**INTRODUCTION**

All metazoan animals synthesize in large quantities a tiny number of highly conserved, specialized proteins as provisions of nutrients to their progeny. These include vitellogenins (Vtg), the precursors of yolk proteins (YP) that are deposited in the eggs of nearly all oviparous animals (Sun & Zhang, 2015). In viviparous mammals, which have no Vtg genes (Zhou et al., 2021), this functional role is taken up in a sense by casein, the major protein component of milk. Interestingly, unlike casein proteins, Vtg are not merely nutrients.
allocated to the young. For example, Vtg affects the division of labor in honey bees, that is, hive bees vs foragers (Amdam et al., 2003). In addition, Vtgs can scavenge free radicals, carry metal ions, and exert immunological activities in insects, fish, and corals (Du et al., 2017; Leipart et al., 2022; Sun & Zhang, 2015). There are reports of Vtg/YP participating in intergenerational signal transduction. In C. elegans, maternal age and early-life starvation experience of the mother affect maternal provision of YPs to the progeny, which in turn affects growth, fecundity, and several other physiological traits of the progeny (Jordan et al., 2019; Perez et al., 2017). Another way by which YPs could influence intergenerational inheritance in C. elegans is to act as carriers of double-stranded RNAs and deposit these messenger molecules from the mother to the progeny (Marré et al., 2016).

There is also a connection between Vtg and aging in perennial social insects. In ants and honey bees, the production of Vtg is negatively correlated with that of juvenile hormone (Amdam et al., 2004; Amsalem et al., 2014), which prevents precocious metamorphosis during development and promotes aging of adults (Jindra et al., 2013). Reducing the honey bee Vtg protein levels by RNA interference (RNAi) elevates juvenile hormone and shortens lifespan (Nelson et al., 2007).

Studies of the nematode C. elegans have found repeatedly that expression of the Vtg genes affects adult lifespan in a negative way. There are six Vtg genes in C. elegans, from vit-1 to vit-6. RNAi of one or more Vtg genes has been shown to extend the lifespan of wild-type (WT) C. elegans by ~20% or less (Ezcura et al., 2018; Murphy et al., 2003; Seah et al., 2016). A loss-of-function mutation of ceh-60, a transcription factor that activates the expression of all six Vtg genes, extends lifespan by 40% (Dowen, 2019). Conversely, overexpression of vit-2::gfp suppresses the longevity phenotype of daf-2, glp-1, and eat-2 mutants, although it has no effect on WT lifespan (Seah et al., 2016).

Mechanistic explanation of this negative relationship between Vtg expression and C. elegans lifespan is provided by a series of in-depth investigations in recent years (Ezcura et al., 2018; Kern et al., 2020; Murphy et al., 2003; Sornda et al., 2019; Wang et al., 2018). It is shown that C. elegans does not shut down Vtg production in the intestine after the worm lays the last eggs, which happens typically by AD 5 under the standard culture condition at 20°C. In fact, the yolk protein levels continue to increase up till AD 60, accompanied by atrophy of the intestine, growth of the so-called uterine tumors, and a notable increase of pseudocoelomic yolk patches (previously called pseudocoelomic lipoprotein pools, renamed because these “pools” are too small in young adults) (Ezcura et al., 2018; Kern et al., 2020; Sornda et al., 2019; Wang et al., 2018). Knocking down the Vtg transcripts is shown to ameliorate all aging phenotypes described above, and to extend lifespan (Ezcura et al., 2018; Sornda et al., 2019; Wang et al., 2018). Therefore, post-reproductive vitellogenin production promotes senescent pathologies and accelerates aging (Ezcura et al., 2018).

Interestingly, it was recently found that this seemingly self-harming act of Vtg production by post-reproductive hermaphrodites is actually beneficial to the reproductive fitness of C. elegans, for the yolk vented by old worms can be consumed by larvae, and thus, promote larval growth (Kern et al., 2021).

