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C. elegans transthyretin-like protein TTR-52 mediates recognition of apoptotic cells by the CED-1 phagocyte receptor

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Abstract

During apoptosis, dying cells are swiftly removed by phagocytes. How apoptotic cells are recognized by phagocytes is not fully understood. Here we report the identification and characterization of the *C. elegans ttr-52* gene, which is required for efficient cell corpse engulfment and encodes a transthyretin-like protein. The TTR-52 protein is expressed in and secreted from *C. elegans* endoderm and clusters around apoptotic cells. Genetic analysis indicates that TTR-52 acts in the cell corpse engulfment pathway mediated by CED-1, CED-6, and CED-7 and affects clustering of the phagocyte receptor CED-1 around apoptotic cells. Interestingly, TTR-52 recognizes surface exposed phosphatidylserine (PS) *in vivo* and binds to both PS and the extracellular domain of CED-1 *in vitro*. Therefore, TTR-52 is the first bridging molecule identified in *C. elegans* that mediates recognition of apoptotic cells by cross-linking the PS "eat me" signal with the phagocyte receptor CED-1.

Phagocytosis and removal of apoptotic cells is an important event in tissue remodeling, suppression of inflammation, and regulation of immune responses[1,2]. During apoptosis, apoptotic cells expose various "eat-me" signals, which are recognized by phagocytes either directly through phagocyte receptors or indirectly through bridging molecules that cross-link

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AUTHOR CONTRIBUTIONS

X.C.W. and W.D.L. performed most of the genetic and cell biological experiments. D.F.Z. performed both PS-binding experiments and *in vitro* protein interaction assays. Y.S. performed immunoprecipitation experiments in *C. elegans*. B.L., B.H.C., P.F.G., and X.G. performed some of the genetic and cell biological experiments. H.W.Y. performed the initial *in vitro* PS binding experiments and E. P. did bioinformatic analysis of TTR family proteins. Z.H.S., E.K.N. and S.M. contributed to the generation of strains. X.C.W. and D.X. designed the experiments and wrote the paper.

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apoptotic cells to phagocytes[3]. The recognition of "eat-me" signals by phagocytes triggers signaling cascades, leading to internalization and degradation of apoptotic cells by phagocytes[3].

In *C. elegans*, phagocytosis of apoptotic cells is controlled by two partially redundant signaling pathways[4]. In one pathway, several conserved intracellular signaling molecules, CED-2/CrkII, CED-5/DOCK180, and CED-12/ELMO, mediate the activation of the small GTPase CED-10/Rac1, leading to cytoskeleton reorganization needed for phagocytosis[5–9]. In the other pathway, three genes, *ced-1*, *ced-6* and *ced-7*, are involved in recognizing and transducing "eat-me" signals. *ced-1* encodes a single-pass transmembrane protein that acts in engulfing cells to promote removal of apoptotic cells[10]. The CED-1::GFP fusion is found to cluster specifically around apoptotic cells[10], indicating that CED-1 plays a role in recognizing apoptotic cells. CED-1 shares sequence similarity with several mammalian cell surface proteins, including Scavenger Receptor from Endothelial Cells, LRP/CD91, and MEGF10 (multiple EGF-like-domains 10), and two *Drosophila* proteins, Draper and Six-microns-under (SIMU), all of which have been implicated in phagocytosis of apoptotic cells[10–15]. Some, like CED-1, are involved in recognition of apoptotic cells[14,16]. MEGF10 can partially substitute for the function of CED-1 in *C. elegans*[12]. Therefore, CED-1 defines a conserved family of phagocyte receptors important for recognition and removal of apoptotic cells.

How CED-1 family proteins recognize apoptotic cells is not clear. One potential signal recognized by CED-1 is phosphatidylserine (PS) exposed on the surface of apoptotic cells, which has been shown to be a conserved "eat-me" signal[17,18]. Indeed, PS is detected on the surface of most *C. elegans* apoptotic cells and found to be important for cell corpse engulfment[19–22]. In animals lacking TAT-1, an aminophospholipid translocase that maintains plasma membrane PS asymmetry, PS is ectopically exposed on the surface of normal cells, which triggers removal of normally cells in a CED-1-dependent manner[22]. Therefore, CED-1 may recognize and mediate removal of cells with surface exposed PS. However, CED-1 or its homologues are not known to bind PS directly and may recognize PS through an intermediate molecule.

Here we report the identification of a secreted protein, TTR-52, that binds surface exposed PS on the apoptotic cell and the CED-1 receptor and acts as a bridging molecule to mediate recognition and engulfment of apoptotic cells by the CED-1 bearing phagocytes.

RESULTS

A new mutant defective in cell corpse engulfment

In a genetic screen for mutations that enhance the weak engulfment defect of the *psr-1(tm469)* mutant (see Methods), which lacks the PS-recognizing PSR-1 receptor[23], we isolated a recessive mutation (*sm211*) that not only enhances the *psr-1* engulfment defect but also results in increased cell corpses on its own (Fig. 1a, b). In fact, the numbers of cell corpses observed in the *sm211* mutant at all embryonic stages and the L1 larval stage are significantly higher than those of the wild-type or *psr-1(tm469)* animals (Fig. 1a, b).

To determine whether *sm211* animals are defective in cell corpse engulfment, we performed a time-lapse analysis to measure the durations of cell corpses in wild type and *sm211* animals[23]. The majority of cell corpses in wild-type animals persisted from 10 to 40 minutes, with an average duration of 28 minutes (Fig. 1c). In contrast, most cell corpses in *sm211* embryos lasted from 30 to 110 minutes, with an average duration almost twice as long (55 minutes; Fig. 1c), indicating that cell corpse engulfment is compromised. Similar delayed and compromised cell corpse engulfment was observed in the *sm211* mutant in three specific cells (C1, C2, and C3; Fig. 1d), which are programmed to die at the mid-embryonic stage[24]. We also counted the number of nuclei in the anterior pharynx of *sm211* animals (see Methods) and found that they do not have any normally living cells missing or undergoing ectopic apoptosis in this region. Instead, a few cells that normally are programmed to die inappropriately survived in some *sm211* animals (Supplementary Information, Table S1), suggesting that *sm211* actually promotes cell survival. Indeed, *sm211* significantly enhances the cell death defect of the weak *ced-3* or *ced-4* loss-of-function (*lf*) mutants (Table S1), a phenomenon also observed with many engulfment-defective mutations such as *ced-1(lf)* mutations[25],[26]. Taken together, these results indicate that the cell corpse engulfment process is severely compromised in the *sm211* mutant.

ttr-52 acts in the *ced-1* pathway

We analyzed double mutants containing *sm211* and strong *lf* mutations in genes involved in cell corpse engulfment to determine the engulfment pathway in which the gene affected by *sm211* acts. *sm211* specifically enhanced the engulfment defect conferred by mutations in the *ced-2*, *ced-5*, *ced-10* and *ced-12* genes, which act in one pathway, but not that caused by mutations in the *ced-1*, *ced-6* and *ced-7* genes, which act in a different engulfment pathway (Fig. 1e). These results indicate that the gene affected by *sm211* likely functions in the same corpse engulfment pathway as *ced-1*, *ced-6* and *ced-7*.

