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Insulin signaling regulates the toxicity of traffic-related $PM_{2.5}$ on intestinal development and function in nematode *Caenorhabditis elegans*†

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Our previous study has demonstrated the adverse effects of traffic-related $PM_{2.5}$ on *Caenorhabditis elegans*, a non-mammalian toxicological model. In this study, we employed the methods of genetics, gene expression pattern, intestinal autofluorescence, oxidative stress, and intestinal permeability to investigate the molecular control of *in vivo* toxicity from traffic-related $PM_{2.5}$ by the insulin signaling pathway in nematodes. Among genes encoding the insulin signaling pathway, prolonged exposure to $PM_{2.5}$ caused a decrease in the expression levels of *daf-16* and *daf-18* genes and an increase in the expression levels of *daf-2*, *akt-1*, and *pdk-1* genes. Meanwhile, $PM_{2.5}$ exposure increased the DAF-16::GFP nuclear localization. Mutations of the *daf-2* gene encoding the insulin receptor restored deficits in both intestinal development and intestinal function in $PM_{2.5}$ exposed nematodes. In contrast, mutations of the *daf-16* gene encoding the FOXO transcription factor caused more severe damage on both intestinal development and intestinal function in $PM_{2.5}$ exposed nematodes. Moreover, DAF-16 might regulate the toxicity of $PM_{2.5}$ on the intestine through the functions of its downstream targets such as SOD-3, a manganese-superoxide dismutase. Therefore, the insulin signaling pathway may act as an important molecular basis for the toxicity of traffic-related $PM_{2.5}$ in nematodes.

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Introduction

Particle matter (PM) consists of a complex mixture of particles. Among the PMs, the chemistry and biological effects of $PM_{2.5}$ have been extensively studied.[1,2] Epidemiological studies carried out in different regions or countries of the world have suggested that there is an association between $PM_{2.5}$ exposure and the occurrence of a series of clinical diseases.[3–8] Recently, the *in vivo* studies performed in mice or rats have confirmed the adverse effects of $PM_{2.5}$ on organisms.[9–12] Traffic-related $PM_{2.5}$ is a well-defined air pollutant, and a series of epidemiological studies have also demonstrated its toxic effects on human health.[13–15]

Caenorhabditis elegans has been widely used for toxicity assessment and the toxicological study of different toxicants.[16–27] Recently, it has been shown that *C. elegans* can be used for toxicity assessment of toxicants at environmentally relevant concentrations.[28–30] Moreover, *C. elegans* has been successfully employed for the evaluation of toxicity in water,[31] or sediment,[32] implying the important value of *C. elegans* in environmental assessment or monitoring. *C. elegans* is one of the important alterative toxicity assay systems,[33–35] because of its properties of short life cycle, ease of generating mass cultures, low cost, and a well-described genetic background.[36] In *C. elegans*, one of the primary targeted organs for toxicants is the intestine, and the secondary targeted organs for toxicants contain at least neurons and reproductive organs.[35,37–39]

More recently, it has been suggested that $PM_{2.5}$ can be released into the environment and may cause toxicity in environmental organisms.[40,41] In *C. elegans*, our previous study has demonstrated that prolonged exposure to traffic-related $PM_{2.5}$ in the range of $\mu g\ L^{-1}$ could result in toxic effects on the development, lifespan, reproduction, locomotion behavior, and intestinal development of nematodes.[42] The toxic effects were further observed in the progeny of traffic-related $PM_{2.5}$ exposed nematodes.[42] The combinational effects of oxidative stress, damage of intestinal barrier, and abnormal

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defecation behavior may contribute greatly to the formation of traffic-related $PM_{2.5}$ toxicity on nematodes.[42] Nevertheless, the molecular mechanism for $PM_{2.5}$ toxicity is still largely unclear in nematodes.

In *C. elegans*, the insulin/IGF-1 receptor (DAF-2) will initiate a cascade of phosphorylation events that activate several kinases: phosphatidiylinositol 3-kinase (PI3K/AGE-1), 3-phosphoinositide-dependent kinase 1 (PDK-1), and serine/threonine-protein kinase (SGK-1).[43] These kinases will phosphorylate and inactivate the FOXO transcription factor DAF-16 and thereby block the transcription of its targeted genes.[43] DAF-16 influences many biological processes including longevity and stress response.[43,44] Considering the fact that the intestine is usually the crucial primary targeted organ for toxicants in nematodes, the main aim of the present study was to investigate the *in vivo* molecular control of toxicity from traffic-related $PM_{2.5}$ on intestinal development and function by the insulin signaling pathway in *C. elegans*. Our results will be useful for understanding the underlying molecular mechanism for $PM_{2.5}$ toxicity. In addition, our results highlight the possible adverse effects of traffic-related $PM_{2.5}$ on the insulin signaling pathway in organisms.

Materials and methods

Sample collection

Traffic-related $PM_{2.5}$ was collected from a traffic dense area located on the Beiyuan highway and orchard of the Beijing 5th ring road in China. The sampling method and characterization of the examined traffic-related $PM_{2.5}$ have been described previously.[42] The enriched elements in the examined traffic-related $PM_{2.5}$ contained S, Cd, Pb, Zn and Cu, and the examined traffic-related $PM_{2.5}$ also contained several kinds of polycyclic aromatic hydrocarbons (PAHs).[42]

Strain preparation

Nematodes used in the present study were wild-type N2, mutants of *daf-16(mu86)*, *daf-2(e1370)*, *sod-3(gk235)*, *daf-16(mu86);daf-2(e1370)*, *daf-16(mu86);sod-3(gk235)*, and the transgenic strain of *zIs356*[P*daf-16::daf-16a/b::GFP* + *rol-6*]. Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C as described.[36] Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 mol L^{-1} NaOH, 2% HOCl). Age-synchronized populations of L1-larval nematodes were obtained by the collection as described.[16] Because the L1-larvae were more sensitive than the L4-larvae or adults,[45,46] we performed a prolonged exposure from L1-larvae to adult day-1 to investigate the $PM_{2.5}$ toxicity on nematodes. Prolonged exposure was performed for $PM_{2.5}$ at different concentrations (0.01–100 mg L^{-1}) in sterile tissue culture plates in a 20 °C incubator in the presence of food (OP50). If not specially indicated, approximately 1000 nematodes were present in each well of the sterile tissue culture plate. The L1-larval nematodes were transferred from NGM plates into a centrifuge tube by washing with M9 buffer. After centrifugation and discarding the supernatant, the L1-larval nematodes were re-suspended in the $PM_{2.5}$ solution and then transferred into the wells of the sterile tissue culture plates.

