NSF- and SNARE-mediated membrane fusion is required for nuclear envelope formation and completion of nuclear pore complex assembly in *Xenopus laevis* egg extracts

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Summary

Despite the progress in understanding nuclear envelope (NE) reformation after mitosis, it has remained unclear what drives the required membrane fusion and how exactly this is coordinated with nuclear pore complex (NPC) assembly. Here, we show that, like other intracellular fusion reactions, NE fusion in *Xenopus laevis* egg extracts is mediated by SNARE proteins that require activation by NSF. Antibodies against *Xenopus* NSF, depletion of NSF or the dominant-negative NSF_{E329Q} variant specifically inhibited NE formation. Staging experiments further revealed that NSF was required until sealing of the envelope was completed. Moreover, excess exogenous α-SNAP that blocks SNARE function prevented membrane fusion and caused accumulation of non-flattened vesicles on the chromatin surface. Under these conditions, the nucleoporins Nup107 and gp210 were fully recruited, whereas assembly of FxFG-repeat-containing nucleoporins was blocked. Together, we define NSF- and SNARE-mediated membrane fusion events as essential steps during NE formation downstream of Nup107 recruitment, and upstream of membrane flattening and completion of NPC assembly.

Key words: Membrane fusion, Nuclear envelope, Nuclear pore, NSF, SNAP

Introduction

The nuclear envelope (NE) is composed of two membranes. The outer membrane and the lumen between the two membranes are continuous with the endoplasmic reticulum (ER), whereas the inner membrane contains a unique set of proteins that interact with nuclear lamina and chromatin (Gerace and Burke, 1988; Newport and Forbes, 1987). The NE is perforated with pores that are gated by nuclear pore complexes (NPC). NPCs contain approximately 30 different nucleoporins, three of which are membrane spanning and several contain FxFG repeats (Hetzer et al., 2005; Mansfeld et al., 2006). In most metazoan cells, both NE and NPCs undergo dramatic changes during cell division as they disassemble at the onset of mitosis and then need to reform in the daughter cells at the end of mitosis (Burke and Ellenberg, 2002).

Reformation of the NE can be recapitulated in a cell-free system based on *Xenopus laevis* egg extract and demembranated sperm chromatin (Lohka, 1998; Newport and Dunphy, 1992). First, membrane vesicles that contain NE proteins are recruited to decondensed chromatin in an energy- and cytosol-independent manner. Subsequent steps, however, require addition of cytosol, adenosine triphosphate (ATP) and guanosine triphosphate (GTP). Chromatin-bound vesicles fuse to initially form tubules and small cisternae on the chromatin surface (Dreier and Rapoport, 2000; Hetzer et al., 2001). Formation of cisternae also involves membrane flattening from the original vesicle diameter of approximately 100-200 nm to approximately 40 nm width of the NE (Lohka and Masui, 1984). The tubules and cisternae then extend laterally to generate larger cisternae that progressively cover the entire chromatin surface and ultimately seal the envelope. Once sealed, the nucleus grows, thereby extending its membrane surface.

Concomitantly with the appearance of flattened cisternae, NPCs assemble from transmembrane nucleoporins and soluble subcomplexes in a defined order (Bodoor et al., 1999). NPC assembly is linked to the formation of flattened cisternae, because NPC do not form, if cisternal formation is prevented by pretreatment of the membranes with the alkylating agent N-ethylmaleimide (NEM) (Goldberg et al., 1997; Macaulay and Forbes, 1996). However, it is unclear whether this broad inhibitor blocks fusion or flattening and what precisely is required for individual NPC assembly steps. Moreover, at least one NPC component, the Nup107-160 subcomplex, binds to the chromatin surface at least in part even in the absence of membranes and is essential for NPC assembly (Boehmer et al., 2003; Walther et al., 2003).

The small GTPase Ran regulates both NPC assembly as well as the formation of flattened cisternae (Hetzer et al., 2005). However, the mechanisms that drive the required membrane fusion are unknown. Membrane fusion in the secretory pathway is mediated by tail-anchored, helical membrane proteins, the SNAREs (soluble NSF attachment protein receptor) (Jahn et al., 2003). Approximately 30 different SNAREs exist in humans that reside and function in different compartments. SNAREs associate to three-helical bundles on

acceptor membranes to form a target-(t-)-SNARE complex. The t-SNARE complex pairs with a vesicle-(v)-SNARE containing a single helix on the apposing membrane to form a trans-SNARE four-helix bundle. Trans-SNARE pairing is believed to bring the membranes into close enough proximity to trigger bilayer fusion. The resulting highly stable cis-complex on the fused membrane is a substrate of the hexameric ATPase NSF (NEM-sensitive factor) (Söllner et al., 1993). It binds through its adapter α-SNAP (soluble NSF attachment protein) and dissociates the SNAREs upon ATP hydrolysis to reactivate them for subsequent fusion events. SNARE dissociation occurs either just prior to fusion as in vacuolar fusion (Mayer et al., 1996) or directly after fusion as in regulated secretion (Jahn et al., 2003).

Whether or not SNAREs and NSF are involved in NE formation is unknown. Earlier results showed that mutations in factors of the protein translocation machinery caused defects in nuclear fusion (Rose, 1996), raising the possibility that fusion of the NE, like that of mitochondria, may be mediated by SNARE-independent mechanisms. More recently, the p97 ATPase was shown to be required for two distinct steps during NE formation that are mediated by different p97-adapter complexes (Hetzer et al., 2001). Together with its heterodimeric Ufd1-Npl4 adapter, p97 is needed for NE sealing. Together with the p47 adapter, however, p97 is required for subsequent growth of the nucleus, but not for the earlier sealing. However, at least in the case of $p97^{Ufd1\text{-}Npl4}$, it is unlikely that p97 mediates membrane fusion directly. The same complex has also been shown to extract ubiquitinated proteins from ER membranes in a process called ER-associated degradation (Meusser et al., 2005; Ye, 2006). We therefore believe that $p97^{Ufd1\text{-}Npl4}$ is part of a ubiquitin-dependent regulatory mechanism that governs NE formation rather than directly mediating membrane fusion (Meyer, 2005; K. R. and H.M, unpublished).

The aim of this study was therefore to clarify whether the formation of a sealed NE involves NSF- and SNARE-driven membrane fusion and how this is coordinated temporally with NPC assembly.

