Depletion of nucleoporins from HeLa nuclear pore complexes to facilitate the production of ghost pores for in vitro reconstitution

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Abstract During cell division, Nuclear Pore Complexes (NPCs) are broken down into protein subcomplexes that are the basis for reassembly in daughter cells. This is the driving force for the establishment of an in vitro reconstitution system to study aspects of NPC reassembly. In this study, nuclear envelope (NE) was isolated from HeLa cells. NE was treated with increasing concentrations of heparin to extract nucleoporins (Nups) for the production of “ghost pores” which are pores severely deficient in Nups, while still containing Pore Membrane proteins (POM) needed to anchor the NPC. Ghost pores have been subjected to incubation with previously stripped Nups and some re-binding has been shown to occur by western blot analysis. This in vitro assay provides a powerful tool to investigate the protein–protein interactions of NPC reassembly from a human cell line. Through a better understanding of the process of NPC reassembly, we can continue to piece together the puzzle of this macromolecular structure. It is most advantageous to establish a straightforward reconstitution procedure at the mammalian level.

Keywords Nuclear pore complexes · Nuclear envelope · Nups · Heparin · Ghost pores

Abbreviations

NE	Nuclear envelope
NPC	Nuclear pore complex
Nups	Nucleoporins
FG-Nup	Phenylalanine–glycine nucleoporins
CE	Cell extract
INM	Inner nuclear membrane
ONM	Outer nuclear membrane
POM	Pore membrane
EM	Electron microscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
PBS	Phosphate buffered saline
DTT	Dithiothreitol
TEA	Triethanolamine

Introduction

Nuclear pore complexes (NPCs) are macromolecular arrangements of protein embedded in the Nuclear Envelope (NE) of eukaryotic cells (Alber et al. 2007; Bilokapic and Schwartz 2012; Brohawn et al. 2009; Lim et al. 2006a, 2008; Rout et al. 2000; Rout and Wentz 1994; Schwartz 2005; Tran and Wentz 2006). They are the sole facilitators of nucleocytoplasmic transport. Ions and small molecules are able to passively diffuse across the NE through these pores

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(Rout and Aitchison 2000; Schwartz 2005; Tran and Wentz 2006). Large biological molecules, such as proteins and RNAs, translocate across this membrane by receptor-mediated transport (Tran and Wentz 2006). NPCs are located at circular openings in the NE created by fusion of the inner nuclear membrane (INM) and outer nuclear membrane (ONM) (Lusk et al. 2007). Cryo-electron tomography reconstructions have revealed the NPC as a cylindrical structure that consists of a central core with an outer diameter of 125 nm and inner diameter of 60 nm (Beck et al. 2004, 2007; Maimon et al. 2012). While the NPC is an enormous molecular trafficking machine, its general architecture is highly systematic on many levels. Mammalian NPCs are ~125 MDa structures. They are composed of a family of ~30 different proteins, collectively referred to as nucleoporins (Nups), with a significant level of protein repetition (Alber et al. 2007; Cronshaw et al. 2002; Rout et al. 2000; Rout and Wentz 1994; Schwartz 2005). Nups are organized into a pseudo-symmetric structure with a twofold plane quasi-parallel to the nuclear envelope and an eight-fold axis of symmetry around the nucleo-cytoplasmic axis (Beck et al. 2004, 2007). Nups can be classified into groups based on their structure and function. Scaffold Nups have structural motifs that interlock (coiled coils, α -solenoids, β -propellers or disordered areas) while phenylalanine–glycine Nups (FG-Nups) contain flexible repeating units of phenylalanine–glycine (forming sea anemone-like fingers within the NPC which are implicated in active nuclear transport) (Alber et al. 2007; Lim et al. 2006b; Rout and Aitchison 2000; Schwartz 2005). These hydrophobic FG-repeat regions directly interact with karyopherins in the facilitated transport of protein cargo (Lim et al. 2006b). FG-Nups are highly responsible for the flexibility and dynamic nature of the NPC because of their lack of a rigid secondary structure (Lim et al. 2006a, 2008; Rout et al. 2000; Rout and Wentz 1994). The three Nups with transmembrane domains, also referred to as Pore Membrane proteins (POM), that act to anchor the NPC to the NE are gp210, NDC1 and POM121 (Cronshaw et al. 2002; Lusk et al. 2007; Shaulov et al. 2011).

