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FoxO/Daf-16 Restored Thrashing Movement Reduced by Heat Stress in *Caenorhabditis elegans*

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Abstract

Many studies on thermotolerance have been done in *Caenorhabditis elegans* in order to extend survival under heat stress; Daf-16, a homolog of FoxO in *Caenorhabditis elegans*, was detected as the key factor in thermotolerance. However, the recovery process from heat stress damage has been seldom discussed. In this study, we analyzed the roles of FoxO/Daf-16 on the recovery from heat stress damage by monitoring thrashing movement. Heat shock reduced the movement, which was restored by culturing at 20°C. Thrashing movement was not restored in the daf-16 mutant, which suggests that Daf-16 is one of the essential factors in repairing the damage. Movement restoration was promoted in the daf-2 mutant, a homolog of Insulin/IGF-1-like receptor, in a daf-16-dependent manner. In addition, heat stress decreased the expression of daf-28 and ins-7, agonists of Daf-2. Taken together, these results revealed that FoxO/Daf-16 removes heat stress damage and restores movement via inhibition of the insulin-like signaling pathway in *C. elegans*, suggesting that FoxO/Daf-16 plays a critical role in thermotolerance.

Key words: *Caenorhabditis elegans*, Thermotolerance, Daf-16, Thrashing movement, Insulin-like signaling pathway

1. Introduction

Heat stress induces protein denaturation or aggregation (Kampinga, 1993) and Ca^{2+} leakage (Kourtis et al., 2012; Lanner et al., 2012); therefore, heat stress can hinder an organism's survival. In order to protect our own health, we need to understand how humans prevent the accumulation of and recover from heat stress damage; therefore, studies on thermotolerance are needed.

Many studies on thermotolerance have been performed in *C. elegans*. Thermotolerance in *C. elegans* is related to the insulin-like signaling pathway. *C. elegans* die quickly under heat stress; however, their survival can be extended by inducing a daf-2 (insulin/IGF receptor homolog) knock out (KO) (Lithgow et al., 1995). Because the daf-2 KO induces the nuclear localization of Daf-16, a homolog of FoxO, (Lin et al., 2001; Henderson and Johnson, 2001; Yen et al., 2011) and extends the lifespan of *C. elegans* in a Daf-16-dependent manner (Kenyon et al., 1993; Murphy et al., 2003; Yen et al., 2011), Daf-16 is considered a key factor in thermotolerance. In a previous study,

it was shown that Daf-16 is localized by heat stress in nucleus (Henderson and Johnson, 2001; Lin et al., 2001; Singh and Aballay, 2009). It was proved that Daf-16 overexpression extends survival under heat stress (Henderson and Johnson, 2001). Our previous study showed that viability is decreased by the daf-16 KO under heat stress (Horikawa and Sakamoto, 2009). In addition, the survival extension by the daf-2 KO is dependent on Daf-16 under heat stress (McColl et al., 2010). Therefore, FoxO/Daf-16 is expected to prevent the accumulation of damage from heat stress.

Although many studies focused on FoxO/Daf-16 and thermotolerance, the recovery process from heat stress damage has been seldom discussed. Likely, *C. elegans* can restore the damage from heat stress, because it was suggested that the stimulation of weak heat stress extends lifespan and induces thermotolerance (Lithgow et al., 1995; McColl et al., 2010; Kourtis et al., 2012) and innate immunity in a Daf-16-dependent manner (Singh and Aballay, 2006). In addition, the daf-2 mutant survives for a long time compared to WT after acute heat shock, which is daf-16 dependent (McColl et al., 2010). Hence, we analyzed the mechanisms underlying the restoration from heat stress damage by using *C. elegans*.

For the recovery, we decided to use thrashing movement because *C. elegans* can be observed for a long time after heat treatment. The movement of *C. elegans* is often used as an index in studies of polyglutamine (PolyQ) diseases. *C. elegans* expressing PolyQ had a larger decline in movement or were paralyzed in an age- and temperature-dependent manner (Morley et al., 2002; van Ham et al., 2010; Haldimmann et al., 2011). Because heat stress induces protein aggregation (Kampinga 1993), it is probable that heat stress alters thrashing movement.

We studied the roles of Daf-16 on altered thrashing movement in *C. elegans* exposed to heat stress and analyzed the activation pathway of Daf-16 under heat stress. We discovered a novel function for Daf-16, which is the focus of much *C. elegans* research (Yen et al., 2011), such as longevity (Kenyon et al., 1993; Murphy et al., 2003; Hashimoto et al., 2010; Kwon et al., 2010), thermotolerance, oxidative stress (Honda and Honda, 1999; Heidler et al., 2010), lipid metabolism (Horikawa and Sakamoto, 2009; Horikawa and Sakamoto, 2010), and innate immunity (Singh and Aballay, 2006; Alper et al., 2007; Kawli and Tan, 2008; Hahm et al., 2011)

2. Materials and methods

2.1 Strains and culture

Wild-type (WT) *Caenorhabditis elegans* Bristol N_2, daf-16 mutant (mgDf-15), daf-2 mutant (e1370), and TJ356 (*daf-16::gfp*) transgenic mutants were provided by the Caenorhabditis Genetics Center (CGC, MN, USA). Each strain was cultured on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 as previously described (Brenner 1974).

2.2 NaClO treatment

To synchronize the growth of *C. elegans*, adult worms were treated with a 10:1 NaClO solution (NaClO [Haiter, KAO, Tokyo, Japan]:10N NaOH [WAKO, Osaka, Japan)]). The eggs were cultured in S-basal (0.1 M NaCl [Kanto Chemical, Tokyo, Japan], 50 mM potassium phosphate buffer [pH 6.0]) until hatching at 20°C.

