A Conserved PHD Finger Protein and Endogenous RNAi Modulate Insulin Signaling in Caenorhabditis elegans

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A Conserved PHD Finger Protein and Endogenous RNAi Modulate Insulin Signaling in Caenorhabditis elegans

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Abstract

Insulin signaling has a profound effect on longevity and the oxidative stress resistance of animals. Inhibition of insulin signaling results in the activation of DAF-16/FOXO and SKN-1/Nrf transcription factors and increased animal fitness. By studying the biological functions of the endogenous RNA interference factor RDE-4 and conserved PHD zinc finger protein ZFP-1 (AF10), which regulate overlapping sets of genes in Caenorhabditis elegans, we identified an important role for these factors in the negative modulation of transcription of the insulin/Pi3 signaling-dependent kinase PDK-1. Consistently, increased expression of pdk-1 in zfp-1 and rde-4 mutants contributed to their reduced lifespan and sensitivity to oxidative stress and pathogens due to the reduction in the expression of DAF-16 and SKN-1 targets. We found that the function of ZFP-1 in modulating pdk-1 transcription was important for the extended lifespan of the age-1(hx546) reduction-of-function Pi3 kinase mutant, since the lifespan of the age-1; zfp-1 double mutant strain was significantly shorter compared to age-1(hx546). We further demonstrate that overexpression of ZFP-1 caused an increased resistance to oxidative stress in a DAF-16-dependent manner. Our findings suggest that epigenetic regulation of key upstream signaling components in signal transduction pathways through chromatin and RNAi may have a large impact on the outcome of signaling and expression of numerous downstream genes.

Introduction

The role of RNA interference (RNAi) in the silencing of transposons and other repetitive elements is well documented [1,2], while the knowledge of its impact on endogenous genes and signaling pathways is limited. In this article, we investigate the remarkable and similar effects of the Caenorhabditis elegans RNAi-promoting factors RNAi DEficient 4 (RDE-4) [3] and Zinc Finger Protein 1 (ZFP-1) on the expression of stress-related genes. We focus on the key gene regulated by RDE-4 and ZFP-1, pdk-1, which encodes 3-phosphoinositide-dependent kinase-1 (PKD-1) [4], a component of a conserved insulin-signaling pathway. We describe a functional connection between zfp-1, rde-4 and insulin signaling in genetic epistasis experiments and demonstrate the significance of pdk-1 regulation by zfp-1 and rde-4 for C. elegans fitness.

ZFP-1, a Plant Homeo Domain (PHD) zinc finger protein, was first identified as a factor promoting RNAi interference in C. elegans [5–7]. It is a homolog of mammalian AF10 (Acute Lymphoblastic Leukemia 1-Fused gene from chromosome 10) [8] and plays a key role in leukemias caused by Mixed Lineage Leukemia MLL-AF10 fusion due to the recruitment of histone methyltransferase Dot1 by the AF10 portion of the fusion protein [9]. The developmental and physiological roles of AF10 are largely unknown. RDE-4 is a double-stranded RNA (dsRNA)-binding protein and a component of the Dicer complex responsible for the production of short interfering RNAs (siRNAs) from exogenous dsRNA [10]. The rde-4(rn299) null mutation was discovered in a screen for RNAi resistant mutants [3]. rde-4(rn299) does not have obvious developmental abnormalities, but shows synthetic phenotypes when combined with the null mutant in C. elegans Retinoblastoma gene lin-35 [11] and appears to be less healthy at elevated temperatures [12]. Also, rde-4 mutants were reported to have a slightly reduced lifespan [13]. The effects of rde-4 loss-of-function are likely to be related to recently identified endogenous siRNAs...
Author Summary

Reduced activity of the insulin-signaling pathway genes has been associated with a longer lifespan and increased resistance to oxidative stress in animals due to the activation of important transcription factors, which act as master regulators and affect large networks of genes. The ability to manipulate insulin signaling and reduce its activity may allow activation of oxidative-stress response programs in pathological conditions, such as neuronal degeneration, where oxidative stress plays a significant role. Here, we describe a new way of inhibiting insulin signaling that exists in the nematode Caenorhabditis elegans. We find that transcription of one of the insulin-signaling genes is inhibited by mechanisms involving chromatin and RNA interference, a silencing process that depends on short RNAs. We demonstrate that mutants deficient in RNA interference are more susceptible to stress due to increased insulin signaling and that increased dosage of a chromatin-binding protein repressing insulin signaling and promoting RNA interference leads to better survival of nematodes grown under oxidative stress conditions. Since there is a clear homolog of this chromatin-binding protein in mammals, it may also act to promote resistance to oxidative stress in human cells such as neurons.

(endo-siRNAs), which perfectly match thousands of genes in C. elegans either in sense or antisense orientation [14–17]. Indeed, the expression of some endo-siRNA is diminished in the absence of rde-4 [14,18].

Our recent genome-wide mRNA expression study has revealed that ZFP-1 and RDE-4 affect the transcript levels of close to 250 overlapping genes [19]. Furthermore, putative target genes of endo-siRNAs [16] showed a significant enrichment among genes upregulated in the rde-4(ne299) null [3] and zfp-1(ok554) [20] loss-of-function mutant larvae [19]. We proposed that ZFP-1 and endo-siRNAs produced in an rde-4-dependent manner cooperate in the repression of target genes in the nucleus. Here, we confirm a direct repressive effect of ZFP-1 on endo-siRNA targets by comparing gene expression changes in zfp-1(ok554) and rde-4(ne299) with genome-wide localization of ZFP-1. Moreover, using functional analysis of misregulated genes we predict a role for RDE-4 and ZFP-1 in modulating insulin signaling and further demonstrate that regulation of pdk-1 transcription by ZFP-1 and endogenous RNAi underlies the oxidative stress sensitivity and short lifespan of zfp-1(ok554) and rde-4(ne299) mutants.

