Programmed elimination of cells by caspase-independent cell extrusion in *C. elegans*

Daniel P. Denning¹, Victoria Hatch¹ & H. Robert Horvitz¹

The elimination of unnecessary or defective cells from metazoans occurs during normal development and tissue homeostasis, as well as in response to infection or cellular damage. Although many cells are removed through caspase-mediated apoptosis followed by phagocytosis by engulfing cells, other mechanisms of cell elimination occur, including the extrusion of cells from epithelia through a poorly understood, possibly caspase-independent, process. Here we identify a mechanism of cell extrusion that is caspase independent and that can eliminate a subset of Caenorhabditis elegans cells programmed to die during embryonic development. In wild-type animals, these cells die soon after their generation through caspase-mediated apoptosis. However, in mutants lacking all four *C. elegans* caspase genes, these cells are eliminated by being extruded from the developing embryo into the extra-embryonic space of the egg. The shed cells show apoptosis-like cytological and morphological characteristics, indicating that apoptosis can occur in the absence of caspases in *C. elegans*. We describe a kinase pathway required for cell extrusion involving PAR-4, STRD-1 and MOP-25.1/-25.2, the *C. elegans* homologues of the mammalian tumour-suppressor kinase LKB1 and its binding partner STRADα and MO25α. The AMPK-related kinase PIG-1, a possible target of the PAR-4–STRD-1–MOP-25 kinase complex, is also required for cell shedding. PIG-1 promotes shed-cell detachment by preventing the cell-surface expression of cell-adhesion molecules. Our findings reveal a mechanism for apoptotic cell elimination that is fundamentally distinct from that of canonical programmed cell death.

The caspase CED-3 is essential for nearly all programmed cell deaths that occur during *C. elegans* development. However, a few cells undergo programmed cell death in ced-3 mutants. We observed that some cells are eliminated from ced-3-mutant embryos by being shed from the developing animal. The eggs of ced-3 mutants but not those of wild-type animals contained on average six shed cells that had detached during the comma stage of embryogenesis (≈300 min after fertilization) (Fig. 1a–c, f and Supplementary Table 1). The shed cells detached at the anterior sensory depression or the ventral pocket (Fig. 2a, b) and remained in the egg (Supplementary Table 1). The shed cells showed apoptosis-like cytological and morphological characteristics, indicating that apoptosis can occur in the absence of caspases in *C. elegans*. We describe a kinase pathway required for cell extrusion involving PAR-4, STRD-1 and MOP-25.1/-25.2, the *C. elegans* homologues of the mammalian tumour-suppressor kinase LKB1 and its binding partner STRADα and MO25α. The AMPK-related kinase PIG-1, a possible target of the PAR-4–STRD-1–MOP-25 kinase complex, is also required for cell shedding. PIG-1 promotes shed-cell detachment by preventing the cell-surface expression of cell-adhesion molecules. Our findings reveal a mechanism for apoptotic cell elimination that is fundamentally distinct from that of canonical programmed cell death.

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The *C. elegans* genome encodes three additional caspase homologues: csp-1, csp-2 and csp-3 (ref. 9). We determined that individual csp mutations did not cause the appearance of shed cells (Supplementary Fig. 1 and Supplementary Table 2). Eggs from quadruple mutants lacking all four caspase genes (csp-1, csp-2, csp-3 and ced-3), like ced-3-mutant eggs, contained on average six shed cells (Fig. 1c, e), indicating that the generation of shed cells is caspase independent.

Although caspase activation can drive apoptosis, recent studies have suggested that caspases are not necessary for apoptosis. We therefore examined csp-3; csp-1; csp-2 ced-3 quadruple-mutant (csp-3; csp-1; csp-2 ced-3) shed cells for apoptotic characteristics, specifically phosphatidylyserine exposure and TdT-mediated dUTP nick end labelling (TUNEL)-reactive DNA fragments. Like floaters that undergo caspase-mediated apoptosis

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1Howard Hughes Medical Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

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(Supplementary Fig. 2)*, the csp-α shed cells were reactive to the phosphatidylserine-binding protein MFG-E8 and to TUNEL staining (Fig. 1h, i). Also, ced-3-mutant shed cells showed chromatin condensation (darkly staining nuclear material) and separation of the nuclear envelope double membrane in transmission electron micrographs (Fig. 1f and Supplementary Fig. 3). These apoptotic features were present in ced-5-mutant floaters (Fig. 1g and Supplementary Fig. 3), although the cytoplasm of ced-5-mutant floaters were more compact. We conclude that the shed cells of embryos lacking caspase activity are in many respects cytologically and morphologically apoptotic, indicating that caspases are dispensable for many cellular changes that occur during apoptosis.

