EAK-7 Controls Development and Life Span by Regulating Nuclear DAF-16/FoxO Activity

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SUMMARY

FoxO transcription factors control development and longevity in diverse species. Although FoxO regulation via changes in its subcellular localization is well established, little is known about how FoxO activity is regulated in the nucleus. Here, we show that the conserved C. elegans protein EAK-7 acts in parallel to the serine/threonine kinase AKT-1 to inhibit the FoxO transcription factor DAF-16. Loss of EAK-7 activity promotes diapause and longevity in a DAF-16/FoxO-dependent manner. Whereas akt-1 mutation activates DAF-16/FoxO by promoting its translocation from the cytoplasm to the nucleus, eak-7 mutation increases nuclear DAF-16/FoxO activity without influencing DAF-16/FoxO subcellular localization. Thus, EAK-7 and AKT-1 inhibit DAF-16/FoxO activity via distinct mechanisms. Our results implicate EAK-7 as a FoxO regulator and highlight the biological impact of a regulatory pathway that governs the activity of nuclear FoxO without altering its subcellular location.

INTRODUCTION

FoxO transcription factors (TFs) promote life span extension, stress resistance, and metabolic homeostasis in diverse species (Accili and Arden, 2004; Arden, 2008; Calnan and Brunet, 2008; Gross et al., 2008; Partridge and Bruning, 2008). FoxO knockout mice develop tumors and exhibit abnormalities in glucose metabolism and bone mineral density (Ambrogi et al., 2010; Dong et al., 2008; Matsumoto et al., 2007; Paik et al., 2007; Rached et al., 2010), suggesting that dysregulation of FoxO TFs may contribute to the pathophysiology of common human diseases associated with aging, such as cancer, type 2 diabetes, and osteoporosis. Intriguingly, FoxO3 polymorphisms are associated with extreme longevity in humans (Flachsbart et al., 2009; Li et al., 2009; Wilcox et al., 2008). Thus, understanding how FoxO TFs are regulated has the potential to yield fundamental insights into both the pathophysiology of human disease as well as the physiology of normal aging.

Activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway by insulin and IGF-1 signaling (IIS) results in direct phosphorylation of FoxO by Akt and its subsequent association with 14-3-3 proteins and nuclear export (Van Der Heide et al., 2004), whereupon it is targeted for ubiquitin-mediated proteasomal degradation (Huang et al., 2005; Matsuzaki et al., 2003). The paradigm of FoxO inhibition by IIS first emerged from genetic analysis in C. elegans, where a conserved IIS pathway controls life span, stress resistance, and entry into a developmentally arrested larval stage known as dauer. The C. elegans insulin-like receptor (InsR), DAF-2 (Kimura et al., 1997), activates the PI3K AGE-1 (Morris et al., 1996), PDK-1 (Paradis et al., 1999), and the AGC family kinases AKT-1, AKT-2, and SGK-1 (Hertweck et al., 2004; Paradis and Ruvkun, 1998). AKT phosphorylation of the C. elegans FoxO TF DAF-16 promotes its binding to the 14-3-3 protein FTT-2 and subsequent nuclear exclusion (Berdichevsky et al., 2006; Li et al., 2007a). The PI3K/Akt pathway is antagonized by the C. elegans phosphatase and tensin (PTEN) ortholog DAF-18 (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999). Reduction of IIS results in life span extension, increased stress resistance, and constitutive dauer arrest, and these phenotypes are suppressed by DAF-16/FoxO loss-of-function mutations (Finch and Ruvkun, 2001; Kenyon, 2005). Thus, DAF-16/FoxO promotes longevity, stress resistance, and dauer arrest and is inhibited by IIS.

DAF-16/FoxO localizes to the nucleus in daf-2/InsR mutants (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001); however, nuclear localization per se is insufficient for full DAF-16/FoxO activation, as a DAF-16/FoxO mutant lacking all AKT phosphorylation sites exhibits constitutive nuclear localization but does not promote dauer arrest or longevity (Hertweck et al., 2004; Lin et al., 2001). Furthermore, although loss-of-function alleles of daf-18/PTEN and gain-of-function alleles of akt-1 or pdk-1 fully suppress the dauer-constitutive phenotype of age-1/pi3k null mutants, they weakly suppress dauer arrest caused by a partial loss-of-function mutation in daf-2/InsR (Gil et al., 1999; Ogg and Ruvkun, 1998; Paradis and Ruvkun, 1998). These data suggest that a second pathway acts in parallel to the PI3K/Akt pathway to regulate the activity of nuclear DAF-16/FoxO (Figure 1A).

To identify genes encoding regulators of nuclear DAF-16/FoxO activity, we performed a screen for mutants that enhance the weak dauer arrest phenotype of an akt-1 null mutant (i.e., eak...
mutants (Hu et al., 2006). Here, we describe the identification and characterization of eak-7, which encodes a conserved protein that regulates development, life span, and stress resistance by controlling nuclear DAF-16/FoxO activity.

