Nicotinamide adenine dinucleotide extends the lifespan of *Caenorhabditis elegans* mediated by *sir-2.1* and *daf-16*

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Abstract It is well understood that sir2 (sirtuin), an NAD-dependent deacetylase, is essential for the extension of lifespan under caloric restriction. However, the mechanism underlying activation of *sir2* is unclear. Life extension through caloric restriction requires the *sir2* ortholog *sir-2.1* in nematodes but occurs independently of the forkhead-type transcription factor DAF-16. We aimed here to elucidate the correlation between life extension in nematodes and NAD-dependent activation of sirtuin by analyzing the relationship between NAD and DAF-16. Lifespan was extended when *C. elegans* were bred using medium containing NAD. An RNA interference experiment revealed that life extension by NAD was *sir-2.1* dependent. However, life extension by NAD did not occur in *daf-16*-RNAi nematodes, suggesting that NAD-dependent longevity requires *daf-16*. This result suggested that different signaling pathways are involved in life extension resulting from caloric restriction and from NAD addition. Expression of *sod-3*, a target gene of *daf-16*, and increased oxidative-stress resistance and adiposity were observed in response to NAD addition, indicating that NAD activated *daf-16* in each phenotype. These results suggest that NAD affected lifespan through the activation of SIR-2.1 and DAF-16 along a signaling pathway, namely insulin-like signalling pathway (at least parts of it), different from that associated with caloric restriction.

Keywords NAD • longevity • sirtuin • daf-16 • nematode

Introduction

It has recently become understood that sir2 (silent information regulator 2, sirtuin) plays an important role under caloric restriction. Sir2 acts as a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase or ADP ribosyltransferase (Tanny et al. 1999), and modulates the activity of many proteins, including histone (Guarente et al. 2000; Smith et al. 2000; Landry et al. 2000). Sir2 was first discovered in budding yeast as a factor involved in the silencing of gene expression (Rusche et al. 2003; Rine et al. 1979; Rine et al. 1987). The presence of the same protein was later confirmed in a wide range of species from *Homo sapiens* to *Drosophila* (Brachmann et al. 1995). Sir2 contributes to the adaptation of lifespan in budding yeasts, because lifespan is extended by overexpression of *sir2* and shortened by loss of function of this gene (Kaeberlein et al. 1999). Sir2 also plays an essential role in life extension under caloric restriction (Lin et al. 2000). The finding that overexpression of s*ir-2.1,* a *sir2* ortholog in *C. elegans,* extends life (Tissenbaum et al. 2001) suggests that *sir2* regulates lifespan in a wide range of living creatures. The main action of sirtuin in yeast is to inhibit increases in homologous recombination of extrachromosomal rDNA with aging (Guarente et al. 2000; Smith et al. 2000; Landry et al. 2000; Rusche et al. 2003). In mammals, sirtuin is thought to suppress gene expression by stabilizing chromatin structure (Vaquero et al. 2004). However, the functions of sirtuin in mammals are diverse, and sirtuin may also control lifespan by regulating apoptosis and adiposity by deacetylating proteins such as p53 or PGC-1α (Luo et al. 2001; Nemoto et al. 2005). In addition, sirtuin is indispensable under caloric restriction in *C. elegans,* which is similar to the situation observed in yeast. Extension of life of *eat-2* (*ad1116*), a mutant that causes caloric restriction through pharyngeal abnormality, is canceled by a deficit in *sir-2.1* (Wang et al. 2006). A similar result was obtained in *Drosophila* (Kopec 1928), suggesting that sirtuin acts under caloric restriction. However, the correlation between sirtuin and insulin signaling pathways is not well understood. Extension of life by caloric restriction reportedly occurs independently of insulin signaling pathways (Lakowski and Hekimi 1998; Bishop et al. 2007; Houthoofd et al. 2003), but life lengthening by *sir-2.1* overexpression in nematodes is dependent on DAF-16, which encodes a protein involved in insulin signaling (Tissenbaum et al. 2001). It was recently reported that caloric restriction requires DAF-16 activity (Greer et al. 2007). However, the role of DAF-16 in this case was exceptional because DAF-16 acts at extranuclear. The mechanism of life extension by caloric restriction is therefore complicated and may be controlled by many factors and signal networks acting together. The mechanism underlying the action of SIR-2.1 acts under caloric restriction is unclear. Our current knowledge of caloric restriction in *C. elegans* is as follows: (1) *sir-2.1* is indispensable to life extension under caloric

restriction; (2) *daf-16* is indispensable to the role of overexpressed *sir-2.1*; and (3) *daf-16* is not necessarily indispensable to life extension under caloric restriction. Furthermore, recent reports suggested that *sir-2.1* is not responsible in life extension under caloric restriction. Removal of bacterial food increases lifespan to a greater extent than partial reduction of food through a mechanism that is distinct from insulin/IGF-like signaling and the Sir2-family deacetylase, SIR-2.1 (Kaeberlein, 2006). Caloric restriction-induced longevity was independent of *sir-2.1*, and was independent of the DAF-2/insulin-like signaling pathway that independently regulates longevity and larval diapause in *C. elegans* (Lee et al, 2006; Houthoofd and Vanfleteren, 2006). If the seemingly contradictory functional mutual relationships between these factors can be clarified, then it may be possible to imitate caloric restriction in the human body in the clinical setting.

Sirtuin is activated by the cofactor NAD, which is essential for electron transfer and plays a central role in oxidative phosphorylation. Because NAD is reduced to NADH along with the oxidation of sugar and fatty acid in the glycolytic pathway and the citric acid cycle, the NAD level and the NAD to NADH ratio are potential indicators of internal energy level and degree of starvation (Lin et al. 2000; Guarente et al. 2005). Under caloric restriction, the glycolytic pathway and citric acid cycle are suspended, and as a result the level of NAD can be expected to increase. Therefore, it is generally thought that NAD-dependent sirtuin is activated in response to diminution of the cellular energy state. The deacetylation activity of SIR-2.1 in vitro increases with an increase in the NAD level (Wood et al. 2004). In bacteria, although increasing NAD levels confer resistance to various stresses, including heat, salt, and nutrient stress, the detailed mechanism is not clear (Foster et al. 1990). Life lengthening under caloric restriction does not occur in response to deletion of *npt-1* (nicotinic acid phosphoribosyltransferase), one of the NAD synthases in budding yeasts (Lin et al. 2000). Conversely, one would expect overproduction of NPT1 to promote NAD production, resulting in the activation of *sir2* and the extension of life. This would suggest that NAD is indispensable to life lengthening under caloric restriction and that NAD production increases under caloric restriction. However, NAD production does not change even if NPT1 is overexpressed (Anderson et al. 2002). In addition, caloric restriction by limiting glucose supply has revealed that intracellular NAD levels do not change in yeast (Lin et al. 2001; Anderson et al. 2003a; Lin et al. 2004). Therefore, it is not apparent how NAD-dependent sirtuin is activated under caloric restriction.

In the present study we aimed to clarify the relationship between life extension and activation of SIR-2.1 in response to the addition of NAD in the nematode *Caenorhabditis elegans*. Our results suggest that NAD extends lifespan through the activation of SIR-2.1 and DAF-16 along a signal course that is different from that of caloric restriction.

