Role of *psr-1* C-terminal domain in mediating apoptotic cell clearance

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Abstract

Programmed cell death plays a major physiological role in cell elimination in order to maintain cell homeostasis. Cell engulfment and clearance are important final steps of programmed cell death. Apoptotic cell clearance in *C.elegans* is triggered by alteration in phospholipid asymmetry. Phosphatidylserine (PS), which exists in the inner leaflet of plasma membrane in dormant cells, is externalized onto the outer leaflet during apoptosis and signals phagocytic cells for engulfment. PSR-1(PS Receptor) in *C.elegans* has been shown as a protein that preferentially binds to PS. PSR-1 was first discovered as a transmembrane protein on phagocytes that engulf apoptotic cells; with the N-terminal region in the cytosol and the C-terminal region in the extracellular space. This implies that the primary function of detecting externalized PS might be performed by the C-terminal portion of the protein. Therefore, single and double mutants of *psr-1* C-terminal domain were generated with *ced-1*, *ced-8* and *acced-8(sm351)*. The C-terminal region of *psr-1* was found to play an important role in maintaining a regulated cell clearance mechanism. Both versions of *psr-1* C-terminal region mutants exhibited an increased cell clearance phenotype, with lower cell corpse numbers compared to the wild type strain. Depending on the length of deletion at C-terminus which includes NLS sequence, the two *psr-1* mutants show different number of somatic cell corpse in each embryonic stage when *ced-8* was activated (acCED-8), while they do not show significant difference without acCED-8.While *acced-8(sm351)* mutant alone exhibited N2-like phenotype, double mutants of *psr-1* with *acced-8(sm351)* exhibited significantly different phenotype when compared to one another. This observation strongly indicates that PSR-1 C-terminal region is critical for cell corpse clearance in coordination with acCED-8.

Chapter 1

Introduction

Apoptosis

Cell death mainly occurs by two processes; namely necrosis and apoptosis. Necrosis is a process in which intracellular proteases and lysozymes are released into the cellular environment by the dying cell to induce inflammatory response and is thus engulfed by macrophages[1]. Meanwhile, apoptosis is a process in which damaged or old cells are removed without affecting surrounding cells. Apoptosis is the major physiological mechanism of cell elimination[1]. Based on studies done on liver atrophy cells to understand how organs die during development and in result of injuries, it was soon understood that cell populations are regulated by cell proliferation and programmed cell death[3].

Apoptosis plays a major role in development and cell homeostasis. This physiological phenomenon is used in removal of excess cells during embryogenesis, tissue remodeling, interdigital spacing and elimination of cells with genotoxic damage [1]. Cells undergo a plethora of morphological changes during apoptosis. This includes chromatin condensation and nuclear fragmentation followed by rounding of the cell and decreased cell volume [8]. The plasma membrane of the dying cell typically stays intact throughout the process. Towards the end of apoptosis, membrane blebbing or puncture of plasma membrane could occur, by when phagocytosis would have usually taken place [8]. Apoptotic cells that do not get engulfed undergo degradation similar to that in the necrotic pathway [8].
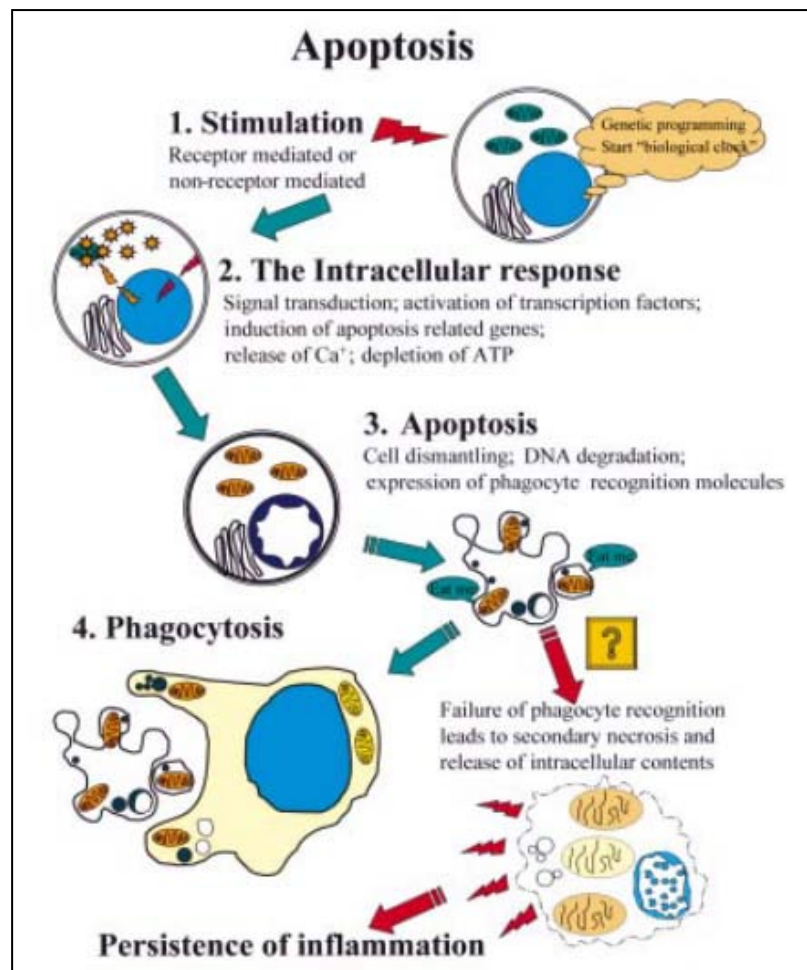
Figure 1: From *Afford and Randhawa* (2000). The process of apoptosis takes place through a few steps; which starts by instructing a cell to undergo apoptosis. The process continues with expression of necessary genes and DNA degradation, finally leading up to phagocytosis by a macrophage or a neighboring cell.

Some developmental cell deaths in mammals are autonomous, where signals for apoptosis are not needed from neighboring cells [4]. However, signals for survival are often provided instead to prevent apoptosis [4]. This is constant with how cell death occurs in a cell-autonomous manner in a nematode worm called *Caenorhabditis elegans* [5] in which most work on apoptotic gene regulation have been done [3]. The Ced (cell death abnormal) genes of *C.elegans* have been extensively cloned and studied in terms of their functions on apoptosis [3]. *C. elegans* hermaphrodites have a fixed final number of 959 cells, after 131 cells of 1090

cells undergo programmed cell death during development [6]. As programmed cell death is not necessary for *C.elegans* survival, it is possible to study the mechanism without disrupting the normal physiological conditions of the model animal [5]. Apoptosis in *C.elegans* takes place through three separate phases; namely the specification phase, killing phase and execution phase [5]. In the specification phase, a cell receives instructions to undergo apoptosis while in the killing phase; the cell death program is activated in the cell. Finally, in the execution phase, the cell is disassembled and is phagocytosed by its neighboring cells [5]. It is common for some dead cells to go unengulfed and these cells are called cell corpses. Cell corpses have a button-like morphology under a DIC field.
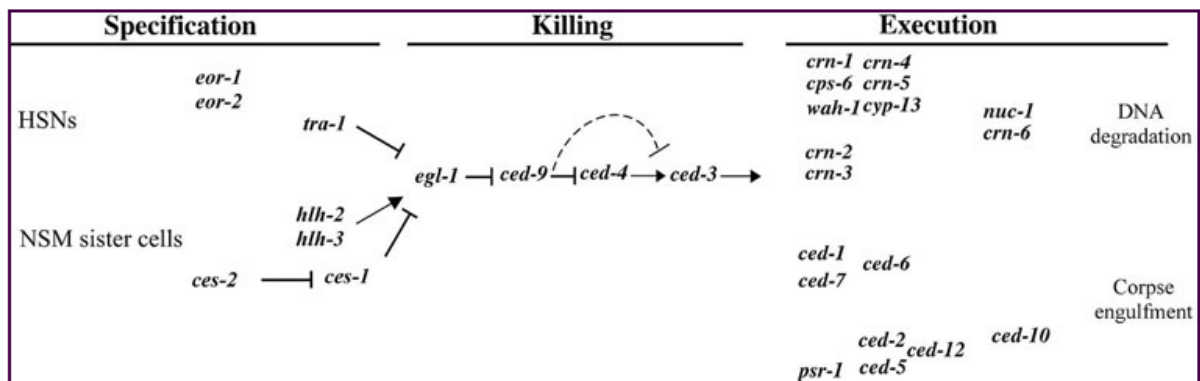

Figure 2: From *Conradt and Xue* (2005). This figure outlines the genetic pathway of the three steps in programmed cell death in *C.elegans*. Mutations in genes involved in the final phase lead to accumulation of unengulfed dead cells; which are referred to as cell corpses.

Defects in apoptosis have been attributed to a number of human diseases. A high frequency of apoptosis has been shown to lead to neuronal diseases such as Parkinson's, Alzheimer's and spinal muscular atrophy while a low frequency of apoptosis has been shown to lead to cancers and autoimmune diseases like diabetes [2]. Experiments on pro-apoptotic genes in the apoptotic pathway have shown their ability to act as tumor suppressors [4]. Most cancers exhibit a defect in pathways that controls cell proliferation in normal cells either by upregulating cell proliferation or inhibition of apoptosis [7]. Apoptosis can be used as an option

to reverse uncontrolled cell proliferation in diseases like cancer. However, before therapeutic measures could be taken against cancer, the process and mechanism of apoptosis has to first be well studied.

Phospholipid asymmetry and its importance in cell corpse engulfment

Phospholipids serve important function in plasma membrane where they create a semi-permeable membrane that separates cells from their external environment. A common characteristics shared by all eukaryotic organisms is their possession of non-random lipid arrangement on plasma membrane, also known as lipid asymmetry. Lipid asymmetry can be altered for crucial cellular processes such as cell fusion and identification and removal of apoptotic cells by phagocytes [9].