We mapped *sm211* very close to the *bli-5* gene on Linkage Group III (Fig. 2a; see Methods). Transformation rescue experiments revealed that one cosmid in the mapped region, F11F1, fully rescued the engulfment defect of the *sm211* mutant. Subclones of F11F1 were made and a 3.7 kb Bam HI-Nhe I genomic fragment was capable of rescuing the *sm211* mutant (Fig. 2a). There is only one gene in this region, *ttr-52* (Transthyretin-related family domain), which encodes a 135 amino-acid protein that shares limited sequence similarity to transthyretin (Fig. 2b), a thyroid hormone-binding protein found in the blood of vertebrates[27]. TTR-52 is one of the 57 transthyretin-like proteins in *C. elegans*[28],[29], all of which contains a transthyretin-like domain (PF01060)(Supplementary Information, Fig. S1). The biological functions of this gene family are unknown and most are predicted to encode secretory proteins (Supplementary Information, Table S2). We identified a G to A transition in the *ttr-52* gene from the *sm211* mutant, which results in substitution of Val 43 by Met, a conserved residue among worm TTR proteins and human transthyretin (Fig. 2b and Supplementary Information, Fig. S1). Expression of a full-length *ttr-52* cDNA under the control of several different *C. elegans* gene promoters fully rescued the *sm211* mutant (Fig. 2a), confirming that *ttr-52* is the gene affected by *sm211*.

TTR-52 is a secretory protein that binds apoptotic cells

Protein sequence analysis reveals that TTR-52 contains a secretion signal at its amino-terminus (Fig. 2b). To determine the cellular localization pattern of TTR-52, we expressed a TTR-52 GFP fusion under the control of the *C. elegans* heat-shock promoters (P_{hsp}TTR-52::GFP), which fully rescues the engulfment defect of the *ttr-52(sm211)* mutant (Fig. 2c). Upon heat-hock treatment, TTR-52::GFP was detected almost exclusively on the surface of apoptotic cells, displaying a bright ring-like staining (Fig. 2c and Fig. 3a). In some embryos, weak GFP staining was also observed on the surface of cells adjacent to the dying cells (Supplementary Information, Fig. S2a). Since heat-shock promoters induce global gene expression in *C. elegans* embryos, this unique, restricted TTR-52 localization pattern indicates that TTR-52 may be a secretory protein that binds rather specifically to the surface of apoptotic cells. Expression of a TTR-52::mCHERRY (monomeric Cherry) fusion under the control of the heat-shock promoters or the *ttr-52* promoter resulted in the same staining pattern (Fig. 3b). The staining of TTR-52::mCHERRY or TTR-52::GFP on the surface of dying cells was abolished by a loss-of-function mutation in the *ced-3* gene (*n717*) (Fig. 3d; data not shown), which blocks almost all apoptosis in *C. elegans*[30], confirming that the cells labeled by TTR-52 were apoptotic cells.

To confirm that TTR-52 is a secretory protein, we generated two mutant TTR-52::GFP fusions, TTR-52(21-135)::GFP and TTR-52(F11D, F12D)::GFP, and expressed them under the control of heat-shock promoters (Fig. 2c and Supplementary Information, Fig. S3). The first one lacks the predicted secretion signal (amino acids 1-20) and the latter contains mutations altering two hydrophobic residues in the signal peptide predicted to be critical for the secretion of the protein (SignalP 3.0 program, www.cbs.dtu.dk/services/SignalP/). In embryos expressing these two mutant TTR-52::GFP fusions, the surface of apoptotic cells was not labeled by GFP. Instead, diffused GFP was observed in the cytosol and nucleus of both apoptotic and non-apoptotic cells, indicating that they are not secreted (Fig. 3c, e and Supplementary Information, Fig. S4a, b). We observed a similar GFP staining pattern with TTR-52::GFP carrying the V43M mutation found in the *sm211* mutant (Fig. 3f and Supplementary Information, Figs. S3e, S4c). All three TTR-52::GFP fusions failed to rescue the engulfment defect of the *ttr-52(sm211)* mutant (Fig. 2c). Therefore, TTR-52 needs to be secreted to function.

We also tested whether TTR-52 could function properly when tethered to the cell surface. We generated a transmembrane TTR-52::GFP fusion (TTR-52::TM::GFP) by inserting the transmembrane domain of CED-1 between TTR-52 and GFP and expressed this fusion in either engulfing cells or dying cells under the control of the *ced-1* or *egl-1* promoter (Fig. 2c)[10,31]. In embryos transgenic for P_{ced-1}TTR-52::TM::GFP or P_{egl-1}TTR-52::TM::GFP, the GFP fusion was found on the surface of normal cells and dying cells, respectively (Fig. 2c, Supplementary Information, Fig. S2c, and data not shown). However, TTR-52::TM::GFP expressed in engulfing cells did not cluster around apoptotic cells like TTR-52::GFP (Supplementary Information, Fig. S2c), suggesting that membrane tethering affects or interferes with recognition of apoptotic cells by TTR-52. Indeed, neither of the constructs alone nor in combination rescued the engulfment defect of the *ttr-52(sm211)* mutant (Fig. 2c; data not shown). In comparison, expression of TTR-52 under the control of the same

promoters (P_{ced-1}TTR-52 or P_{egl-1}TTR-52) fully rescued the *ttr-52 (sm211)* mutant (Fig. 2a), indicating that the membrane-tethered TTR-52 cannot substitute for a secreted TTR-52.

To examine where *ttr-52* is expressed in *C. elegans*, we generated a *ttr-52* transcriptional fusion with mCHERRY (P_{ttr-52}mCHERRY) and found that the *ttr-52* promoter drove mCHERRY expression specifically in intestine cells, which completely overlapped with the GFP expression pattern of P_{ges-1}GFP, an intestine-specific reporter construct (Fig. 3g)[32]. Therefore, the intestine cells, which do not undergo programmed cell death in *C. elegans*[33],[34], synthesize TTR-52, which likely is secreted, diffuses, and binds to apoptotic cells, promoting their engulfment by neighboring phagocytes. Consistent with this notion, when TTR-52::mCHERRY and GFP were co-expressed under the control of the endogenous *ttr-52* promoter (P_{ttr-52}TTR-52::mCHERRY and P_{ttr-52}GFP), GFP expression was restricted to the gut, whereas TTR-52::mCHERRY was seen mostly outside the gut region, labeling apoptotic cells that either were close to or away from the gut (Supplementary Information, Fig. S2d).

