The C-terminal domain of Nup93 is essential for assembly of the structural backbone of nuclear pore complexes

Ruchika Sachdev[a], Cornelia Sieverding[a], Matthias Flötenmeyer[b], and Wolfram Antonin[a]
[a]Friedrich Miescher Laboratory of the Max Planck Society and [b]Max Planck Institute for Developmental Biology, 72076 Tuebingen, Germany

ABSTRACT **Nuclear pore complexes (NPCs) are large macromolecular assemblies that control all transport across the nuclear envelope. They are formed by about 30 nucleoporins (Nups), which can be roughly categorized into those forming the structural skeleton of the pore and those creating the central channel and thus providing the transport and gating properties of the NPC. Here we show that the conserved nucleoporin Nup93 is essential for NPC assembly and connects both portions of the NPC. Although the C-terminal domain of the protein is necessary and sufficient for the assembly of a minimal structural backbone, full-length Nup93 is required for the additional recruitment of the Nup62 complex and the establishment of transport-competent NPCs.**

Monitoring Editor
Martin Hetzer
Salk Institute for Biological Studies

Received: Sep 7, 2011
Revised: Nov 4, 2011
Accepted: Dec 8, 2011

INTRODUCTION

Nuclear pore complexes (NPCs) are the gatekeepers of the nuclear envelope. They mediate all transport of proteins and nucleic acids between the cytoplasm and the nucleoplasm (for review see Brohawn *et al.*, 2009; Hetzer and Wente, 2009). At the same time, they restrict access to the nucleus by forming a permeability barrier. NPCs are among the largest cellular complexes. Despite their enormous size—60 MDa in vertebrates and 40 MDa in yeast—they are composed of only ~30 distinct proteins named nucleoporins (Nups), which, because of the eightfold symmetry of NPCs, are present in 8, 16, or 32 copies per NPC (Rout *et al.*, 2000; Cronshaw *et al.*, 2002; Alber *et al.*, 2007).

The majority of nucleoporins are organized in discrete subcomplexes both in yeast and metazoa. These subcomplexes are defined biochemically and reflect a stable interaction among nucleoporins. During the open mitosis used by metazoans the nuclear envelope and NPCs break down, but several nucleoporin subcomplexes remain intact. At the end of mitosis NPCs assemble from these subcomplexes in a defined order (Dultz *et al.*, 2008). First, the Nup107–160 complex binds to chromatin, acting as a seeding point for NPC assembly (Harel *et al.*, 2003; Walther *et al.*, 2003). Membranes subsequently associate with chromatin causing enrichment of nuclear envelope/NPC–specific membrane proteins (Antonin *et al.*, 2005; Anderson *et al.*, 2009). Once nuclear envelope membranes have been recruited, the Nup93 complex, followed closely by the Nup62 complex, which is largely made up of phenylglycine (FG) repeat–containing nucleoporins, associates with the assembling pore to form its central channel. Finally, peripheral nucleoporins, including Nup214, TPR (translocated promoter region), and the largest pools of Nup153 and Nup50 associate with the NPC (Dultz *et al.*, 2008).

The Nup93 complex—the second major subcomplex recruited to the assembling NPC—has been implicated in several structural aspects of the pore. Nup53—one member of this complex—as well as the corresponding yeast homologues Nup53p and Nup59p, interacts with the transmembrane nucleoporin Ndc1, potentially linking the NPC to the pore membrane (Mansfeld *et al.*, 2006; Onischenko *et al.*, 2009). Nup53 interacts with two other members of the complex—Nup155 and Nup93—in vertebrates (Hawryluk-Gara *et al.*, 2008) and the corresponding proteins in yeast (Fahrenkrog *et al.*, 2000; Onischenko *et al.*, 2009). Nup93 in turn tightly interacts with the remaining two members of the subcomplex—Nup188 and Nup205 (Grandi *et al.*, 1997; Miller *et al.*, 2000; Theerthagiri *et al.*, 2010). Of interest, a subfraction of Nup93 interacts with the FG-repeat nucleoporin Nup62, as does the yeast homologue Nic96p with Nsp1p (Grandi *et al.*, 1997; Grandi *et al.*, 1993), thus potentially linking the structured part of the NPC to the unstructured FG repeat–containing nucleoporin complexes that form the central channel.

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-09-0761) on December 14, 2011.

Address correspondence to: Wolfram Antonin (wolfram.antonin@tuebingen.mpg.de).

Abbreviations used: aa, amino acids; FG, phenylglycine; NPC, nuclear pore complex; Nup, nucleoporin; TPR, translocated promoter region.

In vertebrates, Nup53 and Nup155 are indispensable for NPC formation (Franz *et al.*, 2005; Hawryluk-Gara *et al.*, 2008; Mitchell *et al.*, 2010). Of interest, Nup93 is an essential gene in most organisms tested, including *Caenorhabditis elegans*, *Danio rerio*, *Saccharomyces cerevisiae*, and *Aspergillus nidulans* (Grandi *et al.*, 1993; Allende *et al.*, 1996; Galy *et al.*, 2003; Osmani *et al.*, 2006), but, surprisingly, not in *Schizosaccharomyces pombe* (Yoon *et al.*, 1997). Quantification of the Nup93 in the NPCs of rat liver cells and the corresponding Nic96p in *S. cerevisiae* suggests that it is present in 32–48 or more copies per NPC and is thus one of the most abundant nucleoporins (Rout *et al.*, 2000; Cronshaw *et al.*, 2002; Alber *et al.*, 2007). Immunodepletion of Nup93 from *Xenopus laevis* extracts followed by in vitro nuclear assembly reactions results in nuclei with reduced NPC staining (Grandi *et al.*, 1997). This suggests that Nup93 has an important function in NPC assembly and function. However, because Nup93 tightly associates with Nup205 and Nup188 (Theerthagiri *et al.*, 2010; Amlacher *et al.*, 2011), it is not clear whether the effect of Nup93 depletion is due to the loss of the protein itself or a codepletion of both Nup205 and Nup188. When depleted individually, neither Nup188 nor Nup205 is essential for NPC formation (Theerthagiri *et al.*, 2010). However, these proteins may have partially redundant functions in NPC assembly, and it has indeed been suggested that they arose, like many other nucleoporins, from duplication and diversification events during evolution (Alber *et al.*, 2007).

To dissect the importance of Nup93, Nup188, and Nup205 in NPC assembly and function, we depleted all three proteins from *Xenopus* egg extracts. We performed add-back experiments using recombinant Nup93, and here we show that Nup93 by itself is essential for NPC formation. Functional NPCs can be assembled in the absence of both Nup205 and Nup188. Although the N-terminal part of Nup93 is important for the recruitment of the Nup62 complex and establishment of the permeability barrier and transport competency of the NPC, the C-terminal region of the protein is sufficient for the assembly of the NPC's structural backbone.

RESULTS

Nup93 is essential for nuclear pore complex assembly

We previously showed that two components of the Nup93 complex—Nup188 and Nup205—both interact separately with Nup93, forming Nup205–Nup93 and Nup188–Nup93 complexes (Theerthagiri *et al.*, 2010). Neither complex is individually essential for NPC formation. However, we could not rule out that these complexes have at least partially redundant functions in NPC formation, as depletion of both complexes together using a combination of antibodies against Nup188 and Nup205 was not technically feasible (Theerthagiri *et al.*, 2010). To overcome this limitation, we raised antibodies against the common protein of both complexes, Nup93. We were able to efficiently immunodeplete cellular extracts derived from *X. laevis* eggs of Nup93 (Figure 1A; note that a slightly slower migrating cross-reactivity detected by the Nup93 antibody by Western blotting (asterisk) is not immunoprecipitated or depleted). Because Nup188 and Nup205 interact tightly with Nup93 (Grandi *et al.*, 1997; Miller *et al.*, 2000; Theerthagiri *et al.*, 2010; Amlacher *et al.*, 2011), both proteins were efficiently codepleted. The levels of other nucleoporins, including Nup155 and Nup53 from the same Nup93 subcomplex, as well as Nup62, Nup98, and Nup160, were not affected by this treatment (Figure 1A).

We analyzed the effect of Nup93 depletion on NPC assembly. In fractionated *Xenopus* egg extracts nuclei are able to form in vitro upon incubation of DNA with cytosolic and membrane components. When sperm chromatin was incubated for 90 min in Nup93-depleted extracts, membrane vesicles bound to the chromatin surface but did not fuse to form a closed nuclear envelope (Figure 1B; see also Grandi *et al.*, 1997). This phenotype was previously observed when depleting nucleoporins that are essential for NPC formation (Franz *et al.*, 2005; Hawryluk-Gara *et al.*, 2008). Consistent with this idea, immunofluorescence staining for mAB414, an antibody that recognizes several FG repeat–containing nucleoporins that localize to different substructures of the NPC, was largely absent from Nup93-depleted nuclei (Figure 2). This observation suggests that NPCs are not properly formed. A similar reduction in mAB414 staining was also observed in HeLa cells, where levels of Nup93 were decreased by RNA interference treatment (Krull *et al.*, 2004). As expected, when Nup93 was depleted, neither Nup188 nor Nup205 was detected on the chromatin (Figure 2), as both proteins were codepleted with Nup93 (Figure 1A).

