Chapter 1

Receptor tyrosine kinase mediated inositol phosphate signaling

Extracellular signals initiate intracelluar signaling pathways to relay information inside the cell to evoke a cellular process, be it development or some behavioral response. Ligand binding to a receptor can lead to activation of signaling pathways to control multiple processes. How then does a cell interpret the information to generate a specific response appropriately? A challenge in signal transduction is to understand how biological specificity can be maintained when activation of the same receptor elicits different cellular responses and, furthermore, to elucidate the regulatory mechanisms that ensure an appropriate biological response. Unique responses can be generated because of quantitative (strength/duration of signaling) or qualitative (different intracellular pathways being activated downstream) differences as seen with the case of receptor tyrosine kinase (RTK) signaling.

The mechanism of action of RTKs and the signaling pathways they regulate provide insights about how a specific biological response can be generated. Upon activation of RTK, tyrosine phosphorylated sites can recruit different docking proteins to diversify signaling. Furthermore, signaling specificity can be achieved through different downstream effectors which regulate distinct functions. Another aspect of signal specificity is signal thresholds. Regulatory mechanisms that down regulate signaling once an optimal level is reached ensure an appropriate response. A twofold decrease in gene dosage of signaling regulatory proteins can have significant consequences on a biological response.

Studies in whole animals provide examples in which specificity can be transmitted during RTK signaling. Using *Caenorhabditis elegans* as a model system, we can address how biological specificity is achieved. It is a genetically tractable organism with a generation time of four days. The complete genome has been sequenced providing us with a complete array of genes to analyze for function within

a pathway (*C. elegans* Sequencing Consortium, 1998). The conservation of signaling pathways across eukaryotes allows extrapolation of data from one organism to predict existance of pathways and their components in another. The RTK-mediated RAS MAPK pathway was one of the first examples in which biochemical and genetic analysis demonstrated this pathway is conserved from nematodes and flies to vertebrates.

RTK signaling in *C. elegans*

Studies of the *C. elegans* LET-23 RTK is an example in which activation of distinct tissue specific effectors downstream of RTK signaling regulates different functions. The LET-23 RTK, the epidermal growth factor (EGF) receptor homolog, mediates multiple functions: viability, vulva induction, male tale development, and fertility (Ferguson and Horvitz, 1985; Han et al., 1990, 1993; Aroian et al., 1990, 1991; Hill and Sternberg, 1992; Chamberlin and Sternberg, 1994; Jiang and Sternberg 1998). In the past ten years, we have made considerable progress dissecting the vulva induction pathway (Figure 1), which consists of a highly conserved RAS signaling cassette: LIN-3, an EGF homolog, signals through LET-23 RTK, SEM-5/Grb-2, and LET-341/SOS, to activate LET-60 ras, LIN-45/ MAPKKK and MAPK protein kinases (Ferguson and Horvitz, 1985; Han et al., 1990, 1993; Aroian and Sternberg, 1991; Clark et al., 1992; Hill and Sternberg,1992; Chang et al., 2000).

Aroian et al. (1994) demonstrated different domains are required for the distinct functions of LET-23. Furthermore, Lesa and Sternberg (1997) demonstrated the distinct SH2 binding sites in the cytoplasmic domain of LET-23 RTK contribute to its distinct functions in vivo. These in vivo studies show that specific information is transmitted at the level of receptor via distinct signaling molecules recruited to and

activated by RTKs. Four of the binding sites on the cytoplasmic domain of LET-23 activate RAS through the SH2-SH3 adaptor SEM-5 to mediate some of the developmental processes. The fifth site mediates fertility and does not act through RAS. Because a comprehensive genetic analysis of LET-23 RTK RAS signaling has been carried out by Clandinin (1998), Lesa (1998), and Chang (2000), this body of work focuses on the genetic analysis of this RAS-independent pathway. The LET-23 effectors that mediate fertility were uncovered in a genetic screen for suppressors of sterility of *lin-3*, which encodes the ligand (Clandinin et al., 1998). Two genes were identified and molecularly cloned: *lfe-1,* which encodes an inositol 1,3,5-triphosphate (IP_3) receptor, and *lfe-2,* which encodes an IP_3 3-kinase. Clandinin's work shows that the fertility function of LET-23 RTK is mediated by tissue-specific effectors in the IP_3-mediated calcium signaling pathway.

