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The *Caenorhabditis elegans* LET-418/Mi2 plays a conserved role in lifespan regulation

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Summary

The evolutionarily conserved nucleosome-remodeling protein Mi2 is involved in transcriptional repression during development in various model systems, plays a role in embryonic patterning and germ line development, and participates in DNA repair and cell cycle progression. It is the catalytic subunit of the nucleosome remodeling and histone deacetylase (NuRD) complex, a key determinant of differentiation in mammalian embryonic stem cells. In addition, the *Drosophila* and *C. elegans* Mi2 homologs participate in another complex, the MEC complex, which also plays an important developmental role in these organisms. Here we show a new and unexpected feature of the *C. elegans* Mi2 homolog, LET-418/Mi2. Lack of LET-418/Mi2 results in longevity and enhanced stress resistance, a feature that we found to be conserved in *Drosophila* and in *Arabidopsis*. The fact that depletion of other components of the NuRD and the MEC complexes did not result in longevity suggests that LET-418 may regulate lifespan in a different molecular context. Genetic interaction studies suggest that *let-418* could act in the germ-cell-loss pathway, downstream of *kri-1* and *tcer-1*. On the basis of our data and on previous findings showing a role for *let-418* during development, we propose that LET-418/Mi2 could be part of a system that drives development and reproduction with concomitant life-reducing effects later in life.

Key words: aging; *C. elegans*; longevity gene; Stress resistance; FoxO transcription factor; germline.

Introduction

The protein Mi2 (CHD-3/CHD-4) is an evolutionarily conserved ATP-dependent chromatin remodeler of the CHD family. It is characterized by an ATPase domain, two chromodomains, and two plant homeodomain (PHD) fingers, which are required for interaction with HDAC (Zhang *et al.*, 1998). Mi2 is a key-component of the multiprotein chromatin-remodeling complex NuRD. This complex is present in vertebrates and invertebrates and unifies three enzymatic activities central to epigenetic regulation: chromatin remodeling, histone deacetylation, and histone demethylation (Ramirez & Hagman, 2009). The Mi2 homologs in *Caenorhabditis elegans* and *Drosophila* participate in another chromatin complex, MEC, which plays also important developmental roles (Wilkinson *et al.*, 1994; Unhavaithaya *et al.*, 2002; Kunert & Brehm, 2009; Kunert *et al.*, 2009; Passannante *et al.*, 2010). Mi2 is mainly involved in transcriptional repression (reviewed in Denslow & Wade, 2007 Kunert *et al.,* 2009), but more recently it has also been shown to be involved in nontranscriptional roles, such as the maintenance of higher-order chromatin structure and the prevention of accumulation of double-stranded DNA damage (reviewed in Li & Kumar, 2010). It is also likely involved in the assembly and/or maintenance of chromatin structures required for proper cell cycle progression (Sims & Wade, 2011).

Mi2 is a major factor controlling differentiation, development, and disease (reviewed in Ramirez & Hagman, 2009). Together with other NuRD components, it is essential for maintaining the balance between pluripotency and self-renewal in embryonic stem cells (ESCs) (reviewed in Hu & Wade, 2012). In *Drosophila*, dMi2 functions during embryo patterning and is essential for embryogenesis and germ cell development (Kehle *et al.*, 1998). In *Arabidopsis*, the Mi2 homolog, Pickle, represses embryonic development to allow the transition to postembryonic development (Ogas *et al.*, 1999).

The genome of *C. elegans* encodes two well-conserved Mi2 homologs, LET-418 and CHD-3. Despite a high degree of similarity shared by the two proteins, the mutant phenotype resulting from loss of function of the respective genes is quite different. While *chd-3* mutants show no obvious phenotype, *let-418*/Mi2 was shown to be essential for development and reproduction. Strong loss-of-function alleles of *let-418* lead to sterility, vulval defects, and, in the absence of maternal contribution, developmental arrest at the mid-L1 stage associated with ectopic expression of P granule components in somatic cells (von Zelewsky *et al.*, 2000; Unhavaithaya *et al.*, 2002; Passannante *et al.*, 2010).

In this study, we describe a new feature of *let-418*. We show that *let-418* mutants are long-lived and stress resistant and that this phenotype requires the activity of the lifespan determinant DAF-16/FOXO. Depletion of other components of the NuRD and the MEC complexes did not enhance lifespan, suggesting that LET-418 acts in a different molecular context. Genetic experiments suggest an interaction of *let-418* with the germ-cell-loss pathway and the insulin signaling cascade. On the basis of our findings, we propose that *let-418* could impact on aging by being part of a system, which is involved in the allocation of resources between reproduction and soma maintenance. This function might be evolutionarily conserved as we found that flies and plants lacking Mi2 activity are long-lived and/or stress resistant.

Results

LET-418 regulates lifespan in adult *C. elegans* worms

The long-lived phenotype caused by decreased insulin-like signaling is (in some mutants) associated with somatic misexpression of germline genes (Curran *et al.*, 2009). As *let-418* mutants also show a soma-to-germline transformation (Unhavaithaya *et al.*, 2002; Passannante *et al.*, 2010),

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we hypothesized that they were long-lived as well. We thus measured the lifespan of *let-418* worms bearing either the loss-of-function allele *let-418(s1617)* or the temperature-sensitive allele *let-418(n3536)* (named hereafter *let-418(lf)* and *let-418(ts),* respectively). Both *let-418 (lf)* mutants and *let-418(ts)* adults at nonpermissive temperature showed a significant lifespan extension compared with control animals (20%, $P < 0.0001$ and 25%, $P < 0.0001$, respectively) (Fig. 1A,B and Table S1). Comparably, we found that *let-418* mutants bearing the alleles *ar113* and *ar114* exhibited an extended lifespan too (17%, $P = 0.0032$ and 28%, $P < 0.0001$) (Table S1). Rescue experiments with a LET-418::GFP fusion protein in two independent transgenic strains were able to restore normal lifespan in *let-418(ts)* mutants (Fig. 1B, $P = 0.8918$, $P = 0.6398$ vs. control, respectively), whereas the same constructs had no influence on the lifespan in a wild-type background (Fig. 1B, $P = 0.5133$, $P = 0.8526$ vs. control, respectively). Altogether, these rescuing experiments further support the idea that the prolonged lifespan was due to the *let-418* deficiency.

let-418 adult worms bearing the alleles *s1617*, *ar113,* and *ar114* have a strongly disorganized germline and are either sterile or produce very few eggs (Table S2). Theoretically, the longevity of *let-418* worms could therefore result from these defects in the germline. This is, however, not likely, as temperature-sensitive *let-418(n3536)* animals, shifted at the restrictive temperature at the L4 stage, develop a normal germline and are fertile, but nevertheless have an extended lifespan (Fig. 1B, Table S2). Furthermore, as mentioned above, we found that somatically expressed extrachromosomal *let-418::gfp* transgenes (Fig. S1) were able to restore normal lifespan (Fig. 1B), but not the germline defects of *let-418* mutants (Table S2). Altogether, these findings indicate that longevity of *let-418* animals does not simply originate from a defective germline or from an altered reproductive state (Table S1, S2).

