ANALYSIS OF EGF SIGNALING IN HEALTHY AGING PROMOTION IN *CAENORHABDITIS ELEGANS*.

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Biology of aging

Aging can be defined as the gradual changes in an organism over time accompanied by progressive loss of physical function and increasing mortality with advancing age. The rapidly increasing elderly population suffers a diminished quality of life and consumes a disproportionate amount of health care resources. Therefore, understanding the biology of aging is centrally important for medical, political, and social reasons.

There are many factors involved in the aging process, including genetic and environmental components. Several conserved genes in model systems from yeast *Saccharomyces cerevisiae* to *C. elegans*, *Drosophila*, and mice exhibit remarkable effects on life span. For example, mutants that reduce activity of the insulin signaling pathway exhibit slowed locomotory decline and extended lifespan (Kenyon, 2010). Likewise, genes or conditions affecting dietary restriction (Lakowski and Hekimi, 1998; Panowski et al., 2007) or mitochondrial function (Dillin et al., 2002b; Feng et al., 2001) can alter lifespan. Furthermore, environmental signals such as stress (An and Blackwell, 2003; Apfeld et al., 2004; Hsu et al., 2003) and nutrient availability can affect organism longevity. Stochastic factors also are significant in aging and can explain how individuals with a uniform genetic background and environment can age either well or poorly (Herndon et al., 2002). Overall, genetic, environmental, and stochastic processes exert interconnected effects on quality of aging.

Sarcopenia is one of the most prevalent conditions in aging and genetic approaches toward understanding

Sarcopenia, the progressive decline of age-related muscle mass and strength with age, is a major problem of functional decline and frailty in the elderly. Sarcopenia initiates at midlife and causes significant loss so that individuals can expect nearly 50% muscle mass loss by age 90 (Evans, 1997). At the cellular level, sarcopenia is associated with disorganized change in muscle fibers, including sarcomere loss, and lipid content increase in the muscle tissue. There are several factors that are correlated with sarcopenia during aging such as contraction-related cellular injury, endocrine changes, reduced regenerative potential, and oxidative stress (Chow et al., 2006). However, the pathophysiology leading to the development of sarcopenia is not well investigated genetically or molecularly and preventative therapies are not available (Fisher, 2004).

The benefit of addressing aging biology in simple organisms

Analyses in invertebrate models have significantly advanced understanding of genetic and environmental influences that modulate aging process. Manipulating aging of higher organisms is difficult and time consuming. However, simple organism models (yeast (*S. cerevisae*), worms (*C. elegans*), flies (*D. melanogaster*), to mice (*M. musculus*)) can be good experimental sources in which to advance understanding of basic biological mechanisms. For instance, *C. elegans* lives only about three weeks in the lab, whereas humans live 10 decades. Many of these simple models share biologically relevant pathways with

mammals. Simple models are also easier to maintain and do not present experimental ethics barriers. Indeed, much insight into the basic biology of aging has been gleaned from studies of conserved pathways in simple model systems.

Caenorhabditis elegans – a powerful model organism for the study of aging

Caenorhabditis elegans is a small free-living, non-parasitic soil nematode that feeds mainly on bacteria. *C. elegans* have a life cycle that progresses through four larval stages: L1, L2, L3, and L4, then molt into reproductive adult (Figure 1). The animal exists primarily as a self-fertilizing hermaphrodite that reproduces the major proportion of *C. elegans* with a low frequency of males. Each hermaphrodite animal is fertile for approximately 4-8 days and can produce over 300 progeny. The progeny from hermaphrodite are genetically identical to the parent, yielding an extremely useful homogeneous genetic tool. However, there is also sexual reproduction between male worms and hermaphrodites to provide facile genetics studies.

C. elegans is a popular and powerful *in vivo* genetic model used to study the process of aging. The entire genome is sequenced and annotated, which facilitates conducting a broad range of molecular and genetic experiments. Many genetic mutants, RNAi knockdown gene libraries (Kamath and Ahringer, 2003), or DNA transgenic animals (for example, generating GFP reporter fusion) are available to manipulate gene expression. Plenty of data has been acquired and

much insight into basic biological mechanisms has been gained. The advantages of using *C. elegans* specifically for aging studies include the relatively short life cycle. Analysis of aging tissues can be simplified since all somatic cells are post-mitotic and there is no tissue regeneration. Numerous experiments have identified mutations that dramatically extend lifespan, and analyses of these mutants has provided a basis for understanding the mechanisms driving the aging process (Antebi, 2007; Kenyon, 2010).

