The ESCRT-II proteins are involved in shaping the sarcoplasmic reticulum in C. elegans

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ABSTRACT

The sarcoplasmic reticulum is a network of tubules and cisternae localized in close association with the contractile apparatus, and regulates Ca²⁺ dynamics within striated muscle cell. The sarcoplasmic reticulum maintains its shape and organization despite repeated muscle cell contractions, through mechanisms which are still under investigation. The ESCRT complexes are essential to organize membrane subdomains and modify membrane topology in multiple cellular processes. Here, we report for the first time that ESCRT-II proteins play a role in the maintenance of sarcoplasmic reticulum integrity in C. elegans. ESCRT-II proteins colocalize with the sarcoplasmic reticulum marker ryanodine receptor UNC-68. The localization at the sarcoplasmic reticulum of ESCRT-II and UNC-68 are mutually dependent. Furthermore, the characterization of ESCRT-II mutants revealed a fragmentation of the sarcoplasmic reticulum network, associated with an alteration of Ca²⁺ dynamics. Our data provide evidence that ESCRT-II proteins are involved in sarcoplasmic reticulum shaping.

KEY WORDS: ESCRT-II, Sarcoplasmic reticulum, Organelle shape, Caenorhabditis elegans

INTRODUCTION

The sarcoplasmic reticulum is an endomembrane network that regulates Ca²⁺ dynamics and controls the excitation–contraction coupling in striated muscle cells. Sarcoplasmic reticulum comprises cisternae and tubular sections that are specifically responsible for the liberation and reuptake of Ca²⁺, respectively, and is tightly linked to both the sarcomere contractile unit and sarcolemma. Recent data show that sarcoplasmic reticulum is maintained close to the myofilibrils, thanks to molecular links made of the sarcomeric giant protein obscurin, the ankyrin sAnk1.5 and the tropomodulin Tmod3 (Bagnato et al., 2003; Lange et al., 2009; Ackermann et al., 2011; Gokhin and Fowler, 2011). The shape of the sarcoplasmic reticulum is also modified in the absence of the ryanodine receptor (RyR) (Maryon et al., 1998), and a recent work suggests that Trisk95, a Triadin isoform, participates in the organization of the sarcoplasmic reticulum (Fourrest-Lieuvin et al., 2012). Additionally, alteration of the phosphoinositide phosphatase myotubularin MTM1, which leads to human centronuclear myopathies, results in sarcoplasmic reticulum remodeling and disorganization of the contact zone between sarcoplasmic reticulum and sarcolemma (Amoasii et al., 2013).

In eukaryotes, the endosomal sorting complexes required for transport (ESCRT-0 to ESCRT-III) were initially identified as key proteins involved in the sorting of ubiquitylated membrane proteins into intraluminal vesicles during multivesicular bodies maturation (Hanson and Cashikar, 2012; McCullough et al., 2013). ESCRT proteins display additional key cellular functions in membrane remodeling during exosome biogenesis (Colombo et al., 2013), enveloped virus budding (Garrus et al., 2001; Carlton and Martin-Serrano, 2007), cell cytokinesis membrane abscission (McCullough et al., 2013), immunological synapse formation (Choudhuri et al., 2014), plasma membrane repair (Jimenez et al., 2014) and in the biogenesis of the nuclear membrane (Webster et al., 2014; Olmos et al., 2015; Vietri et al., 2015). Interestingly, ESCRT-II, a heterotetramer complex comprising VPS-22/EAP30, VPS-36/EAP45 and two VPS-25/EAP20 subunits, has also been associated with regulation of gene expression (Irion and St Johnston, 2007; Kamura et al., 2001; Jin et al., 2005). Studies of the ESCRT functions during Caenorhabditis elegans development indicate that ESCRT mutant phenotypes are heterogeneous. It is noteworthy that ESCRT proteins are required early during C. elegans embryogenesis (Roudier et al., 2005; Michelet et al., 2009), albeit ESCRT-II mutants have late developmental defects (Djeddi et al., 2012). This observation supports a hypothesis of a different role for ESCRT-II components compared to that of other ESCRTs in C. elegans. Here, we used a combination of imaging and genetic techniques to test the cellular function of ESCRT-II in C. elegans. We present the first direct evidence that ESCRT-II, but not ESCRT-0, ESCRT-I and ESCRT-III complexes, localize to the sarcoplasmic reticulum, and that ESCRT-II and UNC-68/RyR localization at the sarcoplasmic reticulum are mutually dependent. We also show that the depletion of ESCRT-II components results in alterations of sarcoplasmic reticulum shape, Ca²⁺ dynamics and muscular contraction. Overall, this study indicates that ESCRT-II contributes to the maintenance of the sarcoplasmic reticulum architecture and function.