This conclusion is also supported by genetic data. Lifespan extension in animals with an ablated germline depends on KRI-1, an ankyrin repeat protein located in the intestine (Berman & Kenyon, 2006). If longevity of *let-418* animals was the result of their germline defects, it should depend on the *kri-1* function. To test whether *kri-1* is required for the extended lifespan of *let-418* worms, we measured the lifespan of sterile *let-418(lf)* worms lacking the *kri-1* function. We found that RNAi depletion of *kri-1* did not significantly change the enhanced lifespan of *let-418(lf)* worms ($P = 0.6164$, Fig. 1C and Table S3).

The *C. elegans* protein TCER-1 is another factor that is required for loss-of-germ cells to increase lifespan. In animals with ablated germ cells or in *glp-1* mutants lacking germ cell proliferation, *tcer-1* is upregulated in somatic tissues and promotes the expression of DAF-16-regulated genes that contribute to longevity (Ghazi *et al.*, 2009). We tested whether *tcer-1* is also upregulated in *let-418* mutants, but found no increase in the *tcer-1* mRNA levels in *let-418* mutants (Fig. S2). Consistent with this observation, we found no significant difference in lifespan between *let-418* and *let-418;tcer-1* animals ($P = 0.3359$, Fig. 1D and Table S3). Taken together, our results indicate that the longevity of *let-418* animals does not depend on their germline defects.

We then sought whether LET-418/Mi2 could control the lifespan of the worm in a NuRD context. However, depletion of *egr-1/MTA*, *lin-53/RbAp48,* or *hda-1/HDAC1*, which encode *C. elegans* NuRD members (Passannante *et al.*, 2010), did not result in an increase in longevity (Table S1, Samuelson, 2007 #2162). Interestingly, *lin-53(n3368)* mutant worms lived significantly shorter than control worms (Table S1). LET-418/Mi2 also exists in a complex with MEP-1 (Unhavaithaya *et al.*, 2002; Passannante *et al.*, 2010). Therefore, we measured the lifespan of *mep-1* mutants, but found no extended longevity (Table S1, Samuelson, 2007 #2162). Thus, the role of LET-418/Mi2 in normal lifespan regulation might not involve a NuRD or an MEC complex, but could depend on a different molecular context.

Loss of *let-418* enhances resistance to environmental stress

Many long-lived mutants are also more resistant to environmental stress (reviewed in (Zhou *et al.*, 2011). Therefore, we addressed the question whether *let-418* also functions in stress resistance. To assay

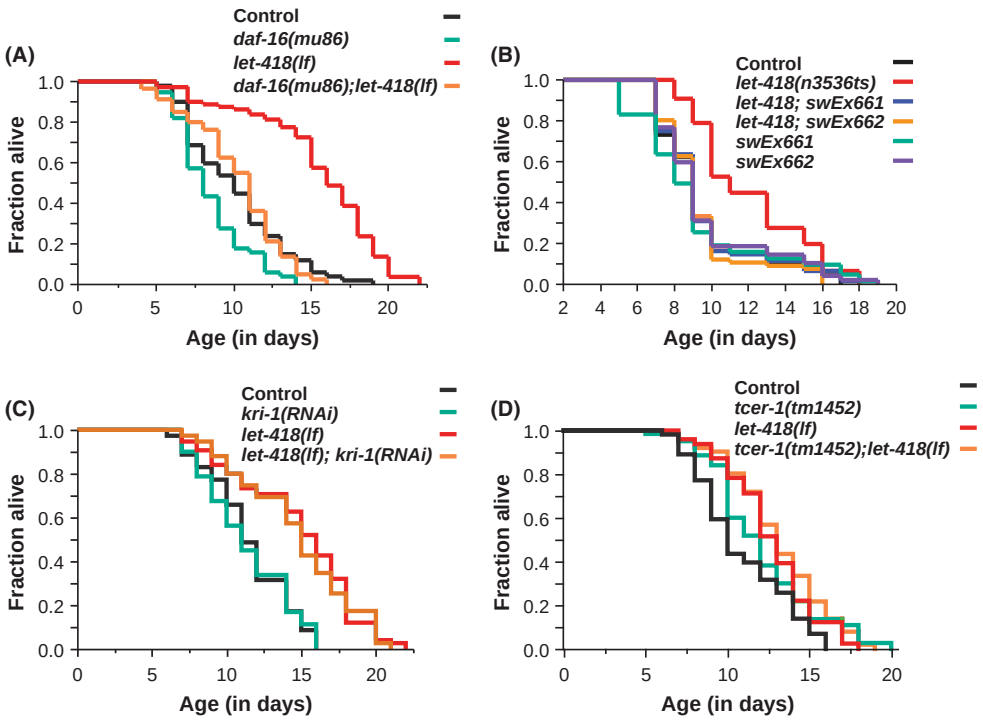

Fig. 1 Loss of *let-418* results in longevity. (A) *let-418(lf)* mutants show a significantly increased lifespan ($P < 0.0001$), which partially depends on *daf-16*. (B) The enhanced lifespan of *let-418(ts)* mutants compared with control worms ($P < 0.0001$) is rescued by two transgenes, *swEx661* and *swEx662*, encoding a translational LET-418::GFP fusion protein ($P = 0.8918$, $P = 0.6398$ vs. control, respectively). Furthermore, the transgenes do not have an effect on lifespan of wild-type worms ($P = 0.5133$, $P = 0.8526$ vs. control, respectively). (C) The extended lifespan of *let-418(lf)* animals does not depend on *kri-1*. The longevity of *let-418(lf);kri-1(RNAi)* worms is not significantly different from the lifespan of *let-418(lf)* worms ($P = 0.6164$). The efficiency of *kri-1* RNA depletion was assessed using *kri-1::gfp* transgenic worms (Fig. S5). (D) The longevity of *let-418(lf)* worms does not need *tcer-1* activity. The lifespan of *let-418(lf);tcer-1(RNAi)* animals is not significantly different from the lifespan of *let-418(lf)* worms ($P = 0.3359$). Additional lifespan experiments and statistics are presented in Table S3.

for a response to oxidative stress, we exposed control and *let-418(lf)* adult worms to high doses of paraquat, a superoxide-inducing agent, and monitored their survival rate. We observed that *let-418* worms displayed an increased resistance to paraquat compared with control animals (47%, $P < 0.0001$) (Fig. 2A and Table S4 for other tests). To test the response to heat stress, we shifted 1-day-old *let-418(lf)* adult worms to 35 °C and monitored their survival rate. We found that *let-418* animals were more resistant to heat stress than control worms (30%, $P < 0.0001$) (Fig. 2B and Table S5 for other tests). In summary, our results revealed that *let-418* depletion produces adults that live longer and are more resistant to oxidative stress and heat exposure.