Phosphoinosital metabolism and cell signaling

Activation of RTKs leads to stimulation of phosphoinositol metabolism to generate second messengers. The phosphoinositol specific phospholipase C γ (PLCγ) is recruited to the membrane through binding of its SH2 domain to phospho-tyrosine sites in the activated receptor which phosphorylates PLCγ (Margolis et al., 1989). Activated PLCγ hydrolyzes phosphatidyl 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG activates members of the protein kinase C (PKC) family, while IP_3 binds to the IP_3 receptor to mobilize intracellular calcium. Thus, stimulus-induced PIP_2 hydrolysis activates intracellular responses through its second messenger breakdown products (rev. by Berridge, 1993). Much of our understanding of IP_3 signaling comes from biochemical and molecular cloning

studies of enzymatic function. We shall review what is known of IP_3 signaling proteins.

Phospholipase C

The first characterization of two functionally distinct PLC enzymes was from sheep seminal vessicals (Hofmann and Majerus, 1982). Since then, multiple PLC isoforms have been biochemically identified that catalyze the hydrolysis of PIP2: isoforms α, β, γ, δ, and most recently, ε. Although there are differences in presence of some structural motifs amongst isoforms (i.e., plectin homology domain, EF domains, and SH2 domains) they all share in common the catalytic X and Y domains, and C2 regulatory domain. Functional differences among them have been difficult to detect based on enzymological studies (rev. by Majerus et al.,1990).

These enzymes all act on the same substrate, PIP2, embedded in lipid membrane bilayers. Thus, activation of these proteins must somehow involve recruitment to the membrane; for example, as discussed above for PLCγ. The biological role of PLC activation is unclear. The in vitro data for PLC's involvement in cell growth is conflicting. In cell culture studies, heterologous overexpression of PLCγ in NIH 3T3 results in increases in PtdIns turnover but is not sufficient for a mitogenic growth response (Margolis et al., 1990). However, microinjection of PLC β or γ, or antibodies to PLCγ in NIH 3T3 cells is sufficient (Smith et al., 1989; 1990). These cell culture studies implicate a role of PLC mediated PtdIns turnover in proliferation.

The inositol triphophate receptor and intracellular IP_3induced calcium signaling

The IP_3 receptor releases calcium from intracellular stores triggering calcium mediated events, including muscular contractions, secretion of neurotransmitters at synaptic terminals and of other secretory products, exocytosis, and gating of channels that regulate other processes. In resting cells, the extracellular calcium concentration is maintained at 10-100 μM levels, whereas inside, calcium is stored in various intracellular compartments so that the intracellular calcium concentration rests at 10-100 μM, and upon stimulation can rise to the micromolar levels. Calcium release can give rise to highly localized calcium signals that can spread throughout the cell. Mobilization of internal calcium stores is coupled with calcium entry from across the plasma membrane to refill the internal stores. This allows for a regenerative wave-like pulse of calcium release. It is thought that the oscillations encode some type of signal, and understanding the regulation of calcium signaling may provide insights to the fine control of various biological processes (rev. by Bootman and Berridge, 1995; Brown et al., 1995).

Release of calcium from intracellular stores is controlled by two different families of receptors: the ryanodine receptor (RYR), which controls release from the sarcoplasmic reticular stores (SR); and the inositol triphosphate receptor (IP_3R), which gates calcium from endoplasmic reticular (ER) stores. Both receptor families are present as tetramers and show significant homology in the calcium channel domain. Different isoforms of each family exist; however, the divergence of the three isoforms within each family and between species is very small, indicating a strict evolutionary requirement for maintaining conservation amongst species.

The IP_3R exhibits the phenomenon of calcium-induced calcium release (CICR), whereby small incremental release units of calcium further potentiate or

facilitate calcium release such that we observe an amplification of calcium release. Calcium eventually negatively feeds back on IP_3R activity. Thus, IP_3 signaling is subject to regulation at the level of the receptor. Understanding the ways in which calcium can negatively feedback on the IP_3R has consequences for calcium oscillations, which may encode specific information (rev. by Clapham, 1995). Thus, studying the regulation of the IP_3R may increase our understanding of the regulation of calcium signaling which contributes to signal specificity. The next section will focus on the IP_3R.

