

NDC1: a crucial membrane-integral nucleoporin of metazoan nuclear pore complexes

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POM121 and gp210 were, until this point, the only known membrane-integral nucleoporins (Nups) of vertebrates and, thus, the only candidate anchors for nuclear pore complexes (NPCs) within the nuclear membrane. In an accompanying study (see Stavru et al. on p. 477 of this issue), we provided evidence that NPCs can exist independently of POM121 and gp210, and we predicted that vertebrate NPCs contain additional membrane-integral constituents. We identify such an additional membrane protein in the NPCs of mammals, frogs, insects, and nematodes as the orthologue to yeast Ndc1p/Cut11p.

Human NDC1 (hNDC1) likely possesses six transmembrane segments, and it is located at the nuclear pore wall. Depletion of hNDC1 from human HeLa cells interferes with the assembly of phenylalanine-glycine repeat Nups into NPCs. The loss of NDC1 function in *Caenorhabditis elegans* also causes severe NPC defects and very high larval and embryonic mortality. However, it is not ultimately lethal. Instead, homozygous NDC1-deficient worms can be propagated. This indicates that none of the membrane-integral Nups is universally essential for NPC assembly, and suggests that NPC biogenesis is an extremely fault-tolerant process.

Introduction

Nuclear pore complexes (NPCs) permit the exchange of metabolites and macromolecules between the nuclear compartment and the cytoplasm. They are embedded in the nuclear envelope (NE) and belong to the largest macromolecular assemblies of the cell. There are two modes of NPC assembly (Maul et al., 1972; Maul, 1977). The first pathway leads to the insertion of NPCs into a closed NE. It represents the only pathway of NPC formation in lower eukaryotes, and it allows the interphase cells of higher eukaryotes to double their NPC number between two mitoses (Maul et al., 1972). The “open mitotic mode” is a pathway that is only used in higher eukaryotic cells, in which NPCs and NEs are disassembled during mitosis. The resulting soluble Nup subcomplexes and vesicular or reticulate membrane structures then reassemble upon mitotic exit, reforming an NPC-perforated NE around chromatin

(Maul, 1977; Drummond and Allen, 2004; Rabut et al., 2004; Burke et al., 2005).

The open mitotic mode is characterized by a synchronous assembly of the entire NPC population of a cell. It has been widely studied in cell culture systems (Maul, 1977; Buendia and Courvalin, 1997; Bodoor et al., 1999) and in an in vitro system based on *Xenopus laevis* egg extracts (Newmeyer et al., 1986; Finlay and Forbes, 1990; Macaulay and Forbes, 1996; Goldberg et al., 1997). Although most of the NPC structure might self-assemble through interactions between individual nucleoporins (Nups), assembly factors probably assist in this process. Importin β , for example, appears to act as a RanGTPase-regulated chaperone, which initially shields certain Nup complexes and releases them in proximity to chromatin (Zhang et al., 2002; Harel et al., 2003; Walther et al., 2003).

The actual pores within the NE can be considered products of local fusion between the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). It is still unclear which mechanisms create them, but two scenarios can be envisaged as to how the special structure of the pore membrane forms during exit from an open mitosis. First, vesicles could fuse around preassembled, chromatin-attached NPC scaffolds and thereby create the pore membrane before, or concomitantly with, the closure of the NE. Alternatively, the assembly of NPCs

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Abbreviations used in this paper: FG, phenylalanine-glycine; INM, inner nuclear membrane; NE, nuclear envelope; NGS, N-glycosylation site; NPC, nuclear pore complex; Nup, nucleoporin; ONM, outer nuclear membrane; SPB, spindle pole body; TMS, transmembrane segment.

The online version of this article contains supplemental materials.

Supplemental Material can be found at:
<http://jcb.rupress.org/content/suppl/2006/05/15/jcb.200601001.DC1.html>

in telophase could follow principles similar to those in interphase, i.e., the double membrane of the NE could form first and, subsequently, be perforated by a local fusion between INM and ONM.

How new NPCs are inserted into a closed NE is still unclear, but, again, two strategies can be envisaged. First, a pre-existing NPC could grow and then split into two daughter pores (Rabut et al., 2004). Intermediates of such a mechanism should be NPCs of higher than the standard eightfold rotational symmetry. Indeed, NPCs with a rotational symmetry of up to 10-fold have been detected (Hinshaw and Milligan, 2003). However, there is no evidence for 16-fold symmetrical intermediates, as predicted for a presplitting NPC or, indeed, for any other plausible combination of pre- and postsplitting symmetry. Furthermore, such pore splitting would also require a membrane fusion event, namely, between opposing sides of the parental pore membrane. In view of the massive NPC structure, the inaccessibility of the lipid bilayers at the pore membrane, and the wide diameter of the pore channel, it is difficult to imagine how a fusion could possibly occur at such a position.

Therefore, it appears more likely that a true *de novo* insertion of NPCs into the NE occurs. Indeed, experiments using the NPC assembly inhibitor BAPTA indicate that such an insertion does not require preexisting NPCs (Macaulay and Forbes, 1996).

A *de novo* insertion of NPCs into the NE must include a local fusion between INM and ONM to yield the actual pore. How this fusion comes about is still unknown. One complication is that INM and ONM are held ~20–25 nm apart; hence, the fusion machinery needs to bring them into a sufficiently close proximity to allow membrane fusion to occur. A second complication is that the pore-forming fusion occurs at the luminal faces of INM and ONM. Therefore, it must use factors other than the classical fusion machineries of the secretory pathway, which catalyze membrane fusions through the cytoplasmic sides of the target membranes. In analogy to membrane fusion events mediated by SNAREs or viral fusion proteins (Söllner, 2004), however, it appears likely that integral membrane proteins play a critical role. Possibly, these integral fusion factors remain stably associated with mature NPCs as membrane-integral Nups. Membrane-integral Nups probably fulfill several additional functions, e.g., the recruitment of other Nups to assembly sites at the nuclear membrane, as (static) anchors of (mature) NPCs within the NE, as part of the rigid NPC structure, and, if equipped with the Nup-typical phenylalanine-glycine (FG)-rich repeats, as constituents of the permeability barrier of nuclear pores.

Given the striking conservation of general NPC architecture, it would be very surprising if the integration of yeast and animal NPCs into the NE traced back to different evolutionary origins. Nevertheless, thus far it appeared that NPCs from yeast and vertebrates are equipped with completely different sets of membrane-integral Nups. POM121 and gp210 (Gerace et al., 1982; Hallberg et al., 1993) have, so far, been the only known membrane-integral constituents of vertebrate NPCs, but they are both absent from fungi.

