SUPPRESSION OF EGL-13 SOX DOMAIN MUTANTS REVEALS A NOVEL FUNCTION FOR SOME MEIOTIC GENES

A Thesis in
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by

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ABSTRACT

The gene egl-13 is needed for the proper development of the C. elegans uterine seam cell, an essential component of the egg-laying apparatus. In order to determine what other genes may be required for uterine seam cell development, a suppressor screen was performed, and two suppressors, him-8 and ku376 were discovered. I have characterized the genetic behavior of these two suppressors to gain insight into the molecular mechanism of their action. Only mutations within the C2H2 zinc fingers of him-8 are able to suppress the egg-laying and connection-of-gonad defects of egl-13 non-null mutants. I have found that wild-type HIM-8 acts in a semi-dominant fashion either upstream of or parallel to egl-13 in an antagonistic manner. The genetic behaviors of him-8 in suppression are different than its previously characterized genetic behavior in meiosis. I have shown that him-8 functions outside of the germ line and independently of its role in meiosis, revealing a novel function for him-8. Also, egl-13 may not be the only target of the him-8 suppressive function, as I have shown that some pop-1 defects can also be suppressed. ku376 also suppresses the defects of non-null egl-13 mutants, acting in a semi-dominant fashion. Whereas him-8 mutants also have a high-incidence-of-males phenotype, ku376 has no phenotype on its own. I have mapped the ku376 locus to the far left end of the fourth chromosome and have identified two candidate genes, but the molecular identity of ku376 remains unknown. While we do not know if ku376 plays any role in meiosis, I have shown that suppression of egl-13 defects can be effected by mutations in other meiotic genes beside him-8, suggested a broader role for meiotic C2H2 zinc finger proteins outside of meiosis.
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ABSTRACT

The *C. elegans* egg-laying apparatus is a useful system to study the fundamental mechanisms of developmental biology. One gene needed for proper development of this system is *egl-13*, a Sox domain transcription factor. In this chapter I present what is currently known about the development of the egg-laying apparatus and the mutant phenotype and putative wild-type function of *egl-13* in this process. Among several possible strategies, we have utilized a screen for mutations that suppress *egl-13* mutant defects to uncover possible *egl-13* interactors. As a result of this screen, we have identified two potential *egl-13* interacting genes, *him-8* and *ku376*, to be discussed in later chapters.
INTRODUCTION

**Model organisms in developmental biology**

Many scientists seek to understand the basic principles and underlying genetic and molecular causes of the natural processes that govern the progression of life. While it is often rewarding to learn about any organism and its inherent idiosyncratic development, certain systems are better suited for addressing a specific fundamental question and/or lend themselves better to more broad application of the principles learned. This enables researchers in these fields to study basic science, accruing understanding for the sake of understanding, while still obtaining powerful clues that could later be applied to addressing human health concerns. Although it is possible in some cases to directly observe and carry out experiments with human cells or even living humans, there are often issues involved with ethics, complexity, and even redundancy of genes that stand as obstacles to the progression of scientific discovery.

In the field of developmental biology, in order to address many of these questions in a practical and ethical manner, scientists have searched out numerous model organisms that may serve as microcosms that can represent and reveal the fundamental biological processes innate to all eukaryotes. Another rewarding aspect of using established model organisms is the wealth of knowledge and technical expertise afforded by a wide array of scientists all studying the same organism. Today due to efforts of discerning pioneers in the field, we have the ubiquitous, well-established eukaryotic systems of *Saccharomyces*
*cerevisiae, Drosophila melanogaster, Danio rerio, Xenopus laevis, Mus musculus,* and *Caenorhabditis elegans.* These systems and their respective fields have been carefully chosen and developed to harness the strengths of each system to move scientific discovery along at a fast pace.

**C. elegans**

One endeavoring scientist in particular, Sydney Brenner, recognized the potential for the tiny soil-dwelling nematode roundworm *Caenorhabditis elegans* (Figure 1.1) as an excellent candidate for such a model system (Brenner, 1974). There are many advantages to using this organism to understand fundamental biological processes as well as to model human health. *C. elegans* has a life-cycle of only four days from time of fertilization to adulthood, allowing large numbers of genetic experiments spanning multiple generations to be accomplished quickly (Brenner, 1974). The hermaphroditic nature of the worm gives rise to “self” progeny which approximate a clonal, homogeneous population useful for studying the effects of genetic mutations in large populations and for carrying out large scale screens for specific mutations. The worm’s transparent body allows visualization of all cells, just under 1000 in the adult hermaphrodite (Sulston and Horvitz, 1977), making the study of these cells and their placements relatively simple and manageable. The complete cell lineage of the worm has been mapped and is well-defined, so that all cells and their progenitors are known (Sulston and Horvitz, 1977; Sulston et al., 1983). This knowledge makes *C. elegans* an excellent tool for studying cell fate decisions, cell-cell interactions, and the formation of
organ-like systems. The complete genome of the worm has been mapped and sequenced (C.elegans-Genome-Consortium and Wilson, 1999), and many of the gene products share a substantial degree of homology with higher eukaryotes all the way up to humans. All of these attributes as well as others not mentioned and the strong and growing community of researchers combine to make C. elegans a very powerful tool in discovering some of the fundamental principles of biology and especially developmental biology while at the same time enabling modeling for addressing human health concerns.

**Development of the C. elegans egg-laying apparatus**

In my research on the developmental biology of C. elegans, I have focused on the development of the egg-laying apparatus and the genes necessary for its function. The egg-laying apparatus is required for the storage and delivery of fertilized eggs from inside the mother to the outside environment (Figure 1.2). The two main components of the egg-laying apparatus are the toroidal vulva and uterus (Figure 1.2). In addition to the cells that make up the structural toroids of the vulva and uterus, there are vulval and uterine muscle cells that attach to the walls of these and enable physical force so that as fertilized eggs pass through the spermatheca and into the uterine lumen, they are pushed out through the uterine lumen into the vulval lumen, and then exit the worm. The uterine and vulval muscles are also innervated with specific neurons that control the contractions of those muscles. In addition to these structural and neuronal cells, there are several cells that are important for the interface between the two major structural components of the egg-laying apparatus. These cells include the anchor cell, which acts much like an
organizing center during vulval and uterine development, coordinating signals to the surrounding cells; the uterine seam cell, which is needed to connect the uterus to the hypodermis and forms a laminar process separating the vulval and uterine lumens; and the uv1 cells, which attach the vulva and uterus to each other.

An important feature of this system is that it is a non-essential structure--the worm can survive without the egg-laying apparatus or its components, and as long as the genetic defects do not disrupt the development of the oocytes or embryos, the progeny are viable. This allows for manageable study and genetic manipulation of the genes and processes involved in its development. Lethal or sterile mutations would lessen the ability to mate these animals with other genotypes or study the combinatorial effect of double mutants. Mutants with egg-laying (Egl) defects also have a striking phenotype that allows easier screening and detection of such mutants. In worms that cannot lay eggs but still have viable progeny, the unlaid eggs accumulate and hatch inside the mother. The hatched progeny then eat the inside of the mother, leaving only the cuticle. This gives the appearance of a “bag of worms” (Bag) phenotype (Figure 1.2). The hatched progeny are subsequently able to eat through the cuticle and are viable.

Cell lineage of the uterus and vulva

After fertilization, the C. elegans zygote divides into an anterior blastomere (AB, a somatic “founder cell” blastomere) and a posterior blastomere (P1). The posterior blastomere divides several times to give rise to the other somatic “founder cell”
blastomeres MS, E, C, and D, as well as the germ-line precursor blastomere P_4 (Sulston et al., 1983). A subset of the MS lineage, the cells Z1 and Z4, combine with Z2 and Z3, the progeny of P_4 to make up the gonadal primordium, but only the progeny of Z1 and Z4 make up the somatic gonad (Kimble and Hirsh, 1979), of which the uterus and anchor cell are a part (Figure 1.3). The vulval precursor cells arise from the AB lineage (Sulston and Horvitz, 1977).

**Specification and execution of uterine and vulval cell fates**

The anchor cell, a central cell of the somatic gonad, helps to coordinate vulval and uterine development by signaling to the adjacent ventral uterine cells and underlying vulval precursor cells to adopt their respective cell fates (Delattre and Felix, 1999; Hill and Sternberg, 1992; Newman et al., 1995). The anchor cell induces the six nearby ventral uterine cells to adopt a π cell fate. The π cells undergo a single round of asymmetric cell division in the dorsal-ventral plane to produce twelve π progeny. In contrast, ventral uterine cells that are not induced adopt the default ρ fate and undergo two rounds of division in the anterior-posterior or left-right plane prior to differentiation (Newman et al., 1995). Eight of the twelve π progeny fuse to form the uterine seam cell and four differentiate as uv1 cells (Newman et al., 1995). The uterine seam cell subsequently fuses with the anchor cell and forms a thin laminar structure at the apex of the vulva (see Fig. 1A). The fusion event is required to clear the anchor cell from the apex of the vulva and allow for the passage of eggs into the vulva during egg-laying.
Some of the genes involved in these processes have already been identified. It is known that the induction of the π lineage requires LIN-12/Notch signaling and is mediated by the presenilin gene *sel-12* (Cinar et al., 2001; Newman et al., 1995). However, much less is known about the fate decisions allowing the lineage to differentiate into two cell types.

**egl-13 is a gene required for the proper development of the egg-laying apparatus**

A mutation of the *egl-13* locus was first isolated in a large-scale screen for mutants with egg-laying defects (Trent et al., 1983). Two alleles of this gene, *n483* and *e1447*, were obtained. The allele *e1447* is no longer available, and only the “canonical” allele, *n483*, was characterized in the original study. *egl-13(n483)* was reported to have a recessive, incompletely penetrant egg-laying phenotype (only 70% did not lay eggs, n=10), and males of this strain were able to mate, suggesting that there were no sperm defects or male mating apparatus structural defects. Also, the ability of *egl-13(n483)* mutants to lay eggs was not altered by the administration of serotonin, imipramine, levamisole, or phentolamine, suggesting that neuronal defects were not responsible for the egg-laying defects (Trent et al., 1983).

**egl-13 is required for maintenance of uterine seam cell fate**

Although *egl-13* was first isolated prior to 1983, no further published study was introduced until the discovery of alleles of a gene that was then designated *cog-2*, which was later discovered to be identical to *egl-13*. Multiple alleles of the gene *cog-2/egl-13*
were isolated in screens for Cog mutants (Hanna-Rose and Han, 1999). Improper specification or maintenance of the uterine seam cell fate can prevent fusion of the anchor cell to the uterine seam cell. When this fusion fails, no connection between the vulval and uterine lumens is formed, which is referred to as a connection-of-gonad (Cog) defect (Cinar et al., 2003; Hanna-Rose and Han, 1999; Newman et al., 1999).

*egl-13* is expressed in the π lineage (Hanna-Rose and Han, 1999). In the absence of *egl-13*, the putative π cells undergo a normal dorsal-ventral division, and they express π lineage markers including *lin-11::GFP* and *egl-13::GFP* as in wild type (Hanna-Rose and Han, 1999). Additionally, the four uv1 cells differentiate as expected in *egl-13(ku207)* mutants (L. Huang and W. Hanna-Rose, unpublished), as well as *egl-13(n483)* mutants (B. Nelms and W. Hanna-Rose, unpublished), all suggesting that π fate specification is not perturbed. However, a subset of the remaining eight nuclei undergoes an extra round of cell division, characteristic of the uninduced ρ fate and the uterine seam cell subsequently fails to fuse to the anchor cell resulting in a Cog defect and suggesting that uterine seam cell fate is not maintained or fully executed (Cinar et al., 2003; Hanna-Rose and Han, 1999). Similarly, in *lin-11* mutants, although the putative π precursors express markers characteristic of the π fate (Newman et al., 1999, L. Huang, unpublished), some divide as if adopting a ρ fate (Newman et al., 1999). Mutations in *lin-11*, which encodes a LIM domain transcription factor (Freyd et al., 1990), cause a more severe defect than mutations in *egl-13* in that neither the uv1 (L. Huang and W. Hanna-Rose, unpublished) nor the uterine seam cell subsequently differentiates properly (Newman et al., 1999), resulting in failure of the anchor cell and uterine seam cell to fuse
and a Cog defect. Also, a recent study in the Hanna-Rose lab by an undergraduate student, Sejal Vyas, has shown that a transgene encoding exc-9::GFP, a uterine seam cell-specific marker, is not expressed in egl-13(ku194) mutants (S. Vyas and W. Hanna-Rose, unpublished).

