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The Conserved Transmembrane Nucleoporin NDC1 Is Required for Nuclear Pore Complex Assembly in Vertebrate Cells

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Summary

Nuclear pore complexes (NPCs) are large proteinaceous channels embedded in the nuclear envelope (NE), through which exchange of molecules between the nucleus and cytosol occurs. Biogenesis of NPCs is complex and poorly understood. In particular, almost nothing is known about how NPCs are anchored in the NE. Here, we characterize vertebrate NDC1—a transmembrane nucleoporin conserved between yeast and metazoans. We show by RNA interference (RNAi) and biochemical depletion that NDC1 plays an important role in NPC and NE assembly in vivo and in vitro. RNAi experiments suggest a functional link between NDC1 and the soluble nucleoporins Nup93, Nup53, and Nup205. Importantly, NDC1 interacts with Nup53 in vitro. This suggests that NDC1 function involves forming a link between the NE membrane and soluble nucleoporins, thereby anchoring the NPC in the membrane.

Introduction

The NE defines the boundary between the nucleus and cytoplasm of a eukaryotic cell. It is composed of two parallel membrane bilayers—the inner nuclear membrane and the outer nuclear membrane, which is continuous with the endoplasmic reticulum (ER). Both nuclear membranes are fused to form holes occupied by NPCs that mediate nucleocytoplasmic transport.

NPC structure and composition are partly conserved between yeast and vertebrates. An NPC consists of multiple copies of about 30 different nucleoporins, many of which are also members of subcomplexes that can be isolated from cell extracts. The modular organization of the NPC into subcomplexes is reflected in its three-dimensional architecture (for review see Schwartz [2005]). The assembly of nucleoporins into the NPC is a fascinating example of a process involving many different protein interactions that occur in a spatially and temporally defined order. In vertebrates, NPC assembly occurs both after mitosis, when the NE reforms, and during interphase, when NPCs have to be inserted into the closed NE.

Postmitotic NE and NPC assembly have been studied in vitro by using fractionated *Xenopus laevis* egg extracts, which assemble nuclei around sperm chromatin. Depletion experiments in this system revealed pivotal roles for two distinct sets of soluble nucleoporins. Whereas the conserved Nup107-160 subcomplex is recruited to chromatin early during mitosis, Nup155 joins the NPC later. The lack of either results in deficiencies in NPC assembly and, in the case of Nup155, also NE assembly (Harel et al., 2003; Franz et al., 2005; Walther et al., 2003). Nup155 is part of a distinct nucleoporin subcomplex in mammals that also contains Nup205, Nup93, Nup53/35, and likely Nup188 (Grandi et al., 1997; Hawryluk-Gara et al., 2005; Miller et al., 2000). Depletion of Nup53 or Nup93 from cultured somatic cells by RNAi leads to a loss from the NPC of both interacting nucleoporins and the group of mAb414-reactive nucleoporins, suggesting an important role of this nucleoporin subcomplex in NPC assembly (Hawryluk-Gara et al., 2005; Krull et al., 2004).

NPCs are assumed to be anchored in the NE by transmembrane nucleoporins, but the nature of the linkage between soluble and transmembrane nucleoporins is presently unclear. Another puzzling aspect of NPC structure and assembly has been the lack of obvious conservation of the transmembrane nucleoporins in evolution. Three distinct pore membrane proteins (poms), Pom152p, Pom34p, and Ndc1p, have been identified in *Saccharomyces cerevisiae* (Chial et al., 1998; Rout et al., 2000; Wozniak et al., 1994). Whereas Pom152p and Pom34p are not essential, the deletion of *NDC1* or its *S. pombe* homolog *cut11*$^{+}$ is lethal (West et al., 1998; Winey et al., 1993). Both Ndc1p and Cut11p are also found at the spindle pole body (SPB) and play a role in SPB duplication, a function that may contribute to their being required for viability (Thomas and Botstein, 1986; West et al., 1998).

The two previously characterized vertebrate transmembrane nucleoporins POM121 and GP210 have no obvious homologs in yeast. POM121 has been implicated in NPC reassembly after mitosis because its depletion blocked NE and NPC assembly in vitro (Antonin et al., 2005). Studies on the role of GP210 in NPC

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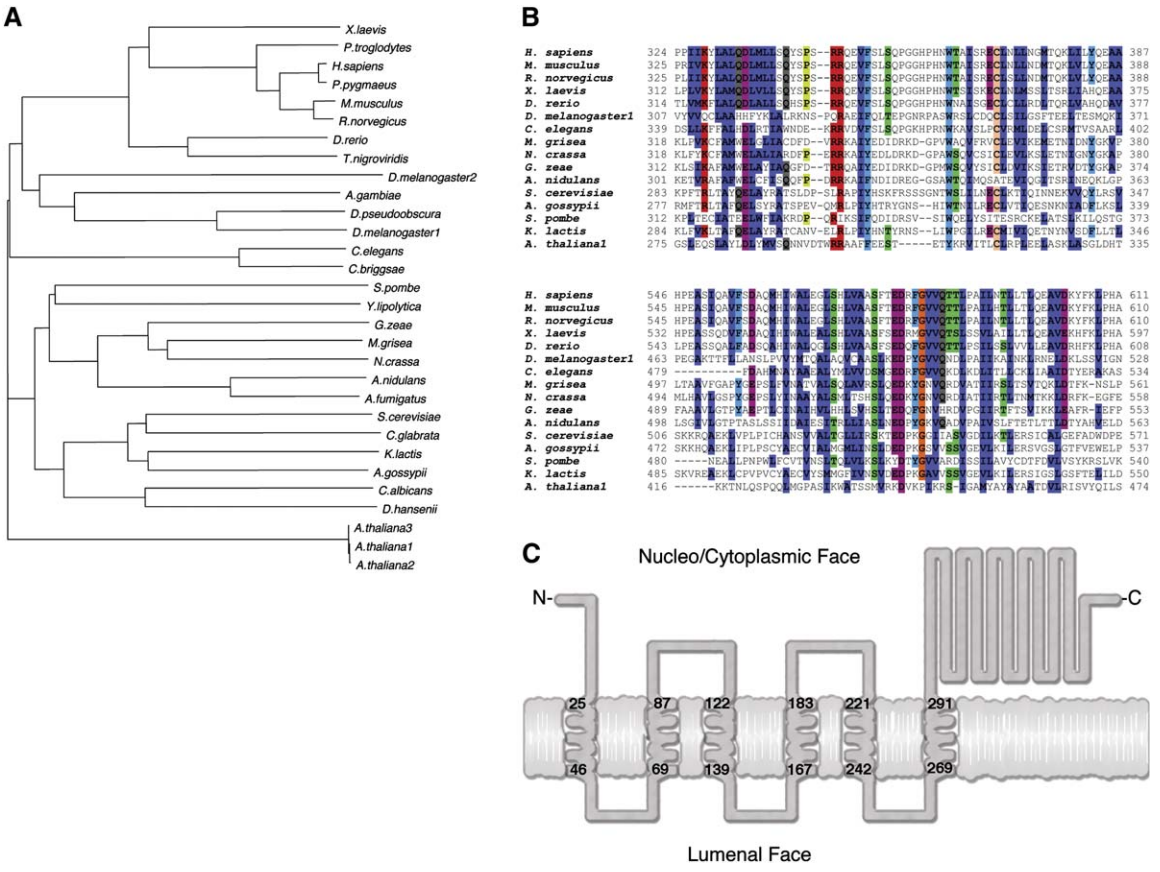

Figure 1. NDC1 Is a Conserved Transmembrane Protein

(A) Phylogenetic tree of NDC1. The phylogenetic relationship between NDC1 homologs from eukaryotic species was derived by using ClustalW.

