Early embryonic requirement for nucleoporin Nup35/NPP-19 in nuclear assembly

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A B S T R A C T
Nuclear pore complexes (NPCs) are gateways for transport between the nucleus and cytoplasm of eukaryotic cells and play crucial roles in regulation of gene expression. NPCs are composed of multiple copies of ~30 different nucleoporins (nups) that display both ubiquitous and cell type specific functions during development. Vertebrate Nup35 (also known as Nup53) was previously described to interact with Nup93, Nup155 and Nup205 and to be required for nuclear envelope (NE) assembly in vitro. Here, we report the first in vivo characterization of a Nup35 mutation, npp-19(tm2886), and its temperature-dependent effects on Caenorhabditis elegans embryogenesis. At restrictive temperature, npp-19(tm2886) embryos exhibit chromosome missegregation, nuclear morphology defects and die around mid-gastrulation. Depletion of Nup35/NPP-19 inhibits NE localization of Nup155/NPP-8, NPC assembly and nuclear lamina formation. Consequently, nuclear envelope function, including nucleo-cytoplasmic transport, is impaired. In contrast, recruitment of Nup107/NPP-5, LEM-2 and nuclear membranes to the chromatin surface is Nup35/NPP-19-independent, suggesting an uncoupling of nuclear membrane targeting and NPC assembly in the absence of Nup35/NPP-19. We propose that Nup35/NPP-19 has an evolutionary conserved role in NE formation and function, and that this role is particularly critical during the rapid cell divisions of early embryogenesis.

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Introduction

Early embryogenesis in many plant and animal species is characterized by rapid cell cycles predominated by S and M phases and with shortened or absent gap phases (O’Farrell et al., 2004; Oegema and Hyman, 2006). Complex structural rearrangements must be executed both precisely and efficiently to prevent developmental abnormalities. In addition to the cytoskeleton, many cell organelles undergo dramatic changes during mitosis, including the Golgi (Colanzi and Corda, 2007), the endoplasmic reticulum (ER) (Lowe and Barr, 2007) and the nucleus (Prunuske and Ullman, 2006). Elucidating the molecular mechanisms controlling these events is necessary to understand early embryonic development. In metazoan systems, the nuclear envelope (NE) is completely disassembled during mitosis and reforms around the chromatin of daughter nuclei in late anaphase and telophase (Hetzer et al., 2005). Analysis of NE dynamics in embryos of the nematode Caenorhabditis elegans revealed that the timing of NE break down (NEBD) relative to anaphase onset depends on embryo age. Cells in young embryos undergo NEBD later than those in older embryos (Lee et al., 2000). The reason behind this asymmetry is unknown, but NEBD and NE reformation may be rate-limiting steps during the fast, early cell divisions.

The NE is crucial to numerous cellular and developmental processes, such as cell compartmentalization and nucleocytoplasmic transport (Terry et al., 2007), regulation of gene expression (Schneider and Grosschedl, 2007), and nuclear positioning (Wilmelsen et al., 2006). The complex nature of the NE has so far limited our understanding of its dynamics. Broadly, the NE can be subdivided into three main components; the nuclear membranes with associated proteins, the underlying nuclear lamina and the nuclear pore complexes (NPCs). NPCs form the only known transport route for RNA and protein between the nucleus and cytoplasm (Terry et al., 2007). In an evolutionarily conserved fashion, the NPCs are composed of approximately 30 different proteins termed nucleoporins (nups), each present in several copies. Recent estimates in Saccharomyces cerevisiae suggest that 456 individual proteins assemble into the ~50 MDa yeast NPC (Alber et al., 2007) whereas predictions for vertebrate NPCs range from ~60 to ~125 MDa (Hetzer et al., 2005). Although most nups appear to be expressed ubiquitously, some are enriched in certain cell types and in specific tissues (Lupu et al., 2008; Olsson et al., 2004; Uv et al., 2000). The role of individual nups in nucleo-cytoplasmic transport has been the focus of many studies, while their contribution to NPC assembly remains largely undefined.
NPC biogenesis is tightly linked to postmitotic NE reformation in organisms that undergo 'open' mitosis, i.e. species in which the NE is disassembled during mitosis. *C. elegans* is an attractive model to study NPC dynamics based on its amenability to genetic manipulation, stereotypic embryonic development, and an amplified RNA interference (RNAi) response (Gorjanacz et al., 2007a). A systematic RNAi-based study of *C. elegans* nups demonstrated that 7 of 20 nups are required for correct nuclear assembly (Galy et al., 2003). Among these nups, Nup35, encoded by the *C. elegans* npp-19 gene, remains one of the most poorly characterized. In rodent cells, Nup35 (also termed Nup53) to emphasize its homology to *S. cerevisiae* Nup53p) tagged with GFP has a relatively long residence time at the NPC, suggesting a possible scaffold function (Rabut et al., 2004). Indeed, vertebrate Nup35 can be co-purified together with Nup93, Nup155 and Nup205, indicating they form a stable NPC subcomplex (Hawryluk-Gara et al., 2005). Partial depletion of Nup35 from HeLa cells reduces the amount of Nup35 and stimulating nuclear assembly in reconstituted Xenopus egg extracts was defined (Hawryluk-Gara et al., 2008), but the role of Nup35 remains unclear. In *S. cerevisiae*, deletion of NUP53 is not lethal (Marelli et al., 1998), although overexpression of Nup53p induces the formation of intranuclear membranes (Marelli et al., 2001).

