RESEARCH ARTICLE

The secreted MSP domain of *C. elegans* VAPB homolog VPR-1 patterns the adult striated muscle mitochondrial reticulum via SMN-1

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INTRODUCTION

VAMP/synaptobrevin-associated proteins (VAPs) comprise an evolutionarily conserved protein family with an N-terminal major sperm protein domain (MSPd), coiled-coil motif, and transmembrane region (Lev et al., 2008). They are synthesized as type II integral membrane proteins with the MSPd residing in the cytosol. The MSPd is named after nematode major sperm proteins, which function as sperm cytokeskeletal elements and secreted signaling molecules (Bottino et al., 2002; Miller et al., 2001; Roberts and Stewart, 2012). In animals, VAPs have two diverse biochemical functions. VAPs act in a cell-autonomous fashion as scaffolding components at intracellular membrane contact sites (Lev et al., 2008; Stefan et al., 2011). In this capacity, there is evidence for roles in lipid transport, Ca\(^{2+}\) homeostasis, the unfolded protein response and other processes. VAPs also have a non-cell-autonomous signaling function (Han et al., 2012, 2013; Tsuda et al., 2008). In neurons and other cells, the MSPd is liberated from the transmembrane domain and unconventionally secreted into the extracellular environment. The MSPd signals through Eph and Lar-like receptors that modulate the actin cytoskeleton. An important MSPd target is striated muscle, where signaling regulates mitochondrial morphology and localization (Han et al., 2012, 2013).

Humans have two VAP paralogs called VAPA and VAPB, which have broad, largely overlapping expression patterns (Gkogkas et al., 2008; Larroquette et al., 2015; Lev et al., 2008). A P56S substitution in the VAPB MSPd is associated with amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Di et al., 2016; Nishimura et al., 2004). ALS clinical symptoms often emerge when the patient is in their fifties and are characterized by progressive muscle weakness, atrophy, and spasticity, resulting from degeneration of upper and lower motor neurons (Rowland, 1998). Most ALS cases occur sporadically without a clear family history. However, mutations in over 20 genes, including *VAPB*, are associated with familial ALS forms (Peters et al., 2015).

The MSPd P56S mutation also causes a late-onset form of SMA, which is characterized by lower motor neuron degeneration (Burbges and Beattie, 2009). Although SMA is more common in infants and children, rare adult-onset cases do occur (Nishimura et al., 2004; Tiziano et al., 2013). Reduced survival of motor neuron 1 (SMN-1) function causes ~95% of all SMA cases (Burbges and Beattie, 2009; Lefebvre et al., 1995). SMN-1 is part of a protein complex that controls the assembly of small nuclear ribonucleoproteins (snRNPs) essential for pre-mRNA splicing (Fallini et al., 2011; Liu et al., 1997). It is not clear whether this function or an alternative function is crucial for SMA pathogenesis (Cauchi, 2010). For instance, SMN-1 localizes to myofilaments in *Drosophila* flight muscles, where it regulates actin dynamics (Rajendra et al., 2007).

Evidence is accumulating that MSPd signaling may be important in sporadic ALS cases. The VAP MSPd is found in human blood and cerebrospinal fluid (CSF), although its circulating function is not understood (Deidda et al., 2014; Tsuda et al., 2008). In an Italian cohort, a majority of sporadic ALS patients had undetectable VAPB MSPd levels in CSF (Deidda et al., 2014). The pathogenic P56S mutation prevents MSPd secretion in cultured cells and animal tissues (Han et al., 2012; Tsuda et al., 2008). EphA4, an ephrin receptor that also interacts with the VAPB MSPd (Lua et al., 2011; Tsuda et al., 2008), modifies pathogenesis in ALS patients and in a zebrafish model (Van Hoecke et al., 2012). Eph and Lar-related...
receptors are expressed in motor neurons and striated muscles. While both cell types are implicated in ALS, their respective roles are not well delineated (Dupuis et al., 2011; Turner et al., 2013; Zhou et al., 2010). Familial ALS patients carry the pathogenic mutation throughout their lives. Disease-causing mutant proteins tend to be expressed early and ubiquitously, potentially triggering secondary effects and compensatory mechanisms that mask the primary pathological event. Unfortunately, defining early pathogenic processes has proven challenging. A better understanding of MSPd function might provide insight into these processes.

C. elegans and Drosophila VAPs have an important signaling function that impacts striated muscle mitochondria (Han et al., 2012, 2013; Tsuda et al., 2008). MSPd signaling to C. elegans body wall muscle remodels the actin cytoskeleton, thereby docking mitochondria to myofilaments, altering fission/fusion balance and promoting energy metabolism (Han et al., 2012). MSPd antagonizes signaling via the CLR-1 Lar-related tyrosine phosphatase receptor. Excess CLR-1 activity promotes actin filament formation in the muscle belly, displacing mitochondria from I-bands. In aging worms, muscle cytoskeletal or mitochondrial abnormalities induce elevated Forkhead Box O (FoxO) transcription factor activity (Han et al., 2013). FoxO promotes muscle triacylglycerol (TAG) accumulation, alters ATP metabolism, and extends lifespan, despite reduced mitochondria electron transport chain activity. Vapb knockout mice also exhibit abnormal muscular FoxO metabolic gene regulation (Han et al., 2013). These data support the model that the MSPd promotes striated muscle energy metabolism.