However, the other members of the Nup93 complex—Nup155 and Nup53—as well as the transmembrane nucleoporins pom121 and gp210, were present, albeit at reduced levels, on the chromatin (Figure 3). In contrast, Nup107, as well as MEL-28, which recruits the Nup107–160 complex to chromatin, could be detected on chromatin. With the exception of gp210, all of these nucleoporins have been implicated in early steps of NPC assembly (Harel *et al.*, 2003; Walther *et al.*, 2003; Antonin *et al.*, 2005; Rasala *et al.*, 2006; Franz *et al.*, 2007). Nup153 and Nup98, both of which interact with the Nup107–160 complex (Vasu *et al.*, 2001), could also be detected. Their presence on chromatin probably reflects recruitment via the Nup107–160 complex, which proceeds in vitro in the absence of Nup93.

These data indicate that Nup93 crucially contributes to nuclear envelope and NPC formation subsequent to chromatin binding of the Nup107–160 complex and the initial recruitment of pom121, Nup53, and Nup155. However, the phenotype observed could be either a direct consequence of Nup93 depletion or the result of codepleting its two interacting partners, Nup205 and Nup188. To distinguish between these two scenarios, we added recombinant Nup93 protein, which was expressed and purified from *Escherichia coli*, to the Nup93-depleted extracts at approximately endogenous levels as detected by Western blotting (Figure 1A). The readdition of Nup93 resulted in the formation of a closed nuclear envelope, as visualized by the membrane stain DiIC18 and electron microscopy (Figure 1, B–D). Recombinant Nup93 was detected at the nuclear rim by immunofluorescence and costained with mAB414 (Figure 2), suggesting that NPC formation is restored and that the recombinant protein integrates into assembling NPCs.

Despite the fact that a closed nuclear envelope formed, Nup188 and Nup205 could not be detected by immunofluorescence when recombinant Nup93 was added back to the depleted extracts (Figure 2). Thus the rescue of the depletion phenotype in the add-back experiment cannot be attributed to residual levels of Nup188 and/or Nup205 in the depleted extracts acting with the recombinant Nup93. Of interest, the nuclei in the add-back experiment were larger in diameter than mock-treated nuclei, a phenotype that was previously observed upon depletion of Nup188 (Theerthagiri *et al.*, 2010). Most likely, this effect is due to the codepletion of Nup188. Structural nucleoporins such as Nup155, Nup53, and Nup107, as well as the transmembrane nucleoporins pom121 and gp210, showed normal recruitment in the add-back experiments (Figure 3). In addition, peripheral nucleoplasmic or cytoplasmic nucleoporins Nup153 and Nup88, respectively, could be detected on these nuclei.

Taken together, these data indicate that Nup205 and Nup188 are not essential for nuclear envelope and NPC assembly. Thus NPCs can form even in the absence of two major components of the

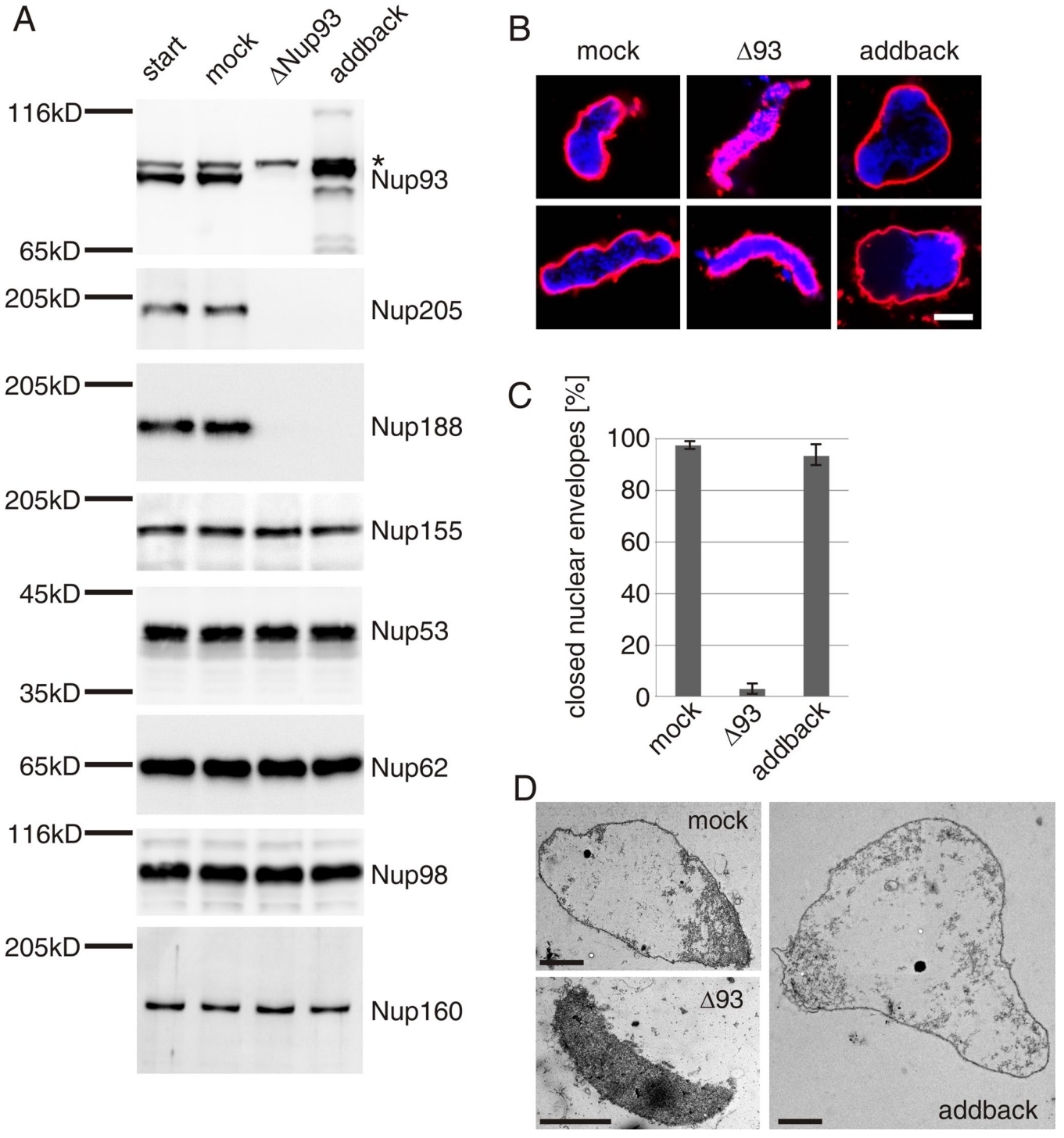

FIGURE 1: Nup93 is essential for NPC formation. (A) Western blot analysis of untreated, mock, Nup93-depleted (Δ93) and Nup93-depleted extracts with full-length recombinant Nup93 (addback), respectively. The Nup93 antibody recognizes a slightly slower migrating cross-reactivity by Western blotting (asterisk), which is neither immunoprecipitated nor depleted. The recombinant Nup93 migrates slightly more slowly than the endogenous protein probably due to absence of eukaryotic posttranslational modifications. (B) Nuclei were assembled in mock, Nup93-depleted extracts (Δ93) or Nup93-depleted extracts supplemented with full-length recombinant Nup93 (addback) for 90 min, respectively, fixed with 2% paraformaldehyde (PFA) and 0.5% glutaraldehyde, and analyzed for chromatin and membrane staining (blue, 4′,6-diamidino-2-phenylindole [DAPI]; red, DiIC18; bar, 10 μm). (C) Quantitation of chromatin substrates with a closed nuclear envelope of reactions done as in B. More than 100 randomly chosen chromatin substrates were counted per reaction. The average of three independent experiments are shown; error bars represent the total variation. (D) Transmission electron micrography of a nucleus assembled in mock, Nup93-depleted extracts or depleted extracts supplemented with full-length Nup93 as in B. Bar is 2 μm.

