Identifying novel suppressors of *memi-1(sb41)* and their possible roles in meiosis

by

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Abstract

Meiosis consists of a highly regulated pair of cell divisions, which ensures haploidization of gametes. In *C. elegans* oogenesis*,* the first meiotic division (MI) proceeds only after the oocyte receives a diffusible signal from the sperm. Meiosis II (MII) proceeds only after fertilization; if the diffusible signal is received but the sperm do not fertilize, the oocytes abort MI, skip MII, and enter mitosis. This suggests that the sperm communicates with the oocyte both before and during fertilization which facilitates proper meiotic progression. The Srayko lab previously identified three highly-similar genes called *memi-1, memi-2,* and *memi-3*, which represent good candidates for "sensing" sperm entry. The *memi* genes are expressed in the female germline and are functionally redundant. When all three genes are targeted via *memi-1/2/3(RNAi),* oocytes are fertilized normally, but they abort MI, skip MII, and enter mitosis. Interestingly, a presumed hypermorphic mutation, *memi-1(sb41)*, results in oocytes that become "stuck" in MII, until they eventually abort and progress into an abnormal mitosis. To find other components of the MEMI pathway important in the meiosis-to-mitosis transition, the Srayko lab conducted a mutagenic suppressor screen that yielded 27 suppressors of *memi-1(sb41)*, of which 10 were found to be intragenic in initial screening. I investigated the remaining 17 *memi-1(sb41)* suppressors. Using classical genetic techniques, I identified 10 novel suppressor mutations of *memi-1(sb41)* and began characterization of the mutations. I also narrowed the number of possible suppressing mutations for the remaining 7 suppressor strains, from several thousand to 84. There is a problem though, in evaluating *memi-1(sb41)* because of the redundant action of *memi-2/3*. Previous experiments have shown that increasing the number of WT copies of *memi-1* in a heterozygous *memi-1(sb41)* background increased the severity of the maternal-effect lethal (Mel) phenotype. This suggests that the Mel phenotype of *memi-1(sb41)* results in an increase in MEMI activity, which would make it a hypermorph. However, as the copies of *memi* act redundantly it is possible that *memi-2/3* compensate for a loss of normal *memi-1(sb41)* function. To investigate if this was the case I lowered the activity of *memi-2/3* through introducing deletions would also suppress *memi-1(sb41)*, and tested the assumption that *memi-1(sb41)* continues to act redundantly in the absences of *memi-2/3*. I confirmed that deletions of different *memi* genes did improve embryonic viability in a *memi-1(sb41)* background. However, I also found that while *memi-1(+) memi-2Δ memi-3Δ* worms were viable, *memi-1(sb41) memi-2Δ memi-3Δ* were not. This indicated that MEMI-1(sb41) protein function, alone, is likely insufficient for meiotic processes, suggesting that it may be a neomorph. The *memi-1(sb41) memi-2Δ memi-3Δ* worms did show an improvement in embryonic viability when mated to males of the same genotype, suggesting that physiological differences between male and hermaphrodite sperm can impact the MEMI pathway, further strengthening the import of sperm derived factors in oocyte meiosis. Together this work provides multiple insights into the *memi-1(sb41)* mutation and potential interactors which will prove useful in future inquiries.

Dedication

I would like to dedicate this thesis to the people whose support made it possible. First, I would like to thank my family, in particular I want to thank my wife Miyu, who endured three years of long distance while supporting my studies. Second, I would like to thank my friends I made in grad school, who have made this a fun and fulfilling experience. In particular: Karen Lange, Katharine Pelletier, and Kacie Norton, without whose support, technical skill, and advice, I would have given up on this so many times.

Acknowledgments

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List of abbreviations

Abbreviation	Definition
+	Wildtype genotype
Δ	Deletion
°C	Degrees Celsius
A	Nucleic acid adenine
Abc	Srayko lab mutation designation
Acy	Adenylate cyclase
Air	Aurora/IpI1 related kinase
AMP	Adenosine monophosphate
APC	Anaphase Promoting Complex
Bli	Blister
C	Nucleic acid cytosine
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
Cdc	Cell division cycle
Cdk	Cyclin dependant kinase
CFS	Cytostatic Factor
CG	Complementation group
cGMP	Cyclic guanine monophosphate synthase
Chs	Chitin Synthase
Cm	Centimeter
Cul	Cullin
DAP	Dacapo
Daz	Human DAZ (deleted in Azoospermia) homolog
DAZL	Deleted in azoospermia like
Df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides used for PCR: GACT
Dpy	Dumpy
Egg	Egg sterile: unfertilizeable
Elgi	Early Girl
EMI	Early mitotic inhibitor
EMS	Ethyl methanesulfanate
EtBr	Ethidium bromide
EV	Embryonic viability
F	Filial hybrid
Fer	Fertilization defective
G	Nucleic acid guanine
GATOR	GAP activity toward rags
GLC7	Yeast Glc Seven
Gld	GermLine Development

Abbreviation	Definition
Glh	Germ Line Helicase
Gsa	G protein Subunit Alpha
Gsk	Glycogen synthase kinase
Gsp	GLC7 Seven like Phosphatase
Gwl	Greatwall
Hecd	Hectd1 ubiquitin ligase homolog
Htas	Histone H2A variant sperm-specific
IP3R	Inositol 1,4,5-triphosphate receptor
Kb	Kilobase
L	Larval stage
LG	Linkage group
LH	Luteinizing Hormone
Lon	Long
M	Molar
MAPK	Mitogen activated protein kinase
MAS	Srayko lab strain designation
MASTL	Microtubule associated serine/threonine kinase like
MBK	Mini-Brain Kinase
MBSU	Molecular Biology Service Unit
Mei	Defective meiosis
Mek	Mitogen-activated protein kinase kinase
Mel	Maternal-effect lethal phenotype
Memi	Meiosis-to-mitosis transition associated
Mex	Muscle excess
Mg	Milligram
MI	Meiosis I
MII	Meiosis II
mL	Milliliter
mM	Millimolar
Mm	Millimeter
Mn	Magnesium
mRNA	Messenger RNA
MSP	Major Sperm Protein
Mtrm	Matrimony
N	Sample size
Ng	Nanograms
NGM	Nutrient growth medium
Nos	Nanos related
Oma	Oocyte maturation defective
P	p-value
P0	Parental generation filial 0

Abbreviation	Definition
PCR	Polymerase chain reaction
Pg	Page
pH	Potential of hydrogen
Pie	Pharynx and intestine in excess
PKA	Protein kinase A
Plk	Polo-like kinase
PP	Protein phosphatase
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Rotations per minute
SKP	S-phase kinase-associated protein
Smz	Sperm meiosis PDZ domain containing proteins
SNP	Single nucleotide polymorphism
Spch	Sperm Chromatin enriched
Spe	Defective spermatogenesis
Spn	eIF4E transporter
Sup	Suppressor
T	Nucleic acid thymine
Taf	TBP-associated transcription factor
Taq	Thermus aquaticus
TORC	Target of rapamycin complex
Unc	Uncoordinated
V	Volts
WGS	Whole genome sequencing
WT	Wildtype
Zif	Zinc finger-interacting protein
Zyg	Zygotic defective: embryonic lethal
μL	Microliter
χ^2	Chi Squared

1 Introduction

1.1 <u>Genetic regulation of meiosis</u>

1.1.1 Meiosis: specialized cell division

Successful meiosis consists of two sequential rounds of cell division, that reduce the ploidy of cells so that they can become gametes. As with mitosis, meiosis is preceded by DNA replication. However, unlike mitosis, the information content of the replicated genome is then reduced in meiosis I (MI) through the separation of homologous chromosomes. Then, in meiosis II (MII), the sister chromatids (the replicated DNA products of S-phase) are separated giving rise to haploid daughter cells. Many of the same proteins participate in both meiosis and mitosis. For example, structural components: tubulin and actin are required for spindle assembly and cortical contractility, in both meiosis and mitosis. Many of the same cell cycle regulators also function during both processes. However, the meiosis and mitosis each require unique proteins with distinct functions to facilitate their specialized features of chromosome segregation and cytokinesis. The genetic regulation of meiosis is not completely understood but there are many proteins that have been implicated in playing a role in different models.
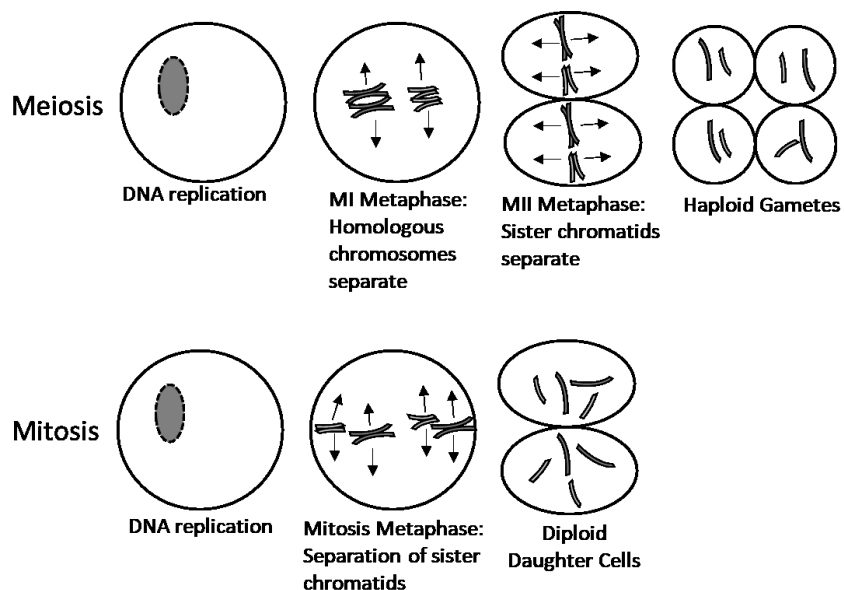
Figure 1: Meiosis and mitosis, showing the divisions that result in four haploid gametes and two diploid daughter cells respectively, following DNA replication

This knowledge pertains to the broadly studied signalling pathways required to regulate the process. There is an absence in many cases, of specific information on the proteins that

differentially regulate meiosis in each model organism which are greatly varied across sexually reproducing species. In the oocyte, because chromosomes are condensed, and the meiotic divisions occur quickly once meiosis resumes, transcription is limited. Instead, the process must rely on existing proteins and maternally-contributed mRNA to regulate the cell divisions (Handel and Schimenti 2010). This process necessitates that the proteins involved in regulating the meiotic divisions exist in the cytoplasm before meiosis is initiated. To complete proper meiotic divisions and produce viable gametes, germline stem cells rely on sequential degradation of proteins throughout meiosis (Von Stetina and Orr-Weaver 2011). In *C. elegans* the process involves precise timing, a diffusible sperm signal MSP primes the oocyte to begin MI, but relies on other sperm factors entering upon fertilization to progress (McNally and McNally 2005). This signals the degradation of proteins like MBK-2 that maintain the meiosis and when degraded stop repressing of mitotic factors such as OMA-1/2 (Stitzel, Pellettieri, and Seydoux 2006). These systems are tailored to individual species and it requires specific components to regulate the transition, as species reproductive environments and life cycles differ greatly allowing for a large diversification of meiotic controls.

1.1.2 Evolutionary divergence of meiotic proteins

Because meiosis relates directly to the reproductive success of an organism, genes involved in the process evolve rapidly. The proteins used to regulate reproduction across species appear to have diverged more quickly than proteins used for other essential processes: mutations that favour reproductive success are easily selected for in systems that allow competition at the gametic level (White-Cooper and Bausek 2010). Many homologous RNA-binding proteins for example, serve different or tweaked functions as their species diverged evolutionarily. Nanos in *Drosophila*, or NOS-1 in *C. elegans*, is a RNA binding protein which is required for embryonic patterning, but is also involved in transcriptional repression during meiosis. Alternatively, mouse Nanos2 is involved in the inhibition of meiosis in male embryos only (Lesch and Page 2012). In this introduction, I will focus on two topics. The first is oocyte meiosis, and the second is the genetic regulation of the cell division mechanisms required in the developing oocyte to undergo successful meiosis and to prepare for a successful mitotic division.

1.1.3 Evolution of anisogamous gametes: oogenesis and spermatogenesis

As a further complication to meiotic cell divisions, anisogamous species produce highly specialized gametes, spermatozoa and oocytes, that are morphologically and functionally distinct (Bell 1978). Because two different types of gametes are produced, further specialization of the genes that regulate meiosis is required, leading to two very different forms of meiosis. Anisogamy evolved to take advantage of the benefits of having small motile gametes in sperm that can find and fertilize oocytes easily, while at the same time relying on large immobile oocytes to provide all the nutrients necessary for successful development (Bulmer and Parker 2002; Bell 1978). This situation leads to dichotomous strategies in gamete production, where sperm are made as small as possible and are energetically inexpensive, so that they can (usually) be produced continuously. Oocytes are made as large as possible to improve the development of the embryos, but are energetically expensive (Bell 1978). As sperm are generally produced in bulk and oocytes are more controlled for quality, this necessitates different genetic mechanisms to regulate these two forms of meiosis (White-Cooper and Bausek 2010). In many species such as humans, oocytes are created by germline stem cells early in development and then sequestered for long periods of time, suspended during a MI prophase arrest, which can last multiple decades before resumption, as it does in humans (Clift and Schuh 2013). This necessitates an external stimulus to resume oocyte meiosis, so that oocyte meiosis completes properly in coordination with fertilization (Von Stetina and Orr-Weaver 2011). Because the timing of meiotic divisions relies in many species on fertilization, the oocyte evolves mechanisms to receives sperm signals from the sperm, tailored to the timeframe of the individual species specific meiotic program.

1.2 The role of sperm in oocyte meiosis

As sperm are produced continuously, without arrests, and do not require external signals, spermatogenesis is simpler than oogenesis. Because sperm production is usually tailored to maximize the number of sperm produced, progenitors undergo two meiotic divisions that are symmetric and produce four identical spermatozoa (White-Cooper and Bausek 2010). In many species, adult males produce sperm continuously, as continued production aids in reproductive success (White-Cooper and Bausek 2010). There are many different types of sperm, but many of the genetic mechanisms that regulate sperm production are conserved (White-Cooper and

Bausek 2010). There are many shared proteins between *C. elegans* and mammals, in which defects lead to male infertility in both organisms. Examples include GLC7 (yeast Glc Seven) like Phosphatases-3/4 (GSP-3/4), a pair of protein phosphatases (PP1), histone related proteins Histone H2A variant Sperm-specific (HTAS-1) as well as Sperm Chromatin enriched-1/2/3 (SPCH-1/2/3), and the RNA helicase: Germ Line Helicase (GLH-2), all of which have been shown to have spermatogenesis function in *C. elegans* and mammals (Chu et al. 2006). Additionally, for all of these genes, knockouts or mutations of the mammalian counterparts have been linked to male infertility (Chu et al. 2006). The role of sperm in oocyte meiosis, is the re-initiating oocyte meiosis by lifting meiotic arrests. Major Sperm Protein (MSP) was first purified from *C. elegans* as a protein that represented about 15% of the total protein content of sperm (Klass and Hirsh 1981). In *C. elegans,* MSP serves two main functions: as a cytoskeletal polymer (Ward, Argon, and Nelson 1981) and as a secreted hormone that can signal the oocyte and surrounding tissues (Miller et al. 2001). The downstream effect of the MSP signal is the activation of MAPK in the oocyte which in turn triggers the oocyte-to-embryo transition (Heger et al. 2010; Lee et al. 2007). In this way sperm signalling is required for lifting the primary meiotic arrest. In most species (except *Echinoderms*), fertilization of the oocyte by required for the completion of oocyte meiosis, making sperm signals integral to oocyte meiosis. Despite widespread differences between sperm in different species, for example *Drosophila* sperm is thin and longer than the organism, while the sperm of *Caenorhabditis* is amoeboid, there are many necessary proteins that are required for sperm meiosis. An example of a gene involved in spermatogenesis in a broad range of species is *Dazl*: deleted in azoospermia, however in different species it has evolved different roles. Mouse *Dazl* and the *D. melanogaster* gene *boule*, are homologues which are required for sperm meiosis (Lesch and Page 2012). Interestingly though, *C. elegans daz-1* is required for oocyte meiosis (Karashima, Sugimoto, and Yamamoto 2000). This demonstrates the evolutionary divergence in proteins to differently regulate meiosis. While the evolution of spermatogenesis and oogenesis has diverged between species, because the two processes have to work in concert with one another any change can create a feedback loop to drive evolution of gametogenesis together (White-Cooper and Bausek 2010).

1.3   Oogenesis
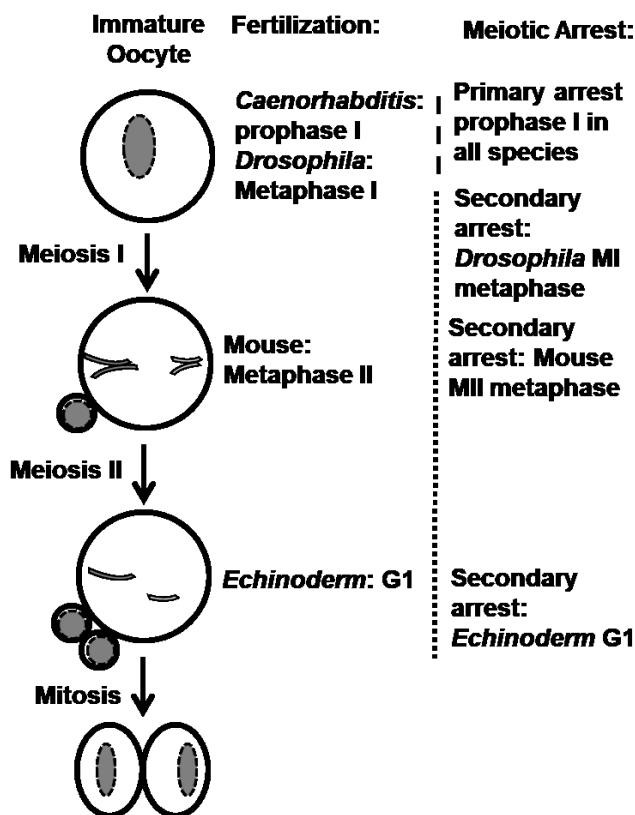
Figure 2: Fertilization points of different species. All oocytes are subject to the primary meiosis arrest in prophase I. The secondary meiosis arrest is lifted after fertilization. Note there is no secondary oocyte meiosis arrest in *C. elegans*.

Oogenesis differs from spermatogenesis in that it produces one large oocyte and two polar bodies in meiosis instead of four equally-sized cells. Because oocytes are energetically expensive to produce, they are often sequestered for long periods of time (Nishiyama, Tachibana, and Kishimoto 2010). The role of the oocyte is also different than that of the sperm as their primary purpose is to contribute genetic material. The egg, on the other hand, needs to provide the proteins and energy in the form of nutrients required for embryogenesis in addition to the contribution of genetic material. In sexually-reproducing animals, the timing of reproduction is linked to the age of the parents, as they reach sexual maturity and temporal cues from the environment (Von Stetina and Orr-Weaver 2011). These are both critical factors that can determine the success of the offspring. Due to these constraints, oocyte meiosis is often put on hold partway through production. The oocyte, then, relies on external signals (*e.g.* hormones) to complete meiosis. All animals exhibit a primary arrest during female meiosis, and many exhibit a secondary arrest to coordinate fertilization with the final stages of meiosis (Nishiyama,

Tachibana, and Kishimoto 2010). This sequence allows oocytes in different species to progress to a fertilization-competent state, which can occur at different stages of meiosis depending on the species (Sagata 1996). For example, oocytes in *Drosophila* and some molluscs are fertilized at metaphase I, whereas, most mammalian oocytes are fertilized at metaphase II (Figure 2, pg5) (Sagata 1996). Only in *Echinoderms*, are oocytes fertilized after oogenesis has completed (Figure 2, pg5) (Kishimoto 2011). While oocyte maturation varies from species to species, the molecular mechanisms at work seem to share many common features. While the primary meiosis hold is lifted in *C. elegans* by major sperm protein (MSP) and in mammals by luteinizing hormone (LH), both affect the Gα-adenylate cyclase pathway (Kim, Spike, and Greenstein 2013). The meiotic hold adds a completely different set of regulatory issues for the oocyte as it needs to be able to receive and interrupt environmental cues, in order to proceed with division and degrade the proteins involved in the correct timing and sequence. This means that, while undergoing meiotic divisions and fertilization, the oocyte is also priming itself for later mitotic divisions and patterning of the early embryo (Lesch and Page 2012).

1.3.1   Regulation of oogenesis

There is a broad range of conserved genes that are important in controlling oocyte meiosis in all species; many of these are involved in controlling the degradation of proteins. During meiosis, the oocyte DNA is condensed, and transcription is generally quiescent. This necessitates that the oocyte-to-embryo transition rely on maternally contributed proteins and maternal mRNA, to regulate meiosis and the first mitosis (Walker et al. 1999). This system requires strict control of the precise timing at which molecules are activated and degraded. Errors at any stage prevent survival of the zygote (Lesch and Page 2012). In species with a secondary meiotic arrest, it is modulated by cytostatic factor (CFS) which inhibits the function of the anaphase promoting complex (APC) (Clift and Schuh 2013). At fertilization, there is a wave of Ca^{2+} that is released from the endoplasmic reticulum, which serves an important role in blocking polyspermy and signalling fertilization to the rest of the oocyte (Clift and Schuh 2013). This wave of Ca^{2+} also causes the exocytosis of cortical granules and promotes the translation of maternal mRNA. However, these broadly conserved cues alone, do not explain all the regulatory differences in oocyte meiosis specific to different species; this requires further inquiry.

1.3.1.1 Primary regulatory pathways of oogenesis

The general, many of the meiotic proteins that are broadly conserved in metazoans that make up the primary meiosis pathway, play similar roles in the regulation of gamete formation. These factors are regulated in a variety of ways, that create the differences in how oocyte meiosis progresses. For example *Echinoderms* complete female meiosis prior to fertilization, but they use many of the same factors to control oocyte meiotic divisions, including Cyclin, mitogen-activated protein kinase (MAPK), and Greatwall (Gwl) (Kishimoto 2011). This makes oocyte maturation proceed relatively autonomously. After the activation signal from 1-MeAde, a starfish specific maturation-inducing hormone, a cascade of activation promotes Cdc2/Cyclin B. This leads to the promotion of MAPK through Mos kinase (oocyte maturation factor) and Mek (MAPK/ERK, a MAPK kinase), as well as Plk1 (polo-like kinase) and Gwl, ultimately inhibiting PP2A (protein phosphatase 2 A) (Kishimoto 2011). Fertilization inhibits the action of Mos, leading to inhibition of MAPK and accumulation of cyclin A and cyclin B through an unknown mechanism (Hara et al. 2009). This accumulation of cyclin A and B, paired with Cdk1 expression, allows for the progression through the first mitotic division (Hara et al. 2009). Cdk1/cyclin A accumulates it becomes active, which in turn activates Cdk1/cyclin B that goes on to promote the first mitosis (Hara et al. 2009). Many of these proteins are shared between species and make up cell division pathways. Amongst these are cyclin and cyclin-dependant kinase (Cdk), mitogen activated protein kinase (MAPK), and cyclic AMP (Von Stetina and Orr-Weaver 2011). In all animals Katanin p60 and p80 subunits, that are paralogues of *C. elegans* MEI-1 and MEI-2 meiosis-specific microtubule cutting proteins trim microtubules during meiosis to control spindle length (Pleuger et al. 2016). Different species adapt many of the same elements to meet their schedule of meiotic events, however the general plan remains the same, using homologous proteins across different species, such as CDK and cyclins, and signalling pathways such as MAPK. This allows for comparative analysis between species in research, which can lead to a broader understanding of oocyte meiosis.

1.3.2 Mammalian oogenesis

1.3.2.1 Mammalian oocyte maturation

In mammals, oocytes are created early in development and enter a prophase I arrest (Hunt and Hassold). The duration of this arrest depends on the species; in mice it can last for months, or for decades in humans (Clift and Schuh 2013). Primary oocytes in mammals grow surrounded by

granulosa cells, which form the secondary follicle. The oocyte, supported by the follicle, only begins maturation with the signal of luteinizing hormone (LH), which triggers the release of the prophase arrest (Peng et al. 1991). In the maturing oocyte, cyclic AMP (cAMP) promotes the activation of protein kinase A (PKA) (Conti et al. 2002). PKA phosphorylates Wee1 a kinase active in oocyte maturation, which acts as an inhibitor of Cdk1 (Han et al. 2005).

1.3.2.2 Mammalian meiosis

As the preovulatory follicle matures, cAMP is inhibited when cGMP (cyclic guanosine monophosphate) enters the oocyte through gap junctions from cumulous granulosa cells during ovulation (Sela-Abramovich et al. 2008). MASTL, the mammalian orthologue of *Drosophila* Greatwall kinase (Gwl), is required to inhibit protein phosphatase 2A (PP2A) activity and release the MI prophase arrest (Zhao et al. 2014; Adhikari et al. 2014; Li et al. 2013). PP2A regulates the activity of cyclin B/Cdc2 which later allow the progression of MII. In MASTL-null mutants, oocytes fail to enter MII after completion of MI and chromosomes become uncondensed (Adhikari et al. 2014). MASTL is required for the oocyte to properly exit MI through activation of the APC/cyclosome to properly exit MI (Adhikari et al. 2014). This allows for a timely increase in Cdk1 activity, which is required for entry into MII (Adhikari et al. 2014). As Cdk1 activity increases, so does the M-phase promoting factor, which triggers the onset of meiosis in conjunction with cyclin B (Sagata 1996). When the first polar body is extruded completing meiosis I, the APC/Cdc20 to targets cyclin B for ubiquitination and degradation by the proteasome so that further division is prevented (Adhikari et al. 2014). This allows the developing oocyte to progress to the secondary meiotic arrest in metaphase II (Von Stetina and Orr-Weaver 2011).

The developing oocyte prepares for meiosis and subsequent mitosis by transcribing maternal mRNAs, which are required later in the process. The translation of many of the mRNAs is prevented in MI, via binding of CPEB1 to the 3'UTR (untranslated region), which sequesters the mRNA (Clift and Schuh 2013). The phosphorylation of CPEB1 activates translation of the multitude of mRNAs that allow expression of maternal proteins (Chen et al. 2011). This includes DAZL, which promotes the translation of other mRNAs that are important for meiotic spindle assembly and oocyte maturation (Clift and Schuh 2013). In mice, an important factor controlling mRNA stabilization is MYS2, which acts by binding to RNAs (Clift and Schuh 2013). Mutations

that inhibit phosphorylation of MYS2, maintain mRNA transcripts in the oocyte beyond the time that they are normally degraded (Clift and Schuh 2013). The oocyte then enters the second meiotic arrest at metaphase of MII. This second arrest is lifted upon fertilization (Sagata 1996). With fertilization the activity of MAPK, and Cdk1/cyclin B are lowered so meiosis can resume (Von Stetina and Orr-Weaver 2011; Sagata 1996). Cdk1 is upregulated after anaphase I and is essential for MII entry, where the activity peaks at metaphase II and remains high until fertilization (Adhikari et al. 2014). The MII metaphase arrest relies on stabilization of Cdk1 by cytostatic factor (CSF) (Tsurumi et al. 2004). MYS2 is phosphorylated in a Cdk1 dependent manner. When MYS2 contains a mutation that mimics phosphorylation, mRNA is degraded prematurely in the fertilized oocyte (Clift and Schuh 2013).

1.3.2.3   Mammalian sperm signalling in oocyte meiosis

In mammals, as in most species, the secondary meiotic arrest of the oocyte is lifted after an external signal is received. In the case of mammals this signal is received from the sperm upon fertilization. The sperm contributes sperm-derived phospholipase, which, through a series of intermediates, causes the binding of a receptor on the endoplasmic reticulum. This causes IP3R receptors to open Ca^{2+} channels that flood the cytoplasm (Clift and Schuh 2013). Ca^{2+}/calmodulin-dependent kinase (CaMKII), together with Polo-like kinase 1 (PLK1), phosphorylate the early mitosis inhibitor (EMI2) (Clift and Schuh 2013). The oocyte MII metaphase arrest is maintained as long as the APC remains inactive; EMI2 associates with the APC and inhibits it to block the oocytes in MII (Ohe et al. 2010). However, when EMI2 is phosphorylated, it is degraded via ubiquitination by the SKP2-cullin-F-box protein, a ubiquitin ligase complex (Clift and Schuh 2013). This removes EMI2 activity allowing the APC to progress through meiosis (Clift and Schuh 2013). Through this process, Ca^{2+} ions induce cortical granule exocytosis, and translation of maternal mRNAs necessary for the meiosis-to-mitosis transition (Clift and Schuh 2013).

1.3.3   *Drosophila* oogenesis

1.3.3.1   *Drosophila* oocyte maturation

Oogenesis in *Drosophila* is different than other forms of oogenesis discussed, because the germline stem cell undergoes four rounds of mitotic cell division in preparation for meiosis. Interestingly, there is no evidence that MAPK, MOS signalling, and cAMP transduction are

involved in oocyte meiosis despite the importance of these pathways in other systems (Von Stetina and Orr-Weaver 2011). Down regulation of target of rapamycin complex 1 (TORC1) which controls cell growth and cell size (Kim et al. 2008), is required for entry into meiosis. This is facilitated by GTPase-activating proteins toward Rags 1 (GATOR1) along with GATOR2 (Wei et al. 2014). Later in oogenesis, GATOR2 inhibits GATOR1 and allows TORC1 to accumulate, allowing for continuation of oocyte growth and development (Wei et al. 2014).

1.3.3.2   *Drosophila* oocyte meiosis

Cytokinesis in the germline stem cell, is halted in the developing oocyte before abscission, leaving a 16-cell network, or cyst, composed of one oocyte and 15 supporting nurse cells (Sugimura and Lilly 2006). This cyst is surrounded by a layer of somatic follicle cells that supports the developing oocyte (Sugimura and Lilly 2006). The nurse cells replicate their DNA without dividing, in a process termed endoreplication. The oocyte is prevented from entering this endocycle through the action of dacapo (DAP), a protein which inhibits Cdk2/cyclin E (Hong et al. 2003). The nurse cells provide the proteins and mRNA required for meiosis while the oocyte is quiescent (Page and Hawley 2001). As the oocyte develops, the protein: muskellin, clears the nuclei from the nurse cells, leaving behind a large oocyte with a single-nucleus (Kronja, Whitfield, et al. 2014). Meiotic regulation relies on the control of Cdk1/cyclin B, which affects the timescale of meiotic maturation (Von Stetina et al. 2008). Mutations in either Cdk1 or cyclin B have been shown to delay meiotic maturation (Von Stetina et al. 2008). During this process, the oocyte enters the primary meiosis arrest in prophase I, which lasts for approximately two days (Von Stetina and Orr-Weaver 2011). The prophase I arrest is maintained by early girl (Elgi), matrimony (Mtrm), and greatwall (Gwl) (Von Stetina and Orr-Weaver 2011). Elgi directly inhibits meiotic maturation (Kronja, Whitfield, et al. 2014; Von Stetina and Orr-Weaver 2011). Elgi and Polo are both controlled by Endos, which inhibits Elgi and promotes Polo so that the net effect is a promotion of meiotic maturation (Von Stetina et al. 2008). Similarly, mutations in endos have been shown to effect the timing of oocyte meiosis and on maturation (Kronja, Whitfield, et al. 2014). Mtrm and Gwl work by inhibiting Polo, which normally promotes Twine/Cdc25-dependent activation of Cdk1/cyclin B. The inhibition of Cdk1/cyclinB activity allows for meiotic maturation (Kronja, Yuan, et al. 2014). PP2A/Twins-mediated dephosphorylation of Gwl is thought to inhibit its activity, thus allowing Polo activity to increase (Wang, Pinson, and Archambault 2011). Lowered levels of Polo and PP2A/Twins due to knock-

down, have been shown to result in embryonic lethality because oocytes fail to progress through meiosis properly (Wang, Pinson, and Archambault 2011). In cases where Gwl is absent, sister chromatids fail to adhere to one another (Archambault et al. 2007). However, it is not known if this is directly caused by Gwl or by unopposed Polo activity (Archambault et al. 2007). Conversely, hypermorphs of Gwl fail to progress through meiosis but can be rescued through increasing Polo expression (Archambault et al. 2007).

1.3.3.3   Progression through *Drosophila* oogenesis arrests

The signal that releases *Drosophila* oocytes from prophase I arrest is unknown. When the arrest is lifted, the oocyte proceeds to the second arrest at metaphase I (Von Stetina and Orr-Weaver 2011). Oocyte activation, and the release of the second meiotic hold, is independent of fertilization in *Drosophila* and requires the degradation of cyclin (Yu et al. 2004). The ovulation of oocytes is induced through rehydration, which creates mechanical pressure and triggers the completion of meiosis (Mahowald, Goralski, and Caulton 1983). At the molecular level, completion of meiosis requires the degradation of proteins by the APC. This is accomplished through the Cdc20/cortex that works to target the proteins for ubiquitination (Kronja, Whitfield, et al. 2014; Von Stetina and Orr-Weaver 2011). By regulating proteins that sequester mRNA and control RNA processing in late meiosis and embryogenesis, the oocyte can be cleared of maternal proteins required for meiosis. Upon completion of oocyte meiosis, female and male pro-nuclei form and fuse in preparation for the mitotic divisions (Kronja, Whitfield, et al. 2014).

