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The stoichiometry of the nucleoporin 62 subcomplex of the nuclear pore in solution

Alexander Ulrich\textsuperscript{a,b}, James R. Partridge\textsuperscript{a}, and Thomas U. Schwartz\textsuperscript{a}
\textsuperscript{a}Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{b}Institut für Chemie und Biochemie, AG Strukturbiochemie, Freie Universität Berlin, 14195 Berlin, Germany

ABSTRACT The nuclear pore complex (NPC) regulates transport between the nucleus and cytoplasm. Soluble cargo-protein complexes navigate through the pore by binding to phenylalanine-glycine (FG)-repeat proteins attached to the channel walls. The Nup62 complex contains the FG-repeat proteins Nup62, Nup54, and Nup58 and is located in the center of the NPC. The three proteins bind each other via conserved coiled-coil segments. To determine the stoichiometry of the Nup62 complex, we undertook an in vitro study using gel filtration and analytical ultracentrifugation. Our results reveal a 1:1:1 stoichiometry of the Nup62 complex, where Nup54 is central with direct binding to Nup62 and Nup58. At high protein concentration, the complex forms larger assemblies while maintaining the Nup62:Nup54:Nup58 ratio. For the homologous Nsp1 complex from \textit{Saccharomyces cerevisiae}, we determine the same stoichiometry, indicating evolutionary conservation. Furthermore, we observe that eliminating one binding partner can result in the formation of complexes with noncanonical stoichiometry, presumably because unpaired coiled-coil elements tend to find a promiscuous binding partner. We suggest that these noncanonical stoichiometries observed in vitro are unlikely to be physiologically relevant.

INTRODUCTION

In the eukaryotic cell, the genetic information is stored and transcribed in the nucleus, while mRNA translation into proteins occurs in the cytoplasm. As a result, a large number and diverse set of molecules has to be transported across the double-layered membrane that is the nuclear envelope (NE). Nuclear pore complexes (NPCs) are the essential transport gates that sit in circular openings in the NE. In all eukaryotes examined, NPCs are built from multiple copies of \textasciitilde 30 nucleoporins (nups; Rout \textit{et al.}, 2000; Cronshaw, 2002), which are arranged around a central eightfold rotational axis (Gall, 1967; Maul, 1971; Unwin and Milligan, 1982; Onischenko and Weis, 2011). The overall architecture of the NPC is conserved between eukaryotes as shown by electron microscopy (EM; Reichelt \textit{et al.}, 1990) and cryoEM (Akey and Radermacher, 1993; Beck \textit{et al.}, 2007; Frenkiel-Krispin \textit{et al.}, 2010). The cylindrical NPC has an outer diameter of \textasciitilde 125 nm surrounding an inner pore of \textasciitilde 50 nm, and a height of 60–95 nm without its asymmetrical nuclear and cytoplasmic extensions, the “nuclear basket” and “cytoplasmic filaments.” Many nups are conserved across all eukaryotes (DeGrasse \textit{et al.}, 2009; Neumann \textit{et al.}, 2010) and can be roughly categorized into three groups: membrane nups anchor the NPC to the NE, scaffold nups form the framework of the NPC, and barrier nups build the permeability barrier for selective transport (Grossman \textit{et al.}, 2012). In recent years, x-ray structures of individual nups and their subcomplexes have been obtained, facilitated by the modular assembly of the complex (Brohawn \textit{et al.}, 2009). These structures revealed common structural features between the NPC scaffold and endomembrane trafficking coats (COPI, COPII, clathrin) that appeared early in the evolution of the eukaryotic endomembrane system (Devos \textit{et al.}, 2004, 2006; Mans \textit{et al.}, 2004; Brohawn \textit{et al.}, 2008).