Although it was known that calcium release from sarcoplasmic stores was one mechanism of generating calcium transients, the mechanism by which calcium was released from other stores remained elusive until the identification of the IP_3R. Whereas voltage changes on the surface membrane convey information to the RYR to trigger intracellular calcium release from the SR, extracellular signals activate either a receptor tyrosine kinase receptor or G protein coupled receptor signaling pathways, both of which activate phospholipase C to hydrolyze phosphatidyl 4,5-bisphosphate to diacyl glycerol (DAG) and inositol triphosphate (IP_3). Ehrlich and Watras (1988) demonstrated that IP_3 induced openings of channels in planar lipid bilayers fused with vesicles from aortic smooth muscle SR. Lipid bilayer experiments measure the probability of open channels in response to a specific stimulus as monitored by cation movement through activated channels embedded in the lipid bilayer separating the cis and trans chambers in the setup apparatus. Single channel analysis of lipid bilayer experiments provided direct evidence that IP_3 activates a channel in smooth muscle SR. This channel differed from the RYR calcium gated channel of striated muscles SR based on the pharmacological profile. Caffeine is a known agonist of the RYR; however, it failed to induce calcium transients in these lipid bilayers fused with

vesicles from the aortic smooth muscle SR whereas IP_3 was able to induce calcium currents but not in the muscular striated SR vesicular preps (Ehrlich and Watras, 1988). These experiments led a search for the identity of that elusive receptor present in smooth muscle.

In addition to being present in smooth muscle, the IP_3R is highly concentrated in the cerebellum, as visualized by autoradiography. Electron microscopy revealed localization to the ER, which further supports the idea that the IP_3R is the receptor that gates calcium from internal ER stores. Biochemists took advantage of the affinity of heparin for the IP_3R to purify this channel from rat cerebellar microsomal preps isolated from cell fractionation experiments. They ran these preparationss over heparin affinity columns and then subsequently a sepharose affinity column to achieve 1000X purification of this receptor. When the purified receptor was inserted into lipid bilayers, it was activated by IP_3 as expected (rev. by Mikoshiba, 1994, 1997).

Besprozvanny et al. (1991) further showed that the IP_3R and other calcium gated channels from the ER vesicles from canine cerebellum in planar bilayers exhibited a bell-shaped calcium response curve. Addition of IP_3 to the IP_3R channels from ER of cerebellum in planar lipid bilayers activated the channel as detected by a conductance. To test whether calcium has a direct effect on the gating of the channel, they monitored the effect of calcium on channel activity. As the calcium was elevated from 0.01 μM to 0.25 μM, the channel open probability increased when IP_3 was added; however, above 1 μM of calcium caused inhibition. Examination of the sequence shows several calcium binding sites. Together, the data suggests that calcium exerts some negative feedback on the receptor, which has consequences for the regenerative calcium waves observed in cells. 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 CICR (rev. by Berridge, 1993) whereby calcium induces further release. The regulation of intracellular calcium allows for fine control of many cellular processes. The regulation of calcium signaling occurs at many levels, one of which is at the receptor level. Next, we examine the other modes by which the receptor seems to be regulated.

Actual binding of the agonist IP_3 exerts some positive effect on the gating, as increasing levels of IP_3 can potentiate or facilitate channel opening (Ehrlich and Watras, 1988). The evidence for cooperativity is still controversial. Since calcium release by IP_3 is positively cooperative, Meyer et al. proposed that IP_3 binding is cooperative (Hill coefficient n=3) and that channel opening depends on sequential binding of IP_3 to four sites on the tetrameric receptor (Meyer et al., 1988;1990). However, others find no evidence for cooperativity (Hill coefficient n=1) suggesting that binding of a single IP_3 molecule is sufficient for channel opening (Watras et al., 1991; Finch et al., 1991). So it still remains to be determined how IP_3 opens channels.

