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Investigation

miR-71 mediates age-dependent opposing contributions of the stress-activated kinase KGB-1 in *Caenorhabditis elegans*

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Abstract

Studying the evolutionary processes that shaped aging offers a path for understanding the causes of aging. The antagonistic pleiotropy theory for the evolution of aging proposes that the inverse correlation between age and natural selection strength allows positive selection of gene variants with early-life beneficial contributions to fitness despite detrimental late-life consequences. However, mechanistic understanding of how this principle manifests in aging is still lacking. We previously identified antagonistic pleiotropy in the function of the *Caenorhabditis elegans* JNK homolog KGB-1, which provided stress protection in developing larvae, but sensitized adults to stress and shortened their lifespan. To a large extent, KGB-1's contributions depended on age-dependent and opposing regulation of the stress-protective transcription factor DAF-16, but the underlying mechanisms remained unknown. Here, we describe a role for the microRNA miR-71 in mediating effects of KGB-1 on DAF-16 and downstream phenotypes. Fluorescent imaging along with genetic and survival analyses revealed age-dependent regulation of *mir-71* expression by KGB-1—upregulation in larvae, but downregulation in adults—and showed that *mir-71* was required both for late-life effects of KGB-1 (infection sensitivity and shortened lifespan), as well as for early life resistance to cadmium. While *mir-71* disruption did not compromise development under protein-folding stress (known to depend on KGB-1), disruption of the argonaute gene *alg-1*, a central component of the microRNA machinery, did. These results suggest that microRNAs play a role in mediating age-dependent antagonistic contributions of KGB-1 to survival, with *mir-71* playing a central role and additional microRNAs potentially contributing redundantly.

Keywords: miR-71; ALG-1; JNK; KGB-1; antagonistic pleiotropy; DAF-16

Introduction

Pleiotropic effects manifesting at different ages are the basis of the antagonistic pleiotropy theory for the evolution of aging. This theory proposes that since the strength of natural selection declines with age, gene variants with late-life deleterious effects can still be positively selected if they have early-life beneficial effects (Williams 1957). This theory explains aging as the result of gene variants that promote early life processes, such as development and reproduction, but delimit lifespan. Examples of antagonistic pleiotropy have been described (and debated) including the target of rapamycin (TOR) pathway, which regulates protein synthesis with contrasting impacts on growth and development *versus* lifespan, and p53, which prevents cancerous cell proliferation, but also promotes cell senescence (Ungewitter and Scrable 2009; Kapahi *et al.* 2010; Rodríguez *et al.* 2017; Long and Zhang 2019). However, a mechanistic understanding of when and how a good contribution becomes bad is still missing.

We previously identified age-dependent contributions for the *Caenorhabditis elegans* c-jun N-terminal kinase homolog KGB-1, which demonstrated characteristics of antagonistic pleiotropy (Twumasi-Boateng *et al.* 2012). KGB-1 is expressed throughout development and adulthood in various tissues, including the gut, epidermis, muscle, neurons, and the gonad (Liu *et al.* 2018). Early work demonstrated its importance for reproduction (Orsborn *et al.* 2007). Work using a hypomorphic allele further showed that KGB-1 also protected from heavy metals and protein misfolding stress (ER stress), enabling development under adverse environmental conditions (Mizuno *et al.* 2004, 2008). More recently, we found that these protective contributions of KGB-1 were limited to developing larvae, whereas its activation in adults (following knock-down of a negative regulator, the phosphatase VHP-1) increased sensitivity to the same stresses, as well as to infection, and shortened lifespan. While VHP-1 is also a negative regulator of P38 MAPK signaling, the described effects on stress resistance were independent of the p38 pathway, activation of which contrasted with that of KGB-1 as invariably beneficial (Twumasi-Boateng *et al.* 2012).

To a large extent, age-dependent contributions of KGB-1 depended on the stress-protective and longevity-associated

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transcription factor DAF-16 (Kenyon *et al.* 1993; Larsen *et al.* 1995; Ogg *et al.* 1997). In larvae, KGB-1 activation promoted DAF-16 nuclear localization and transcriptional output, while the same activation in young adults attenuated nuclear localization and output (Twumasi-Boateng *et al.* 2012). A second transcription factor, FOS-1, was found to be important for gene expression downstream of KGB-1, but its contributions were age-invariant (Zhang *et al.* 2017).

How KGB-1 modulated DAF-16 was not clear. Previous results indicated that this modulation could be achieved cell nonautonomously, with neuronal KGB-1 modulating intestinal DAF-16 nuclear localization (Liu *et al.* 2018), but to date, no molecular mechanism was identified that mediated these effects. Here, we show that the microRNA *miR-71*, and potentially additional microRNAs, is regulated by KGB-1 in an age-dependent manner and is required to mediate effects on DAF-16, as well as for a subset of KGB-1 age-dependent contributions to stress resistance and lifespan.