Materials and methods

Worms and culture

Wild type Bristol N2 and mutant strain of *Caenorhabditis elegans* (Caenorhabditis Genetics Center, Minnesota, USA) were cultured at 20 °C on NGM (Nematode Growth Medium) agar plated with *E. coli* OP50 (Brenner 1974). The mutants used were *daf-16* (*mgDf50*), *sir-2.1* (*ok434*), and *eat-2* (*ad1116*).

Construction of feeding-RNAi plasmid

Because two unidentified nematode genes, *W06B3.1* and *F26H9.4* (wormbase, www.wormbase.org), are homologous to the gene encoding mammalian nicotinamide mononucleotide adenylyl transferase (*nmnat*), we supposed that these genes are homolog of *nmnat* (*nmnat-1* and *nmnat-2*, respectively). PCR was performed to amplify the each cDNA regions (*daf-16*, *sir-2.1, nmnat)* using PCR-2720 (Applied Biosystems**,** Foster City, CA, USA). Each primer was designed to have restriction site (*Bam*H1 or *Xho*I, Takara, Otsu, Shiga, Japan) at each 5'-end, and to make PCR products with 700 ntds in length. The amplified PCR products were digested by restriction enzymes (Takara) and were subcloned into plasmid L4440 (provided from Fire laboratory) designed for Bacteria-mediated feeding-RNAi (RNAi plasmid). The nucleotide sequences of plasmid DNA were analyzed by using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI PRISM 377 DNA Sequencer (Perkin Elmer Biosystems, Massachusetts, USA).

Each genetic sequence referred to wormbase (www.wormbase.org). PCR (95 °C for 5 min; 25–30 cycles of 95 °C for 1 min, 52 °C for 30 sec, 72 °C for 90 sec 72 °C for 7 min) was performed using the primers (Supplement 1).

Bacteria-mediated RNAi (Timmons and Fire 1998)

Escherichia coli strain HT115 was transfected with RNAi plasmid and treated with 35 mM IPTG (Isopropyl-β-D-thiogalactopyranoside, Wako, Chuo, Osaka, Japan) to induce the RNA expression. This transformants were plated on NGM agar containing 1 mM IPTG and incubated at 37 °C for 1 h (RNAi-plate). L4440, an empty vector, was used for a negative control of RNAi. Because *E. coli* HT115 transformant and the

RNAi-plate lose RNAi efficiency along with time course, they were prepared newly in every experiment.

To synchronize the growth stage of nematode, adult worms were treated with 10% NaClO solution (10 N NaOH/NaClO (10:1), and eggs were collected and cultured overnight in S-basal (0.1 mol/l NaCl, 50 mmol/l potassium phosphate buffer) at 20 °C until the hatching. Hatched L1 larvae were then cultured on NGM RNAi-plate at 20 °C.

RNA preparation and cDNA synthesis

Batches of 400 worms were collected after 72 hrs culture on RNAi-plate, and RNA was extracted by the acid-GTC-phenol method (Klass 1977). Genomic DNA was digested by treatment with DNase I (Takara) for 60 min at 37 °C, and RNA was repurified by repeating the acid-GTC-phenol extraction. cDNA was synthesized by using M-MLV Reverse Transcriptase (Takara) and used for PCR.

Lifespan analysis

A series of experiment was performed at 20 °C. The synchronized-nematodes (15 worms/plate) were bred on standard NGM plate or RNAi-plate till they grow up to young adult (3rd day), they were moved to new NGM plate (RNAi+/-) containing NAD (Wako) (Hsin and Kenyon 1999). The day when young adult was moved was counted as day 0, and lifespan were measured. The living population was counted every 2 days, and it was moved to new plate every 4 days. On day 4 and 8 after the start of RNAi feeding, worms were treated with 0.5 mg/ml fluorodeoxyuridine (FUdR, Wako) to prevent the next genetical birth. Worms were transferred to new plates every 4 days to analyze viability. Life or death of a nematode was judged by its response to tapping on the plate.

Body size analysis

L1 larvae were bred on RNAi-plate containing NAD for 96 hrs. After the worms had been fixed with PFA (paraformaldehyde, Kanto Chemical, Tokyo, Japan) solution (0.2% PFA / 10% EtOH / S-basal), they were photographed under a microscope (DMRXA, Leica Microsystems, Inc., Bannockburn, IL, USA), and their individual lengths were measured using image analysis software Lia32 (Dr. Kazukiyo Yamamoto, Nagoya

University).

Egg laying analysis

Adult worm (synchronized) which was bred with NGM plate including NAD was moved to new NGM plate (NAD+) one by one on the first day of adult (the third day on RNAi-plate), and number of eggs was measured every 24 hrs. The number of eggs laid by each worm was counted beginning 72 hrs after the start of culture of the worms on RNAi-plate.

RT-PCR analysis

cDNA was synthesized by using M-MLV Reverse Transcriptase (Takara), and quantity of cDNA was corrected by using *gpd-1* (glyceraldehyde 3-phosphate dehydrogenase) as an internal standard. PCR (95 °C for 5 min; 23–35 cycles of 95 °C for 1 min, 52 °C for 30 sec, 72 °C for 90 sec; 72 °C for 7 min) was performed using the primers (Supplement 2).

Nile red assays

Nematode was bred on NGM plate including 50 ng/ml nile red (MP Biomedicals, CA, USA) with *E. Coli*. After 72 hrs culture, worms were collected, treated with 0.2% PFA solution, and observed under a fluorescence microscope (DMRXA, Leica) with N3 filter (565 nm, Leica) (Ashrafi et al. 2003; Horikawa et al., 2008).

Transcriptional activity of DAF-16 (Essers et al. 2005)

The promoter region of *sod-3* gene (-994~1120 ntds), was amplified by PCR using primers (Supplement 3), and was subcloned into GFP expression plasmid pPD95.77. This construct, P_{sod-3}::gfp, was injected into *lin-15* mutant worm (*n765ts*) with *lin-15* expression plasmid, pDLH98, to have worm which stably expresses GFP in response to transcriptional activity of DAF-16. The P_{sod-3}::gfp worm at L1 stage was bred on NGM agar plate including 100 μM NAD. The expression level of GFP was analyzed after 72 hrs incubation, fixed in PFA solution, and was observed under fluorescent microscope (DMRXA, Leica) with L5 filter (505 nm, Leica).

Paraquat assays

Young adult worms (synchronized) were bred on NGM plate including 5 mM paraquat (1, 1'-Dimethyl-4, 4'-bipyridinium Dichloride, TCI ORGANIC CHEMICALS, Tokyo, Japan) and NAD (100 μM, 1 mM), and the living population was counted after 6 days culture. Life or death of a nematode was judged by its response to tapping on the plate.

Results

Effect of NAD on lifespan

To determine whether NAD influences the lifespan of the nematode *C. elegans*, worms were bred on NGM plates containing NAD at 0, 10, 100, or 1000 μM, and the number of the living nematodes was counted every 2 days. Addition of NAD at all concentrations extended life (Fig. 1A); in particular, 100 μM of NAD caused the highest increase in life extension of 15% compared with that of the untreated controls. Because NAD induces the deacetylase activity of sirtuin by acting as a cofactor (Landry et al., 2000), life extension in *C. elegans* may occur through the activation of SIR-2.1 by NAD. We therefore examined the effect of NAD on RNAi worms in which *sir-2.1* expression was inhibited. For this experiment we used NAD at 100 μM, the dose giving the highest level of life extension. NAD did not cause lifespan extension in *sir-2.1*-RNAi worms (Fig. 1B). We performed a similar experiment using a *sir-2.1* deletion mutant (*ok434*) and again found that NAD had no effect on lifespan (Fig. 1B). These results suggest that NAD increases nematode longevity by activating SIR-2.1.