RESULTS

At 25°C, akt-1 null mutants exhibit nuclear enrichment of DAF-16/FoxO, but do not arrest as dauers (Hu et al., 2006; Zhang et al., 2008). We mutagenized akt-1 null mutant animals and identified rare F2 progeny that arrested as dauers at 25°C. akt-1 mutants were defined as those mutants whose constitutive dauer arrest phenotype required the presence of the akt-1 mutation (Hu et al., 2006; Zhang et al., 2008). The eak-7 gene is defined by the missense allele mg338 and the independently isolated deletion allele tm3188, which is a null allele (Figures S1A and S1B).

EAK-7 Acts in Parallel to AKT-1 to Inhibit Dauer Arrest via DAF-16/FoxO and DAF-12

daf-2/InsR mutants arrest as dauers at 25°C (Gems et al., 1998; Kimura et al., 1997). In contrast, whereas eak-7 mutants undergo dauer arrest at 27°C on plates lacking supplemental cholesterol (Figures 1B and 1C), they develop into adults on standard NGM plates at 25°C (Figures 1D–1F). However, eak-7 mutation strongly enhances dauer arrest in an akt-1 null mutant (Figures 1D–1F) as well as in age-1/Pi3K and pdk-1 partial loss-of-function mutants (Figure S1C) and in daf-2(e1370) mutants (data not shown). The enhancement of the dauer arrest phenotype of an akt-1 null mutant by an eak-7 null mutation indicates that EAK-7 acts in parallel to AKT-1 to regulate dauer arrest.

EAK-7 Acts in the EAK Pathway to Regulate Dauer Arrest

Four other EAK proteins act in a single pathway in parallel to AKT-1 to regulate dauer arrest (Hu et al., 2006; Zhang et al., 2008). To determine whether EAK-7 also acts in this pathway, we examined the effect of eak-7 mutation on the dauer arrest phenotype of other eak mutants. eak-7 mutation did not enhance the dauer arrest phenotype of eak-3, sdf-9/eak-5, or eak-6 mutants (Figure S1D).

Dauer arrest is also regulated by dafachronic acids (DAs), which are steroid hormone ligands for the nuclear receptor DAF-12 (Motola et al., 2006). Mutations in the DA biosynthetic proteins DAF-9 and DAF-36 induce dauer arrest (Gerisch et al., 2001; Jia et al., 2002; Rottiers et al., 2006), and eak-3 mutations enhance...
the dauer arrest phenotype of daf-9 and daf-36 mutants (Zhang et al., 2008). Similarly, eak-7 mutation strongly enhanced the dauer arrest phenotype of daf-9 and daf-36 mutants (Figure S1E). Taken together, these data suggest that EAK-7 functions in the same pathway as other EAK proteins in dauer regulation.

EAK-7 Regulates Dauer Arrest via DAF-16/FoxO

IIS and EAK proteins regulate dauer arrest by inhibiting DAF-16/FoxO activity (Gottlieb and Ruvkun, 1994; Hu et al., 2006; Ohkura et al., 2003; Vowels and Thomas, 1992; Zhang et al., 2008). To determine whether EAK-7 inhibits DAF-16/FoxO, we tested whether dauer arrest phenotypes in eak-7 mutants were daf-16/FoxO dependent. Dauer arrest phenotypes of eak-7;akt-1 double mutants and eak-7 single mutants were fully suppressed by a daf-16/FoxO null mutation (Figures 1B, 1D, and 1E). Dauer arrest in eak-7 single mutants was also suppressed by a gain-of-function mutation in akt-1 (Paradis and Ruvkun, 1998) and a loss-of-function mutation in daf-18/PTEN (Ogg and Ruvkun, 1998) (Figure S1F), both of which are predicted to inhibit DAF-16/FoxO via increased AKT activity. Thus, EAK-7 regulates dauer arrest by inhibiting DAF-16/FoxO activity.

eak-7 Mutation Does Not Enhance Dauer Arrest in akt-2 Mutants

AKT-1, AKT-2, and SGK-1 physically associate with each other (Hertweck et al., 2004) and are thought to inhibit DAF-16/FoxO activity by phosphorylating DAF-16/FoxO at canonical Akt/PKB phosphorylation sites (Hertweck et al., 2004; Lee et al., 2001; Lin et al., 2001). Since EAK-7 acts in parallel to AKT-1 to regulate dauer arrest (Figures 1D–1F), we sought to determine whether EAK-7 also acts in parallel to AKT-2 and/or SGK-1. To this end, we constructed eak-7;akt-2, akt-1;akt-2, akt-2;sgk-1, and akt-1;sgk-1 double mutants using the candidate null alleles akt-2(ok393) (Hertweck et al., 2004) and sgk-1(mg455) (Soukas et al., 2009). If EAK-7 acts in parallel to AKT-2 or SGK-1, then eak-7 mutation should enhance the dauer arrest phenotype of akt-2 or sgk-1 mutants.

sgk-1 mutants did not undergo significant dauer arrest at 25°C or 27°C on standard NGM plates containing cholesterol and did not enhance the dauer arrest phenotype of akt-1 mutants at either temperature (Figures 1F and 1G). Thus, in agreement with previous results (Hertweck et al., 2004), SGK-1 plays a minor role in dauer regulation. sgk-1 mutation weakly enhanced the dauer arrest phenotype of eak-7 mutants at both temperatures (Figures 1F and 1G), suggesting that SGK-1 acts in parallel to EAK-7.

