Effects of *Caenorhabditis elegans* sgk-1 mutations on lifespan, stress resistance, and DAF-16/FoxO regulation

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**Summary**

The AGC family serine–threonine kinases Akt and Sgk are similar in primary amino acid sequence and in vitro substrate specificity, and both kinases are thought to directly phosphorylate and inhibit FoxO transcription factors. In the nematode *Caenorhabditis elegans*, it is well established that Akt-1 controls dauer arrest and lifespan by regulating the subcellular localization of the FoxO transcription factor DAF-16. SGK-1 is thought to act similarly to Akt-1 in lifespan control by phosphorylating and inhibiting the nuclear translocation of DAF-16/FoxO. Using sgk-1 null and gain-of-function mutants, we now provide multiple lines of evidence indicating that AKT-1 and SGK-1 influence *C. elegans* lifespan, stress resistance, and DAF-16/FoxO activity in fundamentally different ways. Whereas AKT-1 shortens lifespan, SGK-1 promotes longevity in a DAF-16/FoxO-dependent manner. In contrast to AKT-1, which reduces resistance to multiple stresses, SGK-1 promotes resistance to oxidative stress and ultraviolet radiation but inhibits thermotolerance. Analysis of several DAF-16/FoxO target genes that are repressed by AKT-1 reveals that SGK-1 represses a subset of these genes while having little influence on the expression of others. Accordingly, unlike AKT-1, which promotes the cytoplasmic sequestration of DAF-16/FoxO, SGK-1 does not influence DAF-16/FoxO subcellular localization. Thus, in spite of their similar in vitro substrate specificities, Akt and Sgk influence longevity, stress resistance, and FoxO activity through distinct mechanisms in vivo. Our findings highlight the need for a re-evaluation of current paradigms of FoxO regulation by Sgk.

**Key words:** aging; *C. elegans*; FoxO; insulin-like growth factor signaling; lifespan; Sgk.

**Introduction**

Akt/protein kinase B (PKB) and Sgk are two highly related members of the AGC family of serine–threonine kinases that act in cellular signaling pathways to modulate survival, growth, proliferation, metabolism, and other processes (Pearce et al., 2010). Akt/PKB has evolutionarily conserved functions in the control of development, growth, metabolism, cell survival, and longevity, and dysregulation of Akt/PKB contributes to the pathogenesis of common human diseases such as cancer and type 2 diabetes (Franke, 2008).

The mechanism of Akt/PKB activation is well established. In response to growth factors, Akt/PKB is activated in a phosphoinositide 3-kinase (PI3K)-dependent manner by phosphorylation at two critical regulatory sites: T308 within its kinase domain and S473 within a C-terminal hydrophobic motif (Alessi et al., 1996a). The 3-phosphoinositide-dependent kinase PDK1 phosphorylates Akt/PKB at T308 (Alessi et al., 1997; Stephens et al., 1998), and members of the PI3K-related kinase (PIKK) family such as TOR complex 2 phosphorylate Akt/PKB at S473 (Feng et al., 2004; Sarbassov et al., 2005; Viniegra et al., 2005).

Activated Akt/PKB phosphorylates several substrates in vivo at sites that lie within RxRxxS/T motifs (Alessi et al., 1996b; Manning & Cantley, 2007). Among these substrates are the FoxO family of transcription factors that control development, metabolism, growth, and aging (Accili & Arden, 2004). Akt/PKB-dependent phosphorylation of FoxO at three conserved RxRxxS/T motifs inhibits FoxO activity by promoting its export from the nucleus and sequestration in the cytoplasm (Brunet et al., 1999). FoxO is a critical substrate of Akt/PKB in vivo, as its inhibition in mice with reduced hepatic Akt/PKB signaling impairs metabolic homeostasis (Dong et al., 2008), and a null mutation in daf-16, which encodes the sole FoxO transcription factor in the nematode *Caenorhabditis elegans*, suppresses the dauer-constitutive and lifespan extension phenotypes of animals with reduced Akt/PKB activity (Paradis & Ruvkun, 1998; Kwon et al., 2010). Thus, Akt/PKB has an evolutionarily conserved function as a direct inhibitor of FoxO transcription factors.

