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C. elegans EAK-3 inhibits dauer arrest via nonautonomous regulation of nuclear DAF-16/FoxO activity

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SUMMARY

Insulin regulates development, metabolism, and lifespan via a conserved PI3K/Akt pathway that promotes cytoplasmic sequestration of FoxO transcription factors. The regulation of nuclear FoxO is poorly understood. In the nematode *Caenorhabditis elegans*, insulin-like signaling functions in larvae to inhibit dauer arrest and acts during adulthood to regulate lifespan. In a screen for genes that modulate *C. elegans* insulin-like signaling, we identified *eak-3*, which encodes a novel protein that is specifically expressed in the two endocrine XXX cells. The dauer arrest phenotype of *eak-3* mutants is fully suppressed by mutations in *daf-16*/*FoxO*, which encodes the major target of *C. elegans* insulin-like signaling, and *daf-12*, which encodes a nuclear receptor regulated by steroid hormones known as dafachronic acids. *eak-3* mutation does not affect DAF-16/FoxO subcellular localization but enhances expression of the direct DAF-16/FoxO target *sod-3* in a *daf-16*/*FoxO*- and *daf-12*-dependent manner. *eak-3* mutants have normal lifespans, suggesting that EAK-3 decouples insulin-like regulation of development and longevity. We propose that EAK-3 activity in the XXX cells promotes the synthesis and/or secretion of a hormone that acts in parallel to AKT-1 to inhibit the expression of DAF-16/FoxO target genes. Similar hormonal pathways may regulate FoxO target gene expression in mammals.

Keywords

C. elegans; dauer formation; insulin signaling; Akt; FoxO; signal transduction; steroid hormones; nuclear receptors

INTRODUCTION

During early postembryonic development of the nematode *Caenorhabditis elegans*, animals respond to unfavorable conditions by arresting in an alternative larval stage called dauer. Dauers are long-lived and equipped to withstand suboptimal environments. Upon improvement of ambient conditions, dauers resume larval development and progress to adulthood. The major influence on dauer arrest is a constitutively synthesized pheromone that induces dauer arrest

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and serves as an indicator of population density. Pheromone responses are modulated by temperature and food availability (Riddle, 1988).

Dauer arrest is regulated by a complex signal transduction network. In the amphid neurons, which are in direct contact with the external environment, the DAF-11 transmembrane guanyl cyclase (GC) prevents dauer arrest (Birnby et al., 2000). In a subset of these sensory neurons, DAF-11/GC promotes the synthesis of DAF-7, a transforming growth factor-β (TGFβ) homolog, and DAF-28, an insulin-like molecule (Li et al., 2003; Murakami et al., 2001; Ren et al., 1996; Schackwitz et al., 1996). DAF-7/TGFβ and DAF-28/insulin are likely secreted and activate TGFβ receptor-like (DAF-1/TGFβRI and DAF-4/TGFβRII) (Estevez et al., 1993; Georgi et al., 1990) and insulin receptor-like (DAF-2/InsR) (Kimura et al., 1997) molecules that are expressed throughout the animal. Each of these receptors activates an evolutionarily conserved signaling cascade that functions in most if not all tissues in the animal. A hormonal pathway also regulates dauer arrest and is defined by the cytochrome P450 homolog DAF-9/CYP27A1 (Gerisch et al., 2001; Jia et al., 2002), the nuclear receptor DAF-12 (Antebi et al., 2000), the Rieske oxygenase homolog DAF-36 (Rottiers et al., 2006), and the Niemann-Pick C disease gene homologs NCR-1 and NCR-2 (Sym et al., 2000).

DAF-2/InsR regulates development, metabolism, and longevity through a conserved PI3K/Akt/FoxO signaling pathway (Finch and Ruvkun, 2001; Kenyon, 2005; Taniguchi et al., 2006). Two Akt/PKB homologs, AKT-1 and AKT-2, have distinct and overlapping functions in this pathway (Hertweck et al., 2004; Paradis and Ruvkun, 1998). Reduction of DAF-2/InsR signaling results in dauer arrest in replete environments (Riddle, 1988). DAF-2/InsR pathway mutants also exhibit increased fat storage (Ashrafi et al., 2003; Kimura et al., 1997) and extended lifespan (Kenyon et al., 1993; Morris et al., 1996; Paradis et al., 1999; Wolkow et al., 2002). All DAF-2/InsR pathway mutant phenotypes require the FoxO transcription factor DAF-16 (Ashrafi et al., 2003; Dorman et al., 1995; Gottlieb and Ruvkun, 1994), indicating that the major target of DAF-2/InsR signaling is DAF-16/FoxO. FoxO transcription factors are regulated by a number of posttranslational modifications, including phosphorylation, ubiquitination, and acetylation (Barthel et al., 2005; Vogt et al., 2005). However, the biological relevance of many of these modifications remains to be demonstrated in intact organisms, and the physiological inputs that regulate many of these events have not been identified.

Several lines of evidence suggest that in *C. elegans*, DAF-16/FoxO regulatory inputs distinct from PI3K/Akt exist. For example, activating mutations in *pdk-1* (Paradis et al., 1999) and *akt-1* (Paradis and Ruvkun, 1998) suppress dauer arrest in *age-1*/PI3K mutant backgrounds more strongly than they do in *daf-2*/InsR mutant backgrounds. Likewise, a weak *daf-18*/PTEN loss-of-function allele suppresses dauer arrest in *age-1*/PI3K mutants but not in *daf-2*/InsR mutants (Gil et al., 1999; Gottlieb and Ruvkun, 1994; Inoue and Thomas, 2000; Ogg and Ruvkun, 1998; Vowels and Thomas, 1992). Furthermore, DAF-16/FoxO that localizes to the nucleus as a consequence of either loss-of-function of the 14-3-3 protein FTT-2 (Berdichevsky et al., 2006) or mutation of the consensus AKT phosphorylation sites (Lin et al., 2001) is not fully active, suggesting the existence of an AKT-independent mechanism of inhibition of nuclear DAF-16/FoxO activity. Multiple genetic screens have failed to reveal the identity of the molecules that comprise this parallel pathway.

We now report the cloning and characterization of *eak-3*, a gene encoding a novel plasma membrane-associated protein that functions in the endocrine XXX cells. Our results define a role for EAK-3 in the nonautonomous regulation of nuclear DAF-16/FoxO activity.

MATERIALS AND METHODS

Strains

The following strains were used: N2 Bristol (wild-type), *daf-2(e1370)*, *akt-1(mg306)*, *eak-3 (mg344)*, *eak-4(mg348)*, *sdf-9(ut187)*, *eak-6(mg329)*, *daf-16(mgDf47)*, *daf-3(mgDf90)*, *osm-5 (p813)*, *sqt-1(sc13) age-1(hx546)*, *daf-18(e1375)*, *akt-1(mg144)*, *daf-9(k182)*, *daf-12 (rh61rh411)*, *daf-36(k114)*, *akt-2(ok393)*, *pdk-1(sa709)*, *ncr-1(nr2022)*, and *ncr-2(nr2023)*. *akt-1(mg306)* is a molecular null allele (Hu et al., 2006), and *akt-1(mg144)* is a gain-of-function allele that was isolated as a suppressor of the dauer arrest phenotype of an *age-1* null allele (Paradis and Ruvkun, 1998). *ncr-1(nr2022)* and *ncr-2(nr2023)* were provided by Dr. Weiqing Li (University of Washington, Seattle, WA, USA), and *daf-9(k182)* and *daf-36(k114)* were provided by Dr. Adam Antebi (Baylor College of Medicine, Houston, TX, USA). Double and triple mutant strains were constructed using standard genetic techniques, and genotypes were confirmed using either restriction fragment length polymorphisms or PCR polymorphisms, with the following exceptions. The presence of the *age-1(hx546)* allele was inferred based on linkage to *sqt-1(sc13)*, and the presence of *pdk-1(sa709)* in the *eak-3;pdk-1* double mutant was inferred from the strong 25°C dauer arrest phenotype.

DAF-16::GFP localization was assayed using strain TJ356 (*zIs356*) IV, which contains a translational fusion of the DAF-16A isoform in-frame to GFP (Henderson and Johnson, 2001). *sod-3*p::GFP activity was assayed using strain CF1553 (*muIs84*), which contains a transgene consisting of the *sod-3* promoter fused to a nuclear-localized GFP reporter (Libina et al., 2003).

eak-3 mutant isolation, SNP mapping, sequencing of mutant alleles, and cDNA isolation

Isolation, mapping, and sequencing of *eak-3* alleles and isolation of an *eak-3* cDNA was performed as described for *eak-4*, *sdf-9*, and *eak-6* (Hu et al., 2006), except that the 5′/3′ RACE kit was purchased from Invitrogen (Carlsbad, CA, USA). The 5′ and 3′ ends of the longest *eak-3* cDNAs identified correspond to nucleotides 19625 and 18124, respectively, of YAC Y92C3A. We did not identify any spliced leader sequences at the 5′ ends.

Dauer and lifespan assays

Dauer and lifespan assays were performed as previously described (Hu et al., 2006). All dauer assays at 27°C were performed in blinded fashion. P-values for differences in mean lifespans were calculated using the two-sided heteroscedastic Student's T-test (Microsoft Excel X).

RNAi

Feeding RNAi was performed as described (Hu et al., 2006).

