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## Nanoplastics at environmentally relevant concentrations activates a germline *mir-240-rab-5* signaling cascade to affect the secreted ligands associated with transgenerational toxicity induction in *C. elegans*†

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Epigenetic regulation plays an important role in regulating the transgenerational toxicity of pollutants. However, the underlying mechanism of microRNAs (miRNAs) in regulating transgenerational nanoplastic toxicity remains largely unclear. We aimed to determine the miRNA-mediated mechanism for the induction of transgenerational nanoplastic toxicity. In *Caenorhabditis elegans*, although germline RNAi of both *mir-240* and *mir-36* suppressed polystyrene nanoparticle (PS-NP) toxicity, exposure to PS-NPs (1–100  $\mu\text{g L}^{-1}$ ) only increased *mir-240* expression. A transgenerational increase in *mir-240* expression was observed after PS-NP exposure at P0 generation (P0-G), and the germline RNAi of *mir-240* suppressed transgenerational PS-NP toxicity. Among the predicted target genes of *mir-240* in the germline, the exposure to PS-NPs (1–100  $\mu\text{g L}^{-1}$ ) decreased *rab-5* and *rab-6.2* expressions, whereas the germline RNAi of *mir-240* only increased *rab-5* expression in PS-NP exposed nematodes. A transgenerational decrease in *rab-5* expression was detected after PS-NP exposure at P0-G, and the germline RNAi of *rab-5* strengthened transgenerational PS-NP toxicity. Moreover, the resistance of *mir-240*(RNAi) to transgenerational PS-NP toxicity in inhibiting locomotion behavior and in reducing the brood size was inhibited by the germline RNAi of *rab-5*. Among the secreted ligands, the germline RNAi of *rab-5* increased the expressions of genes encoding insulin peptides (*ins-3*, *ins-39*, and *daf-28*), FGF ligand (*egl-17*), and ephrin ligand (*efn-3*) in PS-NP exposed nematodes and their corresponding receptor genes (*daf-2*, *egl-15*, and *vab-1*) in the offspring of PS-NP exposed nematodes. Therefore, an increase in germline *mir-240* mediated transgenerational PS-NP toxicity through insulin, FGF, and ephrin signals by affecting its target RAB-5. Our data demonstrated the important involvement of germline microRNA in mediating nanoplastic toxicity across multiple generations in organisms.

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### Environmental significance

The release of nanoplastics is ubiquitous in the environment, which causes their exposure risk and poses health risk to humans. *Caenorhabditis elegans* is sensitive to pollutants at environmentally relevant concentrations (ERCs). Using *C. elegans* as the animal model, nanopolystyrene at ERCs induced a transgenerational increase in the expression of germline microRNA/*mir-240*, suggesting the activation of this epigenetic signal. Germline *mir-240* mediated transgenerational nanoplastic toxicity through insulin, FGF, and ephrin signals by affecting its target RAB-5. Our findings demonstrated the transgenerational dysregulation of certain microRNAs by nanoplastics, which was associated with their toxicity induction across multiple generations.

### Introduction

In our daily lives, along with the tremendous applications of plastic-based products, plastic pollution has been considered as a global concern.<sup>1</sup> This pollution is not only due to the existence of wide and vast waste plastic products, but also due to the limited biodegradation of waste plastics.<sup>2</sup> Environmental degradation through microorganisms, photo-oxidation, and mechanical abrasion causes the formation of microplastics and even nanoplastics.<sup>3,4</sup> In several ecosystems

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(such as the soil ecosystem), nanoplastics have been frequently detected.<sup>5,6</sup> Besides this, various commercial applications result in the release of nanoplastics into the environment.<sup>7</sup> Largely owing to these facts, the environmental fate, transport, and risk of microplastics and nanoplastics have received increasing attention.<sup>8-13</sup>

Through the food chain, nanoplastics are bioavailable to humans.<sup>14,15</sup> After ingestion, nanoparticles will encounter the intestinal barrier and then reach system circulation and other tissues.<sup>16,17</sup> This form of translocation of nanoplastics leads to corresponding toxicity.<sup>18-20</sup> Nanoplastic exposure results in the disruption of the normal reproductive function, development, nervous-system function, and immune response.<sup>21-24</sup> In mammals, nanoplastics could induce genotoxicity, oxidative stress, and damage on biological membranes.<sup>25,26</sup> Nanoplastic toxicity can be affected by several factors, such as dose, size, and charge.<sup>27,28</sup>

*Caenorhabditis elegans* have tiny size and high sensitivity to pollutants.<sup>29-34</sup> Due to the short life-cycle, it is useful for the evaluation of transgenerational toxicity of pollutants.<sup>35,36</sup> This animal model can be applied to assess the transgenerational toxicity of nanoplastics (such as polystyrene nanoparticle (PS-NP)),<sup>37-40</sup> which was affected by the size and modification of PS-NP.<sup>41,42</sup> Moreover, some secreted ligands, including insulin, Wnt, FGF, Notch, ephrin, and their receptors acted together to regulate transgenerational PS-NP toxicity.<sup>43-47</sup>

For underlying molecular basis, besides transgenerational signal communication, alteration in some histone methylation-related molecules were associated with transgenerational PS-NP toxicity.<sup>39,48</sup> microRNA (miRNA) having 19–22 nucleotides can regulate the gene expression after binding to the 3'-UTR of targeted genes.<sup>49</sup> At P0-generation (P0-G), some miRNAs have been identified to function in different tissues (intestine, neurons, and germline) to regulate the PS-NP toxicity.<sup>50-54</sup> Moreover, the decrease in germline *mir-38* controlled transgenerational PS-NP toxicity by inhibiting some targets, such as the hedgehog ligand of WRT-3.<sup>55</sup> In the germline, some other miRNAs may also be involved in regulating the transgenerational PS-NP toxicity. Thus, we first identified other dysregulated germline miRNAs induced by PS-NP exposure. Moreover, we determined the underlying mechanism for candidate germline miRNA(s) in controlling the transgenerational PS-NP toxicity. Our results provided further epigenetic regulation mechanism for the transgenerational toxicity of nanoplastics in organisms.

