A Dissertation presented

By

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REGULATION OF LIFE SPAN BY *DAF-16*/FORKHEAD TRANSCRIPTION FACTOR IN *CAENORHABDITIS ELEGANS*

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My journey was a fearful one.

I did not reach here until 4 o'clock yesterday morning.

Lacking horses the post-coach chose another route, but what an awful one

at the stage before the last I was warned not to travel at night.

I was made fearful of a forest, but that only made me the more eager

and I was wrong.

.

Yet I got some pleasure out of it,

as I always do when I successfully overcome difficulties

Now a quick change to things internal from things external.

.

My heart is full of so many things to say to you

- ah - there are moments when I feel that speech amounts to nothing at all.

Ludwig van Beethoven

- from a Love Letter

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감사의 글

가끔은 글이라는 것이 참 초라해 보일 때가 있다. 특히, 내가 표현하고 싶은 것이 많으면 많을수록, 그걸 담아내지 못하는 언어의 한계를 느끼곤 한다. 하물며, 영어로 쓴 감사의 글 따위에 나의 마음을 다 담기는 역시 역부족이란 생각이다.

박사학위 논문이라는 거창한 이름을 생각하면 뭔가 감동적이고 찡한 그런 감사의 글이 먼저 떠오르지만, 지금 내 머릿속엔 온통 지난 6 년동안 내가 살아온 시간들과 그 굴곡들이 떠오를 뿐이다. 그리고 그 시간들을 채워왔던 수많은 사람들과 기억들과 추억들이 지금 이 글을 쓰는 나의 마음을 빽빽히 메워버리고 있다. 무슨 배부른 소리냐고 할 지 모르겠지만, 사실상 내가 개인적으로 느끼는 학문적인 성취감이나 뿌듯함 뭐 그런건 그리 크지 않다. 그냥 난 내가 선택했던 지난 6 년의 시간들을 그저 무사히 마무리했다는 것에 감사할 뿐이다.

쉽지 않았던 20 대 후반과 30 대 초반을 보내면서, 어쩌면 나뿐만 아니라 모든 사람들이 비슷하게 느꼈을지도 모를 그런 감정들이 학위과정과 꽉 엇물려있다. 가끔 어떤 음악을 들으면, 그 음악을 들으며 떠났던 언제 어디로의 여행이 생각난다. 그 길에 함께 했던 사람들이 생각나고, 그 때의 날씨가 생각나고, 그 때 생겼던 추억이 생각난다. 지난 6 년동안의 수많은 기억들이 아마도 이 학위과정이라는 걸 평생동안 잊을 수 없는 한 편의 긴 음악으로 남게 해줄 것 같다.

처음으로 경험해본 외국생활.. 길게만 느껴졌던 대학원 생활.. 이 긴 시간동안 많다면 많았던 어려움들이 결과적으론 더 큰 어려움이 아닐 수 있게 도와준 많은 분들께 짧은 지면을 통해서나마 감사를 드리고 싶다. 이게 무슨 미스코리아 시상식이 아닌만큼.. 내가 아는 감사하지 않으면 안될 모든 사람을 거명하고 싶진않다. 다만, 지난 6 년의 시간.. 나의 삶에 힘이 되어준 사람들이 결국은 학위과정을 마치는데 도움을 준 사람들일 거란 생각에 그렇게 하고 싶을 뿐이다. 어쩌면 우리 지도교수나 같이 실험했던 실험들 동료들만큼이나 나의 이 보잘것 없는 논문을 완성시키는데 도움을 준 사람들일 거다.

먼저, 나의 가족들... 영어로도 많이 썼지만, 나에겐 항상 이런 좋은 분들이 나의 가족이라는 것이 즐거움이었고 행복이었다. 할머니, 어머니, 아버지, 장모님, 두 형과 동생, 두 처남, 처형.. 사랑하는 조카들.. 그 가족분들.. 그 모든 분들의 염려와 걱정.. 매일 매일 느낄 수 있는 그런 것은 아니겠지만, 모든 것의 밑바닥에서 보이지 않게 나를 떠받쳐준 힘이었음을 깨닫는다. 특히, 부모님들께는 이 학위논문이 단순한 논문이 아닌 훨씬 더 큰 의미를 지님을 잘 알기에, 모처럼만에 좋은 선물 하나 해드린거 같아서 즐거울 뿐이다. 사랑하는 가족 모두 다 항상 건강하고 행복하시길..

혜원이와 산하.. 나에겐 언제부턴가 모든 것의 의미가 되어버린 두 사람이다. Countless Love... 나같이 성격 별로 안좋은 사람이 받기에는 너무나 과분한 선물이었다. 그 사랑의 빚을 평생이라도 갚을 수 있을지... 모르겠다.

지난 6 년 동안 수많은 우여곡절 속에서도, 종종 힘들었던 나에게 큰 힘이 되어주었던 사랑하는 안디옥교회의 모든 분들.. 남들이 보기엔 어쩌면 한없이 초라한 모습의 조그마한 시골교회일 수도 있겠지만, 오히려 다른 ' 잘나가는 교회' 를 통해서는 절대 배울 수 없는 너무나 소중한 것들을 나에게 가르쳐준 교회.. 하나님의 사랑을 느끼게 해주었던 사랑들.. 이 학위 과정의 마지막 순간에 감사하지 않으면 안될 분들이다. 내 마음 속에 평생토록 남을 좋은 기억들을 주셨다.

이곳 우스터에서의 6 년동안 항상 곁에서 온갖 즐거움과 유희의 원천이 되어준 UMASS 에 같이 있었던 모든 한국분들께 감사드린다.

공부하려고 유학 온 나를 골프의 구렁텅이로 몰아넣은, 하지만 나에게 하나님을 알게 하신 정박사님(어색해!).. 형수님, 연우, 한나 모두 다 항상 행복하시길.. 나의 유학생활을 망친(!) 또 한 명의 주동자이자 ^^ 학교에서 본 시간보단 골프장에서 만난 시간이 훨씬 더 많았던 진균씨, 싱글칠려면 백스윙을 줄여야되.. 아짐마, 종헌, 종성 모두 건강하시길.. 역시 학교에서 본 시간보단 술먹으며 보낸 시간이 더 많은 성규씨, 성애씨.. 꼭 좋은 곳으로 job 잡아 가시고 준호와

함께 항상 행복하시길.. 마찬가지로 학교건물 안에서보단 잔디밭에서 노닥거린 시간이 더 많은 상묵씨.. 노닥거리다 골프치고 삼겹살에 쏘주.. 참 코스 좋았는데.. 정민엄마, 정민, 경환이 모두 행복하시길..

인간미 철철 넘치는 오박사님.. 오박사님 표현대로 이젠 제발 눈좀 깔고 결혼 좀 해야 될 차박사님 아니 Professor Cha.. 좋은데로 job 잡아 가신 정미영 박사님.. 바로 옆에서 많은 조언을 아끼지 않으신 심해홍박사님.. 일 년 찐하게 같이 논 유진이, 유진이엄마, 그리고 문제의 그 남편 손판.. 역시 또 좋은 동네로 job 잡아 가신 목소리의 마술사 성권씨와 현수씨.. 얼마전에 결혼해서 요새 한참 실험 안하고 있을 Tom.. 나처럼 벌레가지고 실험한, 역시 눈 깔고 시집좀 가야할 소영씨.. 옆집으로 이사와서 재밌게 놀았던 성휴씨, 지연씨, 이안이.. 그 외에 이 곳을 거쳐갔거나 있는 많은 UMASS 식구들..

돌아보면 scientific 한 기억은 거~의 떠올리기 힘들지만, 지치고 힘들 때마다 항상 나에겐 너무나 커다란 위안이었고, 즐거움이었고, 어떨땐 일주일을 사는 목표(!)가 되기도 했던^^.. 이 모든 분들께 정말로 감사하다는 말을 꼭 하고 싶다. 항상 건강과 기쁨이 함께했으면 좋겠다.

버클리 음대를 통해 알게 된 수많은 뮤지션들.. 나에게는 그들과 함께 들은 음악뿐만 아니라 그들 스스로가 너무나 신선한 감동이었고, 휴식이었고, 또 다른 설레임의 이유가 되어주었다.

Westborough 여관의 1 기 멤버들.. 도무지 다른 호칭이 어울리지 않는 나나언니와 요러스.. 항상 행복하시고, 그 둘 만큼이나 재밌는 2 세를 갖게 되길.. 한국을 대표하는 재즈피아노계의 거장이자, 정말 겁나게 웃기신 임여사 (본인은 그런거 알까 몰라).. 2 집은 부디 희귀음반 안되시길.. 힙합걸이자 북경소녀이자 나의 영원한 팬인 진영씨.. 좋은 남자와 행복하고 찐~한 사랑 나누길^^.. 한국 가스펠과 재즈계의 통합챔피언을 노리는 영주씨.. 보고있으면 무조건 기분이 좋아지는 분.. 부디 좋은 남자 만나시길.. 나의 세상과 음악을 바라보는 시각을 무지하게 넓혀준 사랑하는 미진씨.. 린든하고 오래오래 서로 사랑하길..

우리 여관 2기 멤버들.. 부부가 쌍으로 수준높은 코메디를 구사하는 범준씨, 인옥씨.. 보고있으면 그냥 미소짓게 만드는 좋은 사람들^^.. 두 분 항상 행복하시길.. 머리엔 머리띠, 얼굴엔 웃음 가득, 잊지못할 스키장의 추억을 함께 나눈 귀여운 민정씨.. 한국가서 귀여운 아들하고 남편하고 행복하고, 좋은 음악 하시길.. 서른 넘은 노처녀가 츄리닝바지만 입고 다녀서는 절대 남자 사귈 수 없다는 통념을 일순간에 무너뜨린 미경씨.. 감동의 여름 리싸이틀.. 그래도 인제 딴 거 입어.. 지난 2년동안 나에게 너무나 좋은 음악들을 들려주고 내가 재즈피아노를 사랑할 수 있게 만든, 나의 음악선생님 지선씨.. 꼭 좋은 뮤지션이 되시길..

위의 모든 분들.. 돈과 거리가 너무 먼 딴따라의 길을 가게 되겠지만, 자기의 감정을 자신만의 언어로 표현할 수 있다는 것.. 누구도 누리지 못하는 큰 행복이 아닐까 싶다. 미소와 눈물과 사랑과 그리움.. 그 모든 것들이 저 분들의 손을 통해 아름다운 예술이 될 수 있길.. 굶어죽지 않게 되길.. 이런 좋은 뮤지션들의 음악이 좀 더 그 가치를 인정받는, 그런 정도의 여유를 우리 사회가 빨리 갖게 되길...

그 외에 내가 참 힘들었던 시절.. 싸이질을 통해 나에게 숨쉴 수 있는 공간을 마련해준 나의 싸이 일촌들.. 위에 계신 분들과 중복이 많이 되지만, 그 분들께도 너무나 고맙다는 말을 하고싶다. 특히, 나를 싸이라는 구렁텅이로 인도한 가영이.. 이제는 그걸 고마워해야 될 거 같군.. 건강하고, 한백이, 신랑 모두 항상 행복하길.. 나의 석사때 대학원 동기이자, 나의 영원한 팬이자^^, 내가 너무나 아끼고 사랑하는 진호처자.. 포스닥 좋은 데로 가게됐으면 좋겠고, 고운 마음씨 만큼이나 아름다운 사랑 하게 되길..

그 외에도 항상 나에게 힘이 되어주고, 정말 형편없고 보잘것 없는 나를 아껴주시고 사랑해주셨던 많은 분들께 너무나 고맙다는 말을 건네며 긴 글을 맺는다.

ABSTRACT

The insulin/IGF-1 signaling pathway plays a pivotal role in life span regulation in diverse organisms. In *Caenorhabditis elegans*, a PI 3-kinase signaling cascade downstream of DAF-2, an ortholog of the mammalian insulin and insulin-like growth factor-1 (IGF-1) receptor, negatively regulates DAF-16/forkhead transcription factor. DAF-16 then regulates a wide variety of genes involved in longevity, stress response, metabolism and development. DAF-16 also receives signals from other pathways regulating life span and development. However, the precise mechanism by which DAF-16 directs multiple functions is poorly understood.

First, in Chapter II, we demonstrate that JNK is a novel positive regulator of DAF-16 in both life span regulation and stress resistance. Our genetic analysis suggests that the JNK pathway acts in parallel with the insulin-like signaling pathway to regulate life span and both pathways converge onto DAF-16. We also show that JNK-1 directly interacts with and phosphorylates DAF-16. Moreover, in response to heat stress, JNK-1 promotes the translocation of DAF-16 into the nucleus. Our findings define a novel interaction between the stress response pathway (JNK) and the master regulator of life span (DAF-16), and provide a mechanism by which JNK regulates longevity and stress resistance.

Next, in Chapter III, we focus on the downstream targets of DAF-16. Here, we used a modified chromatin immunoprecipitation (ChIP) method to identify direct target promoters of DAF-16. We cloned 103 target sequences containing consensus DAF-16

binding sites and randomly selected 33 targets for further analysis. The expression of majority of these genes is regulated in a DAF-16-dependent manner. Moreover, inactivation of more than 50% of these genes significantly altered DAF-16-dependent functions such as longevity, fat storage and dauer diapause. Our results show that the ChIP-based cloning strategy leads to greater enrichment of DAF-16 target genes, compared to previous studies using DNA microarray or bioinformatics. We also demonstrate that DAF-16 is recruited to multiple promoters to coordinate regulation of its downstream target genes.

In summary, we identified the JNK signaling pathway as a novel input into DAF-16 to adapt animals to the environmental stresses. We also revealed a large number of novel outputs of DAF-16. Taken together, these studies provide insight into the complex regulation by DAF-16 to control diverse biological functions and eventually broaden our understanding of aging.

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CHAPTER I

INTRODUCTION

I) Introduction to Aging Studies

a) Longevity and Aging

As people get older, they all face an inevitable question at certain point of their lives: "Am I aging? When and how will I die?" and then, "Why?" These have been the most fundamental questions throughout the entire human history, both philosophically and religiously. Despite numerous scientific progresses made in the last century, these questions have remained unsolved or even untouched for a long time. With the recent advances in biological science as well as the improvement of basic life needs, which have resulted in the remarkable increase in the elderly population, these questions started gaining more attention of biologists. Now, people try to answer these questions in a scientific manner and in fact, have slowly begun to understand this long-standing mystery. After all, 'Aging' is among the most frequently appearing words in scientific journals today.

Aging is the process of growing old or maturing. Biologically, aging is the cumulative changes in an organism, organ, tissue, or cell leading to a decrease in functional capacity, whereas senescence is generally restricted to the cellular level. In fact, there is not a well-defined aging process, rather there are multiple processes contributing to aging. Among the universally shared characteristics of aging, are the progressive deterioration of cells, tissues and organs, decline of the proliferative potential and metabolic activity, decreased fecundity and an increased risk of injury and death. In

humans, aging is also associated with degenerative changes in the skin, bones, heart, blood vessels, lungs, nerves, and other organs and tissues (Braeckman et al., 2001).

Life span has long been used as a measurement for aging since no other molecular marker for aging is established yet. Life span is defined as the length of the life of an animal or plant, whereas longevity can be interchangeably used for life span but is more often used to indicate an unusual long life. On the other hand, life expectancy is the number of years that an individual is expected to live as determined by statistics. Life span has dramatically increased over the last few centuries of human history. The average life span in the United States in 1901 was 49 years and has increased to 77 years by the end of the 20th century (more than 50% increase) (Encyclopedia, 2005). Similar gains have been observed throughout the world. These changes are largely due to the improvements in public health, medicine and nutrition, which mainly increased the numbers of people living beyond childhood, with less effect on overall life expectancy in the adult. However, many scientists believe that the life expectancy can be further increased by advances in medical research in future (Encyclopedia, 2005).

There are large variations in average life span worldwide, mainly due to the large differences in hygienic and nutritional status from country to country. There are also variations between races and sexes, with women outliving men. The occupation and living style can also make a significant difference to life expectancy. Currently, the longest-lived person authenticated lived 122 years 164 days. With natural diversity, the average life span differs greatly by species as well. Among animals, the giant tortoise lives about 177 years whereas the gastrotrich (a minute aquatic animal) lives as short as 3

days. Interestingly enough, even between two close species, the squirrel-like rodent lives two to three times longer than the mouse-like rodent. Among plants, the bristlecone pine of California has the greatest longevity, over 4,600 years (Encyclopedia, 2005).

Mortality rate is defined as the ratio of the number of deaths of a given age group to the total population in that age group. Mortality rates increase nearly exponentially in species when close to the average life span, but decrease at very advanced age (Figure 1). The common rigid pattern of mortality curve and the huge variation of life span among species have stimulated the search for a 'gerontogene', a gene that determines when an individual starts to age and dies (Sinclair et al., 1998).

b) Causes of Aging and Its Implication in Other Human Diseases

Whether such a 'gerontogene' exists has been a topic of debate for a long time and is still unclear. However, the recent advances in aging studies have suggested that the aging process is not as complex as once thought, but is quite accessible to molecular analysis and even inheritable. Life span has been shown to be 15 to 25% inheritable in humans (Miller, 2001) and as much as 18 to 79% inheritable in mice (Goodrick, 1975; Myers, 1978), indicating that both genetic and environmental factors affect the aging process and that it is subject to manipulation (not fixed). Many theories exist to explain why we age, and most of them agree that this process is largely determined by genetic factors (Aging Theories will be discussed later in this chapter). Thus far, more than 100 genes have been found to affect the life span of *Caenorhabditis elegans* (Patterson, 2003). Recent studies have shown that the life span of *C. elegans* and *Drosophila Melanogaster*

can be more than doubled by genetic manipulation (Table 1 and 2), and that mice harbouring similar mutant genes have been observed to have a considerable effect in life span extension as well as slowing down aging (Table 3). The genes affecting life span of model organisms are summarized in Table 1 to 3 (Aigaki et al., 2002; Quarrie and Riabowol, 2004). In addition, telomere maintenance and genomic instability have been suggested to contribute to the senescence and death of the cell. Recent studies suggest that they might be involved in the life span control of organisms as well (Hasty et al., 2003).

In addition to the genetic influences, environmental factors also have been observed to affect aging. For instance, the restriction of caloric intake (CR) can extend life span in diverse organisms, from yeast to mice (Guarente and Picard, 2005). Caloric restriction also postpones age-dependent deterioration and prevents number of age-related diseases in mice and primates (Longo and Finch, 2003).

Aging is also implicated in numerous major human diseases. Many age-related diseases, e.g. heart diseases, arteriosclerosis, cancer or neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, account for more than 60 % of natural human death today (Table 4). These diseases mostly develop after an individual ages to some extent and have been related to the age-dependent decline of important biological functions. For instance, basic processes involved in aging of the central nervous system contribute to several prevalent neurodegenerative diseases (Maccioni et al., 2001). The decline of proteasomal function with age well correlates with the accumulation of intra- or extra-cellular plaques, which is the common pathology in most of age-related diseases

(Merker et al., 2001). Therefore, better understanding of the aging process would also provide important clues to cure those late-onset human diseases. By slowing down the aging process, we might expect collective benefits that certainly exceeds the advance by curing individual disorders, and find ways to extend human life span (Figure 2) (Martin et al., 2003).

c) Model Systems

Several model systems have been used to study aging including yeast, worm, fly and mouse. First, mouse model systems are widely studied since they are likely to be more relevant for understanding aging-related processes that occur in humans, which makes mice the ultimate system to test various hypotheses on biological research. A wide variety of established inbred mouse strains also exist, many of which have been extensively characterized for background phenotypes and common pathologies (Silver, 1995). In addition, the mouse is the first mammalian model organism to have its genome completely sequenced. Although mouse models provide numerous advantages, several drawbacks limit general use of mice as a model system in aging study. First of all, relatively long life span (2 to 3 years) and low brood size makes it difficult to test number of emerging hypotheses. Mice are relatively expensive to maintain which is particularly important for aging studies since they need to be kept around for several years. In addition, mice are difficult to manipulate genetically compared to lower model organisms.

The budding yeast *Saccharomyces cerevisiae*, the roundworm *C. elegans* and the fruit fly *D. Melanogaster* have been used as useful systems in aging studies as they have

very short life span (approximately 4 hours for budding yeast, 16 days for worm and 40 days for fruit fly) and are readily amenable to genetic and molecular analyses. Moreover, the pattern of the mortality curves of these model systems are similar to that of human and major biological processes are highly conserved from yeast to human (Figure 1). In fact, genetic studies in yeast, *C. elegans*, and *Drosophila* have identified many genes that influence life span, which has led to insights into the relevant biochemical and physiological pathways (Warner, 2003).

S. cerevisiae is the easiest model organism to perform genetics studies with its simple biology and fully established genomic and proteomic tools. However, in yeast, life span is not measured by chronological age, but by the number of divisions that each mother cell can undergo (Tissenbaum and Guarente, 2002). Moreover, as a single cell organism, yeast is not suitable for testing systematic features of life span regulation, such as endocrinal regulation by insulin/insulin-like peptides.

Drosophila has many biological properties suitable for aging research, such as short life span, high brood-size, easy maintenance, and availability of genetic tools. However, a systematic analysis of longevity genes has not been executed thus far and relatively limited number of genes are known to regulate life span (Aigaki et al., 2002).

C. elegans is an excellent model organism and has been extensively used in aging research, largely due to its short and reproducible life span (approximately 16 days at 20°C), low maintenance cost and handiness of genetic manipulation. Life cycle of *C. elegans* is illustrated in Figure 3 (Braeckman et al., 2001). Worms develop from eggs through four larval stages, each separated by molt, and become adult hermaphrodites

within 3 days at 20 to 25°C. Life span is measured by number of days that worm lives (or practically, number of days that worm can respond to the tapping with the platinum wire pick). Since the entire genome is sequenced and many molecular and genetic tools are available including RNA interference library, large number of genome-wide screening has been performed to identify the genes that affect aging in *C. elegans*. A number of genes that affect *C. elegans* life span have already been isolated and successfully shown to affect life span in other model systems as well.

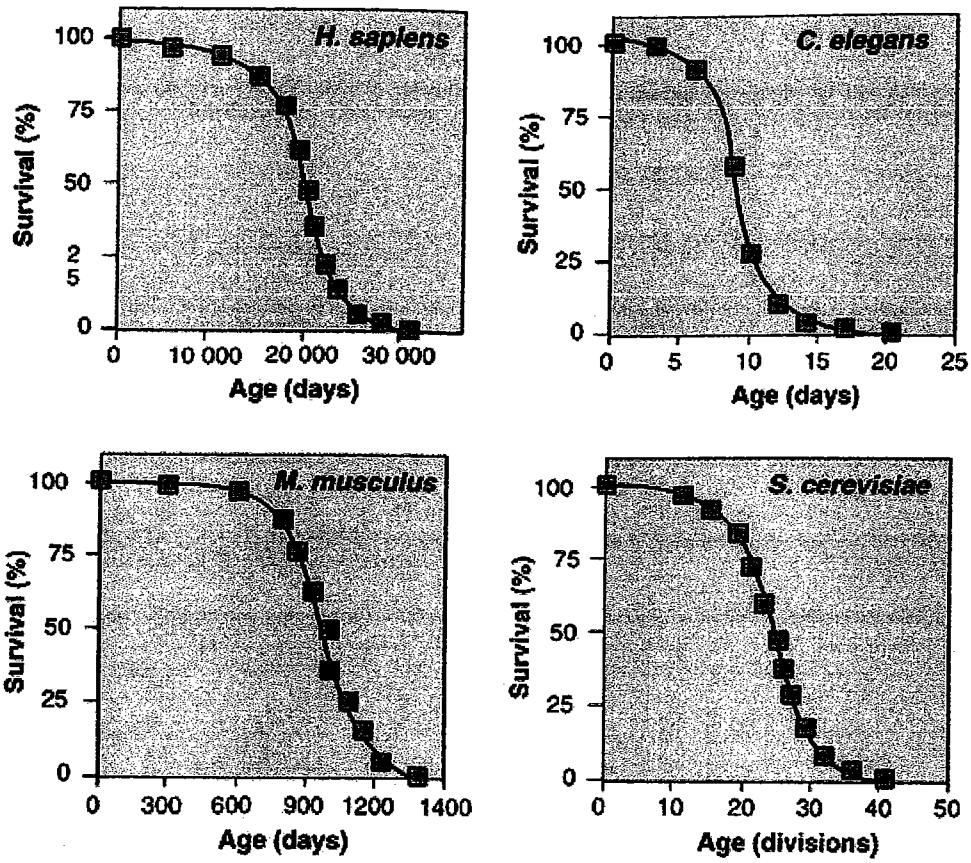


Figure 1. Mortality curves of various species (Sinclair et al., 1998).

Gene	Biological function	% increase in life span	Type of mutation
daf-2	Insulin-like receptor	>100	hypomorph
age-1	PI 3-kinase	40	hypomorph
clk-1	Coenzyme Q synthesis	20-90	hypomorph
eat-2	Pharyngeal pumping	50	hypomorph
isp-1	Electron transport (complex III)	65	hypomorph

Table 1. Genes extending life span in *C. elegans*. The percent increase in life span is measured at 20°C. The life span increase is variable according to the growth temperature and mutant alleles used. For example, *age-1* can extend life span at 25°C up to 65% and *clk-1;clk-2* double mutant can augment life span increase by 90% of wild-type (Arantes-Oliveira et al., 2003; Feng et al., 2001; Friedman and Johnson, 1988; Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998b).

Mutation/gene	Gene product	Percent increase in longevity	Type of mutation	References
Mth	G-protein coupled receptor	35	Hypomorph	Lin et al. (1998)
Indy	Cotransporter	100	Hypomorph	Rogina et al. (2000)
Chico	Insulin receptor substrate	48	Null	Clancy et al. (2001)
InR	Insulin-like receptor	50	Hypomorph	Tatar et al. (2001)
Cu/Zn SOD, Catalase	Cu/Zn SOD and Catalase	33	Overexpression	Orr and Sohal (1994)
hsp70	Molecular chaperone	7.9	Overexpression	Tatar et al. (1997)
Cu/Zn SOD	Cu/Zn SOD	48	Overexpression	Sun and Tower (1999)
DPOSH	Scaffold protein	14	Overexpression	Seong et al. (2001a,b)

Table 2. Genes extending life span in *Drosophila* (Aigaki et al., 2002).

