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Short running title: LIN-35 is important for the stress response in *C. elegans*

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Abstract

The pocket protein family controls several cellular functions such as cell cycle, differentiation, and apoptosis, among others; however, its role in stress has been poorly explored. The roundworm *Caenorhabditis elegans* is a simple model organism whose genes are highly conserved during evolution. *C. elegans* has only one pocket protein, LIN-35; a pRB-related protein similar to p130. To control the expression of some of its targets, LIN-35 interacts with E2F-DP transcription factors and LIN-52, a member of SynMUV (Synthetic Muv complex). Together, these proteins form the DRM complex, which is also known as the DREAM complex in mammals. In this review, we will focus on the role of LIN-35 and its partners in the stress response. It has been shown that LIN-35 is required to control starvation in L1 and L4 larval stages, and to induce starvation-induced germ apoptosis. Remarkably, during L1 starvation, insulin/IGF-1 receptor signaling (IIS), as well as the pathogenic, toxin, and oxidative stress-responsive genes, are repressed by LIN-35. The lack of *lin-35* also triggers a downregulation of oxidative stress genes. Recent works showed that *lin-35* and *hpl-2* mutant animals showed enhanced resistance to UPR^{ER}. Additionally, *hpl-2* mutant animals also exhibited the upregulation of autophagic genes, suggesting that the SynMuv/DRM proteins participate in this process. Finally, *lin-35(n745)* mutant animals overexpressed *hsp-6*, a chaperone that participated in the UPR^{mt}. All of these data demonstrate that LIN-35 and its partners play an important role during the stress response.

Introduction

Nearly 50 years ago, the retinoblastoma protein (pRB) was discovered as the first tumor suppressor and it proved to possess an essential role in cell cycle regulation (Chellappan *et al.*, 1991, Knudson, 1971, Sellers and Kaelin, 1997). Since then, its function in cell cycle control and cancer has been widely studied. Furthermore, many more biological aspects have been attributed to pRB, such as apoptosis, differentiation, and quiescence. Excellent reviews have been published on the role of pRB and its partner proteins in these aspects (Fischer and Muller, 2017, Kipreos and van den Heuvel, 2019, Sadasivam and DeCaprio, 2013, van den Heuvel and Dyson, 2008). However, not much is known about the role of *RB1* in stress.

pRB belongs to a family denominated pocket proteins, and in mammals, there are other two members: p130, and p107. Like pRB, these proteins also control cell cycle, proliferation, and differentiation (Stengel *et al.*, 2009). Pocket proteins interact with E2F proteins and with DP proteins (reviewed by (van den Heuvel and Dyson, 2008)). Two of these pocket proteins, p130 and p107, interact with LIN52, a protein part of the Muv sub-complex (<u>mu</u>ltiple <u>v</u>ulva), which is also formed by LIN9, LIN37, LIN52, LIN53/RBBP4, and LIN54 (Guiley *et al.*, 2015, Korenjak *et al.*, 2004, Litovchick *et al.*, 2007). Together, this group of proteins is known as the <u>d</u>imerization partner, <u>R</u>B-like, <u>E</u>2F <u>a</u>nd <u>m</u>ulti-vulval class B (DREAM) complex (Litovchick *et al.*, 2007). LIN52 and p130 interaction stabilizes the DREAM complex and is controlled by DYRK1A phosphorylation (Guiley *et al.*, 2015, Litovchick *et al.*, 2007). The DREAM complex is considered a master regulator of cycle-cell and is necessary to progress through the G0/S or G2/M (Fischer and Muller, 2017).

The DREAM complex is conserved from invertebrates to mammals (Litovchick *et al.*, 2007). In this review, we will focus on LIN-35, the sole pocket protein in *Caenorhabditis elegans* and its role in the stress response (Lu and Horvitz, 1998). LIN-35 is the closest homolog to p130 and is part of the worm