Aging in *C. elegans*

Although multiple factors are involved in aging process in *C. elegans*, many single longevity related mutations known in yeast, flies, and mice dramatically extend the *C. elegans* lifespan (Longo and Finch, 2003)(Figure 2). In addition, there is a major stochastic component to how well animals age that is distinct from standard genetic programming and standard environmental factors (Herndon et al., 2002).

C. elegans has a basic biology of muscle structure and function that is remarkably similar to human skeletal muscle. Moreover, nematode sarcopenia bears striking similarity to human sarcopenia at the cell and phenomenological levels such as mid-life onset, sarcomere degeneration, and fat accumulation (Herndon et al., 2002). Interestingly, *C. elegans* muscle integrity deteriorates dramatically while the nervous system shows little obvious gross structural change in aging worms. Given the power of *C. elegans* genetic screens to

identify genes required for specific aging phenotypes, it is plausible to screen for genes that influence muscle healthspan by identifying those that accelerate or delay mobility decline (Iwasa et al., 2010; Schreiber et al., 2010).

Evaluation of healthy aging in *C. elegans*

As a focus of aging research progresses from concentration on the longevity endpoint to increased healthspan evaluation, the issue of how to measure healthy aging becomes a front-and-center challenge. One metric that most likely indicates healthspan at the organism level is measurement of survival of an aging population at middle and middle/late life--scores such as the 50% survival time point or mean lifespan. Genetic or drug manipulations that extend mean survivorship but not necessarily maximum lifespan are likely to be of value for extending the period of healthy life prior to decline. Analysis of mortality curves is another option for analysis of changes in the "rate of aging" (Johnson, 1987).

Several *C. elegans* behaviors decline with age (Bolanowski et al., 1981; Chow et al., 2006; Garigan et al., 2002; Glenn et al., 2004; Herndon et al., 2002; Huang et al., 2004) and the extent of change can be used as a measure of healthspan. Pumping of the pharynx, an organ through which the animal ingests its bacterial food, declines fairly precipitously with age (Chow et al., 2006; Huang et al., 2004). A two-day-old adult pumps on the order of 300 contractions/minute, a rate that progressively slows to essentially zero at about 12 days of adult life (Collins et al., 2008). Pharyngeal contractions are easily observed through the transparent

cuticle, and can be scored directly or with the aid of video analysis. Because the pharynx pumps by some autonomous mechanisms and includes specialized muscle that resembles mammalian heart muscle, the *C. elegans* pharynx is sometimes considered to model mammalian heart (Mango, 2007), and its age-associated decline to model mammalian cardiac aging.

The vigor of locomotion on solid support (agar plates in the lab) or in liquid (swimming) declines during *C. elegans* adult life. Diminished rates of movement can be measured by eye (body bends per unit time) or with computer programs that track locomotion (Huang et al., 2006; Schreiber et al., 2010; Tsechpenakis et al., 2008). Interestingly, locomotory behavior decline correlates with deterioration of body wall muscle, with fewer sarcomere units present over time and increased fat infiltration, reminiscent of mammalian sarcopenia (Garigan et al., 2002; Glenn et al., 2004). Although muscle exhibits markedly more dramatic cellular deterioration than do neurons, some neuronal influence on *C. elegans* sarcopenia appears operative (Glenn et al., 2004; Murakami et al., 2008).

Another conserved trait that tracks with age is the accumulation of fluorescent lipofuscin and advanced glycation end products (Gerstbrein et al., 2005), collectively referred to as age pigments. In *C. elegans* this fluorescence is concentrated in gut granules that appear to be secondary lysosomes (Clokey and Jacobson, 1986). Since extensively cross-linked lipofuscin cannot be degraded by lysosomal machinery, lysosomes accumulate this fluorescent material and are

thought to become progressively impaired as adults age. Interestingly, animals that age well (by locomotory criteria) tend to have low age pigment accumulation and those that age poorly accumulate relatively high levels (Gerstbrein et al., 2005). Likewise, animals that are long-lived (for example the *daf-2(rf)* insulin receptor mutant) tend to have lower age pigment scores than do progeric mutants such as *daf-16*/FOXO transcription factor. Thus, high age pigment scores suggest a poor healthspan and relatively low age pigments scores suggest a strong healthspan. Age pigments can be scored using an image analysis program that measures relative intensity of fluorescence in the 340nm range; a more precise approach is to use a fluorometer that can scan samples over a range of input wavelengths and quantitate emission spectra (typically peak emission is ~342 nm).

