

Supplemental Data

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The Amyotrophic Lateral Sclerosis 8 Protein VAPB Is Cleaved, Secreted, and Acts as a Ligand for Eph Receptors

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Supplemental Figures

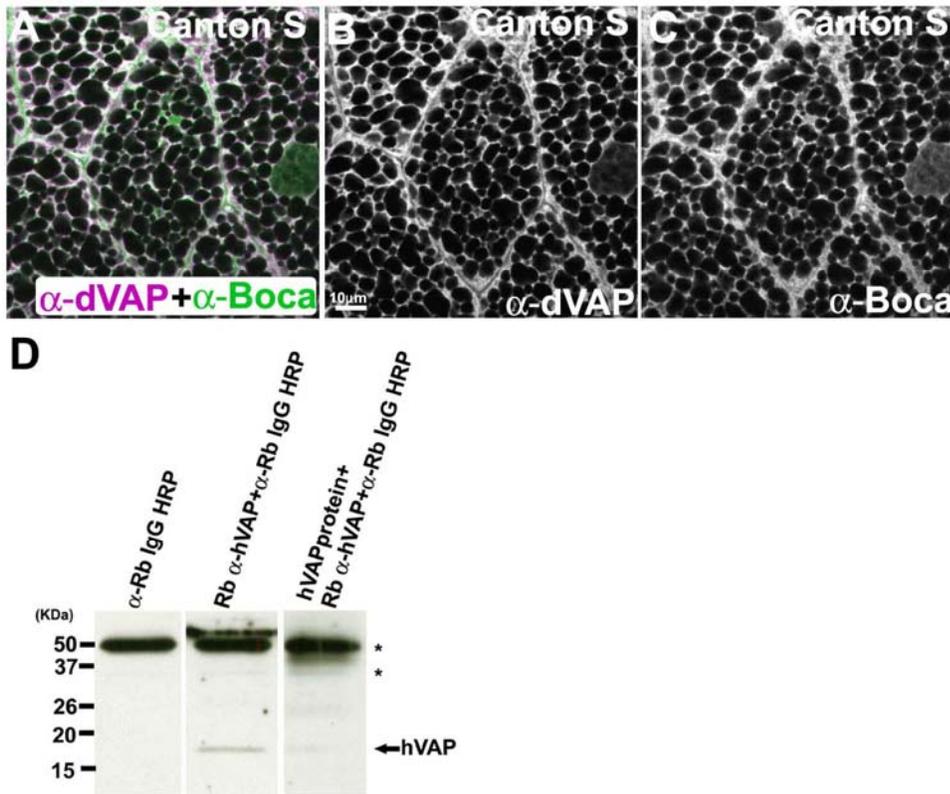


Figure S1. VAP expression

(A-C) dVAP is colocalized with Boca, an ER marker (Culi and Mann, 2003), in wild type salivary gland cells.

(D) Competition assays showing the specificity of the positive band with the hVAP antibody (arrow). The band disappeared when the antibody was incubated with purified hVAP protein. * indicates non-specific band.

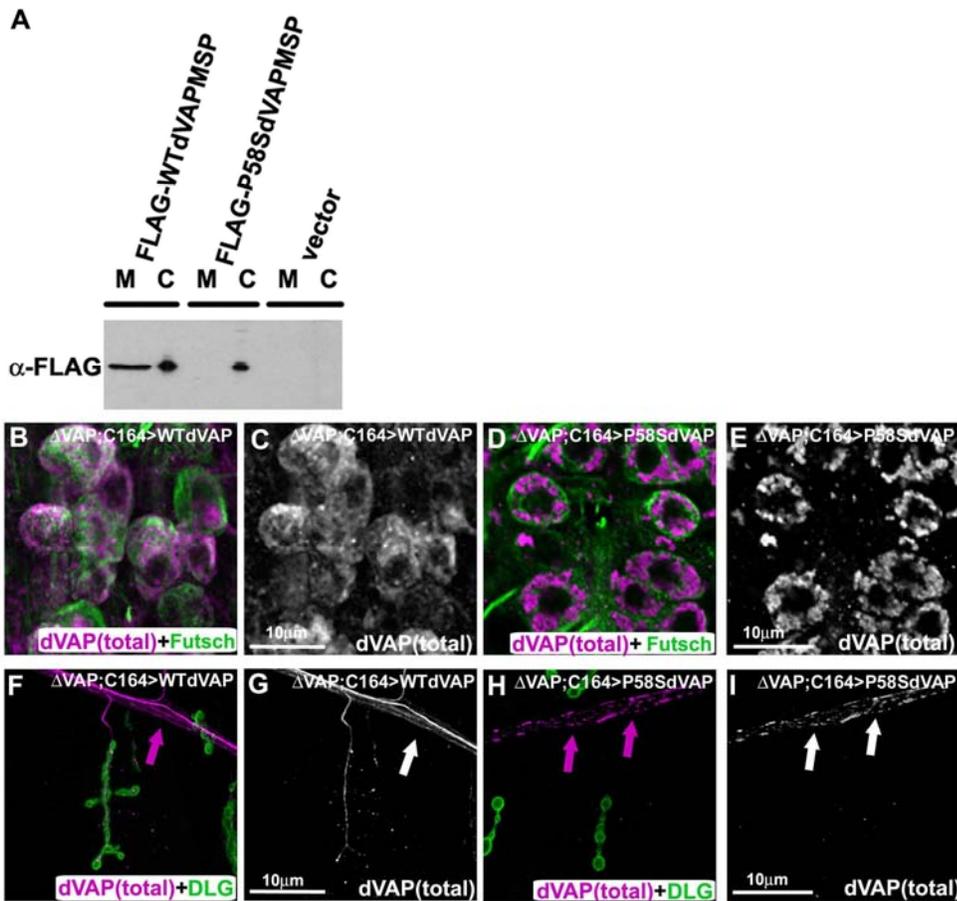


Figure S2. P58S mutation causes dVAP protein to be mislocalized in the inclusions and suppresses its secretion.