The typical phospholipid constituents of plasma membrane are phosphatidylcholine and sphingomyelin on the exoplasmic leaflet while phosphatidylserine, phosphatidylethanolamine and aminophospholipid are maintained on the cytoplasmic leaflet[9]. Asymmetrical distribution of phospholipids is regulated by proteins by either maintaining them on a leaflet or by translocating them[10]. The translocation event involves phospholipid 'scrambling' from one leaflet of plasma membrane to the other; which is usually regulated by specific molecules such as flippases, floppases and scramblases [10]. Flippases move phospholipids to the inner leaflet while floppases do the opposite. Scramblases on the other hand mediate transbilayer migrations in energy-independent manner [10]. It was soon discovered that the asymmetry is a result of ATP-dependent translocation of phospholipids known as phosphatidylserine (PS) and phosphatidylethanolamine (PE) between the leaflets, asserting that maintenance of lipid asymmetry is of utmost importance to cells[11].

Phospholipid asymmetry has very important roles in maintaining cellular processes. One such process is the engulfment of apoptotic cells. Apoptotic cells have to be cleared as a

measure to regulate immune responses and this involves externalization of phosphatidylserine from the cytoplasmic leaflet which alters lipid asymmetry and serves as a signal to activate phagocytosis by allowing interactions between apoptotic cells and phagocytic cells.

Importance of phosphatidylserine (PS) in apoptosis and cell clearance

Phosphatidylserine (PS) is a relatively minor component among phospholipids, making up about 10% of total phospholipids [12]. This type of phospholipid is typically maintained in the inner leaflet membrane in dormant cells. However, during apoptosis, PS is exposed to the outer membrane of the dying cell and serves as a trigger to signal neighboring cells for phagocytosis [12, 13, 14, 15]. Interestingly, this process of PS externalization is well conserved and has been studied in various organisms [13]. A phosphatidylserine receptor (PSR) was identified in the recent times[15]. This receptor was shown to preferentially bind to exposed phosphatidylserine on apoptotic cells through an ELISA done with recombinant PSR-1[15]. PSR was first discovered as a transmembrane protein on phagocytes that engulf apoptotic cells[13]. The human homolog of this receptor protein is known as PSR while the *C.elegans* homolog is called PSR-1. A number of studies have shown the role of PSR-1 in cell engulfment through knockdown of the gene[13]. However there were also studies which reported otherwise. Inconsistencies in these studies clearly represent the need to further analyze this gene to better understand its function in apoptotic cell clearance.

PSR-1 is a protein with a molecular weight of 46.6 kD and 400 amino acids. It has a 72% sequence alignment with human PSR gene[15]. The protein PSR-1 has been found to oligomerize and aggregate upon detecting externalized PS on an apoptotic cell[13]. Besides, the membranous orientation of this transmembrane protein has also been predicted; with the N-terminal region in the cytosol and its C-terminal region in the extracellular space[13]. This

evidence suggested that the primary function of detecting externalized PS might be performed by the C-terminal portion of the protein that stays on the extracellular space.

A few mutants of the *psr-1* gene in *C.elegans* were generated to better study the function and mechanism of function of the protein. The first mutant generated was *psr-1(tm469)*, which is a 968 base-pair deletion of the gene after the first 14 amino acids[15]. This mutant was generated through UV mutagenesis and the right strain was identified using nested primers for PCR screening[15]. A double mutant of this strain with *ced-1* loss of function was built to understand the effects of *psr-1* deletion towards cell corpse engulfment[15]. CED-1 is a part of the apoptotic pathway and functions in commencing a signaling pathway in phagocytic cells to engulf apoptotic cells[16]. Loss of function mutation in *ced-1(e1735)* leads to increased number of cell corpses due to poor engulfment process[16]. The double mutant, *ced-1(e1735);psr-1(tm469)* showed a significantly higher cell corpse number compared to *ced-1* loss of function mutants[15]. This result showed that the deletion of *psr-1* did have an effect on apoptotic cell engulfment pathway. However, it was not sufficient to understand which specific region of the gene was necessary for its function as well as how the protein localizes in a *C.elegans* embryo.

 Background

In order to better understand the importance of *psr-1* C-terminal region in apoptotic cell clearance, two new mutant strains were created using the CRISPR/Cas9 protocol. The first mutant strain's *psr-1* gene was truncated after the 349th amino acid (addressed as *psr-1(1-349)*) while the other mutant's *psr-1* gene was truncated after the 380th amino acid (addressed as *psr-1(1-380)*). Both strains were tagged with knocked-in GFP continuing from the truncated *psr-1* gene. The *psr-1(1-349)* mutant was built as it is known to be the minimal

length of *psr-1* needed to bind well with PS[13] while the *psr-1(1-380)* mutant was built to study how the presence of Nuclear Localization Signal affects the function of this protein.

Mutant 1: *psr-1 (1-349)* GFP KI

amino acid 1-349	GFP
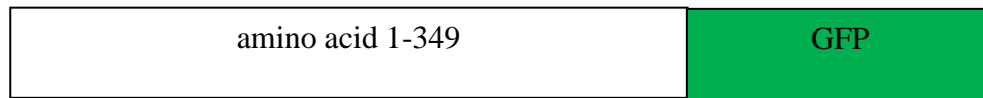
Mutant 2 : *psr-1 (1-380)* GFP KI
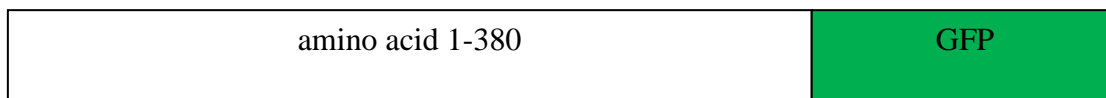
amino acid 1-380	GFP

Figure 3: Illustration of the two *psr-1* mutant strains created and used in this study. Mutant 1 is *psr-1(1-349)* with GFP knock-in (GFP KI) while mutant 2 is *psr-1(1-380)* also with GFP knock-in (GFP KI).

These mutants created with GFP tag were useful to detect how the variation in C-terminal domain of the PSR-1 protein affected its localization in live *C.elegans* embryos. Besides, it was also possible to investigate if the variations in C-terminal domain of either of the mutant and its resulting protein localization have an effect on the protein function. This was done by performing a cell corpse count and comparing it to control strains to find out if the protein function was altered.

Furthermore, to build on the information obtained from the first part of the project, double mutants of *psr-1* and genes that are known to be involved in the apoptotic cell clearance namely; *ced-1(e1735), ced-8(n1891)* and *acced-8(sm351)* were built. These double mutants will be discussed in detail in their specific chapters.

Hypothesis

Based on a preliminary data generated by members of the Xue lab, over expressed *psr-1(1-349)* GFP under heat shock promoter was found to localize in the plasma membrane while over expressed *psr-1(1-380)* GFP under similar conditions was found to localize in the nucleus. This led to a hypothesis regarding the presence of NLS in *psr-1(1-380)* which could have influenced the localization in the nucleus. To gain a better insight, C-terminal mutants with GFP knock-in were generated. We hypothesized that the truncation of the C-terminal region of the *psr-1* gene will demonstrate adverse effects on the cell corpse engulfment process and thus accumulate apoptotic cells. Besides, a difference in phenotype between the two mutants was also expected. This assumption was made based on the orientation of C-terminal region of PSR-1 in the extracellular space; which depicts the possibility of this region being an important part of PS recognition.

Chapter 2

Comparison of *psr-1(1-349)GFP KI* and *psr-1(1-380)GFP KI*

The strains compared in this experiment contain GFP knock-in and a mutated version of the *psr-1* gene. The C-terminal domains of both strains have been truncated as described in the introduction. Mutant *psr-1(1-380)* contains the Nuclear Localization Signal sequence while *psr-1(1-349)* does not. To better understand how mutations in the *psr-1* gene affect apoptotic cell clearance, cell corpse count of *psr-1(tm469)* was compared to that of these two strains. A higher number of cell corpses indicate a defect in apoptotic cell clearance machinery. The N2 wild type *C.elegans* strain was used as a control for this experiment.

