# Chapter 2

## Suppressors of ovulation defective sterile mutants due to increased $IP_3$ signaling

**Introduction**

*let-23,* which encodes an EGF receptor tyrosine kinase (RTK) homolog (Aroian et al., 1990), promotes viability, vulva induction, development, and fertility in *C. elegans* (Aroian and Sternberg,1991) upon activation by the EGF-like molecule, LIN-3 (Hill and Sternberg, 1992). Distinct domains of *let-23* have been shown to mediate viability, vulva development, and fertility (Aroian et al., 1994; Lesa and Sternberg, 1997). Genetic studies indicate that *let-23* mediates viability, vulva induction and development through RAS signaling (Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991; Hill and Sternberg, 1992; Han *et al.,* 1990, 1993; Chamberlin and Sternberg, 1994; Jiang and Sternberg 1998, Chang et al., 2000). However, mutations in RAS signaling components that suppress vulva and viability defects of *let-23* mutations cannot rescue fertility (Han et al., 1990; Clark et al., 1992), suggesting that the fertility function of *let-23* is mediated through a RAS-independent pathway. In a genetic screen, Clandinin et al. (1998) isolated mutations in two loci that can suppress the sterile phenotype of *lin-3* and *let-23: **l**et-23 **f**ertility **e**ffectors-1* and *l**et-23 f**ertility e**ffectors-2*. Cloning and molecular characterization identified a gain-of-function (gf) in LFE-1, *sy290*, an inositol 1,4,5-triphosphate receptor ($IP_3$ R) homolog,which acts as a positive effector of LET-23, and a loss-of-function in (lf) LFE-2/$IP_3$ kinase, which acts as a negative effector to mediate fertility by converting $IP_3$ to $IP_4$ (Figure 1). This, is consistent with the fertility function of *let-23* being mediated through a RAS-independent $IP_3$ signaling pathway (Clandinin et al., 1998).

The sterility defect associated with reduction-of-function mutations in *lin-3* or *let-23* (Horvitz and Sulson, 1980; Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991) results because these mutants fail to ovulate (McCarter et al., 1997;

Clandinin et al., 1998). Normally, ovulation occurs every half hour in the adult hermaphrodite. Oocytes align in the proximal gonad and mature in an assembly line fashion as they proceed proximally towards the spermatheca, a bivalved muscular compartment that stores the sperm (Figure 2). During ovulation, the myoepithelial sheath lining the proximal gonad contracts, and the spermatheca dilates to envelop a mature oocyte from the gonad into the spermatheca. The oocyte is fertilized and exits through the spermathecal uterine valve into the uterus where the egg is eventually laid through the vulva.

Ovulation in *C. elegans* hermaphrodites is a rhythmic motor pattern dependent on LET-23/EGF receptor tyrosine kinase (RTK) signaling, which proposedly stimulates $IP_3$-induced calcium release from internal stores (McCarter et al. 1997; Clandinin et al. 1998). $IP_3$ also regulates other worm behavioral rhythms. P. Dal Santo et al. (1999) has characterized two mutations in the *itr-1* locus, which was shown to be allelic to LFE-1: a reduction-of-function allele, *itr-1(sa73)*, and loss-of-function allele, *itr-1(n2559)*. Throughout the text, we refer to the LFE-1 locus which encodes the *C. elegans* $IP_3R$ as *itr-1*. She has shown that these mutations in *itr-1* disrupt the normal 50-second defecation behavioral rhythm. In ovulation behavior, the levels of $IP_3$ and possibly $IP_4$ are important for regulating intracellular calcium levels, which most likely control spermathecal dilation and hence ovulation. *lfe-2(lf); itr-1(sy290)(gf)* double mutants are semi-sterile. This phenotype perhaps results from excess $IP_3$ signaling due to the simulaneous presence of both the gain-of-function in the $IP_3R$ and loss-of-function in the $IP_3K$, a negative regulator. However, the exact physiologic defect in calcium signaling that causes sterility is unclear. We therefore characterized further the fertility defect associated with this double mutant. To this end, in collaboration with Minqin Wang, I screened for suppressors of the semi-sterile

*lfe-2(lf); itr-1(sy290 gf)* double mutant to find downstream effectors that control calcium signaling and the rhythmic ovulation pattern. I expect to find loss-of-function mutations in positive components of the signaling system as well as revertants of the gain-of-function *itr-1(sy290).* The *itr-1* gene is a relatively big locus composed of 32 exons and spans 15.5 kb and thus, poses a large target for mutational hits.

Suppression of this phenotype will yield other significant components that regulate ovulation through calcium signaling. Because $IP_3$-induced increases in intracellular calcium concentrations are important for diverse signaling pathways in many cell types (Berridge, 1993), identifying new genes downstream of *itr-1* and *lfe-2* may increase our general understanding of calcium signaling and how various components respond to and regulate calcium mobilization.

**Materials and Methods**

**Strains**

Strains were maintained at 20˚ C and handled according to standard protocol (Brenner, 1974). Ethylmethanesulfonate (50 mM) was used as a mutagen (Brenner, 1974).

The following alleles were used:

for LGI, *unc-57(ad592), lfe-2(sy326*), *unc29(e1072), dpy-5(e61);*

for LGII, *unc-4(e120), let-23(sy10), let-23(sa62)*;

for LGIV, *unc24(e138), lin-3(n1058), unc-44(e362), deb-1(st555), unc-82(e1223), itr-1(sy290), itr-1(sa73), itr-1(n2559), dpy-20(e1282);*

for LGX, *him-5(e1490).*

*mnC1[dpy-10(e128) unc-52(e444)]* is a rearrangement balancer chromosome on LGII. *DnT1[nT1[unc(n745dm)let]* is a reciprocal IV;V translocation balancer.