RESULTS

ESCR-T-II proteins localize to muscle sarcomeres

C. elegans ESCRT mutants have heterogeneous phenotypes, which could be explained by the functional pleiotropy of ESCRT genes. However, ESCRT-II mutants display a rather homogenous phenotype with late developmental defects (Djeddi et al., 2012), suggesting a particular role for ESCRT-II components in this organism. To further investigate C. elegans ESCRT-II function, we analyzed the localization of the ESCRT-II proteins VPS-22, VPS-25 and VPS-36. We generated GFP-tagged transgenic strains (Fig. 1A), which could rescue the corresponding null mutants.
Contrary to previously characterized ESCRT proteins (Roudier et al., 2005; Michelet et al., 2009; Djeddi et al., 2012), the ESCRT-II components were enriched in pharyngeal, vulval and body wall muscles (BWM), and were globally detected in punctate structures (Fig. 1B, C). In BWM cells, ESCRT-II puncta were regularly aligned in parallel lines along the main axis of the cells (Fig. 1C). In vulval muscles, the ESCRT-II puncta were both cortical and cytoplasmic, but VPS-22::GFP expression was weaker and VPS-25::GFP was more diffuse. In pharyngeal muscles, ESCRT-II puncta localized at the sites of attachment of actin cables (Fig. 1D, E; Fig. S1D), similar to the localization of the actin attachment protein paxillin/PXL-1 (Warner et al., 2011). We thereafter focused our study on BWMs, which are functionally and structurally similar to mammalian striated muscle (Waterston, 1988; Benian and Epstein, 2011).

**ESCRT-II localizes at the sarcoplasmic reticulum attachment zones**

The sub-cellular localization of ESCRT-II in BWMs was investigated by performing co-localization experiments with muscle cell markers, namely ATN-1/α-actinin, DEB-1/vinculin and actin (Fig. 2A–C; Fig. S2A, B). VPS-36::GFP shows two sites of localization in the sarcoceme. The majority of the signal largely co-localized with ATN-1/α-actinin (Fig. 2B; Fig. S2A) or DEB-1/vinculin (Fig. S2B), suggesting that it is strongly associated with dense bodies (Fig. 2A), the actin attachment structures corresponding to both vertebrates Z discs and costameres. However, VPS-36::GFP was also present in small patches localized between aligned dense bodies, probably apposed to the sarcolemma (Fig. 2B; Fig. S2B). Correlative light-electron microscopy (CLEM) on BWMs confirmed that VPS-36 was associated with both dense bodies and an electron-dense structure that was localized between the plasma membrane and the lattice of contractile filaments (Fig. 2C). This pattern corresponds to the organization of the sarcoplasmic reticulum network within BWMs (Fig. 2A), which forms thin tubular structures along the dense bodies and the plasma membrane (Hammarlund et al., 2000). A similar localization to that of VPS-36 was also detected for the ESCRT-II subunits VPS-22::GFP and VPS-25::GFP (Fig. S2B). The localization of endogenous ESCRT-II was further confirmed using two antibodies directed against VPS-36 (this study) and the whole ESCRT-II complex (Schuh et al., 2015), respectively (Figs 2B and 3). Moreover, the co-localization analysis with the RyR UNC-68, involved in sarcoplasmic reticulum Ca^{2+} release, indicated that ESCRT-II localized to the sarcoplasmic reticulum (Fig. 2B). To further support the link between sarcoplasmic reticulum and ESCRT-II proteins, we analyzed the localization of ESCRT-II in the unc-68(e540) mutant, which exhibits an alteration of the sarcoplasmic reticulum (Maryon et al., 1998; Cho et al., 2000). In unc-68
animals, the subcellular localization of both VPS-36 and VPS-22 was strongly decreased in BWMs (Fig. 2D), albeit unaffected in pharyngeal and vulval muscles (Fig. S2C). This data shows that the localization of ESCRT-II subunits at the sarcoplasmic reticulum is dependent on UNC-68.

