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Two Membrane-Associated Tyrosine Phosphatase Homologs Potentiate C. elegans AKT-1/PKB Signaling

Patrick J. Hu1,2*, Jinling Xu1, Gary Ruvkun1*

1 Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts, United States of America, 2 Division of Hematology/Oncology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts, United States of America

Akt/protein kinase B (PKB) functions in conserved signaling cascades that regulate growth and metabolism. In humans, Akt/PKB is dysregulated in diabetes and cancer; in Caenorhabditis elegans, Akt/PKB functions in an insulin-like signaling pathway to regulate larval development. To identify molecules that modulate C. elegans Akt/PKB signaling, we performed a genetic screen for enhancers of the akt-1 mutant phenotype (eak). We report the analysis of three eak genes. eak-6 and eak-5/sdf-9 encode protein tyrosine phosphatase homologs; eak-4 encodes a novel protein with an N-myristoylation signal. All three genes are expressed primarily in the two endocrine XXX cells, and their predicted gene products localize to the plasma membrane. Genetic evidence indicates that these proteins function in parallel to AKT-1 to inhibit the FoxO transcription factor DAF-16. These results define two membrane-associated protein tyrosine phosphatase homologs that may potentiate C. elegans Akt/PKB signaling by cell autonomous and cell nonautonomous mechanisms. Similar molecules may modulate Akt/PKB signaling in human endocrine tissues.

Introduction

Conserved signaling cascades activated by insulin and insulin-like growth factors (IGFs) are critical to the normal development, growth, and physiology of many organisms. In mice, insulin signaling regulates embryonic growth and glucose uptake [1], and insulin-like growth factor-1 (IGF-1) signaling regulates normal prenatal and postnatal growth [2]. Insulin-like signaling regulates cell size in Drosophila [3–5] and development, metabolism, and longevity in Caenorhabditis elegans [6–9].

In humans, dysregulation of insulin and IGF-1 signaling plays a prominent role in disease pathogenesis. Patients with type 2 diabetes mellitus exhibit resistance to insulin [10]; similar insulin resistance is observed in mice harboring mutations in the insulin receptor and downstream components of insulin signaling [11–14]. Downstream components of IGF-1 signaling have been implicated in cancer pathogenesis based on the identity of homologous transforming retroviral oncoproteins [15,16] as well as the existence of gene amplifications [17–19] and somatic mutations [20–23] in primary tumors and tumor cell lines.

Binding of IGFs to their cognate transmembrane receptors activates a cascade that is conserved throughout metazoan phylogeny [24–26]. In C. elegans this pathway includes 38 insulin-like proteins [9,27], an insulin/IGF-1-receptor-like molecule (DAF-2 [7]), PI 3-kinase catalytic (AGE-1 [28]) and adaptor (AAP-1 [29]) subunits, a phosphoinositide-dependent kinase (PDK-1 [30]), two Akt/protein kinase B (PKB) homologs (AKT-1 and AKT-2 [31]), and a serum- and glucocorticoid-inducible kinase homolog (SGK-1 [32]) (see later). Although the biological role of most of the insulin has not been established, a mutation in the daf-28 insulin gene causes decreased insulin signaling [8], implicating DAF-28 as a candidate ligand for DAF-2/InsR (insulin receptor homolog) [9]. Additionally, the C. elegans INS-6 insulin can bind to and activate the human InsR tyrosine kinase [33]. Analogous to insulin and IGF-1 signaling in mammals [34,35], activation of DAF-2/InsR leads to the phosphorylation, cytoplasmic retention, and inhibition of the FoxO transcription factor DAF-16 [36–40]. DAF-2/InsR signaling is likely downregulated by the PTEN (phosphatase and tensin homolog) tumor suppressor homolog DAF-18 [41–44].

daf-2/InsR mutants were first identified based on their increased tendency to enter an alternative larval developmental stage called the dauer stage (daf refers to a dauer formation phenotype) [45]. In replete growth conditions, C. elegans undergoes four larval molts prior to reaching

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Abbreviations: GFP, green fluorescent protein; IGF, insulin-like growth factor; PDK, phosphoinositide-dependent kinase; PI, phosphatidylinositol; PKB, protein kinase B; PNP, p-nitro-phenylphosphate; PTP, protein tyrosine phosphatase; SGK, serum- and glucocorticoid-inducible kinase; SNP, single nucleotide polymorphism; TGF, transforming growth factor

* To whom correspondence should be addressed. E-mail: ruvkun@molbio.mgh.harvard.edu
† Current address: Division of Hematology/Oncology, Department of Internal Medicine, and Department of Cell and Developmental Biology, University of Michigan Medical School, Life Sciences Institute, University of Michigan, Ann Arbor, Michigan, United States of America
Synopsis

Insulin and insulin-like growth factor (IGF) signaling regulates critical physiological processes in a wide variety of multicellular organisms. In humans, dysregulation of IGF signaling underlies the pathogenesis of cancer and diabetes. In the nematode Caenorhabditis elegans, the DAF-2 insulin-like pathway regulates development, metabolism, and longevity. All known components of DAF-2 insulin-like signaling are structurally and functionally conserved in mammals, suggesting that insights gained from studying this pathway in C. elegans may shed light on pathogenetic mechanisms underlying cancer and diabetes. In this study, the authors describe a genetic screen designed to identify novel components of DAF-2 insulin-like signaling in C. elegans. They have characterized three genes that may encode parts of a novel multimolecular membrane-associated complex that potentiates DAF-2 insulin-like signaling in two neuroendocrine cells, the XXX cells. Two of these genes encode proteins similar to mammalian protein tyrosine phosphatases. These results suggest that protein tyrosine phosphatase-like molecules may transduce IGF signals in mammalian endocrine cells and highlight the role of endocrine circuits in the pathogenesis of cancer and diabetes.

reproductive adulthood [46]. Under conditions of high population density, high temperature, or starvation, early larvae bypass the normal second and third larval stages and instead develop into the alternative dauer larva. Dauers are morphologically distinct from normal L3 larvae, exhibiting radial and pharyngeal constriction, decreased pharyngeal pumping, and cuticular specializations called alae. In addition, they increase intestinal fat storage and exhibit extended longevity. Upon improvement of ambient conditions, dauers recover to the L4 larval stage and proceed to reproductive adulthood [47].

Genetic screens have defined three signaling pathways that normally function to inhibit dauer arrest under replete environmental conditions [45,48–50]. In addition to DAF-2/InsR inputs, dauer arrest is regulated by conserved DAF-7/tob transforming growth factor (TGF)-β-like [51] and DAF-11/guananyl cyclase [52] signaling pathways. Insulin and TGF-β-like ligands are expressed in small subsets of head neurons, and this expression is regulated by various environmental inputs and by cyclic guanosine monophosphate signaling [9,27,51,53]. These ligands bind to cognate receptors that are expressed widely in target tissues throughout the animal [54]. The major targets of DAF-7/TGF-β signaling are the SMAD4 tumor suppressor homolog DAF-3 [55] and its binding partner DAF-5/SNO [56,57]. Mutations in daf-16/FoxO specifically suppress dauer formation of daf-2/InsR pathway mutants, indicating that DAF-16/FoxO is the major target of DAF-2/InsR signaling in C. elegans [49,50]. Genetic analysis indicates that these pathways function in parallel to promote normal larval development [48,49].

