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A Fasting-Responsive Signaling Pathway that Extends Life Span in *C. elegans*

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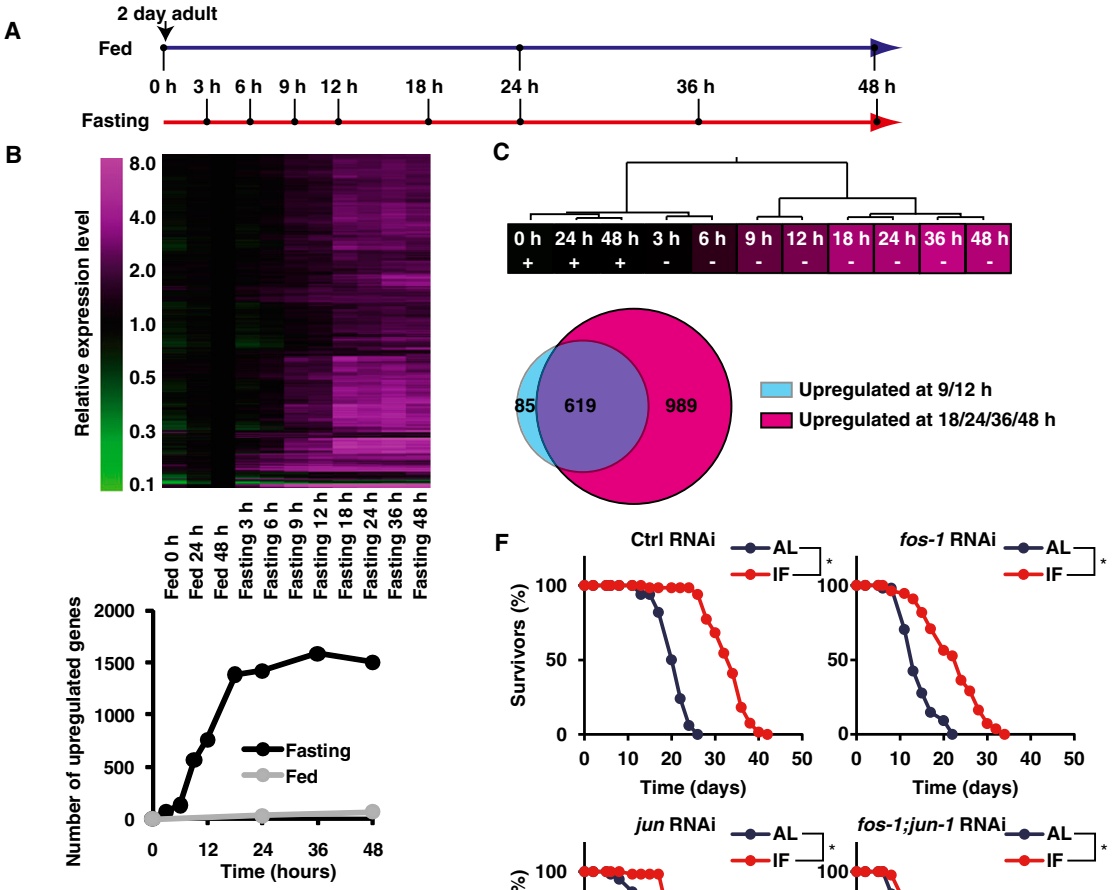
SUMMARY

Intermittent fasting is one of the most effective dietary restriction regimens that extend life span in *C. elegans* and mammals. Fasting-stimulus responses are key to the longevity response; however, the mechanisms that sense and transduce the fasting stimulus remain largely unknown. Through a comprehensive transcriptome analysis in *C. elegans*, we find that along with the FOXO transcription factor DAF-16, AP-1 (JUN-1/FOS-1) plays a central role in fasting-induced transcriptional changes. KGB-1, one of the *C. elegans* JNKs, acts as an activator of AP-1 and is activated in response to fasting. KGB-1 and AP-1 are involved in intermittent fasting-induced longevity. Fasting-induced upregulation of the components of the SCF E3 ubiquitin ligase complex via AP-1 and DAF-16 enhances protein ubiquitination and reduces protein carbonylation. Our results thus identify a fasting-responsive KGB-1/AP-1 signaling pathway, which, together with DAF-16, causes transcriptional changes that mediate longevity, partly through regulating proteostasis.

INTRODUCTION

Environment can influence life span in many ways. One environmental factor, food, has a well-documented effect on the rate of aging. It has been shown that dietary restriction, a reduction in food intake that does not cause malnutrition, can increase the healthy life span of laboratory model organisms, including yeasts, flies, worms, fish, rodents, and rhesus monkeys (Anderson et al., 2009; Colman et al., 2009; Fontana et al., 2010; Kenyon, 2010). Dietary restriction protects rodents and rhesus monkeys from age-related disorders, and it reduces risk factors for diabetes, cardiovascular disease, and cancers in humans (Anderson et al., 2009; Fontana et al., 2010). In mammals, there are two ways of restricting diet, chronic calorie restriction and intermittent fasting (IF), both of which have proven effective in increasing life span and disease resistance (Anderson et al., 2009; Fontana and Klein, 2007). During the standard IF in mice, food is available ad libitum (AL) every other day. Compared to AL-treated animals, IF-treated animals eat more (sometimes twice as much as AL-treated ones) on the day they can access food, and thus IF can extend the life span even if there is little or no overall decrease in calorie intake (Fontana and Klein, 2007; Froy and Miskin, 2010; Mattson and Wan, 2005). Even in *C. elegans*, an IF regimen (an alternate 2 days AL/2 days fasting regimen) has been shown to be one of the most effective dietary restriction regimens for extending life span (Honjoh et al., 2009). Interestingly, even under chronic fasting conditions, worms live longer than under AL conditions (Kaeberlein et al., 2006; Lee et al., 2006), suggesting the importance of fasting-stimulus responses in IF-induced longevity. Fasting induces profound changes, including transcriptional, posttranscriptional, and metabolic changes. The maximal longevity response to IF is shown to be mediated by the Rheb/TOR pathway, which is required for fasting-triggered nuclear translocation of DAF-16 (*C. elegans* FOXO) as well as transcriptional changes (Honjoh et al., 2009). These findings suggest the importance of fasting-induced transcription changes in IF-induced longevity. Because DAF-16 is partially responsible for IF-induced longevity (Honjoh et al., 2009), it is possible that, besides DAF-16, there would be other transcription factors responsible for fasting-induced transcriptional changes and therefore IF-induced longevity.

In this study, we performed a comprehensive transcriptome analysis of fasting-stimulus response in *C. elegans*. Subsequent promoter analysis has shown that the AP-1-binding site, together with the FOXO-binding site, is highly enriched in the promoter regions of fasting-induced genes. We then showed that KGB-1, one of the *C. elegans* c-Jun N-terminal kinases (JNKs), acted as a direct activator of AP-1 and was activated in response to fasting and that KGB-1 and AP-1 were involved in IF-induced longevity. We have identified an upstream kinase cascade for the KGB-1/AP-1 axis. Detailed analyses have then demonstrated that two fasting-responsive signaling pathways, which lead to DAF-16 nuclear translocation and KGB-1/AP-1 activation, respectively, play a central role in mediating fasting-induced transcriptional changes. Moreover, we have identified important transcriptional targets of these signaling pathways, which function in IF-induced longevity. These results reveal



D Genes upregulated at 9/12 h (704 genes)

GO term	p-value
aging	5.94E-07
cellular response to chemical	4.01E-05
oxidoreductase activity	4.14E-05
ER-nuclear signaling pathway	9.78E-05
cellular response to protein stimulus	1.26E-04

Genes upregulated at 18/24/36/48 h (989 genes)

GO term	p-value
regulation of cellular process	1.19E-05
neuropeptide signaling pathway	4.81E-04
binding	1.78E-03
signal transduction	2.04E-03
transcription regulator activity	5.02E-03

E

9/12 h	
Matrix_name	p_value
V_FOXO1_02	2.94E-05
V_XFD3_01	6.78E-05
V_AP1_Q2	2.10E-04
V_AP1_Q2_01	2.60E-04
V_HFH1_01	2.78E-04
V_GATA_C	3.14E-04
V_FREAC2_01	4.66E-04
V_AP1_01	7.60E-04
V_AP1_C	9.04E-04
V_AP1_Q4	9.41E-04

Figure 1. Aging-Related Genes Are Induced Early in Response to Fasting

(A) The sampling scheme is shown. At day 2 of adulthood, wild-type N2 worms were transferred to the plate in the presence or absence of food. At the indicated time, worms were collected and total RNAs were extracted.

(legend continued on next page)

a fasting-responsive signaling network that extends life span in *C. elegans*.

