Matefin/SUN-1 is a nuclear envelope receptor for CED-4 during *Caenorhabditis elegans* apoptosis

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In *Caenorhabditis elegans*, the antiapoptotic protein CED-9 is localized at the mitochondria, where it binds the proapoptotic protein CED-4. Induction of apoptosis begins when the proapoptotic protein EGL-1 is expressed and binds CED-9. The binding of EGL-1 to CED-9 releases CED-4 from CED-9 and causes the activation of the caspase CED-3. Upon its release from CED-9, CED-4 rapidly translocates to the nuclear envelope (NE) in a CED-3-independent manner. However, the identity of the NE receptor for CED-4 and its possible role in the execution of apoptosis has remained unknown. Here, we show that the inner nuclear membrane SUN-domain protein matefin/SUN-1 is the NE receptor for CED-4. Our data demonstrate that matefin/SUN-1 binds CED-4 and is specifically required for CED-4 translocation and maintenance at the NE. The role of matefin/SUN-1 in the execution of apoptosis is further suggested by the significant reduction in the number of apoptotic cells in the organism after matefin/SUN-1 down-regulation by RNAi. The finding that matefin/SUN-1 is required for the execution of apoptosis adds an important link between cytoplasmic and nuclear apoptotic events.

inner nuclear membrane | nuclear lamina | SUN-domain 1 lamin | programmed cell death

Programmed cell death (apoptosis) is essential for normal development and homeostasis in metazoans (1–3). In the nematode *Caenorhabditis elegans*, the mitochondria-associated Bcl-2 homologue CED-9 binds the Apaf-1 homologue CED-4. Upon apoptosis initiation, the Bcl-2 homology domain 3-only (BH3-only) protein EGL-1 is expressed and binds CED-9, releasing CED-4, which activates the caspase CED-3 (4). This core apoptotic pathway is conserved in higher eukaryotes (4). Once released, CED-4 translocates from the mitochondria to the nuclear envelope (NE) (5). The NE is composed of inner and outer nuclear membranes that join at the nuclear pore complexes. Underneath the inner nuclear membrane (INM), there is a meshwork of proteins, termed the nuclear lamina, which is composed of lamins and lamin-associated proteins, most of which are integral proteins of the INM (6, 7). The *C. elegans* INM harbors two LEM-domain proteins (Ce-emerin and Ce-MAN1) (8) and two SUN-domain proteins (UNC-84 and matefin/SUN-1) (9, 10). Matefin/SUN-1 colocalizes with Ce-lamin *in vivo* and binds Ce-lamin *in vitro* but does not require Ce-lamin for its NE localization. Matefin/SUN-1 is present in all embryonic cells until mid/late embryogenesis and thereafter in germline cells. Matefin/SUN-1 is essential for embryogenesis and germ-line proliferation and maintenance (10). Matefin/SUN-1 is also required for centrosome attachment to the nuclear periphery via an interaction with ZYG-12 (11). It was suggested that CED-4 translocation from the mitochondria to the NE plays an important role in apoptosis (5). We report that matefin/SUN-1 is specifically required for CED-4 localization at the NE. Furthermore, we show that this localization is probably required for executing apoptosis.

Results

CED-4 Binds Matefin/SUN-1 *in Vitro*. Because CED-4 lacks a classic transmembrane domain (hydrophobic analysis, data not shown), we hypothesized that its translocation from the mitochondria to the NE involves binding of one or more NE proteins. To determine whether CED-4 binds directly to a known nuclear lamina protein *in vitro*, we used ^{35}S-labeled CED-4 and examined its binding to various recombinant nuclear lamina proteins immobilized on blots (Fig. 1*a*). ^{35}S-labeled CED-4 bound specifically to matefin/SUN-1 but not to Ce-lamin, Ce-emerin, or Ce-MAN1 (12). As expected, ^{35}S-labeled CED-4 bound its known partner CED-3 (13, 14) but not a control BSA (Fig. 1*a*). A control probe of ^{35}S-labeled thymidine kinase did not bind matefin/SUN-1 in a blot overlay assay, suggesting that CED-4 binding to matefin/SUN-1 is specific (Fig. 1*b*). The binding of CED-4 to matefin/SUN-1 was further confirmed in His-tag pulldown experiments. Matefin/SUN-1 and CED-3 specifically pulled down ^{35}S-labeled CED-4, whereas Ce-lamin, Ce-emerin, or Ce-MAN1 did not (Fig. 1*c*). Likewise, CED-4 and Ce-lamin (a known matefin/SUN-1 partner) (10) specifically pulled down ^{35}S-labeled matefin/SUN-1 but not Ce-emerin or buffer (Fig. 1*d*).

