Caspase-activated phosphoinositide binding by CNT-1 promotes apoptosis by inhibiting the AKT pathway

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Inactivation of cell-survival factors is a crucial step in apoptosis. The phosphoinositide 3-kinase (PI3K)-AKT signaling pathway promotes cell growth, proliferation and survival, and its deregulation causes cancer. How this pathway is suppressed to promote apoptosis is poorly understood. Here we report the identification of a CED-3 caspase substrate in *Caenorhabditis elegans*, CNT-1, that is cleaved during apoptosis to generate an N-terminal phosphoinositide-binding fragment (tCNT-1). tCNT-1 translocates from the cytoplasm to the plasma membrane and blocks AKT binding to phosphatidylinositol (3,4,5)-trisphosphate, thereby disabling AKT activation and its prosurvival activity. Our findings reveal a new mechanism that negatively regulates AKT cell signaling to promote apoptosis and that may restrict cell growth and proliferation in normal cells.

One major unanswered question in apoptosis, an essential process in animal development and homeostasis, is how the cell-suicide program is executed in a highly coordinated fashion so that the dying cell is disassembled and removed swiftly without causing any deteriorating outcomes. The cell death–execution process is activated by caspases, which are aspartate-specific cysteine proteases that cleave multiple substrates to orchestrate cellular disassembly, including chromosome fragmentation, nuclear membrane breakdown and phagocytosis of apoptotic cells. Numerous caspase substrates have been identified in mammals, and some are involved in cell-death execution. For example, the PAK2 kinase is activated by caspase-3 cleavage, which is required for the fragmentation of dying cells into apoptotic bodies. In *C. elegans*, many apoptosis regulators and executors are conserved, including the CED-3 caspase and CED-3 activator CED-4 (homologous to the human caspase activator Apaf-1), both of which are essential for apoptosis in *C. elegans*. How CED-3 kills cells by proteolytic cleavage is poorly understood. Recently, the Dicer RNase (DCR-1) was found to be converted into a DNase upon CED-3 cleavage during apoptosis, to initiate apoptotic chromosome fragmentation. Similarly, CED-3 cleavage of the mitochondrial fission protein DRP-1 and the multipass transmembrane protein CED-8 generates C-terminal cleavage products that promote mitochondria elimination and phosphatidylinerine externalization, respectively, in apoptotic cells. Thus, CED-3 cleavage of DCR-1, DRP-1 and CED-8 activates three different cell-disassembly events that facilitate cell killing. In this study, we report a new CED-3 substrate, CNT-1, whose cleavage by CED-3 activates an N-terminal cleavage product, truncated CNT-1 (tCNT-1), that promotes apoptosis by suppressing the AKT prosurvival activity.

In mammals, the PI3K-AKT pathway is critical for cell growth, proliferation, survival and metabolism. Hyperactivation of this pathway results in cancers, thus revealing the oncogenic potential of PI3K-AKT signaling components, whereas impaired signaling in this pathway causes diabetes and cardiovascular disease. In *C. elegans*, the AKT homologs AKT-1 and AKT-2 act in the insulin and insulin-like growth factor signaling (IIS) pathway to regulate lifespan, development, metabolism and stress resistance. In the IIS pathway, the insulin receptor–like protein DAF-2 activates the PI3K complex AGE-1–AAP-1 to lead to the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the inner leaflet of the plasma membrane. PIP3 may then recruit the serine/threonine kinases PDK-1, AKT-1, AKT-2 and SGK-1 to the plasma membrane by engaging their pleckstrin homology (PH) domains, as has been shown with mammalian AKT. PDK-1 probably phosphorylates AKT kinases PDK-1, AKT-1, AKT-2 and SGK-1, which negatively regulate the activity of the forkhead transcription factor DAF-16 (refs. 24,25) and prevent its translocation from the cytosol to the nucleus. The PI3K-AKT pathway is negatively regulated by the lipid phosphatase PTEN, which dephosphorylates and converts PIP3 to phosphatidylinositol 4,5-bisphosphate (PIP2) and blocks recruitment of AKT kinases to the plasma membrane. Mutations in components of this pathway lead to changes in lifespan, development, metabolism and stress responses in *C. elegans*. In addition, loss-of-function mutations in the *akt-1* and *akt-2* genes cause hypersensitivity to ionizing radiation–induced germ-cell apoptosis, thus suggesting that AKT kinases also have a prosurvival role in *C. elegans*.