TTR-52 mediates recognition of apoptotic cells by CED-1

ced-1 encodes a phagocyte receptor that clusters around apoptotic cells through an unknown mechanism[10]. The observations that TTR-52, a secreted protein, similarly clusters around apoptotic cells and acts in the same engulfment pathway as CED-1 suggest that TTR-52 may function to mediate recognition of dying cells by CED-1. Indeed, in a strain expressing both TTR-52::mCHERRY (P_{hsp}TTR-52::mCHERRY) and CED-1::GFP (P_{ced-1}CED-1::GFP), TTR-52::mCHERRY frequently co-localized with CED-1::GFP, as 69% of apoptotic cells clustered by CED-1::GFP were also surrounded by TTR-52::mCHERRY (n=183). TTR-52::mCHERRY and CED-1::GFP either formed an overlapping mCHERRY/GFP ring around the apoptotic cell (indicated by an arrow, Fig. 4a) or a mCHERRY/GFP ring inside a larger CED-1::GFP ring, indicative of an internalized apoptotic cell in a phagocyte (indicated by an arrowhead, Fig. 4a). TTR-52::mCHERRY rings were also seen alone (indicated by a blue arrowhead, Fig. 4a) or accompanied by a partial or incomplete CED-1::GFP ring (Fig. 5, b–e), indicating that formation of the TTR-52::mCHERRY ring precedes the formation of CED-1::GFP ring on apoptotic cells. By time-lapse microscopy analysis, we observed that a complete TTR-52::mCHERRY ring was formed rapidly around the dying cell early during apoptosis (indicated by an arrowhead, Fig. 5b), whereas only trace amounts of CED-1::GFP were seen nearby (indicated by an arrow, Fig. 5b). CED-1::GFP continued to circularize (Fig. 5, c–e) and reached a complete circle overlapping with the TTR-52::mCHERRY ring within 30 minutes (Fig. 5f). In 37 apoptotic cells from 8 embryos that we monitored, the TTR-52::mCHERRY ring was always formed prior to the CED-1::GFP ring, indicating that TTR-52 may induce the formation of the CED-1::GFP ring around apoptotic cells.

We thus examined whether loss of *ttr-52* affects clustering of CED-1::GFP around apoptotic cells by analyzing *C. elegans* embryos expressing CED-1::GFP (*smIs34:* P_{ced-1}*ced-1::gfp)*. Approximately 64% of cell corpses were labeled by CED-1::GFP in wild-type 1.5-fold stage embryos. By contrast, in *smIs34; ttr-52(sm211)* 1.5-fold embryos, only half (34%) of the cell corpses were labeled (Fig. 4b), indicating that TTR-52 is important for mediating the

clustering of CED-1 around apoptotic cells. Since clustering of apoptotic cells by TTR-52::mCHERRY was not affected by loss of *ced-1* (Fig. 4c), these results indicate that TTR-52 is independent of and precedes CED-1 in binding to apoptotic cells.

We examined whether TTR-52 directly interacts with CED-1 *in vitro*, using a Glutathione-S-Transferase (GST) fusion protein pull down assay. Recombinant TTR-52 interacted with purified GST-CED-1(Extra), which contains the extracellular domain of CED-1, but not with either GST or GST-CED-1(Intra), which contains the intracellular domain of CED-1 (Fig. 4d). None of these GST fusion proteins bound SYCT (specific Yop chaperone), a control protein, suggesting that TTR-52 interacts specifically with the extracellular domain of CED-1. We also examined the interaction of TTR-52 with CED-1 by co-immunoprecipitation (co-IP) assays using a *C. elegans* strain that co-expressed CED-1::GFP from *smIs34* and TTR-52::FLAG and SUR-5::GFP from a second integrated transgene *smIs118* (carrying both P_{hsp}TTR-52::FLAG and $P_{sur\text{-}5}$SUR-5::GFP)(Fig. 4e, lane 1). Using an antibody to the FLAG epitope, CED-1::GFP but not SUR-5::GFP was specifically co-precipitated with TTR-52::FLAG (Fig. 4e, lanes 2–3). Together, these results indicate that TTR-52 interacts specifically with the CED-1 receptor to mediate recognition and binding of apoptotic cells by CED-1.

TTR-52 recognizes surface-exposed PS

To identify the apoptotic cell signal recognized by TTR-52, we performed a genetic screen to search for mutations that altered the staining of TTR-52::mCHERRY to apoptotic cells. One mutation, *qx30*, resulted in TTR-52::mCHERRY staining of virtually all cells in *qx30* mutant embryos, including non-apoptotic cells that normally are not labeled by TTR-52 (Fig. 6a, b). *qx30* turns out to be an allele of *tat-1* (see Methods), which encodes an aminophospholipid translocase that prevents appearance of PS in the outer leaflet of plasma membrane[22]. Because in *tat-1(lf)* animals PS is ectopically exposed on the surface of many living cells[22], this unexpected finding suggests that TTR-52 may bind surface-exposed PS.

We employed a yeast-based PS binding assay[35] to test the binding of TTR-52 to PS. In this assay, the C2 domain of lactadherin (Lact-C2), which binds specifically to PS[36], associates predominantly with plasma membrane that contains PS in its inner leaflet in wild-type yeast cells (Fig. 6d)[35]. In *cho1* mutant cells that are deficient in PS synthesis, GFP::Lact-C2 becomes cytosolic (Fig. 6e), due to loss of PS in yeast plasma membrane[35]. Like GFP::Lact-C2, TTR-52::mCHERRY labeled plasma membrane in wild-type yeast cells but failed to do so in the *cho1* cells (Fig. 6f, g), indicating that TTR-52 binds PS in plasma membrane.

To identify the region of TTR-52 important for PS binding, we generated several TTR-52 mutants with mutations or small deletions (data not shown). One mutant, TTR-52(M5), in which residues 50-55 were replaced by Alanines, failed to associate with yeast plasma membrane (Fig. 2b; Fig. 6h), presumably due to loss of PS binding. *In vivo*, TTR-52(M5)::mCHERRY failed to rescue the engulfment defect of the *ttr-52(sm211)* mutant and did not cluster around apoptotic cells in wild-type embryos (Fig. 2c, Fig. 6c, and Supplementary Fig. 3i), although it was secreted normally and accumulated in embryo cavity.

We also examined whether TTR-52 directly binds PS and apoptotic cells. Recombinant TTR-52::mCHERRY::FLAG was purified from human 293T cells and tested for binding to a membrane strip spotted with 16 different phospholipids (see Methods). TTR-52 showed strong and specific binding to PS but not to other phospholipids such as PC, PE, PA and various phosphoinositides, with the exception of a weak binding to PtdIns(4)P (Fig. 6i). In contrast, the binding of TTR-52::mCHERRY(M5)::FLAG to PS was barely detectable. Thus, TTR-52 binds specifically to PS *in vitro*.