In this report, we characterize a temperature-sensitive allele of the *C. elegans* npp-19 gene, providing the first mutational analysis of a metazoan Nup35 during development. At restrictive temperatures, nearly all mutant embryos die before completing gastrulation. Microscopy-based studies revealed several defects in the 1-cell zygote. Most notably, pronuclear size and positioning were abnormal, and chromosomes failed to properly align or segregate. Whereas npp-19 mutant embryos displayed clear temperature-dependent phenotypes, RNAi-mediated depletion of NPP-19 induced strong NE and chromosome segregation defects and demonstrated a general requirement for NPP-19 activity.

**Materials and methods**

**Nematode strains**

The wild type strain was the *C. elegans* Bristol strain N2. npp-19 (tm2886) was provided by Dr. Shohi MiTani at the Japanese National Bioresource Project and backcrossed to the wild type six times followed by self-fertilization to obtain the homozygous strain BN38 npp-19(tm2886), BN7 unc-119(ed3) bqls07[unc-119(+); Ppie-1::IAP:: npp-19] and BN51 unc-119(ed3) bqls51[unc-119(+); Ppie-1::GFPl:5] were obtained by microparticle bombardment of DP38 unc-119(ed3) using plasmids pDP#MM051 (Maduro and Pilgrim, 1995) and pPGLv.1 npp-19 (this study) or pDP#MM051 and pPAG1 (Franz et al., 2005).

Subsequently, BN7 was crossed with OD83 gfp::lem-2; cherry::his-58 (Audhya et al., 2007) to generate BN14 gfp::npp-19; cherry::his-58, which was further crossed with BN38 to generate BN46 npp-19(tm2886); gfp::npp-19 and BN47 npp-19(tm2886); gfp::npp-19; cherry::his-58. Similarly, BN51 was crossed with OD57 gfp::bta-2; cherry::his-58 (McNally et al., 2006) to generate BN68 gfp::npp-5 cherry::his-58, which was further crossed with BN38 to generate BN79 npp-19(tm2886); gfp::npp-5 cherry::his-58. BN78 npp-19 (tm2886); gfp::lem-2; cherry::his-58 was obtained by crossing BN38 with OD83. BN13 gfp::npp-8; cherry::his-58, BN16 gfp::hmr-1; cherry::his-58, and BN27 gfp::pcn-1; cherry::his-58 were generated by crossing XA3546, XA3541 (Franz et al., 2005) and a GFP-PCNA/PCN-1 expressing strain (Brauchle et al., 2003) with OD83, respectively. JH1327 gfp::pie-1 was described previously (Reese et al., 2000). All strains were cultured using standard *C. elegans* methods (Stiernagle, 2006).

**Plasmids and RNAi**

Plasmid pGEX-GST-His for cloning and expression of C-terminal GST-tagged and C-terminal His-tagged fusion protein was generated by removal of a vrk-1-containing BamHI fragment of pGEX-VRK-1-His (Gorjanacz et al., 2007b). Full length and truncated npp-19 cDNAs were generated by RT-PCR using forward primer 5'-caggatc-cATGTTCCTCGCATCTAAACCA and either 5'-caggatcGGTGAATCCGATCGTTA or 5'-caggatccCAACAAAAATCGACTTGCCTGG as reverse primer. PCR fragments were digested with BamHI and inserted into pGEX-GST-His. Plasmid pGVLv.1 npp-19 was generated by Gateway cloning (Invitrogen) using a genomic npp-19 sequence (Franz et al., 2005) and pGVLv.1 (Gorjanacz et al., 2007b).

npp-3, npp-8, npp-13, npp-19 RNAi constructs and RNAi-by-feeding conditions were described previously (Galy et al., 2003), maf-2 and att-1 RNAi constructs were from the Drhringer library (Kamath et al., 2003). As negative control, the empty pPDF129.36 vector was used. RNAi was performed at 20 °C unless indicated otherwise.

**Western blot**

Embryos for Western blot analysis were obtained by hypochlorite treatment, disrupted by boiling and vortexing in SDS sample buffer together with 0.5 mm diameter glass beads and separated by 10% SDS-PAGE. Proteins were transferred to Immobilon P membrane (Millipore), which was probed with antibodies diluted in PBS containing 0.05% Tween-20 and 3% low-fat milk. The following primary antibodies were used: mouse monoclonal antibody DM1alpha (Sigma-Aldrich, 1:1000); rabbit polyclonal antiserum GLC (α-NPP-10C, 1:500), BTM6 (α-NPP-5, 1:300), BU84 (α-NPP-7, 1:500), BS08 (α-NPP-8, 1:300), OWYL (α-NPP-19, 1:1000) (Galy et al., 2003) and α-NPP-19 antibodies affinity purified from OWYL serum with an NPP-19 antigen fragment consisting of aa (amino acid residues) 1–215 (this study; 1:100). Secondary antibodies were peroxidase conjugate antibodies (Sigma-Aldrich, 1:5000–1:10,000).