LET-418 modulates lifespan and stress resistance in a *daf-16*-dependent manner

To better understand how *let-418* modulates lifespan and stress resistance, we asked whether it is genetically interacting with the well-characterized longevity factor DAF-16/FOXO, which promotes enhanced lifespan in response to many inputs such as decreased insulin signaling (reviewed in Landis & Murphy, 2010). We found that depletion of *daf-16* significantly reduced the lifespan of *let-418* animals ($P < 0.0001$, Fig. 1A and Table S3). Similarly, paraquat-treated or heat-shocked *daf-16(mu86);let-418(s1617)* worms were significantly less resistant than *let-418(s1617)* single-mutant worms ($P < 0.0001$, Fig. 2A,B and Tables S4 and S5). Altogether, this supports the idea that loss of *let-418* acts through DAF-16 to achieve longevity and stress modulation. Nevertheless, we observed that *let-418;daf-16* double mutants still lived longer ($P = 0.0003$, Fig. 1A and Table S1) and were more resistant to stress ($P < 0.0001$, Tables S4 and S5) than *daf-16* single-mutant animals, suggesting that, besides DAF-16, other factors must also be involved.

let-418 could influence lifespan and stress resistance through DAF-16 by changing its nuclear localization, its expression levels, or its transcriptional activity. However, we found no evidence for nuclear accumulation of a DAF-16::GFP reporter protein in 1-day-old *let-418* adults in any tissue (Fig. S3A). Moreover, only a very modest increase in the *daf-16* mRNA levels, but no obvious differences in protein levels, was observed (Fig. S3B and data not shown). This situation is reminiscent of that observed with the host cell factor HCF-1. HCF-1 forms an inhibitory complex with DAF-16, thereby limiting its access to the target gene promoters (Li *et al.*, 2008). Mutations in *hcf-1* lead to an enrichment of DAF-16 at its target gene promoters and result in a *daf-16*-dependent lifespan extension and enhanced stress resistance without changing the subcellular localization or the expression levels of DAF-16. We tested whether LET-418 could physically interact with DAF-16, but co-immunoprecipitation experiments revealed no evidence for an inhibitory complex between the two proteins (data not shown).

To ask more directly whether depletion of *let-418* modifies the transcriptional activity of DAF-16, we monitored the mRNA levels of individual genes upregulated in 1-day-old *let-418* mutants, which had been identified in a comparative transcriptome analysis (data not shown). The list included the superoxide dismutase gene *sod-3* (a direct transcriptional target of DAF-16 Oh *et al.*, 2010), the heat shock protein gene *hsp-16.2,* and the glutathione S-transferase gene *gst-4*. All three genes have previously been associated with stress resistance or longevity (Murphy *et al.*, 2003; Kahn *et al.*, 2008; Tullet *et al.*, 2008). We also analyzed the F-box gene *fbxa-75*, because it was highly upregulated in *let-418* mutant animals (Fig. 3). Our qPCR analyses revealed that the enhanced transcript levels of *sod-3* in *let-418* mutants depended entirely, and those of *fbxa-75* partially, on DAF-16 (Fig. 3). These results confirmed that LET-418 somehow influences the transcriptional activity of DAF-16. The transcript levels of the heat shock protein *hsp-16.2*, however, were completely independent of DAF-16 (Fig. 3). Altogether, these findings were consistent with the previous observation that mutations in *daf-16* only partially reduced longevity and stress resistance of *let-418* mutant animals (Figs 1A and 2A,B) and suggested that other transcription factors participate in the regulation of the *let-418* target genes. This was confirmed by the fact that overexpression of *hsp-16.2* in *let-418* mutants depended almost entirely on the heat shock factor (HSF-1), whereas upregulation of the glutathione S-transferase-coding gene *gst-4* required, at least partially, the activity of the Nrf2-like transcription factor SKN-1 (Fig. 3). SKN-1 and HSF-1 are, like DAF-16, longevity- and stress-resistance- promoting transcription factors (reviewed in Kenyon, 2010b). Correspondingly, we found that longevity of *let-418* mutants was also dependent of *skn-1* and *hsf-1* (Table S3). Taken together, our data suggest that LET-418 modulates the transcriptional activity of DAF-16 and that of other lifespan-regulating transcription factors at a subset of target genes. Besides gene repression, LET-418 seems also to be involved in gene activation. This is shown by the fact that we could identify genes that are downregulated in a *let-418(lf)* background (Fig. S4). At present, we do not know, however, whether these genes are directly or indirectly regulated by LET-418.

LET-418 is not required for *eat-2*- and *clk-1*-induced lifespan extension

We next tested whether *let-418* interacts with pathways that regulate lifespan of the worm. Dietary restriction extends lifespan not only in worms, but also in flies and rodents (Mair & Dillin, 2008). In *C. elegans*,

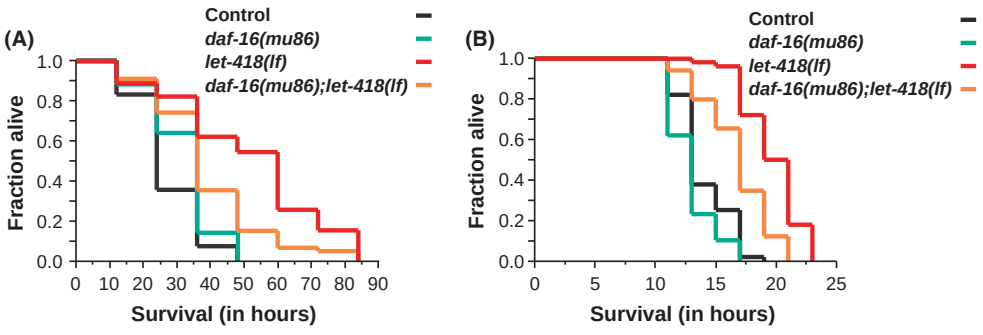

Fig. 2 Loss of *let-418* results in stress resistance. (A) *let-418(lf)* mutants show an increased resistance to 30 mM paraquat ($P = 0.0001$), which is partially dependent on *daf-16*. (B) *let-418(lf)* mutants exhibit an enhanced resistance to a 35 °C heat shock ($P < 0.0001$), which is partially dependent on *daf-16*. Additional experiments and statistics are presented in Tables S4 and S5.