*egl-13 encodes a member of the Sox family of transcriptional regulators*

The *cog-2* locus was mapped to the X chromosome, at a distance of 4.4 cM to the left of the genetic “center” of the chromosome. A cosmid corresponding to that genetic region, T22B7, showed rescue of the phenotype and the reading frame corresponding to the locus, T22B7.1, was found to encode the gene responsible for the observed mutant phenotype (Hanna-Rose and Han, 1999). This gene is now known as *egl-13* because it later was determined to be the same locus affected by the original *egl-13* mutants, which had already long been established in the nomenclature.

The molecular identity of the gene can tell us a great deal about its function. EGL-13 is a Sox domain (SRY-related HMG box) protein (Hanna-Rose and Han, 1999). The HMG box can bind to DNA in the minor groove and induce a severe bend in the DNA target (Connor et al., 1994), perhaps acting as an architectural factor to promote the ability of other transcriptional regulators to bind the target DNA or interact with one another once bound. Sox proteins are found throughout metazoa and have been classified into groups labeled A-J according to HMG box homology (Bowles et al., 2000; Cinar et al., 2003; Hanna-Rose and Han, 1999; Prior and Walter, 1996). *egl-13* is the only group D Sox
gene in the *C. elegans* genome. The group D Sox proteins Sox5, Sox6, and Sox13 are the mammalian proteins most related to EGL-13. Murine Sox5 and Sox6 have well-studied roles in notochord formation and chondrogenesis (Smits et al., 2004; Smits and Lefebvre, 2003; Smits et al., 2001). It has been hypothesized that EGL-13 targets a gene or genes required for terminal uterine seam cell fate, perhaps acting directly or indirectly to allow recognition of the utse cell fusion partner, the AC, or otherwise promote fusion. Little is known about the targets of *egl-13* regulation or about interactions of *egl-13* with other transcriptional regulators. In order to better understand what else may be involved, we set out to look for genetic and biochemical interactions of other factors with *egl-13*.

One potential subset of interactors that we could find are EGL-13 binding partners or other direct protein interactors. Sox proteins do not generally have their own transactivation domain, and it is thought that they require a binding partner to activate targets (Kamachi et al., 2000). Protein-protein interactions could occur through several conserved but unidentified domains toward the EGL-13 N-terminus where EGL-13 homologs often have a coiled-coil domain hypothesized to be important for interactions with other factors or dimerization. Alternatively, there is evidence that the C-terminal portion of the HMG box can also participate in protein-protein interactions (Wissmuller et al., 2006), particularly with other transcription factors. Selection of Sox binding partners through protein-protein interactions in different cell types could modulate Sox function by giving the Sox protein varied functions depending on the cellular environment (Kamachi et al., 2000). We could have also discovered targets of *egl-13*. The HMG box will bind to a septamer consensus sequence. However, due to the
degeneracy of the consensus sequence and the short length, it is hard to predict targets based on promoter elements.

The elucidation of other factors that function in concert with EGL-13 in the process of uterine seam cell maintenance will give us insight into Sox factor function at Sox targets, transcriptional regulation of Sox genes, potential Sox binding partners, and how the maintenance of cell fate in general is controlled.

**Searching for egl-13 interactors**

We would like to better understand the molecular pathways that control the development of the uterus and vulva. To uncover other factors that are involved in uterine or vulva development, I set out to identify and characterize factors that interact with *egl-13*. I used several strategies to discover other factors that may be playing a role in the same pathway or a similar, parallel pathway to that of *egl-13*. These strategies were either genetic or biochemical in nature. The two genetic strategies I employed were a screen for suppressor mutations and a screen for *egl-13*-like mutations, while the two biochemical strategies were a yeast two-hybrid screen and a co-immunoprecipitation assay. While I first set out to try all four of these methods, I had the most success with the screen for suppressor mutations. As a result of this screen, I obtained two distinct suppressor mutations.
RESULTS

*Biochemical analysis of EGL-13 interactions*

I first set out to test if I could detect a biochemical interaction between EGL-13 and the protein LIN-11, also expressed in the uterine seam cell and necessary for proper development of the egg-laying apparatus, in a yeast two-hybrid system. I created a construct, pBLN1, which contained the *lin-11* gene within the pACT2 activation domain vector. A second construct, pWH39, was created by Wendy Hanna-Rose to contain the *egl-13* gene within pAS2, a DNA-binding vector. Unfortunately, even though our yeast two-hybrid system was working and all other controls were good, the *egl-13* prey vector on its own exhibited a large amount of self-activation. This could be due to inherent activation of the reporter construct promoter by EGL-13. Swapping the *egl-13* and *lin-11* fragments so that *egl-13* is within the bait vector and *lin-11* is within the prey vector has the potential to resolve this problem, but was not pursued.

*A screen for egl-13-like mutants reveals sterile mutants and more alleles of egl-13*

One genetic approach that has been pursued is a mutagenesis-based screen for mutants with a phenotype similar to *egl-13* mutants. Wendy Hanna-Rose developed an efficient screening method for discovering mutants with an *egl-13*-like connection-of-gonad phenotype. We utilized a strain expressing a transgenic marker (*kuIs38[cdh-3::gfp]*) specifically in the anchor cell (as well as two flanking vulva cells). *kuIs38;egl-13(ku207)*
animals show a pattern distinct from kuIs38 in an otherwise wild-type background in that a very bright green GFP-expressing spot (the anchor cell) can be detected under a dissecting scope equipped for epi-fluorescence because the anchor cell does not fuse to the uterine seam cell and sits at the apex of the vulva. Mutants like egl-13, in which the anchor cell does not fuse, should show GFP in a three-spot pattern, not only in the two vulval cells, but also in the unfused anchor cell. This screen, which I first performed but was subsequently performed several times by various others in our lab, produced mostly sterile mutants and other alleles of egl-13. Over 30,000 mutagenized genomes have been screened. Lethal mutations, by the nature of the screen, were avoided. The uniqueness of egl-13 and the apparent saturation of our screen speaks to the idea that egl-13 may be one of the most crucial players in proper uterine seam cell development or anchor cell–uterine seam cell fusion that is not also crucial in some other process needed for fertility.

As described above, for various reasons I did not have a large degree of success in elucidating interactions by using the yeast two-hybrid system or a screen for egl-13-like mutants. (Similarly, an effort to create a FLAG-tagged EGL-13 fusion protein was not pursued due to early problems in creating the tagged protein). However, the greatest reason that none of these methods lead to the uncovering of new interactions was the abandonment of these methods in the face of the successful generation and subsequent pursuit of characterization of two interesting egl-13 suppressors.
**Generation of the ku376 mutation as a suppressor**

One common method for identifying interacting factors is by a forward genetics approach, using mutagenesis to generate mutations and then screening for suppressor mutations. Prior to my arrival in the lab, Wendy Hanna-Rose carried out a suppressor screen of approximately 2200 ethyl methyl sulfonate (EMS)-mutagenized *egl-13(ku207)* animal genomes (Figure 1.5). The *egl-13(ku207)* background was chosen in order to avoid informational suppressors such as amber or ochre suppressors that might arise in a nonsense mutation background such as *egl-13(ku194)* (Figure 1.4).

This screen yielded one mutant that showed a significantly higher incidence of egg-laying animals among its progeny. To rule out the possibility that *ku376* was a revertant mutation or an informational suppressor, I sequenced the *egl-13* gene at the site of the *egl-13(ku207)* lesion and found the sequence to be identical to the *egl-13(ku207)* mutant sequence (not shown). To further confirm that *ku376* is a second-site mutation, I mapped the suppressor activity to the fourth chromosome, as discussed in Chapter 3.

**Discovery of him-8 as a second suppressor of the egl-13(ku207) egg-laying defect**

During the course of preparing strains for mapping the original suppressor of the *egl-13(ku207)* egg-laying defect, denoted *ku376*, a second suppressor was serendipitously discovered. The strain him-8(e1489);mIs10;egl-13(ku207), created to test if *ku376* was on the fourth or fifth chromosomes, was found to have a higher proportion of egg-laying...
animals than a strain containing the egl-13(ku207) mutation alone. Caenorhabditis elegans is naturally hermaphroditic, with less than one percent male progeny within a wild-type population, so the him-8(e1489) mutation is commonly used as a background mutation because of its high-incidence-of-males (Him) phenotype, making directed genetic crosses easier. mls10 is a transgene integrated onto the fifth chromosome that expresses green fluorescent protein (GFP) driven by a pes-10 promoter, and is used as a genetic marker for the fifth chromosome. I backcrossed the him-8(e1489);mls10;egl-13 strain into a wildtype (N2) strain and found that green mls10 animals without the him-8(e1489) mutation did not retain suppressor activity, whereas non-green animals that still had the him-8(e1489) mutation maintained suppressor activity.

To confirm that him-8(e1489) as opposed to a second mutation in the strain was indeed responsible for the egl-13 suppression we observed, I mapped the Egl suppressor activity to linkage group IV, narrowing the suppressor to the region corresponding to the location of the him-8 gene, between dpy-20 and unc-24 (data not shown), and confirmed that additional him-8 alleles suppress egl-13 mutants (shown in Chapter 2).
The nematode C.elegans and vulval-uterine development. Portions of this figure were modified from [www.wormatlas.org](http://www.wormatlas.org). White dots represent the nuclei of the syncytial uterine seam cell, shown in red.
Figure 1.2

Failure of the anchor cell and uterine seam cell to fuse (a connection of gonad, or “Cog”) results in a bag-of-worms, or “Bag” phenotype. Top panels: Micrographs using Nomarski differential interference contrast optics. The thin, laminar uterine seam cell is identified with a red arrow. The unfused anchor cell is identified with a green circular outline approximating the shape and position of the anchor cell. Middle panels: A schematic showing blockage between the uterine and vulval lumens, causing the accumulation of eggs. Lower panels: Photographs of adult worms as seen through a dissecting stereoscope, taken with a common digital camera through one ocular.
The embryonic lineage of C.elegans. The uterus and the rest of the somatic gonad is derived from the Z1 and Z4 lineages, while the vulva is derived from the AB lineage.
Lesions of the EGL-13 protein. Relative locations of the mutations within the 470 amino acid protein are designated by asterisks. The light blue and blue boxes correspond to non-null, incompletely penetrant alleles *ku207* and *n483*. The red boxes correspond to the putative null, completely penetrant alleles *ku241* and *ku194*. The specific molecular lesion for each allele is specified in the same box. The *ku241* allele is a 3' end splice site mutation at amino acid residue 158. *ku194* is a nonsense mutation changing amino acid 179 from glutamine to a stop codon. *ku207* and *n483* are missense mutations causing an alanine-to-threonine and a proline-to-leucine change, respectively. The small chart at far right shows corresponding egg-laying percentages for each allele on its own.
EMS mutagenesis and screen for suppressors of the *egl-13(ku207)* egg-laying defect. A pilot screen of approximately 3600 genomes by Wendy Hanna-Rose was performed by soaking a population of early adult *egl-13(ku207)* worms in a solution of the mutagen ethyl-methyl sulfonate (EMS) for a period of four hours. After this time, animals were placed onto new plates to recover from the mutagenesis and healthy, mobile early adult worms were picked. F2 progeny derived from isolated healthy mutagenized F1 animals were picked onto separate plates and then examined for an increase in egg-laying ability. One suppressor, *ku376*, was obtained from this pilot-scale screen.
CHAPTER 2: HIM-8 MUTANTS SUPPRESS EGL-13 DEFECTS
FOREWORD

A majority of the work and ideas presented in this chapter have been published in the journal *Developmental Biology* (Nelms and Hanna-Rose, 2006), [Full citation: Nelms B.L. and Hanna-Rose W. (2006). *C. elegans* HIM-8 functions outside of meiosis to antagonize EGL-13 Sox protein function. *Developmental Biology* 293, 392-402.] That text has been altered and extended here, but in many cases large sections are almost duplicated, so I will not cite the paper elsewhere.
ABSTRACT

HIM-8 is a C2H2 zinc finger protein that has a previously studied role in meiosis. I have found that mutations in him-8 can suppress both the egg-laying and connection-of-gonad defects of non-null alleles of egl-13. Unlike the meiotic phenotype that gives rise to the high-incidence-of-males, which is recessive, suppression of egl-13 defects by him-8 is semi-dominant due to haploinsufficiency of the him-8 locus. Suppression is not a result of a general meiotic non-disjunction defect, and suppression occurs independently of meiosis, revealing a novel function for him-8.
INTRODUCTION

I first identified the mutation *him-8(e1489)* to be a suppressor of the *egl-13* egg-laying defect when I was using this mutation as a tool to aid the mapping of the *ku376* suppressor of *egl-13* defects. Because this mutation results in a higher percentage of males (explained below), this mutation, as well as others that result in a higher percentage of males phenotype, is commonly used to make the exchanging of different genotypes (including mapping markers) between animals that are normally hermaphroditic easier.

