RESEARCH ARTICLE

The DEP domain-containing protein TOE-2 promotes apoptosis in the Q lineage of C. elegans through two distinct mechanisms

Mark Gurling, Karla Talavera and Gian Garriga*

ABSTRACT

Neuroblast divisions in the nematode Caenorhabditis elegans often give rise to a larger neuron and a smaller cell that dies. We have previously identified genes that, when mutated, result in neuroblast divisions that generate daughter cells that are more equivalent in size. This effect correlates with the survival of daughter cells that would normally die. We now describe a role for the DEP domain-containing protein TOE-2 in promoting the apoptotic fate in the Q lineage. TOE-2 localizes at the plasma membrane and accumulates in the cleavage furrow of the Q.a and Q.p neuroblasts, suggesting that TOE-2 might position the cleavage furrow asymmetrically to generate daughter cells of different sizes. This appears to be the case for Q.a divisions where loss of TOE-2 led to a more symmetric division and to survival of the smaller Q.a daughter. Localization of TOE-2 to the membrane is required for this asymmetry, but, surprisingly, the DEP domain is dispensable. By contrast, loss of TOE-2 led to loss of the apoptotic fate in the smaller Q.p daughter but did not affect the size asymmetry of the Q.p daughters. This function of TOE-2 required the DEP domain but not localization to the membrane. We propose that TOE-2 ensures an apoptotic fate for the small Q.a daughter by promoting asymmetry in the daughter cell sizes of the Q.a neuroblast division but by a mechanism that is independent of cell size in the Q.p division.

KEY WORDS: Asymmetric cell division, TOE-2, Apoptosis, Programmed cell death, Cell fate, Neuroblast

INTRODUCTION

Cysteine proteases, called caspases, mediate apoptosis. When activated, caspases cleave specific substrates, leading to the initiation of the various cellular processes that kill the cell (reviewed by Elmore, 2007). Of the four Caenorhabditis elegans caspases, only ced-3 plays a major role in apoptosis (Denning et al., 2013). In mammals, multiple caspases regulate apoptosis (Shaham, 1998; Elmore, 2007). In certain contexts, these mammalian caspsases can be activated in response to external signals (Ashkenazi and Dixit, 1998); by contrast, we know less about how the apoptotic fate is specified in C. elegans. Most of what was previously known about the assignment of the apoptotic fate concerns transcription factors that regulate the expression of the proapoptotic gene egl-1 (Potts and Cameron, 2011).

Both caspase-dependent and caspase-independent pathways regulate apoptosis, and genetic studies suggest that PIG-1, a member of the AMP-activated protein kinase family, acts in parallel to CED-3 (Cordes et al., 2006). The demonstration that a C. elegans homolog of the Sp1 transcription factor regulates both egl-1 and pig-1 transcription in specific cells that are fated to die supports the hypothesis that PIG-1 and CED-3 act in parallel (Hirose and Horvitz, 2013). Divisions that generate apoptotic cells are asymmetric, producing a larger cell that survives and a smaller cell that dies. Loss of PIG-1 leads to daughter cells that are more symmetric in size, suggesting that cell size contributes to the apoptotic fate (Cordes et al., 2006; Ou et al., 2010). In the C. elegans Q lineage, both the anterior (Q.a) and posterior (Q.p) daughter cells divide to generate a smaller apoptotic cell, but the two divisions employ distinct mechanisms to generate this asymmetry: a spindle-dependent mechanism generates Q.p asymmetry, and a spindle-independent mechanism generates Q.a asymmetry (Ou et al., 2010).

Here, we describe a role for TOE-2 in the regulation of the apoptotic fate. TOE-2 is a poorly understood DEP (domain found in Dishevelled, EGL-10 and Pleckstrin) domain-containing protein that is a target of the worm ERK ortholog MPK-1, a negative regulator of germline apoptosis (Arur et al., 2009).

DEP domains can promote localization to the plasma membrane (Axelrod et al., 1998; Wong et al., 2000), and this localization allows DEP domain-containing proteins to regulate signals that are sent from cell surface receptors to downstream effectors. For example, regulator of G-protein signaling proteins (RGSs) regulate heterotrimeric GTPases, which are involved in transducing signals from various extracellular factors (Neves et al., 2002). RGSs are GTPase activating proteins (GAPs) that modulate G-protein signaling by enhancing the hydrolytic activity of Gα, thus reducing the amount of time that the G-protein subunits are dissociated from one another – the time when Gα is active (Chen and Hamm, 2006). In addition to their interaction with G proteins, RGSs also probably bind, through their DEP domains, to G-protein-coupled receptors (GPCRs). The yeast RGS Sst2 binds to the C-terminal tail of the GPCR Ste2, leading to an attenuation of trimeric G-protein activity (Ballon et al., 2006).

We provide evidence that TOE-2 functions differently in the Q.a and Q.p divisions. Although DEP domains are thought to facilitate membrane localization, we find that the DEP domain is not required for the cortical localization of TOE-2 but is required for its function in promoting apoptosis in the Q.p division. In contrast with the loss of other regulators of the apoptotic fate in the Q lineage, loss of TOE-2 does not affect the daughter cell size asymmetry of the Q.p division. TOE-2 localizes to the nuclei of the Q-lineage cells, and we speculate that function of TOE-2 in the nucleus is to promote the cell-death fate in the Q.p division. By contrast, loss of TOE-2 affects the size asymmetry of Q.a divisions, and cortical localization might regulate this TOE-2 activity. This TOE-2 function appears to require MPK-1 activity but not the DEP domain of TOE-2. The finding that the Q.a and Q.p cells divide asymmetrically by using two distinct mechanisms (Ou et al., 2010) raises the interesting possibility that these two functions of TOE-2 contribute to the differences between these cells.
RESULTS

Loss of toe-2 results in the production of extra A/PVM-like cells

The Q,p neuroblast divides to form a larger anterior cell that survives (Q.pa) and a smaller posterior cell (Q.pp) that dies. The larger cell divides again to form an AVM or PVM mechanosensory neuron and an SDQ interneuron (Fig. 1A). Mutations in genes that positively regulate programmed cell-death – e.g. ced-3 – cause Q.pp to survive and to occasionally express the differentiation markers expressed by one of its nieces (Cordes et al., 2006). Mutations in these genes do not affect the Q.p division plane.