Results

NSF activity is needed for NE fusion in *Xenopus* egg extracts

To analyze membrane fusion during NE formation, we studied the process in *Xenopus laevis* egg extracts. Progression of the reaction can easily be monitored by epifluorescence microscopy after staining of the membranes with the lipid dye 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$). During the reactions, the initial rough coat of chromatin-recruited vesicles first transforms into smooth cisternae that subsequently extend to seal the particle (Dreier and Rapoport, 2000). We visually scored closed NEs when the entire chromatin surface was covered with a smooth NE, which is associated with rounding up of the nucleus and decondensation of the chromatin. The visual analysis for quantification was done by manually focusing through individual particles.

We initially asked whether NSF had any role during NE formation and applied three different approaches to address this question. First, we used the dominant-negative NSF_{E329Q} variant, which cannot hydrolyze ATP in the first of two ATPase domains and thus can bind but not dissociate SNARE complexes (Whiteheart et al., 1994). Addition of bacterially expressed NSF_{E329Q} to nuclear formation reactions at a concentration of 1.2 μM (hexamer) had a dramatic effect on membrane fusion as revealed by fluorescence light microscopy after fixation of the reaction and staining of chromatin and membranes. NE formation was reduced by 90% compared with control reactions with wild-type NSF or buffer alone (Fig. 1A,B). Second, we cloned and expressed *Xenopus laevis* NSF (xNSF) to raise antisera in rabbits. One serum specifically recognized only one band on western blots of egg extracts that migrated, consistent with the predicted molecular mass of 83 kDa of xNSF (Fig. 2C). Fractionation of the extract revealed that most of xNSF was soluble in the 200,000 ***g*** supernatant, and a smaller fraction bound to membranes corresponding to the distribution of p97. Addition of affinity-purified xNSF antibodies, but not purified preimmune immunoglobulin G (IgG), again had a strong effect on NE fusion, and formation of closed NEs was reduced by approximately 75% compared with control reactions (Fig. 2A,B).

In a third approach, we used the antibody to immunodeplete NSF from cytosol. Although depletion of cytosolic NSF was efficient (Fig. 2D), it alone did not significantly inhibit NE formation (Fig. 2F). We reasoned that membrane-bound NSF may be sufficient to mediate fusion (Fig. 2C) and therefore briefly incubated the membranes in the absence of ATP, which irreversibly inactivates NSF (Block et al., 1988). This treatment did not generally damage the membranes, because NE formation was not impaired when treated membranes were incubated with mock-depleted cytosol. By contrast, NE formation was strongly

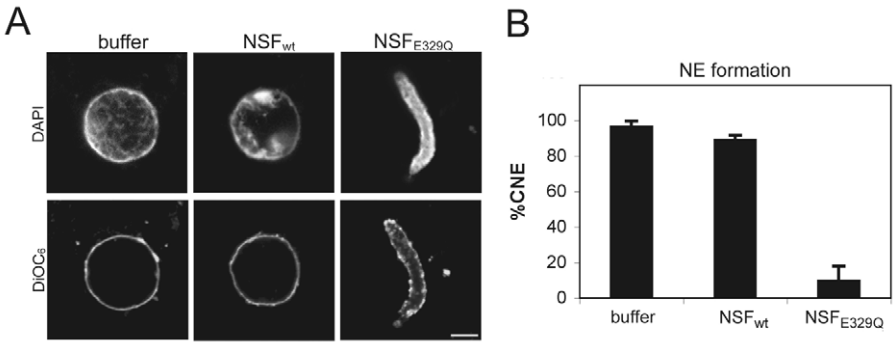

Fig. 1. NSF is required for NE formation. (A) NEs were formed by incubating *Xenopus laevis* egg cytosol and membranes with demembranated sperm chromatin. Reactions were performed in the presence of 1.2 μM (hexamer) bacterially expressed wild-type NSF or the dominant-negative E329Q variant, or NSF buffer as control. After 90 minutes, the samples were fixed. Chromatin and membranes were stained with 4',6-diamidino-2-phenylindole (DAPI) and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$), respectively, and imaged by confocal fluorescence microscopy. Bar, 5 μm. (B) The percentage of chromatin particles with closed NEs (CNE) was determined visually by light microscopy in reactions performed as in A. Shown are means from three independent experiments (n=3) with >50 particles counted in each (error bars indicate ± s.d.).