The yeast *S. cerevisiae* possesses three membrane-integral Nups: Pom152p, Pom34p, and Ndc1p (referred to as Cut11p in *Schizosaccharomyces pombe*). Pom152p and Pom34p are not essential, and they lack obvious orthologues in higher eukaryotes (Wozniak et al., 1994; Miao et al., 2005). In contrast, Ndc1p is essential (Thomas and Botstein, 1986; Winey et al., 1993; Chial et al., 1998; West et al., 1998). It is, however, not only a Nup but also a constituent of spindle pole bodies (SPBs), which are the NE-embedded form of centrosomes that is typical of yeast.

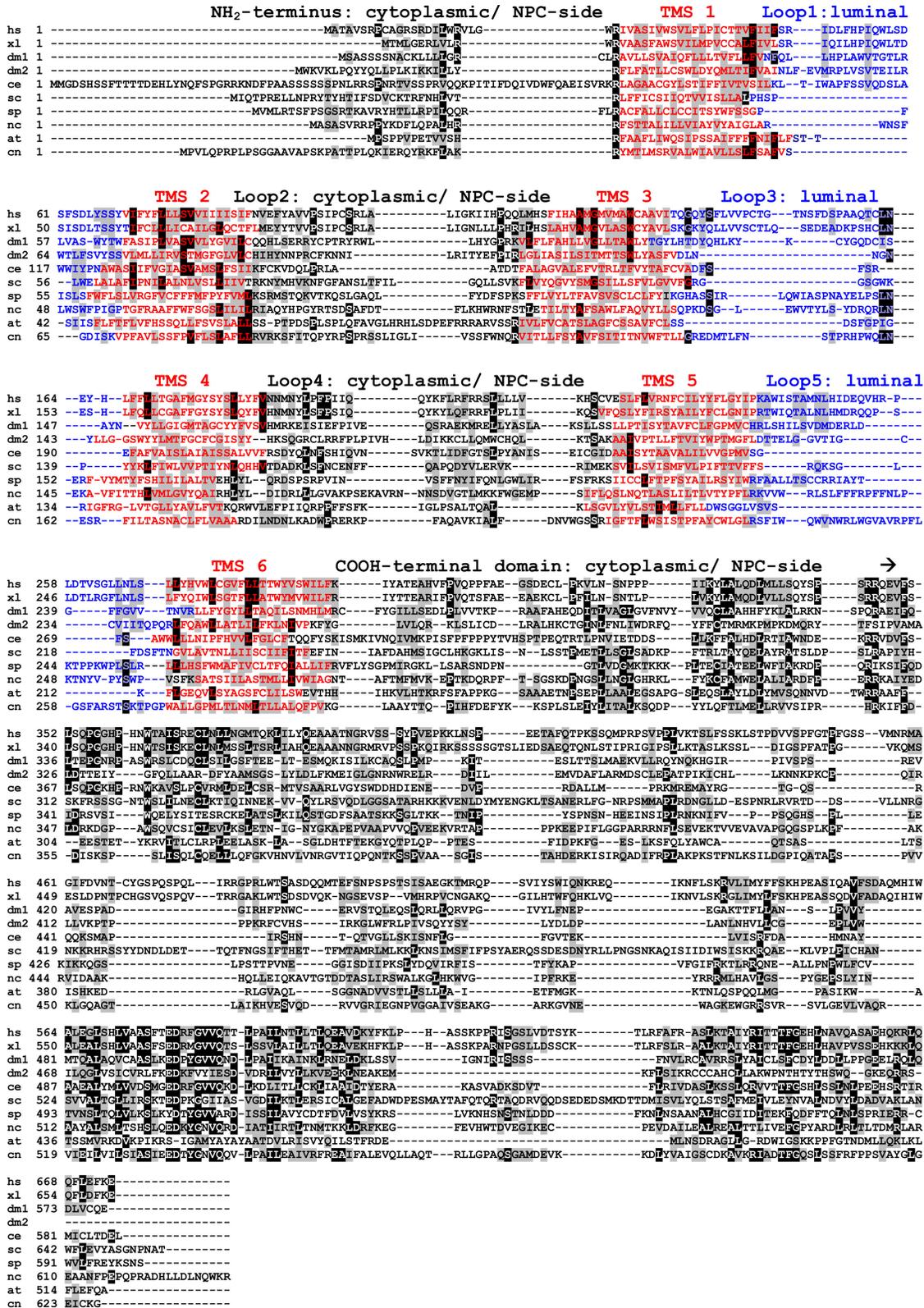
Nuclear pore and SPB membrane exhibit analogous topological features. Nevertheless, NPCs and SPBs represent distinct structures, apparently sharing just a single component, which is Ndc1p (Chial et al., 1998). Ndc1p is required for inserting newly formed SPBs into the NE, and this function is clearly essential for the viability of yeast (Winey et al., 1993; West et al., 1998). So far, however, a role for Ndc1p in NPC biogenesis is only indicated by genetic interactions with Nic96p and by the *ndc1-39* mutant, which, at the nonpermissive temperature, fails to properly incorporate Nup49p into otherwise functional NPCs (Lau et al., 2004). It is still unclear if the function of Ndc1p in NPC biogenesis goes beyond anchoring individual Nups to the NPC scaffold. However, if it had a fundamental function in NPC biogenesis, then it should be conserved across eukaryotic kingdoms and should also be present in those eukaryotes that have an open mitosis and, thus, lack NE-embedded SPBs.

In the accompanying study (see Stavru et al. on p. 477 of this issue), we report the observation that functional mammalian NPCs can assemble in cells that are devoid of gp210 and severely depleted of POM121. This suggested that, to date, at least one crucial membrane-integral Nup of mammals must have escaped detection. We confirm this assumption and demonstrate that metazoan NPCs contain an additional constituent, which is orthologous to yeast Ndc1p. Depletion of human NDC1 (hNDC1) from HeLa cells causes severe NPC-assembly defects. Loss of NDC1 function in *Caenorhabditis elegans* also causes severe phenotypes, but it is not ultimately lethal. This leads to the conclusion that none of the membrane-integral Nups is essential under all conditions for NPC biogenesis, and points to an extreme flexibility and robustness of the NPC assembly process.

Results

Identification of Ndc1p orthologues in higher eukaryotes

So far, POM121 and gp210 were the only known membrane-integral Nups found in vertebrates. In the accompanying study (Stavru et al., 2006), however, we report the surprising finding that both nucleoporins are not limiting to, and are even dispensable in, NPC biogenesis. Although the formal possibility exists that peripheral membrane proteins shape the pore membrane to its characteristic topology and serve as primary anchors for the NPC, it appears more likely that Nups with membrane-spanning segments fulfill this function. In the latter case, a crucial component of vertebrate NPCs must so far have escaped identification.