The NPC scaffold consists of four major subcomplexes. The best-characterized of these is the multimeric, \textasciitilde 0.5-MDa Y complex, so named because of its characteristic shape (Lutzmann, 2002; Kampmann and Blobel, 2009; Bilokapic and Schwartz, 2012; Fernandez-Martinez \textit{et al.}, 2012). The Y complex consists of...
7–10 members, depending on the organism, and is the main scaffolding unit of the NPC, essential for its assembly (Harel et al., 2003; Walther et al., 2003). The Ndc1 subcomplex contains membrane-bound nups that anchor the NPC scaffold into the circular openings of the NE (Onischenko et al., 2009). It binds directly to the Nup93 subcomplex (Nehrbass et al., 1996), which acts as an adaptor between the NE and the central pore facing the Nup62 subcomplex (Nsp1 subcomplex in Saccharomyces cerevisiae; Marelli, 1998; Amlacher et al., 2011).

The most central, pore-facing subcomplex is the trimeric Nup62 complex, which connects to the NPC scaffold by direct coiled-coil interaction with Nup93 (Nic96 in S. cerevisiae; Grandi et al., 1995b; Bailier et al., 2001; Schrader et al., 2008). The three Nup62 complex members—Nup62, Nup54, and Nup58—have been localized to the center of the NPC by immunoelectron microscopy (Guan et al., 1995; Hu et al., 1996). The yeast homologues Nsp1 (Carmo-Fonseca et al., 1991; Buss and Stewart, 1995), Nup57, and Nup49 are essential for cell viability (Hurt, 1988; Wente et al., 1992; Grandi et al., 1995b). Each of the three Nup62-complex members contains a coiled-coil domain that connects the binding partners (Bailer et al., 2001; Melcák et al., 2007). The coiled-coil domain is flanked N-terminally (Nup54, Nup62) or both N- and C-terminally (Nup58) by unstructured phenylalanine–glycine (FG)-repeat regions (Wente et al., 1992; Hu et al., 1996). The sequences of the coiled-coil domains are relatively well conserved, in contrast to the FG-repeat regions (Supplemental Figure S1). The FG repeats are necessary for the main function of the NPC: the selective transport through the nuclear membrane (Mohr et al., 2009). The central transport pore of the NPC is filled with protein filaments containing FG repeats, which are essential for nuclear transport (Finlay et al., 1991). In yeast, a total of ~160 individual FG nucleoporins are located at the inner pore of the NPC (Alber et al., 2007). Small molecules (<40 kDa) can diffuse freely through the pore. Larger cargoes require active transport, facilitated by soluble nuclear transport receptors that engage in temporary binding to FG repeats (Cook et al., 2007; Choob and Suel, 2011).