Early embryos from both strains were imaged to visualize the protein localization. Cell corpse number from 4 different embryo stages was quantified as described in the methods section.
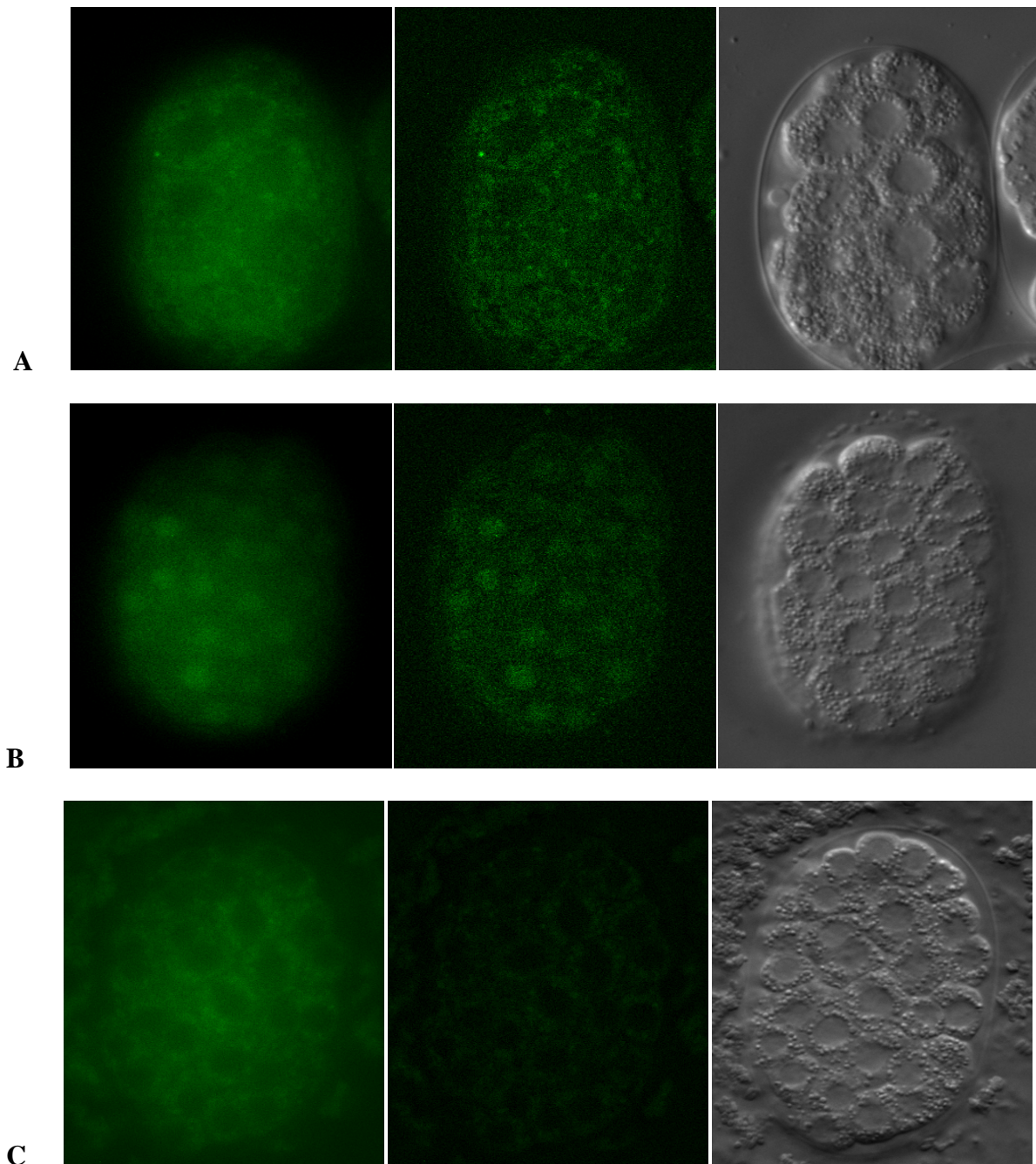
Results and Discussion

Figure 4: Figure 4A is a series of images of *psr-1(1-349)* GFP KI. The first picture in 4A was taken under the FITC channel. The second image is a deconvoluted version of the first to remove background while the third was taken using the Differential Interference Contrast (DIC) channel. Figure 4B is a series of images of *psr-1(1-380)* GFP KI, in the same order as described for 4A while figure 4C is a series of images of N2, also in the same order.

Based on the 4D microscopy images presented in Figure 4, it is evident that PSR-1 localizes in the nucleus in the *psr-1(1-380)* GFP KI mutant. However, in the *psr-1(1-349)* mutant, localization of PSR-1:GFP is unclear although it seems to mostly localize in the cytosol. Ectopically expressed *psr-1(1-349):GFP* has been previously shown to localize in the plasma membrane (data not shown). This difference of localization among the two *psr-1* mutants might have been due to the presence of Nuclear Localization Signal (NLS) sequence in *psr-1(1-380)* GFP KI. This observation led to an inference about the localization of wild type PSR-1. It might be possible that the wild type protein would also localize in the nucleus as it would have the NLS sequence. However, this inference could not be validated due to the unavailability of a wild type PSR-1 GFP knock-in strain.

It is interesting to see how the *psr-1 (1-380)* mutant, which lacks the last 20 amino acid residues in the C-terminal region, localizes in the nucleus (Figure 4). As it has already been shown that PSR-1 is a transmembrane protein[13], it would be safe to hypothesize that the last 20 residues are important for localization in the plasma membrane. These 20 residues could either act as signals to lead PSR-1 to the membrane or have a cleavage site; which, upon cleavage activates PSR-1 when needed, leading to its localization in the plasma membrane.

The *psr-1(1-349)* GFP KI mutant showed PSR-1 localization in the cytosol that looked very similar to the pattern of localization seen in N2 due to auto-fluorescence (Figure 4). This raised a concern regarding the identity of the mutant strain. However, based on the nuclear localization of PSR-1 in *psr-1(1-380)* GFP KI due to the presence of NLS, it is possible that PSR-1 in *psr-1(1-349)* GFP KI localized in the cytosol or in the plasma membrane based on the ectopic expression data as there were no signals to direct it elsewhere.

We expected the cell corpse numbers in both strains to be different when compared to one another and to control strains N2 and *psr-1(tm469).*
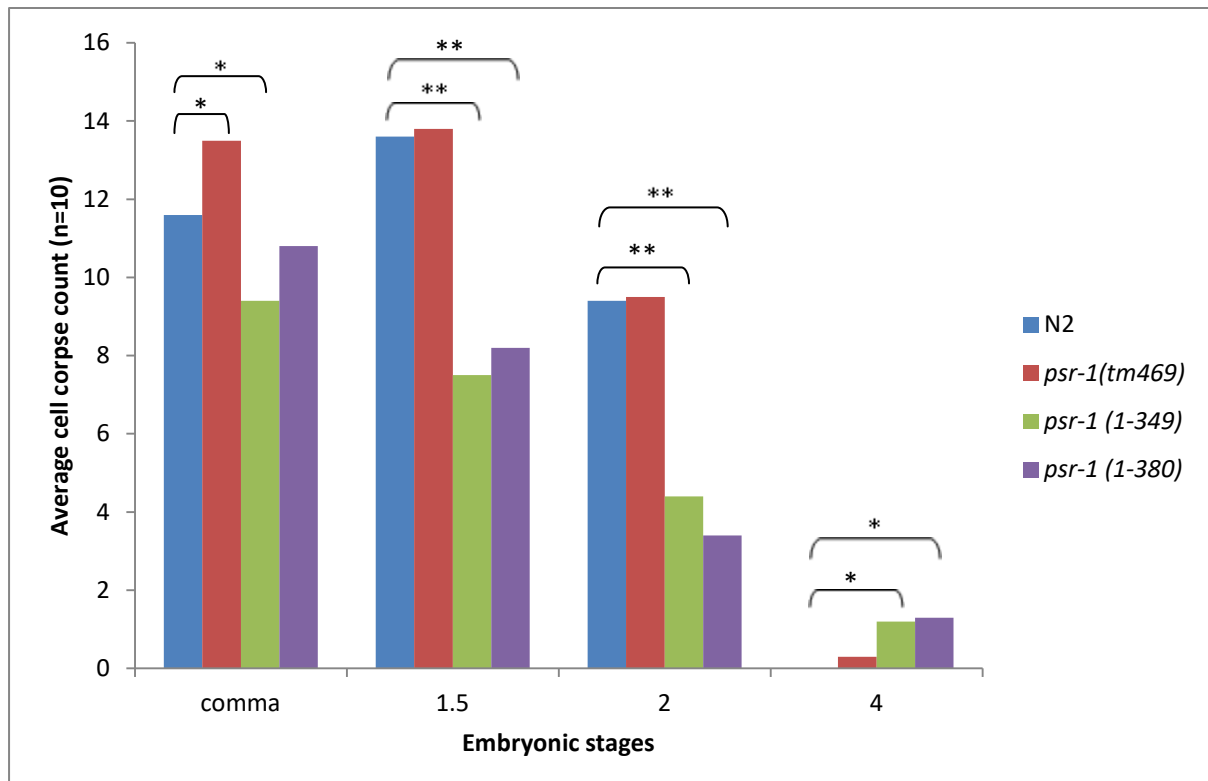

Chart 1: This chart depicts the quantification of cell corpses in four different strains; N2, *psr-1(tm469)*, *psr-1(1-349)* and *psr-1(1-380)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages and were compared to N2 to test the significance in difference. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. *$P < 0.05$ and **$P<0.0001$. All other p-values were >0.05.

As compared to N2, a typical pattern of cell corpse number was seen in all three *psr-1* mutant strains; where the mean number of cell corpses decreases as embryo development proceeds. The cell corpse number in *psr-1(1-349)* and *psr-1(1-380)* was lower than that in N2. The difference in cell corpse numbers between either of the two *psr-1* GFP KI strains and N2 was significant, with p-values less than 0.0001 for embryo stages 1.5-fold and 2-fold.

This might possibly mean that mutations in the C-terminal domain of both of these mutants triggered activation of cell clearance as the cell corpse numbers were lower than that in N2.

The difference in mean number of cell corpses between N2 and *psr-1(tm469)* was significant with a p-value below 0.05 in comma stage only (Chart 1); with a higher number in *psr-1(tm469).* This led us to infer that deletion of almost the entire *psr-1* gene does not cause a defect significant enough in apoptotic cell clearance; indicating the possibility of a redundant gene. The *psr-1* gene in *psr-1(tm469)* consists of sequence that code for only the first 14 amino acids[16].

As a future direction, it would be useful to have a *psr-1* wild type strain with GFP knock-in and a *psr-1(tm469)* strain with a GFP knock-in to enable a complete comparison of PSR-1 localization *in situ*. The visualization of PSR-1 localization in these strains will be useful to understand how wild type copy of *psr-1* and an almost complete deletion of *psr-1* behave and function in cell corpse clearance.

Methods

Strains

Strains used in this experiment were *psr-1(1-349)* GFP KI, *psr-1(1-380)* GFP KI, N2 and *psr-1(tm469).* The first two strains were generated using the CRISPR/Cas9 method by researchers in Tsinghua University. N2 and *psr-1(tm469)* animals were readily available in the Xue lab.

Strain maintenance

C.elegans strains used were maintained using standard methods on NGM plates[17].