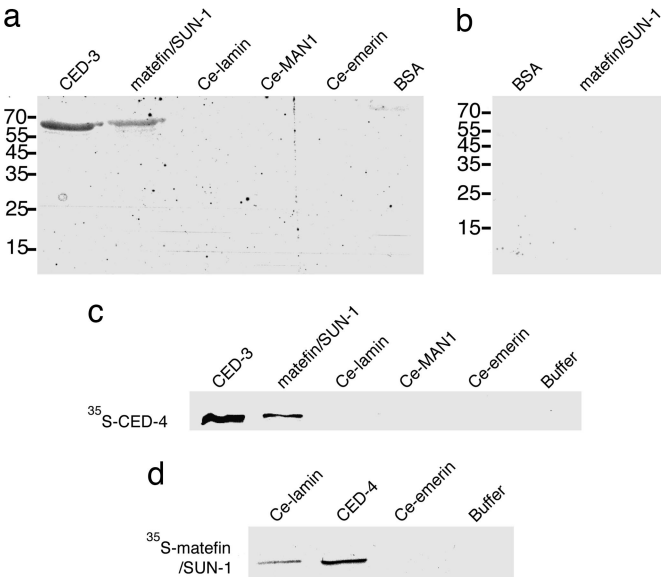

Fig. 1. CED-4 binds matefin/SUN-1 *in vitro*. (*a* and *b*) Equal amounts of bacterially expressed and purified proteins (55 pmol) were resolved by 12% SDS/PAGE, transferred to nitrocellulose membranes and probed with ^{35}S-labeled CED-4 (*a*) or ^{35}S-labeled thymidine kinase (*b*). The positions of the size markers are shown on the left side of each panel. (*c* and *d*) Equal amounts of bacterially expressed and purified His-tagged proteins (440 pmol) were coupled to Ni-NTA agarose beads and used to precipitate *in vitro*-translated ^{35}S-labeled CED-4 (*c*) or ^{35}S-labeled matefin/SUN-1 (*d*). Autoradiograms of the pellet fractions are shown.

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Abbreviation: NE, nuclear envelope.

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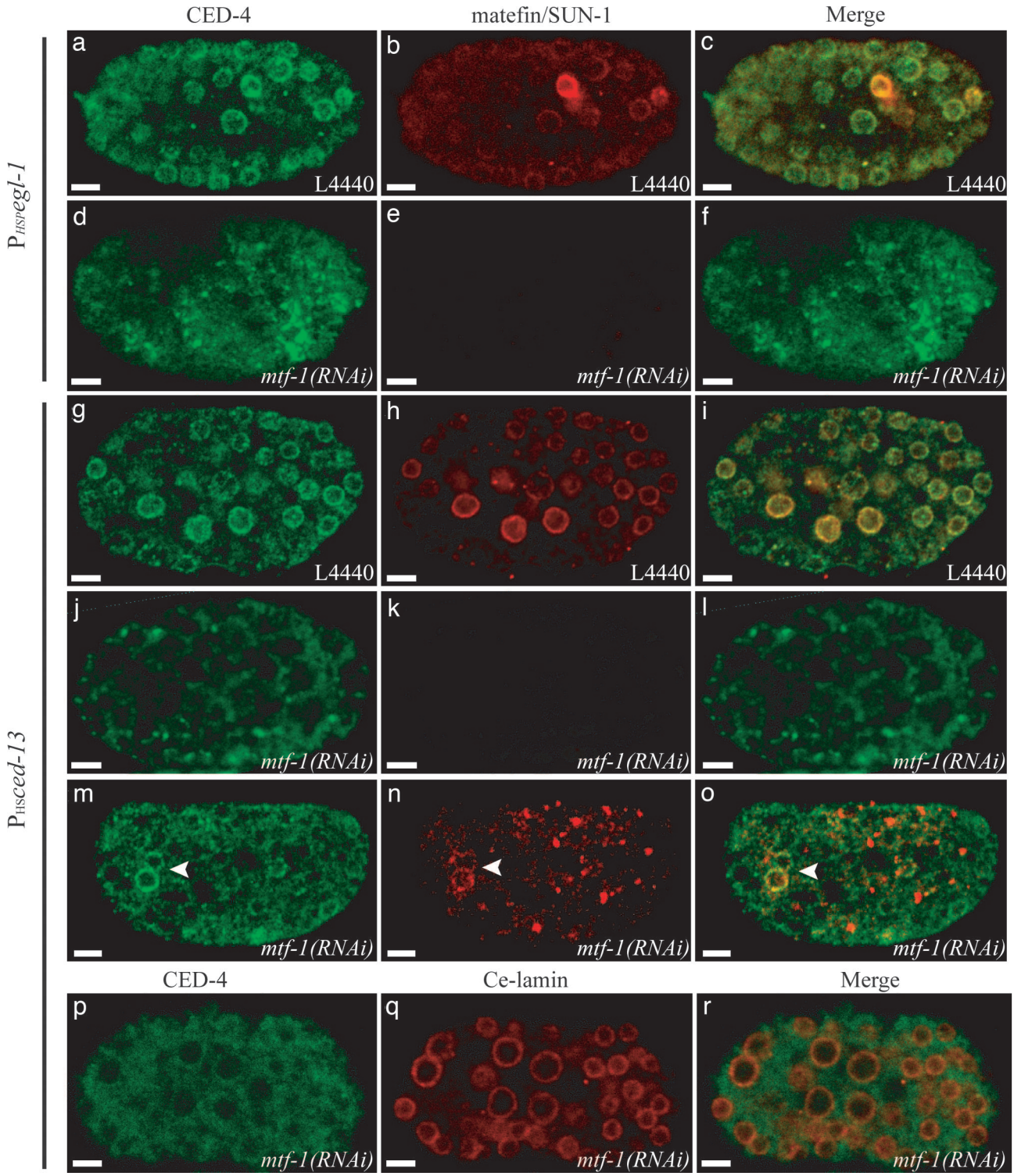