Here we use C. elegans to further investigate the VAP-related 1 (VPR-1) signaling mechanism. Our results support the model that neurons and germ cells secrete the MSPd into the pseudocoelom, where it acts on CLR-1 receptors expressed throughout the muscle plasma membrane. Although vpr-1 mutant muscle mitochondrial defects initiate early in larval development, MSPd–to–CLR-1 signaling is sufficient during the L4 stage and adulthood to localize mitochondria to I-bands. In a suppressor screen, we identified SMN-1 as a crucial MSPd downstream mediator in muscle, where it regulates mitochondrial morphology and localization. We propose that VAPB homologs have an evolutionarily conserved signaling function to pattern the mitochondrial reticulum in striated muscle. This signaling activity is essential during adulthood and requires SMN-1 in muscle.

RESULTS

Muscle mitochondrial defects in vpr-1 mutants emerge in larval development

In adult central body wall muscles, mitochondrial tubules lie in parallel arrays on top of (or beneath, depending on dorsal or ventral orientation) dense bodies along myofilament I-bands (Fig. 1A,B). Muscle mitochondria are visualized using a mitoGFP reporter expressed under the muscle-specific myo-3 promoter (Fig. 1B), as well as dyes such as Rhodamine 6G and MitoTracker CMXRos (Han et al., 2012, 2013). Mitochondria localize along actin-rich thin filaments (Fig. 1C), where they undergo fission and fusion with adjacent tubules (Han et al., 2012). Myofilaments appear normal in vpr-1 mutants, but ectopic Arp2/3-dependent actin network reorganization in the adult muscle belly displaces most mitochondria from I-bands (Han et al., 2012, 2013).

To investigate the origin of vpr-1 mutant mitochondrial defects, we conducted a developmental timecourse starting at the L1 larval stage. Shortly after hatching, mitochondria in wild-type body wall muscle are predominantly peri-nuclear and extend thin tubules into the muscle cytoplasm or belly (Fig. 1D). As larvae develop, muscle mitochondria become progressively associated with I-bands, forming parallel arrays of mitochondrial tubules. By the early adult stage, most tubules are closely associated with I-bands (Fig. 1D) (Han et al., 2012). Thus, mitochondria in muscle develop their stereotypical positioning during larval development.

At hatching, muscle mitochondria in vpr-1(tm1411) mutants look similar to those in the wild type. The tm1411 allele is a molecular null mutation that deletes the translational start site and entire MSPd (Han et al., 2012, 2013; Tsuda et al., 2008). vpr-1 mutant mitochondria are predominantly peri-nuclear with tubules branching into the cytoplasm (Fig. 1E). However, mitochondria in vpr-1 mutant muscles fail to target I-bands during larval development and adulthood. They largely remain in the muscle belly, forming branched networks as the muscle grows in size (Fig. 1E). These mitochondrial networks increase in complexity as the worm ages. Rhodamine 6G and MitoTracker CMXRos dyes stain vpr-1(tm1411) mutant muscle mitochondria well during early larval development. Staining is less efficient at the L4 and adult stages, perhaps reflecting changes in transmembrane potential or tubule architecture (Han et al., 2012). During adulthood, mitochondrial tubules are hyperfused and fat droplets accumulate in the belly (Han et al., 2012, 2013). Consistent with fission/fusion imbalance, transgenic lines expressing the fission mediator DRP-1::mCherry and fusion mediator FZO-1::mCherry show abnormal localization in adult vpr-1 mutant muscle (Fig. S1). We conclude that the vpr-1 mutant muscle mitochondrial defects initiate in larval development, resulting in abnormal mitochondrial fission/fusion dynamics in adults.

In neurons, synapses have a high energy demand that depends on closely associated mitochondria (Hollenbeck and Saxton, 2005). To determine if vpr-1 loss affects neuronal mitochondrial localization, we generated wild-type and vpr-1(tm1411) transgenic lines expressing mitoGFP and the synaptic vesicle marker mCherry::RAB-3 (Ding et al., 2007; Nonet et al., 1997) in motor neurons. There was no statistical difference between the two lines in the percentage of mitochondria associated with synapses (Fig. S2). We observed a possible increase in mCherry::RAB-3 puncta size in vpr-1(tm1411) mutants, suggesting that synaptic size or vesicle density is increased. Therefore, vpr-1 loss causes mitochondrial localization defects in larval and adult muscles, but not in motor neurons. Whether vpr-1 loss causes functional or subtle trafficking defects in neuronal mitochondria is not clear with the present data.