Nups are stably organized into subcomplexes of proteins that are the foundation for the re-assembly of NPCs in daughter cells following open mitosis (Hetzer et al. 2005; Kutay and Hetzer 2008). Isolation of one Nup member by immunoprecipitation from cell

extracts, under non-denaturing conditions, facilitates the isolation of other subcomplex members (Belgareh et al. 2001; Cristea et al. 2005; Fontoura et al. 1999; Glavy et al. 2007). In preparation for cell division, NE surface area greatly increases and the number of NPCs embedded in the NE is doubled. In open mitosis, the mammalian NE breaks down (NEBD) (Anderson and Hetzer 2008; D'Angelo et al. 2006; Doucet and Hetzer 2010; Hetzer 2010; Kutay and Hetzer 2008; Muhlhauser and Kutay 2007; Shaulov et al. 2011). During this process, NPCs disassemble and subcomplex units are distributed throughout the cytoplasm, ER, and kinetochore (Hetzer 2010). POM proteins localize to the ER and kinetochore (Hetzer 2010). During reassembly, NPCs are formed by recruitment of subcomplexes, which were previously disassembled, to membrane bound states in the NE in a step-wise manner (Huang 2005; Tran and Wentz 2006). Several of these subcomplex components have been proven to be essential for complete rebuilding of an NPC. For example, the depletion of the scaffolding protein, Nup107 has been shown to prevent the proper attachment of its binding partner, Nup133 and other FG-Nups (Anderson and Hetzer 2008; Boehmer et al. 2003; Hetzer et al. 2005; Kutay and Hetzer 2008). Additionally, NDC1 and POM121 have been shown to be crucial for the reformation of NE in vertebrates (Anderson and Hetzer 2008; Huang 2005). Even after formation of the NE and reconstitution of NPCs, the nuclear membrane will undergo a series of structural changes and the points at which NPCs become available for transport is not clearly determined (Anderson and Hetzer 2008; Huang 2005). Residence time of Nups varies and possibly only a subset of Nups may be stably integrated into the NPC, while others are dynamic (Rabut et al. 2004). Furthermore, subsets of the same Nup may vary in their associations (Rabut et al. 2004).

The mechanisms by which NPCs are reformed in the NE of daughter cells remain unknown, though it is clear that the process is very carefully regulated by cellular machinery (Anderson and Hetzer 2008). The sequential order in which NPC subcomplexes are recruited to the NE remains to be determined. In an endeavor to learn more about the chronological order of addition and release, we attempt to develop a system for *in vitro* reconstitution. The basis for this reconstitution is the successful production of Nup extracted ghost pores. A ghost pore is defined as the intact NE that has been stripped of peripheral Nups

and most scaffolding Nups. The intact NE should thus contain POM proteins and lamina associated proteins. In our study, the stripping of Nups to form these ghost pores is achieved by heparin treatment of isolated HeLa cell NE. This is followed by the incubation of ghost pores with excess NPC subcomplex units. Our results provide evidence for the feasible development of an in vitro reconstitution system that will test protein–protein interactions.

Methods

Mammalian cell culture

HeLa cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) with 10 % Fetal Bovine Serum, 1 % Penicillin and Streptomycin. These monolayer cells were sub-cultured every 3–4 days. At 70–80 % confluence cells were collected in Phosphate Buffered Saline (PBS) with protease inhibitors (Sigma, St. Louis, MO, USA) for nuclei preparation.

Reagents

DNase (D-5319), RNase (R-4642), Low MW 3 kDa heparin sodium salt (H-3400) and protease inhibitors (P-8340) were purchased from Sigma. SulfoLink Coupling Resin (20401) for antibody affinity purification was purchased from Pierce Thermo Scientific (Rockford, IL, USA).