Fig. 2. Matefin/SUN-1 is required for CED-4 NE localization. $P_{hsp}egl$-1 (*a–f*) and $P_{HS}ced$-*13* (*g–r*) embryos after RNAi treatment with either control L4440 vector (*a–c* and *g–i*) or *mtf-1*/*sun-1* dsRNA (*d–f* and *j–r*). Embryos were heat-shocked at 34°C, fixed, and costained with antibodies against CED-4 (*Left*, green) and either matefin/SUN-1 (*b*, *e*, *h*, *k*, and *n*) or Ce-lamin (*q*). Only in cells with residual matefin/SUN-1 did CED-4 remain localized at the NE (*m–o*, arrowheads). (Scale bars, 5 μm.)

Matefin/SUN-1 Is Required for CED-4 NE Localization. CED-4 displayed the expected web-like cytoplasmic labeling in wild-type embryos, typical of mitochondrial localization (Fig. 6*a*, which is published as supporting information on the PNAS web site) (5). In the $P_{hsp}egl$-*1* transgenic strain, a heat-shock promoter drives the expression of *egl-1*. Thus, heat-shocking embryos of this strain ectopically invokes apoptosis in many cells and CED-4 translocation to the NE (5) (Figs. 2*a* and 6*c*), confirming that the NE localization of CED-4 is specific to the induction of apoptosis (5). Web-like cytoplasmic labeling of CED-4, similar to wild type, was obtained