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Figure 1. Multiple alignment of selected NDC1 orthologues. Predicted TMSs (1–6) are indicated in red, luminal loops are indicated in blue, and cytoplasmic parts remained uncolored. Cytoplasmic localization of the NH₂ and COOH termini, as well as the luminal localization of loops 1, 3, and 5, is supported by experimental evidence (see Fig. 3 and Fig. 4 A, as well as Fig. S3). Residues were shaded in black when identical and in gray when similar in >40% of the sequences. hs, *H. sapiens*; xl, *X. laevis*; dm1 and dm2, *D. melanogaster* NDC1 variants 1 and 2; ce, *C. elegans*; sc, *S. cerevisiae*; sp, *S. pombe*; nc, *N. crassa*; at, *A. thaliana*; cn, *C. neoformans*. Fig. S3 is available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>.

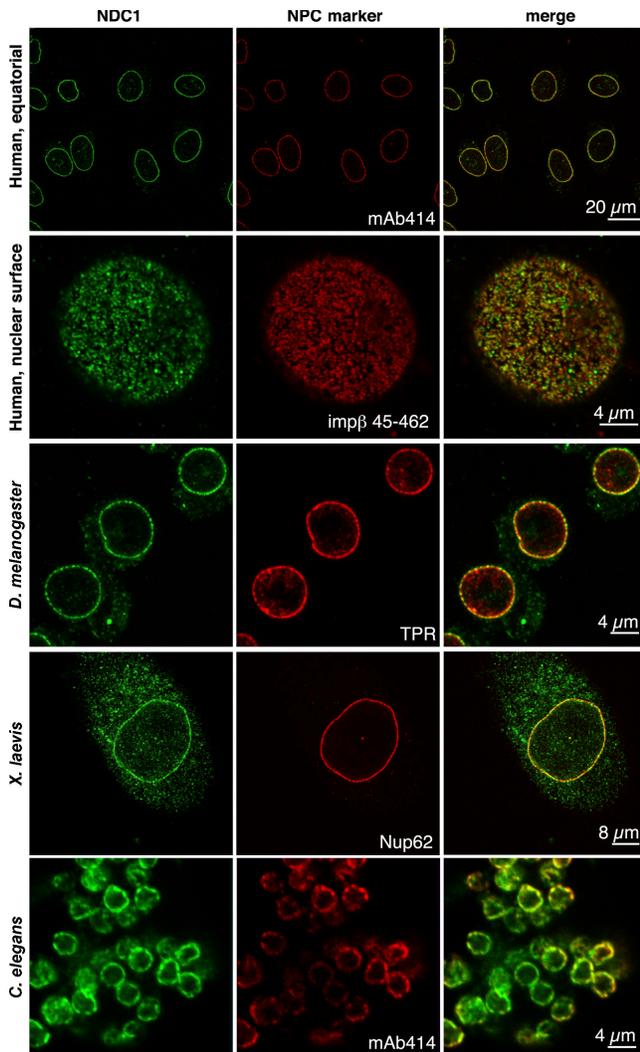


Figure 2. Animal NDC1 proteins localize to NPCs. Antibodies were raised against NDC1 from *H. sapiens*, *X. laevis*, *D. melanogaster* (variant 1), and *C. elegans*, and then affinity purified and used to localize NDC1 in human HeLa cells, *D. melanogaster* Schneider cells, *X. laevis* XL177 cells, or cells isolated from *C. elegans*. The following NPC markers were used: mAb414, antibodies against *D. melanogaster* TPR, anti-*X. laevis* Nup62, and the fluorescently labeled importin β^{45-462} fragment. Optical sections through the nuclear equator and the nuclear surface are shown.

In search of the missing component, we reasoned that a membrane-integral constituent of yeast NPCs might have an as yet unidentified orthologue in higher eukaryotes. Searches with *S. cerevisiae* Pom152p or Pom34p did not yield any convincing hits. However, we found Ndc1p orthologues not only in other ascomycetous fungi (e.g., *Pichia pastoris*, *Yarrowia lipolytica*, *Aspergillus nidulans*, and *Neurospora crassa*) but also in basidiomycetes (e.g., *Ustilago maydis*, *Cryptococcus neoformans*) and viridiplantae (e.g., *Pinus taeda*, *Solanum demissum*, *Arabidopsis thaliana*, *Oryza sativa*, and *Chlamydomonas reinhardtii*), as well as in nematodes (e.g., *C. elegans*), insects (e.g., *Drosophila melanogaster*), cnidarians (e.g., *Hydra magnipapillata*), tunicates (e.g., *Ciona intestinalis*), amphibians (e.g., *X. laevis*), fish (e.g., *Fugu rubripes*), birds, and mammals (Fig. 1 and Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>).

NDC1 was, thus, an excellent candidate for constituting a widely conserved membrane anchor of NPCs.

NDC1 also localizes to NPCs in higher eukaryotes

hNDC1 was previously identified in a proteomics screen as NE transmembrane protein 3 (Net3; Schirmer et al., 2003). To determine its intracellular localization at a higher resolution, we expressed NH₂- and COOH-terminal GFP fusions of hNDC1 in HeLa cells (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>). At low or moderate expression levels, a clear colocalization with NPCs was observed, suggesting that NDC1 is also a Nup in human cells.

To localize the endogenous hNDC1, we raised antibodies against two regions of the protein and used them for immunofluorescence on HeLa cells. For both sets of antibodies, a clear NPC staining was evident (Figs. 2 and 3). In Fig. 2, we used either mAb414, which recognizes several FG repeat Nups (Sukegawa and Blobel, 1993), or the fluorescently labeled Imp β^{45-462} fragment (Kutay et al., 1997) to decorate NPCs, and we observed conspicuous colocalization with the hNDC1 signal.

As already mentioned, we identified Ndc1 orthologues in numerous other eukaryotes and, hence, wanted to know if localization at NPCs represents a general feature of NDC1 family members. Therefore, we raised antibodies against *X. laevis* NDC1, against the more widely expressed isoform of the two *D. melanogaster* paralogues (variant 1; Fig. 1), and against *C. elegans* NDC-1. Again, colocalization with the respective nuclear pore markers, i.e., *X. laevis* Nup62, *D. melanogaster* TPR, or mAb414, was observed (Fig. 2). NDC1 is, thus, a widely conserved constituent of NPCs.

Topology of hNDC1

hNDC1 clearly behaves like an integral membrane protein; it fractionates with membranes and withstands membrane extraction at pH 12.0 (unpublished data). The number and orientation of the transmembrane segments (TMSs) determine which parts of hNDC1 are exposed to the cytoplasmic/NPC side of the membrane and, hence, are available for interaction with other Nups. Therefore, we decided to resolve its topology. An in silico analysis was used to generate a topology model (see Materials and methods), which was subsequently tested experimentally. The model predicted six putative TMSs and cytoplasmic exposure for the NH₂ and COOH termini, as well as for loops 2 and 4, whereas loops 1, 3, and 5 are predicted to face the lumen of the NE (Fig. 1 and Fig. 4 B).