Antibodies

Affinity purified polyclonal antibodies against human Nup160, Nup133, Nup107, Nup75, Nup43, Nup93, NDC1, POM121 and gp210 were prepared as described (Glavy et al. 2007; Kaur et al. 2010) (Colcalico Biologicals, Reamtown, PA, USA) and used for western analysis. Antibodies Lap2 β and MAb414 were purchased from AbCam (ab2738, Cambridge, MA, USA) and Covance (MMS-120P, Emeryville, CA, USA), respectively. MAb414 antibodies specifically target FG-Nups by recognizing their repeating FG units (Davis and Blobel 1986, 1987).

Western analysis

Proteins were solubilized in sample buffer at 65 °C for 5 min, separated by sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS/PAGE) using 4–20 % Tris–glycine gels (Invitrogen), and transferred to nitrocellulose membranes. Membranes were blocked in 5 % non-fat milk. Specific proteins were probed with indicated primary antibodies then detected with peroxidase-conjugated secondary antibodies by chemiluminescence (34094, Super Signal West Femto Chemiluminescent Kit, Pierce, Thermo Scientific).

Buffer list for nuclei, NE, ghost pore preparation, and NPC reconstitution

Buffer A: 50 mM Tris (pH 7.5), freshly added protease inhibitors.

Buffer B: 0.25 M Sucrose, 50 mM Tris (pH 7.5), 25 mM KCl, 5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Dithiothreitol (DTT), freshly added protease inhibitors.

Buffer C: 2.3 M Sucrose, 50 mM Tris (pH 7.5), 25 mM KCl, 5 mM MgCl₂, 0.5 mM PMSF, 2 mM DTT, freshly added protease inhibitors.

Buffer D: 0.1 mM MgCl₂, 0.2 mM PMSF, 1 mM DTT, 0.5 μ l DNase/1 ml and 0.5 μ l RNase/1 ml

Buffer E: 10 % Sucrose, 0.1 mM MgCl₂, 0.2 mM PMSF, 1 mM DTT, 20 mM TEA (pH 8.5)

Buffer F: 30 % Sucrose, 0.1 mM MgCl₂, 0.2 mM PMSF, 1 mM DTT, 20 mM TEA (pH 7.5)

Buffer G: 10 % Sucrose, 0.1 mM MgCl₂, 0.2 mM PMSF, 1 mM DTT, 20 mM TEA (pH 7.5)

Preparation of HeLa nuclei

HeLa cells were cultured in preparation for isolation of nuclei on a sucrose bed (Blobel and Potter 1966a, b; Bornens and Courvalin 1978; Chardonnet and Dales 1970b, 1972; Cronshaw et al. 2002; Matunis 2006). Cells were lifted from plates by scraping in ice-cold PBS containing protease inhibitors. Cells were collected by scraping and pelleted by centrifugation (4 °C, 5 min, 1,300 g). The pellet (~80 million cells) was washed with PBS, resuspended in 7.5 ml hypotonic Buffer A and incubated on ice for 40 min. Cells were then ruptured by douncing and centrifuged (4 °C, 13 min, 2,100 g). Pellets were resuspended in Buffer B and centrifuged (4 °C, 13 min, 2,100 g). Pellets were resuspended in 3.5 ml mixture of Buffer 2C:1B and underlayered with 1.5 ml Buffer C in an ultracentrifuge tube to form a sucrose bed beneath the cell

lysate. The sample was ultra-centrifuged (35 min, 4 °C, 17,000g, SW55 rotor). Nuclear pellets were carefully isolated from the bottom of the tube, removing excess sucrose residue and extra-nuclear components. Nuclei were resuspended in Buffer B.

Preparation of HeLa NE

Nuclear pellets were resuspended by drop-wise addition of 1 ml Buffer D (containing DNase/RNase) with vortexing, followed by 4 ml of Buffer E and incubated at room temperature for 15 min. The digested nuclei were then underlaid with ~4 ml of Buffer F and centrifuged (4 °C, 15 min, 3,200 g). NE pellets were collected and resuspended in 200 µL Buffer G (Bornens and Courvalin 1978; Chardonnet and Dales 1970b, 1972; Matunis 2006). The nuclei and NE pellets were prepared for EM analysis as previously described (Chardonnet and Dales 1970a, 1972; Cronshaw et al. 2002; Pain et al. 1990).