Results

Gene expression signatures suggest a role for ZFP-1 and RDE-4 in modulating insulin signaling

In order to elucidate the common biological roles of ZFP-1 and endogenous RNAi we analyzed gene sets misregulated in zfp-1(ok554) and rde-4(ne299) mutants [19]. We found that genes with lowered expression in the mutants compared to the wild type were enriched in metabolic, oxidative stress-related and anti-pathogenic factors present in the intestine (Table S1). Since insulin signaling mutations lead to increased expression of factors important for defense against oxidative stress and pathogens [21–23], we decided to compare the lists of genes downregulated in zfp-1(ok554) and rde-4(ne299) with longevity-promoting “Class 1” genes found upregulated in the daf-2 mutant in a daf-16-dependent manner [23].

Insulin-like signaling in C. elegans via the DAF-2 insulin receptor and phosphatidylinositol 3-kinase (PI3K) negatively regulates the DAF-16/FOXO [24,25] and SKN-1/Nrf [26] transcription factors. When insulin signaling is reduced, the enhanced DAF-16 and SKN-1 activities contribute to longer lifespan and stress resistance in worms due to concerted regulation of many of their targets [21–23,27,28]. DAF-16 and SKN-1 are negatively regulated in part at the level of their nuclear localization; therefore, mutants in this pathway are long-lived due to a higher level of the active nuclear DAF-16 and SKN-1 and appropriate transcriptional activation or repression of their direct targets. Our analyses revealed that genes downregulated in the zfp-1 and rde-4 mutants significantly overlapped with “Class 1” longevity-promoting genes upregulated in the daf-2 mutant (a condition when DAF-16 and SKN-1 are activated) [23] (Figure 1, Table 1, Table S1). Examples of genes whose expression is negatively regulated by daf-2 and positively regulated by zfp-1 and/or rde-4 include glutathione transferases gst-4 and gst-38, and aquaporin (aqp-1) (Table 1, Figure 2A). Since RNAi is a gene-silencing phenomenon and gene sets expressed lower in zfp-1(ok554) and rde-4(ne299) are not enriched in endo-siRNA targets [19], we predict that “Class 1” longevity-promoting genes are regulated by ZFP-1 and RDE-4 indirectly. Consistently, genome-wide localization data showed no enrichment of ZFP-1 at longevity-promoting genes (Figure 1).

A higher level of pdk-1 expression in zfp-1(ok554) and rde-4(ne299) correlates with lower expression of DAF-16 target genes

We considered the possibility that a direct target gene negatively regulated by rde-4 and zfp-1 would be de-repressed in the mutants to account for the reduced expression of the secondary targets, which may therefore be regulated by these factors indirectly. Indeed, a component of the insulin-signaling pathway, the kinase PDK-1, was among the most upregulated genes in zfp-1 and rde-4 [19] (Figure 2A). Although our microarray study was performed on zfp-1 and rde-4 mutant larvae (L1–L2), we found that pdk-1 expression was increased in these mutants at other developmental stages as well (Figure 2A).

The zfp-1 gene was shown to be a direct target of DAF-16 by chromatin immunoprecipitation (ChIP) combined with sequencing [29] and, more recently, using chromatin profiling by DNA adenine methyltransferase identification (DamID) [30]. However, it was not clear whether DAF-16 had a significant role in the regulation of zfp-1. We found that zfp-1 mRNA expression in the daf-2 mutant background was influenced by daf-16 and was 2-fold lower in the daf-2; daf-16 double mutant strain (Figure 2B). Therefore, DAF-16 appears to enhance transcription of zfp-1, although not nearly to the same extent as other prominent DAF-16 targets, such as sod-3 (Figure 2B).

The analyses of gene expression described above suggest a model where ZFP-1 and RDE-4 modulate the insulin-signaling pathway by repressing pdk-1 and that a DAF-16-dependent enhancement of zfp-1 expression under conditions of low insulin signaling may contribute to a positive-feedback loop enhancing the effect of DAF-16 on other targets (Figure 2C).

Nuclear localization of DAF-16::GFP conferred by the pdk-1(sa709) mutation persists in zfp-1; pdk-1 and rde-4; pdk-1 double mutants

Next, we determined a molecular lesion in the weak loss-of-function pdk-1 allele sa709 [4] and tested whether the pdk-1(sa709) mutant mRNA was still regulated by ZFP-1 and RDE-4. We found that sa709 affects pdk-1 mRNA splicing and leads to the incorporation of intron three into the mature pdk-1 mRNA with
a very low expression level of the correctly spliced mRNA in the mutant (Figure 3A, 3B). We combined pdk-1(sa709) with zfp-1(ok554) and found the level of mutant pdk-1 mRNA expression to be elevated in the double mutant compared to pdk-1(sa709) alone (Figure 3C). The pdk-1(sa709) mRNA expression was also elevated in rde-4(ne299); pdk-1(sa709) (Figure 3C). Therefore, regulation of pdk-1(sa709) mRNA expression by ZFP-1 and RDE-4 was similar to that of wild type pdk-1 mRNA.

Since loss-of-function mutations in insulin-signaling components lead to increased nuclear localization of DAF-16::GFP [31], we tested the pdk-1(sa709) allele in this assay and found that DAF-16::GFP persisted in pdk-1; zfp-1 and pdk-1; rde-4 double mutant animals (Figure 3D, 3E). These results demonstrate that pdk-1(sa709) is epistatic to zfp-1(ok554) and rde-4(ne299) and support a model where ZFP-1 and RDE-4 affect expression of DAF-16 targets through regulation of pdk-1.

