RAL-1 controls multivesicular body biogenesis and exosome secretion

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Exosomes are secreted vesicles arising from the fusion of multivesicular bodies (MVBs) with the plasma membrane. Despite their importance in various processes, the molecular mechanisms controlling their formation and release remain unclear. Using nematodes and mammary tumor cells, we show that Ral GTPases are involved in exosome biogenesis. In Caenorhabditis elegans, RAL-1 localizes at the surface of secretory MVBs. A quantitative electron microscopy analysis of RAL-1–deficient animals revealed that RAL-1 is involved in both MVB formation and their fusion with the plasma membrane. These functions do not involve the exocyst complex, a common Ral guanosine triphosphatase (GTPase) effector. Furthermore, we show that the target membrane SNARE protein SYX-5 colocalizes with a constitutively active form of RAL-1 at the plasma membrane, and MVBs accumulate under the plasma membrane when SYX-5 is absent. In mammals, RaA and RaB are both required for the secretion of exosome-like vesicles in cultured cells. Therefore, Ral GTPases represent new regulators of MVB formation and exosome release.

Introduction

Most cells secrete extracellular vesicles (EVs), which are released outside of the organism or within internal fluids. When EVs are taken up by a distant cell, their content (proteins, mRNAs, and microRNAs) can induce a cellular response (Raposo and Stoorvogel, 2013). Over the last decade, EVs have been implicated in a growing number of processes, such as signal transduction during development, extracellular matrix generation, host–pathogen communication, and tumor–stroma interactions (Sabatier and Saurin, 2013), including the PLD2–syntenin–ALIX axis (Ghossoub et al., 2014), whereby four complexes control the inward budding of the MVB limiting membrane, leading to ILV formation. The second type consists of mechanisms unrelated to the ESCRT machinery, such as the ceramide pathway (Trajkovic et al., 2008).

Thus far, eight different RAB GTPases have been linked to exosome secretion (RAB-2B, RAB-5A, RAB-7, RAB-9A, RAB-11, RAB-27A, RAB-27B, and RAB-35). Among them, RAB-27 is the best characterized (Savina et al., 2005; Hsu et al., 2010; Ostrowski et al., 2010; Baietti et al., 2012), although the precise mechanism of action of these GTPases remains elusive (Kowal et al., 2014). In addition, the SNARE proteins VAMP-7 and YKT-6 are required for exosome release (Fader et al., 2009; Gross et al., 2012; Hong and Lev, 2014) and act...
at a step that remains to be defined. Finally, we found that the V0 complex of the H+-vacular ATPase promotes exosome secretion by epithelial cells in the nematode Caenorhabditis elegans independently of its role as a proton pump (Liégeois et al., 2006). Indeed, mutations in VHA-5, the largest subunit of the V0 complex, induce abnormally enlarged MVBs to accumulate, suggesting that this complex controls the final steps of MVB docking and fusion with the plasma membrane.

Here, we first identify C. elegans RAL-1 as a new regulator of MVB formation and exosome secretion. We show that RAL-1 acts independently of its common effector, the exocyst, but through the t-SNARE SYX-5 at the plasma membrane. Furthermore, we demonstrate the conservation of RAL GT-Pase function in mammals.

Results and discussion

RAL-1 and the exocyst are required for alae formation

The C. elegans cuticle is a highly organized extra-cellular matrix mainly composed of cross-linked collagens, insoluble glycoproteins, and lipids, which is renewed at the end of each larval stage (Page and Johnstone, 2007). Two types of epithelial cells located below the cuticle, the Hyp and seam cells, secrete cuticular components. We previously showed that exosomes are secreted by epidermal Hyp cells and contribute to the formation of a specific cuticular structure, the alae (Fig. 1 A; Liégeois et al., 2006). The epithelial seam cells located under the alae also contribute to alae formation. To identify new genes required for exosome secretion, we conducted a RNAi-based screen for alae defects. We screened over a thousand genes predicted or previously linked to vesicular trafficking in C. elegans (Frand et al., 2005; Balklava et al., 2007; Kinchen et al., 2008), as well as all kinases and phosphatases for which a RNAi-inducing clone was available. We identified 73 genes affecting alae formation (Fig. 1 B and Table S1). Although some of them might indirectly affect alae formation (e.g., by impairing seam cell division), several others encode homologues of proteins found to affect exosome biogenesis in mammalian cultured cells (ESC RT components; RAB GT-Pases RAB-2, RAB-11, RAB-27, and RAB-35; Savina et al., 2005; Hsu et al., 2010; Ostrowski et al., 2010; Colombo et al., 2013).