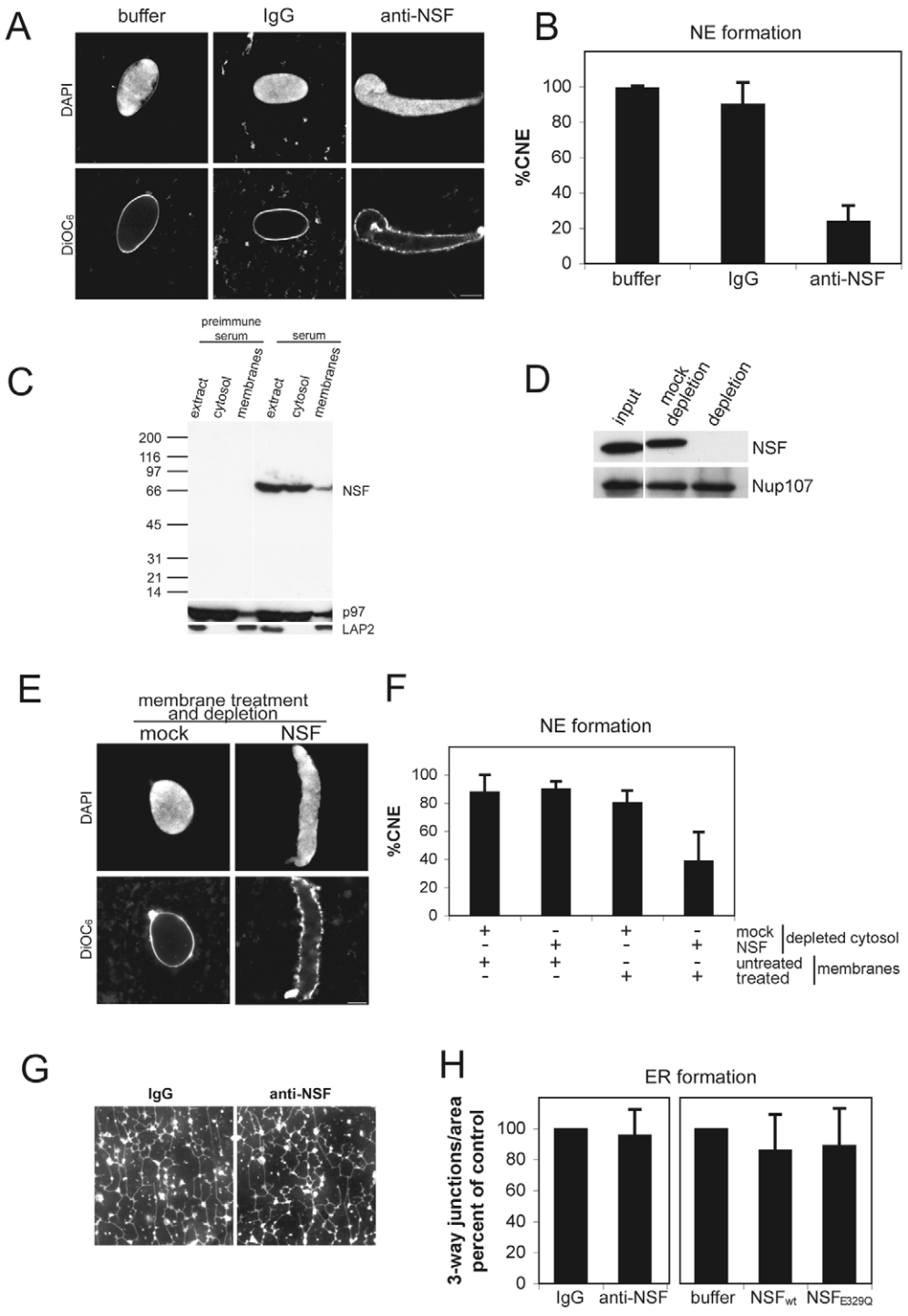

Fig. 2. (A,B) Reactions were performed as in Fig. 1 after addition of either 7 μg/μl affinity-purified antibodies raised against *Xenopus laevis* NSF, purified preimmune IgG or antibody buffer as indicated. Samples were fixed and imaged as in Fig. 1. The percentages of CNE were determined as in Fig. 1B. Bar, 5 μm. (C) Equal fractions of total egg extract, cytosol and light membranes were analyzed by western blotting with either preimmune or anti-NSF serum. Migration of molecular mass markers in kDa is indicated. (D) *Xenopus laevis* egg cytosol was immunodepleted with anti-xNSF or preimmune (mock) antibodies. Depletion efficiency was analyzed by western blotting as indicated with Nup107 as control. (E,F) Membranes were either incubated without ATP for 5 minutes at 19°C to inactivate membrane-bound NSF (treated) or mock treated (untreated). Reactions were performed for 90 minutes with treated or untreated membranes in combinations with either mock- or NSF-depleted cytosol as indicated. Imaging and quantification was performed as in Fig. 1A and Fig. 1B, respectively. Note that only the combination of NSF inactivation on membranes and NSF depletion from cytosol inhibits envelope formation. Bar, 5 μm. (G,H) ER formation assays with egg extracts were performed in flow chambers in the presence of 7 μg/μl purified preimmune IgG or anti-NSF antibodies, or 1.2 μM NSF_{wt} or NSF_{E329Q} for 30 minutes. Samples were carefully washed, stained with $DiOC_6$ and imaged by epifluorescence microscopy. ER formation was quantified by counting the number of three-way junctions per area. Results are presented as percentage of control. All results are presented as means (n=3, error bars indicate ± s.d.).

reduced when treated membranes were incubated with NSF-depleted cytosol (Fig. 2E,F), again showing that NSF is required for NE formation and that both membrane-bound and cytosolic NSF can mediate NE fusion. The egg extract also promotes the formation of ER (Dreier and Rapoport, 2000). We therefore tested the effect of the antibody or the NSF mutant (Fig. 2G,H) at the same concentrations on ER formation, but could not observe any effect as judged by the number of three-way junctions as a measure of network complexity.

These results suggest that NE formation, but not ER formation, requires NSF. Given the function of NSF in SNARE dissociation, they further imply that NE fusion is mediated by SNAREs.

NSF and p97 are required until completion of envelope sealing

We next asked whether NSF-mediated fusion during NE formation was required throughout the reaction even during cisternal extension and sealing of the NE. To address this question, we performed staging experiments, in which we

tested the sensitivity of the reaction to NSF_{E329Q} at different time points during NE formation. We compared it with the requirement for p97, the other ATPase needed for NE sealing, in complex with its Ufd1-Npl4 adapter. In an analogy to NSF_{E329Q}, we used the dominant-negative $p97_{\Delta D2}$, which lacks the second ATPase domain and can bind substrates but cannot hydrolyse ATP (Ye et al., 2003). As a control, we used the double-mutant $p97_{\Delta D2\text{-}K251A}$. It is also inactive, but not dominant negative, because it cannot bind substrates (Ye et al., 2003). As expected, $p97_{\Delta D2}$ inhibited NE formation. By contrast, $p97_{\Delta D2\text{-}K251A}$ inhibited much less, showing that the effect caused by $p97_{\Delta D2}$ was specific (Fig. 3A).

To perform staging experiments, we incubated three different series of samples in parallel for 75 minutes at a slightly lower temperature of 16°C to increase time resolution.