2.3 Preparing cDNA

Adult worms were collected by S-basal and washed with dDW to remove *E. coli*. RNA was extracted with RNAiso PLUS (Takara, Shiga, Japan) from *C. elegans* extract and treated with DNase I (Takara). cDNA was synthesized by using M-MLV Reverse Transcriptase (Takara) or PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara).

2.4 Feeding RNAi

Plasmid DNA L4440 (Fire Laboratory), which has a daf-16 cDNA fragment in the multicloning site, was transformed into *Escherichia coli* HT115 treated with 50 mM $CaCl_2$ (WAKO). Primers for preparing the cDNA insert are listed in Table 1. The HT115 strain transformed with the L4440 plasmid was treated with isopropyl-β-D-thiogalactopyranoside (IPTG; WAKO) to induce dsRNA expression. After treatment, HT115 was seeded onto NGM RNAi medium plates (Timmons and Fire 1998). Age-synchronized L1 larvae were transferred onto an RNAi plate.

2.5 Pharynx pumping assay under heat stress

Age-synchronized L1 larvae were transferred onto a plate and cultured for 4 days at 20°C. After 4 days, the *C. elegans* adult worms were transferred onto a new NGM plate seeded with OP50 and cultured at 35°C for 0–4 h. Ten *C. elegans* were chosen randomly, and pharynx pumping was counted for 15 s every h.

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137 *2.6 Thrashing movement assay under heat stress*

138 Age-synchronized L1 larvae were transferred onto a plate and cultured for 4 days at
139 20°C. After 4 days, the *C. elegans* adult worms were transferred onto an NGM plate
140 without food and cultured at 35°C for 4 h. After heat treatment, the *C. elegans* were
141 transferred onto a new plate and cultured for 0–24 h, after which 10 *C. elegans* chosen
142 randomly were moved to S-basal on an NGM plate without food and the thrashing
143 movement was counted for 15 s. However, we did not choose worms that still showed
144 remarkable weakness 3 h after heat treatment.

145

146 *2.7 Observation of Daf-16::GFP*

147 Age-synchronized TJ356 (*daf-16::gfp*) was collected by S-basal and fixed in 1%
148 paraformaldehyde (PFA) solution ((2% PFA [WAKO], 20% EtOH [WAKO], 25 mM
149 potassium phosphate buffer, 50 mM NaCl [Kanto Chemical]):S-basal = 1:1). GFP
150 fluorescence was observed under a BZ8000 fluorescence microscope (KEYENCE Japan,
151 Osaka, Japan).

152

153 *2.8 Analysis of gene expression*

154 *C. elegans* exposed to heat stress for 4 h were collected by S-basal. cDNA was prepared
155 and amplified on an ABI-7300 system (Applied Biosystems, CA, USA) using
156 Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan). Primers are listed in Table 1.

157

158 *2.9 Statistical analysis*

159 Significant differences were analyzed with t-test, Games-Howell test and Dunnett's T3
160 test with statistical differences represented by $*p < 0.05$ and $**p < 0.005$.

161

162 **3. Results**

163

164 *3.1 C. elegans can restore the thrashing movement reduced by heat stress*

165 First, we observed pharynx pumping under heat stress, because it was reported that
166 feeding of *C. elegans* is totally inhibited at 32°C (Jones and Candido, 1999). As a result,
167 pumping was remarkably decreased by heat stress (Figure 1A). Because starvation
168 stress affects the insulin signaling pathway and the translocation of Daf-16 (Henderson
169 and Johnson, 2001; Weinkove et al., 2006), we compared movement under heat stress

conditions to normal conditions on an NGM plate without food to remove the quantity of food ingested as a factor. We analyzed the altered thrashing movement under heat stress. *C. elegans* adult worms were cultured at 20°C for 4 h (20°C, [+]), or cultured on an NGM plate without food at 20°C (20°C, [-]) or 35°C for 4 h (35°C, [-]). After 4 h, *C. elegans* were transferred to a new NGM plate seeded with OP50 and cultured at 20°C for 0–24 h. We found no change in movement between the NGM plate with food and the *E. coli*-free plate; however, heat stress remarkably reduced the thrashing movement (Figure 1B). After 24 h, the movement of *C. elegans* under heat stress (35°C, [-]) increased by 80%. Therefore, *C. elegans* can restore the thrashing movement reduced by heat stress.

3.2 Daf-16 is activated immediately after heat shock

Next, we observed Daf-16 activation. First, we used the TJ356 strain (*daf-16::gfp*) to observe the behavior of Daf-16 under heat stress. Adult TJ356 worms were cultured at 20°C for 4 h (20°C, [+]), or cultured on an NGM plate without food at 20°C (20°C, [+]) or 35°C for 4 h (35°C, [-]). As a result, Daf-16 was localized by heat stress in nuclear (Figure 2A). Although, the previous study suggested that Daf-16 is localized in nuclear under starvation stress for 1 day in TJ356 (Bamps et al., 2009), starvation stress for 4 h didn't induce Daf-16 nuclear translocation (Fig. 2A). Therefore, it is suggested that heat stress induces Daf-16 nuclear translocation more than starvation stress. Heat-treated TJ356 was transferred to a new NGM plate seeded with OP50 and cultured at 20°C for 24 h. GFP localization of worms treated with heat stress (35°C, [-]) at 24 h was decreased compared to that of 0 h (Fig. 2A), as found in previous studies (Singh and Aballay, 2009). Even though, nuclear localization ratio of worms treated with heat stress (35°C, 24 h) was still much higher than those of other conditions (20°C, 24 h). In addition, we analyzed the expression of hsp-12.6, a daf-16 downstream gene, by using qRT-PCR. The results showed that heat stress increased hsp-12.6 expression (Figure 2B) as previously reported (Hsu et al., 2003; McColl et al., 2010). These results showed that the transcriptional activity of Daf-16 was enhanced immediately after heat treatment.