Experimental procedures

Strains

Strains used included *kgb-1(km21)*, *alg-1(tm492)*, *alg-2(ok304)*, TJ356 (*zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]*), *drsh-1(ok369)/hT2 [bli-4(e937) let-?(q782) qIs48]*), *pash-1(mj100); mjEx331[eft-3p::pash-1::GFP::unc-54 3′UTR; myo-2p::mCherry::unc-54 3′UTR]*, *mir-71(n4115)*, *mir-71*o/e: *nIs286[mir-71(+) + sur-5::GFP]*, *daf-12(rh61rh412)*, *kri-1(ok1251)* and the N2 wild-type strain. All were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). Homozygous *pash-1(mj100)* and *drsh-1(ok369)* mutants were isolated from genetically rescued or balanced strains, respectively, by picking non-fluorescent animals. Strains containing combinations of the aforementioned mutations and transgenes were generated by mating and verified by PCR and sequencing. *unc-119(ed3); maIs352[mir-71p::GFP; unc-119(+)]* worms were gratefully received from Dr. Zachary Pincus, Washington University.

Bacterial strains included *Escherichia coli* OP50-1 and *Pseudomonas aeruginosa* PA14 (Shapira and Tan 2008).

Imaging

Worms were picked onto unseeded 2% agarose plates, paralyzed with 25 mM levamisole (Sigma, St. Louis, MO), and imaged using a Leica MZ16F fluorescent stereoscope (Leica, Wetzlar, Germany) equipped with a MicroPublisher 5.0 RTV (QImaging, BC, Canada). Images were processed and quantified using ImageJ (National Institutes of Health, Bethesda, MD).

RNA interference-mediated knock-down

Knock-down by feeding was performed using standard protocols, with clones from the Ahringer RNAi library (Kamath and Ahringer, 2003), except for the *vhp-1* clone, which was from the Open Biosystems Library (Reboul *et al.* 2003). Bacteria harboring an empty RNAi vector (EV) served as control. Larval knock-down was achieved by exposing worms from the egg stage to larval L3 or L4, as described; knock-down in adults was carried out from L4 to day 2 of adulthood, unless otherwise mentioned. All experiments were performed at 20°C, unless otherwise specified. *cdc-25.1* RNAi was used to disrupt germline proliferation, as described elsewhere (Shapira and Tan 2008), sterilizing worms, and promoting DAF-16 nuclear localization downstream of gonad signaling. Young adult parents were fed *cdc-25.1* RNAi for 8 h and their progeny further grown on *cdc-25.1* RNAi until the L4 larval stage, and then transferred to plates containing a 1:1 mixture of *cdc-25.1* RNAi with vhp-1 RNAi or empty vector clones.

Acute cadmium toxicity resistance assays

Worms were fed EV or *vhp-1* RNAi from hatching until the L3 stage, then transferred to K media plates (1.55 g NaCl, 1.19 g KCl, 8.5 g Bacto Agar, H_2O to 1 L; sterilized by autoclaving) with 10 mM $CdCl_2$ and seeded with OP50-1. Survival was scored after 11 h, at which point the majority of EV fed worms were dead.

Tunicamycin development assays

Eggs were transferred to NGM plates containing 1 μg/ml tunicamycin. After 3 days at 20°C, worms were staged and counted, and the percentages at different developmental stages, as well as dead worms, were calculated.

Survival assays

Survival experiments were carried out at 20°C for lifespan and at 25°C for infection assays. Worms were exposed to RNAi from L4 to day 2 of adulthood. For infection assays, they were then transferred to plates pre-seeded with *P. aeruginosa* PA14. For lifespan assays, they were transferred to NGM plates with 100 μg/ml kanamycin seeded with kanamycin-killed OP50-1. Alternatively, worms were kept on RNAi plates to maximize *vhp-1* knock-down and its effects. Survival was scored every 1 or 2 days. The statistical significance of differences between survival curves was determined using a log-rank test in GraphPad Prism 7.

Quantitative RT-PCR

Total RNA was extracted from 100 to 500 animals using TRIzol (Invitrogen). RNA was treated with DNase to remove genomic DNA contamination (QIAGEN, Hilden, Germany), and cDNA was synthesized using iScriptTM (Bio-Rad). SYBR Green quantitative (q)RT-PCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a StepOnePlus system (Applied Biosystems, Foster City, CA). Gene-specific threshold cycle (Ct) values were normalized by subtracting the respective values for measurements of actin gene expression. Statistical significance was calculated with a t-test based on actin-normalized Ct values. Primers, whose sequences are listed below, were used with an annealing temperature of 60°C.

mtl-1 forward: GAGGCCAGTGAGAAAAAATGCT
mtl-1 reverse: GCTCTGCACAATGACAGTTTGC
actin forward: TCGGTATGGGACAGAAGGAC
actin reverse: CATCCCAGTTGGTGACGATA
mir-71 forward: CGACGGCGAAAAACAGAATA
mir-71 reverse: GTCTGCTCTGAACGATGAAAG