Cell corpse quantification

Somatic cell corpses were quantified on embryos of stages comma, 1.5 fold, 2 fold and 4 fold. About 20-40 *C.elegans* embryos were mounted on slides with agar pads in 1-2µL of 25mM NaN_3 and cover slips[14]. Embryos were visualized under Nomarski optics[13] with a 100X magnification and cell corpse number was scored.

4D fluorescence microscopy

C.elegans embryos of 32-cell to 64-cell stages were picked and mounted on slides with agar pads in 1-2µL of 25mM NaN_3 and cover slips[14]. Images were captured in z-series with 1µm per plane for a total of about 20-25 planes using a Zeiss Axioplan 2 microscope with a Cohu CCD camera and Slidebook 5 software[14]. DIC and FITC channels were used, with 10ms exposure for the former and a 2000ms exposure for the latter.

Statistical analysis

The significance of difference between two strains was compared by employing a Student's *t*-test.

Chapter 3

Comparison of *ced-1(e1735);psr-1(1-349)* GFP KI and *ced-1(e1735);psr-1(1-380)* GFP KI

Strains compared in this experiment are partially the same with those compared in Chapter 2; this time with loss of function mutation in the *ced-1(e1735)* gene. This variation of the mutation called *ced-1(e1735)* presents a high number of accumulated cells that have undergone programmed cell death but have not been engulfed[18]. This phenotype allowed for selective observation of how mutation in the *psr-1* C-terminal region affects apoptotic cell engulfment and also to intuitively investigate if the *psr-1* and *ced-1* genes work in the same pathway towards clearing apoptotic cells.

Early embryos from both strains were imaged to visualize the protein localization. Cell corpse number from 4 different embryo stages was quantified as described in the methods section.
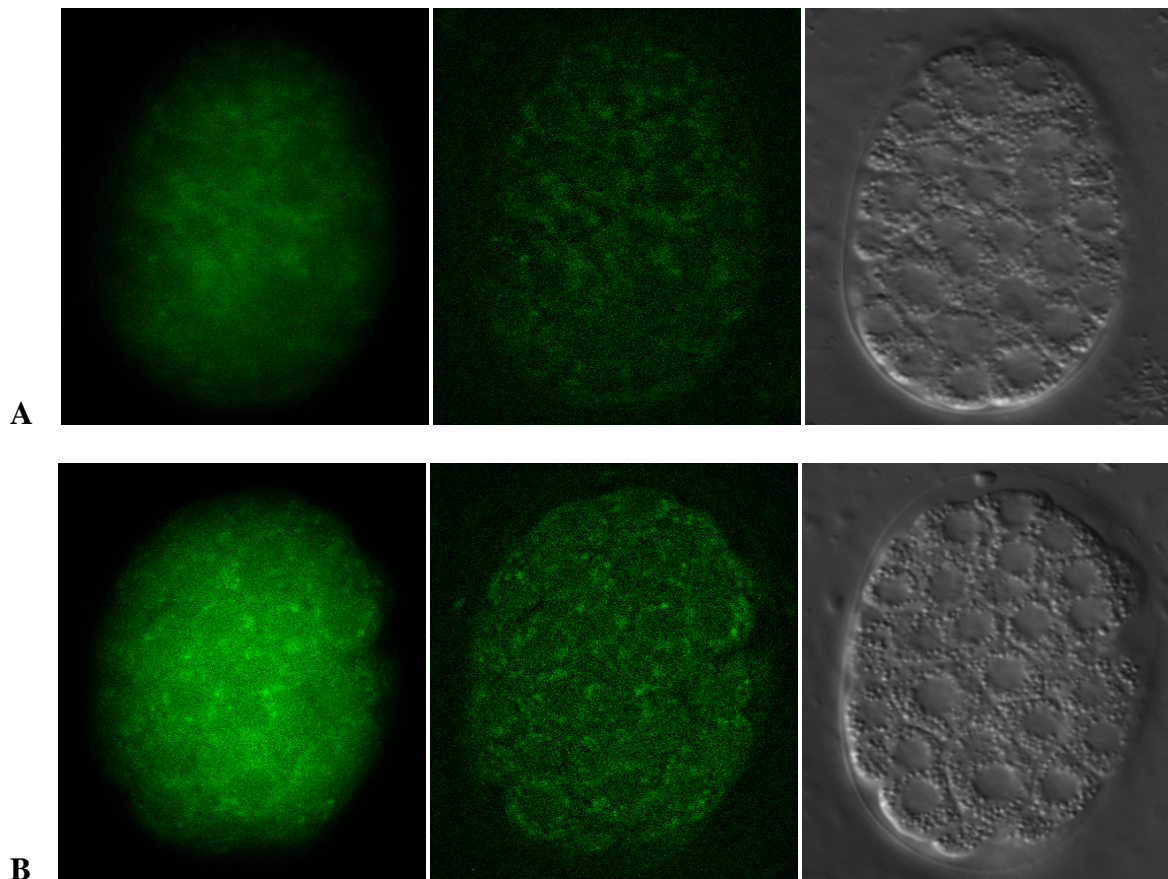
Results and Discussion

Figure 5: Figure 5A is a series of images of *ced-1(e1735);psr-1(1-349)* GFP KI. The first picture in 5A was taken under the FITC channel. The second image is a deconvoluted version of the first to remove background while the third was taken using the DIC channel. Figure 5B is a series of images of *ced-1(e1735);psr-1(1-380)* GFP KI, in the same order as described for 5A.

Fluorescence images depicted in figure 5 show conservation of PSR-1 localization in the double mutant *ced-1(e1735);psr-1(1-349)* GFP KI when compared to the *psr-1(1-349)* GFP KI strain in Figure 4. This shows that the coupling of *psr-1(1-349)* mutant with *ced-1* loss of function mutation does not alter the localization of PSR-1 in that strain.

Meanwhile, the *ced-1(e1735);psr-1(1-380)* strain did have a change of localization. The addition of *ced-1* loss of function mutation led PSR-1 in *psr-1(1-380)* to no longer

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localize in the nucleus (Figure 5B). The genes *ced-1(e1735)* and *psr-1* undeniably work together in identifying apoptotic cells and engulfing them. While PSR-1 in phagocytic cells senses the presence of externalized PS, CED-1 commences a signaling pathway in the same phagocytic cells to engulf apoptotic cells[16]. It is not entirely clear as to why PSR-1 localization would have changed from nucleus to what seems like cytosol. If this was caused by the loss of function in *ced-1(e1735)*, then *ced-1* is probably upstream of *psr-1*, leading to a change in PSR-1 localization. However, the next question would be regarding why PSR-1 would localize in the cytosol and not the plasma membrane since it is a transmembrane protein. Again, a complete comparison with a wild type *psr-1* GFP knock-in is needed to formulate a comprehensive inference.
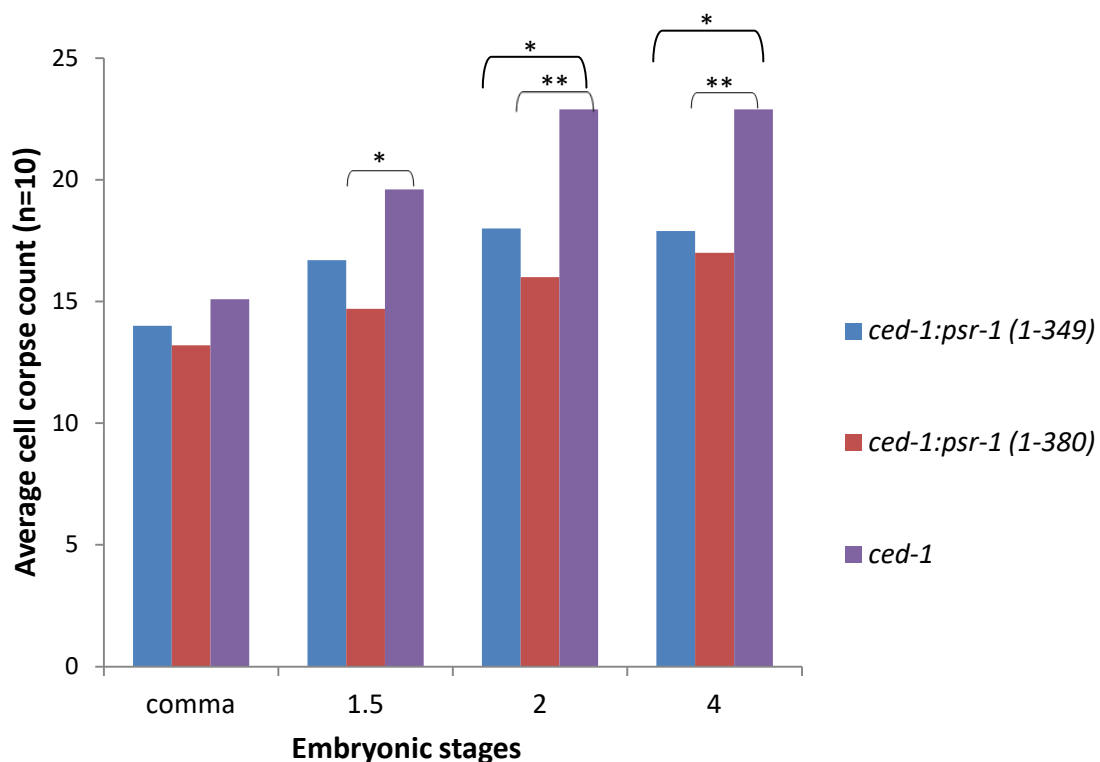

Chart 2: This chart depicts the quantification of cell corpses in three different strains; *ced-1(e1735);psr-1(1-349), ced-1(e1735);psr-1(1-380)* and *ced-1(e1735)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of

mean cell corpse number was done by comparing each double mutant with *ced-1(e1735)* strain. *P<0.05 and **P < 0.0001. All other p-values were >0.05.