## Experimental

### PS-NP properties

PS-NPs with a particle size of 20 nm was purchased from New-Materials Co., Ltd. Before the experiments, PS-NPs morphology was confirmed with a regular spherical shape and the particle size was  $20.42 \pm 2.4$  nm using transmission

electron microscopy (TEM) (Fig. 1a). Raman spectroscopy was used to reflect the plastic property of PS-NP. PS-NPs have three peaks at  $1031.44\text{ cm}^{-1}$  (symmetric extension vibration of  $-\text{C}-\text{C}-$  in the benzene ring),  $1000.04\text{ cm}^{-1}$  (indicating the respiratory vibration of the benzene ring), and  $1602.11\text{ cm}^{-1}$  (indicating the asymmetric stretching vibration of the benzene ring carbon atoms) (Fig. 1b). The FTIR spectrum of PS-NP was described previously.<sup>42</sup>

### Maintenance of animals

Nematodes were cultured on nematode growth medium (NGM) fed with *Escherichia coli* (*E. coli*) OP50 at  $20\text{ }^{\circ}\text{C}$ .<sup>56</sup> Strain information is provided in Table S1.<sup>f</sup> Eggs were isolated using lysis buffer from pregnant nematodes.<sup>57</sup> After that, eggs were cultured to develop into L1-larvae nematodes for following experiments.

### PS-NP exposure

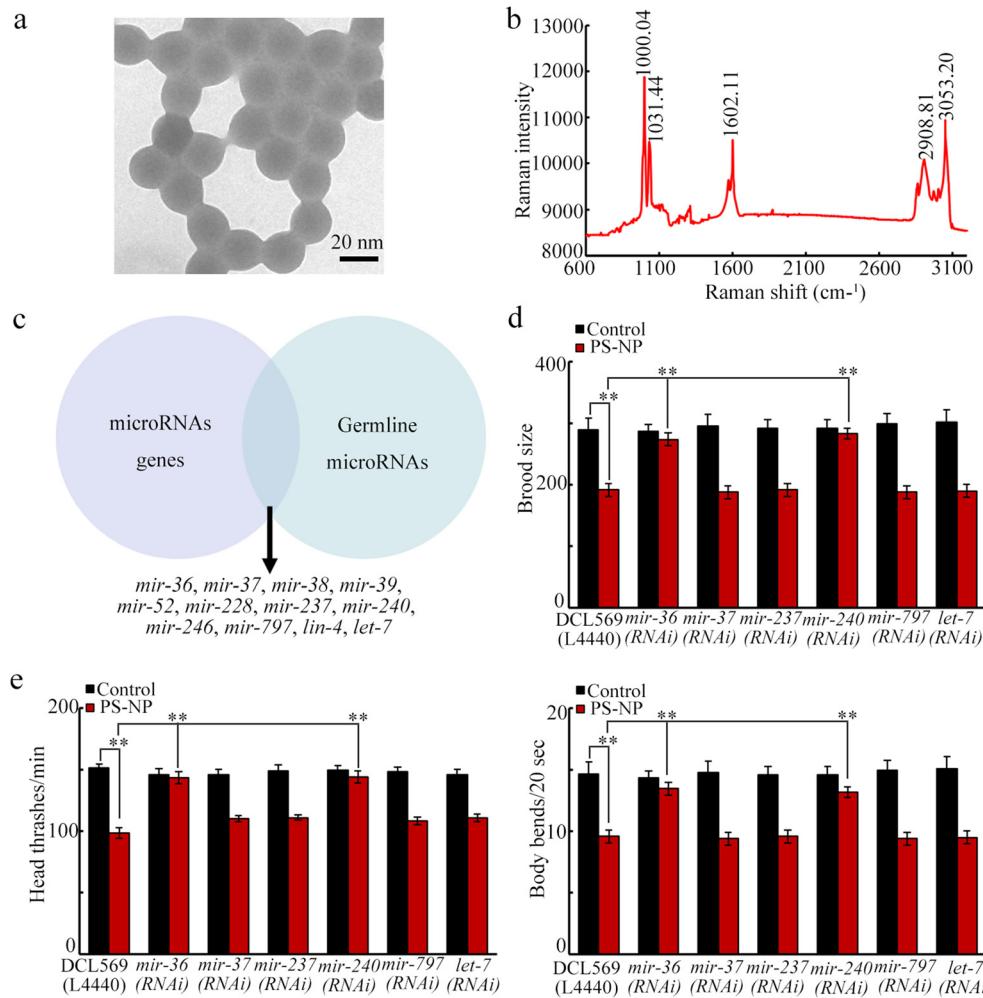
According to our previous study, the exposure concentrations of PS-NPs exposure were 1, 10, and  $100\text{ }\mu\text{g L}^{-1}$ .<sup>42</sup> It was reported that the environmentally relevant concentrations (ERCs) of nanoplastics are in the range of  $\text{ng L}^{-1}$  to  $\text{μg L}^{-1}$ .<sup>58</sup> To evaluate the toxicity, animals were exposed to PS-NPs solutions at concentrations of  $1\text{--}100\text{ }\mu\text{g L}^{-1}$  from L1-larvae to adult day-3, which is called P0-G. During the exposure process, PS-NPs exposure solutions were refreshed daily. After that, P0-G nematodes were removed, while their eggs were transferred to NGM plate to develop into adults, which is called F1-G. Using the same procedure, the following F2–F5 generation was obtained. All generations of nematodes were used for the subsequent determination of endpoints.