Results

mir-71 is required for detrimental effects of KGB-1 activation in adults

Previous results have shown that KGB-1 activation following *vhp-1* knock-down in adults decreased infection resistance and lifespan (Twumasi-Boateng *et al.* 2012). This was shown to be due to attenuation of DAF-16 nuclear localization in adults, particularly apparent following germline disruption, where *vhp-1* knock-down abolishes an otherwise prominent DAF-16 nuclear localization (Liu *et al.* 2018). Effects of KGB-1 activation on the survival of germline-disrupted animals are reproducible, but their extent variable, likely due to variable efficiency of RNAi-mediated gene knock-down. In some cases, *vhp-1* knock-down suppressed the lifespan-extension effect of germline disruption such that sterile

and fertile worms had similar lifespans (Figure 1A). This observation suggested that KGB-1 might regulate DAF-16 through pathways activated by germline disruption. Candidates known to contribute to lifespan extension following germline disruption and putatively affected by KGB-1 activation included the dafachronic acid-binding nuclear hormone receptor DAF-12 and the ankyrin-repeat protein KRI-1, both previously shown to promote DAF-16 nuclear localization in germline ablated animals (Berman and Kenyon 2006; Gerisch *et al.* 2007), and miR-71, a microRNA shown to promote DAF-16 nuclear localization and extend lifespan, putatively by inhibiting insulin signaling (de Lencastre *et al.* 2010; Boulias and Horvitz 2012). We tested these candidates for involvement in KGB-1-dependent susceptibility to *Pseudomonas aeruginosa* infection following *vhp-1* knock-down, which mirrors KGB-1's effects on lifespan (Twumasi-Boateng *et al.* 2012). Only *mir-71* disruption reproducibly prevented the detrimental effects of KGB-1 activation in germline-disrupted adults (Figure 1B; Supplementary Table S1). Instead of detrimental effects, *vhp-1* knock-down in *mir-71* mutants resulted in a small, yet reproducible, increase in resistance. This is attributed to the parallel activation of the p38 kinase ortholog PMK-1, a central contributor to *C. elegans* immunity, by *vhp-1* knock-down. The protective effects of this activation become apparent upon disruption of deleterious KGB-1 signaling in adults (Twumasi-Boateng *et al.* 2012).

Lifespan of *mir-71* mutants, while shorter to begin with, attesting to miR-71's beneficial contributions, showed no further decrease upon *vhp-1* knock-down (Figure 1C; Supplementary Table S2). This was prominent in sterile animals (Figure 1C), but was observed also in fertile animals (Supplementary Table S2). Furthermore, *vhp-1* knock-down in longer-lived worms overexpressing miR-71 from an integrated multicopy *mir-71p::mir-71* transgene reduced lifespan to a similar level as in *vhp-1* RNAi-treated wild-type animals, supporting the involvement of miR-71 in mediating detrimental effects of KGB-1 (Figure 1C; Supplementary Table S2).

Argonaute-like proteins are required for microRNA processing and binding, and the two main *C. elegans* homologs, *alg-1* and *alg-2*, were reported to have opposite effects on lifespan, with *alg-1* extending it and *alg-2* restricting it, both through interactions with IIS (Aalto *et al.* 2018). We found that *alg-1* mutants were unaffected by KGB-1 activation, whereas *alg-2* mutants responded as wild-type animals (median lifespan of *vhp-1* RNAi-treated animals was 77% of control RNAi-treated animals; Figure 1D). Furthermore, disruption of the *pash-1*, encoding a nuclear RNAase III co-factor necessary for microRNA processing (but not siRNA/RNAi), also abolished the detrimental effects of KGB-1 activation. In contrast, animals with disruption of the RNAase III gene itself, *drsh-1*, which is short-lived to begin with, still showed significant detrimental effects following *vhp-1* knock-down (median lifespan in *vhp-1* RNAi-treated animals was 70% of controls).

KGB-1 activation modulates *mir-71* gene expression in an age-dependent manner

To examine how *mir-71*, with its beneficial contributions to lifespan, was involved in the detrimental contributions of KGB-1 in adults, we used transgenic worms expressing GFP from the *mir-71* promoter (Martinez *et al.* 2008) to monitor its expression following KGB-1-activation. KGB-1 activation following *vhp-1* knock-down in adults resulted in a significant decrease in

