The membrane interaction of Nup53 and its implication in nuclear pore complex assembly

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1. Summary

The unifying feature of all eukaryotes is the presence of intracellular membrane structures. One of the most distinctive structures is the nucleus. This organelle is defined by the two continuous membrane layers of the nuclear envelope. Nuclear pores are established at fusion points between both membranes and stabilized by the proteins of the nuclear pore complex (NPC). These huge protein assemblies form the selective gate which allows a bidirectional transport, and also the scaffold maintaining the highly bent pore membrane structure.

Although the last years have seen advances in the determination of the NPC structure and its components, the necessary factors and processes for the formation of the nuclear pore are still ill-defined. NPCs consist of multiple copies of only 30 different proteins called nucleoporins. The assembly of these complexes is a highly regulated, multi-step process which occurs at different stages during the metazoan cell cycle. After chromosome segregation in mitosis, NPCs are established concomitantly with the re-formation of the nuclear envelope. Moreover, NPCs are incorporated into the intact nuclear envelope during interphase. Although the two processes probably differ in their requirements for stabilization and deformation of the nuclear membranes, the components of NPCs are assumed to play a pivotal role in both. Nevertheless, the molecular details of these processes as well as the involved proteins are not known.

In the course of my PhD work I studied the involvement of the nucleoporin Nup53 in the formation of the nuclear pore and the assembly of NPCs. Nup53 is part of the Nup93 complex which is one of two subcomplexes that forms the structural backbone of NPCs. In addition to the formation of protein-protein interactions within the Nup93 complex, Nup53 was suspected to anchor this complex to the pore membrane via an interaction with the transmembrane nucleoporin Ndc1 and by a direct membrane binding. By the use of *in vitro* systems and the application of recombinant proteins, I was able to prove a direct membrane binding of Nup53. This interaction is mediated by two separate membrane binding sites that I characterized biochemically. Furthermore, I demonstrated that the homodimerization of Nup53 is a prerequisite for membrane binding. Additionally, I found that Nup53 is also able to deform membrane surfaces by its C-terminal membrane binding site. I used the gained information to study the relevance of Nup53s membrane binding ability for NPC assembly with *in*

vitro nuclear assembly reactions featuring Xenopus laevis egg extracts. This revealed that at least one membrane binding site of Nup53 is necessary during NPC assembly after mitosis to recruit other members of the Nup93 complex to the reforming nuclear envelope and to stabilize the curvature of the nuclear pore membrane. This permits the assembly of NPCs and the re-establishment of the nuclear envelope. Interestingly, I could verify that the C-terminal membrane binding site of Nup53 is specifically required for the mode of interphasic NPC assembly. Here, the membrane deformation performed by Nup53 probably induces the fusion of the two nuclear envelope membranes, thereby helping to form and stabilize the nuclear pore.

The *in vitro* assay system to test for membrane binding also proved useful to verify the membrane binding capability of other soluble proteins. In this context, a direct membrane interaction of the esterase domain of the non-LTR retrotransposon ZfL2-1 ORF1p and a trimeric LINE-1 ORF1p was validated.

Taken together this study demonstrates the advantages of *in vitro* test systems to confirm direct membrane interactions. Using these methods, Nup53 could be identified as the first peripheral membrane protein of the NPC which has the ability to deform membranes. It further describes the function of Nup53 as a crucial interaction point for the assembly of the Nup93 complex. In addition, this investigation demonstrates that the membrane interaction of Nup53 is an essential prerequisite for the formation of NPCs and it identifies mechanistic differences in the formation of NPCs depending on the cell cycle stage.

2. Zusammenfassung

Ein Merkmal, das alle Eukaryoten verbindet, ist das Vorhandensein intrazellulärer Membranstrukturen. Dabei ist der Zellkern eine der markantesten dieser Strukturen. Dieses Organell wird von zwei miteinander verbundenen Membranschichten der Kernhülle gebildet. Die Kernporen formen Fusionspunkte zwischen diesen Membranen, welche durch die Proteine der Kernporencomplexe stabilisiert werden. Diese riesigen Proteinkomplexe sichern zum Einen den selektiven Transport in und aus dem Kern und bilden außerdem das Gerüst, welches die stark gekrümmte Porenmembran stabilisiert.

Obwohl in den letzten Jahren Fortschritte bei der Bestimmung der Struktur der Kernporenkomplexe und deren Komponente gemacht wurden, sind die Faktoren und Prozesse, die zur Bildung der Kernpore führen, weitestgehend unbekannt. Mehrere Kopien 30 verschiedener Proteine, genannt Nukleoporine, bilden einen Kernporenkomplex. Der Aufbau dieser Komplexe ist ein streng regulierter, mehrstufiger Prozess, der in verschiedenen Phasen des Zellzyklus von Mehrzellern abläuft. Nach der Teilung der Chromosomen während der Mitose werden die Kernporenkomplexe parallel zum Wiederaufbau der Kernhülle zusammengesetzt. Darüber hinaus werden sie auch in der Interphase in die intakte Kernhülle eingebaut. Obwohl sich beide Arten wohl in den Anforderungen für die Stabilisierung und Verformung der Kernmembran unterscheiden, spielen die Komponenten des Kernporenkomplexes wahrscheinlich in beiden eine wichtige Rolle. Dennoch sind sowohl die molekularen Details als auch die daran beteiligten Proteine unbekannt.

Im Rahmen meiner Doktorarbeit untersuchte ich die Beteiligung des Kernporenproteins Nup53 an der Bildung der Kernpore und am Aufbau des Kernporenkomplexes. Nup53 ist ein Mitglied des Nup93 Komplexes, welcher einer von zwei Unterkomplexen ist, die das strukturelle Rückgrat des Kernporenkomplexes bilden. Zusätzlich zu der Funktion Protein-Protein Interaktionen innerhalb des Nup93 Komplexes herzustellen, wurde vermutet, dass Nup53 diesen Komplex über eine Interaktion mit dem Transmembranprotein Ndc1 und über eine direkte Membranbindung in der Porenmembran verankert.

Mit Hilfe von in vitro Testsystemen und unter Verwendung rekombinanter Proteine konnte ich Nup53 eine direkte Membranbindung nachweisen. Diese Interaktion wird durch zwei separate Bindungsstellen vermittelt, die ich biochemisch genau charakterisierte. Zudem konnte ich zeigen, dass eine Dimerisierung des Proteins notwenig für die Membranbindefähigkeit ist. Zusätzlich zur Membranbindeaktivität konnte ich nachweisen, dass Nup53 auch eine Membranbeugungsaktivität besitzt, die auf der Funktion der C-terminalen Membranbinderegion beruht. Mit Hilfe eines in vitro Systems, das durch die Verwendung von Xenopus laevis Eiextrakten den Aufbau des Zellkerns nachstellt, konnte ich die gesammelten Informationen verwenden, um die Relevanz der Membranbindung von Nup53 für den Aufbau von Kernporenkomplexen nachzuweisen. Hier zeigte sich, dass nach der Mitose

mindestens eine Membranbindestelle notwendig ist, um die weiteren Mitglieder des Nup93 Komplexes an die sich schließende Kernhülle zu rekrutieren und um die Krümmung der Kernpore zu stabilisieren. Dies ermöglicht den Aufbau der Kernporenkomplexe und den Wiederaufbau der Kernhülle. Interessanterweise konnte ich zeigen, dass die C-terminale Bindestelle von Nup53 insbesondere für den Modus des interphasischen Kernporenkomplexaufbaus erforderlich ist. Die Membranbeugungsaktivität von Nup53 ist in diesem Zusammenhang wahrscheinlich notwendig, um die Fusion der zwei Kernmembranen einzuleiten und die sich dabei formende Kernpore zu stabilisieren.