**Immunofluorescence**

Embryos were processed for immunofluorescence as described (Gorjanacz et al., 2007b). The following primary antibodies were used: mouse monoclonal antibody (mAb) 414 (Covance, 1:300); rabbit polyclonal α-MEL-28 antiserum BUD3 (Galy et al., 2006) (1:500); rabbit polyclonal α-NPP-8 antiserum BUF9 (1:200); rabbit polyclonal α-NPP-19 antiserum OWYL (Galy et al., 2003) (1:300) and α-NPP-19 antibodies affinity purified from OWYL serum with an NPP-19 antigen fragment consisting of aa 1–215 (this study; 1:25). Secondary antibodies were Alexa Fluor 546-conjugated goat anti-mouse antibodies (Invitrogen, 1:1000) and Alexa Fluor 633-conjugated goat anti-rabbit antibodies (Invitrogen, 1:1000). Hoechst 33258 (Hoechst, 5 μg/ml) was used to detect chromatin. Confocal images were obtained with Leica AOBs SP2 and SPE microscopes and processed with ImageJ and Adobe Photoshop.

**Live embryo imaging**

Embryos were mounted in M9 buffer between a cover slip and a 2% agarose pad. Epifluorescence and transmitted light were recorded with a Leica Confocal Microscope TCS SP2 through a HCX PL APO 63×/1.4 objective. Images were captured using integrated Leica software and processed with ImageJ and Adobe Photoshop. The laser intensity was adjusted so that no effect on development was observed. Images were collected at 5 s (Fig. 8) or 20 s (Figs. 3–6) intervals for a total of 20–40 min. For extended recordings up to 14 h (Suppl. Fig. 3), samples were sealed with VALAP and z-stacks were acquired at 15 min intervals.
the amount of mutant NPP-19 protein present in lacking amino acid residues (aa) 217 analyses showed that embryos from homozygous Dr. Shohei Mitani). Reverse transcriptase PCR and Western blot
reduced to approximately 20
animals can be propagated under standard conditions but tm2886
we characterized a novel mutant allele, combined RNAi experiments with other genetic approaches. Initially,
propose that embryos require additional NPP-19 activity at 25 °C as
compared to 20 °C. Alternatively, mutant NPP-19 protein may not

Transmission electron microscopy

C. elegans hermaphrodites fed bacteria expressing either control or npp-19 dsRNA were cryo-immobilized using a Leica EM PACT high-pressure freezer (Leica, Vienna, Austria) and processed as described (Franz et al., 2005).

Results

Characterization of Nup35/NPP-19 mutant allele tm2886

To investigate the function of Nup35/NPP-19 in C. elegans we combined RNAi experiments with other genetic approaches. Initially, we characterized a novel mutant allele, tm2886 (kindly provided by Dr. Shohei Mitani). Reverse transcriptase PCR and Western blot analyses showed that embryos from homozygous npp-19(tm2886) hermaphrodites (hereafter termed tm2886 embryos) express NPP-19 lacking amino acid residues (aa) 217–286 (Figs. 1A, B). Interestingly, the amount of mutant NPP-19 protein present in tm2886 embryos is reduced to approximately 20–25% of wild type levels suggesting that the mutant mRNA and/or the truncated protein is unstable (Fig. 1B). tm2886 animals can be propagated under standard conditions but show 43–56% embryonic lethality at 15–20 °C (Table 1). At 25 °C the embryonic lethality increases significantly to 86%, which is similar to the lethality observed when expression of npp-19 is knocked down by RNAi (96%; Table 1, Fig. 1D; Galy et al., 2003). Western blot analysis of tm2886 embryos after incubation for 10 h at either 20 °C or 25 °C revealed that the amount of mutant NPP-19 protein is temperature-independent (Fig. 1C), ruling out that increased lethality observed at 25 °C is caused by a further reduction in NPP-19 levels. Instead, we propose that embryos require additional NPP-19 activity at 25 °C as compared to 20 °C. Alternatively, mutant NPP-19 protein may not

properly fold at elevated temperatures. Expression of NPP-19 fused to GFP restored embryonic viability of tm2886 embryos at 20 °C and partially at 25 °C (Table 1). These data demonstrate that the phenotypes observed in tm2886 are due to the deletion in the npp-19 gene and indicate that GFP-NPP-19 can functionally replace endogenous NPP-19. Finally, we note that the tm2886 embryos that