DREAM complex known as DRM (Goetsch *et al.*, 2017, Lu and Horvitz, 1998) (Figure 1). In *C. elegans*, the DRM complex is also composed of three E2F proteins encoded by *efl-1*, *efl-2*, and *efl-3* genes (Ceol and Horvitz, 2001, Winn *et al.*, 2011), by *dpl-1* gene (Page *et al.*, 2001), and the Muv orthologous genes: *lin-9*, *lin-37*, *lin-53*, *lin-54,* and lin-53/RBAP48 (Harrison *et al.*, 2006) (Figure 1). There is no clear ortholog in *C. elegans* for MYB (Harrison *et al.*, 2006, van den Heuvel and Dyson, 2008). The nematode *C. elegans* has been useful for understanding different aspects of the pocket proteins and the DRM protein complex function (Goetsch *et al.*, 2017, van den Heuvel and Dyson, 2008). As in other organisms, the DRM complex in *C. elegans* is also involved in the repression of tissue-dependent gene expression (Kipreos and van den Heuvel, 2019, Kudron *et al.*, 2013, Latorre *et al.*, 2015, Petrella *et al.*, 2011).

One of the best-known functions of the DRM complex is during the *C. elegans* vulval development (Figure 2A). The *C. elegans* vulva is formed during larval development, and in the adult hermaphrodite, it is necessary for egg-laying and for copulation with males (reviewed by (Sternberg, 2005)). Defects in genes that regulate vulval development can have a variety of phenotypes. No vulval formation is known as an egl phenotype (egg laying defect), in which worms accumulate embryos inside themselves because they cannot lay them. In the multiple vulva phenotype (muv phenotype) many vulvas are visible in the worms, such as protusions present along the ventral side of the animal, although not all these are necessarily functional.

Vulval development involves cell cycle regulation to specify the vulval cell fate. A single cell of the somatic gonad, the anchor cell, organizes the development of the vulva by signaling the vulval precursor cells, which were formed between worm´s larval stages L1 and L2. During the L3 larval stage, the anchor cell induces nearby epidermal precursor cells to generate vulval cells via an epidermal growth factor (EGF)-like ligand (LIN-3) signaling (Figure 2A). The expression of LIN-3, driven by a heat-shock promoter, is sufficient to induce vulval formation in the absence of the anchor cell or even the gonad (reviewed by (Sternberg, 2005)). LIN-3 activates the Ras-MAPK pathway in the cells that structure the vulva. Two

transcription factors, LIN-31 and LIN-1, are likely targets of the MAPK pathway. Mutant animals in *lin-1* or *lin-31* reveal another muv phenotype. The increased expression of LIN-31 or of a non-phosphorylated LIN-31 protein gives rise to vulva-less animals (egl phenotype). synMuv (synthetic multi-vulva) genes are divided into three classes (A, B, and C) of broadly expressed nuclear proteins that can inhibit vulval precursors from adapting to their vulval fates (Kipreos and van den Heuvel, 2019). Class B synMuv genes encode for the proteins LIN-35 and EFL-1. Class B synMuv genes act redundantly with class A genes in the transcriptional repression of *lin-3* (Figure 2A).

The DRM protein complex has recently been linked with gene regulation during stress conditions. Herein we wish to review the most recent findings on *lin-35/RB1* function during stress and how the DRM is involved in this response.

LIN-35/pRB possesses an essential role for L1 larval survival during starvation

In the wild, animals are exposed to constant changes in food availability; thus they have developed survival strategies to counteract with this stress. The *C. elegans* life cycle lasts for 3 days and is composed of embryogenesis, four larval stages (L1-L4), and adulthood. Worms can live up to 20 days in the laboratory. When worms are faced with fasting, they can suspend their growth for several days in four different stages of development. Later, when worms encounter favorable conditions, they can continue their normal life cycle, produce offspring, and experience a normal life span. Animals arrested in the L1 larval stage can suspend their growth for up to 10 days, at dauer stage for up to 4 months (reviewed in (Baugh, 2013)), at L4 larval stage for up to 10 days, and at the Adult Reproductive Diapause (ARD) (occurring when animals are starved anytime between the L4 larval stage through adulthood) for up to 1-month (Angelo and Van Gilst, 2009, Carranza-Garcia and Navarro, 2019, Carranza-Garcia and Navarro, 2020).