In contrast, although akt-2 mutants did not arrest as dauers at 25°C or 27°C (Figures 1F and 1G), akt-2 mutation strongly enhanced dauer arrest in an akt-1 mutant (Figure S1G), as previously reported (Oh et al., 2005). In fact, akt-1;akt-2 double mutants undergo nonconditional dauer arrest and do not recover (Oh et al., 2005) (Figure S1G and data not shown). Thus, like eak-7 mutations, akt-2 mutations are strong enhancers of the dauer arrest phenotype of akt-1 mutants. The severity of the akt-1;akt-2 double mutant dauer arrest phenotype, i.e., nonconditional constitutive dauer arrest, is similar to that observed in age-1/pi3k and daf-2/lnsr null mutants (Morris et al., 1996; Patel et al., 2008) and suggests that AKT-1 and AKT-2 constitute the major dauer regulatory output of IIS.

In contrast to eak-7;akt-1 double mutants, which have a strong dauer arrest phenotype at 25°C (Figures 1D–1F), eak-7;akt-2 double mutants did not arrest as dauers at 25°C or 27°C (Figures 1F and 1G). Thus, eak-7 mutation does not enhance the dauer arrest phenotype of akt-2 mutants. This result is consistent with at least two models of EAK-7 action. EAK-7 could act in the same pathway as AKT-2 to inhibit DAF-16/FoxO activity. Alternatively, since akt-2 mutation has a relatively modest effect on DAF-16::GFP nuclear localization in an akt-1 wild-type background (Hertweck et al., 2004), the amount of DAF-16/FoxO present in nuclei of eak-7;akt-2 double mutant animals may be below the threshold necessary for eak-7 mutation to have a phenotypic effect.

EAK-7 Regulates Dauer Arrest via DAF-12

DAF-12 is required for dauer arrest in all dauer-constitutive mutants studied to date (Fielenbach and Antebi, 2008; Hu, 2007). Dauer-constitutive phenotypes of eak-7 single mutants and eak-7;akt-1 double mutants were fully suppressed by a daf-12 null mutation (Figures 1C and 1H), indicating that EAK-7 promotes reproductive development by inhibiting DAF-12 activity.

EAK-7 Controls Life Span via DAF-16/FoxO

IIS mutants also exhibit DAF-16/FoxO-dependent life span extension and stress resistance during adulthood (Gems et al., 1998; Honda and Honda, 1999; Kenyon et al., 1993; Morris et al., 1996; Murakami and Johnson, 1996; Paradis et al., 1999). eak-7 mutants also live longer than wild-type animals (Figures 2A–2F and S2A–S2E and Table S2), and this phenotype requires daf-16/FoxO (Figures 2B and S2A and Table S2) as well as the protein phosphatase 4 regulatory subunit SMK-1 (Kim et al., 2007; Wolff et al., 2006) and the heat shock TF HSF-1 (Hsu et al., 2003) (Figures S2B and S2C and Table S2), both of which are required for increased longevity in daf-2/lnsr mutants. eak-7 mutants also exhibited DAF-16/FoxO-dependent resistance to ultraviolet, heat, and oxidative stress (Figures S2F–S2H and Table S2). Thus, EAK-7 controls life span and stress resistance by inhibiting DAF-16/FoxO activity.

EAK-7 Acts in Parallel to AKT-1 and AKT-2 to Control Life Span

In contrast to the strong enhancement of dauer arrest in akt-1 mutants caused by eak-7 mutation (Figures 1D–1F), eak-7 mutation enhanced the extended life span phenotype of akt-1 mutants modestly (mean life spans ±SD of 24.0 ± 3.3 for akt-1 versus 25.5 ± 4.9 for eak-7;akt-1, p < 0.0001 by the log rank test) (Figure 2A and Table S2). eak-7 mutation enhanced the life span extension phenotype of akt-2 mutants to a comparable extent (mean life spans ±SD of 21.8 ± 3.8 for eak-7;akt-2 versus 25.7 ± 7.4 for eak-7;akt-2, p < 0.0001 by the log rank test) (Figure 2C and Table S2). akt-1;akt-2 double mutants exhibited a profound extension of life span compared to akt-1 and akt-2 single mutants (Figure 2G). This is reminiscent of the extreme longevity of adult age-1/pi3k null mutants (Ayyadevara et al., 2008) and suggests that as is the case for dauer regulation, AKT-1 and AKT-2 are the major outputs of IIS in life span control.

Taken together, these results suggest that whereas EAK-7 acts in parallel to AKT-1 to regulate dauer arrest, EAK-7 acts in
parallel to both AKT-1 and AKT-2 to control adult life span. Notably, the magnitude of the enhancement of akt-1 and akt-2 mutant life span extension phenotypes by eak-7 mutation is substantially smaller than the effect of eak-7 mutation on the akt-1 mutant dauer arrest phenotype.

