# Investigating the Role of LIN-35 and the DREAM Complex in Preserving Fertility Under Stress in C. elegans

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INVESTIGATING THE ROLE OF LIN-35 AND THE DREAM COMPLEX IN
PERSERVING FERTILITY UNDER STRESS IN *C. ELEGANS*

By

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ABSTRACT

INVESTIGATING THE ROLE OF LIN-35 AND THE DREAM COMPLEX IN PERSERVING FERTILITY UNDER STRESS IN *C. ELEGANS*

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Organisms are subjected to environments that can fluctuate and cause them stress. Therefore, organisms must have the ability to adapt to the stress to survive and propagate. Temperature stress is becoming an increasingly relevant type of stress due to climate change and is known to affect fertility in many types of organisms including the model organism *Caenorhabditis elegans.* Here we examine the role of LIN-35, the single *C. elegans* homolog of the tumor suppressor Retinoblastoma (pRB), in fertility under moderate temperature stress. We found that *lin-35* mutants lose fertility more drastically under moderate temperature stress than wildtype worms, and that this loss of fertility is dependent on both somatic and germline expression of LIN-35. A common cellular response to stress is programmed cell death via apoptosis. In *C. elegans,* the only adult tissue that exhibits apoptosis is the germline and while germline apoptosis eliminates ~50% of all oogenic germ cell nuclei during normal oogenesis, the induction of higher levels of germline apoptosis appears to be a general response to stressful conditions. LIN-35 was previously shown to be important for germline apoptosis but because it cannot bind to DNA by itself it must work with other partners to regulate genes. A group of proteins LIN-35 works with in some contexts is the DREAM complex, a conserved multi-subunit transcriptional repressor complex. We found that there is an induction of germline apoptosis under moderate temperature stress and that LIN-35 and the DREAM complex are important for this induction. The DREAM complex and LIN-35 are also important for DNA damage induced apoptosis. Together this indicates a general role for LIN-35 and the DREAM complex in the induction of germline apoptosis in response to stress. This role in germline apoptosis may be the role in the germline that LIN-35 has that helps it to preserve fertility.

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**CHAPTER 1: INTRODUCTION**

**Overview**

Fertility is the capacity of an individual to produce offspring and pass along their genetic information to the next generation. This capacity is one of the most important ones in biology because without the ability to pass along genetic information to a new generation there is no ability for new generations to exist or evolve. While fertility is a critical trait it is susceptible to stresses. A stress that especially affects fertility is heat stress. The temperature sensitivity of the germline is conserved from nematodes to mammals (Petrella 2014, Hanson 2009, Takahashi 2012, Yaeram et al 2006). In mammals it is known that fertility is affected on several different levels by heat stress in both males and females (Takahashi 2012, Yaeram et al 2006). In invertebrates like *Caenorhabditis elegans,* which rely on the environment to control their body temperatures, the effect of temperature on fertility is even more pronounced (Petrella 2014). There are still several unknowns surrounding the relationship between fertility and temperature. First, we currently do not know which aspects of fertility, especially during oogenesis, are impacted by temperature stress. Second, it is unknown how temperature affects fertility on the molecular level.

**THE *C. elegans* GONAD**
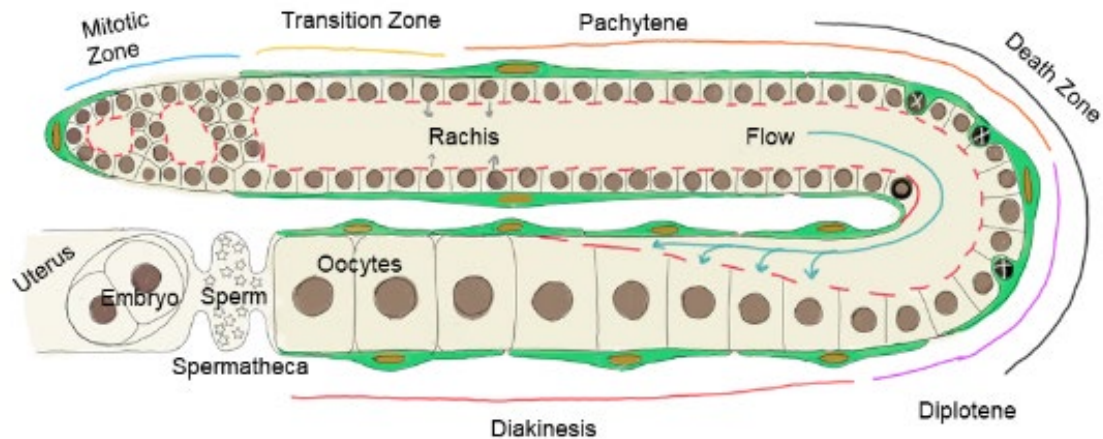


**Figure 1.1: *C. elegans* hermaphrodite gonad.** Figure adapted from Raiders et al 2017. Green cells are somatic gonad cells, blacked out nuclei represent apoptotic cells.

The *C. elegans* hermaphrodite germline is a highly structured tissue that has a stem cell pool at one end and maturing oocytes and sperm at the other (Figure 1.1). The end of the germline with the stem cell pool is referred to as the distal germline, the other end with the differentiated oocytes is the proximal germline (Hubbard and Greenstein 2005). Proliferation of the germline occurs in the mitotic zone from the stem cells, then the cells are pushed out of the mitotic zone into the transition zone. The germline mitotic zone is the only place in the adult animal where mitosis actively occurs. In the transition zone the cells stop undergoing mitosis and instead star making the switch to meiosis (Hubbard and Greenstein 2005). Subsequently once cells leave the transition zone they enter pachytene stage followed by the diplotene stage as the cells round the gonadal

bend and move towards the proximal gonad (Hubbard and Greenstein 2005). The distal arm of the gonad through the gonadal bend of the germline exist in a syncytium with the nuclei sharing cytoplasm in the rachis (Hubbard and Greenstein 2005). As a developing oocyte approaches the proximal arm of the gonad cytoplasm starts to stream into the oocyte from the rachis and it begins to pinch off membrane around its nucleus and cellularizes (Wolke et al 2007). Once cellularized the germ cell moves into the proximal arm and continues to grow in volume, taking in proteins and lipids from the intestine, as it finishes meiosis and matures prior to ovulation (Greenstein 2005). Finally, the oocyte is ovulated into the spermatheca, fertilized, and the resulting embryo is pushed out into the uterus of the hermaphrodite (Greenstein 2005). The sperm stored in the spermatheca are a result of spermatogenesis in the hermaphrodite during the L4 stage or from insemination by a male (Hubbard and Greenstein 2005).

The entire gonad arm is surrounded by five pairs of somatic gonadal sheath cells and a somatic distal tip cell at the distal end. These cells make up entirety the somatic portion of the gonad and contribute to the regulation and execution of many processes in the gonad. The distal tip cell and the most distal sheath cells move their processes across the germ cells to help specify how asymmetric cell division occurs in the distal gonad (Hall et al 1999). The sheath cells that span the gonadal bend phagocytose the germ cell nuclei that have undergone apoptosis. Yolk lipoproteins that come from the intestine and are transported through the gonadal basal lamina are then deposited into the

developing oocytes through the gap junctions in the sheath cells around the proximal gonad (Hall et al 1999).

**Oogenesis**

After the late L4 stage, the germline makes a switch from spermatogenesis to oogenesis in *C. elegans.* At this point the germline proliferates more and then those subsequent germ cells differentiate into oocytes rather than sperm (Hubbard and Greenstein 2005). A potential oocyte is born near the distal tip cell from the pool of germline stem cells (Hubbard and Greenstein 2005). The potential oocyte moves from the stem cell niche to the transition zone, where it transitions from performing mitosis to meiosis (Hubbard and Greenstein 2005). As the cell moves along the gonad arm it enters the pachytene stage of meiosis forming the synaptonemal complex to allow for homologous recombination to occur (Hubbard and Greenstein 2005). About 50% of all potential oocytes undergo apoptosis as they travel through the gonadal loop and get engulfed by the sheath cells that cover that region of the gonad (Greenstein 2005). Around this time Ras/MAPK signaling also begins to be important and helps to direct oocyte differentiation (Greenstein 2005). After cells exit pachytene they begin to cellularize out of the syncytium of the rachis. Cells destined to become oocytes start to enlarge whereas the other cells undergo apoptosis. Once cells begin to enlarge due to cytoplasmic streaming they started on the path to becoming an oocyte and go from being quite transcriptionally active to transcriptional quiescent (Wolke et al 2007). The transcriptionally active

cells produce many RNAs and proteins that are then pulled into the oocytes by the streaming of the cytoplasm from the rachis (Wolke et al 2007). The cytoplasm streams through cytoskeletal traction anchored in the developing oocyte. Yolk lipoproteins are also received by the oocyte from the proximal sheath cell (Greenstein 2005).As oocytes differentiate, they undergo diplotene where the meiotic chromosomes condense and then in diakinesis the chromosomes become highly visible as the 6 bivalents (Greenstein 2005). From there the oocytes mature as directed by signals from the proximal gonadal sheath cells and then are ovulated into the spermatheca where they get fertilized and then pushed out into the uterus as embryos (Greenstein 2005).

**Temperature stress and oogenesis**

Fertility is known to be temperature sensitive. While most of the time the temperature sensitivity of fertility is attributed to the temperature sensitivity in sperm, there is also evidence that oogenesis is temperature sensitive (Petrella et al 2011, Poullet et al 2015). When wild type *C. elegans* hermaphrodites are raised and maintained at 27°C they have decreased fertility (Petrella 2011). In some wild type strains fertility is partially rescued by mating with a male, indicating that there is a sperm defect, but because they do not recover full fertility this indicates that there is also an oogenic defect (Petrella 2011). Additionally, when hermaphrodite worms that were raised at 27°C were downshifted back to 20° and mated to males, there was still a slight decrease in

fertility that could be explained by an oogenic defect that was recovered after downshift since oogenesis is ongoing throughout adulthood (Petrella 2011).

Other evidence that temperature stress impacts oogenesis is that there are multiple types of oogenic germline defects that arise in worms that are grown at elevated temperatures. Some wildtype strains including the lab adapted N2 have been shown to have endomitotic oocytes at 26.5°C, which can result from defects in oocyte maturation, ovulation, and/or fertilization (Poullet et al 2015). N2 and another wildtype strain also occasionally show a phenotype where the oocytes differentiate at different locations along the germline at elevated temperatures (Poullet et al 2015). Other wildtype nematode strains both *C. elegans* strains and other nematode species, show other oogenic defects such as bulges in the germline, germline outgrowth, and the presence of undifferentiated cells in the proximal germline (Poullet et al 2015). These results indicate that oogenesis can be greatly affected in different ways by temperature stress resulting in a loss of fertility. It is still unknown exactly what parts of oogenesis are temperature sensitive and how they are affected by temperature stress.

**LIN-35 AND OTHER SynMuv B PROTEINS**

LIN-35 was first identified as a synthetic Multivuvla type B (synMuv B) protein. SynMuv B proteins are a class of proteins that are redundantly required with synMuv A proteins to maintain normal vulval morphogenesis in *C. elegans* (Fay and Yochem 2007). When a synMuv A gene and a synMuv B gene are

mutated in the same animal it leads to the synthetic multivulva phenotype in which additional nonfunctional vulva like protrusions develop on the ventral side of the worm in addition to the true vulva (Fay and Yochem 2007). These genes are known to be generally involved in transcriptional regulation (Fay and Yochem 2007).

LIN-35 is the single worm homolog of the Retinoblastoma (Rb) protein, which in mammals, along with p130 and p107, make up the pocket protein family (Fay and Yochem 2007). LIN-35 canonically represses transcription of genes through association with E2F and DP, similarly to how Rb and the other pocket proteins associate with E2Fs and DP to regulate cell cycle genes (Boxem and van den Heuvel 2002; Sadasivam and DeCaprio 2013). In the hypodermal cells near the vulval precursor cells, LIN-35 works with other proteins including E2F, DP, and additional synMuv B proteins LIN-37, LIN-52, and LIN-54 to repress the transcription of genes involved in Ras signaling that promote development of the vulva (Thomas et al 2003, Harrison et al 2006). This helps to regulate vulval formation so that only one vulva develops and prevents the multivulva phenotype (Meyers and Greenwald 2005).

LIN-35 has other synthetic roles in addition to the role in vulval morphogenesis (Table 1.1). For instance, in *lin-35; xnp-1* double mutants there are severe defects seen in the development of the gonad that often result in sterility (Bender et al 2004). These mutants have short and narrow gonads that had markedly fewer spermatogenic and oogenic germ cells. These gonads are also distorted in their morphology due to defects in the elongation of the gonad

during development (Bender et al 2004). In addition, in *lin-35; fzr-1* double mutants, there is hyperproliferation of several tissues during larval development including the somatic gonad, vulva, and hypodermal seam cells (Fay et al 2002). *lin-35; fzr-1* double mutants also have extraneous distal tip cells (DTCs), which leads to a bifurcation of the gonad arms (Fay et al 2002). These double mutants have hyperproliferation in the intestine as well (Fay et al 2002). LIN-35 also appears to have a possible role in the germline here because in the *lin-35; fzr-1* double mutants there is hyperproliferation of the germ cells as they reenter mitosis after failing to complete meiotic prophase (Fay et al 2002).

LIN-35 also has canonical roles in regulating cell cycle genes with the E2F complex (Figure 1.2) (Boxem and van den Heuvel 2002).  It appears to be targeted by cyclins and cyclin dependent kinases involved in the transition of the cell cycle from G1 into S phase (Boxem and van den Heuvel 2002). The cyclins and cyclin dependent kinases that are involved in the transition between G1 and S are *cyd-1* and *cdk-4* respectively in *C. elegans* and these genes appear to inhibit *lin-35*, *efl-1* and *dpl-1* thus enabling the transition to S phase by lifting the repression of the S phase targets of the E2F complex (Boxem and van den Heuvel 2002).