The sequence of the IP_3R suggests other factors involved in its regulation. The IP_3R can be divided into three domains: the ligand binding domain, the regulatory domain, and the channel domain. The regulatory domain has several consensus cAMP cyclic dependent kinase (PKA) sites. Mutation of these residues no longer confers PKA sensitive down regulation. Although PKA phosphorylation seems to shut down the receptor, this actually potentiates calcium release over longer periods of time since the pools of intracellular calcium stores remain higher. Chemical antagonists that inhibit PKC also potentiates IP_3-induced calcium release, suggesting protein kinase C also negatively regulates the receptor. The specific sites

that are phosphorylated have not yet been determined. Addition of ATP increases the probability of channel opening, indicating that ATP positively cooperates with IP_3 to activate the IP_3R (Bezprozvanny and Ehrlich, 1993). ATP's effect on calcium release maybe twofold, as ATP also is required for the SERCA pumps on the ER membrane which actively pump calcium back to refill the stores. Refilling of internal calcium stores allows for regenerative waves to occur (rev. by Mikoshiba, 1994).

Calcium may exert its negative effect on the receptor through calmodulin (Michikawa et al., 1999). The cytoplasmic calcium dependency of the purified IP_3R, was investigated by varying calcium concentrations from 0.15-200 μM. The purified receptor reconstituted in lipid bilayers behaved differently from microsomal IP_3R (vesicles fused with lipid bilayers) in that the purified preps did not show calcium-dependent inactivation as did the microsomal preps. This observation suggested that some other protein component in the microsome was responsible for the negative regulation. The IP_3R regulatory domain has a calmodulin binding domain suggesting that calmodulin may play a regulatory role. Planar lipid bilayer experiments reconstituted with a mostly homogenous prep of IP_3R1 demonstrate that the steep calcium dependence is due to calmodulin. Upon addition of calmodulin in high calcium medium, we observe an inhibition of the current in the lipid bilayers reconstituted with the purified IP_3R. Calmodulin antagonists could reverse this inhibition observed at high calcium further demonstrating that calmodulin directly plays an inactivating role in gating of the IP_3R.

The cell employs various control measures to regulate the intracellular calcium levels as calcium is involved in multiple processes. It makes use of two families of receptors that are often colocalized to control calcium release from internal stores: the RYR and the IP_3R. Upon stimulation, these tetrameric channels

open to release calcium in a wavelike fashion across the cell. 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 IP_3-mediated calcium release.

Metabolism of IP_3 -A mechanism of negative regulation

Because IP_3 acts as a key second messenger for many cellular processes, its production and metabolism must be tightly regulated to mediate normal responses dependent on IP_3 signaling. Thus, regulating the formation and metabolism of IP_3 is one way in which to modulate cellular processes dependent on this second messenger as the balance between formation and metabolism of IP_3 will likely determine the amount of signaling through the IP_3R. Mechanisms of metabolizing IP_3 thereby attenuating signaling rely on two negative regulators which are likely to be essential for proper cell function: the IP_3 kinase (IP_3 3K) and the inositol 5-polyphosphate phosphatase (5-Ptase).

Inositol 3-kinase

The first route of IP(1,4,5)3 metabolism is via IP_3 3-kinase, which phosphorylates IP_3 into IP(1,3,4,5)4. The role of IP_4 as a second messenger still remains inconclusive (rev. by Irvine 1995). IP_3 3-kinase activity was initially reported in *Xenopus* oocytes, upon microinjection of [^{3}H] IP_3 (Irvine et al., 1986). Subsequent biochemical studies show that the enzyme is soluble, while the 5-phosphatase activity is found in the particulate membrane fraction (rev. by Communi et al., 1995). It appears to be

regulated by calcium-dependent calmodulin (Lee et al., 1990; Communi et al., 1994). Activity of IP_3 3K is stimulated by calcium, however the degree of stimulation is cell type specific (rev. by Communi et al., 1995). Phosphorylaton of IP_3 3K by PKC shows a decrease in the 3-kinase activity, suggesting regulation via PKC (Sim et al., 1990).

Molecular cloning studies indicate two isoforms of IP_3 3K exist. Using the rat cDNA IP_3 3K isoform A to probe a human hippocampus cDNA library, a human IP_3 3K isoform A was isolated, along with a novel isoform B (Takazawa et al., 1991). The rat IP_3 3K isoform A has limited expression in the brain and testis, whereas isoform B is expressed in multiple tissues. Differential expression of IP_3 3K isoforms has been proposed to add an additional layer of regulation for IP_3 levels.

Additional biochemical expression studies of rat IP_3 3K have identified various domains. Site-directed mutagenesis studies identified key residues, Lys-197 and Asp-414, that are conserved in both isoforms among rat and human and are critical for ATP/Mg^{2+} binding within the C terminal catalytic. Furthermore, the calmodulin binding regulatory domain is localized to the regions of Ser156-Leu189 in the N terminus domain (Communi et al., 1993).