**Mutagenesis**

Strain PS2718 *lfe-2 unc-29; itr-1(sy290) dpy-20; syEx227 [R06H2, pMH86]* was mutagenized with 50 mM EMS. We screened 20, 000 haploid genomes. Suppressors of *lfe-2(lf); itr-1(sy290 gf)* sterility were identified by looking for fertile F2 Dpy Unc animals that no longer carried the transgene containing the wild-type copy of *itr-1(sy290)* and the phenotypic marker, pMH86, which rescues *dpy-20*.

Four candidates were isolated and backcrossed twice. It was determined that three candidates, *sy580, sy602, and sy604,* were linked to either *lfe-2* or *itr-1(sy290)*, while *sy597* is unlinked.

**Mapping**

To check for linkage of the suppressors (mut X) to *itr-1(sy290)*, we crossed *lfe-2 unc-29; itr-1(sy290) dpy20; mut X* to N2 wild-type males. We cloned F1 non-Dpy non-Unc progeny from two crosses. We then cloned F2 Dpy Unc animals and assayed their brood size to determine the frequency with which the suppressor was inherited. If suppressor X is unlinked, we expect 25% of the F2s to be fertile, whereas if it is linked, than >25% will be fertile. For *sy602*, 97.2% of F2 Dpy Uncs were fertile (n=71). For *sy604*, 86% of F2s were fertile (n=42). For *sy580,* 100% of F2s were fertile (n=25). The high frequency of F2 animals carrying the mutation which suppresses the sterility, indicates that *sy602*, *sy604*, and *sy580* are linked to either *lfe-2* or *itr-1(sy290)*. For *sy597,* 36% of F2s were fertile (n=55), suggesting the

suppressor mutation is unlinked, and segregates independently of *itr-1(sy290)* and *lfe-2*. Sequencing around the *sy290* region, shows *sy580* reverts the sy290 mutation to wild-type, while the *sy290* mutation is intact in *sy602* and *sy604*.

3X mapping

It is likely that *itr-1* poses a big target for mutagenesis, and thus these linked mutations may be second site mutations in the *itr-1* locus (map position 3.47) on chromosome IV or a second candidate gene, that encodes a 5-phosphatase (map position 3.18 on LGIV).

For the two mutations, *sy602* and *sy604*, I then performed a three factor mapping cross in a 2.31 map unit interval between *unc-44* and *dpy-20* that includes the *itr-1* locus. I first needed to build the recombinant mapping strain: *unc-44 mut X? itr-1 dpy-20*.

I heat shocked the hermaphrodite strain *unc-44 deb-1/unc82 unc-24* to generate a male stock. Subsequently *unc-44 deb-1/unc82 unc-24* males were crossed *to sy602 itr-1 dpy-20*. Cross progeny that are non-Dpy were subcloned. I tossed plates that segregrated unc-24 and those plates that did not were kept. The genotype was *unc-44 deb-1/ sy602 itr-1 dpy-20*. On these plates, I looked for recombinants between *unc-44* and *deb-1* which span an interval of 0.5 map units and are thus likely rare. Because *deb-1* is lethal, I identified recombinants that picked up*unc-44* and *dpy-20*. I identified 7 recombinants for *sy602* and 5 recombinants for *sy604*. These recombinants will include the *itr-1* and possibly the suppressor mutation if it maps within that region. They are of the genotype *unc-44 mut x? itr-1 dpy-20*.

The presence of the suppressor mutation in the recombinants must be assayed in the *lfe-2; itr-1* background. Thus, I rebuilt the strain crossing each of the different recombinants into *unc-57 lfe-2* using the following genetic scheme. N2 males were

crossed into *unc-57 lfe-2* hermaphrodites. Cross males of the genotype *unc-57 lfe-2*/+ were mated into each recombinant strain *unc-44 sy602? itr-1 dpy-20*. F1 cross progeny that were non-Unc-44 and non-Dpy were subcloned. I looked for plates that segregated *unc-57* (sluggish s-shaped worms) and cloned *unc-5* non-Unc-44 non-Dpy L4 hermaphrodites. Among these plates, I looked for those which segregated Dpy animals and cloned as many Unc-44 (paralyzed crescent moon shaped) Dpy animals of the genotype *unc-57 lfe-2; unc-44 mut X? itr-1 dpy-20* as I could find. I assayed the fertility/brood size in the next generation for presence of *sy602*. If *sy602* is present, then I expect suppression of the *lfe-2; itr-1* sterile phenotype. I tested 7 recombinants by rebuilding this double mutant strain. None of the recombinants picked up the suppressor as they were all semi-sterile /sterile. (I expected broods of 40 progeny, if the suppressor mutation is present.) Thus, the suppressor *sy602* maps to the left of *unc-44* based on the mapping data. The 3X mapping cross was performed using the same strategy for *sy604*. Five *unc-44 sy604?? itr-1dpy-20* recombinants were isolated. Crossing these recombinants into *unc-57 lfe-2,* n=2/5 recombinants picked up *sy604* indicating that *sy604* does map within the interval (see Results).

**Construction of *let-23(sa62)* with *itr-1(sy290)* or *lfe-2***

Construction of *let-23(sa62); lfe-2 dpy-5*

*lfe-2 dpy-5*/++ heterozygous males were mated into *let-23 unc-4* hermaphrodites. Twenty four F1 non-Unc cross progeny were picked individually, each from two independent crosses. From plates that segregrate *dpy-5*, I cloned Muv Dpy Unc animals of the genotype *let-23(sa62) unc-4(e120); lfe-2 dpy-5*. Animals of this

genotype were subsequently picked singly and scored for fertility by assaying brood size.