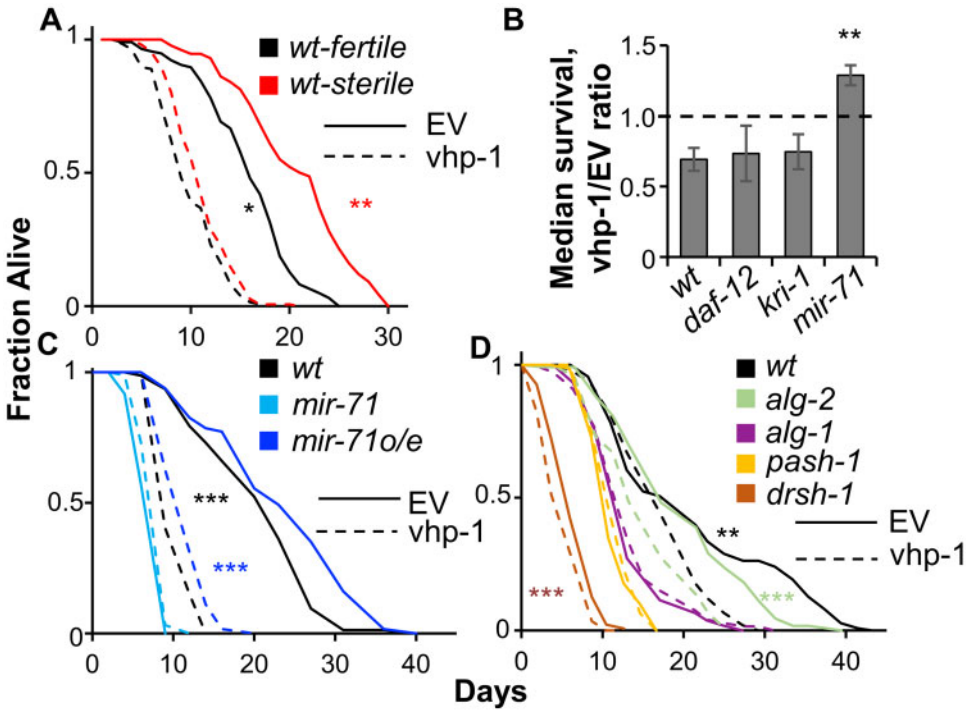

Figure 1 Detrimental effects of KGB-1 depend on *mir-71*. (A) Lifespan trajectories for wild-type animals, either fertile or rendered sterile by development on *cdc-25.1* RNAi, fed control (EV, empty vector) or *vhp-1* RNAi for 2 days, starting at L4 ($N = 108–137$ worms per group; $^{*}P < 0.05$, $^{**}P < 0.01$, log-rank test). (B) Ratios of the median infection survival time of *cdc-25.1* RNAi-sterilized animals of the designated strains exposed to *vhp-1* RNAi during early adulthood and shifted to *Pseudomonas aeruginosa*, over that of respective animals exposed to control (EV) RNAi before transfer. Averages ± SD for four independent experiments (see Supplementary Table S1 for individual assays); $^{**}P < 0.01$ compared with wild-type. (C) Lifespan assays for sterile wild-type, *mir-71*, and *mir-71* overexpressing (o/e) animals ($N = 55–75$ worms per group; $^{***}P < 0.0001$); shown is a representative experiment of three (see Supplementary Table S2). (D) Lifespan assays of fertile microRNA processing mutants treated with control or *vhp-1* RNAi as described above. Asterisks, as above ($N = 48–135$ per group).

expression from the *mir-71* promoter, particularly in the intestine (Figure 2). This was observed as early as 2 days from the beginning of RNAi exposure but was more prominent following longer exposure, until day 5 of adulthood, when *mir-71p*-driven GFP expression is maximal (Pincus *et al.* 2011). Although suppression of *mir-71* expression was most prominent in *cdc-25.1* RNAi-sterilized worms (Figure 2), it was also apparent in the pharynx and intestine of fertile animals (not shown). In all cases, *kgb-1* disruption dramatically reduced the extent of *mir-71* suppression following *vhp-1* knock-down, supporting the notion that suppression of *mir-71* expression was KGB-1 dependent, and suggesting that this suppression underlay the detrimental effects of KGB-1.

Previous results demonstrated that KGB-1 contributed to downstream gene expression and phenotypes mostly through cell nonautonomous regulation. This was found to be true also for *mir-71* expression, with activation of neuronal KGB-1 potently repressing intestinal *mir-71* expression, while activation of the intestinal KGB-1 demonstrated marginal effects, similar to those observed in *kgb-1* mutants (Supplementary Figure S1).