Although the senescent pathologies related to Vtg/YP have been investigated in detail in the C. elegans system by means of genetics or molecular biology, they have not been examined systematically using immuno-electron microscopy (immuno-EM). In the previous EM studies of C. elegans yolk proteins, the lipid membrane structures were not preserved in the best way, and the somatic tissues were missed as the focus was placed on the gonad and the pseudocoelom (Britton & Murray, 2004; Hall et al., 1999; Herndon et al., 2002; Paupard et al., 2001).

Here, using high-pressure freezing to preserve membrane structures and immuno-gold labeling, we inspected age-dependent changes of vitellogenin vesicles (VVVs), pseudocoelomic yolk patches (YPPs), and yolk organelles (YOs) in multiple tissues. We find that in post-reproductive hermaphrodites of AD 6 and AD 9, intestinal VVs, which are 0.2 μm in diameter on AD 2, fuse with one another at high frequencies and form VVs that are 3–4 μm in median diameter. Occasionally, intestinal VVs of AD 6 and AD 9 worms can exceed 10 μm in diameter and fill up the cytoplasmic space of intestinal cells. For PYPs, we identified two subtypes based on the density of anti-YP170B gold particles. Only the high-density ones accumulate in post-reproductive animals. YOs in oocytes become slightly smaller, from ~0.5 μm in diameter on AD 2 to ~0.4 μm on AD 6 and AD 9. Unexpectedly, YOs, which should be limited to oocytes, are found mislocalized in the hypodermis, uterine cells, and the gonad sheath in post-reproductive worms. Both YOs and the membrane-less yolk are seen in high abundance in the tumor-like masses or oocyte clusters in the uterus, confirming the notion that YP complexes fuel the growth of uterine tumors. Graphical summary and Table 1 summarize the age-dependent changes of Vtg/YP-containing structures as found in this and the earlier EM studies.

The immuno-EM documentation of Vtg/YP-related senescent pathologies in this study confirms and extends earlier studies (Ezcura et al., 2018; Wang et al., 2018) at the ultrastructural level. Our data indicate that Vtg/YP-related senescent pathologies affect more tissues than previously thought and that vesicular fusion is a prominent and previously unknown aspect of those pathological phenotypes. The increase of VV-occupied regions in the intestine and the accumulation of PYPs suggest that gut-to-yolk biomass conversion occurs both inside and outside of the intestine.

2 | RESULTS

2.1 | The size of intestinal VVs increases dramatically with age

We examined the age-associated morphological changes of vitellogenin/yolk protein (Vtg/YP) containing structures in wild-type C. elegans by immuno-EM. Specifically, we used an anti-VIT-1/2 antibody and indirectly conjugated colloidal gold particles to label vitellogenin vesicles (VVVs), PYPs, and yolk organelles (YOs). We compared these
three types of Vtg/YP structures seen in reproductive young adults (adult day 2 or AD 2) with their counterparts in post-reproductive adults (AD 6 and AD 9) and quantified comprehensively the ultrastructural changes.

For context, we illustrate the known developmental relationships between these three Vtg/YP structures in Figure 1. Briefly, vitellogenins are synthesized in the adult intestine, packed into VVs, and then exocytosed out of the intestine to become PYPs (Zhai et al., 2022). Through openings in the gonad sheath, oocytes take up pseudocoelomic yolk and store it in YOs (Hall et al., 1999). The diameter of YOs decreases slightly to ~400 nm (Figure 5a–d). YOs are still present (Figure S3e,g).