Although many components of DAF-2/InsR signaling have been characterized, at least three lines of evidence indicate that undiscovered pathway components that act downstream of DAF-2/InsR but parallel to AGE-1/PI3K may exist. Gain-of-function mutations in pdk-1 and akt-1 suppress the dauer-constitutive phenotype of age-1/PI3K null mutants more efficiently than they do that of daf-2/InsR mutants [30,31]. The weak daf-16/PTEN(e1375) allele suppresses dauer arrest in age-1/PI3K null mutants [42,43,49] but does not suppress the dauer-constitutive phenotype of daf-2(e1370) mutants [42,43,50]. Last, a functional DAF-16:GFP fusion protein harboring point mutations in all four putative Akt/PKB phosphorylation sites localizes to the nucleus but does not increase life span or induce dauer formation in a daf-2 wild-type background [38].

The tumor suppressor function of the 3-phosphoinositide phosphatase PTEN [20,21], the frequent somatic mutation of phosphatidylinositol (PI) 3-kinase in human cancers [23], and the discovery of a germline Akt2 loss-of-function mutation in a family with autosomal dominant insulin-resistant diabetes [58] underscore the central role of this pathway in human disease. In view of the striking structural and functional conservation of insulin-like signaling throughout metazoan phylogeny [59], it is likely that the identification of novel DAF-2/InsR signaling components will illuminate not only mechanisms of developmental regulation in C. elegans but also the pathogenesis of common human diseases such as cancer and diabetes.

To identify genes encoding such molecules, we performed a genetic screen for mutants that enhance the dauer-constitutive phenotype of an akt-1 null mutant (Eak screen). Seven genetic loci enhance akt-1 when mutated; we report the molecular identity of three eak loci here. These genes may encode elements of a membrane-associated complex that functions in two endocrine cells to potentiate insulin-like signaling. A similar activity may modulate insulin and IGF-1 signals in human endocrine tissues.

Results

A Sensitized Genetic Screen for Akt/PKB Signaling Components

We hypothesized that a genetic screen performed in a weak dauer-constitutive daf-2/InsR pathway mutant background might allow the identification of DAF-2/InsR signaling components which, when mutated alone, might not have phenotypes. akt-1(mg306) was isolated in a genetic screen for mutations that affect the production of or response to elevated 3-phosphoinositide levels and contains a C→T transition that generates a nonsense mutation in the pleckstrin homology domain (see Materials and Methods). In contrast to daf-2/InsR, age-1/PI3K, and pdk-1 loss-of-function (lf) mutants, which form dauers at 25 °C or lower [28,30,60], akt-1(lf) alleles have a Hid (high-temperature-induced dauer) phenotype [61], developing reproductively at 25 °C but forming dauers at 27 °C (unpublished data and Figure 1). Consistent with a role for AKT-1 in DAF-2/InsR signal transduction [31,43], the 27 °C dauer-constitutive phenotype of akt-1 mutants is suppressed by a daf-16/FoxO (lf) mutation [61].

We mutagenized akt-1(mg306) animals and screened for dauer arrest at 25 °C, a temperature at which akt-1(mg306) mutants do not form dauers (Figure 1A). Among 30 independent mutants isolated from approximately 21,000 haploid genomes screened, 26 were suppressible by daf-16/FoxO feeding RNAi (unpublished data), suggesting that their dauer-constitutive phenotypes were either dependent upon the presence of the akt-1(mg306) mutation or the result of mutations in the daf-2/InsR pathway [49,50], or both. Twenty-one of these 26 mutants were true Eak mutants, exhibiting dauer arrest at 25 °C only in a homozygous akt-1(mg306)
background (unpublished data and Figure 1A). Mapping and complementation analysis indicate that these 21 mutants define seven Eak genes. All eak;akt-1(mg306) double mutants formed a high percentage of dauers at 25 °C (Figure 1A), exhibiting a dauer-constitutive phenotype as strong as the canonical daf-2/InsR mutant daf-2(e1370). However, in contrast to daf-2(e1370) dauers, which have constricted pharynxes and dauer-specific cuticular alae, all eak and eak;akt-1(mg306) dauers were partial dauers, exhibiting characteristic alae but failing to undergo pharyngeal remodeling (Figure S1). eak mutants also enhanced dauer arrest phenotypes of weak alleles of the daf-2/InsR pathway components age-1/PI3K and pdk-1; whereas age-1(hx546) and pdk-1(sa709) did not exhibit dauer arrest at 25 °C, age-1;eak and eak;pdk-1 double mutants had strong dauer arrest phenotypes at 25 °C (Figure S2A). Notably, eak mutants did not enhance dauer arrest phenotypes of akt-2(ok393), a deletion allele of akt-2 (Figure S2B). Therefore, eak gene products may function in the same pathway as AKT-2.

In an akt-1(+) background, eak mutants had minimal dauer-constitutive phenotypes at 25 °C (Figure 1A) and relatively weak dauer-constitutive phenotypes in comparison to akt-1(mg306) animals at 27 °C (Figure 1B). The ability of eak mutants to enhance dauer arrest phenotypes of age-1/PI3K, akt-1, and pdk-1 loss-of-function mutants is consistent with their functioning in a parallel pathway, and their low penetrance dauer arrest phenotype at 25 °C would explain why these mutants were not isolated in previous screens for dauer arrest mutants performed at this temperature.

**The eak Genes Act in the C. elegans Insulin-like Pathway**

Loss-of-function mutations in daf-16/FoxO specifically suppress the dauer arrest phenotype of daf-2/InsR pathway mutants [49,50]. To determine whether eak gene products function in the daf-2/InsR pathway, we performed epistasis analysis on daf-16/eak double mutants and daf-16/eak;akt-1 triple mutants. For epistasis analysis of eak-6(mg329), we used feeding RNAi of daf-16/FoxO; close linkage of eak-6 and daf-16 on Chromosome I prevented construction of an eak-6 daf-16
double mutant. A daf-16/FoxO null mutation, mgDf47 [36], fully suppressed dauer-constitutive phenotypes of all eak single mutants tested (Figure 1C) and both eak-akt-1 double mutants tested (Figure S3A), whereas a daf-3/SMAD null mutation did not [62]. RNAi of daf-16/FoxO also suppressed akt-1(mg306), eak-4(mg348), and eak-6(mg329) dauer-constitutive phenotypes, as did RNAi of the nuclear hormone receptor gene daf-12 (Figure 1D; loss-of-function daf-12 mutations suppress dauer arrest caused by most known dauer-constitutive mutants [48,50,61,63–65]). These findings suggest that eak genes function in the daf-2/InsR pathway.

