Fatty Acid Metabolism is Involved in Stress Resistance Mechanisms of *Caenorhabditis elegans*

Makoto Horikawa [a, b], Kazuichi Sakamoto [a, 1]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan.

[b] Department of Molecular Genetics of Ageing, Max Planck Institute for Biology of Ageing, Cologne D-50931, Germany.

[1] To whom correspondence should be addressed: Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan. Fax: +81-29-853-4676; E-mail: sakamoto@biol.tsukuba.ac.jp

Abstract

Fatty acids are the major components of the phospholipid bilayer and are involved in several functions of cell membrane. We previously reported that fatty-acid metabolism is involved in the regulation of DAF-2/insulin signal in *Caenorhabditis elegans*. In this study, we investigate the role of fatty-acid metabolism in stress resistance with respect to *daf-16* in nematode. We found that fatty-acid metabolism regulates heat, osmotic, and oxidative-stress resistance in *C. elegans*. RNA interference (RNAi) of *fat-6*, *fat-7,* and *elo-2* enhanced heat resistance but decreased oxidative-stress tolerance. RNAi of *fat-2* strongly increased osmotic-stress resistance, whereas *nhr-49*-RNAi remarkably reduced osmotic and oxidative-stress tolerance. In *daf-16* mutants (*mgDf50*), RNAi of *fat-2* and *fat-7* increased viability under osmotic stress, while RNAi of *fat-6*, *fat-7*, and *elo-2* enhanced heat resistance. Exposure of saturated fatty acids to RNAi-worms of *fat-1*, *fat-7*, and *nhr-49* increased osmotic resistance. On the other hand, polyunsaturated fatty acids (PUFAs) reduced osmotic-stress tolerance in *fat-2*-RNAi worms, whereas PUFAs enhanced it in *nhr-49*-RNAi worms. Heat-stress resistance in *fat-6*- and *fat-7*-RNAi worms was suppressed by oleic acid.

These results suggest that stress-resistance mechanisms are regulated by fatty-acid metabolism with or without DAF-16 activity.

Keywords: fatty acid desaturase, fatty acid elongase, stress resistance, DAF-16

Abbreviations: DAF: Dauer-Defective Form, ELO: Fatty Acid Elongase, FAT: Fatty Acid Desaturase, NGM: Nematode Growth Medium, HNF: Hepatocyte Nuclear Factor, NHR: Nuclear Hormone Receptor, PQ: Paraquat, PUFA: Polyunsaturated Fatty Acid

Introduction

Phospholipids are the main components of the cell membrane, and the proportions of fatty acids in the phospholipid bilayer are closely related to the regulation of cell-membrane function. The fatty-acid composition in the cell membrane is strictly controlled by temperature [1-9]. In response to cold stress, bacteria activate fatty acid unsaturation and increase the fluidity of the cell membranes to adapt themselves to low temperatures [1-3]. In contrast, the proportion of unsaturated fatty acids is low in thermophilic and hyperthermophilic bacteria, which contributes to the stability of the cell membrane [4]. Animals and plants that live in cold climates or show cold tolerance also have a specific mechanism to activate fatty acid unsaturation or increase the proportion of unsaturated fatty acids [6-9]. However, the role of fatty acid unsaturation in stress resistance, and the interaction between fatty-acid metabolism and other stress responses is still unclear.

Caenorhabditis elegans has been used as the model organism for studies related to aging, aging-related diseases, and stress-response mechanism [10-12]. Insulin signaling is one of the most important pathways involved in the regulation of aging and stress resistance in *C. elegans* [10-15]. DAF-16, the transcription factor regulated by insulin signaling, is known to increase tolerance to various stresses, including heat, osmotic, oxidative, pathogenic, heavy-metal, and radioactive stress [10-20]. However, the genes involved in aging and stress resistance, which are controlled by DAF-16 activity, have not been elucidated.

Recent studies have reported that DAF-16 regulates the expression of fatty acid desaturase (*fat*) and fatty acid elongase (*elo*) genes [15,21]. Moreover, we have previously demonstrated that RNAi of *fat* and *elo* activates the transcription of DAF-16 [22], which indicates that fatty-acid metabolism and insulin signaling are interdependent. Therefore, we hypothesized that fatty-acid metabolism regulates stress resistance because RNAi of *fat, elo*, and *nhr* genes also regulates longevity in the worm.

In this report, we investigated the role of fatty-acid metabolism in the stress-response mechanisms of *C. elegans*. We analyzed the effect of RNAi of *fat, elo*, and *nhr* genes on heat, osmotic, and oxidative stress resistance. We also analyzed the physiological interaction between fatty-acid metabolism and insulin signaling in stress-resistance mechanisms.

