The *C. elegans* VAPB homolog VPR-1 is a permissive signal for gonad development

Pauline A. Cottee‡, Tim Cole‡, Jessica Schultz, Hieu D. Hoang, Jack Vibbert, Sung Min Han* and Michael A. Miller§

**ABSTRACT**

VAMP/synaptobrevin-associated proteins (VAPs) contain an N-terminal major sperm protein domain (MSPd) that is associated with amyotrophic lateral sclerosis. VAPs have an intracellular housekeeping function, as well as an extracellular signaling function mediated by the secreted MSPd. Here we show that the *C. elegans* VAP homolog VPR-1 is essential for gonad development. vpr-1 null mutants are maternal effect sterile due to arrested gonadogenesis following embryo hatching. Somatic gonadal precursor cells and germ cells fail to proliferate fully and complete their respective differentiation programs. Maternal or zygotic vpr-1 expression is sufficient to induce gonadogenesis and fertility. Genetic mosaic and cell type-specific expression studies indicate that vpr-1 activity is important in the nervous system, germ line and intestine. VPR-1 acts in parallel to VAB-1 Eph receptor and other receptors expressed on oocyte and gonadal sheath cell membranes (Brisbin et al., 2009; Cheng et al., 2008; Miller et al., 2003); this binding modulates G-protein signaling in sheath cells that triggers oocyte meiotic maturation and sheath contraction (Govindan et al., 2006).

Sperm MSPs contain a single MSPd that is related to the N-terminal MSPd from VAMP/synaptobrevin-associated proteins (VAPs) (Fig. 1A) (Lev et al., 2008; Skehel et al., 1995). Although sperm MSPs appear to be unique to nematodes, VAPs are widely conserved among animal species. Mammals have two VAP paralogs called VAPA and VAPB, which both contain an N-terminal MSPd. Mutations in the VAP MSPd are associated with amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA), two motor neuron degeneration diseases (Nishimura et al., 2004). VAPs are broadly expressed type II transmembrane proteins that function as scaffolding components at intracellular membrane contact sites, such as those bridging the endoplasmic reticulum and mitochondria or peroxisomes (Costello et al., 2017; Dong et al., 2016; Gomez-Suaga et al., 2017; Hua et al., 2017; Lev et al., 2008; Stefan et al., 2011; Stoica et al., 2016). This cell-autonomous activity affects actin nucleation, endocytic trafficking, lipid transfer, Ca2+ dynamics and other processes. In addition, the VAP MSPd is cleaved from the transmembrane domain and secreted in a cell type-specific fashion (Deidda et al., 2014; Han et al., 2013, 2012; Tsuda et al., 2008). The secreted MSPd binds to Eph protein tyrosine kinase and LAR-like protein tyrosine phosphatase receptors, which are important for neuron development and striated muscle energy metabolism, respectively (Han et al., 2013, 2012; Tsuda et al., 2008). Hence, VAPs also have an important non-cell-autonomous signaling function.

To better understand essential roles of animal VAPs, we have been studying the nematode model *C. elegans*. The *C. elegans* genome encodes numerous proteins that contain an MSPd (Tarr and Scott, 2005). Only *vpr-1* encodes the N-terminal MSPd, coiled-coil motif and single transmembrane region characteristic of VAPs (Fig. 1A) (Miller et al., 2001; Tsuda et al., 2008). *C. elegans vpr-1* mutants share phenotypes in common with *Drosophila Vap* (Vap33) and mouse *Vapb* mutants (Han et al., 2013, 2012; Larroquette et al., 2015, 2008). Moreover, these VAPs are functionally interchangeable and their MSPds have conserved signaling and receptor binding activities (Han et al., 2012, 2011; Tsuda et al., 2008). *vpr-1* null mutants exhibit incompletely penetrant embryonic lethality and 100% sterility, whereas *Drosophila Vap* null mutants die as second or third instar larvae (Pennent et al., 2002; Tsuda et al., 2008). By contrast, mouse *Vapb* mutants are viable and fertile (Kabashi et al., 2013; Larroquette et al., 2015), perhaps due to redundancy or division of function with *Vapa*.

Here we characterize the role of *vpr-1* in gonadogenesis. *vpr-1* null mutants are maternal effect sterile due to arrested somatic gonadal precursor cell and germ cell development. We show that *vpr-1* expression is crucial in neurons and germ cells to induce...
gonadogenesis. Moreover, transgenic vpr-1 overexpression is sufficient for gonadogenesis induction in several cell types accessible to the pseudocoelom, a primitive circulatory cavity. The most likely target tissue is the sheath cell precursors, which are required for germline proliferation and differentiation independent of GLP-1 Notch receptor signaling (Killian and Hubbard, 2005; McCarter et al., 1997; Sulston et al., 1983). Consistent with this idea, vpr-1 acts independently of glp-1 at a stage when sheath precursors are starting to divide. Collectively, our data support the model that the VPR-1 MSPd acts as a permissive signal for gonadogenesis early in postembryonic development.