Construction of *let-23(sa62); dpy-5* *dpy-5 him-5*/++ heterozygous males were mated into *let-23 unc-4* hermaphrodites. F1 L4 non-Uncs animals were picked singly from three independent crosses. Subsequently, F2 Muv Dpy Unc animals of the genotype *let-23 unc-4; dpy-5* were picked singly to plates. Animals of this genotype were then individually picked and scored for fertility by assaying brood size.

Construction of *let-23(sa62) unc-4(e120)/mnC1; itr-1(sy290) dpy-20*

*sa62 e120/mnC1; him-5* males were mated *to itr-1(sy290) dpy-20* hermaphrodites. Cross male progeny were mated to *sa62 e120/mnC1* hermaphrodites. I then subcloned many F1 L4 progeny. Cross progeny hermaprhodites were identified as those plates that segregated Dpy, in addition to s*a62* (MUV), *unc-4*, and *mnC1*. From these plates, I picked individually non-Unc Dpy animals of the genotype *sa62 e120/mnC1; itr-1 dpy-20*. I then picked singly Muv Unc Dpy animals of the genotype *sa62 e120; itr-1 dpy-20* and assayed their brood size.

*let-23(sa62) e120 /mnC1; sy602 itr-1(sy290) dpy-20* was built using the same strategy except *sy602* itr-1(sy290) dpy-20 hermaphrodites were used.

*let-23(sa62) e120/mnC1); sy602 itr-1(sy290) dpy-20* was built using the same strategy except *sy604 itr-1(sy290) dpy-20* hermaphrodites were used.

*let-23(sa62) unc-4(e120); dpy-20* was received from N. Moghal.

**Construction of *mutant X itr-1(sy290)* with *let-23(sy10)***

Construction of *let-23(sy10) unc-4(e120); him-5*

*him-5* males were crossed into *sy10 e120/mnC1* hermaphrodites. Cross male progeny were then mated into *sy10 e120/mnC1* hermaphrodites. F1 L4 hermaphrodites were individually subcloned and cross progeny were identified by those which segregated him-5, indicated by presence of males. From 4 independent plates that also segregated *mnC1* and *unc-4,* I picked individually non-Unc L4 hermaphrodites and looked in the next generation for males to verify *him-5* homozygosity. I identified four different independent lines of the this genotype *let-23(sy10) unc-4(e120)/mnC1; him-5*. These animals were used for other crosses.

N2 males were mated to *sy10 e120/mnC1* hermaphrodites. Cross males were mated to *mut X itr-1(sy290) dpy-20*. The male cross progeny were mated to *sy10 e120/mnC1* hermaphrodites. F1 non-Unc animals were picked singly. Cross progeny were identified by looking for plates that segregate *dpy-20, mnC1, unc-4*, and *sy10* (*sy10* homozygotes were expected to display a lethal phenotype of rod-like larvae). From these plates, F2 *dpy-20* animals were then picked individually, having the genotype *sy10 e120/mnC1; mut X itr-1(sy290) dpy-20*. Subsequently, Unc Dpy animals of the genotype *sy10 e120; itr-1(sy290) dpy-20* were subcloned individually and scored for fertility. As a positive control, *sy597,* which was shown to be unlinked to *itr-1(sy290),* was also tested. The strain *itr-1(sy290) dpy-20* was derived from the parental mutant *sy597; lfe-2 unc-29; itr-1(sy290) dpy-20*, and used to test for suppression of *sy10* in the manner described above.

**Complementation of *mutant X itr-1(sy290)* with *itr-1(n2559)***

Construction of *itr-1/ mutX itr-1(sy290) dpy-20*

*sy604 itr-1(sy290) dpy-20; him-5* males were mated into *itr-1(n2559)/lin-45 unc-24* hermaphrodites. L4 F1's were then picked individually. Cross progeny were recognized as those plates that segregated Dpy and did not segregate uncoordinated animals and dead rods (indicative of *lin-45*). These animals were of the genotype *itr-1(n2559)/ sy604 itr-1(sy290) dpy-20*. I subsequently looked at the fertility of these animals by assaying brood size. As *itr-1(n2559)* homozygotes are sterile, I verified the presence of *n2559* by pickings singly constipated looking worms and checking that they were sterile. The complementation test for *sy602* and *sy580* was performed in the same manner.

Construction of *itr-1(n2559)/itr-1(sa73)*

*itr-1(sa73)/dpy-20* males were mated into *n2559/DnT1* hermaphrodites. I individually subcloned non-Unc F1 L4 hermaphrodites (n=53) and looked in the next generation to identify cross progeny or self. Of those animals that were fertile, n= 28 segregated *dpy-20* and non-Unc and were of the genotype *itr-1(n2559)/dpy-20*; n=3 segregated *dpy-20* and dead eggs and were of the genotype *DnT1/dpy-20*; n=2 segregated dead eggs and non-Dpy and were of the genotype *DnT1/ sa73*; n=2 were self progeny and segregated dead eggs and constipated animals. Of the 53 F1's subcloned, 19 were sterile and were presumably of the genotype *n2559/sa73*.

**Brood assay**

L4 larvae hermaphrodites were serially transferred to fresh plates every 12 hrs for 4 days at 20°C and progeny counted two days after eggs hatched.

**Sequencing the sy290 region in mutX *itr-1(sy290)***

The presence of *sy290* mutation in the *lfe-2; itr-1(sy290)* suppressors was tested by PCR and DNA sequence analysis. I amplified a region encompassing the *sy290* mutation using primers YBRec9F (ggg ttc atg caa cta atg caa c) and TCRec8R (gcg gat ccg caa gat gat cag agg). The PCR products were purified and sequenced.

**Microscopy**

Worms were anesthetized for 30 min in a solution of M9 with 0.1% tricaine and 0.01% tetramisole (Sigma Inc.) before mounting (McCarter et al., 1999). Under anesthetic, body wall muscles are paralyzed while oocyte ovulation occurs. Animals were mounted on 5% agarose pads with 20 μl of anesthetic, covered with an 18 mm glass cover slip, and the edges of the cover slip were sealed with Vaseline. Observations were made at 20°- 23°C.

