Developmental basis for intestinal barrier against the toxicity of graphene oxide

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Abstract

Background: Intestinal barrier is crucial for animals against translocation of engineered nanomaterials (ENMs) into secondary targeted organs. However, the molecular mechanisms for the role of intestinal barrier against ENMs toxicity are still largely unclear. The intestine of Caenorhabditis elegans is a powerful in vivo experimental system for the study on intestinal function. In this study, we investigated the molecular basis for intestinal barrier against toxicity and translocation of graphene oxide (GO) using C. elegans as a model animal.

Results: Based on the genetic screen of genes required for the control of intestinal development at different aspects using intestine-specific RNA interference (RNAi) technique, we identified four genes (erm-1, pkc-3, hmp-2 and act-5) required for the function of intestinal barrier against GO toxicity. Under normal conditions, mutation of any of these genes altered the intestinal permeability. With the focus on PKC-3, an atypical protein kinase C, we identified an intestinal signaling cascade of PKC-3-SEC-8-WTS-1, which implies that PKC-3 might regulate intestinal permeability and GO toxicity by affecting the function of SEC-8-mediated exocyst complex and the role of WTS-1 in maintaining integrity of apical intestinal membrane. ISP-1 and SOD-3, two proteins required for the control of oxidative stress, were also identified as downstream targets for PKC-3, and functioned in parallel with WTS-1 in the regulation of GO toxicity.

Conclusions: Using C. elegans as an in vivo assay system, we found that several developmental genes required for the control of intestinal development regulated both the intestinal permeability and the GO toxicity. With the focus on PKC-3, we raised two intestinal signaling cascades, PKC-3-SEC-8-WTS-1 and PKC-3-ISP-1/SOD-3. Our results will strengthen our understanding the molecular basis for developmental machinery of intestinal barrier against GO toxicity and translocation in animals.

Keywords: Graphene oxide, Intestinal barrier, Intestinal permeability, Molecular basis, PKC-3, Caenorhabditis elegans

Background

Graphene and its derivatives are two-dimensional carbon engineered nanomaterials (ENMs) with a single layer of sp²-bonded carbon atoms. They have the properties of chemical stability, high coefficient of thermal conduction, amphipathicity, large surface area, and ease of functionalization [1]. Graphene oxide (GO) is one of the important graphene derivatives. Due to its unique physical and chemical properties, GO is potentially applied in the fields of drug delivery, biosensor, and environmental remediation [2–5]. Considering the potential of increasing production and various applications [2], a large amount of GO would be released into the environment. In the recent years, the cytotoxicity of GO in inducing oxidative stress, cell division inhibition, apoptosis, or mutagenicity has been observed in different human cell lines [6–10]. Additionally, at least pulmonary and reproductive toxicities could be detected in mammals [11, 12]. The GO distribution assay has further demonstrated the potential of GO translocation into different targeted organs, such as lung, liver, and kidney, in mammals [11, 13].

Caenorhabditis elegans is a widely used non-mammalian animal model for toxicity assessment and toxicological study of various toxicants, including the ENMs [14–17]. Besides properties of short life-cycle and lifespan,
transparent body, self-fertilization, and ease of culture [18]. *C. elegans* has been shown to be very sensitive to environmental toxicants [14, 19, 20], and frequently used in toxicological study of different ENMs, including the GO [21–27]. In *C. elegans*, GO exposure could cause toxic effects on the functions of both primary targeted organs (such as the intestine) and secondary targeted organs (such as the neurons and the reproductive organs) [22, 28–33]. During the control of ENMs toxicity, bioavailability plays a crucial role in the toxicity induction of ENMs in nematodes [34, 35]. More importantly, intestinal barrier is very important for nematodes against ENMs toxicity and to block translocation of ENMs into secondary targeted organs [30, 36–38]. Nevertheless, the molecular basis for intestinal barrier against ENMs toxicity is still largely unclear in animals. We hypothesized that deficit in intestinal development at certain aspects may alter the function of intestinal barrier and affect the toxicity and the translocation of GO in nematodes.

In *C. elegans*, intestine is a powerful experimental system for different aspects of biological studies, including the stress response [39]. The intestine comprises approximately one third of the total somatic mass in nematodes. In *C. elegans*, intestine is structurally organized by several cellular domains: apical domain including brush border and terminal web, basolateral domain including basement membrane, and apical junctions joining enterocytes to their partners or to adjacent ints into the intestinal structure [39]. The molecular basis for different cellular domains of intestine was summarized in the Table 1. In the apical domain, ACT-5 is required for microvilli development [40], IFB-2 is required for terminal web development [41], ERM-1 is required for connection between actin cytoskeleton and plasma membrane [42], EPS-8 is required for microvilli length control and actin-capping function [43], PAR-3, PAR-6, and PKC-3 are required for apical-basal polarity [44, 45], GEM-4 is required for brush border development [46], and MTM-6 and EAT-3/dynamin are required for endocytosis [47, 48]. In the basolateral domain, LET-413 is required to confine the apical domain [49], NFM-1 acts as a cytoskeletal linker [50], INX-7 is required for cell adhesion [51], and UNC-64/syntaxin, a plasma membrane receptor for intracellular vesicles, regulates vesicle secretion [52]. In the apical junctions, DLG-1 is required for organization of epithelium to a coherent tube [53], AJM-1 is required for junctional integrity [54], EGL-8 is required for vesicle secretion [55], LIN-7 functions as an organizational center for large macromolecular complexes [56], and HMP-1, HMP-2, and HMR-1 are