GFP and RFP reporter constructs

The *eak-3*p::GFP promoter fusion was generated as follows: a 1203-bp genomic DNA fragment corresponding to nucleotides 19605-20807 of YAC Y92C3A (annotated at the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) was PCR-amplified using primers tailed with BglII linkers. The BglII-digested PCR product was subcloned into BamHI-digested pPD95.67 (a gift from Dr. Andrew Fire, Stanford University, Palo Alto, CA, USA) to generate *eak-3*p::GFP. Fragment orientation was confirmed by restriction digestion. Plasmids were purified using columns from Qiagen (Venlo, The Netherlands). The *sdf-9*p::RFP construct marking the cytoplasm of the XXX cells has been described (Hu et al., 2006). The EAK-3::GFP translational fusion was generated using overlap extension PCR (Hobert, 2002). A genomic DNA fragment corresponding to nucleotides 18161-20807 of YAC Y92C3A (encompassing the *eak-3* promoter and open reading frame up to but not including the

translation termination codon) was PCR-amplified and fused to a PCR product containing GFP and the *unc-54* 3′ UTR that was amplified from pPD95.75 (also a gift from Dr. Andrew Fire). The EAK-3::GFP G2A N-myristoylation mutant was constructed using overlap extension PCR by incorporating nucleotide changes resulting in mutation of glycine at residue 2 of EAK-3 to alanine. The final fusion PCR product was sequenced to confirm the presence of the G2A mutation. Fusion PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Venlo, The Netherlands).

Transgenic animals were generated and colocalization studies performed as described previously (Hu et al., 2006). In colocalization experiments (Fig. 4A and B), animals were visualized using a Zeiss Axioplan 2 upright microscope and analyzed using OpenLab imaging software (Improvision, Inc., Lexington, MA, USA). Animals harboring wild-type and G2A mutant EAK-3::GFP (Fig. 4C) and animals harboring DAF-16::GFP and *sod-3*p::GFP reporter constructs (Figs. 5 and S3) were visualized using an Olympus BX61 upright microscope and analyzed using SlideBook 4.1 digital microscopy software (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Animals were mounted on 2% agarose pads in the presence of PBS with 10 mM sodium azide, visualized, and photographed immediately after mounting.

Real-time PCR

Synchronized wild-type and mutant animals grown at 25°C were harvested 26 hours after egg prep, and total RNA was prepared from ~300–400 animals per strain using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript™ III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. *sod-3* mRNA was quantified with SYBR® Green-based detection using an Eppendorf realplex^2 Mastercycler (Eppendorf North America, Westbury, NY, USA) according to the manufacturer's protocol. 40 cycles of PCR (95°C for 15 seconds followed by 60°C for one minute) were performed on cDNA template. Each sample was assayed in duplicate. *sod-3* mRNA levels were normalized to *act-1* mRNA levels. *sod-3* primers used were 5′ TATTAAGCGCGACTTCGGTTCCCT 3′ and 5′ CGTGCTCCCAAACGTCAATTCCAA 3′. *act-1* primers have been described (Li et al., 2007).

RESULTS

eak-3 mutants exhibit enhanced dauer arrest

In *C. elegans*, DAF-2/InsR signaling prevents dauer arrest, and the degree of reduction in DAF-2/InsR signaling in any given pathway mutant is proportional to the strength of the dauer arrest phenotype; *i.e.*, animals with greater reductions in DAF-2/InsR signaling are more likely to arrest as dauers. AKT-1 functions downstream of DAF-2/InsR and AGE-1/PI3K (Paradis and Ruvkun, 1998) and promotes development to adulthood by phosphorylating DAF-16/FoxO and promoting its cytoplasmic sequestration (Hertweck et al., 2004; Lee et al., 2001; Lin et al., 2001). *akt-1(mg306)* is a molecular null allele that has a weak dauer arrest phenotype (Hu et al., 2006), indicating that *akt-1* mutation reduces but does not fully abolish DAF-2/InsR signaling.

To identify novel genes that function in parallel to AKT-1 to regulate dauer arrest, we performed a genetic screen for mutants that enhance the *akt-1(mg306)* dauer arrest phenotype (*eak* mutants). This screen, as well as three genes identified in this screen, *eak-4*, *sdf-9*, and *eak-6*, has been described elsewhere (Hu et al., 2006). The *eak-3* gene is defined by three alleles, *mg331*, *mg335*, and *mg344*, that complement *eak-4*, *sdf-9*, and *eak-6* mutants but do not complement each other.

eak-3 mutants have a very weak dauer arrest phenotype at 25°C but strongly enhance the dauer arrest phenotype of *akt-1(mg306)* (Fig. 1A). *eak-3;akt-1* double mutants arrest as dauers at 25° C to the same extent as *daf-2(e1370)* mutants. The *eak-3* mutant phenotype is comparable to that of *eak-4*, *sdf-9*, and *eak-6* mutants that emerged from the same genetic screen (Hu et al., 2006). These phenotypes have been confirmed with a second *eak-3* allele (data not shown) and are consistent with the hypothesis that EAK-3 functions in parallel to AKT-1. *eak-3* mutants did not enhance the dauer arrest phenotype of *eak-4(mg348)*, *sdf-9(ut187)* (Fig. 1A), or *eak-6 (mg329)* (Fig. S1A), suggesting that EAK-3 functions in the same pathway or complex as EAK-4, SDF-9, and EAK-6 (Hu et al., 2006).

eak-3 mutants interact genetically with the DAF-2/InsR pathway

Genetic analysis suggests that EAK-3 functions in the DAF-2/InsR pathway. The 25°C dauer arrest phenotype of *eak-3;akt-1* double mutants was suppressed by a null mutation in *daf-16*/*FoxO* (Fig. 1B). *daf-16*/*FoxO* mutations specifically suppress phenotypes caused by DAF-2/InsR pathway mutations (Gottlieb and Ruvkun, 1994;Vowels and Thomas, 1992). *daf-16*/*FoxO* mutation also suppressed the 27°C dauer arrest phenotype of *eak-3* single mutants (Fig. 1C). Furthermore, *daf-18*/*PTEN* loss-of-function and *akt-1* gain-of-function mutations, which also suppress the dauer arrest phenotype of insulin-like pathway mutations (Gil et al., 1999;Gottlieb and Ruvkun, 1994;Ogg and Ruvkun, 1998;Paradis and Ruvkun, 1998;Vowels and Thomas, 1992), suppressed *eak-3(mg344)* dauer arrest at 27°C (Fig. 1D). Mutations in *daf-3*/*SMAD* (Patterson et al., 1997), which specifically suppress the dauer arrest phenotype of *daf-7*/*TGFβ* pathway mutants (Gottlieb and Ruvkun, 1994;Thomas et al., 1993;Vowels and Thomas, 1992), and *osm-5*/*Tg737* (Haycraft et al., 2001), which specifically suppress the dauer arrest phenotype of *daf-11*/*GC* mutants (Vowels and Thomas, 1992), did not suppress dauer arrest in *eak-3;akt-1* double mutants (Fig. 1B), suggesting that EAK-3 does not impinge upon those pathways. Consistent with the placement of *eak-3* in the DAF-2/InsR pathway, *eak-3 (mg344)* enhances the weak dauer arrest phenotype of *age-1*/*PI3K* and *pdk-1* partial loss-of-function mutants (Fig. S1B). *eak-3(mg344)* also enhanced dauer arrest of *daf-2(e1370)* at 15° C (Fig. S1C). *eak-3(mg344)* did not enhance the dauer arrest phenotype of an *akt-2* null mutant (Fig. S1B), suggesting that EAK-3 may function in the same pathway as AKT-2.

eak-3 mutants interact genetically with the DAF-9/CYP27A1 hormonal pathway

Since DAF-9/CYP27A1 overexpression suppresses dauer arrest in *sdf-9* mutants (Ohkura et al., 2003) and EAK-3 and SDF-9 may function in the same pathway (Fig. 1A), we determined whether *eak-3* interacts genetically with components of the DAF-9/CYP27A1 hormonal pathway. We were not able to assess the effect of *eak-3* mutation in a *daf-9* null background, as *daf-9* null mutants undergo non-conditional dauer arrest (Gerisch et al., 2001; Jia et al., 2002). *eak-3(mg344)* did enhance dauer arrest of the partial loss-of-function allele *daf-9 (k182)*, which has a weak dauer arrest phenotype at 25°C (Fig. 2A) (Gerisch et al., 2001). As is the case for all dauer-constitutive mutants, a *daf-12* null mutation fully suppressed dauer arrest in *eak-3;akt-1* and *eak-3(mg344)* mutants (Figs. 2B and C). *daf-36*/*Rieske* mutants exhibit weak dauer arrest at 25°C (Figs. 2D and E) (Rottiers et al., 2006), and *ncr-1* and *ncr-2* single mutants do not arrest as dauers at 25°C (Fig. S1D) (Li et al., 2004). *eak-3 (mg344)* enhanced the dauer arrest phenotype of a *daf-36*/*Rieske* null allele, although not to the extent that it enhanced *akt-1(mg306)* dauer arrest (Fig. 2D). *eak-3(mg344)* also enhanced the dauer arrest phenotype of a *ncr-1* null allele but did not enhance the dauer arrest phenotype of a *ncr-2* null allele (Fig. S1D). These results suggest that *eak-3* may act in parallel to *ncr-1* and *daf-36*/*Rieske* to inhibit dauer arrest.