In mammalian cell culture, Sgk inhibits FoxO3 activity (Liu et al., 2000; Brunet et al., 2001), and in *C. elegans*, SGK-1 is thought to limit lifespan by inhibiting DAF-16/FoxO activity (Hertweck et al., 2004). Taken together with the known role of Akt/PKB in FoxO regulation, these studies have established a paradigm whereby Akt/PKB and Sgk are thought to act via similar mechanisms to inhibit FoxO activity (Fielenbach & Antebi, 2008; Bruhn et al., 2010; Pearce et al., 2010).
We and others recently reported that in contrast to the lifespan extension phenotype observed after RNAi knockdown of sgk-1 (Hertweck et al., 2004), sgk-1 null mutations shorten C. elegans lifespan (Soukas et al., 2009; Alam et al., 2010; Kwon et al., 2010). This phenotype is the opposite of that observed for akt-1 null mutations (Soukas et al., 2009; Alam et al., 2010; Kwon et al., 2010) and is inconsistent with prevailing models implicating Sgk as a FoxO inhibitor. In light of these results, we have performed a detailed phenotypic analysis of sgk-1 null and gain-of-function mutants. Our results indicate that in C. elegans, Akt/PKB and Sgk influence lifespan, stress resistance, and FoxO transcription factor activity through distinct mechanisms. These surprising findings call into question current paradigms of FoxO regulation by Sgk and reveal that the interaction between Sgk and FoxO transcription factors may be more complex than previously appreciated.

**Results**

**Effects of sgk-1 mutations on lifespan**

We and others have shown that the sgk-1(mg455) mutation shortens lifespan (Soukas et al., 2009; Alam et al., 2010). The mg455 allele is a nonsense mutation that is predicted to result in truncation of SGK-1 within its kinase domain (Soukas et al., 2009); therefore, this is likely to be a null mutation. A third group has shown that the sgk-1(ok538) deletion mutation, which is predicted to remove half of the SGK-1 kinase domain and is also probably a null mutation (Hertweck et al., 2004), also reduces lifespan (Kwon et al., 2010). We confirmed these results by measuring the lifespans of both sgk-1(ok538) and sgk-1(mg455) null mutants in the same assay (Fig. 1B and Table S1). sgk-1(ok538)(hereafter referred to as ‘null #1’) and sgk-1(mg455)(hereafter referred to as ‘null #2’) each shorten mean lifespan by at least 27.5% and median lifespan by at least 19.0% and 33.3%, respectively ($P<0.0001$ by the log-rank test). The observation that two outcrossed strains harboring independently isolated sgk-1 null mutations both have short lifespans compared with wild-type animals strongly suggests that these short lifespan phenotypes are a consequence of reduced SGK-1 activity. These results contrast with the reported lifespan extension induced by sgk-1 RNAi (Hertweck et al., 2004) and are consistent with a model whereby SGK-1 promotes longevity.

One possible explanation for the discrepancy between the lifespans of animals harboring sgk-1 loss-of-function mutations and animals subjected to sgk-1 RNAi is that strong loss-of-function mutations could cause developmental abnormalities that shorten adult lifespan by reducing general fitness; such abnormalities can be avoided by initiating RNAi during late larval or early adult stages. To address this possibility, we assayed the lifespans of animals harboring the sgk-1(ft15) gain-of-function mutation.

