Molecular identification of *let-336* and analysis of ionizing radiation induced lethal mutations within *Caenorhabditis elegans eT1*(*III*;*V*)

by

Aalton Rylan Fernandes
B.Sc., Simon Fraser University, 2005

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Department of Molecular Biology and Biochemistry

SIMON FRASER UNIVERSITY

Spring 2011

APPROVAL

Name: **Aalton Rylan Fernandes**

Degree: **Master of Science**

Title of Thesis: **Molecular identification of *let-336* and analysis of ionizing radiation induced lethal mutations within *Caenorhabditis elegans eT1*(*III*;*V*)**

Examining Committee:

Chair: **Dr. Nancy Hawkins**
Associate Professor, Department of Molecular Biology and Biochemistry

_____

Dr. David L. Baillie
Senior Supervisor
Professor, Department of Molecular Biology and Biochemistry

_____

Dr. Jack N. Chen
Supervisor
Associate Professor, Department of Molecular Biology and Biochemistry

_____

Dr. Barry Honda
Supervisor
Professor, Department of Molecular Biology and Biochemistry

_____

Dr. Fiona S. L. Brinkman
Internal Examiner
Professor, Department of Molecular Biology and Biochemistry

Date Defended/Approved: 20 April 2011

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ABSTRACT

let-336 was one of 194 EMS induced lethal mutations generated within the *eT1*(*III*;*V*) balanced region on the left arm of *Caenorhabditis elegans LGV*. Using data obtained from complementation tests with the deficiency strain *hDf36*, sequencing candidate genes within this region, as well as performing a fosmid rescue, I determined the molecular identity of *let-336* to be *F56E10.4* (*rps-27*).

Using *eT1*(*III*;*V*) as a mutational capture system, I screened 13,218 individuals and determined the spontaneous lethal mutation rate within the balanced region to be 0.03%. Furthermore, I analyzed the lethal mutation response of *C. elegans* when exposed to low doses of ionizing radiation. Exposure to a cumulative low dose of X-ray radiation was shown to produce less mutations when compared to an equivalent single high dose exposure. Increasing the time between exposures, irradiating dauers, as well as administering a low dose before a second high dose resulted in less lethals being produced.

Keywords: *let-336*; *rps*-27; Ribosomal small subunit S27; ionizing radiation; X-ray; *eT1*(*III*;*V*) genetic balancer

This work is dedicated to Sixteen

ACKNOWLEDGEMENTS

First and foremost I would like to express my thanks to my senior supervisor, Dr. David Baillie, who has supported and encouraged me throughout my graduate career. Dr. Baillie was a constant source of valuable knowledge and allowed me to pursue research on my own yet was always available for guidance and encouragement. I would also like to thank the other members of my supervisory committee, Dr. Jack Chen and Dr. Barry Honda, who provided advice and guidance over the years. I would also like to thank Dr. Johnsen for his advice as well as help in preparing my thesis. I would also like to thank Martin Jones for providing the oaCGH data, Yang Zhao for supplying complementation test data, and Domena Tu for injecting fosmids. I would also like to thank my volunteers, Nihar Bhattacharyya, Vicky Wu, and Byoungjoo Yoo for helping set up the background mutation screens. I would also like to acknowledge past and present members of the Baillie lab, especially Dr. Jeff Chu and ShuYi Chua who offered invaluable guidance along the way. Finally, I would like to thank my close friends and family who were always there to encourage me, and helped me prepare this manuscript.

I am grateful to Simon Fraser University, especially the labs that make up the MBB department for providing a rich learning environment and a memorable learning experience.

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LIST OF ACRONYMS AND ABBREVIATIONS

DNA	Deoxyribonucleic acid
DPY	*C. elegans* Dumpy
DSB	Double-strand break
EMS	Ethyl methanesulfonate
LG	Linkage group
mRNA	Messenger RNA
oaCGH	oligonucleotide array Comparative Genomic Hybridization
PCR	Polymerase chain reaction
rad	Radiation absorbed dose
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RNAi	RNA interference
tRNA	Transfer RNA
UNC	*C. elegans* UNCoordinated

CHAPTER 1: GENERAL INTRODUCTION

1.1 Genetic blueprint of life

Life forms are inherently specified by their genome. Every organism's genome contains all the biologically meaningful information needed to build and maintain a living example of that particular organism (Ridley 2006). In 1958 Francis Crick first envisioned the central dogma of molecular biology, which states that the biological information contained in a genome is encoded in DNA, transcribed into RNA, and then translated into proteins (Crick 1970). All living organisms are made up of cells, each of which contains this inherited genetic information packaged in the form of genes. Evolution occurs through the differential survival of competing genes; resulting in an increase in the frequency of alleles whose combined phenotypic effects increases the overall fitness of an individual (Futuyma 2005). The mechanisms of evolution are mutation, natural selection, genetic drift, recombination, and gene flow. All of these mechanisms are fundamental to create genetic variation upon which selective forces can act.

Genes can be classified as essential or non-essential based on their indispensability for a living organism. When categorizing genes as essential, one must also be aware of the environmental conditions that the organism was grown in, as these conditions may determine if certain genes are essential or not (Papp, Pal et al. 2004). Essential genes are required for an organism to survive and produce viable offspring (Kemphues 2005). It is therefore not surprising that

throughout evolution there is strong selection pressure to conserve these genes. Mutant alleles of essential genes can cause three types of discontinuous phenotypes:

1. Zygotic lethal mutations, known as *let*hals, prevent growth to adulthood when individuals are homozygous for the mutation, and are defined by arresting at either embryonic or larval stages.

2. *M*aternal-*e*ffect *l*ethal mutations, known as *mel* mutations, are a special form of sterile mutations. These mutants define genes that are expressed in the mother and are required for embryonic development. *Mel* mutations present in the P_o generation prevent F2 progeny from developing to fertile adults.

3. Sterile mutations prevent production of fertilized eggs. Sterile mutants define defects in germ line or somatic gonad development, oogenesis, spermatogenesis, ovulation or fertilization.

In order to understand the biology of any organism it is important to identify and analyze essential genes that make up that particular organism. Studies on essential genes in one organism can also further our understanding of evolution, as well as development in other organisms. This is because evolutionarily conserved genes will show homology to genes within other organisms that descended from a common ancestor. Therefore, studies using model organisms can ultimately give insight into human biology and development.

1.2 *Caenorhabditis elegans* a model organism

In 1963, Sydney Brenner identified a model organism that was ideal for the study of eukaryotic cellular development. He chose the soil nematode *Caenorhabditis* elegans because it is one of the simplest multi-cellular eukaryotes (Brenner 1974; Kenyon 1988). It is a transparent, free-living, non-parasitic nematode that can easily be maintained, observed and analyzed by various genetic and molecular techniques (Riddle, Blumenthal et al. 1997). Because of its transparency, internal structures can be detected using a compound microscope. As a model organism for the study of molecular genetics and development, *C. elegans* meets several requirements: firstly, it is a small multi-cellular organism with an adult length of 1 mm, thus permitting large cultures to be cultivated in the laboratory. Secondly, it has a short life cycle of approximately 4 days at 20^{o}C, allowing for rapid genetic analyzes. Thirdly, the nematode has two sexes, hermaphrodite and male. Hermaphrodites allow easy strain maintenance by self-fertilization, and males allow crossing for genetic analyzes.

C. elegans was the first multi-cellular organism to have its complete genome sequenced. When sequencing was essentially completed in 1998 (with very few remaining gaps), the genome was thought to contain about 97 Megabases and approximately 20,000 genes (Consortium 1998), of which about fifteen to thirty percent are believed to be essential genes (Johnsen 1997). Five years later, in 2003, the Human Genome Project (HGP) was completed. It was determined that the entire human genome, comprising of 3 billion bases, still only

accounts for 20,000 – 25,000 protein-coding genes (Stein 2004). Sequencing data allows for the identification of all genes, however it does not provide information about the biological functions of the genes. *C. elegans* genes are mapped into six linkage groups, which correspond to the six haploid chromosomes. There are five autosomes and one sex chromosome, all of which are roughly equal in size. Hermaphrodites are diploid for all six chromosomes (XX), whereas males are diploid for the autosomes but only have one X chromosome (XO). Genetic crosses can be carried out using these males.

1.3 Effects of ionizing radiation

Previous studies have shown that the nematode *C. elegans* is an excellent model organism with which to study the biological effects and mechanisms of ionizing radiation (Luo, Shah et al. 2009; Suzuki, Sakashita et al. 2009). Ionizing radiation is electromagnetic radiation with enough energy that when it interacts with an atom it can remove tightly bound electrons from the orbit, causing the atom to become charged or ionized (Blakely and Fry 1995). Particles with energies above a few electron volts (eV) are ionizing (Tisne 1995). Only the high frequency short wavelength portion of the electromagnetic spectrum, which includes X-rays and gamma rays, is ionizing (U.S.NRC 2004). X-rays are electromagnetic waves with a wavelength smaller than about 10 nm. The biological effects begin with the ionization of atoms within an organisms tissue or genetic material. When the electron shared by two atoms to form a molecular bond is dislodged by ionizing radiation, the bond is broken and the molecule falls apart. In this manner, ionizing radiation can cause double-strand breaks (DSBs)

within chromosomes (Povirk 2006). However, there are still very effective repair mechanisms which are conserved among organisms to repair this cellular or chromosomal damage (Lemmens and Tijsterman 2010), (van Haaften, Romeijn et al. 2006). Not all cells are equally sensitive to radiation damage. Cells which divide rapidly tend to show effects at lower dosages of radiation than cells which are less rapidly dividing and are more specialized (Cerutti 1974).

The biological effects of ionizing radiation depends on the dosage as well as how fast a radiation dose is received. An acute radiation dose can be defined as a large dose of radiation delivered during a short period of time. The effects caused by acute doses are deterministic and therefore based on the amount of dose received (Anno, Young et al. 2003). Deterministic effects have a threshold level – below which, the effect will not occur, but when the dose is above the threshold the severity of the effect increases as the dose increases (Pierce and Preston 2000). A chronic dose is a relatively small amount of radiation received over a long period of time. Organisms are better equipped to tolerate a chronic dose then an acute dose since there is more time to repair any damage that has occurred (Ikushima, Aritomi et al. 1996; Luckey 2008). The biological effects of high levels of radiation exposure are fairly well known, but the effects of low levels of radiation are more difficult to determine because the deterministic effects do not occur at these levels. Although radiation is known to cause cancers at high doses and high dose rates (Vrijheid, Cardis et al. 2007), currently there is no data to unequivocally establish the occurrence of cancer following exposure to low doses and low dose rates. Even so, the International

Commission on Radiological Protection (ICRP *Publication 60*, *paragraph 123*) assumes that any amount of radiation may pose some risk for causing cancer, and that the risk is higher for higher radiation exposures. A linear, no-threshold dose response relationship is used to describe the relationship between radiation dose and the occurrence of cancer (Cardis, Vrijheid et al. 2007). This collective dose model suggests that any increase in dose will result in an incremental increase in risk.

1.4 Research Objectives

This thesis concentrates on furthering our current knowledge of *Caenorhabditis elegans* genomic organization as well as response to mutational damage.

To fully understand the complex features of mammalian development, one must start with the analysis of smaller less complex genomes of model organisms. With this goal in mind, several researchers in the *C. elegans* research community initiated the *C. elegans* genome sequencing project with the arduous task of determining the complete nematode sequence (Sulston, Du et al. 1992). With this task completed over twelve years ago, researchers have now moved onto sequencing other nematode species, such as *Caenorhabditis briggsae*, with the goal of comparing genomes of closely related species (Hillier, Miller et al. 2007). Our lab has aided to complement the genome project by systematically creating *C. elegans* strains transformed with sequenced cosmids and making them available to the research community. With this information, several aspects of *C. elegans* growth and development have been intensively researched with

the ultimate goal of completely understanding the genome. One approach to completely understanding *C. elegans* growth and development is to identify and study all the essential genes. With this goal in mind, several researchers in the community have focused on one or a few specific genes within a region. In order to aid researchers, the Sanger Institute established, updates, and curates the *C. elegans* genome database called WormBase (www.wormbase.org). WormBase stores mapping, sequencing, and phenotypic information for *C. elegans* as well as some other nematode species.

In the second chapter of this thesis I focus on determining the molecular identity of *let-336*. This was one of 194 lethal mutations on the left arm of *LGV*, generated via a large EMS mutagenesis screen (Johnsen and Baillie 1991), and balanced by the reciprocal translocation *eT1*(*III*;*V*) (Fig.1). *let-336* is an interesting gene in that it is a decent size EMS target, with three alleles being generated, and it is an essential gene that mapped close to the telomere end of *LGV*(left). In this chapter, from complementation tests, sequencing data, as well as fosmid rescue experiments I determined that the molecular identity of *let-336* is *F56E10.4*.

F56E10.4 (*rps*-27) encodes a small ribosomal subunit S27 protein. Ribosomes are vital cellular components of all living cells; required to make proteins, they are made from complexes of approximately 60% ribosomal RNA (rRNA) and 40% proteins. The ribosomes in eukaryotic cells have a Svedberg value of 80S and are comprised of 60S and 40S subunits. The large 60S subunit is responsible for binding to transfer RNA (tRNA) and the amino acids, and the

small 40S subunit is required to bind messenger RNA (mRNA). *rps*-27 is one of the 31 *rps* genes that that make up the ribosomal components of the 40S small subunit.

In the third chapter of this thesis I focus on lethal mutations in essential genes generated by ionizing radiation. My screen encompasses mutations generated within the whole region balanced by *eT1*(*III*;*V*). More specifically I concentrate on the effects low dosages of ionizing radiation has on the mutation rate in *C. elegans*.

The discovery of X-ray mutagenesis originally started with the work of Hermann Joseph Muller in the 1920's. In 1927, Muller published the first paper demonstrating the power of X–rays to induce lethal and visible mutations in Drosophila (Muller 1927). In subsequent years, several studies in other model organisms have shown that the frequency of induced mutations is a function of X-ray dose (King 1954; Ono, Ikehata et al. 1997). These experiments revealed that there was a linear relationship between X-ray dose and induced mutation level, that there was no threshold or "safe" dose of radiation and that all doses are significant. Previously our lab developed a system to measure the accumulated mutation rate (Rosenbluth and Baillie 1981), I have adapted the *eT1* system to measure the mutation rate caused by administering low doses of ionizing radiation.

The objectives of this thesis will be to:

1. Determine the molecular identity of *let-336*.
2. Investigate the lethal mutation rate of *C. elegans*, concentrating on the effects low dose ionizing radiation has on mutation levels.