*him-8 background*

The gene *him-8* was originally identified in a screen for mutants with defects in the pairing and segregation, or disjunction, of X chromosome homolog pairs during meiosis (Hodgkin et al., 1979). Wild-type *C. elegans* are typically hermaphroditic, with less than one-half of a percent of a wild-type population male (Hodgkin et al., 1979). Sex determination in wild-type *C. elegans* is decided by the presence of either one or two copies of the X chromosome; one X chromosome for males, two X chromosomes for females. Meiotic non-disjunction of the X chromosome results in X chromosome aneuploidy, leading to an increase in the percentage of males (or “high-incidence-of-males”, from which the gene designation derives) in a mutant versus a wild-type population. The presence of extra males is an easy phenotype to score and is thus suitable for screening mutagenized populations to obtain mutations that affect X
chromosome number. Many alleles of Him-phenotype genes have been uncovered through such screens.

*him*-8 has been previously partially characterized and studied for its effect on X chromosome pairing and segregation (Broverman and Meneely, 1994). As a result of these studies, *him*-8 is hypothesized to be required for a process initiating at the far left end of the X chromosome and affecting the meiotic disjunction of X chromosome homolog pairs. In correlation with the meiotic non-disjunction phenotype, a polar decrease in levels of recombination between the X chromosome pairs is observed, with recombination levels decreasing more and more for loci closest to the right end of the X chromosome, while an increase in recombination is actually observed at the very left end of the X chromosome. *him*-8 mutations only disrupt the disjunction of the sex chromosome, X, and the five remaining autosomes undergo seemingly normal disjunction. *him*-8 mutants have homolog pairs containing normal synaptonemal complexes (Goldstein, 1986).

**HIM-8 is a C2H2 zinc finger protein**

At the same time that I was beginning to attempt cloning *him*-8, we learned that two other labs studying the meiotic role of *him*-8 had been in the process of cloning *him*-8 for several years (A. Dernburg and P. Meneely, personal communication). *him*-8 had previously been mapped to the fourth chromosome, and combined three-point mapping data for *him*-8 from several different labs was compiled on the WormBase website.
(www.wormbase.org) and gave very specific clues to the genetic map location of him-8.

I had ordered several cosmids covering the predicted physical map location corresponding to the genetic map location in order to attempt rescue of the him-8 suppression and high-incidence-of-males phenotypes, but we soon learned that one of the other labs had recently gotten rescue with a specific fragment of one of the cosmids we had just obtained, corresponding to a predicted gene called T07G12.12, effectively discovering the sequence and molecular identity of the him-8 gene (Phillips et al., 2005). They kindly shared their data with us prior to publication, and to confirm their identification, I sequenced the three alleles of him-8 (e1489, ec56, and mn253) that we had with primers specific to T07G12.12. The predicted gene T07G12.12 (now him-8) encodes a C2H2 zinc finger protein with two C-terminal zinc finger domains and no other apparent conserved domains (Figure 2.1). The him-8(e1489) and him-8(ec56) mutations were found to be the same lesion, affecting a conserved residue of the C-terminal most zinc finger domain. The him-8(mn253) lesion was a missense mutation affecting the more N-terminal zinc finger domain (Figure 2.1).

After learning that him-8 encoded the C2H2 zinc finger protein predicted by T07G12.12, we were able to obtain a fourth him-8 allele from the C. elegans Knockout Consortium in Japan. This allele, him-8(tm611), is a large deletion that disrupts the C-terminal most zinc finger domain. We subsequently received a fifth him-8 allele from Abby Dernburg, him-8(me4), which is a missense mutation affecting the N-terminal portion of the protein (Figure 2.1).
Along with the publication of the molecular identity of him-8, a detailed characterization of the meiotic function of him-8 was disclosed. HIM-8 was found to bind the X chromosome, concentrating at the X chromosome meiotic pairing center, and localize these bound sites to the nuclear envelope, a process apparently required for the stability of homolog pairs. However, other aspects of HIM-8 function must also be required for stabilization of pairing and promotion of synapsis, because the him-8(me4) mutation does not affect X chromosome binding but still results in meiotic disjunction defects (Phillips et al., 2005).
RESULTS

*Mutation of him-8 suppresses the Cog and Egl defects of egl-13(ku207)*

As reviewed in Chapter 1, in wild-type (N2) animals the anchor cell fuses with the uterine seam cell and migrates away from the apex of the vulva leaving the thin uterine seam cell cytoplasm visible as a line at the apex (Figure 1.2). In contrast, in egl-13 mutants, the anchor cell does not fuse to the uterine seam cell. Instead, it remains at the apex of the vulva causing a connection of gonad defective (Cog) phenotype (Figure 1.3) and resulting in a subsequent egg-laying defective (Egl) phenotype (Hanna-Rose and Han, 1999). Only 6.5% of egl-13(ku207) animals have a normal vulval-uterine connection, indicating that ku207 has a strong albeit not fully penetrant Cog phenotype (Figures 2.3 and 2.4). In contrast, 45% of egl-13(ku207) animals carrying two copies of the e1489 allele of him-8 have a normal vulval-uterine connection (Figure 2.3 and 2.4), signifying that mutation of him-8 can suppress the Cog defect caused by mutation of egl-13. Morphological suppression results in a functional suppression as well. Although only 2% of egl-13(ku207) animals can lay eggs, 26% of him-8(e1489); egl-13(ku207) double mutants are Egl+ (Figure 2.2). It is interesting that proper morphology is restored to a higher percentage of the population than is egg-laying function (compare data in Figures 2.2 and 2.4). There may be another subtle or previously undetected egl-13 defect that contributes to a failure to lay eggs in some portion of animals that have regained normal morphology by executing anchor cell-uterine seam cell fusion. The fact that the egg-laying defect corresponds to the underlying defect suggests that the rescue of egg-
laying ability is not caused by alteration of any of the other components of the egg-laying apparatus such as the uterine or vulval muscles or neurons.

**Multiple alleles of him-8 suppress incompletely penetrant alleles of egl-13**

I tested various allelic combinations of *him-8* and *egl-13* to determine if multiple *him-8* alleles could suppress multiple alleles of *egl-13* and to gain insight into the mechanism of suppression. The *egl-13* alleles *ku207* and *n483* cause missense mutations (A335T and P394L, respectively) at conserved residues within the Sox domain (Cinar et al., 2003; Hanna-Rose and Han, 1999). *ku207* has a highly penetrant Egl phenotype as noted above, while *n483* is somewhat weaker; 24% of *egl-13(n483)* mutants lay eggs (Figure 2.2). In contrast, *ku241* and *ku194* are presumptive null alleles that are completely penetrant for the Egl defect; 0% of animals in either population can lay eggs (n>100 for both alleles) (Figure 2.5). *ku241* is a splice-site mutation between the fifth and sixth exons of *egl-13*, prior to the Sox domain, and *ku194* is a nonsense mutation within the sixth exon, also upstream of the Sox domain (Hanna-Rose and Han, 1999). (Also see Chapter 1, Figure 1.4)

There are multiple alleles of the *him-8* gene, which encodes a protein containing two C2H2 zinc fingers in tandem near the C-terminus (Phillips et al., 2005). The *e1489* allele of *him-8* results in a C281Y substitution in the second conserved cysteine of the second zinc finger (Phillips et al., 2005). *tm611* is a deletion allele that removes 52 internal amino acids toward the C-terminus, disrupting the second zinc finger (Phillips et al.,
2005). *mn253* results in a G259R substitution within the first zinc finger (Phillips et al., 2005), and the *me4* allele contains a missense mutation much closer to the N-terminus (S85F) and outside of either zinc finger (Phillips et al., 2005) (Figure 2.1).

I tested all four *him-8* alleles for suppression of the Cog and Egl defects of *egl-13* mutants. Like *e1489, tm611* and *mn253* also suppressed the Cog (Figure 2.4) and Egl (Figure 2.2) defects of *egl-13(ku207)*. *e1489, tm611, and mn253* also suppress *egl-13(n483)* (Figures 2.2 and 2.4). In contrast, *me4* does not suppress either allele of *egl-13* (Figures 2.2 and 2.4). Although all four *him-8* alleles have a similarly penetrant Him phenotype (Broverman and Meneely, 1994; Phillips et al., 2005) they exhibit variance with regard to the strength of suppression. *e1489* and *tm611* are more potent suppressors than *mn253*, while *me4* does not suppress at all (Figures 2.2 and 2.4). As with the *him-8(e1489);egl-13(ku207)* strain, suppression of the underlying Cog morphology defect by *him-8* mutants is consistently more effective than the functional suppression of the Egl defect. The ability of multiple alleles of *him-8* to suppress *egl-13* defects confirms that our original suppression in the *him-8(e1489);egl-13(ku207)* strain was due to the *him-8* mutation and not a hidden background mutation.

I also assayed egg-laying percentages for *him-8* mutant combinations with the putative null alleles of *egl-13, ku194* and *ku241*, and found that they could not be suppressed. Egg-laying percentages were still 0% for both the *him-8(e1489);egl-13(ku194)* and *him-8(mn253);egl-13(ku194)* strains (n=63 and 64, respectively), as well as for *him-8(e1489);egl-13(ku241)* and *him-8(mn253);egl-13(ku241)* (n=58 and 114 respectively)
(Figure 2.5). Because *him-8* mutations do not suppress presumptive *egl-13* nulls, we can conclude that the suppression induced by mutation of *him-8* is unlikely to act by bypassing the requirement for EGL-13 protein. Some amount of albeit less functional EGL-13 protein might be required for suppression to be effective. RNAi experiments confirm this conclusion. RNAi of *egl-13* eliminates suppression in a *him-8(e1489); egl-13(ku207)* strain, reducing the percentage of animals that lay eggs from the suppressed level of 26% Egl+ to 0% Egl+ (*n*=41), reiterating the inability to suppress a null-like allele (Figure 2.5). Thus, *him-8* is likely to act upstream of or in parallel to EGL-13 protein.