(A) The WT dVAP MSP protein is secreted from S2 cells, but P58S dVAP MSP is not secreted. WT dVAP MSP protein and P58S dVAP MSP protein were expressed in the S2 cells. After two days, conditioned medium and cells were harvested for western blot analysis. WT dVAP MSP is secreted into the medium, but P58S dVAP MSP is not secreted. M: medium; C: cells.

(B-I) The P58S mutation causes dVAP protein to be mislocalized in intracellular inclusions in motor neurons. WT dVAP (B, C, F, G) and P58S dVAP (D, E, H, I) are expressed in the motor neurons in the *dVAP* null background (ΔVAP). Co-staining of motor neurons with anti-dVAP (magenta) and anti-Futsch (green) (B, D) and with anti-dVAP (magenta) and anti-DLG (green) (F, H). Staining with anti-dVAP only (C, E, G, I). WT dVAP is widely expressed in the soma, axon, and NMJ (B, C, F, G). In contrast, P58S dVAP accumulates in inclusions in the soma and axons, but not at the NMJ (D, E, H, I). Note that only exogenous WT or P58S dVAP protein is labeled in the *dVAP* null background. Arrows (F, G, H, I) indicate axons.

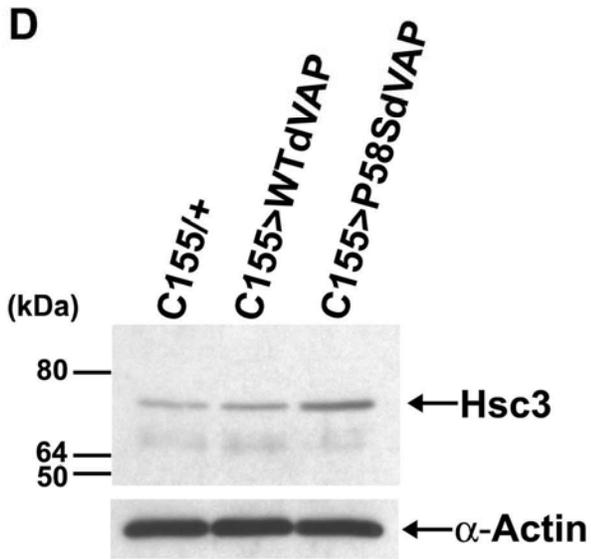
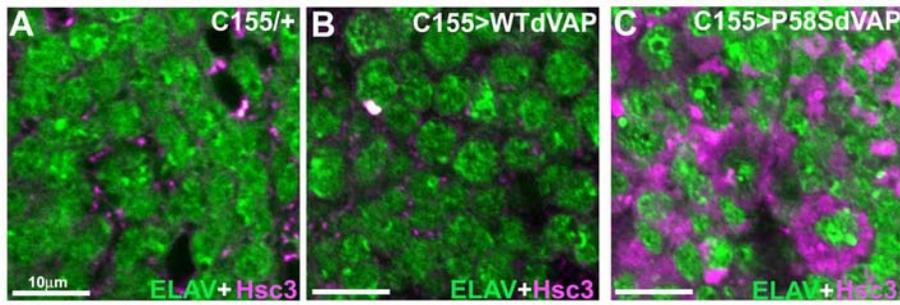


Figure S3. P58S mutation induces an UPR

(A-C) Overexpression of P58S dVAP causes an UPR. Anti-Hsc3 and anti-Elav co-staining of adult brains of control flies (A), flies expressing WT dVAP (B) or P58S dVAP (C). Flies overexpressing P58S dVAP exhibit higher levels of BiP/Hsc3 (C).

(D) The extracts of control flies and flies expressing WT dVAP or P58S dVAP were immunoblotted with anti BiP/Hsc antibody. Flies overexpressing P58S dVAP exhibit increased levels of BiP/Hsc3.