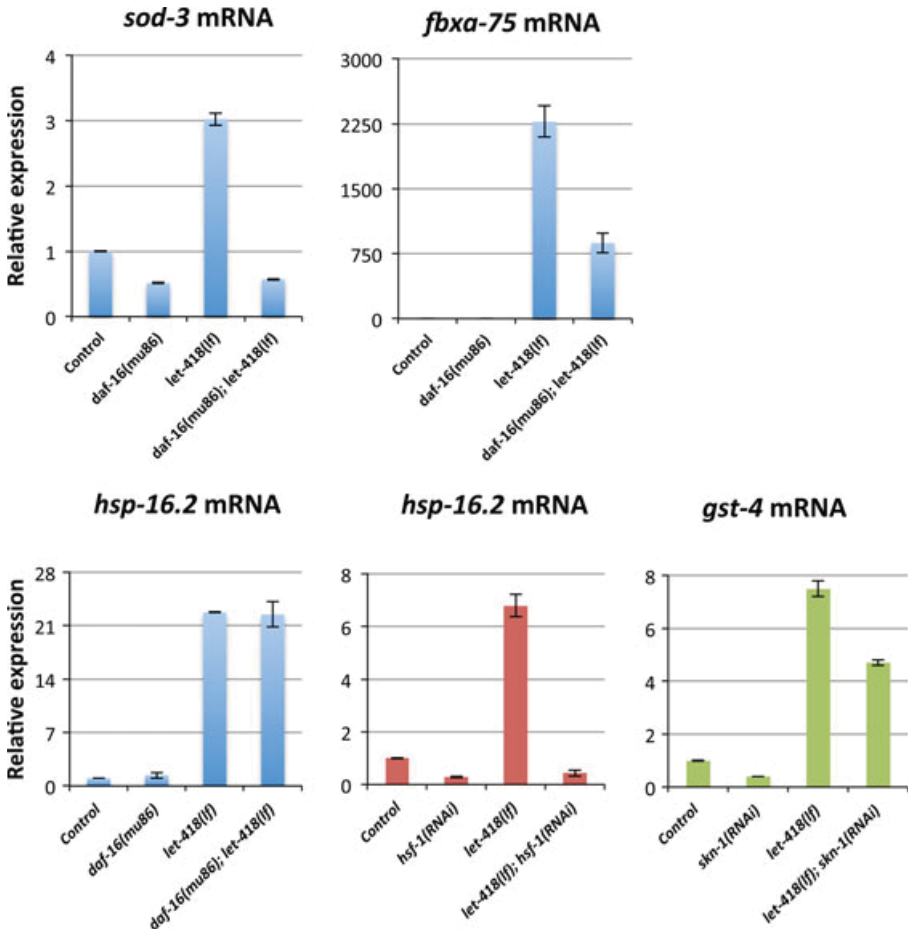

Fig. 3 The upregulation of longevity and stress-resistance genes in *let-418* animals depends on DAF-16, HSF-1, and SKN-1. The mRNA levels of *sod-3*, *fbxa-75*, *hsp-16.2,* and *gst-4*, as determined by qPCR, were all significantly increased in the *let-418(lf)* mutant. The upregulation of *sod-3* was completely, that of *fbxa-75* partially, and that of *hsp-16.2* not at all dependent on DAF-16. Instead, upregulation of *hsp-16.2* required HSF-1 and *gst-4* upregulation was partially dependent on SKN-1. The data from at least three independent experiments were pooled, and the mean normalized RNA levels and SEM for each gene in the indicated strains are shown. The RNA level of each gene was normalized to the *ama-1* level.

there are many methods for inducing dietary restriction (Greer & Brunet, 2009). One method is achieved through mutation in the acetylcholine receptor gene *eat-2*. Lack of *eat-2* activity causes a reduced pharyngeal pumping rate, resulting in dietary-restricted worms (Lakowski & Hekimi, 1998). To test whether *let-418* functions in the same pathway as *eat-2*, we generated *eat-2(ad1116);let-418(lf)* double mutants and measured their lifespan (Fig. 4A). Both *eat-2* and *let-418* worms showed an extended lifespan compared with control animals ($P < 0.0001$), but the double mutants *eat-2(ad1116);let-418(lf)* lived even longer than either of the single mutants (*P* value vs. *let-418* = 0.0002, *P* value vs. *eat-2* < 0.0001). This indicates that *let-418* and *eat-2* do not function in the same pathway to regulate lifespan.

We also tested the respiration pathway. *clk-1* encodes a demethoxy-ubiquinone hydroxylase that participates in the biosynthesis of ubiquinone and mutations in this gene result in defects of mitochondrial oxidative phosphorylation, a reduced respiration rate of the worms and an extended lifespan (Wong *et al.*, 1995). To test whether *let-418* interferes with the respiration rate of the worms, we analyzed the lifespan of *let-418(lf);clk-1(RNAi)* worm. In this test, both *let-418* and *clk-1(RNAi)* worms had an extended lifespan ($P < 0.0001$), but the *let-418 (lf);clk-1(RNAi)* double mutants showed even a greater lifespan extension compared with the *let-418* mutant or the *clk-1*-depleted worm (*P* value vs. *let-418* = 0.0024, *P* value vs. *clk-1* < 0.0001, Fig. 4B). Altogether, these results indicated that LET-418 does not participate in the dietary-restriction pathway or the respiration pathway to control aging.

Low-insulin-signaling animals require *let-418* to live long

Given the epistatic relationship between *let-418* and *daf-16*, we wondered whether *let-418* modulates lifespan by functioning in the insulin/IGF-1 pathway. *daf-2* encodes the ortholog of the mammalian insulin/IGF-1 receptor (Kimura *et al.*, 1997). We generated a *daf-2 (e1370);let-418(lf)* double mutant and measured its lifespan. *daf-2* strongly increased the lifespan of *let-418* (65%, $P < 0.0001$), indicating a synthetic effect of both mutations. However, *let-418* knockdown did not further extend the long lifespan of *daf-2(e1370)* worms (Fig. 5A, Table S3). On the contrary, the lifespan of the double mutants was even slightly, but significantly shorter than that of *daf-2(e1370)* animals (−17%, $P = 0.0007$, Table S3 for other tests), suggesting that *daf-2* mutants require the *let-418* function for maximal lifespan. We also measured the lifespan of *let-418* mutants, in which *age-1* was inhibited by RNAi. The PI3 kinase AGE-1 acts downstream of the DAF-2/insulin