In this study, we set out to understand how caspases inactivate cell-survival factors to promote apoptosis, and we demonstrate a new mechanism that inactivates AKT kinases. CNT-1, upon cleavage by CED-3, generates tCNT-1, which in turn acquires a potent phosphoinositide (PI)-binding activity and translocates from the cytoplasm to the plasma membrane, where it outcompetes AKT kinases for binding to PIP3, suppresses the PI3K-AKT cell-survival pathway and promotes apoptosis.

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RESULTS
CNT-1 promotes apoptosis downstream of CED-3

To identify genes encoding proteins acting downstream of CED-3 to kill cells, we performed a genetic screen to isolate suppressors of ectopic neuronal death induced by activated CED-3 (acCED-3) and isolated a recessive mutation, sm8, which defines a new gene cps-2 (CED-3 protease suppressor) on linkage group (LG) II (Online Methods).

We investigated whether cps-2 affects apoptosis by examining embryonic cell death in cps-2(sm8) animals. Compared with wild type N2 embryos, cps-2(sm8) embryos had fewer apoptotic cell corpses in the early stages of embryogenesis (comma and 1.5-fold stages) and more cell corpses in later stages (Fig. 1a), displaying a characteristic delay-of-cell-death phenotype observed in mutants defective in genes encoding proteins acting downstream of CED-3 (refs. 7, 35–37).

Three-point mapping and single-nucleotide polymorphism (SNP) mapping placed cps-2(sm8) at a position of 12103626 bp on LGII (Fig. 1b). Because there are no available fosmid or cosmid clones in this region for transformation rescue experiments, we performed an RNA interference (RNAi) screen on nine candidate genes in this region and found that RNAi knockdown of cps-2 resulted in more cell corpses in later stages (N2 embryos, Fig. 1c).

To identify genes encoding proteins acting downstream of CED-3 to induce apoptosis and is mainly expressed in dying cells. When we placed smIs111 in the ced-4(n1162) mutant, in which almost all physiological cell death is blocked, acCED-3 still induced ectopic cell death (Fig. 1c). cnt-1(tm2313) significantly reduced the number of ectopic deaths in smIs111; ced-4(n1162) embryos, thus indicating that CNT-1 is likely to act downstream of CED-3 to promote apoptosis.

CNT-1 is cleaved sequentially at two sites by CED-3

Given that CNT-1 acts downstream of CED-3, we tested whether CNT-1 is a substrate of CED-3 protease. We incubated an 35S-methionine–labeled glutathione-S-transferase (GST) fusion of the longer CNT-1 isoform, GST–CNT-1a, with CED-3 and found that it was cleaved by CED-3 to yield two species of approximately 68 and 24 kDa (Fig. 2a).

We investigated whether CNT-1 functions downstream of CED-3, using an integrated transgene (smIs111) expressing acCED-3 from the egl-1 promoter. The cell death–initiating EGL-1 functions upstream of CED-3 to induce apoptosis and is mainly expressed in dying cells. When we placed smIs111 in the ced-4(n1162) mutant, in which almost all physiological cell death is blocked, acCED-3 still induced ectopic cell death (Fig. 1c). cnt-1(tm2313) significantly reduced the number of ectopic deaths in smIs111; ced-4(n1162) embryos, thus indicating that CNT-1 is likely to act downstream of CED-3 to promote apoptosis.

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Figure 1 Cloning and characterization of cps-2. (a) Embryonic cell corpses counted in animals with the indicated genotypes. (b) Mapping and cloning of cps-2. Top, genetic map with two genetic markers (dpy-10 and rol-1) and three SNPs (pkP2069, CE2-196 and Y43F11A[1]) used for mapping cps-2(sm8). Numbers below are the numbers of recombination events (out of 105 total) that occurred between two genetic markers and three SNPs. Bottom, the ced-4 coding region, the sm8 mutation, the tm2313 deletion and the results of rescue of the cps-2(sm8) mutant by the P CNT-1a::3xFlag and P cis CNT-1a::3xFlag constructs. Three independent transgenic arrays (numbers inside parentheses) were examined for each rescue experiment, and the number of arrays that rescued the cell-death defect of cps-2(sm8) is indicated. ∆Δ indicates the A nucleotide deleted in cps-2(sm8). (c) Embryonic cell corpses counted in animals with the indicated genotypes. In a and c, average number of cell corpses ± s.d. (n = 15 embryos at each stage) are shown. *P < 0.001; **P < 0.05 by two-way ANOVA followed by Bonferroni comparison.
has the same 564-amino acid C terminus, including a PH domain, but a different 177-residue N-terminal sequence (Asp298, Asp424 and Asp525). We observed similar CED-3–cleavage patterns with GST–CNT-1b and GST–CNT-1b mutants carrying D1E, D2E or D3E (Fig. 2b). These data indicate that D1 is the primary CED-3–cleavage site in CNT-1 that is different from CNT-1a.

