Nup53 Is Required for Nuclear Envelope and Nuclear Pore Complex Assembly

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Transport across the nuclear envelope (NE) is mediated by nuclear pore complexes (NPCs). These structures are composed of various subcomplexes of proteins that are each present in multiple copies and together establish the eightfold symmetry of the NPC. One evolutionarily conserved subcomplex of the NPC contains the nucleoporins Nup53 and Nup155. Using truncation analysis, we have defined regions of Nup53 that bind to neighboring nucleoporins as well as those domains that target Nup53 to the NPC *in vivo*. Using this information, we investigated the role of Nup53 in NE and NPC assembly using *Xenopus* egg extracts. We show that both events require Nup53. Importantly, the analysis of Nup53 fragments revealed that the assembly activity of Nup53 depleted extracts could be reconstituted using a region of Nup53 that binds specifically to its interacting partner Nup155. On the basis of these results, we propose that the formation of a Nup53–Nup155 complex plays a critical role in the processes of NPC and NE assembly.

INTRODUCTION

The nuclear envelope (NE) provides a physical barrier between the nucleus and cytoplasm and their unique metabolic tasks. The NE is defined by three morphologically distinct regions. The outer nuclear membrane (ONM) is continuous with the rough endoplasmic reticulum (ER) and contains a similar set of proteins (reviewed in Mattaj, 2004). The inner nuclear membrane (INM) lies adjacent to the nucleoplasm and contains a unique repertoire of proteins that, in part, mediate its interactions with the nuclear lamina and chromatin. Finally, at numerous locations along the NE, the ONM and INM are interrupted by pores where the INM and the ONM are bridged by a connecting membrane termed the pore membrane (POM) domain. Within these pores reside the nuclear pore complexes (NPCs), aqueous channels that provide portals for both passive and active transport of macromolecules.

An NPC contains ~30 nucleoporins (Nups), many of which are evolutionarily conserved in structure and function (Tran and Wente, 2006). In many cases these Nups are organized into subcomplexes that are present in multiple copies, and they are distributed around the central axis of the NPC, contributing to its characteristic eightfold symmetry. Different groups of Nups and their respective subcomplexes contribute to distinct structural components of the NPC. For example, Nup214/Nup84 are components of the cytoplasmic fibrils (Kraemer *et al.*, 1994; Bastos *et al.*, 1997), the Nup53/Nup155 complex is part of the central core (Marelli

et al., 1998), and Nup153 and Tpr contribute to the nucleoplasmic ring and fibrils (Krull *et al.*, 2004).

When metazoan cells enter mitosis, their NE is disassembled allowing for the mitotic spindle to access the condensed chromatin. Several models based on previous findings have been proposed to explain the mechanism by which this occurs (reviewed in Hetzer *et al.*, 2005). NE disassembly is accompanied by the phosphorylation of multiple NE proteins including the lamins and several Nups (Burke and Ellenberg, 2002, and references therein). There are several fates for the disassembled NE components. The disassembled lamins and Nups either become dispersed in the cytoplasm or, as is the case for integral membrane Nups, are redistributed within the mitotic ER network (Yang *et al.*, 1997; Daigle *et al.*, 2001). In addition, Nups belonging to the Nup107-160 complex have been shown to interact with kinetochores during M phase of the cell cycle (Belgareh *et al.*, 2001; Loiodice *et al.*, 2004).

In the final stages of telophase, the NE and NPCs reassemble around the chromosomes. This process has been extensively studied, but the mechanistic basis remains largely unclear. Reformation of the NE membrane is initiated by the binding of vesicles to the chromatin surface followed by fusion and the formation of a double membrane NE. Direct interactions between integral membrane proteins such as the lamin B receptor (LBR), NDC1, POM121, and chromatin, including interactions with DNA, are responsible for the initial docking of NE membrane vesicles to chromatin (Ulbert *et al.*, 2006). Membrane fusion steps resulting in a closed and continuous NE involve the AAA-ATPase p97 (Hetzer *et al.*, 2001) and GTP hydrolysis by Ran (Hetzer *et al.*, 2000; D'Angelo *et al.*, 2006). Concomitant with vesicle binding are the initial steps in NPC formation. Experiments examining cultured cells and using *in vitro* *Xenopus* NE assembly assays have established a general ordering of events that lead to NPC assembly. An early step in this process is recruitment of the Nup107-160 complex to chro-

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matin, thereby potentially serving as seeding sites for recruitment of subsequent Nups (Belgareh *et al.*, 2001; Harel *et al.*, 2003; Walther *et al.*, 2003). The Nup107-160 complex, together with POM121, whose depletion results in nuclei lacking both a continuous NE membrane and NPCs (Antonin *et al.*, 2005), have been proposed to participate in a checkpoint that functions to monitor NE and NPC formation. In this model, progression of NE vesicle fusion is inhibited until such time as the presence of POM121, presumably through interactions with the “pre-pore” Nup107-160 structure on the surface of chromatin, is detected (Antonin *et al.*, 2005).

More recently, other Nups have been implicated in NE and NPC assembly. For example, depletion of Nup155 from *in vitro* nuclear assembly reactions not only resulted in a block in accumulation of Nups at the nuclear periphery but also prevented the formation of a closed NE, halting the process at the vesicle docking stage (Franz *et al.*, 2005). This suggests that Nup155, like POM121 and the Nup107-160 complex, plays a critical role both in NE assembly and in the proposed NPC/NE assembly checkpoint. In addition, *in vitro* depletion of NDC1, a conserved transmembrane Nup, results in identical NE assembly defects as observed for POM121 and Nup155, where the NE membrane vesicles are docked to the chromatin but not fused (Mansfeld *et al.*, 2006). Although studies examining the depletion of NDC1 from cultured mammalian cells suggest a critical role for NDC1 on NPC assembly, they further imply that the protein is not absolutely essential for NE formation (Mansfeld *et al.*, 2006; Stavru *et al.*, 2006).