To help determine whether eak genes function in parallel to AKT-1, we constructed eak2daf-18 and eakakt-1 gain-of-function double mutants and performed epistasis analysis. The weak daf-18/PTEN allele e1375 suppresses dauer arrest in age-1/PI3K null mutants but does not suppress dauer arrest in daf-2(e1370) [42,43,49,50], and the akt-1 gain-of-function allele mg144 exhibits stronger suppression of age-1/PI3K null phenotypes than daf-2/InsR loss-of-function phenotypes [31], suggesting that DAF-18/PTEN and AKT-1 function in an AGE-1/PI3K–specific branch of DAF-2/InSR outputs. An inability of daf-18(e1375) or akt-1(mg144) to suppress eak dauer arrest would indicate that eak gene products function in parallel to and independently of AGE-1/PI3K. Both daf-18(e1375) and akt-1(mg144) strongly suppressed dauer arrest in eak-4, eak-5/sdf-9, and eak-6 mutants (Figure S3B and S3C), suggesting that eak gene products function either upstream of or in parallel to AGE-1/PI3K and AKT-1.

To address the issue of whether all three eak gene products function in the same pathway, we constructed eak2eak-3 double mutants and tested them for dauer arrest at 25 °C and 27 °C. Lack of enhanced dauer arrest in double mutants would suggest that eak gene products function together in the same complex or pathway. eak-4/sdf-9 and eak-6/sdf-9 double mutants did not arrest as dauers at 25 °C (Figure 1A) and did not exhibit enhanced dauer arrest at 27 °C (Figure 1B), indicating that the EAK proteins likely function together in the same complex or pathway.

Most dauer-constitutive mutants in the daf-2/InsR pathway also have extended organismal longevity [6,29,30,66]. Therefore, we performed longevity assays on all eak mutants. Consistent with a recent report [32], the akt-1(mg306) mutation extended median life span by less than 10%, which is a smaller extension of life span than has been seen with other loss-of-function dauer-constitutive mutants in the daf-2/InsR pathway. Interestingly, no eak mutants tested extended median or maximum life span significantly (Figure S4). Furthermore, eak alleles did not enhance life span extension of akt-1(mg306), although they all strongly enhanced the dauer formation phenotype of akt-1(mg306) (Figure 1A). Thus, eak signaling does not subserve longevity control.

Two eak Genes Are Related to Protein Tyrosine Phosphatases

Nine mutants define the eak-5 gene. Single nucleotide polymorphism (SNP) mapping [67] localized eak-5 to an approximately 480-kb genomic interval between cosmid F48F5 and the right telomere of Chromosome V. sdf-9, identified in a screen for enhancers of the dauer-constitutive phenotype of unc-31(e169) [62], lies within this interval. Sequencing of sdf-9 exons and splice junctions in all nine eak-5 alleles identified eight distinct point mutations (Table 1), and an akt-1 sdf-9 double mutant constructed using the sdf-9(u187) allele identified in the unc-31(e169) enhancer screen [62] had a strong dauer-constitutive phenotype at 25 °C (Figure 1A), indicating that eak-5 is allelic to sdf-9.

The eak-6 gene is defined by one allele, mg329. SNP mapping localized eak-6(mg329) to an approximately 210-kb genomic region on Chromosome I between cosmids F52F12 and B0379. BLASTP analysis of SDF-9 against the C. elegans Wormpep database (http://www.sanger.ac.uk/Projects/C__elegans/WORMBASE/current/wormpep.shtml) identified a predicted homolog encoded by open reading frame F10G8.4. This open reading frame lies within the eak-6 genomic interval defined by our SNP mapping. Sequencing of predicted exons and splice junctions of F10G8.4 in eak-6(mg329) identified a G→A transition resulting in an opal nonsense mutation near the predicted amino-terminus of the protein (Figure 2A). Two of nine transgenic lines containing a genomic PCR fragment including the putative promoter, open reading frame, and three untranslated regions (UTR) of F10G8.4 exhibited rescue of the dauer arrest phenotype of an eak-6(mg329);akt-1(mg306) double mutant (unpublished data), supporting the argument that F10G8.4 is eak-6.

Table 1. eak-5 Is Allelic to the Synthetic Dauer Formation Gene sdf-9

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<td>mg324</td>
<td>C(17090)→A</td>
<td>G129R</td>
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<tr>
<td>mg325</td>
<td>C(17890)→T</td>
<td>P219S</td>
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<td>mg330</td>
<td>C(17879)→T</td>
<td>P215L</td>
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<tr>
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<td>C(17694)→A</td>
<td>G168E</td>
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<td>First exon splice donor mutation</td>
</tr>
<tr>
<td>mg341</td>
<td>C(17879)→T</td>
<td>P215L</td>
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<tr>
<td>mg342</td>
<td>(G16113)→A</td>
<td>Promoter mutation</td>
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<tr>
<td>mg343</td>
<td>(C17912)→A</td>
<td>G226E</td>
</tr>
<tr>
<td>mg349</td>
<td>(C17121)→T</td>
<td>A139V</td>
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</table>

SDF-9 residues mutated in each eak-5 allele are numbered according to the annotated sequence of YAC Y4446D (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=3217937). Predicted amino acid changes are numbered according to the predicted primary sequence of SDF-9 [62].

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PTP Homologs Potentiate Akt Signaling

Nine mutants define the eak-5 gene. Single nucleotide polymorphism (SNP) mapping [67] localized eak-5 to an approximately 480-kb genomic interval between cosmid F48F5 and the right telomere of Chromosome V. sdf-9, identified in a screen for enhancers of the dauer-constitutive phenotype of unc-31(e169) [62], lies within this interval. Sequencing of sdf-9 exons and splice junctions in all nine eak-5 alleles identified eight distinct point mutations (Table 1), and an akt-1 sdf-9 double mutant constructed using the sdf-9(u187) allele identified in the unc-31(e169) enhancer screen [62] had a strong dauer-constitutive phenotype at 25 °C (Figure 1A), indicating that eak-5 is allelic to sdf-9.

The eak-6 gene is defined by one allele, mg329. SNP mapping localized eak-6(mg329) to an approximately 210-kb genomic region on Chromosome I between cosmids F52F12 and B0379. BLASTP analysis of SDF-9 against the C. elegans Wormpep database (http://www.sanger.ac.uk/Projects/C__elegans/WORMBASE/current/wormpep.shtml) identified a predicted homolog encoded by open reading frame F10G8.4. This open reading frame lies within the eak-6 genomic interval defined by our SNP mapping. Sequencing of predicted exons and splice junctions of F10G8.4 in eak-6(mg329) identified a G→A transition resulting in an opal nonsense mutation near the predicted amino-terminus of the protein (Figure 2A). Two of nine transgenic lines containing a genomic PCR fragment including the putative promoter, open reading frame, and three untranslated regions (UTR) of F10G8.4 exhibited rescue of the dauer arrest phenotype of an eak-6(mg329);akt-1(mg306) double mutant (unpublished data), supporting the argument that F10G8.4 is eak-6.