The ability of *eak-3(mg344)* to enhance dauer arrest phenotypes of both *akt-1* and *daf-36*/*Rieske* mutants suggests that AKT-1 and DAF-36/Rieske could conceivably act in the same pathway. To test this possibility, we determined whether inactivation of *akt-1* by RNAi would

enhance the dauer arrest phenotype of a *daf-36*/*Rieske* loss-of-function mutant (the proximity of *akt-1* and *daf-36*/*Rieske* genes in the *C. elegans* genome prevented the construction of a *daf-36 akt-1* double mutant). *akt-1* RNAi enhanced dauer arrest in both *eak-3* and *daf-36*/*Rieske* mutants to a comparable extent (Fig. 2E). Since this *daf-36*/*Rieske* allele is a null allele (Rottiers et al., 2006), AKT-1, EAK-3, and DAF-36/Rieske likely act in parallel pathways.

eak-3 regulation of lifespan

DAF-2/InsR and DAF-9/CYP27A1 pathways regulate organismal lifespan (Broue et al., 2007; Finch and Ruvkun, 2001; Gerisch et al., 2007; Gerisch et al., 2001; Jia et al., 2002; Kenyon, 2005; Rottiers et al., 2006). Since *eak-3* interacts genetically with both DAF-2/InsR and DAF-9/CYP27A1 pathways, we assayed *eak-3* mutants for lifespan phenotypes. In contrast to *daf-2(e1370)*, which exhibits approximately 2-fold lifespan extension compared to wild-type animals, neither *eak-3(mg344)* nor *eak-3;akt-1* double mutants lived longer than wild-type animals (Figs. 3A and C, Table 1). As previously reported (Hu et al., 2006), *akt-1 (mg306)* animals lived slightly longer than wild-type animals, and other *eak* single mutant and *eak;akt-1* double mutants had lifespans comparable to *eak-3* single and *eak-3;akt-1* double mutant animals (Table 1). Although mutation of *eak* genes did not significantly affect lifespan in wild-type backgrounds, *eak-3;akt-1* animals had slightly shorter lifespans than *akt-1 (mg306)* single mutants (Fig. 3A and Table 1), as did other *eak;akt-1* double mutants (Table 1).

To further investigate the role of *eak-3* in lifespan regulation, we determined the effect of *eak-3* mutation on lifespan in *daf-2*/*InsR* and *daf-16*/*FoxO* mutant backgrounds. At 20°C, *eak-3 daf-2* double mutants exhibited extended longevity compared to *daf-2* single mutants (Fig. 3B). This is consistent with the enhancement of *daf-2(e1370)* dauer arrest by *eak-3* mutation (Fig. S1C). *eak-3* mutation did not affect the lifespan of *daf-16*/*FoxO* mutants (Fig. 3C), suggesting that the lifespan extending effects of *eak-3* mutation in a *daf-2(e1370)* background (Fig. 3B) are mediated by DAF-16/FoxO.

Cloning of *eak-3*

We used single nucleotide polymorphism mapping (Wicks et al., 2001) to localize *eak-3* to a ~210 kb genomic interval on the left arm of chromosome III. Rescue assays with pools of overlapping ~10–12 kb PCR products (Winston et al., 2002) that spanned the *eak-3* interval indicated that *eak-3* likely corresponds to the open reading frame Y92C3A.3. Sequencing of predicted exons and splice junctions of Y92C3A.3 in three *eak-3* alleles identified distinct point mutations in each allele (Fig. S2). The Y92C3A.3 open reading frame lies within a large intron of *cdh-12*, a cadherin gene that lies on the opposite genomic strand. A genomic PCR fragment corresponding to this intron and containing the predicted Y92C3A.3 promoter, open reading frame, and 3′ untranslated region rescued the *eak-3* mutant phenotype in 1 of 4 transgenic lines (data not shown).

The *eak-3* promoter drives transcription specifically in the XXX cells

To determine where *eak-3* is expressed in intact animals, we generated transgenic animals harboring a construct consisting of the predicted *eak-3* promoter fused to GFP (*eak-3*p::GFP). These animals exhibited specific GFP expression in two cells in the head, the position and morphology of which suggested that they were the XXX cells (Fig. 4A). Colocalization of GFP and RFP in animals coexpressing *eak-3*p::GFP and *sdf-9*p::RFP, which marks the cytoplasm of the XXX cells (Hu et al., 2006;Ohkura et al., 2003), confirmed that the *eak-3* promoter is specifically active in the XXX cells (Fig. 4A).

An EAK-3::GFP fusion protein localizes to the plasma membrane of the XXX cells

We isolated *eak-3* cDNAs from total *C. elegans* RNA and confirmed the predicted cDNA structure (WormBase; www.wormbase.org; Fig. S2). EAK-3 has no statistically significant amino acid similarity to known proteins. Strikingly, 4 of the first 5 predicted amino-terminal amino acid residues are identical to EAK-4 (Hu et al., 2006), including a glycine residue at position 2 that is invariant in N-myristoylated proteins (Maurer-Stroh et al., 2002). However, the sixth amino acid residue, which is conserved in most N-myristoylated proteins (Maurer-Stroh et al., 2002), is not conserved in EAK-3.

We generated transgenic animals harboring a C-terminal EAK-3::GFP translational fusion construct to determine the subcellular localization of EAK-3::GFP. EAK-3::GFP localized to the plasma membrane of the XXX cells. This localization was confirmed by visualization of animals coexpressing EAK-3::GFP and *sdf-9*p::RFP (Fig. 4B).

To examine the role of the N-myristoylation motif in EAK-3::GFP plasma membrane localization, we tested the effect of mutagenizing the glycine at residue 2 that is absolutely required for N-myristoylation on the subcellular localization of EAK-3::GFP. We generated transgenic animals expressing wild-type EAK-3::GFP and mutant EAK-3::GFP with glycine at residue 2 mutated to alanine (G2A). Whereas wild-type EAK-3::GFP localizes to the plasma membrane, the G2A mutation abrogates membrane association and results in relocalization to the cytoplasm (Fig. 4B and C). Thus, the glycine that is absolutely required for N-myristoylation is also required for EAK-3::GFP plasma membrane association. Five of six amino acids immediately following the putative N-myristoylation motif in EAK-3 are either lysine or arginine (residues 6–11; Fig. S2) and may contribute to the membrane localization of EAK-3.

EAK proteins do not regulate DAF-16::GFP subcellular localization

Insulin-like signaling pathways inhibit FoxO transcription factors via a conserved mechanism of Akt-mediated phosphorylation and subsequent cytoplasmic sequestration (Barthel et al., 2005; Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001; Vogt et al., 2005). The suppression of *eak-3* mutant phenotypes by *daf-16*/*FoxO* mutation suggests that in wild-type animals, EAK-3 also inhibits DAF-16/FoxO function. To determine whether EAK-3 regulates DAF-16/FoxO nuclear translocation, we examined the subcellular localization of a DAF-16::GFP translational fusion (Henderson and Johnson, 2001) in wild-type, *akt-1 (mg306)*, and *eak-3(mg344)* mutant animals.

In wild-type animals, DAF-16::GFP is predominantly cytoplasmic (Fig. 5A), consistent with the presence of intact DAF-2/InsR signaling. Mutation of *akt-1* results in nuclear translocation of DAF-16::GFP, consistent with previous results (Hertweck et al., 2004). In contrast, *eak-3* mutation has no effect on DAF-16::GFP subcellular localization, indicating that EAK-3 does not regulate the subcellular distribution of DAF-16/FoxO.

Since *sdf-9* and *eak-6* likely function in the same pathway as *eak-3* (Figs. 1A and S1A), we also assayed DAF-16::GFP subcellular localization in *sdf-9* and *eak-6* mutant backgrounds. As expected, neither *sdf-9* nor *eak-6* mutation affected DAF-16::GFP subcellular localization (Fig. S3A).

EAK proteins regulate DAF-16/FoxO target gene expression in an *akt-1* mutant background

In *C. elegans*, nuclear translocation of DAF-16/FoxO is not sufficient for full DAF-16/FoxO activation (Berdichevsky et al., 2006; Hertweck et al., 2004; Lin et al., 2001), indicating that nuclear DAF-16/FoxO activity may be regulated independently of Akt. We tested the ability of EAK-3 to regulate the activity of nuclear DAF-16/FoxO by constructing wild-type and

mutant animals harboring a *sod-3*p::GFP reporter construct (Libina et al., 2003). DAF-16/FoxO directly activates transcription of *sod-3* (Oh et al., 2006), and the *sod-3*p::GFP reporter serves as a readout for DAF-16/FoxO activity (Libina et al., 2003). Wild-type animals exhibited baseline expression of *sod-3*p::GFP in the head and the tail without significant GFP expression in the intestine or body wall muscle (Fig. 5B). *eak-3* mutation did not affect *sod-3*p::GFP expression significantly in a wild-type background. Since DAF-16/FoxO remains in the cytoplasm in *eak-3* mutants (Fig. 5A), this result demonstrates that in the absence of nuclear DAF-16/FoxO, EAK-3 does not regulate *sod-3*p::GFP transcription. *akt-1* mutation increased *sod-3*p::GFP transcription above baseline in the body wall muscle. However, intestinal *sod-3*p::GFP expression remained comparable to that seen in wild-type animals. This indicates that in body wall muscle, AKT-1 may be the primary regulator of DAF-16/FoxO-mediated activation of the *sod-3* promoter. Interestingly, in the *eak-3;akt-1* double mutant background, *sod-3*p::GFP expression was induced in both body wall muscle and intestine, and intestinal GFP expression was substantially greater in *eak-3;akt-1* double mutants compared to *akt-1* single mutants (Fig. 5B). This indicates that in *akt-1* mutant animals, EAK-3 inhibits *sod-3* transcription, perhaps in a tissue-specific manner. Since the *eak-3* promoter drives expression specifically in the XXX cells (Fig. 4), EAK-3 functions nonautonomously to inhibit nuclear DAF-16/FoxO activity in the intestine. We observed similar *sod-3*p::GFP expression patterns in *sdf-9*, *eak-6*, *akt-1 sdf-9*, and *eak-6;akt-1* mutants, consistent with SDF-9, EAK-6, and EAK-3 functioning in the same pathway or complex in the XXX cells (Fig. S3B).