RESULTS

vpr-1 null mutants are maternal effect sterile

The C. elegans hermaphrodite gonad develops postembryonically from a primordium consisting of two somatic gonadal precursor cells, Z1 and Z4, and two germline precursors, Z2 and Z3 (Fig. 1B) (Hubbard et al., 2013; Pazdernik and Schedl, 2013). During larval development, Z1 and Z4 descendants form the distal tip cells (DTCs), anchor cell, gonadal sheath cells, spermathecae and uterus. Z2 and Z3 start proliferating during L1, forming a germline syncytium with a central core, called the rachis, during subsequent larval stages (Fig. 1B) (Amini et al., 2014). A stem cell population at the distal gonad tip gives rise to sperm and oocyte precursors (Kimble and Crittenden, 2005). Sperm develop first at the L4 stage, followed by oocytes during early adulthood (Hubbard et al., 2013; Pazdernik and Schedl, 2013). Germ cell development and meiosis proceed from the distal to proximal ends (Fig. 1B). As a result, the adult hermaphrodite has two U-shaped gonad arms that connect to a common uterus.

The vpr-1 (tm1411) null mutation eliminates the first two vpr-1 exons, which encode the MSPd and part of the coiled-coil motif.
(Tsuda et al., 2008). Homozygous F1 vpr-1(tm1411) mutants derived from P0 vpr-1(tm1411)/hT2 heterozygous hermaphrodites exhibit limited fertility, with an average brood size of ~30 F2 progeny (Fig. 1C). These progeny give rise to completely sterile vpr-1(tm1411) hermaphrodites lacking maternal (M−) and zygotic (Z−) vpr-1 expression (Fig. 1C). F1 vpr-1(tm1411) mutant adults with maternal vpr-1 mRNA (M+ Z−) contain functional sperm and oocytes (Fig. 1D). By contrast, M− Z− vpr-1 mutant adults contain stunted gonads without mature gametes (Fig. 1D). To investigate gonad development, we monitored staged animals using differential interference contrast (DIC) microscopy. The 4-cell gonad primordium appears similar in freshly hatched wild-type and vpr-1 mutant hermaphrodites (Fig. 1E). However, the distal portion of each gonad stops developing in vpr-1 mutants (Fig. 1E). We mated wild-type males to M+ Z− vpr-1 mutant hermaphrodites to test whether zygotic vpr-1 expression is sufficient to induce gonadogenesis. Male sperm provide a wild-type vpr-1 copy to oocytes completely lacking vpr-1. The M− Z+ vpr-1(tm1411)/+ progeny produce functional gametes, similar to wild-type and M+ Z− vpr-1 mutant hermaphrodites (Fig. 1D). We conclude that vpr-1 is a maternal effect sterile gene and that maternal or zygotic vpr-1 expression is sufficient for gonadogenesis.

**vpr-1 loss arrests germ cell and somatic gonadal precursor cell development**

M− Z− vpr-1 mutant adult gonads are much smaller than control adult gonads, suggesting that germ cell development is abnormal. To investigate germ cells, we used DAPI to assess chromosome morphology. In the wild type, germ cell chromosomes exhibit characteristic features in the mitotic, meiotic transition, and meiotic pachytene zones (Fig. 2). Mature sperm have highly condensed chromosomes, whereas growing oocytes in diakinesis have dispersed chromosomes (Fig. 2A,C). M− Z− vpr-1 mutant adult gonads contain fewer than 80 germ cells and are without sperm or oocytes (Fig. 2B,D). Germ cell chromosomes more closely resemble those in the mitotic or transition zone, although

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**Fig. 2. vpr-1 is essential for germ cell expansion during larval development.** (A,B) Adult wild-type (A) and vpr-1 M− Z− (B) hermaphrodites stained with the DNA dye DAPI to identify germ cell stages. Gonads are outlined in white. In wild-type gonads, mitotic zone (MZ) precursor nuclei are observed at the distal end. From the distal to proximal end, meiotic transition zone (TZ) nuclei, pachytene stage (PS) nuclei, and spread diakinesis stage chromosomes of oocyte (Oo) nuclei are indicated. Highly condensed sperm (Sp) chromatin is seen at the most proximal end. Differentiating germ cells are not observed in vpr-1 M− Z− gonads (B). Scale bars: 10 µm. (C,D) DAPI staining of dissected wild-type (C) and vpr-1 M− Z− (D) gonads improves imaging of germ cell chromosome morphology. Three different vpr-1 M− Z− gonads are shown. (E,F) Counting germ cell numbers during wild-type larval development (E) shows exponential expansion that is not observed in vpr-1 M− Z− larva (F). Germ cell number was counted in DAPI-stained larva from 1 µm z-stacks imaged through the body. Total germ cell number was divided by two in cases where both anterior and posterior gonads were counted (nearly all cases). Differentiating spermatocytes were not included in L4 stage wild-type gonads and are not observed in vpr-1 null gonads. Larval development is represented on the x-axis as total worm body length instead of time post-hatching because vpr-1 M− Z− hermaphrodites grow more slowly than wild-type hermaphrodites (Han et al., 2013, 2012). Dotted line indicates best-fit polynomial equation.
chromosome morphology is often abnormal (Fig. 2D). Next, we counted the number of germ cell nuclei during larval stages. Wild-type gonads exhibit a robust increase in germ cell numbers at L3 and L4 stages (Fig. 2E), which is due to GLP-1 Notch receptor signaling and a parallel pathway requiring gonadal sheath cell precursors (Killian and Hubbard, 2005; Kimble and Crittenden, 2005; McCarter et al., 1997). In contrast to the wild type, vpr-1 null mutants fail to expand the germ line during larval development (Fig. 2F). These data indicate that vpr-1 is essential for germ cell expansion and differentiation.

