Rab11 is required for synchronous secretion of chondroitin proteoglycans after fertilization in *Caenorhabditis elegans*

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Summary

We previously identified a novel type of caveolin-enriched secretory vesicle in *Caenorhabditis elegans* oocytes. These vesicles undergo synchronous fusion with the plasma membrane immediately after fertilization, suggesting that they could be cortical granules that have been described in diverse animal species. Here, we report that these vesicles are indeed cortical granules, delivering essential chondroitin proteoglycans and mucin-like glycoproteins to the early embryonic extracellular matrices (ECMs). Furthermore, we have found that the small GTPase RAB-11 and the target-SNARE SYN-4 are required for cortical granule excoytosis after fertilization. In oocytes, SYN-4 localizes mainly to the plasma membrane, whereas GFP::RAB-11 accumulates transiently on the cortical granules during ovulation, immediately prior to fertilization. Importantly, cytokinesis defects in early embryos are commonly observed after depletion of either *rab-11* or *syn-4*, producing a phenotype very similar to that observed after blockade of chondroitin synthesis. Taken together, our results indicate that at least part of the essential role for RAB-11 and SYN-4 in early embryogenesis is in the targeting of cortical granules to the plasma membrane during the precisely regulated secretion of ECM components.

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Key words: Chondroitin, *C. elegans*, Exocytosis, Fertilization, Cortical granules

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Introduction

Fertilization triggers rapid and synchronous exocytosis of a number of secretory vesicles (cortical granules) from the newly fertilized embryo. These vesicles deliver components that have essential functions in blocking polyspermy, promoting egg activation and enabling embryonic development by restructuring the extracellular environment around the embryo (Wong and Wessel, 2006). Despite the fundamental nature of these events, the molecular machinery that mediates exocytosis of these vesicles is largely unknown.

C. elegans has emerged as a highly amenable model system for the study of oogenesis, fertilization and embryogenesis. One of the key advantages of this system is the ability to observe directly all of these processes within the intact living animal. In *C. elegans*, the germ cells of adult hermaphrodites are contained within a U-shaped tubular gonad (McCarter et al., 1999) (see Fig. 1D). 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 grow as they move towards the region proximal to the spermatheca. Such oocytes are arrested in diakinesis of meiotic prophase I and do not re-enter meiosis until they receive signals from the sperm and the overlying gonadal sheath cells, which promote oocyte maturation and ovulation. Upon receiving these signals, the mature oocyte in the most proximal region ovulates and enters the spermatheca, followed by immediate fertilization in the spermatheca. After fertilization, embryos complete meiosis I and meiosis II, and start zygotic development in the uterus.

Caveolin is the major protein component required for the formation of caveolae on the plasma membrane (PM) of mammalian cells (Drab et al., 2001). *C. elegans* has two caveolin homologues, the CAV-1 and CAV-2 proteins (Tang et al., 1997). Endogenous CAV-1 is expressed strongly in the germ line of adult hermaphrodites (Scheel et al., 1999). To analyze caveolin dynamics in vivo, we previously imaged a fusion of CAV-1 with the green fluorescent protein (CAV-1::GFP) in living animals, and showed that trafficking of CAV-1::GFP is dynamically regulated during the development of the germ line and embryos (Sato et al., 2006) (see Fig. 1D). CAV-1::GFP is first localized primarily to the PM in the distal germ cells, and then as oocytes grow it also gradually appears in small intracellular vesicles and unique ring-shaped structures (CAV-1 bodies) derived from the Golgi in an ARF-1-dependent manner. We found that immediately after fertilization, the CAV-1 bodies synchronously fuse with the PM and that this exocytosis is tightly linked to meiotic cell cycle progression in embryos. Thus, the CAV-1 bodies behave like cortical granules.

After fertilization, *C. elegans* zygotes secrete components of the chitinous eggshell and peri-vitelline fluid, specialized types of extracellular matrices (ECMs) that form an osmotic and mechanical barrier around the embryo. The eggshell contains three distinct layers, with each layer formed at different times (Rappleye et al., 1999). Peri-vitelline fluid contains abundant glycoproteins and fills the extra-embryonic space between the PM of zygotes and the chitinous eggshell. The proper formation of these ECMs is required for meiotic fidelity, polar body extrusion and embryonic polarization

(Johnston et al., 2006). Recent studies in many systems have revealed that ECMs not only provide structural support but also actively affect cell behaviors by regulating the micro-environment. Among these ECMs, chondroitin proteoglycans are widely expressed in the various tissues of animals and play important roles in development, cell adhesion, cell proliferation and morphogenesis (Schwartz and Domowicz, 2002). Strikingly, in *C. elegans*, chondroitin is abundant in the extra-embryonic space in fertilized eggs and is essential for embryonic cytokinesis, as well as normal eggshell formation (Hwang et al., 2003; Mizuguchi et al., 2003).

Here, we identify chondroitin and mucin-like glycoproteins as physiological cargos of the CAV-1 bodies, indicating that CAV-1 bodies are cortical granules of *C. elegans*. Moreover, we show that the small GTPase RAB-11.1 and the syntaxin 1 homolog SYN-4 are required for the exocytosis of the CAV-1 bodies/cortical granules and the delivery of these ECM components after fertilization.