3.3 Daf-16 is needed to restore thrashing movement reduced by heat stress

We analyzed the relation of Daf-16 to altered thrashing movement by using a daf-16 mutant (mgDf50). WT and daf-16 mutants were transferred to a new NGM plate and

cultured at 20°C or 35°C for 4 h. After 4 h, each strain was transferred to a new NGM plate seeded with OP50 and cultured at 20°C for 0, 12, and 24 h. The movement of the daf-16 mutant could not be restored (Table 2 and Figure 3A). We also observed the movement of daf-16 RNAi under heat stress. *C. elegans* treated with daf-16 RNAi also displayed restoration inhibition (Table 2 and Figure 3B). Additionally, daf-16 RNAi remarkably decreased the GFP fluorescence of the TJ356 strain (Figure 3C). Therefore, daf-16 was knocked down by our plasmid DNA. These results suggest that Daf-16 restores the thrashing movement reduced by heat stress.

3.4 Daf-2 promotes restoration of thrashing movement in dependent of Daf-16

To analyze altered movement under conditions of Daf-16 activation, we used a daf-2 mutant (e1370). WT or daf-2 mutants were transferred to a new NGM plate and cultured at 20°C or 35°C for 4 h, after which each strain was transferred to a new NGM plate seeded with OP50 and cultured at 20°C for 0, 3, and 6 h. As a result, the movement of the daf-2 mutant was greater than the WT 0 h after heat treatment, and it was almost restored 6 h later (Table 3 and Figure 4A).

Next, we treated the daf-2 mutant with daf-16 RNAi. The results showed that the movement of the daf-2 mutant was unchanged between the empty vector and the daf-16 RNAi at 0 h. In addition, thrashing movement was not increased by daf-16 knockdown (Table 3 and Figure 4B). Previous study suggested heat stress increase hsp-12.6 expression in daf-2 mutant in a Daf-16 dependent manner (McColl et al., 2012). We analyzed the hsp-12.6 expression of WT and daf-2 mutant in recovery process. As a result, expression of hsp-12.6 in daf-2 mutant was still high compared to that of WT in recovery process (Fig. 4C). And, hsp-12.6 expression was reduced due to restoration of thrashing movement (Fig. 4A and Fig. 4C). Therefore, these results suggested that daf-2 KO promotes restoration of thrashing movement via induction of Daf-16 activation.

3.5 Heat stress suppresses the activity of the insulin-like signaling pathway

Daf-16 restored the thrashing movement in the WT strain (Figure 3). Additionally, Daf-16 promoted restoration in the daf-2 mutant (Figure 4). Therefore, heat stress may affect the activity of the ILS pathway. To analyze the activity of the ILS pathway under heat stress, we measured gene expression of daf-28, a homolog of insulin, and ins-7, a homolog of IGF-1, which are Daf-2 agonists (Wormbase: http://www.wormbase.org). Therefore, we focused on their insulin-like peptides. The results showed that the ins-7

238 and daf-28 expression was remarkably decreased by heat stress (Figure 5). Therefore, it
239 was suggested that heat stress inactivates the insulin-like signaling pathway.
240
241 **4. Discussion**
242
243 We found that a novel function of Daf-16 was to restore the thrashing movement that
244 was decreased by heat stress (Figure 3 and Figure 4). Daf-16 activity was enhanced
245 immediately after heat treatment (Figure 2) and was suppressed by a daf-16 KO or
246 RNAi, resulted in the increase of the stress resistance genes expression (Hsu et al.,
247 2003; McColl et al., 2010). Therefore, enhancement of Daf-16 downstream genes is
248 necessary for restoring the thrashing movement that was reduced by heat stress.
249 Downstream genes of Daf-16, such as sod-3 and hsp-12.6, preserve cells from stress,
250 including heat stress. In fact, it was reported that heat stress increased the fluorescent
251 flux of DCF in *C. elegans* (Kampk¨otter et al., 2007) and remarkably increased Sod-3
252 expression (Wolf et al., 2008). Research on poly-glutamine (PolyQ) diseases showed
253 that knockdown of Daf-16 or Hsp-12.6 accelerated the aggregation of the PolyQ protein
254 (Hsu et al., 2003) and daf-16 RNAi accelerated paralysis in a *C. elegans* of PolyQ
255 disease model (Haldimann et al., 2011). And, hsp-12.6 expression was decreased due to
256 restoration of thrashing movement in daf-2 mutant (Fig. 4A and Fig. 4C). Therefore, it
257 is expected that Daf-16 activity possibly relate to removal of heat stress damage and
258 restoration of the thrashing movement.
259 This restoration was promoted in a daf-2 KO in a daf-16-dependent manner (Figure 4).
260 In the daf-2 mutant, Daf-16 is localized in the nucleus (Lin et al., 2001) and enhances
261 the expression of stress resistance genes (Hsu et al., 2003; McElwee et al., 2003;
262 Murphy et al., 2003). Therefore, it is expected that Daf-16 nuclear localization is
263 maintained before and after heat shock in the daf-2 mutant. In other words, downstream
264 genes of Daf-16 may be enhanced at all times in the daf-2 mutant. In fact, hsp-12.6 level
265 of daf-2 mutant was still high in recovery process compared to that of WT (Fig. 4C).
266 Therefore, these results suggest that the consecutive activation of FoxO/Daf-16
267 accelerates heat stress damage recovery. However, daf-2 KO prevented the decline of
268 thrashing movement in a Daf-16-independent manner (Fig. 4B). So, Daf-16 and its
269 downstream genes are involved in restoration of thrashing movement only.
270 Heat stress decreased daf-28 and ins-7 gene expression, agonists of Daf-2 (Figure 5).
271 Our findings suggest that heat stress suppresses the activity of the ILS pathway. Daf-16