### Locomotion behavior and reproduction

To evaluate the toxicity, the locomotion behavior and reproduction were studied. Locomotion behavior was used to reflect the functional state of motor neurons.<sup>59</sup> Brood size was used to reflect the reproductive capacity.<sup>60</sup> In detail, locomotion behavior was evaluated by the frequencies of head thrashes and body bends.<sup>61</sup> At the end of exposure, fifty nematodes were picked randomly in P0-G, and F1–F5 generations were used to count the number of head thrashes for 1 minute and number of body bends for 20 seconds under a stereomicroscope.<sup>62</sup> The reproduction was evaluated using the endpoint of the brood size.<sup>63</sup> The offspring number was counted until the end of spawning. Thirty animals were examined for the assessment of the brood size. Three independent experiments were conducted.

### Transcriptional expression analysis

Nematodes obtained from P0-G to F5-G were collected in centrifuge tubes, followed by the addition of Trizol to isolate the total RNA. The cDNA was synthesized using M-MuLV reverse transcriptase with the following reaction:  $42\text{ }^{\circ}\text{C}$  for 60 min and  $70\text{ }^{\circ}\text{C}$  for 10 min. Quantitative real-time polymerase chain



**Fig. 1** Identification of germline microRNAs involved in regulating PS-NP toxicity. (a) TEM image of PS-NP before sonication. (b) Raman spectrum of PS-NP. (c) microRNAs expressed in the germline of nematodes. (d) Effect of germline RNAi of candidate microRNAs on the brood size in PS-NP exposed nematodes. (e) Effect of germline RNAi of candidate microRNAs on the locomotion behavior in PS-NP exposed nematodes. Exposure concentration of PS-NP was 1  $\mu\text{g L}^{-1}$ . \*\*P < 0.01.

reaction (qRT-PCR) was conducted to assess the transcriptional expression of the examined genes. The expression of *mir-240* was normalized by the expression of *F35C11.9*.<sup>64</sup> For other genes' expression, *tba-1* is the reference gene.<sup>65</sup> Primers are given in Table S2.<sup>†</sup> Three replicates were performed.

#### RNA interference (RNAi)

To analyze the function of the examined genes in transgenerational toxicity, nematodes were fed with *E. coli* HT115 expressing dsRNA.<sup>66</sup> In detail, the gene construct for RNAi was generated using L4440, an empty vector, and subsequently transformed into HT115.<sup>67</sup> Before the experiment, RNAi cells were cultured on NGM plates containing 1 mM isopropylthiogalactoside for 24 h to induce double-stranded RNA expression. For acquiring a higher RNAi efficiency, the F1-G of RNAi nematodes were collected for subsequent experiments. Meanwhile, the RNAi efficiency was determined to measure the expression of the determined

gene or miRNAs by qRT-PCR, which were presented in Fig. S1.<sup>†</sup> The control consisted of HT115 expressing L4440.<sup>68</sup>

#### Data analysis

Data were presented by mean  $\pm$  standard deviation (SD). Significance was assessed by analysis of variance (ANOVA). In detail, one-way ANOVA was performed between the control and exposure groups. Two-way ANOVA was performed comparing multiple factors. The probability level threshold of 0.01 was used to define the statistical significance.

## Results

#### Identification of germline microRNAs required for the control of the PS-NP toxicity

Twelve miRNAs are expressed in the germline, including *mir-38* (Fig. 1c) (<https://wormbase.org/>). Among these 12 miRNAs, *mir-39*, *mir-52*, *mir-228*, *mir-246*, and *lin-4* were not required for controlling the toxicity of pollutants.<sup>64,69,70</sup> Besides *mir-*

38, we further identified whether some other miRNAs controlled the PS-NP toxicity. The germline RNAi of *mir-37*, *mir-237*, *mir-797*, and *let-7* did not affect the PS-NP toxicity (Fig. 1d and e). However, the germline RNAi of *mir-36* and *mir-240* inhibited the PS-NP toxicity (Fig. 1d and e).

#### Induction of transgenerational increase in the *mir-240* expression after PS-NP exposure at P0-G

For candidate *mir-36* and *mir-240*, we first investigated their expression at P0-G after PS-NP exposure. Exposure to PS-NP (1–100  $\mu\text{g L}^{-1}$ ) did not affect the germline *mir-36* expression but significantly increased germline *mir-240* expression (Fig. 2a). Moreover, the increase in germline *mir-240* expression was detected from P0-G to F2-G after PS-NP exposure (Fig. 2b). After PS-NP exposure at P0-G, the *mir-240* expression was recovered to the control at F3-G (Fig. 2b).

Previous study has indicated that the expression of certain miRNAs could be changed by the RNAi of genes (*alg-1-5*) encoding Argonaute proteins.<sup>55</sup> In PS-NP exposed nematodes, *alg-2* and *alg-3* expressions could be affected by PS-NP.<sup>55</sup> In PS-NP exposed nematodes, *mir-240* expression was decreased by the germline RNAi of *alg-2* and increased by the germline RNAi of *alg-3* (Fig. 2c).

