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Understanding Anti-viral Immunity Mechanisms in *C. elegans*

A Thesis submitted in partial satisfaction
of the requirements for the degree of

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in

Cell, Molecular, and Developmental Biology

by

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ABSTRACT OF THE THESIS

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Shruthi Satish

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C. elegans, a free-living nematode, forms an excellent model system to study several aspects of biology including host-pathogen interactions. In this study, we looked at factors involved in anti-viral immunity mechanisms in *C. elegans* with a prospect of being able to extrapolate into other systems. A reporter worm strain was used (MS1395), in which GFP expression directly indicates the extent of viral replication in the system. A feeding RNAi screen was performed to identify genes in anti-viral immunity and a total of 11,700 *C. elegans* genes were knocked down and screened for the presence/ absence of GFP. Of these genes, 2180 genes were screened by me. A total of 691 genes (6% of total number of genes) were found to be positive. The screen was repeated to eliminate false positives and 260 genes (2.2% of the total number of genes) were found to be positive. Meanwhile, discovery of a naturally infecting *C. elegans* virus, Orsay, by Felix et al., helped us further our research. A

subset of genes positive from the secondary screen was tested for susceptibility to Orsay infection. Chromosomal mutations for two of the genes identified by RNAi, *him-14(it44)* and *nhr-68(gk708)* showed increased susceptibility to Orsay infection. qRT-PCR, *in situ* hybridization and Northern blots showed higher levels of Orsay RNA1 molecules in these two mutants compared to the wild-type strain. We are currently in the process of understanding the roles played by these two genes in anti-viral immunity. *him-14* gene encodes a protein that is required for promoting crossing over between homologous chromosomes and therefore is expressed in the germ-line. Evidence suggests that the increased susceptibility to Orsay infection in *him-14(it44)* mutants is germ-line dependent. Prior reports have linked the germline to longevity, suggesting that *him-14* indirectly affects virus susceptibility by modifying some aspect of body physiology. *nhr-68* belongs to nuclear hormone receptor family and are transcription factors typically regulated by lipophilic molecules like steroids, retinoids, bile and fatty acids. *nhr-68* mutants also have increased fat content compared to wild-type animals. We hypothesize that the increase to Orsay susceptibility in these worms is due to increased lipid molecules in the body.

Currently, the lab is trying to understand the mechanism of action of these two genes in anti-viral immunity. We believe this study will provide a better understanding of the nature of host- pathogen interaction and will open a wide area of interesting biology for further investigation.

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CHAPTER 1. Introduction

1.1. RNA Silencing

RNA silencing is a mechanism through which gene expression is silenced and the silencing depends on the nucleotide sequence of RNA molecules (Carthew and Sontheimer, 2009). Long dsRNA molecules are recognized by a set of proteins in cells, leading to the formation of smaller dsRNA molecules called small RNAs (21-30 nucleotides). This process is called 'dicing'. These small RNAs are recognized by another set of proteins called the effector complex, essentially involving Argonaute protein (AGOs) and its complex, and they bring about RNA silencing in several ways such as inhibition of transcription/translation, promoting heterochromatin formation, RNA/DNA degradation. (Carthew and Sontheimer, 2009).

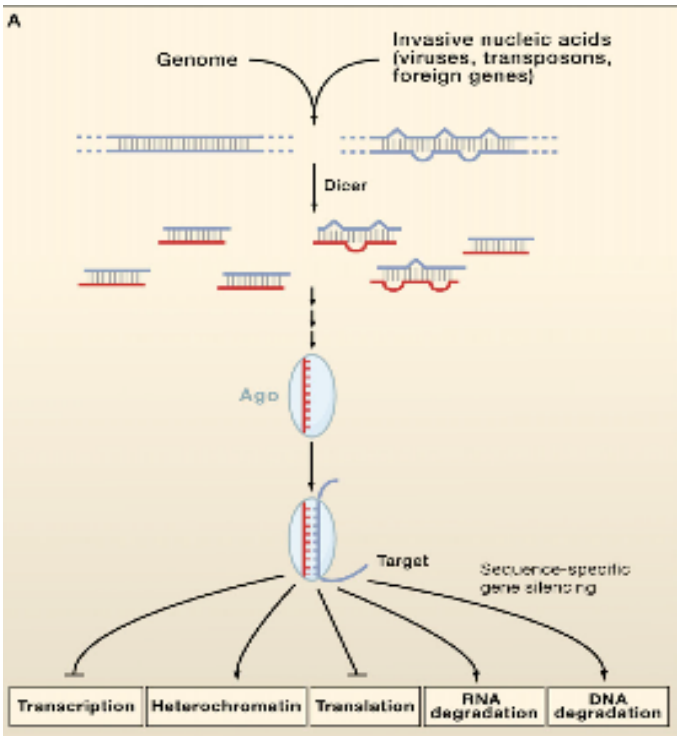


Figure 1: Types of RNA silencing (Carthew and Sontheimer, Cell, 2009)

There are mainly three types of small RNAs: micro RNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) (Carthew and Sontheimer, 2009). siRNAs and miRNAs are seen in somatic cells whereas piRNAs are primarily found in the germline cells of animals (Carthew and Sontheimer, 2009). The biogenesis of miRNAs and siRNAs in cells are different. siRNA is activated by long double stranded RNAs whereas single stranded RNAs with stem loops activate miRNA pathway (Carthew and Sontheimer, 2009). *Figure 2* shows the different types of small RNAs and their biogenesis. miRNA encoding genes, when transcribed, generate primary miRNAs. They get recognized by RNAi proteins to form precursor miRNAs and mature miRNAs. The RISC complex recognizes these miRNAs and brings about silencing of mRNAs complementary to it. siRNAs on the other hand are produced from long dsRNA and biogenesis of piRNAs is not very clear. piRNAs are mostly complimentary to transposon transcripts and are seen mostly in animal germline cells. In our studies, we performed a reverse genetic screen where we employed siRNA pathway to silence the genes of interest.

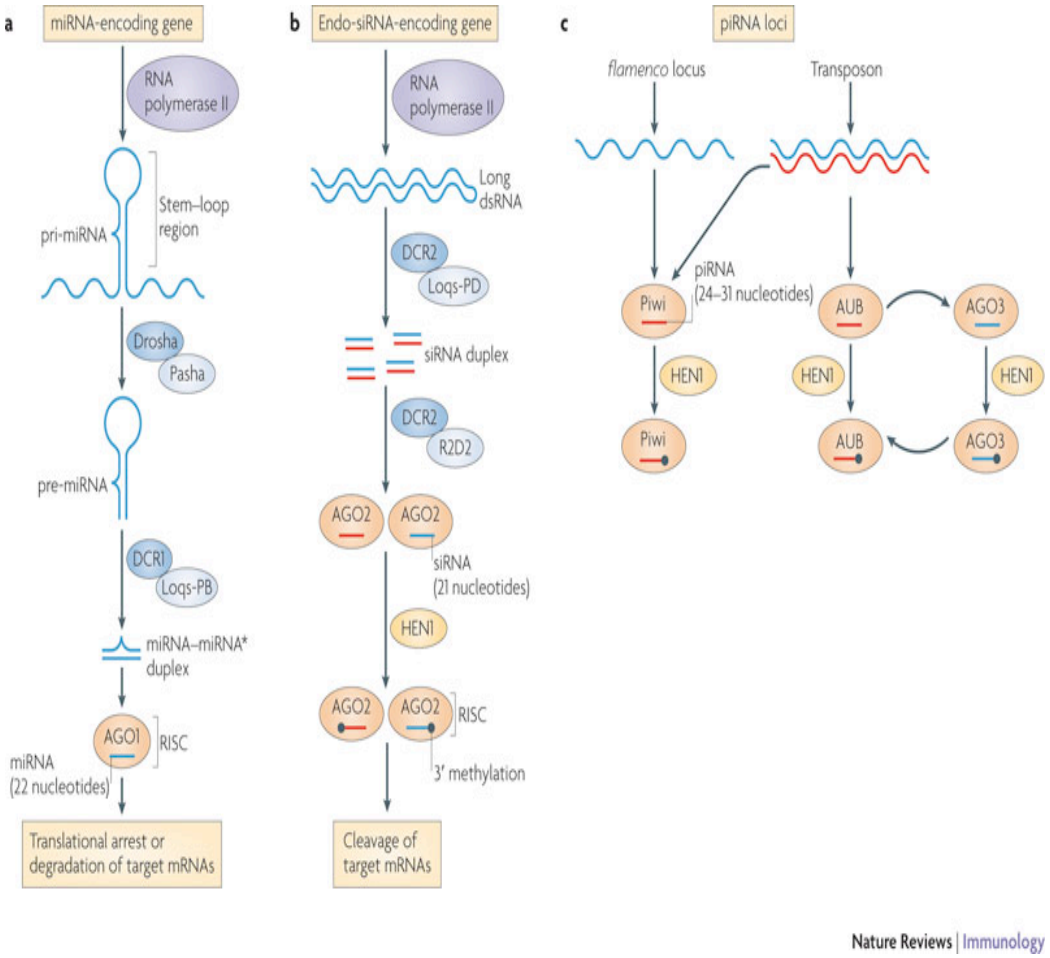


Figure 2: Types of small RNA pathways seen in Drosophila that are highly conserved in mammals (S.W.Ding, 2010)

There are several proteins involved in the RNA silencing pathway. Most of these components were discovered by performing forward genetic screens in model systems like *C. elegans* and *Drosophila* (Tabara et al., 1999).

1. TypeIII ribonucleases (dicer) – biogenesis of siRNAs and miRNAs.
2. Argonaute proteins (AGOs)- cleavage or translational repression of the mRNA targets.
3. RDE-4, R_2D_2, Pasha, TRBP- biogenesis and loading of small RNAs.

4. RNA dependent RNA polymerase-amplifies RNAi by secondary siRNA production. It is seen in plants, nematodes, fungi (Voinnet, 2005).

RNAi in *C. elegans*: The RNAi pathway was first discovered and characterized in *C. elegans* (Fire, Mello, 1998). They showed that introduction of double stranded RNA molecules into *C. elegans* brought about RNA silencing. *Figure 3* shows the different steps involved in RNA silencing in *C. elegans.*

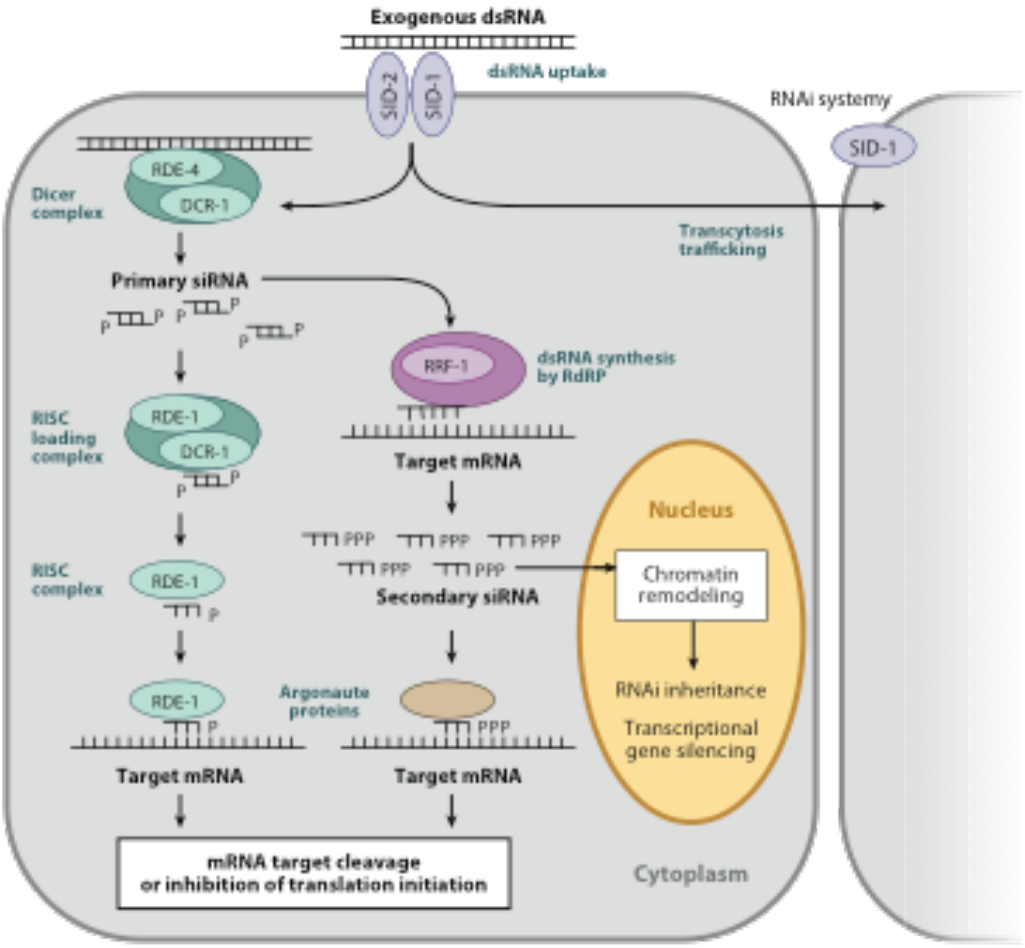


Figure 3: Exo-RNAi pathway in C. elegans (Rosso, 2009, Annual Review)

RNAi in *C. elegans* can be induced by: (Grishok, 2005)

1)Direct injection of dsRNA into the gonad or body cavity of the worms (Fire, Mello, 1998)

2)Soaking worms in a high concentration of dsRNAs (Tabara, Mello, 1998)

3)Feeding the worms with *E. coli* that produce dsRNA (Timmons, Fire, 1998)

1.2. RNA silencing as an anti-viral defense

The concept of RNA silencing playing a natural role in viral defense came about by studies done in plants. It was shown that viral infection of plants brought about activation of RNA silencing pathway (Covey, 1997). Subsequently, several other features of antiviral silencing were discovered such as detection of virus derived siRNAs in infected plants (Hamilton and Baulcombe, 1999), detection of viral suppressors of RNAi (Anandalakshmi et al., 1998), and RNAi defective plant mutants showing increased disease susceptibility (Boutet et al., 2003).