In contrast with the egl-1, ced-3 and ced-4 cell-death mutants, loss of the MELK ortholog PIG-1 (Cordes et al., 2006), or the ArfGAP CNT-2 (Singhvi et al., 2011), causes Q.pp to adopt a mitotic fate, producing neurons like its sister Q.pa. The mitotic and apoptotic fates are somewhat independent from one another: in pig-1 mutants, the newly adopted mitotic fate of Q.pp can be masked by its apoptotic fate. Accordingly, the penetrance of the mitotic-fate defects in these mutants is enhanced in a cell-death mutant background. Mutations in these genes also alter the position of the mitotic furrow, producing daughter cells that are more equivalent in size.

To identify genes that regulate asymmetric cell divisions in the Q.p lineage, we mutagenized ced-3(n2436) mutant worms that carried zdIs5, a transgene that drives expression of green fluorescent protein (GFP) in the A/PVMs (Clark and Chiu, 2003), and screened for mutants with extra A/PVM-like cells. It has been previously shown that the ced-3(n2436) mutation results in few extra A/PVM-like cells that express GFP from the zdIs5 transgene but sensitizes the genetic background to mutations that alter the Q.p division (Cordes et al., 2006; Singhvi et al., 2011). We isolated the mutant gm396. As with other mutations that alter the Q.pp fate, gm396 produced extra A/PVM-like cells at a lower penetrance in the absence of the ced-3 mutation (Fig. 1B-D and Fig. 2E). Through a combination of single-nucleotide polymorphism (SNP) mapping and whole-genome sequencing, we found that gm396 mutants carry a missense mutation within the toe-2 locus. To determine whether the mutation in toe-2 was responsible for the extra-cell phenotype that we observed in gm396 mutants, we used RNA interference (RNAi) against toe-2 in zdIs5; rrf-3(pk1426); ced-3(n2436) worms and saw extra A/PVMs in approximately 36.0% (n=89) of the lineages scored, whereas RNAi using the empty L4440 vector produced extra neurons in only 2.3% of lineages (n=44). The rrf-3 mutation renders animals more sensitive to RNAi (Simmer et al., 2002). RNAi against toe-2 in the absence of the ced-3 and rrf-3 sensitizing mutations produced extra cells in 10.5% of lineages (Fig. 1D).

TOE-2 has a DEP domain near its N-terminus. The toe-2(gm396) allele is defined by a point mutation that changes tyrosine residue 159, found at the C-terminal end of the DEP domain, to an aspartate. One additional allele of toe-2, ok2807, was available (Fig. 1D). This mutant had a much less penetrant extra-neuron phenotype than toe-2 (gm396) (Fig. 1D). This difference in phenotype could be caused by the nature of the ok2807 allele, which carries an in-frame deletion that might allow for the production of a less stable, or partially functional, protein.

Fig. 1. toe-2 mutants have extra A/PVM-like cells. (A) The Q.a and Q.p neuroblasts each give rise to a large neuron (A/PQR) or a neuronal precursor (Q.pa), and a small cell that dies. The right Q, Q.a and Q.aa migrate forward and result in anteriorly positioned AVM, SDQ and AQR neurons, while the same cells on the left side migrate backward and generate posteriorly positioned PVM, SDQL and PVM neurons. The AVM and PVM neurons are together referred to as A/PVM neurons, the AQR and PQR neurons as A/PQR neurons, and the SDQ and SDQL neurons as SDQ neurons. (B,C) Fluorescence photomicrographs of wild-type (B) and extra (C) PVM mechanosensory neurons. All photomicrographs are oriented with the anterior to the left. (D) Quantification of extra and missing A/PVM neurons using zdIs5 [mec-4::gfp]. The percentage of lineages that exhibited extra cells is indicated in the positive y axis. The percentage of lineages that were lacking cells is indicated in the negative y axis. The number of lineages scored is indicated on each bar. Scale bars: 5 μm.
We generated two additional alleles of toe-2 – gm407 and gm408ok2807 – using a TAL effector nuclease (TALEN)-mediated genome-editing approach (Wood et al., 2011) and saw that these mutants also had extra A/PVMs, at a frequency similar to that seen in the original gm396 mutant (Fig. 1D). The gm407 allele contained an in-frame six base-pair deletion that removed the codons for phenylalanine residue 84 and lysine residue 85, residues that are found within the DEP domain. The phenotype of this deletion, together with the changes caused by the gm396 allele, suggests that the DEP domain is important for TOE-2 function. We generated the gm408 allele in an ok2807 mutant, and it contained an 8 bp deletion that caused a frame shift, replacing phenylalanine residue 84 with the amino acid sequence LHIROAQL. This sequence was followed by a premature stop codon, suggesting that this allele is a molecular null. We placed the gm408 allele over the deficiency mnDf52, which uncovers the toe-2 locus, and observed extra and missing A/PVM-like neurons in 44% and 4% of lineages scored (n=50), respectively, a penetrance that is not significantly different from what we observe in gm408ok2807 homozygous mutants in experiments that were performed in parallel (38% extra and 4% missing A/PVMs, n=50, see Materials and Methods; P>0.5). The similar phenotypes of toe-2 hemizygous and toe-2 homozygous mutants support the hypothesis that gm408ok2807 either severely reduces, or eliminates, toe-2 function.