Interestingly, we found that loss of the GT-Pase RAL-1, which plays key roles in secretion in various models (Kawata et al., 2008; Lopez et al., 2008), induces alae defects (Fig. 1 C). We confirmed the RNAi results using a previously characterized null ral-1 mutant, ral-1(tm5205) (Armenti et al., 2014), which displayed severe alae defects in 94% of the animals (Fig. 1 C, Table S1). To determine whether the GT-Pase activity of RAL-1 is involved in alae formation, we expressed constitutively active (CA; G23V) and dominant negative (DN, G23A) RAL-1 (Fig. 2 E) at the external surface of MVBs (Fig. 2, G–G’). Consistently, ral-1(tm5205) null mutants, light MVBs that are likely to originate (Liégeois et al., 2006), dark MVBs (putative intermediate toward the lysosomes), and endolysosomes (Fig. S2). We quantified the density of each of these organelles (number per epidermal cell surface), their distance to the apical plasma membrane, their diameter, and the number and diameter of ILVs (Fig. 3 A). Strikingly, in ral-1(tm5205) null mutants, light MVBs were severely affected: (a) their density was drastically reduced (Fig. 3 B), (b) their mean size was increased (Fig. 3, D and E), and (c) the number of ILVs per MVB was decreased (Fig. 3, D and F). Consistent with a decrease in MVB density, that of VHA-5 fluorescent puncta also decreased in transgenic animals homozygous for ral-1(tm5205) compared with heterozygotes (Fig. 3 C). These results suggest that RAL-1 could either promote MVB formation or inhibit MVB degradation through the Pase activity is required.

In addition to RAL-1, we found four members of the exocyst complex in our screen (Fig. 1, B, D, and E; and Table S1). The exocyst has been shown to control the secretion of different types of vesicles downstream of RAL in many species, including C. elegans (Brymora et al., 2001; Moskalenko et al., 2002; Sugihara et al., 2002; Armenti et al., 2014). We confirmed the screen results, using previously characterized mutants for five of the eight exocyst subunits: sec-3, sec-5, sec-8, exoc-7, and exoc-8 (Jiu et al., 2012; Armenti et al., 2014). Indeed, all of them displayed alae defects, suggesting that they could affect exosome secretion (Fig. 1, E, Fig. S1, and Table S2).

RAL-1 directly controls MVB formation and exosome secretion

We first analyzed RAL-1 localization in C. elegans, showing that it has a punctate distribution in the epidermis (Fig. 2 A). Because no purely specific marker of the exosomal pathway has been identified thus far in any system, we used VHA-5, a V-ATPase component found on MVBs and at apical plasma membrane foldings called membrane stacks (Liégeois et al., 2006), as a nonexclusive marker of the exosomal pathway. We found that almost 100% of RAL-1 apical puncta colocalize with VHA-5 in the epidermis (Fig. 2, A and D), suggesting that it could localize at the surface of MVBs.

To assess whether the GT-Pase activity of RAL-1 influences its localization, we analyzed the localization of RAL-1 mutants within the epidermis. We observed that the GTP-bound form RAL-1(CA) did not colocalize with VHA-5 as well as the WT form RAL-1(WT) or the GDP-bound form RAL-1(DN) (Fig. 2, A–D). This suggests that RAL-1 needs to cycle between GTP- and GDP-bound forms to properly localize in the epidermis. To establish that RAL-1 localizes to MVBs in the epidermis, we used the recently developed APEX tag, which catalyzes the local oxidation of DAB into an electron dense deposit visible by electron microscopy (Martell et al., 2012). After chemical fixation and DAB treatment, electron microscopy revealed a specific staining of animals expressing APEX-tagged RAL-1 (Fig. 2 E) at the external surface of MVBs (Fig. 2, G–G’). Collectively, our results clearly demonstrate that RAL-1 can localize at the surface of MVBs.