To analyze whether the localization to sarcoplasmic reticulum is specific for ESCRT-II complex, we then performed immunostaining using antibodies directed against VPS-27 (ESCRT-0), VPS-23 (ESCRT-I), VPS-20 and VPS-32 (ESCRT-III) (Fig. 3; Fig. S2D). We observed that neither VPS-27, nor VPS-
23 or VPS-32 localized in muscle cells, similar to ESCRT-II subunits. However, VPS-20 (ESCRT-III), which directly interacts with ESCRT-II (Schuh et al., 2015), was also detected at the sarcoplasmic reticulum. These observations indicate that the localization to the sarcoplasmic reticulum is not a common characteristic of all ESCRT components, but specific for ESCRT-II and VPS-20.

The structure of the sarcoplasmic reticulum network is altered in ESCRT-II mutants

To decipher the function of ESCRT-II proteins on sarcoplasmic reticulum, we performed transmission electron microscopy (TEM) and analyzed the subcellular structure of BWM cells in vps-36 (tm1483) and vps-22(tm3200) mutants. In both mutants, the sarcomeric contractile apparatus is only slightly affected, with few misaligned filaments (Fig. S3A). These data confirmed our previous observation that myosin and actin staining appears normal in mutant worms, and indicate that mutations in ESCRT-II do not profoundly alter the ultrastructure of BWM cell contractile filaments.

However, we observed defects in the structure of the sarcoplasmic reticulum in vps-36(tm1483) and vps-22(tm3200) BWMs. TEM pictures revealed that the sarcoplasmic reticulum was significantly fragmented, whereas the size of the dense bodies was unaffected (Fig. 4A; Fig. S3). Quantitative analysis of TEM pictures showed that, in vps-36 and to a lesser extent in vps-22 mutants, the size of sarcoplasmic reticulum tubules parallel to dense bodies was decreased, but their number was higher compared to that in wild-type animals (Fig. 4B,C). The sarcoplasmic reticulum that was associated with sarcolemma was also fragmented in vps-36 animals (Fig. S3E,F).

To support the TEM-based observations, we then analyzed in ESCRT-II mutants two well-characterized sarcoplasmic reticulum components – UNC-68/RyR and SCA-1/SERCA, a Ca\textsuperscript{2+} ATPase pump required to refill sarcoplasmic reticulum. In BWM sarcomeres, the localization of both SCA-1 and UNC-68 was similar to that of the ESCRT-II proteins (Fig. 5A,B). No strong modification of the localization of SCA-1 was detected in vps-36 mutants (Fig. 5A), confirming that a sarcoplasmic reticulum network was still present in vps-36 BWMs. However, the localization of UNC-68/RyR protein was affected in both vps-36 (gk427) and vps-22(tm3200) mutants (Fig. 5B). UNC-68::GFP expression was reduced in the muscle cell, and almost no signal could be observed in the sarcomere.

Together with the TEM analysis, our data indicate that the sarcoplasmic reticulum network is present in ESCRT-II mutants but shows characteristic alterations. Moreover, these experiments indicate that UNC-68/RyR and ESCRT-II are involved in the correct localization of each other in a reciprocal manner at sarcoplasmic reticulum.

ESCRT-II mutants have defects in Ca\textsuperscript{2+} dynamics and locomotion behavior