Both Nup155 and NDC1 form evolutionarily conserved interactions with the nucleoporin Nup53 (Hawryluk-Gara *et al.*, 2005; Mansfeld *et al.*, 2006). These interactions suggest Nup53 may function as a bridge point between the NPC core and the pore membrane, a strategic location to influence NE and NPC biogenesis. Consistent with these proposed roles for Nup53, RNA interference (RNAi)-mediated depletion of *Caenorhabditis elegans* Nup53 produces defective postmitotic nuclear formation and embryonic lethality (Galy *et al.*, 2003). Similarly, depletion of human Nup53 results in an inability of interacting Nups (namely, Nup93, Nup205, and Nup155) to incorporate into NPCs (Hawryluk-Gara *et al.*, 2005). Nup53 has also been linked to NE biogenesis and NPC formation in yeast. Overproduction of yeast Nup53p induces a massive proliferation of the inner nuclear membrane and the formation of incompletely assembled NPCs (Marelli *et al.*, 2001).

In this study, we investigated the role of vertebrate Nup53 in NPC and NE formation. In our initial experiments, we identified the regions of Nup53 necessary for its interactions with the neighboring nucleoporins Nup93, Nup155, and the membrane protein NDC1. These experiments established that Nup53 interacts with its binding partners through distinct regions. This information was applied to the analysis of the functions of these regions in mediating the NPC association of Nup53 in HeLa cells and their role in NPC assembly and NE formation by using the *Xenopus* nuclear reconstitution assay. Using this latter assay, we showed that Nup53 is required for both NE and NPC assembly. Depletion of Nup53 results in docking of membrane vesicles to chromatin but a block in NE membrane fusion and NPC formation, reminiscent of the defect observed after depletion of Nup155, POM121, or NDC1. Our data further suggest that the interaction between Nup53 and Nup155 is critical for proper NPC and NE assembly.

MATERIALS AND METHODS

Antibodies

Antibodies against Nup53 (Hawryluk-Gara *et al.*, 2005), Nup155 (Franz *et al.*, 2005), and Nup205 (Krull *et al.*, 2004; Mansfeld *et al.*, 2006) have been described previously. Other antibodies used were mAb414 (BAbCO, Richmond, CA), anti-glutathione transferase (GST) (Abcam, Cambridge, MA), and fluorescently labeled secondary antibodies used for immunofluorescence (Jackson ImmunoResearch Laboratories, West Grove, PA).

GST-Pulldown Assay

GST-Nup53 chimeras were constructed precisely as described previously (Mansfeld *et al.*, 2006). Expression and purification have been previously described in detail (Hawryluk-Gara *et al.*, 2005). Purified rat liver nuclear envelope extracts were used in subsequent GST-pulldown assays as described previously (Hawryluk-Gara *et al.*, 2005).

Cell Imaging

Green fluorescent protein (GFP)-Nup53 chimeras were constructed by using a human fetal kidney cDNA library (Clontech, Mountain View, CA) as a polymerase chain reaction (PCR) template. PCR products were inserted into the EcoRI site of pEGFP-C1 (Clontech). HeLa S3 cells at 30% confluence in a 100-mm culture dish were transfected with 2.0 μ g of pEGFP-Nup53 chimeras by using TransIT-HeLaMONSTER transfection kit (Fisher Scientific, Nepean, ON, Canada) as instructed by the manufacturer. After 24 h of transient expression, the transfected cells growing on coverslips were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature. Duplicate coverslips were permeabilized with 0.1% Triton X-100 in PBS before formaldehyde fixation. Nuclear DNA was visualized with the DNA-specific Hoechst No. 33342 Dye (Sigma-Aldrich, Oakville, ON, Canada). For subsequent visualization of all immunofluorescent images, coverslips were mounted in Fluoromount G mounting medium (Electron Microscopy Sciences, Hatfield, PA) before confocal microscopy. All microscopic images were obtained on an Axiovert 100M coupled with a Zeiss LSM510 laser scanning system (Carl Zeiss, Jena, Germany) by using a 63 $\times$ plan-apochromat objective.

Xenopus Egg Extract Preparation

Cytosol from *Xenopus* eggs was prepared as described previously (Hartl *et al.*, 1994) with the following changes. After the first low-speed centrifugation, the crude low-speed extract was centrifuged twice at 225,000 $\times g$ for 40 min at 4°C to obtain the cytosol as a clear supernatant. Floated membranes were prepared as described previously (Wilson and Newport, 1988), and the two lowest density membrane fractions were combined. Sperm heads were prepared from *Xenopus* testis (Gurdon, 1976).

For NE assembly reactions, 10 μ l of cytosol was incubated with 0.5 μ l of sperm heads (3000 sperm heads/ μ l) for 10 min at 20°C to allow for chromatin decondensation. The reaction was then continued by the addition of 0.5 μ l of floated membranes, 0.8 μ l of energy mix (50 mM ATP, 500 mM creatine phosphate, 10 mg/ml creatine kinase), and 0.2 μ l of oyster glycogen (20 mg/ml USB; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and incubation at 20°C for 2 h. Membrane labeling with DiI C18, and fixation at the end of the reaction was carried out as described previously (Antonin *et al.*, 2005). Samples to be processed for immunofluorescence were fixed in freshly prepared 3.7% paraformaldehyde in PBS. Samples were subsequently centrifuged through a cushion of 30% (wt/vol) sucrose in PBS onto poly-L-lysine-coated coverslips. Samples were either further processed for immunofluorescence using methods described previously (Antonin *et al.*, 2005), or they were mounted directly for fluorescence microscopy by using Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA). Images were acquired using a widefield optical sectioning deconvolution microscope (Delta Vision; Applied Precision, Issaquah, WA). Most images were acquired with 100 $\times$ 1.4 Plan-apochromat objective. Three-dimensional images were acquired and deconvolved. Single optical sections or maximum intensity projections were subsequently used.