| Tissues | AD 1/AD 2 | AD 6/AD 9 | AD 18 |
|---------|-----------|-----------|-------|
| Intestine | VITs are found localized in the rough ER, the Golgi apparatus, and VVs. The median diameter of VVs: 200 nm (Figure 2a,b,e). Exocytosis likely mediates VITs secretion from the intestine to the pseudocoelom (Zhai et al., 2022). | The median diameter of VVs increases to 3–4 μm, occasionally >10 μm (Figure 2c–g). VVs frequently fuse with one another and likely expand in this way (Figure 3). | Almost no detectable VVs in the intestine (Figure S3e). |
| Pseudocoelom | Weak and diffuse VIT-2::GFP and VIT-1/3/6::mCherry signals can sometimes be seen in the pseudocoelom (Zhai et al., 2022). By DIC and fluorescence imaging, most PYPs look like droplets, <5 μm in diameter (Figure S2). | PYPs increase dramatically (Figure S2). PYPs can fuse with each other (Video S1). | PYPs are dispersed throughout the pseudocoelomic space (Figure S3e). |
| Sheath cell | No YPs found inside sheath cells (Hall et al., 1999). | Occasionally, YOs are found inside sheath cells (Figure 6e). | Not seen in sheath cells |
| Oviduct | YPs are barely detectable | YPs accumulate in the oviduct (Figures 6d,e, S3e,g). | |
| Oocyte | Mature oocytes have more YOs than less mature ones, ~500 nm in diameter (Zhai et al., 2022). | The diameter of YOs decreases slightly to ~400 nm (Figure 5a–d). | YOs are still present (Figure S3e,g). |
| Eggs or uterine tumors in the uterus | YOs in eggs look the same as YOs in oocytes, with a diameter of ~500 nm (Zhai et al., 2022). | There are YOs and amorphous yolk in uterus tumors (Figure 5e–g, and Figure S3h,i), and the diameter of YOs inside uterine tumors is ~400 nm (Figure S3j). | |
| Uterine cells | Not seen | Yolk substance frequently seen inside the uterine cells of WT worms on AD 6/9/18 (Figures 6f, 7d,e, S3h). | |
| Distal gonad | Not seen | In worms expressing vit-2::gfp and mCherry-tagged vit-1/3/6, weak (Figure S3a–c) or no (Figure S3d) fluorescent signals were detected in the distal gonad on AD 6–9. Immuno-EM did not detect YPs in the distal gonad of WT worms of the same age. | |
| Hypodermis | Not seen | YOs present inside the hypodermal cells of WT worms on AD 6, 9, and 18 by immuno-EM and conventional EM (Figures 4f, 6a–c, S3e,f). | |
| Muscles | Yolk proteins were not detected by immuno-EM in the body wall muscles of WT worms of any age. This is in disagreement with a published study using a GFP reporter (Turek et al., 2021). | | |

2.2 | Fusion of intestinal VVs

Fusion events between two or multiple VVs are readily detectable in post-reproductive worms (Figure 3a–d). Quantification of the occurrence frequency of VVs caught in the middle of a fusion event, AD 2 VVs look minuscule, whereas AD 6 and AD 9 VVs appear gigantic (Figure 2a–d). In the extreme case, AD 6 and AD 9 VVs can reach above 10 μm across (Figure 2e) and fill up almost the entire cellular space of an intestinal cell (Figure 2f,g).
event indicates that on AD 6 and AD 9, 49.4% and 29.7% of the VVs captured in micrographs are, respectively, in the act of coalescing with one another (Figure 3e). In comparison, this number is only 6.2% for the VVs captured in micrographs on AD 2 (Figure 3e). These findings suggest that intestinal VVs grow by fusion in older worms.