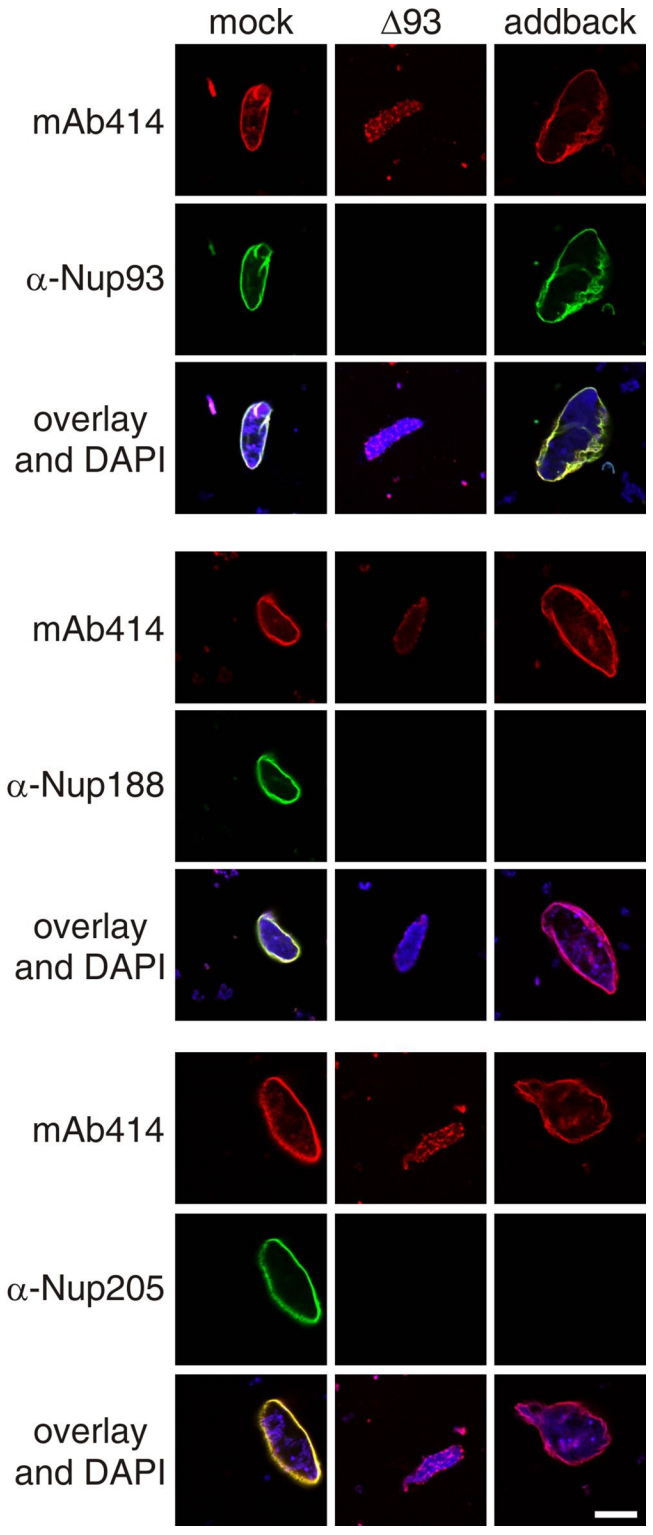

FIGURE 2: Nup188 and Nup205 together are not essential for NPC formation. Nuclei were assembled in mock, Nup93-depleted extracts (Δ93) or Nup93-depleted extracts supplemented with full-length Nup93 (addback) for 90 min, fixed with 4% PFA, and analyzed with Nup93-, Nup188-, or Nup205-specific antibodies, respectively (green), and the monoclonal antibody mAb414, which recognizes FG repeat nucleoporins (red). Chromatin is stained with DAPI; scale bar, 10 μm.

Nup93 subcomplex. However, Nup93 is essential for NPC formation, and addition of Nup93 alone is sufficient to compensate for the loss of the Nup188–Nup93 and Nup205–Nup93 complexes.

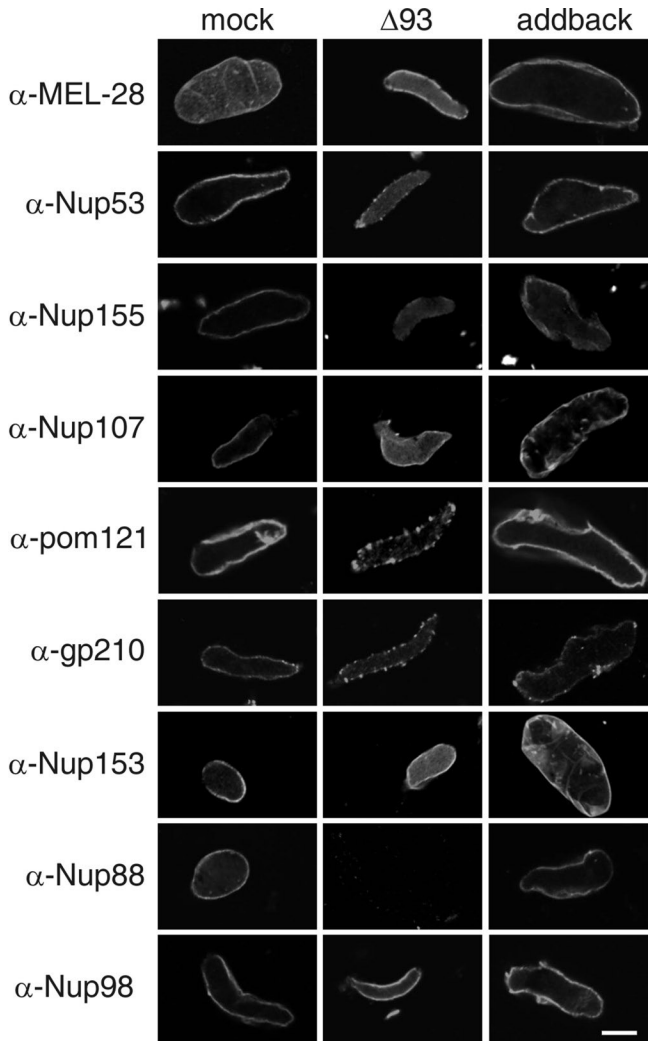

FIGURE 3: Nuclei lacking Nup188 and Nup205 have a normal NPC composition. Nuclei were assembled in mock, Nup93-depleted extracts (Δ93) or Nup93-depleted extracts supplemented with full-length Nup93 (addback) for 90 min, fixed with 4% PFA, and stained with the respective antibodies. Scale bar, 10 μm.

The C-terminal domain of Nup93 is sufficient for assembly of a minimal pore

Because Nup93 is essential for NPC formation but neither its interaction with Nup188 nor Nup205 is necessary in this regard, we sought to determine which regions of the protein contribute to NPC assembly and function. The amino terminus of Nup93 and its yeast homologue Nic96p is predicted to form a coiled-coil domain (Grandi *et al.*, 1995, 1997). In yeast, this region is important for the interaction to Nsp1p, the Nup62 homologue (Grandi *et al.*, 1995). The remainder of the protein consists of an elongated, mostly α-helical structure (Jeudy and Schwartz, 2007; Schrader *et al.*, 2008) that can be subdivided into a middle part (amino acids [aa] 197–583 in *S. cerevisiae*, corresponding to aa 183–579 in *Xenopus*) and C-terminal part (aa 617–839, corresponding to aa 608–820 in *Xenopus*). To analyze the function of the different regions of Nup93, we generated fragments of the protein comprising the N-terminal (aa 1–175), middle (aa 183–579), and C-terminal (aa 608–820) portions, as well as fragments lacking either the N-terminal or C-terminal domain (aa 183–820 or 1–579, respectively) and expressed and purified them in *E. coli* (see Figure 4A for a schematic representation of

