Secreted VAPB/ALS8 Major Sperm Protein Domains Modulate Mitochondrial Localization and Morphology via Growth Cone Guidance Receptors

Sung Min Han,1 Hiroshi Tsuda,2,5 Youfeng Yang,1 Jack Vibbert,1 Pauline Cottie,1 Se-Jin Lee,1 Jessica Winek,1 Claire Haueter,3 Hugo J. Bellen,2,3,4 and Michael A. Miller1,∗

1Department of Cell Biology, University of Alabama School of Medicine, Birmingham, AL 35294, USA
2Department of Molecular and Human Genetics
3Howard Hughes Medical Institute
4Program in Developmental Biology
5Present address: Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec H3A 2B4, Canada
∗Correspondence: mamiller@uab.edu
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SUMMARY

The VAPB/ALS8 major sperm protein domain (vMSP) is implicated in amyotrophic lateral sclerosis and spinal muscular atrophy, yet its function in the nervous system is not well understood. In Caenorhabditis elegans and Drosophila, the vMSP is cleaved from its transmembrane anchor and secreted in a cell type-specific fashion. We show that vMSPs secreted by neurons act on Lar-like protein-tyrosine phosphatase and Roundabout growth cone guidance receptors expressed in striated muscle. This signaling pathway promotes Arp2/3-dependent actin remodeling and mitochondrial localization to actin-rich muscle I-bands. C. elegans VAPB mutants have mitochondrial localization, morphology, mobility, and fission/fusion defects that are suppressed by Lar-like receptor or Arp2/3 inactivation. Hence, growth cone guidance receptor pathways that remodel the actin cytoskeleton have unanticipated effects on mitochondrial dynamics. We propose that neurons secrete vMSPs to promote striated muscle energy production and metabolism, in part through the regulation of mitochondrial localization and function.

INTRODUCTION

Mitochondria are obligate endosymbionts that generate ATP for cellular energy through oxidative phosphorylation and play central roles in metabolism, calcium homeostasis, and apoptosis (Parekh, 2003; Suen et al., 2008). They are often distributed nonrandomly in differentiated cells like neurons and muscle, presumably to provide energy to regions high in metabolic demand. In neurons, mitochondria are enriched at synapses (Hollenbeck and Saxton, 2005), whereas in mammalian skeletal muscle, mitochondria are positioned in pairs near the I-bands (Vendelin et al., 2005). Motor proteins that move along microtubules drive mitochondrial transport over long distances (Hollenbeck and Saxton, 2005). However, the actin cytoskeleton can facilitate short-range mitochondrial movement and docking of mitochondria to specific sites (Boldogh and Pon, 2007; Pathak et al., 2010). In chicken sensory neuron cultures, Nerve Growth Factor-conjugated beads promote mitochondrial accumulation and docking by a mechanism dependent on F-actin (Chada and Hollenbeck, 2004). These data raise the possibility that growth factors may affect mitochondrial localization in vivo.

The VAPs (VAMP/synaptobrevin-associated proteins) comprise a highly conserved protein family with an N-terminal MSP (major sperm protein) domain, a coiled-coil motif, and transmembrane-spanning region anchored in endoplasmic reticulum (ER) membranes. The ~120 amino acid MSP domain is named after C. elegans MSPs, which function as secreted ligands that induce oocyte maturation and ovarian muscle contraction (Han et al., 2010; Miller et al., 2001). MSPs also have an intracellular cytoskeletal function, but this function is independent of actin and myosin, depends upon MSP polymerization into filaments, and is not conserved in VAPs (Bottino et al., 2002; Han et al., 2010; Lev et al., 2008). VAPs have been implicated in diverse processes, including regulation of lipid transport, ER morphology, and membrane trafficking (Lev et al., 2008). In addition, VAPs and MSPs have a conserved function as a secreted signaling molecule (Tsuda et al., 2008). We have shown that VAP MSP domains (vMSPs) are cleaved from the transmembrane domain and secreted into the extracellular environment, where they bind to Eph receptors (EphRs) and other unidentified receptors (Miller et al., 2003; Tsuda et al., 2008). The secretion mechanism appears to be unconventional and cell type specific.

A P56S mutation in the human VAPB MSP domain causes amyotrophic lateral sclerosis (ALS) and late-onset spinal muscular atrophy (SMA), two neuropathies characterized by progressive muscle atrophy and motor neuron degeneration (Funke et al., 2010; Miliecampes et al., 2010; Nishimura et al., 2004). VAPP56S causes VAP ubiquitination, recruitment of wild-type and mutant VAPs to cytoplasmic inclusions (Teuling et al.,
impaired MSP domain secretion (Tsuda et al., 2008). VAPB levels are reduced in sporadic ALS patients, 
\textit{sod1} mutant mice, and ALS8 patient motor neurons derived from induced pluripotent stems cells, suggesting that VAPB plays a widespread role in pathogenesis (Anagnostou et al., 2010; Mitne-Neto et al., 2011; Teuling et al., 2007).

Here, we present evidence that MSP domains comprise a conserved ligand class that modulates mitochondrial localization and morphology in muscle and oocytes. Neurons secrete vMSPs to promote mitochondrial docking at actin-rich I-bands of \textit{C. elegans} striated muscle. vMSPs transduce signals through muscle Roundabout and Lar-like receptors that modulate the actin-related protein 2/3 (Arp2/3) complex. VAP loss causes aberrant Lar receptor and Arp2/3 activity that displaces mitochondria from I-bands, influences the fission/fusion balance, and decreases transmembrane potential. We propose that neurons secrete vMSPs to promote mitochondrial localization and function important for energy metabolism in muscles.

