However, the amino acid residues that allow for MSP polymerization in nematode sperm are not conserved in the MSPd [15]. In fact, independent MSP genes have not been identified outside nematode genomes [14, 16]. It is therefore generally believed that nematode MSP genes are derived from a duplication event of VAP genes and acquired sperm-specific modification and functional deviation during the evolution of the nematode [16]. The

conservation between the ubiquitous VAP MSPd and the nematode sperm-specific MSP and the ability of both proteins to signal extracellularly in worm gonad ([9] and see below) suggest a common secretory mechanism and/or downstream receptor(s).

VAP MSPd, unlike nematode sperm-specific MSP, is attached to a transmembrane domain (TMD) (Figure 1) [10-12]. The boundaries of VAP TMD are not well defined but a common transmembrane motif, GXXXG, is conserved near the C-terminus of the protein across species [15]. The longest uninterrupted hydrophobic string of amino acids is a stretch of 18 amino acids between L221 and G238 [15]. The TMD is predicted to play a role in helix-helix interactions among themselves within the membrane bilayer [15]. After Triton X-114 extractions, mouse VAP protein was reported to form SDS-stable dimers [15]. Skehel et al. proposed that this might be due to the interaction of the hydrophobic C-termini. Immunoprecipitation assays with full length and truncated VAP that lacks the TMD confirmed that the TMD is necessary for VAPA and VAPB homo- and heterodimerization [10].

Linking the VAP MSPd and TMD is a coiled-coil domain (Figure 1) [10, 12]. This coiled-coil domain is less conserved across species in comparison to VAP MSPd and TMD [15]. Little is reported on the function of the VAP protein coiled-coil domain. This domain is similar to the coiled-coil domains found in t-SNAREs, the group of proteins involved in membrane vesicle tethering and membrane fusion, but VAP proteins do not participate in the SNARE complex [10, 17]. One group suggested that the coiled-coil domain may be a site for protein binding, specifically with VAMP/synaptobrevin proteins [12]. VAMP/synaptobrevin was identified as a binding patterner of various VAP homologs by in vitro binding and yeast two-hybrid assays [10, 12, 15]. However, the

necessary interacting amino acids within the coiled-coil domain for protein binding have not been identified. Further experimentation, such as a structural-functional assay, is needed to reveal whether this coiled-coil domain has a functional role either for interaction with other partner proteins or for modulation of cleavage and/or secretion of VAP protein MSPd.

1.3 VAP localization and topology

Early studies characterizing VAP proteins in various model systems concluded that VAPs are integral ER transmembrane proteins. VAP was isolated in the membrane fraction of cell lysates and most abundantly extracted from the ER fraction [11, 15, 17]. Conditions under which peripheral proteins are extracted (i.e. high salt concentrations, sodium carbonate, or urea) left VAP proteins associated within the membrane. Conversely, treatment with Triton X-100 or Triton X-114 allowed VAP to solubilize into the detergent phase, consistent with the characteristics of integral membrane proteins [11, 15, 17].

Single-spanning ER transmembrane proteins, such as VAPs, can be categorized into four classes or types based on their orientation and mechanism of insertion into the membrane (Figure 2) [18, 19].

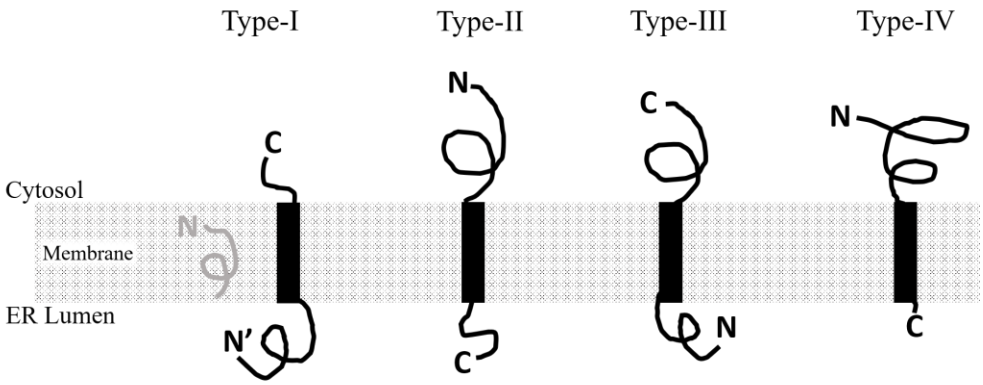

Figure 2: The four types of single-spanning transmembrane proteins.

Type-I membrane proteins have a cleavable N-terminal signal sequence that targets the polypeptide to the ER during translation. They are anchored into the membrane by a stop-transfer sequence that stops the translocation of the polypeptide [19]. The N-terminal signal peptide is cleaved in the ER lumen by signal peptidase to allow for a new N-terminus in the ER lumen and the C-terminus facing the cytosol [19]. Type-II transmembrane proteins have a stretch of non-cleaved hydrophobic residues that allow the protein to be targeted, inserted, and anchored into the ER membrane with its C-terminus in the ER lumen and the N-terminus into the cytosol [19]. Type-III proteins are like type II proteins regarding insertion but have the opposite orientation with a N-terminus in the ER lumen and the C-terminus extending into the cytosol [19]. The fourth type of transmembrane proteins are anchored into the membrane by a C-terminal sequence with much of the protein in the cytosol [19]. Synaptobrevin and SNARE proteins are examples of type-IV membrane proteins and these proteins are thought to be inserted into the membrane post-translationally [19].

Immunofluorescent staining of VAP in yeast demonstrated its colocalization with ER markers [11, 15]. Immunoelectron microscopy further revealed that VAP is present predominantly on the cytosolic side of the ER, specifically on the rough ER cisternae near the cis-Golgi [17]. Protease protection assays of yeast and rat VAP protein indicated that the VAP N-terminus extends into the cytosol and is anchored into the ER membrane by a short hydrophobic C-terminus (TMD) [11, 17]. These experiments confirms that VAP proteins are a type-II transmembrane protein. Immunogold electron microscopic analysis of mouse hippocampal slices showed that VAP proteins are associated with microtubules, membranes of vesicles, and the point of contact between the microtubule

and vesicles [15]. Moreover, in rat, anti-VAP antibodies masking the functionality of VAP proteins resulted in an accumulation of COPI vesicles from the Golgi [17]. Taken together with the evidence from a cell-free intra-Golgi transport system, VAP proteins are thought to participate in the early secretory pathway [17]. Nonetheless, the orientation of the VAP protein calls into question the mechanism by which the cytosolic N-terminus containing the MSPd is secreted to function as an extracellular signal.

1.4 Secretion of the VAP protein N-terminal peptide

Conventionally secreted proteins traverse the ER-Golgi secretory pathway. A signal peptide stops ribosomal translation of the nascent peptide to initiate translocation of the protein into the ER lumen. This is followed by a series of vesicle transport steps to the Golgi and then the plasma membrane. Although extracellular signaling of VAPs implies that it is secreted outside the cells, the topology of VAPs with a cytosolic N-terminus is peculiar and points to a possible secretion route through an unconventional secretion mechanism.

Unconventional secretion mechanisms consist of a group of routes that deviate from the track most secreted eukaryotic proteins take through the ER-Golgi to the plasma membrane [20-23]. There are four principal routes that can be characterized into non-vesicular and vesicular pathways [21, 23]. Non-vesicular mechanisms are utilized by proteins that can translocate across the plasma membrane unassisted or with the help of ABC-transporters. The vesicular pathways involve proteins that utilize autophagosomes or proteins that bypass the Golgi and are generally integral membrane proteins. Of these mechanisms, the one in which VAP N-terminal MSPd peptides are secreted by is not yet known.

Evidence for the cleavage and secretion of the VAP N-terminal peptide was first demonstrated in *Drosophila* and *C. elegans*. *Drosophila* VAP (dVAP) in the imaginal wing discs was tagged at the N-terminus with a FLAG tag and at the C-terminus with an HA tag. The N-terminal tag was detected extracellularly by immunofluorescence whereas the C-terminal HA tag was not detected unless the membranes were permeabilized [9]. This suggests the separation of the N- and C-termini of dVAP prior to extracellular secretion of the VAP N-terminal peptide. Western blots of protein lysate from fly larvae expressing FLAG-dVAP-HA revealed a number of bands between 13-18 kDa that corresponded with the potentially N-terminal peptides, indicating the cleavage event [9]. Human leukocytes have also been shown to express the full length VAP protein (25 kDa) as well as a smaller protein band at 18 kDa while human serum only harbors the 18 kDa presumably cleaved N-terminal peptide [9]. *C. elegans* that expressed mCherry::VPR::GFP fusion protein under the *unc-119* pan-neuronal promoter revealed localization of mCherry and GFP in the neuron cell bodies, but just mCherry in the axons, coelomocytes, body wall muscles, and vulval muscles [24]. Taken together, these results provide evidence of VAP cleavage and the secretion of the N-terminal peptide in various organisms.

1.5 *C. elegans* as a model system

The *C. elegans* is an unsegmented roundworm with a simple anatomy. The germline and the intestine are the two largest organs within the nematode's body cavity. The intestinal cells form a long tube-like structure that extends from the pharynx in the head to the anus at the tail (Figure 3). The gonad takes the form of two U-shaped arms

that wrap around the intestinal tube (Figure 3). Surrounding these two organs is the pseudocoelom that serves as the *C. elegans* circulatory system (Figure 3).

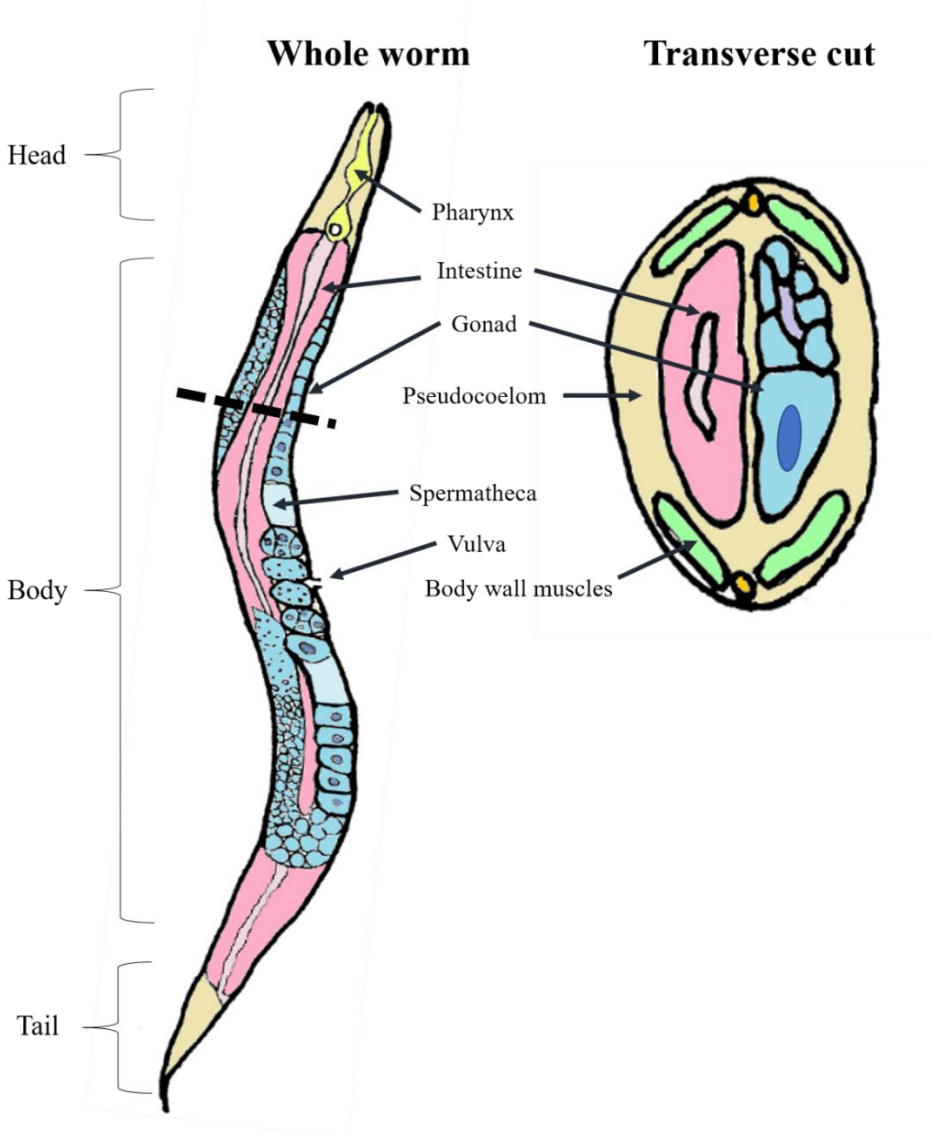

Figure 3: The anatomy of *C. elegans*. Transverse cut taken from the whole worm across the dotted line.

Most *C. elegans* are self-fertilizing hermaphrodites, but 0.1% of a wild population are males. An uninterrupted line of dividing cells is found within the adult hermaphroditic gonad. Starting at the distal end, germ cells are in a mitotically dividing syncytium. These germ cells develop into oocytes as they progress towards the proximal

end of the gonad. Oocytes are fertilized at the spermatheca where hermaphroditic sperm is stored. Once a developing embryo reaches the 24-cell stage, the fertilized egg is laid through the vulva. A single hermaphrodite can produce a brood size of 300 offspring from self-fertilization and nearly 1,000 progenies when cross-fertilized with a male.

There are many advantages to using *C. elegans* as the genetic model in this study. Among these advantages is that *C. elegans* have a transparent epidermis. This permits the visualization and manipulation of several internal processes. In relation to work described in this thesis, the transparent epidermis allows for easy visualization of the gonad syncytium. Visualizing the syncytium under a light microscope permits in-house microinjections of DNA plasmids to make various transgenic and genome-edited lines, such as those described in Chapter 2. The ability for an injected hermaphrodite to self-fertilize and produce hundreds of clones results in multiple lines from one injection session. Although *C. elegans* produce large brood sizes, maintaining large quantities of roundworms is relatively inexpensive and does not require much space. This makes large genetic screens feasible. Therefore, in Chapter 3 of this thesis, 422 genes were screened by RNAi knockdown in *C. elegans*. While knockdown of desired genes can be done by injecting RNAi constructs into the roundworm syncytium, a simpler feeding method, as described in Chapter 3, is possible in *C. elegans*. Finally, of particular benefit to understanding how the VAP N-terminal peptide is cleaved and secreted, much of the groundwork for VAP N-terminal cleavage and secretion has been done in *C. elegans* (see below).

1.6 Cleavage, secretion, and signaling of MSPd VPR-1

Compelling evidence for the cleavage and secretion of the N-terminal peptide of VAP proteins can be found in *C. elegans* studies of the MSP signaling pathway. *C. elegans* has one copy of the VAP gene known as *vpr-1* [9]. *C. elegans* containing the *tm1411* allele in *vpr-1*, which is a deletion of the first two exons of *vpr-1* that leads to complete loss of the VPR-1 protein, have two phenotypes: maternal effect sterility and a muscle mitochondria defect [4, 24]. The sterility is due to defects in distal-tip cell (DTC) migration that result in a truncated gonad with undefinable germ cells [4]. Cell specific expression of *vpr-1* in the neurons, germ cells, or intestinal cells is sufficient for proper gonadogenesis while expression of *vpr-1* under a somatic gonad promoter is not [4]. In Chapter 2 of this work, N-terminal FLAG tagged VPR-1 expressed in the intestinal cells was detected on the distal gonad. Taking together, this indicates that VPR-1 plays a non-cell-autonomous role in gonadogenesis, presumably by the cleavage and secretion of its N-termini. *C. elegans* sperm MSP has been shown to target and bind Ephrin receptors (Eph) expressed on oocytes and sheath cells [25, 26]. Similarly, the VPR-1 MSP N-terminal domain has been shown to compete with Ephrin ligands to bind the Eph receptor extracellular domain [9]. In *C. elegans*, the Eph receptor is known as VAB-1. Absence of *vab-1*, or the ephrin ligand EFN-2, results in DTC migration defects similar to *vpr-1* mutants [9]. While the role of MSP signaling by binding Eph receptors in the gonad is not fully understood, the conserved Eph receptor binding between sperm MSP and VPR-1 MSP further suggests a downstream role for the N-terminal peptide of VPR-1 in gonad development.