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**Fig. 1** Effects of sgk-1 mutations on lifespan. (A) Schematic of the sgk-1 genomic locus (not to scale). Locations of the ft15 missense gain-of-function, ok538 deletion, and mg455 nonsense mutations are shown. Exons encoding the kinase domain are colored red. (B) Lifespans of sgk-1 mutants ft15 (gf), ok538 (null #1), and mg455 (null #2). (C) Effect of the daf-16(mu86) null mutation on the lifespans of sgk-1(gf) animals. (D) Effect of sgk-1(gf) on the lifespan of akt-1(mg306) null mutant animals. (E) Effect of sgk-1(gf) on the lifespan of hcf-1(pk924) null mutant animals. Raw data and statistics are presented in Table S1.
sgk-1(fft15) emerged from a genetic screen for suppressors of the developmental delay phenotype of animals harboring a loss-of-function mutation in lpo-6/rist-1, which encodes the C. elegans ortholog of the TOR complex 2 component Rictor (Jones et al., 2009; Soukas et al., 2009). sgk-1(fft15) suppresses both the developmental delay and small body size phenotypes of lpo-6/rist-1 loss-of-function mutants, and this suppression is abrogated by sgk-1 RNAi (Jones et al., 2009). Taken together with the observations that lpo-6/rist-1 and sgk-1 act in the same genetic pathway (Jones et al., 2009; Soukas et al., 2009) and mammalian TOR complex 2 activates Sgk by promoting its phosphorylation (Garcia-Martinez & Alessi, 2008), these data strongly suggest that sgk-1(fft15) is a gain-of-function allele.

We reasoned that if sgk-1 null mutants are short-lived because SGK-1 plays a role in promoting longevity, then animals harboring sgk-1(fft15) (hereafter referred to as ‘sgk-1(gf)’) should live longer than animals with wild-type sgk-1. However, if sgk-1 null mutants are short-lived because they are sick, then sgk-1(gf) animals would not be expected to live long. sgk-1(gf) animals consistently lived ~15–20% longer than nonsibling wild-type animals (Table S1B). When siblings harboring wild-type sgk-1 were used as controls, sgk-1(gf) animals exhibited a modest but statistically significant extension in median and mean lifespan in eight of ten experimental trials (Fig. 1B–E and Tables S1A,C). In Fig. 1B, sgk-1(gf) increased mean and median lifespan by 17.5% and 9.5%, respectively, compared with wild-type siblings (P = 0.0008). This lifespan extension was suppressed by a null mutation in daf-16/FoxO (Fig. 1C and Table S1A).

In C. elegans, DAF-16/FoxO activity is regulated through at least two mechanisms. Phosphorylation of DAF-16/FoxO by kinases such as AKT-1 or HCF-1 to influence lifespan is not clear. It is already activated. As the thermotolerance of Escherichia coli HB101-derived HT115 strain used in experiments demonstrating that sgk-1 RNAi extends lifespan (Hertweck et al., 2004) differs from the OP50 strain used in our experiments (Fig. 1B–E), we sought to determine the influence of E. coli strain differences on the lifespans of sgk-1 mutants. Therefore, we assayed the lifespans of sgk-1(null) and sgk-1(gf) mutants grown on HT115. As observed in experiments using OP50 as a food source, sgk-1(null) shortened and sgk-1(gf) extended lifespan in animals feeding on either HT115 or HB101 (Fig. 1F and Table S1C). Therefore, the longevity activity of SGK-1 is not significantly influenced by differences between E. coli OP50 and HT115/ HB101.

Taken together, these results suggest that in contrast to existing paradigms of FoxO inhibition by Sgk (Brunet et al., 2001; Hertweck et al., 2004), SGK-1 promotes C. elegans longevity in a DAF-16/FoxO-dependent manner.