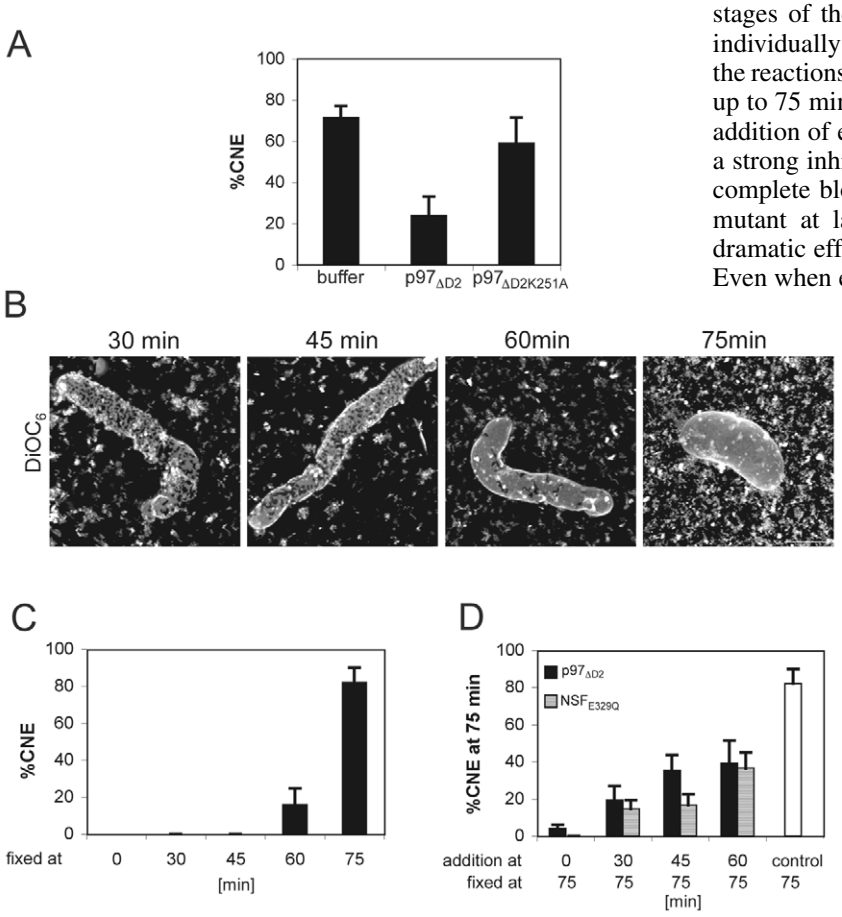

Fig. 3. NSF and p97 are required throughout NE formation. (A) The dominant-negative mutant $p97_{\Delta D2}$ inhibits NE formation specifically. NE formation reactions were performed in egg extract in the presence of buffer, or 12.5 μM (hexamer) of $p97_{\Delta D2}$ or the double-mutant protein $p97_{\Delta D2\text{-}K251A}$. The percentage of closed NE was determined after 75 minutes (n=3, error bars indicate ± s.d.). (B-D) Staging experiments. Three sets of NE formation reactions were performed in parallel. From the first, samples were taken, fixed and stained at indicated time points. Representative intermediates were imaged by confocal microscopy and presented as maximum intensity projections (B), and the percentage of closed NEs was determined (C). The other two sets were supplemented with the dominant-negative mutants NSF_{E329Q} (2.3 μM) or $p97_{\Delta D2}$ (12.5 μM), respectively, at the indicated times. The reactions were further incubated for a total of 75 minutes, then fixed and the percentage of CNE determined (D). Note that the reactions remained sensitive to addition of the mutants until completion of NE formation. Results are presented as means (n=3, error bars indicate ± s.d.).

The first sample was incubated solely as a control to illustrate the progress of NE formation throughout the reaction. Samples of the control were taken and fixed at different time points, and the percentage of closed NEs was determined (Fig. 3C). Furthermore, representative intermediates were imaged by confocal microscopy and reconstructed in three dimensions (Fig. 3B). At the 30- and 45-minute time points, most chromatin particles in the control were covered with membrane cisternae, although they still contained excessive holes and no closed NE had formed. At 60 minutes, the chromatin surfaces were mostly covered with membrane, but still only 20% of chromatin particles had a fully sealed envelope. Sealing was completed, however, in more than 80% of the particles at 75 minutes.

The second and third sets of samples were incubated to test the sensitivity to NSF or p97 mutants, respectively, at different stages of the reaction. The NSF or p97 mutants were added individually to the reactions at the indicated time points, and the reactions were then incubated further for the rest of the time up to 75 minutes, fixed and quantified (Fig. 3D). As expected, addition of either NSF or p97 mutants right from the start had a strong inhibitory effect on the reaction, leading to an almost complete block of NE formation. However, addition of either mutant at later time points during the reaction also had a dramatic effect on completion of NE formation at 75 minutes. Even when either of the mutants was added close to the end of the reaction at 60 minutes, completion of NE formation at 75 minutes was reduced by more than 50%. These results show that the reaction remains sensitive to either mutant until the chromatin is fully covered with membrane, indicating that both the NSF and the p97 pathways are needed up to the end of the reaction when the NE is completely sealed.

NE formation is SNARE-dependent

High concentration of the NSF-adapter α-SNAP inhibits yeast vacuole fusion in vitro by binding and blocking available SNAREs (Wang et al., 2000). We reasoned, therefore, that if SNAREs were involved in NE fusion, their function should be inhibited by excess wild-type α-SNAP, but not by a variant that lacks the N-terminal 80-residue SNARE-binding site (ΔN80) (Hayashi et al., 1995). We generated these proteins in bacteria along with a variant harboring an L294A mutation, which fails to stimulate NSF ATPase activity (Barnard et al., 1997). Addition of wild-type or L294A α-SNAP at a concentration of 37.5 μM had a dramatic effect on fusion, leading to an almost complete block of NE formation, whereas $\alpha\text{-SNAP}_{\Delta N80}$ affected fusion only slightly even at concentrations of up to 100 μM (Fig. 4A,B). A three-dimensional reconstruction of confocal images of the $\alpha\text{-SNAP}_{L294A}$ reaction revealed an apparent accumulation of non-fused vesicles and absence of any cisternae or tubules on the chromatin surface (Fig. 4C). The effect was reversible as addition of fresh cytosol after 45

