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C. elegans Nuclear Envelope Proteins Emerin, MAN1, Lamin, and Nucleoporins Reveal Unique Timing of Nuclear Envelope Breakdown during Mitosis

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Emerin, MAN1, and LAP2 are integral membrane proteins of the vertebrate nuclear envelope. They share a 43-residue N-terminal motif termed the LEM domain. We found three putative LEM domain genes in *Caenorhabditis elegans*, designated *emr-1*, *lem-2*, and *lem-3*. We analyzed *emr-l*, which encodes Ce-emerin, and *lem-2*, which encodes Ce-MAN1. Ce-emerin and Ce-MAN1 migrate on SDS-PAGE as 17- and 52-kDa proteins, respectively. Based on their biochemical extraction properties and immunolocalization, both Ce-emerin and Ce-MAN1 are integral membrane proteins localized at the nuclear envelope. We used antibodies against Ce-MAN1, Ce-emerin, nucleoporins, and Ce-lamin to determine the timing of nuclear envelope breakdown during mitosis in *C. elegans*. The *C. elegans* nuclear envelope disassembles very late compared with vertebrates and *Drosophila*. The nuclear membranes remained intact everywhere except near spindle poles during metaphase and early anaphase, fully disassembling only during mid-late anaphase. Disassembly of pore complexes, and to a lesser extent the lamina, depended on embryo age: pore complexes were absent during metaphase in >30-cell embryos but existed until anaphase in 2- to 24-cell embryos. Intranuclear mRNA splicing factors disassembled after prophase. The timing of nuclear disassembly in *C. elegans* is novel and may reflect its evolutionary position between unicellular and more complex eukaryotes.

INTRODUCTION

In multicellular animals (metazoans), the nuclear envelope consists of the inner and outer nuclear membranes, the nuclear pore complexes (NPCs), and lamina. The lamina is mainly composed of nucleus-specific intermediate filament proteins named lamins (Stuurman *et al.*, 1998). The nuclear lamina has important but poorly understood roles in organizing chromatin structure, in creating an environment permissive for DNA replication and other nuclear activities (Goldberg *et al.*, 1999a; Gotzmann and Foisner, 1999), and in nuclear disassembly (Collas, 1998). Some lamins are located inside the nucleus as part of the nuclear matrix (Ellis *et al.*, 1997; Dechat *et al.*, 1998; Broers *et al.*, 1999; Vlcek *et al.*, 1999) and colocalize with sites of DNA replication (Spann *et al.*, 1997). The single B-type lamin in *Caenorhabditis elegans* (Riemer *et al.*, 1993) is essential, and its loss-of-function phenotype suggests that lamins have critical roles in nuclear shape, mitotic progression, spacing of pore complexes, and chromosome segregation (J. Liu, T.R. Ben-Shahar, D. Reimer, M. Treinin, P. Spann, K. Weber, A. Fire, and Y. Gruenbaum; unpublished results).

Several integral proteins of the inner nuclear membrane have been identified in mammalian cells, including the lamina-associated polypeptide 1 (LAP1), LAP2, emerin, MAN1, LBR, and nurim (reviewed by Goldberg *et al.*, 1999b; Wilson, 2000). Most of these proteins interact with type-A or type-B lamins during interphase and are differentially phosphorylated during mitosis, when the nuclear envelope disassembles (Stuurman *et al.*, 1998). Binding to lamins A/C during interphase is important for the proper localization and retention of emerin, and perhaps other proteins, at the inner nuclear membrane (Sullivan *et al.*, 1999; reviewed by Gruenbaum *et al.*, 2000). LAP2, emerin, and MAN1 are defined as a family because they share a region of ~43 residues, termed the LEM (LAP2-emerin-MAN1) domain, at or near their N termini (Lin *et al.*, 2000). The LEM domain of LAP2 mediates binding to BAF, a small novel

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DNA-binding protein of unknown function (Cai *et al.*, 1998; Lee and Craigie, 1998; Furukawa, 1999).

In addition to their potential roles in nuclear dynamics, nuclear envelope proteins are likely to have unique functional roles during interphase. People who lack emerin develop a rare X-linked disease named Emery-Dreifuss muscular dystrophy (Morris and Manilal, 1999). A clinically identical disease is caused by heterozygous mutations in the gene encoding lamins A and C (Bonne *et al.*, 1999). Unexpectedly, specific mutations in lamin A/C can alternatively cause two other diseases: Dunnigan-type familial partial lipodystrophy (Cao and Hegele, 2000; Shackleton *et al.*, 2000) and dilated cardiomyopathy (Fatkin *et al.*, 1999). The mechanism by which mutations in emerin and lamin A/C cause disease is unknown, but it is proposed to involve changes in gene expression (Wilson, 2000).

In mammals, the nucleus is completely disassembled during mitosis, a process known as "open" mitosis (Gerace and Burke, 1988). The lamina depolymerizes, and nuclear membranes disperse into the endoplasmic reticulum network during prometaphase (Ellenberg *et al.*, 1997; Yang *et al.*, 1997). Physical disruption of the nuclear envelope, caused by spindle microtubules during mid-late prophase (Georgatos *et al.*, 1997), may also contribute to the release of intranuclear contents. By metaphase, the vertebrate nuclear envelope is completely disassembled. The envelope reassembles onto chromosomes during late anaphase and telophase (Haraguchi *et al.*, 2000). LAP2, lamin B receptor, and lamins have been proposed to help target reforming nuclear membranes to chromosomes or to mediate nuclear envelope assembly or growth (Gant and Wilson, 1997). The open mitosis of higher eukaryotes contrasts with the "closed" mitosis of single-celled eukaryotes such as *Saccharomyces cerevisiae* (Heath, 1980; Gerace and Burke, 1988). During closed mitosis, the nucleus remains intact and chromosomes are segregated by an intranuclear spindle apparatus. *Drosophila* early embryos undergo a morphologically intermediate mitosis in which pore complexes disassemble during prophase and prometaphase, leaving behind open holes, whereas nuclear membranes remain largely intact and the lamina partially disassembles: some lamins delocalize to the cytoplasm, but a fraction of them remain in place through early-mid anaphase (Harel *et al.*, 1989; Paddy *et al.*, 1996).

To begin determining the functions of LEM domain proteins in vivo, we chose the genetically tractable nematode *C. elegans*. We report here the identification and characterization of the LEM domain proteins MAN1 and emerin in *C. elegans* and the discovery that the timing of nuclear envelope breakdown may be unique in *C. elegans* relative to other studied eukaryotes.