The nervous system and germ line are major origins of VPR-1 signaling activity

Previous experiments showed that driving vpr-1 cDNA expression pan-neuronally using the unc-119 promoter rescued ∼30–40% of the muscle mitochondrial defects in vpr-1(tm1411) mutants (Han et al., 2012). We found that vpr-1 genomic sequence, including the 3′ UTR, is more efficient than the cDNA with the unc-54 3′ UTR in rescuing the vpr-1 mutant gonadogenesis defect (Cottee et al., 2017). Transgenes containing vpr-1 genomic DNA driven by pan-neuronal (unc-119p), GABA motor neuron (unc-25p), cholinergic motor neuron (unc-17p), head interneuron (glr-5p) or sensory neuron (osm-6p) specific promoters rescue the vpr-1 mutant muscle mitochondrial defects in about half the muscles, with variation apparent among animals (Fig. 2; data not shown). By contrast, the endogenous vpr-1 promoter and genomic locus provide complete rescue (Han et al., 2013). These results indicate that vpr-1 expression in diverse neuron classes is sufficient to promote muscle mitochondrial localization, but additional sources of VPR-1 might be involved.
Genetic mosaic analysis using the \textit{vpr-1} genomic locus indicates that \textit{vpr-1} expression is essential in the nervous system and germ line but not in muscle to promote muscle metabolism (Han et al., 2013). The germ line is a source of maternal mRNAs provided to the embryo, as well as zygotic gene expression in developing and adult gonads. The germ line and muscle can exchange signaling molecules and other factors through the pseudocoelom (Fig. 2A). Maternal \textit{vpr-1} mRNA is sufficient to weakly rescue the \textit{vpr-1} (tm1411) mitochondrial defects in adult muscle (Fig. 2B). To investigate zygotic germline expression, we generated single-copy integrated transgenic lines that express \textit{vpr-1} under the pie-1 promoter (Seydoux and Dunn, 1997). Two independent integrated transgenes were crossed into the \textit{vpr-1} (tm1411) background. Both transgenes completely rescued the muscle mitochondria (Fig. 2B) and gonad development defects (Cottee et al., 2017). We used two strategies to evaluate zygotic germline \textit{vpr-1} expression (see Materials and Methods). These \textit{vpr-1} mutant hermaphrodites lack maternal \textit{vpr-1} mRNA and contain a single copy of the pie-1p::\textit{vpr-1} transgene provided in the paternal genome. In both experiments, the \textit{vpr-1} mutant muscle mitochondrial defects were rescued in about half the muscle, with variability among animals (Fig. 2B). Therefore, zygotic germline \textit{vpr-1} expression is sufficient to promote body wall muscle mitochondrial localization. In summary, the results of these genetic mosaic and transgenic expression studies are consistent with the nervous system and germ line acting together as major sources of VPR-1 MSPd signaling activity to body wall muscle.

**Endogenous CLR-1 receptor is expressed throughout the sarcolemma in larval and adult worms**

The VPR-1 MSPd might signal to muscle from the pseudocoelom or via the neuromuscular junction during larval development, adulthood, or both. MSPd signals are transduced in muscle through the CLR-1 receptor (Han et al., 2012, 2013). The CLR-1...
expression pattern should provide clues as to where and when MSPd signal transduction initiates. To determine endogenous CLR-1 expression, we used Cas9 to fuse tdTomato to the *clr-1* genomic locus, creating a C-terminal fusion protein (Fig. 3A). Reduced *clr-1* function in the hypodermis causes fluid to accumulate throughout the pseudocoelom, arresting development and causing gonad degeneration (Huang and Stern, 2004; Kokel et al., 1998). tdTomato insertion did not disrupt CLR-1 function, as shown by growth to adulthood, fertility, and the absence of fluid accumulation. We observe endogenous CLR-1::tdTomato expression in muscle and a variety of other cell types, including somatic gonad, hypodermis and neurons (Fig. 3B and Fig. S3). In larval and adult body wall muscle, CLR-1 is expressed throughout the plasma membrane, called the sarcolemma, and in puncta within the muscle cytoplasm (Fig. 3B). We did not detect enrichment or absence at post-synaptic plasma membrane sites near the nerve cord, showing that CLR-1 is uniformly expressed. Cell surface CLR-1 expression did not significantly change in the vpr-1(tm1411) background (Fig. S4). Therefore, the CLR-1 extracellular domain is accessible to MSPd signals secreted from adjacent motor neurons or from more distant neurons and germ cells via the pseudocoelom.