toe-2 mutations affect multiple divisions within the Q lineage

In toe-2 mutants, A/PVM and SDQ neurons appeared to be missing in some Q lineages (Fig. 1D and Fig. 2E). This absence of visible neurons might be the result of a loss of expression of neuronal markers or a failure to produce the neuronal precursor Q.p. To better characterize the missing-cell phenotype, we used the transgene gmIs81, which labels each of the Q-derived differentiated neurons with a different fluorescent marker. We noticed that when A/PVM and SDQ neurons were both missing in a toe-2 mutant lineage, the lineage usually produced two or more A/PQR-like cells (see Fig. 1A; 16 out of 19 lineages for toe-2(gm408ok2807) and 24 out of 28 for toe-2(gm396) mutants). We observed a similar phenomenon in the toe-2(gm396); ced-3(n717) double mutant. It has been shown previously that this mutation ensures that Q.aa survives (data not shown). As expected, we frequently observed a total of two A/PQR-like cells in the Q lineages of the ced-3 mutant (80.0%, n=110) because Q.aa failed to die. A total of two A/PQR-like cells was observed at a similar frequency (76.3%, n=160) in toe-2(gm396); ced-3 double mutants (Fig. 2E); however, we also observed lineages with three or four A/PQR-like cells. These observations and the data for the toe-2 single mutants are consistent with the notion that Q.p transforms into its sister Q.a, where extra A/PQR-like cells are produced at the expense of A/PVM and SDQ. In a toe-2 single mutant, this defect usually results in an additional Q.a neuroblast and, therefore, one additional A/PQR-like cell. In a toe-2; ced-3 double mutant, the defect results in an extra Q.a, and because of the ced-3 mutation, one or both of these neuroblasts will give rise to two neurons for a total of three or four A/PQR-like cells.

We also observed extra A/PQR-like cells in toe-2 mutants that were not caused by Q.p to Q.a transformations. In these mutant lineages, the Q.a neuroblast produced a total of two A/PQR-like cells, but the Q.p neuroblast retained its fate. These extra A/PQR cells were observed in 5.1% and 12.4% of lineages (n=158 and n=177) in toe-2(gm408ok2807) and toe-2(gm396) mutants, respectively. The presence of extra A/PQR-like cells is consistent with the survival of Q.aa – the daughter of Q.a that normally dies. Overall, we observed extra A/PQR-like cells in 15.2% and 26.0% of lineages (n=158 and n=177) in toe-2(gm408ok2807) and toe-2(gm396) mutants, respectively. These extra neurons appear to be the result of two separate defects – a defect in the ability of Q.aa to adopt the apoptotic fate, and a defect that causes Q.p to adopt the fate of its sister Q.a.

Mutations in toe-2 also affect the fate of Q.pp, the posterior daughter of Q.p that normally adopts the apoptotic fate.
We observed one extra A/PVM- or SDQ-like cell in 17.1% of lineages (n=158) in toe-2 (gm408ok2807) mutants, suggesting that Q.pp failed to adopt the apoptotic fate and expressed either the A/PVM or SDQ marker (Fig. 1C and Fig. 2B,E). We observed two extra cells, one extra A/PVM-like cell and one extra SDQ-like cell, in 7.0% of lineages (n=158) in toe-2 (gm408ok2807) mutants, suggesting that Q.pp survived and adopted the fate of the neuronal precursor Q.pa (Fig. 1A and Fig. 2E). Overall, Q.pp survived and adopted a neuronal, or neuronal precursor, fate in 24.1% of lineages (n=158) in toe-2 (gm408ok2807) mutants. We made similar observations in toe-2 (gm396) mutants (Fig. 2E).

Loss of TOE-2 function prevents Q.pp from adopting the apoptotic fate in some lineages. In ced-3 mutants, we observed extra A/PVM- or SDQ-like cells in 29.1% of lineages (n=110), a penetrance that was not significantly different from the penetrance of this phenotype in toe-2 mutants (Fig. 2E). If TOE-2 were simply promoting apoptosis in Q.pp, then we would not expect the penetrance of the Q.p extra-cell phenotype to increase in a toe-2; ced-3 double mutant; however, in toe-2 (gm396); ced-3 (n717) double mutants we did see an additive effect on the overall penetrance of the Q.p extra-cell phenotype (44.4% of lineages had extra neuron-like cells; Fig. 2E), suggesting that toe-2 and ced-3 play different roles in promoting the apoptotic fate in Q.pp.

Although the overall penetrance of the Q.p extra-cell phenotype among ced-3 and toe-2 mutants was similar, there were differences in the frequency at which a particular fate was adopted by the surviving Q.pp. As stated above, we observed extra cells in 29.1% (32 out of 110) of ced-3 (n717) mutant lineages. In those 32 lineages, 62.5% of Q.pp cells expressed the SDQ fluorescent marker – the posterior daughter of Q.pa – whereas the remaining 37.5% adopted the fate of either Q.pa (the anterior daughter of Q.p) or A/PVM (the anterior daughter of Q.pa). Conversely, we observed extra cells in 24.1% (38 out of 158) of toe-2 (gm408ok2807) mutant lineages, but in those 38 lineages, only 23.7% of Q.pp cells expressed the marker of SDQ, whereas the remaining 76.3% adopted an anterior fate – either the Q.pa or A/PVM fate. This was also the case for the Q.pp (gm396); ced-3 (n717) mutant lineages that produced extra Q.pp-derived cells, only 16.9% expressed the SDQ marker, and the remaining 83.1% had an anterior fate (Fig. 2F). Mutations in toe-2 appear to be epistatic to mutations in ced-3 with regard to the frequencies at which the different fates were observed in the surviving Q.pp cell.

TOE-2 acts autonomously in the Q lineage

Where does toe-2 function to regulate Q.pp apoptosis? We first asked where TOE-2 is expressed – by expressing GFP from the toe-2 promoter (from –2 kb to the start codon). This promoter drove expression of GFP in the Q, Q.a and Q.p cells (data not shown), in the daughters of Q.a and Q.p (Fig. 3B,C) and in the mature neurons A/PVM, SDQ and A/PQR (Fig. 3A), suggesting that TOE-2 plays an autonomous role in the Q lineage.