**Effects of sgk-1 mutations on dauer arrest**

Because DAF-16/FoxO promotes developmental arrest in the dauer larval stage in animals with reduced DAF-2 insulin-like signaling (Vowles & Thomas, 1992; Gottlieb & Ruvkun, 1994), we tested the effect of sgk-1(null) and sgk-1(gf) on dauer arrest. In agreement with a previous report (Hertweck et al., 2004), neither sgk-1(null) nor sgk-1(gf) had significant effects on dauer arrest at 27°C (Table 1A). Although a significant percentage of sgk-1(null) animals arrested during larval development (Table 1A), analysis of these animals using Nomarski microscopy revealed no evidence of dauer alae or pharyngeal constriction (Fig. S3), indicating that these animals were nondauer larvae. In contrast and as previously reported (Hu et al., 2006), an akt-1 null mutation had a strongly penetrant DAF-16/FoxO-dependent dauer-constitutive phenotype under the same assay conditions. Neither sgk-1(null) nor sgk-1(gf) significantly influenced the dauer-constitutive phenotype of daf-2(e1368) (Table 1B). Therefore, SGK-1 does not function in dauer regulation.

**Effects of sgk-1 mutations on stress resistance**

In light of our observations on the effects of sgk-1 mutations on lifespan (Fig. 1), we tested sgk-1(null) and sgk-1(gf) for their sensitivity to oxidative stress, ultraviolet radiation (UVR), and heat. akt-1 null mutants were slightly more resistant to hydrogen peroxide than wild-type animals, although this difference was only statistically significant in one of four assays (Fig. 2A,B and Table S2). akt-1 null mutants were significantly more resistant to UVR and heat than wild-type animals (Fig. 2C–F and Tables S3 and S4). In contrast, both sgk-1 null mutants were more sensitive to hydrogen peroxide (statistically significant in 2 of 3 trials for each mutant) and UVR (statistically significant in 3 of 3 trials) than wild-type animals (Fig. 2A,C and Tables S3–4), consistent with their short lifespans (Fig. 1B and Table S1). sgk-1(gf) did not significantly influence sensitivity to any of the three stressors tested (Fig. 2B,D,F and Tables S3–5).

Both sgk-1 null mutations enhanced thermotolerance to at least the same extent that an akt-1 null mutation did (Fig. 2E and Table S4). This result is consistent with a previous report examining thermotolerance of the sgk-1(ok538) null mutant (Hertweck et al., 2004). Taken together with our observation that the sgk-1(gf) mutation extends lifespan (Fig. 1 and Table S1), this enhanced thermotolerance phenotype of sgk-1 null mutants strengthens the argument that the short-lived phenotype of sgk-1 null mutants is not simply a consequence of frailty secondary to developmental abnormalities. In contrast to AKT-1, which promotes general sensitivity to environmental stress, SGK-1 is protective against oxidative stress and UVR but enhances sensitivity to heat.

As the thermotolerance of sgk-1(ok538) is thought to require DAF-16/FoxO (Hertweck et al., 2004), we tested the effect of a daf-16 null mutation on the thermotolerance of both sgk-1 null mutants. Surprisingly, daf-16 null mutation did not significantly influence the thermotolerance of either sgk-1 null mutant (Fig. 2G and Table S4). Therefore, our results suggest that SGK-1 promotes sensitivity to heat in a DAF-16/FoxO-independent manner.

**Effects of sgk-1 mutations on DAF-16A:GFP subcellular localization**

As our lifespan data are consistent with a model in which SGK-1 promotes longevity by activating DAF-16/FoxO, we sought to determine the influence of sgk-1 mutations on the subcellular localization of
In vivo activity data suggest that unlike AKT-1, SGK-1 does not control DAF-16/FoxO influence on the nucleocytoplasmic distribution of DAF-16A::GFP. These data indicate that AKT-1 and SGK-1 control DAF-16/FoxO in a DAF-16/FoxO-dependent manner in the context of akt-1 null mutation (Fig. 3B and Table S6). Thus, null mutations in sgk-1 and akt-1 have opposite effects on sod-3 expression. Neither sgk-1 null mutation nor sgk-1(gf) reproducibly influenced the expression of nnt-1 and sip-1 (Fig. 3C, D and Table S6). Expression of both sod-3 and mtl-1 was elevated in a DAF-16/FoxO-dependent manner in the context of sgk-1 null mutation in five of six trials (Fig. 3E, F and Table S6), suggesting that SGK-1 and AKT-1 may act similarly to regulate these two DAF-16/FoxO target genes.