The somatic cell lineage of C. elegans is essentially invariant\(^{10,11}\), allowing the precise identification of cell origins and fates. To determine the cellular identities of ced-3-mutant shed cells, we recorded time-lapse videos of developing ced-3-mutant embryos and traced the lineages of extruded cells in reverse (Fig. 2a, b and Supplementary Movie 1). We identified seven different cells eliminated by shedding from ced-3-mutant embryos (Fig. 2c), all of which are cells that normally die during wild-type embryogenesis. This finding is consistent with our observation that ced-3-mutant shed cells expressedegl-1 (Fig. 2d), the transcription of which initiates programmed cell death\(^{12}\), and with a previous report in which ABAlppapa (a cell fated to die) was observed detaching from a ced-3-mutant embryo\(^{7}\). The cells that can be shed are among the first to die in the wild-type embryo: 14 cells die within the first 300 min of development, and 7 of the 8 identified shed cells are among this group of 14 cells\(^{11}\). Thus, specific cells fated to die early in embryogenesis can be eliminated by either canonical caspase-dependent apoptosis or by caspase-independent shedding.

To identify factors required for cell shedding, we tested genes involved in different cell-death processes and found that the generation of ced-3-mutant shed cells did not require genes that mediate germline apoptosis, cell-corpus engulfment, necrosis, autophagy or lin-24-mediated cell death (Supplementary Tables 1 and 3). We therefore developed a screening strategy based on the hypothesis that ABplpapp (one of the shed cells we identified; Fig. 2a, c) might survive and adopt a fate associated with a lineally related cell in animals doubly defective in the canonical cell-death pathway and cell shedding. The sister cell of ABplpapp generates RMEV (a GABAergic (γ-aminobutyric acid-containing) neuron) and the excretory cell (Fig. 3a), which functions in osmoregulation\(^{13}\). We generated a ppg-12:cNLS:GFP transgene to express green fluorescent protein (GFP) specifically in the excretory cell and observed that wild-type and ced-3-mutant animals contain a single GFP-positive cell (Fig. 3b, d). Using this reporter, we screened mutagenized ced-3-mutant animals and identified double mutants with a single-excretory-cell (Tex) phenotype. Two such Tex isolates, n5433 and n5437, are alleles of the gene pig-1 (Supplementary Fig. 4). Single mutants defective in a null allele of pig-1(gm344A) had one excretory cell (Fig. 3d), whereas 89% of pig-1(gm344) ced-3(n3692) double mutants contained two GFP-positive nuclei that resembled the large nucleus of the excretory cell (Fig. 3c, d and Supplementary Fig. 4). Double mutants with pig-1 and ced-4, ced-9 (gain-of-function) oregl-1 mutations were similar to pig-1 ced-3 mutant animals (Supplementary Table 4). Inactivation of pig-1 by RNA interference (RNAi) treatment phenocopied gm344, n5433 and n5437, confirming that loss of pig-1 function caused the Tex phenotype in these mutants (Supplementary Table 7).

The LIN-3 epidermal growth factor ligand is expressed embryonically by the excretory cell\(^{14}\) and, pig-1 ced-3-mutant embryos contain an extra cell that expressed lin-3 (Supplementary Fig. 5a). Furthermore, the heads of pig-1 ced-3 mutant animals contained large cysts (Fig. 3e) similar to those of mutants with defective excretory cell function\(^{15}\). Thus, pig-1 ced-3 mutants generated an ectopic excretory cell, albeit one that was defective in osmoregulation or interfered with the function of the endogenous excretory cell.