To examine gonad architecture, we used the pie-1p::gfp::ph transgene, which drives germline expression of a GFP::PH fusion protein that acts as a plasma membrane marker (Audhya et al., 2005). In the wild type, germ cells in the distal gonad surround and open into the rachis, which forms during larval development (Amini et al., 2014). A well-defined rachis is not observed in M– Z– vpr-1 mutant gonads (Fig. 3A,B). Instead, germ cells with fully enclosed basement membrane during L2, before turning 180° in L3 (Hubbard et al., 2005). In the wild type, germ cells in the distal gonad surround and open into the rachis, which forms during larval development (Amini et al., 2014). A well-defined rachis is not observed in M– Z– vpr-1 mutant gonads (Fig. 3A,B). Instead, germ cells with fully enclosed plasma membranes are disordered throughout the core region. We previously showed that vpr-1 loss causes DTC migration failure (Tsuda et al., 2008). The DTCs are generated from Z1 and Z4 divisions during L1 (Fig. 3C) and start migrating along the ventral basement membrane during L2, before turning 180° in L3 (Hubbard and Greenstein, 2000). We used the lag-2p::gfp transgene to monitor DTC development in vpr-1 mutants (Byrd et al., 2014). In M– Z– vpr-1 mutant gonads, the DTCs are similar in morphology to those in control L2 or L3 gonads, although they lack the membranous processes seen in adult gonads (Fig. 3D). The vulva, which is induced by the anchor cell during the L3 stage, appears to form normally (Fig. 1D,E). Similar to the DTCs, the anchor cell is derived from Z1 or Z4 divisions during L1 and early L2 (Fig. 3C). Thus, two of the earliest differentiating somatic gonadal cell types, namely the DTCs and anchor cell, appear to form during early larval development in vpr-1 mutants.

Gonadal sheath cells arise from sheath/spermatheca (SS) blast cells formed during L1 and early L2 (Fig. 3C) (Hubbard and Greenstein, 2000). To investigate sheath cells, we used the lim-7p:: gfp transgene (Hall et al., 1999). In adult controls, GFP is observed in the four most distal sheath cell pairs, tightly encircling the germ cells and developing oocytes (Fig. 3E). We observe GFP expression in vpr-1 null gonads, but sheath cells fail to completely cover the germ cells, suggesting that sheath proliferation and development are incomplete (Fig. 3E). Further support comes from the lim-7p::yfp:: act-5 transgene, which labels the sheath cell actin cytoskeleton (Kinchen et al., 2005). vpr-1 mutant sheath cells do not exhibit the actin filament organization seen in control sheath cells (Fig. 3F). SS blast cells give rise to 10 sheath cells and 18 spermathecal cells from early L3 to mid-L4 stages (Fig. 3C). The adult spermatheca comprises 24 cells in total, which derive from SS and a sister lineage

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**Fig. 3. vpr-1 is essential for postembryonic somatic gonad development.** (A) Diagrams of gonad orientation and structure in wild-type (left) and vpr-1 M– Z– (right) hermaphrodites. (B) The germline plasma membrane GFP::PH marker (Audhya et al., 2005) reveals overall gonad structure in transgenic wild type (WT) and vpr-1 mutants. WT distal gonads contain a central cytoplasmic core called the rachis (arrow), which shares germ cell cytoplasmic contents. A vpr-1 mutant gonad (outlined in yellow) shows disorganized structure and poorly defined or absent rachis. (C) Diagram showing somatic gonad lineages (Z1 is shown) that form the DTC, 10 sheath cells, 24 spermathecal cells, 18 uterine cells, and the anchor cell (AC). The AC is necessary for vulva development (Hubbard and Greenstein, 2000). Z4 forms the other gonad. Approximate L1/L2 and L3/L4 transition periods are indicated. (D) Transgenic WT and vpr-1 mutants expressing GFP in the DTC driven by the lag-2 Notch ligand promoter (Bieloch et al., 1999). (E) Transgenic WT and vpr-1 mutants expressing GFP in the gonadal sheath cells driven by the lim-7 promoter (Hall et al., 1999). (F) Transgenic WT and vpr-1 mutants expressing YFP::ACT-5 actin in the sheath cells (Kinchen et al., 2005). Note that the actin filaments seen in WT distal sheath cells are not observed in vpr-1 mutant sheath cells. (G-J) The sur-5p::NLS-GFP transgenic reporter, which expresses GFP in the nuclei of all somatic cells (Yochem et al., 1998), was used to visualize and quantify somatic gonadal cells from 1 day posthatch to 10 days in young adult animals. (G) A single z-stack image showing numerous spermathecal cell (arrowheads) and sheath cell (arrows) nuclei in vpr-1(tm1411) null mutants expressing a transgene containing the vpr-1 genomic locus (fosmid DNA). (H) A similar image from vpr-1 M– Z– gonads shows fewer spermatheca and sheath nuclei. Gonad orientation is shown in A. Quantification of sheath nuclei (I) and spermatheca nuclei (J) from the z-stacks. White numbers indicate number of gonads scored. Error bars are s.d. *P<0.001 (Student’s t-test). DTC, distal tip cell; SS, sheath/spermatheca precursors; v, vulva. Scale bars: 10 μm.
that also forms the uterus (Fig. 3C) (Hubbard and Greenstein, 2000). We used the sur-5p::NLS-GFP marker that expresses nuclear GFP in all somatic cells (Yochem et al., 1998) and serial imaging in 1 μm z-stacks to count the number of sheath and spermathecal cells in young adult control and vpr-1 M− Z− gonads (Fig. 3G-J). vpr-1 (tm1411) null mutants transgenically expressing vpr-1 from fosmid DNA contain ∼10 sheath cells (Fig. 3G,I) and 24 spermathecal cells per gonad (Fig. 3G,J). By contrast, vpr-1 M− Z− gonads contain severely reduced numbers of sheath (∼4) and spermathecal (∼6) cells (Fig. 3H-J). The uterine lineage is also likely to be affected, although we did not count these cells. We conclude that vpr-1 is essential for proliferation and differentiation of somatic gonadal precursors that form sheath and spermathecal lineages. These precursors are essential for germ cell expansion and differentiation (Killian and Hubbard, 2005; McCarter et al., 1997).