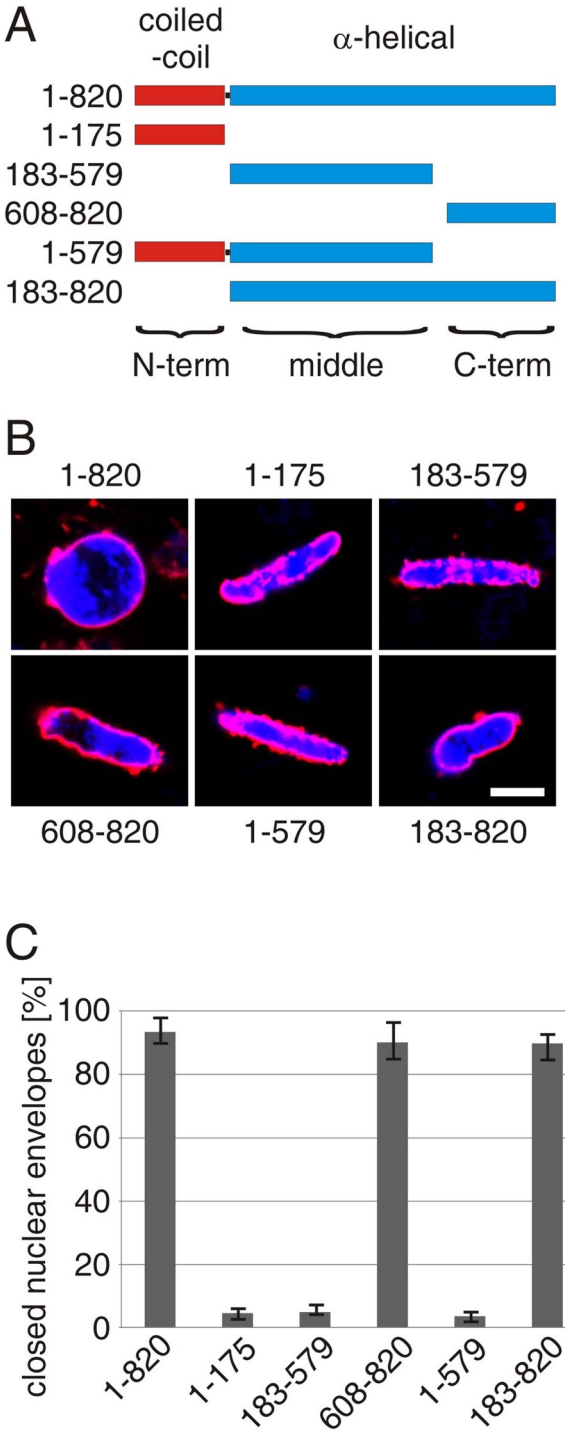

FIGURE 4: The C-terminal Nup93 fragment supports formation of a closed nuclear envelope. (A) Schematic representation of the domain structure of *Xenopus* Nup93 and the fragments used. The N-terminal coiled-coil region is marked in red, the α-helical region in blue. Numbers indicate the amino acids of the respective constructs. (B) Nuclei were assembled in Nup93-depleted extracts supplemented as indicated either with full-length recombinant Nup93 (1–820) or the respective fragments for 90 min, fixed with 2% PFA and 0.5% glutaraldehyde, and analyzed for chromatin and membrane staining (blue, DAPI; red, DiIC18; bar, 20 μm). (C) Quantitation of chromatin substrates with a closed nuclear envelope of reactions done as in B. More than 100 randomly chosen chromatin substrates were counted per reaction. The average of three independent experiments is shown; error bars represent the total variation.

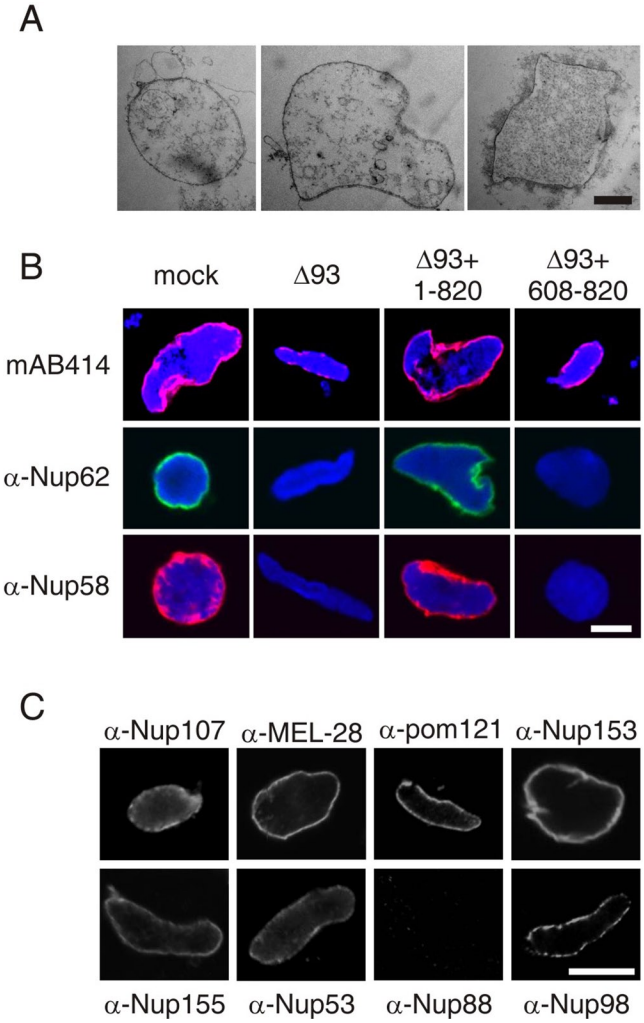

FIGURE 5: The C-terminal Nup93 fragment supports formation of the structural part of the NPC but not incorporation of the Nup62 complex. (A) Transmission electron micrographs of nuclei assembled in Nup93-depleted extracts supplemented with the C-terminal Nup93 fragment containing aa 608–820. Note the presence of a closed nuclear envelope. Bar, 2 μm. (B) Nuclei were assembled in mock, Nup93-depleted extracts (Δ93) or Nup93-depleted extracts supplemented with either full-length recombinant Nup93 (1–820) or the C-terminal fragment (608–820) for 90 min, fixed with 4% PFA, and analyzed with the antibody mAB414 (top), an antibody against Nup62 (middle), or one against Nup58 (bottom). Overlays with DAPI staining (blue) are shown. Scale bar, 10 μm. (C) Nuclei were assembled in Nup93-depleted extracts supplemented with the C-terminal fragment (aa 608–820) for 90 min, fixed with 4% PFA, and stained with the respective antibodies. Scale bar, 10 μm.

the generated fragments). When added to extracts depleted of endogenous Nup93, all fragments lacking the C-terminal region of Nup93 did not support formation of a closed nuclear envelope (Figure 4, B and C). In contrast, fragments containing the middle and C-terminal regions (183–820) or, surprisingly, only the C-terminal region of Nup93 (608–820) formed small nuclei with closed nuclear envelopes as visualized by membrane staining.

Thus an astonishingly small part of Nup93 of ~200 amino acids is sufficient to seemingly compensate for the loss of the endogenous protein in nuclear assembly. It allows for formation of a closed nuclear envelope, as confirmed by electron microscopy (Figure 5A). We therefore analyzed these nuclei in detail. First, we checked for

the presence or absence of nucleoporins. Immunofluorescence using the antibody mAB414 as a marker for FG-containing nucleoporins revealed a weaker nuclear rim staining after readdition of the C-terminal fragment (608–820) compared with control nuclei or nuclei formed when full-length Nup93 (1–820) was added back to the depleted extracts (Figure 5B).

The antibody mAB414 recognizes a subset of nucleoporins, including Nup62. Because Nup62 is known to interact with the N-terminal region of Nup93, we tested for the presence of Nup62 on nuclei formed in the presence of the extreme C-terminal Nup93 fragment (Figure 5B). The antigen was absent, indicating that the Nup62 complex, a major constituent of the NPC, is missing. The absence of Nup62 is not due to a codepletion of the protein with Nup93 from the egg extracts (Figure 1A). Consistent with this, the readdition of full-length Nup93 results in detectable Nup62 at the nuclear rim (Figure 5B). Similarly, Nup58, which is also part of the Nup62 subcomplex, was only detected in the presence of full-length Nup93 and not in nuclei formed in the presence of the C-terminal Nup93 fragment (Figure 5B).

Of interest, structural nucleoporins such as Nup107, Nup53, Nup155, and MEL-28 could be detected in nuclei formed in the presence of the C-terminal Nup93 fragment (Figure 5C). Nup153, which is a part of the nuclear basket of the NPC and interacts with the Nup107–160 subcomplex (Vasu *et al.*, 2001), was also detected at the NPCs of these nuclei. The weak mAB414 staining observed might therefore be Nup153, which is also recognized by this antibody. Furthermore, the FG-repeat containing nucleoporin Nup98 was detected on these nuclei, albeit at reduced levels. At least a subfraction of this protein interacts with the Nup107–160 complex (Vasu *et al.*, 2001), and its recruitment to the chromatin probably reflects this feature. Together, these data suggest that nuclei in which endogenous Nup93 is replaced by the C-terminal fragment form a closed nuclear envelope and the structural part of NPCs but lack Nup62 complexes—a substantial portion of the unstructured FG repeat–containing nucleoporins.

Because the Nup62 subcomplex is implicated in nuclear transport and establishment of the permeability barrier of the NPC, we tested whether either function was impaired in nuclei that had been assembled with the C-terminal Nup93 fragment instead of the endogenous protein. When an enhanced green fluorescent protein–fused nuclear import substrate was added to in vitro assembly reactions it was quickly imported and enriched in the nucleoplasm in the mock control as well as when full-length Nup93 was added back to depleted extracts (Figure 6). In contrast, only faint or no nuclear staining could be detected in nuclei assembled in the presence of the C-terminal Nup93 fragment only. The same results were obtained when a fragment comprising the middle and C-terminal regions of Nup93 and thus missing the N-terminus was used. Thus the NPCs in the nuclei assembled in the presence of Nup93 fragments lacking the N-terminus were not competent for nuclear import. This defect could explain why these nuclei are small (Figure 4B), as nuclear growth requires nuclear import after initial nuclear envelope enclosure.

