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Dynamic Regulation of Caveolin-1 Trafficking in the Germ Line and Embryo of *Caenorhabditis elegans*[D] [V]

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Caveolin is the major protein component required for the formation of caveolae on the plasma membrane. Here we show that trafficking of *Caenorhabditis elegans* caveolin-1 (CAV-1) is dynamically regulated during development of the germ line and embryo. In oocytes a CAV-1-green fluorescent protein (GFP) fusion protein is found on the plasma membrane and in large vesicles (CAV-1 bodies). After ovulation and fertilization the CAV-1 bodies fuse with the plasma membrane in a manner reminiscent of cortical granule exocytosis as described in other species. Fusion of CAV-1 bodies with the plasma membrane appears to be regulated by the advancing cell cycle, and not fertilization per se, because fusion can proceed in *spe-9* fertilization mutants but is blocked by RNA interference–mediated knockdown of an anaphase-promoting complex component (EMB-27). After exocytosis, most CAV-1-GFP is rapidly endocytosed and degraded within one cell cycle. CAV-1 bodies in oocytes appear to be produced by the Golgi apparatus in an ARF-1–dependent, clathrin-independent, mechanism. Conversely endocytosis and degradation of CAV-1-GFP in embryos requires clathrin, dynamin, and RAB-5. Our results demonstrate that the distribution of CAV-1 is highly dynamic during development and provides new insights into the sorting mechanisms that regulate CAV-1 localization.

INTRODUCTION

Caveolae are lipid-raft-enriched, flask-shaped invaginations present on the plasma membrane of many eukaryotic cell types (Anderson, 1998). Caveolae are enriched in glycosphingolipids and cholesterol and are thought to play a role in signaling, cholesterol homeostasis, clathrin-independent endocytosis, transcytosis, and potocytosis (Matveev *et al.*, 2001; Parton and Richards, 2003). Caveolae are also implicated in internalization of some bacterial toxins, viruses, and bacteria into cells (Parton and Richards, 2003).

The major structural components of caveolae are caveolins, which are integral membrane proteins with carboxyl and amino termini located in the cytosol and a hydrophobic loop inserted into the membrane (Glenney and Soppet, 1992; Rothberg *et al.*, 1992). Caveolins bind to cholesterol (Murata *et al.*, 1995) and oligomerize to form filamentous structures that are thought to stabilize the membrane and to define the size and shape of caveolae (Fernandez *et al.*, 2002). In mammalian cells, caveolin-1 and -2 are ubiquitously expressed, whereas caveolin-3 is specifically expressed in muscle tissues (Parton, 1996). Expression of caveolin-1 is sufficient to generate caveolae in cells previously lacking caveolae (Lipardi *et al.*, 1998), and caveolin knockout mice show a remarkable loss of caveolae (Drab *et al.*, 2001), indicating an essential role of caveolins in caveolar biogenesis. Caveolin knockout mice also show defects in diverse physiological processes (Le Lay and Kurzchalia, 2005). Previous studies in *C. elegans* found that CAV-1 is strongly expressed in the germ line and suggested that CAV-1 plays a role in meiotic progression as a negative regulator of Ras signaling (Scheel *et al.*, 1999). Studies of caveolin in mammalian systems reached similar conclusions regarding its role in Ras signaling (Roy *et al.*, 1999).

Caveolin-GFP fusions expressed in cultured cell lines have been extensively used to study caveolin dynamics in vivo (Pelkmans *et al.*, 2001, 2002, 2004, 2005; Tagawa *et al.*, 2005). Some groups studying caveolae in cultured cell lines have reported that caveolae are highly immobile structures that do not show a high turnover rate at the plasma membrane (Thomsen *et al.*, 2002), whereas other groups indicate that a subset of surface caveolae are highly mobile under standard culture conditions (Pelkmans and Zerial, 2005). However, all groups agree that various stimuli can lead to greatly increased internalization of caveolae. Antibody cross-linking of MHC class I molecules or glycosyl phosphatidyl inositol–anchored proteins results in clustering of these molecules in caveolae and subsequent internalization of caveolae (Huet *et al.*, 1980; Parton *et al.*, 1994). Simian virus 40 (SV40) infection also induces a massive internalization of caveolae containing SV40 (Pelkmans *et al.*, 2001). Recently, it has been reported that cell detachment from the extracellular matrix triggers internalization of lipid rafts and caveolin-1 from the plasma membrane to an intracellular compartment to down-regulate various signal transduction

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pathways (del Pozo *et al.*, 2005). Furthermore, caveolins are redistributed from the plasma membrane to lipid bodies in early stages of liver regeneration after partial hepatectomy (Pol *et al.*, 2004). These observations strongly suggest that the function and localization of caveolins are highly regulated in the cell. However, relatively little is known of caveolin trafficking in intact animal systems, and a genetically tractable system for the analysis of the molecular machinery that regulates the dynamic behavior of caveolins has been lacking.

To analyze caveolin dynamics, we have imaged CAV-1-GFP in living animals under a variety of experimental conditions. Here, we report that trafficking of *C. elegans* caveolin-1 is dynamically regulated during the development of the worm germ line and embryo. In the syncytial gonad CAV-1-GFP localizes to the plasma membrane and to punctate Golgi ministacks in the cytoplasm. As oocytes form, a significant fraction of the CAV-1-GFP in the cell begins to appear in large ring-like membrane compartments (CAV-1 bodies) in the cytoplasm that remain closely apposed to the Golgi. The formation of the CAV-1 body is clathrin-independent but ARF-1–dependent. Just after ovulation and fertilization, the large ring-like organelles positive for CAV-1-GFP apparently fuse with the plasma membrane. Shortly thereafter most of the CAV-1-GFP is internalized via clathrin-mediated endocytosis and is rapidly degraded. Our results demonstrate that the distribution of CAV-1 is highly dynamic during development, with at least two distinct mechanisms regulating CAV-1 localization at different developmental stages. Our results also suggest new potential functions for caveolin in development.

MATERIALS AND METHODS

General Methods and Strains

Methods for the handling and culturing of *C. elegans* were essentially those described by Brenner (1974). All strains were grown at 20°C unless otherwise stated. The wild-type parent for all strains was *C. elegans* var Bristol strain N2. Mutations used were LGIII, *unc-119 (ed3)* (Maduro and Pilgrim, 1995); LGIV, *rme-2 (b1008)* (Grant and Hirsh, 1999); LGI, *spe-9 (hc52)* (Singson *et al.*, 1998); LGV, *fog-2(q71)* (Schedl and Kimble, 1988); and unmapped pwIs61 [*GFP::cav-1, unc-119*(+)] and pwIs281 [*CAV-1-GFP, unc-119(+)*] (this work). *arf-1(ok796)* was obtained from the *C. elegans* Gene Knockout Consortium.