The involvement of the RNAi pathway in anti-viral immunity was established in animal cells (Li et al., 2002) and in fungi and *C. elegans* (Lu et al., 2005). Very recently, it has been shown that even prokaryotes produce virus-derived small RNAs (Andersson, Banfield, 2008). We believe studies on anti-viral mechanisms in non-mammalian systems may contribute to the understanding of human viral immunity mechanisms.

1.3. *C. elegans* as a model system

C. elegans is a free-living nematode that feeds on bacteria and therefore is constantly exposed to pathogens. Hence it forms an excellent model to study host-pathogen interactions. Its average life cycle is 2.5 days at 25^0C (Russell et al., 1976). Worms exist as hermaphrodites and males. Hermaphrodites are females that make sperm for a short while, then switches to oogenesis for the remainder of the lifespan. The hatched embryos go through four larval stages before they become adults (Russell et al., 1976). The short life cycle is of great advantage and it is very easy to study physiological changes brought about in the animals as a result of change in conditions. Also, it has been shown that *C. elegans* can be used to study replication of DNA and RNA viruses (Liu et al, 2006). All of the above features make it an excellent system for the study of anti-viral immunity mechanism and host pathogen interactions.

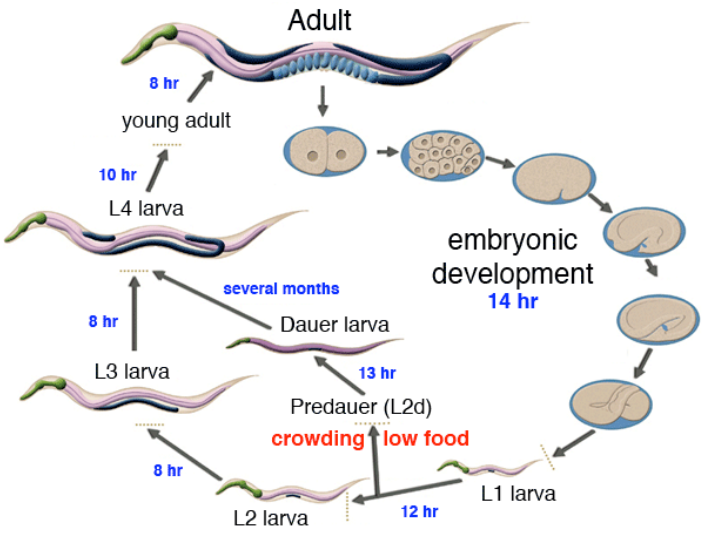


Figure 4: C. elegans life cycle at 22˚C (Altun and Hall, Wormatlas)

1.4. Flock House Virus as a model system

Flock House Virus was originally isolated from grass grubs in New Zealand (Dearing SC, 1980). It is a non-enveloped, icosahedral virus belonging to the family *Nodaviridae* (Schneemann, 2005). It has a genome consisting of two molecules of single-stranded, positive sense RNAs- RNA1 and RNA2, which are packaged into single virion. RNA1 (3107 nucleotides) encodes protein A, the RNA-dependent RNA polymerase (RdRP). RNA2 (1400 nucleotides) encodes protein alpha, the precursor of the coat protein (Krishna NK, 1999). A sub-genomic RNA, RNA3 (387 nucleotides) derived from 3' end of RNA1 encodes two small structural proteins B1 and B2. The precise function of B1 is not known, B2 however is a suppressor of RNA silencing in animal and plant cells (Li et al, 2002).

When FHV infects the host, the particles release their RNAs into the host cells. RNA1 is first translated to RdRP protein and this in turn synthesizes more RNA1 and RNA2 molecules through negative strand intermediates (Ball LA, 1998). RdRP then sub-genomically encodes for B2 protein using 3' end of RNA1 as template. Progeny RNA serves as template for additional rounds of replication and synthesis of viral proteins. These RNAs are then incorporated into new viral particles.

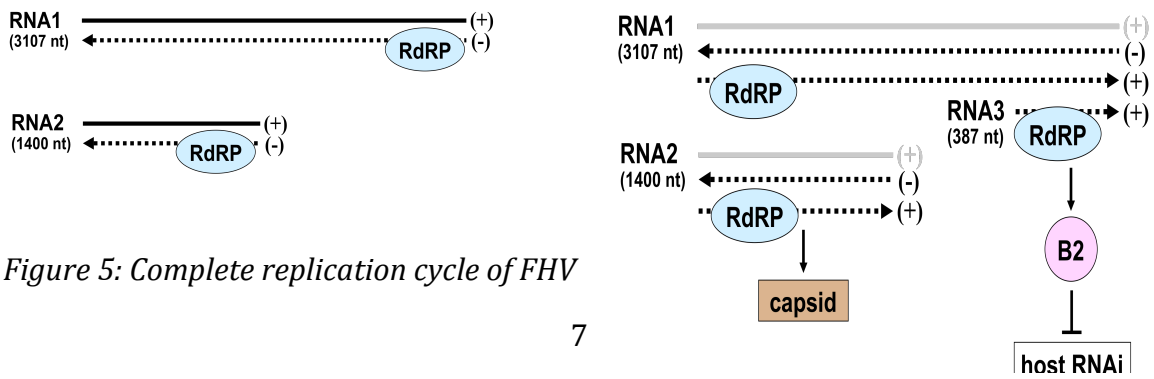


Figure 5: Complete replication cycle of FHV

1.5. Discovery of Orsay Virus

In January 2011, Marie Ann Felix et al. reported the discovery of a strain of virus that naturally infects *C. elegans* and called it Orsay virus (based on the geographic region in which the virus was discovered). They also identified another similar virus that infects *C. briggsae* and called it Santeuil virus. Both these viruses have similarities to viruses belonging to *Nodaviridae* family and have a bipartite, positive strand RNA genome (Felix et al., 2011).

JU1580, the *C. elegans* strain sensitive to Orsay infection showed several symptoms of infection including abnormal distribution of intestinal granules, nuclear elongation and degradation, fluid cytoplasm and cell fusion in the intestine. The infection was not vertically transmitted. Also, they showed that wild-type strains deficient in the RNAi pathway were readily infected.

In our research, Orsay infection system will be used to validate the involvement of genes that were positive from the secondary screen in anti-viral immunity. Orsay viral extracts were prepared as described by Felix et al. Since the viral concentration in the extract is unknown and varies with every batch, it is not possible to estimate the number of viruses infecting the worms in our experiments. The Orsay infection protocol is described under Material and Methods and we have standardized the protocol towards minimizing the variables like number of viruses, number of worms and duration of infection.

1.6. Immunity mechanisms

In vertebrates, upon infection of the host by viruses, bacteria or parasitic worms, both innate and adaptive immune systems are activated (Bartl, 1994). Innate immunity is generic and the cells of the innate immune system are non-specific in their response to the nature of infecting particles. The response is immediate but short-lived. The major functions of the vertebrate innate immune system include activation of cytokines, complement system, adaptive immune system, removal of foreign substances by WBCs (Charles et al., 2002). There are several pathways involved in *C. elegans* immunity including p38 MAPK, Insulin signaling, TGF-B, Wnt/Hox, autophagy and ERK MAPK pathways (Engelmann and Pujol, 2010).
The adaptive immune response in vertebrates on the other hand is very specific to the invading particle and the response is stronger and long-lived than innate immunity. This system mainly involves B and T lymphocytes. B-lymphocytes are associated with humoral immune response and produce antibodies against the antigens and T lymphocytes are intimately involved in cell-mediated immune response where specific infecting particles are targeted and killed (Engelmann and Pujol, 2010).

1.7. Genome-wide RNAi screen

A complete genetic sequence of *C. elegans* was available as early as 1998 (*C. elegans* sequencing consortium 1998). In the same year, RNAi was discovered in *C. elegans* (Fire, Mello et al., 1998). Since then RNAi has become an important tool in studying

gene function in animals (Kamath et al, 2003). A genome-wide genetic screen can be performed where the genes can be knocked down using RNAi and the effects studied. This is termed reverse genetic screen and this technique reveals functions of genes in the genome. Timmons and Fire et al in 1998 showed that when *C. elegans* were fed with *E. coli* containing dsRNA for a specific gene, knock down of the gene could be brought about in animals and termed this 'feeding RNAi'.

For our study to identify genes involved in anti-viral immunity, a reverse-genetic screen using RNA interference was performed. The screen protocol was standardized by the others in the lab and the protocol is described under Materials and Methods.

1.7.1. Resources for screen

Several resources were required to carry out the above-mentioned screen.

Reporter worm strain:

The reporter strain MS1395, has a chromosomally integrated FR1*gfp* reporter transgene (Lu et al., 2005). FR1*gfp* consists of the FHV RNA1 sequence driven by a heat shock promoter. The sequence encoding the B2 protein (protein inhibiting host RNAi pathway) at the 3' end of the RNA is replaced by the GFP coding sequence. In a wild-type background, B2 function is absent and as a result, the host RNAi pathway is able to degrade the viral RNAs. Due to this, RNA1 does not replicate leading to lack of RNA3 transcripts and hence absence of GFP expression. On the other hand, in a sensitized background where RNAi or other unknown pathways are blocked, RNA1 and RNA3 transcripts are produced and this leads to higher GFP levels.

Therefore, any increased GFP expression implies enhanced viral replication in the animal. This transgene allows a rapid identification of conditions that permit viral replication (such as RNAi, mutation or environmental treatment) and is a powerful means to study host-pathogen interactions.

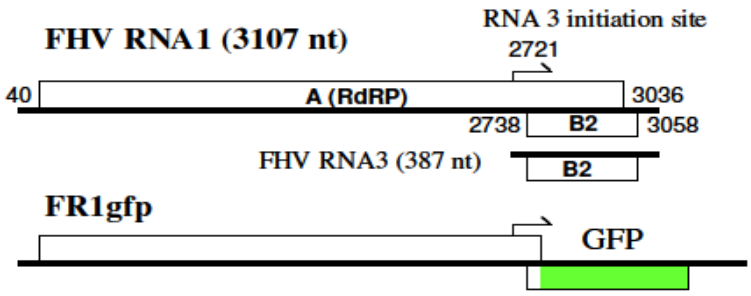


Figure 6: FR1gfp construct: Top: FHV RNA sequence with RNA1 and RNA2 regions encoding RdRP and B_2. Bottom: FR1gfp construct where B_2 region is replaced by GFP sequence.

Feeding RNAi library:

Feeding RNAi library is a collection of *E. coli* strains expressing dsRNA for a particular gene of *C. elegans*. When fed to worms, the dsRNA molecules enter the worms and knock down the gene through RNA silencing. *C. elegans* ORFeome version 1.1 feeding RNAi library was ordered from Geneservice Ltd. (Cambridge, UK). This feeding RNAi library contains *E. coli* strains representing ~60% (11,700 genes) of *C. elegans* genes. Each strain carries a unique plasmid expressing IPTG-inducible sense and antisense RNA. A fragment from the gene of interest is amplified by PCR and cloned into the L4440 double-T7 vector, which has two T7 promoters in inverted orientation flanking the multiple cloning sites. Cloned plasmids are

transformed into HT115(DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible expression of T7 polymerase (L. Timmons and A. Fire, 1998).