1.3.4   *Caenorhabditis elegans* oogenesis

1.3.4.1   *C. elegans* oocyte maturation

In *C. elegans* hermaphrodites, the ovaries are found at the end of two reflexed gonad arms that make up the ovotestes. Germline stem cells divide mitotically at the distal tip of the gonad where nuclei progress proximally through the gonad to become mature oocytes (Kim, Spike, and Greenstein 2013). The gonad of *C. elegans* is syncytial at the distal end. This is where the DNA enters pachytene, and the cell membrane starts to engulf the germ cell nuclei, leaving a connection to the syncytial gonad through ring canals. Before cellularizing, many maternal mRNAs important for oocyte meiosis and mitotic divisions, are repressed by GLD-1 (defective in GermLine Development) (Biedermann et al. 2009). As the oocytes mature, they encounter a

series of signal gradients that control mRNA expression so that only maternal factors are present (Jones, Francis, and Schedl 1996). GSA-1 (G protein Subunit Alpha 1) is also present and inhibits the premature entry into meiosis (Govindan et al. 2009). While developing, the EGG-4/5 (EGG sterile: unfertilizeable) proteins bind MBK-2 (Mini-brain kinase) to prevent a premature entry into meiosis (Cheng et al. 2009), as it plays multiple important roles later in meiosis (Stitzel, Pellettieri, and Seydoux 2006). When the developing oocyte nucleus passes the bend in the ovary, it cellularizes and comes in contact with the gonadal sheath (Kim, Spike, and Greenstein 2013). Once the oocyte is adjacent to the spermatheca, it receives a diffusible signal from the sperm in the form of major sperm protein (MSP) (Robertson and Lin 2013). This releases the oocyte from the meiotic arrest, which is characterized by the breakdown of the germinal vesicle (Von Stetina and Orr-Weaver 2011; Heger et al. 2010).

1.3.4.2 *C. elegans* oocyte meiosis and fertilization

The MSP signal causes activation of GSA-1, which activates adenylate cyclase (ACY-4), which in turn elevates cyclic AMP (cAMP) levels (Von Stetina and Orr-Weaver 2011). The oocyte is normally fertilized as the oocyte passes through the spermatheca. This is where a calcium wave passes over the fertilized oocyte (Robertson and Lin 2013). Fertilization in nematodes is extremely efficient, and it usually occurs within minutes of the oocyte entering the spermatheca. Perhaps due to selection for fast reproduction, a second meiotic arrest is not present in *C. elegans*. However, oocyte meiosis is still dependant on fertilization as hermaphrodites mated with males with fertilization-defective sperm, will fail to complete meiosis I and begin mitotic divisions (McNally and McNally 2005). There are many proteins that are involved in *C. elegans* oocyte meiosis. There are sperm proteins necessary for fertilization, as well as oocyte proteins that control the progression of meiosis, eggshell formation, and the polarization of the fertilized oocyte in preparation for the mitotic divisions (Marcello, Singaravelu, and Singson 2013). During oogenesis, before the MSP signal is received, proteins are already being sequestered in the oocyte, so that there is no delay in activation. EGG-3, a cortical binding protein, sequesters two key proteins at the cortex of the oocyte, chitin synthase I (CHS-1) important in eggshell formation, and mini-brain kinase 2 (MBK-2). MBK-2 plays several roles in relation to the regulation of MEI-1/MEI-2 and OMA-1/2, both of which are important in the oocyte to embryo transition (Nishi and Lin 2005; Johnston, Krizus, and Dennis 2010). After fertilization, this sequestration ends as EGG-3 is degraded by the proteasome directed by the APC (Jones, Francis,

and Schedl 1996). This must occur quickly as the degradation of EGG-3 allows CHS-1 to begin synthesizing the eggshell (Johnston, Krizus, and Dennis 2010). Most of the coordination of the other mechanics of meiosis are thought to be directed by MBK-2 (Robertson and Lin 2013).

1.3.4.3   *C. elegans* oogenesis protein degradation pathways

MBK-2 is activated via phosphorylation by CDK-1, although other kinase(s) could be involved (Cheng et al. 2009). Early in meiosis, MBK-2 phosphorylates other proteins to activate them (Guven-Ozkan et al. 2008), but, later in mitosis, it has a role in marking other proteins for degradation once meiosis is complete (Nishi and Lin 2005; Guven-Ozkan et al. 2008). MEI-1/MEI-2 control the length of the meiotic spindle by severing microtubules, trimming the meiotic spindle and seeding new microtubules (Beard et al. 2016). Similarly, to other proteins, MEI-1/MEI-2 need to be cleared from the fertilized oocyte prior to the first mitosis. In the developing oocyte, *mei-1* mRNA binds to oocyte maturation defective (OMA-1) when paired with a eukaryotic translation initiation factor SPN-2, and the complex localizes them to the developing p-granules (Li et al. 2009). In this way, *mei-1* translation is inhibited before meiosis. Unsurprisingly, decreased SPN-2 function increases MEI-1 expression, resulting in mitotic spindle defects (Li et al. 2009). MEI-1 and MEI-2 are Katanin subunits of the microtubule severing complex that is important in regulating the spindle length and assembly in meiosis (Srayko et al. 2000; Clark-Maguire and Mains 1994). During meiosis, the HECT domain E3 ubiquitin ligase subunit, HECD-1, activates MEI-1, but in mitosis, HECD inhibits MEI-1 (Beard et al. 2016). MEI-1 is also regulated by PP4 which increases microtubule severing during meiosis (Dow and Mains 1998; Han et al. 2009). MEI-1/MEI-2 needs to be degraded quickly after meiosis to ensure proper mitosis (Beard et al. 2016). To facilitate mitosis, MEI-1/MEI-2 is degraded by two parallel pathways, one using both CUL-2/MBK-2 and the other CUL-3/MEL-26 (Johnson et al. 2009). Ubiquitin ligase degradation is facilitated by ubiquitin ligases, which attach ubiquitin to the substrate specified by the substrate specific adaptor, which then targets it for degradation by the proteasome (Pellettieri et al. 2003). This degradation requires a regulatory shift for CUL-2. It normally targets the maternal effect lethal (MEL-26), a substrate specific adaptor of E3 ubiquitin ligases. MEL-26 is targeted in meiosis to prevent premature degradation of MEI-1/MEI-2, but then it works with CUL-3 to target MEI-1 in mitosis (Beard et al. 2016; Johnson et al. 2009). Mutations to genes in this system, such as *mel-26(null)*, results in the

persistence of MEI-1/MEI-2 into mitosis. This persistence is lethal to the embryo (Lu and Mains 2007).

OMA-1/2 are important in regulating the degradation proteins that have been shown to be cell fate determinants, and play a role in maternally inherited patterning and polarization of the embryo (Guven-Ozkan et al. 2008; Robertson and Lin 2013). OMA-1/2 requires activation by MBK-2 which phosphorylates them after fertilization (Guven-Ozkan et al. 2010). OMA-1/2 double mutants are sterile: their oocytes arrest in prophase I, and they are not released from the primary meiotic hold (Robertson and Lin 2013). This is due to the failure of OMA-1/2 to activate MAPK and AIR-2 (Robertson and Lin 2013). SPN-2 and OMA-1/2 associate with each other to repress the translation of *zif-1* by binding the mRNA (Guven-Ozkan et al. 2010). ZIF-1 opposes PIE-1, which is a cell fate determinant localized to the P-granules (Guven-Ozkan et al. 2010). ZIF-1 is also important in marking cells that will become the germline in developing embryos (Chi and Reinke 2009). OMA-1/2 represses transcription in the fertilized oocyte and early embryo by sequestering TAF-4, a transcription factor required in the early embryo (Guven-Ozkan et al. 2008). OMA-1/2 then is degraded after the first mitotic division (Guven-Ozkan et al. 2010). This is facilitated through secondary phosphorylation of OMA-1/2 by GSK-3 (Nishi and Lin 2005) which marks it for ubiquitination by CUL-2 (Shirayama et al. 2006; Nishi and Lin 2005). Mutations in MBK-2, GSK-3, ZYG-11, and CDK-1 have been shown to stabilize OMA (Shirayama et al. 2006). Once OMA is degraded, ZIF-1 is able to accumulate and promotes the degradation of maternally-contributed PIE-1 to pattern the developing embryo (Robertson and Lin 2013). MBK-2 also plays a role in the phosphorylation of MEX-5/6, which is required to establish polarity in the embryo (Beard et al. 2016). Requires MEX-5/6 to be secondarily phosphorylated by PLK-1/2 (Beard et al. 2016). MBK-2 is required for the degradation of MEI-1/MEI-2, OMA-1/2, PIE-1 as well as other patterning proteins (Nishi and Lin 2005). Finally, MBK-2 needs to be removed quickly before mitotic cleavage (Beard et al. 2016), however the specific factors that regulate the meiosis-to-mitosis transition are unknown.

1.3.4.4   *C. elegans* meiosis-to-mitosis transition

Through this regulation, the oocyte-to-embryo transition is completed: formation of the eggshell through CHS-1, regulation of the meiotic spindle by MEI-1/MEI-2, and clearing of maternal proteins required to pattern the embryo and prepare it for mitosis by MBK-2. Although MEI-1, CHS-1, MBK-2 are all members of well-characterized pathways that define the meiotic and

mitotic cell division, it is unclear how all these events are coordinated upon fertilization. This requires a sperm signal, contributed through fertilization, to be sensed by some mechanism in the oocyte. Many genes such as *spe-11*, *oma-1/2*, *mbk-2*, and *mei-1/mei-2* are required for proper progression through meiosis. Each of these have been proposed at different times to be the mechanism for regulating the meiosis-to-mitosis transitions. However, to date none of these candidates were shown to be responsible for oocytes "sensing" fertilization. The Srayko lab has proposed that the MEMI proteins are likely involved in sensing fertilization and activating the proper meiotic program of division after MI. This is based on many observations, but includes: i) depletion of MEMI via *RNAi* results in a phenotype similar to that which occurs when oocytes are not fertilized and fail to enter meiosis II, and ii) a hypermorphic mutation *memi-1(sb41)* causes oocytes to fail to exit meiosis II properly. Together these results indicate that MEMI is required for normal progression into MII after fertilization, and that excess MEMI activity results in embryos becoming "stuck" in MII (Ataeian et al. 2016).

1.4 *Caenorhabditis elegans* **as a model organism**

Caenorhabditis elegans is a well-established model organism for genetic research, with decades of study and a well annotated and mapped genome, as well as fate maps for the entire organism specific to individual cells. The nematode worm has many advantages for studying oocyte meiosis, as the hermaphroditic worms reproduce continuously for the majority of their adulthood. The species is androdioecious, with hermaphrodites either self-fertilizing oocytes which are developed in their double branched ovotestes, or by mating with males who continuously produce sperm. The early development of *C. elegans* can be easily observed *in utero*, because both the adult hermaphrodite and the embryo being transparent under the microscope. The entire meiosis-to-mitosis transition occurs in about 1 hour after the lifting of the primary meiotic arrest. *C. elegans* has five autosomes and a single sex chromosome. Each chromosome exhibits a maximum recombination rate close to 50% (Brenner 1974). Therefore, each chromosome is a single linkage group. This simplifies the mapping of unknown suppressor mutations. In particular, *C. elegans* is useful in forward genetic screens attempting to identify novel suppressors, short generation time allow for quick crosses, a small number of linkage groups allows fast identification, and the highly annotated genome allows for the identification of protein altering mutations once linkage is established. These reasons in addition to their high

fecundity, make *C. elegans* an ideal organism for studying the genetic regulation of oocyte meiosis.

1.5   <u>MEMI</u>

The *memi* gene family was identified by the Srayko lab as potential regulators of the meiosis-to-mitosis transition in *C elegans* (Ataeian et al. 2016). Three homologous genes, *memi-1*, *memi-2*, and *memi-3*, exhibit 87% identical nucleotides between copies, and they encode highly similar proteins as there is further 92% conservation of amino acids. They are collectively referred to as MEMI proteins. To date, homologues have not been detected outside of the *Caenorhabditis* genus, and are likely a product of the fast evolution of the *Caenorhabditis* genome where genes regularly are duplicated and then repurposed for alternate functions, then evolve to become unrecognizable when compared to their original predecessor (Long 2001). The MEMI paralogs function redundantly, as strains containing deletions of any one or two *memi* gene are still viable (Ataeian et al. 2016). While the presence of a single copy of any *memi* gene is sufficient for survival of embryos, worms containing only *memi-3* have decreased embryonic viability (Ataeian et al. 2016). However, when all copies of *memi* are deleted, hermaphrodites do not produce viable embryos (Ataeian et al. 2016). Similarly, the *memi-1/2/3(RNAi)* treatment, which knocks down transcription of MEMI, results in oocytes that fail to complete MI (Figure 3: b, pg17). In these oocytes the polar body is not extruded and meiosis II (MII) is skipped. They also undergo several rounds of mitotic divisions with an improper complement of chromosomes (Ataeian et al. 2016). This is interesting, because the phenotype is similar to that exhibited by unfertilized oocytes in *fer-1* mutants (Figure 3: c, pg17). *fer-1* hermaphrodites have defective sperm that cannot fertilize but still prompt MI by releasing oocytes from the primary meiotic hold (McNally and McNally 2005). The *fer-1* oocytes do not extrude the first polar body; instead they abort MI in anaphase and begin to divide mitotically (McNally and McNally 2005). There is evidence that *memi* transcription is limited to late oogenesis, as the previous studies have shown that *memi-3* mRNA is bound to germline development 1 (GLD-1), which is expressed distally in the ovary (Johnston and Dennis 2012). GLD-1 is also important in repressing the translation of other important proteins that regulate the oocyte-to-embryo transition, such as *chs-1*, *egg-1/2*, and *egg-4/5* (Johnston and Dennis 2012). *memi-1* was originally identified by a dominant mutation: *sb41*, that was isolated in a genetic screen for temperature-sensitive

maternal-effect lethal (Mel) alleles. It was originally referred to as *mel-43* (Mitenko et al. 1997). When grown at 25 °C, *memi-1(sb41)* homozygous hermaphrodites produce dead embryos, with less than 0.1% survivors. The strain can be maintained at 15 °C (Mitenko et al. 1997). Embryos from *memi-1(sb41)* mothers do not exit MII and fail to extrude the second polar body. After a delay (~10 minutes), the embryos proceed to mitosis, with both meiotic and mitotic spindle structures co-existing in the cell (Figure 3: d, pg17) (Ataeian et al. 2016). The *sb41* mutation acts as a hypermorph as the Mel phenotype is more severe in homozygotes than heterozygotes (Ataeian et al. 2016).
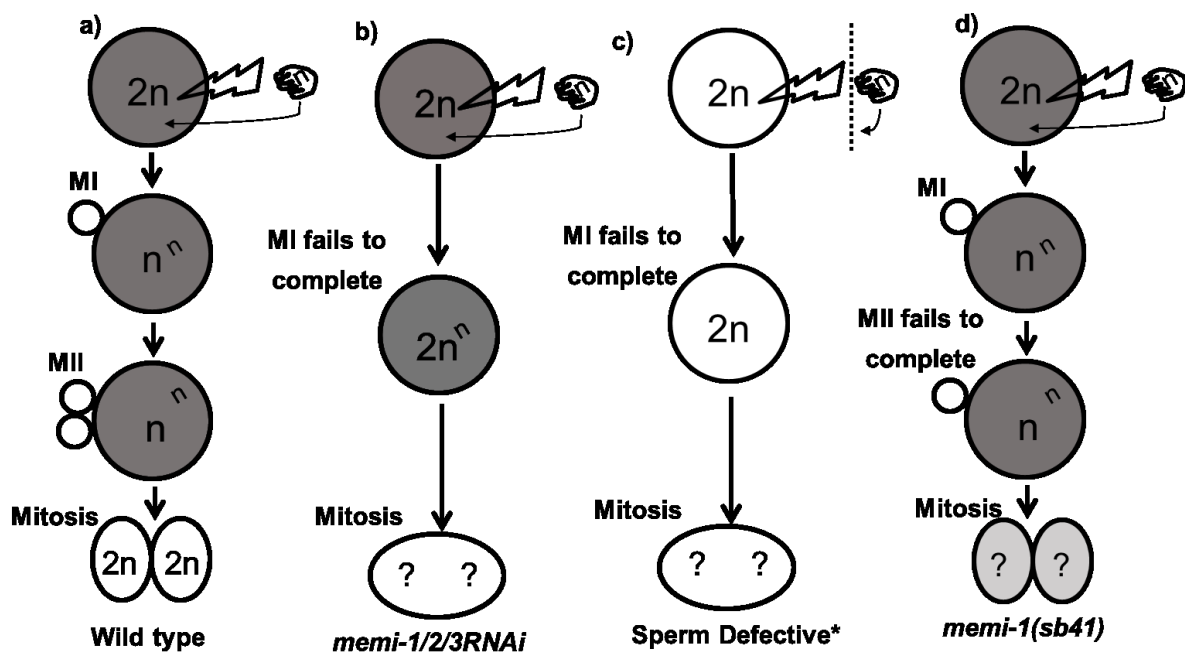
Figure 3: MEMI degradation in different genetic backgrounds with MEMI levels in shading.
Note: * indicates unknown progression of MEMI degradation. a) In normal progression of meiosis, MEMI is partially degraded after MI and completely degraded by mitosis. b) Oocytes with MEMI depleted through *memi-1/2/3(RNAi)* fertilized oocytes fail to complete MI and begin an abortive mitosis. c) Fertilization of defective sperm that cannot fertilize the oocytes fail to progress through MI. Note that the MEMI state of MEMI degradation is unknown, but female pronuclei form and attempt mitosis. d) Worms with the *memi-1(sb41)* mutation that causes MEMI-1 to not be degraded over the course of meiosis. This leads to a failed MII, and the beginning of an unsuccessful mitotic, as it leads to severe aneuploidy.

Furthermore, heterozygotes that have a duplication of wild-type *memi-1* (*i.e., sb41/+/+*) exhibit a more severe phenotype than heterozygotes without the duplication (*sb41/+)*, which in Muellers morphs classification would make it a hypermorph (Ataeian et al. 2016). The *sb41* mutation is a single nucleotide polymorphism, C220T, which causes a P74S amino acid substitution at a putative proline-directed phosphorylation site. The location of the mutation is in a conserved

region that is shared between *memi-1* and *memi-2/*3. The mutation prevents MEMI-1 from being degraded properly at the end of meiosis (Ataeian et al. 2016). Via western blot analysis, MEMI-1 is normally not detected in mitotic WT embryo extracts. However, MEMI-1 is detected in mitotic embryo extracts from *memi-1(sb41)* worms, indicating that the mutation interferes with the degradation of the protein (Ataeian et al. 2016). Proteolytic degradation of MEMI is also dependent on *zyg-11*, a targeting subunit for CUL-2-E3 ubiquitin ligase, suggesting that MEMIs are potential substrates for ubiquitin-mediated proteolysis at the end of meiosis II (Ataeian et al. 2016). Immunostaining against MEMI in meiotic oocytes shows that MEMI is degraded over the course of meiosis, partially in MI and completely in MII (Ataeian et al. 2016). Thus, as with many other proteins that are essential for female meiosis, the MEMI proteins must be removed prior to mitosis.

1.5.1 MEMI as a candidate for "sensing" fertilization

The evidence to date suggests that MEMI could function as a sensor for fertilization. First, oocytes that are activated by MSP, but not fertilized, abort MI anaphase and proceed directly into mitosis. *memi-1/2/3(RNAi)* embryos can be fertilized. However, they exhibit phenotypes that are strikingly similar to the unfertilized, activated oocytes (Figure 3: b, pg17)*.* Furthermore, while loss of MEMI results in a skipped meiosis II phenotype, hyperactive MEMI results in a failure to properly transition from meiosis II to mitosis (Figure 3: d, pg17) (Ataeian et al. 2016). To identify candidate genes that interact with MEMI in regulating the progression through MII, an RNAi screen was performed. This screen recovered two genes, *gsp-3 and gsp-4*, which have 95% DNA similarity and 98% protein similarity. The two genes encode phosphatases of the protein phosphatase 1 (PP1) family. In addition, they were shown to be important for sperm meiosis, sperm activation, and motility in both flagellate and amoeboid sperm (Wu et al. 2012). Because *gsp-3* and *gsp-4* are so similar, they are believed to act redundantly (Wu et al. 2012), and cannot be knocked down individually through RNAi (Ataeian et al. 2016). In *C. elegans* GSP-3/4 is restricted to the male germline, where it localizes to the chromosomes during spermatogenesis (Chu et al. 2006). GSP-3/4 is also important within activated spermatozoa. It plays a role in localizing MSP to the tips of the pseudopods for motility of the amoeboid sperm (Chu et al. 2006). Consistent with GSP-3/4 having a sperm-specific function, suppression of *memi-1(sb41)* by *gsp-3/4(RNAi)* was also observed at 25 °C in *memi-1(sb41)* hermaphrodites that were mated with males treated with *gsp-3/4(RNAi)* (Ataeian et al. 2016). One of the

limitations of genome-wide RNAi, is that false negatives are difficult to eliminate due to variability in knock-down efficacy for different genes. Furthermore, *C. elegans* sperm are notoriously poor targets of dsRNA methods (Fraser et al. 2000). Hence, an alternate strategy using ethyl methane sulfonate (EMS) was employed to identify more components of the MEMI pathway.

1.5.2 Identifying suppressor mutations of *memi-1(sb41)*

To find other proteins that are important for oocyte meiosis, an EMS mutagenesis screen for more suppressors of *memi-1(sb41)* was conducted. This screen yielded 27 individual suppressors strains of *memi-1(sb41)*. Upon initial screening, 10 of these strains contained a second mutation in the coding region of *memi-1,* and were determined to likely be intragenic suppressors (Lange et al. 2013). The focus of my work was to identify the responsible mutation in the remaining 17 suppressor strains. In my investigation I employed complementation testing, genetic mapping, and whole genome sequencing. From these 17 suppressors, I have found the suppressing mutation in 10 of the strains, and have limited the number of candidate mutations in the remaining 7 strains to notable potential candidates. Each stage of the investigation was used stochastically to further eliminate more candidate mutations from consideration, this had the benefit of finding suppressing mutations while narrowing the number of candidates without the completion of complementation testing or mapping for strains where the suppressing mutation was found. Because some of these investigations occurred concurrently there is a degree of overlap between some complementation and mapping which provided further insights into the characterization of the involved suppressor strains.

For the purpose of this thesis the suppressor mutations investigated were limited to those that altered amino-acid sequences of proteins, because the promoters of most *C. elegans* genes and other regulatory sequences are not fully understood. Of the known suppressors I found, one is caused by an intragenic secondary mutation in *memi-1(sb41)*, that was not checked in the initial screen. The other 16 suppressing mutations are extragenic, of which 9 have been found. Five suppressors contained mutations in *gsp-4,* which encodes one of the two sperm-specific PP1 phosphatases identified in the RNAi-suppressor screen (Ataeian et al. 2016). The suppressing mutations occur in conserved domains of *gsp-4*, and affect amino acids that have been shown to be necessary for PP1 catalytic function in other organisms (Peti, Nairn, and Page 2013). While it

is possible that the lack of mutations found in *gsp-3* is due to random chance, because all of these domains are conserved between the two genes it suggests that there are some differences in expression or regulation that may be determine why the same mutations in *gsp-3* do not appear in the suppressor screen. The four other suppressor strains contained a mutation in *R03D7.5,* which is one of 7 orthologues of glycogen-synthase kinase 3 (GSK-3) in *C. elegans*. While *R03D7.5* is not directly linked to control of oocyte meiosis, a different member of the GSK family, GSK-3, has been shown to phosphorylate OMA-1 for its degradation prior to mitosis (Nishi and Lin 2005), among other important functions in meiosis. This suggests that *R03D7.5* may play a similar role in regulating the meiosis-to-mitosis transition (Nishi and Lin 2005) however, it is difficult to explain why its loss would suppress *memi-1(sb41)* as it prevents MEMI degradation. As one of the mutations in *R03D7.5* is likely a null, introducing a premature termination codon early in the gene, it is likely that knocking out the gene rescues the *memi-1(sb41)* phenotype. The remaining 7 suppressor strains that have not been attributed to a single mutation collectively contain 410 protein altering mutations in total. These have been limited to 84 possible mutations through linkage analysis. 3 of the suppressor strains have been mapped to a single chromosome with less than 7 gene altering mutations. Of these, one occurs in a gene known to play an important role in *C. elegans* in meiosis, another is implicated in meiosis in mammals, and the last is a sperm specific protein. In the remaining 4 suppressor strains, the remaining 66 possible suppressing mutations can be narrowed down through further linkage analysis and SNP mapping. This has the benefit of finding suppressing mutations for any of the remaining unknown suppressor strains if they do not alter amino-acid sequence of proteins, and will be found through future investigation.

1.5.3   Confirming the function of *memi-1(sb41)* and clarifying its classification as a hypermorph

A second question that was addressed in my research was the functionality of *memi-1(sb41)*. It was already established that *memi-1* alone (*i.e., memi-1(+) memi-2Δ, memi-3Δ*) is sufficient for nearly wild-type levels of embryonic viability (Ataeian et al. 2016). Although the *memi-1(sb41)* hypermorph causes phenotypes that are distinct from *memi-1/2/3(RNAi)*, it is not clear whether the MEMI-1(sb41) protein is functional during meiosis. The simplest model that fits the data is that MEMI-1(sb41) is not degraded at the end of meiosis, but is otherwise functional. However, if MEMI-1(sb41) also results in a loss-of-function during meiosis, this would be masked by

meiotic functions provided by MEMI-2 and MEMI-3. Distinguishing between these possibilities would allow a better understanding of the mechanism of suppression of *memi-1(sb41)*. If suppression modulated through *gsp-3/4* works by decreasing the overall activity of MEMI in *memi-1(sb41)*, then reducing *memi-2* and/or *memi-3* would also be expected to suppress *memi-1(sb41)*. However, *gsp-3/4(RNAi)* might specifically act on MEMI-1(sb41), and play a limited role (or no role) in the function of wild-type MEMI. In this case, reducing *memi-2* and/or *memi-3* would not be expected to suppress *memi-1(sb41)*. Using deletions of *memi-2* and *memi-3*, I was able to assay for the function of *memi-1(sb41)* in the absence of other *memi* activity, and test the ability of *memi-2* and *memi-3* deletions to suppress *memi-1(sb41)*. From this testing, I confirmed that embryonic viability of *memi-1(sb41)* worms was improved by reducing the number of WT copies of *memi*. However, some WT MEMI activity was required, as, even at the restrictive temperature of 15 °C, *memi-1(sb41)* worms that lacked any WT copies of *memi* had less than 1% embryonic viability, and could not be maintained. This showed that *memi-1(sb41)* seems to act as a neomorph, as the Mel phenotype is improved by deleting WT copies of *memi*, and that *memi-1(sb41)* is not sufficient as the sole copy of *memi* to fulfill the normal function of the gene. The low embryonic viability of worms containing only *memi-1(sb41)* and no WT copies of *memi-2/3* was alleviated when hermaphrodites were mated to males, suggesting that, although *memi-1(sb41)* is a strict maternal-effect lethal phenotype, loss of WT MEMI activity created a sensitivity to differences in male vs. hermaphrodite sperm. One possibility is that this could be related to the potency of male sperm. The oocytes of hermaphrodites mated with males are preferentially fertilized by the male sperm, which are larger and more motile (Ting et al. 2014). This indicates that the male sperm are not equivalent to the hermaphroditic sperm and so their contribution to the oocyte that could differ due differences in gene regulation and expression. While this data shows that *memi-1(sb41)* acts as a neomorph, the rescue of embryonic viability by male sperm further implicates the importance of sperm factors in contributing to oocyte meiosis.

2 Materials and Methods

2.1 Worm Strains

Strain	Suppressor	Mutations
CB1893	N/A	*unc-17(e113) dpy-13(e184)LGIV*
MAS135	N/A	*memi-1(sb41) memi-2(tm2638)Δ memi-3(tm3158)ΔLGIV*
MAS138	N/A	*memi-1(sb41) ruIs57[GFP::tubb-2]LGIV; his-58::mCherry*
MAS182	N/A	*memi-1(sb41) dpy-20(e1282)LGIV*
MAS199	*abc36*	*memi-1(sb41) dpy-20(e1282)LGIV; abc36*
MAS202	*abc39*	*gsp-4(abc39)LGI; memi-1(sb41)dpy-20(e1282)LGIV*
MAS203	*abc40*	*gsp-4(abc40)LGI; memi-1(sb41) dpy-20(e1282)LGIV*
MAS208	*abc41*	*R03D7.5(abc41)LGII; memi-1(sb41) dpy-20(e1282)LGIV*
MAS209	*abc42*	*memi-1(sb41) dpy-20(e1282)LGIV; abc42*
MAS211	*abc44*	*gsp-4(abc44)LGI; memi-1(sb41) dpy-20(e1282)LGIV*
MAS214	*abc47*	*gsp-4(abc47)LGI; memi-1(sb41) dpy-20(e1282)LGIV*
MAS215	*abc48*	*gsp-4(abc48)LGI; memi-1(sb41) dpy-20(e1282)LGIV*
MAS217	*abc50*	*memi-1(sb41) dpy-20(e1282)LGIV; abc50*
MAS218	*abc51*	*R03D7.5(abc51):GII; memi-1(sb41) dpy-20(e1282)LGIV*
MAS219	*abc52*	*memi-1(sb41) dpy-20(e1282)LGIV; abc52*
MAS220	*abc53*	*memi-1(sb41) dpy-20(e1282)LGIV; abc53*
MAS221	*abc54*	*memi-1(sb41) dpy-20(e1282)LGIV; abc54*
MAS222	*abc55*	*memi-1(sb41abc55) dpy-20(e1282)LGIV*
MAS223	*abc56*	*memi-1(sb41) dpy-20(e1282)LGIV; abc56*
MAS224	*abc57*	*R03D7.5(abc57)LGII; memi-1(sb41) dpy-20(e1282)LGIV*
MAS226	*abc59*	*R03D7.5(abc59)LGII; memi-1(sb41) dpy-20(e1282)LGIV*
MAS246	*abc47*	*gsp-4(abc47)LGI; memi-1(sb41)LGIV*
MAS247	*abc40*	*gsp-4(abc40)LGI; memi-1(sb41)LGIV*
MAS250	*abc48*	*gsp-4(abc48)LGI; memi-1(sb41)LGIV*
MAS251	*abc51*	*R03D7.5(abc51)LGII; memi-1(sb41)LGIV*
MAS260	*abc36*	*memi-1(sb41)LGIV; abc36*
MAS261	*abc44*	*gsp-4(abc44)LGI; memi-1(sb41)LGIV*
MAS262	*abc55*	*memi-1(sb41abc55)LGIV*
MAS266	*abc50*	*memi-1(sb41)LGIV; abc50*
MAS267	*abc59*	*R03D7.5(abc59)LGII; memi-1(sb41)LGIV*
MAS270	*abc41*	*R03D7.5(abc41)LGII; memi-1(sb41)LGIV*
MAS271	*abc42*	*memi-1(sb41)LGIV; abc42*
MAS272	*abc57*	*R03D7.5(abc57)LGII; memi-1(sb41)LGIV*
MAS276	*abc56*	*memi-1(sb41)LGIV; abc56*
MAS277	*abc52*	*memi-1(sb41)LGIV; abc52*
MAS281	*abc55*	*memi-1(sb41abc55)LGIV*
MAS283	*abc54*	*memi-1(sb41)LGIV; abc54*
MAS297	*N/A*	*unc-17(e113) memi-2(tm2638)Δ memi-3(tm3158)ΔLGIV*
MAS321	*N/A*	*memi-1(sb41)/+ +/unc-17(e113) memi-2(tm2638)Δ memi-3(tm3158)ΔLGIV*
MAS322	*N/A*	*memi-1(sb41) memi-2(tm2638)Δ LGIV*
MAS323	*N/A*	*memi-1(sb41) memi-2(tm2638)Δ memi-3(tm3158)ΔLGIV with males*
MAS324	*N/A*	*dpy5(e61)LGI; bli-2(e786)LGII; unc-32(e189)LGIII; memi-1(sb41)LGIV*
MAS325	*N/A*	*memi-1(sb41)LGIV; dpy-119(e224)LGV; lon-2(e678)LGX*
MAS326	*N/A*	*memi-1(sb41)LGIV; lon-2(e678)LGX*
MAS327	*abc39*	*gsp4-(abc39)LGII*
MAS91	*N/A*	*ruIs57[GFP::tubb-2]LGIV; his-58::mCherry*
N2	*N/A*	*C. elegans wildtype*

Table 1: List of Worm Strains

2.2   Bacteria Strains

Worms were fed one of two different strains of *E. coli*. OP50 is a slow-growing uracil auxotroph that was used for general strain maintenance on standard nutrient growth medium (NGM) plates. The *E. coli* strain DH5α is a fast-growing wild-type that was used to grow large populations of worms for DNA extraction and WGS analysis.

2.3   Strain Maintenance

Worms were cultured on 50 mm standard NGM agar plates seeded with OP50. Plates were checked every 3-4 days and worms were re-plated if OP50 was depleted to prevent the worms from starving. Each strain was kept in triplicate to prevent issues related to contamination and to ensure that all larval stages and adults were consistently present. Homozygous viable strains were maintained by picking hermaphrodites to a fresh plate or chunking. Strains that were heterozygous for one or more alleles were checked each time that they were re-plated to ensure that they carried the same phenotypic markers.

2.3.1   Re-plating of worms

Worms were transferred to a new plate using a worm pick, consisting of a platinum wire housed in an ergonomic stick. The wire was sterilized by an ethanol flame until white hot before and after each transfer. This minimized the chances of contamination by bacteria, or mold, and ensured that no worms or eggs were unintentionally transferred to the wrong plates.

2.3.2   Chunking of worm plates

To maintain strains with males or starved plates, re-plating was done through chunking. A flamed scalpel was used to cut out 1 cm^2 of agar from the old plate, and transfer it to a freshly-seeded plate. Because chunking transfers eggs intact, this ensured larger numbers of worms being kept on plates. This helped maintain slow-growing strains and male stocks.

2.3.3   Worm incubation

As the *memi-1(sb41)* mutation is temperature-sensitive, strains carrying the mutation were maintained at the permissive temperature of 15 °C. Strains that contained *memi-1(sb41)* and a suppressor mutation were kept at 25 °C. Experiments looking at embryonic viability of *memi-1(sb41)* were done at 25 °C, unless otherwise noted.

2.4   Generating Males for Crosses

Males were generated by exposing L4 hermaphrodites to 30 °C for 4 hours. The elevated temperature increases the chances of chromosomal non-disjunction in meiosis, giving rise to rare gametes with no X chromosome and, subsequently, XO zygotes that develop as males (Madl and Herman 1979). Heat-shocked L4s were transferred to a new plate and returned to their maintenance temperature. Males that hatched on these plates were transferred to mating plates with hermaphrodites to generate more males through mating. In strains that contained phenotypes that decreased male mating efficiency, such as *dpy-20* (Hodgkin 1983), Dpy hermaphrodites were mated to WT or heterozygous males.