When we incubated purified TTR-52::mCHERRY::FLAG with dissected gonads from animals treated with *gla-3* RNAi that causes increased germ cell deaths[37], TTR-52::mCHERRY labeled specifically apoptotic germ cells on the surface of the dissected gonad (Fig. 6j)[19]. This TTR-52 labeling was abolished by the *ced-3(n717)* mutation (Fig. 6k), indicating that TTR-52 binds apoptotic germ cells. TTR-52::mCHERRY also stained many germ cells in the *tat-1(qx30)* mutant (Fig. 6l), in which PS is ectopically exposed on the surface of normal germ cells[22]. In contrast, purified TTR-52(M5)::mCHERRY failed to label apoptotic germ cells in *gla-3(RNAi)* animals and normal germ cells in the *tat-1(qx30)* mutant (Fig. 6m, n). Taken together, these results indicate that TTR-52 binds surface exposed PS, and as such, mediates recognition of apoptotic cells by the phagocyte receptor CED-1.

TTR-52 mediates engulfment of cells with surface-exposed PS

One physiological consequence of ectopic PS exposure on the surface of normal cells in *tat-1(lf)* animals is random removal of these cells through a CED-1-dependent phagocytic mechanism[22]. For example, in *bzIs8* animals, six touch-receptor neurons are labeled by GFP expressed from the $P_{mec\text{-}4}$GFP construct carried by the integrated *bzIs8* transgene and none of the *bzIs8* animals lost touch cells (Fig. 7a). By contrast, 15–16% of *tat-1(qx30); bzIs8* or *tat-1(tm1034); bzIs8* animals lost at least one touch cell. This missing cell phenotype was strongly suppressed by the *ced-1(e1735)* mutation (Fig. 7a), suggesting that CED-1 recognizes and mediates removal of cells with surface-exposed PS. Interestingly, the missing cell phenotype of the *tat-1(lf)* mutants was also strongly suppressed by *ttr-52(sm211)* (Fig. 7a), despite being a weaker engulfment-blocking mutation than *ced-1(e1735)*. This result suggests that TTR-52 solely mediates recognition of surface exposed PS by CED-1, which could be the only engulfment signal expressed by touch cells in *tat-1(lf)* animals. Consistent with this finding, TTR-52::mCHERRY labeled the surface of touch cells in *tat-1(tm1034); bzIs8* animals, but not touch cells in *bzIs8* animals (Fig. 7b).

DISCUSSION

How the CED-1 family of phagocyte receptors recognizes apoptotic cells is unknown and is a subject of intense study. In this study, we identify a new gene, *ttr-52*, that encodes a secretory protein and acts specifically in the CED-1 signaling pathway to mediate engulfment of apoptotic cells in *C. elegans*. Interestingly, the secreted TTR-52 protein clusters around apoptotic cells and precedes CED-1 in binding to apoptotic cells *in vivo*. Moreover, TTR-52 is important for efficient binding of CED-1 to apoptotic cells and interacts specifically with the extracellular domain of CED-1. These findings together

provide strong evidence that TTR-52 is a new extracellular bridging molecule that mediates the binding and recognition of apoptotic cells by the phagocyte receptor CED-1.

How does CED-1 or TTR-52 recognize apoptotic cells? We found that TTR-52 binds plasma membrane PS in a yeast-based PS binding assay (Fig. 6f, g) and binds PS specifically *in vitro* (Fig. 6i), indicating that it is a PS-binding protein. Moreover, recombinant TTR-52 labeled specifically apoptotic germ cells and the surface of many germ cells in the *tat-1(lf)* mutant *ex vivo* (Fig. 6j–l), providing direct evidence that TTR-52 recognizes and binds surface exposed PS. A TTR-52 mutant, TTR-52(M5), that fails to bind PS *in vitro* (Fig. 6i), loses its ability to bind apoptotic cells in *C. elegans* and its activity to rescue the engulfment defect of the *ttr-52(sm211)* mutant (Fig. 2 and Fig. 6), indicating that the ability to bind PS is critical for TTR-52's function in phagocytosis. Like CED-1, TTR-52 is required for removing normal cells with inappropriately exposed PS in the *tat-1(lf)* mutants (Fig. 7), which presumably do not express other "eat-me" signals seen on the surface of apoptotic cells[22]. Therefore, TTR-52 most likely recognizes and binds surface exposed PS to mediate cell corpse engulfment. Given that surface exposed PS is the only conserved engulfment signal identified thus far in multiple organsims[18], it may serve as a conserved recognition signal for the CED-1 receptor family.

In mammals, extracellular bridging molecules such as thrombospondin (TSP), β2 glycoprotein I, and the collectin family proteins[38]–[44], some of which recognize and bind surface exposed PS, play an important role in cross-linking apoptotic cells to macrophages, which often are not in close contact with their targets. For invertebrate animals such as *Drosophila* and *C. elegans*, it is unclear whether bridging molecules are needed to mediate removal of apoptotic cells, especially in *C. elegans*, where phagocytes are neighboring cells already in close contact with apoptotic cells. Our finding that TTR-52, an extracellular bridging protein, is important for mediating recognition and binding of apoptotic cells by the CED-1 phagocyte receptor suggests that this is a conserved and important mechanism for clearance of apoptotic cells, although the identities of bridging molecules could differ significantly across the species.

TTR-52 is a member of the transthyretin-like protein family, a subfamily of the larger transthyretin-related protein family (TRPs) that has sequence and structural similarity with transthryretin in the signature transthyretin-like domain and that has been found in a broad range of species, including bacteria, plants, invertebrates, and vertebrates[45],[46]. The functions of TRPs are largely unknown, although some have been implicated in purine catabolism in mice and regulation of the brassinosteroid receptor in plants[45]–[48]. There are 57 transthyretin-like proteins in *C. elegans*, whose biological functions have not been characterized. TTR-52 is the first of this protein family with a clearly defined cellular function. Since many of the nematode transthyretin-like proteins are predicted to be secretory proteins (Supplementary Information, Table S2), it seems likely that one potential important function of this protein family is to act extracellularly to mediate cell-cell interaction, although individual RNAi knockdown of 57 worm transthyretin-like genes, including *ttr-52*, fails to reveal an obvious defect (data not shown). Since *ttr-52(sm211)* only partially blocks the clustering of CED-1 around apoptotic cells and causes a weaker engulfment defect than *ced-1(lf)* mutations, additional bridging molecule(s) and/or "eat-me"

signal(s) could act in parallel to TTR-52 to mediate recognition of apoptotic cells by CED-1. Furthermore, given the presence of multiple PS-recognizing receptors in mammals[49], additional PS-recognizing receptors, including PSR-1[23], could act in parallel to TTR-52/CED-1 in *C. elegans* to mediate removal of apoptotic cells with surface exposed PS.