With the presence of *ced-1* loss of function mutation, a different pattern of cell corpse number was seen in all three *psr-1* mutant strains. The mean number of cell corpses was high in all embryonic stages and were significantly different from *psr-1* single mutant strains (Figures S1 and S2). This is consistent with what can be seen in the *ced-1(e1735)* strain. The accumulation of cell corpse occurred because all of these mutants have a loss of function in *ced-1*[14]. Surprisingly, the cell corpse count of *ced-1(e1735);psr-1(1-380)* was significantly lower than that of *ced-1(e1735)* in embryonic stages 1.5-fold, 2-fold and 4-fold (Chart 2). We expected *ced-1(e1735);psr-1(1-380)* to have a very high cell corpse number due to the addition of *ced-1* loss of function mutation and the alteration in PSR-1 localization seen in Figure 5B. While *ced-1(e1735);psr-1(1-380)* did have a higher cell corpse number compared to *psr-1(1-380)* (Figure S2), the lower number compared to *ced-1(e1735)* led us to infer that *ced-1(e1735);psr-1(1-380)* did manage to rescue the defect in apoptotic cell clearance caused by *ced-1*.

The cell corpse count of *ced-1(e1735);psr-1(1-349)* in 2-fold and 4-fold embryos (p-value<0.05) was also significantly lower than that of *ced-1(e1735)* although they were higher than cell corpse numbers of *psr-1(1-349)*GFP KI alone (Figure S1). This means that although both of these *psr-1* mutant strains have a deletion in their C-terminal domains, they were able to rescue the *ced-1(e1735)* phenotype. Considering that the *ced-1(e1735)* single mutant has a wild type copy of *psr-1*, this led to an interesting discussion about the importance of *psr-1* C-terminal domain. It is possible that the truncation of C-terminal region in both these mutants could have activated PSR-1 and promoted cell clearing; leading to lower cell corpse numbers (Chart 2).

Methods

<u>Strains</u>

The *psr-1(1-349)* GFP KI and *psr-1(1-380)* GFP KI strains from Tsinghua University were used to build the *ced-1* double mutant strains needed for this experiment. L4 male animals of the *psr-1* mutants were mated with L4 *ced-1(e1735)* hermaphrodite animals in a ratio of 12:3. The resulting F1 progeny was cloned out into separate NGM plates (at least 25). The F1 animals were allowed to lay about 20-30 embryos before performing a genetic screen using PCR with primers specific for *psr-1* and GFP sequences. Upon obtaining a heterozygote animal for *psr-1(1-349)* GFP or *psr-1(1-380)* GFP, F2 progeny from the respective animal were cloned out and screened again for *psr-1* mutant homozygosity and for increased cell corpse number as a phenotype for *ced-1* loss of function mutation. Strain homozygosity for both *psr-1* and *ced-1(e1735)* mutations were checked multiple times to confirm their identity.

<u>Strain maintenance</u>

C.elegans strains used were maintained using standard methods on NGM plates[17].

<u>Cell corpse quantification</u>

Refer to methods in Chapter 2.

<u>4D fluorescence microscopy</u>

Refer to methods in Chapter 2.

<u>Statistical analysis</u>

The significance of difference between two strains was compared by employing the Student's *t*-test.

Chapter 4

Comparison of *psr-1(1-349) GFP KI;ced-8(n1891)* and *psr-1(1-380) GFP KI;ced-8(n1891)*

Strains compared in this experiment are partially the same with those compared in Chapter 2; this time with loss of function mutation in the *ced-8* gene. CED-8 has been found to be important for PS externalization in apoptotic cells[20]. This variation of the mutation called *n1891* presents a delayed appearance of cell corpse[19]. A *ced-8(n1891)* mutant *C.elegans* shows a different pattern of cell corpse number in the developmental stages with L4 animals having the most number of cell corpse compared to comma stage embryos in wild type animals[19]. This phenotype allowed for a selective observation of how mutation in the *psr-1* C-terminal region affects apoptotic cell engulfment and also to intuitively investigate if the *psr-1* and *ced-8* genes work in the same pathway towards clearing apoptotic cells.

Early embryos from both strains were imaged to visualize the protein localization. Cell corpse number from 4 different embryo stages was quantified as described in the methods section.
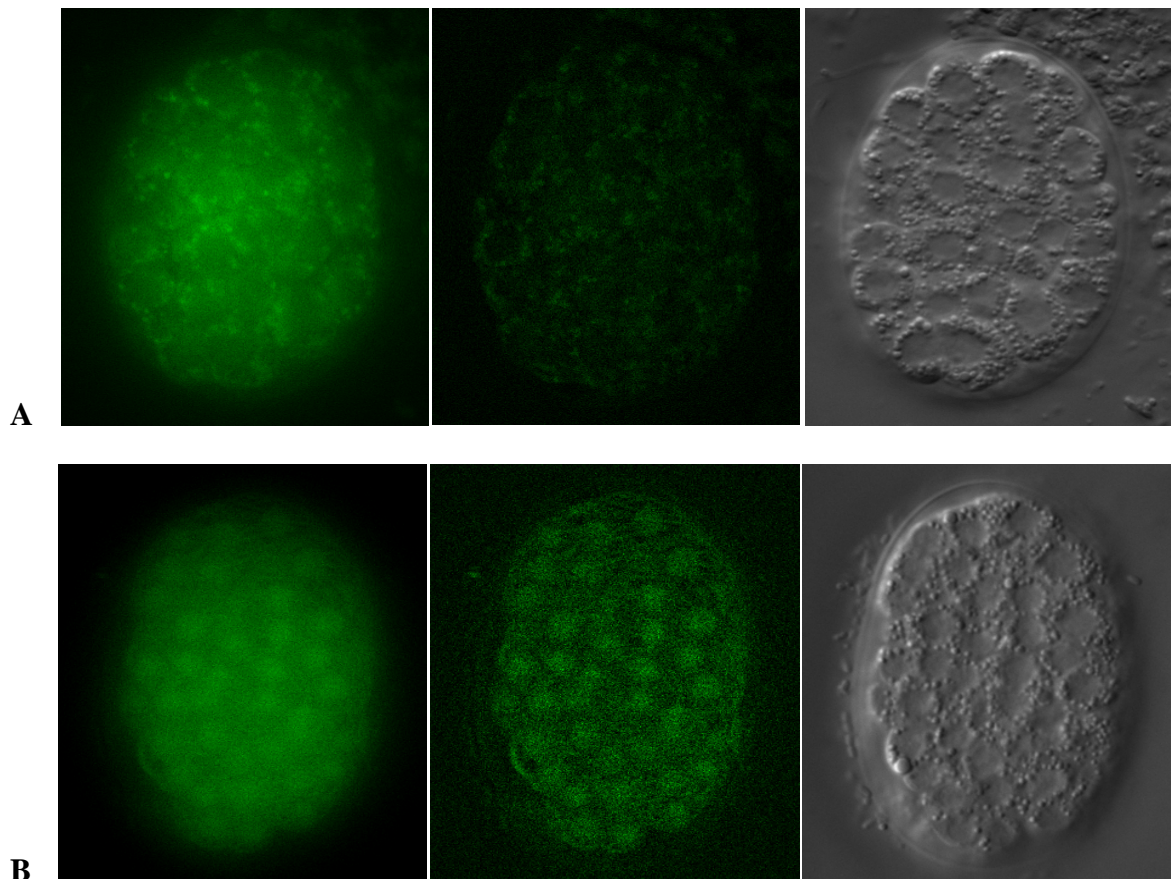
Results and Discussion

Figure 6: Figure 6A is a series of images of *psr-1(1-349) GFP KI;ced-8(n1891)*. The first picture in 6A was taken under the FITC channel. The second image is a deconvoluted version of the first to remove background while the third was taken using the DIC channel. Figure 6B is a series of images of *psr-1(1-380) GFP KI;ced-8(n1891)*, in the same order as described for 6A.

The coupling of *ced-8* loss of function mutation to either of the *psr-1* mutant GFP knock-in strains did not alter the localization of PSR-1. The same pattern seen in *psr-1(1-349)* GFP KI and *psr-1(1-380)* GFP KI was seen again in *ced-8(n1891)* double mutants. This shows that the loss of function in *ced-8* does not affect the localization of PSR-1 in either of these *psr-1* mutant GFP knock-in strains. Thus, we inferred that there might not be a significant difference in cell corpse number between *ced-8(n1891)* and either of the double mutants generated. We were led to this inference because localization of PSR-1 is important

for its function of recognizing PS. Since similar localization is seen in Figure 4 and Figure 6, it was safe to extrapolate that there might not be a significant change in cell corpse number.
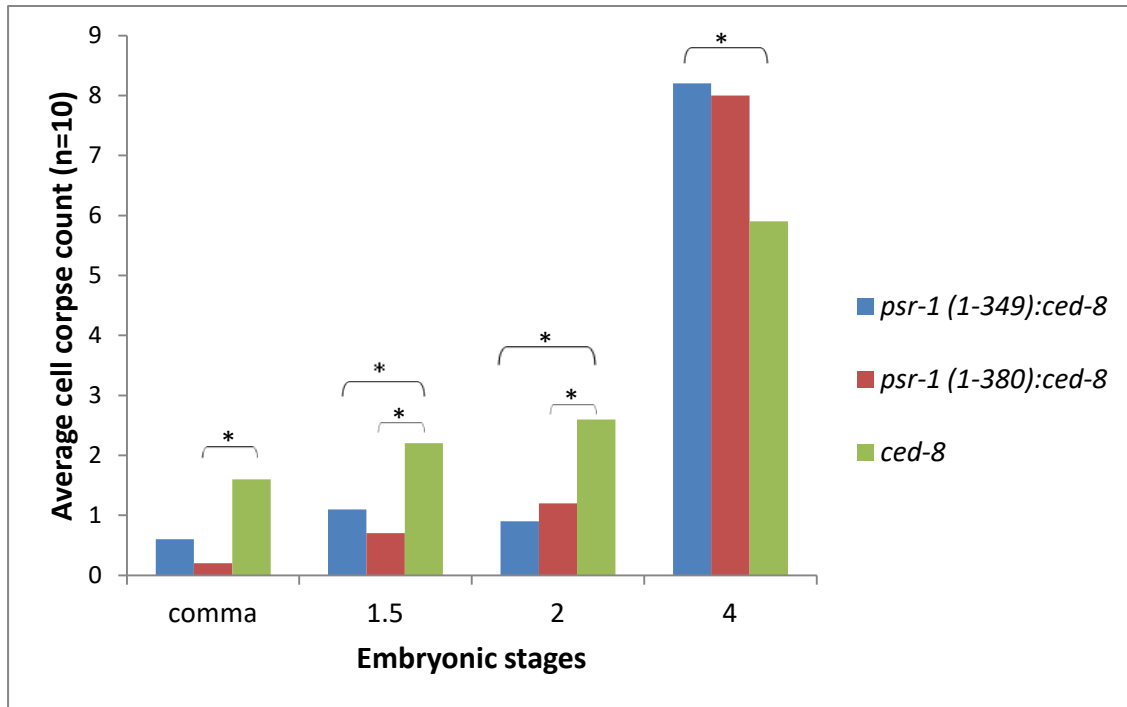