2.5   Freezing Newly Generated Strains

To save newly generated strains the worms were frozen. Worms were washed off plates depleted of OP50 with M9 to collect L1 and L2s. Worms were poured into 5 separate 1.5 mL tubes to which equal volumes of freezing solution was added. 5 plates of worms were used to ensure a high enough number survived. The tubes were insulated in a Styrofoam box and placed in the -80 °C freezer to decrease the rate of cooling. After freezing, one tube was thawed to test that the worms survived, and the remaining tubes were split between a -80 °C fridge and liquid nitrogen. At this point, if worms were confirmed to have been frozen successfully the strain could cease to be maintained. Frozen strains were recorded in the lab database. To resurrect strains a tube would be unfrozen at room temperature, and poured onto fresh agar plates seeded with OP50.

2.6   Crossing Worms

Crosses were performed by plating 10-12 adult males and 5 L4 hermaphrodites on a mating plate, with a small lawn of OP50 in the center. Males were picked and transferred to a separate plate, before being transferred to the mating plate. This insured that no L1 and L2 worms or eggs were accidentally transferred to the mating plates. When possible, multiples of the same cross were set up at the same time to increase the chances of success. These plates were

incubated at 15 °C and checked for eggs every 24 hours. Hermaphrodites were transferred to new plates when eggs were observed, usually 72 hours.

2.7   Scoring embryonic viability of worms

2.7.1   Preparing hermaphrodites for scoring

Unless otherwise indicated, maternal-effect lethality (Mel) was scored by plating L4 hermaphrodites onto a plate and then transferring them to a second plate at least 24 hours before their offspring reached adulthood. When transferring hermaphrodites, they were not kept out of the incubators for longer than 30 minutes to minimize the oocytes fertilized outside of the correct temperature range so that the Mel phenotype of *memi-1(sb41)* was not heavily affected. When transferring large numbers of worms, plates were taken out of incubators in batches of 12. Lower temperatures slow the *C. elegans* lifecycle (Byerly, Cassada, and Russell 1976) so hermaphrodites kept at higher temperatures were re-plated faster. Hermaphrodites at 25 °C were kept on each plate for 24 hours before being re-plated. For testing where worms were incubated at 15 °C or 20 °C, they were kept on plates for up to 48 hours, but were transferred to the second plate if L1-L2 worms were observed at 24 hours, because their lifecycle was slowed (Byerly, Cassada, and Russell 1976). After the hermaphrodites were removed from each plate the eggs were incubated at 25 °C so that they would hatch quickly, and the Mel phenotype of *memi-1(sb41)* only affects meiotic oocytes (Ataeian et al. 2016). Worms were scored after 24 hours so that all fertilized eggs had a chance to hatch. For quality assurance, plates were checked for mold or foreign bacteria that could affect brood counts; contaminated plates were discarded.

2.7.2   Scoring plates

For scoring plates, the bottoms of the plates were divided into columns using a fine tipped marker. All worms and unhatched embryos were counted in each column using a tally counter. Worms or embryos that lay on a line dividing a column were counted the first time they were encountered. If the phenotype or sex of the worms was relevant to the test, they were kept for another day and then scored when the phenotype or sexual dimorphism was apparent. In this way, hatching rates were scored first, and the late-expressing phenotypes were scored later, when they became more clearly distinguishable.

2.8 <u>**Calculating Embryonic Viability**</u>

Embryonic viability was calculated as the percentage of hatching offspring out of all fertilized eggs laid. Worms were only used to calculate embryonic viability if they laid 20 or more eggs. This was to counteract the possibility of artificially creating outliers who had higher or lower embryonic viability because in smaller broods the chances of all or none of their offspring hatching is higher.

2.9 <u>**PCR Protocol**</u>

2.9.1 Worm lysis

DNA for PCR was collected by placing worms in 6 µL aliquots of 1 part 20 mg/mL proteinase-K to 30 parts worm lysis buffer. When preparing samples from multiple worms between 5 and 10 gravid hermaphrodites were selected when possible. Samples were heated for 1 hour at 95 °C and 15 minutes at 65 °C. These prepared samples were then used as the template DNA for the PCR amplification.

2.9.2 PCR samples

PCR reactions were performed using components provided in 5 PRIME PerfectTaq DNA polymerase (MAT#2900659). All reagents that were frozen were thawed on ice before being used. A master mix was made for all the same PCRs in an Eppendorf tube to ensure the reaction was the same for the experimental and control samples. While being made, the reagents and the master mix remained on ice. The master mix was made by adding 0.25 µL of 10 mM of each (GACT) dNTPs together to 2.5 µL of 10x PCR buffer from the 5 PRIME kit. 1 µL of forward and of reverse primer were added, before sterile deionized water was added. That would bring the final volume of the PCR to 25 µL, after the addition of Taq polymerase and template DNA. 0.3 µL of Taq polymerase in glycerol was added quickly and the whole mixture was vortexed to homogenize the solution. The master mix being complete was then aliquoted so that the final volume would be 25 µL after the addition of the template DNA. Depending on the number of PCRs required per sample, 2 -5 µL were used. If the PCR product was used as a template for sequencing, the initial PCR reaction volume used was 50 µL.

2.9.3   PCR reaction

PCR reactions started with heating at 95 °C for 2 minutes. The reaction then proceeded through several cycles: 30 for multiple worms, 35 for a single worm. Each cycle started with denaturation for 30 seconds at 95 °C, followed by 30 seconds at 58 °C for annealing, and finally a 1-minute extension at 72 °C. Once the cycles were completed the reaction was held at 4 °C and used immediately or was frozen overnight. A list of primers can be found in the appendices (Table 10, pg97).

2.10 Gels and PCR sequencing

PCR products were confirmed by agarose gel electrophoresis. Gels were poured with 2.5 µL of EtBr per 100 mL of agarose. Samples were separated in agarose at 85V for 45-60 minutes and then verified under an ultraviolet light. PCR length was confirmed by comparing to controls and GeneRuler 1kb plus. Standard gels for imaging used 0.7% agarose, but 0.5% agarose was used to improve yields when extracting sequencing templates. Correct-sized bands were cut from the gels to obtain DNA of amplified sequences and then purified using the Qiagen gel extraction kit Cat. No. 28706 using the kit procedure. The kit procedure was followed exactly except that the DNA was eluted in 35 µL of elution buffer to increase concentration. Concentration of the gel extraction was determined by a spectrophotometer (Nano-View). Sequencing samples (10 µL) were submitted to MBSU at 22.5 ng/µL with 0.25 µL of sequencing primer.

2.11 Assessing suppressors as dominant or recessive

Hermaphrodites that were either homozygous or heterozygous for their suppressors were assessed to determine whether they could be used for complementation tests. Each hermaphroditic suppressor strain was maintained by self-crossing over several generations and assumed to be isogenic and homozygous for the suppressor mutation, due to the propensity for self-fertilizing hermaphrodites to become homozygous over multiple generations (Anderson, Morran, and Phillips 2010), selective pressure as suppressors increase embryonic viability. Strains were first tested for their embryonic viability and then outcrossed to males from a *memi-1(sb41)* background. The MAS182 strain that was originally used for the mutagenesis was used as a control for *memi-1(sb41)* as well as N2 for WT.

2.11.1 Homozygous Suppressor Scoring

Homozygous suppressor strains from the original suppressor background were scored for their embryonic viability at 25 °C. All strains that had been generated by the original EMS mutagenesis for the forward genetic screen, but were not identified by initial screening within the *memi-1* gene (Caitlin Slomp, *unpublished data*), were included.

2.11.2 Heterozygous Suppressor Scoring

Homozygous suppressor strains were crossed to MAS138 *memi-1(sb41)* males to generate F1 worms heterozygous for the suppressor but homozygous for *memi-1(sb41)*. Because the original suppressor strains carried *dpy-20* as a phenotypic marker, outcrossed F1 hermaphrodites were identifiable as non-Dpy. F1 heterozygotes were scored as previously described.

2.12 Complementation Testing of Suppressor Alleles

Suppressors that were recessive were used for complementation tests, as well as semi-dominant suppressors that could be distinguished when heterozygous from homozygotes. All of the suppressors strains were either recessive or semi-dominant, so each suppressor strain was used. Failure of two suppressor mutations to complement was indicated when the *trans* heterozygote displayed an increase in embryonic viability relative to *memi-1(sb41)* without any suppressor. A failure of two heterozygous suppressors to suppress *memi-1(sb41)* in this context was interpreted to mean that the two suppressors belonged to the same complementation group, and were likely alleles of the same gene.

2.12.1 Generating males for complementation tests
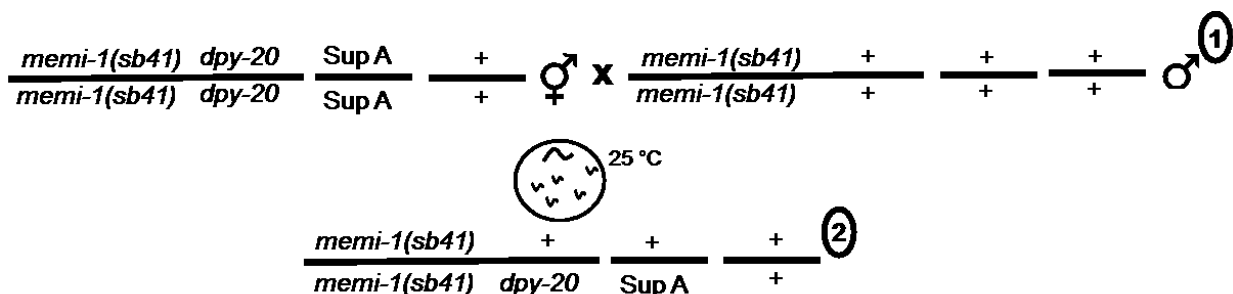
Figure 4: Cross Diagram heterozygous suppressors
This diagram shows the cross to get worms heterozygous for the hypothetical suppressor Sup A. The F1 progeny (2) were generated by mating the original suppressor worms with males in a *memi-1(sb41)* background (1). Incidentally, this also created worms that were heterozygous for *dpy-20*. F1 hermaphrodites were used to score heterozygous suppressor embryonic viability, while F1 males were used in complementation crosses.

Worms were crossed to determine complementation between different suppressor strains. The originally suppressors of *memi-1(sb41)* were generated in a *dpy-20* background, a convenient phenotypic marker close to *memi-1*. Dpy-20 males also mate poorly, which made it difficult to maintain male stocks. To circumvent these problems, males were instead generated by outcrossing hermaphrodites from suppressor strains to non-Dpy males in a *memi-1(sb41)* background (Figure 4, pg28). The resultant heterozygous suppressor males of these crosses were then crossed to other suppressor strains to determine complementation (Figure 4: 2, pg28). Similarly, heterozygous hermaphrodites were used to score heterozygous embryonic viability of suppressor alleles (Figure 4: 2, pg28). These heterozygotes were also used to generate non-Dpy suppressor strains to determine which linkage group (LG) the suppressor alleles belonged to. By incubating the heterozygous hermaphrodites (Figure 4: 2, pg28) at 15 °C for a generation and then shifting their offspring to 25 °C, non-Dpy worms that were homozygous for recessive suppressor alleles could be selected.

2.12.2 Complementation cross
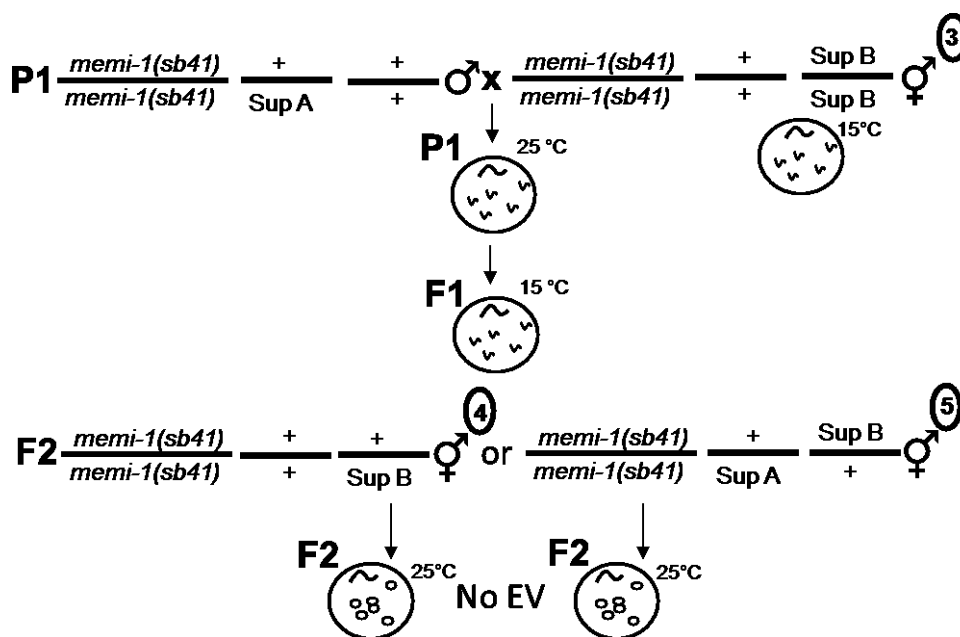
Figure 5: Hypothetical cross showing complementation
Outcome of the cross between males heterozygotes for Sup A (refer to (2) in Figure 4), and hermaphrodites for a second hypothetical suppressor Sup B (3). Because Sup A is heterozygous there are two equally likely progeny generated: the F1 worm inherits Sup A or does not (4 and 5). If the F1 did or did not inherit the Sup A suppressor there is no embryonic viability EV. It should be noted that because the heterozygous suppressor Sup A (2) is also heterozygous for dpy-20 and can be used as a phenotypic marker to confirm mating in the F2 worms phenotype.

All worms were mated for 72 hours at 15 °C. In cases where a recessive morphological marker was not available, mating was confirmed if the F1 generation displayed a 1:1 ratio of males to hermaphrodites. If either of these indicators were not fulfilled, then the F1 progeny were not used to assess complementation. As the males used in complementation tests, were generated as heterozygotes (Figure 4: 2, pg28), only half of the F1 worms would inherit the suppressor; complementation would be evident, only if all F1 worms were not suppressed for sb41 (Figure 5: 5, pg29, Figure 6: 6, pg30). In cases where the heterozygous male parent was generated using a suppressor in a *dpy-20* background, the re-emergence of the Dpy phenotype in the F2 generation was also used to confirm successful mating.
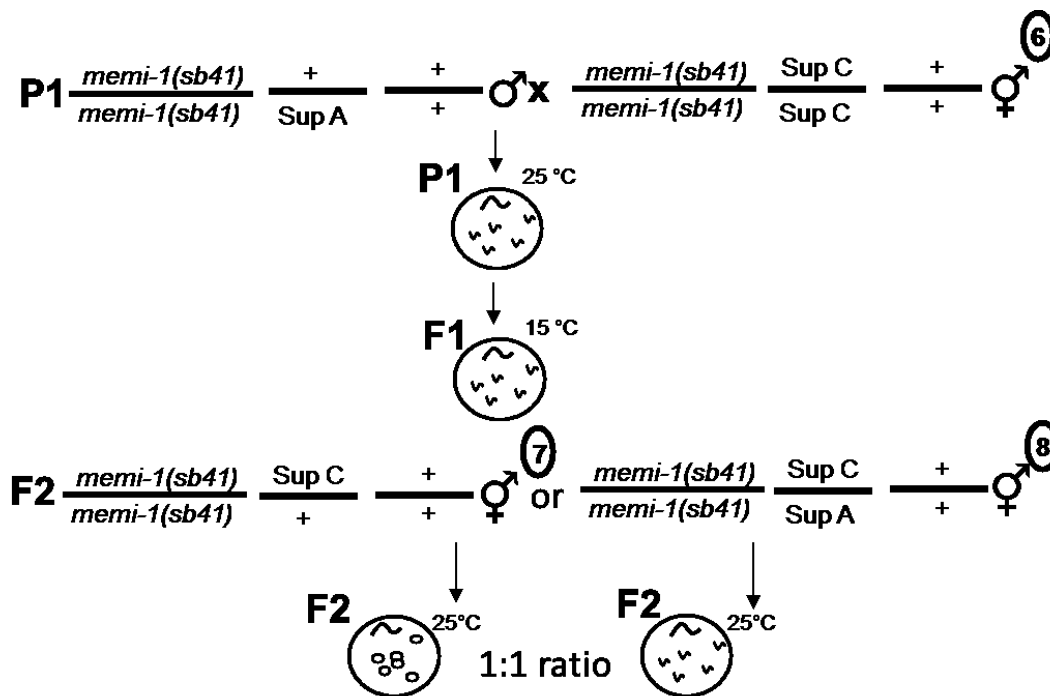
Figure 6: Hypothetical cross showing non-complementation
Cross shows the outcome of the cross between males heterozygotes for Sup A (refer to (2) in figure 4), and hermaphrodites for a third hypothetical suppressor Sup C (6). Because Sup A is heterozygous there are two equally likely classes of progeny that are generated where the F1 worm inherits Sup A or does not (7 and 8). As both Sup A and Sup C are found in the same gene if Sup A is inherited (8) The F1 will have some embryonic viability as *memi-1(sb41)* is suppressed. However, if the Sup A allele is not inherited there will be no embryonic viability (7). It should be noted that in because the heterozygous suppressor Sup A (refer to (2) in figure 4) is also heterozygous for *dpy-20* and can be used as a phenotypic marker to confirm mating in the F2 worms phenotype.

2.12.3 Complementation Assessment

Hermaphrodites were then divided into two categories, to assess whether the Mel phenotype of *memi-1(sb41)* was suppressed. Because males used in the mating cross were heterozygous for the suppressor allele, if the alleles failed to complement, there would be a 1:1 ratio of suppressed to unsuppressed offspring (Figure 6, pg30). Whereas in cases of complementation where the suppresosrs in the two parental suppressor strains did not act together to rescue the embryonic viability caused by the *memi-1(sb41)* Mel, the F1 would have no embryonic viability (Figure 5, pg29). For the purposes of finding instances of complementation worms with embryonic viability higher than 5% at 25 °C were considered suppressed. Consistent with the methods for assessing embryonic viability previously discussed, results were obtained from worms that laid 20 or more eggs. χ^2 tests were performed on a minimum of 8 hermaphrodites to determine if the ratio of suppressed hermaphrodites to unsuppressed supported non-complementation. If the χ^2 was not rejected using an alpha of 0.05, suggested that suppression of *memi-1(sb41)*. In these cases, the hatching rates for hermaphrodites above 5% embryonic viability was compared to that of both homozygous parental strains by two-tailed t-test to determine whether they were significantly different. After both these criteria were met the strains were found to complement as they did not rescue embryonic viability. The same comparison of F1 worms with embryonic viability >5% was done with worms with n<8 and compared to the embryonic viability of the homozygous parental strains to check for non-complementation.

2.12.4 Creating Complementation Groups

Complementation groups were established when any single pair of suppressors failed to complement. Other members were added if they failed to complement any single member of a group. Thus, complementation between individuals of a group was often inferred. In a few of these tests, there were instances where a complementation test contradicted the expected relationship, based on previous groupings. In these instances, each member of the complementation group that showed non-complementation with the others was given equal weight even if they contradict other tests in the complementation group. Complementation between members of different complementation groups can be inferred, when members of each group complemented each other. This made it unnecessary to test each individual combination of suppressor alleles once they were found to belong to an established complementation group.

2.13 Mapping Suppressor Alleles

Each suppressor mutation was mapped to determine its chromosomal location. Because each chromosome in *C. elegans* is a maximally 50 map units (Brenner 1974), genes on the same chromosome exhibit genetic linkage. Using a different phenotypic marker for each chromosome, suppressor strains were crossed to different mapping strains that contained *memi-1(sb41)*. Linkage was confirmed by running a χ^2 test, that under the hypothesis that the suppressor was unlinked to each of the phenotypic markers. If the suppressors were linked to the LG marker, then because the phenotypic marker and the suppressor start on different chromosomes suppression should be inherited at a lower rate than expected through independent assortment of chromosomes in worms homozygous for the phenotypic marker. As the suppressors are all recessive or semi-dominant homozygous worms for suppressors can be distinguished and linkage can be established.

2.13.1 Mapping

2.13.1.1 Phenotypic markers

Two mapping strains were used to cross phenotypic markers into the *memi-1(sb41)* suppressor worms to assess linkage. These were MAS324 *dpy5(e61)LGI; bli-2(e786)LGII; unc-32(e189)LGIII; memi-1(sb41)LGIV* and MAS325 *memi-1(sb41)LGIV; dpy-119(e224)LGV; lon-2(e678)LGX*. Mapping for linkage to chromosome IV was accomplished by assessing linkage to the marker *dpy-20(e1282),* which was present in the stock used to create suppressors through mutagenesis, and so was in the background of all suppressor strains. All the phenotypic markers used were recessive and could be reliably identified as homozygotes.

2.13.1.2 Mapping cross setup

Mapping crosses were performed by mating MAS138 males, to hermaphrodites of mapping strains to generate heterozygous males for each of the phenotypic markers, while keeping them in a *memi-1(sb41)* background. These heterozygous males, were then mated to hermaphrodites of suppressor strains, that did not contain any phenotypic markers. The cross was set up this way so that the same stock of males could be crossed to any of the suppressor strain hermaphrodites

as needed, instead of attempting to generate males in each of the suppressor strains. The hermaphrodites were re-plated after mating and allowed to lay their eggs at 25 °C. After 48 hours, the hermaphrodite was removed, and the phenotype of the offspring observed. If there was a 1:1 ratio of males to hermaphrodites, confirmed through a χ^2 test, the hermaphrodite was considered to have been outcrossed successfully and F1 hermaphrodites were re-plated in groups of 10, and incubated at 15 °C. After 48 hours of incubation at 15 °C the plates were shifted up to 25 °C and the L4 and adult worms were scored for phenotypes consistent with their mapping marker. Worms homozygous for each marker were removed and plated individually to score embryonic viability. A few idiosyncrasies in working with a two of the mapping marker phenotypes should be noted. The Bli phenotype of the *bli-2(e786) II* is apparent only in adults, and blistering is masked by other phenotypes, such as Dpy. When LGII was being assessed, many WT hermaphrodites were individually plated and only those that exhibited a Bli phenotype were considered for further assessment of suppression of *memi-1(sb41)*. For assessing linkage to LGIV because the *dpy-20(e1282)* allele was already in the suppressor background when they were originally generated, these suppressor strains were outcrossed with MAS138 so that the heterozygotes for the suppressor and the *dpy-20(e1282)* alleles could recombine and Dpy F2 hermaphrodites could be assessed for linkage.

2.13.1.3 Linkage assessment

The mapping crosses generated F1 worms, heterozygous for both the suppressor and the phenotypic markers used for linkage. Through self-fertilization the F2 generated offspring should inherit the suppressor and the phenotypic marker at a 3:1 ratio as both are recessive. If unlinked then for worms homozygous for the phenotypic marker there should be a 3:1 ratio of suppressed to unsuppressed worms, this could then be assessed through a χ^2 to determine if the difference is significant. F2 hermaphrodites expressing one of the phenotypic markers from the mapping cross were individually plated and scored for embryonic viability to check whether the Mel *memi-1(sb41)* phenotype was suppressed. The cut-off for suppression was >5% hatching with the standards for scoring as previously described. The number of suppressed and unsuppressed F2 hermaphrodites were compared to the expected values according to whether they were dominant or recessive and determined if the χ^2 value was larger than the critical value of for p=0.05, df=2 which is 3.841 for crosses of n=8 or more. If the χ^2 value was larger than the critical, then the suppressor was determined to be linked to the morphological marker. If the χ^2

value was lower than the critical value, then the allele was determined to be unlinked to the LG. For example, if 20 Dpy F2 hermaphrodites were scored in a *dpy-5LGI* background, if the genes suppressor was unlinked to LGI I would expect 5 suppressed hermaphrodites and 15 unsuppressed as the suppressor is inherited independently. If the suppressor was linked to LGI, then I would expect less than 5 hermaphrodites to be suppressed as a crossover would have to occur between *dpy-5LGI* and the suppressor on both copies of chromosome I that the F2 worm inherited from their dihybrid F1 parent.

2.14 Whole Genome Sequencing

Whole genome sequencing (WGS) was done to find all polymorphisms induced by the EMS mutagenesis that gave rise to each of the suppressors. DNA was purified for whole genome sequencing from strains containing the *memi-1(sb41)* suppressor alleles. Wherever possible, strains were used that had been outcrossed once to remove as many background mutations as possible, and background strains were also sequenced for comparison.

2.14.1 DNA Extraction and Purification

2.14.1.1 DNA extraction

DNA extraction was done following the protocol developed by Keith Reidy (2015, *personal comm.*). To prepare a large amount of DNA for sequencing, worms were cultured on three 10 cm plates seeded with DH5α. After the bacteria had been eaten away, worms were washed off plates with distilled water and concentrated by letting the worms sink to the bottom of an Eppendorf tube in 100 μL. Concentrated worms were added to 600 μL of worm lysis buffer and 20 μL of 20 mg/mL of proteinase K. The solution was incubated in a rotating hybridization oven overnight at 60 °C.

2.14.1.2 DNA purification

After the overnight incubation, 2.7 μL of 10 mg/mL RNase A and 400 μL of phenol chloroform were added. This was mixed for 5 minutes by inversion and centrifuged at 13200 rpm for 5 minutes. The supernatant was then added to a fresh Eppendorf tube with 400 μL of chloroform/isoamyl alcohol and mixed and centrifuged as before. The supernatant was then

transferred to a fresh Eppendorf tube containing 3M sodium acetate (pH 5.2). Two volumes of 95% ethanol were added to precipitate the DNA. Tubes were then kept at -20 °C for 1 hour or until DNA precipitated. Precipitated DNA was again centrifuged for 5 minutes and the excess ethanol poured off without disturbing the pellet. The pellet was washed with 1 mL of 70% ethanol, centrifuged and the ethanol removed. This process was repeated twice before the tube was inverted on a paper towel to dry for 10 minutes. The DNA pellet was then re-suspended in 50 μL of TE buffer (pH 8) and stored at 4 °C. DNA concentration and purity were assessed for a 5 μL sample using a spectrophotometer (NanoDrop, ThermoFisher).

2.14.2 Sequencing

DNA samples (150 ng/μL) were sent to Delta Genomics to create DNA libraries. The libraries were then sent to Genome Quebec for HiSeq analysis. The HiSeq data was interpreted by Paul Stothard and Xiaoping Liang (University of Alberta). Genomes were assembled for each of the strains using a program from Xiaoping Liang which matched individual reads to the *C. elegans* reference genome. These reads were then compared to the reference genome in order to identify where mutations had occurred, and noted single nucleotide polymorphisms and indels. These variants were catalogued for each of the suppressor strains as well as the two background strains sequenced. Finally, a program produced by Paul Stothard compared each of these variants to the reference genome in order to determine first whether the mutation was in a coding region of the genome, second whether the class of mutation resulted in a predicted change to protein: missense mutation, insertions, or deletions in coding regions, as well as changes to splice sites. This was done by comparing mutations to the known published genes in *C. elegans* that are publicly available on www.wormbase.org. Unique mutations acting as suppressors in potential genes were then found by comparing them to the reference genome and other mutations in the strains. MAS138 and MAS182 were also sequenced because they were used to outcross and generate the *memi-1(sb41)* suppressors, respectively. This allowed filtering of pre-existing mutations that were present in the strains prior to EMS mutagenesis, which differed between suppressor strains, as they each contained a different combination of MAS138 and MAS182 background mutations. All mutations specific to any single strain were assessed if they occurred in or near a known and

predicted locus of a gene. These mutations were sorted by type and whether the change was predicted to alter the protein product.

2.14.3 Assessment of Potential Suppressor Genes

2.14.3.1 Identifying suppressor mutations

Suppressors strains of *memi-1(sb41)* were subjected to screening criteria and tests to determine the putative suppressing mutations. First all strains were checked for mutations within genes known to be related to *memi-1* including, *memi-1*, *memi-2*, and *memi-3*. Strains that did not contain *memi* mutations were then assessed by the type of change that they would have on other proteins. For this analysis, only mutations expected to alter a protein sequence were considered, because the regulatory sequences for the majority of the genome is unknown. Any suppressing mutations that occurred outside of coding regions of genes or splice sites, can still be found by SNP mapping after the protein altering mutations have been considered. The WGS analysis was also able to distinguish homozygous and heterozygous alleles for each mutation. In cases where the suppressors were recessive or semi-dominant, so that homozygous suppressor mutations could be distinguished, only homozygous mutations were considered from the WGS. Mutations occur randomly in the genome with EMS mutagenesis so larger genes are more likely to be mutagenized than other areas (Anderson 1995). Homozygous mutations were tested for shared genes within the same complementation group, or mapping data for linkage to specific chromosomes where available. This would likely identify suppressor strains where the suppression was due to mutations in the same gene, while eliminating commonly mutated genes as they would not fail to complement consistently. Finally, if any members of complementation groups did not contain a mutation within the same gene, then the list of potential candidates were considered based on what is known in the published literature to determine if they were known to have known interactions in other systems.

2.14.3.2 Confirmation of locus of suppressor mutations

In cases where multiple alleles in a single gene were identified by WGS, the mutations were confirmed through PCR amplification of the gene and Sanger sequencing of the region (MBSU,

University of Alberta). Samples were prepared the PCR procedure previously described using a 50 μL preparation doubling the reaction. Once amplified, the PCR product was separated by agarose gel electrophoresis (0.5% agarose gel) for 45-60 minutes at 85 V. Bands of the correct PCR product size were cut out and DNA samples were purified (Qiagen gel extraction kit).

2.15 Characterization of discovered suppressor alleles

Once suppressor alleles had been found they were further characterized. Multiple suppressor strains were assessed for embryonic viability, while heterozygous in a *memi-1(sb41)/+* background. One suppressor allele: *abc39*, was crossed with WT N2 worms to remove the *memi-1(sb41)* background, so that the embryonic viability could be assessed. The genotypes were confirmed through screening of F2 worms for *memi-1* and the suppressing gene through PCR amplification and Sanger sequencing. Suppressing mutations that were found in genes represented in the literature, were compared to their homologues in *C. elegans* and their orthologues should they exist in other species, wherever possible.

2.16 Isolating memi-1(sb41) from memi-2 and memi-3

The creation of the *memi-1(sb41)*; *memi-2Δ*; *memi-3Δ* strain was done using two crosses. This was done using strains containing the *memi-2(tm2638)Δ memi-3(tm3158)Δ* alleles, which were originally acquired from the National Bioresource Project, Tokyo, Japan. The first strain created contained a phenotypic marker on chromosome IV in a deletion strain containing *memi-2Δ* and *memi-3Δ* (Figure 7, (3) pg38). This was done by crossing a worm carrying two phenotypic markers *unc-17(e113)* and *dpy-13(e184)* to the deletion strain containing *memi-2Δ* and *memi-3Δ* (Figure 7, (2) pg38). A crossover between *unc-17* and *dpy*-13 was identified as F2 progeny that were non-Dpy Unc, (Figure 7: (1-2) dashed-line, pg38). Because *dpy-13* is between *unc-17* and *memi-3Δ* on the same chromosome, the crossover would result in a chromosome containing *unc-17(e113)*, *memi-3Δ*, and *memi-2Δ* (Figure 7, (3) pg38). The progeny of these non-Dpy Unc worms that shared their parental phenotype were tested to identify individuals homozygous for *memi-2Δ* and *memi-3Δ* via PCR. The deletions were easily distinguishable as the PCR fragments they create are both several hundred base pairs shorter than WT, which can easily be resolved when run on a gel. The *unc-17 memi-3Δ memi-2Δ* (Figure 7, (3) pg38) worms were crossed with

a strain containing *memi-1(sb41)* (Figure 7, (4) pg38) to find a crossover that put *memi-1(sb41)* on the same chromosome as *memi-2Δ* and *memi-3Δ*. By screening F2 worms that were non-Unc, but homozygous for *memi-2Δ* and *memi-3Δ* could identify worms where a crossover had occurred between *unc-17* and *memi-3* (Figure 7: (3-4) dot-dashed line, pg38). These worms were identified as heterozygotes that were non-Unc but gave Unc progeny, while being homozygous for *memi-2Δ* and *memi-3Δ*. Based on the recombination distances, the F2 generation should have a recombination frequency of 8.09%. Because *memi-1* is 4.09 map units from *memi-3* approximately half of these crossovers should put *memi-1(sb41)* on a chromosome with the deletions. From candidate recombinants, *memi-1(sb41)* was confirmed by sequencing. Performing the cross this way has the additional benefit of having *memi-1(sb41)* in the deletion background with *unc-17* and WT *memi-1* on the other chromosome.
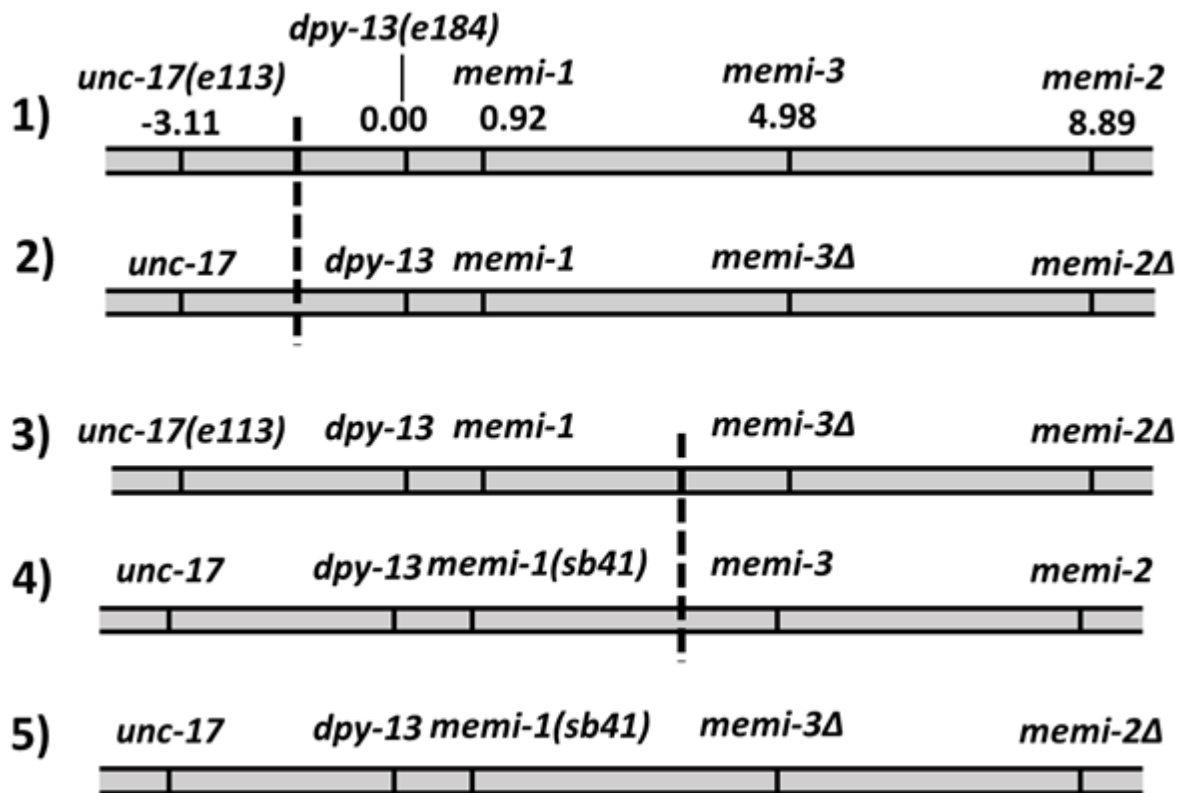
Figure 7: Schematic of chromosome IV and genes used to create *memi-1(sb41) memi-2Δ/3Δ* worms
Spacing of *memi-1/2/3* and phenotypic markers important in creating a *memi-1(sb41)*; *memi-2Δ*; *memi-3Δ* strain. *unc-17(e113)* and *dpy-13(e184)* are recessive phenotypic markers which were used to detect crossovers. The dashed lines are the regions where crossovers occurred for the creation of the intermediate chromosome (3) and the final chromosome (5). The intermediate chromosome (3) was created from the two parental strains (1-2), it was then crossed to *memi-1(sb41)* (4), to create the desired *memi-1(sb41)*; *memi-2Δ*; *memi-3Δ* strain.