Das in vitro test System für den Nachweis von Membraninteraktionen erwies überdies als nützlich um die Membranbindefähigkeit anderer löslicher Proteine zu belegen. In diesem Zusammenhang konnte eine direkte Membranbindung der Esterase Domäne des non-LTR Retrotransposons ZfL2-1 ORF1p und des trimären LINE-1 ORF1p gezeigt werden.

Zusammenfassend demonstriert diese Studie die Vorteile von in vitro Testsystemen, um direkte Membraninteraktionen zu verifizieren. Mit Hilfe dieser Methode wurde Nup53 als erstes membranbindendes Kernporenprotein, das die Fähigkeit besitzt Membranen zu verformen, identifiziert. Ferner erweitert sie das Wissen über die essentielle Funktion von Nup53 als Interaktionspunkt für den Aufbau des Nup93 Komplexes. Die untersuchte Membranbindefunktion von Nup53 ist dabei eine notwendige Voraussetzung für den Aufbau von Kernporenkomplexen und zeigt, dass es mechanistische Unterschiede zwischen den verschiedenen Modi des Kernporenaufbaus im Zuge des Zellzyklus gibt.

3. Introduction

3.1. The nuclear envelope - part of the intracellular membrane network that forms the nucleus

The last common ancestor of modern eukaryotes represents the prototype of the eukaryotic cell. These primitive eukaryotic ancestors or prekaryotes are separated from prokaryotes by means of an innovative feature, which is the compartmentalisation of the cytoplasm by intracellular membranes. The most prominent organelle formed by this endomembrane system is the nucleus.

The membrane structure which defines the nucleus is the nuclear envelope (NE). Two membranes, namely the outer nuclear membrane (ONM), which is continuous with the endoplasmatic reticulum (ER), and the inner nuclear membrane (INM), that surrounds the chromatin, form the NE. By the separation of the nucleoplasm from the cytoplasm, an elaborate regulation of diverse cellular functions like transcription and translation is achieved (for review see Dultz & Ellenberg, 2007). The NE, however, does not establish an impermeable barrier as it is interrupted by pores representing fusion points of the INM and ONM. Nuclear pore complexes (NPCs) comprised of roughly 30 different proteins called nucleoporins (Nups) reside in these pores as gatekeepers that control the transport through and maintenance of the nuclear pore (Cronshaw et al, 2002; Rout et al, 2000). The integrity and structure of the nucleus is supported by a protein meshwork called the nuclear lamina. This supportive layer is located at the INM facing the chromatin and is anchored via interactions with transmembrane proteins of the INM and nucleoporins (reviewed in Burke & Stewart, 2013; Gruenbaum et al, 2005).

3.2. Biogenesis of the nuclear envelope during cell cycle

The NE undergoes dramatic morphological changes in the course of the cell cycle. Depending on the mode of mitosis, these changes vary considerably between different organisms.

3.2.1. Open, closed and semi-open mitosis

Open mitosis is found in higher eukaryotes. In the course of this process the membrane system of the NE, as well as NPCs, are completely disassembled (for (reviewed in Kutay & Hetzer, 2008). NE breakdown starts with the onset of

prometaphase. After synchronous dissociation of several Nups (Dultz et al, 2008), the nuclear lamina is disintegrated (Gerace & Blobel, 1980) and parts of the NE containing transmembrane proteins are retracted into the ER network (Ellenberg et al, 1997). The process of membrane removal from the chromatin surface is also aided by microtubule induced tearing of the NE (Beaudouin et al, 2002). This triggers the formation of the mitotic spindle followed by the capture of kinetochores by microtubules and the subsequent segregation of chromosomes. As a result, all of these processes happen in the cytoplasm due to the absence of an intact NE (Zheng, 2010).

In contrast to the process of open mitosis, the situation in lower eukaryotes like *Saccharomyces cerevisiae* is different. In these organisms the NE stays intact not only during chromosome separation but also during cell division (Zhang & Oliferenko, 2013). This form of closed mitosis requires the incorporation of the microtubule-organizing centres in the NE and the import of tubulin to establish the mitotic spindle (Guttinger et al, 2009).

Between those two extreme forms of open and closed mitosis, several modes of semi open and semi closed mitosis exist. One example is found in the filamentous fungus *Aspergillus nidulans*. In this organism the NE and NPCs are only partially disassembled during mitosis (De Souza & Osmani, 2007; Guttinger et al, 2009). The dissociation of the nuclear structure in the different forms of open mitosis is believed to be triggered by phosphorylation. Parts of the NPC (Blethrow et al, 2008; Favreau et al, 1996; Glavy et al, 2007; Laurell et al, 2011; Lusk et al, 2007b; Macaulay et al, 1995; Mansfeld et al, 2006) and in some organisms members of the lamina (Gajewski et al, 2004; Heald & McKeon, 1990; Hocevar et al, 1993; Peter et al, 1990) are phosphorylated by CDK1 (Muhlhausser & Kutay, 2007) and by members of the NIMA-related kinase family (De Souza et al, 2004; Lu & Hunter, 1995).

3.3. NE reformation after mitosis

The reformation of the NE starts at the end of anaphase (Ellenberg et al, 1997). By the interaction of INM proteins with the de-condensing chromatin, the membrane of the NE is efficiently attached to re-establish the nuclear structure (Anderson et al, 2009). Interestingly, two transmembrane proteins of the NPC are also implicated in this task. The decrease of Pom121 and Ndc1 by RNAi delayed the formation of the

NE. In contrast, this process was reversed and accelerated by the overexpression of these proteins (Anderson et al, 2009). This process could be mediated either by a direct interaction with DNA (Ulbert et al, 2006) or via interacting nucleoporins residing on the chromatin (Fernandez & Piano, 2006; Franz et al, 2007). The membrane structure of the outgrowing ER that encloses the chromatin is still a matter of debate (reviewed in Wandke & Kutay, 2013). It remains to be resolved whether the ER membranes approach the chromatin as a network of tubules, which subsequently flatten (Anderson & Hetzer, 2007; Puhka et al, 2007) or as membrane sheets from ER cisternae (Lu et al, 2009; Lu et al, 2011). Instead of a general mode of NE reformation, the observed difference could also be due to cell type specific variations (Puhka et al, 2012). The way in which the NE reforms could furthermore influence the process of NPC assembly after mitosis.

