Studies on Physiological Function of FoxO/DAF-16 and HSF-1 on Thermotolerance in Caenorhabditis elegans

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Studies on Physiological Function of FoxO/DAF-16 and HSF-1 on Thermotolerance in *Caenorhabditis elegans*

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[ABSTRACT]

Heat stress hinders the survival of organisms. In order to protect the survival from heat stress, it is important to understand the mechanisms of thermotolerance. 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*, and HSF-1, regulator of heat shock responses, were detected as the key factors in thermotolerance.

Although many researches have focused on the studies in related to the effects of DAF-16 on thermotolerance, the recovery process from heat stress damage has been seldom discussed. In CHAPTER 1, I analyzed the roles of FoxO/DAF-16 on the recovery from heat stress by monitoring thrashing movement. Heat shock reduced the movement, which was restored by culturing at normal temperature. However, thrashing movement was not restored by silencing of *daf-16*. Activity of DAF-16 is negativily regulated by insulin/IGF-1-like signaling pathway. To analyze alteration of thrashing movement under DAF-16 activated condition, knock out (KO) mutant of *daf-2*, a homolog of Insulin/IGF-1-like receptor, was treated with heat stress. As a result, reduction of thrashing movement was prevented and restoration of the movement reduced by heat stress was promoted by *daf-2* KO. Furthermore, restoration of *daf-2* mutant was suppressed by a *daf-16* RNAi. However, *daf-16* RNAi didn't affect to the prevention of movement reduction by *daf-2* KO under heat stress. Because *daf-2* KO promotes thermotolerance *via* DAF-16, it is expected that Insulin/IGF-1-like singaling is inactivated by heat stress. Then, I analyzed the mRNA expression of DAF-2 agonists, *daf-28* and *ins-7*. As a result, expression of *daf-28* and *ins-7* was decreased by heat stress. Taken together, these results revealed that FoxO/DAF-16 is activated *via* inhibition of the insulin/IGF-1-like signaling pathway under heat stress and restores thrashing movement reduced by heat stress in *C. elegans*.

HSF-1 is activated by heat stress and induces the expression of heat shock proteins (HSPs). However, the role of HSF-1 in thermotolerance remains unclear. In CHAPTER 2, I analyzed the function of HSF-1 on thermotolerance by monitoring thrashing movement. *hsf-1* RNAi inhibited the restoration of thrashing reduced by heat stress. In

contrast, *hsf-1* knockdown cancelled prevention of movement reduction by *daf-2* KO, which activates HSF-1, but didn't suppress thrashing restoration in *daf-2* mutant. In addition, *hsf-1* RNAi accelerated the reduction of thrashing in heat-shocked wild-type (WT) *C. elegans*. And, *daf-16* KO didn't accelerate the reduction of thrashing by heat stress. In CHAPTER 1, it was shown that expression of DAF-2 agonists was decreased by heat stress. Taken together, my results suggest that HSF-1 is activated *via* inhibition of the insulin/IGF-1-like signaling pathway and prevents the reduction of thrashing caused by heat shock.

As a conclusion, it was suggested that FoxO/DAF-16 and HSF-1 are activated by heat stress through inactivation of Insulin/IGF-1-like singaling and restore or prevent movement disorder induced by heat stress in *C. elegans*, suggesting that FoxO/DAF-16 and HSF-1 play a critical role in thermotolerance.

[ABBREVIATIONS]

DAF: **Abnormal Dauer Formation**

FoxO: **Fork Head Box, Subtype O**

HSF: **Heat Shock Factor**

HSP: **Heat Shock Protein**

IGF: **Insulin Like Growth Factor**

INS: **Insulin Like Peptide**

ROS: **Reactive Oxygen Species**

SOD: **Superoxide Dismutase**

UNC: **UNCoordinated**

[GENERAL INTRODUCTION]

When organisms are exposed to high temperature, reactive oxygen species (ROS) generation (Zuo *et al*., 2000), protein denaturation and aggregation of denatured protein (Kampinga, 1993; Sorger, 1991) are induced. In addition, high temperature triggers Ca^{2+}-induced necrosis (Lanner *et al*., 2012; Kourtis *et al*., 2012). Disorder by high temperature is called as heat stress and can hinder the survival of organisms. In order to protect survival from heat stress, we need to understand how organisms prevent or recover heat stress damage. Then, I paid attention to the studies of thermotolerance.

Traditionally, heat shock responses have been paid attention in thermotolerance study. Nevertheless heat shock responses generally are understood as increase of heat shock proteins (HSPs), which work as molecular chaperone, under high temperature (Richter *et al*., 2010); heat shock factor (HSF) was discovered as one of the regulators of heat shock responses (Kampinga, 1993; Sorger, 1991). HSF binds heat shock element (HSE) region of HSP promoter (Sorger, 1991) and induces transcription of HSPs under high temperature (Kampinga, 1993; Sorger, 1991). These phenomena are conserved in wide species (Sorger, 1991). Meanwhile, it was reported that DAF-16, homolog of FoxO, translocates in nucleous under high temperature in *Caenorhabditis elegans* (Henderson and Johnson, 2001; Lin *et al*., 2001). In addition, it was shown that DAF-16 can increase expression of small heat shock proteins (sHSP) under high temperature (Hsu *et al*., 2003). Then, many studies on thermotolerance have been performed in *C. elegans*. Because *C. elegans* is poikilotherm, researcher can conclude that heat-induced phenomena are caused by high temperature but not thermoregulation.

Previous studies showed that thermotolerance in *C. elegans* is related to the insulin/IGF-1-like signaling pathway. In fact, *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). Then, DAF-16, homolog of FoxO and HSF-1, one of HSF in *C. elegans*, are considered as key factor in thermotolerance, because *daf-2* KO extends the lifespan of *C. elegans* in DAF-16 (Kenyon *et al*., 1993; Murphy *et al*., 2003; Yen *et al*., 2011) and HSF-1-dependent manner (Hsu *et al*., 2003; Morley *et al*., 2004; Chiang *et al*., 2012). In addition, it was reported that heat stress

induces nuclear translocation of DAF-16 (Henderson and Johnson, 2001; Lin *et al*., 2001; Singh and Aballay, 2009) and HSF-1 (Chiang *et al*., 2012).

Previous studies of DAF-16 showed that overexpression of DAF-16 extends survival under heat stress (Henderson and Johnson, 2001), viability is decreased by *daf-16* KO (Horikawa and Sakamoto, 2009), and survival extension of *daf-2* mutant is in dependent on DAF-16 (McColl et al., 2012). Therefore, it is expected that DAF-16 can prevent heat stress damage. However, the recovery process from heat stress damage has been seldom discussed. Meanwhile, previous studies of HSF-1 showed that viability is increased by overexpression of HSF-1 (Kourtis *et al*., 2012). However, roles of HSF-1 on thermotolerance are still remained unclear. In fact, it was shown that HSF-1 KO doesn't affect survival of *C. elegans* under heat stress and *daf-2* knock down by RNA interference extends survival under heat stress in independent of HSF-1 (McColl *et al*., 2010).

To solve the questions of roles of DAF-16 and HSF-1 on thermotolerance, I analyzed thrashing movement, that *C. elegans* frequently bends its body from side to side in water, as an index of thermotolerance. It is possible to analyze prevention of heat stress and restoration from heat stress by using movement. The movement including thrashing is often used as an index in studies of proteotoxic diseases. For example, *C. elegans* expressing polyglutamine (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; Haldimann et al., 2011). And, *C. elegans* expressing β-amyloid also involved movement disorder in an age-dependent manner (Cohen et al., 2006). Because heat stress induces protein aggregation (Kampinga 1993), it is probable that heat stress alters the movement of *C. elegans*. My study is the first research which was used movement as an index of thermotolerance and made it possible to divide into prevention assay and restoration assay in thermotolerance study.

In CHAPTER 1, I analyzed the physiological function of DAF-16 on recovery from heat stress by monitoring thrashing movement. As a result, I found that DAF-16 restores thrashing movement reduced by heat stress. In CHAPTER 2, I analyzed the physiological function of HSF-1 on thrashing movement altered by heat stress. As a result, I found that HSF-1 prevents reduction of thrashing movement under heat stress.

CHAPTER 1

DAF-16 restores Thrashing Movement reduced by Heat Stress in *Caenorhabditis elegans*

[Introduction]

Previously, a lot of studies were performed to find the function of DAF-16 on thermotolerance. As a result, it was proved that *daf-16* overexpression extends survival under heat stress (Henderson and Johnson, 2001). And, previous study of my laboratory showed that viability is decreased by *daf-16* KO under heat stress (Horikawa and Sakamoto, 2009). In addition, the survival extension by *daf-2* KO is dependent on DAF-16 under heat stress (McColl *et al*., 2010). Therefore, FoxO/DAF-16 has important roles for thermotolerance.

However the recovery process from heat stress damage has been seldom discussed, although many studies focused on FoxO/DAF-16 and thermotolerance. 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, *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, I analyzed the mechanisms underlying the restoration from heat stress damage by using movement of *C. elegans*.

In the CHAPTER 1, I 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. I 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).

[Materials and Methods]

Strains and culture

Wild-type (WT) *Caenorhabditis elegans* Bristol N_2, *daf-16* mutant (mgDf50), *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 *Escherichia coli* OP50 as previously described (Brenner 1974).

NaClO treatment

To synchronize the growth of *C. elegans*, adult worms were treated with 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.

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) to prevent contamination of genomic DNA. cDNA was synthesized by using M-MLV Reverse Transcriptase (Takara). Or, genomic DNA was broken and cDNA was synthesized by PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara).