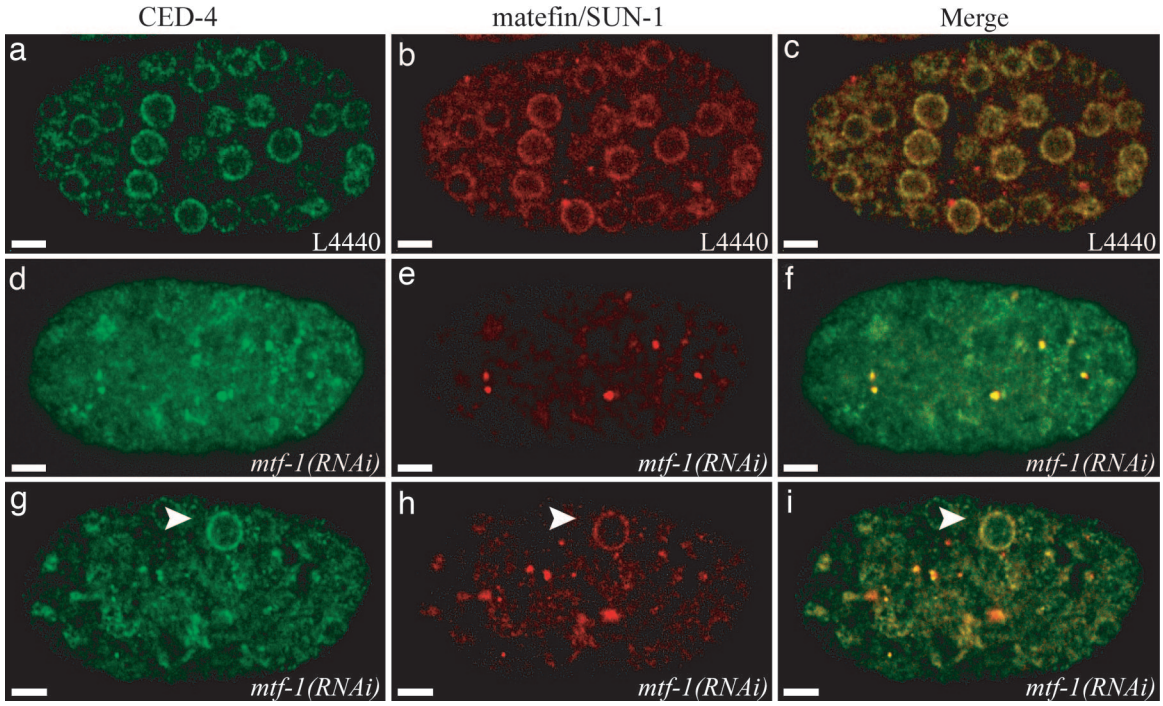

Fig. 3. Matefin/SUN-1 is required to maintain CED-4 at the NE. *ced-9(n2812); ced-3(n717)* embryos after RNAi treatment with either control L4440 vector (*a–c*) or *mtf-1/sun-1* dsRNA (*d–i*) were costained with antibodies against CED-4 (*Left*, green) and matefin/SUN-1 (*Center*, red). Only in cells with residual matefin/SUN-1 did CED-4 remain localized at the NE (*g–i*, arrowheads). (Scale bars, 5 μm.)

after heat shock in embryos carrying a P_{HS}*gfp* construct (GFP driven by a heat-shock promoter), confirming that heat shock alone does not affect CED-4 localization (ref. 5 and data not shown). CED-4 was continuously present at the NE in a strain that carries loss-of-function mutations in both *ced-9* and *ced-3* [*ced-9(n2812); ced-3(n717)*], as was reported (5) (Figs. 3 *a–c* and 6*b*).

To directly test the role of matefin/SUN-1 in CED-4 NE localization *in vivo*, the expression of *mtf-1/sun-1* was down-regulated in P_{hsp}*egl*-1 embryos by using *mtf-1/sun-1* dsRNA [*mtf-1(RNAi)*]. Western blot analysis of protein lysates derived from a total embryonic population showed an ≈70% reduction in matefin/SUN-1 protein levels after *mtf-1(RNAi)* (Fig. 7, which is published as supporting information on the PNAS web site). Immunostaining revealed that a fraction of the P_{hsp}*egl-1* embryos in which matefin/SUN-1 was down-regulated had very little or no detectable matefin/SUN-1 labeling (Fig. 2*e*). In heat-shocked P_{hsp}*egl-1* embryos, the down-regulation of matefin/SUN-1 caused CED-4 to disperse in the cytoplasm (Fig. 2 *d–f*).

CED-13 is another BH3-only protein that, like EGL-1, binds CED-9, releases CED-4 *in vitro*, and ectopically activates apoptosis (15, 16). In the P_{HS}*ced-13* strain, a heat-shock promoter drives the expression of a stably integrated transgene of *ced-13*, causing apoptosis in many cells (16). In P_{HS}*ced-13* embryos kept at 16°C, CED-4 displayed a web-like cytoplasmic labeling, similar to the CED-4 pattern in wild-type embryos (5) (Fig. 6*d*). After induction of CED-13 expression by heat shock at 34°C, CED-4 was detected at the NE (Figs. 2 *g–i* and 6*e*), similarly to CED-4 localization in heat-shocked P_{hsp}*egl-1* or *ced-9(n2812); ced-3(n717)* embryos (5). To verify the P_{hsp}*egl-1* results, we down-regulated matefin/SUN-1 expression in P_{HS}*ced-13* embryos. The down-regulation of matefin/SUN-1 expression in P_{HS}*ced-13* embryos prevented CED-4 translocation to the NE after ectopic expression of CED-13 by heat shock. The diffused cytoplasmic CED-4 labeling that was detected in these embryos (Fig. 2 *j–l*) was similar to that observed in P_{hsp}*egl-1* (Fig. 2 *d–f*). In some of the *mtf-1(RNAi)* embryos, few cells retained residual matefin/SUN-1 at the NE. In these cells (but not in the surrounding cells that did not show staining of matefin/SUN-1), CED-4 was found at the NE (Fig. 2 *m–o*, arrowheads). The lack of CED-4 association with the NE in *mtf-1(RNAi)* embryos was not due to a general change in nuclear lamina organization, because the major nuclear lamina protein, Ce-lamin, retained its normal perinuclear localization (Fig. 2 *p–r*). We conclude that matefin/SUN-1 is specifically required for CED-4 translocation to the NE.