The short life span and enhanced sensitivity to oxidative stress of zfp-1(ok554) and rde-4(ne299) depend on PI3K signaling

Since longevity-promoting genes have lower expression in the zfp-1 and rde-4 mutants, it is expected that they may live shorter than wild type worms. Indeed, a decrease in lifespan of zfp-1(ok554) [29] and rde-4(ne299) [13] has been reported, with the zfp-1 mutant exhibiting a stronger phenotype than rde-4. In order to test whether upregulation of PDK-1 and therefore increased insulin signaling may contribute to the short lifespan of zfp-1, we conducted epistasis experiments with a reduction-of-function mutation in the PI3 kinase AGE-1, age-1(hx546) [32,33]. We found that the short lifespan phenotype of zfp-1(ok554) was suppressed by age-1(hx546) (Figure 4A), i.e. the reduction in lifespan of the mutant was dependent on the active insulin signaling. Also, the extended lifespan of age-1(hx546) was dependent on ZFP-1 function, consistent with the possibility that enhanced PDK-1 dosage may suppress the defect in signaling.
conferred by the non-null age-1 mutation (Figure 4A). Indeed, increased pdk-1 dosage suppresses the constitutive dauer phenotype of age-1(mg44) [4]. In order to show that high levels of pdk-1 expression contributed to the short lifespan of zfp-1(ok554) we attempted to combine zfp-1(ok554) with a strong loss-of-function mutation pdk-1(sa680) [4] for genetic suppression analyses. We were not able to recover zfp-1(ok554); pdk-1(sa680) and assume that this double mutant is not viable. Therefore, all epistasis analyses described below were performed with the sa709 allele. zfp-1 and rde-4 affect expression of multiple target genes, and some phenotypes of zfp-1(ok554), such as dauer promotion [29],

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*aFunctional annotation of genes is done based on TOPOMAP classification [73]  
*bThese genes are prominently regulated by SKN-1 [74], gst-38 is not listed among “class 1” longevity-promoting genes defined by [23].  
doi:10.1371/journal.pgen.1002299.001
are similar rather than opposite to the phenotypes of insulin-signaling mutants. We have found that \textit{zfp-1; pdk-1} and \textit{rde-4; pdk-1} double mutants display some egg-laying deficiency, which complicates the longevity assays that we conduct in the absence of drugs inducing sterility. However, although \textit{zfp-1; pdk-1} and \textit{rde-4} worms were undoubtedly sicker than \textit{zfp-1} or \textit{rde-4} single mutants, we found that the reduction of \textit{pdk-1} function significantly suppressed the decreased lifespans of \textit{zfp-1(ok554)} and \textit{rde-4(ne299)} (Figure 4B, 4C). These results further support the idea that ZFP-1 and RDE-4 affect insulin signaling through the negative regulation of \textit{pdk-1}.

The gene expression signatures of \textit{zfp-1} and \textit{rde-4} mutants suggested that they could be deficient in oxidative stress response. We induced oxidative stress by soaking L4 animals in 100mM paraquat and found that the \textit{zfp-1(ok554)} mutant strain was much more sensitive to this treatment compared to the wild type (Figure 4D), similarly to \textit{daf-16(mu86)} (Figure 4D), while \textit{rde-4(ne299)} showed moderate sensitivity (Figure 4D, Figure S1A). These results further support the idea that ZFP-1 and RDE-4 affect insulin signaling through the negative regulation of \textit{pdk-1}.

In order to determine whether increased \textit{pdk-1} expression may be sufficient to cause a stress sensitivity phenotype, we tested the SP940 strain, which contains the free duplication mmDp (II;X;f) that includes the \textit{pdk-1} locus. We found that \textit{pdk-1} mRNA levels are increased about 2.5-fold in this strain (Figure S1C), close to that observed in \textit{rde-4(ne299)}, and it shows a comparable sensitivity to paraquat (Figure S1A, S1B). These data are consistent with the idea that regulating \textit{pdk-1} dosage is important for animal fitness.

Increase in ZFP-1 expression promotes resistance to oxidative stress in a DAF-16–dependent manner

We generated transgenic lines expressing ZFP-1::GFP and ZFP-1::FLAG fusion proteins by introducing tags into the C-terminal region of ZFP-1 through fosmid recombineering in bacteria [34]. The resulting genes are expressed from the 30kb fosmid and are subject to the same regulatory inputs as the endogenous \textit{zfp-1} locus; the transgenes fully rescued the stress sensitivity and reduced lifespan phenotypes of the \textit{zfp-1} mutant (Figure 5A, 5B). \textit{zfp-1} mRNA expression was about two-fold greater in ZFP-1 transgenic lines compared to the control line generated by a similar technique of \textit{unc-119} mutant rescue but not containing the ZFP-1
Figure 3. *pdk-1* loss-of-function mutation *sa709* is epistatic to *zfp-1(ok554)* and *rde-4(ne299)*. (A) Schematic of the *pdk-1* gene with numbered boxes for exons and lines for introns, location of the *sa709* mutation and predicted effects of the mutation on mRNA and protein; exons