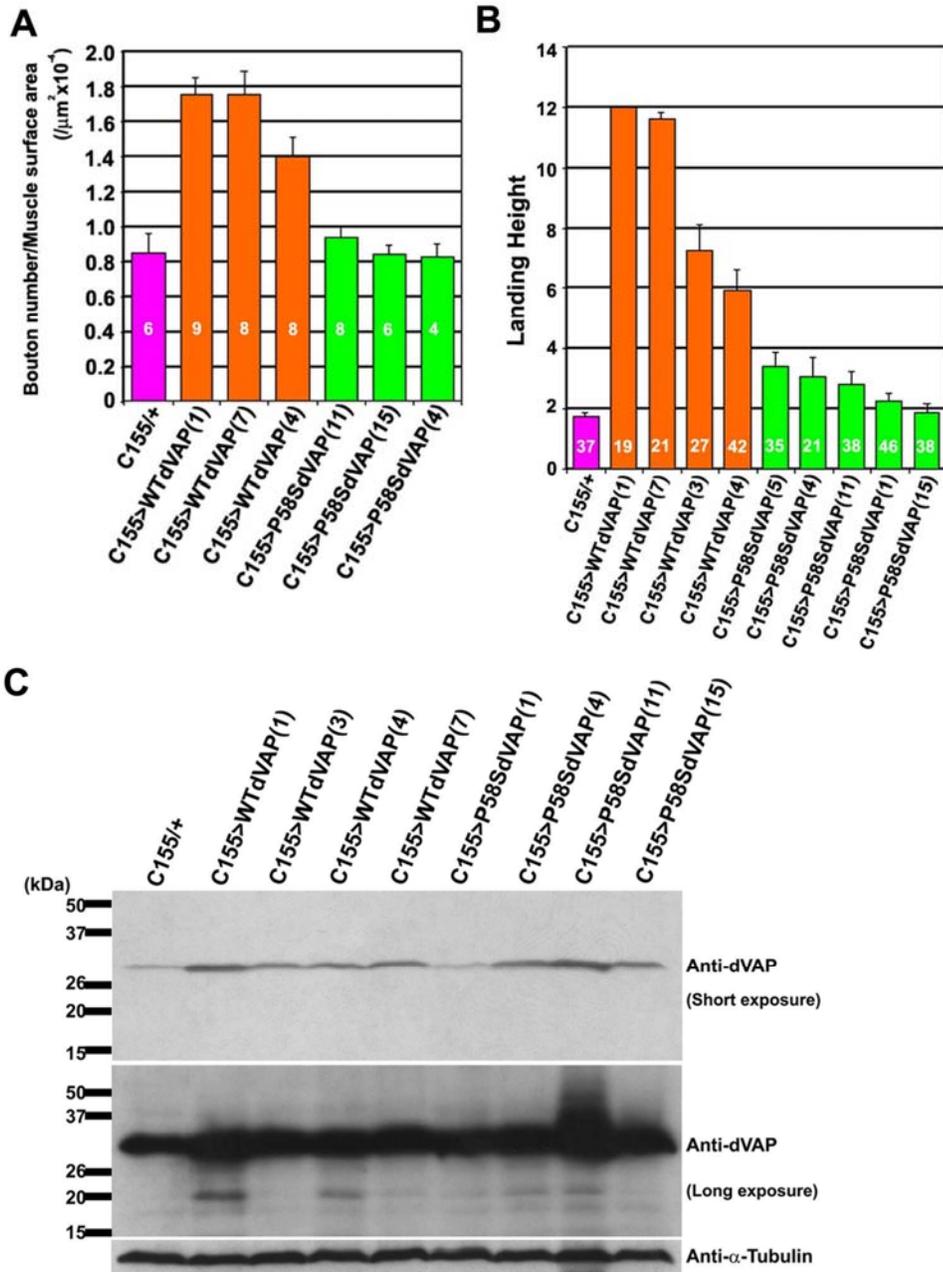


Figure S4. The mutation P58S reduces dVAP activity

(A) Overexpression of WT dVAP increases the number of boutons at NMJs ($P < 0.01$, C155/+ vs. each insertion line expressing WT dVAP). In contrast, the P58S dVAP does not cause this

phenotype. ($P > 0.05$, *C155/+* vs. flies expressing P58S dVAP). The quantification was performed at muscles 6 and 7 of segment A3. Error bars represent SEM.

(B) Flight test. Overexpression of WT dVAP causes a severe flight defect ($P < 0.01$). However, overexpression of P58S dVAP shows a mild phenotype compared to control flies ($P < 0.05$). Flies were individually dropped into a plastic cylinder, and the height at which they landed was recorded. The greater the distance from the top to their landing point, the worse their ability to fly. Error bars represent SEM.

(C) To ensure that all transgenes permit expression of dVAP at similar levels we performed western blots. As shown in this figure, all transgenes produce comparable levels of dVAP when driven by *C155-GAL4*. The proteins from the controls as well as flies expressing WT dVAP or P58S dVAP were extracted using SDS sample buffer. Immunoblotting was performed with anti-dVAP (GP33) antibody. Anti- α -Tubulin was used as a loading control (bottom).