Inositol poly 5-phosphatase

The second mode of IP_3 metabolism is mediated by the inositol 5-phosphatase. The inositol 5-phosphatase removes the 5'-position phosphate from the inositol ring of Ins(1,4,5)P3 and Ins(1,3,4,5)P4 and from PtdIns(4,5)P2 and PtdIns(3,4,5)P3 to form Ins(1,4)P2, Ins(1,3,4)P3, PtdIns(4,)P and PtdIns(3,4,)P2, respectively. The first isoform of a 5-phosphatase was cloned by screening a pooled bacterial lysates from a λgt11 cDNA library for proteins which bind Ins(1,3,4,5)P4 (Ross et al., 1991). Since

the molecular cloning of this 75 kDa isoform, other isoforms have been biochemically identified and are classified based on their substrate specificity. The type I 5-phosphatases (43 kDa) act on the water soluble inositols IP_3 and IP_4, while the type II 5-phosphatases act on the phospholipids, PIP2 and PIP3 (rev. by Verjans et al., 1994). To date, these 5-phosphatases family members have been biochemically cloned from the bovine (Palmer et al., 1994), dog (Verjans et al.,1994), and human (De Smedt et al., 1994). Using degenerate oligonucleotides primers coding for a highly conserved region, other cDNAs encoding novel 5-phosphatases have been obtained (Mitchell et al.,1996).

Sequence comparison among family members of 5-phosphatases define two conserved signature motifs shared among all 5-phosphatases (rev. by Drayer et al., 1996). Futhermore, mutagenic studies have directly shown these domains function in catalysis (Jefferson and Majerus, 1996).

Genetics of IP_3 signaling

For many of these proteins in the inositol signaling pathway, biochemical studies via expression in heterologous systems have elucidated its enzymatic properties. However, these studies do not give us a full understanding of its physiological function in an intact organism. Thus, it is important to characterize its function in its native physiological context. Studying biological function in genetically tractable organisms allows the identification of physiological and behavioral responses in which these proteins are involved. For some of the components, similar proteins have been identified (see Table 1) and studied using genetic methodologies, which then gives us insights into their biological function. In the following section, I briefly

present what is known about the few genetic studies of IP_3 signaling proteins and the cellular functions that use IP_3 as a second messenger.

IP_3 signaling in mice

Extensive pathway analysis of IP_3 signaling in mice has not yet been performed, possibly because of functional overlap of multiple isoforms of proteins complicating functional anaylsis. Still, knock out studies have proved enlightening. The IP_3 type I receptor knock out in mice results in adult neuronal phenotypes of epilepsy and ataxia, suggesting it modulates motor control (Matsumoto et al., 1996). This is not surprising, considering the localization studies show its widespread presence in the brain. Because three isoforms exist, genetic studies in simpler model systems, whose genome encodes one subtype, may prove easier to dissect physiological function of IP_3 signaling.

Other studies of IP_3 metabolizing enzymes have implicated IP_3 in various processes. Deletions of mammalian 5-phosphatases in mice suggest that the enzymes have non redundant functions. The deletion of the SHIP type III 5- phosphatase, which expresses in hematopoietic cells, causes a severe myeloid proliferation and infiltration of the lungs (Helgason, et al., 1998). Deletion of synaptojamin type II 5-phosphatase causes defects in neuronal signaling along with early death (Cremona, et al., 1999). Double mutant mice with deletions of the OCRL 5- phosphatase (which when mutated in humans causes Lowes' oculocerebrorenal syndrome) and another type II 5- phosphatase result in embryonic lethality (Janne et al., 1998), while the single mutant shows no phenotype. Although these knock out studies implicate processes dependent on IP_3 signaling, more studies are needed to elucidate more components to fully understand its regulation in a physiologic context.