To examine whether CNT-1 is a CED-3 substrate in vivo, we probed for CNT-1–cleavage products in worm lysates by using an antibody raised against the N-terminal cleavage product of CNT-1b. We observed similar CED-3–cleavage patterns with GST–CNT-1b and GST–CNT-1b mutants carrying D1E, D2E or D3E (Fig. 2b). These data indicate that D1 is the primary CED-3–cleavage site in CNT-1 and is required for subsequent CED-3 cleavage at D3.

Cleavage of CNT-1 activates tCNT-1 proapoptotic activity

We determined whether CNT-1 cleavage by CED-3 is important for apoptosis (Fig. 3). Expression of CNT-1a or CNT-1b from a globally active dpy-30 promoter (P\(_{\text{dpy-30}}\)) fully rescued the delay-of-cell-death defect in cnt-1(tm2313) animals (Fig. 3a and Supplementary Fig. 2a). However, P\(_{\text{dpy-30}}\)CNT-1a(D1E) and P\(_{\text{dpy-30}}\)CNT-1b(D1E), expressing CNT-1 mutants resistant to CED-3 cleavage, did not rescue the cnt-1(tm2313) mutant (Fig. 3b and Supplementary Fig. 2b), thus indicating that cleavage of CNT-1 by CED-3 is critical for its proapoptotic function. Expression of tCNT-1a or tCNT-1b at 20% of the endogenous CNT-1 level (Supplementary Fig. 2c–d) led to increased cell death in most embryonic stages compared to that in wild type and cnt-1(tm2313) embryos (Fig. 3c and Supplementary Fig. 2e). The increased numbers of cell corpses observed in animals carrying P\(_{\text{dpy-30}}\)FN2)CNT-1a or P\(_{\text{dpy-30}}\)FN2)CNT-1b were due to ectopic cell death, because some pharyngeal cells that normally live were randomly lost in transgenic larvae (Supplementary Table 3). In comparison, coexpression of the other two CNT-1a CED-3–cleavage fragments, CNT-1a F2 and CNT-1a F3 (Fig. 2a), did not induce ectopic cell death or rescue the cnt-1(tm2313) mutant (Fig. 3d), whereas coexpression of all three CED-3–cleavage products did (Supplementary Fig. 2f). These results indicate that tCNT-1, activated by CED-3 cleavage, is responsible for CNT-1’s proapoptotic activity.

CNT-1 promotes cell death by inhibiting the AKT pathway

Because the only recognizable motif in tCNT-1 is the PH domain (Fig. 2), a potential PI-binding domain\(^{41}\), and because one of the PI species, PIP\(_2\), activates AKT kinases (also containing a PH domain) to promote cell survival\(^{39}\), we asked whether C. elegans AKT kinases participate in regulating apoptosis and genetically interact with cnt-1. An activating mutation in akt-1, akt-1(mg144gf)\(^{22}\), caused a delay-of-cell-death defect similar to that of the cnt-1(tm2313) mutant.
Because the ced-3(n2438) double mutant had more extra undead cells than ced-3(n2438) or akt-1(mg144gf) single mutants (Supplementary Table 4), this suggests that AKT-1 inhibits cell death. Epistasis analysis with animals carrying a P_dpy-30CNT-1a transgene showed that akt-1(mg144gf) completely blocked ectopic cell death induced by P_dpy-30 tCNT-1a (Fig. 4b), a result indicating that AKT-1 probably acts downstream of CNT-1 to inhibit cell death. In agreement with this result, akt-1(mg144gf) suppressed ectopic cell death induced by smls111, a transgene expressing acCED-3, to the same extent as ced-1(tm2313) (Fig. 1c), thus indicating that AKT-1 can act downstream of CED-3 to promote cell survival.

We also examined animals carrying a if mutation in akt-1, akt-2 or sgk-1, encoding three AKT kinases acting in parallel in the IIS pathway,22,23 akt-1(tm399), akt-2(tm1075) and sgk-1(ok538) each did not cause a detectable cell death defect, whereas all three combinations of double mutations and the triple mutation combination caused increased cell death throughout embryogenesis (Supplementary Fig. 3a), thus indicating that the presence of at least two AKT and SGK kinases is important for cell survival. Because ced-1(tm2313) failed to suppress ectopic cell death in the triple mutant (Fig. 4c and Supplementary Table 5), these results suggest that CNT-1 functions upstream of AKT-1, AKT-2 and SGK-1 to promote cell death.