#### Germline RNAi of *mir-240* confers resistance to transgenerational PS-NP toxicity

In nematodes, the germline RNAi of *mir-240* inhibited transgenerational PS-NP toxicity in inhibiting the locomotion

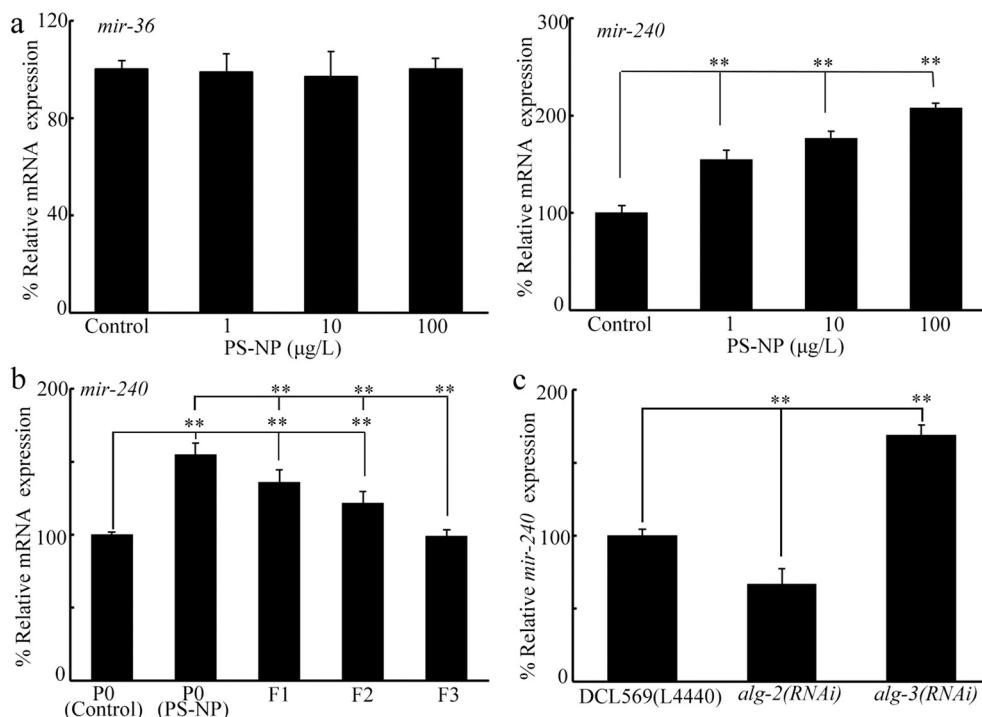
behavior and in reducing the brood size (Fig. 3a and b). Therefore, the resistance to transgenerational PS-NP toxicity could be observed in *mir-240(RNAi)*.

#### Identification of targets of germline *mir-240* in regulating transgenerational PS-NP toxicity

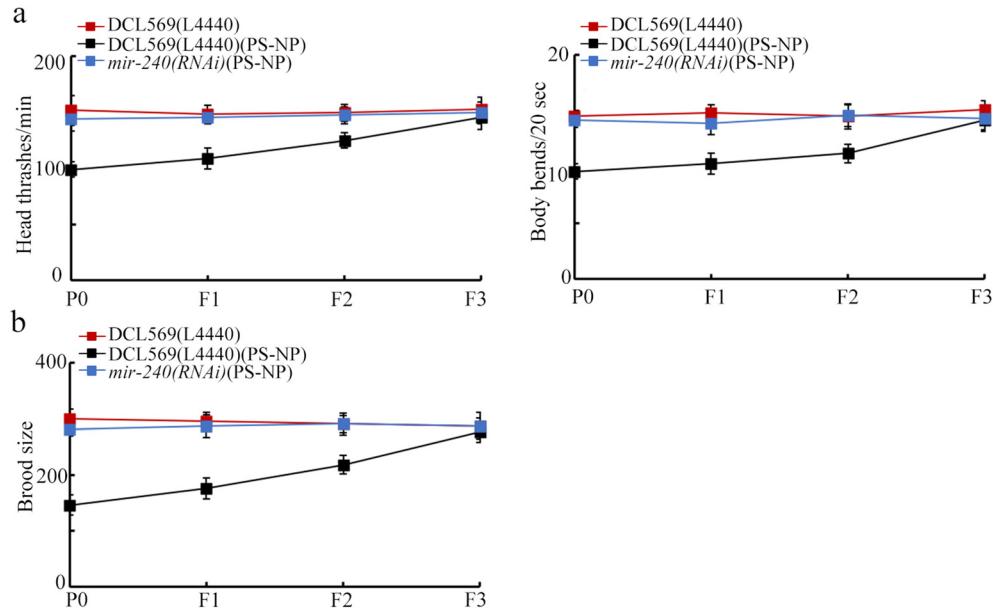
Using TargetScan ([http://www.targetscan.org/worm\\_52/](http://www.targetscan.org/worm_52/)), 20 target genes are predicted for *mir-240*. Among these 20 genes, 11 genes are expressed in the germline (<https://www.wormbase.org>) (Fig. 4a). Among these 11 germline genes, exposure to PS-NP (1–100  $\mu\text{g L}^{-1}$ ) decreased the germline expressions of *rab-5* and *rab-6.2* (Fig. 4b). After PS-NP exposure, the transgenerational decrease in germline expressions of *rab-5* and *rab-6.2* was further observed at F1-G and F2-G (Fig. 4c). Nevertheless, in PS-NP exposed nematodes, the germline RNAi of *mir-240* did not affect *rab-6.2* expression, whereas *rab-5* expression was increased by the germline RNAi of *mir-240* (Fig. 4d).