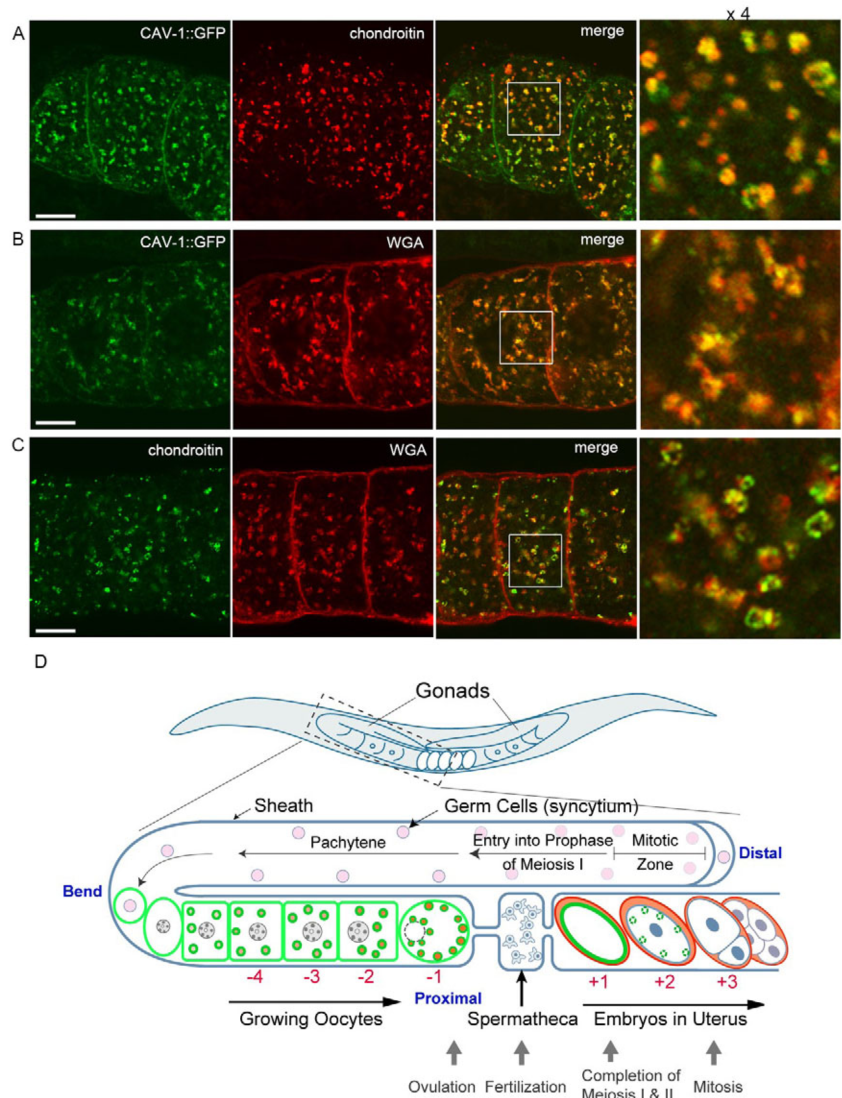

Fig. 1. CAV-1 bodies/cortical granules deliver chondroitin proteoglycans to extracellular space after fertilization. (A,B) The gonads of transgenic animals expressing CAV-1::GFP were stained with an anti-chondroitin antibody (A) or WGA-TRITC (B). CAV-1 bodies were stained with the anti-chondroitin antibody and WGA-TRITC, indicating that these structures contain chondroitin proteoglycans and mucin-like glycoproteins. (C) The gonads of N2 wild-type worms were stained with anti-chondroitin antibody and WGA-TRITC. The right panels are 4× enlargements of the boxed area indicated in merged images. Scale bars: 10 μm. (D) Stylized drawing of one gonad arm connected to the spermatheca and the uterus. Oocytes and embryos are numbered according to their positions from the spermatheca. Schematic localization of CAV-1::GFP is indicated in green. Chondroitin proteoglycans and mucin-like glycoproteins are indicated in orange. Developmental events are assembly-line-like processes and take place at indicated positions of the germline.

Results

CAV-1 bodies are cortical granules that deliver chondroitin proteoglycans to the extracellular space after fertilization

To address the physiological role of the CAV-1 body, we sought to identify its cargo. Our previous experiments showed that CAV-1 bodies are not labeled by FM4-64 or Nile Red, which are markers for endocytic compartments and neutral lipids, respectively (Sato et al., 2006). Rather, our previous work indicated that CAV-1 bodies are regulated secretory vesicles whose exocytosis appears to be coincident with eggshell formation. Furthermore, as described below, conditions that inhibit CAV-1 body exocytosis result in the production of osmotically sensitive embryos, a phenotype associated with a defective eggshell (supplementary material Fig. S1A-C). Thus, we hypothesized that the CAV-1 bodies might be *C. elegans* cortical granules, delivering glycoproteins to the egg surface required for eggshell formation and/or filling of the extra-embryonic space between the zygote and eggshell. Morphologically, the *C. elegans* eggshell consists of three layers: an outer layer derived from the vitelline membrane of the oocytes, a middle chitinous layer and an inner lipid-rich layer (Rappleye et al., 1999). The chitinous layer is formed around embryos immediately after fertilization by chitin synthase CHS-1, which resides at the oocyte PM and catalyzes the polymerization of UDP-N-acetylglucosamine to produce the extracellular chitin layer (Maruyama et al., 2007; Zhang et al., 2005). The inner proteolipid layer is formed later, immediately before the first zygotic cell division. The *C. elegans* eggshell not only functions as an osmotic and mechanical barrier, but also provides a necessary framework for early embryogenic processes. Defects in the eggshell are known to cause loss of meiotic fidelity, polar body extrusion and embryonic polarization (Johnston et al., 2006).

We directly examined whether the CAV-1 bodies contain glycoproteins consisting of the eggshell and the peri-vitelline space. Chondroitin proteoglycans are known to be abundant components of the *C. elegans* eggshell and the peri-vitelline space, and as such represent candidate cargo molecules for CAV-1 bodies (Mizuguchi et al., 2003). Chondroitin is synthesized by the germ cells and gradually accumulates in oocytes as they grow (Mizuguchi et al., 2003). Consistent with chondroitin proteoglycans residing with CAV-1 bodies prior to their secretion, we found that an anti-chondroitin antibody stained ring-shaped structures in the oocytes that colocalized extensively with CAV-1::GFP (Fig. 1A). Mucin-

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like glycoproteins are also known to be abundant constituents of the eggshell and the peri-vitelline space of embryos, and can be detected by wheat germ agglutinin (WGA) (Johnston et al., 2006; Natsuka et al., 2005). WGA also stains rings in the oocytes (Bembenek et al., 2007) and exhibited extensive colocalization with CAV-1::GFP (Fig. 1B). Immunostaining of dissected gonads from wild-type hermaphrodites with anti-chondroitin antibody and WGA showed that chondroitin and mucin-like glycoproteins are present in the same large vesicles in wild-type oocytes, indicating that these regulated secretory granules exist in the wild type and are not dependent on CAV-1::GFP expression (Fig. 1C). Taken together, we conclude that CAV-1 bodies are *C. elegans* cortical granules that deliver chondroitin proteoglycans and mucin-like glycoproteins as intrinsic cargos. It should be noted that, in the gonad, we detected mucin-like glycoproteins in both the CAV-1 bodies and on the oocyte surface, while staining with anti-chondroitin antibody appeared to be specific to the CAV-1 bodies prior to fertilization, implying that the timing of chondroitin secretion is highly regulated.

RAB-11.1 and SYN-4 are required for exocytosis of cortical granules

In wild-type zygotes, CAV-1 bodies/cortical granules undergo nearly synchronous exocytosis during anaphase of meiosis I (Sato et al., 2006; Bombenek et al., 2007). After exocytosis, most CAV-1::GFP is rapidly endocytosed and degraded within one cell cycle (Fig. 2B; supplementary material Movie 1) (Sato et al., 2006). To understand the molecular mechanisms mediating delivery of cortical granules to the PM, we examined the effects of RNAi-mediated depletion of each *C. elegans rab* family GTPase on CAV-1::GFP localization in oocytes and embryos (Sato et al., 2006). Rab GTPase family proteins play pivotal roles in vesicular transport, and each step of vesicular transport is thought to be regulated by at least one Rab protein (Zerial and McBride, 2001). Among the 30 *rab* genes in *C. elegans*, we found that knockdown of *rab-11.1* affected cortical granules exocytosis without apparently affecting their biogenesis (Fig. 2C,D). *rab-11.1* is a homologue of Rab11, which is implicated in the Golgi-to-PM transport and endocytic recycling in mammalian systems. As complete knockout of the *rab-11.1* gene (*tm2341*) resulted in zygotic lethality (see Materials and Methods), we used *rab-11.1(RNAi)* animals for further experiments. In oocytes of *rab-11.1(RNAi)* animals, CAV-1::GFP and mucin-like glycoproteins accumulated together in characteristic ring-shaped structures, suggesting that cortical granule formation was not severely impaired (Fig. 3A). However, the intracellular distribution of cortical granules was aberrant after *rab-11.1(RNAi)*. Cortical granules labeled by CAV-1::GFP appeared abnormally clustered around the nuclear envelope in the proximal oocytes of *rab-11.1(RNAi)* animals, less evenly dispersed in the cytoplasm than in wild-type oocytes (Fig. 2C, arrowheads). Knockdown of *rab-11.1* strongly inhibited exocytosis of the cortical granules, resulting in retention of cortical granules in embryos long after their entry into the uterus (Fig. 2D). These CAV-1::GFP-positive vesicles in arrested *rab-11.1(RNAi)* embryos were stained with WGA, indicating that they are persisting cortical granules rather than endocytic intermediates that are involved in degrading previously exocytosed CAV-1::GFP (Fig. 3C).