AKT kinases phosphorylate proteins to transduce signals in various pathways.22,23,24 To examine whether the kinase activity of AKT-1 is important for its antiapoptotic activity, we expressed kinase-defective mutants of AKT-1 and AKT-2, AKT-1(K222M) and AKT-2(K209M), in the akt-1(tm399); akt-2(tm1075) mutant.22 Expression of wild-type AKT-1 or AKT-2 rescued the increased-cell-death phenotype of the akt-1(tm399); akt-2(tm1075) mutant, whereas expression of AKT-1 (K222M) or AKT-2 (K209M) did not (Supplementary Fig. 4a,b), thus indicating that the kinase activity of AKT-1 is required for its prosurvival function.

A key substrate of AKT kinases in the IIS pathway is DAF-16, a homolog of the FOXO transcriptional factor.25,26,27 A daf-16 if mutation, mu868, caused a delay-of-cell-death defect similar to that of ced-3(n2313) or akt-1(mg144gf) animals but did not enhance their cell-death defects (Supplementary Fig. 3b,c), thus indicating that DAF-16, AKT-1 and CNT-1 function in the same pathway to affect apoptosis. Moreover, daf-16(mu868) fully suppressed ectopic cell death induced by tCNT-1a (Supplementary Fig. 3d) and increased the number of extra undead cells in ced-3(n2438) animals (Supplementary Table 4), suggesting that DAF-16 acts downstream of CNT-1 to promote cell death.

CNT-1 acts downstream of PI3K to promote cell death

C. elegans AKT kinases are activated by PIP3, and therefore are regulated by AGE-1, a PI3K that phosphorylates PIP3 to generate PIP4 (refs. 19,20). An age-1(mg44) if mutation caused an increased-cell-death phenotype similar to that of akt-1(tm399); akt-2(tm1075) sgk-1(ok538) animals (Fig. 4c,d) but did not enhance the increased-cell-death phenotype caused by P_dpy-30CNT-1a (Supplementary Fig. 3e), thus suggesting that AGE-1 inhibits and tCNT-1 promotes apoptosis in the same pathway. Conversely from what we observed with the akt-1(0f); akt-2(0f) sgk-1(0f) triple mutant (Fig. 4c), ced-1(tm2313) blocks both the increased cell death and the missing-cell phenotypes caused by age-1(mg44) (Fig. 4d and Supplementary Table 5), thus indicating that CNT-1 probably acts downstream of AGE-1 to promote apoptosis.

PI3P is dephosphorylated and converted to PIP2 by the PTEN phosphatase5,9, which is encoded by daf-18 in C. elegans.28-31 The daf-18(e1375) if mutation caused a delay-of-cell-death phenotype similar to that of ced-1(tm2313) animals (Fig. 4e) and did not exacerbate the ced-1(tm2313) cell-death defect, thus suggesting that DAF-18 and CNT-1 act in the same pathway. daf-18(e1375) also increased the number of extra cells in ced-3(n2438) animals (Supplementary Table 4), daf-18(e1375), however, did not block ectopic cell death induced by tCNT-1a (Fig. 4f), a result indicating that DAF-18 acts upstream of, or in parallel to, CNT-1 to promote cell death.
death, possibly at the level of PIP3 regulation. When taken together, our results suggest that CNT-1 and DAF-18 act in the same pathway to promote cell death and that CNT-1 operates downstream of AGE-1 and upstream of AKT-1, AKT-2, SGK-1 and DAF-16.

Cleavage of CNT-1 activates its PI binding
Given that AGE-1 activates AKT kinases through PIP3, and DAF-18 inactivates this pathway through dephosphorylation of PIP3, we hypothesized that tCNT-1, with a PH domain, promotes cell death by interfering with the binding of PIP3 by AKT kinases. We examined whether tCNT-1 binds PIP3 by using an in vitro lipid binding assay. 35S-methionine–labeled GST–CNT-1a on its own did not bind any lipid but showed strong binding to PIP3, PIP2 and PIP3 and weak binding to phosphatidic acid and cardiolipin after pretreatment with CED-3 (Fig. 5a). GST–tCNT-1a displayed an identical lipid binding pattern to that of CED-3–treated GST–CNT-1a (Fig. 5a), and so did GST–CNT-1b pretreated with CED-3 and GST–tCNT-1b (Fig. 5a). When we altered one of the highly conserved PI-binding lysine residues in the PH domain of CNT-1a (K284A)44, neither CNT-1a(K284A) pretreated with CED-3 nor tCNT-1a(K284A) showed any lipid binding activity (Fig. 5a). Moreover, expression of CNT-1a(K284A) or tCNT-1a(K284A) in ced-1 (tm2313) animals did not rescue the cell-death defect or cause ectopic cell death (Fig. 3e,f). Because the K284A mutation did not alter the expression levels of CNT-1a proteins in vivo or in vitro (Supplementary Fig. 5), these results indicate that the PH domain is required for tCNT-1 to acquire PI binding after CED-3 cleavage and to promote apoptosis.