Materials and Methods

Nematodes and culture conditions. Bristol N2 (wild-type) nematodes, *daf-2* mutants (*e1370*), and *daf-16* (*mgDf50*) mutants were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). These strains were grown at 20 °C on nematode growth medium (NGM) agar plated with *E. coli* OP50 using the protocol described by Brenner [23]; the *Daf-2* (*e1370*) strain was maintained at 15 °C.

Bacteria-mediated RNAi. Plasmid constructs designed for RNAi of individual genes (*elo-2*, *fat-1~7*, and *nhr-49*) were introduced into the nematodes via bacteria [22]. The *E. coli* strain HT115 was transfected with RNAi plasmids and plated on NGM agar (RNAi plate) [24]. To analyze the effect of fatty acids, we added 0.01% (w/vol) of palmitic, stearic, oleic, linoleic, and α-linolenic acid to NGM agar with 0.1% Nonidet P (NP)-40; the controls contained 0.1% NP-40 only. After treating adult worms with 10% NaClO solution (10 N NaOH/NaClO, 10:1), the eggs were collected and cultured overnight in S-basal medium (0.1 mol/l NaCl, 50 mmol/l potassium phosphate buffer; [pH 6.8]) at 20 °C. Hatched L1 larvae were then grown on RNAi plates with or without each fatty acid at 20 °C.

Osmotic-stress tolerance assay. A series of experiments was performed at 20 °C. Age-synchronized N2 worms were grown on RNAi plates with or without each fatty acid until they reached the young-adult stage, and the worms were treated with 0.5 mg/ml 5-fluoro-2'-deoxyuridine (FUdR) overnight. Twenty-four hours after the treatment, the worms were transferred to new RNAi plates containing 500 mM NaCl; life or death of a nematode was judged by its response to tapping on the plate 72 h after FUdR treatment. *Daf-2* and *daf-16* mutants were grown to the young-adult stage at 20 °C and were treated with FUdR for 24 h at 25 °C. Subsequently, the worms were transferred to 500 mM NaCl plates and bred at 25 °C; the survival rate of the worms was calculated at 36, 60, and 84 h after FUdR treatment.

Heat-tolerance assay. The age-synchronized worms were bred on RNAi plates with or without a fatty acid for 72 h. Worms were washed with S-basal buffer, transferred to bacteria-free NGM plates, and incubated at 35 °C for 13 h. After incubation, the worms were immediately cooled at 10 °C for 30 min, and the survival rate of the worms was calculated.

Oxidative-stress tolerance assay. The age-synchronized worms were bred on RNAi plates until they reached the young-adult stage, and worms were treated with 0.5 mg/ml FUdR overnight. After FUdR treatment, the worms were transferred to NGM plates containing 10 mM paraquat. The survival rate was calculated after 6 days.

Analysis of DAF-16 localization. Worms of the *TJ356* strain, which carries the *daf-16*::*gfp* construct, were used for the localization analysis of DAF-16. *TJ356* strain worms were grown on RNAi plates with or without a fatty acid for 96 h and observed under a fluorescence microscope. The location of DAF-16 was classified as follows: Nuc, Nuclear; Nuc/Cyt, Nuclear and Cytoplasm; and Cyt, Cytoplasm.

Results

Fatty-acid metabolism affected several stress-resistance mechanisms.

We investigated the effect of fatty-acid metabolism on 3 different stresses: osmotic stress, heat stress, and oxidative stress. RNAi of the *fat-2* gene strongly enhanced osmotic-stress tolerance (Fig 1A). In contrast, RNAi of *nhr-49* remarkably reduced osmotic-stress tolerance (Fig 1A). RNAi of other *fat* genes and *elo-2* had no effect on worm viability under high osmotic stress (Fig 1A). More than 85% of the worms that underwent RNAi of *fat-6*, *fat-7*, and *elo-2* endured heat treatment at 35 °C (Fig 1B). RNAi of *fat-2*, *fat-3*, *fat-4*, and *fat-5* increased worm viability after heat treatment by 20% when compared to the viability of control-RNAi worms. RNAi of *fat-1* and *nhr-49* had no effect on heat-stress tolerance (Fig 1B). Oxidative-stress tolerance was slightly enhanced in the worms that underwent RNAi of *fat-2* (Fig 1C). RNAi of *fat-3* reduced the viability of worms by 20%, whereas RNAi of *fat-6*, *fat-7*, *elo-2*, and *nhr-49* caused strong sensitivity to oxidative stress (Fig 1C).

Fatty-acid metabolism is involved in the regulation of DAF-2/insulin signaling.