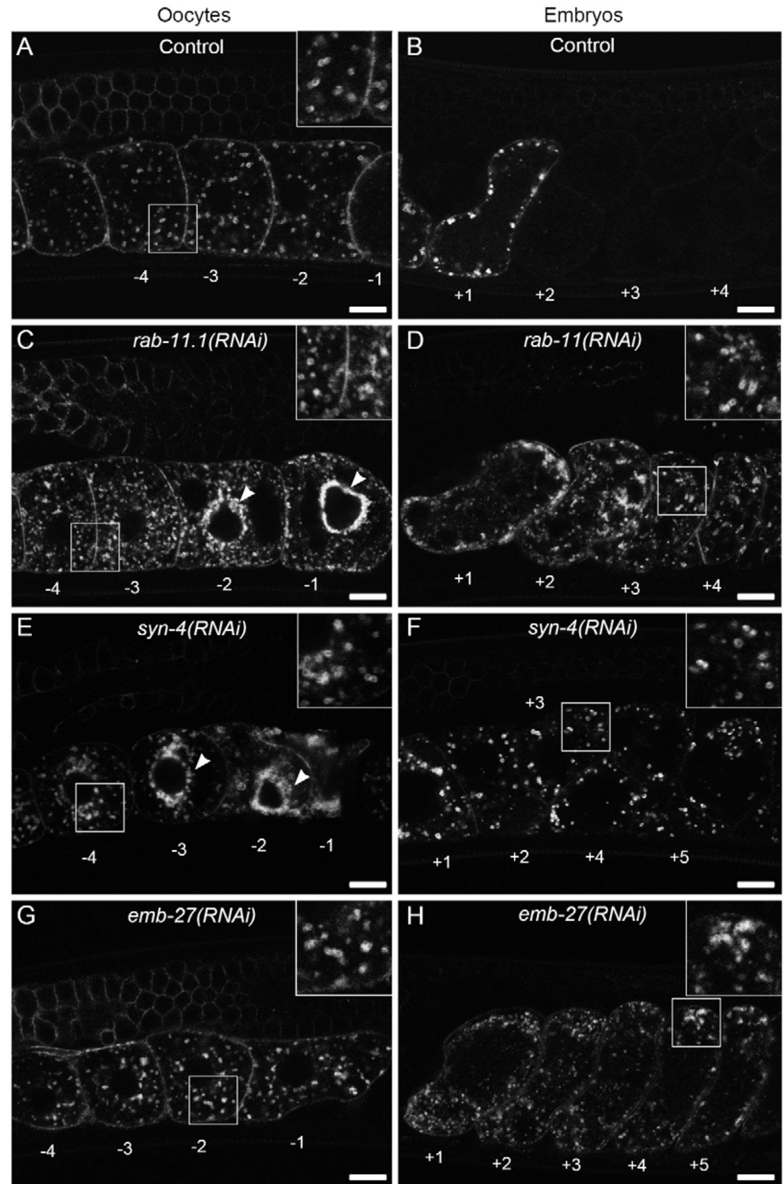

Fig. 2. RAB-11.1 and SYN-4 are required for exocytosis of cortical granules. The subcellular localization of CAV-1::GFP was determined in mock (A,B), *rab-11.1(RNAi)* (C,D), *syn-4(RNAi)* (E,F) and *emb-27(RNAi)* (G,H) animals by confocal laser microscopy. (A) In mock RNAi-treated animals, CAV-1::GFP gradually appears in ring-shaped structures (CAV-1 bodies/cortical granules, the inset) as oocytes grow. (B) The cortical granules are synchronously exocytosed in fertilized embryos and CAV-1::GFP targeted to the PM is then internalized for degradation within one-cell cycle. (C-F) RNAi of *rab-11.1* or *syn-4* causes accumulation of cortical granules around the nuclear envelope in oocytes (C,E, arrowheads) and inhibits fusion of cortical granules with the PM in embryos after fertilization (D,F). In the *emb-27(RNAi)* worm, the localization of CAV-1::GFP is normal in oocytes (G) but cortical granules remain in embryos (H). Oocytes and embryos are maturated from left to right. The numbers indicate the positions of oocytes and embryos from the spermatheca. The insets show 2× enlargements of the boxed area. Scale bars: 10 μm.

In addition to Rab GTPases, docking and fusion of vesicles with their target membranes is regulated by the assembly of vesicle-

SNARE (v-SNARE) and target-localized SNARE (t-SNARE) proteins (Hong, 2005). Among the 10 genes encoding syntaxin related t-SNAREs in *C. elegans,* knockdown of *syn-4* led to a very similar phenotype to that observed in the *rab-11.1(RNAi)* animals (Fig. 2E,F). By sequence similarity, SYN-4 is predicted to belong to the syntaxin 1 subfamily, t-SNARE proteins that are involved in the Golgi-to-PM transport in mammals (Lafont et al., 1999). Complete knockout of the *syn-4* gene (*ok372*) caused a maternal effect sterile phenotype, presumably owing to abnormal gonadogenesis (supplementary material Fig. S2). In *syn-4(RNAi)* animals, cortical granules containing mucin-like glycoproteins were generated but appeared clustered around the nucleus in the proximal oocytes (Fig. 2E; Fig. 3B). In *syn-4(RNAi)* embryos, the cortical granules remained in the cytoplasm (Fig. 2F; Fig. 3D). These results suggest that RAB-11.1 and SYN-4 both function in targeting of the cortical granules to the PM.

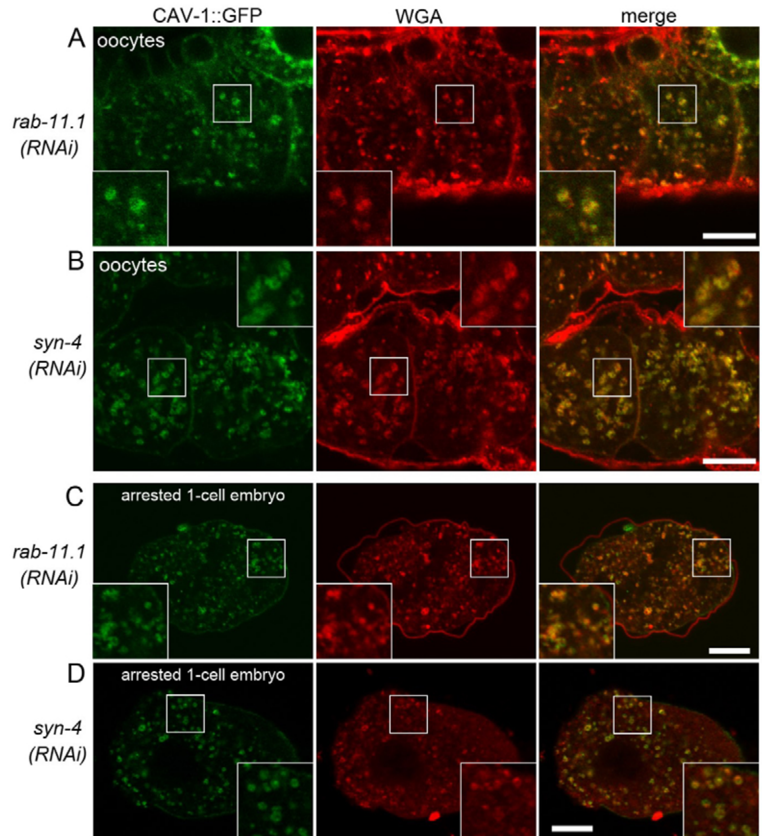

Fig. 3. Cortical granule exocytosis requires RAB-11.1 and SYN-4. (A,B) Cortical granule formation is not impaired by *rab-11.1(RNAi)* and *syn-4(RNAi)*. The gonad of *rab-11.1(RNAi)* or *syn-4(RNAi)* animals were stained with WGA-TRITC. (C,D) Cortical granule exocytosis is inhibited in *rab-11.1(RNAi)* and *syn-4(RNAi)* embryos. Embryos were dissected out from the adult worms and stained with WGA-TRITC. Multinucleated embryos arrested at the one-cell stage are shown. The insets show 2× enlargements of the boxed area. Scale bars: 10 μm.