The E2F complex also seems to work independently of LIN-35 in some contexts. In embryonic development the E2F complex helps to regulate the duplication of the centriole during S phase (Miller et al 2016). In the germline E2F is vital for oogenesis in a way that seems to be independent of LIN-35 because the genes that are up- or down- regulated in *efl-1* and *dpl-1* mutant germlines are

a distinct set from those up- or down- regulated in *lin-35* mutant germlines (Chi

and Reinke 2006). Additionally, E2F appears to have a separate function in

germline apoptosis as compared with LIN-35 since different core apoptotic

machinery genes are upregulated in *efl-1* and *dpl-1* mutant germlines than in *lin-*

*35* mutant germlines (Schertel and Conradt 2007).
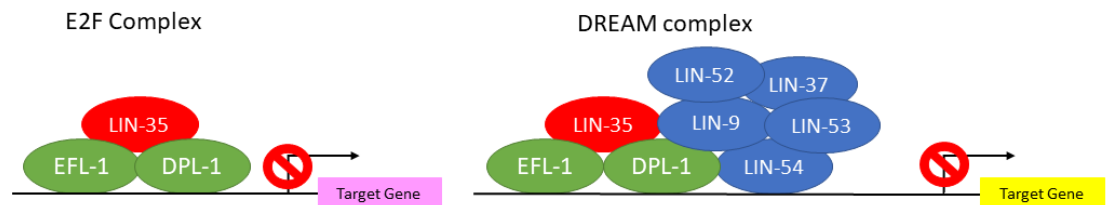


**Figure 1.2: The E2F complex and the DREAM complex**: This figure shows a representation of the E2F complex and the DREAM complex. The E2F members of both complexes are shown in green, the pocket protein in red, and the MuvB core in blue. The line represents chromatin in front of the promoter of a target gene. The arrow represents the promoter of the target gene. The different colors of the target genes represent the fact that the two complexes have different targets.

Another complex that LIN-35 often associates with is the DREAM complex

(Figure 1.2). DREAM stands for DP, Rb, E2F, and MuvB. The DREAM complex

is a conserved multi-subunit complex that represses the transcription of target

genes. In *C. elegans*, the DREAM complex is made up two subcomplexes;  E2F-

DP (DPL-1 and EFL-1) and the MuvB core (LIN-54, LIN-37, LIN-9, LIN-52, and

LIN-53), which are connected by LIN-35, (Goestch et al 2017; Harrison et al

2006). The DPL-1/EFL-1 part of the complex and LIN-54 in the MuvB part of the

complex have DNA binding capabilities to allow for the complex to bind to the

promoters of genes and repress them (Schmit et al 2009; Marceau et al 2016).

Within the DREAM complex, LIN-35 helps to stabilize the association between

DPL-1/EFL-1 and the MuvB core (Goestch et al 2017). Without LIN-35, DREAM

binding is decreased, and this affects expression of DREAM target genes;

DREAM target genes get expressed at higher levels when DREAM is not bound

(Goestch et al 2017). In the soma, DREAM and LIN-35 repress germline genes

(Petrella et al 2011). With the E2F complex LIN-35 seems to have more of an

effect on cell cycle; whereas with DREAM complex, which also contains the E2F

complex, there seem to be more diverse effects on different pathways such as

maintaining cell fate, cell cycle progression, proliferation, and as I show in this

thesis in cell death (Figure 1.2). It should also be noted that the mutant

phenotypes of *lin-35* mutants more closely resemble mutants of the  MuvB

component mutants than *efl-1* and *dpl-1* mutants (Chi and Reinke 2006, Goestch

et al 2017).

**Table 1.1: Phenotypes associated with LIN-35**

| Phenotype | Type of Phenotype[a] | Synthetic? | Known tissue | TS[b] | Reference |
|---|---|---|---|---|---|
| synMuv | M | Yes | Vulval precursor cells and hypodermis | yes | Ferguson and Horvitz 1989 |
| Defect in endoreduplication | P | Yes/No (depends on the paper) | Intestine | Yes | Grishok and Sharp 2005; Ouellet and Roy 2007; Fay et al 2001; Boxem and van den Heuvel 2001; Boxem and van den Heuvel 2002 |
| Gonadal development issues | M, P | Yes | Distal Tip cell and somatic gonad | ? | Bender et al 2004; Fay et al 2001; |
| Pharyngeal morphogenesis | M | Yes | Pharynx | ? | Fay et al 2003 |
| RNAi enhancement | F | No | Somatic tissues; neurons | ? | Lehner et al 2006 |
| Germline Gene Misexpression | F | No | Somatic tissues; Intestine | Yes | Petrella et al 2011; Kudron et al 2013, Wang et al 2005 |
| Germline Apoptosis | D | No | Germline | Yes* | Schertel and Conradt 2007 |
| Fertility Defect | ? | No | ? | Yes | Chi and Reinke 2006 |

[a]M = morphogenesis, P = proliferation, F = cell fate, D = cell death
[b]Phenotype has been shown to be high temperature sensitive
*data shown in this thesis

**APOPTOSIS AND THE *C. elegans* GERMLINE**

Apoptosis is a type of programmed cell death that occurs during development and maintenance of tissue homeostasis. The key hallmarks of apoptosis include cytoplasmic shrinkage, nuclear condensation, and retention of membrane and organelle integrity, as opposed to other types of programmed cell death where the membrane and organelles are compromised, such as necrosis (Fuchs and Stellar 2015). In *C. elegans* the only tissue that actively undergoes apoptosis in the adult animal is the germline. In the gonad approximately 20 cells are born near the distal tip cell per hour and only 3 oocytes are ovulated with in the same time-period. Under non-stress conditions the rest of the cells undergo what is known as physiological apoptosis through a mechanism that is not fully understood and for reasons that are still debated (Gartner et al 2008).

**Figure 1.3: Conserved Apoptosis Pathways:** This figure represents the conserved portions of the apoptosis pathway from *C. elegans* to mammals with the same color oval indicating homology

**GENERAL APOPTOSIS**
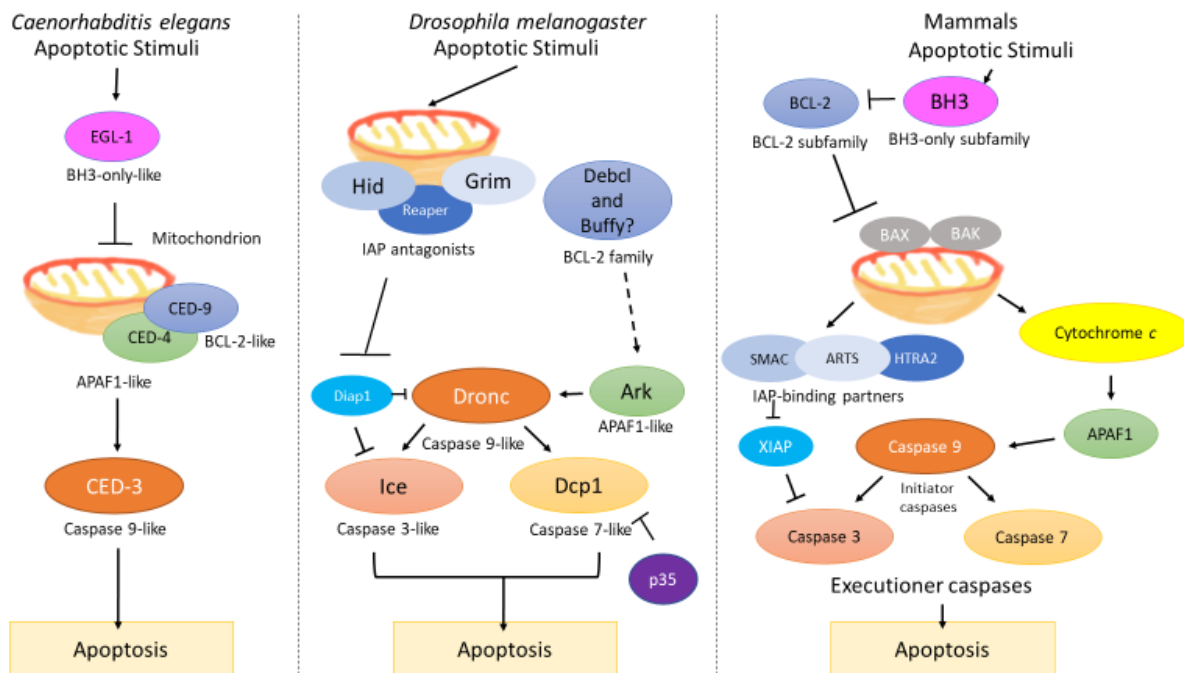
The general genetic pathway that leads to apoptosis is conserved across *C. elegans, D. melanogaster,* and mammals (Fuchs and Stellar 2015) (Figure 1.3). The conserved cascade starts with a stimulus that triggers the initiation of apoptosis and culminates in the deployment of caspases, which act as the cellular executioners. In *C. elegans* and mammals the cascade starts through the inhibition of CED-9/BCL-2, which lifts its inhibition of apoptosis. Then through the course of the cascade CED-4/APAF1 is activated to activate the CED-3/Caspase

9 family members and cause apoptosis. Caspases are a class of cysteine proteases that are expressed as inactive zymogenes almost ubiquitously in all cells (Fuchs and Stellar 2015). The main subclass of caspases that is conserved and involved in apoptosis is the initiator caspases, which have long amino-terminal pro-domains (Fuchs and Stellar 2015). In *Drosophila* and mammals there is an additional opposing pathway to the conserved APAF1 activation pathway that inhibits caspase activity. In all these organismal pathways the mitochondria are involved as well but in slightly different ways. Overall, the cell must reach multiple apoptosis checkpoints to be killed and even then, some cells can recover at late stages of apoptosis through the process of anastasis.

In *C. elegans* there are two main types of apoptosis that occur, somatic apoptosis and germline apoptosis. Somatic apoptosis occurs in an invariant pattern during both embryonic and larval development, and it is not induced by environmental stresses (Wang and Yang 2016). On the other hand, physiological germline apoptosis leads to the death of approximately half of oogenic-germ cells in what appears to be a random manner, but this apoptosis can be induced by genotoxic or non-genotoxic stresses (Wang and Yang 2016). Both types of apoptosis involve the same core death machinery; *ced-3, ced-4,* and *ced-9* (Figure 1.4)

When a cell is designated to die CED-9 undergoes a conformational change and causes CED-4 to localize perinuclearly (Wang and Yang 2016). The CED-4 dimer further oligomerizes, which promotes the activation of CED-3 (Wang and Yang 2016). CED-3 previously exists in the cell as a zymogen, but

when activated it auto-cleaves itself into p17 and p15 (Wang and Yang 2016).

Activated and cleaved CED-3 remains associated with CED-4, which stimulates

the proteolytic cleavage of CED-3 protein targets, which is thought to be how

CED-3 kills cells (Wang and Yang 2016). Only a few of the targets of CED-3

have been characterized, but CED-9 is known to be one of them. Cleaving CED-

9 prevents the cascade from being turned off. Other protein targets help to

initiate chromosome fragmentation and to help eliminate the mitochondria, which

are key stages of apoptosis. CED-3 also helps to promote apoptosis by

promoting the exposure of eat me signals and the antagonization of pro-survival

signals.

One difference between somatic and germline apoptosis lies in the role of

*egl-1*. Somatic cell death is usually dependent on *egl-1,* which is the BH3-only

homolog in *C. elegans and* is transcriptionally activated in the individual cells that

are destined to die. Somatic apoptosis can only be induced endogenously

through the course of the invariant development that *C. elegans* embryos exhibit,

this type of apoptosis does not occur in the adult animal. Other signals can also

induce germline apoptosis under different conditions discussed below.

**GERMLINE APOPTOSIS**

Germline apoptosis in *C. elegans* can be either endogenously or

exogenously induced, and it occurs during the process of oocyte differentiation

and development. It is typically independent of *egl-1* but rather MAPK signaling

plays an important role where it may negatively regulate *ced-9* expression

(Gumienny et al 1999) Other proteins can also play a role in germ cell death by

up- or down-regulating *ced-9* expression or activity.

The last stage of apoptosis is for the dying cell to present "eat me" signals

to its neighbors to entice them to engulf the cell corpse. In the *C. elegans* gonad

the somatic sheath cells are the cells that phagocytose the germ cell corpse

(Wang and Yang 2016). Engulfment mutants sometimes allow for some cells that

are programmed to die to escape death indicating that corpse engulfment is a

critical step in the cell death process (Wang and Yang 2016). The main "eat me"

signal that apoptotic cells project is phosphatidyl serine (PtdSer) (Wang and

Yang 2016). This molecule is normally expressed on the inner membrane of

living cells and gets flipped outside in dying cells (Wang and Yang 2016).

Exposure of ectopic PtdSer causes excessive and random cell death (Wang and

Yang 2016). Caspase cleavage promotes the externalization of PtdSer in dying

cells. Reduction in the ability to expose PtdSer can compromise corpse

engulfment (Wang and Yang 2016). CED-1/MEGF10/11 is a conserved

phagocytic receptor expressed on the membrane of the sheath cells in the gonad

that has an extracellular domain that associates with PtdSer *in vitro,* which may

mean that this association allows it to recognize cell corpses by directly binding

to PtdSer on the cell that is being engulfed (Wang and Yang 2016). CED-1

signaling is transduced through to signals that help to organize the Phagosome

in the sheath cell and then other downstream factors help to reorganize the

cytoskeleton to engulf the cell (Figure 1.4).
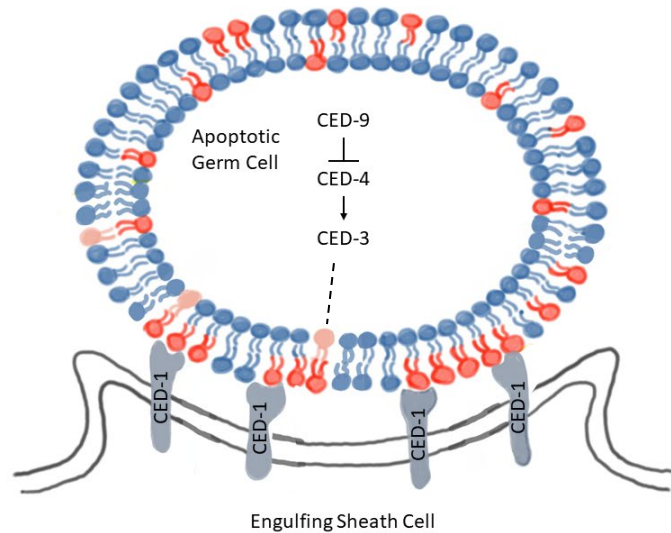
**Figure 1.4: Germline Apoptosis and Engulfment:** General pathway leading to germline apoptosis. PtdSer is shown as the red phospholipids. The grey transmembrane proteins on the engulfing cell are CED-1. CED-9 inhibits CED-4, which activates CED-3. CED-3 leads to the flipping of PtdSer from the inside membrane towards the outside of the cell, which then gets recognized by CED-1 on the engulfing cell. Figure adapted from Wang and Yang 2016.