We used the Ca\textsuperscript{2+} bio-sensor indicator GCaMP3.35 to analyze the Ca\textsuperscript{2+} dynamics in BWMs of vps-36 animals (Schwarz et al., 2012). Time-lapse analyses and quantifications revealed that the intensity of GCaMP3.35 was globally reduced in vps-36 mutants (Fig. 5C,D; Movies 1 and 2). However, the intensity of GCaMP3.35 could locally reach levels similar to those in the control, suggesting that BWMs are still able to produce Ca\textsuperscript{2+} transients, but less efficiently. These results demonstrate that ESCRT-II is essential for the integrity of Ca\textsuperscript{2+} homeostasis in muscle cells. We then asked whether the sarcoplasmic reticulum structure and muscle Ca\textsuperscript{2+} dynamic defects of ESCRT-II mutants result in a modification of the muscle physiology. We therefore analyzed pharyngeal contractions and locomotion of vps-36 (tm1483), vps-36(gk427) and vps-22(tm3200) animals (Fig. 6). Macroscopic observation of vps-36 and vps-22 animals revealed pharyngeal pumping and locomotion defects (Fig. 6A) (data not shown) only during late larval development. To further study and quantify the locomotion defect, we performed an electrotaxis analysis (Manière et al., 2011) to check that vps-22 and vps-36 heterozygous animals had wild-type locomotion (Fig. 6B). The speed locomotion of synchronized populations of heterozygous and homozygous vps-22(tm3200) and vps-36(tm1483) animals was then analyzed during larval development. From the third larval stage, we observed a decreased velocity of both vps-22 and vps-36 mutants compared to that of the controls (Fig. 6C). Additionally, the locomotion defect further increased during development. To check that the locomotion defect of ESCRT-II mutants was due to the function of ESCRT-II in BWMs, muscle-specific RNA interference (RNAi) (Kashyap et al., 2012) was performed against vps-36, vps-22 and vps-32. vps-36 (muscleRNAi), vps-22(muscleRNAi) but not vps-32(muscleRNAi)
animals displayed a reduced velocity compared to the controls (Fig. 6D). This result indicates that ESCRT-II mutants show a reduction of locomotion behavior, which correlates with the sarcoplasmic reticulum ultrastructural and Ca$^{2+}$ dynamic defects observed in muscle cells.

**DISCUSSION**

Altogether, the data in our study describe a previously unidentified function for ESCRT-II components in controlling the maintenance of sarcoplasmic reticulum shape. We present here the first direct evidence that ESCRT-II components VPS-36, VPS-22 and VPS-25 share a specific sarcoplasmic reticulum sub-cellular localization in *C. elegans* muscle cells. The lack of colocalization with ESCRT subunits other than VPS-20 suggests a role that is not linked to endosome maturation. Interestingly, a genomic screen performed in *Drosophila*, indicates that muscle-specific knockdown of Vps25 and Vps36 genes are lethal at the late pupal and pharate stages, respectively, supporting that both genes are involved in muscle morphogenesis and function in *Drosophila* (Schnorrer et al., 2010). These data suggest that ESCRT-II function in muscle cells could be evolutionarily conserved between *Drosophila* and *C. elegans*. Vaccari et al. (2009) have obtained *Drosophila melanogaster* null mutants for most of the genes encoding the four ESCRT complexes. They show that vps22- and vps25-mutant germline cells frequently lack subcortical actin skeleton, with alterations of the plasma membrane integrity. These data suggest that ESCRT-II could be involved in maintaining membrane integrity.

Several clues from the literature could explain how ESCRT-II is recruited to the sarcoplasmic reticulum membrane. The ESCRT-II complex contains several protein–protein-interaction and phosphoinositide-binding domains that could mediate its localization to the sarcoplasmic reticulum membrane. The predicted N-terminal helix structure (H0) of VPS-22 participates in membrane binding, together with the VPS-36 GLUE domain (Im and Hurley, 2008). Importantly, both domains mediate the binding of the whole ESCRT-II complex to phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate phosphoinositide membranes, and therefore could be involved in the localization of ESCRT-II at sarcoplasmic reticulum membranes, where tight regulation of phosphoinositide content could have a key structural role (Amoasii et al., 2013). The VPS-20 ESCRT-III subunit is able to bind to ESCRT-II (Fyfe et al., 2011) in a curvature-dependent manner, and VPS-20 decreases the ESCRT-II affinity for flat lipid bilayers. It is therefore possible that ESCRT-II and ESCRT-II associated with VPS-20 could be localized to flat and curved sarcoplasmic reticulum membrane, respectively. Interestingly, the ESCRT subunit CHMP7 has been recently shown to be involved in endoplasmic reticulum dynamics in yeast (Bauer et al., 2015) and during the process of nuclear envelop reformation in metazoans (Vietri et al., 2015). Noticeably,