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</table>

*Figure 3. pdk-1* loss-of-function mutation *sa709* is epistatic to *zfp-1(ok554)* and *rde-4(ne299).* (A) Schematic of the *pdk-1* gene with numbered boxes for exons and lines for introns, location of the *sa709* mutation and predicted effects of the mutation on mRNA and protein; exons.
coding for the kinase domain and the pleckstrin homology domain (PH) according to [4] are indicated. (B) Expression levels of correctly spliced pdk-1 mRNA and intron 3-containing mRNA were determined by RT-qPCR, and ratios of spliced/unspliced isoforms in the indicated mutants were calculated and presented on a graph. The forward primer used spanned the exon 1/exon 2 junction, the reverse primer for the spliced isoform spanned the exon 3/exon 4 junction and the reverse primer for the intron 3-containing isoform was intron 3-specific (see Materials and Methods). (C) Intron 3-containing pdk-1 mRNA levels were measured by real-time RT-qPCR in indicated mutants (L4 stage animals) and normalized to pdk-1(sa709). Results of two biological replicates are shown; error bars represent standard deviation. (D, E) DAF-16::GFP nuclear localization in indicated mutants was assessed as described in Materials and Methods. Representative epifluorescence images of intestinal cells (D) were taken on a Zeiss AxioImager Z1 microscope at 630x total magnification; white arrowheads point to the nuclei.
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ZFP-1 functions to protect the animals against pathogenic challenge

An example of an acute stress response is the response of animals to pathogens. The human pathogenic bacterium Pseudomonas aeruginosa (PA14) inhibits DAF-16 nuclear localization and therefore downregulates the expression of defense factors that are dependent on DAF-16 [33]. We tested the effect

![Figure 4. Functional connection between zfp-1, rde-4 and insulin/PI3K signaling.](image)

(A-C) Life span of indicated mutant strains (see Materials and Methods). (A) Mean life spans were significantly different between wild type and all mutants (P < 0.0001 age-1, P < 0.01 daf-16, P < 0.0001 zfp-1, P < 0.001 zfp-1; age-1). Data shown is from one representative experiment that has been performed three times. (B) Mean life spans were significantly different between wild type and both pdk-1 and zfp-1 mutants (P < 0.002 pdk-1, P < 0.0001 zfp-1) while zfp-1 was found to be significantly different from zfp-1; pdk-1 (P < 0.01). Data shown is from one representative experiment that has been performed three times. (C) Mean life spans were significantly different between wild type and all mutants (P < 0.0001 pdk-1, P < 0.01 rde-4, P < 0.0001 rde-4; pdk-1). Data shown is from one representative experiment that has been performed two times. (D) Survival of L4 larva (n = 120) from indicated strains after 20 hour incubation period in 100mM paraquat: *** indicates significance of P < 0.001, ** - P < 0.01 and * - P < 0.05 compared to wild type, # indicates significance of P < 0.05 and ## - P < 0.01 compared to respective single mutant.
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of the loss of ZFP-1 function on innate immunity by assaying the survival of zfp-1(ok554) animals. Upon exposure to PA14 under the standard infection assay conditions [36], we observed that the zfp-1(ok554) mutants were significantly more susceptible to P. aeruginosa infection-mediated killing (Figure 6A, 6B). The pathogen sensitivity seen in zfp-1(ok554) mutants was due to loss of ZFP-1 function as was confirmed using a ZFP-1::GFP transgene that rescued the mutant phenotype (Figure 6B).

Next, we tested whether the increased susceptibility of zfp-1(ok554) to PA14 was dependent on insulin signaling. We confirmed that age-1(hx546) was more resistant to the infection (Figure 6C) and tested age-1; zfp-1 double mutants. The results were similar to those found in the longevity assays: age-1 and zfp-1 suppressed each other’s phenotypes (Figure 6C). The survival of the double mutant was closer to that of zfp-1(ok554) than age-1(hx546), although age-1 significantly suppressed the sensitivity of zfp-1 to PA14 killing. We conclude that PI3K signaling contributes to the pathogen-sensitivity of zfp-1(ok554).

ZFP-1 localizes to the pdk-1 promoter but not to the promoters of DAF-16 targets

Consistent with our expression and genetic epistasis data suggesting a direct role of ZFP-1 in repressing pdk-1 transcription, a strong peak of ZFP-1 function was found at the pdk-1 promoter in ChIP/chip experiments conducted by the modENCODE (model organism ENCyclopedia Of DNA Elements) project (Figure 7A). We confirmed ZFP-1 localization to the pdk-1 promoter by ChIP/PCR with antibodies specific to endogenous ZFP-1 (Figure 7B) as well as with anti-FLAG antibodies in experiments with ZFP-1::FLAG transgenic lines (Figure 8A).

ZFP-1 was not localized to the promoters of other genes of the insulin signaling pathway (daf-2, age-1, akt-1, sgk-1); it was also not present at DAF-16 target genes that have reduced expression in zfp-1(ok554) and appear to be positively regulated by this factor, as discussed earlier (Figure 1, Figure 7B, Figure 8A, and Table S1).

There was no enrichment in direct ZFP-1 targets among the longevity-promoting genes (P-value 0.83) (Figure 1). Therefore,
ZFP-1 is likely to target directly only genes whose transcription it inhibits (Table S1).

Endogenous siRNAs map to the pdk-1 promoter

We reported previously a very significant overlap between genes negatively regulated by zfp-1 and rde-4 and endogenous siRNA target genes [19]. Consistently, we find that direct ZFP-1 target genes are overrepresented among genes expressed higher in the rde-4 mutant (Figure 7C), pdk-1 is repressed by ZFP-1 and is also negatively regulated by RDE-4, which is a dsRNA-binding protein required for the biogenesis of siRNAs in the exogenous RNAi pathway [10] and contributing to the biogenesis of some endo-siRNAs [14,18]. Knowing this, we searched available deep sequencing data [37–40] for endo-siRNAs mapping to the pdk-1 locus. There were few endo-siRNAs corresponding to the coding region of pdk-1, and more siRNAs mapped to the promoter region of the gene (5kb upstream of the transcription start site), including a predicted open reading frame, H42K12.2. (Figure 7A). However, for this open reading frame, no transcriptional evidence exists, neither from EST collections nor from deep sequencing runs undertaken in the context of the modencode project, and it therefore appears to be a mis-annotated gene [41,42]. We were able to detect ~100–250 nt transcripts at the pdk-1 promoter produced from both the plus and minus DNA strands, consistent with the possibility of dsRNA production and processing by RDE-4 and Dicer (Figure 7D and Text S1). Moreover, we detected an elevated level of this RNA in the rde-4 mutant (Figure 7E), further supporting the possible involvement of RDE-4 in the dsRNA processing.