**Physiological Germline Apoptosis**

In *C. elegans* about 50% of all oogenic germ cell nuclei that are born undergo apoptosis under non-stressed conditions. This is known as physiological apoptosis (Gartner et al 2008). The cause for physiological apoptosis is still unknown and it is not required for fertility as mutants that do not have physiological apoptosis still beget some offspring (Gartner et al 2008). There are a few hypotheses for why there is so much apoptosis in the *C. elegans* germline if it is not essential for fertility.

The leading hypothesis as to why germ cells proliferate so much for so few oocytes as payoff is that the extra germ cells act as "nurse" cells to the cells that do not undergo apoptosis (Gartner et al 2008). Nurse cells supply cytoplasm and cytoplasmic components to the cells destined to become oocytes. Indeed, in other systems nurse cells do just that to increase the quality of the oocytes that are made (McCall 2004, Alexandrova et al 2005). There are other lines of evidence that provide support for this hypothesis as well. For instance, apoptosis occurs in the region of the germline where developing oocytes begin to enlarge. Additionally, developing sperm, which require less cytoplasm do not undergo apoptosis (Gartner et al 2008). In fact, the female germline sufficient for germline apoptosis to occur regardless of the sex of the somatic tissue, i.e. feminized male germlines and normal hermaphrodite germlines undergo germline apoptosis but masculinized hermaphrodite germlines and normal male germlines do not. An issue that comes up with this hypothesis is that while physiological apoptosis removes around half of all the germ cell nuclei in the gonad, loss of physiological apoptosis in young unstressed animals does not affect fertility (Andux and Ellis 2008). Older animals lacking physiological apoptosis have smaller oocytes that are correlated with increased embryonic lethality (Gartner et al 2008, Andux and Ellis 2008). Another line of evidence for the apoptotic cells being nurse cells is because these apoptotic cells eject their mitochondria and cytoplasm into the rest of the syncytium before being completely enclosed in the membrane of the sheath cells (Raiders et al 2018).

A second hypothesis for the role of apoptosis in the germline is to cull nuclei that have defects in them to preserve genomic integrity to the next generation. Apoptosis could be the solution to having damaged nuclei or binucleated cells (Raiders et al 2018). While it was determined that germline apoptosis does get rid of the odd cell with serious nuclear damage or are binucleated, under physiological conditions these are sufficiently rare for it to be unlikely that this alone explains the need for physiological germline apoptosis (Raiders et al 2018).

Some proteins involved with germline apoptosis are RNA-binding proteins or are involved in mRNA metabolism. These proteins might influence expression of apoptosis proteins by perhaps limiting translation of the mRNAs (Gartner et al 2008). Another way these proteins may be controlling apoptosis is through policing a "checkpoint" for the proper distributing of RNAs, proteins, and other components from the cytoplasm into developing oocytes (Gartner et al 2008). This gives rise to a third model that may regulate germline apoptosis. This model is that the frequency of germline apoptosis is regulated by the cellularization of the oocytes. Perhaps if an apoptotic cell is not fully enclosed in the plasma membrane it distributes some if its apoptotic signals to the neighboring cells. This could lead to the clustering of apoptotic cells that is often seen particularly when there is an elevated level of apoptosis (Garner et al 2004). Some additional evidence for this includes that apoptosis appears to increase when proteins involved in the endosome and protein trafficking are impaired. However, this

increase in apoptosis could be a delay in cell corpse clearance (Gartner et al 2008).

Other players in the regulation of germline apoptosis include the P-granule proteins PGL-1 and PGL-3 (Min et al 2016). P-granules are perinuclear complexes of protein and RNAs that are only present in germ cells in wildtype animals. Germ cells fated to die lose their P-granules and then begin to undergo apoptosis (Min et al 2016). Cells that lose P-granule localization show highly condensed chromatin, which is characteristic of late stage apoptosis (Min et al 2016). Mutants with perturbed P-granules such as *glh-1* single and *glh-1; glh-4* double mutants, which have a significant proportion of PGL-1 that was dissociated from the nuclear envelope, show excessive germline apoptosis (Min et al 2016). Additionally, ectopic PGL-1 and PGL-3 expression in the soma inhibits developmental apoptosis in larval stages, indicating that the presence of P-granules may be critical to the inhibition of apoptosis (Al-Amin et al 2016).

**Germline Apoptosis Under Stress Conditions**

Exogenously induced apoptosis occurs in response to stresses such as DNA damage, osmotic stress, oxidative stress, starvation, and moderately elevated temperature stress (Figure 1.5) (Gartner et al 2000, Salinas et al 2006, Láscarez-Lagunas et al 2014; this document). In the case of DNA damage CEP-1/p53 upregulates the transcription of *egl-1* promoting cell death (Gartner et al 2008). MAPK activity is important for osmotic and oxidative stress (Salinas et al 2006). LIN-35 and insulin signaling appear to affect cell death signals in the case

of starvation (Navarro 2018 insulin signaling paper). Endogenously induced

apoptosis can result from failure in chromosomal synapsis or defects in the repair

of DNA double strand breaks (DSBs) during meiosis. DSBs caused by SPO-11

that are not repaired can trigger *egl-1* dependent apoptosis through the same

pathway as exogenous DNA damage (Wang and Yang 2016). Synaptic failure

cause by defects in the pairing center or by mutations in the synaptonemal

complex trigger apoptosis through PCH-2, which is homologous to the yeast

Pch2 and acts as a synapsis checkpoint. BUB-3 is also part of the synapsis

checkpoint (Wang and Yang 2016). Loss of *bub-3* reduces germline apoptosis

that is induced by the synapsis checkpoint. Genome stability regulators are also

important, as mutations in these genes can also lead to an induction of apoptosis

through either of the endogenously induced apoptosis pathways (Wang and

Yang 2016).

DNA damage induced apoptosis occurs in response to double strand

breaks that cannot be repaired in time and while it mostly relies on the same

apoptotic machinery it also depends on CEP-1/p53 and EGL-1. DNA damage

leads to more cells without PGL-1 localization at the nuclei, which is thought to

allow SIR-2.1 to translocate from the nucleus to the cytoplasm. SIR-2.1 is a *C.

elegans* Sirtuin and it is known to be involved in apoptotic signaling pathways

that result from DNA damage (Greiss et al, 2008). The translocation of SIR-2.1

results in a transient colocalization with CED-4, which is thought to allow the

activation of CED-3 leading to apoptosis (Greiss et al 2008). In *pgl-1* mutants

SIR-2.1 is more commonly translocated to the cytoplasm even without DNA

damage indicating that PGL-1 expression may be what prevents SIR-2.1 from

constituently being translocated (Min et al 2016). Additionally, while LIN-35 is

known to be important for DNA damage induced germline apoptosis, it is

unknown exactly where in the pathway it acts (Schertel and Conradt 2007).

Under other stress conditions, like starvation, LIN-35 acts upstream of *ced-9* and

represses its expression (Láscarez-Lagunas et al 2014). EFL-1 and DPL-1

however work at the *ced-4* point in the pathway as they do not modulate *ced-9*

expression at all (Schertel and Conradt 2007, Láscarez-Lagunas 2014).

Therefore, it is unknown what partners LIN-35 is working with to repress *ced-9*

expression.
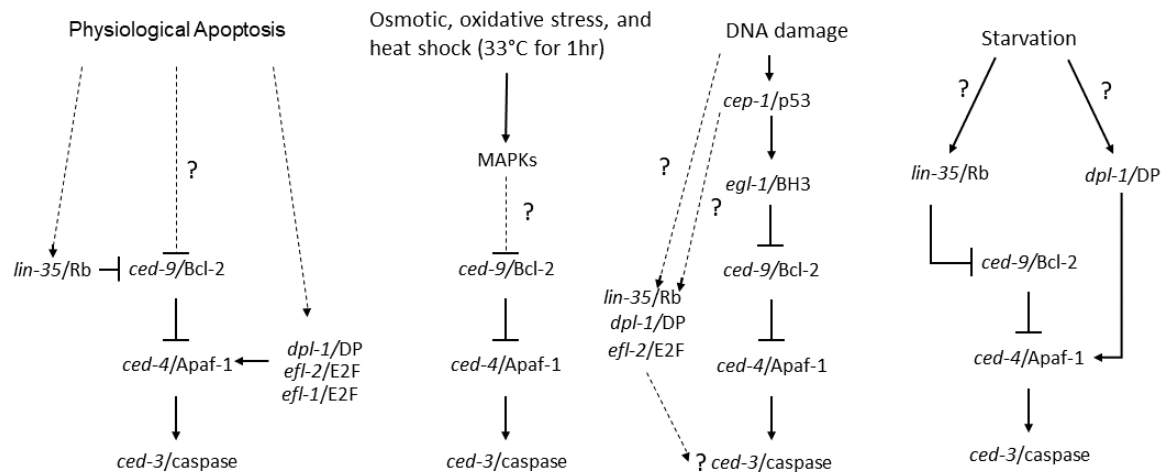


**Figure 1.5: Genetic Pathways Leading to Germline Apoptosis:** Pathway figure showing the general genetic pathways that lead to germline apoptosis in different contexts. CED-9, CED-4, and CED-3 are common to all the pathways as the core apoptotic machinery. Question marks represent where the relationship is unknown. Figure adapted from Láscarez-Lagunas et al 2014.

**CHAPTER 2: MATERIALS AND METHODS**

**Strains and nematode culture**

Unless otherwise stated all worm strains were grown at 20°C on NGM plates seeded with *E. coli* strain AMA1004 (Casadaban et al, 1983). All animals were scored as stated in the text. The CGC provided some strains, the CGC is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). All strains used are listed in Table 2.1, the allele of each gene is indicated in the text as appropriate.

**Table 2.1: Strain List**

| Genotype | Strain Name | Source |
|---|---|---|
| *N2; wildtype* | N2 | Caenorhabditis Genetics Center |
| *lin-35(n745)* | MT10430 | Caenorhabditis Genetics Center |
| *lin-54(n2331)* | MT8841 | Caenorhabditis Genetics Center |
| *lin-37(n768)* | MT5470 | Caenorhabditis Genetics Center |
| *lin-35(ea75)* | QP1863 | Gift from Judith Yanowitz |
| *bcls39* | MD701 | Caenorhabditis Genetics Center |
| *lin-35(n745); bcls39(lim-7p::CED-1::GFP)* | LNP0089 | This thesis |
| *lin-54(n2331); bcls39(lim-7p::CED-1::GFP)* | LNP0091 | This thesis |
| *lin-37(n768); bcls39(lim-7p::CED-1::GFP)* | LNP0092 | This thesis |
| *lin-35(ea75); bcls39(lim-7p::CED-1::GFP)* | LNP0093 | This thesis |
| *petEx1(let-858p::lin-35::GFP, rol-6+)* | LNP0044 | This thesis |
| *lin-35(n745); petEx1(let-858p::lin-35::GFP, rol-6+* | LNP0043 | This thesis |
| *bnEx56(elt-2p::lin-35::GFP + rol-6)* | SS0991 | This thesis |
| *lin-35 (n745); bnEx56(elt-2p::lin-35::GFP + rol-6)* | LNP0016 | This thesis |

| | | |
|---|---|---|
| *petEx3(inx8p::lin-35::GFP, myo-3::mCherry)* | LNP0087 | This thesis |
| *bnEx56(elt-2p::lin-35::GFP + rol-6);* *petEx3(inx8p::lin-35::GFP, myo-3::mCherry)* | LNP0099 | This thesis |
| *lin-35(n745); bnEx56(elt-2p::lin-35::GFP + rol-6); petEx3(inx8p::lin-35::GFP, myo-3::mCherry)* | LNP0086 | This thesis |
| *bnEx56(elt-2p::lin-35::GFP + rol-6) ;* *petEx3(mir786p::lin-35::GFP, myo-3::mCherry)* | LNP0082 | This thesis |
| *lin-35(n745); bnEx56(elt-2p::lin-35::GFP + rol-6); petEx3(mir786p::lin-35::GFP, myo-3::mCherry)* | LNP0081 | This thesis |
| *lin-35(n745); vrIs56 (pie-1p::lin-35::GFP::FLAG::lin-35 3'UTR + unc-119(+))* | LNP0022 | This thesis |
| *lin-35(n745); vrIs93 (mex-5p::lin-35::GFP::FLAG::lin-35 3'UTR + unc-119(+))* | LNP0023 | This thesis |
| *vrIs56 (pie-1p::lin-35::GFP::FLAG::lin-35 3'UTR + unc-119(+))* | LNP0031 | This thesis |
| *vrIs93 (mex-5p::lin-35::GFP::FLAG::lin-35 3'UTR + unc-119(+))* | LNP0038 | This thesis |

**Brood size assays:**

20°C: L4s of each genotype were put onto individual plates and moved to fresh individual plates every 24 hours for 3 days to give a total of 4 plates per individual worm. Progeny on each plate were counted once most of the progeny reached the L4 stage on each plate. The total progeny on the four plates were then compiled into the brood size.   n = 28-98 worms

Upshift 20° → 26°: L4s of each genotype were picked to individual plates and upshifted to 26°C and then moved to fresh individual plates every 24 hours for three days to give a total of 4 plates per worm. Progeny on each plate were counted when most of the progeny on the plate were L4s. Total progeny on the four plates were then added to get the brood size for each worm. n = 15-59 worms

26°C: L4s of each genotype were picked to one plate per genotype and upshifted to 26°C, then L4 progeny from those upshifted worms were chosen and picked to individual plates. They were then moved every 24 hours for three days to give a total of 4 plates per worm. Progeny on each plate were counted once most of the worms on that plate were L4s. Total progeny on the four plates were added together to get the brood size for each worm. n = 13-51 worms