| Gene    | Encoded protein                      | Function                                                | Reference                                    |
|---------|--------------------------------------|---------------------------------------------------------|---------------------------------------------|
| act-5   | Actin                                | Microvilli development                                  | MacQueen et al., [73]                       |
| ifb-2   | Intermediate filament protein        | Terminal web development                                | Carberry et al., [41]                       |
| erm-1   | Ezrin-radixin-moesin protein         | Connection between actin cytoskeleton and plasma membrane | Gobel et al., [42]                          |
| eps-8   | Cell signaling adaptor protein       | Microvilli length control                               | Croce et al., [43]                          |
| par-3   | PDZ-domain-containing protein        | Apical-basal polarity                                   | Nance and Priess, [44]                      |
| par-6   | PDZ-domain-containing protein        | Apical-basal polarity                                   | Nance and Priess, [44]                      |
| pck-3   | Proein kinase C                      | Apical-basal polarity                                   | Wu et al., [45]                             |
| mtm-6   | Myotubularin                         | Endocytosis                                             | Xue et al., 2003                            |
| eat-3   | Dynamin                              | Endocytosis                                             | Labrousse et al., [48]                      |
| gem-4   | Ca^{2+}-dependent phosphatidyserine  | Bush border development                                 | Church and Lambie, [46]                     |
| let-413 | Scribble                             | Confinde apical domain                                  | Legouis et al., [49]                        |
| nfm-1   | Neurofibrilin                        | Cytoskeletal linker                                      | Culetto and Sattelle, [50]                   |
| unc-64  | Syntaxin                             | Vesicle secretion                                       | Saife et al., [52]                          |
| inx-3   | Gap junction channel                 | Cell adhesion                                           | Altun et al., [51]                          |
| dlb-1   | MAGUK protein                        | Organization of epithelium to a coherent tube           | Segbert et al., [53]                        |
| ajm-1   | Apical junction molecule             | Junctional integrity                                    | Koppen et al., [54]                         |
| egl-8   | Phospholipase Cβ                     | Vesicle secretion                                       | Lackner et al., [55]                        |
| lin-7   | PDZ-domain-containing protein        | Organization of large macromolecular complexes          | Feng et al., [56]                           |
| hmp-2   | β-catenin                            | Tissue integrity of intestinal tube                     | Segbert et al., [53]                        |
| hmp-1   | α-catenin                            | Tissue integrity of intestinal tube                     | Segbert et al., [53]                        |
| hmr-1   | Cadherin                             | Tissue integrity of intestinal tube                     | Segbert et al., [53]                        |
required for tissue integrity of intestinal tube [53]. The aim of this study was to investigate the molecular basis for intestinal barrier against toxicity and translocation of GO using the C. elegans as a model animal. In this study, we first performed a genetic screen of genes required for the function of intestinal barrier against GO toxicity using the technique of intestine-specific RNA interference (RNAi). And then, we focused on the candidate gene of pkc-3 to examine the underlying molecular mechanism for its role in regulating the function of intestinal barrier against GO toxicity. Our results will strengthen our understanding the molecular basis for developmental machinery of intestinal barrier against environmental ENMs in animals.

