Published in final edited form as:
Cell Metab. 2010 July 4; 12(1): 30–41. doi:10.1016/j.cmet.2010.05.004.

EAK-7 controls development and lifespan 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 new regulatory pathway that governs the activity of nuclear FoxO without altering its subcellular location.

INTRODUCTION

FoxO transcription factors (TFs) promote lifespan 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 (Ambrogini 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; Willcox 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.

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The authors disclose no conflicts of interest with this study.

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 lifespan, 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 transcription factor 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 lifespan 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 et al., 1999; 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, lifespan, 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 F_2 progeny that arrested as dauers at 25°C. *eak* 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–B).

EAK-7 acts in parallel to AKT-1 to regulate dauer arrest

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–C), they develop into adults on standard NGM plates at 25°C (Figures 1D–F). However, *eak-7* mutation strongly enhances dauer arrest in an *akt-1* null mutant (Figures 1D–F) as well as in *age-1/PI3K* and *pdk-1* partial loss-of-function mutants

(Figure S1C) and *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–F), 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*, *eak-7;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–G). 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–G), 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–G), *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.*, non-conditional constitutive dauer arrest, is similar to that observed in *age-1/PI3K* and *daf-2/InsR* 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–F), *eak-7;akt-2* double mutants did not arrest as dauers at 25°C or 27°C (Figures 1F–G). 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 lifespan *via* DAF-16/FoxO

IIS mutants also exhibit DAF-16/FoxO-dependent lifespan 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–F, S2A–E, and Table S2), and this phenotype requires *daf-16/FoxO* (Figures 2B, 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 transcription factor HSF-1 (Hsu et al., 2003) (Figures S2B–C and Table S2), both of which are required for increased longevity in *daf-2/InsR* mutants. *eak-7* mutants also exhibited DAF-16/FoxO-dependent resistance to ultraviolet, heat, and oxidative stress (Figures S2F–H and Table S2). Thus, EAK-7 controls lifespan and stress resistance by inhibiting DAF-16/FoxO activity.

EAK-7 acts in parallel to AKT-1 and AKT-2 to control lifespan

In contrast to the strong enhancement of dauer arrest in *akt-1* mutants caused by *eak-7* mutation (Figures 1D–F), *eak-7* mutation enhanced the extended lifespan phenotype of *akt-1* mutants modestly (Figure 2A and Table S2; mean lifespans in days +/− s.d. of 24.0+/−3.3 for *akt-1 vs.* 25.5+/−4.9 for *eak-7;akt-1*, $p < 0.0001$ by the log-rank test). *eak-7* mutation enhanced the lifespan extension phenotype of *akt-2* mutants to a comparable extent (Figure 2C and Table S2; mean lifespans +/− s.d. of 21.8+/−3.8 for *akt-2 vs.* 25.7+/−7.4 for *eak-7;akt-2*, $p < 0.0001$ by the log-rank test). *akt-1;akt-2* double mutants exhibited a profound extension of lifespan 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 lifespan 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 lifespan. Notably, the magnitude of the enhancement of *akt-1* and *akt-2* mutant lifespan 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 lifespan

Germline ablation extends lifespan 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 lifespan 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–F) 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 lifespan extension in animals lacking a germline, in which relative concentrations of nuclear 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). Lifespan extension caused by *eak-7* mutation was comparable at 20°C and 25°C (compare Figures 2C–F to Figures 2A–B; Table S2). The magnitude of lifespan 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 lifespan.

SGK-1 is required for lifespan 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 lifespan extension in *eak-7* mutants, we assayed the lifespans 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 2D and Table S2), indicating that SGK-1 is necessary for lifespan extension in *eak-7* mutants. Surprisingly, SGK-1 was also required for lifespan extension in *akt-1* mutants (Figure 2H and Table S2). Thus, the requirement for SGK-1 in lifespan extension is not specific to *eak-7* mutants. SGK-1 may either be required for full DAF-16/FoxO activation in lifespan control, or SGK-1 may promote lifespan extension independently of DAF-16/FoxO.

Lifespan extension in *eak-7* mutants does not require other EAK proteins

Although EAK-7 acts in the same pathway as other EAK proteins to regulate dauer arrest (Figure S1D), *eak-7* mutants are distinct from other *eak* mutants in that they are long-lived (Figure 2F and S2D–E; Hu et al., 2006; Zhang et al., 2008). To determine whether the activity of other *eak* genes is required for lifespan extension in *eak-7* mutants, we performed lifespan assays on various *eak-7;eak* double mutants. Mutations in *eak-3*, *sdf-9*/*eak-5*, and *eak-6* did not suppress lifespan extension in *eak-7* mutants (Figures 2F and S2D–E; Table S2). Thus, other EAK proteins are not required for lifespan extension caused by *eak-7* mutation.