tCNT-1 blocks PIP3 binding by AKT and SGK kinases
We tested whether tCNT-1 interferes with binding of AKT and SGK kinases to PIP3 (refs. 21–23). 35S-methionine–labeled CNT-1 alone bound strongly to PIP3 and weakly to cardiolipin, and this binding was not affected by addition of unlabeled GST–CNT-1a or GST–tCNT-1b (Fig. 5b). Addition of GST–CNT-1a or GST–tCNT-1b pretreated with CED-3 completely blocked PIP3 binding by AKT-1 (Fig. 5b), whereas incubation of AKT-1 with CED-3 did not alter AKT-1 lipid binding (Fig. 5b). Likewise, both AKT-2 and SGK-1 bound strongly to PIP3, and this binding was blocked by GST–CNT-1a pretreated with CED-3 (Fig. 5c). To probe how tCNT-1 inhibits PIP3 binding by AKT-1, we compared their PIP3 binding affinity. We first determined the concentrations of 35S-methionine–labeled GST–tCNT-1a and AKT-1 synthesized in rabbit reticulocyte lysate (RRL) by comparing them with known concentrations of recombinant GST–tCNT-1a and AKT-1 proteins, which were purified from bacteria and showed no lipid binding activity (Fig. 6a). Labeled GST–tCNT-1a and hexahistidine (His6)-tagged AKT-1 displayed comparable radioactive-signal intensity at the same concentrations (Fig. 6b). GST–tCNT-1a displayed strong PIP3 binding at 0.04 nM, weak binding at 0.04 nM and no binding at 0.004 nM (Fig. 6c). By contrast, AKT-1 showed strong PIP3 binding at 4 nM, weak binding at 0.4 nM and no binding at 0.04 nM (Fig. 6c), results indicating that tCNT-1a binds PIP3 with an affinity two orders of magnitude higher than that of AKT-1. Because the expression level of CNT-1 in C. elegans is approximately 70% higher than that of AKT-1 (Supplementary Fig. 6), the combination of higher CNT-1 concentrations and much higher binding affinity to PIP3 than those of AKT-1 allows tCNT-1 to block PIP3 binding by AKT kinases and thus block their activation.

tCNT-1 translocates to the plasma membrane upon CED-3 cleavage
Several proteins containing the PH domain translocate from the cytosol to the plasma membrane via a PIP3–mediated mechanism45–47. We analyzed the subcellular localization of CNT-1 by using an antibody raised against tCNT-1b (Supplementary Fig. 1d). CNT-1 localized in the cytoplasm of all cells in wild-type embryos (Fig. 7a) but was not detectable in cnt-1(tm2313) embryos (Fig. 7b), thus demonstrating the specificity of the antibody.
embryos in which we induced global apoptosis and widespread CED-3 activation through heat-shock treatment, we observed CNT-1 remaining in the plasma membrane of all cells in addition to staining in the cytoplasm (Fig. 7d and Supplementary Fig. 7c,d), results indicating that a portion of CNT-1 translocated from the cytosol to the plasma membrane upon apoptosis activation. By contrast, in smIs82 embryos without heat-shock treatment or in smls82; cnt-1(tm2313) embryos with heat-shock treatment, CNT-1 remained in the cytoplasm (Fig. 7e and Supplementary Fig. 7a,b,f), thus indicating that CNT-1 translocation from the cytosol to the plasma membrane is dependent on apoptosis and CED-3. In cnt-1(tm2313); smls82 embryos expressing CNT-1a(D1E), the CED-3–uncleavable form of CNT-1a, CNT-1a(D1E) did not translocate to the plasma membrane after heat-shock treatment (Supplementary Fig. 7g–i). In contrast, in cnt-1(tm2313); smIs82 embryos expressing CNT-1a, we observed CNT-1a on plasma membranes after heat-shock treatment (Supplementary Fig. 7j–l). These results confirm that CED-3 cleavage is required for CNT-1 translocation to the plasma membrane during apoptosis. Consistently with this, loss of cnt-1 partially suppressed and expression of ICNT-1a enhanced ectopic cell death induced by smls82 (Supplementary Note and Supplementary Fig. 4c,d), thus providing further support to the finding that cleavage of CNT-1 and activation of tCNT-1 is an important downstream event of CED-3 activation and apoptosis.
tCNT-1 inhibits association of AKT-1 with the plasma membrane