Eak-6 and SDF-9 both have amino acid similarity to PTPs (Figure 2B and [62]). Conservation is strongest in the ten motifs that are conserved among 37 human PTPs [68]. Whereas SDF-9 does not retain the canonical catalytic cysteine residue found in all known PTPs and is therefore predicted to be catalytically inactive [62,69], the catalytic cysteine is conserved in EAK-6. Despite this difference, sdf-9 and eak-6 mutants have a similar phenotype (Figure 1A and 1B). Among 19 amino acid residues that are invariant among 113 vertebrate PTPs [68], 15 are conserved in EAK-6 and 13 are conserved in SDF-9 (Figure 2B). PTP activity assays on both epitope-tagged EAK-6 expressed in and immuno precipitated from cultured human cells and a GST-EAK-6 fusion protein expressed in Escherichia coli revealed no hydrolytic activity on the substrate p-nitro-phenylphosphate (PNPP), a phosphotyrosine analog (unpublished data). Thus, EAK-6 and SDF-9 may be inactive phosphatase homologs that bind to tyrosine phosphoproteins. However, we cannot rule out the possibility that EAK-6 has PTP activity that is not detectable in the PNPP assay.
Three alleles, mg326, mg328, and mg348, define the eak-4 locus. SNP mapping narrowed the eak-4 interval to an approximately 560-kb genomic region on Chromosome IV between cosmids B0001 and T23B5. Cosmid rescue assays identified F53B2 as the rescuing cosmid, and rescue experiments with genomic PCR fragments corresponding to

Figure 2. Predicted Primary Amino Acid Sequence of EAK-6 and Similarity with PTPs

(A) EAK-6 amino acid sequence. EAK-6 sequence was derived from full-length cDNA amplified by RT-PCR from wild-type C. elegans total RNA. The PTP domain is denoted in boldface. The residue mutated in eak-6(mg329) is boxed and the predicted change indicated above the mutated residue. Amino acids encoded by an alternatively spliced exon present in EAK-6L but not in EAK-6S (see Materials and Methods) are underlined.

(B) EAK-6 homology with PTPs. The PTP domain of EAK-6 is aligned with that of SDF-9 and three human PTPs. Sequences used in the alignment are based on domains defined by Pfam [101] and correspond to amino acids 40 to 276 of PTPN1 (PTP1B), 57 to 308 of EAK-6, 31 to 283 of SDF-9, 265 to 500 of PTPRA (receptor-type PTPa), and 273 to 520 of PTPN11 (SHP-2). Alignment was performed using ClustalX 1.8 and MacBoxshade 2.15. Conserved and identical residues are shaded. Ten motifs conserved among 37 vertebrate PTPs [68] are underlined, and 19 residues that are invariant among 113 vertebrate PTP domains are boxed. The four invariant residues that are not identical in EAK-6 (R45, Q85, H214, and G220, numbered according to the PTPN1 primary sequence) are denoted with dots. The catalytic cysteine residue [69] is denoted by an asterisk. Two conserved residues that are not conserved in EAK-6, D181 and Q262, are denoted by arrowheads.

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**eak-4 Encodes a Novel Protein with an N-Myristoylation Signal**

Three alleles, mg326, mg328, and mg348, define the eak-4 locus. SNP mapping narrowed the eak-4 interval to an approximately 560-kb genomic region on Chromosome IV between cosmid B0001 and T23B5. Cosmid rescue assays identified F53B2 as the rescuing cosmid, and rescue experiments with genomic PCR fragments corresponding to...
open reading frame, and 3′ UTR rescued the PCR fragment containing the predicted F53B2.3 promoter, independent point mutations in all three predicted exons and splice junctions in F53B2.3 identified N at residue 6 in the indicated above the box. The conserved G at residue 2 and S/T/A (boxed) alleles of eak-4 indicate amino acid identity and conservation. Mutated residues in three constructed using ClustalX 1.8 and MacBoxshade 2.15. Shaded residues C. elegans by RT-PCR from wild-type five genes. EAK-4 sequence was derived from full-length cDNA amplified Sequences represent the entire predicted amino acid sequences of all Homologs F53B2.3 as a candidate gene for eak-4 (Figure 3). EAK-4 Amino Acid Sequence and Alignment with Four C. elegans Homologs

Sequences represent the entire predicted amino acid sequences of all five genes. EAK-4 sequence was derived from full-length cDNA amplified by RT-PCR from wild-type C. elegans total RNA. Alignment was established data), a common site of artifactual GFP expression specifically in XXX, as was an SDF-9::GFP fusion protein [62]. Coexpression of translational GFP fusions with an sdf-9p::RFP promoter fusion was expressed exclusively in two head cells with position and morphology consistent with their identification as the XXX cells [62]. In both strains containing GFP and RFP reporter constructs, GFP and RFP colocalized (Figure 4A), indicating that eak-4, sdf-9, and eak-6 are all expressed in XXXLR.

EAK-4, SDF-9, and EAK-6::GFP Fusion Proteins Localize to the Plasma Membrane

In order to visualize the subcellular localization of EAK proteins, we made full-length translational GFP fusion constructs and expressed them in wild-type animals. Both EAK-4::GFP and EAK-6::GFP fusion proteins were expressed specifically in XXX, as was an SDF-9::GFP fusion protein [62]. They were also variably expressed in the intestine (unpublished data), a common site of artificial GFP expression [74,75]. Coexpression of translational GFP fusions with an sdf-9p::RFP promoter fusion indicated that all three GFP fusions localize to the plasma membrane of the XXX cells (Figure 4B). Membrane localization was also apparent in intestinal cells (unpublished data).

We determined the role of the N-myristoylation consensus motif in EAK-4 plasma membrane localization by constructing an EAK-6::GFP mutant in which the invariant glycine residue at position 2 is mutated to alanine (G2A). In contrast to the specific expression of EAK::GFP proteins in the XXX cells (Figure 4A and 4B), a functional AKT-1::GFP fusion protein is expressed widely in postem-
To determine the importance of AKT-1 function specifically in XXXL/R, we expressed the akt-1 open reading frame and 3' UTR under the control of the eak-4 promoter and asked whether this transgene could rescue dauer arrest in an eak-4(mg326);akt-1(mg306) double mutant. In three of three transgenic lines assayed, animals harboring the eak-4p::AKT-1 transgene bypassed dauer arrest and grew reproductively, whereas nontransgenic siblings formed davers (Figure 5). Control transgenic animals expressing an eak-4p::GFP transcriptional fusion construct did not bypass dauer (unpublished data). These results show that AKT-1 expression in the XXX cells is sufficient to rescue the akt-1(mg306) dauer arrest phenotype and indicate that the XXX cells are a major site of AKT-1 function in C. elegans.

Figure 4. EAK-4, SDF-9, and EAK-6 Localize to the Plasma Membrane of the XXX Cells
(A) eak-4, sdf-9, and eak-6 promoters drive expression in the same cells. Wild-type animals harboring an extrachromosomal array with eakp::GFP and sdf-9p::RFP constructs were analyzed by fluorescence microscopy. Representative photographs are shown. Animals are oriented anterior left and dorsal up.
(B) EAK-4::GFP, SDF-9::GFP, and EAK-6::GFP fusion proteins localize to the plasma membrane of XXX. Animals harboring EAK::GFP translational fusion constructs and an integrated sdf-9p::RFP array were analyzed using fluorescence microscopy. Representative photographs of a XXX cell are shown.
(C) Mutation of the invariant glycine in the N-myristoylation motif of EAK-4 abrogates plasma membrane localization. Animals harboring either a wild-type EAK-4::GFP construct or an EAK-4::GFP construct with the glycine at position 2 mutated to alanine (G2A) were analyzed using fluorescence microscopy.
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In order to assess whether eak gene products regulate DAF-16/FoxO cell autonomously, we first determined whether a functional DAF-16A::GFP fusion protein under the control of the daf-16a promoter is expressed in XXXL/R. We constructed a strain harboring integrated DAF-16A::GFP and sdf-9p::RFP transgenes and assayed for colocalization of GFP and RFP.
Surprisingly, although DAF-16A::GFP is widely expressed [40], it did not colocalize with RFP (Figure 6), indicating that it is not expressed at high levels in XXXL/R. Therefore, eak gene products may regulate DAF-16/FoxO nonautonomously.