In aggregate, these data indicate that AKT-1 and SGK-1 control DAF-16/FoxO target gene expression through distinct mechanisms. The heterogeneity of the influence of sgk-1 mutations on DAF-16/FoxO target gene expression suggests that the molecular basis for SGK-1 regulation of DAF-16/FoxO activity is significantly more complex than has been appreciated.

**Discussion**

Akt/PKB inhibits FoxO transcription factors via a well-established and evolutionarily conserved mechanism involving phosphorylation of FoxO at three sites that lie within conserved RxRxxS/T motifs (Manning & Cantley, 2007; Franke, 2008). Based on both its similarity in primary structure (Webster et al., 1993) and substrate specificity (Kobayashi &
Cohen, 1999) to Akt/PKB as well as data from mammalian cell culture (Liu et al., 2000; Brunet et al., 2001) and C. elegans (Hertweck et al., 2004), Sgk is also thought to inhibit FoxO by promoting its phosphorylation at RxRxxS/T motifs. Our data challenge this model of FoxO regulation by Sgk and support the notion that in C. elegans, Akt/PKB and Sgk regulate FoxO activity in fundamentally different ways. Our conclusions are at odds with those of the only study in the literature that has focused on Sgk action in C. elegans lifespan control and FoxO regulation (Hertweck et al., 2004). This study showed that sgk-1 RNAi extends lifespan in a DAF-16/FoxO-dependent manner. One possible explanation for this discrepancy is that the E. coli strain used for RNAi (the HB101-related strain HT115) is different from the standard strain used for growth and maintenance of C. elegans (the E. coli B-related OP50) that we used in our experiments. Indeed, wild-type C. elegans grown on HT115 live nearly 20% longer than wild-type animals grown on OP50 (Maier et al., 2010). However, we have shown that sgk-1(null) and sgk-1(gf) animals are respectively short-lived and long-lived when cultured on E. coli OP50, HT115, or HB101 (Fig. 1 and Table S1), indicating that the lifespan phenotypes of sgk-1(null) and sgk-1(gf) are not significantly influenced by differences between OP50 and HT115/HB101 per se.

We did confirm the previously reported finding that sgk-1 null mutant animals are thermotolerant compared with wild-type animals (Hertweck et al., 2004). This suggests that sgk-1 null mutant animals are not short-lived due to general frailty or sickness, as such animals would be expected to be generally hypersensitive to environmental stresses. Intriguingly, daf-16/FoxO was not required for thermotolerance in sgk-1(null) animals, suggesting that although AKT-1 and SGK-1 both promote thermosensitivity, they likely do so through distinct mechanisms. Our results dissociate thermotolerance from longevity and suggest that divergent molecular pathways act downstream of SGK-1 to influence lifespan and responses to increased ambient temperature.

Our results also contrast with a detailed analysis of mammalian FoxO3 regulation demonstrating that both Sgk and Akt/PKB can inhibit FoxO3 activity in cell culture by promoting the phosphorylation of all three RxRxxS/T motifs.
conserved sites that lie within RxRxxS/T motifs (Brunet et al., 2001). This discrepancy may be due in part to differences in experimental context; these experiments were performed in cell culture, where growth factors are frequently added in excess of physiologic concentrations, and overexpressed proteins may exhibit activities that are not discernible when they are expressed at endogenous levels. The effect of Sgk knockdown or deletion on the activity of endogenous FoxO transcription factors has not been investigated in mammals. Although it is conceivable that Sgk regulates FoxO activity through distinct mechanisms in mammals and C. elegans, this is unlikely in light of the conservation of mechanisms of FoxO regulation by insulin-like growth factor signaling pathways (Kenyon, 2010).

