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Endogenous RNAi and adaptation to environment in *C. elegans*

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Abbreviations: dsRNA, double-stranded RNA; endo-siRNA, endogenous short interfering RNA; ZFP-1, zinc finger protein 1; AF10, acute lymphoblastic leukemia 1-Fused gene from chromosome 10; RDE-4, RNAi deficient 4; PDK-1, 3-phosphoinositide-dependent kinase 1; RdRP, RNA-dependent RNA polymerase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; DAF-16, DAF-18, abnormal dauer formation; SKN-1, skinhead; PPTR-1, protein phosphatase 2A regulatory subunit; CSR-1, chromosome-segregation and RNAi deficient; WAGO, worm-specific AGO (argonaute); NRDE, nuclear RNAi defective; piRNA, piwi-interacting RNA

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The contributions of short RNAs to the control of repetitive elements are well documented in animals and plants. Here, the role of endogenous RNAi and AF10 homolog ZFP-1 in the adaptation of *C. elegans* to the environment is discussed. First, modulation of insulin signaling through regulation of transcription of the PDK-1 kinase (Mansisidor et al., PLoS Genetics, 2011) is reviewed. Second, an siRNA-based natural selection model is proposed in which variation in endogenous siRNA pools between individuals is subject to natural selection similarly to DNA-based genetic variation. The value of *C. elegans* for the research of siRNA-based epigenetic variation and adaptation is highlighted.

Introduction

RNA interference was discovered in *C. elegans* as a gene silencing phenomenon induced by double-stranded RNA (dsRNA) that was introduced by injection or by the feeding of bacteria that express dsRNA.[1,2] Shortly, RNAi was shown to exist in organisms ranging from fission yeast to humans and to be similar to the phenomenon of repetitive transgene silencing discovered in plants.[3,4] The two major steps in the RNAi process are: (1) generation of short 21–30 nt interfering RNAs (siRNAs) and (2) targeting of specific cellular RNAs by siRNAs complementary to these targets, resulting in gene silencing through a variety of different mechanisms.

The first mutants deficient in the RNAi response, *rde*, did not have obvious developmental abnormalities.[5,6] However, some of them exhibited mobilization of transposons in the germline,[5,6] a phenotype consistent with the view of RNAi as a defense mechanism against viruses and repetitive DNA elements. Later, it was discovered that some RNAi factors, such as the dsRNA-specific ribonuclease Dicer, do have a role in development.[7-9] This role was shown to be in the processing of the hairpin precursors of the short RNAs *lin-4* and *let-7*,[7,8] which were known to regulate developmental timing.[10,11] *lin-4* and *let-7* were the first examples of a class of endogenous RNAs derived from hairpin precursors and named microRNAs (miRNAs).[12-14] It became evident that miRNAs are not the only endogenous small RNAs in *C. elegans*[15] with the discovery of increasing numbers of endogenous siRNAs (endo-siRNAs) similar to the siRNAs generated during experimental dsRNA treatment.[15-20] Endo-siRNAs are short interfering RNAs that are largely generated by RNA-dependent RNA polymerases (RdRP) and are perfectly antisense to the sequences of thousands of coding genes.[15-20] Recent studies have identified endo-siRNAs perfectly complementary to coding mRNAs in flies[21-24] and mammals,[25,26] which means that these short RNAs are not limited to organisms containing RdRP genes, such as *C. elegans*. Despite the progress of RNAi research and the discovery of increasing numbers of pathways regulated by microRNAs, our understanding of the biological roles of RNAi processes mediated by endo-siRNAs is limited.

RDE-4 and ZFP-1 Regulate Endo-siRNA Targets

In order to find genes potentially regulated by endogenous RNAi, we conducted an mRNA expression profiling study[27] using mutants that affect RNAi-induced transcriptional gene silencing.[28] We chose

loss-of-function mutants in two genes: *rde-4* and *zfp-1,* which are predicted to act in the initiation of RNAi and downstream in the pathway, respectively. RDE-4 is a dsRNA-binding protein and a component of the Dicer complex required for siRNA production.[29] It acts upstream in the RNAi pathway.[30] ZFP-1 is a chromatin factor homologous to human AF10[31] and may mediate the repressive effect of siRNAs on their target genes directly.