RESULTS

Transcriptome Analysis of the Fasting-Stimulus Response Identifies AP-1 as an Important Regulator of IF-Induced Longevity

We reasoned that transcriptional changes in response to fasting stimulus should play an important role in the longevity response to IF. To delineate the whole picture of transcriptional changes in response to fasting, we performed genome-wide gene expression analysis of 2 days (48 hr) fasting stimulus, as outlined in Figure 1A. To identify transcription factors responsible for fasting-induced transcriptional changes and thus IF-induced longevity, we analyzed the promoter regions of selected genes. As genes related to aging and stress response are concentrated in the fasting-induced upregulated genes (see below), we focused on them. We found that a large portion of them began to be upregulated between 6 and 9 hr of fasting (Figure 1B, lower panel). The hierarchical clustering analysis for these 1,708 upregulated genes showed that 3 and 6 hr fasting samples belong to the same group as the fed samples when classified into two groups, and that the other fasting samples are further classified into two groups; 9 and 12 hr fasting and 18–48 hr fasting groups (Figure 1C). Gene ontology (GO) analysis showed that genes related to aging and stress response are much more highly concentrated in the upregulated genes of the 9 and 12 hr fasting samples than in those of the 18–48 hr fasting samples (Figure 1D; Table S1). These analyses thus suggested that the crucial changes in gene expression, which could be related to longevity, should occur rapidly within 9 or 12 hr of fasting.

We previously reported that 24 and 48 hr of fasting result in 40% and 57% life-span extension, respectively (Honjoh et al., 2009). Thus, 48 hr of fasting extended life span more efficiently than 24 hr of fasting; however, even 24 hr fasting has considerable life-span extension effect. It should be pointed out all of the genes listed as aging by GO analysis, which were upregulated at 9/12 hr of fasting (34 genes), were kept upregulated at later time points at 24 and 48 hr of fasting (see Figure 1C). During the period from 12 to 24 or 48 hr of fasting, only 12 genes listed as aging by GO analysis were additionally upregulated despite the fact that fasting-induced genes were more than doubled during this period (Table S1). Thus, aging genes were much more highly enriched in the 9/12 hr fasting samples than in later time points' samples.

We aimed to identify the transcription factors responsible for fasting-induced transcriptional changes and thus IF-induced longevity; therefore, we performed promoter analysis for the fasting-induced genes at 9 and 12 hr fasting (Table S1). To this end, we used the TRANSFAC database, which catalogs vertebrate and worm transcription factors and their known binding sites (Kel et al., 2003). Our analysis has shown that the binding sites of the forkhead transcription factors (FOXO1, XFD3, and HFH1) and the AP-1 (activator protein 1)-binding site are enriched in the promoter regions of the fasting-induced genes at 9 and 12 hr fasting (Figures 1E and S1B). The forkhead transcription factors (DAF-16, *C. elegans* FOXO, and PHA-4, *C. elegans* FOXA) have previously been shown to be involved in dietary-restriction-induced longevity (Greer et al., 2007; Honjoh et al., 2009; Panowski et al., 2007), indicating the validness of our prediction of transcription factors responsible for IF-induced longevity. As the involvement of the forkhead transcription factors in dietary-restriction-induced longevity has already been studied (Greer et al., 2007; Honjoh et al., 2009; Panowski et al., 2007), we focused our attention to AP-1 transcription factor. In mammals, c-Jun and c-Fos constitute the AP-1 transcription factor complex, whose activity is strongly induced by various kinds of stresses (Eferl and Wagner, 2003). In *C. elegans*, JUN-1 (*C. elegans* c-Jun) and FOS-1 (*C. elegans* c-Fos) are also shown to form a heterodimer that binds to the AP-1-binding site (TPA response element [TRE]) (Hiatt et al., 2009). We hypothesized that JUN-1 and FOS-1 should have a role in IF-induced longevity. To test this hypothesis, we first examined the effect of our IF regimen on the life span of the loss-of-function mutant of *jun-1* [*jun-1(gk-557)*]. When the IF regimen increased the life span of N2 (wild-type worms) by 77%, the same IF regimen increased that of *jun-1(gk557)* by 32% (Figure S1C). The IF-induced increase in life span was markedly suppressed with statistical significance in the *jun-1(gk557)* mutant. These results indicate the involvement of JUN-1 in IF-induced longevity. It was possible, however, that the suppression of IF-induced longevity in the *jun-1* mutant could be due to some developmental abnormality of this mutant, as their life span under AL conditions was shorter than that of N2 (Figure S1C; Table S2). To avoid the potential developmental abnormality, we then suppressed *jun-1* expression after completion of development by feeding RNAi. The IF-induced life-span extension of *jun-1* RNAi-treated worms was then reduced as

(B) Expression profiles of genes whose expression was upregulated during 48 hr of fasting. Average expression profiles of two independent experiments are shown (upper). Numbers of upregulated genes, whose expression at the indicated time point was upregulated more than 2-fold as compared to 0 hr fed (both in fed and fasting conditions), are shown (lower).

(C) Hierarchical clustering analysis for the total fasting-induced upregulated genes. Venn diagram for fasting-upregulated genes is shown. Blue circle or red circle indicates genes upregulated at 9/12 hr or 18/24/36/48 hr of fasting.

(D) Top five terms in the GO analysis for 704 upregulated genes at 9 or 12 hr fasting (upper) and 989 upregulated genes after 18 hr fasting, which were not upregulated until 12 hr (lower), respectively, are shown.

(E) Promoter analysis for the upregulated genes at 9 or 12 hr fasting. Top five enriched binding sites are shown. V, vertebrate.

(F) Survival curves of control RNAi- (upper left, n = 50 [AL] and 66 [IF]), *fos-1* RNAi- (upper right, n = 55 [AL] and 54 [IF]), *jun-1* RNAi- (lower, left, n = 59 [AL] and 61 [IF]), or *fos-1;jun-1* RNAi- (lower right, n = 67 [AL] and 44 [IF]) treated worms in AL and IF. *$p < 0.05$ log rank test ($p = 1.2E-20$, 1.6E-9, 6.5E-12, and 7.1E-4 in control RNAi-, *jun-1* RNAi- or *fos-1* RNAi- treated worms, respectively). Mean life-span extension by IF (±SEM) (lower far left) of three independent experiments is shown. *$p < 0.05$ t test.

See also Tables S1, S2, and Figure S1.

compared to that of control RNAi-treated worms with statistical significance (Figure 1F). Moreover, IF-induced longevity was also suppressed in *fos-1* RNAi- and *fos-1;jun-1* RNAi-treated worms with statistical significance (Figure 1F). These results demonstrate that *C. elegans* AP-1 (JUN-1/FOS-1) is involved in IF-induced longevity and suggest that it exerts its longevity effect through inducing gene expression changes in response to fasting. As knockdown of *jun-1* and *fos-1* slightly reduced the life span under AL conditions, knockdown of these genes might make animals generally sick instead of interfering specifically with IF-induced life span. To examine this point, we performed life-span measurement analysis for knockdown animals of *jun-1*, *fos-1*, or both in the well-known long-lived mutant *daf-2(e1370)*. Interestingly, the life span of the *daf-2(e1370)* was not reduced by knockdown of *jun-1*, *fos-1*, or both (Figure S1D), suggesting that depletion of *jun-1* and *fos-1* does not make animals sick but interferes specifically with IF-induced longevity.

The KGB-1/AP-1 Signaling Pathway Is Required for IF-Induced Longevity

We then explored the upstream of AP-1 in a signaling pathway that induces the longevity response to IF. As an important mechanism that induces AP-1 activation is phosphorylation of c-Jun by JNK, which is a well-known stress-activated mitogen-activated protein kinase (MAPK) family member (Eferl and Wagner, 2003; Weston and Davis, 2007), we thought that *C. elegans* JNKs could also function in IF-induced longevity. To test whether *C. elegans* JNKs (there are three JNKs in *C. elegans*, JNK-1, KGB-1, and KGB-2) are required for the IF-induced longevity, we measured the life span of the loss-of-function mutants of *jnk-1*, *kgb-1*, and *kgb-2* [*jnk-1(gk7)*, *kgb-1(km21)*, and *kgb-2(km16)*, respectively] under both AL and IF conditions. Only in the *kgb-1* mutant, the IF-induced increase in life span was markedly suppressed with statistical significance (Figure 2A). These results suggest that KGB-1 is involved in the regulation of IF-induced longevity. Similar to the *jun-1* mutant, life span of the *kgb-1* mutants under AL conditions was, however, shorter than that of N2 (Figure 2A). To avoid the potential developmental abnormality, we suppressed *kgb-1* expression after completion of development by feeding RNAi. The life span of *kgb-1* RNAi-treated worms under AL conditions was almost the same as that of control RNAi-treated worms (Figure 2B). The IF-induced life-span extension of *kgb-1* RNAi-treated worms was then reduced as compared to that of control RNAi-treated worms with statistical significance (Figure 2B). Collectively, these results strongly suggest that KGB-1 functions in IF-induced longevity. Our analysis with a *kgb-1* promoter::gfp reporter construct showed that KGB-1 is ubiquitously expressed (Figure S2).