We confirmed these results by examining the effects of *eak-3*, *eak-4*, *sdf-9*, and *eak-6* mutations on the expression of endogenous *sod-3*. We isolated total RNA from wild-type and mutant animals and performed quantitative real-time reverse transcription PCR using gene-specific primers for *sod-3*. Whereas relative endogenous *sod-3* mRNA levels were modestly increased in *eak* and *akt-1* single mutants compared to wild-type animals, they were dramatically elevated in all *eak;akt-1* double mutant animals compared to single mutants (Fig. 6A). These results indicate that EAK proteins function in parallel to AKT-1 to inhibit DAF-16/FoxO activity on endogenous promoters.

In light of the observation that dauer arrest in *eak-3;akt-1* double mutants requires both *daf-16*/*FoxO* (Fig. 1) and *daf-12* (Fig. 2), we measured endogenous *sod-3* mRNA expression in *eak-3;akt-1* double mutants after reduction of *daf-16*/*FoxO* or *daf-12* activity by RNAi. RNAi of either *daf-16*/*FoxO* or *daf-12* reduced endogenous *sod-3* expression approximately seven-fold (Fig. 6B), indicating that in *eak-3;akt-1* double mutants, DAF-12 is also required for the expression of certain DAF-16/FoxO targets. *daf-9* RNAi had no effect on *sod-3* expression levels.

Since DAF-36/Rieske may function in parallel to both EAK-3 (Fig. 2D) and AKT-1 (Fig. 2E) to inhibit dauer arrest, we measured endogenous *sod-3* mRNA levels in *eak-3;daf-36* double mutants and in *daf-36* single mutants after inactivating *akt-1* by RNAi. *sod-3* mRNA levels were significantly increased in *eak-3;daf-36* double mutants compared to *eak-3* and *daf-36* single mutants (Fig. 6A). *akt-1* RNAi dramatically increased *sod-3* mRNA levels in both *eak-3* and *daf-36* mutants (Fig. 6C). Taken together with the synthetic dauer-constitutive phenotype of *eak-3;daf-36* double mutants at 25°C (Fig. 2D) and the enhancement of dauer arrest in *daf-36*/*Rieske* mutants by *akt-1* RNAi (Fig. 2E), this result suggests that DAF-36 and EAK-3 act in parallel to AKT-1 and to each other to inhibit *sod-3* expression.

DISCUSSION

EAK-3 may function in an endocrine pathway

The endocrine nature of EAK-3 function is supported by the observation that *eak-3* mutation enhances a systemic phenotype, namely dauer arrest (Figs. 1 and 2), in spite of the fact that the

eak-3 promoter is only active in the XXX cells (Fig. 4). Furthermore, *eak-3* mutation enhances DAF-16/FoxO target gene expression nonautonomously (Fig. 5). These results are consistent with at least two models of EAK-3 function. EAK-3 could inhibit the activity of a hormone secreted by the XXX cells that activates nuclear DAF-16/FoxO. However, the fact that laser ablation of the XXX cells results in a dauer-constitutive phenotype suppressed by loss of *daf-16*/*FoxO* activity in the rest of the animal (Ohkura et al., 2003) rather than a dauer-defective phenotype suggests that the XXX cells normally inhibit DAF-16/FoxO. This leads us to favor an alternative model whereby EAK-3 promotes the activity of a hormone secreted from the XXX cells that inhibits nuclear DAF-16/FoxO activity (Fig. 7).

One plausible model of nonautonomous DAF-16/FoxO regulation by EAK-3 invokes the secretion of insulin-like peptide hormones that activate DAF-2/InsR on the surface of target cells. If EAK-3 inhibits DAF-16/FoxO by promoting the activity of a DAF-2/InsR agonist ligand, then *eak-3* loss-of-function should phenocopy *daf-2* loss-of-function. However, in contrast to the extended lifespan exhibited by sixteen independent *daf-2* loss-of-function alleles (Gems et al., 1998), *eak-3* loss-of-function mutants have normal lifespans (Fig. 3 and Table 1). Furthermore, whereas *daf-2* loss-of-function promotes DAF-16::GFP nuclear localization (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), *eak-3* loss-of-function mutation has no effect on DAF-16::GFP subcellular distribution (Fig. 5A). These observations suggest that EAK-3 does not regulate the synthesis or secretion of DAF-2/InsR ligands.

Genetic interactions of *eak-3* with the DAF-9/CYP27A1 hormonal pathway (Figs. 2 and S1D) suggest that EAK-3 may regulate the synthesis and/or secretion of a steroid hormone. Similar interactions have been described for *sdf-9*, a gene that is also specifically expressed in the XXX cells (Hu et al., 2006;Ohkura et al., 2003) and that also emerged from our genetic screen (Hu et al., 2006). Intriguingly, *daf-9*/*CYP27A1* is expressed in the XXX cells (Ohkura et al., 2003) as well as in other tissues (Gerisch et al., 2001;Jia et al., 2002). Overexpression of *daf-9*/*CYP27A1* can rescue the *sdf-9* mutant phenotype (Ohkura et al., 2003), suggesting that DAF-9/CYP27A1 functions either parallel to or downstream of SDF-9. The lack of phenotypic enhancement in an *eak-3;sdf-9* double mutant (Fig. 1A) and the colocalization of EAK-3 and SDF-9 in the XXX cells (Fig. 4) suggest that DAF-9/CYP27A1 may also function parallel to or downstream of EAK-3. These results would be consistent with EAK-3 and SDF-9 functioning in the XXX cells to promote the DAF-9/CYP27A1-dependent synthesis of a steroid hormone that inhibits DAF-16/FoxO activity in target tissues. In this scenario, DAF-9/CYP27A1 expressed in the XXX cells would be predicted to have a function upstream of DAF-16/FoxO. The observation that *daf-16*/*FoxO* mutations do not suppress the dauer arrest phenotype of *daf-9*/*CYP27A1* mutants (Gerisch et al., 2001;Jia et al., 2002) suggests that DAF-9/CYP27A1 also functions in parallel to or downstream of DAF-16/FoxO in other tissues.

eak-3 and *sdf-9* mutations enhance dauer arrest in *ncr-1* mutants but do not influence dauer arrest in *ncr-2* mutants (Fig. S1D) (Ohkura et al., 2003). The observation that the *ncr-2* promoter is only active in the XXX cells during early larval stages (Li et al., 2004) suggests that *eak-3* and *sdf-9* mutations may fail to enhance dauer arrest in *ncr-2* mutants by virtue of EAK-3 and SDF-9 acting in the same pathway as NCR-2 in the XXX cells. Alternatively, this may be a consequence of the stronger phenotype of *ncr-1* mutants compared to *ncr-2* mutants (Li et al., 2004). It is not known whether the difference in *ncr-1* and *ncr-2* mutant phenotypes is a consequence of the distinct expression patterns of *ncr-1* and *ncr-2* or of intrinsic differences in the function of NCR-1 and NCR-2 proteins.

EAK-3 decouples DAF-16/FoxO regulation of dauer arrest and lifespan

The physiological consequences of DAF-16/FoxO activation are determined at least in part by where in the animal and when during its life history DAF-2/InsR signaling is inhibited. For example, DAF-16/FoxO activity in neurons has disproportionate effects on dauer arrest

compared to lifespan, whereas intestinal DAF-16/FoxO activity affects lifespan more prominently than dauer arrest (Libina et al., 2003). Also, transient activation of DAF-16/FoxO during early larval development promotes dauer arrest without impacting organismal lifespan, whereas DAF-16/FoxO activation during early adulthood promotes extended longevity (Dillin et al., 2002). Although some proteins have been implicated in the regulation of lifespan-specific DAF-16/FoxO functions (Berman and Kenyon, 2006; Wolff et al., 2006), to our knowledge, EAK-3 is the first protein described that primarily regulates DAF-16/FoxO dauer-inducing activity. The ability of *eak-3* mutation to extend the lifespan of *daf-2(e1370)* mutants at 20°C (Fig. 3B) is consistent with its enhancement of dauer arrest in *daf-2(e1370)* mutants at low temperatures (Fig. S1C) and suggests that EAK-3 can regulate lifespan in certain contexts.

The mechanisms underlying EAK-3 decoupling of the dauer arrest and lifespan regulatory functions of DAF-16/FoxO remain obscure. The *eak-3* promoter is active from late embryogenesis through young adulthood (data not shown), suggesting that *eak-3* expression is not limited to the developmental stages during which the dauer arrest decision is made. Furthermore, although intestinal DAF-16/FoxO activity correlates with lifespan extension (Libina et al., 2003), *eak-3;akt-1* mutants also exhibit increased intestinal DAF-16/FoxO activity during late larval stages (data not shown) but do not have extended longevity (Fig. 3A and Table 1). This suggests that activation of intestinal DAF-16/FoxO *per se* is not sufficient to extend organismal lifespan.

EAK-3 regulates DAF-16/FoxO activity

Since *daf-16*/*FoxO* loss-of-function fully suppresses *eak-3* dauer arrest phenotypes (Fig. 1), DAF-16/FoxO is a major genetic target of EAK-3. This is consistent with the observation that loss-of-function mutations in *daf-18*/*PTEN* and gain-of-function mutations in *akt-1* also suppress *eak-3* mutant phenotypes (Fig. 1D). The design of the genetic screen from which *eak-3* was isolated (Hu et al., 2006) and the regulatory effects of EAK-3 on DAF-16/FoxO activity (Figs. 5B and 6) suggest that EAK-3 functions in parallel to AKT-1 to inhibit DAF-16/FoxO activity.