To test directly whether TOE-2 functions autonomously in the Q lineage, we expressed a Pmab-5:: toe-2::gfp transgene (gmEx674, gmEx675) in toe-2 (gm396) mutants. It has been shown previously that the mab-5 promoter is active in cells near the right and left Q cells before they migrate and that its activity is required in the QL neuroblast for its posterior migration; however, it is not active in the QR neuroblast (Costa et al., 1988; Cowing and Kenyon, 1992; Salser and Kenyon, 1992). If TOE-2 acts non-autonomously, we would expect rescue of the extra-cell phenotype in the Q lineages, on both the right and left sides of the worm, or no rescue of either side. However, if TOE-2 acts in the Q lineage, rescue would be observed on the left side, but not on the right. We compared siblings from mothers that carried the gmEx674 (data not shown) and gmEx675 transgenes, which are independent lines of the Pmab-5:: toe-2::mCherry transgene (Fig. 3D). In siblings that no longer carried the transgene, we observed extra cells at a frequency similar to that seen in toe-2 mutants (Fig. 3F). In siblings that still carried the transgene, we saw a significant rescue of the extra-PVM phenotype on the left side, but not of the extra-AVM phenotype on the right (Fig. 3D), suggesting that TOE-2 acts autonomously within the Q lineage. We then expressed the toe-2::gfp fusion from the egl-17 promoter, which is expressed in both the QL and QR cells. In toe-2 mutant worms that carried either of two Pegl-17:: toe-2::gfp transgenes, non-transgenic siblings had extra cells on both sides, but the extra-neuron phenotype was rescued on both sides of their transgenic siblings (Fig. 3D), confirming that toe-2 acts in the Q lineage.

---

Fig. 3. TOE-2 acts autonomously in the Q lineage to promote the apoptotic fate.

(A-C) Fluorescence photomicrographs of GFP that was expressed from the toe-2 promoter in mature Q-lineage neurons (A) and in the daughters of Q.a (B) and Q.p (C). The promoter of toe-2 was also active in the Q, Q.a and Q.p neuroblasts (data not shown).

(D) Quantification of the toe-2 extra (positive y axis) and missing (negative y axis) A/PVM phenotype, and the rescue of these phenotypes with transgenes expressing toe-2::gfp from either the mab-5 or egl-17 promoter (Pmab-5 and Pegl-17, respectively). A/PVMs were observed using zds5, gmEx675, gmEx678, gmIs86. The number of lineages scored is indicated on each bar. 

---
TOE-2 localizes dynamically during the divisions of Q and Q.p

Using the rescuing TOE-2::GFP fusion expressed from the egl-17 promoter (gmIs86), we observed dynamic subcellular localization of TOE-2::GFP in the Q lineage (Fig. 4A). Before the Q division, TOE-2::GFP localized diffusely in the cytoplasm and was concentrated in the nucleus (Fig. 4D). Around metaphase, TOE-2::GFP localized to the centrosomes (Fig. 4A; supplementary material Movie 1). The protein remained at the centrosomes throughout the rest of the division and was inherited by both daughter cells (supplementary material Movie 1). Before anaphase, TOE-2 also localized near the region of the membrane that eventually forms the furrow (Fig. 4A; supplementary material Movie 1). As anaphase continued, TOE-2 concentrated within the furrow and remained there, eventually localizing to the midbody. During telophase, TOE-2 localized to chromatin (Fig. 4A). After abscission of the daughter cells, TOE-2 was again diffuse in the cytoplasm and highly concentrated in the nucleus (Fig. 4D).

In Q.a and Q.p – as in the Q cell – TOE-2 localized to centrosomes, to the region of the cortex in which the furrow forms, to the furrow and, at a later stage, to the midbody (Fig. 4B,C). In contrast with the localization of TOE-2::GFP to the chromatin of Q daughter cells, the protein did not appear to accumulate near chromatin, or in nuclei, of the daughter cells of Q.a and Q.p during, or after, telophase (Fig. 4D).

A C-terminal region, but not the DEP domain, of TOE-2 is required for cortical localization

The function of most DEP domains is not well understood; however, they can facilitate localization of a protein to the plasma membrane. To determine whether the DEP domain is required for membrane localization of TOE-2, we analyzed the distribution of a tagged TOE-2 construct that lacked the DEP domain (TOE-2ΔDEP, gmEx681). Surprisingly, this protein still localized to centrosomes, to the cortex, to the cleavage furrow, and to the nuclei of Q, Q.a and Q.p (Fig. 5A-C;H; supplementary material Movies 2, 3). In addition to the DEP domain, TOE-2 has two D motifs and a DEF motif, which are predicted docking sites for MPK-1 – the worm ortholog of mammalian ERK (Arur et al., 2009). Tagged TOE-2 that lacked the region containing all three MAPK docking sites and intervening sequence (TOE-2ΔDOCK, gmEx721) localized to the nuclei and centrosomes of the Q, Q.p and Q.a neuroblasts but failed to localize to the cortex, or furrow, of these cells when they divided (Fig. 5D,E; supplementary material Movies 4-6; data not shown). We quantified the cortical and furrow localization of the tagged TOE-2, TOE-2ΔDEP and TOE-2ΔDOCK proteins by calculating the ratio between the intensities of the pixels found at the edge of the furrow and of the pixels found within the cytoplasm of the intercellular bridge of dividing cells. TOE-2ΔDOCK localized consistently in the cytoplasm as opposed to the furrow during anaphase (Fig. 5H; supplementary material Movies 4-6), whereas the opposite was true for the tagged TOE-2 and tagged TOE-2ΔDEP proteins (Fig. 5H; supplementary material Movies 1-3). It could have been possible that TOE-2ΔDOCK localized to the furrow, but the reason we saw a low ratio of furrow to cytoplasmic localization was that the protein was expressed at much higher levels than the other two transgenes. We looked at the distribution of all of the pixels measured in all of the time-lapse movies that we collected for each transgene and noted that all three transgenic proteins were present in the cytoplasmic bridges at similar levels (Fig. 5G).

TOE-2 requires the DEP domain to function in Q.p, and the DEF motif for its role in Q.a

The DEP domain is the only recognizable domain that has been identified in TOE-2. We observed that the TOE-2ΔDEP transgene (Fig. 6A) did not rescue the extra- or missing-cell phenotypes of toe-2(gm408ok2807) mutants (Fig. 6B). These findings, along with the localization data (Fig. 5A-C,H), indicate that the DEP domain is required for TOE-2 function but not for its localization. We also tested the function of the TOE-2 MAPK docking sites. The toe-2(ok2807) mutation deletes the region of toe-2 that codes for a region containing both of the D motifs (Fig. 6A). Extra A/PVM and SDQ neurons were seen at a low frequency in toe-2(ok2807) mutants (Fig. 1D and Fig. 5C), suggesting that MAP kinases (MAPK) could play a minor role in promoting the apoptotic fate of Q.pp. However, to assess the role of MAPK directly, we looked for extra and missing cells that were derived from the Q.p and Q.a neuroblasts in MAPK mutants, and double mutants that comprised these mutations and toe-2(ok2807). We did not observe significant differences in cell number in single or double mutants of pmk-1(lm25) (p38 family MAPK) or kgb-2(gk361) (JNK family MAPK) (data not shown). We also looked at mpk-1(ga117) single mutants and saw no significant change in cell number (Fig. 6C). There was no significant difference in the number of Q.p-derived neurons between toe-2(ok2807) and toe-2(ok2807); mpk-1(ga117) mutants.