Heparin treatment for ghost pore preparation

50 µL NE was mixed with 24 µL Buffer G with heparin to yield a total reaction volume of 74 µL. Heparin concentrations ranged from 0.1 mg/ml to 5 mg/ml. The reaction mixture was vortexed quickly and incubated on ice for 15 min. This was mixed by pipet, an aliquot was removed as total fraction and the reaction was centrifuged (4 °C, 5 min, 11,000 g). Pellets were washed with Buffer G without heparin. Samples were resuspended in sample buffer for immunoblotting.

Reconstitution of the NPC in HeLa cells

Heparin was previously removed from the pellet by washing. Heparin was removed from the supernatant fraction through microdialysis at 4 °C for 3 h against Buffer G without heparin. (Dialysis tubing, Carolina, with molecular weight cut off of 13 kDa) Dialyzed supernatant, in three-fold excess, was incubated with the stripped and washed 0.7 mg/ml heparin treated pellets. Heparin treated pellets were resuspended in Buffer G without heparin as a blank control. Reconstitution reaction was incubated for 30 min at room temperature with shaking. 10 % of the reconstitution and control reactions were aliquoted for the total fraction and the rest of the sample was then centrifuged

(4 °C, 5 min, 11,000 g). Products of the reconstitution and control reactions were subjected to western blot analysis.

Results

Preparation of HeLa nuclei and NE

Our attempts to initiate the in vitro reconstitution of HeLa cell NPCs were carried out by a ten-step procedure (Fig. 1) (Blethrow et al. 2008; Blobel and Potter 1966a, b; Bornens and Courvalin 1978; Chardonnet and Dales 1970b, 1972; Cronshaw et al. 2002; Matunis 2006). Each step was performed in the presence of protease inhibitors to reduce degradation. Nuclei were obtained from HeLa cells by incubating harvested cells in hypotonic buffer and rupturing by douncing (Chardonnet and Dales 1970b, 1972). Ruptured cells were centrifuged to obtain a crude nuclear pellet (Chardonnet and Dales 1970b, 1972). Nuclei were purified by ultracentrifugation over a 2.3 M sucrose cushion (Fig. 1). Figure 2 shows negative stain electron microscopy (EM) image of HeLa cell nuclei obtained by this procedure. Nuclear structures including NE, NPCs, nucleolus and heterochromatin regions are visible in this image. NE was obtained by DNase/RNase treatment of purified nuclei and further centrifuged through a sucrose cushion (Fig. 1). Figure 3 shows a negative stain EM image of HeLa NE treated with low levels of heparin. Intact NE is visible with clearly identifiable INM and ONM, chromatin regions and NPCs embedded in the NE. Proteins from rat liver NE, heparin treated rat liver NE, HeLa NE and heparin treated HeLa NE were separated using a 4–20 % gradient SDS/PAGE gel and compared by coomassie stain (Fig. 4a). This demonstrates the release of chromatin from its INM association by the reduced amount of histone proteins detected in the NE fractions as compared to those that have been heparin treated. Clearly visible are the nuclear lamins at around 67 kDa (Fig. 4a) and Nups were enriched in nuclear extract over cell extract as shown with MAb414 (Fig. 4b).

Heparin treatment for ghost pore preparation

Ghost pores were obtained by the classic heparin treatment of NE to remove chromatin association

Fig. 1 Experimental scheme **Fig. 2** Negative stain EM image of HeLa cell nuclei. Nuclear structures, NE and NPCs are visible. Bar, 500 nm

Heparin Treated HeLa Nuclear Envelope

Fig. 3 Negative stain EM image of HeLa NE treated with low levels of heparin (0.3 mg/ml). Intact NE with embedded NPCs are visible. Bar, 100 nm