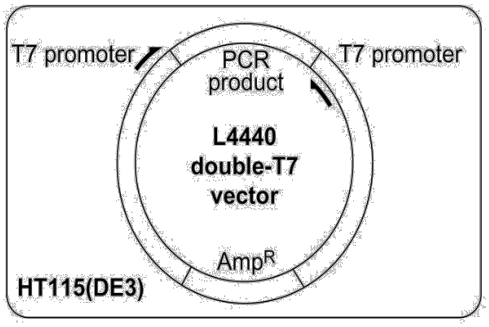


Figure 7: L4440 double-T7 vector inside HT115 RNase-deficient E. coli.

We used the reporter worm strain MS1395 in an *rrf-3(pk1426)* mutant background (MS1576) to enhance the feeding RNAi effect (Simmer et al., 2002) and used the feeding library to knock down one gene at a time and screened for increased GFP expression in worms indicating increased FHV replication in them.

A pilot screen to identify genes involved in anti-viral immunity was performed in Ding lab. They discovered the role of *drh-1* (Dicer related helicase) in immunity, acting downstream of viral siRNAs (Lu et al., 2009). This discovery proved that feeding RNAi screen was a robust system to identify genes involved in immunity and a genome-wide screen was performed to do the same.

Chapter 2: Results

The results of the screen are as shown below.

2.1. Primary Screen

Feeding and screening was performed as described under Materials and Methods. 11,700 *C. elegans* genes were knocked down one at a time and GFP expressions were scored. Several people participated in the screen and 2180 genes out of 11,700 were screened by me. The total number of animals in each plate was counted. Among them, the number of animals expressing GFP (pharynx- whole body) was counted and the percentage of animals expressing GFP on each plate was computed. Any plate that had a higher percent of GFP expression than the negative control (*rrf-3(pk1426); irIs91* worms with no RNAi) was considered positive. In the primary screen, 691 genes were scored as positive (~6% of total genes in the library). The positives were grouped into several categories as shown in the pie chart below *(Figure 8).*

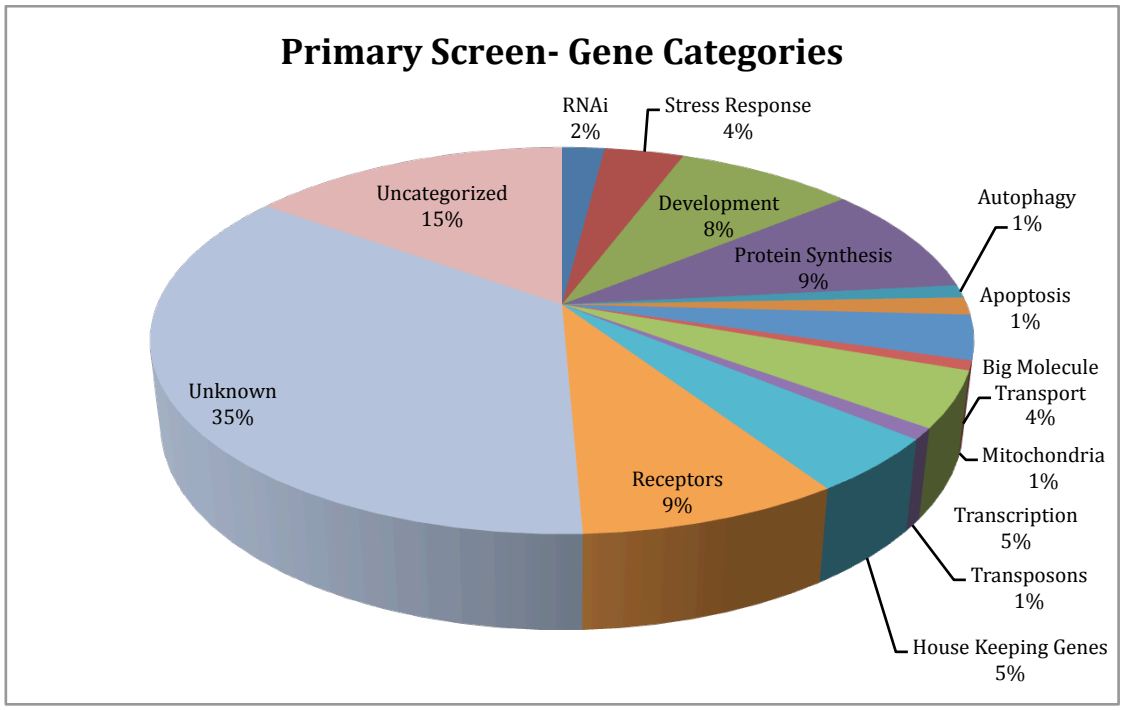


Figure 8: Distribution of primary screen positive genes into different categories

2.2. Secondary Screen

A secondary screen was performed using candidates from the primary screen, this time in triplicate, to eliminate false positives that could arise due to heat-shock conditions and other environmental changes. 150 genes of the 691 positive genes were screened by me. Out of the 691 genes, 250 were seen to be consistently positive. They were grouped into several categories as shown in the pie chart below *(Figure 9).*

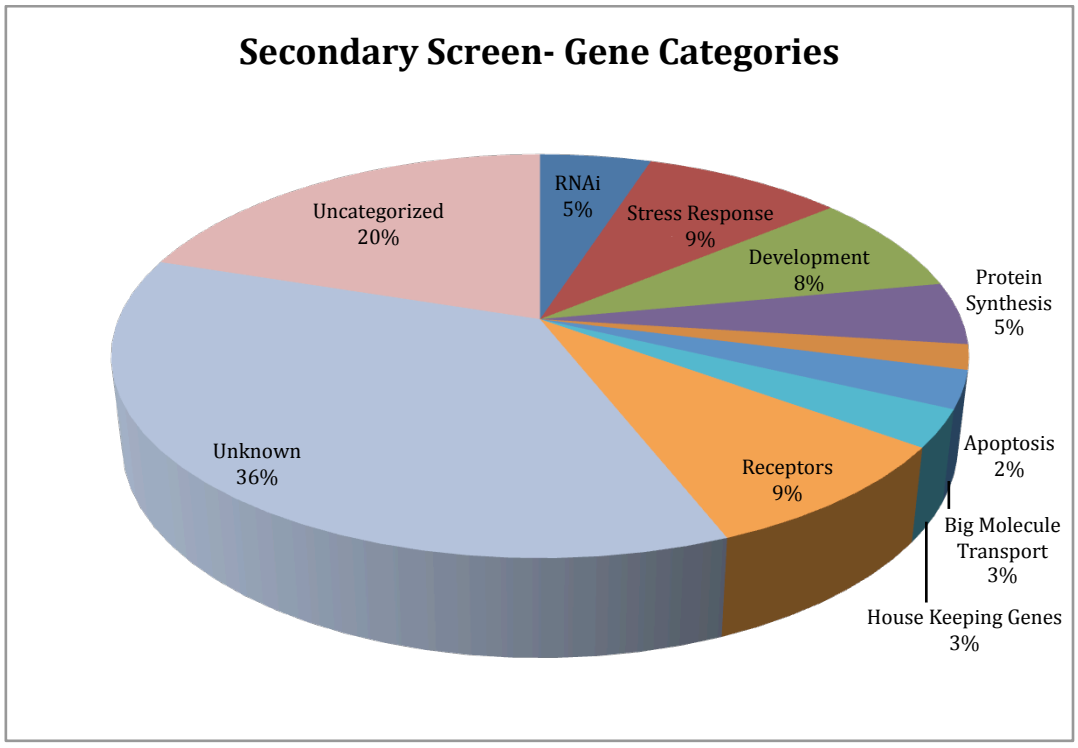


Figure 9: Distribution of secondary screen positive genes into different categories

Among the hits from the secondary screen, a few RNAi genes were identified. This confirmed the efficacy of our technique to identify genes involved in anti-viral immunity. The genes that were positive from the secondary screen which were screened by me are listed below.

Sl. No.	Gene ID	Gene name	Gene function
1	ZC239.15		BTB/POZ domain; voltage-gated potassium channel complex
2	ZK945.4		Zinc ion binding; down regulated in *dcr-1*
3	F58E1.2	*fbxb-25*	F-box B protein; Predicted transposase
4	F49E12.9		C-4 sterol methyl oxidase; fatty acid hydroxylase; Endoplasmic Reticulum
5	F52C6.10	*bath-7*	Uncharacterized protein; contains BTB/POZ and MATH domain
6	Y17G7B.4		Deoxyhypusine synthase ;embryonic development
7	Y110A2AL.14	*sqv-2*	Glycosaminoglycan galactosyltransferase
8	ZK1320.9		Acetyl-CoA hydrolase

9	R06F6.8		Cellular response to DNA damage; Ribosome Control Protein 1 domain
10	T08H4.1	*rhgf-2*	RHo Guanine nucleotide exchange Factor
11	Y116A8C.12	*arf-6*	GTP-binding protein of the ADP-ribosylation factor (ARF) family
12	F36H12.14		Unnamed protein
13	Y43C5B.2		Protein tyrosine kinase
14	F42C5.3		Unknown
15	F13E9.6	*sdz-13*	SKN-1 Dependent Zygotic transcript
16	F47C10.4	*nhr-187*	Nuclear Hormone Receptor family
17	C03G6.19	*srp-6*	Functional serpin (serine protease inhibitor)
18	T26H2.6	*str-81*	Seven TM Receptor; embyronic development
19	H12C20.3	*nhr-68*	Nuclear Hormone Receptor family; lipid storage
20	F53F8.5		Uncharacterized Conserved Protein
21	R08A2.2		Serine/threonine specific protein phosphatase
22	C03G6.8	*nhr-147*	Nuclear Hormone Receptor family
23	F11A5.7		Predicted Receptor; Recepter L domain
24	F25E5.10	*try-8*	Trypsin-like protease
25	F16B4.9	*nhr-178*	Nuclear Hormone Receptor family
26	T06A1.1		Extracellular protein with conserved cysteines; domain of unknown function DUF23
27	R11G11.1	*nhr-132*	Nuclear Hormone Receptor family
28	K11D12.3	*srr-4*	Serpentine Receptor
29	C06H5.2	*fbxa-157*	F-box A protein
30	F28H7.7		Extracellular protein with conserved cysteines; domain of unknown function DUF23
31	F44A2.2		Voltage-gated K+ channel KCNB/KCNC
32	C18G1.6		Unnamed Protein
33	F48G7.12		Predicted small molecule kinase
34	F41B5.7		Cytochrome P450 family; heme binding
35	F56A4.10		Permease of the major facilitator superfamily
36	ZC196.6		Unknown; positive regulation of growth rate
37	R08F11.3		Cytochrome P450 family
38	F39G3.2		Unnamed Protein
39	B0213.16		Cytochrome P450 family
40	C04F5.7	*ugt-63*	UDP-glucuronosyl and UDP-glucosyl transferase
41	C35A5.7		Unnamed Protein; G-protein coupled receptor protein signaling pathway
42	F41F3.2	*soc-1*	Suppressor Of Co 1
43	K12B6.8		Predicted E3 ubiquitin ligase
44	T11A5.6		Cytosolic Ca^{2+}dependent cysteine protease
45	F46F3.2	*lgc-44*	Ligand-gated ion channel
46	Y37H2C.1		Unnamed protein
47	T19B10.4	*pqn-70*	Contains a glutamine/asparagine (Q/N)-rich (prion) domain; RNA interference

48	F36D3.4		Uncharacterized protein, contains major sperm protein (MSP) domain
49	K12F2.2	*vab-8*	Kinesin-like motor domain for cell migrations; contains a kinesin-related domain
50	ZC376.7		Regulation of transcription, DNA-dependent
51	C35A11.4		Permease of the major facilitator superfamily
52	F52E1.4	*gcy-7*	Guanylyl Cyclase
53	T27C4.4	*lin-40*	Abnormal cell Lineage; homolog of mammalian MTA (Metastasis-Associated) proteins
54	Y42A5A.1		Puromycin-sensitive aminopeptidase and related aminopeptidases
55	F44C4.4	*gon-14*	Abnormal Gonad development; growth
56	F52H2.3		Unknown
57	C47D2.1		Unknown
58	F53B1.6		Uncharacterized conserved protein
59	ZK899.3		Unknown
60	C31E10.4		Unknown
61	F49E2.4	*glb-17*	Globin related; heme binding
62	T22B7.3		Unknown
63	C18A11.4		Unknown
64	R04A9.3		Unknown
65	F42F12.3		Steroid reductase
66	W06B11.3	*dct-11*	DAF-16/FOXO Controlled
67	T24D5.3		Unnamed Protein; heme binding
68	F52D10.4		Unknown
69	T01B10.2	*grd-14*	Hedgehog-like protein
70	F48B9.2		Unknown

Table 1: List of genes positive from secondary screen tested by me.