Although more detailed cellular phenotypes than those listed above can be characterized, use of the aforementioned relatively easily determined measures can indicate whether a population appears to be aging well or poorly relative to wild-type controls. Note that animals may exhibit system-wide evidence of a strong healthspan (all measures) or extended healthspan of individual tissues (only pharynx, body wall muscle, intestine, for example) for a given genetic or drug intervention.

The Insulin/IGF signaling pathway

Insulin/IGF signaling has multiple roles in biology functions. The insulin/IGF pathway affects cellular metabolism, development, and aging of living organisms. Importantly, the insulin signaling pathway is conserved from nematodes to humans. Dauer formation defects and longevity are the most observed phenotypes caused by insulin signaling pathway mutations in *C. elegans*. Some insulin signaling pathway molecules such as insulin-like receptor DAF-2, and downstream transcription factor FOXO/DAF-16 can regulate dauer formation (Gottlieb and Ruvkun, 1994). Interestingly, mutations involved in the insulin signaling pathway induce a conserved mechanism that promotes longevity in yeast, worms, fruit flies, and mammals (Kenyon, 2001). Previous studies have shown that down-regulation of *daf-2, age-1*, *pdk-1,* and *akt-1/akt-2* in the insulin/IGF-1 signaling pathway extends lifespan (Kimura et al., 1997; Morris et al., 1996; Paradis et al., 1999; Paradis and Ruvkun, 1998); while *daf-16* inactivation shortens life span (Lin et al., 1997; Ogg et al., 1997). In *C. elegans*, the insulin-like signaling pathway features 40 insulin-like ligands, the DAF-2 insulin like receptor, PI3 (phosphatidylinositol-3-OH) kinase AGE-1, kinases PDK-1, AKT-1/AKT-2, SGK-1 and downstream DAF-16/FOXO transcription factor (Figure 3). Mutations in the genes upstream of *daf-16* lower DAF-16 phosphorylation and DAF-16 translocates into the nucleus (Lin et al., 2001). In the nucleus DAF-16 affects longevity by turning on or repressing genes that regulate stress responses, development and metabolism. Some other signaling such as germline signaling (Hsin and Kenyon, 1999) and JNK signaling (Oh et al.,

2005) can also affect life span through DAF-16 only, but not the whole insulin signaling pathway.

EGF signaling is a conserved pathway in many organisms

The epidermal growth factor receptor (EGFR) is transmembrane receptor tyrosine kinase that belongs to the mammalian ErbB subfamily (EGFR, Erb-2/HER-2; Erb-3/HER-3; Erb-4/HER-4). When mammalian EGF family ligands bind (EGF, TGF-α, HB-EGF, amphiregulin, epiregulin, epigen, betacellulin, neuregulin) (Figure 4), EGFRs regulate cellular signal transduction pathways that regulate cell proliferation, survival, and migration (Citri and Yarden, 2006). Although different ligands can bind to different receptors to induce the signaling network, the ligands contain an EGF-like consensus sequence which consists of six cysteines, and the receptors include extracellular ligand binding domain (leucine-rich region; responsible for ligand binding; termed as L domain), transmembrane domain, and kinase domain.

EGF signaling is a conserved pathway and several genetic studies have studied EGF signaling in model organisms such as *Drosophila* and *C. elegans*. In *Drosophila,* EGF family signaling is mediated by TGFα-related ligands Spitz (ventral ectoderm patterning (Schweitzer et al., 1995)), Keren (Reich and Shilo, 2002), Gurken (oogenesis (Nilson and Schupbach, 1999)), and Vein (oogenesis role, neuregulin homolog (Schnepp et al., 1996)) and EGFR homolog DER (Shilo, 2003)(Figure 4). One secreted EGF-binding protein, Argos, is known to

sequester Spitz ligand to limit signaling (Klein et al., 2004). During embryogenesis, the ligands regulate the receptor activation temporally and spatially, maintaining gonad homeostasis and growth (Gilboa and Lehmann, 2006; Shilo, 2005).