Zygotic vpr-1 is important in the germ line, nervous system and intestine

vpr-1 is broadly expressed in most tissues (Tsuda et al., 2008). We used genetic mosaic analysis to investigate where vpr-1 functions (i.e. in which cell type) to promote gonad development. Transgenic vpr-1(tm1411) null mutants were generated using a DNA fosmid containing the vpr-1 genomic locus and a plasmid containing the sur-5p::NLS-GFP lineage marker (Yochem et al., 1998). Transgenic DNA is spontaneously lost at low frequency during gonadogenesis. Thus, the mosaic lines provide a readout of zygotic vpr-1 activity only. The loss of vpr-1 from the AB lineage, which produces the somatic gonad (i.e. Z1, Z4, and their descendants; Fig. 3C), or C lineage, which gives rise to specific body wall muscle and hypodermal cells, did not disrupt development (Fig. 4). vpr-1 loss from the EMS and E lineages that form the intestine did not prevent germ cell expansion or gamete differentiation. However, 12/27 mutants were sterile due to either ectopic germ cell proliferation in the proximal gonad (i.e. the Pro phenotype) or failed ovulation, two defects consistent with abnormal somatic gonad development or function (Fig. 4) (Hubbard and Greenstein, 2000). The remaining 15/27 mutants produced a small number of fertilized eggs. These mosaic data suggest that zygotic vpr-1 expression from the germ line, nervous system and intestine is important for fertility. Therefore, multiple tissue sources contribute vpr-1 genetic activity that promotes gonad development.

vpr-1 can induce gonadogenesis non-cell-autonomously

Genetic mosaic worms lacking vpr-1 in the somatic gonad are fertile (Fig. 4), consistent with vpr-1 acting non-autonomously in these

Expressing the vpr-1 genomic locus in vpr-1(tm1411) worms rescued the gonad defects (Fig. 4) in three independent transgenic lines. These lines were maintained as transgenic vpr-1 mutant homozygotes. Importantly, complete transgene loss in F1 progeny resulted in sterile adults, indicating that maternal vpr-1 expression from the extrachromosomal arrays was insufficient for gonadogenesis. Thus, the mosaic lines provide a readout of zygotic vpr-1 activity only. The loss of vpr-1 from the AB lineage, which gives rise to the nervous system, or the P lineages, which give rise to the germ line, caused arrested gonadogenesis (Fig. 4). These gonads often appeared larger than M− Z− vpr-1 gonads (Figs 1–3), but rarely contained visible oocytes. vpr-1 loss from the EMS lineage, which produces the somatic gonad (i.e. Z1, Z4, and their descendants; Fig. 3C), or C lineage, which gives rise to specific body wall muscle and hypodermal cells, did not disrupt development (Fig. 4). vpr-1 loss from the EMS and E lineages that form the intestine did not prevent germ cell expansion or gamete differentiation. However, 12/27 mutants were sterile due to either ectopic germ cell proliferation in the proximal gonad (i.e. the Pro phenotype) or failed ovulation, two defects consistent with abnormal somatic gonad development or function (Fig. 4) (Hubbard and Greenstein, 2000). The remaining 15/27 mutants produced a small number of fertilized eggs. These mosaic data suggest that zygotic vpr-1 expression from the germ line, nervous system and intestine is important for fertility. Therefore, multiple tissue sources contribute vpr-1 genetic activity that promotes gonad development.

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cells. If MSPd signaling promotes gonadogenesis, then overexpressing vpr-1 in various tissues should rescue the vpr-1 null defect. To test this hypothesis, we first drove a vpr-1 genomic fragment containing introns and 3’ UTR (vpr-1g) in the germ line using the pie-1 promoter (pie-1p::vpr-1g). Single-copy integrants on chromosome II were used to avoid germline silencing mechanisms. Two independently generated integrated transgenes completely rescued the vpr-1(tm1411) gonad and body wall muscle mitochondrial defects (Fig. 5A-D) (Schultz et al., 2017), and are likely to contribute maternal and zygotic vpr-1. To test whether zygotic vpr-1 germine expression is sufficient for rescue, we crossed vpr-1 null mutant males expressing pie-1p::vpr-1g to M+ Z− vpr-1 hermaphrodites. The resulting M− zygotic germine vpr-1 M− Z− mutant progeny were largely fertile with proliferating germ cells and differentiating gametes (Fig. 5B). The ~20% of mutant hermaphrodites that were sterile exhibited a novel phenotype whereby oocytes differentiate near the vulva, a defect apparently due to delayed spermathecal and uterine development (Fig. 5E). As sheath cells are essential for germ cell proliferation, meiosis and ovulation (Hubbard and Greenstein, 2000; Killian and Hubbard, 2005; McCarter et al., 1997), sheath development must occur in the rescuing vpr-1 mutant pie-1p::vpr-1g transgenics. These data suggest that germine vpr-1 expression is sufficient to promote gonadogenesis in vpr-1 null mutants. An important difference between this experiment and the mosaic is that the latter contained the entire vpr-1 genomic region in an extrachromosomal transgene (see Discussion).