Expression of Nup53 Full-Length and Nup53 Fragment Proteins

Xenopus Nup53 full-length, Human Nup53 full-length, and Human Nup53 truncations were expressed in *Escherichia coli* BL21 DE3 strain, as GST fusion proteins. They were subsequently purified by GST affinity chromatography by using immobilized glutathione (Glutathione Sepharose Fast Flow; GE Healthcare), and the GST moiety was cleaved by PreScission protease. For use in the NE assembly reactions, purified proteins were dialyzed in S250 buffer (250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, and 20 mM HEPES, pH 7.5).

Nup53 Depletion from Xenopus Cytosol

Saturating amounts of nonimmune rabbit IgG or rabbit *Xenopus* Nup53 antibody sera were cross-linked to protein A-Sepharose (Pharmacia, Uppsala,

Sweden) in the presence of 10 mM dimethylpimelimidate (Sigma-Aldrich) to produce resins for mock or Nup53 depletion from *Xenopus egg* extracts. High-speed cytosol was incubated with the antibody column for two rounds of 30 min each, at 4°C. Cytosol was used directly for NE assembly reactions or frozen with 3% (wt/vol) glycerol.

Transmission Electron Microscopy (TEM)

In vitro nuclear assembly was performed as described above, scaled up to 60 μ l and processed for TEM as described previously (Franz *et al.*, 2005).

RESULTS

Mapping the Interactions between Nup53 and Neighboring Nups

Vertebrate Nup53 is tightly associated with the NE membrane where it seems to interact, either directly or indirectly, with several neighboring Nups, including Nup93, Nup155, and the pore membrane protein NDC1 (Hawryluk-Gara *et al.*, 2005; Mansfeld *et al.*, 2006). Here, it is strategically positioned to function in mediating the interactions between the pore membrane, and the core structures of the NPC and potentially participate in NE and NPC assembly. To more clearly define the function of Nup53 in these processes, we have mapped the regions of Nup53 that mediate its interactions with other Nups. For these experiments we have used an in vitro binding assay using recombinant forms of Nup53 and Nups derived from extracts of rat liver NEs. Using this assay we have previously reconstituted interactions between full-length recombinant Nup53 and rat liver NE-derived Nup93, Nup155, and NDC1 [(Hawryluk-Gara *et al.*, 2005); Nup53¹⁻³²⁶ in Figure 2A]. Similar binding experiments were performed with a series of NH₂- and COOH-terminal deletions of Nup53. These truncated forms of Nup53 were synthesized in *E. coli* as GST fusions and bound to glutathione-Sepharose. The bead-bound fusions were then incubated with rat liver NE extract containing solubilized Nups and the bound fractions analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. As predicted, full-length Nup53 (GST-Nup53¹⁻³²⁶) bound Nup93, Nup155, and NDC1, whereas no binding was detected to GST alone. Removal of the last 26 amino acid residues of Nup53 (GST-Nup53¹⁻³⁰⁰), which contains a predicted amphipathic helix, eliminates NDC1 binding (Mansfeld *et al.*, 2006). However, the GST-Nup53¹⁻³⁰⁰ fusion continues to bind Nup93 and Nup155 (Figure 2A). Surprisingly, Nup155 was observed in increased amounts in the Nup53¹⁻³⁰⁰ bound fraction when compared with full-length Nup53¹⁻³²⁶ (Figure 2A). Further truncating the COOH terminus to amino acid residues 203 and 143 (Nup53¹⁻²⁰³ and Nup53¹⁻¹⁴³, respectively) abolished Nup155 binding but not the ability of these GST fusions to interact with Nup93, albeit with reduced efficiency compared with full-length Nup53. Binding to these latter Nups was finally lost when the COOH-terminal truncations extended to residue 83 (Nup53¹⁻⁸³).

Similar experiments were performed with NH₂-terminal Nup53 truncations. As shown in Figure 2B, deletion of the NH₂-terminal 83 residues of Nup53 (Nup53⁸⁴⁻³²⁶) did not affect its ability to bind Nup93, Nup155, and NDC1. However, further deleting residues 1-203 (Nup53²⁰⁴⁻³²⁶) eliminated the binding to all but NDC1. As predicted, removal of residues 1-166 (Nup53¹⁶⁷⁻³²⁶) resulted in an inability of Nup53 to interact with Nup93, but binding to Nup155 and NDC1 was not altered.

Our data were consistent with a model in which Nup93 binds to a region within amino acid residues 1-143 and NDC1 binds within residues 204-326. In contrast, the region of Nup53 required for binding Nup155 seemed to lie more centrally in Nup53. Consistent with this idea, we detected

	Interacting nups			Localization	Rescue of NE/NPC assembly defect
	Nup93	Nup155	Ndc1		
1 ————— 326	+	+	+	NR	yes
1 ————— 300	+	+	-	NR/C	yes
1 ————— 203	+	-	-	NR/C	no
1 ————— 143	+	-	-	NR/C	
1 ————— 83	-	-	-	N/C	
167 ————— 326	-	+	+	NR/C	
84 ————— 326	+	+	+	NR/C	
204 ————— 326	-	-	+	NR/C	no
167 ————— 300	-	+	-	NR/C	yes

Figure 1. Summary of human Nup53 truncation characterization. Compilation of Nup53 truncation interaction, localization, and depletion phenotype rescue data. NH₂- and COOH-terminal human Nup53 truncations were constructed based on regions that are well conserved among orthologues with considerations of predicted secondary structure. Helical stretches from amino acid residues 181–193, 216–226, and 314–326 are represented by light gray bars. Interaction data were obtained either by silver staining, Western blot, or both. Interactions detected (+) or not detected (–) between indicated Nups and Nup53 truncations are shown. Localization was scored as either nuclear rim (NR), nuclear (N), or cytoplasmic (C). The ability of the various Nup53 truncations to rescue the Nup53 depletion phenotype was monitored by DiIC₁₈ NE membrane staining.

binding of Nup155 to Nup53¹⁶⁷⁻³⁰⁰ (Figure 2C). This fragment, however, failed to bind Nup93 and NDC1. These data, coupled with the observations that recombinant Nup53 directly interacts with both recombinant Nup93 (Hawryluk-Gara *et al.*, 2005) and Nup155 (data not shown) but that Nup155 and Nup93 do not seem to directly interact with each other, support the conclusion that Nup93 and Nup155 bind to unique sites on Nup53. Cumulatively, our data support a model in which the binding regions for Nup93, Nup155, and NDC1 in Nup53 lie primarily within residues 1–143, 167–300, and 204–326, respectively (Figure 1).