Intestinal autofluorescence and reactive oxygen species (ROS) production

The methods were performed as described previously.[47,48] Intestinal autofluorescence is caused by lysosomal deposits of lipofuscin, which can accumulate over time in aging nematodes or nematodes exposed to specific toxicants.[47,49] Images were collected for fluorescence in the endogenous intestine using a 525 nm bandpass filter and without automatic gain control to preserve the relative intensity of fluorescence of different animals. Nematodes were photographed on the same day to avoid the effects of variance in light sources on fluorescence intensity. Fluorescence was recorded, and color images were taken for the documentation of results using the Magnafire® software (Olympus, Irving, TX, USA). Lipofuscin levels were measured using the ImageJ Software (NIH Image, Bethesda, MD, USA) by determining the mean pixel intensity in the intestine of each nematode. Three independent experiments with thirty nematodes per treatment were examined.

To examine the intestinal ROS production, the nematodes were transferred to 1 mL of M9 buffer containing 1 μmol L^{-1} 5′,6′-chloromethyl-2′,7′dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes) in 12-well sterile tissue culture plates to pre-incubate at 20 °C for 3 h in the dark. CM-H_2DCFDA can specially detect the presence of various intracellular produced ROS species. The intracellular ROS can oxidate H_2DCF without fluorescence to generate DCF with green fluorescence. The nematodes were then mounted on 2% agar pads for examination with a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany) at 488 nm of excitation wavelength and 510 nm of emission filter. The relative fluorescence intensity of the intestine was semi-quantified, and the semi-quantified ROS were expressed as relative fluorescence units (RFUs). Three independent experiments with twenty nematodes per treatment were examined.

DAF-16 nuclear translocation assay

DAF-16 nuclear translocation was scored by exposing the transgenic strain of *zIs356* to $PM_{2.5}$. DAF-16::GFP was scored as nuclear localization in the anterior part of the body. Images were taken with a Zeiss Imager A2 fluorescence microscope. Three independent experiments with twenty nematodes per treatment were examined.

Intestinal permeability assay

We used the Nile Red staining method to determine whether the nematodes have a hyper-permeable intestinal barrier. If the nematodes have a hyper-permeable intestinal barrier, a significantly increased Nile Red staining would be observed in them.[50,51] Nile Red staining was performed as previously described.[50,51] Nile Red (Molecular Probes, Eugene, OR) was

dissolved in acetone to produce a 0.5 mg mL^{-1} stock solution and stored at 4 °C. The stock solution was freshly diluted in 1 × PBS buffer to 1 μg mL^{-1}, and 150 μL of the diluted solution was poured onto the NGM plates seeded with OP50. Nematodes were cultured on the plates for 3 days before observation. Three independent experiments with thirty nematodes per treatment were examined.

Considering the fact that Nile Red can be used to label fat storage in nematodes,[42] we also examined whether the observed increased Nile Red staining in nematodes is due to the enhanced fat storage by determining the triglyceride amount. To analyze the triglyceride content, lipids of nematodes were extracted by the Bligh and Dyer method.[52] Triglyceride content was measured using an enzymatic kit (Wako Triglyceride E-test, Wako Pure Chemical Ltd, Osaka, Japan). Ten plates of nematodes were used for each triglyceride content assay. Ten plates of nematodes were transferred into a well of sterile tissue culture plates for $PM_{2.5}$ exposure. Ten replicates were examined per treatment.

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

Total RNA was extracted using an RNeasy Mini Kit (Qiagen). Total nematode RNA (~1 μg) was reverse-transcribed using a cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative reverse transcription PCR was run at an optimized annealing temperature of 58 °C. The relative quantification of targeted genes in comparison with the reference *tba-1* gene encoding a tubulin protein was determined. The final results were expressed as the relative expression ratio (between the targeted gene and the reference *tba-1* gene). The designed primers for targeted genes and the reference *tba-1* gene are shown in Table S1.†

Antioxidant enzyme (superoxide dismutase (SOD) and catalase (CAT)) activity assay

Antioxidant enzyme activities (SOD and CAT) in nematodes were determined using standard photometric assay kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Protein contents were determined using the standard bicinchoninic acid method. To analyze the SOD activity, nematodes were suspended in a cold homogenized buffer (0.01 mol L^{-1} Tris-HCl, 0.0001 mol L^{-1} EDTA-2Na, 0.01 mol L^{-1} sucrose, and 0.8% NaCl), and homogenized using a homogenizer for 6 min on ice. The mixture was centrifuged at 367*g* for 10 min at 4 °C. The upper aqueous layer containing the enzyme was transferred into a new tube for the SOD enzymatic assay. An SOD unit is defined as the amount of SOD corresponding to the inhibition rate of 50% per mg tissue protein in 1 mL of SOD in the reaction solution. To analyze the CAT activity, the enzyme extract was incubated in H_2O_2 for 1 min at 37 °C. The reaction was stopped by the addition of ammonium molybdate. A CAT unit is defined as the decomposition of 1 μmol H_2O_2 per second. The SOD or CAT activity was then measured at an absorbance of 550 or 405 nm, respectively, using a microplate reader and the SOFTmax software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

All data in this article were expressed as means ± standard error of the mean (S.E.M.). Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). The probability levels of 0.05 and 0.01 were considered statistically significant.

Results

Prolonged exposure to $PM_{2.5}$ altered the permeable state of intestinal barrier in nematodes

Our previous study has demonstrated that prolonged exposure to $PM_{2.5}$ at concentrations of 0.1–100 mg L^{-1} caused a significant induction of both intestinal autofluorescence and intestinal ROS production, and prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$ influenced the expression patterns of genes required for intestinal development, suggesting altered intestinal development in $PM_{2.5}$ exposed nematodes.[42] In the present study, we further investigated the possible adverse effects of $PM_{2.5}$ on intestinal function. After prolonged exposure, we used the lipophilic fluorescent dye of Nile Red to stain $PM_{2.5}$ exposed nematodes. We found that prolonged exposure to 0.1–100 mg L^{-1} of $PM_{2.5}$ could significantly increase the relative fluorescence intensity of Nile Red in the intestine of nematodes compared with the control (Fig. 1A and 1B). Meanwhile, there was no significant difference in triglyceride amounts between the control and $PM_{2.5}$-exposed nematodes (Fig. 1C). Therefore, $PM_{2.5}$-exposed nematodes may have a hyper-permeable intestinal barrier rather than an increased lipid accumulation. That is, besides altered intestinal development, damaged intestinal function would also be found in $PM_{2.5}$ exposed nematodes.