METHODS

Methods and associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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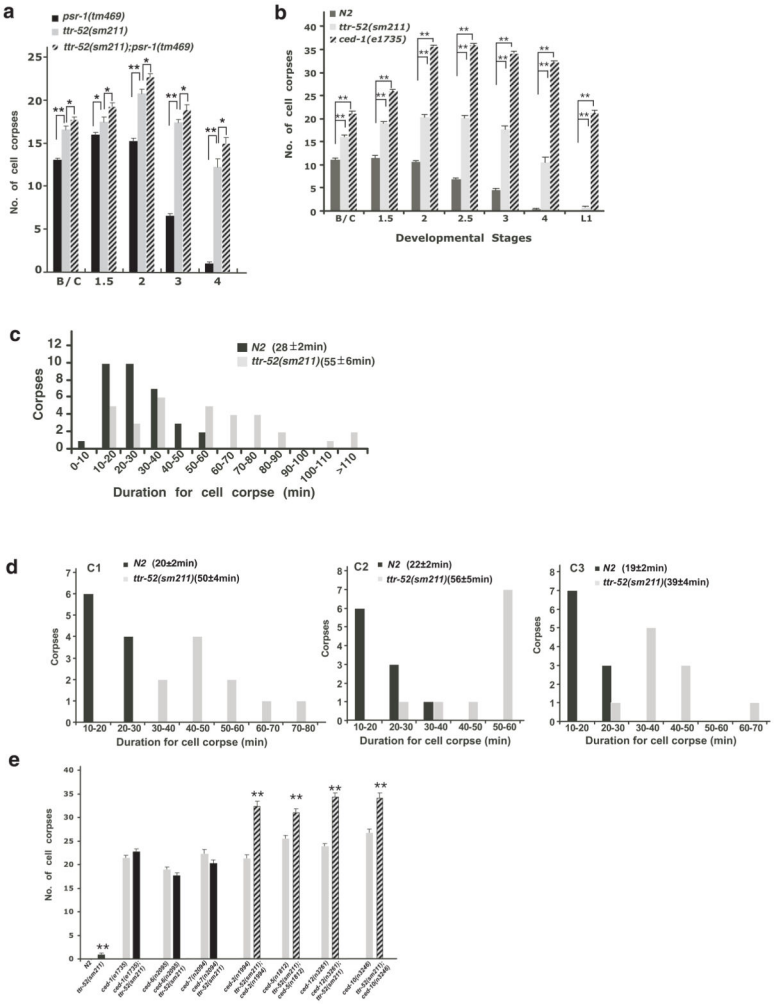


Figure 1. *ttr-52* is important for cell corpse engulfment in *C. elegans*

(**a, b)** Time-course analysis of cell corpses during development. Cell corpses from the indicated strains were scored at six embryonic stages [bean/comma (B/C), 1.5-fold, 2-fold, 2.5 fold, 3 fold, 4-fold] and the early L1 larval stage (L1). The y axis represents the mean number of cell corpses scored at the head region of embryos or L1 larvae (15 animals at each stage). Error bars represent the standard error of mean (SEM). ** $P < 0.0001$, * $P < 0.05$ (see Methods). (**c**) Four-dimensional microscopy analysis of cell corpse durations in the *ttr-52(sm211)* mutant. The durations of 33 cell corpses from wild-type (N2) embryos (n=3, black bars) and 32 cell corpses from *ttr-52(sm211)* embryos (n=3, gray bars) were monitored. The numbers in parentheses indicate the average durations of cell corpses (±SEM). The y-axis indicates the number of cell corpses within a specific duration range as shown on the x-axis. (**d**) Corpse durations of C1, C2 and C3 cells were monitored as described in **c**. 10 corpses each in wild-type and *ttr-52(sm211)* embryos were followed for each cell. (**e)** *ttr-52(sm211)* enhances the engulfment defect of the *ced-2*, *ced-5*, *ced-10*, and *ced-12* mutants. Cell corpses from the indicated strains were scored at the head region of early L1 larvae (15 animals each). Error bars represent SEM. ** $P < 0.0001$, all other points had P value > 0.05.

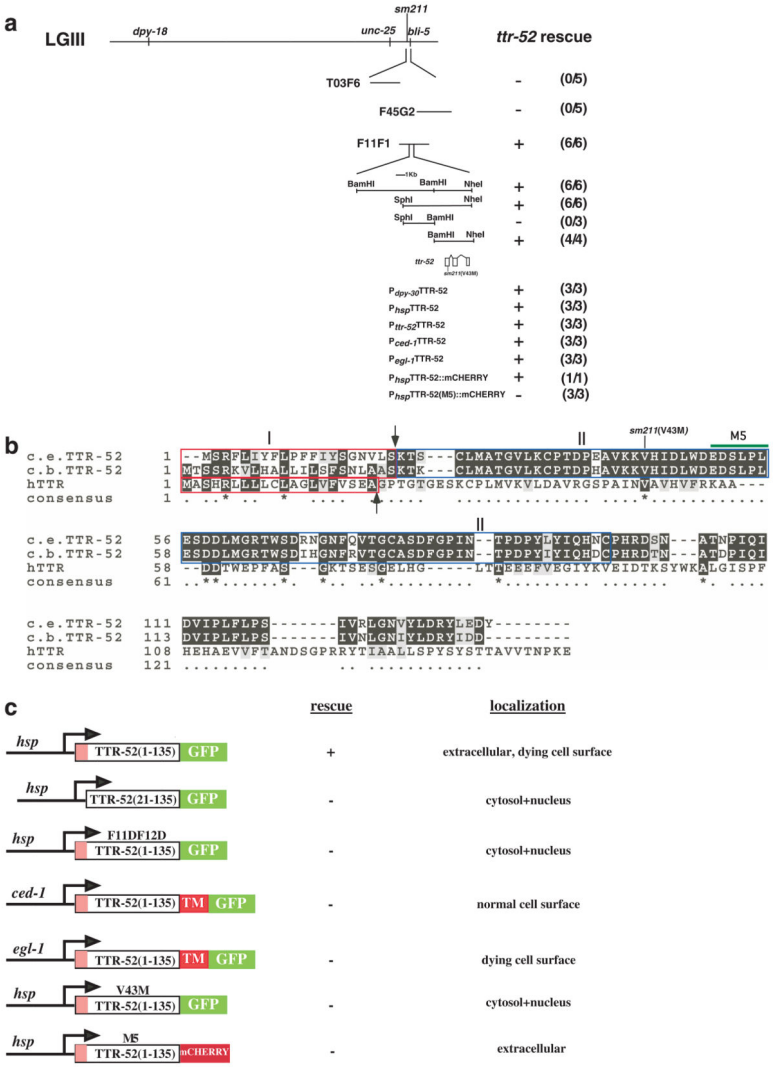


Figure 2. *ttr-52* encodes a secreted, transthyretin-like protein important for cell corpse engulfment