<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Embryonic lethality</th>
<th>Hatched embryos</th>
<th>→-alpha-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 wild type, 15 °C</td>
<td>555</td>
<td>0.6±0.5%</td>
<td>N.D.*</td>
<td></td>
</tr>
<tr>
<td>N2 wild type, 20 °C</td>
<td>1206</td>
<td>0.6±1.0%</td>
<td>N.D.†</td>
<td></td>
</tr>
<tr>
<td>N2 wild type, 25 °C</td>
<td>748</td>
<td>2.2±2.4%</td>
<td>N.D.‡</td>
<td></td>
</tr>
<tr>
<td>npp-19(tm2886), 15 °C</td>
<td>419</td>
<td>55.6±18.0%*†</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>npp-19(tm2886), 20 °C</td>
<td>1133</td>
<td>42.5±15.7%*†</td>
<td>86.8±7.2%*</td>
<td></td>
</tr>
<tr>
<td>npp-19(tm2886), 25 °C</td>
<td>632</td>
<td>86.2±12.1%*†</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>npp-19(tm2886); gfp::npp-19, 20 °C</td>
<td>243</td>
<td>4.7±6.2%*†</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>npp-19(tm2886); gfp::npp-19, 25 °C</td>
<td>138</td>
<td>21.6±7.1%*†</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>N2 control RNAi, 20 °C</td>
<td>431</td>
<td>0.4±0.4%</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>N2 npp-19(RNAi), 20 °C</td>
<td>662</td>
<td>96.0±5.6%*</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Young gravid hermaphrodites were incubated for 1 h on NGM plates at indicated temperatures. The number of embryos analyzed, percentage of embryonic lethality and percentage of hatched embryos that developed into fertile adults were determined after 0 h, 24 h and 96 h, respectively. *Standard deviation. †Not determined. Significant differences by Chi-square test: *different from the wild type or control RNAi at same temperature (p<0.001); †different from npp-19(tm2886) at 20 °C (p<0.001); ‡different from npp-19(tm2886) at 25 °C (p<0.001).
nuclei in (Fernandez and Piano, 2006; Galy et al., 2006). More than 95% of MEL-28, a nup discovered recently based on its role in NE assembly embryos with reduced NPP-19 activity. For all strains (including RNAi and chromatin markers to characterize in greater detail the defects in Live imaging of tm2886 and npp-19(RNAi) embryos

Right). As expected from the Western blot analysis above, NPP-19 localization to the nuclear periphery was strongly reduced in tm2886 embryos (n=100) obtained from nematodes grown at 20 °C showed normal mAb414 and α-MEL-28 staining (Fig. 2A, top; Suppl. Fig. 1). In contrast, tm2886 and npp-19(RNAi) embryos developing at 25 °C and 20 °C, respectively, displayed severe defects in nuclear morphology and mAb414 staining (Figs. 2A, bottom right; B, right). As expected from the Western blot analysis above, NPP-19 localization to the nuclear periphery was strongly reduced in tm2886 embryos as well as in npp-19(RNAi) embryos (Fig. 2; Suppl. Fig. 2). However, the remaining NPP-19 detected at the nuclear rim in tm2886 embryos indicates that NPP-19 aa 217–286 are dispensable for NE targeting. Strikingly, during cytokinesis we observed chromatin associated with the cleavage furrow in more than half of npp-19 (RNAi) and tm2886 embryos at restrictive temperature (n=100), suggesting that wild type NPP-19 activity is required for correct chromosome segregation during mitosis (Figs. 2A, bottom right; B, right).

Live imaging of tm2886 and npp-19(RNAi) embryos

We next crossed tm2886 with strains expressing fluorescent NE and chromatin markers to characterize in greater detail the defects in embryos with reduced NPP-19 activity. For all strains (including RNAi experiments described below), at least five embryos were observed by confocal time-lapse microscopy and the most frequent phenotype (≥80%) is reported here. Initially, we focused on Nup107/NPP-5 as a marker for the Nup107–160 NPC subcomplex, which is essential for NPC formation (Franz et al., 2005; Harel et al., 2003; Walther et al., 2003). In wild type embryos, GFP-NPP-5 accumulated at the NE prior to mitosis and relocalized to kinetochores subsequent to NEBD (Fig. 3A, left; Suppl. Movie S1; Franz et al., 2005). Completion of oocyte meiosis and polar body extrusion appeared normal in tm2886 zygotes developing at 25 °C (data not shown). GFP-NPP-5 still localized to the nuclear periphery but nuclear morphology and behavior was abnormal: Indicative of NE defects, pronuclei were considerably smaller than in wild type embryos and failed to juxtapose properly (Fig. 3A, middle, 3:40; Suppl. Movie S1) (Askaer et al., 2002). Frequently, the sperm-derived pronucleus was more severely affected than its oocyte-derived counterpart (arrow in Fig. 3A, middle, 3:40; 66%, n=32). Moreover, alignment of chromosomes (visualized by mCherry-histone H2B/HIS-58) on the metaphase plate was defective and chromosomes failed to be properly incorporated into the mitotic spindle of 1-cell stage tm2886 embryos (arrow in Fig. 3A, middle, 0:00). Unsuccessful segregation of chromosomes caused chromatin to become trapped in the cytokinetic furrow and resulted in an abnormal nuclear appearance (Fig. 3A, middle, 6:00). Similarly, in npp-19(RNAi) embryos, GFP-NPP-5 continued to localize to chromatin, but recruitment to the nuclear periphery in interphase cells was irregular (Fig. 3B, right, Suppl. Movie S2). Moreover, upon extended periods of RNAi treatment (∼40 h) we frequently observed that the pronuclei failed to properly interact and centrosomes often detached from the sperm-derived pronucleus. Together, these defects likely prevented normal chromosome congression during mitosis (Fig. 3B, right, 4:40–0:00).