Fig. 4. TOE-2 localizes to the centrosomes and plasma membrane of dividing neuroblasts and concentrates in the cleavage furrow as division proceeds. (A-C) TOE-2::GFP expression (gmIs86) localized near the membrane and centrosomes of Q, Q.a and Q.p (A, B and C, respectively) during their divisions. Images were captured every 20 seconds. Arrows, centrosomes; arrowheads, furrows; empty arrowheads, chromatin. (D) Still images of TOE-2::GFP (gmIs86) localization in the nuclei of Q.a and Q.p before division. Nuclear localization was not observed in the daughters of Q.a and Q.p. Scale bars, 1.5 μm.
However, we did observe an increase in the number of extra Q.a-derived neurons in the double mutants over that observed in toe-2(ok2807) single mutants. Furthermore, the penetrance of this defect was 6.0%, very near the frequency with which we saw extra A/PQR cells that were not the result of transformation of Q.p into Q.a in toe-2(gm408ok2807) mutants (5.1%). Taken together with the localization data for the TOE-2ΔDOCK protein, these data suggest a role for MPK-1 in the regulation of TOE-2 through the DEF motif specifically in the Q.a division.

We also observed that expression of TOE-2ΔDOCK rescued the extra-A/PVM phenotype of toe-2(gm408ok2807) mutants (Fig. 6B). Taken together with the low-penetrance extra-cell phenotype of the toe-2(ok2807) allele, these results suggest that MPK-1 does not regulate TOE-2 during the development of the Q.p division.

**The cell size asymmetry between Q.p daughter cells is maintained in a toe-2 mutant**

Previous work has shown a correlation between the size of sister cells and their competency to execute the apoptosis pathway. Although the Q.p division is normally asymmetric with respect to size in cell-death mutants, in mutants where Q.pp survives and becomes a neuron or neuronal precursor – e.g. pig-1 and cnt-2 – the Q.p cell divides more symmetrically (Cordes et al., 2006; Singhvi et al., 2011). The localization of TOE-2 to centrosomes, and to the cleavage furrow, suggested that TOE-2 might position the cleavage furrow so as to produce daughter cells of different sizes. This idea was tempting because TOE-2 localized at the cortex where the furrow forms, before the furrow itself is visible (Fig. 4A-C; supplementary material Movie 1). To test whether TOE-2 functions to regulate furrow positioning, we used rdvIs1, a transgene that expresses a histone 1 linker-mCherry fusion protein that localizes to chromatin and a myristoylated mCherry protein that localizes to the plasma membrane of the cells in the Q lineage (Ou et al., 2010). This marker allowed us to observe and measure the relative sizes of the anterior and posterior Q.p daughter cells in wild-type and toe-2(gm408ok2807) mutant animals. We reasoned that if TOE-2 were important for the asymmetric placement of the cleavage furrow, the Q.p daughters, which are usually of unequal size, would be more symmetric with respect to size in a toe-2 mutant. To the contrary, we found that the cell size asymmetry between the daughters was not significantly altered (Fig. 7A).

In contrast with the division of Q.p, we found that the division of Q.a was more symmetric in toe-2 mutants, resulting in daughter...
cells that were more equivalent in size. We tested whether cortical and furrow localization were important for this function by using the TOE-2ΔDOCK mutant transgene that failed to localize to the membrane of the dividing cell. Although expression of the transgenes that encoded the full-length TOE-2 and TOE-2ΔDEP proteins rescued the Q.a size asymmetry defect of toe-2(gm408ok2807) mutants, the transgene encoding TOE-2ΔDOCK did not (Fig. 7B). We also observed that the TOE-2ΔDEP and TOE-2 transgenes caused some Q.a cells to divide more asymetrically with respect to size than any of the divisions that we observed in wild-type animals (Fig. 7B). The ability of TOE-2ΔDOCK to rescue the toe-2 Q.p defect indicates that the protein was expressed at levels necessary for function, and its failure to localize to the membrane suggests that this localization is necessary for its function in Q.a asymmetry. We propose that TOE-2 has two distinct activities: a DEP-dependent function that regulates the apoptotic fate of Q.pp and a membrane localization function that regulates the asymmetry of the Q.a division.

Fig. 6. The DEP domain, but not the sequence containing the MAPK docking sites, is required for TOE-2 function in determining the apoptotic fate of Q.pp. (A) Representation of the full-length TOE-2, TOE-2ΔDEP and TOE-2ΔDOCK transgene products, as well as the putative product of toe-2(ok2807) (ΔL237-V431). (B) The quantification of toe-2(gm408ok2807) extra- and missing-cell phenotypes in the presence or absence of transgenes that expressed TOE-2ΔDEP (gmEx681) and TOE-2ΔDOCK (gmEx721). (C) The quantification of extra- and missing-cell phenotypes in toe-2, mpk-1 and toe-2; mpk-1 double mutants by using gmls81. In B,C, the percentage of lineages that exhibited extra cells is indicated in the positive y axis, and the percentage of lineages that were lacking cells is indicated in the negative y axis. The number of lineages scored is indicated on each bar. **P<0.01, ****P<0.0001. NS, not significant. Chi-squared test.