**Discussion**

We have used an akt-1 enhancer screen to identify three genes encoding proteins that potentiate AKT-1 signaling. It is noteworthy that this screen has yielded neither weak alleles of known dauer-constitutive genes encoding components of DAF-2/InsR, DAF-7/TGF-β, or DAF-11/GC pathways nor strong alleles of the many mutants with weak dauer-constitutive phenotypes [61,63,76]. The fact that we have isolated multiple alleles of five of the seven genes identified in our screen (Figure 3, Table 1, and unpublished data) without identifying alleles of most mutants with weak dauer phenotypes argues that the target for akt-1 enhancement is very specific and that most mutants with weak dauer phenotypes do not strongly enhance akt-1 (mg306). Therefore, eak-4, sdf-9, and eak-6 likely do not enhance akt-1 (mg306) simply by virtue of their weak dauer-constitutive phenotype; rather, these genes probably encode components of a complex or pathway that cooperates specifically with AKT-1.

sdf-9 was also identified in a genetic enhancer screen using the weak dauer arrest mutant unc-31(e169) as a genetic background [62]. unc-31 encodes a homolog of CAPS, a protein required for calcium-induced dense core vesicle exocytosis [77]. Since insulin is stored in and secreted from dense core vesicles in pancreatic islet beta cells [78], it is likely that UNC-31/CAPS and AKT-1 function in the same insulin signaling pathway. This is underscored by the suppression of the weak dauer arrest phenotypes of akt-1 and unc-31 mutants by daf-16/FoxO mutations (Figure 1C and 1D [61]) and may explain why sdf-9 mutants were isolated in both screens. akt-1 sdf-9 and unc-31 sdf-9 double mutants have similar dauer arrest phenotypes at 25 °C (Figure 1A and [62]).

Surprisingly, we have not identified any akt-2 mutants in this screen. We expected akt-2 mutants to emerge from this screen, given that akt-1/akt-2 double mutants [79] as well as animals subjected to simultaneous RNAi of akt-1 and akt-2 [31,32] undergo dauer arrest. Furthermore, eak mutants do not enhance dauer arrest of akt-2 mutants (Figure 2B), indicating that they may act in the same pathway. However, none of the 21 alleles isolated in the Eak screen is X-linked (the akt-2 gene lies on the X chromosome). In the course of constructing an akt-1(mg306)/akt-2(ok393) double mutant, we noted that akt-1/akt-2 animals formed nonconditional dauers in the F2 generation but were maternally rescued for dauer arrest in the F2 generation (unpublished data), explaining why akt-2 alleles were not isolated in this F2 screen. It is possible that other components of DAF-2/InsR signaling would emerge from an F3 akt-1 enhancer screen.

In contrast to previous results demonstrating a strong dauer arrest phenotype for multiple sdf-9 alleles [62], we have only observed weak dauer phenotypes for all eak-4, sdf-9, and eak-6 alleles tested (Figure 1 and unpublished data). The alleles tested included the ut163 and ut187 alleles of sdf-9 previously described (Figure 1A and 1B; [62]). As dauer assays at 27 °C are exquisitely sensitive to small changes in environmental conditions [63], we attribute these differences to small discrepancies between assay conditions in different laboratories. This may also explain the disparate effects of XXX laser ablation on dauer formation reported in the literature [62,65,80].

**The XXX Cells as a Site of DAF-2/InsR Function**

The XXX cells are annotated as hypodermal cells in the head [81] that abut the pseudocoelom and have neuronal characteristics [62,80,82]. Laser ablation of the XXX cells causes partial dauer arrest [62], indicating that the XXX cells normally function to inhibit dauer arrest. The specific expression of EAK-4, SDF-9, and EAK-6 in the XXX cells (Figure 4), taken together with our finding that AKT-1 expression in XXX suffices to rescue the dauer arrest phenotype of an eak-3 akt-1 double mutant (Figure 5), indicates that the XXX cells may be a major site of DAF-2/InsR function in *C. elegans*. However, the output of the XXX cell in the regulation of dauer arrest also depends on insulin signaling in other cells, since the dauer arrest phenotype caused by ablation of the XXX cells is suppressed by mutations in daf-16/FoxO but not by mutations in daf-3/SMAD4 [62]. This is also consistent with our observation that DAF-16::GFP expressed from the daf-16a promoter is not expressed in XXX (Figure 6). It is not known whether DAF-2/InsR is expressed in XXX.

As reported previously for sdf-9 [62], we have observed that eak-4, sdf-9, and eak-6 single mutants form partial dauers that have cuticular alae but have not undergone pharyngeal remodeling (Figure S1). Indeed, some daf-2/InsR mutant alleles also cause partial dauer arrest [60]. It is possible that signals from the XXX cells control dauer arrest and extrapharyngeal remodeling, whereas DAF-2/InsR signaling outputs from other cells, for example, the pharyngeal tissues themselves, may affect pharyngeal remodeling. Phenotypes of rare daf-2 mosaics that lack daf-2 activity in both XXX cells and various portions of the ABa cell lineage [83] suggest that descendants of the ABalpa cell may regulate pharyngeal remodeling during dauer formation.
EAK-4, SDF-9, and EAK-6 May Be Novel Components of DAF-2/InsR Signaling

Enhancement of akt-1 phenotypes could occur either through further attenuation of DAF-2/InsR signaling or by derangement of DAF-7/TGF-β or DAF-11/GC signaling [56]. As is the case for all known components of the DAF-2/InsR pathway [30–32, 48, 49], eak-4, sdf-9, and eak-6 phenotypes are fully suppressed by mutations in daf-16/FoxO (Figure 1C and 1D [62]), the major target of DAF-2/InsR signaling [37,49]. This supports the contention that EAK-4, SDF-9, and EAK-6 are components of DAF-2/InsR signaling. Indeed, the strong, nonconditional dauer phenotype of some eak;akt-1 double mutants (unpublished data) is reminiscent of the phenotype of strong loss-of-function alleles of daf-2/InsR [60].

Results of epistasis experiments with the weak daf-18/PTEN allele e1375 [42,43] and the akt-1 gain-of-function allele mg144 [31] (Figure S3B and S3C) implicate EAK-4, SDF-9, and EAK-6 function upstream of or in parallel to AGE-1/PI3K and AKT-1. Since one of the molecular consequences of either DAF-18/PTEN loss-of-function or AKT-1 gain-of-function is phosphorylation and inhibition of DAF-16/FoxO [38–40], it is not surprising that daf-18(e1375), akt-1(mg144), and daf-16(mgDf47) are all epistatic to eak mutants.