In agreement with the model, we found the COOH-terminal domain (NDC1²⁹²⁻⁶⁷⁴), as well as the extreme NH₂ and COOH termini, to be accessible for antibodies from the cytoplasmic side of the membrane (for data and experimental description see Fig. 3 and Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>). In contrast, antibodies against loop 5 recognized their epitope only when the internal membranes had been solubilized by Triton X-100 (Fig. 3). This is expected, if loop 5 is located in the lumen of ER or NE.

In a second set of experiments, we introduced N-glycosylation sites (NGSs) into loops 1, 3, or 5. Indeed, we observed the

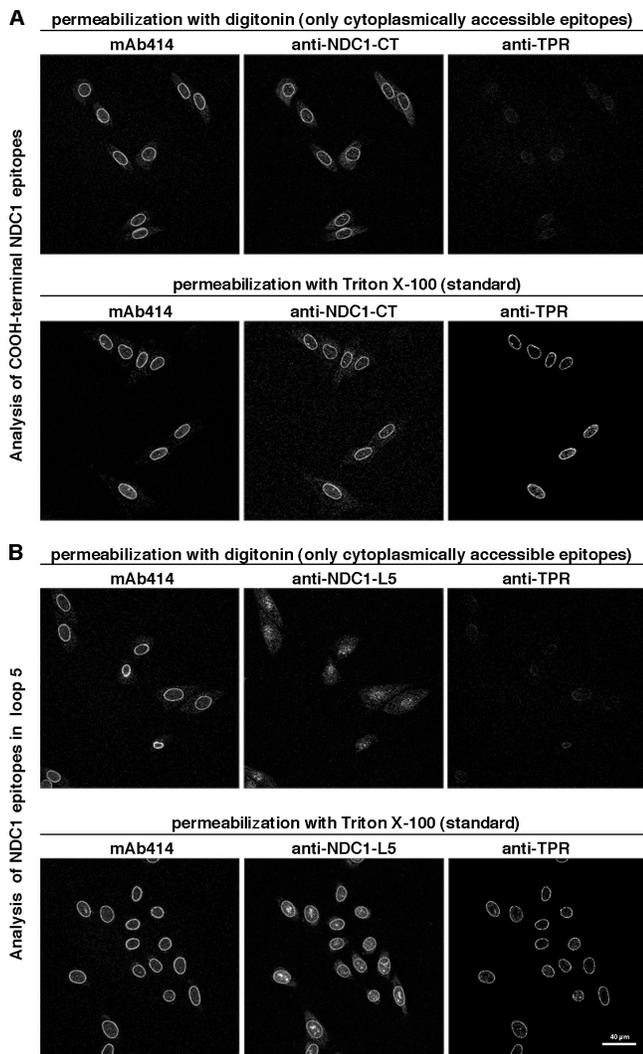


Figure 3. Membrane topology of endogenous hNDC1 probed with anti-hNDC1 antibodies. (A) HeLa cells were fixed with paraformaldehyde and permeabilized with 0.05% digitonin, which perforates the plasma membrane but leaves ER and NE intact. Nuclear or luminal epitopes, hence, remain inaccessible for antibodies. Yet, antibodies clearly recognized the COOH-terminal domain of NDC1 under these conditions (top). The COOH terminus of NDC1 is, therefore, extraluminal, i.e., exposed to cytoplasm or the central pore channel. The procedure was verified by two internal controls; as expected, mAb414 also recognized NPCs from the cytoplasmic side, whereas recognition of TPR, which is located on the nuclear NPC side, required complete permeabilization of internal membranes by 0.25% Triton X-100. Detection of bound primary antibodies was carried out with Alexa Fluor 488 anti-rabbit (for anti-NDC1), Alexa Fluor 647 anti-mouse (for mAb414), and Alexa Fluor 568 anti-guinea pig (for anti-TPR). Images were taken by confocal laser scanning microscopy. (B) Analysis was analogous to Fig. 3 A, the difference being that an antibody against loop 5 of NDC1 was used. Loop 5 is inaccessible from the cytoplasm and recognized by the antibody only after dissolving internal lipid bilayers with Triton X-100.

selective glycosylation of these sites, when *in vitro* translation was performed in the presence of RER membranes (Fig. 4 A). As this modification occurs only in the RER lumen (Nilsson and von Heijne, 1993), one can conclude that loops 1, 3, and 5 are indeed luminal. This experiment also indicates that NDC1 is initially integrated into RER membranes before its assembly into NPCs. Such intermediates in the RER can indeed be

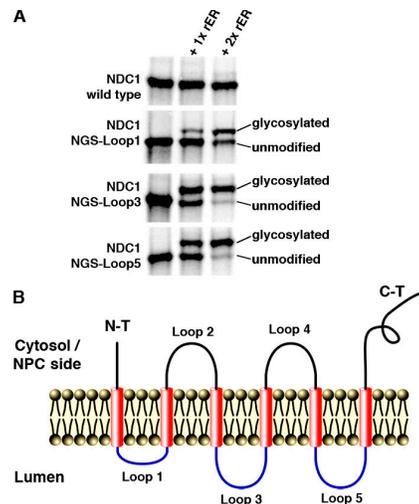


Figure 4. hNDC1 topology probed by N-glycosylation site tagging. (A) Indicated forms of hNDC1 were translated *in vitro* in the presence of [³⁵S]methionine and signal recognition particles, and subsequently detected by autoradiography. NGSs introduced into loops 1, 3, or 5 were indeed efficiently glycosylated, provided that RER membranes were present during translation. As N-glycosylation activity is RER-luminal, loops 1, 3, and 5 must also be luminal. (B) Scheme of the proposed membrane topology of human NDC1.

detected microscopically, when GFP-tagged hNDC1 is over-expressed (Figs. S2 and S3).

The experimental data, thus, support the topology model, at least for the human member of the NDC1 family. Its ~45-kD COOH-terminal domain (NDC1²⁹²⁻⁶⁷⁴), which includes the most conserved part of this protein (Fig. 1), is therefore entirely extraluminal and available for interactions with other Nups.

EM localization of hNDC1

In the next step of our analysis, we used postembedding immunogold EM to localize the conserved COOH-terminal domain of hNDC1. The antibodies gave a very specific labeling along the NE, with >95% of the gold decorating NPCs (see representative EM images in Fig. 5 B). The positions of the gold labels are consistent with the assumption that the COOH-terminal domain resides within the body of the NPC proper and is part of the NPC scaffold.

Mitotic modification of hNDC1

Sequence analysis of the conserved COOH-terminal domain of NDC1 predicted several consensus phosphorylation sites for mitotic kinases. Because mitotic phosphorylation plays a key role in disassembling NPCs (Onischenko et al., 2005), it was tempting to assume that a mitotic modification of NDC1 might contribute to this disassembly process. Such modifications often change the mobility of protein species in SDS gels, and, indeed, immunoblots revealed a prominent slow-migrating NDC1 species that was specific for HeLa cells arrested in M phase (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>). We are currently investigating the nature of these modifications and their positions within the NDC1 sequence.