To determine how RAL-1 could affect MVBs and exosome secretion, we used a systematic quantitative analysis of MVBs by electron microscopy after high-pressure freezing (HPF; Fig. 3 A). We considered three types of compartments: light MVBs from which exosomes are likely to originate (Liégeois et al., 2006), dark MVBs (putative intermediate toward the lysosomes), and endolysosomes (Fig. S2). We quantified the number density of each of these organelles (number per epidermal cell surface).
lysosomal pathway. We favor the first possibility, because the density of dark MVBs and endolysosomes also decreased in ral-1(tm5205) mutants compared with controls (Fig. S2). Thus, we suggest that the entire pathway from MVBs to lysosomes was affected, and that RAL-1 functions early in the process of MVB formation and generally in ILV biogenesis.

To examine whether RAL-1 can also control later steps of MVB fate, we used RNAi to induce a mild depletion of ral-1, which induced lighter alae defects (Fig. 1 C) and reduced ral-1 transcripts by almost 60% based on quantitative RT-PCR assays (Fig. S1C). We found that, in this case, the density of MVBs was increased compared with either WT or control(RNAi) animals (Fig. 3 B and Fig. S2). The discrepancy between the null mutant and the RNAi phenotype was confirmed using a VHA-5::RFP integrated line. In the total absence of ral-1, the density of VHA-5 puncta decreased by 30%, whereas after depletion by RNAi, it remained comparable to the control (Fig. 3 C). Furthermore, electron microscopy analysis showed that MVBs were closer to the apical plasma membrane in ral-1(RNAi) animals compared with both controls (Fig. 3 G and Fig. S2N). A careful analysis of the MVBs revealed that 45% of them have a direct connection with the apical plasma membrane (ranging from a single link to a hemifusion diaphragm; Fig. 3 I), compared with 9% in WT and 11% in control(RNAi) animals (Fig. 3 H and Fig. S2 O). Therefore, we suggest that when RAL-1 becomes limiting, MVB fusion with the apical plasma membrane is hampered, likely because additional fusion factors are not recruited, but membrane attachment remains possible. Altogether, our data show that RAL-1 affects MVBs at different steps: in their formation and in the last steps before exosome secretion.

To directly assess the effect of RAL on exosome secretion (which is not possible in our system, because the cuticle prevents their collection), and to determine whether RAL function is conserved throughout evolution, we analyzed the role of the two ral-1 homologues in mammals, RalA and RalB. We first purified

Figure 1. **RAL-1 GTPase or exocyst deficiency induces alae defects.** (A) *C. elegans* epidermal cells contain MVBs, which can fuse with the apical plasma membrane and liberate exosomes. These exosomes are integrated in the cuticle and contribute to the formation of the alae. (B) An RNAi-based screen identified 73 genes required for alae formation. (C) Disruption of ral-1 by RNAi, or by the null allele ral-1(tm5205), leads to alae defects. The number of animals is shown at the top of the graph. **, P < 0.05; ***, P < 0.01. (D) Schematic representation of the exocyst complex involved in plasma membrane attachment of secretory vesicles. Subunits found in the screen are in black. (E) Alae defects observed after disruption of several members of the exocyst complex in mutants or by RNAi.
the EVs secreted by 4T1 mammary tumor cells by differential centrifugation. Combining electron microscopy and Western blot analysis, we observed that they display an exosomal size (mean diameter $84 \pm 0.8$ nm, $n = 2,386$ vesicles; Fig. 3, J and J″) and are enriched in four exosomal markers (Fig. 3 L). 4T1 cells knocked down with shRNA for either RalA or RalB (Fig. S1 D) secreted significantly fewer exosome-like vesicles than cells expressing a control shRNA (Fig. 3, K–M). This directly demonstrates that, like in *C. elegans*, the Ral GTPase is required for secretion of exosome-like vesicles in mammals. The precise mechanism of action of RalA and RalB remains to be determined in mammals.