**MSPd signaling via CLR-1 is sufficient during adulthood**

The MSPd interacts with the CLR-1 extracellular domain to antagonize CLR-1 receptor tyrosine phosphatase signaling (Han et al., 2017). MSPd signaling via CLR-1 is necessary and sufficient for glucose uptake and fat accumulation in neurons and muscle cells (Han et al., 2017). Therefore, we tested whether the expression of the MSPd receptor, and the MSPd binding site, are sufficient to rescue the phenotype of the *clr-1* mutant. We found that the expression of the MSPd receptor, and the MSPd binding site, are sufficient to rescue the phenotype of the *clr-1* mutant.
et al., 2012). Accordingly, CLR-1 loss suppresses the vpr-1 (tm1411) mitochondrial defects (Han et al., 2012). To investigate the temporal requirements of MSPd signaling, we took advantage of the clr-1(e1745ts) temperature-sensitive allele (Kokel et al., 1998). Shifting clr-1(e1745ts) worms from the permissive temperature (16°C) to the restrictive temperature (25°C) reduces CLR-1 function, resulting in fluid accumulation. To avoid confounding issues due to fluid, we generated vpr-1(tm1411); clr-1(e1745ts) transgenic animals that express clr-1 specifically in the hypodermis using the rol-6 promoter. The rol-6p::clr-1 transgene largely rescued the clr-1(e1745ts) fluid accumulation defect, allowing for development (Fig. S5). At the permissive temperature, muscle mitochondria in transgenic double mutants exhibited either the highly branched networks seen in vpr-1 null muscle or more fragmented networks with no apparent organization (Fig. 4A). We shifted the double mutants to the restrictive temperature at various times during development, thereby simulating MSPd signaling through CLR-1 inactivation. When the mutants were shifted at embryonic or early larval stages (L1-L2) and grown to adulthood, mitochondria aligned at myofilaments, but with abnormal morphology (Fig. 4B). However, shifting them at L4 and early adulthood rescued the muscle mitochondrial defects scored 3 days later (Fig. 4C). These data suggest that MSPd signaling via CLR-1 is sufficient in late larval and adult stages, when a major source of MSPd signals, the germ line, is expanding and differentiating. Temporally inducing vpr-1 expression in head neurons using the binary Q system (Wei et al., 2012) also promoted muscle mitochondrial alignment (Cottle et al., 2017).

In 1-day adult vpr-1 mutants, the muscle mitochondrial reticulum is strongly disrupted due to excess CLR-1 activity (Fig. 1E) (Han et al., 2012). To test whether this reticulum can be remodeled, we shifted 1-day adult worms to the restrictive temperature and observed their mitochondria 24, 48 and 72 h later. After 24 h, the mitochondria are still largely disorganized and surround fat droplets in the muscle belly (Fig. 4D). Mitochondria disorganization and increased fat droplets are both due to abnormal Arp2/3 activity (Han et al., 2012, 2013). Shifting for 24 h is sufficient to induce fluid accumulation in nontransgenic mutants, indicating that CLR-1 function is compromised. At 48 h, most mitochondrial tubules are aligned at myofilaments, although morphology is still abnormal (Fig. 4E). By 72 h, mitochondria are positioned correctly at I-bands with morphology similar to those in wild-type muscle (Fig. 4F). Very few fat droplets are observed in the cytoplasm. These data suggest that MSPd signals continuously instruct muscle mitochondria to remodel via an active process throughout adulthood.

**A vpr-1 mutant mitochondrial suppressor screen identifies smn-1**

We next sought to understand how MSPd signals are transduced in muscle. To identify downstream mediators, we developed a vpr-1 (tm1411) RNAi suppressor screen based on prior work using arx-2 and clr-1. arx-2 (also known as arp-2) encodes a component of the Arp2/3 complex (Roh-Johnson and Goldstein, 2009). Arp2/3 loss in vpr-1 mutants suppresses the muscle mitochondrial defects (Han et al., 2012). Transgenic lines that express functional ARX-2::mCherry (see below) in wild-type muscle show punctate localization between thin filaments, slightly above mitochondrial tubules (Fig. 5A,B). Little ARX-2::mCherry is found in the muscle belly (Fig. 5B). In vpr-1 mutants, ARX-2::mCherry is observed at myofilaments and throughout the belly (Fig. 5B), where actin filaments and most mitochondria exist. These data support the model that excess CLR-1 signaling promotes Arp2/3 activity and/or localization in the muscle cytoplasm, Preventing mitochondria from targeting I-bands. Disrupting this pathway suppresses the vpr-1 (tm1411) muscle mitochondrial defects, which is likely to be because a redundant mechanism localizes mitochondria to I-bands.

Our suppressor screen should identify those gene products specifically required for abnormal mitochondrial localization caused by MSPd deficiency. As a pilot investigation, we tested 31 RNAi clones corresponding to *C. elegans* homologs of genes implicated in ALS and SMA (Table S1). Three potential suppressors were identified: the nuclear export receptor *xpo-1*, the *Gemin3* homolog *mel-48*, and the survival of motor neuron 1 gene *smn-1*. In this paper, we focus on *smn-1*. Similar to inactivation of *clr-1* or *arx-2* (Fig. 4D and Fig. 5C), *smn-1* RNAi largely suppresses the muscle mitochondrial defects in vpr-1 mutant animals (Fig. 5C). *smn-1* RNAi initiated in the parental generation often causes larval arrest in vpr-1 mutant progeny and muscle mitochondria to localize to I-bands with more globular morphology. *smn-1* RNAi initiated in L2-L3 larva is sufficient to partially suppress the vpr-1 mutant mitochondrial defect, suggesting that *smn-1* activity is essential
during late larval development and adulthood. smn-1 RNAi also partially suppresses vpr-1 null mutant muscle fat droplet accumulation, as measured using fluorescent BODIPY fatty acid analogs (Fig. S6). We did not observe mitochondrial or fat droplet suppression following RNAi of C. elegans homologs of Gemin2, Gemin6, Gemin7 or other genes crucial for pre-mRNA splicing (Table S1 and Fig. S7). Hence, smn-1 function may be independent of its role in spliceosome assembly.