In contrast to its interactions with *akt-1* mutants, *eak-3* mutation did not enhance dauer arrest in an *akt-2* mutant background (Fig. S1B). This could be secondary to the weaker phenotypic effects of *akt-2* loss-of-function compared to *akt-1* loss-of-function (Hertweck et al., 2004;Paradis and Ruvkun, 1998;Quevedo et al., 2007). Alternatively, EAK-3 may inhibit DAF-16/FoxO by activating AKT-2. This would not be inconsistent with the lack of effect of *eak-3* mutation on DAF-16::GFP subcellular distribution (Fig. 5A), as AKT-2 does not strongly influence DAF-16::GFP localization (Hertweck et al., 2004). Reporters driven by *akt-1* and *akt-2* promoters are both widely expressed (Paradis and Ruvkun, 1998), suggesting that differences in spatial expression patterns do not account for the differential phenotypic effects of *eak-3* mutation in *akt-1* and *akt-2* mutant backgrounds.

Mechanisms of DAF-16/FoxO regulation

Most proteins known to regulate DAF-16/FoxO do so by controlling its subcellular distribution. For example, the kinases JNK-1 (Oh et al., 2005) and CST-1 (Lehtinen et al., 2006) and the intestinal protein KRI-1 (Berman and Kenyon, 2006) promote DAF-16/FoxO nuclear translocation. Conversely, AKT-1 (Hertweck et al., 2004) and the 14-3-3 proteins PAR-5 (Berdichevsky et al., 2006) and FTT-2 (Berdichevsky et al., 2006; Li et al., 2007) promote the cytoplasmic sequestration of DAF-16/FoxO. Intriguingly, although FTT-2 inhibits DAF-16/FoxO nuclear localization, it appears to be required for SIR-2.1-dependent activation of nuclear DAF-16/FoxO (Berdichevsky et al., 2006; Wang et al., 2006).

Two proteins, SMK-1 (Wolff et al., 2006) and SIR-2.1 (Berdichevsky et al., 2006), have been shown to potentiate DAF-16/FoxO activity without affecting DAF-16/FoxO subcellular localization; interestingly, both regulate DAF-16/FoxO-dependent lifespan extension without strongly impacting DAF-16/FoxO-mediated dauer arrest (Tissenbaum and Guarente, 2001; Wolff et al., 2006). It is conceivable that EAK-3 prevents dauer arrest by inhibiting molecules that function analogously to SMK-1 and SIR-2.1 in dauer regulation.

DAF-12 may regulate a subset of DAF-16/FoxO target genes

We have shown that DAF-12 is required for maximal endogenous *sod-3* expression in *eak-3;akt-1* double mutants (Fig. 6B). DAF-12 is also required for full expression of intestinal *sod-3*::GFP in *eak-3;akt-1* double mutants (data not shown). To our knowledge, this is the first demonstration that DAF-12 is required for the expression of a specific DAF-16/FoxO target gene. The requirement of DAF-12 for the dauer arrest and *sod-3* expression phenotypes of *eak-3* mutants (Figs. 2 and 6B) raises the possibility that EAK-3 may regulate DAF-16/FoxO target gene expression indirectly via DAF-12. For example, nuclear DAF-16/FoxO and unliganded DAF-12 could act in parallel to activate intestinal *sod-3* expression by converging on the *sod-3* promoter. *sod-3* expression would be inhibited by activation of either AKT-1 (resulting in DAF-16/FoxO phosphorylation and cytoplasmic sequestration) or EAK-3 (resulting in dafachronic acid synthesis and binding to DAF-12). *daf-12* RNAi had no effect on DAF-16::GFP nuclear localization in wild-type, *akt-1* mutant, or *eak-3* mutant animals, and *daf-9* RNAi did not affect DAF-16::GFP localization in wild-type or *akt-1* mutants and resulted in DAF-16::GFP nuclear localization in less than 10% of *eak-3* mutants (data not shown), suggesting that the major regulatory effect of the hormonal pathway on DAF-16/FoxO target gene expression does not occur via changes in DAF-16/FoxO subcellular distribution.

This model also accommodates the observation that DAF-36/Rieske may act in parallel to both AKT-1 and EAK-3 (Figs. 2D, 2E, 6A, and 6C), as DAF-36/Rieske is thought to participate in the biosynthesis of dafachronic acids (Rottiers et al., 2006). Although both EAK-3 and DAF-36/Rieske may act in dafachronic acid biosynthetic pathways, they may function in parallel by virtue of their expression in distinct cells; expression of *daf-36* in the XXX cells has not been observed (Rottiers et al., 2006).

A model for EAK-3 function

Based on our data as well as previously published work, we propose a model for EAK-3 function (Fig. 7). In the XXX cells, EAK-3 acts together with other EAK proteins (Hu et al., 2006) to promote the synthesis and/or secretion of a hormone that regulates the expression of DAF-16/FoxO target genes nonautonomously. XXX-specific expression of *akt-1* rescues the dauer arrest phenotype of an *eak-4;akt-1* double mutant (Hu et al., 2006), suggesting that AKT-1 functions in the XXX cells to prevent dauer arrest. AKT-1 may target a protein distinct from DAF-16/FoxO in the XXX cells, since the major isoform of DAF-16/FoxO that regulates dauer arrest is not expressed at detectable levels in the XXX cells (Hu et al., 2006). In the XXX cells, EAK-3, other EAK proteins, and AKT-1 may regulate one or more proteins involved in hormone synthesis and/or secretion, such as DAF-9/CYP27A1 or other undiscovered proteins (indicated by the orange shape).

In target cells, AKT-1 functions to promote DAF-16/FoxO cytoplasmic sequestration, and the steroid hormone (or hormones) produced in the XXX cells acts in parallel to AKT-1 to inhibit the transcription of DAF-16/FoxO target genes, possibly via DAF-12. In wild-type animals, DAF-16/FoxO is sequestered in the cytoplasm, and DAF-12 is bound to steroid hormone ligands (Fig. 7A). When *akt-1* is mutated, DAF-16/FoxO translocates to the nucleus; however, certain DAF-16/FoxO target genes such as *sod-3* are not fully induced in the presence of liganded DAF-12. In *eak-3;akt-1* double mutants, nuclear DAF-16/FoxO and unliganded

DAF-12 cooperate to promote dauer arrest by regulating the expression of a subset of DAF-16/FoxO target genes (Fig. 7B). This model does not exclude the possibility that EAK-3 may also inhibit the expression of DAF-16/FoxO target genes through DAF-12-independent mechanisms.

It is noteworthy that the genetic screen that yielded *eak-3* also identified three other genes that remain to be characterized. These genes could encode components of the hormone biosynthetic or regulatory machinery in the XXX cells as well as mediators of hormonal effects in target tissues. Their molecular identities and functions will illuminate the EAK-3 endocrine pathway and may also shed light on how FoxO transcription factors are regulated in mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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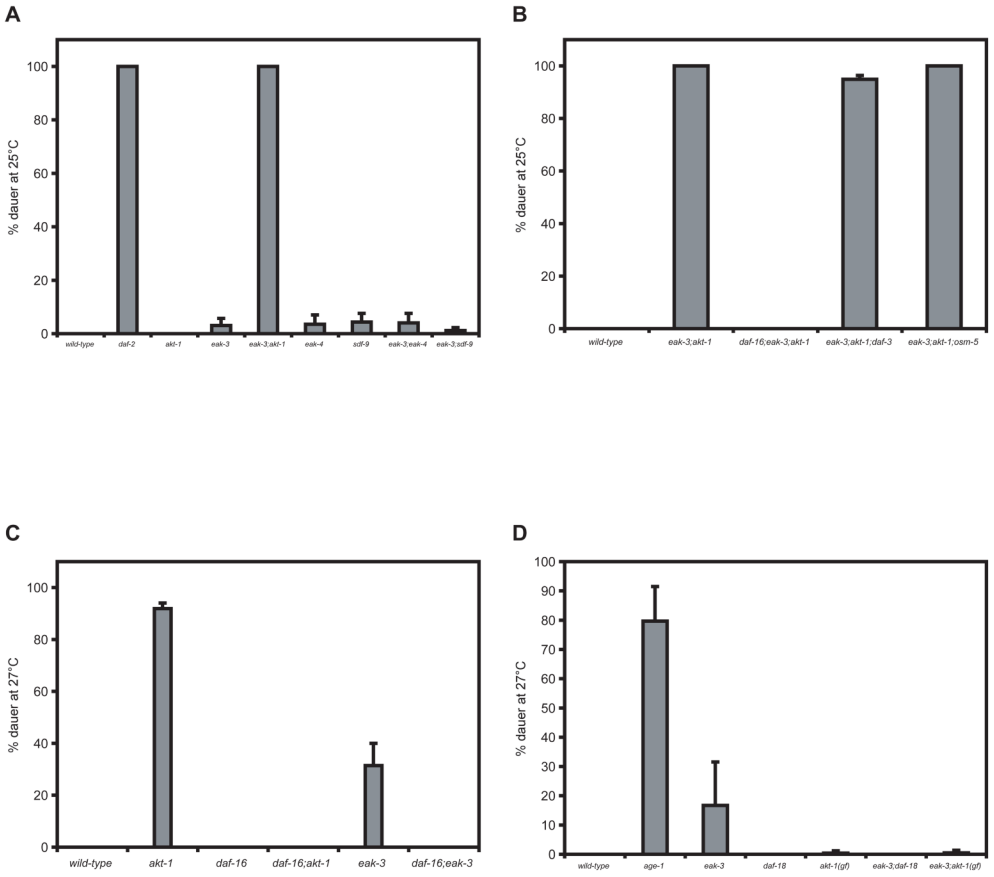


Fig. 1. *eak-3* interacts with the *daf-2* insulin-like signaling pathway

Animals were assayed for dauer arrest phenotypes at 25°C (A. and B.) or 27°C (C. and D.). A. *eak-3* mutation enhances the dauer arrest phenotype of *akt-1* mutants but not of *eak-4* or *sdf-9* mutants. B. Dauer arrest of an *eak-3;akt-1* double mutant is suppressed by a mutation in *daf-16*/*FoxO* but not by mutations in *daf-3*/*SMAD* or *osm-5*/*Tg737*. C. Dauer arrest of an *eak-3* mutant at 27°C is suppressed by a mutation in *daf-16*/*FoxO*. D. Dauer arrest of an *eak-3* mutant at 27°C is suppressed by *daf-18*/*PTEN* loss-of-function and *akt-1* gain-of-function mutations. Data are represented as mean + s.d. The number of animals scored is documented in Table S1.