2.16.1 Crosses Generating *memi-1(sb41)* Deletion Strains

Genotype				Worms containing the desired genotype
unc-17	memi-1	memi-3	memi-2	
e113	+	Δ	Δ	MAS297
e113	+	Δ	Δ	
+	sb41	+	+	MAS138
+	sb41	+	+	
+	sb41	+	Δ	F1 progeny of MAS322 x MAS138
+	sb41	+	+	
+	sb41	Δ	Δ	F1 progeny of MAS321 x MAS138 and confirmed through PCR amplification of memi-2/3 and no Unc F2
+	sb41	+	+	
+	sb41	+	Δ	MAS322
+	sb41	+	Δ	
+	sb41	Δ	Δ	F1 of MAS321 crossed with a heterozygous male generated through MAS322 x MAS138 confirmed by no Unc F2 and PCR of memi-2/3
+	sb41	+	Δ	
+	sb41	Δ	Δ	F1 of MAS321 self fertilized and confirmed through sequencing of memi-1(sb41)
+	sb41	Δ	Δ	
+	sb41	+	+	F1 progeny of MAS138 x N2
+	+	+	+	
+	sb41	Δ	Δ	F1 progeny of MAS321 x N2 with no Unc F2
+	+	+	+	
+	sb41	Δ	Δ	F1 of MAS321 crossed with heterozygous male generated through MAS322 x MAS138 confirmed by a 3:1 non-Unc:Unc F2 and PCR of memi-2/3
e113	+	+	Δ	
+	sb41	Δ	Δ	MAS321 self fertilized and confirmed by a 3:1 non-Unc:Unc progeny
e113	+	Δ	Δ	

Table 2: Genotypes of various *memi-1(sb41)* strains in *memi-2/3* deletion backgrounds used to score embryonic viability

The *memi-1(sb41)* deletion strains were crossed together to generate a variety of deletion backgrounds for *memi-1(sb41)* in both heterozygous and homozygous form (Table 2, pg39). This was done to determine the effect of *memi* gene dosage on *memi-1(sb41)*-dependent

maternal-effect lethality. The crosses were performed using the strains: N2, MAS138, MAS297, MAS321, and MAS322, all of which carry different combinations of *memi-2Δ/3Δ* and are either homozygous or heterozygous for *memi-1(sb41)*. The *C. elegans* WT reference strain N2 was used to provide WT alleles of *memi-1/2/3*. MAS138 *memi-1(sb41)* was used to replace *memi-2Δ/3Δ* with wild-type alleles, without altering homozygous *memi-1(sb41)*. MAS297 was used as the *memi-2Δ memi-3Δ* control. MAS321 had the genotype: *+ memi-1(sb41) memi-3Δ memi-2Δ / unc-17(e113) + memi-3Δ memi-2Δ* (Table 2, pg39). The inheritance of *memi-1(sb41)* was observed through the appearance of F2 Unc worms and confirmed through Sanger sequencing of a PCR product. MAS322 was used to place *memi-1(sb41)* on the same chromosome as the *memi-2Δ*. Crosses were setup using N2 and MAS138 males or males generated by outcrossing the strains with MAS138 (Table 2, pg39). All combinations of *memi-1(sb41)*, *memi-2Δ*, and *memi-3Δ* were individually plated and scored at 15, 20, and 25 °C.

2.16.2 Mated memi-1(sb41) memi-2Δ memi-3Δ Embryonic Viability

MAS321 (*+ memi-1(sb41) memi-3Δ memi-2Δ / unc-17(e113) + memi-3Δ memi-2Δ*) were difficult to maintain over multiple generations, as they had very low embryonic viability at 15 °C. Therefore, morphologically wild-type worms were plated individually and checked for dead eggs characteristic of *memi-1(sb41)* as well as Unc offspring. To ensure that the strain was being maintained in the heterozygous state, non-Unc single worms were selected from plates where not all the worms were Unc but there were Unc siblings, and dead eggs associated with *memi-1(sb41)*. Non-Unc worms were plated and scored, then the genotype for *memi-1* was determined through Sanger sequencing. If no crossovers occurred between *memi-1* and *unc-17,* of the non-Unc worms selected, 1/3 would be homozygous for *memi-1(sb41)*, *memi-2Δ*, and *memi-3Δ*.

2.16.2.1 Maintaining memi-1(sb41) memi-2Δ memi-3Δ worms

Maintenance of *+ memi-1(sb41) memi-3Δ memi-2Δ / unc-17(e113) + + +* worms required replating Non-Unc worms and checking for Unc progeny. Because the heterozygous *memi-1(sb41)* phenotype is less severe than the homozygous *memi-1(sb41)* worms (Mitenko et al. 1997), the *memi-1(sb41) memi-2Δ memi-3Δ* worms could be distinguished. However, these plates were very difficult to maintain even at 15 °C but it was noticed that plates containing worms of this genotype had higher embryonic viability when males were present.

2.16.2.2 Mating memi-1(sb41) memi-2Δ memi-3Δ to males

To test whether outcrossed sperm increased the embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ* hermaphrodites, the genotype was confirmed through Sanger sequencing of *memi-1* and checking *memi-2/3* for deletions via PCR. Plates of the worms were set up to freeze the strain with the male worms present, and the strain was designated MAS323. Hermaphrodites of this strain were tested for their embryonic viability while unmated, mated to males of the same genotype, and mated to *memi-1(sb41) memi-3(+) memi-2(+)* males. Embryonic viability was scored for the hermaphrodites as previously described for worms incubated at 15 °C.

3 Results

3.1 EMS Suppressors of *memi-1(sb41)* rescue maternal effect embryonic lethality
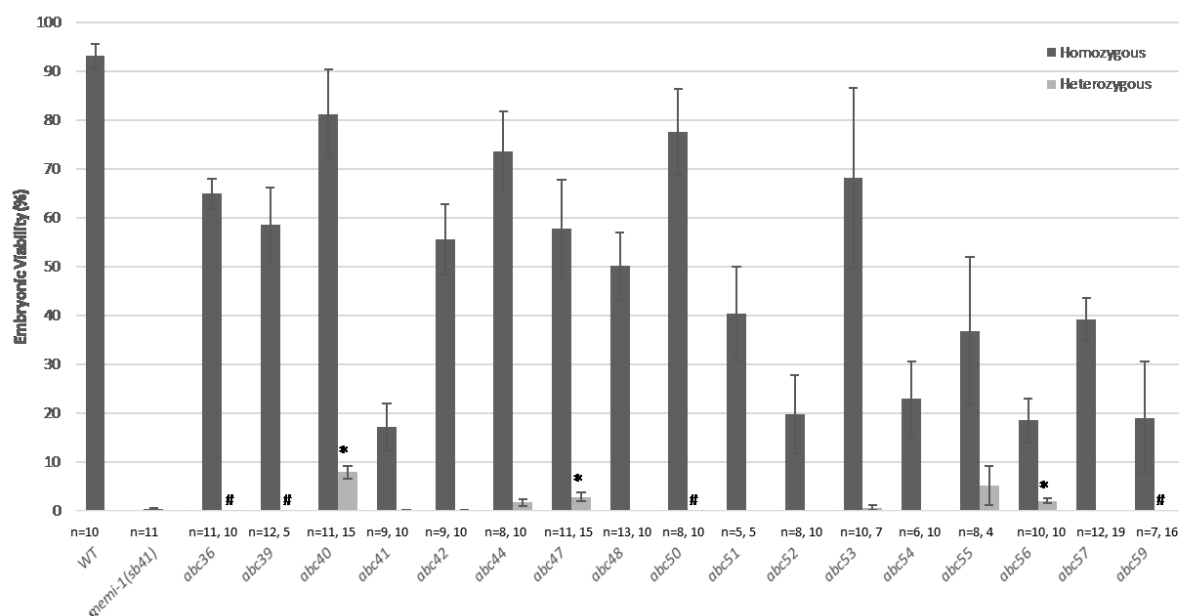
Figure 8: Embryonic viability of *memi-1(sb41)* suppressor strains at 25 °C
Average embryonic viability of homozygous and heterozygous *memi-1(sb41)* suppressors incubated at 25 °C. Error bars show standard error. * denote heterozygous embryonic viability significantly higher than the *memi-1(sb41)* control determined by two-tailed t-test (p<0.05). # indicate the embryonic viability is zero.

To test the magnitude of the *memi-1(sb41)* suppression the embryonic viability of unknown suppressors was measured. To determine whether the suppressors were dominant or recessive, worms heterozygous for each suppressor were also tested. All the suppressors tested had significantly higher embryonic viability as homozygotes than as heterozygotes (Figure 8, pg42). The embryonic viability of the homozygous suppressors was also highly variable, with each homozygous suppressor strain having a higher standard error than the WT control (Figure 8, pg42). Only three suppressors *abc40*, *abc47*, and *abc56* exhibited significant suppression of maternal-effect embryonic lethality when heterozygous (Figure 8: see *, pg42). The *abc55* suppressor strain appears to have higher heterozygous embryonic viability, however it is due to one individual having higher embryonic viability while the others had none, creating a large standard deviation, and was found not to be statistically different from *memi-1(sb41)*. The rescue of the *memi-1(sb41)* Mel phenotype by these heterozygotes was very low and inconsistent compared to their effect as homozygotes, indicating that these suppressors were semi-dominant (Figure 8, pg42). Because it was possible to distinguish between their homozygous and

heterozygous states, these suppressors were included with the recessive alleles for complementation testing.

3.2   Complementation tests

3.2.1.1   Complementation results summary

Between the 17 suppressor alleles, there were 136 possible pairings to be tested for complementation; 41 of these were directly tested (Figure 10, pg46). Because the males used in the complementation crosses were heterozygous for the suppressor allele, the worms scored for complementation would inherit the allele half the time. If the alleles failed to complement, this would be reflected by a 1:1 ratio of suppression of *memi-1(sb41)* in the F1 (Figure 4, pg28). Allele pairings that did not significantly violate this ratio and whose embryonic viability was not significantly lower than the parental alleles were found to fail to complement. The results of the complementation tests yielded 31 instances of complementation and 10 instances of non-complementation (Table 3, pg44). The complementation tests indicated a minimum of four different complementation groups and a maximum of seven (Figure 11, pg47). However, a further 61 complementation relationships can be inferred based on which complementation groups form (Table 3, pg44). Untested combinations of alleles were inferred, where possible, by eliminating pairings where one of the allele's complementation was known for members of a complementation group (Figure 10, pg46). When the instances of complementation and non-complementation are assessed together, 53 instances of complementation and 8 instances of non-complementation were inferred (Figure 10, pg46). The last 34 pairings remain unknown as they were not tested (Figure 10, pg46), or testing did not have a high enough n-number, a list of incomplete complementation tests with preliminary data can be found in the appendices (Table 11, pg98).

3.2.1.2   Semi-dominant suppressors in complementation testing

While the semi-dominant suppressors *abc40*, *abc47*, and *abc56* had significantly higher embryonic viability while heterozygous in complementation testing, they were never tested to one another (Table 3, pg44), as they were found to be members of the same complementation group and further testing was deemed redundant (Figure 11, pg47). Each of the suppressors though was shown both to complement other alleles and fail to complement others. In all cases

of failed complementation, the embryonic viability was not significantly different than one of the homozygous parents.

Complementation test				n	Mel		χ^2	Avg. of non-Mel	St. Dev. of non-Mel	Complements
Allele 1	Avg. EV	Allele 2	Avg. EV		No	Yes				
abc36	64.87	abc51	40.42	15	15	0	15.00	none		Yes
abc36	64.87	abc52	19.81	11	11	0	11.00	none		Yes
abc36	64.87	abc54	22.93	14	14	0	14.00	none		Yes
abc36	64.87	abc57	39.23	26	26	0	26.00	none		Yes
abc39	58.54	abc40	81.17	6	2	4	0.67	51.89	0.23	No: high EV
abc39	58.54	abc41	17.25	27	26	1	23.15	11.36	0.00	Yes
abc39	58.54	abc47	57.84	6	3	3	0.00	51.33	10.42	No: high EV
abc39	58.54	abc48	50.10	6	2	4	0.67	41.79	19.66	No: high EV
abc39	58.54	abc51	40.42	11	11	0	11.00	none		Yes
abc39	58.54	abc52	19.81	8	8	0	8.00	none		Yes
abc39	58.54	abc53	68.15	14	14	0	14.00	none		Yes
abc39	58.54	abc54	22.93	33	33	0	33.00	none		Yes
abc39	58.54	abc55	36.87	9	5	4	0.11	30.17	12.62	No
abc39	58.54	abc56	18.55	8	8	0	8.00	none		Yes
abc41	17.25	abc44	73.48	23	16	7	3.52	12.02	4.57	No
abc41	17.25	abc53	68.15	12	12	0	12.00	none		Yes
abc41	17.25	abc54	22.93	22	12	10	0.18	19.36	13.68	No
abc41	17.25	abc55	36.87	17	14	3	7.12	7.55	1.71	Yes
abc41	17.25	abc56	18.55	47	15	32	6.15	13.01	8.19	Yes
abc42	55.58	abc50	77.59	18	18	0	18.00	none		Yes
abc42	55.58	abc54	22.93	8	8	0	8.00	none		Yes
abc44	73.48	abc50	77.59	10	10	0	10.00	none		Yes
abc44	73.48	abc51	40.42	8	8	0	8.00	none		Yes
abc48	50.10	abc54	22.93	12	12	0	12.00	none		Yes
abc50	77.59	abc51	40.42	14	14	0	14.00	none		Yes
abc50	77.59	abc52	19.81	36	35	1	32.11	15.00		Yes
abc50	77.59	abc54	22.93	14	10	4	2.57	13.39	4.73	No
abc51	40.42	abc53	68.15	30	13	17	0.53	54.09	17.85	No
abc51	40.42	abc54	22.93	16	14	2	9.00	47.63	21.03	Yes
abc51	40.42	abc55	36.87	30	9	21	4.80	12.93	4.93	Yes
abc51	40.42	abc59	19.06	17	9	8	0.06	31.54	18.83	No
abc52	19.81	abc53	68.15	8	8	0	8.00	none		Yes
abc52	19.81	abc54	22.93	8	6	2	2.00	8.25	0.12	Yes
abc52	19.81	abc55	36.87	22	20	2	14.73	7.00	0.04	Yes
abc52	19.81	abc57	39.23	23	23	0	23.00	none		Yes
abc52	19.81	abc59	19.06	12	12	0	12.00	none		Yes
abc53	68.15	abc54	22.93	34	33	1	30.12	6.67	0.00	Yes
abc53	68.15	abc55	36.87	42	26	16	2.38	12.19	4.94	Yes: low EV
abc54	22.93	abc56	18.55	32	25	7	10.13	12.95	5.05	Yes
abc54	22.93	abc57	39.23	13	13	0	13.00	none		Yes
abc55	36.87	abc56	18.55	19	6	13	2.58	27.17	18.32	No

Table 3*: Complementation tests between suppressing alleles of *memi-1(sb41)*
EV stands for embryonic viability. The embryonic viability of tested hermaphrodites was calculated expecting a 1:1 ratio for strains that fail to complement at 25 °C as males used were heterozygous for the suppressor. Strains not deviating from the expected ratio as tested by χ^2 test (χ^2>critical value p=0.05 df=2) were confirmed to fail to complement if their embryonic viability was not significantly lower than that of their homozygous parental suppressor strains. Dark grey shows strains that complemented (*i.e.,* different genes) and white show strains that failed to complement (*i.e.,* alleles of the same gene).
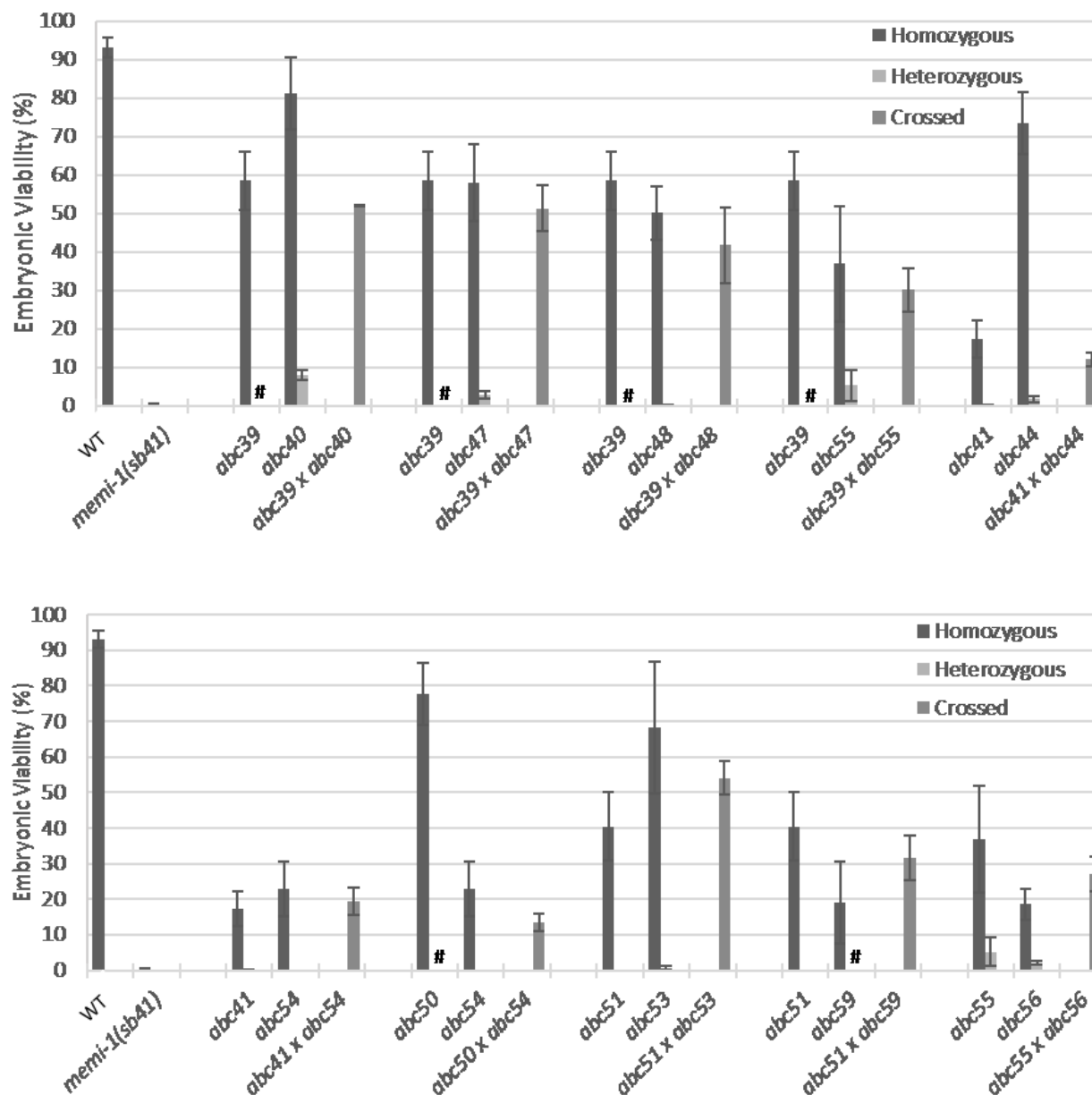
Figure 9: Embryonic viability of failed complementation tests at 25 °C

Displayed are all embryonic viability averages of worms that were heterozygous for two suppressor alleles that were not significantly lower than the lower of their parental embryonic viability determined using a two-tailed t-tests (p=0.05). Error bars show standard error. # indicate the value is zero.

3.2.1.3   Complementation groups

The four complementation groups encompass 14 of the 17 suppressor alleles tested (Figure 11, pg47). Complementation group 1 which contains the most suppressors, clustered around *abc39*: which was directly shown to fail to complement *abc40*, *abc44*, *abc48*, and *abc55*. The final member of the group *abc56* was shown to fail to complement *abc55* (Figure 11, pg47). There

was only one other test between the members of complementation group 1 between *abc39* and *abc56* and have the alleles complement. This contradicts the other tests which indicate that the strains should fail to complement (Figure 11, pg47). Complementation group 2 is made up of four suppressors all of which have been directly tested to two other members of the complementation group (Figure 11, pg47). *abc41* was shown to fail to complement both *abc44* and *abc54*. *abc50* was tested with *abc54* which failed to complement, however it was also tested *abc44* which it complemented. This test is the only other contradictory complementation test. Complementation group 3 is centered on *abc51* which fails to complement with *abc53* and *abc59* (Figure 11, pg47). The final complementation group is the lone allele *abc52* which has been tested directly to all other known complementation groups and complements with members of each one (Figure 11, pg47).

Alleles	abc57	abc42	abc36	abc52	abc59	abc53	abc51	abc54	abc50	abc44	abc41	abc56	abc55	abc48	abc47	abc40	abc39
abc39				Yes		Yes	Yes	Yes			Yes	Yes[A]	No	No	No	No	abc39
abc40												[C]				abc40	
abc47												[C]			abc47		
abc48								Yes				[C]		abc48			
abc55				Yes		Yes	Yes				Yes	No[A]	abc55				
abc56				[C]		[C]	[C]	Yes			Yes	abc56					
abc41						Yes		No[B]		No[B]	abc41						
abc44							Yes		Yes[B]	abc44							
abc50		Yes		Yes			Yes	No[B]	abc50								
abc54	Yes	Yes	Yes	Yes		Yes	Yes	abc54									
abc51			Yes		No	No	abc51										
abc53				Yes		abc53											
abc59				Yes	abc59												
abc52	Yes		Yes	abc52													
abc36	Yes		abc36														
abc42		abc42															
abc57	abc57																

Complementation Group 1 (abc39, abc40, abc47, abc48, abc55, abc56)
Complementation Group 2 (abc41, abc44, abc50, abc54)
Complementation Group 3 (abc51, abc53, abc59)
Complementation Group 4 (abc52)
Unknown Complementation (abc36, abc42, abc57)

Legend:
- Complement (dark grey)
- Untested (light grey)
- Fail to Complement (white)

Total:	
Yes	31
	53
No	10
	9
	33
Total:	136

Complementation Groups:
1) abc39 abc40 abc47 abc48 abc55 abc56
2) abc41 abc44 abc50 abc54
3) abc51 abc53 abc59
4) abc52

Figure 10: A summary of complementation results involving suppressors of *memi-1(sb41)*
Each box corresponds to a heteroallelic combination of suppressors, each of which is shown along the top or side of the matrix. The results of the complementation are indicated yes and no for combinations that were tested, and by light grey (unknown), dark grey (complementation), and white (non-complementation), found through complementation groups. [A] and [B] denotes contradictory complementation tests. [C] notes untested combinations of alleles where inferences on complementation would be contradictory because [A].

The four complementation groups include 14 of the 17 suppressor strains (Figure 10, pg46). Complementation groups 1 and 2 contain some inconsistencies (Figure 10: C. pg46), which may be explained by non-allelic non-complementation which will be discussed later. This was also considered when complementation data was used to determine likely suppressing mutations that were shared between members of the same complementation groups. The remaining suppressors that cannot be ruled out from being members of each of the previously established complementation groups were partially tested to the other groups and each other (Table 4, pg47).
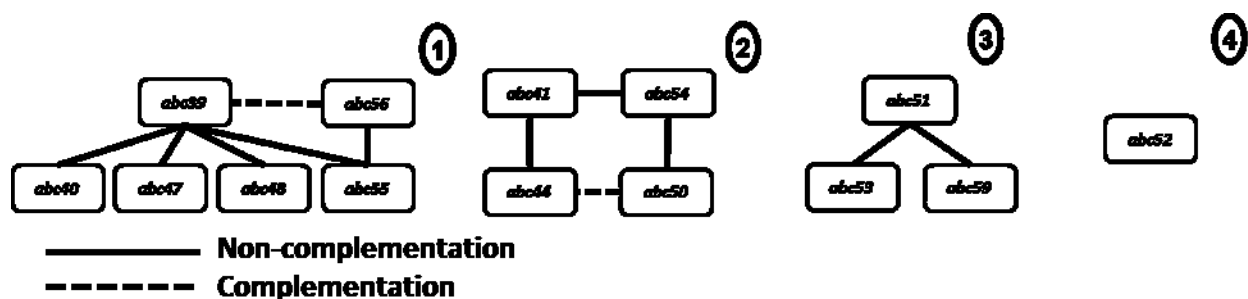
Figure 11: Complementation Groups
Suppressor alleles were placed into four complementation groups, where each allele failed to complement at least one other allele in the group. Lines represent a complementation test between two alleles: solid lines indicate failure to complement, dashed lines indicate complementation. The remaining suppressors that are not shown here: *abc36*, *abc42*, and *abc57*, have not been shown to complement all other existing complementation groups, but have not been shown to fail to complement with these groups either.

Single Alleles	Complementation Group			
	1	2	3	4
abc36		Tested	Tested	Tested
abc42		Tested		
abc57			Tested	Tested

Table 4: Complementation testing of individual suppressors to each complementation group
Tested notation indicates that the suppressor complements with at least one member of the complementation group. Because each of them has not been tested to the existing complementation groups, it is unknown whether they are representatives of additional independent complementation groups.

3.2.1.4 Unfinished complementation testing

The alleles outside of complementation groups *abc36*, *abc42*, and *abc57* were all partially tested to members of each complementation group (Table 3, pg44). Between these single alleles, though, only *abc36* and *abc57* have been directly shown to complement one another (Table 1, pg22). The *abc36* allele was also shown to complement members of complementation group 2, 3, and 4 (Table 4, pg47). *abc42* was shown to complement members of complementation group

2 (Table 4, pg47). *abc57* was shown to complement members of complementation group 3 and 4 (Table 4, pg47). Further testing was discontinued once whole genome sequencing data became available.

3.3   Mapping

LG	I				II				III			
	Non-Mel	Mel	χ2	n	Non-Mel	Mel	χ2	n	Non-Mel	Mel	χ2	n
abc36												
abc39												
abc41	5	11	0.33	16					8	3	13.36*	11
abc42	12	8	13.06*	20	1	2	0.11	3	18	10	23.05*	28
abc50	8	15	1.17	23	9	6	9.8*	15	0	22	7.33	22
abc51	3	5	0.67	8					3	8	0.03	11
abc52									1	4	0.07	5
abc53**												
abc54	2	15	1.59	17	0	1	0.33	1	2	15	1.59	17
abc55												
abc56	2	5	0.05	7	1	0	3.00	1	1	1	0.67	2

LG	IV				V				X			
	Non-Mel	Mel	χ2	n	Non-Mel	Mel	χ2	n	Non-Mel	Mel	χ2	n
abc36					2	4	0.22	6	0	13	4.33	13
abc39	0	2	0.67	2								
abc41	0	1	0.33	1	6	14	0.27	20	3	9	0.00	12
abc42					1	16	3.31	17	4	17	0.40	21
abc50	15	37	0.41	52	15	55	0.48	70	16	40	0.38	56
abc51					12	21	2.27	33	13	28	0.98	41
abc52	4	10	0.10	14	6	26	0.67	32	3	5	0.07	8
abc53**	7	5	7.33	12								
abc54	14	33	0.57	47	11	41	0.41	52	18	42	0.80	60
abc55	44	4	113.78	48	4	19	0.71	23	15	16	9.04*	31
abc56	27	4	63.75	31					20	53	0.22	73

Table 5: Linkage group analysis for *memi-1(sb41)* Suppressor Strains

χ^2 ratio calculated based on the expected 3:1 ratio of Non-Mel:Mel, which is reversed for LGIV because the suppressor and phenotypic marker were present in the strain background. * denotes instances where the χ^2 values was larger than the critical value but were determined to be unlinked because there were more worms Non-Mel worms than the expected ratio would allow. If there was no linkage where if the suppressor was linked it would result in fewer and not less and so these are also considered unlinked. All the alleles noted are recessive suppressors. Dark grey boxes note tests with no linkage, light grey boxes are undetermined, and white shows instances where suppressors are linked to the LG.

Mapping crosses were performed so that linkage analysis could be used to find suppressing mutations, easily in the WGS data. Because the mapping tests began while the complementation analysis was ongoing, some duplication in testing occurred. Of the 17 suppressor alleles, 10 were mapped successfully to show a linkage relationship for at least one linkage group (LG) (Table 5, pg48). The results of the 32 mapping tests showed 4 instances where alleles were linked to a LG and 28 instances where the alleles were not linked to the LG. The linkage groups for 5 suppressor alleles were found; *abc36*, *abc50*, *abc53*, *abc55* and *abc56* (Table 6, pg49). *abc50* was shown to be linked to LG III (Table 5, pg48). *abc53*, *abc55*, and *abc56* were all shown to be linked to LGIV (Table 5, pg48). *abc54* was shown to be unlinked with all other linkage groups, except LGII (Table 5, pg48). The alleles, *abc41*, *abc42*, and *abc51* were shown to be unlinked with all the linkage groups except LGII and LGIV. Finally, *abc52* was shown to not be linked to LGIV, LGV, and LGX (Table 6, pg49).

LG	One Possible LG	Multiple Possible LG
I		*abc52*
II	*abc54*	*abc41, abc42, abc51, abc52*
III	*abc50*	*abc52*
IV	*abc53, abc55, abc56*	*abc41, abc42, abc51, abc52*
V		
X	*abc36*	

Table 6: Linkage group possibilities from linkage analysis for mapped alleles
Table shows a summary of the linkage analysis from the mapping tests. If the allele is linked to one possible LG either because it was tested directly, or all other LG are unlinked for the allele it appears once in the first column. If the number of possible LG is reduced but not limited to one chromosome all possible LG are noted in the second column.

3.3.1.1 Mapping data in consideration of complementation testing
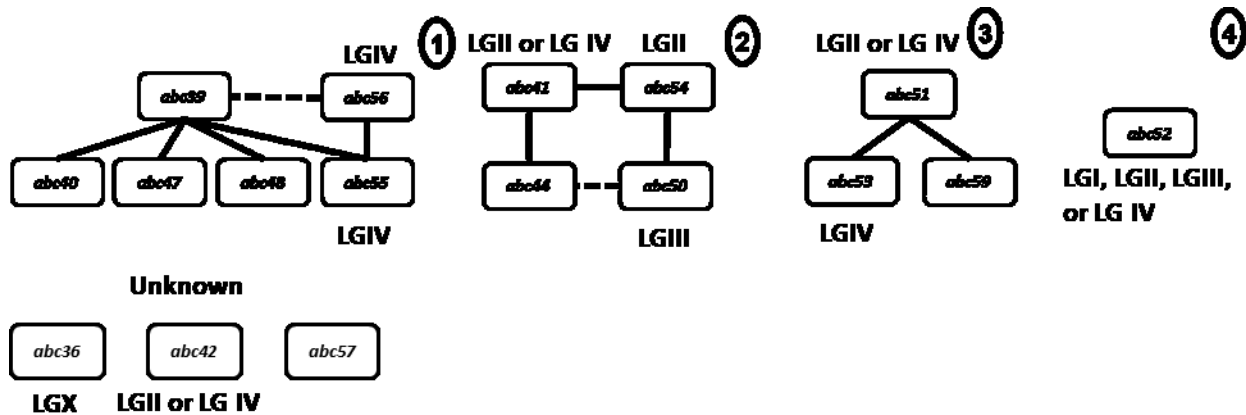
Figure 12: Linkage groups of suppressor strains for each complementation group
The linkage groups are labeled 1-4, and the LG group is indicated where limited to a few or one candidate.