We investigated the role of fatty-acid metabolism on DAF-2/insulin signaling. DAF-2/insulin signaling is known to be involved in stress-resistance mechanisms. Figure 2 shows that RNAi of *fat-2* and *elo-2* facilitated the nuclear translocation of DAF-16, whereas RNAi of other *fat* genes and *nhr-49* did not affect the intracellular localization of DAF-16 (Figs 2).

Role of daf-16 on stress tolerance in elo-, fat-, and nhr-RNAi worms.

We demonstrated that fatty-acid metabolism regulates DAF-2/insulin signaling and stress resistance. Next, we studied whether fatty-acid metabolism regulates stress resistance in a *daf-16*-dependent manner. First, we investigated the role of *daf-16* on osmotic-stress tolerance in the *daf* mutants that underwent RNAi of *fat*, *elo*, and *nhr* genes; we found that almost all RNAi worms were viable under osmotic stress conditions (Fig 3A). *Daf-2* mutants showed very high viability under osmotic stress with or without RNAi. Interestingly, RNAi of *fat-2* and *fat-7* increased the osmotic-stress tolerance of *daf-16* mutants 36 h treatment, but reduced worm viability 60 and 84 h after treatment (Fig 3A). Viability was much lower in control- and *fat-1*-RNAi of *daf-16* mutants. The loss of *daf-16* remarkably reduced osmotic-stress resistance in *nhr-49*-RNAi worms (Fig 3A). Secondly, we investigated the role of *daf-16* on heat tolerance. The loss of *daf-16* caused heat-stress sensitivity, but RNAi of *fat-6, fat-7*, and *elo-2* also increased heat-stress resistance in *daf-16* mutants (Fig 3B).

Role of fatty-acid on stress tolerance in elo-, fat-, and nhr-RNAi worms.

The effects of fatty-acid metabolism on stress resistance in *fat*-, *elo*-, and *nhr*-RNAi worms were analyzed. The treatment of worms with saturated fatty acids (such as palmitic and stearic acid) enhanced osmotic-stress tolerance in the control-, *fat-1*-, *fat-7*-, and *nhr-49*-RNAi worms (Fig 4A). Treatment with unsaturated fatty acids, such as oleic, linoleic, and α-linolenic acid also increased osmotic-stress resistance in *nhr-49*-RNAi worms, whereas there was no effect on control-, *fat-1*-, and *fat-7*-RNAi worms. Further, treatment with polyunsaturated fatty acids (PUFAs), such as linoleic and a-linolenic acid, significantly reduced the survival rate in *fat-2*-RNAi worms (Fig 4A). We had previously demonstrated that RNAi of *fat-2* increased the nuclear localization of DAF-16, and therefore, we analyzed the effects of fatty acids on

the localization pattern of DAF-16 in *fat-2*-RNAi worms. Interestingly, PUFAs suppressed the nuclear localization of DAF-16 in *fat-2*-RNAi worms (Fig. 4B). We also analyzed the effect of fatty acids on heat-stress resistance. The addition of oleic acid clearly reduced heat-stress tolerance in *fat-6*- and *fat-7*-RNAi worms (Fig. 4C). However, the addition of palmitic acid slightly increased heat-stress resistance. The addition of NP-40 remarkably decreased the survival rates of *elo-2*-RNAi worms, and both palmitic and oleic acid had no effect on heat-stress resistance. RNAi of *elo-2* suppressed nematode growth, but additional NP-40 restored the growth (data not shown). Furthermore, the addition of fatty acids did not affect heat-stress resistance in control-RNAi worms (Fig. 4C).

Discussion

Fatty acid metabolism is involved in adaptation to temperature changes [1-9]. Generally, unsaturated fatty acids increase cell-membrane fluidity at cold temperatures, whereas saturated fatty acids play a role in cell-membrane stability at high temperatures. We have demonstrated that RNAi of *fat-6*, *fat-7*, and *elo-2* enhanced heat-stress tolerance. Fat-6 and fat-7, both stearic acid desaturases, regulate the first step of the fatty acid desaturation pathway. Elo-2 is involved in the elongation of palmitic acid to stearic acid and regulates the desaturation by dat-6 and fat-7. Therefore, RNAi of *fat-6*, *fat-7*, and *elo-2* possibly caused the reduction in the proportion of unsaturated fatty acids and enhanced heat tolerance. The treatment of the worms with oleic acid reduced viability (Fig. 4C). However, it is unclear why NP-40 reduced heat-stress resistance in *elo-2*-RNAi worms. DAF-16 enhances heat tolerance by inducing the gene expression of heat shock proteins [25]. Further, RNAi of *fat-6, fat-7*, and *elo-2* enhanced heat tolerance in *daf-16* mutants (mgDf50) (Fig. 3B). Our results suggest that fatty-acid metabolism partially regulates heat-tolerance mechanisms without DAF-16 involvement.