**CED-1::GFP apoptosis assay**:

*Upshift at L4 assess 24 hours post L4*

20°C: L4s were put onto a plate for each genotype and aged for 24 hours then mounted on a 2% agarose pad in 10uM levamisole in 1X egg buffer and scored for GFP positive apoptotic cells using Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 31-54 gonad arms

26°C: L4s were put onto a plate, upshifted to 26°C and aged for 24 hours. Then the adult worms were mounted on a 2% agarose pad in 10uM levamisole in 1X egg buffer and scored for GFP positive apoptotic cells using the Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 40-58 gonad arms

*Upshift at L1 assess 24 hours post L4*

20°C: L4s were put onto a plate for each genotype and aged for 24 hours then mounted on a 2% agarose pad in 10uM levamisole in 1X egg buffer and

scored for GFP positive apoptotic cells using Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 28-36 gonad

26°C: 10-20 L4s were placed onto a plate per genotype and allowed to age for 24 hours. Young adult worms were placed onto new plates and allowed to lay embryos for 3 hours. These plates were upshifted to 26°C 24 hours post being laid. The L4s that resulted from those embryos were picked to new plates, aged for 24 hours and then mounted on 2% agarose pads in 10uM levamisole and 1X egg buffer and scored for GFP positive apoptotic cells using a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 52-56 gonad arms

*Upshift at L1 (HT) and assess 48 hours post L4*

20°C and no UV: L4s were put onto a plate for each genotype and aged for 48 hours then mounted on a 2% agarose pad in 10uM levamisole and 1X egg buffer and scored for GFP positive apoptotic cells using a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 61-160 gonad arms

20°C and UV: L4s were put onto a plate for each genotype and aged 24 hours before being subjected to 400 J/m$^2$ of UV in a CL-1000 Ultraviolet Crosslinker and then allowed to recover for 24 hours before mounting them on 2% agarose pads in 10 uM levamisole and 1X egg buffer. They were then scored for GFP positive apoptotic cells using a Nikon Eclipse TE2000-S inverted

microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 50-98 gonad arms

26°C no UV: 10-20 L4s were placed onto a plate per genotype and allowed to age 24 hours. Then they laid embryos on new plates for 3 hours. These plates were upshifted to 26°C 24 hours later. The resulting L4s on the plates were picked to new plates, aged for 48 hours and then mounted on 2% agarose pads in 10uM levamisole and 1X egg buffer and scored for GFP positive apoptotic cells using a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 41-54 gonad arms

**Acridine Orange apoptosis assay:**

no UV: L4s were put onto a plate for each genotype and aged for 48 hours. 50ug/mL AO in M9 was applied to the bacterial lawn and the worms were allowed to stain for 2 hours. After staining the worms were picked to fresh plates and allowed to crawl around for 10 minutes before being picked to new plates to finish out 2 hours of destaining recovery. They were then mounted on a 2% agarose pad in 10uM levamisole and 1X egg buffer and scored for AO positive cells using a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 40 gonad arms

UV: L4s were put onto a plate for each genotype and aged for 24 hours before being subjected to $400L/m^2$ of UV in a CL-1000 Ultraviolet Crosslinker.

They were then aged for 24 more hours before 50ug/mL AO in M9 was applied to the bacterial lawn. The worms were allowed to stain for 2 hours. After staining the worms were picked to fresh plates and allowed to crawl around for 10 minutes before being picked to new plates to finish out 2 hours of destaining recovery. They were then mounted on a 2% agarose pad in 10uM levamisole and 1X egg buffer and scored for AO positive cells using a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics, Plan Apo 60x/1.25 numerical aperature oil objective. n = 38-40 gonad arms

**CHAPTER 3: ROLE OF *LIN-35* IN BOTH THE SOMA AND THE GERMLINE FOR BUFFERING FERTILITY UNDER MODERATE TEMPERATURE STRESS**

**Introduction**

The goal of this chapter was to determine whether the role of LIN-35 in fertility is temperature sensitive and in which tissues this role is important. LIN-35 is expressed in virtually every cell in *C. elegans* and *lin-35* mutants have phenotypes that affect many tissues in the worm. In the somatic gonad, *lin-35* is redundantly required with *xnp-1* for normal sheath cell and spermatheca development (Bender et al 2004). Loss of *lin-35* enhances the hyperproliferation phenotypes seen in *fzr-1* mutants where various aspects of the somatic gonad and germline morphologies are disrupted (Fay et al 2001). In *lin-35* mutants there appear to be defects in intestinal endoreduplication that would normally lead to polyploid intestinal nuclei, instead of endocycling there are cycles of DNA replication that lead to nuclear division leading to multinucleate intestinal cells (Grishok and Sharp 2005; Ouellet and Roy 2007). This phenotype is further enhanced by the loss of *fzr-1* (Fay et al 2001). Loss of *lin-35* on its own can also lead to post-embryonic increases in the number of intestinal nuclei and is known to enhance this phenotype in *cki-1* mutants (Fay et al 2001,  Ouellet and Roy 2007, Boxem and van den Heuvel 2001, Boxem and van den Heuvel 2002). These tissues are important for fertility in the worm. The germline is the source of the sperm and egg cells that allow for fertilization to occur. The somatic gonad is the conduit between the somatic tissues and the germline helps regulate

signaling that contributes to the ability of the worm to create and maintain germ cells, it also engulfs the corpses of apoptosing meiotic germ cell nuclei and the most proximal somatic gonad cells help to cause ovulation of maturing oocytes. The intestine contributes important nutrients to the developing oocytes in the gonad. Having a phenotype in any of these tissues could impact fertility because of disruption in the processes by which germ cells are made, maintained, or culled to contribute to fertility.

*lin-35* mutants also have several temperature sensitive phenotypes. The synMuv phenotype seen in *lin-35* mutants is temperature sensitive as it becomes more pronounced and penetrant with increasing temperature (Ferguson and Horvitz 1989). Additionally, *lin-35* mutants show a high temperature larval arrest phenotype when they are upshifted to 26°C (Petrella et al 2011). Germline genes including P-granule components are ectopically expressed in the soma of *lin-35* mutants, particularly at 26°C (Petrella et al 2011). Additionally,  P-granule components in the primordial germ cells (PGCs) of *lin-35* mutants are not localized to the puncta in the nuclear periphery of the cells but rather show diffuse staining in the cytoplasm specifically at 26°C (unpublished observations). Disruption of the P-granules in the PGCs indicates there may be a temperature sensitive role for LIN-35 in the germline as well as the soma.

We sought to determine whether *lin-35* is important for buffering fertility in response to temperature stress and to determine which tissues *lin-35* expression is important in to ensure fertility. *lin-35* mutants have reduced fertility at 20°C when compared to wild type but are not sterile (Chi and Reinke 2006). Our data

shows that *lin-35* is critical for buffering fertility under moderate temperature stress because in *lin-35* mutants there is a much sharper decrease in fertility than in wildtype animals under the same conditions. To try to elucidate which tissues were critical to this buffering of fertility we used transgenes that rescued *lin-35* expression in all somatic tissues, the intestine alone, the somatic gonad alone, and the germline alone; we also put the intestine and somatic gonad transgenes into the same worm together to rescue in both tissues. At 20°C we found that any of the somatic expression transgenes partially rescue fertility in *lin-35* mutants, and germline expression of *lin-35* also partially rescues fertility in these mutants indicating that *lin-35* expression is required in both the soma and the germline to buffer fertility. At 26°C the pan-somatic transgene significantly rescues fertility, but the somatic gonad and intestine specific transgenes do not, indicating that under moderate heat stress these are not the critical somatic tissues that play a role in buffering fertility.

**Results:**

***lin-35 buffers fertility in response to moderate temperature stress***

*lin-35* mutants have been shown to have a reduced brood size at 20°C (Chi and Reinke 2006), but it was unknown how moderate temperature stress would impact fertility in *lin-35* mutants. We compared N2 and *lin-35(n745)* mutant brood sizes at 20° to worms that were upshifted to 26°C as L4 larvae. We used the upshift as an elevated temperature treatment because *lin-35* mutants cannot be maintained at 26°C due to the HTA phenotype. Fertility in wildtype is reduced

from around 230 offspring at 20° to around 200 offspring under the upshift condition, whereas in *lin-35* mutants have around 60 offspring at 20°C and only about 20 offspring under the upshifted condition (Figure 3.1A). Fertility is thus more affected by the increased temperature in *lin-35* mutants than in wildtype worms. There is a sharper decline in fertility between the temperature schemes in *lin-35* mutants than we see in wildtype worms (Figure 3.1B). These data indicate that *lin-35* helps maintain fertility in response to moderate temperature stress.
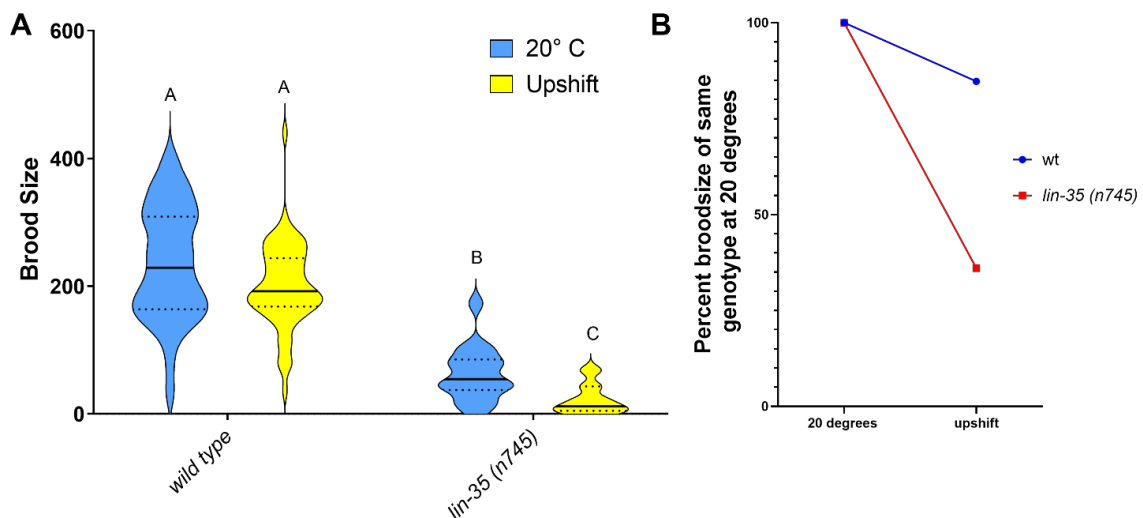


**Figure 3.1: *lin-35* mutants have reduced fertility in response to moderate temperature stress** A) A violin plot showing the distribution of the brood sizes of wildtype and *lin-35(n745)* worms. The plot shows that while the brood size of N2 worms does not significantly change when upshifted, the brood size of *lin-35(n745)* mutants does significantly decrease. The upshift condition worms were upshifted from 20°C to 26°C at the L4 stage of development. Significance is indicated by the letters above the violins with the same letter indicating no significant difference between the means of the data. Brown-Forsythe and Welch ANOVA tests were used to compare the data with a p<0.05. (B) Plot shows the relative brood size of worms in response to moderate temperature stress in comparison to their brood size at 20°C. This graph shows that the relative brood

size of *lin-35(n745)* declines much more with moderate temperature stress than the relative brood size of N2.

**lin-35 is important in the soma for buffering fertility**

To determine if somatic expression of *lin-35* was important for fertility we rescued *lin-35* expression in somatic tissues using four different transgenes on multicopy extrachromosomal arrays (Figure 3.2). First, the *let-858p::lin-35::GFP* transgene expresses in all somatic tissues, which would help us answer whether somatic expression of *lin-35* was important for fertility. Second, the *elt-2p::lin-35::GFP* transgene expresses specifically in the intestine, which is important as the intestine directly provides nutrients to the developing oocytes (Petrella et al 2011, Greenstein et al 2005). Third and fourth, *inx-8p::lin-35::GFP* and *mir-768p::lin-35::GFP* express in the somatic gonad, which is the tissue that assists in the regulation of many aspects of germline development and germline processes. The *elt-2p::lin-35::GFP* and *let-858p::lin-35::GFP* transgenes were able to rescue the HTA phenotype. Rescue of the HTA phenotype allowed for the worms to develop into adulthood and have offspring at 26°C. The HTA phenotype in *lin-35* mutants is 100% penetrant so this bypass is essential to assess brood sizes of *lin-35* mutants under moderate heat stress (Petrella et at 2011). Because of this the *inx-8p::lin-35::GFP* and *mir-768p::lin-35::GFP* were assessed in a background with *elt-2p::lin-35::GFP* also present.

Pan-Somatic
*lin-35(n745); let-858p::lin-35::GFP*

Intestinal Specific
*lin-35(n745); elt-2p::lin-35::GFP*

Intestinal Specific + Somatic Gonad Specific
*lin-35(n745); elt-2p::lin-35::GFP,  mir-768::lin-35::GFP*
*Or*
*lin-35(n745); elt-2p::lin-35::GFP,  inx-8::lin-35::GFP*

Germline Specific
*lin-35(n745); mex-5::lin-35::GFP*
*Or*
*lin-35(n745); pie-1:: lin-35::GFP*

Wildtype

Mutant
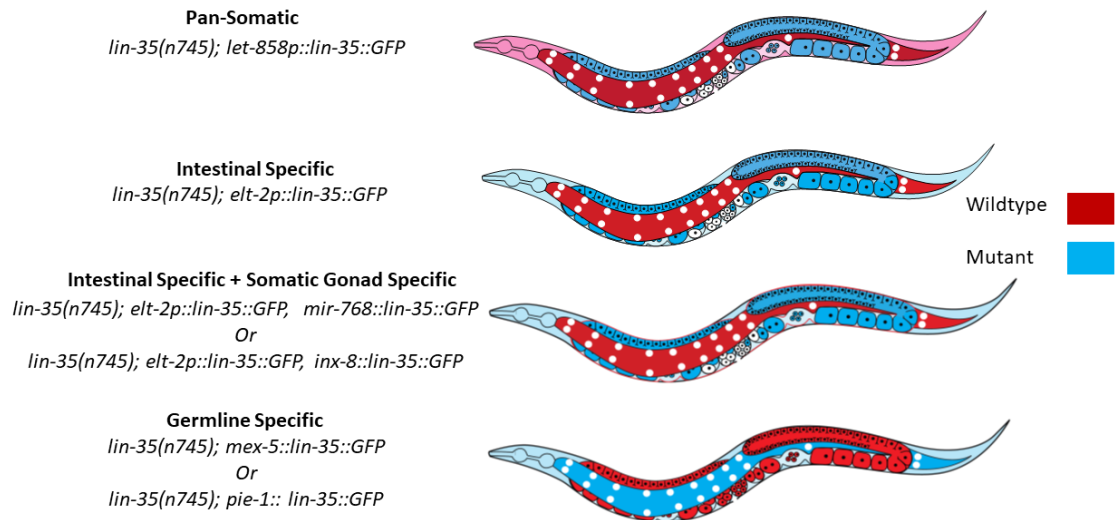
**Figure 3.2: Expression of Transgenes used in this study:** Above are cartoons representing worms with the expression patterns of the transgenes used in this study. In the cartoons any tissue that is red is expressing wildtype LIN-35 and any tissue that is blue is mutant for *lin-35.*

Pan-somatic expression of *lin-35* in a *lin-35(n745)* mutant background partially rescued fertility at 20°C (Figure 3.3A). Intestinal expression of *lin-35* with or without somatic gonad expression in a *lin-35(n745)* mutant background also partially rescues fertility at 20°C (Figure 3.3A).  Therefore, any somatic expression of *lin-35* at 20°C in a *lin-35(n745)* background partially rescues fertility.