RAB-11.1 transiently localizes to cortical granules in oocytes

In order to determine whether RAB-11.1 is directly involved in cortical granules exocytosis, we analyzed the subcellular localization of RAB-11.1 in live animals by using a transgenic line expressing low-levels of GFP::RAB-11.1 in the germline. In growing oocytes GFP::RAB-11.1 mainly localized to tubulovesicular structures beneath the PM, probably representing endocytic recycling compartments (Fig. 4A,B, –2 and –3 oocytes). Some GFP::RAB-11.1 localized to the Golgi-like punctate structures deeper in the cytoplasm (Fig. 4A, arrowheads). Interestingly, we observed that in ovulating oocytes the cortical localization of GFP::RAB-11.1 disappeared and GFP::RAB-11.1 accumulated transiently in characteristic ring-shaped structures (the oocyte labeled as –1 in Fig. 4A,B). GFP::RAB-11.1 was observed on similar ring-shaped structures in early one-cell stage embryos, but was only detected on smaller punctate structures and the PM in later stage embryos (Fig. 4C).

To verify localization of RAB-11.1 on the cortical granules, we examined the behavior of GFP::RAB-11.1 under conditions that prevent entry into anaphase I of meiosis, the cell cycle stage during which cortical granules are exocytosed. *C. elegans emb-27* encodes an ortholog of the cdc16p subunit of the anaphase-promoting complex (APC) that is essential for meiotic cell cycle progression (Golden et al., 2000). Knockdown of *emb-27* results in accumulation of one-cell stage embryos arrested in metaphase of meiosis I (Golden et al., 2000) and blocks the fusion of cortical granules to the PM (Sato et al., 2006). In *emb-27(RNAi)* embryos, the cortical granules resided close to the PM but never fused with the PM (Fig. 2G,H). We found that in *emb-27(RNAi)* embryos GFP::RAB-11.1 localized to the ring-shaped structures for a prolonged period after fertilization, suggesting that the GFP::RAB-11.1-positive structures are the cortical granules (Fig. 4D). In aged *emb-27(RNAi)* embryos, some of the GFP::RAB-11.1 population was released from the ring-shaped structures and redistributed to large cytoplasmic foci (Fig. 4D, asterisks). Finally, we confirmed that GFP::RAB-11.1 localized to WGA-positive vesicles in the early one-cell stage wild-type embryos (Fig. 4E-H). These results demonstrate that GFP::RAB-11.1 transiently accumulates on the cortical granules shortly before their synchronous exocytosis, suggesting a direct role for RAB-11.1 in cortical granule exocytosis.

SYN-4 and SNB-1, a synaptobrevin homolog, localizes to cortical granules

It has previously been reported that endogenous SYN-4 localizes mainly to the PM in oocytes, consistent with a predicted function for SYN-4 in secretion (Fig. 5A) (Jantsch-Plunger and Glotzer, 1999). We re-evaluated this using the original SYN-4 antibodies, and found that SYN-4 is also present in cortical granules, especially in the most proximal oocytes (Fig. 5A, arrowheads).

We also sought a corresponding v-SNARE, which localizes on the cortical granules. We found that endogenous VAMP1 homolog SNB-1 colocalized with CAV-1::GFP and WGA on ring-shaped structures in oocytes, demonstrating that SNB-1 is expressed in the germline and localizes on the cortical granules (Fig. 5B,C). In oocytes, SNB-1 was also found on cytoplasmic small vesicles and on the PM. Although SNB-1 is one of the worm homologues of synaptobrevin that is required for normal neuronal functions (Nonet et al., 1998), its localization and role in the germline has not been previously addressed. We further confirmed that GFP::SNB-1 or mCherry::SNB-1 expressed in the germline showed a very similar localization pattern to that of endogenous

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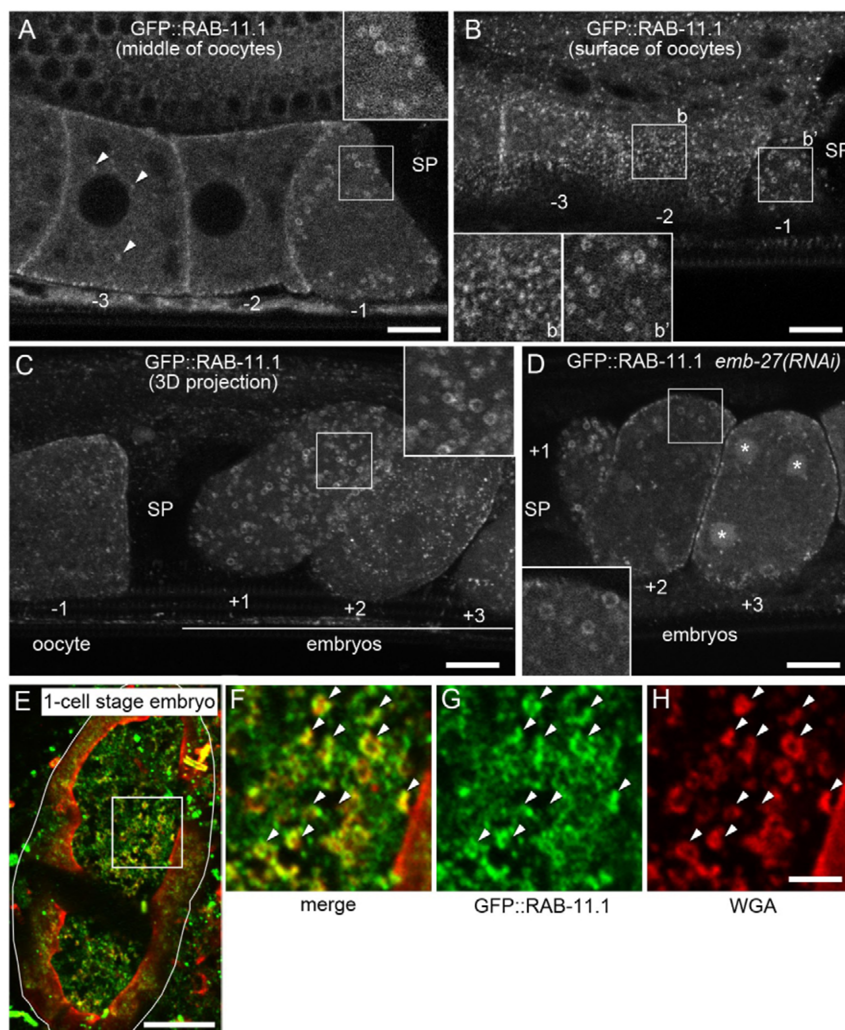

Fig. 4. Dynamic behavior of GFP::RAB-11.1 during oocyte-embryo transition. (A,B) Subcellular localization of GFP::RAB-11.1 in wild-type oocytes. GFP::RAB-11.1 localizes to the cortical region in growing oocytes (–3 and –2 oocytes) but transiently accumulates on the ring-like structures in the ovulating oocyte (–1 oocyte). Arrowheads indicate Golgi-like punctate structures deeper in the cytoplasm. Middle (A) and top (B) focal planes are shown. (C) Subcellular localization of GFP::RAB-11.1 in wild-type embryos. A projected image of confocal *z*-stacks is shown. GFP::RAB-11.1 localizes on the ring-shaped structures in the early one-cell stage embryo (+1 embryo). (D) Subcellular localization of GFP::RAB-11.1 in *emb-27(RNAi)* embryos. GFP::RAB-11.1 stably localizes on the ring-shaped structures in +1 and +2 embryos arrested at the one-cell stage. Some GFP::RAB-11.1 localizes on large cytoplasmic foci in +3 embryo (asterisks). Insets in A-D show 2× enlargements of the boxed areas. (E-H) GFP::RAB-11.1 localizes on cortical granules in wild-type one-cell stage embryos. Embryos were dissected from animals expressing GFP::RAB-11.1 and stained sequentially with WGA-TRITC and an anti-GFP antibody. Enlarged images of the boxed area in E are shown in F-H. Arrowheads show colocalization of GFP::RAB-11.1 and WGA. Scale bars: 10 μm in A-E; 2.5 μm in H. SP, spermatheca.