The next step was to validate the involvement of genes positive from secondary screen in the anti-viral immunity mechanism. Several techniques such as qRT-PCR, *in situ* hybridization and Northern blot were employed to validate the roles of these genes. Once their involvement in anti-viral immunity was confirmed, the next step would be to understand the mechanism of action of these genes.

2.3. Validation of genes identified

A subset of the above 250 genes positive from the secondary screen was tested and validated for their roles played in anti-viral immunity. The hits from the secondary screen were grouped together based on their function and a few categories were chosen for further investigation. Since Maduro lab studies development and differentiation of cells in developing *C. elegans* embryos, the genes belonging to developmental pathway were chosen. Since there were several genes belonging to lipid metabolism pathway and ubiquitin pathway in the secondary hits, we speculated a high possibility of involvement of these genes in anti-viral immunity, hence chosen for further investigation.

2.3.1. qRT-PCR- Orsay infection

A total of 14 genes were first tested for susceptibility to Orsay infection. The necessary existing mutants were ordered from Caenorhabditis Genetics Center, University of Minnesota. The following mutants were ordered for further studies.

Gene	**Allele**	**Description**
him-14	*it44*	High incidence of males (MutS familty)
egl-18	*ok290*	Egg laying defective (GATA transcription factor)
puf-9	*ok1136*	RNA binding protein
lin-40	*ku285*	Abnormal cell lineage(Mammalian metastasis associated protein homolog)
grd-14	*ok3254*	Groundhog family
sur-7	*ku119*	Suppressor of activated *let-60* Ras(Cation diffusion facilitator family)
nhr-68	*gk708, gk755*	Nuclear hormone receptor
nhr-178	*gk1005, gk1158*	Nuclear hormone receptor
clp-4	*ok2808*	Calpain family(Mammalian muscle specific Calpain3 Protein homolog)
mef-2	*gk633*	Sole *C. elegans* member of the MEF2 subgroup

Table 2: List of mutants ordered for further investigation

Orsay viral extract was prepared as described under Materials and Methods section. Synchronized worms were inoculated with Orsay viral extract and grown on plates for 3 days. qRT-PCR was performed for the detection of Orsay RNA1 molecules.

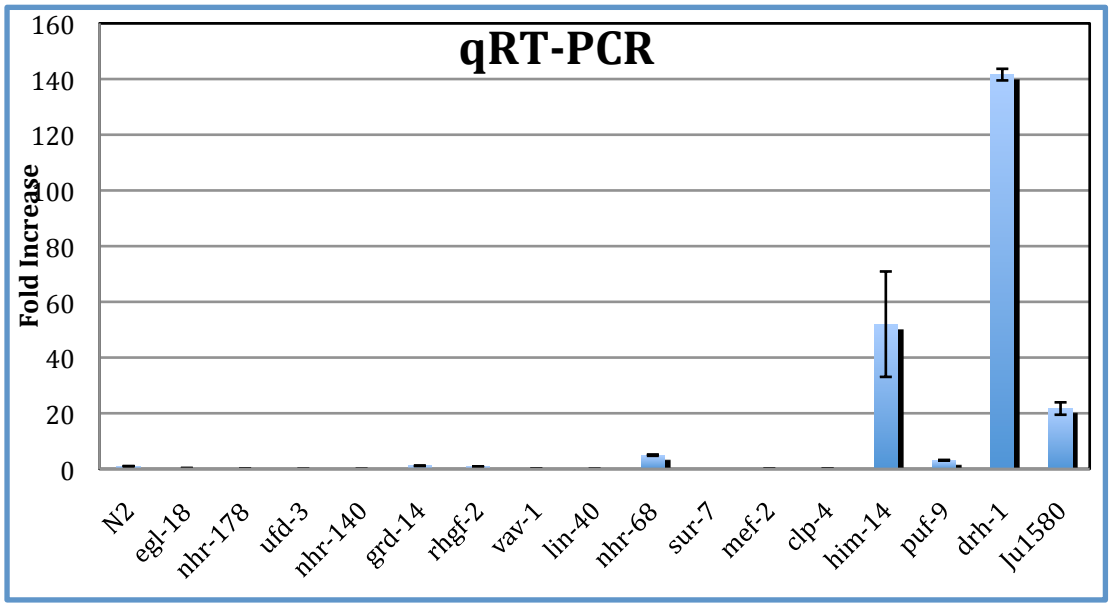


Figure 10: qRT-PCR detecting Orsay RNA1 molecules.

The above qRT-PCR data indicates that *nhr-68(gk708)* and *him-14(it44)* mutant worms' susceptibility to the virus are significantly higher than that of N2 strain and therefore were chosen for further investigation. *drh-1* encodes a dicer related helicase and it is already established that it is involved in the RNAi pathway (Tabara H., 2002). In our study, we use *drh-1(ok3495)* as a positive control for increased susceptibility to Orsay infection.

2.3.2. Percent GFP expression

The GFP expression pattern in *nhr-68(RNAi)* and *him-14(RNAi)*/*him-14(it44)* mutants was studied more closely. Table 1 summarizes the GFP expression pattern of the strains under different conditions. The total number of worms in each plate and the number of worms expressing GFP in pharynx were counted and a percent of worms expressing GFP was calculated.

	Strains (n~100)	**Percent GFP**
1.	*nhr-68 (RNAi)* *rrf-3(pk1426); irIs91 worms*	77%
2.	*him-14 (RNAi)* *rrf-3(pk1426); irIs91 worms*	52%
3.	*rrf-3(pk1426); irIs91 worms*	3%
4.	*him-14 (it44); irIs91*	40%
5.	*irIs91*	9%

Table 3: Quantification of GFP expression

When *him-14* and *nhr-68* genes are knocked down using *RNAi* in *rrf-3(pk1426); irIs91* worms, they show increased FHV replication as against *rrf-3(pk1426); irIs91* worms. Also, *him-14(it44); irIs91* strains were constructed and they showed increased FHV replication compared to *irIs91* worms. *Figure 11* shows GFP

expression pattern in the above-mentioned strains. GFP expression was seen in the pharynx in the strains that were tested.

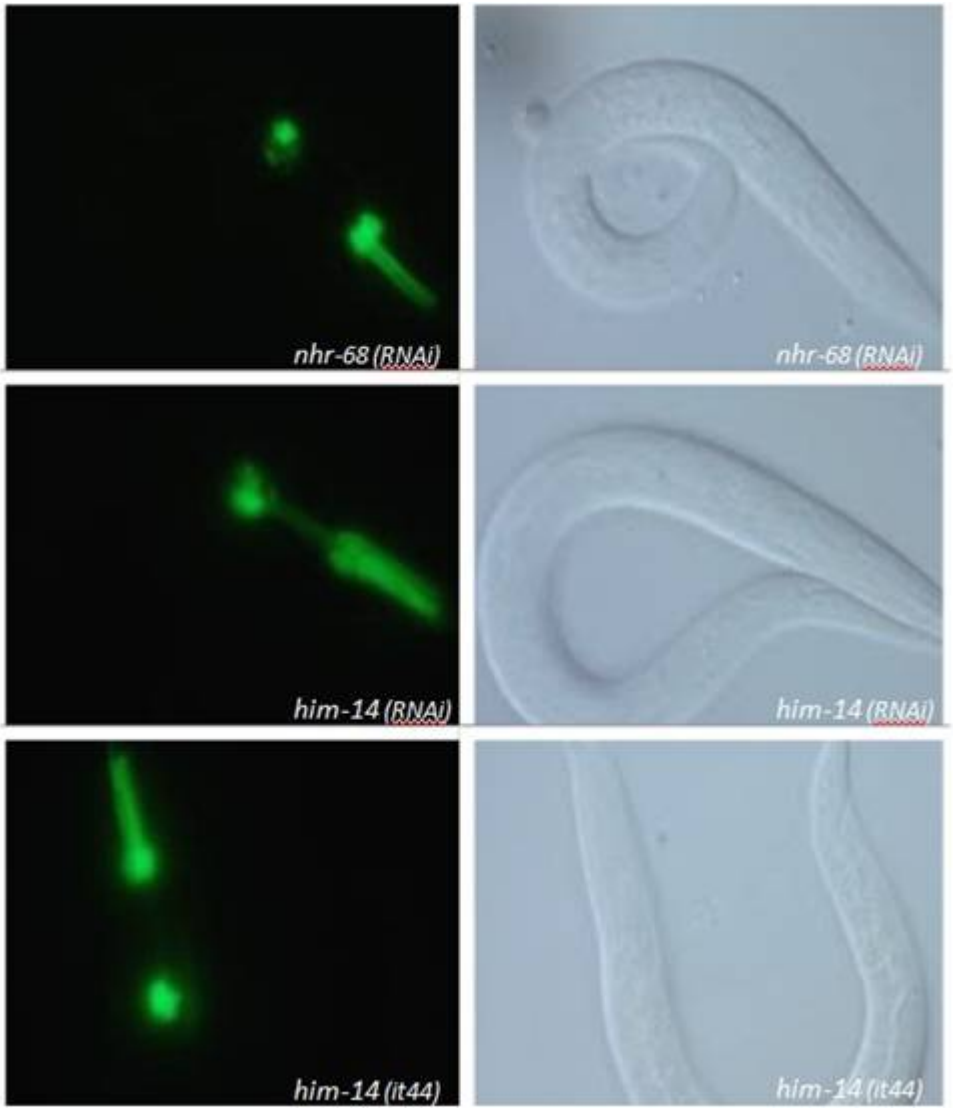


Figure 11: nhr-68 and *him-14* strains expressing GFP in pharynx.

2.3.3. A test for effect of *him-14* and *nhr-68* on heat shock transgene expression

One possibility for increased FR1*gfp* in strains where *him-14* and *nhr-68* are knocked down could be due to increased RNA1 transgene expression in those

worms. Due to some unknown mechanism, when these particular genes are knocked down, there could be increased transcription of the initial transgene and hence increased GFP molecules after translation. To rule out an effect at the level of transgene induction, a heat shock-activated GFP transgene (hs::GFP strain MS1434) was tested with *nhr-68 (RNAi)* and *him-14 (RNAi)*, L4 worms heat shocked for 4hours at 34^0C and expression of GFP screened.

The result obtained is shown in the Figure 12 below. It is clear that GFP is expressed at the same time during the three treatments indicating that there is no over-expression of transgene upon feeding. GFP observed during the screen is due to increased FR1*gfp* replication in the worms.

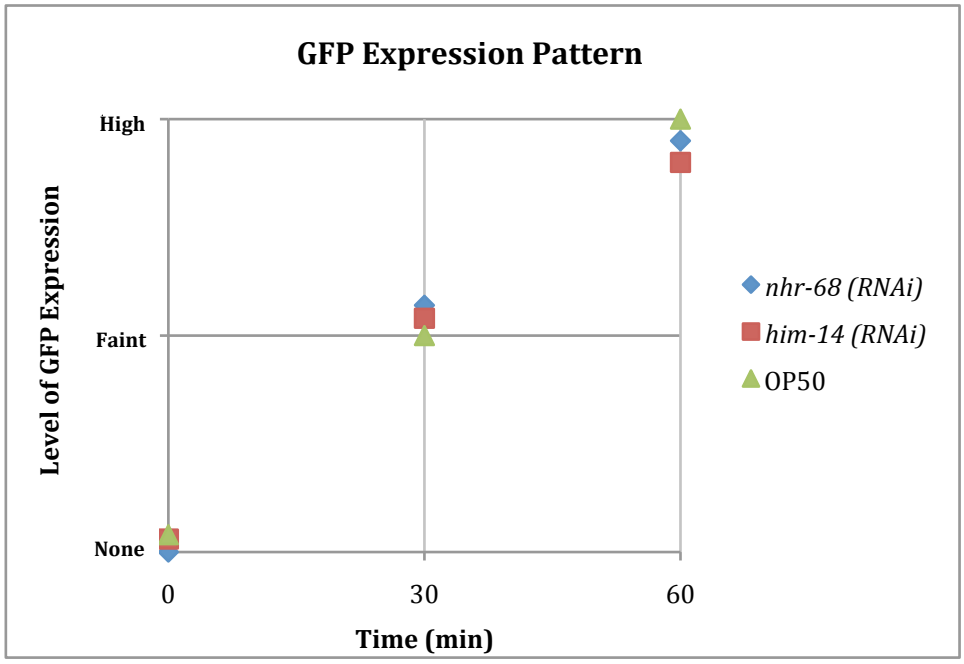


Figure 12: GFP expression pattern of MS1434 strain

2.3.4. *In situ* hybridization- Orsay infection

Another method used to validate the involvement of *him-14* and *nhr-68* in anti-viral immunity is by performing an *in situ* hybridization assay and testing for increased concentrations of Orsay RNA1 molecules in the two strains in comparison to the wild-type strain. The two strains were infected with Orsay virus using a standard protocol as described in the Materials and Methods chapter. N2 strains were infected at the same time as a negative control and *drh-1* and *rde-4* as positive controls. *In situ* hybridization for detection of single molecules of Orsay RNA1 molecules was performed (Raj et al., 2010). The worms were observed for fluorescence and some sample pictures of fluorescence due to Orsay infection are shown below in Figure 13.