Feeding RNAi

Plasmid DNA L4440 (Fire Laboratory), which has a *daf-16* cDNA fragment in the multicloning site, was transformed into *E. coli* HT115 treated with 50 mM $CaCl_2$ (WAKO). Primers for preparing the cDNA insert are listed in CHAPTER 1-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; Timmons *et al*., 2001). Age-synchronized L1 larvae were transferred onto RNAi plate.

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 worms were chosen randomly, and pharynx pumping was counted for 15 s every hour.

Thrashing movement assay under heat stress

Age-synchronized L1 larvae were transferred onto a plate and cultured for 4 days at 20^{o}C. After 4 days, adult worms were transferred onto NGM plate (*E. coli* (-)) and cultured at 20^{o}C or 35^{o}C for 4h. After heat stress, the worms were transferred onto a new plate and cultured for 0-24 h, and then ten worms were picked and moved into S-basal at random to count the movement for 15s. These methods have been previously described (Furuhashi and Sakamoto, 2014).

Observation of DAF-16::GFP

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

Quantitative RT-PCR

cDNA was prepared and amplified on an ABI-7300 system (Applied Biosystems, CA, USA) using Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan). Primers are listed in CHAPTER 1-Table 1.

Statistical analysis

Statistical analyses were performed using the analysis software SPSS (IBM, NY, USA). Statistical significance was analyzed with t-test, Games-Howell test and Dunnett's T3 test with statistical differences represented by $*p < 0.05$ and $**p < 0.005$.

[Results]

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

First, I observed pharynx pumping under heat stress, because it was reported that feeding of *C. elegans* is totally inhibited at 32^{o}C (Jones and Candido, 1999). As a result, pumping was remarkably decreased by heat stress (CHAPTER 1-Fig. 1A). Because starvation stress affects the insulin signaling pathway and the translocation of DAF-16 (Henderson and Johnson, 2001; Weinkove *et al*., 2006), I used NGM plate without food for heat treatment to avoid the difference of food intake between heat stress conditions and normal temperature conditions. I analyzed the difference of thrashing movement under heat stress. *C. elegans* adult worms were cultured at 20°C for 4 h (20°C, [+]), or cultured on NGM plate without food at 20°C (20°C, [-]) or 35°C for 4 h (35°C, [-]). After 4 h, worms were transferred to a new NGM plate seeded with OP50 and cultured at 20°C for 0–24 h. I found no change in movement between the NGM plate with food and without food; however, heat stress remarkably reduced the thrashing movement (CHAPTER 1-Figure 1B). After 24 h, the movement of worms under heat stress (35°C, [-]) recovered by 70 to 80% of the movement of worms under normal temperature conditions (20^{o}C, [-]). Therefore, worms can restore the thrashing movement reduced by heat stress.

DAF-16 activates immediately after heat shock

Next, I observed DAF-16 activation. First, I used the TJ356 strain (*daf-16::gfp*) to observe the intracellular localization of DAF-16 protein under heat stress. Adult TJ356 worms were cultured at 20°C for 4 h (20°C, [+]), or cultured on NGM plate without food at 20°C (20°C, [+]) or 35°C for 4 h (35°C, [-]). As a result, DAF-16 protein was tentatively accumulated in nucleus by heat stress (CHAPTER 1-Fig. 2A). Nevertheless, 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 (CHAPTER 1-Fig. 2A). Therefore, it was suggested that heat stress induces DAF-16 nuclear translocation more efficiently than starvation stress. Heat-treated TJ356 was transferred to a new NGM plate seeded with OP50, and

cultured at 20°C for 24 h. As a result, the GFP localization was decreased compared with that at 0 h (CHAPTER 1-Fig. 2A), as found in previous studies (Singh and Aballay, 2009). In addition, I 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 (CHAPTER 1-Fig. 2B) as previously reported (Hsu *et al*., 2003; McColl *et al*., 2010). These results suggested that the transcriptional activity of DAF-16 was enhanced immediately after heat treatment.

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

I analyzed the relation of DAF-16 and thrashing activity by using a *daf-16* mutant (mgDf50). WT and *daf-16* mutant 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. As a result, sequential restoration of thrashing activity was inhibited by *daf-16* KO (CHAPTER 1-Fig. 3A). I also observed the thrashing activity of worms treated with *daf-16* RNAi under heat stress. As a result, worms treated with *daf-16* RNAi also displayed restoration inhibition (CHAPTER 1-Fig. 3B). Additionally, *daf-16* RNAi remarkably decreased the GFP fluorescence of the TJ356 strain (CHAPTER 1-Fig. 3C). Therefore, *daf-16* was knocked down by *daf-16* RNAi. These results suggested that DAF-16 is important for restoration of the thrashing movement reduced by heat stress.

Knock out of *daf-2* promotes restoration of thrashing movement in dependent on DAF-16

To analyze the change of thrashing activity under the conditions of DAF-16 activation, I used a *daf-2* mutant (e1370). WT and *daf-2* mutant 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 thrashing movement of *daf-2* mutant was greater than that of WT at 0 h after heat treatment, and it was almost restored at 6 h later (CHAPTER 1-Fig. 4A).

To analyze the relation of DAF-16 and phenotype in *daf-2* KO, *daf-2* mutant was treated with *daf-16* RNAi. The results showed that the movement of *daf-2* mutant was

unchanged between the empty vector and *daf-16* RNAi at 0 h. However restoration of thrashing movement was inhibited by *daf-16* knockdown in *daf-2* mutant (CHAPTER 1-Fig. 4B). Previous study suggested heat stress increases *hsp-12.6* expression in *daf-2* mutant in a DAF-16 dependent manner (McColl *et al*., 2010). I analyzed *hsp-12.6* expression in WT and *daf-2* mutant during the recovery process. As a result, expression of *hsp-12.6* in *daf-2* mutant was still higher than that of WT. (CHAPTER 1-Fig. 4C). And, *hsp-12.6* expression was reduced due to restoration of thrashing movement (CHAPTER 1-Fig. 4A and Fig. 4C). Therefore, these results suggested that *daf-2* KO promotes the restoration of thrashing movement *via* activation of DAF-16.

Heat stress suppresses the activity of the insulin/IGF-1-like signaling pathway

DAF-16 restored the thrashing movement in WT strain (CHAPTER 1-Fig. 3). Additionally, DAF-16 promoted restoration in *daf-2* mutant (CHAPTER 1-Fig. 4). Therefore, heat stress may affect the activity of the insulin/IGF-1-like signaling pathway. To analyze the activity of the insulin/IGF-1-like signaling pathway under heat stress, I measured gene expression of *daf-28* and *ins-7*, which are the agonists of DAF-2 (Wormbase: http://www.wormbase.org). The results showed that *ins-7* and *daf-28* expression was remarkably decreased by heat stress (CHAPTER 1-Fig. 5). Therefore, it was suggested that heat stress inactivates the insulin/IGF-1-like signaling pathway.

[Discussion]

I found one of the potential functions of DAF-16, which restored the thrashing movement decreased by heat stress (CHAPTER 1-Fig. 3 and 4). This is the first finding which was obtained through analyzing the physiological function of DAF-16 on thermotolerance in the viewpoint of restoration.

DAF-16 activity was enhanced immediately after heat treatment (CHAPTER 1-Fig. 2) and was suppressed by a *daf-16* KO or RNAi, resulted in the increase of the stress resistance genes expression (Hsu *et al*., 2003; McColl *et al*., 2010). Therefore, it was expected that increase of genes whose expression is positively regulated by DAF-16 can restore the thrashing movement that was reduced by heat stress. The genes, such as *sod-3* and *hsp-12.6,* preserve cells from stress, including heat stress. For example, it was reported that heat stress increased the fluorescent flux of DCF in *C. elegans* (Kampkötter *et al*., 2007, Arch. Toxicol; Kampkötter *et al*., 2007, Toxicology) and remarkably increased SOD-3 expression (Wolf *et al*., 2008). Furthermore, research on poly-glutamine (PolyQ) diseases showed that knockdown of *daf-16* or *hsp-12.6* accelerated the aggregation of the PolyQ protein (Hsu *et al*., 2003) and *daf-16* RNAi accelerated paralysis in a *C. elegans* of PolyQ disease model (Haldimann *et al*., 2011). And, *hsp-12.6* expression was decreased due to restoration of thrashing movement in *daf-2* mutant (CHAPTER 1-Fig. 4A and C). Therefore, it is expected that DAF-16 removes heat stress damage and restores the thrashing movement.

This restoration was promoted in a *daf-2* KO in a *daf-16*-dependent manner (Figure 4). In *daf-2* mutant, DAF-16 is localized in the nucleus (Lin *et al*., 2001) and enhances the expression of stress resistance genes (Hsu *et al*., 2003; McElwee *et al*., 2003; Murphy *et al*., 2003). Therefore, it is expected that DAF-16 nuclear localization is maintained before and after heat shock in *daf-2* mutant. In other words, expression of genes whose expression is positively regulated by DAF-16 may be enhanced at all times in *daf-2* mutant. In fact, *hsp-12.6* level of *daf-2* mutant was still high in recovery process compared with that of WT (CHAPTER 1-Fig. 4C). Therefore, these results suggest that the consecutive activation of FoxO/DAF-16 accelerates restoration of movement disorder induced by heat stress. On the other hand, *daf-2* mutant prevented

the decline of thrashing movement in a DAF-16-independent manner (Fig. 4B), whereas heat stress increases expression of *hsp-12.6* in WT (Fig. 2B) (Hsu *et al*., 2003; McColl *et al*., 2010) and *daf-2* mutant (McColl *et al*., 2010). So, it was expected that DAF-16 and genes whose expression is positively regulated by DAF-16 can only restore the thrashing movement that was lowered by heat stress.