To address whether the ectopic excretory cell of pig-1 ced-3 mutants is derived from the un-shed ABplpapp cell, we examined directly the fate of ABplpapp in pig-1 and pig-1 ced-3-mutant embryos (Supplementary Movies 2 and 3). In pig-1-mutant embryos (and as in wild type), ABplpapp became a highly refractile cell corpse within 45 min of its generation (Fig. 3f; data not shown). By contrast, ABplpapp survived and divided approximately 115 min after it was generated in pig-1 ced-3-mutant embryos (Fig. 3g). In the three pig-1 ced-3-mutant embryos we examined, neither ABplpapp nor its descendants detached from the embryo, suggesting that an ABplpapp descendant gives rise to the ectopic excretory cell in pig-1 ced-3-mutant larvae. As reported previously\(^{15}\), pig-1 ced-3 animals also contain ectopic RME-like neurons (Supplementary Fig. 5b and Supplementary Table 5), suggesting that when ABplpapp survives it generates both an ectopic RME-like and an ectopic excretory-like cell.

pig-1 inactivation by mutation or RNAi treatment reduced the number of shed cells in ced-3-, ced-4- or ced-9 (gain-of-function)-mutant embryos by nearly 75% (P < 0.5 × 10⁻⁷ for each pair-wise comparison, Student’s t-test; Fig. 3h; data not shown), demonstrating that pig-1 is generally required for the generation of shed cells. Given this observation, the effects of pig-1 on cell shedding are in several ways comparable to the effects of ced-3 on programmed cell death: (1) ced-3 affects most programmed cell deaths and pig-1 similarly affects most shed cells, indicating that ced-3 and pig-1 act generally to drive programmed cell death and generate shed cells, respectively; (2) like programmed cell deaths, extruded cells share morphologic and genetic properties and can be viewed as expressing a specific cell fate; and (3), like mutations in ced-3, mutations in pig-1 cause cells that should die to express the fates of cells that normally survive.

pig-1 encodes a homologue of MELK, an AMPK-related serine-threonine kinase required cell autonomously for the asymmetric cell divisions of many C. elegans neuroblast\(^{15,16}\). Mammalian AMPK-related kinases control metabolism and cell polarity\(^{17}\) and are activated through phosphorylation of a conserved threonine within their T-loop domains by the kinase LKB1 (also known as STK11) (ref. 18) and its complex partners STRAD\(^{18}\) and MO25\(^{19}\) (also known as CAB39) (refs 19, 20). The PIG-1 T-loop threonine (T169) was necessary for a comparable to the effects of pig-1 on cell shedding outlined above.
**Figure 3** | The LKB1 homologue PAR-4 and the AMPK-related kinase PIG-1 are required for cell shedding from ced-3 embryos. a. The sub-lineage that produces the shed cell ABplpappap, which is the lineage aunt of the neuron RME-1 and the excretory cell (exc. cell). b, c. Merged DIC and fluorescence micrographs of wild-type (b) and pig-1(gm344) ced-3(n3692) (c) larvae containing the transgene nls434[P_{pig-1:zgfp}], which expresses GFP in the excretory cell. Arrowheads indicate excretory and ectopic excretory-like cells. d. Percentage of L3 larvae with ectopic excretory cells. All genotypes contained nls434[P_{pig-1:zgfp}]. e. The head of a larval pig-1(gm344) ced-3(n3692) animal containing large cysts (asterisks). f, g. The fate of the cell ABplpappap in pig-1(gm344) and pig-1(gm344) ced-3(n3692) embryos. i. ABplpappap in a pig-1 mutant embryo shown 5 min after its generation and shortly after it underwent programmed cell death 45 min later. j. ABplpappap in a pig-1 ced-3 mutant embryo shown 5 min after its generation and immediately after it divided 115 min later. k, l. pig-1 and strd-1 are required for cell shedding. Mutation of pig-1 and of strd-1 reduced the number of shed cells in ced-3, ced-4 or ced-9 (gain-of-function (gf)) eggs. Error bars denote s.d. **P < 5 x 10^-7**, Student’s t-test. i. The T-loop threonine (T169) of PIG-1 is required for the elimination of ABplpappap. Average percentage of larvae with ectopic excretory cells from multiple pig-1(gm344) ced-3(n3692) lines carrying the following pig-1 transgenes: pig-1(wt), the wild-type pig-1 genomic locus (three lines, n = 42, 47 and 58); pig-1(null), two STOP codons in the first exon (three lines, n = 40, 43 and 45); pig-1(T169A), threonine 169 changed to alanine (three lines, n = 40, 41 and 48); and pig-1(T169D), threonine 169 changed to aspartic acid (five lines, n = 31, 32, 43, 48 and 50). Error bars denote s.e.m. **P > 0.05** (Student’s t-test). NS, not significant (P < 10^-3). j-I. Merged DIC and fluorescence micrographs of ced-3(n3692) par-4(RNAi) (j), ced-3(n3692) strd-1(RNAi) (k), and mop-25.2(ok2073); ced-3(n3692); mop-25.1(RNAi) (l) larvae carrying nls434[P_{pig-1:zgfp}]. Arrowheads indicate excretory and ectopic excretory-like cells. m. Redundant pathways mediate the elimination of ABplpappap and other cells shed from ced-3-mutant embryos. Mammalian counterparts of proteins involved in cell elimination are shown in orange and blue. Scale bars, 10 μm.