LIN-35 is required for L1 larval survival during starvation. Under this condition, LIN-35 controls the expression of StarvUP genes, which are expressed

during starvation (Baugh *et al.*, 2009, Cui *et al.*, 2013). By means of microarray analyses, Cui and collaborators compared the gene expression of starved L1 *lin-35* (n745) mutant animals against well-fed ones. These authors found that, during starvation, the expression of 1,200 genes was affected in *lin-35(n745)* mutant animals when compared with the wild-type animals; specifically, 57% of these genes were downregulated (Cui *et al.*, 2013). Among them, two genes encoding insulin-like   proteins, *ins-24* and *ins-30,*   were   down-regulated   in starved   *lin-35(n745)* L1 larvae and were upregulated in wild-type animals under the same conditions, suggesting that LIN-35 may function as an antagonist of insulin/IGF-1 receptor signaling (IIS) (Figure 2B) (Cui *et al.*, 2013). In *C. elegans*, more than 40 peptides related to insulin bind to the insulin receptor DAF-2 to negatively regulate the DAF-16/FoxO transcription factor (reviewed by (Murphy and Hu, 2013)). The expression of two other genes that participate in the insulin pathway, *unc-31* to *daf-16*, was not affected in starved *lin-35(n745)* animals. Therefore, these authors concluded that *lin-35* partially represses the IIS pathway in the L1 larvae (Cui *et al.*, 2013).

LIN-35 possibly regulates L1 starvation-survival by both IIS-dependent and -independent   mechanisms.   Using   an   overexpression   allele   of   *daf-16*,   the transcription   factor   downstream   of   the   Insulin   pathway,   in a   *lin-35*   mutant background, there was a partial improvement of the L1-stage survival-starvation rate compared to *lin-35(745)* single mutants in starved animals (Cui *et al.*, 2013). This observation suggests that LIN-35 controls this pathway at least partially. This could be due to that DAF-16 controls a large number of genes, and these targets are not all controlled for LIN-35. In agreement with this observation, similar results were   found   in   *lin-35(745),   unc-31(lf),* or *lin-35(745),   age-1(rf)* double mutants of genes that are upstream of the insulin pathway.

Ciu *et al.* (Cui *et al.*, 2013) also compared the data set of their microarray analysis with previously published data (Baugh *et al.*, 2011, Shapira *et al.*, 2006, Troemel   *et al.*,   2006),   and   observed   that   there   is   an   upregulation   of   many pathogenic, toxin or oxidative stress-responsive genes in starved *lin-35(n745)*

animals (Cui *et al.*, 2013). These results suggest that LIN-35 is required to repress genes that participate in pathogen infection and oxidative stress.

Interestingly, other SynMuv genes such as: *efl-1*, *dpl-1*, *lin-52*, *lin-9*, *hpl-2*, *lin-36* and *lin-15B* are not required for L1-stage survival during starvation, suggesting that this effect is likely specific to the pocket protein LIN-35 and not for all DRM complexes (Figure 2B) (Cui *et al.*, 2013). Since LIN-35 can interact with several proteins, more experiments are necessary to discover which proteins participate in this pathway. The function of *lin-35* during starvation in L1 is mainly controlled in two tissues: the intestine and neurons (Cui *et al.*, 2013). Specific rescue experiments demonstrated that a major role for starvation survival in L1 stage derives from the neurons.

LIN-35 is more abundant during L4-stage acute starvation

A study using SILAC mass spectroscopy showed that the protein profile of the animals at the mid-L4 larval stage animals has significant changes during the time exposed to starvation (Larance *et al.*, 2015). Employing an inclusion threshold of at least a two-fold change in the abundance of protein, the authors identified around 500 proteins that changed their expression when mid-L4-stage larvae were exposed to starvation. Notably, LIN-35 was more abundant in this analysis (Larance *et al.*, 2015), suggesting that this pocket protein is important for the mid-L4 larval starvation response (Figure 2C). Proteome analysis results highlight the importance of the transcriptional regulation during the starvation response (Larance *et al.*, 2015). It will be interesting to understand how this affects the chromatin landscape in response to starvation and to prove whether the DRM complex plays a role during this larval stage.

The results of the gene ontology analysis of Larence and collaborators showed that the most essential upregulated proteins are in categories such as "feeding behavior", "determination of adult lifespan," and "chromatin organization", while, the downregulated proteins fell, as expected, into the categories "cell cycle," "DNA replication," "regulation of meiosis," and "genitalia development" (Larance *et*

al., 2015). Remarkably, the starvation response in the L4 stage appears to be regulated by chromatin regulators such as PHA-4 or DAF-16, and by histone variants that affect chromatin accessibility (Larance *et al.,* 2015).