Heat stress decreased *daf-2*8 and *ins-7* gene expression, the agonists of DAF-2 (CHAPTER 1-Fig. 5). These findings suggest that heat stress suppresses the activity of 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 at 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 *daf-2* mutant (CHAPTER 1-Fig. 4). These findings strongly suggest that DAF-16 is activated by heat stress *via* inactivation of the insulin/IGF-1-like signaling pathway.

FoxO families are the common transcription factor which existed in the wide varieties of organisms, and have similar functions on oxidative stress and longevity (Kenyon, 2010). In fact, it is generally understood that FoxO increases the expression of genes related to anti-oxidant (Welker et al., 2013) in response to oxidative stress; *daf-2* KO increases *sod-3* expression (Honda and Honda, 1999 and Yoshinaga *et al*., 2003) and promotes oxidative stress tolerance dependent on DAF-16. Furthermore, FoxO also induces longevity in yeast (Postnikoff *et al*., 2012), *C. elegans* (Kenyon *et al*., 1993) and *Drosophila melanogaster* (Slack *et al*., 2011). In addition, it is reported that knock out 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 and Kenyon, 2010). Therefore, although the functions 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 the wide 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 is involved in, at least, the function of removing and recovering damage. It is expected that my findings are applied to the studies of mammals.

Overall, I concluded that FoxO/DAF-16 was activated by inhibiting the insulin/IGF-1-like signaling pathway under heat stress and recovered movement reduced by heat stress in *C. elegans*. And, it is expected that FoxO/DAF-16 can remove the heat stress damage.

CHAPTER 2

HSF-1 Prevents the Reduction in Thrashing Due to Heat Shock in *Caenorhabditis elegans*

[Introduction]

In CHAPTER 1, I investigated thermotolerance in *Caenorhabditis elegans* by analyzing thrashing movement as an index and found that: (1) heat stress reduced thrashing, (2) lack of *daf-16*, a homolog of FoxO, failed to restore thrashing movement reduced by heat stress, and (3) knock out of *daf-2*, a homolog of insulin/IGF receptor, promoted restoration of the movement reduced by heat stress in a *daf-16*-dependent manner. However, *daf-2* mutants retained the movement immediately after heat stress in a *daf-16*-independent manner. Therefore, it was suggested that DAF-16 is not the only inducer of thermotolerance and that some other factors prevent the reduction of thrashing movement during heat stress. One protein that was of interest in this phenomenon was heat shock factor 1 (HSF-1).

In previous studies using *C. elegans*, it was shown that exposure of worms to low levels of heat stress for a few hours promotes thermotolerance (Lithgow *et al*., 1995) and these phenomena depend on HSF-1 (McColl *et al*., 2010; Kourtis *et al*., 2012). Thus, HSF-1 is thought to be a key factor in thermotolerance. Although a number of studies represented that HSF-1 has very important roles on thermotolerance, functions of HSF-1 remain unclear in *C. elegans*. In fact, the survival time of *C. elegans* under heat stress is not affected by *hsf-1* KO (McColl *et al*., 2010). In addition, *hsf-1* is not associated with extension of survival time in *daf-2* RNAi-treated worms under heat stress (McColl *et al*., 2010), whereas lifespan extension in response to *daf-2* KO depends on HSF-1 (Hsu *et al*., 2003; Morley *et al*., 2004; Chiang *et al*., 2012).

In CHAPTER 2, I analyzed thrashing movement an index of thermotolerance and investigated the roles of HSF-1 and HSPs in *C. elegans*. I found a novel function of HSF-1.

[Materials and Methods]

Strains and culture

WT *C. elegans* Bristol N_2, *daf-2* (*e1370*), and *daf-16 (mgDf50)* were provided by the Caenorhabditis Genetics Center (CGC). Each strain was cultured on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 as previously described (Brenner, 1974).

NaClO treatment

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

Preparation of cDNA

Adult worms were washed with S-basal media followed by double-distilled water to remove *E. coli*. RNA was purified from whole-cell extracts of worms using RNAiso PLUS (Takara, Shiga, Japan) and was treated with DNase I (Takara) to prevent contamination of genomic DNA. cDNA was synthesized using Moloney murine leukemia virus (MML-V) reverse transcriptase (Takara), PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara) or PrimeScript® RT Master Mix (Perfect Real Time) (Takara).

Feeding RNAi

Plasmid DNA L4440 (Fire Laboratory) containing *hsf-1* cDNA (primers used to obtain the cDNA are shown in CHAPTER 2-Table 1) was transfected into *E. coli* HT115 treated with 50 mM $CaCl_2$ (WAKO). The transfected cells were then treated with isopropyl-β-D-thiogalactopyranoside (IPTG; WAKO) to induce dsRNA expression. After treatment, the transfected cells were seeded onto NGM plates for RNAi (Timmons and Fire 1998; Timmons et al., 2001). Age-synchronized L1 larvae were cultured for 3 days at 20°C on NGM plates seeded with *E. coli* OP50. After 3 days, worms were

transferred onto RNAi plates and cultured for 24 h at 20°C.

Determination of restoration of thrashing after exposure to heat stress

Age-synchronized L1 larvae were transferred onto a plate and cultured for 4 days at 20^{o}C. After 4 days, adult worms were transferred onto NGM plate (*E. coli* (-)) and cultured at 20^{o}C or 35^{o}C for 4 h. After heat stress, the worms were transferred onto a new plate and cultured for 0-24 h, and then ten worms were picked and moved into S-basal at random to count the movement for 15s. These methods have been previously described (Furuhashi and Sakamoto, 2014).

Determination of inhibition of thrashing

Adult worms were transferred onto NGM plates (*E. coli* (-)) and cultured at 35°C for 1 h. Then, the plates were incubated at room temperature for 10 min after which the worms were observed for thrashing for 15 s.

RT-PCR

cDNA was amplified using an ABI-2720 Thermal Cycler (Applied Biosystems, CA, USA) and Taq DNA Polymerase (Ampliqon, Herlev, Denmark). The cycling conditions were as follows: 94°C/5 min, (94°C/30 s, 55 or 57°C/30 s, 72°C/30 s) × 21–25, and 72°C/7 min. *gpd-1* was used as the internal control. Primers are shown in Table 1.

Quantitative RT-PCR

cDNA was amplified using Thermal Cycler Dice® Real Time System Lite (Takara) and Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. *actin* was used as the internal control. cDNA samples for quantitative RT-PCR were prepared as previously described (Furuhashi and Sakamoto, 2014). Primers are shown in Table 1.

Statistical analysis

Statistical analyses were performed using the analysis software SPSS (IBM, NY, USA). Statistical significance for the values plotted on the graph were analyzed using

the *t*-test, Games-Howell test and Dunnett's T3 test with statistical differences represented by *$p < 0.05$ and **$p < 0.005$.

[Results]

HSF-1 induces thermotolerance *via* HSPs expression

My previous result showed that thrashing movement of *C. elegans* was reduced by heat stress (35°C for 4 h) and recovered after culturing at 20°C (CHAPTER 1-Fig. 1B; Furuhashi and Sakamoto, 2014). First, to determine the role of HSF-1 in the change of thrashing movement, I applied heat stress to worms treated with *hsf-1* RNAi. I observed that *hsf-1* RNAi interferes with the sequential restoration of thrashing after heat stress (CHAPTER 2-Fig. 1A). To know whether RNAi specifically interfere the mRNA expression of *hsf-1*, I analyzed mRNA expression of *hsf-1*. Based on the result of RT-PCR, *hsf-1* mRNA expression is suppressed specifically by *hsf-1* RNAi (CHAPTER 2-Fig. 1B) and *daf-16* level wasn't knocked down by *hsf-1* RNAi (CHAPTER 2-Fig. 1B). I next analyzed expression of *hsp-16.2* and *hsp-70*. It is known that mRNA expression of these genes is positively regulated by HSF-1, the transcription factor which is inducible by heat stress and *daf-2* KO (Chiang *et al*., 2012). It was found that heat stress increases the expression of both genes temporarily (CHAPTER 2-Fig. 2A and 2B). These results suggest that HSF-1 induces thermotolerance and that HSP expression may trigger the thermotolerance.

Activated HSF-1 prevents heat stress-induced reduction of thrashing

It has been reported that the insulin/IGF-1-like signaling pathway negatively regulates HSF-1 activity (Hsu *et al*., 2003; Morley *et al*., 2004; Chiang *et al*., 2012). In addition, *daf-2* mutant promotes restoration of thrashing movement reduced by heat stress in a *daf-16*-dependent manner, and retains the movement immediately after heat stress in a *daf-16*-independent manner (Furuhashi and Sakamoto, 2014). Then, to analyze the physiological relation of *hsf-1* to *daf-2* mutant phenotype, I applied RNAi of *hsf-1* to *daf-2* mutants. As a result, *daf-2* mutants treated with *hsf-1* RNAi could not retain the movement under heat stress (CHAPTER 2-Fig. 3). Furthermore, thrashing activity was restored to the level that is close to that observed in *daf-2* mutants treated with empty vector control (CHAPTER 2-Fig. 3). These results indicate that activated HSF-1 prevents thrashing reduction by heat stress but does not accelerate restoration.

HSF-1 prevents thrashing reduction due to heat stress

Although the previous results showed that HSF-1 was not involved in restoration of thrashing in heat-stressed *daf-2* mutants (CHAPTER 2-Fig. 3), *hsf-1* RNAi inhibited restoration of thrashing in WT worms (CHAPTER 2-Fig. 1A). In addition, *hsf-1* RNAi suppressed the prevention of thrashing in *daf-2* mutants (CHAPTER 2-Fig. 3). Therefore, I hypothesized that the extent of restoration is associated with the extent of reduction in thrashing under heat stress. I applied heat stress for 1 h to worms treated with *hsf-1* siRNA. As a result, thrashing activity of worms treated with *hsf-1* RNAi was lower than that of worms treated with control vector (CHAPTER 2-Fig. 4A). This result shows that *hsf-1* RNAi accelerates the reduction in thrashing due to heat stress. Furthermore, thrashing activity wasn't changed between WT and *daf-16* mutant after heat shock for 1 h (CHAPTER 2-Fig. 4B). Therefore, it was suggested that HSF-1 prevents the reduction of thrashing caused by heat stress.