SNB-1 in oocytes, and that mCherry::SNB-1 also colocalized with CAV-1::GFP on the cortical granules (Fig. 5D; supplementary material Fig. S3). As *snb-1* knockout animals (*js124*) die at the L1 larval stage and never become adult (Nonet et al., 1998), we examined the effect of RNAi knockdown or a partial loss of function mutant of *snb-1(md247)* on the cortical granule exocytosis. However, we could not detect any obvious defects in the cortical granule exocytosis under these conditions (supplementary material Fig. S4A,B). RNAi of *snb-1* did not phenocopy the L1 lethality of *snb-1(js124)*, suggesting some residual SNB-1 activity after RNAi. To examine the efficiency of RNAi-mediated depletion of SNB-1 in the germline, animals expressing GFP::SNB-1 in the germline were subjected to *snb-1* RNAi (supplementary material Fig. S4C). The level of GFP-SNB-1 was reduced to about 5% of that in mock-treated control animals. The lack of the phenotype might be due to residual SNB-1 activity or redundancy among v-SNAREs.

In fertilized embryos, CAV-1::GFP is targeted to the PM and quickly internalized for degradation within one cell cycle (Sato et al., 2006) (Fig. 2B). Interestingly, GFP::SNB-1 stably localized to the PM and small cytoplasmic vesicles in embryos (Fig. 5E). This observation suggests that degradation of maternal CAV-1::GFP in fertilized embryos is a selective process rather than a bulk degradation of membrane proteins.

RAB-11.1 and SYN-4 are required for proper eggshell formation and cytokinesis

As RAB-11 and SYN-4 are key trafficking regulators and we found that they are enriched on cortical granules prior to and during their exocytosis, we sought to determine whether these proteins are required for secretion of cortical granule components. Genome-wide RNAi analysis has previously reported that *rab-11.1(RNAi)* and *syn-4(RNAi)* embryos are osmotically sensitive (Sonnichsen et al., 2005). We confirmed these findings (supplementary material Fig. S1B,C). This phenotype indicates that the innermost 'lipid-rich' eggshell layer, which is known to provide an osmotic barrier function, is probably defective in these embryos (Rappleye et al., 1999). The cortical granule cargo chondroitin and mucin-like glycoproteins are eggshell components, and failure to secrete these components could explain the osmotic sensitivity that was observed.

To test directly the integrity of the eggshell barrier to small molecules, we examined permeability of embryos to the lipophilic dye FM4-64. FM4-64 cannot normally pass through the mature eggshell, preventing staining of the cells within. Conversely, mutant embryos with a defective eggshell inner layer are known to stain with FM4-64 (Johnston et al., 2006; Rappleye et al., 1999). Although the immature eggshell surrounding the embryo just after fertilization is permeable to FM4-64 (Fig. 6A, an embryo labeled as +1), embryos with a complete eggshell are protected from contact

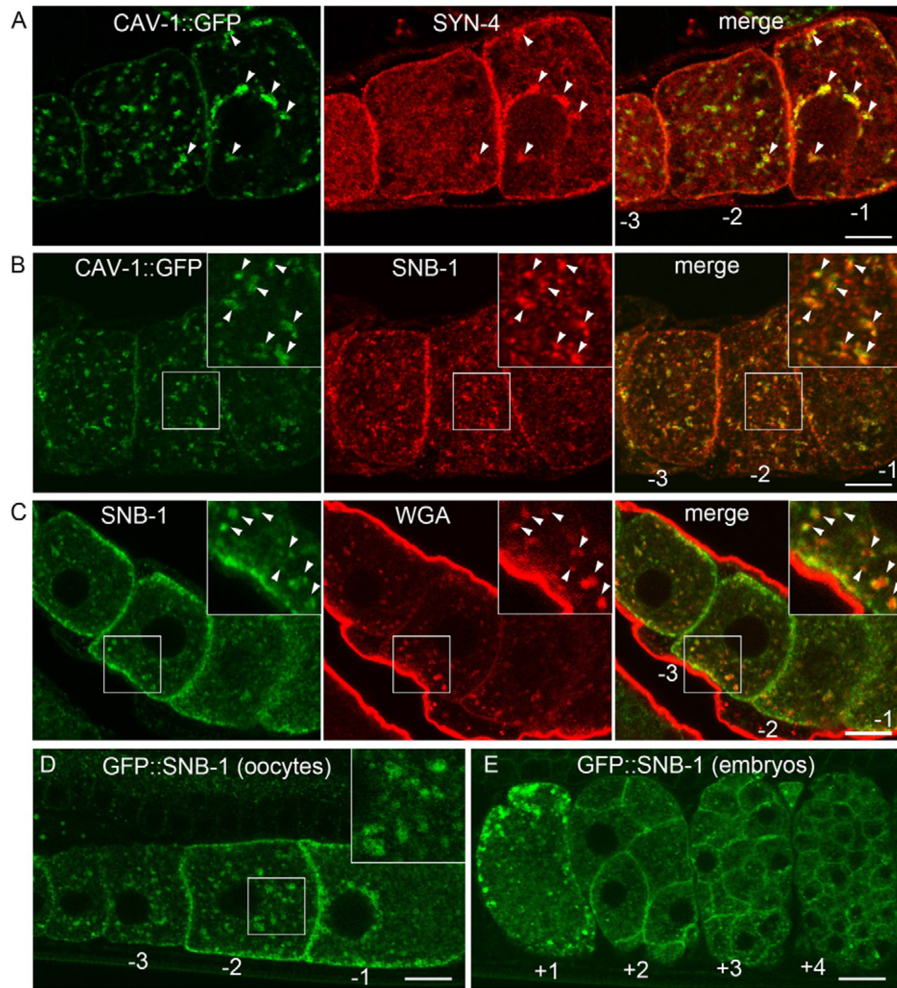

Fig. 5. Subcellular localization of SYN-4 and SNB-1 in oocytes and embryos. (A,B) The gonads of animals expressing CAV-1::GFP were stained with anti-SYN-4 (A) or anti-SNB-1 (B) antibodies. SYN-4 localizes mainly on the PM but a part of SYN-4 is detected on cortical granules (arrowheads in A). SNB-1 localizes on the cortical granules (arrowheads in B) and small cytoplasmic vesicles. (C) The gonads of wild-type animals were stained with anti-SNB-1 antibody and WGA-TRITC. SNB-1 localizes to cortical granules labeled with WGA (arrowheads). (D,E) Subcellular localization of GFP::SNB-1 in the germline. In oocytes, GFP::SNB-1 localizes to ring-shaped structures (D). Unlike CAV-1::GFP, GFP::SNB-1 is not degraded in embryos and the GFP signal is stably detected in developing embryos (E). The insets show 2× enlargements of the boxed areas. Scale bars: 10 μm.

with the dye and remain unlabeled (Fig. 6A, +2 and +3 embryos). *emb-27(RNAi)* and *rab-11.1(RNAi)* embryos, which fail in cortical granule exocytosis, stained readily with FM4-64, indicating a defective eggshell (Fig. 6B,C). However the FM4-64 staining pattern appeared somewhat different in *emb-27(RNAi)* and *rab-11.1(RNAi)* embryos, implying that although both depletions lead to permeable eggshells, *emb-27(RNAi)* and *rab-11.1(RNAi)* differ to some extent in their defective osmotic support or cellular osmoregulation.