Fig. 4 Nuclear Envelope Preparation. **a** Lane 1 HeLa NE, Lane 2 heparin treated HeLa NE, Lane 3 Rat NE and Lane 4 heparin treated Rat NE were separated on 4–20 % SDS/PAGE and coomassie stained. **b** Four cell fractions, Lane 1 CE cell extract, Lane 2 CY cytoplasm, Lane 3 NP nucleoplasm and Lane 4 NE Nuclear Envelope, were separated on 4–20 % SDS/PAGE, transferred to nitrocellulose and probed with MAb414 (Against FG-Nups)

with the INM and force the release of membrane bound NPC subcomplexes (Fig. 1) (Blethrow et al. 2008; Bornens and Courvalin 1978). Heparin is a

polysaccharide capable of interacting electrostatically with a large number of different proteins. It has the highest negative charge density of any known biomolecule and because of this it takes on a linear conformation in solution. An aliquot of heparin treated total protein prior to centrifugation, and pellet and supernatant fractions from centrifugation of heparin reactions, ranging from 0 mg/ml up to 5 mg/ml heparin, were analyzed (Fig. 5–7). Under these conditions the POM proteins: gp210, POM121, and NDC1 maintained their association with the NE (Figs. 5, 7). Western analysis with α -Lap2 β , an INM associated protein, indicates the integrity of NE is maintained up to 5 mg/ml heparin by the detection of Lap2 β in the pellet (Fig. 5). We believed an isomer of POM121 is found in the supernatant, while the rest of POM121 remains constant throughout heparin treatment (Fig. 5) (Blethrow et al. 2008; Cronshaw et al. 2002). This form of supernatant isomer POM121 runs as a doublet upon gel separation and may be less embedded at the NE (Funakoshi et al. 2007). We found the stability of the remaining POM proteins and α -Lap2 β to be consistent through increasing heparin levels.

Heparin treated NE was probed with α -Nup antibodies to determine the heparin concentration required to free respective Nups from their membrane bound state (Fig. 5–7). Probing with MAb414 confirmed the release of several Nups, including Nup358, Nup153 and a

Fig. 5 Heparin treated HeLa NE probed for POM and NE proteins. HeLa NE was treated with increasing levels of heparin (0 mg/ml to 5 mg/ml) then separated into pellet (bound) and supernatant (unbound) under low speed centrifugation conditions. Samples were separated on an 8 % SDS-PAGE gel and

transferred to nitrocellulose for western analysis. Top panel probed for POM proteins: gp210, middle panel POM121 and bottom panel probed for NE protein with α -LAP2 β antibody. S Supernatant, P Pellet

majority of Nup62, from their membrane bound state starting at 0.7 mg/ml heparin (Fig. 6). Nup133 and Nup107 are associated with the NE and with a fraction in the supernatant (Fig. 7), which may reflect a nuclear pool of these proteins. Other scaffold Nups: Nup160, Nup75, Nup43 and Nup93 are completely dissociated from 0.7 mg to 1 mg heparin treatments (Fig. 7). Nup43 is easily dissociated at 0.1 mg/ml while our western analysis reveals that α -NDC1 maintains NE association throughout heparin exposure (Fig. 7). These results

clearly reflect the production of Nup depleted NPCs which we refer to as ghost pores. All further treatments in the preparation of ghost pores and isolation of released NPC subcomplexes were carried out at 0.7 mg/ml heparin. FG-Nups were not further depleted beyond 0.7 mg/ml, hence this represents the optimal concentration, while maintaining the POM proteins anchoring setting (Fig. 5). Furthermore, we examined several time points for heparin treatment and determined the 15 min treatment as optimal (data not shown).

Fig. 6 Heparin treated HeLa NE. HeLa NE was treated with increasing levels (Panels A–C) of heparin (0 mg/ml to 5 mg/ml) then separated into pellet (bound) and supernatant (unbound) under low speed centrifugation conditions. Samples were separated on a 4–20 % SDS/PAGE gel and transferred to nitrocellulose for western analysis. Probed with MAb414 (FG-Nups). S Supernatant, P Pellet and T Total, an aliquot removed before centrifugation

Fig. 7 Heparin treated HeLa NE probed for scaffold Nups. HeLa NE was treated with increasing levels of heparin (0 mg/ml to 1 mg/ml) (Lanes 1–12) then separated into pellet (bound) and supernatant (unbound) under low speed centrifugation conditions. Samples were separated on 8 % SDS-PAGE gel and

transferred to nitrocellulose for western analysis. Top panel probed for scaffold Nups with α -Nup160, α -Nup133, α -Nup107, α -Nup75 and α -Nup43 antibodies, bottom panels probed with α -Nup93 and NDC1 antibodies. *S* Supernatant, *P* Pellet