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–D 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–C). DAF-16/FoxO target gene expression in *eak-7;akt-1* double mutants was approximately 50–100-fold greater than in wild-type animals and approximately 6–8-fold greater than in *akt-1* single mutants (Figures 3A–C). 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 lifespan extension phenotypes by *eak-7* mutation (Figures 1D–F 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–C). 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–F to Figure 2A and Figure 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 the possibility that EAK-7 inhibits nuclear DAF-16/FoxO activity in more detail, 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–G), 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–G) 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-16^{AM}; Lin et al., 2001). As previously reported (Lin et al., 2001), wild-type animals expressing DAF-16^{AM} did not arrest as dauers (Figure 4C). In contrast, *eak-7* mutation promoted highly penetrant dauer arrest in animals expressing DAF-16^{AM} 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–F, 3A–C, 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–F) as well as relative levels of DAF-16/FoxO target gene expression in the same mutants (Figures 3A–C and 3E).

eak-7 encodes a novel 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–B and S4A–B). The *mg338* allele is a missense mutation in the TLDc domain (Figures 5A–B 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 lifespan extension phenotype of an *eak-7* null mutant (Figure S5). GFP was first expressed during embryogenesis (Figure S6A). At all stages of post-embryonic 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, S6B–G, and data not shown). This expression pattern is consistent with that reported for a *K08E7.1* promoter fusion constructed 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 lifespan nonautonomously

To determine the site of EAK-7 action in the control of dauer arrest and lifespan, 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 lifespan 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 expressed specifically 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 under the control of the XXX-specific *eak-4* promoter (Hu et al., 2006). Transgenic lines harboring these constructs exhibited fluorescence patterns consistent with the reported promoter specificity (data not shown).

XXX- and neuron-specific EAK-7::GFP expression rescued the dauer arrest phenotype of *eak-7;akt-1* mutants, whereas intestinal EAK-7::GFP expression failed to rescue dauer arrest (Figures 6A–C and Table S3). In contrast, expression of EAK-7::GFP in the XXX cells, neurons, or the intestine was sufficient to rescue the lifespan extension phenotype of *eak-7* mutants (Figures 6D–F and Table S4). Thus, similar to DAF-2/InsR (Apfeld and Kenyon,

1998; Wolkow et al., 2000), EAK-7 acts nonautonomously to control both dauer arrest and lifespan.

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-1, AKT-2, and the germline in adults) to control DAF-16/FoxO-dependent dauer arrest and lifespan 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 and 4B–C) and enhances lifespan 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 lifespan 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 lifespan extension but not dauer arrest in *eak-7* mutants (Figures 6C and 6F), consistent with the role of intestinal DAF-16/FoxO in lifespan 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 rescues both dauer arrest and lifespan 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 transcription factors 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 lifespan 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), eak-7(tm3188), akt-1(mg306)* (Hu et al., 2006), *daf-16(mgDf47)* (Ogg et al.,

1997), *daf-16(mu86)* (Lin et al., 2001), *akt-2(ok393)* (Hertweck et al., 2004), *sgk-1(mg455)* (Soukas et al., 2009), *daf-12(rh61rh411)* (Antebi et al., 2000), *glp-1(e2141)* (Priess et al., 1987), *eak-3(mg344)* (Zhang et al., 2008), *rle-1(tm2447)* (Li et al., 2007b), TJ356 (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 *mu86*. 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.

Lifespan assays

Lifespan 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 seeded with 20× 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 24h after a two-hour 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 four days later. qPCR primer sequences are available upon request.

*sod-3*p::GFP and DAF-16::GFP localization assays

sod-3::GFP (*muIs84*) (Libina et al., 2003) and DAF-16::GFP (*zIs356*) (Henderson and Johnson, 2001) were introduced into various mutant backgrounds and visualized at 200× 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 *ftt-2* RNAi plasmid. Plates were allowed to dry overnight at room temperature. Gravid animals cultured on standard NGM plates containing *E. coli* OP50 were picked to RNAi plates for egglays. Dauers were scored after progeny had been incubated at 25°C for 48–60 hours.

For lifespan assays, 6-cm plates containing NGM + 5 mM IPTG + 25 μg/ml carbenicillin + 25 μg/ml FUDR + 10 μg/ml nystatin were spotted with 400 μl of 5× concentrated 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^{AM} 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 non-transgenic siblings were identified using a

fluorescence dissecting microscope and/or the Rol phenotype. Measurements represent the percentage of Rol animals that arrested as dauers.