**Suppression by *him-8(e1489)* is semi-dominant due to haplo-insufficiency of the *him-8* locus**

Suppression of *egl-13* was first characterized in a homozygous *him-8(e1489); egl-13(ku207)* strain. Although *him-8(e1489)* has a fully recessive Him phenotype (Broverman and Meneely, 1994), I tested whether heterozygosity at the *him-8* locus could suppress *egl-13*. Suppression of the Cog (Figure 2.8) and Egl (Figure 2.6) defects of *egl-13(ku207)* by *him-8(e1489)/+* is roughly half that of *him-8(e1489)/him-8(e1489)*, suggesting a genetic model of semi-dominance in which each additional gene copy has an equal effect. To determine if the semi-dominant suppression by *him-8(e1489)* was due to haplo-insufficiency, I used a deficiency (*sDf2*) that includes the *him-8* locus (Hodgkin, 1983). Animals heterozygous for the deficiency (*sDf2/+*) suppressed *egl-13(ku207)* functional egg-laying activity at a level similar to that of *him-8(e1489)/+*, and *him-
8(e1489)/sDf2;egl-13(ku207) mutants were suppressed at a level similar to homozygous him-8(e1489);egl-13(ku207) (Figure 2.6). Therefore, we can conclude that the semi-dominant suppression by him-8(e1489) is due to haplo-insufficiency at the him-8 locus and that him-8(e1489) is a reduction-of-function mutation. This strongly suggests that the normal role of him-8 is antagonistic to that of egl-13.

**Suppression of egl-13 defects is unique to him-8 among many him mutants**

To begin to decipher the molecular mechanism of him-8 suppression, I wanted to determine if suppression is a broader characteristic of genes with a Him phenotype, perhaps as a result of meiotic non-disjunction, or if suppression is limited to mutations in him-8. Therefore, I tested additional him genes for suppression. We specifically chose to test him-1 and him-5 because of their phenotypic similarity to him-8, especially with regard to their affects on the X chromosome (Broverman and Meneely, 1994). Both him-1 and him-5, much like him-8, specifically affect pairing of the X chromosome homologs, and cause a polar decrease in X chromosome recombination levels (Broverman and Meneely, 1994).

I also examined him-3, him-6, and him-10. We found that mutations in none of these five genes [him-1(e879), him-3(e1256), him-5(e1490), him-6(ok412), and him-10(e1511)] suppress the Cog (Figure 2.7) or Egl defects (not shown) of egl-13(ku207) or egl-13(n483) mutants. Therefore, not only is suppression by him-8 not an indirect result of any general meiotic non-disjunction effect, it is also likely not due to the polar decrease
in recombination.

\textit{egl-13(ku207) mutants lacking a functional germ line can still be suppressed by him-8(e1489)}

Because \textit{him-8} has known effects on chromosome segregation during meiosis, we wanted to determine if suppression of the somatic gonad morphological defect was an indirect effect of loss of \textit{him-8} in meiotic germ line cells or if suppression reflected a new role for \textit{him-8} outside of meiosis. To begin to explore these distinct hypotheses, we assayed suppression in a mutant that lacks the germ line and, thus, lacks meiotic cells. \textit{glp-1(q46)} is a severe loss-of-function allele (Kodoyianni et al., 1992) that inhibits production of a germ line by blocking proliferation of the germ line stem cells (Austin and Kimble, 1987). As a result, the few germ cells produced in early larval stages undergo meiosis and form sperm at the beginning of the third larval stage. In \textit{glp-1} mutants during late L3 and L4 larval stages, the period of vulval and uterine morphogenesis, there are no meiotic cells present. In contrast, wild type animals have a large number of meiotic cells present during all of L3 and throughout the rest of the reproductive life (Austin and Kimble, 1987). We created \textit{glp-1(q46); him-8(e1489); egl-13(ku207)} animals to assay suppression in the absence of meiotic cells. We cannot assay egg-laying in these germ line-defective, sterile mutants, but we directly examined morphology of the vulval-uterine connection compared to a control \textit{glp-1(q46); egl-13(ku207)} strain to see if the \textit{him-8} mutation was still able to partially suppress the Cog defect of \textit{egl-13(ku207)} in the absence of the germ line. The presence of \textit{him-8(e1489)} significantly increased the
incidence of normal morphology relative to the control glp-1; egl-13(ku207) strain (Figure 2.3 and Figure 2.8). Furthermore, suppression of the Cog phenotype by him-8(e1489) was just as effective in the absence of the germ line as it was in the presence of the germ line (Figure 2.8). These data clearly indicate that a severe reduction in the germ cell population, where him-8 is thought to function in promoting proper chromosome segregation, does not prevent suppression of egl-13(ku207) by him-8(e1489), demonstrating a likely role for him-8 outside of coexisting meiotic cells in antagonizing egl-13 function.

**Suppression by him-8(e1489) does not depend on the genotype of the mother**

We also addressed the question of whether the suppression phenotype was a consequence of unpaired X chromosomes during the meiotic events in the hermaphrodite that gives rise to the suppressed animals. We took advantage of the semi-dominant nature of the suppression phenotype but the fully recessive nature of the meiotic phenotype to address this question. In wild type or him-8(e1489)/+ hermaphrodites, the X chromosomes pair appropriately and no male self-progeny are produced. However, him-8(e1489) homozygous hermaphrodites have X-chromosome pairing defects, leading to a high percentage of nullo-X gametes and a high percentage of male self-progeny (Him phenotype). If X chromosome pairing defects were related to suppression, only homozygous him-8(e1489) hermaphrodites should give rise to suppressed animals. In contrast, we assayed suppression by Egl percentage in him-8(e1489)/+ progeny segregating from +/+; him-8(e1489)/+ and him-8(e1489)/him-8(e1489) hermaphrodites.
and found no differences; all were suppressed to similar degrees (Figure 2.6). Thus, abnormal X chromosome pairing does not correlate with suppression, and we suggest that \textit{him-8} has a previously undetected function outside of meiosis.

\textit{him-8 does not suppress the egg-laying defects of lin-11}

Mutation of \textit{lin-11} results in a uterine seam cell defect, similar to mutation of \textit{egl-13}, and a vulval cell fate defect, which could each contribute to its Egl phenotype (Freyd et al., 1990; Newman et al., 1999). To determine if mutation of \textit{him-8} could suppress other mutants with uterine seam cell defects, we asked whether \textit{him-8} could suppress a non-null mutation in \textit{lin-11}. \textit{ps1} is an allele of \textit{lin-11} with an incompletely penetrant \(\pi\) lineage defect (L. Huang and W. Hanna-Rose, unpublished) and it is not predicted to be a molecular null. \textit{ps1} would result in a missense mutation (V286M) with a possible affect on splicing, since codon 286 overlaps a splice acceptor/donor site (K. Clemens and W. Hanna-Rose, unpublished). \textit{lin-11(ps1); him-8(e1489)} animals have no increase in egg-laying ability (0%) relative to \textit{lin-11(ps1)} (0%), indicating that at least one of the \textit{lin-11} defects remains unsuppressed. We specifically scored morphology of the vulval-uterine connection to look for suppression of the \(\pi\) lineage defect. We observed a normal thin uterine seam cell in 33\% (n=27) of \textit{lin-11(ps1)} animals and in 17\% (n=24) of \textit{lin-11(ps1); him-8(e1489)} animals. Thus, we conclude that the uterine defects of \textit{lin-11(ps1)} are not suppressed by \textit{him-8}. This observation supports our hypothesis that mutations in \textit{him-8} do not somehow bypass the need for the processes involved in \(\pi\) lineage cell fate maintenance, and in particular, \textit{lin-11} or \textit{egl-13}. 

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Extra copies of EGL-13 mutant protein can partially rescue the egl-13(ku194) egg-laying defect

One attractive model to explain the ability of him-8 mutants to suppress only non-null egl-13 mutants is that mutation of him-8 leads to up-regulation of EGL-13, which is encoded on the X chromosome, and that a higher level of partially functional protein leads to higher activity. To simulate this scenario, we used recombinant PCR to introduce the ku207 lesion (A335T) into a plasmid encoding the EGL-13::GFP translational fusion, then injected this construct into null mutant egl-13(ku194) animals at a concentration of 2 ng/µL. We recovered one strain that carried this extrachromosomal array, designated psEx38. Animals from this egl-13(ku194);psEx38 strain that kept the array were 6% Egl\(^+\) (n=148), while the population of animals that lost the array was 0% Egl\(^+\) (n>100) (Figure 2.9), showing that extra EGL-13(A335T) mutant protein was able to partially rescue the completely penetrant egl-13(ku194) egg-laying defect. (Note: as a negative control, an egl-13 C-terminal deletion construct did not show any rescue ability (data not shown)). We then crossed the egl-13(ku194);psEx38 strain with him-8(e1489) to obtain egl-13(ku194);him-8(e1489);psEx38 animals. Direct comparison of egg-laying ability between egl-13(ku194);psEx38 animals (6% Egl\(^+\), n=148) and egl-13(ku194);him-8(e1489);psEx38 animals (7% Egl\(^+\), n=88) showed that mutation of him-8 was unable to significantly increase egg-laying ability in the context of egl-13(ku207) on an extrachromosomal array (Figure 2.9).
Up-regulation of egl-13 transcript by him-8(e1489) cannot be detected via semi-quantitative RT-PCR

If mutations in him-8 lead to an upregulation of egl-13 transcript, we expect to be able to detect these differences. We used a semi-quantitative RT-PCR approach to approximate egl-13 transcript levels in wild-type, mutant, and suppressed mutant strains to test for up-regulation of transcript. Although we were able to clearly detect increased message in a control transgenic strain with multiple copies of a full-length egl-13 transgene (kuIs27), we could detect no significant difference in egl-13 transcript levels among wild-type, mutant (ku207), and suppressed populations (e1489; ku207) in duplicate experiments (Figure 2.10).

Extra copies of the egl-13 promoter antagonize suppression by him-8(e1489)

The integrated transgene kuIs29 contains multiple copies of a transcriptional fusion of the egl-13 promoter (5000 base pairs upstream of the egl-13 coding region and the first nineteen egl-13 codons) to green fluorescent protein (GFP) (Hanna-Rose and Han, 1999). In the presence of the homozygous kuIs29 transgene, him-8(e1489) suppression of egl-13(ku207) is blocked., i.e., him-8(e1489); kuIs29; egl-13(ku207) is 0% Egl+ (n=100) (Figure 2.11). In him-8(e1489); kuIs29/+; egl-13(ku207) animals, where only half the amount of additional egl-13 promoter is present, suppression of the Egl phenotype by him-8 is weakened (8%, n=26), but not completely abrogated. The low-level egg-laying ability (2% Egl+) of egl-13(ku207) animals is also eliminated in the presence of extra
copies of the *egl-13* promoter on the *kuIs29* transgene. Introduction of other arrays containing the same transcriptional *egl-13::gfp* fusion can weakly phenocopy an *egl-13* mutant; that is, a small portion of otherwise wild-type animals expressing similar arrays have an Egl phenotype (W. Hanna-Rose, unpublished). We considered two mechanisms that would fit this observation: The *egl-13* promoter may be capable of titrating the EGL-13 protein itself, effectively phenocopying a null allele. Alternatively, the array with extra copies of the promoter may titrate a factor necessary for *egl-13* expression. Either mechanism would result in an effective *egl-13* null and, thus, prevent suppression by *him-8(e1489)*. We attempted to test the model that *kuIs29* might titrate a factor or factors necessary for *egl-13* expression via our PCR assay (above). However, the *egl-13* transcript level in a transgenic (*kuIs29*) strain containing extra copies of *egl-13* promoter was not detectably lower than in wild-type animals, as would be predicted if the promoter titrated a positive regulator of *egl-13* transcription.