**RESULTS**

**VAP Loss Causes Muscle Mitochondrial Defects in \textit{Drosophila} and \textit{C. elegans}**

We previously documented that neuronal overexpression of dVAP, a \textit{Drosophila} VAPB homolog (Pennetta et al., 2002), caused myofibrillar defects in the indirect flight muscle (IFM) of adult flies (Tsuda et al., 2008). This defect could be suppressed by Eph receptor knockout, suggesting that neurons secrete the dVAP MSP domain. The IFMs are massive and provide most of the energy needed for flight. To test whether VAP loss affects muscle, we examined the IFM in wild-type and dvap null mutant flies using transmission electron microscopy (TEM). In wild-type adults, TEM shows that the mitochondria are arranged in columns between the myofibrils (Figure 1A). In contrast, most mitochondria in dvap null mutant muscle are small and have abnormal cristae, whereas others are larger and have vacuole-like structures (Figure 1A; 84% of the...
mitochondria lack lamellar cristae, whereas 7% are enlarged, lack electron density, and are highly aberrant \( (n = 123) \). The myofibrils, on the other hand, appear similar to control myofibrils. Hence, dVAP loss causes severe mitochondrial morphology defects in adult fly muscles.

Next, we examined adult \textit{C. elegans} body wall muscle to test whether the role of VAPs (vpr-1 in worms) is evolutionarily conserved. Mitochondria were visualized in live muscle by expressing mitochondrial matrix-targeted GFP (mitoGFP) under control of the \textit{myo-3} muscle promoter, as well as by feeding worms the dyes MitoTracker CMXRos and Rhodamine 6g. In wild-type adults, muscle mitochondria form largely unbranched tubules within the belly (Figures 1B and 1C). 84.7% of these tubules in mitoGFP transgenic worms form linear arrays that are regularly spaced, correlating with the spacing between I-bands (Figures 1C and 1D; Table S1, line 1 available online). Indeed, the mitochondrial arrays overlap with the dense bodies, which occupy the middle of the I-band and appear as small bumps organized in the same orientation as the myofilaments (Figure 1D). Identical results are observed with MitoTracker CMXRos and Rhodamine 6g. Additional support for the association of mitochondria with I-bands comes from muscle TEM cross-sections (Figure 2A).

In contrast to wild-type, mitochondria in \textit{vpr-1(tm1411)} mutants are not arranged in parallel arrays and are rarely associated with dense bodies (Figures 1D and 1E; Table S1, lines 1 and 2). Three-dimensional microscopy indicates that mitochondria form thin and highly branched tubular networks within the muscle belly (Figure 1E). TEM cross-sections confirm that mitochondrial tubules in \textit{vpr-1} mutant muscle are smaller in diameter than controls and mostly displaced from the myofilaments (Figure 2B; Table S1, lines 1 and 2, \( p < 0.001 \)). The muscle belly is expanded, in part due to abnormal actin branching (see below) and mitochondria rarely localize to I-bands. Muscle myofilaments, ER, and motor neuron positions in \textit{vpr-1} mutants appear similar to the wild-type (Figure 2B; data not shown). Moreover, \textit{vpr-1} mutants do not exhibit abnormal muscle ER homeostasis (S.M.H. and M.A.M., unpublished data).

We conclude that \textit{vpr-1} is required for formation of tubular and largely unbranched mitochondrial arrays positioned along the I-bands. Collectively, these data indicate that VAP loss in \textit{Drosophila} and \textit{C. elegans} causes specific defects in muscle mitochondrial morphology.

**VAP Mutants Have Interconnected Mitochondrial Networks**

The mitochondrial tubules in \textit{vpr-1} mutant muscle appear more fused than tubules in wild-type muscle (Figure 1E). RNAi of the outer membrane fission mediator \textit{drp-1} and the inner membrane fusion mediator EAT-3/OPA1 can be used to shift the fission/fusion balance in mitochondrial networks (Kanazawa et al., 2008; Labrousse et al., 1999). Shifting the balance toward fusion in wild-type animals by \textit{drp-1} RNAi causes the formation of elongated and branched networks similar to those in \textit{vpr-1} mutants, except that the matrix often accumulates in large aggregates (Figure 3A). \textit{drp-1} RNAi in \textit{vpr-1} mutants also causes accumulation of identical aggregates. Shifting the balance toward fission in wild-type and \textit{vpr-1} mutant animals by \textit{eat-3/opa1} RNAi causes mitochondrial fragmentation (Figure 3A). These data suggest that muscle mitochondria in \textit{vpr-1} mutants have low fission/fusion balance.

We tested whether or not the mitochondrial tubules in \textit{vpr-1} mutants are physically connected to each other, consistent with their appearance in confocal axial scans. Fluorescence loss in photobleaching (FLIP) can be used to assess connectivity among muscle mitochondria. Our fluorescence recovery after photobleaching experiments (data not shown) and a previous study demonstrate that mitoGFP rapidly diffuses between contiguous compartments (Labrousse et al., 1999). In FLIP experiments, a mitochondrial tubule is repeatedly photobleached...
every 5 s followed by a scan of the surrounding area. Contiguous mitochondria lose fluorescence during each cycle, as mitoGFP diffuses into the bleached area. Thus, interconnected mitochondrial networks will exhibit rapid reduction in fluorescence outside of the bleached spot. To assess mitochondrial connectivity with FLIP, we used conditions that stabilize mitochondria and prevent

![Diagram](image.png)

**Figure 3. vpr-1 Loss Influences Muscle Mitochondrial Connectivity and Function**

(A) Genetic relationships between vpr-1 and the mitochondrial fission mediator drp-1 or the fusion mediator eat-3. Asterisks indicate nucleus. Bar, 5 μm.

(B) MitoGFP fluorescence loss in photobleaching of wild-type control and vpr-1 mutant muscle. The red circle indicates the area of laser bleaching. Fluorescence was measured throughout the field after each 5 s cycle. Intensities of numbered spots are shown in (C) and (D). Bar, 5 μm.

(C and D) Quantification of fluorescence intensities in indicated mitochondria (B) of control (C) and vpr-1 mutant (D) muscles.