$PM_{2.5}$ exposure altered expression patterns of genes encoding the insulin signaling pathway in nematodes

In *C. elegans*, insulin signaling plays a key role in regulating the stress response.[53] At least 8 genes (*daf-16*, *daf-2*, *age-1*, *daf-18*, *akt-1*, *akt-2*, *pdk-1*, and *sgk-1*) encode the insulin signaling pathway in nematodes (Table S2†). We next selected a concentration of 100 mg L^{-1} to investigate the effect of $PM_{2.5}$ exposure on the expression patterns of genes encoding the insulin signaling pathway and the role of the insulin signaling pathway in regulating $PM_{2.5}$ toxicity in nematodes.[42] After prolonged exposure, we found that 100 mg L^{-1} of $PM_{2.5}$ resulted in a significant decrease in the expression levels of the *daf-16* and *daf-18* genes, and a significant increase in the expression levels of the *daf-2*, *akt-1*, and *pdk-1* genes (Fig. 2A). Because the insulin signaling pathway regulates biological processes such as lifespan by limiting DAF-16 nuclear localization,[54] we postulated that $PM_{2.5}$ exposure may also affect DAF-16 nuclear localization in nematodes. Moreover, prolonged exposure to 100 mg L^{-1} of

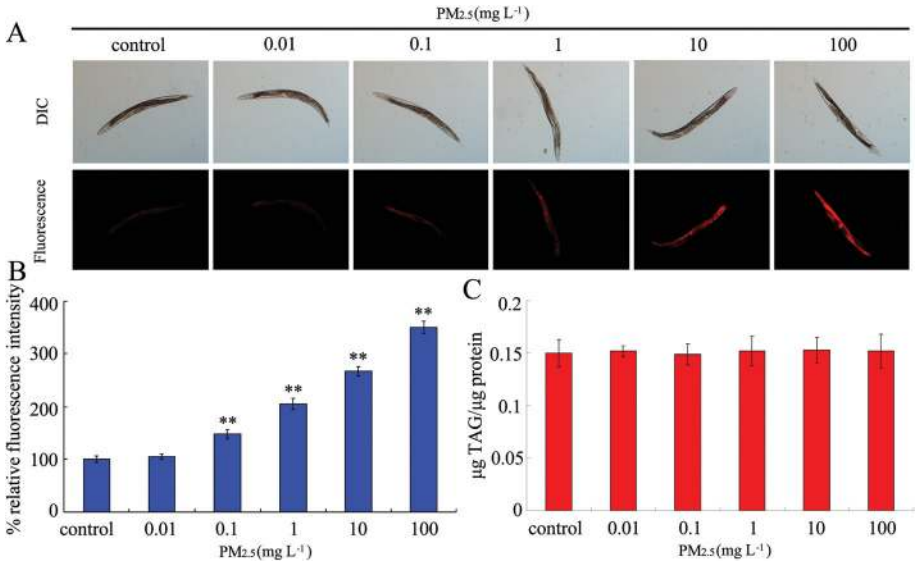

Fig. 1 Effects of $PM_{2.5}$ exposure on intestinal permeability in wild-type nematodes. (A) Nile Red staining of nematodes exposed to $PM_{2.5}$. (B) Comparison of relative fluorescence intensity of Nile Red in nematodes exposed to $PM_{2.5}$. (C) Comparison of the triglyceride amount in nematodes exposed to $PM_{2.5}$. Exposure was performed from L1-larvae to adult day-1. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. control.

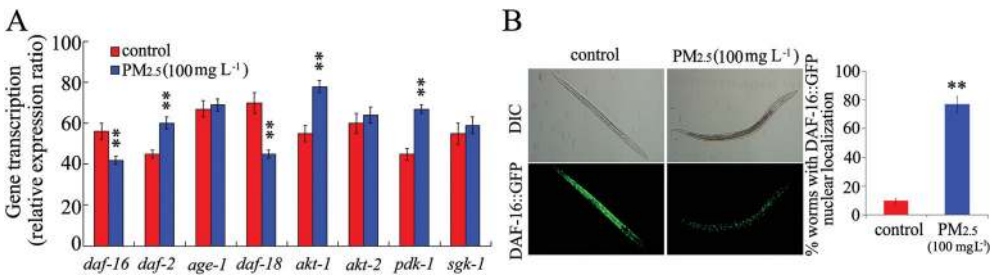

Fig. 2 Effects of $PM_{2.5}$ exposure on expression patterns of genes encoding the insulin signaling pathway in wild-type nematodes. (A) $PM_{2.5}$ altered expression levels of some genes encoding the insulin signaling pathway in wild-type nematodes. (B) $PM_{2.5}$ influenced the nuclear translocation of DAF-16::GFP in nematodes. Exposure was performed from L1-larvae to adult day-1. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. control.

$PM_{2.5}$ caused a significant increase of DAF-16::GFP in the nucleus compared with the control (Fig. 2B). Therefore, $PM_{2.5}$ exposure may not only influence the transcriptional activities of genes encoding the insulin signaling pathway, but also affect the nuclear–cytoplasm translocation of DAF-16 in nematodes.

Mutations of the *daf-2* or *daf-16* gene altered the intestinal development in $PM_{2.5}$ exposed nematodes

In *daf-2* mutants, the blockage of DAF-16 activity is inhibited and the nematodes exhibit the long-lived and stress-resistant phenotypes.[43] We employed the *daf-2* and *daf-16* null mutants to investigate the possible role of the insulin signaling pathway in regulating the toxicity of $PM_{2.5}$ on the intestinal development in nematodes. Mutations of the *daf-2* or *daf-16* gene did not induce a significant induction of intestinal autofluorescence or intestinal ROS production (Fig. 3). After prolonged exposure, we found that the $PM_{2.5}$ (100 mg L^{-1}) exposed *daf-2(e1370)* mutants showed a decreased intestinal autofluorescence compared with the $PM_{2.5}$ exposed wild-type N2 nematodes, and the $PM_{2.5}$ exposed *daf-16(mu86)* mutants exhibited an increased intestinal autofluorescence compared with the $PM_{2.5}$ exposed wild-type N2 nematodes (Fig. 3A). Similarly, after prolonged exposure, we found that the $PM_{2.5}$ (100 mg L^{-1}) exposed *daf-2(e1370)* mutants showed a decreased intestinal ROS production compared with the $PM_{2.5}$ exposed wild-type N2 nematodes, and the $PM_{2.5}$ exposed *daf-16(mu86)* mutants showed an increased intestinal ROS production compared with the $PM_{2.5}$ exposed wild-type N2 nematodes (Fig. 3B). These results suggest that both DAF-2 and DAF-16 regulate $PM_{2.5}$ toxicity on the intestinal development in nematodes.