(B) Multiple sequence alignment of the most conserved parts of the C-terminal domain of hNDC1 from selected eukaryotic species. The alignment was generated by ClustalW. Conserved positions were highlighted in color if at least 50% of the sequences were conserved with respect to the presence of the following groups of amino acids: AILMV, blue; WFY, cyan; RK, red; DE, magenta; ST, green; P, yellow; C, pink; NQ, gray; and G, orange.

(C) Predicted membrane topology of hNDC1. Transmembrane segments were predicted by using the TMPRED software.

biogenesis in different experimental systems have not yielded uniform conclusions ([Cohen et al., 2003; Galy et al., 2003; Antonin et al., 2005]; see Hetzer et al. [2005] for discussion).

We report here on the identification and characterization of vertebrate NDC1, the homolog of the essential yeast integral membrane nucleoporin Ndc1p. Depletion experiments performed by RNAi in cultured somatic cells in vivo and in the NE assembly system in vitro show that NDC1 is required for NPC assembly and NE formation. NDC1 interacts with Nup53 in vitro and may therefore recruit the Nup53-Nup93 nucleoporin subcomplex to the assembling NPC. Our data provide insight into NPC assembly and, in particular, suggest how the central NPC framework is anchored in the NE via a transmembrane nucleoporin.

Results

NDC1 Is Conserved in Evolution

To identify potential homologs of the yeast transmembrane nucleoporins in higher eukaryotes, we performed blast searches with 200 amino acid segments of each protein. Using the C-terminal domain of *S. pombe NDC1* (Cut11p), we were able to identify a *Caenorhabditis elegans* homolog, which in turn allowed us to find potential vertebrate NDC1s (Figure 1A). All these proteins contain an N-terminal segment with several predicted transmembrane anchors, which are poorly conserved in primary sequence, and a C-terminal domain displaying higher conservation (Figures 1B and 1C). Notably, human NDC1 (hNDC1, FLJ10407) was among 67 novel proteins identified in a comprehensive proteomic study of potential NE membrane proteins (Schirmer et al., 2003). A recent comparative genomics study of NPC proteins identified Ndc1p-like proteins in many eukaryotic species (Mans et al., 2004).

Characterization of hNDC1

To gain insight into the subcellular localization of hNDC1, we performed immunofluorescence in HeLa cells by using an antibody raised against the C-terminal 19 amino acids of hNDC1. The antibody was specific by immunoblotting (Figure 2A). hNDC1 localized to the NE in a punctate pattern, indicative of NPC localization. p62, a central component of the NPC, colocalized with hNDC1 at the nuclear rim. Colocalization was not perfect, possibly due to the harsh extraction conditions

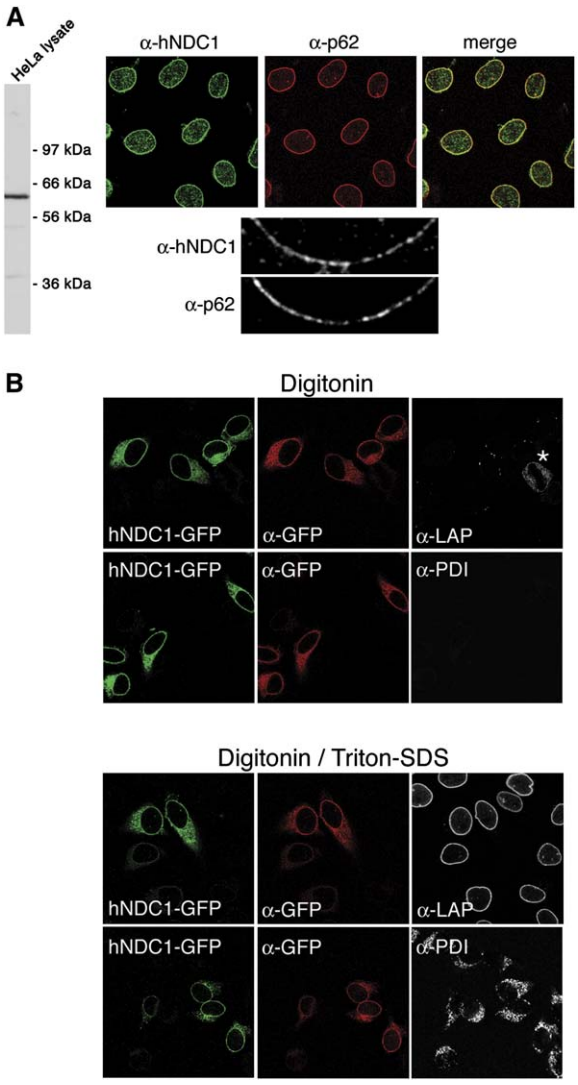

Figure 2. Characterization of Human NDC1

(A) hNDC1 colocalizes with nucleoporins at the nuclear rim. Western blot of total HeLa cell protein with α-hNDC1 (left). HeLa cells were fixed with paraformaldehyde (PFA), extracted with guanidinium hydrochloride, and subjected to immunostaining with α-hNDC1 (green) and α-p62 (red) antibodies (right). The overlay shows colocalization at the nuclear rim (yellow). The magnifications reveal colocalization of both antigens in many spots at the nuclear rim. Colocalization is not perfect, probably due to preextraction (see text).

(B) The C-terminal domain of hNDC1 faces the cytoplasm. HeLa cells transiently transfected with p-hNDC1-EGFP were fixed and then permeabilized with either digitonin or digitonin followed by Triton X-100/SDS. Cells were probed with α-GFP antibodies in combination with either α-LAP2 or α-PDI antibodies. GFP fluorescence is green, the α-GFP signal red, and the α-LAP2 or α-PDI signals are white. Note that the GFP moiety is fully accessible to the antibody in digitonin semipermeabilized cells, whereas LAP2 and PDI are only recognized upon full permeabilization. A single nontransfected cell that is in mitosis is stained by the α-LAP2 antibody (star) because the epitope is accessible after NE breakdown.

required to reveal the hNDC1 epitope that might partially extract p62. Intranuclear staining was also observed, but because this was not reduced after efficient depletion of NDC1 by RNAi (see below), this is probably nonspecific.

The most conserved part of the Ndc1p homologs is the C-terminal domain, which is predicted to be exposed to the cytoplasm/NPC (Figure 1C). To test this, we produced a fusion of hNDC1 with GFP at the C terminus and examined its accessibility to anti-GFP antibodies in semipermeabilized cells. The hNDC1-GFP fusion protein localized to the nuclear rim and also to the ER, likely due to overexpression. Permeabilization of cells with digitonin allowed detection of the GFP epitope on hNDC1, whereas membrane extraction with Triton X-100/SDS was required to provide access for antibodies to LAP2 in the nucleoplasm or PDI in the perinuclear space and ER lumen (Figure 2B).

Many nucleoporins are phosphorylated during mitosis. hNDC1 has several predicted phosphorylation sites for mitotic kinases such as CDK1 or Plk1 (Figure S1A available in the Supplemental Data with this article online). To examine whether hNDC1 might be a target for mitotic phosphorylation, we analyzed the electrophoretic mobility of hNDC1 from cells arrested either in interphase or in mitosis. We observed a significant retardation of hNDC1 from mitotic extracts as compared to hNDC1 from interphase extracts on Western blots (Figure S1B). Treatment of mitotic cell extracts with protein phosphatase converted hNDC1 to the faster-migrating form, demonstrating that hNDC1 is indeed phosphorylated during mitosis.