2.3 | Yolk accumulates in the expanded pseudocoelom in older worms

Turning from the intestine to the pseudocoelom, we found that AD 2 PYPs were categorically distinct from the AD 6 and AD 9 counterparts. Although gold particle labeling is found throughout the pseudocoelom regardless of the age of the adult worm, the density of immuno-gold particles attached onto AD 2 PYPs is markedly lower compared with AD 6 and AD 9 PYPs (median value: 19, 130, and 90 gold particles/μm² for AD 2, AD 6, and AD 9, respectively) (Figure 4a–d). This suggests that the concentration of yolk proteins of AD 6 and AD 9 PYPs is more than four times as much as that of AD 2 PYPs. This confirms the previous observations of pseudocoelomic yolk accumulation in old worms by fluorescence microscopy (Ezcurra et al., 2018; Garigan et al., 2002; Herndon et al., 2002). Supporting this conclusion, analysis of the pseudocoelom by conventional EM showed that they have relatively low electron density (expressed as gray value in micrograph) on AD 2 and high electron density on AD 6 and AD 9 (median value 13.1, 31.3, and 31.6, respectively) (Figure 4e–h). Drawing a cutoff of 50 gold particles/μm² for immuno-EM (Figure 4d) or a gray value of 20 for conventional EM (Figure 4h), we classified PYPs into two categories: the low-density ones are predominant on AD 2 and the high-density ones are predominant on AD 6 and AD 9.

To characterize the dynamic process of pseudocoelomic yolk accumulation with age, we examined PYPs in worms expressing VIT-2::GFP and mCherry-tagged VIT-1/3/6. From AD 1 to AD 4, most PYPs look like droplets, and they can fuse to become bigger ones (Figure S2 a–i, and Video S1). Video S1 shows that fusion is fast and dynamic. In post-reproductive worms, PYPs are milk-like and dispersed throughout the pseudocoelom (Figure S2 j–o). Video S2 shows that milk-like PYPs slosh back and forth as the worm moves.

As more PYPs accumulate in the pseudocoelom of older animals, the pseudocoelomic space expands. Using longitudinal EM sections, we quantified the pseudocoelomic area relative to the area occupied by the worm and found that from AD 2 to AD 6 and AD 9, the relative pseudocoelomic area increased from 4% to 20% and 26%, respectively (Figure 4i–j).

To summarize, in post-reproductive C. elegans, while the intestine continues to produce vitellogenins and secret YP complexes to the pseudocoelom, large amounts of high-density PYPs accumulate in and expand the pseudocoelom.
2.4 | YOs in oocytes hardly change with age

In contrast to the dramatic changes of VVs in the intestine and of yolk in the pseudocoelom, YOs found in oocytes remain unchanged by and large. The diameter of YOs decreases only very slightly, from an average of 0.5 μm on AD 2 to 0.4 μm on both AD 6 and AD 9 (Figure 5a–d).

Post-reproductive worms frequently develop uterine tumors, which originate from oocytes (Wang et al., 2018). We detected in uterine tumors immuno-gold labeling in two types of structures: those that looked exactly like YOs and those that were amorphous and not enclosed by a membrane, resembling PYPs (Figure 5e–g). Although speculative, it seems plausible that these amorphous patches may originate from YOs after membrane rupture.

2.5 | Mislocalized yolk in the hypodermis and somatic gonad

Apart from pseudocoelom, we observed that yolk substances also accumulated in the oviduct, which suggested that the yolk flood was overwhelming or the yolk endocytic capacity of old oocytes was compromised in old worms (Figure 6d,e). In AD 6 and AD 9 but not AD 2 hermaphrodites, we observed YO-like structures in the hypodermal cells (Figure 6a–c), the gonad sheath cells (Figure 6e), and uterine cells (Figures 6f, 7d,e). We verified that these mislocalized Vtg/YP structures are enclosed by a lipid bilayer membrane (Figure 6c). These ectopic YOs in the hypodermis and uterine cells resemble the YOs found in oocytes, but can be much larger, sometimes reaching several micrometers in diameter (Figure 6a,b, Figure 7a,b,d,e).
2.6 | Absence of YO fusion in oocytes

We observed fusion of YOs not only in the intestine, but also in hypodermal cells and uterine cells (Figure 7a,b,d,e). As worms age, the frequency of YO appearing in hypodermal cells increases, as does the frequency of fusion of hypodermal YOs, from 0% on AD 2 to 20% on AD 6 and then to 27% on AD 9 (Figure 7c). The frequencies of YO appeared in uterine cells and sheath cells are low, and there are not enough images for quantification.