![Fig. 4. The structure of the sarcoplasmic reticulum network is altered in vps-36 and vps-22 mutants.](image-url)

(A) TEM analysis of transverse sections of control, vps-36(tm1483) and vps-22(tm3200) animals (left panels). Middle and right panels show a higher-magnification image of one dense body with surrounding sarcoplasmic reticulum (SR) and their corresponding schematics, respectively. Images are representative of the analyses of at least 12 dense bodies (DB). Scale bar: 1 µm. (B) Quantification of the number of sarcoplasmic reticulum tubules associated to the dense bodies (upper panel) and the mean height of the sarcoplasmic reticulum tubules (lower panel) in control, vps-36 and vps-22 animals. *n* indicates the number of measurements, based on 12 to 29 dense bodies from two or three animals. Boxplots illustrate the measure distribution as indicated. *, ** and *** indicate a statistically significant difference between mutant and control animals by a Mann–Whitney-Wilcoxon test with *P*<0.01, *P*<0.001 and *P*<0.0001, respectively. (C) Schematic representation of the sarcoplasmic reticulum defect in vps-36 and vps-22 mutants compared to control animals.
CHMP7 appears to be an in-frame fusion between VPS20-like and VPS25-like domains.

In vitro studies have shown that ESCRT-II together with ESCRT-I can induce deformation of the membrane surface of giant unilamellar vesicles, and one can imagine a similar function for ESCRT-II at the level of the sarcoplasmic reticulum. By analogy with the known functions of ESCRT in regulating membrane topology, three molecular mechanisms could explain how ESCRT-II controls sarcoplasmic reticulum shape. Firstly, VPS-22 has a cationic amphipathic helix that can insert into a membrane and modify its curvature (Im and Hurley, 2008). Secondly, in vitro studies have shown that ESCRT-II can assemble to form clusters of 10–100 molecules on a lipid bilayer and induce a lipid-phase separation, which could modify the membrane and lead to fragmentation (Boura et al., 2012). Thirdly, the partially curved shape of ESCRT-II and its high capacity for membrane interactions...
could mean that it acts as a scaffold for the sarcoplasmic reticulum membrane curvature (Mercker and Marciniak-Czochra, 2015). Depletion of ESCRT-II from the sarcoplasmic reticulum would affect membrane topology and lead to defective maintenance of the sarcoplasmic reticulum. Further studies will be necessary to identify the precise function of the ESCRT-II complex in relation to other proteins involved in sarcoplasmic reticulum shaping.

MATERIALS AND METHODS

C. elegans strains

General methods for the maintenance of C. elegans have been described previously (Brenner, 1974). Worms were cultured on nematode growth medium (NGM) agar plates, plated with Escherichia coli OP50 strain, at 20°C unless otherwise indicated. We used the N2 (Bristol) wild-type strain. The following mutant alleles and transgenic markers were used: HT1593 unc-119(ed3)III; CB2065 dpy-11(e224)unc-76(e911)V; VC947 vps-36(gk427)V/nT1 [qIs51](IV;V); TM1483 vps-36(tm1483)V; TM3200 vps-22(tm3200)III; CB540 unc-68(e540)V; DM7335 pxl-1(ok1483)III; raEx335 [pxl-1a::GFP; rol-6(su1086)]; mvl-12(ok3482)IV; RB674 pqs-19(ok406)I; unc-29(x29) (gift from Jean-Louis Bessereau, Université Claude Bernard Lyon1, CGphiMC, Lyon, France). The mutant strain RD116 vps-36(gk427)V/dpy-11(e224)unc-76 (e911)V was obtained by crossing VC947 with CB2065. The mutant strain RD117 was obtained by crossing TM1483 with CB2065. The mutant strain RD139 vps-22 (tm3200) III/unc-119(ed3)III was obtained by crossing TM3200 (Shohei Mitani, National BioResource Project) with HT1593. RD116, RD117 and RD139 were outcrossed four to six times to N2 before any phenotype analysis. RD323 vps-36(gk427)V/goels3[pmyo-3::GCamP3.35::unc-54–3′utr;unc-119(+)]V was obtained by crossing RD116 with HBR4 goels3[pmyo-3::GCamP3.35::unc-54–3′utr; uncle-68(+)]V.