**Neither the proximal nor distal halves of the *egl-13* promoter are sufficient for abrogating suppression**

In an attempt to map the region of the promoter responsible for abrogating suppression, I created two constructs, both derived from the pWH17 construct, that each contain approximately half of the *egl-13* promoter. I injected these constructs into *him-8(e1489);egl-13(ku207)* worms to obtain the extrachromosomal array transgenes *psEx21* (proximal *egl-13* promoter) and *psEx22* (distal *egl-13* promoter). Neither transgene showed a significant abrogation of expression (Figure 2.11). 25% of the distal
psEx22; him-8(e1489); egl-13(ku207) animals (n=102) and 20% of the proximal
psEx21; him-8(e1489); egl-13(ku207) animals (n=64) had egg-laying ability. As a control, I injected the full pWH17 construct to obtain the extrachromosomal array transgene psEx19. Only 1% of these psEx19; him-8(e1489); egl-13(ku207) animals (n=66) were able to lay eggs (Figure 2.11)
DISCUSSION

I have identified several *him-8* mutations as suppressors of non-null mutations of *egl-13*. Specifically, mutation of the C2H2 zinc fingers of *him-8* partially restores morphogenesis of the vulval-uterine connection in *egl-13* mutants, suggesting that uterine seam cell fate maintenance or at least the competency of the uterine seam cell to fuse with the anchor cell is re-established. Consequently, egg-laying ability is also partially restored. Interestingly, the percentage of animals with restored morphology is higher than the percentage with restored egg-laying function. The discrepancy may reflect the existence of another defect in the morphogenesis or the function of the egg-laying apparatus that contributes to the egg-laying defect. This additional defect could be caused by either the *egl-13* or *him-8* mutation. However, mutation of *him-8* alone has no obvious vulval or uterine morphology defect or Egl defect; thus, I suggest that a second, previously undetected defect affecting egg-laying may result from mutation of *egl-13*.

My genetic dissection of the suppression mechanism has helped to refine potential molecular models. *egl-13(ku207)* and *egl-13(n483)* have mutations within the Sox domain, which is responsible for DNA-binding, and the coupled incomplete penetrance of the Egl phenotype is likely due to weak binding of the Sox domain to its target. Because suppression is specific for these non-null alleles of *egl-13*, I hypothesize that mutation of *him-8* is not acting as a bypass suppressor; some amount of mutant EGL-13 protein must be present in order for the defects to be suppressed. *him-8(e1489)* is also unable to suppress a non-null allele of *lin-11*, which further supports the hypothesis that I
have not identified a bypass suppressor that restores function downstream of uterine seam cell fate determination, but rather a suppressor that affects a process upstream of or in parallel to egl-13 function.

**him-8 function in suppressing abnormal uterine morphogenesis is independent of its role in meiosis**

*him-8* has a well-studied role in meiosis (Broverman and Meneely, 1994; Phillips et al., 2005), where it is required for efficient pairing of the X chromosomes (Phillips et al., 2005). HIM-8 binds to the pairing center of the X chromosome and localizes to the nuclear periphery (Phillips et al., 2005). To determine if suppression of somatic gonad defects is an indirect result of compromising the function of *him-8* in meiotic cells, I performed two experiments. First, I showed that *him-8* mutations suppress the *egl-13(ku207)* defects in animals that have a severe reduction in the number of meiotic cells, suggesting that the germ line of an animal plays no role in suppression of somatic gonad morphogenesis defects in the same animal. Second, I showed that *him-8(e1489)* suppresses *egl-13(ku207)* independently of whether *him-8*-mediated X-chromosome pairing was compromised in the germ line of the mother giving rise to the suppressed animals, suggesting that an indirect, post-meiotic effect of *him-8* meiotic function is not responsible for suppression. Based on these results, I conclude that *him-8* likely functions outside of meiosis to antagonize EGL-13. The recent study by Phillips et al, 2005, determined the molecular identity of *him-8* and examined its expression pattern in the germline using antibodies directed against HIM-8. However, to obtain optimal
staining for the germline, only dissected gonads were stained, so no information can currently be gleaned about somatic expression of HIM-8.

Consistent with the proposal that \textit{him-8} has a meiotic and a non-meiotic function, \textit{him-8} alleles display a different set of genetic behaviors when comparing the Him and suppression phenotypes. All four alleles of him-8 that I have examined here have a similarly penetrant (almost 40%), fully recessive Him phenotype (Broverman and Meneely, 1994; Phillips et al., 2005) In contrast, \textit{me4} does not suppress, and \textit{mn253} is a consistently weaker suppressor than \textit{tm611} or \textit{e1489}. Furthermore, \textit{e1489} behaves as a semi-dominant \textit{egl-13} suppressor. Since \textit{e1489} behaves similarly to a deletion, I have concluded that semi-dominance is due to haplo-insufficiency for the non-meiotic function, that \textit{e1489} is a reduction-of-function allele and that the wild-type \textit{him-8} gene product acts antagonistically to \textit{EGL-13}.

\textit{Not all aspects of him-8 activity are shared between its suppression and meiotic functions}

Phenotypic differences between the \textit{him-8} alleles hint at a two-step process for \textit{him-8} function. Mutation of either HIM-8 C2H2 zinc finger, such as in mutants \textit{e1489}, \textit{tm611} and \textit{mn253}, leads to loss of chromosome binding and loss of chromosome association with the nuclear envelope in the first step (Phillips et al., 2005). Mutation of the zinc fingers is also associated with suppression. Thus, the zinc fingers of HIM-8 are important for both HIM-8 functions, and chromosome binding is likely important for
HIM-8 function in suppression of egl-13 mutant defects. The stronger suppression phenotypes of e1489 and tm611 relative to mn253 suggest that the more C-terminal zinc finger plays a slightly more prominent role in the mechanism of him-8 function during suppression of egl-13 defects. In contrast, the me4 allele affects the N-terminus of HIM-8 but not the zinc fingers. me4 produces protein that binds the chromosome. Furthermore, the chromosome still associates with the nuclear periphery in the me4 mutants, but me4 mutants are nonetheless defective in X chromosome pairing (Phillips et al., 2005), a second step of him-8 meiotic function. Thus, an N-terminal mediated function of HIM-8 is required for meiosis but dispensable for suppression since the non-null me4 alleles retains wild type activity in the suppression assay (fails to suppress).

him-1 (Mito et al., 2003) and him-5 (Broverman and Meneely, 1994; Goldstein, 1986), like him-8, affect disjunction of only the X chromosome. Furthermore, him-5 mutants display a polar reduction in X-chromosome recombination similar to that of him-8 (Broverman and Meneely, 1994). These him genes may share functions in the second step of the meiotic process executed by him-8, but they do not share in the suppression phenotype. A wide array of genes have him phenotypes; they encode proteins including kinesins, cohesins, kinetochore proteins, synaptonemal complex proteins and gene products of unknown function (Kamath et al., 2003; Oishi et al., 2001; Pasierbek et al., 2001; Piano et al., 2002), and they have molecular roles in a variety of processes including recombination, pairing and segregation. Mutations in many him genes, including him-3 (Zetka et al., 1999), him-6 (Wicky et al., 2004) and him-10 (Howe et al., 2001), cause nondisjunction of all chromosomes and those tested here do not suppress egl-13 mutants. In addition, it is worth noting that the egl-13 suppressor that we
originally isolated, *ku376*, does not have a Him phenotype (discussed in Chapter 3), further evidence that meiotic disjunction activity and suppression are not necessarily intertwined.

_Mutation of him-8 may lead to an increase in effective egl-13 activity_

Conceivably, suppression could be due either to increased levels of EGL-13 or enhanced effective activity of EGL-13. One possible role for normal *him-8* function could be to directly or indirectly repress transcription of *egl-13*, which happens to be encoded on the X chromosome, possibly making it a fitting target for *him-8* function. A reduction-of-function mutation in *him-8* could alleviate repression of *egl-13(ku207)* and lead to a larger pool of albeit less functional EGL-13(A335T) protein. With an increase in the available pool of mutant EGL-13, more of the altered transcription factor could find and/or interact with its target(s), leading to partial rescue of the mutant phenotype. Null mutants that produce no protein would not be affected by de-repression. I attempted to address this hypothesis in two ways. First, I simulated upregulation of mutant protein by artificially expressing a multi-copy plasmid containing a translational EGL-13(A335T)::GFP fusion and found that this could indeed rescue a null *egl-13* mutant. I also tested for such up-regulation of *egl-13* transcription using RT-PCR. Although higher levels of transcript could be detected in a control transgenic strain, I did not observe increased transcript levels in strains with the suppressor (Figure 2.10). However, there are a few possible limitations to this approach due to possible contaminating *egl-13* expression from non-π cells and the presence of multiple alternatively spliced forms of
egl-13 (shown at www.wormbase.org), only some of which may be preferentially upregulated. The effect of him-8 in up-regulating EGL-13 could also be post-transcriptional or post-translational; I have not ruled out these scenarios. However, I also do not detect up-regulation of an EGL-13::GFP translational fusion protein in response to mutation of him-8, based on the intensity of the GFP reporter signal.

Given that him-8 can interact with and affect X chromosome behavior during meiosis, that alleles which eliminate chromosome binding cause suppression, and that I can detect no upregulation of egl-13 transcript, I suggest another potential model in which mutation of him-8 may affect chromosome structure directly or indirectly and thus promote access of mutant EGL-13(A335T or P394L) protein to its targets, possibly without affecting egl-13 expression levels. There is certainly much evidence for chromatin silencing occurring at the nuclear periphery (Andrulis et al., 1998; Cockell and Gasser, 1999; Feuerbach et al., 2002). If mutant HIM-8 prevents association of the X chromosome with the nuclear envelope, normally silenced targets would now be more accessible to transcription factors such as EGL-13, allowing upregulation of factors needed for uterine seam cell fate maintenance.

In the upregulation model, we know that the target of regulation, egl-13, lies on the X chromosome, consistent with him-8 functioning on the X chromosome in meiosis. The model of greater accessibility of EGL-13 to its targets would suggest that if the X-specific nature of him-8 function in meiosis were conserved in its non-meiotic role, the affected downstream target of the EGL-13 transcription factor would lie somewhere
along the X chromosome. Outside of meiosis, I cannot rule out that him-8 might act more globally on autosomes as well. In this scenario, access of EGL-13 to autosome-encoded targets might be promoted by mutation of a him-8 gene that affects chromosome structure in general. To address whether the context of the X chromosome is important for the suppression effect, I compared egg-laying ability between two strains that were overexpressing an EGL-13(A335T)::GFP fusion protein in a null egl-13(ku194) background, one of which had the him-8(e1489) mutation, and one of which that did not. I observed that the presence of the him-8 mutation was unable to increase the low-level egg-laying ability present in the partially rescued ku207 overexpression strain (6% to 7% Egl\(^+\)), unlike the ability of him-8 mutations to increase the low-level egg-laying ability in egl-13(ku207) mutants (2% to 26% Egl\(^+\)). I conclude from this that suppression of egl-13 defects by him-8 mutations likely requires the context of the X chromosome.

I am continuing to investigate these predictions based on the proposed models and expect these results to contribute to elucidation of the possible role of him-8 in somatic cells and morphogenesis as well as meiosis. Some of the strategies used to address the question of the importance of chromosomal context will be discussed in Chapter 4. Such questions include what may other targets of him-8 regulation be, and if other meiotic genes similar to him-8 are able to exert a similar effect on egl-13.
METHODS

RNAi

Using primers flanking the coding region, I amplified 1.4 kilobase pairs from egl-13 cDNA. The primers incorporated the restriction sites for Xma I and Apa I (sequence in bold) to either end of the egl-13 amplicon for cloning purposes.

\[
e_{gl-13} + Xma \text{ I forward primer} \\
5' {GC}CCCGGG{ATGAGCG}CGTAGACGAAAAGCG 3'
\]

\[
e_{gl-13} + Apa \text{ I reverse primer} \\
5' {GC}GGGCCC{TTATTAGCGT}TTTGTAGG 3'
\]

I digested and ligated pPD129.36 (A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication) and the PCR product to form the egl-13 RNAi feeding vector pBLN2. pBLN2 and a control vector, pLT61.1, were separately transformed into the E. coli strain HT115. Cultures were spotted onto M9 minimal media plates with 0.02% lactose to create an RNAi feeding source (Timmons et al., 2001). Animals of the following genotypes were grown on both types of RNAi-feeding plates: wild type (N2), rrf-3(pk1426), and him-8(e1489):egl-13(ku207).
Scoring of egg-laying ability

I placed individual animals at the L4 stage or younger on culture plates and scored them Egl+ if I saw any deposited eggs on the plate within two days after reaching adulthood. I excluded animals that released eggs solely due to ruptured at the vulva, which happened at a very low rate in a few strains (less than 1%, data not shown). Null alleles of egl-13 did not lay a single egg during my observations.