#### Germline RNAi of *rab-5* confers susceptibility to transgenerational PS-NP toxicity

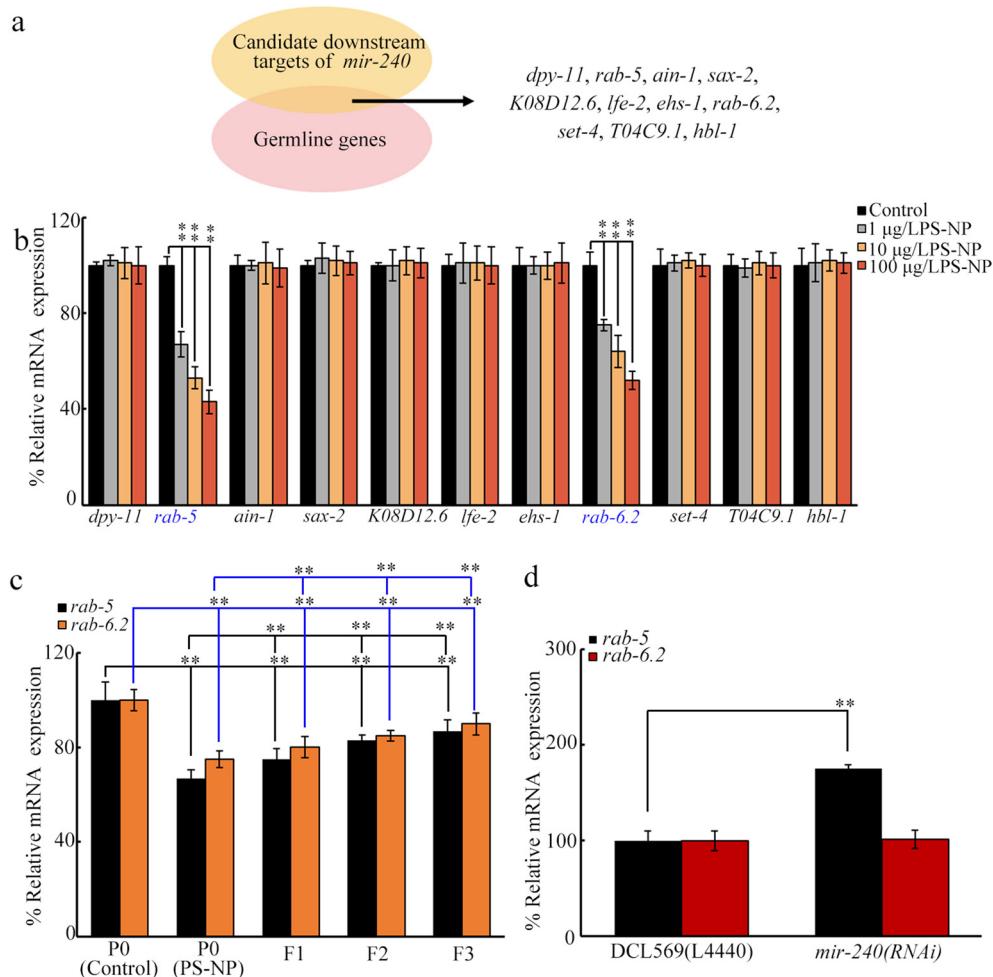
Compared with PS-NP toxicity from P0-G to F2-G in DCL569(L4440), the PS-NP toxicity was detected from P0-G to F4-G in *rab-5(RNAi)* (Fig. 5a and b). This suggested the susceptibility of *rab-5(RNAi)* to transgenerational PS-NP toxicity.



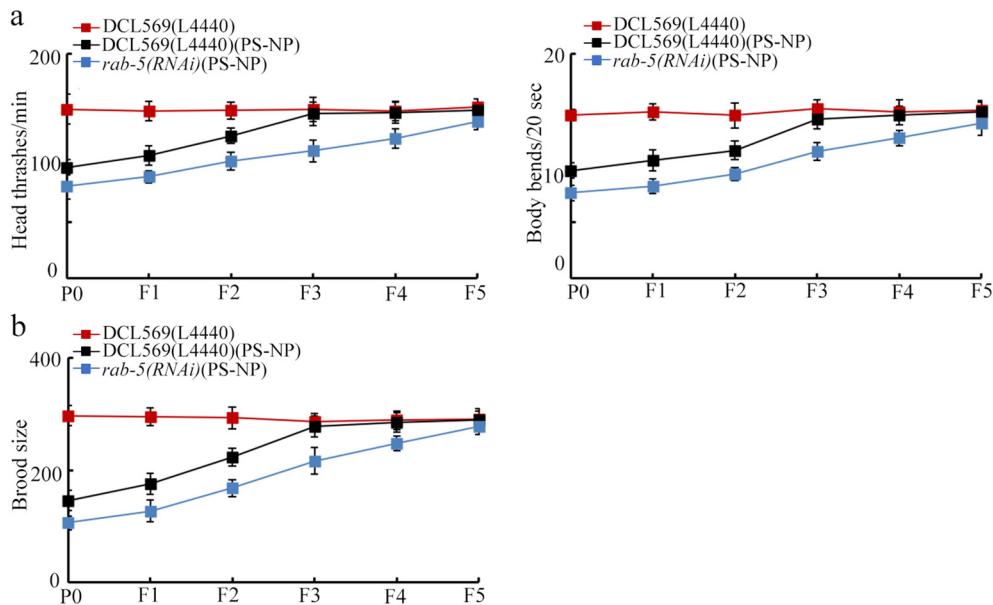
**Fig. 2** Effect of PS-NP exposure on the expressions of *mir-36* and *mir-240*. (a) Effect of PS-NP exposure on the germline expressions of *mir-36* and *mir-240* at P0-G. (b) Transgenerational expression of germline *mir-240* after PS-NP exposure. Exposure concentration of PS-NP was 1  $\mu\text{g L}^{-1}$ . (c) Effect of germline RNAi of *alg-2* and *alg-3* on the *mir-240* expression in PS-NP exposed nematodes. Exposure concentration of PS-NP was 1  $\mu\text{g L}^{-1}$ . \*\* $P < 0.01$ .