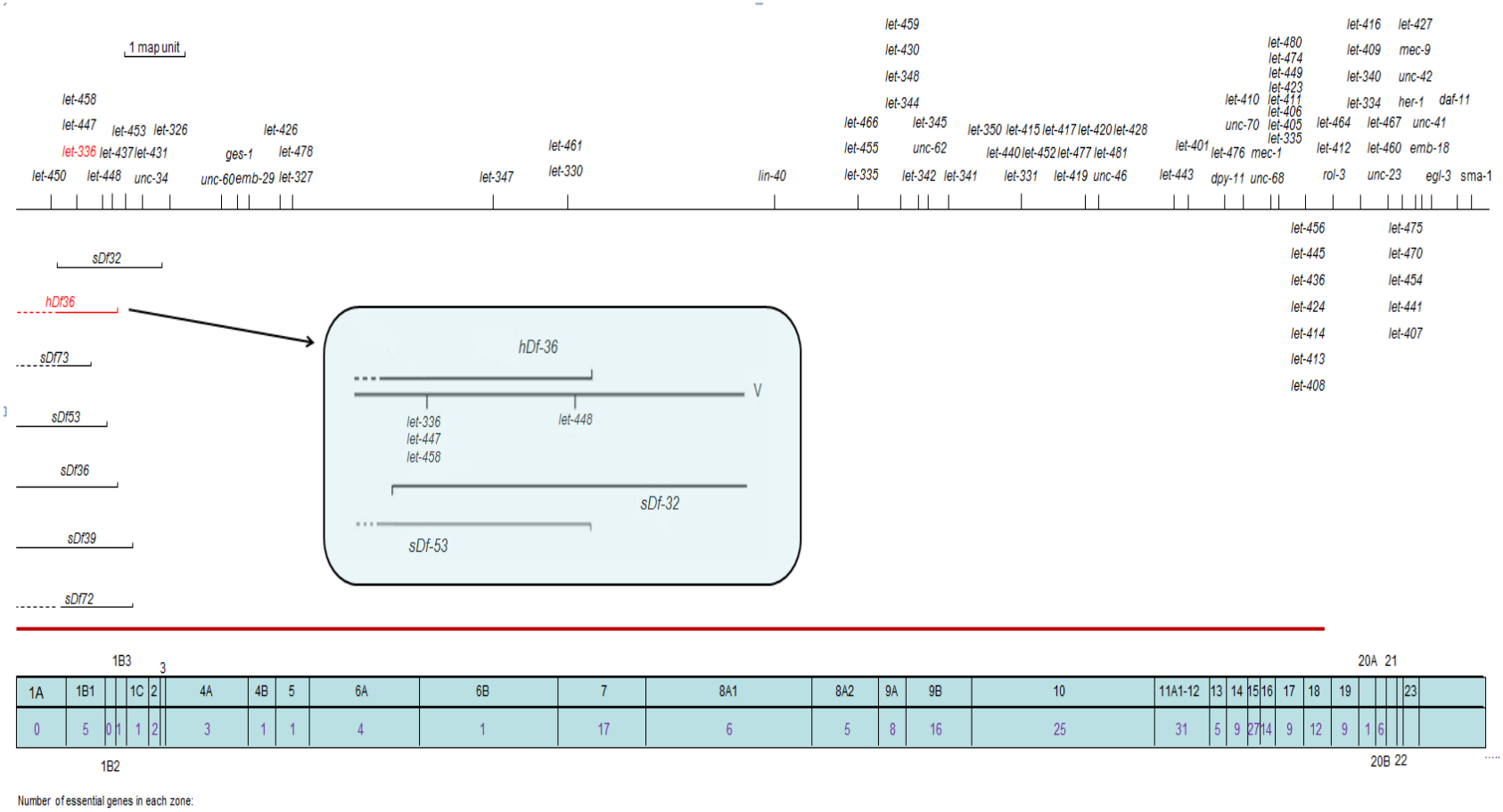

Figure 1 Genetic map of *LGV* (left) showing location of lethals generated by EMS. Adapted from Johnsen *et al.*, (1991).

***let-336* and the deficiency *hDf36* (shown in red) can be seen clearly in insert. Thick red line represents area balanced by *eT1*.**

CHAPTER 2: MOLECULAR IDENTIFICATION OF *let-336*

2.1 Introduction

The entire *C. elegans* genome consists of 10.3 x 10^7 base pairs (bp) contained within six chromosomes (Gerstein, Lu et al. 2010). Each chromosome is roughly the same size with the smallest, Chromosome *III*, containing 13,783,700 bp and the largest, Chromosome *V*, containing 20,924,149 bp (WormBase WS*218*). In order to maintain and study lethal mutations, a mutation must be allowed to be passed on to subsequent generations without recombination occurring. This can be accomplished by using genetic balancers that suppress crossing over within certain regions. One such balancer is the reciprocal translocation *eT1(III;V)* (Fig. 2), in which the left end of *LGV* and the right end of *LGIII* has been balanced, the balanced region spans approximately 5.6 x 10^6 bp on *LGIII* (right) and 8.9 x 10^6 bp on *LGV* (left), suppressing cross over within this region (Zhao, Lai et al. 2006).

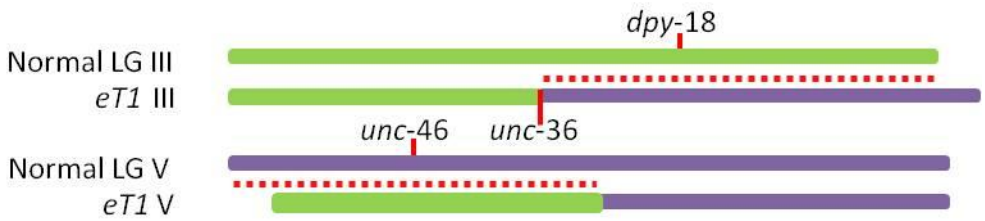

Figure 2 Genetic map of *eT1(III;V)*showing breakpoint of *eT1* within *unc-36* gene. Balanced region represented by dashed line. *dpy-18* and *unc-46* marker position shown.

hDf36 is a deficiency that extends from the left end of *LGV* and deletes a region approximately 164 Kbp (Fig. 1 and 3). Complementation tests performed by Y. Zhao have determined that the deficiency *hDf36* fails to complement *let-336* (Yang Zhao *Pers. Comm*), narrowing down this lethals location to the left arm of *LGV* on the physical map, in the region deleted by *hDf36* (Zhao, Lai et al. 2006). Another tool that can be used for the molecular characterization of deficiencies as well as duplications within strains is an *O*ligonucleotide *A*rray *C*omparative *G*enome *H*ybridization (oaCGH) chip (Jones, Maydan et al. 2007). Using this technique the deficiency right endpoint of *hDf36* was determined to be located approximately *18*4,204- 194,476 bp from the left end of *LGV*. The deficient region of *hDf36* was shown to be between 20,729 bp and *18*4,204 bp (Martin Jones *Pers. Comm.*), and is shown in figure 3. Using these results there is in total twenty four confirmed and predicted genes that lie in this region (Table 1). These twenty four genes make up the list of candidate genes for *let-336*.

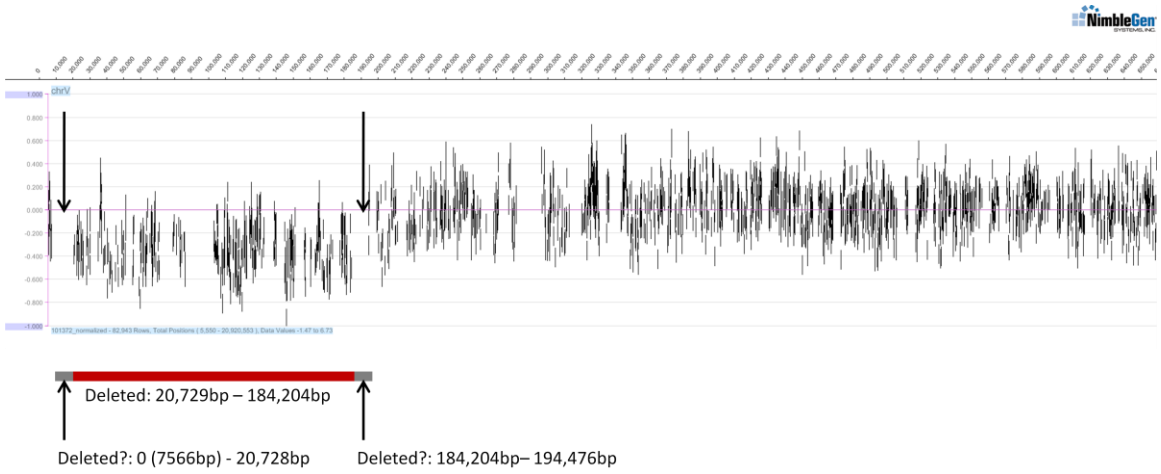

Figure 3 oaCGH data *LGV* (left) showing deletion breakpoints of *hDf36*. [~20.7-*184* Kbp] Red area represents confirmed deletion, grey area represents highly repetitive elements with no probe coverage (Data provided by Martin Jones).

Table 1 Genes known to lie in the region covered by *hDf36*.

Sequence Name	Gene Name	Start (bp)	End (bp)
B0348.4	*egl-8*	20825	43694
B0348.2		45*46*7	*46*880
B0348.1		48922	49619
Y38C9B.2	*srt-52*	49956	560*46*
Y38C9B.1	*cyp-29A3*	59242	63509
Y38C9B.3		63726	64503
F56E10.2	*fhod-2*	64564	71006
F56E10.3		79362	86*46*0
F56E10.4	*rps-27*	103385	104070
F56E10.1		104830	105964
Y38C9A.1		107402	1*18*162
Y38C9A.2	*cgp-1*	1*18*039	122966
W03F9.1		124*46*	126745
W03F9.2		127051	127982
W03F9.10		128237	131739
W03F9.3		132553	132998
W03F9.11		137660	141302
W03F9.9		138892	140225
W03F9.6	*srh-82*	145194	1*46*931
W03F9.4		147538	151384
W03F9.5	*ttb-1*	156825	166863
Y108G3AL.3		163842	172211
Y108G3AL.2		166994	176762
Y108G3AL.1	*cul-3*	177422	*18*4873

2.1.1 Analysis of a single gene

A classical approach commonly used to identify essential genes is through random mutagenesis followed by screens for non-conditional lethals and steriles within balanced genomic regions. *E*thyl *m*ethane*s*ulfonate (EMS) is a commonly used mutagen that randomly creates point mutations through guanine alkylation, the ethyl group of EMS reacts with guanine forming O-6-ethylguanine. DNA polymerase places thymine instead of cytosine opposite the abnormal base and a subsequent round of replication results in a G/C to A/T transition (Anderson 1995). *let-336* is an essential gene in which all three alleles were generated in a

previous study by EMS treatment (Johnsen and Baillie 1991). I initially determined the molecular identity of *let-336* by sequencing the genomic region of the candidate genes, *W03F9.10*, *F56E10.2*, and *F56E10.4* in the strain BC2214 that contains *let-336* (*s1413*). Genomic DNA was then extracted from heterozygous individuals by single worm lysis. Sequence data was assembled and analyzed for G/C to A/T transitions. Once a C to T transition was found in the candidate gene *F56E10.4* (*rps*-27) I sequenced rps-27 in the second allele BC2296 (*s1495*), and found another C to T transition. Protein alignment of *rps*-27 with several orthologs in *Homo sapiens, Drosophila melanogaster,* and *Saccharomyces cerevisiae* resulted in mutations in evolutionarily conserved residues. Sequencing results were supported by rescuing *let-336* with the fosmid *WRM0629cC03* that spans the genomic region containing *rps*-27 (Fig. 4). The fosmid *WRM0629cC03* includes transcripts from the following genes: *F56E10.4, F56E10.1, Y38C9A.1, Y38C9A.2, W03F9.1, W03F9.2, W03F9.3, W03F9.10,* and *W03F9.11.*

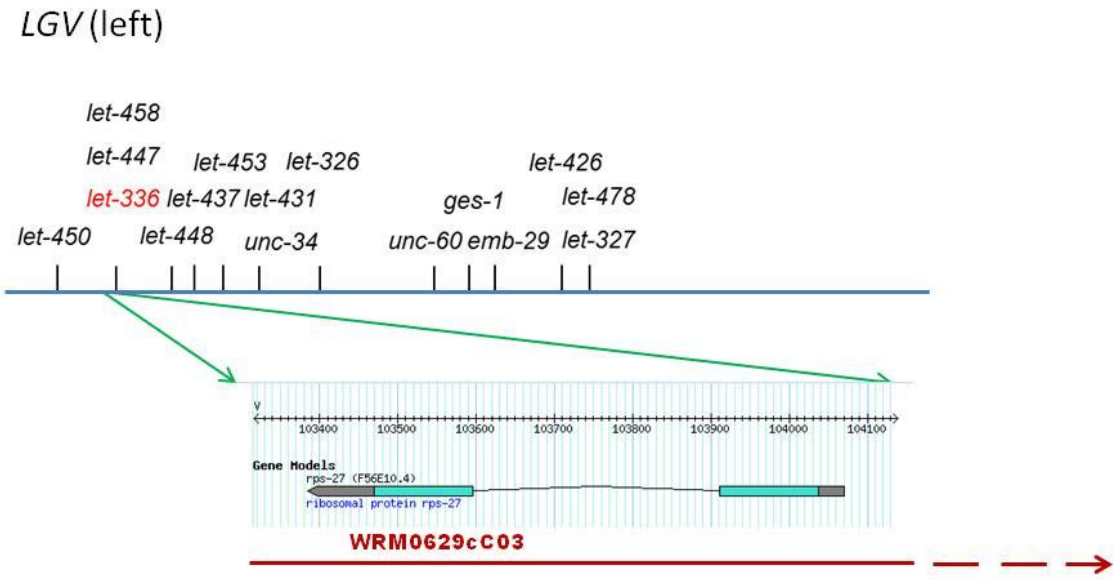

Figure 4. Physical map and genetic map in *let-336* region. *rps*-27 genetic location shown below. WRM0629cC03 was used for fosmid rescue of *let-336*.

2.2 Materials and Methods

2.2.1 Maintenance of *C. elegans*

All nematode strains were cultured on 60 x 15 mm Petri Plates containing Easiest Worm Plate Agar [Easiest Worm Plate Base Mix: 55 g Tris-HCl, 24 g Tris-OH, 310 g Bacto Peptone, 800 mg Cholesterol, 200 g NaCl. Easiest Worm Plate Agar: 11.8 g Easiest Worm Plate Base Mix, 36.0 g agar, dissolve in 2 L dH_2O] and seeded with approximately 0.05 mL of *Escherichia coli* OP50 (Brenner 1974). The wild-type strain N2 (var. Bristol, BC49) and mutant strains used were obtained from our own laboratory. All experiments as well as matings were carried out at 20^{o}C. Mutant strains used in this study with the *let-336* genotype were previously induced on *LGV* (left). To prevent recombination and loss of lethal mutations all alleles were balanced over *eT1(III;V)*. Three strains used in this study: BC2214, BC2221, and BC2296 all had *let-336* linked to the marker *unc-46* (*e177*). For a list of all *let-336* strains refer to Appendix A. These stocks were maintained by picking wild type progeny that did not give rise to viable DpyUnc nematodes. To view the genotype and phenotype of selfed *let-336* progeny refer to Appendix B.