3.4. Nuclear pore complexes

3.4.1. Purpose & Function

The presence of intracellular self-contained membrane compartments necessitates strategies for an efficient transport across these membranes. In eukaryotic cells several different mechanisms for the translocation of cargo can be found. The transport of ions and small molecules like metabolites is mediated by channel and transporter proteins (Payandeh et al, 2013). The translocation of proteins into membrane separated organelles such as the ER or mitochondria requires the presence of protein complexes like the Sec61 complex (Hegde & Keenan, 2011) or the TOM complex (Dudek et al, 2013). All of these transport proteins and complexes have a common feature: the formation of a channel that crosses one lipid bilayer. In this respect, the transport across the nuclear envelope stands out as it fuses the transport processes across two separate membranes by one transport machinery (Figure 1). In contrast to other transport proteins and complexes, the lipid bilayers of the two nuclear membranes are not interrupted, but fused to form a membranous pore which bridges the outer and the inner nuclear membrane. This fusion is accompanied by several concomitants that have to be considered. The membrane topology of the pore exhibits regions of high convex as well as concave curvature (Antonin & Mattaj, 2005). To create this unusual membrane conformation, the two membranes of the NE have to be bent in order to bring them in close proximity for a

subsequent fusion (Shibata et al, 2009). The resulting pore is unlikely to be stable in its conformation and this would probably lead either to a reclosing of the opening or to a collapse of the membranes resulting in the breakdown of the NE. The latter scenario is seen during nuclear envelope breakdown when holes in the NE, resulting from membrane tearing by microtubules, rapidly expand (Beaudouin et al, 2002). In order to maintain the pore membrane, NPCs have to act as scaffolds which stabilize this membrane conformation in the NE (Schwartz, 2013). In principle, the fusion of INM and ONM would lead to an unrestricted passage between the nucleoplasm and the cytoplasm. Consequently, NPCs have to form a diffusion barrier that restricts the free movement of proteins in and out of the nucleus but at the same time supplies a selective transport mechanism to translocate cargo across the NE membranes (reviewed in Tran & Wente, 2006). Hence nucleoporins can be roughly categorized into two functional groups: those necessary for the formation and maintenance of the structural backbone of the pore and those involved in the transport function of the NPC.

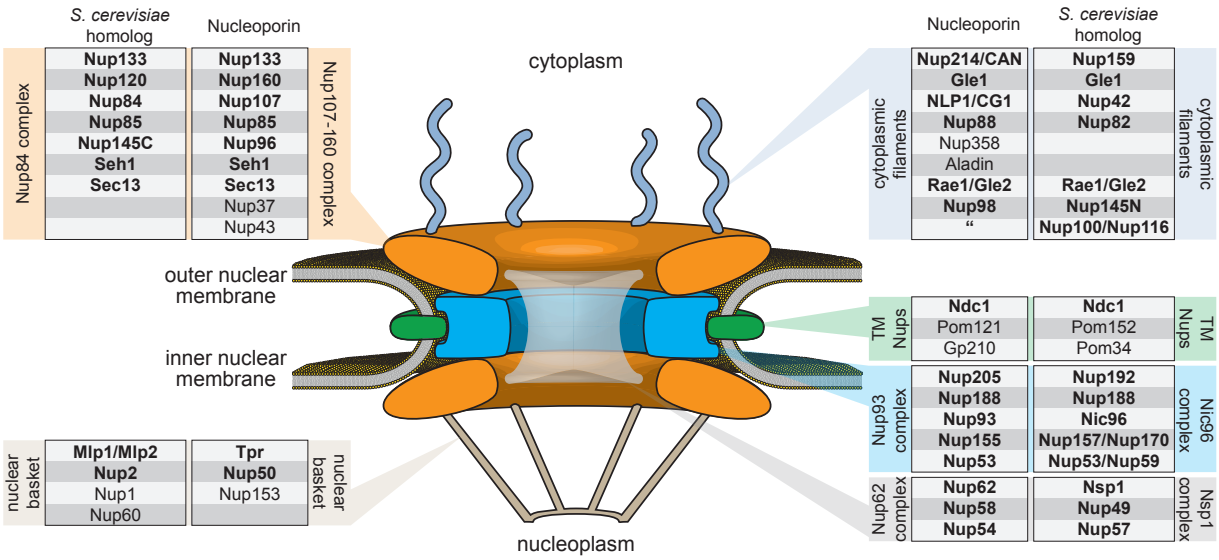

Figure 1 Schematic representation of the nuclear pore complex and its components

Major subcomplexes are shown in a simplified representation. Membranes of the outer (ONM) and inner (INM) nuclear membrane are shown in yellow. The cytoplasmic and nucleoplasmic rings are mainly formed by the Nup107-160/Nup84 complexes (orange). The inner ring is formed in most part by the Nup93/Nic96 complexes (cyan) and is connected to the transmembrane Nups (green) residing in the pore membrane. Nucleoporins and corresponding homologues from Saccharomyces cerevisiae are grouped in their corresponding subcomplexes. Homologous proteins are written in bold. Shape sizes are not proportional to molecular weights. Adapted from (Brohawn et al, 2009).

3.4.2. Soluble transport via the NPC

Nups involved in transport are defined by the presence of repetitive motifs of Phe-Gly (FG) amino acid (aa) residues which are separated by variable polar spacers (Rout & Wente, 1994). These domains are directly involved in active transport as they are able to interact with transport receptors like importins, exportins or transportins. As the detailed description of the different transporters and their characteristics would go beyond the scope of this introduction, the underlying principle will be described by one of the best characterized transport pathways which depends on the transport receptor importin beta. While loading of cargo is mediated indirectly via importin alpha, the interaction with the NPC is accomplished by binding to different Nups (reviewed in Pemberton & Paschal, 2005). The directionality that drives the transport through the pore is ensured by the small GTPase Ran. After translocation through the NPC, importin beta is bound by RanGTP causing the disassembly of the cargo complex. The presence of RanGEF proteins located in the nucleus provides an excess of RanGTP in the nucleoplasm. RanGTP bound importin beta is thus driven along the gradient back into the cytoplasm where RanGAP proteins mediate the hydrolysis of the Ran bound nucleotide to RanGDP. In the GDP bound form the interaction with importin beta is weakened and the transport receptor can be bound again by importin alpha (reviewed in Fried & Kutay, 2003). Interestingly, RanGTP also plays an important role in NPC formation, as it dissociates essential components that are bound by importin and thereby regulating distinct steps of NPC assembly (Walther et al, 2003b).