Reconstitution of the NPC in HeLa cells

Ghost pores were washed and pelleted to remove heparin (Fig. 1) (Blethrow et al. 2008). Supernatant containing released Nups was dialyzed to remove heparin. Released Nups were incubated, in three-fold excess, with ghost pores for 30 min at RT. This reaction was centrifuged and any re-association of Nups with NE was investigated by western analysis (Fig. 8). Ghost pore pellets were incubated with a control Nup free buffer (Lanes 1 and 2 under control heading) and treated by the same approach as ghost pore pellets incubated with stripped NPC components (Lanes 3 and 4 under reconstitution heading). Control gp210 showed no difference under these experimental conditions. The higher intensity of the bands in Lane 3 compared to those of Lane 1 indicates re-association of the particular Nup with the ghost pore pellet. A fraction of FG-Nups: Nup153, Nup62 and Nup54 are found to be associated with NE following heparin treatment (Lane 1, pellet) and in even higher amounts after reconstitution (Lane 3, pellet). Scaffold Nups: Nup93 and Nup107 showed similar results. An interesting result was found with regard to Nup43 in that less of this protein was shown to be associated with NE after reconstitution incubation. Yet, cytosolic

filament FG-Nups; Nup358, and Nup214, showed no re-association after reconstitution reaction (Data not shown). These results are initial evidence that an in vitro system for the reconstitution of HeLa NPCs can be developed.

Discussion

Intact NE with embedded NPCs was obtained by DNase/RNase treatment of nuclei. HeLa cell NE was treated with heparin at a series of concentrations and the associations of Nups and other NE associated proteins with the NE were investigated. With respect to FG-Nups, it was shown that Nup358, Nup214, Nup153 and Nup62 are fully associated with the HeLa NE prior to heparin treatment and that they are released in a sequential manner upon addition of heparin. Nup358 and Nup153 were shown to lose their NE association with a minimum concentration of 0.7 mg/ml heparin (Fig. 6). Figure 6b shows that 0.7 mg/ml heparin treatment of HeLa NE sufficiently strips the removable Nup62 from the NPC and that there is some intimate association of this Nup to the NPC that cannot be disrupted, up to concentrations as high as 5 mg/ml heparin. These findings may reflect a

Fig. 8 Reconstitution of heparin treated NPCs. HeLa NE was treated with 0.7 mg/ml heparin then separated into pellet (bound) and supernatant (unbound) under low speed centrifugation conditions after removing a total aliquot. Samples were separated on 8 % SDS/PAGE gel and transferred to nitrocellulose for western blot analysis. Heparin treated pellet was incubated with heparin treated supernatant containing Nups that were previously removed from NE. Lanes 1 (*P* Pellet) and 2 (*S* Supernatant) represent controls incubated in buffer alone. Under reconstitution heading, Lane 3 (*P* Pellet) is the pellet after incubation with 3X volume of supernatant containing stripped Nups. Lane 4 (*S* Supernatant) is depleted supernatant. From Top to bottom panels were probed with α -gp210 antibodies, MAb414 (for FG-Nups Nup153 and Nup62), α -Nup54, α -Nup93 antibodies, α -Nup107 antibodies and α -Nup43 antibodies

lack of FG-Nup uniformity and/or subpopulations within the POM region (Bilokapic and Schwartz 2012).

The POM protein gp210 was shown to strictly maintain its NE association under heparin concentrations as high as 5 mg/ml (Fig. 5). POM121 appears to exist in multiple versions at the NE due to alternatively spliced variants (Funakoshi et al. 2011; Funakoshi et al. 2007). One of these POM proteins is predominately associated with the membrane at heparin concentrations from 0 mg/ml to 5 mg/ml, though some amount of this protein was released from NPCs with the addition of any amount of heparin (Funakoshi et al. 2011; Funakoshi et al. 2007). Lap2 β is consistently present in the NE, up to 5 mg/ml heparin (Fig. 5).