is translocated to the nucleus from the cytoplasm by ins-7 RNAi (Murphy et al., 2007; Kawli and Tan, 2008). It has also been shown that Daf-16 is localized in the nucleus by daf-28 RNAi in the L2 larval stage (Li et al., 2003). Decreasing daf-28 and ins-7 promoted the *C. elegans* innate immunity against bacteria by Daf-16 (Kawli and Tan, 2008; Hahm et al., 2011). Additionally, the thrashing movement was restored via Daf-16 in the daf-2 mutant (Figure 4). These findings strongly suggest that Daf-16 is activated by heat stress via inactivation of the insulin-like signaling pathway.

FoxO families are the common transcription factor existed in wide varieties of organisms, and have the similar functions on oxidative stress and longevity (Kenyon 2010). In fact, it is generally understood that FoxO increases expression of genes related to anti-oxidant (Welker et al., 2013) in response to oxidative stress; Daf-16 increases sod-3 expression under oxidative stress and promotes oxidative stress tolerance in *C. elegans* (Honda and Honda, 1999; Yoshinaga et al., 2003). Furthermore, FoxO also induces longevity in yeast (Postnikoff et al., 2012), *C. elegans* (Kenyon et al., 1993) and *D. melanogaster* (Slack et al., 2011). In addition, it is reported that deletion of insulin receptor extends lifespan in mice (Selman et al., 2008) and FoxO3a relates to longevity in human too (Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009; Li et al., 2009; Kenyon 2010). Therefore, although the functions and the behaviors of FoxO family under heat stress in other organisms are still unclear, it is expected that FoxO families play the similar functions on thermotolerance in side varieties of species including human. It was reported that keratinocyte-specific FoxO1 KO inhibits wound healing in mice (Ponugoti et al., 2013). And, FoxO3a maintains the expression of pro-autophagic genes and rescued the hematopoietic stem cells from apoptosis induced by metabolic stress (Warr et al., 2013). Therefore, FoxO of mammals has, at least, the function of removing and recovering damage. It is expected that our findings are important for further analysis of thermotolerance and give the idea to establish the treatment of heat stroke and summer weakness.

Overall, we concluded that FoxO/Daf-16 was activated by inhibiting the insulin-like signaling pathway under heat stress, removed heat stress damage, and recovered movement in *C. elegans*. In addition, we showed that consecutive activation of FoxO/Daf-16 accelerated the heat stress damage recovery. Our findings not only provide new knowledge of thermotolerance but also provide a strategy toward studying disorders related to heat stress, such as heat stroke and summer weakness.

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Legends

Figure 1. Alteration of movement under heat stress. (A) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to a new NGM plate seeded with OP50 and cultured at 35°C for 0–4 h. Ten *C. elegans* were chosen randomly and pumping was counted for 15 s every h. 3 independent experiments were performed and these data were combined for graph. Significant differences were analyzed with t-test. N = 30, mean ± SE, **P* < 0.05, ***P* < 0.005. (B) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an NGM plate seeded with OP50 (+) or an *E. coli*-free NGM plate (-) and cultured for 4 h at 20°C or 35°C. After 4 h, *C. elegans* was transferred to a new NGM plate seeded with OP50 and cultured for 0–24 h. After 0–24 h, 10 *C. elegans* chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. 3 independent experiments were performed and these data were combined for graph. Significant differences were analyzed with t-test. N = 30, mean ± SE, **P* < 0.05, ***P* < 0.005.

Figure 2. Activity of Daf-16 under heat stress. (A) An age-synchronized L1 larva of TJ356 was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an NGM plate seeded with OP50 (+) or an *E. coli*-free NGM plate (-) and cultured for 4 h at 20°C or 35°C. TJ356 cultured in each

condition were transferred to new NGM plates seeded with OP50. TJ356 were fixed in 1% PFA solution. Fluorescence emitted by GFP was observed using fluorescence microscopy. The number of worms that GFP was localized in the nucleus (Nuc), cytosol (Cyto) or both of them was counted. 3 independent experiments were performed and these data were combined for making graph. N = 3, mean ± SE, Scale = 100 μm. (B) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an NGM plate seeded with OP50 (+) or an *E. coli*-free NGM plate (-) and cultured for 4 h at 20°C or 35°C. RNA was extracted and cDNA was synthesized. Expression of hsp-12.6 was measured using qRT-PCR. 2 independent experiments were performed and these data were combined for graph. In each trial, gene expression was analyzed by 3 different wells. Significant differences were analyzed with Dunnett's T3 test. N = 6, mean ± SE, $*P < 0.05$, $**P < 0.005$.

Figure 3. Alteration of thrashing movement by Daf-16 KO under heat stress. (A) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, *C. elegans* were transferred to a new NGM plate seeded with OP50 and cultured for 0–24 h. After 0, 12, or 24 h, 10 *C. elegans* chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the movement of *C. elegans* treated by heat stress divided by the movement of *C. elegans* untreated by heat stress. 3 independent experiments were performed and these data were combined graph. Significant differences were analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. Details are shown in Table 2. (B) An age-synchronized L1 larva was transferred to an RNAi plate and cultured for 4 days at 20°C. Adult worms were transferred to an *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, *C. elegans* were transferred to a new NGM RNAi media plate cultured for 0–24 h. After 0, 12, or 24 h, 10 *C. elegans* chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. *C. elegans* treated by heat stress divided by movement of *C. elegans* untreated by heat stress. 3 independent experiments were performed and these data were combined for graph. Significant differences were analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. Details are shown in Table 2. (C) An age-synchronized L1 larva of TJ356 was transferred to an RNAi plate

and cultured for 4 days at 20°C. Adult worms were fixed in 1% PFA solution, and fluorescence was observed via fluorescence microscopy. Scale = 100 μm.