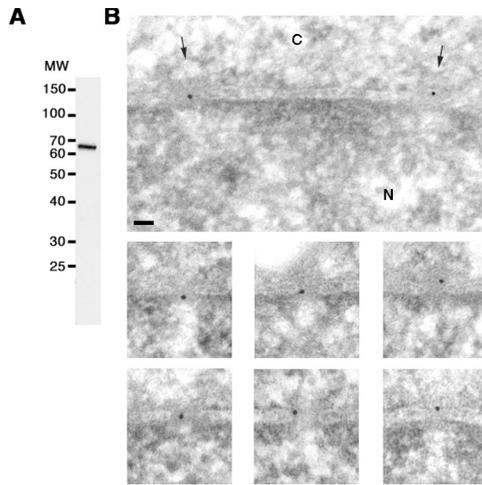


Figure 5. hNDC1 is a central component of NPCs. (A) An immunoblot of total HeLa cells probed with affinity-purified antibodies raised against the COOH-terminal domain of hNDC1 (hNDC1²⁹²⁻⁶⁷⁴). Typical of a multimembrane-spanning protein, hNDC1 migrates faster on SDS-gels than expected from its calculated molecular mass (76 kD). (B) HeLa cells were fixed in 2% formaldehyde + 0.1% glutaraldehyde, embedded into LR white and processed for postembedding immunogold-labeling as described previously (Krull et al., 2004). hNDC1 was detected by the antibodies characterized in A. Arrows in the representative EM images point at two neighboring NPCs in cross section. Bar, 50 nm. C, cytoplasmic; N, nuclear side of the NE.

Depletion of hNDC1 from human cells causes a severe NPC assembly defect

Having established that all tested metazoan NDC1 proteins are constituents of NPCs, we wanted to elucidate the consequences of a loss of NDC1 function. For this we used the RNAi approach to knockdown hNDC1 in human cell lines (Elbashir et al., 2001). The four different siRNA duplexes that were efficient (see Materials and methods) all gave a similar phenotype (Fig. 6 and not depicted). The reduction of NDC1 correlated with a proportional loss of the NPC signal for the mAb414-reactive FG repeat Nups, or for Nup88, which anchors Nup214 and Nup358 to NPCs (Bernad et al., 2004). Therefore, the assembly defects caused by the NDC1 depletion might not be

restricted to the vicinity of the pore membrane, but extend to NPC structures that are distant from the pore membrane.

There is, apparently, highly selective pressure against NDC1-depleted cells. This is indicated by the observation that the fraction of cells that showed significant depletion of the target protein was consistently smaller in hNDC1 knockdowns than in parallel RNAi experiments against POM121 or gp210.

To address the question of whether eukaryotes can assemble at least rudimentary NPCs without NDC1, we switched the model organism and analyzed NDC-1 genetically in *C. elegans*.

Loss of NDC-1 function in *C. elegans*

Database searches pointed to the *C. elegans ndc-1* $-/-$ strain (*tm1845*), generated by S. Mitani at the Japanese *C. elegans* deletion consortium. The strain carries a mutation at the B0240.4 locus, which is predicted to disrupt the ORF of ceNDC-1 just after the second membrane-spanning segment. If expressed, the resulting deletion would still comprise $\sim 25\%$ of the protein sequence, but would lack all parts of the protein that are conserved and potentially exposed toward the NPC.

The mutant strain has so far been propagated only in the heterozygous form because the phenotype of the homozygous mutant is so severe that it was initially listed as sterile or lethal. However, we were able to detect rare cases of homozygous mutant worms that not only developed until adulthood but also produced a few offspring. The homozygous mutant *ndc-1* $-/-$ genotype was confirmed by single-worm PCR (not depicted), as well as by immunoblots showing that the ceNDC-1 protein is, indeed, absent in homozygous mutant worms (Fig. 7). We have now maintained these homozygotes for >15 generations and can therefore exclude the possibility that their survival is only attributable to a maternal ceNDC-1 mRNA pool inherited from a heterozygous progenitor.

Immunofluorescence also confirmed the absence of the ceNDC-1 protein from the mutant worms (unpublished data). In addition, it revealed a significantly reduced mAb414 signal of the NE, as compared with wild-type worms. However, this staining for FG repeat-containing Nups was not completely lost, indicating that rudimentary NPCs can assemble and persist in the absence of ceNDC-1 (Fig. 8).

Depletion of NDC1 from HeLa cells

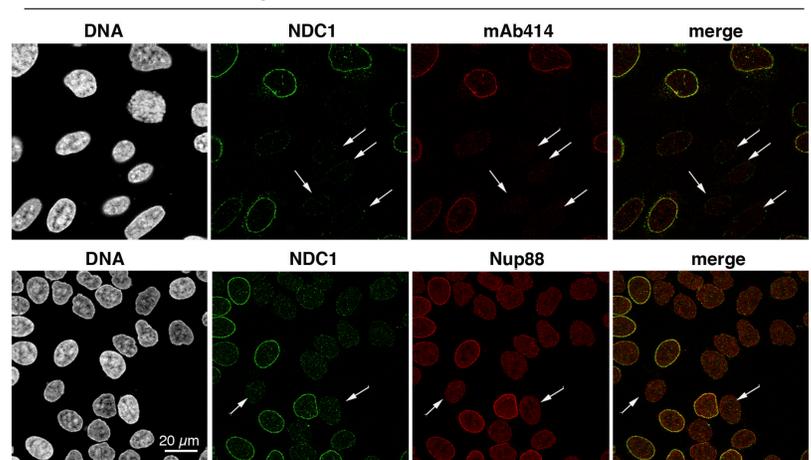


Figure 6. Depletion of hNDC1 from HeLa cells causes severe NPC assembly defects. HeLa cells were transfected with hNDC1-specific siRNAs and analyzed 96 h later by double immunofluorescence with indicated antibody combinations. Depletion of hNDC1 from transfected cells (arrows) resulted in a proportional decrease in mAb414 and Nup88 stain.

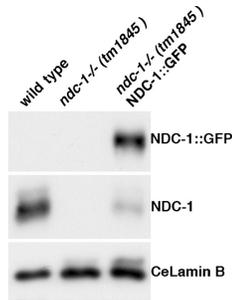


Figure 7. **Immunoblot analysis of the *ndc-1* $-/-$ (*tm1845*) *C. elegans* mutant.** Total protein extracts from wild-type worms, the homozygous *ndc-1* (*tm1845*) strain, and ceNDC1::GFP rescue strain were prepared and analyzed by immunoblotting with affinity-purified antibodies against ceNDC-1 and antibodies against lamin B, which served as a loading control.