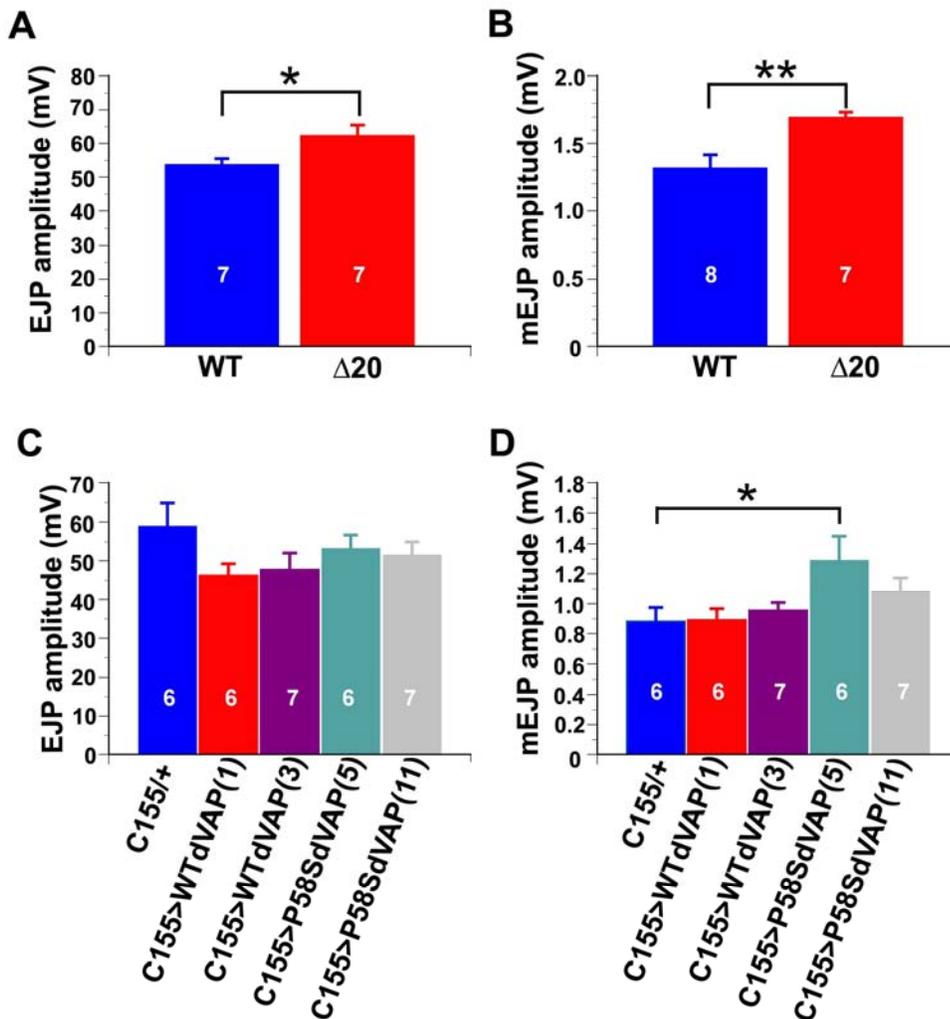


Figure S5. Electrophysiological properties of dVAP null mutants and transgenic animals overexpressing dVAP and P58S based on intracellular recordings (Error bars represent SEM)

(A-B) Average amplitudes of excitatory junction potentials (EJPs) and miniature EJPs (mEJPs) of *dvap* null mutant.

(A) EJPs of control (precise excision of *P* element) and *dvap* null mutant animals ($\Delta 20$). Averaged compound EJPs recorded from muscle 6 (segment 3 or 4). Note that mutant animals have a subtle but significantly higher EJP ($P < 0.05$).

(B) Similarly, the mEJPs are also increased in mutants when compared to controls ($P < 0.005$). Note that Chai et al., 2008 obtained similar data and showed that glutamate receptor clustering was increased in *dvap* mutants (Chai et al., 2008).

(C-D) Average amplitudes of EJPs and mEJPs of transgenic animals.

(C) EJP amplitudes were not significantly different between control and different genotypes ($P > 0.05$) overexpressing WT or P58S dVAP.

(D) There were no significant differences in amplitudes of mEJPs between control and wild type animals overexpressing WT dVAP with *C155-GAL4*. However, we observed an increase in mEJP when mutant P58S was overexpressed. Although MT5 is statistically significantly different from WT ($P < 0.05$), MT11 is not ($P = 0.114$). These data are again consistent with the observation that loss of function mutations and P58S proteins display similarities in phenotypes, further arguing that P58S is a dominant negative protein.