EAK-7 Acts in Parallel to the Germline to Control Life Span
Germline ablation extends life span by inducing the nuclear translocation of DAF-16/FoxO in intestinal cells (Berman and Kenyon, 2006; Hsin and Kenyon, 1999; Libina et al., 2003). Thus, one possible explanation for the relatively modest effect of eak-7 mutation on life span extension in akt-1 and akt-2 mutants (Figures 2A and 2C) compared to its effect on dauer arrest in akt-1 mutants (Figures 1D–1F) is that in eak-7;akt-1 and eak-7;akt-2 double mutant adult animals, signals from the germline that are not present in early larvae inhibit DAF-16/FoxO nuclear translocation. Therefore, we wished to determine whether eak-7 mutation enhances life span extension in animals lacking a germline, in which relative concentrations of nuclear

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**Figure 2. Genetic Interactions of eak-7 Mutants in Life Span Control**
(A) Life span phenotype of eak-7 null mutants at 25°C.
(B) daf-16/FoxO RNAi suppresses life span extension in eak-7 null mutants.
(C) eak-7 null mutation enhances the life span extension observed in akt-2 null mutants.
(D) sgk-1 activity is required for life span extension in eak-7 null mutants.
(E) eak-7 null mutation enhances life span extension of glp-1(e2141) mutants.
(F) Life span extension in eak-7 null mutants does not require eak-3 activity.
(G) akt-1;akt-2 double mutants are extremely long-lived compared to single mutants.
(H) sgk-1 activity is required for life span extension in akt-1 null mutants. See Table S2 for numbers of animals assayed and p values for all life span experiments.
DAF-16/FoxO are increased. To this end, we constructed double mutants with glp-1(e2141) animals, which lack a germline when raised at 25°C (Priess et al., 1987). Life span extension caused by eak-7 mutation was comparable at 20°C and 25°C (compare Figures 2C–2F to Figures 2A and 2B, Table S2). The magnitude of life span extension caused by the glp-1(e2141) mutation was consistent with previous reports (Berman and Kenyon, 2006) and greater than that caused by eak-7 mutation (Figure 2E and Table S2). Strikingly, glp-1;eak-7 double mutants lived nearly twice as long as glp-1 single mutants (Figure 2E and Table S2). Thus, EAK-7 acts in parallel to germline signals to control life span.

SGK-1 Is Required for Life Span Extension in eak-7 Mutants

Culturing worms on E. coli HT115 that express sgk-1 double-stranded RNA promotes long life due to a food avoidance behavior induced by reduction of sgk-1 activity that results in dietary restriction (Hertweck et al., 2004; Soukas et al., 2009). In contrast, sgk-1 null mutants are short-lived when cultured on the standard E. coli OP50 strain (Soukas et al., 2009). To determine whether SGK-1 is required for life span extension in eak-7 mutants, we assayed the life spans of eak-7;sgk-1 double mutants. The double mutant lived slightly longer than sgk-1 single mutants and substantially shorter than eak-7 single mutants (Figure 2F and Table S2), indicating that SGK-1 is necessary for life span extension in eak-7 mutants. Surprisingly, SGK-1 was also required for life span extension in akt-1 mutants (Figure 2H and Table S2). Thus, the requirement for SGK-1 in life span extension is not specific to eak-7 mutants. SGK-1 may be required for full DAF-16/FoxO activation in life span control, or SGK-1 may promote life span extension independently of DAF-16/FoxO.

EAK-7 Inhibits DAF-16/FoxO Target Gene Expression

To determine whether eak-7 mutation influences DAF-16/FoxO activity, we assayed endogenous transcript levels of three DAF-16/FoxO target genes: sod-3, mtl-1, and dod-3 (Murphy et al., 2003; Oh et al., 2006; Figures 3A–3D and S3). In early larval stages, both eak-7 and akt-1 single mutants had increased DAF-16/FoxO target gene mRNA levels relative to wild-type animals. akt-1 mutation caused a consistently larger increase than eak-7 mutation did. Strikingly, eak-7;akt-1 double mutants exhibited a synergistic increase in mRNA levels of all three DAF-16/FoxO target genes that either was comparable to or exceeded that observed in daf-2/InsR mutants (Figures 3A–3C). DAF-16/FoxO target gene expression in eak-7;akt-1 double mutants was approximately 50- to 100-fold greater than in wild-type animals and approximately 6- to 8-fold greater than in akt-1 single mutants (Figures 3A–3C). As expected, DAF-16/FoxO target gene expression in this context was completely dependent upon daf-16/FoxO.
Whereas adult eak-7 and akt-1 mutant animals exhibited increases in sod-3 mRNA comparable to increases observed in larvae, adult eak-7;akt-1 double mutant animals did not exhibit a synergistic increase in sod-3 transcript levels (Figure 3D). Furthermore, sod-3 transcript levels in adult eak-7;akt-1 double mutants were substantially lower than those observed in adult daf-2/InsR mutants (Figure 3D). This difference in DAF-16/FoxO target gene expression in distinct life stages of eak-7;akt-1 double mutants is commensurate with the magnitude of enhancement of akt-1 mutant dauer arrest and life span extension phenotypes by eak-7 mutation (Figures 1D–1F and 2A) and may be a consequence of the inhibitory effect of the adult germline on DAF-16/FoxO activity (Lin et al., 2001).