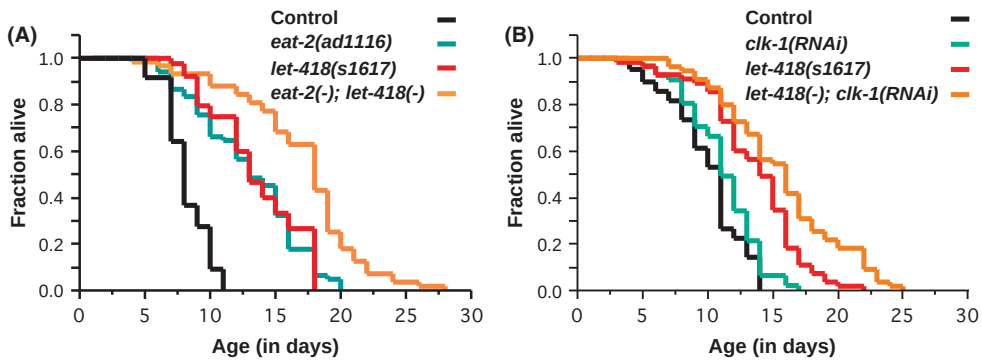

Fig. 4 LET-418 is not required for *eat-2*- and *clk-1*-induced lifespan extension. (A) *let-418* knockdown further extends the lifespan of long-lived *eat-2(ad1116)* mutants ($P < 0.0001$) and that of control worms ($P < 0.0001$). (B) *let-418(lf)* further increases the longevity of *clk-1(RNAi)* worms ($P < 0.0001$) and that of control worms ($P < 0.0001$). Additional lifespan experiments and statistics are presented in Table S3.

receptor in lifespan regulation (Tissenbaum & Ruvkun, 1998). The type of interaction was similar to the one we found with *daf-2*: *age-1* depletion further increased the lifespan of *let-418* (10%, $P = 0.0042$), but *let-418 (lf);age-1(RNAi)* worms lived less longer than *age-1* worms alone (−13%, $P < 0.0001$, Fig. 5B, Table S3). We also investigated the lifespan changes upon knockdown of the Akt/PKB kinase homologs *akt-1* and *akt-2*, which function redundantly downstream of *daf-2* to regulate the activity of the insulin pathway (Paradis & Ruvkun, 1998). *akt-1* RNAi, combined with a mutation in *akt-2*, extends lifespan more than the single mutants alone (data not shown), and we used this double knockout to further investigate the interaction between *let-418* and the DAF-2/insulin pathway. *let-418* worms lived longer upon depletion of both *akt-1* and *akt-2* (31%, $P < 0.0001$), but the activity of *let-418* was necessary for maximal lifespan extension of *akt-1(RNAi);akt-2(ok393)* worms (−11%, $P = 0.0012$, Fig. 5C, Table S3). Contrary to what is observed in wild-type background, the absence of *let-418* has a life-shortening effect in the low-insulin-signaling mutant, indicating that LET-418 is required in this pathway when insulin signaling is reduced.

let-418 may function in the germ-cell-loss pathway

Ablation of germline precursor cells in the worms leads to a lifespan extension that also depends on the longevity determinant DAF-16 (Hsin & Kenyon, 1999). This effect can be reproduced genetically using mutations in *glp-1*, which encodes a notch receptor (Arantes-Oliveira *et al.*, 2002). *glp-1* activity is required for germ cell proliferation. If *let-418* modulates lifespan by acting in this pathway, loss of *let-418* activity should have no major impact on the lifespan of worms lacking *glp-1* activity. To test this, we generated *let-418(lf);glp-1(q224)* double mutants. Loss of *glp-1* activity in worms grown at the restrictive temperature, from the time when germline precursor cells were born until adulthood, prevented germ cell proliferation and extended lifespan (41%, $P < 0.0001$, Fig. 5D) (Arantes-Oliveira *et al.*, 2002). Remarkably, *glp-1(q224);let-418(lf)* double mutants did not live longer than *glp-1(q224)* or *let-418(lf)* single mutants ($P = 0.5953$, $P = 0.0885$, respectively, Fig. 5D), suggesting that *let-418* may function in the germ-cell-loss pathway. In this case, LET-418 should act downstream of or in parallel to KRI-1 and TCER-1, as longevity of *let-418* mutants did not depend on these two factors. Furthermore, a small fraction of the *glp-1(q224)* worms lived longer than the *let-418(lf)* and the *glp-1 (q224);let-418(lf)* animals (Fig. 5D). The same observation was made with another allele of *glp-1* (Table S3). This suggests that the prolongevity function of *let-418*, already observed in *daf-2;let-418* double mutants, may also be required late in life for the maximum lifespan of *glp-1* worms.

The regulation of oxidative stress resistance and lifespan by Mi2 orthologs is evolutionarily conserved between *C. elegans*, *Drosophila,* and *Arabidopsis*

Given that the protein LET-418/Mi2 is remarkably conserved among multicellular eukaryotes, we wondered whether its function regarding the regulation of stress resistance and longevity may also be evolutionarily conserved. To address this question, we first investigated whether dMi2, the *Drosophila* melanogaster homolog of LET-418 (Kunert & Brehm, 2009), displays comparable functions in longevity in the fruit fly. To knockdown *dMi2*, we expressed UAS-RNAi against *dMi2* under the control of the ubiquitously expressed *tubulin*-Gal4. Comparable to the results obtained from *C. elegans,* flies expressing UAS-*dMi2*$^{RNAi\ 107204}$ displayed an increased lifespan compared with the parental control groups (Fig. 6A). The same fly strain was assayed for its stress resistance to oxidative stress, and it also showed an increased resistance to oxidative stress compared with parental control (Fig. 6B).

The genome of the plant *Arabidopsis thaliana* encodes two Mi2 homologs, namely PICKLE (PKL) and *PICKLE RELATED 1* (*PKR1*) (Ogas *et al.*, 1999). Seeds from *pkl* or *pkr1* single mutants were treated with paraquat and allowed to germinate. We found that *pkr1*, but not *pkl*, exhibited an increased tolerance to oxidative stress compared with wild-type (Fig. 6C). The situation in *Arabidopsis* is reminiscent to that of *C. elegans*, which also encodes two almost identical Mi2 orthologs (LET-418 and CHD-3), of which only LET-418 functions in longevity and stress resistance (Table S1). Taken together, these results indicate that the regulation of oxidative stress resistance and/or lifespan by Mi2 orthologs is conserved between *C. elegans*, *Drosophila,* and *Arabidopsis,* revealing that it might constitute a very ancient mechanism.

Discussion

Here we show that the evolutionarily conserved chromatin-remodeling protein LET-418/Mi2 is a new longevity determinant in *C. elegans*. Depletion of LET-418/Mi2 results in enhanced lifespan and stress resistance, which depend at least partially on the transcription factor DAF-16/FOXO. Further genetic interaction studies suggested that *let-418* could control longevity by acting in the germ-cell-loss pathway, downstream of *kri-1* and *tcer-1*. On the other hand, we found that LET-418 has also a longevity-promoting function in low-insulin-signaling mutants, where its activity is required for full lifespan. Finally, we show that the function of LET-418/Mi2 in lifespan and stress-resistance regulation is conserved in *Drosophila* and in *Arabidopsis*.