System affected	Mouse strain	Mutation	Regular function	Life span extension	Other phenotypes	References
Pituitary	Ames	*prop1*$^{-/-}$	Transcription factor in anterior pituitary embryonic development	40-50%	Dwarfism (1/3 normal size); stunted growth; lowered core body temperature; GH,TSH, prolactin, IGF-1, insulin deficiencies; lowered IRS-2-associated PI3K activity; increased expression of Cu/Zn SOD and catalase; variable fertility in males but females infertile (treatable with prolactin); delayed reproductive maturity; retarded age-related increases in bone mineral density and percent body fat; hypersensitive glucose response; reduced or delayed tumour development, renal pathology, changes in collagen, and declines in immune function, locomotor activity, learning and memory; CR further extends life span	*43, 52-59*
	Snell	*pit1*$^{-/-}$	Pituitary-specific transcription factor	40-50%	Dwarfism (1/3 normal size); stunted growth; GH,TSH, prolactin, IGF-1, insulin deficiencies; lowered IRS-2-associated PI3K activity; preferentially form IRS-2-p85α-p110α complexes over IRS-2-p85α-p110β; most symptoms of decreased aging seen in Ames mice; also slower immune, joint, and connective tissue senescence; increased resistance to UV, heat, ROS, toxic metal-induced stress	*52, 53, 60, 61, 63*
GH/IGF-1 axis	GH tg	*GH* overexpression	Growth and development	Decreased	Shortened reproductive life span; increased astrogliosis and plasma corticosterone levels; early onset of aging-related effects on cognitive function	*61*
	Little	*ghrhr*$^{-/-}$	Release of GH	23-25% when on low-fat diet	Dwarfism (1/2 normal size); incomplete GH deficiency; IGF-1 insufficiencies especially in the peripheral circulation; normal TSH and prolactin levels	*52, 64, 65*
	Laron	*GHR*$^{-/-}$ *GHBP*$^{-/-}$	Growth hormone receptor	37-55%	Dwarfism (1/2 normal size); GH-resistant; very low to undetectable IGF-1 levels; greater circulating GH levels and elevated prolactin production; lowered plasma insulin and glucose levels; augmented insulin responsiveness and lowered glucose tolerance; low thyroid hormone levels; lowered core body temperature; fertile but delayed reproductive maturity; deficient reproductive function and endocrine control of the gonads; delayed age-related cognitive decline	*54, 56, 64, 66-69*
	IGF-1R	*IGF-1R*$^{-/-}$	IGF-1 receptor	26% (overall) 33% (females) 16% (males; change is not significant)	Virtually no growth effect (6-8% size reduction); no effect on metabolism, fertility, development; regular onset and decline of reproductive maturity; absence of age-related diseases; life span extension not significant in males; augmented resistance to oxidative stress in both genders but especially in females; unaltered metabolism and caloric intake; 50% reduced phosphorylation of IRS-1, p66Shc, p52Shc; 50% reduced p66Shc binding of Grb2, ERK1/2, Akt; homozygous KO lethal	*65, 76-78*
	p66shc	*p66shc*$^{-/-}$	IGF-1R effector	30% (approx. equivalent to 40% reduction in caloric intake)	Normal size, development, and fertility; incapable of being serine-phosphorylated upon SOS binding due to cellular oxidative stress; differential promoter histone acetylation and methylation; p21 down-regulated; resistant to UV and ROS but not gamma irradiation; catalase overexpression; reduced apoptosis; increased vascular fitness	*70-74, 78*

Table 3. Genes extending life span in mouse (Quarrie and Riabowol, 2004).

Table 3 (continued).

System affected	Mouse strain	Mutation	Regular function	Life span extension	Other phenotypes	References
Oxidative metabolism	$\Delta IR_{adipose}$	*IR*$^{-/-}$ (adipose tissue only)	Insulin receptor	18%	No effect on caloric intake or metabolism; reduced fat mass, less age-related obesity, fewer obesity-related metabolic abnormalities; indicates that some effects of CR may be separable from those of leanness	*75, 83-88*
	Peroxiredoxin	*Prdx1*$^{-/-}$	Antioxidant enzyme	Significantly reduced	Develop severe hemolytic anemia and several types of malignant cancers at ~9 months	*96*
	Thioredoxin (TRX-Tg)	*TRX* overexpression	Electron donor for peroxiredoxin	22-35%	Increased resistance to oxidative stress	*94, 95*
	Bcl-2 tg DC	*Bcl-2* overexpression in dendritic cells	Anti-apoptotic and antioxidative effects	Increased DC longevity	Elevated $CD4^+$ T cell and humoral immune responses	*97, 98*
Other	Klotho	*Klotho*$^{-/-}$	Membrane protein of unknown function; homologous to β-glucosidases and a mammalian hydrolase	Greatly decreased (<100 days)	Many aging-associated phenotypes: decreased activity, infertility, osteoporosis, arteriosclerosis, skin atrophy, emphysema; greatly reduced subcutaneous fat; atrophied thymus; slightly atrophic GH-, FSH- and LH-secreting cells in pituitary; lowered pancreatic insulin; lowered glycogen/lipid storage	*91-93*
	p53 mut	Increased expression/ activity	Tumor suppressor	Decreased 17-19%	Decreased cancer incidence; premature aging phenotypes: osteoporosis, generalized organ atrophy, diminished stress tolerance	*133-136*
	XPD TTD	R^{722}→W mut	5'→3' DNA helicase; part of NER pathway	Decreased >50%	Premature aging phenotypes: osteoporosis, osteosclerosis, kyphosis, cachexia, early graying, infertility, early cessation of development, cachectic dwarfism	*139*

FORMAL NAME	INFORMAL NAME	% ALL DEATHS
(1) Diseases of the heart	heart attack (mainly)	28.5%
(2) Malignant neoplasms	Cancer	22.8%
(3) Cerebrovascular disease	Stroke	6.7%
(4) Chronic lower respiratory disease	emphysema, chronic bronchitis	5.1%
(5) Unintentional injuries	Accidents	4.4%
(6) Diabetes mellitus	Diabetes	3.0%
(7) Influenza and pneumonia	flu & pneumonia	2.7%
(8) Alzheimer's Disease	Alzheimer's senility	2.4%
(9) Nephritis and Nephrosis	kidney disease	1.7%
(10) Septicemia	systemic infection	1.4%
(11) Intentional self-harm	Suicide	1.3%
(12) Chronic Liver/Cirrhosis	liver disease	1.1%
(13) Essential Hypertension	high blood pressure	0.8%
(14) Assault	Homicide	0.7%
(15) All other causes	Other	17.4%

[Source: National Vital Statistics Report, Volume 53, Number 5 (October 2004)]

Table 4. Causes of human death in USA, 2002

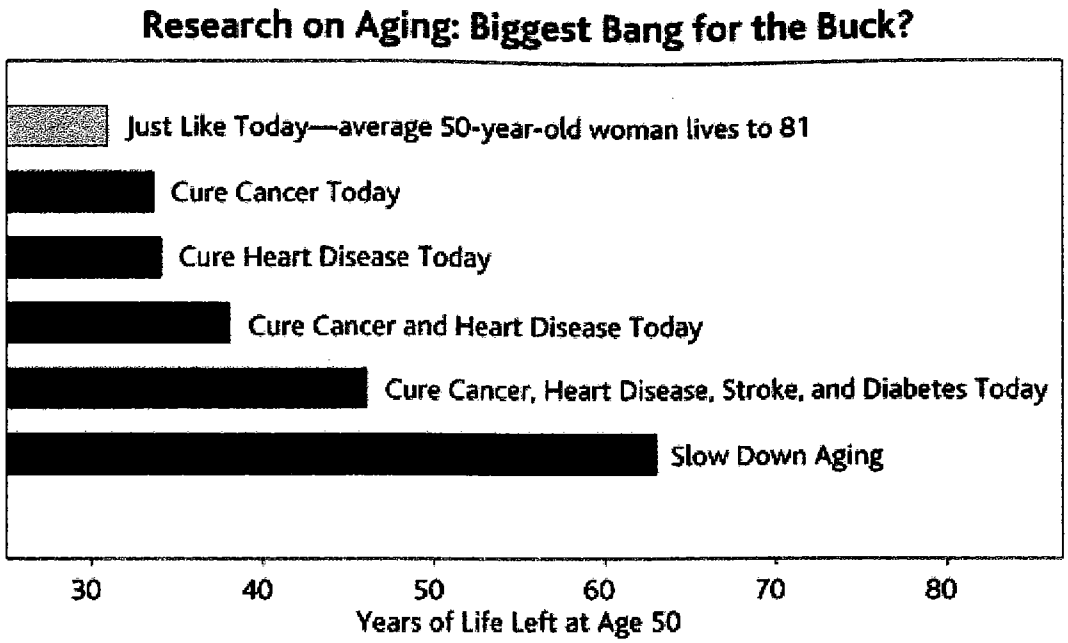


Figure 2. Impact of aging study on human life expectancy (Martin et al., 2003).

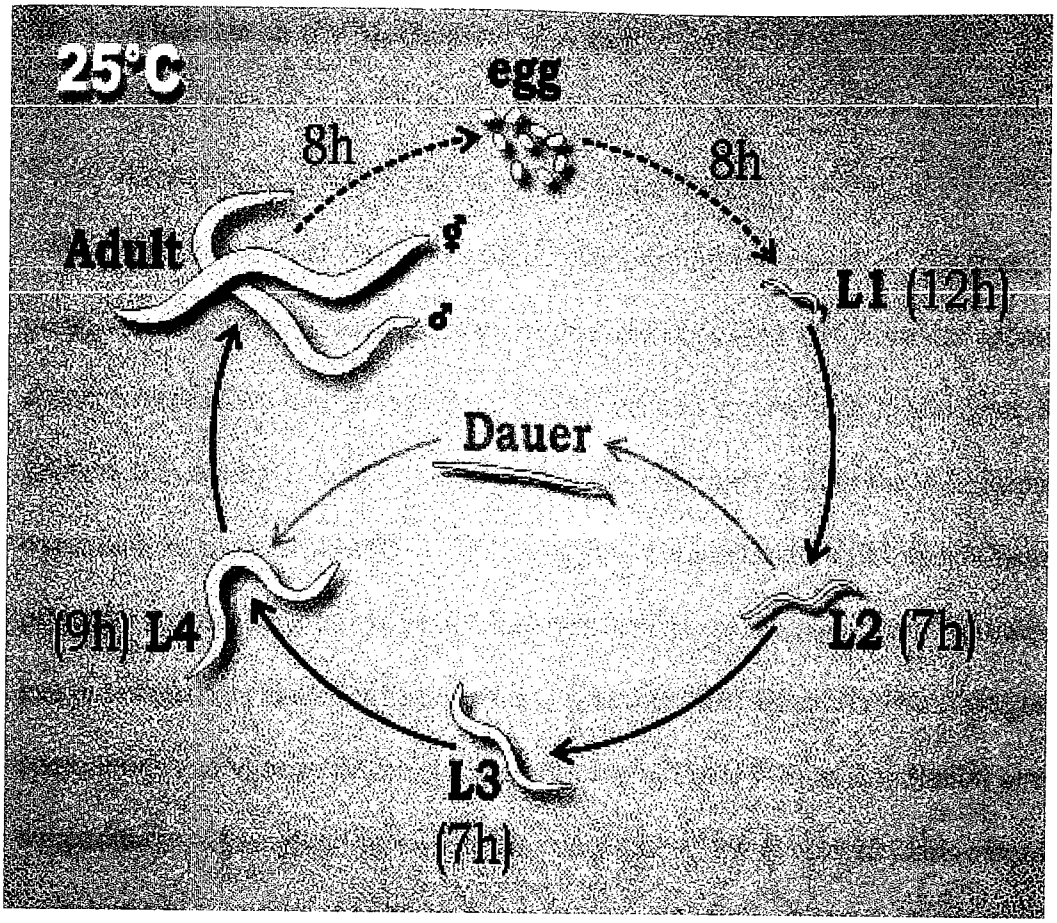


Figure 3. Life cycle of *C. elegans* at 25°C (Braeckman et al., 2001).

II) Theories on the Mechanism of Aging

a) Free Radical Theory and Metabolic Rates Theory

The free radical theory originally proposed by Harman *et al.* (Harman, 1956) postulated that free radical reactions are the cause of aging and therefore accumulation of damage by reactive oxygen species (ROS) is critical in determining life span. ROS are generated in multiple compartments by multiple enzymes within the cell. Important contributions include NADPH oxidases in plasma membrane, lipid metabolism within the peroxisomes and various cytosolic enzymes such as cyclooxygenases. Additionally, exogenous factors can contribute to the generation of ROS, such as oxidant generating compounds and various physical influences including UV-light, irradiation or elevated temperature. Although all these sources contribute to the overall oxidative burden, the mitochondria is responsible for the vast majority of cellular ROS (estimated at approximately 90%) (Balaban et al., 2005). The generation of mitochondrial ROS is a consequence of oxidative phosphorylation, a biological process that oxidizes NADH or FADH, to generate a potential energy for protons across the mitochondrial inner membrane for ATP production. Along the electron transport chain (ETC), electrons derived from NADH or FADH can directly react with oxygen or other electron acceptors to generate free radicals (Figure 4).

ROS generated inside the cell can be climinated by several ROS scavenging enzymes such as supcroxide dismutase (SOD), catalase, peroxiredoxin (Prx) and glutathione (GSH) (Figure 4) (Balaban et al., 2005). Either increase in ROS production or

decrease in scavenging activity results in residual intracellular ROS, which have detrimental effects on various cellular processes for its highly reactive chemical property. ROS can directly damage mitochondrial and nuclear DNA, leading to activation of p53 and other DNA damage pathways and eventually cell death. ROS can also oxidize and denature proteins and lipids directly (Balaban et al., 2005). In the past, the generation of ROS or other free radicals was only considered as an unproductive and toxic side reaction. More recently, it has been proposed that mitochondrial ROS may actually be important in various redox-dependent signaling processes as well (Dada et al., 2003; Nemoto et al., 2000; Werner and Werb, 2002).

Studies in mammalian cell culture have shown many evidences supporting the role of ROS as an important determinant of the life span. Cellular senescence has long been used to uncover the molecular mechanisms of aging in organisms. Lowering the ambient oxygen concentration can significantly extend the life span of primary cells in culture (Packer and Fuehr, 1977). Similar extension of cellular life span was achieved by augmenting antioxidant levels such as SOD (Serra et al., 2003). Conversely, knockdown of SOD using RNAi was demonstrated to induce senescence (Blander et al., 2003).

The link between mitochondrial metabolism and longevity is also supported by several studies in *C. elegans*. One of the long-lived mutants, *isp-1*, has a defect in a component of complex III of the ETC and shows a reduction in oxygen consumption (Feng et al., 2001). Another long-lived mutant, *clk 1*, lacks an enzyme required in the biosynthesis of ubiquinone (Ewbank et al., 1997), also known as coenzyme Q, an important and well-conserved electron acceptor for both complex I- and II-dependent

respiration. Long-lived *clk-1* mutants accumulate a precursor of coenzyme Q and show a decrease in cytoplasmic ROS levels (Shibata et al., 2003). Interestingly, the withdrawal of coenzyme Q from the diet of wild-type worms can increase life span by 60% (Larsen and Clarke, 2002). Similarly, a systematic RNAi screen revealed that the large fraction of genes affecting life span was implicated in the mitochondria metabolism (Lee et al., 2003b). Conversely, a short-lived mutant, *mev-1* was initially isolated for its increased sensitivity to the ROS generator paraquat (methyl viologen). Subsequent studies have demonstrated that the mutation maps to a subunit of complex II (Ishii et al., 1998). *mev-1* mutants have significant mitochondrial structural abnormalities and indirect evidence of increased ROS generation (Senoo-Matsuda et al., 2001). This idea is also supported by observations in mice that are heterozygous for mitochondrial superoxide dismutase (Sod2), one of the ROS scavenging enzymes. These mice demonstrate an increased incidence of nuclear DNA damage as well as a significant increase in tumor formation (Van Remmen et al., 2003).

A theory very closely related to the free radical theory is the concept called the rate of living hypothesis. This theory stems from the observation that species with higher metabolic rates have shorter maximum life span potential and age faster (Smeal and Guarente, 1997). The basic assumption that more energy consumption by mitochondria would result in larger amount of toxic ROS production linked the free radical theory and the rate of living theory. Evidences supporting this theory also exist in simple model organisms. Growing worms or flies at a lower temperature slows the metabolic rate and

also results in a concomitant extension of life span (Miquel et al., 1976). Similarly, in yeast, the reduction of glucose in the media results in life span extension.

Thus far, the free radical theory along with the metabolic rates theory seems to be the most widely accepted aging theory. However, it still remains unclear whether ROS causes or merely correlates with aging and what governs the relationship between overall metabolic rate and the production of ROS.

b) Telomere Shortening Theory

Another theory is the 'telomere-shortening theory'. Telomeres are the repetitive sequence and specialized proteins located at the ends of linear eukaryotic chromosomes, protecting them from degradation, fusion, and recombination. The DNA sequences at the end of linear chromosomes are not replicated as same as the rest of the genome, but are synthesized by an telomere maintaining enzyme, telomerase, that creates random variation in the number of repeats of the telomeric DNA sequence (McEachern et al., 2000). The telomere and its maintenance have been suggested to contribute to aging process since Hayflick et al. proposed this hypothesis in 1975 (Hayflick, 1975). Telomere length of somatic cells tends to decrease as individuals age as shown in the studies comparing young and old human fibroblast (Iwama et al., 1998). Cellular senescence has been closely related to a progressive reduction in the number of telomeric repeats. In contrast, overexpression of the telomerase can prevent human cells in culture from undergoing senescence (Bodnar et al., 1998). In accordance with this observation,

overexpression of HRP-1, a telomere-binding protein, which gradually increase telomere length, extends the life span of *C. elegans* (Joeng et al., 2004).

Recent studies in human also support this theory of aging. The telomere length has been found to correlate with longevity and disease resistance in humans (Cawthon et al., 2003). Moreover, defects in genes responsible for human progeria (accelerated aging), such as WRN (Werner's syndrome) and ATM (ataxia telangiectasia), exacerbate premature aging phenomenon in telomerase dysfunctional mice (Chang et al., 2004; Wong et al., 2003). These suggest that telomere maintenance is involved in human aging.

Additionally, the ROS and genomic instability have connection with the telomere shortening. It has been shown that oxidative stress modulates the shortening rate of telomeres and induces telomere single-strand breaks followed by losses of bigger parts of the telomere in cell division (Sitte et al., 1998). The telomere also participates in DNA repair and the aging phenotype in telomerase defective mice often manifests with genome maintenance defects (Hasty et al., 2003). However, it is still controversial whether telomere shortening can be a general cause of aging since there also exist some contradictory findings: Mice age and die with long telomeres and loss of telomerase in mice has no effect on aging for several generations (Blasco et al., 1997; Kenyon, 2005).

c) Other Theories

The 'somatic mutation theory' proposed by Harman, Szilard and Muller focuses on somatic mutations in DNA. These mutations gradually accumulate non-functional proteins that do not only fail to fulfill their essential functions, but also produce

detrimental outcomes. The accumulation of mutations leads to structural disabilities and finally to the death of the organism (Merker et al., 2001).

Another theory is the 'lipofuscin theory of aging'. Lipofuscin is a non-degradable granule which becomes more abundant with age and in some diseases. It is characterized by three different compounds: lipids (20–50%), proteins (30–50%) and hydrolysis resistant residues (10–30%) (Yin, 1996) and is thought to consist of the membranes of cells, that are mainly composed of proteins and phospholipids. According to this theory, aging is the result of various side-reactions to essential biological processes. For instance, free radical-induced peroxidation, protein cross-linking, nucleotide modifications and other biological side reactions lead to the formation of age-pigments, and contribute to the aging process.

W. Denckla proposed the 'death-hormone theory'. In this theory, there exists an aging hormonal factor and this factor dictates the aging process. However, supporting data is greatly insufficient (Denckla, 1975).

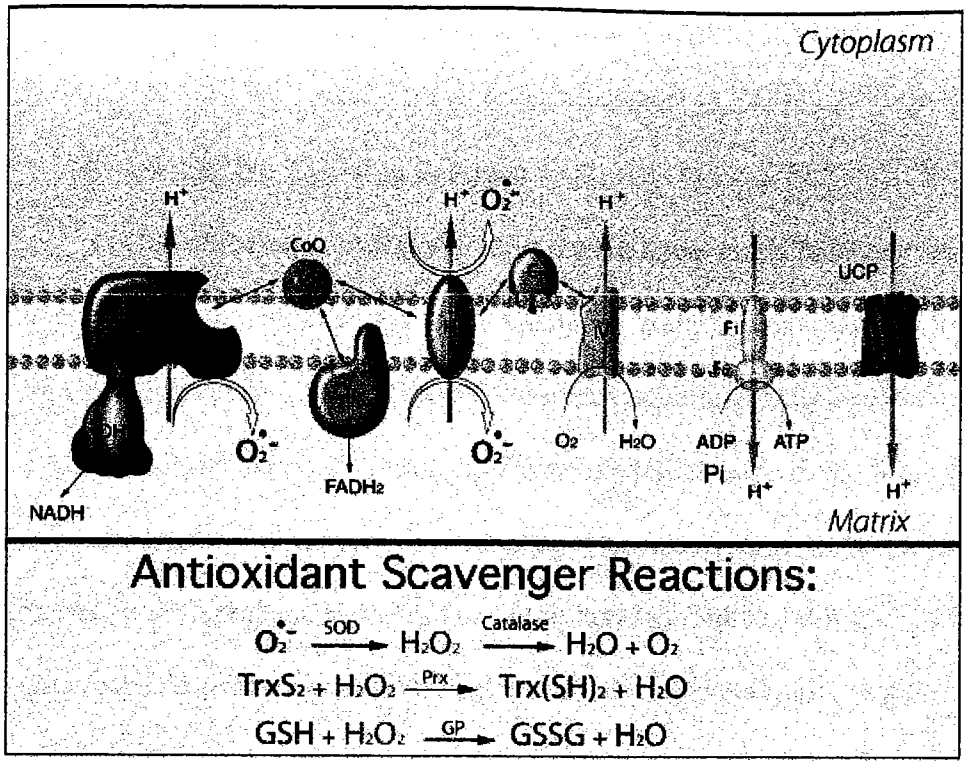


Figure 4. ROS production by the mitochondrial electron transport chain. The scavenging pathways include superoxide dismutase (SOD), catalase, thioredoxin (Trx), peroxiredoxin (Prx) and glutathione (GSH) (Balaban et al., 2005).

III) Genetic Pathways Regulating Longevity in *C. elegans*

a) Insulin-like Signaling Pathway

One of the most prominent advances in aging study has been made about a decade ago when strains of *C. elegans* with mutations in *daf-2* were found to live exceptionally long (Kenyon et al., 1993). The *daf-2* mutants were originally isolated by their dauer-constitutive phenotype. Dauer is an alternative larval stage in the life cycle of *C. elegans* to be designed to survive harsh conditions. Under normal growth conditions, worms go through four larval stages to become adult hermaphrodites. However, when worms face unfavourable environment such as scarce food, overpopulation or elevated temperature during early developmental stage, they undergo dauer diapause to enter this dormant stage and wait until growth conditions become improved. Thus far, more than 15 reduction-of-function mutations of *daf-2* were isolated and shown to increase life span (Gems et al., 1998). Among those, *daf-2(e1370)* allele, one of the strongest hypomorphic allele, can extend life span up to 2- to 3-fold of wild-type worm and even further when *daf-2* RNAi was used concomitantly (Arantes-Oliveira et al., 2003). A few years after this breakthrough finding, *daf-2* gene was cloned in *C. elegans* and named as insulin-like receptor due to its homology to both mammalian insulin receptor and insulin-like growth factor-1 (IGF-1) receptor (Kimura et al., 1997). Analyses of downstream genes of *daf-2* revealed that the whole insulin/IGF-1 signaling pathway is well conserved in nematode and that the *C. elegans* insulin-like pathway (named after *daf-2*, insulin-like receptor) is a key element in life span regulation as well as dauer formation (Figure 5).

The activity of the insulin-like signaling pathway is regulated by insulin-like ligands that bind to the insulin/IGF-1-like receptor DAF-2. The *C. elegans* genome contains 37 insulin-like ligands that are mainly expressed in neurons, but are also found in intestine, muscle, epidermis, and gonad (Kawano et al., 2000; Pierce et al., 2001; Tatar et al., 2003). Activation of the receptor results in auto-phosphorylation and subsequently the phosphorylation of the PI-3-kinase heterodimer, which is composed of the regulatory p85 subunit AAP-1 and the catalytic p110 subunit AGE-1 (Morris et al., 1996; Wolkow et al., 2002). The PIP_2 and/or PIP_3 formed recruit AKT-1, AKT-2, SGK-1 and PDK-1 to the plasma membrane where PDK-1 activates the AKT and SGK-1 kinases by phosphorylation (Alessi et al., 1997; Hertweck et al., 2004; Paradis and Ruvkun, 1998). AKT and SGK-1 then phosphorylate and sequester the DAF-16/forkhead transcription factor (FOXO) in the cytosol (Figure 6) (Hertweck et al., 2004; Lin et al., 2001; Oh et al., 2005). However, in the absence of ligands or with a mutation in one of the genes in this pathway, DAF-16 is released from the phosphorylation-mediated suppression and translocates into the nucleus (Figure 6) (Henderson and Johnson, 2001; Lin et al., 2001). Once DAF-16 enters nucleus, it is assumed that DAF-16 binds to and transactivates numerous genes involved in life span regulation, stress response, dauer formation and metabolism.

In accordance with this molecular mechanism, the reduction-of-function mutations of the upstream genes (*daf-2, age-1, aap-1, pdk-1, sgk-1* and *akt-1/2*) that down-regulate DAF-16 result in life span increase (Friedman and Johnson, 1988; Hertweck et al., 2004; Kawano et al., 2000; Kenyon et al., 1993; Lin et al., 1997; Murphy

et al., 2003; Ogg et al., 1997; Oh et al., 2005; Pierce et al., 2001; Wolkow et al., 2002). In contrast, a null allele of *daf-16(mu86)* completely abolishes life span extension by mutations of upstream genes as well as decreases life span slightly but significantly on its own (Figure 5 and 7) (Lin et al., 1997; Ogg et al., 1997). Until very recently, the insulin-like signalling pathway was the only known pathway to modulate DAF-16 activity to regulate life span. The downstream targets of DAF-16 were also largely unknown (will be discussed later in this chapter).

b) Caloric Restriction

Caloric restriction (CR) has remarkably broad effects on increasing life span and attenuating chronic diseases of aging in diverse organisms. McCay's pioneering work 60 years ago showed that CR increases life-span by about 35% and also results in a lower incidence of tumors, kidney disease, vascular calcification, and chronic pneumonia (Longo and Finch, 2003). Monkeys show similar effects of CR on glucose and insulin, as well as indications of reduced mortality (Roth et al., 2001). In a small group of healthy non-obese humans, CR also causes physiologic, hormonal, and biochemical changes resembling those caused by CR in rodents and monkeys (Walford et al., 2002).