EAK Proteins May Inhibit DAF-16/FoxO Nonautonomously

eak-4, sdf-9, and eak-6 mutants strongly enhance the akt-1 null phenotype (Figure 1A), suggesting that EAK proteins function in parallel to AKT-1. In addition, eak-4;sd9 and eak-6;sd9 double mutants do not exhibit more severe phenotypes compared to the respective single mutants (Figure 1A and 1B), indicating that EAK-4, SDF-9, and EAK-6 function in the same pathway or complex. Parallel signaling of AKT-1 and EAK proteins could occur either at the organellar level, whereby AKT-1 signaling in non-XXX cells would converge with EAK signals in XXX to inhibit DAF-16/FoxO cell autonomously, or at the intracellular level, whereby AKT-1 signaling would converge with EAK signals in XXX to inhibit DAF-16/FoxO cell autonomously. This suggests that the AKT-1 signaling pathway in non-XXX cells would converge with EAK signals in XXX to inhibit DAF-16/FoxO cell autonomously. The ability of AKT-1 to rescue a eak-1;akt-1 double mutant (Figure 5) supports the notion that AKT-1 functions at least in part by signaling in parallel to EAK-4, SDF-9, and EAK-6 in XXX. However, the observation that dauer arrest caused by XXX laser ablation is suppressed by a daf-16 loss-of-function mutant [62] and the lack of DAF-16A::GFP expression in XXX (Figure 6) suggest that EAK proteins regulate DAF-16A nonautonomously. It is possible that AKT-1 phosphorylates critical substrates in XXX distinct from DAF-16/FoxO. Alternatively, since there are at least three DAF-16/FoxO isoforms that may be transcribed from distinct promoters (WormBase Web site, http://www.wormbase.org, release WS150, November 30, 2005), a DAF-16/FoxO isoform distinct from DAF-16A may be a target of AKT-1 and EAK signals in XXX.

Mechanisms of SDF-9 and EAK-6 Membrane Localization

In contrast to EAK-4, which likely localizes to the plasma membrane through N-myristoylation (Figure 4C), the mechanisms underlying the membrane localization of SDF-9 and EAK-6 are not clear. Neither SDF-9 nor EAK-6 possesses an N-myristoylation motif or a predicted transmembrane domain. SDF-9 and EAK-6 could associate with the plasma membrane by binding to membrane-associated proteins such as EAK-4. Alternatively, given that SDF-9 (and possibly EAK-6) has an inactive PTP domain that might bind to phosphotyrosine residues, they could bind to membrane-associated tyrosine phosphoproteins such as DAF-2/InsR. The ability of the DAF-18 ortholog PTEN to hydrolyze both phosphotyrosines [84] and phosphoinositides [85] suggests a third possible mechanism of SDF-9 and EAK-6 membrane localization via direct binding to phospholipids.

We addressed each model experimentally. First, coprecipitation assays on N-terminal epitope-tagged EAK-4, SDF-9, and EAK-6 expressed in all pairwise combinations in cultured 293T cells failed to reveal direct physical interactions among the three proteins despite high levels of protein expression (unpublished data). Furthermore, epitope-tagged SDF-9 and EAK-6 did not coprecipitate tyrosine phosphoproteins after exposure of transfected cultured 293T cells to IGF-1 (unpublished data). Finally, radiolabeled SDF-9 and EAK-6 synthesized in vitro did not bind to phosphoinositides immobilized on nitrocellulose (Seth Field and Lewis Cantley, personal communication). The observation that SDF-9::GFP exhibits plasma membrane localization in the absence of intact DAF-2/InsR signaling (Figure S6) is consistent with the lack of SDF-9 binding to tyrosine phosphoproteins in 293T cells and suggests that SDF-9 membrane association is independent of tyrosine phosphorylation. We cannot rule out the possibility that biologically relevant EAK protein–protein or protein–lipid interactions require additional components that exist in vivo but were not present in the assays described. Notably, one (or more) of the four eak genes that remain to be cloned (eak-1, eak-2, eak-3, and eak-7) may encode such a molecule.

SDF-9 and EAK-6 Are Similar to Tyrosine Phosphatases

The similarity of SDF-9 and EAK-6 amino acid sequences to PTPs (Figure 2B and [62]) suggests that SDF-9 and EAK-6 may comprise a PTP that activates downstream signaling through dephosphorylation of tyrosine residues. Although SDF-9 lacks the cysteine residue that is critical for PTP catalytic activity [69], EAK-6 retains this cysteine (Figure 2B) and may possess phosphatase activity. Many receptor-type PTPs harbor...
tandem catalytic domains, one of which frequently does not have PTP activity [68]. Similarly, SDF-9 and EAK-6 might function as a heteromeric PTP associated with the plasma membrane. The finding that EAK-6 expressed in mammalian cells or bacteria lacks PTP activity on the substrate PNPP does not preclude the possibility that it may have activity on specific phosphotyrosine residues in the context of a full-length protein.

Alternatively, it is possible that EAK-6 has phosphohydrolase activity on nonphosphotyrosine or nonprotein substrates. Among 19 invariant residues in the catalytic domains of 113 vertebrate PTPs [68], 15 are conserved in EAK-6 (Figure 2B). Two of the four invariant residues not conserved in EAK-6, R45 and G220 (PTPN1/PTP1B numbering), are thought to be involved in phosphotyrosine binding [68]. In EAK-6 these are replaced with isoleucine and alanine, respectively. Changes in these residues could reflect changes in EAK-6 substrate specificity.

A third possibility is that EAK-6 does not possess catalytic activity. One of the four invariant residues not conserved in EAK-6, H214, is a glutamine residue in EAK-6 (Figure 2B). This histidine is proposed to lower the pK_a of the catalytic cysteine [68]; mutation of H214 to alanine reduces the catalytic activity of PTPN1/PTP1B approximately 80-fold [86]. Two other conserved residues, D181 and Q262, are replaced by glutamate and leucine, respectively, in EAK-6.

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**Figure 7. Models of EAK-4, SDF-9, and EAK-6 Function**

(A) Cell autonomous signaling in XXX. A schematic of one XXX cell is shown. EAK proteins function in parallel with AKT-1 and in the same pathway as AKT-2 to promote nondauer development by potentiating DAF-9/CYP27A1 function either directly or indirectly. DAF-9/CYP27A1 synthesizes 3-keto-7(5α)-cholestenoic acid, a ligand that promotes reproductive development by inhibiting the nuclear hormone receptor DAF-12 [91]. DAF-16/FoxO is denoted with dashed lines, since DAF-16A does not appear to be expressed in XXX. It is not known whether other DAF-16/FoxO isoforms or DAF-2/InsR are expressed in XXX.

(B) Nonautonomous signaling from XXX. A schematic of the anterior portion of an animal is shown with the head pointing left. The pharynx is also shown. XXX cells are denoted by red ovals, and DAF-16/FoxO-expressing cells are denoted by green ovals. EAK proteins in the XXX cells generate signals that regulate the synthesis or secretion of a hormone that inhibits DAF-16/FoxO function in other cells.

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tyrosine phosphoproteins, and structure. Detailed analysis of EAK-6 catalytic activity, binding to activation. Resolution of these uncertainties awaits a more local concentration of associated proteins during cascade activation. Resolution of these uncertainties awaits a more detailed analysis of EAK-6 catalytic activity, binding to tyrosine phosphoproteins, and structure.