We then asked whether KGB-1 is activated in response to fasting. As the activation of KGB-1 is accompanied by its dual phosphorylation, we can determine the extent of KGB-1 activation by using anti-dual phosphoKGB-1 antibody (Mizuno et al., 2008). Immunoblotting analysis with this antibody showed that some KGB-1 activation was detected within 30 min after fasting and clear activation occurred within 60 min (Figure 2C, upper panel). As a large portion of the fasting-induced genes began to be upregulated between 6 and 9 hr of fasting (Figure 1B, lower panel), we tested whether KGB-1 is activated for a longer period. Our results showed that KGB-1 activation was maintained at 4.5 and 9 hr of fasting. Control experiments demonstrated that the KGB-1 activation level induced by arsenite, which is a well-known activator of KGB-1, was comparable to that induced by fasting (Figure 2C, lower panel) and that KGB-1 activation was abolished in the mutant of MEK-1 and MLK-1, which are upstream kinases for KGB-1 (see Figure 3). These results indicate that the KGB-1 pathway is activated in response to fasting and therefore strongly suggest that the KGB-1 signaling pathway functions to extend life span through sensing and transducing fasting stimulus in IF.

To test whether JUN-1 and FOS-1 lie downstream of KGB-1, we coexpressed JUN-1 or FOS-1 with KGB-1 in mammalian cells. When coexpressed with wild-type KGB-1, a mobility-shifted band of JUN-1 or FOS-1 appeared (Figure 2D). In contrast, when coexpressed with a kinase-dead form of KGB-1 (KR) or a nonactivatable form of KGB-1 (ADF), no mobility-shifted bands of JUN-1 or FOS-1 appeared (Figure 2D). These results suggest that JUN-1 and FOS-1 are phosphorylated by KGB-1, and are consistent with the idea that KGB-1 is a direct activator of AP-1 (JUN-1/FOS-1 heterodimer).

It has previously been shown that MEK-1 and MLK-1, which are *C. elegans* ortholog of mammalian MKK7 and MLK-1, respectively, act as MAPKK (MAPK kinase) and MAPKKK (MAPKK kinase) for KGB-1, respectively, in the heavy metal stress response pathway (Mizuno et al., 2004). We then asked whether these two protein kinases also function in IF-induced longevity. Our life-span measurements showed that the IF-induced increase in life span in the loss-of-function mutant of *mek-1* [*mek-1(ks54)*] or that of *mlk-1* [*mlk-1(km19)*] was significantly suppressed as compared to that in wild-type N2 (Figure 3). The extent of reduction in IF-induced longevity in *mlk-1(km19)*, however, was smaller than that in *mek-1(ks54)* or *kgb-1(km21)*. So we considered the possibility that other MAPKKKs might also be involved in IF-induced longevity. We tested loss-of-function mutants of NSY-1 and MTK-1, *C. elegans* orthologs of mammalian ASK1 and MEKK4, respectively [*nsy-1(ky400)* and *mtk-1(km14)*]. The reduction in the IF-induced life-span extension in *nsy-1(ky400)* was statistically significant, whereas that in *mtk-1(km14)* was not, suggesting that NSY-1, but not MTK-1, also partially mediates IF-induced longevity. We then made a double-knockout mutant of *mlk-1* and *nsy-1* (*mlk-1;nsy-1*) and subjected it to IF. IF-induced life-span extension in *mlk-1;nsy-1* was significantly reduced as compared to that in wild-type worms (statistically significant, Figure 3). These results taken together demonstrate that a signaling module consisting of MLK-1 and NSY-1 MAPKKKs, MEK-1 MAPKK, and KGB-1 MAPK functions in IF-induced longevity.

As NSY-1 is shown to act also as MAPKKK for PMK-1, one of *C. elegans* p38 MAPKs, in the oxidative stress response pathway (Inoue et al., 2005), it is possible that p38 MAPKs are also involved in IF-induced longevity. Then, we examined the effect of our IF regimen on the life span of each of the loss-of-function mutants of three *C. elegans* p38 MAPKs, *pmk-1*, *pmk-2*, and *pmk-3* (*pmk-1(km25)*, *pmk-2(km37)*, and *pmk-3(ok169)*, respectively). The IF-induced increase in life span was not suppressed with statistical significance in these three mutants (Figure S3),

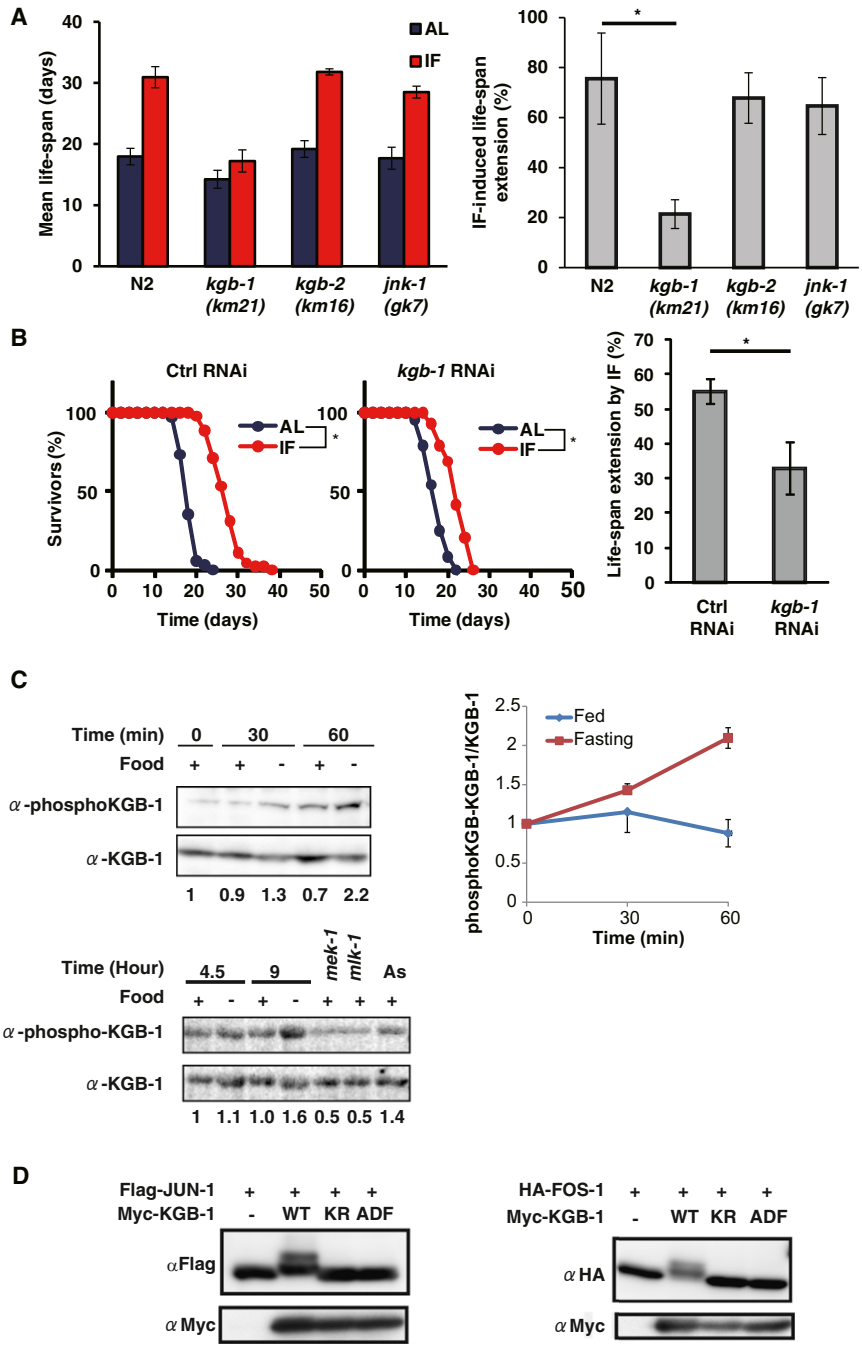


Figure 2. KGB-1 Is Required for IF-Induced Longevity and Lies Upstream of AP-1(JUN-1/FOS-1) in the Fasting-Stimulus Response

(A) Mean life span (±SEM) (left) and mean life-span extension by IF (±SEM) (right) of four independent experiments are shown. *p < 0.05 t test.

(B) Survival curves of control RNAi- (left) and *kgb-1* RNAi- (middle) treated worms in AL and IF are shown. *p < 0.05 log rank test (p = 2.0E-13, and 2.1E-5 in control- (n = 34 [AL] and 29 [IF]) and *kgb-1*-RNAi- (n = 34 [AL] and 45 [IF]) treated worms, respectively). Mean life-span extension by IF (±SEM) of three independent experiments is shown (right). *p < 0.05 t test.