Deficiency genetics

We crossed lon-2(e678) egl-13(ku207)/0 males to let-51(s41) unc-22(s7)/sDf2 hermaphrodites and selected Lon F₂ progeny, indicative of lon-2(e678) egl-13(ku207) homozygotes. We allowed these animals to self-fertilize and kept only those cultures that failed to segregate Unc F₃ progeny, indicating the selection of the heterozygous deficiency (sDf2/+) rather than the heterozygous balancer (let-51(s41) unc-22(s7)/+).

The self-progeny of these sDf2/+; lon-2(e678) egl-13(ku207) animals will consist of one-third +/+; lon-2(e678) egl-13(ku207) progeny and two-thirds sDf2/+; lon-2(e678) egl-13(ku207) progeny (sDf2/sDf2; lon-2(e678) egl-13(ku207) animals die as embryos). If the suppression by sDf2/+ is equivalent to suppression by him-8/+ (normally 12%), we expect approximately 9% of these self-progeny to lay eggs (2/3*12% Egl+ + 1/3*2%Egl+ = 9%). Instead, a slightly higher percentage (10.5%, n=151) laid eggs, which when calculated as follows gives us the predicted 14.75% for sDf2/+ suppression (If 2/3 * true heterozygous suppression % + 1/3 * 2% leaky egl-13(ku207) egg-laying = 10.5%, then
true heterozygous suppression = 14.75%.

**glp-1 genetics**

We created ruIs38[myo-2::GFP]/glp-1(q46) unc-32(e189); him-8(e1489); egl-13(ku207) and ruIs38[myo-2::GFP]/glp-1(q46) unc-32(e189); egl-13(ku207) strains. We selected L4 progeny with an uncoordinated, germ-line-deficient, non-GFP phenotype and examined vulval and uterine morphology of these animals by differential interference contrast (DIC) microscopy.

**Heterozygous suppression and maternal effect genetics**

We created him-8(e1489)/mIs11; egl-13(ku207) to examine morphological suppression of him-8(e1489)/+ heterozygotes. From this strain, we selected him-8(e1489)/mIs11; egl-13(ku207) progeny based on their fluorescence intensity (mIs11 is detectably brighter when homozygous) and examined them via DIC microscopy. After examination, the animals were washed off the slide and recovered individually. As expected, all were heterozygous (n=57) for mIs11 and him-8(e1489), as determined by correct phenotypic segregation of their progeny, confirming the accuracy of selecting the heterozygotes according to fluorescence intensity. Note that these heterozygous progeny come from mothers that are also heterozygous for him-8(e1489).

To examine the maternal requirement of him-8, we used two different mating schemes to produce him-8(e1489)/+;egl-13(ku207) mutants from mothers that were either wild-type
or mutant for *him*-8. To produce *him*-8(e1489) heterozygotes from a wild-type mother, we mated *him*-8(e1489);*mIs10;egl-13(ku207) males to *egl-13(ku207)/lon-2(e678) unc-6(e78)* hermaphrodites to produce *him*-8(e1489)/+;*mIs10/+;egl-13(ku207) animals, as well as *him*-8(e1489)/+;*mIs10/+;egl-13(ku207)/lon-2(e678) unc-6(e78) animals. Every animal was scored for Egl, but animals that were not homozygous for *egl-13(ku207)* were subtracted from the count after they were revealed by the presence of Lon Unc progeny.

To produce *him*-8(e1489) heterozygotes from a homozygous mutant *him*-8 mother, we mated *mIs11;him-5(e1490);egl-13(ku207)/0* males to *him*-8(e1489);egl-13(ku207) hermaphrodites, to produce *him*-8(e1489)/mIs11;him-5(e1490)/+;egl-13(ku207) hermaphrodite progeny.

**Overexpression of EGL-13(A335T)**

We swapped an *ApaI*-HindIII fragment from pWH14, which encodes a functional EGL-13::GFP translational fusion (Hanna-Rose and Han, 1999), with a fragment containing a single base pair change at EGL-13 codon 335 (GCA to ACA) to create pBLN11, encoding EGL-13(A335T). We created the mutated fragment by recombinant PCR and sequenced the vector after subcloning. We injected pBLN11 into the null *egl-13(ku194)* animals at a concentration of 2 ng/µL with *sur-5::gfp* (pTG96) at 75 ng/µL to form the transgene *psEx38*, and pBLN11 at a concentration of 20 ng/µL with *sur-5::gfp* (pTG96 (Yochem et al., 1998) at 75 ng/µL to form the transgene *psEx52*. A control vector, a construct containing a C-terminal deletion of EGL-13 does not show any level of rescue (data not shown).
**Semi-quantitative RT-PCR**

Total RNA was extracted from whole, mixed stage wild-type and mutant animals using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 0.1 µg of total RNA was used as template for a 30-minute cDNA synthesis via SuperScript II RNase H- Reverse-Transcriptase immediately followed by 25 cycles of polymerase chain reaction (PCR) via Platinum Taq DNA polymerase (both enzymes from Invitrogen). We visualized PCR products with ultraviolet light after agarose gel electrophoresis and uniform staining with ethidium bromide and quantified using an ImageQuant software package. Intron-exon border-spanning primers to *egl-13* and *ama-1* (used as an internal control) (Bird and Riddle, 1989, Johnstone, 1996 #124, Larminie, 1996 #125) are as follows:

*egl-13* forward primer

5' TCCACTATCTGGATTTCGGC 3'

*egl-13* reverse primer

5' CGGGATCCGCGGCCGCTTCAGCTGTTTGTAGGAGATGTGA 3'

*ama-1* forward primer

5' TTCCAAGCGCCGCTGCGCATTGTCTC 3'

*ama-1* reverse primer

5' CAGAATTTCAGCACTCGAGGAGCGGA 3'
him-8 encodes a C2H2 zinc finger protein (Phillips et al., 2005). We have obtained four alleles of this gene: me4, mn253, e1489, and tm611. A fifth allele, ec56, was found to be the same lesion as e1489. As shown, all mutants have roughly equivalent percentages of male progeny, whereas only mutations of the zinc fingers (mn253, e1489, tm611), and not me4, show suppression of egl-13 defects.
Figure 2.2

Mutations affecting the zinc finger region of *him-8* suppress the egg-laying (Egl) defect of non-null alleles of *egl-13*. Histogram illustrating the egg-laying percentage of various single (*egl-13*) or double (*him-8; egl-13*) mutants. “+” indicates a wild-type chromosome. Mutants are in the background of *egl-13* mutations as indicated in the vertical text (left) of each data set. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
*him-8(e1489)* restores the morphology defect of *egl-13* mutants. Differential interference contrast photomicrographs of the mid to late L4 stage ventral uterus and vulva. (A) N2 wild type. The anchor cell (AC) has fused with the thin uterine seam cell (utse), indicated by arrow. U, uterus; V, vulva. (B) *egl-13(ku207)*. A thin laminar utse is not observed, and the AC (arrowhead) at the apex of the vulva has not fused with the utse. (C) Suppressed *him-8(e1489); egl-13(ku207)* animals exhibit restored AC-utse fusion. For panels B and C, see Figure 2 for quantification of this rescued morphology. (D) Suppressed *glp-1(q46) unc-32(e189); him-8(e1489); egl-13(ku207)* animals exhibit restored AC-utse fusion. See Figure 5 for quantification of this rescued morphology.
Mutations affecting the zinc finger region of *him-8* suppress the connection-of-gonad (Cog) defect of non-null alleles of *egl-13*. Histogram illustrating the restored connection-of-gonad percentage of various single (*egl-13*) or double (*him-8; egl-13*) mutants. “+” indicates a wild-type chromosome. Mutants are in the background of *egl-13* mutations as indicated in the vertical text (left) of each data set. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
Suppression of the egl-13 egg-laying defect by him-8 mutations is specific to non-null alleles of egl-13. Histogram illustrating the egg-laying percentage of various single (egl-13) or double (him-8; egl-13) mutants. "+" indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
Figure 2.6

*Percentage of sDf2/+ egg-laying animals calculated as described in Materials and Methods.

him-8(e1489) is a semi-dominant suppressor of the egl-13 egg-laying defect due to haplo-insufficiency and suppression is independent of maternal him-8 genotype. Histogram illustrating the egg-laying percentage of various single (egl-13) or double (him-8; egl-13) mutants. "+" indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
Survey of *him* genes for suppression of *egl-13*. Histogram showing the percentage suppression of the *egl-13* connection-of-morphology defect in double mutants between either *egl-13(ku207)* or *egl-13(n483)* and alleles of *him-1, him-3, him-5, him-6, and him-10*. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
him-8(e1489) is a semi-dominant suppressor of the egl-13 Cog defect and suppression is unaffected by loss of the germline. Histogram illustrating the restored connection-of-gonad percentage of various single (egl-13) or double (him-8; egl-13) mutants. him-8(e1489)/+ mutants examined here are from him-8(e1489)/+ mothers. "+" indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
EGL-13(A335T) expressed from an extrachromosomal transgene can rescue null egl-13(ku194), but rescue is not enhanced by him-8(e1489). Histogram showing the egg-laying percentage for various genotypes, all in a null egl-13(ku194) background.

psEx38[2 ng/µL EGL-13(A335T) vector] and psEx52[20 ng/µL EGL-13(A335T) vector] increase egg-laying percentage to 6% and 44%, respectively. The presence of him-8(e1489) does not significantly enhance the egg-laying percentage of psEx38;egl-13(ku194). Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
Semi-quantitative RT-PCR to detect *egl-13* transcript levels. RNA template isolated from wild-type (WT) and mutant animals was treated with (+) or without (-) reverse transcriptase (RT) and then amplified by PCR. In order to normalize our transcript detection by RT-PCR, we used the RNA polymerase II gene *ama-1* as an internal control in these experiments (Bird and Riddle, 1989, Johnstone, 1996 #124, Larminie, 1996 #125). The 597 base pair product amplified from *egl-13* cDNA template and the 355 base pair product amplified from *ama-1* cDNA template are indicated. This figure shows a representative experiment using 0.1 µg of template RNA and 25 cycles of amplification. These amplification conditions were well below those for producing a saturating amount of product because ten times more RNA template (1.0 µg) and seven more cycles (32 total) still resulted in less product than 1.0 µg of template amplified for 36 cycles (not shown). This exact experiment was performed twice with similar results. The values listed at the bottom are the average ratios for both experiments of *egl-13* to *ama-1* transcript normalized to the wild type ratio, which was given a value of 1. A number of other experimental reactions under a variety of conditions failed to ever detect
differences in *egl-13* transcript levels among wild type, *him-8(e1489), egl-13(ku207)* and *him-8(e1489);egl-13(ku207)* populations. *kuIs27* is a translational fusion of the EGL-13 full-length protein and was used as a positive control.
Extra copies of the *egl-13* promoter can abrogate suppression of the egg-laying defect of *egl-13(ku207)* by *him-8(e1489)*. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
CHAPTER 3: THE KU376 MUTATION SUPPRESSES EGL-13 DEFECTS
ABSTRACT

Using a forward genetics, mutagenesis screening approach to identify suppressors of *egl-13* mutant defects, a mutation at an unknown locus, temporarily designated *ku376*, was identified as one suppressor (Hanna-Rose, unpublished). I have characterized the genetic behaviors of suppression by *ku376*, and found that it behaves similarly to *him-8* despite having no similar Him or other chromosome pairing phenotypes. The *ku376* mutation can also interact genetically with a *him-8* mutation. I have mapped this locus to the far left end of the fourth chromosome in close association with the *dpy-9* locus and I continue to pursue the molecular identity of *ku376*. I have also isolated the *ku376* mutation away from the *egl-13(ku207)* background for future analysis.
INTRODUCTION

Using the EMS mutagenesis screen discussed in Chapter 1, the *ku376* mutation was found to be a suppressor of the *egl-13(ku207)* egg-laying defect. This strain was backcrossed with a strain that still had an *egl-13(ku207)* background in order to isolate the *ku376* mutation in an *egl-13* mutant background and ensure that the suppression was not due to multiple unlinked mutations. After outcrossing, the *ku376;egl-13(ku207)* animals were inspected to determine if the *ku376* mutation had any other effects on the morphology or behavior of the worm. No other obvious phenotype could be detected.
RESULTS

*ku376 is a semi-dominant suppressor of the egl-13(ku207) egg-laying and connection-of-gonad defects*

I quantified the percentage of *ku376;egl-13(ku207)* egg-laying animals using the same standards for egg-laying analysis described in the Methods section of Chapter 2. The suppression level of *ku376;egl-13(ku207)* animals was greater than the suppression level of *him-8(e1489);egl-13(ku207)*, with 36% of the population able to lay eggs (n=415) (FIGURE 3.1). I also looked at the connection-of-gonad morphology to see if the underlying defect was also suppressed by the *ku376* mutation, and found that the anchor cell had fused with the uterine seam cell in 53% of the animals (n=45) (Figure 3.1), also higher than the *him-8* suppression of the Cog defect. Other morphological defects of the vulva were seen at low frequency. It is interesting to note here that, much like *him-8*, the ability of *ku376* to suppress the underlying connection-of-gonad defect is greater than the ability to suppress the egg-laying defect.