Consistent with the assumption that these rudimentary NPCs are functionally impaired, we observed a very high embryonic and larval mortality rate for the homozygote *ndc-1* $-/-$ (*tm1845*) mutant. The few surviving individuals developed very slowly until adulthood, and most of them remained sterile. These phenotypes culminated in a strongly reduced brood size (Table I and Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>). The surviving homozygous animals displayed additional pleiotropic phenotypes (unpublished data), such as the “clear” phenotype, which indicates the failure of properly developing internal structures and organs. Homozygous adults were smaller than the heterozygote *ndc-1* $-/-$ (*tm1845*) mutants or wild-type worms. This also held true for the eggs and embryos.

To prove that these phenotypes were indeed the consequences of the *ndc-1* mutation, in the *ndc-1* $-/-$ (*tm1845*) homozygous background we generated a transgenic worm that expresses NDC-1::GFP from the endogenous *ndc-1* promoter. The NDC-1::GFP fusion protein was detectable by anti-ceNDC-1 antibodies (Fig. 7) and localized to NPCs (Fig. 9). Because we had introduced the NDC-1::GFP fusion in the form of an extra-chromosomal array, which typically gives a mosaic expression,

Table I. **Complementation of *ndc-1* $-/-$ (*tm1845*) phenotypes by NDC-1::GFP**

Incubation time on plate at 20°C	<i>ndc-1</i> $-/-$ (<i>tm1845</i>)	<i>ndc-1</i> $-/-$ (<i>tm1845</i>) NDC-1::GFP
2 d	0–13 eggs	19 to 93 eggs
3 d	0–32 eggs 0 larvae	35 to >160 eggs and larvae
4 d	0–32 eggs 0–8 larvae	numerous adult hermaphrodites

10 L2/L3 larvae of each strain were singled out and grown at 20°C. The offspring per plate were counted at the indicated times. The variability in *ndc-1* $-/-$ (*tm1845*) NDC-1::GFP worms is attributable to the mosaic expression of the transgene and germline silencing of the extrachromosomal array from which the transgene is expressed.

the GFP signal was not observed in all cells of the embryos. Nevertheless, expression of the transgene complemented the ceNDC-1 loss-of-function phenotypes and dramatically improved fertility of the homozygous *ndc-1* $-/-$ (*tm1845*) mutant (Fig. S5 and Table I). The phenotypes of the *ndc-1* $-/-$ (*tm1845*) strain, therefore, are caused by the ceNDC-1 gene disruption and are not the consequences of secondary mutations.

Discussion

Ndc1p in NPCs and SPBs

The fungal SPBs and NPCs share Ndc1p as a common component, and both reside within giant pores of the NE that originate from local fusions between INM and ONM. Ndc1p is clearly required for SPB insertion into the NE, and this alone explains why the *Ndc1* gene is essential. The question as to whether Ndc1p is also essential for NPC assembly remained unresolved so far.

SPBs and NPCs are distinct structures; therefore, Ndc1p must cooperate with distinct sets of components to create either nuclear or SPB pores (Araki et al., 2006). The SPB- and NPC-relevant interactions of Ndc1p even appear to be in competition, as indicated by the observation that the deletion of the membrane-integral Nup POM152 suppresses SPB assembly

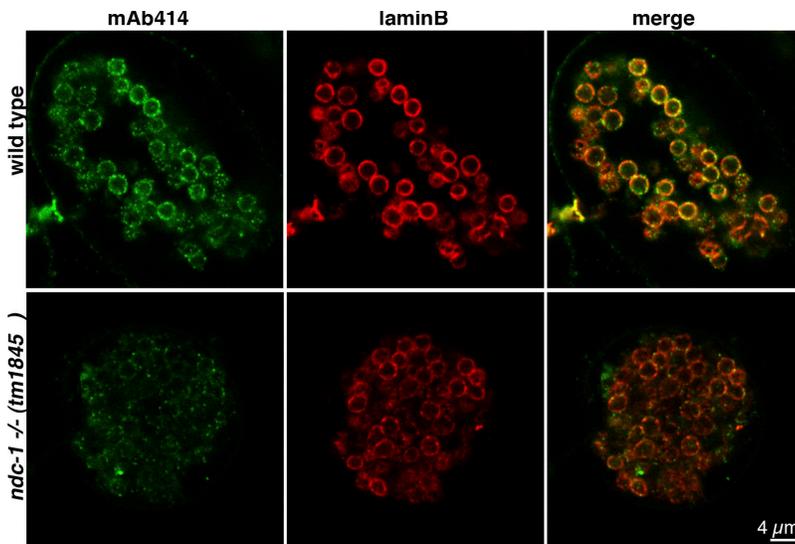


Figure 8. **Reduced mAb414 signal at NPCs of homozygous *ndc-1* $-/-$ (*tm1845*) mutant *C. elegans* embryos.** Wild-type embryos or embryos lacking ceNDC-1 were stained in parallel with mAb414 (green) and anti-laminB (red). Note that NPCs of the mutant embryos stain only weakly with the NPC marker mAb414. The increased mAb414 signal outside the NE suggests that a significant proportion of FG repeat Nups failed to assemble into NPCs. The smaller size and altered morphology of the mutant embryos is probably a secondary effect of impaired NPC function.

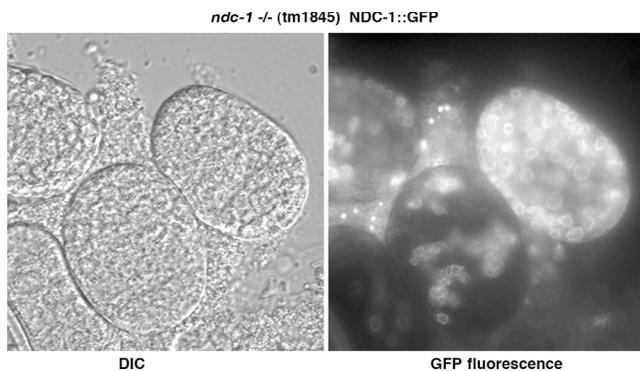


Figure 9. Localization of the ceNDC1::GFP rescue protein. Figure shows analysis of the homozygous *ndc-1* $-/-$ (*tm1845*) strain rescued by an extrachromosomal array that allows expression of a ceNDC1::GFP fusion. Adult hermaphrodites were mounted on a slide and imaged by differential interference contrast or conventional fluorescence microscopy in the GFP channel. The NDC1::GFP fusion shows the expected NPC stain. However, because of the extrachromosomal array, it is not expressed in every cell.

defects that are caused by certain *Ndc1p* mutations (Chial et al., 1998). The extreme sensitivity of yeast cells to any change in *Ndc1p* dosage (Chial et al., 1999) indicates how delicate the equilibria in these interactions might be.