Results
Identification of intestinal-development related proteins required for the regulation of GO toxicity
VP303 strain is a tool for intestine-specific RNAi of certain genes [57]. Using the VP303 as an intestine-specific RNAi knockdown tool, we tried to identify the possible intestinal-development related genes required for the regulation of GO toxicity. Our previous study has indicated that acute exposure (from L4-larvae for 24 h) to GO at concentrations more than 10 mg/L could result in significant induction of intestinal reactive oxygen species (ROS) production and decrease in locomotion behavior in nematodes [28]. We here first selected the 10 mg/L as the working concentrations for GO exposure. L2-larvae were used to perform the RNAi treatment until the nematodes became L4-larvae. And then, the L4 larvae with RNAi knockdown of certain gene were exposed to GO for 24 h. We used the endpoint of intestinal ROS production to assess the potential toxic effect of GO exposure on the intestinal function. The data and the related information on the efficiency of RNAi of examined genes were shown in Additional file 1: Figure S1 and Table S1. Treatment with paraquat, a ROS generator, was used as a positive control of assay on intestinal ROS production (Additional file 1: Figure S2). Under normal conditions, intestine-specific RNAi knockdown of any examined gene required for the control of development of intestinal apical domain did not induce the significant intestinal ROS production (Fig. 1a). After acute exposure to GO, among the examined genes required for the control of development of intestinal apical domain, intestine-specific RNAi knockdown of ifb-2, eps-8, par-3, par-6, mtm-6, eat-3, or gem-4 did not significantly affect the induction of intestinal ROS production (Fig. 1a). However, after acute exposure to GO, intestine-specific RNAi knockdown of erm-1 or pkc-3 significantly inhibited the GO/Rho B in the body of nematodes (Fig. 1b). The UV/Vis spectral data on GO/Rho B, GO, and Rho B demonstrated the binding of Rho B to GO in the obtained GO/Rho B, since we could observe both the UV/Vis peak of GO and the UV/Vis peak of Rho B in the obtained GO/Rho B (Additional file 1: Figure S3).

Effects of intestine-specific RNAi knockdown of act-5, erm-1, pkc-3, or hmp-2 on distribution and translocation of GO
We next examined the effects of intestine-specific RNAi knockdown of act-5, erm-1, pkc-3, or hmp-2 on distribution and translocation of GO. After GO/Rho B exposure, GO/Rho B could be severely accumulated in the body of nematodes, including the pharynx, intestine, spermatheca, and tail in VP303 nematodes (Fig. 2). Compared with the accumulation and translocation of GO/Rho B in VP303 nematodes, intestine-specific RNAi knockdown of erm-1, pkc-3, or hmp-2 significantly enhanced the accumulation of GO/Rho B in the body of nematodes; however, intestine-specific RNAi knockdown of act-5 significantly inhibited the GO/Rho B in the body of nematodes (Fig. 2). The UV/Vis spectral data on GO/Rho B, GO, and Rho B demonstrated the binding of Rho B to GO in the obtained GO/Rho B, since we could observe both the UV/Vis peak of GO and the UV/Vis peak of Rho B in the obtained GO/Rho B (Additional file 1: Figure S3).

We next focused on the PKC-3 to investigate the underlying cellular and molecular mechanisms for its function in the regulation of toxicity and translocation...
We employed the molecular probe of Nile Red to analyze the possible effect of intestine-specific RNAi knockdown of \( pkc-3 \) on intestinal permeability. Under normal conditions, the Nile Red signals were mainly located in the intestinal lumen in VP303 nematodes, whereas intestine-specific RNAi knockdown of \( pkc-3 \) could cause the significant translocation of Nile Red into the intestinal cells in VP303 nematodes (Fig. 3a).

Moreover, after GO exposure, we observed the more severe translocation and accumulation of Nile Red signals into the intestinal cells and even the body cavity in nematodes with intestine-specific RNAi knockdown of \( pkc-3 \) compared with that in VP303 (Fig. 3b). Meanwhile, under normal conditions, intestine-specific RNAi knockdown of \( pkc-3 \) did not obviously affect the fat storage, because we observed the similar Sudan Black staining.
results between nematodes with intestine-specific RNAi knockdown of pck-3 and VP303 nematodes (Fig. 3b). Additionally, after GO exposure, intestine-specific RNAi knockdown of pck-3 also did not obviously influence the fat storage (Fig. 3b). It has been reported that GO exposure could not alter the fat storage in wild-type nematodes [28]. Considering the fact that the Nile Red can also be used to label the fat storage [58], our results suggest that PKC-3 is required for the regulation of intestinal permeability in nematodes.

Identification of downstream targets for intestinal PKC-3 in the regulation of toxicity and translocation of GO

In *C. elegans*, PAR-3, PAR-6, LGL-1, LIN-5, NLP-29, and SEC-8 may act as the potential targets for PKC-3 [59–64]. We further examined whether these proteins can act as the downstream targets for intestinal PKC-3 in the regulation of GO toxicity. After GO exposure, intestine-specific RNAi knockdown of pck-3 did not significantly affect the transcriptional expressions of par-1, par-6, lin-5, and nlp-29 (Fig. 4a). In contrast, after GO

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**Fig. 2** Effects of intestine-specific RNAi knockdown of intestine-developmental related genes on distribution and translocation of GO. The pharynx (*) and the intestine (**) were indicated by asterisks. Single arrowhead indicates the spermatheca, and double arrowhead indicates the tail. Acute exposure was performed from L4-larvae for 24 h. GO exposure concentration was 10 mg/L. Bars represent means ± SD. **P < 0.01 vs VP303.