2.2.2 Extraction of *C. elegans* genomic DNA

Genomic DNA was isolated from heterozygous nematodes using single worm lysis. One nematode was placed into a single 0.2 mL PCR tube containing 2 µl of worm lysis buffer, the nematodes were spun down at 14,000 RPM in a micro centrifuge for one minute, and then freeze-cracked twice in liquid nitrogen. A MJ Research- Peltier Thermal Cycler was used to incubate the nematodes at

60^{o}C for one hour, followed by a 15 minute incubation at 95^{o}C to deactivate proteinase K. Lysis buffer was prepared by mixing: 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% DNA free gelatine and 100 ug/ml proteinase K. This mixture was then used as the template (*let* genomic DNA) for subsequent PCR reactions. Genomic DNA samples were then stored at -20^{o}C until needed as a template for a 20 µl PCR reaction. 1 µl of genomic DNA was then used as template for the 20 µl PCR reaction.

2.2.3 PCR protocol for sequencing candidate genes

The DNA sequence for candidate genes were obtained from WormBase and used as a template to create amplification and sequencing primers. All primers were designed using the Primer 3 (v0.4.0) program (Rozen and Skaletsky 2000). *F56E10.4* PCR amplification primers: external forward: GATG AAAT TGCT GGG CCTA A, external reverse: GGGT TTGA TTGG GAAA AGGT, internal forward: GCAA TCGT GTTT TTCC GTTT, internal reverse: GAAG CGCC AATT ACCT TTCA. *F56E10.4* sequencing primers: first forward: GCAA TCGT GTTT TTCC GTTT, second forward: TGCT CCGA GAAG TAGC CATT, first reverse: GGGT GTTG AACA AGAC GCTT A, second reverse: CAAA TGGA AGTC GAAC ACAC A. Primer sequence properties, identity, and location for amplification and sequencing are shown in Appendix C. Samples were sent to Macrogen Korea for sequencing.

2.2.4 Agarose gel electrophoresis

PCR products were resolved by running samples through a one percent electrophoresis agarose gel in 1X TAE (Tris-acetate-EDTA) buffer. 0.05 µg/mL ethidium bromide was added to the agarose gels and a 1 kb ladder was used as a marker to compare band sizes. DNA bands were visualized by exposure to UV light.

2.2.5 Sequence alignment and protein sequence analysis

To assemble and analyze the sequencing results for EMS induced mutations and the corresponding amino acid changes I utilized the BioEdit (v7.0.9) sequence alignment editor (www.mbio.ncsu.edu/BioEdit/BioEdit.html). To further analyze the protein sequence of *rps*-27 I utilized InterProScan (www.ebi.ac.uk/Tools/pfa/iprscan/) as a tool to scan protein signatures against the amino acid sequence of *rps*-27.

2.2.6 Preperation of fosmid *WRM0629cC03*

The fosmid *WRM0629cC03* was kindly provided by Andreas Steimel (Hutter Lab Simon Fraser University). Bacterial cultures containing this fosmid were grown overnight in LB CAM media (containing 12.5 mg/mL chloramphenicol), in a 37^{o}C incubator with vigorous shaking for 16 hours. After initial incubation, 4.5 mL of fresh LB CAM was inoculated with 0.5 mL of the overnight culture along with 5 µl 1000X fosmid induction solution (Copy-Control Induction Solution 10% L-arabinose in H_2O) to promote high copy replication. These samples were incubated for 5 hours in a 37^{o}C incubator with vigorous

shaking. QIAprep Spin Miniprep Kit was used to extract the fosmid. Samples were eluted in 30 µl autoclaved ddH_20 heated to 70^oC. Concentrations of samples were then determined using a Nanodrop ND-1000 Spectrophotometer (*WRM0629cC03* - 26.4 ng/µl). A sample of the original liquid cultures (700 µl culture + 300 µl 100% Glycerol) was frozen away in -80^oC freezer.

2.2.7 Microinjection

Microinjection needles were made by pulling 1.0 mm, 6" filament capillary tubes (World Precision Instruments) with a Sutter P-97 horizontal needle puller and subsequently mounted into a Leitz Wetzlar micromanipulator. Domena Tu generated transgenic strains by injecting the fosmid *WRM0629cC03* into the nematodes gonad using these needles.

Injection mix was prepared using 100 ng/µL *pCeh361* containing *dpy-5*$^+$ rescue, 10 ng/µL p[myo-2]::GFP fusion reporter gene, and 10 ng/µL fosmid, and injected into *dpy-5*$^-$(e907). From the injected worms, F1 progeny that displayed the wild type phenotype were individually plated and grown in 23^oC for 3 days. F2 wild type progeny were screened for with the fosmid by searching for nematodes that displayed non-Dpy phenotype as well as GFP expression. These nematodes were picked under a ZEISS Stemi SV11 dissecting microscope fitted with a GFP filter. Those worms that displayed GFP expression were plated individually and reset to create several lines. Single worms from all lines were lysed using the single worm lysis protocol mentioned above to ensure they contained fosmids. Fosmid PCR check was conducted using PCR primers, 600 bp apart, designed by ShuYi Chua and specific to the fosmid backbone.

Fosmid-F: GCGA CCAC GTTT TAGT CTAC G, Fosmid-R: TCAA TACT TGCC CTTG CCCT TGAC AGG. The line containing the fosmid and having the highest transmission rate were used for subsequent matings, as well as being frozen away in the Baillie Laboratory stocks. (BC7961 sEX1753; *dpy*-5(*e907*)/*dpy*-5(*e907*)[*WRM0629cC03+pCeh361+ myo*-2::GFP])

2.2.8 *let-336* fosmid rescue using *WRM0629cC03*

The fosmid *WRM0629cC03* was chosen to rescue *let-336* (*s1413*), as it covers *F56E10.4* (*rps-27*). Fosmid DNA was prepared and injected into nematodes as described in previous sections. The resulting genotype was created (dpy-5(e907)/dpy-5(e907) [WRM0629cC03+myo-2::GFP+pCeh361]), crossed to +/+ N2 males, which resulted in generating males with the genotype dpy-5(e907)/+ [WRM0629cC03+myo-2::GFP+pCeh361]. Fosmid carrying GFP expressing males were crossed to *let-336* (*s1413*). The F1 hermaphrodites were singly plated and allowed to self. F2 progeny were screened for the presence of viable Dpy-Unc's, indicating a successful rescue. The line was retained and designated BC7902 (sEX1744).

2.3 Results

2.3.1 *F56E10.4* Sequencing Results

Initially all three alleles of *let-336 (s1413), (s1420),* and *(s1495),* under the same conditions, were lysed and genomic templates were obtained as previously described. However, in an attempt to save time determining the molecular identity of *let-336* primarily through sequencing, only one allele, *s1413,* was used

as a template. I designed primers to PCR amplify the *F56E10.4* gene in the *let-336* (*s1413*) mutant. Since *F56E10.4* is a fairly small gene, I designed one set of external primers to amplify 1679 bases, and also a set of nested primers that amplify 835 bases within the external PCR product. The nested primers amplified approximately 150 bases surrounding the gene, and were used to produce a purified sample that could be sent out for sequencing. Initially only forward primers were used for sequencing. A clean contiguous sequence was obtained and assembled using the program BioEdit, and aligned to the reference sequence obtained from WormBase. A single mutation, consistent with EMS mutagenesis, was found. The C to T transition was discovered at nucleotide position 508.

After a mutation was found in *s1413*, the other two alleles of *let-336 (s1495* and *s1420)* were PCR amplified using the same primers, and sent out for sequencing. Both forward and reverse sequencing reads were obtained and aligned together in BioEdit as done previously.

Unfortunately, no mutation was found in *s1420*. *s1495*, on the other hand, was found to have another single C to T transition at nucleotide position 517 from the start site. When analyzing sequencing results, both mutations had multiple coverage and are known to lie within the second exon of the gene.

Nucleotide sequences for both mutants *s1413* and *s1495* were then translated into a protein sequences. In *s1413* a Q to Stop mutation was found, truncating the protein prematurely at amino acid residue 65, and in *s1495* a R to

Stop mutation was found, truncating the protein prematurely at amino acid residue 68.

2.4 Discussion

2.4.1 Identification of *let-336*

In an EMS screen for essential genes, our lab previously isolated three recessive lethal alleles of a *C. elegans* gene *let-336* (*s1413*, *s1420*, *s1495*). By crossing to several deficiency strains, *let-336* was eventually mapped to the left arm of *LGV* within the region deleted by the deficiency *hDf36* (Yang Zhao *Pers. Comm*). oaCGH analysis to determine the exact base pair where the molecular breakpoint of *hDf36* occurs was done by Martin Jones. However the deficiency breaks in an area of low probe coverage, therefore the right breakpoint of *hDf36* was only narrowed down to about a 10kb region, and similarly for the left end of the deficiency. This data was used to generate a list of candidate genes that potentially could be the identity of *let-336*. Of the 24 genes that lie within the region covered by *hDf36* the list was narrowed down to first search for essential genes with known RNA interference (RNAi) phenotypes. The list of candidate genes was then further narrowed down to seven genes. Out of the seven candidate genes three (*W03F9.10*, *F56E10.2*, and *F56E10.4*) have RNAi phenotypes similar to the molecular phenotype of *let-336*. These were the strongest candidates for the molecular identity of *let-336* and were tested first. Published RNAi data from previous studies which targeted *F56E10.*4 have reported larval arrest (Rual, Ceron et al. 2004), and embryonic lethal RNAi phenotypes (Sonnichsen, Koski et al. 2005). By sequencing *F56E10.4* in two *let-*

336 alleles I determined mutations that would cause truncated proteins to be made. I further provided evidence that *F56E10.4* was the molecular identity of *let-336* by rescuing *let-336* (*s1413*) by crossing it to the strain BC7961 *(dpy-*5(*e907*)/*dpy*-5(*e907*)[*WRM0629cC03+pCeh361+ myo*-2::GFP]) carrying the fosmid *WRM0629cC03*.

The gene *rps*-27 in *C. elegans* encodes a small ribosomal subunit S27 protein. This protein is known to be a structural component of the 40S small ribosomal subunit, and is one of 31 genes that make up the *rps* gene family, which are responsible for making ribosomal proteins. Each ribosome is made up of ribosomal RNA (rRNA) and 76 different proteins. Some ribosomal proteins are involved in the assembly or stability of ribosomes, others help carry out ribosomal functions: to read genetic instructions encoded in messenger RNA (mRNA) and build new proteins by combining appropriate amino acids. Because ribosomal proteins interact with one another and with rRNA, it can be difficult to determine the specific functions of individual proteins within the ribosomes. Ribosomal protein levels can vary as a function of organism growth and development (Hansen, Taubert et al. 2007). Several studies have also suggested that some ribosomal proteins may have other cellular functions in addition to their roles in protein production. These additional functions include regulating cell division (Chiocchetti, Zhou et al. 2007), participating in chemical signalling pathways within the cell (Li, Tan et al. 2007), and triggering cellular apoptosis (He and Sun 2007).

The *C. elegans* RPS-27 protein sequence is quite short, consisting of only 83 amino acids. A signature recognition search tool, InterProScan, was used to determine that RPS-27 contains a C4-type zinc finger domain. The sequence of this class of zinc finger binding domain is: CX(2)CX(14-16)CX(2)C, where X can be any amino acid. Zinc fingers are small protein domains that utilize one or more zinc ions to help stabilize folds, they typically function as interaction molecules that bind DNA, RNA, or other small molecules. The amino acid sequence of *C. elegans* RPS-27 is shown below; the exact location of the zinc finger domain is underlined in bold.

MPLAV DLLHP EPQRE IRCHK LKRLV QHPNS YFMDV K**CSGC** FKIST

VFSHA TTVVV **CVGC**N TVLCQ PTRGK AKLTE GCSFR KKQ

Essential genes, vital for an organisms survival, experience a lot of selective pressure to be conserved evolutionarily. RPS-27 is shown to be conserved across multiple species, and shows high sequence similarity and conservation between *C. elegans* and several distantly related species such as *Homo sapiens*, *Drosophila melanogaster*, as well as *Saccharomyces cerevisiae* (Fig. 5). The EMS induced lethal mutation generated in *s1413* occurred in the 65th amino acid residue. This residue is highly conserved across several species. The resulting C to T mutation converted a glutamine into an amber stop codon, and resulted in a truncated protein. The EMS induced lethal mutation generated in *s1495* occurred in the 68th amino acid residue. The resulting C to T mutation converted an arginine into an opal stop codon, and also resulted in the formation of a truncated non-functional protein.

Figure 5 RPS-27 conservation and location of amino acid mutations in *let-336* alleles *s1413* Q→Stop (65th amino acid), and *s1495* R→ Stop (68th amino acid).

There are 31 *rps* genes in *C. elegans* distributed across five of the six linkage groups. *LGIV* contains the most, containing ten *rps* genes in total: *rps-24, rps-28, rps-25, rps-4, rps-8, rps-2, rps-5, rps-11, rps-23,* and *rps-18*. *LGIII* contains nine genes in total: *rps-29, rps-22, rps-1, rps-0, rps-12, rps-3, rps-13, rps-14,* and *rps-21*. *LGI* contains eight genes: *rps-10, rps-17, rps-15, rps-19, rps-7, rps-6, rps-20,* and *rps-26*. *LGV* contains three genes: *rps-27, rps-30,* and *rps-16*. And finally, *LGII* only contains one gene: *rps-9*. There are no *rps* genes on *LGX* (Fig. 6). A possible explanation for the absence of *rps* genes on the X chromosome is due to the fact that X-linked genes are transcriptionally silenced during mitosis and early meiosis (Kamath, Fraser et al. 2003). Therefore, genes that are essential for cellular processes are expected to be absent from the X chromosome. All *rps* genes have lethal RNAi phenotypes (Table 2).

If we analyze *rpl* genes distribution across *C. elegans* linkage groups we see a similar pattern. There are 45 *rpl* genes in *C. elegans* distributed across all six linkage groups. *LGI* contains the most, containing 13 *rpl* genes in total: *rpl-7, rpl-17, rpl-27, rpl-1, rpl-13, rpl-24.1, rpl-4, rpl-19, rpl-25.2, rpl-24.2, rpl-14, rpl-30*, and *rpl-31*. *LGII* contains nine genes in total*: rpl-22, rpl-33, rpl-10, rpl-5, rpl-26, rpl-32, rpl-42, rpl-41*, and *rpl-43*. *LGIII* also contains nine genes in total: *rpl-16, rpl-37, rpl-3, rpl-23, rpl-36, rpl-6, rpl-9, rpl-35*, and *rpl-21*. *LGIV* contains seven genes: *rpl-15, rpl-7A, rpl-20, rpl-34, rpl-12, rpl-18*, and *rpl-29*. *LGV* contains five genes: *rpl-11.1, rpl-39, rpl-28, rpl-38*, and *rpl*-2. And *LGX* contains two genes: *rpl-11.2*, and *rpl-25.1* (Fig. 7).

With regards to genes essential for cellular processes, silencing of X-linked genes during germ-line development would place constrains on gene activity. One possible solution to X chromosome silencing of these cell-essential genes is that germ-line-active copies of the genes are also located on an autosome (McKee and Handel 1993). There are two autosomal *rpl* genes: *rpl-25.2* (*LGI*) and *rpl-11.1*(*LGV*), both of which have paralogs on the X chromosome, *rpl-11.2* (*LGX*) and *rpl-25.1*(*LGX*). The functions of duplicated genes may change over evolutionary time, or both genes may maintain functionality and subfunctionalization can occur (Thomas 1993; Lynch and Conery 2000). A previous study examining the RNAi phenotypes of the *rpl* genes *rpl-11* and *rpl-25* has determined that the zygotic germ-line function is carried by the autosomal copy (*rpl-11.1* and *rpl-25.2*), while somatic function is carried by the X-linked copy (*rpl-11.2* and *rpl-25.1*) (Maciejowski, Ahn et al. 2005).

