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Whole genome microarray analysis of *C. elegans rrf-3* and *eri-1* mutants

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Abstract **We performed genome wide gene expression analysis on L4 stage *Caenorhabditis elegans rrf-3*(*pk1426*) and *eri-1*(*mg366*) mutant strains to study the effects caused by loss of their encoded proteins, which are required for the accumulation of endogenous secondary siRNAs. Mutant *rrf-3* and *eri-1* strains exhibited 72 transcripts that were co-over-expressed and 4 transcripts co-under-expressed (>2-fold, $P < 0.05$) compared to N2 wild type strain. Ontology analysis indicated these transcripts were over represented for protein phosphorylation and sperm function genes. These results provide additional support for the hypothesis that RRF-3 and ERI-1 act together in the *endo*-siRNA pathway, and furthermore suggests their involvement in additional biological processes.**

Keywords: RNA interference; RRF-3; ERI-1; Endogenous; RNAi; Gene expression; *C. elegans*

1. Introduction

Gene silencing through RNA interference (RNAi) pathways are mediated by small RNAs of ~21–24 nt in length found in virtually all eukaryotes [1,2]. This evolutionary conserved mechanism has likely evolved to protect organisms against invading exogenous nucleic acids but its endogenous roles are only beginning to be better understood [3–6]. Studies on *Caenorhabditis elegans* have revealed a wide variety of microRNAs (miRNAs) and endogenous small interfering RNAs (*endo*-siRNAs) with an apparent regulatory role for animal health and development [2,6–8].

In *C. elegans*, miRNAs enter their own pathway, while siRNAs enter specific pathways depending on the origin and molecular structure of the initial trigger molecule and specific downstream processing enzymes. The pathways for exogenous siRNA (*exo*-siRNA) and endogenous siRNA (*endo*-siRNA) uses in part, separate enzymes while sharing others including dicer (DCR-1) [7,8]. siRNA amplification has been shown to use alternative RNA-dependent RNA polymerases (RdRPs) for synthesis of secondary siRNAs to produce RNAi effects [7–9]. According to current models, the *endo*-siRNAs pathway uses the RdRP homolog RRF-3, while the *exo*-siRNA pathway uses RRF-1 [7,8]. Germ cells use a third RdRP homolog, EGO-1, for *exo*-siRNAs [7,8,10].

RRF-3 associates, in a large complex, physically with ERI-1, a conserved protein with DEDDh-like exonuclease and SAP/SAF-box nucleic acid binding domains in the *endo*-siRNA pathway [8,11]. Both of these proteins are required for accumulation of at least some *endo*-siRNAs [7,8]. Both mutants are sterile in 25 °C, exhibit high incidence of males (Him-phenotype) due to X-chromosome non-disjunction, and are hypersensitive to exogenous RNAi (enhanced RNAi, ERI-phenotype) [11,12]. The enhanced sensitivity to exogenous RNAi has been exploited in a number of whole genome RNAi screens and has thus led to widespread use of both *rrf-3* (RNA-dependent RNA polymerase-3) and *eri-1* (enhanced RNAi-1) mutants in gene silencing experiments [13]. It has been proposed that the ERI-phenotype is caused by release of limiting components from the endogenous to the exogenous RNAi pathway in these mutants [7,8].

Genome wide gene expression analysis was performed on *C. elegans rrf-3* and *eri-1* mutant strains to study transcriptional effects caused by loss of their encoded proteins in the endogenous RNAi pathway and to provide further insight into their functions. Mutant strains exhibited highly similar expression patterns and co-regulation of over-expressed transcripts was observed for genes encoding proteins associated with protein phosphorylation and sperm function. These results provide additional support for the model where RRF-3 and ERI-1 act in the same pathway, and suggests that they affect additional biological functions.

2. Materials and methods

2.1. Sample preparations

rrf-3(*pk1426*) mutant worms (NL2099), *eri-1*(*mg366*) mutant worms (GR1373), and wild type worms (N2) were grown on culturing plates containing NGM agar media with OP50 *Escherichia coli* (*E. coli*) as a food source [14]. Animals were synchronized by hatching purified eggs using 1% hypochlorite solution with 250 mM KOH. Worms were grown at 20 °C and harvested at the fourth larval (L4) stage. RNA isolation was made using Ribopure Total RNA isolation-kit (Ambion, Foster City, CA). Animals were grown separately and RNA isolated separately for each biological replicate (three for microarrays, three for quantitative real-time PCR). RNA samples were isolated separately for microarray, qRT-PCR, and Northern blot experiments. Worm stains were genotyped by PCR to confirm their identities (data not shown).