Body wall muscle mitochondria, both their form and function, are also defective in *vpr-1(tm1411)* mutants. Wild-type muscle mitochondria are largely found as linear parallel arrays running along the length of the myofibril in *C. elegans* [16]. The spacing between the arrays corresponds with the spacing between the I-bands [24]. In *vpr-1(tm1411)* mutants, the muscle mitochondrial network is thin and branched implicating VPR-1 in mitochondrial localization and balance between fission and fusion [24]. *vpr-1(tm1411)* mutant body wall muscles also contain lipid droplets of triglycerides (TAGs) in young adult worms [27]. These mutant worms have metabolic defects, such as reduced oxygen consumption, reduced ATP production, and slower growth rates [24]. Expression of *vpr-1* under a pan-neuronal promoter rescues both the muscle mitochondrial morphology and function as well as decreases the level of muscle fat droplets in *C. elegans* [24, 27]. Interestingly, expression of *vpr-1* under the myo-3 muscle promoter fails to rescue these defects [24, 27]. The loss of *vpr-1* in the EMS lineage, one of the cell lineages from which muscle cells are derived, does not result in the accumulation of TAG droplets in the body wall muscles. However, *vpr-1* loss in the AB lineage, which generates neurons, and the germ cell lineage does result in lipid accumulation in the muscles, indicating that VPR-1 signals in a non-cell-autonomous way from the neurons and germ cells to the muscles [27]. An RNAi screen in *C. elegans* looking for muscle receptors that may bind the VPR-1 derived signal identified SAX-3/Robo and CLR-1/Lar-like receptors as potential receptors [24]. SAX-3/Robo and CLR-1/Lar-like receptors are expressed in oocytes, motor neurons, and body wall muscles [5, 28, 29]. *sax-3 (ky123)* mutants have an incomplete penetrant muscle mitochondrial phenotype that mimics the mitochondrial phenotype found in *vpr-1(tm1411)* mutants. Intriguingly, loss

of another known SAX-3 ligand, Slit, does not cause the same mitochondrial phenotype, suggesting that binding of the VPR-1 N-terminal peptide containing MSPd to the SAX-3 receptor specifically regulates mitochondrial morphology and localization in the body wall muscles [24]. Loss of *clr-1* was shown to suppress the muscle mitochondrial defects of *vpr-1* and *sax-3* mutants [24]. Therefore, it is proposed that SAX-3/Robo helps the cleaved VPR-1 N-terminal peptide that contains the MSPd to bind to and antagonize CLR-1 signaling in the muscle to modulate mitochondrial positioning along the I-bands [24].

1.7 VAP mutations in human diseases

Several mutations within the human *VAPB* gene have been implicated in cases of familial amyotrophic lateral sclerosis (ALS) [6-8, 30, 31]. ALS is a lethal neurodegenerative disease of the upper and lower motor neurons with an estimated life expectancy of 2 to 3 years after symptom onset [32]. At the present, there is no specific diagnostic test for the disease prior to the onset of symptoms and no known cure [32]. About 90% of cases rise sporadically, but about 10% of the cases are familial [32]. The first ALS-associated mutation within the VAPB gene was discovered in a Brazilian extended family and segregated with the members who were diagnosed with ALS and spinal muscular atrophy (SMA) [6, 33, 34]. This mutation is a point mutation in exon 2 of the *VAPB* gene and lies within the N-terminal MSPd [6]. At the DNA level, a cytosine is substitute for a thymine causing the proline at the 56^{th} amino acid position to be replaced by a serine [6]. This serine substitution removes the kink between two β strands and disrupts hydrogen bonding between β strands on the same β sheet making the sheet more flexible [6]. While the mechanistic relationship between P56S and ALS is not known, it

has been shown that P56S negatively affects the ability of VAPB to mediate the unfolded protein response (UPR) by localizing the protein to non-ER components [35, 36]. The result of misfolded protein build-up in the ER may be an underlying cause of motor neuron degeneration in ALS. P56S VAPB also sequesters wild-type VAPB in insoluble aggregates in a dominant negative fashion [9, 35-37]. A recent study utilized these ER-aggregates as a potential diagnostic biomarker of sporadic cases of ALS [38]. P56S also prevents the cleavage of the N-terminal MSPd peptide from VAPB [37]. This aggregation and protection against proteolytic cleavage may explain why VAPB N-terminal MSPd fragment could not be detected in the cerebral spinal fluid of some sporadic and most bulbar ALS patients [39]. This association between VAPB N-terminal peptide circulation and ALS pathology provides clinical relevance to our understanding of VAPB N-terminal cleavage and secretion mechanism.

1.8 Contribution of this dissertation

This collection of work aims at bridging the gap between our understanding of the intracellular role of VAP and extracellular VAP signaling. The gaps to be addressed center on how the VAP N-terminus is 1) cleaved and 2) secreted. In Chapter 2, a genome edited *C. elegans* line is presented to identify the cleavage sequence in VPR-1 and to provide direct evidence for the secretion of the N-terminal peptide in non-cell-autonomous signaling from the intestine to the distal gonad. In Chapter 3, an RNAi screen in *C. elegans* uncovers potential players in VAP cleavage and secretion. The candidate genes identified include *ykt-6*, a v-SNARE that may play a role in VAP N-terminal peptide secretion, and several proteasomal components that may mediate VAP cleavage. These data provide important insight into extracellular signaling mechanism by

VAP MAPd and may lay groundwork for further investigation into targeting VAP secretion as a therapeutic target for ALS treatment.

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THE TYPE II INTEGRAL ER MEMBRANE PROTEIN VAP-B HOMOLOG IN *C. ELEGANS* IS CLEAVED TO RELEASE THE N-TERMINAL MSP DOMAIN TO SIGNAL NON-CELL-AUTONOMOUSLY.

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ABSTRACT

VAMP/synaptobrevin-associated protein B (VAP-B) is a type II ER membrane protein, but its N-terminal MSP domain (MSPd) can be cleaved and secreted. Mutations preventing the cleavage and secretion of MSPd have been implicated in cases of human neurodegenerative diseases. The site of VAP cleavage and the tissues capable in releasing the processed MSPd are not understood. In this study, we analyze the *C. elegans* VAP-B homolog, VPR-1, for its processing and secretion from the intestine. We show that intestine-specific expression of an N-terminally FLAG-tagged VPR-1 rescues underdeveloped gonad and sterility defects in *vpr-1* null hermaphrodites. Immunofluorescence studies reveal that the tagged intestinal expressed VPR-1 is present in the distal gonad. Mass spectrometry analysis of a smaller product of the N-terminally tagged VPR-1 identifies a specific cleavage site at Leu156. Mutation of the leucine results in loss of gonadal MSPd signal and reduced activity of the mutant VPR-1. Thus, we report for the first time the cleavage site of VPR-1 and provide direct evidence that intestinally expressed VPR-1 can be released and signal in the distal gonad. These results establish foundation for further exploration of VAP cleavage, MSPd secretion, and non-cell-autonomous signaling in development and diseases.

INTRODUCTION

VAMP/synaptobrevin-associated proteins (VAPs) are endoplasmic reticulum (ER) integral proteins that are evolutionarily conserved from yeast to mammals (1). Two homologous genes, VAP-A and VAP-B, exist in mammalian genomes (2). VAP proteins

contain three conserved domains: an N-terminal major sperm protein domain (MSPd), a central coiled-coil domain (CCD), and a C-terminal transmembrane domain (TMD) (1-3). The MSP domain is named after the nematode major sperm protein (MSP), a cytosolic protein that can polymerize to form the filamentous cytoskeleton required for sperm motility in *C. elegans* (1, 4). MSP also has a hormone-like activity to stimulate oocyte maturation and sheath cell contraction, but it is not understood how MSP is released from sperm to act non-cell-autonomously (5-7). In VAP, the amino acid residues responsible for MSP polymerization are not conserved in MSPd, though the domain might contribute to dimerization of VAP (1, 4). The MSPd folds into an immunoglobin-like, seven-stranded β sandwich and is involved in interaction with its protein partners (4, 7, 8). The CCD also participates in binding to other proteins, whereas the TMD anchors the VAP protein into the ER membrane (1). VAP proteins assume a type II membrane protein topology with the N-terminal MSPd extending into the cytosol rather than the ER lumen (9). This allows VAP proteins to function in various intracellular processes such as lipid transport and metabolism, membrane trafficking, the ER unfolded protein response, and microtubule organization (8, 10).

Although most studies focus on intracellular activities of VAP, recent work has suggested that VAP MSPd can function as a secreted non-cell-autonomous signal in *C. elegans*, *Drosophila*, and mammals (11-14). In *Drosophila*, the VAP homolog is cleaved and the N-terminal MSPd is secreted to regulate boutons at neuromuscular junctions. In human, a short, processed form of VAP, but not full length VAP, is detected in serum samples of healthy individuals (11). In *C. elegans*, the MSPd of the VAPB homolog, VPR-1, functions as a permissive signal for gonadogenesis (14). *C. elegans* null for *vpr-*

1 are maternal effect sterile due to the underdevelopment of their gonad. The defects in gonadogenesis can be rescued by transgenically expressing *vpr-1* in the germ line or the nervous system, and to a lesser extent other tissues, indicating a non-cell-autonomous role for VPR-1 in gonad development (14). VPR-1 is also shown to modulate mitochondrial localization and fission/fusion in striated muscles by binding to SAX-3 and Lar-like surface receptors (12, 15). Genetic mosaic analyses and tissue-specific transgenic expression of *vpr-1* demonstrate that VPR-1 from the nervous system and the germ line, but not the muscles, maintains muscle mitochondrial localization and metabolism (12, 15). The results again support the notion that VPR-1 acts in a non-cell-autonomous fashion to regulate development of other tissues.

The relevance of VAP processing and signaling in normal physiology is inferred from studies of VAP-B mutants identified in human patients with amyotrophic lateral sclerosis (ALS) or Lou Geghrig's disease (16-19). While most ALS cases are sporadic, a small percentage of cases have been traced genetically through large extended families (20). Studies of these extensive pedigrees identified several genes, including VAP-B, that segregate with cases of ALS (16-18, 21-25). Among the point mutations in MSPd of VAP-B that are associated with ALS, a proline to serine change at the amino acid 56, known as P56S, is best studied. P56S is a dominant negative mutation that disrupts hydrogen bonding between two β-sheets of MSPd (16). Unlike the wild type VAP, a mutant that mimics P56S substitution in *Drosophila* prevents secretion of MSPd and leads to protein aggregation (11). Moreover, though VAP MSPd is detected in cerebral spinal fluid of healthy individuals, it is largely absent in sporadic ALS patients with bulbar onset (19). These data imply that MSPd of VAP-B might function as a circulating

hormone to signal to and regulate other cells. However, despite this evidence, a direct demonstration of the role of VAP cleavage has not been provided by any of the studies.

In this study, we present *in vivo* evidence for cleavage and secretion of the N-terminal MSPd of VPR-1 from intestinal cells in *C. elegans*. We identify the cleavage site of VPR-1 and show that mutation of this site reduces the activity of VPR-1 to rescue the sterility defects of *vpr-1* mutant. We demonstrate that MSPd released from intestinal cells can reach the distal gonad in *C. elegans* and might regulate gonadal cell development. Our results show for the first time the biochemical evidence of VPR-1 processing site and the importance of VRP-1 cleavage for its long distance signaling and function.

MATERIALS AND METHODS

C. elegans genetics and strains

C. elegans strains were maintained at 20° C and fed NA22 *E. coli* bacteria (26, 27). N2 Bristol (wild type) and VC1478 *vpr-1 (tm1411)/hT2 [bli-4 (e937) let-? (q782) qIs48] (I; III)* were the two strains used in this study. A list of the strains made for this study can be found in supplemental table 1. Phenotypes of *vpr-1 (tm1411)* mutants and transgenic lines were evaluated in *vpr-1 (tm1411)* homozygous F2 progeny from *vpr-1 (tm1411)/hT2* heterozygotes (F0) due to the maternal effect of *vpr-1* (14).

Molecular cloning

All transgenic expression plasmids were made in a TOPO vector backbone with an insert that included *ges-1p* intestinal specific promoter, genomic *vpr-1,* and *vpr-1*

UTR. PCR was used to amplify insert sequences from genomic DNA with primers designed for Gibson Assembly. All constructs were ligated by Gibson Assembly (New England Biolabs). FLAG and HA tags were inserted by designing overlapping primers that included the desired tag sequence and by adding corresponding forward and reverse oligos in the Gibson Assembly mix. Constructs were transformed into TOP10 cells for amplification. All constructs were confirmed by sequencing.

The Cas9 DNA repair template to insert a FLAG tag at the N-terminus of the endogenous *vpr-1* loci was a construct that contained a 2kb left homology arm of *vpr-1*::double-FLAG tag sequence:: 2kb right homology arm of *vpr-1*. The Cas9 targeting guide sequence was 5'- CTACCCACTAAGCACTGGCC -3'. The Cas9 DNA repair template to insert an HA tag at the C- terminus of the endogenous *vpr-1* loci contained an insert of 2kb left homology arm of *vpr-1*:: double-HA tag sequence:: 2kb right homology arm of *vpr-1*. The Cas9 targeting guide sequence was 5'- CTCTCCTCATCGGGCTTATT -3'. The single guide RNA (sgRNA) plasmid was derived from Addgene plasmid 46169. PCR was used to amplify the entire sgRNA backbone except for the 20 bp guide sequence. The 20 bp guide sequence was incorporated into the sgRNA backbone by overlapping primers designed for Gibson Assembly. Gibson Assembly was used to ligate all constructs. Constructs were transformed into TOP10 cells for amplification. All constructs were confirmed by sequencing.

CRISPR/Cas9

CRISPR/Cas 9 methods were performed as previously described (28). Cas9 plasmid (75 ng/µl), *vpr-1* sgRNA plasmid (50 ng/µl), *vpr-1* repair template plasmid (100

ng/µl), co-CRISPR *unc-119* sgRNA plasmid (50 ng/µl), co-CRISPR *unc119* repair template (50 ng/µl), and *myo-3p::mitoGFP* co-injection marker (30 ng/µl) were injected into young adult *unc119(ed3)* hermaphrodite gonads. Progeny was screened for rescue of the *unc-119* movement defect and loss of *myo-3p::mitoGFP*. Individual worms were isolated repeatedly to ensure 100% segregation. PCR and sequencing were used to confirm the insertion of FLAG and HA tags.

Transgenics

Desired plasmids (50µl) were injected into young adult *vpr-1 (tm1411)/hT2* hermaphrodite gonads to generate transgenic *C. elegans* lines. The *myo-3p::mitoGFP* transgene was co-injected (30µl) as a marker for successful injections. *C. elegans* transgenic strains that transmitted at ≥60% were used for assays. Multiple independent transgenic lines for each plasmid combination were analyzed.

Fertility and Brood size Assays

One L3-L4 staged *C. elegans* hermaphrodite was placed to an agar plate seeded with NA22 *E. coli* and transferred to a fresh plate with bacteria every 24 hours for 6 days. Live progeny was counted on preceding plate after 48 hours of incubation at 20°C. Brood counts from *C. elegans* that died before day 5 were eliminated. *C. elegans* that produced no progeny were considered sterile.

Statistical Tests

Two-tailed Student's *t*-tests without the assumption of equal variance were conducted using Prism. Chi-Square tests were conducted using Microsoft Excel.

Immunofluorescences and Imaging

Adult *C. elegans* were washed in M9 buffer and decapitated with a syringe needle to expel the intestine. Tissues were fixed overnight at 4°C in 4% formalin (Sigma-Aldrich HT5011) then blocked for 1 hour at room temperature in PBS+ (1x PBS with 0.1% Triton X-100, 1% bovine serum albumin, 1% donkey serum, and 0.02% sodium azide). Rabbit anti-FLAG primary antibody (Thermo Fisher Scientific, Cat. No. 740001) was diluted in PBS+ at a 1:8,000 dilution and tissues were incubated occurred overnight at 4°C. Secondary antibody incubation with Alexa Fluor 555 goat anti-rabbit IgG (Thermo Fisher Cat no. A21428; 1;10,000 dilution) was for 1 hour at room temperature. Tissues were washed three times for 5 minutes each with PBS with 0.1% Triton X-100 in between antibody incubation periods. The final wash contained Hoechst nuclear stain (Thermo Fisher 33258) at 1:1,000 dilution to visualize nuclei. Confocal images were taken with a Nikon Ti2 spinning disk confocal with a Yokohama X1 disk and an Orca Flash4.0 sCMOS (Hamamatsu). Images were acquired in Nikon Elements AR 5.0.

Immunoprecipitation

Unsynchronized *C. elegans* were collected to prepare the worm lysate for immunoprecipitation. *C. elegans* were washed with M9 buffer to remove any external trace of *E. coli* that would affect imaging quality. A 5mL pellet of *C. elegans* carcasses was frozen down at -80°C for up to one week. An equal amount of worm lysis buffer containing 25mM Hepes-NaOH, 150mM NaCl, 0.2mM DTT, 10% glycerol, and 1% Triton X-100 supplemented with protease inhibitor cocktail (Roche Applied Sciences, Germany) was added to the frozen carcass pellet. To homogenize the worm carcasses into

a lysate, the worm carcasses and worm lysis buffer mixture was blended using 0.5 mm zirconium oxide beads and a Bullet Blender 5 homogenizer (Next Advance Inc., NY, USA) at speed 10 for 5 minutes. The homogenate was then transferred to a 15 mL Falcon tube and centrifuged at 4,500 × g for 10 min at 4 °C. The clarified supernatant (10mL) was incubated with either EZview Red Anti-FLAG M2 Affinity Gel (Sigma-Aldrich F2425) or EZview Red Anti-HA Affinity Gel (Sigma-Aldrich E6779) beads (500µl) for 2 hours at 4°C. Beads with bound proteins were washed three times with 1x TBST buffer. Proteins bound to the anti-FLAG gel beads were eluted by 4mg/mL 3x FLAG peptide (Sigma Aldrich F4799). Proteins bound to the anti-HA gel beads were eluted by 2x SDS sample buffer.