Table 2 *rps* gene location, protein size and observed RNAi phenotype

Ribosomal Enzyme	LG Location	Sequence Name	Protein Size	Observed RNAi Phenotype
rps-10	I	D1007.6	149 aa	1,2,4
rps-17	I	T08B2.10	130 aa	1,2,4
rps-15	I	F36A2.6	151 aa	1,2,4
rps-19	I	T05F1.3	1*46* aa	1,2,4
rps-7	I	ZC434.2	194 aa	1,2,3,4
rps-6	I	Y71a12B.1	2*46* aa	2,4
rps-20	I	Y105E8A.16	117 aa	1,2,4
rps-26	I	F39B2.6	117 aa	1,2,4
rps-9	II	F40F8.10	*18*9 aa	1,2,3,4
rps-29	III	B0412.4	56 aa	1,2,4
rps-22	III	F53A3.3	130 aa	2,3
rps-1	III	F56F3.5	257 aa	1,2,4
rps-0	III	B0393.1	276 aa	2,4
rsp-12	III	F54E7.2	140 aa	2
rps-3	III	C23G10.3	247 aa	1
rps-13	III	C16A3.9	151 aa	1
rps-14	III	F37C12.9	152 aa	1,2
rps-21	III	F37C12.11	88 aa	1,2
rps-24	IV	T07A9.11	131 aa	2
rps-28	IV	Y41D4B.5	65 aa	4
rps-25	IV	K02B2.5	117 aa	1
rps-4	IV	Y43B11AR.4	259 aa	1
rps-8	IV	F42C5.8	208 aa	2,4
rps-2	IV	C49H3.11	272 aa	1,2,4
rps-5	IV	T05E11.1	210 aa	1,4
rps-11	IV	F40F11.1	155 aa	1,4
rps-23	IV	F28D1.7	143 aa	4
rps-18	IV	Y57G11C.16	154 aa	4
rps-27	V	F56E10.4	83 aa	1,2
rps-30	V	C26F1.4	130 aa	4
rps-16	V	T01C3.6	144 aa	1,4

All *rps* genes have a lethal RNAi phenotype (WormBase WS224) RNAi phenotypes: 1. Embryonic lethal 2. Larval lethal 3. Sterile 4. Maternal sterile

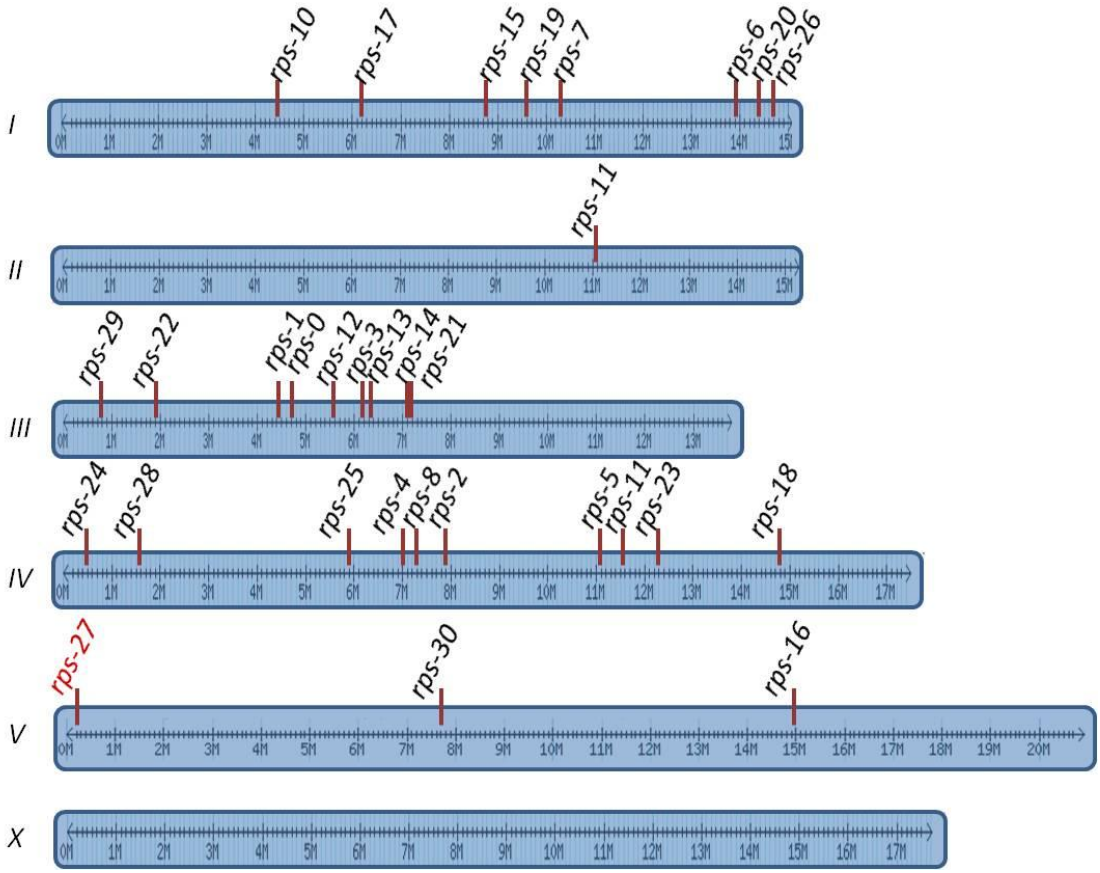

Figure 6 Distribution of *rps* genes within *C. elegans* genome, *rps-27* location shown in red.

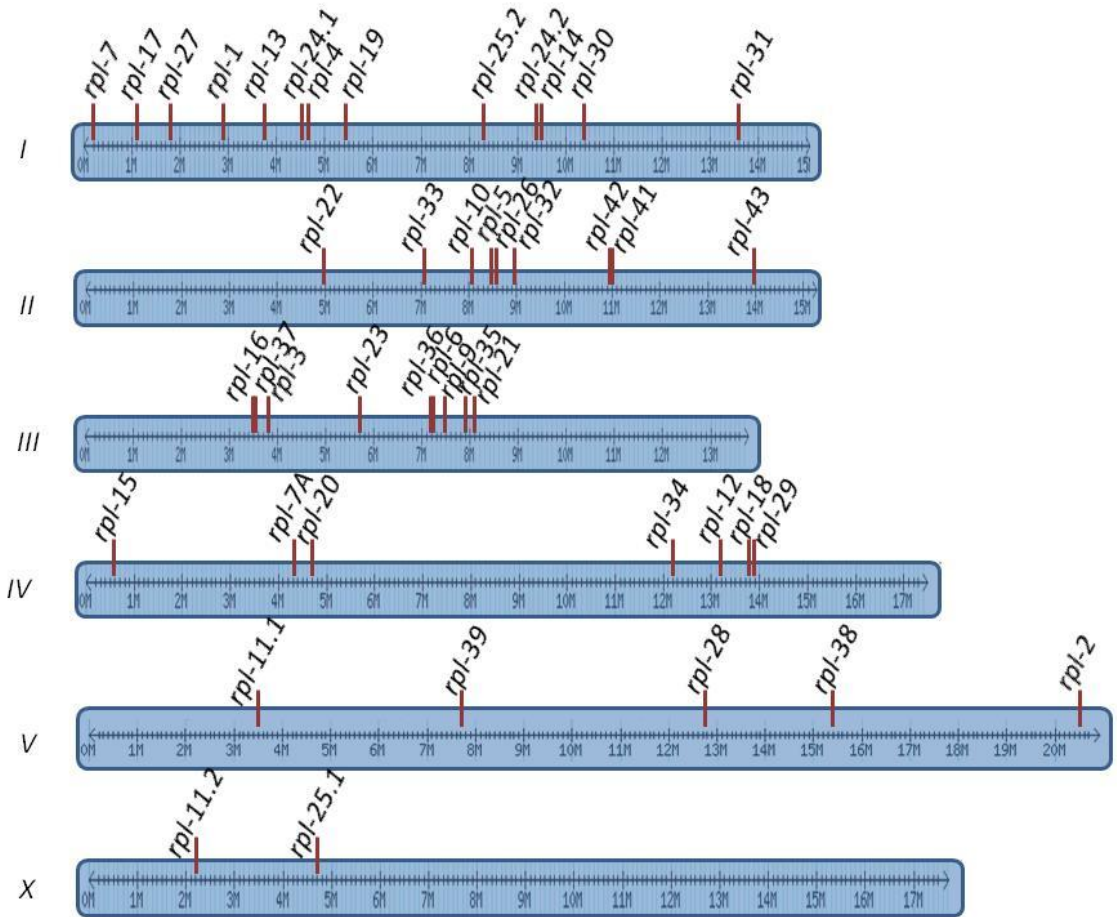

Figure 7 Distribution of *rpl* genes within *C. elegans* genome.

2.4.2 Distribution of genes on the genetic map

In 1974, Sydney Brenner noticed a feature of *C. elegans* genetic map where there was the strong tendency for clustering of genes on the autosomes (Brenner 1974). If we look at all the *rps* genes across all linkage groups the genes appear to have a non-uniform genome distribution. However, if we analyze the distribution of lethals generated of *LGV* (left) (Fig.1), there is evidence that essential genes on *LGV* (left) tend to cluster into two groups: A large group of essential genes near the centre of the chromosome, and a smaller cluster of essential genes near the telomere end. From WormBase (WS190), I obtained a list of all the protein coding genes on *LGV*. I divided the chromosome up into 164 Kbp regions starting from *LGV* (left) and counted the number of protein coding genes in each region (Fig. 8). It is clearly evident that there are gene rich regions and gene sparse regions spanning the chromosome. The maximum number of genes in a 164 Kbp region was 64, located near the last third of the chromosome. The minimum number of genes was 16, located near the first third region of the chromosome. The average number of genes was 39. If we compare these results to the 24 genes that lie within the 164 Kbp *hDf36* deficiency region (Table 1), it is evident that the essential gene *rps*-27 lies in a gene poor region close to the telomere end. In figure 8, I also plotted the location of all the essential genes on *LGV*. The maximum number of essential genes in a 164 Kbp region was 11, located near the centre of the chromosome. The minimum number of essential genes was 0, located in several places, mainly located towards the ends of the chromosome. The average number of essential genes was 3. Recently a paper by Rockman (2009) analyzed the recombination

landscape of *C. elegans* and found that it was not only the centre of the chromosome but also the terminal domain did not effectively display recombination. Over time, these regions with essentially no recombination would allow highly conserved essential genes, including *rps*-27, to cluster near the telomeric ends of the chromosomes.

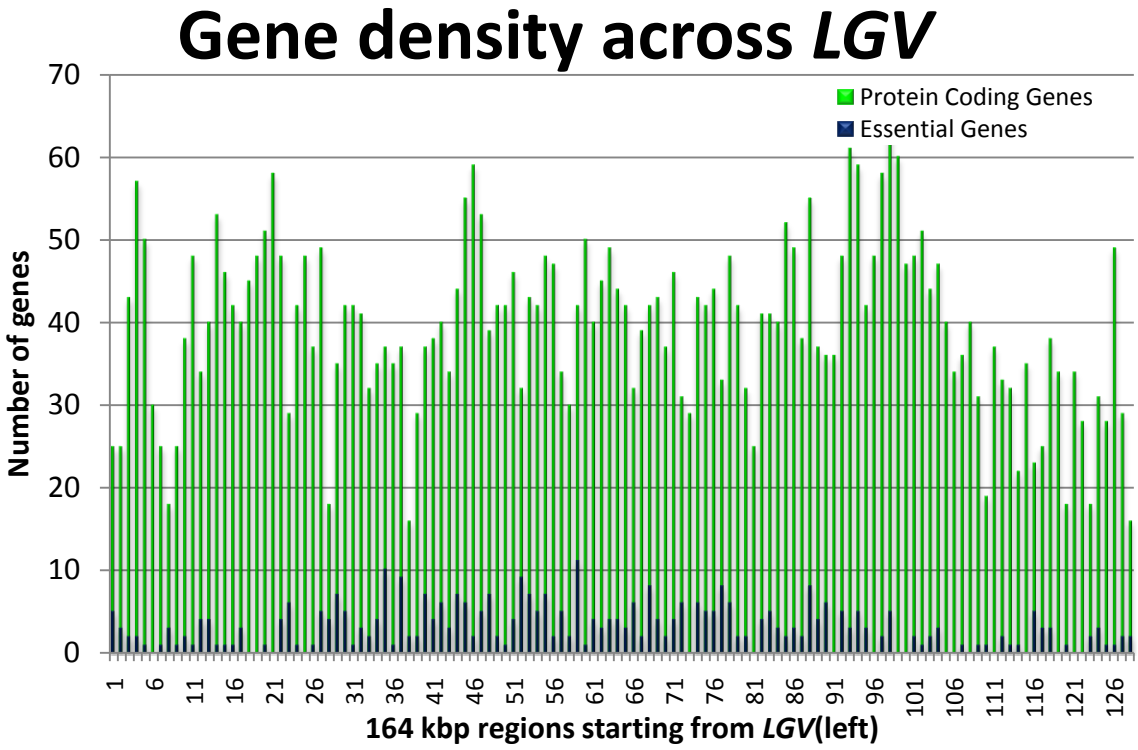

Figure 8. Number of genes in 164 Kbp regions spanning *LGV*. X axis represents 164 Kbp bin numbers spanning *LGV* from left to right side. Protein coding genes: Total=5051, Max=64, Min=16, Mean=39. Essential genes: Total=388, Max=11, Min=0, Average=3.

CHAPTER 3: ANALYSIS OF CORRELATION BETWEEN EXPOSURE TIME TO X-RAY RADIATION AND MUTATION RATE IN THE *eT1* BALANCED REGION

3.1 Introduction

Genome integrity is essential to the health of an individual organism and to the reproductive success of the species. The stability of the genome is affected by internal and external factors; it is a selective balance between maintenance of genetic stability and elimination of mutational change, leading to loss of evolutionary potential. Throughout evolution there have been a number of mechanisms that have occurred to maintain genomic integrity. There are known to be several steps in DNA replication, repair, as well as during meiosis/mitosis that all together combine to ensure genomic integrity (Hassold and Hunt 2001). Most of our knowledge about factors that influence genome instability comes from previous research on model organisms. *C. elegans* provides an excellent model in which to study DNA damage because most of the genes involved in response to damage and repair are conserved between nematodes and other eukaryotes such as humans (van Haaften, Romeijn et al. 2006). In a previous genome-wide RNAi study by Gijs van Haaften (2006), forty-five *C. elegans* genes were identified for increased sensitivity to ionizing radiation in germ cells. These genes are known to be involved with: double strand break repair, chromatid cohesion, mitosis/ meiosis, protein degradation, as well as RNA processing and

trafficking in *C. elegans,* and several of these genes are also orthologs of well-known human cancer predisposition genes (van Haaften, Romeijn et al. 2006).