3.4.3. Transport of transmembrane proteins

The INM harbours a distinct set of transmembrane proteins that are involved in various functions like chromatin binding or lamina anchorage (Burke & Stewart, 2006; Gruenbaum et al, 2005; Lusk et al, 2007a). As the ER is physically connected to the NE these proteins can, in principle, move within the membrane to the INM after their synthesis at the rough ER. During interphase the only membrane connection that links the ONM to the INM is the pore membrane. Consequently, these transmembrane proteins have to pass through the NPC (Antonin et al, 2011; Lusk et al, 2007a) which should restrict the size of extralumenal domains to NPC's passive

permeability barrier limit of about 2.5 nm (Mohr et al, 2009). These proteins that freely diffuse in the plane of the membrane can be anchored at the INM via interactions with the lamina or the chromatin (Ohba et al, 2004; Wu et al, 2002). In addition to this diffusion-retention model, transmembrane proteins can also be actively transported via karyopherin-mediated import (King et al, 2006). In recent years some nuclear localisation sequences in INM proteins have been found (King et al, 2006; Turgay et al, 2010), but it is still unclear if the karyopherin-cargo complex utilizes the central channel of the NPC for trafficking. A more likely alternative would be a route via the small peripheral channels that bypass the core structure of NPCs (Bui et al, 2013; Maimon et al, 2012; Theerthagiri et al, 2010). This assumption is in accordance with observations which indicate an upper size limit of 60 kDa for extralumenal domains of INM proteins (Ohba et al, 2004; Soullam & Worman, 1993).

3.4.4. Structure & Composition of NPCs

The tasks of transport through as well as stabilization of the nuclear pore are both accomplished by the NPCs. The necessary complexity to fulfil these functions is reflected in the size of this complex that ranges from 50 MDa in yeast (Rout et al, 2000; Yang et al, 1998) to 125 MDa in vertebrates (Ori et al, 2013; Reichelt et al, 1990) making it one of the biggest protein complexes in eukaryotic cells. The multiple copies of the ~30 different Nups result in a total number of ~1000 proteins that form one NPC (Bui et al, 2013). Due to the eightfold rotational symmetry of the complex, Nups are usually present in copy numbers of eight or multiples thereof (Bilokapic & Schwartz, 2012).

The overall structural backbone of the NPC is arranged in three stacked rings (Figure 1). The central or spoke ring establishes the connection to the transmembrane Nups which reside in the pore membrane. The nuclear ring and the cytoplasmic ring are linked to long extended structures forming the nuclear basket and the cytoplasmic filaments respectively. This overall composition is conserved from yeast to vertebrates (Alber et al, 2007; Bui et al, 2013; Frenkiel-Krispin et al, 2010) (Figure 1). The proteins mainly responsible for the maintenance and probably also for the formation of this overall structure are clustered in two evolutionary conserved subcomplexes. The Nup107-160 and Nup93 complexes in metazoa or in yeast the Nup84 and Nic96 complexes respectively.

3.4.4.1. The Nup107-160/Nup84 complex

The cytoplasmic ring, which is connected to the cytoplasmic filaments, and the nucleoplasmic ring, which anchors the nuclear basket to the NPC, are both mainly formed by the Nup107-160/Nup84 complex (Alber et al, 2007; Bui et al, 2013; Szymborska et al, 2013). In the last years structural and biochemical data revealed the interactions and arrangements for most parts within the Nup107-160/Nup84 complex (Bilokapic & Schwartz, 2012; Debler et al, 2010; Kampmann & Blobel, 2009). Each nucleoporin engaged in this nonameric complex (heptameric in yeast) is present in a single copy giving rise to an overall Y shape. Recent data from cryo electron tomograms and super resolution microscopy coupled with particle averaging demonstrated the arrangement of the Nup107-160 complexes within the NPC structure in a yet unprecedented resolution (Bui et al, 2013; Szymborska et al, 2013). The presented data suggest that 16 copies of the Nup107-160 complex are present in each ring, resulting in a total number of 32 copies per NPC. This finding is in accordance with previous data that determined the stoichiometry of Nups within the NPC (Ori et al, 2013). The single complexes are arranged on the cytoplasmic and on the nucleoplasmic side in two concentric rings parallel to the NE surface slightly tilted to the centre of the pore. Interestingly, computational and structural analyses suggest that this complex is related to vesicle coats (Brohawn et al, 2008; Devos et al, 2004; Mans et al, 2004). Accordingly, it is possible that nucleoporins of the Nup107-160/Nup84 complex form a coat-like assembly that stabilizes the curved pore membrane of the NPC in a manner analogous to clathrin or COPI and II during vesicle formation (Field et al, 2011; Onischenko & Weis, 2011).

3.4.4.2. The Nup93/Nic96 complex

3.4.4.2.1. Structure of the Nup93/Nic96 complex and its components

The central spoke ring is mainly formed by the Nup93/Nic96 complex (Alber et al, 2007; Krull et al, 2004) that links a part of the FG-repeat Nups to the NPC centre (Grandi et al, 1997; Grandi et al, 1995; Sachdev et al, 2012; Schlaich et al, 1997). The complex itself is anchored in the pore via interactions with the pore membrane. This connection is mediated by members of the transmembrane Nups and probably by a direct interaction with the membrane (Marelli et al, 2001; Mitchell et al, 2010; Onischenko et al, 2009; Patel & Rexach, 2008). The proteins associated with the

Nup93 complex in metazoa are Nup53; Nup93; Nup155; Nup188 and Nup205. Although Nup53 and Nup155 have two orthologues in *Saccharomyces cerevisiae* the overall architecture is probably similar as these proteins emerged by gene duplication and have redundant functions (Brohawn et al, 2009). The composition in yeast is therefore Nup53/Nup59; Nic96; Nup157/Nup170; Nup188 and Nup192 (Figure 1). Like the scaffolding Nups of the Nup107-160/Nup84 complex, proteins of the Nup93/Nic96 complex are mainly composed of alpha helical domains or of combinations of the latter with beta propellers (Brohawn et al, 2009).

Only Nup53 and its yeast counterparts Nup53/Nup59 do not fall into this category. These proteins contain a noncanonical RRM domain located in the centre of the protein that, instead of interacting with RNA, causes the formation of homodimers via hydrophobic interactions (Handa et al, 2006). The N- and C-terminal regions of the proteins are not structurally determined, but unlikely to be largely alpha-helical. Interestingly, Nup53 is suspected to contain an amphipathic helix at its conserved C-term (Marelli et al, 2001).

Vertebrate Nup155 and its yeast homologues Nup170 and Nup157 feature an N-terminal beta propeller that matches the canonical seven-bladed beta propeller fold. The remaining C-terminal part is composed of an alpha helical domain that forms in connection with the beta propeller a curved shape followed by an extended stack of helices (Seo et al, 2013). This structural arrangement is also found in Nup133 which could be due to a common ancestry (Whittle & Schwartz, 2009).