In *C. elegans*, LIN-3 and LET-23 are the homologs of EGF ligand and EGFR, respectively (Aroian et al., 1990; Hill and Sternberg, 1992). There are three distinct downstream signal transduction pathways for EGF signaling (Figure 5). EGFR acts through the RAS-MAPK (*let-60-mek-1*) pathway to affect several cell fates, including the well-studied vulva development and male spicule formation (Moghal and Sternberg, 2003). In addition, EGFR acts through PLC-γ (*plc-3*) and IP3-inositol (1,4,5) trisphosphate (*itr-1*) signaling to affect ovulation and defecation (Clandinin et al., 1998; Yin et al., 2004). EGFR (LET-23) signaling also induces behavioral quiescence at the molt through *plc-3*, *unc-13*(diacylglycerol binding protein), and *tpa-1*(diacylglycerol binding protein). *unc-7* (gap junction innexin) is required for inhibition of feeding at the molt, and *egl-4* (cGMP dependent protein kinase) is required for inhibition of locomotion (Van Buskirk and Sternberg, 2007). However, no aging related information has ever been published on this pathway in nematodes.

Summary of thesis work

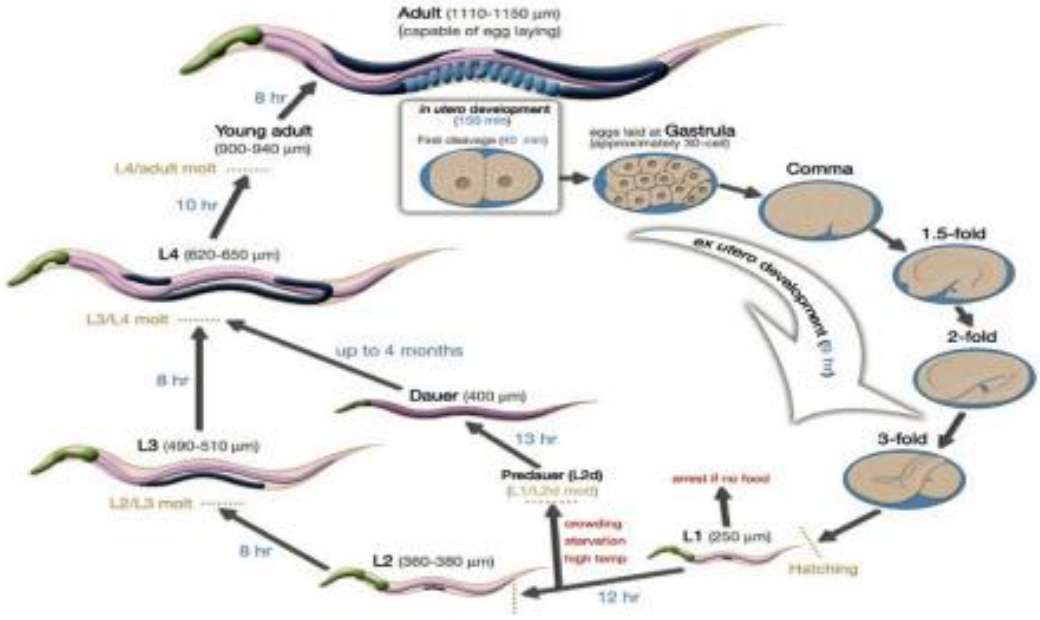
In this thesis I describe our efforts to identify factors that delay *C. elegans* sarcopenia, and how that work unexpectedly led us to discover that the EGF

pathway is a strong promoter for healthy aging in *C. elegans* (Chapter 2). Our initial work also showed identified novel negative regulators of EGF healthspan signaling, the HPA genes, which have primary sequences that suggest they might bind EGF ligand to limit signaling. We also defined the ITR-1 branch of the EGFR signal transduction pathways as critical for promoting healthspan.