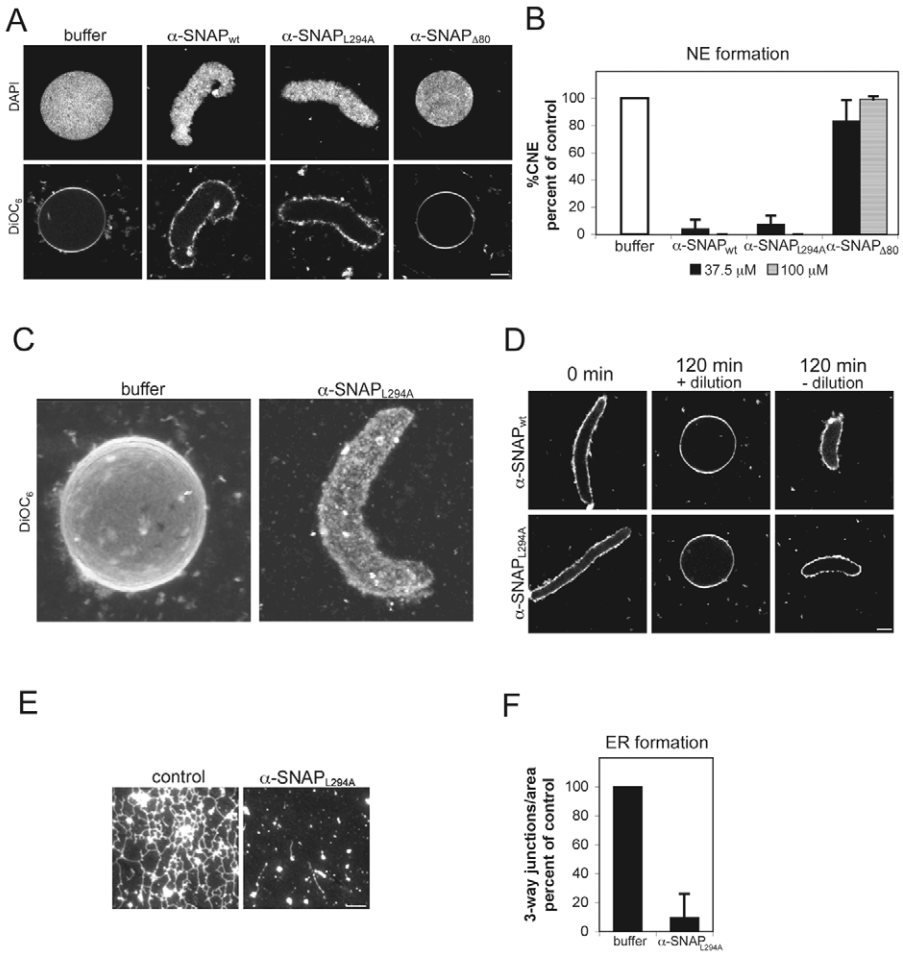

Fig. 4. NE formation is SNARE-dependent. (A) Nuclear formation reactions were performed in egg extracts. Reactions contained either buffer or 37.5 μM or 100 μM exogenous, bacterially expressed α-SNAP$_{wt}$, α-SNAP$_{L294A}$ or the α-SNAP$_{ΔN80}$ variant that lacks the SNARE-binding site. Samples were processed and imaged as in Fig. 1A. Note that only the full-length variants with SNARE-binding activity blocked NE formation. Bar, 5 μm. (B) Quantification of three experiments performed as in Fig. 1B. Results are presented as percentage of control. (C) Maximum intensity projections of confocal sections taken as in Fig. 1A. Note that excess α-SNAP$_{L294A}$ efficiently blocked the formation of tubules or cisternae on the chromatin surface. (D) Nuclear formation reactions were performed in the presence of 50 μM wild-type or L294A α-SNAP for 45 minutes. Samples were taken and fixed (0 minutes). The remaining reaction was split and either diluted 1:2 in fresh cytosol (+ dilution) or not (– dilution) and incubated for a further 120 minutes, followed by fixation. NE membranes were imaged as before. Note that the effect of both α-SNAP variants was equally well restored with fresh cytosol, which was confirmed by quantification (data not shown). (E,F) ER formation was performed for 60 minutes in the presence of 70 μM α-SNAP$_{L294A}$ or buffer and quantified as in Fig. 1E. Bar, 10 μm. All results are presented as means (n=3, error bars indicate ± s.e.m.).

minutes led to restoration of fusion activity and nuclear formation, whereas reactions without cytosol addition remained inhibited (Fig. 4D). Surprisingly, the sample containing α-SNAP$_{L294A}$ was also restored, suggesting that the mutant did not act as a strong dominant-negative at lower concentrations and that restoration was mostly because of dilution. We also tested the effect of excess α-SNAP$_{L294A}$ on ER formation and observed an almost complete block of tubule and network formation (Fig. 4E,F). These results show that both NE and ER fusion are SNARE-mediated.

Excess α-SNAP inhibits fusion and flattening, but not membrane attachment

Next, we analyzed the effect of excess α-SNAP on the ultrastructural level using transmission electron microscopy (Fig. 5). In control reactions, the typical NE was generated with flattened cisternae of approximately 40 nm width, studded with nuclear pores (arrowheads). By contrast, in the presence of excess α-SNAP the chromatin was covered with a layer of vesicles. On none of the chromatin particles could we observe any cisternae or vesicles larger than the original size of 100-200 nm in diameter, again suggesting that fusion was completely blocked. Strikingly, however, the vesicles were very often tightly attached to each other, in many cases with contact sites covering large areas of their surface (arrows). Pretreatment of membranes with NEM also inhibits fusion of vesicles on chromatin (Macaulay and Forbes, 1996). For direct comparison, we repeated these experiments and confirmed accumulation of round vesicles on the chromatin surface. The effect of NEM could not be because of inhibition of NSF, because the majority of NSF in the untreated cytosol fraction should have restored NSF activity (Fig. 2C,F). Intriguingly, unlike vesicles inhibited by excess α-SNAP, NEM-treated membranes did not tightly attach to each other, indicating that attachment prior to fusion may be NEM-sensitive. Both α-SNAP as well as NEM treatment also prevented membrane flattening. This suggests that membrane fusion is required prior to flattening of the vesicles.

Block of membrane fusion arrests nuclear pore assembly at a defined stage

Previous work showed that inhibition of cisternal formation by NEM reduced recruitment of FxFG-repeat-containing nucleoporins, as monitored by light microscopy with the FxFG-repeat-specific monoclonal antibody mAb414 (Macaulay and Forbes, 1996). We wanted to clarify whether specific inhibition of the membrane fusion step had any effect on the quality and quantity of nucleoporin recruitment. We performed nuclear formation in the absence and presence of excess α-SNAP, and also in the presence of α-SNAP but absence of sperm chromatin as a control. Afterwards, chromatin was recovered by centrifugation through a sucrose

cushion. Western blot analysis revealed that equal amounts of nuclear membranes were recruited as judged by the co-isolation of the inner nuclear membrane proteins lamina-associated protein 2 (LAP2) and lamin-B receptor (LBR), as well as the transmembrane nucleoporin gp210 (Fig. 6A). Strikingly, also the soluble nucleoporin Nup107 was recruited to the same extent as in fully assembled nuclei. As Nup107 also binds to chromatin alone (Walther et al., 2003), we asked whether binding was increased by the presence of fused or non-fused membranes. However, we observed no difference in the amount of Nup107 between chromatin alone, chromatin decorated with vesicles, whose fusion was blocked by excess α-SNAP, or control nuclei (Fig. 6B), showing that Nup107 can be fully recruited independently of membranes. In sharp contrast to Nup107, recruitment of the FxFG-repeat nucleoporins p62, Nup153, Nup214 and Nup358 was prevented when membrane fusion was blocked, as detected with mAb414 in western blots (Fig. 6A). We confirmed the differential recruitment of Nup107 and FxFG-nucleoporins by immunofluorescence microscopy on fixed samples (Fig. 6C,D).