SDS-PAGE and western blotting

Protein samples were boiled in 2x SDS sample buffer for 5 minutes and loaded into 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad 456). If used for Western Blots, SDS gels where transferred to immobilon-P PVDF membranes (Milipore). Immunoblots were blocked for 1 hour with 1% non-fat dry milk in 1x TBS buffer containing 0.1% Tween-20 (TBST). The membranes were then probed overnight at 4°C with rabbit anti-FLAG antibody (Thermo Fisher Scientific 740001; 1:8000) diluted in 1% non-fat dry milk. Membranes were then washed three times with TBST and incubated with IRDye 800CW Donkey anti-Rabbit secondary antibodies (Li-cor 925-32213) for 1 hour at room temperature. The signal was visualized by Licor Odyssey CLx. If SDS gel was submitted for in-gel digestion and mass spectrometry (below), then gel was incubated in Coomassie blue stain overnight at room temperature and destained with DI water.

In-gel digestion

SDS gel bands were excised and excess stain was removed by an overnight wash of 50% 100 mM ammonium bicarbonate/50% acetonitrile. After destaining, disulfide bonds were reduced by 25 mM dithiothreitol at 50°C for 30 minutes. Alkylation of the free thiol groups was carried out with 55 mM iodoacetamide for 30 minutes in the dark. The excess alkylating agent was removed, and the gel pieces were washed twice with a 100 mM ammonium bicarbonate for 30 minutes. The gel pieces were evaporated to dryness in a SpeedVac (Savant) before enzymatic digestion. A 12.5 ng/ml concentration of trypsin (Promega Gold Mass Spectrometry Grade, which is modified to be autolytic resistant and includes TPCK to inactivate any chymotrypsin activity.) was added to each gel sample and incubated overnight at 37°C. Peptides were extracted from the gel pieces using a 1:1 mixture of 1% formic acid and acetonitrile twice for 15 minutes. Extracts were pooled and evaporated to dryness. The samples were then resuspended in 30 mL of a 0.1% formic acid prior to mass spectrometry analysis.

NanocHiPLC-tandem mass spectrometry

An aliquot (5 mL) of each digest was loaded onto a Nano cHiPLC 200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å reverse-phase trap cartridge (Eksigent, Dublin,CA) at 2 mL/min using an Eksigent autosampler. After washing the cartridge for 4 minutes with 0.1% formic acid in dd$H_2$0, the bound peptides were flushed onto a Nano cHiPLC column 200 µm x 15 cm ChromXP C18-CL 3 µm 120 Å (Eksigent, Dublin,CA) with a 45 minutes linear (5-50%) acetonitrile gradient in 0.1% formic acid at 1000 nL/min using an Eksigent Nano1D+ LC. (Dublin, CA). The column was washed with 90% acetonitrile-

0.1% formic acid for 10 minutes and then re-equilibrated with 5% acetonitrile-0.1% formic acid for 10 minutes. The SCIEX 5600 Triple-Tof mass spectrometer (AB-Sciex, Toronto, Canada) was used to analyze the protein digest. The IonSpray voltage was 2300 V and the declustering potential was 80 V. Ionspray and curtain gases were set at 10 psi and 25 psi, respectively. The interface heater temperature was 120°C.

Eluted peptides were subjected to a time-of-flight survey scan from 400-1250 m/z to determine the top twenty most intense ions for MSMS analysis. Product ion time-of-flight scans at 50 milliseconds were carried out to obtain the tandem mass spectra of the selected parent ions over the range from m/z 100-1500. Spectra are centroided and de-isotoped by Analyst software, version TF (Applied Biosystems). A beta-galactosidase trypsin digest was used to establish and confirm the mass accuracy of the mass spectrometer.

Protein Pilot 4.5 Search Queries

The tandem mass spectrometry data were processed to provide protein identifications using an in-house Protein Pilot 4.5 search engine (SCIEX) using the Homo sapiens UniProt protein database and using a trypsin plus missed cleavage digestion parameter. Potential novel cleavage site peptide spectra were evaluated via de novo sequencing to verify the validity of the search results.

RESULTS

Expression of *vpr-1* in intestinal cells rescues *vpr-1* null sterile phenotype

C. elegans null for *vpr-1*, or *vpr-1(tm1411)*, are maternal effect sterile (Figure 1A-C). The *vpr-1 (tm1411)* line is maintained as heterozygous hermaphrodites containing a hT2 balancer chromosome, a translocation between chromosomes I and III that includes a GFP pharyngeal marker (Figure 1A). Homozygous *vpr-1* null progenies are identified by the loss of the GFP pharyngeal marker during self-fertilization. These F1 *vpr-1* null worms are viable and fertile due to material deposit of VPR-1. However, the second generation of *C. elegans* from these homozygous *vpr-1* null hermaphrodites produce no progeny due to maternal effect sterility (Figure 1A-C), and the gonad of these F2 worms is underdeveloped (Figure 1E) when compared with age-matched wild type animals (N2, Figure 1D). We have previously shown that expression of *vpr-1* in the intestinal cells is sufficient to rescue the *vpr-1* defects in gonadogenesis (14). Here we further characterized the level of rescue with intestine-specific transgenic expression of *vpr-1*. We showed that expression of *vpr-1* in intestinal cells by the *ges-1* promoter results in a significant rescue in the F2 population with fertility detected in 60% of the worms (Figure 1B), and the brood size is also increased significantly (Figure 1C). These fertile hermaphrodites have no defects in gonad morphology at adulthood (Figure 1F). Expressing *vpr-1* in one cell lineage to rescue an unrelated phenotype of another cell lineage led us to previously propose a non-cell-autonomous signaling function of VPR-1 (11-14). Our results here support a non-cell-autonomous role of VPR-1 in gonad and germ cell development.

VPR-1 from intestinal cells travels to the surface of the distal gonad

Different mechanisms might account for the non-cell-autonomous function of VRP-1. The protein might be release from intestine to signal in the gonad directly, or it

might regulate a secondary extracellular signal to modulate worm sterility. To further explore the mechanism of VPR-1 function, we proceeded to examine the localization of VPR-1. For this purpose, we tagged *vpr-1* with a double-FLAG epitope at the N-terminus and transgenically expressed it using the intestinal *ges-1* promoter (referred to as *ges-1p::FLAG vpr-1*). This N-terminal tag sits adjacent to the MSPd-coding region of *vpr-1*. Functional assay revealed that construct does not show significant difference to rescue *C. elegans* fertility or brood size when compared with the untagged *ges-1p::vpr-1* transgenic worm lines (Figure 1B-C). Moreover, no noticeable defects were detected in gonad morphology in *vpr-1* null adults rescued by the tagged VPR-1 (Figure 1G).

Immunofluorescent staining of *C. elegans* intestinal cells with an anti-FLAG antibody revealed that the N-terminal FLAG-tagged VPR-1 localizes to the basolateral membrane of the intestinal cells (Figure 2). No localization was found around the apical membrane that lines the intestinal lumen (Supplemental Video 1). This basolateral localization implies that FLAG-VPR-1 is concentrated at subcellular positions that are close to the pseudocoelom or the circulatory system of the worm. This localization reveals that if VRP-1 is secreted from intestinal cells, it will likely enter the pseudocoelom, which is adjacent to the gonad, rather than into the intestinal lumen.

To further investigate possible secretion of FLAG-VPR-1 from *C. elegans* intestinal cells, we examined its localization around other cell types in *ges-1p::FLAG vpr-1* transgenic worms. Previous work showed that the VPR-1 N-terminal MSPd binds to an unknown receptor on the somatic gonad to signal for proper gonad development (14). This suggest that the FLAG-tagged N-terminus of VPR-1 might reach the gonad if it is indeed released from the intestinal cells.

Immunofluorescent imaging revealed FLAG signal on the distal gonad of dissected *ges-1p::FLAG vpr-1* transgenic worms (Figure 2). Z-stack imaging showed that the N-terminal FLAG signal of VPR-1 is restricted to the surface of the somatic gonad (Supplemental Video 2). No FLAG staining was detected inside the syncytium, on oocytes, fertilized embryos, or in the proximal gonad. Taken together, the results provide strong evidence that N-terminal FLAG-tagged VPR-1 is secreted from intestinal cells and travel to the distal gonad to modulate gonadal development.

The N-terminal VPR-1 is cleaved at residue leucine 156

Previous work from our lab and others suggest that the VAP N-terminal MSP domain is cleaved and secreted whereas the full length VAP remains as an intracellular protein (11, 12). Therefore, we expected that the FLAG staining that we detected on the distal gonad of transgenic *ges-1p:: FLAG vpr-1* hermaphrodites might reflect a cleaved product of VPR-1 that was expressed in the intestine. However, as a type II ER membrane protein, it is not clear where the cleavage might occur. We therefore set out to investigate the issue.

To distinguish the full length and the cleaved protein products, we applied CRISPR/Cas9 technology to tag the endogenous VPR-1 with a double-FLAG tag at its N-terminus and a double-HA tag at its C-terminus (*vpr-1(xm17)*; Figure 3A). Tagging of the endogenous *vpr-1* allele did not result in obvious defects in gonad morphology (Figure 3B-C), though the resulting hermaphroditic *C. elegans* did show slightly decreased brood size (Figure 3D). Nevertheless, as the worms are fertile and appear

healthy, we believe that these terminal tags did not drastically impair endogenous function of VPR-1.

Using the endogenously tagged *vpr-1* line, we cultured the worms and made protein extract from unsynchronized adults. We then performed immunoprecipitation (IP) assay using either α-HA or α-FLAG antibody-conjugated agarose beads and analyzed the protein containing the N-terminal FLAG tag in the pulldown by Western Blot analysis. A protein band corresponding to the full sized VPR-1 at 37kDa was detected from the elutes using either antibody (Figure 3E-F). With α-FLAG beads, we also observed a smaller protein band at less than 25kDa that contained the N-terminal FLAG tag (Figure 3F). This band was not detected in the precipitate from α-HA IP (Figure 3E). The result indicates that the smaller band is the cleaved N-terminal product. To biochemically characterize this product, we excised this smaller band from the protein gel and subjected it to mass spectrometry analysis using conventional trypsin digestion (Figure 4). We recovered several peptides that matched the sequences within the N-terminal MSPd, but none matched within the CCD or the TMD. The most C-terminal peptide that we recovered has the amino acid sequence of NEDSFASSGQAQEL with a 99% confidence level using the Protein Pilot 4.5 search engine (29) (Figure S1). This sequence ends at leucine 156 (Leu156), which is an amino acid at the start of the predicted CCD. The amino acid adjacent to the cleavage site are highlighted to illustrate the homology in this region (Figure S2). The result suggests that VPR-1 is cleaved after Leu156 to produce the N-terminal peptide that contains the MSP domain.

Cleavage at Leu156 is required for VPR-1 function

To test the functionality of VPR-1 cleavage at Leu156, we made a point mutation changing leucine 156 to an alanine (L156A) in the *ges-1p::FLAG vpr-1* construct. Two independently derived transgenic *C. elegans* lines were obtained. The fertility of these worms, the gonad morphology, and the localization of the N-terminal FLAG tag of the VPR-1 protein were then analyzed. Unlike the wild type FLAG:VPR-1 transgenic hermaphrodites, all of the hermaphrodites from the first line and the majority of the hermaphrodites from the second line were sterile (Figure 5A). An underdeveloped gonad, similar to that seen in *vpr-1* null *C. elegans*, was observed and likely underlay the sterility phenotype (Figure 5B and C). The small percentage of fertile hermaphrodites from the second line developed a U-shaped somatic gonad but presented slight defects in the germ line shown by the lack of a continuous stream of developing oocytes (Figure 5D). Protein expression of FLAG:VPR-1 with L156A substitution was confirmed by immunofluorescent staining with anti-FLAG antibodies. Similar to the wild type FLAG:VPR-1 protein, the FLAG:VPR-1(L156A) localized to the basolateral membrane of intestinal cells (Figure 5C; Supplemental Video 3). The defective gonad from the first line prevented us from further examination of gonadal FLAG signal. However, inspection of the distal gonad of the fertile hermaphrodites from the second line showed no FLAG signal. These results suggest that cleavage at leucine 156 is required for the release of VPR-1 from intestinal cells to travel to and function in the gonad in order to regulate gonadal development and worm fertility.

DISCUSSION

As type II ER membrane proteins, VAPs regulate diverse intracellular processes, such as lipid transport, membrane trafficking, and microtubule organization. However,

recent studies from our lab and several other groups also reveal an unexpected function of VAP in extracellular signaling (11, 12, 14, 15). This non-cell-autonomous activity seems to be mediated by the conserved N-terminal MSP domain, which is proposed to be cleaved and secreted to signal to other cells. However, VAP proteins lack of a signal peptide characteristic of proteins secreted through the ER-Golgi pathway, and the topology of VAP proteins posits the MSPd in the cytosolic side rather than facing the ER lumen (9). This indicates that VAPs do not utilize the conventional protein secretion pathway through the vesicular network, but instead adopt an unconventional mechanism to release its N-terminal domain after cleavage.

The evidence for secreted, non-cell-autonomous function of VPR-1 comes from genetic studies in *C. elegans*. VPR-1 functions as a permissive signal for gonad development (14). Using tissue-specific transgene to rescue *vpr-1* null mutants, our lab has shown previously that expression of *vpr-1* in either the nervous system or the germ line strongly rescues deficiencies in gonadogenesis. However, expression of *vpr-1* in muscle or hypoderm does not lead to phenotypic rescue (14). This suggests that secretion of VPR-1 might be regulated in a cell type-specific fashion. This notion is supported by work performed in *Drosophila* where a VAP homolog is also processed and secreted in specific populations of cells (11). In both *Drosophila* and *C. elegans*, the MSPd of VAP homologs can be released from the neurons (11, 12). However, no study has been reported to examine whether intestinally expressed *vpr-1* also rescues the gonad defects in the *vpr-1* null mutants via direct cleavage and secretion of the MSPd. Our immunofluorescent staining experiment shown in this study reveals that the intestinal FLAG-tagged VPR-1 is indeed secreted and travels to the distal gonad. No staining is

observed in the proximal gonad, implying that retaining of tagged VPR-1 at the distal gonad is likely mediated by specific binding of VPR-1 N-terminal peptide to the surface receptor(s) expressed in cells at distal gonad. Though our interpretation of the observed FLAG signal is that it represents the MSPd of VPR-1 based on our data on VPR-1 cleavage, it is possible, though unlikely, that the staining represents a larger fragment or the full length VPR-1. A congruent C-terminal tag in the transgenic *ges-1p::FLAG:vpr-1* line could help in distinguishing the cleaved N-terminal peptide from the whole VPR-1 protein in intestinal cells. Unfortunately, *vpr-1* tagged simultaneously with double-FLAG on the N-terminus and double-HA on the C-terminus and transgenically expressed in either the intestine using the *ges-1* promoter (Figure 1C) or neurons using the *glr-5* promoter did not rescue the sterile phenotype of *vpr-1* null worms. Our preliminary observations of these transgenic worms suggest that adding the C-terminal HA tag causes transgenically expressed *vpr-1* to aggregate. This result differs from that when we tag the endogenous protein in a similar way using CRISPR/Cas9-mediated knock-in of the epitopes, as the hermaphrodites expressing the tagged protein are fertile (Figure 3D). The difference in VPR-1 protein behaviors might due to distinct expression levels of the protein from the endogenous locus versus from the regulatory elements of an intestinal gene. As VAP proteins can form homo- and hetero-dimers, high level expression might drive the protein to form aggregates. This notion agrees with previous reports on gain-of-function point mutations in the N-terminus of mammalian VAPB which cause protein aggregation and prevent secretion (11, 30-34). Because of protein aggregation of C-terminally HA-tagged VPR-1, we will not be able to examine the localization of C-terminal tag. The ubiquitous expression of endogenously tagged VPR-1 protein also

prevents us from distinguishing between cleaved or full length VPR-1 in mediating non-cell-autonomous function of VPR-1. Although our N-terminally FLAG-tagged VPR-1 that is expressed in the intestine rescues gonadogenesis, the effect on the brood size of these vpr-1 null mutants bearing the transgene is quite modest compared to when the endogenous gene is tagged in vivo. The result might suggest that expression of *vpr-1* in multiple tissue types might be required to achieve optimal extracellular VPR-1 levels to rescue the brood size to a greater extent. Future work involving developing transgenic lines that simultaneously express differentially tagged *vpr-1* under both intestinal and neuronal promoters can provide additional insight into regulation of MSPd signaling from different tissues.