pig-1 transgene to rescue the ectopic excretory cell defect of pig-1 ced-3 mutants, whereas changing T169 to a phosphomimetic aspartic acid bypassed this requirement (Fig. 3i), indicating that PIG-1 is probably activated by phosphorylation of its T-loop. We therefore tested the C. elegans homologues of LKB1, STRAD and MO25 (par-4, strd-1 and paralogues mop-25.1, mop-25.2 and mop-25.3, respectively) for roles in the elimination of ABplpappap, par-4 or strd-1 inactivation caused the Tex phenotype in ced-3 mutants (Fig. 3j, k and Supplementary Table 6). Furthermore, the ok2283 deletion allele of strd-1 reduced the number of shed cells in ced-3-mutant embryos by 67% (P = 1.9 x 10^-10, Student’s t-test; Fig. 3h). The inactivation of both mop-25.1 and mop-25.2 was necessary to cause the Tex defect in ced-3 mutants (Fig. 3l and Supplementary Table 6), indicating redundant function. We conclude that both PIG-1 and the PAR-4–STRAD–MO25 complex are required for cell shedding in C. elegans.

Our genetic analyses suggest that PIG-1 and PAR-4 function in the same pathway. The deletion mutation ok2283, a putative null allele of strd-1 (Supplementary Table 8), failed to enhance any of the pig-1 or pig-1 ced-3 defects (Fig. 4i, h and Supplementary Fig. 6 and Supplementary Tables 8 and 9). Although, par-4 and strd-1 can act independently^21, this result suggests that PIG-1 and PAR-4 and its binding partners function in the same pathway. We believe that PIG-1 is the phosphorylation target of PAR-4 in cell shedding because inactivation of PIG-1 but of no other AMPK-related kinase caused the survival of ABplpappap in ced-3 mutants (Supplementary Table 7). The Tex defect of pig-1(gm344) ced-3(n3692) animals (89%) was higher than that of strd-1(ok2283); ced-3(n3692) animals (43%) (Fig. 3d and Supplementary Table 4). Thus, STRD-1 and possibly the entire PAR-4 complex are required partially for PIG-1 activation, indicating that other factors, or PIG-1 itself through autophosphorylation, also stimulate PIG-1 activity. Indeed, MEK phosphorylates itself in vitro^18.

To investigate how PIG-1 regulates cell shedding, we reasoned that shed cells must be deficient in adhesive contacts with the embryo and explored whether pig-1 modulates cell-adhesion complexes, specifically adherens junctions. In C. elegans, adherens junctions comprise a cadherin–catenin complex and a complex including DLG-1 (a homologue of the Drosophila discs large protein) and AJM-1. Both complexes participate in the enclosure of the embryo within a layer of epidermal cells^22, a major morphogenetic event that coincides with the detachment of shed cells.