We also examined chitin layer formation in the eggshell that is directly synthesized by chitin synthase CHS-1 on the PM. When wild-type animals were fixed and incubated with a fluorescently labeled chitin-binding domain (ChBD), a commercial chitin-specific probe, the eggshell of one-cell stage embryos was stained (Zhang et al., 2005) (Fig. 7A). However, most embryos at the two-cell or later stages remained unstained, suggesting that the accessibility of the ChBD probe to the chitinous middle eggshell layer is blocked in fully matured eggshells (Zhang et al., 2005). We also observed that very early embryos, as identified by the cortical granules that had not yet been exocytosed, were stained with ChBD, indicating that chitin deposition precedes deposition of the chondroitin-rich lipid layer (data not shown). In *emb-27(RNAi)* embryos in which cortical granule exocytosis was blocked, ChBD labeled the embryo next to the spermatheca, indicating that chitin synthesis and remodeling are independent of cortical granule exocytosis and occurs prior to or during metaphase I (Fig. 7C). As in wild type, older *emb-27(RNAi)* embryos were not labeled, indicating that maturation of the eggshell necessary for ChBD exclusion also occurs without cortical granule exocytosis or proper cell-cycle progression. However, we found that in *rab-11.1(RNAi)* animals, most embryos were uniformly stained with ChBD, suggesting that *rab-11.1* is also involved in a cortical granule exocytosis-independent pathway that normally confers ChBD inaccessibility to the chitin layer of the eggshell (Fig. 7B).

It has been previously reported that both *rab-11.1* and *syn-4* are required for embryonic cytokinesis; RNAi of ether gene results in multinucleated embryos (Jantsch-Plunger and Glotzer, 1999; Skop et al., 2001). We confirmed this observation for *rab-11.1* and *syn-4,* finding multinucleated embryos after RNAi for either gene (supplementary material Fig. S1D-F). Notably, deficiency of chondroitin proteoglycans, which are the cargo of the cortical granules, is known to result in a similar cytokinesis defects in early embryos (Hwang et al., 2003; Mizuguchi et al., 2003; Olson et al., 2006). Thus, our results suggest that the cytokinesis defects caused by depletion of *rab-11.1* or *syn-4* is due, at least in part, to the failure in cortical granule exocytosis, resulting in failure to deliver chondroitin proteoglycans to the extra-embryonic space.

CAV-1 is not essential for eggshell formation

Given how well CAV-1 marks the cortical granules, we considered that it might be required for cortical granule biogenesis or function. To address this issue, we first analyzed a deletion mutant, *cav-1(ok2089),* that lacks the entire coding region of the *cav-1* gene. We found that this mutant produces a normal number of embryos without any detectable defect in eggshell formation (supplementary material Table S1, data not shown). These embryos were not stained with FM4-64 beyond the one-cell stage, and were not sensitive to changes in the ionic strength of the media (data not shown). When the gonads were dissected from the mutant animals and stained with WGA, we observed normal cortical granule morphology and number in the oocytes (Fig. 8B). We considered that CAV-1 might be functionally redundant with the only other worm caveolin, CAV-

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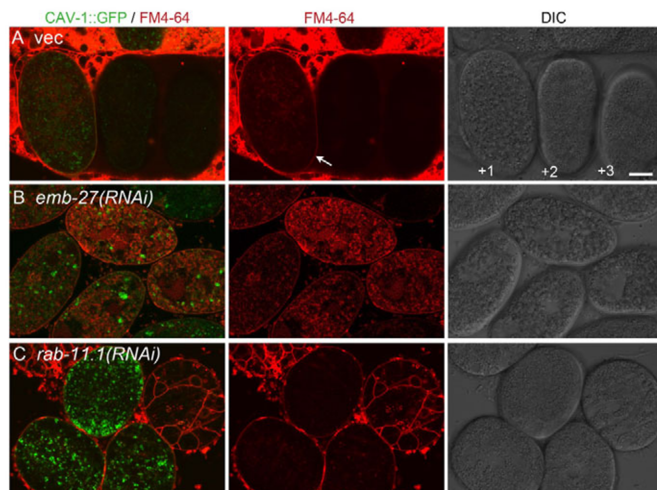

Fig. 6. RAB-11.1 and EMB-27 are required for proper eggshell formation after fertilization. The permeability of embryos to FM4-64 was examined in mock (A), *emb-27(RNAi)* (B) and *rab-11.1(RNAi)* (C) animals expressing CAV-1::GFP. A newly fertilized wild-type embryo is permeable to FM4-64 (+1 embryo in A, an arrow indicates the PM stained with FM4-64) but blastomeres are not (+2 and +3 embryos in A). The *emb-27(RNAi)* and *rab-11.1(RNAi)* embryos are permeable to FM4-64. Nomarski image is on the right in each pair. Scale bar: 10 μm.

2. However, we likewise found that double-knockout mutants lacking both caveolins [*cav-1(ok2089); cav-2(hc191)*] displayed a nearly normal brood size and normal cortical granule morphology and number, and the double mutant eggshell was impermeable to FM4-64 (Fig. 8D, supplementary material Table S1, data not shown). These results indicate that worm caveolins themselves are not essential components of cortical granule biogenesis or exocytosis, although CAV-1::GFP is an abundant cargo molecule of these vesicles. However, *cav-1(ok2089)* and the *cav-1(ok2089); cav-2(hc191)* double mutants showed a slightly slower rate of egg laying compared with that of wild-type animals, implying that CAV-1 has some function to maintain the normal rate of egg production.

Discussion

In our previous work, we have identified large vesicles in the oocyte that are enriched in CAV-1::GFP protein (CAV-1 bodies). The fusion of these vesicles with the embryo PM immediately after fertilization, in a nearly synchronous wave, suggested that these vesicles could be cortical granules, as defined in the oocytes of other animals (Sato et al., 2006). In this study, we further demonstrate that these vesicles deliver chondroitin and mucin-like glycoproteins to the extracellular space, molecules that are required for eggshell formation and/or cytokinesis in the early embryo (Hwang et al., 2003; Mizuguchi et al., 2003). We now also show that RAB-11.1 and SYN-4, a GTPase and a syntaxin 1 homolog, respectively, are each required for regulated cortical granule (CAV-1 body) exocytosis.

Cortical granules transport extracellular matrix components essential for early embryogenesis

Proteoglycans play important roles in animal development by modulating the extracellular environment. In *C. elegans*, chondroitin is known to be required for early embryogenesis and vulva morphogenesis (Hwang et al., 2003; Mizuguchi et al., 2003). Chondroitin proteoglycans consist of core proteins and covalently attached chondroitin chains. Chondroitin is synthesized on a unique tetrasaccharide linker structure (GlcAβ1,3Galβ1,3Galβ1,4Xyl1β) that is built onto specific serine residues of the core proteins, followed by the polymerization of the chain by the addition of N-acetylgalactosamine (GalNac) and glucuronic acid (Hwang et al., 2003; Mizuguchi et al., 2003). Chain initiation and elongation of chondroitin is catalyzed by chondroitin synthase, SQV-5. Loss of function of *sqv-5,* which is specifically involved in chondroitin synthesis, causes cytokinesis defects in embryos, resulting in multinucleated cells (Hwang et al., 2003; Mizuguchi et al., 2003). Recently, CPG-1 and CPG-2, chitin-binding proteins, were identified as core proteins in *C. elegans* that receive chondroitin modification (Olson et al., 2006). Consistent with our direct observation of chondroitin in cortical granules, simultaneous depletion of CPG-1 and CPG-2 reduces the size of cortical granules (Bembenek et al., 2007) and produces a cytokinesis phenotype identical to *sqv-5* knockdown (Olson et al., 2006), suggesting that chondroitin chains on these proteins are important. Strikingly, RNAi of *rab-11.1* or *syn-4* leads to a similar cytokinesis defect that results in multinucleated embryos that continue karyokinesis without undergoing cytokinesis (Jantsch-Plunger and Glotzer, 1999; Skop et al., 2001). Given the observation that chondroitin is an intrinsic cargo of the cortical granules, the impaired secretion of chondroitin proteoglycans after *rab-11.1* or *syn-4* depletion explains, at least in part, the cytokinesis defects of these embryos. However, we found that RNAi of *rab-11.1* or *syn-4* causes a more severe eggshell defect than does chondroitin deficiency. *sqv-5(RNAi)* (Hwang et al., 2003) and *emb-27(RNAi)* embryos are orderly and oval-shaped in the uterus, whereas knockdown of *rab-11.1* or *syn-4* produces irregular-shaped embryos that appear to lack all mechanical support from an eggshell. The pattern of FM4-64 staining was also different between *rab-*