KGB-1 activation in larvae also affected *mir-71* expression. However, in agreement with its age-dependent antagonistic contributions, larval activation of KGB-1, contrasting with its activation in adults, increased *mir-71* expression (Figure 3, A and B). This was supported by qRT-PCR measurements demonstrating increased expression of the endogenous non-processed pri-mir-71 (Figure 3C). In conclusion, *mir-71* expression reflects the age-dependent antagonistic contributions of KGB-1—induced by KGB-1 activation in larvae but repressed by KGB-1 activation in adults.

mir-71 is required for KGB-1-dependent attenuation of DAF-16 nuclear localization in adults

KGB-1 activation was previously shown to modulate DAF-16 nuclear localization in an age-dependent and antagonistic manner, and *daf-16* was shown to be required for most of the KGB-1-dependent phenotypes (Twumasi-Boateng *et al.* 2012). miR-71 was also shown to modulate DAF-16 nuclear localization (Boulias and Horvitz 2012). Thus, we examined whether miR-71 was involved in modulating DAF-16 nuclear localization following KGB-1 activation, specifically the attenuation of DAF-16 nuclear localization following KGB-1 activation in adults. To this end, we used transgenic strains expressing a DAF-16::GFP fusion protein from the *daf-16* promoter (Henderson and Johnson 2001). Germline proliferation disruption following development on *cdc-25.1* RNAi led to nuclear localization of DAF-16 (Figure 4A). This nuclear localization was significantly attenuated following *vhp-1* knock-down in wild-type, but not in *kgb-1* mutants, indicating a role for KGB-1 (Figure 4B; Supplementary Figure S2). A thwarted effect was also observed in *mir-71* mutants, but this is more difficult to interpret since these mutants show little nuclear localization to begin with. However, qRT-PCR measurements of *mtl-1* gene expression, a DAF-16 target and a surrogate for its output, showed loss of KGB-1-dependent repression, both in *mir-71*, as well as in *alg-1* mutants, supporting a role for miR-71 in mediating the detrimental effects of KGB-1 activation on DAF-16 output (Figure 4C). These results suggest that miR-71 mediates KGB-1-dependent DAF-16 regulation in adults and places it upstream of DAF-16.

In contrast to the importance of *mir-71* in KGB-1-dependent DAF-16 regulation in adults, *mir-71* appeared to be dispensable for enhanced nuclear localization of DAF-16 following KGB-1 activation in larvae (Supplementary Figure S3). In agreement, expression of the DAF-16 target gene *mtl-1* was induced following KGB-1 activation in *mir-71* mutant larvae, as in wild-type animals. Thus, while *mir-71* was inversely regulated by KGB-1 in larvae and adults, its contribution to mediating KGB-1 effects on DAF-16 activity was apparent only in the adult stage.

MicroRNA processing and *mir-71* are required for a subset of KGB-1's protective contributions in developing larvae

In developing larvae, KGB-1 activation was shown to protect animals from protein-folding stress in the endoplasmic reticulum (ER stress) and against heavy metals. Protection from heavy metals was shown to be dependent on DAF-16 (Zhang *et al.* 2017), which was also suggested to protect from ER stress (Henis-Korenblit *et al.* 2010; Safra *et al.* 2014). To examine the involvement of microRNA processing and *mir-71* in these protective contributions, we evaluated the development of *alg-1* and *mir-71* mutants in the presence of tunicamycin, which causes ER stress by inhibiting N-linked protein glycosylation, and the resistance of mutant larvae to acute heavy metal stress in the form of 10 mM cadmium. We found that *alg-1* mutants were somewhat

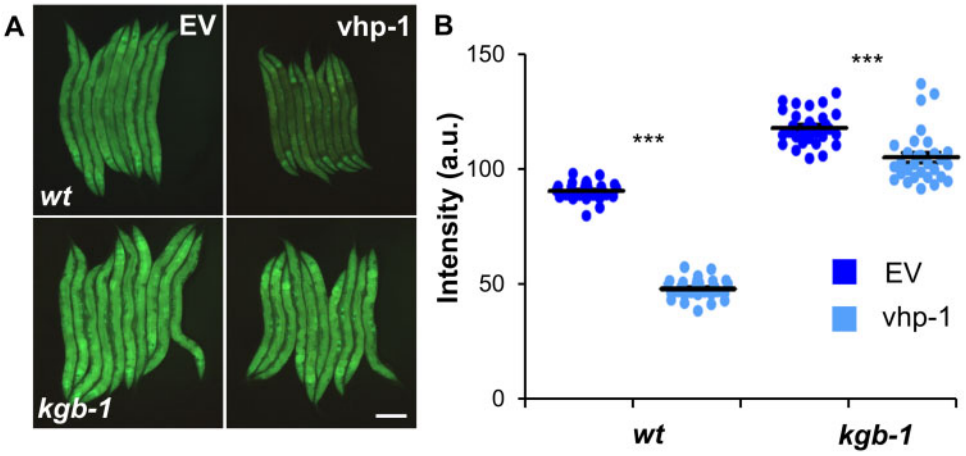

Figure 2 KGB-1 activation in adults suppresses *mir-71* expression. (A) Representative images of *cdc-25.1*(RNAi)-sterilized transgenic animals expressing GFP from the *mir-71* promoter, in a wild-type or *kgb-1(km21)* background, and following a 5-day exposure, beginning at L4, to control (EV) or vhp-1 RNAi. Scale bar, 200 μm. (B) Quantification of signal intensity from worms as in (A). Lines mark averages ± SE; individual measurements shown in dots, 29–30 worms per group; $^{***}P < 0.001$, *t*-test.