When animals are starved between the L4 larval stage and adulthood, they enter into a stage of diapause that is characterized by delayed in reproduction and slow growth, this stage is known as Adult Reproductive Diapause (ARD) (Carranza-Garcia and Navarro, 2020). In this hypometabolic stage, the hermaphrodite gonad reveals a remarkable change; it shrinks progressively, losing germ cells, and this event is known as the oogenic germline response (Carranza-Garcia and Navarro, 2020, Seidel and Kimble, 2011). Notably, gonad shrinking and delayed in reproduction are reversible. Carranza-Garcia and Navarro showed that *lin-35(n745)* mutant animals are capable of recovering their gonad size after starvation, suggesting that LIN-35 is not required for oogenic germline response during ARD (Carranza-Garcia and Navarro, 2019). However, these authors did not tested for whether *lin-35(n745)* were able to survive other aspects of ARD. There remains so much to learn about ARD and its regulation.

LIN-35 is required to induce germ cell apoptosis during starvation

During *C. elegans* oogenesis, physiological apoptosis eliminates one half of the germ cells in order to maintain homeostasis in the germline (Gumienny *et al.,* 1999). LIN-35 partially controls physiological apoptosis by repressing the expression of *ced-9* (Figure 2D) (Schertel and Conradt, 2007). *ced-9* encodes the worm orthologous of the human anti-apoptotic protein Bcl2*.* Additionally, *dpl-1*/DP, *efl-1*/E2F, and *efl-2*/E2F also promote physiological apoptosis by inducing the expression of the pro-apoptotic genes *ced-4*/APAF1 and *ced-3* (the pro-caspase) (Figure 2D) (Schertel and Conradt, 2007).

One-day-old adult animals exposed to 6 h of starvation increased germ cell apoptosis by nearly two-fold (Salinas *et al.,* 2006). Starvation-induced apoptosis also depends on LIN-35 because animals that lack this protein are unable to increase germ cell apoptosis during fasting (Figure 2E) (Lascarez-Lagunas *et al.,*

2014). Furthermore one-day-old adult animals exposed to 6 hours of starvation undergo the upregulation of the *lin-35* gene expression and its protein (Lascarez-Lagunas *et al.*, 2014). Similarly to physiological apoptosis, *ced-9* expression is downregulated during starvation; *ced-9* gene expression decreases considerably during fasting, and this downregulation is dependent on *lin-35* (Lascarez-Lagunas *et al.*, 2014). These data suggests that *lin-35* represses *ced-9* expression during starvation.

During ARD, germ cell apoptosis increases considerably (Carranza-Garcia and Navarro, 2019). *lin-35* is also required to maintain high levels of germ cell apoptosis under this condition (Figure 2E) (Carranza-Garcia and Navarro, 2019). These data suggest that *lin-35* plays a key role in the regulation of germ cell apoptosis (Lascarez-Lagunas *et al.*, 2014, Schertel and Conradt, 2007). However, more experiments are necessary to understand how *lin-35* regulates *ced-9* expression.

During starvation, one-day-old starved animals exhibited slowed down translation levels, supporting the idea that starvation reduces overall global expression (Lascarez-Lagunas *et al.*, 2014). Unexpectedly, *lin-35* mRNA avoids this state of translational repression, supporting its important role under this condition.

Heterodimeric partners E2F-DP encoded by *efl-1, efl-2,* and *dpl-1* induce *ced-4* expression to trigger physiological apoptosis during oogenesis (Figure 2D) (Schertel and Conradt, 2007). However, during starvation, E2F transcription factors *efl-1* and *efl-2* are not required to control *ced-4* expression (Figure 2E) (Lascarez-Lagunas *et al.*, 2014). In fact, the translation efficiency of their mRNA is diminished during starvation, supporting the idea that *efl-1* or *efl-2* are not required to control *ced-4* expression. In contrast, during starvation, the expression of *dpl-1* is higher and its mRNA continues to be efficiently translated; accordingly, DPL-1 is required to control *ced-4* expression, demonstrating that DPL-1 is important for *ced-4* upregulation during starvation-induced apoptosis (Figure 2E) (Lascarez-Lagunas *et al.*, 2014).