Further, we found that fatty-acid metabolism is involved in osmotic-stress resistance. RNAi of *fat-2* remarkably enhanced osmotic-stress tolerance and activated DAF-16 (Fig. 1B and 2). In contrast, RNAi of *nhr-49* strongly reduced worm viability under high-salt conditions. *Daf-2* mutants showed very high viability under osmotic stress with or without RNAi, but *daf-16* mutants showed very low viability (Fig. 3A). These results suggest that RNAi of *fat-2* increased osmotic-stress resistance by mediating DAF-16

transcription. In fact, the PUFAs linoleic acid and α-linolenic acid suppressed the nuclear localization of DAF-16 in *fat-2*-RNAi worms and decreased their viability on salt plates (Fig 4A and B). However, RNAi of *fat-2* and *fat-7* enhanced osmotic-stress tolerance in *daf-16* mutants (Fig 3A), which suggested that fatty-acid metabolism regulates osmotic-stress tolerance mechanisms with and without DAF-16 activity. The addition of any fatty acid increased the viability of *nhr-49*-RNAi worms (Fig 4A). Nhr-49 is involved in the maintenance of fatty acid homeostasis through fatty acid synthesis and expenditure [26-28]. Although fatty acid homeostasis plays an important role in stress-resistance mechanisms, the mechanism underlying the regulation of osmotic resistance by fatty acids is unclear.

Paraquat treatment decreased the viability of *fat-6*-, *fat-7*-, *elo-2*-, and *nhr-49*-RNAi worms, but RNAi of *fat-2* increased oxidative-stress tolerance (Fig. 1C). DAF-16 was activated by *fat-2*-RNAi; therefore, RNAi of *fat-2* enhanced oxidative-stress resistance through the activation of DAF-16 (Fig. 2). Fat-6, fat-7, and elo-2 are involved in fatty acid desaturation; NHR-49 is involved in the gene expression of *fat-6 and fat-7* [26], and it regulates fatty acid desaturation. Unsaturated fatty acids are readily oxidized by intercellular reactive oxygen species [29]. It suggests that unsaturated fatty acid can act as intracellular scavengers. Therefore, the reduction in the ratio of unsaturated fatty acids by RNAi of *elo-2*, *fat-6*, *fat-7*, and *nhr-49* may be responsible for an increase in oxidative-stress sensitivity.

We have demonstrated that fatty acids are involved in the regulation of insulin signaling and several stress-resistance mechanisms (Figs. 1 and 2). The insulin-signaling pathway controls certain stress-response mechanisms via fatty-acid metabolism, and other fatty acids directly regulate stress-resistance mechanisms without DAF-16 activity (Figs 3). These results suggest that fatty acids can regulate DAF-16-mediated stress-resistance mechanisms by mediating insulin signaling; fatty acids can also play a role in direct defense against several stresses.

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Figure Legends

Fig. 1. Fatty-acid metabolism affected several stress-resistance mechanisms.

(A) Worms were cultured on RNAi media for 72 h and treated with 0.5 mg/ml FUdR for 24 h. Worms were transferred to RNAi plates containing 500 mM NaCl for 72 h and survival rate was calculated (N = 10). (B) Worms were cultured on RNAi media for 72 h and washed once with S-basal buffer. Then the worms were transferred to bacteria-free NGM plates and incubated at 35 °C for 13 h. After cooling at 10 °C for 30 min, the survival rate was calculated (N = 12). (C) Worms were cultured on RNAi media for 72 h and treated with 0.5 mg/ml FUdR for 24 h. Worms were transferred to RNAi plates containing 10 mM paraquat (PQ), and the survival rate was calculated after 6 days (N = 6). The error bar indicates the standard error (SEM).

Fig. 2. Fatty-acid metabolism is involved in the regulation of DAF-2/insulin signaling.

(A) Fluorescence microphotograph obtained using an L5 filter (Leica) showing *TJ356* worms (*daf-16*::*gfp*) bred on RNAi plates at 20 °C for 96 h. (B) The localization patterns of DAF-16 in the worms were classified as "Nuc", Nuclear; "Nuc/Cyt", Nuclear and Cytoplasm; and "Cyt", Cytoplasm. The number of worms of each strain was measured (N = 5). The error bar indicates the standard error (SEM).

Fig. 3. Role of *daf-16* on stress tolerance in *elo*-, *fat*-, and *nhr*-RNAi worms.