Unfortunately, pdk-1 promoter-specific endo-siRNAs are expressed at a very low level, and we were not able to reliably detect them by RT-qPCR. Nevertheless, additional evidence for pdk-1 regulation by endogenous RNAi comes from the observation that pdk-1 mRNA levels are increased in drh-3(ne2453), a loss-of-function mutant in dicer-related helicase 3 [38], (Figure 8B). DRH-3 is thought to participate in multiple branches of endogenous RNAi in C. elegans [38].

RNA polymerase II occupancy at the pdk-1 coding region is increased in zfp-1(ok554) and rde-4(ne299)

We have shown that both zfp-1 and rde-4 affect the longevity of C. elegans and its ability to resist oxidative stress and that pdk-1 mRNA levels are elevated in zfp-1(ok554) and rde-4(ne299) ([19] and Figure 1 and Figure 3). Furthermore, we have demonstrated that ZFP-1 binds the pdk-1 promoter and that endogenous siRNAs also have a potential to regulate pdk-1 directly. Next, we analyzed RNA polymerase II (Pol II) occupancy at the pdk-1 promoter and coding region by ChIP in wild type, zfp-1(ok554) and rde-4(ne299) L3-L4 animals and found it to be significantly increased in both mutants (Figure 8C, 8D). Consistent with transcriptional regulation, pdk-1 pre-mRNA levels were elevated in both mutants as well (Figure 8E). RDE-4, and therefore rde-4-dependent endo-siRNA production, did not affect ZFP-1 localization to the pdk-1 promoter (Figure 8A). It is possible that ZFP-1 and the RNAi machinery are independently recruited to the same targets and cooperate in inhibiting their transcription. Alternatively, ZFP-1 may help stabilize downstream RNAi factors at the endo-siRNA target genes.

Pol II levels increased only at the promoters, but not at the coding regions of indirect target genes expressed lower in zfp-1(ok554) and rde-4(ne299) (Figure 8C, 8D), a signature consistent with a slower rate of transition from transcriptional initiation to elongation [43]. This finding reflects the lower expression of these genes in the mutants, although they are not regulated directly by ZFP-1 and do not belong to the group of prevalent endo-siRNA targets ([19] and Table S1).

We have previously described a very significant overlap between genes misregulated in zfp-1(ok554) and genes misregulated in rde-4(ne299) and noted that the level of expression of zfp-1 mRNA did not change in rde-4(ne299) and vice versa [19]. Since the rde-4 mutation has milder effects on gene expression than zfp-1(ok554), they could potentially be due to zfp-1 misregulation. Therefore, we further confirmed that protein levels of ZFP-1 are not decreased in rde-4(ne299) (Figure S3).

ZFP-1 localizes to the pdk-1 promoter and both the pdk-1 mRNA level and Pol II occupancy at the pdk-1 gene are increased
Figure 7. ZFP-1, dsRNA, and siRNAs localize to the promoter of pdk-1. (A) A screen shot of the pdk-1 locus generated using the UCSC browser and indicating ZFP-1 localization peaks (ChIP/chip modENCODE data), cloned endo-siRNAs from [38] - (WT1) and [37] - (WT2), and promoter regions 1 and 2 with detected bi-directional transcription shown in (D). Antisense siRNAs are indicated in red, sense siRNAs in blue. (B) ZFP-1 ChIP/RNAi Modulates Insulin Signaling in C. elegans.
in zfp-1(ok554). These findings strongly suggest that transcription of pdk-1 is directly and negatively modulated by ZFP-1. Our genetic and molecular data also clearly demonstrate that rde-4 has a role in the transcriptional regulation of pdk-1. Several lines of evidence provide correlative support for a possible direct role of endo-siRNAs in pdk-1 regulation: endo-siRNAs match the pdk-1 promoter in a region also targeted by ZFP-1, dsRNA production is detected at the promoter and is increased in rde-4(ne299), and pdk-1 mRNA levels are elevated in at least two RNAi pathway mutants. However, since the endo-siRNAs targeting pdk-1 are not very abundant, we were not able to determine whether they change in rde-4(ne299), and there is a possibility that rde-4 affects pdk-1 transcription indirectly. In either case, RDE-4 is most likely involved in gene regulation through endo-siRNA production since this is the only known molecular function of this protein. The relatively more abundant endo-siRNAs matching the pdk-1 promoter (Figure 7A) are not unique and correspond to the repeat sequences. The Argonaute proteins that bind endo-siRNAs and work downstream in the RNAi pathways have been described and include at least two separate branches: the CSR-1 branch [37].
and the WAGO branch [38]. Although CSR-1-bound endo-
siRNAs are enriched in sequences antiseen to protein-coding
genes, they also include endo-siRNAs matching repeats [37], while
the WAGO system appears to preferentially target repeats and
pseudo genes [30]. Both the WAGO and CSR-1 systems have
been shown to have some connection to the RDE-4-regulated genes
[36,44], and ZFP-1 ChIP/chip targets are enriched in both WAGO and
CSR-1-dependent endo-siRNA target gene sets (G. Cecere, M. Jensen, et al., manuscript in preparation). Therefore,
we think that regulation of some endogenous genes, such as pdk-1,
which contain simple repeats in their promoters, may have evolved
to depend on the RNAi surveillance system, either WAGO or
CSR-1-based.