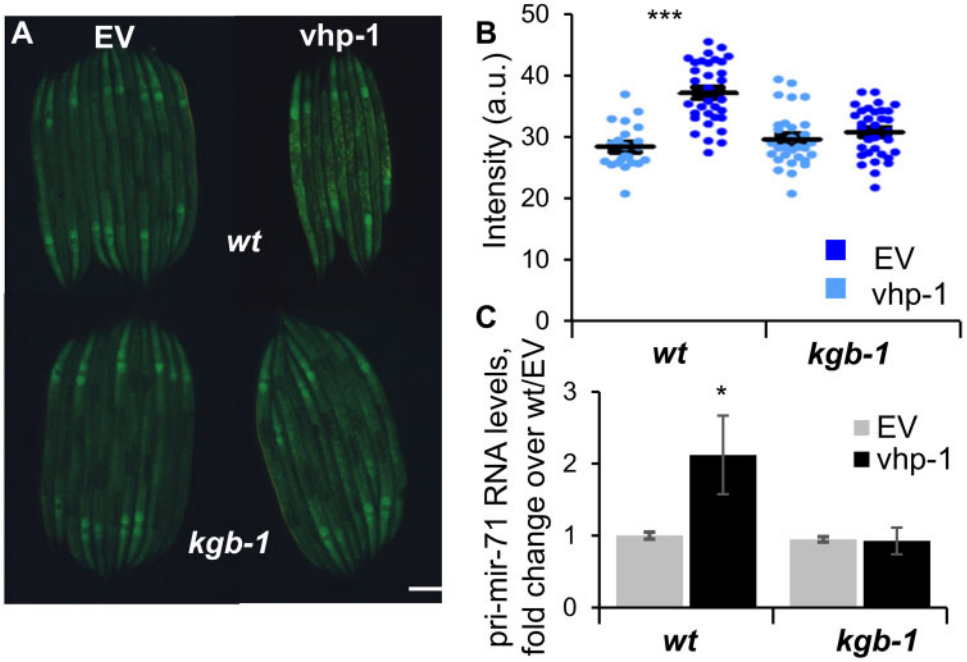

Figure 3 KGB-1 activation in larvae induces *mir-71* expression. (A) Representative images of transgenic *mir-71p::GFP* animals and *mir-71p::GFP*; *kgb-1(km21)* mutants raised from the egg stage to L3 on control (EV) or *vhp-1* RNAi. Scale bar, 100 μm. (B) Quantification of signal intensity in worms as in (A). Lines mark averages ± SE; individual measurements shown in dots, 22–33 worms per group; $^{***}P < 0.001$, *t*-test. (C) qRT-PCR measurements of *mir-71* expression (unprocessed pri-mir-71) in strains and RNAi treatments as designated. Shown are averages and SDs for two independent experiments; $^{*}P < 0.05$.