In order to determine whether EAK-7 regulates DAF-16/FoxO target gene expression by influencing promoter activity, we examined the effect of eak-7 mutation on the expression of a GFP reporter under the control of the sod-3 promoter (Libina et al., 2003) in early-stage larvae. Whereas mutations in eak-7 and akt-1 alone did not result in substantial changes in sod-3::GFP expression, eak-7;akt-1 double mutants exhibited a dramatic increase in GFP expression throughout the animal, relative to wild-type animals (Figure 3E). These findings are consistent with the effects of eak-7 and akt-1 mutations on endogenous DAF-16/FoxO target gene expression (Figures 3A–3C). Thus, EAK-7 inhibits DAF-16/FoxO target gene transcription, and in larvae, the effect of eak-7 mutation on DAF-16/FoxO target gene transcription is magnified in an akt-1 mutant background.

EAK-7 inhibits nuclear DAF-16/FoxO activity

Because eak-7 mutation enhances akt-1 mutant phenotypes and DAF-16/FoxO target gene expression more strongly during larval development than in adulthood (compare Figures 1D–1F to Figure 2A and Figures 3A to 3D), we explored the mechanism by which EAK-7 inhibits DAF-16/FoxO activity in larvae. Since IIS inhibits DAF-16/FoxO by promoting its cytoplasmic sequestration (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), we assayed the subcellular localization of a functional DAF-16::GFP fusion protein (Henderson and Johnson, 2001) in various mutant backgrounds (Figure 4A). As expected, we observed diffuse fluorescence corresponding to cytoplasmic localization in wild-type animals and increased punctate fluorescence corresponding to nuclear localization in akt-1 mutants (Figure 4A) (Hertweck et al., 2004; Zhang et al., 2008). In contrast to akt-1 mutants, eak-7 mutant animals exhibited diffuse fluorescence indistinguishable from that observed in wild-type animals (Figure 4A). These results suggest that unlike AKT-1, which inhibits DAF-16/FoxO by promoting its translocation from the nucleus to the cytoplasm, EAK-7 inhibits nuclear DAF-16/FoxO activity without inducing DAF-16/FoxO nuclear exclusion.

To examine in more detail the possibility that EAK-7 inhibits nuclear DAF-16/FoxO activity, we determined the effect of 14-3-3 protein loss of function on the dauer arrest phenotype of eak-7 mutants. The 14-3-3 protein FTT-2 binds to AKT-phosphorylated DAF-16/FoxO and sequesters it in the cytoplasm, and reduction of FTT-2 function promotes DAF-16/FoxO nuclear localization (Berdichevsky et al., 2006; Li et al., 2007a). Since ftt-2 deletion is lethal (Berdichevsky et al., 2006; Li et al., 2007a), we inactivated ftt-2 using RNAi. To clarify whether EAK-7 acts in the same pathway as AKT-2 in DAF-16/FoxO regulation (see Figures 1F and 1G), we also tested the effect of ftt-2 RNAi on the dauer arrest phenotype of akt-2 mutants. If EAK-7 and AKT-2 act in the same pathway to regulate DAF-16/FoxO activity, then ftt-2 RNAi should have the same effect on the dauer arrest phenotype of both eak-7 and akt-2 mutants.

As expected, ftt-2 RNAi did not induce dauer arrest in wild-type or akt-1 mutant animals (Figure 4B). Whereas ftt-2 RNAi strongly promoted dauer arrest in eak-7 mutant animals, it did not induce dauer arrest in an akt-2 null mutant. Thus, EAK-7 and AKT-2 act in distinct pathways to regulate DAF-16/FoxO activity. The lack of phenotypic enhancement observed in eak-7;akt-2 double mutants (Figures 1F and 1G) is likely a consequence of the relatively modest effect of akt-2 mutation on DAF-16/FoxO nuclear translocation in a wild-type akt-1 background (Hertweck et al., 2004). Although this result supports a model whereby EAK-7 inhibits nuclear DAF-16/FoxO activity, it does not exclude the possibility that EAK-7 may contribute to DAF-16/FoxO cytoplasmic retention in an FTT-2-independent manner.

To further investigate this issue, we determined the effect of eak-7 mutation on dauer arrest in animals expressing a constitutively nuclear DAF-16/FoxO mutant fused to GFP that lacks all consensus Akt/PKB phosphorylation sites (DAF-16AM) (Lin et al., 2001). As previously reported (Lin et al., 2001), wild-type animals expressing DAF-16AM did not arrest as dauers (Figure 4C). In contrast, eak-7 mutation promoted highly penetrant dauer arrest in animals expressing DAF-16AM but not in animals expressing a wild-type DAF-16::GFP transgene (Figure 4C). This result provides further support for the hypothesis that EAK-7 inhibits nuclear DAF-16/FoxO activity and indicates that the ability of eak-7 mutation to enhance dauer arrest in akt-1 mutants is a function of nuclear enrichment of DAF-16/FoxO caused by akt-1 mutation as opposed to the dysregulation of other AKT-1 targets, such as SKN-1 and CEP-1/p53 (Quevedo et al., 2007; Tullet et al., 2008). Furthermore, it demonstrates that EAK-7 inhibition of DAF-16/FoxO activity does not require the canonical Akt/PKB phosphorylation sites present in wild-type DAF-16/FoxO.

Thus, EAK-7 inhibits the activity of DAF-16/FoxO that localizes to the nucleus by virtue of akt-1 mutation (Figures 1D–1F, 3A–3C, and 3E), depletion of FTT-2 activity (Figure 4B), or mutation of its Akt/PKB phosphorylation sites (Figure 4C).