A caveat with integrated transgenes is that ectopic expression in unwanted tissues may result from surrounding regulatory sequences. To avoid this issue, we tested a panel of well-characterized neuron-specific promoters using high-copy extrachromosomal arrays (Kelly et al., 1997). Overexpressing vpr-1g throughout the nervous system using the unc-119 promoter (Maduro and Pilgrim, 1995) largely rescues the vpr-1(tm1411) gonad defects (Fig. 5B,F). Similar results are observed using the glr-5 promoter, which drives expression in ~56 cholinergic neurons (Brockie et al., 2001), the unc-25 promoter, which drives expression in 26 GABAergic motor neurons (Jin et al., 1999), and the unc-17 promoter, which drives expression in ~80 cholinergic neurons (Lickteig et al., 2001) (Fig. 5B,G). These overexpressing transgenic lines were fertile and could be maintained in a vpr-1(tm1411) background. A small percentage (~20%) of transgenic mutants exhibited the novel gonad phenotype described above. In addition to the neuronal promoters, overexpressing vpr-1g specifically in the intestine using the ges-1 promoter (Kennedy et al., 1993) also rescues the vpr-1 mutant gonad defect (Fig. 5B). The body wall muscle-specific myo-3 promoter (Okkema et al., 1993) and hypodermis-specific rol-6 promoter (Kramer et al., 1990) are sufficient for partial rescue (Fig. 5B), but the transgenic lines could not be stably propagated – in contrast to the mutant lines expressing vpr-1 in neurons, the intestine or germ line. Body wall muscle and hypodermis may have limited capacity for vpr-1 expression or MSPd secretion. vpr-1 introns and 3’ UTR appear to contain key regulatory sequences, as transgenes containing the vpr-1 cDNA and unc-54 3’ UTR exhibit less activity (data not shown). These results show that vpr-1 overexpression in diverse cell types is sufficient to promote gonadogenesis.

The VPR-1 MSPd has been shown to interact with two broadly expressed receptors, CLR-1 and VAB-1, as well as an unidentified receptor(s) (Han et al., 2013, 2012; Tsuda et al., 2008). This interaction is required for MSPd signaling and gonadogenesis. The vpr-1 promoter drives expression in diverse cell types and in a variety of tissues that are essential for gonadogenesis. In the nervous system, the vpr-1 promoter drives expression in 80 cholinergic neurons, the mec-7 promoter drives expression in ~5000 neurons, and the rol-6 promoter drives expression in ~5000 neurons. In the intestine, the vpr-1 promoter drives expression in ~56 head interneurons, the ges-1 promoter drives expression specifically in 26 GABAergic motor neurons, and the unc-17 promoter drives expression specifically in ~80 cholinergic neurons. In the hypodermis, the vpr-1 promoter drives expression specifically in ~150 hypodermal cells. In the gonad, the vpr-1 promoter drives expression specifically in ~5000 gonadal cells. In summary, overexpression of vpr-1 in a variety of cell types is sufficient to rescue the vpr-1 null defect.
encodes a Lar-like receptor protein tyrosine phosphatase that is essential for larval fluid homeostasis and survival (Kokel et al., 1998). In adults, CLR-1 acts in body wall muscle to regulate actin remodeling and mitochondrial localization. The MSPd antagonizes muscle CLR-1 signaling to target mitochondrial tubules to myofibrils (Han et al., 2012). We considered the possibility that this pathway might constitute a checkpoint mechanism for gonadogenesis. Although clr-1(e2530) null and clr-1(e1745ts) temperature-sensitive mutants have degenerating gonads (Fig. S2A-D), genetic mosaic and transgenic expression studies indicate that this degenerative defect is a consequence of hypodermal clr-1 loss, which causes massive fluid accumulation and larval lethality (Fig. S1) (Kokel et al., 1998). Adult vpr-1(tm1411) clr-1 RNAi hermaphrodites and transgenic vpr-1(tm1411); clr-1(e1745ts) hermaphrodites expressing clr-1 in the hypodermis exhibit correct muscle mitochondrial localization, yet have arrested gonads (Fig. S2A-D). Therefore, the clr-1 muscle pathway is unlikely to influence gonadogenesis. The MSPd also antagonizes the VAB-1 Eph receptor, which is expressed throughout the nervous system (George et al., 1998; Miller et al., 2003; Tsuda et al., 2008). vab-1(dx31); vpr-1(tm1411) double-null mutants have arrested gonads (Fig. S2E), indicating that excess VAB-1 signaling does not cause arrested gonadogenesis. Collectively, the results are consistent with MSPd signaling being important for gonad development, but independent of excess CLR-1 or VAB-1 signaling individually.

vpr-1 and Notch act in parallel genetic pathways

Signals from SS blast cells and the DTCs act in independent pathways to promote germline expansion during larval development (Hubbard and Greenstein, 2000; Killian and Hubbard, 2005; McCarther et al., 1997). Although the sheath pathway is not well understood, Notch signaling from the DTCs to germline stem cells controls proliferation and maintains stem cell fate (Hansen and Schedl, 2013; Kimble and Crittenden, 2005). Loss of the glp-1 Notch receptor causes germ cells to exit mitosis and differentiate into sperm. By contrast, gain of glp-1 function causes unregulated germ cell mitosis. To test whether glp-1 acts in the same pathway as vpr-1, we first generated vpr-1(tm1411); glp-1(ar202ts) double mutants. glp-1(ar202ts) is a temperature-sensitive (ts) gain-of-function mutation (Pepper et al., 2003). DAPI and fluorescence deconvolution microscopy were used to visualize germ cell nuclei in wild-type, vpr-1(tm1411) single-mutant, glp-1(ar202ts) single-mutant, and vpr-1(tm1411); glp-1(ar202ts) double-mutant hermaphrodites grown at the restrictive temperature (25°C) from L1 (Fig. 6A-D). vpr-1(tm1411); glp-1(ar202ts) gonads (Fig. 6D) lack the germ cell tumor-like phenotype seen in glp-1(ar202ts) gonads (Fig. 6C) and instead closely resemble vpr-1(tm1411) gonads (Fig. 6B). This result indicates that vpr-1 either acts downstream of glp-1 or in an independent parallel pathway.