To unravel the composition and organization of the FG network that fills the central pore of the NPC, it is very important to gain a more detailed picture of the transport process. Questions concern the anchor points for different FG-proteins, the nature of the FG repeats (cohesive, noncohesive, etc.), and the copy numbers and stoichiometries of the individual FG-nups. For the Nup62 complex, various studies have attempted to address its composition, but the results are inconsistent. Using native Nup62 complex purifications from rat liver nuclei and subsequent size exclusion chromatography and gel electrophoresis, researchers reported Nup58:Nup54:Nup62 ratios of 1:4:4 (Finlay et al., 1991), 1:2:1 (Kita et al., 1993), and 2:4:2 (Buss and Stewart, 1995). Guan and colleagues also used purified native rat protein but applied cross-linking and scanning transmission electron microscopy (STEM) analysis to estimate a molar ratio of 1:1:1:1 (including the Nup58 splice variant Nup45; Guan et al., 1995). In Xenopus egg extracts, an equimolar ratio of purified Nup62 subcomplex was reported (Macaulay et al., 1995). Other studies reported ratios based on purifications of entire rat nuclei of Nup45:Nup58:Nup54:Nup62 equal to 2:3:2:3 by quantitative two-dimensional gel electrophoresis (Cronshaw, 2002). In yeast nuclei, the Nup49:Nup57:Nsp1 complex stoichiometry was measured to be 1:1:2 by quantitative immunoblotting (Rout et al., 2000); the same ratio was reported by a recent targeted proteomic measurement of the human NPC (Ori et al., 2013). Taking into account that Nup62:Nsp1 is also present in the Nup62-Nup88-Nup214 subcomplex (Nsp1-Nup82-Nup159 in yeast), two studies support an isostoichiometric occurrence of Nup62:Nup54:Nup58 within the Nup62 subcomplex (Grandi et al., 1995a; Belgareh et al., 1998). In a computational model of the yeast NPC, an Nsp1-complex stoichiometry of 1:1:1 was predicted (Alber et al., 2007). The first crystal structure of a member of the Nup62 complex was published in 2007 (Melcák et al., 2007). An 85-residue fragment of the coiled-coil region of rat Nup58 (aa 327–411) formed a tetrameric helical bundle in the crystal, consisting of a dimer of dimers. Structural differences between Nup58 crystallized in two independent crystal forms, of which only one is publicly available in the Protein Data Bank (PDB 2OSZ), were interpreted as possibly representing a sliding mechanism of Nup58 that alters the diameter of the transport channel. The ability of individual FG-nups to form oligomers was also observed for Nup62, forming linear fibers visible by EM (Buss et al., 1994), and for a 35-residue Nup54 coiled-coil fragment (aa 460–494) observable by a crystal structure (Solmaz et al., 2013). Solmaz and colleagues also reported the crystal structures of isolated coiled-coil fragments of a Nup54 (aa 346–402)–Nup62 (aa 364–419) complex and of a Nup58 (aa 327–412)–Nup54 (aa 456–494) complex (Solmaz et al., 2011). In both cases, the asymmetric unit contained a 1:2 heterotrimer. Taking all the crystallographic data together, an overall ratio of Nup58:Nup54:Nup62 of 1:2:4 was suggested; however, it was never confirmed using an independent approach.

Because the biochemically obtained Nup62 complex ratios (Finlay et al., 1991; Kita et al., 1993; Buss and Stewart, 1995; Guan et al., 1995; Rout et al., 2000; Cronshaw, 2002) are not consistent among each other and also differ substantially from the ratio deduced from the crystal structures (Melcák et al., 2007; Solmaz et al., 2011, 2013), we set out to solve this ambiguity. We determined the stoichiometry of the Nup62 complex in solution; we purified recombinant trimeric Rattus norvegicus (Rn) Nup62 complex and the homologous S. cerevisiae (Sc) Nsp1 complex and analyzed its molecular weight and oligomerization state by size exclusion chromatography and sedimentation equilibrium analytical ultracentrifugation.

RESULTS

Composition of the Nup58-Nup54-Nup62 complex

To determine the molecular composition of the mNup58-Nup54-Nup62 complex, we coexpressed and copurified the predicted α-helical portions of the three proteins. To study the evolutionary conservation, we also studied the homologous mScNup49-Nup57-Nsp1 complex (Figures 1 and S1). The “long” mNup58-Nup54-Nup62 complex contains the entire predicted α-helical portions of Nup58 (aa 239–415), Nup54 (aa 346–494), and Nup62 (aa 322–525). N-terminally truncated Nup58 (aa 327–415) can still form a trimeric “short” mNup58-Nup54-Nup62 complex, which exhibits higher solubility. A homologous “short” mScNup49-Nup57-Nsp1 construct was designed based on the high-solubility “short” mNup58-Nup54-Nup62 complex (Figures 1 and S1). All three heterotrimeric complexes were analyzed by gel filtration (Figure 2A). They elute as single peaks from a gel-filtration column, without any significant signs of complex disassembly during the experiment. Based on visual observation of the peak fractions on Coomassie blue-stained SDS–PAGE gels, all samples appear homogenous in composition and have a protein ratio of 1:1:1 (Figure 2A; mScNup57 is partially proteolyzed).