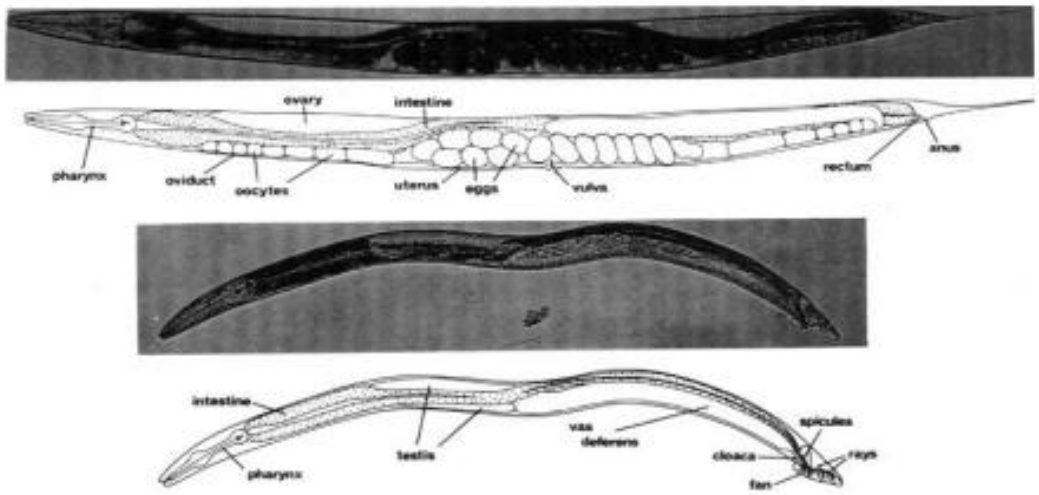
I further investigated details of the mechanism by which EGF signaling promotes healthspan (Chapter 3). I identified splice isoforms EGF-S and EGF-XL as factors that can promote healthy aging, I showed that EGF acts both in development and during adulthood to influence aging, and I showed that EGFR(gf) expression in muscle, neurons and intestine can promote healthy aging. I also identified additional molecules that act in the pathway to extend healthspan, and I found some candidate proteins that might directly link cellular calcium changes to healthy aging. Overall, my thesis work began when the field did not know anything about EGF signaling effects on aging in any organism and ended with the identification of multiple gene activities in one specific sub-pathway that promote successful aging. Other recent discoveries in the field, including the establishment of a role of a different EGF subpathway for maintaining protein folding homeostasis (Liu et al., 2011) and a role of EGF in protecting against late-onset polyglutamine expansion disorders (Fryer et al., 2011), support that EGF signaling in general plays an important role in late-age maintenance.