The N-terminal domain of Nup93 or Nic96 in yeast is formed by a coiled coil domain which establishes the connection to the central Nup62 complex. This domain is followed by an elongated region consisting of an alpha helical stack that provides the binding sites for the interacting proteins Nup188 and Nup205 or Nup188 and Nup192 in yeast (Amlacher et al, 2011; Jeudy & Schwartz, 2007; Schrader et al, 2008). The uniqueness of this structure is underlined by the conservation of architecturally significant residues that are found in all homologues (Amlacher et al, 2011; Jeudy & Schwartz, 2007).

Although not closely related in sequence, the two interacting partners of Nup93, Nup205 or Nup192 in yeast and Nup188 are thought to originate from early gene duplication (Mans et al, 2004) which is reflected in their common overall structure

(Andersen et al, 2013). Both exhibit an alpha solenoid like fold formed by alpha helical tandem repeats (Andersen et al, 2013; Sampathkumar et al, 2013). Several hinges provide the flexibility to exist in an open and closed conformation. This flexibility and the overall structure exhibit a closer relation to karyopherins than to any other alpha helical nucleoporin (Andersen et al, 2013; Sampathkumar et al, 2013). Interestingly, Nup188 and Nup192 from the thermophilic fungus *Myceliophthora thermophila* have the ability to interact with FG-repeats *in vitro* and can mimic the behaviour of transport receptors when transfected in HeLa cells (Andersen et al, 2013).

3.4.4.2.2. NPC assembly and the role of the Nup93/Nic96 complex in it

Unlike the Nup107-160/Nup84 complex which stays intact (Glavy et al, 2007), members of the Nup93/Nic96 complex are detached at the onset of mitosis. In accordance with this model, proteins of the Nup93 complex have been detected in screens for mitotic phosphorylation in human cell lines (Daub et al, 2008; Dephoure et al, 2008; Guttinger et al, 2009). Additionally in *Saccharomyces cerevisiae* and also *Xenopus laevis* egg extracts, specific phosphorylation of Nup53 during mitosis was demonstrated (Lusk et al, 2007b). Nevertheless it is not clear whether this phosphorylation is necessary or sufficient for the detachment of the complex or if other modifications are involved. The disassembly of the NPC in prophase is a rapid process that is probably not just a reversal of the assembly steps. In contrast to the early attachment during assembly the Nup93 and Nup107-160 complexes dissociate early and rapidly during NPC disassembly (Dultz et al, 2008).

3.4.4.2.3. NPC assembly after mitosis

After mitosis members of the Nup93/Nic96 complex are individually recruited to the reforming NPC (reviewed in Antonin et al, 2008). Nuclear assembly after mitosis can be performed *in vitro* using *Xenopus laevis* egg extracts (Lohka, 1998) and this allows the determination of necessary factors by biochemical manipulation. Single proteins can be specifically depleted using bead coupled antibodies to determine their contribution for nuclear and NPC assembly. This method has been extensively used in order to find the essential components and to define the order of events of NPC assembly. The formation of new NPCs starts from the chromatin where the

DNA interacting protein MEL28/ELYS localizes via its AT-hook (Franz et al, 2007; Galy et al, 2006; Rasala et al, 2006; Rasala et al, 2008). This allows the recruitment of the Nup107-160 complex (Franz et al, 2007) that in turn can interact with the transmembrane protein Pom121 (Antonin et al, 2005; Mitchell et al, 2010). The formation of the NE membrane on the chromatin is promoted by this interaction. In contrast to the *in vivo* situation, in the nuclear assembly using *Xenopus laevis egg* cytosole, the membranes are recruited as vesicles to the chromatin (Gant & Wilson, 1997). After docking on the DNA surface the vesicles start to fuse forming the continuous NE double membrane (Hetzer et al, 2005). The simultaneous assembly of the Nup93 complex is initiated on this forming nuclear membrane. Nup53 is believed to interact directly (Marelli et al, 2001; Patel & Rexach, 2008) and via the transmembrane nucleoporin Ndc1 with the nuclear membrane providing the binding site for Nup155 (Makio et al, 2009; Mansfeld et al, 2006; Mitchell et al, 2010; Onischenko et al, 2009). The C-terminal domain of Nup93 further strengthens this interaction (Sachdev et al, 2012). After Nup93 is anchored at the complex, Nup188 and Nup205 interact in a mutually exclusive fashion with Nup93 (Amlacher et al, 2011; Theerthagiri et al, 2010). In the last steps the Nup62 complex forms the diffusion barrier by providing part of the FG nucleoporins that fill the central channel together with Nup98 (Dultz et al, 2008; Grandi et al, 1997; Sachdev et al, 2012). This experimental setup also revealed that except Nup188 and Nup205 (Theerthagiri et al, 2010) all other components of the Nup93 complex, namely Nup53 (Hawryluk-Gara et al, 2008; Hawryluk-Gara et al, 2005), Nup155 (Franz et al, 2005) and Nup93 (Grandi et al, 1997; Sachdev et al, 2012), are essential for this process. The depletion of the individual proteins does not interfere with the recruitment of membrane vesicles but leads to a block in NPC assembly and membrane fusion. The established order of events is not necessarily universally applicable but to be specific for NPC assembly after open mitosis.

3.4.4.2.4. NPC assembly during interphase

The formation of new NPCs is also seen during interphase by de novo formation of pores into the NE (D'Angelo et al, 2006; Maul et al, 1972). Although the final protein composition of the NPC is the same, there seem to be differences in the order, requirements as well as the velocity of assembly (Doucet & Hetzer, 2010; Doucet et

al, 2010; Dultz & Ellenberg, 2010). One example is MEL28/ELYS which is essential for post-mitotic, but dispensable in interphasic NPC assembly (Doucet et al, 2010). As for organisms undergoing closed mitosis this assembly mode is the only way to form new NPCs in the NE (Winey et al, 1997) this can give insightful information about interphasic pore assembly. In *Saccharomyces cerevisae* the transmembrane nucleoporin Ndc1 forms interactions with two other transmembrane proteins Pom152 and Pom34 as well as with the soluble Nup53 and Nup59 proteins. This creates the platform to recruit Nup157 and Nup170 by direct binding to Nup53, Nup59 and Pom152 (Makio et al, 2009; Onischenko et al, 2009). Single and multiple deletions have led to the model that the transmembrane interacting network starts the formation of new NPCs. By the anchorage of the peripheral membrane proteins Nup53 and Nup59 the formation of the membrane pore is initiated, further supported by members of the reticulon family that shape and maintain the tubular ER network (Dawson et al, 2009). The subsequently recruited Nup157/Nup170 proteins would act as scaffolds which stabilize the membrane and also bind Nic96 together with Nup188 and Nup192 to the complex. The final fusion of the membranes would be mediated mainly by the transmembrane proteins Pom152 and Pom34 while interactions with FG-repeat containing Nups form the diffusion barrier in the emerging aqueous channel (Flemming et al, 2009; Makio et al, 2009; Onischenko et al, 2009). In a final step the Nup84 complexes would stabilize the structure on the nuclear and the cytoplasmic side (Rexach, 2009). It is not clear if these steps and the general assembly order are applicable to the interphasic NPC assembly in organisms undergoing open mitosis as two of the three transmembrane Nups are not evolutionary conserved. In vertebrates the non-conserved transmembrane nucleoporin Pom121 is assumed to play an important role in pore formation (Doucet et al, 2010). As this protein precedes the early recruitment of the Nup107-160 complex (Doucet et al, 2010; Dultz & Ellenberg, 2010). Interestingly, one member of this complex Nup133 contains an amphipathic helix that preferentially binds to membrane regions of high curvature (Drin et al, 2007). Furthermore the curvature sensing mechanism is specifically required for interphasic NPC assembly, probably targeting the complex to the highly curved pore membrane of newly formed membrane fusion points (Doucet et al, 2010). Nevertheless it is not clear if Pom121 is mediating the membrane fusion or if other proteins are necessary. Using *Xenopus laevis* extracts an involvement of members of the ER bending and maintaining