[Discussion]

The results in this chapter indicate that HSF-1 prevents the reduction of thrashing caused by heat stress (CHAPTER 2-Fig. 3 and 4A). This is the first finding which was obtained through analyzing the physiological function of HSF-1 on thermotolerance in viewpoint of prevention.

Previous studies have reported that the movement reduction caused by aging is accelerated by *unc-15* KO, the gene that encodes paramyosin, in *C. elegans*, and that this movement reduction is induced by the mislocalization of paramyosin (Ben-Zvi *et al*., 2009), which is caused by protein misfolding (Gidalevitz *et al*., 2006). In addition, it was shown that paramyosin misfolds at 25°C in the *unc-15* mutant (Gidalevitz *et al*., 2006), that *hsf-1* RNAi exacerbates the reduction in movement seen in *unc-15* mutant (Ben-Zvi *et al*., 2009), and that overexpression of *hsf-1* prevents mislocalization of paramyosin in the *unc-15* mutant (Ben-Zvi *et al*., 2009). Therefore, I speculated that HSF-1 prevents the reduction of thrashing caused by heat stress by preventing protein misfolding or denaturation. Consequently, HSP, whose expression is positively regulated by HSF-1, may be important for preventing the reduction of movement caused by heat stress.

I found that heat stress increased the expression of *hsp-16.2* and *hsp-70* (CHAPTER 2-Fig. 2A and 2B) (Hsu *et al*., 2003; McColl, *et al*., 2010; Chiang *et al*., 2012). And, these mRNA are increased by heat stress or *daf-2* KO in dependent on HSF-1 (Chiang *et al*., 2012). A previous study showed that HSP-70 family could prevent the aggregation of denatured protein (Kampinga, 1993). In addition, it was shown that overexpression of HSP-70 prevents protein denaturation and promotes the refolding of denatured protein (Souren *et al*., 1999). Nevertheless, in *C. elegans*, paralysis caused by accumulation of β-amyloid is promoted by *hsf-1* RNAi (Cohen *et al*., 2006; Cohen *et al*., 2010), whereas overexpression of *hsp-16.2* prevents aggregation of β-amyloid and subsequent paralysis (Fonte *et al*., 2008). In addition, it was reported HSP-16.1 increased by HSF-1 prevents cell death resulting from Ca^{2+} leakage from the golgi to the cytosol through prevention the denaturation of PMR-1, a Ca^{2+} golgi channel (Kourtis *et al*., 2012). Therefore, it was expected that HSF-1 and HSPs may prevent

damage from heat stress.

hsf-1 RNAi did not suppress the restoration of thrashing activity reduced by heat stress in *daf-2* mutants (CHAPTER 2-Fig. 3). This result indicates that HSF-1 is not associated with the restoration of thrashing movement. In CHAPTER 1, I indicated that DAF-16 is a key factor for the restoration of thrashing after heat stress (Furuhashi and Sakamoto, 2014). However, *daf-16* KO didn't affect the reduction of thrashing under heat stress (CHAPTER 2-Fig. 4B). Therefore, it was suggested that HSF-1 and DAF-16 induced thermotolerance *via* different mechanisms.

However, *hsf-1* RNAi inhibited the restoration of thrashing after heat stress (CHAPTER 2-Fig. 1A). This may be because *hsf-1* RNAi exacerbates damage caused by heat stress. In fact, *hsf-1* RNAi accelerates the reduction of thrashing under heat stress in WT (CHAPTER 2-Fig. 4A). Therefore, it is likely that damage aggravated by *hsf-1* interferes with the restoration of thrashing after heat stress.

In CHAPTER 1, heat stress significantly decreased the expression of *daf-28* and *ins-7*, which encode DAF-2 agonists (CHAPTER 1-Fig. 5). In addition, HSF-1 activity was negatively regulated by the insulin/IGF-1-like signaling pathway (Hsu *et al*., 2003; Morley *et al*., 2004; Chiang *et al*., 2012). Moreover, *hsf-1* RNAi suppressed the prevention of the heat stress-induced reduction in thrashing observed in *daf-2* mutants (CHAPTER 2-Fig. 3). Therefore, it was expected that HSF-1 is activated by heat stress *via* inactivation of the insulin/IGF-1- like signaling pathway.

Above all, it was suggested that HSF-1 is activated by heat stress *via* inactivation of insulin/IGF-1-like signaling pathway and prevents movement disorder induced by heat stress. And it is expected that HSF-1 can prevent damage accumulation of heat stress.

[GENERAL DISCUSSION]

I found that silencing of *daf-16* inhibits restoration of thrashing movement reduced by heat stress in CHAPTER 1 and knock down of *hsf-1* suppresses prevention of reduction of thrashing movement under heat stress in CHAPTER 2. Meanwhile, it was shown that DAF-16 doesn't involve prevention of movement reduction under heat stress and HSF-1 doesn't rescue thrashing movement decreased by heat stress. Therefore, it was suggested that DAF-16 and HSF-1 induced thermotolerance *via* different mechanisms. Probably these differences are appeared by difference of genes whose expression is positively regulated by DAF-16 and HSF-1. And, it was expected that DAF-16 and HSF-1 are activated by heat stress through inactivation of insulin/IGF-1 signaling, because activity of DAF-16 and HSF-1 are negatively regulated by insulin/IGF-1 signaling and heat stress decreased mRNA expression of *ins-7* and *daf-28*, agonists of DAF-2, a homolog of insulin/IGF-1 receptor (CHAPTER 1-Fig. 5). Therefore, it was suggested that FoxO/DAF-16 and HSF-1 are activated by heat stress through inactivation of insulin/IGF-1-like signaling and involve thermotolerance through different functions, which are prevention and restoration of movement disorder induced by heat stress, *via* increasing expression of different kinds of genes (GENERAL DISCUSSION-Fig. 1). These are the first findings which were obtained through analyzing the physiological function of FoxO/DAF-16 and HSF-1 on thermotolerance in different viewpoint of restoration and prevention.

Heat stress increased mRNA expression of HSPs regulated by DAF-16 or HSF-1. Nevertheless movement disorder of *C. elegans* is induced by PolyQ expression (Morley *et al*., 2002; van Ham *et al*., 2010; Haldimann *et al*., 2011), β-amyloid expression (Cohen *et al*., 2006) and mislocalization of paramyosin (Ben-Zvi *et al*., 2009); DAF-16 and HSF-1 prevent these movement disorder. Therefore, it is expected that movement disorder is caused by heat induced-protein denaturation. On the other hand, ROS level is increased by heat stress and PolyQ expression (Zuo *et al*., 2000; Bertoni *et al*., 2011). And, DAF-16 regulates not only sHSP but also ROS scavenging gene, such as *sod* and catalase family (Murphy *et al*., 2003; McElwee *et al*., 2003). In addition, DAF-16 and HSF-1 involve oxidative stress tolerance (Honda and Honda 1999; Sagi *et al*., 2012). So,

ROS is also candidate of causes of movement disorder under heat stress. As prospect, we need to define the real essence of heat stress through analyzing the effects of genes, whose expression is positively regulated by DAF-16 and HSF-1, on thermotolerance.

Heat stress may cause movement disorder through damaging muscle or neuron. Although nervous system involves resistance on RNAi (Timmons *et al*., 2001), movement of *C. elegans* treated by heat stress is changed by *daf-16* or *hsf-1* RNAi. Therefore, it is expected that movement disorder is induced by damage of muscle under 35^{o}C. However, heat stress at 39^{o}C induces Ca^{2+}-induced necrosis in neuron (Kourtis *et al*., 2012). So, acute heat shock has a potential to decrease movement through neural damage. As a prospect, we need to perform thermotolerance studies under different temperature.

FoxO families of mammals are expected to have similar function to DAF-16 on oxidative stress tolerance and lifespan (Kenyon 2010). And, HSF-1 induced thermotolerance in not only *C. elegans* but also yeast and colony-forming unit-granulocyte-macrophage derived from mice (Smith and Yaffe, 1991; Zhang *et al*., 2002). Therefore, my findings can be applied to thermotolerance studies in a wide variety of species including mammals. It is considered as social problems that heat stress from environmental high temperature threatens human health, such as heat stroke (Nakai *et al*., 1999), and economy of livestock industries through reduction in production of meat, milk and egg (St-Pierre *et al*., 2003). My results showed that knock out of *daf-2* prevents reduction of thrashing movement under heat stress *via* HSF-1 activation and restores the movement reduced by heat stress through activation of DAF-16. Therefore, in the future, it might be necessary to develop the medicines or functional foods that can activate FoxO and HSF-1 in mammals to solve these problems by heat stress.

I concluded that FoxO/DAF-16 and HSF-1 are activated by heat stress through inactivation of insulin/IGF-1-like signaling and involve thermotolerance through different functions, which are prevention and restoration of movement disorder induced by heat stress, *via* expression of stress tolerance genes (GENERAL DISCUSSION-Fig. 1). My findings contribute to thermotolerance studies and may give one of the prospects to solve social problems in related to heat stress.