Genetic interaction of DAF-2 and DAF-16 in regulating $PM_{2.5}$ toxicity on intestinal development

To confirm the role of insulin signaling in regulating $PM_{2.5}$ toxicity on intestinal development, we investigated the intestinal autofluorescence and intestinal ROS production in the

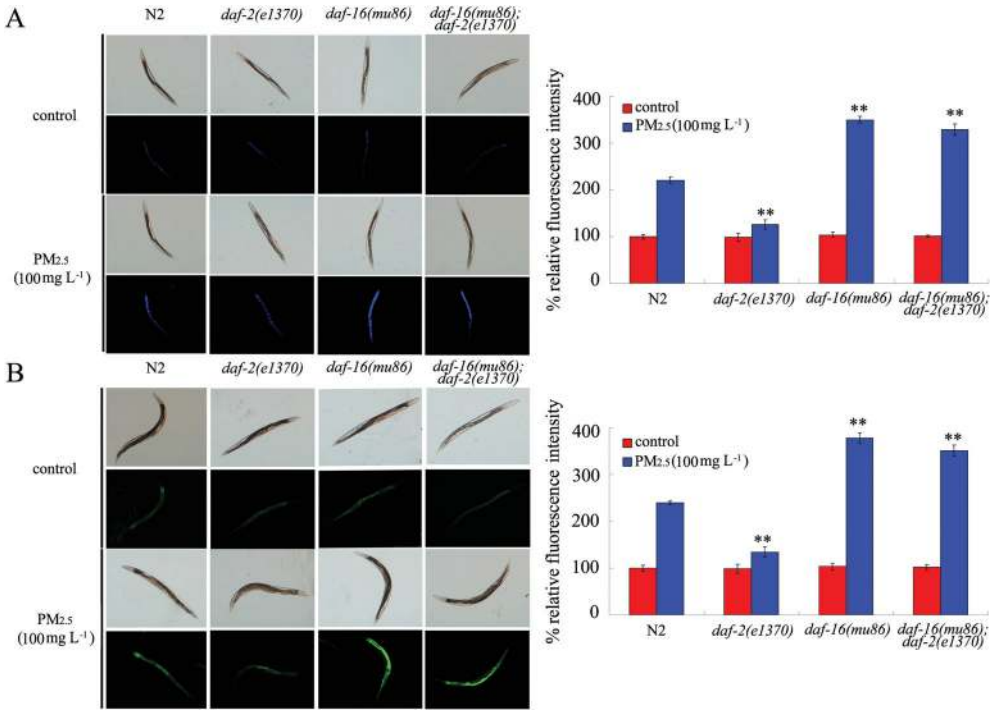

Fig. 3 Effects of mutation of the *daf-2* or *daf-16* gene on intestinal autofluorescence (A) or intestinal ROS production (B) in $PM_{2.5}$ exposed nematodes. Exposure was performed from L1-larvae to adult day-1. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2.

double mutant of *daf-16(mu86);daf-2(e1370)* exposed to 100 mg L^{-1} of $PM_{2.5}$. The double mutant of *daf-16(mu86);daf-2(e1370)* showed a similar intestinal autofluorescence or intestinal ROS production as that of wild-type N2 (Fig. 3). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the double mutant of *daf-16 (mu86);daf-2(e1370)* showed a similar intestinal autofluorescence or intestinal ROS production as that of the *daf-16(mu86)* mutant (Fig. 3). That is, the effects of *daf-2* mutation on intestinal autofluorescence and intestinal ROS production were suppressed by the *daf-16* mutation in nematodes exposed to $PM_{2.5}$. Thus, DAF-16 may function downstream of DAF-2 to regulate $PM_{2.5}$ toxicity on intestinal development in nematodes.

Mutations of the *daf-2* or *daf-16* gene influenced the expression patterns of genes required for intestinal development in $PM_{2.5}$ exposed nematodes

Our previous study has indicated that prolonged exposure to $PM_{2.5}$ could significantly decrease the expression levels of some genes (*mtm-6*, *vha-6*, *gem-4*, *ajm-1*, *act-5*, *nfm-1*, *par-3*, and *gtl-1*) required for intestinal development in wild-type N2 nematodes.[42] In contrast, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the *daf-2(e1370)* mutants showed significantly increased expression levels of *mtm-6*, *vha-6*, *gem-4*, *act-5*, *par-3*, *gtl-1*, *nfm-1*, and *ajm-1* genes compared with those in the wild-type N2 nematodes (Fig. 4). In contrast, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the *daf-16(mu86)* mutants showed more severely decreased expression levels of *mtm-6*, *vha-6*, *gem-4*, *act-5*, *par-3*, *gtl-1*, *nfm-1*, and *ajm-1* genes compared with those in the wild-type N2 nematodes (Fig. 4). Moreover, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the *daf-16(mu86)* mutants further showed significantly decreased expression levels of *nhx-2*, *par-6*, *pkc-3*, *ifb-2*, and *inx-3* genes (Fig. 4). In *C. elegans*, the *mtm-6* gene encodes a myotubularin lipid phosphatase, the *vha-6* gene encodes a vacuolar proton-translocating ATPase, the *gem-4* gene encodes a Ca^{2+}-dependent phosphatidylserine binding protein, the *act-5* gene encodes a cytoplasmic actin, *par-3* and *par-6* genes encode the PDZ domain-containing protein, the *gtl-1* gene encodes a TRPM subfamily member of the TRP channel family, the *nfm-1* gene encodes a homolog of human merlin/schwannomin (NF2), the *ajm-1* gene encodes a member of the apical junction molecule class, the *nhx-2* gene encodes a sodium/proton exchanger, the *pkc-3* gene encodes an atypical protein kinase, the *ifb-2* gene encodes a nonessential intermediate filament protein, and the *inx-3* gene encodes a gap protein (Table S2†). Mutation of the *daf-16* or *daf-2* gene did not significantly alter the expression patterns of genes required for intestinal development in nematodes (data not shown). These data further imply the involvement of the insulin signaling pathway in the control of $PM_{2.5}$ toxicity on the intestinal development in nematodes.