EAK-7 reduces steady-state DAF-16/FoxO protein levels

To further elucidate the mechanism by which EAK-7 inhibits DAF-16/FoxO activity, we determined the effect of eak-7 mutation on endogenous DAF-16/FoxO transcript and protein levels. Mutation of eak-7 and akt-1, either alone or in combination, did not significantly affect daf-16/FoxO mRNA levels (Figure 4D). Endogenous DAF-16/FoxO protein was undetectable in lysates from wild-type and eak-7 single mutants (Figure 4E). In contrast, DAF-16/FoxO was detectable in lysates from akt-1 single mutants, and eak-7;akt-1 double mutants exhibited a synergistic increase in DAF-16/FoxO protein levels compared to eak-7 and akt-1 single mutants (Figure 4E). Increased DAF-16/FoxO protein levels were also observed in daf-2/InsR mutants (Figure 4E). Thus, EAK-7 reduces steady-state DAF-16/FoxO protein levels in akt-1 mutants without influencing daf-16/FoxO mRNA levels.
Relative DAF-16/FoxO protein levels in lysates from various mutants correlated with both the magnitude of dauer arrest (Figures 1D–1F) as well as relative levels of DAF-16/FoxO target gene expression in the same mutants (Figures 3A–3C and 3E).

**eak-7 Encodes a Conserved Protein**

*eak-7* encodes a conserved protein with a consensus N-myristoylation motif (Farazi et al., 2001) and a TLDc (TBC and LysM domain-containing) domain (Doerks et al., 2002), the function of which is obscure (Figures 5A, 5B, S4A, and S4B). The *mg338* allele is a missense mutation in the TLDc domain (Figures 5A, 5B, and S4A). The *eak-7(tm3188)* null mutation harbors a deletion that spans the entire first exon and part of the second exon of K08E7.1 (Figure 5A). The *eak-7* gene lies in an operon downstream of *hsb-1* (Figure 5A), which encodes a conserved protein that binds to and inhibits HSF-1 (Satyal et al., 1998).

**EAK-7 Is Expressed in Multiple Tissues**

To determine the expression pattern and subcellular localization of EAK-7, we analyzed transgenic animals that expressed an EAK-7::GFP fusion protein under the control of the *hsb-1* operon promoter (*hsb-1*p::EAK-7::GFP). This transgene rescued the life span extension phenotype of an *eak-7* null mutant (Figure S5). GFP was first expressed during embryogenesis (Figure S6A). At all stages of postembryonic development, transgenic animals exhibited fluorescence in the pharynx, nervous system, intestine, body wall muscle, hypodermis, vulva, and a group of cells near the anus (Figures 5C and S6B–S6G and data not shown).
expression pattern is consistent with that reported for a K08E7.1 promoter fusion construct as part of a high-throughput analysis of C. elegans gene expression (Hunt-Newbury et al., 2007). EAK-7::GFP is also expressed in the XXX cells (Figure S6H), where eak-3, eak-4, sdf-9/eak-5, and eak-6 are specifically expressed (Hu et al., 2006; Ohkura et al., 2003; Zhang et al., 2008).

Consistent with the presence of an N-myristoylation motif, EAK-7::GFP localized to the plasma membrane (Figures 5D and S6H). Mutation of the glycine residue (G2A) that is required for N-myristoylation (Farazi et al., 2001) resulted in diffuse cytoplasmic fluorescence (Figure 5D), suggesting that the motif is functional. The G2A EAK-7::GFP mutant partially rescued dauer arrest in eak-7;akt-1 double mutants (data not shown), indicating that N-myristoylation is not an absolute requirement for EAK-7 function.

**EAK-7 Controls Dauer Arrest and Life Span Nonautonomously**

To determine the site of EAK-7 action in the control of dauer arrest and life span, we generated tissue-specific EAK-7::GFP expression constructs and tested their ability to rescue eak-7 mutant phenotypes. Since both EAK-7 and DAF-16/FoxO are expressed in neurons and intestine (Figure 5C) (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), and DAF-16/FoxO activity in neurons and intestine promotes dauer arrest and life span extension (Apfeld and Kenyon, 1998; Libina et al., 2003; Wolkow et al., 2000), we expressed EAK-7::GFP using neuron (ric-19p) and intestine (ges-1p)-specific promoters (Aamodt et al., 1991; Pilon et al., 2000). Since EAK-7 is also expressed in the XXX cells (Figure S6H) and acts in the same pathway as other EAK proteins that are specifically expressed in the XXX cells (Figure S1D) (Hu et al., 2006; Ohkura et al., 2003; Zhang et al., 2008), we also tested an EAK-7::GFP transgene expressed in the XXX cells (Figure S1D) (Hu et al., 2006; Ohkura et al., 2003; Zhang et al., 2008).

**DISCUSSION**

Our results indicate that EAK-7 defines a conserved pathway that acts in parallel to signals that inhibit DAF-16/FoxO nuclear translocation (primarily AKT-1 in early larvae and AKT-2, and the germline in adults) to control DAF-16/FoxO-dependent dauer arrest and life span extension (Figure 7). Since an eak-7 null mutation promotes dauer arrest in three distinct contexts, characterized by increases in relative nuclear concentrations of DAF-16/FoxO (Figures 1, 4B, and 4C), and enhances life span extension in germline-deficient animals (Figure 2E), which exhibit increased DAF-16/FoxO nuclear localization (Lin et al., 2001), we favor a model whereby EAK-7 inhibits the activity of nuclear DAF-16/FoxO.