Nup160 is initially localized to the NE and begins to be released with low levels of heparin added. At a heparin concentration of 0.7 mg/ml most of the Nup160 is released, with a small amount continually maintaining its NE interaction up to 5 mg/ml heparin. Nup133/Nup107 appear to maintain strong interactions with the NE at most heparin concentrations, however a significant amount of these Nups were present in the supernatant prior to heparin treatment. The similar behavior of these two Nups in this series of reactions is indicative of their strong binding affinity for one another (Boehmer et al. 2003; Boehmer et al. 2008). Nup75 is fixed in its NPC location in the absence of heparin and begins to be released with the addition of low amounts. Nup75 is completely dissociated from NE at 0.7 mg/ml heparin. A majority of Nup43 is removed from NE at concentrations as low as 0.1 mg/ml. Nup93 is mostly NPC associated prior to heparin addition. It begins to be released with heparin at low concentrations and is completely NPC dissociated at 0.7 mg/ml heparin. These results do not correlate with measurements of Nup43 and Nup93 having more stable *in vivo* residence times compared to other Nups (Ellenberg et al. 1997).

The results of heparin treatment at varied concentrations indicate that the optimal conditions for ghost pore production is 0.7 mg/ml heparin treatment of NE. This work provides initial groundwork necessary for *in vitro* reconstitution of HeLa NPCs and indicates that it is achievable. Ghost pores were successfully prepared and used as a foundation for NPC reconstitution by combination with previously stripped NPC subcomplexes. Reconstitution was carried out by 30 min incubation of washed ghost pore pellets with three-fold excess of previously removed NPC components at RT. Western analysis showed that Nup62, Nup54, Nup93 and Nup107 were all re-congregated into the NPC. The strong affinities of both scaffold Nups and FG-Nups may reflect their significance in reassembly to establish structure and basic function. Nup107 is part of the Nup107-160 subcomplex considered the linchpin of the NPC (Boehmer et al. 2003; Doye 2011; Glavy et al. 2007; Walther et al. 2003). In the “protocoatome” hypothesis, the Nup107–160 subcomplex has been proposed to stabilize the sharp bend between the inner and outer nuclear envelope membrane. Like the proteins of other coatome complexes (clathrin, COPI, and COPII), the proteins of the Nup107–160 subcomplex

predictably contain β -propellers and α -solenoids, either alone or in combination. Proteins of the yeast Nup84 subcomplex have been expressed recombinantly, in both *Escherichia coli* and yeast, assembled into a Y-shaped complex (Kampmann and Blobel 2009; Lutzmann et al. 2002). However, Nup43, another member of the subcomplex, present in the ghost pore foundation was displaced in reconstituting NPCs, perhaps by members of its subcomplex. The internal scaffold protein Nup93 re-associates which may reflect a key step in assembly. Nup358 and Nup214 did not reassemble into their NPC bound locations but a significant amount of Nup153 was re-built into ghost pores. This reattachment of a nuclear basket protein may also reflect the need to establish binding or recognition area on the nuclear side of the NPC while additional cytosolic factors may be necessary for proper re-association of Nup358 and Nup214.

Conclusion

Based on our observations, we see a re-association of FG-Nups and scaffold Nups to the ghost pores. A portion of transport proteins like FG-Nups, Nup62 and Nup54 were found well embedded within the pore region, nevertheless we have shown affinity for re-attachment either to ghost pores or to one another for these proteins. The surprising stability of the FG-Nups may reflect their dual roles in transport and structural functions within the NPC. Also nuclear basket FG-Nup Nup153 re-associates, presumably at the nuclear side of the NPC, perhaps as a foundation for re-formation of the nuclear basket. Scaffold Nups are removed by low-level heparin treatments. Several scaffold Nups from the Nup107–160 and Nup93 subcomplexes can re-associate to potentially restore the structural integrity of the NPC.

Our establishment of a mammalian tissue culture based system to isolate HeLa NE, produce ghost pores, and attempt reconstitution is a step forward. These sets of experiments are straightforward and reproducible. With the production of ghost pores, we are able to examine protein–protein interactions during assembly with human proteins. One would benefit from investigation of individual proteins and subcomplexes (Funakoshi et al. 2007). And finally, the production of ghost pores and reconstitution steps can be utilized

for visualization of an individual component's re-association. As further investigations with ghost pores will lead to a better understanding of the process of NPC reassembly, we can begin to engage the challenge of this macromolecule assembly.

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