Fig. 7. Loss of TOE-2 affects the asymmetric cell size of Q.a, but not Q.p, daughters. (A) A box-and-whisker plot of the log2 of the ratio of the size of the Q.pa cell to that of Q.pp in wild-type and toe-2(gm408ok2807) animals. (B) A box-and-whisker plot of the log2 of the ratio of the size of the Q.a cell to that of Q.ap in wild-type and toe-2(gm408ok2807) animals. Rescue of the defect in the size of the Q.a cell was attempted with TOE-2, TOE-2ΔDEP and TOE-2ΔDOCK transgenes. 1gmls86, 2gmEx681, 3gmEx721. All cells were observed using rdvIs1. (A,B) The box indicates quartiles 1 through 3, i.e. the interquartile range (IQR), the whiskers indicate the highest and lowest points within 1.5 times the IQR above or below the median, respectively; the line represents the median. The number of lineages scored is indicated underneath each plot. *P<0.05, **P<0.01. NS, not significant. Mann–Whitney U test.
DISCUSSION
TOE-2 regulates cell fate
TOE-2 normally functions in the Q lineage to promote a consistent pattern of asymmetric cell division and fate assignment. TOE-2 is required for fate asymmetry in Q neuroblast daughters. In strong loss-of-function toe-2 mutants, the posterior daughter of Q often adopts the fate of the anterior Q daughter. The reverse does not seem to occur. We observed a similar phenomenon in the fates of Q.p daughters of toe-2 mutants. When Q.pp survives in a toe-2 mutant, it is more likely to have an anterior fate (AVM or Q.pa) than a posterior fate (SDQ). The reverse is true in a ced-3 mutant. The toe-2 single and toe-2; ced-3 double mutants have the same phenotype, and this epistatic interaction suggests that TOE-2 normally promotes a posterior differentiation program that is required for Q.pp to undergo apoptosis. These observations suggest that the normal function of TOE-2 is either to promote posterior fates or to inhibit anterior fates in the daughters of Q and Q.p.

These defects all occur in spite of the lack of any change in the relative sizes of Q.p daughter cells in toe-2 mutants. The transformation of cells that have posterior fates to those of anterior sister cells is reminiscent of the fate changes that occur in mutants with impaired Wnt signaling (reviewed in Sawa, 2012), and in two lineages, Wnt signaling regulates divisions that produce cells that are fated to die (Bertrand and Hobert, 2009). Further work will be required to determine whether there is a connection between TOE-2 and Wnt signaling in asymmetric cell divisions.

C. elegans cells that are fated to die are smaller than their surviving sisters due to the asymmetric position of the cleavage furrow along the axis of division (Hatzold and Conradt, 2008; Frank et al., 2005; Cordes et al., 2006; Ou et al., 2010; Singhvi et al., 2011). The similar sizes of Q daughters in wild-type and toe-2 mutant animals, and the lack of cell size defects in the Q.p division of the mutants, indicate that the Q.p and Q.pp cell fate changes do not result from altered cell sizes. These defects of toe-2 mutants, in which only the cell fate is changed, are different from those of cnt-2 and pig-1 mutants, which have defects in both the fates and sizes of the Q.p daughter cells (Cordes et al., 2006; Ou et al., 2010; Singhvi et al., 2011). The ability of TOE-2ΔDOCK to rescue the defects in the Q.pp fate raises the interesting possibility that either nuclear or centrosomal TOE-2 regulates this fate.

TOE-2 and MPK-1
TOE-2 was first described as a target of MPK-1, the C. elegans ortholog of ERK, and was shown to inhibit apoptosis in the germ line (Arur et al., 2009). This role of TOE-2 and MPK-1 is opposite to that which we observed in the Q lineage – where TOE-2 promoted the apoptotic fate. This discrepancy is not unprecedented as ERK has been shown to have both anti-apoptotic (Vaudry et al., 2002; Leeds et al., 2005; Ortega et al., 2011; Jan et al., 2013) and pro-apoptotic (Yamagishi et al., 2005; Chen et al., 2009) activities in mammalian cells.

The ability of TOE-2ΔDOCK to rescue Q.p defects, but not the Q.a size asymmetry defect, together with the apoptosis defect of the Q.a cell of mpk-1(ga117); toe-2(ok2807) mutants, suggests that MPK-1 can act through the DEP motif of TOE-2 and that this activity is specific to the function of TOE-2 during Q.a development.

TOE-2 regulates the asymmetry of the Q.a division
TOE-2 appears to play different roles in the regulation of the Q.a and Q.p divisions. Like Q.pp, Q.aa can survive and adopt the fate of its sister. But, unlike the Q.p division, the asymmetry of the Q.a division is altered in toe-2 mutants. We propose that the changed fate of the Q.aa cell is a consequence of an altered furrow position, which results in daughter cells that are more equivalent in size. Unlike the role that TOE-2 plays in the Q.p lineage to regulate fate, the activity of TOE-2 that promotes the asymmetric size of Q.a daughter cells does not require the DEP domain. This finding was surprising because there are extra Q.a-derived neurons in toe-2 (gm396) mutants that are probably the product of a more symmetric Q.a division, yet the only lesion in toe-2(gm396) is a missense mutation in the DEP domain. This change could alter TOE-2 function as opposed to simply disrupting DEP function.

The different roles of TOE-2 in the anterior and posterior branches of the Q lineage are interesting because Q.a and Q.p have been observed previously to use different mechanisms for asymmetric division (Ou et al., 2010). Our observations suggest that TOE-2 is involved in generating asymmetry in both divisions – cell size in Q.a and cell fate in Q.p. It is noteworthy that pig-1 and toe-2 mutants both have more symmetric Q.a divisions and a low penetrance of extra A/PQR-like neurons (Cordes et al., 2006), suggesting that these two genes could act together to regulate the position of the Q.a cleavage furrow.

The TOE-2 DEP domain
Extra Q.lineage neurons were seen much less frequently, or not at all, in the weak loss-of-function mutant toe-2(ok2807). One prominent difference between the strong toe-2 mutants and this weak mutant is that the DEP domain remains intact in the weak mutant, whereas the DEP domain is either not expressed (gm408ok2807) or is mutated (gm396, gm407) in strong mutants. These findings raise questions about the function of the DEP domain in Q lineage divisions. DEP domains are generally thought to be important for plasma membrane localization (Axelrod et al., 1998; Wong et al., 2000), suggesting that TOE-2 functions at the membrane – which is consistent with the localization of TOE-2 that we observed in the Q. Q.a and Q.p cells. However, we also observed that TOE-2ΔDEP localized normally, suggesting a different role for the DEP domain of TOE-2.