These results show that NPC assembly is initiated but does not proceed in the absence of membrane fusion. It was recently reported that FxFG-nucleoporins could be recruited to chromatin independently of membranes in the presence of the constitutively active Ran variant Ran_{Q69L} (Walther et al., 2003). This would suggest that nucleoporin recruitment is not dependent on the topology of membranes. Instead, it would be regulated by a Ran-mediated checkpoint that monitors fusion, and Ran_{Q69L} would override this checkpoint. We reproduced this result and observed increased FxFG-nucleoporin recruitment from high-speed extract supernatant in the presence of Ran_{Q69L} (Fig. 7). However, we noticed that Ran_{Q69L} also increased recruitment of small amounts of membranes, which had not been removed by the 200,000 ***g*** centrifugation, to the site of nucleoporin assembly. Moreover, Ran_{Q69L}-induced recruitment of FxFG-repeat nucleoporins was sensitive to excess α-SNAP as well as to the addition of 0.1% of the detergent Triton X-100. This implies that complete NPC assembly is dependent on nuclear membrane formation and relies on a particular membrane topology, most likely flattened cisternae, whose generation requires prior vesicle-vesicle fusion (Fig. 6A).

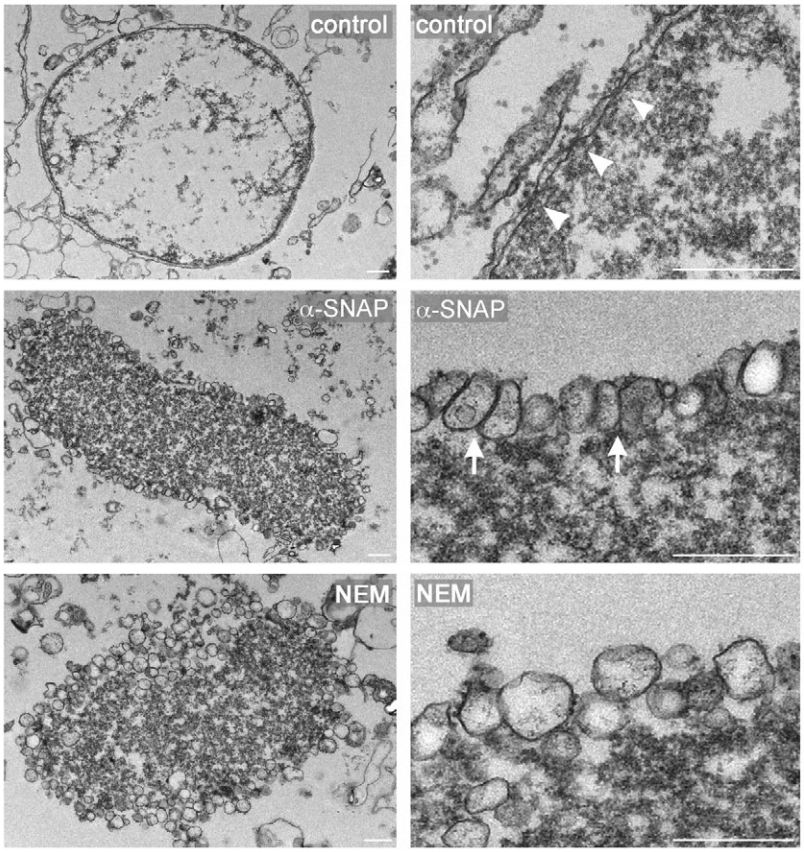

Fig. 5. Ultrastructural analysis. Nuclear formation was performed as before with cytosol and light membranes. Reactions were run with mock-treated membranes along with either buffer (control) or in the presence of 100 μM α-SNAP. Shown are results for $\alpha\text{-SNAP}_{L294A}$ that were identical to $\alpha\text{-SNAP}_{wt}$ (data not shown). In parallel, a reaction was performed with membranes pretreated with NEM, but without exogenous α-SNAP (NEM). Samples were fixed, processed and imaged by transmission electron microscopy. Note the flattened cisternae and nuclear pores (arrowheads) in control reactions and the tight attachment of vesicles (arrows) upon inhibition with α-SNAP, but not with NEM. Bars, 500 nm.

Discussion

NSF and SNAREs mediate NE formation

The work presented in this study suggests that membrane fusion during NE formation is mediated by SNARE proteins that need to be activated by the NSF ATPase. This is based on two different lines of evidence.

The first one addresses the role of NSF with three independent experimental approaches. First, we used a dominant-negative variant of NSF that prevents the function of endogenous NSF. Furthermore, we directly blocked endogenous NSF with inhibitory antibodies. Inhibition required a relatively large concentration of antibody. However, given that the generation of inhibitory antibodies to vertebrate NSF has been proven difficult, it is likely that only a small fraction of the added polyclonal antibodies was inhibitory. Finally, we removed NSF from the cytosol by immunodepletion. NSF depletion inhibited NE formation, but only when combined with inactivation of membrane-bound NSF, showing that the effect was specific. All three independent approaches demonstrated that NSF is required for the formation of the NE, implying that the fusion events that form the double nuclear membrane depend on SNARE proteins and that NSF needs to dissociate them prior to membrane fusion events, as it does in vacuolar fusion reactions (Mayer et al., 1996).

The involvement of SNAREs was confirmed in the second set of data based on the inhibitory effect of high concentrations of the NSF adapter α-SNAP. Full-length α-SNAP, but not a variant lacking the SNARE-binding site, prevented NE membrane fusion, suggesting that inhibition was specific to binding to SNAREs. At physiological