AKT-1 also contains a PH domain, and its human homolog translocates to the plasma membrane through binding to PIP3 (ref. 48). Given the limited amount of PIP3 on the plasma membrane, the higher concentration of CNT-1 than AKT-1 in vivo and the much-stronger PIP3 binding affinity of tCNT-1 than AKT-1, we examined whether tCNT-1 blocks AKT-1 translocation to the plasma membrane. We fused the first 180 residues of AKT-1, including its PH domain (residues 15–118), to GFP and expressed it from the sur-5 gene promoter (P_{sur-5}AKT-1-PH::GFP). In wild-type embryos carrying P_{sur-5}AKT-1-PH::GFP, GFP was diffuse within the cells (Fig. 7f). When we crossed this transgene into daf-18(e1375) animals, which are deficient in converting PIP3 to PIP2 and thus have elevated levels of PIP3 on the plasma membrane, we saw bright GFP signals on the plasma membrane, thus suggesting that AKT-1-PH::GFP translocated to the plasma membrane through binding to PIP3 (Fig. 7g). Expression of tCNT-1a abolished AKT-1-PH::GFP localization to the plasma membrane in daf-18(e1375) embryos (Fig. 7h), thus indicating that tCNT-1 inhibited association of AKT-1 with the plasma membrane. Similarly, in smls82; daf-18(e1375) animals carrying P_{sur-5}AKT-1-PH::GFP, some AKT-1-PH::GFP localized to the plasma membrane in the absence of heat-shock treatment (Fig. 7i). After heat-shock treatment, AKT-1-PH::GFP disappeared from the plasma membrane (Fig. 7j), a result indicating that endogenous tCNT-1 induced by smls82 is sufficient to block AKT-1 association with the plasma membrane and thus block AKT-1 activation and function.

DISCUSSION

The physiological relevance of caspase substrates is an under-studied area that limits the understanding of apoptosis. We have identified multiple components acting downstream of CED-3 to promote apoptosis, using a sensitized CED-3 protease suppressor (cps) screen (ref. 35). The cell-death defects of each cps mutant are mild or undetectable because multiple pathways downstream of CED-3 act in parallel to kill the cell (refs 5, 7, 35). In this study, we characterized one of the cps genes, cps-2 (cnt-1), whose inactivation delays cell death and suppresses apoptosis in sensitized genetic backgrounds. Importantly, CNT-1 is a substrate of CED-3 both in vivo and in vitro. A mutation blocking CNT-1 cleavage by CED-3 abolishes its proapoptotic activity, whereas tCNT-1 alone is sufficient to induce apoptosis. These results establish that CNT-1 is an in vivo CED-3 target.

Despite containing a PH domain, a potential PI-binding motif, CNT-1 does not bind any phospholipids in vitro or associate with the plasma membrane in vivo. Upon activation by CED-3 cleavage during apoptosis, tCNT-1 acquires strong binding to PIP3 and translocates to the plasma membrane, where it outcompetes AKT kinases for PIP3 binding to block recruitment and activation of AKT kinases at the plasma membrane, thereby inactivating the AKT survival pathway to accelerate cell killing (Fig. 8). Therefore, CED-3 activates a downstream death-execution event by cleaving CNT-1, which then inactivates the AKT cell-survival pathway. Our study elucidates a previously unknown regulatory link between cell-death execution and cell-survival signaling.

Given the critical roles of the AKT kinases in cell growth, survival and metabolism, the activities of AKT kinases are tightly regulated at several different levels (ref. 8). Association of AKT with PIP3 generated by PI3K at the plasma membrane is the critical first step. This is followed by sequential phosphorylation of AKT by PDK1 and mTORC2, leading to full activation of AKT. At each step, negative regulators are in place to fine-tune the level of AKT activation. The PTEN phosphatase converts PIP3 back to PIP2, reducing or blocking AKT activation (ref. 49). Dephosphorylation of AKT by phosphatases, including protein phosphatase 2A and phosphatase PHLPP, also reduces or blocks AKT activation (ref. 51). In this study, we report a new mechanism that could rapidly
shut down AKT at the level of PI3 binding and plasma-membrane recruitment, through proteolytic activation of a PH-containing protein to generate a competing PI-binding activity. Given the presence of hundreds of PH-domain proteins with unknown functions in eukaryotes, this could be a general and conserved mechanism for regulating membrane-associated cell signaling or activity. There are several potential human CNT-1 homologs, ACAP1, ACAP2 and ACAP3, but none has been reported to have a role in apoptosis. It will be interesting to investigate whether these ACAP proteins might act like CNT-1 to inhibit AKT survival signaling in humans.