The increase in endogenous DAF-16/FoxO protein levels caused by eak-7 mutation in an akt-1 mutant background (Figure 4E) suggests that EAK-7 inhibits nuclear DAF-16/FoxO activity by reducing steady-state DAF-16/FoxO protein levels. This could occur via regulation of DAF-16/FoxO synthesis or turnover. Our data suggest that the DAF-16/FoxO E3 ubiquitin ligase RLE-1 (Li et al., 2007b) and the cullins CUL-5 and CUL-6 (Ghazi et al., 2007) do not mediate potential effects of EAK-7 on DAF-16/FoxO turnover (Figure S7 and data not shown). At this time, we cannot exclude the possibility that EAK-7 also...
inhibits DAF-16/FoxO activity through mechanisms that are independent of DAF-16/FoxO protein levels.

EAK-7 controls life span and dauer arrest nonautonomously (Figure 6), as do DAF-2/InsR and DAF-16/FoxO (Apfeld and Kenyon, 1998; Libina et al., 2003; Wolkow et al., 2000). Intestinal EAK-7::GFP expression rescued life span extension but not dauer arrest in eak-7 mutants (Figures 6A and 6D), consistent with the role of intestinal DAF-16/FoxO in life span control (Libina et al., 2003). Interestingly, expression of EAK-7::GFP in the XXX cells, where a DAF-16::GFP fusion protein is not expressed (Hu et al., 2006), suffices to rescue both dauer arrest and life span extension in eak-7 mutants (Figures 6A and 6D). Thus, EAK-7 may regulate DAF-16/FoxO activity via both cell-autonomous and cell-nonautonomous mechanisms. Since a functional EAK-7::GFP fusion protein localizes to the plasma membrane (Figures 5D and S6H), cell-autonomous regulation of nuclear DAF-16/FoxO by EAK-7 is likely to be indirect.

The conservation of EAK-7 and IIS throughout animal phylogeny (Figure S4B) suggests that mechanisms by which EAK-7 regulates DAF-16/FoxO activity may also be conserved in mammals. In light of recent reports demonstrating that FoxO TFs are tumor suppressors and critical regulators of bone mass and glucose homeostasis in mice (Ambrogini et al., 2010; Dong et al., 2008; Matsumoto et al., 2006; Paik et al., 2007; Rached et al., 2010), dysregulation of human EAK-7 may play a role in the pathogenesis of type 2 diabetes, osteoporosis, and cancer. EAK-7 may also play a role in human longevity control, as FoxO3 polymorphisms are associated with extreme longevity in three independent cohorts of long-lived individuals.
(Flachsbart et al., 2009; Li et al., 2009; Willcox et al., 2008). Thus, functional human EAK-7 polymorphisms could influence metabolism, tumor survival, bone mineral density, and life span based on their effect on nuclear FoxO activity.

**EXPERIMENTAL PROCEDURES**

**Strains and Reagents**

The following strains were used: N2 Bristol (wild-type), daf-2(e1370) (Kimura et al., 1997), eak-7(mg338), akt-1(mg306) (Hu et al., 2006), daf-16(muDf47) (Ogg et al., 1997), daf-16(mu66) (Lin et al., 2001), akt-2(ok393) (Hertweck et al., 2004), sgk-1(mu455) (Soukas et al., 2009), daf-12(tm6114d11) (Antebi et al., 2000), gfp-1(e2141) (Press et al., 1987), eak-3(mg344) (Zhang et al., 2008), nls1(tm2447) (Li et al., 2007b), J356 (Henderson and Johnson, 2001), CF1553 (Libina et al., 2003), CF1371, and CF1330 (Lin et al., 2001). Unless otherwise indicated, the akt-1 mutant allele used was mg306, the eak-7 mutant allele used was tm3188, and the daf-16/FoxO mutant allele used was mu66. Double and triple mutants were constructed using standard genetic techniques. Genotypes were confirmed using either restriction fragment length or PCR polymorphisms.

**Dauer Arrest Assays**

Dauer arrest assays were performed as described (Zhang et al., 2008). 27°C dauer assays were performed using NGM plates with or without supplemental cholesterol as indicated.

**Life Span Assays**

Life span assays were performed at 20°C or 25°C as described (Zhang et al., 2008). Briefly, L4 larvae were placed onto NGM plates containing 25 μg/ml fluorodeoxyuridine (FUDR) and 10 μg/ml nystatin that had been seed with 20x concentrated E. coli OP50. Animals were assayed for viability visually or with mild prodding. GraphPad Prism (GraphPad Software; La Jolla, CA) was used for graphing and statistical analysis.

**Quantitative RT-PCR**

For L2 animals, wild-type and mutant animals grown at 25°C were harvested 24 hr after a 2 hr egg lay, and total RNA was prepared from 300–400 animals per strain using TRIzol (Invitrogen; Carlsbad, CA). For adults, 50 L4 animals were placed on FUDR/nystatin plates at 25°C and harvested 24 hr after a 2 hr egg lay, and total RNA was prepared from 300–400 animals per strain using TRIzol (Invitrogen; Carlsbad, CA). For adults, 50 L4 animals were placed on FUDR/nystatin plates at 25°C and harvested 4 days later. Quantitative PCR primer sequences are available upon request.