Mutations of the *daf-2* or *daf-16* gene regulated the intestinal permeability in $PM_{2.5}$ exposed nematodes

We further used the *daf-2* and *daf-16* null mutants to investigate whether the insulin signaling pathway regulates $PM_{2.5}$ toxicity on the intestinal function in nematodes. Mutation of

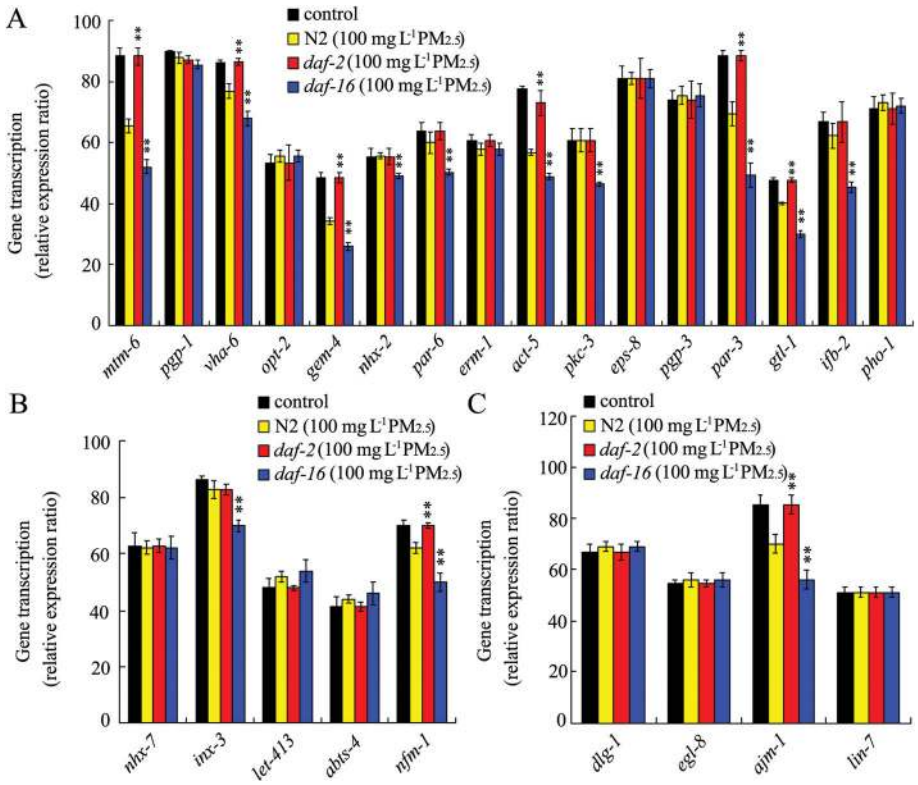

Fig. 4 Effects of mutation of the *daf-2* or *daf-16* gene on the expression patterns of genes required for intestinal development in $PM_{2.5}$ exposed nematodes. (A) Effects of mutation of the *daf-2* or *daf-16* gene on expression patterns of genes required for intestinal microvilli development in $PM_{2.5}$ exposed nematodes. (B) Effects of mutation of the *daf-2* or *daf-16* gene on the expression patterns of genes required for regulation of the baso-lateral domain in the intestine of $PM_{2.5}$ exposed nematodes. (C) Effects of mutation of the *daf-2* or *daf-16* gene on the expression patterns of genes required for regulation of the apical junctions in the intestine of $PM_{2.5}$ exposed nematodes. Exposure was performed from L1-larvae to adult day-1. Control, wild-type N2 without $PM_{2.5}$ exposure. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2 (100 mg L^{-1} $PM_{2.5}$).

the *daf-2* gene induced an increase in the fluorescence signals of Nile Red staining (Fig. 5A and 5B). In contrast, mutation of the *daf-16* gene could not cause a significant increase in the fluorescence signals of Nile Red staining (Fig. 5A and 5B). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, we did not observe an obvious alteration of the fluorescence signals of Nile Red staining in $PM_{2.5}$ exposed *daf-2(e1370)* mutants compared with that in the *daf-2(e1370)* mutants without $PM_{2.5}$ exposure (Fig. 5A and 5B). In contrast, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, we observed a significant increase in the fluorescence signals of Nile Red staining in $PM_{2.5}$ exposed *daf-16(mu86)* mutants when compared with the *daf-16(mu86)* mutants without $PM_{2.5}$ exposure (Fig. 5A and 5B). Moreover, the increase in the fluorescence signals of Nile Red staining in $PM_{2.5}$ exposed *daf-16(mu86)* mutants was more severe than that in the $PM_{2.5}$ exposed wild-type N2 (Fig. 5A and 5B). Meanwhile, we observed that exposure to 100 mg L^{-1} of $PM_{2.5}$ did not significantly alter the triglyceride amount in *daf-2(e1370)* or *daf-16(mu86)* mutant nematodes (Fig. 5C). The triglyceride amount in the *daf-2(e1370)* mutant without $PM_{2.5}$ exposure (Fig. 5C) suggests that the increased fluorescence signals of Nile Red staining were due to fat accumulation.

Furthermore, we investigated the effects of double mutations of *daf-2* and *daf-16* genes on the intestinal function in nematodes. The double mutant of *daf-16(mu86);daf-2(e1370)* showed similar fluorescence signals of Nile Red staining and triglyceride amounts to those of wild-type N2 (Fig. 5). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the fluorescence signals of Nile Red staining in the $PM_{2.5}$ exposed double mutant of *daf-16(mu86);daf-2(e1370)* were similar to that in the $PM_{2.5}$ exposed *daf-16(mu86)* mutant (Fig. 5A and 5B). Meanwhile, prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$ also did not significantly alter the triglyceride amount in *daf-16(mu86);daf-2(e1370)* mutant nematodes (Fig. 5C). Therefore, the insulin signaling pathway may also play an essential role in the control of $PM_{2.5}$ toxicity on the intestinal function in nematodes.

