Title: 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.

Keywords: VAPB, VPR-1, Major Sperm Protein, MSP, YKT-6, 20S proteasome, 19S proteasome, *Caenorhabditis elegans*, UBXD1, ALS

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 immunoglobulin-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), 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 (26). HT115(DE3) bacterial feeding strains were obtained from the Ahringer library (27). 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, 28). *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-1p::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 (29). 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 (30). 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 (31). p97/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 (32, 33). 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, 32, 34, 35). 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 VAPB/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) (36). This classification is attributed to its N-terminal profilin-like domain, known as a longin domain (LD), that helps regulate YKT-6 binding (36). Unlike most SNAREs, YKT-6 lacks a transmembrane domain (37, 38). 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 (37-39), as well as a reversible post-translation modification, palmitoylation (39, 40). Farnesylation itself is insufficient for YKT-6 to insert into the membrane, as most YKT-6 is present as a depalmitoylated, farnesylated, cytosolic protein (37-39, 41-43). 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 (44, 45). 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 (36, 39, 41, 44). 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 (37, 38, 41-43, 46-52).

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 (53). 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 (50). 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, 41). 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 (54). 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 β 2 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, β 1 and β 5, 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 eIF3a and eIF4G (61), Y-box RNA/DNA binding protein (62), and Δ 40p53 (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 (54). 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)

1 MSEKHSLLQV TPNRELVFTG PFSDVVTSHM TLKNTSTNPV CFKVKTAPK
 51 QYCVRPNSGL LKSGDSKQIT VMLQPLEGIP SDAGRHKFMV QSCVAPSEDL
 101 PDLESVWKII DPAELTYSKL MVTFVDKRDP ASGDDTKTFA VRNEDSFASS
 151 GQAQELGSSY SGTPSHDGTV NSLRKSLKST VDEKEELQKK VHGLEQEIEV
 201 MLKKNRKLQQ SHSDGALVDG AYPTLQIFLI AVAALLIGLI VGRLF

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).

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

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