To investigate if the failure to properly congress chromosomes on the metaphase plate may be a consequence of nuclear morphology defects, we took advantage of the temperature-sensitive properties of tm2886. Mutant embryos were incubated at 20 °C until approximately 2 min before NEBD, at which point embryos were shifted to 25 °C. Pronuclei in these embryos were only slightly smaller than in wild type embryos and were juxtaposed correctly (Fig. 3A, right, 3:40). Importantly, chromosome alignment on the metaphase plate as well

Fig. 2. Perturbation of NPP-19 activity results in nuclear morphology and chromatin segregation defects. Control, tm2886 (A) and npp-19(RNAi) (B) embryos were fixed and stained using α-NPP-19 antiserum (green) and monoclonal antibody mAb414 (red). Chromatin was detected using Hoechst 33258 (blue). Boxed regions in the merged panels are shown at higher magnification to the left, illustrating NPP-19 and mAb414 staining individually. Scale bars, 10 μm.
as kinetochore and NE recruitment of GFP-NPP-5 were normal (Fig. 3A, right). Nuclear growth after mitosis was however reduced. Thus, defects in chromosome alignment and segregation appear to correlate with defects in pronuclear morphology. Shifting \( \text{tm2886} \) embryos from 25 °C to 20 °C at the 2-cell or 4-cell stage did not improve viability as compared to embryos kept constantly at 25 °C (Table 2). Conversely, up-shift of 2–4-cell stage \( \text{tm2886} \) embryos from 20 °C to 25 °C did not increase lethality as compared to embryos kept constantly at 20 °C (Table 2). This demonstrates that wild type NPP-19 activity is most critically required in the 1-cell zygote and possibly also during oocyte maturation. Despite the dramatic defects at the 1- and 2-cell stages, cells of \( \text{tm2886} \) embryos continued to divide for approximately 100 min (4–5 division rounds) after which

### Table 2

Temperature shift of \( \text{tm2886} \) reveals an early embryonic npp-19 requirement

<table>
<thead>
<tr>
<th>Temperature pre-dissection</th>
<th>Temperature post-dissection</th>
<th>Embryonic stage</th>
<th>n</th>
<th>Embryonic lethality (average ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C</td>
<td>20 °C</td>
<td>&gt;4-cell</td>
<td>382</td>
<td>44.1 ± 18.3%</td>
</tr>
<tr>
<td>25 °C</td>
<td>20 °C</td>
<td>&gt;4-cell</td>
<td>137</td>
<td>33.8 ± 10.5%</td>
</tr>
<tr>
<td>20 °C</td>
<td>20 °C</td>
<td>2–4-cell</td>
<td>138</td>
<td>87.6 ± 7.9%</td>
</tr>
<tr>
<td>25 °C</td>
<td>25 °C</td>
<td>&gt;4-cell</td>
<td>445</td>
<td>90.0 ± 11.0%</td>
</tr>
</tbody>
</table>

*Gravid \( \text{tm2886} \) hermaphrodites were incubated for 6 h at indicated temperature prior to dissection. Embryos were separated into two groups: 2–4-cell stage and >4-cell stage embryos (1-cell stage embryos were discarded) that were incubated at either 20 °C or 25 °C. Embryonic lethality was determined after 24 h. *Standard deviation. Significant differences by Chi-square test: *different from control embryos \((p<0.001)\); †different from embryos incubated constantly at 20 °C \((p<0.001)\); ‡different from embryos shifted from 20 °C to 25 °C at 2–4-cell stage \((p<0.001)\).
development slowed down and eventually arrested in mid-gastrulation after ∼200 min (Suppl. Fig. 3; Suppl. Movie S3).

Lack of NPP-19 affects asynchronous cell division

Monitoring the second round of division in tm2886 and npp-19 (RNAi) embryos revealed that NPP-19 is required for proper cell cycle timing in both daughter cells of the P0 zygote. Measured from first cytokinesis, anaphase onset in the anterior AB cell was delayed 8% in tm2886 embryos and 29% in npp-19(RNAi) embryos (Table 3). For the posterior P1 cell, the effect was more pronounced with a delay of 42% in tm2886 embryos and 63% in npp-19(RNAi) embryos. AB and P1 have different sizes and fates and AB divides normally ∼2.5 min before P1 (Bao et al., 2008; Deppe et al., 1978). However, this asynchrony was dramatically increased in both tm2886 embryos (∼7 min) and npp-19 (RNAi) embryos (∼7.5 min) (Figs. 4A, B; Table 3). In 79% of tm2886 and npp-19(RNAi) embryos (n = 33), lagging chromosomes were detected during division of the AB cell whereas this phenotype was observed in only 10% of dividing P1 cells (Fig. 4A). Thus, the longer cell cycle delay observed in P1 as compared with AB correlated inversely with defects in chromosome segregation.

The delay in cell cycle timing upon depletion of NPP-19 suggested that a checkpoint(s) may be activated in the absence of this nup. To investigate the role of the spindle assembly checkpoint we depleted tm2886 embryos for MDF-2, the C. elegans homolog of yeast Mad2p (Encalada et al., 2005). Compared to control embryos, tm2886; mdf-2 (RNAi) embryos did not exhibit significant delays in either AB or P1 cell divisions, although the asynchrony between the divisions remained slightly elevated (Fig. 4B; Table 3). We conclude that the spindle assembly checkpoint is activated in the AB and P1 cells in the absence of NPP-19 and contributes to delaying cell division. We next examined the role of the DNA replication checkpoint by depleting ATL-1, which is required to both protect cells against damage arising from defective DNA replication and repair and establish asynchrony between the AB and P1 divisions (Brauchle et al., 2003). In accordance with this latter function, depletion of ATL-1 in tm2886 embryos specifically affected