The DRM complex participates in the unfolded protein response of the endoplasmic reticulum and autophagy

Another type of stress is induced in the cell when misfolded protein accumulates at the endoplasmic reticulum (ER), triggering the <u>u</u>nfolded <u>p</u>rotein <u>r</u>esponse (UPR^{ER}). There are three UPR signaling pathways (PKR-like ER kinase, activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE-1), and their principal goal is to reestablish ER homeostasis and promote survival via the upregulation of ER chaperones (Hetz, 2012). In *C. elegans*, the *ire*-1/xbp-1 pathways control transcriptional regulation in response to acute ER stress, while *pek-1* and *atf-6* play only minor roles (Kozlowski *et al.*, 2014).

hpl-2(tm1489) mutant animals demonstrated enhanced resistance to UPR^{ER} stress when exposed to Tunicamycin and Thapsigargin. *C. elegans hpl-2* gene encodes for the heterochromatin protein 1 homolog whose protein is a SynMuv B gene that interact with LIN-35 (Kudron *et al.*, 2013). Additionally, *hpl-2(tm1489)* mutant animals showed overexpression of *xbp-1* and of other ER chaperones genes, that is, *hsp-3* and *hsp-4,* under baseline conditions (Figure 2F) (Kozlowski *et al.*, 2014). Interestingly, *lin-13* or *lin-35* RNAi animals showed a similar phenotype on UPR^{ER} stress resistance, suggesting that *hpl-2* and the genes of the SynMuv complex participate together to regulate this response (Figure 2F) (Kozlowski *et al.*, 2014).

Additionally, *hpl-2(tm1489)* mutant animals exhibited high expression of the autophagic genes *lgg-1* and *lgg-2*, which encode for their human homolog of LC3/Atg-8 (microtubule-associated protein light-chain 3), a crucial autophagy-related protein (Figure 2F) (Alberti *et al.*, 2010). These results suggest that HPL-2 regulates the expression of *lgg-1* and *lgg-2* and might participate in the regulation of autophagy (Kozlowski *et al.*, 2014).

LIN-35 participates in the unfolded mitochondrial protein response

The mitochondrial Unfolding Protein Response (UPR[mt]) is activated when the balance of protein-folding in this organelle is disrupted by the presence of reactive oxygen species and/or difficulties associated with the assembly of the electron transport chain (reviewed in (Haynes and Ron, 2010)). As a result, some transcriptional factors, such as CHOP, C/EBPb and Jun are activated (Horibe and Hoogenraad, 2007, Zhao *et al.*, 2002). The CHOP transcription factor then activates the expression of HSP60; a specific chaperone that participates in this response. Remarkably, the involvement of stress-responsive transcription factor JUN during the UPR[mt] response suggests the potential for crosstalk between UPR[mt] signaling and other stress responses (Haynes and Ron, 2010).

Cui and collaborators found that in *lin-35(n745)* mutant animals, mitochondrial respiratory chain (MRC) proteins are largely misregulated (Cui *et al.*, 2013). Among the genes found in this experiment is *hsp-6,* whose expression is highly upregulated in *lin-35(n745)* L1 starved compared to the wild-type animals (Figure 2G) (Cui *et al.*, 2013). HSP-6 is a mt-associated protein chaperone that is activated during the mitochondrial unfolded protein response (Durieux *et al.*, 2011). Supporting this data starved *lin-35* L1 mutant animals are sensitive to potassium cyanide, a potent inhibitor of MRC (Cui *et al.*, 2013). These results suggest that LIN-35 plays an important role in the regulation of the MRC in the UPR[mt] response.

LIN-35 role in oxidative stress remains poorly explored

The role of *lin-35* in the oxidative stress response in *C. elegans* has been poorly investigated. However, as we have previously mentioned *lin-35(n745)* mutant animals show changes in their metabolism that could affect the oxidative state. In agreement with this, several glutathione transferase genes, such as *gst-6*, *gst-10*, *gst-20*, *gst-23*, *gst-27*, and *gst-30* are downregulated in *lin-35(n745)* mutant animals (Figure 2H) (Cui *et al.*, 2013). Furthermore, *lin-35* (n745) mutant animals

are very sensitive to treatment with paraquat (a potent oxidant that interferes with electron transfer) (Cui *et al.,* 2013). These data suggest that *lin-35* might be involved in oxidative stress, and more experiments are necessary to elucidate the role of LIN-35 and the DREAM complex in the oxidative stress response.