Next, we tested whether matefin/SUN-1 is required for not only localizing but also maintaining CED-4 at the NE. CED-4 lost its NE localization in *ced-9(n2812); ced-3(n717)* embryos in which matefin/SUN-1 was down-regulated (Fig. 3 *d–f*). CED-4 was localized at the NE only in *ced-9(n2812); ced-3(n717)* cells that retained residual matefin/SUN-1 at their NE but not in the surrounding cells lacking detectable matefin/SUN-1 (Fig. 3 *g–i*, arrowheads). The specific requirement of matefin/SUN-1 for CED-4 localization at the NE was confirmed by analyzing the subcellular localization of CED-4 in *ced-9(n2812); ced-3(n717)* embryos in which the inner nuclear membrane proteins Ce-MAN1 (Fig. 8 *a–f*, which is published as supporting information on the PNAS web site), Ce-emerin, or UNC-84 (data not shown) were down-regulated. In all these cases, CED-4 retained its NE localization. Most nuclear lamina proteins, including Ce-emerin, Ce-MAN1, and UNC-84, depend on Ce-lamin for their NE localization (12, 17, 18). To exclude the possibility that Ce-lamin is also required for CED-4 NE localization, we analyzed CED-4 localization in *ced-9(n2812); ced-3(n717)* embryos down-regulated for Ce-lamin [*lmn-1 (RNAi)*] (Fig. 8 *g–l*). Although the knockdown of Ce-lamin resulted in severely abnormal nuclei, early embryonic arrest, and mortality (19, 20), CED-4 was still detected at the NE (Fig. 8 *j–l*). These results are in agreement with the findings that matefin/SUN-1 does not require Ce-lamin for its NE localization (10). We therefore conclude that matefin/SUN-1 is specifically required for CED-4 NE localization, not depending on LEM-domain proteins (Ce-emerin and Ce-

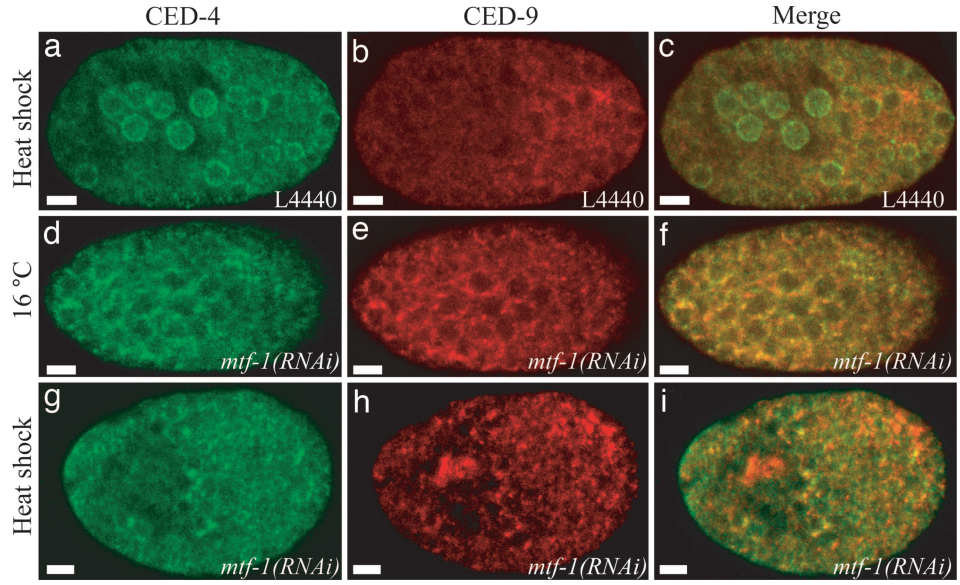

Fig. 4. Matefin/SUN-1 does not affect CED-4 dissociation from CED-9. P_{HS}*ced-13* embryos treated with either control L4440 (*a–c*) or with *mtf-1/sun-1* dsRNA (*d–i*) were either kept at 16°C (*d–f*) or induced to initiate apoptosis by heat shock at 34°C (*a–c* and *g–i*). The embryos were costained with antibodies against CED-4 (*Left*, green) and CED-9 (*Center*, red). (Scale bars, 5 μm.)

MAN1), UNC-84, or Ce-lamin. We also analyzed whether ZYG-12, which is a hook protein that interacts with matefin/SUN-1 (11), is required for CED-4 localization at the NE. The *zyg-12(ct350)* allele causes temperature-sensitive lethality (11). *zyg-12(ct350);* P_{HS}*ced-13* embryos at either permissive (16°C) or restrictive (26°C) temperatures were induced to start apoptosis. In both permissive and restrictive conditions, CED-4 was localized at the NE (Fig. 9, which is published as supporting information on the PNAS web site). We conclude that ZYG-12 is not essential for CED-4 translocation to the NE.