Discussion

ZFP-1/AF10 and resistance to oxidative stress

This work has revealed a direct repression of pdk-1 transcrip-
tion by C. elegans AF10 homolog ZFP-1 and the significance of
this transcriptional regulation in modulating insulin signaling.
We have demonstrated that overexpression of ZFP-1 leads to
enhanced resistance to oxidative stress in nematodes in a DAF-
16-dependent manner. The role of DAF-16/FOXO in longevity
and stress response is conserved in animals [45], and it would be
interesting to see whether AF10 has a role in promoting stress
resistance through the activation of FOXO. FOXO proteins have
been shown to cause a neuroprotective effect in C. elegans, Drosophila
and mammalian models of neurodegeneration [46].
Another transcription factor involved in the antioxidant response,
Nrf2 – a homolog of C. elegans SKN-1 - has been implicated in the
neuroprotection of motor neurons in a mouse model of ALS [47],
while SKN-1 was shown to be important for protection of
dopamine neurons against methylnitrogen-induced degeneration
in C. elegans [48]. Since both DAF-16 and SKN-1 are negatively
regulated by insulin/P13K signaling in C. elegans [45] (Figure 2C),
perhaps inhibition of this signaling pathway in mammalian
neurons will lead to activation of both FOXO3α and Nrf2. Our
work suggests that the homolog of ZFP-1, AF10, may have a
neuroprotective effect by indirectly activating FOXO3α and
Nrf2 if the regulation of pdk-1 by ZFP-1/AF10 is conserved in
animals.

Endogenous RNAi in gene expression regulation

RNAi was discovered in C. elegans as a response to exogenously
introduced dsRNA [3,49] and was considered to be primarily an
anti-viral mechanism also directed against repetitive elements [50],
especially since the first RNAi-resistant mutants did not have
obvious developmental phenotypes [3]. The discovery of mutants
in RNA-dependent RNA polymerase (RdRP) genes that displayed
developmental phenotypes [51,52] and were either RNAi-resistant
[51] or more sensitive to exogenous RNAi [52], highlighted the
possibility that RNAi may be used for regulating endogenous
genes. Indeed, endogenous siRNAs antiseen to protein-coding
genes and similar to those produced during exogenous RNAi were
discovered first in the worm [15] and then in other animals [53]. It
became apparent that in mutants lacking specific endo-siRNAs,
corresponding mRNAs become upregulated [14,34,53], and
microarray and deep sequencing approaches have been used for
identifying genes that change expression in the RNAi mutants
[13,14,19,37–39,44,56–58]. However, the significance of mis-
regulation of specific genes for the biology of the worm has not been
clearly demonstrated and phenotypes described for RNAi-related
mutants [13,37,51,54–61] were not connected to specific targets by
functional epistasis experiments. This study interprets the
microarray signature of zfp-1 and nde-4 mutants, demonstrates
short lifespan and stress sensitivity phenotypes consistent with the
signature, and provides functional evidence that pdk-1 is a major
target responsible for these phenotypes through genetic epistasis,
RNA expression and CHIP analyses.

RNAi in C. elegans has the potential to cause both post-
transcriptional [49] and transcriptional [6,62] gene silencing. It is
possible that endogenous RNAi utilizes multiple mechanisms and
that some genes are subject mostly to post-transcriptional
regulation while others are regulated at the transcriptional level;
the latter are likely to have fewer matching endo-siRNAs to the
coding region and relatively more promoter-specific endo-siRNAs.
like pdk-1. We surveyed the genes upregulated in rde-4 (n299) for
an endo-siRNA signature similar to that of the pdk-1 locus and
found a number of examples (Figures S4 and S5). Interestingly,
most of these types of genes, including pdk-1, have repetitive
elements at the promoters and endo-siRNAs matching them. It
appears that a modulating effect of RDE-4 on the transcription of
some endogenous genes is linked to the control of repetitive
elements.

RNAi-dependent silencing of long terminal repeats (LTR) and
non-coding RNA genes located in euchromatic regions that
functions with trace amounts siRNAs has been described recently
in S. pombe [63]. The lack of abundant siRNA species was
remarkable, considering that Dicer and RdRP interacted physi-
cally with the loci and that LTR transcript levels were significantly
elevated in the dcr-1, ago1 and rdpl mutants. This type of RNAi-
based regulation appears to be very similar to that operating on the
pdk-1 gene in C. elegans that we describe here.

Examples of genes regulated by RNAi through repetitive
elements in promoters already exist in Arabidopsis and include the
FWA gene, which affects flowering time [64,65] and, more
recently, an extracellular peroxidase Ep5C gene [66]. High levels
of Ep5C promote susceptibility to Pseudomonas syringae and mutation
in the Argonate 4 gene was recovered in an unbiased screen for
increased susceptibility to infection [66]. It is interesting that both
in plants and animals regulation of endogenous genes by RNAi has
evolved to promote fitness.

Materials and Methods

C. elegans mutant and transgenic strains

Strains were maintained at 20 °C unless otherwise noted, using
standard methods [67]. The following mutants were used: LGE:
daf-16(mu86), daf-16(mgDf50), LGH: age-1(hx546), LGII: daf-
2(e1370), rde-4(n299), zfp-1(ok554), LGX: pdk-1(sa709).