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Fig. 4. Depletion of NPP-19 slows cell cycle progression and increases asynchrony between the AB and P1 cell divisions. (A) Control and npp-19(RNAi) embryos co-expressing GFP-α-tubulin/TBA-2 (green) and mCherry-HIS-58 (red) were imaged using time-lapse confocal microscopy as described in Fig. 3. Boxed regions in merged panels are shown at higher magnification on the left, illustrating chromatin morphology. Arrows highlight defects in chromosome segregation. Scale bars, 10 μm. (B) Asynchrony between the AB and P1 cell divisions in control, npp-19(RNAi), tm2886, mdf-2(RNAi), and tm2886; atl-1(RNAi) embryos expressed as the ratio of time between P0 cytokinesis and P1 anaphase over the time between P0 cytokinesis and AB anaphase. Probability values (p) from two-tailed t-tests are shown.
timing of P1 cell division (Table 3), thereby restoring normal degree of asynchrony between AB and P1 division (Fig. 4B; Table 3). This suggests that the P1 cell is particularly sensitive to decreased NPP-19 activity and that depletion of NPP-19 leads to activation of an ATL-1-dependent checkpoint in this blastomere.

Depletion of NPP-19 induces structural NE defects

To characterize the structural and molecular defects induced by NPP-19 depletion, we analyzed NE dynamics in further detail. Nup155/NPP-8 is essential for NPC formation (Franz et al., 2005) and has been shown to interact physically with Nup35 in vertebrates (Hawryluk-Gara et al., 2008, 2005). We therefore decided to investigate the behavior of NPP-8 in the absence of NPP-19. GFP-NPP-8 localization to the NE in late anaphase was disrupted following NPP-19 depletion (Fig. 5A, 3:00; Suppl. Movie S4). Later in interphase, GFP-NPP-8 eventually appeared at the nuclear periphery in npp-19(RNAi) embryos at significantly reduced levels (Fig. 5A, 9:20). Analysis of endogenous NPP-8 by immunofluorescence likewise demonstrated a decreased NE localization in npp-19(RNAi) embryos (Suppl. Fig. 4A). We have previously observed that depletion of NPP-8 prevents nuclear rim accumulation of NPP-19 (Franz et al., 2005). Thus, NPP-19 and NPP-8 show a mutual dependency for NE localization. Depleting NPP-19 from embryos expressing GFP-NPP-4 induced an identical phenotype as obtained for GFP-NPP-8 (data not shown). From these experiments, we conclude that NPP-19 regulates NPP-8 and Nup45/58/NPP-4 recruitment. Additionally, NPP-19 is required for the correct distribution of NPP-5, suggesting an important role for NPP-19 in NPC assembly.

In addition to NPCs, the NE consists of inner and outer nuclear membranes and an underlying nuclear lamina (Hetzer et al., 2005). To analyze if NPP-19 is required for assembly of these essential structures, we monitored the behavior of the inner nuclear membrane protein LEM-2 and lamin/LMN-1 fused to fluorescent proteins. Similar to the defects in NPP-8 and NPP-4 recruitment, YFP-LMN-1 levels at the nuclear periphery were strongly reduced in npp-19(RNAi) embryos, implying that nuclear lamina assembly is NPP-19-dependent (Fig. 5B). In contrast, GFP-LEM-2 recruitment was unaffected in npp-19(RNAi) (Fig. 5C; Suppl. Movie S5) and tm2886 embryos (Suppl. Fig. 5; Suppl. Movie S6). Thus, NPP-19 appears to be dispensable for targeting of nuclear membrane to the chromatin surface after mitosis.

NE function depends on NPP-19

The alterations in nup recruitment and distribution in the absence of NPP-19 prompted us to investigate if a functional NE is formed in npp-19(RNAi) embryos. We initially analyzed whether a cytoplasmic protein, α-tubulin/TBA-2, fused to GFP, is excluded from the nuclear space upon NPP-19 knockdown. In control embryos, the NE assembled while the chromatin was still highly compacted (see Fig. 5A, left, 3:00), and as nuclei grew in size, exclusion of GFP-TBA-2 was observed (Fig. 5A, 9:20). In contrast, nuclear exclusion was not observed in npp-19(RNAi) embryos (Fig. 5A, right). To analyze if nuclear protein import also depends on NPP-19, we determined the localization of GFP fused to either PCNA/PCN-1, a DNA replication co-factor, or PIE-1, an RNA polymerase II repressor essential for germ-line specification. Nuclear accumulation of GFP-PCN-1 was observed significantly earlier in control embryos than in npp-19(RNAi) embryos (180±14 s vs. 288±36 s [average±standard deviation]) and reached higher concentrations (Fig. 6B), strongly suggesting that nuclear protein import is impaired in the absence of NPP-19. Moreover, we speculate that nuclear GFP-PCN-1 observed in npp-19(RNAi) embryos may reflect the direct association of GFP-PCN-1 to chromatin in cells with

Fig. 5. Depletion of NPP-19 inhibits NPC and nuclear lamina formation. Control and npp-19(RNAi) embryos co-expressing mCherry-HIS-58 with various GFP/YFP fusion proteins were imaged using time-lapse confocal microscopy as described in Fig. 3. Higher magnification views of boxed regions shown in merged panels highlight localization of (A) GFP-NPP-8, (B) YFP-LMN-1, and (C) GFP-LEM-2. Scale bars, 10 μm.
increased NE permeability. In support of this notion, nuclear accumulation of GFP-PIE-1 was inhibited in npp-19(RNAi) embryos. In control embryos, GFP-PIE-1 was present in the P2 cell, displaying strong nuclear enrichment (Fig. 6C, left). Asymmetric inheritance of GFP-PIE-1 was maintained in npp-19(RNAi) embryos, indicating that NPP-19 does not regulate early cell specification. However, in embryos lacking NPP-19, GFP-PIE-1 failed to concentrate in the nucleus (Fig. 6C, right).