(E) MitoTracker CMXRos staining of wild-type and vpr-1(tm1411) mutant muscle. Bar, 5 μm.

(F and G) Oxygen consumption rates of wild-type and mutant hermaphrodites. Consumption rates were normalized by protein content (F) or number of worms (G). *p < 0.001. Error bars represent SD.

(H) ATP concentration in wild-type and mutant hermaphrodite extracts. *p < 0.001 compared to wild-type. Error bars represent SD.

(I) Paraquat sensitivity in wild-type and vpr-1(tm1411) hermaphrodites. Error bars represent SD.
theirs mobility. FLIP in control muscles causes rapid fluorescence loss in the targeted area, but the vast majority of surrounding mitochondria retain their fluorescence, even after 48 cycles (Figures 3B and 3C). In 5 of 11 experiments, a single neighboring tubule lost fluorescence. The average bleached area in the 11 experiments was 7.8 μm², indicating that individual tubules have limited connectivity in wild-type muscle. In contrast to the wild-type, FLIP in vpr-1 mutant muscles resulted in rapid loss of fluorescence in the targeted area and numerous surrounding mitochondrial tubules within 20 s of photobleaching initiation (Figures 3B and 3D). The average bleached area in nine experiments was 32.6 μm² (p < 0.001 compared to the control). We calculated that each vpr-1 mutant muscle contains between 20 and 25 contiguous subpopulations of mitochondria, whereas each wild-type muscle contains more than 100 subpopulations (based on an average muscle area of 800 μm²). Therefore, mitochondrial networks in vpr-1 mutant muscle exhibit increased connectivity relative to networks in wild-type muscle. This increase in connectivity may be due to an increase in fusion, reduction in fission, or both.

Mitochondrial dynamics can be observed in live animals using time-lapse fluorescence microscopy. Mitochondria in wild-type muscle appear stably docked at I-bands (Movie S1). Shape changes and fission gradually reduce tubule length over time during imaging. In vpr-1 mutants, mitochondrial tubules exhibit increased mobility (Movie S2) and occasionally form ring-shaped structures that join other tubules or collapse (Figure S1A; Movie S3). Tubules move at speeds between 0 and 2.0 μm/min, although only a fraction of tubules are mobile under our conditions. We noticed that fission sites in wild-type muscle are different than sites in vpr-1 mutant muscle. In the control, fission primarily occurs within elongated tubules (29/29 fission events from eight videos). However, fission rarely occurs within elongated tubules in vpr-1 mutants (1/12 fission events from eight videos) and instead occurs at connection sites between tubules (Movie S4). We conclude that mitochondria in vpr-1 mutants are more mobile and have different fission sites relative to mitochondria in wild-type muscle. Taken together, the results support the model that VPR-1 influences the fission/fusion balance.

VAP Mutants Have Impaired Mitochondrial Function

The abnormal mitochondrial morphology in vpr-1 mutants raises the possibility that their function is impaired. To evaluate muscle mitochondrial function, we used MitoTracker CMXRs, which concentrates in the mitochondrial matrix depending on transmembrane potential (Pendergrass et al., 2004). MitoTracker accumulation in vpr-1 mutant muscle mitochondria is reduced compared to accumulation in controls (Figure 3E). We found that drp-1 RNAi could increase MitoTracker accumulation, suggesting that matrix aggregation caused by DRP-1 loss can partially restore mitochondrial transmembrane potential in vpr-1 mutants (Figure S1B). Importantly, this MitoTracker accumulation defect can be fully suppressed (see below), indicating that vpr-1 mutants are not deficient in dye uptake or muscle transport.

Worms with reduced mitochondrial respiration should consume less oxygen and generate less ATP. Indeed, vpr-1 mutants consume less oxygen than controls, whether the data are normalized to protein content (Figure 3F) or worm number (Figure 3G). ATP concentration in 1-day-old adult vpr-1 mutants is significantly reduced compared to adult controls (Figure 3H). We previously showed that sperm-derived MSPs promote reactive oxygen species (ROS) production in oocytes (Yang et al., 2010). vpr-1 mutants are more resistant than the wild-type to paraquat, which generates intracellular ROS that cause a concentration-dependent toxicity depending on endogenous ROS levels (Figure 3I). Hence, vpr-1 mutants have reduced ROS, which could be due to decreased production or increased breakdown. These independent metabolic assays support the hypothesis that vpr-1 mutants have altered mitochondrial function.

Inhibition of mitochondrial electron transport chain (ETC) activity causes defects such as sluggish motility, reduced swimming rate in liquid, prolonged defecation cycle, reduced brood size, slow development, and larval arrest (Tsang and Lemire, 2003; Wong et al., 1995a). In particular, muscle-specific ETC inhibition affects motility, brood size, and possibly defecation and development rate (Durieux et al., 2011). Thus, vpr-1 mutants should exhibit some of these defects if their mitochondria have reduced respiration, as indicated by the metabolic assays. Indeed, vpr-1 null mutants exhibit sluggish motility, reduced swimming rate (99.7 ± 9.4 thrashes/min for vpr-1(tm1411) versus 125.3 ± 11.8 thrashes/min for wild-type; p < 0.005), prolonged defecation cycle (59.9 ± 7.2 s for vpr-1(tm1411) versus 55.0 ± 7.1 s for wild-type; p < 0.01), sterility, and slow development (57.7 ± 3.9 hr for vpr-1(tm1411) versus 42 hr for wild-type; p < 0.001). We conclude that vpr-1 mutants have defects consistent with abnormal mitochondrial function in muscle and possibly other cell types.