**Fig. 3** Effect of intestine-specific RNAi knockdown of pck-3 on intestinal permeability in nematodes. a Effect of intestine-specific RNAi knockdown of pck-3 on intestinal permeability as indicated by the signals of Nile Red. The right shows the comparison of fluorescence intensity of Nile Red signals in intestinal cells. Bars represent means ± SD. **P < 0.01 vs VP303. b Effect of intestine-specific RNAi knockdown of pck-3 on fat storage labeled by Sudan Black. Acute exposure was performed from L4-larvae for 24 h. GO exposure concentration was 10 mg/L.
Fig. 4 (See legend on next page.)
exposure, intestine-specific RNAi knockdown of \textit{pkc-3} significantly increased the transcripational expression of \textit{lgl-1}, and decreased the transcripational expression of \textit{sec-8} (Fig. 4a).

We next focused on the \textit{LGL-1} and \textit{SEC-8} to determine the effects of intestine-specific RNAi knockdown of \textit{lgl-1} or \textit{sec-8} on toxicity and translocation of GO. After GO exposure, intestine-specific RNAi knockdown of \textit{sec-8} significantly enhanced the GO toxicity in inducing intestinal ROS production (Fig. 4b). However, intestine-specific RNAi knockdown of \textit{lgl-1} did not signiﬁcantly affect the GO toxicity in inducing intestinal ROS production (Fig. 4b). After acute exposure to GO, RNAi knockdown of \textit{sec-8} did not affect the survival of nematodes. Moreover, we observed that intestine-specific RNAi knockdown of \textit{sec-8} could noticeably strengthen the accumulation of GO/Rho B in the body of nematodes (Fig. 4c).

Furthermore, under normal conditions, we found that intestine-specific RNAi knockdown of \textit{sec-8} caused the obvious translocation of Nile Red signals into the intestinal cells (Fig. 4d). Additionally, after GO exposure, intestine-specific RNAi knockdown of \textit{sec-8} induced the more severe translocation and accumulation of Nile Red signals into the intestinal cells and even the body cavity (Fig. 4d). Meanwhile, intestine-specific RNAi knockdown of \textit{sec-8} did not noticeably affect the fat storage under the normal conditions, and intestine-specific RNAi knockdown of \textit{sec-8} also did not obviously influence the fat storage after GO exposure (Additional file 1: Figure S4). Therefore, like the \textit{PKC-3}, \textit{SEC-8} may also function in the maintenance of normal intestinal permeability.

To further confirm the potential role of \textit{SEC-8} as the downstream target of \textit{PKC-3}, we investigated the genetic interaction between \textit{PKC-3} and \textit{SEC-8} in the regulation of toxicity and translocation of GO. After GO exposure, the GO toxicity in inducing intestinal ROS production in nematodes with intestine-specific RNAi knockdown of both \textit{pkc-3} and \textit{sec-8} was similar to that observed in nematodes with intestine-specific RNAi knockdown of \textit{pkc-3} and \textit{sec-8} (Fig. 4e). Additionally, the accumulation and translocation of GO in nematodes with intestine-specific RNAi knockdown of both \textit{pkc-3} and \textit{sec-8} was also similar to that observed in nematodes with intestine-specific RNAi knockdown of \textit{pkc-3} or \textit{sec-8} (Fig. 4f). Therefore, \textit{PKC-3} and \textit{SEC-8} may act in the same genetic pathway in the intestine to regulate the toxicity and the translocation of GO.
Fig. 5 (See legend on next page.)
suggest that SEC-8 and WTS-1 may further act in the same genetic pathway in the intestine to regulate the toxicity and the translocation of GO.

Effects of intestine-specific RNAi knockdown of pkc-3 on molecular basis for oxidative stress

In *C. elegans*, mev-1 encodes a subunit of mitochondrial complex II [66], gas-1 encodes a subunit of mitochondrial complex I [67], isp-1 encodes a subunit of the mitochondrial complex III [68], and clk-1 encodes a ubiquinone biosynthesis protein COQ7 [69]. Under normal conditions, intestine-specific RNAi knockdown of pkc-3 did not significantly affect the transcriptional expressions of mev-1, gas-1, isp-1, and clk-1 (Fig. 6a). After GO exposure, although intestine-specific RNAi knockdown of pkc-3 still did not significantly influence the transcriptional expressions of mev-1, gas-1, isp-1, and clk-1, intestine-specific RNAi knockdown of pkc-3 significantly increased the transcriptional expression of isp-1 (Fig. 6a). In *C. elegans*, the isp-1 mutant shows a decreased sensitivity to ROS [68].