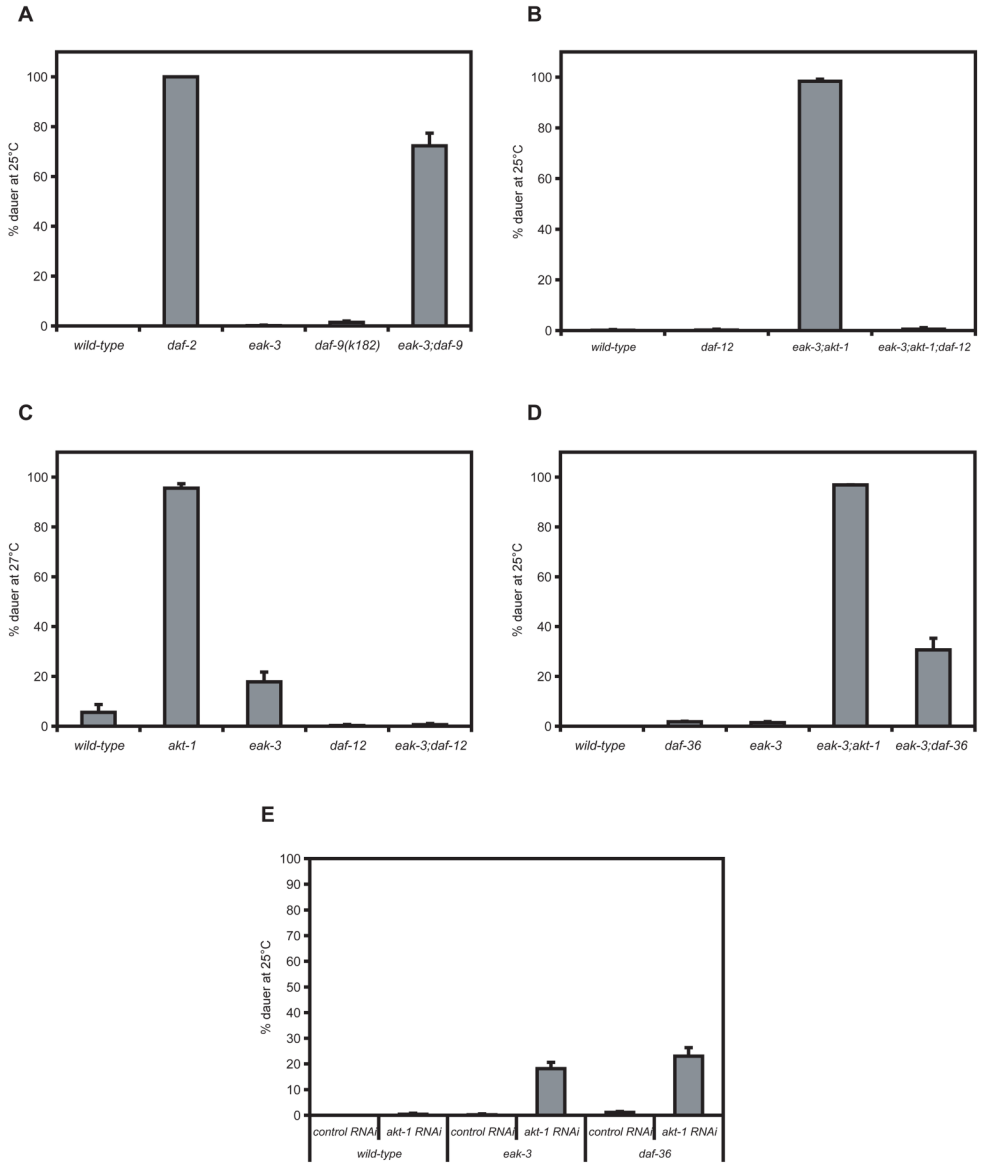


Fig. 2. *eak-3* interacts with the *daf-9*/*CYP27A1* hormonal pathway

Animals were assayed for dauer arrest phenotypes at 25°C (A., B., D., and E.) or 27°C (C.). A. *eak-3* mutation enhances the dauer arrest phenotype of a partial loss-of-function allele of *daf-9*. B. Dauer arrest of an *eak-3;akt-1* double mutant is suppressed by a mutation in *daf-12*. C. Dauer arrest of an *eak-3* mutant at 27°C is suppressed by a mutation in *daf-12*. D. *eak-3* mutation enhances the dauer arrest phenotype of a *daf-36* null mutation. E. *akt-1* RNAi enhances the dauer arrest phenotype of *eak-3* and *daf-36* mutants. Data are represented as mean + s.d. The number of animals scored is documented in Table S1.

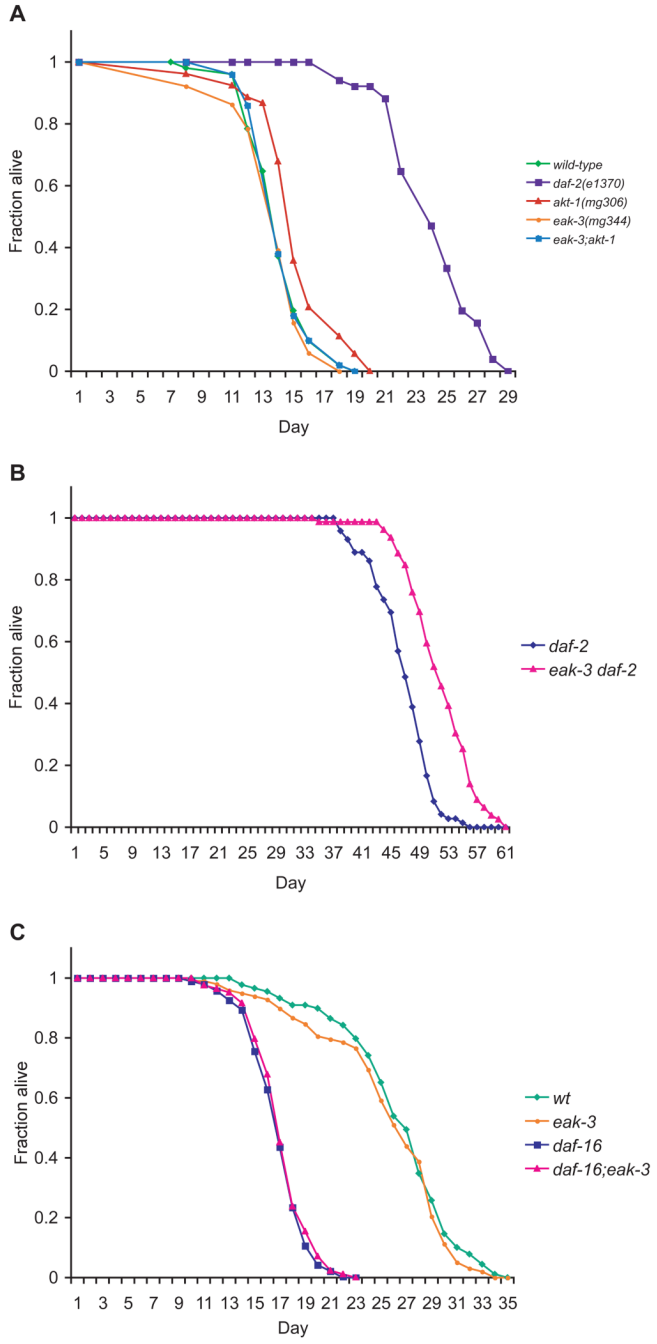


Fig. 3. Effects of *eak-3* mutation on lifespan

Lifespans were assayed at 25°C (A.) or 20°C (B. and C.). The number of animals scored is documented in Table S1. Mean lifespans (days) +/− standard deviation are as follows: A. (p-value vs. wild-type): wild-type, 12.56 +/− 1.99; *daf-2(e1370)*, 22.54 +/− 2.89 ($p = 4.53 \times 10^{-35}$); *akt-1(mg306)*, 13.49 +/− 2.92 (p = 0.06); *eak-3(mg344)*, 11.84 +/− 2.97 (p = 0.16); *eak-3;akt-1*, 12.64 +/− 1.79 (p = 0.83). For *akt-1* vs. *eak-3*, p = 0.01; *akt-1* vs. *eak-3;akt-1*, p = 0.08; *eak-3* vs. *eak-3;akt-1*, p = 0.11. B. *daf-2*, 45.82 +/− 4.06; *eak-3 daf-2*, 50.85 +/− 4.58 ($p = 3.67 \times 10^{-11}$). C. wild-type, 25.47 +/− 4.61; *eak-3*, 24.54 +/− 5.22 (p = 0.27); *daf-16*, 15.97 +/− 2.26; *daf-16;eak-3*, 16.24 +/− 2.25 (p = 0.42 *vs. daf-16*).