We examined the expression of AJM-1, DLG-1, HMP-1 (β-catenin) and JAC-1 (p120 catenin) fused to GFP in shed cells. AJM-1, DLG-1
and HMP-1 were not visible at the surface of ced-3- or ced-4-mutant shed cells that had recently detached from either the anterior sensory depression (Fig. 4a, c and Supplementary Fig. 7b) or the ventral cleft (Fig. 4b and Supplementary Fig. 7a) (JAC-1::GFP was detectable in adjacent epidermal cells (Fig. 4a–c and Supplementary Fig. 7). ABplpappap in ced-3-mutant embryos remained at the ventral surface of the embryo from its generation until its detachment and never expressed HMP-1::GFP (Fig. 3m). These results demonstrate that the delay in corpse clearance was after engulfment and reflected a defect in corpse degradation, a process that requires endocytic pathway components23.

Interestingly, a recent study showed that two GTPase genes, grp-1 and arf-6, and a gene coding for an ARF GTPase-activating protein, ctn-2, have functions in cell-fate determination similar to those of pig-1 as well as roles in receptor-mediated endocytosis24, 25. Mammalian ARF GTPases function in endocytosis25 and can remove cadherin complexes from the cell surface26. We noted that inactivation of ARF GTPase genes arf-1.2 or arf-3 or the ARF guanine exchange factor gene grp-1 also produced ectopic excretory cells in ced-3 mutants (Fig. 4k and Supplementary Table 10).

Taken together, our observations suggest that the PAR-4 complex, pig-1 and ARF GTPases promote the detachment of shed cells through the endocytosis-mediated removal of cell-adhesion molecules from the cell surface. Thus, the programmed elimination and apoptosis of at least eight C. elegans cells can be accomplished through either canonical caspase-mediated apoptosis involving the engulfment of dying cells or a caspase-independent shedding mechanism that also results in apoptosis and that requires the PAR-4 complex and the AMPK-related kinase PIG-1 (Fig. 3m). These two mechanisms are functionally redundant, as ABplpappap and the other shed cells survive only in mutants in which both pathways are disrupted.

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Several lines of evidence suggest that pig-1 promotes shed-cell detachment through endocytosis. Many cell corpses, including the ABplpappap corpse, were not cleared efficiently in pig-1-mutant embryos (Fig. 4h, i). The pig-1-mutant cell corpses were encircled rapidly by the engulfment receptor CED-1 and stained positively with acridine orange, a marker of internalized corpses (Fig. 4j and Supplementary Table 10). Cells expressing the caspase inhibitor CED-9 were efficiently engulfed by the excretory cell, indicating that a loss of PAR-4 might facilitate cell shedding by preventing the expression of cell-adhesion molecules on the surfaces of shed cells.
We propose that cell shedding is an evolutionarily conserved mechanism of cell elimination. Many epithelia extrude cells constitutively to maintain tissue homeostasis, and the shed epithelial cells of vertebrates share features with the shed cells of caspase-deficient C. elegans embryos. First, like other shed epithelial cells, shed intestinal enterocytes frequently show apoptotic markers,27,28, including caspase-3 activation and TUNEL reactivity. Second, despite the apoptotic appearance of shed enterocytes, the intestinal epithelia of Casp3−/−, Apg1−/−, Bax−/−, Bak1−/− or Bcl2-overexpressing mice are not grossly abnormal,29, suggesting that cell extrusion is not dependent on caspase-mediated cell killing. Third, LKB1 mutations cause Peutz–Jeghers syndrome,30 which is characterized by intestinal hamartomas (polyps) containing excess epithelial cells. It is possible that LKB1 mutations contribute to polyp formation by causing a defect in the extrusion of epithelial cells. On the basis of our observations of C. elegans, we predict that the PIG-1 homologue MELK could be a target of LKB1 in the gastrointestinal tract and that mutations of MELK might also impair enterocyte shedding and cause a polyposis phenotype in the mammalian intestine.