Effect of NAD on body length and egg-laying

Next, we compared the effects of caloric restriction and NAD addition on body size and egg-laying. It is well known that body length and the laying rate are reduced by caloric restriction (Klass 1977). We first investigated the influence of NAD on body length in wild-type (WT) worm. The *eat-2* (*ad1116*) mutant, which has a pharyngeal movement defect, was used as a control of caloric restriction. The body length of worm continues to increase from hatching through to early adulthood but this rate of increase is sensitive to factors such as

nutritional status. Therefore, the nematodes were bred on NGM plates containing NAD and *E. coli*. Although average body length was reduced in the *eat-2* mutant compared with that of the WT worm, 70 hrs after hatching NAD didn't affect the body length of the WT worm (Fig. 2A).

Next, we counted the number of eggs to examine the influence of NAD on worm reproduction. Body length and egg-laying are independent phenomena, because egg-laying is specific to the adult worm. In the presence of NAD, the number of eggs laid per individual was counted for 3 days. The *eat-2* worms laid fewer eggs than the WT control worms. Addition of NAD did not influence the period of egg laying or the number of eggs in the WT control worms (Fig. 2B).

Effect of NAD on DAF-2/insulin signaling

We examined the influence of NAD on life extension in the *eat-2* (*ad1116*) mutant. If caloric restriction and NAD addition were to act by the same pathway, then NAD would not extend lifespan in this mutant. However, NAD caused dose-dependently a further extension of life in the *eat-2* mutant (Fig. 3A). These results suggest that extension of life by NAD worked along a signaling pathway different from that of caloric restriction. Next, we analyzed the action of NAD on DAF-2/insulin signaling, which is known to regulate lifespan. The function of the DAF-2 signal depends on the activity of the forkhead type transcription factor DAF-16. To determine whether life extension by NAD depends on the DAF-2 signal, *daf-16* mRNA was suppressed by administering RNAi. NAD had no effect on lifespan in *daf-16*-RNAi worms (Fig. 3B). As with the RNAi analysis, lifespan analysis of *daf-16* mutants (*mgDf50*) revealed that NAD caused no life extension at all (Fig. 3B). These results suggest that the lifespan effect of NAD depends on both *sir-2.1* and *daf-16*.

Effect of NAD on DAF-16 transcriptional activity

Next, we analyzed the action of NAD on the transcriptional activity DAF-16. Activation of the transcriptional activity of DAF-16 induces downstream gene expression, resulting in increased stress resistance (Honda et al. 1999) and accumulation of lipid (Lee et al., 2003). First, we analyzed the action of NAD on the expression of superoxide dismutase *sod-3*, a gene that is essential for oxidative stress resistance in nematodes. *Sod-3* is a typical scavenger enzyme in oxidative stress and catalyzes the active superoxide anion (Giglio et al 1994). The binding domain of DAF-16 is located in a transcriptional promoter region of *sod-3*, and expression of *sod-3*

depends on DAF-16 activity (Furuyama et al. 2000). To examine the expression of *sod-3* we generated a transgenic nematode in which we introduced a GFP expression plasmid carrying the *sod-3* promoter (Psod-3::gfp). Fluorescence microscopy revealed an increase in the expression of *sod-3* by the addition of NAD. However, the expression of *sod-3* did not change in *daf-16* or *sir-2.1*-RNAi worms (Fig. 4A) indicating that activation of DAF-16 by NAD depends on SIR-2.1. We used the pesticide paraquat as a source of oxidative stress to examine whether oxidative stress resistance was affected by a change in expression of *sod-3*. After 6 days, the addition of NAD had increased the survival rate of WT worms treated with paraquat by 30% (Fig. 4B). In contrast, survival rates of *daf-16* mutant (*mgDf50*) and *sir-2.1* mutant (*ok434*) did not change with or without NAD (Fig. 4B). The finding that NADH (100 μM), a reduced form of NAD, did not affect oxidative resistance suggests that the increase in oxidative stress resistance is NAD-specific.

Effect of NAD on adipogenesis

We analyzed fat accumulation, one of the phenotypes that may occur with activation of DAF-16, by using Nile red, which can directly stain nematode body fat. Adiposity increased with the addition of 100 μM NAD in WT worms, whereas no increase was observed in the *daf-16* mutant (*mgDf50*) or *sir-2.1* mutant (*ok434*) (Fig. 5A). RT-PCR revealed that NAD didn't affect the expression of *daf-16* and *sir-2.1* (Fig. 5B). Next, we analyzed the expression of *sod-3* and *fat-7*, which is regulated by DAF-16. Fat-7, one of the fatty acid desaturases, desaturates stearic acid to produce oleic acid; a rise in *fat-7* expression induces life extension as well as fat accumulation (Brock et al. 2006). RT-PCR showed that the expression of these genes increased markedly with the addition of NAD (Fig. 5B). We then analyzed the characteristics of *sod-4* (Panowski et al. 2007), the expression of which is controlled by caloric restriction. Five types of *sod* gene are found in nematodes, and all are controlled by different mechanisms that regulate expression and protein localization (Panowski et al. 2007; Giglio MP et al. 1994; Suzuki et al. 1996; Fujii et al. 1998). Transcription of *sod-1* (Cu/Zn type, perikaryon localization), *sod-3* (Fe/Mn type, mitochondrial localization), and *sod-5* (Cu/Zn type, localization unknown), is controlled by the transcription factor DAF-16. By comparison, the transcription of *Sod-1*, *sod-2* (Fe/Mn type, mitochondria localization), *sod-4* (Cu/Zn type, extracellular localization), and *sod-5* is controlled by transcription factor PHA-4 (Panowski et al. 2007). Panowski *et al.* (2007) reported that the mRNA levels of *sod-2*, *sod-4*, and *sod-5* rise via activation PHA-4 under caloric restriction, without influencing the mRNA level of *sod-3*. Expression of *sod-4* rose markedly with the addition of NAD (Fig. 5B). We also examined the expression of *sod-2*, which is not

controlled by DAF-16, but *sod-2* expression was not detected (data not shown). Expression analysis of the *daf-16* mutant revealed that NAD upregulated the expression of *sod-4* without affecting the expression of *daf-16*, *sir-2.1*, *sod-3*, or *fat-7* (Fig. 5B). NAD had no effect on the expression of these genes in the *sir-2.1* mutant.