In *C. elegans*, sod genes encode superoxide dismutases (SODs), which are required for the animals defending the oxidative stress [70, 71]. Under normal conditions, intestine-specific RNAi knockdown of pkc-3 did not significantly affect the transcriptional expressions of all examined sod genes (Fig. 6b). After GO exposure, although intestine-specific RNAi knockdown of pkc-3 still did not significantly influence the transcriptional expressions of sod-1, sod-2, sod-4, and sod-5, intestine-specific RNAi knockdown of pkc-3 significantly increased the transcriptional expression of sod-3 (Fig. 6b). In *C. elegans*, sod-3 encodes a mitochondrial manganese SOD (Mn-SOD).

Moreover, we observed that intestine-specific RNAi knockdown of isp-1 significantly suppressed the GO toxicity in inducing intestinal ROS production, whereas intestine-specific RNAi knockdown of sod-3 enhanced the GO toxicity in inducing intestinal ROS production (Fig. 6c). After acute exposure to GO, RNAi knockdown of sod-3 did not affect the survival of nematodes. These results imply that intestinal PKC-3 may further regulate the GO toxicity by affecting the functions of ISP-1 and SOD-3.

To determine the underlying mechanism for the function of ACT-5 in regulating GO toxicity and translocation, we further investigated the genetic interaction between

![Figure 5](See figure on previous page.)

**Fig. 5** Identification of downstream targets for intestinal SEC-8 in the regulation of GO toxicity and translocation. a Effects of intestine-specific RNAi knockdown of sec-8 on expressions of wts-1 in GO-exposed nematodes. b Effects of intestine-specific RNAi knockdown of wts-1 on GO toxicity in inducing intestinal ROS production. c Effects of intestine-specific RNAi knockdown of wts-1 on distribution and translocation of GO/Rho B. The pharynx (‘) and the intestine (”) were indicated by asterisks. Single arrowhead indicates the spermatheca, and double arrowhead indicates the tail. d Effects of intestine-specific RNAi knockdown of wts-1 on intestinal ROS production as the toxicity assessment endpoint to investigate the effect of RNAi knockdown of wts-1, sod-3, or both on GO toxicity in nematodes. e Genetic interaction between SEC-8 and WTS-1 in the regulation of GO toxicity and translocation of GO induced intestinal ROS production. f Genetic interaction between SEC-8 and WTS-1 in the regulation of GO toxicity and translocation of GO/Rho B. The pharynx (‘) and the intestine (”) were indicated by asterisks. Single arrowhead indicates the spermatheca, and double arrowhead indicates the tail. Acute exposure was performed from L4-larvae for 24 h. GO or GO/Rho B exposure concentration was 10 mg/L. Bars represent means ± SD. *P < 0.01 vs VP303 (if not specially indicated).
ACT-5 and PKC-3 in the regulation of the toxicity and the translocation of GO. After acute exposure to GO at the concentration of 10 mg/L, intestine-specific RNAi knockdown of *pkc-3* significantly suppressed the resistance of nematodes with intestine-specific RNAi knockdown of *act-5* to GO toxicity in inducing ROS production (Fig. 8a). Moreover, intestine-specific RNAi knockdown of *pkc-3* could obviously disrupt the protection function for intestine-specific RNAi knockdown of *act-5* against translocation and accumulation of GO in targeted organs (Fig. 8b). These results suggest that, in intestinal cells, ACT-5 may act upstream of PKC-3 to regulate the toxicity and the translocation of GO in nematodes.

**Discussion**

In this study, with the aid of VP303 as an intestine-specific RNAi tool for certain genes, we identified the potential intestinal-development related genes required for the control of GO toxicity and translocation by performing a large screen. Based on the assays on the toxicity and translocation, we found that intestine-specific RNAi knockdown of *erm-1, pkc-3*, or *hmp-2* enhanced the GO toxicity and the accumulation of GO in the body of nematodes (Figs. 1 and 2). Our previous study has demonstrated that prolonged exposure to GO from L1-larvae to young adults could significantly decrease the transcripational expression of *pkc-3* [28]. In contrast, prolonged exposure to GO did not significantly alter the transcripational expression of *erm-1* [28]. Although prolonged exposure to GO significantly decreased the transcripational expression of *par-6* [28], intestine-specific RNAi knockdown of *par-6* did not obviously affect the GO toxicity in inducing intestinal ROS production (Fig. 1a). These results imply that the altered expression of some genes, such as *pkc-3*, may provide an important molecular basis for the involvement of developmental state of intestine in the regulation of GO toxicity and translocation.