Plasmids and Transgenic C. elegans

A genomic fragment containing the ORF in *cav-1* was amplified by PCR and cloned into the Entry vector pDONR221 by Gateway recombinational cloning technology. The ORF of *cav-1* was then cloned into pID3.01 (Pellettieri *et al.*, 2003) to create an amino-terminal GFP fusion. pID3.01 utilizes *pie-1* 5′ and 3′ UTR sequences to drive expression of the transgene in the maternal germ line. To create a carboxy-terminal GFP fusion with CAV-1, a genomic fragment containing the ORF in *cav-1* was amplified by PCR with cav-1Gw+ and cav-1Bgl2R [5′-GGGGACAACTTTGTACAAGAAAGTTGTTAagatctGACGCATGGAGCAGTAGTTTC-3′] and cloned into the Entry vector pDONR221, resulting pDONR221cav-1BglII. A DNA fragment encoding GFP was amplified using pID3.01 as a template and inserted into a BglII site of pDONR221cav-1BglII. The ORF of *CAV-1-GFP* was then cloned into pID2.01 (Pellettieri *et al.*, 2003). GFP fusion expressing transgenic lines were created by the microparticle bombardment method as described previously (Praitis *et al.*, 2001).

For RNA interference (RNAi) experiments, we cloned genomic DNA corresponding to F54C9.10, *rab-27*, *rab-30*, *rab-33*, *rab-38*, *rabY4*, *rabY6*, and *chc-1* by PCR using purified N2 genomic DNA and inserting them into RNAi vector L4440. cDNAs corresponding to Y57G11C.13, Y116A8C.12, *rab-3*, *rab-5*, *rab-7*, *rab-8*, *rab-11.1*, *rab-14*, *rab-18*, *rab-19*, and *rab-35* were prepared from EST clones provided by Yuji Kohara (National Institute of Genetics, Japan) and subcloned into L4440. All other feeding RNAi constructs were obtained from the Ahringer genomic RNAi library (Kamath and Ahringer, 2003).

Antibody Production

To generate antibodies directed against SQV-8, the nucleotides encoding amino acids 150–349 of SQV-8 were amplified from a cDNA library and inserted into pGEX6P-1 (GE Healthcare, Piscataway, NJ). The purified GST fusion protein was outsourced for injection into rabbits (Sigma Genosys), and affinity purification of anti-SQV-8 antibodies was performed as described previously, after removal of the GST by cleavage of the antigen with Precision protease (Precision Systems, Natick, MA; Audhya *et al.*, 2005).

RNA-mediated Interference

RNAi experiments in this study were performed using the feeding method (Timmons *et al.*, 2001). L4 larvae were placed on plates containing NGM agar with 5 mM IPTG and HT115 (DE3) bacteria carrying double-stranded RNA expression constructs and allowed to grow for 48 h at 20°C. P0 animals were transferred to a new plate and allowed to lay eggs for 1–2 h. Then, P0 animals were removed from the plate and observed by fluorescence microscopy. F1 progeny were further incubated for 4 d and observed by fluorescence microscopy.

Microscopy and Immunostaining

To observe live worms expressing transgenes, worms were mounted on agarose pads with 10 mM levamisole in M9 buffer. Fluorescence images were obtained using an Axiovert 200M (Carl Zeiss MicroImaging, Oberkochen, Germany) microscope equipped with a digital CCD camera (C4742-95-12ER, Hamamatsu Photonics, Hamamatsu, Japan) and deconvolved with AutoDeblur software (AutoQuant Imaging, Watervliet, NY). Confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope system (Bio-Rad, Tokyo, Japan) and an Olympus confocal microscope system FV1000 (Olympus, Tokyo, Japan). Movies of the gonad were made using a spinning disk confocal microscope (Nikon Eclipse TE2000-E, Tokyo, Japan) equipped with a Hamamatsu Orca-ER CCD camera at 20°C using a Nikon 60×, 1.4 NA Planapo oil objective lens.

Immunostaining of dissected gonads was performed as described previously (Grant and Hirsh, 1999; Sato *et al.*, 2005). Images of fixed worms stained with anti-SQV-8 antibody were acquired on a DeltaVision deconvolution Olympus IX70 microscope (Applied Precision, Issaquah, WA) equipped with a CoolSnap CCD camera (Roper Scientific, Tucson, AZ) at 20°C using a 100×, 1.35 NA Olympus U-Planapo oil objective lens.

RESULTS

CAV-1-GFP Displays Highly Dynamic Behavior during Development

To visualize caveolar trafficking in the context of a living animal, we expressed caveolin-GFP fusion proteins in transgenic nematodes. *C. elegans* has two caveolin genes, encoding the CAV-1 and CAV-2 proteins (Tang *et al.*, 1997). Endogenous CAV-1 is known to be strongly expressed in the germ line of adult hermaphrodites and is thought to influence Ras/MAP-kinase–dependent progression through the pachytene-stage of the meiotic cell cycle (Scheel *et al.*, 1999). To examine the dynamics of meiotic CAV-1 protein, we expressed CAV-1 with an amino- or carboxy-terminal GFP tag in the *C. elegans* germline using the germ line-specific *pie-1* promoter (Pellettieri *et al.*, 2003). We used both CAV-1-GFP fusions for the experiments presented here, but show the results for only one CAV-1-GFP reporter, because both fusions behaved identically in all cases.

The germ cells of adult hermaphrodites are contained within a U-shaped tubular gonad (McCarter *et al.*, 1999; see Figure 7G). Germ cells in the most distal region of the gonad arm are in mitosis and enter meiosis as they move away from the distal tip. Oocytes first appear near the "bend" region of the gonad arm and are arrested in diakinesis of meiotic prophase I. The oocytes increase dramatically in size as they move from the bend proximally toward the spermatheca, but do not reenter meiosis until they receive signals from the overlying sheath and adjacent sperm cells to mature and ovulate. On receiving these signals, the first signs of maturation are nuclear envelope breakdown and cortical rearrangement. A mature oocyte in the proximal arm will then ovulate, entering the spermatheca for fertilization. After fertilization, embryos complete meiosis I and meiosis II and start zygotic development.