Depletion of Transmembrane Nucleoporins from Cultured Somatic Cells

To gain insight into the contribution of individual integral membrane nucleoporins to NPC biogenesis, we first used RNAi. Four different hNDC1 siRNAs effectively decreased cellular levels of hNDC1 in HeLa K cells as judged by immunoblotting and indirect immunofluorescence of the nuclear rim (Figure 3A and data not shown). Two particularly effective siRNAs (see Figure S2 for quantitation of depletion) were selected for further use.

The effect of depletion of NDC1, POM121, and GP210 on NPCs in HeLa K cells was analyzed by using mAb414 staining as a read out. mAb414 recognizes a group of FG repeat-containing nucleoporins (Davis and Blobel, 1986) that constitute a significant proportion of the NPC by mass. To control the efficiency of the individual siRNA treatments, cells from the same batch were costained with combinations of POM121/mAb414, GP210/mAb414, and Nup107/lamin A/C antibodies (Figure 3B). Treatment of cells with either of the two hNDC1 siRNAs robustly impaired the localization of mAb414 nucleoporins to the nuclear rim, and many cells displayed defects in nuclear morphology (Figure 3B). The NE association of Nup107, a component of the Nup107-160 subcomplex essential for NPC biogenesis (Harel et al., 2003; Walther et al., 2003) was slightly reduced, that of GP210 appeared to be largely unaffected.

The RNAi-mediated depletion of POM121 also reduced mAb414 staining, as previously observed in NRK cells (Antonin et al., 2005), and the nuclei of affected cells were on average smaller. We consistently found that POM121 had to be very efficiently depleted to observe these effects. Cells that still displayed traces of residual POM121 antibody staining at the nuclear rim showed no change in the mAb414 signal (data not shown). Downregulation of GP210 slightly reduced

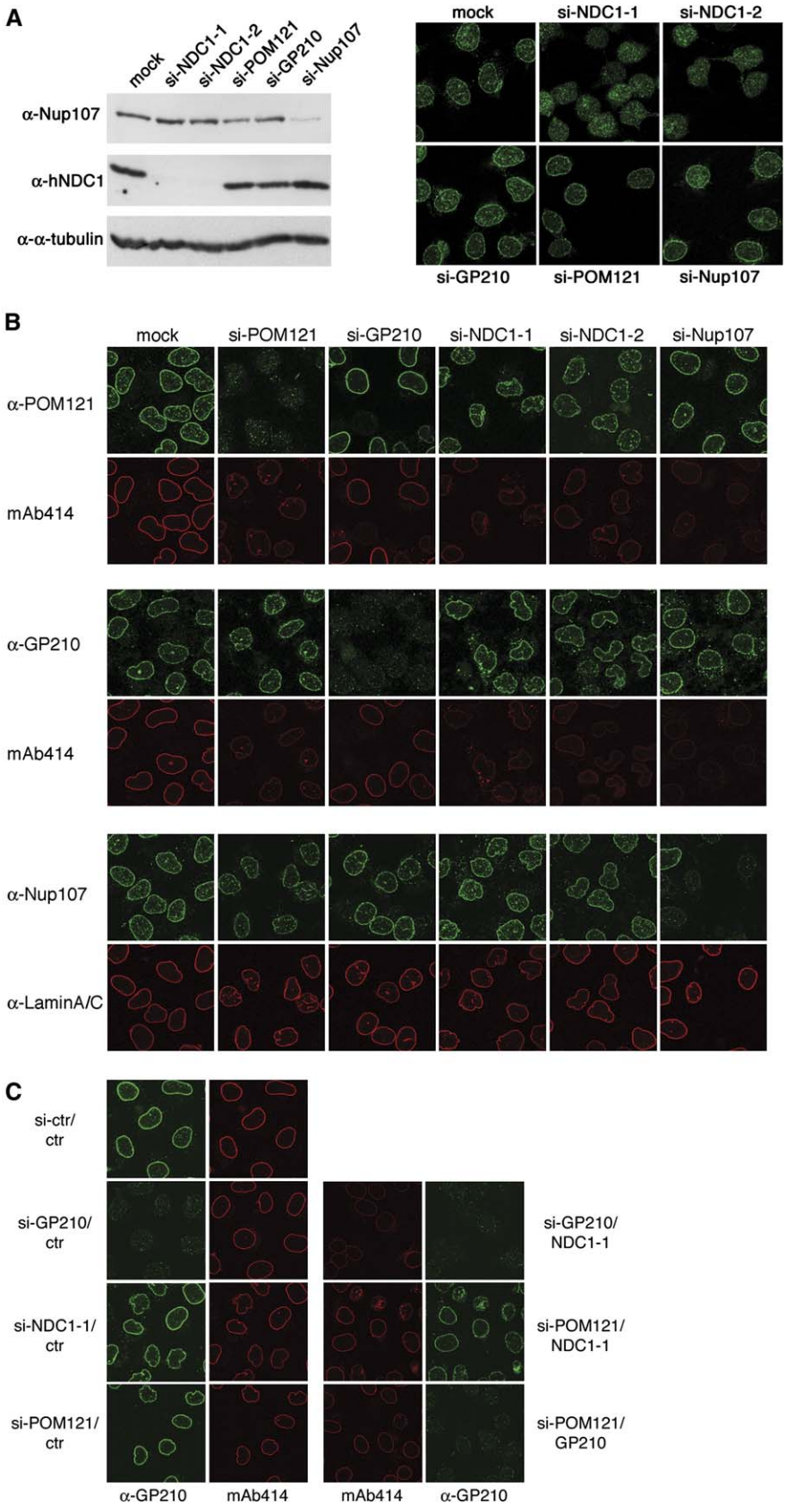

Figure 3. Depletion of NDC1 from HeLa K Cells by RNAi

(A) Depletion of hNDC1 by RNAi is efficient. HeLa K cells were either mock treated or transfected with siRNAs (at 35 nM) to POM121, GP210, NDC1, and Nup107. Cells were harvested 72 hr later and subjected to Western blot analysis with NDC1, Nup107, and α-tubulin antibodies

mAb414 staining after 3 days of RNAi. Knockdown of Nup107 served as positive control and, as expected, drastically reduced mAb414 staining (Figure 3B).

To obtain additional evidence for a role of hNDC1 in NPC biogenesis, we repeated these RNAi experiments in human U2OS cells, which like HeLa cells are very susceptible to siRNA treatment. Similar to the results with HeLa cells, knockdown of NDC1, POM121, and Nup107 decreased the staining of mAb414-reactive nucleoporins at the nuclear rim (Figure S3B). We consistently observed that the effect of downregulation of NDC1 and POM121 was stronger in U2OS cells than in HeLa K cells. Interestingly, Western blot analysis revealed that U2OS cells contained less nucleoporins relative to total protein and to cytoskeletal markers than HeLa K cells (Figure S3C). This might explain why U2OS cells show a stronger response to treatment with NDC1 and POM121 siRNAs.