MATERIALS AND METHODS

Antibodies

To obtain polyclonal antibodies against Ce-MAN1 and Ce-emerin, mice and rabbits were immunized at 3-week intervals with synthetic peptides conjugated to keyhole limpet hemocyanin (KLH). Immunizations and serum production were performed by Covance Research Products (Denver, PA). The following KLH-conjugated peptides were used: CAVWKWIGNQSQKRW-COOH (named Ce-MAN-C peptide; mouse 3268 antiserum used for Western blotting and indirect immunofluorescence), which corresponds to the last 14 residues of Ce-MAN1 plus an N-terminal Cys residue; and CQLKLVAETNPEDTI-COOH (named Ce-Emer-C peptide; mouse 3272 antiserum used for immunoblots and indirect immunofluorescence), which corresponds to the last 14 residues of emerin plus an N-terminal Cys residue. All peptides were synthesized, purified by reverse-phase HPLC with the use of a C18 analytical column, and conjugated to KLH by Boston Biomolecules (Woburn, MA).

Rabbit polyclonal antibodies to Ce-lamin were produced against a bacterially expressed polypeptide consisting of residues D-217 to F-550 of lamin and were affinity purified (Chen *et al.*, 2000). mAb414, which recognizes a subset of nucleoporins, was purchased from BAbCO (Richmond, CA). mAb104, which recognizes conserved small nuclear ribonucleoproteins (snRNPs) (Roth *et al.*, 1990; Zahler *et al.*, 1993), was provided by Dr. Geraldine Seydoux (Johns Hopkins Medical School, Baltimore, MD). Cy3-conjugated goat anti-mouse and goat anti-rabbit antibodies, and FITC-conjugated goat anti-rabbit antibodies, were purchased from Jackson Laboratories (West Grove, PA). mAbs against tubulin were purchased from Sigma Chemical (catalog number T-9026; St. Louis, MO).

Immunostaining

Immunostaining was performed essentially as described (Miller and Shakes, 1995). Mixed-stage animals or isolated wild-type (N2) adult *C. elegans* were placed on polylysine-treated slides, and 60-mm coverslips were placed above the nematodes. The slides were placed in liquid N_2 or dry ice, and the coverslips were immediately removed. The nematodes were fixed for 4 min at −20°C in methanol and then incubated for 30 min at 22–24°C in PBST (PBS containing 0.1% Tween 20) containing 3.7% formaldehyde. Nematodes were then washed once in PBST, incubated for 10 min at room temperature in PBST containing 5% nonfat dry milk, washed once again with PBST, and incubated overnight at 4°C with the primary antibody diluted in PBST (1:200 for Ce-MAN1 and Ce-emerin, 1:400 for lamin, and 1:1000 for mAb414). Excess primary antibody was removed by washes in PBST: once for 1 min, once for 10 min, and twice for 30 min each. The nematodes were then incubated for 2 h at 22°C with the Cy3-conjugated goat anti-rabbit antibodies (for Ce-lamin) or Cy3-conjugated goat anti-mouse antibodies (for Ce-MAN1, Ce-emerin, and mAb414) diluted in PBST. Double-label immunostaining for snRNPs (or tubulin) and Ce-lamin was performed as follows. Animals were first stained with antibodies to Ce-lamin, followed by FITC-conjugated anti-mouse secondary antibody, and then washed in PBST (once for 1 min, once for 10 min, and twice for 30 min each); the animals were then incubated for 2 h at 22°C with mAb104 (for snRNPs) or anti-tubulin antibodies, rewashed as described above, and incubated for 2 h with Cy3-conjugated anti-mouse antibodies. For both double- and single-label immunostaining, excess secondary antibody was then removed by washes in PBST: once for 1 min, once for 10 min, and twice for 30 min each. Nematodes were then incubated for 10 min in PBS containing 1 μg/ml Hoechst 33258, washed once with PBS, and mounted in glycerol containing 2% *n*-propyl gallate. Nematodes were viewed with a Zeiss (Thornwood, NY) Axioskop microscope equipped with epifluorescence illumination with the use of a 63×/numerical aperture 1.4 Apochromat objective lens.

Confocal samples were acquired with the Noran Oz confocal laser scanning microscope system with the use of Intervision Software (version 6.3) on a Silicon Graphics Indy R5000 platform (Silicon Graphics Inc, Mountain View, CA). A krypton–argon laser (Omnichrome series 43, Noran Instruments, Inc, Middleton, WI) that excites at wavelengths of 488 and 568 nm was used to obtain optical sections. Narrow-band emission filters (525 and 605 nm) were used to eliminate channel cross-talk, and 0.5-μm z-plane sections (as determined by full-width half-maximum intensity values) were collected with the use of a 10-μm fixed slit. Slides were imaged with the use of a 100× oil-immersion planar apochromatic objective lens (numerical aperture 1.35) through an Olympus (Tokyo, Japan) IX-50 inverted microscope.

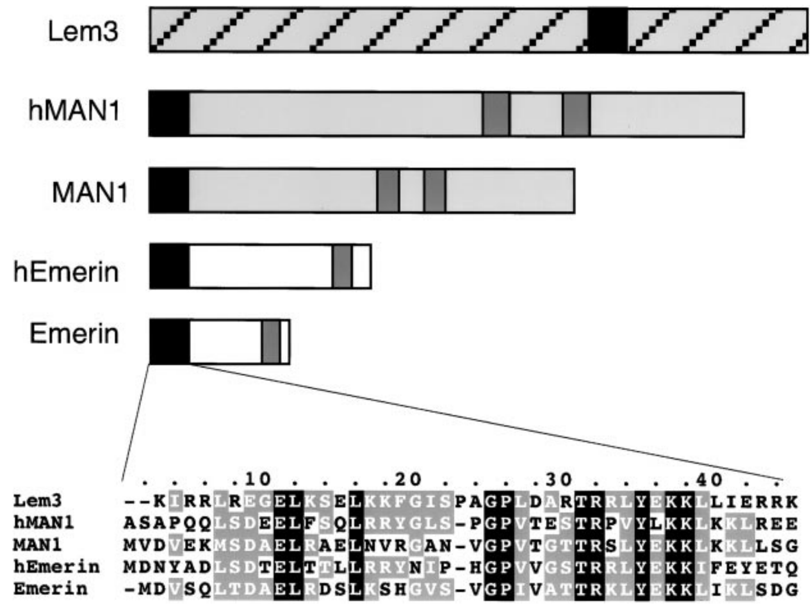


Figure 1. Schemes of the three LEM domain proteins in *C. elegans* showing the positions of LEM domains (black) and transmembrane domains (dark gray). Also shown are the amino acid sequences of the LEM domains of Ce-Lem3 (Lem3), human MAN1 (hMAN), Ce-MAN1, human emerin (hEmerin), and Ce-emerin (Emerin). Numbering of the amino acids is arbitrary. Black shading indicates identical residues, gray shading indicates conserved residues, and dashes indicate gaps introduced to optimize homology.