Non-complementation usually indicates that two suppressor mutations are allelic. In this case, the alleles should map to the same chromosome. To verify the complementation results, I also used genetic mapping data. In some instances though, suppressors belonging to the same complementation group did not map to the same LG. This indicates that the suppressors are likely due to suppressing mutations in different genes, and not different alleles of the same gene. For example, *abc50* was linked to LGIII and unlinked to the other linkage groups (Figure 12, pg50), while exhibiting non-complementation to *abc54*, as a member of group 2 (Figure 12, pg50). There disparate results are likely the result of non-allelic non-complementation, which will be examined further in a later section. Similarly, *abc41* and *abc54* both show that they are not linked to all but LGII. This should be noted with the contradiction in complementation group 2 as and *abc50* complement (Figure 12, pg50). CG3 containing *abc51*, *abc53*, and *abc59* also contained a contradiction in linkage analysis between the two alleles tested, *abc51* exhibited linkage to LGIV while *abc53* exhibited linkage to LGIV (Figure 12, pg50). The instances where mapping and complementation data suggested non-allelic non-complementation, as the suppressors would be unlinked for these strains from one another. This relationship was confirmed when WGS data became available for both cases.

3.4   Whole Genome Sequencing

Ethyl methanesulfanate (EMS) is a commonly used mutagen to induce mutations in *C. elegans* (Sarin et al. 2010). EMS mutagenesis creates thousands of mutations throughout the genome, typically resulting in G/C to A/T transitions (Sarin et al. 2010). The EMS mutagenesis was performed to find suppressor of *memi-1(sb41)*, that were not found through RNAi screening. Mutations could also be generated in *gsp-3/4*, the only suppressors found in the RNAi screen, providing alleles to work with in the future. The mutagenesis screen identified 27 strains that contained suppressor mutations of *memi-1(sb41)*, and were stable enough to be maintained. To identify the mutations responsible for suppression, whole genome sequencing of the suppressor strains was performed. Because EMS mutagenesis generates hundreds or thousands of mutations, mapping data and complementation testing was used to limit the number of possible candidates. Since complete loss of *memi-1* activity itself (*ie., memi-1(RNAi)*) completely suppresses the *sb41* phenotype (Ataeian et al. 2016), the EMS-generated suppressors were first sequenced for second site mutations in *memi-1(sb41)* by Caitlin Slomp (*pers. comm.*), which initially found that 10 strains contained 8 different second site mutations. The remaining 17 strains were submitted for whole genome sequencing (WGS), in order to identify lesions responsible for extragenic suppression.

3.4.1   Narrowing the possible *memi-1(sb41)* suppressor mutation candidates

In the WGS of 17 suppressor strains and the 2 background strains, 13,741 unique mutations were identified overall, with an average of 808 mutations in each suppressor strain. The majority of the lesions were single G/C to A/T transition mutations, but some deletions were also observed. Mutations shared by the background strains were filtered out, leaving 6,527 mutations generated by the EMS mutagenesis, however only 1,032 of these mutations were predicted to alter protein structure, either altering the amino acids, or translation of the protein. A full list of these genes that contain protein altering mutations can be found for each suppressor strain in the appendices (Table 12, pg102).

Strain	Allele	Mutation in Protein / LG							Notes
		I	II	III	IV	V	X	Total	
MAS182	N/A	0	3	2	0	8	1	14	Original strain, *memi-1(sb41) dpy-20*
MAS138	N/A	0	0	0	0	0	0	0	Strain used to outcross suppressors. *memi-1(sb41)*
MAS260	*abc36*	19	4	12	4	5	4	48	
MAS249	*abc39*	11	11	13	3	12	0	50	
MAS247	*abc40*	13	6	9	2	9	2	41	
MAS270	*abc41*	10	25	22	17	16	16	106	
MAS271	*abc42*	0	14	12	3	20	6	55	
MAS261	*abc44*	11	16	6	1	28	25	87	
MAS241	*abc47*	16	13	4	4	3	6	46	
MAS250	*abc48*	23	6	1	3	3	4	40	Suppressor strains generated by EMS mutagenesis of MAS182. Strains were outcrossed with MAS138.
MAS266	*abc50*	4	4	12	1	3	11	35	
MAS251	*abc51*	4	10	2	6	1	2	25	
MAS253	*abc52*	2	7	3	7	2	8	29	
MAS283	*abc54*	30	25	22	5	8	1	91	
MAS281	*abc55*	2	2	2	21	2	3	32	
MAS276	*abc56*	1	14	2	7	5	4	33	
MAS272	*abc57*	8	9	23	3	18	9	70	
MAS267	*abc59*	11	25	4	1	1	1	43	
MAS220	*abc53*	26	22	17	7	18	29	119	Suppressor not outcrossed with MAS138.

Table 7: Homozygous protein altering mutations found through WGS
Dark grey boxes indicate no linkage between the suppressor and the LG, light grey boxes indicate that linkage is unknown, white cells linkage. Background mutations found in MAS138 and MAS182 are not included so these strains only show mutations that are not shared with any of the suppressors.

Some of the mutations found in the WGS were heterozygous. These were eliminated because in all cases the suppressor mutations were recessive or semi-dominant, as they were distinguishable as homozygotes. Using these criteria, the number of candidate mutations was reduced to 964. Some of the same mutations were found in multiple strains, but not in the background. Mutations generated by EMS mutagenesis can occur anywhere in the genome and so larger genes are more likely to be mutagenized than other areas (Anderson 1995), and so as expected some many mutations were found in commonly mutagenized genes. *ttn-1*, the *C. elegans* copy of Titin, the largest known protein was found to be have incurred protein altering mutations in 9 different suppressors strains. To distinguish suppressor mutations that could be found through complementation analysis from non-suppressing mutations complementation of the respective suppressor strains were reviewed. If these repeat mutations which are contraindicated to being suppressors by complementation testing are also excluded, the final number becomes 846. The number of mutations in each of the suppressor screens sequenced was highly varied. This is

likely due to the single round of outcrossing performed leaving just an average of 56 mutations per strain, with 9 mutations per chromosome (Table 7, pg52).

3.4.1.1   Whole genome sequencing data in consideration of mapping data

When the mapping data for suppressor strains was considered, the mutations on the chromosomes that were unlinked to suppression can be eliminated, and those on chromosomes that show linkage can be isolated. With the inclusion of the linkage analysis, the total number of possible suppressor mutations being considered was reduced from 964 to 540 (Table 7, pg52). For the strains where linkage was narrowed down to a single chromosome, there were only a few mutations that needed to be considered; *abc36* only has 4 possible suppressing mutations on LGX, *abc50* has 12 on LGIII, *abc53* has 7 on LGIV, *abc54* has 25 on LGII, *abc55* has 21 on LGIV, and *abc56* has 7 on LGIV (Table 7, pg52). Where linkage was limited to a few chromosomes, the number of possible suppressing mutations was similarly reduced; *abc41*, *abc42*, and *abc51* all can only be linked to LGII or LGIV, leaving 42, 17, and 16 possible suppressing mutations respectively (Table 7, pg52). The WGS data was examined to determine the likely location of suppressors of *memi-1(sb41)*. One suppressor, *abc55,* contained a second site mutation in *memi-1(sb41)* (Table 12, pg102), which was not found in initial screening for intragenic mutations. The possibility of this mutation being the suppressor was corroborated by the linkage analysis that showed that *abc55* was linked to LGIV (Table 6, pg49).

3.4.1.2   Whole genome sequencing data in consideration of complementation testing

For the remaining suppressors, it was important to first identify any genes that contained a mutation in multiple strains. Because EMS mutagenesis creates mutations randomly for each strain, a gene that can become a suppressor may be mutated multiple times. Any genes that had mutations in different strains that belonged the same complementation group were considered good suppressor candidates. Amongst all the mutations found in the WGS, 57 occurred in the same genes in multiple strains (Table 8, pg55). Of these genes, 45 mutations were in strains that were shown to complement one another (Table 8, pg55). The remaining 12 genes either failed to complement one another or were not tested in the complementation analysis (Table 8, pg55). 10 of these genes were located on a chromosome that did not match the linkage group for the suppressor (Table 7, pg52). Thus, only the remaining two genes fit the criteria for a potential suppressor with multiple alleles: *gsp-4* and *R03D7.5* (Table 5, pg48 and Table 8, pg55).

Gene	LG	Suppressor Backgrounds	Complementation
C55C2.4	I	*abc50, abc54*	No: *abc50* x *abc54*
fasn-1	I	*abc36, abc47*	Not tested
gip-2	I	*abc36, abc39, abc40, abc41, abc44, abc47, abc48, abc53, abc54, abc59*	Contradictory tests
gsp-4	I	*abc39, abc40, abc44, abc47, abc48*	No: *abc39* x *abc40*, *abc47*, and *abc48*
lron-9	I	*abc40, abc44, abc48, abc57*	Yes: inferred through other tests
lrp-2	I	*abc47, abc53*	Yes: inferred through other tests
mfap-1	I	*abc54, abc59*	Yes: inferred through other tests
nas-30	I	*abc39, abc55*	No: *abc39* x *abc55*
oac-13	I	*abc36, abc39, abc40, abc47, abc48, abc51, abc53, abc54, abc59*	Contradictory tests
sig-7	I	*abc36, abc39, abc40, abc41, abc44, abc47, abc48, abc53, abc54, abc59*	Contradictory tests
smd-1	I	*abc36, abc39, abc40, abc47, abc48, abc52, abc53, abc54, abc55*	Contradictory tests
tba-1	I	*abc54, abc59*	No: *abc54* x *abc59*
Y47H9C.9	I	*abc48, abc53*	No: inferred through other tests
ZC247.1	I	*abc44, abc48, abc52, abc53*	Yes: *abc52* x *abc53*, others inferred
ZK909.3	I	*abc36, abc39, abc40, abc44, abc47, abc48, abc51, abc53, abc54*	Contradictory tests
C33C12.1	II	*abc41, abc44*	No: *abc41* x *abc44*
C38C6.3	II	*abc39, abc40, abc41, abc42, abc47, abc48, abc51, abc53, abc54, abc56, abc57, abc59*	Contradictory tests
clec-122	II	*abc39, abc51, abc53*	Contradictory tests
cpna-2	II	*abc54, abc56*	Yes: *abc54* x *abc56*
F10E7.2	II	*abc39, abc41*	Yes: *abc39* x *abc41*
K09E4.4	II	*abc39, abc40, abc41, abc42, abc47, abc48, abc51, abc53, abc54, abc56, abc57, abc59*	Contradictory tests
oac-4	II	*abc39, abc42, abc44, abc50, abc52, abc57*	Contradictory tests
pqn-87	II	*abc41, abc42*	Not tested
ptc-2	II	*abc39, abc40, abc41, abc42, abc44, abc48, abc53, abc54, abc55, abc57, abc59*	Inferences contradictory
R03D7.5	II	*abc41, abc51, abc57, abc59*	No: *abc51* x *abc59*
trr-1	II	*abc41, abc59*	Yes: inferred through other tests
xrn-2	II	*abc40, abc41, abc42, abc47, abc48, abc53, abc54, abc56, abc57, abc59*	Contradictory tests
zyg-9	II	*abc41, abc54*	No: *abc41* x *abc54*
F40H6.2	III	*abc41, abc53*	Yes: *abc41* x *abc53*
H14E04.2	III	*abc36, abc53*	Yes: inferred through other tests
let-716	III	*abc36, abc50*	Yes: inferred through other tests
lin-12	III	*abc42, abc57*	Not tested

Gene	LG	Suppressor Backgrounds	Complementation
numr-1	III	*abc36, abc39, abc40, abc41, abc42, abc47, abc51, abc52, abc53, abc54, abc55, abc59*	Contradictory tests, mutation loci not sequenced in background strains
Y39A1A.9	III	*abc55, abc56*	No: *abc55* x *abc56*
Y82E9BR.18	III	*abc41, abc53, abc57*	Yes: *abc41* x *abc53* and *abc57*. Inferred *abc53* and *abc57* contradictory.
Y82E9BR.23	III	*abc40, abc41*	Yes: inferred through other tests
cdh-8	IV	*abc41, abc55*	Yes: *abc41* x *abc55*
eel-1	IV	*abc41, abc55*	Yes: *abc41* x *abc55*
tag-80	IV	*abc36, abc39, abc40, abc41, abc42, abc44, abc47, abc48, abc50, abc51, abc52, abc54, abc55, abc56, abc57, abc59*	Inferences contradictory, mutation loci not sequenced in background strains
tpa-1	IV	*abc40, abc48, abc51, abc56*	Yes: inferred through other tests
Y116A8A.6	IV	*abc41, abc42, abc47, abc53, abc57*	Yes: *abc41* x *abc53*, others inferred through other tests
C05C8.2	V	*abc39, abc40, abc41, abc42, abc44, abc53, abc57*	Contradictory tests
F15H10.8	V	*abc36, abc47, abc48, abc53*	Yes: inferred through other tests
H39E23.3	V	*abc39, abc52*	Yes: *abc39* x *abc52*
K09H11.11	V	*abc36, abc39, abc42, abc44, abc53*	Yes: *abc39* x *abc53*
ttn-1	V	*abc36, abc39, abc40, abc41, abc42, abc44, abc47, abc48, abc50, abc51, abc52, abc55, abc57, abc59*	Contradictory tests
6R55.2	X	*abc44, abc47, abc50, abc52, abc53, abc57*	Yes: *abc44* x *abc50*, *abc52* and *abc50*, *abc53*, *abc57*
C30E1.4	X	*abc41, abc44, abc47, abc50, abc52, abc53, abc57*	Contradictory tests
C46H3.3	X	*abc42, abc53, abc55*	Yes: *abc53* x *abc55*. Others not tested
C53C11.1	X	*abc41, abc44, abc47, abc50, abc52, abc53, abc57*	Contradictory tests
F16H11.2	X	*abc36, abc53*	Yes: inferred through other tests
F43B10.1	X	*abc41, abc44, abc48, abc50, abc52, abc53, abc57*	Yes: *abc53* and *abc41*, *abc52*. *abc50* and *abc44*, *abc52*. *abc52* x *abc57*
F16H11.2	X	*abc36, abc53*	Yes: inferred through other tests
F43B10.1	X	*abc41, abc44, abc48, abc50, abc52, abc53, abc59*	Yes: *abc53* and *abc41*, *abc52*. *abc50* and *abc44*, *abc52*. *abc52* x *abc59*
F52E10.3	X	*abc41, abc44, abc47, abc50, abc52, abc53, abc57*	Yes: *abc53* and *abc41*, *abc52*. *abc50* and *abc44*, *abc52*. *abc52* x *abc57*
H03A11.2	X	*abc42, abc48, abc56*	Yes: inferred through other tests
osm-11	X	*abc41, abc44, abc47, abc50, abc52, abc53, abc57*	Yes: *abc53* and *abc41*, *abc52*. *abc50* and *abc44*, *abc52*. *abc52* x *abc57*

Table 8: Homozygous Protein Altering Mutations Found in the Same Gene in Multiple Suppressor Strains
Complementation lists all suppressor alleles with protein altering mutations in the gene complement or fail to complement either directly or inferred through other tests. Dark grey: complementation. Light grey: undetermined because strains were not tested or tests were contradictory. White: fail to complement. Inferred complementation denotes that the alleles involved were not directly tested but are part of complementation groups where the relationship is not directly tested but can be inferred.

3.4.2 Identified *memi-1(sb41)* suppressor mutations

Gene	Mutation	DNA		Amino Acid		Function
		Change	Position	Change	Position	
gsp-4	*abc39*	C to T	352	G to E	89	Missense mutation
gsp-4	*abc40*	C to T	747	G to R	221	Missense mutation
gsp-4	*abc44*	G to A	276	H to Y	64	Missense mutation
gsp-4	*abc47*	A to T and C to T	235 and 406	L to H and C to Y	50 and 107	Missense mutations
gsp-4	*abc48*	C to T	268	G to E	61	Missense mutation
memi-1(sb41)	*abc55*	T to A	859	R to S	286	Missense mutation
R03D7.5	*abc41*	G to A	673 and 705	210 and 221	T to I and V to I	Missense mutations
R03D7.5	*abc51*	G to A	bp 1 of exon 6	AHKLCGSGR to GLEAYRG-stop	235-243	Splice acceptor variant
R03D7.5	*abc57*	CGTCACCTA deletion	225-232	ITGVFGYPT to TVKNWKT W-stop	21-29	Frameshift deletion
R03D7.5	*abc59*	G to A	762	G to R	240	Missense mutation

Table 9: Known suppressing mutations found through whole genome sequencing and their effects on protein

The molecular lesions in *gsp-4* and *R03D7.5* in each of the relevant suppressor strains were subjected to PCR and Sanger sequencing to confirm the WGS data. *abc39*, *abc44*, *abc47*, and *abc*48 were confirmed within *gsp-4*. *abc40* was not able to be confirmed by Sanger sequencing due to the unavailability of good sequencing primer candidates in that portion of the gene, and so the data is reliant on the WGS alone. All the suppressing alleles in *R03D7.5* were confirmed through Sanger sequencing. The same was done for *abc55* in *memi-1(sb41)*, in total confirming the presence of the 10 putative suppressor mutations.

3.4.2.1 Identified suppressing mutations considering mapping and complementation data
While the alleles of *gsp-4* and *R03D7.5* both were shown to fail to complement both complementation group 1 and 3 (Figure 11, pg47), which show failure to complement of *gsp-4* and *R03D7.5* respectively, contain suppressors that do not share mutations in those genes. Complementation group 1 contains *abc39*, *abc40*, *abc47*, and *abc48*, all of which contain mutations in *gsp-4*, but also *abc55* and *abc56* which do not *abc55* instead contains an intragenic mutation in *memi-1(sb41)* and *abc56* remains unknown (Figure 11, pg47). This suggests that there are multiple instances of non-allelic non-complementation to be found in the results of the complementation testing. The last strain containing a mutation in *gsp-4*, *abc44* was not directly tested to any of the other *gsp-4* alleles, as it was found to fail to complement member of group 2 (Figure 11, pg47). The final two members of complementation group 1; *abc55* and *abc56* both

do not contain mutations in *gsp-4* but are linked to LGIV (Table 6, pg49). The non-allelic non-complementation is not consistent for all tested members of complementation group 1 as *abc39* was determined to complement *abc56* (Table 3, pg44). There is another instance of non-allelic non-complementation involving the putative *gsp-4* suppressor *abc44*; fails to complement *abc41* as part of complementation group 2 (Table 3, pg44).

Strains containing mutation in *R03D7.5* occur in complementation groups 2 and 3 (Figure 11, pg47). containing *abc41* and *abc51* and *abc59* respectively. WGS analysis indicates that none of the members of complementation group 2 have any mutated genes in common with each other (Table 8, pg55). This indicates that there is non-allelic non-complementation between the members the complementation group 2. However, two other members of complementation group 2: *abc44* and *abc50* complement. Non-allelic non-complementation can also be seen in complementation group 3, as *abc53* does not have any mutated genes in common with *abc51* or *abc59*, which both contain mutations in *R03D7.5*.

3.4.3   Unidentified *memi-1(sb41)* suppressor mutations

For the remainder of the *memi-1(sb41)* suppressor strain *abc36*, *abc42*, *abc50*, *abc52*, *abc53*, *abc54*, and *abc56* it is possible to restrict the number of possible candidate mutations using linkage data. The remaining 7 suppressor strains contain 84 mutations located on chromosomes that are not ruled out by linkage analysis (Table 7, pg52). Because none of these suppressors share any mutations with other suppressors that they fail to complement, or if they do the suppressors are not linked to the same LG. This indicates that if the suppressing mutations occur in the coding regions of genes, the remaining 7 suppressors are caused by mutations in independent genes (Table 8, pg55). This indicates that there are at least 9 different genes that can become suppressors of *memi-1(sb41)* including *gsp-4* and *R03D7.5*.

3.5   **Characterization of Mutations**

C. elegans contains two nearly identical sperm specific genes *gsp-3* and *gsp-4* with 97% DNA similarity which are required for sperm meiosis and motility. *gsp-3/4* encode catalytic subunits of protein phosphatase 1 (PP1), which is broadly conserved across all eukaryotes where it is

required in sperm development (Varmuza et al. 1999), but has also been shown to play a role in cell division (Sivakumar et al. 2016) in both meiosis and mitosis (Fisher et al. 2014). Previous research in the Srayko lab has shown that *gsp-3/4(RNAi)* rescues the embryonic viability of *memi-1(sb41)* worms (Ataeian et al. 2016). With the discovery of mutations in *gsp-4* that were confirmed to be suppressors through complementation testing, I began a characterization of the alleles in different genetic backgrounds to probe the functional significance of the mutations.

3.5.1 Characterization of *gsp-4* mutations
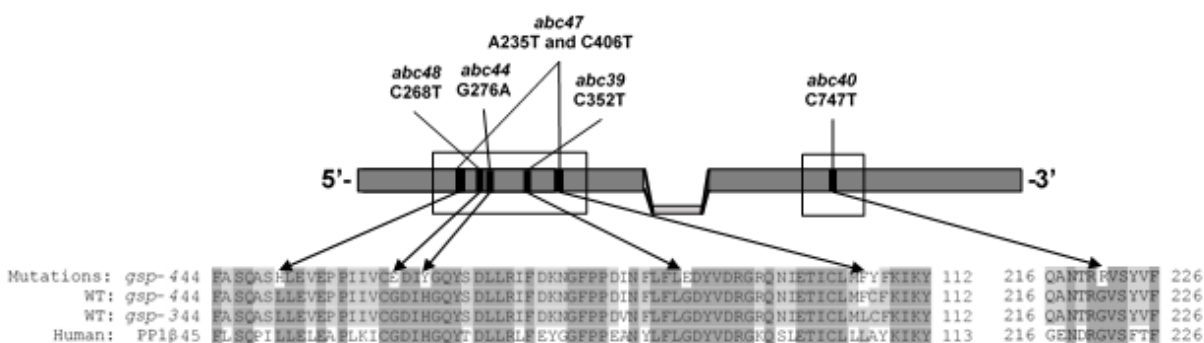
Figure 13: *gsp-4* Mutations
The sites of mutations found in *gsp-4* found through WGS. Effects of the mutation on protein are shown below compared to WT *gsp-4*, the paralogue *gsp-3* and the orthologue, human PP1β. Matching amino acids are indicated by colour: dark grey are the same in all three proteins, light grey are the same for two, and white for no match in the others.

The *gsp-4* mutations are all missense mutations caused by changes in the nucleic acids that lead to a single amino acid substitution in *abc39*, *abc40*, *abc44*, and *abc*47, and two substitutions in *abc48*. Each of the amino acid substitutions is caused by a SNP typical of EMS mutagenesis. All the mutations occur in conserved regions of amino acids that are identical amongst *gsp-3*, *gsp-4*, and human PP1β, except the second mutation of *abc48* G320A, which is only present in *gsp-3* and *gsp-4* (Figure 13, pg58). This group of *gsp-4* mutations overlaps with complementation group 1 (Figure 11, pg47), except that it does not include *abc55* and *abc56*. Because *abc55* was linked to LGIV (Table 5, pg48) and contains an intragenic mutation on *memi-1* which is on chromosome IV, it was determined that suppression was likely due to this second site mutation as a protein altering mutation was found in the *memi-1* coding region. *abc56* fails to complement *abc55* but complements *abc39* has no mutations in either *gsp-4* or *memi-1* despite mapping to LGIV, indicating that suppression of *abc56* is due to a different mutation in LGIV. There are no mutations shared by *abc55* and *abc56* or any of the other

complementation group 1 alleles, except *Y39A1A.9* on chromosome III (Figure 13, pg58) while both suppressors show linkage to LGIV indicating that it is not responsible for suppression (Table 7, pg52).

3.5.1.1 Embryonic viability of suppressor strains containing *gsp-4* mutations in different *memi-1(sb41)* backgrounds

Figure 14: Embryonic viability of *gsp-4* suppressor alleles in different *memi-1(sb41)* backgrounds
Error bars show standard error. * indicates a statistically significant difference between the trial and the *memi-1(sb41)/+* control. Direct comparisons show the result of two-tailed t-tests between homozygous and heterozygous *memi-1(sb41)* for each suppressor background where significant (p<0.05). # indicate the value is zero. All worms were incubated at 25 °C.

Three of the *gsp-4* suppressor alleles, while heterozygous, were scored for embryonic viability in different genetic backgrounds involving *memi-1(sb41)*, *i.e.*, *sb41/sb41*, vs. *sb41/+*. All the mutations suppressed embryonic lethality while heterozygous in a *memi-1(sb41)/+* background, as compared with control *memi-1(sb41)/+* worms (Figure 14: see *, pg59). However, each of the tested alleles of *gsp-4* had a different embryonic viability to the homozygous suppressor allele in a *mem-1(sb41)* background, one higher one lower and one the same. The *abc39/+ memi-1(sb41)/+* had significantly lower embryonic viability when compared to *abc39 memi-1(sb41)* (Figure 14, pg59). The embryonic viability of the *abc40 memi-1(sb41)* dihybrid was not significantly different than the homozygote. While it is already known that heterozygous *memi-*

1(sb41) is less severe Mel than homozygous *memi-1(sb41)* (Ataeian et al. 2016), this does indicate that the *gsp-4* suppressors are able to suppress this less severe phenotype.

3.5.1.2 Embryonic viability of *gsp-4(abc39)* in the absence of *memi-1(sb41)*

	n=10	n=8	n=12	n=6
Average	93.10	0.00	58.54	26.23
Standard error	2.51	0.00	7.61	5.47
memi-1	+/+	sb41	sb41	+/+
gsp-4	+/+	+/+	abc39	abc39
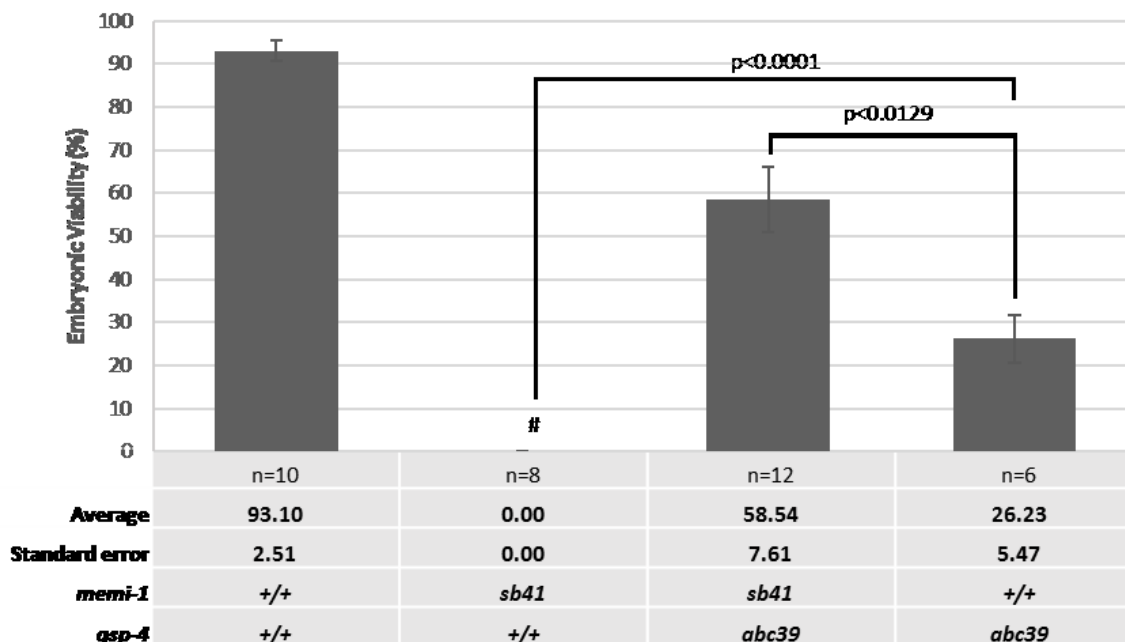
Figure 15: Embryonic viability of suppressor *gsp-4(abc39)* outcrossed from *memi-1(sb41)*
Error bars show standard error. # indicate the value is zero. All worms scored at 25 °C. Statistical comparison done by two-tailed t-test.

The *gsp-4(abc39)* mutation was successfully outcrossed from *memi-1(sb41)*. The embryonic viability of *gsp-4(abc39)* worms was significantly lower than *gsp-4(abc39) memi-1(sb14)* worms (Figure 15, pg60). However, they still have significantly higher embryonic viability than the *memi-1(sb41)*, indicating that the worms are viable (Figure 15, pg60).

3.5.2 Characterization of *R03D7.5* suppressor mutations

R03D7.5 is currently uncharacterized gene in *C. elegans,* which is part of a group of seven genes in the species that are paralogues of GSK3. GSK3 is a multifunctional serine/threonine kinase, which is known to be involved in cell-cycle regulation, amongst other roles (Doble and Woodgett 2003). From the WGS analysis, *R03D7.5* seems to be a likely candidate gene containing four putative suppressor mutations in the *abc41*, *abc51*, *abc57*, and *abc59* suppressors. The only pair of suppressor strains containing *R03D7.5* mutations that were tested to one another were *abc51* and *abc59*, which fail to complement (Figure 10: 3, pg46). The *abc41* allele, though, is part of complementation group 2 and *abc51* and *abc59* are in

complementation group 3. Both *abc41* and *abc51* were tested to members of the opposite complementation group, but were not tested to each other, as they were presumed to complement based on the complementation with other members of each complementation group. There are three instances where paired complementation tests separate these alleles: *abc41* complements with *abc53* while *abc51* fails to complement, and *abc41* fails to complement with *abc44* and *abc54* while *abc51* complements. This shows three instances where *abc41* and *abc51* complement suppressors that the other strain has been shown directly to fail to complement (Figure 10: 3, pg46). These instances where one *R03D7.5* allele fails to complement a suppressors containing a mutation in another gene but the other *R03D7.5* allele does not may be evidence of a genetic interaction.
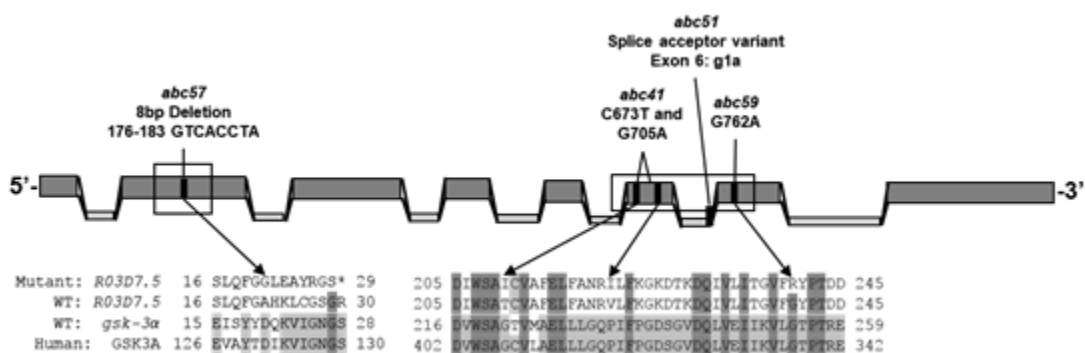
Figure 16: *R03D7.5* Mutations
The sites of all the *R03D7.5* mutations found through WGS. The alignment shows the amino acid changes caused by the mutations compared to WT *R03D7.5*. For the *abc57* deletion the χ^2 amino acids are displayed following the mutation until the first stop codon, the results of *abc51* cannot be predicted as the result of the mutation could result in splice readthrough, alternate splicing, or nonsense mediated decay. The *C. elegans R03D7.5* paralogue; *gsk-3α* and the orthologue human *gsk-3α* are used to show the broadly conserved amino acid sequences of the gene. In the alignment the dark grey regions show amino acids are the same for all three proteins, light grey where two are the same, and white for those that do not match any. * indicate an introduced stop codon.

3.5.2.1 Embryonic viability of *R03D7.5* suppressors in homozygous and heterozygous *memi-1(sb41)* backgrounds

Differences between the embryonic viability of heterozygous suppressors in a *memi-1(sb41)/+* background were also observed for suppressors with a mutation in *R03D7.5*. The *abc41/+ memi-1(sb41)/+* was not statistically different than the homozygote or *memi-1(sb41)/+* (Figure 17, pg62). The opposite was true for *abc57/+ memi-1(sb41)/+* which had significantly higher embryonic viability that of *abc57 memi-1(sb41)* and the *memi-1(sb41)/+* control (Figure 17, pg62).
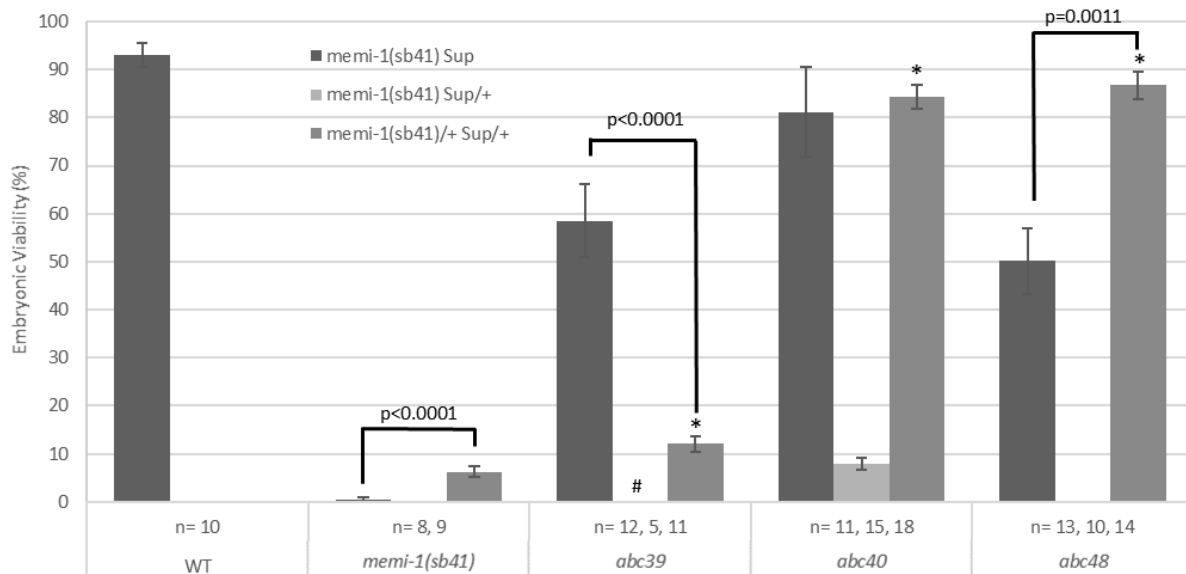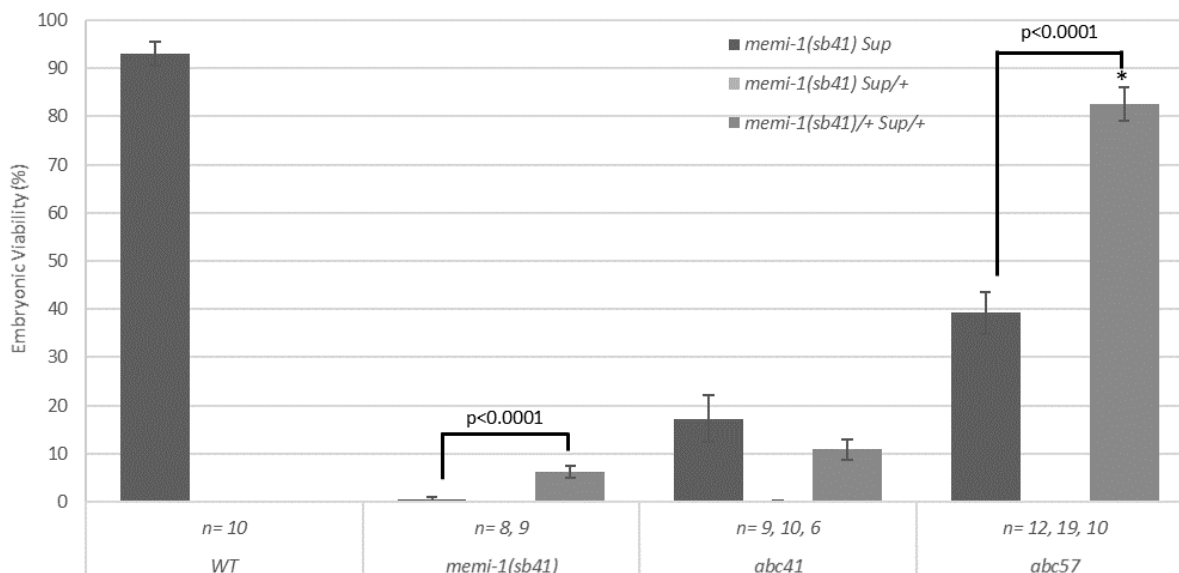
Figure 17: Embryonic viability of R03D7.5 suppressor alleles in different *memi-1(sb41)* backgrounds
Error bars show standard error. * indicates a statistically significant difference between the trial and the *memi-1(sb41)/+* control (two tailed t-test; p<0.05). Direct comparisons show the result of t-tests between homozygous and heterozygous *memi-1(sb41)* for each suppressor background where significant (p<0.05). # indicate the value is zero. All worms incubated at 25 °C.