Immunoblotting

L2 larvae grown at 25 °C were harvested 24h after a two-hour 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 Labs (Bar Harbor, MA). The EAK-7 antibody was raised in rabbits against a peptide corresponding to the last 19 residues of *C. elegans* of EAK-7 (Proteintech Group Inc., Chicago, IL).

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*p::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 K08E7 encoding the *hsb-1* promoter, a 1884 bp genomic fragment corresponding to nucleotides 3090–4973 of cosmid K08E7 that includes the open reading frame of *K08E7.1* 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 Dr. Andrew Fire). The EAK-7::GFP G2A N-myristoylation mutant was also constructed using overlap extension PCR by incorporating nucleotide changes into the primers resulting in mutation of glycine at residue 2 of EAK-7 to alanine.

Tissue-specific EAK-7::GFP transgenes contained the same *K08E7.1* genomic sequences as the *hsb-1*p::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 *eak-4* start site amplified from cosmid F53B2 for the XXX-specific construct, 1547 nucleotides upstream of the *ric-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/μl mixture containing the transgene construct (25 ng/μl), the *rol-6*(pRF4) coinjection marker (12.5ng/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*p::EAK-7::GFP was injected into worms carrying an integrated *sdf-9p::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 non-transgenic siblings were distinguished using a fluorescence dissecting microscope and/or the Rol phenotype.

Highlights for Alam *et al.*, D-10-00082

- *eak-7* emerged from a *C. elegans* screen for enhancers of the *akt-1* mutant phenotype
- EAK-7 controls larval development and adult lifespan by inhibiting DAF-16/FoxO
- EAK-7 and AKT-1 inhibit DAF-16/FoxO *via* distinct mechanisms
- EAK-7 inhibits nuclear DAF-16/FoxO without promoting its cytoplasmic translocation

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank John Kim, Shawn Xu, and Lois Weisman for discussions and comments on the manuscript, Sylvia Lee for useful suggestions, Cheng-Yu Lee for assistance with confocal microscopy, Yuji Kohara for *eak-7* cDNAs, Andrew Fire for pPD95.75, John Kim for pJK325 and pJK332, Alex Soukas for *sgk-1(mg455)*, and Muazzum Shah for outcrossing *eak-7(tm3188)*. Some strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). These studies were supported by the Life Sciences Institute at the University of Michigan as well as by grants from the Nathan Shock Center for the Biology of Aging and the Michigan Diabetes Research and Training Center at the University of Michigan Medical School, the Sidney Kimmel Foundation for Cancer Research, NIH grants DK62884 and DK78183 (P.J.H.), and an American Cancer Society postdoctoral fellowship (#113680) to H.A.

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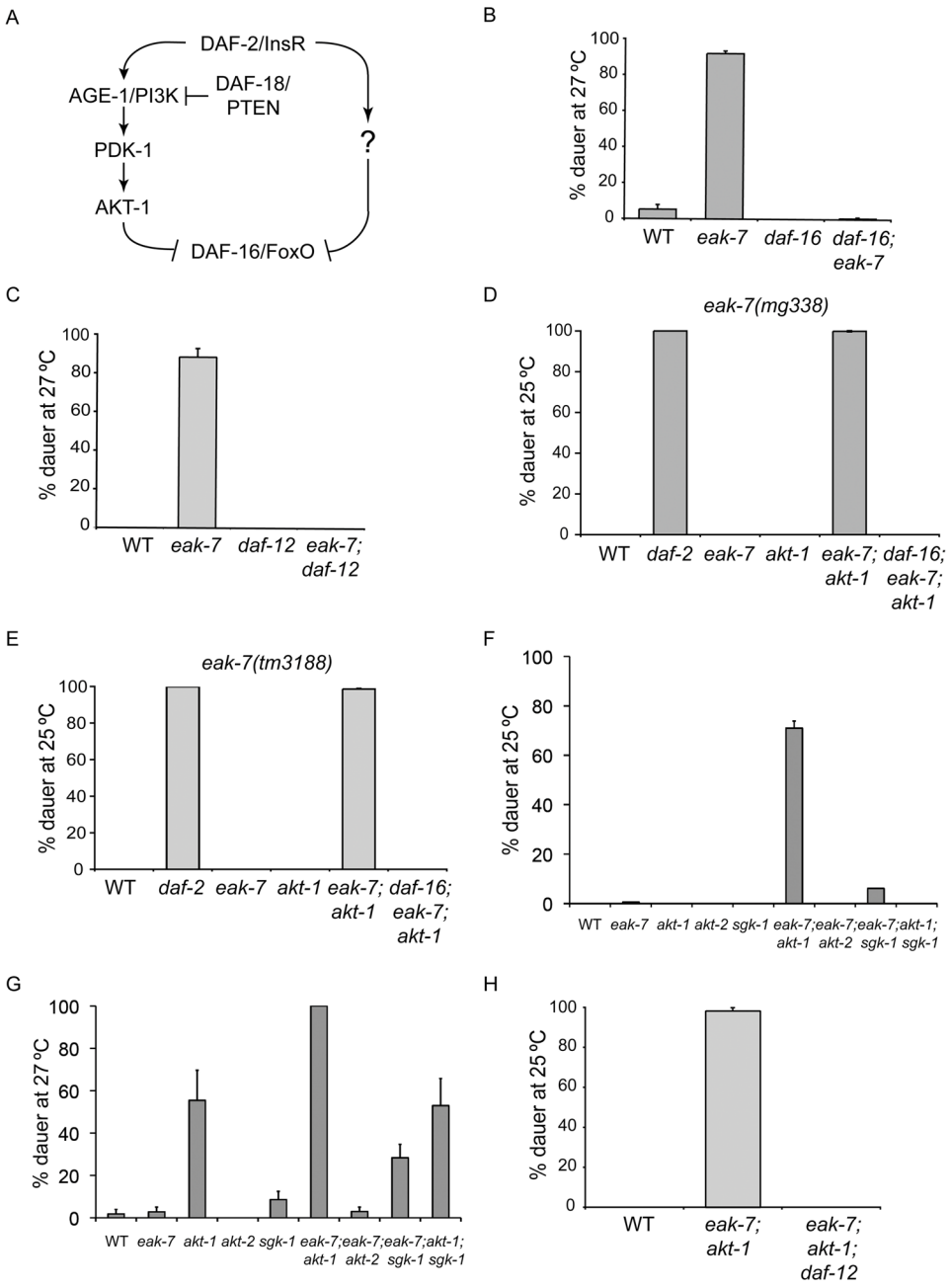