For accurate mass determination, we assayed the three trimeric complexes by sedimentation equilibrium analytical ultracentrifugation (SE-AUC). Fitted data with residuals for all samples are shown in Figure S2. The long mNup58-Nup54-Nup62 complex was fitted about equally well with monomer-dimer (M-D), monomer-trimer

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FIGURE 1: Protein complexes used in this study. Definitions of all Nup58-Nup54-Nup62, Nup58-Nup54, and Nup54-Nup62 complexes used in our study. Narrow tubes represent FG-repeat regions; broad cylinders represent α-helical, predicted coiled-coil domains. Numbers indicate residue positions in the R. norvegicus proteins. Numbers in italics indicate protein residues in the S. cerevisiae homologues. Regions present in the particular complex are colored. The scNup49-Nup57-T4 Lysozyme complex contains, in addition to the residues of the scNup49-Nup57 complex, T4 Lysozyme N-terminally fused to Nup57.
that the investigated rmNup62/scNsp1 complexes are organized in a 1:1:1 ratio, with rmNup58-Nup54-Nup62 exhibiting a modest tendency to former higher-order oligomers. This difference in oligomerization behavior might explain why the size exclusion peak of the yeast complex appears sharper than the peaks of the rat complexes (Figure 2A).

Binary subcomplexes reveal direct interactions within the Nup62/Nsp1 complex

To find how the three Nup62/Nsp1 components directly interact, we coexpressed and copurified all three binary combinations and tested for complex formation. rmNup54 can form a stable complex both with rmNup62 alone as well as with rmNup58 alone (Figure 2, B and C). On the other hand, rmNup58 and rmNup62 do not form a stable complex. The same behavior was seen when we assayed the yeast homologues (unpublished data). Thus we conclude that Nup54 in metazoa and Nup57 in yeast are in the center of the complex bridging Nup62 (scNsp1) and Nup58 (scNup49).

Composition of an incomplete Nup58-Nup54 complex

To identify the molecular ratio of the binary Nup58-Nup54 subcomplex, we eliminated Nup62 from the trimeric Nup58-Nup54-Nup62 complex (Figure 1). We also tested the published Nup58-Nup54 crystal construct (Solmaz et al., 2011). Both constructs were purified and both eluted in a single peak in gel filtration (Figure 2B). We further designed a minimal scNup49-Nup57 construct, stable on a size exclusion column. Interestingly, although the ends of the...
rather than 1:2, as seen in the published crystal structure (PDB code 3T98). This 2:1 ratio is also supported by the relative band intensities on SDS–PAGE gels (Figure 2B). Thus, depending on how the fragments are chosen, different Nup58:Nup54 ratios can be obtained in vitro. Finally, the yeast Nup49-Nup57 complex is experimentally determined as 32.3 kDa, which could be explained by either a 2:1 (34.7 kDa) or a 1:2 stoichiometry (32.0 kDa) (Table 1). To increase the weight differences and thus better distinguish between the two stoichiometries, we fused T4 Lysozyme to the N terminus of scNup57. In this configuration, the complex size is most consistent with a 2:1 ratio (scNup49:scNup57), the same composition we also observed for the rat Nup58-Nup54 crystal construct. We conclude that, in absence of Nup62/scNsp1, the two remaining proteins Nup58/scNup49 and Nup54/scNup57 still form a trimeric complex, where Nup62/scNsp1 is substituted by either of the two remaining components, depending on the sequence context.