Fig. 3 Effects of sgk-1 mutations on DAF-16A::GFP subcellular localization and DAF-16/FoxO target gene expression. (A) Subcellular localization of DAF-16A::GFP in akt-1 and sgk-1 mutants. Nuclear localization is increased by akt-1(mg306) null mutation (two-way ANOVA, $F = 14.47, P < 0.0001$), but not by sgk-1(ok538) null mutation ($F = 1.825, P = 0.1733$) or by sgk-1(ft15) gain-of-function mutation ($F = 0.869, P = 0.5037$). Error bars represent SEM for 3 cohorts of 20–30 animals per genotype imaged separately. All animals also harbored the daf-16(mu86) null allele, so no endogenous DAF-16/FoxO is present. Representative images are shown in Figure S2. Raw data and statistics are presented in Table S5. (B,F) Representative experiments measuring sod-3 (B), nnt-1 (C), sip-1 (D), dod-3 (E), and mtl-1 (F) transcript levels using quantitative RT-PCR on total RNA isolated from young adult animals. Values are normalized to expression levels in wild-type animals. Columns represent mean ± SEM of three technical replicates. Raw data and statistics for biological replicates are summarized in Table S6. (G) Summary of statistically significant gene expression changes ($P < 0.05$; Table S6; unpaired two-tailed t-test with Welch’s correction) in akt-1 and sgk-1 mutants and their dependence on DAF-16/FoxO. The asterisk indicates that dod-3 expression was increased significantly in eight of twelve trials. The number sign indicates that daf-16(null) significantly reduced expression of dod-3 and mtl-1 in sgk-1(null) mutants in five of six trials. See Table S6 for details.
Although the increased lifespan phenotypes caused by akt-1 null mutation and the sgk-1(gf) both require daf-16/FoxO (Fig. 1C and Table S1), the expression of DAF-16/FoxO target genes was influenced by these two mutations in starkly discordant ways (Fig. 3B-F). Whereas the expression of five DAF-16/FoxO target genes is induced in a DAF-16/FoxO-dependent manner in akt-1 null mutants, sgk-1(null) and sgk-1(gf) mutations had distinct and varying influences on the expression of specific DAF-16/FoxO target genes. This difference is likely a reflection of underlying differences in the molecular basis for DAF-16/FoxO regulation by AKT-1 and SGK-1.

These observations suggest that the underlying mechanisms of lifespan control by AKT-1 and SGK-1 are fundamentally different. In contrast to AKT-1, which inhibits DAF-16/FoxO by promoting its nuclear export and cytoplasmic retention (Hertweck et al., 2004; Zhang et al., 2008; Alam et al., 2010; Dumas et al., 2010), SGK-1 may promote longevity by regulating other proteins that functionally and/or physically interact with DAF-16/FoxO, such as SKN-1 (Tullet et al., 2008), HSF-1 (Hsu et al., 2003), or HCF-1 (Li et al., 2008). In this regard, DAF-16/FoxO may play a permissive role in lifespan control by SGK-1 without being directly regulated by SGK-1. Alternatively, SGK-1 may directly regulate DAF-16/FoxO activity in a small number of cells, which in turn could control lifespan by influencing other cells in a DAF-16/FoxO-independent manner.

In summary, we have shown that the AGC kinase family members Akt/PKB and Sgk control C. elegans lifespan and stress resistance in fundamentally different ways, and they likely influence FoxO transcription factor activity through distinct mechanisms in vivo. Our findings challenge existing paradigms of FoxO regulation by Sgk and should engender a reassessment of the role of Sgk in FoxO transcription factor regulation.