In *C. elegans*, mutation in *eat* reduces pharyngeal pumping (feeding) rate and thus confers caloric restriction (Figure 7). Some *eat* mutants extend life span by up to 50%. Moreover, the severity in pharyngeal function well correlates with life span increase, which makes this mutant a great model for CR (Lakowski and Hekimi, 1998a). Several observations in axenic medium (synthetic growth medium without bacteria) also indicate

that calorically restricted worms have advantage in longevity; life span in axenic medium is twice as long as on agar plate with bacterial lawn (Houthoofd et al., 2002). In contrast, life span decreases considerably with increasing food supply (bacterial concentration) (Hosono et al., 1989; Klass, 1977). Interestingly, *eat-2*; *daf-2* double mutants live longer than either single mutant and life span extension by *eat-2* is not suppressed by *daf-16* null mutation, which completely suppresses that by insulin-like signalling pathway mutants (Lakowski and Hekimi, 1998a). It indicates that *eat-2* (caloric restriction) extends life span in a manner independent of the insulin-like signaling pathway.

c) Mitochondrial Mutation

As previously described in the free radical theory, the toxicity of ROS is thought to accelerate aging. Consistent with this, growth in low oxygen concentration extends the life span of *C. elegans,* but shortens in higher oxygen concentration (Honda et al., 1993). In the same context, several mitochondrial genes have been shown to have significant effect on longevity (Figure 7). The first isolated mitochondrial long-lived mutant, *clk-1* has a defect in the biosynthesis of coenzyme Q, an electron acceptor for both complex I- and II in ETC (Lakowski and Hekimi, 1996). Another long-lived mutants, *isp-1*, defective in a iron sulfur protein of complex III of ETC, has been isolated from a genetic screen for Clk-like gene (Feng et al., 2001). On the other hand, a short-lived *mev-1* mutant has a defect in the enzyme succinate dehydrogenase cytochrome b, component of complex II (Ishii et al., 1998).

A systematic RNAi screen also identified group of mitochondrial genes associated with longevity (Lee et al., 2003b). 15% of genes that extended life span upon RNAi inactivation were assigned to mitochondrial function, half of which are directly involved in the electron transport chain. Another independent study also showed that inactivation of genes functioning in ETC by RNAi extended life span (Dillin et al., 2002). The long-lived worms with impaired mitochondrial function in these studies show lower ATP content and oxygen consumption as well as apparent morphological abnormality in mitochondria.

The mechanism underlying life span extension by mitochondrial genes seems to be more complicated. It is generally accepted that these mitochondrial mutants or inactivation of mitochondrial ETC genes by RNAi increase life span by modulating ROS level produced by mitochondria. Consistent with this idea, RNAi inactivation of those genes and *isp-1* mutant show significant reduction in respiration rate, assessed by measuring ATP content and oxygen consumption. Thus, decreased mitochondrial activity would produce less ROS. In accordance with this observation, a short-lived mutant, *mev-1*, is thought to generate more ROS and highly sensitive to oxidative stress. However, *clk-1* mutants show a normal level of respiration but decrease in ROS. Moreover, *isp-1* and *clk-1* mutants have normal body size and fertility whereas RNAi inactivation of ETC genes generally results in severe developmental defects such as small body size, sterility and structural abnormality of mitochondria. Interestingly, RNAi inactivation of ETC genes in adulthood lowers ATP contents but does not increase life span any longer whereas inactivation in larval stages are sufficient to extend life span (Dillin et al., 2002).

Therefore, it is suggested that mitochondrial function in early development might play a critical role in life span determination (Hekimi and Guarente, 2003).

In addition, *clk*-1 mutants and RNAi of ETC genes further extend the life spans of *daf-2* mutants, but *isp-1* mutants do not. However, the life span extension by all these genes seems to be *daf-16* independent since it is only partially suppressed by *daf-16* null mutation and still extends life span significantly compared to the wild-type in *daf-16* mutant background. Taken together, it is difficult to infer a common mechanistic view of life span regulation by these genes as of today.

d) Sensory Perception

C. elegans senses environmental signals through ciliated sensory neurons located in head (amphid neurons) and tail (phasmid neurons). Many mutants have been isolated by their defects in sensory perception (response to the food/chemotaxis) in these sensory organs and shown to be long-lived (Figure 7) (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). This life span extension is largely *daf-16* dependent (Lin et al., 2001). Moreover, a number of sensory neurons seem to secret insulin-like peptides (Pierce et al., 2001). Therefore, it is suggested that the sensory perception may affect longevity by controlling the activity of the insulin-like signalling pathway. Interestingly, each subsets of sensory neurons, such as gustatory neuron and olfactory neuron, seems to play a distinct or even an opposite role in life span regulation (Alcedo and Kenyon, 2004). Whether the sensory perception can control life span of higher eukaryotes is unknown (Kenyon, 2005).

e) Germline Signaling

The reproductive system has been demonstrated to influence the life span of *C. elegans* (Figure 7). When the germ line precursors are removed by laser ablation, life span is extended by 60% (Hsin and Kenyon, 1999). This is not simply due to sterility because removal of the entire reproductive system (germ line plus somatic gonad) has no effect on life span. The germ line signaling acts through insulin-like signalling pathway since life span extension by germ line ablation is dependent on *daf-16*. This life span extension also requires the nuclear hormone receptor DAF-12 and DAF-9, a cytochrome P450 homolog involved in the synthesis of a DAF-12 ligand (Gerisch and Antebi, 2004). Interestingly, the somatic gonad must be intact in order for germ line ablation to extend life span. However, germ line ablation further extends life span of *daf*-2 mutants as long as 4-fold of wild-type regardless of presence of the somatic gonad. Thus, somatic gonad may signal through DAF-2 to counterbalance germ line signal that regulate DAF-16 probably in parallel to DAF-2. Further study showed that neither oocytes nor sperm are required for the germ line to extend life span, but preventing germ line stem cell division in the adult extends life span (Arantes-Oliveira et al., 2002). In addition, the germ cell ablation causes DAF-16 nuclear localization primarily in the intestine/adipose tissue (Lin et al., 2001), and the activity of DAF-16 in these tissues is sufficient to account for the entire life span extension produced by removal of the germ line (Libina et al., 2003).

Studies in higher eukaryotes also demonstrate that the reproductive system influences life span. When the ovaries of either young or old mice are transferred into

age-matched ovariectomized mice, life span is not affected. However, when young ovaries are transplanted into old recipients, life span is extended 40%–60% (Cargill et al., 2003). Reproductive signaling influences *Drosophila* life span as well. A mutation that kills oocytes extends life span, as does low-dose X-irradiation, which kills germ cells (Sgro and Partridge, 1999). The life span regulation by the reproductive system suggests the coordination between the rate of aging and the timing of reproduction. This probably ensures that the animals are in their prime time when it reproduce (Kenyon, 2005). However, the mechanism underlying this regulation by reproductive system is not clear.

f) Others

Sir2:

A silent information regulator (Sir) gene is originally known by its function in gene silencing in yeast. Providing additional copies of yeast Sir2, a histone deacetylase, can extend yeast life span (Guarente and Picard, 2005). Similarly, the overexpression of Sir2 orthologs extends life span in both worms and flies (Figure 7) (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). In *C. elegans*, genetic analysis suggests that *sir-2.1* extends life span by functioning through the *daf-2* insulin-like signaling pathway. Interestingly, Sir2 has a NAD-dependent histone deacetylase activity. Thus, Sir2 might sense and respond to the metabolic state of organisms. Moreover, life span extension by caloric restriction (CR) requires Sir2 activity both in yeast and *Drosophila,* suggesting a potential link between Sir2 and CR. However in *C. elegans*, it is not clear whether *sir-2.1* can mediate the life span extension by CR since the life span extension produced by *sir-2*

overexpression is *daf-16* dependent, whereas that produced by CR (i.e. in case of *eat*-2) is not. In mammals, SirT1, a Sir2 ortholog, has many intriguing roles in the regulation of metabolism and hormone signaling. Recent evidences show that SirT1 deacetylates FOXO and modulate the downstream targets of FOXO (Brunet et al., 2004; Motta et al., 2004). It is also speculative that SirT1 might be involved in life span extension by CR in mammals since SirT1 expression is induced by CR in mice and its activity is required for cultured mammalian cells to undergo additional divisions following CR (Guarente and Picard, 2005; Kenyon, 2005).

TOR signalling:

The highly conserved target-of-rapamycin (TOR) protein kinases control cell growth in response to nutrients and growth factors. The TOR kinase phosphorylates ribosomal S6 kinase and translation initiation factor 4E binding protein 1, an inhibitor of the eukaryotic initiation factor 4E, in response to nutrients, which in turn promotes growth (Inoki et al., 2005). *daf-15* (raptor, TOR-interacting protein) and *let-363* (CeTOR) mutants extend life span (30%) in *C. elegans* (Figure 7) (Jia et al., 2002; Kapahi et al., 2004; Ramsey et al., 2002). Interestingly, the *daf-15* transcription is regulated by DAF-16, indicating that the insulin-like signaling and nutrient signaling coordinate to regulate C. elegans life span. Mutations that decrease TOR activity also extend the life span in *Drosophila* (13%–30%) (Jia et al., 2002; Kapahi et al., 2004; Ramsey et al., 2002). Moreover, the life spans of the mutant flies cannot be further extended by CR, suggesting that TOR signalling is involved in the life span regulation by CR.

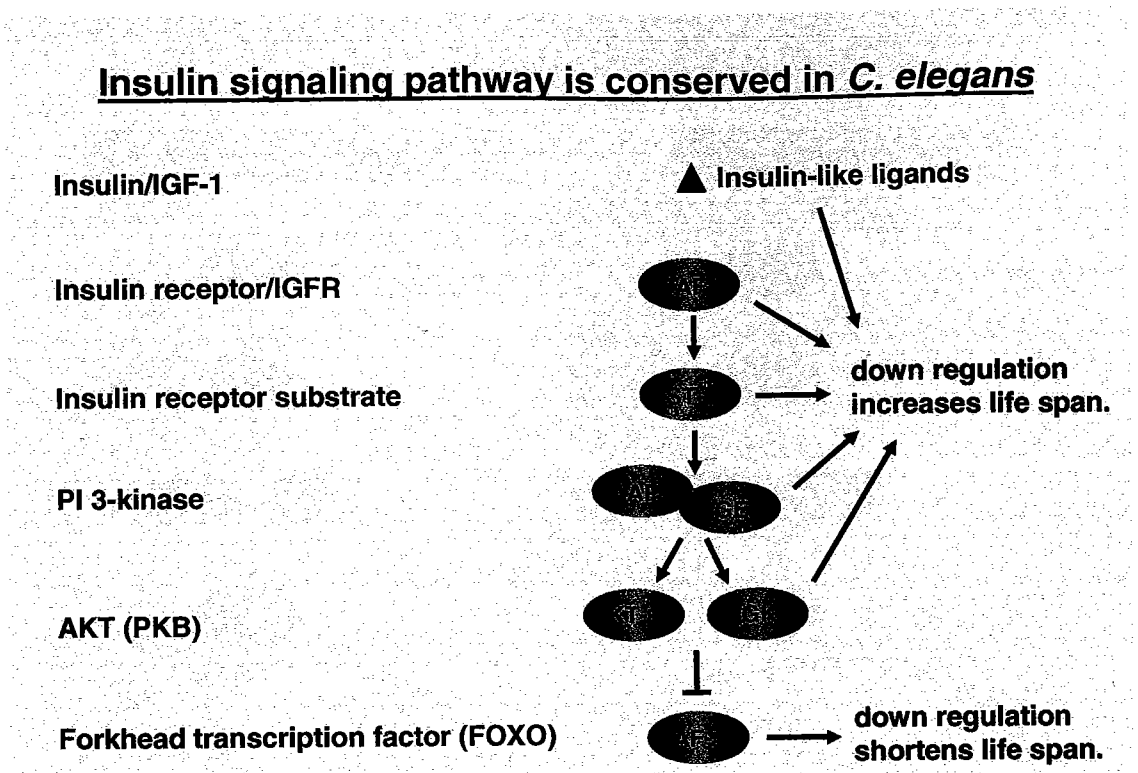


Figure 5. Life span regulation by the insulin-like signalling pathway.

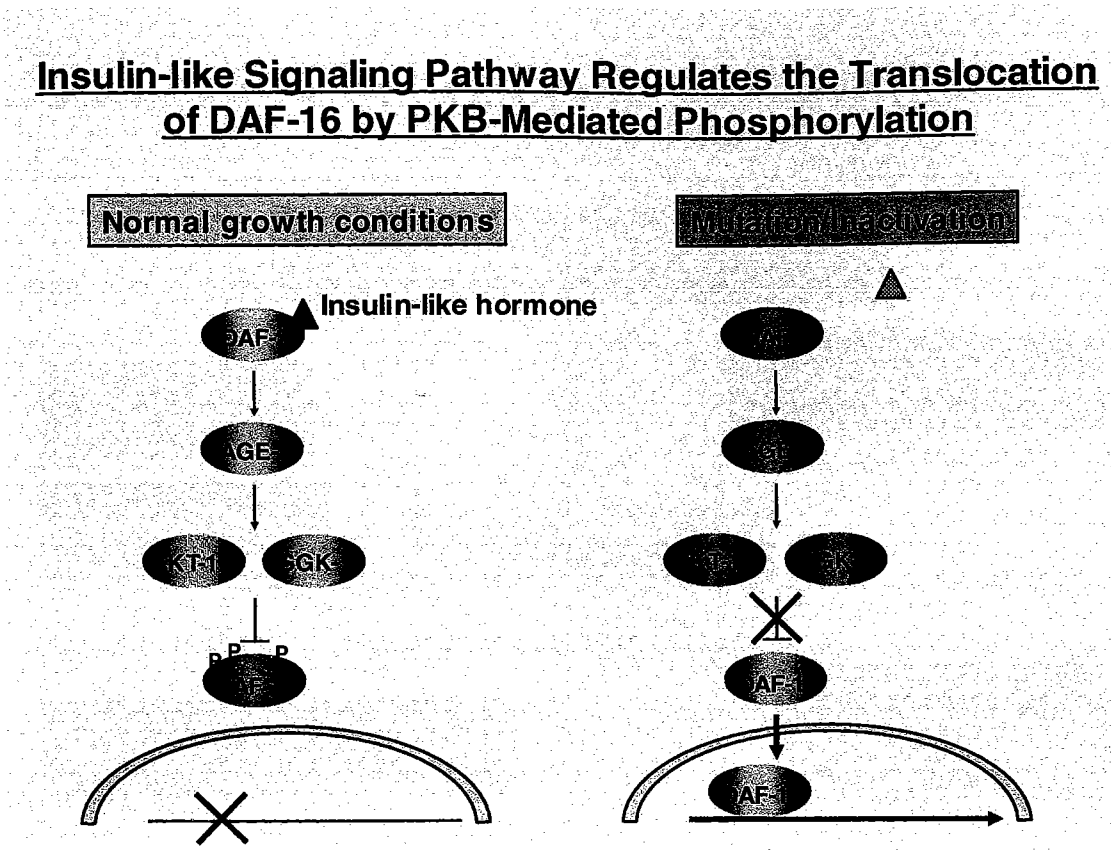


Figure 6. Regulation of DAF-16 by the insulin-like signalling pathway.

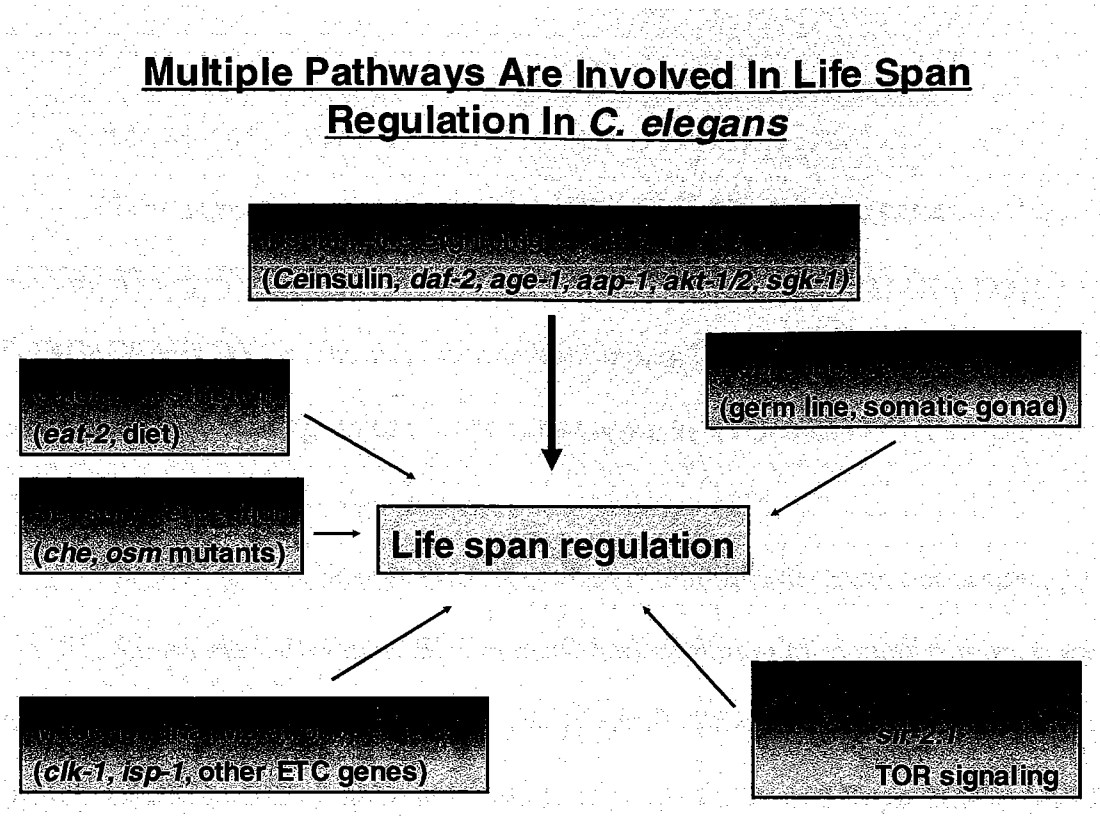


Figure 7. Genetic pathways regulating life span in *C. elegans*.

IV) The Role of DAF-16/FOXO in Regulation of Life Span

a) Regulation of DAF-16 Activity

The forkhead transcription factors (FOX factor) include the proteins AFX, FKHR and FKHR-L1; FKHR is now known as FOXO1, FKHR-L1 as FOXO3a and AFX as FOXO4. The FOXO is a transcription factor binding DNA as a monomer and the consensus DNA-binding sequence has been known as TTG/ATTTAC (Furuyama et al., 2000).

The FOXOs are phosphorylated *in vivo* on multiple threonine and serine residues (T1, T2, S1, S2 and S3) (Figure 8). Three of these residues (T1, S1 and S2) are known as consensus phosphorylation sites by protein kinase B (PKB)/Akt and shown to be phosphorylated by PKB both *in vitro* and *in vivo* (Burgering and Kops, 2002). A PKB-mediated phosphorylation on these consensus residues inhibits transcriptional activity of the FOXO members. A detailed molecular mechanism for this inhibition is suggested in mammal. As illustrated in Figure 9, The FOXO has the DNA-binding domain (DBD) and the nuclear-localization signal (NLS). FOXO proteins shuttle continuously between the nucleus and the cytosol with the help of nuclear transporter proteins to facilitate this shuttling such as α- and β-importins (import) and exportins like Crm1 (export). Under conditions of low PKB activity (e.g. by serum deprivation in culture or low insulin level *in vivo*), the rate of import exceeds that of export to leave FOXO proteins predominantly in the nucleus. Upon activation of PKB (e.g. by serum addition in culture or high insulin level *in vivo*), PKB phosphorylates FOXO proteins on consensus phosphorylation sites

(T1, S1 and S2) probably in the nucleus, and phosphorylated FOXO proteins have increased binding affinity to 14-3-3 protein, which results in the release of the FOXO protein from the DNA and relocalization to the cytosol. Following translocation to the cytosol, the bound 14-3-3 protein prevents re-entry of FOXO into the nucleus by masking the NLS and inhibiting importin binding until FOXO proteins are modified (e.g. dephosphorylation) (Burgering and Kops, 2002). Although all three FOXO members harbors a putative NLS and NES, little is known about its *in vivo* function and the importin that binds to the NLS remains unidentified.

The regulation of the transcription activity of FOXO can also be achieved by other ways than translocation. First, the binding of the 14-3-3 protein to phosphorylated FOXO proteins can inhibit binding to the target promoter DNA (Cahill et al., 2001). Second, the phosphorylation might dampen FOXO interaction with cofactors that are essential for transcriptional activation such as the recently described histone acetylase p300/CREB-binding protein (CBP) (Nasrin et al., 2000).

The activity of DAF-16/FOXO can be regulated by other mechanisms than PKB-mediated phosphorylation as well. Several observations in mammal revealed that the transcriptional activity of FOXO is subject to the deacetylation/acetylation status on its lysine residues, controlled at least in part by Sir2 homologs, Sirtuins (SirT1-7) (Burgering and Kops, 2002). The expression of SirT1 causes deacetylation of FOXO which result in transcriptional up- or down-regulation of its downstream targets (Brunet et al., 2004; Motta et al., 2004). These results are consistent with the observation in *C. elegans*, suggesting that DAF-16 is downstream of SIR-2.1 (Tissenbaum and Guarente,

2001). The functional consequences of FOXO deacetylation remain unclear. The expression of *p27kip*, a well-studied FOXO target in the regulation of cell cycle progression is suppressed by SirT1-mediated deacetylation (Motta et al., 2004). However, other study showed that the expression of pro-apoptotic genes (Fas ligand and BIM) is inhibited, whereas that of genes that promote survival (GADD45 and MnSOD) is increased by SirT1 (Brunet et al., 2004).

Although the Sir2/SirT1-mediated deacetylation of FOXO has been generally thought to decrease its activity, there is an opposite case in *C. elegans* where increased dosage of SIR-2.1 appears to lead to increased activity of DAF-16 (Tissenbaum and Guarente, 2001). Thus far, it is not clear whether the functional result of deacetylation of mammalian FOXO proteins is different from that of nematode DAF-16, or whether deacetylation by Sir2 homologs can lead to either gain-of function or loss-of-function of FOXO/DAF-16. On the other hand, although FOXO is largely known as a transcriptional activator, there are examples of FOXO-dependent transcriptional suppression (our unpublished data, elaborated in Chapter III) (Kitamura et al., 2002). Therefore, the molecular consequence of increased activity of FOXO should be interpreted with caution.

b) Downstream Targets and Physiological Roles of DAF-16/FOXO

At least in mammal, a variety of genes are known as transcriptional targets of FOXO: genes involved in the oxidative stress response (*MnSOD*), DNA repair (*GADD45*), cell-cycle arrest (*p27KIP1*) and apoptosis (*BIM* and *Fas ligand*) (Birkenkamp and Coffer, 2003; Van Der Heide et al., 2004). Studies in mammalian cell

have shown that the overexpression of FOXO members generally induces either cell cycle arrest or apoptosis. The FOXO proteins are also implicated in tumorigenesis. The FOXO members have been found as a part of chromosomal translocations in certain types of tumors. In addition, the inactivation of FOXO (cytosolic localization of FOXO) is suggested to be an important part of the mechanism of transformation in PTEN (tumor suppressor, DAF-18 in worm) negative renal and prostate carcinoma cells (Burgering and Kops, 2002). However, the *in vivo* role of FOXO proteins remains unclear since apoptosis, one of the most ascribed results of higher FOXO activity, do not occur in transgenic mice overexpressing constitutively active FOXO (Nakae et al., 2002), and most of the FOXO studies are restricted to certain types of cell lines.

In *C. elegans*, DAF-16 seems to play distinct roles from mammalian FOXO. DAF-16 has caught attention of many biologists for its role in life span regulation. Both in *C. elegans* and *Drosophila*, the activity of DAF-16, controlled by components of upstream insulin signalling pathway, well correlates with longevity phenotype of animals. The down-regulation of upstream genes results in long-lived phenotype, which is completely dependent on DAF-16 or DFOXO activity (Kenyon, 2005). Until recently, only a few genes had been suggested to be DAF-16 targets from individual studies on each gene in *C. elegans*: superoxide dismutase (*sod-3*), transmembrane tyrosine kinase (*old-1*), metallothioneine (*mtl-1*), catalase (*ctl-1*), SCP-like extracellular protein (*scl-1*) and heat shock proteins (*hsp-12.6, hsp-16.1, hsp-16.49*) (Hsu et al., 2003; Lithgow and Walker, 2002; Munoz, 2003; Ookuma et al., 2003). They are based on the requirement of intact DAF-16 for their expression and/or phenotypic correlation of deletion or

overexpreesion of those genes with mutants in insulin-like signaling pathway. These genes are generally implicated in the stress response, supporting the connection between stress resistance and longevity.

To ultimately answer how insulin-like signaling pathway regulates life span, more systematic analyses have been performed and identified array of genes as putative downstream targets of DAF-16. A combination of cDNA microarray and RNAi library has led to the identification of a number of downstream targets of DAF-16 whose expression level is dependent on DAF-16 (McElwee et al., 2003; Murphy et al., 2003). In another study, the functional significance of a set of genes with consensus DAF-16 binding site in the promoter was assayed for their involvement in DAF-16 dependent phenotypes (Lee et al., 2003a). Many of these genes influenced life span including: antioxidant genes such as superoxide dismutase, metallothioneine, catalase, and glutathione S-transferase, metabolic genes including apolipoprotein genes, glyoxylate-cycle genes, and genes involved in amino acid turnover, and small heat shock protein genes, and antibacterial genes. Some of antioxidant genes and heat shock protein genes were previously suggested to be downstream targets of DAF-16 and important regulators of life span (Kenyon, 2005). The antibacterial genes are also interesting since bacterial proliferation contributes to the death of *C. elegans* (Garigan et al., 2002; Gems and Riddle, 2000), and *daf-2* mutants are resistant to pathogens (Garsin et al., 2003). These results generally agree with the concepts that increase in cellular defence results in life span extension. Likewise, mammalian FOXO proteins activate stress-response genes when insulin or IGF-1 levels are reduced (Kops et al., 2002; Nemoto and Finkel, 2002;

Tran et al., 2002). It also suggests that insulin-like signaling pathway/DAF-16 is a master regulator for longevity and control a wide variety of downstream genes with diverse functions that act together in a cumulative fashion to regulate life span. Many of these downstream genes might influence longevity in humans as well.