Figure 1. EAK-7 acts in parallel to AKT-1 to inhibit dauer arrest *via* DAF-16/FoxO and DAF-12

A. Schematic of the DAF-2/InsR pathway. B. *daf-16/FoxO* null and C. *daf-12* null mutations suppress the dauer arrest phenotype of an *eak-7* null mutant at 27°C on NGM plates lacking supplemental cholesterol. D. Dauer arrest phenotypes of *eak-7(mg338)* at 25°C. The null allele *daf-16(mgDf47)* was used in this experiment. E. Dauer arrest phenotypes of the null allele *eak-7(tm3188)* at 25°C. F. and G. Genetic interactions of *eak-7* null mutation with *akt-1*, *akt-2*, and *sgk-1* null mutations at F. 25°C and G. 27°C on standard NGM plates containing supplemental cholesterol. H. A *daf-12* null mutation suppresses the dauer arrest phenotype of *eak-7;akt-1* double mutants at 25°C. All measurements in this figure represent the mean + S.D. See Table S1 for raw data and numbers of animals scored in each dauer assay.

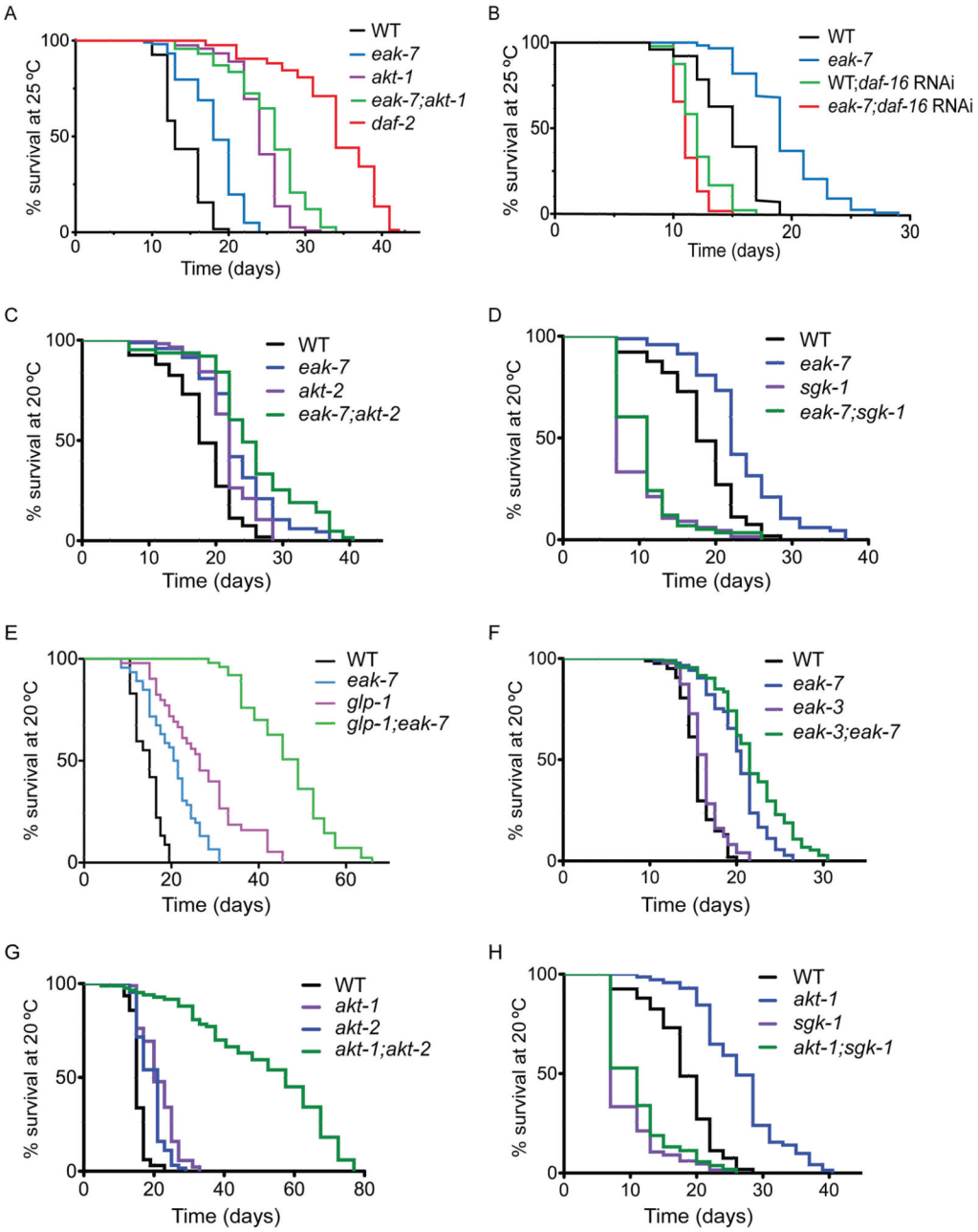

Figure 2. Genetic interactions of *eak-7* mutants in lifespan control

A. Lifespan phenotype of *eak-7* null mutants at 25°C. B. *daf-16/FoxO* RNAi suppresses lifespan extension in *eak-7* null mutants. C. *eak-7* null mutation enhances the lifespan extension observed in *akt-2* null mutants. D. *sgk-1* activity is required for lifespan extension in *eak-7* null mutants. E. *eak-7* null mutation enhances lifespan extension of *glp-1(e2141)* mutants. F. Lifespan 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 lifespan extension in *akt-1* null mutants. See Table S2 for numbers of animals assayed and p-values for all lifespan experiments.