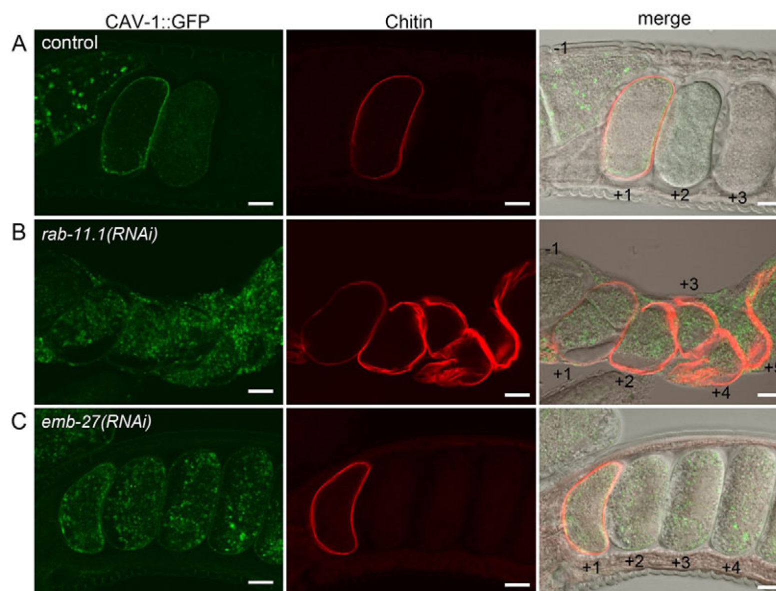

Fig. 7. Chitin layer formation is independent of cortical granule exocytosis. Embryos of animals expressing CAV-1::GFP were stained with rhodamine-conjugated chitin-binding domain (ChBD). (A) Mock; (B) *rab-11.1(RNAi)*; (C) *emb-27(RNAi)*. Nomarski images merged with fluorescent images are also shown on the right in each pair. Scale bars: 10 μm.

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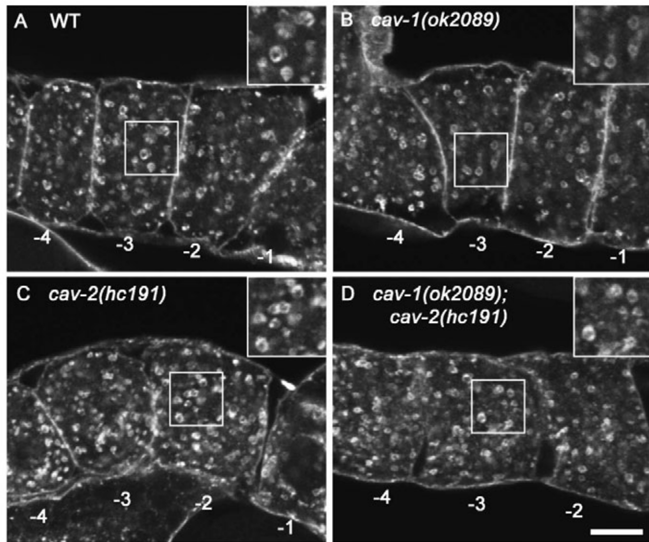

Fig. 8. Caveolins are not required for cortical granule biogenesis. The gonads were dissected and stained with WGA-TRITC. (A) Wild type; (B) *cav-1(ok2089)*; and (C) *cav-1(ok2089); cav-2(hc191)*. The insets show 1.5× enlargements of the boxed areas. Scale bar: 10 μm.

11.1(RNAi) and *emb-27(RNAi)* embryos. Furthermore, *rab-11.1(RNAi)* embryos showed abnormal ChBD accessibility. These observations imply that, in addition to the cortical granule exocytosis, RAB-11.1 and SYN-4 are involved in an additional route of exocytosis that delivers different cargos required for complete eggshell formation. These proteins may also contribute to membrane traffic at the cleavage furrow itself. In addition to exocytosis, RAB-11 could further affect microtubule dynamics, as reported recently (Zhang et al., 2008). We observed that knockdown of *syn-4* inhibits formation of the chitin layer around embryos, implying that *syn-4(RNAi)* also contributes to the earlier chitin deposition directly, or may affect other developmental effects such as fertilization or spermatogenesis (data not shown).

RAB-11.1 and SYN-4 regulate developmentally-controlled exocytosis

Previous reports indicate that, in sea urchin, Rab3 and a VAMP homologue localize to cortical granules, and injection of a peptide corresponding to the Rab3 effector domain interferes with cortical granule exocytosis (Conner et al., 1997; Conner and Wessel, 1998). In *C. elegans*, however, RAB-3 is predominantly expressed in neurons, and a null mutant, *rab-3(js49)*, is viable and fertile (Nonet et al., 1997). Our work indicates that the exocytosis of the cortical granules depends upon the function of RAB11.1 and SYN-4 in *C. elegans*. In mammalian cells, Rab11 is best known for its role in maintaining the endocytic recycling pathway (Wilcke et al., 2000). Indeed, in growing *C. elegans* oocytes, GFP::RAB-11.1 is concentrated in peripheral tubular recycling endosomes (Fig. 4). RNAi-mediated depletion of *rab-11.1* causes a defect in yolk endocytosis by oocytes due to impaired recycling of the yolk receptor (Grant and Hirsh, 1999; Sato et al., 2008). However, we show here that RNAi of *rab-11.1* blocked cortical granule exocytosis, and, moreover, that the subcellular localization of GFP::RAB-11.1 shifts from cortical endosomes to the cortical granules during ovulation. Thus, RAB-11.1 seems to have dual roles, i.e. endocytic recycling in oocytes and regulated exocytosis of the cortical granules in embryos, and these two activities are developmentally controlled.

In agreement with our findings, recent studies have suggested that Rab11 can be involved in exocytosis (Chen et al., 1998; Satoh et al., 2005; Ward et al., 2005). For example, Rab11b localizes to mature synaptic vesicles in the brain and functions as a GTP-dependent switch between the constitutive and Ca^{2+}-regulated exocytic pathway (Khvotchev et al., 2003). Rab11 is also associated with exocytic vesicles that undergo regulated secretion in cytotoxic T lymphocytes (Menager et al., 2007). Thus, Rab11 could be involved in regulated exocytic pathways in many cell types. Interestingly, in *emb-27(RNAi)* embryos, GFP::RAB-11.1 localized to the cortical granules but fusion was still tightly inhibited. This suggests that recruitment of RAB-11.1 to the cortical granules is not sufficient to drive the cortical granules to fuse with the PM. At least one additional step must be required to allow fusion with the membrane. Consistent with this hypothesis, direct observation indicates that the cortical granules redistribute underneath the PM after fertilization, remain quiescent there for a period of time and then fuse with the PM (supplementary material Movie 1). It is possible that the APC complex regulates the activity of RAB-11.1, or some other later exocytic components, to couple transition into anaphase of meiosis I with the secretion of the cortical granules.

In other systems, a family of Rab11-interacting proteins (FIPs), which share the Rab11-binding domain (RBD), and the exocyst complex are known Rab11 effectors (Junutula et al., 2004; Wu et al., 2005; Zhang et al., 2004). We find that RNAi-mediated knockdown of exocyst subunits, or of F55C12.1, the sole RBD protein in *C. elegans*, did not produce any effect on cortical granule dynamics (M.S., B.D.G., A.H. and K.S., unpublished). Thus, cortical granules associated RAB-11 might function through as yet unidentified effectors to promote this regulated exocytosis.