CED-4 Dissociation from CED-9 Does Not Require Matefin/SUN-1. We next tested whether down-regulation of matefin/SUN-1 has an effect on the release of CED-4 from CED-9. *mtf-1(RNAi)* P_{HS}*ced-13* embryos were costained with antibodies against CED-4 and CED-9 (Fig. 4). At 16°C, CED-4 and CED-9 were mostly colocalized in a web-like pattern (Fig. 4 *d–f*). After the heat-shock-derived expression of CED-13, CED-4 was dispersed in the cytoplasm and lost its colocalization with CED-9 (Fig. 4 *g–i*). We conclude that matefin/SUN-1 is involved in CED-4 translocation only after CED-4 releases from CED-9.

Knockdown of Matefin/SUN-1 Dramatically Reduces the Number of Apoptotic Events in *C. elegans* Embryos. Because matefin/SUN-1 is required for apoptotic CED-4 localization, we next analyzed the role of matefin/SUN-1 in the execution of apoptosis. Matefin/SUN-1 knockdown embryos die at the ≈300-cells stage, they have deformed cells, and show abnormal nuclear morphology (10). *mtf-1(RNAi)* embryos outside the most potent window of down-regulation escape early embryonic lethality (10). Scoring the number of cell corpses in 1.5- and 2-fold *C. elegans* wild-type embryos revealed an average of 12 or 10 corpses, respectively (Fig. 5*a*), a finding that is in agreement with previous studies (21, 22). In contrast, scoring the number of cell corpses in 1.5- and 2-fold *C. elegans* embryos that were down-regulated for matefin/SUN-1 showed an average of 2 or 1 cell corpses, respectively (Fig. 5*a*). These results were further verified by TUNEL or acridine orange staining (both label apoptotic nuclei) (23–25). TUNEL-positive staining of apoptotic nuclei in embryos with mutations in the *nuc-1* gene, a *C. elegans* homologue of the mammalian DNase II gene, persists for a much longer period than in wild-type embryos, resulting in the accumulation of TUNEL-positive nuclei (25). TUNEL staining at the 1.5-, 2-, and 3-fold stages of *nuc-1(e1392 a.m.)* embryos that escaped *mtf-1/sun-1 (RNAi)* early embryonic lethality showed a significant reduction in the number of TUNEL-positive nuclei compared with control

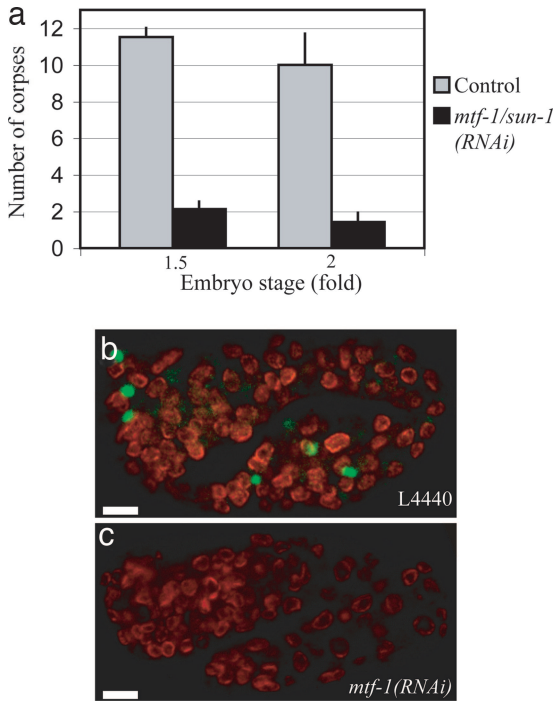

Fig. 5. Matefin/SUN-1 knockdown reduces the number of apoptotic cells. (*a*) Cell corpses were scored in wild-type embryos treated with either control L4440 vector (gray) or *mtf-1/sun-1* dsRNA (black). Numbers represent the average ± SEM of cell corpses. At least 29 embryos were examined for each developmental stage. Control *nuc-1(e1392 a.m.)* embryos (*b*) or *nuc-1(e1392 a.m.)* embryos in which matefin/SUN-1 was down-regulated (*c*) were costained for TUNEL (green) and Ce-lamin (red) and viewed with a confocal microscope. (Scale bars, 5 μm.)