**Results**

We performed a screen to isolate new downstream effectors involved in the $IP_3$ mediated ovulation pathway downstream of *itr-1(sy290)*and *lfe-2*. The screen in Figure 3 was designed to identify recessive mutations that suppress the sterility of *lfe-2; itr-1(sy290)*. The double mutant *lfe-2 unc-29; itr-1(sy290) dpy-20* can be maintained by carrying a transgene containing the wild-type copy of *itr-1(sy290)* and a plasmid rescuing *dpy-20* to phenotypically identify transgenic animals. Upon mutagenesis, Dpy Unc animals that are fertile will have picked up a suppressor mutation. In a screen of 20,000 gametes, we found four suppressors: *sy580, sy 597, sy602, sy604*. Based on preliminary mapping data, *sy597,* is unlinked to *itr-1(sy290)*,

while *sy580, sy602*and *sy604* are linked to *itr-1(sy290)* (See Materials and Methods). Because the *itr-1* locus spans 15 kb and thus poses a large target for mutational hits, we suspect that linked suppressors may be intragenic mutations of *itr-1*.

**Complementation with *itr-1(lf)***

To determine whether the three mutations are allelic to *itr-1(sy290),* I performed a complementation test using a loss-of-function allele of *itr-1*, *n2559,* which was shown to be a more severe allele than *itr-1(sa73)* (Dal Santo et al., 2000). *itr-1(n2559)* homozygotes are sterile but heterozygotes in trans to wild type are fertile (Dal Santo et al., 2000). As a control, we confirmed prior observations (Figure 4 Dal Santo et al., 2000) that a reduction-of-function allele of the *C. elegans* $IP_3$ receptor, *itr-1(sa73),* fails to complement the sterility of the null allele, *itr-1(n2559),* as *n2559/sa73* animals are sterile (n=19; Table 1). Additiionally, as a control *itr-1(sy290) dpy-20* derived from the parental extragenic suppressor *lfe-2 unc-29; itr-1(sy290) dpy-20; sy597* complements the sterility of *itr-1*. I tested three mutations (*sy602, sy604, sy580)* for allellism with *itr-1*. The three mutations complement the sterility of *itr-1*: *n2559/ mutX itr-1(sy290) dpy-20* animals are fertile, indicating they are not intragenic loss-of-function mutations in *itr-1* (Table 1). As fertility may be sensitive to *itr-1* gene dosage (Dal Santo et al., 2000; Clandinin et al., 1998), it is possible that these three mutants are wild-type revertants, or second site intragenic mutations that lower the gain-of-function activity (gf) of *itr-1(sy290).* Alternatively, they may be extragenic suppressors tightly linked nearby the *itr-1(sy290)* locus.

**Mapping of *sy602* and *sy604***

We tested whether *sy602* and *sy604* map within a 2.31 map unit interval encompassing the *lfe-1* locus. We preformed a 3-factor mapping cross using *unc-44* and *deb-1* as the marker strain to isolate recombinants in order to map *sy602* and *sy604* (see Methods ). The results show that *sy602* maps to the left of *unc-44* as none of the seven recombinants tested picked up *sy602*. All animals of the genotype *unc-57 lfe-2; unc-44 sy602? lfe-1* were sterile. Results for mapping of *sy604* tells us it maps within this interval. Two of the five recombinants tested picked up *sy604* as indicated by its suppression of *lfe-2; itr-1* sterility (Table 2). The mapping results are complicated because of the potential synergistic interaction of the marker *unc-44* with the other genes. We observed larval arrest in the test mutants *unc-57 lfe-2; unc-44 mut X ? lfe-1*. This poses a caveat to our mapping data, and thus, the interpretation of the mapping data may not be entirely correct. Sequencing the *lfe-* locus should resolve this issue of whether *sy602 and sy604* are intragenic second site mutations. Additional genetic tests can examine the gain-of-function activity of *lfe-1* in the background of these mutations.

**Suppression of *let-23(sy10)***

As *itr-1(sy290)* is a large locus presenting higher frequency for mutational hits, it is possible that the three suppressors linked to *itr-1(sy290)* may be intragenic mutations that either revert *sy290* to wild type or second-site mutations that compromise *itr-1(sy290)* gain-of-function (gf) activity. We performed a genetic test to see whether these suppressors affect the gf activity of *itr-1(sy290),* as measured by its ability to suppress the sterility of a reduction-of-function mutant *let-23(sy10). let-23(sy10)* is vulvaless and sterile (Aroian and Sternberg, 1991). However, *itr-1(sy290)* can bypass

the requirement for LET-23 signaling in the gonad; *let-23(sy10); itr-1(sy290)(sy290)* are fertile and have a "bag of worms" phenotype where the eggs accumulate in the uterus and hatch inside the mother, since there is no vulva through which the eggs can be expelled (Clandinin et al., 1998). Partial lethality by *let-23(sy10)* also occurs. We constructed the double mutant strains, *let-23(sy10); mut X itr-1(sy290).* As a positive control, *sy290 dpy-20* derived from the parental extragenic suppressor *lfe-2 unc-29; sy290 dpy-20; sy597* suppresses the sterility of *let-23(sy10)* as 100% of the animals are fertile, forming a bag of worms phenotype (n= 68; Table 3). Similarly, *sy602 itr-1(sy290)* and *sy604 itr-1(sy290)* can both suppress the sterility of *sy10,* although to a lesser extent. However, *sy580 itr-1(sy290)* no longer suppresses *sy10* sterility; 100% of the animals are sterile (n=98; Table 3 ). Thus, mutation *sy580* likely reverts *itr-1(sy290)* to wild-type, while *sy602* and *sy604* are not wild-type revertants as the gf activity of *itr-1(sy290)* to suppress *sy10* is intact.