**sod-3::GFP and daf-16::GFP Localization Assays**

sod-3::GFP (mul84) (Libina et al., 2003) and DAF-16::GFP (zls356) (Henderson and Johnson, 2001) were introduced into various mutant backgrounds and visualized at 200x magnification immediately after mounting.

**RNAi**

Feeding RNAi was performed using standard procedures. For dauer assays, 6 cm plates containing NGM + 5 mM IPTG + 25 μg/ml carbenicillin were spotted with 500 μl of overnight culture of E. coli HT115 harboring either control L4440 vector or hts-2 RNAi plasmid. Plates were allowed to dry overnight at room temperature. Gravid animals cultured on standard NGM plates were spotted with 500 μl of overnight culture of E. coli HT115 harboring either control L4440 vector or smk-1 RNAi plasmid. Plates were allowed to dry overnight at room temperature. Young adult animals were picked to plates and scored for viability as described.

**DAF-16**

**DAF-16 Dauer Assay**

CF1371 and CF1330 (Lin et al., 2001) carry extrachromosomal arrays containing either a GFP::DAF-16A transgene with all AKT phosphorylation sites mutated (CF1371) or a wild-type GFP::DAF-16A transgene (CF1330) with the rol-6 co-injection marker (Lin et al., 2001). Transgenic animals and their nontransgenic siblings were identified using a fluorescence dissecting microscope and/or the rol phenotype. Measurements represent the percentage of Rol animals that arrested as dauer.

**Immunoblotting**

L2 larvae grown at 25°C were harvested 24 hr after a 2 hr egg lay and washed three times in M9 buffer. Protein lysates were prepared by boiling 600–800 animals per strain in sample buffer (Bio-Rad; Hercules, CA). Proteins were separated by SDS-PAGE, and immunoblots were performed using standard procedures. Anti-DAF-16/FoxO antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-actin antibodies from Millipore (Billerica, MA), anti-mouse-HRP from GE (Piscataway, NJ), and anti-goat-HRP from Jackson Laboratory (Bar Harbor, ME). The EAK-7 antibody was raised in rabbits against a peptide corresponding to the last 19 residues of C. elegans EAK-7 (Proteintech Group, Inc.; Chicago).

**Cloning of eak-7**

Isolation, mapping, and sequencing of eak-7(mg338) were performed as described for eak-3 (Zhang et al., 2008).

**Transgenes and Transgenic Rescue Experiments**

The hsb-1::EAK-7::GFP translational fusion was generated using overlap extension PCR (Hobert, 2002). The following fragments were amplified and fused: a 3391 bp genomic fragment corresponding to nucleotides 6205–9595 of cosmid K08B7 encoding the hsb-1 promoter, a 1884 bp genomic fragment corresponding to nucleotides 3090–4973 of cosmid K08B7 that includes the open reading frame of K08B7. 1 up to but not including the translation termination codon, and a DNA fragment containing GFP and the unc-54 3′ UTR that was amplified from pPD95.75 (a gift from Andrew Fire). The EAK-7::GFP 02A N-myristoylation mutant was also constructed using overlap extension PCR by incorporating nucleotide changes into the primers, resulting in mutation of glycine to alanine at residue 2 of EAK-7.

Tissue-specific EAK-7::GFP transgenes contained the same K08B7.1 genomic sequences as the hsb-1::EAK-7::GFP construct and were also generated by overlap extension PCR. The following regions were used in place of the hsb-1 promoter: 1054 nucleotides upstream of the hsb-4 start site amplified from cosmid F53B2 for the xxx-specific construct, 1547 nucleotides upstream of the rct-19 start site amplified from pJK325 for the neuron-specific construct, and 2256 nucleotides upstream of the ges-1 start site amplified from pJK332 for the intestine-specific construct. All fusion products were verified by restriction digest or sequencing. Fusion PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN; Venlo, The Netherlands).

Transgenic animals were generated and localization studies performed as described previously (Zhang et al., 2008). Animals were injected with a 100 ng/ml mixture containing the transgene construct (25 ng/μl), the rol-6 (pRF-4) co-injection marker (12.5 ng/μl), and pBluescript. Transgenic animals were visualized using an Olympus BX61 upright microscope and analyzed using SlideBook 4.1 digital microscopy software (Intelligent Imaging Innovations, Inc.; Denver, CO). To determine if EAK-7 was expressed in the XXX cells, hsb-1::EAK-7::GFP was injected into worms carrying an integrated sdp-9::RFP promoter fusion (Hu et al., 2006). Animals were visualized using a Leica DM6000 confocal microscope and analyzed using Leica LAS AF Version 1.8.2. Animals were mounted on 2% agarose pads in the presence of 10 mM levamisole or 10 mM sodium azide, visualized, and photographed immediately after mounting. Transgenic animals and their nontransgenic siblings were distinguished using a fluorescence dissecting microscope and/or the Rol phenotype.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, seven figures, and four tables and can be found with this article online at doi:10.1016/j.cmet.2010.05.004.

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