### Table 1: Results of SE-AUC experiments.

| Complex                        | Expected MW in kDa (various protein ratios) | Calculated MW in kDa | Fitting model | Rotor speeds | SD of residuals |
|-------------------------------|--------------------------------------------|----------------------|---------------|--------------|----------------|
| R. norvegicus trimeric Nup62 long | 63.8 (1:1:1)                               | 59.1                 | M-D           | 1-4          | 6.5711E-03     |
| R. norvegicus trimeric Nup62 short | 53.8 (1:1:1)                               | 61.3                 | M-D           | 1-4          | 8.5899E-03     |
| S. cerevisiae trimeric Nsp1    | 56.9 (1:1:1)                               | 51.7                 | Single        | 1-4          | 6.1343E-03     |
| R. norvegicus Nup58-Nup54 long | 30.1 (1:1)                                | 42.8 (2:1)           | Single        | 1-4          | 7.1562E-03     |
| R. norvegicus Nup58-Nup54 short | 18.6 (1:1)                                | 31.3 (2:1)           | Single        | 2-4          | 5.6959E-03     |
| S. cerevisiae Nup49-Nup57      | 22.2 (1:1)                                | 34.7 (2:1)           | Single        | 2-4          | 6.5178E-03     |
| S. cerevisiae Nup49-T4-L-Nup57 | 40.7 (1:1)                                | 53.2 (2:1)           | Single        | 2-4          | 6.3640E-03     |
| R. norvegicus Nup54-Nup62 long | 31.0 (1:1)                                | 49.9 (2:1)           | Single        | 2-4          | 8.5302E-03     |
| R. norvegicus Nup54-Nup62 short | 16.4 (1:1)                                | 25.2 (2:1)           | Single        | 2-4          | 5.2776E-03     |
| S. cerevisiae Nup57-Nsp1       | 35.8 (1:1)                                | 56.7 (2:1)           | Single        | 2-4          | 6.2001E-03     |

See Table S1 for more details. Data fit and residual distribution diagrams are presented in Figure S2.

The expected molecular weight (MW) was calculated from the protein sequences. Protein ratios are indicated in parentheses.

Molecular weight (MW) was calculated from SE-AUC data analyzed with UltraScan II (Demeler, 2013).

Selected fitting model used for analysis of AUC raw data. Single, single-species model; M-D, monomer-dimer model; M-Tri, monomer-trimer model; M-Tetr, monomer-tetramer model.

Selected rotor speed for analysis. Speeds were included in analysis if data were between 0.1 and 0.9 AU 280 nm, contained a gradient of at least 0.4 AU 280 nm and at least a channel radius fraction of 0.07 cm. Speed 1 = 10,000 rpm; speed 2 = 15,000 rpm; speed 3 = 20,000 rpm; speed 4 = 25,000 rpm.

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mNup58/scNup49 fragment are very similar between the crystal structure and our minimal yeast construct, the mNup54/scNup57 fragments are shifted (Figures 1 and S1). When aligned, the m-Nup54 and the scNup57 fragments share a common core of only 25 residues. The minimal yeast constructs extends N-terminally by 39 additional residues, while the rat construct extends by 24 residues C-terminally (Figures 1 and S1). Purification of a yeast complex analogous to the published rnNup58-Nup54 crystal structure failed due to poor protein expression.

We again performed SE-AUC experiments with the three binary mNup58-Nup54 and scNup49-Nup57 complexes. Fitted data with residuals for the samples are shown in Figure S2. Data of all three complexes fit a single-species model. The calculated molecular weight of the long mNup58-Nup54 complex was 53.2 kDa. A 1:1 stoichiometry (30.1 kDa) was expected, assuming the binary complex is organized like the trimeric complex but without Nup62. However, the calculated mass is closest to a Nup58:Nup54 ratio of 1:2, suggesting that Nup54 can substitute for the missing Nup62. In contrast, the crystal Nup58-Nup54 construct has a measured molecular weight of 29.9 kDa, which fits best with a Nup58:Nup54 ratio of 2:1 (31.3 kDa), rather than 1:2, as seen in the published crystal structure (PDB code 3T98). This 2:1 ratio is also supported by the relative band intensities on SDS–PAGE gels (Figure 2B). Thus, depending on how the fragments are chosen, different Nup58:Nup54 ratios can be obtained in vitro. Finally, the yeast Nup49-Nup57 complex is experimentally determined as 32.3 kDa, which could be explained by either a 2:1 (34.7 kDa) or a 1:2 stoichiometry (32.0 kDa) (Table 1). To increase the weight differences and thus better distinguish between the two stoichiometries, we fused T4 Lysozyme to the N terminus of scNup57. In this configuration, the complex size is most consistent with a 2:1 ratio (scNup49:scNup57), the same composition we also observed for the rat Nup58-Nup54 crystal construct. We conclude that, in absence of Nup62/scNsp1, the two remaining proteins Nup58/scNup49 and Nup54/scNup57 still form a trimeric complex, where Nup62/scNsp1 is substituted by either of the two remaining components, depending on the sequence context.