To test if *ku376* also had any dominant effects like *him-8*, I mated homozygous *ku376;egl-13(ku207)* hermaphrodites to GFP-marked *egl-13(ku207)* males. I checked the first generation progeny (F1s) to test heterozygous suppression while avoiding the possibility of losing the *ku376* mutation due to recombination. 19% of heterozygous *ku376/+;egl-13(ku207)* (n=43) animals laid eggs, roughly half the level of homozygous suppression, demonstrating that *ku376* is also semi-dominant. To confirm this for the
connection-of-gonad defect, I mated a marked homozygous ku376 strain (see Methods for further explanation), mis11 ku376;egl-13(ku207), to him-10(e1511);egl-13(ku207) or him-5(e1490);egl-13(ku207). Heterozygous progeny also had a normal connection-of-gonad phenotype 34% of the time (n=67), a value between the homozygous wildtype and homozygous ku376 mutant levels, confirming semi-dominance (Figure 3.1).

Suppression by ku376 is specific to non-null alleles of egl-13

I proceeded to test the ability of ku376 to suppress the egg-laying defects of the three other alleles of egl-13. I mated ku376; lon-2 ku207 worms to him-8(ec56);egl-13(n483), him-8(ec56);egl-13(ku241), and him-8(ec56);egl-13(ku194), and then selected F2 progeny that did not have the lon-2 marker or him-8 to get the genotypes ku376;egl-13(n483), ku376;egl-13(ku241), and ku376;egl-13(ku194). ku376;n483 animals showed suppression, with 42% (n=121) laying eggs (Figure 3.2). I created several independent lines each of the presumptive genotypes ku376;egl-13(n483), ku376;egl-13(ku241), and ku376;egl-13(ku194). Multiple independent lines were created to guard against the possibility of losing the ku376 allele due to recombination. None of these independent lines for either putative null allele showed any egg-laying ability. ku376 was also unable to suppress the egl-13(ku207) defect in the presence of RNAi against egl-13, further supporting the idea that ku376, like him-8, is not a bypass suppressor.
Genetic interaction of ku376 and him-8

To help dissect potential differences between suppression by him-8 and suppression by ku376, I created mutant animals heterozygous for each gene in an egl-13 mutant background (ku376/him-8(e1489);egl-13( ku207)) and then analyzed the egg-laying and connection-of-gonad defects in these mutants. I examined only first generation cross-progeny to again protect against the possibility of losing ku376 due to recombination events. ku376/him-8 animals showed a level of suppression in between the him-8 homozygous mutant and ku376 homozygous mutant suppression levels (Figure 3.1). Two possibilities can explain this data. First, him-8 and ku376 could be acting at distinct steps during development, and each mutation would have an additive effect. Alternatively, him-8 and ku376 could be acting at the same process to exert the suppression effect, but the process could be dosage dependent, so that one mutant copy of him-8 plus one mutant copy of ku376 is similar to two mutant copies of one of these two genes (we would expect the difference in strength between heterozygous him-8(e1489) and heterozygous ku376, although small, to be averaged out in the combined effect.)

The latter is the hypothesis we favor. We know that mutations of either gene (it is worth restating that ku376 and him-8 are distinct loci) act semi-dominantly, lending credence to the significance of gene product dosage. The experiment does rule out the possibility that him-8 and ku376 are affecting the same process in a non-dosage dependent manner, where we would expect a non-additive interaction between mutations in both genes.
Genetic mapping of the ku376 locus

*Caenorhabditis elegans* has a set of six chromosome pairs: five autosomes, designated linkage groups one through five (LG I – V), and one sex chromosome, designated LG X. By examining exclusion of separate genetic markers for each chromosome, the *ku376* locus was found to reside on LG IV. I created a set of strains with genetic markers, usually a dominant transgene encoding green fluorescent protein specific to each chromosome (Table 3.1). I used the markers to test if their presence could exclude the presence of *ku376*, suggesting a shared chromosome. My data shows an ability to exclude *ku376* by *mIs11*, a marker for chromosome IV, with only a low percentage of inclusion of *ku376*, suggesting that *ku376* is on the fourth chromosome, but perhaps not very close to the locus corresponding to *mIs11* (Table 3.1).

To further pinpoint the location of the *ku376* mutation along the fourth chromosome, I used the following strains containing pairs of genetic markers on the fourth chromosome to perform three-point mapping analysis: *dpy-20 unc-24, unc-17 dpy-13,* and *dpy-9 unc-17* (Table 3.2). I found that *ku376* was located outside of the region bounded by the markers *dpy-20* and *unc-24*, and outside of the region bounded by the markers *unc-17* and *dpy-13*, but inside the region bounded by the markers *dpy-9* and *unc-17* (Figure 3.4), and much closer to the marker *dpy-9*. In fact, none of the recombinants that I examined had a *dpy-9 ku376* recombinant; the only recombinants were *unc-17 ku376; egl-13(ku207)* animals. I repeated a similar experiment on a larger scale (n>600) using only the marker *dpy-9* and could not obtain any *dpy-9 ku376* recombinants,
suggested that *ku376* is located very close to *dpy-9* on the genetic map (Table 3.2, Figure 3.4)).

**Genomic DNA rescue of the ku376 phenotype**

The *C. elegans* genome sequence is covered by a series of overlapping yeast artificial chromosomes (YACs) harboring large fragments of genomic *C. elegans* DNA. These yeast artificial chromosomes can be used to inject into the syncytial gonad of the worm to create an extrachromosomal transgene with the given DNA fragment. One can then test to see if the injected DNA is capable of rescuing the mutant phenotype, suggesting mutation of a sequence contained within the known, injected DNA. I used injected YAC DNA from the YAC Y38C1 ([www.wormbase.org](http://www.wormbase.org)) and found low-level rescue (in this case abrogation of suppression) of the *ku376;egl-13(ku207)* phenotype with one of these transgenic lines. Similarly to the YACs, the genome is also covered by a series of smaller DNA fragments from cosmids (~30-40 kb) that can be used to test for rescue. Examining the region corresponding to the far left end of chromosome IV and the YAC Y38C1, there are two candidate cosmids that each contain a C2H2 zinc finger protein. I will test these two cosmids for rescue first.
Further phenotypic analysis of ku376;egl-13(ku207) mutants

*ku376* has no plainly observable phenotype on its own. The only effect we see is its effect in relieving the inability of *egl-13(ku207)* and *egl-13(n483)* to lay eggs. However, I perceived a possible longer length in some of the *ku376;egl-13(ku207)* animals versus their non-suppressed *egl-13(ku207)* counterparts. To analyze this, I compared body length along the mid-plane of the worm from the anterior tip of the pharynx to the posterior tip of the tail in late L4 stage worms in *egl-13(ku207)* and *ku376;egl-13(ku207)* worms, but found no significant difference. It is still possible that a difference in length could arise later in the adult stage.

*ku376 mutants have no visible chromosome pairing defects*

Given that *him-8* and *ku376* mutations behave similarly with regard to suppression, I set out to examine chromosome pairing in *ku376;(egl-13)ku207* mutants. *him-8* mutants give rise to a higher frequency of XO progeny due to a failure of X chromosome pairing. Although *ku376* does not lead to a higher frequency of male progeny, my hypothesis was that *ku376* could affect autosomal chromosome pairing, since I had previously shown suppression activity to be independent of increased incidence of males. The common denominator could be a chromosome-binding effect, where *ku376* would affect chromosomes other than the X chromosome while *him-8* only would affect the X chromosome. I examined N2 (wildtype), *him-8(e1489);egl-13(ku207)*, and *ku376;egl-13(ku207)* strains for pairing defects by fixing worms with Carnoy’s fixative then
staining with DAPI and visualizing chromosome pairs within unfertilized oocyte nuclei via DIC fluorescence microscopy. All 106 unfertilized ku376;egl-13(ku207) oocytes that were examined showed completely normal chromosome pairing. The control N2 oocytes were also normal, whereas him-8(e1489);egl-13(ku207) oocytes showed the abnormal chromosome pairing (7 chromosome “foci” present rather than the normal 6) in at least 25% of the worms examined (n=50).

**Isolation of ku376**

In order to better assess the phenotype of our ku376 mutation in future studies, I isolated the mutation from the egl-13(ku207) mutant background by mating wild-type males to egg-laying hermaphrodites from a GFP-marker-linked ku376 strain, mIs11ku376;egl-13(ku207). The resulting mIs11ku376/+;egl-13(ku207)/+ heterozygous hermaphrodite F1 progeny were selected, and then F2 progeny that retained the marker. Because of the large genetic distance between mIs11 and ku376, I expected a significant number of recombination events. To avoid an undetected loss of the ku376 suppressor by recombination, I crossed the lines presumptively containing ku376 back into an egl-13(ku207) background and then tested for suppression, eliminating any original lines that did not show suppression when crossed back to egl-13(ku207). Through this method, I obtained at least one line of the mIs11 ku376 genotype isolated away from the egl-13(ku207) background.
The *ku376* mutation can suppress both the egg-laying and connection-of-gonad defects in a semi-dominant manner. "+" indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right. *ku376/him-8* worms also show a level of suppression similar to both *ku376* and *him-8* homozygotes.
Suppression of the egg-laying defect of *egl-13* mutants by *ku376* is allele-specific. "+" indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right. *ku376/him-8* worms also show a level of suppression similar to both *ku376* and *him-8* homozygotes.
Figure 3.3

*ku376* mutants display no meiotic pairing defects. DAPI staining after treatment of adult worms with Carnoy’s fixative. Top: Whole worm staining. Bottom left: A *ku376;egl-13(ku207)* adult gonad. Bottom right panel: a *ku376;egl-13(ku207)* oocyte displaying six intact chromosome pairs.
The *ku376* mutation resides at the far left end of chromosome IV. Other genes shown (*dpy-9, unc-17, dpy-20, and unc-24*) are genetic markers used in three-point mapping of the *ku376* locus. The bottom portion of the figure shows yeast artificial chromosomes (YACs) and cosmids that cover the region shown in red at the left end of the fourth chromosome.
CHAPTER 4: Effects beyond him-8 and egl-13
ABSTRACT

In order to gain more insight into the mechanism of egl-13 suppression, I looked for other genes that share similar features with egl-13 to test if they could also be suppressed by him-8 mutations. Defects due to a missense mutation in an HMG-box containing protein, pop-1, were partially suppressed. The pop-1 locus is on the first chromosome, suggesting that the effect of him-8 may not be limited to the X chromosome. I also wanted to examine other genes like him-8 to see if those genes were capable of suppressing egl-13 defects. The him-8 gene resides in an operon containing three additional C2H2 zinc-finger encoding genes. Preliminary data shows that mutations in at least two of the other C2H2 zinc finger proteins can suppress the defects of egl-13(ku207). These two results suggest a broader role for the function of multiple meiotic genes outside of meiosis with the potential to act on multiple targets, not just egl-13.
INTRODUCTION

*pop-1 encodes an HMG-box protein like EGL-13*

I have been looking for other transcription factors whose levels may be regulated by HIM-8 or have easier access to their targets because of mutations in HIM-8. Candidates for such genes include other factors that have a Sox domain or HMG box. One factor I found is a gene designated *pop-1* (for posterior pharynx defects). POP-1 is the only member of the TCF/LEF transcription factor family in *C. elegans* (Lin et al., 1995). It has a conserved HMG box similar to the HMG box in the Sox domain of EGL-13. However, unlike *egl-13*, *pop-1* is located on the first chromosome. I expect the analysis of factors like *egl-13* that are not on the X chromosome to help answer the question of the importance of chromosomal context in the suppression mechanism.