(**a**) Cloning of the gene affected by the *sm211* mutation. The mapped position of *sm211* on Linkage Group III (LGIII) and results of transformation rescue of the *sm211* mutant by various cosmids and constructs are shown. Cell corpses in 2-fold transgenic embryos were scored (15 embryos each line). "+" indicates rescue and "−" for no rescue. For each construct, the number of independent transgenic lines that show rescue and the number of transgenic lines tested are shown in parentheses. For P_{hsp}TTR-52::mCHERRY, the rescue was scored using an integrated array (*smIs119*) carrying this construct. (**b**) Sequence alignment of *C. elegans* (c.e.) and *C. briggsae* (c.b.) TTR-52 and human Transthyretin (hTTR). Residues that are identical are shaded in black and residues that are similar in gray. Residues that are identical in all three proteins are marked with "*". Box I indicates the predicted secretion signal with arrows pointing to the putative cleavage sites. Box II delineates the transthyretin-like domain. The mutation identified in the *ttr-52(sm211)* mutant and the residues mutated in TTR-52(M5) are indicated. (**c**) Secretion of TTR-52 is crucial for its function in cell corpse engulfment. The GFP or mCHERRY fusion constructs shown

on the left were injected into wild type and *ttr-52(sm211)* animals. The subcellular localization patterns of the fusion proteins and their ability to rescue the *ttr-52* mutant are shown on the right. 15 animals each from three independent transgenic lines were scored for each construct.

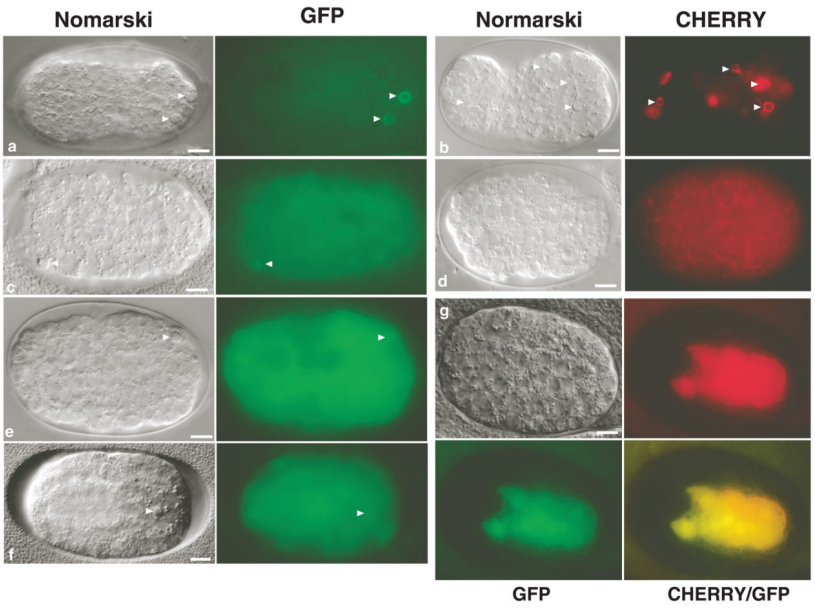


Figure 3. TTR-52 is expressed in and secreted from intestine cells and binds to the surface of apoptotic cells

(**a**–**f**) Localization patterns of various TTR-52 GFP or mCHERRY fusions. Nomarski and GFP or mCHERRY images of a wild type *C. elegans* embryo transgenic for P_{hsp}TTR-52::GFP (**a**), P_{hsp}TTR-52::mCHERRY (**b**), P_{hsp}TTR-52(21-135)::GFP (**c**), P_{hsp}TTR-52(F11D F12D)::GFP (**e**), or P_{hsp}TTR-52(V43M)::GFP (**f**) or a *ced-3(n717)* embryo carrying P_{hsp}TTR-52::mCHERRY (**d**) are shown. Apoptotic cells, displaying raised disc-like morphology in Nomarski images, are indicated with arrowheads. Exposure times were 2000 ms (**a**), 3000 ms (**c, e, f**), and 500 ms (**b, d**), respectively. (**g**) *ttr-52* is expressed in intestine cells. Nomarski, mCHERRY, GFP images and the merged image of a wild type embryo transgenic for both P_{ttr-52}mCHERRY and P_{ges-1}GFP are shown. Scale bars represent 5 μm. 3 independent transgenic lines were examined for each experiment.