Secreted Neuronal vMSPs Modulate Muscle Mitochondrial Position and Morphology

Our previous studies showed that VAP MSP domains are secreted in a cell type-specific fashion (Tsuda et al., 2008). However, VAPs can also have cell autonomous functions. To determine the site(s) where VAPs function, we first examined Drosophila. Expressing dVAP in muscle of dvap null mutant flies using the MHC-GAL4 driver causes 100% lethality and does not rescue dvap mutant defects. In contrast, expressing dVAP using neuronal drivers, including C164-GAL4 and Elav-GAL4, rescues the lethality (Chai et al., 2008; Tsuda et al., 2008) and nearly 100% of muscle mitochondrial defects associated with dvap loss (Figure 1A; data not shown). These data indicate that dVAP functions in a cell nonautonomous fashion, consistent with a signaling role.

Next, we examined the mechanism by which VPR-1 acts on muscle in C. elegans. Transgenic GFP reporters driven by upstream vpr-1 genomic sequence show broad expression in adults, including the ventral nerve cord and body wall muscle (Figure S2), similar to Drosophila dVAP. However, vpr-1 expression in muscles using the myo-3 promoter or intestine using the ges-1 promoter does not affect muscle mitochondrial shape or distribution in the mutants (Figure 1E; Figure S1C). In contrast, vpr-1 expression in neurons with the unc-119 pan-neuronal promoter rescues mitochondrial morphology, distribution, and I-band position (Figures 1E) in ∼30%–40% of muscles, while most remaining muscles exhibit improved phenotypes. The incomplete rescue may be due to transgene expression mosaicism, overexpression, or missing untranslated regulatory
sequences, as neuronal expression in flies rescues nearly 100% of mitochondrial defects and neuron-specific VPR-1 inhibition causes mitochondrial defects in nearly all muscle (data not shown and see below). We conclude that VAPs are required in neurons to control muscle mitochondrial position and shape.

The above data are consistent with neurons secreting vMSP domains to regulate muscle mitochondria. To further test this model, we inhibited neuronal vMSP secretion in wild-type worms. The VAPP56S mutation acts as a dominant negative, sequestering wild-type VAP in neuronal aggregates (Ratnaparkhi et al., 2008; Teuling et al., 2007) and preventing secretion of the wild-type protein (Tsuda et al., 2008). Neuronal VPR-1P56S overexpression causes muscle mitochondria to form thin, branched, and abnormally distributed networks within the muscle belly, similar to vpr-1 null mutants (Figure 1E). When we overexpressed neuronal VPR-1P56S in vpr-1 null mutants, muscle mitochondria were identical to those seen in nontransgenic vpr-1 mutant controls. These data confirm that VPR-1P56S acts as a dominant negative and support the model that neurons secrete vMSPs to regulate muscle mitochondria.

C. elegans, Drosophila, and mammalian VAPs are cleaved between the MSP domain and the transmembrane domain (Gkogkas et al., 2011; Tsuda et al., 2008). Previously, we documented vMSP secretion in Drosophila wing disc cells by expressing a dVAP fusion protein with distinct N-terminal and C-terminal tags (Tsuda et al., 2008). Only the N-terminal tag containing the MSP domain was secreted. We used a similar strategy to test whether C. elegans neurons secrete the VPR-1 MSP domain. mCherry was fused to the N-terminus of VPR-1 and GFP to the C terminus and this mCherry::VPR-1::GFP fusion protein was expressed in neurons using the unc-119 promoter (Figure 4A). While partial colocalization of mCherry and GFP was observed in motor neuron cell bodies (and other neuron bodies), mCherry was observed within axons and in the extracellular environment (Figures 4B–4D). GFP does not colocalize with most axonal mCherry, nor does it colocalize with extracellular mCherry, indicating that the VPR-1 fusion protein is cleaved. Secreted mCherry/MSP fragments were observed at the body wall and vulval muscles (Figures 4C and 4D). mCherry also accumulated in the coelomocytes, mesodermal cells that nonspecifically endocytose fluid, and secreted molecules within the body cavity (data not shown) (Fares and Greenwald, 2001). Taken together, these results provide strong evidence that neurons secrete vMSPs.

An MSP Receptor Screen Identifies SAX-3 Robo and CLR-1 Lar Receptors

An important prediction based on above data is that secreted vMSPs regulate a muscle receptor pathway(s) that transduces signals to mitochondria. vMSPs bind to EphRs and other unidentified receptors (Miller et al., 2003; Tsuda et al., 2008). However, EphR loss in C. elegans or Drosophila does not influence muscle mitochondria or mitochondria-related phenotypes (data not shown), suggesting that an unknown receptor mediates this signaling mechanism. We previously developed an MSP receptor identification assay based on the ability of recombinant MSP domains conjugated to fluorescein (MSP-FITC) to specifically bind to receptors expressed in oocytes (Miller et al., 2003; Figures S3A and S3B). In the worm gonad, sperm secrete MSPs to induce oocyte maturation (Figure 5A), which involves

![Figure 4. VAP Cleavage and Secretion in C. elegans Neurons](image-url)
metabolic changes (Han et al., 2010; Yang et al., 2010). Under conditions where all MSP receptor sites are occupied, MSP-FITC binding to oocytes is reduced by ~35% when the VAB-1 EphR is absent (Miller et al., 2003). The remaining binding is due to unidentified MSP domain receptors. Sperm-derived MSPs and VAP MSP domains bind to identical receptors expressed in oocytes (Miller et al., 2003; Tsuda et al., 2008). We hypothesized that both oocytes and muscle express MSP domain receptors that transduce signals to mitochondria. Support for this hypothesis comes from examining the effects of extracellular MSP addition to oocyte mitochondria. Microinjecting MSPs and human vMSPs into the extracellular spaces of spermless reproductive tracts induces a rapid transition (<15 min) in oocyte mitochondria from ring-shaped to branched, tubular forms (Figures 5A and 5B; Figures S4A–S4D). These shape changes, as well as MSP-induced mitochondrial transport into growing oocytes (Govindan et al., 2009; Wolke et al., 2007), are dependent on dpr-1 (Figures S4E and S4F). Identical shape changes in oocyte mitochondria occur when sperm presence is manipulated through mating (Figure 5B; Figures S4B–S4D). Thus, oocytes likely express MSP receptors that rapidly regulate mitochondrial shape.