3.5.3 Intragenic suppressor *memi-1(sb41:abc55)*
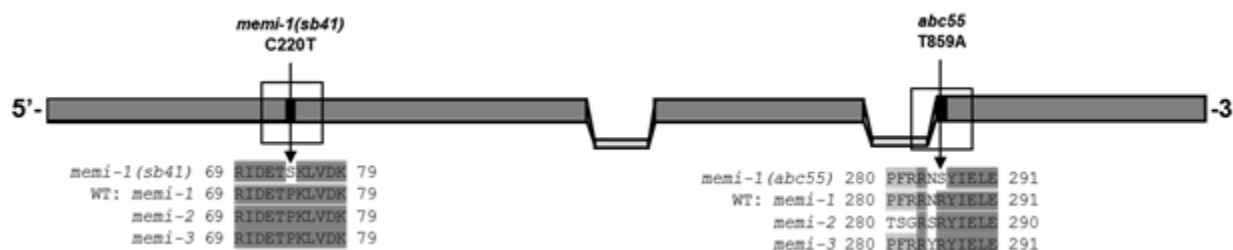
Figure 18: *memi-1(sb41)* and *abc55*
The *abc55* intragenic mutation is shown in conjunction with *sb41*. The alignment below the show the amino acid changes caused by the mutations compared to WT *memi-1/2/3*. The dark grey regions show where amino acids are the same for all three proteins, light grey where two are the same, and white for those that do not match.

The suppressing mutation of *abc55* does not occur in the immediate vicinity of the original *sb41* mutation (Figure 18, pg62). As the protein structure and functional domains of MEMI are unknown, the effect of the second mutation is not clear. The mutation does not occur at the same site as any other intragenic *memi-1(sb41)* suppressor (Martin Srayko, *pers. comm.*). The specific amino acid that is mutated is conserved across all MEMI proteins (Figure 18, pg62).

3.5.3.1 Intragenic *memi-1(sb41:abc55)* and complementation with other known suppressors
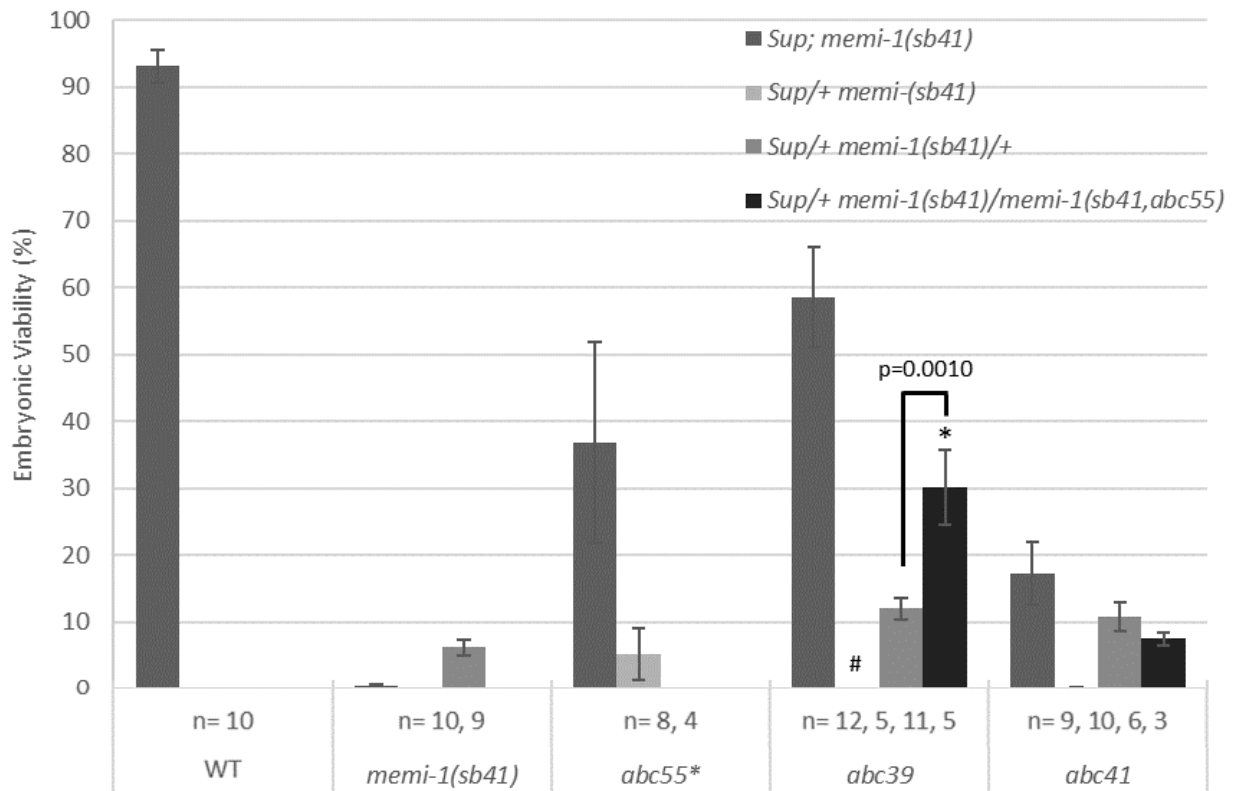
Figure 19: Embryonic viability of extragenic suppressor alleles of *memi-1(sb41)* that were crossed to *abc55* Error bars show standard error. # indicate the value is zero. * show significant difference in embryonic viability between the strain and *memi-1(sb41abc55)/memi-1(sb41)* determined by two-tailed t-test with p<0.05. All embryonic viability is for worms incubated at 25 °C.

The *abc55* suppressor was found to contain a mutation within the *memi-1* gene, in addition to the original *sb41* lesion, indicating that this allele is an intragenic suppressor (Lange et al. 2013). The location of the suppressor on chromosome IV, was also confirmed through linkage analysis (Table 6, pg49). However, certain complementation tests indicated that *abc55* failed to complement other suppressors that do not have a mutation within the *memi-1* gene. Based on previous experiments that showed that loss of *memi-1* activity completely suppresses *memi-1(sb41)* (Ataeian et al. 2016), it is possible that *abc55* also results in a loss of *memi-1* function. From initial strain-testing, the embryonic viability of homozygous and heterozygous *abc55* that the intragenic suppressor is recessive (Figure 8, pg42). As the mutation is intragenic the heterozygotes would be *memi-1(sb41)/memi-1(sb41abc55)*, with one unmodified copy of *memi-*

1(sb41). The *memi-1(sb41)/memi-1(sb41abc55)* worms do not have a significantly different embryonic viability than the *memi-1(sb41)/+* (Figure 19, pg63). Complementation testing indicated *abc55* failed to complement *abc39*, despite the two strains not sharing any mutated genes (Figure 19, pg63). Despite this, the *gsp-4(abc39)/+ memi-1(sb41abc55)/memi-1(sb41)* hermaphrodites had higher embryonic viability than either *memi-1(sb41abc55)/memi-1(sb41)* worms or *gsp-4(abc39)/+ memi-1(sb41)/+* worms (Figure 19, pg63). This indicates that the *abc39* and *abc55* suppressors have an additive effect on the *memi-1(sb41)* Mel phenotype. The *abc41* suppressor showed the opposite result when crossed with *abc55*: The *abc41/+ memi-1(sb41abc55)-memi-1(sb41)* worms did not have a statistically higher embryonic viability than the *memi-1(sb41abc55)/memi-1(sb41)* worms or *abc41/+ memi-1(sb41)/+* worms (Figure 19, pg63).

3.6   An analysis of the functionality of *memi-1(sb41)*

The *memi-1(sb41)* mutation was generated in worm's WT for *memi-2/3,* this makes it possible that the homologous WT genes acting redundantly compensate for a possible functional loss of activity in MEMI-1. As *memi-1(sb41)* has been found to be a hypermorph as the Mel phenotype becomes more severe with additional copies of *memi-1*. The natural hypothesis then is that the converse should be true: by lowering the amount of WT MEMI by introducing *memi-2Δ* and *memi-3Δ* in a *memi-(sb41)* background should improve the embryonic viability of hermaphrodites incubated. If the *memi-1(sb41)* mutation impaired the function of MEMI-1 in a way unrelated to its persistence in the oocyte this could be compensated for by the redundant WT copies of *memi-2/3*. Through the creation and testing of deletions strains I attempted to understand the relationship between *memi-1(sb41)* and embryonic viability in a variety of *memi-2Δ* and *memi-3Δ* backgrounds.

Figure 20: Embryonic viability of *memi-1(sb41)* in different *memi-2/3* deletion backgrounds
Error bars show standard error. # indicate the value is zero. * shows significant difference from *memi-1* WT *memi-2Δ memi-3Δ* and ** shows significant difference from *memi-1(sb41) memi-2 memi-3* at the same temperature. All statistics determined by two-tailed t-test (p<0.05).

The *memi-1(sb41)* control with WT *memi-2* and *memi-3* had significantly reduced embryonic viability at 15 °C and less than 1% at 20 and 25 °C demonstrating the maternal-effect lethal phenotype when compared to a *memi-1 memi-2Δ memi-3Δ* control (Figure 20: see *, pg65). The different worm strains tested contained different numbers of WT copies of *memi-2* and *memi-3*, depending on the number of deletions each of the strains carried. In multiple deletion strains both for *memi-1(sb41)* homozygotes and heterozygotes, there was an increase in embryonic viability when compared to their respective controls. *memi-1(sb41) memi-2Δ/+*, as well as the *memi-1(sb41) memi-2Δ memi-3Δ/+*, and *memi-1(sb41) memi-2Δ memi-3Δ/+* all had significantly higher embryonic viability than the *memi-1(sb41)* control at 20 °C (Figure 20: see **, pg65). This shows that, with the reduction of WT copies of *memi-2/3,* the *memi-1(sb41)* Mel phenotype can be alleviated. The exception to this trend is the *memi-1(sb41) memi-2Δ/+ memi-3Δ/+* worms which does not have a significant increase in embryonic viability when compared to *memi-*

1(sb41). When compared to the other deletion strains the *memi-1(sb41) memi-2Δ/+ memi-3Δ/+* worms have significantly lower embryonic viability than the *memi-1(sb41) memi-2Δ/+* as well as the *memi-1(sb41) memi-2Δ* and the *memi-1(sb41) memi-2Δ memi-3Δ* worms (Figure 20, pg65). This is interesting, as it indicates that, at 20 °C, the *memi-1(sb41) memi-2Δ/+ memi-3Δ/+* worms do worse than worms that only contain one deletion of *memi-2Δ* and that, if *memi-3Δ* is also deleted, these worms do worse. These worms can be improved however with the addition of a second deletion in *memi-2* making *mem-1(sb41) memi-2Δ memi-3Δ/+*. This seems to indicate a peculiarity either with *memi-3* or the specific deletion allele. The *memi-1(sb41) memi-2Δ* worms had significantly higher embryonic viability than all other deletion strains at 15 °C (Figure 20, pg65). Finally, *memi-1(sb41) memi-2Δ memi-3Δ* worms had very little embryonic viability at all temperatures, and could not be maintained at the permissive temperature of 15 °C (Figure 20: see **, pg65).

I observed that *memi-1(sb41)/+* worms had significantly higher embryonic viability *memi-1(sb41)* worms (Figure 22, pg68), as originally described by Mitenko et al, (1997). To determine the effect of altering the dosage of *memi-2* and *memi-3* in strains with a *memi-1(sb41)/+* background, the deletion strains were compared to the *memi-1(sb41)* control (Figure 22: see **, pg68). All three deletion strains (*memi-2Δ/+ memi-3Δ/+*, the *memi-2Δ memi-3Δ/+*, and the *memi-2Δ memi-3Δ* strains) had significantly higher embryonic viability at 20 °C than the *memi-1(sb41)* control (Figure 22, pg68). The only other instance where embryonic viability was significantly different was for *memi-1(sb41)/+ memi-2Δ/+ memi-3Δ/+* worms at 25 °C which was found to be significantly lower than the control (Figure 22, pg68).
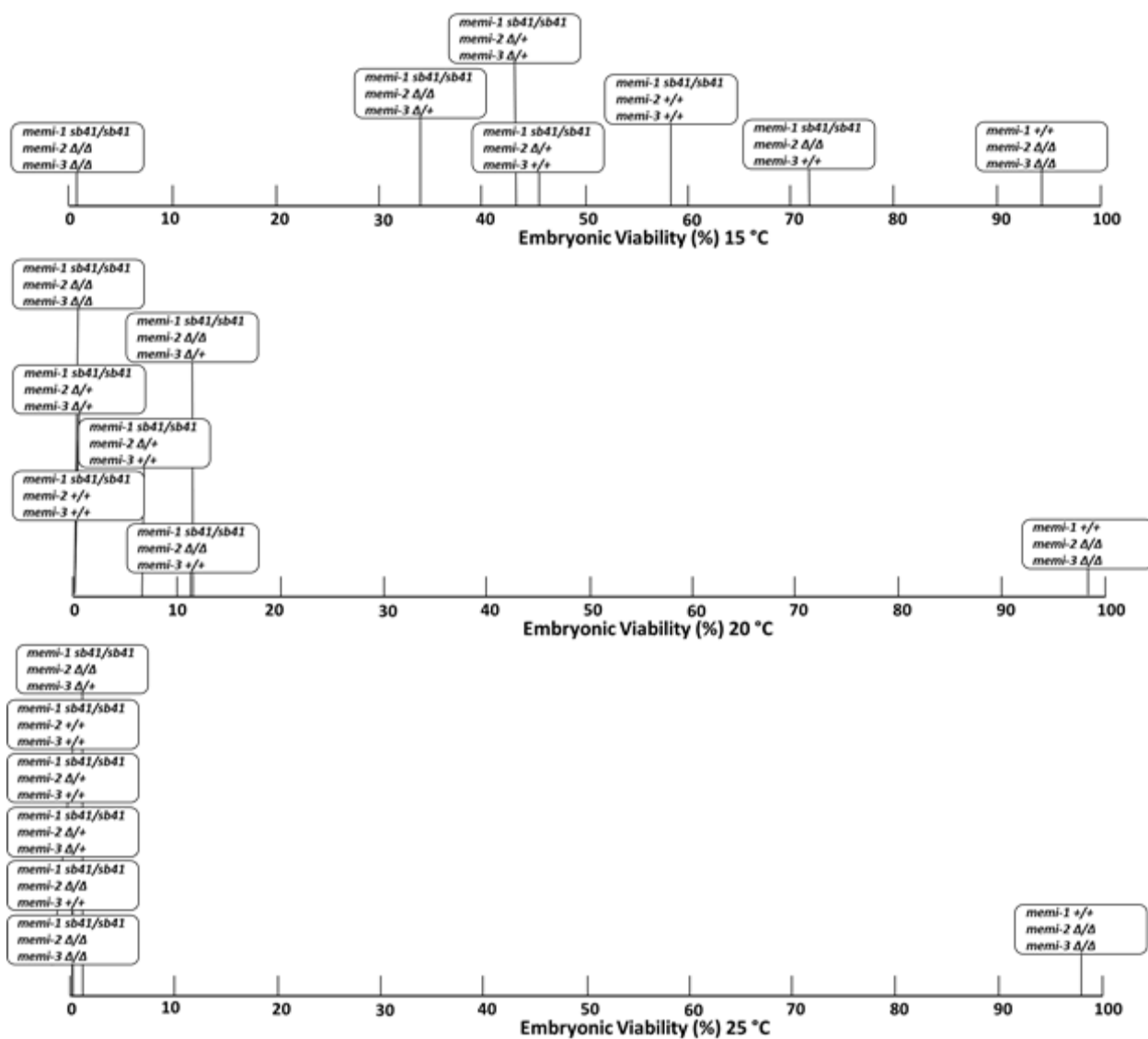
Figure 21: Relative embryonic viabilities of *memi-1(sb41)* worms in different *memi-2Δ memi-3Δ* backgrounds

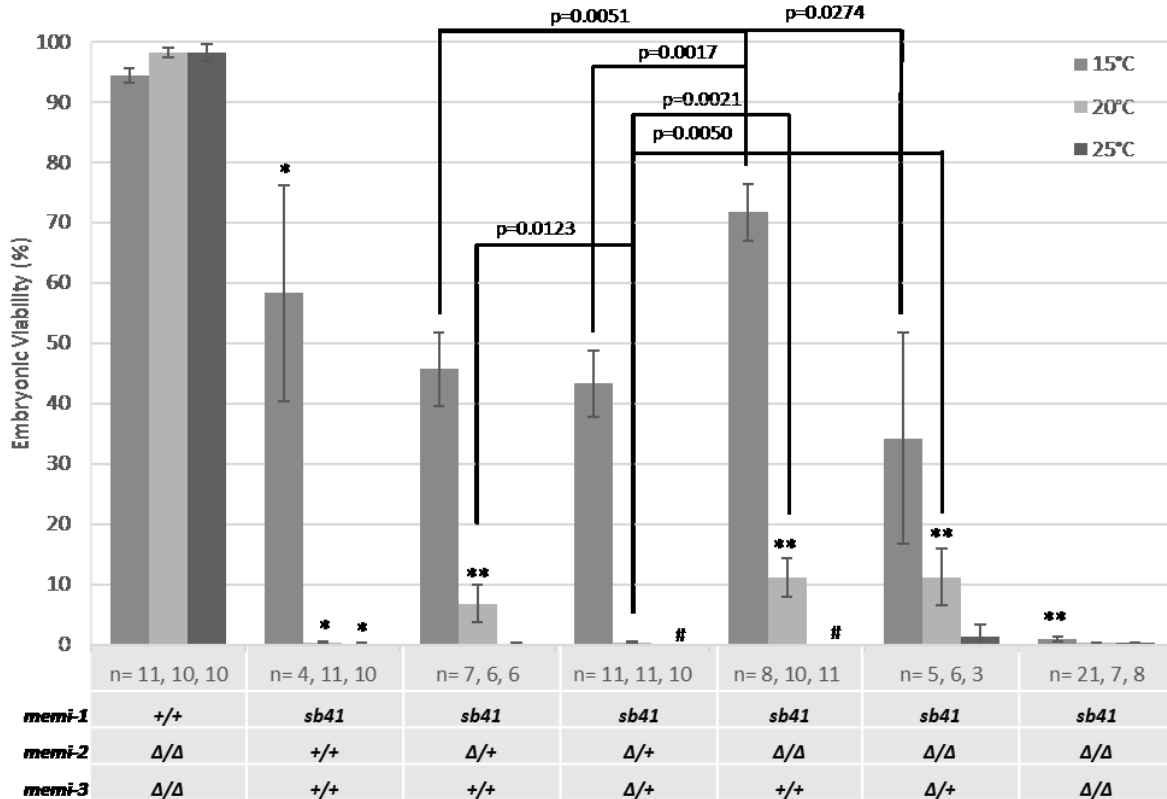### 3.6.1 Homozygous versus heterozygous *memi-1(sb41)* in different *memi-2/3* deletion backgrounds
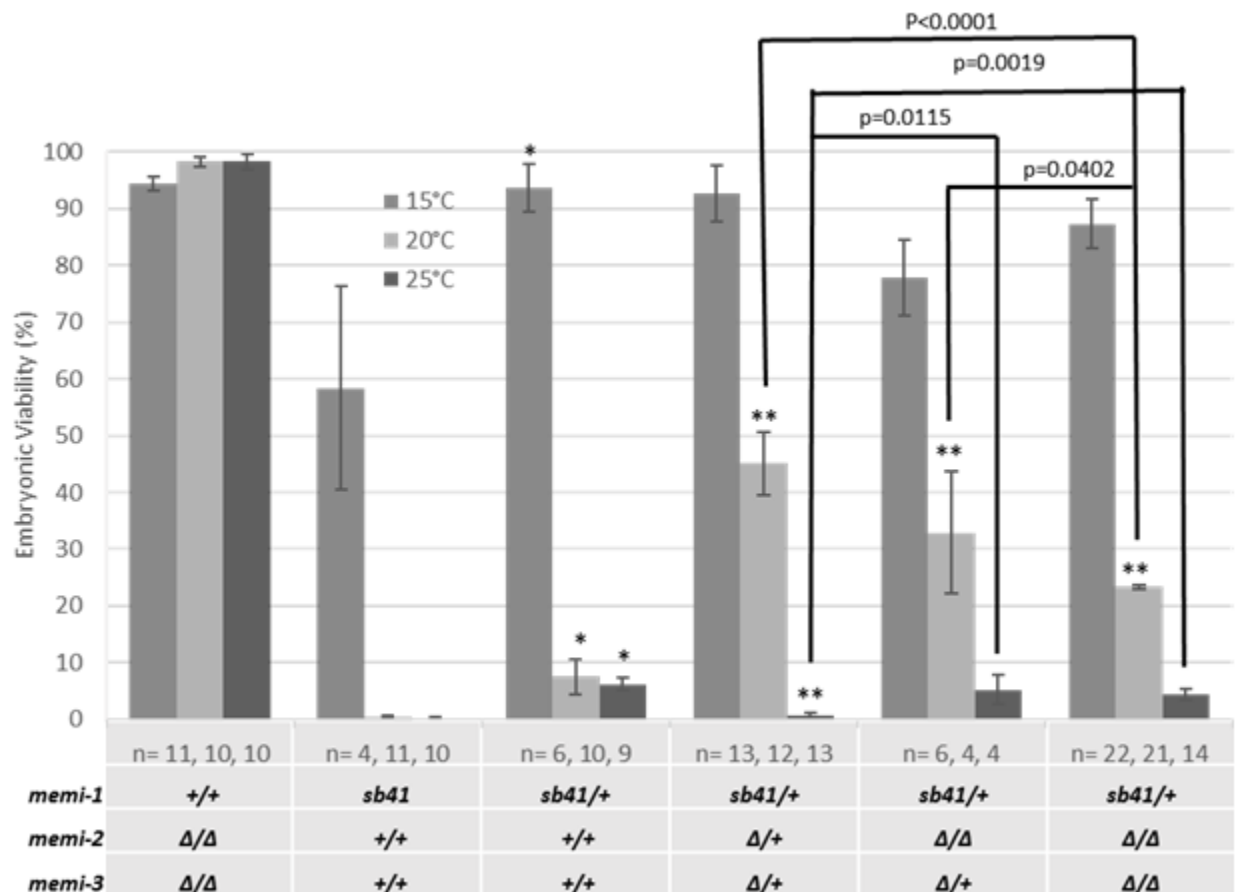
Figure 22: Embryonic viability of Heterozygous *memi-1(sb41)* in Different *memi-2/3* Deletion Backgrounds
Error bars show standard error. *shows significant difference with *memi-1(sb41) memi-2 memi-3* and ** shows significant difference with *memi-1(sb41)/+ memi-2 memi-3* for the same temperature determined by two-tailed t-test (p<0.05).

When comparing the heterozygous *memi-1(sb41)/+* worms to homozygous *memi-1(sb41)* worms in the same deletion backgrounds, the heterozygous worms had significantly higher embryonic viability in most cases (Figure 20, pg65 and Figure 22, pg68). In almost all instances the embryonic viability of the deletion strains was significantly higher when in combination with *memi-1(sb41)/+,* than with *memi-1(sb41/memi-1(sb41)* at 15 and 20 °C. The exceptions to this are the 15 and 20 °C comparison between the homozygous *memi-2Δ*, heterozygous *memi-1Δ* worms. In the 15 °C test the heterozygote did have a significantly higher embryonic viability (Figure 20, pg65 and Figure 22, pg68), but not as definitive as the other tests. In the 20 °C test

the heterozygous worms did not have a statistically significant increase in embryonic viability (p=0.685).
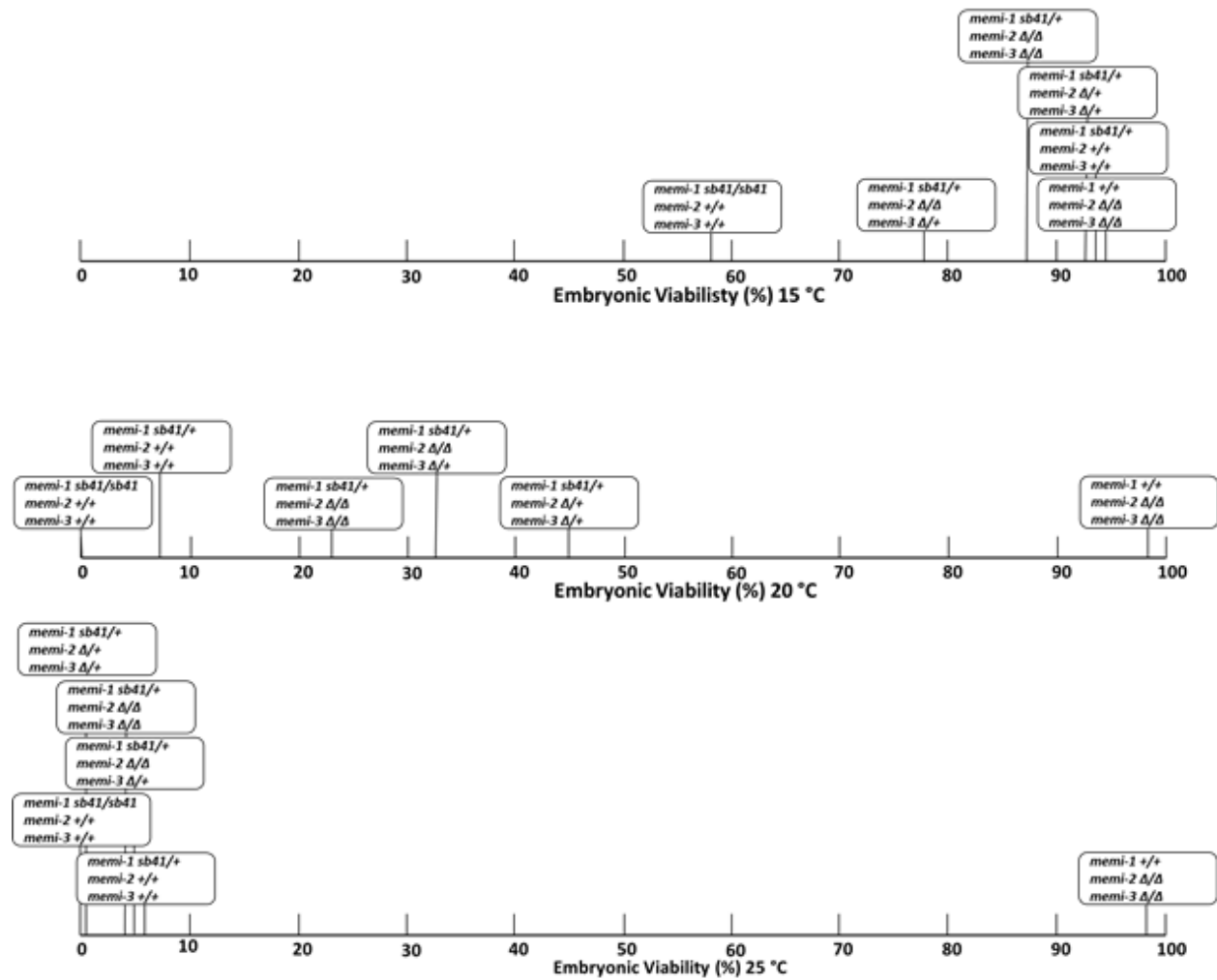
Figure 23: Relative embryonic viabilities of *memi-1(sb41)/+* worms in different *memi-2Δ memi-3Δ* backgrounds
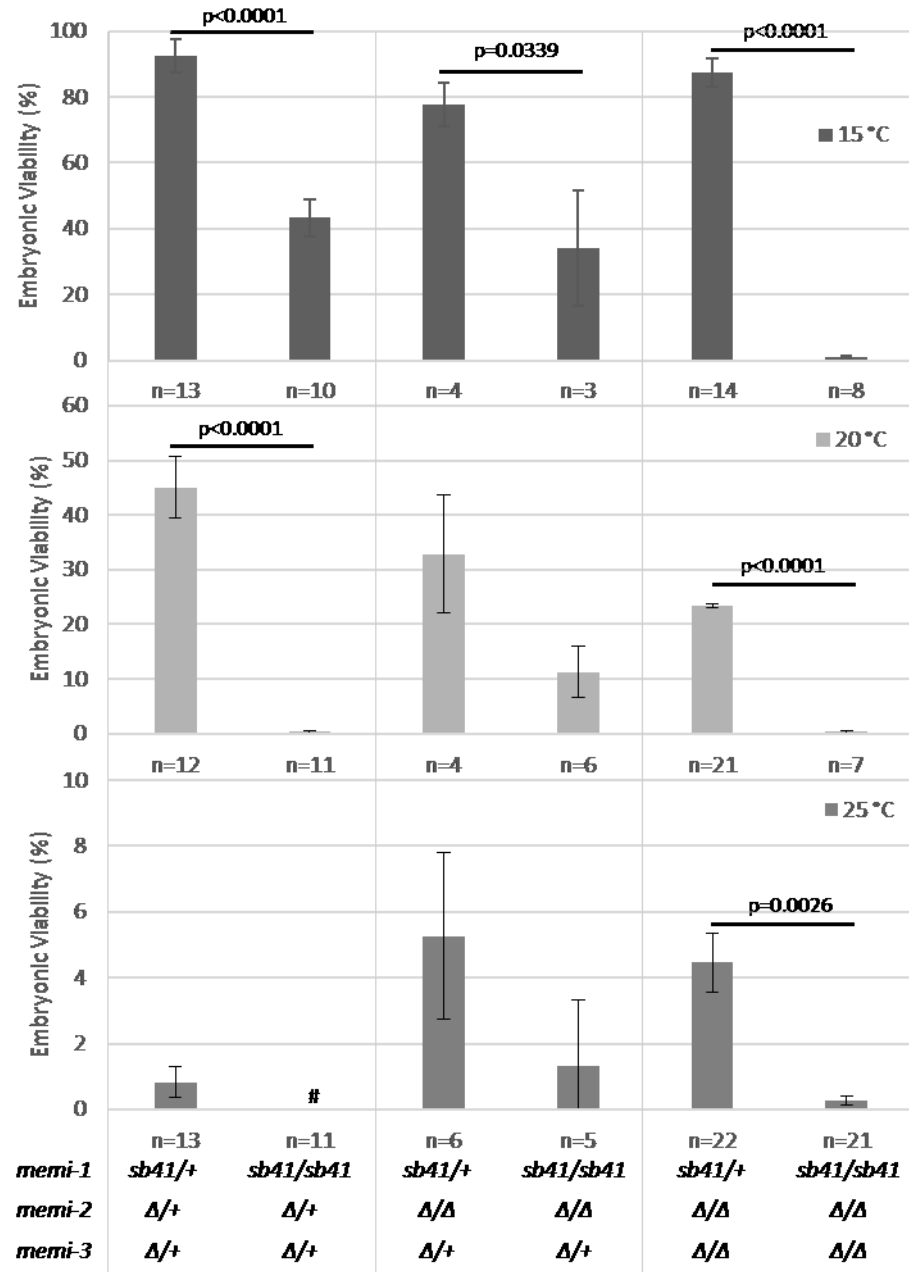
Figure 24: Comparison of *memi-1(sb41)/+* to *memi-1(sb41)* in *memi-2Δ memi-3Δ* backgrounds
Error bars show standard error. # notes values that are zero. Significance determined by two-tailed t-test (p<0.05).