smn-1 is broadly expressed and smn-1(ok355) null mutants arrest development around the L3 stage, after maternal smn-1 mRNA is depleted (Briese et al., 2009). During this stage, mitochondria in smn-1 knockout body wall muscle are largely globular in morphology and abnormally positioned throughout the belly (Fig. 6A). To test whether smn-1 functions in muscle, we generated smn-1(ok355) transgenic worms that express smn-1::mCherry specifically in muscle using the myo-3 promoter. The myo-3p::smn-1::mCherry transgene rescued the muscle mitochondrial defects of smn-1(ok355) worms (Fig. 6B). In addition, muscle smn-1 expression partially suppresses the larval arrest and sterility phenotypes in a subset (<15%) of smn-1(ok355) mutants (Fig. 6B). We conclude that the smn-1::mCherry transgene is functional and smn-1 expression in muscle is sufficient to regulate mitochondria.

To test whether smn-1 is necessary in muscle, we specifically depleted smn-1 in body wall muscle of wild-type animals using an RNAi mosaic strategy (Durieux et al., 2011; Esposito et al., 2007). sid-1 (systemic RNA interference deficiency-1) mutants are defective for siRNA transport between cells, thereby preventing systemic RNAi effects. However, producing siRNA within tissues still induces cell-autonomous RNAi (Winston et al., 2002). We expressed smn-1 sense and antisense RNAs in body wall muscle of sid-1(pk3321) mutants. Muscle smn-1 RNAi causes mitochondrial morphological defects similar to those seen in smn-1 mutants or systemic RNAi hermaphrodites (Fig. 6A,C). smn-1 reduction of function suppresses the vpr-1 null muscle mitochondrial defects, whereas smn-1 loss throughout development causes more globular mitochondrial morphology. In summary, a vpr-1 mitochondrial suppressor screen identified smn-1, which is necessary and sufficient in muscle to control mitochondrial morphology.

SMN-1 and ARX-2 colocalize at muscle myofilaments
SMN-1 expressed from the rescuing smn-1::mCherry transgene is seen in the nucleus and cytoplasm (Fig. 7A). In the cytoplasm, most SMN-1::mCherry is found at myofilaments in regularly spaced puncta. These puncta appear more ordered in adult
muscle as compared with younger muscle (Fig. 7A). The SMN-1 expression pattern at muscle myofilaments resembles that of ARX-2::mCherry (Fig. 5A). To test whether ARX-2 and SMN-1 colocalize, we generated transgenic animals expressing both smn-1::GFP and arx-2::mCherry in muscle. SMN-1::GFP colocalizes with ARX-2::mCherry at the thin filaments (Fig. 7B). However, only SMN-1 is observed in the nucleus. We occasionally observed SMN-1::GFP and ARX-2::mCherry in peri-nuclear puncta (data not shown). SMN-1::GFP and ARX-2::mCherry also colocalize in the vpr-1(tm1411) background, indicating that MSPd signaling does not influence their association (Fig. 7B). We conclude that SMN-1 and ARX-2 colocalize at muscle myofilaments adjacent to elongated mitochondrial tubules.

SMN-1 may promote Arp2/3 activity in muscle
The above data and dependence of mitochondrial localization on actin remodeling raise the possibility that SMN-1 promotes Arp2/3 activity. In this case, smn-1 deletion should cause a similar muscle mitochondrial phenotype as arx-2 depletion. Although arx-2 null worms are embryonic lethal, restricting arx-2 inactivation to wild-type larval and adult stages via RNAi feeding causes muscle mitochondria to adopt globular morphologies (Fig. 6A) (Han et al., 2012). Muscle mitochondria in smn-1(ok355) and smn-1 RNAi hermaphrodites exhibit similar globular morphologies (Fig. 6 and Fig. 8A). Moreover, smn-1(ok355) heterozygotes also contain abnormally shaped mitochondria (Fig. 6A), suggesting that the smn-1 expression level is important. Thus, reduced smn-1 or arx-2 function causes similar muscle mitochondrial phenotypes.

**Fig. 6. SMN-1 functions in body wall muscle.** (A) Mitochondria visualized with mitoGFP in smn-1 (ok355) mutant muscle. smn-1(ok355) hermaphrodites arrest development around the L3 stage when maternal smn-1 runs out (Breise et al., 2009). (B) Muscle mitochondria in smn-1(ok355) mutants expressing smn-1::mCherry specifically in muscle. The top two panels are from the same L4 stage hermaphrodite; the bottom three panels are from different adult hermaphrodites. Some transgenic mutants are able to progress to L4 and young adult stages and contain embryos (E), which are not seen in nontransgenic smn-1(ok355) mutants. (C) Mitochondria in adult muscle following muscle-specific smn-1 RNAi using sid-1 mutants. Asterisk, nucleus. Scale bars: 5 µm in A,C; as indicated in B.