Experimental procedures

Strains and reagents

The following strains were used: N2 Bristol (wild-type), sgk-1(ft15) (Jones et al., 2009), akt-1(mg306) (Hu et al., 2006), sgk-1(ok538) (Hertweck et al., 2004), sgk-1(md455) (Soukas et al., 2009), daf-16 (mu86) (Lin et al., 1997), hcf-1(pk924) (Li et al., 2008), and TJ356 (zts56) (Henderson & Johnson, 2001). Because sgk-1(ft15) was isolated after mutagenesis of animals harboring the linked akt-2(tm812) mutation (Jones et al., 2009), we confirmed the absence of akt-2(tm812) prior to further analysis. Throughout the manuscript, sgk-1(ft15) is referred to as ‘sgk-1(gf)’, akt-1(mg306) as ‘akt-1(null)’, sgk-1(ok538) as ‘sgk-1(null) #1’, and sgk-1(md455) as ‘sgk-1(null) #2’. sgk-1 mutant strains were outcrossed with N2 at least twice in the seven outcross period to genotype specific. Wild-type siblings of sgk-1(ft15) from the seventh outcross with N2 Bristol were used as controls for phenotypic comparison to sgk-1(ft15). This sibling is labeled ‘wild-type’ in all figures, in contrast to ‘N2 wild-type’. Double and triple mutants were generated using standard genetic techniques. For maintenance and all assays, animals were grown in Percival I-30NL or I-36NL incubators (Percival Scientific, Inc., Perry, IA, USA).

Lifespan assays

Lifespan assays were performed at 20°C as described (Alam et al., 2010; Dumas et al., 2010). Briefly, animals were treated with alkaline hypochlorite and grown for at least three generations at 15°C. A synchronized egg lay was then performed to yield animals for the lifespan assay. These were grown at 20°C until the L4 larval stage, at which time they were picked to separate plates and grown until they were day 2 adults. They were then transferred to NGM plates (10–15 animals per plate) containing 25 µg/mL (100 µM) 5-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/mL nystatin (Sigma-Aldrich) and seeded with 20 x concentrated OP50. Animals were incubated at 20°C and scored every 1–2 days. Animals that were not moving, did not respond to prodding, and did not exhibit pharyngeal pumping were scored as dead and removed. Animals that died due to desiccation on the side of the plate, a compromise in vulval integrity, or bagging were censored. Statistical significance was assessed using the standard chi-square-based log-rank test in GRAPHPAD Prism (GraphPad Software, La Jolla, CA, USA).

Dauer assays

Dauer assays were performed at 25°C or 27°C as previously described (Hu et al., 2006). Briefly, animals were synchronized in a 4- to 6-h egg lay and grown at 25° or 27°C on NGM plates. Dauers were scored when wild-type animals were gravid adults and daf-2(e1368) or akt-1 (mg306) mutant animals were arrested as dauers (approximately 60–84 h after egg lay). sgk-1 null mutant animals were plated twelve hours prior to other strains to compensate for developmental delay. Plates were observed for two additional days after initial scoring to account for possible dauer arrest in animals with severe developmental delay.

Stress resistance assays

Animals were grown at 20°C for 48 hours after a 4- to 6-h egg lay until most animals were L4 larvae. sgk-1(null) animals were grown starting 12 h earlier than other strains for L4 synchronization due to developmental delay (Hertweck et al., 2004; Jones et al., 2009; Soukas et al., 2009). Young adults, L3 larvae, and males were removed by suction. Cohorts were sufficiently large to allow for thermotolerance, oxidative stress, and UV assays to be performed in parallel. All assays were performed in triplicate.

For oxidative stress assays, L4 larvae were transferred to fresh seeded NGM plates, grown for an additional 18 h, washed two or three times with M9 buffer, and diluted to a concentration of ~50 animals ml−1 of M9. 0.5 ml of animals was dispensed to Eppendorf tubes and rocketed for ~20 min to allow animals to digest E. coli. Four tubes were used per genotype per concentration of H2O2. 0.5 ml of H2O2 dissolved in M9 was then added to each tube to the final concentration, followed by rocking for 2 h protected from light. The H2O2 solution was then removed, and the animals were washed with M9. Animals were then pipetted back onto fresh NGM plates and scored after an 18-h recovery period at 20°C. Two-way ANOVA was conducted using GraphPad Prism, with survival of animals on each plate as the dependent variable and H2O2 dose and genotype as independent variables.