### Composition of an incomplete Nup54-Nup62 complex

To analyze the molecular composition of an incomplete Nup54-Nup62 complex, we first eliminated Nup58 from the short
mNup58-Nup54-Nup62 complex. Although we were able to copurify the binary construct, the amounts were not sufficient for AUC analysis (Figure S3). However, we had previously determined a minimal scNup57-Nsp1 construct containing the full α-helical portion of scNup57 and a C-terminally truncated scNsp1 compared with scNsp1 in the scNup49-Nup57-Nsp1 construct (Figure 1). We translated this construct to rat (Figure S1) and expressed both complexes and obtained them in sufficient purity and amount for AUC analysis (Figure 2C). In addition, we used a construct equal to the mNup54-Nup62 crystal construct previously used by Solmaz et al. (2011; Figure 1).

We performed SE-AUC experiments with these three subcomplexes. Fitted data with residuals for all samples are shown in Figure S2. The long mNup58-Nup54-Nup62 complex was successfully fitted to monomer-trimer and monomer-tetramer models. The resulting molecular weights were 48.0 kDa (M-Tri) and 48.3 kDa (M-Tetr). This matches a Nup54:Nup62 ratio of either 2:1 (49.9 kDa) or 1:2 (43.1 kDa) but not of 1:1 (31.0 kDa) (Table 1). Band intensities on SDS–PAGE gels support a Nup54:Nup62 ratio of 1:2 (Figure 2C). The crystal construct was successfully fitted with the single-species model. A molecular weight of 26.0 kDa was calculated, also indicating a Nup54:Nup62 ratio of 2:1 (25.2 kDa) or 1:2 (24.1 kDa) but not a 1:1 ratio (16.4 kDa). The composition of the mNup54-Nup62 subcomplex is, in analogy to the mNup58-Nup54 subcomplex, different from the composition of the three-protein complex. The mNup54 surface involved in binding of the missing protein mNup58 might be occupied by an additional copy of mNup54 or, more likely, mNup62. The yeast homologue of the long mNup54-Nup62 complex was fitted to a single-species model and has a calculated molecular weight of 27.0 kDa. Different from the rat complex, this is indicating a 1:1 ratio with 35.8 kDa or 33.6 kDa (see degradation issue of Nup57, discussed above) rather than a 2:1 or 1:2 ratio. Taken together, incomplete mNup54-Nup62 and scNup57-Nsp1 complexes exhibit different behavior in compensating for the missing mNup58/scNup49 component.

**DISCUSSION**

In this study, we examined the oligomerization state of the Nup62 complex. We find that the Nup62 complex is organized in a 1:1 stoichiometry, as shown by our gel filtration, SDS–PAGE, and SE-AUC data. This ratio is also observed for the homologous Nsp1 complex from *S. cerevisiae*, separated by an estimated ~1.5 billion
years of evolution (Hedges et al., 2004). Therefore the 1:1:1 stoichiometry appears to be a common, well-conserved design principle of the Nup62/Nsp1 complex (Figure 4). Fittingly, the amino acid sequences of the α-helical portions of Nup58, Nup54, and Nup62 are each relatively well conserved between distant eukaryotic species, more so, for example, than the large class of scaffold nucleoporins with stacked α-helical architecture (Figure S1). This observation suggests that the specific interactions between the three complex members are important. It possibly also indicates interaction sites with other proteins. Nup93 is a known candidate, but there might be others yet to be identified.