Moreover, we found that intestine-specific RNAi knockdown of *act-5* could inhibit the GO toxicity and suppress the accumulation and translocation of GO in the body of nematodes (Figs. 1 and 2). This observation suggests that different deficits in intestinal development may have different or even opposite effects on toxicity and translocation of GO. ACT-5 is required for the microvilli development in the apical domain of intestine [73], implying that certain alterations in intestinal microvilli development caused by *act-5* mutation may be helpful for nematodes against the toxicity and the translocation of GO.
translocation of GO. In nematodes, it has been found that the ACT-5 can form coats around the membrane-bound vesicles containing environmental toxicants or pathogens to enhance their endocytosis into the targeted cells [74]. Therefore, the resistance of act-5 mutant to GO toxicity may be formed by suppressing this coating around the membrane-bound vesicles containing environmental ENMs and the endocytosis process in nematodes. In the intestinal cells of nematodes, ACT-5 may further regulate the toxicity and the translocation of GO by suppressing the function of PKC-3 in nematodes (Fig. 8).

Among the identified four genes (erm-1, pck-3, hmp-2, and act-5), erm-1, pck-3, and act-5 are required for the control of connection between actin cytoskeleton and plasma membrane, apical-basal polarity, or microvilli development in the apical domain of intestine [42, 44, 73], and hmp-2 is required for the control of tissue integrity of intestinal tube in the apical junctions [53]. However, among the examined four genes (let-413, nfm-1, unc-64, and inx-3) required for the control of different aspects of development of basolateral domain in the intestine, intestine-specific RNAi knockdown of any of these genes did not obviously affect the GO toxicity in inducing intestinal ROS production (Fig. 1b). Thus, at least the data obtained in this study do not support the potential involvement of developmental state of intestinal basolateral domain in the regulation of GO toxicity.

In C. elegans, pck-3 encodes an atypical protein kinase C, which is required for the normal progression of embryogenesis and viability [45]. In this study, we further observed that intestine-specific RNAi knockdown of pck-3 enhanced the intestinal permeability (Fig. 3). Moreover, after GO exposure, intestine-specific RNAi knockdown of pck-3 resulted in the more severe enhancement of intestinal permeability compared with VP303 nematodes (Fig. 3). In C. elegans, PKC-3 is localized to the outer, apical surfaces of intestinal epithelia [75]. These results imply that the integrity of intestinal epithelium in apical domain of intestine is necessary for the maintenance of normal intestinal permeability in nematodes. Once the nematodes lack the normal
function of PKC-3, the development in the apical surfaces of intestinal epithelia may be disrupted, and the normal intestinal permeability may be difficult to be further maintained.

In this study, we provide several lines of evidence to prove the role of SEC-8 as the downstream target of PKC-3 in regulating toxicity and translocation of GO. Firstly, intestine-specific RNAi of pkc-3 altered the transcriptional expression of sec-8 (Fig. 4a). Secondly, intestine-specific RNAi of sec-8 resulted in the enhanced GO toxicity in inducing intestinal ROS production and GO accumulation in the body of nematodes (Fig. 4b and c). Thirdly, under the normal conditions, the nematodes with intestine-specific RNAi of sec-8 showed the increased intestinal permeability, which was similar to that observed in nematodes with intestine-specific RNAi of pkc-3 (Fig. 4d). Moreover, genetic interaction assay indicated that PKC-3 and SEC-8 acted in the same genetic pathway in the intestine to regulate the toxicity and the translocation of GO (Fig. 4e and f). In C. elegans, sec-8 encodes a subunit of exocyst complex, and may be involved in the endocytosis [76]. Therefore, our results imply that PKC-3 may regulate the intestinal permeability by affecting the function of SEC-8 in the regulation of the process of endocytosis, which in turn influences the response of nematodes to GO exposure.

Moreover, we provide several lines of evidence to further demonstrate the role of WTS-1 as the downstream of SEC-8 in the regulation of toxicity and translocation of GO. Intestine-specific RNAi of sec-8 decreased the transcriptional expression of wts-1 (Fig. 5a). Additionally, intestine-specific RNAi of wts-1 could also induce the enhanced GO toxicity in inducing intestinal ROS production and the more severe GO accumulation in the body of nematodes compared with those in VP303 nematodes (Fig. 5b and c). Moreover, nematodes with intestine-specific RNAi of wts-1 also exhibited the increased intestinal permeability, as observed in nematodes with intestine-specific RNAi of pkc-3 or sec-8 (Fig. 5d). Furthermore, SEC-8 and WTS-1 can act in the same genetic pathway in the intestine to regulate the toxicity and the translocation of GO (Fig. 5e and f). Therefore, in this study, we raise the intestinal signaling cascade of PKC-3-SEC-8-WTS-1 required for the maintenance of intestinal permeability and the regulation of toxicity and translocation of GO. In C. elegans, wts-1 is required for the integrity of the apical intestinal membrane by affecting localization of newly synthesized apical actins, and SEC-8-mediated exocyst complex is required for the mislocalization of apical actin in wts-1 mutant [65]. In this raised intestinal signaling cascade of PKC-3-SEC-8-WTS-1, PKC-3 may regulate the intestinal
permeability and GO toxicity and translocation by affecting the function of SEC-8-mediated exocyst complex in controlling the role of WTS-1 in the maintenance of integrity of the apical intestinal membrane (Fig. 7b).