2.2. Microarray analysis

Purified total RNA was labeled for chip experiments using One cycle cDNA synthesis followed by cRNA synthesis according to the manufacturer's protocol (Affymetrix, Palo Alto, CA). cRNA from each

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mutant was hybridized to Gene Chip *C. elegans* whole genome arrays containing 22 500 transcripts (Affymetrix) as three biological replicates (3 chips each for N2, *rrf-3*, *eri-1*). Microarray hybridization and scanning was performed at the Biomedicum Biochip Core Facility (Helsinki, Finland). GeneSpring-software (Agilent, Palo Alto, CA) was used to carry out data-analysis. For the probe intensity values generated by the Affymetrix scanner, Robust Multichip Average (RMA) – algorithm was used for normalization and statistical processing. Data were then filtered to remove genes with low expression values (<10). *T*-test was performed to sequentially filter out genes with unreliable signal level between replicates ($P < 0.05$) and then to collect genes with significant signal level between samples ($P < 0.05$). Finally, genes were filtered for fold change (>2-fold up or <0.5-fold down).

2.3. Functional analysis

Annotations for the regulated genes were obtained from Affymetrix and supplemented with additional annotations from David 2.1 [15]. In the case that no known annotations existed, manual annotations were extracted from Wormbase. Lists of up- and down-regulated genes were uploaded to David 2.1. Calculations of overrepresented GO biological process annotations using functional annotation clustering was performed using the GOchart function. Significance cutoff was set at $P < 0.05$.

2.4. Quantitative real-time PCR (qRT-PCR) and Northern blot

qRT-PCR was performed using Power SYBR® Green PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturers instructions. Amplifications were performed on ABI Prism 7700 (Applied Biosystems). *Act-1* was used as a control to normalize transcript levels. PCR primers designed by Primer 3 [16] were as follows: *act-1* (left, 5′tcggtatgggacagaaggac, right 5′catcccagttggtgacgata); *ssp-16* (left, 5′ttaacggaggtgccgataag, right, 5′ tttgtcctcctttggtgctc); T16G12.7 (left, 5′tatggagcaaagggtggaac, right, 5′ccagagcagtgtacggcata); C35E7.9 (left, 5′gatggattctcgctggatgt; right, 5′attctccacaggcggtttc); F25B3.4, (left, 5′ctgacacgtcttattccacctg, right, 5′gtgagttgacctcaatgagca); *clec-69*, (left, 5′tggtggtgacagttcagagc; right, 5′agctggaagattggttgtgc). For the northern blot, an antisense probe for *ssp-16* transcript (*ssp-16*: 5′TGGTGCGAAATGAACGACAAGTTTGTCCTCCTTTG) and (*act-1*: 5′GGTGGTTCCTCCGGAAAGAACAGTGTTGGCGTACA) was labeled with α-[^{33}P]-dATP using terminal deoxytransferase, and hybridized to Northern blots containing total RNA (10 μg per lane) isolated from each strain. Northern blots were hybridized overnight at 42 °C in 5× SSPE, 50% formamide, 5× Denhardt's solution, 10% dextran sulfate, 1% SDS, and washed thrice at room temperature for 2 min in 4× SSC, 0.1% SDS, and thrice at room temperature for 1–2 min in 0.2× SSC, 0.1% SDS until there was no longer background signal, dried, and exposed on a phosphor screen overnight.

3. Results

3.1. Microarray and GO analysis

All microarray data used in this experiment is available in MIAME format at GEO database (www.ncbi.nih.gov/geo) (Accession: GSE8659). In the *rrf-3* mutant, we observed a total of 112 over- and 17 under-expressed genes, while in the *eri-1* mutant, 199 over- and 66 under-expressed genes were found (Fig. 1). An overlap of 72 over- and 4 under-expressed genes were identified. The list of overlapping over-expressed genes is shown in Table 1. Twenty-five co-over-expressed genes matched previously cloned siRNAs. Complete gene lists are shown in supplementary material Table S1 (available at http://www.uku.fi/aivi/neuro/genomics/rrf-3.shtml). Gene ontology analysis was performed with DAVID 2.1 using Affymetrix probe set identifiers to calculate statistically enriched GO (gene ontology) molecular function annotations for the overlapping over-expressed genes (Table 2). Complete GO annotations (molecular function, biological process, and cellular component) for all regulated gene lists are available