Transgenesis

The transgenes Ppys-22::vps-22::gfp, Ppys-25::vps-25::gfp and Ppys-36::gfp were constructed using a PCR-based protocol (Hobert, 2002). C. elegans transgenic strains RD163; ppEx163[Ppys-22::vps-22::gfp; rol-6 (su1006)], RD112; ppEx112(Ppys-36::vps-36::gfp; rol-6(su1006)] and RD324 ppEx324[Ppys-25::vps-25::gfp; rol-6(su1006)] were obtained by microinjection of the PCR products of interest associated with a plasmid expressing the rol-6(su1006) phenotypic markers (Mello and Fire, 1995). F1
p-toluidine. Thin sections of 200 nm were cut and collected on 200 mesh acetate in 95% acetone-5% water. Solvent was exchanged for glycol instrument (Leica) and prepared as described previously (Watanabe et al., 2010). Ultrathin sections of 80 nm were cut on an ultramicrotome, and were blocks further infiltrated with 100% EPON and then embedded in fresh freezing in the EMPACT-2 HPF apparatus (Leica Microsystems) and cryo-into M9 buffer with 20% BSA to 200-µm-deep flat carriers, followed by washes in 1 ml 1× BO3, worms were incubated in 1 ml 1× BO3 10% 0.1 M DTT for 15 min. After three washes in 1 ml 1× BO3, worms were incubated in 1 ml 1× BO3 buffer supplemented with 75 ml 30% H2O2 for 15 min. After two washes in 1× BO3 buffer, worms were incubated for 20 min in 1 ml AbB (1× PBS pH 7.4, 0.1% BSA, 0.5% Triton X-100, 1 mM EDTA), washed in 1 ml AbA (1× PBS pH 7.4, 1% BSA, 0.5% Triton X-100, 1 mM EDTA) and incubated with primary antibodies against the following proteins: ATN-1/α-actinin, 1/200 (MH35, a kind gift from Kathrin Gieseler, Université Claude Bernard LyonI, CéphelMC, Lyon, France); DEB-1/vinculin, 1/25 [MH24, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA]; Myo-3/Myxosin, 1/100 (5-6, DSHB); whole ESCRT-II (1/200) and VPS-20 (1/200) (gifts from Anjon Audhya, University of Wisconsin, WI); VPS-36, 1/200 (this study); VPS-23, 1/100; VPS-32, 1/ 100; and GFP, 1/200 (Invitrogen). Incubation was performed overnight at 4°C with constant agitation in the dark. Worms were washed four times, incubated with the appropriate secondary antibodies conjugated with Alexa-Fluor-488 or Alexa-Fluor-568 (1/500; Molecular Probes) for 2 h at room temperature. After four washes with 1 ml of AbB, worms were mounted in DABCO medium.

For actin immunostaining, worms were fixed in 1% formaldehyde in phosphate buffer (0.1 M pH 7.4) for 2 h, permeabilized in 1 ml of frozen acetone for 2 min and washed in 1 ml of 1× PBS. Worms were then incubated with Alexa-Fluor-568–phallolidin, 1/200 (Invitrogen), for 2 h.

Routinely, fluorescent expression and phenotypic analyses were performed with an Axioskop 2 Plus microscope (Zeiss) equipped with differential interference contrast (DIC) Nomarski optics coupled to a camera (CoolSNAP, Roper Scientific). Confocal images were captured on a confocal Leica (SP8) instrument. Image analyses and quantifications were performed with ImageJ.

**Ca2+ imaging using GCaMP**

Genetic crosses were made to transfer the genetically encoded Ca2+ indicator GCaMP3.5 (Schwarz et al., 2011) into the vps-36 (kgy427) background. Heterozygous males vps-36(kgy427)/+ were crossed with homozygous HBR4: goeels3/Pmyo-3::GCaMP3.5::unc-54-3’UTR: unc- 119(+); +. By genetic recombination, vps-36(kgy427)/HBR4/vps-36(+); HBR4 worms were recovered and maintained. GCaMP signal was analyzed in vps-36(kgy427) homozygous progeny and compared with that in heterozygous animals.