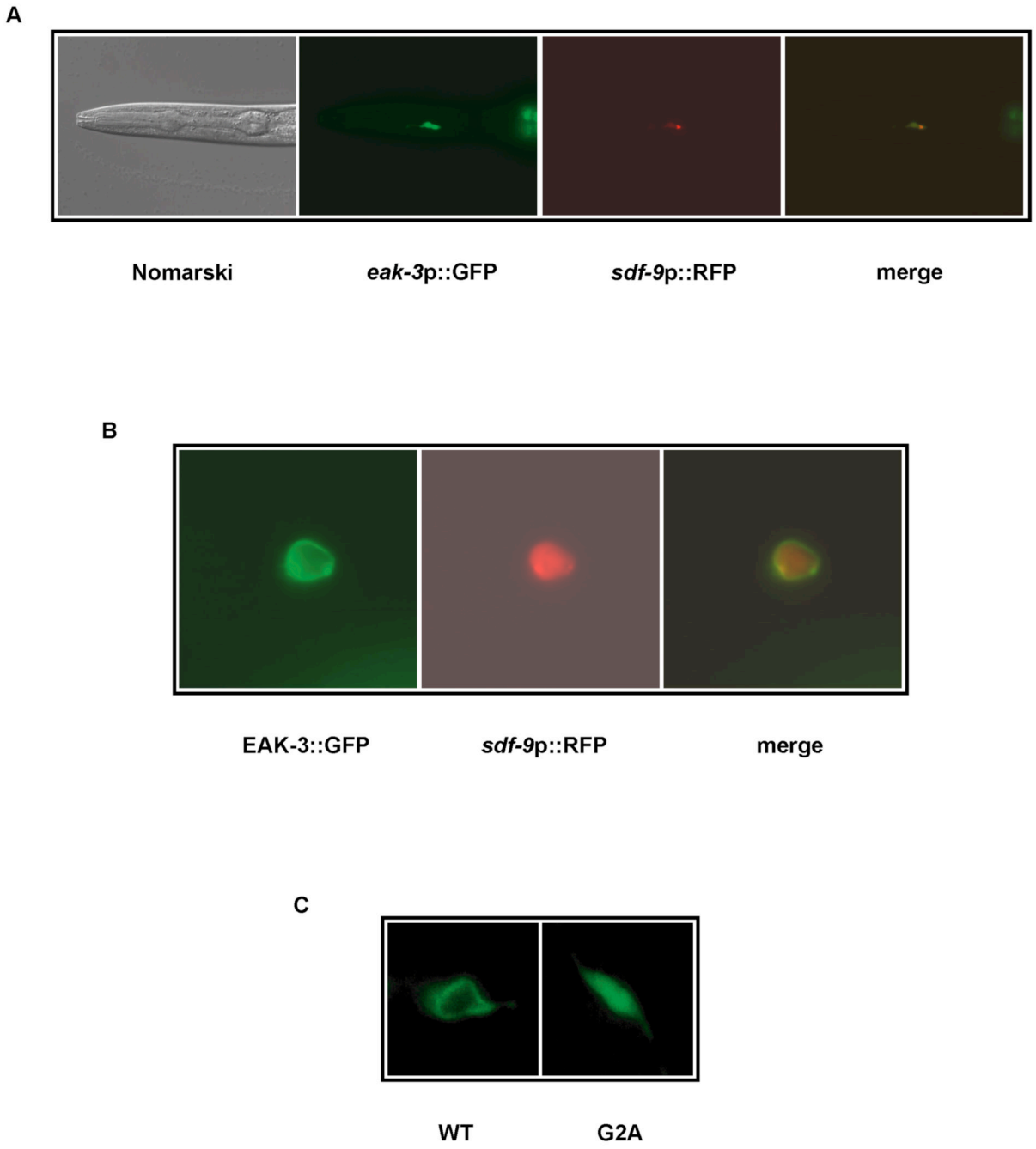


Fig. 4. Expression pattern of *eak-3*::GFP fusion constructs

A. Fluorescent protein expression in animals harboring *eak-3*p::GFP and *sdf-9*p::RFP promoter fusions. B. Fluorescent protein expression in animals harboring EAK-3::GFP translational and *sdf-9*p::RFP promoter fusions. C. GFP localization in animals expressing wild-type EAK-3::GFP or EAK-3::GFP containing a mutation at the conserved glycine of the N-myristoylation motif (G2A). Representative images are shown.

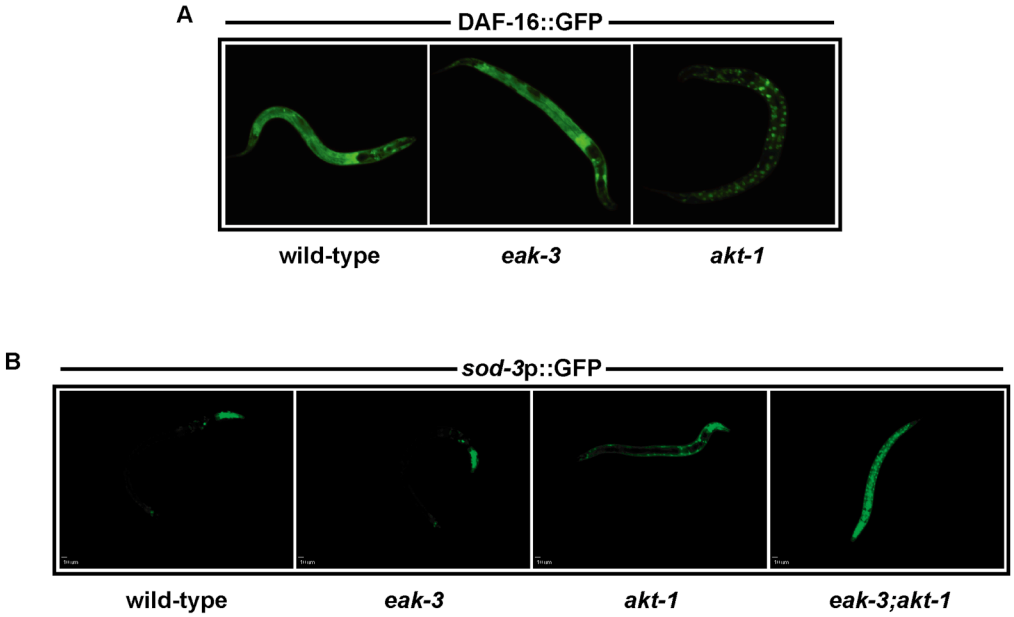


Fig. 5. Effects of *eak-3* mutation on DAF-16/FoxO subcellular localization and activity *in vivo*

A. Wild-type and mutant animals (late L1 to early L2 larvae) harboring a DAF-16::GFP translational fusion were assayed for DAF-16::GFP subcellular localization. B. Wild-type and mutant animals (late L1 to early L2 larvae) harboring a *sod-3*p::GFP promoter fusion were assayed for GFP expression. Identical exposure times were used to photograph all animals harboring a specific GFP reporter. Representative images are shown.