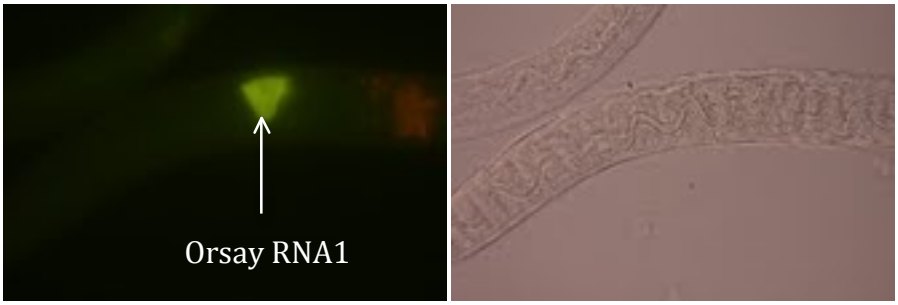


Figure 13: drh-1; irIs91 worm showing Orsay infection in an intestinal cell

The number of worms showing fluorescence was counted and also a record was made of the number of cells positive for fluorescence in each worm. *Figure 14* shows the infection rate in different strains.

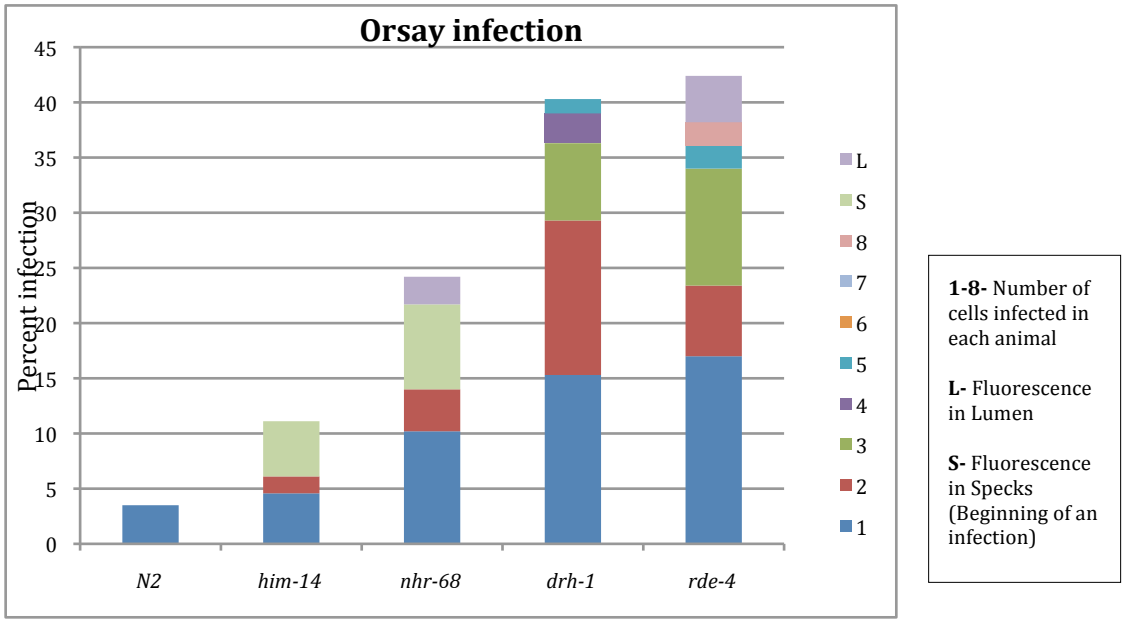


Figure 14: In situ hybridization showing Orsay infection rate in different mutants

From the above data, it is evident that *nhr-68(gk708)* and *him-14(it44)* are more susceptible to Orsay infection compared to the negative control N2 and less susceptible than the positive control *drh-1.*

2.3.5. Northern blot- Orsay infection

Northern blot is one of the techniques used to detect the presence of RNA of interest (Zalevsky et al., 1999). A standard Northern blot experiment was performed and probes specific to RNA1 molecules of Orsay were used for detection. *Figure 15* shows the data obtained from the Northern blot.

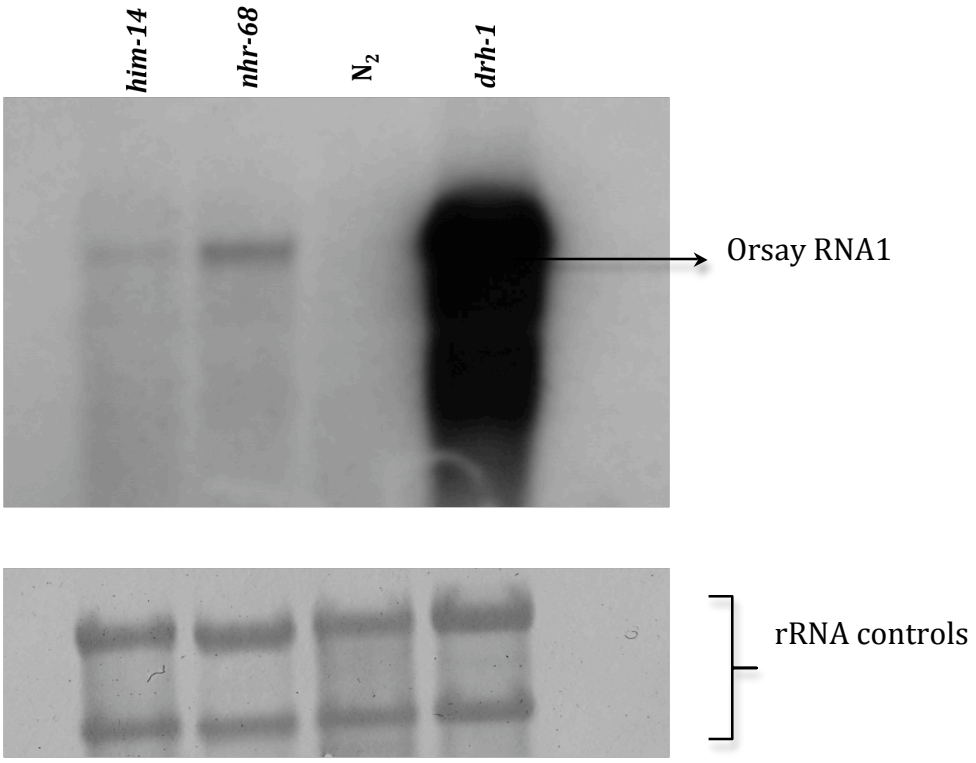


Figure 15: Northern blot showing Orsay RNA1 bands

From the above Northern blot data and also from GFP expression and *in situ* hybridization data, it can be concluded that mutants for *him-14(it44)* and *nhr-68(gk708)* show increased susceptibility to Orsay virus infection. However, the mechanism of action is unknown and have a good potential for further investigation in anti-viral immunity mechanisms.

The functions of the two genes and the possible mechanisms through which they could be involved in immunity are discussed in the following two chapters.

2.4. *him-14 and nhr-68*

2.4.1. him-14

Introduction:

Proper segregation of homologous chromosomes at meiosis I is dependent on the formation of crossovers between two homologous chromosomes (Hawley, 1988). HIM-14 protein is required for the formation of crossovers during the pachytene stage of meiosis I in *C. elegans*. Loss of *him-14* function reduces crossover, resulting in lack of chiasmata between homologous chromosomes and consequently resulting in missegregation (Zalevsky et al., 1999).

him-14 is present on chromosome II and the gene location and features are as shown in *Figure 16.*

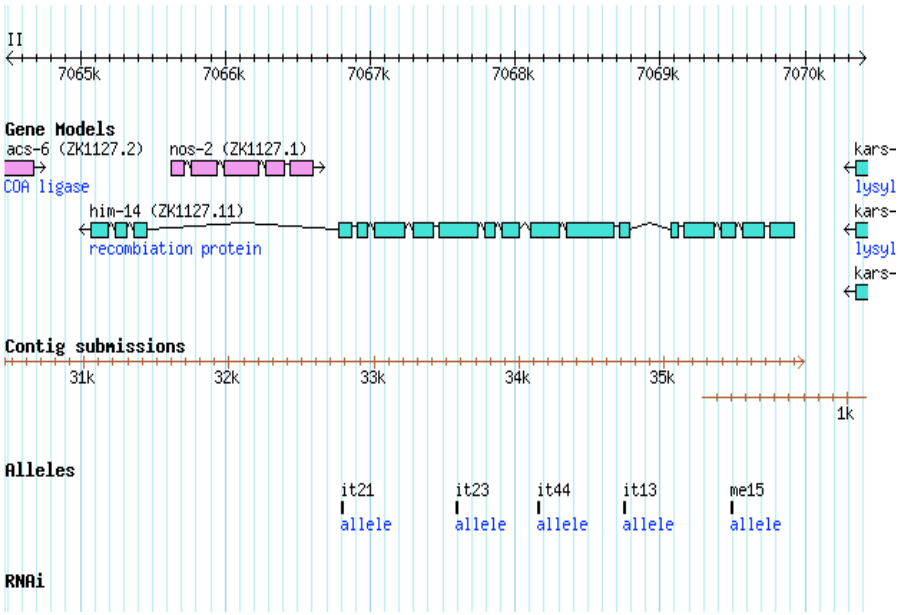


Figure 16: him-14 gene environment (Wormbase)

The expression of *him-14* gene is seen only in the germline. A Northern blot experiment performed for the detection of *him-14* mRNA in *glp-4 (bn2)* worms that lacked germline showed no *him-14* mRNA band and wild type animals showed the band, indicating that *him-14* expression was germline specific (Zalevsky et al., 1999).

Considering the expression of *him-14* is only seen in germline cells, the possible role of the gene in anti-viral immunity is not very obvious. In our experiments, Flock House Virus GFP expression in *him-14(it44)* mutants is seen in the pharynx and Orsay viral replication is seen in the intestinal cells of these worms. It is not clear how such events in the soma can occur when a gene expressed in the germline is mutated. However, there have been reports supporting the above relation between germline and soma. In one report, upon killing of germline cells using a laser beam, an extension of lifespan in those worms was observed (Hsin et al., 1999). This pathway included *daf-16*, a gene expressed in the soma. We hypothesize that changes in the germline such as mutation in *him-14* brings changes in soma, resulting in increased viral replication in soma.

Effect of germline depletion:

Since *him-14* expression is restricted to germline in *C. elegans*, we wanted to study viral replication in *him-14(it44)* mutant worms in combination with a defect in the germline. It has been previously established that mutations in the gene *glp-4* leads to hermaphrodite sterility due to lack of germ cell proliferation (Beanan MJ, Strome S, 1992). GLP-4 protein is required in germ cells to progress through mitotic

prophase and proliferate to give rise to a normal germline (Beanan MJ, Strome S, 1992).