**Fig. 3** Effect of germline RNAi of *mir-240* on the transgenerational toxicity of PS-NP on the locomotion behavior (a) and brood size (b). Exposure concentration of PS-NP was  $1 \mu\text{g L}^{-1}$ .



**Fig. 4** Identification of potential targets of germline *mir-240* in regulating transgenerational PS-NP toxicity. (a) Predicted germline targets of *mir-240*. (b) Effect of PS-NP exposure on the germline expressions of predicted germline target genes. \*\* $P < 0.01$  vs. control. (c) Transgenerational expressions of germline *rab-5* and *rab-6.2* after PS-NP exposure. Exposure concentration of PS-NP was  $1 \mu\text{g L}^{-1}$ . \*\* $P < 0.01$ . (d) Effect of the germline RNAi of *mir-240* on the expressions of *rab-5* and *rab-6.2* in PS-NP exposed nematodes. Exposure concentration of PS-NP was  $1 \mu\text{g L}^{-1}$ . \*\* $P < 0.01$ .



**Fig. 5** Effect of the germline RNAi of *rab-5* on transgenerational PS-NP toxicity on the locomotion behavior (a) and brood size (b). Exposure concentration of PS-NP was  $1 \mu\text{g L}^{-1}$ .

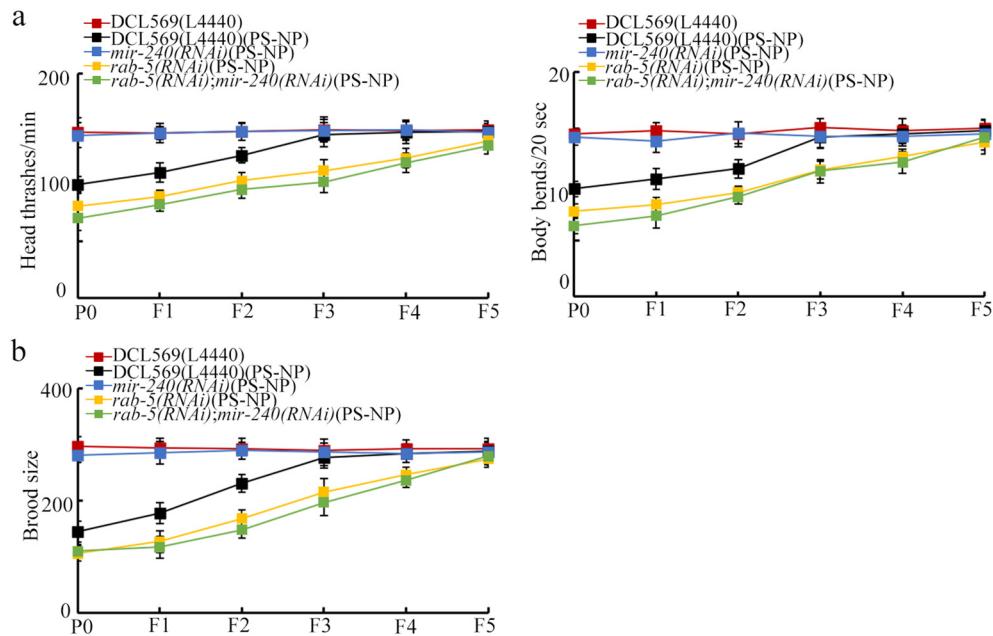
### Genetic interaction between *mir-240* and *rab-5* in controlling transgenerational PS-NP toxicity

We performed double germline RNAi of *mir-240* and *rab-5* in PS-NP exposed nematodes. After PS-NP exposure, *rab-5(RNAi)*; *mir-240(RNAi)* showed the similar phenotype of transgenerational PS-NP toxicity to that of *rab-5(RNAi)* (Fig. 6a and b). Therefore, the resistance of *mir-240(RNAi)* to transgenerational PS-NP toxicity in inhibiting the locomotion

behavior and in reducing the brood size could be suppressed by the RNAi of *rab-5*.

### Identification of targets of germline RAB-5 in regulating transgenerational PS-NP toxicity

In *C. elegans*, RAB-5 regulates neuropeptide release,<sup>71</sup> suggesting the potential of RAB-5 to regulate the secreted ligands. In the germline, insulin peptides (INS-3, INS-39, and



**Fig. 6** Genetic interaction between *mir-240* and RAB-5 for regulating the transgenerational PS-NP toxicity on the locomotion behavior (a) and brood size (b). Exposure concentration of PS-NP was  $1 \mu\text{g L}^{-1}$ .

DAF-28), Wnt ligand (LIN-44), FGF ligand ( EGL-17), Notch ligand (LAG-2), hedgehog ligand (WRT-3), and ephrin ligand (EFN-3) were involved in controlling transgenerational PS-NP toxicity.<sup>43-48</sup> Although the germline RNAi of *rab-5* did not influence *lin-44*, *lag-2*, and *wrt-3* expressions, *ins-3*, *ins-39*, *daf-28*, *egl-17*, and *efn-3* expressions were increased by *rab-5* RNAi at P0-G of the PS-NP exposed nematodes (Fig. 7a). DAF-2 is an insulin receptor, EGL-15 is an FGF receptor, and VAB-1 is an ephrin receptor.<sup>43,45,47</sup> Moreover, after the RNAi of *rab-5* at P0-G, at F1-G of PS-NP exposed *rab-5(RNAi)* nematodes, *daf-2*, *egl-15*, and *vab-1* expressions were further increased (Fig. 7b).