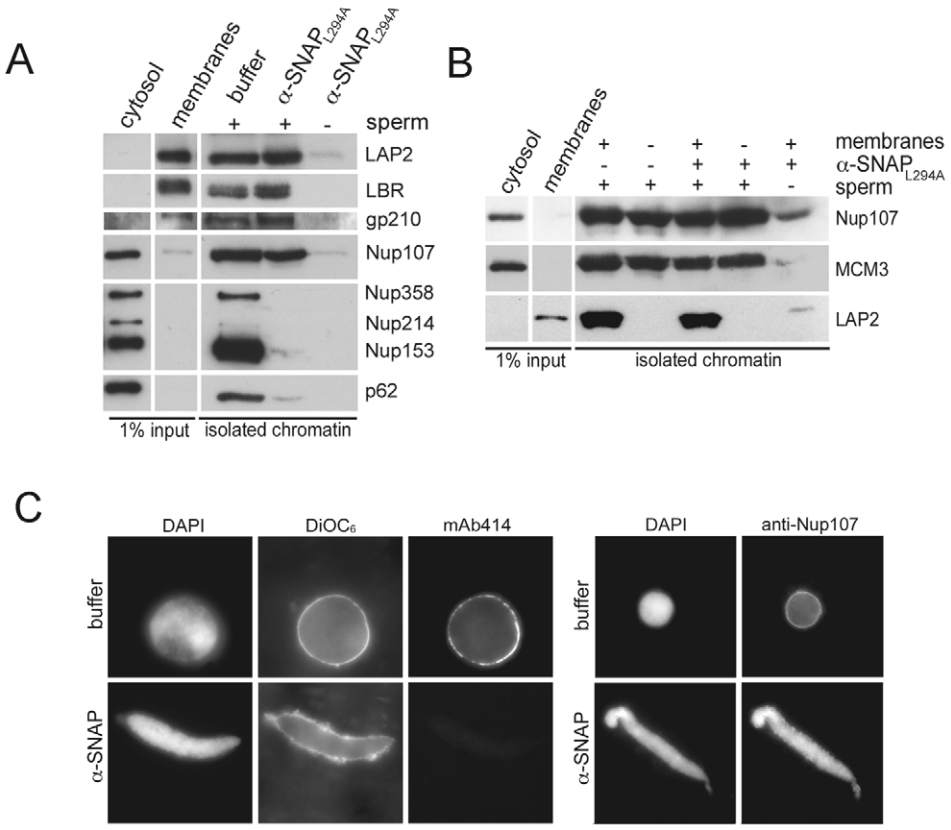

Fig. 6. Block of membrane fusion arrests NPC assembly at a defined stage. (A) Nuclear formation assays were performed as before with extract and sperm chromatin. Reactions were run after addition of buffer or 100 μM α-SNAP$_{L294A}$ with or without sperm chromatin, as indicated. After 60 minutes, reactions were diluted, chromatin recovered by centrifugation through a sucrose cushion and analyzed by western blotting with antibodies specific to proteins indicated on the right. (B) The experiment as shown in A was repeated, but this time including a reaction lacking membranes. Nup107 was detected as indicated. LAP2 served as a membrane marker and the DNA-binding protein MCM3 as a control for equal chromatin recovery. (C) Samples from an identical experiment to that in A were fixed. Nup107- and FxFG-containing nucleoporins were detected by immunofluorescence microscopy using Nup107-specific or mAb414 antibodies, respectively, as indicated.

concentrations, α-SNAP binds cis-SNARE complexes together with NSF. We assume that at high concentrations, α-SNAP binds SNAREs independently of NSF or their oligmerization state possibly even after SNARE dissociation and thereby interferes with their function. The inhibitory effect appears to be different from the one observed by Wickner and colleagues for vacuolar SNAREs (Wang et al., 2000). Whereas restoration of vacuolar fusion required NSF activity, block of NE fusion was reversed by dilution even in the presence of α-SNAP$_{L294A}$ that does not stimulate NSF activity. Inhibition of fusion during NE formation was apparently complete as revealed by ultrastructural analysis. This implies that no other dominant, SNARE-independent fusion mechanism contributes to NE fusion. Inhibition of fusion also prevented flattening of vesicles, showing that flattening depends on prior fusion.

Excess α-SNAP blocked ER formation, too, suggesting that this process also relies on SNARE-mediated membrane fusion. However, ER formation was insensitive to inhibition of NSF. This finding is consistent with results from other in vitro assays. Fusion of ER microsomes isolated from yeast does not require the activity of the NSF orthologue Sec18 (Latterich et al., 1995), and ER forms in frog egg extract independently of cytosolic or peripheral membrane proteins (and therefore NSF) (Voeltz et al., 2006). The differential requirement for NSF may point to an interesting difference between fusion events during formation of the NE and the ER. It is possible that ER fusion is mediated by SNAREs that have already been dissociated prior to the reaction. This may be the same pool that mediates retrograde transport to the ER as these SNAREs are constantly being dissociated from cis-SNARE complexes to allow vesicular transport. NE formation, however, may require dissociation of a different pool of SNAREs just prior to fusion.

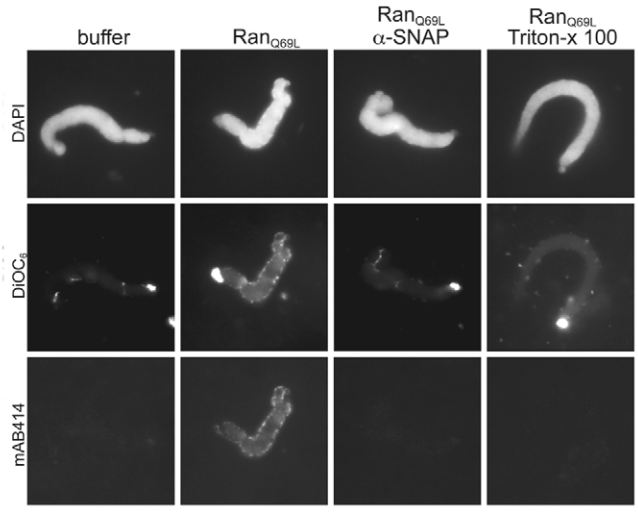

Fig. 7. Ran$_{Q69L}$ addition cannot overcome the arrest of NPC assembly caused by inhibition of membrane fusion. Sperm chromatin was incubated with the 200,000 ***g*** supernatant of egg extract. Reactions were performed after addition of buffer, 5 μM RanQ69L, Ran$_{Q69L}$ plus 100 μM α-SNAP$_{L294A}$ or Ran$_{Q69L}$ plus 0.1% Triton X-100, as indicated. Samples were fixed and chromatin, membranes and FxFG-containing nucleoporins were visualized with DAPI, DiOC$_6$ or by fluorescently labeled mAb414 antibody, respectively. Note the increased recruitment of membrane and FxFG-nucleoporin in the presence of Ran$_{Q69L}$, but not with Ran$_{Q69L}$ in combination with detergent or excess α-SNAP.

It will require further work to identify the exact set of SNAREs involved in either ER or NE fusion.