In a series of recent publications, the rat Nup62 complex was analyzed using a crystallographic approach (Melcák et al., 2007; Solmaz et al., 2011, 2013). Using rather small, -4- to 10-kDa coiled-coil fragments of dimeric subassemblies, the authors hypothesized that the in vivo composition of the Nup58-Nup54-Nup62 complex would be 1:2:4, inconsistent with our data. Coiled-coil regions in proteins are, by and large, interaction domains (Lupas and Gruber, 2005), and understanding the specificity of these represents an intense research field (Grigoryan and Keating, 2008). It is well known that the use of fragmented coiled-coil portions, taken out of their natural context, can yield non-cognate assemblies. We tested this by using six incomplete binary assemblies of Nup62 components. These tests reveal that mNup54/scNup57 is the central helical element in the trimeric assembly, with direct interaction with the two remaining proteins, mNup62/scNsp1 and rnNup58/scNup49, which do not directly interact in our experiments. We further observe that, if we take one binding partner out, the now-unoccupied binding site on the remaining protein does not necessarily stay vacant but is often bound promiscuously. Consequently, the resulting incomplete complex might have a 1:1 stoichiometry, but can also generate 1:2 or 2:1 complexes, depending on the sequence context. The fact that we do not see any of these noncanonical interactions occurring when we copurify the trimeric mNup62/scNsp1 complex strongly supports that the physiologically relevant stoichiometry is 1:1:1.

One prominent aspect of the hypothesis that the Nup62/Nsp1 complex might assemble in various stoichiometries is that it could potentially explain opening and closing of the NPC as suggested by EM and tomographic studies (Kiseleva et al., 1998; Beck et al., 2004). The fact that we observe a uniform stoichiometry of the Nup62/Nsp1 complex in solution neither supports nor challenges the observation that NPCs might dilate. Most current models consider the Nup62/Nsp1 complex anchored to the main NPC scaffold via Nup93/Nic96. The main scaffold itself is largely composed of the latticework generated by β-propellers and the different types of stacked α-helical domains, which are prominently represented in the Nup93/Nic96 complex and the Y complex. Because some of these scaffold proteins have intrinsic flexibility, observed structural dynamics within the assembled NPC can easily be explained by this characteristic alone. It is also important to ask what role a postulated dilation mechanism might play in the NPC transport function. At this point, it is still an open question whether the NPC scaffold needs to be dynamically arranged or whether it can simply function as a rigid entity once it is assembled. Because transport of most substrates through the wide central channel occurs simultaneously in both directions, flexibility of the scaffold is not an obvious necessity.

Another interesting consequence of a stable 1:1:1 stoichiometry of the Nup62/Nsp1 complex is that it supports the notion that the distribution of the various FG repeat–containing Nups within the NPC is organized and not arbitrary. FG repeats can be classified in different categories, and their behavior is not uniform (Patel et al., 2007; Frey and Görlich, 2009; Yamada et al., 2010). That the
stoichiometry of the Nup62/Nsp1 complex is highly conserved suggests that the FG network has a rather specific composition whose functional significance we have yet to fully understand. In support of a discrete composition of the Nup62/Nsp1 complex, it was recently shown that hydrogels made up of various Nup62-Nup54-Nup58 combinations exhibit substantially different transport characteristics (Labokha et al., 2013).

In conclusion, our analysis suggests that the universally conserved trimeric Nup62/Nsp1 complex arranges in a 1:1:1 stoichiometry. It will now be interesting to see where exactly this complex is anchored to the NPC scaffold. The organization of the different FG-nups within the transport channel is still largely unclear but needs to be understood to arrive at an accurate description of facilitated transport through the NPC.