Compound mutant strains and transgenes used are as follows:
CF1595: daf-16(mu64); daf-1/e1370III, AGK138: zfp-1(ok554)III;
pdk-1(sa709)X, AGK241: rde-4(n299)III; pdk-1(sa709)X, AGK25:
age-1(hx546)II; zfp-1(ok554)III, AGK264: age-1(hx546)II; rde-4
(ne299)III, AGK72: daf-16(mgDf50); armEx5, T356: zls536 IV,
AGK30: zfp-1(ok554)III; zls536 IV, AGK262: zfp-1(ok554)III;
zls536 IV; pdk-1(sa709)X, AGK377: rde-4(n299)III; zls536 IV,
AGK265: rde-4(n299)III; zls536 IV; pdk-1(sa709)X, AGK267: zfp-
1(ok554) unc-119(ed3)III; armEx5, AGK284: rde-4(n299) zfp-1
(ok554) unc-119(ed3)III; armEx5, AGK260: zls536 IV; pdk-1
(sa709)X, SP940: unc-32(e444)II; UNC-1(e539)X; maDp11;III(Xi).

Transgenic worms were created by microparticle bombardment
using a PDS-1000 Hepta Apparatus (Bio-Rad) [68]. All strains
were made by co-bombardment of both a fosmid of interest and
plasmid pMM016b (AddGene) for unc-119(ed3)III rescue. Strains
created are as follows: AGK29: armEx2 [unc-119+] – control
strain, AGK128: armEx5 [zfp-1::FLAG,unc-119+], AGK26:
armEx5 [zfp-1::GFP,unc-119+].

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Recombinant fosmid construction

The WRM0629 GD09 fosmid containing the ZFP-1 locus was obtained from the *C. elegans* fosmid library generated by *C. elegans* Reverse Genetics Core Facility, Vancouver, B.C., Canada.


We generated derivative fosmid constructs to express recombinant ZFP-1 protein tagged with GFP or FLAG at the C-terminal portion of the protein by a fosmid recombineering method as described by [34].

Oxidative stress assays (paraquat sensitivity)

Paraquat sensitivity assays were done essentially as described by [69]. L4 animals were transferred from NGM agar plates into 24-well plates (10 per well) containing 300 μL of 100 mM paraquat dissolved in M9. Worms were then incubated at 20°C and scored for survival after 20 hours. Dead animals were scored by their continuous absence of swimming movements and pharyngeal pumping. A t-test between two means was used to calculate statistical significance.

Lifespan assays

Assays were performed as described by [70]. Worms were kept at 20°C on NGM plates (10 animals per plate). Day of hatching was used as the first time point. Dead animals were scored as dead when they refused to move after repeated prodding with a pin. Animals that crawled away from the plate, exploded, or contained internally hatched worms were excluded from the analysis. Life spans were determined in parallel for all strains shown together on graphs. Statistical significance was determined by a log-rank analysis using Prizm software.

*P. aeruginosa* infection

*C. elegans* survival assays were performed as described earlier [36]. To avoid the confounding effects of varying brood sizes, egg laying rates and progeny hatching within the infected worms on worm mortality, we used worms rendered sterile by RNAi of *pos-1*, loss of which results in inviable embryos [71,72]. Worms that died due to desiccation on the walls of the Petri dish or due to bursting vulva were censored from further analysis. Kaplan-Meier non-parametric survival analysis was performed using the software Statview (Version 5.0.1 SAS Institute Inc.). *P*<0.001 was considered significantly different than wild type.

Analysis of the nuclear localization of DAF-16::GFP

Since the addition of the DAF-16::GFP transgene to the *zfp-1* (ak354); *pdk-1* (sa709) double mutant strain led to a penetrant dauer phenotype at 20°C, all DAF-16::GFP strains were maintained at 16°C. L4 and adult stage worms were used for scoring nuclear localization. Worms were mounted on agarose pads and DAF-16::GFP localization was assessed in 10–20 worms at a time using 200X magnification on a Zeiss AxioImager Z1 immediately, higher magnification images of DAF-16::GFP at a time using 200X magnification on a Zeiss AxioImager Z1 were done for 40 cycles in a two-step cycling, according to the manufacturer’s instructions, with 25 μL of reaction containing 12.5 μL SYBR master mix, 0.15 μL of 100 μM primers, 5 μL of diluted cDNA, and 7.2 μL dH2O. Each PCR reaction was performed in triplicate. We used the ΔΔCt method to quantify the change in mRNA expression in the mutant samples compared to wild type and act-3 mRNA was used as a reference gene. The primers used were as follows: Forward CAGCAGACCTTCATTACAACTGCC and Reverse GCATACTGTCAGGAATTC for act-3 mRNA detection, Forward AGCCATCAACCCGTCTTACAG and Reverse CGAAATGCGCCGCGTGTCTTC for *pdk-1* mRNA detection, Forward GGCGACTGTCAGGAGT and Reverse GCAGAGAAGCAGCAGCAAACG for *aqp-1* mRNA detection, Forward ATGCCTGTCGTCTCTGC and Reverse GACTGACCGGACCTTC for *pdk-1* mRNA detection, Forward TACGCAGAGTAGTGGGAAGA and Reverse CGAATTCGCGAGAAGATA for *gst-3* mRNA detection, Forward TTTCGAAATACGACGACAC and Reverse TGGCGATACATGGTCAGAAGA for *zfp-1* mRNA detection, Forward ACTAATTAAGCGGCGAAC and Reverse AGTGGCGCAATCTCCCAAATGC for sod-3 mRNA detection, Forward pdk-1 ex2-ex3 junction CCTGATCCGAGGGATTCG and Reverse pdk-1 intron 3 ACAAGTGATTTTTGGTGGG-TCC for detecting the mutant sa709 pdk-1 mRNA and pre-mRNA and Reverse pdk-1 ex3-junction GATCAGCGAAATATTTGCGCTGGA for detecting the wild-type pdk-1 mRNA.