Chart 3: This chart depicts the quantification of cell corpses in three different strains; *psr-1(1-349);ced-8(n1891)*, *psr-1(1-380);ced-8(n1891)* and *ced-8(n1891)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing each double mutant with *ced-8(n1891)* strain. *P<0.05. All other p-values were >0.05.

The presence of *ced-8* loss of function mutation led to an interesting pattern of cell corpse number in both *psr-1* mutant strains. The mean number of cell corpses was low in the early embryonic stages and was high in the late embryonic stage of 4-fold. This was consistent with what is usually seen in a *ced-8(n1891)* strain[20]. In comma, 1.5-fold and 2-fold embryos, the cell corpse count of *psr-1(1-380);ced-8(n1891)* was significantly lower than that of *ced-8(n1891)* with p-value< 0.05 (Chart 3). Meanwhile, the cell corpse count of *psr-1(1-349);ced-8(n1891)* was significantly different than that of *ced-8(n1891)* in 1.5-fold, 2-

fold and 4-fold embryos (p-value<0.05). Although minor significance of difference was seen, it was not sufficient to conclude anything about the role of *ced-8* when coupled with *psr-1* mutants. This was because the changes seen were not consistent with embryonic development. Therefore, the next chapter analyzes how a different variant of CED-8 could possibly interact with *psr-1* mutant strains.

Methods

Strains

The *psr-1(1-349)* GFP KI and *psr-1(1-380)* GFP KI strains from Tsinghua University were used to build the strains needed for this experiment. L4 male animals of the *psr-1* mutants were mated with L4 *ced-8(n1891)* hermaphrodite animals in a ratio of 12:3. The resulting F1 progeny was cloned out into separate NGM plates (at least 25). The F1 animals were allowed to lay about 20-30 embryos before performing a genetic screen using PCR with primers specific for *psr-1* and GFP sequences. Upon obtaining a heterozygote animal for psr-1(1-349) GFP or psr-1(1-380) GFP, F2 progeny from the respective animal were cloned out and screened again for *psr-1* mutant homozygosity. The presence of *ced-8* loss of function mutation was checked by looking for reduced cell corpse number in early embryos and increased cell corpse number in late embryos. Strain homozygosity for both *psr-1* and *ced-8* mutations were checked multiple times to confirm their identity.

Strain maintenance

C.elegans strains used were maintained using standard methods on NGM plates[17].

Cell corpse quantification

Refer to methods in Chapter 2.

4D fluorescence microscopy

Refer to methods in Chapter 2.

Statistical analysis

The significance of difference between two strains was compared by employing the Student's *t*-test.

Chapter 5

Comparison of *psr-1(1-349) GFP KI;acced-8(sm351)* and *psr-1(1-380) GFP KI; acced-8(sm351)*

Strains compared in this experiment are again partially the same with those compared in Chapter 2; this time with a cleavage in the *ced-8* gene which activates it into *acced-8(sm351)*. This variation of the protein called acCED-8 has been shown to be necessary and adequate to carry out the pro-apoptotic roles of CED-8[20].Besides, acCED-8 has also been found to induce ectopic externalization of PS in living cells[20].This characteristic of the activated *ced-8* allowed for selective observation of how mutation in the *psr-1* C-terminal region would interact with the ectopic exposure of PS in living cells. Previous experiments performed by members of the Xue lab have shown that extrachromosomal overexpression of *psr-1(1-349):GFP* driven by heat shock promoter on an extrachromosomal *acced-8(sm351)* background led to PSR-1 localization in the plasma membrane and very high cell corpse numbers. This was the finding that served as a foundation for this experiment. We wanted to know if similar observations will be seen in a knock-in mutant strain.

Early embryos from both strains were imaged to visualize the protein localization. Cell corpse number from 4 different embryo stages was quantified as described in the methods section.
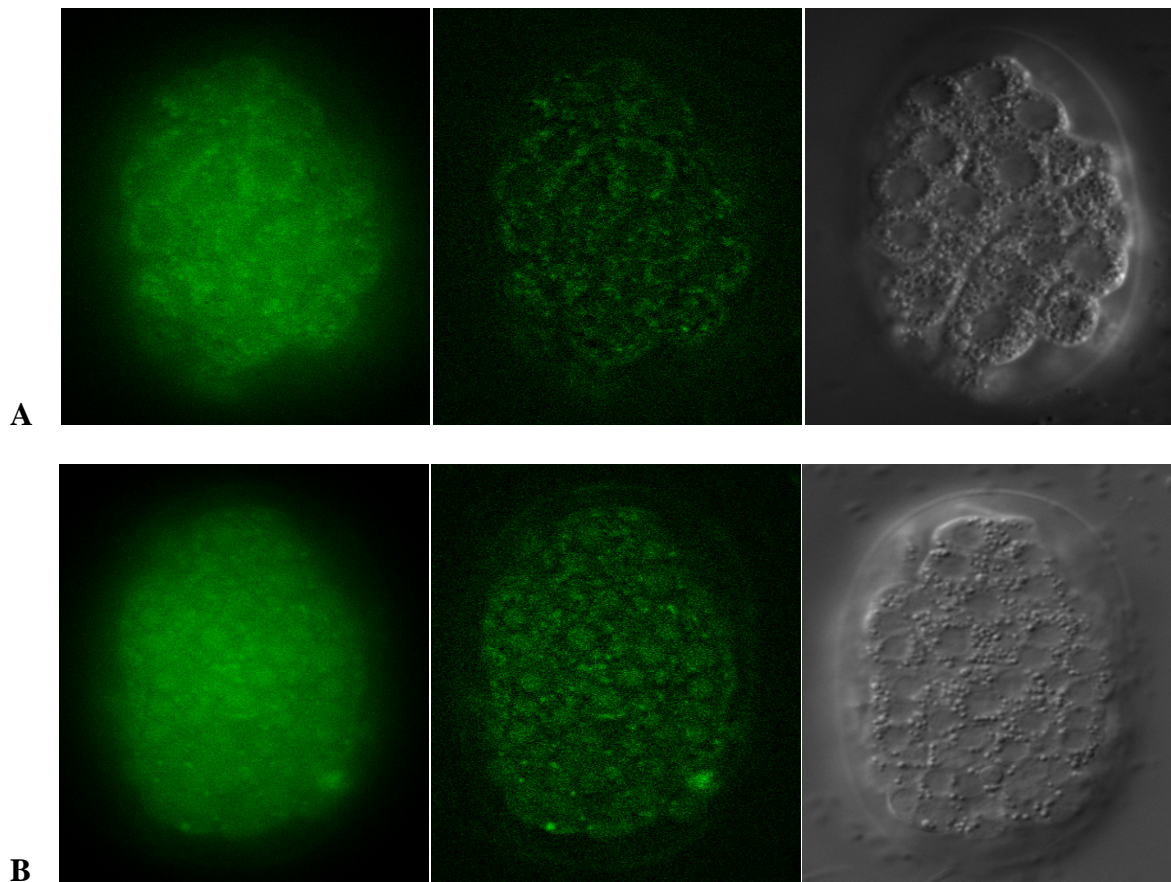
Results and Discussion

Figure 7: Figure 7A is a series of images of *psr-1(1-349) GFP KI;acced-8(sm351)*. The first picture in 7A was taken under the FITC channel. The second image is a deconvoluted version of the first to remove background while the third was taken using the DIC channel. Figure 7B is a series of images of *psr-1(1-380) GFP KI;acced-8(sm351),* in the same order as described for 7A.