3.6.1.1   Embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ* hermaphrodites mated to males
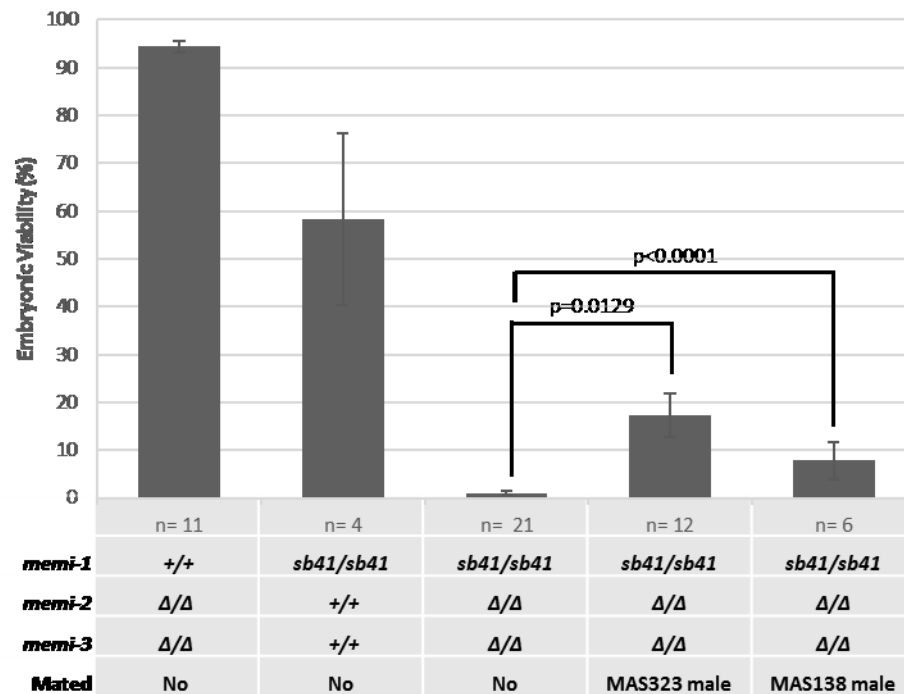
Figure 25: Embryonic viability of *memi-1(sb41)* Worms with *memi-2Δ* and *memi-3Δ*
p value if for two-tailed t-tests. Error bars show standard error. All worms incubated at 15 °C.

memi-1(sb41) memi-2Δ memi-3Δ worms showed significantly lower embryonic viability at 15 °C (Figure 20, pg65). However, further observations indicated that there was a significant increase in embryonic viability at 15 °C when the *memi-1(sb41)* in the *memi-2Δ*, *memi-3Δ* worms were mated to males of the same genotype (Figure 25, pg71). This recovery of embryonic viability of *memi-1(sb41)* in the *memi-2Δ*, *memi-3Δ* worms (Figure 25, pg71). When the two mated strains were compared to one another the differences were found to be insignificant at 15 °C again using a two-tailed t-test (Figure 25, pg71).

4   Discussion

4.1   <u>Summary of important findings</u>

4.1.1   Identification of *memi-1(sb41)* suppressors

The purpose of my investigation was to identify the 17 suppressors of *memi-1(sb41)* generated by EMS mutagenesis, which remained unknown after the initial screening for intragenic *memi-1* mutations. Using complementation testing, mapping, and whole-genome sequencing, I found nine putative extragenic suppressor mutations in two genes *gsp-4* and *R03D7.5*, as well as one intragenic mutation *abc55* which was not sequenced in initial screening for intragenic mutations. While incomplete, the mapping and complementation data narrows down the number of possible suppressor mutations in the remaining 7 suppressor strains, leading to some interesting candidates that make a compelling case for being possible suppressors.

4.1.1.1   Complementation testing of *memi-1(sb41)* suppressors

When tested for their effect on embryonic viability of *memi-1(sb41),* all the suppressor alleles were found to be recessive or semi-dominant. This allowed me to use the alleles in complementation tests and determine possible relationships between alleles, because all of the heterozygous suppressor strains were had significantly lower embryonic viability than when homozygous. Not every possible complementation pairing was tested. While the great deal of non-allelic non-complementation found in the complementation groups is not useful for finding suppressor mutations, it did indicate that two sets of mutations were suppressors. The tests were useful in determining the identity of two suppressing genes *gsp-4* and *R03D7.5.* It also provided some interesting incidental information in some cases of non-allelic non-complementation, however due to the frequency of non-allelic non-complementation, further testing of all possible complementation pairings may be useful. In total four individual complementation groups were found, with three suppressors not being eliminated from all complementation groups. This indicates that there is a minimum of four complementation groups and a maximum of seven that suppress *memi-1(sb41).*

4.1.1.2   Identifying *memi-1(sb41)* suppressing mutations

From analysis of the whole genome sequencing (WGS) data, it was found that there were two genes that were commonly mutated that were confirmed as suppressors through complementation testing and mapping of the suppressor alleles: *gsp-4* and *R03D7.5*.

Additionally, the *abc55* strain was revealed to contain a be an intragenic second-site mutation in *memi-1(sb41)*. Firstly, *gsp-4,* which had 5 different alleles: *abc39*, *abc40*, *abc44*, *abc47*, and *abc48*. *gsp-4* was known to be a suppressor of *memi-1(sb41)* through RNAi testing (Ataeian et al. 2016). The *R03D7.5* suppressor was completely new, with 4 different alleles: *abc41*, *abc51*, *abc57*, and *abc59*. The remaining 7 alleles that were determined to be extragenic suppressor, as they lacked mutations in *memi-1* were all determined to be caused by mutations in different genes. While a few of these unknown strains did share mutated genes with one another, the strains either did complement, or the mutation was found in a linkage group that was unlinked to suppression of *memi-1(sb41)*. This strongly indicated that the remaining extragenic suppressing mutations all occur in different genes, suggesting that there are at least 7 genes that have the potential to suppress *memi-1(sb41)* in addition to *gsp-4* and *R03D7.5*. Identification of these remaining genes will be key to understanding the MEMI pathway and the *C. elegans* oocyte meiosis-to-mitosis transition.

4.1.1.3 Narrowing down other candidates for *memi-1(sb41)* suppression

An important point to consider when isolating suppressor mutations created by EMS mutagenesis, is that many other molecular lesions will also be present in the strain, and these can contribute to related or unrelated phenotypes. During my work, I outcrossed many of the suppressor strains, to remove other mutations that were not responsible for suppression of *memi-1(sb41)*. Despite this, there are still many candidate mutations that could be responsible for the suppression of *memi-1(sb41)* amongst the suppressor strains: *abc36*, *abc42*, *abc52*, *abc53*, *abc54*, and *abc56*. These can be narrowed to a few potential candidates through linkage mapping. A few of these candidates have been implicated in oocyte meiosis in previous studies. These candidates could be confirmed as suppressors, through SNP mapping and further study could determine the role these genes play in controlling oocyte meiosis progression.

4.1.2 The *memi-1(sb41)* in different *memi-2/3* deletion backgrounds

Previous experiments have shown that the *memi-1(sb41)* mutation behaves as a hypermorph as adding more copies of *memi-1* to a *memi-1(sb41)* background increased the severity of the Mel phenotype (Ataeian et al. 2016). Finding suppressor mutations of the *sb41* Mel phenotype then could be instrumental in discovering other biologically relevant proteins involved in the genetic regulation of the meiosis-to-mitosis transition, as we presume anything that lowers the activity of

MEMI should negate the effect of the presumed hypermorph. However, *memi-1(sb41)*, and the newly discovered suppressors have always been tested in a background containing WT *memi-2* and *memi-3*. This is problematic as it is unknown whether the *sb41* renders MEMI-1 unable to function during meiosis, because it is compensated for by MEMI-2/3. This is also important for assessing the suppressors of *memi-1(sb41)*, because they could be acting on WT MEMI-2/3 in order to suppress MEMI-1(*sb41*). I showed that the Mel phenotype of *memi-1(sb41)* could be partially rescued through the deletion of WT copies of *memi-2/3* decreasing the total MEMI activity. This was shown though only for *memi-1(sb41)* strains containing deletions of either or both *memi-2Δ* and *memi-3Δ* at 20 °C. However, this is dependant on the strain containing at least one WT copy of *memi*. When all copies of WT *memi* were removed in *memi-1(sb41) memi-2Δ memi-3Δ* worms, there was no embryonic viability which is inconsistent with the mutation acting as a hypermorph. This suggests that *memi-1(sb41)* does not retain normal WT *memi-1* function giving it the properties of a neomorph in a *memi-2Δ memi-3Δ* background. This should be considered in all future research into the *memi-1(sb41)* allele as although it behaves as a hypermorph, this is only when the allele is assessed in a WT background where the two redundant copies of the gene may be compensating for it loss of WT function.

4.1.2.1   Embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ*

Additional experiments into the nature of the *memi-1(sb41)* showed that there was little to no embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ*, as worms with no WT copies of *memi* had no embryonic viability, even at the regularly permissive temperature of *memi-1(sb41)*, 15 °C. This indicates that *memi-1(sb41)* is not sufficient alone to maintain embryonic viability unlike WT *memi-1(sb41)* (Ataeian et al. 2016). While this is an important distinction between WT and *memi-1(sb41)*, what is more curious is that this effect is somewhat diminished when the *memi-1(sb41) memi-2Δ memi-3Δ* worms' oocytes are fertilized with male sperm, as opposed to self-fertilized by the hermaphrodites endogenous sperm. This insight may contribute to future understanding of the interaction between sperm contributed factors and the ability of MEMI to detect fertilization.

4.2   Confirmed suppressor mutations of *memi-1(sb41)*

4.2.1   Novel *memi-1(sb41)* suppressors alleles identified in *gsp-4*

The most common suppressors found were mutations in *gsp-4*: a paralogue of the catalytic subunit of protein phosphatase 1 (PP1). PP1 is a serine/threonine phosphatase, and is a multi-role phosphatase important in sperm meiosis, sperm activation, and sperm motility (Shirayama et al. 2006; Schlesinger et al. 1999). The role of PP1 in sperm motility is very broadly conserved in flagellate sperm as well as the amoeboid sperm of *Caenorhabditis* (Wu et al. 2012). In *C. elegans*, *gsp-3/4(RNAi)* results in non-motile and polyploid sperm, which exhibit bridged nuclei during spermatogenesis MII (Wu et al. 2012). To regulate phosphorylation of its many targets, the PP1 catalytic subunit interacts with many different regulatory subunits, and is estimated to be guided by more than 200 regulatory subunits (Peti, Nairn, and Page 2013). Several amino acids in PP1 are highly conserved across all eukaryotes, that are important in promoting the catalytic action of the protein or binding domains. Essential to the proper function of PP1 are amino acids that allow the protein to associate with two Mn^{2+} ions, which are required for in the catalytic site in order for the phosphatase to function (Peti, Nairn, and Page 2013).

Based on the suppression of *memi-1(sb41)* by *gsp-3/4(RNAi)* it was hypothesised that because *memi-1(sb41)* acted as a hypermorph, lowering the signal contribute to the oocyte from the sperm would lower the Mel phenotype (Ataeian et al. 2016). In this respect if the *gsp-4* suppressor mutations were acting the same way, we would expect to find mutations that lower GSP-4 function, similar to knockdown by RNAi. Amino acids needed to bind Mn^{2+} ions required for catalytic action of the enzyme, then would naturally be good candidates for incurring mutations, which is was observed. However, it should be noted that all mutations were only recovered occurred in *gsp-4*, suggesting some functional difference to *gsp-3*.

4.2.1.1   The significance of *gsp-4* alleles in the absence of *gsp-3* alleles

From a genome-wide RNAi screen for suppressors of *memi-1(sb41),* only two genes rescued the Mel phenotype, *gsp-3* and *gsp-4* (Ataeian et al. 2016). *gsp-3* and *gsp-4* have 97% DNA similarity and cannot be knocked down separately by RNAi methods (Ataeian et al. 2016), thus, it is likely that the suppression involves the reduction of both *gsp-3* and *gsp-4* activity, as knocking down the genes through RNAi would have a similar effect. Previous work indicated that GSP-3 and GSP-4 are enriched in the male germline and that the two highly-similar proteins

likely act redundantly for sperm motility and male meiosis (Chu et al. 2006). Therefore, it is likely that the suppression of *memi-1(sb41)* requires a reduction, and not complete loss, of *gsp-3/4* activity. If *gsp-3* and *gsp-4* are completely redundant in their functions, it was not known if suppressor mutations within either single gene alone would suppress *memi-1(sb41)*. However, the RNAi results indicated that sb41 is very sensitive to dosage of GSP-3/4 activity; the RNAi conditions that conferred suppression of *memi-1(sb41)* did not result in any obvious phenotypes in wild-type worms (Ataeian et al. 2016). My analysis of the WGS data revealed mutations within *gsp-4* in five different suppressor strains, each the result of a different single nucleotide polymorphism (SNP) causing missense mutations. Ethyl methane sulfonate (EMS) mutagenesis most commonly introduces G/C→A/T transition mutations throughout the genome randomly (Jansen et al. 1997), suggesting that mutations within *gsp-3* should have been equally likely. However, my results showing that mutations in *gsp-4* alone were sufficient to suppress the *memi-1(sb41)* Mel phenotype. This could imply that *gsp-3* and *gsp-4* may have some specialized functions that differ from one another, at least with respect to the *memi* pathway. However, the two genes are highly similar with 98% DNA identity, so it may be more likely that the difference could be due to differences in the expression of the two genes. Because the knockdown of *gsp-3/4RNAi* is imprecise it could be possible that either knocking out *gsp-3*, decreases expression too much for the worms to be viable, or too little for *memi-1(sb41)* to be suppressed.

4.2.1.2   Possible results of *gsp-4* suppressor mutations on protein function

As the functional domains of PP1 are known, it is possible to use this information to postulate the effect on GSP-4 function. One element of PP1 structure involves two metal coordination regions that are highly conserved across PP1, PP2A, and PP2B in humans and across eukaryotes (Peti, Nairn, and Page 2013). Three mutations were found within the active site and could directly interfere with catalytic activity of the PP1 enzyme. *abc44* is a G276A substitution that results in a H54Y amino acid change, and *abc39* is a C352T substitution that results in a G89E amino acid change (Figure 13, pg58). Similarly, the glycine at position 89 in the protein could affect the metal coordination of the protein: the amino acid is not noted to affect metal coordination, however, the adjacent aspartic acid at amino acid position 90 has been (Peti, Nairn, and Page 2013).

Only one other mutation of *gsp-4* in a different recognized region of PP1 is *abc40*. The mutation is a C to T substitution at position 221 that introduces a missense mutation by changing a G to R at amino acid 221 (Figure 13, pg58). This residue is noted to be important in interacting with other molecules (Peti, Nairn, and Page 2013). *abc47* contains two missense mutations. The first one is a G235A substitution causing a L50H change in the amino acid (Figure 13, pg58). The L50H missense mutation in *abc47* occurs at the boundary of an alpha-helix and beta-sheet, where it could affect protein folding. The first mutated amino acid is likely important, as it is conserved between GSP-3, GSP-4, and human PP1. The second mutation in *abc47* is a C406T substitution, changing amino acid C107Y. This mutation occurs in a locus that is only conserved between GSP-4 and GSP-3, but not with human PP1. In *abc48* a missense mutation causes a C268T substitution, changing a G61E change in the amino acid (Figure 13, pg58). As these regions are broadly conserved, they likely are important for proper protein function (Peti, Nairn, and Page 2013).

In complementation testing, the alleles *abc40*, *abc47*, and *abc48* failed to complement *acb39*. This indicates that these *gsp-4* mutations likely suppress *memi-1(sb41)* in the same manner, as all the mutations are predicted to impair proper function of GSP-4. As at least one of the mutation *abc39* would disrupt Mn^{2+} binding within the phosphatase active site, and would be expected to exhibit null or hypomorphic behaviour. This corroborates the previously described *gsp-3/4(RNAi)*, which suppresses *memi-1(sb41)* through a partial knockdown of GSP-3/4 levels (Ataeian et al. 2016). The *abc39* mutation is recessive, though, indicating that a single copy of the allele is not sufficient to decrease GSP-4 activity enough to suppress *memi-1(sb41)*, however when paired with the other mutations they fail to complement indicating that they likely suppress in the same manner by decreasing the activity of GSP-4. To increase the sensitivity of the assay for suppression, each allele was also tested as a heterozygote in the background of heterozygous *memi-1(sb41)/+* at 25 °C. At 25 °C, *memi-1(sb41)/+* typically yields ~6% hatching in the absence of suppression. For those tested, *abc39/+* had no effect on *memi-1(sb41)/+*, however *abc40/+ memi-1(sb41)/+* did suppress as well as *abc40 memi-1(sb41)* and *abc48/+ memi-1(sb41)/+* showed suppression and it was also significantly higher than *abc48 memi-1(sb41)* (Figure 14, pg59). This analysis allowed a stratification of the relative suppressor strengths, and it suggested that the different alleles have different effects on the function of the GSP-4 protein.

To further characterize the *gsp-4* alleles, I attempted to separate the *gsp-4* mutations from the original *memi-1(sb41)* mutation. I isolated *gsp-4(abc39)* from the *memi-1(sb41)* background and found that the worms exhibited 26% embryonic viability. *abc39* causes a missense in the amino acid adjacent to a residue involved in interacting with Mn^{2+}, which likely abolishes phosphatase activity. It should be noted that this is a separate amino acid than that of *abc44* which is also noted to have an affect on binding Mn^{2+} ions. Relative to the other *gsp-4* suppressors tested in the less severe *memi-1(sb41)/+* background, *abc39* was the weakest. Therefore, the ability of *gsp-4* mutations to suppress *sb41* may not correlate with changes in phosphatase activity. Alternatively, the decrease in embryonic viability might be unrelated to the *gsp-4* mutation, as other mutations are likely still present in this strain.

4.2.1.3   Complementation of *gsp-4* alleles

Four of the *gsp-4* suppressor strains were directly tested for complementation (*abc39* with *abc40*, *abc47*, and *abc48*), and all three pairs failed to complement (Figure 11: 1, pg47), indicating that the suppressor mutations likely affected the same gene. Five different mutations in *gsp-4* affected conserved amino acids that are present in GSP-3 and human PP1. The *abc47* allele contained a second SNP that results in a missense mutation in an amino acid is only shared with GSP-3.

4.2.1.4   Possible functional differences between *gsp-3* and *gsp-4*

Despite the high degree of conservation between GSP-3 and GSP-4, it is interesting that all suppressor mutations were in *gsp-4*. This may suggest a difference in function between *gsp-3* and *gsp-4,* although it may be due to chance or a differential probability that each gene become mutated through EMS mutagenesis. Assuming the chances of mutations occurring in *gsp-3* and *gsp-4* are the same there would only be a 3.125% chance that all five suppressors would appear in *gsp-4* as if the chances of a mutation causing suppression of *memi-1(sb41)* in each were equally likely there is a very small chance that all the mutations would occur in the same gene. This suggests that there is a functional difference between *gsp-3* and *gsp-4,* which, due to their similarity, likely arises in the level of protein that is expressed. One possibility for why *gsp-3* was not found in the EMS suppressor screen is that disrupting GSP-3 is not sufficient to suppress *memi-1(sb41)* when knocked out because normal levels are not high enough. Or the second possibility; disruption of GSP-3 does not result in viable embryos. While *gsp-3* deletion alleles

that represent likely nulls have been tested and are viable (Wu et al. 2012), it has not been confirmed in a *memi-1(sb41)* background. Testing of whether the same mutations in *gsp-3* are viable and are sufficient to suppress *memi-1(sb41)* could be confirmed through introducing the same mutations to a *gsp-3* in a *memi-1(sb41)* background using CRISPR-Cas9 gene editing.

4.2.2   Novel *memi-1(sb41)* suppressor alleles in *R03D7.5*

The second gene found containing multiple suppressor mutations was *R03D7.5*, one of seven GSK3 paralogues found in *C. elegans*. *R03D7.5* is likely suppressor because four different suppressor strains contain a different mutation in this gene. Thus far, I have not been able to confirm that these suppressors map to LG II (where *R03D7.5* resides). However, for two strains where mapping data is available, *abc41* and *abc51*, the data indicates that these suppressors are likely not on LG I, III, V, or X. Furthermore, *abc51* and *abc59* failed to complement each other, suggesting that these two strains have a mutation in the same gene (Figure 11, pg47). As one of the seven paralogues of GSK3 in *C. elegans, R03D7.5* is not as conserved as *C. elegans gsk-3*. In *C. elegans* GSK-3 is involved in Wnt signalling and important for organising the EMS cells in early mitotic divisions and innervation (Schlesinger et al. 1999). However, little is known about *R03D7.5,* except that it is enriched in the male germline (Grun et al. 2014). Interestingly, phosphorylation of GSK3 isoforms by PP1 has been observed in other systems (Hernandez et al. 2010), suggesting that *R03D7.5* could interact with *gsp-4*. If there is a functional relationship between the two genes it could explain why potentially hypomorphic mutations in either of the genes results in suppression of *memi-1(sb41)*.

4.2.2.1   Possible results of *R03D7.5* suppressor mutations on protein function

Two of the strains containing mutations in *R03D7.5* result in a premature termination codon, one due to a frameshift and the other due to a alteration of a splice acceptor site. The frameshift caused by the deletion of 8 nucleic acids in *abc57* which leads to the inclusion of only the first 17% of the protein. This mutation likely impairs the function of the protein as it results in the removal of many functional domains of the protein (Dajani et al. 2001). The splice acceptor mutation in *abc51* is created by a G to A substitution in the last nucleic acid of exon 6. This mutation likely causes a read-through of the splice acceptor site. If splicing continued at the next available splice acceptor site, leaving out the intron this alternate splicing would include 65% of the WT protein followed closely by a premature termination codon due to a frameshift.

However, it is impossible to confirm without molecular data. It is also possible though, that the mRNA is degraded through nonsense-mediated decay, as it contain multiple premature termination codons which the mechanisms of nonsense-mediated decay specifically target in mRNA (Chang, Imam, and Wilkinson 2007).

Two other strains contain sequence alterations in *R03D7.5* that result in missense mutations. *abc41* contains two SNPs; are G673A and G705A causing T210I and V221I changes in the amino acids. Neither of these amino acids is conserved in *C. elegans* GSK-3 or human GSK3A. *abc59* is a G to A substitution at position 762, leading to a G240T amino acid change. This amino acid is conserved in both *C. elegans gsk-3* and human GSK3A.

4.2.2.2   Embryonic viability of *R03D7.5* alleles in different *memi-1(sb41)* backgrounds
Amongst the suppressor alleles of *R03D7.5, abc41* and *abc57* were also analyzed in a *memi-1(sb41)/+* background to determine their relative ability to supress *sb41*. The *memi-1(sb41)/+ abc41/+* worms do not have significantly different embryonic viability than either the control or *memi-1(sb41) abc41* worms (Figure 17, pg62). The *memi-1(sb41)/+ abc57/+* worms however have significantly higher embryonic viability than the *memi-1(sb41)/+* control and the worms homozygous for the same mutations (Figure 17, pg62). This suggests that *abc57* is dominant to the less severe *memi-1(sb41)/+* Mel phenotype while *abc41* is not. This shows that *abc57* is more effective at rescuing the Mel phenotype in the sensitive background of *memi-1(sb41)/+*.

4.2.3   *abc55* is an intragenic suppressor of *memi-1(sb41)*
Similar to 10 other suppressor strains that had intragenic mutations, *abc55* contains a second site mutation in *memi-1* but was not found in initial testing of *memi-1* (Caitlin Slomp, *pers. comm.*). Further evidence of the suppressor being intragenic was provided through linkage analysis which found the suppressor to be linked to LGIV, the same chromosome as *memi-1*. As an intragenic mutation *abc55* gives us more information as to the nature of the intragenic mutations as none of the other intragenic strains were tested beyond homozygous embryonic viability. The second site mutation in *memi-1* that causes the *abc55* allele is a T to A substitution at position 859, changing an R to S at amino acid 286. Testing of *abc55* indicated that the second-site mutation does not just disrupt the MEMI-1(sb41) protein as the allele as the heterozygote; *memi-1(sb41)/memi-1(sb41abc55)* did not have significantly higher embryonic viability than *memi-1(sb41)* homozygous worms, leading the suppressor to be classified as recessive.

4.3   Potential *memi-1(sb41)* suppressor candidates

The remaining seven suppressors did not contain mutations within the coding regions, introns, or UTRs of *memi-1*, *gsp-4*, or *R03D7.5*. By integrating data from WGS, complementation-testing and genetic mapping, many of the potential EMS mutations in these strains were ruled out as suppressors of *sb41*.   All the suppressors were recessive or semi-dominant and the embryonic viability of the sequenced strains indicated that the suppressors were homozygous, and any heterozygous mutations can be dismissed. There is linkage data for the remaining suppressors, which limits the possible mutations that cause suppression. There are three candidate genes in different suppressor strains, where the mutation occurs in an orthologue of a gene implicated in regulating meiosis in *C. elegans* or other organisms, including *Drosophila* and humans. These mutations present the most likely suppressors as they were the only mutations shared in genes that occurred in multiple strains and were not ruled out by complementation. In all cases where alleles were tested together, they failed to complement, rescuing the *memi-1(sb41)* Mel phenotype. It is important to note that these preliminary considerations are limited to examining only the protein altering mutations of each linkage group because the regulatory domains of most of the genes are unknown.

4.3.1   A candidate gene for the suppressor: a*bc36*

In the *abc36* strain, three mutations were identified on LG X, consistent with genetic mapping data. All the candidates on this chromosome are uncharacterized genes: *D1025.1*, *F39C12.1*, and *K09C4.5,* which have not been studied in depth in *C. elegans*. Of these, the most likely candidate is *F39C12.1,* as it is a paralogue of human MIOS (meiosis regulator for oocyte development). MIOS and its paralogues in *Drosophila* and other organisms has been found to be necessary for meiosis (Wei et al. 2014). The protein is important in mTORC1 regulation, as one of two subunits of GATOR2 (Yao, Jones, and Inoki 2017).

4.3.2   A candidate gene for the suppressor: *abc53*

In the *abc53* strain, seven candidate genes were found on LG IV, consistent with genetic mapping data. Most of these genes are uncharacterized, except for *C25G4.6*, which encodes *smz-1* (*sperm meiosis PDZ domain*-containing). Recent work in the lab indicates that feeding *smz-1* dsRNA can suppress *memi-1(sb41)* (J. Chum, *pers. comm.*), suggesting that the *abc53* suppressor mutation is in *smz-1*. SMZ-1 is required for meiotic segregation of chromosomes in

the spermatocyte (Chu et al. 2006). Like *gsp-4*, *smz-1* also has a highly similar paralogue, *smz-2* although they do not act redundantly, as they each yield a defective sperm meiosis when individually knocked down with RNAi (Chu et al. 2006). *smz-1(RNAi)* worms also exhibit many of the same phenotypes as *gsp-3/4(RNAi)* worms, including failed sperm meiosis (Chu et al. 2006).

4.3.3 A candidate gene for the suppressor: *abc56*

In the *abc56* strain, seven candidate genes were identified on LG IV, consistent with genetic mapping data. One gene, *mbk-2,* stands out as a potential suppressor because it has been implicated in regulating oocyte meiotic divisions. *mbk-2* encodes a DYRK dual-specificity Yak1-related kinase that is required for proper oocyte meiosis-to-mitosis transition. *mbk-2* has been implicated in a variety of processes important to the oocyte-to-embryo transition, where it is required for proper P-granule formation and degradation of oocyte factors prior to the first meiosis (Shirayama et al. 2006), as well as coordinating the breakdown of proteins required for the oocyte-to-embryo transition (Guven-Ozkan et al. 2010). MBK-2 acts as a substrate specific adaptor of CUL-2 in meiosis, directing it to breakdown the proteins required to allow meiosis to finish and the first mitotic division to begin being key to the breakdown of OMA and MEI, both of which are key to a successful meiosis-to-mitosis transition (Beard et al. 2016; Johnson et al. 2009). Ataeian et al (2016) showed that MEMI is downstream of CUL-2, thus if MBK-2 normally controls the timing of degradation of proteins, it is conceivable that certain mutations in MBK-2 could accelerate the degradation of MEMI, and thus suppress the effects of MEMI-1(sb41), which is resistant to proteolytic degradation during the transition to mitosis.

4.3.4 Remaining candidate mutations in the last unknown suppressor strains

The remaining four suppressor mutations remain unknown; however, the total number of possible mutations can be restricted by incorporating the mapping data. For example, *abc42* has 17 candidates on either LG II (14 possible) or LG IV (3 possible), *abc50* has 12 candidates on LG III, *abc52* has 12 candidates on LG III, and *abc54* has 25 candidates on LG II. Because the WGS data did not reveal a mutation within any common gene amongst these four strains, these suppressors likely represent four different genes. There were not more than one strain containing these possible suppressor mutations they cannot be confirmed through complementation testing. The best way to confirm this for each of the strains is to map the strains to a single LG and then

follow this up with SNP mapping for each of the individual mutations to determine how the suppression is inherited, or RNAi if the candidate has not previously been tested in the initial RNAi suppressor screen.

4.4 <u>Non-allelic non-complementation</u>

In all the complementation testing, there were 6 instances of non-allelic non-complementation. This was determined by comparing the WGS data for strains found in the same complementation groups and cross referencing this with linkage data from mapping crosses. In each of these 6 instances, the two strains that failed to complement had no genes mutated in common.

4.4.1 Complementation group 1

In complementation group 1, *abc39* and *abc55* failed to complement, as did *abc55* and *abc56*. When *abc39* and *abc56* were tested to one another, the two alleles complemented, which is contradictory to what is expected of two alleles in the same complementation group. However, neither of the two alleles had mutated genes in common in the linkage groups that they mapped to. The conflicting data was resolved by the discovery that *abc39* was an allele of *gsp-4,* and *abc55* was a second-site mutation in *memi-1(sb41)*. Non-allelic non-complementation in this instance could point to a common molecular function in the fertilized embryo. Although the molecular relationship between GSP-3/4 and MEMI is not known, co-immunoprecipitation data from Ataeian et al (2016) indicated that GSP-3/4 and MEMIs are capable of physically interacting with each other. Thus, if MEMIs act with GSPs in the fertilized embryo, it is conceivable that loss of either component would reduce overall MEMI activity and explain the suppression by trans heterozygous combinations. *mbk-2* has been implicated in protein degradation by targeting different proteins for ubiquitination (Cheng et al. 2009). Considering the importance of MEMI degradation to the completion of MII, this could suggest that *mbk-2* plays a role in this, however it is premature to say as *mbk-2* is also involved in many other aspects of the meiosis-to-mitosis transition.

4.4.2 Complementation group 2

Another case of conflicting data within a complementation group occurred in complementation group 2. Testing indicated that *abc41* failed to complement *abc44*, *abc41* failed to complement *abc54*, and *abc50* failed to complement *abc54*. These tests put all four suppressor alleles in the

same complementation group, however, analysis of the WGS data indicated that none of these suppressor strains had a mutation within a single gene that was shared by two suppressor strains, so there are none that were common to all. This does explain why *abc44* and *abc50* complemented when they were directly tested to one another because they are only linked in the same complementation group through three instances of non-allelic non-complementation. However, these cases do not explain why these alleles failed to complement one another.

4.4.3 Complementation group 3

The final case of non-allelic non-complementation in complementation group 3: *abc51* which is caused by a mutation in *R03D7.5* and *abc53* which is proposed to be found in *smz-1*. There are no obvious links between *smz-1* and *R03D7.5*[GSK3], other than that both genes are expected to be expressed during spermatogenesis (Chu et al. 2006).

4.4.4 Non-allelic non-complementation involving intragenic suppressor *memi-1(sb41 abc55)*

Interestingly the non-allelic non-complementation between *gsp-4(abc39),* and *memi-1(sb41 abc55)* shows that there are additive effects of each of the suppressors when in the same background. This could be indicative of an interaction between the two proteins or that they function in the same genetic pathway. The *gsp-4(abc39)* and *memi-1(abc55)* alleles are both recessive in a homozygous *memi-1(sb41)* background. However, as *abc55* is a second site mutation in *memi-1(sb41)* it could counteract the disruptive Mel phenotype causing these worms to be same as *memi-1(sb41)/+*. This may not necessarily be the case though as *memi-1(sb41)/+* ; *abc39/+* worms do not have equal or greater embryonic viability than when the genes are homozygous, but as *abc39* and *abc55* fail to complement in *memi-1(sb41)/memi-1(sb41abc55); abc39/+* worms had the embryonic viability was not significantly different from *memi-1(sb41) abc39*. Because both *memi-1(sb41)/memi-1(sb41 ab55)* worms and *memi-1(sb41)/+ abc39/+* worms are recessive and have low embryonic viability the *memi-1(sb41)/memi-1(sb41abc55); abc39/+* clearly has an increase in embryonic viability. This shows that in the presence of *abc39* the deleterious effects of the *sb41* allele are decreased. This suggests that there is a direct interaction between MEMI-1 and GSP-4, because embryonic viability of *abc39/+* worms improves in the case of *abc55:sb41/sb41* compared to *+/sb41*. This could be explored in the future through testing with the *abc39* and *abc55* alleles.

There is another instance of non-allelic non-complementation between *memi-1(sb41 ab55)* and *abc56* but the significance is less certain, because it is not clear which mutated gene causes the suppression in *abc56*. It is likely that the *mbk-2* mutation in *abc56* is responsible for suppression, however it needs to be confirmed through additional testing. It could be the same case where the two suppressors enhance the effects of each other, but it is not certain because *memi-1(sb41)/+ abc56/+* worms were never tested. The *abc56* allele may be dominant over *memi-1(sb41)* which would almost certainly guarantee that it fails to complement *abc55*. Again, if this were born out through additional testing this could show a direct interaction between MEMI-1 and MBK-2 however at this point any predictions are highly speculative.