To identify MSP domain receptors important for mitochondria, we compared genome-wide DNA microarray data sets of adult hermaphrodites undergoing oogenesis to those undergoing spermatogenesis (Reinke et al., 2004). MSPs bind to oocyte plasma membranes, but not to sperm plasma membranes (Miller et al., 2003). From the top 3000 oogenesis/spermatogenesis-enriched genes, we identified 40 genes that encode cell surface receptors (Table S2). These genes are not specifically expressed in oocytes; many are also expressed in neurons and muscle. We screened RNAi clones corresponding to the 40 predicted receptors to identify clones that cause reduced MSP-FITC binding to oocytes and mitochondrial shape or positioning defects in oocytes and muscle (Table S2). The only RNAi clones that affected both MSP binding and muscle mitochondria corresponded to the SAX-3 Roundabout (Robo) and CLR-1 Lar-like (Lar) receptors (Figures 5C and 5D; Table S2; Kokel et al., 1998; Zallen et al., 1998). Analysis of clr-1 and sax-3 mutants confirmed that both receptors are required for MSP-FITC binding and muscle mitochondrial morphology (see below). Previous studies have shown that SAX-3 Robo and CLR-1 Lar are expressed in oocytes, motor neurons, and body wall muscle (Chang et al., 2004; Kohara, 2001; Zallen et al., 1998).

SAX-3 or CLR-1 loss causes a significant reduction in MSP-FITC binding to oocytes, similar to loss of the MSP/ephrin receptor VAB-1 (Figures 5C and 5D). To test whether CLR-1 Lar or SAX-3 Robo expression is sufficient to promote MSP binding, we expressed these receptors alone and in combination in cultured HEK293 cells (Figures 5E–5G). Expressing the VAB-1 EphR in cultured cells confers increased MSP-FITC cell surface binding and rapid internalization in live cells (Miller et al., 2003). We found that CLR-1 Lar expression resulted in the same significantly increased level of MSP-FITC and vMSP-FITC binding as the VAB-1 EphR positive control, whereas Robo expression resulted in a weak increase relative to the negative control (Figures 5E–5G). However, expressing both SAX-3 Robo and CLR-1 Lar together caused a synergistic increase in MSP and vMSP binding, which could be inhibited by incubating cells with an excess of unlabelled MSP (Figures 5E–5G). These data support the model that Robo and Lar function together to promote vMSP binding. Consistent with this idea, SAX-3 and CLR-1 loss synergistically affects human vMSP binding to C. elegans oocytes (Figures 5C and 5D). In summary, our screen identified SAX-3 Robo and CLR-1 Lar, perhaps acting via a receptor complex, as candidates for mediating VAP signaling to muscle mitochondria.

**SAX-3 Robo and CLR-1 Lar Function in Muscle to Influence Mitochondria**

To specifically evaluate muscle mitochondria in sax-3 Robo and clr-1 Lar mutants, we generated myo-3p::mitoGFP transgenic lines. sax-3(ky123) mutants have incompletely penetrant and variably expressed mitochondrial defects [63.7% of sax-3(ky123) muscles affected (n = 55) versus 100% for vpr-1 mutants (n > 400)]. The affected muscles have mitochondrial networks nearly identical to vpr-1 mutants, including abnormally positioned elongated mitochondria with excess branching (Figure 6A; Table S1, lines 1–3). TEM cross-sections of sax-3 mutant muscle show that mitochondria are smaller in diameter than controls and mostly displaced from the I-bands, similar to vpr-1 mutants (Figures 2A–2C). However, the muscle belly is less swollen than vpr-1 mutants and not all sax-3 mutant muscles are affected, consistent with the presence of a second vMSP receptor. The sax-3(ky123) mutant defects were rescued with a fosmid containing the sax-3 genomic locus, indicating that the defects are due to loss of SAX-3 Robo (Figure 6A). Temperature shift experiments using the sax-3(ky200)ts hypomorphic temperature sensitive (ts) allele indicate that sax-3 is required postembryonically (Figure 6A). We also tested the gene encoding the Slit ligand for SAX-3 Robo and did not detect mitochondrial defects in slt-1(eh15) null mutants (Figure 6A). As slit-1 is the only Slit homolog present in C. elegans (Hao et al., 2001), SAX-3 does not require Slits for regulating muscle mitochondrial morphology. Therefore, sax-3 Robo influences muscle mitochondrial shape and positioning independent of Slit.

Next, we evaluated metabolic status in sax-3 Robo mutants. MitoTracker CMXRos staining indicated that sax-3(ky123) mutants have a variably expressed transmembrane potential defect (Figure 6B) where the more severe cases resemble vpr-1 mutants. Adult sax-3 mutants consumed less oxygen than controls, but contained less total protein, so the interpretation depends on the normalization method (Figure 6C). Compared to wild-type controls, sax-3 mutants had reduced ATP concentration (Figure 6D), increased resistance to paraquat (Figure 6E), and slower growth [53.8 ± 3.6 hr for sax-3(ky123) versus 42 hr for wild-type; p < 0.001]. These data indicate that sax-3 mutants have metabolic or mitochondrial defects that are similar to vpr-1 mutants, but most defects are less severe or occur with reduced frequency.

sax-3 Robo is expressed in motor neurons and body wall muscle (Chang et al., 2004; Kohara, 2001; Zallen et al., 1998). To test whether sax-3 functions in muscle, we conducted two experiments. In the first, we expressed SAX-3 specifically in muscle of sax-3(ky123) mutants using the myo-3 promoter. Muscle-specific SAX-3 expression strongly rescued the sax-3(ky123) muscle mitochondrial defects (Figure 7A; rescue observed in 27/31 transgenic worms). Next, we specifically
Figure 5. An MSP Receptor Screen Identifies SAX-3 Robo and CLR-1 Lar-Like Receptors

(A) Diagram of the *C. elegans* adult gonad. Mitochondrial morphology and MSP domain binding were quantified in oocytes colored green (B–D and Figure S4). DTC, distal tip cell; Sp, spermatheca.