Many different forms of radiation, including UV light, X-rays, γ-rays, and ionizing particles, can result in DNA mutation. Non-ionizing radiation such as UV light at a wavelength of 260 nm is known to be strongly absorbed by bases and can photochemically fuse two adjacent pyrimidines on the same strand. This type of mutation causes thymine dimers which are incapable of base pairing and result in blocking replication. Ionizing radiation such as X-rays, gamma rays, and other ionizing particles are more hazardous since they can attack the DNA backbone directly, causing double strand breaks (Little 2000). Radiation can also damage DNA indirectly by generating highly reactive free radicals within the cell (Povirk 2006).

C. elegans provides an excellent model for studying DNA damage and repair components. The *C. elegans* genome has already been completely sequenced, and since 2003 there has been an accumulation of sequence data available for the related species *C. briggsae,* providing the platform for comparative genomics (Stein, Bao et al. 2003). *C. elegans* has a large number of DNA repair genes that function in highly conserved pathways. The protein sequences of DNA repair genes have been found to be conserved both within yeast, as well as humans (O'Neil 2006). *C. elegans* has many DNA repair and checkpoint pathways including excision repair, mismatch repair, as well as double strand break repair.

The first study on the effects of ionizing radiation in *C. elegans* was carried out over three and a half decades ago (Herman, Albertson et al. 1976). In this study, the researchers analyzed chromosome rearrangements induced by X-rays to produce a balancing lethal system using chromosome balancers. A later study obtained the first radiosensitive mutants in *C. elegans*, *rad-1* and *rad-2* (Hartman 1985). Following this, more radiation-induced mutations were analyzed and various mutants were obtained. Recently, interest was expanded to use *C. elegans* as an "*in vivo"* model to study radiation-induced DNA damage and repair.

Analyzing mutational events is an important step towards further understanding DNA damage and repair pathways. Strains carrying lethal mutations cannot be maintained as homozygotes, while heterozygous mutations are easily lost through segregation unless there is a means to identify individuals that carry them. This is possible by using visible markers linked to lethal mutations, which subsequently aids in analysis. In *C. elegans* the use of genetic balancers can be adapted to isolate a large number of mutational events over large genomic regions. There are two types of genetic balancers: firstly, ones that eliminate recombination between a mutant allele on one chromosome and the wild type allele on the homologous chromosome, and secondly, ones that provide an extra chromosomal or integrated wild type allele of a homozygous lethal mutation (Edgley 2006).

The first *C.* elegans translocation in which reciprocal exchange of chromosome segments was shown is *eT1*(*III*;*V*). This reciprocal translocation is a

genetic balancer that eliminates recombination across the translocated regions; it is viable exhibiting an Unc-36 phenotype as a homozygote (Rosenbluth and Baillie 1981). *eT1* has the left half of *LGV* translocated to the left portion of *LGIII*, and the right half of *LGIII* translocated to the right portion of *LGV* (Fig. 2). The translocated portion of each chromosome is balanced and hence meiotic recombination is suppressed from *unc*-36 to the right end of *LGIII*, and from the *eT1*(*V*) breakpoint to the left end of *LGV*. The regions that segregate from their normal homologues are not balanced and hence recombination is not suppressed in this region. When used as a balancer, the non-translocation chromosomes are marked with the visible mutations *dpy-18*(*III*) and *unc-46*(*V*) in the recombination-suppressed region. In this way, heterozygotes (which have a wild type phenotype) can be distinguished from non-*eT1* homozygotes (which have a Dpy Unc phenotype). Self progeny of *eT1* heterozygotes are (4/16) wild type heterozygotes; (1/16) *eT1* homozygotes (which have an Unc-36 phenotype); (1/16) visible mutation marked homozygotes (which have a Dpy Unc phenotype); and the rest (10/16) are aneuploid progeny that arrest development as embryos or early larvae (Fig. 9) (Adames, Gawne et al. 1998). If a strain carries a lethal mutation in the recombination-suppressed region of either normal homologue, the animals homozygous for the non-*eT1* chromosome will not be viable, which results in the absence of progeny with the Dpy Unc phenotype. If a strain carries a lethal mutation in the recombination-suppressed region of either *eT1* homologue, the animals homozygous for the *eT1* chromosome will not be viable, which results in the absence of progeny with the Unc-36 phenotype. In this way,

lethal mutations can be maintained in the heterozygotes and easily identified in a visual screen of the progeny. In this chapter I utilize the *eT1* balancer system to measure mutational response to different administered doses of ionizing radiation.

BC2200: *dpy-18/ eT1(III); unc-46/ eT1(V)*

	dpy-18; unc-46	***dpy-18; eT1*(V)**	***eT1*(III); *unc-46***	***eT1*(III); *eT1*(V)**
dpy-18; unc-46	*dpy-18; unc-46/ dpy-18; unc-46* **dpy, unc**	*dpy-18; unc-46/ dpy-18; eT1*(V) **Arrested**	*dpy-18; unc-46/ eT1*(III); *unc-46* **Arrested**	*dpy-18; unc-46/ eT1*(III); *eT1*(V) **Wild Type**
***dpy-18; eT1*(V)**	*dpy-18; eT1*(V)/ *dpy-18; unc-46* **Arrested**	*dpy-18; eT1*(V)/ *dpy-18; eT1*(V) **Arrested**	*dpy-18; eT1*(V)/ *eT1*(III); *unc-46* **Wild Type**	*dpy-18; eT1*(V)/ *eT1*(III); *eT1*(V) **Arrested**
eT1*(III); *unc-46	*eT1*(III); *unc-46/ dpy-18; unc-46* **Arrested**	*eT1*(III); *unc-46/ dpy-18; eT1*(V) **Wild Type**	*eT1*(III); *unc-46/ eT1*(III); *unc-46* **Arrested**	*eT1*(III); *unc-46/ eT1*(III); *eT1*(V) **Arrested**
***eT1*(III); *eT1*(V)**	*eT1*(III); *eT1*(V)/ *dpy-18; unc-46* **Wild Type**	*eT1*(III); *eT1*(V)/ *dpy-18; eT1*(V) **Arrested**	*eT1*(III); *eT1*(V)/ *eT1*(III); *unc-46* **Arrested**	*eT1*(III); *eT1*(V)/ *eT1*(III); *eT1*(V) **unc-36**

Figure 9 Punnett square showing genotypes and phenotype of selfed BC2200 *eT1* nematodes.

3.2 Materials and Methods

3.2.1 Strains and culture conditions

Nomenclature used in this paper follows the system adopted for *C. elegans* (Horvitz, Brenner et al. 1979). Two strains were used in this chapter: wild-type N2 (var. Bristol, BC49) and BC2200 (*dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*)) which were thawed from stocks maintained in our lab. Nematode strains were maintained on Easiest Worm Plate Agar streaked with *E. Coli* (OP50) (Brenner 1974). All strains were stored and maintained at 20^{o}C for the duration of the experiment.

3.2.2 Strain maintenance

All stock strains as well as mutant strains generated in this work were catalogued with a unique Baillie Lab (BC) strain designator and then placed in liquid freezing solution before being frozen away in liquid nitrogen. Three plates for each strain were grown until the nematodes have just exhausted the *E. coli* OP50 lawn and are full of freshly starved L1-L2 larvae. These nematodes were then washed into three 1.5 mL Cryotube Vials with internal threads, using premade liquid freezing solution [129 mL S Buffer(0.05 M K_2HPO_4, 871 mL 0.05 M KH_2PO_4, 5.85 g NaCl] with a 15% final volume of glycerol, and then frozen in liquid nitrogen (-196^{o}C) (Brenner 1974).

3.2.3 Generation of Dauers

Dauer pheromone was prepared using the Cori Bargmann and Brenda Reinhart 1992 protocol. The pheromone was extracted by growing up a large

mass of nematodes in a 1 liter culture and then spinning the worms down and filtering the supernatant. The supernatant was then placed on the surface of Easiest Worm Mix Agar plates after nematodes were transferred.

3.2.4 X-ray generation protocol

Nematodes were exposed to X-ray radiation administered by a Torrex 150D X-ray Inspection System from EG&G Astrophysics Research Corporation. The Torrex 150D uses a thin 0.6 mm Beryllium window X-ray tube with a stationary anode which can produce X-ray energies up to 150 kVp. For this study, a 50 radiation-absorbed dose (rad) was produced by placing the samples 25.4 cm away from the X-ray source; operating the X-ray machine at 145 kV, 5 mA, for 4.5 seconds. A 1450 rad dose was produced by placing the samples 25.4 cm away from the X-ray source; operating at the following parameters: 145 kV, 5 mA, shelf 7, for 130.5 seconds. A 1500 rad dose was administered by placing the samples 25.4 cm away from the X-ray source; operating at the following parameters 145 kV, 5 mA, shelf 7, for 135 seconds.

3.2.5 Screening for mutations using the *eT1* system

Screening methods which measure the frequency of recessive lethal mutations induced with EMS and γ-radiation within the *eT1* balanced region of *C. elegans* have been described previously (Rosenbluth 1983). Synchronized populations of gravid adult BC2200 phenotypically wild-type looking nematodes (*dpy-18/eT1*; *unc-46*/*eT1*) were exposed to X-ray radiation. These nematodes were then incubated for one hour at 20^{o}C to allow the P_o generation time to rest

before transferring as well as expel eggs that were far advanced in development before X-ray exposure. The P_o generation was then singly plated on Easiest Worm Plate Agar plates and incubated at 20^oC for four days. From each P_o plate, ten F_1 progeny were singly plated and kept in 20^oC for four days. The F_2 progeny of these worms were screened for the absence of gravid Dpy-Unc worms. The absence of mature Dpy-Uncs indicates the presences of a lethal mutation linked to either *dpy-18* or *unc-46* within the balanced region of *eT1*. Each plate that contained a lethal mutation was reset by picking phenotypically looking wild-type nematodes, which were subsequently frozen away in liquid nitrogen.

3.2.6 Mapping lethal mutations to *LGIII* or *LGV*

Lethal mutations generated can be mapped to either *LGIII* or *LGV* by replacing the *eT1* balancer with the wild-type chromosome. Lethal bearing strains were crossed to N2 males, and wild-type F_1 progeny were singly plated. If the ratio of the progeny of the lethal bearing *dpy-18*/+ ; *unc-46*/+ was approximately 3 (wild-type) : 1 (Dpy-*18*) then the lethal mutation was linked to *LGV*. If the ratio of the progeny of the lethal bearing *dpy-18*/+ ; *unc-46*/+ was approximately 3 (wild-type) : 1 (Unc-*46*) then the lethal mutation was linked to *LGIII*.

3.3 Results

3.3.1 Background mutation rate of *eT1* (*III*;*V*)

Several studies in the past have utilized the *eT1* balancer system to measure the frequency of mutations in *C. elegans* when exposed to different

mutagenesis methods (Rosenbluth 1983; Rosenbluth, Cuddeford et al. 1985; Nelson, Schubert et al. 1989). In the heterozygous *eT1*, meiotic crossing over is suppressed in the balanced regions, which makes it useful for capturing and maintaining mutations in *C. elegans*. The *eT1* balancer system used in this study (BC2200 (*dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*)) can recover mutations in the crossover-suppressed regions that span approximately 6 Mbp on *LGIII* and approximately 9 Mbp on *LGV*. The screening process was simplified by only scanning for recessive lethal mutations in either *LGIII* or *LGV* balanced regions that were detected by the absence of Dpy-Unc individuals in the F2 generation. The spontaneous mutation frequency of *C. elegans* cultured on plates has been previously shown to be approximately 0.06% with a sample size of 3198 F1's tested (Rosenbluth 1983). In order to increase the accuracy of the background mutation rate, this study increased the sample size to 13 2*18* individual F1's screened. To obtain a sample size this large, the number of F1 individuals that can be tested conveniently was maintained at around 100 F1's screened by two people over 2-2.5 weeks. Simultaneous screens were staggered approximately every four days in order to maintain constant populations of screened F2's and plated F1's per week. The results of all screens, including previous results published by Rosenbluth (1983) are shown in Appendix D. In total 4 spontaneous lethal mutations were discovered. With this larger sample size the spontaneous background mutation rate within the *eT1* balanced region is shown to be 0.03%. By crossing to wild type N2, as outlined in section 3.2.6, I then further analyzed the two new lethal mutations captured in this study to determine which

chromosome the mutations mapped to. Both mutations were found to be linked to *LGIII.* The strains were given the following BC designation numbers and frozen away in our stocks:

BC9172 (*s3374*) (*let*) *dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*) and

BC9173 (*s3375*) (*let*) *dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*).

3.3.2 Cumulative low dose exposure of X-ray radiation

To investigate if the mutational effects of ionizing radiation are additive, I exposed the *C. elegans eT1* balanced strain BC2200 *dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*) to 30 exposures of low dosage (50 rad) X-ray radiation, for a total cumulative dose of 1500 rads. Initially, repeated 50 rad exposures (4.5 seconds long) were administered every 5 minutes, lasting 150 minutes in total. The nematodes were then allowed 1 hour to rest to allow mature eggs to be expelled, and then plated and screened for lethal mutations as previously described. I determined the lethal mutation rate for a 5 minute resting time to be 2.8±.6%, which is less than the 4.5±.6% mutation rate published earlier for a single exposure to 1500 rads (Rosenbluth 1983).

I then proceeded to determine the mutational response by changing the resting time between 50 rad exposures. I determined the lethal mutation rate for a 2.5 minute resting time to be 4.2±.4%, for a 10 minute resting time to be 2.4±.4%, for a 15 minute resting time to be 1.8±.3%, and finally for a 30 minute resting time to be 1.1±.3%. These results as summarized in table 3 as well as

shown in figure 10. All strains carrying recessive lethal mutations were given BC designator numbers and frozen away in our stocks.

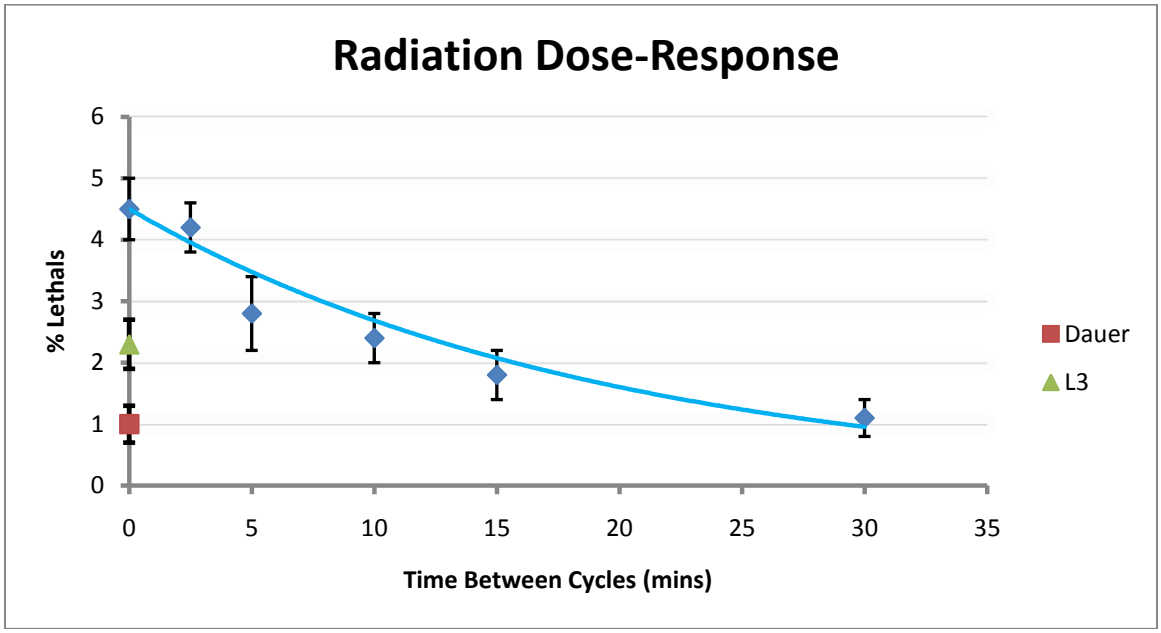

Figure 10 Radiation dose-response. Blue dots represent cumulative 50 rad exposures over varying time scales. Red square represents dauer mutation response to a single 1500 rad exposure. Green triangle represents L3 mutation response to a singe 1500 rad exposure.