glp-1(bn18ts) is a temperature-sensitive los-of-function mutation (Kodyoianni et al., 1992). Gonads from both glp-1(bn18ts) and vpr-1(tm1411) single mutants are small, but with an important difference. Germ cells in glp-1(bn18ts) gonads differentiate as sperm, whereas germ cells in vpr-1(tm1411) gonads fail to differentiate. If GLP-1 signaling is independent of vpr-1, then vpr-1(tm1411); glp-1(bn18ts) double-mutant gonads should contain sperm. To test this prediction, we grew single- and double-mutant hermaphrodites at 25°C from L1, stained them with DAPI, and performed fluorescence deconvolution microscopy. Characteristic highly condensed sperm chromosomes are not observed in vpr-1(tm1411) gonads (Fig. 6B), but are clearly observed in glp-1(bn18ts) single-mutant (Fig. 6E) and vpr-1(tm1411); glp-1(bn18ts) double-mutant (Fig. 6F) gonads. This result indicates that GLP-1 signaling prevents sperm differentiation in vpr-1 mutants. These genetic data support the model that vpr-1
and glp-1 act in independent genetic pathways to promote germ cell expansion and differentiation.

**Neuronal vpr-1 activity is crucial shortly after embryo hatching**

SS blast cells form during L1 and early L2, but do not complete development until mid to late L4 (Hubbard and Greenstein, 2000; Killian and Hubbard, 2005). To determine the critical time period for vpr-1 activity we used the Q system, a drug-inducible binary gene expression system (Potter et al., 2010; Wei et al., 2012). The QF transcriptional activator binds a 16 bp sequence called QUAS to activate gene transcription (Fig. 7A). A transcriptional repressor called QS blocks QUAS-dependent transcription mediated by QF. The small molecule quinic acid (QA), which is added to plates, inhibits QS repressor activity, thereby activating gene expression. We used the glr-5 promoter to drive QF and QS expression in ∼56 interneurons (Fig. 7A). Seven independent transgenic lines were generated expressing glr-5p::QF, glr-5p::QS and QUASp::vpr-1g in vpr-1(tm1411) mutants (see Materials and Methods). Six lines grew robustly in the presence of QA. All seven lines exhibited minimal QA-independent vpr-1 expression, as indicated by gamete development in a small percentage of transgenic vpr-1(tm1411) hermaphrodites. The Q system was more tightly regulated than the heat shock promoter (data not shown). Providing QA produced functional VPR-1, as evidenced by rescue of the muscle mitochondrial defect (Fig. S3). We selected line 3 for further characterization because it grew very slowly without QA but grew rapidly with QA. An advantage of this line is that sufficient numbers of transgenic vpr-1(tm1411) homozygotes could be generated for staging. Similar results were observed for lines that failed to grow without QA.

Three phenotypic classes were used to quantify the data (Fig. 7B). Class 1 gonads lack germ cell expansion and gamete differentiation, closely resembling nontransgenic vpr-1 mutant gonads. Class 2 gonads contain visible oocytes or an expanded germ cell population, but are sterile with no fertilized eggs in the uterus. Class 3 gonads contain differentiated gametes and fertilized eggs. In the absence of QA, 57% of transgenic vpr-1 mutant gonads were class 1 and 19% were class 3 (Fig. 7C). When transgenic mutant adults (P0) were added to QA plates, their adult F1 progeny contained 17% class 1 gonads and 63% class 3 gonads (Fig. 7C). These class 3 gonads were larger and contained more fertilized eggs than class 3 gonads from worms lacking QA (Fig. 7B). The number of class 2 gonads did not significantly change in the presence or absence of QA. Therefore, inducing vpr-1 expression in head interneurons throughout development is sufficient to promote gonadogenesis.

Next, we exposed worms at different stages of development to QA plates and analyzed their gonads 3 days later. Adding late-stage embryos or fresh hatchlings to QA plates is sufficient to induce a 3-fold increase in class 3 gonad percentage (Fig. 7D). However, adding L2 stage or older worms to QA plates did not affect gonadogenesis. These data suggest that vpr-1 activity is needed shortly after embryogenesis, when QA first becomes available to hatchlings. Maternal vpr-1 mRNA provided in vpr-1(tm1411) embryos is sufficient for gonad induction. As this maternal contribution is not renewable, we considered the possibility that vpr-1 is sufficient transiently, early in development. To investigate this idea further, we provided QA to transgenic vpr-1 mutant adults, let their progeny hatch on QA plates, and then moved the L2 worms to plates lacking QA. We found little difference in class 3 gonad percentage in these experiments compared with controls grown continuously on QA (Fig. 7E). These data support the model that

![Fig. 7. Neuronal vpr-1 expression is sufficient for gonadogenesis early in postembryonic development.](image-url)
vpr-1 activity is crucial for gonadogenesis during a short time period between hatching and the early L2 stage.