In this work, we identify for the first time the cleavage site of VPR-1 in *C. elegans*. By mass spectrometry, we show that VPR-1 is cleaved at leucine 156 to release an N-terminal peptide that consists of the entire MSPd and a short linker between MSPd and CCD. The alignment of VAP sequences across multiple species reveals that amino acids around L156 seem to be conserved. The mammalian homologs of VPR-1 in human and mouse harbor a conserved leucine residue at the amino acid position 157 (Figure S2). Highlighted in this figure, the residues surrounding the cleavage site are similar in the other species (Figure S2). Future work involving isolation and mass spectrometry of the short VAP products from these organisms will promise to provide additional insight into possible conservation of VAP cleavage during evolution. Although we attempted to provide biochemical evidence to show an absence or dramatic decrease of MSPd 25 kDa protein due to the L156A substitution mutation, this was technically challenging given

the maternal effect of *vpr-1* and the small brood size of the rescued *ges-1p::FLAG:vpr-1(L156A)* animals.

The identification of the cleavage site of VPR-1 in *C. e*legans offers a great opportunity to gain further understanding of possible enzymes involved in VAP/VPR-1 processing. Unlike ligand processing in the secretory pathway where Furin family of proteases are often involved to recognize di-basic residues, the cleavage site at leucine does not conform to any consensus protease recognition motifs. This implies that the enzyme(s) involved might recognize certain amino acid features, such as bulky hydrophobic amino acids, rather than particular amino acid residues. Among the cytosolic proteases, chymotrypsin seems to have such characteristics. It is possible that two processing events occur to generate the N-terminal peptide ending at L156. *C. elegans* have 9 identified serine-type proteases known as the TRY family of proteins. The best studied of these proteases is TRY-5, which was identified as a secreted serine-protease that is required for the activation of male derived sperm in *C. elegans* (35). One of TRY proteins might act in the cytosol to cleave the N-terminal MSPd of VPR-1 in the intestine. The potential cleavage by TRY might persist in VPR-1(L156A) though with reduced efficiency, hence the mutant hermaphrodites might have impaired, but not null, cleavage of VPR-1 in the intestinal cells. Therefore, though the released N-terminal MSPd cannot be detected in our immunofluorescence studies, it might be sufficient to function in rescue of gonadogenesis and fertility in a small percentage of animals.

Our data also bring up the issue of cell-specific regulation of VPR-1 cleavage and secretion. In theory, both steps can be regulated in a cell type specific fashion. Chymotrypsin-like enzymes exist in many cell types in *C. elegans*, but non-cell-

autonomous rescue of *vpr-1* null phenotypes occurs only when the protein is expressed in particular tissues. This suggests that either tissue-specific proteases participate in the cleavage event, or the activity of the protease is regulated by a tissue-specific factor, or secretion of cleaved MSPd is controlled in a tissue-specific manner. It is interesting to note that the MSP protein after which the domain is named can also have non-cell-autonomous hormonal function to stimulate oocyte maturation, even though it is a cytosolic protein with no signaling peptide. The mechanism for MSP secretion is not understood, but it is tempting to speculate that MSP and MSPd might utilize a similar process to exit the cell and enter the extracellular space. If this is true, we may expect that cleavage of VAP is the rate limiting step, which might then be followed by constitutive secretion of the MSPd. This model can be tested by expression of a synthetic construct containing the N-terminal fragment only and followed by examination of its secretion and function. Alternatively, VAP cleavage and secretion might be linked intimately and both require tissue-specific regulation. Further studies will help to resolve the different models.

In summary, we present *in vivo* results in this work to show that the N-terminus of *C. elegans* VPR-1 is cleaved at a specific site at leucine 156 to release an N-terminal peptide that encompasses the MSPd. The MSPd-containing product is secreted from the intestinal cells into the pseudocoelom and bind the distal gonad to function as a non-cell-autonomous signal to regulate gonad development and hermaphrodite fertility. We demonstrate that mutation of the cleavage site results in absence of the gonadal MSPd signal and a much reduced ability of the mutant VPR-1 to rescue the *vpr-1* null mutant. Our identification of the novel cleavage sequence in VPR-1 in *C. elegans* paves the way

for investigation of the cleavage enzyme(s) responsible for the unconventional processing and secretion of VAP. These results, together with our previously published work, open the opportunity to further explore the role of MSPd cleavage and secretion in neurodegenerative diseases such as ALS.

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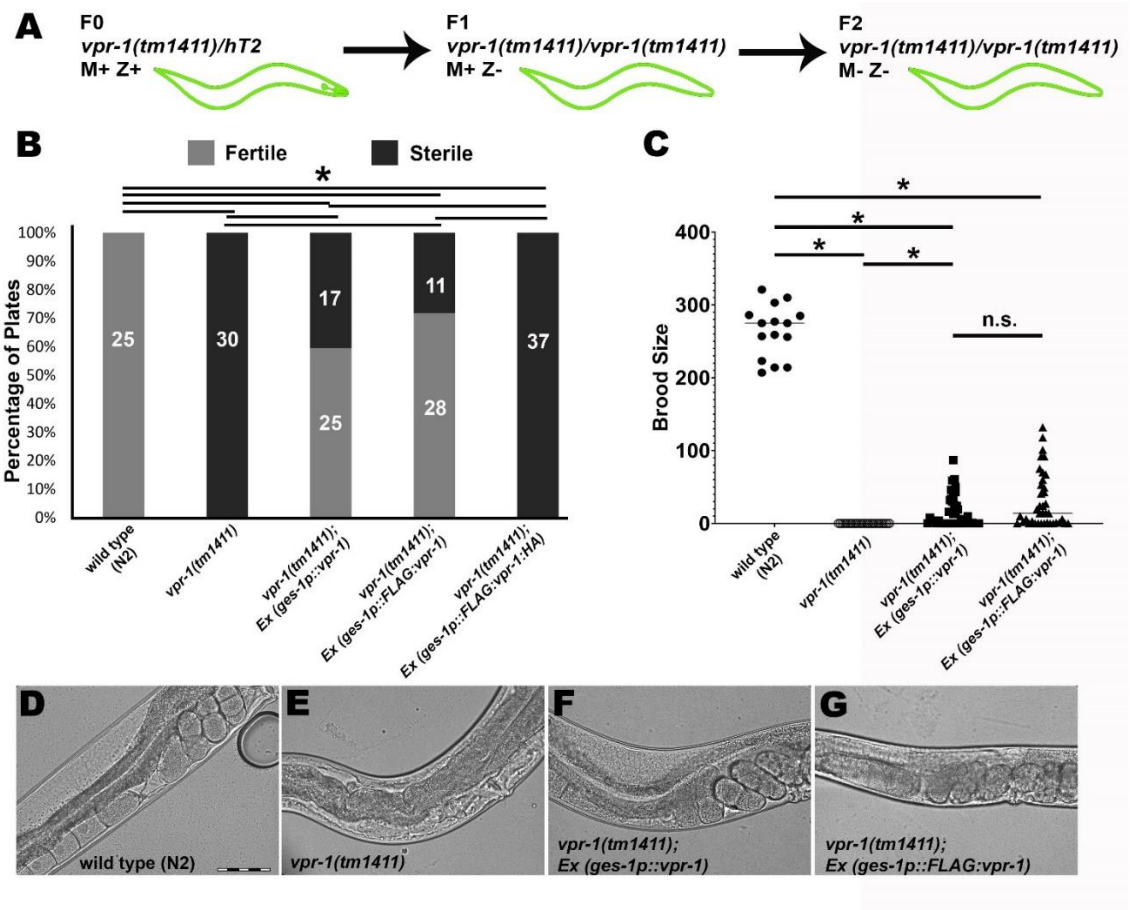

Figure 1: *vpr-1(tm1411)* maternal effect sterility in *C. elegans* can be rescued by intestinal expression of *vpr-1*. A) The *vpr-1(tm1411)* deletion allele is maintained by the hT2 balancer. The sterility phenotype of null *vpr-1 C. elegans* is assessed in F2 *vpr-1(tm1411)* hermaphrodites due to the maternal effect of *vpr-1*. B) Graph represents fertility assay. N2 wild type *C. elegans* are 100% fertile (25/25) while 0% of *vpr-1(tm1411)* hermaphrodites are fertile (0/30). Majority of *vpr-1(tm1411); Ex (ges-1p::vpr-1*) (25/42) and *vpr-1(tm1411); Ex (ges-1p:: FLAG:vpr-1)* (28/39) hermaphrodites are fertile and brood sizes are not significantly different. *C. elegans* expressing *Ex (ges-1p::FLAG:vpr-1:HA)* in the *vpr-1(tm1411)* null background are completely sterile (0/37) (*p>0.01, Chi-Square Test) C) Graph represents brood size count. Brood size of transgenic worms was compared to wild type. *vpr-1(tm1411)* hermaphrodites produce no progeny while intestinal expression of untagged or FLAG-tagged *vpr-1* rescues this sterility phenotype (*p>0.0001, Student's t-test) D-G) *C. elegans* adult gonad images of the various genotypes assayed. Images captured with a 40x Plan Fluor 1.3NA objective. Scale bar: 50μm.

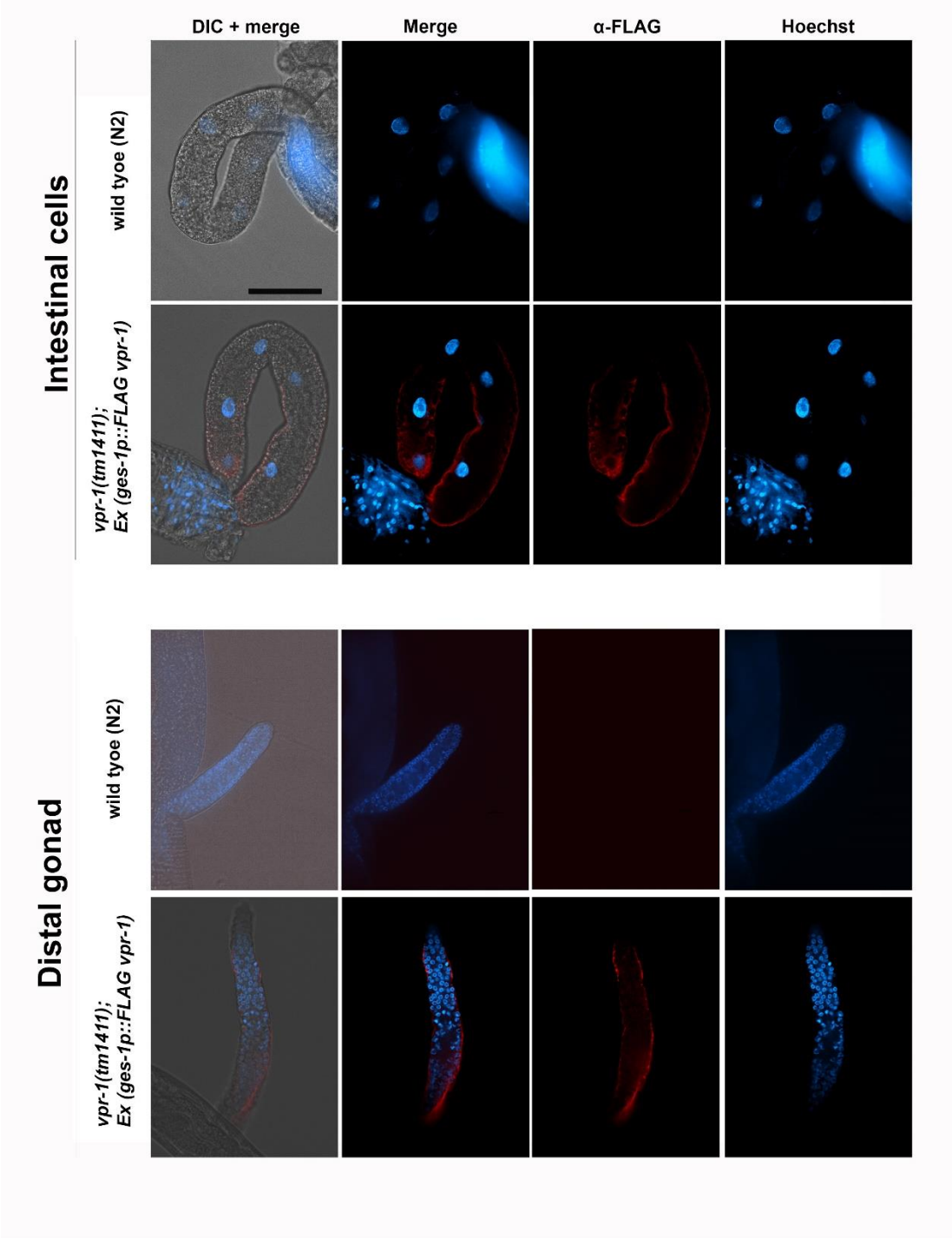

Figure 2: VPR-1 is secreted from the intestine and binds to the distal gonad. VPR-1 localization is visualized by its N-terminal FLAG tag. In the intestine, VPR-1 localizes along the basolateral membrane of intestinal cells. VPR-1 was also detected along the surface of the distal gonad indicating protein secretion from the intestinal cells. Images captured with a 60x APO Tirf 1.49NA objective. Scale bar: 50μm

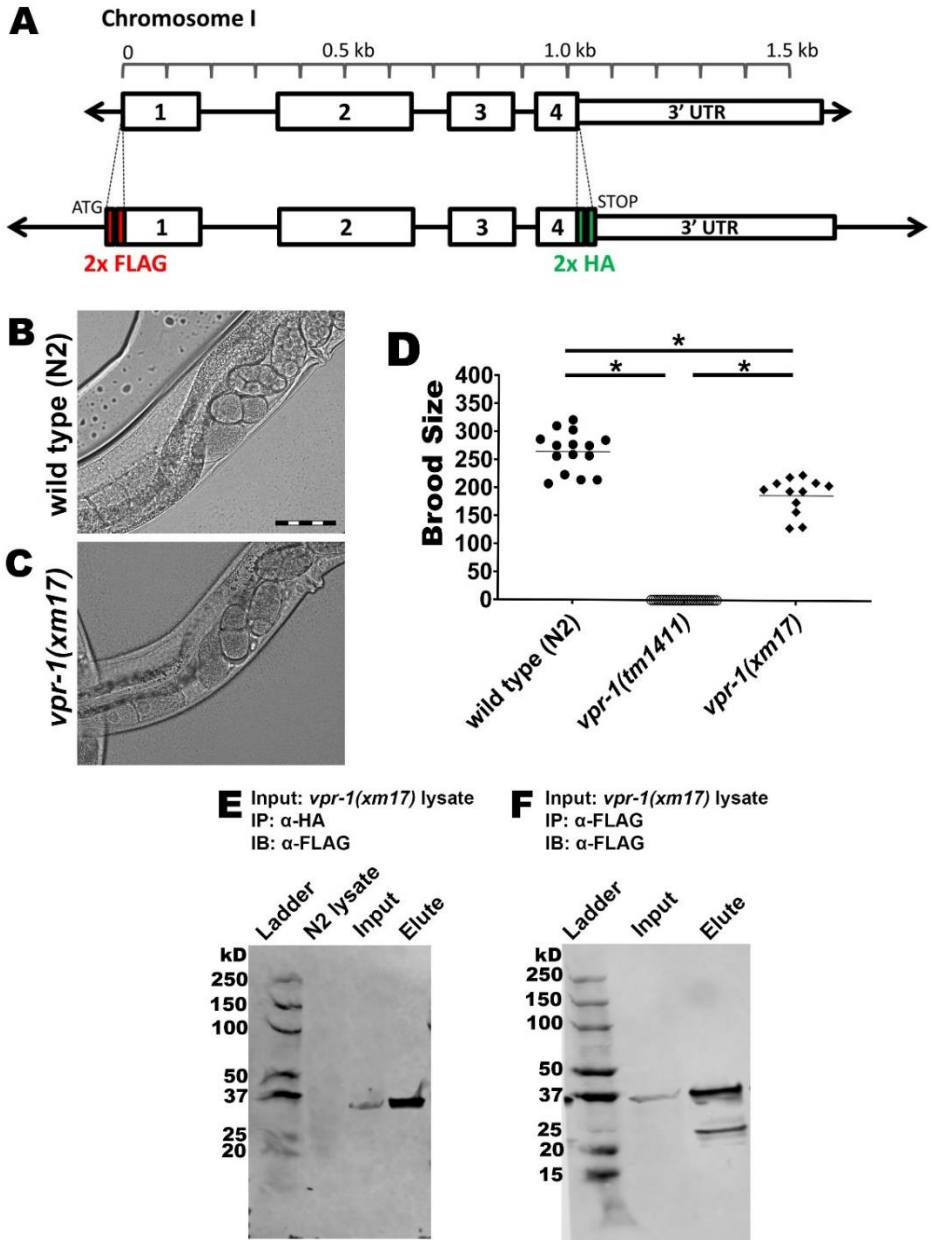

Figure 3: VPR-1 is cleaved into a <25kDa peptide. A) Diagram of CRISRP/Cas9 schematic to make the *vpr-1(xm17)* line by endogenously tagging the N-terminus of *vpr-1* with double-FLAG and the C-terminus with double-HA tag. B-C) Endogenously tagged line, *vpr-1(xm17)*, develops a normal gonad like wild type (N2) *C. elegans*. Images captured with a 40x Plan Fluor 1.3NA objective. Scale bar: 50µm. D) *vpr-1(xm17) C. elegans* have a reduced brood size, but are still significantly more fertile than the null. (*p>0.0001, Student's t-test) E) Immunoprecipitation with anti-HA beads pulls down full size VPR-1 protein at 37kD. F) Immunoprecipitation with anti-FLAG beads pulls down the full size VPR-1 protein at 37kD and the cleaved N-terminal peptide at <25kD.