Although the downregulation of hNDC1 was very efficient in both HeLa K and U2OS cells, there was still residual mAb414 staining, suggesting that either remaining NDC1 levels are sufficient to allow some NPC assembly or that other transmembrane nucleoporins might partially substitute for the loss of hNDC1. To address the second possibility, we performed combinatorial RNAi with limiting amounts of the individual siRNAs in HeLa K cells. At the chosen low concentration of siRNAs, the levels of FG containing nucleoporins remained unaffected by application of individual siRNAs directed against NDC1, GP210, or POM121, each mixed with a control siRNA. The combination of GP210 and POM121 siRNAs had only a slight effect on mAb414 staining under these conditions. When the GP210 and NDC1 siRNAs were combined, mAb414 staining was severely reduced (Figure 3C). This may indicate a contribution of GP210 to NPC assembly that is partly overlapping with that of NDC1 or that GP210 contributes to other aspects of NPC function or dynamics. The combined application of NDC1 and POM121 siRNAs had a different phenotype. Although mAb414 staining did not change in the majority of the cells, about 35% of the nuclei were very small and displayed altered morphology, suggesting a defect in post-mitotic nuclear formation.

NDC1 Is Required for Postmitotic NPC and NE Assembly In Vitro

Xenopus NDC1 (xNDC1) was next characterized. xNDC1 antibodies recognized a 65 kDa protein in the membrane fraction of frog egg extracts and stained the nuclear rim in *Xenopus* XL177 cells (Figure S4) or in vitro-assembled nuclei (Figure 6B). Immuno-electron microscopy of *Xenopus* oocyte nuclei showed localization to NPCs (Figure S5). NDC1, like GP210, behaved as an integral membrane protein in phase separation experiments compared to Importin α, which exists in both cytosolic and membrane attached pools (Hachet et al., 2004; Figure S4A).

POM121 and GP210 enrich in different vesicle populations, which differ in their ability to bind chromatin (Antonin et al., 2005). To investigate whether NDC1 coenriches with either population we immunoisolated membrane vesicles containing NDC1, POM121, GP210, or lamin B receptor (LBR). After separation of bound from unbound material, the fractions were analyzed by Western blotting (Figure 4A). When compared to the starting material, each vesicle fraction was enriched for the respective antigen, which was therefore depleted from the unbound material. Strikingly, NDC1, POM121, and LBR were codepleted by each of the three antibodies, but not by GP210 antibodies. In contrast, GP210 was not enriched by NDC1, POM121, or LBR antibodies, only by anti-GP210. This indicates that NDC1, POM121, and LBR reside on similar or overlapping vesicle populations, whereas GP210 is in a different population.

We next examined whether, like POM121 (Antonin et al., 2005), NDC1 would be enriched on vesicles binding to chromatin. We incubated membranes with sperm chromatin that had been pretreated with nucleoplasmin and histones to promote decondensation. Under these conditions, i.e., without cytosol, docking of vesicles occurs but nuclear assembly cannot proceed further. Chromatin was isolated by centrifugation and the bound and unbound material analyzed by Western blotting (Figure 4B). When increasing numbers of sperm heads were used, NDC1-containing membranes bound efficiently to chromatin and became depleted from the unbound fraction, similarly to POM121. In contrast, little GP210 was bound to chromatin under these conditions. As previously reported (Antonin et al., 2005), much higher numbers of sperm heads had to be used to observe binding of GP210-containing membranes. These data show that a significant fraction of NDC1-containing vesicles bind to chromatin and confirm that POM121 and NDC1 localize to the same vesicle population.

We next investigated the potential function of NDC1 in NE and NPC assembly in vitro. First, we tested the ability of anti-xNDC1 antibodies to inhibit nuclear assembly. To do so, the membrane fraction was preincubated with Fab fragments derived from two different anti-xNDC1 antibodies, and these membranes were then used in assembly reactions together with cytosol and sperm chromatin. After membrane staining, the samples were fixed and analyzed by fluorescence microscopy (Figure 5A). Control reactions using membranes pretreated with PBS produced 96% closed nuclei (Figure 5B). Fab

(left) or fixed with PFA, extracted with guanidinium hydrochloride, immunostained with α-hNDC1 antibodies, and analyzed by confocal fluorescence microscopy (right).

(B) Depletion of hNDC1 reduces the presence of FG-containing nucleoporins at the nuclear rim. HeLa K cells were grown on coverslips in 6-well plates and either mock treated or transfected with siRNAs to POM121, GP210, NDC1, and Nup107. Coverslips from the same well were harvested 72 hr later, fixed, and costained with combinations of POM121/mAb414, GP210/mAb414, and Nup107/lamin A/C antibodies. Note that NDC1 depletion from the nuclear rim was confirmed in parallel by immunofluorescence (shown in [A]) on coverslips from the same well after guanidinium hydrochloride treatment to reveal the NDC1 epitope.

(C) Combined depletion of transmembrane nucleoporins. HeLa K cells were treated with combinations of control (ctr) siRNAs and siRNAs to transmembrane nucleoporins, or combinations of siRNAs directed to transmembrane nucleoporins (17 nM each). Note that the concentration of the siRNAs was chosen such that their individual application had no striking effect on mAb414 antibody staining. After 3 days, cells were fixed and analyzed by mAb414 and α-GP210 antibody staining.

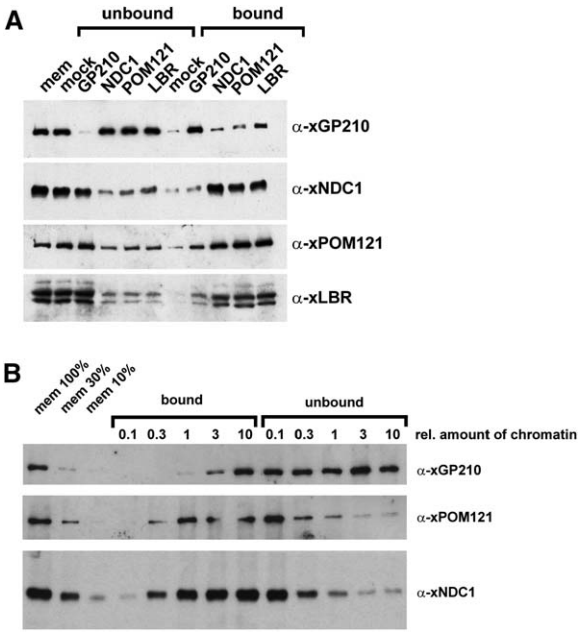

Figure 4. NDC1 Is Coenriched with POM121

(A) Immunoisolation of different vesicles. Membranes were incubated with control (mock), α-GP210-, α-POM121-, α-NDC1, or α-LBR affinity beads. Unbound membranes and bound material as well as starting membranes (mem) were analyzed by SDS-PAGE and Western blotting for the presence of NDC1, GP210, POM121, and LBR.

(B) Chromatin binding of vesicles. Membranes were incubated with increasing amounts of decondensed chromatin. Chromatin bound and unbound membrane fractions were separated by centrifugation and analyzed by SDS-PAGE and Western blotting for GP210, POM121, and NDC1. 100%, 30%, and 10% of the starting membrane material was loaded on the left.