A second major function of FG-nucleoporins is the establishment of a diffusion barrier within the NPC. Small molecules are able to diffuse between cytoplasm and nucleoplasm, but substances larger 2.5 nm in radius are excluded from the nucleus (Mohr *et al.*, 2009) and are translocated only by signal-mediated transport. The exclusion of nonnuclear factors larger than 30 kDa from the nucleoplasm is important for the establishment of the distinct environments of the cytosol and nuclear compartments. We assayed the integrity of the nuclear envelope barrier by adding fluorescently labeled dextrans to the in vitro–reconstituted nuclei (Figure 7). Control nuclei, as well as nuclei assembled in the presence of full-length Nup93, excluded 70-kDa dextrans. However, nuclei assembled in the presence of the C-terminal Nup93 fragment (608–820) did not exclude dextrans of this size. These nuclei form an apparently closed nuclear envelope (Figure 5A), indicating that the loss of permeability barrier function is not due to a block in nuclear envelope formation.

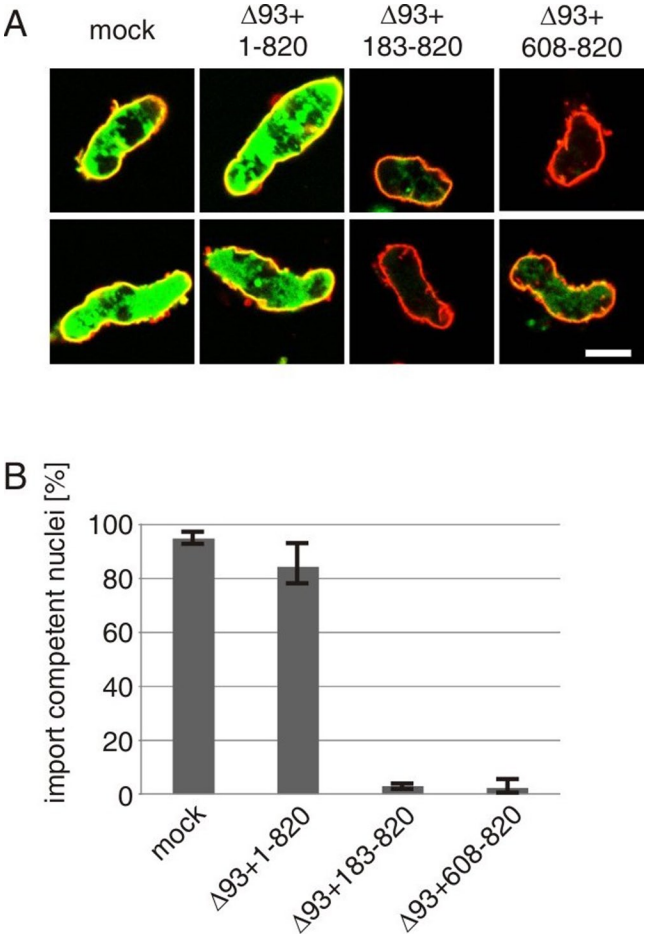

FIGURE 6: The N-terminal coiled-coil region of Nup93 is required to assemble import-competent nuclei. (A) Nuclei were assembled in mock or Nup93-depleted extracts supplemented with the either full-length Nup93 (1–820), a fragment lacking the N-terminal coiled-coil region (183–820), or the C-terminal fragment (608–820). After 50 min an enhanced green fluorescent protein–fused nuclear import substrate was added. After 120 min nuclei were isolated and analyzed by confocal microscopy. Membranes are stained with DiIC18 (red). Bars, 20 μm. (B) Quantitation of import reactions performed as in A. More than 100 randomly chosen chromatin substrates were counted per reaction. The average of three independent experiments is shown; error bars represent the total variation.

Together these data indicate that Nup93 is important for the assembly of import- and exclusion-competent nuclei. Our data support the view that in vertebrates, similar to yeast (Grandi *et al.*, 1995), the N-terminal coiled-coil region is important for recruiting the Nup62 complex and thus establishing the barrier function of NPCs. The C-terminal Nup93 fragment (608–820) does not support recruitment of the Nup62 complex or assembly of import- and exclusion-competent nuclei. However, this fragment is sufficient for formation of a closed nuclear envelope and at least partially assembled NPCs. These NPCs contain structural nucleoporins (Figure 5), including Nup53 and Nup155.

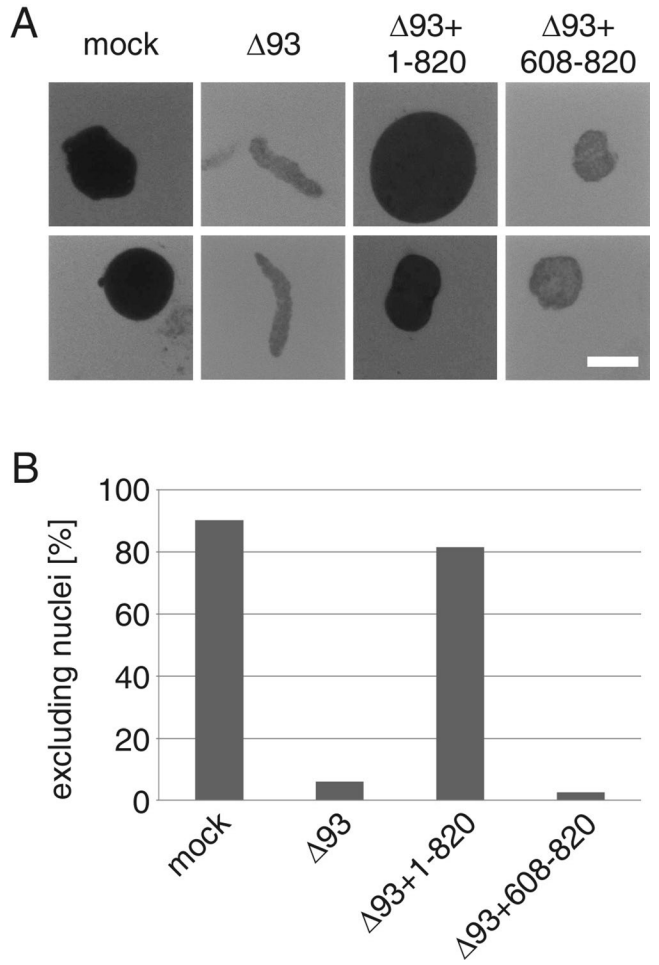

FIGURE 7: The N-terminal-coiled coil region of Nup93 is required to assemble exclusion-competent nuclei. (a) Nuclei were assembled in mock or Nup93-depleted extracts supplemented with the either full-length Nup93 (1–820) or the C-terminal fragment (608–820). After 120 min fluorescein-labeled, 70-kDa dextran was added. DNA was stained with DAPI to identify nuclei. Samples were analyzed by confocal microscopy. Representative images of the dextran staining are shown. Scale bar, 10 μm. (B) Quantitation of the size exclusion assays with 70-kDa dextran performed as in A. For each condition more than 40 nuclei from at least two independent experiments were analyzed.

The C-terminal domain of Nup93 promotes the Nup155–Nup53 interaction

Nup53 is known to interact with Nup93 and Nup155 via its N- and C-terminal regions, respectively (Fahrenkrog *et al.*, 2000; Hawryluk-Gara *et al.*, 2008; Amlacher *et al.*, 2011), linking these two protein in an interaction network (Onischenko *et al.*, 2009; Amlacher *et al.*, 2011). We wondered how the C-terminal region of Nup93 could contribute to this interaction. Previous immunoprecipitation experiments in *Xenopus* egg extracts did not reveal detectable interactions between Nup93, Nup53, or Nup155 (data not shown, but see Figure 1A in Theerthagiri *et al.*, 2010). However, using glutathione *S*-transferase (GST) pulldowns with recombinant Nup53 that was incubated with *Xenopus* egg extracts, we could detect its interaction with Nup155 (Figure 8A). This interaction was strengthened in the presence of the C-terminal Nup93 fragment (aa 608–820, expressed as a SUMO fusion to facilitate detection) but not the middle part of Nup93 (aa 183–579). Of note, when we performed GST pulldowns using the C-terminal Nup93 fragment, its interaction with Nup155 could not be detected (data not shown). This result is consistent with the fact that Nup53 is necessary for the formation of a trimeric complex, as detected with recombinant proteins from the fungus *Chaetomium thermophilum* (Amlacher *et al.*, 2011). Indeed, if we replace *Xenopus* egg extracts in our GST-pulldown experiments by bacterial lysates containing recombinant *Xenopus* Nup155, we detect an interaction with Nup53 that is strengthened in the presence of the C-terminal Nup93 fragment (aa 608–820), supporting the existence of direct interactions between these proteins. Thus our data indicate that the C-terminus of Nup93 is sufficient for its binding to Nup53, which in turn promotes the interaction between Nup53 and Nup155.