Cell Extracts

C. elegans nuclei were prepared essentially as described (Dixon *et al.*, 1989). The quality of each preparation was analyzed by staining aliquots with Hoechst 33258 and viewing with a Zeiss Axioskop microscope. For chemical extraction, 1 volume of nuclei was either used directly or thawed on ice, washed once in PBS-Inh (PBS containing 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), centrifuged at 4000 $\times$ *g* for 1 min at 4°C, and then extracted for 30 min at 4°C in 10 volumes of PBS-Inh plus the extraction reagent (e.g., 1 M NaCl). Extraction at pH 11 was performed in NaOH. After extraction, the residual nuclear pellet was separated from the supernatant by centrifugation at 9000 $\times$ *g* for 1 min at 4°C. The nuclear pellet was washed in PBS. The supernatant was further purified by centrifugation at 14,000 $\times$ *g* for 5 min at 4°C.

To prepare protein samples for SDS-PAGE, we boiled each sample (nuclei, salt/detergent supernatants, or wild-type N2 *C. elegans* animals) for 5 min in 2$\times$ SLB solution (25 mM Tris-HCl, pH 6.8, 20% glycerol, 0.2 M β-mercaptoethanol, 4% SDS, 0.001% bromphenol blue) and then passed the extract through a 25-gauge, five-eighth–inch syringe. Protein extracts were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with specific antibodies.

RESULTS

The C. elegans Genome Encodes Three Putative LEM Domain Proteins: Ce-emerin, Ce-MAN1, and Ce-Lem3

We searched the nearly complete *C. elegans* genome for sequences encoding conserved LEM domain residues and found three ORFs that we designated *emr-1* (GenBank accession number AAB58065.1), *lem-2* (accession number CAA21599), and *lem-3* (accession number CAB05722.1), which encode the putative proteins Ce-emerin, Ce-MAN1, and Ce-Lem3, respectively. Based on the presence of expressed sequence tags that match these genes in the Kohara database (16 for Ce-emerin, 5 for Ce-Lem3, 1 for Ce-MAN1; www.ddbj.nig.ac.jp/htmls/c-elegans/html/ce-index.html), we deduced that all three ORFs are transcribed and that Ce-emerin might be more abundant than Ce-MAN1. Based on their amino acid sequence similarity (our unpublished results) and the positions of their transmembrane domains (Figure 1), we concluded that *lem-2* and *emr-1* corresponded to human MAN1 (Lin *et al.*, 2000) and emerin (Bione *et al.*, 1994), respectively. Ce-Lem3 was unique in two respects: hydropathy analysis predicted that it had no transmembrane domain (our unpublished results), and its LEM domain was located near the middle of the protein rather than at the N terminus (Figure 1). Ce-Lem3 had no obvious homology to either Ce-emerin or Ce-MAN1 outside the LEM domain, nor with any human proteins in the database. Further experiments were focused on Ce-emerin and Ce-MAN1.

Polyclonal antibodies were raised in mice against synthetic peptides corresponding to the N or C termini of Ce-MAN1 or Ce-emerin (see MATERIALS AND METHODS). These antibodies were used to determine the mass of the endogenous Ce-MAN1 and Ce-emerin proteins on immunoblots of whole-protein extracts from mixed-stage wild-type (N2) *C. elegans*. Both proteins migrated on SDS-PAGE close to their predicted masses, 52 kDa for Ce-MAN1 (predicted mass, 55 kDa) and 17 kDa for Ce-emerin (predicted mass, 18 kDa; Figure 2). On immunoblots of whole *C. elegans* extracts, specific recognition of each protein was abolished by preincubating the antibodies with 1 mg/ml peptide antigen (Figure 2, Im + pep), confirming that the bands detected on immunoblots were specific.

To determine if Ce-MAN1 and Ce-emerin behaved as integral membrane proteins of the nuclear envelope, we tested their resistance to extraction by detergents, salt, and chaotrophic agents (Singer, 1974). *C. elegans* nuclei were isolated and extracted with PBS containing each reagent. Supernatants and pellets were then separated and analyzed by immunoblotting. Ce-MAN1 (Figure 3) and Ce-emerin (our unpublished results) gave the same results. The majority of Ce-lamin was extracted by treatment with 1 M NaCl or 8 M urea (Figure 3) as expected, because lamins are not

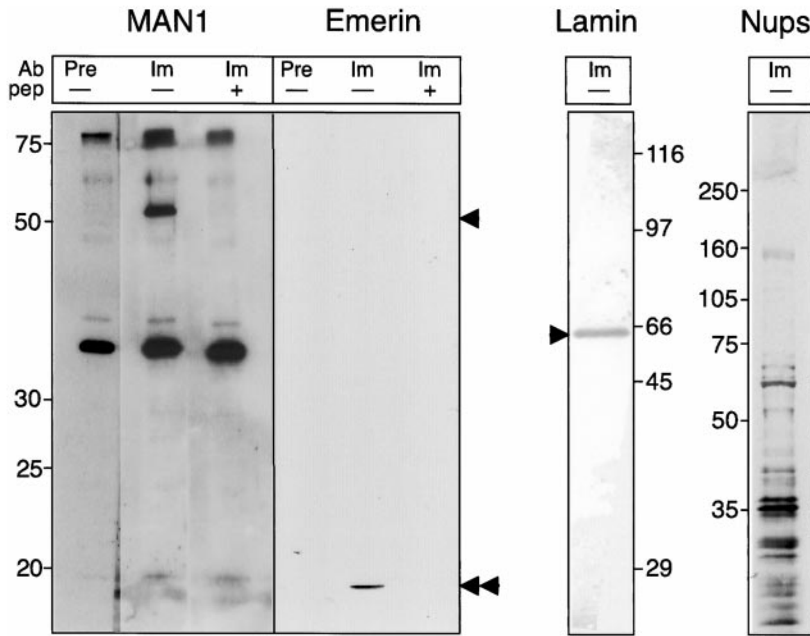


Figure 2. Protein blot analysis of whole *C. elegans* proteins probed with antibodies to Ce-MAN1, Ce-emerin, Ce-lamin, and nuclear pore complex proteins (nucleoporins; Nups). Protein blot analysis was performed on total protein extracts of mixed-stage *C. elegans* with the use of mouse polyclonal antibodies raised against a C-terminal peptide from Ce-MAN1 (serum 3268) or a C-terminal peptide from Ce-emerin (serum 3272). Ce-lamin was detected with the use of affinity-purified rabbit anti-lamin antibodies. Nucleoporins were detected with the use of mAb414, which recognizes a subset of nucleoporins that contain FG repeats. For the Ce-MAN1 and Ce-emerin blots, total *C. elegans* proteins were probed with preimmune serum (Pre), immune serum (Im), or with immune serum preincubated with an excess of peptide antigen to compete for specific binding (Im + pep). Ce-MAN1 (arrowhead) and Ce-emerin (double arrowhead) migrate on SDS-PAGE as 52- and 17-kDa proteins, respectively. Ce-lamin migrates at 64 kDa (arrowhead). mAb414 recognizes one major protein migrating at 60 kDa and additional proteins (arrowheads). Size markers in kDa are indicated for each blot.