Table 1. Matefin/SUN-1 knockdown reduces the number of TUNEL-positive nuclei

	nuc-1(e1392am) mtf-1/sun-1 (RNAi)		*nuc-1(e1392am)* control	
Embryo stage	TUNEL-positive nuclei	*n*	TUNEL-positive nuclei	*n*
1.5-fold	5.7 ± 2.0	17	23.4 ± 3.8	11
2-fold	6.2 ± 1.6	42	25.8 ± 2.4	39
3-fold	8.7 ± 1.3	38	19.1 ± 1.7	42

Control *nuc-1(e1392am)* embryos or *nuc-1(e1392am)* embryos in which matefin/SUN-1 was down-regulated were costained for TUNEL. Average ± SEM of TUNEL-positive nuclei are presented. *n*, number of examined embryos.

nuc-1(e1392 a.m.) embryos (Table 1). These embryos had normal lamin distribution, as viewed by Ce-lamin staining, demonstrating the specific role of matefin/SUN-1 in apoptosis (Fig. 5 *b–c*). Similar results were observed in *ced-5(n1812)* embryos stained with acridine orange (24) (data not shown). We conclude that matefin/SUN-1 is required for apoptosis in *C. elegans*.

Discussion

In this article, we provide evidence that matefin/SUN-1 is required for CED-4 translocation to the NE during apoptosis. The direct binding of CED-4 to matefin/SUN-1 *in vitro* and the requirement of matefin/SUN-1 for CED-4 localization at the NE suggest that matefin/SUN-1 is the CED-4 receptor at the NE. The significant reduction in the number of apoptotic cells in embryos in which matefin/SUN-1 was down-regulated indicates that the binding between CED-4 and matefin/SUN-1 is necessary for the progression of apoptosis. Because the apoptosis-detection essays were performed in embryos that escaped *mtf-1/sun-1 (RNAi)* early embryonic lethality and, thus, contained residual levels of the matefin/SUN-1 protein, it is likely that the small number of cells that underwent apoptosis had enough matefin/SUN-1 to bind CED-4 at the NE. Although the binding of CED-4 to matefin/SUN-1 strongly suggests that matefin/SUN-1 has a direct role in the progression of apoptosis, we cannot exclude the possibility that the reduction in the number of apoptotic cells resulted from an indirect effect on cell fates.

Matefin/SUN-1 is an essential protein required for several other cellular functions, including early embryonic development, centrosome binding to the NE in early embryos, and germ-line proliferation and maturation (10, 11). We hypothesize that the other essential roles of matefin/SUN-1 prevented its discovery in the general screens for genes involved in apoptosis (26, 27). Because matefin/SUN-1 is not detected in the soma after mid/late embryogenesis (10), it is possible that other proteins replace the matefin/SUN-1 function as CED-4 NE receptors at these stages. Alternatively, at later developmental stages, matefin/SUN-1 may be present in the NE of apoptotic cells at undetectable levels that are sufficient to bind CED-4 at the NE.

Studies have demonstrated that, in contrast to mammalian and *Drosophila* cells, the *C. elegans* nuclear lamina is not a primary target for caspases (23). One possible explanation is that the nuclear lamina is required for CED-4 apoptotic localization. UNC-84 is the other *C. elegans* SUN-domain nuclear lamina protein (17). It links the nucleus to microtubule and actin cytoplasmic filaments and is required for nuclear positioning (28). Our data support the model that, in addition to the structural roles of SUN-domain proteins (7), they can function as receptors that link cytoplasmic components to the NE, thus connecting cytoplasmic processes to the nucleus.

Why would CED-4 translocate to the NE during apoptosis? One possibility is that the matefin/SUN-1-dependent localization of CED-4 targets the death machinery to doomed nuclei. This homing device is required because some apoptotic cells begin to be engulfed before they are completely divided from their sister cell (29). Moreover, during germ-cell development, apoptosis is initiated only in certain nuclei within the syncytium (30). The *C. elegans* apoptotic genetic program is conserved through evolution, and the intracellular apoptotic process goes through similar steps of activation (4). Because both SUN-domain proteins and the apoptotic machinery are conserved in evolution, it is tempting to suggest that these proteins are the link for apoptotic processes between the cytoplasm and the nucleus in higher eukaryotes as well. For example, in mammalian cells, SUN-domain proteins could have similar roles as receptors for "death-homing" complexes in specific doomed nuclei within multinucleate myotubes (31).