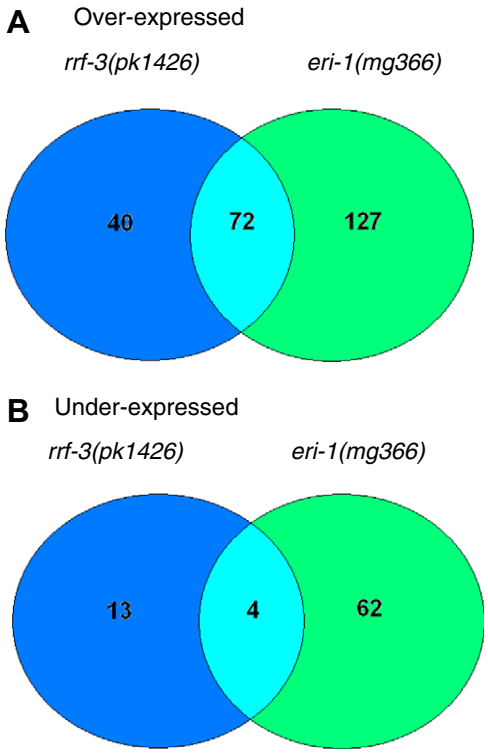

Fig. 1. Venn-diagram of >2-fold over-expressed (A) and under-expressed genes (B) in *rrf-3*(*pk1426*) and *eri-1*(*mg366*) mutants compared to N2.

in Table S2 (available at http://www.uku.fi/aivi/neuro/genomics/rrf-3.shtml). From the list of overlapping genes, we observed a statistically significant enrichment of both protein tyrosine ($P < 10^{-4}$) and serine/threonine kinases ($P < 10^{-11}$). From the list of down regulated genes, significant enrichment ($P < 0.01$) of oviposition and reproductive behavior were observed in *rrf-3* mutants and sugar/carbohydrate binding ($P < 0.05$) in *eri-1* mutants.

3.2. Confirmation with quantitative RT-PCR

Five genes were selected for confirmation by quantitative RT-PCR. These genes were chosen based on magnitude of change and common over-expression in *rrf-3* and *eri-1* mutants, and representing sperm specific proteins, phosphatases, and a lectin type protein. *Act-1* was used as a control. The microarray changes correlated well with the qRT-PCR, although the magnitudes of the changes were not always identical (Table 3). We also performed Northern blot analysis on *ssp-16* using *act-1* as a loading control and obtained results similar to those from qRT-PCR (Fig. 2).

4. Discussion

Recent studies indicate that RRF-3, an RNA-dependent RNA polymerase, and ERI-1, a conserved exonuclease are required for accumulation of endogenous siRNAs. Both proteins are physically linked in a complex with DCR-1 [7,8,17], and mutants share identical enhanced RNAi (ERI) phenotypes. To study gene expression effects caused by loss of these pro-

Table 1
List of 72 commonly over-expressed genes in *rrf-3*(*pk1426*) and *eri-1*(*mg366*) mutants vs. N2