We also upshifted worms at the L4 larval stage to 26°C (Upshift condition) and compared their brood sizes. *lin-35* mutants have a much smaller brood size when upshifted than when raised at 20°C but they also have a sharper drop off in fertility when compared to wildtype (Figure 3.3A). Wildtype upshifted worms

maintain a brood size that was about 80% of that wildtype worms raised and assessed at 20°C whereas in *lin-35* mutants the relative size of their brood size when upshifted was about 30% of that of *lin-35* mutants at 20°C (Figure 3.3B). Fertility was rescued by the pan-somatic transgene but not back to wildtype levels. *lin-35* mutants with the *let-858p::lin-35::GFP* transgene that were upshifted had a relative brood size that was about 60% of what they have at 20°C, which is improved but still a sharper drop in fertility than exhibited by wildtype (Figure 3.3B). When somatic expression of *lin-35* is rescued by the intestinal with or without somatic gonad expressed transgenes, fertility was not rescued.

The final condition we tested was raising worms at 26°C. To do this we upshifted P0 worms to 26°C at the L4 larval stage and then assessed the brood sizes of their F1 progeny. This was previously impossible in *lin-35* mutants due to the HTA phenotype. Wildtype worms born and raised at 26°C have a relative broodsize that is 25% of that of worms born and raised at 20°C (Figure 3.3B). In *lin-35* mutants with the pan-somatic transgene there was a full rescue of fertility back to wildtype levels (Figure 3.3A). Worms with the *let-858p::lin-35::GFP* transgene had a better relative brood size at 26°C as compared to the same genotype at 20°C than wildtype did, *let-858::lin-35::GFP* in a *lin-35* background at 26°C was 55% of *let-858p::lin-35::GFP* in a *lin-35* background at 20°C while wildtype at 26°C is only about 25% the size of the brood size of wildtype worms at 20°C (Figure 3.3B). In *lin-35* mutants with either the intestinal transgene alone or the intestinal transgene with one of the somatic gonad transgenes there was

no rescue of fertility, most of these worms were essentially sterile or had only one or two progeny (Figure 3.3A). This indicates that somatic expression of *lin-35* is important in part for buffering fertility in response to temperature stress, but that the tissues we chose, the intestine and somatic gonad, are not the critical tissues where *lin-35* expression buffers fertility.
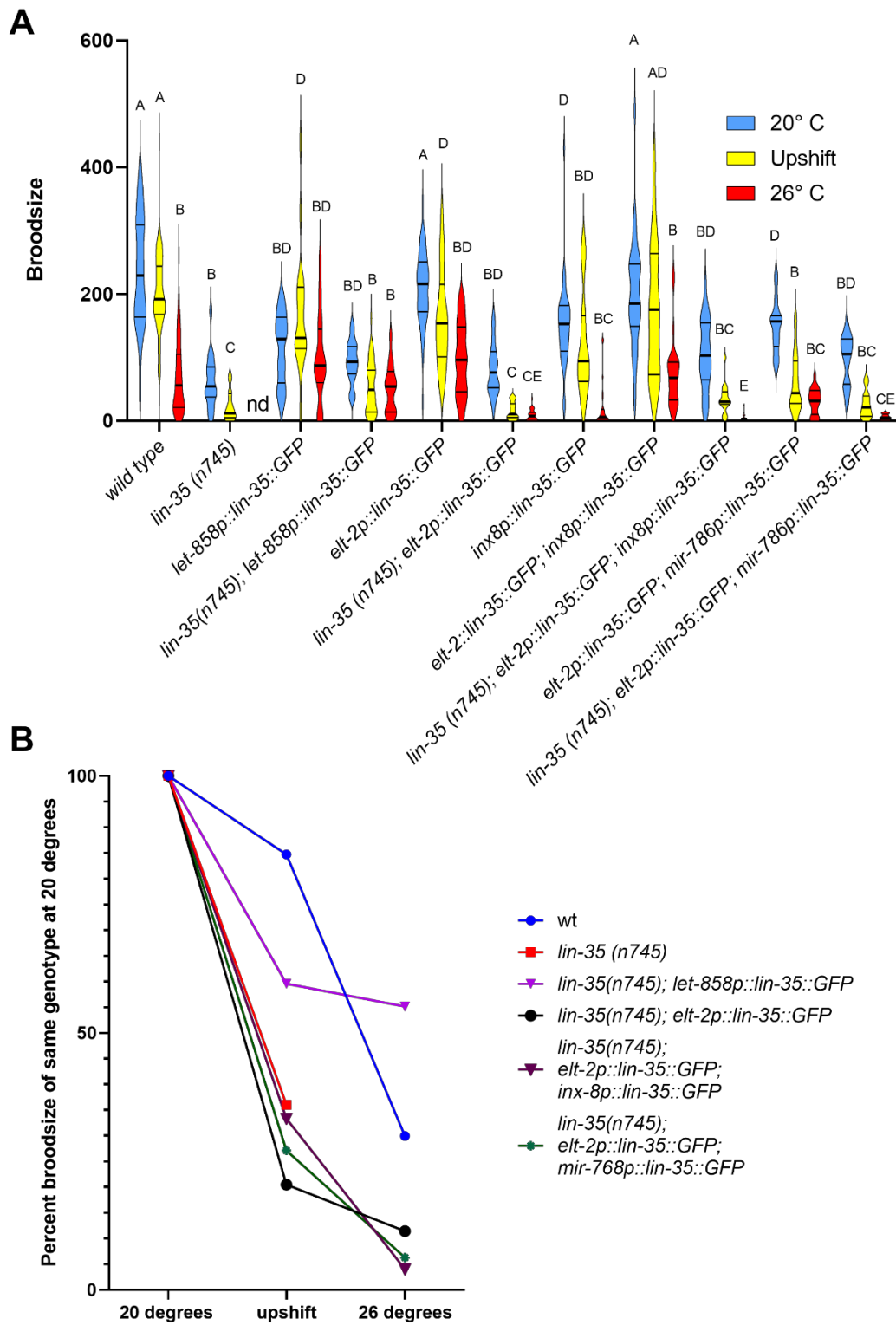
**Figure 3.3: Any somatic expression of *lin-35* partially rescues fertility at 20°C but only Pan-somatic expression of *lin-35* improves fertility under moderate temperature stress** (A) A violin plot showing the brood sizes of worms under the different temperature schemes. This plot shows that at 20°C somatic expression of *lin-35* using any of the transgenes partially rescues fertility but at 26°C only the pan-somatic transgene rescues fertility. Upshift refers to raising worms at 20°C until the L4 stage when they were upshifted to 26°C. For the 26°C condition P0 worms were upshifted at the L4 stage and then the brood sizes of the F1 worms were counted. Significance is represented by the letters above the violins where when two violins have the same letter above them there is no statistical difference between them. Significance was determined using a Brown-Forsythe and Welch's ANOVA with $p < 0.05$. (B) A plot showing the relative brood sizes of wildtype worms, *lin-35* mutants, and *lin-35* mutant worms with the somatic transgenes as compared to the same genotype at 20°C.

***lin-35* is important in the germline for buffering fertility**

There is likely a role for *lin-35* in the germline that could pertain to fertility because *lin-35* is expressed in the germline, *lin-35* mutants have disrupted P-granules, and *lin-35* mutants have smaller brood sizes than wildtype animals (Chi and Reinke 2006, Petrella et al 2011). To test whether germline expression of *lin-35* was critical for fertility we used two low copy 3' UTR tagged integrated transgenes to rescue germline expression of *lin-35*, *pie-1p::lin-35::GFP* and *mex-5p::lin-35::GFP* (Figure 3.2). These transgenes were produced using gene bombardment by the Reinke lab (Kudron et al 2013). We assessed the brood sizes of worms with each of these transgenes at 20°C and found that germline expression of *lin-35* partially rescues fertility in *lin-35* mutants but it does not rescue all the way back to wildtype (Figure 3.4A).

When we upshifted the worms to 26°C at the L4 stage and compared their brood sizes we found that again the transgenes rescued some fertility but not

back to wildtype (Figure 3.4A). However, the expression of *lin-35* in the germline

was sufficient to rescue fertility back to the same relative fertility as wildtype

(Figure 3.4B). Wildtype upshifted worms have 60% of the brood size of wildtype

at 20°C whereas *lin-35* mutants have about 35% of the 20°C brood size when

they are upshifted (Figure 3.4B). The mutants with the transgenes land in the

middle but closer to wildtype at around 50%-55% of the brood size seen at 20°C

in worms that are upshifted (Figure 3.4B). This shows that germline expression of

*lin-35* is critical for fertility particularly at higher temperature in order to buffer

fertility in response to this type of stress.

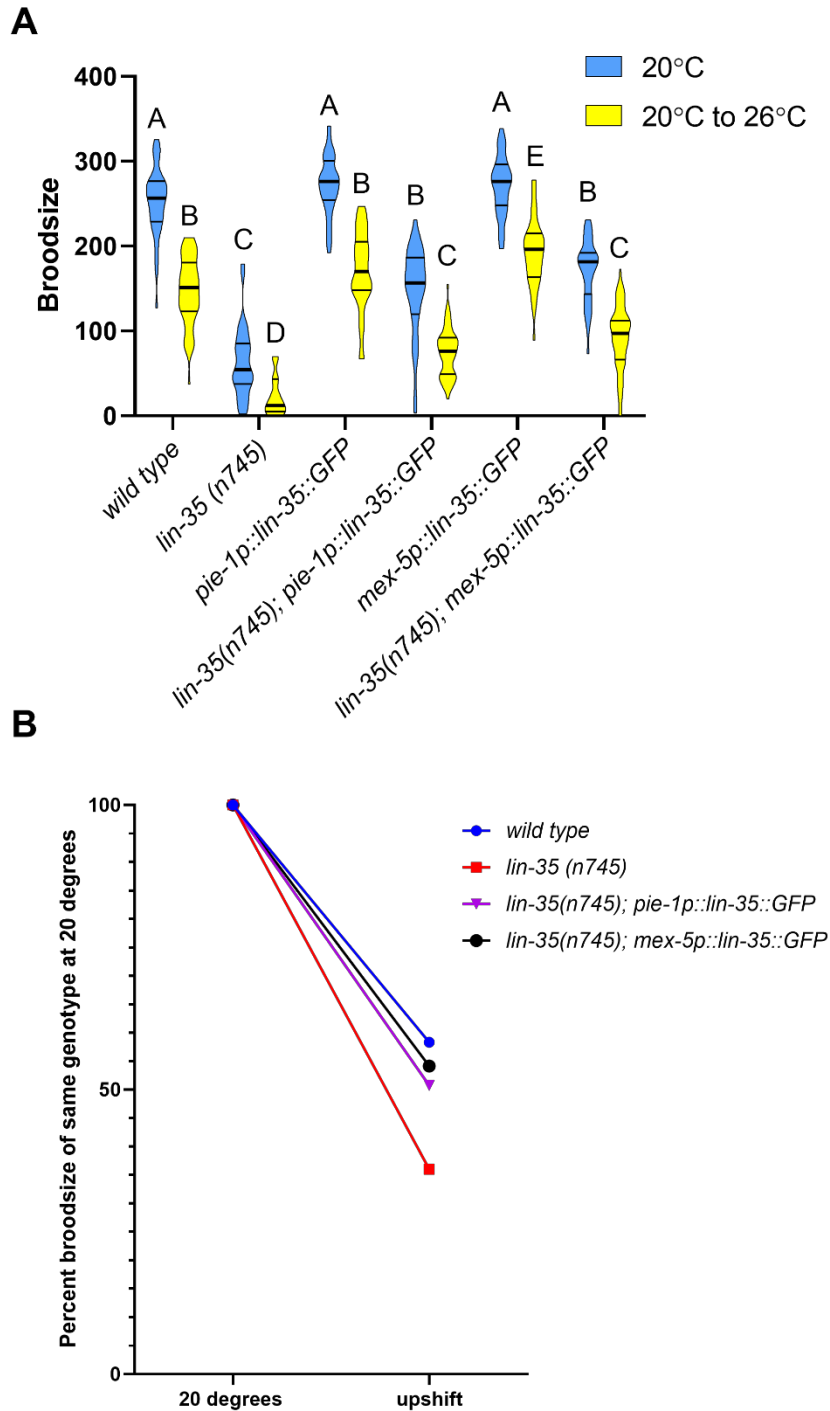**Figure 3.4: Germline expression of *lin-35* partially rescues fertility in *lin-35* mutants.** (A) Violin plot showing the brood sizes of wildtype and *lin-35* mutant worms with and without the germline expression of *lin-35* transgenes. Under both temperature schemes the fertility of *lin-35* mutants is improved by the germline transgenes. Upshift refers to worms that were upshifted to 26°C at the L4 stage

and then analyzed for brood size. Significance is indicated by the letters above the violins and was determined using a Brown-Forsythe and Welch ANOVA p<0.05 (B) Plot that shows the relative brood size of wildtype, *lin-35* mutants, and *lin-35* mutants with the germline expression transgenes that were upshifted as compared to the same genotype at 20°C. The transgenes rescued some of the relative fertility of *lin-35* mutants nearly back to wildtype.