UV stress assays were performed as described (Wolff et al., 2006). Briefly, animals were transferred to plates containing 25 µg ml−1 FUDR on day 1 of adulthood. After four days, they were transferred to plates lacking bacteria and irradiated with 1200 J m−2 UV-C using a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA, USA). They were then transferred onto NGM plates with food and scored daily for survival. Statistical significance was assessed using the standard chi-square-based log-rank test.

Thermotolerance assays were performed essentially as described (Kwon et al., 2010). Briefly, L4 larvae were transferred to fresh seeded NGM plates and placed on an incubator set at either 25°C or 27°C. Dauer were scored every 2 days for a period of 8 days. Statistical significance was assessed using the standard chi-square-based log-rank test.
NGM plates (~20 per plate) and then grown for an additional 18 h prior to shifting them to an incubator set at 35°C. Four plates were used per genotype per time point. At each time point, plates to be scored were removed and incubated further for 18 h at 20°C, after which living and dead animals were scored. Two-way ANOVA was conducted using GRAPHPAD PRISM, with survival of animals on each plate as the dependent variable and time at 35°C and genotype as independent variables.

DAF-16A::GFP localization assays

Animals were mounted onto slides in M9 with 10 mM sodium azide. Approximately ten young adults were picked to each slide, and the anterior segment of each animal was imaged within five minutes after mounting. Images were scored according to the criteria shown in Figure S1. Both imaging and scoring were performed in a blinded manner. Images were scored according to the criteria shown in Figure S1. Both imaging and scoring were performed in a blinded manner. Two-way ANOVA was used to test statistical significance in GRAPHPAD PRISM.

Quantitative RT–PCR

Animals from a 4.5-h egg lay were grown at 20°C for 48 h until most animals were L4 larvae. sgk-1(null) animals were grown starting 12 h earlier than other strains for L4 synchronization due to developmental delay (Hertweck et al., 2004; Jones et al., 2009; Soukas et al., 2009). Young adults and L3 larvae were removed by suction, and the remaining animals were grown for an additional 12 h. Total RNA was isolated from 600–1000 young adults per strain per biological replicate using TRIzol (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy Kit (QIAGEN Inc., Valencia, CA, USA). cDNA was synthesized using a Superscript III Reverse Transcriptase Kit (Invitrogen). SYBR Green (Applied Biosystems, Warrington, UK) Real-Time PCR was then performed using primers corresponding to the DAF-16/FoxO target genes sod-3, nnt-1, sip-1, dod-3, and mtl-1. act-1 was used as an internal control. Quantitative PCR primer sequences are listed in Table S7. Statistical analysis was performed in GraphPad Prism by unpaired two-tailed t-test with Welch’s correction.

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Author contributions

A. T.-Y. C., K.A., and P.J.H. conceived the experiments; A. T.-Y. C., C.G., and K.J.D. performed the experiments; A. T.-Y. C. and P. J. H. analyzed the data and wrote the manuscript.

Conflict of interest

The authors have no conflict of interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. 51 Criteria for scoring of DAF-16A::GFP subcellular localization.

Fig. 52 Representative photographs of wild-type, sgk-1(null), sgk-1(gf), and akt-1(null) animals expressing DAF-16A::GFP.

Fig. 53 Photograph of arrested sgk-1(null) nondauer larva.

Table 51 Lifespan data and statistics relevant to Fig. 1.

Table 52 H2O2 survival data and statistics for each replicate of Fig. 2A,B.

Table 53 UV survival data and statistics for each replicate of Fig. 2C,D.

Table 54 Thermotolerance data and statistics for each replicate of Fig. 2E–G.

Table 55 Raw DAF-16A::GFP subcellular localization data and statistics for Fig. 3A.

Table 56 qPCR data and statistics for each replicate of Fig. 3B–F.

Table 57 qPCR primers for Fig. 3B–F.