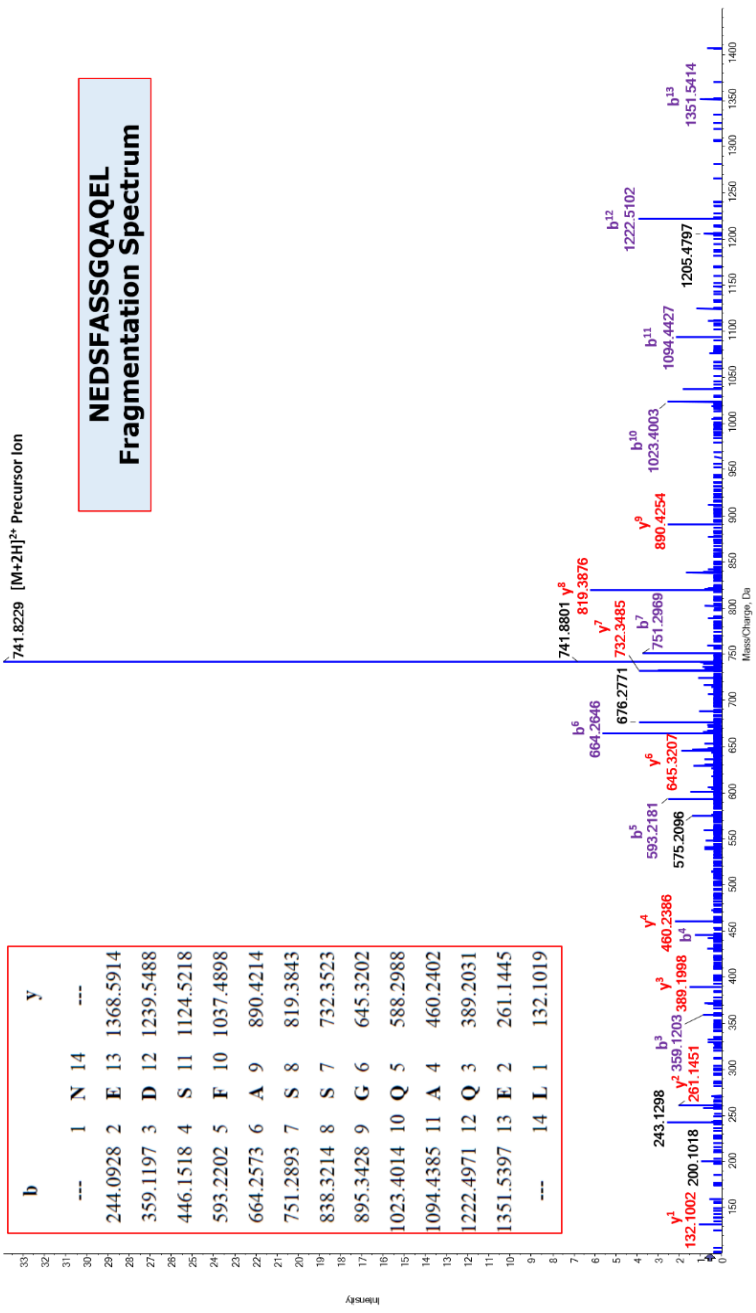

Figure 4: The amino acid sequence NEDSFASSGQAQEL was identified as the end the VPR-1 25kDa band of by mass spectrometry.

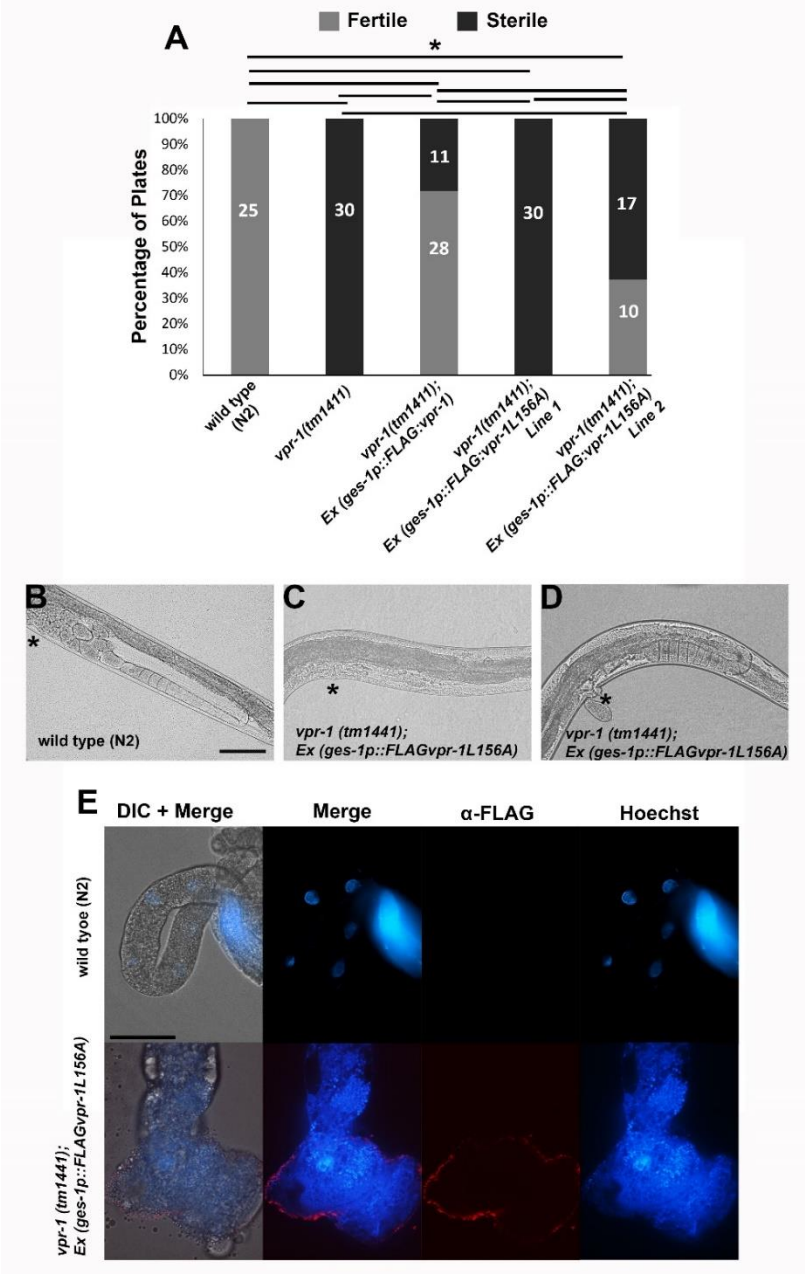

Figure 5: L156A point mutation in *vpr-1* causes sterility in *C. elegans*. A) Graph represents fertility assay. N2 wild type *C. elegans* are 100% fertile (25/25) while 0% of *vpr-1(tm1411)* hermaphrodites are fertile (0/30). Majority of *vpr-1(tm1411); Ex (ges-1p:: FLAG:vpr-1)* (28/39) hermaphrodites are fertile. Two lines of *C. elegans* expressing *Ex (ges-1p::FLAG:vpr-1L156A)* were developed. All line 1 (0/30) and most of the line 2 hermaphrodites are sterile (10/27) and there is no significant difference between both lines and *vpr-1(tm1411).* (*p>0.01, Chi-Square Test) B-D) Most *C. elegans* that transgenically express ges-1p::*FLAG:vpr-1* with a L156A point mutation fail to develop a gonad like wild type (C). A few, however, do develop a somatic U-shaped gonad and are fertile. Asterisk indicates vulva. Images captured with a 20x multi-immersion Plan Fluor

0.8NA objective. Scale bar: 100µm. E) VPR-1 localization is visualized by immunofluorescence of its N-terminal FLAG tag. FLAG:vpr-1 with a L156A point mutation localizes along the basolateral membrane of the intestine. Images captured with a 60x APO Tirf 1.49NA objective. Scale bar: 50µm

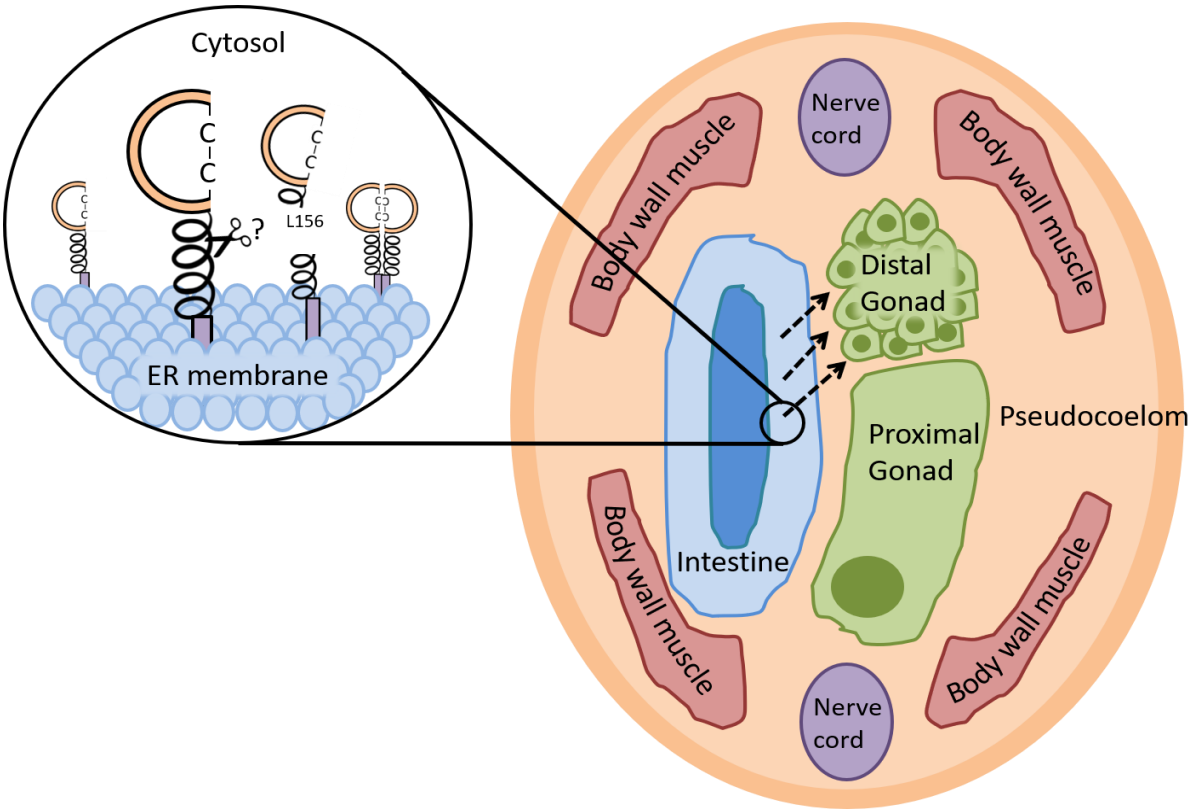

Figure 6: VPR-1 MSPd secretion and non-cell autonomous signaling model. In the intestinal cells of *C. elegans*, MSPd is cleaved off VPR-1, which is ER bound, at amino acid L156. The cleaving enzyme not known. This cleaved MSPd is secreted out of the intestinal cells and into the pseudocoelom to travel and binds to the cell membrane of the distal gonad (dashed arrows).

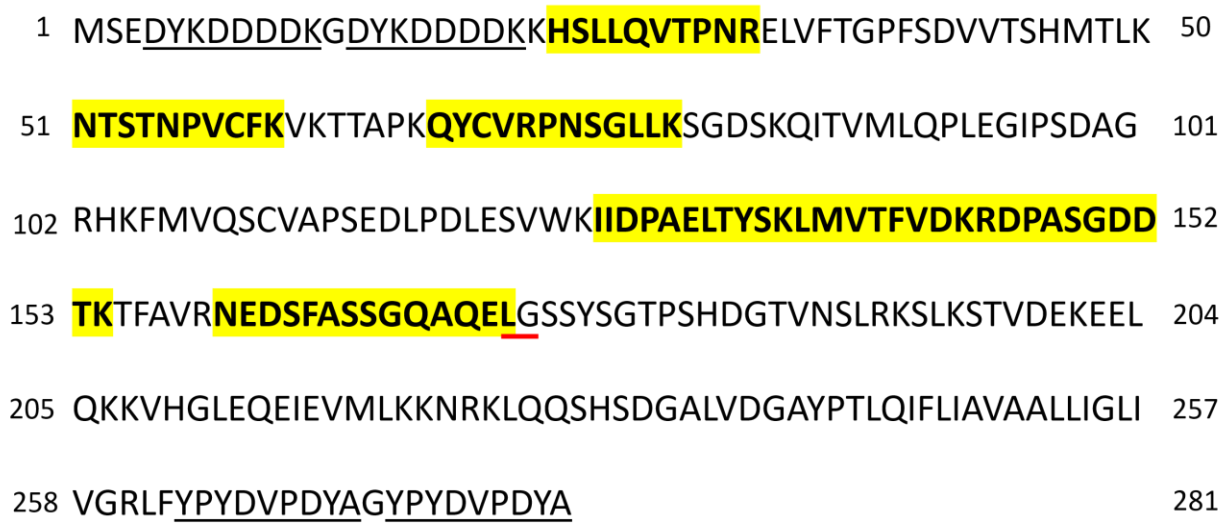

Figure S1: The FLAG:VPR-1:HA amino acid sequence. Highlighted in yellow are the peptide sequences detected by mass spectrometry after trypsin digest of the <25kDa band. Underlined in black are the double-FLAG tag at the N-terminus and the double-HA tag at the C-terminus. The predicted cleaved site is underlined in red and corresponds to Leu156-Gly157 in an untagged VPR-1 protein. VPR-1 MSPd is predicted to span a conservative sequence to mammalian VAP MSPd from aa008-aa126 in an untagged protein.