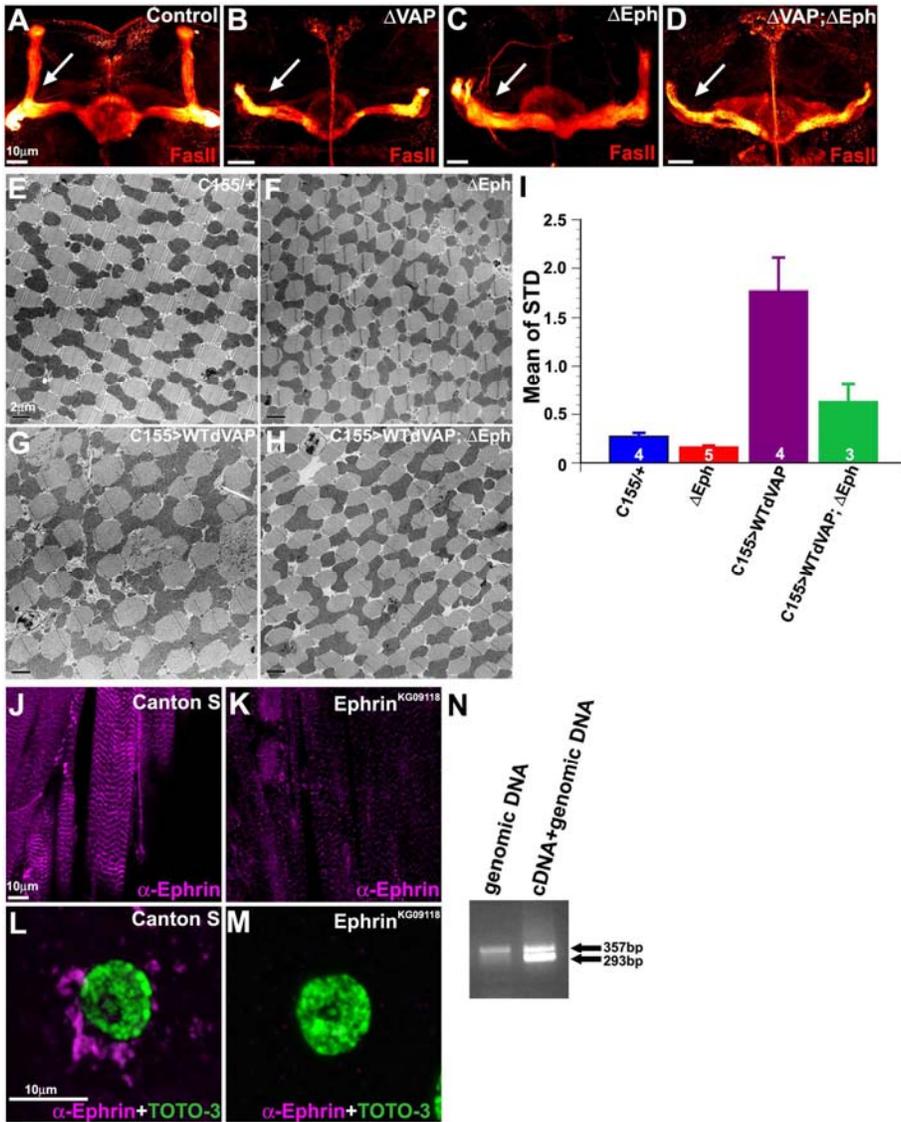


Figure S6. Genetic interaction between *Eph* receptor and *dVAP*

(A-D) *dVAP* mutants show defects in mushroom bodies similar to those documented for *Eph* receptor mutants (Boyle et al., 2006). Mushroom bodies in control (A), *dVAP* mutant (B), *Eph* receptor mutant (C), and *dVAP*; *Eph* receptor double mutants (D) were stained with anti-FasII antibody. (A) FasII labels the α -lobes (arrow) and β -lobes. (B) *dVAP* null mutants display a loss of α lobes (100%, n=10). (C) *Eph* receptor mutants also display an absence of α lobes (70%, n=13). (D) *dVAP*; *Eph* receptor double mutants also lack α -lobes (100%, n=10).

(E-H) *Eph* receptor mutants suppress the muscle phenotype induced by overexpression of WT *dVAP* in the nervous system. TEM analysis of the dorsal longitudinal muscle (DLM) in the control

animals (E), *Eph receptor* mutant (F), control flies overexpressing WT dVAP in the nervous system (G) and *Eph receptor* mutant flies overexpressing WT dVAP in the nervous system (H).

(I) Average standard deviation of the area of myofibrils in each section of the DLM. Flies overexpressing neuronal WT dVAP (G) in the wild type background cause a heterogeneous diameter of myofibrils ($P < 0.01$, when compared to control *C155/+* flies). In contrast, flies expressing WT dVAP in *Eph* receptor mutants show a much reduced frequency of heterogeneous size of myofibrils ($P < 0.02$, when compared to flies expressing WT dVAP in a wild type background). More than 30 myofibrils/animal were examined. The numbers of animals of each genotype are shown. Error bars represent SEM.

(J-M) Ephrin (Bossing and Brand, 2002) is expressed in the muscle. The muscles of 3rd instar larvae of *Canton S* (J, L) and *Ephrin* hypomorphic mutants, *Ephrin*^{*KG09118*} (Boyle et al., 2006) (K, M). Ephrin is present on the muscle surface (J) and perinuclear regions (L).

(N) RT-PCR shows the presence of *Eph* receptor mRNA in *Eph*^{*x652*} mutants, suggesting that this may not be a null allele (Boyle et al., 2006). PCR was performed using primers corresponding to sequences located in exon 12 and 13 of the *Eph* receptor gene. PCR using genomic DNA and cDNA shows a 293bp band derived from the cDNA. The amplified product was confirmed by sequencing.