NDC1 in higher eukaryotes

We identify metazoan orthologues to *Ndc1p* and show that they constitute an integral component of NPCs in mammals, amphibians, insects, and nematodes. Metazoan NDC1 is presumably fully dedicated to its function at NPCs because metazoa lack NE-embedded SPBs. NDC1 is now the third known membrane-integral Nup in vertebrate NPCs, and its presence may be one possible explanation as to why NPCs can still form in the virtual absence of POM121 and gp210, which are the other two integral constituents (Stavru et al., 2006). The crucial contribution of NDC1 to the NPC assembly process is indicated by the severe NPC biogenesis defects that occurred when the protein was either depleted by RNAi or when the ORF had been disrupted genetically.

NPC assembly: a robust and fault-tolerant process

The biogenesis of NPCs is a very elaborate process. It requires not only the self-assembly of ~ 700 individual polypeptide chains (representing multiple copies of the ~ 30 different Nups) into a single giant protein complex but also a local fusion between INM and ONM to create the actual pore, as well as the implantation of the NPC scaffold into this pore. NPCs are essential structures, and their failure to assemble would be lethal. Therefore, it is not surprising that the assembly process is robust and fault tolerant. This resistance toward disturbances becomes particularly apparent in the fact that more than half of the yeast Nups can be singly deleted without causing deleterious defects (Rout and Aitchison, 2001). For most deletions of such nonessential Nups, however, synthetic-lethal interactions with loss-of-function alleles of other Nups have been found (Doye et al., 1994; Aitchison et al., 1995; Miao et al., 2005). This illustrates that many of the crucial protein-protein interactions are

backed by more than one player. Such inherent flexibility probably contributes greatly to the intrinsic fault tolerance of the NPC assembly process.

Based on experiments in the *X. laevis* egg extract system, a different explanation for the fidelity of assembling an NPC-perforated NE has been given, namely a surveillance of the process by a POM121-dependent checkpoint system (Antonin et al., 2005). For several reasons, we view this concept with some caution. Bona fide checkpoints allow active intervention into those cellular processes that could result in uncorrectable errors (Murray and Hunt, 1993). The mitotic spindle checkpoint, for example, reduces the probability of an uncorrectable aneuploidy by delaying sister chromatid segregation until each of the chromosomes is properly attached to the mitotic spindle. Nuclei enclosed by a pore-free membrane, however, are not uncorrectable dead-end products. Instead, NPCs can still be integrated into them at later time points (Macaulay and Forbes, 1996). In addition, the great number of NPCs, which become embedded into an NE, should make the nuclear assembly process tolerant against occasional failures to assemble individual NPCs. Considering further that all crucial checkpoints, such as the DNA damage and mitotic spindle checkpoints, are disabled during the early cell cycles in the developing *X. laevis* embryo (Murray and Hunt, 1993), we find it hard to understand why an NPC assembly checkpoint should be kept in operation. Finally, we observed that POM121-depleted human cells formed functional NPCs and showed no uncoupling between NE formation and NPC assembly (Stavru et al., 2006).

Multiple membrane-integral Nups

Consistent with the concept of redundancy and robustness, NPCs appear not to rely on just a single anchor within the NE. Instead, they typically contain several membrane-integral Nups (e.g., three different ones in either yeast or mammals). Genomic data indicate that two of them, gp210 and NDC1, are evolutionary conserved (Mans et al., 2004). The fact that both are found in metazoans as well as in plants, clearly suggests that they evolved before the unikont/bikont bifurcation, which is considered as the oldest time point of a major evolutionary diversification of known eukaryotes (Richards and Cavalier-Smith, 2005). Primordial NPCs were therefore probably equipped with both gp210 and NDC1. However, it appears that some lineages (e.g., all fungi) lost gp210, whereas other lineages (e.g., *Dictyostelium discoideum* or other protozoa) lost NDC1 from their genomes. This brings us to the unexpected conclusion that none of the integral Nups is—generally and in all cellular settings—essential for NPC assembly and function. This also explains why the nematode *C. elegans* can live, although miserably, in the absence of NDC1, why many mammalian cell types, such as fibroblasts, assemble fully functional NPCs without gp210, and why POM121 can be depleted from human cells without deleterious defects.

Do membrane-integral Nups play a direct role in the nuclear pore-forming membrane fusion?

How could membrane proteins of very different topology and domain structure possibly substitute for each other?

One possible explanation would be that membrane-integral Nups act at the nuclear membrane only as nucleation sites for attracting soluble Nups, which then self-assemble further and form the entire rigid scaffold of the pore complex. Their presumed, and apparently redundant, role in creating the membranous pore is, however, difficult to comprehend. The integration of an NPC into a closed NE requires a fusion between the luminal faces of ONM and INM, which have a distance of 20–25 nm. For fusion, this distance needs to be bridged. gp210 had been an excellent candidate for this function in the vertebrate system because it has such a giant luminal domain. However, fusion clearly also occurs in the absence of gp210. Therefore, we now face the puzzling problem that none of the remaining membrane-integral Nups possesses any significant luminal parts: the luminal loops of NDC1 are so short that they will hardly protrude from the membrane. For POM121, it is even unlikely that any part is exposed to the lumen. A possible solution to the problem is that soluble luminal components bridge the distance between INM and ONM. Possibly, they use the luminal loops of NDC1 or the luminal domain of gp210 as docking sites.

Studies in viral systems and in the secretory pathway have clearly established that a controlled membrane fusion requires energy (Söllner, 2004). In all of the cases characterized so far, it is conformational energy stored in fusion-promoting proteins that forces the opposing lipid bilayers to such a short distance that they can eventually coalesce. Viral fusion proteins can release their conformational energy only once, and such a single-use fusion factor would be sufficient to explain NPC biogenesis in yeast. In higher eukaryotes with open mitosis, however, the situation is more complex. NE and NPC disassemble here once per cell cycle and, subsequently, reassemble from the existing membrane and protein components. Of course, the still elusive fusion factor could be degraded and resynthesized during every cell cycle and, in this case, it might not remain associated with mature NPCs. This would explain why no such activity has been found so far. Otherwise, multiple cycles of NPC formation and disassembly would require an additional recycling machinery, which converts the fusion factor from a postfusion to a prefusion conformation. Such recycling machinery would have to reside in the lumen of the NE, and it will be very interesting to see whether it exists or whether nuclear pore formation relies on “disposable” fusion proteins.

Materials and methods

Homology searches and analysis

Ndc1p orthologues were identified by BLAST from public databases. cDNAs comprising the coding regions of human, mouse, *D. melanogaster*, *X. laevis*, and *C. elegans* NDC1 were obtained from the German Resource Center for Genome Research or amplified from total RNA by RT-PCR. Coding regions were verified by DNA sequencing.

Multiple alignments were performed with the ClustalW algorithm (Thompson et al., 1994). Membrane-spanning segments were predicted by combining the results of different algorithms and the hydrophobicity profiles of the respective sequences. The multiple alignments of predicted TMSs were manually corrected.