Our analysis revealed that *zfp-1* and *rde-4* mutant animals have strikingly similar profiles of misregulated genes; close to 250 genes are commonly regulated by both *zfp-1* and *rde-4*.[27] This functional link between upstream and downstream RNAi factors indicated the possibility of a significant role for RNAi-induced chromatin silencing in the regulation of endogenous genes. Additional analysis of the microarray data further supported a direct role for RDE-4 and ZFP-1 in the regulation of RNAi targets: several studies had reported the cloning of hundreds of endogenous siRNAs antisense to the protein coding genes,[15,19,20] and we found a statistically significant enrichment of genes with siRNAs in the sets of genes upregulated in *zfp-1* (p value 10^{-22}) and *rde-4* (p value 10^{-19}) mutants, but not among the downregulated genes.[27] These data strongly suggested that the genes upregulated in the studied mutants may represent direct targets repressed by RNAi. The downregulated genes might be affected by the mutations indirectly. The genome-wide localization of ZFP-1 is consistent with its direct role in negatively regulating endo-siRNA targets[32] (Cecere et al., in preparation).

Modulation of Insulin Signaling by ZFP-1 and RDE-4

Functional analysis of genes misregulated in the *zfp-1* and *rde-4* mutants revealed a connection to stress response and longevity. First, translation-related genes targeted by endogenous siRNAs were notably upregulated in *rde-4* and *zfp-1* mutant animals.[27] Second, metabolic genes expressed in the intestine, which promote longevity and resistance to oxidative stress,[33] were downregulated in the same mutants.[27,32] Inhibition of translation[34-36] and the activation of genes encoding proteins combating oxidative-stress damage, such as superoxide dismutase,[37] are essential for fitness in unfavorable conditions. Since the gene expression signature in the *rde-4* and *zfp-1* mutants was the opposite of that favored during stress, it suggested that these mutants should be deficient in stress responses. Indeed, we found them to be short-lived,[32] consistent with previous reports,[38,39] and sensitive to oxidative stress (paraquat) and pathogens (*P. aeruginosa*).[32]

Although ZFP-1 and endogenous RNAi factors inhibit a number of genes whose regulation may contribute to increased fitness, we found that downregulation of the 3-phosphoinositide-dependent kinase-1 (PDK-1)[40] by ZFP-1 and RDE-4 is most significant for the normal life span and stress resistance of *C. elegans*.[32] Indeed, the short lifespan and increased stress sensitivity of the *zfp-1* and *rde-4* mutants is fully suppressed by the loss-of-function mutation in *pdk-1*.[32] PDK-1 is a conserved kinase activated in response to insulin and phosphatidylinositol (PI3), whose major targets are AKT kinases[40] (**Fig. 1**). In *C. elegans*, activation of AKT-1/2 through the insulin-signaling pathway leads to the phosphorylation and inactivation of DAF-16/FOXO[41,42] and SKN-1/Nrf,[43] the key transcription factors promoting stress response and longevity (**Fig. 1**). Therefore, modulation of the insulin-signaling pathway has a large impact on the global transcription profile of an organism. The best-known factor antagonizing insulin and PI3K signaling is the lipid phosphatase PTEN (DAF-18 in *C. elegans*).[44] In addition, a serine/threonine protein phosphatase PPTR-1 has been shown recently to antagonize AKT-1 phosphorylation and activation[45] (**Fig. 1**). Our work demonstrates that transcriptional modulation of signal transduction components has a potential for inducing significant biological effects as well. The *zfp-1* gene has been previously identified as a direct target of DAF-16,[38] and we find that DAF-16 has a modest positive effect on *zfp-1* expression.[32] This connection suggests a possibility of a positive feed-forward loop, which can be induced in response to initial DAF-16 activation during stress (**Fig. 1**).[32]