IP_3 signaling in yeast

To date, a yeast IP_3 receptor has not been identified; however, IP_3 metabolizing proteins have been genetically characterized, suggesting IP_3's involvement in various biological processes. Flick et al. (1993) identified a phosphospecific phospholipase C in yeast. Additionally, four 5-phosphatases have been identified: INP51, INP52, INP53, INP54 (Stolz, et al., 1998). Knock out technology to generate deletion mutants in yeast implicates their diverse cellular functions. Deletion of INP51 defines a role for INP51 in maintaining PIP2 homeostasis, as well as growth tolerance at cold temperatures (Stolz et al., 1998). Deletion analysis of INP51, INP52, and INP53 suggests these phosphatases may have roles in vessical trafficking, membrane structure and cell wall formation (Stolz, et al., 1998). Deletion of INP54 results in a 2-fold increased secretion of a reporter protein relative to wild-type, and likely plays a role in modulating secretion (Wiradjaja et al., 2001). Currently, the deletion analyses in various organisms have examined function of type II or type III 5-ptases; however, there are no genetic studies on type I phosphatases. My work in Chapter 3 presents genetic analysis of a type I 5-ptase to fill in the gap and expand our knowledge of biological function of 5-phosphatases and how they modulate IP_3 signaling.

IP_3 signaling in *Drosophila*

The IP_3R has been implicated in development. However, the physiologic processes it affects are not known. IP_3 R null mutants, *itpr*, were generated in flies, resulting in delayed larval moulting and lethality in the larval stages (Acharya et al. 1997; Venkatesh and Hasen,1997). Feeding ecdysone to mutant larvae partially rescues the *itpr* phenotypes, implicating this steroid hormone, which is involved with insect

moulting, is somehow linked to inositol signaling. Mutants have lower levels of ecdysone, indicated by reduced transcripts of the ecdysone inducible gene, E74. The authors conclude that the peptide hormone, which regulates synthesis and release of ecdysone, requires IP_3 signaling. Other key components of the signaling pathway for moulting have not yet been characterized.

A PLCβ-like gene, *norpA* has been cloned in *Drosophila* (Bloomquist, et al., 1988). Light activation of rhodopsin activates Gq, which stimulates PLCβ-mediated PIP2 hydrolysis to yield IP_3. Biochemical and genetic studies of the PLCβ fly homolog, *norpA,* provide definitive evidence for a cellular role in vision. *norpA* mutants lack PLC activity in the eye and result in complete blindness, indicating PLC is a critical component of the fly phototransduction pathway. Mosaic flies that are mutant for *itpr* have normal vision, indicating the IP_3 receptor does not seem to be essential for vision (Acharya et al 1997.). Thus, the IP_3 signaling pathway is not required. We now turn to the nematode.

<u>IP_3 signaling in *C. elegans*</u>

Ovulation in *C. elegans* is a prime example of how RTK-mediated signaling can generate a specific response because of activation of different downstream pathways. Ovulation is mediated by ras-independent signaling downstream of LET-23 RTK. Two proteins have been identified as ***L***ET-23 ***f***ertility ***e***ffectors. LFE-1/ITR-1, which encodes an inositol triphosphate receptor homolog, acts as a positive effector, while LFE-2, which encodes and IP_3 3-kinase, acts as a negative effector (Clandinin et al., 1998). Previously, our knowledge of IP_3 3-kinase function was limited to in vitro biochemical data; however, this recent genetic study has elucidated

a physiological function in *C. elegans* ovulation, modulating spermatheca contraction behavior.

The *C. elegans* IP_3 receptor, *itr-1*, was first identified based on homology to the mammalian homolog (Baylis personal comm. 1997). From these molecular cloning studies, we had no idea of the biological processes dependent on IP_3 signaling. Since then, an elegant genetic screen looking for suppressors of *lin-3* sterility has uncovered the essential function of IP_3 signaling in ovulation, which occurs every 30 minutes in the adult hermaphrodite.

In an adult hermaphrodite, oocytes align the proximal gonad oviduct, which is encased by a myoepithelial sheath. The sheath consists of an array of longitudinal filaments (Strome, 1986) that contract during ovulation to help expel an oocyte into the spermatheca (McCarter et al. , 1999). Oocytes mature in an assembly line fashion as they proceed proximally towards the spermatheca. The spermatheca is a bi-valved muscular structure that contains the sperm. The spermatheca can be anatomically divided into three sections: the distal spermatheca, the proximal spermatheca, and spermatheca-uterine junction. Eight distal spermathecal cells align in two rows to form a valve that separates oocytes in the oviduct from the sperm in the proximal spermatheca, consisting of 16 cells. The microfilaments of the distal cells are arranged circumferentially (Strome, 1986), and dilate during ovulation. Figure 2 depicts a schematic drawing of the proximal gonad. For a detailed review of the sheath and spermatheca structures, see McCarter et al.1997. Work in Chapter III implicates a critical function of a 5-phosphatase in negatively regulating dilation/contraction behavior of the distal spermatheca. Six cells form the spermatheca uterine (sp-ut) junction, which also acts as a valve through which fertilized oocytes exit into the uterus. Analysis presented in Chapter IV defines a role

for a novel phosphoinositol-specific phospholipase C in regulating sp-ut valve contractile behavior.