(C) Activation of KGB-1 in response to fasting. KGB-1 activation was analyzed by immunoblot analysis at 30 and 60 min (upper left), and at 4.5 and 9 hr (lower). Mean KGB-1 activation (phosphoKGB-1/total KGB-1) ±SEM of two independent experiments is shown (upper right). In these experiments, worms at 2 days adult in the presence of food were collected at time 0 and moved into plate with (food, +) and without food (food, −), respectively. The value at time 0 is set to 1.

(D) Phosphorylation of JUN-1 and FOS-1 by KGB-1. 293T cells were transfected with Myc-KGB-1 (WT), KGB-1(K67R) (KR), Myc-KGB-1(S198A and Y200F) (ADF), Flag-JUN-1, and HA-FOS-1 as indicated.

See also Figure S2 and Table S2.

indicating that *C. elegans* p38 MAPKs are dispensable for IF-induced longevity.

Evidence that the KGB-1/AP-1 Signaling Pathway Collaborates with DAF-16 to Mediate Fasting-Induced Transcriptional Changes

In mammals, the interaction between JNK signaling and INS/IGF signaling has been reported (van der Horst and Burgering, 2007; Taguchi and White, 2008). In *Drosophila*, JNK signaling can extend life span by antagonizing the INS/IGF signaling pathway (Wang et al., 2003, 2005). In *C. elegans*, JNK-1, one of the other JNKs, has been reported to be able to regulate life span through the INS/IGF signaling effector DAF-16 (Oh et al., 2005), and DAF-16 is shown to be involved in IF-induced longevity (Honjoh et al., 2009). We thus examined whether the KGB-1 signaling pathway affects DAF-16 function. We first tested the effect of the loss of *kgb-1* on fasting-induced upregulation of DAF-16 transcriptional target genes. We measured the expression levels of six genes, which are well-identified DAF-16 target genes during fasting (*aqp-1*, *dod-6*, *hil-1*, *hsp-12.6*, *mtl-1*, and *sod-3*) (Honjoh et al., 2009; Murphy et al., 2003). While their mRNA levels were markedly increased in response to fasting in wild-type worms, their increases were significantly suppressed in the loss-of-function mutant of *kgb-1* (*kgb-1(km21)*) (Figure 4A), suggesting the possibility that KGB-1 signaling is required for the fasting-induced activation of DAF-16. Because DAF-16 translocates to the nucleus in response to fasting (Henderson and Johnson, 2001) and because the Rheb pathway, which also mediates IF-induced longevity, is shown to be required for fasting-induced nuclear translocation of DAF-16 (Honjoh et al., 2009), we then considered the possibility

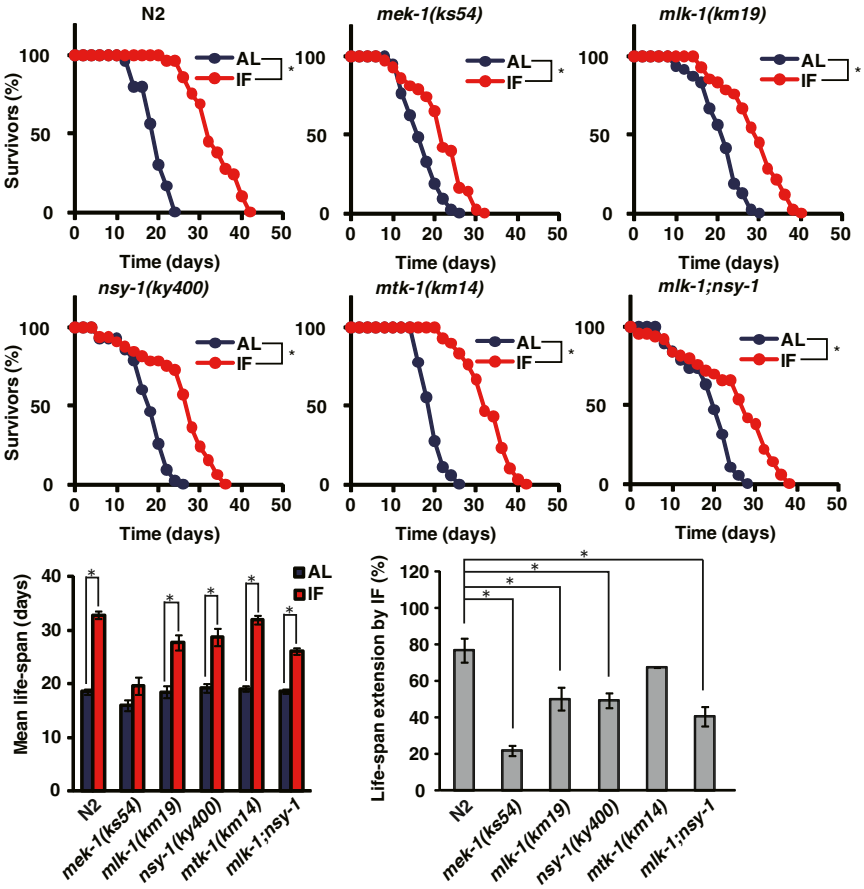


Figure 3. The KGB-1 JNK Cascade Is Required for IF-Induced Longevity

IF-induced increase in life span was suppressed in *mek-1(ks54)*, *mlk-1(km19)*, and *nsy-1(ky400)* mutants. Survival curves of N2 (upper left, n = 34 [Al] and 29 [IF]), *mek-1(ks54)* (upper middle, n = 43 [AL] and 43 [IF]), *mlk-1(km19)* (upper right, n = 44 [AL] and 38 [IF]), *nsy-1(ky400)* (lower left, n = 43 [AL] and 33 [IF]), *mtk-1(km14)* (lower middle, n = 49 [AL] and 48 [IF]), and *mlk-1;nsy-1* (lower right, n = 29 [AL] and 50 [IF]) in AL and IF are shown. *p < 0.05 log rank test (p = 1.9E-19, 5.4E-5, 3.1E-7, 1.1E-6, 7.0E-10, and 3.6E-4 in N2, *mek-1(ks54)*, *mlk-1(km19)*, *nsy-1(ky400)*, *mtk-1(km14)*, and *mlk-1;nsy-1*, respectively). Mean life span ±SEM (lower left) and mean life-span extension by IF (±SEM) (lower right) of three independent experiments is shown (bottom). *p < 0.05 t test.
See also Figure S3 and Table S2.

that KGB-1 is also required for fasting-induced nuclear translocation of DAF-16. Surprisingly, however, it was not suppressed in *kgb-1* RNAi-treated worms (Figure 4B), indicating that KGB-1 is not involved in fasting-induced DAF-16 nuclear translocation. It is likely, therefore, that the two signaling pathways, the KGB-1 and Rheb pathways, regulate fasting-induced transcriptional changes in DAF-16 target genes through different mechanisms.

To explore the relationship between DAF-16 and KGB-1 further, we made a double-knockout mutant of *daf-16* and *kgb-1* and performed life-span measurement. The double knockout of *daf-16* and *kgb-1* further suppressed IF-induced longevity, compared to the *daf-16* mutant (Figure 4C), suggesting that KGB-1 and DAF-16 do not lie linearly in a linear signaling pathway, but may constitute a signaling network that functions to extend life span. On the other hand, the double knockout did not significantly reduce IF-induced longevity of the KGB-1 mutant, suggesting that KGB-1 should somehow regulate DAF-16 function.

We then reasoned that the KGB-1 signaling effector AP-1 (JUN-1/FOS-1) should interact with DAF-16 in the nucleus. Consistent with this idea, our quantitative RT-PCR (qRT-PCR) analysis showed that, like in *kgb-1*-null mutants, the fasting-induced increases in the six DAF-16 target genes were markedly suppressed in *jun-1* RNAi- or *fos-1* RNAi-treated worms (Figure 4D) and in the loss-of-function mutant of *jun-1* [*jun-1(gk557)*] (Figure S4A). These results suggest that AP-1(JUN-1/FOS-1) should collaborate with DAF-16 in the nucleus to mediate fasting-induced transcriptional changes. In fact, our analysis demonstrates that all these fasting-responsive DAF-16 target genes have both the DAF-16-binding elements (Furuyama et al., 2000; Murphy et al., 2003; Oh et al., 2006) and TRE in their promoter region (Figure S4B; Table S3).

To investigate the relationship among KGB-1, JUN-1, and DAF-16 in fasting-stimulus responses in more detail, we examined the genome-wide gene expression changes by 2 days of fasting in N2, *kgb-1(km21)*, *jun-1(gk557)*, and *daf-16(mu86)*. While there were 539 genes whose expression was upregulated more than 2-fold by 2 days of fasting with statistical significance in wild-type N2, such genes were decreased to 64, 170, and 291 in *kgb-1(km21)*, *jun-1(gk557)*, and *daf-16(mu86)*, respectively (Figure S5; Table S4; statistical analysis was performed by two-way ANOVA with a Benjamini and Hochberg false discovery rate [BH-FDR-0.1] and multiple testing corrections followed by Tukey post hoc tests using log-transformed data), suggesting participation of KGB-1, JUN-1, and DAF-16 in the fasting-induced upregulation of genes. In contrast, the number of fasting-repressed genes was significantly decreased only in *kgb-1(km21)* as compared to that in N2 (Figure S5), suggesting that a hitherto-unidentified KGB-1 effector, which is different from JUN-1, might function in the fasting-induced downregulation of genes.