(B) Quantification of ring-shaped mitochondria per mm² oocyte area in the presence and absence of extracellular MSP domains introduced by mating and microinjection. MSP domains were injected through the vulva into the reproductive tract of unmated females lacking sperm. See Figures S4A–S4D for representative pictures of oocyte mitochondria. *p < 0.001 compared to unmated female controls. Error bars represent SEM.

(C) Two hundred nanomolar MSP-FITC binding to oocyte plasma membranes. In the compete panels, a 20-fold molar excess of unlabelled MSP or hVAP MSP was added before the assay. Quantitative data is shown in (D). Proximal is to the right, as shown in (A) (green). Bar, 20 μm.
depleted SAX-3 in body wall muscle of wild-type worms using an RNAi mosaic strategy (Durieux et al., 2011; Esposito et al., 2007). sid-1 mutants are defective for systemic RNAi, yet undergo cell autonomous RNAi normally (Winston et al., 2002). We specifically expressed sax-3 sense and antisense RNAs (creating dsRNA) in sid-1(pk3321) muscle and examined their mitochondria. Muscle-specific sax-3 RNAi caused mitochondrial defects nearly identical to those seen in sax-3 mutants (Figure 7A). These results indicate that sax-3 functions cell autonomously in body wall muscle to influence mitochondria.

Our RNAi screen also identified the CLR-1 Lar receptor as a muscle mitochondrial regulator. In clr-1 RNAi and clr-1(e1745) mutants mutant adults, most mitochondria are positioned at the I-bands, but tubule length is much shorter than in the wild-type (Figure 6A; Table S1, lines 1 and 4). Despite the abnormal appearance of mitochondria, MitoTracker CMXRos staining did not show decreased transmembrane potential (Figure 6B). clr-1 is an essential gene required in hypodermal cells for fluid balance and the e1745 allele is temperature sensitive (Huang and Stern, 2004; Kocel et al., 1998). We conducted temperature shift experiments to assess the temporal requirement of clr-1 function. At the permissive temperature, clr-1(e1745)ts adult muscles contain mostly tubular mitochondria similar to the wild-type. When young adults were shifted to the restrictive temperature for 12 hr, globular mitochondria were similar to clr-1 RNAi animals (Figure 6A). These results demonstrate that CLR-1 Lar is required in adults to maintain mitochondrial elongation on the I-bands. To test whether clr-1 functions in muscle, we specifically depleted clr-1 in body wall muscle using the sid-1 mutant RNAi mosaic strategy. Muscle-specific CLR-1 depletion caused the same mitochondrial defects as those seen in clr-1 RNAi and clr-1(e1745) mutants (Figure 7A). In contrast, hypodermis-specific clr-1 RNAi caused fluid accumulation, but not muscle mitochondrial defects (data not shown). Taken together, the data indicate that SAX-3 Robo and CLR-1 Lar function in muscle to influence mitochondria.

VPR-1 and SAX-3 Robo Antagonize CLR-1 Lar Signaling

Our data indicate that SAX-3 Robo and CLR-1 Lar function in muscle, yet their mutant phenotypes are clearly different (Figure 6A). The sax-3 mutant mitochondrial defects are nearly identical to the vpr-1 mutant defects. To examine the signaling hierarchy between vpr-1 and sax-3, we compared single and double null mutant strains. TEMs show that mitochondrial shape, including cross-sectional area, and positions in the double mutants are identical to single null vpr-1 mutants (Table S1, lines 2 and 5, p > 0.1; data not shown). In addition, ATP concentration in vpr-1(tm1411); sax-3(ky123) double mutants is not significantly different than sax-3(ky123) null mutants (Figure 6D). Unfortunately, we were unable to generate mitoGFP transgenic lines in the double mutants. Taken together with the binding data, the results support the model that vMSPs positively regulate SAX-3 Robo.

Robo receptors antagonize Lar receptor signaling during C. elegans and Drosophila growth cone guidance decisions (Chang et al., 2004; Sun et al., 2000). Consistent with this relationship, the globular mitochondrial morphology in clr-1 Lar mutants contrasts with the elongated morphology in VAP and Robo mutants (Figures 1E and 6A). To examine the signaling hierarchy, we compared vpr-1(tm1411); clr-1 RNAi mutants to vpr-1(tm1411) mutants using the mitoGFP marker. clr-1 loss strongly suppresses the muscle mitochondrial defects of vpr-1 mutants (Figure 7A; Table S1, lines 6 and 2) and muscle mitochondria in vpr-1(tm1411); clr-1 RNAi are similar in morphology and distribution to mitochondria in wild-type hermaphrodites. Importantly, they are also positioned at muscle I-bands (Table S1, line 6). MitoTracker CMXRos staining and time-lapse imaging indicate that clr-1 loss suppresses the mitochondrial transmembrane potential and mobility defects of vpr-1 mutants (Figure 7B and data not shown). Thus, muscle mitochondria in vpr-1(tm1411); clr-1 RNAi are largely normal. This important result indicates that excess CLR-1 activity specifically causes the vpr-1 mutant muscle mitochondrial defects. Hence, VPR-1 antagonizes Lar signaling in muscle. Moreover, a redundant mechanism must exist that positions mitochondria in the absence of VPR-1 and CLR-1.