**Discussion:**

LIN-35 is important for buffering fertility under temperature stress. It is currently unclear what role LIN-35 is playing to buffer fertility. It is possible that it could be regulating genes that contribute to one of the many important processes in the germline. One such process that LIN-35 could be influencing to protect fertility in response to stress is germline apoptosis. Previous work has shown that stress can induce higher levels of germline apoptosis and that LIN-35 is important to at least some germline apoptosis pathways (Schertel and Conradt 2007, Láscarez-Lagunas et al 2014). It is possible that LIN-35 may be influencing germline apoptosis under moderate temperature stress to protect fertility.

LIN-35 function in the germline is more important than soma for fertility but the somatically expressed LIN-35 does play some role. Here we show that the somatic tissues we chose to test were not the key somatic tissues that help to protect fertility under moderate temperature stress, so it remains unclear which somatic tissue or tissues are these key somatic tissues. It is possible that LIN-35 is necessary in the nervous system or muscle for full fertility.

**CHAPTER 4: MODERATE TEMPERATURE STRESS INDUCES GERMLINE APOPTOSIS IN A PARTIALLY LIN-35 AND DREAM COMPLEX DEPENDENT MANNER**

**Introduction:**

We have shown that LIN-35 has a function in buffering fertility in response to temperature stress , but the way in which it does this is unknown, the goal of this chapter was to determine if LIN-35 and the DREAM complex have a role in germline apoptosis under moderate temperature stress. LIN-35 clearly has a role in both the germline and the soma that helps to protect fertility in response to moderate temperature stress that does not cause gross defects in germline (this work and Chi and Reinke 2006).  The goal of this chapter is to….

Apoptosis increases in response to temperature stress in wildtype animals (Poullet et al 2015). This increase in apoptosis leads to a smaller pool of meiotic oocyte precursors (Poullet et al 2015). Apoptosis has the potential to regulate fertility levels by allowing the where the remaining oocytes may take on additional resources from the dying cells, termed the nurse cell hypothesis. This could help to ensure the survival of the remaining cells and/or confer more fitness upon the offspring. Alternatively, this elevation in apoptosis could eliminate nuclei that have issues with meiosis that could be triggered by moderate temperature stress (Poullet et al 2015).

LIN-35 has known roles in physiological apoptosis as well as in stress induced apoptosis. LIN-35 has been shown to be important for physiological

apoptosis where it helps maintain normal levels of apoptosis through regulating

*ced-9* expression (Schertel and Conradt 2007). LIN-35 has also been shown to

be key in the regulation of starvation induced apoptosis, where it was also shown

to repress *ced-9* expression leading to an induction of apoptosis (Láscarez-

Lagunas et al 2014).

LIN-35 does not seem to work with the E2F complex in the germline

because it does not regulate the same genes as the other members of the

complex in the germline (Chi and Reinke 2006). LIN-35 regulates apoptosis at a

different level in the core apoptotic pathway than the rest of the E2F complex,

which regulate apoptosis at the level of *ced-4* expression and seems to promote

the expression of *ced-4* rather than repressing the expressing of *ced-9* (Schertel

and Conradt 2007). LIN-35 does not have a DNA binding domain and thus

cannot transcriptionally repress genes on its own. Therefore, it needs a partner in

order to do so. Another complex that could be such a candidate is the DREAM

complex, specifically the Muv B core of the DREAM complex. The DREAM

complex is a multi-subunit transcriptional repressor complex that is stabilized by

LIN-35 to help prevent the expression of germline genes in the soma (Goestch et

al 2018). Additionally, the DREAM complex DNA binding component LIN-54 has

been shown to localize to the promoter of the *ced-9* operon by ChIP from whole

animal mixed stage samples (Goestch et al 2018).  Therefore, it is possible LIN-

54 may also bind to this operon in the germline.

**Results:**

**The induction of germline apoptosis in response to temperature stress is dependent on LIN-35 and the DREAM complex**

Previously it had been shown that at 26.5°C germline apoptosis increases in wildtype animals, peaking around 24 hours post L4 (Poullet et al 2015). We sought to determine if this induction of germline apoptosis still occurred at 26°C and whether it is dependent on *lin-35* and the DREAM complex. To do this I constructed single mutant strains of *lin-35(n745), lin-35(ea75), lin-54(n2231),* and *lin-37(n758)* with the CED-1::GFP transgene. CED-1 is a transmembrane receptor expressed on the gonadal sheath cells that engulf apoptotic nuclei, and the CED-1::GFP transgene allows the visualization of apoptotic cells as they become engulfed by the sheath cells.

Using these strains, I assessed numbers of CED-1::GFP positive cells that were counted as apoptotic germ cells. I did this using a few different temperature schemes and assessed the number of apoptotic cells at a couple of different points during adulthood (Figure 4.1A). First, I upshifted worms to 26°C at the L1 stage to bypass the HTA phenotype seen *lin-35* and DREAM complex mutants and assessed the worms for apoptosis 48 hours post L4. When upshifting at L1, the nearly the entire development of the germline occurs at the elevated temperature ensuring that any effect on the germline that could occur due to moderate temperature stress would be captured. Next, I upshifted worms at L1 stage and then assessed them at only 24 hours post the L4 stage. This was done

to try and capture the window where the worm has the highest induction of germline apoptosis because previous work had shown that temperature stress induced the highest apoptosis in wildtype *C. elegans* at 24 hours post L4 (Poullet et al 2015). Finally, I upshifted the worms at the L4 stage and then assessed them at 24 hours post the L4 stage. The reason I upshifted the worms at the L4 stage rather than an earlier stage is that one of the mutants, the *lin-35(ea75)* mutant, has an even more potent HTA phenotype such that even when upshifted at L1 the worms would still arrest (data not shown). The germline development that occurs before L4 is mainly spermatogenic whereas the oogenic germline development occurs during late L4 and throughout adulthood so that development would still be captured in the time that the worm spends at the elevated temperature.

First, I assessed apoptotic germ cells in worms at the 48 hrs post L4 stage that were upshifted to 26°C after hatching at the L1 stage. Upshifting post embryonic development is important because *lin-35* and DREAM complex mutants have a High Temperature larval Arrest (HTA) phenotype if they are subjected to moderate temperature stress before hatching (Figure 4.1A). This first developmental time point and temperature scheme showed that there was an induction of germline apoptosis in wildtype worms under moderate temperature stress, where instead of ~2 apoptotic cells per gonad arm that are seen at ideal temperature there were ~4 apoptotic cells (Figure 4.1B). In *lin-35(n745)* mutants there was no induction of germline apoptosis under moderate temperature stress under these conditions. The members of the MuvB core of

DREAM, *lin-54(n2231)* and *lin-37(n758)* also showed no induction of apoptosis under these conditions. This indicates that DREAM complex and *lin-35* likely have roles in the induction of germline apoptosis in response to temperature stress.

Because of the previous data showing that the peak of germline apoptosis occurs at 24 hours post L4 I also upshifted L1s and assessed them at 24 hours post L4 (Figure 4.1A). Wild type again showed an induction of germline apoptosis (Figure 4.1C). *lin-35(n745)* and *lin-54(n2231*) mutants did not show a significant induction of germline apoptosis in response to moderate temperature stress under these conditions.  On the other hand, *lin-37(n758)* mutants did show a slight induction of germline apoptosis where the level of germline apoptosis in this mutant at 26°C that was significantly higher than that of the *lin-37(n758)* mutant at 20°C, but was not significantly different than wildtype at 20°C.

Finally, in order to assess the *lin-35(ea75)* allele, which still shows the HTA phenotype even when upshifted at L1, I upshifted worms at the L4 stage and then assessed them 24 hours later (Figure 4.1A). The *lin-35(ea75)* allele is a CRISPR allele with the entire gene including part of the promoter deleted. There has been some disagreement about whether the *lin-35(n745)* allele, which is a single point mutation resulting in a premature stop codon at amino acid 151, is a true null allele even though there is no LIN-35 protein on a western blot from this allele (Lu and Horvitz 1998), Therefore, we also included the *lin-35(ea75)* allele in this study to see if there was an enhancement of the germline apoptosis phenotype. In this last case I saw the highest induction of germline apoptosis in

wild type of ~8 cells per gonad arm in wildtype at 26°C vs ~ 2 cells at 20°C (Figure 4.1D). I also saw a small but significant induction of apoptosis in *lin-35(n745), lin-54(n2231),* and *lin-37(n758)* at 26°C when compared to the same genotype at 20°C. However, it was significantly smaller than level of apoptosis that was seen in wildtype 26°C. There was no induction of apoptosis in *lin-35(ea75)*. Together these results show that *lin-35* and the DREAM complex are important for the induction of germline apoptosis under moderate temperature stress.
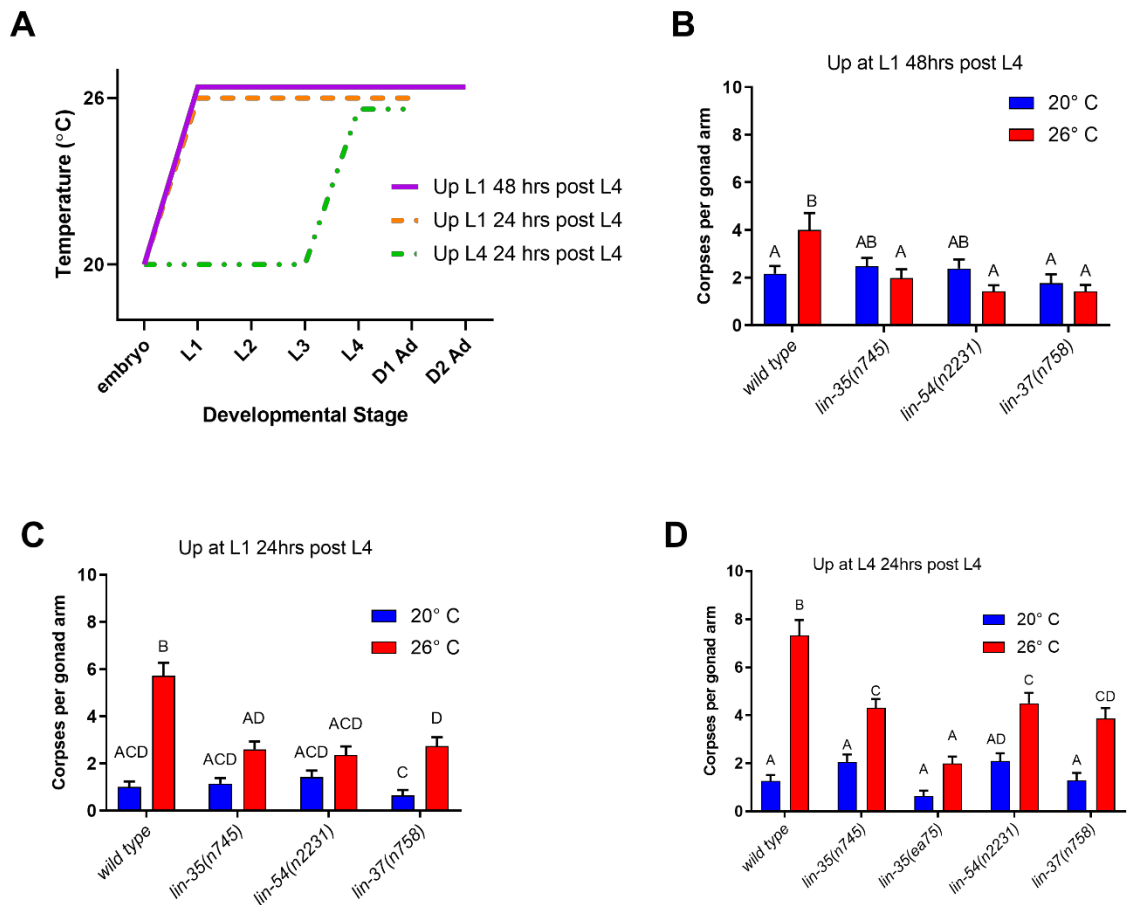
**Figure 4.1: Moderate temperature stress induces germline apoptosis in a LIN-35 and DREAM complex dependent manner.** (A) This is a schematic figure showing the different temperature schemes that were used in the respective experiments. (B) Bar graph representing the average number of CED-1::GFP positive cells found per gonad arm in worms that were upshifted at L1 and then assessed 48 hours post L4. Error bars indicate SEM. Significance is indicated by the letters above the bars where the same letter indicates that there is no statistical difference. Significance was calculated using a Tukey 2way ANOVA with p < 0.05. (C) Bar graph representing the average number of CED-1::GFP positive cells found per gonad arm in worms that were upshifted at L1 and assessed 24 hours post L4. Error bars indicate SEM. Significance is indicated by the letters above the bars where the same letter indicates that there is no statistical difference. Significance was calculated using a Tukey 2way ANOVA with p < 0.05. (D) Bar graph representing the average number of CED-1::GFP positive cells found per gonad arm in worms that were upshifted at L4 and assessed 24 hours post L4. Error bars indicate SEM. Significance is indicated by the letters above the bars where the same letter indicates that there is no statistical difference. Significance was calculated using a Tukey 2-way ANOVA with p < 0.05.

**Discussion:**

In this work we have shown that in wildtype animals, moderate temperature stress induces germline apoptosis. This is consistent with the previous data that was collected (Poullet et al 2015). Also consistent with the previously published data is that the peak of this induction of apoptosis occurs 24 hours post L4. What was previously unknown in wildtype animals is that the developmental stage at which the animals are upshifted does not make a difference in the level of induction of germline apoptosis. In this work we showed that worms that were upshifted to 26°C at the L4 stage and then assessed at 24 hours post L4 had the same level of germline apoptosis statistically as those

which were upshifted earlier in development at the L1 stage (Welch's t-test with p≤0.05).