To validate the involvement of KGB-1, JUN-1, and DAF-16 in the fasting-induced transcriptional changes, we compared the induction rate of the 539 fasting-induced upregulated genes in the mutants with that in wild-type worms. Then, we identified 470, 356, and 256 genes as KGB-1-, JUN-1-, and DAF-16-dependent genes, respectively: the rate of their induction in response to fasting in mutants (*kgb-1(km21)*, *jun-1(gk577)*, and *daf-16(mu86)*, respectively) was less than half compared to that in wild-type (the genes that were plotted under the black line of the scatterplots in Figure 5A; Table S4). Thus, 87% and 66% of

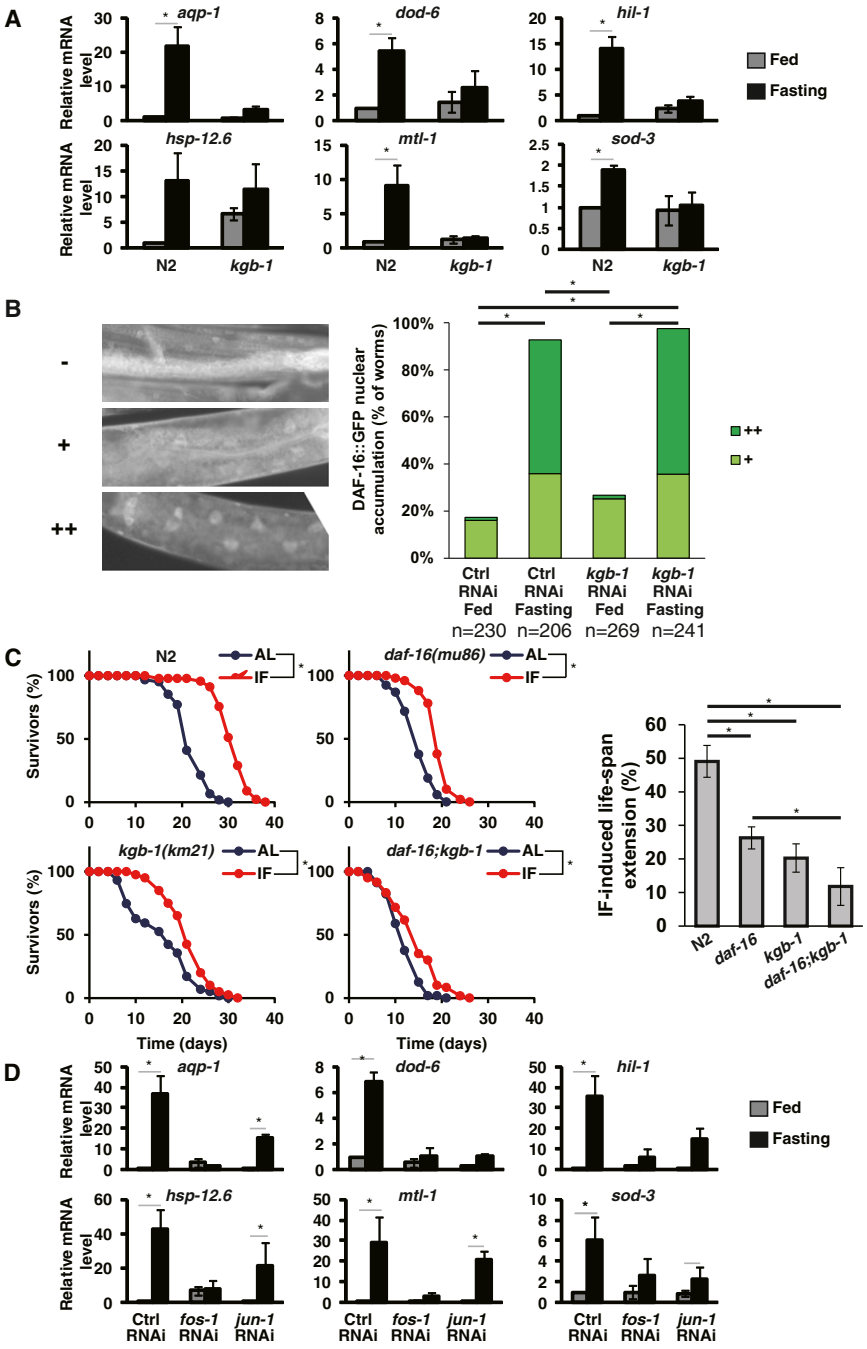


Figure 4. KGB-1-JUN-1/FOS-1 Signaling Regulates Fasting-Induced Upregulation of DAF-16 Target Genes without Affecting Fasting-Induced DAF-16 Nuclear Translocation

(A and D) The expression levels of six DAF-16 target genes (*aqp-1*, *dod-6*, *hil-1*, *hsp-12.6*, *mtl-1*, and *sod-3*) in N2 or *kgb-1(km21)* (A) and in control RNAi-, *fos-1* RNAi-, or *jun-1* RNAi-treated worms (D) are shown. At day 2 of adulthood, worms were transferred to the plate in the presence (Fed) or absence (fasting) of food. After incubation for 2 days, worms were collected and total RNA was extracted. The expression levels of six DAF-16 target genes were determined by qRT-PCR. Each value was normalized to *act-1*, and the value in wild-type (N2) (Fed) was set to 1. Mean mRNA levels ±SEM of three independent experiments are shown. *p < 0.05 t test.

(B) Representative images of DAF-16 localization (little or no nuclear accumulation [−], weak nuclear accumulation [+], and strong nuclear accumulation [++]) in control RNAi; fasting are shown (left). Percentages of worms with nuclear accumulation of DAF-16::GFP in intestine after 15 hr of fasting were scored in three independent experiments (right). *p < 0.05 Fisher's exact test.

(C) Survival curves of N2 (upper left, n = 41 [AL] and 52 [IF]), *daf-16(mu86)* (upper right, n = 53 [AL] and 50 [IF]), *kgb-1(km21)* (lower left, n = 59 [AL] and 40 [IF]), or *daf-16;kgb-1* (lower right, n = 56 [AL] and 60 [IF]) worms in AL and IF are shown. *p < 0.05 log rank test [p = 6.5E-13, 1.4E-6, 5.3E-3, and 4.0E-3 in N2, *daf-16(mu86)*, *kgb-1(km21)*, and *daf-16;kgb-1* worms, respectively]. Mean life-span extension by IF (±SEM) of three independent experiments is shown (far right). *p < 0.05 t test. See also Figure S4 and Table S2.

539 fasting-induced genes were dependent on KGB-1 and JUN-1, respectively, and 47% was dependent on DAF-16 (Figures 5A and 5B). Remarkably, there were 323 overlapping genes between 470 KGB-1-dependent genes and 356 JUN-1-dependent ones, 228 overlapping genes between KGB-1-dependent genes and 256 DAF-16-dependent ones, and 210 overlapping between JUN-1-dependent genes and DAF-16-dependent ones, respectively (Figure 5B). These results are consistent with our idea that the KGB-1-JUN-1/FOS-1(AP-1) signaling pathway senses and transduces fasting stimulus and collaborates with DAF-16 to mediate fasting-induced transcriptional changes. Then we did hierarchical clustering analysis for 539 fasting-induced genes under 17 conditions (Figure 5C). Strikingly, all the conditions of mutants (conditions; [*kgb-1/jun-1/daf-16*: fed/fasting 48 hr]) belong to the same group as (N2:fed 0–48 hr) and (N2:fasting 3 hr/6 hr), when classified into two groups, the other of which consists of (N2:fasting 9–48 hr) (Figure 5C). As described before, a significant portion of the fasting-induced genes starts to increase between 6 and 9 hr of fasting (Figure 1B, lower panel), and these genes are related to aging and stress responses in the GO analysis (see Figure 1D). Collectively, these results strongly suggest that KGB-1 and JUN-1, together with DAF-16, play a central role in the fasting-stimulus response pathway.