METHODS SUMMARY

DIC and epifluorescence micrographs were obtained using an Axioskop II (Zeiss) compound microscope, an ORCA-ER CCD camera (Hamamatsu) and OpenLab software (Agilent) and modified using ImageJ software (National Institutes of Health). For the time-lapse experiments, early (two- or four-cell stage) embryos were dissected from gravid adults, mounted on a slide with a 4% agar pad and covered with a cover slip sealed to the slide with petroleum jelly to prevent the preparation from drying. The developing embryos were imaged every 4 min for a total of 300 min, and at each time point a Z-stack of 50 images spaced at 0.6 μm was acquired. Confocal microscopy was performed using a Zeiss LSM 510 instrument, and the resulting images were modified using ImageJ software. Shed cells or floaters were counted in eggs between the 2-fold and 3.5-fold stages of development (approximately 450–600 min after the first cell division) using a ×100 objective equipped with DIC optics. The numbers of excretory cells and excretory-like cells were counted in L3 larvae carrying the Ppgp-12::gfp transgenic reporter using a ×100 objective. A cell was scored as being ‘excretory-cell-like’ if it was located in the anterior third of the animal and its nucleus strongly expressed GFP.

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

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METHODS

Strains. All C. elegans strains were cultured as described previously31,32 and maintained at 20 °C unless noted otherwise. We used the Bristol strain N2 as the wild-type strain. The mutations used in our experiments were as follows: LGI: ced-1(e1735), cep-1(k1138), cep-3(s4872), daf-19(gk219), gap-2(ad541), lin-35(n745), nls33[Ppar-4::gfp, unc-76(+)]; LII: ced-1(1), ced-30(h476), mop-25.2(ko2037); LGIII: alf-1(ak796), ced-4(n1162), ced-5(n8183), ced-7(n8187), ced-9(n1950, n1950 n2077), ced-12(m3261), grp-1(tm5196), strain-1(ok2283), nls400[Ppar-4::gfp, ppy-4::zIsRed]; LGIV: ced-2(e1752), ced-3(n717, n2452, n3692), ced-5(n8183), ced-10(n1993), ham-1(n3458), lin-24(n2094), lin-33(n4514), pig-1(gm344, n5343, n5437), jcs-1[ajm-1::gfp, rol-6su1006]; LGV: unc-76(e911), para-4(is17), ced-1(n1084, n3082, n3330), ced-3(n948), unc-86(e58), unc-851(e369), nls342[Ppar-4::gfp, unc-24(a160)], LGX: nuc-1(e1392), lin-15A(n765), nls433[Ppar-4::gfp, unc-76(+)]; unknown linkage: nls398[Ppar-4::nmgf8e::Venus, ppy-4::zIsRed], jcs1[hmp-1::gfp, d1::ZIsRed, rol-6(su1006)], nls201[Ppar-4::zIsRed, mStrawberry, pln-15E(K)], sxs17[rlg-1::gfp, rol-6(su1006)], syls1[3079[pig-1::gfp, unc-54(3′ UTR)], rol-6(su1006)]; extrachromosomal arrays: nEx1747, nEx1748 and nEx1749 [pig-1(w1u1), Ppar-4::zIsRed, nEx1758, nEx1759 and nEx1760 [pig-1(1-STOP), Ppar-4::zIsRed, nEx1755, nEx1756 and nEx1757 [pig-1(T696A), Ppar-4::zIsRed, nEx1831, nEx1832, nEx1833, and nEx1834 [pig-1(T1696D), Ppar-4::zIsRed]].

Plasmids. The Ppar-4::gfp transcriptional reporter and the Ppy-4::zIsRed constructs were used in this study. Plasmids are fully described in the Supplemental Information.

Transmission electron microscopy. Gravid worms were prepared for transmission electron microscopy. The worms were fixed in 1% glutaraldehyde, post fixed in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed in a JEOL 1200 electron microscope.

Microscopy. Nomarski DIC and epifluorescence micrographs were obtained using an Axioskop II (Zeiss) compound microscope and OpenLab software (Agilent). Merged DIC and epifluorescence images were generated using ImageJ software (National Institutes of Health). For the time-lapse experiments, early, late or larval stage embryos were dissected from gravid adults, mounted on a slide with a 4% agar pad and covered with a cover slip that was sealed to the slide with petroleum jelly to prevent the preparation from drying. The resulting embryos were imaged every 4 min for a total of 300 min, and at each time point a Z-stack of 50 images spaced at 0.6 µm was acquired. Confocal microscopy was performed using a Zeiss LSM 510 instrument, and the resulting images were prepared and modified using ImageJ software.

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