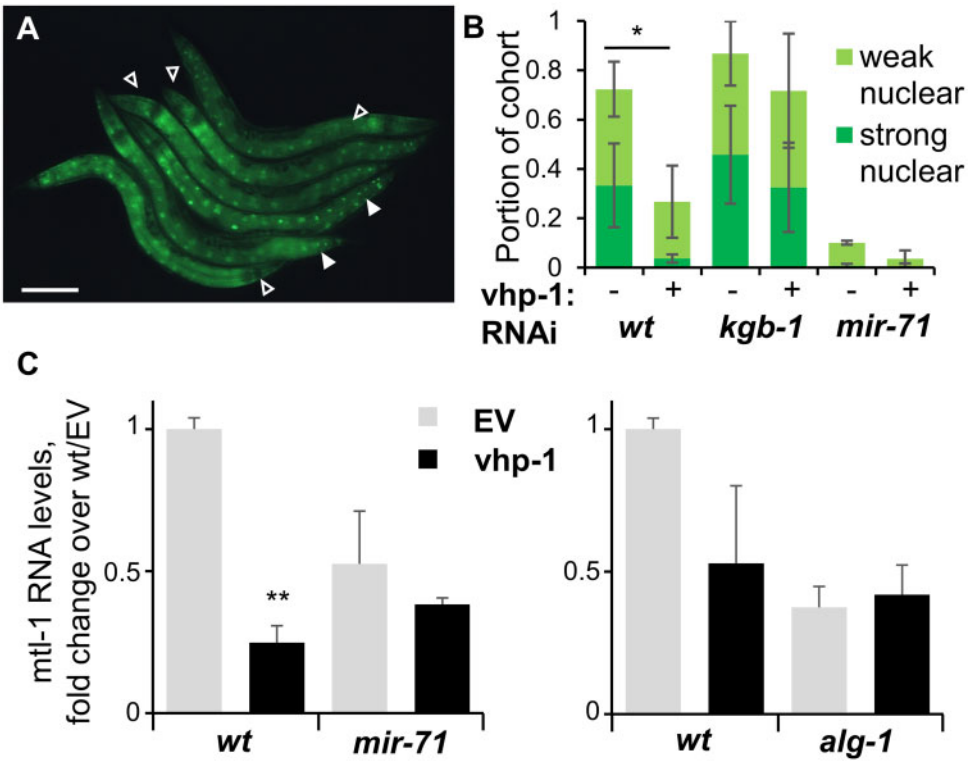

Figure 4 *mir-71* mediates effects of KGB-1 activation on DAF-16 output. (A) A representative image of *cdc-25.1* RNAi-sterilized transgenics expressing a DAF-16::GFP fusion protein following a 2-day exposure, beginning at L4, to control RNAi (EV). Filled arrowheads mark worms with strong nuclear localization, empty arrowheads mark weak nuclear localization. Scale bar, 200 μm. (See Supplementary Figure S2 for representative images of each group). (B) Portion of worms with DAF-16::GFP nuclear localization as in (A), with genetic backgrounds as designated, and 2-day exposure to the designated RNAi. Shown are averages ± SDs of three independent experiments ($N = 219–329$ worms total per group); $^{*}P < 0.05$, *t*-test. (C) qRT-PCR measurements of *mtl-1* gene expression in worms of the designated strains, following a 2-day exposure to the designated RNAi. Shown for each graph are averages ± SDs for two independent experiments; $^{**}P < 0.01$.

susceptible to ER stress, showing retarded development similar to that observed for *daf-16* mutants, although not as dramatic as in *kgb-1* mutants (Figure 5A). *mir-71* mutants, on the other hand, were as resistant to ER stress as wild-type animals. This suggested that the microRNA processing machinery was involved in developmental ER stress resistance; however, *mir-71* was dispensable. *mir-71* was also dispensable for basal resistance to cadmium, as was *alg-1*; however, both were essential for enhanced larval resistance to cadmium conferred by KGB-1 activation (Figure 5B). Together, these results indicate a role for miR-71 and the microRNA processing machinery in mediating a subset of the protective contributions of KGB-1 activation in larvae. The redundancy of miR-71 for developmental ER stress resistance, in contrast to the involvement of *alg-1*, suggests that additional microRNAs may be involved in protecting against ER stress during development. It remains to be determined whether such additional microRNAs might also act downstream of KGB-1.

Discussion

Previous work identified an age-dependent switch in the contributions of KGB-1 to stress resistance, which was associated with age-dependent and opposing effects on DAF-16. However, what causes this switch and how KGB-1 activity affected DAF-16 remained a mystery. The results described here offer a clue by identifying the microRNA miR-71 as a downstream mediator of KGB-1, showing age-dependent regulation by KGB-1 and linking KGB-1's activity both to DAF-16 output and downstream phenotypes.

Our results suggest that the interactions between KGB-1 and miR-71 are pivotal for their respective contributions in adults. Disruption of *mir-71*, which shortens lifespan, prevented detrimental effects of KGB-1 activation. Reciprocally, KGB-1 activation largely abolished the lifespan extension seen in worms overexpressing miR-71 (Figure 1C; Supplementary Table S2). This is presumably due to the strong repression of miR-71 expression by upstream KGB-1 activation (Figure 2). Nevertheless, we cannot rule out additional negative contributions of KGB-1 through effects on other targets. In vertebrates, JNK proteins have been shown to have numerous protein targets. This is likely true also in *C. elegans*, although only a few direct targets have been described to date (Egge 2019).

The importance of miR-71 as a downstream mediator of KGB-1 is not similar at different ages. Whereas it was fully required for activated KGB-1's detrimental consequences in adult animals (*i.e.* increasing sensitivity to infection, shortening lifespan, and reducing DAF-16-dependent gene expression), it was in part dispensable for the beneficial contributions of KGB-1 in developing larvae (*i.e.* it was required for larval resistance to acute cadmium stress following KGB-1 activation, but not for KGB-1-dependent DAF-16 nuclear localization in larvae or for basal level resistance to tunicamycin-induced ER stress). While *mir-71* was not required for ER stress resistance in larvae, disruption of the argonaute gene *alg-1*, involved in microRNA processing and binding, did compromise ER stress resistance, indicating that its function was required independently of miR-71 and suggesting an involvement of other microRNAs besides miR-71. Redundancy in microRNA contributions in developing worms is well-described, thought to ensure developmental robustness (Miska *et al.* 2007; Weaver and Han 2018). The redundancy of *mir-71* for certain facets of KGB-1-dependent larval stress resistance may be explained by such redundancies. However, the observation that *mir-71* disruption was sufficient to abolish KGB-1-dependent resistance to acute cadmium exposure may suggest a more central role for *mir-71* compared with other microRNAs in mediating KGB-1-dependent regulation of stress resistance.