**DISCUSSION**

VAPs are broadly expressed proteins that function as secreted signaling molecules and intracellular scaffolds between the endoplasmic reticulum and other cytoplasmic structures (Dong et al., 2016; Han et al., 2013, 2012; Lev et al., 2008; Stoica et al., 2016; Tsuda et al., 2008). The first function often requires VAP synthesis in cells distinct from the effector cells (i.e. a non-cell-autonomous function), whereas the second, housekeeping function requires VAP synthesis only in the effector cell. The extent to which these diverse functions contribute to animal development and physiology is an open question. Here we present evidence in C. elegans that VPR-1 acts as a permissive signal for postembryonic gonadogenesis. Taken together with previous studies, our results support the model that neurons, germ cells, and intestinal cells secrete the MSPd into the pseudocoelom. A key signaling target appears to be somatic gonadal sheath cell precursors, which are essential for germ cell expansion and differentiation during larval development. Below we discuss evidence for this model, along with implications for reproduction and motor neuron diseases.

vpr-1 mutants are maternal effect sterile due to arrested gonadogenesis. We found that germ cells fail to expand and differentiate into sperm or oocytes during larval development. Importantly, the somatic gonad is also affected, as the SS lineages that form the sheath cells and spermatheca do not fully proliferate and differentiate. Genetic mosaic and transgenic overexpression studies indicate that the vpr-1 mutant SS lineage defect is non-cell-autonomous. Previous SS laser ablations showed that these cells are crucial for germ cell expansion and gametogenesis (Killian and Hubbard, 2005; McCarter et al., 1997). By contrast, laser or genetic ablation of germ cells does not appear to impact somatic gonad development (Beanan and Strome, 1992; Kimble, 1981; Sulston et al., 1983). The vpr-1 mutant gonad defect could be largely explained by abnormal SS, or SS precursor, cells. Consistent with this idea, SS blast cells (McCarter et al., 1997) and vpr-1 act in parallel to GLP-1 Notch receptor signaling to regulate germ cell proliferation and differentiation. There are differences between SS-ablated and vpr-1 mutant gonads. In particular, germ cells enter meiotic pachytene following SS ablation and sometimes differentiate into sperm (McCarter et al., 1997), whereas germ cell nuclei in vpr-1 mutants do not show pachytene or sperm chromosomal morphologies. Timing in these experiments could be important. Neuronal vpr-1 expression is sufficient to induce gonadogenesis shortly after embryo hatching, but not after the L1 stage. The SS ablations were performed at the L2-L3 molt (Killian and Hubbard, 2005; McCarter et al., 1997). VPR-1 could also have an important role in the germ line that is independent of somatic gonadal cells. In any event, our results are consistent with the VPR-1 MSPd acting as a permissive signal at least in part on the developing somatic gonad.

Genetic mosaics indicate that zygotic vpr-1 activity is important in the nervous system, germ line and intestine for fertility. Transgene loss in either the AB (primarily neuronal) or P4 (germ line) lineage caused gonads to arrest, although gonad development often appeared more advanced than that of gonads in M–Z~ vpr-1 animals. Intestinal vpr-1 loss also caused sterility in some animals. Gonads from these mosaics produced oocytes but exhibited ovulation defects or other defects consistent with an abnormal somatic gonad. Our mosaics used fosmid DNA containing the entire vpr-1 genomic locus and likely most gene regulatory sequences. Although transgenes are typically silenced in the germ line due to their repetitive nature (Kelly et al., 1997), fosmids are more likely to at least partially escape germline silencing and better reflect endogenous gene expression (Govindan et al., 2009; Yochem and Herman, 2003). However, our mosaic lines lacked sufficient maternal vpr-1 to induce gonadogenesis and therefore provided a zygotic vpr-1 activity readout. Using smaller DNA transgenes to overexpress vpr-1 specifically in neuron subsets, the intestine or germ cells was sufficient to induce gonadogenesis. These transgenes lacked many endogenous gene regulatory sequences and contained promoter fragments that drive high-level expression. Including vpr-1 introns and 3’ UTR in the constructs enhanced fertility in the transgenic lines, allowing for stable propagation in a vpr-1 null background. Based on these data we speculate that, in wild-type hermaphrodites, multiple cell types contribute to a secreted MSPd pool. Neurons, germ cells and maternal vpr-1 mRNA might be particularly important sources early in postembryonic development. As germ cell ablation does not impact somatic gonad development (Beanan and Strome, 1992; Kimble, 1981; Sulston et al., 1983), we consider it likely that there is some redundancy among MSPd secretion cell types. At present, genes that are essential for MSPd processing and secretion have yet to be discovered.

An important missing element to the VPR-1 secretion model is the MSPd receptor, which is presumably expressed on SS blast or precursors cells. The MSPd receptors VAB-1 and CLR-1 are both expressed in gonadal and peripheral tissues (Brisbin et al., 2009; George et al., 1998; Han et al., 2012; Koke et al., 1998). However, these receptors individually do not appear to mediate the (early) role of VPR-1 in gonadogenesis, as vab-1 loss or clr-1 loss does not suppress the vpr-1 null mutant gonadogenesis defect. It is possible that eliminating both receptors together could be suppressive, but the triple mutants are likely to exhibit severe embryonic lethality. We consider it unlikely that muscle relays a metabolic checkpoint signal to the gonad, because suppressing the vpr-1 mutant muscle mitochondrial defect does not trigger gonadogenesis. During adulthood, MSPs secreted by sperm induce oocyte maturation via gonadal sheath cell heteromeric G-protein pathways (Ellis and Stanfield, 2014; Govindan et al., 2006). Extracellular VPR-1 MSPd binds to sheath cells and is sufficient to induce oocyte maturation in the absence of sperm (Tsuda et al., 2008). Therefore, a similar signaling mechanism might also mediate VPR-1 MSPd signaling earlier in development. Additional work is necessary to clarify the mechanism by which VPR-1 induces gonadogenesis.