Wormbase gene	Fold change *rrf-3* vs. N2	Fold change *eri-1* vs. N2	Annotation
Y106G6G.3	9.39	8.43	Dynein light chain motif
F59A6.2	7.49	5.57	
Y39E4B.11	7.02	5.74	
Y54G11A.13	6.13	6.83	*ctl-3*, catalase
T27A3.3*	5.93	6.38	*ssp-16*, sperm-specific class P protein 16
T16G12.7*	5.61	5.71	Protein phosphatase 1
W08E3.4	5.14	4.53	
ZK666.8	4.54	4.02	Serine/threonine kinase
F25B3.4*	4.54	4.52	Serine/threonine protein phosphatase
C38C10.3	4.52	3.88	
T01C3.5	4.51	4.23	
Y54G11A.6	4.47	5.01	*ctl-1*, catalase
F09C12.8*	4.35	3.68	Phosphoglyserate mutase motif
Y6E2A.9	4.28	3.00	Proton or iron transport? Sideroflexin?
F47B3.5*	4.27	3.96	
Y38H6C.15	4.24	3.40	
F09G8.4	4.11	3.95	*ncr-2*, homolog of human NPC1, which when mutated leads to Niemann-Pick disease
B0218.5	4.07	3.88	Kinase activity
C35E7.9*	3.88	4.09	H5 and major sperm protein domain protein
C25A8.5	3.88	3.84	Protein-tyrosine kinase
K07F5.6*	3.77	3.84	Protein-tyrosine phosphatase
ZC581.7*	3.69	3.35	Protein-tyrosine kinase
Y53F4B.45	3.66	3.90	
ZK1010.5*	3.53	3.48	
ZK484.7	3.49	3.17	Protein-tyrosine phosphatase
C49C8.1*	3.48	2.81	Serine/threonine kinase
C54G4.3*	3.46	3.39	
F58D5.7	3.38	3.22	
C47A4.5	3.37	3.30	
C05C12.1	3.30	3.14	Serine/threonine kinase
C10C6.3	3.27	2.28	
C33F10.8*	3.19	3.41	Protein-tyrosine phosphatase
F53C3.1	3.10	3.01	protein kinase
K07F5.4	3.07	2.86	*kin-24*, protein KINase family member
R05H5.4*	3.05	2.52	Tyrosine-protein kinase (FES/FPS subfamily)
ZC168.6	3.04	3.01	Sperm-specific protein
ZK507.1	3.03	3.92	Serine/threonine-protein kinase
F02C9.4	3.01	2.98	Protein-tyrosine kinase, EGF receptor activity
ZK973.8*	3.00	2.59	
C29E6.3*	2.99	3.09	*pph-2*, Protein PHosphatase family member
W03C9.1*	2.92	2.51	
F56F3.4	2.90	2.48	Ubiquitin family
ZC412.5*	2.86	2.61	
F54C8.1	2.82	2.78	Probable 3-hydroxyacyl-CoA dehydrogenase
F26F4.2	2.82	2.75	spt-2, novel protein with high similarity to *C. elegans* F37A8.1
W01B6.2	2.79	3.19	Serine/threonine kinase
F07F6.1	2.73	3.21	Nucleotide binding
ZK354.6	2.73	2.60	Protein kinase
F11G11.8*	2.71	2.06	*nspd-4*//*nspd-5*
F54A3.4	2.68	3.58	Homolog of the human gene CBS, which when mutated leads to homocystinuria
C24D10.1	2.68	2.54	Protein-tyrosine phosphatase
K01D12.15*	2.68	3.02	
F58D2.2	2.67	2.92	
Y71F9AL.2	2.62	2.78	Protein kinase
F59A3.8	2.58	2.88	Protein kinase
E03H12.7	2.55	2.82	
C39H7.1	2.47	2.26	Casein kinase, serine/threonine kinase
C04G2.8*	2.43	2.38	
F31E8.5	2.38	2.26	
C25G4.6*	2.34	2.56	PDZ domain (also known as DHR or GLGF)
C41H7.6	2.33	2.10	
ZK637.12	2.28	2.05	
C25G4.4	2.24	2.05	*tag-347*, SAND domain
T22B3.2	2.21	2.81	
F13G11.2	2.20	2.13	
F56A11.6*	2.16	2.06	
C24D10.7*	2.14	2.03	
Y38H8A.4	2.13	2.17	Serine/threonine kinase

Table 1 (*continued*)

Wormbase gene	Fold change *rrf-3* vs. N2	Fold change *eri-1* vs. N2	Annotation
B0205.10*	2.11	2.42	
C34F11.1	2.05	2.17	Protein and zinc ion binding
T21G5.4*	2.00	2.16	Protein binding
Y95B8A.4	2.00	2.20	

Values for fold change are the average from 3 independent biological replicates. Annotations were provided by Affymetrix when available and were supplemented manually by Wormbase and DAVID 2.1 gene ontologies. *, indicates match with cloned siRNA [7,20].

Table 2
Over-represented Gene Ontology (GO) molecular function terms associated with co-over-expressed gene list from *rrf-3* and *eri-1* mutant animals

GO term (molecular function)	Count	%	*P*-value
Protein kinase activity	17	22.08	3.43E−11
Phosphotransferase activity, alcohol group as acceptor	17	22.08	1.59E−10
Kinase activity	17	22.08	3.65E−10
Transferase activity, transferring phosphorus-containing groups	17	22.08	5.21E−09
Catalytic activity	32	41.56	5.32E−08
ATP binding	16	20.78	1.49E−07
Adenyl nucleotide binding	16	20.78	2.31E−07
Phosphoprotein phosphatase activity	9	11.69	2.64E−07
Nucleotide binding	17	22.08	1.53E−06
Purine nucleotide binding	16	20.78	1.57E−06
Phosphoric monoester hydrolase activity	9	11.69	1.69E−06
Phosphoric ester hydrolase activity	9	11.69	4.18E−06
Transferase activity	17	22.08	1.20E−05
Protein tyrosine phosphatase activity	6	7.79	6.81E−05
Protein-tyrosine kinase activity	7	9.09	1.48E−04
Protein serine/threonine kinase activity	7	9.09	2.56E−04
Hydrolase activity, acting on ester bonds	9	11.69	5.05E−04

GO term annotations were assigned and statistically evaluated using David 2.1. The count indicates the number of observations from the input of the 72 genes. Terms are listed in decreasing order of significance (*P*-value).