Figure 1

A



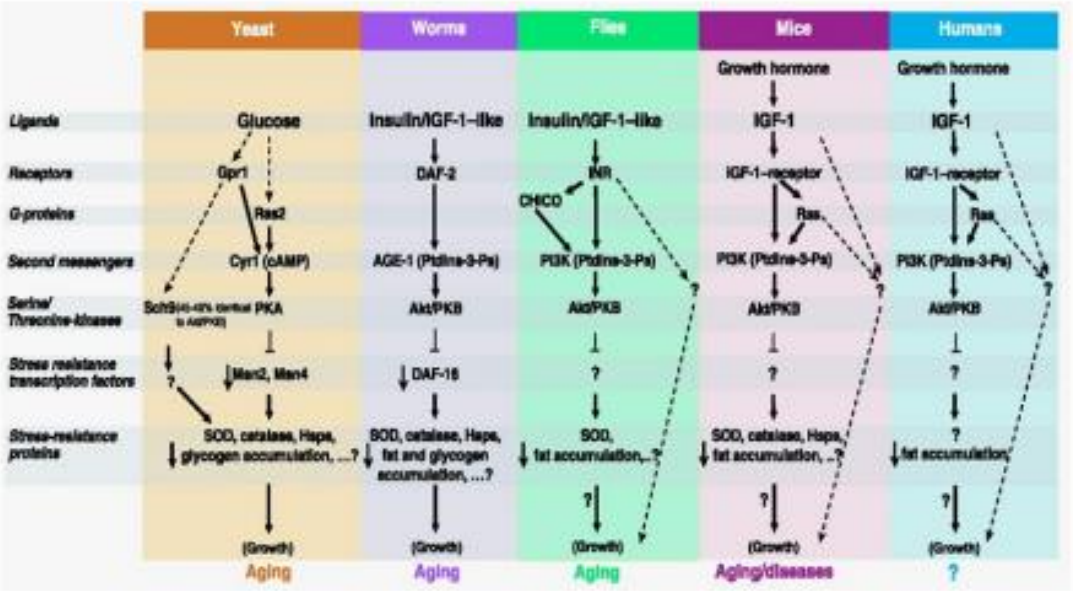
B



A. The life cycle of *C. elegans* (worm atlas)

B. The anatomy of *C. elegans* (*C.elegans II*)

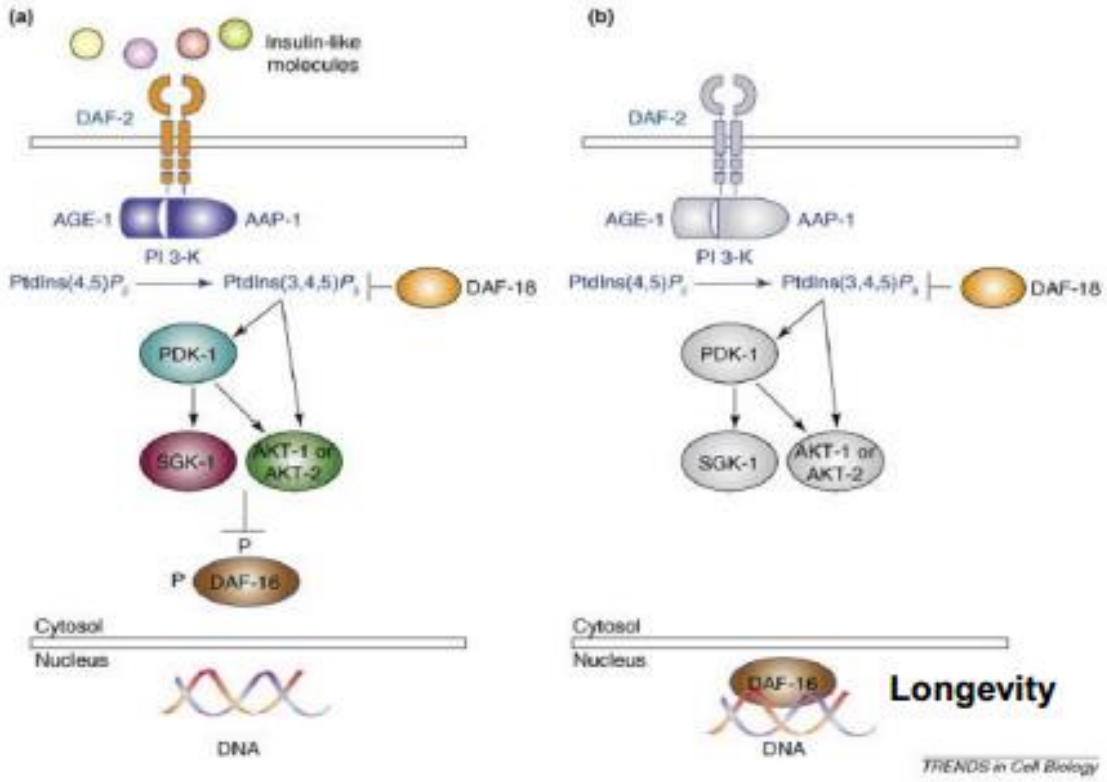
Figure 2



(Longo and Finch, 2003)

A conserved insulin-mediated pathway regulating lifespan. Certain components are still unknown in some simple and higher organisms but the general pathway is similar across species.