For detection of bi-directional transcription at the *pdk-1* promoter the primers used were as follows. Region 1 RT primers: detecting (-) strand transcript CCGAGGTATATAATTTTGGCTAAAACTT; detecting (+) strand transcript ATCAAGAGATACGGCGGGAG. Region 1 PCR primers: forward- CCGAGTTATAAACAGCACCA reverse- GGTGTCACTTGGAATATCAGCGG

Region 2 RT primers: detecting (-) strand transcript CTCGCCGTGTATCTTTGATT detecting (+) strand transcript GTACGTTTGTATTGCTTTCAAGG

Region 2 PCR primers: forward - GAATGTTCGAAAACCTTAAAGG reverse – AGGGATAATGGAGGTAGCAGTG

Chromatin immunoprecipitation (ChiP)

Chromatin immunoprecipitation was performed following the modENCODE Protocol from the Lieb Lab with the following modification: 2.5–3mg of cross-linked extract from L3 or adult worms was incubated for 1h at 4°C with the specific antibody and the immune complexes were then incubated with 60 μl IgG Dynabeads (Invitrogen) for 1h at 4°C. DNA was cleaned up with the Qiagen PCR purification kit. For the FLAG ChiP, we incubated the cross-linked extract with ANTI-FLAG M2 Affinity Gel (Sigma) for 2h at 4°C and, after the washing steps, eluted with 300 μg/ml of FLAG peptide (Sigma) for 30min at 4°C. The other antibodies used were anti-ZFP-1 (generated by the Lieb Lab) and anti-Poly II 8WG16 (Covance).

The immunoprecipitated DNA was quantified by qPCR using the ΔΔCt method to calculate the percentage of immunoprecip-
iation relative to the input. We used the following specific primers:
Forward AAACAACATAGACTTGTGCC and Reverse GTACGGTGTATGCTCTTC to amplify the promoter region of the pdk-1 gene; Forward pdk-1 ex2 GCAAGTGAATCGGAGAAGAG and Reverse pdk-1 ex2 TGAAGAAACATGAGTGCTTGG to amplify the coding region of the pdk-1 gene; Forward TTCAGAATTATCATGCGAGG and Reverse TCTCTGAGGCAAACATTGGAGG to amplify the promoter region of the app-1 gene; Forward app-1 ex5 TTGCAAGCATATC- CATCTCACA and Reverse app-1 ex5 CTCCTGCAATATTAA- CAAACCTAG to amplify the coding region of the app-1 gene; Forward TTAGATAGAAGATTGGCGAGG and Reverse CAAGTAGCAAAGCGATAAACC to amplify the promoter region of the. For

modENCODE protocols
To investigate the function of ZFP-1 with ChiP we first developed an antibody (termed JL00006_ZFP1) to the C-terminal portion of the protein. Alternative transcription start sites give rise to two ZFP-1 protein isoforms with identical C-terminal domains. As expected, both isoforms are recognized by the JL00006_ZFP1 antibody. The protocols used for generating ZFP-1::FLAG and ZFP-1::GFP transgenic strains. (TI)

Determining genes bound by ZFP-1
C. elegans genes (refSeq id) from genome build CE4 (ws170) were extracted from the UCSC genome browser’s refGene table. A gene was called bound by ZFP-1 if the center base pair of a ZFP-1 peak overlapped the ORF or the 1,500 bp upstream region. Overlap calls were done using the Galaxy web tool. Of the total 24,901 genes, 3,598 were bound by ZFP-1. Genome-wide ZFP-1 localization data are available at modENCODE: http://intermine.modencode.org/.

Supporting Information
Figure S1 Increase in pdk-1 expression correlates with susceptibility to oxidative stress. (A, B) Survival of L4 larva (n = 90) from indicated strains after 20 hour incubation period in 100 mM paraquat; ** indicates significance of P<0.01 and * - P<0.05 compared to wild type. (C) RT-qPCR detecting an increase in pdk-1 expression in SP940, results of three biological replicates are shown, error bars represent standard deviation. (TI)

Figure S2 RT-qPCR confirming enhanced expression of zfp-1 mRNA in ZFP-1::FLAG and ZFP-1::GFP transgenic strains. (TI)

Figure S3 ZFP-1 levels do not change in rde-4(ne299). Western blot analysis with anti-ZFP-1 C-terminal antibody; actin levels and Poncet S staining of the membrane are shown as loading controls. (TI)

Figure S4 An example of a gene with promoter siRNAs matching repetitive elements and expressed higher in rde-4(ne299) according to [19]. A screen shot from the UCSC browser and PollChIP results showing enhanced occupancy in rde-4(ne299). (TI)

Figure S5 Examples of genes with promoter siRNAs matching repetitive elements and expressed higher in rde-4(ne299) according to [19]. Screen shots from the UCSC browser. (TI)

Table S1 Dataset table allowing identification of overlaps between the following datasets: microarray data listing genes misregulated in rde-4(ne299) and zfp-1(ok554) from [19], micro-array data of Class 1 and Class 2 genes acting downstream of daf-16 from [23], rde-4-dependent siRNA target genes identified by [38], and ZFP-1 target genes where the ZFP-1 peak was found in the 1,500bp promoter window identified by ChiP/chip; functional mount and category groups from [73]. (XLSX)

Text S1 Sequencing results for RNA sequences produced from the pdk-1 promoter and shown in Figure 7D. (DOC)

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Author Contributions
Conceived and designed the experiments: AG ARM GC MBJ TK M-WT JDL. Performed the experiments: ARM GC MBJ TK LMK VC AG. Analyzed the data: SH MBJ ARM GC AG. Contributed reagents/materials/analysis tools: MBJ JDL. Wrote the paper: AG.

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RNi Modulates Insulin Signaling in C. elegans