Yolk organelles are abundant in the oocytes of post-reproductive adult worms, but fusion between oocyte YOs was not observed. Among all the cell types examined, it seems that oocytes have a mechanism to prevent YO fusion, while somatic cells do not.

2.7 | Intestinal atrophy and deterioration of older worms

Intestinal atrophy during aging was previously measured by the relative intestinal width, that is, subtracting the width of the intestinal lumen from the width of the intestine and then normalizing it against the width of the worm body (Ezcurra et al., 2018; Kern et al., 2020). Here, using stereological analysis, we quantified age-associated changes of the intestinal volume in both absolute and relative terms (Figure 8b).

Stereology is a methodology for quantifying three-dimensional characteristics by examining evenly spaced, two-dimensional sections that sample through an entire three-dimensional object (Ferguson et al., 2017). We take advantage of the Cavalieri principle to quantify the absolute volume of the tissue of interest. By analyzing the micrographs of 16–17 cross-sections that were evenly spaced from the head to the tail of a worm, we measured the absolute volume of the body, the pseudocoelom, and the intestine (excluding the luminal space). Two worms each were examined on AD 2, AD 6, AD 9, and AD 18 (Figure 8a,b).

As shown, the absolute volumes of the worm body (with or without the pseudocoelomic space subtracted), the pseudocoelom, and the intestine all peaked on AD 6 (Figure 8a,b). The relative volume of the intestine kept declining, from 24% on AD 2 to 12% on AD 18 (Figure 8b). Hence, intestinal atrophy is evident after AD 6, but arguable from AD 2 to AD 6 because the absolute volume of the intestine increases (from 6.1E5 to 7.3E5 μm³) whereas the relative volume decreases.

Knowing that intestinal VVs grow dramatically from AD 2 to AD 6, with a 15-fold increase in diameter or >3000-fold increase in volume (Figure 2), we wondered whether this underlies the increase of the absolute volume of the intestine. From a random selection of immuno-EM sections, we quantified the total intestinal area and the summed area of VVs in each section and calculated the relative VV-free area. On AD 2, AD 6, and AD 9, the mean value of the percentage of VV-free intestinal area is 99.4%, 86.6%, and 84.8%, respectively (Figure 8c). If the intestinal volume is corrected with the percentage of VV-free intestinal area, then the enlargement of the intestine on AD 6 becomes marginal (6.1E5 and 6.3E5 μm³ for AD2 and AD 6, respectively). This suggests that the apparent enlargement of the intestine on AD 6 can be accounted for by the expansion of VVs. In other words, the external enlargement reflects internal deterioration.

Some of the EM sections recorded impressive examples of intestinal deterioration. In Figure 8d, a representative EM section of an AD 2 worm shows the normal structures, and the top right, of an AD 6 worm, displays the increased intestinal area in a cross-section compared with the one in AD 2. At the bottom left, a cross-section of AD 6 indicated the VV enlargement counted for the age-related increment in the absolute intestinal volume, while the VV-free volume did not get increase. The AD 6 micrograph features two large VVs and an expanded pseudocoelom (Figure 8d). The two VVs almost fill up the entire cross-section of the intestine. At the bottom right, the intestine shrinks dramatically in worms at AD 18. What is shown in the EM images of Figure 8d is consistent with the quantified results (Figure 8a–c).