For GCaMP analysis, progeny of vps-36(kgy427)HBR4/vps-36(+); HBR4 were immobilized on a 2% agarose pad in 5 µl of M9 buffer between the slide and coverslip. Worms were imaged every 100 ms for 20 s, then removed from the pad for phenotyping. For each acquisition, the fluorescence signal for each frame was monitored in a region of interest (ROI) with ImageJ software (http://rsb.info.nih.gov/ij/). The intensity of GCaMP3.5 was analyzed in three different ROIs defined and used for each analyzed animal: one was located in the ventral muscle cell next to the vulva, one in the BWM cell in the dorsal quadrant just opposite to the previous one, and one in the head either on the ventral or the dorsal side. The fluorescence records were first plotted as fluorescence signal over time. Then, values were plotted as AF/F0 using Excel. F0 is an average baseline value, which is the number of pharyngeal contractions counted under the dissecting microscope.
mean intensity of the 20 lowest fluorescence intensities recorded during the time acquisition (Kerr, 2006). The ΔF/F0 series were compared between control and vps-36 mutant using a Mann–Whitney–Wilcoxon test.

**Statistical analysis**

Data visualization and statistical analyses were performed with the R software (https://www.r-project.org). Unpaired two-tailed Student’s t-test was used to compare the means of two samples for which there were assumptions about normality and similar variances. A Shapiro–Wilk’s test was used to evaluate the normal distribution of the values within each sample. Similar variances were checked with Hartley Fmax test. When samples did not assume equal variances, a Welch’s t-test was performed. In case the sample did not assume normality, a Mann–Whitney–Wilcoxon non-parametric rank test was performed. We did not use statistical methods to predetermine sample size. We did not use any method of randomization to select data. The investigators were not blinded to allocation during experiments and outcome assessment. Statistics source data can be obtained upon request.

**Acknowledgements**

The authors would like to thank M. Jospin, K. Gieseler and C. J. Merrifield for critical reading of the manuscript. We are grateful to V. Scarcelli for technical help. We also thank Shohei Mitani (National Bioresources Project for the nematode, Japan) for providing vps-22(tm3200) and vps-36(tm1483) mutant alleles. J. Aythys for giving us antibodies against the whole ESCRT-II complex and VPS-20, Maëlle Jospin (Université Claude Bernard Lyon1, CGPhIMC, Lyon, France) for giving us the GCaMP3.35 strain, Kenneth R. Norman (Albany Medical College, NY) for sending us antibodies against the whole ESCRT-II complex and VPS-20, Maelle Jospin and Shohei Mitani (National Bioresource Project for the nematode, Japan) for providing ESCRT-II negative C. elegans. We are grateful to V. Scarcelli for technical help. We also thank Shohei Mitani for providing ESCRT-II negative C. elegans.

**Funding**

The Legous group is supported by the Agence Nationale de la Recherche (project EAT) [grant number ANR-12-BSV2-018] and the Association pour la Recherche sur le Cancer [grant number FSI20111203826].

**Supplementary information**

Supplementary information available online at http://jcs.biologists.orglookup/suppl/doi:10.1242/jcs.178467/-/DC1

**References**


Supplementary figures
Fig. S1. VPS-36::GFP and VPS-22::GFP rescue null vps-36(gk427 or tm1483) and vps-22(tm3200) mutants respectively. (A) Light microscope images of 4 days old wild-type animal (adult stage, upper panel), mutant animal (blocked at the late L4 stage, middle panel) and mutant animal rescued by transgene expression (adult stage, lower panel). Bar, 100 µm. (B) Percentage of fertile animals amongst the F1 progeny and mean number of progeny of control, vps-22(tm3200), vps-36(gk427 or tm1483) mutants and mutant animal rescued by transgene P_{vps-22::vps-22::gfp} and P_{vps-36::vps-36::gfp}, error bars are SD and n is the number of animals. (C) PCR genotyping of wild-type animals, heterozygous and homozygous vps-22(tm3200) and vps-36(tm1483) mutants and rescued mutant animals for the presence of wild type and deletion mutant alleles of vps-22 and vps-36 genes. MW, DNA marker indicated in base-pairs. (D) Confocal images of the pharyngeal terminal bulb from worms expressing VPS-22:GFP, VPS-25::GFP and PXL-1a::GFP (green signal) and immunolabelled for F actin filaments (red signal). The insets show higher magnification of the selected area of the pharyngeal terminal bulb. Arrows point to the actin attachment structure localized to the basal membranes of pharyngeal cells, arrowheads point to the signal localized between the F actin bundles. Bar, 5 µm.
**Fig. S2. Subcellular localization of ESCRT proteins in control and unc-68 animals (A)**