Also, from this work it is clear that the DREAM complex and *lin-35* are important for the induction of germline apoptosis in response to moderate temperature stress. I showed that there is no statistically significant induction of germline apoptosis in response to moderate temperature stress in *lin-35* mutants or in mutants of the MuvB core of DREAM when worms were upshifted at the L1 stage. While I showed that there is some induction of germline apoptosis in response to moderate temperature stress in the *lin-35(n745), lin-54(n2231),* and *lin-37(n758)* mutants when worms were upshifted at the L4 stage. Thus LIN-35 and the DREAM complex may not be necessary for induction of germline apoptosis. However, these mutants still have a blunted induction of apoptosis. Additionally, the *lin-35(ea75)* mutant did not have a statistically significant induction of germline apoptosis when upshifted at the L4 stage. Since this mutant is a true null allele, unlike any of the other mutants used, this suggests that it may be the potential residual function left in the mutants that allows for the small level of apoptotic induction.

**CHAPTER 5: THE DREAM COMPLEX AND *LIN-35* HAVE ROLES IN THE INDUCTION OF GERMLINE APOPTOSIS IN RESPONSE TO DNA DAMAGE**

**Introduction**

The goal of this chapter was to determine if the DREAM complex has a role in the induction of germline apoptosis in response to DNA damage. It is known that LIN-35 is important for the induction of germline apoptosis in response to DNA damage (Schertel and Conradt 2007). It is unknown which partners LIN-35 is working with in the induction of germline apoptosis in response to DNA damage.     The DREAM complex is a possible partner for LIN-35 in this context. In the previous chapter I showed that LIN-35 and the DREAM complex MuvB components are important for the induction of germline apoptosis under moderate temperature stress. The DREAM complex mutants and *lin-35* mutants have a highly reduced or lack of induction of apoptosis in response to moderate temperature stress. Because the DREAM complex has a role in moderate temperature stress it may also have a role here with DNA damage induced apoptosis. One thing to note is that DNA damage induced germline apoptosis does not rely on the same players as other stress induced apoptosis. It depends more on CEP-1/p53 and EGL-1/BH3 to induce apoptosis than other pathways that generally depend more heavily on MAPK pathways or proteins involved in synapsis. It is unknown whether LIN-35 and CEP-1/p53 work in the same pathway or in parallel pathways to regulate *ced-9* expression.

**Results:**

**Apoptosis is not induced in response to DNA damage in *lin-35* or DREAM complex mutants**

To investigate whether members of the DREAM complex had a role in DNA damage induced germline apoptosis we first used Acridine Orange vital dye to stain apoptotic cells. Acridine Orange works by being fed to the worms, crossing the intestine into the gonad where it is selectively taken up by germ cells undergoing apoptosis, intercalates into the fragmented DNA of these cells and fluoresces green. I used AO in wildtype worms as well as *lin-35(n745), lin-54(n2231),* and *lin-37(n758)* mutants. Worms were isolated as L4 larvae then subjected to either 0 J/m$^2$ or 400 J/m$^2$ of UV 24 hours post L4. At 48 hours post L4 the worms were stained with AO for 2 hours in the dark then destained on fresh seeded NGM plates for 2 hours in the dark. 20 minutes into the destaining procedure the worms were moved to fresh plates to help reduce background staining. After destaining worms were mounted on 2% agarose pads in 10mM levamisole and scored for green fluorescence. As was expected in wildtype there was a very low level of physiological germline apoptosis in the absence of DNA damage (Figure 5.1A). In the presence of DNA damage from the UV irradiations there was an induction of apoptosis, doubling the number of AO positive cells present in the gonad arms (Figure 5.1A). As has been previously published, in *lin-35(n745)* mutants there was a similar level of physiological apoptosis to wildtype, but there was no induction of apoptosis in response to DNA damage

(Figure 5.1A) (Schertel and Conradt 2007). In *lin-54(n2231)* mutants and *lin-37(n758)* there was also no induction of apoptosis in response to DNA damage (Figure 5.1A). This indicates that these genes are important for the DNA damage induced germline apoptosis response.
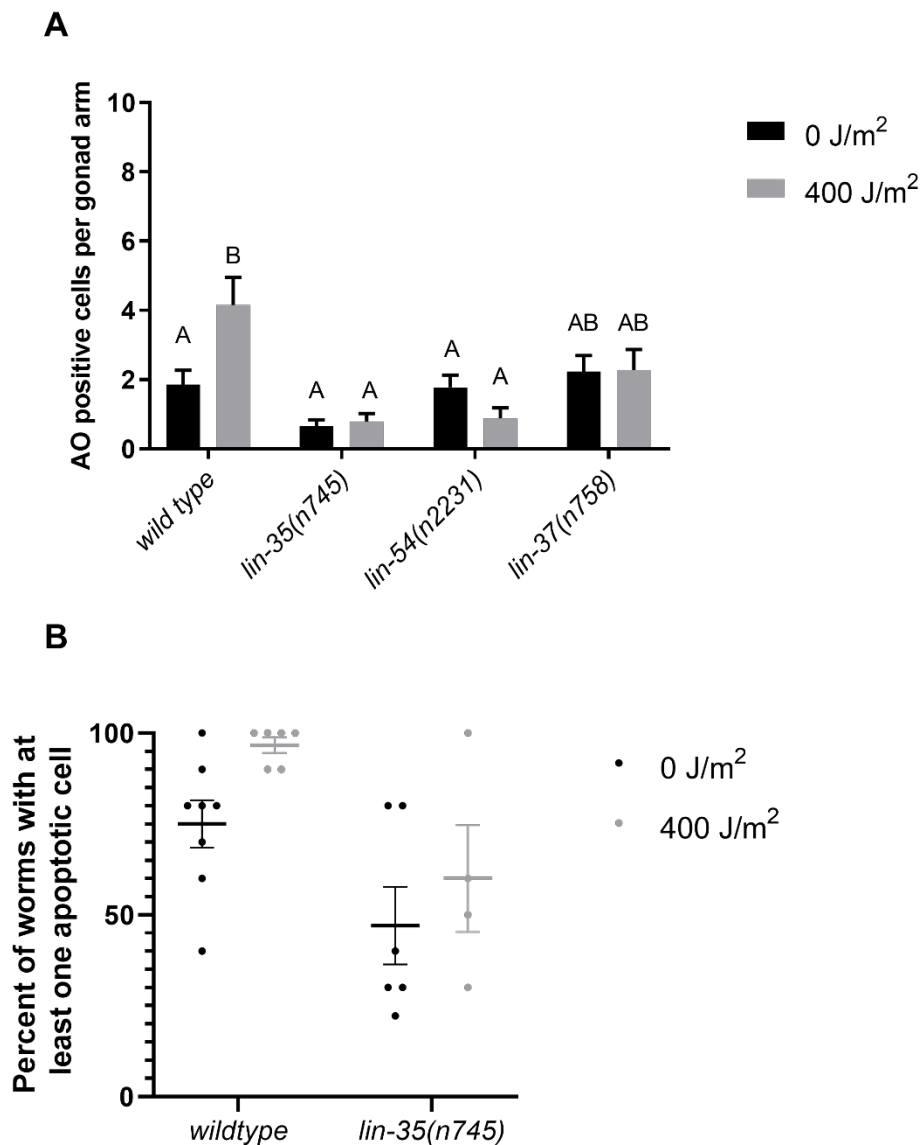
**A**



**B**



**Figure 5.1: *lin-35* and DREAM complex mutants do not have an induction of germline apoptosis in response to DNA damage and AO shows some**

**variability in DREAM complex mutants.** (A) Bar graph showing the average number of AO positive cells per gonad arm with an n≥38 gonads per genotype. This shows that there is no induction of germline apoptosis in response to DNA damage in the gonads of *lin-35(n745)* mutants or DREAM complex mutants. The letters over the bars indicate significance where if two bars have the same letter they are not statistically significant from each other at p<0.05. The worms were isolated at the L4 stage then irradiated 24 hours post L4 and then 48 hours post L4 they were stained for 2 hours with AO and destained for 2 hours then scored for AO positive cells. Significance was found using a Tukey 2way ANOVA. (B) Scatter plot showing the percentage of worms with at least one apoptotic cell stained with AO, each dot represents a trial, error bars show SEM. n≥20 for all genotypes.

However, it should be noted that AO staining showed a high level of variation in the number of cells stained between experiments, especially in mutants (Figure 5.1B). Some experiments with the mutants would have 80% or more of the worms having at least one apoptotic cell, while others would have less than 60% of the worms having at least one stained cell. There was less of this variation in wildtype particularly when worms were subjected to DNA damage (Figure 5.1B).

Because of the inconsistencies we saw with AO staining, I constructed *lin-35(n745)*, *lin-35(ea75), lin-54(n2231),* and *lin-37(n758)* single mutant strains with the CED-1::GFP *bcIs39* to use CED-1::GFP as a means of scoring germline apoptosis. These strains were then used to quantify germline apoptosis. CED-1::GFP relies not on the intestine absorbing a dye but rather on a fusion protein that expresses on the engulfing cells (the sheath cells of the gonad) to show the apoptosing cells. Because *lin-35* mutants and DREAM complex mutants have intestinal defects this method may be more accurate in showing germline

apoptosis. CED-1::GFP was less variable between treatments and genotypes than AO (Figure 5.2B).

Using the CED-1::GFP we saw an increase in DNA damaged induced apoptosis in wild type as expected (Figure 5.2A).  Neither the *lin-35(n745)* allele nor the *lin-35(ea75)* allele showed an induction of apoptosis in response to DNA damage (Figure 5.2A). *lin-54* mutants and *lin-37* mutants also did not show an induction of germline apoptosis in response to DNA damage (Figure 5.2A). Interestingly with the *lin-35(n745)* allele we saw wildtype levels of physiological apoptosis (the non-UV treated state) with an average of about 2 corpses per gonad arm in this allele, but with the *lin-35(ea75)* allele there was a significant reduction of physiological apoptosis to about 1 apoptotic cell corpse per gonad arm (Figure 5.2A). Neither *lin-54* mutants nor *lin-37* mutants showed less physiological apoptosis.
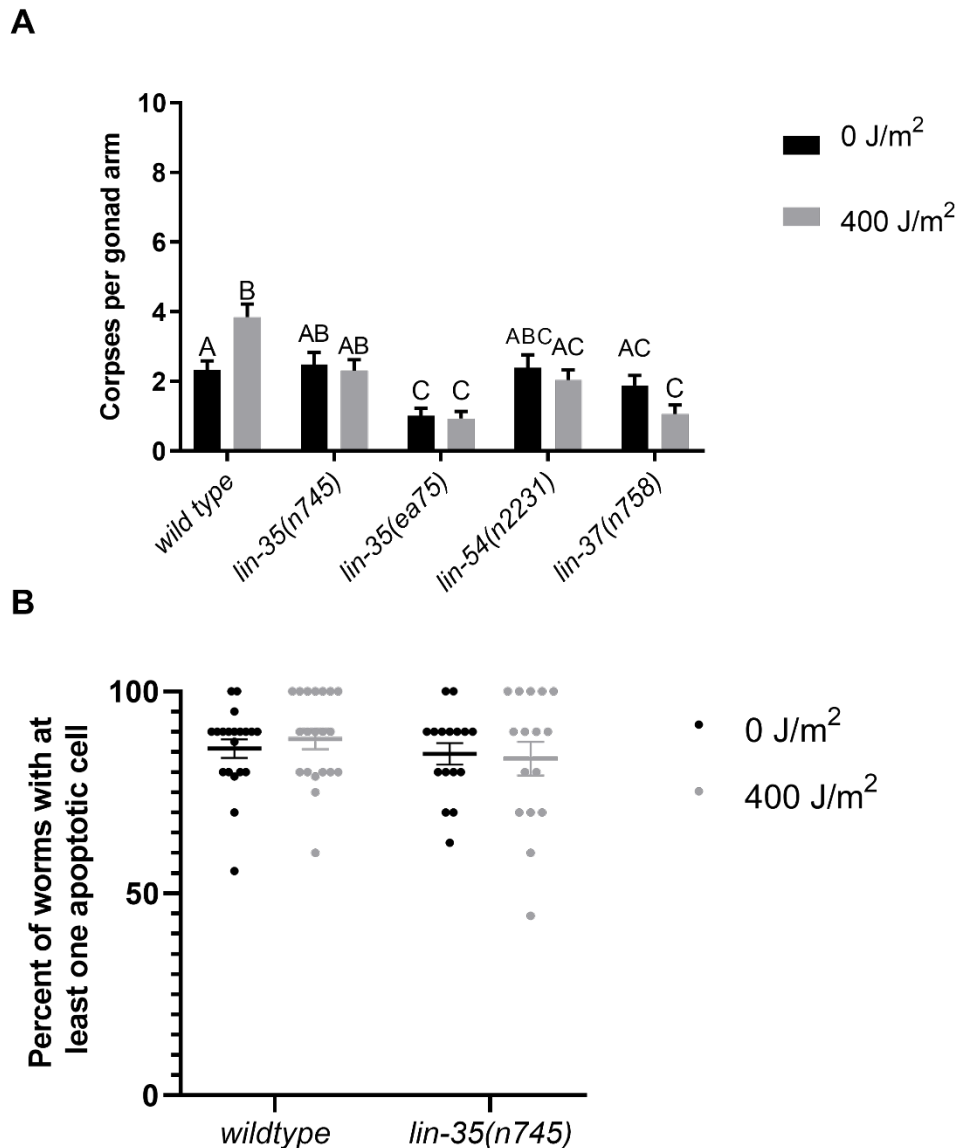
**A**



**B**



**Figure 5.2: CED-1::GFP is less variable than AO and the DREAM complex is important to DNA damage induced apoptosis.** (A) Bar graph that shows the average number of CED-1::GFP positive cell corpses per gonad arm of n≥60 gonad arms. *lin-35* mutants and DREAM complex mutants do not have an induction of germline apoptosis in response to DNA damage. Significance is indicated using the letters above the bar where if two bars have the same letter then they are not statistically different from each other. Significance was calculated using a Brown-Forsythe and Welch ANOVA with p<0.05. Worms were isolated as L4s then subjected to UV radiation of the appropriate dose 24 hours post L4 and scored 48 hours post L4. (B) Scatter plot showing the average percentage of worms with at least one CED-1::GFP positive cell between their

two gonad arms, each dot represents a trial, the error bars represent SEM. CED-1::GFP showed less variability across genotypes and treatments than AO.