It is likely that the DEP domain mediates the interaction between TOE-2 and other proteins that regulate asymmetric cell division in the neuroblasts of the Q lineage. The DEP domain of Sst2, an RGS in yeast, binds to the C-terminal tail of the GPCR Ste2 (Ballon et al., 1996; Ross and Wilkie, 2000). TOE-2 is distantly related to vertebrate proteins that contain a GAP domain in addition to their DEP domain (Dohlman et al., 1996; Ross and Wilkie, 2000). TOE-2 lacks a GAP domain, it can bind to the worm ortholog of GRAF, which contains a RhoGAP domain (Xin et al., 2009, 2013). It is not known whether this interaction is relevant in the context of the Q lineage, but it suggests that TOE-2 might regulate Rho-family GTPases.
LET-99 is a C. elegans protein that has both a DEP domain and a RhoGAP domain, and it has been shown to regulate asymmetric cell division in the one-cell embryo. LET-99 localizes at the cortex of the one-cell embryo in a lateral-posterior band that excludes the G-protein regulators GPR-1 and GPR-2 from this region and concentrates them at the posterior pole of the embryo (Tsou et al., 2002, 2003). Concentration of GPR-1 and GPR-2 at the posterior pole causes the mitotic spindle to move towards the posterior to produce a smaller posterior, and a larger anterior, daughter. The localization of TOE-2 at the membrane, the more symmetric Q.a division in a toe-2 mutant and the increased difference in the asymmetry of division when TOE-2 is overexpressed are all consistent with a role, like that of LET-99, in promoting size-asymmetric cell division. If this is true, the localization and function consistent with a role, like that of LET-99, in promoting size asymmetric cell division. If this is true, the localization and function of TOE-2 shown here might aid the study of related proteins in mammals, which presently have no known functions.

MATERIALS AND METHODS

Strains and genetics

Worms were cultured as described previously (Brenner, 1974). All experiments were conducted using worms that were cultured at 20°C unless otherwise noted. The following strains were used:

- LG I: zds5[mp3e-4::gfp; lin-15(+)]; (Clark and Chiu, 2003).
- LG II: toe-2(gm396, gm407, gm408); (this study), toe-2(ok2807); C. elegans Gene Knockout Project at OPMF, rrlf-pk1426 (Simmer et al., 2002), wu-4 (e120) mndf52/mnc1 dp-10(e128) unc-52(e444) (Sigurdson et al., 1984), mnl1[mnl4 dp-10(e128)] (Edgley and Riddle, 2001).
- LG III: mpsk-1(ga111) (Leacock and Reinke, 2006), rvd611[peg7-17::myristoylated mCherry::pie-1 3′ UTR; Pegl-17::mCherry-TEV-S::his-24; Pegl-17::mit-10::YPF::unc-54 3′ UTR; pRF4] (Ou et al., 2010).
- LG IV: ced-3(n2436) (Shaham et al., 1999), ced-3(n771) (Ellis and Horvitz, 1986), pSg-1(gm301) (Cordes et al., 2006), gmsl86[peg7-17::toe-2a::gfp; pmyo-2::mCherry] (this study).
- LGX: gmsl87[peg4-4::mCherry; Pegl-17::ebfp2; peg7-17::gfp] (a gift from Gérome Teulière and John Chien, University of California, Berkeley, USA).

Extra-chromosomal arrays: gmEx674, gmEx675[pmab-5::toe-2a::mCherry; pmyo-2::gfp], gmEx676[peg7-17::toe-2a::gfp; pmyo-2::mCherry], gmEx678[peg7-17::adek-toe-2a::gfp; pmyo-2::mCherry], gmEx720[ppeg7-17::gfp] (this study).

To generate toe-2(gm408 ok2807)/mndf52 animals, we crossed zds5+/--; mnl1+/+ males to mndf52/mnc1 hermaphrodites. The mndf52/mnl1 hermaphrodites were crossed with zds5; toe-2(gm408 ok2807)/mnl1 males. The toe-2(gm408 ok2807)/mndf52 males were scored for the number of AVMs and PVMs. As a control, we scored the numbers of AVMs and PVMs that were generated by toe-2(gm408 ok2807) homozygote males produced from zds5; toes-2(gm408 ok2807)/mnl1 animals. Both the toe-2(gm408 ok2807)/mndf52 hemizygous and toe-2(gm408 ok2807) homozygous males were raised at room temperature in parallel in these experiments.

Molecular biology and transgene construction

The toe-2 cDNA was isolated from a cDNA library, which had been primed using random hexamers, with the primers 5′-ATGAGTTCCTGCTCGT-CACCTCA-3′ and 5′-TTATATCATCCTCGGAAAAGTCGTT-3′. The PCR fragment was subcloned into the pCR8 TOPO vector. The sequence in the cDNA that encodes the DEP domain was removed from the cDNA using the primers 5′-CTACAAGACAGATCCGTTGCTCGT-CAGTTAT-ATTGTG-3′ and 5′-ACAATATATCTAGAAGAACTGGGAGT-GTTGCTCGT-3′ and the Quick Change II site-directed mutagenesis kit (Agilent Technologies). The sequence coding for the region that contained the MAPK docking sites was removed in the same way, using the primers 5′-CAAAATTGAAATCGAGGCTTGGTACACCCGGCCAACAATTG-3′ and 5′-CAAATATTGCGCCTGGGTACCCCACCTGAGTCAAAATTG-3′. The transgenic constructs were generated from pre-existing multi-site Gateway entry clones and entry clones that were made using the toe-2 cDNAs described above. TOE-2 transgenes were generated by injecting the construct and co-injection marker DNA into the gonads of young adult worms (Mello et al., 1991).