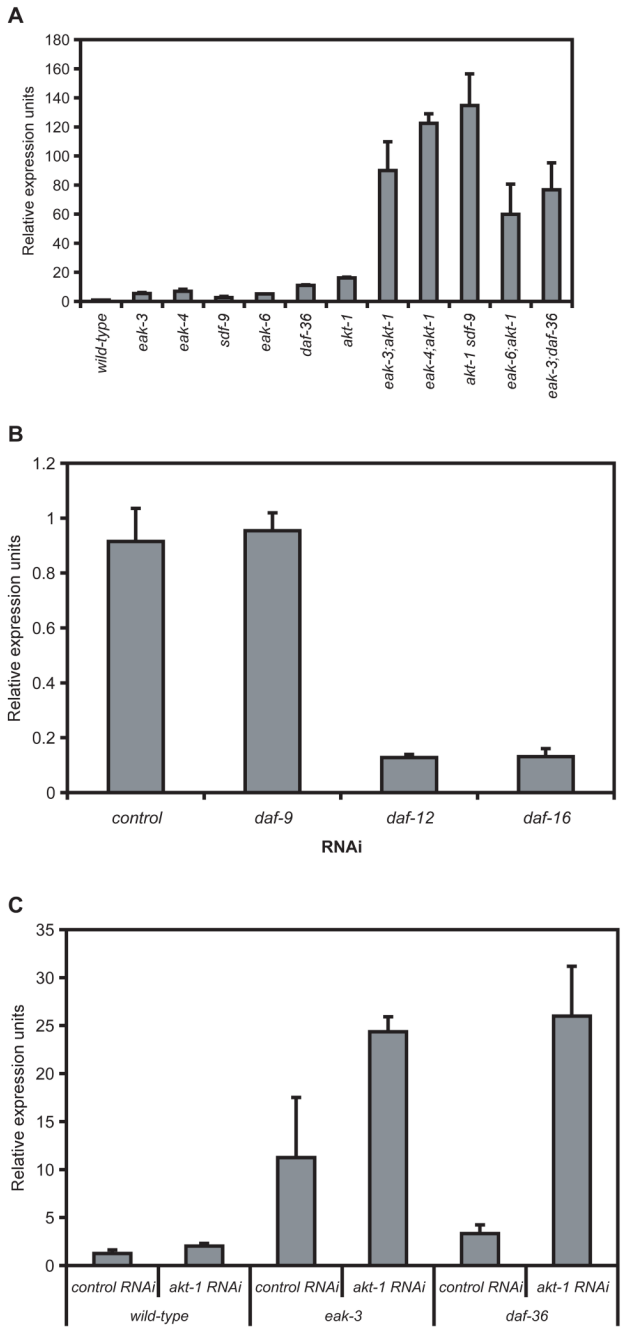
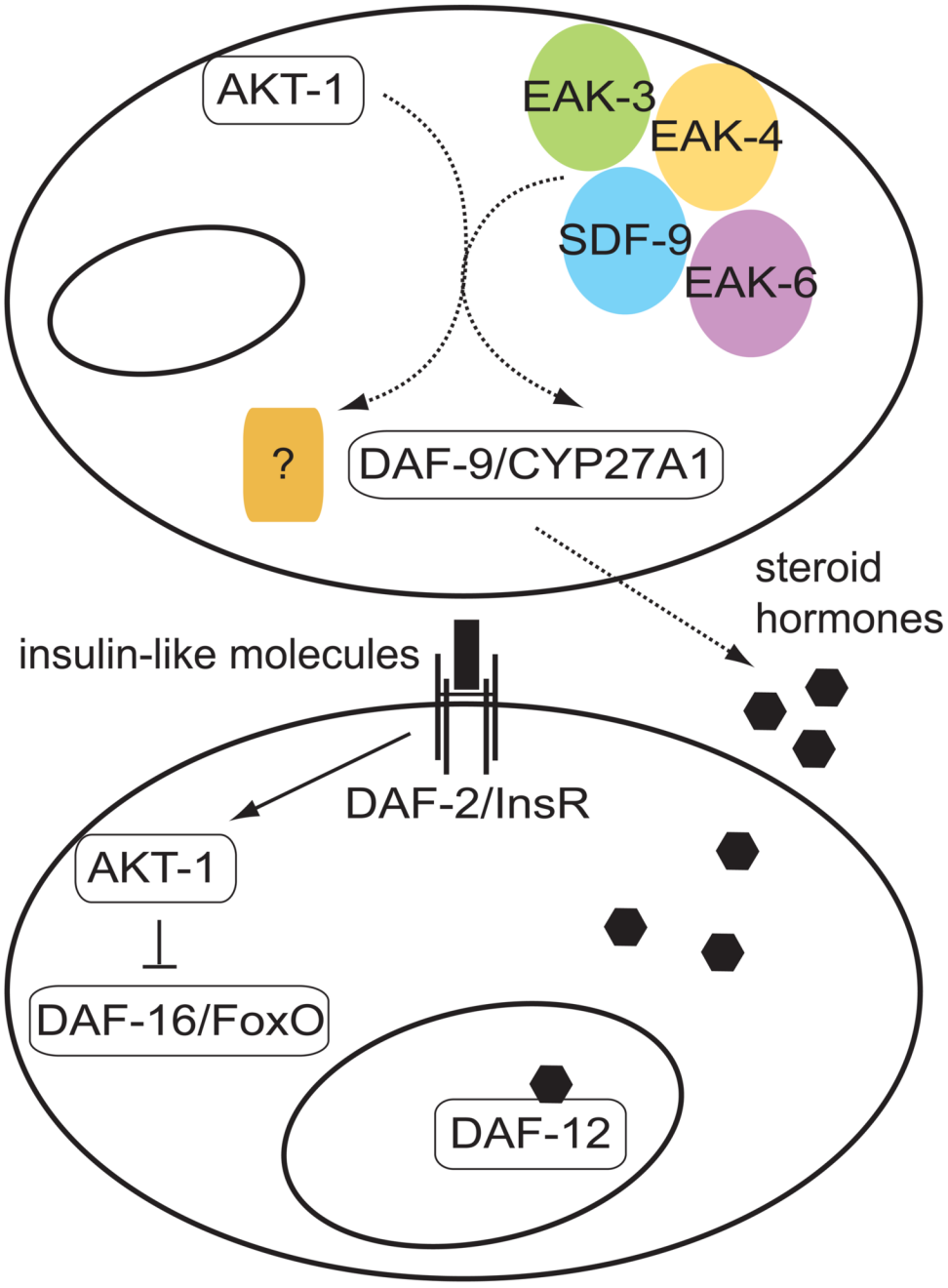


Fig. 6. Quantification of endogenous *sod-3* mRNA levels in wild-type and mutant L2 stage animals by real-time reverse transcription PCR

A. *sod-3* mRNA levels in single and double mutants. B. *sod-3* mRNA levels in *eak-3;akt-1* double mutants after RNAi. C. *sod-3* mRNA levels after *akt-1* RNAi in wild-type, *eak-3* mutant, and *daf-36* mutant animals. Relative expression units are shown. Data are represented as mean + s.d.



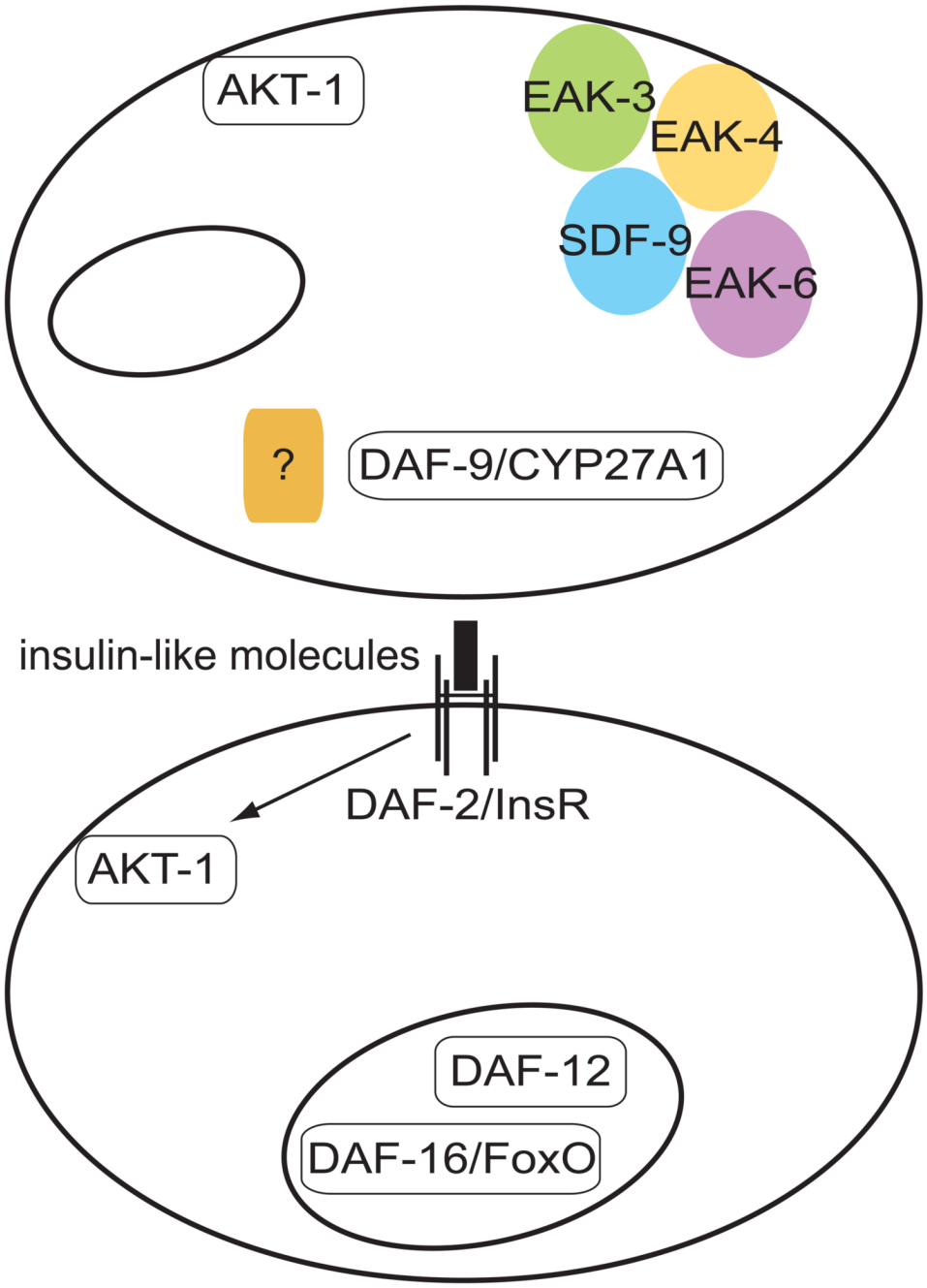


Fig. 7. Model of EAK-3 regulation of DAF-16/FoxO target gene expression in A. wild-type and B. *eak-3;akt-1* double mutant animals

Schematics of an XXX cell (top of figure) and a target cell (bottom of figure) are shown. Dashed arrows and lines denote hypothesized relationships between molecules. See text for details.

Table 1

Mean lifespans of *eak* single and *eak;akt-1* double mutants.

Strain	Mean +/− s.d.	P vs. N2	P vs. *akt-1*	N
N2	12.7 +/− 2.1	—	0.002	101
daf-2(e1370)	23.5 +/− 2.7	7.5×10^{-79}	4.8×10^{-68}	103
akt-1(mg306)	13.7 +/− 2.6	0.002	—	105
eak-3(mg344)	12.6 +/− 2.6	0.90	0.003	105
eak-4(mg348)	12.9 +/− 1.9	0.40	0.014	97
sdf-9(ut187)	12.9 +/− 2.0	0.39	0.017	102
sdf-9(mg337)	12.9 +/− 1.9	0.39	0.014	101
eak-6(mg329)	12.9 +/− 1.6	0.42	0.008	100
eak-3;akt-1	13.0 +/− 2.1	0.29	0.029	99
eak-4;akt-1	13.1 +/− 2.2	0.19	0.058	101
akt-1 sdf-9(ut187)	12.7 +/− 2.5	0.95	0.003	99
akt-1 sdf-9(mg337)	13.0 +/− 2.5	0.40	0.033	101
eak-6;akt-1	12.8 +/− 2.1	0.77	0.005	97

Combined results from two independent experiments are shown. Lifespans from one of these experiments have been reported previously (Hu et al., 2006). Abbreviations: s.d., standard deviation.