Taken together with the nuclear assembly experiments, these data suggest that the C-terminal region of Nup93 induces or stabilizes an interaction between Nup155 and Nup53 as central components of the structural part of the NPC and that this region is sufficient for formation of the structural backbone of the NPC.

DISCUSSION

In summary, we found that Nup93 is essential for NPC formation and function in *Xenopus* egg extracts, whereas both Nup188 and Nup205 are dispensable. Unexpectedly, the C-terminal domain of Nup93 is able to replace the full-length protein for the assembly of the structural part of the NPC. Our data indicate that it does so by strengthening the interaction between Nup155 and Nup53, which are core components of the structural part of NPCs.

We previously showed that two large vertebrate nucleoporins—Nup205 and Nup188—can be individually depleted without compromising NPC assembly. This is surprising when considering that both proteins are localized to the structural part of the pore and believed to participate in central ring formation (Alber *et al.*, 2007; Amlacher *et al.*, 2011). It was proposed that both proteins arose during evolution from a common ancestor (Alber *et al.*, 2007). Such duplication and diversification of nucleoporins could well explain the redundancies observed among other NPC components (Stavru *et al.*, 2006). However, here we demonstrate that NPCs, which are functional with regard to nuclear transport and exclusion properties, can form in vitro in the absence of both proteins. These experiments rule out the possibility that each protein compensates for the loss of the other when nuclei are assembled in vitro. Of course, we cannot exclude that other nuclear functions are affected in vitro or in vivo by the codepletion of Nup188 and Nup205. Indeed, in vitro–assembled nuclei lacking both proteins grow larger. This phenotype was previously observed upon depletion of Nup188 and was found to be caused by an enhanced passage of integral membrane proteins through NPCs (Theerthagiri *et al.*, 2010; Antonin *et al.*, 2011).

In contrast to Nup188 and Nup205, the nucleoporin Nup93 is essential for the formation of in vitro functional NPCs. In the absence of Nup93, NPC assembly and nuclear envelope closure are blocked. Our add-back experiments unambiguously demonstrate that this phenotype is caused by the absence of Nup93 and not by the codepletion of Nup205 and/or Nup188. Nic96, the yeast Nup93 homologue, is essential, and a thermosensitive Nic96p protein leads to a decrease in NPC density at restrictive temperature (Grandi *et al.*, 1993; Zabel *et al.*, 1996) suggesting that its function in NPC assembly is conserved during evolution.

Fragments lacking the N-terminal domain of Nup93 can substitute for the endogenous protein in NPC assembly. However, these NPCs lack the Nup62 complex and are not functional for nuclear import or exclusion of nonnuclear factors. The amino-terminal coiled-coil region of yeast Nic96p is known to interact with the

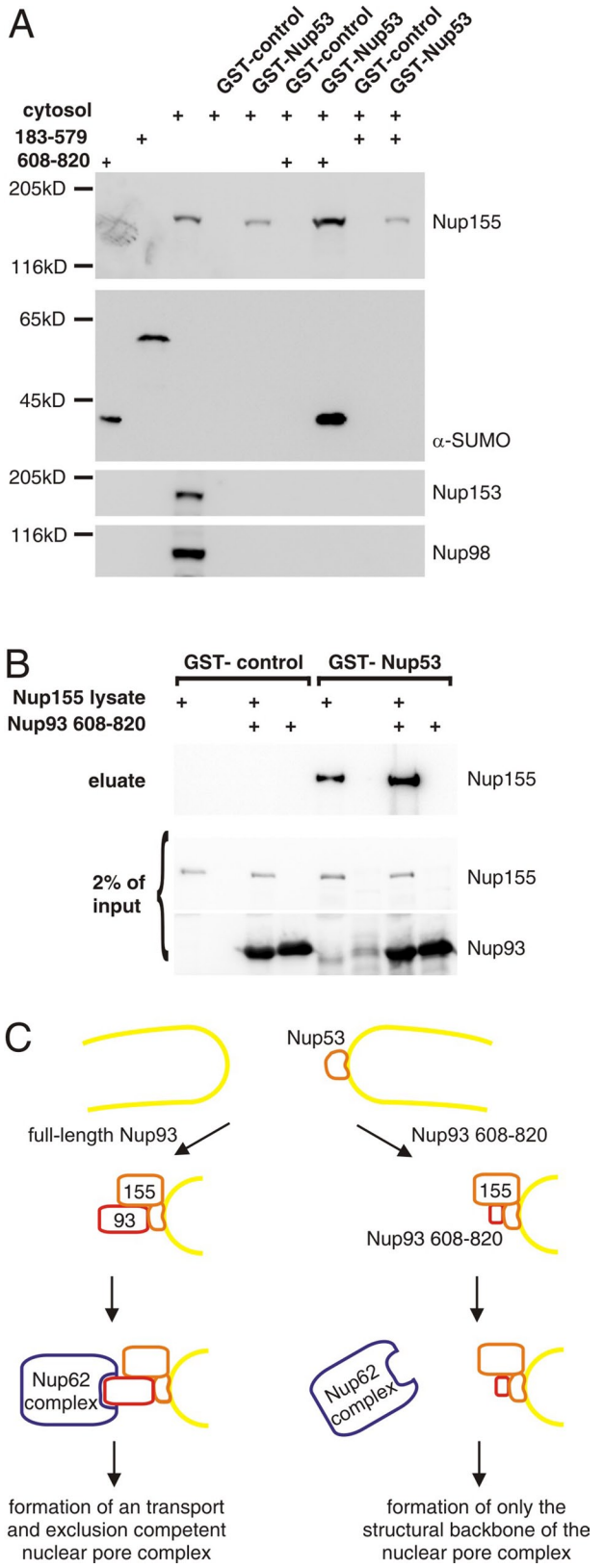

FIGURE 8: The C-terminus of Nup93 stabilizes the Nup53–Nup155 interaction. (A) GST fusions of the nucleoplasmic domain of gp210 (control) or Nup53 were incubated with cytosol from *Xenopus* egg extracts alone or, where indicated, in the presence of SUMO fusions of the Nup93 middle fragment (183–582) or Nup93 C-terminal fragment (608–820), respectively. Eluates were analyzed by Western blotting with antibodies against SUMO to detect the Nup93 fragments or antibodies against Nup155, Nup153, and Nup98 to detect the respective protein. In the left three lanes 5% of the input of the Nup93 fragments and 2% of the cytosol were loaded. (B) Purified recombinant GST or GST-Nup53 fusion protein was incubated with lysates from bacteria expressing recombinant *Xenopus* Nup155 and supplemented with the C-terminal fragment Nup93 (608–820) where indicated. Eluates and 2% of the input were analyzed by Western blotting using antibodies against the His6 tag. Because of the removal of the His6 tag from the Nup93 fragment during TEV protease elution, it is not possible to detect this protein in the eluate. (C) Model for Nup93 function in postmitotic NPC assembly. The C-terminal region of Nup93 stabilizes the Nup155–Nup53 interaction, allowing the assembly of the structural part of the NPC. This function of Nup93 can be substituted by a fragment comprising amino acids 608–820 (right). The N-terminal coiled-coil region of Nup93 recruits the Nup62 complex to the structural part of the NPC. This requires in the assembly reaction full-length Nup93 and leads to the association of the central channel and formation of functional NPCs (left). Note that for the sake of simplicity, transmembrane nucleoporins and the Nup107–160 complex are not shown, although our data indicate that they are present in both assembly lines.

Nup62 complex (Grandi *et al.*, 1995). Recent data from the Hurt lab suggest that the Nup62 complex is synergistically recruited by this region and interactions with Nup188 and Nup192—the yeast homologues of vertebrate Nup188 and Nup205, respectively (Amlacher *et al.*, 2011). Deletion of an α-helix in Nic96p, which is known to be important for its interaction with Nup188 and Nup192 and probably its recruitment to NPCs, showed a mild growth defect. In contrast, deletion of the coiled-coil region important for the interaction of Nic96p with the Nup62 complex retarded cell growth more severely. If both interacting regions were lacking, an extremely slow growth phenotype was observed. This could indicate that the Nup62 complex is recruited to NPCs by a direct and more important interaction to Nic96p and an indirect interaction via Nup188p and/or Nup192p. In support of this view, our add-back experiments with full-length Nup93 and fragments show that in vertebrates the N-terminus of Nup93 is sufficient to recruit the Nup62 complex in the absence of Nup188 and Nup205.