**RAL-1 localizes at the surface of MVBs.** (A–D) WT (RAL-1(WT); A) and dominant negative (RAL-1(DN); C) versions of RAL-1, but not the constitutively active version (RAL-1(CA); B), colocalize fully with VHA-5 in the epidermis at the time of alae formation, as shown in the quantification (D). In D, the numbers inside the bars indicate the number of puncta (number of animals). (E–H) APEX::RAL-1(DN) shows DAB staining both at apical membrane stacks (E and F, arrowheads) and at the external surface of MVBs (E and G–G″, arrows). Animals expressing no APEX tag treated with DAB show no staining (H). The star indicates a MVB. APM, apical plasma membrane.

**RAL-1 controls exosome secretion independently of the exocyst**

To assay whether RAL-1 and the exocyst act together, we analyzed the properties of MVBs present in null mutants of two exocyst subunits, *sec-5(pk2358)* and *sec-8(ok2187)*. In contrast with the situation observed in *ral-1(tm5205)* null mutants, we found that the density of MVBs in these mutants was similar to that in control animals (Fig. 4 A). Occasionally, we observed some MVBs attached to the plasma membrane, indicating that this attachment occurred independently of the exocyst tethering complex (Fig. 4 B and Fig. S2 O). In addition, we found an
Figure 3. RAL-1 affects different steps of exosome secretion. (A) Quantitative electron microscopy analysis of MVBs in epidermal cells: (1 and 2) density (1) and diameter (2) of MVBs, (3) number of ILVs per MVB, (4) ILV diameter, and (5) distance between MVB and the APM. (B) MVB density is decreased in ral-1(tm5205) compared with the WT and is increased in ral-1(RNAi) compared with control(RNAi). (C) The density of VHA-5::RFP puncta is decreased in ral-1(tm5205) compared with the WT but is unaffected in ral-1(RNAi). (D–F) In ral-1(tm5205) mutants, MVBs have an abnormal size (E) and ILV content (F). (G) In ral-1(RNAi) animals, 57% of MVBs are within 50 nm of the apical plasma membrane, compared with 20% in control animals. (H and I) Two MVBs in proximity of the apical plasma membrane from control (H) and ral-1(RNAi) (I) animals. MVBs from ral-1(RNAi) animals can form a hemifusion diaphragm (I) with the apical plasma membrane. (J and J’) EVs purified from 4T1 mammalian cells and observed by electron microscopy. (K) Depletion of either RalA or RalB by shRNA leads to a decrease in the number of EVs observed by electron microscopy compared with control shRNA (P < 0.0001 between sh control and either sh RalA or sh RalB; pool of four independent purifications, Mann-Whitney test). (L and M) Western blot of cell lysates and secreted EVs. One representative experiment (L) and pooled quantification (M) of four independent purifications (P < 0.03 between sh control and either sh RalA or sh RalB for each marker, Mann-Whitney test). Numbers in or above the bars indicate the number of animals (C), MVBs (E, F, and M) or fields (K) analyzed. APM, apical plasma membrane; CL, cell lystate; Cu, cuticle; Cy, cytoplasm; MVBm, MVB outer membrane. Errors bars, SEM.
accumulation of small vesicles (<100 nm diameter) under the plasma membrane of sec-5 and sec-8 mutants (Fig. 4 C), which are likely to be exocytic vesicles unable to attach to the plasma membrane, according to the known functions of the exocyst.

To determine whether a complete lack of the exocyst would mask its requirement at other steps in MVB biogenesis, we used a mild depletion of two exocyst subunits. We found that depletion of sec-8, but not sec-15, by RNAi leads to a significant increase in MVB density compared with either WT or control(RNAi) animals, in an extent similar to that observed in ral-1(RNAi) animals (Fig. 3 A and Fig. S2). However, after RNAi against sec-8 or sec-15, the MVBs were not enriched in the vicinity of the apical plasma membrane, in contrast with what we observed after ral-1(RNAi) (Fig. S2 N).