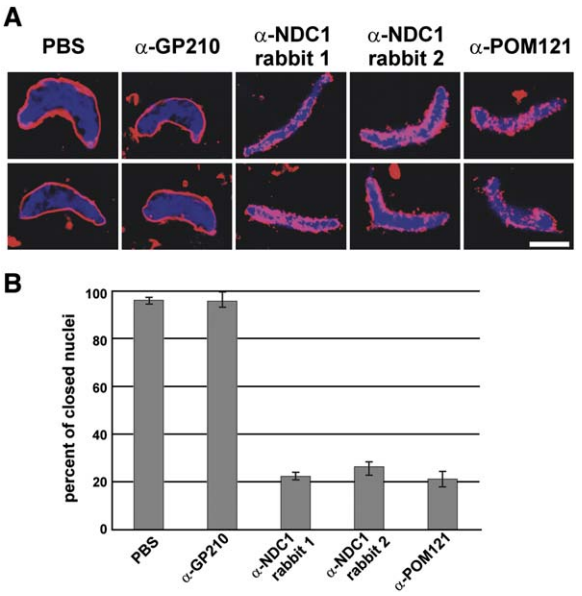

Figure 5. Fab Fragments Specific for NDC1 Inhibit Formation of a Closed NE

(A) Membranes were preincubated with PBS or Fab fragments derived from α-GP210, α-POM121, or two different α-NDC1 antisera, respectively, and used in nuclear assembly reactions. Membranes were stained with $DiIC_{18}$ and chromatin with DAPI and samples analyzed by confocal microscopy.

(B) More than 100 randomly chosen chromatin substrates per reaction from samples from (A) were analyzed for closed NEs. The average of three independent experiments are shown, error bars represent the total variation over the three experiments.

fragments specific for GP210 did not inhibit NE formation, as reported previously (Antonin et al., 2005). In contrast, when membranes were preincubated with Fab fragments specific for NDC1, nuclear assembly was mostly arrested at the stage when vesicles bind to chromatin: only 22% or 26% of chromatin substrates formed closed nuclei after treatment with anti-xNDC1 Fab fragments (Figures 5A and 5B). This was in the same range as the inhibition observed with POM121 Fabs (20%). Combining POM121 and NDC1 Fab fragments did not give stronger inhibition nor did the use of intact IgGs (data not shown).

In the next experiments, NDC1 was depleted from the membrane fraction used for nuclear assembly reactions. Membranes were solubilized with octylglucopyranoside, and the resulting detergent extract was either mock depleted or depleted of NDC1 by using antibodies. Membranes were then reconstituted by removing the detergent via size exclusion chromatography (Antonin et al., 2005). This procedure efficiently removes NDC1 without affecting the other transmembrane nucleoporins (Figure 6A). When reconstituted membranes were added to membrane-free cytosol and sperm heads, mock-depleted membranes formed closed NEs that could be stained with mAb414 and xNDC1 antibodies with an efficiency of over 80%, indicating that NPC assembly was not greatly affected by the solubilization-reconstitution procedure (Figures 6B and 6C). Moreover, these nuclei were still competent for nuclear import of fluorescently labeled BSA-NLS. Electron microscopic analysis of the reconstituted nuclei confirmed that a closed NE and NPCs were formed (Figure 6D). In contrast, when NDC1-depleted membranes were employed, membranes still bound to chromatin but no closed NE formed (Figures 6B and 6C). mAb414 and xNDC1 staining were very strongly reduced.

In order to confirm that the effect of NDC1 depletion was specific, an add-back experiment was performed. N- or C-terminally His-tagged NDC1 was purified from baculovirus-infected insect cells by using reconstitution into liposomes as the last enrichment step (Figure S6). These NDC1 containing liposomes were resolubilized by adding them to the depleted detergent extract before final reconstitution. It was possible to add back enough purified NDC1 to reach endogenous levels (Figure 6A). When these membranes were utilized in an assembly reaction, closed nuclei containing NPCs were formed, as judged by confocal and electron microscopy (Figures 6B–6D). NE formation could, however, be blocked by pretreating the reconstituted membranes with antibodies specific for NDC1 (data not shown). As a control, POM121 purified according to the same purification procedure was added back. It should be noted that this purification is not optimal for POM121 and yielded low amounts of protein and a variety of degradation products (Figure 6A). When POM121 from this "mock" purification was added to the detergent extract depleted for NDC1, no restoration of closed NE formation was observed. Importantly, this POM121 fraction could rescue

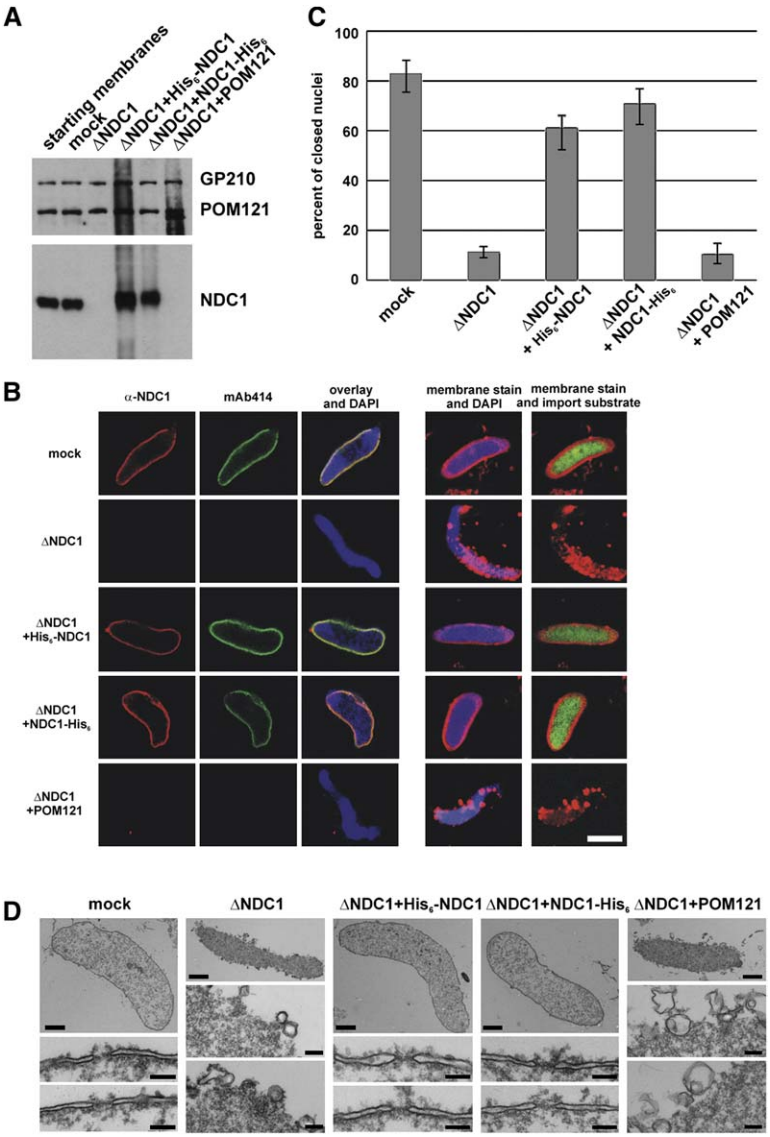

Figure 6. Removal of NDC1 from Membranes Blocks Formation of a Closed NE

(A) Membranes were solubilized, and incubated with control (mock) or α-NDC1-affinity beads to deplete NDC1 and reconstituted. For the add-back experiment, purified N-terminal- or C-terminal-tagged recombinant NDC1 was added to the depleted membranes before reconstitution (ΔNDC1 + His_6-NDC1 or ΔNDC1 + NDC1-His_6). Recombinant xPOM121 addback was the negative control. Starting material and reconstituted membranes were analyzed by SDS-PAGE and Western blotting for GP210, POM121, and xNDC1.

(B) Nuclear assembly reactions were performed by using reconstituted membranes from (A). Reactions in the left three columns were analyzed by confocal microscopy after immunofluorescence using α-xNDC1 (red) and mAb414 (green). In the right two columns, a nuclear import reaction was performed for 20 min before fixation. Membranes are visualized by using the membrane dye $DiIC_{18}$ (red); the import substrate (fluorescently labeled BSA-NLS) is shown in green. Chromatin was stained with DAPI (blue in the overlays). Bar, 10 μm.