CAV-1-GFP expressed in the germ line displayed highly dynamic behavior during oocyte formation, ovulation, fer-

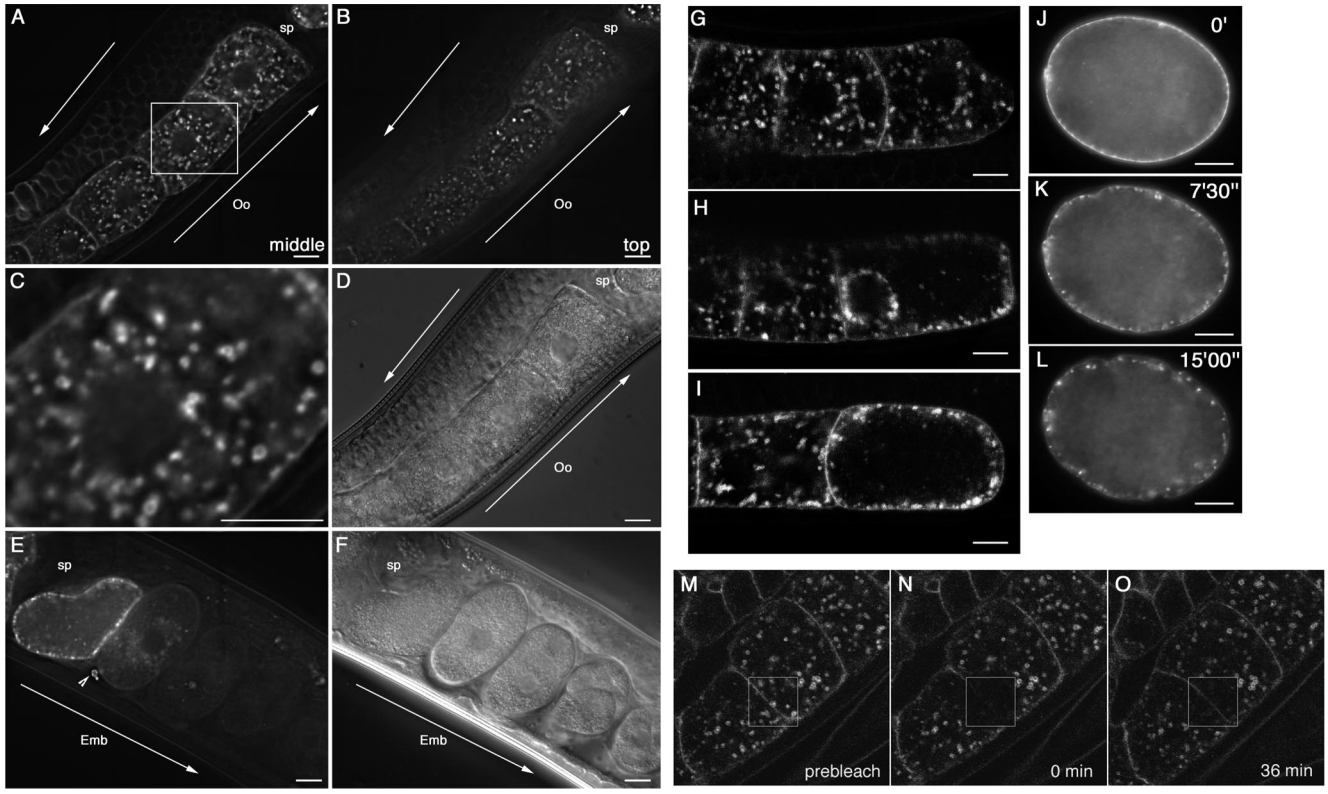

Figure 1. Dynamic behavior of CAV-1-GFP during development. (A–D) Subcellular localization of CAV-1-GFP during oogenesis. CAV-1-GFP–labeled ring-like structures (CAV-1 bodies) and puncta. CAV-1-GFP puncta first appear in early oocytes in the bend region of gonad, near the plasma membrane and deeper in the cytoplasm (A and B). (C) An enlargement of the square indicated in A. (D) A Nomarski image of the oocytes. (E) Degradation of CAV-1-GFP after fertilization. After fertilization, CAV-1-GFP accumulates on the plasma membrane and then largely disappears before the first cell division of the embryo (E). CAV-1-GFP partitioned to the polar body of embryos remains highly fluorescent and does not disappear (arrowhead). (F) A Nomarski image of the embryos. (G–I) CAV-1-GFP changes localization dynamically, accumulating in the cortex and clustering around the nucleus (H). Perinuclear CAV-1-GFP is lost during nuclear breakdown (I). (J–L) Internalization of GFP-CAV-1 in the embryo. Plasma membrane localized GFP-CAV-1 (J, 0′) is then internalized (K and L). Oo, oocyte; emb, embryo; sp, spematheca. Arrows indicate the direction of maturation of oocytes and embryos. Scale bars, 10 μm. (M–O) CAV-1-GFP on the plasma membrane and in CAV-1 bodies was photobleached, and the recovery of fluorescence was followed as a function of time. From left to right, before (Prebleach), immediately after (0 min), and 36 min after bleaching are shown. The squares show the photobleached areas. Note that little fluorescence recovery occurred in CAV-1 bodies. Partial recovery occurred on the plasma membrane.

tilization, and the first embryonic cell cycle. In mitotic and early meiotic cells of the distal germline CAV-1-GFP is mainly localized to the plasma membrane. As oocytes form in the bend region CAV-1-GFP additionally accumulates in small vesicles, and in larger oocytes appears in large ring-like structures deeper in the cytoplasm (Figure 1, A, C, and G).

To determine if these CAV-1–positive vesicular structures define a unique compartment as is the case for mammalian caveosomes, we compared CAV-1-GFP localization with several membraneous organellar markers in oocytes (see Supplementary Material, Supplementary Figure S1). First we compared CAV-1-GFP with RME-2, the *C. elegans* yolk receptor (Grant and Hirsh, 1999). RME-2 is first expressed in very early oocytes in the bend region of the gonad where it appears primarily in the ER and Golgi. Soon thereafter it reaches the plasma membrane, and in large nearly full-grown oocytes is primarily found cycling through early endosomes and recycling endosomes (Grant and Hirsh, 1999; Grant *et al.,* 2001; Sato *et al.,* 2005). CAV-1-GFP partially colocalizes with RME-2 in early stage oocytes, probably in the ER and Golgi, but in larger oocytes did not overlap significantly with RME-2 in the endosomal system (Supplementary Figure S1, A and B). In addition CAV-1-GFP did not exhibit colocalization with a marker for early endosomes, EEA-1 (Supplementary Figure S1, C and D), or a marker for recycling endosomes, RME-1 (Supplementary Figure S1, E and F), at any stage of oogenesis. CAV-1-GFP–labeled organelles did not accumulate Lysotracker-Red, a marker for acidified organelles such as late endosomes and lysosomes, nor did they label with Nile Red, a marker for neutral lipid bodies (Supplementary Figure S1G). Thus at least in their unique labeling the CAV-1-GFP–labeled ring-like structures in large nearly full-grown oocytes are reminiscent of caveosomes (Pelkmans *et al.,* 2001). Mammalian caveosomes are defined as caveolin-1–rich, intracellular organelles with a near neutral pH (Pelkmans *et al.,* 2001). We refer to these CAV-1-GFP–labeled ring-like structures in *C. elegans* oocytes as CAV-1 bodies. The large CAV-1 bodies were relatively immobile, whereas the smaller CAV-1-GFP–labeled vesicles in the cytoplasm moved actively (Supplementary Video 1). CAV-1-GFP was also found in small, scattered spots on the plasma membrane (Figure 1B) that presumably represent individual caveolae or small caveolar vesicles beneath the plasma membrane.