In this study, we further identified the ISP-1 and SOD-3 as the downstream targets for PKC-3 in the regulation of GO toxicity (Fig. 6). Moreover, we found that RNAi knockdown of both wts-1 and sod-3 led to a more severe GO toxicity in inducing intestinal ROS production than RNAi knockdown of wts-1 and sod-3 (Fig. 7a), suggesting a synergistic effect between WTS-1 and SOD-3 was formed in the regulation of GO toxicity (Fig. 7b). This observation further implies that, in nematodes with RNAi knockdown of pkc-3, the decrease in sod-3 could further enhance the susceptibility to GO toxicity caused by the expressional suppression of wts-1.

It is normally considered that the environmentally relevant concentrations of ENMs were in the range of 1.0 mg/L or μg/L [77, 78]. In this study, we even found the significant induction of intestinal ROS production in nematodes with RNAi knockdown of both wts-1 and sod-3 after acute exposure to GO (100 μg/L) (Fig. 7a). Therefore, our results imply that acute exposure to GO in the range of μg/L may potentially cause the toxic effects on environmental organisms under certain genetic background(s).

Conclusions

In conclusion, we investigated the developmental basis for intestinal barrier against environmental GO toxicity using C. elegans as a model animal. Based on the genetic screen of genes required for intestinal development at different aspects, we identified four developmental genes (erm-1, pck-3, hmp-2 and act-5) necessary for the function of intestinal barrier against GO toxicity. Erm-1, pck-3, and act-5 are required for the connection between actin cytoskeleton and plasma membrane, apical-basal polarity, or microvilli development in apical domain of intestine, and hmp-2 is required for the tissue integrity of intestinal tube in the apical junctions. With the focus on the PKC-3, an atypical protein kinase C, we raised a signaling cascade of PKC-3-SEC-8-WTS-1 that is required for the regulation of both intestinal permeability and GO toxicity. ISP-1 and SOD-3, two proteins required for the control of oxidative stress, were further identified as downstream targets for PKC-3, and a synergistic effect between WTS-1 and SOD-3 was formed in the regulation of GO toxicity. Using the strain with RNAi knockdown of both wts-1 and sod-3 as a tool, we could detect the toxicity of GO in the range of μg/L after acute exposure. Our results will aid our understanding molecular mechanisms for the organization of intestinal barrier to defense the translocation and the toxicity of environmental ENMs in animals.

Methods

Preparation and characterization of GO

GO was prepared from natural graphite as described previously [79]. GO was finally obtained by ultrasonication of as-made graphite oxide in water for 1 h. GO was characterized by atomic force microscopy (AFM, SPM-9600, Shimadzu, Japan), Raman spectroscopy (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK), and X-ray photoelectron spectrum (XPS) (AXIS Ultra instrument, Kratos, UK) [34]. Based on the AFM assay, GO thickness was approximately 1.0 nm in topographic height, and sizes of most of the GO in K-medium after sonication (40 kHz, 100 W, 30 min) were mainly in the range of 40–60 nm [34]. Based on Raman spectroscopy measurement using a 632 nm wavelength excitation, a G band at 1573.7 cm$^{-1}$ and a D band at 1350.2 cm$^{-1}$ were detected in GO sheet [34]. Based on XPS assay, different oxygen functional groups exist in the GO structures (e.g. carbonyl, epoxy, hydroxyl groups), suggesting the considerable degree of oxidation of GO sheets [34].

C. elegans strains and culture

Nematode strains used in this study were wild-type N2 and transgenic strain of VP303/kkbIs7[nhx-2p::rde-1], which were from Caenorhabditis Genetics Center. Nematodes were maintained on normal nematode growth medium (NGM) plates seeded with Escherichia coli OP50 as a food source at 20 °C [18]. Gravid hermaphrodite nematodes were collected and lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl) in order to separate the eggs from the worms. The collected eggs were used to prepare the age synchronous L2-larvae populations.

Exposure and toxicity assessment

The stock solution of GO (1 mg/mL) was prepared in K medium by sonication for 30 min (40 kHz, 100 W). In this study, 0.1, 1, 10 mg/L were selected as the working concentrations for GO exposure. GO at the working concentrations were prepared by diluting the stock solution with K medium. Acute exposure (from L4-larvae for 24 h) to GO was performed in the liquid at 20 °C in the presence of food (OP50).