**Fig. 7. SMN-1 and ARX-2 localization in body wall muscle myofilaments.** (A) The rescuing myo-3p::smn-1::mCherry transgene shows SMN-1 expression in the nucleus (asterisk) and in puncta at myofilaments. Expression is sometimes observed in peri-nuclear puncta in the muscle belly. (B) Confocal images showing SMN-1::GFP and ARX-2::mCherry localization at myofilaments of adult control and vpr-1 mutant muscles. Boxed areas are magnified on the right. Puncta are also observed in the muscle belly of vpr-1 mutants (see Fig. 5B). Scale bars: 5 µm.
Current data suggest that \textit{arx-2} and \textit{smn-1} act in the same pathway. To further investigate their relationship, we expressed the \textit{myo-3p::arx-2cDNA::mCherry} transgene in \textit{smn-1(ok355)} null mutants (Fig. 8B-F). \textit{arx-2} expression may escape endogenous RNA regulatory interactions because the transgene lacks \textit{arx-2} introns and 3' UTR (Fig. 8C). We found that ARX-2::mCherry localizes to myofilament puncta in \textit{smn-1(ok355)} muscle (Fig. 8B), similar to the localization in wild-type muscle (Fig. 5). Importantly, ARX-2::mCherry overexpression partially suppresses the \textit{smn-1} mutant mitochondrial defect, as mitochondria adopt elongated morphologies often along I-bands (Fig. 8D,E). ARX-2::mCherry overexpression also mildly, but significantly, increased \textit{smn-1} mutant mobility in the thrashing assay (Briese et al., 2009), which measures the ability to contract body wall muscles in liquid medium (Fig. 8F). Taken together, the results support the model that SMN-1 and Arp2/3 promote actin remodeling events needed to localize mitochondria in muscle.

\section*{DISCUSSION}

The results presented here, together with those in prior studies, suggest that VAPB proteins have a noncanonical function as an endocrine factor to pattern the striated muscle reticulum. The primary mechanism hinges on a secreted VAP proteolytic fragment, the MSPd (Tsuda et al., 2008). In \textit{C. elegans}, the secreted MSPd antagonizes the CLR-1 Lar-like phosphatase receptor, triggering changes in muscle mitochondrial localization, fission/fusion and function (Han et al., 2012). Disrupting MSPd signaling causes energy deficit in adult muscle, along with compensatory metabolic changes during aging (Han et al., 2013). Here we show that muscle mitochondria target myofilament I-bands during late larval development and adulthood. This mitochondrial patterning mechanism fails in \textit{vpr-1} null mutants. Our results support the model that neurons and germ cells secrete the MSPd into the pseudocoelomic fluid, where it interacts with CLR-1 throughout the body wall muscles and other internal organs (Hall et al., 1999). We show that endogenous CLR-1 receptor is expressed throughout the muscle plasma membrane in larval and adult worms, where it is accessible to the pseudocoelomic fluid. These results are consistent with the MSPd having endocrine and paracrine signaling activity to muscle.

A temperature-sensitive \textit{clr-1} mutation (Kokel et al., 1998) was used to temporally induce MSPd signaling in \textit{vpr-1} null animals. MSPd signaling at the L4 stage or during adulthood is sufficient to localize mitochondrial tubules to muscle I-bands. Earlier inductions promote mitochondrial localization, but morphology is abnormal. It is possible that MSPd levels rise throughout larval development as germ cells increase in number, further antagonizing CLR-1 signaling. In wild-type animals, reducing muscle \textit{clr-1} function throughout larval development causes globular instead of tubular mitochondrial morphology (Han et al., 2012). A balance in CLR-1 signaling might be important early in development, whereas CLR-1 is largely inactive during adulthood. Muscle mitochondrial networks are already disorganized in young adult...
vpr-1 mutants, yet CLR-1 inactivation at this time induces remodeling events that target mitochondria to I-bands within 48 to 72 h. Therefore, MSPd activity on muscle appears to be instructive.

In muscle, CLR-1 activity triggers actin remodeling in the muscle belly that is dependent on the Arp2/3 complex (Han et al., 2012). These actin networks prevent mitochondria from associating with actin-rich I-bands, where ATP and Ca²⁺ levels fluctuate (Moerman and Williams, 2006). The MSPd attenuates CLR-1 signaling, thereby restricting Arp2/3 activity to the I-band. Mitochondria then align along the I-band, alter their fission/fusion properties, and alter function (Han et al., 2013). Whether Arp2/3 restriction occurs by controlling localization or actin nucleation activity is not clear. We identified smn-1 in an RNAi screen for vpr-1 mutant mitochondrial suppressors. SMN-1 acts in muscle, where it colocalizes with the Arp2/3 complex. Genetic studies are consistent with SMN-1 promoting Arp2/3 activity. This function is sensitive to gene dosage, suggesting that the SMN-1 expression level is important. It might also require the Gemin3 homolog MEL-46, but appears independent of other gemins, SM proteins, and other proteins involved in snRNP assembly. Prior studies have implicated SMN-1 in mRNA transport and actin remodeling independent of its snRNP role, although the mechanism is not well understood (Burghes and Beattie, 2009; Fallini et al., 2012; Rajendra et al., 2007).