To confirm that MEK-1 and MLK-1 lie upstream of KGB-1 in response to fasting, we performed the genome-wide gene expression changes by 2 days of fasting in *mek-1(ks54)* and

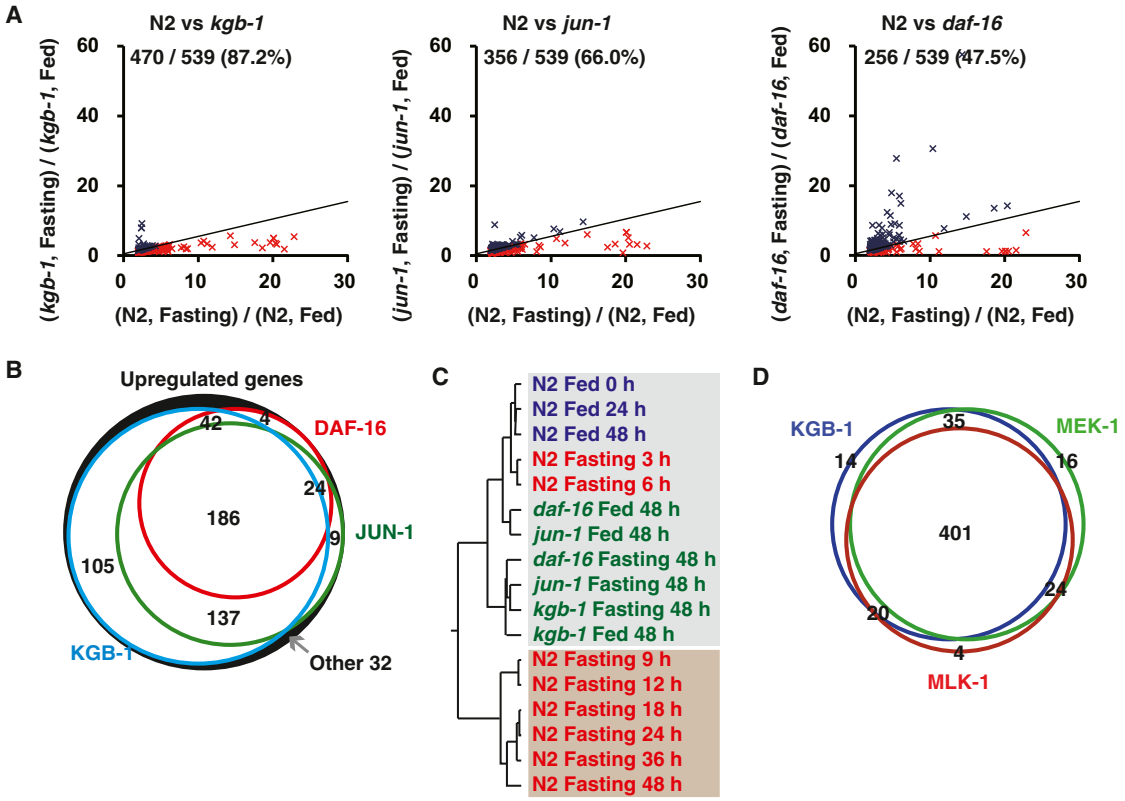


Figure 5. The KGB-1/AP-1 Module Plays a Central Role in Mediating Fasting-Induced Gene Expression Changes

(A) Scatterplots of fold changes of the fasting-induced upregulated genes for *kgb-1(km21)* (left), *jun-1(gk557)* (middle), or *daf-16(mu86)* (right). A black line indicates (Y – 1) = 0.5 × (X – 1). When the (Y – 1) value becomes less than half of the (X – 1) value in each fasting-induced gene (i.e., each gene below a linear black line), we consider it as "dependent on an indicated mutant." These "dependent genes" are shown by red crosses in each plot.

(B and D) Venn diagram for fasting-induced 539 genes, whose expression is upregulated in response to fasting with statistical significance in wild-type worms, is shown. Blue, red, or green circle indicates KGB-1-, DAF-16-, or JUN-1-dependent genes, respectively (B). Blue, green, or red circle indicates KGB-1-, MEK-1-, or MLK-1-dependent genes, respectively (D).

(C) Hierarchical clustering analysis for 539 fasting-induced genes.

See also Tables S1, S4, and Figure S5.

mlk-1(km19). To validate the involvement of MEK-1 and MLK-1 in the fasting-induced transcriptional changes, we compared the induction rate of the 539 fasting-induced upregulated genes in the mutants with that in wild-type worms. Then, we identified 476 and 449 genes as MEK-1- and MLK-1-dependent genes, respectively. Interestingly, there were 436 overlapping genes between 470 KGB-1-dependent genes and 476 MEK-1-dependent ones, 421 overlapping genes between KGB-1-dependent genes and 449 MLK-1-dependent ones, and 425 overlapping between MEK-1-dependent genes and MLK-1-dependent ones, respectively (Figure 5D). These results strongly suggest that KGB-1, MEK-1, and MLK-1 function in the same pathway in response to fasting.

The Components of the SCF E3 Ligase Complexes Are Induced by Fasting through the Fasting-Responsive Signaling Pathways and Are Involved in IF-Induced Longevity

The above results suggested that there should be prolongevity genes that play a role in the longevity response to IF within the fasting-induced upregulated genes whose induction was dependent on both KGB-1/AP-1 signaling and DAF-16. To identify such candidate genes, we focused on 198 genes whose expression levels after 2 days of fasting in wild-type worms were more than 2-fold of those in every mutant of *kgb-1*, *jun-1*, and *daf-16* (the genes that were plotted under the red line of the scatterplots in Figure S6A [Figures S6B; Table S4]). It should be noted that this Venn diagram (Figure S6B) was based on the expression levels of each gene after 2 days of fasting, because the absolute expression levels after fasting should be important, whereas the previous diagram (Figure 5B) was based on the induction rate by fasting in each mutant. Interestingly, GO analysis revealed that genes related to aging and ubiquitin-dependent protein catabolic process are enriched in the 198 genes (Figure S6C). In the enriched 198 genes, there were seven genes that belong to the GO term "ubiquitin-dependent protein catabolic process" (Table S4). Surprisingly, these seven genes were all Skp1-related protein-encoding genes. Skp1-related protein is the components of Skp1-Cullin-F-box (SCF) E3 ligase complex. There are 21 Skp1-related proteins, 322 F-box proteins, and six cullin

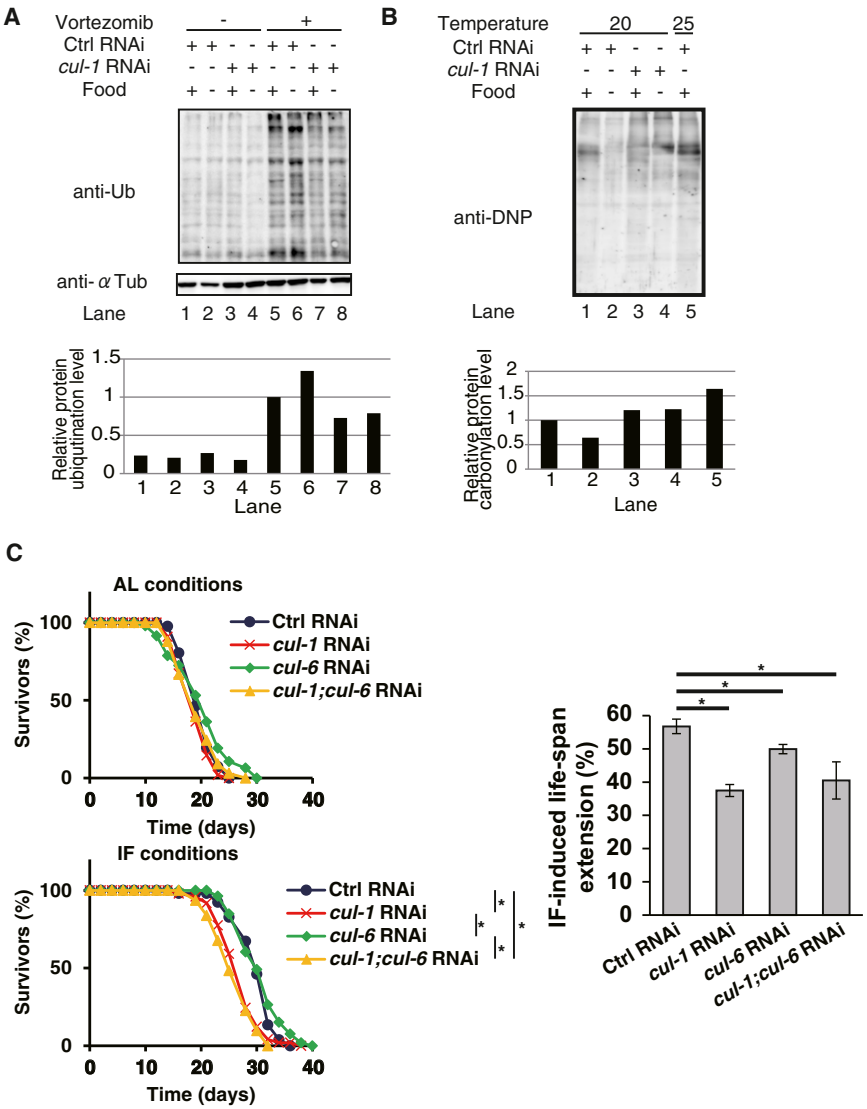


Figure 6. The Components of the SCF E3 Ligase Complexes Are Induced by Fasting through the Fasting-Responsive Signaling Pathways and Are Involved in IF-Induced Longevity

(A) Immunoblotting analysis (antiubiquitin antibody) to detect protein ubiquitination in the indicated worms. Worms were treated with *cul-1* RNAi or control (Ctrl) RNAi in the presence or absence of proteasome inhibitor bortezomib (20 μM). Food + (plus) and – (minus) show fed and fasting (2 days), respectively. Relative ubiquitination levels are shown (lower). The value of lane 5 is set to 1.