3 | DISCUSSION

The nematode C. elegans employs a precise mechanism to turn on the vit genes, so that their expression starts exactly at the
beginning of adulthood and is usually limited to the intestine of a worm with a female gonad (Kimble & Sharrock, 1983; Klass et al., 1979). This makes sense because the purpose of vit genes is to generate a nutrient supply for the progeny, but this is costly for the mother. Analogously, expression of vitellogenins of mated C. elegans males may promote post-mating death of those animals (Shi et al., 2017). Another intriguing phenomenon is that C. elegans hermaphrodites do not turn off these genes after the task of reproduction is completed. Post-reproductive mothers continue to make yolk at the cost of intestinal atrophy and shortened lifespan,
which has been characterized in detail (Ezcurra et al., 2018; Sornda et al., 2019), albeit not at the EM level. Here, using immuno-EM, we observed a previously unreported aspect of this intestine-to-yolk biomass conversion: the intestinal atrophy starts internally before the intestine shrinks visibly. It has been shown that the relative intestinal width decreases by about one-third from AD 1 to AD 7 (by ~22% from AD 1–AD 4, and by ~25% from AD 1–AD 8) (Sornda et al., 2019), and by half or more after AD 11 (Ezcurra et al., 2018). The intestinal atrophy occurs internally in a concealed manner in addition to the visible shrinkage. In other words, the
intestinal atrophy is worse than how it looks on the outside, as VVs grow huge from ~0.2 to 3–4 μm across and occupy more and more space inside the intestine (Figure 8c).

In contrast, YOs in oocytes are able to maintain a nearly constant size, with a diameter of 0.5 μm on AD 2 and 0.4 μm on AD 6 and AD 9. This seems to be a unique property of oocytes, because mislocalized YOs in AD 6 and AD 9 hypodermal cells or uterine cells can be several micrometers across, almost as big as the VVs in old intestinal cells.

We find that VVs can grow bigger by fusion with one another (Figure 3). Fusion between mislocalized YOs in somatic tissues was also seen (Figure 7). In contrast, no fusion events were detected for YOs in oocytes, nor for YOs in uterine tumors, which originate from oocytes. We thus conclude that oocytes have a mechanism to prevent fusion between YOs, which is worth investigating in the future.

RME-2 is the only yolk protein receptor so far identified in C. elegans (Grant & Hirsh, 1999). Only oocytes express rme-2 and only late-stage oocytes have an abundance of RME-2 on the cell surface (Grant & Hirsh, 1999). YOs form through RME-2-mediated endocytosis of yolk from the pseudocoelom. Normally, YOs are present only in oocytes and after fertilization, in embryos. It is unclear how the somatic cells of old worms acquire YOs. It could result from misexpression of RME-2 in the hypodermis, gonad sheath, and uterine cells of old worms, or through an RME-2 independent mechanism.

Compared with the dramatic aging pathologies associated with Vtg/YP, there is only a modest lifespan extension of 20% or so by knocking down the vitellogenin genes did not make worms more resistant to oxidants (Sornda et al., 2019), in those experiments, RNAi started at L4 and the treated worms were assayed on adult day 1 (Sornda et al., 2019). Mutations of multiple vit genes have been shown to cause Vtg accumulation and ER stress in the intestine and also sensitivity to pathogenic P. aeruginosa (Singh & Aballay, 2017), but the immunity defects are likely a secondary phenotype of ER stress. In any case, it remains to be tested whether post-reproductive Vtg production affords protection to the mother from oxidants or pathogens.

4 | MATERIALS AND METHODS

4.1 | Worm culture and strains

Caenorhabditis elegans was fed with E. coli OP50 on nematode growth medium (NGM) plates and cultured at 20°C. To produce synchronized cohorts of worms, 25 gravid hermaphrodites were put on a plate and allowed to lay eggs for 4 h before being taken away. Worms were regarded as one-day-old within 24 h after reaching sexual maturity. In this study, five worm strains were used, including wild-type (N2), BCN9071 vit-2(crg9070[vit-2::gfp])[X] MQD2798 vit-1(hq503[vit-1::mCherry]) vit-2(crg9070[vit-2::gfp]) [X] MQD2775 vit-2(crg9070[vit-2::gfp]) vit-3(hq485[vit-3::mCherry])[X] MQD2774 vit-6(hq486[vit-6::mCherry])[IV] vit-2(crg9070[vit-2::gfp])[X].