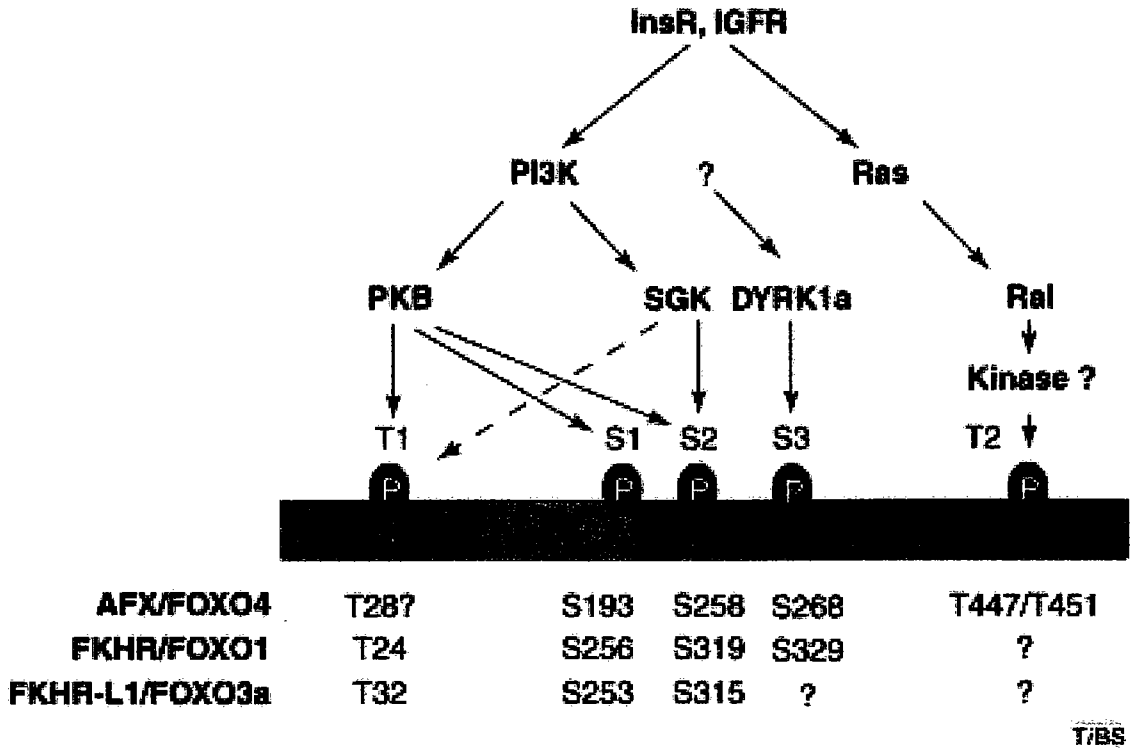


Figure 8. Phosphorylation of FOXO by upstream protein kinases (Burgering and Kops, 2002).

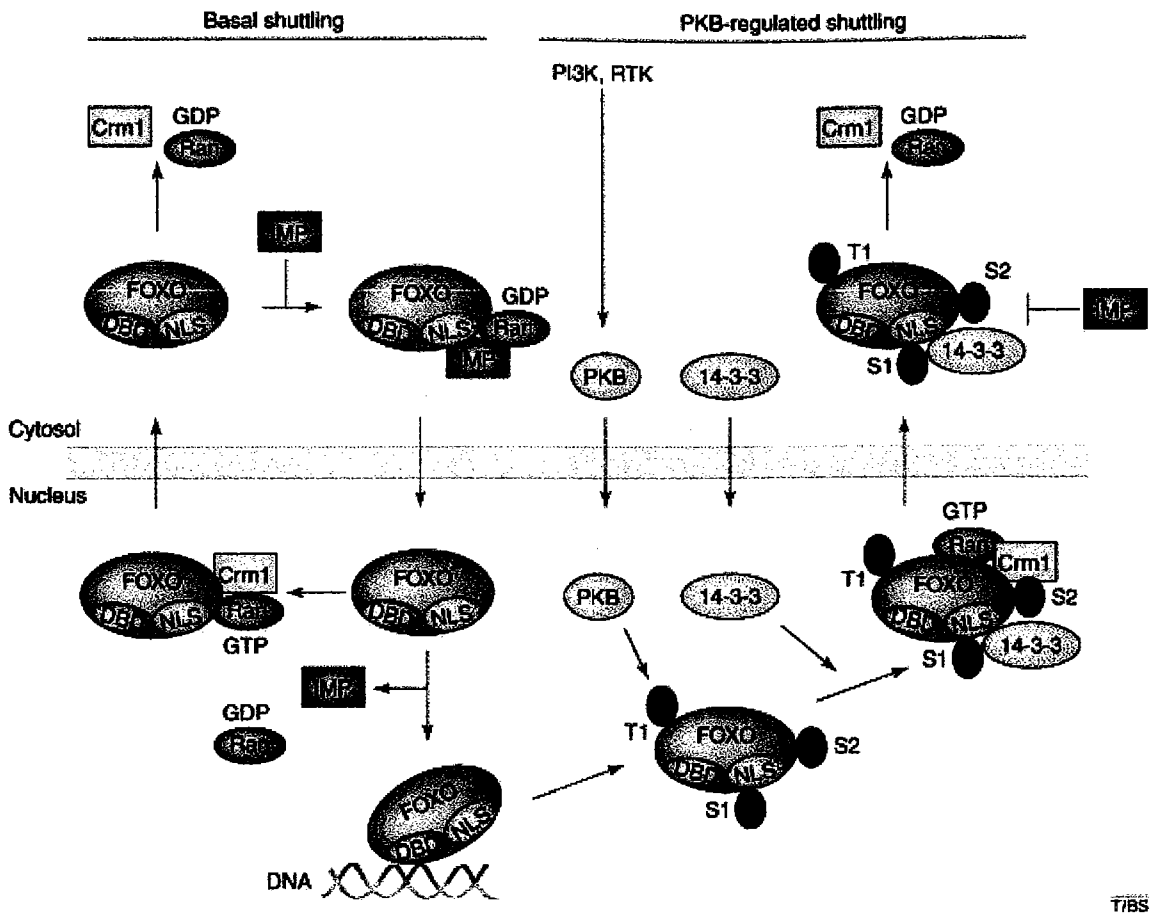


Figure 9. Shuttling of FOXO between the nucleus and the cytosol (Burgering and Kops, 2002).

CHAPTER II

JNK REGULATES LIFESPAN IN *CAENORHABDITIS ELEGANS* BY MODULATING NUCLEAR TRANSLOCATION OF FORKHEAD TRANSCRIPTION FACTOR/DAF-16

I) Abstract

DAF-16/FOXO transcription factor, the downstream target of the insulin-like signaling in *C. elegans*, is indispensable for both life span regulation and stress resistance. Here we demonstrate that JNK is a novel positive regulator of DAF-16 in both processes. Our genetic analysis suggests that the JNK pathway acts in parallel with the insulin-like signaling pathway to regulate life span and both pathways converge onto DAF-16. We also show that JNK-1 directly interacts with and phosphorylates DAF-16. Moreover, in response to heat stress, JNK-1 promotes the translocation of DAF-16 into the nucleus. Our findings define a novel interaction between two well-conserved proteins, JNK-1 and DAF-16, and provide a mechanism by which JNK regulates longevity and stress resistance.

II) Introduction

The insulin signaling pathway plays a pivotal role in life span regulation and stress resistance in many diverse organisms (Barbieri et al., 2003). In *C. elegans*, the PI 3-kinase signaling cascade downstream of *daf-2,* an ortholog of the mammalian insulin and insulin-like growth factor-1 (IGF-1) receptor (Kimura et al., 1997), targets DAF-16/FOXO transcription factor (Lin et al., 1997; Ogg et al., 1997). DAF-16 then regulates a wide variety of genes involved in longevity, stress responses, metabolism and development (Lee et al., 2003a; Murphy et al., 2003). In mammalian systems, other signal transduction pathways including the c-Jun N-terminal kinase (JNK) signaling pathway have been shown to couple to the insulin/IGF-1 pathway (Aguirre et al., 2000; Kim et al., 2003; Kim et al., 2002).

The JNK family, a subgroup of the MAPK superfamily, is part of a signal transduction cascade that is activated by cytokines, including TNF and IL-1, and by exposure to environmental stresses (Davis, 2000). The JNK pathway has been implicated in critical biological processes such as cancer, development, apoptosis and cell survival (Davis, 2000). In mammalian cell culture, components of the JNK signaling pathway have been shown to interact with the insulin signaling pathway through interaction with either the insulin receptor substrate-1 (IRS-1) (Aguirre et al., 2000) or the AKT protein kinase (Kim et al., 2003; Kim et al., 2002). A recent observation in *Drosophila* also suggested that the JNK signaling pathway is involved in life span regulation and stress resistance, but the mechanism of JNK signaling was not established (Wang et al., 2003).

Therefore, we investigated the role of the JNK signaling pathway in life span regulation in *C. elegans* and its possible connection to the insulin-like signaling pathway.

III) Results and Discussion

Several orthologs of JNK pathway components have been characterized in *C. elegans* including: a mammalian JNK ortholog, *jnk-1*, two MKK4/7 (JNK kinase) orthologs, *jkk-1* and *mek-1*, and a JIP3 (scaffold protein) ortholog, *unc-16* (Byrd et al., 2001; Kawasaki et al., 1999; Koga et al., 2000; Villanueva et al., 2001). We started by examining the life span of loss-of-function mutants of *jnk-1* and *jkk-1*. Both mutants show a statistically significant decrease in life span (wild-type: 16.8 ± 0.2 days, *jnk-1(gk7)*: 13.8 ± 0.2 days (P<0.0001) and *jkk-1(km2)*: 13.9 ± 0.2 days (P<0.0001)) (Figure 10). However, mutation in the two other components of the *C. elegans* JNK pathway, *mek-1(ks54)* and *unc-16(e109)* show little effect on life span (Figure 11). These data indicate that part of the JNK signaling pathway is required to maintain normal life span in *C. elegans*.

We next asked whether the life span regulation by *jnk-1* is dose-dependent. We amplified 9.3 kb of *jnk-1* genomic DNA including 3 kb of the promoter region, the entire coding region, and 500 bp of the 3'-UTR by PCR. The entire PCR fragment was injected into the gonad of wild-type worms along with the co-injection marker, *rol-6(su1066)* (Mello et al., 1991), to create *jnk-1* overexpression transgenic worms. From one of several extrachromosomal lines (*lpEx1*), we derived two independent integrated lines (*lpIn1*, *lpIn2*). Both *lpIn1* and *lpIn2* exhibit similar increases in expression of the *jnk-1* transcript, as determined by RT-PCR (Figure 12). *lpIn1* and *lpIn2* extend life span by 40% compared to the control implying that *jnk-1* is a positive regulator of life span (N2 +

pRF4 (*rol-6(su1066)* plasmid) control: 15.2 ± 0.3 days, *lpIn1*: 20.9 ± 0.6 days (P<0.0001), and *lpIn2*: 18.8 ± 0.5 (P<0.0001)) (Figure 13).

Dual phosphorylation of JNK on conserved Thr and Tyr by an upstream kinase is critical for its function (Davis, 2000). Therefore, we investigated whether the phosphorylation of JNK is required for life span regulation. We crossed *jnk-1* transgenic worms with loss-of-function mutants of upstream kinases *jkk-1(km2)* and *mek-1(ks54)*, and analyzed phosphorylation status of JNK-1 using a phospho-specific antibody that binds activated JNK. The phosphorylation of JNK-1 is considerably increased in the *jnk-1* overexpression strain (*lpIn2*) (Figure 14). However, it is not detectable in either *jnk-1(gk7)* or *jkk-1(km2)* mutants. In addition, mutation in *jkk-1* completely suppresses the phosphorylation of JNK-1 in *jnk-1* overexpression worms (*lpIn2*) (Figure 14). In accordance with this result, life span extension by *jnk-1* overexpression is also suppressed by mutation in *jkk-1* (*lpIn2*: 18.8 ± 0.5 days, *jkk-1*; *lpIn2*: 14.9 ± 0.4 days, N2 + pRF4 control: 15.2 ± 0.3 days) (Figure 15). However, mutation in *mek-1(ks54),* alone or in combination with *jnk-1* overexpression (*lpIn2*), does not affect JNK-1 phosphorylation (Figure 14). These results show that JKK-1 is the upstream kinase of JNK-1 and phosphorylation of JNK-1 is required for life span extension.

In *C. elegans*, *daf-16* plays a central role in life span regulation such that a mutation in *daf-16* suppresses the life span extension of *daf-2*, *age-1* or other long-lived mutants (Braeckman et al., 2001; Hekimi et al., 2001; Kenyon, 2001; Nelson and Padgett, 2003). Therefore, we asked whether life span extension by *jnk-1* overexpression is also dependent on *daf-16*. We performed life span analysis of *jnk-1* overexpression worms on

daf-16 RNAi. In agreement with previous work (Lee et al., 2003b), *daf-16* RNAi shortens life span (wild type on control RNAi: 17.6 ± 0.4 days, on *daf-16* RNAi: 14.4 ± 0.4 days) (Figure 16). Life span extension by *jnk-1* overexpression is completely suppressed by *daf-16* RNAi (*lpIn1* on control RNAi: 19.1 ± 0.6 days, on *daf-16* RNAi: 14.7 ± 0.4 days) (Figure 16). This suggests that *daf-16* is required for life span extension by *jnk-1* overexpression.

If *jnk-1* exerts its effect through the insulin-like pathway, *jnk-1* overexpression would not have an additional effect on life span in the background of mutations in the insulin-like pathway. However, if combining a hypomorphic mutation in the insulin-like pathway with *jnk-1* overexpression produces a further increase in life span, then this would be consistent with the possibility that *jnk-1* acts in a parallel pathway. We crossed a *jnk-1* overexpression line (*lpIn1*) with the standard allele of *daf-2(e1370)* and found that the combination significantly extends life span beyond *daf-2(e1370)* (*daf-2*: 44.0 ± 0.7 days, *daf-2*; *lpIn1*: 53.3 ± 1.7 days (P<0.0001)) (Figure 17). Further downstream of *daf-2* in the insulin-like signaling pathway are the kinases AKT-1 and AKT-2 (Paradis and Ruvkun, 1998). Since experiments in mammalian system have shown an interaction between the AKT and JNK pathways (Kim et al., 2003; Kim et al., 2002), we examined the life span of *lpIn1* in combination with *akt-1/akt-2*. We generated a double mutant strain containing null mutations (Hertweck et al., 2004) in both *akt-1(ok525)* and *akt-2(ok393)*. However, 100 % of the *akt-1(ok525); akt-2(ok393)* double mutants arrest at dauer larval stage at all temperatures. To circumvent this problem, we grew *akt-1(ok525); akt-2(ok393)* double mutants and the *akt-1(ok525); akt-2(ok393); lpIn1* strain

on *daf-16* RNAi to bypass the dauer larval stage and then tested life span on regular plates. *akt-1(ok525); akt-2(ok393); lpIn1* strain shows a life span extension beyond the *akt-1(ok525); akt-2(ok393)* double mutant *alone* (wild-type (N2): 14.9 ± 0.4 days, *akt-1(ok525); akt-2(ok393)*: 34.2 ± 0.8 days, *akt-1(ok525); akt-2(ok393); lpIn1*: 38.8 ± 0.9 days) (Figure 18). Similar results were obtained with *akt-1/2* RNAi (Figure 19). In addition, life span extension by either *daf-2* mutation alone or in combination with *jnk-1* overexpression (*lpIn1*) is completely suppressed by *daf-16* (Barbieri et al., 2003; Lin et al., 1997; Ogg et al., 1997); data not shown). These results suggest that *jnk-1* regulates life span in parallel to the insulin-like pathway but both converge onto *daf-16*. Alternatively, it is also possible that *jnk-1* acts in a linear pathway through other members of the insulin-like pathway.

Based on our genetic studies, we next examined whether JNK-1 interacts physically with DAF-16. COS-7 cells were transfected with plasmids encoding either Flag-tagged DAF-16 alone or in combination with Xpress-tagged JNK-1. Following co-immunoprecipitation with anti-Xpress antibody, we find that JNK-1 binds to DAF-16 (Figure 20). We then examined if DAF-16 could serve as a substrate for JNK-1. COS-7 cells were transfected with plasmids encoding Xpress-tagged JNK-1 that was then immunoprecipitated from the cell lysate with anti-Xpress antibody and incubated with bacterially expressed N- or C-terminal portion of DAF-16 as a substrate in an *in vitro* kinase assay. Upon activation of JNK-1 by UV, JNK-1 phosphorylates DAF-16 as well as the mammalian c-Jun protein used as a positive control (Figure 21). The phosphorylation was only observed with the N-terminal (83-307) fragment of DAF-16,

but not with the C-terminal (255-470) fragment (data not shown), implying that the JNK-1 phosphorylation site resides in the N-terminal region of DAF-16. Previously, JNK-1 was shown to be activated by dual phosphorylation on Thr276 and Tyr278 (Kawasaki et al., 1999; Villanueva et al., 2001). Therefore, we created kinase-dead JNK-1 construct to determine specificity by replacing TPY residues with APF, and performed the *in vitro* kinase assay using N-terminal portion of DAF-16 as a substrate. The kinase-dead JNK-1 (APF) fails to phosphorylate DAF-16 (Figure 22). This confirms the specificity of JNK-1 phosphorylation of DAF-16 as a substrate. These results demonstrate that JNK-1 directly interacts with and phosphorylates DAF-16 as a separate input from AKT-1/2 and therefore supports a parallel pathway model suggested by our genetic data.

JNK is a stress responsive gene in diverse organisms (Davis, 2000; Wang et al., 2003). Therefore we examined whether overexpression of *jnk-1* confers stress resistance. We monitored survival of young adult worms at 35 °C. Mutation in *jnk-1(gk1)* results in stress sensitivity when compared to wild-type (Figure 23). *jnk-1* transgenic lines show significantly increased resistance to heat stress (mean survival time N2 + pRF4 control: 10.8 ± 0.2 hours, *lpIn1*: 15.3 ± 0.3 hours, *lpIn2*: 14.4 ± 0.2 hours ($P<0.0001$)) (Figure 24). In addition, *jnk-1* transgenic lines significantly increase resistance to oxidative stress (Figure 25).

In *C. elegans*, mutation in *daf-2* or *age-1* confers stress resistance and this resistance is dependent on *daf-16* (Johnson, 2002; Lithgow and Walker, 2002; Munoz, 2003). We also found that the stress resistance observed in the *jnk-1* overexpression strains were also dependent on *daf-16* (Figure 26). Additionally, in response to stress,

DAF-16 translocates from the cytoplasm to the nucleus (Henderson and Johnson, 2001). Therefore, we crossed *jnk-1* overexpression lines to a strain containing a *daf-16::gfp* reporter construct to visualize the nuclear translocation *in vivo*. We compared *daf*-16::*gfp* alone with *lpIn2*; *daf-16::gfp* or *jkk-1*; *lpIn2*; *daf*-16::*gfp* following 30 minutes of heat shock. The extent of nuclear translocation was categorized as Cyt (cytosolic), Nuc (nuclear) or Int (intermediate) (see Materials and Methods) (Figure 27). The number of worms with nuclear localization signal (Nuc) is increased in the *jnk-1* overexpression (*lpIn2*; *daf-16::gfp)* strain whereas that with cytosolic localization (Cyt) is decreased compared to the control (*daf-16::gfp)*. However, mutation of *jkk-1* suppresses the enhancement in nuclear localization in *jnk-1* overexpression worms (*daf*-16::*gfp* (Cyt): 16.7 ± 1.4 %, *lpIn2*; *daf-16::gfp* (Cyt): 4.3 ± 0.9 %: *jkk-1; lpIn2; daf*-16::*gfp* (Cyt): 21.0 ± 2.4 %, *daf*-16::*gfp* (Nuc): 13.6 ± 1.8 %, *lpIn2*; *daf-16::gfp* (Nuc): 30.4 ± 2.1 %: *jkk-1; lpIn2; daf*-16::*gfp* (Nuc): 19.4 ± 2.5 %) (Figure 27). Together with biochemical evidence, our genetic analysis suggests that JNK-1 interacts directly with DAF-16 and modulates its nuclear translocation in response to stress. Previous studies have also shown that *daf-18* a phosphatase in the insulin-like signalling pathway is also required for *daf-16* translocation in response to stress (Lin et al., 2001). Our data would suggest that this is a novel input into *daf-16* for regulation of life span and stress.

The JNK signaling pathway serves as a molecular sensor for various stresses. Upon detecting environmental cues, JNK-1 might deliver the signal to DAF-16 by direct interaction and modulation of its nuclear translocation. Once DAF-16 enters the nucleus, it enhances the expression of numerous target genes to prevent damage from any harmful

stresses. This would then confer increased stress resistance and help to maintain normal life in *C. elegans* (Figure 28). In addition, it has recently been shown that life span extension by *Drosophila* JNK is also dependent on DFOXO (Wang et al., 2005). In summary, we present results that uncover a new role of JNK as a life span regulator in *C. elegans*. We demonstrate a direct interaction between JNK-1 and DAF-16 that modulates the nuclear translocation of DAF-16. These findings provide important clues on underlying mechanism by which an animal can respond and adapt to environmental fluctuations to preserve homeostasis.

IV) Materials and Methods

Strains

All strains were maintained and handled as described in (Brenner, 1974; Sulston and Hodgkin, 1988).

Strain construction

For *jkk-1; lpIn2* and *mek-1; lpIn2: jkk-1* and *mek-1* males were obtained by heat shock at 30 °C for 6 hr or spontaneously on the plate and mated to the *lpIn2 hermaphrodites*. From the mating plate, twenty putative F1 cross progeny were singled to individual plates and allowed to have progeny. From at least 2 individual F1 plates that segregated both rollers and non-rollers (indicating cross progeny), 20-30 F2 rollers were singled to individual plates and allowed to have progeny. Once the parents had progeny (F3), the F2 parents were tested for hypersensitivity to $CuSO_4$ for *mek-1* or for the presence of the *jkk-1* deletion mutation (5'-AGGAGAAAAGCAAGTTGTCG, 3'-GCAGCAGCTTCTCACAACAC) and *jnk-1* overexpression (5'-ACAGTGGAACAGGAGGAGG, 3'-ATGCCTATCTGCCTGAGAGC) by PCR. Then, the plates that segregated 100% rollers (F3) were kept to establish the strain. The crosses were done at 20 °C.

For *lpIn2; daf-16::gfp* and *jkk-1; lpIn2; daf-16::gfp:* Spontaneously obtained *daf-16::gfp* males were mated to either *lpIn2* or *jkk-1; lpIn2* hermaphrodites. The crosses

were done as described above except that F2 worms were screened for both GFP and the roller phenotype.

For *daf-2; lpIn1: daf-2(e1370)* males were mated to *lpIn1* hermaphrodites. About 20 putative F1 roller cross progeny were transferred to 25 ℃ and allowed to have F2 progeny. From plates segregating rollers and non-rollers, wild type and dauers, 15-20 rolling dauers were singled to individual plates and allowed to recover at 15 ℃. The plates were scored for 100 % roller progeny in F3. The rollers were then retested for dauer formation at 25℃.

Transgenic worms

jnk-1 genomic DNA including 3 kb of the promoter region, the entire coding region, and 500 bp 3'-UTR was amplified from N2 genomic DNA by PCR (5'-GCGTCCTCCTGTGCTCACTC, 3'-CCCACGACAACTGCTACAAC). After gel extraction (Qiagen), the 9.3 kb fragment was injected at 50 ng/µl into the gonad of N2 worms along with pRF4, (*rol 6(su1066)* plasmid) as a co-injection marker (100 ng/µl) (Mello et al., 1991) to generate stable extrachromosomal transgenic lines. Two independent integrated lines (*lpIn1* and *lpIn2*) were generated from one extrachromosomal line (*lpEx1*). Several extrachromosomal and integrated lines using at least two different markers were established and showed similar results (data not shown).

Life span analysis

Life span assays were performed at 20 °C. Adult hermaphrodites from each strain were transferred to fresh nematode growth medium (NGM) plates at 20 °C and allowed to undergo one full generation to ensure the worms were well fed and had not gone through dauer. L4s or young adults were then transferred to new NGM plates containing 0.1 mg/ml of 5'flourodeoxyuridine (FUDR) to prevent the growth of progeny (Hosono et al., 1982). Animals were tapped every 2-3 days and scored as dead when they did not respond to the platinum wire pick. Life span is defined as the number of days that worms are alive after they were transferred to FUDR plate (day 1). All the life span assays were repeated at least three times. For life span analysis on RNAi plates, a single colony from each RNAi clone was grown in LB broth containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline to OD 0.5-1.0. The bacteria were then seeded onto an NGM plate containing 1 mM isopropylthiogalactoside (IPTG), 50 μg/ml of ampicillin, and 0.1 mg/ml FUDR. Seeded plates were dried overnight at room temperature and then stored at 4 °C for subsequent use.

Plasmid construction and transfection

Full-length *daf-16 a1* and *jnk-1α* cDNAs were amplified by RT-PCR (Invitrogen) using total RNA isolated from N2 worms (Ambion). The cDNAs were cloned into the mammalian expression vector with either Flag-tag (p3XFLAG-myc-CMV^{TM}-26, Sigma) or Xpress-tag (pcDNA3.1B, Invitrogen) to obtain Flag-tagged DAF-16 and Xpress-tagged JNK-1. Plasmids were transfected into COS-7 cells and harvested after 48 hours. For determining whether DAF-16 could serve as a substrate for JNK-1, N- and C-

terminal portion of DAF-16 were amplified by PCR using N2 total RNA and cloned into pET-24b vector (Novagen) between *HindIII* and *XhoI*. The His-tagged DAF-16 fusion protein was expressed in *E. coli* (BL-21) and purified under native condition with Ni-NTA His-Bind resin (Novagen).

Antibody production

A c-terminal portion of JNK-1α(228 – 451) was cloned into a His-tagged expression vector (pET-24b, Novagen), expressed in bacteria (BL21(DE3)) and purified using Ni-NTA His-bind resin (Novagen). Polyclonal antiserum was raised against the recombinant protein in rabbit (Capralogics Inc.).

Immunoblotting, immunoprecipitation and kinase assay

For phospho-JNK immunoblotting, worms were grown on 10 cm NGM plates and ground with stainless steel Dounce homogenizer in lysis buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 10 % Glycerol, 1 % Triton X-100, 25 mM ß-glycerophosphate, 1 mM $NaVO_3$, 1 mM PMSF, 10 ug/ml Leupeptin, 10 ug/ml Aprotinin). Proteins were separated by SDS-PAGE and immunoblotted with phospho-JNK antibody (Cell signaling) or JNK-1 antibody (raised against *C. elegans* JNK-1). For immunoprecipitation, COS-7 cells were lysed in the same lysis buffer and following the centrifugation at 14,000 g for 10 min, the supernatant was pre-cleared with 50 µl of protein G-Sepharose bead (Amersham) and then incubated with anti-Xpress antibody (Sigma) along with fresh bead O/N at 4 ℃. After several washes, lysates were boiled

with sample buffer. Proteins were separated by SDS-PAGE and immunoblotted with anti-Flag antibody (Invitrogen). For kinase assay, Protein G-Sepharose (Pharmacia-LKB Biotechnology) beads were incubated for 3 to 4 h at 4°C with anti-Xpress antibody, washed twice with the lysis buffer as described above, and then incubated with lysates of COS-7 cells transfected with Xpress-JNK-1 O/N at 4 °C. Complexes were washed three times with the lysis buffer and once with kinase buffer (25 mM HEPES (pH 7.4), 25 mM ß-glycerophosphate, 25 mM MgCl2, 0.1 mM $NaVO_3$, 2 mM DTT). The kinase activity of JNK-1 was measured by adding 20 µl of kinase buffer containing 50 µM [γ-^{32}P]ATP (10 Ci/mmol) and 1 µg of N- or C- terminal portion of DAF-16 or GST-c-Jun (1-79) and incubating at 30 °C for 30 min. The reactions were terminated by boiling in sample buffer. Proteins were resolved by SDS-PAGE and analyzed by autoradiography.