NSF is required until chromatin is fully covered with NE

Nuclear formation involves NE sealing that is followed by nuclear growth. NE sealing, however, may be further divided into three steps. The first one clearly requires membrane fusion to generate small cisternae and a tubular network from chromatin-bound vesicles. The second involves extension of these structures to completely cover the chromatin surface, with only small holes remaining. This process may require fusion of additional vesicles. Alternatively, cisternae could simply extend with additional membrane that is being provided by the ER (Burke and Ellenberg, 2002; Mattaj, 2004). Indeed, the ER forms at the same time in the test tube (Voeltz et al., 2006) and is probably in contact with the NE (Paiement, 1984). In our assay, cisternal extension remained sensitive to the NSF mutant until the chromatin was fully covered by NE, implying that membrane fusion needs to occur even after chromatin-bound vesicles have fused. At this point, however, we cannot discriminate between whether this represents fusion of additional vesicles or fusion of the rims of the cisternae. Intriguingly, the activity of p97 is also required during the late stages of NE formation. This suggests that NSF-mediated membrane fusion and the p97 function may be intimately linked. It should be noted that the third and final step of closing the remaining small holes involves annular fusion or rather membrane fission events, in which outer and inner membranes need to be separated (Burke, 2001; Stegmann et al., 1989). It therefore requires a molecular machinery that is different from the fusion molecules.

NPC assembly depends on membrane fusion-mediated NE formation

The specific and efficient inhibition of fusion by high concentrations of α-SNAP allowed us to analyze the coordination of NPC and NE formation quantitatively. In the absence of membrane fusion, transmembrane proteins such as LAP2, LBR and gp210, but also the soluble Nup107-160 complex are recruited to chromatin in the absence of membrane fusion to the same extent as in fully assembled nuclei. Membrane-independent recruitment of Nup107-160 has been described before (Walther et al., 2003). However, it is still remarkable that, as we show here, it is recruited in full before NPC are being assembled, given that it localizes also to the cytoplasmic ring of fully assembled NPCs (Belgareh et al., 2001). By contrast, other NPC components such as the FxFG-containing Nup358, Nup214, Nup153 and p62 are not recruited to the forming nuclei when NE formation is prevented by inhibition of membrane fusion.

One explanation is that the recruitment of FxFG-containing nucleoporins marks the assembly of the NPC in the membrane and that this assembly depends on a particular membrane topology. This topology is most likely a flattened cisterna as inhibition of vesicle-to-vesicle fusion also prevents flattening of chromatin-attached membranes. Furthermore, NPCs can form de novo in preformed cisternae or NE (D'Angelo et al., 2006; Macaulay and Forbes, 1996). The notion that lack of SNARE-mediated fusion imposes a topological constraint to NPC assembly, rather than inducing a block through signaling events, is further supported by our observation that NPC assembly cannot be restored by constitutively active Ran in the absence of membrane fusion. Together, our findings stress the importance of SNARE-mediated fusion for the formation of double-membrane NE. We further show that without this double-membrane structure, NPC assembly cannot proceed past early initiation steps.

Materials and Methods

Nuclear and ER formation assays

Crude extract, cytosol, light membranes (Macaulay and Forbes, 1996) and demembranated sperm chromatin (Lohka, 1998) were prepared as described. NEM treatment was as described elsewhere (Macaulay and Forbes, 1996). A typical 20 μl nuclear formation reaction contained 11 μl cytosol, 1.5 μl membranes, 2 μl 10× ATP-regenerating system (20 mM ATP, 100 mM creatine phosphate, 1 mM GTP, 0.2 mg/ml creatine kinase), 1 μl sperm (1000 spermheads/μl) and 4.5 μl buffer, recombinant proteins or antibodies. Reactions were incubated at 19°C for indicated times, except for the staging experiment reactions that were incubated at 16°C to increase the time resolution. For chromatin isolation, a 60 μl nuclear assembly reaction was diluted in 1 ml sperm isolation buffer (20 mM Tris-HCl, pH 7.4, 70 mM KCl, 10 mM EDTA, 2 mM DTT and 2% polyvinylpyrolidone) and chromatin was recovered by centrifugation through a 1.3 M sucrose cushion at 5000 ***g*** for 10 minutes. ER formation was performed as described elsewhere (Dreier and Rapoport, 2000). For immunodepletion, cytosol was incubated with preimmune or anti-xNSF antibodies preadsorbed to protein G-Sepharose in two subsequent rounds for 30 minutes and 60 minutes at 4°C. For NSF inactivation, membranes were incubated at 19°C for 5 minutes in buffer lacking ATP or kept on ice as control before addition to the reactions.

Microscopy and immunofluorescence

Samples were processed for electron microscopy as described elsewhere (Macaulay and Forbes, 1996), except that samples were post-fixed with 2% OsO_4, embedded in epon and imaged with an FEI Morgagni 286 microscope. For fluorescence microscopy, reactions were fixed with 2.5% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) containing $DiOC_6$ (18.75 μg/ml, Sigma) and 4',6-diamidino-2-phenylindole (DAPI) (7.5 μg/ml, Roche). To detect FxFG-repeat nucleoporins, mAb414 (Covance) was labeled with Alexa-Fluor 568 and incubated with fixed samples quenched with 0.1 M glycine in PBS. For Nup107 detection, fixed samples were spun through a 1.5 M sucrose cushion in PBS onto a poly-L-lysine-coated coverslip for 5 minutes at 180 ***g***, and processed for indirect immunofluorescence staining. Imaging was done with a Zeiss Axiovert 100 TV or confocal Leica SP2 AOBS microscope.

Cloning and proteins

IMAGE clone 4757552 coding for *Xenopus laevis* NSF was identified through homology searches and fully sequenced (xNSF, GeneBank DQ841552). The peptide sequence is 90.1% identical and 5.6% similar to Chinese hamster NSF. The xNSF open reading frame (ORF) was cloned into pTrcHisA (Invitrogen) and expressed in *Escherichia coli* for immunization. Chinese hamster NSF_{wt} and NSF_{E329Q} (Whiteheart et al., 1994), and $\alpha\text{-SNAP}_{wt}$, $\alpha\text{-SNAP}_{L294A}$ and $\alpha\text{-SNAP}_{\Delta N80}$ (Hayashi et al., 1995) and $p97_{\Delta D2}$ (Ye et al., 2003) were generated and purified as described. Ran_{Q69L} was kindly provided by Ulrike Kutay (ETH Zurich, Switzerland).

Antibodies

Serum number 98145790 was raised against His-xNSF in a rabbit. After heat inactivation of complement, antibodies were purified using immobilized His-xNSF or protein G-Sepharose and desalted into storage buffer (150 mM KCl, 25 mM HEPES, pH 7.4, 5% glycerol). Other antibodies were very kindly provided by Katherine Wilson (LAP2) (Drummond and Wilson, 2002), Georg Krohne (LBR) (Gajewski and Krohne, 1999), Wolfram Antonin (gp210) (Antonin et al., 2005), Ulrike Kutay (xNup107) and Peter Jackson (Stanford University, CA) (MCM3). mAb414 was purchased from Covance. Anti-p97 was described previously (Meyer et al., 2000).

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