Mutations of the *daf-2* or *daf-16* gene influenced the expression patterns of genes required for oxidative stress in $PM_{2.5}$ exposed nematodes

Our previous study has suggested that $PM_{2.5}$ exposure could induce a significant induction of ROS production.[42] We next investigated the effects of mutations of the *daf-2* or *daf-16* gene on the expression patterns of genes required for oxidative stress in $PM_{2.5}$ exposed nematodes. Prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$ caused a significant increase in the expression levels of *sod-1*, *sod-2*, *sod-3*, and *isp-1* genes and a

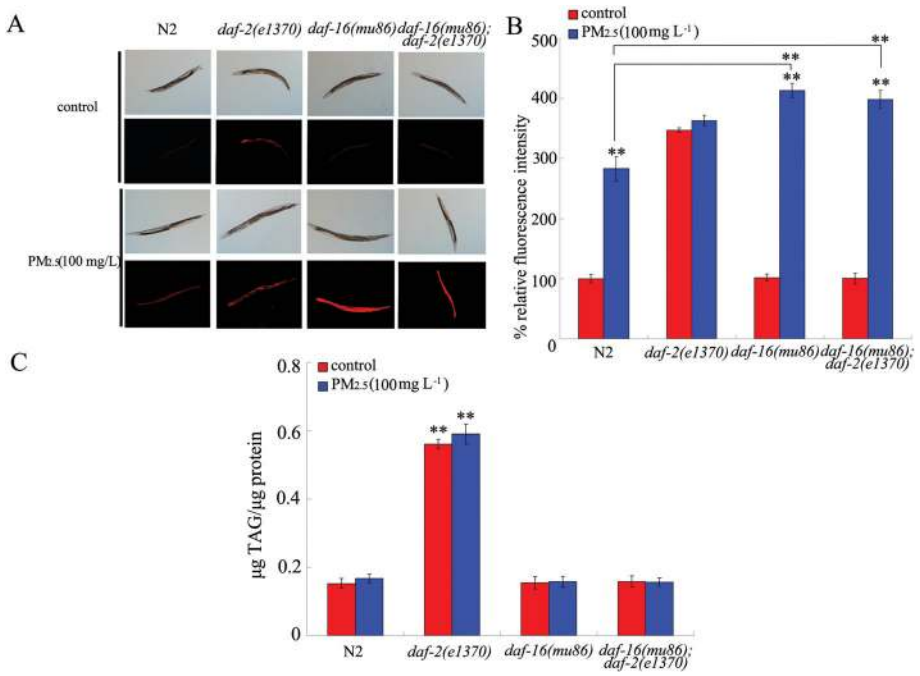

Fig. 5 Effects of mutation of the *daf-2* or *daf-16* gene on intestinal permeability in $PM_{2.5}$ exposed nematodes. (A) Nile Red staining of wild-type or mutant nematodes exposed to $PM_{2.5}$. (B) Comparison of relative fluorescence intensity of Nile Red in nematodes exposed to $PM_{2.5}$. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. control (if not specially indicated). (C) Comparison of the triglyceride amount in nematodes exposed to $PM_{2.5}$. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2. Exposure was performed from L1-larvae to adult day-1.

significant decrease in the expression level of the *gas-1* gene in nematodes (Fig. 6A). In contrast, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, we found that the *daf-2(e1370)* mutants exhibited significantly decreased expression levels of *sod-1*, *sod-2*, *sod-3*, and *isp-1* genes and a significantly increased expression level of the *gas-1* gene compared with those in the wild-type N2 nematodes (Fig. 6A). In contrast, after prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the *daf-16(mu86)* mutants showed more severely increased expression levels of *sod-1*, *sod-2*, *sod-3*, and *isp-1* genes and a more severely decreased expression level of the *gas-1* gene compared with those in the wild-type N2 nematodes (Fig. 6A). In *C. elegans*, the *sod-1* gene encodes a copper/zinc SOD, *sod-2* and *sod-3* genes encode manganese-SODs, the *isp-1* gene encodes a "Rieske" iron–sulfur protein which is a subunit of mitochondrial complex III, and the *gas-1* gene encodes a subunit of mitochondrial complex I (Table S2†). Mutation of the *daf-16* or *daf-2* gene did not significantly alter the expression pattern of genes required for oxidative stress in nematodes (data not shown).

Meanwhile, we observed that $PM_{2.5}$ (100 mg L^{-1}) exposed *daf-2(e1370)* mutants showed a significant increase in SOD or CAT activity compared with $PM_{2.5}$ (100 mg L^{-1}) exposed wild-type N2; however, $PM_{2.5}$ (100 mg L^{-1}) exposed *daf-16(mu86)* mutants exhibited a significant decrease in SOD or CAT activity compared with $PM_{2.5}$ (100 mg L^{-1}) exposed wild-type N2 (Fig. S2†). The $PM_{2.5}$ (100 mg L^{-1}) exposed double mutant of *daf-16(mu86);daf-2(e1370)* showed a similar SOD or CAT activity as that of $PM_{2.5}$ (100 mg L^{-1}) exposed wild-type N2 (Fig. S2†). These results further support the important role of the insulin signaling pathway in regulating the oxidative stress induced by $PM_{2.5}$ in nematodes.

Mutations of the *sod-3* gene affected the intestinal development in $PM_{2.5}$ exposed nematodes

In *C. elegans*, among the examined oxidative stress-related genes with altered expression levels in $PM_{2.5}$ exposed animals, the *sod-3* gene is one of the primary targets for *daf-16* to regulate the downstream biological processes.[55] We used the *sod-3* null mutants to further investigate their possible role in regulating the toxicity of $PM_{2.5}$ on the intestinal development in nematodes. Mutations of the *sod-3* gene could not induce a significant induction of intestinal autofluorescence or intestinal ROS production (Fig. 6B and 6C). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, like the *daf-16(mu86)* mutants, the $PM_{2.5}$ exposed *sod-3(gk235)* mutants showed a significantly increased intestinal autofluorescence or intestinal ROS production compared with the $PM_{2.5}$ exposed wild-type N2 nematodes (Fig. 6B and 6C), suggesting that SOD-3, the primary target of DAF-16, also regulates $PM_{2.5}$ toxicity on intestinal development in nematodes.

Moreover, we investigated the effects of double mutations of *sod-3* and *daf-16* genes on the intestinal development in nematodes. The double mutant of *daf-16(mu86);sod-3(gk235)* showed a similar intestinal autofluorescence or intestinal ROS production as that of wild-type N2 (Fig. 6B and 6C). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the double mutant of *daf-16(mu86);sod-3(gk235)* showed a similar intestinal auto-