More recently, another genetic study has uncovered an essential role of the IP_3 R in the defecation behavior motor program in *C. elegans* (Dal Santo et al. 1999). This behavior consists of a 50-second contraction cycle of three distinct sets of muscles: the anterior body muscles, the posterior body muscles and the enteric muscles. Mutants in the IP_3 R, previously identified in a screen for an abnormal defecation rhythm, have been named *dec* for **de**fecaton **c**ycle defective: *dec-4 (sa73)* or *itr-1(n2559)* after the **i**nositol **t**riphosphate **r**eceptor (Iwasaki et al., 1995; Dal Santo et al., 1999). Figure 3 shows a summary of the structure of the ITR-1/LFE-1 and mutations isolated. To date, IP_3 signaling has been implicated in control of behavioral rhythms in *C. elegans* defecation and spermathecal contraction in ovulation.

Thesis overview

Powerful technologies which enable targeted gene knock outs, along with completed genomes, have facilitated the undertaking of genetic studies of IP_3 signaling in mice, flies, and worms to define some of the biological processes that require IP_3 signalling. Still, all of the key components of the inositol signaling pathway have not been characterized in the present genetic studies to give us a complete picture of how IP_3 signaling modulates a particular response. The advantage of having IP_3 signaling mutants is the possibility of designing genetic screens to identify other interacting genes to elucidate the signaling pathway. In Chapter 2, I describe a screen to identify genes that suppress the ovulation defective sterile phenotype of the double mutant *lfe-2;lfe-1,* which are defective for the IP3R and IP_3 3K. Many aspects of regulation of

IP_3 signaling via metabolism of IP_3 remain undefined. We do not know which of the two metabolizing enzymes is most important in regulating levels of IP_3 and IP_4 in the intact cell. In Chapter 3, I describe the genetic characterization of a *C. elegans* 5-phosphatase type I and its primary role in negative regulation of spermatheca contraction behavior in ovulation. Our genetic analysis of other mutants with perturbed IP_3 signaling provides evidence that levels of IP_3 signaling are crucial for proper ovulation, demonstrating specific information is imparted by thresholds. In Chapter 4, I present the genetic characterization of a new member of the PLC family, whose function is also critical for ovulation.

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Table 1. IP3 signaling proteins across species

	flies	worms	yeast	mammals
IP3 Kinase	CG18854	LFE-2	----	IP3Kb IP3Ka
IP3R	ITPR-83A	LFE-1/ITR-1	----	IP3R1,IP3R2, IP3R3
5-phosphatase	CG7613	IPP-5 C01B10.3	---	5-phosphatase type I
		C16C2.3	INP51 INP52 INP53 INP54	5-phosphatase type II
				OCRL type II SHIP type III
PLC	NORPA	EGL-8 T01E.8	GI:295640	PLC Beta PLC gamma
		PLC-210	----	PLC Epsilon PLC sigma PLC alpha

Legend. Summary of IP3 signaling proteins identifed in flies, worms, yeast, and mammals: IP3 Kinase, IP 3 receptor, 5-phosphatase, phosphospecific phospholipase C. For some, actual genes have not been identified but merely predicted to exist in the completed genome based on homology: fly, CG18854, CG7613 (Adams et a.l 2000); worm C01B10.3, C16C2.3, T01E.8 (C. elegans Sequencing Consortium, 1998); yeast, GI295640 (Goffeau et al. 1996).

Figure 1. Two pathways originate from the LET-23 RTK. A Ras MAPK pathway mediates vulva induction, viability, and development. While an IP3-mediated pathway that signals through the IP3Receptor, *lfe-1,* mediates fertility.