Table 3 Recessive lethal response to doses of X-ray radiation

Sample	# of F_1's Tested	# of Lethals	Lethals (%)
Control	13218	4	0.03
1500_{rad} (Rosenbluth 1983)	364	22	6.0
	485	17	3.5
	787	35	4.4
Total	1636	74	4.5±.6
Dauer	638	6	0.9
	412	5	1.2
Total	1050	11	1.0±.3
L3	439	13	2.7
	582	10	1.7
Total	1021	23	2.3±.4
50_{rad} /1450_{rad}	1146	47	4.1
	499	9	1.8
	603	14	2.3
Total	2248	70	3.1±1.1
2.5 min	536	24	4.5
	512	20	3.9
Total	1048	44	4.2±.4
5 min	506	11	2.1
	576	19	3.2
Total	1082	30	2.8±.6
10 min	610	15	2.5
	394	9	2.3
Total	1004	24	2.4±.4
15 min	545	12	2.2
	513	7	1.4
Total	1058	19	1.8±.3
30 min	627	8	1.3
	602	5	0.8
Total	1229	13	1.1±.3

3.3.3 Mutational response of a single high dose exposure after initial exposure to a low dose of X-ray radiation

I next determined what effect the initial exposure to X-ray radiation had on the mutation rate in the cumulative dose experiment. To accomplish this, a population of adult BC2200 *dpy-18*(*e364*)/*eT1*(*III*); *unc-46*(*e177*)/*eT1*(*V*) nematodes were initially pulsed with a low dose (50 rad) exposure of X-ray radiation. After initial exposure, these nematodes were allowed to rest on the plate for one hour at 20^{o}C, before being exposed to a high dose (1450 rad) of X-ray radiation. These nematodes were then allowed to rest for another hour before being singly plated and screen as described previously. The amount of lethal mutations within this population of *C. elegans* was determined to be 3.1±1.1%, which is a decrease when compared to the 4.5±.6% lethal mutations arising from a single exposure of 1500 rads. All mutant strains carrying recessive lethals were given a BC designator number, maintained, and frozen away in our stocks.

3.3.4 Irradiation of dauer larvae

After egg bleach was performed to create a synchronized population of BC2200 L1s nematodes, I plated these individuals on either seeded plates containing *e. coli* OP50 or plates containing *e. coli* OP50 and dauer pheromone. To determine the mutational response of dauer larvae when exposed to X-ray radiation, I irradiated both the L3 population, as well as the dauer population of nematodes that arose with a single exposure of 1500 rads. L3 nematodes were then incubated at 20^{o}C and allowed to grow up to young adults before being singly plated and screened for recessive lethals. The dauer population remained

on the plate for one hour after exposure to X-ray radiation, dauers were then singly plated on plates containing only *e. coli* OP50 and allowed to exit the dauer stage before I proceeded to screen the F2 generation for lethal mutations. I determined the L3 lethal mutation rate when exposed to a single exposure of 1500 rads to be 2.3±.4% and the lethal mutation rate of the dauer population to be 1.0±.3%. All mutant strains carrying recessive lethals were given a BC designator number, maintained, and frozen away in our stocks.

3.4 Discussion

Ionizing radiation is extremely harmful for an organism's cells, and can cause DNA double-strand breaks (DSBs) which are considered to be the main lesions induced (Little 2000). Faithful repair of DSB's is vital for the organism to develop, as inappropriate repair can cause chromosomal alterations that may potentially lead to death. To avoid this, organisms have an elaborate and evolutionarily well-conserved network of DSB repair pathways. In *C. elegans* the DNA damage response, including most of the known DNA repair pathways are conserved between the nematodes and humans (O'Neil 2006). Faithful repair of DSB's is vital for the organism to develop, as inappropriate repair can cause chromosomal alterations that may potentially lead to death. To avoid this, organisms have an elaborate and evolutionarily well-conserved network of DSB repair pathways. In *C. elegans* the DNA damage response, including most of the known DNA repair pathways are conserved between the nematodes and humans (Rosenbluth 1983; Nelson, Schubert et al. 1989). Scientists have also used *C. elegans* as a tool for measuring the biological consequences of exposure to

cosmic radiation (Zhao, Lai et al. 2006). In this chapter I utilized the same mutational capture system, the *eT1* balancer, as a biological dosimeter for analyzing the effects of exposure to low dosages of ionizing X-ray radiation.

3.4.1 Mutational effects of exposure to low dosage X-ray radiation

Using the *eT1* mutational capture system, Rosenbluth (1983) previously reported the dose-response curve for the induction of recessive lethals generated by different doses of gamma radiation. The results are shown in figure 11. It is clearly evident that as the dosage of ionizing radiation exposure is increased the amount of lethals produced also increases. With high doses of ionizing radiation (>500 rads), the amount of lethal mutations produced increases in a linear fashion. However, when dealing with low dosages of ionizing radiation (<500 rads), there is a lag where the lethal mutation response does not follow the trend for high dosages. In order to gain further insight into the effects of low dose ionizing radiation in contrast to high dose, I compared the amount of lethal mutations produced by each when the total amount of radiation exposure was equal. A total (high) dosage of 1500 rads was chosen, as this dosage lies within the middle of the linear region (Fig. 11), and a low dosage of 50 rads was chosen as it falls well below the linear region. In order to obtain a total dosage of 1500 rads from the low dose, 30 repeated exposures of 50 rads was performed.

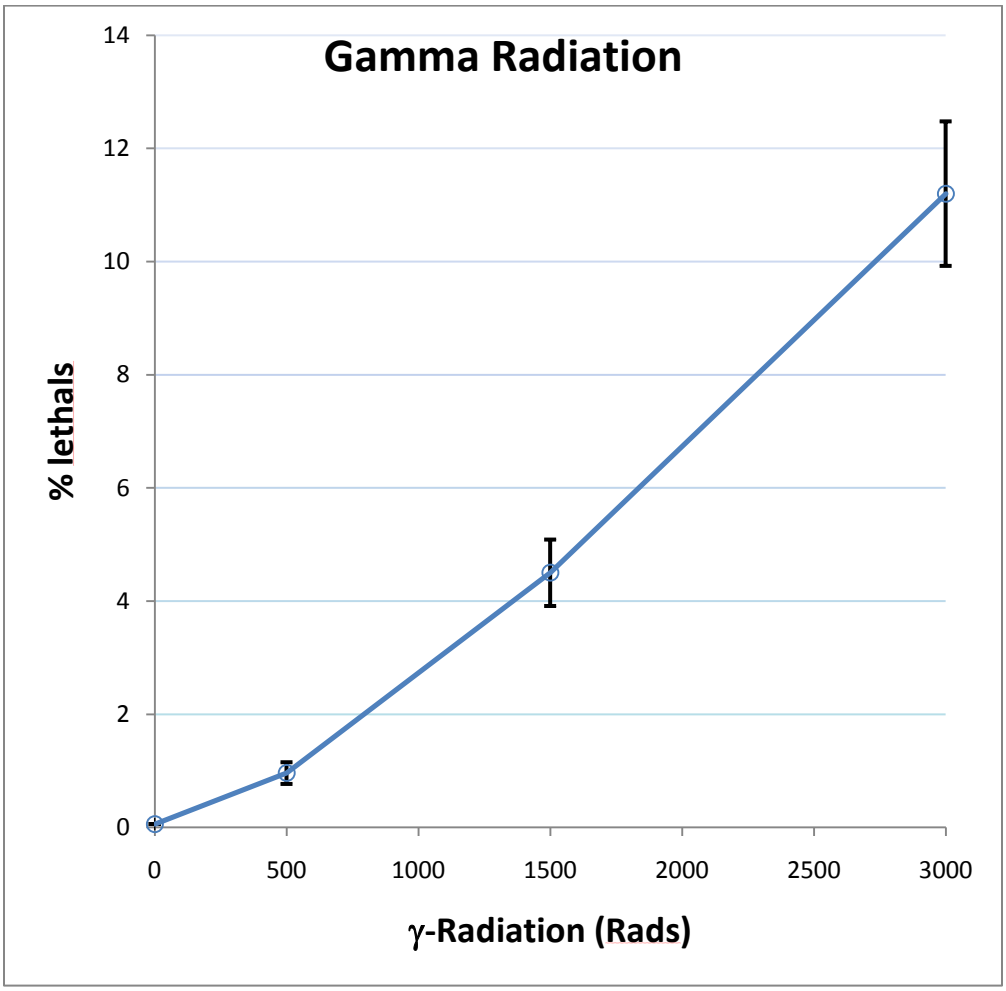

Figure 11. Dose-response curve for the induction of recessive lethals in the region balanced by *eT1*(*III*;*V*). Exposure to gamma radiation from a ^{60}CO source. Bars indicate standard error. Adapted from Rosenbluth 1983.

As evident in figure 10, the amount of lethal mutations produced by a cumulative exposure to low dose X-ray radiation was reduced compared to a single exposure of 1500 rads. This is strong evidence that mutational damage captured within the *eT1* balanced region is not additive. In *C. elegans* the cellular response to radiation is known to involve several overlapping pathways, which can trigger cell-cycle arrest and provide time for DNA repair (Lukas, Lukas et al. 2004). I have determined that there is a trend between rest time after low dose exposures and the mutation rate. As the rest time between 50 rad dosages increases in 5 minute intervals, the amount of lethal mutations quickly decreases. This observation then led me to test for an induced DNA repair response, which would reduce the amounts of mutants in runs with multiple exposures. To determine if there was an induced response, an initial dosage of 50 rads was administered followed by a one hour rest period and then a second high exposure of 1450 rads. There was a reduction in the amount of lethal mutations in nematodes exposed to an initial low dose exposure compared to nematodes given a single high dose of 1500 rads.

3.4.2 Dauer mutation response

In 1975 it was discovered that during harsh conditions *C. elegans* larvae can enter a dauer arrest at the second molt (Cassada and Russell 1975). These dauers are known to display morphological and behavioural characteristics that differ from nematodes that have not entered into the dauer state. In addition, they are easily distinguishable from L3 larvae under a dissecting microscope. It has been shown previously that *C. elegans* dauers induced by stresses such as

starvation or high temperatures, can remain developmentally arrested for months (Riddle, Swanson et al. 1981). I subjected BC2200 dauer larvae to 1500 rads X-ray radiation in order to study the mutational response of the nematodes within the dauer state compared to nematodes in the alternate L3 state. The dauer lethal mutation rate was half that of L3 nematodes grown from the same synchronized population. Dauer larvae also exhibited the least amount of lethal mutations out of all screens completed in this study. Previous studies have looked at dauer gene expression as they exit the dauer state (Dalley and Golomb 1992). When exiting the dauer state, DNA repair gene regulation may be increased. This may possibly explain the decreased number of lethal mutations observed.

CHAPTER 4: GENERAL DISCUSSION

Every organism's genome contains essential genes that are vital for that individual to grow and develop into a fertile adult. An organism also needs to preserve the informational content of the genetic material for both its own health and successful reproduction of the species. Both internal and external factors affect the stability of the genome. Studying essential genes allows one to gain knowledge about the minimal genetic requirement for an organism to survive. Essential genes also tend to be highly conserved in terms of evolution. Therefore, research using model organisms such as *C. elegans* can ultimately provide a greater understanding of the human genome.

In the first half of this thesis, I focus on determining the molecular identity of the essential gene *let-336*. *let-336* was one of 194 lethal mutations picked up in a *C. elegans* EMS mutagenesis screen (Johnsen and Baillie 1991). This lethal proved to be a fairly large target as three alleles were picked up: *s1413*, *s1420*, and *s1495*. Interestingly, the essential lethal genes that mapped to *LGV*(left) tended to cluster either near the telomere end of the chromosome or near the centre of the chromosome. *let-336* was one of a few lethals that mapped close to the telomere end. *let-336* was shown to be an essential gene as homozygous mutants arrested in an early larval stage. In order to maintain the lethal mutant *let-336* was balanced over the reciprocal translocation *eT1*(*III*;*V*).

I determined the molecular identity of *let-336* by sequencing candidate genes which are known to map to the far end of *LGV*. The lethal was previously shown to map within the region deleted by the deficiency *hDf36*. The initial list of candidate genes was generated by looking at all the genes which were known to lie within the *hDf36* region. I further narrowed down the list of candidate genes by looking at genes that had similar published RNAi phenotypes as *let-336*. *F56E10.4* showed a similar RNAi phenotype and was chosen as a possible candidate. Sequencing *F56E10.4* in the first allele *let-336* (*s1413*) resulted in a C→T transition indicative of a mutation caused by EMS mutagenesis. I subsequently sequenced the second allele *let-336* (*s1495*) and found another C→T transition. Both mutations cause a premature stop codon which results in a truncated protein being made. When looking at the sequencing results from the heterozygous template of the third allele *let-336* (*s1420*) I could not find any mutation. To further confirm the sequencing results, I performed a complementation test and rescued *let-336* (*s1413*) with the fosmid WRM0629cC03 which is known to span the genomic region of *F56E10.4* (*rps*-27).

The gene *rps*-27 in *C. elegans* encodes a small ribosomal subunit S27 protein. This gene is one of 31 *rps* genes that together make up the 40S small subunit of the ribosome. Ribosomes are vital cellular components of all living cells, and are required to make proteins. RPS-27 is a small protein consisting of only 83 amino acids. Even though the protein is small in size, it poses a large mutational target as the sequence is highly conserved across multiple species

and does not tolerate much variation. RPS-27 is an important structural scaffolding protein containing a C4-type zinc finger domain. In *C. elegans* all *rps* genes can be found distributed on the autosomal chromosomes, however no *rps* gens can be found on the sex chromosomes. This can be attributed to the fact that X-linked genes are transcriptionally silenced during mitosis and early meiosis (Kamath, Fraser et al. 2003). When looking at the genome organization of all the genes on *LGV* (left) there is evidence of gene rich regions and gene poor regions (Fig. 8). However, when looking at essential genes in figure 1, there are two gene clusters, one near the centre of the chromosome and one near the telomeric end. A study published in 2009 characterized the landscape of recombination across the chromosomes and discovered that the terminal domains of each chromosome exhibited effectively no recombination (Rockman and Kruglyak 2009). These results explain why essential genes are found clustering in regions near the chromosome centres as well as near the telomeric ends in *LGV*.