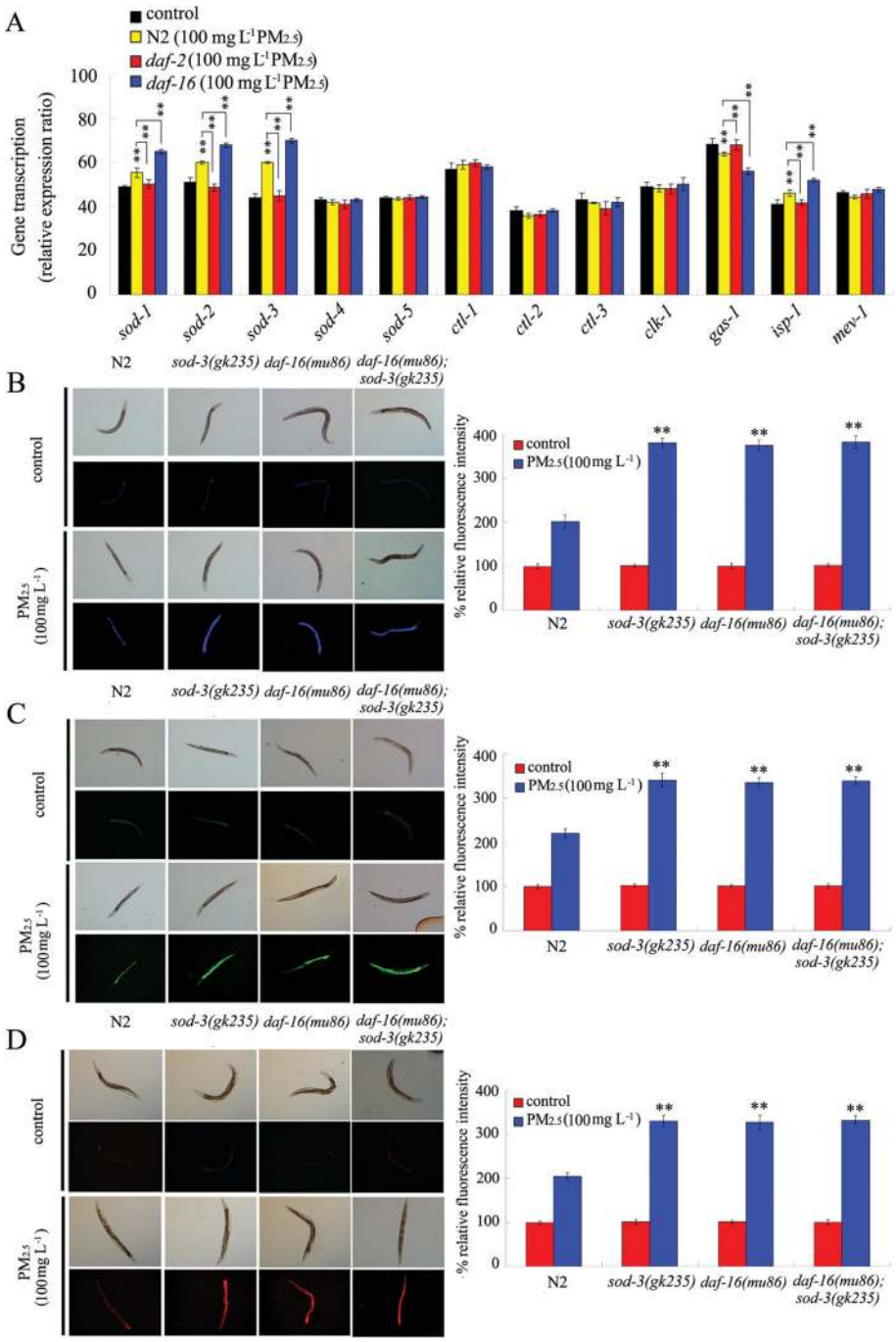

Fig. 6 Effects of mutation of the *sod-3* gene on intestinal development and function in $PM_{2.5}$ exposed nematodes. (A) Expression patterns of genes required for oxidative stress in control and $PM_{2.5}$ exposed nematodes. Control, wild-type N2 without $PM_{2.5}$ exposure. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. control (if not specially indicated). (B) Effects of mutation of the *sod-3* gene on intestinal autofluorescence in $PM_{2.5}$ exposed nematodes. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2. (C) Effects of mutation of the *sod-3* gene on intestinal ROS production in $PM_{2.5}$ exposed nematodes. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2. (D) Effects of mutation of the *sod-3* gene on intestinal permeability in $PM_{2.5}$ exposed nematodes. Bars represent means ± SEM. $^{**}P < 0.01$ *vs*. N2. Exposure was performed from L1-larvae to adult day-1.

fluorescence or intestinal ROS production as that of the *sod-3(gk235)* or *daf-16(mu86)* mutant (Fig. 6B and 6C). Therefore, DAF-16 and SOD-3 may function in the same genetic pathway to regulate the toxicity of $PM_{2.5}$ on the intestinal development in nematodes.

Mutations of the *sod-3* gene influenced the intestinal function in $PM_{2.5}$ exposed nematodes

We further employed the *sod-3* null mutants to investigate whether SOD-3 is involved in the control of $PM_{2.5}$ toxicity on

the intestinal function in nematodes. Mutations of the *sod-3* gene did not result in a significant increase in Nile Red staining (Fig. 6D). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, we found more severely increased Nile Red staining in *sod-3(gk235)* mutants compared with that in the wild-type N2 nematodes (Fig. 6D). Meanwhile, we observed a similar triglyceride amount in $PM_{2.5}$ exposed wild-type N2 or *sod-3(gk235)* mutants as that in control nematodes (Fig. S1†). Therefore, SOD-3 may also play an important role in regulating $PM_{2.5}$ toxicity on the intestinal function in nematodes.

Moreover, we investigated the effects of double mutations of *sod-3* and *daf-16* genes on the intestinal function in nematodes. The double mutant of *daf-16(mu86);sod-3(gk235)* showed a similar fluorescence signal of Nile Red staining or triglyceride amount as that of wild-type N2 (Fig. S1†). After prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$, the fluorescence signal of Nile Red staining in the $PM_{2.5}$ exposed double mutant of *daf-16(mu86);sod-3(gk235)* was similar to that in the $PM_{2.5}$ exposed *sod-3(gk235)* or *daf-16(mu86)* mutant (Fig. 6D). Meanwhile, prolonged exposure to 100 mg L^{-1} of $PM_{2.5}$ also did not significantly alter the triglyceride amount in *daf-16 (mu86);sod-3(gk235)* mutant nematodes (Fig. 6D). Therefore, DAF-16 and SOD-3 may also function in the same genetic pathway to regulate the toxicity of $PM_{2.5}$ on the intestinal function in nematodes.

Discussion

Previous studies have demonstrated that traffic-related $PM_{2.5}$ could cause toxic effects on human health.[13–15] Our previous study has further indicated the environmental toxicity of traffic-related $PM_{2.5}$ with the aid of *C. elegans* as the *in vivo* assay system.[42] It has also demonstrated that prolonged exposure to traffic-related $PM_{2.5}$ could result in adverse effects on the intestinal development of nematodes.[42] The results of our present study further provide direct data to indicate the adverse effects of prolonged exposure to traffic-related $PM_{2.5}$ at concentrations of 0.1–100 mg L^{-1} on the intestinal function as reflected by the severely enhanced intestinal permeability (Fig. 1). Therefore, prolonged exposure to traffic-related $PM_{2.5}$ may induce damage on both the development and the function of intestinal barriers in nematodes. Considering that the crucial role of intestinal barrier is to prevent the adverse effects of toxicants,[35,37–39] our data imply that severe damage to the intestinal barrier may be the key reason to induce a series of toxic effects on $PM_{2.5}$ exposed nematodes.