The increased lifespan and stress resistance observed in *let-418* mutants depends, at least partially, on the well-known prolongevity

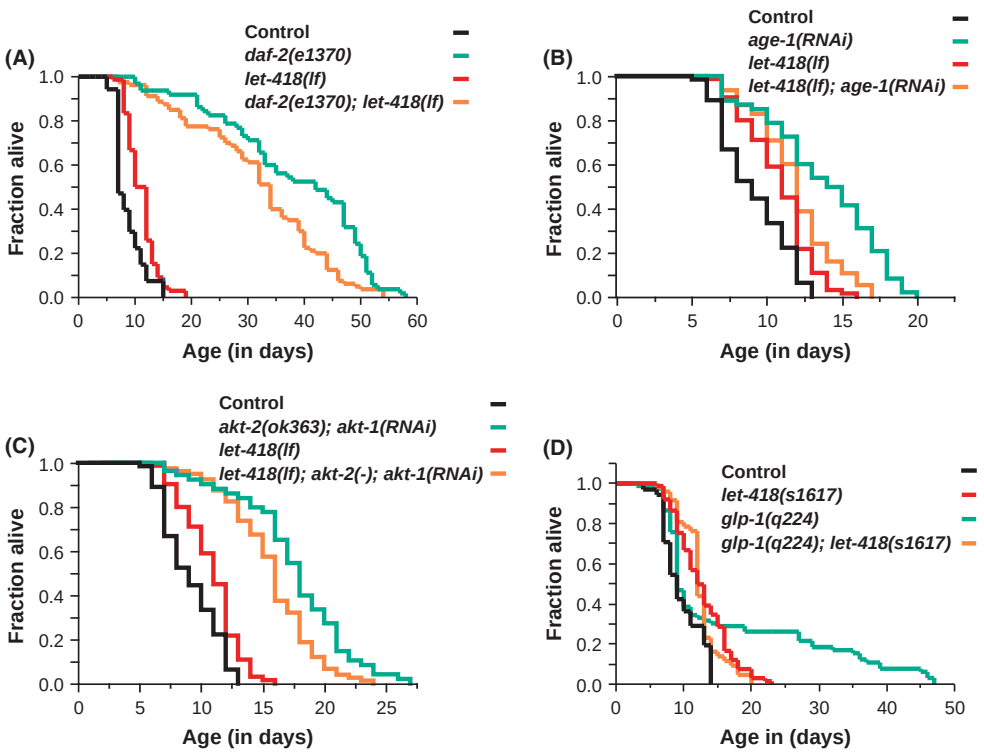

Fig. 5 *let-418* interacts with the insulin signaling and the germ-cell-loss pathways. (A) *daf-2* significantly increases the lifespan of *let-418* worms ($P < 0.0001$). The double mutants *daf-2(e1370);let-418(lf)*, however, live less longer than *daf-2 animals* ($P = 0.0007$). (B) *let-418(lf)* and *age-1* RNAi-depleted worms live longer than control worms ($P = 0.0001$, $P < 0.0001$, respectively). *let-418(lf);age-1(RNAi)* worms show a reduced lifespan compared with *age-1(RNAi)* worms ($P < 0.0001$), but live significantly longer than *let-418(lf)* mutants ($P = 0.0042$). (C) *let-418(lf)* and *akt-2(ok393);akt-1(RNAi)* worms live longer than control worms ($P = 0.0001$, $P < 0.0001$, respectively). *let-418(lf);akt-2(ok393);akt-1(RNAi)* worms live slightly but significantly less longer than *akt-2(ok393);akt-1(RNAi)* worms ($P = 0.0012$), but they still show an extended lifespan compared with *let-418(lf)* ($P = 0.0012$). Additional experiments and statistics are presented in Table S3. (D) *glp-1*, *let-418(lf)* and *glp-1;let-418(lf)* mutants and control worms were shifted from the permissive (15 °C) to the restrictive temperature (25 °C) at the L1 stage and grown to adulthood. The lifespan of the adults was then measured at 20 °C. Both *glp-1(q224)* and *let-418(lf)* worms lived longer compared with control worms ($P = 0.0486$ and $P = 0.0006$, respectively). *let-418(lf)* did not further extend the lifespan of *glp-1(q224)* worms ($P = 0.5953$). Analysis by a two-way ANOVA confirmed that there was no interaction between *let-418* and *glp-1* ($P = 0.6280$).

determinant DAF-16/FOXO. Previously, it was shown that DAF-16 promotes longevity upon phosphorylation and subsequent translocation from the cytoplasm to the nucleus. Interestingly, however, in *let-418* mutants, we found no evidence for nuclear accumulation of DAF-16 in any tissue, although we cannot completely exclude that this may happen in a few cells that escaped discovery. Besides DAF-16, longevity of *let-418* animals also requires SKN-1 and HSF-1, two other transcription factors that have been associated with longevity (Kenyon, 2010b). So far, it remains unclear how LET-418 interacts with DAF-16, SKN-1, or HSF-1 to regulate longevity and stress resistance. The role of LET-418 in normal lifespan regulation might not involve a NuRD or an MEC complex, as depletion of the other NuRD members EGR-1/MTA, LIN-53/RbAp48, and HDA-1/HDAC1 or the MEC-complex component MEP-1 did not result in an increase in longevity. A possible interaction of LET-418 with other proteins that modify chromatin and regulate aging in *C. elegans* (Greer *et al.*, 2010; Maures *et al.*, 2011; Ni *et al.*, 2012) remains to be tested.

Our genetic data indicate that LET-418 participates neither in the *eat-2*-mediated dietary restriction nor in the respiration pathway to control aging. We also tested the interaction between *let-418* and the insulin signaling pathway. We found that depletion of *daf-2* or the downstream-acting genes *age-1, akt-1,* and *akt-2* further prolonged the lifespan of *let-418(lf)* animals, suggesting a synthetic interaction. Unexpectedly, however, all double mutants lived significantly less longer than *daf-2, age-1, or akt-1;akt-2* single mutants, indicating that LET-418 is required for the maximal lifespan extension of low-insulin-signaling mutants. A similar finding was made upon depletion of *egr-1* encoding another NuRD component, which also shortens the lifespan of *daf-2* mutants (Samuelson *et al.*, 2007). The lifespan promoting function of LET-418 and EGR-1 in low-insulin-signaling animals may be associated with a NuRD complex. This hypothesis is supported by the recent finding that depletion of individual NuRD components in human Hutchinson–Gilford progeria syndrome (HGPS) cells results in chromatin defects associated with aging (Pegoraro *et al.*, 2009). Thus, LET-418/Mi2 could have two opposite functions in lifespan determination. As a member of the NuRD complex, it might prevent age-shortening chromatin defects, whereas in a NuRD-independent manner, it controls the normal rate of aging. Interestingly, an additive lifespan was observed in *daf-2;let-418 (ts)* double mutants (Table S3). The *let-418(ts)* is a non-null allele, which produces a stable protein (data not shown). This mutated protein may have retained the ability to interact with NuRD components and consequently the capacity to mediate maximal lifespan of low-insulin-signaling mutants. However, because of the hypomorphic nature of the *let-418(ts)* allele, this interpretation must be taken with caution.