In the second half of this thesis I utilised the *eT1* balancer system to measure recessive lethal mutations induced by low dosage X-ray radiation. This system was previously used to measure the frequency of mutations for commonly used mutagens (Rosenbluth 1983; Rosenbluth, Cuddeford et al. 1985), and incorporates a very easy screening protocol that can be quickly analyzed for strains carrying lethal mutations. A benefit of using this system is that it does not require crossing strains to screen progeny.

Meiotic crossing over is suppressed within the balanced region which allows lethal mutations to be captured and maintained over several generations

without loss of the mutation. When exposed to varying doses of ionizing radiation a clear mutational effect can be observed by screening F2 progeny for loss of the Dpy-Unc phenotype. Since *C. elegans* has a short life cycle screening can be done within two weeks of initial exposure. Freezing the strains carrying mutations was done as soon as possible to avoid the chance of spontaneous mutations accumulating within the balanced region. For this reason, when determining the background mutation rate of *eT1* I thawed a fresh copy of BC2200 every six months. After a total sample size of 13,2*18* F1's I determined the spontaneous mutation rate of *eT1* to be 0.03%.

Using the same mutational capture system, Rosenbluth (1983) has clearly shown that high dosages of ionizing radiation causes an increase in the amount of lethal mutations observed. This increase in lethal mutations increased linearly with a subsequent increase in radiation dosage. However, the nature of genome instability and damage caused by low dose exposure is still poorly understood. I have demonstrated that thirty cumulative low dosages of 50 rads X-ray radiation administered every 5 minutes does not result in the same amount of lethal mutations produced by a single exposure to 1500 rads. The mutation rate of this cumulative low dose experiment was compared to the single high dosage mutation rate published by Rosenbluth (1983). These results prove that damage caused by repeated exposure to low dose ionizing radiation is not directly additive. Ionizing radiation is known to induce several responses in *C. elegans* cells. These responses include apoptosis, cell cycle arrest, and DSB repair to maintain genome integrity. After initial exposure to the low dose radiation

nematodes may have already initiated cell cycle arrest or increased DSB repair mechanisms. Both of these scenarios would decrease the amount of lethal mutants present.

An organism would take some time to switch from actively dividing cell state to an arrested state. Also when DNA lesions are first detected it would take some time before protein complexes sense the damage and subsequently trigger “transducer” systems to eventually coordinate “effector” components of the response pathway (Kurz and Lees-Miller 2004). In order to test if repair mechanisms were more efficient with a longer lag period between exposures I increased the amount of time between low dose exposures by 5 minute intervals. I determined that the amount of lethal mutations captured by the *eT1* balancer system decreases as the amount of resting time between exposures increases. The amount of lethal mutations seems to rapidly decrease between the 2.5 minutes and 5 minutes resting time, as shown in figure 10, and then levels off near the same mutation rate of dauers exposed to radiation. It is possible that at this total radiation dosage nematodes are incapable of repairing all the damage and a basal mutation rate is established. If this is the case, dauers which were exposed to a single high dose of 1500 rads and still only had a 1.0±.3% mutation rate, show the optimal amount of DNA damage repair. To further analyze the effect of prolonging exposures, I exposed nematodes to an initial 50 rad dosage and then waited a whole hour before exposing them to a subsequent 1450 rad dosage. The results of this experiment showed that the initial exposure did result in a reduction in the amount of lethals being produced compared to a single

exposure of 1500 rads. These results show that the time between exposures to low dosages of ionizing radiation greatly affects the mutational response.

APPENDIX A STRAINS USED AND GENERATED

Strains used in this study showing alleles, genotype, type of mutagen used and blocking stage/ molecular phenotype.

Strain	Genotype	Mutagen	Blocking Stage
N2 ♂	Wild type	None	**None**
BC2214	*dpy-18*(e364)/eT1(III);*let-336*(*s1413*)*unc-46*(e177)/eT1(V)	EMS	**Early Larval**
BC2221	*dpy-18*(e364)/eT1(III);*let-336*(*s1420*)*unc-46*(e177)/eT1(V)	EMS	**Early Larval**
BC2296	*dpy-18*(e364)/eT1(III);*let-336*(*s1495*)*unc-46*(e177)/eT1(V)	EMS	**Early Larval**
BC7961	sEX1753;dpy-5(e907)/dpy5(e907) [WRM0629cC03+pCeh361+ myo-2::GFP]	DNA	**None**
BC7902	sEX1744;*dpy-18*(e364)/eT1(III);*let-336*(*s1495*)*unc-46*(e177)/eT1(V)[WRM0629cC03+pCeh361+myo-2::GFP]	DNA	**None**

Strain	Genotype	allele	Screen/ Trial #
BC7625	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3304 (let)*	**2.5 min/ Trial 1**
BC7626	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3305 (let)*	**2.5 min/ Trial 1**
BC7627	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3306 (let)*	**2.5 min/ Trial 1**
BC7628	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3307 (let)*	**2.5 min/ Trial 1**
BC7629	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3308 (let)*	**2.5 min/ Trial 1**
BC7630	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3309 (let)*	**2.5 min/ Trial 1**
BC7631	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3310 (let)*	**2.5 min/ Trial 1**
BC7632	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3311 (let)*	**2.5 min/ Trial 1**
BC7633	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3312 (let)*	**2.5 min/ Trial 1**
BC7634	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3313 (let)*	**2.5 min/ Trial 1**
BC7635	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3314 (let)*	**2.5 min/ Trial 1**
BC7636	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3315 (let)*	**2.5 min/ Trial 1**
BC7637	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3316 (let)*	**2.5 min/ Trial 1**
BC7638	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3317 (let)*	**2.5 min/ Trial 1**
BC7639	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3318 (let)*	**2.5 min/ Trial 1**
BC7640	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3319 (let)*	**2.5 min/ Trial 1**
BC7641	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3320 (let)*	**2.5 min/ Trial 1**
BC7642	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3321 (let)*	**2.5 min/ Trial 1**
BC7643	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3322 (let)*	**2.5 min/ Trial 1**
BC7644	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3323 (let)*	**2.5 min/ Trial 1**
BC7645	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3324 (let)*	**2.5 min/ Trial 1**
BC7646	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3325 (let)*	**2.5 min/ Trial 1**
BC7647	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3326 (let)*	**2.5 min/ Trial 1**
BC7648	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3327 (let)*	**2.5 min/ Trial 1**
BC7649	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3328 (let)*	**2.5 min/ Trial 2**
BC7650	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3329 (let)*	**2.5 min/ Trial 2**
BC7651	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3330 (let)*	**2.5 min/ Trial 2**
BC7652	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3331 (let)*	**2.5 min/ Trial 2**
BC7653	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3332 (let)*	**2.5 min/ Trial 2**
BC7654	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3333 (let)*	**2.5 min/ Trial 2**
BC7655	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3334 (let)*	**2.5 min/ Trial 2**
BC7656	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3335 (let)*	**2.5 min/ Trial 2**
BC7657	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3336 (let)*	**2.5 min/ Trial 2**
BC7658	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3337 (let)*	**2.5 min/ Trial 2**
BC7659	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3338 (let)*	**2.5 min/ Trial 2**
BC7660	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3339 (let)*	**2.5 min/ Trial 2**
BC7661	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3340 (let)*	**2.5 min/ Trial 2**
BC7662	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3341 (let)*	**2.5 min/ Trial 2**
BC7663	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3342 (let)*	**2.5 min/ Trial 2**
BC7664	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3343 (let)*	**2.5 min/ Trial 2**
BC7665	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3344 (let)*	**2.5 min/ Trial 2**
BC7666	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3345 (let)*	**2.5 min/ Trial 2**
BC7667	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3346 (let)*	**2.5 min/ Trial 2**
BC7668	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3347 (let)*	**2.5 min/ Trial 2**
BC7330	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3088 (let)*	**5 min/ Trial 1**

BC7331	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3089 (let)*	**5 min/ Trial 1**
BC7332	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3090 (let)*	**5 min/ Trial 1**
BC7333	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3091 (let)*	**5 min/ Trial 1**
BC7334	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3092 (let)*	**5 min/ Trial 1**
BC7335	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3093 (let)*	**5 min/ Trial 1**
BC7336	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3094 (let)*	**5 min/ Trial 1**
BC7337	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3095 (let)*	**5 min/ Trial 1**
BC7338	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3096 (let)*	**5 min/ Trial 1**
BC7339	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3097 (let)*	**5 min/ Trial 1**
BC7340	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3098 (let)*	**5 min/ Trial 1**
BC7341	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3099 (let)*	**5 min/ Trial 2**
BC7342	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3100 (let)*	**5 min/ Trial 2**
BC7343	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3101 (let)*	**5 min/ Trial 2**
BC7344	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3102 (let)*	**5 min/ Trial 2**
BC7345	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3103 (let)*	**5 min/ Trial 2**
BC7346	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3104 (let)*	**5 min/ Trial 2**
BC7347	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3105 (let)*	**5 min/ Trial 2**
BC7348	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3106 (let)*	**5 min/ Trial 2**
BC7349	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3107 (let)*	**5 min/ Trial 2**
BC7350	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3108 (let)*	**5 min/ Trial 2**
BC7351	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3109 (let)*	**5 min/ Trial 2**
BC7352	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3110 (let)*	**5 min/ Trial 2**
BC7353	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3111 (let)*	**5 min/ Trial 2**
BC7354	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3112 (let)*	**5 min/ Trial 2**
BC7355	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3113 (let)*	**5 min/ Trial 2**
BC7356	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3114 (let)*	**5 min/ Trial 2**
BC7357	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3115 (let)*	**5 min/ Trial 2**
BC7358	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3116 (let)*	**5 min/ Trial 2**
BC7359	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3117 (let)*	**5 min/ Trial 2**
BC7378	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3133 (let)*	**10 min/ Trial 1**
BC7379	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3134 (let)*	**10 min/ Trial 1**
BC7380	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3135 (let)*	**10 min/ Trial 1**
BC7381	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3136 (let)*	**10 min/ Trial 1**
BC7382	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3137 (let)*	**10 min/ Trial 1**
BC7383	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3138 (let)*	**10 min/ Trial 1**
BC7384	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3139 (let)*	**10 min/ Trial 1**
BC7385	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3140 (let)*	**10 min/ Trial 1**
BC7386	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3141 (let)*	**10 min/ Trial 1**
BC7387	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3142 (let)*	**10 min/ Trial 1**
BC7388	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3143 (let)*	**10 min/ Trial 1**
BC7389	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3144 (let)*	**10 min/ Trial 1**
BC7390	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3145 (let)*	**10 min/ Trial 1**
BC7391	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3146 (let)*	**10 min/ Trial 1**
BC7392	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3147 (let)*	**10 min/ Trial 1**
BC7393	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3148 (let)*	**10 min/ Trial 2**
BC7394	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3149 (let)*	**10 min/ Trial 2**

BC7395	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3150 (let)*	**10 min/ Trial 2**
BC7396	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3151 (let)*	**10 min/ Trial 2**
BC7397	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3152 (let)*	**10 min/ Trial 2**
BC7398	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3153 (let)*	**10 min/ Trial 2**
BC7399	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3154 (let)*	**10 min/ Trial 2**
BC7400	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3155 (let)*	**10 min/ Trial 2**
BC7401	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3156 (let)*	**10 min/ Trial 2**
BC7361	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3118 (let)*	**15 min/ Trial 1**
BC7362	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3119 (let)*	**15 min/ Trial 1**
BC7363	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3120 (let)*	**15 min/ Trial 1**
BC7364	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3121 (let)*	**15 min/ Trial 1**
BC7365	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3122 (let)*	**15 min/ Trial 1**
BC7366	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3123 (let)*	**15 min/ Trial 1**
BC7367	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3124 (let)*	**15 min/ Trial 1**
BC7368	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3125 (let)*	**15 min/ Trial 1**
BC7369	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3126 (let)*	**15 min/ Trial 1**
BC7370	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3127 (let)*	**15 min/ Trial 1**
BC7371	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3128 (let)*	**15 min/ Trial 1**
BC7372	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3129 (let)*	**15 min/ Trial 1**
BC7373	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3130 (let)*	**15 min/ Trial 2**
BC7374	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3131 (let)*	**15 min/ Trial 2**
BC7375	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3132 (let)*	**15 min/ Trial 2**
BC7376	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3133 (let)*	**15 min/ Trial 2**
BC7377	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3134 (let)*	**15 min/ Trial 2**
BC7378	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3135 (let)*	**15 min/ Trial 2**
BC7379	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3136 (let)*	**15 min/ Trial 2**
BC7708	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3348 (let)*	**30 min/ Trial 1**
BC7709	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3349 (let)*	**30 min/ Trial 1**
BC7710	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3350 (let)*	**30 min/ Trial 1**
BC7711	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3351 (let)*	**30 min/ Trial 1**
BC7712	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3352 (let)*	**30 min/ Trial 1**
BC7713	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3353 (let)*	**30 min/ Trial 1**
BC7714	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3354 (let)*	**30 min/ Trial 1**
BC7715	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3355 (let)*	**30 min/ Trial 1**
BC7716	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3356 (let)*	**30 min/ Trial 2**
BC7717	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3357 (let)*	**30 min/ Trial 2**
BC7718	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3358 (let)*	**30 min/ Trial 2**
BC7719	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3359 (let)*	**30 min/ Trial 2**
BC7720	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3360 (let)*	**30 min/ Trial 2**
BC7462	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3217 (let)*	**L3/ Trial 1**
BC7463	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3218 (let)*	**L3/ Trial 1**
BC7464	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3219 (let)*	**L3/ Trial 1**
BC7465	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3220 (let)*	**L3/ Trial 1**
BC7466	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3221 (let)*	**L3/ Trial 1**
BC7467	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3222 (let)*	**L3/ Trial 1**
BC7468	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3223 (let)*	**L3/ Trial 1**