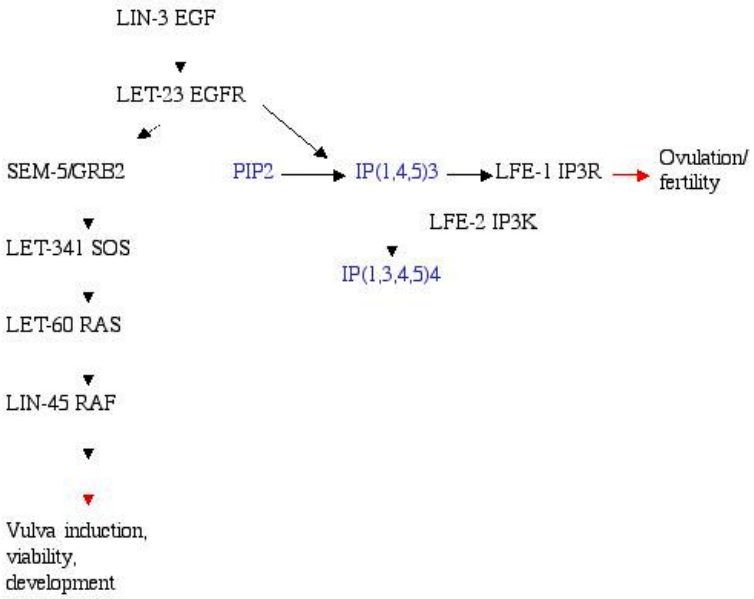


Figure 2. Schematic drawing of the proximal gonad of a *C. elegans* hermaphrodite. The spermatheca (denoted Sp) can be structurally subdivided in three regions (inferred from McCarter et al. 1997). Oocytes align the proximal gonad on the right. The distal spermatheca consists of eight cells (depicted in yellow) which form an entry gate through which oocytes enter into the proximal spermatheca (16 cells in grey), where fertilization occurs. After fertilization, the egg exits through the spermatheca-uterine junction (consisting of six blue cells) into the uterus where the eggs are eventually expelled through the vulva.

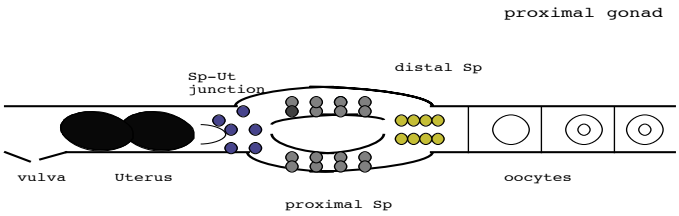
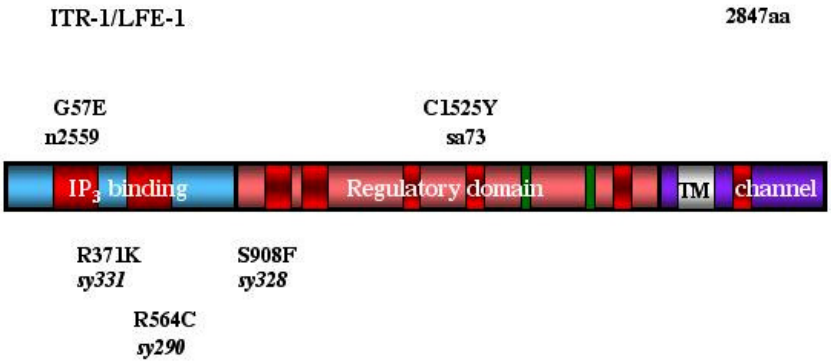


Figure 3 Summary of structure of IP_3 receptor and Mutations Identified.
The IP_3R consists of three functional domains: an IP_3 binding domain (depicted in blue), a regulatory domain (in pink) followed by a channel domain (purple) which includes the transmembrane domain (white). The domains are inferred from Mikoshiba et al. 1994. The red boxes depict calcium binding sites that are proposed to regulate channel opening. The identified point mutations in *itr-1*/*lfe-1* are depicted with the amino acid residue subsitution. *sy290, sy331,* and *sy328* are gain of function mutations (Clandinin et al. 1998), while *sa73* is a reduction of function and *n2559* is a null (Dal Santo et al. 1999).


ITR-1/LFE-1
2847aa
G57E
n2559
C1525Y
sa73
IP3 binding
Regulatory domain
TM
channel
R371K
sy331
S908F
sy328
R564C
sy290