Finally, we investigated the localization of two exocyst members, one on the target membrane side (SEC-8) and the other on the vesicle side (SEC-15), using integrated transgenic lines, which were previously shown to rescue mutant phenotypes and thus be functional (Armenti et al., 2014). Interestingly, although both proteins are expressed in the epidermis with a punctate localization (Fig. 4, D and E), neither SEC-8 nor SEC-15 significantly colocalized with VHA-5, whether in larval or adult animals (2%–5% colocalization in >50 animals, two independent strains in each case).

Altogether, the fact that exocyst depletion leads to MVB phenotypes different from RAL-1 depletion, added to the absence of colocalization between the exocyst and VHA-5, argues that RAL-1 and the exocyst function independently in alae formation.
We propose that the exocyst is not directly involved in tethering MVBs at the plasma membrane. Thus, the alae defects observed in exocyst mutants reflect either an indirect effect on exosome secretion or a different secretory function, independent of exosome secretion.

**Syntaxin-5 is involved in the fusion between the MVB membrane and the plasma membrane, downstream of RAL-1**

We next wanted to elucidate the molecular mechanisms acting downstream of RAL-1 to allow MVB fusion with the apical plasma membrane and exosome secretion. In our screen, we identified **syx-5**, a gene belonging to the syntaxin family (Table S1), whose closest mammalian homologue, syntaxin-5 (41% identity with SYX-5), is a t-SNARE mediating vesicle fusion in anterograde ER-Golgi trafficking (Hardwick and Pelham, 1992; Malsam and Söllner, 2011). Accordingly, we found that **SYX-5** localizes in large round structures potentially corresponding to the epidermal Golgi apparatus. In addition, we observed more discrete puncta localizing at the most apical side of the epidermis (Fig. 5A). These puncta occasionally colocalized with VHA-5 (Fig. 5B). To determine whether **SYX-5** could function downstream of RAL-1, we assessed its localization with RAL-1(CA) and RAL-1(DN) mutant forms. We found that **SYX-5** small apical puncta partially colocalize with RAL-1(CA), but not with RAL-1(DN) (Fig. 5, C–E). Thus, an active form of **RAL-1** could activate or recruit **SYX-5** at the level of the apical plasma membrane to promote MVB fusion.

To further address the function of **SYX-5**, we generated two **syx-5** mutants using CRISPR-Cas9 technology (Dickinson et al., 2013). We targeted the SNARE domain of **SYX-5**, the characteristic coiled-coil motif responsible for bringing opposing membranes together and catalyzing their fusion (Hong and Lev, 2014). We recovered two alleles, **syx-5**(mc50) (in-frame deletion of two amino acids, including a methionine conserved throughout evolution) and **syx-5**(mc51) (frameshift adding 17 ectopic amino acids and a premature stop codon; Fig. 5F). Both mutant alleles induced a premature larval arrest, likely because of an absence of molting, precluding a study of the role of **SYX-5** in alae formation in adults. However, because **L1** stage larvae also have alae, we tested whether **syx-5** alleles could affect their formation. We found that 100% of **syx-5** L1 mutants have absent or defective alae (Fig. 5G and Fig. S1). This defect was partially rescued by the expression of a **WT** version of **SYX-5** fused to GFP (Fig. S1 and Table S2). In addition, overexpression in **WT** animals of **syx-5** or mutant **SYX-5** forms induced alae defects at the adult stage (Fig. S1 and Table S2). Finally, electron microscopy analysis on **L1 syx-5**(mc51) larvae showed that 62% of the MVBs present in the epidermis are within 50 nm of the apical plasma membrane (77 MVBs in three animals), compared with 24% in **WT** larvae (67 MVBs in four animals; Fig. 5H). Accordingly, the mean distance between MVBs outer membrane and the apical plasma membrane is reduced in **syx-5**(mc51) mutants (68 ± 14 nm) compared with **WT** animals (152 ± 30 nm; P < 0.01, Student’s t test). This result suggests that in the absence of **syx-5**, the MVB outer membrane can no longer fuse with the plasma membrane.