In our epistatic analyses, we found that loss of *let-418* activity had no impact on the lifespan of worms lacking *glp-1* activity. These results provide some evidence that LET-418 could function in the germ-cell-loss pathway downstream of or in parallel to KRI-1 and TCER-1 (Berman & Kenyon, 2006; Ghazi *et al.*, 2009). LET-418 could attenuate the expression of target genes of this signaling system that adjusts the

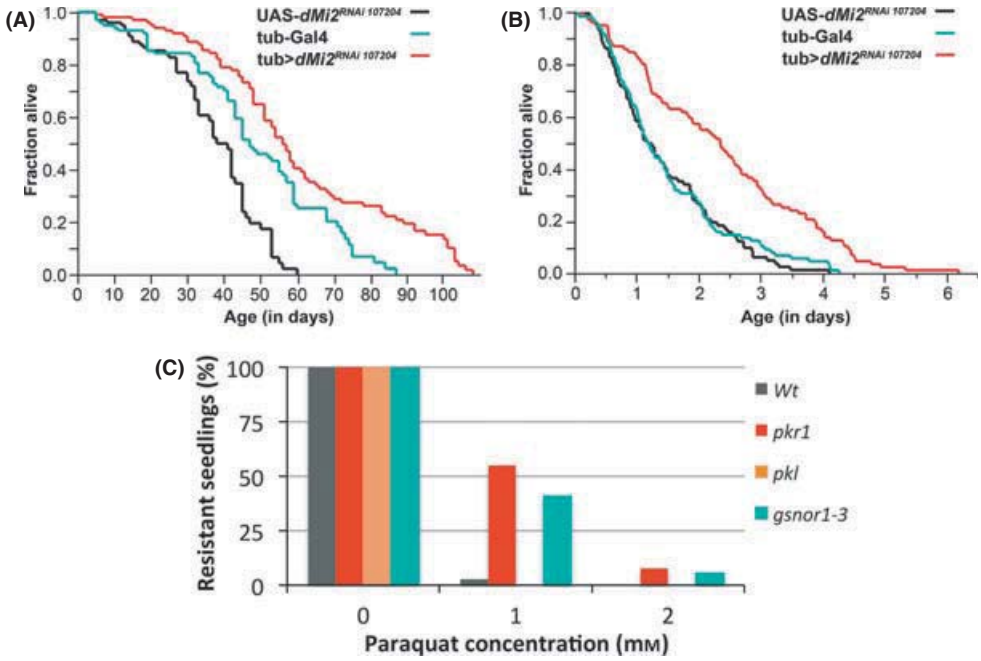

Fig. 6 The involvement of Mi-2 in regulating lifespan and stress resistance is evolutionarily conserved. (A) A reduced amount of dMi2 in *tub* > dMi2$^{RNAi\ 107204}$ (n = 104) leads to a significantly increased lifespan compared with the control groups UAS-dMi2$^{RNAi\ 107204}$ (P < 0.0001; n = 101) and *tub*-Gal4 (P < 0.007; n = 100). A Wilcoxon test was used to assess the statistical significance among groups.(B) A reduced amount of *Drosophila* dMi-2 in tub > dMi-2$^{RNAi\ 107204}$ (n = 87) leads to a significant increase in resistance to oxidative stress compared with the control groups UAS-dMi-2$^{RNAi\ 107204}$ (P < 0.0001; n = 82) and tub-Gal4 (P < 0.0001; n = 88). (C) *Arabidopsis* plants lacking *pkr1* are more resistant to paraquat (P < 0.001 and P = 0.0068 with respect to paraquat concentration of 1 μM and 2 μM). Scoring was made by counting the percentage of germinated plants after 10 days on paraquat. *pkl*-deficient plants are not more resistant than wild-type (P = 1). As positive control, *gsnor1-3* mutants, which lack a *S*-nitrosoglutathione reductase, were used (P < 0.0001, P = 0.0228 in 1 μM and 2 μM paraquat, respectively) (Chen *et al.*, 2009). The data were pooled from two independent experiments.

rate of aging according to the state of the reproductive system. Without LET-418, the target genes are not properly repressed, and the nuclear amounts of DAF-16 and other transcription factors might be sufficient to turn on their expression and to cause longevity, even in the absence of the proper signaling inputs. LET-418 may act directly on the target genes, for example, by changing their chromatin structure. Alternatively, LET-418 could act indirectly by modulating the expression of additional regulatory factors or coregulators that act downstream of this signaling pathway together or in parallel with DAF-16.

The signals from the reproductive system are not thought to program aging *per se* but rather to reallocate resources between reproduction and somatic maintenance to allow the development of the reproductive system (Kirkwood & Melov, 2011) and to coordinate the rate of aging with the timing of reproduction (Kenyon, 2010a). The chromatin-remodeling protein Mi2 has been associated with developmental decisions in a variety of different contexts (reviewed in Clapier & Cairns (2009) and Ramirez & Hagman, 2009), and in *C. elegans,* LET-418/Mi2 is essential for several aspects of development and reproduction (von Zelewsky *et al.*, 2000; Unhavaithaya *et al.*, 2002). LET-418/Mi2 could play a general role in the regulation of the allocation of resources, and this function may be evolutionarily conserved in flies and plants. Early in life, it may epigenetically program cells for development and make them by using their resources remained programmed. The idea that aging would be pushed by the drift of developmental pathways due to the decline of the force of natural selection (antagonistic pleiotropy) was already proposed by Williams (1957). Thus, LET-418/Mi2 may function in the control of normal lifespan and stress resistance later in life, as a continuation of its role during development. It might keep the organism epigenetically programmed for reproductive development and the concomitant use of resources, accompanied by life-controlling effects late in life. Depletion of *let-418* in adults would then result in longevity and stress resistance.

Experimental procedure

Lifespan assays

Caenorhabditis elegans

L4 larvae were grown at 15 °C and put on plates seeded with either OP50 or HT115 bacteria expressing various RNAi constructs. For some experiments (Table S3), 30 μM 5-fluorodeoxyuridine (FUdR) was added to the plate. Adults were incubated at 25 °C, unless indicated, and assessed for life daily. The animals that bagged, exploded, or crawled off the plate were censored. The statistical analysis (log rank) was carried out using the JMP-IN 5.1 statistical software.