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[TABLES AND FIGURES]

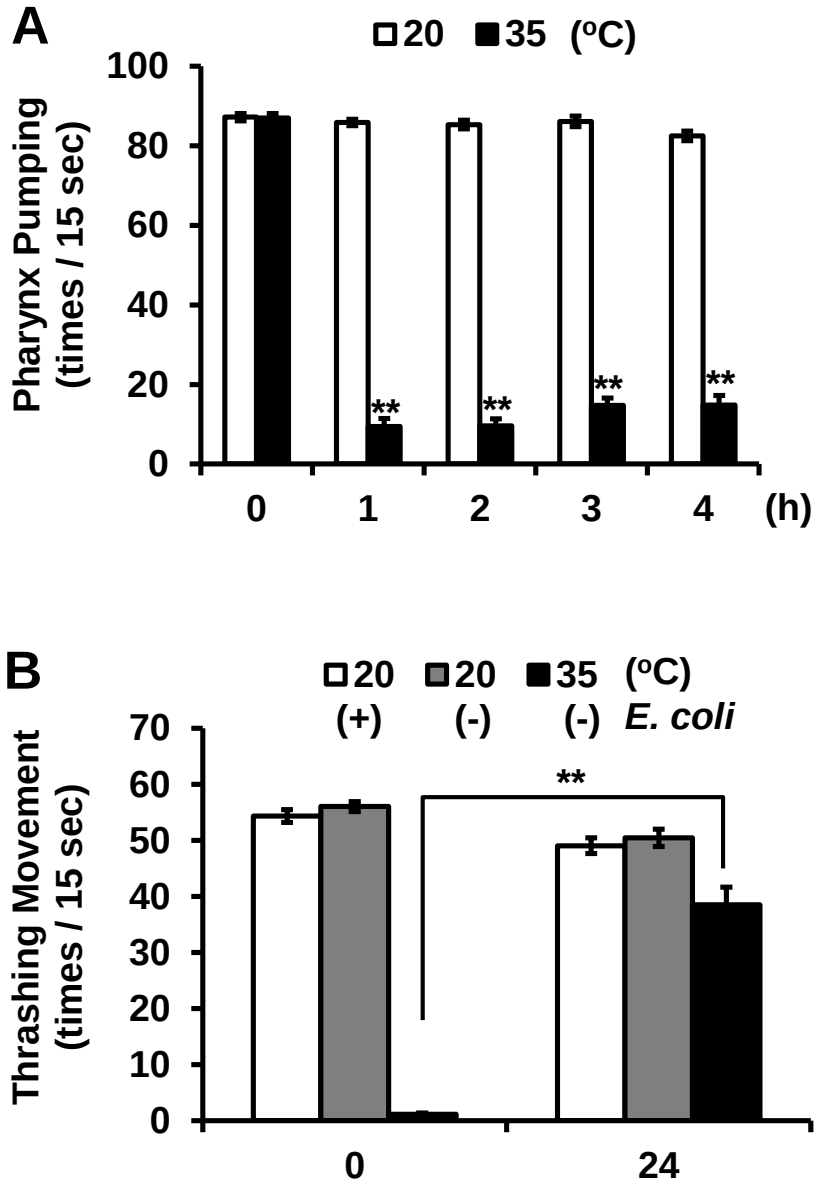
CHAPTER 1-Table 1

Gene	Sense	Antisense	Reference
(qRT-PCR)			
*actin**	TCGGTATGGGACAGA AGGAC	CATCCCAGTTGGTGACG ATA	Kawli and Tan, 2008
hsp-12.6	TGGAGTTGTCAATGT CCTCG	GACTTCAATCTCTTTTGG GAGG	Kwon *et al*., 2010
ins-7	CATGCGAATCGAATAC TGAAG	GAAGTCGTCGGTGCATT C	Kawli and Tan, 2008
daf-28	TTCCGTATGTGTGGAG TGTC	TTTGTATATACTCGGCAG TGC	Hahm *et al*., 2011
(RNAi)			
daf-16	CATGGATCCATCCAGA TGCAAAGCCAG	CATGGATCCGTATGCTGT GCAGCTACA	Hashimoto *et al*., 2010

qPCR was performed by ABI 7300 with the default cycling condition [50^{o}C/2 min, 95^{o}C/10 min, (95^{o}C/15 s, 60^{o}C/1 min) x 40]. Pan-actin was used as internal control.

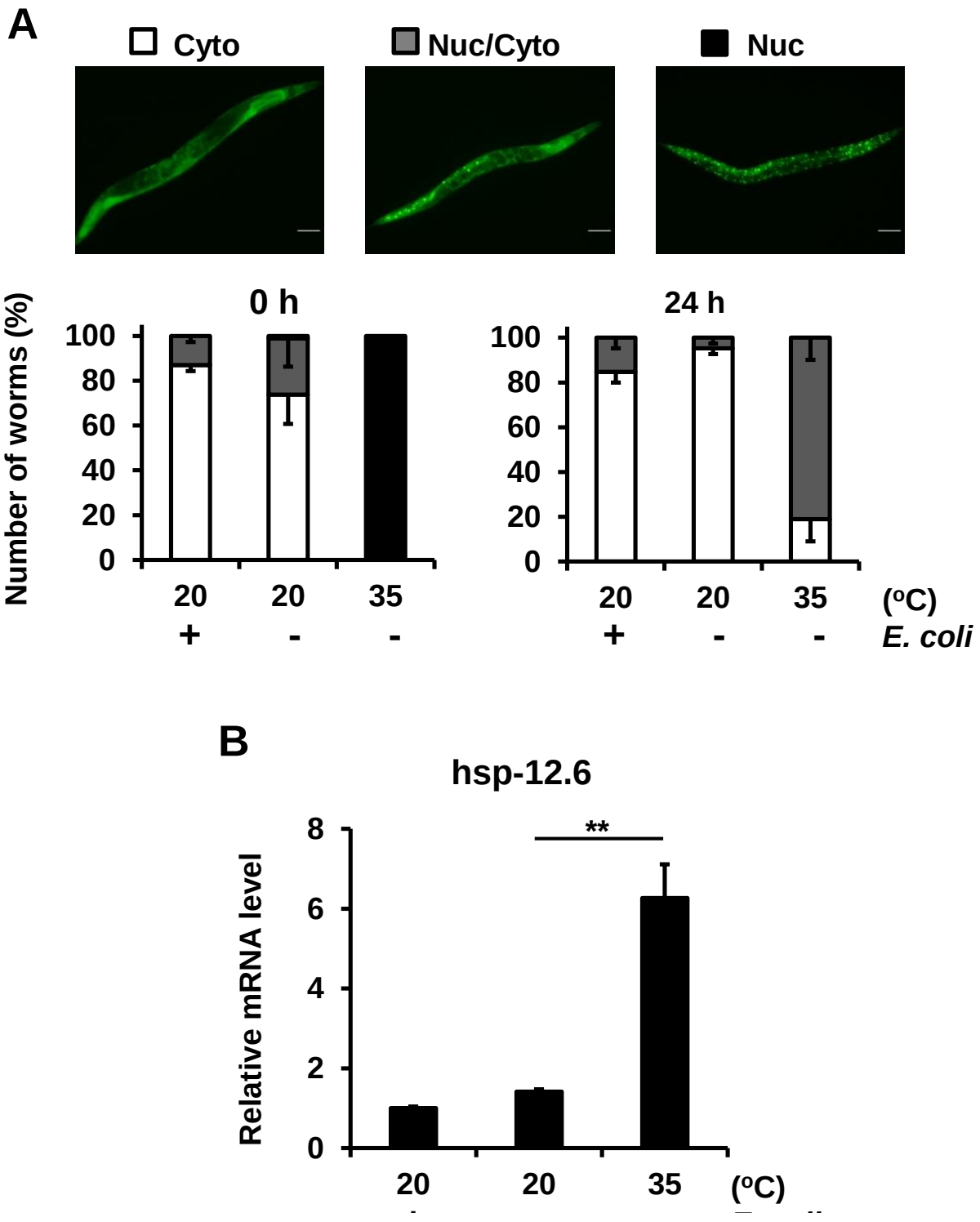
CHAPTER 1-Fig. 1 Alteration of movement under heat stress. (A) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to new NGM plate seeded with OP50 and cultured at 35°C for 0–4 h. Ten worms were chosen randomly and pumping movement was counted for 15 s in every h. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. (B) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to NGM plate seeded with OP50 (+) or *E. coli*-free NGM plate (-) and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred to new NGM plate seeded with OP50 and cultured for 0–24 h. After 0–24 h, ten worms chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$.