(A) N2 (WT), *daf-2* (*e1370*), and *daf-16* (*mgDf50*) worms were cultured on RNAi media for 72 h and treated with 0.5 mg/ml FUdR for 24 h at 25 °C. The worms were transferred to RNAi plates containing 500 mM NaCl and incubated at 25 °C. The survival rates were calculated 36, 60, and 84 h after treatment with FUdR (N = 5). (B) N2 and *daf-16* (*mgDf50*) were cultured on RNAi media for 72 h and washed with S-basal buffer. Then the worms were transferred to bacteria-free NGM plates and incubated at 35 °C for 13 h. After cooling at 10 °C for 30 min, the survival rate was calculated (N = 8). The error bar indicates the standard error (SEM).

Fig. 4. Role of fatty-acid on stress tolerance in *elo*-, *fat*-, and *nhr*-RNAi worms.

(A) Worms were cultured on RNAi media containing 0.01% (w/v) fatty acids (Pal: palmitic, Ste: stearic, Ole: oleic, Lin: linoleic, and Ala: α-linolenic) and 0.1% (w/v) NP-40 for 72 h and treated with 0.5 mg/ml FUdR for 24 h. Then, the worms were transferred to RNAi plates containing 500 mM NaCl and incubated at 25 °C. After 72 h of culture, the survival rate was calculated (N = 9). (B) The *TJ356* (*daf-16*::*gfp*) worms were bred on *fat-2*-RNAi plates containing 0.01% fatty acids (Ole: oleic acid, Lin: linoleic acid, Ala: α-linolenic acid) and 0.1% NP-40. After 96 h of incubation, GFP-fluorescence was observed under the microscope using an L5 filter. (C) Worms were cultured on RNAi media containing 0.01% (w/v) fatty acids (Pal: palmitic and Ole: oleic) and 0.1% (w/v) NP-40 and washed with S-basal buffer. The worms were then transferred to bacteria-free NGM plates and incubated at 35 °C for 13 h. After cooling at 10 °C for 30 min, the survival rate was calculated (N = 8). The error bar indicates the standard error (SEM)