In Vivo Localization of Nup53 Truncations

Truncation mutants of Nup53 similar to those used to define its interactions with binding partners were also used to construct GFP fusion genes for expression in mammalian cells. These experiments were designed to define regions of Nup53 that play a critical role in mediating its NPC association and to correlate important targeting regions with those required for specific Nup interactions. Various GFP-Nup53 chimeric genes were introduced into HeLa cells. The resulting GFP-tagged Nup53 truncations were then examined by confocal microscopy 36 h after transfection. Importantly, Western blot analysis of extracts derived from the transfected HeLa cells revealed that each of GFP-Nup53 chimeras exhibited a molecular mass consistent with that predicted from its sequence (data not shown). As shown in Figure 3A, full-length GFP-Nup53 (Nup53¹⁻³²⁶) localized to the nuclear rim in a characteristic pattern exhibited by Nups, including endogenous Nup53 (Hawryluk-Gara *et al.*, 2005). This pattern was observed in cells exhibiting a range of expression levels within the transfected cultures. Similarly, GFP-Nup53¹⁻³⁰⁰, which lacks the COOH-terminal region required for NDC1 binding but contains the Nup93 and Nup155 binding sites, was also visible at the nuclear rim. However, its ability to concentrate at the NE seemed to be compromised as GFP-Nup53¹⁻³⁰⁰ was also detected diffusely distrib-

Figure 2. Nup53 domain interaction analysis using GST-pulldown assays. (A–C) Purified recombinant GST, GST-Nup53, or various GST-Nup53 truncations immobilized on glutathione-Sepharose 4B beads were incubated with extracts from purified rat liver NEs (+) or buffers alone (–). After binding and washing, bead-bound proteins were eluted, separated by SDS-PAGE, and detected with either silver staining (SS) or Western blotting (WB) by using antibodies against the indicated Nups. Positions of GST-fusions (white asterisks), relevant Nups (black asterisks and name), and mass markers are indicated in kilodaltons.

uted throughout the cytoplasm and in the nucleoplasm. Further COOH-terminal deletion mutants, including GFP-Nup53¹⁻²⁰³, GFP-Nup53¹⁻¹⁴³, and GFP-Nup53¹⁻⁸³ failed to visibly accumulate at the NE, and they were distributed diffusely throughout both the nucleus and the cytoplasm. Because both GFP-Nup53¹⁻²⁰³ and GFP-Nup53¹⁻¹⁴³ fusions contain the Nup93 binding region, we tested whether these fusions might partially associate with NPC but that this binding might be masked by nuclear and cytoplasmic levels of the fusions. Indeed, both the GFP-Nup53¹⁻²⁰³ and GFP-Nup53¹⁻¹⁴³ fusions, but not the GFP-Nup53¹⁻⁸³, could be detected at the NE following mild extraction with Triton X-100 to release the soluble pool of fusion protein (Figure 3A). Because the cellular levels of these fusion proteins were less than GFP-Nup53 (data not shown), we conclude that increased cytoplasmic and nucleoplasmic signal was not related to their overproduction but rather their reduced ability to target to or associate with binding sites at the NPC.

The localization patterns of several NH₂-terminal deletion mutants were also examined (Figure 3B). Nup53⁸⁴⁻³²⁶, which contains all of the identified Nup binding regions, was concentrated primarily at the NE, seeming similar to the full-length protein but with a slightly higher cytoplasmic signal.

Figure 3. In vivo localization of enhanced green fluorescent protein (EGFP)-Nup53 truncations. (A and B) Plasmids encoding either full-length Nup53 or the indicated truncations fused to EGFP were transfected into HeLa cells. Cellular distribution of the indicated GFP chimeras (GFP) and merged images of the GFP fusions and nuclear DNA were detected by staining with the DNA binding dye Hoechst (GFP-DNA) was determined by fluorescence microscopy following 36 h of transient expression. The transfected cells growing on coverslips were processed for fluorescence microscopy either by formaldehyde fixation followed by Triton X-100 permeabilization (F/T) or Triton X-100 permeabilization before formaldehyde fixation (T/F). Bar, 10 μ m.

On further deletion of the NH₂ terminus to residues 167 or 204, the resulting fusions (GFP-Nup53¹⁶⁷⁻³²⁶ and GFP-Nup53²⁰⁴⁻³²⁶) showed an increase in cytoplasmic signal and, in the case of GFP-Nup53²⁰⁴⁻³²⁶, no NE accumulation. Similarly, expression of GFP-Nup53¹⁶⁷⁻³⁰⁰ (containing the Nup155 binding region) revealed that this fusion protein is diffusely distributed throughout the nucleus and cytoplasm (Figure 3B). However, as was seen with several of the COOH-terminal truncations, extraction with Triton X-100 revealed that each of these fusions bind to the NE (Figure 3B). Cumulatively, these results, together with those obtained using the COOH-terminal deletions, suggest that each of the three Nup binding regions of Nup53 is capable of binding the NPC, presumably through interactions with its cognate binding partner (summarized in Figure 1). Together, each of the Nup binding regions contributes to optimal NPC binding. Of note, the lamin B binding described previously for Nup53 (Hawryluk-Gara *et al.*, 2005) does not seem to contribute to its NE association, because a fragment of Nup53 that bound only lamin B in vitro did not concentrate at the nuclear periphery (data not shown).