Table 3
Comparison of microarray and quantitative real-time PCR (qRT-PCR) results

Gene	Annotation	Microarray *rrf-3*(*pk1426*)	qRT-PCR	Microarray *eri-1*(*mg366*)	qRT-PCR
T27A3.3	Sperm-specific class P protein 16 (*ssp-16*)	5.9	12.5 ± 4.5	6.4	10.3 ± 5.5
T16G12.7	Protein phosphatase 1	5.6	8.9 ± 1.6	5.7	5.3 ± 1.5
C35E7.9	Sperm specific domain protein	3.8	5.7 ± 1.7	4.1	3.6 ± 0.9
F25B3.4	ser/thr protein phosphatase	4.5	4.9 ± 1.3	4.5	3.1 ± 1.7
F56D6.15	c-type lectin family member (clec-69)	Non-regulated	0.7 ± 0.9	0.2	0.19 ± 0.07

Values shown are normalized ratios ±S.D. from 3 biological replicates comparing N2 and *rrf-3(pk1426)* and *eri-1*(*mg366*) strains. Independent biological replicates were used for microarray and qRT-PCR experiments.

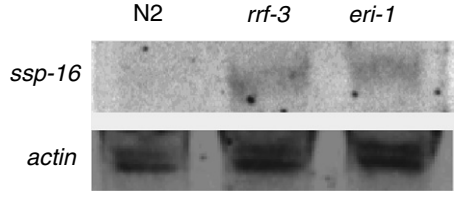

Fig. 2. Northern blot was performed to evaluate expression levels of the *ssp-16* gene. Total RNA (10 μg) from the indicated wild type (N2) or mutant strains (*rrf-3*(*pk1426*), *eri-1*(*mg366*)) was isolated, and probes were prepared, hybridized, and exposed as described in Section 2. *Act-1* was used as a control. Estimated sizes for the bands were 0.5 kb (*ssp-16*) and 2.0 kb (*act-1*).

teins, we performed microarray experiments on *rrf-3* and *eri-1* mutant *C. elegans* strains.

We observed relatively uniform gene expression patterns with a large amount of shared over-expressed genes in L4 stage *rrf-3* and *eri-1* mutants, suggesting a common biological function. Tyrosine and threonine/serine protein phosphatases and kinases were shown to be over-represented in genes at least 2-fold over-expressed in the mutants. This suggests a novel role for posttranslational protein modifications. The targets of these phosphatases and kinases have yet to be identified and could be within the *endo*-siRNA pathway or elsewhere. Secondary siRNAs are known to be triphosphorylated at the 5′ ribose moiety distinguishing them from DCR-1 processed primary siRNAs with 5′ monophosphates, indicating the importance of phosphorylation status in siRNA function [18,19].

Both strains share at least three over-expressed genes that are sperm specific and is consistent with the sperm defective high temperature sterility of these mutants [8,12]. Among the commonly over-expressed transcripts were three genes with Major Sperm Protein (MSP)-domain (*ssp-16*, C35E7.9 and ZC168.6). While the study here was performed on L4 worms raised at 20 °C, some germ or sperm cell perturbation may oc-

cur despite their superficially wild type appearance. This can be under consideration when using these strains for RNAi screens [13].

Loss of key *endo*-siRNA pathway proteins causes up-regulation of target mRNAs, likely due to the loss of complementary siRNA production [7,8,20]. Our results appear to support this notion, since at least 25 genes that were increased in *rrf-3* and *eri-1* vs. N2 have been cloned as *endo*-siRNAs [7,20]. This number is likely to be an underestimation and could increase when more *endo*-siRNAs are cloned and annotated. Alternatively, over-expression could occur through indirect non-siRNA mediated mechanisms.

While this manuscript was in preparation Lee et al. [7] reported the expression profiling of mixed stage population *rrf-3* and *eri-1* mutants along with several additional RNAi pathway related proteins. Comparison of 2-fold elevated transcripts revealed only a few matches between their and our study (*rrf-3*, 6/112; *eri-1*, 1/199; data not shown). The small number of common over-expressed genes from the previous and current study suggests large differences in gene expression in these strains that depend upon the developmental stage.

Our results suggest that the majority of gene expression levels are not significantly changed in *rrf-3* and *eri-1* mutant strains. Future RNAi screening studies can likely be performed with these strains without major concern for background effects. Co-regulation of a large number of genes provides supporting evidence that RRF-3 and ERI-1 function together, likely in the *endo*-siRNA pathway. Finally, our data suggests their novel involvement in additional biological processes including protein phosphorylation.

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