To verify if *sy580* is a reversion mutation, I designed primers to amplify the genomic region encompassing the *sy290* region of *itr-1* in the suppressor strains. Sequencing the PCR products amplified from each mutant confirmed the genetic evidence that *sy580* is actually a wild-type revertant of *itr-1(sy290)*, while the *sy290* mutation is present in *sy602* and *sy604* animals. It is, however, possible that *sy602* and *sy604* are intragenic second-site mutations that subtly decrease the gf activity of *itr-1(sy290).* The *lfe-1* suppression of *sy10* in the background of these suppressor mutations in no longer 100% (Table 3). Atlhough these suppressors appear to decrease the gf activity, it is not enough to compromise its ability to suppress *let-23(sy10).* The other possibility is that they are extragenic mutations tightly linked to the *itr-1(sy290)* locus. If the former hypothesis is true, such second-site mutations of *itr-1* would be useful in understanding regulating of calcium gating in the $IP_3R$.

**Genetic interactions of *itr-1(sy290)* or *lfe-2* with *let-23(sa62)***

As noted above, *lin-3* EGF and *let-23* RTK reduction-of-function mutants are sterile because they fail to ovulate (Clandinin et al., 1998; McCarter et al., 1999). Mutant oocytes become trapped in the gonad arm and undergo multiple rounds of **e**ndo**m**it**o**tic DNA synthesis causing the Emo phenotype. A gain-of-function (gf) in the $IP_3$ receptor, *itr-1(sy290),* or a loss-of- function (lf) in the $IP_3$ kinase, *lfe-2,* can suppress *lin-3* or *let-23* sterility (Figure 1), indicating ovulation is dependent on $IP_3$ mediated signaling downstream of LET-23 RTK (Clandinin et al., 1998). *itr-1(sy290gf)* or *lfe-2(lf)* single mutants have a slightly reduced brood size and ovulate normally; however, *itr-1(sy290);lfe-2* double mutants display a much greater fertility defect than either single mutant; these animals are semi-sterile and have the Emo phenotype (Clandinin et al., 1998).

To investigate further if increasing signaling from molecular components upstream of *itr-1* and *lfe-2* also compromises ovulation, we have constructed double mutants of either the *lfe-2* or *itr-1(sy290)* genes with a gain-of-function mutant in *let-23(sa62)* (Katz et al., 1996). *sa62; dyp-5* animals have a reduced brood (average brood 15.8 ± 20.4, n=40; Table 4), which may in part be attributed to the sickness of the *sa62* homozygotes and to *dpy-5*, the isogenic control phenotypic marker. *sa62* mutants have an ovulation defect where the spermatheca constricts inappropriately tearing the oocyte as it enters (Bui and Sternberg, 2002). *lfe-2 dpy-5* synergizes with *sa62* to cause a further reduction in brood size and a more severe ovulation defect. The animals display the Emo phenotype (ave. brood 9.1± 7.6, n=50; Table 4). Similarly, *itr-1(sy290)* synergizes with *sa62* resulting in decreased brood size and partial lethality (ave. brood 13.4± 23.2, n=25; Table 4) relative to the isogenic control *sa62; dpy-20* (ave. brood 67.9 ± 22.2, n=20; Table 4). In these double mutants, we

also observe the Emo phenotype. Interestingly, the fertility defect of *let-23(sa62); itr-1(gf )* is partially suppressed in the background of either *sy602* or *sy604*. The brood is nearly doubled (Table 4). These genetic data provide further evidence that both *sy602* and *sy604* modify the gain-of-function activity of *itr-1(sy290)* by decreasing it.

We next examined whether a reduction-of-function in the $IP_3R$, *itr-1 (sa73),* could partially suppress the fertility defect from increased RTK signaling due to a gf in *let-23*. Surprisingly, a reduction of $IP_3$ signaling could not ameliorate the phenotype and restore the brood size to normal. *let-23(sa62); itr-1(sa73)* animals have a reduced average brood of 49.7 ± 30.4 (n=38) relative to the average brood of 83.1 ± 40.9 (n= 41) for *sa62* (see Table 4). In summary, these results suggest that ovulation and fertility are sensitive to levels of *let-23* mediated $IP_3$ signaling levels.

**Discussion**

We have performed a genetic screen looking for suppressors of *itr-1(sy290); lfe-2* sterility to identify novel downstream components of the fertility pathway. From 20,000 EMS mutagenized haploid genomes, we identified four suppressors, *sy580, sy597, sy602, sy604*. *itr-1(sy290)* and *lfe-2* were originally identified in a suppressor screen for *lin-3* sterility and found to encode an inositol 1,4,5-triphosphate receptor ($IP_3R$) and an inositol (1,4,5) triphosphate 3-kinase ($IP_3$ 3K) respectively (Clandinin et al., 1998). $IP_3$ binds the $IP_3R$ to release calcium from intracellular stores which serves as a second messenger for diverse cellular functions (Berridge, 1993). The $IP_3K$ converts $IP_3$ into inositol 1,3,4,5-tetraphosphate ($IP_4$), serving as one mechanism for down regulating $IP_3R$ signaling. Molecular cloning of these loci indicates LIN-3/EGF activation of LET-23 RTK signals downstream through $IP_3$ to presumably

release calcium to regulate spermathecal dilation during ovulation (Clandinin et al., 1998).