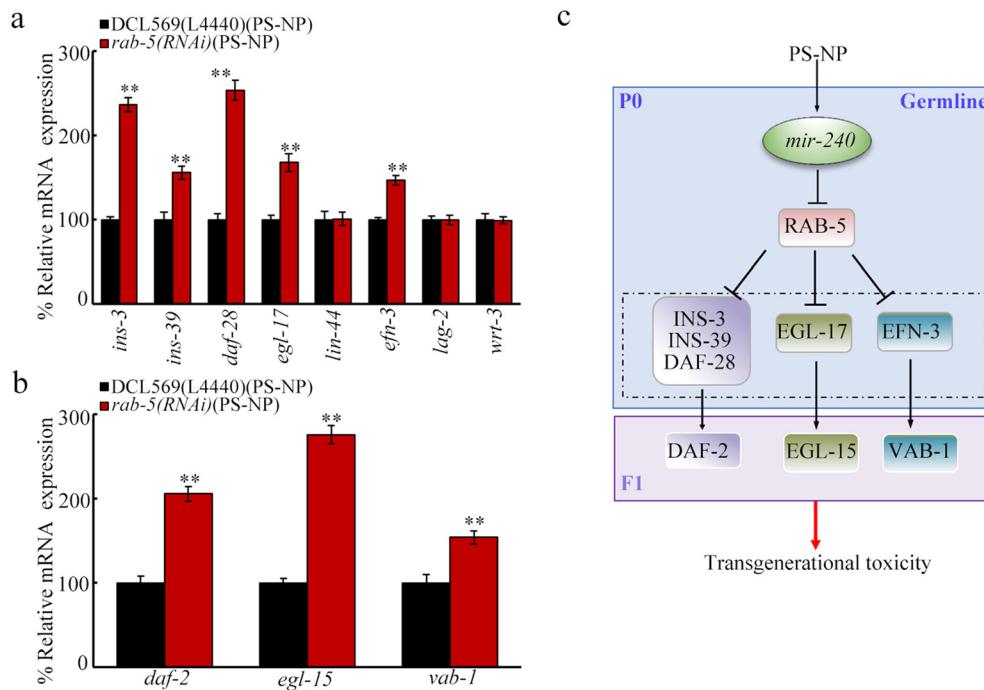
## Discussion

Nanoplastics are widely distributed in the environment.<sup>72,73</sup> Meanwhile, after being released or formed, nanoplastics are bioavailable to humans and organisms.<sup>74,75</sup> After exposure, not only at P0-G, the nanoplastic toxicity could also be detected in the offspring of different organisms.<sup>76,77</sup> The transgenerational toxicity of nanoplastics was further observed in *C. elegans*.<sup>78,79</sup> Exposure to nanoplastics could cause epigenetic signatures associated with transgenerational impairment.<sup>80</sup> Histone methylation regulation, a form of epigenetic regulation, was required for transgenerational toxicity induction of both pristine and aged nanoplastics.<sup>38,39</sup> miRNA regulation is also a form of epigenetic regulation.<sup>81,82</sup> We further found that the activation of miRNA could mediate the induction of transgenerational nanoplastic toxicity.

We here found the role of activated germline *mir-240* to mediate transgenerational PS-NP toxicity. Firstly, the germline RNAi of *mir-240* conferred resistance to PS-NP toxicity at P0-G (Fig. 1d and e). Secondly, a transgenerational increase in the germline *mir-240* expression was detected after PS-NP exposure (Fig. 2b). Thirdly, the germline RNAi of *mir-240* caused resistance to transgenerational PS-NP toxicity (Fig. 3). Besides the involvement of the activated germline *mir-240*, the inhibited germline *mir-38* also mediated transgenerational PS-NP toxicity. Germline *mir-38* expression was decreased by PS-NP, and germline *mir-38* overexpression inhibited transgenerational PS-NP toxicity.<sup>55</sup> Therefore, nanoplastics can activate or inhibit certain germline miRNAs that lead to toxicity toward multiple generations. Our results demonstrated the crucial role of germline miRNAs in mediating an epigenetic mechanism to regulate the transgenerational toxicity of nanoplastics.

Besides germline *mir-240*, we also observed the resistance of *mir-36(RNAi)* to PS-NP toxicity (Fig. 1d and e). However, although PS-NP at 1–100  $\mu\text{g L}^{-1}$  could induce the toxicity, the induced toxicity of PS-NP (1–100  $\mu\text{g L}^{-1}$ ) was not associated with the germline *mir-36*. The reason was that the germline *mir-36* expression was not affected by exposure to 1–100  $\mu\text{g L}^{-1}$  PS-NP (Fig. 2a). In other words, PS-NP at higher concentrations or other pollutants with more severe toxicity may induce transgenerational toxicity by activating germline *mir-36* in nematodes.

miRNAs biogenesis is governed by Argonaute proteins in *C. elegans*.<sup>83,84</sup> Among genes encoding germline Argonaute



**Fig. 7** Identification of targets of germline RAB-5 in regulating transgenerational PS-NP toxicity. (a) Effect of germline RNAi of *rab-5* on expressions of *ins-3*, *ins-39*, *daf-28*, *egl-17*, *lin-44*, *efn-3*, *lag-2*, and *wrt-3* in PS-NP exposed nematodes. Exposure concentration of PS-NP was 1  $\mu\text{g L}^{-1}$ . \*\* $P < 0.01$  vs. DCL569(L4440). (b) Effect of germline RNAi of *rab-5* on the expressions of *daf-2*, *egl-15*, and *vab-1* in the F1-G of PS-NP exposed nematodes. Exposure concentration of PS-NP was 1  $\mu\text{g L}^{-1}$ . \*\* $P < 0.01$  vs. DCL569(L4440). (c) A diagram showing the molecular basis for *mir-240* and *RAB-5* in the germline to regulate transgenerational PS-NP toxicity.