AKT kinases and PI3K are required for the IIS pathway in C. elegans. Inactivation of these genes increases life span and stress resistance. Interestingly, loss of cnt-1 does not affect these two processes (Supplementary Fig. 8), thus indicating that CNT-1 is an apoptosis-specific inhibitor of the PI3K-AKT pathway. AKT is a major drug target in treating cancer; however, clinical development of AKT inhibitors has been restricted by AKT’s involvement in multiple important cellular events, which inevitably will lead to numerous on-target or off-target side effects. Targeted inactivation of AKT in cancer cells to induce apoptosis through a CNT-1–like mechanism could present a new therapeutic strategy when combined with other anticancer therapies.

METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.N. and D.X. conceived the research and designed experiments. A.N. carried out and analyzed experiments. K.D.S. assisted in some experiments. A.N. and D.X. wrote the paper.

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Isolation of the cps-2(sm98) mutation. Ethyl methane sulfonate (EMS) mutagenesis was performed on animals carrying an integrated transgene (sm98) containing both P_mec-3::GFP and P_mec-3::GFP. P_mec-3::GFP is an integrated transgene which was also labeled by GFP (P_gfp::GFP). Progeny of the mutagenized animals were screened for mutants with increased survival of the fluorescent mechanosensory neurons. From a screen of 3,000 mutagenized haploid genomes, we isolated several recessive mutations, including sm98, which define a new gene, cps-2 (CDS-3 protease suppressor) on linkage group (LG) II.

Quantification of cell corpses and extra cells. The number of cell corpses in living embryos and the number of extra cells in the anterior pharynx of L4 larvae were scored with Nomarski optics as described previously.

CND-1 antibody. Amino acids 1–298 of CND-1 (CNT-1b) fused with glutathione S-transferase (GST) were expressed in Escherichia coli strain BL21(DE3) and affinity purified from the soluble fraction of the bacterial lysate with glutathione Sepharose 4B beads (GE Healthcare). Rats were immunized with purified GST–CNT-1a proteins (Spring Valley Laboratories). CND-1 antibodies were affinity purified (Spring Valley Laboratories) from terminal bleeds with purified GST–CNT-1a proteins as described previously.

Immunoblotting analysis. C. elegans embryos were harvested with M9, centrifuged at 500 g for 30 min, and then sonicated in PBS buffer. After centrifugation at 25,400 g for 30 min, 2x SDS loading buffer was added, and samples were heated at 95 °C for 3 min. The samples were resolved with 15% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was first blocked with 5% nonfat dry milk in PBS-T (PBS plus 0.1% Tween 20) and washed twice with PBS-T for 5 min each. The membrane was then incubated with affinity-purified anti–CNT-1 antibody in 1:1,000 dilution for 1 h at room temperature and washed three times with PBS-T for 5 min each. CND-1 antibodies were affinity purified (Spring Valley Laboratories) from terminal bleeds with purified GST–CNT-1a proteins as described previously.

Transgenic worms. Transgenic animals were generated as described previously, except for the expression vector Punc-1 (141). See the ON的方法 section for details.