A small portion of Nup93 in the extreme C-terminus, ~200 amino acids, is sufficient for the assembly of a minimal pore. This finding is surprising in light of results in *S. cerevisiae*, where the C-terminus was regarded as of minor importance (Grandi *et al.*, 1995; Schrader *et al.*, 2008). However, one of two highly conserved surface patches identified in Nic96p is located in this C-terminal region and likely represents an important site for protein–protein interactions (Jeudy and Schwartz, 2007). Our data suggest that this region interacts with Nup53, but we cannot exclude that a different region is involved in the Nic96p–Nup53p interaction in yeast. Although it has been suggested that yeast Nic96p and vertebrate Nup93 are likely to exhibit the same overall structure (Jeudy and Schwartz, 2007; Schrader *et al.*, 2008), Nup93 is predicted to have a much smaller dipole moment than Nic96p, which might hint at a yeast-specific particularity (Schrader *et al.*, 2008). Certainly, we cannot exclude that the discrepancy observed in the importance of the Nic96p and Nup93 C-terminal domains reflects differences in the assembly of NPC between yeast and vertebrates. In yeast, where closed mitosis is used, NPCs assemble only into the intact nuclear envelope. In metazoa, NPC assembly also occurs postmitotically on the chromatin surface concomitantly with formation of a closed nuclear envelope (for review see Antonin *et al.*, 2008; Kutay and Hetzer, 2008). This process is particularly emphasized in the *Xenopus* egg extract nuclear assembly system and probably has distinct requirements of

nucleoporin interactions. The fact that the N-terminal domain of Nic96p is sufficient to substitute for the loss of the endogenous protein in yeast (Jeudy and Schwartz, 2007) but not in our system might reflect such a differences in NPC assembly pathways and mechanisms.

Our pulldown data suggest not only that the C-terminal region of Nup93 is interacting with Nup53, but also that this interaction stabilizes the Nup155–Nup53 interaction, probably by inducing a conformational change in Nup53. Both Nup155 and Nup53 are essential for NPC formation in vertebrates (Franz *et al.*, 2005; Hawryluk-Gara *et al.*, 2008; Mitchell *et al.*, 2010) and are most likely central components of the structural backbone of the NPC (Alber *et al.*, 2007). Establishing and/or stabilizing the Nup53–Nup155 interaction might be a key event in assembling the structural backbone of the NPC (Onischenko *et al.*, 2009). Of interest, depletion of Nup53 blocks Nup93 recruitment to chromatin substrates (Hawryluk-Gara *et al.*, 2008), whereas depletion of Nup93 reduces but does not abolish Nup53 staining on chromatin (Figure 3). This could indicate that Nup53 recruitment not only precedes but is also required for Nup93 binding to the assembling NPC at the end of mitosis, a hypothesis consistent with models for yeast nuclear pore complex assembly (Rexach, 2009).

On the basis of our findings, we propose a model (Figure 8B) in which Nup53 and Nup155 are recruited to the assembling NPC, probably via their interactions with the transmembrane nucleoporins ndc1 and pom121 (Mansfeld *et al.*, 2006; Onischenko *et al.*, 2009; Mitchell *et al.*, 2010). The Nup53–Nup155 interaction is stabilized by the C-terminal region of Nup93 interacting with Nup53. This leads to the assembly of the structural part of the NPC (Figure 8C, right). Neither Nup188 nor Nup205 is required for the stabilization of Nup53 at the pore. In the presence of full-length Nup93 the N-terminus of the protein recruits the Nup62 complex (Figure 8C, left). This recruitments allows for formation of import- and exclusion-competent NPCs. Our data suggest that Nup93 is a key factor in the ordered assembly of NPCs during postmitotic nuclear envelope reassembly, completing the formation of the structural part of the NPC via its C-terminal domain and recruiting, via its N-terminus, the Nup62 complex and thus the central channel to the pore.

MATERIALS AND METHODS

Materials

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC18), fluorescein-labeled dextran, and secondary antibodies (Alexa Fluor 488 goat α-rabbit immunoglobulin G [IgG] and Cy3 goat α-mouse IgG) were obtained from Invitrogen (Carlsbad, CA).

Antibodies

For the generation of antibodies, an amino-terminal fragment of *Xenopus* Nup93 (aa 1–230) or full-length Nup58, respectively, was cloned into a pET28a vector (EMD, San Diego, CA) and purified using Ni-nitrilotriacetic acid agarose (Qiagen, Valencia, CA), dialyzed to phosphate-buffered saline (PBS), and injected into rabbits.

Antibodies against pom121 and gp210 (Antonin *et al.*, 2005), Nup155 (Franz *et al.*, 2005), Nup160 and MEL-28 (Franz *et al.*, 2007), and Nup153 and Nup107 (Walther *et al.*, 2003), as well as Nup188, Nup205, Nup98, and Nup53 (Theerthagiri *et al.*, 2010), were as described. mAB414 was obtained from BAbCO (Richmond, CA), antibodies against yeast SUMO (SMT3) are from Acris (Herford, Germany), Nup88 is from BD Biosciences (San Diego, CA), 6-histidine (His6) antibody is from Roche (Indianapolis, IN), and Nup62 is from Birthe Fahrenkrog (Université Libre de Bruxelles, Belgium; Schwarz-Herion *et al.*, 2007).

Nuclear assembly

Nuclear assembly reactions, dextran exclusion, nuclear transport assays, and transmission electron microscopy were performed as described (Theerthagiri *et al.*, 2010). Generation of affinity resins for protein depletion and preparation of sperm heads and floated membranes were described previously (Franz *et al.*, 2005). For depletions, high-speed extracts were incubated twice with a 1:1.2 bead-to-cytosol ratio for 20 min (Franz *et al.*, 2007). Prelabeled membranes were prepared as in Antonin *et al.* (2005) using DiIC18.

All fluorescence microscopy images were recorded on the confocal microscope (FV1000 [Olympus, Center Valley, PA] equipped with a photomultiplier [model R7862; Hamamatsu, Hamamatsu, Japan]) with 405-, 488-, and 559-nm laser lines and a 60× numerical aperture 1.35 oil immersion objective lens using the FluoView software (Olympus) at room temperature with Vectashield (Vector Laboratories, Burlingame, CA) as a mounting medium.

Generation of Nup93 and fragments

Expression constructs for full-length *Xenopus* Nup93 and fragments were generated from a synthetic DNA optimized for codon usage and expression in *E. coli* (Geneart, Regensburg, Germany; see Supplemental Information for DNA sequence) and cloned into a modified pET28a vector (EMD) with a yeast SUMO (SMT3) as solubility tag, followed by a recognition site for the tobacco etch virus (TEV) protease upstream of the Nup93 fragments. The proteins were expressed in *E. coli*, purified using Ni-nitrilotriacetic acid agarose (Qiagen) via N-terminal His6 tag, and dialyzed to sucrose buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 50 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose, pH 7.5) and the His6 and SUMO tag was cleaved using TEV protease.

Pulldown experiments

Full-length *Xenopus* Nup53 or the nucleoplasmic domain of gp210 (Antonin *et al.*, 2005) was cloned into a modified pET28a vector (EMD) with GST tag, followed by a recognition site for thrombin protease and purified via an N-terminal His6 tag as described. In a 800-µl volume, 2 µM of the respective proteins was incubated with *Xenopus* egg extracts (diluted 1:1 with 10 mM HEPES, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.5, and cleared by centrifugation for 10 min at 100,000 rpm in a TLA110 rotor [Beckman Coulter, Brea, CA]) and 5 µM SUMO-tagged Nup93 (aa 183–582) or Nup93 (aa 608–820), respectively (except for the TEV cleavage generated and purified as in the preceding section). After 2 h, 50 µl of GSH–Sepharose (GE Healthcare, Piscataway, NJ) was added and the sample incubated for another 90 min. After six washes with PBS the samples were eluted by cleavage with thrombin (0.1 mg/ml) for 1 h and analyzed by SDS–PAGE and Western blotting.

For bacterial lysate pulldown experiments, full-length *Xenopus* Nup155 was cloned into a modified pET28a vector containing an N-terminal NusA solubility tag and a C-terminal His6 tag. In a volume of 800 µl, 2 µM GST or GST-Nup53 was incubated with 500 µl of lysates from bacteria expressing Nup155 or from untransformed bacteria and 10 µM recombinant Nup93 (608–820), After 1 h, 50 µl of GSH–Sepharose (GE Healthcare) was added, and the sample was further incubated for 1 h. After six washes with 20 mM Tris and 50 mM NaCl, pH 7.4, the samples were eluted by cleavage using 30 µl of TEV protease (0.5 mg/ml) for 1 h and analyzed by SDS–PAGE and Western blotting.