Ultrastructural NE analysis in npp-19(RNAi) embryos

The defects in nup recruitment and nuclear protein import observed in npp-19(RNAi) embryos by immunofluorescence and live-cell microscopy raised the question of whether NPCs are assembled in the absence of NPP-19. To address this issue, we fixed npp-19(RNAi) embryos by high pressure freezing followed by transmission electron microscopy (TEM). NPCs were detected at high density and with regular spacing in control embryos (Fig. 7, left). In contrast, nuclei of npp-19(RNAi) embryos contained fewer NPCs, separated by long stretches of intervening nuclear membranes (Fig. 7, middle and right; note the different scale of control and npp-19(RNAi) images). Moreover, several gaps larger than NPCs were observed in the nuclear membranes in npp-19(RNAi) embryos (data not shown) as well as chromatin surfaces devoid of nuclear membranes (Fig. 7, middle). Live observation of tm2886 and npp-19(RNAi) embryos revealed that chromatin initially trapped in the cytokinetic furrow eventually separates and forms spherical nuclei (see e.g. Suppl. Movie S6). Thus, the two nuclei from npp-19(RNAi) embryos shown in Fig. 7 most

![Fig. 6. NPP-19 is required for NE function and nuclear protein import. Control and npp-19(RNAi) embryos co-expressing various fluorescent fusion proteins were imaged using time-lapse confocal microscopy as described in Fig. 3. Scale bars, 10 μm. Higher magnification views of boxed regions shown in merged panels highlight localization of (A) GFP-TBA-2, (B) GFP-PCN-1, and (C) GFP-PIE-1.](image)

![Fig. 7. Depletion of NPP-19 leads to lower NPC density. Control and npp-19(RNAi) embryos were analyzed by transmission electron microscopy to visualize nuclear membranes and NPCs. Higher magnification views of boxed regions are shown below. Arrows highlight NPCs and dashed lines indicate regions of chromatin that lack nuclear membranes. White scale bars, 1 μm in upper panels; 100 nm in lower panels.](image)
likely represent early (Fig. 7, middle) and late (Fig. 7, right) time points after mitosis. In summary, TEM analysis showed that depletion of NPP-19 causes a strong decrease in NPC formation whereas nuclear membranes are still able to cover and seal over most of the chromatin surface, consistent with our observations using live microscopy.

**Discussion**

Using *C. elegans* as a model system, we provide the first in vivo characterization of a Nup35/NPP-19 mutant, demonstrating that NPP-19 is important for embryonic development. *npp-19(tm2886)* mutant embryos incubated at restrictive temperature died during gastrulation, whereas RNAi-mediated depletion of NPP-19 revealed that this nup plays a crucial role in NPC and nuclear lamina assembly, which are necessary for proper NE function. Combined with previously characterized functions of Nup35, our data support a model in which Nup35/NPP-19 acts together with Nup155/NPP-8 at a late but nevertheless essential step of NPC assembly (Hawryluk-Gara et al., 2008).

The complexity of NPCs and their involvement in fundamental cellular processes has hampered delineation of how they are assembled (Antonin et al., 2008; Hetzer et al., 2005; Tran and Wente, 2006). In organisms undergoing ‘open’ mitosis, NPCs presumably assemble by two distinct mechanisms: (1) association of nups with membrane-free chromatin surfaces at the end of mitosis and (during pronuclear envelope formation) and (2) insertion of NPCs into existing nuclear membranes during interphase. In the former mechanism, the earliest known step involves recruitment of the Nup107–160 NPC subcomplex to the chromatin surface by MEL-28/ELYS (Franz et al., 2007; Galy et al., 2006; Rasala et al., 2006, 2008). In fact, MEL-28/ELYS and the Nup107-160 subcomplex both associate with chromatin throughout the cell cycle and are essential to initiate NPC assembly (Belgareh et al., 2001; Fernandez and Piano, 2006; Galy et al., 2006; Harel et al., 2003; Rasala et al., 2006; Walther et al., 2003). The subsequent steps include recruitment of transmembrane nups POM121 and NDC1 together with ER-derived membranes (Antonin et al., 2005; Mansfeld et al., 2006; Rasala et al., 2008; Stavru et al., 2006). Finally, soluble nups are recruited to complete NPC assembly and nuclear membrane fusion. Among the nups in this last class, so far only Nup155/NPP-8 and Nup35/NPP-19 have been ascribed essential roles in the assembly process (Franz et al., 2005; Hawryluk-Gara et al., 2008; this study).