*lin-3* or *let-23* reduction-of-function mutants, which have insufficient signaling, are sterile because the spermatheca fails to dilate, hence ovulation is blocked. These animals have an Emo phenotype where the oocytes remained trapped in the gonad arm and the DNA undergoes multiple rounds of synthesis (Clandinin et al., 1998, McCarter, et al., 1999). *itr-1(sy290); lfe-2* double mutants are also sterile and have an Emo phenotype similar to *lin-3(rf)* or *let-23(rf)*. Double mutant animals with a gain-of-function (gf) in *itr-1(sy290* in combination with a loss-of-function in *lfe-2(sy326)* are ovulation defective, presumably due to excess $IP_3$ signaling. The $IP_3R$ can respond to a rise in the level of $IP_3$ or can release calcium in the presence of constant $IP_3$, through a process of calcium-induced calcium release (rev. Berridge, 1993), whereby calcium induces further release. This may have consequences for additional calcium release, as increased levels of $IP_3$ and calcium exert negative feedback on the $IP_3R$ (Besprozvanny et al., 1991; Berridge, 1993). The physiological affect on calcium release in *lfe-1; itr-1(sy290)* double mutant animals causing the sterility is unclear. In our screen, we expected to identify wild-type revertants of the $IP_3R$ or loss-of-function mutations in other positive components that regulate calcium signaling; these would suggest that the defect associated with *lfe-2;itr-1(sy290)* results from excess $IP_3$ signaling causing continual elevated calcium levels, which prevents spermathecal dilation and blocks ovulation. On the other hand, uncovering loss-of-function mutations in negative components in calcium signaling would indicate that excess $IP_3$ signaling inhibits further calcium release. Of the four candidate suppressors of *lfe-2;itr-1(sy290)* that were identified, sequencing confirmed genetic data that *sy580* is indeed a wild-type revertant. Cloning of the other

suppressors will help us better understand the physiological defect underlying sterility of *itr-1(sy290); lfe-2,* as well as mechanisms used to regulate calcium signaling.

The regulation of calcium signaling occurs at many levels, one of which is at the level of the $IP_3R$. Two other mutations are linked to *itr-1(sy290).* Three factor mapping placed *sy604* within a 2.31 map unit interval encompasing the *lfe-1* locus while *sy602* maps to the left of this interval. The caveat of the mapping data lies with the marker *unc-44* used and its potential synergistic interaction with the other genes, which affect viability and fertility. Sequencing the entire *lfe-1* locus in these suppressor strains will resolve this issue of whether these are second-site mutations. It would be interesting if we found additional second-site mutations of *itr-1(sy290)* to better understand how these mutations may alter the calcium gating properties of the $IP_3R$. Understanding the basis of positive and negative regulation is important as this feedback may determine the complex spatial and temporal pattern of calcium release. Fine control of the calcium levels is required to maintain specificity and understanding calcium regulation may increase our understanding of the control of the many diverse processes that depend on calcium.

We expect that other double mutants that have increased $IP_3$ signaling would result in ovulation defects causing a reduced brood size or, in more severe cases, a sterile Emo phenotype, like that of the *lfe-2; itr-1(sy290)* double mutant. We observed genetic interactions in double mutant animals with a gain-of-function in *let-23(sa62)* and either *itr-1(sy290)* or *lfe-2(sy326).* An activating mutation in *let-23* is expected to induce more production of $IP_3$. The cooperative effects of *sa62* with *lfe-2(sy326)*, which should increase $IP_3$ since $IP_3$ cannot be converted to $IP_4$, dramatically increases signaling, prevents ovulation, and results in the Emo phenotype. A similar situation likely occurs with *sa62* and a gf in *itr-1(sy290),* where the $IP_3R$ is more

sensitive to $IP_3$. These results are consistent with excess $IP_3$ signaling preventing spermathecal dilation causing sterility.

Based on our genetic data, a model for ovulation in *C. elegans* is presented in which $IP_3$ mediated intracellular calcium release, downstream of LET-23 RTK activation, modulates spermathecal dilation (Figure 4). Consistent with this, LET-23, ITR-1 and LFE-2 are expressed in the spermatheca. (Stuart Kim, personal comm., Clandinin et al., 1998; Dal Santo et al., 1999; Gower et al., 2001). Ovulation occurs every half hour in the adult hermaphrodite and is likely regulated by cyclic intracellular calcium release that occurs as LIN-3 builds up over the cycle and activates LET-23 mediated intracellular calcium release. The proximal oocyte appears to trigger ovulation (Mc Carter, et al., 1999). In this model, LIN-3 is expressed in the oocyte, and is then packaged and released to the cell surface to activate LET-23 and calcium release in the spermatheca, causing dilation (Clandinin, T.R. 1998). EMO-1 is a candidate protein for packaging LIN-3 (Mc Carter, et al., 1999; Iwaski et al., 1996).

*emo-1* encodes a homolog of the Sec61p gamma subunit, a protein necessary for translocation of secretory transmembrane proteins into the endoplasmic reticulum, and is required in the germline for fertility. It is a member of the class of sterile mutations that produce endomitotic oocytes in the gonad arm (Iwaski et al., 1996). Mutations that reduce activity in *lin-3* or *let-23* that would result in inadequate $IP_3$ signaling and hence insufficient calcium release for spermathecal dilation also result in the Emo sterile phenotype. Likewise, double mutants of *lfe-2; itr-1* have more severe defects on ovulation and fertility than would single mutants, because presumably excessive $IP_3$ signaling and calcium release block ovulation. Other proteins must then be employed to decrease the calcium levels to resting states.

In conclusion, we believe that the genetic screen presented will identify such components, elucidating mechanisms to regulate $IP_3$ signaling in the context of ovulation.