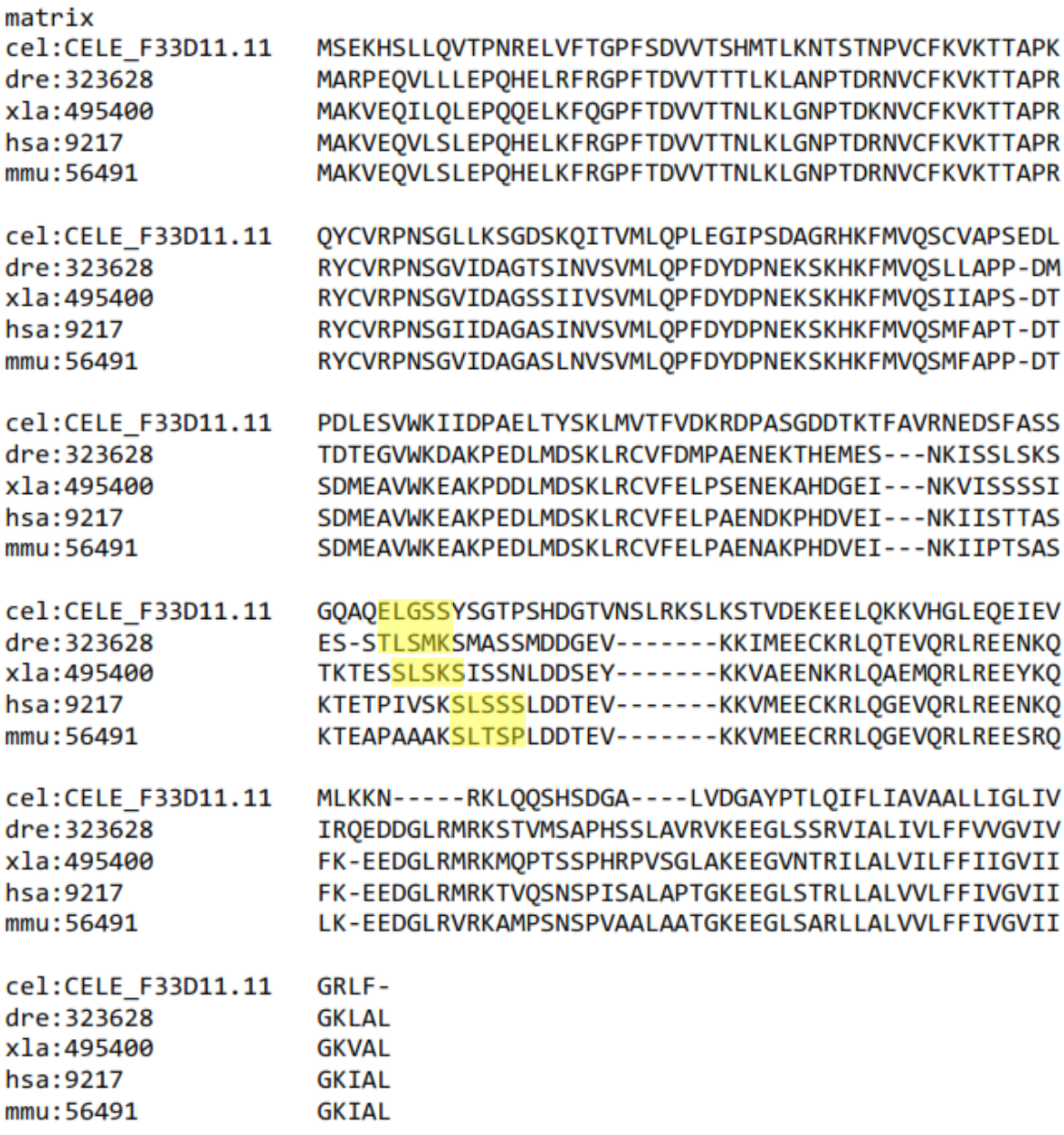

Figure S2: Leu156 is relatively conserved across species. Amino acid sequences of proteins homologues to VAP-B were aligned by Clustral Omega with NEXUS parameters. Cel= *Caenorhabditis elegans*, dre= *Danio rerio*, xla= *Xenopus laevis*, has= *Homo sapiens*, mmu= *Mus musculus*. The predicted cleavage sequences, including the conserved leucine, across species are highlighted in yellow.

Supplemental Table 1. List of *C. elegans* strains used in this study

Strain Name	Genotype
N2	Bristol N2, wild type
VC1478	*vpr-1 (tm1411)/hT2 [bli-4 (e937) let-? (q782) qIs48] (I; III)*
XM9914	*vpr-1 (tm1411); Ex ges-1p::vpr-1*
XM9915	*vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1*
XM9916	*vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1:HA*
XM9917	*FLAG:vpr-1:HA* or *vpr-1(xm17)*
XM9918	*vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1(L156A)*
XM9919	*vpr-1 (tm1411); Ex glr-5p::FLAG:vpr-1:HA*

Supplemental Video 1: Z-stacks through *vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1 C. elegans* dissected intestine. Corresponds to Figure 2. α-FLAG signal is in read and Hoechst staining is in blue. Sections captured with a 60x APO Tirf 1.49NA objective.

Supplemental Video 2: Z-stacks through *vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1 C. elegans* dissected distal gonad. Corresponds to Figure 2. α-FLAG signal is in read and Hoechst staining is in blue. Sections captured with a 60x APO Tirf 1.49NA objective.

Supplemental Video 3: Z-stacks through *vpr-1 (tm1411); Ex ges-1p::FLAG:vpr-1(L156A) C. elegans* dissected intestine. Corresponds to Figure 5E. α-FLAG signal is in read and Hoechst staining is in blue. Sections captured with a 60x APO Tirf 1.49NA objective.

AN RNAI SCREEN IN *C. ELEGANS* FOR GENES THAT PLAY A ROLE IN SECRETION AND CLEAVAGE OF VAPB MSP DOMAIN.

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SUMMARY

The MSP domain (MSPd) of a type-II ER transmembrane protein called VAPB is cleaved and secreted to function as a non-cell-autonomous signal. The topology of VAPB positions MSPd in the cytosol. It is thus unclear how MSPd is cleaved from VAPB and released extracellularly. Using *C. elegans*, we screened 422 genes by RNAi to identify potential candidates regulating MSPd cleaving and secretion. We identified the Golgi v-SNARE YKT-6 and several components of the 20S and 19S proteasome that may mediate MSPd trafficking and cleaving, respectively. These results have promising implications in advancing our understanding of MSPd signaling.

ABSTRACT

VAPB (VPR-1 in *C. elegans*) is a type-II ER transmembrane protein whose N-terminal Major Sperm Protein domain (MSPd) is cleaved and secreted. Mutations in the MSPd of human VAPB cause impaired secretion and are associated with Amyotrophic Lateral Sclerosis (ALS). In *C. elegans*, the secreted MSPd signals non-cell-autonomously to regulate striated muscle mitochondrial morphology and gonad development. As VAPB/VPR-1 does not have a signal peptide and its MSPd extends into the cytosol, it is unclear how the protein is proteolytically cleaved and secreted. To identify genes that are involved in VPR-1 cleavage and unconventional secretion, we performed an RNA interference (RNAi) screen in *C. elegans*. Worms null for *vpr-1* are sterile and have striated muscle mitochondrial abnormalities. These defects can be rescued by *vpr-1* expression in the neurons, germline, or intestine, implying that these three tissues share a common machinery to cleave and secrete the MSPd. Examination

of shared gene expression in these tissues revealed a list of 422 genes, which we targeted with RNAi. *vpr-1* null worms expressing *vpr-1* from intestine were used in the screen, and the brood size of these worms after RNAi knockdown was scored. Disruption of factors involved in VPR-1 MSPd processing and/or secretion should revert fertility phenotypes in these worms. We identified many genes that induce compromised fertility when knocked down in these but not wild type worms, including a V-SNARE, several proteasome components, stress response molecules, and mitochondrial genes. Our screen thus identified many potential players involved in MSPd processing and/or secretion.

INTRODUCTION

Human VAPB is a member of the VAP (VAMP/synaptobrevin associated proteins) family of proteins. VAPs are endoplasmic reticulum (ER) membrane proteins with roles in membrane trafficking, ER unfolded protein response, and lipid transport at intracellular membrane contact sites (1, 2). Three domains make up the VAPB protein: a cytosolic N-terminal MSP domain (MSPd), a central coiled-coil domain, and a C-terminal ER-anchoring transmembrane region (1, 3-5). The MSPd with an immunoglobin-like β-sheet structure is named for its homology with a well-characterized nematode protein known as the Major Sperm Protein, or MSP (1, 3, 6). Nematode MSP is an abundant sperm cytoskeletal protein required for sperm motility (7, 8). In *C. elegans*, it is secreted from sperm in an unknown fashion to regulate oocyte maturation (9-11). The MSPd from VAPB is cleaved and can also be released to the extracellular space via an unconventional pathway in a cell type-specific fashion (12-

15). However, the mechanism by which the MSPd is proteolytically processed and unconventionally secreted is not yet understood.

In humans, the MSPd has been detected in blood and cerebral spinal fluid (CSF) and implicated in fatal neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS) and spinal muscular atrophy (SMA) (12, 16). An amino acid substitution of proline 56 with serine (P56S) in the MSPd of VAPB prevents MSPd secretion and precisely segregates with familial cases of ALS (12, 17-20). In *C. elegans* and *Drosophila*, the MSPd secreted from neurons binds to receptors on striated muscles and in gonadal cells to modulate mitochondria positioning to the myofilaments and gonad development, respectively (12-15, 21). *C. elegans* null for *vpr-1*, the VAPB homolog, are sterile and have body wall muscle mitochondrial abnormalities (15, 21). These phenotypes can be rescued by expression of *vpr-1* solely in the germline, or intestine, or the nervous system (15). These data thus support a model of an endocrine, non-cell-autonomous role for the MSPd in signaling.

To identify genes essential for VAPB/VPR-1 MSPd processing and unconventional secretion, we conducted an RNAi screen in *C. elegans*. Based on the rationale that the cleavage and the unconventional secretion machinery is shared among the three tissues whose expression of *vpr-1* can rescue the null phenotypes, we assembled a list of 422 genes which are expressed commonly in the germline (22), intestine (23), and nervous system (24). RNAi was performed to knockdown these genes in a rescue line expressing *vpr-1* driven by an intestinal promoter, *ges-1p* (*ges-1p::vpr-1*). Brood size was scored to identify genes that decreased the fertility of the rescue line but did not affect the fertility of wild type worms. Many genes without any previously

reported function in regulating protein processing or secretion have been uncovered in our study.

METHODS AND MATERIALS

C. elegans strains

C. elegans strains were propagated at 20°C and fed with NA22 *E.coli* (25, 26), unless otherwise indicated. N2 Bristol was the wild type strain used in the RNAi screen. The transgenic line used in this screen (*ges-1p::vpr-1*) is VC1478 *vpr-1(tm1411)/hT2 [bli-4(e937) let-?(q782) qIs48] (I; II)* transgenically expressing extrachromosomal arrays of *ges-1p::vpr-1* (originally made in Cottee et al., 2017).

RNAi screen

RNAi knock down of genes was performed by the feeding method (27). HT115(DE3) bacterial feeding strains were obtained from the Ahringer library (28). PCR and sequencing (UAB Heflin Center for Genomic Sciences) were used to confirm that bacterial strains contained the correct clones. Each RNAi bacterial feeding strain was grown to feed parallel plates of N2 and the transgenic *ges-1p::vpr-1* worms (Figure 1). Five L4 staged worms of each genotype were maintained on respective RNAi plates at 25°C. Progeny was counted after 7 days on RNAi plates at 25°C. RNAi knockdown was replicated three times for *ykt-6*, *rpn-1*, *rpn-10*, and *pbs-2*. RNAi constructs for *pas-4* and empty L4440 backbone were used as positive and negative controls, respectively. Student's t-test statistical analysis was conducted using Prism software. Pie chart was made with Microsoft Excel.

Data availability

Any worm strain or reagent used in this screen is available upon written request to the corresponding author. All data sets and results from this screen are included in this report.

RESULTS AND DISCUSSION

C. elegans muscle mitochondria and gonadogenesis are regulated non-cell-autonomously by the cleavage and secretion of MSPd from VAPB/VPR-1 (12-15, 21). However, genes that play a role in the cleaving or secretion of the MSPd have yet to be identified. This screen aimed to take an unbiased approach to identify potential players in these processes. Identifying such players can have implications for neurodegenerative diseases like ALS that has been linked to the lack of MSPd circulation in human blood and cerebral spinal fluid (12, 16).

The design of this screen is based on previous work from our lab. We showed *vpr-1* expression in neuronal, intestinal, or germline cells is sufficient to rescue the *vpr-1* null phenotypes of sterility and muscle mitochondrial abnormalities in *C. elegans* (15, 21). This result suggested that these three cell types may share a common mechanism that detaches the MSPd from VPR-1 and releases the MSPd extracellularly into the pseudocoelom to be accessed by the body wall muscles and somatic gonad. Therefore, gene expression data sets of *C. elegans* nervous system (24), intestine (23), and germline (22) were compared. These data sets revealed 422 overlapping genes that became the targets of this screen (Table S1).

C. elegans null for *vpr-1* are maternal effect sterile (15). Of the three strongest rescue lines, intestinal transgenic expression of *vpr-1* in a *vpr-1* null background (*ges-1p::vpr-1*) was chosen to be used in this screen. A strength of using the *ges-1p::vpr-1*

line is that it provides a non-redundant system of VAPB/VPR-1 MSPd cleaving and secretion that is RNAi-sensitive. Transgenic expression exclusively in the germline would require integration into the genome to avoid silencing and neurons are resistant to the effect of RNAi (15, 29). *vpr-1* null worms are maternal effect sterile, but the majority of *ges-1p::vpr-1* worms are fertile (15). Taken together, this screen was designed to knock down a gene that plays a role in VAPB/VPR-1 MSPd cleaving or secretion in order to decrease non-cell-autonomous MSPd signaling such that the otherwise fertile *ges-1p::vpr-1* line reverts back to sterility.

Of the 422 selected genes, 71 candidates were not found in the Ahringer RNAi library or did not grow in culture (Table S1). RNAi constructs of the remaining genes were fed in parallel to wild type N2 Bristol and transgenic *ges-1::vpr-1* worms (Figure 1). Any gene whose RNAi knockdown resulted in a decrease of the N2 brood size was not considered a hit because of its direct implication in fertility or gonadogenesis in a wild type background (Figure 1; Table S1). First-tier hits were arbitrarily categorized as RNAi knockdown genes that did not affect the brood size of the N2 worms while the *ges-1p::vpr-1* plates had a count of 20 or less live progeny (Figure 1; Table S1). The number of live *ges-1p::vpr-1* progeny between 20-50 worms after RNAi knockdown constituted the second tier hits. The screen was not designed to identify any gene, such as a negative regulator, that when knocked down enhanced the fertility of the rescued *ges-1p::vpr-1* line.

First and second tier hits were categorized based on their known functions (Figure 2). A portion of these hits (18%) could not be classified or remain uncharacterized. One-third of the hits are involved in RNA/DNA binding. These genes

alongside the 4% comprising nucleoporin genes may be upstream players that regulate the transcription, mRNA processing, and mRNA transport of proteins responsible for VAPB/VPR-1 MSPd cleaving or secretion. 13% are involved in cell division and could not be explained with regards to VAPB/VPR-1 MSPd cleaving and secretion. 11% are mitochondrial proteins of which three genes encode mitochondrial ribosomal proteins. These proteins may regulate expression of mitochondria outer membrane proteins that can interact with VAPB/VPR-1 and influence the conformation or accessibility of VAPB/VPR-1 for efficient cleavage or secretion.

In the protein degradation category, *ubxn-6* is a member of the ubiquitin regulatory X (UBX) domain proteins and is the *C. elegans* homolog for UBXD-1 in humans (30). UBXD-1 functions as one of several adaptor proteins to an AAA (ATPase associated with various activities) ATPase known as p97/VCP, or CDC-48 (31). One of two CDC-48 isoforms in *C. elegans* was among the 422 genes screened (C06A1.1) but it did not rank in the top two tiers (Table S1). p97/VCP/CDC-48 has different functions based on the binding of various adaptor proteins (32). 97/VCP/CDC-48 binds UBXD1 to mediate ubiquitinated protein trafficking into endolysosomes as well as the formation of ERGIC-53 positive vesicles between the ER-Golgi and potentially the plasma membrane (33, 34). Interestingly, mutations in p97/VCP/CDC-48 that negatively affect UBXD1 binding have been found to segregate with cases of familial ALS, as is the case with the P56S mutation in VAPB that prevents MSPd cleavage and secretion (12, 17, 33, 35, 36). Whether VAPB/VPR-1 MSPd is being trafficked or processed by p97/UBXD1 needs to be further investigated.

Of the 18 first-tier hits, five genes have been previously reported to play a role in protein trafficking or processing (Table 1). Knock down of the v-SNARE *ykt-6* or proteasome components *rpn-1*, *rpn-10*, and *pbs-2* significantly decreased the brood size of *ges-1p::vpr-1* rescue line, but did not affect N2 fertility (Figure 3). How these candidate genes may be playing a role in VABP/VPR-1 MSPd cleaving or secretion is further discussed below:

A SNARE gene: *ykt-6*

SNAREs, or soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors, play a crucial role in mediating membrane fusion between vesicles and cellular compartments of the secretory pathway. YKT-6 is a vesicle SNARE, or R/v-SNARE protein, that is further classified as a longin, or long vesicle-associated membrane protein (VAMP) (37). This classification is attributed to its N-terminal profilin-like domain, known as a longin domain (LD), that helps regulate YKT-6 binding (37). Unlike most SNAREs, YKT-6 lacks a transmembrane domain (38, 39). Instead, YKT-6 is anchored into the membrane of various organelles by lipid modification. For example, to anchor into the Golgi membrane, a lipid moiety is attached to the C-terminal CAAX motif by an irreversible post-translational modification, farnesylation (38-40), as well as a reversible post-translation modification, palmitoylation (40, 41). Farnesylation itself is insufficient for YKT-6 to insert into the membrane, as most YKT-6 is present as a depalmitoylated, farnesylated, cytosolic protein (38-40, 42-44). Cytosolic YKT-6 assumes a closed conformation in which the N-terminal LD folds back and binds the SNARE motif to tuck away and shield the hydrophobic farnesyl anchor (45, 46). This conformation maintains YKT-6 in

a stable, soluble state and serves to autoinhibit the SNARE motif from binding to and negatively affecting membrane bound SNAREs from forming complexes between vesicles and target membranes (37, 40, 42, 45). This accessible pool of cytosolic YKT-6 may be the reason that YKT-6 is a multifaceted SNARE that functions in various intracellular trafficking routes and is found bound to the Golgi, endosomes, exosomes, lysosomes-autophagosomes, and vacuole membranes in yeast (38, 39, 42-44, 47-53).