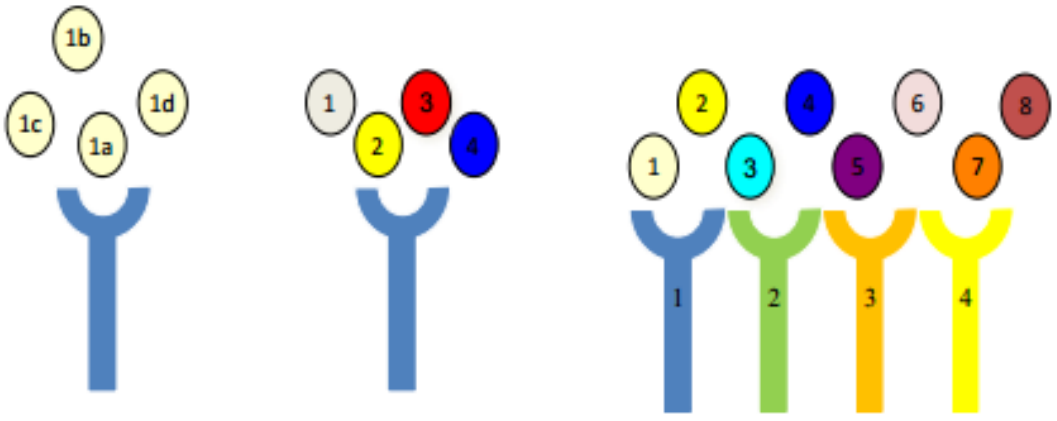
Figure 3



(Mukhopadhyay and Tissenbaum, 2007)

Insulin/IGF-like signaling pathway in *C. elegans*. The insulin signaling pathway is conserved from nematodes to humans. Panel (a) shows the complete signaling when insulin ligands binds to the receptor DAF-2 to transduce downstream signaling under favorable growth/reproduction conditions. Panel (b) shows how DAF-16 translocates into the nucleus during unfavorable environments associated with stresses.

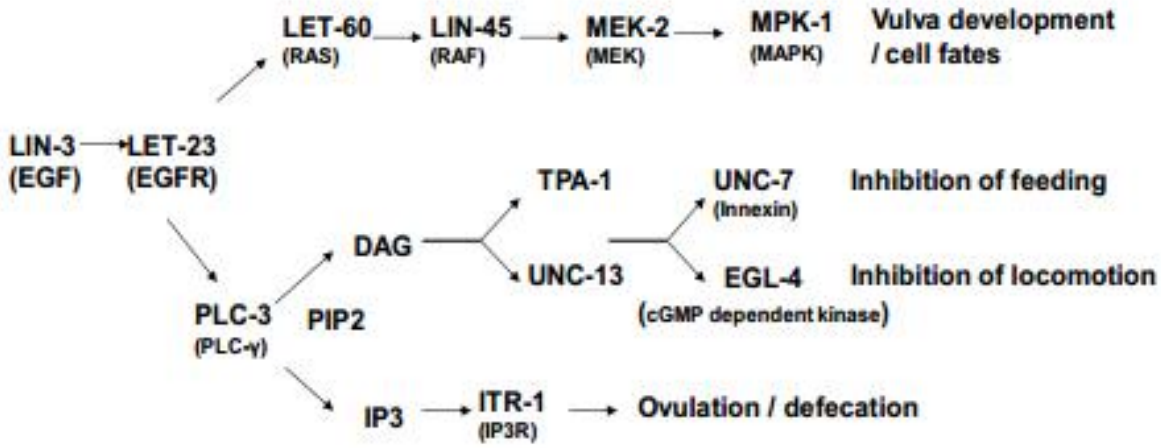
Figure 4



(Yu and Driscoll, 2011)

Summary of ligands and receptors in the EGF signaling pathways in *C. elegans*, *Drosophia melanogaster*, and humans. There is only one EGF ligand gene, *lin-3*, encoded by the *C. elegans* genome, although 4 splice-generated isoforms can be produced (noted a-d) (Van Buskirk et al., 2007). Four ligand genes are found in *Drosophila:* 1. Keren, 2. Spitz (TGF-α related), 3. Gurken, 4. Vein (neuregulin homolog). There are eight ligands in human: 1. EGF, 2. TGF-α, 3. Amphregulin, 4. Neuregulin, 5. Epigerulin, 6. Epigen, 7. Betacellulin, 8.HB-EGF. Both *C. elegans* and *Drosophila* genomes encode single EGFR receptors, LET-23 and DER respectively. Four ErbB subfamily receptors in human are: 1. EGFR , 2. HER-2 , 3. HER-3 , 4. HER-4. Human ligands cross-bind to multiple receptors.

Figure 5



(Modified from Van Buskirk and Sternberg, 2007)

The EGFR signaling pathway in *C. elegans*. LIN-3 is the EGF ligand, and LET-23 is the EGF receptor. There are two separate signaling pathways downstream of *let-23*. The RAS signaling regulates cell fates such as vulva development. The PLC-γ signaling regulates ovulation/defecation through IP3 and behavioral quiescence through DAG.