We have demonstrated that NPP-19 is required for efficient NE accumulation of Nup45/58/NPP-4 and Nup155/NPP-8 but not for recruitment of Nup107/NPP-5 to chromatin. Moreover, electron microscopy revealed that nuclei of *npp-19(RNAi)* embryos contain dramatically fewer NPCs. This supports recent observations in which depletion of Xenopus Nup35 from egg extracts inhibited *in vitro* NPC and NE assembly (Hawryluk-Gara et al., 2008). Furthermore, the study by Hawryluk-Gara et al. defined a human Nup35 fragment containing aa 167–300 (Nup35217–300) that was able to reconstitute nuclear assembly in extracts depleted for endogenous Nup35. Interestingly, this fragment overlaps with a portion of NPP-19 absent in the *tm2886* mutant allele (*C. elegans* NPP-19217–286 corresponding to human Nup35217–252). At restrictive temperature, we observed severe nuclear morphology and NPC phenotypes in *tm2886* mutants. Together, these data suggest that the central portions of human Nup35 and *C. elegans* NPP-19 have critical roles in NE reformation. At semi-permissive temperature, *tm2886* mutant embryos exhibited no obvious NE defects. However, approximately half of the mutant embryos arrested during embryogenesis. We speculate that the embryonic lethality observed at 15–20 °C may be caused by cumulative defects arising from perturbations in nucleocytoplasmic transport. We note that mutant NPP-19217–286 possesses weak NE targeting capacity, which may contribute to NE assembly and NPC function under semi-permissive condition.

**Fig. 8.** NPP-8 is essential for NE localization of NPP-19. The P1 cells of 2-cell stage control RNAi, *npp-3(RNAi)*, *npp-13(RNAi)*, *npp-3(RNAi); npp-13(RNAi)* and *npp-8(RNAi)* embryos expressing GFP-NPP-19 (green) and mCherry-HIS-58 (merge) were imaged using time-lapse confocal microscopy. Scale bars, 5 μm.
Our observation that NPP-19 is required for efficient recruitment of NPP-8 is to our knowledge the first demonstration that Nup35/NPP-19 regulates Nup155/NPP-8 localization in vivo. The mutual dependency between these two nups (Franz et al., 2005; this study) thereby places them together in the NPC assembly pathway downstream of POM121 and NDC1. In agreement with this idea, both yeast Nup35p and vertebrate Nup35 interact with Ndc1p/NDC1 (Mansfeld et al., 2006; Uetz et al., 2000).

The inhibition of NPC assembly caused by NPP-19 depletion has severe consequences for NE function. Nuclear proteins exhibited reduced nuclear accumulation. Moreover, formation of the nuclear lamina was inhibited in npp-19(RNAi) embryos, likely a result of reduced nuclear import of lamin/LMN-1 protein. In contrast, recruitment of the transmembrane nuclear protein LEM-2 to chromatin during NE reformation was found to be NPP-19-independent. Consistent with this observation, ultrastructural analysis revealed that the majority of chromatin in npp-19(RNAi) embryos was enclosed by nuclear membranes. These data suggest that nuclear membrane fusion can proceed in the absence of NPP-19. In a similar manner, in vitro nuclear assembly in extracts immunodepleted for the Nup107–160 subcomplex leads to enclosure of chromatin by NPC-free NEs (Harel et al., 2003; Walther et al., 2003).

The analysis of nups has almost exclusively focused on their functions at the biochemical or cellular level. Few studies have addressed their roles in specific cell types or in a developmental context (Lupu et al., 2008; Olsson et al., 2004; Tran and Wente, 2006; Uv et al., 2000). While NPCs are essential for nucleocytoplasmic transport in all nucleated cells, tissue-specific requirements for certain nups may reflect specialized transport events in those tissues. Alternatively, regulated expression of nups may coincide with their involvement in other processes, such as activation/inactivation of specific gene loci (Schneider and Grosschedl, 2007). Our finding that NPP-19 is particularly critical in the 1-cell stage C. elegans embryo represents a further specialization of nup and NPC function. Several features distinguish the initial cell divisions of C. elegans embryos from later cell cycles: (1) pronuclear formation involves repackaging of sperm-derived DNA, which is presumably devoid of classical histone-containing nucleosomes (Schaner and Kelly, 2006); (2) early embryonic cells are larger, and (3) the rate of cell cycle progression is significantly faster in 1–8-cell stage embryos (Bao et al., 2008; Deppe et al., 1978). We propose that all three features relate to the phenotypes exhibited by tm2886 and npp-19(RNAi) embryos. First, the sperm-derived pronucleus displayed more severe defects as compared to its oocyte-derived counterpart. Second, pronuclei were more frequently observed in mutant embryos. Finally, defects in chromosome segregation and centrosomes will inevitably be more threatening for larger cells, since the 1-cell zygote. Finally, defects in chromosome segregation and abnormal nuclear appearance were more frequently observed during mitosis in the more quickly dividing AB cell as compared to the P1 cell.

In summary, we have demonstrated that NPP-19 is essential for NPC and NE assembly, providing the first mutational analysis of Nup35/NPP-19 in a metazoan organism. We propose that altered NE structure and function lead to severe chromosome segregation defects following perturbation of NPP-19 activity. However, a more direct involvement of NPP-19 in mitotic spindle assembly may exist (louk et al., 2002) and deserves future study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2008.12.024.

References