(B) Immunoblotting analysis (DNP antibody) to detect protein carbonylation in the indicated worms. Total protein extracted from same number of worms is blotted. As an aged animal control, we use 4 days adult grown at 25°C (lane 5). Worms were treated with *cul-1* RNAi or control (Ctrl) RNAi. Food + (plus) and – (minus) show fed and fasting (2 days), respectively. Relative protein carbonylation levels are shown (lower). The value of lane 1 is set to 1.

(C) Survival curves of control RNAi- (n = 41 [AL] and 52 [IF]), *cul-1* RNAi- (n = 55 [AL] and 49 [IF]), *cul-6* RNAi- (n = 47 [AL] and 53 [IF]), and *cul-1;cul-6* RNAi- (n = 33 [AL] and 31 [IF]) treated worms in AL (upper left) or IF (lower left) are shown. *$p < 0.05$ log rank test. Mean life-span extension by IF (±SEM) of three independent experiments is shown (right). *$p < 0.05$ t test.

See also Tables S1, S2, S4, and Figure S6.

proteins in *C. elegans*. Thirty-two of these genes were upregulated in response to fasting, and the inductions were mostly dependent on DAF-16, JUN-1, and KGB-1 (Figure S6D). We then inquired whether the fasting-induced increase of the components of the SCF E3 ligase complex leads to enhanced ubiquitination. Immunoblotting analysis demonstrated that the amount of ubiquitinated proteins in the presence of a proteasome inhibitor was increased in fasting worms compared to that in fed worms (Figure 6A, compare lanes 5 and 6). This indicates that fasting enhances protein ubiquitination and thus suggests that fasting activates the ubiquitin proteasome system. Next, we investigated protein carbonylation, which is thought to be a widespread indicator of oxidative damage and disease-derived protein dysfunction (Levine, 2002). Remarkably, the total protein carbonylation in wild-type worms was significantly decreased by 2 days of fasting (Figure 6B, compare lanes 1 and 2). We then asked whether the enhanced ubiquitination plays a role in IF-induced longevity. To this end, we knocked down cullin proteins. RNAi of each of six cullin protein genes from L4 stage showed that IF-induced life-span extension was significantly suppressed in *cul-1* RNAi-treated worms (Figures 6C and S6E). CUL-1 is shown to be a major cullin protein in the SCF complex (Petroski and Deshaies, 2005). It should be noted that RNAi of any of cullin genes did not affect normal life span under AL conditions (Figure 6C, left upper panel), in complete agreement with the previous report (Ghazi et al., 2007). As CUL-6 is shown to be a CUL-1 paralog (Yamanaka et al., 2002), we double knocked down *cul-1* and *cul-6* and examined the life span. Double knockdown of *cul-1* and *cul-6* did not result in further suppression of IF-induced longevity compared to single knockdown of *cul-1*, suggesting that CUL-6 does not have a role in life-span regulation in response to IF. Moreover, quantitative PCR (qPCR) analysis revealed that the induction of *cul-1* mRNA by fasting was suppressed in *daf-16*, *jun-1*, and *kgb-1* mutants (Figure S6F). Importantly, both the fasting-induced enhancement of protein ubiquitination and the fasting-induced reduction of protein carbonylation were abolished by *cul-1* RNAi (Figure 6A, compare lanes 7 and 8, and Figure 6B, compare lanes 3 and 4). These results taken together suggest that the increased expression of the SCF complex

components in response to fasting has a role in the longevity response to IF.

DISCUSSION

In this study, we have uncovered a pivotal role for a signaling network consisting of two fasting-responsive pathways in fasting-induced transcriptional changes and IF-induced longevity. The idea that the full understanding of responses to fasting in adult worms should help uncover the molecular mechanisms underlying IF-induced longevity prompted us to perform transcriptome analysis of fasting-stimulus responses. Then, our promoter analysis has identified FOXO, FOXA, and AP-1 transcription factors as major candidate transcription factors responsible for fasting-induced gene expression. DAF-16, *C. elegans* FOXO, and PHA-4, *C. elegans* FOXA, have previously been shown to mediate IF- and calorie-restriction-induced longevity, respectively (Greer et al., 2007; Honjoh et al., 2009; Panowski et al., 2007), indicating the accuracy of our analysis. We have here shown that *C. elegans* AP-1 (JUN-1/FOS-1) is also involved in life-span extension by IF. Our results have then shown that KGB-1, one of the *C. elegans* JNKs, acts as a direct activator of AP-1, is activated in response to fasting, and plays an important role in IF-induced longevity. Transcriptome analysis in *kgb-1*-, *jun-1*-, and *daf-16*-null mutants has clearly shown that KGB-1, JUN-1, and DAF-16 play a central role in mediating fasting-induced transcriptional changes. There are 323 overlapping genes between 470 KGB-1-dependent genes and 356 JUN-1-dependent ones, supporting the idea that KGB-1 and JUN-1 lie in a linear fashion in fasting-stimulus responses. Surprisingly, the number of KGB-1- and/or JUN-1-dependent genes is larger than that of DAF-16-dependent genes, suggesting the importance of the KGB-1/AP-1 signaling pathway in the fasting-induced transcriptional response.

Our results showed that fasting-induced KGB-1 activation is sustained for a longer period (at least until 9 hr after fasting. Figure 2C). Thus, we suspect that AP-1-dependent gene expression may require sustained activation of KGB-1, as it has previously been shown that growth-factor-induced AP-1 activation in mammalian cultured cells requires sustained activation of ERK, another MAP kinase family member (Murphy et al., 2002).

In this study, we identified a signaling module consisting of MLK-1 and NSY-1 MAPKKKs, MEK-1 MAPKK, and KGB-1 MAPK, which functions in longevity response to IF. The extent of reduction in IF-induced longevity in *mlk-1(km19)* and *nsy-1(ky400)*, however, was smaller than that in *mek-1(ks54)* or *kgb-1(km21)*. Double knockout of *mlk-1* and *nsy-1* did not result in further reduction in IF-induced longevity compared to single knockout. We can assume that there should be another MAPKKK that contributes to fasting-induced activation of KGB-1. We then speculate that if the total activity, which is supported by the three kinases, MLK-1, NSY-1, and the third kinase, is reduced below a certain level of activity, due to the double knockout of *mlk-1* and *nsy-1*, a feedback compensation mechanism, such as activation of the third kinase, would operate, and that the single knockout, which should not reduce the total activity below the certain level, would not activate the feedback compensation mechanism. This speculation may partially explain why the double knockout does not show additive effects. In addition, there may be still a possibility that MLK-1 and NSY-1 cooperatively function, for example, by physically interacting with each other, to activate the KGB-1 pathway, as it has been well known that the best well-characterized MAPKKKs, Raf kinases, form homo- and heterodimers to regulate the MAP kinase pathway. If so, double knockout may not produce additive effects.

It has previously been shown that JNK signaling interacts with the insulin/IGF signaling pathway components in the cytoplasm and regulates the insulin/IGF signaling pathway effector FOXO in response to environmental stresses in many eukaryotes (van der Horst and Burgering, 2007; Taguchi and White, 2008). In *C. elegans*, JNK-1, one of the *C. elegans* JNKs, has been shown to deliver the signal to DAF-16 through promoting its nuclear translocation by possibly phosphorylating it in response to environmental cues (Oh et al., 2005). Our presented study identifies the interaction of JNK signaling with the insulin/IGF signaling effector in the nucleus. Thus, our results show that KGB-1 is not involved in fasting-induced DAF-16 nuclear translocation, and thus inactivation of KGB-1 does not suppress fasting-induced DAF-16 nuclear translocation. Our life-span measurements using *daf-16* and *kgb-1* suggest that KGB-1 and DAF-16 do not lie linearly in a linear signaling pathway, but may constitute a signaling network that functions to extend life span, and that KGB-1 should somehow regulate DAF-16 function. Moreover, almost all of the DAF-16-dependent genes are dependent on KGB-1 and/or JUN-1, suggesting that the KGB-1/AP-1 axis is required for the transcriptional changes induced by DAF-16. These results taken together demonstrate that AP-1 collaborates with DAF-16 in the nucleus at the promoters of DAF-16 target genes to mediate fasting-induced gene expression changes, and/or the KGB-1/AP-1 module regulates cofactors of DAF-16.