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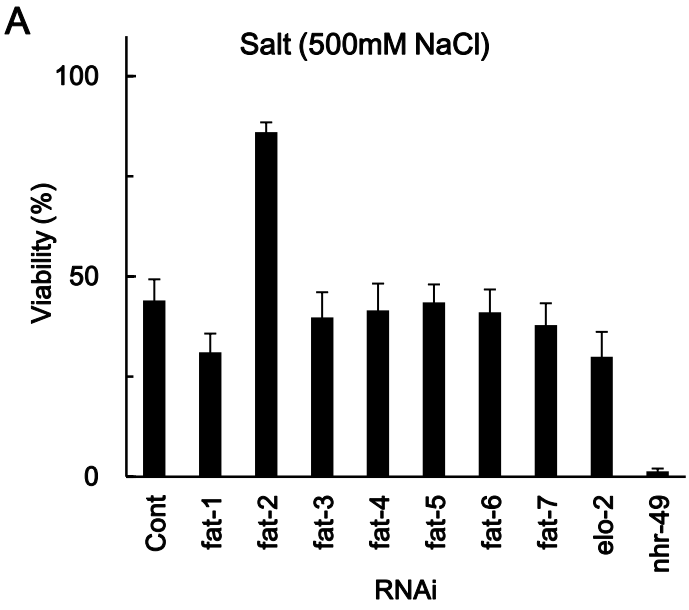
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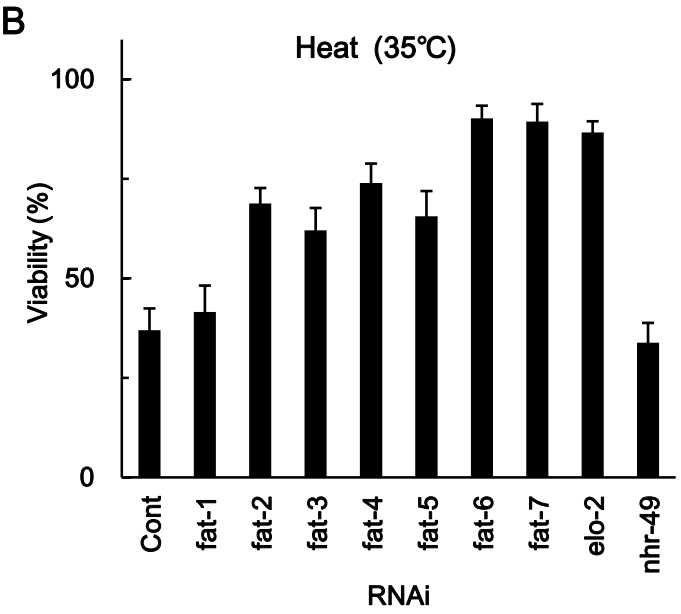
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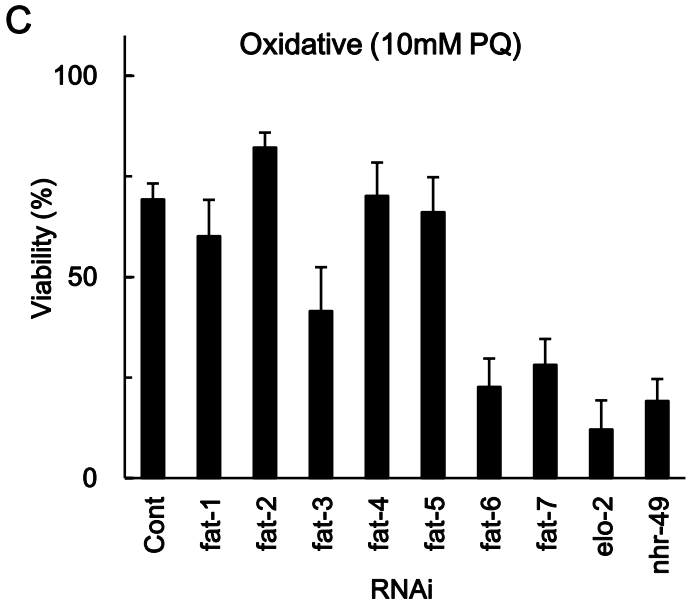
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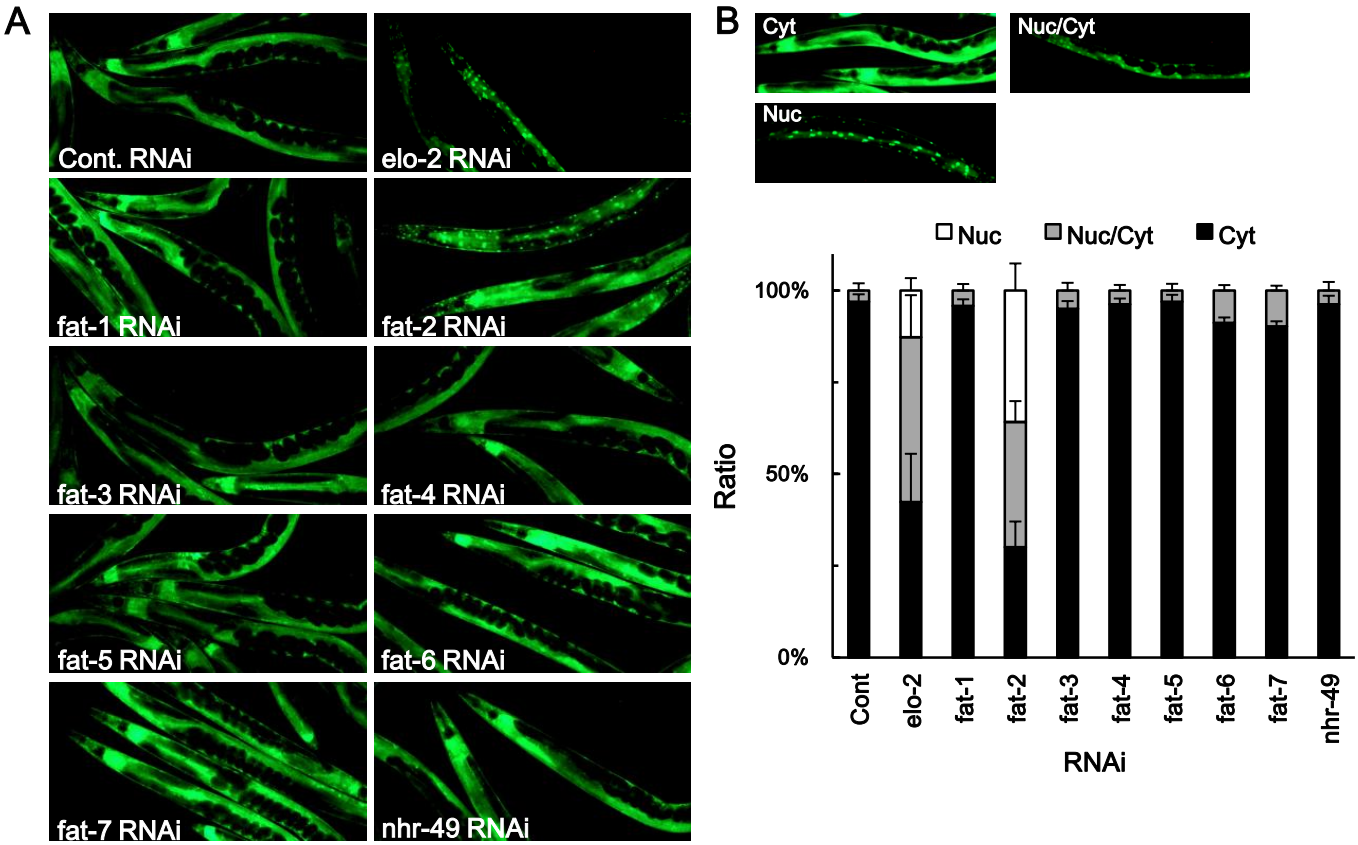