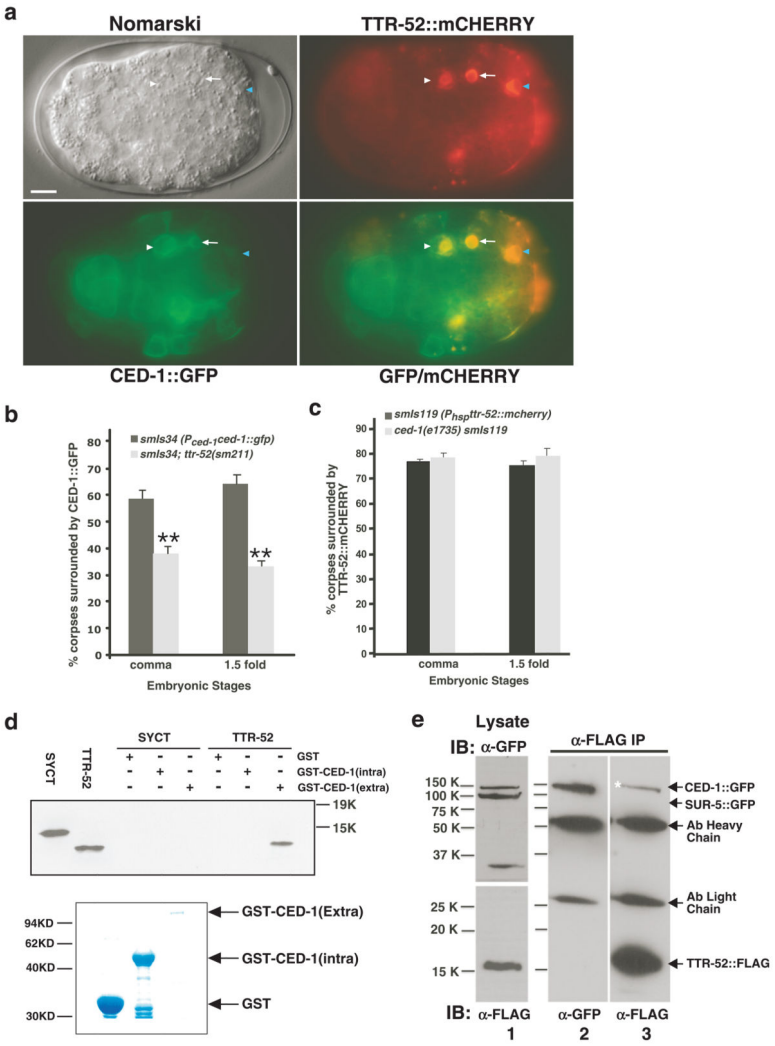


Figure 4. TTR-52 and CED-1 interact and co-localize to apoptotic cells

(**a**) Nomarski, mCHERRY, GFP images and the merged image of an early N2 embryo carrying both P_{hsp}TTR-52::mCHERRY and $P_{ced\text{-}1}$CED-1::GFP. TTR-52::mCHERRY and CED-1::GFP formed a completely overlapping ring surrounding dying cells (arrow), which sometimes was already internalized by a phagocyte (arrowhead). TTR-52::mCHERRY could label a dying cell alone (blue arrowhead). Scale bar indicates 5 μm. **(b)** *ttr-52* mediates in part the binding of CED-1 to apoptotic cells. The percentage of cell corpses surrounded by CED-1::GFP was determined in the indicated strains by analyzing serial optical sections of embryo (see Methods). ** $P < 0.0001$. (**c**) The binding of TTR-52 to apoptotic cells was not affected by loss of *ced-1*. The percentage of cell corpses surrounded by TTR-52::mCHERRY was scored in the indicated strains as described in **b**. 15 embryos each at the comma and 1.5-fold embryonic stages were scored (**b** and **c**). Error bars indicate SEM. (**d**) TTR-52 interacts with the extracellular domain (Extra) of CED-1. Purified GST, GST-CED-1(Extra) and GST-CED-1(Intra) (1 μg each) immobilized on glutathione-agarose beads were incubated with TTR-52(21-135)-His_6 or a control protein SYCT-His_6. The bound proteins were resolved on a 15% SDS-polyacrylamide gel and visualized by immunoblotting

using antibodies to a six Histidine tag. Purified GST fusion proteins stained by Coomassie Blue are shown underneath. Four independent experiments were performed. (**e**) CED-1 interacts with TTR-52 *in vivo*. Co-IP experiment was performed in *ced-5(n1812)* animals co-expressing CED-1::GFP, TTR-52::FLAG, and SUR-5::GFP (see Methods). An antibody to the FLAG epitope pulled down CED-1::GFP, but not SUR-5::GFP, with TTR-52::FLAG, which were visualized by immunoblotting (IB) first using an anti-GFP antibody (lane 2) and then reprobing with an anti-FLAG antibody after the same blot was stripped of antibodies (lane 3; see Methods). In lane 3, the residual CED-1::GFP band observed (indicated by *) is due to incomplete stripping of antibodies. Lane 1, the expression levels of three fusion proteins in the worm lysate used for IP. The blot was cut into two halves, one used for anti-GFP immunoblotting (top) and one used for anti-FLAG immunoblotting (bottom). Three independent experiments were performed.

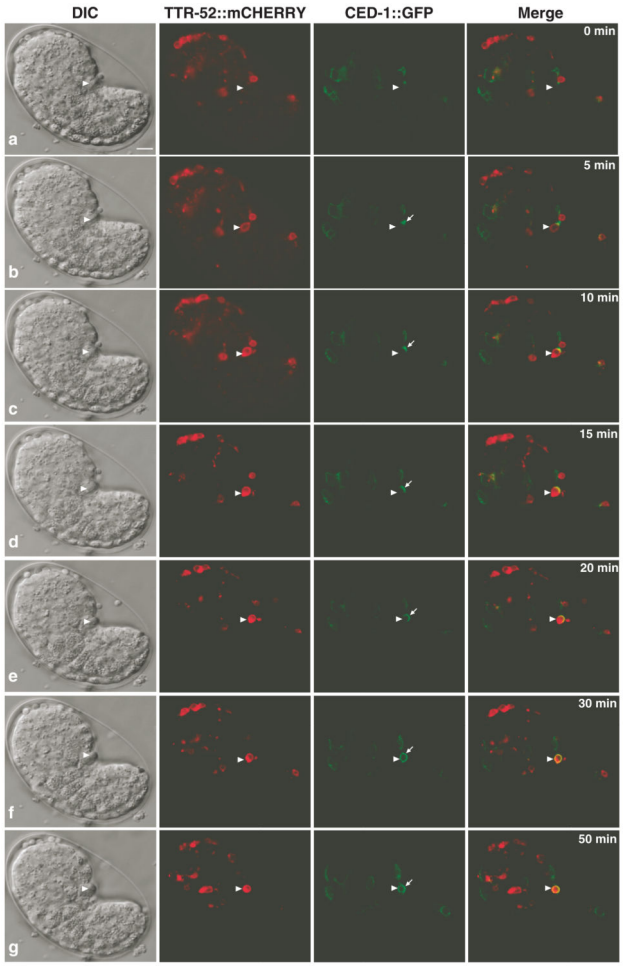


Figure 5. Clustering of TTR-52 and CED-1 around apoptotic cells monitored by time-lapse microscopy

(**a**–**g**) Confocal images of Nomarski (DIC), TTR-52::mCHERRY, CED-1::GFP and the merged images of mCHERRY and GFP of a wild type embryo carrying both P_{hsp}TTR-52::mCHERRY and P_{ced-1}CED-1::GFP at various time points. TTR-52::mCHERRY formed a complete ring surrounding the dying cell early during apoptosis (arrowhead in **b**), whereas a CED-1::GFP ring (indicated by an arrow) was formed gradually (**b** to **e**) and completed 25 min later (**f**). Scale bar represents 5 μm. Similar sequential clustering of TTR-52 and CED-1 around apoptotic cells was observed in 37 cell corpses (8 embryos) by time-lapse recordings.

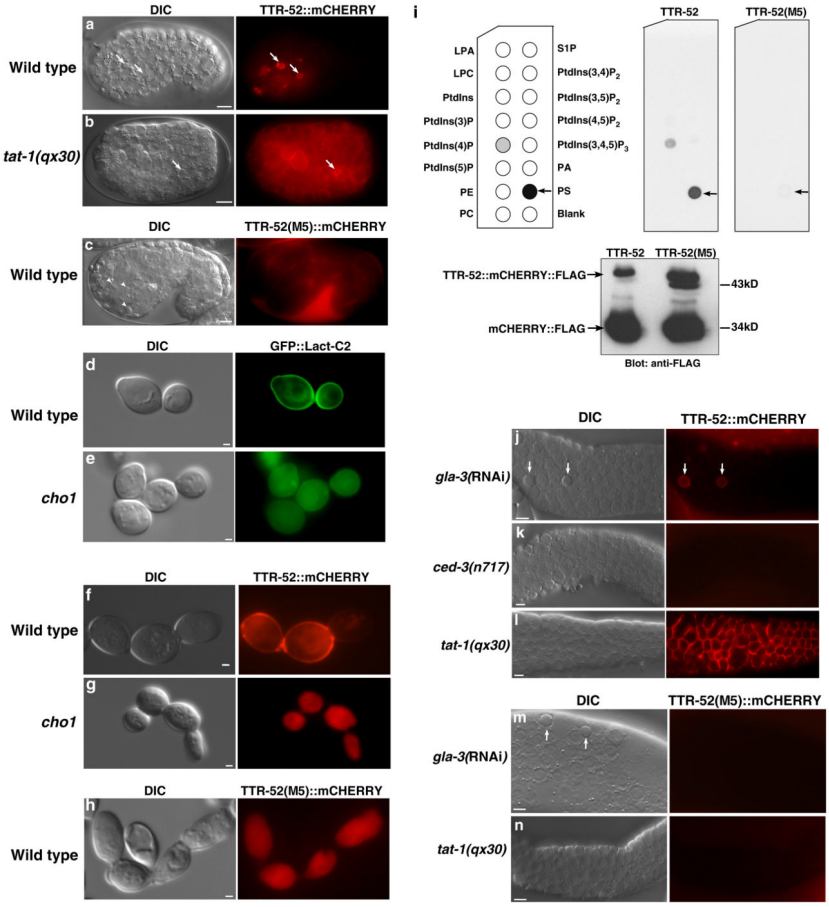
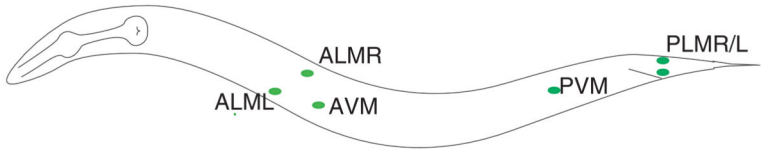