Knockdown of NAD synthetase by RNAi

We investigated the role of intravital NAD on lifespan by examining the synthesis of NAD. Plural synthases contribute to the synthesis of NAD. In particular, nmnat plays a pivotal role in NAD synthesis in both the de novo and salvage pathways and synthesizes NAD or deamido-NAD from nicotinic acid mononucleotide and ATP (Rongvaux et al. 2003). Overexpression of *nmnat* promotes NAD synthesis and activation of SIRT1 (silent information regulator two ortholog 1), the mammalian ortholog of sir2 (Araki et al., 2004; Revollo et al., 2004). The pathway of NAD synthesis varies between mammals and invertebrate, but lifespan is extended by overexpression of one of the NAD synthases, *NPT1* (Magni et al. 2004; Revollo et al. 2004). However, we do not know how *nmnat* influences the lifespan of a multi-cellular organism. So far, no functional *nmnat* gene is identified in nematode. Because of their sequence homologies, we supposed *F26H9.4* and *W06B3.1* as homolog of *nmnat* of nematode (*nmnat-1* and *nmnat-2*, respectively). So, we examined whether *nmnat*-RNAi influences the nematode lifespan. RNAi of *nmnat-1* (*F26H9.4*) caused embryonic death (data not shown), whereas *nmnat-2* (*W06B3.1*)-RNAi reduced lifespan by 13% by comparison to the lifespan of control nematodes (Fig. 6A). However, the survival rate of *nmnat-2*-RNAi worms on paraquat plates on 6 days exposure did not differ from that of controls (Fig. 6B).

Discussion

The finding that NAD extends nematode life suggests that NAD has the capacity to extend lifespan regardless of species. The intracellular concentration of NAD in *Homo sapiens* is 400 to 700 μM (Revollo et al. 2004), and it in yeasts it is about 500 μM (Magni et al. 2004). However, the intracellular NAD concentration in nematodes has not been reported. Because NAD at 10 μM had a life-lengthening effect in our studies, we suspect that the intracellular NAD concentration in the nematode is lower than in other animals. However, it is unclear how addition of NAD influences the nucleus, where SIR-2.1 acts, and the mitochondria, where the NAD/NADH ratio changes markedly. It is also thought that exogenous NAD influences specific cells or organelles. Because

the extension of life by NAD is dependent on *sir-2.1*, it is possible that sirtuin, an NAD-dependent enzyme, regulates the lifespan of the nematode by responding to an increase in the concentration of NAD. It is generally thought that an increase in NAD level and activation of sirtuin are caused by caloric restriction. However, the fact that our study revealed different phenotypes from NAD addition and caloric restriction suggests that NAD addition acts by a signal cascade other than that used by caloric restriction. This agrees with reports (Anderson et al. 2003a; Lin et al. 2004; Klass 1977) that increased NAD levels are absent in caloric restriction in yeast.

Life extension by NAD addition required the action of DAF-16 downstream of the DAF-2 signal cascade, which acts independently of the caloric restriction pathway. In addition, NAD had an effect on the expression of other genes, oxidative stress resistance, and adiposity through the activation of DAF-16. The requirement for both *sir-2.1* and *daf-16* for life extension by NAD and the loss of activation of DAF-16 through suppression of SIR-2.1 suggest that these factors act in the same signal cascade (Fig. 4). These findings are consistent with previous reports (Tissenbaum et al. 2001, Yang et al. 2005; van der Horst et al. 2004; Brunet et al. 2004; van der Horst et al. 2007) and suggest that NAD activates SIR-2.1, prior to activating the transcriptional activity of DAF-16.

RNA levels of *daf-16* and *sir-2.1* did not vary with addition of NAD. By comparison, the expression of *sod-3* and *fat-7*, which are controlled by DAF-16, rose markedly. These results suggest that addition of NAD induces expression of these genes through the activation of DAF-16 to alter stress resistance, adiposity, and lifespan. We consider that DAF-16 was activated by NAD through activation of SIR-2.1. Interestingly, transcription of *sod-4*, which is upregulated under caloric restriction, was induced by addition of NAD. This result suggests that the NAD-induced signal cascade is not completely independent of the pathway by which caloric restriction acts. An increase in NAD level may activate caloric restriction signals partly through the action of sirtuin. In addition, expression of *sod-4* rose on exposure of the *daf-16* (*mgDf50*) mutant to NAD. This result suggests that NAD activates the caloric restriction pathway and DAF-2/insulin signal pathway independently, and that *daf-16* did not contribute to the signaling pathway of caloric restriction. Because the lifespan of the *daf-16* mutant (*mgDf50*) was not extended by exposure to NAD and the level of expression of *sod-4* increased, it is not clear whether sod-4 plays an essential role in lifespan. Additionally, partial activation of the caloric restriction pathway by NAD does not influence lifespan. Furthermore, *sir-2.1* is necessary for expression of *sod-4*, because the expression level of *sod-4* did not change with exposure of the *sir-2.1* mutant (*ok434*) to NAD. Expression of *sod-4* is controlled by the transcription factor PHA-4 (Panowski et al. 2007). Together, these results strongly suggest that the transcriptional activity of PHA-4 is controlled by SIR-2.1. In this report, our results suggest that

NAD affected lifespan and oxidative resistance through the activation of SIR-2.1 and DAF-16 along a signaling pathway, namely insulin-like signalling pathway (at least parts of it), different from that associated with caloric restriction. However, because NAD increased the expression of sod-4 through the activation of SIR-2.1, there is some possibility that NAD partially affect lifespan and oxidative resistance through the activation of caloric restriction pathway.

The mechanism underlying the change in NAD levels *in vivo* is unknown. The level of NAD increases under stress in yeast (Anderson et al. 2003b). Previous reports have revealed a 50% increase in the level of hepatic NAD with starvation with mammals, whereas others have reported that the level of pancreatic NAD is reduced by starvation (Bordone et al. 2006). Therefore, different tissues may utilize different mechanisms to control of the level of NAD, but these mechanisms are unknown. Recently, the pathway of NAD synthesis has become clear in mammals and yeasts (Fulco et al. 2003). By comparison, there are no reports of the mechanism underlying NAD synthesis in nematodes. Therefore, we performed RNAi of the genes considered to be associated with NAD synthase in the nematode, and examined the resultant phenotypes. RNAi specific for *F26H9.4* (*nmnat-1*) caused embryonic death, whereas RNAi specific for *W06B3.1* (*nmnat-2*) shortened the nematode lifespan, although oxidative stress resistance was not affected (Fig. 6). Because oxidative-stress resistance was examined only for 6 days from day 3 of development, it is possible that the decrease in lifespan induced by RNAi specific for *W06B3.1* was supplemented functionally by *F26H9.4*. In contrast, because the lifespan analysis was performed for about 3 weeks, it is possible that this longer time allowed the effect of RNAi to appear. An increase in NAD level may activate caloric restriction signals partly through the action of sirtuin. Our results suggest that activation of sirtuin in caloric restriction possibly be caused only partly by an increase in the level of NAD. The molecular mechanism activated by sirtuin under caloric restriction is not yet clarified. For example, as is the case with resveratrol, there may be an unidentified molecule that activates sirtuin as well as NAD. Direct addition of NAD resulted in both oxidative stress resistance and life extension in the nematode, but we are not sure whether there is an equal effect in higher animals such as mammals. However, there are reports that NAD acts as a co-factor of SIRT1 in mammals, and that sirtuin is activated in response to an increase of NAD/NADH ratio in muscle (Fulco et al. 2003). Therefore, it is possible that the control of SIRT1 by NAD levels has a beneficial effect in higher organisms. Synthesis of NAD and control of the NAD/NADH ratio play important roles in the control of sirtuin activity. If SIRT1 functions to extend the lives of mammals under caloric restriction, the control of sirtuin activity by NAD may be used to improve the outcome of diseases and abnormalities associated with aging.