(C) Quantitation of closed NE formation of experiments performed as in (B). More than 100 randomly chosen chromatin substrates per reaction were counted. The averages of four independent experiments are shown, error bars represent the total variation over the four experiments.

(D) Transmission electron microscopy of nuclear assembly reactions. Bar in upper row 2 μm, lower rows 100 nm.

the POM121 depletion phenotype (Figure S7). Quantitation showed that with mock-depleted membranes, 83% of the chromatin was surrounded by a closed NE (Figure 6C). This number was reduced to 11% by NDC1 depletion. The effect of NDC1 depletion was reversed, with 61% or 71% closed NE formation, when recombinant N- or C-terminally tagged NDC1 were added, but not by POM121 addition (10%) (Figure 6C).

Although the phenotype of NDC1 depletion in vitro is similar to that previously observed for POM121 depletion, i.e., in both cases NE and NPC formation are blocked, the proteins were not codepleted from detergent extracts by their respective antibodies (Figure 6A and Figure S7A). More importantly, adding excess recombinant NDC1 to POM121-depleted membranes did not rescue the phenotype of POM121 depletion (Figure S7B).

A Link between NDC1 and the Nup93-53 Complex

Having characterized the effect of NDC1 depletion on NPC assembly in vitro and in vivo, we asked whether NDC1 might directly connect to "soluble" nucleoporins of the central NPC framework. Previous experiments had shown that RNAi-mediated knockdown of components of two nucleoporin subcomplexes, namely the Nup107-160 and the Nup93-53 complexes, led to failures in NPC assembly (Harel et al., 2003; Hawryluk-Gara et al., 2005; Krull et al., 2004; Walther et al., 2003). To investigate if NDC1 function is linked to one of these two nucleoporin subcomplexes, we again performed combinatorial RNAi targeting Nup93 and Nup107 in combination with hNDC1. Codepletion of Nup93 and NDC1 resulted in nuclei displaying a distorted morphology and reduced mAb414 staining (Figure 7A). Cells treated with combinations of NDC1 and Nup107 siRNAs showed unchanged levels of FG-repeat containing nucleoporins at the NE and normal nuclear shape at these limiting siRNA concentrations. One possible interpretation of the synergistic effect is that NDC1 interacts with members of the Nup93-53 complex, but not the Nup107-160 complex.

To address the role of hNDC1 in NPC targeting of members of the Nup93-53 subcomplex, we investigated the effect of NDC1 RNAi on localization of subcomplex proteins. Downregulation of hNDC1 in HeLa K cells

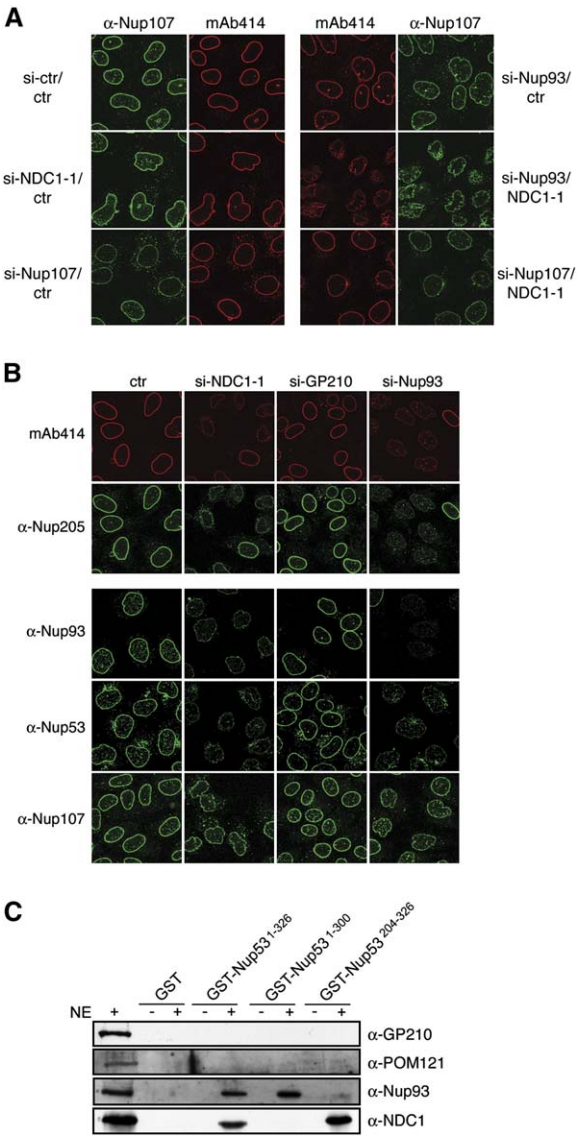

Figure 7. NDC Interacts with the Nup93-53 Complex

(A) Combined depletion of Nup93 and hNDC1 by RNAi reveals a functional link between hNDC1 and the Nup93 complex. HeLa K cells were treated with combinations of control (ctr) siRNAs, Nup93 siRNAs (4.2 nM), or Nup107 siRNAs (8.5 nM). Control siRNAs were always added such that the final concentration of the siRNA mixture was 35 nM. After 3 days, cells were fixed and analyzed by mAb414 and Nup107 antibody staining as in Figure 3.

(B) NDC1 depletion by RNAi affects binding of Nup53, Nup93, and Nup205 to the NPC. HeLa K cells were transfected with siRNAs as in Figure 3B, subjected to immunofluorescence analysis with mAb414/ α-Nup205, α-Nup93, α-Nup53, and α-Nup107 antibodies, and analyzed by confocal microscopy.

(C) In vitro binding of NDC1 from detergent extracts of rat liver nuclei to immobilized recombinant Nup53. Rat liver NEs extracts in buffer containing 0.3% Triton X-100 and 106 mM NaCl were incubated with purified recombinant GST, GST-Nup53-1-326, GST-Nup53-1-300, or GST-Nup53-204-326 immobilized on glutathione Sepharose 4B beads. After binding and washing, the bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE, and analyzed by Western blotting using antibodies directed against POM121, GP210, Nup93, and hNDC1.

reduced NPC association of Nup53, Nup93, and Nup205 as shown by immunofluorescence (Figure 7B). In contrast, Nup107 targeting was only slightly affected by hNDC1 depletion.

Yeast and vertebrate Nup53 interact closely with the pore membrane (Marelli et al., 2001; Hawryluk-Gara et al., 2005). Using in vitro binding assays previously employed to identify interactions between members of the rat Nup93-53 complex (Hawryluk-Gara et al., 2005), we tested whether NDC1 was capable of binding this complex. Recombinant GST-Nup53 was immobilized on glutathione Sepharose beads and incubated with rat liver NE extracts containing solubilized nucleoporins. Western blot analysis of the bound fractions showed that GST-Nup53 specifically interacted with rat NDC1, but not GP210 or POM121 (Figure 7C and Hawryluk-Gara et al. [2005]). NDC1, however, did not bind to a C-terminal truncation of Nup53 that lacks the last 26 amino acid residues, suggesting that this region is necessary for their association. This observation is consistent with our previous data showing that the tight association of yeast Nup53p with the NE membrane is dependent on this conserved C-terminal region (Marelli et al., 2001; Lusk et al., 2002). In contrast, full-length and C-terminally truncated Nup53 bound to Nup93, whereas the C-terminal fragment of Nup53 bound NDC1, but not Nup93. These results suggest that the binding of NDC1 to Nup53 does not occur through Nup93. Instead, their interaction is either direct or mediated by an as yet undefined protein. Taken together, our data are consistent with the notion that NDC1 facilitates recruitment of the Nup93-53 complex to the assembling NPC.