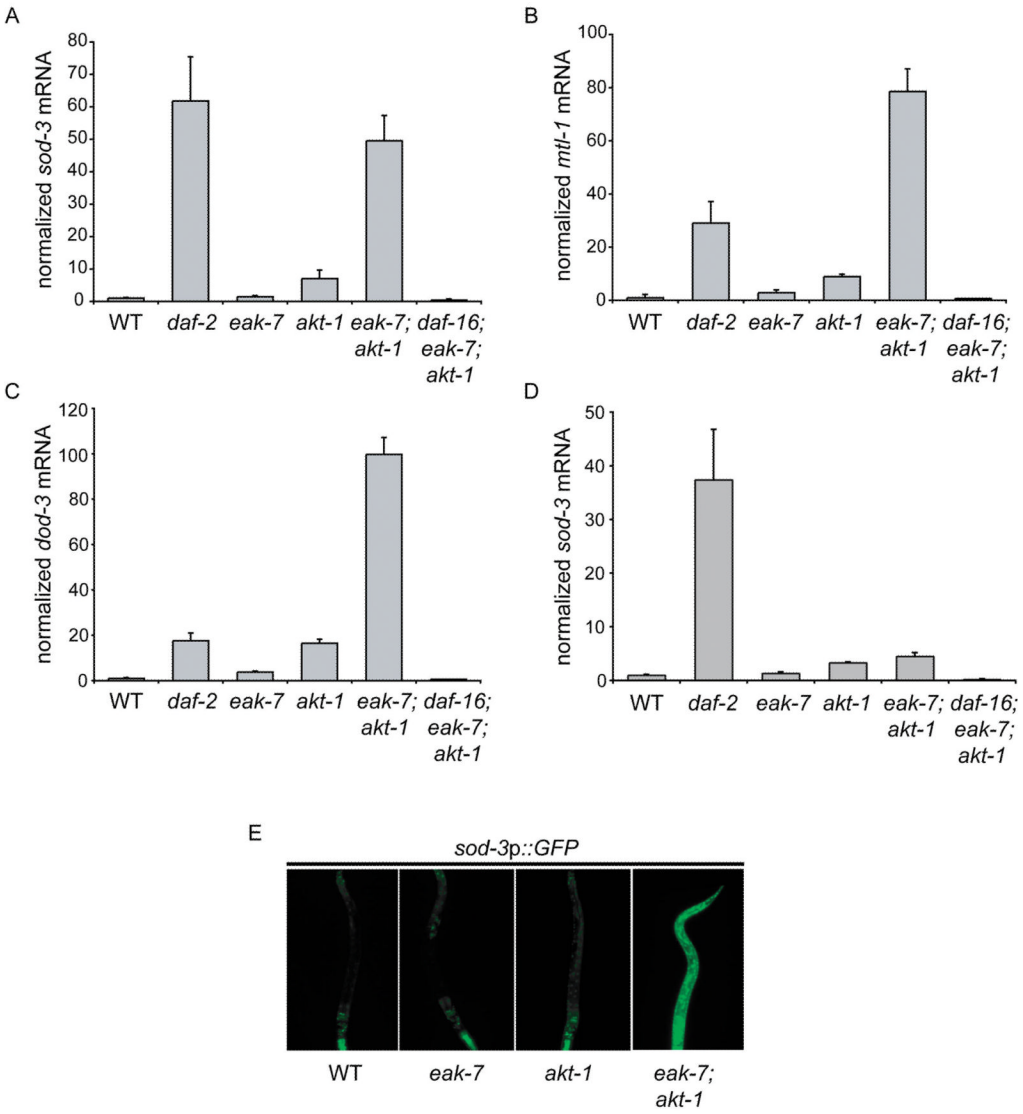

Figure 3. DAF-16/FoxO target gene expression in *eak-7* null mutants

A. *sod-3*, B. *mtl-1*, and C. *dod-3* mRNA quantification using qRT-PCR on total RNA from L2 larvae. Measurements represent the mean + S.D. D. *sod-3* mRNA quantification in adult animals. E. *sod-3p*::GFP expression in various strains. The anterior of the animal is facing down in all images.