    While no difference was seen in the *psr-1(1-349);acced-8(sm351)* double mutant localization, an interesting result was seen in *psr-1(1-380) GFP KI;acced-8(sm351).* Although not entirely clear, PSR-1 localization could be seen in both nucleus and plasma membrane of the embryo; with the cytosol being negative for the fluorescence. Due to acCED-8's role in inducing ectopic externalization of PS in living cells[20], it is understandable why considerable amount of PSR-1 localized in the plasma membrane. This figure showed a surprising result that although *psr-1(1-380)* lacked the last 20 residues, some of the PSR-1

molecules were still able to localize in the plasma membrane as a result of ectopic PS externalization. However, this was also puzzling as to why PSR-1 molecules in one cell would localize in different regions. It is possible that not all PSR-1get employed to the plasma membrane as a response to externalized PS. Based on this image and previous results from extrachromosomal overexpression of *psr-1(1-349):GFP* and *acced-8(sm351)*, we expected the cell corpse numbers to be higher in *psr-1(1-380);acced-8(sm351)* and otherwise in *psr-1(1-349);acced-8(sm351).*
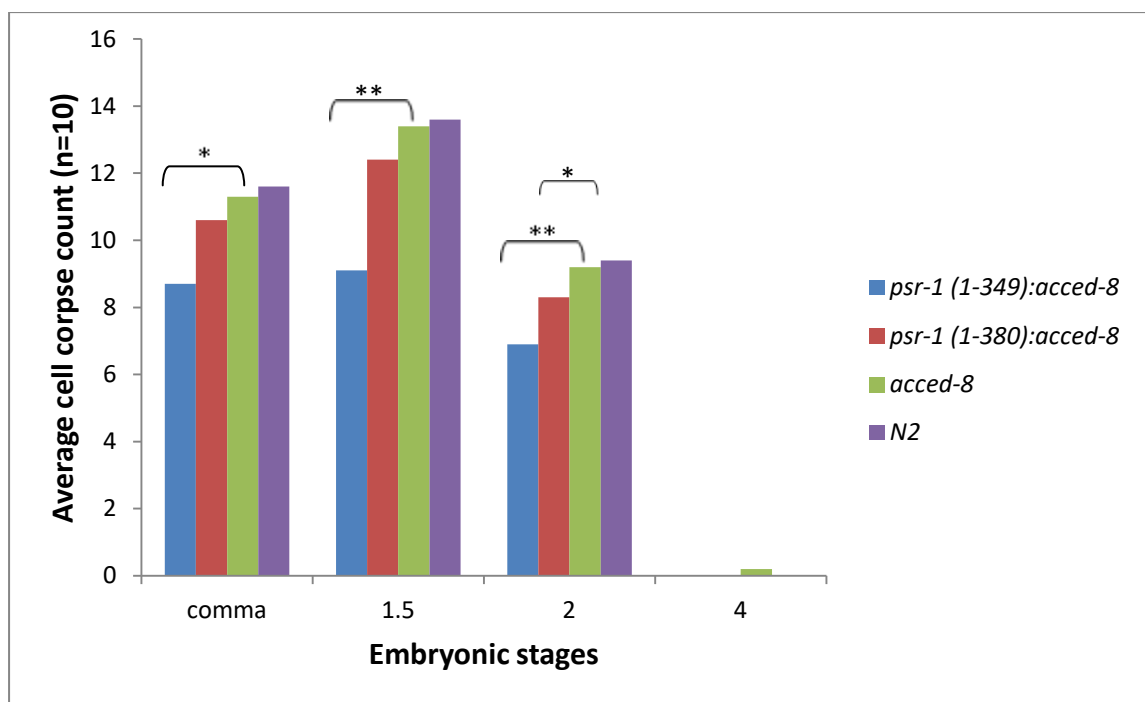
Chart 4: This chart depicts the quantification of cell corpses in four different strains; *psr-1(1-349);acced-8(sm351)*, *psr-1(1-380);acced-8(sm351)*, *acced-8(sm351)* and N2. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing each double mutant with the *acced-8 (sm351)* strain. *P<0.05 and **P<0.0001. All other p-values were >0.05.

For both *psr-1(1-349)* and *psr-1(1-380),* addition of *acced-8(sm351)* variably affected their cell corpse numbers, with no significance difference when compared to the single *psr-1*

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mutant strains (Figures S3 and S4). The cell corpse number of *psr-1(1-380);acced-8 (sm351)* was significantly lower than *acced-8(sm351)* only in 2-fold embryos. Meanwhile, cell corpse number of *psr-1(1-349);acced-8(sm351)* was significantly lower than *acced-8(sm351)* in comma, 1.5-fold and 2-fold stage embryos.

This finding depicts that *psr-1(1-349);acced-8(sm351)* is more active than *psr-1(1-380)* in terms of cell clearance, but only when it is coupled with *acced-8(sm351)*. As shown in Chart 1 and Chart 4, *psr-1(1-349)* GFP KI alone and *acced-8(sm351)* alone do not exhibit any significant difference phenotypically. However, the generation of their double mutant has shown to be effective in clearing cell corpses with numbers lower than N2. It is possible that *psr-1(1-349);acced-8(sm351)* might be recognizing ectopic PS due to acCED-8. We propose that *psr-1(1-349);acced-8(sm351)* is more effective at clearing cell corpses than *psr-1(1-380);acced-8(sm351)* and that residues 350-380 of *psr-1* which contains the NLS might be the reason preventing similar phenotype from being seen in *psr-1(1-380);acced-8(sm351)*.

Methods

Strains

The *psr-1(1-349)* GFP KI and *psr-1(1-380)* GFP KI strains from Tsinghua University were used to build the strains needed for this experiment. L4 male animals of the *psr-1* mutants were mated with L4 *acced-8(sm351)* hermaphrodite animals in a ratio of 12:3. The resulting F1 progeny was cloned out into separate NGM plates (at least 25). The F1 animals were allowed to lay about 20-30 embryos before performing a genetic screen using PCR with primers specific for *psr-1* and GFP sequences and another with primers specific for mutations in *acced-8(sm351)*. Upon obtaining a heterozygote animal for *psr-1(1-349)* GFP or *psr-1(1-380)* GFP, F2 progeny from the respective animal were cloned out and screened again for *psr-1* and *acced-8(sm351)* mutant homozygosity. Strain homozygosity for both *psr-1* and *acced-8(sm351)* mutations were checked multiple times to confirm their identity.

Strain maintenance

C.elegans strains used were maintained using standard methods on NGM plates[17].

Cell corpse quantification

Refer to methods in Chapter 2.

4D fluorescence microscopy

Refer to methods in Chapter 2.

Statistical analysis

The significance of difference between two strains was compared by employing Student's *t*-test.

Conclusion

Based on all of the experiments carried out in this honors thesis project, one obvious finding would be that both *psr-1(349)* GFP KI and *psr-1(1-380)* GFP KI presented an increased cell clearance phenotype. This was evident when the cell corpse numbers were compared to that of N2 and also the control groups in each chapter. Thus, we present that deletion of *psr-1* C-terminal domain in both mutants analyzed in this thesis promoted cell clearing regardless of the mutations in *ced-1* or *ced-8* genes.

Both the *psr-1* double mutants with *ced-1(e1735)* exhibited a rescue of *ced-1(e1735)* phenotype. Interestingly, the different *psr-1* mutants did not exhibit any significant differences among themselves in terms of their cell corpse numbers except when coupled with *acced-8(sm351)* mutant. The double mutant *psr-1(1-349);acced-8(sm351)* had a more effective cell clearing phenotype than *psr-1(1-380);acced-8(sm351)*; indicating that PSR-1 function was activated through the *psr-1(1-349)* truncation and ectopic PS exposure caused by *acced-8(sm351)*. As a future direction, the mechanism by which *acced-8(sm351)* affects *psr-1* function should be studied.

This finding demonstrates that the truncation of *psr-1* C-terminal domain affected the function of PSR-1 favorably; causing it to be activated and leading to lower cell corpse numbers. Another surprising observation was the inconsistency between PSR-1 localization and the cell corpse number quantified. We expected the localization of PSR-1 to be closely related to the cell corpse number; with low numbers when PSR-1 localizes in the plasma membrane. However, this was not the pattern seen in this thesis. To completely test this inference, it would be necessary to build a *psr-1* wild type strain with GFP knock-in. This strain will provide a complete understanding as to how PSR-1 localization affects or does not affect apoptotic cell clearance.