In our study, *glp-4 (bn2)* was used to cross with *irIs91* strain in order to obtain *glp-4(bn2); irIs91*. This strain was again crossed with *him-14 (it44)* to obtain *him-14 (it44); glp-4 (bn2); irIs91*. The details of the crosses are mentioned in the Materials and Methods chapter. We wanted to test the extent of viral replication in these mutants and study the cumulative effect of mutation in *him-14* and *glp-4* genes. Both *him-14(it44) and glp-4(bn-2)* mutants are temperature sensitive. They lack function at 25^0C but function at 15^0C. So animals had to all be raised at 15^0C then shifted up to 25^0C when performing the experiment. Experiment to screen for GFP was performed and the protocol is described in the Materials and Methods chapter. The localization of GFP in different regions of the body was recorded *(Figure 17)*.

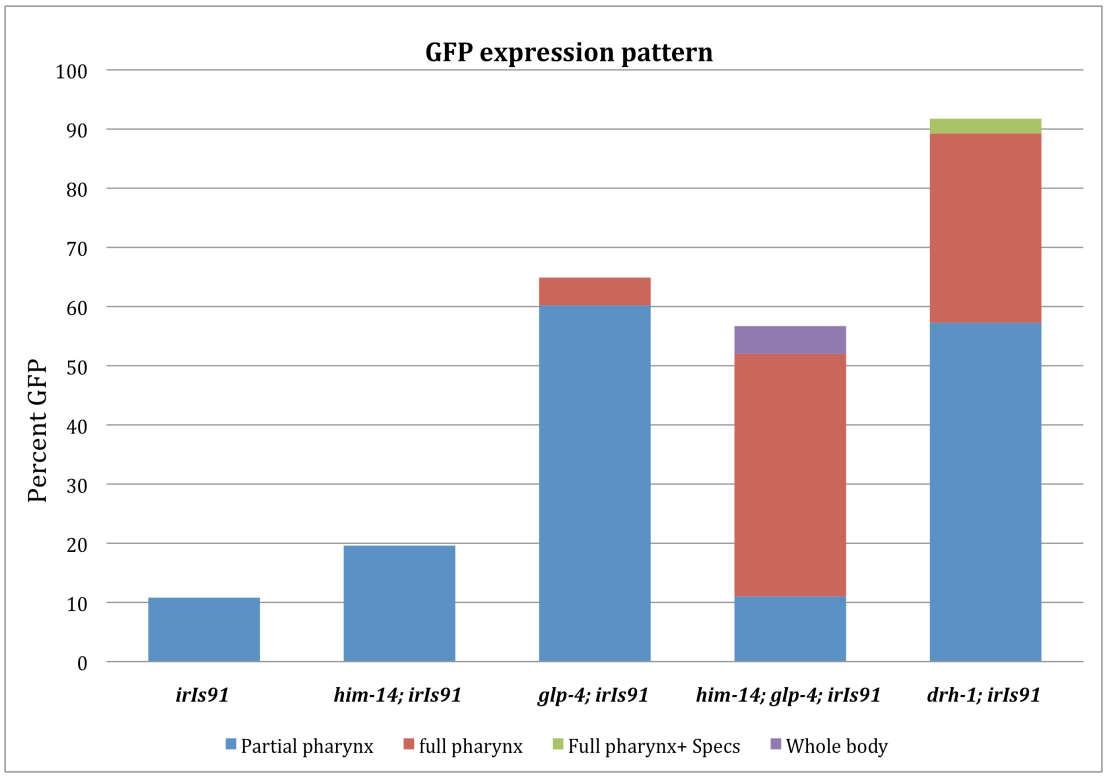


Figure 17: Replication of FR1gfp in wild-type and germline mutant backgrounds

From the above data it is evident that a mutation in *glp-4(bn2)* that lacks germline allows higher FHV replication and this effect is similar to *him-14(it44)* mutation. *him-14(it44); glp-4(bn2)* double mutant does not show an increased viral replication compared to *glp-4* or *him-14* single mutant. This is evident as there is no synergy in susceptibility to viral replication in the *him-14; glp-4* double mutant compared to either of the single mutants. Also, viral replication in *glp-4* mutant is similar to that in *him-14; glp-4* double mutant. This indicates the higher viral replication could be due to the defect in the germline and not loss of function of *him-14 per se*.

2.4.2. *nhr-68*

Introduction:

nhr-68 belongs to the nuclear hormone receptor family of proteins. NHRs are transcription factors typically regulated by lipophilic molecules like steroids, retinoids, bile and fatty acids and they coordinate metazoan metabolism, development and homeostasis (Arda et al., 2010). In the presence of ligands, NHRs bind to their hormone response elements and assemble co-activator complexes and turn on gene expression (Arda et al., 2010). There are a total of 284 NHRs in *C. elegans*, 48 in humans and 21 in flies (Maglich et al., 2001). 15 NHRs in *C. elegans* have homologs including mammalian HNF4 and Vitamin D receptor. The other 279 receptors arose from ancestral HNF4 (Robinson- Rechavi et al., 2005).

The gene location and features of *nhr-68* are as shown in Figure 18.

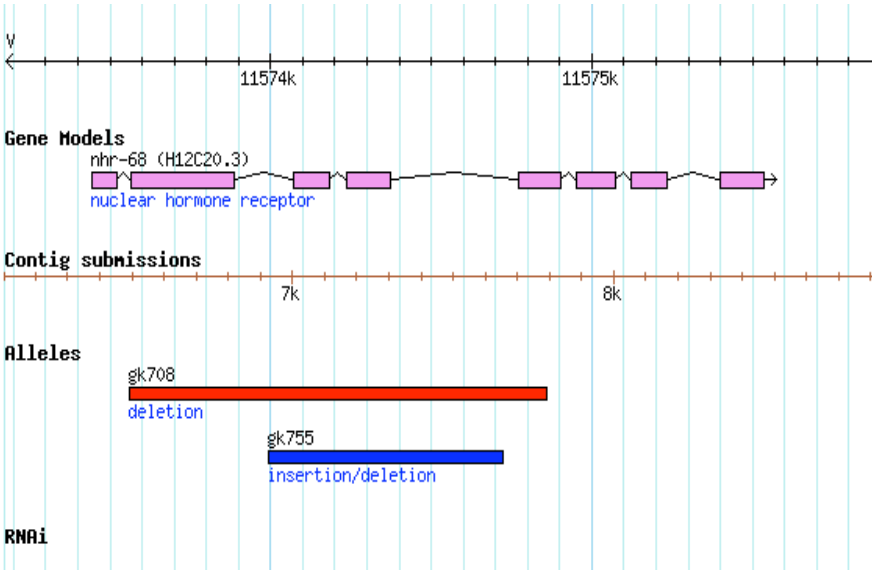


Figure 18: nhr-68 gene environment (Wormbase)

nhr-68 is expressed in embryos and in adults in hypodermal and intestinal nuclei throughout the worm (Antebi, Nuclear hormone receptors in *C. elegans*, Wormbook). In an attempt to identify genes necessary for normal fat storage, a genome-wide RNAi screen was performed by Ashrafi et al. and *nhr-68 (RNAi)* showed increased fat content in worms (Ashrafi et al., 2003). They performed a Nile Red fat assay to study the concentration of lipid molecules in the body.

It has been well established that viruses use lipid molecules for efficient replication in the host. They act as receptors for viral entry into the cells (Tsai B et al., 2003). Positive strand RNA viruses form replication complexes using different organelles like endoplasmic reticulum, golgi complex and mitochondria (Miller S et al., 2008). Once viruses enter cells, they bring about several metabolic changes in the host cell. In particular, a group reported increase in metabolites entering the fatty acid biosynthetic pathway (Munger J. et al., 2006). Lipids also play an important role in virion assembly and budding (Miyanari et al., 2007).

We hypothesize that knockdown of *nhr-68* allows increased FHV and Orsay replication because of increased fat content in these worms.

Oil Red O staining

Nile red is a phenoxazone, lipophilic dye used extensively to stain lipid molecules in cells. It was also used to detect and quantify lipid content in living worms (Ashrafi et al., 2003, Mak et al., 2006). However, it was reported in 2009 that Nile Red stained acidified cellular compartments in worms instead of staining major fat stores (Ruvkun et al., 2009). They also reported inconsistencies between Nile Red and

triglyceride levels. They established that Oil Red O, a fat-soluble dye used for staining neutral triglycerides and lipids, is a better dye for staining and quantifying lipid content in worms.

In our study, we wanted to check if *nhr-68(gk708)* mutant worms have higher fat content in their bodies. Oil Red O dye was used to stain N2 and *nhr-68(gk708)* worms and the staining protocol is described in Materials and Methods chapter.

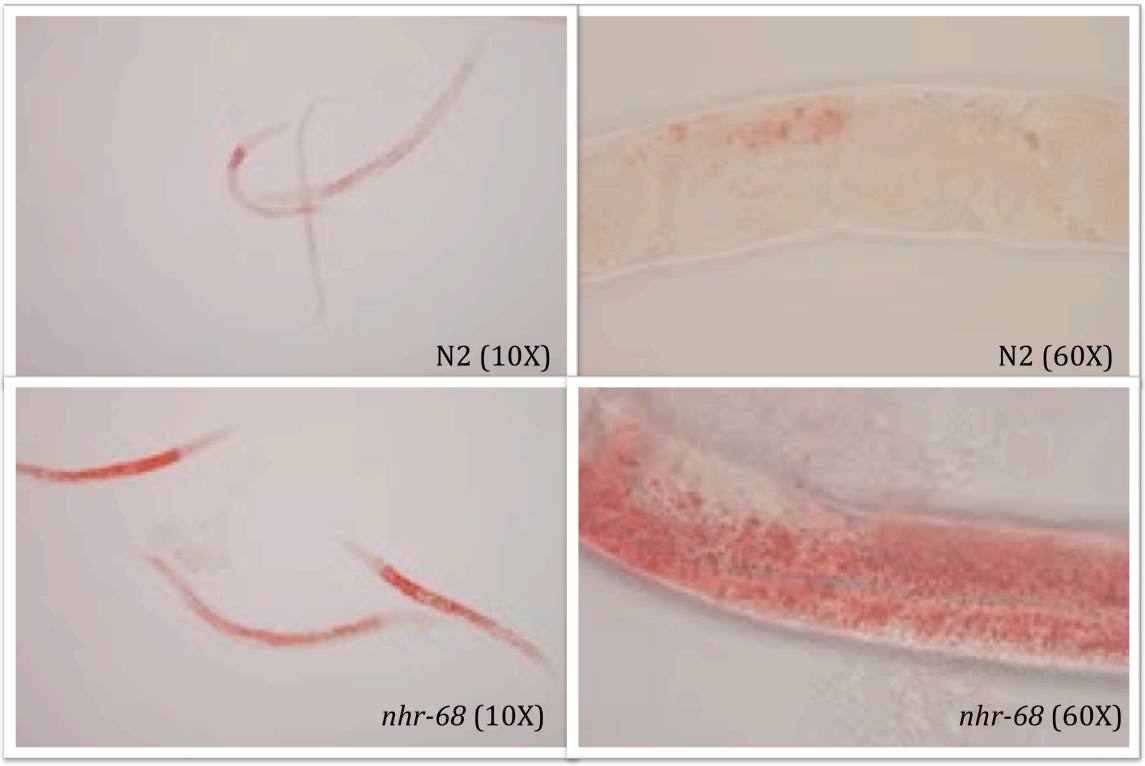
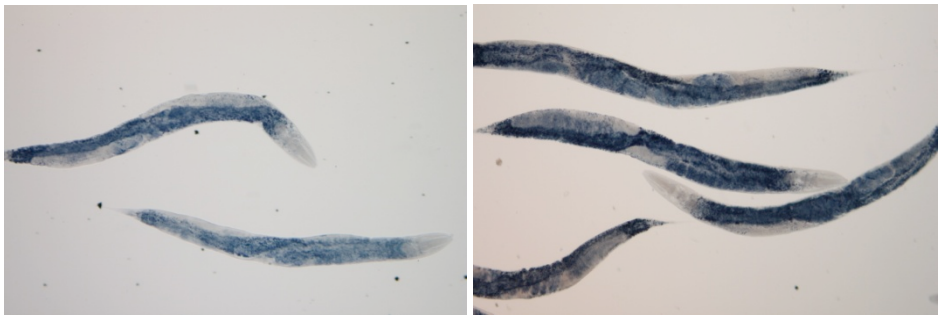


Figure 19: Worms stained with Oil Red O dye for comparison of lipid contents

Oil Red O dye stains lipid molecules red and from the above figure, it is evident that *nhr-68(gk708)* worms have higher fat content compared to wild type worms.