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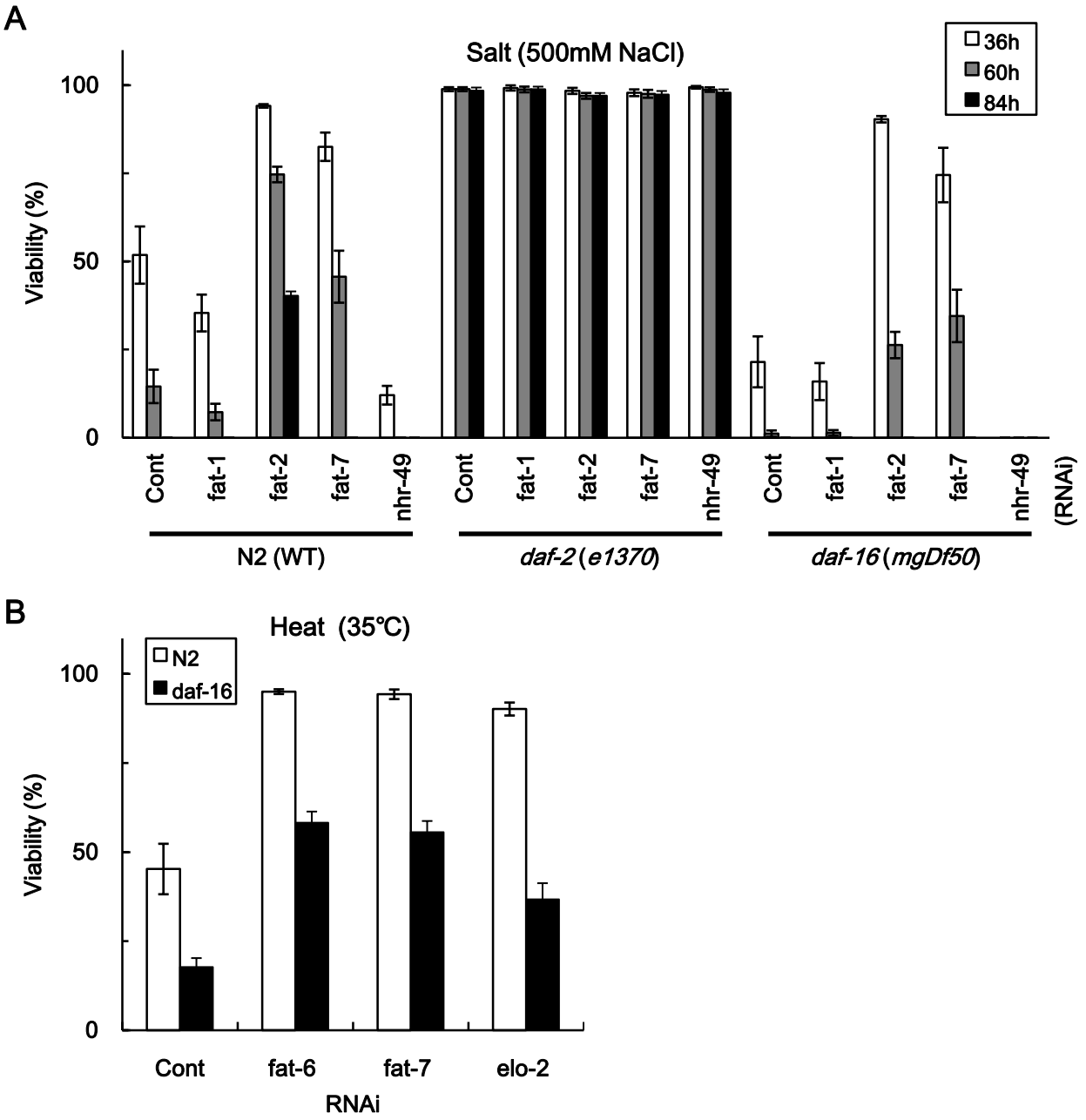
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Salt (500mM NaCl)
36h
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Viability (%)
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daf-16 (mgDf50)
B
Heat (35℃)