4.2 | Antibodies

The rat polyclonal anti-VIT-2 antibody (diluted 1:100 for immuno-EM labeling) was kindly provided by Dr. Xiao-Chen Wang (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) (Liu et al., 2012). The epitope of the antibody is a recombinant protein VIT-2 (83–620 amino acid):>6xHIS. The rabbit-derived second antibody (anti-rat) conjugated with 10-nm colloidal gold (Sigma) is available as a commercial product.
4.3 | Immuno-EM methods

The immuno-EM workflow including sample preparation, sectioning, immuno-labeling, and transmission electron microscopy (TEM) imaging are described clearly before (Zhai et al., 2022).

4.4 | Conventional EM sample preparation

Methods of conventional EM sample preparation were developed by Li et al., (2017). Based on the differences in worm samples, we adjusted the methods slightly. High-pressure freezing and
freeze-substitution were used for sample fixation and dehydration as described before (Zhai et al., 2022). The one difference is that the component of the substitution solution contains 1% OsO₄, 0.1% uranyl acetate (UAc), and 98.9% acetone. After that, samples were put into 1 ml UAc saturated acetone solution and stained for 3.5 h at room temperature on a shaker.

After dehydration, samples were infiltrated in SPI-PON 812 resin mixture. The pure resin mixture was made by mixing 19.5 g SPI-PON 812 (SPI-CHEM), 10 g DDSA (SPI-CHEM), 12 g NMA (SPI-CHEM), and 1.5% (v/v) BDMA (SPI-CHEM). Pure resin mixture and acetone were mixed as 1:3, 1:1, 3:1, and pure resin for sample filtration for 2 h, overnight, 24 h, and 48 h. The worm cakes were taken out from carriers carefully using a pair of needles on a 1 ml syringe under a stereo microscope. Then, nematodes were separated, and individually transferred into a cell of the embedding plate that was already filled with the pure resin mixture. Polymerization was performed in a 60°C oven for 48 h.

4.5 | Serial sectioning and scanning electron microscopy imaging

Serial sectioning and scanning electron microscopy (SEM) imaging were conducted using the methods developed by Li et al., (2017). Before SEM imaging, the tapes carrying sections were adhered to SEM Cylinder Specimen Mounts (Electron Microscopy China, Cat. #DP16232) by carbon conductive double-faced adhesive tape (NISSHIN EM Co. Ltd, Japan). The specimen mounts carrying samples were transferred under the SEM (FEI Helios NanoLab 600i) equipped with a CBS detector. Images were acquired by the software xT microscope control (FEI, version 5.2.2.2898) and iFast (FEI) with parameter settings of 2 kV accelerating voltage, 0.69 nA current, and 5 μs dwell time.

4.6 | Image analysis

All quantitative data came from the manual measurement of cellular structures by ImageJ software. The pixel size of the TEM images was calibrated using a standard sample (diffraction grating replica with latex spheres, TED PELLA, INC, prod. #673) at different magnifications, and the pixel size has been reported before (Zhai et al., 2022). The quantitative data were analyzed by GraphPad Prism 8.4.3.

4.7 | SEM image reconstruction

The reconstructed intestine and VVs in Figure 2g were based on 200 serial 70 nm-thick sections. The methods for image alignment were described before (Li et al., 2017), and the aligned continuous images were processed with Imaris (version 9.0.1) for 3D reconstruction.