Sudan Black staining

Sudan black is a fat-soluble dye used for staining triglycerides and lipids and stains them blue-black. It was first used in 1997 to stain fixed worms (Kimura et al., 1997). Ruvkun et al in 2009 established that Oil Red O is a better dye over Nile Red to measure lipid content in worms. In addition to Oil Red O, we used Sudan Black dye to measure lipid content in these worms. The staining protocol for Sudan Black is described in the Materials and Methods chapter. N2 and *nhr-68(gk708)* worms were tested and the results obtained are shown in Figure 20.



N2 *nhr-68(gk708)*

Figure 20: Worms stained with Sudan Black dye for comparison of lipid contents.

In the above pictures, *nhr-68(gk708)* worms are stained darker indicating higher fat content in these worms.

We hypothesize there is a strong relation between high viral replication rate in *nhr-68(gk708)* mutants and high lipid content in these mutant worms. Further experiments are being performed in the lab to test if the two variables are linked.

2.5. Discussion

C. elegans forms a robust model system for the study of several aspects of biology such as development (Brenner et al., 2002) , sex determination (Sommer et al., 2004), immunity mechanisms: anti-bacterial (M.W. Tan et al. 1999, Aballay et al., 2000), anti- fungal (Pujol et al., 2008) and recently anti-viral immunity mechanisms (Liu et al. 2005, Liu et al., 2006). Studies using bacteria such as *Pseudomonas* and *Salmonella* have revealed innate immunity pathways in *C. elegans*. These pathways include p38MAPK (Kim D.H. et al., 2002), TGF-B (Mallo G.V. et al., 2002), ERK MAPK (Nicholas HR et al., 2004) and autophagy (Jia K. et al., 2009). However, studies on anti-viral immunity have been limited due to the absence of a natural virus infecting *C. elegans*. With the discovery of Orsay virus in 2011 by Felix et al., it is now possible to naturally infect *C. elegans* with virus and many groups have begun to explore mechanisms involved in anti-viral immunity (Felix et al., Miska et al., Maduro et al., Ding et al.).

With a broad scope of identifying genes involved in anti-viral immunity in *C. elegans*, our lab, in collaboration with Ding lab employed an artificial system of viral infection of *C. elegans.* It involved constructing transgenic worms that have multiple copies of chromosomally integrated FHV RNA1 molecules driven by a heat shock promoter hsp-16.41. The lab also obtained feeding RNAi library containing genes specific to 11700 *C. elegans* genes. Several people in the lab and I participated in the screening process to identify genes that when knocked down, facilitated viral replication. We shortlisted the genes to 250 as they were consistently allowing

increased FHV replication when knocked down. I validated the involvement of a sub-set of genes in anti-viral immunity using Northern blot, *in situ* hybridization and RT-PCR. Strains *him-14*(it44), *him-14 (RNAi)* and *nhr-68(RNAi)* allowed higher levels of viral replication when tested using all the above methods. Thus they had a great potential for further investigation.

him-14 is a gene involved in proper segregation of homologous chromosomes during meiosis I in *C. elegans*. Loss of *him-14* reduces crossovers, eventually resulting in missegregation. Given its function, it is not a surprise that the expression of *him-14* is limited to the germline. How does a gene expressed in the germline alone, when knocked down, allow increased viral replication in the soma? Perhaps this effect is brought about through interactions between germline and soma.

There have been several reports on germline having its effect on soma in *C. elegans.* In 1999, it was reported that ablation of germline cells results in increase of lifespan of these animals through insulin/ IGF-1 pathway that is known to be involved in dauer formation (Antebi et al., 2008), fat metabolism (Tan et al., 2011), stress response (Holzenberger et al., 2003) and this activity involves *daf-16*. It was shown that in germline-less worms, DAF-16 moves from cytoplasm to nucleus of cells in the intestine. This indicated that the intestine was the site where the signals from the germline concentrated. DAF-16 in turn changes the expression of a wide set of genes such as stress response genes, chaperones, apolipoproteins and lipases (Ruvkun et al., 2008). They showed that the lifespan extension upon removal of

germline requires LIPL-4 activity, a predicted triglyceride lipase, which is regulated by *daf-16.* Also, autophagy is a critical pathway that regulates several different pathways. Autophagy is important in aging and higher autophagy level leads to a longer lifespan (Levine et al., 2003). Autophagosomes also provide a physical scaffold for RNA viruses to replicate inside host cells (Dreux et al., 2010). More recently, there has been a report relating autophagy, lipid content and lifespan in germline-less worms (Hansen et al., 2011). Here they showed that *glp-1* animals that lacked germline showed higher autophagy levels. Higher autophagy levels in animals also showed higher LIPL-4 activity. They also showed that autophagy and LIPL-4 are interdependent in germline-less worms. These interactions between different pathways are represented in the figure 21.

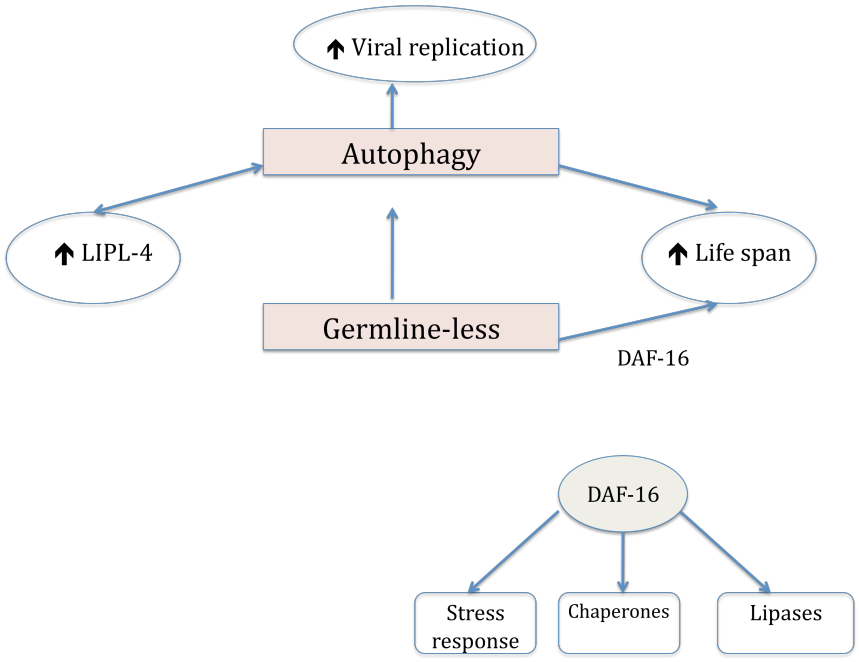


Figure 21: A diagram showing interaction between pathways.

From the above reports, it can be concluded that there is a strong interaction among germline, autophagy, lipid metabolism, lifespan pathways and viral replication. Higher viral replication in *him-14(it44)* and *nhr-68(708)* mutants could be related to one or all the above pathways. In order to better understand the roles played by these two genes, one can ask questions in the following directions.

i) Does higher lipid content in the body always corelate with higher LIPL-4 activity? In germline-less mutant such as *glp-1,* it has been shown that they have higher lipid molecules and also show high LIPL-4 activity. This relation is not very direct since LIPL-4 is a lipase protein that is involved in hydrolysis of lipid molecules. One would then expect lower lipid content when there is high lipase activity. The reason could be related to 'high demand- high supply' theory. If there is high lipid content, there is a greater demand to hydrolyze them and hence increased lipase activity. This way lipid homeostasis could be established. It must be first tested if this theory holds good in worms that are not germline mutants. The next step would be to upregulate LIPL-4 expression using a heat shock promoter or downregulate using RNAi and observe the lipid levels in their bodies. This would tell us the effect of disturbing the lipid homeostasis. Simultaneously, studying FHV/ Orsay viral replication under such conditions would reveal a relation between lipid levels and viral replication. One hypothesis would be that when lipid molecules increase, more sites for attachment of RdRP and the replication complex are made available, hence leading to higher replication. On the other hand, it could be that LIPL-4 is the signaling molecule that

eventually leads to higher/ lower viral replication rate depending on whether the molecule is over-expressed or under-expressed.

One can also study the lipid levels of all the positives from the secondary screen and also mutants in RNAi pathway by performing Oil Red O staining procedure. Preliminary data indicates the presence of high lipid content in mutants that show high viral replication. This could establish a strong relation between lipid content and high viral replication in *C. elegans.*

ii) What would be the effect of altering the signals sent from the germline-less worms to increase lifespan? This type of signaling shows interaction between the germline and soma. It is believed that such signals are concentrated in the intestinal cells. One can alter the levels of DAF-16 molecules and study its effects on lifespan extension and viral replication. If this signal from the germline is responsible for increased viral replication in *him-14(it44)* or *glp-4* mutants, under-expressing DAF-16 might result in reduced signaling, which would undo the higher viral replication rate. Interestingly, there are reports showing germline-less worms showing higher resistance to bacterial infections. This effect is insulin signaling pathway dependent (Ruvkun et al., 2003) and p38MAPK independent (Alper et al., 2010). Why do we see opposite effects in immunity against bacteria and viruses when the germline is removed? Perhaps, it could be due to the presence of a large number of bacteria infecting *C. elegans* compared to viruses. Worms are constantly evolving to fight against any invading microorganism. As a result, they have developed good immune

response against bacterial and fungal invasions. However the response to viral infection is not so robust due to limited existence of viruses that can infect worms.

iii) Autophagy is an important cellular mechanism that works both in favor and against viral infection in host cells. There has been a report showing increased *S. typhimurium* infection upon deletion of a gene involved in autophagy (Jia K. et al., 2009). In mice, knockout of autophagy gene results in increased susceptibility to *T. gondii* (Zhao Z. et al., 2008). On the other hand, microbes are evolving constantly to evade autophagy mechanisms. Viruses can prevent initiation of autophagy and also evade recognition by autophagosomes (Levine et al., 2009). Autophagosomes may act as physical scaffolds for membrane-associated replication of RNA viruses (Dreux et al., 2010). Several autophagy proteins such as Beclin 1, ATG4B, ATG5 are important for translation of viral RNA (Dreux et al., 2009). In our study, one can alter the levels of different autophagy genes and study the change in replication rate of FHV or the infection rate of Orsay virus.

Finally, our study employs two different viruses, Flock House Virus and Orsay virus. They are both positive strand RNA viruses with similar genetic constitutions. In our study to understand mechanisms of anti-viral immunity, the two viruses can bring in very different effects. FHV is an artificial system of viral infection. FHV replication, when activated by a heat shock promoter, makes thousands of copies of FHV RNA1 strand. This most likely is not the case in a natural infection such as Orsay viral infection. This could be both advantageous and disadvantageous. Through studies using FHV, we might uncover mechanisms that may not be evident by Orsay

infection. Certain anti-viral mechanisms may get activated only beyond a certain threshold of RNA1 virus. This condition can be extrapolated to a human condition where the immune system is compromised. On the other hand, studying Orsay virus reveals mechanisms involved in a more natural system of infection including viral recognition, viral entry, identification of exogenous epitopes/ particles by the host cell, viral replication, spread, virion assembly and egress. One can study different stages of viral infection using Orsay. Employing both viruses for anti-viral immunity mechanisms would give us a complete picture of several different factors involved in the process and would be more robust than one over the other. We can reveal parallel contributions of genes to virus resistance by removing multiple pathways at once.

CHAPTER 3. Materials and Methods

3.1. Feed and Screen Protocol:

Feeder plates were prepared as follows.

For 3 Litres:

NaCl……9 g

Agar……51 g

Peptone……7.5 g

dH_20……2925mL

Uracil (2g/L in dH_2O)……3 mL

$CaCl_2$……0.44 g

Cholesterol (10g/L in EtOH)….1.5mL

The mixture was autoclaved and let cool. Then the following were added.

Filtered Phosphate buffer (1M)..7.5mL

Filtered $MgSO_4$ (1M).....3mL

IPTG (1M)……3mL

Carbenicillin (25mg/mL)……3mL

Phosphate buffer (1M KH_2PO_4) (pH 6.0) was prepared as follows:

A= 1M KH_2PO_4……136.09g/L

B= 1M K_2HPO_4……174.18g/L

5-7 parts of A was mixed with 1 part B until pH is 6.0

The solution was autoclaved and let cool.

The media was then poured onto plates and were allowed to solidify. The plates were stored at 4^0C for further use.