Heat resistance assay and DAF-16 translocation assay

For heat stress resistance, 50 young adult worms were picked onto NGM plates and kept at 35 °C. Animals were tapped every hour and scored as dead when they did not respond to the platinum wire pick. Assays were repeated at least 3 times. For DAF-16 translocation assay, 10 ~ 15 L4s were placed on NGM plates at 35 °C. After 30 minutes, worms were immediately mounted onto the slide with 0.1 % of sodium azide in S-basal buffer. Nuclear translocation of DAF-16 was visualized with a fluorescence microscope (Zeiss) equipped with a Hamamatsu digital camera and analysed by Openlab software. The extent of nuclear translocation was categorized as Cyt, Nuc and Int: Cyt (cytosolic) when there is no visible nuclear localization, Nuc (nuclear) when the nuclear localization

is observed throughout the entire body from head to tail, or Int (intermediate) when there is a visible nuclear localization but not as complete as Nuc. The number of worms with each level of nuclear translocation was counted. The translocation assay was repeated at least 5 times by two different individuals.

PCR primers used for cloning

Flag-DAF-16: 5'-GAAGATCTGGAGATGCTGGTAGATCAGGG, 3'-CGGGGTACCTTACAAATCAAAATGAATAT; Xpress-JNK-1: 5'-CGGGATCCGGAGGAACGATTATCCACAAC, 3'-GCTCTAGATCAGGAATAAATGTCATGGG; Xpress-JNK-1 (APF): 5'-GAGGCATTCATGATGGCTCCTTTCGTTGTGACAAGATAC, 3'-GTATCTTGTCACAACGAAAGGAGCCATCATGAATGCCTC; His-DAF-16 (N-terminal fragment): 5'-CCCAAGCTTGGCCTATACGGGAGCAATGAGC, 3'-CCGCTCGAGCGGACGGAAAGATGATGGAACG; IIis-DAF-16 (C-terminal fragment): 5'-CCCAAGCTTGGCGGAGCCAAGAAGAGGATA, 3'-CCGCTCGAGCGCAATTGGAAGAGCCGATGAA

V) Figures

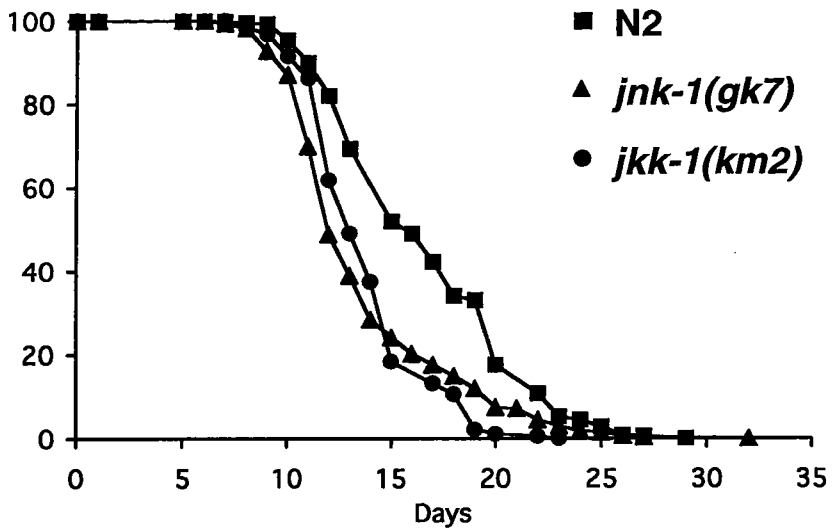


Figure 10. Life span analysis of *jnk-1(gk7)* and *jkk-1(km2)*. All life span data is presented as mean life span ± standard error (total number of animals scored). N2, 16.8 ± 0.2 (386); *jnk-1(gk7)*, 13.8 ± 0.2 (333); *jkk-1(km2)*, 13.9 ± 0.2 (189). The life span curves were plotted from the pooled data of individual experiments.

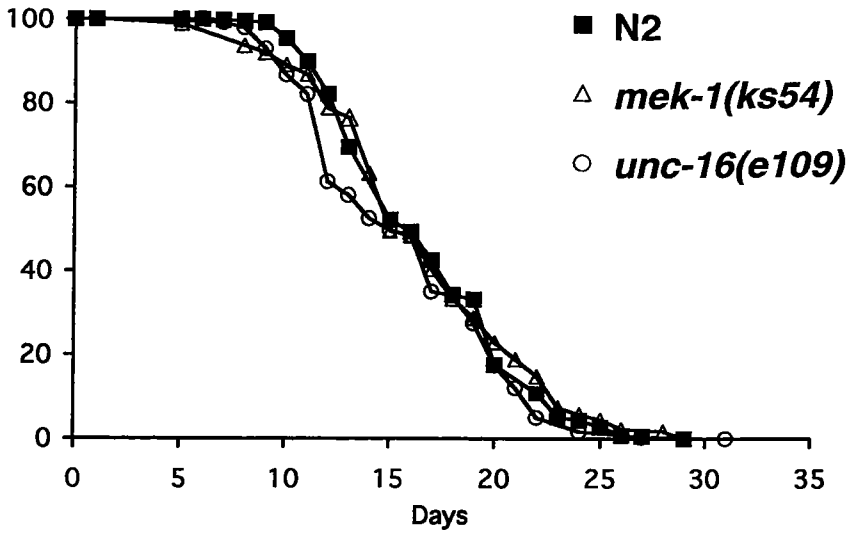


Figure 11. Life span analysis of *mek-1(ks54)* and *unc-16(e109)*. Mean life span ± standard error (total number of animals scored): wild-type (N2), 16.8 ± 0.2 (386); *mek-1(ks54)*, 16.6 ± 0.4 (174) (P=0.7089); *unc-16(e109)*, 15.7 ± 0.3 (240) (P=0.0023).

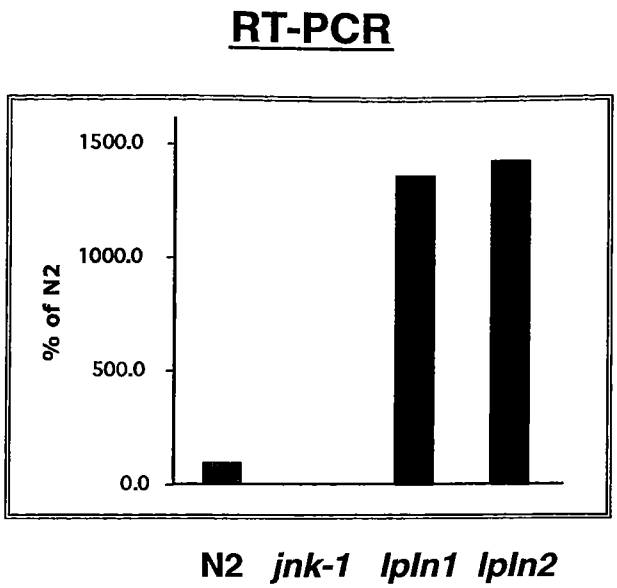


Figure 12. RT-PCR analysis of *jnk-1* overexpression lines (*lpIn1* and *lpIn2*). The transcription level was measured in two independent integrated transgenic lines by RT-PCR (5'-ACAGTGGAACAGGAGGAGG, 3'-ATACGGAAGTGGAGGTGGAG) and compared to the wild-type. Data for each strain (% of N2): wild-type (N2), 100 %; *jnk-1*, 0 %; *lpIn1*, 1368 %; *lpIn2*, 1432 %.

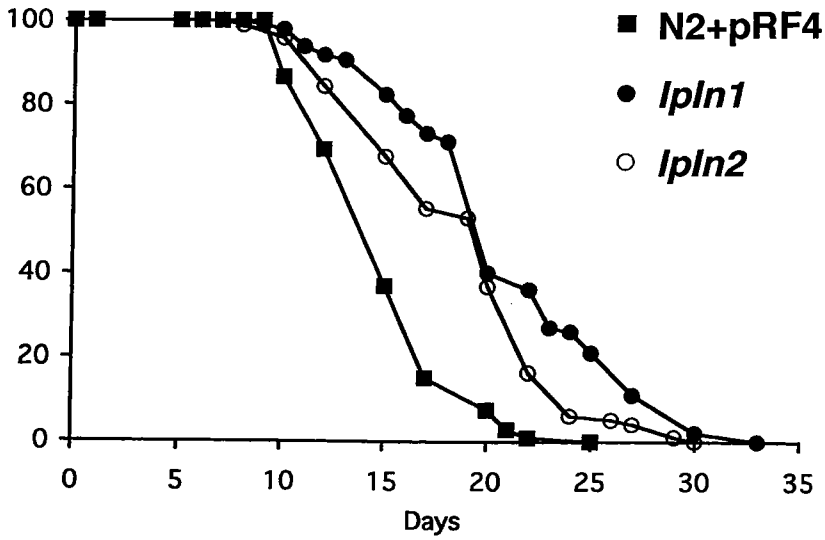


Figure 13. Life span analysis of *lpIn1* and *lpIn2*. Mean life span ± standard error (total number of animals scored): N2 + pRF4, 15.2 ± 0.3 (105); *lpIn1*, 20.9 ± 0.6 (99); *lpIn2*, 18.8 ± 0.5 (97).

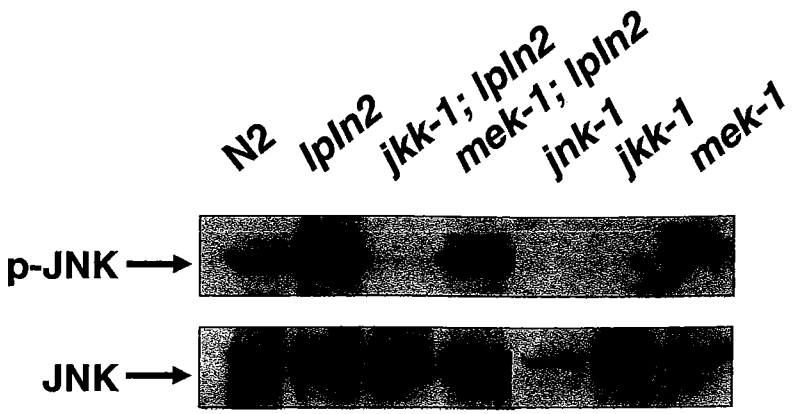


Figure 14. Immunoblotting with phospho-JNK antibody (upper panel). The membrane was reprobed with JNK antibody (lower panel).

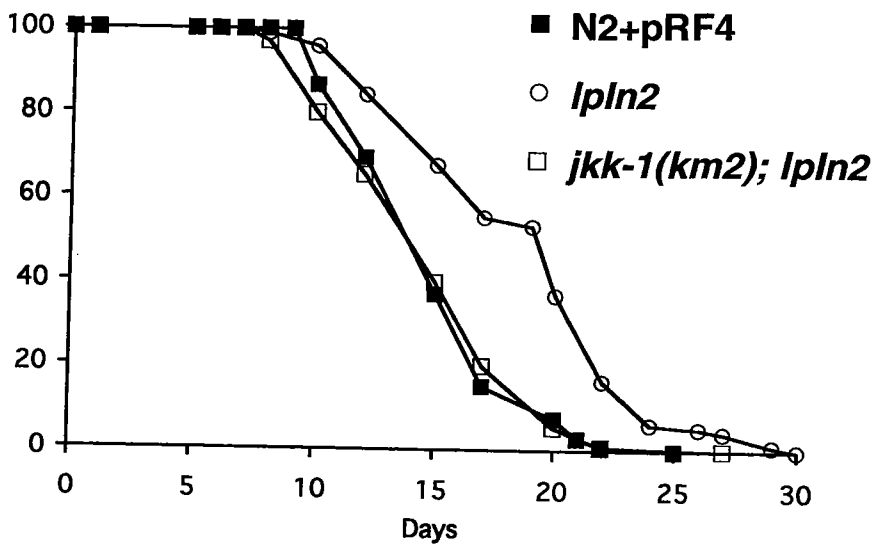


Figure 15. Life span analysis of *jkk-1; lpIn2*. Mean life span ± standard error (total number of animals scored): N2 + pRF4, 15.2 ± 0.3 (105); *lpIn2*, 18.8 ± 0.5 (97); *jkk-1*; *lpIn2*, 15.0 ± 0.3 (130).

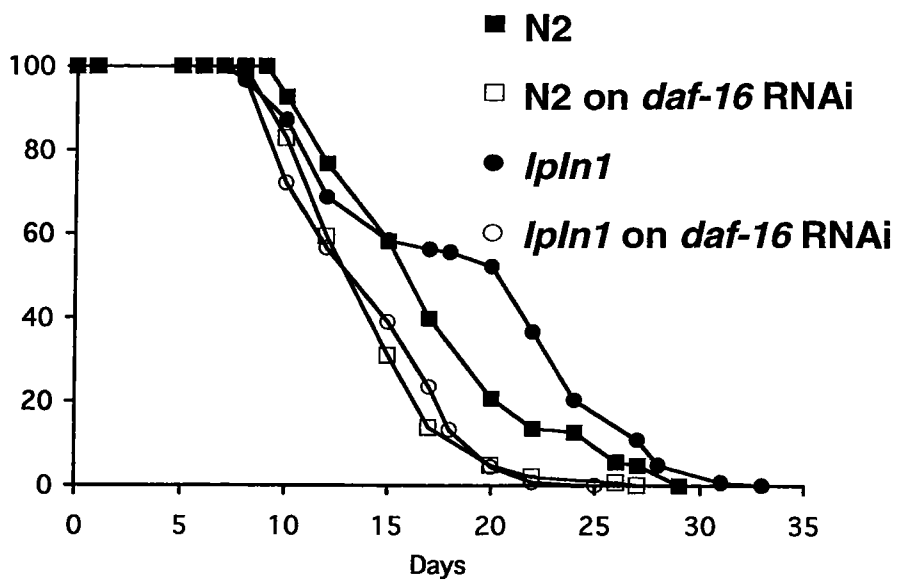


Figure 16. Life span analysis of *lpIn1* on *daf-16* RNAi. Mean life span ± standard error (total number of animals scored): N2 on control RNAi, 17.6 ± 0.5 (125); N2 on *daf-16* RNAi, 14.6 ± 0.3 (145); *lpIn1* on control RNAi, 19.1 + 0.6 (147); *lpIn1* on *daf-16* RNAi, 14.5 ± 0.3 (136).

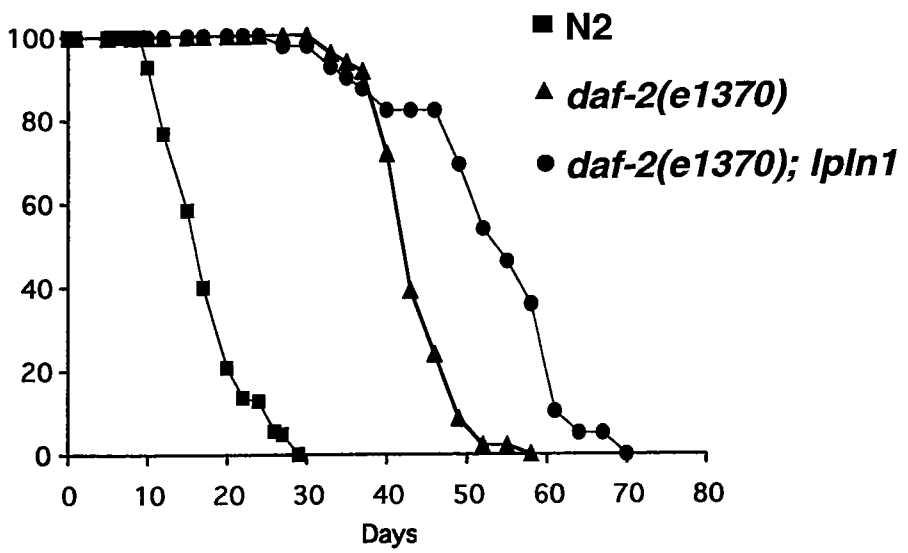


Figure 17. Life span analysis of *daf-2(e1370); lpIn1*. Mean life span ± standard error (total number of animals scored): N2, 17.6 ± 0.5 (125); *daf-2(e1370)*, 44.0 ± 0.7 (46); *daf-2(e1370); lpIn1*, 53.3 ± 1.7 (39).

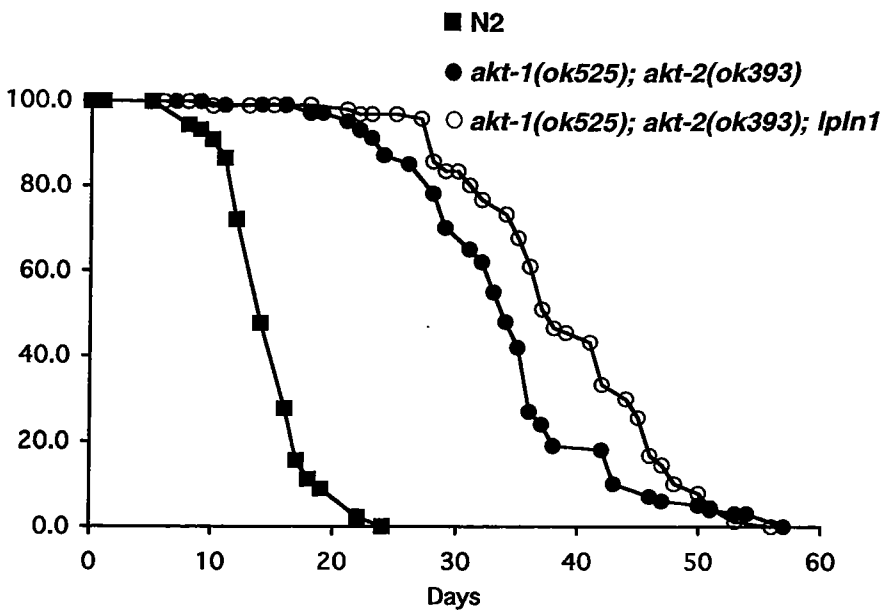


Figure 18. Life span analysis of *akt-1(ok525); akt-2(ok393)* and *akt-1(ok525); akt-2(ok393); lpIn1*. Mean life span ± standard error (total number of animals scored): wild-type (N2), 14.9 ± 0.4 (90); *akt-1(ok525); akt-2(ok393)*, 34.2 ± 0.8 (100); *akt-1(ok525); akt-2(ok393); lpIn1*, 38.8 ± 0.9 (90). The representative life span curve is shown here, which has been repeated with similar results.

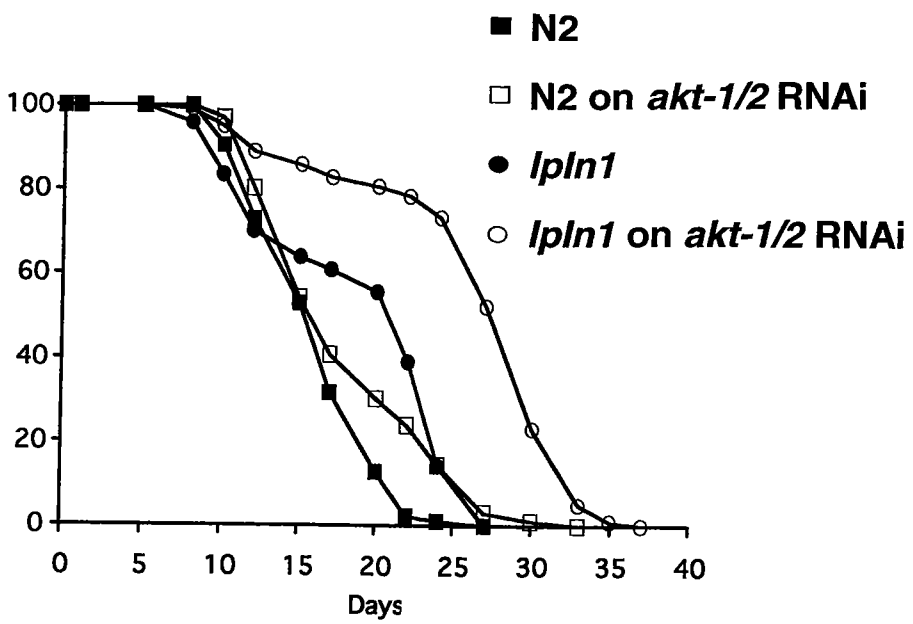


Figure 19. Life span analysis of *lpIn1* on *akt-1/akt-2* RNAi. Mean life span ± standard error (total number of animals scored): N2 on control RNAi, 16.4 ± 0.4 days; N2 on *akt-1/akt-2* RNAi, 18.3 ± 0.6 days; *lpIn1* on control RNAi, 19.1 ± 0.6 days; *lpIn1* on *akt-1/akt-2* RNAi, 26.5 ± 0.7 days ($P<0.0001$).

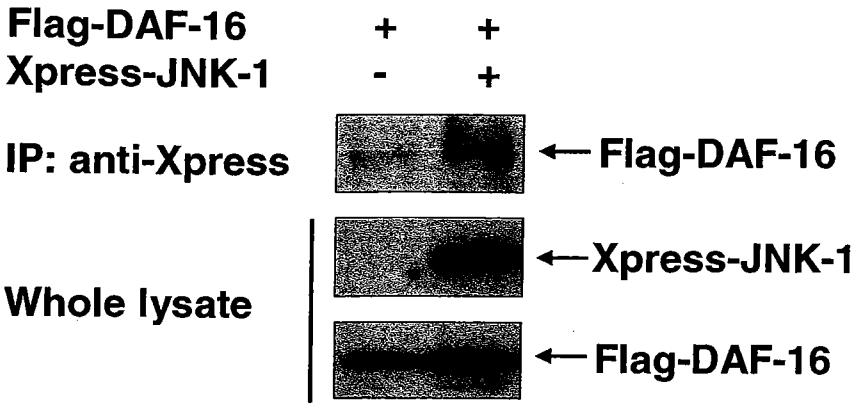


Figure 20. Co-immunoprecipitation assay. Following transfection with either Flag-DAF-16 alone or in combination with Xpress-JNK-1, cell lysates were immunoprecipitated with anti-Xpress antibody and immunoblotted with anti-Flag antibody. The expression of protein was confirmed by immunoblotting with anti-Flag or anti-Xpress antibody.

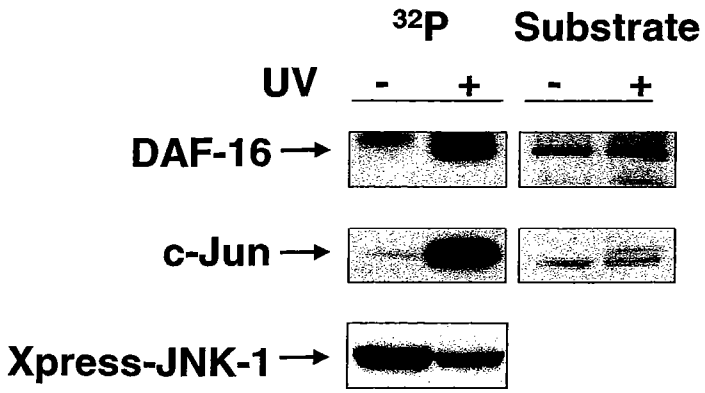


Figure 21. *in vitro* kinase assay using DAF-16 as a substrate. Cells were transfected with Xpress-JNK-1, activated by UV, followed by immunoprecipitation with anti-Xpress antibody and incubation with His-DAF-16 (N-terminal fragment) in kinase buffer. Loading of the substrates (His-DAF-16 (N-terminal fragment) and c-Jun) is shown in the right panel stained with Coomassie Blue. The expression of protein was confirmed by immunoblotting with anti-Flag or anti-Xpress antibody.

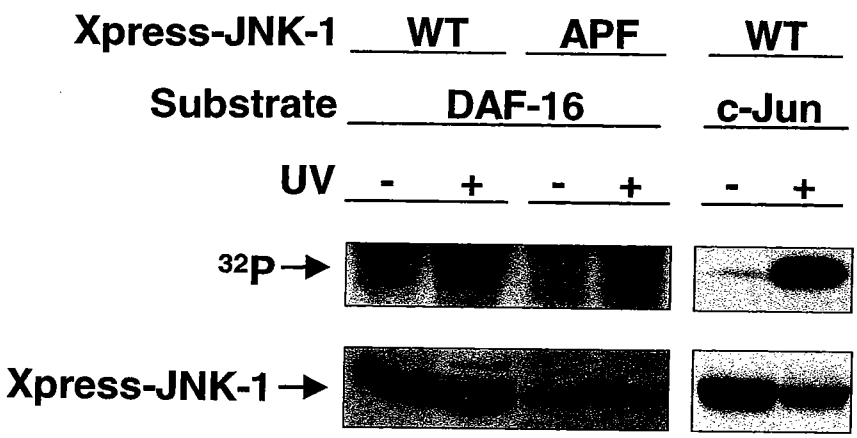


Figure 22. *in vitro* kinase assay using kinase-dead JNK-1 (APF). We created kinase-dead JNK-1 construct by replacing TPY residues with APF, and performed the *in vitro* kinase assay using N-terminal portion of DAF-16 as a substrate. The expression of protein was confirmed by immunoblotting with anti-Xpress antibody.

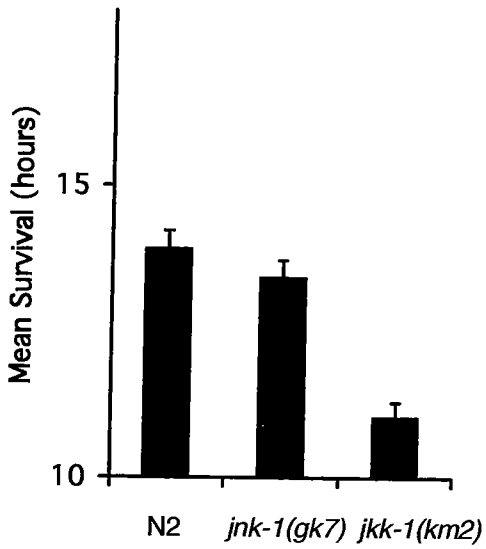


Figure 23. Heat stress assay of *jnk-1(gk7)* and *jkk-1(km2)*. 50 young adult worms were picked onto NGM plates and kept at 35 °C. Animals were tapped every hour and scored as dead when they did not respond to the platinum wire pick. Mean survival time ± standard error (total number of animals scored): N2, 13.9 ± 0.3 (90); *jnk-1(gk7)*, 13.5 ± 0.3 (86); *jkk-1(km2)*, 11.1 ± 0.2 (37).

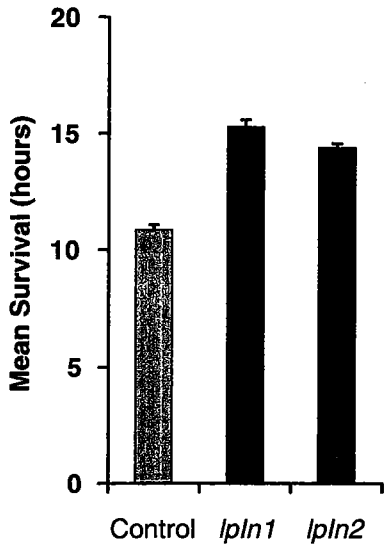


Figure 24. Heat stress assay *lpIn1* and *lpIn2*. Mean survival time ± standard error (total number of animals scored): N2 + pRF4, 10.8 ± 0.2 (138); *lpIn1*, 15.3 ± 0.3 (147); *lpIn2*, 14.4 ± 0.2 (249).