The orientation of the TMSs was predicted from the constraints (a) that the cytoplasmically flanking region of a membrane anchor is typically more positively charged than the lumenally flanking one (Hartmann et al., 1989) and (b) that adjacent TMSs must have opposite orientation.

The complete coding sequences of NDC1 were given the following accession numbers (available from GenBank/EMBL/DBJ): *Mus musculus*, DQ141695; *Homo sapiens*, DQ141696; *D. melanogaster* (variant 1), DQ141697; and *X. laevis*, DQ191159.

Antibody production

Antibodies were newly raised in rabbits or guinea pigs against the following protein fragments: hNDC1^{242–264} (anti-loop 5), hNDC1^{292–674} (anti-COOH-terminal domain), dmNDC1^{268–578}, ceNDC-1^{315–457}, hPOM121^{448–660}, human gp210^{1,828–1,887}, xNDC1^{280–660}, and dmTPR^{1,168–1,177}.

Antibodies against human Nup62, Nup358, Nup96, and Nup107 (Hase and Cordes, 2003) have been described earlier. All polyclonal antibodies were affinity purified on their respective antigen columns. The mAb against *X. laevis* p62 was also previously described (Cordes et al., 1995). mAb414 was obtained from Eurogentec, and the mAb against Nup88 was obtained from BD Bioscience. The antibody against *C. elegans* lamin was a gift from G. Krohne (Biozentrum, Universität Würzburg, Würzburg, Germany).

Cell culture

Human HeLa cells were maintained in DME low glucose supplemented with 10% FCS, 1× nonessential amino acids (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin.

X. laevis XL-177 cells were cultivated in 65% Leibovitz' L-15 (Sigma-Aldrich) supplemented with L-glutamine, 15% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

D. melanogaster S2 cells were obtained from the American Type Culture Collection (ATCC CRL-1963) and cultivated in *D. melanogaster* serum-free medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and L-glutamine, according to the manufacturer's instructions.

All cell culture products were obtained from Invitrogen, unless otherwise stated. Synchronized HeLaS3 cells were a gift of B. Petrova (Zentrum für Molekulare Biologie der Heidelberg, Heidelberg, Germany).

C. elegans work

Worm cultures were maintained using standard techniques (Brenner, 1974). The heterozygous *ndc1* ^{-/-} (*tm1845*) strain was obtained from S. Mitani at the Japanese *C. elegans* deletion consortium (Tokyo Women's Medical University School of Medicine, Tokyo, Japan). To select for homozygous *ndc1* ^{-/-} (*tm1845*) mutants, sick-looking hermaphrodites were singled out on plates. Most animals died without generating any offspring. The remaining animals had few offspring. The animals were selfed for at least 15 generations. Single-worm PCRs were performed on homozygous and heterozygous animals to confirm the homozygosity of the mutants. The NDC-1::GFP fusion was created by cloning the genomic copy of *ndc-1*, including the putative promoter region into pPD95.81 (Fire et al., 1990). This reporter construct (20 ng/µl) was coinjected with 80 ng/µl pRF4 (*rol-6(su1006)*) into the gonads of *ndc1(tm1845)* homozygous worms. Extrachromosomal arrays were selected by the *rol* phenotype, and the ability of NDC-1::GFP to rescue the *ndc-1* ^{-/-} (*tm1845*) phenotype. NDC-1::GFP was visualized with an epifluorescence microscope (Axioplan 2; Carl Zeiss Microimaging, Inc.). Images were collected with an Axiocam (Carl Zeiss Microimaging, Inc.) and the contrast was adjusted with Photoshop (Adobe). Pictures of worm plates were taken with a digital camera (Coolpix; Nikon) mounted onto the binocular.

RNAi

Transfection of cultured human cells with siRNAs was carried out essentially as previously described (Hase and Cordes, 2003). Annealed siRNAs were purchased from Dharmacon. Antisense strands were complementary to nucleotides 1,915–1,935, 405–425, or 1,569–1,596 of the hNDC1 ORF. In addition, we performed RNAi with stealth siRNAs (Invitrogen), whose sense strand modification is thought to reduce nonspecific effects. Its antisense strand was complementary to nucleotides 1,085–1,109 of the hNDC1 ORF. For each of these four siRNAs, we observed the same correlation between hNDC1 knockdown and depletion of mAb414-reactive Nups from the NE. Fig. 6 shows results with the stealth oligo duplex.

DNA transfections

DNA transfections for expression of EGFP-tagged NDC1 were performed with Fugene6 (Roche), according to the manufacturer's instructions. Cells were analyzed 24–60 h after transfection.

Immunofluorescence and microscopy

For immunofluorescence, cultured cells were washed briefly with PBS, fixed for 4 min in 3% paraformaldehyde that was freshly dissolved in PBS,

washed in PBS, quenched with 50 mM NH₄Cl in PBS for 5 min, permeabilized with 0.25% Triton X-100 in PBS, and blocked for at least 30 min in 1% BSA, 10% goat serum, and 0.1% Triton X-100. Primary antibodies were applied for 60 min in blocking buffer. Nonbound antibodies were washed off with PBS. Alexa Fluor–labeled secondary antibodies were purchased from Invitrogen. The secondary antibodies and the DNA stain Hoechst 33342 were applied for 30–60 min in blocking solution, followed by extensive washing and mounting in Vectashield (Vector Laboratories). Immunofluorescence on *C. elegans* embryos was performed after freeze-fracturing and formaldehyde fixation.

Confocal microscopy was performed with a laser scanning microscope (model SP2; Leica) using 405-, 488-, 561-, or 633-nm laserlines for excitation. All pictures were taken at 22–25°C with Leica PlanApo Oil objectives (100×, 1.4 NA, and 63×, 1.32 NA); for pictures involving the 405-nm laser, lambda blue objectives were used. Images were assembled in Photoshop (version CS) or Illustrator (version CS; Adobe).

Elucidation of the topology of hNDC1

For in vitro translation, the hNDC1 coding region was cloned downstream of a T7 promoter. The following NGSs were inserted: SSNGTS after residue 155 of hNDC1 (NGS-loop 3), SSNGTS after residue 253 (NGS-loop 5). The glycosylation site SSNGTS inserted after residue 61 in loop 1 was not glycosylated, probably because it is too close to the membrane (Nilsson and von Heijne, 1993). However, when extended to the sequence TSGSG-NSSNGSGT, it was glycosylated to 70–80%.

Online supplemental material

Fig. S1 shows the evolutionary conservation of NDC1. Fig. S2 shows EGFP-tagged hNDC1 is targeted to NPCs. Fig. S3 shows the membrane topology of hNDC1 probed by epitope tagging. Figure S4 shows that hNDC1 is heavily modified during mitosis. Fig. S5 shows that NDC-1::GFP rescues the high mortality and sterility phenotype of the *ndc-1* *–/–* (*tm1845*) allele. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200601001/DC1>.

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