Molecular biology. Full-length cnt-1a cDNA and cnt-1b cDNA were amplified with reverse-transcription PCR (RT-PCR) and then subcloned into the PET-41b vector via its SpeI and NotI sites to generate the pet-41b-CNT-1a and pet-41b-CNT-1b expression vectors. For the various pet-41b–CND-1 mutant expression vectors (pet-41b-CNT-1a(D1E), pet-41b-CNT-1a(D2E), pet-41b-CNT-1a(D3E), pet-41b-CNT-1b(D1E), pet-41b-CNT-1b(D2E), pet-41b-CNT-1b(D3E), and pet-41b-CNT-1a(K284A)), pet-41b-CNT-1a or pet-41b-CNT-1b was used as a DNA template to make the indicated amino acid substitutions with a QuikChange mutagenesis kit (Stratagene).
pET-41b-cNT-1a and pET-41b-cNT-1b were constructed by subcloning of a PCR fragment encoding amino acids 1–382 of CNT-1a and 1–298 of CNT-1b into pET-41b via SpeI and EcoRV sites. For P\textsubscript{dpy-30}cNT-1a ∼ 3×Flag, a 1,944-bp SphI–NheI PCR fragment containing the cnt-1 promoter was fused with a 2,640-bp NheI–SmaI PCR fragment containing the full-length cnt-1a cDNA, which was then subcloned into a modified pGEM7.79 vector in which the gfp coding region was replaced with a 3× Flag sequence. For the P\textsubscript{act} promoter of the AKT-1 gene, cDNAs were PCR amplified and subcloned into a modified pPD95.79 vector in which the NheI–SmaI PCR fragment containing the full-length cNT-1a and cNT-1b cDNAs were PCR amplified and subcloned into pS235, which contains the promoter of the C. elegans dpy-30 gene, via Sphtl and Xmal sites. QuikChange mutagenesis was then performed to obtain P\textsubscript{dpy-30}cNT-1a (D1E) and P\textsubscript{dpy-30}cNT-1b (D1E). P\textsubscript{dpy-30}cNT-1a and P\textsubscript{dpy-30}cNT-1b were constructed by subcloning of a PCR fragment encoding amino acids 1–382 of CNT-1a and 1–298 of CNT-1b into pS235 via its NheI and Xmal sites. For P\textsubscript{dpy-30}cNT-1a F2 and P\textsubscript{dpy-30}cNT-1a F3, PCR fragments encoding amino acids 383–609 and 610–826 of CNT-1a were subcloned into pS235 via its NheI and Xmal sites. P\textsubscript{dpy-30}cNT-1a(K284A) and P\textsubscript{dpy-30}cNT-1a(K284A) were generated by QuikChange mutagenesis. For the P\textsubscript{et-21b} AKT-1, P\textsubscript{et-21b} AKT-2, and P\textsubscript{et-21b} SGK-1, PCR fragments encoding full-length CNT-1a and 1–298 of CNT-1b into pS235 via its NheI and XmaI sites. QuikChange mutagenesis was then performed to obtain P\textsubscript{et-21b} AKT-1, P\textsubscript{et-21b} AKT-2, and P\textsubscript{et-21b} SGK-1, full-length cDNAs were PCR amplified and subcloned into pET-41b through its NheI and XhoI sites. For the P\textsubscript{dpy-30} AKT-1-1β′-HIS, which contains the CNT-1a promoter, negatively regulates LET-60 Ras activity during vulval induction. Nat. Cell Biol. 9, 541–549 (2007).

Determination of protein expression levels in C. elegans. 200 wild-type N2 C. elegans embryos at 1.5-fold stage were collected in PBS buffer and sonicated before addition of 2× SDS buffer. The N2 embryonic lysate and 1 pmol of GST–CNT-1a or AKT-1–His6 proteins purified from bacteria were resolved by SDS–PAGE and subsequently subjected to immunoblotting with an affinity-purified anti–CNT-1 antibody at 1:500 dilution in PBS for 1 h. After the embryos were washed three times with PBS, they were stained with Alexa Fluor 488–conjugated anti-rat IgG antibody (Molecular Probes, cat. no. A-11006; validation provided on the manufacturer’s website) at 1:1,000 dilution in PBS for 1 h. They were then washed three times with PBS and visualized with an Axioplan 2 Nomarski Microscope (Carl Zeiss MicroImaging).

RT-PCR. The primer sequences for cnt-1 were 5′-CTCTTGCTGCAAGAC TGGATGC-GGCC-3′ and 5′-CGTTGCTGGGAACGGCGGAC-3′, and the primer sequences for rpl-26 were 5′-ATGAAGTCAATCCCCTGCTGTT-3′ and 5′-AGGACAGTCCAGTGTTCC-3′.

Lifespan and thermotolerance assay. For lifespan analysis, animals were grown at 20 °C until the L4 larval stage and then transferred to new plates (ten animals per plate) at 25 °C. Animals were scored every day subsequently and moved periodically to keep growth conditions mild free. Animals were scored as dead if they failed to respond to a gentle tap on the head and tail with a platinum wire. For thermotolerance assay, animals were grown at 20 °C until the L4 larval stage and then transferred to new plates (ten animals per plate) at 33 °C. Animals were scored every 2 h for the first 24 h and subsequently scored every 12 h until 60 h. Animals were scored as dead if they failed to respond to a gentle tap on the head and tail with a platinum wire.

RNAi experiments. RNAi experiments were carried out with a bacteria-feeding protocol described previously.

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