reticulon family was demonstrated underlining the importance of membrane shaping proteins for this process (Dawson et al, 2009).

3.5. Will it bend? – Different ways to induce membrane curvature in the cell

There exist several cellular mechanisms to deform membranes. This can be achieved by proteins that either directly or indirectly influence the surface of the membrane. Proteins of both categories could be involved in the formation of the highly bent pore membrane.

An indirect way to induce membrane deformation is by changing the lipid composition of a lipid bilayer. Enzymes like flippases can transfer phospholipids between the monolayers thereby changing the membrane topology (Farge et al, 1999; Hua & Graham, 2003). A similar mechanism is carried out by lipid transfer proteins. By non-vesicular trafficking, lipid monomers are exchanged between intracellular membranes. These lipid transfer proteins are assumed to act as catalysts by decreasing the energy barrier for the lipid monomers to transfer between membranes (Lev, 2010). Although presumable it is currently unclear if this mechanism is involved in regulating the shape of cellular membranes. A more conceivable process involves phospholipids of different shapes that can be found in various cellular membranes (reviewed in van Meer et al, 2008). By enzymatic modification the acyl chains or head groups of phospholipids can be altered, causing a change of the overall geometry and the occupied area (Hammond et al, 1984). Phospholipase A2 can hydrolyse cone shaped phospholipids like phosphatidic acid (PA) to form lysophosphatidic acid (LPA) that exhibits a funnel shape (Brown et al, 2003). The combination of these different mechanisms with transmembrane proteins that prohibit a lateral diffusion could limit these membrane curving mechanisms to induce local deformations.

A way to generate membrane curvature by a direct interaction of proteins with the membrane is by the insertion of hydrophobic side chains into one membrane leaflet. This hydrophobic insertion is achieved by amphipathic helices. This special form of an alpha helix features one side where polar residues are positioned opposing the hydrophobic residues that are clustered on the other side. In solution these domains are often unstructured and only form upon binding to membranes (Gallop et al,

2006). The hydrophobic side of the amphipathic helix can be inserted into the head-group-hydrocarbon interface of one membrane leaflet (Campelo et al, 2008) causing a local expansion of the monolayer in relation to the other. This asymmetric distribution generates a local deformation resulting in a positive curvature of the membrane. A variety of different proteins have been shown to contain amphipathic helices like amphiphysin, endophilin or epsin (Ford et al, 2002; Gallop et al, 2006; Masuda et al, 2006; Peter et al, 2004) which are all involved in vesicle trafficking and are all members of the BAR (Bin/Amphiphysin/Rvs)-superfamily. Proteins of this family have the ability to form elaborate membrane scaffolds and often feature different membrane binding domains within one protein. BAR domains are rigid, largely alpha helical domains that form antiparallel homodimers (Peter et al, 2004). This dimeric BAR module has an overall crescent shape that can interact with the membrane surface via positively charged residues. For N- and F-BAR proteins these residues are positioned on the concave side of the structure imposing a positive curvature on the interacting membrane (Gallop et al, 2006; Li et al, 2007; Weissenhorn, 2005). For F-BAR proteins this is further supported by the formation of higher oligomers by lateral and tip-to-tip interactions (Frost et al, 2008). In contrast, proteins of the I-BAR family where positively charged residues are located on the convex surface induce a negative curvature (Lee et al, 2007; Saarikangas et al, 2009). Most BAR domains are joined by additional membrane interacting domains that provide the initial membrane binding. Examples are the N-terminal amphipathic helix in epsin (Ford et al, 2002) or the PH-domain in centaurins (Jackson et al, 2000; Peter et al, 2004) that binds the head group of phosphoinositides. The necessity for a combination of curvature inducing and scaffolding domains is reflected in the fact that the minimal BAR domain per se is a curvature sensor rather than an inducer (Peter et al, 2004). Some of the aforementioned proteins are involved in vesicle trafficking. It is noteworthy that the scaffolds that are formed by clathrin and COP are not able to deform cellular membranes into vesicles on their own (Kirchhausen, 2000), but depend on curvature-inducing proteins. These proteins are all soluble and are only transiently associated with membranes (Shibata et al, 2009). This view is challenged though by recent *in vitro* data that describes another mechanism for membrane deformation. By local protein concentration, also known as protein crowding, membranes of liposomes and giant unilamellar vesicles are deformed. This was only dependent on membrane recruitment of proteins, but not on additional factors like

Epsin which are supposed to induce membrane curvature in the process of vesicle formation (Dannhauser & Ungewickell, 2012; Kirchhausen, 2012; Stachowiak et al, 2012). Membrane deformation was even induced by soluble proteins that were artificially tethered to a membrane surface, as soon as a critical threshold of ~20% membrane coverage by the protein was reached (Stachowiak et al, 2012). Nevertheless it remains to be proven if this mechanism is relevant for membrane deformation *in vivo*.

In contrast also integral membrane proteins can form curved lipid bilayers by the intrinsic shape of their transmembrane domain. This effect is seen with the ER tubule inducing DP1/Yop1p and reticulon proteins that are also implicated in NPC assembly. It is assumed that these proteins insert hydrophobic hairpins causing a displacement of the lipids in one monolayer that leads to a local deformation analogous to the insertion of amphipathic helices (Hu et al, 2008). This process also referred to as wedging (Shibata et al, 2009), is also aided by the formation of protein scaffolds. For reticulons and DP1/Yop1p this is achieved by the formation of immobile oligomers (Shibata et al, 2008). A similar transmembrane domain topology is found in atlastins. These proteins are necessary for the formation of the tubular ER and interestingly can interact with proteins of the cytoskeleton (Hu et al, 2009). As tubules can be pulled out of membranes by kinesins *in vitro* (Roux et al, 2005) and it has been observed that tubular structures of the ER and mitochondria move along microtubules (Allan & Vale, 1994; Rodriguez-Boulan et al, 2005) this represents another possibility to deform intracellular membranes.