Importantly, the components comprising the fasting-responsive signaling pathways that play a central role in mediating fasting-induced genes expression are shown to function in IF-induced longevity, suggesting that fasting-induced transcriptional changes make key contribution to IF-induced longevity. We found that SCF E3 ligase complexes are important transcriptional targets of these signaling pathways and play a role in the fasting-induced enhancement of ubiquitination and IF-induced longevity. The SCF E3 ligase complexes may function as prolongevity genes by reducing the amount of damaged proteins that are harmful to the organism and/or by degrading proteins that are no longer needed under fasting conditions to obtain amino acid sources. Although it has been reported that fasting enhances ubiquitin proteasome system (UPS) in rat heart and brown adipose tissue (Nakai et al., 2008; Razeghi et al., 2006), it has been unknown that fasting-induced enhancement of UPS-mediated protein degradation is involved in fasting-induced longevity. Our study identifies the requirement of UPS in the longevity response to IF. Moreover, it has been reported that repeated fasting stress causes JNK activation in rat liver (Nishio et al., 2002). Because the JNK/AP-1 signaling axis, FOXO transcription factors, and UPS are all evolutionarily conserved from *C. elegans* to mammals and because IF is effective in inducing longevity in both *C. elegans* and mammals, there is

a tantalizing possibility that this signaling network also plays a role in the fasting-induced transcriptional changes and IF-induced longevity in mammals.

In summary, through analyzing the fasting-induced transcriptional response, we have here uncovered the fasting-responsive signaling network, which plays an essential role in sensing and transducing fasting stimuli and extending life span. Because IF effectively extends life span in many organisms and delays the onset of age-related diseases in mammals, this study not only greatly increases our molecular understanding of aging but also will help the development of fasting mimetics that have the potential to improve our health by delaying the onset of multiple age-related diseases.

EXPERIMENTAL PROCEDURES

C. elegans Strains

All nematodes were cultured using standard *C. elegans* methods (Brenner, 1974). The strains we analyzed were as follows: wild-type N2, *jnk-1(gk7)* (3), *kgb-1(km21)* (3), *kgb-1(um3)* (3), *kgb-2(km16)* (3), *mek-1(ks54)* (3), *mlk-1(km19)* (3), *nsy-1(ky400)* (3), *pmk-1(km25)* (2), *pmk-2(km37)* (0), *pmk-3(ok169)* (2), *jun-1(gk577)* (3), *daf-16(mu86)* (2), *daf-2(e1370)* (2), and TJ356 *zIs356*[*daf-16*::gfp; *rol-6*] (2). The numbers of outcrossing are shown in parentheses.

Intermittent Fasting

Approximately 100 synchronized young adults worms raised on nematode growth media plates with live OP50-1 were picked to FUDR-containing plates with live OP50-1. At day 2 of adulthood, worms were divided into AL and IF. Worms in AL were fed with kanamycin-killed OP50-1 throughout their life span. Worms in IF were on plate with (2 days) or without (2 days) kanamycin-killed OP50-1 as food alternately. All worms were transferred to new plates every other day.

Promoter Analysis

As a promoter region of all the genes, we used 1 kbp of a 5′ upstream sequence region in each gene. The promoter regions of selected genes were scanned with MATCH (Kel et al., 2003) using 566 vertebrate and seven nematode position weight matrices (cutoff = minFP). For each matrix, the distribution of hits in the promoters was compared with that in the promoters of all genes. The p values are from one-sided Fisher's tests.

RNA Interference

RNAi was performed by feeding methods (Kamath et al., 2001) as described. The 500 nucleotides of the coding region of *kgb-1*, *jun-1*, *fos-1*, *cul-1*, *cul-2*, *cul-3*, *cul-4*, *cul-5*, and *cul-6* complementary DNA were used for RNAi. The primers used were as follows: *kgb-1* left, 5′-AGATCTAACATCAAGTTACAATTAGG-3′ and *kgb-1* right, 5′-CCGCGGCGCCCGAATTGTGAAGATGC-3′; *jun-1* left, 5′-CCGCGGTCTGGCCTCGTCTGCCATTC-3′ and *jun-1* right, 5′-GAGCTCCACGAATCGAATTGTTCGGG-3′; *fos-1* left, 5′-GAGTCGCGAAGAACGAACAC-3′ and *fos-1* right, 5′-GAGACGGAAAGGCCTGCTGG-3′; *cul-1* left, 5′-CTTGTTCAATGATCTTAAGG-3′ and *cul-1* right, 5′-GTCCATTCGGCACTCTATCG-3′; *cul-2* left, 5′-TCCGGATCTGTTGCTTCAGG-3′ and *cul-2* right, 5′-GCACTATAACCTGTTCCATG-3′; *cul-3* left, 5′-TCAAATTGGATCGAAAATCG-3′ and *cul-3* right, 5′-TGTTCGAGCCGGTTTCGTTG-3′; *cul-4* left, 5′-GGAGTCGTTAAATGCGGTTG-3′ and *cul-4* right, 5′-GTTGCCTGAACATCTTTTAG-3′; *cul-5* left, 5′-CTTTGTGGCACTGAACGATC-3′ and *cul-5* right, 5′-CACAGAATCCTTCTCGAACC-3′; *cul-6* left, 5′-CAGTTGTCGAATGTCTGACG-3′ and *cul-6* right, and 5′-CATTAGCTTCGATAAAGTCC-3′.

Transfection

Complementary DNAs (cDNAs) of *kgb-1*, *jun-1* (T24H10.7a), and *fos-1* (F29G9.4) were isolated by PCR and ligated into pcDNA3 containing myc, Flag, and HA tag, respectively. 293T cells were transfected by using Lipofectamin 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

Generation of Transgenic Animals

To generate a *kgb-1* promoter::gfp reporter construct, a fragment containing a 5′ upstream sequence region (3 kbp) of *kgb-1* was isolated by PCR. This fragment was cloned into the green fluorescent protein (GFP) vector pPD95.75. Transgenic worms were generated by microinjecting this plasmid (50 ng/μl) into wild-type N2 worms with pRF4 *rol-6* (50 ng/μl) as a transformation marker. Three independent lines were recovered.

qRT-PCR

Total RNA was extracted with Sepasol(R)-RNA I Super (Nakalai tesque) or TRizol (Invitrogen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) with dT primer, according to manufacturers' protocols. cDNA was subjected to qPCR analysis using the ABI 7300 Real-Time PCR system (Applied Biosystems) with SYBR Green PCR kit (Roche). Each value was normalized to *act-1*, and the value in control RNAi-fed was set to 1. Primer sequences are available on request.

Fluorescence Microscopy

In DAF-16 localization assays, worms expressing DAF-16::GFP were synchronized and grown in the following conditions: control RNAi fed, control RNAi fasting, *kgb-1* RNAi fed, and *kgb-1* RNAi fasting. After 15 hr fasting, worms were fixed with 3% formaldehyde in PBS for 5 min at room temperature and observed with Axioplan2. For *kgb-1* promoter::gfp transgenic worms, worms were anesthetized with 10 mM sodium azide in M9 buffer at day 1 of adulthood.

Microarray Experiments

Two and three independent biological replicates were performed in the time course and *mlk-1* mutants (Figures 1 and 5) and other mutants (Figure 5), respectively. In the time course analysis, we identified fasting-induced upregulated and downregulated genes, whose gene expression is upregulated more than 2-fold and downregulated less than half as compared to control (fed 0 hr for fasting 3, 6, 9, and 12 hr; fed 24 hr for fasting 18, 24, and 36 hr; fed 48 hr for fasting 48 hr), respectively. Total RNA was extracted with TRizol (Invitrogen). Other procedures were performed according to Affymetrix protocols. Hybridized arrays were scanned using an Affymetrix GeneChip Scanner. Scanned chip images were analyzed with Affymetrix GeneChip Command Console version 2.0 (AGCC) and processed using default settings. The Affymetrix outputs (CEL files) were imported into GeneSpring GX 11.0.2 (Agilent Technologies) microarray analysis software for both statistical analysis and presentation of expression profiles (average expression profiles). Expression signals of probe sets were calculated using PLIER (probe logarithmic intensity error, as implemented in GeneSpring GX). The log of ratio mode was used for all analyses (GeneSpring GX). Statistical analysis was performed by two-way analysis of variance (ANOVA) with a Benjamini and Hochberg false discovery rate (BH-FDR-0.1) multiple testing corrections followed by Tukey post hoc tests using log-transformed data (GeneSpring GX). Hierarchical clustering analysis was done with squared Euclidean as the distance metric and average linkage as the cluster method by using GeneSpring GX. GO analyses were performed using GeneSpring GX, and GO terms were provided by the GO Ontology Consortium on their website (http://geneontology.org) (corrected p value < 0.1).

ACCESSION NUMBERS

The microarray data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with series accession numbers GSE27677 and GSE42689.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.12.018.

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