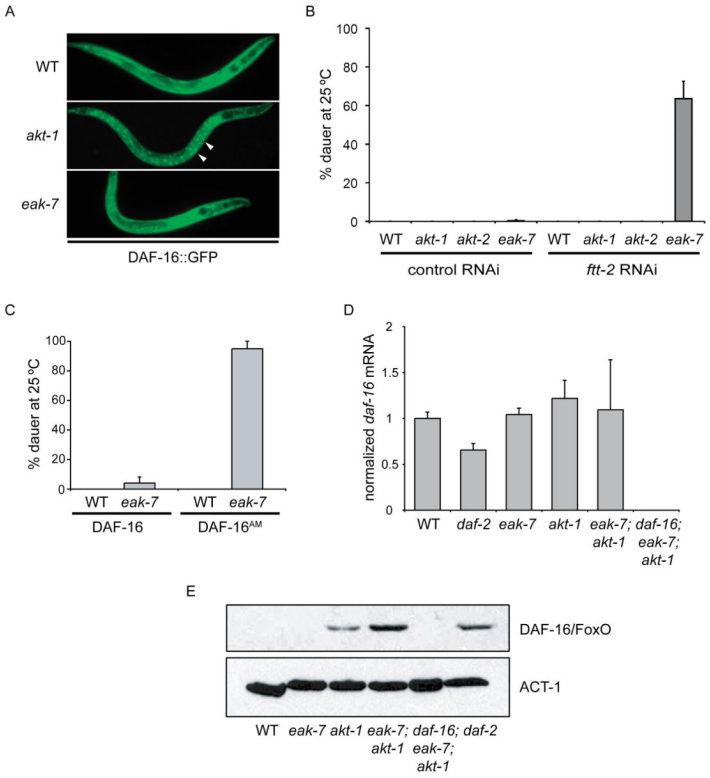

Figure 4. EAK-7 regulation of DAF-16/FoxO

A. An *eak-7* null mutation does not affect the subcellular localization of DAF-16::GFP. White arrows denote nuclear fluorescence in *akt-1* mutants. The anterior of the animal is facing right in all images. B. *ftt-2* RNAi enhances dauer arrest at 25°C in an *eak-7* null mutant but not in *akt-1* or *akt-2* null mutants. C. An *eak-7* null mutation enhances dauer arrest at 25°C in animals harboring DAF-16^{AM}, a constitutively nuclear DAF-16/FoxO mutant GFP fusion protein lacking all canonical Akt/PKB phosphorylation sites. D. *eak*-7 null mutation does not affect endogenous *daf-16/FoxO* mRNA levels in L2 larvae. E. An *eak-7* null mutation increases endogenous DAF-16/FoxO protein levels in an *akt-1* null mutant. DAF-16/FoxO protein was quantified by anti-DAF-16/FoxO immunoblot of whole worm lysates. Anti-ACT-1 immunoblotting was used to confirm equal protein loading. Measurements in dauer assays represent the mean + S.D.

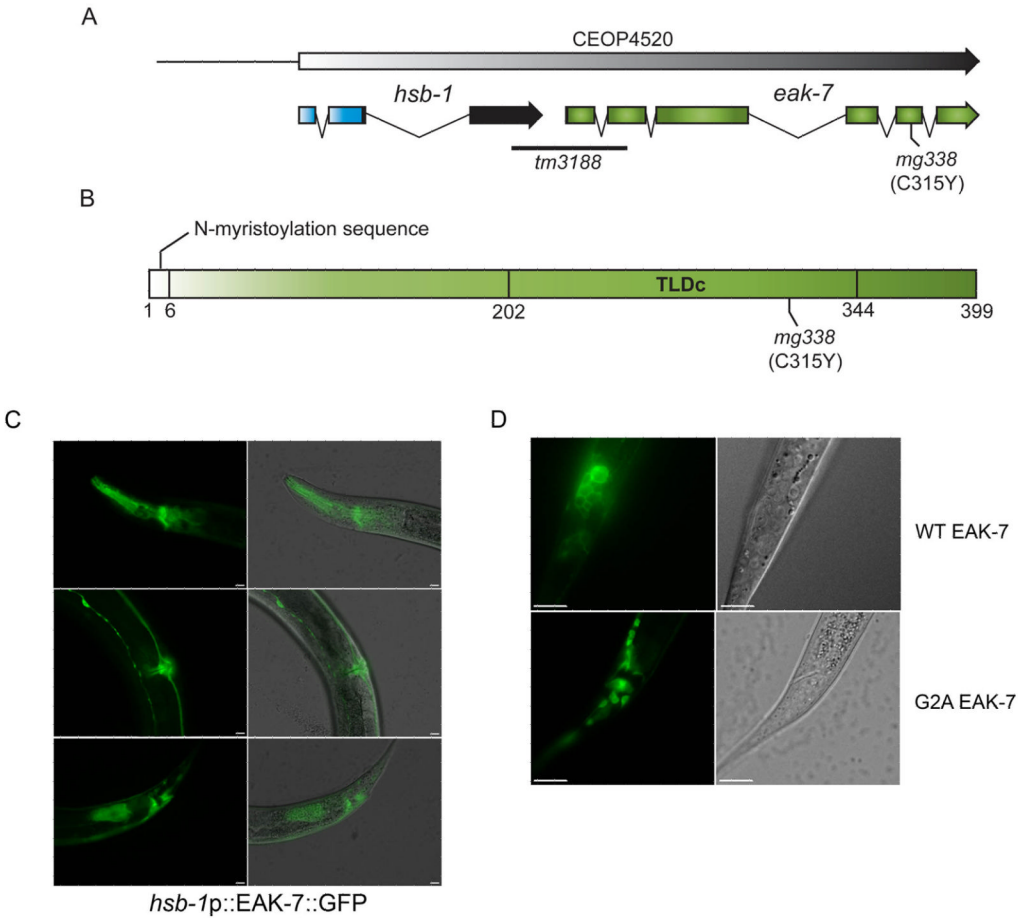

Figure 5. *eak-7* gene structure, protein domain organization, and expression pattern

A. *eak-7* gene structure. *eak-7* is the downstream gene in an operon with *hsb-1*. The deletion in the *eak-7(tm3188)* allele begins 249 bp upstream of the *eak-7* initiator methionine codon and deletes portions of exons 1 and 2 of *eak-7*. The *mg338* allele is a C/T transition that results in a cysteine-to-tyrosine mutation at amino acid 315 (C315Y). B. EAK-7 domain organization. The N-myristoylation motif and TLDc domain are shown. C. *hsb-1p*::EAK-7::GFP expression pattern in L4 larvae. *hsb-1p*::EAK-7::GFP is strongly expressed in the pharynx and nerve ring (top panels), the vulva and ventral nerve cord (middle panels), and in intestinal cells and cells surrounding the anus (bottom panels). Images were taken at 400× magnification. The scale bar is 10 μm. D. Subcellular localization of wild-type EAK-7::GFP (top panels) and EAK-7::GFP harboring a mutation of glycine 2 to alanine (G2A; bottom panels) at 1000× magnification. The scale bar is 10 μm.