Split images corresponding to Fig.2B. Single confocal sections of BWM expressing either VPS-36::GFP (green) and stained with dense body markers α-actinin (red) or UNC-68::GFP (green) and stained with antibody directed against the whole ESCRT-II complex (red). White arrow points to VPS-36::GFP signal localized between DB and just below the sarcolemma. Bar, 5 µm.

**Fig. S2. Subcellular localization of ESCRT proteins in control and unc-68 animals (B)**

Colocalization of the three ESCRT-II subunits. Confocal images of body wall muscle cells from worms expressing VPS-22::GFP, VPS-25::GFP and VPS-36::GFP (green signal) and
immunolabelled for either F actin filaments or vinculin (red signal). Merged images indicate a similar localization of VPS-36, VPS-22 and VPS-25 in the I band and at the interface between the I and A bands. Bar, 5 µm. (C) Confocal images of worms pharyngeal muscle cells expressing either VPS-36::GFP or VPS-22::GFP in control and unc-68 mutants. Bar, 5 µm. (D) Larger field of images corresponding to Fig.3. Immunostaining of wild-type animals with antibodies directed against ESCRT subunits (green signal) and myosin (red signal). Upper panels: α-VPS-36, α-whole ESCRT-II and α-VPS-20 antibodies labelled regions corresponding to the I band and the interface between I and A bands. Insets are split images of higher magnifications of regions labelled with white rectangle showing both XY and YZ views for both signals. Lower panels show immunostaining of BWM cells and epidermis with α-VPS-27(ESCRT-0), α-VPS-23(ESCRT-I) and α-VPS-32(ESCRT-III) antibodies. Green signal corresponding to ESCRT subunits is not or barely detectable in BWM cell whereas green dotted structures corresponding to endosomes are observed in the epidermis. Insets are higher magnification of the region within white rectangle showing XY and YZ views. Bar, 5 µm.
Fig. S3. Structure of BWM cells in *vps-36(tm1483)* and *vps-22(tm3200)* mutants. (A) Representative TEM images of cross sections of the body wall muscle sarcomeres showing rare structure alteration. Bar, 0.5 µm. (B) Schematic representation of the SR structure (white tubules) next to DB (in black) or sarcolemma (grey bar) in wild type and *vps-36(tm1483)* L4 animals. Quantitative analyses are done by measuring DB height (DBh) and SR height (SRh). (C-D) Boxplots of DB height (C) and SR height relative to DB height (D) from wild type, *vps-36(tm1483)* and *vps-22(tm3200)* L4 animals. There is no statistical difference between means; n is the number of analyzed DB from 2 or 3 animals. (E-F) Boxplots of length (E) and number (F) of sarcolemmal membrane SR tubules; n is the number of analyzed DB from 2 or 3 animals. ***, indicates a statistically significant difference by a Mann-Whitney-Wilcoxon test (P<0.01). (G) TEM image showing an extreme case of SR remodelling, only observed in *vps-36* mutant. Bar, 0.5 µm.
Movies

**Movie 1**: Ca$^{2+}$ dynamics in BWM of wild type animal. Time lapse epifluorescence microscopy images showing wild-type animals expressing GCaMP3.35 under the control of *myo-3* promoter in BWM during worm locomotion. Frames have been collected every 100 ms for 20 seconds and displayed with ImageJ Fire LUT. The brightness of the signal reflects the calcium release in body wall muscle cell.
Movie 2: Ca$^{2+}$ dynamics in BWM of vps-36(gk427) mutant. Time lapse epifluorescence microscopy images showing homozygote vps-36 (gk427) mutant expressing GCaMP3.35 under the control of myo-3 promoter in BWM during worm locomotion. Frames have been collected every 100 ms for 20 seconds and displayed with ImageJ Fire LUT. The brightness of the signal reflects the calcium release in body wall muscle cell.