Removal of Nup53 Leads to a Block in NPC Formation and NE Membrane Fusion

Observations made across a wide range of species suggest that human Nup53, and its counterparts are positioned at or near the pore membrane where it interacts with components of the NPC, including Nup155 (Marelli *et al.*, 1998; Marelli *et al.*, 2001; Hawryluk-Gara *et al.*, 2005). This model places Nup53 at a location where it might play a role in NPC assembly. Consistent with this idea, RNAi studies in *C. elegans* have shown that Nup53 is required for nuclear assembly after mitosis (Galy *et al.*, 2003).

To study the function of Nup53 in more detail, we took advantage of the *Xenopus* nuclear reconstitution system. The *Xenopus* in vitro system recapitulates pronuclear assembly and mimics the events of postmitotic NE assembly (Forbes *et al.*, 1983; Lohka and Masui, 1983; Newport, 1987). Mock and Nup53-immunodepleted cytosolic fractions were prepared. After two rounds of depletion, >95% of soluble Nup53 was removed (Figure 4A). Like Nup155 (Franz *et al.*, 2005), Nup53 was also present in the total membrane fraction, and it could not be easily extracted by salt washes (data not shown). This suggested that like Nup155, Nup53 might be pelleted with membranes as part of a large but soluble membrane complex. Floating the total membrane fraction through a sucrose step gradient (Wilson and Newport, 1988) and using the lowest density membrane fraction for the assembly reaction allowed us to prepare membranes devoid of Nup53 (data not shown). The immunodepleted cytosol was then mixed with sperm chromatin, floated membrane vesicles, and an ATP regeneration system to assemble nuclei in vitro. Nuclei were allowed to form for 2 h at 20°C. Membranes were immediately stained with the lipophilic dye DilC₁₈ before fixation. Nuclei assembled using mock-depleted extracts were able to form a smooth and continuous NE membrane with an efficiency of >80% (Figure 4E). These mock-depleted nuclei were competent for nuclear import of fluorescently labeled bovine serum albumin (BSA)-NLS (Figure 6A), and chromatin decondensation was observed. Furthermore, mAb414-reactive Nups (Nup62, Nup153, Nup214, and Nup358) were detected on the nuclear rim (Figure 5B, mock). In contrast, when Nup53 was depleted from cytosol, membrane vesicles associated with chromatin, but they failed to form a smooth and continuous NE (Figure 4D). At the same time, due to the inability to form a closed NE, the sperm chromatin remained condensed and mAb414 staining was dramatically reduced (Figure 5B, depleted), suggesting a block to NPC formation.

To confirm the specificity of the Nup53 depletion phenotype, full-length human Nup53 was purified under native conditions and used to complement the depleted cytosolic extracts. The purified protein was added back to roughly endogenous levels (Figure 4B; data not shown). When complemented extracts were used in an assembly reaction, nuclei with closed NEs were formed (Figure 4D), mAb414 staining was restored (Figure 5B), and nuclei were competent to import fluorescently labeled BSA-NLS (Figure 6A). The efficiency of both the mock and full-length human Nup53 rescue experiments was quantified. In mock-depleted extracts, >80% of sperm templates were surrounded by a closed NE. This value was dramatically reduced to ~20% in the Nup53-depleted extracts, and it was restored to ~80% upon addition of the full-length Nup53 protein (Figure 4E). The same efficiency of rescue was observed after addition of full-length *Xenopus* Nup53 (data not shown).

The incorporation of recombinant Nup53 into the reformed nuclei was confirmed by immunofluorescence. Due

Figure 4. Depletion of Nup53 from cytosol blocks formation of a closed NE. (A) Amount of xNup53 in mock-depleted (Mock) and Nup53-depleted (Δ Nup53) cytosolic extracts (equal volumes) were compared by SDS-PAGE and western blotting by using α -Nup53 antibodies. Varying amounts of untreated cytosol were loaded for comparison. A cross-reacting band indicated with an asterisk serves as a loading control. (B) For add-back experiments, amounts of recombinant proteins corresponding to approximately endogenous levels of *Xenopus* Nup53 (extract) were used. Purified full-length Nup53¹⁻³²⁶ and truncation fragments (Nup53¹⁻³⁰⁰, Nup53¹⁻²⁰³, Nup53²⁰⁴⁻³²⁶, and Nup53¹⁶⁷⁻³⁰⁰) were analyzed by SDS-PAGE and Western blotting by using anti-Nup53 antibodies. Mass markers are indicated in kilodaltons. (C) Equal amounts of mock-depleted (Mock) or Nup53-depleted (Δ Nup53) cytosolic extracts were analyzed by SDS-PAGE and Western blotting by using anti-Nup155, anti-Nup93, and anti-Nup205 antibodies. (D) Nuclei assembled in vitro in Mock, Δ Nup53 extracts, or Δ Nup53 extracts after addition of full-length Nup53. Membranes are visualized by using the membrane dye DilC₁₈ (red). Chromatin was stained with 4,6-diamidino-2-phenylindole (DAPI) (blue). Bar, 10 μ m. (E) Quantitation of closed NE formation of experiments performed as in D. More than 100 randomly chosen chromatin substrates per reaction were counted. Average of three independent experiments are shown, error bars represent the SD over the three experiments.

to technical difficulties, we were unable to successfully use the anti-hNup53 antibodies for immunofluorescence analysis of the in vitro assembled nuclei. For this reason, the full-length GST-Nup53 protein was also used in the add-back reaction to confirm the incorporation of the Nup53 at the NE. Using anti-GST antibodies, we were able to visualize the incorporation of GST-Nup53 in the reformed NE by immunofluorescence (Figure 6B).