It is unclear which of the possible membrane curving processes are necessary for the formation of the pore during NPC assembly. The pore membrane surface exhibits regions of unusual positive and negative curvature (Antonin & Mattaj, 2005). Therefore it is possible that a combination of different mechanisms for the curvature and subsequent stabilisation of the membrane is necessary. It is conceivable that these functions are, at least in part, executed by proteins of the nuclear pore complex.

4. Objectives of the thesis / doctoral research

NPCs are located at fusion points of the inner and outer nuclear membrane. To introduce these pores the two membranes have to undergo unusual bending and curvature. Although several parallels can be seen in mechanisms of cell biological processes like vesicle budding, it is mainly unknown how this bent nuclear pore membrane can be formed and stably maintained (Antonin, 2009; Antonin & Mattaj, 2005).

Nup53 is an essential component for the formation of NPCs in vertebrates (Hawryluk-Gara et al, 2008). Furthermore this protein is conserved from yeast to metazoa (Mans et al, 2004; Neumann et al, 2010) and was suspected to be a peripheral membrane protein (Marelli et al, 2001). Interestingly, overexpression of the Nup53 homolog in *Saccharomyces cerevisiae* resulted in the formation of intra-nuclear double membrane sheets and structural analyses predicted an amphipathic helix at its conserved C-term (Marelli et al, 2001). A strong interaction with the NE and the nuclear lamina was also demonstrated for vertebrate Nup53 (Hawryluk-Gara et al, 2005). Therefore Nup53, as a promising candidate that could be involved in the formation of the pore membrane, was investigated in detail for its ability to interact with membranes and its contribution to nuclear pore assembly.

The first step was to verify the membrane binding of Nup53. The interaction with several other proteins at the NPC required the use of an *in vitro* system. Therefore recombinant Nup53 was purified and tested for membrane binding. To investigate the contribution of a possible membrane interaction for nuclear assembly the next step was to pinpoint the responsible residues. The use of different fragments and mutants of Nup53 also allowed the biochemical characterization of the membrane interaction. In addition this information proved to be useful to dissect the function of membrane association from other protein-protein interactions. As the formation of the pore membrane requires the induction of membrane curvature the putative membrane binding ability of Nup53 was also tested for membrane deformation.

The nuclear assembly assay featuring *Xenopus laevis* egg extracts was used to test the contribution of the membrane binding and shaping function of Nup53 for NPC assembly. Thus endogenous Nup53 was depleted and replaced by recombinant mutant versions or fragments created according to the information obtained from the

biochemical analyses. This experimental setup, not only allowed the investigation of the role of Nup53 in post-mitotic, but also interphasic NPC assembly.

5. List of publications included in the thesis

All results described in this thesis have been published or submitted in one of the following articles or manuscripts.

a) Accepted papers

I. Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly.

Vollmer B, Schooley A, Sachdev R, Eisenhardt N, Schneider AM, Sieverding C, Madlung J, Gerken U, Macek B, **Antonin** W.

EMBO J. 2012 Oct 17;31(20):4072-84. doi: 10.1038/emboj.2012.256.

II. Structure and properties of the esterase from non-LTR retrotransposons suggest a role for lipids in retrotransposition.

Schneider AM, Schmidt S, Jonas S, **Vollmer B**, Khazina E, Weichenrieder O.

Nucleic Acids Res. 2013 Dec;41(22):10563-72. doi: 10.1093/nar/gkt786.

III. Building a nuclear envelope at the end of mitosis: coordinating membrane reorganization, nuclear pore complex assembly, and chromatin de-condensation.

Schooley A, **Vollmer B**, Antonin W.
Chromosoma. 2012 Dec;121(6):539-54. doi: 10.1007/s00412-012-0388-3. Epub 2012 Oct 27. Review.

IV. The diverse roles of the Nup93/Nic96 complex proteins - structural scaffolds of the nuclear pore complex with additional cellular functions.

Vollmer B, Antonin W.

Biol Chem. 2014 May 1;395(5):515-28. doi: 10.1515/hsz-2013-0285.

b) Submitted manuscripts

A single herpesvirus protein mediates vesicle formation in the nuclear envelope

Lorenz M, **Vollmer B**, Klupp B, Mettenleiter T, Antonin W
submitted

6. Personal contribution to collaborative publications

I. Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly

Vollmer B, Schooley A, Sachdev R, Eisenhardt N, Schneider AM, Sieverding C, Madlung J, Gerken U, Macek B, Antonin W

EMBO J. 2012 Oct 17;31(20):4072-84. doi: 10.1038/emboj.2012.256.

As first author of this publication, I designed the study under supervision of WA. Furthermore I optimized methods for Nup53 protein purification, liposome floatation and tubulation and designed and performed all liposome and nuclear assembly experiments under the supervision of WA. Except for the proteins used for GST-pulldowns, I expressed and purified all recombinant proteins used in this study (Fig. S2 and Tab. S2). I prepared all the figures for the manuscript which I wrote together with WA and with whom I also analysed all data. AS analysed nuclei of the interphasic NPC assembly assay quantitatively for their NPC content (Figure 5B). RS did GST-pulldown experiments for Nup93 and Nup205 (Fig. S1A) and verified the highly phosphorylated state of Nup53 during mitosis (Fig. S3A). Together with NE I established the techniques to prepare liposomes by rehydration of a dried lipid film and subsequent extrusion used in this study (Fig. 6 and S7). NE performed GST-pulldown experiments to analyse the interactions of Nup53 with Ndc1 and Nup155 (Fig. S1B), including expression and purification of the GST-bait proteins. AMS did analytical size-exclusion of the Nup53 RRM domain and mutant (Fig. 2A). CS cloned all protein constructs and helped to prepare *Xenopus laevis* egg extracts. JM and BM did mass spectra analyses for mitotic specific phosphorylations on Nup53 (Fig. S3B). UG performed light scattering measurements of liposome radii (Fig. S4). All authors proofred the manuscript and WA supervised the study.

II. Structure and properties of the esterase from non-LTR retrotransposons suggest a role for lipids in retrotransposition

Schneider AM, Schmidt S, Jonas S, **Vollmer B**, Khazina E, Weichenrieder O

Nucleic Acids Res. 2013 Dec;41(22):10563-72. doi: 10.1093/nar/gkt786.

For this publication I introduced AMS to the experimental work flow of the liposome flotation assay to test direct membrane binding of recombinant proteins, which I established in the lab. I prepared all membrane vesicles used in the flotation assays (Fig. 4&S3). In addition I purified and provided a fragment of Nup133 that was used as a positive control for membrane interaction, verifying the functionality of the assay (Fig. 4&S3). I was proofreading the paper draft with special focus on the experimental description of the liposome assay, as well as the results of the assay. AMS and OW designed the experiments and wrote the manuscript together. AMS performed all experiments described and determined the crystal structures. SS did bioinformatical analyses of the data. EK purified all proteins of the human LINE-1 element. SJ helped with establishing the kinetic measurements and analyzed data.