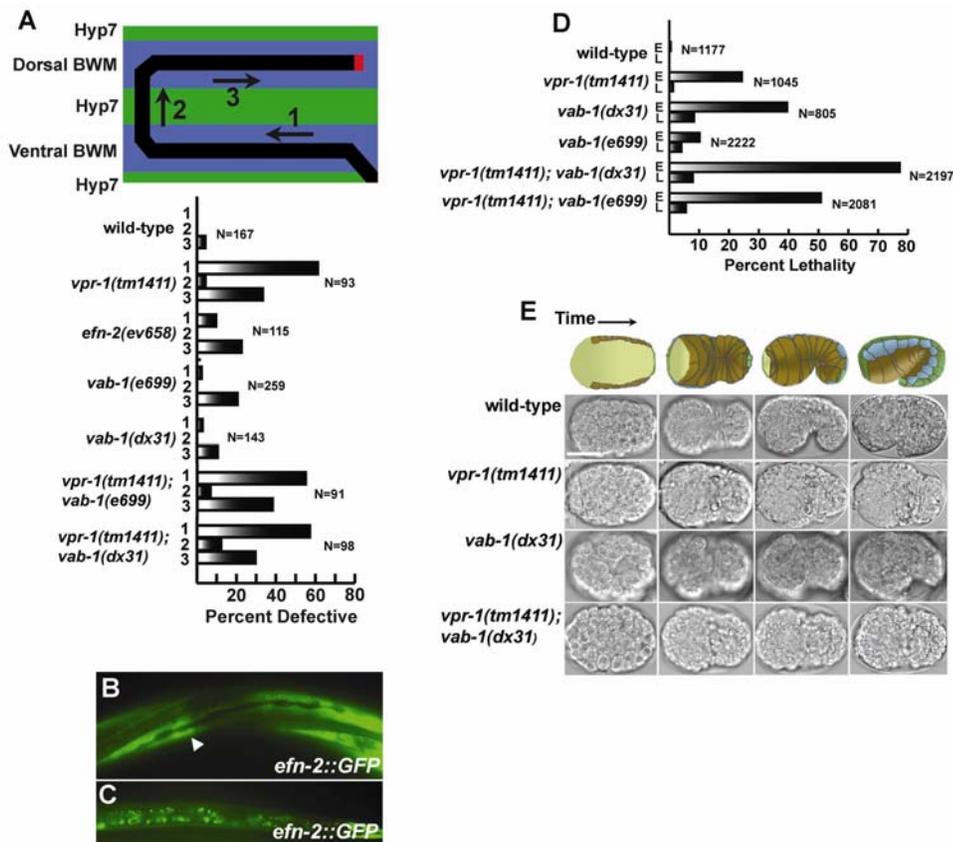


Figure S7. Phenotypic studies of wild type and mutant hermaphrodites.

(A-C) DTC migration and EFN-2 expression. (A) The DTC migrates along the ventral body wall muscle (blue) between the hyp7 hypodermal syncytium (green) during phase 1. The DTC then turns and crosses hyp7 during phase 2. In phase 3, the DTC turns again and migrates along the dorsal body wall muscle. Its final resting position is shown in red. Wild type and mutant hermaphrodites were scored for defects in each phase of DTC migration. (B-C) An *efn-2::GFP* transcriptional reporter (Wang et al., 1999) shows GFP expression in hyp7 (arrowhead) during the time of DTC migration (B), but not before DTC migration (C). The bright spots in panel C are nonspecific gut autofluorescence. These results are consistent with EFN-2 functioning in hyp7 to repel the DTC, keeping it migrating along the body wall muscle.

(D) Embryonic [E] and larval [L] lethality of wild type and mutant hermaphrodites.

(E) 4D time-lapse DIC micrographs of wild-type and mutant embryos undergoing ventral enclosure. During enclosure, the ventral hypodermal cells (brown cells, diagram) migrate and meet at the ventral midline. The blue cells and green cells are the seam and dorsal hypodermal cells, respectively. *vpr-1(tm1411)* embryos have incompletely penetrant and variably expressed enclosure defects that are similar to *vab-1(dx31)* null embryos. *vpr-1(tm1411); vab-1(dx31)* double mutants exhibit similar defects as the single mutants, although the double mutants exhibit increased embryonic lethality (D).

Supplemental Experimental Procedures

Fly strains

The following strains were used:

Canton S

w, *Ubx-FLP*, *tub-GAL80*, *FRT19A*; *Actin-GAL4*, *UAS-CD8::GFP/CyO*

DVAP-33A^{Δ448}, *FRT19A/FM7*, *Kr-GAL4*, *UAS-GFP[dVAP^{Δ448}]*

DVAP-33A^{Δ20} / FM7, *Kr-GAL4*, *UAS-GFP [dVAP^{Δ20}]* (Pennetta et al., 2002)

w¹¹¹⁸; *P{PTT-GA}Pdi^{G00198}/TM3*, *Sb¹ Ser¹* (Herpers and Rabouille, 2004)

P{w^{+mW.hs}=GawB}elav^{C155} (C155-GAL4, elav-GAL4) (Lin and Goodman, 1994)

y¹ w^{67c23}; *P{w^{+mC}=dpp-GAL4.PS}6A/TM6B* (dpp-GAL4/TM6B) (G.Mardon, Houston)

P{w^{+mW.hs}=GawB}C164 (C164-GAL4) (Torroja et al., 1999)

P{w^{+mW.hs}=GawB}C5 (C5-GAL4) (Yeh et al., 1995)

*w**; *P{GAL4-da.G32}UHI* (da-GAL4) (Wodarz et al., 1995).