Figure 5. Depletion of Nup53 from cytosol blocks NPC assembly. (A) Transmission electron microscopy of nuclear assembly reactions of Mock, Δ Nup53 extracts, or Δ Nup53 extracts after addition of full-length Nup53. Black bar, 2 μ m; white bar, 210 nm. (B) Nuclear assembly reactions of Mock, Δ Nup53 extracts, or Δ Nup53 extracts after addition of full-length Nup53. Reactions were analyzed by immunofluorescence against mAb414 (green). Chromatin was stained with DAPI (blue). Bar, 10 μ m.

We have also analyzed the in vitro-reconstituted nuclei by TEM. In mock-depleted extracts, nuclei with a continuous double bilayer perforated by NPCs were observed (Figure 5A). On Nup53 depletion, docked vesicles were visualized on the chromatin surface together with short regions of fused membranes but neither complete membrane fusion nor NPCs were detected. Again, both membrane fusion and NPC insertion were restored upon addition of recombinant human Nup53 (Figure 5A).

The Nup53–Nup155 Interaction Is Required for Nuclear Envelope Formation

Many of the characteristics of Nup53 depletion phenotype are similar to those observed previously upon removal of

Figure 6. Characterization of in vitro-assembled nuclei. (A) Nuclei assembled in vitro in Mock, Δ Nup53 extracts, or Δ Nup53 extracts after addition of full-length Nup53. Nuclear import reaction was performed using fluorescein isothiocyanate (FITC)-labeled BSA-NLS 20–30 min before fixation. Membranes are visualized by using the membrane dye DiIC₁₈ (red). FITC BSA-NLS (green). Chromatin was stained with DAPI (blue). Bar, 10 μ m. (B) Nuclei assembled in vitro in Mock, Δ Nup53 extracts, or Δ Nup53 extracts after addition of GST-Nup53. Localization of the recombinant protein was analyzed by immunofluorescence using α -GST (red). Chromatin was stained with DAPI (blue).

Nup155 (Franz *et al.*, 2005). Because Nup53 has been shown to interact with Nup155, and Nup93, in both yeast and vertebrate cells (Marelli *et al.*, 1998; Hawryluk-Gara *et al.*, 2005), it is possible that the observed Nup53 depletion phenotype arises from the disruption of a single function linked to one or more of these interactions. These interactions are likely established or stabilized during reassembly of the NPC, because we observed no codepletion of Nup93, Nup155, or Nup205 upon removal of Nup53 from the *Xenopus* extract (Figure 4C). Moreover, the recruitment of Nup93, Nup205, and Nup155 to sperm chromatin is dependent on Nup53. Depletion of Nup53 from the soluble extracts inhibits the association of these Nups with the assembling NEs, and this can be reversed by the addition of recombinant full-length Nup53 (Supplemental Figure S1). In contrast, depletion of Nup53 did not affect the chromatin association of Mel-28 (Supplemental Figure S2), a protein that, together with other Nup107 complex components, forms an early NPC assembly intermediate (Franz *et al.*, 2007).

To study the functions of these Nup interactions in more detail and understand their role in NE and NPC formation, we tested the ability of various truncation mutants of Nup53 to reconstitute NE assembly in the Nup53 depleted *Xenopus* extracts. Truncations were chosen based on the characterization of their Nup binding properties and their targeting to the NPC as defined above. The Nup53¹⁻³⁰⁰, Nup53¹⁻²⁰³, Nup53²⁰⁴⁻³²⁶, and Nup53¹⁶⁷⁻³⁰⁰ truncations were selected for analysis, because each of these fragments was capable of interacting with a specific subset of Nups, thereby allowing us to dissect which interaction domains are necessary for the formation of a closed and functional NE. As human Nup53 is 72% identical to its *Xenopus* counterpart (data not shown), the human versions were used to supplement the depleted *Xenopus* extracts.

Nup53 deletion fragments were purified under native conditions and added to the Nup53-depleted cytosolic ex-

Figure 7. Nup53 fragments that interact with NDC1 or Nup93 alone are not sufficient to restore NE formation. (A) Nuclei assembled in vitro in Mock, ΔNup53 extracts, or ΔNup53 extracts after addition of Nup53¹⁻³⁰⁰, Nup53¹⁻²⁰³, and Nup53²⁰⁴⁻³²⁶. Membranes are visualized by using the membrane dye DilC₁₈ (red). Chromatin was stained with DAPI (blue). Bar, 10 μm. (B) Quantitation of closed NE formation of experiments performed as described in A, including the truncation fragment Nup53¹⁶⁷⁻³⁰⁰. More than 100 randomly chosen chromatin substrates per reaction were counted. Average of three independent experiments is shown. Error bars represent the SD over the three experiments.

tracts. Similar to the addition of full-length Nup53, the purified fragments were added in amounts approximately equal to endogenous levels (Figure 4B). First, we asked whether the Nup53 interaction with NDC1 is required for the formation of a closed NE. When depleted extracts were supplemented with Nup53¹⁻³⁰⁰, the fragment lacking the NDC1 interaction domain, and used in an assembly reaction, nuclei with closed NEs were formed (Figure 7A) at a similar frequency to that seen with full-length Nup53 (Figures 4E). On the basis of these results, it seems that the COOH-terminal region of Nup53, previously shown to interact closely with the pore membrane, is not essential for Nup53 recruitment to membranes and NE and NPC assembly (Marelli *et al.*, 2001; Hawryluk-Gara *et al.*, 2005; Mansfeld *et al.*, 2006). Consistent with this idea, a Nup53 fragment containing the NDC1 interaction domain, Nup53²⁰⁴⁻³²⁶, failed to rescue the NE and NPC assembly activity of the Nup53-depleted extracts (Figure 7, A and B), further suggesting that interaction between NDC1 and Nup53 is neither required nor sufficient to rescue NE and NPC formation.