In humans, reduced VAPB MSPd function is associated with sporadic and familial ALS cases (Deidda et al., 2014; Kabashi et al., 2013; Larroquette et al., 2015; Mitne-Neto et al., 2011; Teuling et al., 2007; Tsuda et al., 2008). Patients with the VAPB P56S mutation present with ALS, atypical ALS forms, or late-onset SMA (Marques et al., 2006; Nishimura et al., 2004). Outside of these patients, the vast majority of late-onset (type IV) SMA cases are due to reduced SMN1 function (Burghes and Beattie, 2009). Delayed onset is thought to result from extra copies of SMN2, which can weakly compensate for SMN1 loss (Butchbach, 2016). SMN1 and SMN2 copy number have also been implicated in ALS (Blauw et al., 2012; Butchbach, 2016). Our results show that C. elegans VAPB and SMN-1 homologs act in a signaling pathway governing a striated muscle mitochondrial transition. Motor neurons expressing the MSPd/ephrin receptor VAB-1 might also participate in some way (Brisbin et al., 2009).

In mammalian fast-twitch muscle fibers, mitochondrial doublets encircle myofibers at I-bands, where they couple to the sarcotubular system (Boncompagni et al., 2009; Pham et al., 2012). Similar to C. elegans, mitochondria acquire this I-band positioning during postnatal development (Boncompagni et al., 2009). These data raise an intriguing model. Reduced VAPB or SMN-1 function might perturb a mitochondrial transition in the postnatal neuromuscular system. Motor neurons innervating fast-twitch fibers are the first to degenerate in ALS mouse models (Van Hoecke et al., 2012; Vinsant et al., 2013a,b). Either muscle mitochondrial dysfunction or compensatory mechanisms could predispose these motor neurons to degeneration during aging. An important implication is that motor neuron disease may initiate many years before clinical symptoms emerge. Therefore, a much larger window could exist for therapeutic interventions and biomarker development to track disease progression.

**MATERIALS AND METHODS**

**C. elegans genetics and strains**

*C. elegans* were maintained at 20°C unless otherwise indicated, and fed with NA22 *E. coli* bacteria (Brenner, 1974; Edmonds et al., 2010; Kubagawa et al., 2006). The following strains were used: N2 Bristol (wild type); CB3241 *clr-1(e1745ts)*; VC1478 vpr-1(tm1411)/hT2 [bli-4(e937) let-2(q782) qsl-488];  *clr-1(e1745ts)*; VC1102 clr-1(e2530)/mln1 [dpy-10(e128) mls14] (II); and LM99 smn-1(ok355)/hT2 [bli-4(e937) let-2(q782) qsl-488] (III).

Studies with the *clr-1(e1745ts)* temperature-sensitive (ts) allele were performed at permissive (16°C) and restrictive (25°C) temperatures. Strain construction involved PCR, sequencing (UAB Heflin Center for Genomics Sciences), and phenotypic analyses. vpr-1(*tm1411*) mutants are maternal effect sterile (Cottee et al., 2017). Phenotypes were evaluated in vpr-1(*tm1411*) homozygous F2 progeny from vpr-1(*tm1411*)/hT2 heterozygotes (P0), unless otherwise indicated. vpr-1(*tm1411*) homozygous F1 progeny contain maternal vpr-1 mRNA.

**Zygotic vpr-1 germline expression**

We used two strategies to evaluate zygotic germline *vpr-1* expression. In the first strain, we mated vpr-1(*tm1411*)/SiL[pie-1p::vpr-1+unc-119(+)] males to F1 vpr-1(*tm1411*) hermaphrodites derived from vpr-1(*tm1411*)/hT2 heterozygous hermaphrodites. All cross progeny were scored. These *vpr-1(*tm1411*) null worms lack maternal vpr-1 and contain a single copy of the male-derived pie-1p::vpr-1 transgene for zygotic germline expression. In the second strategy, we mated vpr-1(*tm1411*)/SiL[pie-1p::vpr-1+unc-119(?)] males to vpr-1(*tm1411*); E[unc-119p::vpr-1+myo-3p::mitoGFP] hermaphrodites. vpr-1(*tm1411*) cross progeny lacking the extrachromosomal array were scored. These progeny contained a single copy of the male-derived pie-1p::vpr-1 transgene. Both experiments show rescue of the vpr-1 mutant muscle mitochondrial defects in about half the muscle, with variability among animals.