Previous studies have shown that miR-71 extended lifespan, primarily through repression of insulin signaling (de Lencastre *et al.* 2010; Zhang *et al.* 2011). In agreement with this, miR-71 was subsequently shown to promote nuclear localization and activity of DAF-16 in the intestine of worms lacking germ cells, with this contribution depending on the neuronal expression of *mir-71* (Boulias and Horvitz 2012). Support for neuronal functions of miR-71 was further provided by studies showing both neuronal cell-autonomous repression of TIR-1/SARM—an upstream regulator of the p38 pathway, as well as cell nonautonomous contributions of neuronal miR-71 and TIR-1 to intestinal proteostasis (Couillault *et al.* 2004; Hsieh *et al.* 2012; Finger *et al.* 2019). TIR-1 or additional targets may account for miR-71's contribution to larval KGB-1-dependent stress resistance without observable effects

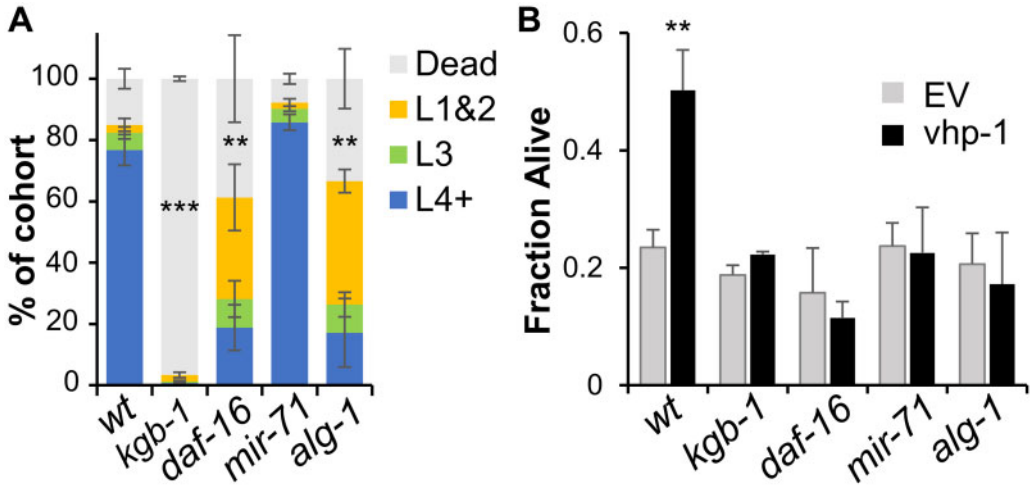

Figure 5 *mir-71* is required for some, but not all of KGB-1's protective effects in larvae. (A) Development in the presence of 1 mg/ml tunicamycin (3 days, 20°C). Shown are averages ± SDs of two experiments, each performed in duplicate; cohorts included 127–521 worms per replicate. $^{**}P < 0.01$, $^{***}P < 0.001$ (paired *t*-test), calculated for L4+ worms, with similar results for dead worms. (B) Resistance of L3 larvae, fed the indicated RNAi upon hatching, to an acute cadmium exposure (10 mM, 11 h). Averages ± SDs of two experiments (except for *daf-16*, which was measured in one experiment only); $N = 232–718$ per group per experiment, $^{**}P < 0.01$ (paired *t*-test).

on DAF-16. Our results support a role for miR-71 in the regulation of DAF-16 and suggest that this regulation can be modulated by KGB-1. Whereas our results do not resolve the tissue from which miR-71 exerted its effects, they do show that it is expressed in the intestine, and that this expression is regulated by KGB-1 in accordance with KGB-1's age-dependent contributions. Whether this intestinal regulation is downstream to the effects of miR-71 on DAF-16 and survival phenotypes (as some sort of a feedback loop), or upstream of these effects, suggesting a causative role, is not clear. Interestingly, our results suggest that *mir-71* is involved in cell nonautonomous regulation but in a different way than those suggested before, as intestinal *mir-71* expression depends primarily on neuronal KGB-1, much like many of the KGB-1-dependent phenotypes (Supplementary Figure S1) (Liu *et al.* 2018).

The work presented here advances our understanding of the antagonistic pleiotropy behavior of KGB-1 one notch forward. It points at miR-71 as a hub for age-dependent regulation—both of DAF-16, and potentially of additional targets. It further demonstrates the age-dependent regulation of miR-71 by KGB-1, but the mechanism responsible for this remains to be determined.

Data availability

Strains used in this study are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplementary material is available at *GENETICS* online at figshare: https://doi.org/10.25386/genetics.10273436.

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Conflicts of interest

The authors declare that no competing interests exist.

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