E. coli clones from the library stored at -80^0C in glycerol stocks in 384-well format were grown overnight in LB media at 37^0C. They were seeded onto feeder plates the next morning. Meanwhile, *rrf-3; irIs91* worms were bleached to obtain a synchronized population. 50-100 bleached L1 worms were inoculated onto feeder plates and were allowed to feed on the *E. coli* strain at 15^0C for 2-3 days. When they reached L4 stage, they were heat shocked at 34^0C for 4 hours in a water bath to induce transcription of viral replicon. They were then transferred to 20^0C incubator for recovery. One day later they were screened for GFP. The total number of worms and the number of worms showing GFP expression in each plate was counted. Percent of worms showing GFP was calculated and any plate that showed higher GFP expression compared to the negative control was scored as positive.

3.2. qRT-PCR

First-Strand cDNA synthesis:

The following 10uL reaction was prepared:

RNA……1000ng

Random primers……..50-250ng

dNTP (10mM)……..0.5uL

RNase free water……..6.5uL

They were mixed by pipetting gently up and down. Mixture was heated at 65^0C for 5 minutes and incubated on ice for at least 1 minute. Then the following was added:

5X First-Strand buffer……..2uL

0.1M DTT……0.5uL

RNaseOUT Recombinant RNase Inhibitor……..0.5uL

Superscript III RT (200units/uL)……..0.5uL

They were mixed by gently pipetting up and down. They were then incubated at 25^0C for 5 minutes and at 50^0C for 60 minutes.

The cDNA was now used as template for amplification in PCR.

qPCR:

cDNA was diluted to 1:100 with ddH_20 for qRT-PCR analysis. Each sample was normalized to *actin-1*, and then viral RNA1 (primers GW194: 5′ ACC TCA CAA CTG CCA TCT ACA and GW195: 5′ GAC GCT TCC AAG ATT GGT ATT GGT) levels in the samples were compared to those in N2 (negative control) and JU1580 and *drh-1* (positive control) strains.

3.3. Crosses

i) him-14(it44); irIs91

Iris91 strain was constructed by others in the Maduro lab. Three plasmids consisting of hsp16-41::RNA1[GFP], unc-119::mCherry, and unc-119(+) were injected into the gonads of adult *C. elegans* worms. F2 worms that were *unc-119* rescued and mCherry positive (red) were selected. It was first made as an extrachromosomal array, then integrated into the genome by gamma rays. It was later mapped to chromosome V, around +5 map units on the genetic map.

him-14 gene is located on chromosome II and *irIs91* on chromosome V. Therefore, a standard protocol for crossing two unlinked genes was followed. 10-15 *him-14 (it44)* young adult males and 5 *irIs-91* hermaphrodites in the L4 stage were transferred to a mating plate and was incubated at 15^0C for 2 days for mating to occur. The hermaphrodites were then transferred to individual plates and were incubated at 15^0C for 4-5 days. 5-10 F1s that showed mCherry expression (indicating presence of *irIs91*) were selected and transferred to a fresh plate and was incubated at 15^0C for 4-5 days. 20 F2s that were mCherry positive were picked and transferred to individual plates and incubated at 15^0C for 1-2 weeks. Plates where subsequent generations were all mCherry positive (homozygous for *irIs91* insertion) and had 5-10% males (indicating mutation in *him-14(it44)* gene) were selected. They were once again confirmed for *him-14(it44)* mutation by sequencing the gene using the primers (*him-14 it44* US: 5'-CAG ATT CAG CCT GTC GAG TCA CG-3' and *him-14 it44* DS: 5'- CGT CCA CAT CAT GGA CTG TTC GCG- 3') and looking for C-T substitution mutation.

ii) glp-4(bn2); him-14 (it44); irIs91

glp-4(bn2); irIs91 was constructed by others in the Maduro lab. This was used to cross with *him-14(it44)* strain. 10-15 *him-14 (it44)* males were mated with 5 *glp-4; irIs91* L4 hermaphrodites. After 1.5 days, *glp-4; irIs91* worms were transferred to individual plates and was incubated for 3-4 days at 15^0C. 5-10 mCherry positive, F1, L4s were transferred to a single plate and was allowed to grow at 15^0C for 3-4 days. 30 mCherry positive L4s from the F2 generation were transferred to individual

plates and were allowed to grow at 15^0C for 3-4 days. Only the plates that gave 3-5% males in the next generation were selected. This indicated that they are *him-14(it44)* mutants. To test for the mutation in *glp-4* gene, the worms from the shortlisted plates were grown at 25^0C and selected for plates with no embryos. If the worms are *glp-4; irIs91* homozygous, since *glp-4* mutation is temperature sensitive, the phenotype is prominent at 25^0C.

3.4. *In situ* hybridization

A new *in situ* hybridization protocol was followed that was capable of detecting individual mRNA molecules within fixed sample (Raj et al., 2010).

Orsay probe:

The Orsay RNA1 probe was ordered from Biosearch Technologies Inc. (www.singlemoleculefish.com). The fluorophore tagged to the 5' end of the oligos was CAL Fluor Orange 560 (Excitation wavelength- 522nm and Emission wavelength- 544nm). To the dry oligo, Tris- EDTA buffer was added to make a 100uM stock solution. This was stored at -20^0C. A further 1:100ul dilution was made with TE buffer to make a working solution of the probe. 5uL of this was mixed with 500uL hybridization buffer. This was incubated with the samples overnight.

In situ hybridization technique essentially involves 3 main steps:

i) Fixation of worms:

The following protocol for fixation and permeabilization of *C. elegans* was used. This protocol is based on the protocol developed in the lab of Robert Singer (Femino et al., 1998)

Fixation solutions:

Fixation solution (3.7% formaldehyde/ 10% formalin, 1X PBS)

40mL RNase free H_2O

5mL 37%(v/v) formaldehyde (100% formalin)

5mL 10X PBS

M9

Na_2HPO_4……5.8g

KH_2PO_4……..3.0g

NaCl……0.5g

NH_4Cl……1.0g

ddH_2O……1 L

Fixation of worms:

The worms were bleached and to ~1000 embryos taken in eppendorf tubes, 100uL Orsay viral extract and 100uL LB containing OP50 *E. coli* were added. This was mixed thoroughly for 24h in a rotisserie at 25^0C. This ensured sufficient and uniform exposure of virus particles to the worms. The infected worms were then transferred to plates containing OP50 and were incubated at 25^0C for another 24h. At the end of 2 days, the worms were washed off from plates using M9 and were rinsed 2-3 times

using M9. M9 was then aspirated and 1mL of fixation solution was added to each tube and was incubated at room temperature for 45 minutes. They were then washed twice using 1X PBS and were resuspended in 1mL 70% ethanol. This was incubated at 4^0C for 1h and further steps were carried out (worms in 70% ethanol can be incubated for days).

ii) Hybridization

Wash buffer (50mL)

40mL RNase free water

5mL Formamide

5mL 20X Saline-Sodium Citrate

Hybridization Solution:

To 100uL hybridization buffer, 1-3uL of probe was added and mixed well.

Fixed samples in ethanol were centrifuged and ethanol was aspirated away. Worms were resuspended in 1mL wash buffer. The sample was then centrifuged and wash buffer was aspirated away. To this, 100uL of hybridization buffer was added and was incubated in dark at 30^0C overnight. Next morning, the samples were washed twice with wash buffer. 1uL of DAPI (4',6-diamidino-2-phenylindole) was added to 1mL wash buffer and 100uL of this solution was added to the samples and were incubated for 30 minutes at 30^0C. The samples were then washed twice with 2X SSC and were resuspended in appropriate volume (~100uL) of 2X SSC. 10-20uL of the sample was added on a slide. A 22X40 cover slip was placed gently on the slide and

excess liquid was absorbed away by small strips of paper towels. They were then screened for Orsay RNA1 molecules under fluorescent microscope.

3.5. Northern Blot

Northern blot method was used to detect the presence of Orsay RNA1 molecules in the mutants of interest. The same infection protocol was followed as described under *in situ* hybridization section in this chapter. After a 2-day infection, RNA molecules were extracted as follows:

Worms were washed off the plates using M9 and were resuspended in Trizol solution in eppendorf tubes. They were then homogenized using a tissue tearer. The samples were incubated at room temperature for 5 minutes. 0.2 mL chloroform was added to 1mL Trizol. The tubes were shaken well and were left at room temperature for 2-3 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 2-8^{0}C. The aqueous phase was transferred to a fresh tube. 0.5mL isopropyl alcohol was added and incubated at room temperature for 10 minutes. The samples were again centrifuged at 12,000g for 10 minutes at 2-8^{0}C. The RNA pellet was washed with 1mL 75% ethanol. The mixture was vortexed thoroughly and spun at 7500g for 5 minutes at 2-8^{0}C. The RNA pellet was allowed to dry. 30-50uL RNase free water was added to the pellet. The tubes were incubated at 55-60^{0}C for 10 minutes to allow pellet to dissolve in water. The concentrations of RNA samples were determined using a nanodrop spectrophotometer.

RNA gel was prepared as follows:

10X MOPS……10 mL

dH_20……90 mL

Agarose……..1.2 g

The solution was heated till all agarose dissolved and was let cool. 1.8 mL of formaldehyde was added slowly, mixed well and was poured onto an electrophoresis well containing comb.

RNA samples were prepared as follows:

Loading buffer:

1 part loading buffer

2 parts Formamide

A drop of Ethidium Bromide

The concentrations of RNA samples were determined using a nanodrop spectrophotometer. To 2ug of RNA samples, equal volume of loading buffer was added. The solution was denatured at 65^0C for 5 minutes. The samples were loaded in the gel and it was run for 1-2 h at 100V.

Blotting:

The pad and membrane used for RNA transfer was first wetted using water. The membrane was then placed on the pad and a plastic sheet was spread over it so that the entire surface is covered. The gel was placed on membrane and was run for 3h at 80^0C. 10X SSC was added on top of the gel so it doesn't dry out. After 3 h, the gel was removed and discarded. RNA was crosslinked to the membrane using an RNA crosslinker. rRNA was then stained using Methylene Blue till rRNA bands were seen.

The membrane was then washed with water to remove excess stain. It was then scanned for rRNA bands, which now acts as a loading control.

Pre-hybridization:

RNA membrane was taken in a hybridization tube. It was made sure that the membrane side was facing up. 7mL hybridization buffer was added to the tube and was rotated in a hybridization oven at 65^0C for at least an hour.

Hybridization:

Preparation of RNA1 probe:

DNA probe was prepared using primers specific to Orsay RNA1 molecules (same as that reported under qRT-PCR section in this chapter). A standard PCR reaction was performed and the RNA1 molecules were amplified. The mixture was then run on a gel, the band obtained was cut and DNA was extracted using a standard DNA gel extraction protocol. The concentration of DNA in sample was measured using a nanodrop.

Probe labeling:

80ng of probe was measured and 45uL dH_2O was added. The mixture was then denatured in boiling water for 5 minutes. It was then incubated on ice for 2 minutes. This solution was added to Amersham ready-to-go DNA Labelling Beads (-dCTP) from GE Healthcare. 5uL of dCTP isotope was also added and was incubated for 10 minutes at 37^0C. The probe was then denatured in boiling water for 5 minutes.

Hybridization:

Labeled probe was added to hybridization buffer. It was then allowed to rotate in hybridization oven at 65^0C for another 3-4 h.

Developing the film:

The membrane was then washed 3 times with 0.1X SSC and 0.1% SDS for 15 minutes each. It was used to develop onto an X-ray film using a developer. The film was then scanned to record the bands obtained.

3.6. Orsay Viral Extract

The same protocol as followed by Felix et al., 2011 for Orsay Viral extract preparation was used. Infected worms were grown on agar plates until just before starvation. The worms were washed off the plates using M9 and were centrifuged at 5000g. The supernatant was centrifuged twice at 21,000g for 5 minutes discarding the pellet each time. The supernatant was then passed through 0.2um filter and the filtrate was aliquot into 1.5mL eppendorf tubes and stored at -20^0C.

3.7. Oil Red O staining

N2 and *nhr-68(gk708)* strains were tested with Oil Red O dye to compare lipid content in these strains. Similar fixation protocol to *in situ* hybridization was followed. Oil Red O stain was prepared by dissolving 0.5 g powder in 100mL isopropanol. This formed the stock solution. This was then diluted to 60% with water to form the working solution. This was then filtered using 0.45um/ 0.22um filter paper.

3.8. Sudan Black staining

Young adults of N2 and *nhr-68(gk708)* strains were washed off from plates using M9. They were then fixed in M9 with 1% paraformaldehyde for 45 minutes at room temperature and then subjected to 3 freeze-thaw cycles. They were washed with 25%, 50% and 70% ethanol. Staining was then performed overnight in the working solution of Sudan Black B. The stained animals were observed under microscope.

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