For the underlying mechanism, we hypothesize here that the insulin signaling pathway may act as an important molecular basis for traffic-related $PM_{2.5}$ toxicity. Among the genes encoding the insulin signaling pathway, we found that prolonged exposure to $PM_{2.5}$ caused a significant decrease in the expression levels of *daf-16* and *daf-18* genes and an increase in the expression levels of *daf-2*, *akt-1*, and *pdk-1* genes (Fig. 2A), suggesting the involvement of insulin signaling in the control of $PM_{2.5}$ toxicity. Moreover, we observed a significant increase in the percentage of animals with DAF-16::GFP nuclear localization in nematodes exposed to 100 mg L^{-1} of $PM_{2.5}$ (Fig. 2B). Our results suggest that both the transcriptional activity of the *daf-16* gene and the nucleus–cytoplasm translocation of the DAF-16 protein are associated with the control of $PM_{2.5}$ toxicity in nematodes. Our data further support the important role of the insulin signaling pathway in the control of stress response.[56,57] More recently, during the molecular control of arsenite toxicity by the insulin signaling pathway, the DAF-16::GFP nuclear localization was also significantly increased in arsenite exposed nematodes.[57] These data imply that the nucleus–cytoplasm translocation of DAF-16 may be a conserved mechanism for the insulin signaling pathway to regulate the stress response in nematodes.

In the present study, we provide the genetic evidence to demonstrate the important function of the insulin signaling pathway in regulating $PM_{2.5}$ toxicity. For the role of the insulin signaling pathway in regulating $PM_{2.5}$ toxicity on intestinal development, we found that mutations of the *daf-2* gene resulted in decreased intestinal autofluorescence and intestinal ROS production in $PM_{2.5}$ exposed nematodes compared with those in the $PM_{2.5}$ exposed wild-type nematodes; however, mutations of the *daf-16* gene caused more severely increased intestinal autofluorescence and intestinal ROS production in $PM_{2.5}$ exposed nematodes compared with those in $PM_{2.5}$ exposed wild-type nematodes (Fig. 3). Meanwhile, we found that the $PM_{2.5}$ exposed *daf-2* mutants showed increased expression levels of *mtm-6*, *vha-6*, *gem-4*, *act-5*, *par-3*, *gtl-1*, *nfm-1*, and *ajm-1* genes compared with those in $PM_{2.5}$ exposed wild-type nematodes; however, the $PM_{2.5}$ exposed *daf-16* mutants showed more severely decreased expression levels of *mtm-6*, *vha-6*, *gem-4*, *act-5*, *par-3*, *gtl-1*, *nfm-1*, and *ajm-1* genes compared with those in $PM_{2.5}$ exposed wild-type nematodes (Fig. 4). Moreover, the $PM_{2.5}$ exposed *daf-16(mu86)* mutants exhibited significantly decreased expression levels of *nhx-2*, *par-6*, *pkc-3*, *ifb-2*, and *inx-3* genes, which were not observed in $PM_{2.5}$ exposed wild-type nematodes (Fig. 4). In *C. elegans*, *gem-4*, *mtm-6*, *nhx-2*, *opt-2*, *pho-1*, *pkc-3*, *par-3*, *par-6*, *pgp-1*, *pgp-3*, *vha-6*, *gtl-1*, *erm-1*, *eps-8*, *act-5*, and *ifb-2* genes are required for the intestinal microvilli development, *let-413*, *nfm-1*, *inx-3*, *nhx-7*, and *abts-4* genes are required for the control of the basolateral domain in the intestine, and *dlg-1*, *ajm-1*, *egl-8*, and *lin-7* genes are required for the control of apical junctions in the intestine.[58] Therefore, mutations of the *daf-2* or *daf-16* gene altered several aspects of intestinal development in $PM_{2.5}$ exposed nematodes. For the role of the insulin signaling pathway in regulating $PM_{2.5}$ toxicity on the intestinal function, we further found that mutations of the *daf-2* gene resulted in a decrease in the fluorescence signal of Nile Red staining in $PM_{2.5}$ exposed nematodes, whereas mutations of the *daf-16* gene caused a more severe increase in the fluorescence signal of Nile Red staining in $PM_{2.5}$ exposed nematodes (Fig. 5), suggesting an altered intestinal permeability in $PM_{2.5}$ exposed nematodes.

In the present study, we further indicate that DAF-16 may at least act through SOD-3, the primary target for DAF-16,[55] to

regulate $PM_{2.5}$ toxicity in nematodes. Mutations of the *sod-3* gene showed similar effects on both intestinal development and intestinal function in $PM_{2.5}$ exposed nematodes (Fig. 6). Oxidative stress is an important mechanism for $PM_{2.5}$ to induce toxic effects on organisms.[59–61] The *sod-3* gene encodes a manganese-superoxide dismutase, and plays a key role in the regulation of oxidative stress by organizing the antioxidation machinery in nematodes.[28] Therefore, the transcription factor DAF-16 may regulate $PM_{2.5}$ toxicity by controlling the function of its downstream targets such as the SOD-3 in nematodes.

Conclusions

In nematodes, prolonged exposure to $PM_{2.5}$ caused adverse effects on both intestinal development and intestinal function. Meanwhile, prolonged exposure to $PM_{2.5}$ induced a significant alteration in the expression patterns of some genes encoding the insulin signaling pathway and an increase in DAF-16 nuclear localization. Mutations of the *daf-2* or *daf-16* gene noticeably influenced both intestinal development and intestinal function in $PM_{2.5}$ exposed nematodes, suggesting that the insulin signaling pathway may act as a key molecular basis for the regulation of $PM_{2.5}$ toxicity. During the control of $PM_{2.5}$ toxicity by the insulin signaling pathway, DAF-16 may regulate the stress response through the functions of its downstream targets such as the SOD-3. Our study will be helpful for understanding the molecular basis of *in vivo* $PM_{2.5}$ toxicity. Our results also highlight the crucial role of the primary target organs of nematodes to prevent damage caused by toxicants.

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Conflict of interest

None of the authors have any conflicting interests.

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