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Legends for Figures

Fig. 1 (**A**) The wild-type nematode N2, and (**B**) *sir-2.1*-RNAi and mutant (*ok434*) worms were cultured on agar plate containing NAD (0, 10, 100, 1000 μM), and a survival population was counted every 2 days, and was plotted on a graph. A survival rate (%) of nematode was shown at a vertical axis, and the individual survival days were shown at horizontal axes. n indicates individual number used for experiment. After the hatching, NAD was given to nematode continuously, and worms were moved to new plate on every 4 days. (Supplement 4).

Fig. 2 The wild-type nematode N2 and *eat-2* mutant *(ad1116*) worm were cultured on agar plate containing NAD (0, 10, 100, 1000 μM), and the body length (**A**) and number of egg (**B**) were measured. n indicates

individual number used for experiment. After the hatching, NAD was continuously given to the worms, and the body size was measured at a young adult stage. After NAD-treated worms started to deliver, number of laid eggs from each worm was counted for 3 days. (Supplement 5).

Fig. 3 (**A**) The wild-type N2 and *eat-2* mutant (*ad1116*), and (**B**) *daf-16*-RNAi worm and *daf-16* mutant (*mgDf50*) were cultured on agar plate containing NAD (0, 100, 1000 μM). The survival population was counted every 2 days, and was plotted on a graph. A survival rate (%) of nematode was shown at a vertical axis, and the individual survival period (days) was shown at horizontal axes. n indicates individual number used for experiment. After the hatching, NAD was given to nematode continuously, and worms were moved to new plate on every 4 days. (Supplement 6).

Fig. 4 (**A**) Fluorescence of Psod-3::gfp-transgenic nematode was observed. The wild type N2 and *daf-16*-RNAi-worm and *sir-2.1*-RNAi-worm were cultured on agar plate containing NAD (0, 10, 100, 1000 μM), and the fluorescence (505 nm) was measured at young adult stage. (**B**) A survival rate (%) of nematode cultured on MGM plate containing 5 mM paraquat for 6 days was measured. The wild type N2 and *daf-16* mutant (*mgDf50*) and *sir-2.1* mutant (*ok434*) were cultured on agar plate containing NAD (100 μM) or NADH (100 μM). After the hatching, NAD (or NADH) was given to nematode continuously, and worms were moved to new plate containing 5 mM paraquat for 6 days. (Supplement 7).

Fig. 5 (**A**) Influence of NAD to adiposity was examined. The wild type N2, *daf-16* mutant (*mgDf50*) and *sir-2.1* mutant (*ok434*) were cultured on agar plate containing nile red. Adipose accumulation of young adult worm was observed under fluorescent microscope (565 nm). After the hatching, NAD (100 μM) was given to nematode continuously. (**B**) Influence of NAD to adipogenesis-related gene expression was examined. The wild type N2, *daf-16* mutant (*mgDf50*) and *sir-2.1* mutant (*ok434*) were cultured on agar plate containing NAD (0, 10, 100, 1000 μM), and RNA was prepared for RT-PCR. *gpd-1* was used as an internal standard.

Fig. 6 (**A**) A survival population of the nematode which introduced RNAi of *nmnat-2* (*W06B3.1*) was measured every 3 days, and it was plotted on a graph. A survival rate (%) of nematode was shown at a vertical axis, and the individual survival periods (days) were shown at horizontal axes. n indicates individual number used for experiment. (**B**) A survival rate of *nmnat-2*-RNAi-worm cultured on MGM plate containing 5 mM paraquat for

6 days was measured. Worm at young adult stage was moved to new plate every 4 days. (Supplement 8).

Fig. 1

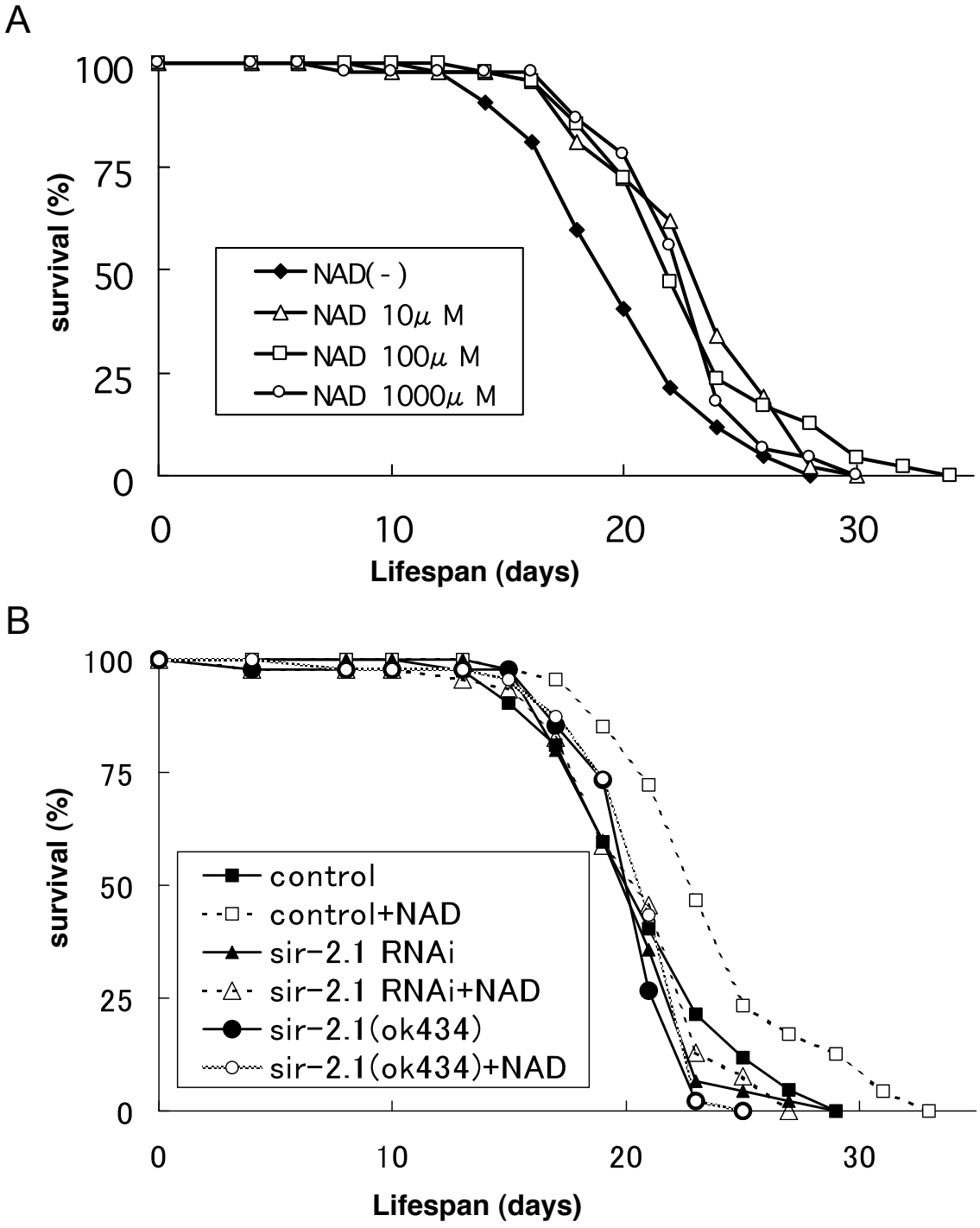
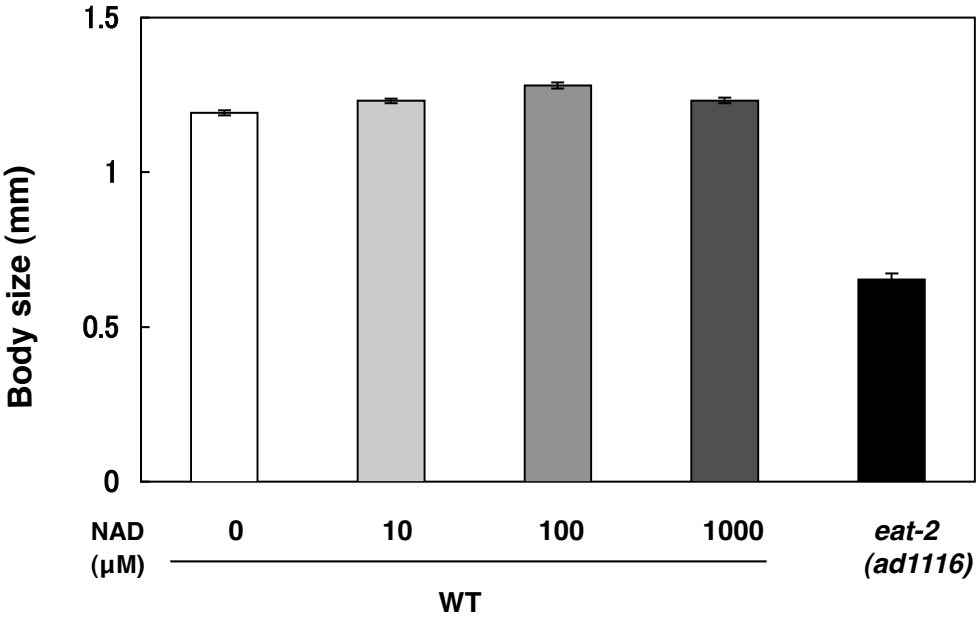