Discussion

Anchoring of NPCs in the NE has been assumed to involve transmembrane nucleoporins. It has been puzzling that transmembrane nucleoporins seemed poorly maintained in evolution. We show here that NDC1 is conserved in most eukaryotes, as also reported in a recent comparative genomics analysis of NPC components (Mans et al., 2004). Interestingly, NDC1 appears to be absent from a few eukaryotes, e.g., *Dictyostelium discoideum*, *Entamoeba histolytica*, or *Plasmodium falciparum* (Mans et al., 2004). It remains to be seen if NDC1 has just been missed so far in these organisms or if they have evolved other mechanisms to compensate for NDC1 loss.

In yeast, in addition to Ndc1p, two nucleoporins containing transmembrane regions are known: Pom152p and Pom34p (Rout et al., 2000; Wozniak et al., 1994). No obvious homologs of these two proteins are found outside fungi (Mans et al., 2004). In addition to NDC1, two transmembrane nucleoporins are known in vertebrates: POM121 and GP210. Neither appears to be related in sequence or membrane topology to yeast Pom152p or Pom34p. Whereas GP210 is quite widespread outside yeast and found in animals, fungi, and plants (Mans et al., 2004), POM121 has only been identified in vertebrates.

This is surprising in light of the fact that we had previously reported an essential function of POM121 in NE assembly in vitro (Antonin et al., 2005). Consistent with this in vitro phenotype, we observe a reduction of

mAb414 staining on depleting the protein from NRK, HeLa, and USO2 cells (Antonin et al., 2005; this paper). It should be noted that the reduction of mAb414 staining is only seen if POM121 expression levels are dramatically reduced, and this is a likely reason that we did not observe this effect in HeLa cells previously (Antonin et al., 2005). Why should such an important function be carried out by a vertebrate-specific protein? POM121 function may either have evolved with vertebrates or there may be functional orthologs in other species that do not show sequence conservation.

Our data suggest that NDC1 fulfills an essential function in nuclear assembly. Depleting the protein from mammalian cells by RNAi caused a severe reduction in mAb414 staining, indicative of defects in NPC assembly and/or stability, whereas we observed a strong inhibition of nuclear formation in vitro on treatment with specific Fab fragments or depletion of the protein. In the absence of NDC1, membrane vesicles bound to the surface of the chromatin but did not fuse to form a closed NE and no NPCs were assembled. Interestingly, NDC1 is found on a population of membrane vesicles that bind with high avidity to chromatin. These vesicles are thought to play an important role in NPC and NE assembly. Consistent with this, the NDC1-containing vesicle population is also enriched in POM121 and LBR. We have previously shown that POM121 and GP210 reside on largely distinct vesicle populations (Antonin et al., 2005), and the data presented here identify two more transmembrane proteins, NDC1 and LBR, which have roles in nuclear formation and/or stability, that cosegregate with POM121.

Depletion of either NDC1 or POM121 induces strikingly similar phenotypes both in vivo and in vitro. In cultured somatic cells, we observe a reduction of mAb414 staining and, in vitro, a block to NE formation. Double RNAi-depletion of the proteins did not result in a synergistic reduction of mAb414-reactive nucleoporins, but in a high percentage of cells containing small nuclei, suggesting that these cells might have defects in the proper assembly or growth of nuclei after mitosis. Consistent with such a function, nuclear assembly in vitro is blocked by depletion of either NDC1 or POM121. However, the phenotype of NDC1 depletion was not rescued by addition of recombinant POM121 or vice versa. This indicates that, at least in the in vitro assembly system, the two proteins are not functionally redundant.

We have previously shown that GP210 is not essential for postmitotic NPC and NE formation in vitro (Antonin et al., 2005). When GP210 was targeted in *C. elegans* by RNAi, viability was slightly reduced, but embryonic development generally continued to an advanced state (Cohen et al., 2003; Galy et al., 2003). Upon depletion of GP210 in mammalian cells by RNAi, we observed here a modest reduction in mAb414 staining. A similar, albeit more pronounced, reduction of mAb414-reactive nucleoporins has been previously described in HeLa cells (Cohen et al., 2003). The differences in mAb414 reduction could be due to differences in the efficiency of GP210 depletion. Interestingly, double depletion of GP210 and NDC1 by RNAi had a pronounced synergistic effect in reducing mAb414 staining at NPCs. The shape of nuclei in cells depleted for both NDC1 and GP210 was normal. These data are consistent with a role for GP210 in NPC assembly during interphase that partly overlaps with NDC1 function, but GP210 could also have other important roles in NPC function or dynamics.

Ndc1p is a component of both the SPB and the NPC in yeast. *NDC1* in *S. cerevisiae* or the homolog *cut11*$^{+}$ in *S. pombe* is essential and required for SPB insertion into the NE (West et al., 1998; Winey et al., 1993). The NPC localization of Ndc1p also suggested a potential role in NPC assembly (Chial et al., 1998). However, a first *ts* mutant of *NDC1*, *ndc1-1*, did not show defects in nuclear protein import or NPC organization (Chial et al., 1998). Therefore, an essential function for Ndc1p in NPC assembly and function seemed unlikely. Recently, another *ts* allele of NDC1, *ndc1-39*, was described. *ndc1-39* strains show defective incorporation of GFP-Nup49 into NPCs at restrictive temperature (Lau et al., 2004). Combining this allele with a nic96 *ts* mutant resulted in synthetic growth defects, which was interpreted as an indication of Ndc1p function in NPC assembly. However, at restrictive temperature, the *ndc1-39* allele did not show abnormalities in NPC structure or nuclear import. NDC1 function in NPC assembly in yeast therefore remains uncertain, whereas its role in SPB insertion is well established (Chial et al., 1998; Winey et al., 1993).

Transmembrane nucleoporins could provide a link between the central NPC framework and the nuclear membrane by interacting with soluble nucleoporins or nucleoporin subcomplexes. In yeast, POM34 genetically interacts with members of the Nup84, Nup188, Nup170, and Nup82 subcomplexes (Miao et al., 2005) and POM152 with the Nup170 and Nup188 subcomplexes (Aitchison et al., 1995; Nehrbass et al., 1996). Our data show that NDC1 interacts with Nup53, a nucleoporin found to associate with Nup93, Nup205, Nup188, and Nup155. RNAi against Nup93 and Nup53 reduces mAb414 staining at NPCs (Hawryluk-Gara et al., 2005; Krull et al., 2004), indicating that they play a role in NPC assembly and depletion of Nup155 from in vitro assembly reactions disrupted both NPC and NE assembly (Franz et al., 2005). Our codepletion of NDC1 and Nup93 from mammalian cells not only led to a reduction in mAb414 staining but also had profound effects on nuclear morphology. These data support a model in which NDC1 provides a docking site for Nup53 and its associated nucleoporins at the nuclear membrane. Three observations suggest that this mechanism of Nup93-53 "subcomplex" recruitment might be conserved in *S. cerevisiae.* First, a two hybrid interaction was detected between yeast Nup53p and Ndc1p (Uetz et al., 2000; Ito et al., 2001). Second, overexpression of Nup53p induced the proliferation of intranuclear membranes that appear to contain both Ndc1p and Pom152p (Marelli et al., 2001). Third, yeast *ndc1-39* mutants show synthetic growth defects in combination with a mutant in the *S. cerevisiae* homolog of Nup93 (Lau et al., 2004).