The data support the model that SAX-3 Robo helps vMSPs antagonize CLR-1 Lar signaling. To further test this model, we generated clr-1(e1745)ts; sax-3(ky123) double mutants that express mitoGFP. The mitochondrial branching and elongation defects caused by sax-3 loss are suppressed in the double mutant adults (Figure 7A). Furthermore, mitochondria in the double mutants localize correctly to the I-bands. Therefore, VPR-1 and SAX-3 Robo antagonize CLR-1 Lar signaling in muscle to position mitochondria at I-bands. The simplest interpretation is that SAX-3 Robo facilitates vMSP binding to CLR-1 Lar or SAX-3/CLR-1 complexes.
within the muscle belly (Figure 8A). These ectopic actin networks occupy the same position as mitochondria, suggesting that their locations are mechanistically linked. Consistent with this idea, RNAi of genes encoding regulators of the actin cytoskeleton, including known downstream mediators of Robo and Lar receptor signaling, caused muscle mitochondrial defects (Figure S5).

The Arp2/3 complex promotes actin nucleation and branching. Given the branched filament networks in vpr-1 mutant muscle bellies, we hypothesized that aberrant Arp2/3 activity in the belly displaces mitochondria from I-bands, contributing to the mitochondrial defects. To test this hypothesis, we used RNAi to deplete Arp2/3 complex components in vpr-1 mutant muscle bellies. RNAi muscle indicate that large mitochondria correctly localize to I-bands (Figure 8B; Table S1, lines 2 and 7). Hence, aberrant Arp2/3 activity causes the VAP mutant mitochondrial defects. The Arp2/3 and CLR-1 Lar suppression data provide unequivocal evidence that these mitochondrial defects are specific and regulatory in nature. Finally, Arp2/3 inactivation in wild-type muscle causes mitochondrial morphology similar to those seen following Lar inactivation (Figure 8B; Table S1, lines 8 and 4). These data support the model that vMSP/Robo/Lar signaling modulates Arp2/3 activity to position mitochondria at I-bands.

**DISCUSSION**

EphR, Robo, and Lar-like receptors are called growth cone guidance receptors because of their established roles in regulating
the actin cytoskeleton during nervous system development. However, these receptors are also expressed after guidance decisions are made, particularly in the adult central nervous system and muscles (Longo et al., 1993; Zabolotny et al., 2001; Zhang and Goldstein, 1991). Here, we show that Robo and Lar-like receptor pathways act in adults to modulate mitochondrial localization and morphology. VAP, Robo, and Lar homologs are present in sponges, animals without neurons and muscles, suggesting that their role in regulating mitochondria is ancestral to their growth cone guidance function (Srivastava et al., 2010). The mechanism by which vMSPs regulate mitochondria appears similar to the mechanism by which Slit repels migrating axons (Chang et al., 2004; Sun et al., 2000). During nervous system development, Slit binding to Robo downregulates Lar receptor signaling that promotes migration. Therefore, Robo acts upstream of Lar. We show that Robo is required for vMSP signaling upstream of Lar. The simplest interpretation of our genetic and binding data is that Robo facilitates vMSP binding to Lar or Robo/Lar complexes, downregulating Lar signaling. However, determining the extent to which high affinity vMSP interactions depend upon receptor complex formation will require further investigation. Given that EphR, Robo, and Lar receptors are broadly expressed throughout the nervous system, it is possible that vMSPs influence mitochondria in neurons. dVAP regulates the presynaptic microtubule cytoskeleton (Pennetta et al., 2002), which controls mitochondrial transport along axons (Hollenbeck and Saxton, 2005). Therefore, neurons may secrete factors like VAPB that regulate mitochondria in muscles and neurons.

The actin cytoskeleton regulates mitochondrial position in C. elegans muscle, cultured Drosophila and chick neurons, cultured mammalian cells, and yeast (Boldogh and Pon, 2007; Chada and Hollenbeck, 2004; Pathak et al., 2010; Quintero et al., 2009; Starr and Han, 2002). The worm ANC-1 protein is thought to couple actin to mitochondria and arc-1 mutants have globular muscle mitochondrial morphology similar to arc-2 RNAi animals (Starr and Han, 2002). We show that vMSPs promote mitochondrial docking at muscle l-bands, actin-enriched sites containing structures analogous to focal adhesions (Lecroyse et al., 2007). CLR-1 Lar and Arp2/3 are required for maintenance or elongation of mitochondrial tubules along l-bands, a process that may facilitate (and/or require) fusion. In vpr-1 mutants, mitochondria mislocalize to the muscle belly along with ectopic actin filaments and form thin elongated branches with increased mobility, increased fusion/fission balance, and reduced transmembrane potential. Lar or Arp2/3 inactivation suppresses these defects, revealing an unexpected role for the actin cytoskeleton in modulating multiple aspects...
of mitochondrial biology. Whether Arp2/3 regulates mitochondria directly or indirectly through positioning or docking effects is not clear. The actin cytoskeleton has been shown to influence mitochondrial fission in cultured cells (De Vos et al., 2005; Pathak et al., 2010). vMSP signals are likely regulatory in nature, as a parallel mechanism promotes mitochondrial positioning in the absence of VAP and Arp2/3 function.

Defects in skeletal muscle mitochondria have been implicated in the pathology of ALS (Dupuis et al., 2004; Zhou et al., 2010). In mutant sod1 transgenic mice, the SOD1 protein aberrantly accumulates in mitochondria (Wong et al., 1995b; Kong and Xu, 1998) and causes skeletal muscle mitochondrial dysfunction that is initiated at the neuromuscular junction (Zhou et al., 2010). Subsarcolemmal aggregates of abnormal mitochondria with low transmembrane potential are found in skeletal muscle of these mice well before the onset of the disease (Zhou et al., 2010).

ALS patients with a SOD1 mutation also show a muscle mitochondrial oxidative defect (Corti et al., 2009) and sporadic ALS patients have been documented with muscle mitochondrial respiratory chain dysfunction (Crugnola et al., 2010). Defects in muscle mitochondria may therefore contribute as a primary cause in ALS pathogenesis. Indeed, uncoupling electron transport from ATP synthesis in muscle mitochondria by overexpressing uncoupling protein 1 is sufficient to initiate motor neuron degeneration (Dupuis et al., 2009).