Given the low mobility of large CAV-1 bodies, we sought to determine if these structures are still actively exchanging molecules with the rest of the cell or are quiescent. As a measure of molecular exchange we subjected the CAV-1-

GFP–labeled CAV-1 bodies to fluorescence recovery after photobleaching (FRAP) analysis (Figure 1, M–O). Interestingly, fluorescence recovery in individual CAV-1 bodies was very slow, indicating that CAV-1-GFP associated with CAV-1 bodies in large, nearly full-grown oocytes has very little exchange with external pools. Significant recovery of fluorescence was noted on the plasma membrane, probably because of the lateral diffusion of CAV-1- GFP on the plasma membrane or influx of newly synthesized CAV-1-GFP to the plasma membrane.

CAV-1-GFP distribution changed rapidly during ovulation. The first change we observed in an oocyte about to ovulate was the clustering of all punctate CAV-1-GFP at the cortex and around the nucleus, clearing the cytoplasm in between (Figure 1H and Supplementary Video 2a). Shortly thereafter, accompanying nuclear breakdown, the perinuclear CAV-1-GFP signal was lost and the plasma membrane proximal CAV-1-GFP signal increased, with apparent fusion of the CAV-1 bodies with the plasma membrane (Figure 1I and Supplementary Video 2a). After fertilization, most CAV-1-GFP (Figure 1E), or GFP-CAV-1 (Figure 1, J–L, and Supplementary Video 2, a and b) was internalized and degraded in the one-cell stage embryo. CAV-1-GFP present in polar bodies persisted much longer and did not appear to be actively degraded (Figure 1E, arrowhead). These results indicate that CAV-1 localization and stability are tightly controlled in the germ line and embryo.

CAV-1-GFP Accumulates on the Plasma Membrane in Oocytes Lacking RME-2

Endogenous CAV-1 was reported to be enriched in cholesterol-rich rafts in *C. elegans*, and cholesterol depletion disrupted the association of CAV-1 with glycosphingolipid-rich rafts (Scheel *et al.*, 1999). The oocytes of *C. elegans* are enriched in cholesterol content relative to most other tissues as a consequence of their uptake of cholesterol-rich yolk particles (Matyash *et al.*, 2001). To examine the effects of cholesterol depletion on CAV-1-GFP localization, we utilized *rme-2* mutants, which lack the yolk receptor and thus fail in yolk uptake by oocytes (Grant and Hirsh, 1999). Because *C. elegans* does not possess the enzymes necessary for de novo sterol synthesis, all sterols enter the worm from their environment, most through ingestion and absorption by the intestine (Matyash *et al.*, 2001). The bulk of the cholesterol entering oocytes is transported from the intestine via vitellogenins and RME-2 (Grant and Hirsh, 1999; Matyash *et al.*, 2001). Thus, lack of RME-2 causes severe depletion of cholesterol in oocytes. As shown in Figure 2, *rme-2(b1008)* null mutant worms display aberrant accumulation of CAV-1-GFP on and near the plasma membrane accompanied by loss of intracellular CAV-1 bodies in the proximal oocytes (Figure 2B), suggesting that yolk uptake via RME-2 is important for correct localization of CAV-1-GFP in oocytes.

Fusion of CAV-1 Bodies with the Plasma Membrane Occurs after Anaphase

To better understand the molecular mechanisms controlling the trafficking of CAV-1 protein, we examined the effects of inactivation or depletion of proteins that regulate fertilization or cell-cycle changes associated with ovulation. In particular the timing of CAV-1 body fusion with the plasma membrane suggested that it might be an event triggered by fertilization. To test this hypothesis we examined CAV-1-GFP trafficking in *spe-9* and *fog-2* mutants. At the restrictive temperature of 25°C, *spe-9(hc52)* mutant sperm are morphologically normal and induce normal oocyte ovulation, but such sperm cannot fertilize the oocyte (Singson *et al.*, 1998; Kadandale and Singson, 2004). *fog-2(q71)* mutants on the other hand develop as females devoid of sperm (Schedl and Kimble, 1988). Because major sperm proteins (MSPs) secreted by the sperm are important for ovulation, *fog-2(q71)* mutants show greatly reduced ovulation rates unless mated to males (Schedl and Kimble, 1988; Miller *et al.*, 2001). Surprisingly, trafficking of CAV-1-GFP was nearly normal in both of these mutants (Figure 3, A–D), indicating that fertilization per se is not required for exocytosis of CAV-1 bodies or degradation of CAV-1-GFP after ovulation. The trafficking of CAV-1-GFP from CAV-1 bodies to the plasma membrane always accompanied ovulation, even in *fog-2* mutant oocytes where ovulation is greatly delayed.

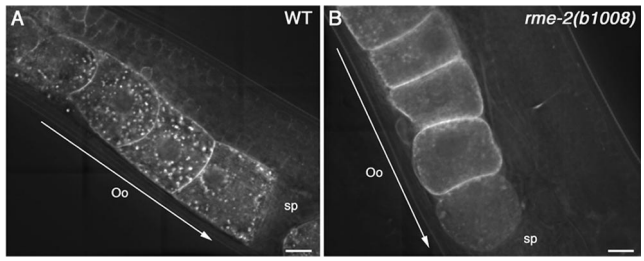

Figure 2. CAV-1-GFP accumulates on the plasma membrane in *rme-2* mutant oocytes. Subcellular localization of CAV-1-GFP in wild-type (A) and *rme-2(b1008)* (B) worms. CAV-1-GFP localizes to the CAV-1 body and the plasma membrane in oocytes of wild-type worms (A). In *rme-2(b1008)*, CAV-1-GFP mainly localized to the plasma membrane, with the number and size of CAV-1-GFP labeled-CAV-1 bodies greatly reduced (B). Arrows indicate the direction of maturation of oocytes and embryos. Oo, oocyte; Sp, spematheca. Scale bars, 10 μm.

Given the consistent timing of the fusion of CAV-1 bodies with the plasma membrane and the apparent fertilization independence of the event, we reasoned that perhaps it was the cell cycle that regulated the trafficking/fusion of CAV-1 at the plasma membrane. To test this hypothesis, we examined the effects of *emb-27* RNAi on CAV-1-GFP trafficking. *emb-27* encodes an ortholog of the cdc16p subunit of the anaphase-promoting complex that functions to target key cellular components for proteolytic destruction, advancing the cell cycle (Golden *et al.*, 2000). RNAi of *emb-27* results in accumulation of one-cell stage embryos arrested in metaphase of meiosis I (Golden *et al.*, 2000). Such arrested embryos fail to transit to anaphase or to produce polar bodies. *emb-27* RNAi did not interfere with CAV-1 body formation in oocytes (Figure 3E), but *emb-27(RNAi)* embryos arrested at the one-cell stage failed to degrade CAV-1-GFP (Figure 3F). CAV-1 bodies remained just beneath the plasma membrane in *emb-27(RNAi)* embryos (Figure 3, G and H), suggesting that fusion with the plasma membrane is inhibited until after metaphase.