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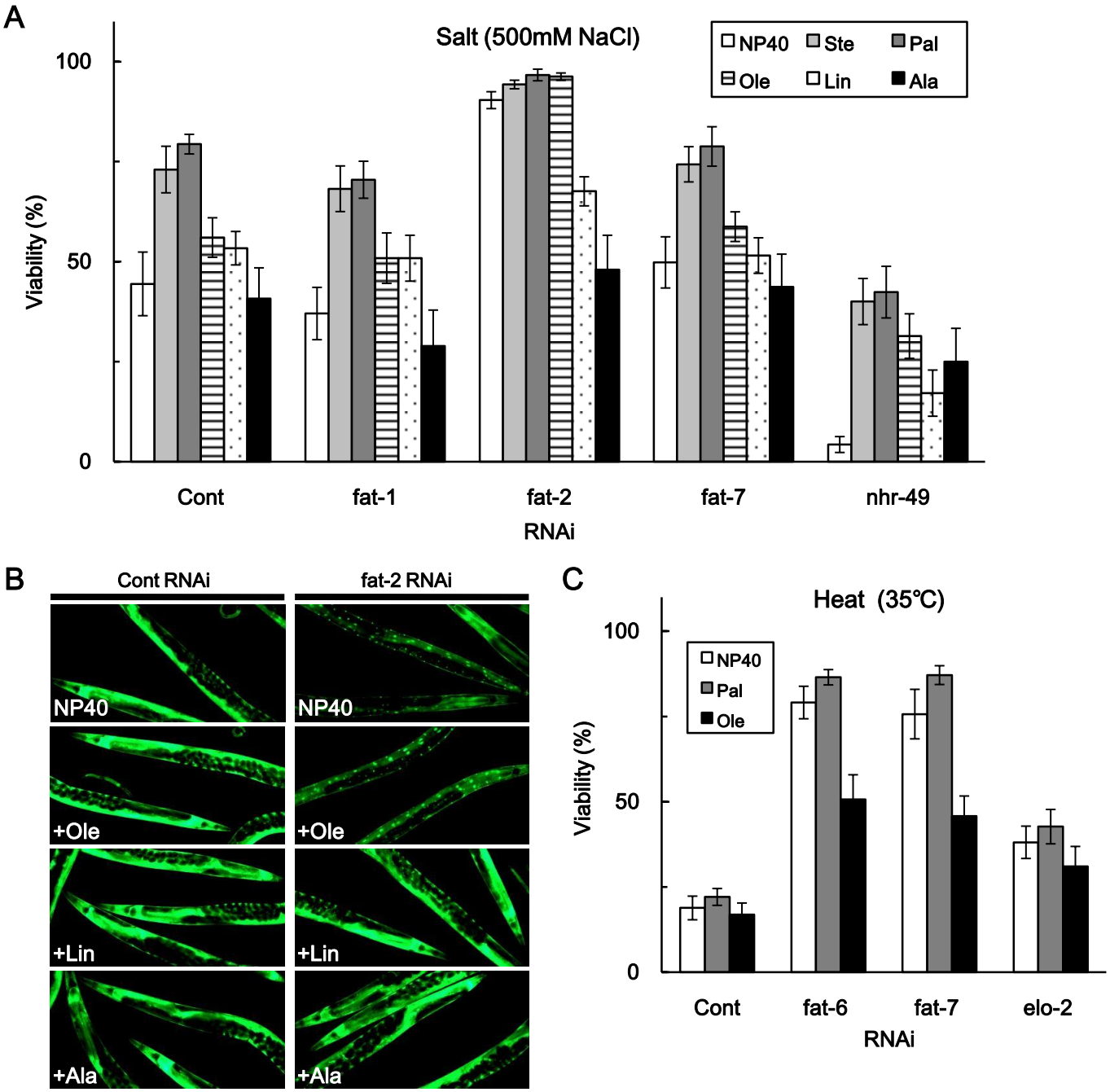
elo-2
RNAi


A
Salt (500mM NaCl)
NP40
Ste
Pal
Ole
Lin
Ala
100
50
0
Viability (%)
Cont
fat-1
fat-2
fat-7
nhr-49
RNAi
B
Cont RNAi
fat-2 RNAi
NP40
NP40
+Ole
+Ole
+Lin
+Lin
+Ala
+Ala
C
Heat (35°C)
NP40
Pal
Ole
100
50
0
Viability (%)
Cont
fat-6
fat-7
elo-2
RNAi