Figure 4. Alteration of thrashing movement by Daf-2 KO under heat stress. (A) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, *C. elegans* were transferred to a new NGM plate seeded with OP50 and cultured for 0–6 h. After 0, 3, or 6 h, 10 *C. elegans* chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the movement of *C. elegans* treated by heat stress divided by the movement of *C. elegans* untreated by heat stress. 3 independent experiments were performed and these data were combined for graph. Significant differences were analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. Details are shown in Table 3. (B) An age-synchronized L1 larva was transferred to an RNAi plate and cultured for 4 days at 20°C. Adult worms were transferred to an *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, *C. elegans* were transferred to a new NGM RNAi media plate cultured for 0–6 h. After 0, 3, or 6 h, 10 *C. elegans* chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the movement of *C. elegans* treated by heat stress divided by the movement of *C. elegans* untreated by heat stress. 3 independent experiments were performed and these data were combined for graph. Significant differences were analyzed with Games-Howell test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. Details are shown in Table 3. (C) An age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an *E. coli*-free NGM plate and cultured for 4 h at 35°C. After 4 h, RNA was extracted from *C. elegans* as 0 h or *C. elegans* were transferred to a new NGM plate seeded with OP50 and cultured for 3–6 h. After 3 or 6 h, RNA was extracted from *C. elegans*. cDNA was synthesized and expression of hsp-12.6 was measured by qRT-PCR. Gene expression was analyzed by 3 different wells. Significant differences were analyzed with t-test. N = 6, mean ± SE, $*P < 0.05$, $**P < 0.005$.

Figure 5. Activity of insulin-like signaling pathway under heat stress. An

age-synchronized L1 larva was transferred to an NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to an NGM plate seeded with OP50 (+) or an *E. coli*-free NGM plate (-) and cultured for 4 h at 20°C or 35°C. RNA was extracted and cDNA was synthesized. Expression of daf-28 and ins-7 was measured by using qRT-PCR. 2 independent experiments were performed and these data were combined for graph. In each trial, gene expression was analyzed by 3 different wells. Significant differences were analyzed with Dunnett's T3 test. N = 6, mean ± SE, *$P < 0.05$, **$P < 0.005$.

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581 **Table 1.** Primer sequences for quantitative PCR.

Gene	Sense	Antisense	Reference
(qRT-PCR)			
Pan-actin*	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA	Kawli and Tan, 2008
hsp-12.6	TGGAGTTGTCAATGTCCTCG	GACTTCAATCTCTTTTGGGAGG	Kwon et al., 2010
ins-7	CATGCGAATCGAATACTGAAG	GAAGTCGTCGGTGCATTC	Kawli and Tan, 2008
daf-28	TTCCGTATGTGTGGAGTGTC	TTTGTATATACTCGGCAGTGC	Hahm et al., 2011
(RNAi)			
daf-16	CATGGATCCATCCAGATGCAAAGCCAG	CATGGATCCGTATGCTGTGCAGCTACA	Hashimoto et al., 2010

582 qPCR was performed by ABI 7300 with the default cycling condition [50^{o}C/2 min,
583 95^{o}C/10 min, (95^{o}C/15 sec, 60^{o}C/1 min) x 40]. Pan-actin was used as internal control.
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Table 2. Times of thrashing movement in Figure 3.

Strain	Time (hr)	N	20°C (mean ± SEM)			35°C (mean ± SEM)		
wild type	0	30	55.17	±	1.44	1.57	±	0.36
	12	30	50.30	±	1.10	26.67	±	2.55
	24	30	42.27	±	1.00	32.63	±	2.10
daf-16 (mgDf50)	0	30	63.63	±	1.75	2.70	±	1.05
	12	30	56.07	±	1.86	13.20	±	2.10
	24	30	55.67	±	1.19	11.13	±	2.54
empty vector	0	30	49.87	±	1.06	6.83	±	1.41
	12	30	49.07	±	1.31	40.07	±	1.39
	24	30	43.03	±	1.34	38.53	±	1.34
daf-16 RNAi	0	30	47.30	±	1.27	6.33	±	1.30
	12	30	50.27	±	1.77	25.47	±	2.31
	24	30	44.47	±	1.27	27.90	±	1.53

These values show times of thrashing movement in Figure 3. N shows the number of *C. elegans* at each temperature.

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623 **Table 3.** Times of thrashing movement in Figure 4.

Strain	Time (hr)	N	20°C (mean ± SEM)			35°C (mean ± SEM)		
Wild type	0	30	53.20	±	1.14	3.43	±	0.88
	3	30	55.10	±	1.42	11.60	±	1.49
	6	30	45.67	±	1.06	13.87	±	1.88
daf-2 (e1370)	0	30	63.97	±	0.84	21.60	±	2.36
	3	30	58.13	±	0.98	45.07	±	1.58
	6	30	53.23	±	1.17	49.07	±	1.52
Wild type + empty	0	30	56.37	±	1.01	10.20	±	2.26
	3	30	49.50	±	1.03	15.73	±	1.67
	6	30	52.07	±	1.31	28.20	±	2.55
daf-2 + empty	0	30	62.90	±	1.25	40.53	±	1.99
	3	30	61.40	±	1.04	59.37	±	1.85
	6	30	61.43	±	1.41	63.20	±	1.45
daf-2 + daf-16 RNAi	0	30	61.60	±	1.40	33.90	±	2.07
	3	30	56.20	±	1.04	33.60	±	1.32
	6	30	54.87	±	1.01	39.27	±	2.10