CHAPTER 1-Fig. 1



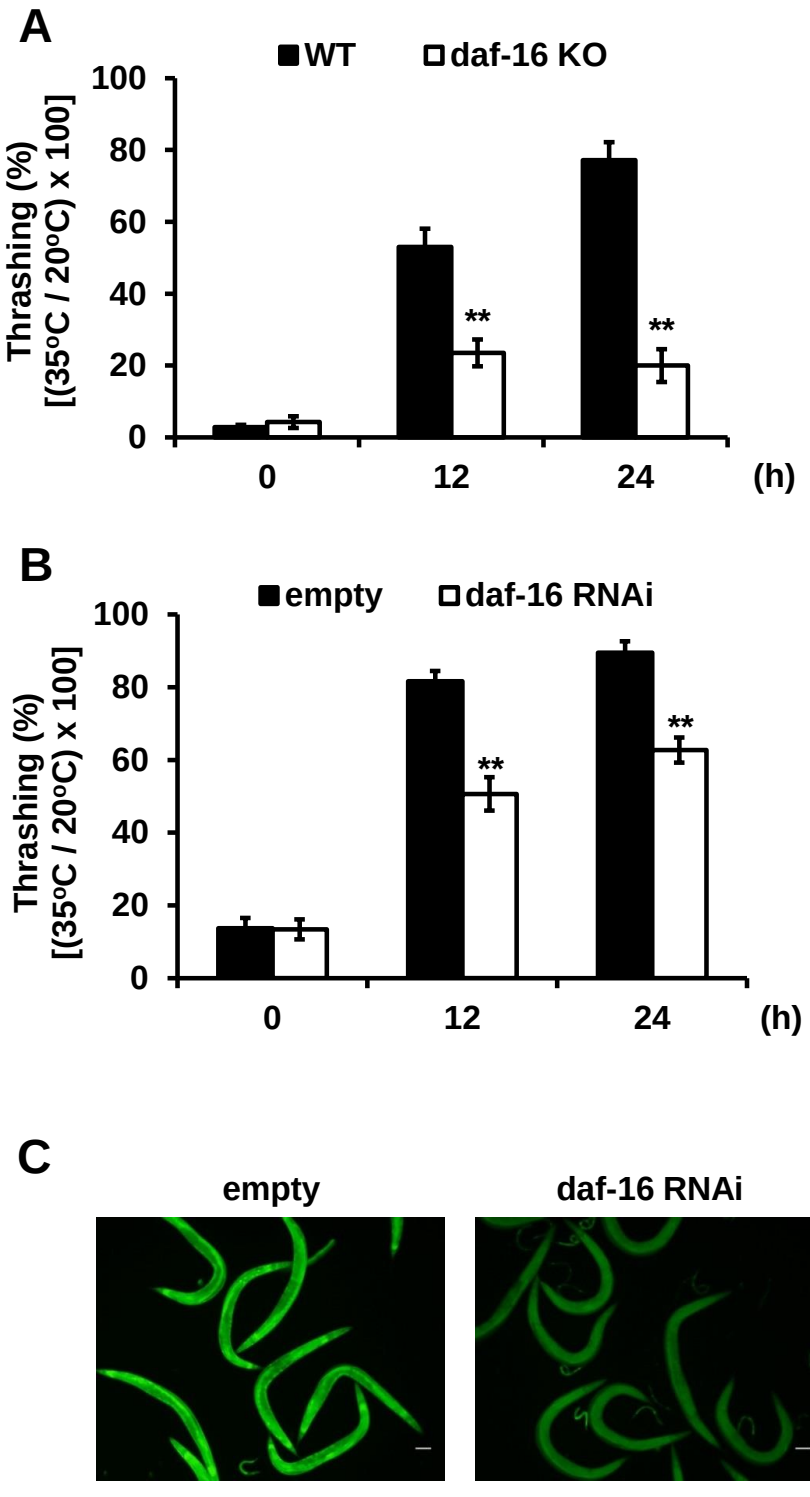
CHAPTER 1-Fig. 2 Activity of DAF-16 under heat stress. (A) Age-synchronized L1 larvae of TJ356 were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to NGM plate seeded with OP50 (+) or *E. coli*-free NGM plate (-), and cultured for 4 h at 20°C or 35°C. The mutants cultured in each condition were transferred to new NGM plates seeded with OP50. The worms were fixed in 1% PFA solution. Fluorescence emitted by GFP was observed using fluorescence microscopy. It was counted that number of worms which is observed GFP nuclear localization (Nuc), cytosol localization (Cyto) or intermediate localization (Nuc/Cyto). Three independent experiments were performed and these data were combined for making a graph. N = 3, mean ± SE, Scale = 100 μm. (B) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to NGM plate seeded with OP50 (+) or *E. coli*-free NGM plate (-), and cultured for 4 h at 20°C or 35°C. RNA was extracted and cDNA was synthesized. The mRNA level of *hsp-12.6* was analyzed using qRT-PCR. Two independent experiments were performed and these data were combined for making a graph. In each trial, gene expression was analyzed by three different wells. Statistical significance was analyzed with Dunnett's T3 test. N = 6, mean ± SE, $*P < 0.05$, $**P < 0.005$.

CHAPTER 1-Fig. 2



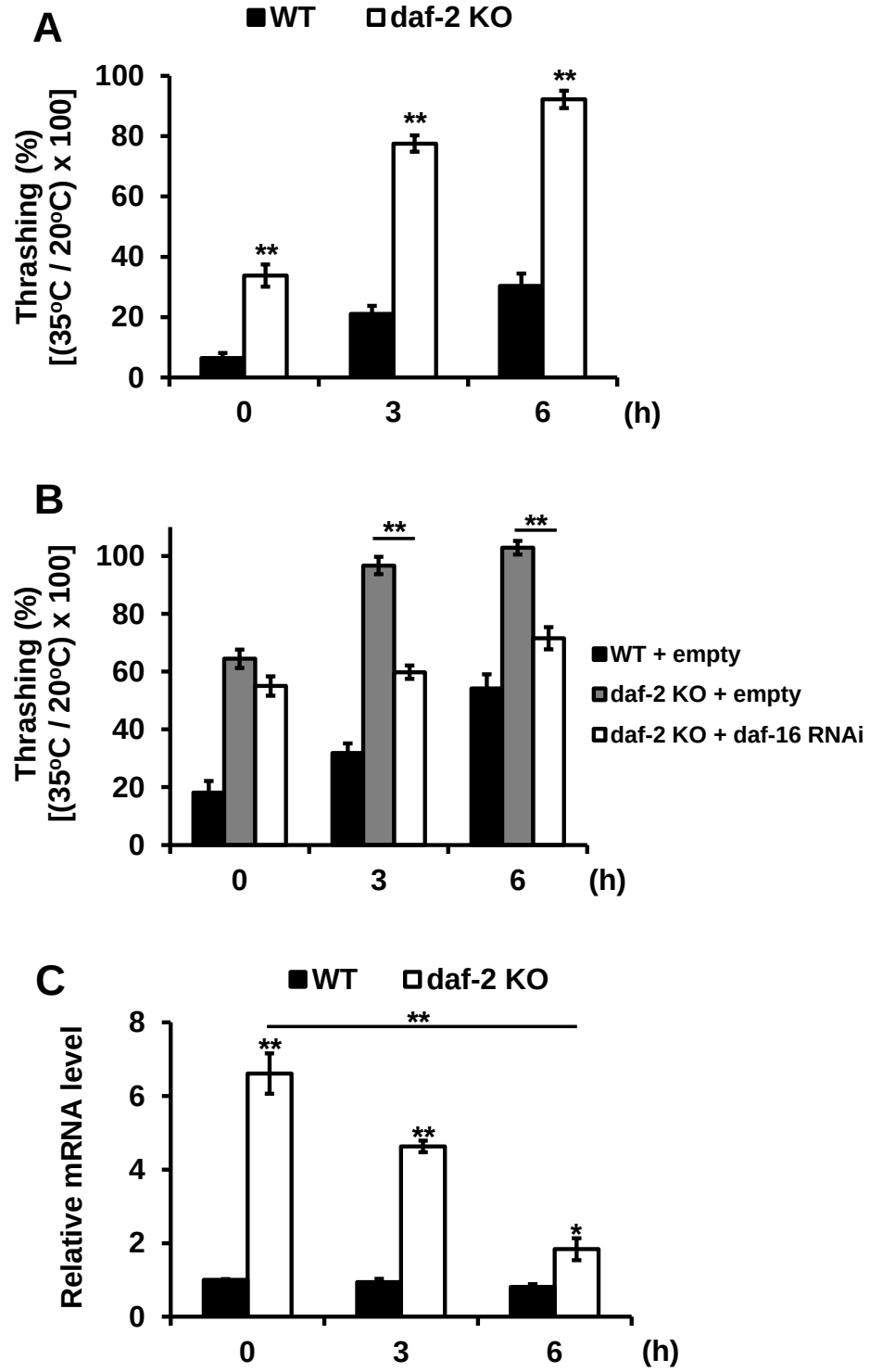
CHAPTER 1-Fig. 3 Alteration of thrashing movement by *daf-16* silencing under heat stress. (A) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred to a new NGM plate seeded with OP50 and cultured for 0–24 h. After 0, 12, or 24 h, ten worms chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. (B) Age-synchronized L1 larvae were transferred to RNAi plate and cultured for 4 days at 20°C. Adult worms were transferred to *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred to new NGM RNAi media plate and cultured for 0–24 h. After 0, 12, or 24 h, ten worms chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. (C) Age-synchronized L1 larvae of TJ356 were transferred to 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.

CHAPTER 1-Fig. 3



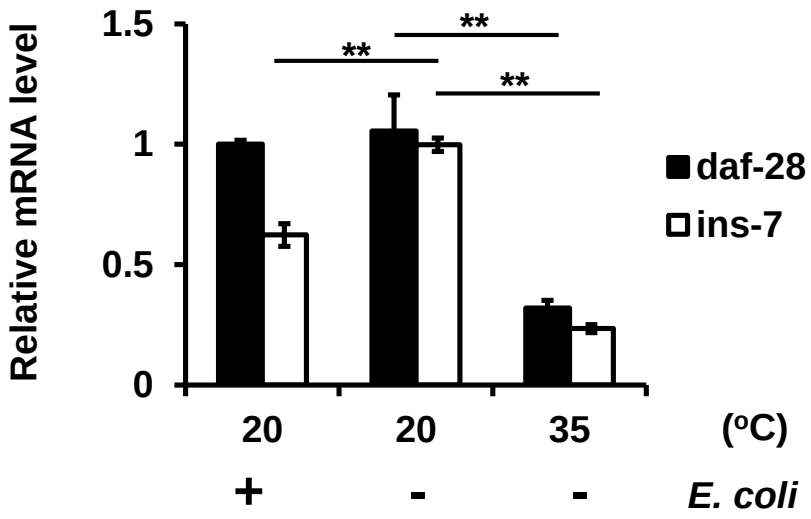
CHAPTER 1-Fig. 4 Alteration of thrashing movement by *daf-2* KO under heat stress. (A) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred to new NGM plate seeded with OP50 and cultured for 0–6 h. After 0, 3, or 6 h, ten worms chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. (B) Age-synchronized L1 larvae were transferred to RNAi plate and cultured for 4 days at 20°C. Adult worms were transferred to *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred to new NGM RNAi media plate and cultured for 0–6 h. After 0, 3, or 6 h, ten worms chosen randomly were transferred to S-basal on *E. coli*-free NGM plates. Thrashing movement was counted for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. N = 30, mean ± SE, $*P < 0.05$, $**P < 0.005$. (C) Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to *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 worms were transferred to 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. Two independent experiments were performed and these data were combined for making a graph. In each trial, gene expression was analyzed by three different wells. Statistical significance was analyzed with t-test. N = 6, mean ± SE, $*P < 0.05$, $**P < 0.005$.