Materials and Methods

Maintenance of Strains and RNAi Experiments. *C. elegans* strains were maintained and manipulated under standard conditions (32). N2 and *zyg-12(ct350)* were obtained from the *C. elegans* Genetic Center at the University of Minnesota (Minneapolis, MN). P_{HS}*ced-13* and P_{HS}*gfp* were a kind gift of Shai Shaham (The Rockefeller University, New York, NY). P_{hsp}*egl-1, ced-9(n2812)*; *ced-3(n717), nuc-1(e1392 a.m.)*; and *ced-5(n1812)* were a kind gift of H. Robert Horvitz (Massachusetts Institute of Technology, Cambridge, MA). Heat shock was induced by incubating the embryos for 1 h at 34°C, and recovery was for 1 h at 20°C. For nonpermissive conditions, *zyg12(CT350);* P_{HS}*ced-13* worms were moved from 16°C to 25°C for 4 h, heat-shocked for 1 h at 34°C, and left to recover for 1 h at 25°C. RNAi feeding experiments were done essentially as described (33). Briefly, NGM plates containing 25 μg/ml carbenicillin and 0.5 mM isopropyl β-D-thiogalactoside were plated with *Escherichia coli* HT115(DE3) cells transformed with the appropriate feeding construct. pJKL483.1 (20) was used to knock down Ce-lamin, and matefin/SUN-1 exon 3 (10) construct was used to knock down matefin/SUN-1. cDNA corresponding to Ce-MAN1 residues 147–385 was cloned into the L4440 vector and used to knock down Ce-MAN1. *emr-1* cDNA was cloned into the L4440 vector for Ce-emerin down-regulation. The *unc-84* feeding construct was described in ref. 34. Empty L4440 was always used as a control (35).

Antibody Staining, TUNEL, Acridine Orange, and Microscopy Procedures. TUNEL analysis, acridine orange, and antibody staining were performed as described (23, 24). CED-4 and CED-9 antibodies were a kind gift of H. Robert Horvitz (5). Ce-MAN1, Ce-emerin, UNC-84, Ce-lamin, and Matefin/SUN-1 antibodies are described in refs. 10, 18, and 23. Cy2 and Cy3-conjugated affinity-purified goat secondary antibodies were purchased from The Jackson Laboratory (West Grove, PA). Embryos were viewed by using an MRC-1024 Bio-Rad (Hercules, CA) confocal scan head coupled to a Zeiss (Thornwood, NJ) Axiovert 135M inverted microscope equipped with a 63× N.A. = 1.3 oil-immersion objective. For Cy2 detection, the excitation wavelength was 488 nm, and an HQ525 ± 20 filter was used to collect the emission. For Cy3 detection, the excitation wavelength was 514 nm, and an HQ570 ± 15 filter was used to collect the emission. Sections were collected every 0.5 μm. Control experiments were performed to verify no bleed through of Cy2 emission into the Cy3 emission channel.

The number of cell corpses in living embryos was scored by using a Zeiss Axioplan II microscope equipped with Nomarski (Edgewater, NJ) optics and Z-sectioning capabilities.

Synthesis of ^{35}S-Labeled Proteins, Blot Overlay, and Protein Blot Assays. The TNT Quick Coupled Transcription/Translation System (Promega, Madison WI), was used according to the manufacturer's protocol to express [^{35}S]methionine-labeled CED-4, matefin/SUN1, and thymidine kinase. cDNAs encoding CED-3

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and -4 were cloned into the pET28a expression vector. Constructs were transformed into *E. coli* BL21 (DE3), and proteins were purified via Ni-NTA agarose (Qiagen, Valencia, CA). Cloning and purification of Ce-emerin (residues 1–130 lacking only the short C-terminal transmembrane domain), full-length Ce-lamin, full-length matefin/SUN-1, and Ce-MAN1 (residues 400–500) were done as described (10). Blot overlay assays were performed as described (12). Protein blot assays were performed as described (23).

His-Tag Pull-Down Assay. Equal amounts (440 pmol, as determined by the Micro BCA protein assay kit, Pierce Biotechnology Rockford, IL) of the His-tagged recombinant proteins were coupled to Ni-NTA-agarose beads by incubation for 1 h at 4°C in 1 ml of wash buffer (20 mM Hepes, pH 7.4/150 mM NaCl/0.5 mM DTT/1% Triton X-100) containing 5 mM imidazole. The ^{35}S-labeled CED-4 or ^{35}S-labeled matefin/SUN-1 were then added to the beads in wash buffer containing 5 mM imidazole and 0.1% FCS and incubated for 3 h at 4°C. The Ni-agarose beads were washed three times in wash buffer with 5 mM imidazole, once with wash buffer containing 15 mM imidazole, and once with wash buffer containing 20 mM imidazole. The beads were precipitated by centrifugation at 2,000 × *g*, and proteins bound to the beads were eluted with SDS sample loading buffer, resolved by 12% SDS/PAGE, and exposed to x-ray film.

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