Currently, mechanistic studies of the components of the multiple RNAi pathways in *C. elegans* are conducted largely independently of investigations of their biological roles. Many reports identify changes in the expression of endo-siRNA target genes in mutants deficient in endo-siRNA production.[17,18,46-51] It is often assumed that specific phenotypes of RNAi mutants are due to the combined effect of misregulation of a multitude of targets. We find that misregulation of just one gene, *pdk-1*, fully explains the reduced lifespan and stress sensitivity of *rde-4* mutant animals since *rde-4; pdk-1* double mutants are long-lived and stress resistant, like *pdk-1(sa709)*.[32] Therefore, it is important to conduct careful epistasis and/or rescue experiments when gene expression changes are thought to cause specific phenotypes.

The role of a dsRNA-binding protein RDE-4 in exogenous RNAi is relatively well understood: it is required for the generation of siRNAs.[29,30,52] However, its contribution to endogenous siRNA production is not so clear, as it is not uniformly required[47,51] and seems to participate in more than one pathway.[48,50] We have connected the biological phenotype of the

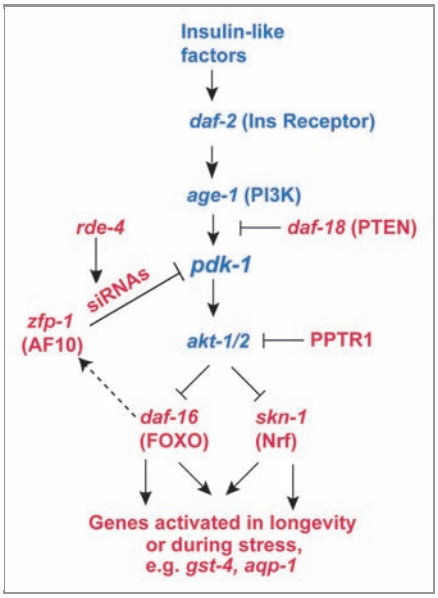

Figure 1. Insulin-signaling pathway and its modulators in *C. elegans* (adapted from Fig. 2C, Mansisidor et al., 2011). Factors promoting longevity and resistance to oxidative stress are shown in magenta font; factors restricting oxidative stress responses are shown in blue.

rde-4 null mutant to the regulation of the *pdk-1* gene. endo-siRNAs targeting *pdk-1* are very unabundant and mostly correspond to the repeat elements present at the *pdk-1* promoter.[32] Repeat-derived promoter endo-siRNAs have been documented for many other genes expressed higher in the *rde-4* mutants.[32] Therefore, repetitive elements in promoters appear to be utilized for gene expression regulation and adaptive responses in *C. elegans* similarly to several instances known in plants.[53,54]

Endo-siRNAs and Epigenetic Inheritance of Fitness

Endogenous RNAi in *C. elegans* targets both repetitive elements and euchromatic, often essential, genes.[16,17,48] Is there gene expression regulation by endogenous RNAi that is not connected to control of repetitive elements? The WAGO system of redundant Argonautes,[17] as well as the specific nuclear RNAi pathway of NRDE proteins,[55] appear to mediate genome surveillance, while gene-specific endo-siRNAs largely exist in a complex with CSR-1 Argonaute.[16] Consistently, we find that endo-siRNA target genes expressed higher in the *rde-4* and *zfp-1* mutant larvae[27] mostly represent CSR-1 targets (Cecere et al., in press).

The inhibition of gene expression by an RNA interference mechanism is the epigenetic equivalent of a genetic mutation. The existence of a large pool of endogenous short RNAs antisense to many genes (CSR-1-bound)[16] may provide a background of random epigenetic mutations present in individuals. If selective pressure is applied to a population of such organisms or cells, not only genetic, but also epigenetic variation could be subject to selection. Selection for epigenetic traits is faster and is also more flexible since it can be easily reversed.

I propose that siRNA molecules represent the effectors of epigenetic variation and selection and that changes in siRNA levels leading to corresponding changes in gene expression can be modulated by the environment to ensure maximum fitness of the organism (**Fig. 2**).