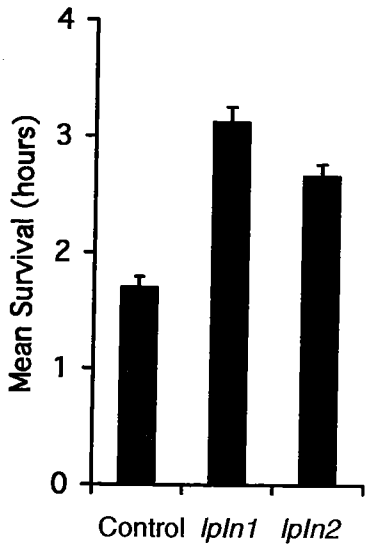


Figure 25. Oxidative stress assay of *lpIn1* and *lpIn2*. 30 ~ 40 young adults were transferred to 96-well plates (5 ~ 6 worms/well) containing 40 µl of 150 mM paraquat at room temperature. Animals were tapped every 30 minutes and scored as dead when they did not respond to the platinum wire pick. Mean survival time ± standard error (total number of animals scored): control (N2 + pRF4), 1.7 ± 0.1 (88); *lpIn1*, 3.1 ± 0.1 (68); *lpIn2*, 2.7 ± 0.1 (118).

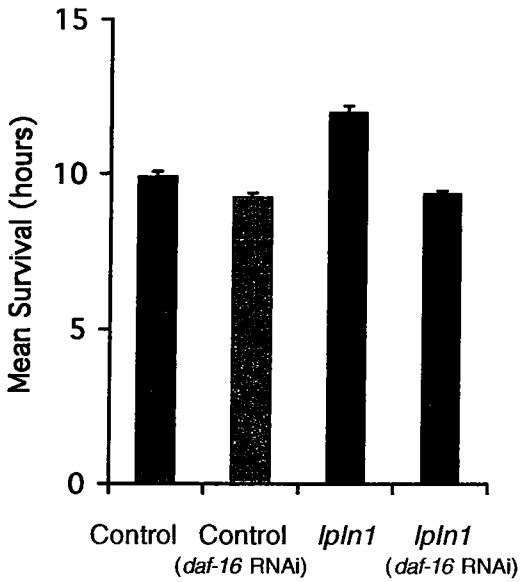
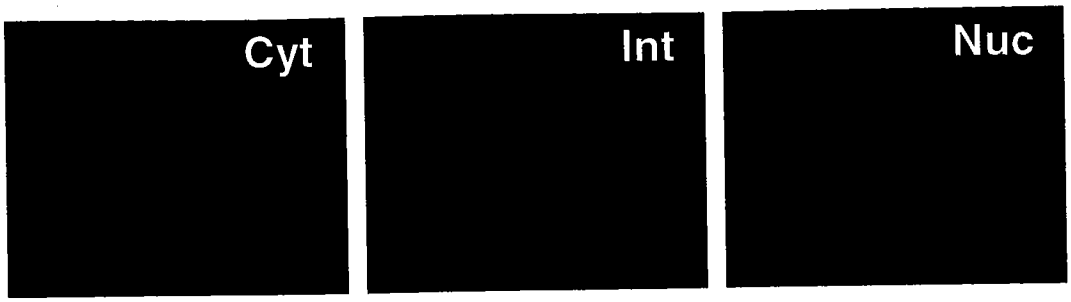


Figure 26. Heat stress assay of *lpIn1* on *daf-16* RNAi. 50 young adult worms were grown and picked onto either control RNAi plates or *daf-16* RNAi plates and kept at 35 °C. Animals were tapped every hour and scored as dead when they did not respond to the platinum wire pick. Mean survival time ± standard error (total number of animals scored): N2 + pRF4 on control RNAi, 9.9 ± 0.2 (60); N2 + pRF4 on *daf-16* RNAi, 9.3 ± 0.1 (84); *lpIn1* on control RNAi, 12.0 ± 0.2 (98); *lpIn1* on *daf-16* RNAi, 9.4 ± 0.1 (100).



30 min. at 35 degree (% of total worms tested)				Number of
Strains	Cytosolic	Intermediate	Nuclear	worms tested
daf-16::gfp	16.7	69.7	13.6	66
lpln2;daf-16::gfp	4.3	65.2	30.4	69
jkk-1;lpln2;daf-16::gfp	21.0	59.7	19.4	62

Figure 27. DAF-16 translocation assay. Following heat shock at 35 °C for 30 min, the nuclear translocation of DAF-16 was measured and the number of worms in each category was counted (see Materials and Methods).

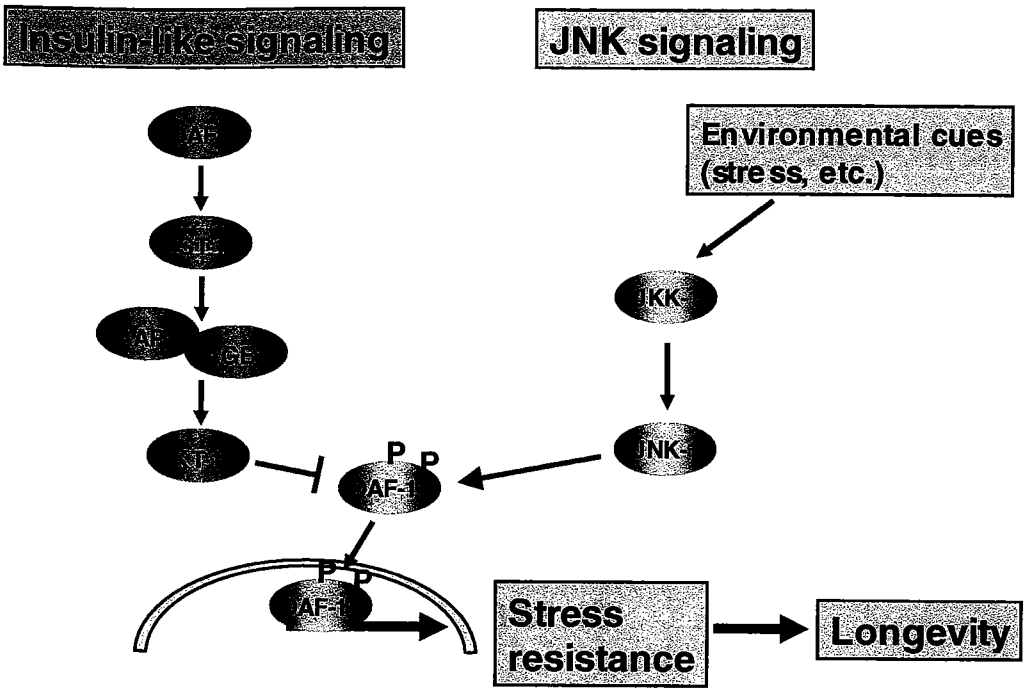


Figure 28. A model for DAF-16 regulation by JNK signalling pathway. JNK signaling pathway presumably serves as a molecular sensor for various stresses. Upon detecting environmental cues, JNK-1 transmits the signal by phosphorylating and modulating the nuclear translocation of DAF-16. Once DAF-16 enters the nucleus, it enhances the expression of numerous target genes to prevent damage from any harmful stresses. This would then confer increased stress resistance and help to maintain normal life in *C. elegans*.

CHAPTER III

IDENTIFICATION OF DIRECT TARGETS OF DAF-16 THAT CONTROL LIFE SPAN, FAT STORAGE AND DIAPAUSE BY CHROMATIN IMMUNOPRECIPITATION

I) Abstract

DAF-16, a forkhead transcription factor, is a key regulator of various biological processes such as life span, metabolism, dauer diapause, development and cell death. The precise mechanism by which DAF-16 directs multiple functions, however, is poorly understood. Putative downstream targets of DAF-16 have been suggested by several studies using DNA microarray and bioinformatics, but it is unclear whether these genes are directly regulated by DAF-16. Here, we used a modified chromatin immunoprecipitation (ChIP) to identify direct target promoters of DAF-16. We cloned 103 target sequences containing consensus DAF-16 binding sites and randomly selected 33 targets for further analysis. The expression of majority of these genes is regulated in a DAF-16-dependent manner. Moreover, inactivation of more than 50% of these genes, either by RNA interference or mutation, significantly altered DAF-16-dependent functions such as longevity, fat storage and dauer diapause. Our results show that the ChIP-based cloning strategy leads to greater enrichment of DAF-16 target genes, compared to previous studies. We also demonstrate that DAF-16 is recruited to multiple promoters to coordinate regulation of its downstream target genes. In addition, the large number of target genes revealed by this powerful technique provides insight into the complex regulation by DAF-16 in controlling diverse biological functions.

II) Introduction

In *Caenorhabditis elegans*, the insulin/IGF-1 receptor (DAF-2) signals through a conserved PI3 kinase/AKT pathway and negatively regulates DAF-16, a forkhead transcription factor (FOXO). This pathway regulates life span and stress response as well as dauer diapause, fertility and metabolism (Barbieri et al., 2003). DAF-16 also receives input from several other pathways that regulate life span (Hsin and Kenyon, 1999; Oh et al., 2005; Tissenbaum and Guarente, 2001). Therefore, DAF-16 is a major target of several pathways that control life span as well as metabolism, stress response and development in *C. elegans*. Identifying direct targets of DAF-16 will help understand how this transcription factor regulates multiple phenotypes.

Thus far, two different approaches have been used to characterize DAF-16 downstream genes: microarray analysis (Murphy et al., 2003) and bioinformatics (Lee et al., 2003a). Both analyses suggested a large number of probable targets, but did not determine whether these are direct *in vivo* targets of DAF-16 or genes affected by downstream pathways.

III) Results and Discussion

Chromatin immunoprecipitation (ChIP) is a powerful assay that can be used to identify the direct target gene(s) of a transcription factor. In mammalian studies, novel direct target genes for several transcription factors have been identified using a powerful combination of ChIP and subsequent cloning (Raha et al., 2005; Weinmann, 2004). We developed a modified ChIP assay for use in *C.elegans* (Figure 29). This assay was performed using wild type, *daf-2(e1370)* or *daf-16(mu86)* strains. Worms were treated with formaldehyde to crosslink the protein-DNA complex. After shearing the chromatin to a desired length (300 bp - 500 bp) by sonication, the DNA-protein complex was immunoprecipitated with an anti-DAF-16 antibody or pre-immune serum as a negative control. To verify the validity of the assay, we monitored DAF-16 binding to the *sod-3* promoter, a well-known target of FOXO/DAF-16 (Furuyama et al., 2000; Honda and Honda, 1999; Murphy et al., 2003). We designed PCR primers to amplify part of the *sod-3* promoter sequence including the predicted DAF-16 binding site (-130 bp) and measured the amount of *sod-3* DNA fragment immunoprecipitated by either the anti-DAF-16 antibody or pre-immune serum. The signal obtained with the anti-DAF-16 antibody was more than 15 times than that with pre-immune serum, and binding was reduced to background level in a *daf-16(mu86)* strain, which harbors a null-allele of *daf-16* (Figure 30). We also designed primers to amplify the coding region, 3'-UTR and distal upstream region (~5 kb) and found that DAF-16 predominantly bound to the *sod-3* promoter (Figure 31). This is the first biochemical evidence of DAF-16 recruitment to

the *sod-3* promoter in *C. elegans* and validates the previously proposed DAF-16 binding site. These results also indicate that DNA specifically bound by DAF-16 can be successfully immunoprecipitated using this technique. Importantly, we did not observe any difference in DAF-16 binding to the *sod-3* promoter between the wild type and *daf-2(e1370)* strains although *sod-3* mRNA expression was elevated in *daf-2(e1370)* (Figure 32). It is possible that DAF-16 binding is only increased in a small subset of cells that cannot be detected using whole worm extract. However, a previous study has shown that *sod-3* is expressed in most tissues in *daf-2(e1370)* (Libina et al., 2003) and in a *daf-16* dependent manner (Honda and Honda, 1999), indicating that DAF-16 binding to this promoter does not change significantly. This suggests that regulation of DAF-16 is not at the level of binding to the promoter and it is also possible that an as yet unidentified cofactor or a post-translational modification may be involved in the transcriptional regulation of *sod-3* by DAF-16.

We next exploited this technique to clone novel target genes of DAF-16 (Figure 33). We used *daf-2(e1370)*, a long-lived strain in which the insulin-like signaling pathway is inactive (Kimura et al., 1997). In this strain, DAF-16 is strongly localized to the nucleus (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001) where it presumably bind its target promoters actively; thus this strain is potentially an enriched source of DAF-16 targets. Following ChIP, the recovered DNA was PCR amplified, size-fractionated and cloned (see Materials and Methods) (De Belle et al., 2003; Raha et al., 2005). We sequenced 320 clones from several rounds of independent cloning. BLAST searches identified 180 *C. elegans* sequences with 100 % identity. The rest of

the clones represented sequences from the bacterial genome, possibly because worms use bacteria as a food source. These 180 clones represent 130 individual genes since certain DNA fragments were cloned multiple times in each trial (Figure 34). However, three target sequences (C14F5.1, *ldb-1* and *rrn-1*) were cloned more than once from several independent cloning experiments, which highlights the abundance of DAF-16 target genes in the *C. elegans* genome (Lee et al., 2003a; McElwee et al., 2003).

We analyzed the promoters and coding regions of these genes for known consensus DAF-16 binding sites (canonical: GTAAAC/TAA or TTG/ATTTAC (Furuyama et al., 2000), predicted: TGATAAG or CTTATCA (Murphy et al., 2003)). Among the 130 genes, 88 (68%) contain at least one consensus DAF-16 binding site within 5 kb of promoter, 15 (11%) have a consensus binding site(s) only in the coding region, and 27 (21%) do not contain any consensus site (Figure 34 and Table 8). The genes without consensus DAF-16 binding sites could be non-specific clones or may contain as yet unidentified DAF-16 binding sites. Alternatively, these could be targets for another transcription factor that interacts with DAF-16 but binds at a distant locus and are thus inadvertently immunoprecipitated by the DAF-16 antibody. Therefore, we considered the 103 genes with a DAF-16 binding site either in the promoter or coding region for further analysis as putative direct binding targets of DAF-16.

These genes represent a variety of biological processes such as metabolism, development, intracellular/extracellular signaling, cell death/apoptosis, transcription/translation as well as detoxification/stress response (Figure 35 and Table 8). Among these, the Mn-superoxide dismutase, *sod-2,* has been previously suggested to be a

putative DAF-16 target similar to *sod-3* (Yanase et al., 2002). Importantly, we identified genes whose functions are directly related to the insulin signaling pathway. These include: neprilysin-like genes (peptidase family M13) which are peptidases that cleave signaling molecules such as insulin (Turner et al., 2001) as well as the haemolysin-III-type/adiponectin receptor that has also been shown to regulate glucose uptake; defects in the receptor lead to insulin resistance in mammals (Kadowaki and Yamauchi, 2005). In addition, we found a gene involved in dauer diapause. The *vha-3* gene encodes the ATP synthase subunit C and is known to be up-regulated specifically in the dauer stage (Oka et al., 1998). Finally, we also identified genes whose human orthologs are implicated in diseases; cell surface glycoprotein/membrane mucin (Treacher Collins Syndrome), *sca-1* (Darier-White disease) and bestrophin 2 (Best disease). Taken together, these results indicate the ChIP-based cloning procedure has led to the identification of predicted and relevant direct downstream targets of DAF-16.

Next, we confirmed the *in vivo* specificity of DAF-16 binding to the identified genes by ChIP PCR assay (see Materials and Methods). Primers were designed to amplify a 300-500 bp region containing the DAF-16 binding site(s) in the promoters. Specificity was ascertained by two different methods. First, the amount of PCR product amplified from DNA samples immunoprecipitated with the anti-DAF-16 antibody was quantitated and compared to that immunoprecipitated with the pre-immune serum using the *daf-2(e1370)* strain. Second, we confirmed the specificity of these genes by comparing the amount of ChIP PCR product from immunoprecipitated DNA obtained using *daf-2(e1370)* versus *daf-16(mu86)*. We tested the first 33 target genes cloned that

contained a DAF-16 binding site(s) in the promoter. A considerably higher amount of target DNA was immunoprecipitated with the anti-DAF-16 antibody compared to that immunoprecipitated with pre-immune serum (Table 5). However, we could only detect minimal binding in *daf-16(mu86)* with the anti-DAF-16 antibody compared to *daf-2(e1370)* (Table 5). Taken together, these results show that the novel promoters identified bind DAF-16 specifically. The rest of the genes remain to be analyzed. Importantly, we found that some regions containing a consensus DAF-16 binding site did not bind DAF-16, as in the case of Y105E8B.9 and D1054.6, whereas DAF-16 binding was observed at binding sites in close proximity within the same promoter (Table 5). This suggests that the presence of a binding site does not guarantee the binding of DAF-16. Alternatively, it is possible that some binding sites are utilized only transiently, during a specific developmental stage or in response to certain stimuli. Although, all the genes we tested showed specific binding, it is possible that some of these might be non-productive. Therefore, they were further characterized for biological relevance.

We next investigated whether the expression of these target genes were modulated in a *daf-16*-dependent manner by comparing mRNA levels in wild type and *daf-16(mu86)* strains. Quantitative RT-PCR showed that more than half of the target genes were either up- or down-regulated in the absence of a functional copy of DAF-16, whereas others did not change significantly (Table 6). These results indicate that DAF-16 acts either as an activator or a repressor as previously suggested (Lee et al., 2003a; Murphy et al., 2003).

Because DAF-16 is known to regulate multiple biological processes, we expected that DAF-16 target genes would affect life span, fat storage and/or dauer entry when inactivated. Therefore, we next analyzed each of the 33 novel DAF-16 target genes for their roles in longevity, fat storage and dauer development by RNAi inactivation or using hypomorphic alleles (Table 6). First, we investigated the DAF-16 target genes for their effects on life span. We found that the hypomorphic alleles of *egl-10* and *lin-2* significantly extended life span compared to wild type at 20 °C (Table 6 and Figure 36). Using RNAi inactivation, we also found that knockdown of *sca*-1 extended life span compared to wild type, whereas knockdown of 4 other genes shortened life span: the zinc-finger domain-containing protein (C01B7.1), neprilysin-B-like gene (F42G10.1), *ldb-1* and *zfp-1* (Table 6 and Figure 37). None of the target genes we identified when inactivated or mutated suppressed life span extension by *daf-2(e1370)* completely like *daf-16(mu86)* or *hsf-1* (data not shown) (Kenyon, 2005). Considering the complexity of life span regulation, one might expect that downstream target genes of DAF-16 partially contribute to life span control by DAF-16. Alternatively, RNAi might have a weaker effect than a null mutant, which is not available for most of new target genes we identified, or that these genes are *daf-2* independent but *daf-16* dependent.

Next, we studied the effect of RNAi knockdown of these genes on fat storage in wild type worms at 20 °C using the vital dye Nile Red. We observed a noticeable decrease in fat storage upon inactivation of 8 genes by RNAi (Table 6; a subset are shown in Figure 38). These include 1 metabolic gene (acetyl CoA synthetase (C36A4.9)), 5 receptor or membrane proteins (*unc-84*, *srh-99,* putative sugar transporter (C01A2.2),

c-type lectin (D1054.6) and cell surface glycoprotein (K06A9.1)) and 2 DNA binding proteins (*glh-1* and *zfp-1*). By contrast, RNAi knockdown of F07F6.1, a gene of unknown function containing a PCI domain, considerably increased the fat content in wild type. Interestingly, the acetyl CoA synthetase (C36A4.9) and *str-254* genes have been previously shown to decrease fat content in both wild type and *daf-2(e1370)* upon RNAi inactivation (Ashrafi et al., 2003).

Finally, we analyzed the effect of RNAi inactivation of these genes on dauer formation of *daf-2(e1370)*. At 25 °C, *daf-2(e1370)* worms are completely arrested at dauer stage, while at 20 °C they temporarily arrest at dauer, however the majority of worms recover to form adults. Therefore, we measured percent dauer after 4 days at the permissive temperature (20 °C) to identify both enhancers and suppressors of the dauer phenotype. RNAi treatment revealed that inactivation of 6 genes resulted in enhancement of dauer formation whereas inactivation of 3 genes suppressed this process (Table 6 and Figure 39). The dauer enhancing genes are *srh-99*, *zfp-1*, zinc-finger protein (C01B7.1), acetyl CoA synthetase (C36A4.9), c-type lectin/CUB/Sushi domain protein (Y38E10A.4) and a voltage-gated potassium channel (Y48A6B.6). On the other hand, inactivation of *aqp*-2, *glh*-1 and *sca-1* caused significant decrease in dauer formation after 4 days. This is an important finding as previous studies have tried to identify suppressors of *daf-2* dauer formation and only yielded *daf-16* alleles (Gottlieb and Ruvkun, 1994; Malone and Thomas, 1994; Ogg et al., 1997).

In total, we detected 18 genes showing phenotypes upon inactivation (Table 6). The remaining genes failed to show any obvious phenotype with regard to life span, fat

storage and dauer formation although they were found to be legitimate binding targets of DAF-16. The lack of detectable phenotype might be due to either the relatively weak suppression of target genes by RNAi, that DAF-16 may act in concert with other proteins such that inactivation of one is not sufficient to produce an obvious phenotype, or that DAF-16 binds non-productively. It is also possible that some DAF-16 targets might not be important for the phenotypes we tested, but may instead be involved in other biological processes that are also regulated by DAF-16. Lastly, these targets may not mediate insulin-like signaling pathway-specific signals since DAF-16 is also the downstream target of several other pathways independent of insulin-like signaling (Hsin and Kenyon, 1999; Oh et al., 2005).

A comparison of our results with those obtained from previous studies (Ashrafi et al., 2003; Hamilton et al., 2005; Lee et al., 2003a; Murphy et al., 2003) indicates that the ChIP-based cloning strategy is an effective way to enrich direct targets of DAF-16 (Table 7). We found that 67.7% of genes we identified (88/130) contain either canonical or new DAF-16 binding sites within 5 kb of promoter, which is significantly higher than that obtained by microarray analysis (Murphy et al., 2003). Using bioinformatics, Lee *et al.* suggested 17 putative DAF-16 targets, out of which 4 (23.5%) showed DAF-16 dependent phenotypes (life span, fat storage or dauer formation) (Lee et al., 2003a). However, we demonstrate that more than 50% of genes we tested show DAF-16 dependent phenotypes. In addition, genome-wide RNAi screening identified 0.5% and 2.5% of the total genome to extend life span or affect fat storage, respectively (Ashrafi et al., 2003; Hamilton et al., 2005), while we obtained more than 10-fold enrichment in

genes affecting these phenotypes by our method. Thus, the ChIP-cloning method we describe here is very powerful and effective for identifying relevant targets of DAF-16.

Using this powerful tool, we have shown for the first time that DAF-16 is directly recruited to a large number of promoters, and as a result, modulates their expression. Some of these target genes affect multiple processes, while others are specific to one (Table 6). The ultimate outcome of the complex regulation of the target genes would determine the phenotypes associated with DAF-16. The cloning of target genes and their systematic analysis provide extensive insight into the complex regulation of diverse biological functions by DAF-16.

IV) Materials and Methods

Preparation of anti-DAF-16 antibody

The C-terminal portion of DAF-16a2 (amino acids 255-467; amplified using 5'-CCCAAGCTTGGCGGAGCCAAGAAGAGGATA-3', 5'-CCGCTCGAGCGCAATTGGAAGAGCCGATGAA-3') was cloned into a His-tagged expression vector (pET-24b, Novagen), expressed in bacteria [BL21(DE3)], and purified using Ni-NTA His-bind resin (Novagen). Polyclonal antiserum was raised against the recombinant protein in rabbit (Capralogics, Hardwick, MA).

ChIP cloning

A modified ChIP protocol was used for ChIP cloning and ChIP PCR (De Belle et al., 2003; Raha et al., 2005). Mixed-stage *daf-2(e1370)* worms were grown on NGM plate seeded with egg/OP50 food (Conte and Mello, 2003) and then washed off the plate with 1XPBS. The worms (500 µl compact worm slurry) were washed twice with 1XPBS and resuspended in cross-linking buffer (1x PBS containing 1% formaldehyde). The worms were partially lysed in a glass homogenizer using 7-8 strokes and incubated in the buffer for 20 min with gentle rocking at room temperature. The cross-linking reaction was quenched with 200 µl of 2.5 M glycine and incubated at RT with gentle rocking. The worm pellet was harvested by centrifugation at 2000 rpm and washed three times with 1X PBS containing proteinase inhibitor cocktail (Sigma, USA). The pellet was snap frozen in liquid nitrogen and stored at -70°C. The pellet was washed once and then

resuspended in 2 ml SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris, pH 8.1) containing proteinase inhibitor cocktail followed by incubation on ice for 10 min. The worm mixture was split into 750 µl aliquots and sonicated 7 times using Misonix sonicator 3000 (New York, NY) with output setting 8 for 10 sec. The lysate was centrifuged (14,000 rpm, 4°C) and supernatant was diluted 4 times using ChIP dilution buffer (0.01% SDS, 1.1% triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1 and 167 mM NaCl). The lysate was split into four 5 ml aliquots and each was precleared with 50 µl salmon sperm DNA/protein A agarose (Upstate, USA) and 20 µl pre-immune serum for 1 hr at 4°C. The precleared supernatant was collected following centrifugation at 2,000 rpm and incubated with 25 µl anti-DAF-16 antibody for 12-16 hr at 4°C. The mixture was then incubated with 50 µl salmon sperm DNA/protein A agarose for 2 hr at 4°C and the complex of DNA-protein-antibody-protein A agarose collected by centrifugation. This complex was washed on a rotating platform with 1 ml of the following solutions: once with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, 500 mM NaCl), once with LiCl immune complex wash buffer (250 mM LiCl, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and three times with 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA-protein complex was eluted with 250 µl elution buffer (1% SDS, 0.1 mM $NaHCO_3$) with brief vortexing followed by incubation at RT with rocking. The supernatant was collected by centrifugation at 2000 rpm. The elution step was repeated once more and the supernatants

were collected and pooled. 50 µl of 5 M NaCl was added and the complexes were reverse-crosslinked at 65°C for 12h. To the mixture, 10 µl 500 mM EDTA, 20 µl 1M Tris-HCl (pH 6.5) and 20 µl of proteinase K (10 mg/ml) was added and incubated at 45°C for 2h. The DNA was then purified using phenol-chlorofom-isoamyalcohol and collected by ethanol precipitation. The DNA was dissolved in 10 µl 10 mM Tris-HCl (pH 8.5) and treated with DNA polymerase I (Roche Biochemicals, USA) for 15 min at 16°C in presence of 40 µM dNTPs. The polymerase was inactivated at 70 °C for 15 min and DNA purified by phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation. The purified DNA was dissolved in 10 µl of 10 mM Tris-HCl (pH 8.5) and *EcoRI* adaptor (Promega, USA) was ligated to the end in an overnight ligation reaction at 16°C. The ligated DNA was PCR amplified using primer designed from the *EcoRI* adaptor (5'-AATTCCGTTGCTGTCG-3') and size fractionated on a 0.8% agarose gel. The DNA fragments (300 bp - 500 bp or 500 bp - 1000 bp) were gel extracted and ligated to pGEM-T Easy vector (Promega, USA). The inserts were amplified using M13 reverse and forward primers and inserts above 300 bp sequenced using the same primers. The sequences were BLASTed against the *C. elegans* genomic database and the region analyzed by bioinformatics for known DAF-16 binding sites.