integral membrane proteins. Ce-MAN1, Ce-emerin, and Ce-lamin all pelleted after treatment with 1% Triton X-100. However, the majority of Ce-MAN1 and Ce-emerin was extracted by a combination of 1 M NaCl plus 1% Triton X-100, demonstrating that they are integral membrane proteins. Both proteins pelleted after extraction at pH 11, as predicted for integral membrane proteins; they also pelleted after extraction with 1 M NaCl or 8 M urea, suggesting that they are attached to insoluble components inside the *C. elegans* nucleus. These results were consistent with the extraction properties of human MAN1 (Lin *et al.*, 2000) and emerin (Manilal *et al.*, 1996). We concluded that Ce-MAN1 and Ce-emerin are integral membrane proteins of the *C. elegans* nuclear envelope and are attached to intranuclear structures, presumably including the nuclear lamina.

Indirect Immunofluorescence Staining of Endogenous Ce-emerin Suggests Novel Timing of Nuclear Membrane Breakdown in C. elegans

To localize Ce-emerin, we stained *C. elegans* embryos by indirect immunofluorescence with the use of antibodies

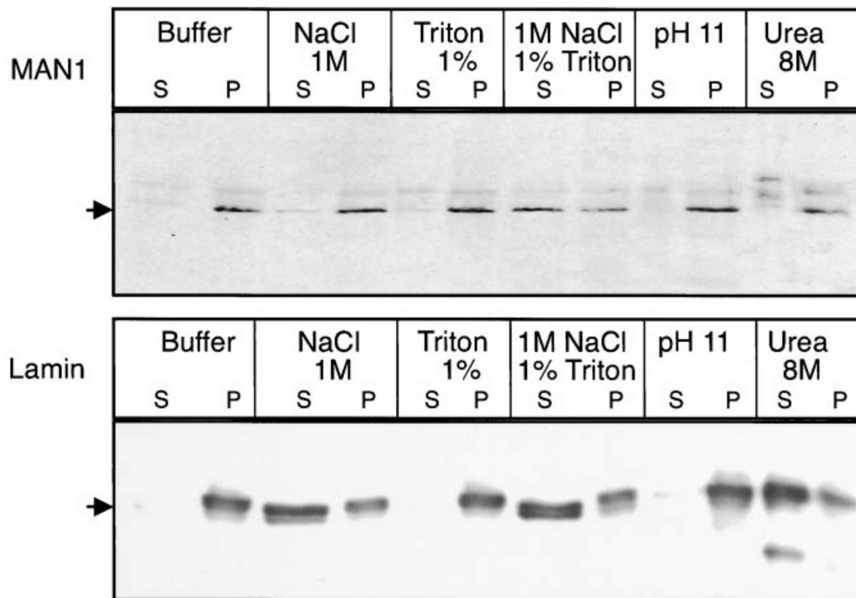


Figure 3. Solubility properties identify Ce-MAN1 as an integral membrane protein. *C. elegans* nuclei were extracted for 30 min in PBS or PBS containing one of the following reagents: 1 M NaCl, 1% Triton X-100, 1 M NaCl plus 1% Triton X-100, NaOH pH 11, or 8 M urea. After extraction, the supernatants (S) and residual pellets (P) were separated by centrifugation, and proteins were separated by 12% SDS-PAGE and analyzed by immunoblotting with the use of antibodies specific for Ce-MAN1 (C-terminal peptide) and Ce-lamin (see MATERIALS AND METHODS). The smaller band in the 8 M urea supernatant lane represents a degradation product of lamin; Ce-MAN1 degradation was not detected in the same extract. The positions of Ce-MAN1 and Ce-lamin are indicated by arrows at left. Similar results were obtained for Ce-emerin.

against Ce-emerin and the DNA dye Hoechst 33258 (Figure 4A; see MATERIALS AND METHODS). During interphase, endogenous Ce-emerin localized at the nuclear envelope and colocalized with lamins (Ce-MAN1 protein also colocalized with the lamina; our unpublished results). To our surprise, nuclear rim staining by Ce-emerin persisted during prophase (Figure 4A, P), metaphase (Figure 4A, M), and early anaphase (see Figure 5). Control embryos stained with preimmune antibodies were negative (our unpublished results). To verify our interpretation of the stages of mitosis, we triple labeled embryos with the use of DNA dye (Figure 4B, left) and antibodies against tubulin (Figure 4B, right, red staining) and Ce-lamin (Figure 4B, right, green staining). Cells designated as early prophase by their DNA morphology (Figure 4B, left, EP) were confirmed by their separated (but not yet opposed) centrosomes and interphase pattern of microtubule staining in the cytoplasm (Figure 4B, right). Cells designated as late prophase (Figure 4B, left, LP) were confirmed because they had nearly opposed centrosomes and a mitotic pattern of microtubule staining. Cells designated as prometaphase by their DNA morphology (Figure 4B, left, PMP) were confirmed by their fully opposed centrosomes, but they still had a potentially intact envelope as judged by exclusion of tubulin staining from the nucleus. Finally, cells designated as metaphase by DNA staining (Figure 4B, left, M) were confirmed because they had an "intranuclear" spindle and a nearly intact lamina that was interrupted only near the spindle poles. Together with the Ce-emerin staining (Figure 4A), these results suggested that the nuclear membranes and lamina remained largely intact during metaphase and that the timing of nuclear envelope disassembly in *C. elegans* might be different from that of other eukaryotes. We tested this prediction systematically with the use of antibodies against nucleoporins, Ce-lamin, Ce-MAN1, and Ce-emerin to monitor each component of the nuclear envelope at each stage of mitosis in *C. elegans* embryos.