Due to the promiscuous nature of YKT-6, it is difficult to explain how YKT-6 might be involved in the cleavage or secretion of VAPB/VPR-1 MSPd. One possibility is that the MSPd is not cleaved from VAPB/VPR-1 until after it reaches the plasma membrane via YKT-6 positive vesicles. While VAPB/VPR-1 does not contain a signal peptide found in conventionally secreted proteins, it does localize to the ER membrane facing the cytosol (4). We suggest that this localization may allow VAPB/VPR-1 to be passively or actively incorporated into COPII vesicles headed for the Golgi, the main site of active bound YKT-6. Transport between the ER and Golgi may be influenced by *ubxn*-6, as discussed above. Once reaching the Golgi, YKT-6 may facilitate further sorting and trafficking of VAPB/VPR-1 through distinct secretory pathways. RNAi knockdown of Ykt-6 in *Drosophila* and human cells revealed that Ykt-6 plays a role specifically in the secretion of Wnt proteins through exosomes (54). VAPB/VPR-1 may be trafficked through a similar exosomal route in a YKT-6-dependent manner. The depletion of YKT-6 in combination with Syb/VAMP3 also resulted in an accumulation of post-Golgi vesicles, indicating that YKT-6 plays an additional role in plasma membrane fusion (51). In neurons, one of the cell types known to secrete the VAPB/VPR-1 MSPd, YKT-6 was reported to localize to unknown membranous

compartments, and not the Golgi, in a punctate fashion (15, 42). Therefore, VAPB/VPR-1 may be trafficked by YKT-6 positive vesicles, whether exosomes or otherwise, to the plasma membrane to have the MSPd cleaved before extracellular release. This hypothesis is consistent with our observation of full length VAPB/VPR-1 protein localization at the basolateral membrane of *C. elegans* intestinal cells (Zein-Sabatto et al., in review). Further studies to assess a direct relationship between VAPB/VPR-1 and YKT-6 positive vesicles are needed to test this model.

Proteasome components: *pbs-2*, *rpn-1*, *rpn-3*, and *rpn-10*

Proteasomes are diverse and have been implicated in the degradation of misfolded or excess protein in order to maintain cellular homeostasis (55). The 20S proteasome has a barrel-like structure with an internal proteolytic core that can function independently or in complex with a regulatory cap referred to as the 19S proteasome to make up the 26S proteasome (56, 57).

The 19S proteasome binds, deubiquitinates, and mediates the translocation of ubiquitinated proteins through the 20S proteasome (58). Two top tier hits from this screen, *rpn-1* and *rpn-3*, encode for two non-ATPase subunits of the 19S proteasome base and lid, respectively (56, 58). Another positive hit, *rpn-10*, encodes for the subunit that links 19S proteasome lid and base, contains two polyubiquitin binding motifs, and functions as one of several ubiquitin receptors (58, 59). These ubiquitin receptors are non-redundant and recognize specific substrates. For example, deleting RPN-10 specifically results in a feminization phenotype in *C. elegans* hermaphrodites due to the accumulation of TRA-2 proteins (60). When knocking down other components of the 19S base from the list of 422 genes (*rpt-1*, *rpt-3*, and *rpt-5*), the N2 brood size was also

affected indicating that these genes may play a broader role in *C. elegans* development. Knocking down other subunits of the 19S lid, such as *rpn-5* and *rpn-12*, had no effect on N2 brood size, but did not decrease the brood size in *ges-1p::vpr-1* as drastically as when knocking down *rpn-1*, *rpn-3*, or *rpn-10*.

Proteolysis, or the breakdown of peptide bonds, occurs in the 20S proteasome. The 20S proteasome is comprised of a stack of two outer rings of α-type subunits and two inner rings of β-type subunits (56). The two inner β-rings encompass the chymotrypsin-like, trypsin-like, and caspase-like proteolytic sites (56). PBS-2 in *C. elegans* is the *β2* subunit, one of three β-subunits that make up these proteolytic sites in the 20S proteasome (56). Knock down of *pbs-2* with RNAi had no effect on wild type *C. elegans* brood size, but rendered the *ges-1::vpr-1* worms sterile (Figure 3). The other two proteolytic subunits, *β1* and *β5*, were not among the 422 genes screened here. Other β-type subunits, *pbs-3*, *pbs-4*, *pbs-6*, and *pbs-7*, and α-type subunit *pas-6* were included in the list of 422 candidates, but knockdown of these genes, unlike *pbs-2*, decreased the wild type brood size (Table S1). This indicates that PBS-2, unlike the other 20S proteasome subunits, may play a specialized role, such as endoproteolytic cleavage of VAPB/VPR-1 MSPd in the 20S proteasome as described below.

While the 20S proteasome degrades whole proteins, it can also cleave proteins at precise sites to produce peptides with independent functions. Such examples can be found in the cleaving of translation initiation factors elF3a and eIF4G (61), Y-box RNA/DNA binding protein (62), and Δ40p53 (57). Common across these cleaved peptides is an internal stretch of amino acids known to form a native unfolded or "disordered" structure (61, 62). Both 26S and 20S proteasomes can recognize and

internally cleave at this motif (63). Using PONDR Protein Disorder Predictor VLXT (http://www.pondr.com/), we identified a disorder motif downstream the MSPd in VPR-1 (Figure 4). In a separate study, we determined the cleave site of VPR-1 in *C. elegans* to be between Leu156 and Gly157, but no known protease was identified (Zein-Sabatto, in review). Interestingly, the Leu156, Gly157 cleavage site lies within the strongest and longest predicted disorder motif (Figure 4), suggesting that VAPB/VPR-1 may be processed by the proteasome to release a functional MSPd peptide.

While 20S and 26S proteasomes are usually found unbound in the cytosol, ~40% of 20S proteasomes have been found to be tightly associated with neuronal plasma membranes in mouse (64). These hydrophilic neuronal membrane proteasomes, or NMPs, are exposed to the extracellular space by interacting with GPM6A/B, a multi-pass transmembrane glycoprotein (64). This plasma membrane localization and access to intra- and extra- cellular spaces confers these NMPs the ability to cleave intracellular protein and release the newly generated peptide extracellularly (64). This may also explain how MSPd is cleaved from VAPB/VPR-1 after being trafficked to the plasma membrane by YKT-6 positive vesicles (as discussed above). In fact, neurons are a major cell type that cleaves and secretes MSPd from VAPB/VPR-1 in *Drosophila* and *C. elegans* (12, 13, 15). While this model involving NMPs is appealing, it is unclear at this time whether similar NMPs exist in intestinal or germline cells as they do in neurons, a very specialized cell type. Though our screen is based on the assumption that MSPd is cleaved and secreted via a similar mechanism in all these cell types, each cell lineage may also utilize slightly different mechanisms. Further investigations are needed to

identify whether NMPs are found in non-neuronal cells and whether they couple cleavage and release of MSPd from these cells.

PERSPECTIVE

This screen aimed to identify players that regulate MSPd cleavage from ER anchored VAPB/VPR-1 and its extracellular secretion. We took an unbiased approach and screened 422 genes in *C. elegans* that were common across the three cell types known to secrete MSPd. One limitation of this screen is that it is based on our understanding of MSPd non-cell-autonomous signaling in *C. elegans*. Mammalian MSPd cleaving and secretion patterns are not known. While various categories of genes came out of this screen, we chose to highlight the v-SNARE (*ykt-6*) and proteasome components (*rpn-1*, *rpn-3*, *rpn-10*, and *pbs-2*) due to their previously described roles in the cleavage and secretion of other proteins. As MSPd cleavage and secretion has been implicated in ALS, the results from this screen may have also uncovered new potential players that have not yet been linked to the neurodegenerative disease.

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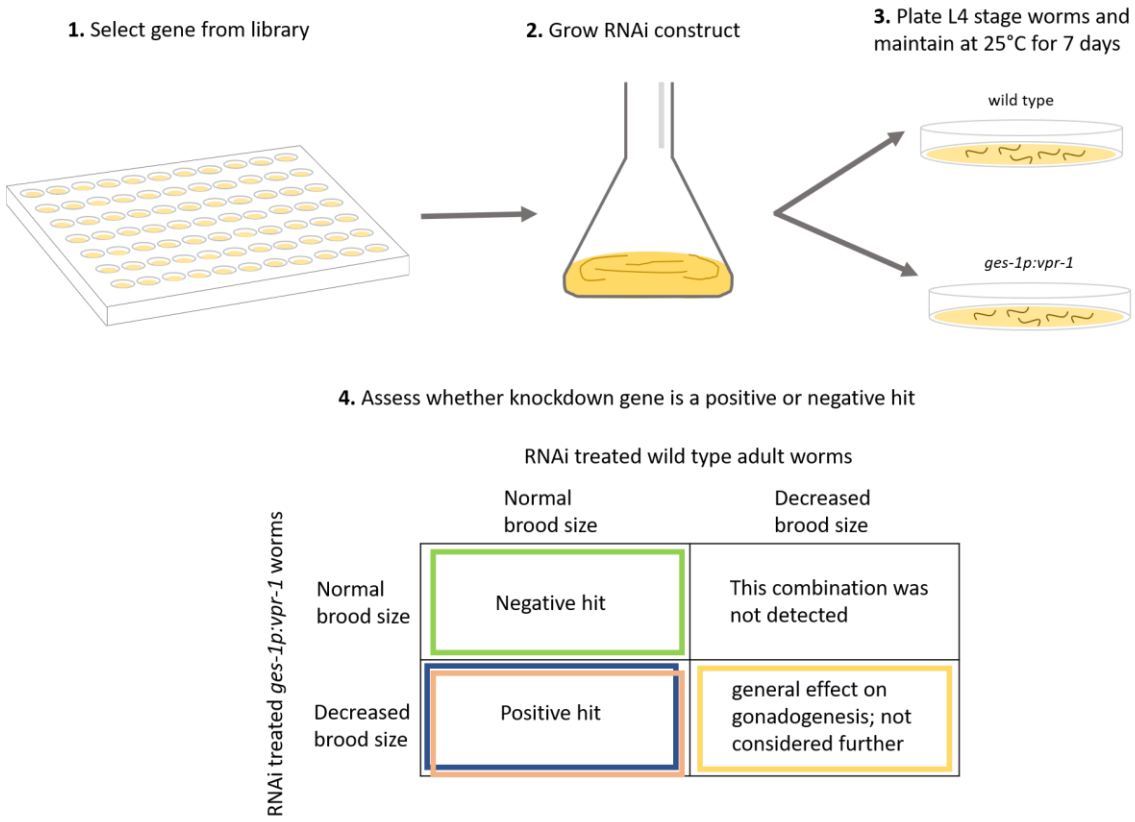

Figure 1: RNAi Screen Paradigm to identify potential effectors of MSPd cleaving and secretion. Colors highlighting hits coordinate with list of genes in Table S1.

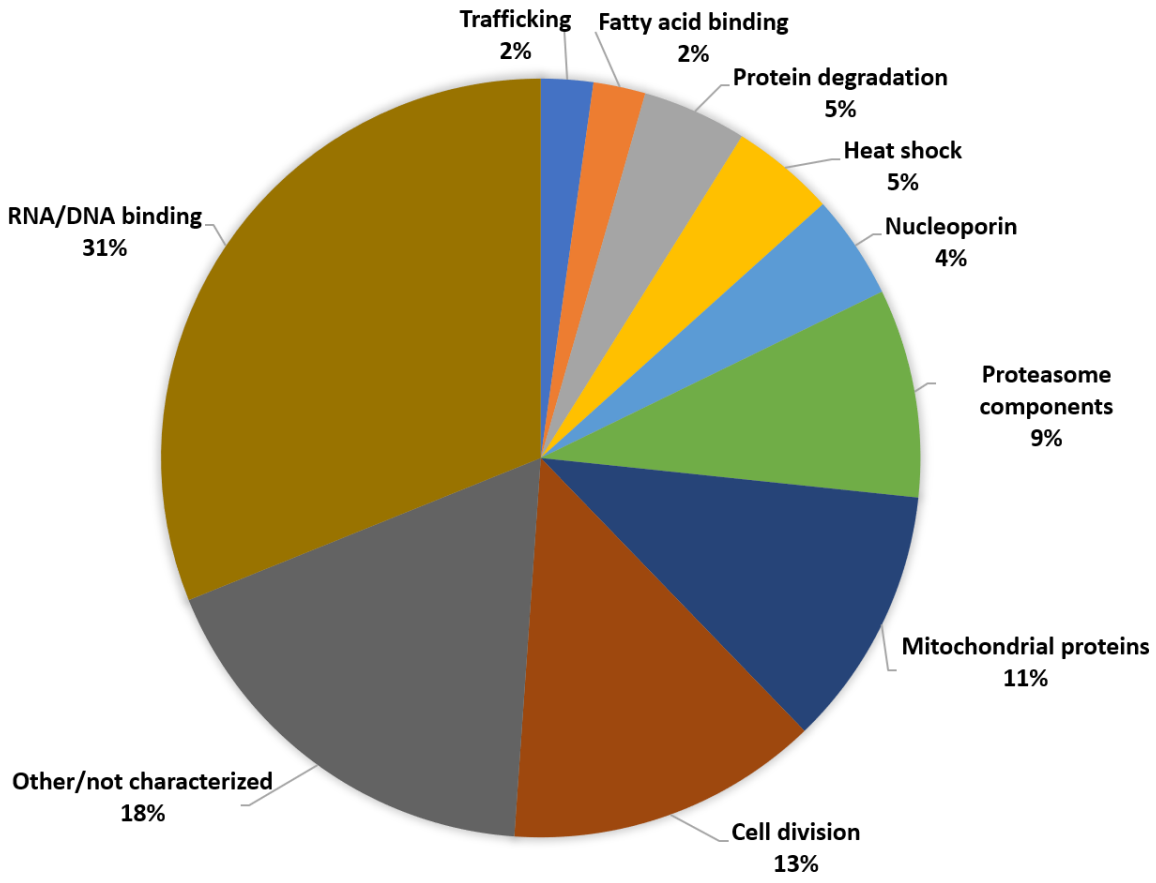

Figure 2: Pie chart depicting the various gene categories identified in this RNAi screen. Genes from the first and second tier of hits were categorized based of off their description on Wormbase.

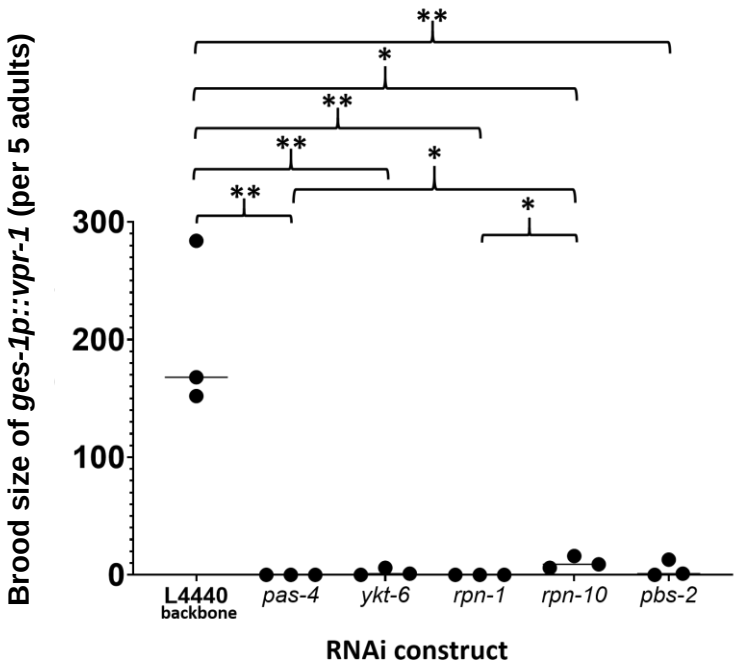

Figure 3: Brood sizes of *ges-1p::vpr-1* decreased after RNAi knockdown of *ykt-6, rpn-1, rpn-10,* and *pbs-2*. Five L4 *ges-1p::vpr-1 C. elegans* were fed RNAi constructs of select gene for 7 days at 25°C. *ges-1p::vpr-1 C. elegans* grown on empty RNAi backbone L4440 produced an average of 200 progeny. The brood size decreased when *ykt-6*, *rpn-1*, *rpn-10*, or *pbs-2* were knocked down in *ges-1p::vpr-1 C. elegans.* Knock down of these four genes did not affect N2 brood sizes (not shown). Knock down of *pas-4* was used as an RNAi control since it caused sterility and lethality in both N2 and *ges-1p::vpr-1 C. elegans.* (*p>0.01 and **p>0.001, Student's t-test)

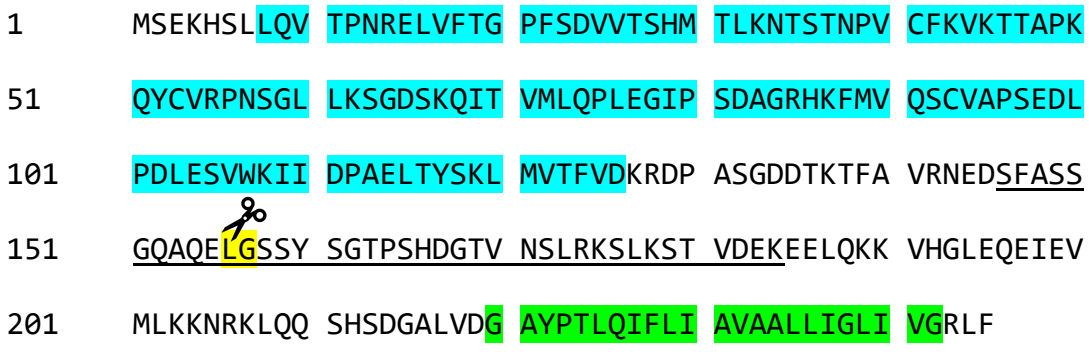

Figure 4: The MSPd cleave site resides in a predicted disordered motif within the VAPB/VPR-1 protein. VPR-1 amino acid sequence was analyzed by PONDR Protein Disorder Predictor VLXT (http://www.pondr.com/) to identify stretches of disordered amino acids. The longest and most likely stretch of disordered amino acids according to the predictor is underlined (aa146-aa184). VPR-1 is cleaved between Leu156 and Gly157 (scissors) to release the MSPd. The MSP domain based on VAPB/VPR-1 MSPd predictions is included the blue highlighted area (11). We predicted the transmembrane domain to be in the highly conserved GXXXG motif (green) (5).