Figure 6. TTR-52 binds surface-exposed PS

(**a**–**c**), Nomarski and mCHERRY images of a wild-type embryo carrying P_{hsp}TTR-52::mCHERRY **(a)** or P_{hsp}TTR-52(M5)::mCHERRY **(c) o**r a *tat-1(qx30)* mutant embryo carrying P_{hsp}TTR-52::mCHERRY **(b)** are shown. TTR-52::mCHERRY formed bright rings specifically around dying cells in the wild type embryo (indicated by arrows in **a)** but appeared on the surface of virtually all cells in the *tat-1(qx30)* embryo (**b**). TTR-52(M5)::mCHERRY failed to label apoptotic cells (arrowheads in **c).** More than 100 embryos were examined for each panel (**a**–**c**). Exposure times were 500 ms (**a**–**c**). Scale bars represent 5 μm. (**d**–**h**), TTR-52 binds PS in yeast plasma membrane. Nomarki, GFP, or mCHERRY images of wild-type yeast cells expressing GFP::Lact-C2 (**d**), TTR-52::mCHERRY (**f**), or TTR-52(M5)::mCHERRY (**h**) and images of PS-deficient yeast cells (*cho1*) expressing GFP::Lact-C2 (**e**) or TTR-52::mCHERRY (**g**) are shown. Three independent experiments were performed for each construct. Scale bars indicate 1 μm. (**i**) TTR-52 binds PS *in vitro.* Affinity-purified TTR-52::mCHERRY::FLAG, but not TTR-52(M5)::mCHERRY::FLAG, bound PS spotted on a membrane strip (indicated by arrows; see Methods). TTR-52 also showed weak binding to PtdIns(4)P. The amounts of purified TTR-52 proteins used in lipid binding were shown by immunoblotting (bottom panel). Two independent experiments were performed. (**j**–**n)** TTR-52 binds apoptotic cells *ex vivo*. Dissected gonads from the indicated strains were incubated with purified TTR-52::mCHERRY::FLAG or TTR-52(M5)::mCHERRY::FLAG (see Methods).

Nomarski and mCHERRY images of dissected gonads are shown. TTR-52 specifically labeled apoptotic germ cells (indicated by arrows) in *gla-3*(RNAi) animals (**j**), but stained many germ cells in the *tat-1(qx30)* mutant (**l**). No TTR-52 labeling was observed in the *ced-3(n717)* mutant, which lacks germ cell death (**k**). TTR-52(M5) failed to label any germ cell in *gla-3*(RNAi) animals (**m**) or *tat-1(qx30)* animals (**n**). Scale bars indicate 5 μm. At least 30 gonads were examined for each experiment.

a



Strain	% animals missing at least one touch cell
bzIs8	0
tat-1(tm1034); bzIs8	15
tat-1(qx30); bzIs8	16
ced-1(e1735); bzIs8	1
ttr-52(sm211); bzIs8	3
ced-1(e1735); tat-1(tm1034); bzIs8	1
ced-1(e1735); tat-1(qx30); bzIs8	2
*tat-1(tm1034) ttr-52(sm211); bzIs8**	2
*tat-1(qx30) ttr-52(sm211); bzIs8**	2

b

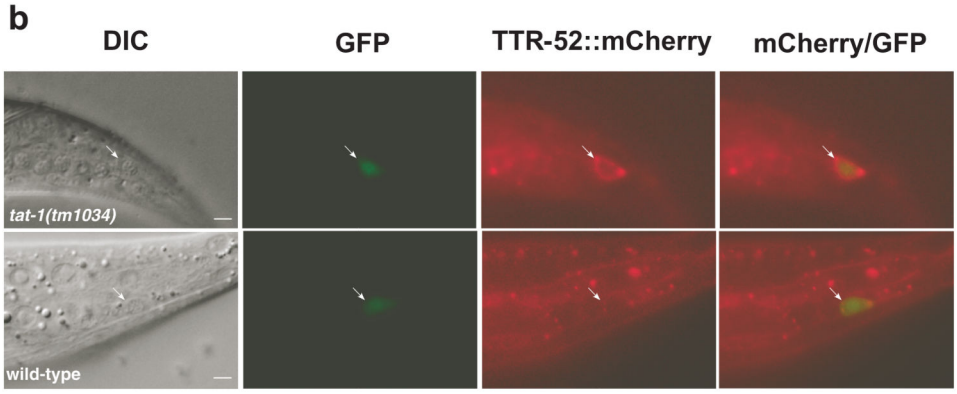


Figure 7. TTR-52 mediates random removal of neurons with surface exposed PS

(a) An integrated GFP reporter line, *bzIs8*, labels six touch-receptor neurons (indicated with green dots). The presence of neurons was scored using a Nomarski microscope with epifluorescence and the percentages of animals missing one or more neurons are shown. 90 animals were scored for each strain. Strains marked with "*" also contain the *dpy-18(e364)* mutation. (**b**) TTR-52 labels the surface of the PLM touch cell in the *tat-1* mutant. Nomarski, GFP, mCHERRY images and the merged images of GFP and mCHERRY of a wild-type or a *tat-1(tm1034)* larva carrying both P_{hsp}TTR-52::mCHERRY (*smIs119*) and $P_{mec\text{-}4}$GFP (*bzIs8*) transgenes are shown. TTR-52::mCHERRY only labeled the surface of the PLM touch cell in the *tat-1(tm1034)* mutant. Scale bars represent 5 μm. 20 animals were examined for each strain.