A
100
75
50
25
0
survival (%)
NAD(-)
NAD 10μ M
NAD 100μ M
NAD 1000μ M
0
10
20
30
Lifespan (days)
B
100
75
50
25
0
survival (%)
control
control+NAD
sir-2.1 RNAi
sir-2.1 RNAi+NAD
sir-2.1(ok434)
sir-2.1(ok434)+NAD
0
10
20
30
Lifespan (days)

Fig. 2

A


1.5
1
0.5
0
Body size (mm)
NAD
(μM)
0
10
100
1000
eat-2
(ad1116)
WT

B

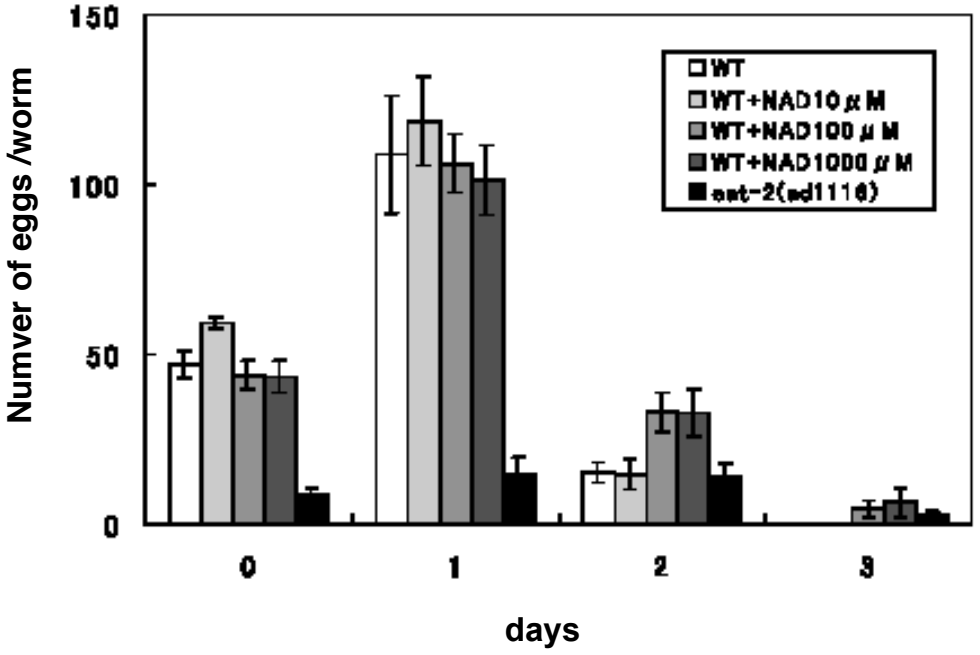

150
100
50
0
Numver of eggs /worm
WT
WT+NAD10μM
WT+NAD100μM
WT+NAD1000μM
eat-2(ad1116)
0
1
2
3
days