ACKNOWLEDGMENTS

We thank Nathalie Eisenhardt, Adriana Magalska, Allana Schooley, and Benjamin Vollmer for critical reading of the manuscript and Birthe Fahrenkrog and Iain Mattaj for the kind gift of antibodies.

REFERENCES

Alber F *et al.* (2007). The molecular architecture of the nuclear pore complex. Nature 450, 695–701.

Allende ML, Amsterdam A, Becker T, Kawakami K, Gaiano N, Hopkins N (1996). Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. Genes Dev 10, 3141–3155.

Amlacher S, Sarges P, Flemming D, van Noort V, Kunze R, Devos DP, Arumugam M, Bork P, Hurt E (2011). Insight into structure and assembly of the nuclear pore complex by utilizing the genome of a eukaryotic thermophile. Cell 146, 277–289.

Anderson DJ, Vargas JD, Hsiao JP, Hetzer MW (2009). Recruitment of functionally distinct membrane proteins to chromatin mediates nuclear envelope formation in vivo. J Cell Biol 186, 183–191.

Antonin W, Ellenberg J, Dultz E (2008). Nuclear pore complex assembly through the cell cycle: regulation and membrane organization. FEBS Lett 582, 2004–2016.

Antonin W, Franz C, Haselmann U, Antony C, Mattaj IW (2005). The integral membrane nucleoporin pom121 functionally links nuclear pore complex assembly and nuclear envelope formation. Mol Cell 17, 83–92.

Antonin W, Ungricht R, Kutay U (2011). Traversing the NPC along the pore membrane: targeting of membrane proteins to the INM. Nucleus 2, 87–91.

Brohawn SG, Partridge JR, Whittle JR, Schwartz TU (2009). The nuclear pore complex has entered the atomic age. Structure 17, 1156–1168.

Cronshaw JM, Krutchinsky AN, Zhang W, Chait BT, Matunis MJ (2002). Proteomic analysis of the mammalian nuclear pore complex. J Cell Biol 158, 915–927.

Dultz E, Zanin E, Wurzenberger C, Braun M, Rabut G, Sironi L, Ellenberg J (2008). Systematic kinetic analysis of mitotic dis- and reassembly of the nuclear pore in living cells. J Cell Biol 180, 857–865.

Fahrenkrog B, Hubner W, Mandinova A, Pante N, Keller W, Aebi U (2000). The yeast nucleoporin Nup53p specifically interacts with Nic96p and is directly involved in nuclear protein import. Mol Biol Cell 11, 3885–3896.

Franz C, Askjaer P, Antonin W, Iglesias CL, Haselmann U, Schelder M, de Marco A, Wilm M, Antony C, Mattaj IW (2005). Nup155 regulates nuclear envelope and nuclear pore complex formation in nematodes and vertebrates. EMBO J 24, 3519–3531.

Franz C, Walczak R, Yavuz S, Santarella R, Gentzel M, Askjaer P, Galy V, Hetzer M, Mattaj IW, Antonin W (2007). MEL-28/ELYS is required for the recruitment of nucleoporins to chromatin and postmitotic nuclear pore complex assembly. EMBO Rep 8, 165–172.

Galy V, Mattaj IW, Askjaer P (2003). *Caenorhabditis elegans* nucleoporins Nup93 and Nup205 determine the limit of nuclear pore complex size exclusion in vivo. Mol Biol Cell 14, 5104–5115.

Grandi P, Dang T, Pane N, Shevchenko A, Mann M, Forbes D, Hurt E (1997). Nup93, a vertebrate homologue of yeast Nic96p, forms a complex with a novel 205-kDa protein and is required for correct nuclear pore assembly. Mol Biol Cell 8, 2017–2038.

Grandi P, Doye V, Hurt EC (1993). Purification of NSP1 reveals complex formation with "GLFG" nucleoporins and a novel nuclear pore protein NIC96. EMBO J 12, 3061–3071.

Grandi P, Schlaich N, Tekotte H, Hurt EC (1995). Functional interaction of Nic96p with a core nucleoporin complex consisting of Nsp1p, Nup49p and a novel protein Nup57p. EMBO J 14, 76–87.

Harel A, Orjalo AV, Vincent T, Lachish-Zalait A, Vasu S, Shah S, Zimmerman E, Elbaum M, Forbes DJ (2003). Removal of a single pore subcomplex results in vertebrate nuclei devoid of nuclear pores. Mol Cell 11, 853–864.

Hawryluk-Gara LA, Platani M, Santarella R, Wozniak RW, Mattaj IW (2008). Nup53 is required for nuclear envelope and nuclear pore complex assembly. Mol Biol Cell 19, 1753–1762.

Hetzer MW, Wente SR (2009). Border control at the nucleus: biogenesis and organization of the nuclear membrane and pore complexes. Dev Cell 17, 606–616.

Jeudy S, Schwartz TU (2007). Crystal structure of nucleoporin nic96 reveals a novel, intricate helical domain architecture. J Biol Chem 282, 34904–34912.

Krull S, Thyberg J, Bjorkroth B, Rackwitz HR, Cordes VC (2004). Nucleoporins as components of the nuclear pore complex core structure and Tpr as the architectural element of the nuclear basket. Mol Biol Cell 15, 4261–4277.

Kutay U, Hetzer MW (2008). Reorganization of the nuclear envelope during open mitosis. Curr Opin Cell Biol 20, 669–677.

Mansfeld J *et al.* (2006). The conserved transmembrane nucleoporin NDC1 is required for nuclear pore complex assembly in vertebrate cells. Mol Cell 22, 93–103.

Miller BR, Powers M, Park M, Fischer W, Forbes DJ (2000). Identification of a new vertebrate nucleoporin, Nup188, with the use of a novel organelle trap assay. Mol Biol Cell 11, 3381–3396.

Mitchell JM, Mansfeld J, Capitanio J, Kutay U, Wozniak RW (2010). Pom121 links two essential subcomplexes of the nuclear pore complex core to the membrane. J Cell Biol 191, 505–521.

Mohr D, Frey S, Fischer T, Guttler T, Gorlich D (2009). Characterisation of the passive permeability barrier of nuclear pore complexes. EMBO J 28, 2541–2553.

Onischenko E, Stanton LH, Madrid AS, Kieselbach T, Weis K (2009). Role of the Ndc1 interaction network in yeast nuclear pore complex assembly and maintenance. J Cell Biol 185, 75–491.

Osmani AH, Davies J, Liu HL, Nile A, Osmani SA (2006). Systematic deletion and mitotic localization of the nuclear pore complex proteins of *Aspergillus nidulans*. Mol Biol Cell 17, 4946–4961.

Rasala BA, Orjalo AV, Shen Z, Briggs S, Forbes DJ (2006). ELYS is a dual nucleoporin/kinetochore protein required for nuclear pore assembly and proper cell division. Proc Natl Acad Sci USA 103, 17801–17806.

Rexach M (2009). Piecing together nuclear pore complex assembly during interphase. J Cell Biol 185, 377–379.

Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, Chait BT (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. J Cell Biol 148, 635–651.

Schrader N, Stelter P, Flemming D, Kunze R, Hurt E, Vetter IR (2008). Structural basis of the nic96 subcomplex organization in the nuclear pore channel. Mol Cell 29, 46–55.

Schwarz-Herion K, Maco B, Sauder U, Fahrenkrog B (2007). Domain topology of the p62 complex within the 3-D architecture of the nuclear pore complex. J Mol Biol 370, 96–806.

Stavru F, Nautrup-Pedersen G, Cordes VC, Gorlich D (2006). Nuclear pore complex assembly and maintenance in POM121- and gp210-deficient cells. J Cell Biol 173, 477–483.

Theerthagiri G, Eisenhardt N, Schwarz H, Antonin W (2010). The nucleoporin Nup188 controls passage of membrane proteins across the nuclear pore complex. J Cell Biol 189, 1129–1142.

Vasu S, Shah S, Orjalo A, Park M, Fischer WH, Forbes DJ (2001). Novel vertebrate nucleoporins Nup133 and Nup160 play a role in mRNA export. J Cell Biol 155, 339–354.

Walther TC *et al.* (2003). The conserved Nup107–160 complex is critical for nuclear pore complex assembly. Cell 113, 195–206.

Yoon JH, Whalen WA, Bharathi A, Shen R, Dhar R (1997). Npp106p, a Schizosaccharomyces *pombe* nucleoporin similar to *Saccharomyces cerevisiae* Nic96p, functionally interacts with Rae1p in mRNA export. Mol Cell Biol 17, 7047–7060.

Zabel U, Doye V, Tekotte H, Wepf R, Grandi P, Hurt EC (1996). Nic96p is required for nuclear pore formation and functionally interacts with a novel nucleoporin, Nup188p. J Cell Biol 133, 1141–1152.