CHAPTER 1-Fig. 4



CHAPTER 1-Fig. 5 Activity of insulin/IGF-1-like signaling pathway under heat stress. Age-synchronized L1 larvae were transferred to NGM plate seeded with OP50 and cultured for 4 days at 20°C. Adult worms were transferred to NGM plate seeded with OP50 (+) or *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. Two independent experiments were performed and these data were combined for making a graph. In each trial, gene expression was analuzed by three different wells. Statistical significance was analyzed with Dunnett's T3 test. N = 6, mean ± SE, **P* < 0.05, ***P* < 0.005.

CHAPTER 1-Fig. 5



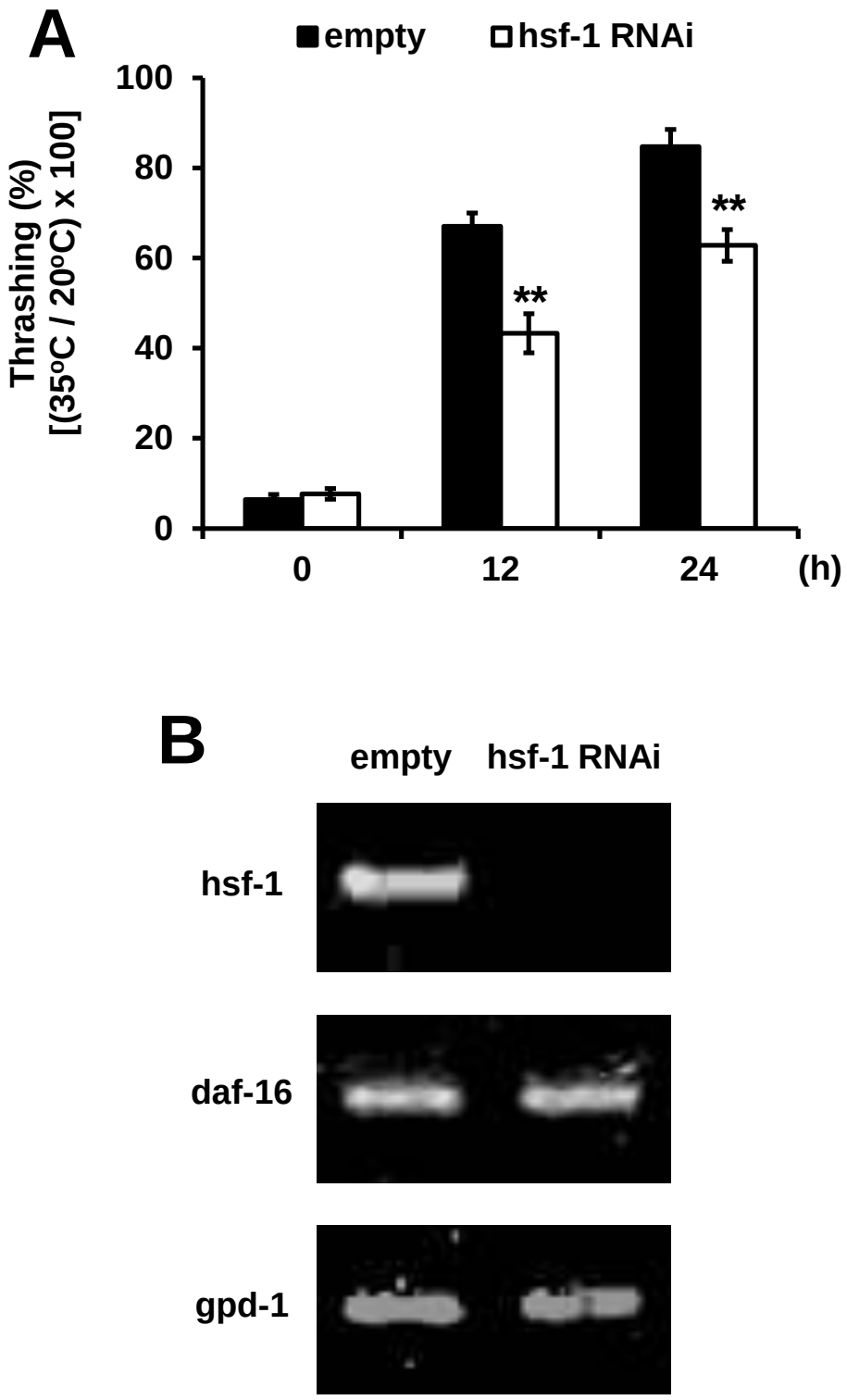
CHAPTER 2-Table 1

Gene	Sense	Antisense	Reference
(qRT-PCR)			
*actin**	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA	Kawli and Tan, 2008
hsp-16.2	TGTTGGTGCAGTTGCTTCGAATC	TTCTCTTCGACGATTGCCTGTTG	
hsp-70	ACCCTTCGTTGGATGGAACG	GCATCCGGAACCTGATGGGC	
(RNAi, RT-PCR)			
hsf-1	CATGAATTCTGATAATGCGTGTTCCG	CATGAATTCATATTGCTGTTGGCGAGC	
daf-16	CATGGATCCATCCAGATGCAAAGCCAG	CATGGATCCGTATGCTGTGCAGCTACA	Hashimoto *et al*., 2010
gpd-1	ATGTCGAAGGCCAACGTC	GTTTTGTCCAGCACCGCG	Nomura *et al*., 2010

qPCR was performed using a Thermal Cycler Dice® Real Time System Lite with the default cycling conditions (95°C/30 s, [95°C/5 s, 60°C/30 s] × 40). *actin* was used as the internal control. PCR was performed using an ABI-2720, and the cycling conditions were as follows: 94°C/5 min, (94°C/30 s, 55 or 57°C/30 s, 72°C/30 s) × 21–25, and 72°C/7 min. *gpd-1* was used as the internal control.

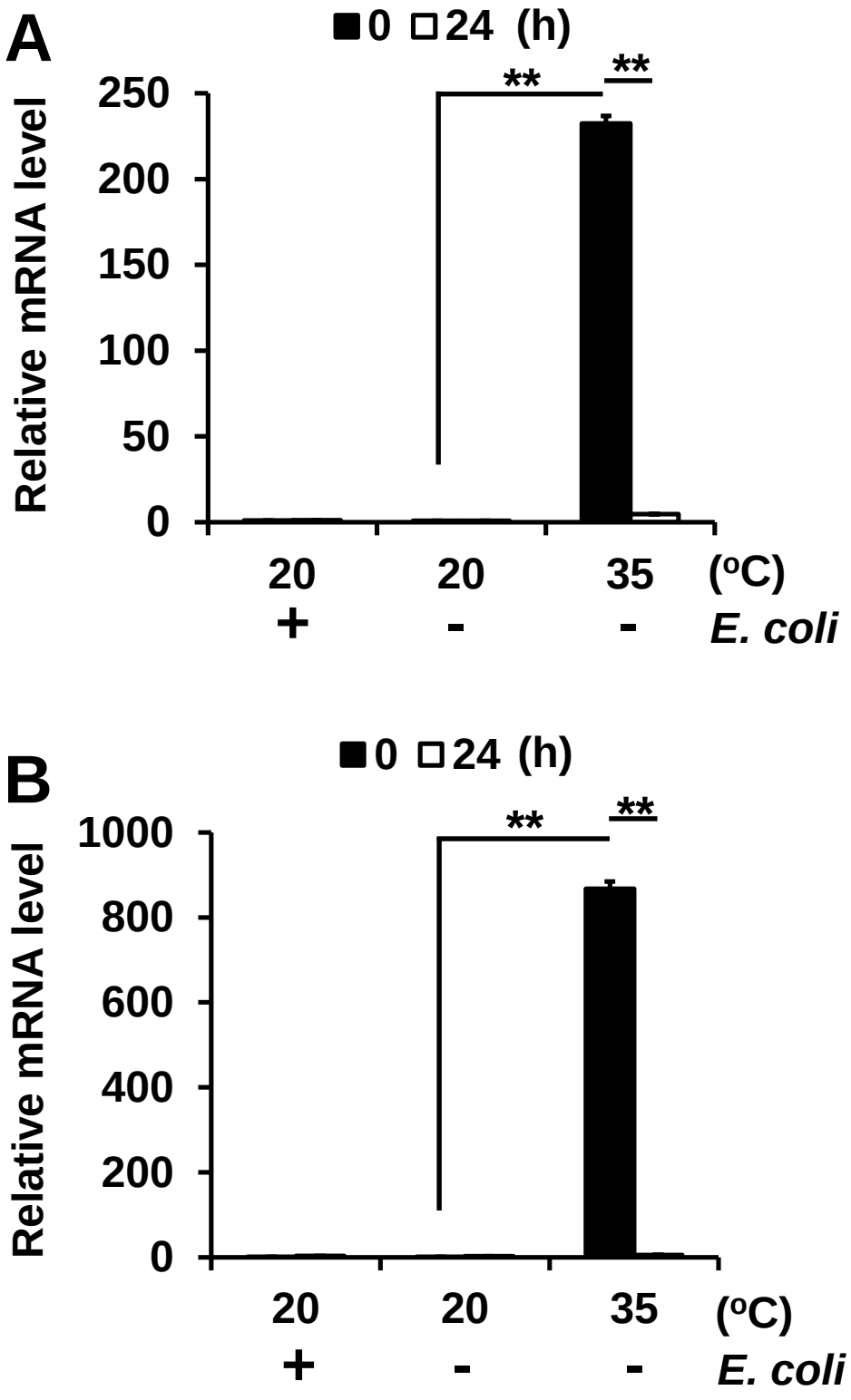
CHAPTER 2-Fig. 1 The role of HSF-1 in restoring thrashing in *C. elegans* exposed to heat stress. (**A**) Worms treated with *hsf-1* siRNA were transferred to *E. coli*-free NGM plate and cultured for 4 h at 20°C or 35°C. After 4 h, worms were transferred onto fresh NGM RNAi media plates and cultured for 0–24 h. After 0, 12, or 24 h, ten worms were observed for thrashing for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. Mean ± SE, $*p < 0.05$, $**p < 0.005$. Three independent trials were conducted and showed similar result. (**B**) RNA was extracted from *C. elegans* treated with siRNA, and cDNA was synthesized. Expression of *hsf-1*, *daf-16*, and *gpd-1* were detected by RT-PCR. Two independent trials were conducted and showed similar result.