Fig. 3

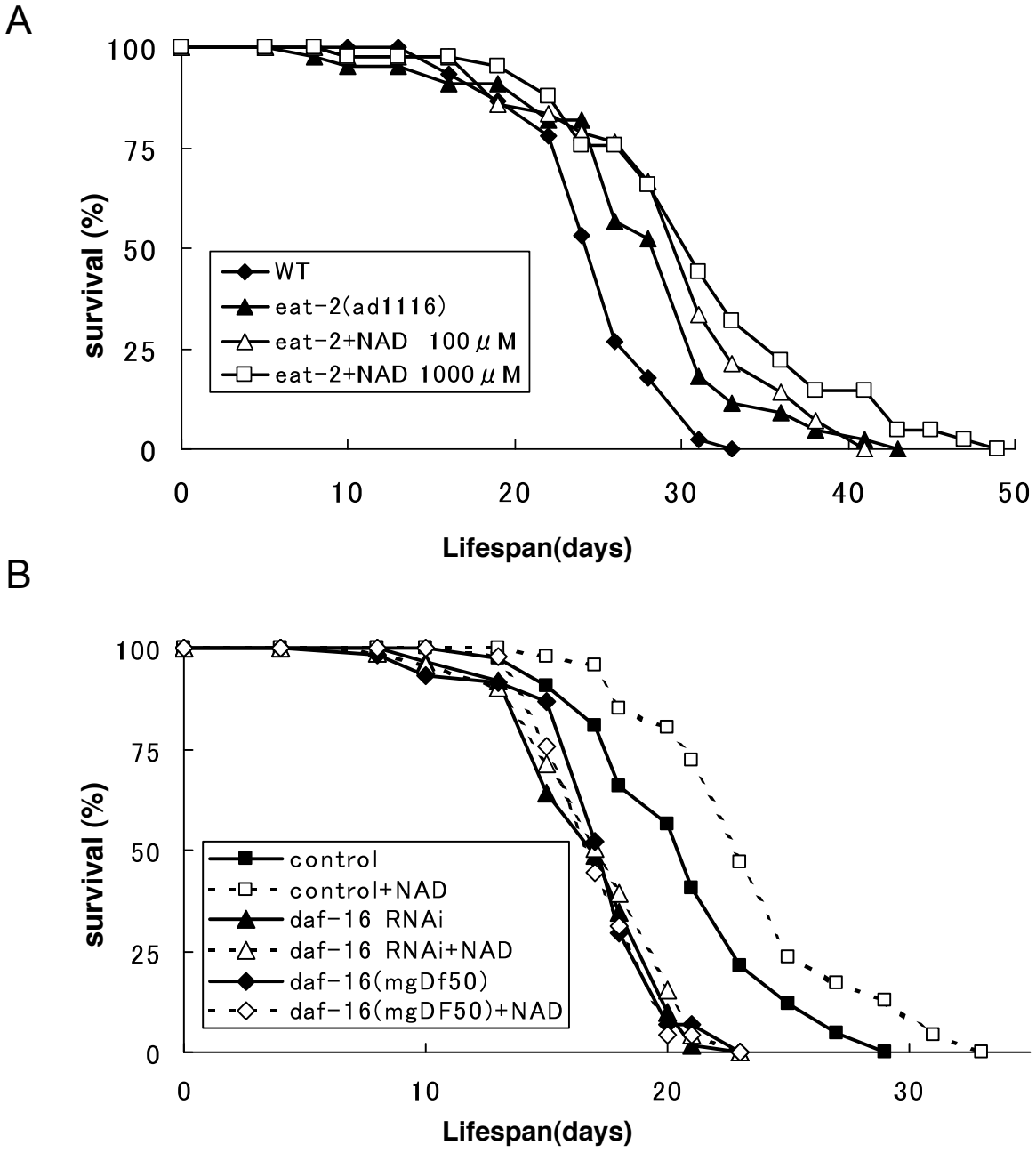
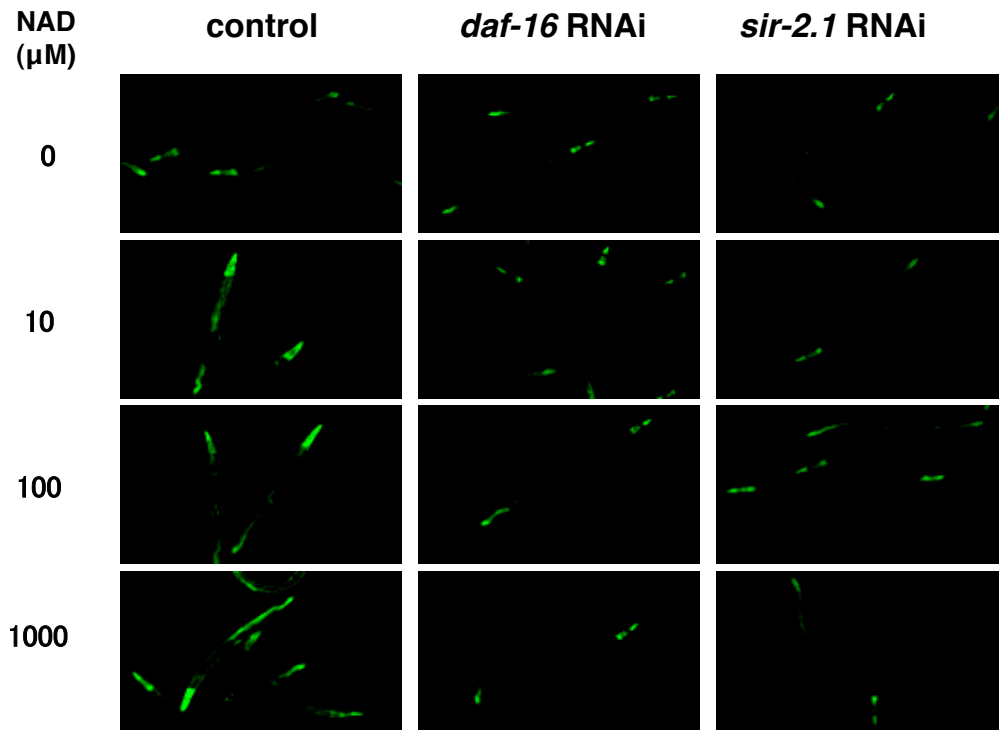

A
100
75
50
25
0
survival (%)
WT
eat-2(ad1116)
eat-2+NAD 100μM
eat-2+NAD 1000μM
0
10
20
30
40
50
Lifespan(days)
B
100
75
50
25
0
survival (%)
control
control+NAD
daf-16 RNAi
daf-16 RNAi+NAD
daf-16(mgDf50)
daf-16(mgDF50)+NAD
0
10
20
30
Lifespan(days)

Fig. 4

A


NAD
(μM)
control
daf-16 RNAi
sir-2.1 RNAi
0
10
100
1000

B

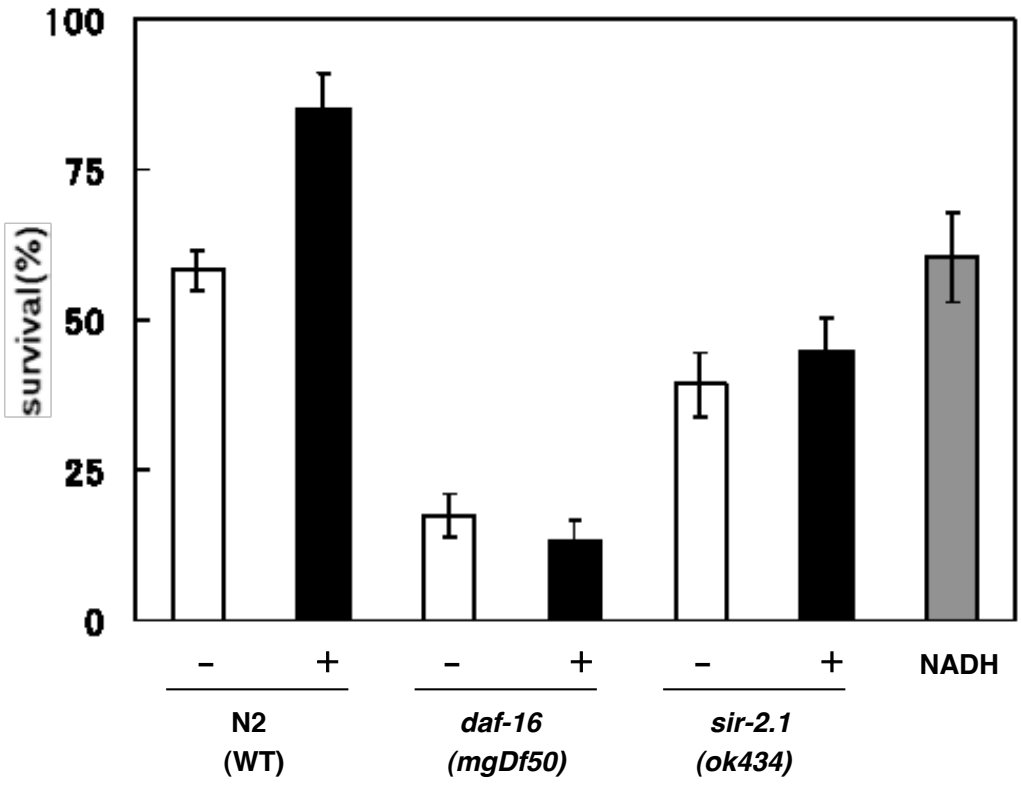
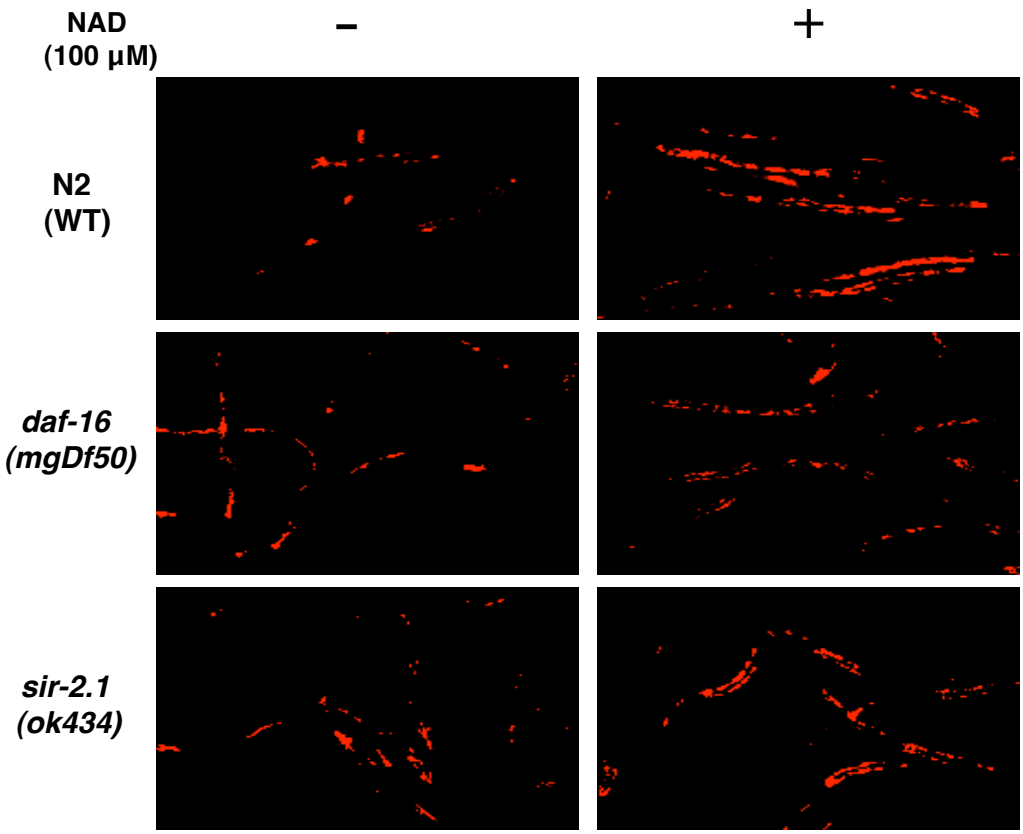