*pop-1(q624)* is a missense allele within the HMG box of POP-1 that, like the missense alleles within the HMG box of *egl-13* (*ku207* and *n483*), has incompletely penetrant phenotypes (Siegfried and Kimble, 2002). *pop-1(q624)* has three described phenotypes: L1 larval lethality, the absence of one or both gonad arms (wild-type hermaphrodites should always have two gonad arms), and the presence of one or more extra anchor cells (wild-type animals should have only one) (Siegfried and Kimble, 2002). The L1 larval lethality is most likely caused by an undetermined maternal requirement during early larval development (Siegfried and Kimble, 2002).
him-8 resides in an operon

Interestingly, the him-8 gene resides in an operon with 3 other genes (Figure 4.2) (www.wormbase.org). These genes are transcribed together and then undergo SL2 splicing to create the individual mRNAs (Spieth et al., 1993). All three of these other genes are also C2H2 zinc finger proteins that have been shown in unpublished results (Carolyn Phillips and Abby Dernburg, UC Berkeley, personal communication) to affect pairing of the five other C. elegans chromosomes during meiosis. None of the three zim genes are thought to affect the X chromosome (C. Phillips and A. Dernburg, personal communication). I am now investigating the interaction of these genes with egl-13, in order to gain further insight into the process responsible for regulation of egl-13 and further address the question of chromosomal context. My preliminary data shows that mutations in at least one of these three genes can suppress the egl-13 defects.

Nature of the zim alleles

zim-1(tm1813) is a 481 base-pair deletion toward the N-terminal end of zim-1, affecting the second exon of zim-1. Animals harboring this mutation are still homozygous viable. zim-1(tm1479) is a 590 base pair deletion and 1 base-pair (T) insertion within a putative intron of zim-1. This mutation results only in a weak loss-of-function and a low level of embryonic lethality. The zim-2(tm574) mutation is a 415 base pair deletion at the N-terminal end of zim-2, much like the deletion in zim-1(tm1813) animals. This mutation results in a higher level of embryonic lethality and a low percentage of male progeny (1-
2\% (www.wormbase.org). The existing allele of \textit{zim-3}, \textit{zim-3(tm756)} was not tested due to lethality problems (Figure 4.2). Neither the \textit{zim-1(tm1813)} mutation nor the \textit{zim-2(tm574)} mutation would result in the downstream sequence being in frame, but splicing could still potentially occur properly.
RESULTS AND DISCUSSION

him-8(e1489) can suppress defects of the HMG-box factor pop-1

To confirm the previously reported phenotypic distribution and get a more accurate comparison between the pop-1(q624) mutants with and without him-8(e1489), I scored the three phenotypes of pop-1(q624), and got data roughly similar to that originally reported (Siegfried and Kimble, 2002) (Figure 4.1). I then analyzed these three phenotypes in pop-1(q624); him-8(e1489) double mutants. In the presence of the him-8(e1489) mutation, the incompletely penetrant gonad arm and anchor cell phenotypes were both partially alleviated (Figure 4.1). However, the level of L1 lethality was not significantly affected (Figure 4.1). This inability to rescue the earliest phenotype may reflect the time-course of the normal function of him-8.

Mutations of zim-1 and zim-2 can suppress the egg-laying and connection-of-gonad defects of egl-13(ku207)

I created strains of the following genotypes: zim-1(tm1813);egl-13(ku207), zim-1(tm1479);egl-13(ku207), and zim-2(tm574);egl-13(ku207). I then tested these double mutants in the same manner as described for other suppressors in Chapters 2 and 3. I found that both alleles of zim-1 were able to suppress the connection-of-gonad (Figure 4.4) and egg-laying defects (Figure 4.3) as well as, and perhaps slightly better than a similar allele of him-8, tm611. zim-2(tm574);egl-13(ku207) mutants were also
suppressed significantly above \textit{egl-13(\textit{ku207})} levels, but were not nearly as strong as the two \textit{zim-1} alleles.

\textbf{Phenotypic differences between \textit{him-8} and the \textit{zim} genes}

Because the \textit{zim} genes occur within the same operon as \textit{him-8}, it would be possible that mutations in any of the upstream \textit{zim} genes could affect transcription of the furthest downstream gene, \textit{him-8}. However, I do not believe this to be the case, because we would expect the disruption of \textit{him-8} by any of the upstream genes to result in a high-percentage Him phenotype (around 36-40\% for \textit{him-8} mutants). In fact, only 1 of the three alleles tested showed a significant level of male progeny, and this was \textit{zim-2(\textit{tm574})}, with only between 1-2\% male progeny. Based on our other studies, it is unlikely that we could affect the ability of \textit{him-8} to suppress \textit{egl-13} without affecting its function in meiotic disjunction by a non-specific mutation.

\textbf{Potential future experiments}

I also obtained from the Caenorhabditis Genetics Center another allele of \textit{pop-1}, which is \textit{q645}, a missense mutation affecting the POP-1 N-terminal beta-catenin binding domain. Testing possible suppression of this allele by \textit{him-8} mutations could give us clues as to HIM-8’s interaction with non-X chromosome partners.
I have found that mutations of \textit{zim-1} and \textit{zim-2} are able to suppress the defects of \textit{egl-13(ku207)}. The remaining \textit{zim} gene, \textit{zim-3}, has not been tested due to embryonic lethality problems with the existing \textit{zim-3(tm756)} allele. A new allele of this gene, \textit{zim-3(tm2303)}, has been created by the same \textit{C.elegans} knockout consortium in Japan, but this allele may also have problems with lethality. However, I can use small interfering RNAs (RNAi) against \textit{zim-3} within an RNAi bacterial feeding vector to deliver the RNAi after embryonic development in the hope of avoiding lethality while still affecting later organogenesis. A similar strategy would allow us to test multiple combinations of the \textit{zim} genes and even him-8 to test for a possible synergistic effect.

Barring differences in post-translational modifications or other interactions affecting localization, we expect that the expression pattern of all three \textit{zim} genes will correspond with the localization of \textit{him-8}, because they are contained in an operon and thus share the same promoter (Blumenthal and Gleason, 2003; Spieth et al., 1993).
METHODS

Scoring L1 lethality

Twenty-five adult hermaphrodites of either *pop-1(q624)* or *pop-1(q624);him-8(e1489)* genotypes were placed onto each of four plates per genotype (for 100 adults total per genotype) and allowed to lay eggs for 90 minutes. I removed all hermaphrodites from the plates after the 90 minutes and carefully counted the number of eggs that had been deposited on each plate. I continued to monitor these plates and carefully counted all animals that progressed beyond the L1 stage. To ensure that the reduction in animals that progressed beyond the L1 stage was not due to delayed development but lethality, I continued to monitor these plates for two more days and could not find any surviving L1s whereas all animals that had made it beyond the L1 stage were then L4 stage or young adult worms.
Figure 4.1

The him-8(e1489) mutation can partially suppress the pop-1 gonad arm and anchor cell phenotypes. Histogram illustrating the egg-laying percentage of the single mutant pop-1(q624) or the double mutant pop-1(q624);him-8(e1489). Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
him-8 resides in an operon. The genes that are comprised by this operon include zim-1, zim-2, zim-3, and him-8, in that order. All four are C2H2 zinc finger genes. For more description of these genes, refer to text.
Mutations in *zim-1* and *zim-2* can partially relieve the egg-laying defects of *egl-13(ku207)*. Histogram illustrating the egg-laying percentage of various single (*egl-13*) or double (*zim; egl-13*) mutants. “+” indicates a wild-type chromosome. Error bars represent 95% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
Mutations in \textit{zim-1} and \textit{zim-2} can partially relieve the connection-of-gonad defects of \textit{egl-13(ku207)} mutants. Histogram illustrating the proper connection-of-gonad percentage of various single (\textit{egl-13}) or double (\textit{zim; egl-13}) mutants. “+” indicates a wild-type chromosome. Error bars represent 95\% confidence intervals. Actual percentage is cited within each bar. Sample sizes (n) are indicated at the right.
CHAPTER 5: SIGNIFICANCE OF FINDINGS
Through my research, I have discovered two new factors that interact, at least genetically, with the Sox domain transcription factor gene *egl-13*. The most important aspect of these results, however, will most likely be the finding of a new role for *him-8* and the evidence that genes playing a role in meiotic chromosome behavior may also play a role in gene regulation in non-meiotic cells.

I have found that *him-8* and a set of related C2H2 zinc finger proteins can effect the development of the uterus, a somatic tissue, and that this happens independently of the germ line. Although several *him-8* mutants had been in circulation among *C. elegans* researchers for the past two and a half decades, and several labs had investigated the meiotic non-disjunction phenotype, the molecular identity of *him-8* was not known until very recently (Phillips et al, 2005). Even after the molecular identity of *him-8* was determined and reagents such as antibodies specific to *him-8* were created, only the role of *him-8* in the germline and its effect on the pairing and segregation of X chromosome homologs during meiosis were examined. My genetic analysis to-date as well as future analysis of the interaction between *him-8* and *egl-13* can serve as a tool to dissect and uncover functions of *him-8* in the soma, perhaps enabling studies of processes that would otherwise go unnoticed or that would otherwise be experimentally unapproachable. My findings have also proven timely, coming at a time when more about the molecular behavior of HIM-8 is being uncovered due to the recent cloning of the gene and more in-depth analysis of its meiotic phenotype with newly available, more powerful reagents.
It remains to be seen whether or not this interaction is direct or solely a genetic interaction elicited by general changes in chromosome architecture caused by mutation of him-8. An important question that my research raises but only begins to answer is how important the chromosome context of the “targets” of the suppressors is. Although him-8 affects the X chromosome in meiosis, and egl-13 resides on the X chromosome, I have shown that him-8 can also affect non-X chromosome genes, as well as showing that genes that are not thought to affect the X chromosome can suppress egl-13 defects.

The identification of other suppressors of egl-13 defects and other “targets” of him-8 opens many doors for investigating the mechanisms by which these genes needed for chromosome behavior during meiosis can affect the chromosomes or genes of somatic tissue in an independent manner. As is often the case, finding answers for one set of questions gives rise to multiple new questions. There are many interesting questions that arise naturally from these findings and which will be enticing for others to begin to answer in future work.
Figure 5.1

Mutation of *him-8* may lead to an increase in effective *egl-13* activity (possibly by upregulation of *egl-13* or easier access to targets)

**ONE POSSIBLE, TESTABLE SCENARIO**

*wildtype:*

- *him-8*
  - Silenced chromatin
  - *egl-13*
  - Normal use

*egl-13(ku207):*

- *him-8*
  - Silenced chromatin
  - *egl-13*
  - Abnormal use

*him-8(e1489); egl-13(ku207):*

- *him-8*
  - More open chromatin
  - *egl-13*
  - Normal use

One potential model for the mechanism of *him-8* function by which mutations of *him-8* lead to an upregulation of EGL-13 protein.
A two-step model for hypothetical him-8 function during suppression.
REFERENCES


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