Pore Complexes Break Down at Different Stages of Mitosis in Early and Late Embryos

The disassembly of NPCs was monitored with the use of mAb414, which recognizes mammalian nucleoporins that contain an FG-repeat motif (Davis and Blobel, 1986; Radu *et al.*, 1995; Shah *et al.*, 1998) and stains nuclear envelope pore complexes in *C. elegans* (Browning and Strome, 1996; Pitt *et al.*, 2000). On immunoblots of *C. elegans* proteins, mAb414 recognized a 60-kDa protein, which we assume to be the orthologue of mammalian nucleoporin p62 (Figure 2, Nups) (Davis and Blobel, 1986). Other cross-reacting bands (including those migrating at 35, 37, and 160 kDa) may represent additional FG nucleoporins in *C. elegans*, but their significance was not determined. Indirect immunofluorescence of *C. elegans* with mAb414 gave strong, slightly punctate staining of the nuclear envelope (Figure 5) (Pitt *et al.*, 2000), typical of nucleoporins in other organisms.

In embryos with <30 cells, mAb414 stained the nuclear rim during interphase, prophase, prometaphase, and metaphase (Figure 5A). Nucleoporin rim staining disappeared only during anaphase and reappeared around chromatin during telophase (Figure 5A). These results suggested that in early embryos of *C. elegans*, the pore complexes remain until after metaphase, strikingly later than their disassembly in mammalian cells and *Drosophila*. We found a different pattern in older embryos (>30 cells): rim staining for pore complexes was diminished in prometaphase and absent during metaphase and anaphase (Figure 5B), closer to the timing in mammalian cells and *Drosophila* and supporting the idea that pore complexes disassemble earlier than other nuclear envelope structures. Nucleoporins reassembled at the same time (telophase) in all *C. elegans* embryos, as in vertebrates and *Drosophila* (Gerace *et al.*, 1982; Davis and Blobel, 1986; Harel *et al.*, 1989).

Lamins Remain in the Nuclear Envelope until Late Anaphase in C. elegans Early Embryos

Affinity-purified polyclonal antibodies against Ce-lamin recognized a protein of 64 kDa on immunoblots of whole-protein extracts from mixed-stage wild-type (N2) *C. elegans* (Figure 2). In both early and later embryos, lamins maintained a nearly complete rim-staining pattern during metaphase and early anaphase (Figure 5, A and B). The specificity of antibody staining for Ce-lamin was confirmed by the lack of signal in preimmune controls (our unpublished results) and by the >40-fold loss of the rim stain signal in nematodes disrupted for Ce-lamin expression (our unpublished results). The exception to rim staining was near the spindle poles, where Ce-lamin staining became progressively weaker starting in prometaphase, with a large gap at both poles during early anaphase (Figure 5, A and B). This local disruption of lamina integrity was consistent with mechanical puncturing by spindle microtubules, as seen in other organisms (Stafstrom and Staehlin, 1984; Paddy *et al.*, 1996; Terasaki, 2000). Elsewhere, the lamina remained apparently intact through early anaphase and was removed only during mid-late anaphase. In later embryos (>30 cells), the lamina appeared to disassemble more extensively at earlier stages (e.g., prometaphase), as deduced from higher levels of cytoplasmic staining at earlier stages of mitosis (our unpublished results). We concluded that in early *C. elegans* embryos, the lamina structure persists much longer than the lamina in vertebrate cells. The intensity of lamin antibody staining during mitosis was always higher than in interphase (see DISCUSSION).

The timing of lamin assembly was similar to that in vertebrate and *Drosophila* lamins (Gerace *et al.*, 1978; Harel *et al.*, 1989); lamins reassociated with chromatin during telophase but did not completely reassemble until G1 phase (Figure 5; our unpublished results).

Staining of Ce-emerin and Ce-MAN1 Reveals That Nuclear Membranes Completely Disassemble Only during Mid-Late Anaphase in C. elegans Embryos

Immune antibodies against Ce-emerin (Figures 4 and 5) and Ce-MAN1 (Figure 5), but not preimmune sera (our unpublished results), specifically stained the nuclear envelope. Identical nuclear envelope rim staining was seen with a total of two independent immune antisera against Ce-emerin and four independent immune antisera against N- and C-terminal peptides from Ce-MAN1 raised in mice and rats; in all cases, preimmune staining of the nuclear envelope was neg-

ative (our unpublished results). This result showed that both Ce-emerin and Ce-MAN1 are localized at the nuclear membrane in *C. elegans* and were suitable markers with which to follow nuclear membrane breakdown in *C. elegans*. We found the same results for both proteins, with no apparent differences between early and late embryos. Ce-emerin and Ce-MAN1 maintained a nuclear rim-staining pattern through early anaphase. Staining for both proteins became weaker near the spindle poles during metaphase and anaphase (Figure 5A, emerin anaphase), but this was less obvious than with lamins, probably because of the lower signal and higher background staining produced by antibodies against Ce-MAN1 and Ce-emerin. Both Ce-MAN1 and Ce-emerin were completely disassembled only during mid-late anaphase (Figure 5, A and B) in both early and late embryos and reassociated with the chromatin periphery at telophase. Antibodies directed against an N-terminal peptide of Ce-MAN1 selectively failed to recognize Ce-MAN1 during telophase or early G1 (our unpublished results). We hypothesized that the N-terminal region of Ce-MAN1 might be covalently modified or masked by protein binding during these stages (see DISCUSSION). We concluded that the staining intensity for the nuclear membranes was strong through early anaphase everywhere except at the spindle poles, with complete breakdown occurring during mid-late anaphase.

Release of Splicing Factors Also Occurs Late during Mitosis in C. elegans

The results described above showed that the timing of nuclear envelope breakdown was significantly later in *C.*