**Discussion:**

Based on the two different types of germline apoptosis assays, it is clear that the Muv B core of the DREAM complex has a role in DNA damage induced apoptosis. The DREAM complex mutants examined here, *lin-54(n2231)* and *lin-37(n758)* mutants, both did not show an induction of germline apoptosis in response to DNA damage. This phenocopies *lin-35(n745)* mutants, which could indicate that the DREAM complex is partnering with LIN-35 to regulate germline apoptosis. The next set of questions to answer would be does LIN-35 still regulate *ced-9* expression in the context of DNA damage induced germline apoptosis and does the DREAM complex also regulate *ced-9* expression. If that were true then it would provide more evidence that the DREAM complex and LIN-35 are working together to regulate *ced-9* expression. Then if they do both regulate *ced-9* expression it would be prudent to do ChIP and CoIP experiments to determine if DREAM complex and LIN-35 bind to the *ced-9* operon in the germline and if they are physically associated in that context. In embryos, LIN-54, the DNA binding component of the DREAM complex does bind to the *ced-9* operon by ChIP indicating it may regulate *ced-9* expression and has the ability to bind to that sequence *in vivo*. It would still be necessary to see if those things were also true in the germline.

Acridine orange appears to be more variable between treatments and genotypes. Whereas all the genotypes and treatments with CED-1::GFP show a similar percentage of worms with no apoptotic cells save for *lin-35(ea75),* Acridine Orange varied across genotypes and between UV and no UV conditions. This could be because of the way that AO works. Acridine Orange is absorbed through the intestine after staining the bacteria that the worms eat. DREAM complex and *lin-35* mutants have defects in their intestines that could make it difficult to absorb the vital dye. CED-1::GFP however relies on the somatic gonad expression of CED-1. CED-1 is a transmembrane receptor on the sheath cells that engulf the corpses of the apoptotic germ cells. Because CED-1::GFP therefore does not rely on the ability of the intestine to absorb the dye it may be a better measure of apoptosis in these mutants.

Finally, the *lin-35(ea75)* mutant appears to have a stronger apoptosis phenotype than the *lin-35(n745)* mutant. The *lin-35(ea75)* allele is a true null allele because it is a CRISPR allele that deletes the entire gene sequence including part of the promoter of *lin-35*. The *lin-35(n745)* allele in contrast is a point mutation that introduces a stop codon early in the sequence of the protein. While *lin-35(n745)* does not produce protein on a Western blot it may still have some sort of activity since the true null allele has less germline apoptosis overall than the *lin-35(n745)* allele. The lack of apoptosis seen in the *lin-35(ea75)* mutant can also explain why even when using CED-1::GFP there are few worms with at least one GFP positive cell. If many of the germlines truly have zero apoptotic

cells, then there would be proportionally fewer worms with at least one GFP

positive cell.

**CHAPTER 6: DISCUSSION**

**Role of Apoptosis in Fertility in Response to Stress**

LIN-35 appears to have a major role in fertility under moderate temperature stress. In worms that are upshifted there is a sharper decline in fertility of *lin-35* mutants than wildtype worms. Any somatic expression of *lin-35* improves fertility of *lin-35* mutants at 20°C but only the pan-somatic *lin-35* transgene improves fertility under the upshift and 26°C temperature schemes. Germline expression of *lin-35* improves fertility more than somatic expression indicating the role of *lin-35* in the germline may be more important for fertility. Previous work has shown that *lin-35* has a role in germline apoptosis, the role of *lin-35* in apoptosis maybe critical for fertility in response to stress as apoptosis increases dramatically under stress conditions. A reason for apoptosis to increase dramatically under stress would be to provide more nurse cells to deliver more cytoplasmic components to developing oocytes. These cytoplasmic components may offer the developing oocytes a better chance at survival under the stressful condition that the mother is enduring. This would help preserve the mother's fertility under stress because it would give her offspring more nutrients and starting materials to survive under the stressful condition.

**Biology Underlying Effects of Temperature**

The biology of the underlying effects of moderate temperature stress are still not well understood. Moderate temperature stress does not engage the heat

shock response the same way severe temperature stress or heat shocks do, but there is evidence that it impacts the organization of chromatin during development (Costello et al 2019). In synMuv B mutants there is a delay in the compaction of chromatin in embryos that are subjected to moderate temperature stress. The delay in chromatin compaction could offer the chance for transcription factors to come in and bind to genes that would not normally be turned on in tissues such as the intestine and thus lead to misexpression of these genes. The misexpression of these genes may lead to the malfunction of critical tissues like the intestine, which is what is thought to ultimately lead to the high temperature larval arrest phenotype seen in the synMuv B mutant worms. By upshifting the worms post L1 for the apoptosis in response to moderate temperature stress experiments we tried to prevent the HTA phenotype while also having as much development as possible occur at the increased temperature. We found that even when upshifted post hatching, the *lin-35(ea75)* mutant worms still arrested. This indicates that this phenotype is more severe in these mutants and to study them we had to modify our upshift temperature scheme. Instead of upshifting at the L1 stage we upshifted worms at the L4 stage. While most of the somatic development is done by the L4 stage and sperm have been created no oocytes are made until the adult stage so upshifting at L4 still allows for the oocyte development to occur under moderate temperature stress. What the data showed between upshifting at L1 and L4 there was a slightly greater induction of apoptosis in wildtype animals that were upshifted at L4. This may indicate that the developmental processes that set the

germline up to be able to induce apoptosis in response to stress are temperature sensitive.

A reason that there might be more induction of apoptosis going on in younger animals than older animals is that the younger animals are more concerned about the quantity of offspring rather than the quality and the induction could balance some of the quality back in times of stress. Day 1 mothers produce about 10% of all of the progeny produced in their lifetimes with the progeny produced during this period being more likely to have defects, have shorter body length, have delays in development, and smaller brood sizes (Perez et al 2017). If stressed mothers are more likely want to have fewer but more fit offspring like we have proposed then it could be logical to have the balance of quantity and quality shifted in the early oocytes being made to make up for how the offspring are likely to be exposed to the same stress as the mother. Instead of wasting resources on lots of offspring that are unlikely to survive to adulthood the germline shifts towards getting rid of more nuclei to donate their cytoplasmic components towards more robust but fewer oocytes.

One thing that may be underlying the effect of temperature stress on germline apoptosis might be synapsis. Synapsis is a temperature sensitive process during meiosis where the synaptonemal complex is formed between the chromosome pairs for crossing over to occur (Bilgir et al 2013). It has been shown previously that it is temperature sensitive, therefore, it could be that under moderate temperature stress, synapsis is not occurring correctly triggering the synapsis checkpoint. The synapsis checkpoint, which is mediated by BUB-3 and

PCH-2, leads to an induction of germline apoptosis once triggered (Bohr et al

2015). Therefore, if synapsis is not occurring correctly because of moderate

temperature stress it could be inducing higher levels of apoptosis in order to cull

nuclei with improperly synapsed chromosomes, which could otherwise ultimately

lead to aneuploidy or polyploidy, which can be disastrous for genomic  integrity.

In order to address that question of whether moderate temperature stress

induced apoptosis is a result of temperature sensitivity in synapsis experiments

could be done using *bub-3* and *pch-2* mutants and upshifting them at the L4

stage to see if there is a normal induction of germline apoptosis or a loss of the

induction. A lack of induction of germline apoptosis under moderate temperature

stress in these mutants would point to there being a triggering of the synapsis

checkpoint that leads to the induction of germline apoptosis in response to
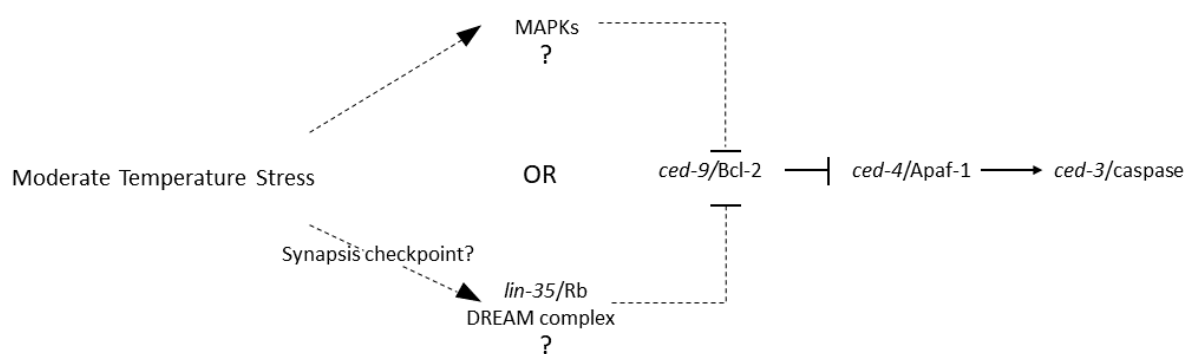
moderate temperature stress.



**Figure 6.1: Possible Pathway Underlying Germline Apoptosis Under Moderate Temperature Stress:** This figure shows the possible pathways that contribute to germline apoptosis induced by moderate temperature stress. Dotted lines indicate that there has not been evidence to show direct regulation yet.

**Roles of LIN-35 and DREAM in DNA damage induced apoptosis**

In accordance with previously published data, LIN-35 appears to have a significant role in the induction of germline apoptosis in response to DNA damage (Schertel and Conradt 2007, this thesis). Additionally, members of the DREAM complex, LIN-54 and LIN-37, appear to have roles in DNA damage induced germline apoptosis as well. In other contexts, LIN-35 works to stabilize the relationship between the two major subcomplexes of DREAM, the MuvB core and the E2F complex, in order to transcriptionally repress genes (Goestch et al 2018). It may be true that LIN-35 does the same in the context of germline apoptosis in order to repress components of the core apoptotic machinery such as *ced-9*. Under starvation conditions *lin-35* is upregulated and promotes germline apoptosis through repressing *ced-9* transcriptionally in the germline. This leads to an increase in apoptosis in response to the starvation, which may promote healthier offspring to be made. The DREAM complex, specifically the DNA binding component LIN-54, binds to the operon in which *ced-9* is in the genome in embryos, indicating that there is potential for it to bind there as well in the germline. In order to test whether the DREAM complex and LIN-35 are directly repressing *ced-9* some more experiments would need to be done. One such experiment would be to analyze the expression of *ced-9* and the other core apoptotic machinery components in *lin-35, lin-54,* and *lin-37* mutants as well as wildtype worms to determine if the apoptosis genes are misregulated in these

mutants. Another important one would be to do ChIP-seq for LIN-35 and L-54 in germlines to see if they bind to the *ced-9* operon in germline tissue. Additionally, experiments would have to be done to show that expression of *lin-35, lin-54,* and *lin-37* increase in the germline in response to DNA damage stress, which would indicate that these proteins are being recruited to the germline in order to promote germline apoptosis.

**Effects of LIN-35 on Physiological Apoptosis**

When we examined the effects of LIN-35 on physiological apoptosis we did not see a physiological apoptosis phenotype in *lin-35(n745)* mutants. We think this is likely a methodological effect rather than a biological effect because unlike in previous work we did not use an engulfment mutant background with which to examine the loss of *lin-35* on physiological apoptosis. Engulfment mutants are mutants that slow down the process of the sheath cells engulfing the apoptotic nuclei, using them allows the apoptotic corpses to persist longer so they can be visualized via Nomarski optics. There were a few reasons we did not use an engulfment mutant background to examine *lin-35* mutants and the DREAM complex mutants. First, we would have had a hard time using the same engulfment mutant background for all of the mutants we were examining due to some of the DREAM complex genes being on the same chromosome as the engulfment genes that are popular for examining this phenotype. Second, we were less interested in the subtle effects of *lin-35* and the DREAM complex on physiological apoptosis and more interested in roles of these genes in response

to stress. Engulfment mutants can have massive amounts of apoptotic cells in response to stress so it is not appropriate to use them in the case of a stress that could cause high amounts of apoptosis like we suspected moderate temperature stress would. Massive amounts of germline apoptosis make it very difficult to score apoptosis (Lant and Derry 2014). Finally, vital dyes like Acridine Orange rely on engulfment signals to work and therefore do not stain effectively in engulfment mutants (Gartner et al 2008).

Beyond the methodological differences between this thesis and previous work we did see evidence, however, that in the true null allele *lin-35(ea75)* had lower physiological apoptosis than wildtype. The true null allele shows significantly lower apoptosis in worms 48 hours post L4 that have not been treated with UV than wildtype under the same condition. Because this allele deletes the entire gene and part of the promoter of the *lin-35* it makes sense for it to have a more severe phenotype versus the *lin-35(n745)* allele, which puts in a premature opal stop codon at amino acid 151. While the *lin-35(n745)* allele does not make protein on a western blot, a similar allele, *lin-35(rr33)*, which has an amber stop codon in the same position does produce *lin-35* mRNAs that are comparable in abundance to *lin-35* mRNA seen in wildtype worms (Lu and Horvitz 1998, Ouellent and Roy 2007). This indicates for the *lin-35(rr33)* allele that the mRNA made is not being degraded, which was unexpected for a transcript with a premature stop codon. This does not appear to occur in the *lin-35(n745)* allele as the transcript levels of *lin-35* are significantly decreased from wildtype in this allele, however some transcripts may be evading decay (Goestch

et al 2018). Generally, the *lin-35(n745)* allele has a more severe phenotype than the *lin-35(rr33)* allele so an even more drastic mutation such as is seen with *lin-35(ea75)* where the entire gene and part of the promoter are gone makes sense to have an even more severe phenotype.

**Role for LIN-35 in the Soma**

The intestine and the somatic gonad do not appear to be the critical somatic tissues where LIN-35 helps to buffer fertility under moderate temperature stress. An idea for why *lin-35* mutants might have decreased germline apoptosis could be that LIN-35 slows down the engulfment process during germline apoptosis. This seems unlikely though because if that were the case then the engulfment machinery would be affected and that would make it difficult to score any sort of apoptosis assay in wildtype. Another somatic tissue where LIN-35 could be important for buffering fertility in response to temperature stress could be in nervous tissue. Nervous tissue closely regulates the germline by regulating signaling to promote fertility or germline senescence depending on the environment in which the animal finds itself (Dálfo et al 2012). Signals from the nervous tissue also can regulate germline apoptosis in a cell non-autonomous manner (Levi-Ferber et al 2014).

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