Antibody generation and immunostaining

For dVAP antibody production, the region N-terminal to the transmembrane domain of the dVAP protein (aa1-248) was expressed using the GST-fusion protein system. A polyclonal rabbit antibody (Rb92) was raised against the fusion protein at Cocalico Biologicals (Reamstown, PA). This antiserum was used at 1:5000 (total staining) or 1:1000 (extracellular staining). Larvae and adult brains were fixed in 4% paraformaldehyde for 20 minutes and washed with PBS containing 0.2% Triton X-100. The following antibody dilutions were used: mouse monoclonals Spectrin- α (3A9) (Byers et al., 1987), 1:20; Elav (7E8A10) (O'Neill et al., 1994), 1:200; DLG(4F3) (Parnas et al., 2001), 1:50; FasII (1D4) (Schuster et al., 1996), 1:10; Futsch(22C10) (Fujita et al., 1982), 1:10; goat polyclonal HRP (Jackson ImmunoResearch), 1:500; mouse monoclonal anti-FLAG (Sigma), 1:50 (extracellular staining), 1:500 (total staining); rat monoclonal anti-HA (Roche), 1:40 (extracellular staining), 1:200 (total staining); guinea pig anti-Boca, 1:500 (Culi and Mann, 2003); guinea pig anti-Hsc3, 1:500 (Ryoo et al., 2007); rabbit anti-Ephrin, 1:500 (Bossing and Brand, 2002) and mouse anti-Ubiquitin (FK1) (BIOMOL), 1:50. Secondary antibodies conjugated to Cy3 or Alexa 488 (Jackson ImmunoResearch, Molecular Probes) were used at 1:250.

Protein chemistry

Secretion assay: S2 cells were cultured in serum free medium (SFM) (Invitrogen) according to the the manufacturer's description. The FLAG tagged (wild type or mutant) dVAP33 MSP domain constructs, were transfected into S2 cells together with an Ubi-Gal4 expression vector. Two days after transfection, the protein present in the medium was precipitated by acetone and re-suspended with 1 \times SDS loading buffer. At the same time, the cells were collected, washed, and loaded with 1 \times SDS loading buffer. The resulting samples were analyzed with western blot.

For the fractionation of proteins into soluble or insoluble extracts, proteins from third instar larvae were extracted with RIPA buffer (50mM Tris base (pH8), 150mM NaCl, 1mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF and Complete protease inhibitor cocktail (Roche)). The detergent-soluble fraction was defined as the supernatant of the cell lysates after centrifugation at 120,000 \times g for 15 minutes. After removal of the supernatant, the pellets were washed with RIPA buffer and then suspended in 8 M urea, 4% SDS, 0.125 M Tris (pH 6.8), 12 mM EDTA, 3% β -mercaptoethanol.

For the immunoprecipitation assay, proteins from third instar larvae were extracted with RIPA buffer. The extracts were incubated with anti-dVAP (GP33) overnight at 4°C, and then incubated with a 40µl bed volume of protein A-Sepharose beads for 2hr at 4°C. The precipitates were then washed five times with RIPA buffer. Guinea pig anti-dVAP antibody (GP33) and mouse anti-Ubiquitin (P4D1) (Covance) antibody were used for immunoblotting of the resulting precipitates. Leukocytes were isolated using dextran as previously described (Skoog and Beck, 1956) and serum was purified with the ProteoSeek Albumin/IgG removal kit (PIERCE). Guinea pig anti-dVAP (GP33) (1:5000), rabbit anti-hVAP (1:1000) (Amarilio et al., 2005), mouse anti-FLAG (Sigma) (1:5000), mouse anti-Actin (MP Biomedicals) (1:10000), rat α -Tubulin (Serotec) (1:500) were used for western blotting.

The constructs of VAP MSP and Eph receptor binding assays: VAB-1 extracellular domain construct, pcVAB-1 Ex V5 His, is a gift from Dr. Greenstein (Govindan et al., 2006). To generate constructs for His and FLAG tagged VPR-1 MSP (His-VPR-1 MSP-Fg), the PCR fragment of the cDNA coding aa 8-120 of VPR-1 and a FLAG-tag fused to the C-terminus was cloned into the pQE30 vector (Qiagen). The PCR fragment coding aa 1-1641 amino acid of mouse EphA4 was cloned into pcTOPO-V5-His vector (Invitrogen) in frame to construct the EphA4 extracellular domain expression vector (pcEphA4Ex-V5His). To generate the construct for His-tagged EphrinB2 expression for the *in vitro* competition assay, we used the cDNA encoding aa 25-187 of mouse EphrinB2 subcloned into the pET 28a vector. To generate the construct for the secreted form of human VAP MSP domain, pcsshVAPMSP, the coding sequence of mouse CD8 signal peptide was fused to the coding sequence of the first 132 amino acids of human VAP by PCR. The resulting fragment was cloned into pcDNA 3.1 vector.

RT-PCR for the *Eph* receptor expression

mRNA was extracted with the Absolutely RNA kit (Stratagene). RT-PCR was performed with SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). The following primers were used: 5'-CTTCAGCATCGGATGTTGGTCGTATGG -3' and 5'-CGTCTGATTCTGGCGAGGGTCTGTTGTC-3'.

Transmission Electron Microscopy

TEM was performed as previously described (Verstreken et al., 2003).

Flight assay

The flight assay was performed as previously described (Fayyazuddin et al., 2006).