Importantly, the Nup53¹⁻³⁰⁰ fragment, which contains both the Nup155 and the Nup93 binding regions (Figure 2A), was capable of replacing Nup53 in the NE reconstitution assays. We next tested whether the domain sufficient for interaction with Nup93 could rescue the depletion phenotype. Supplementing Nup53-depleted extracts with Nup53¹⁻

Figure 8. A Nup53 fragment that interacts with Nup155 is sufficient to restore NE formation. (A) Nuclei assembled in vitro in Mock, ΔNup53 extracts, or ΔNup53 extracts after addition of full-length Nup53, and the Nup53¹⁶⁷⁻³⁰⁰ fragment. Maximum intensity projections of three-dimensional stack are shown. Membranes are visualized by using the membrane dye DilC₁₈ (red). Chromatin was stained with DAPI (blue). (B) Nuclei assembled as described in A were analyzed by immunofluorescence by using Nup155 antibodies (red). Chromatin was stained with DAPI (blue). (C) Nuclei assembled as in A and B were analyzed by immunofluorescence using Nup93 antibodies (red). Chromatin was stained with DAPI (blue). Bar, 10 μm.

203 did not rescue the depletion phenotype. Membranes associated with chromatin but did not fuse to form a closed NE and the percentage of the nuclei that did have a closed NE was similar to that observed with the depleted extracts (Figure 7, A and B).

Finally, we examined whether the Nup155 interacting region of Nup53 alone was sufficient to restore proper NE and NPC assembly by supplementing depleted extracts with Nup53¹⁶⁷⁻³⁰⁰. This construct binds Nup155 but neither NDC1 nor Nup93. When Nup53¹⁶⁷⁻³⁰⁰ was added to the depleted extracts, nuclei were formed that had both a closed NE and NPCs as judged by membrane staining with DilC₁₈ (Figure 8A), by mAb414 staining (data not shown), and electron microscopy analysis (Supplemental Figure S3). Furthermore, quantification revealed that 60–80% of sperm templates formed closed NEs (Figure 7B). Importantly, the nuclei that did have a closed nuclear envelope showed similar chromatin decondensation and overall nuclear size to the

nuclei rescued with the full-length Nup53. The Nup53¹⁶⁷⁻³⁰⁰ reconstituted nuclei were also able to recruit Nup155 as judged by immunofluorescence (Figure 8B). In contrast, these nuclei failed to efficiently accumulate Nup93, showing lower levels of NE association than the mock-depleted samples (Figure 8C). Together, these data suggest that the interaction between Nup53 and Nup155 is sufficient to reverse the depletion phenotype and results in the formation of a closed NE.

DISCUSSION

Using both *in vivo* and *in vitro* approaches, we have shown that Nup53 plays an essential role in nuclear pore complex assembly and nuclear envelope formation. Through our analysis, we have identified the regions of Nup53 that are necessary for its interactions with its neighbors Nup93, Nup155, and the transmembrane protein NDC1, and we have defined the roles of these regions of Nup53 in mediating its targeting to the nuclear rim *in vivo* and its function in NPC assembly and NE formation *in vitro*. Each of the nup interacting regions of Nup53 can associate with the NPC *in vivo*. However, all three regions are required for the efficient targeting of Nup53 to the NPC. The involvement of NDC1 in Nup53 localization is consistent with our previous conclusion that the interaction of Nup53 subcomplex with the membrane is mediated by NDC1 via its association with the COOH-terminal region of Nup53 (Mansfeld *et al.*, 2006). In addition, we have shown that the region of Nup53 required for binding to Nup155 plays a critical role in NPC and NE assembly in the *Xenopus* system, suggesting the formation of a Nup53/Nup155 complex is a key step in this process.

The NPC is a multisubunit complex composed of several structurally defined components positioned at specific locations within the NPC (Schwartz, 2005; Fahrenkrog, 2006). A major challenge in the field is to develop a model for how individual Nups within the subcomplexes are locally organized and how, in turn, these subcomplexes interact with one another and the pore membrane. This portrait of the NPC is, however, only the initial step in our understanding of the complex and dynamic steps that lead to its assembly. As a step toward addressing this process, we have focused on defining interactions between Nup53 and several of its evolutionarily conserved binding partners, including Nup93 and Nup155. This subcomplex is a component of the NPC core, and interactions of two members of this subcomplex, Nup53 and Nup155, with pore membrane proteins [(Mansfeld *et al.*, 2006); Figure 2; Mitchell and Wozniak, unpublished data] suggest it lies adjacent to the membrane.

In this study, we assessed the interactions between members of the Nup53-containing subcomplex using human Nup53 truncations. Minimal binding regions of Nup53 were defined that are required for maintaining interactions with its known partners. Interestingly, as shown in Figure 2, these regions are separable and not entirely interdependent. Nup93 binds an NH₂-terminal segment (amino acids residues 1-143) of Nup53, likely together with its binding partner Nup205 as this nup was universally detected in fractions containing Nup93 (data not shown). Nup155 interacts specifically with a more central region (residues 167-300) of Nup53, whereas NDC1 binds a COOH-terminal segment that contains an amphipathic helix located in the last 26 amino acid residues of Nup53. It is noteworthy that the Nup53¹⁻³⁰⁰ fragment, lacking the NDC1 interaction domain, binds better to Nup155 than full-length Nup53. We interpret these data to suggest that although the Nup155 and NDC1 binding sites on Nup53 are distinct, the binding of Nup53 to

the two partner Nups is not entirely independent. One possible explanation for the increased binding of the Nup53¹⁻³⁰⁰ fragment to Nup155 is that the COOH-terminal 26-amino acid residues of Nup53 inhibits binding to Nup155. *In vivo* this could function to inhibit their interaction until the COOH-terminal region of Nup53 engages the membrane, binds NDC1, or interacts with an as yet unidentified protein. In our *in vitro* binding assays these factors would be absent (in the case of membrane) or potentially present in substoichiometric amounts (i.e., free NDC1), thus leading to the reduced binding of Nup155 to full-length Nup53 compared with the Nup53¹⁻³⁰⁰ fragment. This model is attractive as it is consistent both with the observation that Nup155 is not removed from Nup53-depleted soluble extracts (Figure 4C) and with the apparent interdependence of Nup53 and Nup155 for incorporation into the assembling NE and NPC (Franz *et al.*, 2005; Supplemental Figure S1).