Our work demonstrates a new role for the Ral GTPase in exosome secretion in mammals and nematodes. It further establishes this GTPase as an important player in cell–cell communication, because RalA was previously shown to promote the formation of tunneling nanotubes (Hase et al., 2009). Our data collectively suggest a model (Fig. 5I) whereby **C. elegans** RAL-1 acts both at the initial step of MVB formation and at the late stage of fusion between the MVB membrane and the plasma membrane. One possibility could be that RAL-1 recruits different effectors involved in ILV budding or membrane fusion (Gentry et al., 2014). In particular, Arf6 and PLD are known RalA targets (Luo et al., 1998; Bhattacharya et al., 2004; Vitale et al., 2005; Corrotte et al., 2010) that were recently shown to modulate ILV budding through Alix–syntenin and, as a consequence, exosome secretion (Laulagnier et al., 2004; Trajkovic et al., 2008; Strauss et al., 2010; Ghossoub et al., 2014). Whether RAL-1 functions with the Alix–syntenin pathway to generate MVBs remains to be determined; an RAL-1–independent mechanism is likely to exist because MVBs still contain ILVs in its absence. One important finding of this study is that RAL-1 functions independently of the exocyst complex in MVB tethering and fusion at the plasma membrane. Instead, we suggest that it recruits a yet-to-be-identified tethering factor or complex, as well as the syntaxin SYX-5 at the site of fusion. How the V0-ATPase (including VHA-5) contributes to membrane fusion together with syntaxins remains to be investigated. Another important finding of this study is that the partial loss of RAL-1 tends to block MVBs attached to the plasma membrane and is sometimes locked in a state resembling hemifusion. We speculate that RAL-1 might respond to the local concentration of SNA RE complexes required to promote fusion (Xu et al., 2005) or orchestrate their action (Hernandez et al., 2014). Alternatively, RAL-1 could be more directly involved in pore fusion resolution by recruiting important fusion partners acting downstream of SNA RE complexes, such as VHA-5. Because MVBs are large flat structures compared with small vesicles, which are likely to oppose a higher energy barrier to fusion (Hernandez et al., 2014; Risselada et al., 2014), and because their ILV content presumably requires a larger fusion pore to be released compared with simple peptides, the final step of MVB fusion could require a tight regulation involving several proteins.

**Materials and methods**

**Strains and CRISPR/Cas9**

Strains were propagated and handled as described previously (Brenner, 1974). A complete list of mutants and fluorescent reagents is presented in Table S3. All mutants were characterized previously (Jiu et al., 2012; Armenti et al., 2014), except **syx-5**(mc50) and **syx-5**(mc51) mutants, which were obtained using CRISPR/Cas9 technology, based on the protocol described by Dickinson et al. (2013). The SNARE coding domain of **syx-5** was targeted using the single guide RNA (sgRNA: 5′-GGATCTCT CAATTTGTCGCCATGG-3′) site present on the reverse DNA strand and covering a restriction site for the NcoI enzyme. The **syx-5** sgRNA sequence was introduced into the pPD162 plasmid by PCR (Dickinson et al., 2013). A DNA mix containing the following plasmids was injected in adult animals: pPD162 (Cas9-sgRNA, 50 ng/µl), PGBH (proB–;mcCherry, 10 ng/µl), PcFI104 (mpyo-3–;mcCherry, 5ng/µl), PCF90 (mpyo-2–;mcCherry, 2.5 ng/µl), and pBluescript (80 ng/µl) (Dickinson et al., 2013). 384 F1 animals carrying the injection markers were screened by PCR followed by NcoI digestion, leading to the identification of two **syx-5** mutant alleles. Both alleles displayed similar phenotypes of larval arrest and were balanced to be maintained as heterozygous. We eliminated the co-injection markers by selecting nonfluorescent animals.
Cloning and transgenesis

All plasmids were generated by PCR, with digestion by restriction enzymes or Gibson cloning (NEB), using a Phusion High-Fidelity DNA polymerase (Thermoscientific). RAL-1(CA) (G23V) and RAL-1(DN) (S28N) forms were generated based on previously reported single amino-acid mutations in the GTPase pocket of RAL in diverse species (Jiang et al., 1995; Ohta et al., 1999; Goi et al., 2000; Moskalenko et al., 2002). Specific expression in the epidermal cells of transgenic animals was obtained using the dpy-7 promoter (Gilleard et al., 1997).