Electrophysiology

All experiments were conducted at room temperature (20–22°C), in hemolymph-like (HL-3) solution (Stewart et al., 1994), with the following ionic composition (in mM): NaCl, 70; KCl, 5; MgCl₂, 20; CaCl, 1; NaHCO₃, 10; Trehalose, 5; HEPES, 5; Sucrose, 115. Wandering third instar female larvae were dissected in ice-cold calcium free HL-3 saline, then thoroughly rinsed with HL-3 containing 1mM Ca²⁺. The preparation was then incubated in the latter solution for at least 5 min before recording. Intracellular recordings were made from body-wall muscles 6 (abdominal segment 3 or 4). Recording electrodes (70-85 M Ω), filled with a 2:1 mixture of 4M potassium acetate to 4M potassium chloride, were used to record excitatory junction potentials (EJPs) and spontaneous miniature excitatory junction potentials (mEJPs). Impalements displaying a resting membrane potential (RMP) lower than -60 mV throughout the course of the experiment were chosen for

analysis. Data were discarded if the RMP shifted by more than ± 5 mV during the experiment. EJPs were evoked by directly stimulating the segmental nerve innervating either hemisegment 3 or 4 through a glass capillary suction electrode (internal diameter, ~ 10 μm) at 0.2 Hz. Stimulus pulses (0.3 ms duration) were generated by pClamp 8.0 software (Axon Instruments Inc). The applied currents were $6 \mu\text{A} \pm 3$, which was 50% larger than that required to recruit both 1b and 1s neurons innervating muscles 6 and 7. Both EJPs and mEJPs were amplified with an Axonclamp 2B amplifier (Axon Instruments, Foster City, CA) under bridge mode, filtered at 10 kHz and digitized at 10 kHz (for EJPs) and 40 kHz (mEJPs), respectively, with pClamp 8.0.

EJP were analyzed with pClamp 8.0. Spontaneous release was analyzed using the Mini Analysis Program (Synaptosoft Inc., Decatur, GA). Plots were made using DeltaGraph 5.6.4 (Red Rock Software Inc.). There was no significant difference in the average RMP between different genotypes muscle cells: control (precise excision of P-element) 70.5 ± 1.826 (n=7), $\Delta 20$ 74.5 ± 2.587 (n=7), C155/+, 72.5 ± 2.634 (n=6); C155/WT1, 66.3 ± 1.826 (n=6); C155/WT3, 67 ± 1.106 (n=7); C155/MT5, 70.5 ± 2.977 (n=6); C155/MT11, 70.9 ± 1.571 (n=7). The EJP amplitude was corrected for non-linear summation (McLachlan and Martin, 1981) using the values of the duration of transmitter action (30 ms) and the membrane time constant (160 ms) (Feeney et al., 1998). Comparisons between genotypes were made using Student's t test (SigmaPlot 10.0, Systat software Inc.). Unless otherwise indicated, $p < 0.05$ was deemed significant.

C. *elegans* experiments

Phenotypes were scored in the F2 generation. *vpr-1* (*tm1411*) was balanced with the translocation *hT2(qIs48)I*, which expresses pharyngeal GFP. Strain construction and marker scoring were performed essentially as previously described using PCR and phenotypic analyses (Miller et al., 2003). *tm1411* was detected by PCR using the primers 5'-ACTCCGAGATAATACGGCGA-3', 5'-TTGGGGGAACGGGAACCAT-3', 5'-ACGCTTGCTCTAGGCACTA-3', and 5'-TAGGCACTAAGCACTGGCCA-3'.

The Fosmid WRM06-24-D13 (provided as an unpublished resource by D. Moerman and colleagues) was microinjected along with the *rol-6(su1006)* dominant marker (Mello et al., 1991) into *vpr-1(tm1411)/hT2(qIs48)* hermaphrodites. *vpr-1* (*tm1411*) homozygous transgenic animals were rescued for the DTC migration, sterility, and embryonic defects.

The following strains were used:

XM 1020 [*vpr-1(tm1441)/hT2(qIs48)I*]

XM1021 [*vpr-1(tm1411)/hT2(qIs48)I; vab-1(dx31)II*]

NW1550 [*efn-2(ev658)IV; him-5(e1490)V*] (Wang et al., 1999)

EM305 [*efn-4(bx80)IV; him-5(e1490)V*] (Chow and Emmons, 1994)

CZ337 [*vab-1(dx31)II*] and CZ414 [*vab-1(e699)III*] (George et al., 1998)

CB4108 [*fog-2(q71)V*] (Schedl and Kimble, 1988)

EFN-2::GFP transcriptional reporter (Wang et al., 1999)

JK2049 [*qIs19(lag-2::GFP)V*] (Blelloch et al., 1999)

ST2 [*ncIs2(ph20::GFP)*] (Shioi et al., 2001)

For VPR-1 MSP domain expression, DNA encoding amino acids 8-120 of VPR-1 was amplified by PCR with the primers 5'-GGATCCCTGCAAGTGACTCCAAATCG-3' and 5'-AAGCTTGCTGTAGGTCAGCTCC-3'. The PCR product was subcloned into PCR2.1-Topo Vector (Invitrogen). Next, the BamHI and HindIII DNA fragment was cloned into the pQE30 vector (Qiagen), which has six histidine residues at the N terminus. The pQE30-VPR-1 MSP domain plasmid was transformed into M15 cells. Purification and FITC conjugation were conducted as previously described (Miller et al., 2001; Miller et al., 2003).

4D video microscopy was performed as described previously with minor modifications (Chin-Sang et al., 2002). Embryos were extruded by decapitating adult worms and mounted on the 2% agarose pads. Embryos were recorded at 5 minutes intervals throughout development.

Supplemental References

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