The model outlined in **Figure 2** predicts several important features of siRNA-based epigenetic adaptation to the environment: (1) background levels of endo-siRNAs specific to virtually any gene; (2) the ability of endo-siRNAs to significantly downregulate the expression of their corresponding gene; (3) heritability of endo-siRNAs; (4) differences between individuals in the composition of endo-siRNA pools. There are several lines of evidence supporting these predictions: (1) Cloned endo-siRNAs correspond to virtually every gene of *C.* elegans,[16-18,48] although some genes have thousands of them and others only a few. (2) An inverse correlation between the amount of endo-siRNA present and the mRNA expression level of the corresponding gene in *C. elegans* has been reported.[17,18,46-51] (3) RNAi is heritable in *C. elegans*[30,56,57] and characteristics of the heritable RNAi agent are consistent with those of siRNAs.[30] In addition, inheritance of functional Drosophila piRNAs specific for transposon sequences has been reported,[58] and a connection between epigenetic inheritance and sperm RNA exists in mice.[59] (4) Although there is no direct evidence to support the variation in endo-siRNA abundance or the variation in the composition of endo-siRNA pools between individuals, our analysis of RNAi inheritance noted a high degree of variation between siblings inheriting RNAi (Grishok 2001, thesis research). Instead of a large number of F2 progeny being affected moderately by RNAi, a relatively small number of individuals demonstrated a very high level of RNAi while others were virtually unaffected.

Epigenetic RNAi-based mechanisms are not likely to be limited to lower organisms and may be involved in the immune escape and drug-resistance of malignant tumors and in other cases where cells evolve to escape the action of therapeutic agents. The revelation that the epigenome in the form of short RNAs is capable of modulating the response of organisms to environmental stress may help elucidate new ways of adaptation to harsh environments, and *C. elegans* promises to be a perfect model organism for future discoveries in this exciting field.

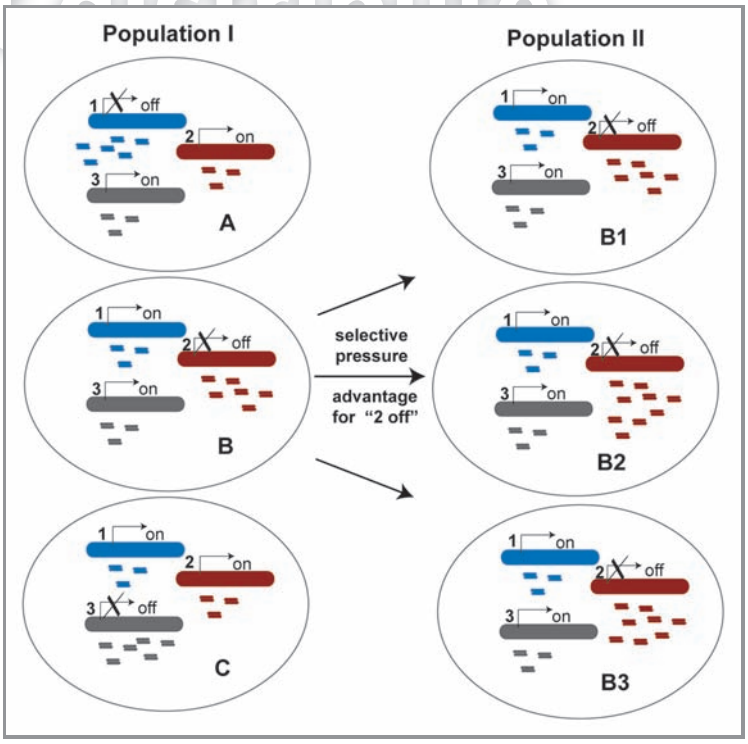

Figure 2. Model for the adjustment of endogenous siRNA pools and epigenetic gene expression regulation to environmental conditions. In population I there is a stochastic production and background variation in siRNA pools targeting genes 1, 2 and 3. In organism/cell A, the expression of gene 1 is reduced, in organism/cell B, gene 2 is, in organism/cell C, gene 3 is. When the environment changes to the advantage of organism/cell B, its progeny will dominate the ensuing population II.

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