BC7469	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3224 (let)*	**L3/ Trial 1**
BC7470	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3225 (let)*	**L3/ Trial 1**
BC7471	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3226 (let)*	**L3/ Trial 1**
BC7472	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3227 (let)*	**L3/ Trial 1**
BC7473	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3228 (let)*	**L3/ Trial 1**
BC7474	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3229 (let)*	**L3/ Trial 2**
BC7475	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3230 (let)*	**L3/ Trial 2**
BC7476	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3231 (let)*	**L3/ Trial 2**
BC7477	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3232 (let)*	**L3/ Trial 2**
BC7478	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3233 (let)*	**L3/ Trial 2**
BC7479	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3234 (let)*	**L3/ Trial 2**
BC7480	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3235 (let)*	**L3/ Trial 2**
BC7481	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3236 (let)*	**L3/ Trial 2**
BC7482	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3237 (let)*	**L3/ Trial 2**
BC7483	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3238 (let)*	**L3/ Trial 2**
BC7405	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3160 (let)*	**Dauer/ Trial 1**
BC7406	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3161 (let)*	**Dauer/ Trial 1**
BC7407	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3162 (let)*	**Dauer/ Trial 1**
BC7408	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3163 (let)*	**Dauer/ Trial 1**
BC7409	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3164 (let)*	**Dauer/ Trial 2**
BC7410	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3165 (let)*	**Dauer/ Trial 2**
BC7411	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3166 (let)*	**Dauer/ Trial 2**
BC7412	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3167 (let)*	**Dauer/ Trial 2**
BC7413	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3168 (let)*	**50/1450 / Trial 1**
BC7414	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3169 (let)*	**50/1450 / Trial 1**
BC7415	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3170 (let)*	**50/1450 / Trial 1**
BC7416	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3171 (let)*	**50/1450 / Trial 1**
BC7417	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3172 (let)*	**50/1450 / Trial 1**
BC7418	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3173 (let)*	**50/1450 / Trial 1**
BC7419	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3174 (let)*	**50/1450 / Trial 1**
BC7420	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3175 (let)*	**50/1450 / Trial 1**
BC7421	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3176 (let)*	**50/1450 / Trial 1**
BC7422	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3177 (let)*	**50/1450 / Trial 1**
BC7423	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3178 (let)*	**50/1450 / Trial 1**
BC7424	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3179 (let)*	**50/1450 / Trial 1**
BC7425	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3180 (let)*	**50/1450 / Trial 1**
BC7426	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3181 (let)*	**50/1450 / Trial 1**
BC7427	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3182 (let)*	**50/1450 / Trial 1**
BC7428	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3183 (let)*	**50/1450 / Trial 1**
BC7429	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3184 (let)*	**50/1450 / Trial 1**
BC7430	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3185 (let)*	**50/1450 / Trial 1**
BC7431	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3186 (let)*	**50/1450 / Trial 1**
BC7432	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3187 (let)*	**50/1450 / Trial 1**
BC7433	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3188 (let)*	**50/1450 / Trial 1**
BC7434	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3189 (let)*	**50/1450 / Trial 1**
BC7435	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3190 (let)*	**50/1450 / Trial 1**

BC7436	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3191 (let)*	**50/1450 / Trial 1**
BC7437	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3192 (let)*	**50/1450 / Trial 1**
BC7438	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3193 (let)*	**50/1450 / Trial 1**
BC7439	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3194 (let)*	**50/1450 / Trial 1**
BC7440	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3195 (let)*	**50/1450 / Trial 1**
BC7441	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3196 (let)*	**50/1450 / Trial 1**
BC7442	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3197 (let)*	**50/1450 / Trial 1**
BC7443	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3198 (let)*	**50/1450 / Trial 1**
BC7444	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3199 (let)*	**50/1450 / Trial 1**
BC7445	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3200 (let)*	**50/1450 / Trial 1**
BC7446	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3201 (let)*	**50/1450 / Trial 1**
BC7447	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3202 (let)*	**50/1450 / Trial 1**
BC7448	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3203 (let)*	**50/1450 / Trial 1**
BC7449	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3204 (let)*	**50/1450 / Trial 1**
BC7450	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3205 (let)*	**50/1450 / Trial 1**
BC7451	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3206 (let)*	**50/1450 / Trial 1**
BC7452	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3207 (let)*	**50/1450 / Trial 1**
BC7453	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3208 (let)*	**50/1450 / Trial 1**
BC7454	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3209 (let)*	**50/1450 / Trial 1**
BC7455	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3210 (let)*	**50/1450 / Trial 1**
BC7456	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3211 (let)*	**50/1450 / Trial 1**
BC7457	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3212 (let)*	**50/1450 / Trial 1**
BC7458	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3213 (let)*	**50/1450 / Trial 1**
BC7459	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3214 (let)*	**50/1450 / Trial 1**
BC9185	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3385 (let)*	**50/1450 / Trial 2**
BC9186	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3386 (let)*	**50/1450 / Trial 2**
BC9187	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3387 (let)*	**50/1450 / Trial 2**
BC9188	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3388 (let)*	**50/1450 / Trial 2**
BC9189	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3389 (let)*	**50/1450 / Trial 2**
BC9190	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3390 (let)*	**50/1450 / Trial 2**
BC9191	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3391 (let)*	**50/1450 / Trial 2**
BC9192	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3392 (let)*	**50/1450 / Trial 2**
BC9193	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3393 (let)*	**50/1450 / Trial 2**
BC9194	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3394 (let)*	**50/1450 / Trial 3**
BC9195	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3395 (let)*	**50/1450 / Trial 3**
BC9196	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3396 (let)*	**50/1450 / Trial 3**
BC9197	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3397 (let)*	**50/1450 / Trial 3**
BC9198	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3398 (let)*	**50/1450 / Trial 3**
BC9199	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3399 (let)*	**50/1450 / Trial 3**
BC9200	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3400 (let)*	**50/1450 / Trial 3**
BC9201	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3401 (let)*	**50/1450 / Trial 3**
BC9202	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3402 (let)*	**50/1450 / Trial 3**
BC9203	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3403 (let)*	**50/1450 / Trial 3**
BC9204	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3404 (let)*	**50/1450 / Trial 3**
BC9205	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3405 (let)*	**50/1450 / Trial 3**
BC9206	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3406 (let)*	**50/1450 / Trial 3**

BC9207	*dpy-18*(*e364*)/*eT1*(*III*); *unc46*(*e177*)/*eT1*(*V*)	*s3407 (let)*	**50/1450 / Trial 3**

Appendix B Punnett square of *let-336* selfed progeny

let*-336*(s1413* and *s1495): dpy*-18 */ eT1(III); let*-336, *unc*-46*/ eT1(V)

	***dpy*-18*; let*-336, *unc*-46	***dpy*-18*; eT1*(V)	*eT1*(III)*; let*-336, *unc*-46	*eT1*(III); *eT1*(V)
***dpy*-18*; let*-336, *unc*-46	*dpy*-18*; let*-336, *unc*-46*/ dpy*-18*; let*-336, *unc*-46 **Arrested**	*dpy*-18*; let*-336, *unc*-46 */ dpy*-18*; eT1*(V) **Arrested**	*dpy*-18*; let*-336, *unc*-46 */eT1*(III)*; let*-336, *unc*-46 **Arrested**	*dpy*-18*; let*-336, *unc*-46 */ eT1*(III)*; eT1*(V) **Wild Type**
***dpy*-18*; eT1*(V)	*dpy*-18*; eT1*(V)*/ dpy*-18*; let*-336, *unc*-46 **Arrested**	*dpy*-18*; eT1*(V)*/ dpy*-18*; eT1*(V) **Arrested**	*dpy*-18*; eT1*(V)*/ eT1*(III)*; let*-336, *unc*-46 **Wild Type**	*dpy*-18*; eT1*(V)*/ eT1*(III)*; eT1*(V) **Arrested**
eT1(III)*; let*-336, *unc*-46	*eT1*(III)*; let*-336, *unc*-46 */dpy*-18*; let*-336, *unc*-46 **Arrested**	*eT1*(III)*; let*-336, *unc*-46 */ dpy*-18*; eT1*(V) **Wild Type**	*eT1*(III)*; let*-336, *unc*-46 */eT1*(III)*; let*-336, *unc*-46 **Arrested**	*eT1*(III)*; let*-336, *unc*-46 */ eT1*(III)*; eT1*(V) **Arrested**
eT1(III)*; eT1*(V)	*eT1*(III)*; eT1*(V)*/ dpy*-18*; let*-336, *unc*-46 **Wild Type**	*eT1*(III)*; eT1*(V)*/ dpy*-18*; eT1*(V) **Arrested**	*eT1*(III)*; eT1*(V)*/ eT1*(III)*; let*-336, *unc*-46 **Arrested**	*eT1*(III)*; eT1*(V)*/ eT1*(III)*; eT1*(V) **unc-36**

Appendix C *F56E10.4* wild type sequence and primers used for sequencing *let-336* alleles.

Primer design for: F56E10.4 (rps-27 Ribosomal Protein Small Subunit)
>V:104070,103385 bp

atttacgataaacacagcgcgctacgacactccgaaaaccgcgccgcaccattacattctccgccccaccgcacggttt
gcggaaggcgatgaaattgctgggcctaaaattttaacccttttttttcccgtttttcaactgttttcccgatttttcg
atatttttttcaggtttttagttagaaaagtatataaaaattgcattatttgtagttttcgctcgtaatttgtgataga
aaacggccaatttgcctagatttgtgattctaccaagtggaatttttgggaaaatatcaatattccacgatttttattc
aaatcataacgtttcgaaattatcagcttaaataaaactaaaataaataataaaatactaaaaaccctgaatttctcgg
ttctcggttcagcttgagttcgttctatacacgttatcgatttttgtcatttcgtttaatcatgggttttgattcattt
taaatatgaaatttgatttattcctcgtttcgaagttggtttgtcttgtccggccggtaaacaaatgttattaaaaaaa
taataaatttcgaaatttttcgtaatgttccgcaatcgtgtttttccgtttatctcgtcttcccttctttcaaatatac
tatttatttttctcaattcacatattttcagccagccccgctcaacaagtggaataaaaatgccttggccgttgaccttt
cttcacccagagccacaaagggagatcagatgccacaagcttaagcgtcttgttcaacacccaaactcctacttcatgg
acgttaagtgctcgggatgcttcaaggtaagattgagcctatgtgcttaatagctgttcatgaaaatttgttgtttcaa
aagtttttgtgcgacaaaccgcccgttttctgagattgaacgattcttgtattcaacttgttaaatctcaacatctaat
gctccgagaagtagccattttcgtttacgaaaatttccttcaatcattatttaggatttgcatttcatgatgattat
gaccttaatttcggtctataaatatatttctcacgtcagatatgctcaaattcacttttttttctaccatgcaatttcc
catactaattcggttttttttttcagatttcgaccgtcttctcgcacgccaccaccgtcgtcgtctgcgttggatgcaac
accgtcctctgccagccaacccgaggaaaggccaagctcaccgagggatgctccttccgcaagaagcaataagtttccc
gtaattgttacataaattgttatttcaaatccttccttttttttggttgaaagaaaaacaatgtgatttttaaaaaaattt
tgtgtgttcgacttccatttgacagacattgagaaattgtttcctaaatctgccaatgaaaggtaattggcgcttctct
tccgttagcaatgtcaaacaactggatcattggctgtctcgtctctggtaatgagggtgaaaacgggggtcgtaatgcc
atttcaattggcaaggtacttgcttctggctagcaattttcaggtgttattcttgatatttatttttcaggattaggt
taccgagtgaaatgagacccccactcaatatacgagtaaatcattttccgcgcataggtaataagaatagtagtgtct
cccctccctcatcggagattatcagtgtacttttccactgatcgctccctcaaattatcataatccttccccactttc
tgtactactacctttttcccaatcaaacccaaaaccttgaaaatagatgagtcggatcaccaccacaagcttctccttta
cgccacacttgaaacaaaaaaggagatacagacgggtaggtaatgagacatgcggaataattagtacagtaattgggcc
catgtttcgggggggggggcaagattcgacgacaagtgtcatatcgaaagtgccaaagtaaatgagaatacgaataggtt
gtggagaaaccagaaaaagcttgtt
Purple Sequence represents introns.

External Primers

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	89	20	60.04	45.00	4.00	2.00	gatgaaattgctgggcctaa
RIGHT PRIMER	1767	20	60.03	45.00	3.00	0.00	gggtttgattgggaaaaggt

PRODUCT SIZE: 1679

Internal Primers

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	585	20	59.98	40.00	3.00	0.00	gcaatcgtgtttttccgttt
RIGHT PRIMER	1419	20	60.21	45.00	4.00	1.00	gaagcgccaattacctttca

PRODUCT SIZE: 835

Forward Sequence Primers

OLIGO	start	len	tm	gc%	any	end	seq
F1	1	20	60.00	40.00	3.00	0.00	**gcaatcgtgtttttccgttt**
F2	364	20	60.00	50.00	5.00	2.00	**tgctccgagaagtagccatt**

Reverse Sequence Primers

OLIGO	start	len	tm	gc%	any	end	seq
R1	*189*	21	60.20	47.60	7.00	2.00	gggtgttgaacaagacgctta
R2	780	21	59.60	42.90	4.00	0.00	caaatggaagtcgaacacaca

Appendix D Spontaneous induction frequency for lethal mutations in the *eT1* balanced region.

Researchers	Number of F_1's tested	Number of lethals	Lethal (%)
Rosenbluth, R.E	*18*12	2	0.11
Rosenbluth, R.E	1386	0	0
Fernandes, A.R	1267	0	0
Fernandes, A.R	1931	0	0
Fernandes, A.R	882	0	0
Fernandes, A.R	1057	1	0.09
Fernandes, A.R	592	0	0
Fernandes, A.R	709	0	0
Fernandes, A.R/ Bhattacharyya, N.	178	0	0
Fernandes, A.R/ Bhattacharyya, N.	93	0	0
Fernandes, A.R/ Bhattacharyya, N.	95	0	0
Fernandes, A.R/ Bhattacharyya, N.	105	0	0

Fernandes, A.R/ Bhattacharyya, N.	107	0	0
Fernandes, A.R/ Bhattacharyya, N.	108	0	0
Fernandes, A.R/ Bhattacharyya, N.	116	0	0
Fernandes, A.R/ Bhattacharyya, N.	100	0	0
Fernandes, A.R/ Bhattacharyya, N.	98	0	0
Fernandes, A.R/ Bhattacharyya, N.	76	0	0
Fernandes, A.R/ Bhattacharyya, N.	116	0	0
Fernandes, A.R/ Bhattacharyya, N.	116	0	0
Fernandes, A.R/ Bhattacharyya, N.	119	0	0
Fernandes, A.R/ Bhattacharyya, N.	98	0	0
Fernandes, A.R/ Bhattacharyya, N.	99	0	0
Fernandes, A.R/ Bhattacharyya, N.	96	0	0
Fernandes, A.R/ Bhattacharyya, N.	99	0	0
Fernandes, A.R/ Bhattacharyya, N.	97	0	0

Fernandes, A.R/ Bhattacharyya, N.	95	0	0
Fernandes, A.R/ Wu, V.	48	0	0
Fernandes, A.R/ Wu, V.	48	0	0
Fernandes, A.R/ Wu, V.	90	0	0
Fernandes, A.R/ Wu, V.	94	0	0
Fernandes, A.R/ Wu, V.	96	1	1.04
Fernandes, A.R/ Wu, V.	96	0	0
Fernandes, A.R/ Wu, V.	95	0	0
Fernandes, A.R/ Wu, V.	94	0	0
Fernandes, A.R/ Yoo, B.	63	0	0
Fernandes, A.R/ Yoo, B.	78	0	0
Fernandes, A.R/ Yoo, B.	97	0	0
Fernandes, A.R/ Yoo, B.	98	0	0
Fernandes, A.R/ Yoo, B.	94	0	0

Fernandes, A.R/ Yoo, B.	100	0	0
Fernandes, A.R/ Yoo, B.	95	0	0
Fernandes, A.R/ Yoo, B.	98	0	0
Fernandes, A.R/ Yoo, B.	95	0	0
Fernandes, A.R/ Yoo, B.	92	0	0
TOTAL	**13 2*18***	**4**	**0.03**

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