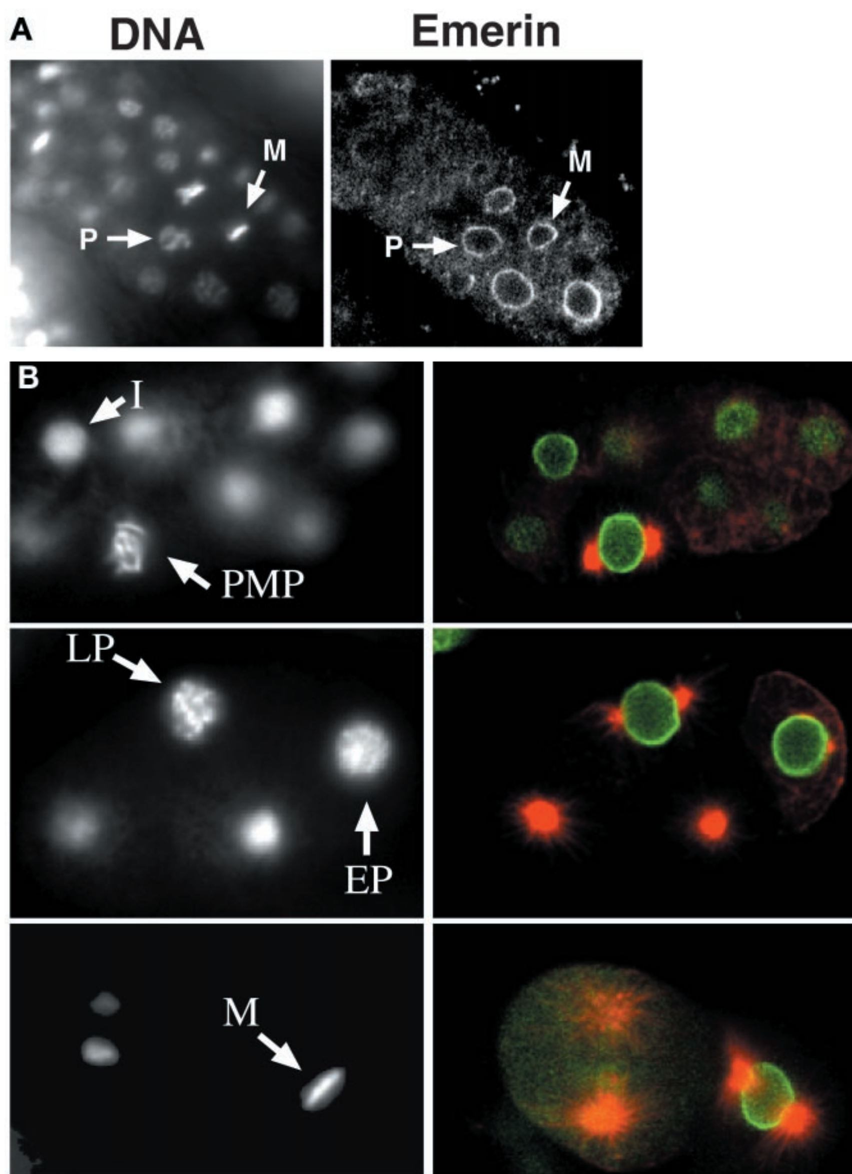
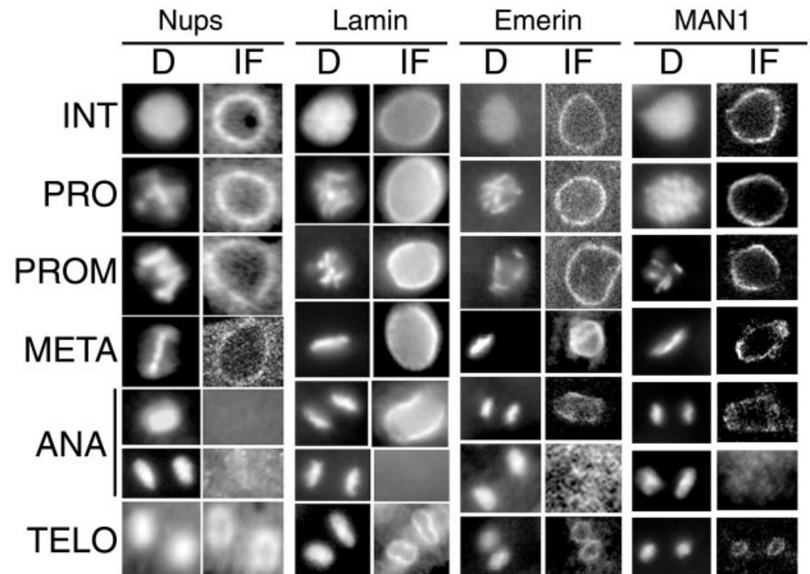


Figure 4. Persistence of nuclear envelope markers during mitosis in *C. elegans*. (A) Indirect immunofluorescence of endogenous Ce-emerin in a *C. elegans* embryo. DNA was stained with Hoechst 33258 (left), and the same embryo was stained for endogenous Ce-emerin with the use of mouse polyclonal serum 3272 (right; imaged by confocal microscopy). Arrows point to nuclei in prophase (P) and metaphase (M). (B) *C. elegans* embryos triple stained for DNA (Hoechst 33258, left) and by indirect immunofluorescence with the use of antibodies against Ce-lamin (green) and tubulin (red; right; imaged by confocal microscopy). The stages of mitosis were determined by chromosome morphology and corroborated by tubulin staining patterns: I, interphase; PMP; prometaphase; LP, late prophase; EP, early prophase; M, metaphase.



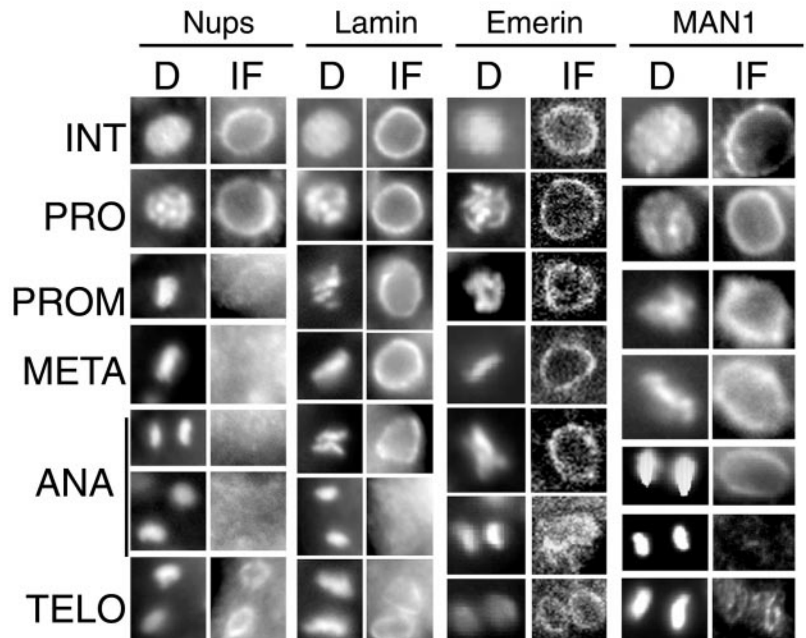


Figure 5. Immunofluorescence localization of endogenous nuclear envelope proteins at different stages of mitosis in 2- to 24-cell *C. elegans* embryos (A) and >30-cell *C. elegans* embryos (B). Embryos were doubly stained for DNA with the use of Hoechst 33258 (D) and by indirect immunofluorescence (IF) with the use of antibodies specific for nuclear pore complexes (mAb414; Nups), Ce-lamin, Ce-emerin, or Ce-MAN1. A representative nucleus from each stage is shown: INT, interphase; PRO, prophase; PROM, prometaphase; META, metaphase; ANA, anaphase; and TELO, telophase. All Ce-emerin immunofluorescence images, plus the panel showing nucleoporin metaphase staining in early embryos and most Ce-MAN1 early embryo images, were obtained by confocal microscopy; all others were imaged by fluorescence microscopy.