Closing remarks

Here we have shown that there is considerably growing evidence demonstrating that the pocket protein LIN-35 and some of its partners participate in different types of stress. LIN-35 participation in the L1 larval survival during starvation is one of the best-known functions of this protein during stress. For L1 larval survival during starvation, it is necessary that LIN-35 repress the expression of genes related to UPR^{mt} and pathogen infection, as well to the MRC. Apparently, LIN-35 protects L1 larvae from starvation independently from the DRM complex. It would be interesting to investigate which proteins participate with this pocket protein to execute its role in this hypometabolic stage. It is possible that LIN-35 plays similar roles during the starvation response of L4 larvae, because its expression is higher under this condition. Furthermore, in adult animals, LIN-35 controls the UPR^{ER} throughout the induction autophagy and the expression XBP-1.

LIN-35 is important to induce starvation-induced apoptosis under short- and long-term fasting conditions, although this pocket protein is not necessary to maintain gonad homeostasis during long-term starvation or ARD. More experiments are necessary to understand the role of LIN-35 and the DRM complex during stress and elucidate how this group of proteins acts to protect animals from stress controlling new biological aspects.

Acknowledgements

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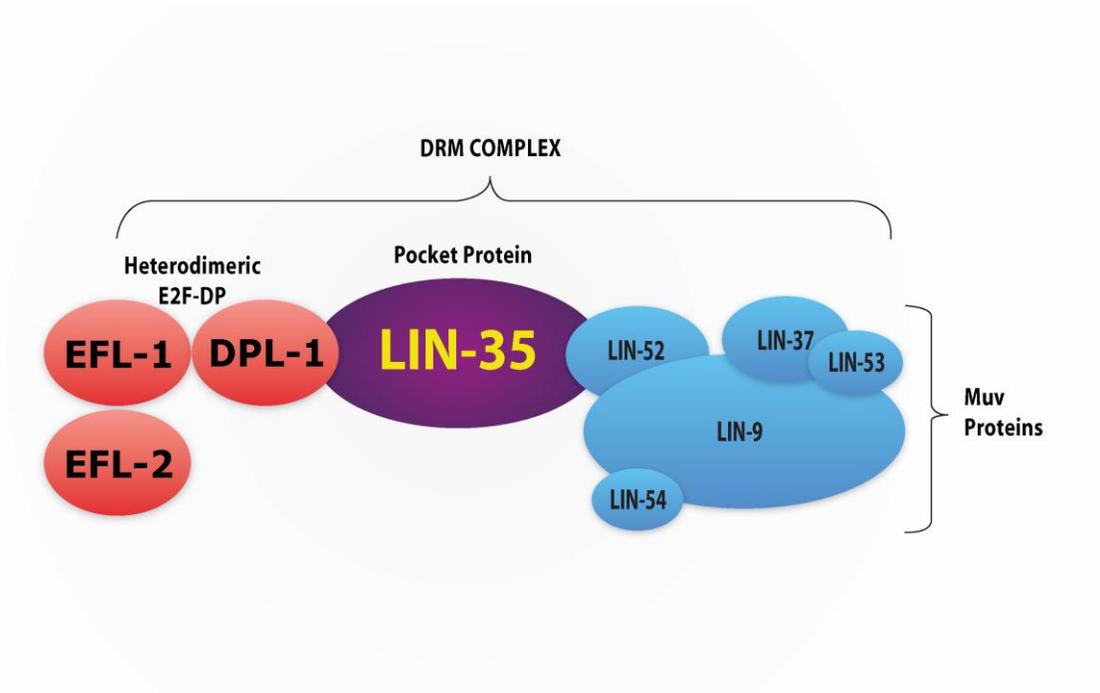
Figure 1. The DRM complex of *C. elegans*.