624 These values show times of thrashing movement in Figure 4. N shows the number of *C.*
625 *elegans* at each temperature.
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FIG 1

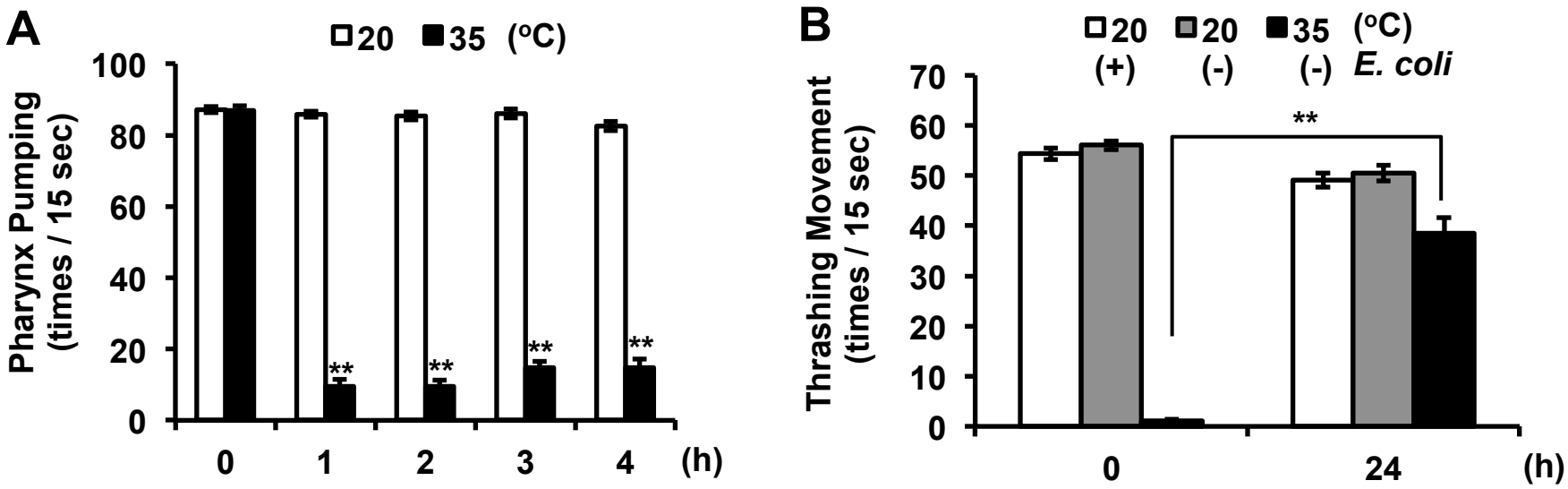
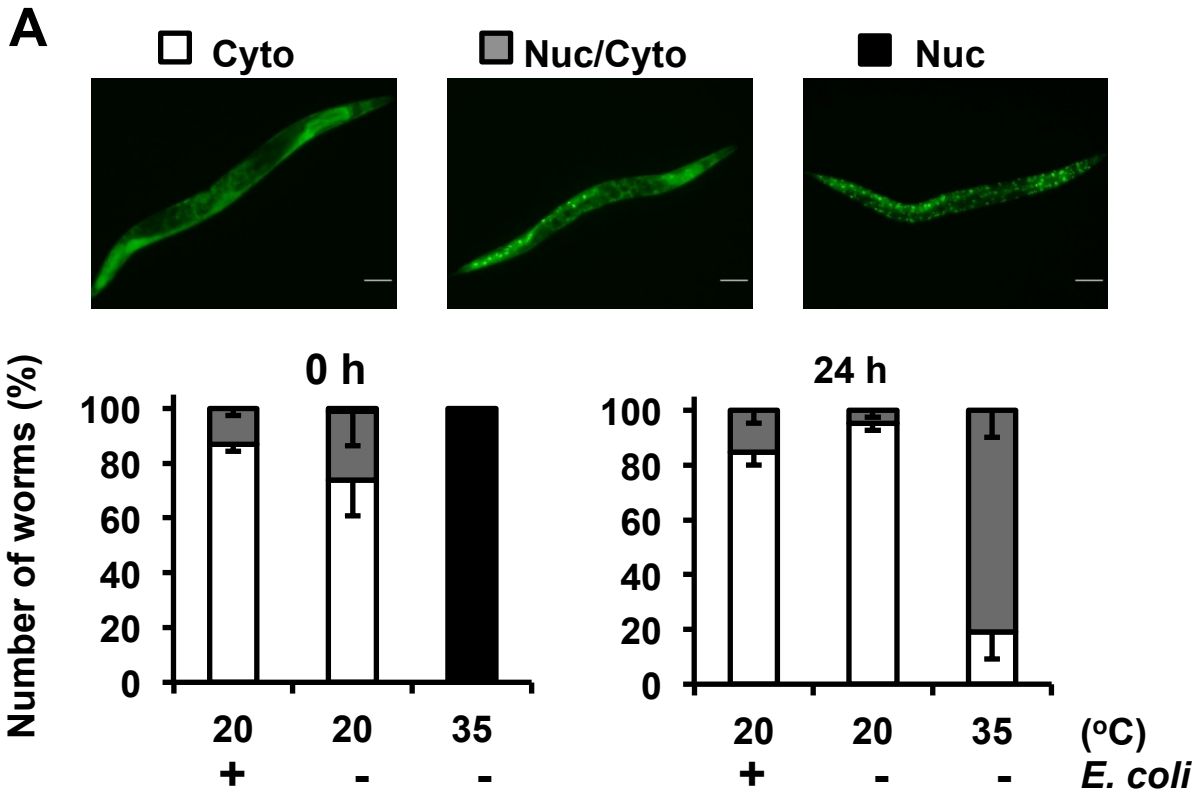