Bifurcation of DAF-2/InsR Signaling into Dauer and Longevity Outputs

Temporal and spatial specificity of DAF-2/InsR signaling may underlie differential effects on dauer formation and longevity. Inhibition of DAF-2/InsR signaling during early development enhances dauer formation but has no effect on organismal longevity, and inhibition of DAF-2/InsR signaling in early adulthood is sufficient to extend life span [88]. eak-4, sdf-9, and eak-6 promoter and translational fusions are all expressed continuously from late embryogenesis through early adulthood (unpublished data and [62]); thus, spatial regulation of EAK-4, SDF-9, and EAK-6 expression is not likely to explain the normal life span of eak-4, sdf-9, and eak-6 mutants. The specificity of eak-4, sdf-9, and eak-6 expression in XXX suggests that DAF-2/InsR signaling in the XXX cells may have specific dauer regulatory functions that have no impact on organismal longevity. This model is supported by a recent analysis of tissue-specific functions of DAF-16/FoxO indicating that longevity is primarily regulated by intestinal DAF-16/FoxO, whereas dauer arrest is regulated by neuronal DAF-16/FoxO [89]. Similarly, neuronal DAF-2/InsR regulates life span, whereas intestinal DAF-2/InsR controls metabolism [90]. In light of the observation that DAF-16A-GFP is not expressed in XXX (Figure 6), it will be of great interest to determine whether DAF-16A-GFP localizes to the nucleus in eak;akt-1 double mutants and, if so, whether there is tissue-specificity of nuclear localization.

A Model for EAK Protein Function

The data presented in this work are consistent with a model whereby EAK-4, SDF-9, and EAK-6 function in a single complex or pathway at the XXX plasma membrane in parallel with AKT-1 to inhibit dauer formation. Overexpression of the steroid hydroxylase DAF-9/CYP27A1, which is normally expressed in XXX, suppresses dauer arrest in sdf-9 mutants [62], suggesting that DAF-9 functions downstream of or parallel to SDF-9. In XXX, SDF-9 may function with DAF-9/CYP27A1 to promote the synthesis and/or secretion of dafachronic acids, which are high-affinity ligands for the nuclear hormone receptor DAF-12 [62,91]. Therefore, high EAK protein activity may inhibit dauer formation by potentiating DAF-9/CYP27A1 activity, either directly or indirectly (Figure 7A). EAK proteins may also have autonomous inhibitory effects on other cells that express DAF-16/FoxO (Figure 7B). EAK regulation of DAF-16/FoxO probably does not occur through DAF-9/CYP27A1, since daf-9 is epistatic to daf-16 for dauer arrest [64,65].

This work identifies two membrane-associated PTP homologs in the endocrine XXX cells that modulate insulin-like signaling in C. elegans. In view of the striking structural and functional similarities in insulin/IGF signaling among metazoans, further studies of insulin/IGF signaling in C. elegans should contribute to our understanding and management of growth factor dysregulation in human disease.

Materials and Methods

eak mutant isolation, SNP mapping, and sequencing of mutant alleles. akt-1(mg306) animals were mutagenized with ethyl methane-sulfonate using standard procedures [92]. Four P0 animals were placed on each of 100 6-cm NGM agar plates [93] and removed after overnight egglay at 25 °C. F2 dauer were picked to new plates for recovery after visualization under a Nikon SMZ800 dissecting microscope. Recovered dauers were retested for the dauer phenotype at 25 °C. Dauers that bred true were then outcrossed once with N2 (wild-type) animals and subjected to secondary assays. Mutants were tested for suppression by feeding RNAi of daf-3/SMAD4 and daf-16/FoxO as described [56]. Those that were suppressed by daf-16/FoxO RNAi but not by daf-3/SMAD4 RNAi were outcrossed again, and F2 dauer were picked for recovery, single for egglay, and picked to worm lys buffer containing 50 mM KC1, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin, and 60 μg/ml protease K. After incubation at −70 °C for at least 10 min, 60 °C for 1 h, and 95 °C for 15 min, 2.5 μl of single worm lysate was PCR-amplified in 100 mM Tris (pH 8.3), 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin with Taq polymerase (Roche, Basel, Switzerland) using primers flanking the akt-1(mg306) point mutation. akt-1(mg306) animals harbor a C-to-T transition at nucleotide 31730 of cosmid C12D8 that creates a TaqI restriction fragment length polymorphism. Mutants for which most or all F2 dauers were homozygous for akt-1(mg306) were considered true Eak mutants and were analyzed further. All mutants were outcrossed at least four times prior to detailed phenotyping.

SNP mapping was performed essentially as described [67]. Mutants were mated with the Hawaiian C. elegans isolate CB4856, and single F1 cross-progeny were allowed to lay eggs overnight at 25 °C. Individual F1 dauers were picked for recovery, subjected to 100 μl of worm lys buffer. Lysates were incubated as described above. Then 2.5 μl of single worm lysate was PCR-amplified as described above using genomic primers (Invitrogen, Carlsbad, California, United States) spanning SNPs between CB4856 and the wild-type N2 strain. SNPs were detected by restriction enzyme digestion or sequencing of PCR products.

Genomic fragments corresponding to exons and predicted splice junctions of eak-4, sdf-9, and eak-6 (WormBase Web site, http://www.wormbase.org, release WS120, March 1, 2004) were amplified by PCR and purified using the QiAquick PCR Purification Kit (Qiagen, Valencia, California, United States). Mutations were confirmed by sequencing both strands of DNA.

Dauer assays. Two or three gravid animals were picked to individual 6-cm NGM plates, allowed to lay eggs for several hours, and removed. Plates were shifted to the assay temperature, and dauers were scored 48 to 60 h thereafter. All assays of 27 °C dauer arrest were scored in blinded fashion.

RNAi. Feeding RNAi was performed as described [94], with minor modifications. Six-well plates containing NGM agar + 5 mM IPTG were spotted with 400 μl of overnight cultures of E. coli HT115 harboring double-stranded RNAi expression plasmid L4440 [95] or L4440 containing daf-12(nhr-); daf-16(FoxO), specific inserts. Overnight cultures were grown in 2XYT medium containing 50 μg/ml carbamycin. After overnight incubation of RNAi plates at room temperature (to allow IPTG induction of double-stranded RNA synthesis), two L4 animals were picked to each well, grown at 15 °C, removed after egglay, and shifted to 27 °C. Dauers were scored approximately 60 h thereafter. Life span assays. L4 animals were picked to seeded NGM agar plates containing 0.1 mg/ml 5-fluorodeoxyuridine (ten to 20 animals per plate), incubated at 25 °C, and scored every 1 to 2 d for vitality as

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described [90]. Animals that did not respond to prodding were scored as dead and removed.

cDNA isolation. *C. elegans* total RNA was isolated as described [96]. cDNA was amplified from total RNA using the SuperScript III RT-PCR Kit (Invitrogen). The 5' and 3' cDNA ends were identified using a 2/5' RACE kit (Roche). PCR products were cloned into pCR4-TOPO (Invitrogen), and plasmid inserts were sequenced using primers flanking the cloned insert.