Drosophila melanogaster

The strain UAS-*dMi-2*$^{RNAi\ 107204}$ was obtained from the VDRC Stock Center and *tubulin*-GAL 4 (*tub*-Gal4) from the Bloomington Stock Center. Flies were raised on standard corn meal medium at 18 °C. After hatching, 100 female flies were posed in small tubes (15 mL) and maintained at 25 °C. Flies were flipped every 2 days into fresh food vials and survival was recorded. The data were analyzed using the DAMFile-Scan106X (TriKinetics Inc., Waltham, MA, USA) and the JMP software (version 8.0, SAS Institute Inc., Cary, NC, USA). A Wilcoxon test was used to assess the statistical significance among groups.

Stress-resistance assays

Caenorhabditis elegans
Worms were grown as for lifespan assay. For thermic stress experiments, plates were put at 35 °C, and living worms were assessed every 2 h until all worms were dead. To measure oxidative stress resistance, worms were transferred to plates containing 30 mM paraquat (methyl viologen, Sigma). Live and dead counts were made every 12 h until all worms were dead. Animals that were missing, exploded, or bagged were censored. The statistical analysis (log rank) was carried out using JMP-IN 5.1 statistical software.

Drosophila melanogaster
Flies were raised as for lifespan assay. The DAMSystem303X (TriKinetics Inc.) was used to record survival under oxidative stress. DAM2 monitor tubes were loaded with 3 to 5-day-old female flies, where they were starved for 4 h. After 4 h, filter paper soaked with a solution of 20 mM paraquat in 1% glucose (Sigma-Aldrich; modified from M. Jimenez-Del-Rio *et al.*, 2008) was added to the tubes and activity was recorded every 5 min. Experiments were performed at 25 °C.

Arabidopsis thaliana
All *A. thaliana* mutants used in this study are in the Columbia background. The *pkr1* and *pkl* alleles are described in Ogas *et al.* (1997) and Aichinger *et al.* (2009), respectively. *gsnor1-3* was described by Feechan *et al.* (2005). *Arabidopsis* seeds were surface sterilized and grown on one half of the MS media containing 1 μM or 2 μM of paraquat. The medium, as described in Ogas *et al.* (1997), is based on Murashige and Skoog (MS) medium supplemented with Gamborg's vitamins solution (Sigma), glycine (2 mg mL^{-1}), biotin (0.1 mg mL^{-1}), myoinsositol (100 mg mL^{-1}), 1% sucrose, and 0.9% Bacto agar (pH 5.6). After stratification for 3 days at 4 °C, plates were incubated in a growth chamber under a 12-h photoperiod with 23 °C/19 °C of day per night temperatures and 80–100 mE m^{-2} sec^{-1} of light intensity. The phenotype was scored after 10 days of germination.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from at least 30 one-day-old adults. The cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the SensiMix® SyBr Kit (Bioline, London, UK) in a Corbett Rotor Gene 6000 machine. Relative mRNA levels were calculated from at least three biological replicates and normalized to *ama-1* mRNA levels. Primers were designed with the primer-BLAST function of the NCBI site to cover at least one exon–exon junction, to have a length of around 130 bp and to have a TM of about 59 °C.

Primer sequences are as follow:

Gene	Forward primer	Reverse primer
ama-1	5′-GAAGGTCGCAGGTGGATG-3′	5′-CCATGATTTTTCGCTCCTG-3′
daf-16	5′-TTGAGACGACTACAAAGGCTCAAC-3′	5′-CATACAAATCGTGAGAAATCGTTTG-3′
fbxa-75	5′-GCGGTCAACCAAAACATTCGAGGG-3′	5′-GCTTGCGCCCAATTTGTTCGATTT-3′
hsp-16.2	5′-AGCTCAACGTTCCGTTTTTG-3′	5′-CAATCTCAGAAGACTCAGATGGAG-3′
sod-3	5′-TCTTGAAGATCGCCACCTGTGCAAAC-3′	5′-GATATTCTTCCAGTTGGCAATCTTCC-3′
gst-4	5-AAAGCTGAAGCCAACGACTC-3	5-AGTTTTTCCAGCGAGTCCAA-3
tbh-1	5-ACG CTG GCG AGA TTG TTG CTG A-3	5-ACA ACA CAT CCG GCG TAC TGG C-3
clec-87	5′-CAG CCG CAT CAA CGA CTG ACG T-3′	5′-TGA TTG ACG AAG GCT GGC GGC-3
vit-3	5′-TCA AGA AGG TCT CTG GAC CAA AGG A-3′	5′-GCC TTG AAT GGG TTG ACC TCA GCC-3′

let-418 transgene

The *let-418* transgene includes 4.8 kb of promoter as well as the coding sequence fused to GFP. The following primers were used to generate the construct according to (Hobert, 2002):

Primer A: 5′-atgtgacgagattggggaag-3′
Primer A*: 5′-tgcaaaatccacgtttgaag-3′
Primer B: 5′-agtcgacctgcaggcatgcaagctcattggttctggctcatctg-3′
Primer C: 5′-agcttgcatgcctgcaggtcgact-3′
Primer D: 5′-aagggcccgtacggccgactagtagg-3′
Primer D*: 5′-ggaaacagttatgtttggtatattggg-3′

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Author contributions

F.M. and C.W. conceived and planned the study. V.D. performed the stress-resistance experiments of Fig. 2 and Tables S4 and S5 and some of the lifespan experiments of Figs 1, 4, and 5. C.P. generated the *let-418* transgenic worms of Fig. 1 and Fig. S1 and performed some of the lifespan experiments of Figs 1 and 5, and Tables S1 and S3. V.D., M.P., and K.B. performed the RT-PCR experiments of Fig. 3. A.v.E. and S.G.S. designed and realized the stress-resistance experiments on *Drosophila.* K.B. planned and performed the stress-resistance experiments with *Arabidopsis*. V.D. and K.B. generated the data on Figs. S2, S3, and S4, and C.P. the data on Fig. S5. C.W. and C.P. completed Table S2. F.M. and C.W. wrote the manuscript. All the authors discussed the results and commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article

Fig. S1 The *let-418::gfp* transgenes, *swEx661* and *swEx662* are not expressed in the germ cells.

Fig. S2 The mRNA level of *tcer-1* was quantified using qRT-PCR.

Fig. S3 Loss of *let-418* does not result in altered DAF-16 subcellular localization or a change in the *daf-16* mRNA levels.

Fig. S4 *tbh-1*, *clec-87* and *vit-3* are downregulated in *let-418(lf)* mutants.

Fig. S5 The efficiency of *kri-1* RNA depletion was assessed using *kri-1::gfp* transgenic worms.

Table S1 Mutations in *let-418* increase lifespan.

Table S2 Reproductive status of *let-418* alleles.

Table S3 *let-418* genetic interactions with other mutations influencing ageing.

Table S4 Mutations in *let-418* enhance oxidative stress resistance.

Table S5 Mutations in *let-418* enhance thermic stress resistance.