elegans than in vertebrates. To determine when intranuclear proteins were released from nuclei during mitosis, we stained for the endogenous serine/arginine-rich family of conserved snRNPs, which are involved in mRNA splicing, with the use of mAb104 (Roth *et al.*, 1990). mAb104 recognizes phosphorylated snRNPs from a wide variety of vertebrate and invertebrate species, including *C. elegans*, during both interphase and mitosis (Roth *et al.*, 1990; Zahler *et al.*, 1993). In mammalian cells, these splicing factors are released into the cytoplasm during early prophase (Roth *et al.*, 1990). In *C. elegans*, this antibody gave punctate intranuclear staining during interphase

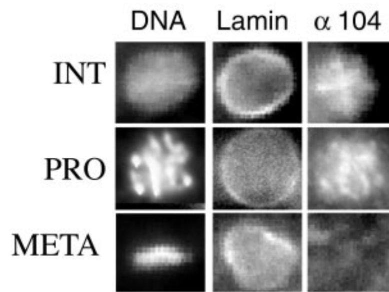


Figure 6. Double-label immunostaining of endogenous snRNPs and Ce-lamin in *C. elegans* embryos. *C. elegans* embryos were double labeled for endogenous snRNPs with the use of mouse mAb104 (Roth *et al.*, 1990) and for endogenous Ce-lamin with the use of affinity-purified antibodies. DNA was stained with Hoechst 33258. Each row shows nuclei from a >30-cell embryo stained for DNA (left), Ce-lamin (middle), and snRNPs (right, α104). Cell cycle stages are abbreviated as in Figure 5.

(Figure 6), as expected. However, the punctate intranuclear signal was still present in late prophase, disappearing in metaphase (Figure 6). Thus, this class of intranuclear proteins also exhibited late disassembly and release from the nucleus during mitosis in *C. elegans*, consistent with our findings for nuclear envelope markers.

DISCUSSION

We characterized two LEM domain proteins, Ce-emerin and Ce-MAN1, and showed that they are integral membrane proteins localized to the nuclear envelope in *C. elegans*, consistent with their mammalian counterparts. We used these proteins, together with Ce-lamin and nucleoporins, to determine the timing of nuclear envelope disassembly in *C. elegans*. We discovered unexpected differences in the timing of nuclear envelope breakdown between *C. elegans* and vertebrates or *Drosophila* and also between early (2- to 24-cell) and later (>30-cell) stages of embryogenesis in *C. elegans*.

LEM Domain Proteins in C. elegans

Ce-emerin is smaller than human emerin (166 versus 254 residues). Most of the residues missing from Ce-emerin corresponded to a serine-rich region of human emerin near the conserved transmembrane domain. As a result, Ce-emerin is much less serine-rich than human emerin (13% versus 34%). We speculate that residues absent from Ce-emerin either might be unnecessary for function or might mediate interactions with partners not found in *C. elegans*. Mammalian emerin has very limited diffusional mobility at the nuclear envelope during interphase (Östlund *et al.*, 1999), consistent with its binding to the lamina/matrix (Squarzoni *et al.*, 1998; Morris and Manilal, 1999), its binding to lamins (Fairley *et al.*, 1999; Clements *et al.*, 2000), and its attachment to the DNA-binding protein BAF (see below; our unpublished results). Mammalian emerin also displays an intriguing pattern of localized reassembly on chromosomes during mitosis (Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000), consistent with specialized roles during nuclear assembly.

Ce-MAN1 is also smaller than its human counterpart (500 versus 754 residues). The level of identity between Ce-MAN1 and human MAN1 is highest in regions near the N and C termini (Lin *et al.*, 2000). Human MAN1 was cloned only recently (Lin *et al.*, 2000), and very little is known yet about its function. Based on its nuclear envelope localization, its conservation during evolution, and the finding that loss of Ce-MAN1 is lethal during *C. elegans* embryogenesis (K.K. Lee, Y. Gruenbaum, and K.L. Wilson, unpublished observations), Ce-MAN1 is likely to have an essential function in the nucleus. Further study of Ce-emerin and Ce-MAN1 in *C. elegans* may yield new insights into their functional roles in humans, in which the loss of emerin causes Emery-Dreifuss muscular dystrophy (Wilson, 2000).

C. elegans has no apparent orthologue to LAP2, the best-characterized vertebrate LEM domain protein (reviewed by Goldberg *et al.*, 1999a; Gotzmann and Foisner, 1999; Wilson, 2000). We excluded Ce-emerin (*emr-1*) as a LAP2 orthologue because Ce-emerin lacks the N-terminal residues (e.g., 1–85 of human LAP2) that are conserved among all LAP2 isoforms (Wilson, 2000) and essential for LAP2 activity in *Xenopus* extracts (D.K. Shumaker, K.K. Lee, Y.C. Tanhehco, R. Craigie, and K.L. Wilson, unpublished observations).

Potential Regulation of Ce-lamin and Ce-MAN1 during Mitosis

The higher intensity of Ce-lamin staining during mitosis suggested that the antigen was either more accessible to antibody or more tightly bound as a result of changes in lamina structure or posttranslational modification(s) of Ce-lamin. Ce-lamin, unlike vertebrate lamins, lacks consensus sites for phosphorylation by the $p34^{cdc2}$ mitotic kinase (*ncc-1* in *C. elegans*; Boxem *et al.*, 1999), which might explain why Ce-lamin disassembles unusually late during mitosis in *C. elegans*. Disassembly of the *C. elegans* lamina may be driven by a kinase other than $p34^{cdc2}$ (Riemer *et al.*, 1993); one candidate is PKC, which is required for lamina disassembly in zebrafish and may act before $p34^{cdc2}$ (Collas, 1999). Further experiments will be needed to determine how the timing of nuclear disassembly is regulated in *C. elegans*.

We hypothesize that Ce-MAN1 might be antigenically masked at its N terminus during telophase and G1, because antibodies against residues 1–14 failed to detect Ce-MAN1 at these stages of the cell cycle (our unpublished results). In contrast, antibodies directed against a C-terminal peptide detected Ce-MAN1 during these stages, when newly assembled nuclei begin to decondense their chromatin and expand. Thus, Ce-MAN1 might be differentially regulated during nuclear growth. Further experiments will be required to determine whether Ce-MAN1 is posttranslationally modified, and if so, to understand the functional significance of the modification(s).