RNAi and alae defects screen

RNAi by feeding was performed using standard procedures, with 100 µg/ml ampicillin/1 mM IPTG (Sigma-Aldrich) using clones from

Figure 5. SYX-5 controls MVB fusion with the apical plasma membrane. (A) In the epidermis, SYX-5 localizes in both large cytoplasmic puncta and smaller apical puncta. (B) SYX-5 displays some colocalization with VHA-5 in the epidermis at the time of alae formation. (C–E) SYX-5 colocalizes with RAL-1(CA) (C), but not with RAL-1(DN) (D), as revealed by quantification (E). The scale bars in B apply to C and D. The numbers inside the bars indicate the number of puncta (number of animals). (F) Generation of two mutant alleles for syx-5 using CRISPR/Cas9 technology. (G) Homozygote mutants for syx-5(mc51) show alae defects at the L1 stage. (H) Electron micrographs of two MVBs showing attachment to the apical plasma membrane in syx-5(mc51) mutant larva. (I) Model for the role of RAL-1 and SYX-5 in exosome biogenesis (see text). Errors bars, SEM.
and endolysosome was taken at high magnification. Measurements of surfaces and lengths were performed using ImageJ software.

For photooxidation of the APEX tag, animals were fixed in 2.5% glutaraldehyde, cut in two pieces, and fixed on ice for at least 2 h. After they were rinsed five times in cold cacodylate buffer (0.1 M, pH 7.4), they were incubated for 30 min in blocking solution (10 mM KCN, 5 mM aminotriazole, and 50 mM glycine in cacodylate buffer) on ice. Photooxidation was performed with freshly prepared DAB (1 mg/ml) combined with 10 mM H2O2 in blocking buffer on ice. Animals were rinsed five times in cacodylate buffer, embedded in epon resin, and processed for thin sectioning and observation. Control animals were processed similarly, but without the DAB treatment.

Mammalian cells and EV purification

4T1 mammary tumor cells were chosen for their ability to secrete exosomes in a rab-27 dependent manner (Bobrie et al., 2012). For stable inhibition of RabA or RabB expression, cells infected with shRNA expressing lentiviruses were selected and maintained in medium containing 1 µg/ml puromycin. Sequences for shRNA (mRabA: 5'-CCGGGTGTCAGTCGATCTGAATGATCGAGATCTAG ATGTCGATCTGACATTTTTT-3'; mRabB: 5'-CCGGCTGGTACCT TCAACAGTTCATCTGAGTAGCCCTTGTAAGTACCGAGTT TTGT-3'; and control scramble: 5'-CCGCAACAGATGAGAAG CACCAACTCGAGTTGCTCTTCTCTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
Online supplemental material

Fig. S1 shows the classification of alae defects in various genetic backgrounds. It also displays the efficiency of ral-1 partial depletion by RNAi in C. elegans and the efficiency of RalA and RalB knock-downs by shRNA in mammalian cells. Fig. S2 shows the quantitative electron microscopy analysis of MVBS and endolysosomes in different nematode strains. Table S1 is a list of the genes identified in the RNAi screen for alae defects. Table S2 is a list of the alae defects in various backgrounds. Table S3 is a list of the nematode strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201504136/DC1.

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Author contributions: V. Hyenne and M. Labouesse planned the project. V. Hyenne designed and conducted most of the experiments. A. Apaydin carried out the initial RNAi screen. D. Rodriguez and V. Hyenne contributed to the cloning. S. Hoff-Yoessle and V. Hyenne generated the syn-5 mutants by CRISPR/Cas9. V. Hyenne carried out most electron microscopy experiments with training and help from Y. Schwab and C. Spiegelhalter. S. Tak carried out ral-1 mRNA quantification. M. Diem helped in cloning and analyzing RAL-1 mutants. O. Lefebvre generated the knocked down mammalian cells. V. Hyenne and M. Labouesse wrote the manuscript. J.G. Goetz contributed to mammalian experiment design and to the manuscript.

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References