Currently, it is largely unclear how different nucleoporins and different NPC subcomplexes interact during NPC assembly. Most nucleoporins are recruited as soluble proteins or subcomplexes from the cytosol. Our data suggest a model in which the interaction between NDC1 and Nup53 is crucial for the assembly of the Nup93-53 subcomplex into the NPC and the subsequent association of the majority of FG-containing nucleoporins that form the NPC channel. Further work will be

needed to incorporate other components, like the transmembrane nucleoporins GP210 and POM121, or other soluble nucleoporins into this scheme. Elucidating the assembly interaction network and the order of the protein contacts necessary to form the NPC in both yeasts and vertebrates remains a challenging task for the future.

Experimental Procedures

Computational Analysis

The accession numbers used to generate the phylogram of NDC1 homologs are listed in the Supplemental Data. Alignments were done with ClustalW (http://www.ebi.ac.uk/clustalw/). Prediction of transmembrane domains in hNDC1 was with TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).

Molecular Cloning and Protein Purification

Human NDC1 was cloned from HeLa cell cDNA into the BamHI/XhoI sites of pEGFP-N3. Full-length *X. laevis* NDC1 was from RZPD (clone IRAKp961H10169Q). A fragment (aa 361–521) was cloned into the vector pGEX-KG as a glutathione-S-transferase fusion protein. Recombinant proteins were expressed in *E. coli* strain BL21(DE3) and purified on GSH Sepharose by standard protocols.

For expression of full-length *Xenopus* POM121 and NDC1 in insect cells, POM121 and NDC1 were cloned into pFAST-BAC HTb with N-terminal His-6 and NDC1 into pFAST-BAC1 with a C-terminal His-6. Proteins were expressed in High Five cells (Invitrogen) per the manufacturer's instructions and purified on Ni-NTA-agarose by standard protocols in the presence of Chaps or Octylglucopyranoside (Calbiochem), the latter purified prior to use with a mixed bed ion exchange resin. After elution from the Ni-NTA-agarose in the presence of 2% detergent, the protein was reconstituted into liposomes.

Antibodies

Peptide-specific antibodies to human NDC1 (CQASAEHQKRLQQ FLEFKE), Nup107 (LLQASQDENFGNTTPRNQVC), POM121 (CGYSIT AEDLDLEKKASLQW), GP210 (CSDRKASPPSGLWSPAYASH), and Nup205 (TPSLSETVNRDGPRQDTQAC) were raised in rabbits by using the peptides as antigens. Affinity purification was performed by using SulfoLink (Pierce). Rabbit antibodies to *Xenopus* NDC1 were against GST-NDC1 (aa 361-521).

Antibodies against Nup53, Importin α, *Xenopus* POM121, GP210, and LBR have been described (Antonin et al., 2005; Hachet et al., 2004; Hawryluk-Gara et al., 2005, Ulbert et al., 2006). Commercial antibodies were anti-p62 (Transduction laboratories), anti-Nup93 (BD Pharmingen), mAb414 (BabCo), anti-LAP2 (Transduction laboratories), anti-PDI (StressGen Biotechnologies), anti-GFP Alexa Fluor 594 (Molecular Probes), anti-γ-tubulin (Sigma), anti-β-actin (Sigma), and anti-α-tubulin (Sigma).

For Fab-fragment generation, affinity-purified antibodies were digested with papain beads (Sigma) for 60 min at 37°C in PBS containing 1 mM EDTA and 10 mM cysteine, pH 7.4. The beads were then pelleted at 14,000 × g for 5 min. Antipain and PMSF were added to the supernatant to 2 μg/ml and 0.5 μM, respectively. The fraction was dialyzed to PBS and passed over protein A Sepharose (Amersham) to remove the Fc moiety and undigested IgGs. The purified Fab fragments exhibited an affinity similar to that of undigested IgGs when tested by serial dilution.

Cell Culture, RNAi, and Immunofluorescence

HeLa K, HeLa Y, and U2OS cells were kind gifts of D. Gerlich, A. Helenius, and W. Krek (ETH, Zurich), respectively.

For RNAi experiments, the following siRNAs (Qiagen) were used (sense sequences): NDC1-1 (5′-CUGCACCACAGUAUUUAUA-3′), NDC1-2 (5′-UCAGUGCCACCAUUAGUUA-3′), Nup107 (5′-GAAAGU GUAUUCGCAGUUA-3′), POM121 (5′-CAGUGGCAGUGGACAUU CA-3′), GP210 (5′-GAACCUCCAUUCACUACAA-3′), Nup93 (5′-GCGCUAAUUUACUACUGCA-3′, [Krull et al., 2004]), and control (5′-CUGUGCAAGCCGUUGUGUA-3′) at 35 nM final concentration unless indicated. Transfection of siRNAs using Oligofectamine (Invitrogen) was performed in six wells containing four to five coverslips for parallel analysis of RNAi samples by immunofluorescence with different antibodies. Cells were fixed with 1% PFA for 10 min and permeabilized with 1× detergent (0.1% Triton X-100/ 0.02% SDS in 1× PBS) for 5 min. Plasmid DNA was transfected with Fugene (Roche). Semipermeabilization of cells was with 40 μg/ml digitonin. Incubation with primary and fluorescently labeled secondary antibodies followed standard procedures (Erkmann et al., 2005).

For hNDC1 immunofluorescence using the hNDC1 antibody, cells were preextracted for 30 s in 1× detergent, fixed in 3% PFA, incubated in 50 mM glycine in PBS (pH 7.5) for 10 min, and again permeabilized for 5 min in 1× detergent. After washing with PBS, cells were treated with 6 M guanidinium HCl (pH 7.5) for 10 min, followed by three washing steps in PBS. After reduction with $NaBH_4$ (0.5 mg/ml in PBS), cells were blocked o/n in 2% BSA/PBS. Incubation with primary and secondary antibodies followed standard procedures.

Reconstitution of Proteoliposomes

To reconstitute xNDC1 and xPOM121 into proteoliposomes, the detergent eluate of the Ni-NTA column was processed as described previously for solublized membranes (Antonin et al., 2005).

GST-Pull-Down Assay

The GST-Nup53-1-300 and GST-Nup53-204-326 chimera were constructed by using a human fetal kidney cDNA library (BD Biosciences Clontech) as a PCR template. The resulting PCR products were inserted into the EcoR1 site of pGEX-6P-1 (Amersham). Subsequent expression and purification were as described for GST-Nup53-1-326 (Hawryluk-Gara et al., 2005). GST-pull-down assays were performed with extracts of purified rat liver nuclear envelopes as described (Hawryluk-Gara et al., 2005).

Miscellaneous

Vesicle isolation, nuclear assembly reactions, antibody inhibition experiments, depletion of membrane proteins, and membrane binding to chromatin were described (Antonin et al., 2005).

Triton X-114 phase separation was as described (Bordier, 1981). TEM was essentially done as in Macaulay and Forbes (1996) with 1.5% K-ferrocyanide added to the postfixation step.

To generate a resin for the depletion from *Xenopus* egg extracts, saturating amounts of affinity-purified antibodies were bound to protein A Sepharose (Pharmacia) and crosslinked with 10 mM dimethyl pimelimidate (Sigma).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at http://www.molecule.org/cgi/content/full/22/1/93/DC1/.

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