In vivo analysis of the Nup53 truncation mutants showed that each of the interaction domains of Nup53 with its neighboring nups (Nup93, Nup155, and NDC1) play a role in the efficient incorporation of Nup53 into the NPC. Individually, these separate regions can bind the NPC to some extent but with reduced efficiency relative to the wild-type protein. This would suggest that the cumulative interactions of Nup53 with its neighbors, rather than one specific binding event, are required for the stable association of Nup53 with the NPC (Figure 3). Studying the dynamics of GFP-Nup53 truncation mutants in more detail in live vertebrate cells and using molecular dynamics simulation approaches, as used to study the binding of importin- β to FG repeat nucleoporins (Isgro and Schulten, 2005), could shed more light on the interactions between the individual members of the Nup53 subcomplex.

Our data suggest that Nup53 plays an important role in nuclear assembly. Previously, depletion of the protein from mammalian cultured cells by RNAi (Hawryluk-Gara *et al.*, 2005) was shown to cause a severe defect in nuclear morphology and reduction of accumulation of Nup93, Nup155, and Nup205 at the nuclear rim suggestive of defects in NPC assembly. Moreover, depletion of the *C. elegans* counterpart of Nup53 caused an embryonic lethal phenotype along with a severe block to nuclear formation after mitosis (Galy *et al.*, 2003). As in the *C. elegans* case, upon depletion of Nup53 from *Xenopus* extracts, we observed a strong inhibition of NE formation. Membrane vesicles were bound to the chromatin surface, but they did not fuse to form a closed NE and no NPC assembly was detected either by immunofluorescence or electron microscopy. Of the nucleoporins thus far tested, depletion of only three have produced a similar defect in NE formation (Finlay *et al.*, 1991; Powers *et al.*, 1995; Grandi *et al.*, 1997; Walther *et al.*, 2001, 2002). Two nucleoporins are the integral pore membrane proteins NDC1 and POM121 (Antonin *et al.*, 2005; Mansfeld *et al.*, 2006) that reside, together with lamin B receptor, in a membrane vesicle population, which has a high avidity for the chromatin surface (Antonin *et al.*, 2005; Ulbert *et al.*, 2006). The third nucleoporin is Nup155 (Franz *et al.*, 2005). The depletion of Nup155 from *C. elegans* embryos also prevents NE formation (Franz *et al.*, 2005). Nup155, although recruited late during NE formation, therefore defines an essential step in NPC and NE assembly.

The similarities in phenotype observed upon depletion of POM121, NDC1, Nup53, and Nup155 might suggest that these proteins function on a similar branch of the assembly pathway. In support of this idea, our analysis of the Nup53 truncation mutants revealed that the region that bound Nup155 (Nup53¹⁶⁷⁻³⁰⁰; Figure 8) was sufficient to restore both

NE and NPC formation and Nup155 recruitment in assembly assays depleted of Nup53. These results suggest that complex formation with Nup155 is critical to the function of Nup53 in NE and NPC assembly. Interestingly, the Nup53¹⁻³⁰⁰ fragment, which, as discussed above, binds Nup155 in vitro at a higher efficiency than full-length Nup53, but it does not interact with NDC1, rescues the depletion phenotype to almost the same extent as full-length Nup53. This implies that although NDC1 interacts with Nup53, and this association is likely to play a role in linking Nup53 to the pore membrane, this specific interaction is not essential for the assembly of the Nup53/Nup155 complex within the NPC. It is possible that functionally redundant mechanisms exist that mediate the association of the Nup53/Nup155 complex with the pore membrane including, for example, the presence of additional interactions interfaces between this complex and NDC1 or other membrane proteins. Recent observations suggest the latter may exist as we have detected a direct interaction between Nup155 and POM121 (Mitchell and Wozniak, unpublished data).

Interestingly, within the Nup53¹⁶⁷⁻³⁰⁰ truncation is an evolutionarily conserved RNA recognition motif (RRM) that is positioned within amino acid residues 167–252 of human Nup53 (Devos *et al.*, 2006). The crystal structure of this RRM has recently been solved for *M. musculus* Nup53 (Handa *et al.*, 2006). These authors proposed that the Nup53 RRM, rather than acting as a nucleic acid binding domain, might be a homodimerization domain or contribute to protein–protein interactions. However, we could not detect any interactions between the RRM (in a Nup53¹⁴⁴⁻²⁶⁵ truncation) and either endogenous Nup53 or other nups, including Nup155, by using our pulldown assay (data not shown). Furthermore, the Nup53¹⁴⁴⁻²⁶⁵ fragment did not rescue the NE and NPC assembly activity of the Nup53 depleted *Xenopus* extracts (data not shown). There was also no detectable effect of adding the RRM fragment in excess to nuclear assembly reactions (data not shown).

This work is the first extensive in vitro and in vivo analysis of nucleoporin truncation fragments. It clearly highlights the importance of defining specific protein interaction domains within a known nucleoporin subcomplex, and it has allowed the analysis of key interactions in the process of NE and NPC assembly (Figure 1). Moreover, we provide further evidence for the proposed checkpoint that links NE and NPC assembly in *Xenopus* egg extracts (Antonin *et al.*, 2005) by demonstrating that the soluble nucleoporin Nup53 must be present and able to interact with Nup155 for NE membrane and NPC assembly to occur. Our data suggest that Nup53, although recruited to chromatin later than the Nup107-160 complex or POM121 (data not shown) plays a key role in the events that bring together the chromatin-associated Nup107-160 complex, vesicle bound transmembrane nucleoporins and soluble Nup155.

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