RT-PCR of wild-type *C. elegans* total RNA revealed the presence of two alternatively spliced *eak-6* mRNAs differing by the absence or presence of a single exon. The presence of the exon in the long cDNA isoform, *eak-6L*, results in a 17-amino-acid-in-frame insertion C-terminal to the PTP domain of EAK-6 (Figure 2A). cDNA analysis also revealed a GeneFinder missprediction in WormBase (WormBase Web site, http://www.wormbase.org; release WS120, March 1, 2004) of the exon/intron boundary between exons 3 and 4 (unpublished data). The 5' and 3' ends of a 5' cDNA and 13 nucleotides upstream of the translation initiation codon and a 3' cDNA end 303 nucleotides downstream of the translation termination codon but did not identify a trans-spliced SL1 leader (unpublished data).

**GFP and RFP reporter constructs.** For promoter fusion constructs, promoter fragments were generated by amplifying 5' upstream sequences between the putative translational start codon and the nearest boundary of the gene immediately upstream. The following promoter fragments were amplified: *eak-6p*: nucleotides 13809 to 14696 [99] (numbering corresponds to contained sequences obtained from the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/]); *akt-1p*: nucleotides 10417 to 10170 of YAC Y44A6D; *eak-6p*: nucleotides 4381 to 5426 of cosmid F10G8. PCR primers were tagged with BglII linkers. Promoter fragments were purified using the Qiapquick PCR Purification Kit (Qiagen) and resubcloned into BglII-digested GFP reporter plasmid pPD95.75 (gift from Ho Yi Mak) to generate *eak-6p-GFP*. Fragments were confirmed by restriction digestion. Plasmids were purified using Qiagen columns.

Protein fusion constructs were made using overlapping extension PCR [97,98]. PCR fragments encompassing the putative promoter and open reading frame upstream to but not including the translation termination codon were fused to a PCR fragment containing GFP and the unc-54 3' untranslated region (amplified from the *C. elegans* promoter expression vector pPD95.75, a gift from Andrew Fire). The following gene-specific fragments were amplified for protein fusions: *EAK-6*: nucleotides 12780 to 14696 of cosmid F10G8. PCR primers were tagged with BglII linkers. Promoter fragments were subcloned into BglII-digested RFP [75] reporter plasmid pPD95.75mRFP (a gift from Ho Yi Mak) to generate *eak-6p-RFP*. Fragment orientation was confirmed by restriction digestion. Plasmids were purified using Qiagen columns.

To generate transgenic animals, fusion constructs were coinjected with 1.3–3ng/ml *pha-1::rescueing plasmid pBX* into *pha-1(e2122)* mutant animals, and transgenic animals were selected and maintained by co-injection marker [100]. Cosmids were prepared using the Qiaprep Miniprep Kit (Qiagen) and injected at concentrations of approximately 1 to 10 ng/ml. Single gene rescue experiments were performed with PCR fragments amplified from genomic DNA that contained predicted promoter, open reading frame, and 3' UTR sequences for *F53B2.5* (*eak-4*) and *F10G8.4* (*eak-6*). Approximately 100 ng/ml of purified PCR product was co-injected with 50 ng/ml SUR-5::GFP into *eak-4(mg326)/akt-1(mg306)* or *eak-6(mg329)/akt-1(mg306)* mutant animals, and transgenic animals were assayed for rescue of dauer arrest at 25°C. A construct containing the *akt-1* gene under the control of the *eak-6* promoter was made using overlap extension PCR [97,98]. An *eak-4* promoter fragment (nucleotides 13809 to 14696 of cosmid F53B2) was fused 5' to the *akt-1* reading frame and 3' untranslated region (nucleotides 30791 to 34802 of cosmid C12D8) to create *eak-4p-akt-1*. Approximately 100 ng/ml of *eak-4p-akt-1* was co-injected with 100 ng/ml SUR-5::GFP into *eak-4(mg326)/akt-1(mg306)* mutant animals, and transgenic (GFP*) animals from three independent lines were assayed for rescue of dauer arrest formation at 25°C. Rescue phenotypes were compared with those of nontransgenic (GFP–) siblings.

**Supporting Information**

**Figure S1.** Pharyngeal Morphology and Alae in akt-1(mg306), *eak*, and eak-1*–*Dauers *daf-2(e1370), akt-1(mg306), eak-6(mg329),* and *eak-4(mg348)/akt-1(mg306)* animals were grown at 25°C (*daf-2* and *eak-akt-1*) or 27°C (*akt-1* and *eak-akt-1*). Animals were grown at 25°C. Dauer morphology comparable to *eak-6* and *eak-akt-1* dauer mutants do not enhance the dauer arrest phenotype of an *akt-1* loss-of-function mutation. All error bars indicate standard deviation. All experiments were performed twice. Refer to Table S1 for numbers of animals scored.

**Figure S2.** *eak* Interactions with Mutants in the DAF-2/InsR Signaling Pathway

(A) *eak* mutants enhance the dauer arrest phenotype of *age-1/p3K and pdk-1* loss-of-function mutants.

(B) *eak* mutants do not enhance the dauer arrest phenotype of an *akt-2* loss-of-function mutant. All error bars indicate standard deviation. All experiments were performed twice. Refer to Table S1 for numbers of animals scored.

**Figure S3.** Epistasis Analysis of *eak* Mutants with Dauer-Defective *daf-2*/*daf-1* Pathway Mutants

(A) *eak-1* 25°C dauer arrest phenotypes are suppressed by a mutation in *daf-16/FoxO*.

(B) *eak* 27°C dauer arrest phenotypes are suppressed by a weak loss-of-function mutation in *daf-18/PTEN*.

(C) *eak* 27°C dauer arrest phenotypes are suppressed by a gain-of-function mutation in *akt-1*. All error bars indicate standard deviation. All experiments were performed twice. Refer to Table S1 for numbers of animals scored.

**Figure S4.** akt-1, *eak*, and eak-1*–*Mutants Have Normal Life Spans at 25°C

This experiment was performed twice. Refer to Table S1 for numbers of animals scored.

**Figure S5.** eak-4 (mg348) Is a Null Allele

*eak-4(mg348)* males were mated with *BC1216 (sd221/hT1; +/hT1)* hermaphrodites, and *F1* cross-progeny were scored for dauer arrest at 27°C, *sd21* is a deficiency on chromosome IV that deletes *eak-4*. (A) *F1* cross-progeny from *eak-4(mg348) X BC1216 do not show enhanced dauer arrest at 27°C compared to *eak-4(mg348)*. (B) Comparison of observed to expected embryonic lethality among progeny confirms the identity of the *BC1216* strain.

**Figure S6.** Plasma Membrane Localization of SDF-9::GFP Does Not Require Wild-type DAF-2/InsR Function

*daf-2* wild-type and *daf-2(e1370) L1* animals harboring an SDF-9::GFP
translational fusion construct were grown at 25 °C and analyzed using fluorescence microscopy.

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Table S1. Numbers of Animals Assayed

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Author contributions. PJH and GR conceived and designed the experiments. PJH and JX performed the experiments. PJH and GR analyzed the data. PJH and GR wrote the paper.

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