A
20
35 (°C)
Pharynx Pumping
(times / 15 sec)
100
80
60
40
20
0
**
0
1
2
3
4
(h)
B
20 (+)
20 (-)
35 (-)
(°C)
E. coli
Thrashing Movement
(times / 15 sec)
70
60
50
40
30
20
10
0
**
0
24
(h)

FIG 2


A
Cyto
Nuc/Cyto
Nuc
0 h
24 h
Number of worms (%)
100
80
60
40
20
0
20
20
35
+
-
-
(°C)
E. coli

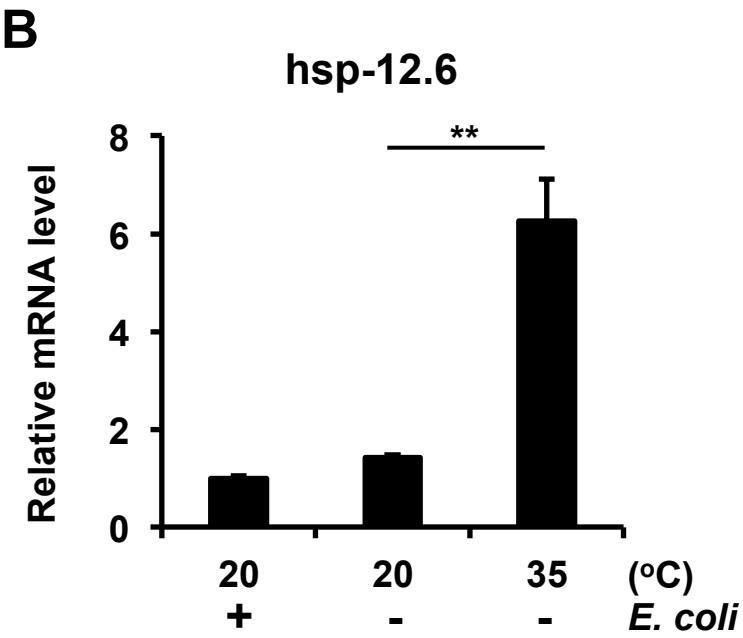

B
hsp-12.6
**
Relative mRNA level
8
6
4
2
0
20
20
35
(°C)
+
-
-
E. coli

FIG 3

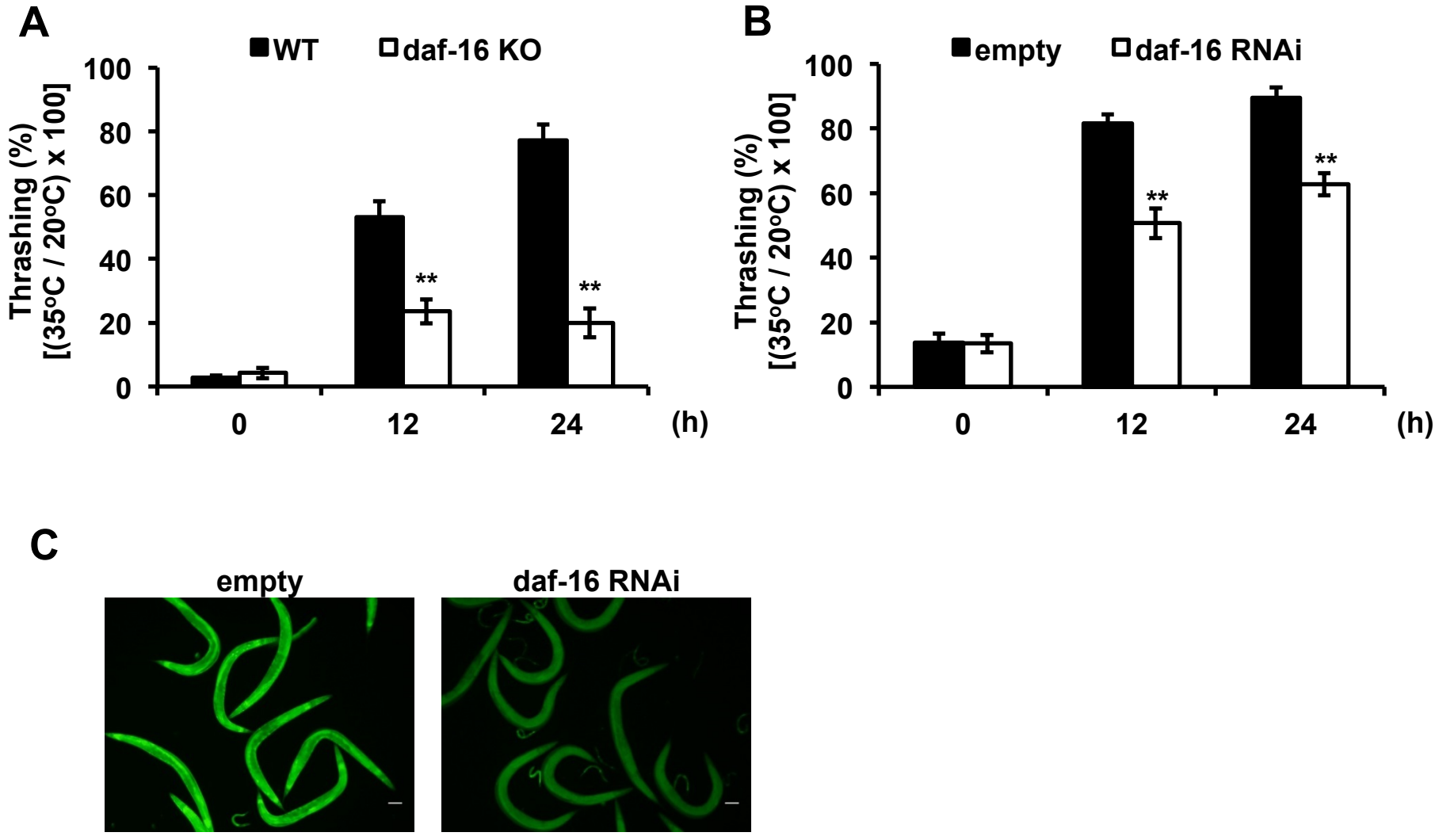

A
WT
daf-16 KO
Thrashing (%)