ChIP PCR

The samples for directed ChIP PCR to validate cloned targets were similar to the ChIP cloning procedure with some modifications. 300-400 µl of frozen compact worm pellet prepared as above was resuspended in HEPES lysis buffer (50 mM HEPES-KOH,

pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF and proteinase inhibitor cocktail) and sonicated 8 times. Following centrifugation, the supernatant was pooled into 750 µl aliquots and precleared with 50 µl salmon sperm DNA/protein A agarose for 1h at 4°C). 0.5% of the supernatant was saved as input sample. Then, 25 µl of DAF-16 antibody or pre-immune serum was added and incubated overnight at 4°C. The beads were washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl, once with LiCl immune complex wash buffer and thrice with 1x TE. The beads were resuspended in 300 µl Proteinase K buffer and 2 µl proteinase K was added followed by incubation at 45°C for 2h. The input was diluted in Proteinase K buffer and incubated with 5 µl proteinase K at 55°C for 4h. All the samples were reverse cross-linked at 65°C overnight, purified by phenol-chloroform extraction and ethanol precipitation. The input was resuspended in 200 µl and ChIP samples in 40 µl of 10 mM Tris-HCl pH 8.5. For validation of targets, PCR was performed in 25 µl using 20 nmol of each primer designed to amplify 300-400 bp of ChIP DNA and 2.5 µCi ^{32}P α-dATP at 95°C for 30 sec, 55°C for 1 min, 72°C for 3 min for 28-29 cycles. The PCR amplicons were size fractionated using 6% acylamide gel, visualized and quantitated using a Fuji Phosphoimager FLA-5000 (Fuji PhotoFilm, Tokyo, Japan). The primers used for ChIP PCR are available on request.

Quantitative RT-PCR

Total RNA was isolated from mixed stage of wild type, *daf-2(e1370)* or *daf-16(mu86)* worms (Ambion, Austin, TX) and reverse transcribed using Superscript II

reverse transcriptase (Invitrogen). PCR reaction was performed in 25 µl with 5 µl of cDNA, 20 nmol of each primer and suggested amount of SYBR Green I dye according to manufacturer's instructions (Applied Biosystems, UK). Cycling parameters were 95°C for 30 sec, 55°C for 1 min and 72°C for 2 min for 40 cycles, followed by a melting curve analysis. The threshold cycle (C_T) was measured and normalized to the endogenous reference gene *act-1* (actin) providing the number of target molecules (X_n) according to manufacturer's manual. The relative expression level of each gene was determined by comparing X_n values obtained from wild type and *daf-2(e1370)* or *daf-16(mu86)*. All the reactions were repeated multiple times with at least 2 biological replicates and 2 endogenous reference replicates for each strain.

Phenotypic analyses

Life span assay: Life span assays were performed at 20°C. RNAi bacterial strains were grown overnight at 37°C in LB medium supplemented with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline. 2ml of this culture was used to inoculate 100 ml culture and allowed to grow to OD_{600} of 0.6. The bacteria was pelleted and resuspended in 10 ml of 1x M9 buffer. The suspension was used to seed nematode growth medium (NGM) plates supplemented with 1mM IPTG and 100 µg/ml ampicillin (RNAi plates). Seeded plates were dried overnight at room temperature and then stored at 4°C for subsequent use. Life span assays of mutant worms (*lin-2* and *egl*-10) were performed using regular NGM plates. Adult hermaphrodites from each strain were transferred to RNAi or NGM plates at 20°C and allowed to undergo one full generation. L4s or young

adults were then transferred to new RNAi or NGM plates containing 0.1 mg/ml of 5'-flourodeoxyuridine (FUDR) to prevent the growth of progeny (Hosono et al., 1982). Animals were tapped every 2-3 days and scored as dead when they did not respond to the platinum wire pick. Life span is defined as the number of days that worms are alive after they were transferred to FUDR plate (day 1). All the life span assays were repeated at least three times.

Fat staining: Nile red staining of stored fat was performed according to Ashrafi et al., (2003) (Ashrafi et al., 2003). Briefly, Nile Red powder (Molecular Probes, USA) was dissolved to a concentration of 0.5 mg/ml in acetone and stored at –20°C. The stock was diluted 1:250 and overlaid on plates seeded OP50 or RNAi bacteria and allowed to dry. Two gravid adults were placed on the Nile red plates and allowed to undergo one full generation. The fat staining was observed in young adult worms prior to starvation under a fluorescence microscope (Zeiss Axioscope 2+ microscope) using a rhodamine filter (emission 560-590 nm). Images were captured and processed using thc OpenLab.3.1.7 software (Improvision Inc. Lexington, MA) under identical settings. For quantitation, similar areas of the animals were selected. The total fluorescence was calculated as the product of mean fluorescence and the area selected.

Dauer test: The percent dauer was measured at 20°C. Two or three *daf-2(e1370)* gravid adults were placed on RNAi plates and allowed to have progeny. After 4 days at 20°C, the number of dauers was scored. The average percent dauer was calculated from 4-6 individual plates. The experiments were repeated at least twice per each RNAi clones.

V) Figures

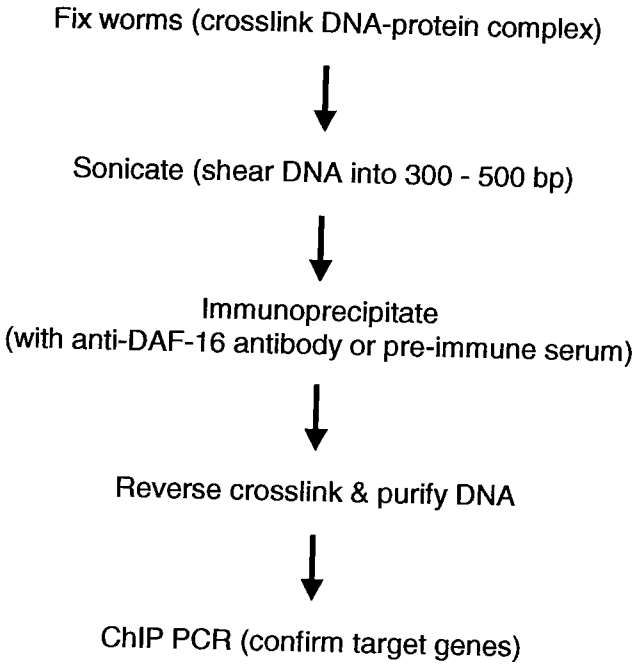


Figure 29. A schematic representation of the modified chromatin immunoprecipitation (ChIP) procedure using the anti-DAF-16 antibody.

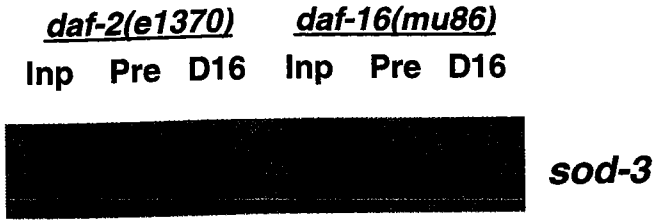


Figure 30. ChIP PCR analysis of the *sod*-3 promoter. The crosslinked DAF-16-DNA complex was immunoprecipitated with either the anti-DAF-16 antibody (D16) or the pre-immune serum (Pre) from either the *daf-2(e1370)* or the *daf-16(mu86)* strain. A *sod-3* promoter fragment containing a consensus DAF-16 binding site was PCR amplified from both samples and visualized by autoradiogram. Input (Inp) samples were loaded as an amount control.

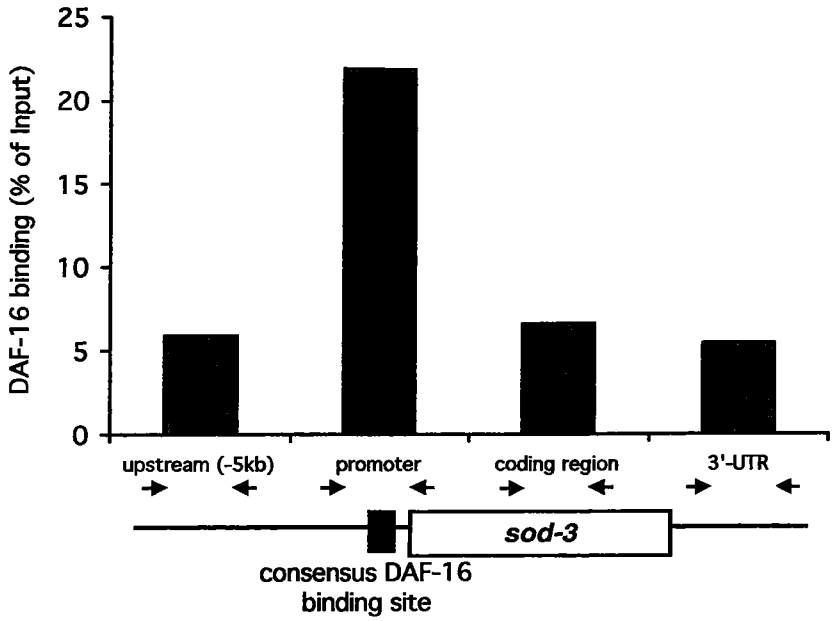


Figure 31. ChIP PCR analysis of the *sod-3* gene. Primers were designed to amplify 300-400 bp of either the promoter, distal upstream region (-5 kb), coding region or 3'-UTR. It shows the specificity of DAF-16 binding to the *sod-3* promoter. The percentage of DNA immunoprecipitated with the anti-DAF-16 antibody relative to input is shown using *daf-2(e1370).*

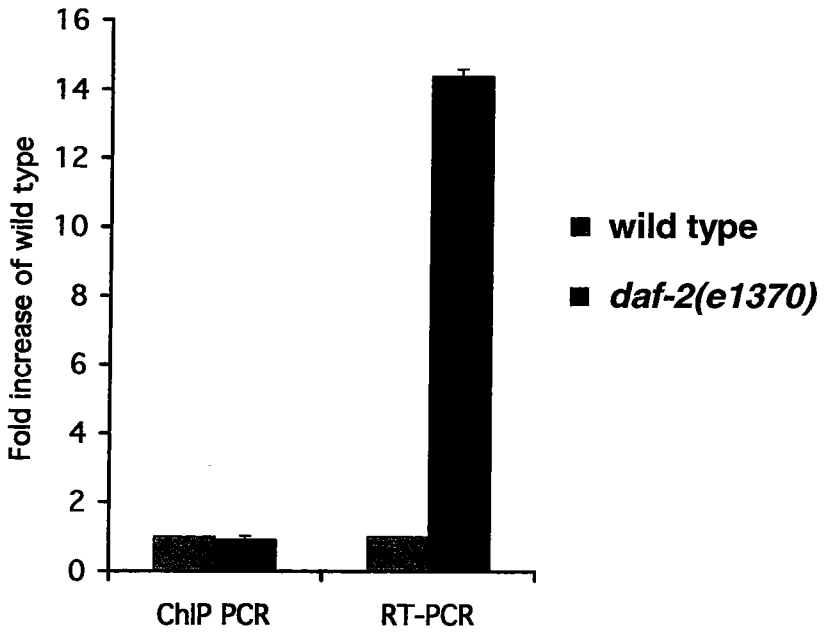


Figure 32. ChIP PCR and RT-PCR analysis of *sod*-3 in *daf-2(e1370)*. DAF-16 binding to *sod*-3 promoter was measured by ChIP PCR and normalized to the amount of input in each strain. The amount of binding in *daf-2(*c1370*)* is shown in fold wild type ± standard error (0.9 ± 0.1). The mRNA expression level of *sod*-3 was obtained by quantitative RT-PCR. The expression level in *daf-2(*e1370*)* is shown in fold wild type ± standard error (14.4 ± 0.2).

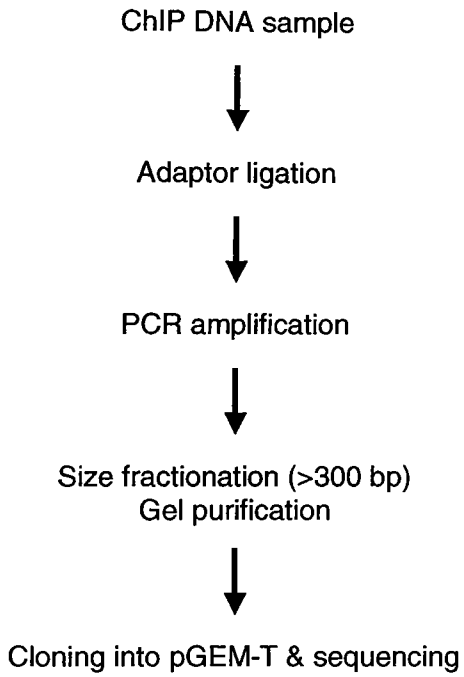


Figure 33. A schematic representation of the ChIP cloning strategy.

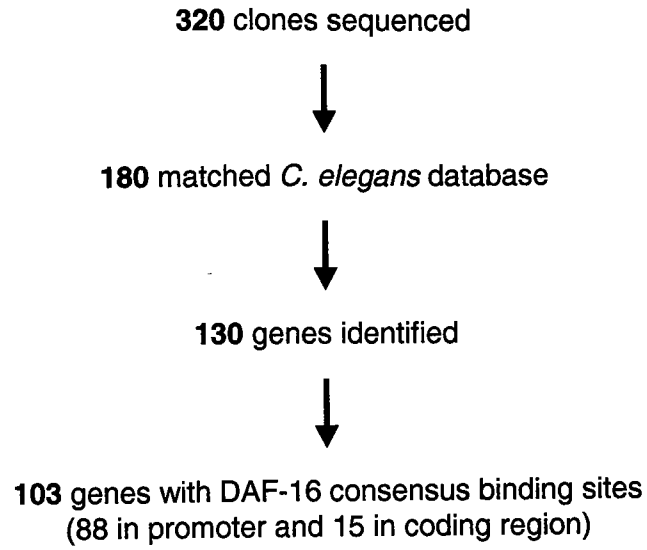


Figure 34. Summary of the cloning results.

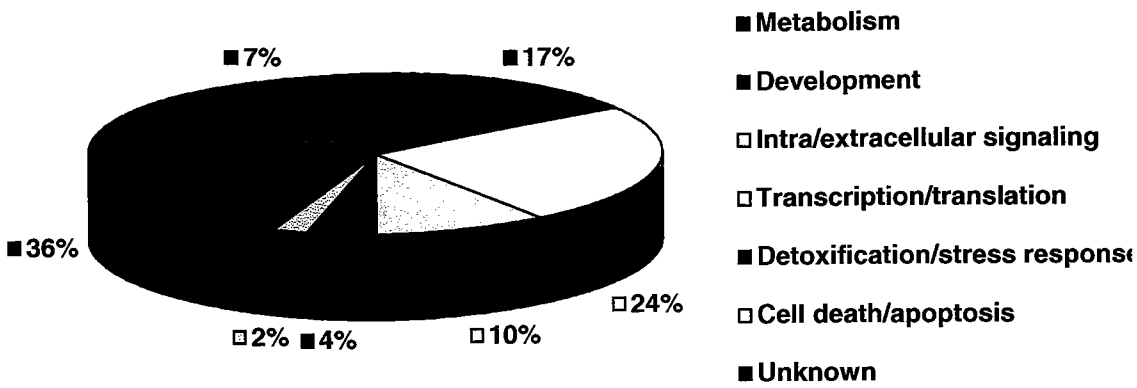


Figure 35. Functional categories of the cloned DAF-16 target genes. The 37 genes without any known function were categorized as Unknown.

gene	homology/description	DAF-16 binding sites (bp)† GTAAAC/TAA, TTG/ATTTAC or TGATAAG/CTTATCA	ChIP PCR ‡	
			daf-2(e1370)	*daf-16(mu86)*
C01A2.2	putative sugar transporter	-30	7.4	1.2
C01B7.1	zinc-finger domain (C2H2 type)	-3300, -3000, +2000	8.5	1.4
C01G6.1 (*aqp-2*)	aquaporin	-3600, **-2100**	9.5	1.9
C14E2.2	aspartate aminotransferase	**-3470, +270, +1410, +2230**	3.0	1.3
C14F5.1	bcl-2/E1B interacting protein-3	**-500**	7.4	1.0
C17D12.6 (*spe-9*)	Notch homolog	**-1070**	3.5	1.0
C29A12.3 (*lig-1*)	ATP-dependent DNA ligase	-1130	3.4	1.1
C36A4.9	acetyl CoA synthetase	-1800, -40, +1400	7.7	1.0
C46E10.7 (*srh-99*)	class H chemoreceptor/olfactory receptor	-3660, -3640	2.3	0.7
D1054.6	c-type lectin/SIV envelope glycoprotein	-2600, **-1600,** -850	7.1 (-1600) *1.6 (-850)	2.0 (-1600) 1.0 (-850)
F07F6.1	plasmodium yoelii PCI domain	**-450**	5.7	1.0
F07F6.5	HOZFP(human ovarian Zn-finger protein) domain	**-1100**	5.7	1.0
F10A3.9 (*str-254*)	7-TM chemoreceptor/olfactory receptor	-1520, **-1160, +840**	2.9	0.8
F10D11.1 (*sod-2*)	Mn-superoxide dismutase	-3750, -2220, **-150, -110**	13.4	0.7
F10D7.5	E3 Ubiquitin ligase/Neuralized	-2240, -600, **+40, +480**	7.0	1.1
F17E5.1 (*lin-2*)	MAGUK family protein kinase	**-1900,** -840	3.0	0.7
F28C1.2 (*egl-10*)	G-protein signaling component	-770, **+610,** +960, **+2260**	5.5	1.2
F31C3.6	death domain associated protein 6	**-100, +390, +460**	6.5	1.8
F37F2.3 (*gst-25*)	glutathione S-transferase	**-230, -10**	3.7	1.2
F42G10.1	Neprilysin-B-like gene (peptidase family M13)	**-4000,** -1900, **+100**	4.6	0.8
F53G12.5 (*mex-3*)	KH domain/RNA binding protein	-60, +5560	3.8	1.4
F54B11.3 (*unc-84*)	nuclear envelope transmembrane protein	**-1300**	7.5	1.0
F54F2.2 (*zfp-1*)	Zinc-finger protein	**-3460,** -3340, +560	17.4	0.9
F58A3.1 (*ldb-1*)	Lim domain binding protein	**-3300**	4.1	1.1
K06A9.1	cell surface glycoprotein/MUC17	-3090, -3030, -2280, **-2230, -1610,** -510	2.1	0.9
K11D9.2 (*sca-1*)	sarco-ER calcium ATPase	-1900, -120	9.7	1.9
M01F1.7	Phosphatidylinositol transfer protein	**-590,** -380	3.5	1.0
T21G5.3 (*glh-1*)	germline helicase	**-720, -700,** +1470	3.5	1.0
T28F2.5 (*ccb-1*)	L-type voltage-gated calcium channel, beta subunit	-2200	2.8	1.0
Y105E8B.9	glutatione transferase zeta 1	**-3600,** -3000, -1200, -60, **+900,** +1200	10.4 (-1200) *1.3 (-60)	1.4 (-1200) 1.5 (-60)
Y38E10A.4	c-type lectin/CUB/Sushi domain	-360, -130, -10	3.1	0.8
Y48A6B.6	voltage-gated potassium channel	**-3320,** -2020, +1440	3.6	1.1
Zk218.8	translation initiation factor 2C (eIF-2C)	-2500	2.7	0.9

† canonical binding sites (bold) and new binding sites (not bold) are shown.

‡ ChIP PCR results are shown as the binding ratio (anti-DAF-16 antibody/pre-immune serum) in each strain.

* two different binding sites were tested for DAF-16 binding in D1054.6 and Y105E8B.9 (the location of binding sites are indicated in paretheses).

Table 5. Analysis of putative DAF-16 target genes.

gene	homology/description	Expression‡	Phenotypes		
		daf-16(mu86)	life span	fat storage	dauer
C01A2.2	putative sugar transporter	104.8 ± 33.1		decreased	
C01B7.1*	zinc-finger domain (C2H2 type)	133.2 ± 24.9	shortened		enhanced
C01G6.1 (*aqp-2*)	aquaporin	209.2 ± 69.2			suppressed
C14E2.2	aspartate aminotransferase	N/D			
C14F5.1	bcl-2/E1B interacting protein-3	201.4 ± 24.3			
C17D12.6 (*spe-9*)	Notch homolog	72.1 ± 16.6			
C29A12.3 (*lig-1*)	ATP-dependent DNA ligase	241.4 ± 40.6			
C36A4.9†	acetyl CoA synthetase	116.4 ± 1.1		decreased	enhanced
C46E10.7 (*srh-99*)	class H chemoreceptor/olfactory receptor	N/D		decreased	enhanced
D1054.6	c-type lectin/SIV envelope glycoprotein	137.2 ± 17.0		decreased	
F07F6.1	plasmodium yoelii PCI domain	82.4 ± 18.2		increased	
F07F6.5	HOZFP(human ovarian Zn-finger protein) domain	100.2 ± 16.3			
F10A3.9 (*str-254*)†	7-TM chemoreceptor/olfactory receptor	52.6 ± 11.2			
F10D11.1 (*sod-2*)	Mn-superoxide dismutase	46.1 ± 1.1			
F10D7.5	E3 Ubiquitin ligase/Neuralized	49.5 ± 26.2			
F17E5.1 (*lin-2*)	MAGUK family protein kinase	48.9 ± 7.8	extended		
F28C1.2 (*egl-10*)	G-protein signaling component	N/D	extended		
F31C3.6	death domain associated protein 6	236.5 ± 2.1			
F37F2.3 (*gst-25*)	glutathione S-transferase	53.2 ± 10.1			
F42G10.1	Neprilysin-B-like gene (peptidase family M13)	30.2 ± 10.3	shortened		
F53G12.5 (*mex-3*)	KH domain/RNA binding protein	146.0 ± 27.0			
F54B11.3 (*unc-84*)	nuclear envelope transmembrane protein	148.5 ± 14.4		decreased	
F54F2.2 (*zfp-1*)	Zinc-finger protein	83.3 ± 3.4	shortened	decreased	enhanced
F58A3.1 (*ldb-1*)	Lim domain binding protein	372.9 ± 19.2	shortened		
K06A9.1	cell surface glycoprotein/MUC17	89.2 ± 22.1		decreased	
K11D9.2 (*sca-1*)	sarco-ER calcium ATPase	105.9 ± 4.9	extended		suppressed
M01F1.7	Phosphatidylinositol transfer protein	51.4 ± 12.4			
T21G5.3 (*glh-1*)	germline helicase	51.5 ± 14.1		decreased	suppressed
T28F2.5 (*ccb-1*)	L-type voltage-gated calcium channel, beta subunit	48.0 ± 7.8			
Y105E8B.9	glutatione transferase zeta 1	314.4 ± 24.9			
Y38E10A.4	c-type lectin/CUB/Sushi domain	58.4 ± 18.2			enhanced
Y48A6B.6	voltage-gated potassium channel	64.4 ± 10.8			enhanced
Zk218.8	translation initiation factor 2C (eIF-2C)	N/D			
sod-3	Mn-superoxide dismutase	30.0 ± 17.2			

* reported as a class I gene that is downregulated in *daf-2(e1370)* by Murphy et al. (2003).

† shown to decrease fat storage when inactivated both in wild type and daf-2(e1370) by Ashrafi et al. (2003)

‡ mRNA expression in *daf-16(mu86)* was compared to N2 and shown as percent N2 ± standard error.

N/D, not-determined because the expression level was too low.

Table 6. Summary of expression and phenotypic analyses of DAF-16 target genes.

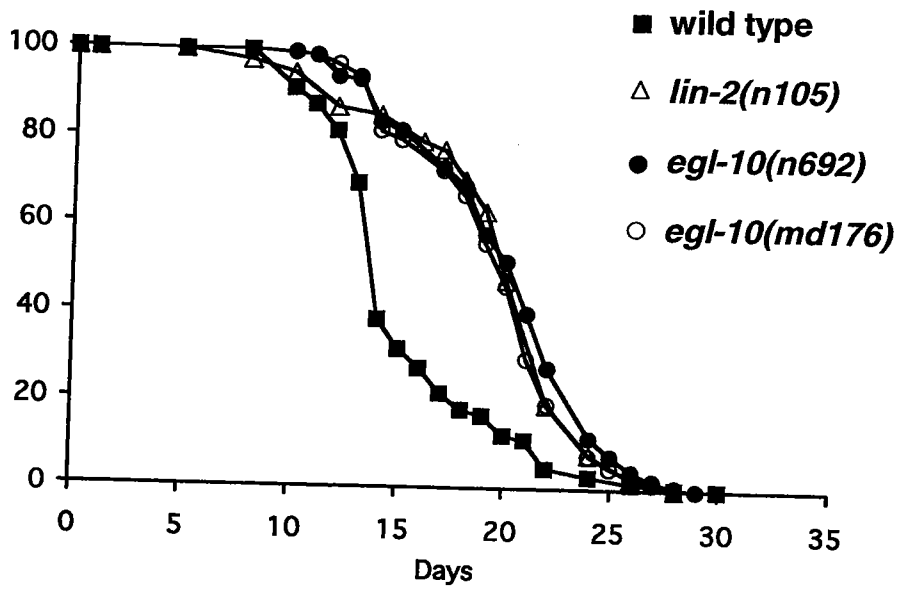


Figure 36. Life span analysis of *egl-10(n692, md176)* and *lin-2(n105)*. Average life span ± standard error (total number of animals scored): wild type, 15.2 ± 0.2 (278); *egl-10(n692)*, 20.0 ± 0.4 (145); *egl-10(md176)*, 19.5 ± 0.3 (145); *lin-2(n105)*, 19.7 ± 0.4 (166).