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**Table 1.** Complementation test for *itr-1*(n2559)

| **Genotype** | **% fertile** | **(n)** | **ave. brood** |
|---|---|---|---|
| *(n2559)/(n2559)* | 0 | 43 | 0 |
| *sa73/sa73* | 100% | 24 | 119.7 ± 47 |
| *n2559/ sa73* | 0 | 19 | 0 |
| *n2559/sy604 itr-1(sy290)* | 100% | 12 | 236.8 ± 30 |
| *n2559/sy602 itr-1(sy290)* | 100% | 20 | 224.4 ± 26 |
| *n2559/sy580 itr-1(sy290)* | 100% | 12 | 235.6 ± 34 |
| *n2559/ itr-1(sy290) ; sy597* | 100% | 17 | 233.1 ± 42 |

Legend. itr-*1(n2559)* is a null mutant of the IP3R (Dal Santo et al., 1999). These animals are sterile as homozygotes. We performed a complementation test of *n2559* sterility using the suppressor mutations that are linked to *itr-1*. *sa73* is a reduction-of-function allele of *itr-1* that fails to complement the sterility of *n2559*. All three suppressors complement *n2559* sterility. *itr-1(sy290)* was marked with *dpy-20(e1282)*.

**Table 2.** Mapping of *sy602* and *sy604*

Mapping was performed for *sy602 lfe-1 dpy-20* and *sy604 lfe-1 dpy-20* within the interval of *unc-44* and *dpy-20*. We isolated recombinants that uncoupled the markers, M1 *unc-44* and M2 *deb-1*. We tested for the presence of the suppressor mutations,*(sy602* and *sy604* ) by crossing the recombinants into *lfe-2* and looking for suppression of the sterile phenotype of *lfe-2; itr-1*. We show the fraction of those recombinants that picked up the suppressor and were thus fertile.

**Table 2** Mapping of *syt602* and *syt604*

| tester strain | Recombinant M1 nonM2 | *lfe-2; unc-44 mnt-x ? lfe-1 dpy-20* fertile (n) |
|---|---|---|
| *syt602 lfe-1 dpy-20/ unc-44 deb-1* | Recomb. *unc-44 syt602? lfe-1 dpy-20* | *lfe-2; unc-44 syt602? lfe-1 dpy-20* |
| | 12D | 0/15 |
| | 1D | 0/6 |
| | 9.2 | 0/21 |
| | 8D | 0/30 |
| | 7D | 0/9 |
| | 2D | 0/22 |
| | 3A | 0/15 |
| *syt604 lfe-1 dpy-20/ unc-44 deb-1* | Recomb. *unc-44 syt604? lfe-1 dpy-20* | *lfe-2; unc-44 syt604? lfe-1 dpy-20* |
| | 4A1 | 0/77 |
| | 9A5 | 3/13 |
| | 6A2 | 0/41 |
| | 8A2 | 5/33 |
| | 3A2 | 0/41 |

**Table 3.** Suppression of *let-23(sy10)*

| genotype | % fertile | (n) |
|---|---|---|
| *let-23(sy10)* | 0 | many |
| *let-23(sy10); lfe-1; sy597* | 100 | 68 |
| *let-23(sy10); sy602 lfe-1* | 83 | 52 |
| *let-23(sy10); sy604 lfe-1* | 96 | 26 |
| *let-23(sy10); sy580 lfe-1* | 0 | 98 |

Legend. *let-23(sy10)* is a reduction of function allele that is completely sterile. A gain-of-mutation, *sy290,* in *lfe-1* can completely suppress the sterile phenotype (Clandinin et al., 1998). Four mutations isolated in *lfe-2; lfe-1* suppressor screen were assayed for their affect on the gain-of-function activity of *lfe-1(sy290),* as measured by its suppression of *sy10*). An extragenic suppressor, *sy597,* and two linked suppressors, *sy602* and *sy604,* do not compromise the gain-of-function activity of *sy290* to suppress *sy10*. *sy580* is a wild-type revertant as this mutation abolishes *sy290* 's ability to suppress of *sy10*. *let-23* was marked with *unc-4(e120)*. *lfe-1* was marked with *dpy-20(e1282)*.

**Table 4.** Genetic interactions with *let-23* and *itr-1* or *lfe-2*

| Genotype | brood | (n) |
|---|---|---|
| *let-23(sa62); dpy-5* | 15.8 ± 20.4 | 40 |
| *let-23(sa62); lfe-2(rf) dpy-5* | 9.1 ± 7.6 | 50 |
| *let-23(sa62); dpy-20* | 67.9 ± 22.0 | 20 |
| *let-23(sa62); itr-1(gf) dpy-20* | 13.4 ± 23.2 | 25 |
| *let-23(sa62); sy602 itr-1(gf) dpy-20* | 28.9 ± 23.2 | 62 |
| *let-23(sa62); sy604 itr-1(gf) dpy-20* | 26.2± 23.3 | 25 |
| *let-23(sa62)* | 83.1 ± 40.9 | 41 |
| *let-23(sa62); itr-1(sa73)* | 49.7 ± 30.4 | 38 |
| *itr-1(sa73)* | 119 ± 47.3 | 24 |
| *itr-1(n2559)* | 0 | 50 |

Legend. *let-23(sa62)* is a gain-of-function allele that has a decreased brood (Katz et al., 1996) and ovulation defects (Bui and Sternberg, 2002). *lfe-2(lf)* and *itr-1(gf)* single mutants increase IP3 signaling, have decreased brood sizes and no ovulation defects. We examined the synergism between *let-23(sa62)* and either *lfe-2* or *itr-1*. Both mutations synergize with *sa62* and cause a further reduction in brood. Two linked mutations to *itr-1* decrease the gf activity of *itr-1* and partially decrease the synergism. A reduction-of-function mutation in the $IP_3R$, *sa73*, also synergizes with *sa62* and reduces the brood. *lfe-2* was marked with *dpy-5(e61)* and *itr-1* was marked with *dpy-20 (e1282)*. *let-23* was marked with *unc-4(e120)*.