CAV-1-GFP Is Degraded via Clathrin-mediated Endocytosis after Fertilization

To gain insight into the mechanisms of both CAV-1 body formation in oocytes and CAV-1 down-regulation in embryos, we tested the importance of candidate trafficking regulators in either process. We began by depleting CAV-1-GFP animals of clathrin heavy chain (*chc-1*), dynamin (*dyn-1*), and *rab-5* using RNAi conditions that we have previously established block endocytosis in oocytes (Grant and Hirsh, 1999; Sato *et al.*, 2005). Clathrin is a major coat protein required for clathrin-mediated endocytosis by which most receptor-ligand complexes are internalized (Brodsky *et al.*,

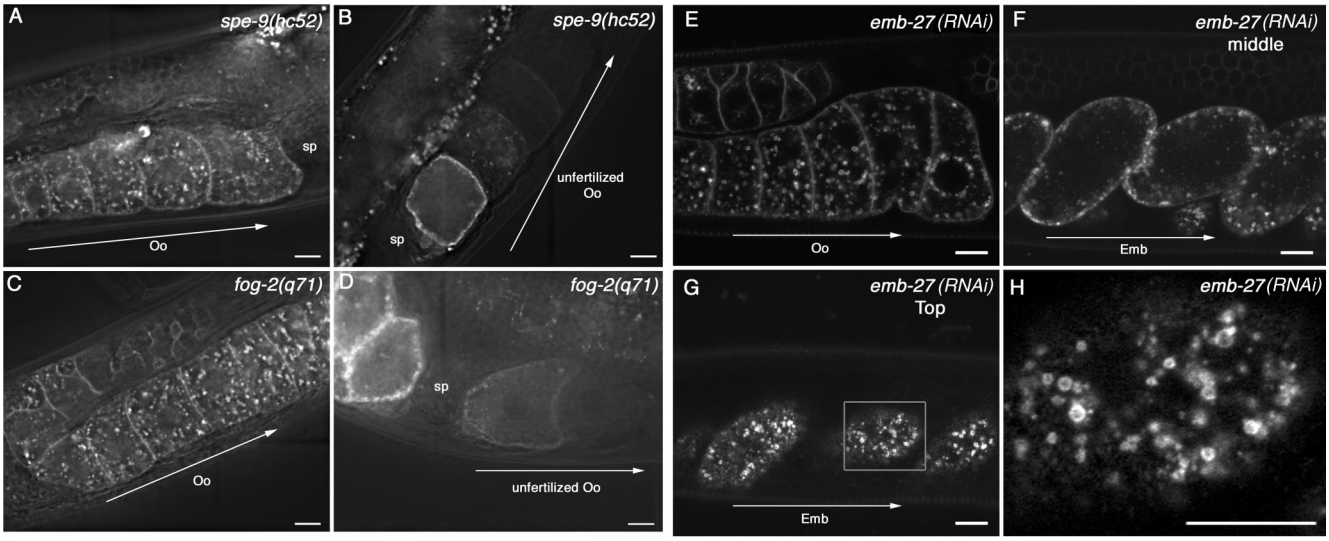

Figure 3. Fusion of CAV-1 bodies with the plasma membrane occurs after anaphase. (A and B) CAV-1-GFP in a fertilization-defective mutant, *spe-9(hc52)*. L4 hermaphrodites of *spe-9(hc52)*-expressing CAV-1-GFP were incubated at 25°C for 24 h and examined by fluorescence microscopy. CAV-1 body formation was normal in oocytes (A), and degradation of CAV-1-GFP occurred with normal kinetics in unfertilized eggs (B). (C and D) CAV-1-GFP in a sperm-deficient mutant, *fog-2(q71)*. CAV-1 body formation was normal in oocytes (C), and the degradation of CAV-1-GFP occurred with normal kinetics in ovulated but unfertilized eggs (D) in *fog-2(q71)*. (E-H) CAV-1-GFP in embryos defective in the metaphase-to-anaphase transition. L4 hermaphrodites expressing CAV-1-GFP were incubated at 20°C for 48 h. *emb-27(RNAi)* embryos are arrested in metaphase of meiosis I. In *emb-27(RNAi)* hermaphrodites expressing CAV-1-GFP, CAV-1 body formation appears normal in oocytes (E), but degradation of CAV-1-GFP in embryos was completely blocked (F). CAV-1 bodies were observed underneath the plasma membrane (G). (H) An enlargement of the square indicated in G. Oo, oocyte; Emb, embryo; Sp, spematheca. Arrows indicate the direction of maturation of oocytes and embryos. Scale bars, 10 μm.

2001). Clathrin is also required for Golgi-to-endosome transport, a pathway utilized by newly synthesized lysosomal hydrolases (Bonifacino, 2004). Caveolar endocytosis is thought to be clathrin-independent. Dynamin is required for pinching off clathrin-coated vesicles and caveolae from the plasma membrane (Takei *et al.*, 2005). The small GTPase RAB-5 is a key regulator of the early clathrin-mediated endocytic pathway and is also required for Simian Virus 40 infection via caveolar endocytosis in mammalian cells (Pelkmans *et al.*, 2004). We did not observe any effect of RNAi-mediated depletion of *chc-1*, *dyn-1*, or *rab-5* on CAV-1-GFP localization in oocytes (Figure 4, C, E, and G), suggesting that targeting of CAV-1-GFP to the CAV-1 bodies is a clathrin-, dynamin-, and RAB-5–independent process. In striking contrast we found that RNAi-mediated knockdown of *chc-1*, *dyn-1*, or *rab-5* blocked degradation of CAV-1-GFP in embryos (Figure 4, D, F, and H). These results indicate that targeting of CAV-1-GFP to CAV-1 bodies in oocytes occurs in a clathrin- and RAB-5–independent-manner, but degradation of CAV-1-GFP in embryos is mediated by clathrin-dependent endocytosis.

ARF-1 Is Required for Targeting of CAV-1-GFP to CAV-1 Bodies in Oocytes

To gain additional insight into the mechanisms of CAV-1 trafficking we examined the effects of RNAi-mediated depletion of each *C. elegans rab* or *sar/arf* family GTPase on CAV-1-GFP localization in oocytes and embryos. Small GTPases of the Rab and Sar/Arf play pivotal roles in vesicular transport and each membrane transport step is thought to be regulated by at least one GTPase of this superfamily (Zerial and McBride, 2001; Nie *et al.*, 2003). *C. elegans* has 1 *sar*, 10 *arfs*, and 29 *rab* genes. Among these genes, we found that RNAi of *rab-1* or *sar-1* caused severe accumulation of CAV-1-GFP in reticular structures, presumably the ER (Figure 5) and resulted in loss of CAV-1-GFP from other structures. These results indicate that CAV-1-GFP is transported through the early secretory pathway in the traditional manner and must exit the ER in a *sar-1*– and *rab-1*–dependent process before reaching the CAV-1 body and plasma membrane.