4.4.5 Non-allelic non-complementation involving known extragenic suppressors of *memi-1(sb41)*

Although non-allelic non-complementation in complementation group 2 occurs between all members of the complementation group the only suppressing alleles involved in non-allelic non-complementation that are confirmed are *abc41* in *R03D7.5* and *abc44* in *gsp-4*. *R03D7.5* is a paralogue of GSK3. In mammalian cell culture GSK3 has been shown to be phosphorylated by PP1, the paralogue of *gsp-4* (Hernandez et al. 2010). Of the alleles tested, only one pair of alleles between these two genes *R03D7.5(abc41)* and *gsp-4(abc44)* failed to complement. The pairings of *gsp-4(abc39)* with *R03D7.5(abc41)*, *gsp-4(abc39)* with *R03D7.5(abc51)*, and *gsp-4(abc44)* with *R03D7.5 (abc51)* all complemented one another. This suggested that the non-allelic non-complementation was due to the specific nature of the two alleles. This is interesting, as *gsp-4(abc44)* likely interferes with Mn^{2+} binding. The complementation relationships of *R03D7.5 (abc41)* relative to *R03D7.5(abc51)* are interesting, as they are three instances where one of the alleles complements another suppressor that the other fails to complement. *gsp-4(abc44)* and *abc54* (unidentified) both fail to complement *R03D7.5(abc41),* but they complement *R03D7.5(abc51)*. *smz-1(abc53)* fails to complement *R03D7.5(abc51)* but complements *R03D7.5(abc41)*. Currently there is no clear reason why this is the case. However, if the extragenic suppressors represent genes that have a positive influence on MEMI activity (or *memi-1(sb41)* activity), then the different non-complementing suppressors could represent members of the same pathway required for MEMI activity. In this case, the dosage would reach a critical level required for suppression of *sb41* hypermorphic activity, with only certain

combinations of alleles but it should be elucidated as further information as to the nature of the suppressor mutations and their interaction is uncovered in the future.

4.4.6   Non-allelic non-complementation involving unknown *memi-1(sb41)* suppressor mutations

There is one case of non-allelic non-complementation between *R03D7.5(abc51)* and *abc53*, where *abc53* is speculated to be caused by a mutation in *smz-1*. SMZ-1 contains a PDZ domain important for anchoring proteins to substrates and protein-protein interactions (Chu et al. 2006). How these two suppressors are related is unclear, especially given that *abc53* complements with *R03D7.5(abc41)*. As the *abc53* suppressor displays the opposite complementation relationship for two different alleles of *R03D7.5*, future study on *abc41* and *abc51* may provide insight into how these genes interact with one another based on their specific mutations.

4.5   **Embryonic viability of *memi-1(sb41)* in *memi-2/3* deletion backgrounds**

The *memi-1(sb41)* mutation has been characterized in the past as a hypermorphic mutation, in part, because *memi-1(RNAi)* is WT and the presence of an extra WT copy of *memi-1(+)* worsens the *memi-1(sb41)* phenotype (Ataeian et al. 2016). One complication with such genetic analyses for this allele is that all characterization has been conducted in the presence of *memi-2(+)* and *memi-3(+)* which are known to compensate for a loss of *memi-1* function (Ataeian et al. 2016). In *memi* deletion strains where two copies of *memi* were deleted leaving only a single copy, *memi-1* and *memi-2* were each sufficient to maintain embryonic viability, while *memi-3* had a small decrease in embryonic viability. This showed that the genes acted redundantly (Ataeian et al. 2016). Some MEMI is still required, though, as triple deletions where all three copies of *memi* were deleted do not yield viable embryos (Ataeian et al. 2016). *memi-1/2/3(RNAi)* oocytes fail to enter meiosis II (Ataeian et al. 2016), which is similar to that of fertilization defective mutants (McNally and McNally 2005). This indicates that MEMI is likely involved in recognizing fertilization, whether directly as a protein that interacts with sperm protein on fertilization, or if it is required somewhere downstream in a pathway that senses fertilization.

4.5.1 The embryonic viability of *memi-1(sb41)* worms increases with the deletion of *memi-2/3*

The *memi-1(sb41)* mutation prevents worms from exiting meiosis II before proceeding to an unsuccessful mitosis. This is likely caused by improper degradation of MEMI-1, which results in a longer active period. Increasing the number of copies of WT *memi* worsened the Mel phenotype which suggested that *sb41* is a hypermorphic mutation. Previous experiments have shown that worms containing a duplication of the *memi-1* region (with the genotype *memi-1(sb41)/+/+,)* had lower embryonic viability than *memi-1(sb41)/+* (Ataeian et al. 2016). Western blotting of *memi-1(sb41)* embryos showed that the degradation of MEMI-1 not complete while it was in WT embryos (Ataeian et al. 2016). In *zyg-11(RNAi)* embryos, the substrate-specific adapter for CUL-2 ubiquitin ligase is knocked-down, and this results in all three MEMI proteins persisting into mitosis (Ataeian et al. 2016). So, without MEMI present, fertilized oocytes cannot enter MII, but when MEMI persists, oocytes cannot exit MII. While it has been firmly established that the *sb41* mutation impairs degradation of MEMI-1, it is unclear if this mutation abolishes the normal meiotic functions. If *memi-1(sb41)* is acting simply as a hypermorph, then decreasing the number of copies of *memi* should improve embryonic viability. Additionally, if MEMI-1(sb41) has normal meiotic activity, and the only effect of sb41 is to prevent its degradation prior to mitosis, then *memi-2Δ memi-3Δ* should not be different from the sb41 mutation with *memi-2(+)* and *memi-3(+)*. If these worms are not viable then it means that some critical function of MEMI-1 is impaired by the *sb41* mutation and it is being compensated for by *memi-2/3* in the WT background, or that by having WT copies of MEMI-2/3 present the normal degradation of MEMI-1(*sb41*) is aided. Understanding the specific action of the *sb41* mutation is important to properly contextualize the suppressor mutants generated in the EMS screen. For instance: by understanding the effect that lowering the amount of MEMI through introducing *memi-2Δ memi-3Δ* into a *memi-1(sb41)* background, we can determine whether it would be possible for a suppressor to rescue embryonic viability by decreasing MEMI-2/3 without affecting MEMI-1(sb41).

4.5.2 Embryonic viability of *memi-1(sb41)* worms in various *memi-2/3* deletion backgrounds suggests the mutation may be a neomorph

By introducing *memi-2Δ memi-3Δ* into the *memi-1(sb41)* background, I attempted to answer whether decreasing the amount of WT MEMI rescued embryonic viability, and whether *memi-*

1(sb41) alone was sufficient for viable embryos. With initial testing of the strains used to construct the *memi-1(sb41) memi-2Δ memi-3Δ* strains, I reconfirmed a few previously discovered characteristics of the *memi* genes. The *memi-1 memi-2Δ memi-3Δ* worms had normal embryonic viability at all temperatures tested (15, 20, and 25 °C). The same was found for the *memi-1(sb41)/+* control, which was performed for the deletion strains heterozygous for *sb41*. As previously reported by Mitenko et al (1997) I found that the *memi-1(sb41)/+* Mel phenotype was less severe than when homozygous.

At the restrictive temperature of 15 °C none of the deletion strains compared had higher embryonic viability than the controls either when homozygous or heterozygous for *memi-1(sb41)*. One strain though was significantly lower: *memi-1(sb41) memi-2Δ memi-3Δ* (Figure 20, pg65). In the absence of any WT MEMI, MEMI-1(sb41) was not sufficient alone to maintain embryonic viability at any of the temperatures tested (Figure 20, pg65). This is interesting as the supposed hypermorphic nature of *memi-1(sb41)* has been attributed to the persistence of MEMI-1 in mitotic embryos as it fails to be degraded by the proteasome over the course of meiosis (Ataeian et al. 2016). This result suggests then that either WT MEMI is required for proper MEMI-1(sb41) degradation, or that MEMI-1(sb41) does not retain all the functions of WT MEMI. In either case this suggests that *memi-1(sb41)* is acting as a neomorph which is masked by redundant copies of *memi-2/3*. It is important to note that *memi-1(sb41)* is not functioning as an antimorph as MEMI is required to enter MII, whereas the *memi-1(sb41)* phenotype prevents the exit from MII from occurring normally. Determining the nature of *memi-1(sb41) memi-2Δ memi-3Δ* worms oocyte meiosis could be observed in a mCherry:histone, GFP;tubulin background so that microtubules and chromosomes could be imaged. This decrease in embryonic viability cannot be attributed to an unknown affect of the deletions themselves as the *memi-1(sb41)/+ memi-2Δ memi-3Δ* worms exhibit embryonic viability at all temperatures, making it more plausible that the increase in embryonic viability is due to the WT copy of *memi-1* (Figure 22, pg68)*.

4.5.2.1   Lowering WT copies of *memi* in *memi-1(sb41)* worms improves embryonic
          viability

There were significant improvements in embryonic viability for some of *memi-1(sb41)* deletion strains at 20 °C. This increase in embryonic viability was seen in almost all the deletion

backgrounds in both the homozygous and heterozygous for *memi-1(sb41)* worms. This corroborates previously reported data that showed the *sb41* mutation acted as a hypermorph (Ataeian et al. 2016). There are however a couple of strains that do not have higher embryonic viability at 20 °C which should be discussed. The first strain is *memi-1(sb41) memi-2Δ/+ memi-3Δ/+*. These worms have significantly lower embryonic viability at 20 °C than all the other homozygous *memi-1(sb41)* deletion strains (Figure 20, pg65). However, there may be something peculiar with the *memi-2Δ/+ memi-3Δ/+* deletion background as the *memi-1(sb41) memi-2Δ/+ memi-3Δ/+* worms have lower embryonic viability at 25 °C than the *memi-1(sb41)/+* control and the other deletions strains (Figure 22, pg68).

4.5.2.2 Embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ* worms in the absence of WT MEMI

It should also be noted that the embryonic viability of *memi-1(sb41) memi-2Δ memi-3Δ* worms was increased in hermaphrodites that were fertilized by males (Figure 25, pg71). This is important, as it supports the hypothesis that *memi-1* responds to a signal in the sperm cytoplasm that enters the oocyte upon fertilization. *C. elegans* male sperm outcompetes hermaphrodite sperm when hermaphrodites mate with males. This is based on physiological differences between male and hermaphrodite sperm. Male sperm are larger and more motile, allowing them to displace the endogenous sperm in the spermatheca after mating. Throughout the course of this work, many of the identified suppressors were reported to be enriched in the male germline and/or involved in sperm motility and meiosis. However, having a stronger more motile sperm signal does not fit with the hypothesis that *memi-1(sb41)* acts as a hypermorph as the *memi-1(sb41)* Mel is rescued by reductions of GSP which is facilitates sperm motility. While the status of *memi-1(sb41)* as a hypermorph is more complicated than originally anticipated, it is also possible that male sperm have cellular differences that allow them to outcompete endogenous hermaphrodite sperm, there may be differences in the proteins that male sperm contain. Particularly as genes known to be involved in sperm motility such as *gsp-4* are suppressors of *memi-1(sb41)*. This could be critical to understanding how the sperm contribution suppresses *memi-1(sb41) memi-2Δ memi-3Δ,* and could be investigated through testing whether *memi1(sb41)* suppressors have the same effect in the *memi-2Δ memi-3Δ* background.

4.6   Future Directions

4.6.1   Following up on *memi-1(sb41)* suppressing mutations

Of the 9 different genes that have the potential to suppress *memi-1(sb41),* only *gsp-4* and *R03D7.5* have been studied previously. While cases can be made for 3 other suppressor alleles, *smz-1*, *mbk-2*, and *F3912.1,* these need to be confirmed. This can be accomplished through SNP mapping of the alleles to determine if they are tightly linked to the suppression phenotype. The remaining 4 suppressors that are each representing different genes could be found in the same manner, but, as many of them still have not been mapped to a single linkage group, further mapping is required for their identification.

4.6.1.1   Recreating *gsp-4* mutations in *gsp-3*

While some initial work has been done to characterize the suppressing mutations, more work needs to be done to determine the consequences the mutations have on protein function. All the mutations were found in *gsp-4,* which exhibits 97% similarity to *gsp-3*, however, would the same mutations in *gsp-3* be able to suppress the Mel phenotype of *sb41*? This could be determined through the creation of *gsp-3* mutants using CRISPR Cas9 gene-editing techniques in a *memi-1(sb41)* background. Although care would have to be taken to construct the guide sequences to only target *gsp-3,* but seeing if the mutations fail to suppress could provide evidence that *gsp-3* and *gsp-4* act differently in the meiosis-to-mitosis transition of oocytes. After this testing of strains containing both mutations in *gsp-3* and *gsp-4* could be tested by crossing them together.

4.6.1.2   Investigating protein interactions by combining *memi-1(sb41)* suppressor strains

The complementation testing revealed that there is a great deal of non-allelic non-complementation between suppressors of *memi-1(sb41)*. However, much of the complementation data was still helpful in finding suppressors, particularly the alleles of *gsp-4,* which make up the majority of complementation group 1. While the other complementation tests only revealed multiple alleles in *R03D7.5*, many instances of complementation were able to eliminate suppressors that shared mutations in the same gene. The non-allelic non-complementation, while not very revealing at this point, will hopefully be useful in the future to determine the nuances of genetic interactions between the suppressor alleles.

4.6.2 Further investigation of *memi-1(sb41) memi-2Δ memi-3Δ* worms

Future work with the *memi-1(sb41) memi-2Δ memi-3Δ* strains should be performed to determine if the suppressor mutations described in this thesis can also suppress the strain that has no memi-2 or memi-3 activity. Given that *memi-1(sb41) memi-2Δ memi-3Δ* worms have no embryonic viability at 15 °C, and *memi-1(sb41)* with some copies of *memi-2/3* deletions rescue embryonic viability at 20 °C, it may be worth checking if there is a balance between the severity of *sb41* phenotype and the number of WT copies of *memi*. This could be investigated by recreating the *sb41* mutation in the conserved regions of *memi-2* or *memi-3* in order to create worms with higher *sb41* content. The easiest test to perform though would be testing *gsp-3/4RNAi* against *memi-1(sb41) memi-2Δ memi-3Δ* worms to see if suppression occurs. This is particularly interesting in lieu of the discovery of multiple mutant alleles in *gsp-4* and the possible suppressor *smz-1* as they are sperm specific, and could also be tested to these *memi-1(sb41) memi-2Δ memi-3Δ* hermaphrodites by mating them to suppressor males. As outcrossed sperm has already been determined to play a role in rescuing the embryonic viability of these worms, these experiments could provide insight into the specific sperm contributed factors and how they interact with MEMI-1(sb41) and WT MEMI differently.

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Appendices

Name	Gene Amplified	Nucleotides	Usage
gsp-4 For PCR	*gsp-4*	GCA TCT TAC TCC CCA AAT C	PCR
gsp-4 Rev PCR	*gsp-4*	TGG TGG TGG CAA TCT ATC G	PCR
Y17G9B.9 F	*memi-1*	TAT TTG ACC TAT TTG ACG TAT TTG ATC CC	PCR
Y17G9B.9 R	*memi-1*	ACA CAC AAA TAA CTT TTC ATG	PCR
memi-2 PCR For	*memi-2*	TGC GTC GTG GTG ATA GC	PCR
memi-2 PCR Rev	*memi-2*	GCC AGG AAG GAA GTC TGA G	PCR
H02I12.5 F	*memi-3*	GCT TCG AAT TCT TAT TTT TCC	PCR
H02I12.5 R	*memi-3*	AGA GCA AGA GTG TAC GTA AAA TAG	PCR
R03D7.5 For PCR	*R03D7.5*	ACG AAA CAC CCA TTA GAT C	PCR
R03D7.5 Rev PCR	*R03D7.5*	CTG TAC CTG ACT ACC GAA AGC	PCR
gsp-4 for seq 1	*gsp-4*	TCA TCA TAC TTT CCG CGC TA	Sequencing
gsp-4 for seq 2	*gsp-4*	GTC AAC TTA CTT CGC TGC G	Sequencing
memi-1 seqR2	*memi-1*	CAT CCC AAC CAA CGA CCA C	Sequencing
R03D7.3 Rev Seq 3	*R03D7.5*	CCA AAC CTT GTC ATT CGC AT	Sequencing
R03D7.5 For seq 1	*R03D7.5*	GAA ACT TTT TCA TCC GGC	Sequencing
R03D7.5 For seq 2	*R03D7.5*	AAT GAG AAG ACA GGA AGT GC	Sequencing
R03D7.5 For seq 3	*R03D7.5*	TTG GAG TAA AAA GGC CTA GA	Sequencing
R03D7.5 Rev seq 1	*R03D7.5*	TCT AAT TTT TCT CTC TAA TTT CCA A	Sequencing
R03D7.5 Rev Seq 4	*R03D7.5*	AGG TCC TGT GGT AAA TAG TCC	Sequencing

Table 10: List of PCR primers

Complementation test				n	Mel		χ2	Avg. of non-Mel	St. Dev. of non-Mel	Complements
Allele 1	Avg. EV	Allele 2	Avg. EV		No	Yes				
abc36	64.87	*abc41*	17.25	7	7	0	7.00	none		n too low
abc36	64.87	*abc47*	57.84	1	1	0	1.00	none		n too low
abc36	64.87	*abc53*	68.15	1	1	0	1.00	none		n too low
abc39	58.54	*abc42*	55.58	5	4	1	1.80	10.67	0.00	n too low
abc39	58.54	*abc57*	39.23	6	6	0	6.00	none		n too low
abc40	81.17	*abc47*	57.84	1	1	0	1.00	none		n too low
abc42	55.58	*abc56*	18.55	5	5	0	5.00	none		n too low
abc42	55.58	*abc59*	19.06	6	4	2	0.67	8.49	0.69	n too low
abc44	73.48	*abc48*	50.10	7	7	0	7.00	none		n too low
abc44	73.48	*abc59*	19.06	6	6	0	6.00	none		n too low
abc47	57.84	*abc53*	68.15	2	2	0	2.00	none		n too low
abc48	50.10	*abc50*	77.59	6	6	0	6.00	none		n too low
abc48	50.10	*abc53*	68.15	1	1	0	1.00	none		n too low
abc51	40.42	*abc52*	19.81	4	4	0	4.00	none		n too low
abc51	40.42	*abc56*	18.55	5	3	2	0.20	11.20	6.42	n too low
abc53	68.15	*abc56*	18.55	4	3	1	1.00	12.82	0.00	n too low
abc53	68.15	*abc57*	39.23	1	1	0	1.00	none		n too low
abc53	68.15	*abc59*	19.06	2	2	0	2.00	none		n too low
abc54	22.93	*abc55*	36.87	7	6	1	3.57	13.68		n too low
abc54	22.93	*abc59*	19.06	1	1	0	1.00	none		n too low

Table 11: Complementation tests with insufficient n to be determined
Supplementary table of complementation tests that did not yield significant conclusions on the complementation. EV stands for embryonic viability.

MAS220abc53

I			X
B0205.1	C38C6.3	srg-3	6R55.2
C34B2.10	clec-122	Y71D11A.3	attf-5
C35E7.10	emb-27	Y82E9BR.18	C28G1.4
F39B2.5	F53C3.6	ZK632.11	C30E1.4
F55H12.3	F55C12.1	**IV**	C46H3.3
fkh-10	fbxa-165	B0035.6	C53C11.1
gip-2	K09E4.4	C25G4.6	che-2
irx-1	ltd-1	cyp-31A5	E01G6.1
K06A5.1	metr-1	F53H1.4	F16H11.2
lrp-2	prx-13	xpc-1	F19C6.3
maea-1	ptc-2	Y116A8A.6	F28H6.6
mel-26	srw-62	Y24D9B.1	F43B10.1
oac-13	T02G5.4	**V**	F52E10.3
oac-34	T22C8.1	atx-3	F55A4.4
pop-1	T13H5.8	C02G6.1	frpr-17
sig-7	xrn-2	C05C8.2	H02F09.3
smd-1	ZC101.1	C50E3.12	H03E18.1
smgl-1	ZK84.2	C50F4.10	H11E01.3
vab-10	**III**	F09G2.1	K09E3.7
W03D8.9	C05D10.4	F15H10.8	nlg-1
W04G5.8	C13B9.2	F26D2.3	osm-11
Y106G6H.5	C23G10.7	F31E9.3	R04E5.2
Y47H9C.9	C27F2.8	F33E11.3	sod-3
Y92H12BL.5	C30A5.10	gsnl-1	sup-10
ZC247.1	dig-1	K09H11.11	T04C10.3
ZK909.3	emb-30	sdc-3	T08D2.7
II	F20H11.5	srb-18	trk-1
acs-15	F25B5.2	srw-55	W07E11.1
bmy-1	F40H6.2	Y38H6C.17	ZC373.3
btb-4	H14E04.2	Y45G5AL.1	
C32D5.7	K01A11.3	ZK856.11	
	numr-1		

MAS241abc47

I			
apr-1	Y92H12BR.3	R53.2	tag-80
cars-1	ZK1053.1	tag-124	Y116A8A.6
F14B4.3	ZK1225.5	xrn-2	**V**
fasn-1	ZK909.3	Y17G7B.3	egl-8
gip-2	**II**	Y51H7C.13	F15H10.8
gsp-4	C06C3.3	**III**	ttn-1
lrp-2	C09E8.1	C02F5.13	**X**
oac-13	C38C6.3	numr-1	6R55.2
pqn-21	decr-1.3	rpn-6.1	C30E1.4
sig-7	F18A1.6	unc-45	C53C11.1
smd-1	jun-1	**IV**	F52E10.3
Y44E3A.4	K05F1.9	acc-1	K09A9.6
	K09E4.4	gcy-23	osm-11

MAS247abc40

I			
C01G8.1	T28B8.3	numr-1	frpr-18
cdt-1	ZK909.3	pdr-1	srj-22
dhhc-2	**II**	rnf-121	srw-21
F16C3.4	C38C6.3	unc-103	str-252
gip-2	E01G4.6	Y102E9.6	str-93
gsp-4	K09E4.4	Y82E9BR.23	T23B12.4
lron-9	M28.2	ZK370.4	ttn-1
oac-13	ptc-2	**IV**	ZC178.1
rrf-2	xrn-2	tag-80	**X**
sig-7	**III**	tpa-1	K10C2.1
smd-1	B0285.4	**V**	lgc-23
	mel-28	C05C8.2	

MAS249abc39

I			
C17H1.5	C38C6.3	F54G8.1	C31G12.4
daf-8	clec-122	nhr-9	col-149
F28H1.5	F10E7.2	numr-1	fbxa-213
gip-2	K09E4.4	R01H2.8	H39E23.3
gsp-4	mig-14	T21C12.8	K09H11.11
nas-30	nhr-257	Y111B2A.19	mrck-1
oac-13	oac-4	Y39A3CL.3	rrbs-1
sig-7	ptc-2	Y75B8A.33	srx-56
smd-1	xrn-2	**IV**	T07F10.1
T05F1.5	**III**	col-105	ttn-1
ZK909.3	arrd-17	tag-80	**X**
II	B0303.4	tbx-40	
best-3	C35D10.7	**V**	
C23H3.9	F14F7.5	acs-10	
	F37C12.1	C05C8.2	

MAS250abc48

I		II	
apb-3	lron-9	C38C6.3	tpa-1
C48E7.1	lrp-1	fbxa-168	**V**
C54G6.2	nmtn-1	K09E4.4	F15H10.8
cdc-6	oac-13	ptc-2	nrx-1
F46F11.1	sig-7	xrn-2	ttn-1
F49B2.3	smd-1	Y54G9A.7	**X**
gip-2	T26E3.8	**III**	F43B10.1
gsp-4	unc-95	fbxa-5	F48E3.2
itx-1	vha-16	**IV**	H03A11.2
K05C4.8	Y47H9C.9	spd-3	nmur-1
lpd-6	ZC247.1	tag-80	
	ZK909.3		

MAS251abc51

I	ing-3	numr-1	V
oac-13	K09E4.4	IV	ttn-1
T27F6.1	lin-42	tag-80	X
ubc-12	pcs-1	tpa-1	gap-1
ZK909.3	R03D7.5	W03G1.2	immt-1
II	spat-1	Y55F3AM.3	
C38C6.3	Y57A10A.25	Y55F3AM.5	
clec-122	III	Y55F3BR.7	
din-1	dat-1		

MAS253abc52

I	Y38E10A.9	unc-44	C53C11.1
smd-1	III	Y37E11AL.2	cah-3
ZC247.1	ant-1.1	Y38C1AA.9	F43B10.1
II	clp-2	V	F52E10.3
aakg-5	numr-1	H39E23.3	F55A4.3
casy-1	IV	ttn-1	osm-11
F09E5.12	clec-85	X	
F15A4.11	fbxc-45	6R55.2	
npp-3	srd-13	C30E1.4	
oac-4	tag-80		

MAS260abc36

I	smd-1	H14E04.2	tag-80
C41G7.8	tag-353	let-716	V
ced-12	vet-6	nhl-2	cyp-35A4
che-3	W06D4.2	numr-1	F15H10.8
dkf-1	xpg-1	snfc-5	gly-4
F36A2.13	ZK909.3	T22F7.4	K09H11.11
F44F1.4	II	T23F11.11	ttn-1
fasn-1	C34C6.3	Y37D8A.4	X
gip-2	lec-5	Y50D7A.3	D1025.1
hsd-1	T02G5.12	Y66D12A.13	F16H11.2
hsr-9	Y54G11A.7	IV	F39C12.1
nhr-85	III	C31H1.8	K09C4.5
oac-13	F59B2.2	fln-1	
sig-7	gly-14	M70.3	

MAS261abc44

I	lact-3	F45F2.9	C44C10.7
axl-1	oac-4	F55C9.14	C53C11.1
cpd-1	ptc-2	fbxa-106	ceh-18
F27D4.6	T06D8.1	fbxa-136	chtl-1
gip-2	ZK673.6	ftr-1	eor-2
gsp-4	III	K09H11.11	F38B6.4
lron-9	ani-1	mig-6	F43B10.1
sig-7	C29F9.5	nhr-57	F48F7.5
Y106G6A.1	cpt-3	oac-7	F52E10.3
Y34D9A.7	gbf-1	R02F11.4	fkh-9
ZC247.1	Y45F3A.1	srh-217	git-1
ZK909.3	Y66D12A.19	srt-15	lam-2
II	IV	str-222	lev-8
B0454.6	tag-80	T01D3.3	osm-11
C05C10.8	V	tmd-2	rig-1
C33C12.1	acs-14	ttn-1	sec-3
C41H7.4	asp-1	unc-41	slo-2
ceh-38	C05C8.2	Y39B6A.1	srh-11
ctns-1	C25D7.5	Y47D7A.3	syd-2
dmd-5	C55A1.11	X	vab-3
F35D11.4	clec-258	6R55.2	Y16B4A.2
F41C3.11	cmd-1	C07A12.2	
F53C3.3	F21D9.4	C30E1.4	
F56D1.1	F40G12.9	C31E10.6	

MAS266abc50

I	III	unc-16	C30E1.4
abtm-1	acdh-11	Y39A3CR.3	C53C11.1
C55C2.4	cdh-4	IV	F43B10.1
spg-7	ced-6	tag-80	F52E10.3
Y39G10AR.21	cul-2	V	F59D12.1
II	dnpp-1	B0331.2	nhr-71
cat-2	F44B9.8	cTel3X.1	osm-11
clec-20	gop-1	ttn-1	ser-1
oac-4	let-716	X	T10B10.8
W09B6.4	stt-3	6R55.2	
	T28D6.3	C09G1.4	

MAS267abc59

I	II	nas-27	III
C15C6.2	arrd-6	ptc-2	klp-20
F43G9.12	bath-1	qua-1	numr-1
frm-4	C28F5.4	R03D7.2	Y22D7AR.7
gip-2	C32B5.7	R03D7.5	Y82E9BR.13
mfap-1	C38C6.3	R12C12.10	**IV**
oac-13	C52E12.4	smu-2	tag-80
prom-1	D2062.6	trr-1	**V**
scpl-1	decr-1.2	ufd-2	ttn-1
sig-7	F07E5.9	vhp-1	**X**
tba-1	fbxb-103	xrn-2	F43B10.1
Y71G12A.2	K09E4.4	Y48B6A.5	
	mrpl-50	ZK938.3	

MAS271abc42

I	Y51H7C.12	tag-80	K09H11.11
	III	Y116A8A.6	ncx-8
II	B0464.4	**V**	sec-23
C18E9.7	C02C2.5	C05C8.2	T19B10.8
C24H12.6	C07H6.4	clec-234	T20B3.1
C34C6.4	C18F10.2	F22B8.3	ttn-1
C38C6.3	C30A5.4	F28C1.3	ZK384.3
F45D11.5	egg-2	F46B6.13	**X**
K09E4.2	lin-12	F47B8.3	C31H2.4
K09E4.4	numr-1	F47H4.2	C46H3.3
K10H10.5	rpia-1	fbxa-112	dhs-25
nep-19	ZC21.9	fbxa-172	F20B6.7
oac-4	zfp-1	fig-1	H03A11.2
pqn-87	ZK418.7	fkb-6	inx-1
ptc-2	**IV**	grd-2	
xrn-2	egl-23	hsp-6	

MAS270abc41

I	R05H10.3	Y82E9BR.18	fbxa-150
F10D11.2	tag-231	Y82E9BR.23	K03B4.2
F27D4.4	trr-1	**IV**	K09H11.1
gip-2	ubxn-3	C01F6.2	let-418
M01E11.3	xrn-2	C08F11.2	nlp-42
pqn-44	Y57G7A.8	cdh-8	srh-125
rad-54	ztf-27	eel-1	ttn-1
sig-7	zyg-9	F21D5.6	ugt-9
T02E1.7	**III**	lst-3	**X**
T25G3.4	B0303.2	oac-22	C07B5.3
Y71G12A.4	C18H2.3	pxf-1	C10E2.2
II	chl-1	R05A10.3	C30E1.4
B0228.9	F02A9.4	sucg-1	C34F6.1
C30B5.4	F40H6.2	T05A12.4	C53C11.1
C33C12.1	F56A8.8	T28F3.8	ckc-1
C38C6.3	F56D2.8	tag-80	F07G6.10
cyn-4	K01A11.1	uso-1	F41C6.7
F10E7.2	K01G5.3	Y116A8A.6	F43B10.1
F26G1.1	K04H4.5	Y116A8C.26	F52E10.3
H12I13.3	lin-13	Y11D7A.19	F55G7.1
him-14	numr-1	**V**	lev-9
hlh-14	pqn-41	abt-4	lim-4
K09E4.4	R155.3	bed-1	osm-11
M195.4	sca-1	C05C8.2	R03A10.5
mdt-26	W03A5.6	C10F3.7	R04E5.8
nhr-72	Y37B11A.2	C39F7.5	
pqn-87	Y54F10AM.11	clec-207	
ptc-2	Y56A3A.7	ehbp-1	
R03D7.5	Y66D12A.14	F15H10.7	

MAS272abc57

I	III	trp-1	F15H10.8
capg-1	C14B9.8	twk-48	F15H10.9
dyf-1	C29F9.4	Y82E9BR.18	F46B6.5
F56C11.6	C48B4.6	ZC155.4	F53F1.4
hpo-11	C56G2.1	ZC84.3	srj-18
lron-9	clu-1	**IV**	srv-3
sydn-1	F44B9.5	cyn-13	tag-293
Y47G6A.30	fbxa-29	tag-80	ttn-1
Y73E7A.1	flt-1	Y116A8A.6	Y39B6A.41
II	gei-4	**V**	**X**
C38C6.3	gsr-1	aat-2	6R55.2
F43G6.5	gst-1	C05C8.2	C18B12.6
K09E4.4	let-756	C06B8.7	C30E1.4
oac-4	lin-12	C07G3.8	C35C5.6
ptc-2	R148.2	C13A2.1	C53C11.1
R03D7.5	R151.4	C13A2.7	cnp-3
R05F9.9	set-3	C50B8.6	F52E10.3
xrn-2	srd-66	C50F4.2	osm-11
Y57A10A.29	T20B12.1	dnj-25	T04G9.7

MAS276abc56

I	math-42	**IV**	srsx-9
srbc-64	srx-102	best-22	str-77
II	T19D12.10	cutl-27	T26E4.7
C08G5.1	xrn-2	dod-24	Y51A2D.15
C38C6.3	ZC239.2	mbk-2	**X**
cpna-2	ZK1127.3	tag-80	C25G6.3
F26C11.3	ZK355.3	tpa-1	H03A11.2
K09E4.4	**III**	Y116A8C.20	npr-19
lgc-34	riok-3	**V**	srd-50
lov-1	Y39A1A.9	F57G4.1	

MAS281abc55

I	**IV**	hot-9	Y69A2AR.18
nas-30	C08F11.12	nhr-264	Y76B12C.9
smd-1	C33H5.1	otpl-1	**V**
II	C46G7.1	srv-24	sri-20
fkh-6	cdh-8	str-173	ttn-1
ptc-2	clec-180	tag-80	**X**
III	cyp-33E2	trap-3	aagr-4
numr-1	D2096.12	unc-82	C46H3.3
Y39A1A.9	eel-1	Y17G9B.9	K10B3.6
	F38A5.7	Y54G2A.44	

MAS283abc54

I	W05H12.1	tag-175	T20B6.3
B0207.5	W09C3.2	wrn-1	trxr-2
B0511.13	Y18D10A.8	xrn-2	W05B2.7
C30H7.2	Y37E3.17	Y17G7B.19	Y53G8AR.7
C54C8.4	Y6B3B.4	Y48C3A.12	Y71H2AR.3
C55C2.4	ZK909.3	ZC239.6	**IV**
DY3.8	**II**	zyg-9	F52C12.1
F46F11.6	C27H5.2	**III**	glb-7
F55D12.5	C38C6.3	C23G10.8	npp-8
F56G4.6	cpna-2	C45G9.9	srt-24
gip-2	F28B12.6	cls-1	tag-80
H25P06.1	F31E8.5	exos-1	**V**
K05C4.2	F39E9.7	F35G12.5	cyp-33C2
mfap-1	F41C3.2	F40H6.5	F13A7.14
nab-1	F45H10.2	F56C9.3	F36D3.1
nhr-2	F49E12.10	faah-4	K03D7.9
oac-13	F54D10.8	gar-2	mlt-11
pad-1	K05F1.6	H14E04.4	str-221
pqn-20	K09E4.4	klp-6	twk-36
R06C7.2	M151.1	ncr-2	Y37H2A.14
sig-7	nep-17	numr-1	**X**
smd-1	ptc-2	pcp-5	C23H4.4
tba-1	rsp-2	polq-1	
uev-3	snrp-200	R17.3	
unc-40	T02G5.11	rgr-1	

Table 12: Homozygous suppressor mutations that alter protein structure for each suppressor strain
Each header shows the linkage group. If a section is blank no mutations meet the criteria. Background mutations from the original strains are not included.