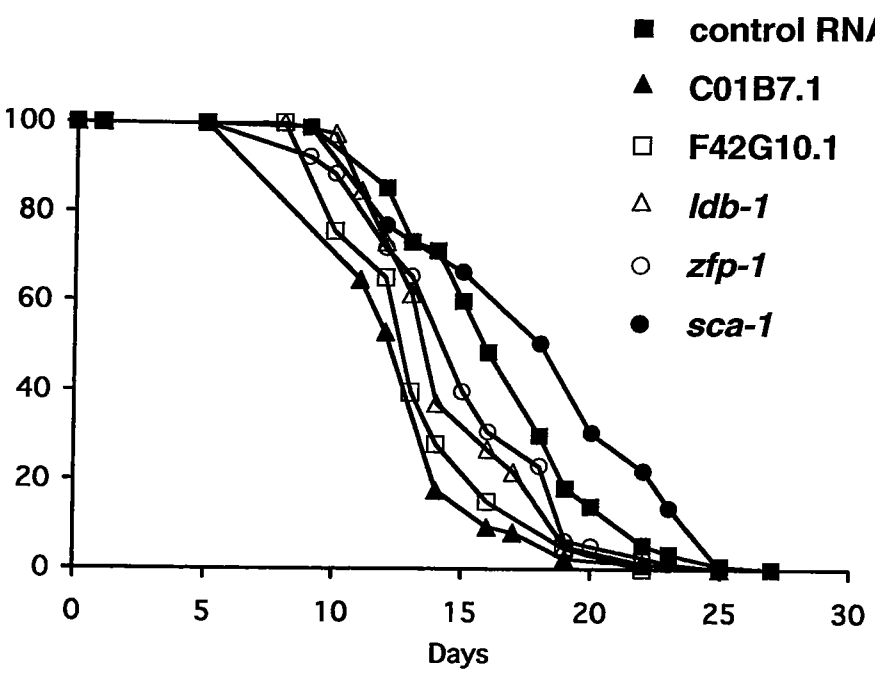


Figure 37. Life span analysis of target genes on RNAi. Average life span ± standard error (total number of animals scored): control RNAi, 16.7 ± 0.2 (264); C01B7.1, 13.4 ± 0.3 (85); F42G10.1, 13.7 ± 0.4 (86); *ldb-1*, 14.8 ± 0.4 (78); *zfp-1*, 15.2 ± 0.4 (78); *sca-1*, 18.4 ± 0.5 (95).

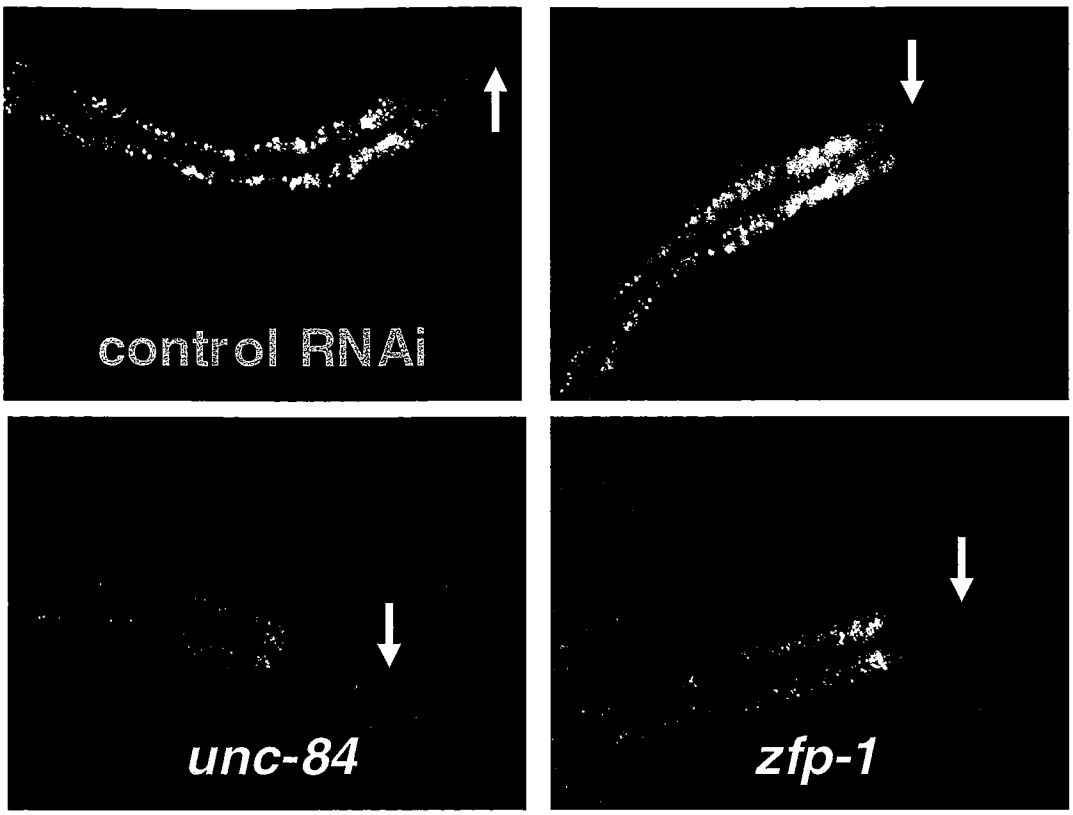


Figure 38. Analysis of fat storage using Nile Red. The worms were grown on RNAi plates in presence of Nile Red and the fat content of young adults was observed. Some representative clones are shown. Inactivation of F07F6.1 (red) increases fat storage whereas others (yellow) result in decrease. Pharynx is indicated with arrows.

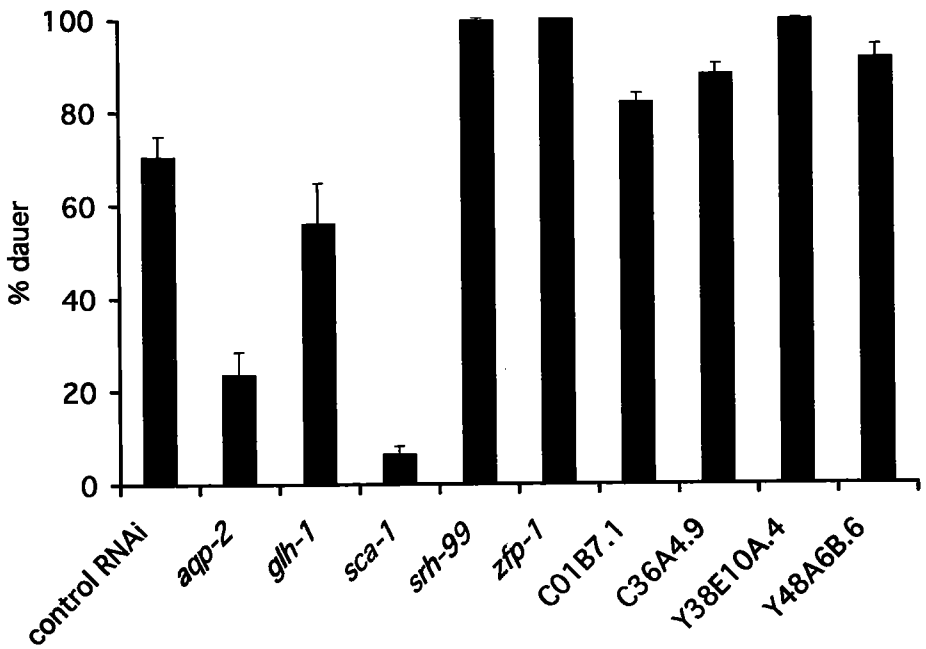


Figure 39. Analysis of dauer formation. Average % dauer ± standard error (total number of animals scored, number of trials): control RNAi, 70.4 ± 4.6 (368, 12); *aqp-2*, 23.7 ± 4.9 (177, 4); *glh-1*, 56.1 ± 8.7 (180, 3); *sca-1*, 6.5 ± 1.8 (154, 5); *srh-99*, 99.4 ± 0.6 (174, 4); *zfp-1*, 100.0 ± 0.0 (332, 8); C01B7.1, 82.0 ± 1.9 (183, 4); C36A4.9, 88.1 ± 2.2 (185, 4); Y38E10A.4, 99.6 ± 0.3 (230, 4); Y48A6B.6, 91.2 ± 2.9 (272, 4).

Categories	Previous studies			This study	
	Genes (Total)	Percent	References	Genes (Total)	Percent
DAF-16 binding sites (5 kb of promoter)	58 (514)	**11.3%**	Murphy *et al.* (2003)	88 (130)	**67.7%**
genes showing DAF-16 dependent phenotypes	4 (17)	**23.5%**	Lee *et al.* (2003)	18 (33)	**54.5%**
genes extending life span upon inactivation	89 (16,475)	**0.5%**	Hamilton *et al.* (2005)	3 (33)	**9.1%**
genes affecting fat storage	417 (16,757)	**2.5%**	Ashrafi *et al.* (2003)	9 (33)	**27.3%**

Table 7. Enrichment of DAF-16 targets by ChIP-based cloning strategy.

Table 8. The list of 103 target genes identified by ChIP cloning

gene	homology/description	consensus DAF-16 binding site
[redacted]		
B0496.4	unknown	**-1850, -1410, -80,** -50
B0513.4	unknown	**-4150, -3430,** -720
B0513.5	proline dehydrogenase/proline oxidase 2	-2830, **-120, +600,** +2610, **+2860**
C01A2.2	putative sugar transporter	-30
C01B7.1	zinc-finger domain (C2H2 type)	-3300, -3000, +2000
C01B7.3	unknown	-4600, +300, +640
C01G6.1 (*aqp-2)*	aquaporin	-3600, **-2100**
C03C10.2	caesin kinase/tau tubulin kinase	-2360, 1930, +760
C03C10.5	probable t-RNA methyltransferase	-4750, -1140
C14E2.2	aspartate aminotransferase	**-3470, +270, +1410, +2230**
C14F5.1	bcl-2/E1B interacting protein-3	**-500**
C17D12.2 (*unc-75*)	RNA binding protein	**-5200**
C17D12.6 (*spe-9*)	Notch homolog	**-1070**

C18E3.9	unknown	-200
C18H7.4	protein tyrosine kinase	-1450, -1350, **+740, +970**
C18H7.6	pancreatic ribonuclease	**-1010, -780,** +1320, +1410
C25E10.1	C4 type zinc finger/nuclear hormone receptor	**-2910,** -2770, **-300**
C29A12.3 (*lig-1*)	ATP-dependent DNA ligase	-1130
C32D5.5	histone tail methylase	-1910, -1820, **-1170**
C36A4.9	acetyl CoA synthetase	-1800, -40, +1400
C41H7.7	c-type lectin/CUB domain	-230, +120
C46E10.3	unknown	-1120, -1100
C46E10.4	F-box domain	**-4150**
C46E10.7 (*srh-99*)	class H chemoreceptor/olfactory receptor	-3660, -3640
C52G5.3	Fragile X mental retardation syndrome related protein 2	**-420**
D1054.6	c-type lectin/SIV envelope glycoprotein	-2600, **-1600,** -850
F02E11.3	unknown	**-1310, +2000**
F07F6.1	plasmodium yoelii PCI domain	**-450**
F07F6.5	HOZFP(human ovarian Zn-finger protein) domain	**-1100**
F10A3.9 (*str-254*)	7-TM chemoreceptor/olfactory receptor	-1520, **-1160, +840**
F10D11.1 (*sod-2*)	Mn-superoxide dismutase	-3750, -2220, **-150, -110**

F10D7.5	E3 Ubiquitin ligase/Neuralized	-2240, -600, **+40, +480**
F17E5.1 (*lin-2*)	MAGUK family protein kinase	**-1900,** -840
F19C6.4	Neprilysin (peptidase family M13)	**-4100,** -480, **+400**
F19C7.1	unknown	**-3370,** -3030, -1830, **+780**
F19C7.4	serine carboxypeptidase S28	**-580,** -250, +950
F20C5.2 (*klp-11*)	kinesin like protein	-5430, -5330, **+5140**
F28C1.2 (*egl-10*)	G-protein signaling component	-770, **+610,** +960, **+2260**
F28C1.3	Rnase inhibitor type leucine rich repeat	-990
F31C3.6	death domain associated protein 6	**-100, +390, +460**
F31C3.7 (*rrn-1*)	18s rRNA	**-90**
F37F2.3 (*gst-25*)	glutathione S-transferase	**-230, -10**
F42G10.1	Neprilysin-B-like gene (peptidase family M13)	**-4000,** -1900, **+100**
F45D3.2	unknown	**-4170,** -3610, -3590, **-3070,** -1390, -830
F48F5.5 (*fce-2*)	farnesylated proteins converting enzyme 2	**-3250,** -1800
F52D2.6	unknown	-2090, +1570, **+4280**
F53G12.5 (*mex-3*)	KH domain/RNA binding protein	-60, +5560
F53G2.6 (*tsr-1*)	human transportin-3	-3230, +9830
F54B11.3 (*unc-84*)	nuclear envelope transmembrane protein	**-1300**
F54F2.2 (*zfp-1*)	Zinc-finger protein	**-3460,** -3340, +560
F58A3.1 (*ldb-1*)	Lim domain binding protein	**-3300**

F58A4.3 (*hcp-3*)	HoloCentric chromosome binding protein/histone	-5920, -5820
F58A4.7 (*hlh-11*)	helix-loop-helix DNA binding protein	-230, -130
K06A9.1	cell surface glycoprotein/MUC17	-3090, -3030, -2280, -**2230, -1610,** -510
K10G4.9 (*srw-30*)	Serpentine receptor class-W	-3090, **-1410**
K11D9.2 (*sca-1*)	sarco-ER calcium ATPase	-1900, -120
M01F1.7	Phosphatidylinositol transfer protein	**-590,** -380
M01H9.3	cell surface glycoprotein	**-360, -260,** +790
M03C11.5	AAA+-type ATPase/peptidase M41 domain	-4870, -2900, +4080, +4290, +4370
M7.2 (*klc-1*)	kinesin light chain	-4060, -3400
T05A10.1 (*sma-9*)	Zinc-finger protein	-2810, **+4090,** +5420, +5520, +6480
T05A8.5	WD domain/cysteine peptidase	-4500, -2700
T12D8.1	putative transcription factor HALR/MLL3	**-3060,** -2880, +3000
T21G5.3 (*glh-1*)	germline helicase	**-720, -700,** +1470
T24B8.3	GTPase regulator-like protein	**-3980, -3050, -770,** -310, -290, +40
T28F2.5 (*ccb-1*)	L-type voltage-gated calcium channel, beta subunit	-2200
W07G4.2	glycosyltransferase/phospholipase A2	**-2990,** -1680, -640
W07G4.3	protein kinase	-2530, -1740, **+2230**

Y105E8B.9	glutatione transferase zeta 1	**-3600,** -3000, -1200, -60, **+900,** +1200
Y110A7A.12	Vacuolar H+ ATPase V1 sector, subunit B (ATP synthase)	-1760, -1320
Y111B2A.3	unknown	-2490, -2280, **-1380, +2150**
Y111B2A.4	myosin heavy chain	**-4400, -870**
Y119D3A.1	cyclin-like F-box protein	**-150**, -120
Y37B11A.2	DNA polymerase zeta, catalytic subunit	-3500, +1280, +3460, +5760
Y38E10A.4	c-type lectin/CUB/Sushi domain	-360, -130, -10
Y38F2AL.4 (*vha-3)*	ATP synthase subunit C	**-2490, -2480,** +160
Y48A6B.6	voltage-gated potassium channel	**-3320,** -2020, +1440
Y48G1BM.5	protein tyrosine kinase	-2130
Y54G11A.7	Immunoglobulin/major histocompatibility complex	**-1710,** +2200
Y59E1B.2	F-box domain/murine adult male testis cDNA	**-1650**
Y67D8A.3	transcription factor *Doublesex*	-4860, -4800, -860
ZC15.6	C-type lectin	-3570, +310, +1240
ZC302.2	WD domain/G-beta repeat	-4900, **-4200**
Zk218.8	translation initiation factor 2C (eIF-2C)	-2500
ZK546.13 (*mdt-4*)	vitamin-D receptor interacting protein complex	+970
ZK546.17	unknown	-1820

ZK673.2	Adenylate kinase	**-2620,** -1160, -710
ZK899.8 (*gap-2*)	GTPase-activator protein	**-4300, -2290**
B0350.2 (*unc-44*)	ankyrin-like protein	+1630, +18230
C17D12.5	ubiquitin conjugating enzyme E2	**+860**
C29A12.1	MEKKK 4	+400
C43G2.1	haemolysin-III type/adiponectin receptor	+320
F17H10.1	esterase/lipase/thioesterase	+1020
F48F5.1	protein tyrosine phosphatase	+4090
R07G3.3	unknown	**+860,** +3450, +4940, +5440
R153.1 (*pde-4*)	3',5'-cyclic nucleotide phosphodiesterase	+2540, +2830, **+3710**
T01A4.1	adenylate guanylate cyclase catalytic domain	**+6520**
T10D4.12 (*sri-32*)	serpentine receptor class I protein	+150, +730, +800
T20G5.4	bestrophin 2	+360, +500, +2190
Y116F11B.14	unknown	+520
Y48G1BM.6	unknown	+1150
Y65B4BL.5	long chain fatty acid acyl CoA synthetase (ligase 1)	+2630
Y71G12B.11	talin	+13130, +14640

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The forkhead transcription factor, DAF-16 plays a central role in longevity, stress response, metabolism and development. Although many signalling pathways are suggested to converge onto DAF-16, at the beginning of this study, the insulin-like signalling pathway, consisting of the insulin-like receptor (*daf-2)*, PI 3-kinase (*age-1*) and AKT (*akt-1/2*), was the only known molecular pathway to modulate DAF-16 activity in *C. elegans*. Therefore, we first investigated whether other molecular pathways exist to regulate DAF-16 activity.

In Chapter II, we identified the JNK signalling pathway as a novel input into DAF-16. We showed that JNK regulates DAF-16 activity by directly binding to and phosphorylating DAF-16. Previously, four putative AKT phosphorylation sites has been identified in *C. elegans* and shown to regulate the nuclear translocation of DAF-16 (Lin et al., 2001). Here, we identified total of 8 putative JNK phosphorylation sites on DAF-16, among which 5 reside in the N-terminus that is phosphorylated by JNK in *in vitro* kinase assay (Figure 21 and 40). Notably, the residues phosphorylated by JNK are separate from those targeted by AKT (Figure 40). Thus, this study identifies a novel molecular input into DAF-16 independent of the insulin-like signalling pathway.

The precise determination of JNK phosphorylation site(s) on DAF-16 will advance understanding of regulatory role of JNK. By mutating the phosphorylation site(s), we will be able to examine whether the phosphorylation by JNK is directly required for nuclear translocation of DAF-16 upon heat shock, and whether there might exist additional molecular pathway(s) to mediate stress signal besides JNK signalling. Alternatively, JNK might regulate DAF-16 indirectly by controlling other components

involved in the translocation of DAF-16 such as 14-3-3 protein, a well-characterized nuclear transporter of FOXO in mammals. Recent observations showed that JNK phosphorylates 14-3-3 proteins upon cellular stress or DNA damages, and antagonizes the effect of AKT signalling (Sunayama et al., 2005; Yoshida and Miki, 2005). Thus, we cannot rule out the possibility that JNK might regulate DAF-16 by a secondary mechanism, not by direct phosphorylation.

It is intriguing that JNK promotes the nuclear translocation of DAF-16, whereas AKT sequesters DAF-16 in the cytosol by phosphorylation. It is possible that the animal possesses several means to regulate this key molecule, DAF-16 as it is involved in number of biological processes. For instance, metabolic changes or developmental signal can be transmitted through AKT, a negative regulator, while environmental stress is mediated by JNK, a positive regulator. In fact, the animal will face more complicated situations in nature where two or more different signals are transmitted to DAF-16 simultaneously, some of which might lead to opposite results on DAF-16 localization like AKT versus JNK. Thus, it will be interesting to investigate how the animal coordinates multiple signals to regulate the translocation and activity of DAF-16 in various circumstances.

This study also demonstrates the connection between stress resistance and longevity, and provides a clue how an animal can adapt to environmental stresses. However, it still remains unclear what accounts for the extra life of worms overexpressing JNK in the molecular level. Does activation of DAF-16 by JNK affect specific target genes (e.g. stress responsive genes), which might play key roles in

determining life span? Are worms overexpressing JNK more tolerant to aging processes, such as damages in proteins or lipids, or decline of major cellular functions? It is also puzzling that overexpression or elevation of the activity of JNK often leads to cell death in mammals (Davis, 2000), but longer life in *C. elegans* and *Drosophila* (Wang et al., 2003). It is possible that JNK plays both beneficial and detrimental roles, but decides whether to protect or kill the cell depending on the level or kind of input signals. Alternatively, JNK might play different roles in lower eukaryotes.

For its importance in many biological processes including life span regulation, various attempts have been made to seek for the downstream components of DAF-16. Although, recent studies suggested a number of probable DAF-16 downstream target genes, none of these studies could determine whether the described targets were direct *in vivo* targets due to the lack of biochemical evidences.

As discussed in Chapter III, we developed a ChIP-based cloning strategy in *C. elegans* and identified more than 100 putative target genes. We show for the first time in *C. elegans* that DAF-16 directly binds to previously known and novel target promoters. We also show that the large portions of the cloned target genes are likely to be functional DAF-16 targets, indicating that the ChIP-based cloning strategy is very powerful and effective for identifying relevant targets of DAF-16. Individual analysis of each of the target genes in depth will allow us to understand the complex regulation of DAF-16 downstream signals.

More thorough analysis of DAF-16 binding sites should also be followed for target promoters cloned. A number of target genes we identified only contain a new

binding site predicted by Murphy *et al.* (Murphy et al., 2003), which, however, has not been determined biochemically. As previously described using canonical DAF-16 binding site (Furuyama et al., 2000), biochemical dissection of the binding site and their binding to DAF-16 protein will be necessary to validate this new binding site. As we also cloned quite a large number of promoters without any known DAF-16 binding site from our cloning (Figure 34), as yet unknown binding site for DAF-16 might exist. These promoters need to be analyzed for the common motif(s) among them. In addition, it will be also interesting to examine how many clones without any known DAF-16 binding site affect DAF-16 dependent phenotypes, and compare with the result obtained from genes containing DAF-16 binding sites (Table 6), which might be a good indication of existence of hidden binding site(s) as well.

Although we show that DAF-16 binds to multiple target promoters, it still remains elusive how DAF-16 selects targets of interest out of hundreds of putative binding targets upon certain input signal and obtains input-specific selectivity. This study suggests that co-activator or repressor might be required for transcriptional activity of DAF-16 (Figure 32). It is possible that various inputs modify DAF-16 differently or activate different set(s) of co-regulators, resulting in specific binding to or modulation of selected target promoters (Figure 41). Therefore, it will be important to seek for co-regulators of DAF-16.

Taken together, this study identifies an additional molecular input into DAF-16 and provides an insight into the underlying mechanism that connects stress resistance with longevity. It also uncovers the novel outputs of DAF-16 and depicts the complexity

of regulation through DAF-16, a key regulator of longevity. In conclusion, our finding will broaden the present knowledge on many biological processes governed by DAF-16 such as life span regulation, metabolism and development.

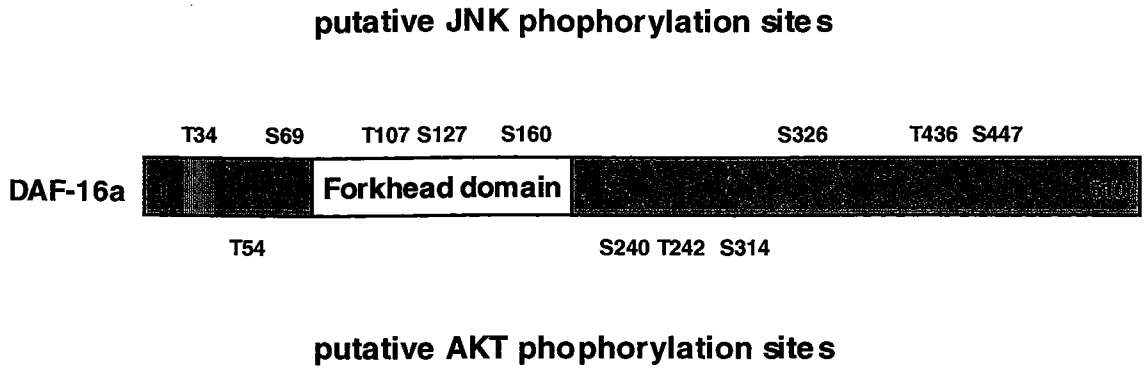


Figure 40. Putative phosphorylation sites of JNK and AKT on DAF-16. Total of 8 and 4 threonine (T) or serine (S) residues are putative JNK (blue) and AKT (red) phosphorylation sites, respectively.

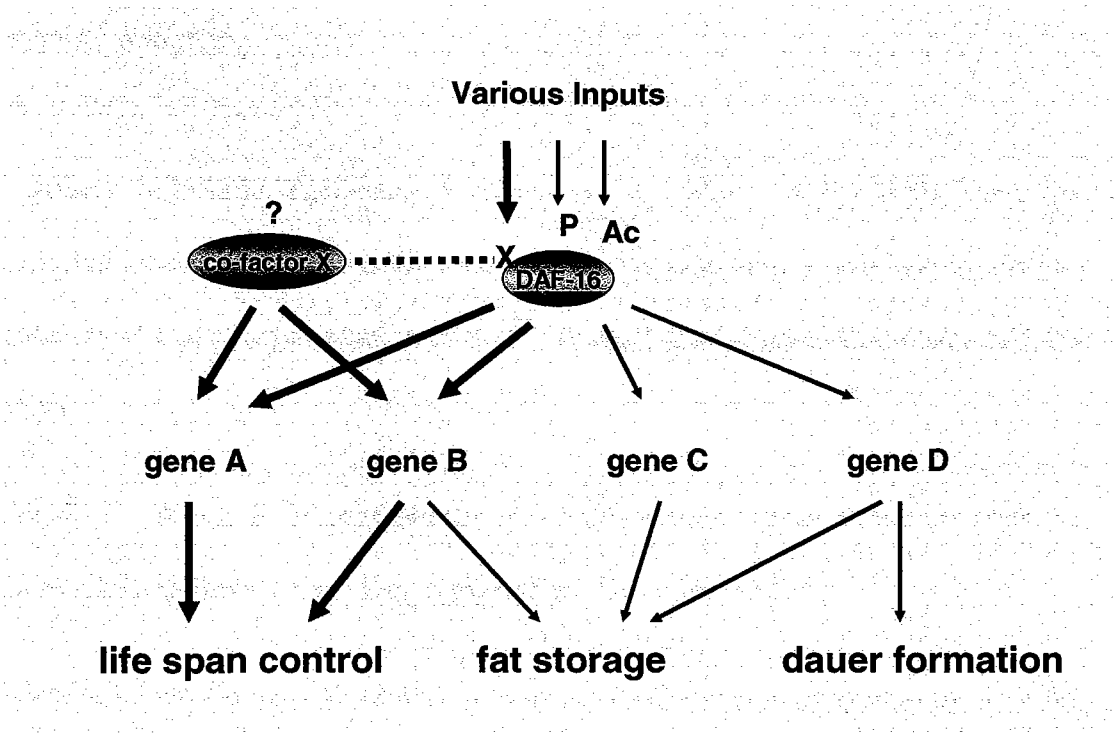


Figure 41. Model: transcriptional regulation by DAF-16. DAF-16 receives inputs from multiple signaling pathways. Depending on the input, DAF-16 might be modified differently (for example, P, phosphorylation; Ac, acetylation; X, unknown modification). This would allow DAF-16 to associate with unknown cofactors or undergo conformational modification to regulate the expression of specific sets of target genes. The modulation of selected target genes will collectively determine the phenotypes associated with DAF-16. Life span regulation is shown as an example.

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