Table 1: Five selected candidate genes from the top-tier list.

Wormbase Sequence	Gene	Short description
B0361.10	*ykt-6*	v-SNARE
T22D1.9	*rpn-1*	Proteasome 26S subunit, non-ATPase 2
C30C11.2	*rpn-3*	Proteasome 26S subunit, non-ATPase 3
B0205.3	*rpn-10*	Proteasome 26S subunit, non-ATPase 4
C47B2.4	*pbs-2*	Ortholog of proteasome 20S subunit beta 7

SUMMARY AND FUTURE DIRECTIONS

4.1 Overview

VAP is a membrane protein that anchors into the ER membrane with its N-terminus facing the cytosol [1, 2]. As a type-II ER transmembrane protein, its well characterized functions involve lipid transport and membrane trafficking at the intracellular membrane contact sites between ER, various organelles, and membrane vesicles [3, 4]. However, recent studies also reveal a non-cell-autonomous signaling capacity of VAP that involves its N-terminal domain. The N-terminal sequence of VAP proteins, which includes the MSPd, is highly conserved and can be cleaved and secreted to regulate various developmental events in different species. In *C. elegans* the MSPd cleaved from VAP is required for gonadogenesis as early as the L2 stage of the nematode developmental cycle [5] while in humans the absence of the MSPd circulating in the cerebral spinal fluid is linked to cases of bulbar ALS [6]. However, the mechanism of VAP cleavage and secretion is not known for any species. In my dissertation studies, I used the *C. elegans* model system to explore molecular control of VAP processing and secretion. In Chapter 2 of this dissertation, I present my work in *C. elegans* to identify the cleavage site of VPR-1, the nematode VAP protein homolog. I provide further evidence for the non-cell-autonomous signaling model of VPR-1 MSPd by detecting intestinally expressed MSPd on the distal gonad. In Chapter 3, I summarize the results of a RNAi screen in *C. elegans* to identify potential players in VPR-1 processing and secretion. Of note, I identified YKT-6 and components of the proteasome as potential genes that play a

role in the trafficking and processing of VPR-1 MSPd, respectively. Collectively, my work offers a promising inroad into a fundamental cell biological question on the cleavage and non-conventional secretion of a type-II membrane protein. This study also helps to shed light on regulation of developmental and pathological processes by extracellular MSPd.

4.2 The cleavage site of VPR-1

Work in *C. elegans* presented in this thesis and previous data in *Drosophila* show that the N-terminal peptide spanning the MSPd is cleaved [7, 8]. However, prior to this work the cleavage site was unknown. Using CRISPR/Cas9-mediated genome editing, I generated a *C. elegans* strain that expressed an endogenously tagged VPR-1. This model allowed for the isolation of the full VPR-1 protein and the smaller cleaved peptide by western blot despite the unavailability of a VPR-1 antibody. This endogenously edited worm line was designed to incorporate different tags at the N- and C- termini. The N-terminal FLAG tag and the C-terminal HA tag allowed for differentiation between the cleaved N-terminal peptide and the full-length VPR-1 protein by western blot. Though we expected to detect the cleaved C-terminal peptide as well, it was never recovered through western blots even after attempts to concentrate the peptide by α-HA immunoprecipitation. This may be because the C-terminal peptide is unstable and quickly degraded upon the cleavage of the N-terminal peptide.

Isolating the cleaved N-terminal peptide (25kD) and full length VPR-1 (37kD) protein by western blot allowed for the identification of the VPR-1 cleavage site by mass spectrometry. Mass spectrometry verified that the 25kD peptide encompassed the N-

terminal MSPd. A leucine at the 156th amino acid position (L156) downstream of the MSPd was the last amino acid of the 25kD peptide as detected by mass spectrometry. Therefore, the cleaved N-terminal peptide of VPR-1 extends beyond the MSPd, which has been estimated to span the first 125 amino acids of VPR-1 [7]. The fact that this cleave site is positioned several amino acids downstream of MSPd offers an opportunity to test directly the importance of MSPd cleavage in relation to the non-cell-autonomous signaling function of VAP. To test this, various VPR-1 isoforms lacking different lengths of amino acids between MSPd and the coiled-coil region can be constructed to examine the consequent effects on VPR-1 cleavage and function. In addition, untethered MSPd that mimics cleaved MSPd can be made and introduced into the nematode to explore whether it is sufficient to rescue *vpr-1* sterility in *C. elegans*. Such experiments would also provide clues on whether MSPd cleavage is required for coupling the secretion of MSPd and its signaling function.

Substituting the 156th leucine to an alanine (L156A) limited the non-cell-autonomous role of VPR-1 in gonadogenesis, but several transgenic worm lines still managed to develop fertile gonads. These fertile worms may have a higher copy number of the transgene and overexpress a greater amount of the VPR-1 protein such that a sufficient amount of N-terminal VPR-1 peptide is cleaved despite the L156A point mutation. This also suggests that the site of VPR-1 cleavage is regulated by more than the presence of L156. It is interesting to note that there is a tyrosine residue downstream of L156 which may serve as an alternative cleavage site. Therefore, cleavage at the leucine residue may be mediated by chymotrypsin-like enzymes, which recognize other bulky hydrophobic amino acids, such as tyrosine, tryptophan, and phenylalanine. Further

examination of the cleavage sequence for other necessary amino acids that may enhance the cleavage event can be performed by a combination of mutagenesis and western blots. In order to achieve this, point mutations, such as L156A, will need to be introduced into the endogenous *vpr-1* gene and balanced to allow for propagation in cases of sterility. Genome-edited lines as such would eliminates variability of protein expression in transgenic lines and allow for worm farming techniques to harvest enough protein for analysis. Western blot analysis of the presence, absence, or reduction of the smaller 25kD band due to L156A or other mutations would then provide a more direct evidence for cleavage site disruption.

A comparison of amino acid sequences across various species showed that L156 and several surrounding amino acids are conserved. While this implies a potentially common cleaving mechanism, future studies are needed to verify the cleavage site in other VAP protein homologs. L156 resides only a few amino acids upstream the predicted coiled-coil domain. This region between the MSPd and the coiled-coil domain is a predicted disordered or unstructured sequence of amino acids. Disordered regions within a protein can be detected and endo-proteolyzed by the proteasome [9]. Consist with this notion, results from the RNAi screen outlined in Chapter 3 of this thesis identified various components of the proteasome.

4.3 The role of the proteasome in VPR-1 processing

The RNAi screen outlined in Chapter 3 of this dissertation uncovered four proteasomal subunits that implicated the proteasome in VPR-1 cleavage and secretion. Of interest is PBS-2 because it is one of the three β-subunits that harbors the proteolytic sites

of the 20S proteasome. The other two β-subunits were not among the 422 genes screened, but their knockdown would reveal whether the proteasome cleaves VPR-1 exclusively through PBS-2.

Data linking the proteasome directly to VPR-1 cleavage is still needed. In Chapter 2, I developed a transgenic line expressing VPR-1 with an N-terminal tag to track MSPd secretion by immunofluorescent imaging. The VPR-1 N-terminal peptide expressed in the intestine was detected on the surface of the distal gonad. This assay can be combined with the RNAi knockdown of the four proteasomal subunits individually or in combination to provide the needed verification of these hits. If proteasome is indeed involved, upon knockdown of any of these proteasomal subunits, no or undetectable levels of VPR-1 should be cleaved and released from the intestinal cells to bind the distal gonad. Since I used the bacteria feeding method to administer the RNAi in the worm to knock down genes in the intestine, where the tagged VPR-1 is expressed, optimal knockdown effect should be achieved.

The RNAi method provided a system where the proteasomal subunits could be knocked down. Analysis of VPR-1 cleavage by western blot in the null mutant lines of these subunits is still needed. However, since the proteasome is generally involved in clearing out misfolded and excess proteins, these null *C. elegans* lines may be lethal and not available for such analysis. Instead, an alternative option to verify the role of the proteasome in cleaving VPR-1 is to treat a cell culture model of VPR-1 cleavage and secretion with proteasome inhibitors such as MG-132. Western blots of concentrated culture media and cell lysates can be analyzed for the presence or absence of the full length and the 25kD cleaved peptide with or without MG-132 treatment.

While the proteasome may play a role in VPR-1 processing, how the proteasome recognizes VPR-1 as a protein to cleave and not degrade is still not understood. Proteasomes usually recognize proteins that have been ubiquitinated. RPN-10, a proteasomal subunit that was identified as a hit in the RNAi screen, is a ubiquitin receptor that contains two polyubiquitin binding motifs [10, 11]. However, preliminary western blots with α-monoubiquitin and pan ubiquitin antibodies revealed that VPR-1 is not ubiquitinated. Ubiquitin alone is not always sufficient for proteasome proteolysis [12]. It has been shown that in the absence of protein ubiquitination, proteasome can access the intrinsically disorder regions for certain proteins and cleave at the internal sites to release folded domains surrounding the region [9]. Ubiquitin and disordered regions can act in cis or trans [12]. While ubiquitin allows for the proteasome to bind to its targets, a disordered region with the protein is needed for proteolysis initiation [13]. As mentioned above, the cleavage site of VPR-1 is within a disordered region. Therefore, while VPR-1 itself is not ubiquitinated, a ubiquitinated adaptor protein may recruit the proteasome to cleave VPR-1. This model may also potentially explain the cell type-specific cleavage and secretion of VPR-1, as VPR-1 interacting partners may be present only in cells that are shown to have non-cell-autonomous signaling capacity. Further studies are needed to test this model.

4.4 The role of YKT-6 in VPR-1 trafficking

The RNAi screen described in Chapter 3 also revealed the potential role of YKT-6 in VPR-1 trafficking. YKT-6 belongs to a class of vesicle SNAREs known as long vesicle-associated membrane proteins, or VAMPs [14]. VAPs were first identified in a screen for VAMP binding partners in *Aplysia* [15], but later shown to not participate in

the formation of the SNARE complex [2]. Therefore, how YKT-6 mediates VAP trafficking and MSPd secretion remains unanswered.

My attempts to provide functional evidence for the role of YKT-6 in VPR-1 MSPd secretion were hindered by the fact that YKT-6 null *C. elegans* are larval lethal. Identifying and cloning the endogenous promoter of *ykt-6* to make a rescue construct was unsuccessful because the gene lies within an operon. Mammalian Ykt-6 is largely expressed in the brain [16] but my attempts to express nematode *ykt-6* in the *C. elegans* nervous system using multiple neuronal promoters did not rescue larval lethality. Alternatively, combining promoter specific expression of *vpr-1* and GFP-mediated degradation of YKT-6 may provide a viable *C. elegans* model. Previous work from our lab [5] and data in Chapter 2 of this thesis show that expression of *vpr-1* in the intestinal cells by the *ges-1* promoter rescues the sterility phenotype of *vpr-1* null *C. elegans*. YKT-6 would need to be degraded in the intestinal cells of the *ges-1p::vpr-1* worms to test whether YKT-6 is necessary in MSPd secretion. To degrade YKT-6 protein, endogenous YKT-6 within the *ges-1p::vpr-1* line would be tagged with GFP using genome-editing techniques and a GFP-degron would be transgenically expressed in the intestine. Given that the GFP tag does not disrupt YKT-6 function, the worm line without the GFP-degron would develop to adulthood and be fertile. If YKT-6 is necessary for VPR-1 MSPd secretion, the worm line expressing the GFP-degron would be sterile.

The transgenic *ges-1p::FLAG:vpr-1* worm line described in Chapter 2 of this thesis serves as a useful tool to understand the YKT-6 and VPR-1 interaction. Confocal images of VPR-1 localization in *C. elegans* intestinal cells show that VPR-1 clusters at cell junctions and the basolateral membrane of intestinal cells. VPR-1 N-terminal

peptides from the intestine were also detected at the distal gonad. If YKT-6 plays a direct role in VPR-1 trafficking and MSPd secretion, then VPR-1 localization patterns within the intestinal cells would be disrupted after RNAi knockdown of YKT-6, and no N-terminal peptide would be detected on the distal gonad. If YKT-6 directly binds VPR-1 or the two proteins are found in a complex, FLAG tagged VPR-1 can be used to pulldown YKT-6 or vice versa.

4.5 Impact of this work

Collectively the work included in this thesis advanced our understanding of VAP protein cleavage and secretion. The data presented supports the theory that the proteasome does not only degrade proteins, but also has a role in regulated protein cleavage at specific sites, specifically those in disordered regions. The type-II topology of VAP proteins across the ER membrane has previously led us to propose that the cleaved N-terminal peptide selects unconventional routes to exit the cell and function as a non-cell-autonomous signal. However, the identification of the Golgi v-SNARE YKT-6 suggests that VAP proteins may be trafficked through the conventional ER-Golgi path to reach the plasma membrane.

There are two possible scenarios that bring the data from the two thesis chapters together. In the first scenario, the proteasome cleaves VPR-1 from the ER membrane to release the 156 amino acid long N-terminal peptide. This N-terminal peptide is then incorporated into YKT-6 vesicles to the plasma membrane for secretion. The second and more evident possibility is that VPR-1 is first trafficked to the plasma membrane by YKT-6 and then cleaved at the plasma membrane by membrane bound proteasomes such as those described by Ramachandran et al. The N-terminal MSPd peptide is released

extracellularly as the C-terminal peptide is degraded completely. This model would explain why attempts to isolate the cleaved C-terminal peptide of VPR-1 were unsuccessful. It also may explain why full length VPR-1 protein clustered heavily at the basolateral membrane and cell junctions of intestinal cells.

The potential role of the proteasome in cleaving VAP proteins has clinical implications in ALS pathology. Proteasome impairment has been linked to various neurodegenerative diseases including ALS, but almost all studies focused on the ubiquitin-proteasome system failing to maintain homeostasis [17, 18]. While it is not clear whether proteasome dysfunction is a cause or effect of neurodegeneration, a recent study showed that protein aggregates can inhibit both ubiquitin-dependent and ubiquitin-independent functions of the proteasome [19]. The substitution mutation P56S has been shown to cause VAPB to oligomerize and recruit wild type VAP proteins into protein aggregates [20]. While cleaved N-terminal MSPd peptides cannot be derived in vivo from $VAPB^{P56S}$ protein aggregates, the mutant protein is not inherently resistant to proteolysis [21]. Therefore, $VAPB^{P56S}$ aggregates may be impairing the function of the proteasome which disrupts MSPd cleavage and thus secretion. As a result, ALS patients do not present with MSPd circulation in the cerebral spinal fluid and blood [6]. Recently, a group of researchers developed a method to use these VAPB aggregates as a biomarker for ALS diagnosis since these aggregates have also been detected in patients with sporadic ALS who do not present with genetic mutations within the VAPB gene [22]. However, linking the role of the proteasome to the cleavage of VAPB situates the proteasome as a potential therapeutic target. Drugs that dissolve or prevent VAP protein aggregation, safe guard the proteasome from impairment by protein aggregates, or

enhance the proteasome cleaving function could be effective treatments for an otherwise untreatable ALS diagnosis.

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