CHAPTER 2-Fig. 1



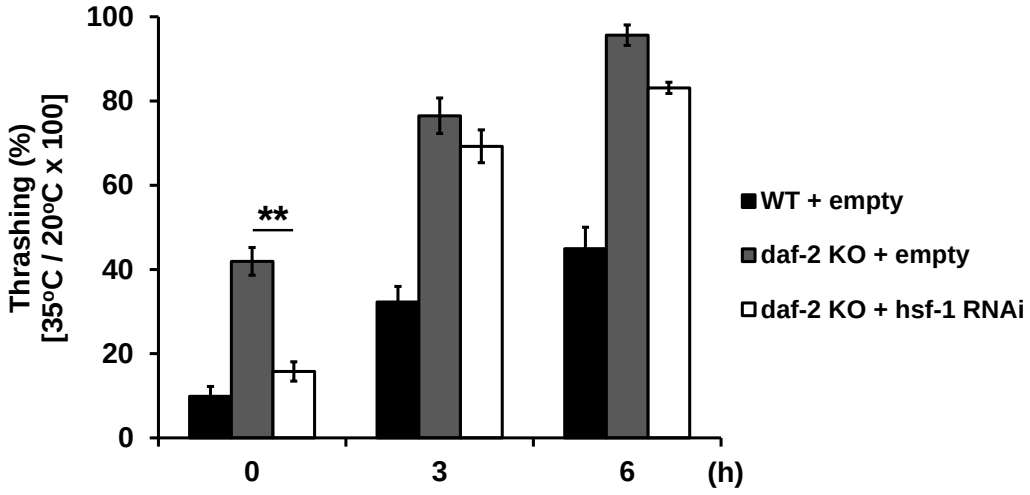
CHAPTER 2-Fig. 2 Expression of genes downstream of HSF-1. Worms were transferred to NGM plates seeded with OP50 (+) or *E. coli*-free NGM plates (-) and cultured for 4 h at 20°C or 35°C. After incubation in each condition, worms were transferred to NGM plates seeded with OP50 and cultured for 24 h at 20°C. Then RNA was extracted, and cDNA was synthesized. Expression of *hsp-16.2* (**A**) and *hsp-70* (**B**) were detected by quantitative RT-PCR. Each cDNA sample was amplified in three wells. Statistical significance was analyzed with Games-Howell test. Mean ± SE, $*p < 0.05$, $**p < 0.005$. Two independent trials were conducted.

CHAPTER 2-Fig. 2



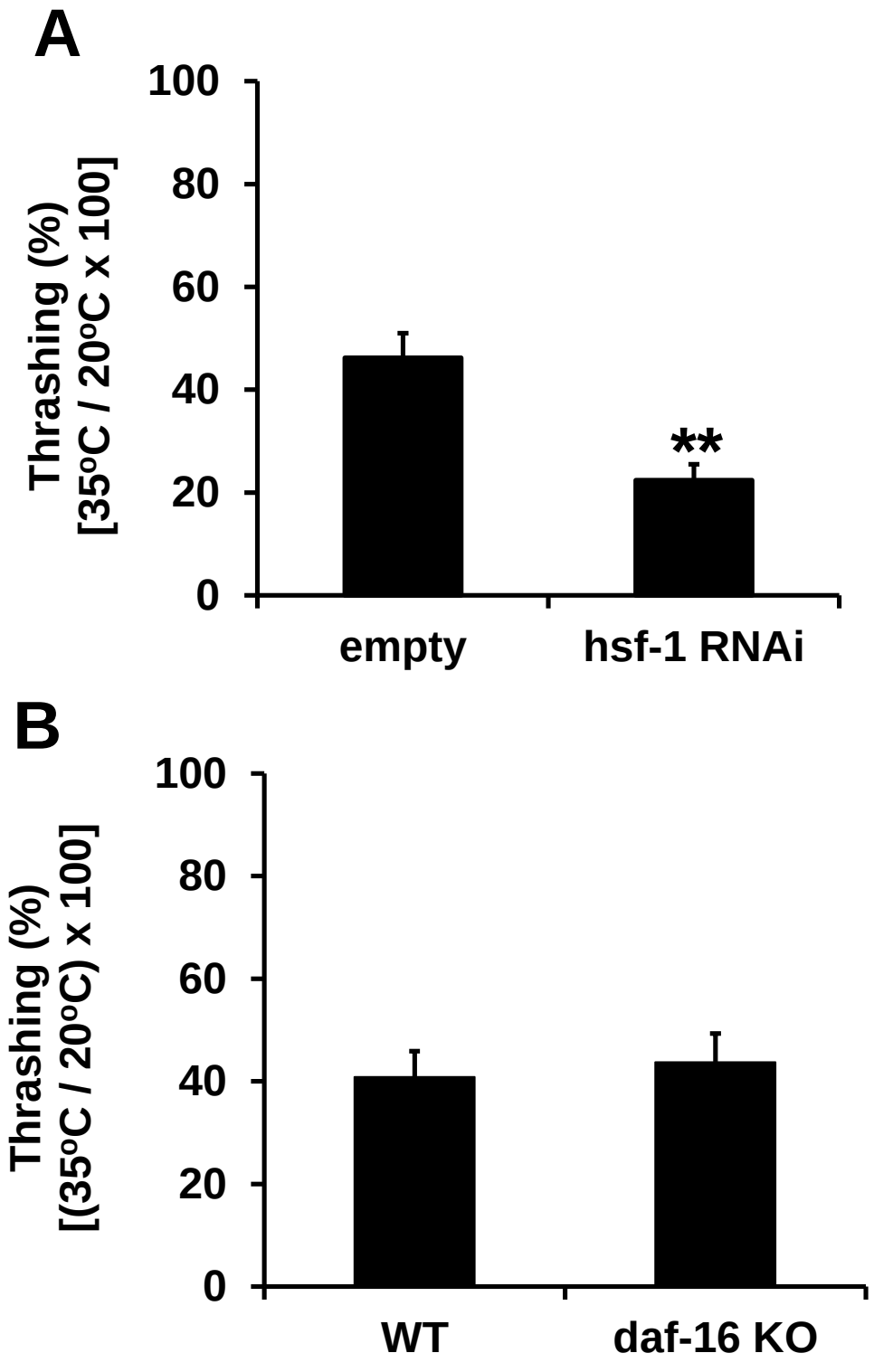
CHAPTER 2-Fig. 3 The role of HSF-1 in the reduction of thrashing by heat stress in *daf-2* mutants. *daf-2* mutant worms treated with *hsf-1* RNAi were transferred to *E. coli*-free NGM plates and cultured for 4 h at 20°C or 35°C. After 4 h, the worms were transferred onto new NGM RNAi media plates and cultured for 0–6 h. After 0, 3, or 6 h, ten worms were observed for thrashing for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with Games-Howell test. Mean ± SE, *$p < 0.05$, **$p < 0.005$.

CHAPTER 2-Fig. 3



CHAPTER 2-Fig. 4 The roles of HSF-1 and DAF-16 in the prevention of thrashing reduction by heat stress. (**A**) Worms treated with *hsf-1* RNAi were transferred to *E. coli*-free NGM plates and cultured for 1 h at 20°C or 35°C. The plates were incubated for 10 min at room temperature, and ten worms were observed for thrashing for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. Mean ± SE, $*p < 0.05$, $**p < 0.005$. At least, three independent trials were conducted and showed similar result. (**B**) Adult worms were transferred onto *E. coli*-free NGM plates and cultured for 1 h at 20°C or 35°C. The plates were incubated for 10 min at room temperature, and ten worms were observed for thrashing for 15 s. The graph shows the ratio of the number of thrashing activity of heat treated worms divided by that of heat-untreated worms. Three independent experiments were performed and these data were combined for making a graph. Statistical significance was analyzed with t-test. Mean ± SE, $*p < 0.05$, $**p < 0.005$.

CHAPTER 2-Fig. 4



GENERAL DISCUSSION-Fig. 1 Summary of my work.

Details are described in GENERAL DISCUSSION.

GENERAL DISCUSSION-Fig. 1