Supplementary figures
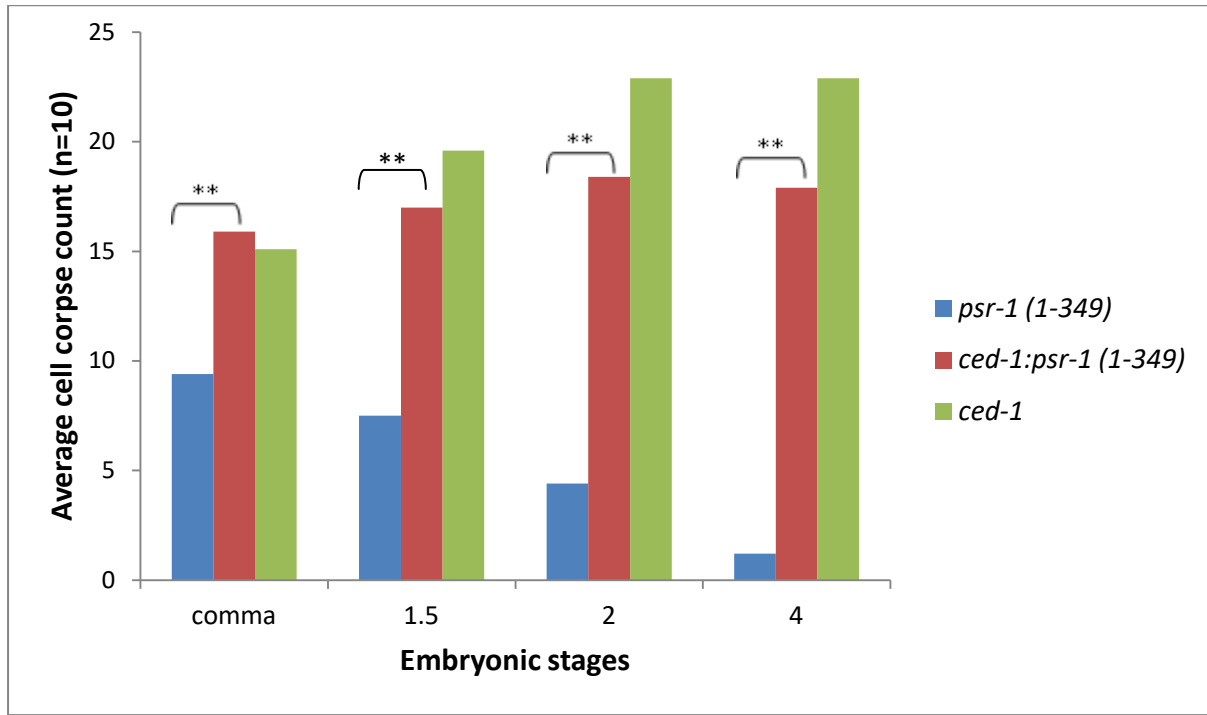

Figure S1: Comparison of cell corpse number between *psr-1(1-349), ced-1(e1735);psr-1(1-349)* and *ced-1(e1735)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing *ced-1(e1735);psr-1(1-349)* with *psr-1(1-349)* strain.**P < 0.0001.
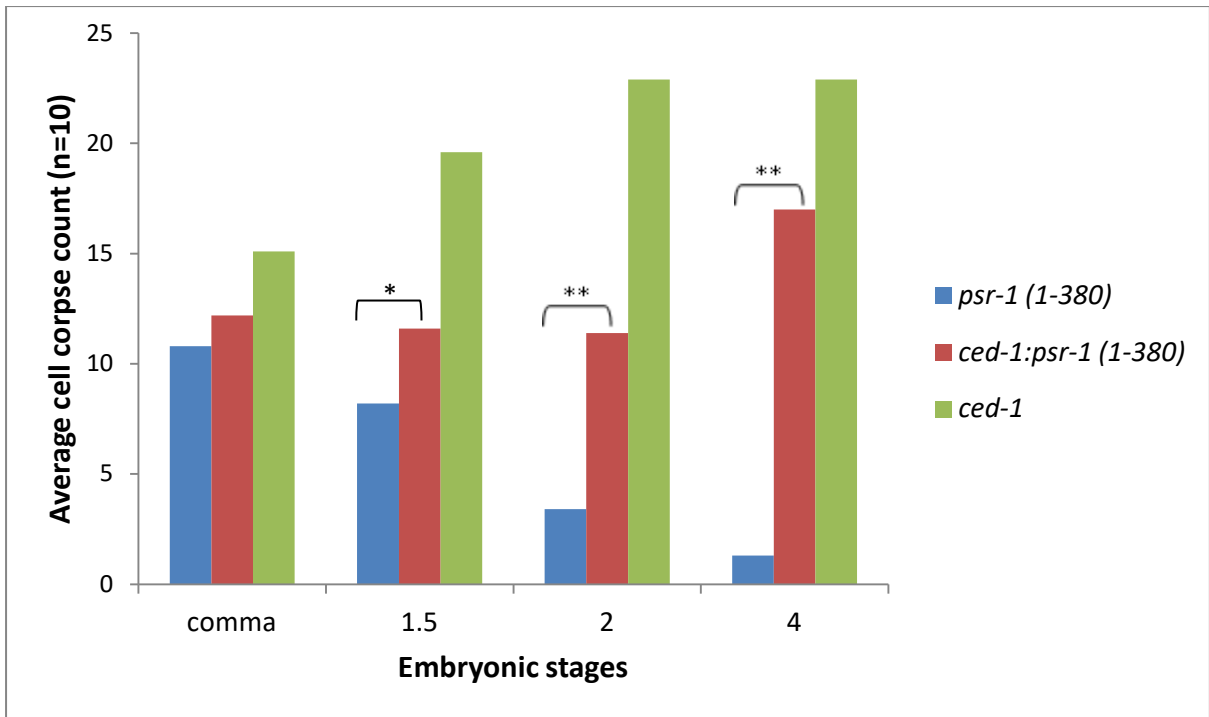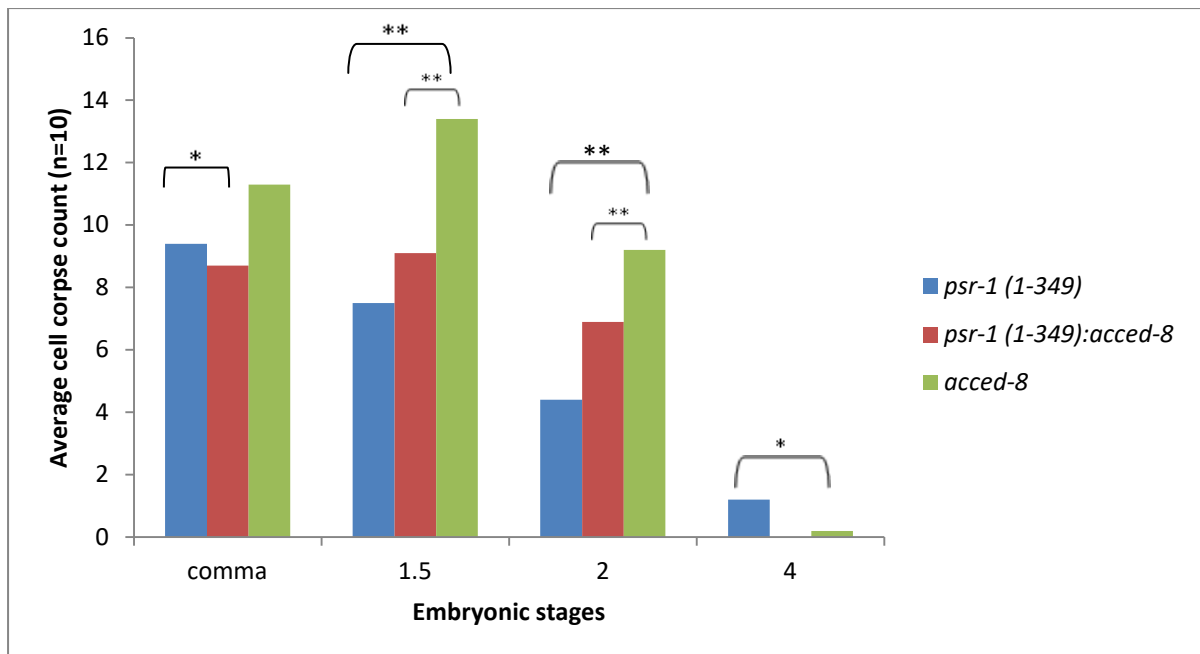
Figure S2: Comparison of cell corpse number between psr-*1(1-380), ced-1(e1735);psr-1(1-380)* and *ced-1(e1735).* Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing *ced-1(e1735);psr-1(1-380)* with *psr-1(1-380)* strain.*P<0.05 and **P < 0.0001. All other p-values were >0.05.

Figure S3: Comparison of cell corpse number between *psr-1(1-349), psr-1(1-349);acced-8(sm351)* and *acced-8(sm351)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing *psr-1(1-349);acced-8(sm351)* with *acced-8(sm351)* strain.*P<0.05 and **P < 0.0001. All other p-values were >0.05.
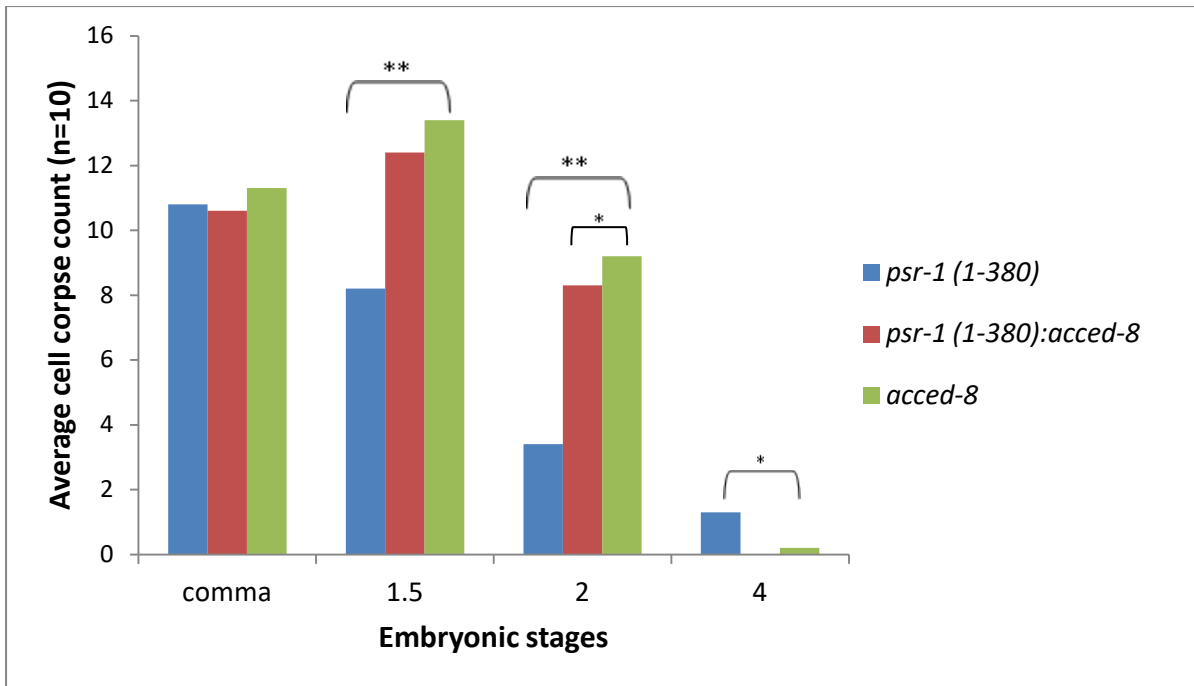
Figure S4: Comparison of cell corpse number between *psr-1(1-380), psr-1(1-380);acced-8(sm351)* and *acced-8(sm351)*. Cell corpses were scored at comma, 1.5-fold, 2-fold and 4-fold stages. The y-axis represents the mean number of cell corpses observed at each stage of embryo development. Statistical significance of mean cell corpse number was done by comparing *psr-1(1-380);acced-8(sm351)* with *acced-8(sm351)* strain.*P<0.05 and **P < 0.0001. All other p-values were >0.05.

<u>Acknowledgements</u>

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