**Figure 1.** *let-23* mediated signaling controls diverse function

Receptor tyrosine kinases mediate several signaling pathways to control a variety of cellular functions. In *C. elegans*, the LET-23 RTK is activated by LIN-3 to mediate a RAS-dependent pathway to mediate vulva induction, viability and development of the male tale. A RAS-independent pathway controls fertility. Upon activation of LET-23, phosphatidyl inositol biphosphate ($PIP_2$) is hydrolyzed into inositol triphosphate ($IP_3$). $IP_3$ binds ITR-1, an $IP_3$ receptor, and likely mobilizes intracellular calcium in the spermethecal cells and consequently the spermetheca dilates during ovulation. $IP_3$ can also be phosphorylated by LFE-2, an $IP_3$ 3-kinase. It remains unclear if $IP_4$ also acts as a signal.

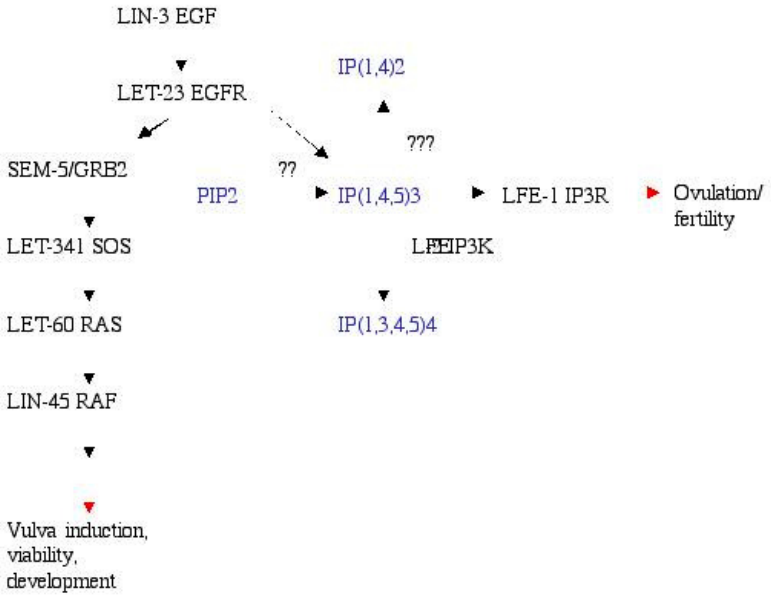
LIN-3 EGF
LET-23 EGFR
IP(1,4)2
???
??
SEM-5/GRB2
PIP2
IP(1,4,5)3
LFE-1 IP3R
Ovulation/
fertility
LET-341 SOS
LFEIP3K
LET-60 RAS
IP(1,3,4,5)4
LIN-45 RAF
Vulva induction,
viability,
development

**Figure 2.** Wild-type ovulation sequence

Nomarki ovulation sequence  Ventral is down.  The white arrow denotes the proximal gonad.  Oocytes mature as they proceed proximally towards the Spermatheca (Sp), outlined in green.  Upon fertilization, the egg exits the Sp into the uterus, denoted by the white bar.

**Ovulation is a rhythmic behavior**

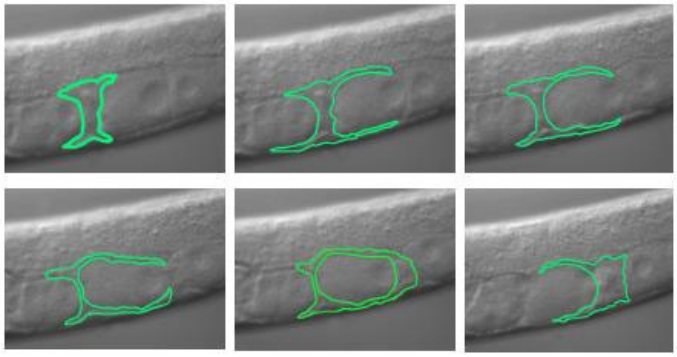

**Figure 3.** Suppressor screen of *lfe-2; itr-1(sy290)* sterility

*lfe-2 unc-29; itr-1 dpy-20* double mutants are sterile, but can be maintained by carrying a transgene of the wild-type copy of *itr-1,* along with a comarker of *dpy-20* which rescues *dpy-20* animals, allowing phenotypic identification of transgenic animals. Upon mutagenesis, an F2 clonal screen was performed looking for Dpy Unc animals no longer carried the transgene are that were fertile due to a new mutation, *m.

**Screen for Suppressors of *lfe-2; itr-(sy290)* sterility**

**EMS**

**P.** ***lfe-2 unc 29; itr-1 dpy-20; syEx*[*itr-1* (+), dpy-20(+)]**

⇓ ⇓

**$F_1$** ***lfe-2 unc 2 ;itr-1 dpy-20; syEx*[*itr-1* (+), pMH86] m*/+** or ***lfe-2 unc-29 ;itr-1 dpy-20*;m*/+**

Clone fertile non-Dpy Uncs — Sterile Dpy Unc

⇓

**$F_2$** ***lfe-2 unc-29 ;itr-1 dpy-20; syEx*[*lfe-1* (+), pMH86] m*/m*** or ***lfe-2 unc-29 ;itr-1 dpy-20*m*/m***

**Clone Dpy Uncs fertile??**

⇓

count $F_3$ progeny

**Figure 4.** A threshold of calcium likely regulates ovulation. Upon $IP_3$ mediated calcium release downstream of LIN-3 activation of LET-23, ovulation occurs. *lin-3* or *let-23* reduction-of-function sterile mutants have reduced signaling resulting in inadequate calcium levels for ovulation. Mutations that increase signaling, such as *let-23(gf)* or *itr-1(gf)* or *lfe-2(gf)* cause a reduction in brood size and ovulation defects. These fertility defects are more severe in double mutants.