The worm DRM complex has only one pocket protein, LIN-35, which is related to p130. 9 proteins composed the DRM complex; LIN-35 (purple), 5 proteins that made the Muv core (blue) and 3 heterodimeric transcription factors EFL-1, EFL-2 and DPL-1 (red). The interaction between LIN-35 and LIN-52 is necessary to stabilize the complex.
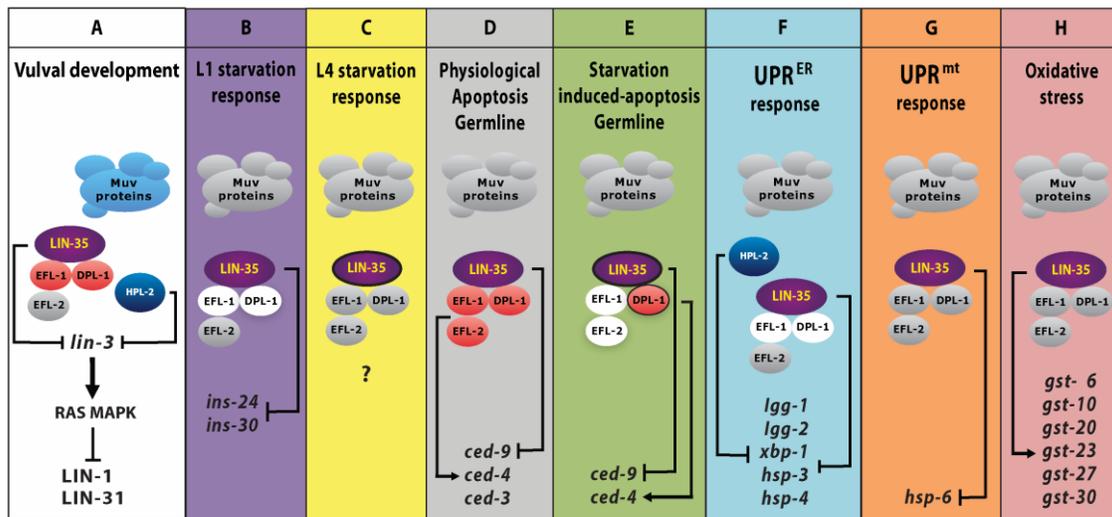
Figure 2. LIN-35 participates in the stress response.

(A) The DRM complex regulates vulval development is *C. elegans*. The DRM complex downregulates the expression of the gene *lin-3*, which encodes for EGF. LIN-3 activates the MAPK signaling that suppresses the expression of the transcription factors LIN-1 and LIN-31 (reviewed by (Sternberg, 2005)). **(B)** In the L1-stage starvation response, LIN-35 partially turns off the insulin/insulin receptor signaling by repressing the expression of the *ins-24* and *ins-30* genes (Cui *et al.*, 2013). **(C)** During the L4-stage starvation, the expression of the LIN-35 protein increases considerably. However is still unknown how LIN-35 or the DRM complex regulates this hypo metabolic stage (Larance *et al.*, 2015). **(D)** In the physiological germ cell apoptosis, LIN-35 downregulates the expression of *ced-9* (the worm homolog of the anti-apoptotic protein Bcl2), while the heterodimeric complex formed by EFL-1 or EFL-2/DPL-1 upregulates the expression *ced-4* (the worm homolog of the pro-apoptotic protein APAF1) (Schertel and Conradt, 2007). **(E)** For the starvation-induced germ cell apoptosis, LIN-35 downregulates the expression of *ced-9* while DPL-1 upregulates the expression of *ced-4*. Under this condition the proteins EFL-1 or EFL-2 are not required (Lascarez-Lagunas *et al.*, 2014). **(F)** During UPR[ER], LIN-35 and HPL-2 repress the expression of some genes involved in autophagy such as *lgg-1* or *lgg-2* or the UPR[ER] response such as *xbp-1 hsp-3*, and *hsp-4*. EFL-1 and DPL-1 also

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participate on this regulation (Kozlowski *et al.*, 2014). **(G)** In UPR^{mt} stress, LIN-35 downregulates the expression of the *hsp-6* gene which encodes an important chaperone involved in this response (Cui *et al.*, 2013). The participation of more proteins of the DRM complex in the UPR^{mt} is still unknown. **(H)** During oxidative stress, LIN-35 and EFL-1/DPL-1 upregulate the expression of some GST genes in oxidative stress (Cui *et al.*, 2013). In all panels, Muv complex in blue means they participate in the process, EFL-1/DPL-1 in red or LIN-35 in purple mean that these proteins are involved in the indicated condition. In grey are proteins whose function is still unknown in the indicated condition, and in white are proteins that are not required for the indicated process.