[(35°C / 20°C) x 100]
100
80
60
40
20
0
**
**
0
12
24
(h)
B
empty
daf-16 RNAi
Thrashing (%)
[(35°C / 20°C) x 100]
100
80
60
40
20
0
**
**
0
12
24
(h)
C
empty
daf-16 RNAi

FIG 4

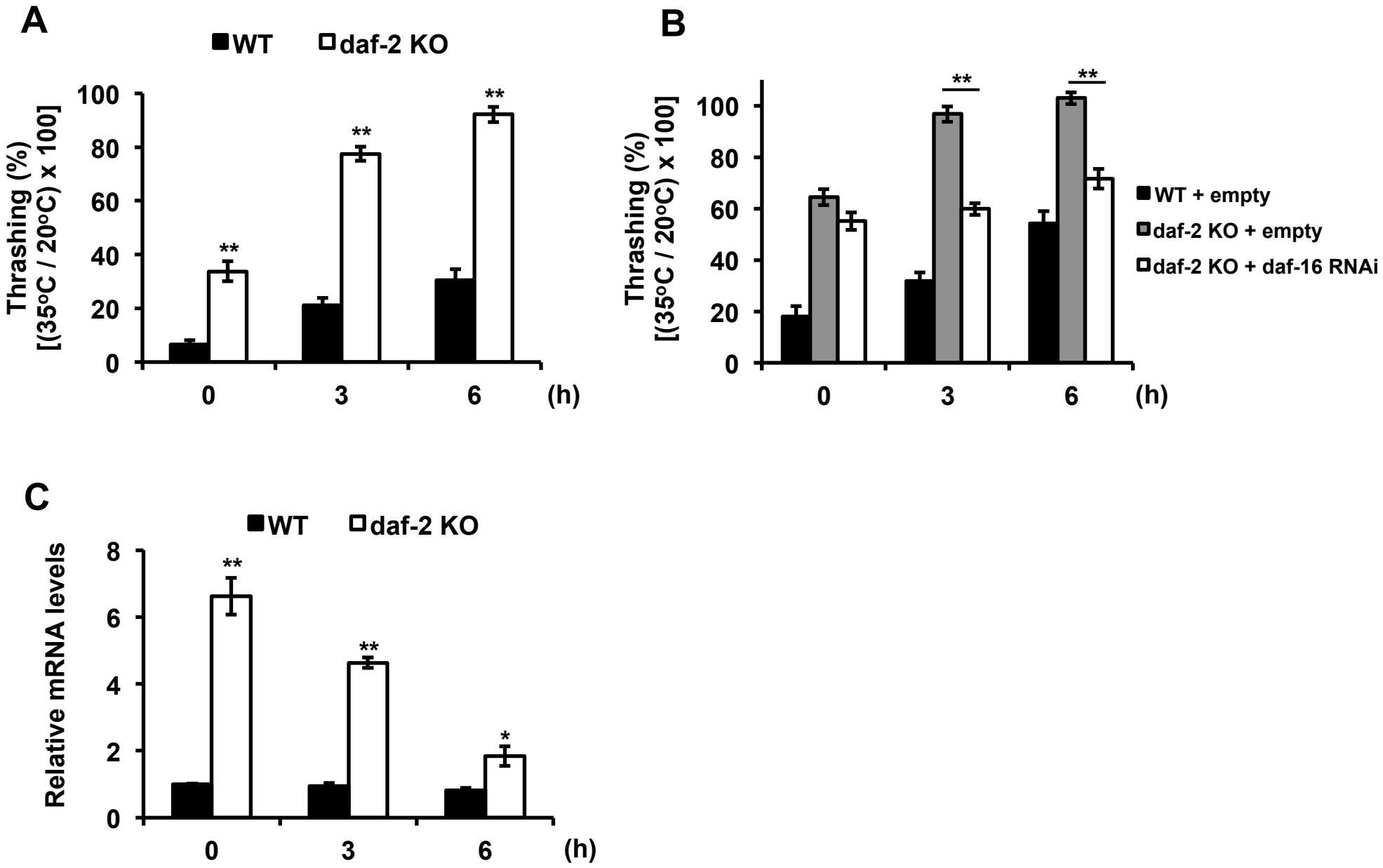

A
WT
daf-2 KO
Thrashing (%)
[(35°C / 20°C) x 100]
100
80
60
40
20
0
**
**
**
0
3
6
(h)
B
Thrashing (%)
[(35°C / 20°C) x 100]
100
80
60
40
20
0
**
**
0
3
6
(h)
WT + empty
daf-2 KO + empty
daf-2 KO + daf-16 RNAi
C
WT
daf-2 KO
Relative mRNA levels
8
6
4
2
0
**
**
*
0
3
6
(h)

FIG 5