**MATERIALS AND METHODS**

**Construct design**

DNA coding for coiled-coil portions of *R. norvegicus* Nup58, Nup54, and Nup62 and *S. cerevisiae* homologues Nup49, Nup57, and Nsp1 was cloned into modified pET-Duet plasmids containing either two or three expression cassettes. In all cases, the first cassette contained an N-terminal 6xHis tag. In addition, the *S. cerevisiae* Nup49-Nup57-Nsp1 construct contained a cleavable 74 Lysozyme fused to the N-terminus of Nsp1; the *S. cerevisiae* Nup49-Nup57-T4 Lysozyme construct contained T4 Lysozyme fused to the N-terminus of Nsp1; and the *S. cerevisiae* Nup57-Nsp1 construct contained a C-terminal 6xArg tag. All tags are cleavable by thrombin or 3C protease. The two Nup49-Nup57-Nsp1 constructs used for N-terminal truncation studies of Nsp1 contained a cleavable 74 Lysozyme fused to the N-terminus of Nup49 (see Table S2).

**Expression and purification of protein complexes**

Proteins were coexpressed in *Escherichia coli* BL21 (DE3) RIL in Luria–Bertani medium and induced with 200 μM isopropyl-β-D-thiogalactopyranoside at 18°C overnight. Cells were resuspended in solubilization buffer (50 mM sodium-phosphate, pH 8.0, 500 mM NaCl, 30 mM imidazole, pH 8.0, and 5 mM β-mercaptoethanol) and lysed using a homogenizer (Constant Systems, Kennesaw, GA). Protein complexes were bound to Ni-affinity resin (GE Healthcare, Waukesha, WI) and eluted with 250 mM imidazole (pH 8.0). After dialysis at 4°C against purification buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, and 1 mM DTT) samples were subjected to ion-exchange chromatography followed by proteolytic removal of tags and size exclusion chromatography. These steps were all performed at 4°C.

**Analysis of concentration-dependent protein oligomerization behavior**

Purified *R. norvegicus* protein complexes containing Nup58, Nup54, Nup62, or their *S. cerevisiae* homologues were each concentrated to 0.5 and 5.0 mg/ml. The long *R. norvegicus* Nup58-Nup54-Nup62 complex could maximally be concentrated to 3.8 mg/ml. The two concentrations of each complex were analyzed on a Superdex 200 10/300 GL (GE Healthcare) size exclusion column, and elution volumes were compared. The peak fractions were analyzed by SDS–PAGE.

**SE-AUC experiments**

Purified protein samples in purification buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, and 1 mM DTT) were concentrated to an absorption of 0.4 at 280 nm, centrifuged for 10 min at 10,000 × g to remove particulates, and filled into two sample chambers of an Epon 6-Channel Centerpiece (Beckman Coulter, Brea, CA). The AUC was performed in an Optima XL-I analytical ultracentrifuge with an An-Ti-50 Rotor (Beckman Coulter). The rotor and samples were incubated in the centrifuge for 1 h at 20°C before the start of centrifugation. Each AUC experiment was performed at 20°C with four rotor speeds: 10,000, 15,000, 20,000, and 25,000 rpm, starting with the lowest. At equilibrium, sample cells were scanned five times at 280 nm, with three replicas of each scan.

**AUC data analysis**

Data analysis of SE data was performed with UltraScan II (Demeler, 2013). Rotor speeds were included in analysis if, after removal of data points <0.1 or >0.9 AU 280 nm, remaining data contained an optical density gradient ≥0.4 AU 280 nm and a channel radius fraction of at least 0.07 cm. The partial specific volume at 20°C (vbar (20°C)) and the molar extinction coefficient at 280 nm (ε(280nm)) were calculated using UltraScan II, based on the protein sequence (Table S1). Data were fitted to multiple models; the most appropriate model was chosen based on visual inspection of the residual maps and best statistical parameters. Fit results contain the calculated molecular weight and statistical parameters (Table 1).

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