Interestingly, *arf-1(RNAi)* caused loss of normal CAV-1 bodies, which were replaced with fewer irregularly shaped CAV-1-GFP–positive vesicles in the cytoplasm (Figure 6C) and on or near the plasma membrane (Figure 6D). Furthermore, we obtained very similar results in *arf-1(ok796)* null mutants (Figure 6, E and F), confirming that ARF-1 is required for normal targeting of CAV-1-GFP to CAV-1 bodies. As a control for effects on general secretion, we also examined trafficking of GFP-tagged RME-2 in *arf-1(RNAi)* and *arf-1(ok796)* mutant animals (see Supplementary Material, Supplementary Figure S2, online). Most of the GFP-tagged RME-2 reached the plasma membrane and endosomes of these mutant oocytes, indicating fairly normal secretion under these conditions (Supplementary Figure S2, B and C). We did observe some extra RME-2-GFP puncta in the cytoplasm of oocytes lacking *arf-1*, however, perhaps indicating some delay in secretion in these strains (Supplementary Figure S2, B and C). We also found that RNAi of *arf-3*, a gene very similar in sequence to *arf-1*, caused a similar but weaker phenotype (our unpublished observation). RNAi of *arf-3* in an *arf-1(ok796)* mutant background resulted in accumulation of CAV-1-GFP in reticular structures, presumably the ER (our unpublished observation), suggesting that ARF-1 and ARF-3 function redundantly in ER-Golgi transport and CAV-1 trafficking, but probably also have unique functions as indicated by their individual phenotypes.

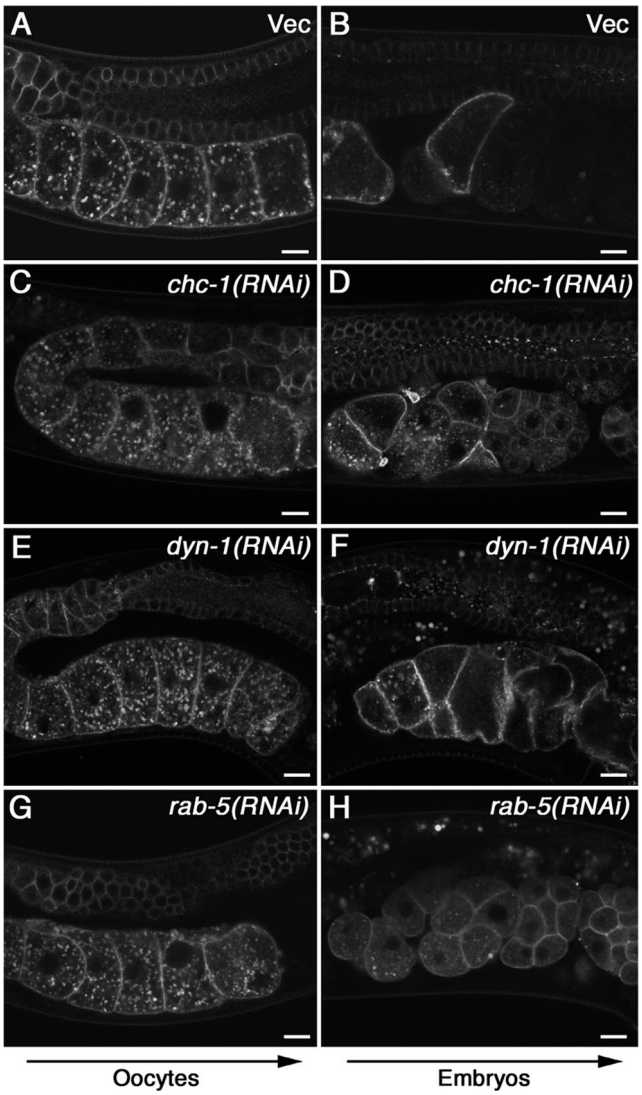

Figure 4. CAV-1-GFP is degraded via clathrin- and RAB-5–dependent endocytosis after fertilization. The subcellular localization of CAV-1-GFP was determined in *chc-1(RNAi)* (C and D), *dyn-1(RNAi)* (E and F), and *rab-5*(RNAi) worms (G and H) by a confocal microscopy. The localization of CAV-1-GFP was normal in mock RNAi-treated animals fed bacteria harboring the empty RNAi vector L4440 (A and B). In the *chc-1(RNAi)*, *dyn-1(RNAi)*, and *rab-5(RNAi)* worms, CAV-1 body formation appeared normal (C, E, and G), but degradation of CAV-1-GFP was blocked (D, F, and H). Arrows indicate the direction of maturation of oocytes and embryos. Scale bars, 10 μm.

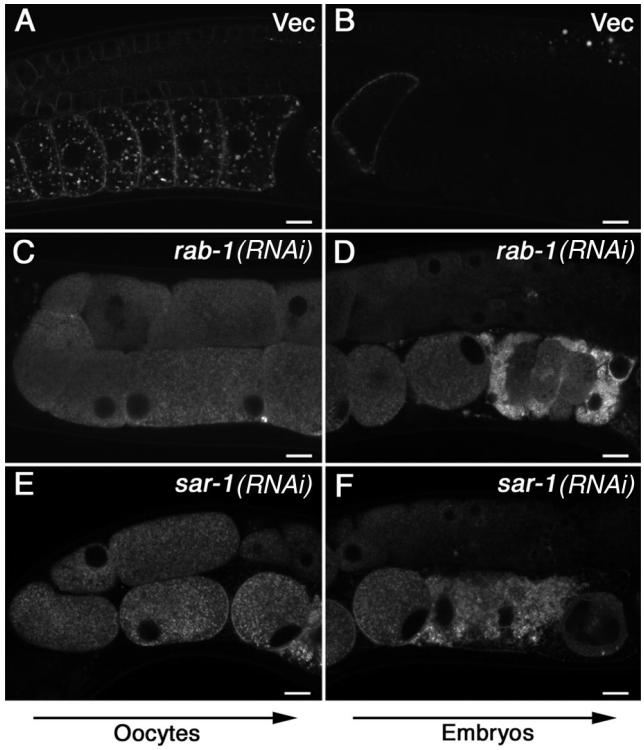

Figure 5. CAV-1-GFP transport via the biosynthetic pathway. The subcellular localization of CAV-1-GFP was determined in *rab-1(RNAi)* (C and D) and *sar-1(RNAi)* (E and F) worms by confocal laser microscopy. The localization of CAV-1-GFP was normal in mock RNAi-treated animals fed bacteria harboring the empty RNAi vector L4440 (A and B). Arrows indicate the direction of maturation of oocytes and embryos. Scale bars, 10 μm.