To construct the Pegl-17::gfp plasmid, the last 5 kb of the egl-17 promoter was cloned upstream of GFP in pPD95.77 (Addgene). The 1 kb promoters of gcy-32, flp-12 and mec-4 were each subcloned into the pCR8 TOPO vector and subsequently placed upstream of GFP, EBFP2 and mCherry, using Gateway cloning, to generate Pgcys-32::gfp, Pflp-12::ebfp2 and Pegl-17::mcherry, respectively. An extrachromosomal transgene was generated by co-injecting Pegl-17::mcherry, Pflp-12::EBFP2, Pgcys-32::gfp and Pegl-17::gfp into the gonads of wild-type hermaphrodites. This transgene was then integrated into the genome by using ultraviolet irradiation to create the gmsb81 transgene, which was backcrossed to wild-type worms eight times.

EMS mutagenesis screen

We mutagenized zds5[peg7-17::gfp]; ced-3(n2436) hermaphrodites with 50 mM ethylmethylsulfonate (EMS), transferred individual F1 progeny to separate plates and screened the F2 progeny for mutants that had extra A/PVMs at a frequency above the background frequency of non-mutagenized zds5; ced-3(n2436) worms.

gm396 SNP mapping

Using the Hawaiian isolate (CB4856) of C. elegans for SNP mapping, we placed gm396 between snp_C34F11[2] and snp_T24B8[1] on LG II (Wicks et al., 2001).

Whole-genome sequencing

Genomic DNA was prepared by using the second method described by Sarin and colleagues (Sarin et al., 2010). Genomic DNA (approximately 5 mg) was sheared to an average length of 300 base pairs, by using a Covaris S2 instrument, and prepared for paired-end sequencing using the Illumina paired-end sample preparation guide. One hundred base-pair single-end reads were obtained using an Illumina Genome Analyzer Ix. Sequencing data were analyzed using MAQ and custom Perl scripts, as described previously (Gerhold et al., 2011).

RNAi knockdown of toe-2

RNAi was performed by feeding worms individual bacterial clones from a library that had been constructed using the C. elegans ORFeome (Rual et al., 2004). RNAi against toe-2 was performed with the clone containing the mv.C56e6.3 cDNA within the L4440 vector. The negative control was a clone containing the empty vector (L4440) (Timmons et al., 2001).

Zeiss imaging and scoring

Worms were anesthetized in 1.25 mM levamisole. A Zeiss Axioskop 2 microscope was used to examine the worms. Images were collected using an ORCA-ER CCD camera (Hamamatsu) and Openlab imaging software (Improvision). When scoring numbers of cells, similar numbers of right and left sides were scored. All micrographs are oriented with the anterior to the left.

Design and use of TALENs

TALENs were used, as described previously (Wood et al., 2011), to create mutant alleles of toe-2. A pair of TALENs was designed to recognize the sequences 5′-TGAGAAATAGCATC-3′ and 5′-TTTGCAGACGG-3′ within the region of the toe-2 ORF that codes for the N-terminal end of the DEP domain, just downstream of an in-frame start codon. TALENS were made using a protocol described elsewhere (Cermak et al., 2011) with a custom vector (Lo et al., 2013) in place of pTAL3.

Confocal and time-lapse imaging

Time-lapse images of Q and Q.p divisions were captured at 20-second intervals on a spinning-disk (CSU-X1; Yokogawa) confocal microscope. Images were captured using an EM CCD camera (Evolve; Photometrics) and SlideBook software (Intelligent Imaging Innovations).
Quantification of cell size asymmetry
The areas of Q.a and Q.p daughter cells were measured in L1 larvae. The areas were measured three times using the ImageJ free-hand selection tool. The ratio of the size of the anterior cell to that of the posterior cell was calculated using average area values for anterior and posterior cells.

Statistics and figures
The Chi-squared test was used to compare extra- and missing-cell defects between genotypes. The Mann–Whitney U test was used to compare the distributions of cell sizes. Figures were generated using matplotlib (Hunter, 2007).

Acknowledgements
We thank J. Chien and J. Teulère for comments on the manuscript and for creating the gmts81 transgene used in this study. We also thank members of the Garriga, Meyer and Dernburg laboratories for helpful discussions. We especially thank T. Lo for help with TALENs and O. Rog for help with confocal microscopy. We thank Gillian Stanfield for the use of her microscope. toe-2(ok2807) was created by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation and was obtained from the C. elegans Genetics Center, which is funded by the National Institutes of Health.

Competing interests
The authors declare no competing financial interests.

Author contributions
M.G. and G.G. designed experiments. M.G., K.T. and G.G. performed all experiments. M.G. and G.G. analyzed all data generated from the experiments. M.G. and G.G. wrote the paper.

Funding
This work was supported by the National Institutes of Health [NS32057 to G.G.]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.110486/-/DC1

References


**Movie 1.**
The localization of TOE-2::GFP is similar among Q, Q.a and Q.p neuroblasts. TOE-2::GFP localizes near centrosomes and near the membrane. It concentrates at the cleavage furrow during anaphase and near chromatin at the end of the division. This movie shows TOE-2::GFP localization during a Q neuroblast division. (20 seconds between frames)

**Movie 2.**
TOE-2ΔDEP::GFP in Q.a (top) and Q.p (bottom) neuroblasts. TOE-2::GFP and TOE-2ΔDEP::GFP always localize to the neuroblast nucleus before metaphase as seen in Q.p. TOE-2ΔDEP::GFP then localizes near centrosomes and becomes diffuse throughout the cytoplasm. TOE-2ΔDEP::GFP localization is indistinguishable from that of TOE-2::GFP. Anterior is to the right, dorsal up. (20 seconds between frames)

**Movie 3.**
TOE-2ΔDEP::GFP localization in Q.p. (20 seconds between frames)

**Movie 4.**
TOE-2ΔDOCK::GFP is absent from the cleavage furrow of the Q neuroblast. During anaphase TOE-2ΔDOCK::GFP begins to concentrate near chromatin. (20 seconds between frames)

**Movie 5.**
TOE-2ΔDOCK::GFP localizes to the nucleus of Q.a. It then diffuses throughout the cell. During anaphase TOE-2ΔDOCK::GFP appears to localize to the chromatin of Q.aa and Q.ap. (20 seconds between frames)

**Movie 6.**
TOE-2ΔDOCK::GFP localization in Q.p. (20 seconds between frames)
Movie 1.

Movie 2.

Movie 3.
Movie 4.

Movie 5.

Movie 6.