Unique Timing of Nuclear Envelope Breakdown in C. elegans

In vertebrates, the nuclear envelope starts disassembling at the prophase–prometaphase transition: NPC subunits are dispersed into the cytoplasm (Gerace *et al.*, 1982; Davis and Blobel, 1986; Snow *et al.*, 1987), the nuclear membrane proteins detach from their substrates and merge into the endoplasmic reticulum network (Ellenberg *et al.*, 1997; Yang *et al.*,

	Nups	Lamin	MAN1	Emerin
Early				
Interphase	+	+	+	+
Prophase	+	+	+	+
Prometaphase	+	+	+	+
Metaphase	+	+	+	+
Early anaphase	-	+	+	+
Mid-late anaphase	-	-	-	-
Telophase	+	+/-	+	+
G1	+	+	+	+
Late				
Interphase	+	+	+	+
Prophase	+	+	+	+
Prometaphase	+/-	+	+	+
Metaphase	-	+/-	+	+
Early anaphase	-	+/--	+	+
Mid-late anaphase	-	-	-	-
Telophase	+	+/-	+	+
G1	+	+	+	+

Figure 7. Summary of immunofluorescence results for endogenous nuclear envelope components during mitosis in early (2- to 24-cell) and later (>30-cell) stage *C. elegans* embryos. + indicates a strong nuclear envelope "rim" fluorescence signal; − indicates the absence of rim staining; and +/− indicates partial nuclear rim staining. The shaded boxes highlight the stages of mitosis when nuclear rim staining was reduced or not detectable.

1997), and the lamina depolymerizes into both soluble and membrane-associated pools (reviewed by Gerace and Burke, 1988; Moir *et al.*, 1995). The lamina also begins to disassemble during prophase in mammalian cells (Georgatos *et al.*, 1997).

We used antibodies directed against three major components of the *C. elegans* nuclear envelope (nucleoporins, nuclear lamina, and nuclear membranes) to determine the fate of the *C. elegans* nuclear envelope during mitosis. Our results are summarized in Figure 7. We found that the timing of nuclear envelope disassembly during mitosis in *C. elegans* was late compared with that in both invertebrates and vertebrates. Notably, the lamins and nuclear membranes remained assembled in a rim-like structure through early anaphase. Rim staining for lamins (and to a lesser extent Ce-MAN1 and Ce-emerin) became weaker near the spindle poles as mitosis progressed. This result suggested that the nuclear envelope was disrupted near the spindle poles, perhaps by mechanical damage from spindle microtubules (Stafstrom and Staehelin, 1984; Paddy *et al.*, 1996; Terasaki, 2000). The NPCs maintained their nuclear rim-staining pattern through metaphase in 2- to 24-cell embryos, disassembling only during anaphase. In later embryos, the pattern changed for the pore complexes and lamina—they started disassembling earlier (at prometaphase and metaphase, respectively), but not quite as early as in vertebrates. Staining for the serine/arginine-rich class of snRNPs further suggested that structures inside the nucleus disassembled and entered the cytoplasm at the same time as NPCs. Despite the variation in the stage at which each nuclear component began to disassemble, one result was consistent in *C. elegans*: all components of the nuclear envelope were completely disassembled only during mid-late anaphase.

We cannot yet explain why the initiation of pore complex (and to a lesser extent lamina) disassembly occurred at different stages in early and late embryos. The speed of cell division is variable in both. Early embryonic cell divisions are not synchronous in *C. elegans*, and there is no obvious equivalent to the midblastula transition, because transcription begins as early as the three- to four-cell stage (Seydoux and Fire, 1994; Newman-Smith and Rothman, 1998) and is lineage-dependent. Even though the progenitor cell for the AB lineage is created at the two-cell stage (Schubert *et al.*, 2000), we note a potentially interesting coincidence: the breakpoint between "early" and "later" embryonic phenotypes occurred around the 24-cell stage, when the progenitor cells for all six major lineages (AB, MS, E, C, D, and P4) have been created. We did not determine the lineages of the cell nuclei examined in this work. Therefore, we speculate that changes in the timing of nuclear envelope disassembly might correlate with specific times in embryonic development.

These findings raise interesting questions about the potential selective advantages of disassembling the nuclear envelope during mitosis. In the yeast *S. cerevisiae*, chromosomes are condensed and segregated within an intact nuclear envelope. In an evolutionarily distant yeast, *Schizosaccharomyces pombe*, the spindle pole body (centrosome) inserts into the nuclear envelope during mitosis to mediate spindle formation inside the nucleus (Ding *et al.*, 1997). The mechanism of centrosome insertion into the nuclear envelope is not understood but might be related to the formation of NPCs (West *et al.*, 1998). More complex eukaryotes have progressively greater extents of nuclear breakdown. Except for the timing of NPC breakdown, our findings in *C. elegans* are similar to *Drosophila* syncytial embryos, where complete breakdown of the nuclear envelope is delayed until mid-late anaphase, and a fraction of lamins and otefin (a lamin-binding peripheral membrane protein in *Drosophila*) persist in a rim-staining pattern until mid-anaphase (Stafstrom and Staehelin, 1984; Harel *et al.*, 1989; Paddy *et al.*, 1996).

Despite differences in timing between early and late *C. elegans* embryos, NPCs are clearly the first component of the nuclear envelope to disassemble in both *C. elegans* and *Drosophila* early embryos. The feature of mitosis that makes *C. elegans* unique among studied eukaryotes, and different from *Drosophila*, is the persistence of NPCs through and including metaphase in early embryos and through prometaphase in later embryos. In *C. elegans*, the disassembly of pore complexes during anaphase (in 2- to 24-cell embryos) or metaphase (in >30-cell embryos) fulfills the minimal requirement for a functionally open mitosis, because it was accompanied by the release of snRNP splicing factors. *C. elegans* achieves fully structurally open mitosis only during mid-late anaphase, when the nuclear lamina and membranes are also disassembled. Thus, *C. elegans* has a fully open mitosis, similar to other metazoans and different from the closed mitosis in single-cell eukaryotes such as *S. cerevisiae* (Heath, 1980). The main difference between *C. elegans* and vertebrates is the stage at which mitosis becomes fully open. *C. elegans* will be a useful organism in which to explore how the timing of nuclear disassembly is regulated.

Our major hypothesis arising from this work is that *C. elegans* appears to represent a unique evolutionary intermediate in which complete nuclear disassembly occurs much later than in higher eukaryotes. We hypothesize that the efficiency of nuclear envelope breakdown may correlate with increasing genome complexity during evolution. It is an open question which aspect(s) of nuclear or chromosome structure or function might benefit during evolution from changes in the efficiency, timing, or extent of nuclear envelope breakdown during mitosis.

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