To corroborate these findings, we further examined the effects of RNAi-mediated depletion of ARF guanine nucleotide exchange factor (GEF) homologues on CAV-1-GFP transport. The exchange of GDP for GTP in ARF family proteins is stimulated by GEFs containing a Sec7 domain (Jackson and Casanova, 2000). *C. elegans* has six genes encoding proteins with predicted Sec7 domains. Among these genes, only RNAi of Y6B3A.1 significantly blocked targeting of CAV-1-GFP to CAV-1 bodies (Figure 6, G and H). RNAi of Y6B3A.1 did not alter the localization of RME-2-GFP on the plasma membrane and endosomes (Supplementary Figure S2D). Y6B3A.1 encodes a homolog of mammalian BIG1 (brefeldin A–inhibited guanine nucleotide-exchange protein 1), which acts as a GEF for class I Arfs, predicted orthologues of *C. elegans* ARF-1 (Togawa *et al.*, 1999). We named this gene *agef-1*, standing for Arf1 GEF homologue. Both Arf1 and BIG1 are known to function at the Golgi apparatus (Donaldson and Honda, 2005). Interestingly, *agef-1* knockdown inhibited CAV-1 body formation more severely than did *arf-1* disruption, suggesting that AGEF-1 might activate both ARF-1 and ARF-3 at the Golgi in *C. elegans*.

These results suggested that *arf-1* and *agef-1* function in CAV-1 body formation at the Golgi. We thus examined colocalization of CAV-1-GFP with the Golgi membrane protein SQV-8 (Herman and Horvitz, 1999). Although SQV-8 showed distinct localization from CAV-1-GFP in the large wild-type oocytes, SQV-8 was generally found directly adjacent to the CAV-1 bodies (Figure 7, A–C). Strikingly, deletion of *arf-1* not only prevents normal CAV-1 body formation (Figure 7D) but also dramatically changed Golgi morphology (Figure 7E). Much of the CAV-1-GFP in *arf-1* mutants was coincident with SQV-8 (Figure 7F), suggesting that ARF-1 is involved in export of CAV-1 from the Golgi to the CAV-1 bodies. Taken together, these results suggest that activation of class I Arfs by AGEF-1 is required for transport of caveolin from the Golgi to the CAV-1 bodies during oogenesis.

Time-lapse movies of CAV-1-GFP trafficking in the gonad of *arf-1* mutants indicated that the abnormal CAV-1 bodies form by clustering of small cytoplasmic vesicles, like wild type, but in the absence of *arf-1*, clustering of small structures continues without reformation into distinct rings, con-

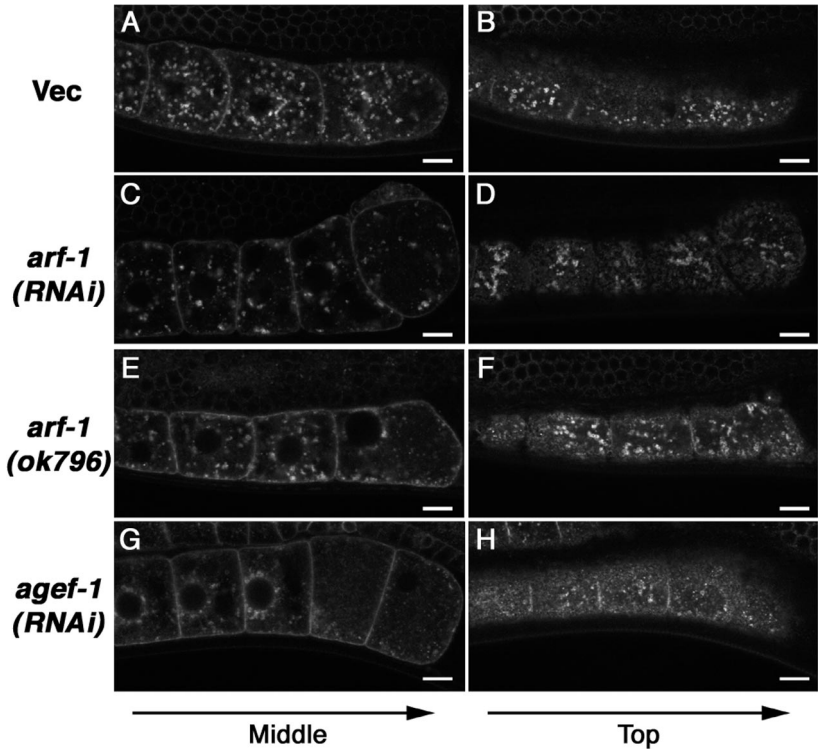

Figure 6. ARF-1–dependent transport of CAV-1-GFP to the CAV-1 body. CAV-1-GFP was normally localized to the CAV-1 body, and the plasma membrane in oocytes of mock RNAi-treated animals fed bacteria harboring the empty RNAi vector L4440 (A and B). In *arf-1(RNAi)* worms, the number of CAV-1 bodies was reduced (C) and large CAV-1-GFP–labeled aggregations were often observed (D, arrowheads). *arf-1(ok796)* displayed a very similar phenotype to *arf-1(RNAi)* (E and F). RNAi of *agef-1*, which encodes the predicted orthologue of a known Arf1 GEF, resulted in a severe defect in CAV-1 body formation (G and H). Scale bars, 10 μm.

sistent with CAV-1 entering Golgi ministacks but failing in the process of exiting (Supplementary Video 3). The defects in *agef-1* RNAi animals were more severe, with apparent clustering of small CAV-1-GFP vesicles in the bend region of the gonad, but no apparent accumulation and no ring formation (Supplementary Video 4).