Our results support the hypothesis that secreted vMSPs modulate mitochondrial position through growth cone guidance receptor pathways. It seems likely that this signaling mechanism plays a role in ALS pathogenesis. The VAPB P56S mutation inhibits wild-type and mutant MSP domain secretion (Tsuda et al., 2008). Reducing vMSP secretion might cause abnormal mitochondrial localization, morphology, and function in ALS patients. Consistent with this model, low VAP levels in sporadic ALS patients and mouse sod1 models correlate with widespread mitochondrial abnormalities (Anagnostou et al., 2010; Dupuis et al., 2004, 2008; Teuling et al., 2007; Wong et al., 1995b). Moreover, analyses of data from whole-genome associated studies have discovered that single nucleotide polymorphisms within genes mediating growth cone guidance, including Robo, Lar, and Cdc42 are associated with susceptibility, survival, or onset of ALS (Lesnick et al., 2008). In addition, the discovery that growth cone guidance pathways influence mitochondria may have implications for other neurodegenerative diseases, such as spinal muscular atrophy and Parkinson’s disease (Lesnick et al., 2007).

**EXPERIMENTAL PROCEDURES**

**Genetics, RNAi, and Plasmids**

Bristol N2 is the wild-type C. elegans strain. Worms were maintained on NGM plates with NA22 bacteria at 20°C except where indicated otherwise. Strain
Developmental Cell

VAPB and Mitochondria

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, Supplemental Experimental Procedures, and four movies and can be found with this article online at doi:10.1016/j.devcel.2011.12.009.

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Metabolic Assays

Resistance to paraguanax (Ultra Scientific, USA). ATP concentration measurement, oxygen consumption, and MitoTracker CMXRos staining methods are described in Supplemental Experimental Procedures. Oxygen consumption was measured at constant temperature (20°C) using the oxygraph system (Hansatech, UK).

Transmission Electron Microscopy

TEM was performed as previously described (Tsuda et al., 2008; Whitten and Miller, 2007). Care was taken to ensure that fixation occurred rapidly and cross-sections were orthogonal to muscle myofilaments. Mitochondria do not consistently localize to I-bands near neuromuscular junctions.

Staining, Microinjection, and Microscopy

Mitochondria were labeled using MitoTracker CMXRos, Rhodamine 6g, and mito::GFP, which targets the matrix. For MitoTracker CMXRos and Rhodamine 6g staining, dyes were added to seeded NGM plates (Labrousse et al., 1999). Imaging was done without anesthetics on dried 2% agarose pads immediately after mounting. Anesthetics can cause mitochondrial fragmentation. Images were taken using a motorized Zeiss Axioskop 2 with MRM AxioCam Hi-Res digital camera and Perkin Elmer Spinning Disc Nikon TE2000 microscope equipped with an EMCCD C9100-50 camera operated by Volog 5.3 software. Purified recombinant MSP domains were microinjected into the gonad using a Zeiss Axiovert 200 microscope. Injected animals were mounted for direct observation after a 15 min to 1 hr recovery period.

Transgenics

To generate transgenic C. elegans, plasmids (60 ng/μl) were mixed with pRF4 [ rol-6] (60 ng/μl) or myo-3p::mito::GFP (60 ng/μl) and injected into young adult hermaphrodite gonads. The myo-3p::mito::GFP plasmid was generously provided by Dr. van der Bliek. Transgenic lines were selected based on the roller phenotype or GFP expression. Multiple independent transgenic lines were analyzed. Two vpr-1(tm1411)/ht2; unc-119p::vpr-1 transgenic lines were integrated by gamma irradiation.

Fluorescence Loss in Photobleaching

Fluorescence loss in photobleaching (FLIP) was achieved with a Perkin Elmer Spinning Disc Nikon TE2000 microscope programmed to cycle between bleaching and scanning every 5 s for 4 min. The fluorescence intensities of indicated areas were analyzed in Velocity software (Perkin Elmer, UK). Animals were immobilized on dried 2% agarose pads under conditions that prevent animal and mitochondrial mobility (i.e., one to three animals per pad). Only muscles with immobile mitochondria were analyzed.

MSP Binding Assays

MSP-FITC and vMSP-FITC binding assays were conducted as previously described (Miller et al., 2003; Tsuda et al., 2008). Briefly, HEK293T cells were cultured on coverslips in 6-well plates and cells (~50% confluence) were transiently transfected with 2 μg of VAB-1, 3xFLAG::SAX-3, CLR-1::V5, or pCDNA3.2 control plasmids (Miller et al., 2003) using FuGENE HD transfection reagent, according to the manufacturer’s instructions (Promega, USA). The 3 × FLAG::SAX-3 construct was generously provided by Dr. Joe Culotti. The CLR-1::V5 construct contained the CLR-1 extracellular and U.S.A. The 3 × FLAG::SAX-3 construct was generously provided by Dr. Joe Culotti. The CLR-1::V5 construct contained the CLR-1 extracellular and transmembrane domains, but not the phosphatase domains. After 24 hr, cells were incubated with MSP-FITC or vMSP-FITC for 30 min at 23°C and washed three times in 50 ml PBS. Transfected cells were preincubated with a 25-fold molar excess of unlabelled MSP to evaluate specific binding. Fluorescence was measured from linear range exposures using Axiosiovision software.

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Resistance to paraguanax (Ultra Scientific, USA). ATP concentration measurement, oxygen consumption, and MitoTracker CMXRos staining methods are described in Supplemental Experimental Procedures. Oxygen consumption was measured at constant temperature (20°C) using the oxygraph system (Hansatech, UK).

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protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell 133, 963–977.