Selective degradation of maternal membrane proteins

Selective degradation of maternal cytoplasmic proteins is an important event for the transition of embryos from meiosis to mitosis, and is strictly controlled by cell cycle progression (Bowerman and Kurz, 2006). Using CAV-1::GFP and GFP::SNB-1, we demonstrated that degradation of membrane proteins after fertilization is highly selective, even if they reached the PM in the same carrier. The yolk receptor RME-2 and the transmembrane fertilization proteins EGG-1 and EGG-2 are also selectively internalized and degraded after fertilization (Audhya et al., 2007; Balklava et al., 2007; Grant and Hirsh, 1999; Kadandale et al., 2005). These observations imply that selective degradation of maternally provided membrane proteins, as well as cytosolic proteins, occurs in early embryos. The selective degradation of cytosolic proteins is regulated by a ubiquitin-dependent process (Pellettieri et al., 2003; Srayko et al., 2000). Mono-ubiquitination of membrane proteins is known to direct their endocytosis (Hicke and Dunn, 2003). Selective ubiquitination of membrane proteins may be the means by which they are targeted for developmentally timed degradation.

Further analysis using this system will offer the opportunity to unveil the molecular mechanisms that regulate membrane trafficking during oogenesis, fertilization and embryogenesis.

Materials and Methods

General methods and strains

Methods for the handling and culturing of *C. elegans* were essentially those described by Brenner (Brenner, 1974). Strains expressing GFP::RAB-11.1, GFP::SNB-1 or GFP::CAV-1 were grown at 25°C, and other strains were at 20°C. The wild-type parent for all strains was *C. elegans* var Bristol strain N2. *unc-119(ed3)* (Maduro and Pilgrim, 1995), *syn-4;*T01B11.4(*ok372*), *snb-1(md247)*, *cav-1(ok2089)* and *cav-2(hc191)* were obtained from the *Caenorhabditis* Genetic

Center. A knockout strain of *rab-11.1(tm2341)* balanced with a rearrangement *hT2[bli-4(e937) let(q782) qIs48]*(I;III) was provided by Shohei Mitani of the Japanese National Bioresource Project for nematodes. This *rab-11.1(tm2341)/hT2* heterozygote produces only viable heterozygotes, dead *hT2* anuploids/homozygotes and dead embryos lacking *hT2*, indicating that *rab-11.1(tm2341)* homozygotes are zygotic lethal. Transgenic strains used were *pwIs281*[P*pie-1*::*CAV-1::GFP*, *unc-119(+)*]; *pwIs61*[P*pie-1::GFP::CAV-1, unc-119(+)*] (Sato et al., 2006); *pwIs33*[P*pie-1*::*GFP::rab-11.1*, *unc-119(+)*]; *dkIs289*[P*pie-1::GFP::snb-1*, *unc-119(+)*]; and *dkIs330*[P*pie-1::mCherry::snb-1, unc-119(+)*] (this study). AZ212 (*unc-119(ed3) ruIs32*[*unc-119*(+) P*pie-1::GFP::H2B*] III) was obtained from the *Caenorhabditis* Genetic Center.

Plasmids and transgenic *C. elegans*

A genomic fragment containing the *snb-1*-coding region and a cDNA fragment of *rab-11.1* were amplified by PCR and cloned into the Entry vector pDONR221 by Gateway recombinational cloning technology (Invitrogen, CA). Then *snb-1* and *rab-11.1* were cloned into pID3.01 (Pellettieri et al., 2003) to create an N-terminal GFP fusion. The genomic *snb-1* fragment was also cloned into a destination vector AZ132_mCherry_Gtwy (a gift from A. Audhya and K. Oegema) to create a mCherry::*snb-1* fusion. pID3.01 and AZ132_mCherry_Gtwy use *pie-1* 5′ and 3′ UTR sequences that drive expression of the transgene in the maternal germ line. Transgenic lines were created by the microparticle bombardment method as described previously (Praitis et al., 2001).

cDNAs of *rab-11.1, syn-4* and *snb-1* were prepared from EST clone provided by Yuji Kohara and subcloned into L4440. As a negative control of RNAi experiments, L4440 harboring cDNA of human transferrin receptor was used. All other feeding RNAi constructs were obtained from the Ahringer genomic RNAi library (Kamath and Ahringer, 2003).

RNA-mediated interference

RNAi experiments in this study were performed using the feeding method (Timmons et al., 2001). Except for RNAi of *syn-4* and *snb-1*, 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 up to young adult worms. L1 or L4 larvae were used for RNAi knockdown of *syn-4* and *snb-1*. Then, P_0 and F1 animals were observed. To examine the efficiency of RNAi-mediated depletion of SNB-1 in the germline, animals expressing GFP::SNB-1 in the germline (*dkIs289*) were subjected to *snb-1* RNAi. Whole-worm lysates were prepared from 40 F1 adults by boiling in Laemmli sampling buffer. Samples were subjected to western blotting using goat anti-GFP antibody (Research Diagnostics, NJ).

Microscopy and immunostaining

To observe live worms expressing transgenes, worms were mounted on agarose pads with 20 mM levamisole in M9 buffer. Staining of dissected gonads was performed as described previously (Grant and Hirsh, 1999; Sato et al., 2005). Briefly, dissected gonads were fixed in 1 ml of 1.25% freshly made paraformaldehyde in egg buffer [118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES (pH7.3)] and washed four times in 1 ml of PTB (1% BSA, 1×PBS, 0.1% Tween20, 0.05% NaN_3, 1 mM EDTA) for 30 minutes at room temperature. For chondroitin staining, the fixed gonads were treated with 10 mU/μl chondroitinase ABC (Seikagaku, Tokyo) at 37°C for 2 hours and incubated with the anti-chondroitin antibody (anti-proteoglycan Di-0S antibody 1-B-5; Seikagaku, Tokyo) diluted 1:1,000 with PTB at 4°C overnight. After washing with PTC (0.1% BSA, 1×PBS, 0.1% Tween20, 0.05% NaN_3, 1 mM EDTA), the gonads were incubated with an antibody against mouse IgG conjugated to Alexa488 or Alexa555 (Molecular Probes) for 3 hours at 20°C. The dissected gonads were washed four times with PTC before observation. Fixed gonads were also stained with anti-SYN-4 and anti-SNB-1 antibodies (Jantsch-Plunger and Glotzer, 1999; Nonet et al., 1998) and rhodamine-conjugated chitin-binding probe (New England BioLabs; 1:500 dilution with PTB). For wheat-germ agglutinin conjugated to tetramethylrhodamine isothiocyanate-dextran (WGA-TRITC) (EY Laboratories, CA) staining, the dissected gonads were fixed in methanol at –20°C for 8 minutes, washed with PTB and incubated with WGA-TRITC diluted 1:100 with the PTB at 4°C overnight. Embryos were fixed and permeabilized by a freeze–crack method (Miller and Shakes, 1995). Then embryos were sequentially stained with WGA-TRITC and an anti-GFP antibody (3E6, Q-BIOgene, CA). For FM4-64 staining, worms were cut in Iwasaki–Teramoto (I–T) solution [136 mM NaCl, 9 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 77 mM glucose, and 5 mM HEPES (pH 7.4)] (Teramoto and Iwasaki, 2006) containing 1 mM FM4-64 (Molecular Probes) and incubated for 10 minutes at 20°C. To examine the osmotic sensitivity of embryos, animals were dissected in 300 mM KCl hypertonic solution and embryos were observed in the same solution. All images were obtained using an Olympus confocal microscope system FV1000 (Olympus, Japan).

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