4.8 | Stereological analysis

The methods of collecting whole worm serial SEM sections and SEM images were described by Li et al., (2017). For adults of different ages, each worm can be cut into over 10,000 serial sections in 50- to 70-nm thick (the exact thickness of every sample and images analyzed are in Table S1 and File S1). Worms at AD 6 and AD 9 could even be cut into about 20,000 serial sections. For stereological analysis, 16 or 17 SEM images (attached in File S1) were selected using the systematic random sampling method. The equation was based on the Cavalieri principle: Volume = \( T \times Area_{point} \times \sum points \); where T means the interval thickness of every adjacent two sections sampled. T values of every worm are in Table S1. Stereological Analyzer (version 4.3.3) software was used to show an evenly distributed point grid covered on an EM image of a cross-cut section. The Area_{point} in the equation means the absolute area of each point represented. Here, Area_{point} is set as 21.34 μm² for worm body volume, and 5.34 μm² for intestinal and pseudocoelomic volume. \( \sum points \) means the total number of points hits on the cellular structures of interest. Relative volume equals the absolute volume over the volume of the whole worm body.

AUTHOR CONTRIBUTIONS
M.-Q.D. and F.S. supervised the project. M.-Q.D. and C.Z. conceived the project, designed the experiments, interpreted data, and drafted this manuscript. C.Z., N.Z., and X.-X.L. performed the EM sample preparation. C.Z. and N.Z. constructed worm strains. C.Z. performed electron microscopy imaging, light microscopy imaging, Western blotting, and data analysis. X.C aligned the continuous images of serial EM sections, and then N.Z reconstructed the 3D model of intestinal cellular structures in Figure 2g. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS
We thank Dr. Xiao-Chen Wang of the Institute of Biophysics, Chinese Academy of Science, for providing the anti-VIT-2 antibody; Dr. Bin Liang of Yunnan University, for providing the vit-2::gfp knock-in worm strain; and the Caenorhabditis Genetics Center, which is supported by the National Institutes of Health Office of Infrastructure Programs (P40 OD010440), for providing the wild-type N2 strain. We thank Drs. Wan-Zhong He of Institute of Chemistry, Chinese Academy of Sciences (ICCAS), and Zhao-Di Jiang of National Institute of Biological Sciences (NIBS), Beijing, for technical guidance in EM sample preparation. We thank Dr. Jian-Guo Zhang, Dr. Gang Ji, and Can Peng of the Institute of Biophysics, Chinese Academy of Science, for guidance in EM imaging. We are grateful to Dr. John Hugh Snyder for critical reading and editing of this manuscript.

FUNDING INFORMATION
This work was funded by National Natural Science Foundation of China (NSFC-ISF 32061143020 to M.-Q.D., 31925026 to F.S., and 31501160 to X.-X.L.), and The Ministry of Science and Technology...
of the People's Republic of China (institutional grants to NIBS, Beijing, a fund of the National High-Level Talents Special Support Program to M.-Q.D.), Beijing Municipal Science and Technology Commission (institutional grants to NIBS, Beijing and a fund for cultivation and development of innovation base to M.-Q.D.).

CONFLICT OF INTEREST
No conflicts of interest exist in the submission of this manuscript.

DATA AVAILABILITY STATEMENT
The authors confirm that the data supporting the findings of this study are available within the supplementary material and corresponding authors, upon reasonable request. The work described is original research that has not been published previously, and is not under consideration for publication elsewhere, in whole or in part.

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How to cite this article: Zhai, C., Zhang, N., Li, X.-X., Tan, X.-K., Sun, F., & Dong, M.-Q. (2022). Immuno-electron microscopy localizes Caenorhabditis elegans vitellogenins along the classic exocytosis route. bioRxiv, 2022.06.27.497668. https://doi.org/10.1101/2022.06.27.497668

Zhou, Y., Shearwin-Whyatt, L., Li, J., Song, Z., Hayakawa, T., Stevens, D., Fenelon, J. C., Peel, E., Cheng, Y., Pajpach, F., Bradley, N., Suzuki, H., Nikaido, M., Damas, J., Daish, T., Perry, T., Zhu, Z., Geng, Y., Rhie, A., ... Zhang, G. (2021). Platypus and echidna genomes reveal mammalian biology and evolution. Nature, 592(7856), 756–762. https://doi.org/10.1038/s41586-020-03039-0