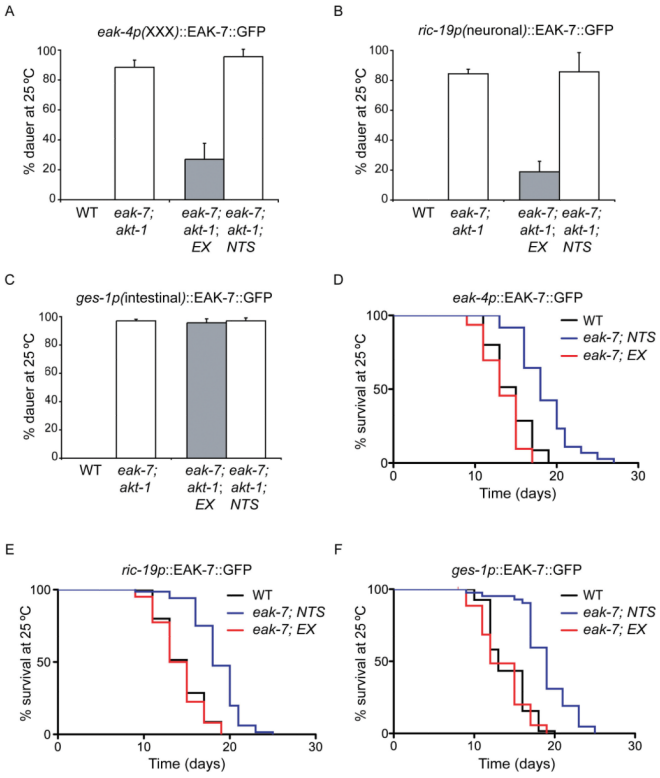

Figure 6. Rescue of *eak-7* mutant phenotypes by tissue-specific EAK-7::GFP expression

Rescue of dauer arrest at 25°C in *eak-7;akt-1* double mutants (A.-C.) and rescue of lifespan extension in an *eak-7* null mutant (D.–F.) by EAK-7::GFP transgenes under the control of *eak-4* (XXX-specific; A. and D.), *ric-19* (neuronal; B. and E.), and *ges-1* (intestinal; C. and F.) promoters. “EX” and “NTS” denote transgenic animals carrying the extrachromosomal array and their non-transgenic siblings, respectively. Measurements represent the mean + S.D. Data are from a single representative transgenic line. Data from additional lines are in Tables S3 and S4.

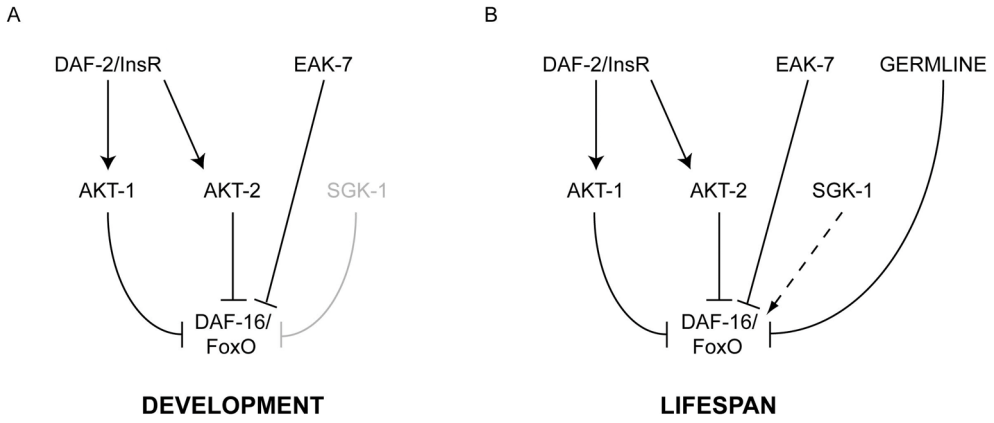

Figure 7. Genetic model of EAK-7 function during development and adulthood

A. During development, EAK-7 acts in parallel to AKT-1 to inhibit DAF-16/FoxO activity. SGK-1 plays a minor role in dauer regulation. B. In adults, EAK-7 acts in parallel to AKT-1, AKT-2, and the germline to inhibit DAF-16/FoxO activity. SGK-1 may be required for maximal DAF-16/FoxO activity.