Why might animal VAPs have such disparate biochemical functions? Animals require signaling mechanisms to coordinate tissue development and physiology. These mechanisms are well conserved in diverse animals, but typically not in unicellular eukaryotes, multicellular fungi or plants (Richter and King, 2013; Srivastava et al., 2010). Ligands and receptors appear to have emerged early in animal evolution through gene duplication, followed by rearrangements and other mutations (Braasch et al., 2009; Markov et al., 2008). In this scenario, the duplication event frees up one gene copy to acquire a new signaling function. The other gene copy continues to perform its original function. However, there might be circumstances when a gene copy retains both functions. We speculate that VAPs are an example of the latter. As the VAP signaling function depends on MSPd cleavage and secretion, it seems likely that the housekeeping function is destroyed. Cells could help overcome this problem by increasing VAP expression, redundancy, or tightly regulating secretion.
The MSPD has functional similarities to testosterone, albeit without the sex-specific features. Both molecules are inducers of gonadogenesis and have metabolic effects on striated muscle (Han et al., 2013, 2012; Kadi, 2008; Wang et al., 2009). The biggest similarity lies in their role in lower motor neuron (LMN) degeneration. AL58 patients contain a F56S substitution in the VAPB MSPD and present with ALS, atypical ALS, or late-onset SMA (Marques et al., 2006; Nishimura et al., 2004). CAG trinucleotide repeat expansion in the androgen receptor causes spinobulbar muscular atrophy (SBMA) (La Spada et al., 1991). A common theme in AL58 and SBMA disorders is the degeneration of LMNs in the spinal cord anterior horn. Recent mouse studies provide compelling evidence that skeletal muscle is crucial to SBMA pathogenesis (Cortes et al., 2014; Katsuno et al., 2012; Lieberman et al., 2014; Monks et al., 2007; Musa et al., 2011). A Cre recombinase strategy to excise polyglutamine-expanded androgen receptor specifically from muscle prevents weight loss, aberrant motor phenotypes, muscle pathology and motor neuropathy, as well as dramatically extends survival (Cortes et al., 2014).

Materials and Methods
C. elegans genetics and strains

C. elegans were maintained at 20°C unless otherwise indicated, and fed with N2 A. coli (Brenner, 1974; Edmonds et al., 2010; Kubagawa et al., 2006). The following strains were used: N2 Bristol (wild type); VC1478 vpr-1(tm1411)/hT2 [bli-4(e937) let-8(q782) qIs48]; glp-1(bn18). Strain construction was performed using PCR, sequencing and phenotypic analyses.

Further work on VAPB and its paralog VAPA could shed new light and SBMA patients could have a significant skeletal muscle aberrant motor phenotypes, muscle pathology and motor neuronopathy, as well as dramatically extends survival (Cortes et al., 2014; Lieberman et al., 2014; Monks et al., 2007; Musa et al., 2011). A Cre recombinase strategy to excise polyglutamine-expanded androgen receptor specifically from muscle prevents weight loss, aberrant motor phenotypes, muscle pathology and motor neuropathy, as well as dramatically extends survival (Cortes et al., 2014).

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To generate transgenic C. elegans, glr-5p::QS-SL2::mCherry::3' UTR-unc-54 (10 ng/µl), glr-5p::QS-SL2::mCherry::3' UTR-unc-54 (50 ng/µl), QUASp::Apes10::vpr-1::SL2::GFP::3' UTR-unc-54 (10 ng/µl) and myo-3p::mito::GFP (30 ng/µl) plasmids were injected into vpr-1(tm1411)/hT2 young adult hermaphrodite gonads. Transgenic lines were selected based on mCherry and myo-3p::mito::GFP expression. Seven independent lines were created. Six responded well to quinic acid (QA) treatment. For treatment, 300 µl 300 mg/ml pH 6.5 QA (Sigma-Aldrich) was mixed with 40 µl M9 buffer and added to NGM plates seeded with NA22 bacteria. All lines exhibited very low vpr-1 expression in the absence of QA, partially rescuing the vpr-1 mutant gonadogenesis defect in a small percentage of transgenic worms. Increasing the glr-5p::QS dosage in the transgenic arrays appeared to limit QA-independent vpr-1 expression.

### Statistics

Two-tailed Student’s t-tests were computed using Excel 2013 (Microsoft) without the assumption of equal variance.

### Acknowledgements

We thank M.A.M. lab members and other colleagues for discussions regarding this work and manuscript comments. Three anonymous reviewers provided valuable criticisms.

### Competing interests

M.A.M. filed a patent application (PCT/US2014/030598) in 2014.

### Author contributions

Methodology: T.C.; Validation: T.C., M.A.M.; Investigation: P.A.C., T.C., J.S., H.D.H., J.V., S.M.H., M.A.M.; Writing - original draft: M.A.M.; Writing - review & editing: S.M.H., M.A.M.; Project administration: P.A.C.

### Funding

This project was funded by the Muscular Dystrophy Association (MDA381893 to M.A.M.) and National Institutes of Health (R01GM08105 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.152207.supplemental

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