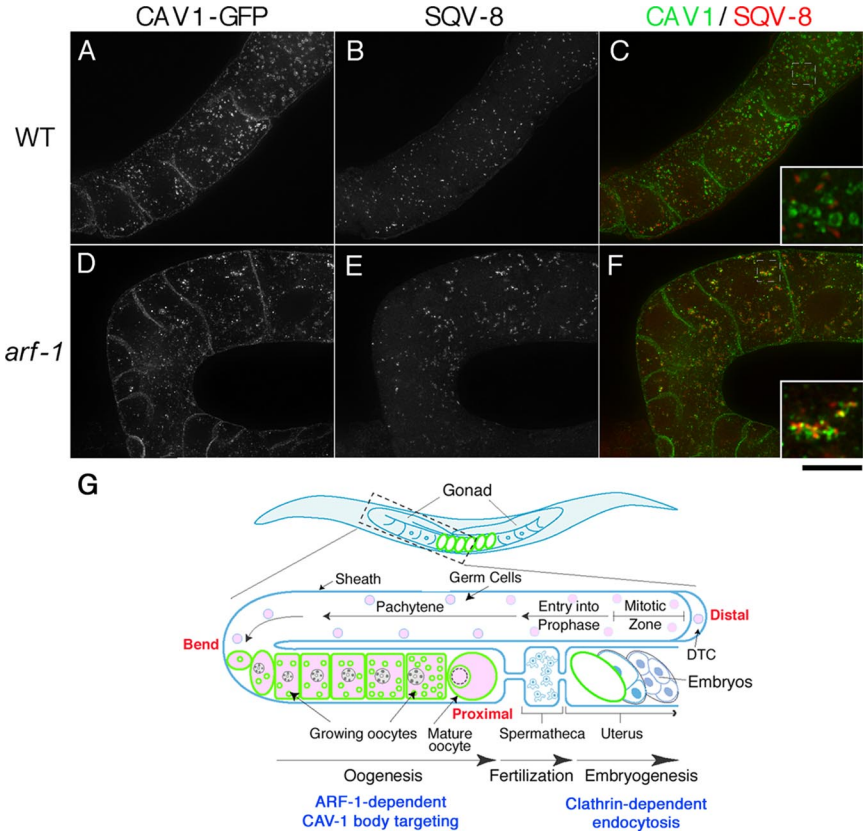

Figure 7. ARF-1 is required for export of CAV-1-GFP from the Golgi. CAV-1-GFP–expressing wild-type (A–C) and *arf-1(ok796)* (D–F) strains were stained with an antibody against a Golgi membrane protein SQV-8. CAV-1-GFP failed to colocalize with SQV-8 in the wild-type strain (C, inset). Deletion of *arf-1* caused a significant alteration in Golgi morphology and a loss of CAV-1 bodies (D–F). Colocalization of CAV-1-GFP with SQV-8 was often observed in *arf-1* mutants (F, inset). Scale bar, 20 μm. The insets (bottom right) are 3× enlargements of the indicated regions in the merged panels. (G) Stylized drawing of one gonad arm connected to the spermatheca and the uterus. Schematic localization of CAV-1-GFP is also indicated with green. In growing oocytes, CAV-1-GFP is transported to the CAV-1 bodies in an ARF-1–dependent manner. After fertilization, CAV-1-GFP is internalized via clathrin-dependent endocytosis.

DISCUSSION

In this study we show that caveolin trafficking is highly regulated during development of the germ line and embryo in *C. elegans*. Caveolin-GFP dynamics have been studied in mammalian cultured cell lines (Pelkmans *et al.*, 2001, 2002, 2004, 2005; Tagawa *et al.*, 2005). However, few of the cultured cell results have been extended to the physiologically relevant context of a living animal. Our findings demonstrate that several distinct mechanisms regulate caveolin trafficking at different developmental stages in a living animal and suggest potential new functions for caveolin in development.

We found that CAV-1-GFP is first transported via the biosynthetic pathway from the ER to the Golgi and is then targeted to a novel membrane compartment, the CAV-1 body, during oocyte formation. We discovered that targeting of CAV-1-GFP to the CAV-1 bodies depends on the *C. elegans* homologues of Arf1 and its putative guanine nucleotide exchange factor BIG1. In *arf-1* mutants CAV-1-GFP becomes trapped in a Golgi-associated structure, possibly an incomplete CAV-1 body that cannot detach from the TGN. In mammalian cells, Arf1 and BIG1 are localized to the Golgi (Stearns *et al.*, 1990; Yamaji *et al.*, 2000) and are required for the recruitment of cytosolic coat complexes to the Golgi membrane (Donaldson *et al.*, 2005). Interestingly, newly assembled caveolar domains also first appear as structures budding from the Golgi in mammalian cells (Tagawa *et al.*, 2005). Arf1 recruits the adaptor protein complex (AP1) and the monomeric Golgi-associated, γ-adaptin ear-containing, Arf-binding (GGA) proteins to the Golgi complex. Both of these coat proteins recognize the cytoplasmic tails of transmembrane cargo proteins and mediate their transport between the TGN and the lysosome in a clathrin-dependent manner (Robinson, 2004). Because targeting of CAV-1-GFP to CAV-1 bodies was not affected by knockdown of clathrin heavy chain, even under conditions stringent enough to redistribute clathrin light chain to the cytosol (our unpublished observation), it is likely that CAV-1 body formation is a novel clathrin-independent process. Strikingly, in *rme-2* mutants defective in cholesterol-enriched yolk uptake, nearly all CAV-1-GFP accumulated on the plasma membrane concurrent with a loss of the CAV-1 bodies. One simple explanation would be that high cholesterol levels are required for correct sorting of CAV-1-GFP at the Golgi.

CAV-1 bodies normally appear to fuse with the plasma membrane after fertilization, suggesting that the CAV-1 body is a regulated secretory compartment derived from the TGN. The fusion of CAV-1 bodies with the plasma membrane is blocked by loss of EMB-27, a subunit of the anaphase-promoting complex required for the metaphase-to-anaphase transition of embryos after fertilization (Golden *et al.*, 2000), indicating that fusion of CAV-1 bodies to the plasma membrane is tightly linked to progression of meiosis I. This process can proceed even if fertilization itself is blocked. In mammalian cells, cytoplasmic caveolar vesicles undergo kiss-and-run–like fusion with the plasma membrane (Pelkmans and Zerial, 2005), suggesting that caveolin-enriched vesicles in general undergo regulated targeting to the plasma membrane. The CAV-1 bodies are quite reminiscent of oocyte cortical granules observed in other animals (Fisher and Rebhun, 1983; Abbott and Ducibella, 2001). In animals, exocytosis of cortical granules located in the cortex of oocytes changes the extracellular environment to prevent additional spermatozoa from penetrating the newly fertilized egg. As such CAV-1 bodies may be a reservoir of cholesterol- and glycosphingolipid-enriched membranes or signaling molecules in the oocyte that are delivered to the plasma membrane to make oocytes resistant to polyspermy. Interestingly, electron microscopy indicates that *C. elegans* zygotes produce a transient "post-ovulation envelope" before eggshell formation (David Greenstein, personal communication). It is conceivable that CAV-1 bodies are involved in forming a postovulation envelope to block polyspermy. Because *C. elegans* embryos secrete their own eggshell, it is also possible that CAV-1 bodies contain components that contribute to eggshell formation (Grant and Sato, 2006).

Strikingly, CAV-1-GFP was internalized for degradation in a clathrin- and RAB-5–dependent manner. Because caveolins in cultured cells produce their own clathrin-independent endocytic pits and vesicles, this was unexpected. However, caveolins are not degraded by uptake into caveosomes, so redirection into the standard endocytosis pathway may be necessary for productive degradation. Regulated degradation of maternal proteins is under strict developmental control and is important for proper embryogenesis. For instance maternal proteins MEI-1 and MEI-2 are degraded in a ubiquitin-dependent reaction that is essential for transition of embryos from meiosis to mitosis (Srayko *et al.*, 2000; Pellettieri *et al.*, 2003). Mono-ubiquitination of membrane proteins is known to direct their endocytosis (Hicke and Dunn, 2003). In addition mono-ubiquitination is recognized by the ESCRT complex of the endosomes and directs transmembrane cargo into the internal vesicles of multivesicular bodies, leading to their degradation and preventing their recycling (Raiborg *et al.*, 2003). Such ubiquitination of CAV-1 might be the means by which it is targeted for degradation.

In this study we reveal that trafficking of *C. elegans* caveolin-1 is dynamically regulated during the development of the germ line and embryo. We do note however that the CAV-1-GFP-fusion proteins examined here were driven by a heterologous promoter, and we were not able to test these fusions for function because no *cav-1* mutants are currently available. Thus, it is not yet certain if our results completely recapitulate the expression and trafficking of the endogenous CAV-1 protein. Further studies will be required to determine the precise mechanisms that drive CAV-1 into this pathway.

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