100
75
50
25
0
survival(%)
-
+
-
+
-
+
NADH
N2
(WT)
daf-16
(mgDf50)
sir-2.1
(ok434)

Fig. 5

A


NAD
(100 μM)
–
+
N2
(WT)
daf-16
(mgDf50)
sir-2.1
(ok434)

B

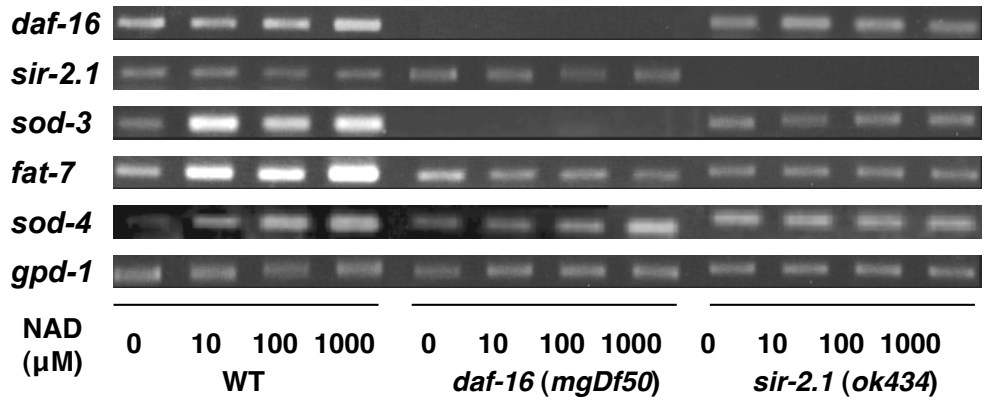
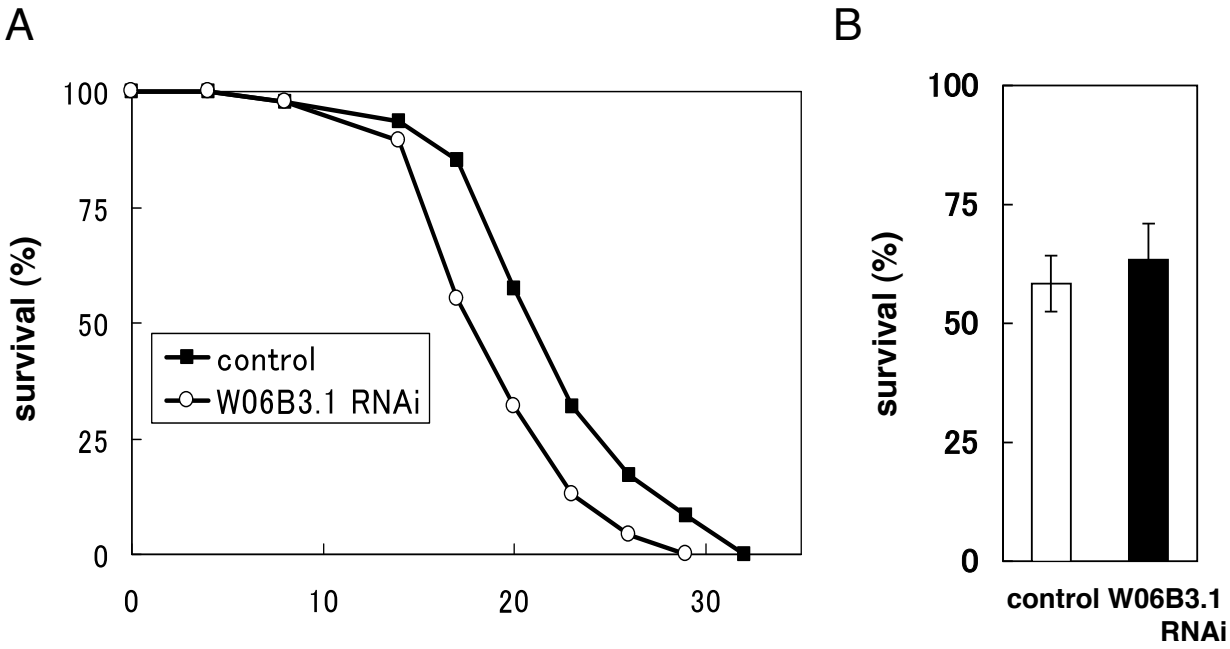

daf-16
sir-2.1
sod-3
fat-7
sod-4
gpd-1
NAD
(μM)
0 10 100 1000
WT
0 10 100 1000
daf-16 (mgDf50)
0 10 100 1000
sir-2.1 (ok434)

Fig. 6


A
100
75
50
25
0
survival (%)
control
W06B3.1 RNAi
0
10
20
30
B
100
75
50
25
0
survival (%)
control
W06B3.1 RNAi