proteins, *alg-2* expression was increased by PS-NP and *alg-3* expression was decreased by PS-NP.<sup>55</sup> The *alg-2(RNAi)* animals showed resistance to PS-NP toxicity, and *alg-3(RNAi)* animals exhibited susceptibility to PS-NP toxicity.<sup>55</sup> In PS-NP-exposed *C. elegans*, germline *mir-240* expression was decreased by *alg-2* RNAi and increased by *alg-3* RNAi (Fig. 2c). The function of some *C. elegans* miRNAs in regulating stresses, such DNA damage and dauer formation, was also dependent on ALG-2.<sup>85,86</sup> Additionally, germline *mir-38* expression was decreased by the RNAi of *C. elegans* *alg-3*.<sup>55</sup> Therefore, PS-NP exposure could activate germline *mir-240* and inhibit germline *mir-38* to induce transgenerational PS-NP toxicity by affecting the expressions of *C. elegans* germline Argonaute genes.

RAB-5 was further identified as the target of germline *mir-240* (Fig. 7c). Firstly, PS-NP exposure caused transgenerational decrease in the expression of germline *rab-5* (Fig. 4c). Secondly, germline *rab-5* expression was decreased by the RNAi of *mir-240* (Fig. 4d). Thirdly, the germline RNAi of *rab-5* induced susceptibility to transgenerational PS-NP toxicity (Fig. 5). More importantly, the resistance of *mir-240(RNAi)* to transgenerational PS-NP toxicity was suppressed by the germline RNAi of *rab-5* (Fig. 6). Therefore, activated germline *mir-240* induced transgenerational nanoplastic toxicity by targeting and inhibiting RAB-5. RAB-5 is an endosomal Rab-type GTPase.<sup>87</sup> RAB-5 was required for modulating the stress resistance and longevity.<sup>88</sup>

After PS-NP exposure, a transgenerational decrease in germline *rab-6.2* was detected (Fig. 4c), suggesting that germline RAB-6.2 was also possibly required for controlling transgenerational PS-NP toxicity. Nevertheless, in PS-NP exposed nematodes, the RNAi of *mir-240* did not alter the germline *rab-6.2* expression (Fig. 4d). This implies that germline RAB-6.2 may participate in regulating the PS-NP toxicity under the control of other miRNAs.

Rab GTPases function as the master regulator for intracellular trafficking by regulating vesicle release.<sup>89,90</sup> In *C. elegans*, Rab GTPases, including RAB-10 and RAB-5, control the release of certain ligands, such as neuropeptides.<sup>71</sup> Among the identified secreted ligands controlling transgenerational PS-NP toxicity, insulin peptide genes (*ins-3*, *ins-39*, and *daf-28*), FGF ligand gene (*egl-17*), and ephrin ligand gene (*efn-3*) were increased by the germline RNAi of *rab-5* after PS-NP exposure (Fig. 7a). Moreover, in the offspring of PS-NP-exposed nematodes, the expressions of corresponding receptor genes of these ligand genes were further increased by the germline RNAi of *rab-5* (Fig. 7b). Thus, the decreased expression of germline *rab-5* caused by PS-NP exposure was associated with the further activation of insulin, FGF, and ephrin ligands (Fig. 7c). In nematodes, the germline RNAi of these ligand genes resulted in resistance toward transgenerational PS-NP toxicity.<sup>43,45,47</sup> In *C. elegans*, the decrease in germline *mir-38* mediated transgenerational PS-NP toxicity by affecting WRT-3, a hedgehog ligand.<sup>55</sup> The increase in the germline *mir-240* mediated transgenerational PS-NP toxicity by affecting insulin peptides, FGF ligand, and ephrin ligand. These implied that germline *mir-240* and *mir-*

*38* acted together to regulate the transgenerational toxicity of nanoplastics by affecting different ligands and their receptors. Unlike the alteration in insulin, FGF, and ephrin ligands, the expressions of ligand genes of Wnt, Notch, and hedgehog were not affected by the germline RNAi of *rab-5* (Fig. 7a). In nematodes, these ligands in the germline also regulated transgenerational PS-NP toxicity.<sup>44,46,55</sup> This implies that the expressions of Wnt, Notch, and hedgehog ligands may be under the control of other germline Rab GTPases in PS-NP exposed nematodes. Besides ligand release, RAB-5 is also required for endocytosis.<sup>71,91,92</sup> The possible association of RAB-5 role in controlling the PS-NP toxicity with its function in modulating endocytosis needs to be further examined.

## Conclusions

Together, we examined the role of germline miRNAs in mediating transgenerational nanoplastic toxicity. The increase in germline *mir-240* expression mediated the transgenerational toxicity of PS-NP at ERCs. In the germline, RAB-5 acted as a downstream target of *mir-240*, and a decrease in germline *rab-5* expression further mediated PS-NP toxicity across generations. Moreover, RAB-5 regulated transgenerational PS-NP toxicity by suppressing the expressions of ligands of insulin, FGF, and ephrin signals and expressions of their receptors in the offspring. Our results suggested the important involvement of activated germline miRNAs in mediating transgenerational nanoplastic toxicity by affecting the release of the secreted ligands across generations.

## Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Southeast University and approved by the Animal Care & Welfare Committee of Southeast University.

## Data availability

The data supporting this article have been included as part of ESL.†

## Conflicts of interest

There are no conflicts of interests.

## Acknowledgements

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