The endpoint of intestinal ROS production was used to reflect the functional state of the primary targeted organ of intestine [80]. ROS production was analyzed as described previously [81, 82]. After GO exposure, the examined nematodes were transferred to 1 μM 5′,6′-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H$_2$DCFDA; Molecular Probes) solution to incubate for 3 h in the dark. After labeling, the nematodes were mounted on a 2% agar pad and examined at 488 nm of excitation wavelength and at 510 nm of emission filter under a laser scanning confocal microscope.
(Leica, TCS SP2, Bensheim, Germany). Relative fluorescence intensity of ROS signals in the intestine was semi-quantified, and expressed as the relative fluorescence units (RFU). Fifty nematodes were examined per treatment.

**Distribution and translocation of GO in the body of nematodes**

To investigate the translocation and distribution of GO in nematodes, Rho B was loaded on GO by mixing Rho B solution (1 mg/mL, 0.3 mL) with an aqueous suspension of GO (0.1 mg/mL, 5 mL) as previously described [72]. Unbound Rho B was removed by dialysis against distilled water over 72 h. The examined nematodes were incubated with GO/Rho B from L4-larvae for 24 h. After washing with M9 buffer for three times, the nematodes were observed under a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany). The UV/Vis spectral measurements were taken on a Perkin Elmer Lambda 25 spectrophotometer to examine the binding property of Rho B with GO.

**Reverse-transcription and quantitative real-time polymerase chain reaction (qRT-PCR) assay**

Total RNAs of nematodes were extracted using RNeasy Mini kit (Qiagen). The prepared total RNAs were reverse transcribed using PrimeScript™ RT reagent kit (Takara, Otsu, Shiga, Japan). After cDNA synthesis, real-time PCR was performed using SYBR Premix Ex Taq™ (Takara) for the aim of amplification of PCR products. Real-time PCR was run at the optimized annealing temperature of 58 °C. Relative quantification of targeted genes in comparison to a reference gene encoding a tubulin protein was determined. The final results were expressed as relative expression ratio between targeted gene and reference gene. All reactions were performed in triplicate. The related primer information for qRT-PCR is shown in Additional file 1: Table S2.

**RNAi assay**

RNAi was performed by feeding nematodes with *E. coli* strain HT115 (DE3) expressing double-stranded RNA that is homologous to a target gene as described [83]. *E. coli* HT115 (DE3) grown in LB broth containing ampicillin (100 μg/mL) at 37 °C overnight was plated onto NGM containing ampicillin (100 μg/mL) and isopropyl 1-thio-β-D-galactopyranoside (IPTG, 5 mM). L2-larvae were placed on RNAi plates for 2 days at 20 °C until the nematodes became L4-larvae. The RNAi efficiency was confirmed by qRT-PCR (Additional file 1: Figure S1). The obtained L4-larvae with RNAi knockdown of certain gene were used for the further exposure to GO.

**Nile red staining**

Nile Red staining method was performed as described previously [35]. Nile Red (Molecular Probes, Eugene, OR) was dissolved in acetone to prepare a stock solution (0.5 mg/mL), and stored at 4 °C. The stock solution was freshly diluted in 1 x PBS buffer to obtain the working solution (1 mg/mL) for the Nile Red staining. Fifty nematodes were examined per treatment.

**Sudan black staining**

Sudan Black staining method was performed as described previously [58]. The examined nematodes were washed in M9 buffer and fixed with 1% paraformaldehyde. The nematodes were subjected to 3 freeze–thaw cycles and dehydrated through an ethanol series. The nematodes were then stained overnight in a 50% saturated solution of Sudan Black in 70% ethanol, rehydrated, and photographed. Fifty nematodes were examined per treatment.

**Statistical analysis**

Data in this article were expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA), and probability level of 0.01 was considered statistically significant. Graphs were generated using Microsoft Excel software (Microsoft Corp., Redmond, WA).

**Additional file**

- **Additional file 1:** Figure S1. Efficiency of RNAi of examined genes based on qRT-PCR assay. L4440, empty vector. Bars represent means ± SD. **P < 0.01** vs L4440. **Figure S2.** Comparison of intestinal ROS production in GO (10 mg/L) and paraquat (2 mM) exposed VP303 nematodes. Acute exposure was performed from L4-larvae for 24 h. Bars represent means ± SD. **P < 0.01** vs control. **Figure S3.** UV/Vis spectral analysis of GO/Rho B, GO, and Rho B. **Figure S4.** Effects of intestine-specific RNAi knockdown of sec-8 on fat storage labeled by Sudan Black. **Figure S5.** Effects of intestine-specific RNAi knockdown of wts-1 on fat storage labeled by Sudan Black. **Table S1.** Primers used for RNAi of certain genes. **Table S2.** Primer information for qRT-PCR. (DOC 1557 kb)

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**Availability of data and materials**

All the data and materials are available.

**Authors’ contributions**

DW conceived and designed the experiments. MR, LZ, XD, NK, and QR performed the experiments, and analyzed the data. DW wrote the paper. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.
Consent for publication
All authors agree for the publication.

Competing interests
The authors declare that they have no competing interests.

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