**Molecular cloning**

To create the Cas9 DNA template for tdTomato insertion into the *clr-1* genomic locus, Gibson assembly was used to construct a plasmid containing *clr-1* 2 kb left homology arm: *cdTomato*:clr-1 3′ UTR::unc-119:2 kb right homology arm. The *unc-119* rescue fragment is from plasmid pCF66 (Addgene plasmid #24981) and includes *C. briggsae unc-119*. The single guide RNA (sgRNA) plasmid was derived from Addgene plasmid 46169. Cas9 targeting sequence was 5′-ACTATATCTTACAGCATAT-3′. PCR was used to amplify the entire sgRNA backbone, except for 20 bp that belong to *unc-119*. DNA fusions were constructed using Gibson assembly. The rol-6p::clr-1 construct was made with clr-1 genomic DNA. 2 kb upstream of the rol-6 start codon was amplified by PCR. *unc-119p::mitoGFP* was created using the *unc-119* promoter (Maduro and Pilgrim, 1995) and mitoGFP sequences (Labrousse et al., 1999). Pan-neuronal (*unc-119p*, 2000 bp), GABA motor neuron (*unc-2p*, 1893 bp) (Jin et al., 1999), cholinergic motor neuron (*unc-17p*, 2003 bp) (Likiteig et al., 2001), head interneuron (*gmr-5p*, 430 bp) (Brockie et al., 2001) and sensory neuron (*osm-6p*, 430 bp) (Collet et al., 1998) promoters were fused upstream of the *vpr-1* genomic locus, which included the 3′ UTR. PCR was used to amplify sequences from genomic DNA. The pie-1p promoter sequence included 1095 bp upstream of the pie-1 translational start site. The *vpr-1* DNA sequence included exons and introns, as well as 745 bp of 3′UTR.

**Gateway recombination (Invitrogen)** was used to generate *myo-3p::moesin::mCherry*, *myo-3p::moesin::GFP*, *myo-3p::ars-2::mCherry*, *myo-3p::smn-1::mCherry*, *myo-3p::smn-1::GFP*, *myo-3p::drp-1::mCherry* and *myo-3p::fzo-1::mCherry*. To generate entry plasmids, *drp-1*, *fzo-1* and *ars-2* entry plasmids were prepared by PCR using a DNA library as the template. smn-1 DNA was amplified by PCR from a plasmid containing the smn-1 ORF (Open Biosystems). *Drosophila Moesin (dmoesin)* was amplified by PCR from pJWZ6 (Addgene plasmid #21744). All PCR products were cloned into pDONRR221 entry vector and sequenced. The entry vectors were subsequently used for the LR recombination reaction. The *unc-54 3′ UTR* was used, except where indicated. Primers (5′-3′; F, forward; R, reverse) were: *drp-1* F1, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG; *drp-1* R1, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG; *fzo-1* F1, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG; *fzo-1* R1, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG; *ars-2* F, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG; *ars-2* R, GGGGACCACTTGTTACAAAAAAGCAGGCTCCATGGA-AATCTTACATCTTGTGCG.
et al., 1998), indicating that tdTomato fusion does not compromise CLR-1.

Knudra Transgenics. Integrated transgenes were crossed into the copy insertion (transgene was a gift from Dr David Miller (Vanderbilt University). 1999). Multiple independent transgenic lines were analyzed. An into young adult hermaphrodite gonads. The

RNA-mediated interference (RNAi) RNAs were performed by the feeding method (Timmons and Fire, 1998). HT115(DE3) bacterial feeding strains were obtained from the genome-wide library (Kamath et al., 2003). PCR and sequencing (UBA Heflin Center for Genomics Sciences) were used to confirm that strains contained the correct clones. RNAi phenotypes were compared with those of null mutants to determine effectiveness.

Transgensics To generate transgenic C. elegans, plasmids (5-60 ng/μl) were injected into young adult hermaphrodite gonads. The myo-3p:mitoGFP transgene was used to evaluate body wall muscle mitochondria (Labrousse et al., 1999). Multiple independent transgenic lines were analyzed. An extrachromosomal array expressing pan-neuronal mitoGFP (unc-119p: mitoGFP) and the synaptic marker RAB-3 (unc-25p: mCherry::rab-3) was integrated into the genome by gamma irradiation. The unc-25p: mCherry::rab-3 transgene was a gift from Dr David Miller (Vanderbilt University). The pie-1p:: vpr-1g transgenic lines were generated using MosSCI single-copy insertion (tTS5605 Mos1 allele, near the center of chromosome II) by Keudra Transgenics. Integrated transgenes were crossed into the vpr-1 (tm1411) background and maintained as transgenic vpr-1 mutant homozygotes.

CRISPR/Cas9 CRISPR/Cas9 methods were performed as previously published (Friedland et al., 2013). DNA template, sgRNA, Cas9, and myo-3p:mitoGFP plasmids were injected into unc-119(ed3) worms. Progeny were 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 (UBA Heflin Center for Genomics Sciences) were used to confirm tdTomato insertion. The clr-1::tdTomato Cas9 line did not exhibit the fluid accumulation phenotype caused by reduced clr-1 function (Kokel et al., 1998), indicating that tdTomato fusion does not compromise CLR-1 activity.

Acknowledgements

We thank Matthew Schultz, M.A.M. lab members and other colleagues for discussions regarding this work and manuscript comments. Three anonymous reviewers provided valuable criticisms. We also thank Sarah Peterson and DavidMiller for the unc-25p:: mCherry::rab-3 plasmid.

Competing interests


Author contributions


Funding

This project was funded by the Muscular Dystrophy Association (MDA381933 to M.A.M.). Financial support for J.S. came from the National Science Foundation Graduate Research Fellowship Program and the University of Alabama at Birmingham Howard Hughes Medical Institute Med-Grad Program. Some strains were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). Deposited in PMC for immediate release.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.152025.supplemental

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Development (2017) 144, 2175-2186 doi:10.1242/dev.152025

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