III. Building a nuclear envelope at the end of mitosis: coordinating membrane reorganization, nuclear pore complex assembly and chromatin decondensation

Schooley A, **Vollmer B**, Antonin W

Chromosoma. 2012 Dec;121(6):539-54. doi: 10.1007/s00412-012-0388-3.

All figures used in this review article were prepared by me. For this I discussed the scientific content with AS and WA and proofred the manuscript. The scope of the article was designed by AS and WA who also wrote the manuscript. The writing of the paper was supervised by WA.

IV. The diverse roles of the Nup93/Nic96 complex proteins – structural scaffolds of the nuclear pore complex with additional cellular functions

Vollmer B, Antonin W

Biol Chem. 2014 May 1;395(5):515-28. doi: 10.1515/hsz-2013-0285.

As first author of this review I designed the outline and scope of the article under supervision of WA. I prepared all figures used in the paper. Together with WA I wrote the manuscript.

V. A single herpesvirus protein mediates vesicle formation in the nuclear envelope

Lorenz M**, Vollmer B**, Klupp B, Mettenleiter T, Antonin W

Submitted manuscript under revision – Nature communications

As a second author I helped establishing the GUV test system to study the vesicle budding function of pUL31. I discussed results with ML and WA, proofred the manuscript and helped preparing figures. ML designed and performed all experiments under the supervision of WA. ML, KB, MT and WA, wrote the manuscript.

7. Results

7.1. Establishing *in vitro* test systems for membrane binding and bending

As membrane interactions can be mediated via protein-protein interactions with peripheral- or transmembrane proteins, an *in vitro* system was established that allows testing for direct membrane binding of proteins in the absence of potential interaction partners. For this, purified, recombinant proteins are used together with artificial membrane vesicles (Fig. 1A left panel (Vollmer et al, 2012)). The lipid composition as well as the size of these unilamellar liposomes can be easily modified. By employing a small percentage of fluorescently labelled lipids ratios of start to floated material can be determined. This allows the quantification of membrane interaction of recombinant proteins by comparison between samples (Bigay & Antonny, 2005). The experimental setup was established, optimized and proved to be useful to verify membrane and lipid interactions for different soluble proteins like nucleoporins (Vollmer et al, 2012), the esterase domain of ZfL2-1 ORF1p and a trimeric LINE-1 ORF1p (Schneider et al, 2013).

To test if membrane binding proteins have an effect on the morphology of the bound membrane surface, liposomes can also be used. The incubation of vesicles with proteins and the subsequent fixation and negative staining for transmission electron microscopic analysis is a widely used technique (Farsad et al, 2001; Frohlich et al, 2013; Henne et al, 2007; Takei et al, 1999). The establishment and optimization of this technique in the lab allowed the investigation of the membrane deforming capability of Nup53 (Fig. 6 (Vollmer et al, 2012)).

By the use of giant unilamellar vesicles (GUVs) the process of membrane binding and deformation can also be visualized on a light microscopic scale. These membrane vesicles with sizes up to several hundred µm can be formed from dried lipids and used for membrane interaction studies ((Keller et al, 2013); Fig. 2 (Lorenz et al, submitted)). The use of fluorescently labelled or tagged proteins in this system, allows the investigation of the localization and involvement of membrane binding and bending proteins in a time resolved manner (Keller et al, 2013; Meinecke et al, 2013; Wollert et al, 2009). The possibility to easily add different factors, like interacting proteins or ions to the reaction allows the evaluation of their influence on membrane binding. Furthermore also in this test system different lipid species can be applied. By

this binding of proteins to specific lipid headgroups can be tested as well as preferences for membrane fluidity ((Betaneli et al, 2012); Fig. 4 (Lorenz et al, submitted)).The use of this assay system proved valuable to verify the membrane deforming ability of a soluble viral protein, which is recruited to the inner nuclear membrane and plays an essential role in nuclear egress (Lorenz et al, submitted).

7.2. The esterase domain of ZfL2-1 can interact with membranes

Mobile genetic elements like non-LTR retrotranposons encode in addition to a reverse transcriptase distinct multi-domain proteins (ORF1ps), but the role of these proteins is often poorly understood (Goodier & Kazazian, 2008). The ORF1p of the *Dani rerio* ZfL2-1 element has an enzymatic function as it forms a lipolytic acylesterase (Schneider et al, 2013). The interaction of the ZfL2-1 esterase domain with negatively charged lipids was verified by the use of lipid strips (Fig. 4A (Schneider et al, 2013)). To test if a lipid interaction of the esterase domain is also possible in the context of a native lipid environment the flotation assay was used. Indeed similar to a fragment of Nup133 that was used as a positive control, ZfL2-1 is detected in the floated fraction together with the liposomes (Fig. 4B (Schneider et al, 2013)). The integrity of the protein is crucial for the membrane interaction as the protein fails to bind liposomes after heat denaturation (Fig. S3 (Schneider et al, 2013)). In contrast, mutations in the active site of the esterase domain did not interfere with the membrane interaction (Fig. S3 (Schneider et al, 2013)). This suggests that the binding of the protein is mediated by charge interactions with negatively charged membranes instead by binding of fatty acid chains. Interestingly, this function could also be evolutionary conserved for non-LTR retrotransposons as the ORF1p of a trimeric human LINE-1 element was also tested positive for membrane binding with the flotation assay (Fig.4B (Schneider et al, 2013)).

7.3. Expression and purification of recombinant Nup53

Nup53 is supposed to be a peripheral membrane protein that is involved in nuclear pore and complex assembly (Hawryluk-Gara et al, 2005; Marelli et al, 2001; Patel & Rexach, 2008). To test for a direct interaction of Nup53 with the membrane, the first step was to optimize the conditions to purify recombinant Nup53. As the expression of Nup53 leads to the formation of insoluble protein aggregates different solubility tags were tested. The small yeast protein SUMO was the best compromise, as it

provides decent solubility, but is also easily separated from the attached protein after protease cleavage. The purification of full length Nup53 constructs or fragments comprising the C-terminal part of the protein resulted in the presence of shorter Nup53 fragments with C-terminal truncations. The optimisation of the expression and purification conditions provided proteins of sufficient concentration and purity for the subsequent *in vitro* experiments (Fig. 1, S2 & Tab. S2 (Vollmer et al, 2012)).

7.4. Nup53 can directly interact with membranes

To test if Nup53 has the ability to interact directly with membranes it was expressed and purified together with several fragments and the two homologous proteins from *Saccharomyces cerevisiae* Nup53 and Nup59. A truncated version of Nup133 was used as positive control as this is able to interact with membranes (Drin et al, 2007). A fragment of Nup98 that showed no membrane binding ability was used as negative control. Membrane binding was verified for vertebrate Nup53 as well as the two yeast homologues (Fig.1 A in (Vollmer et al, 2012)). By testing the different fragments of Nup53 in the assay two independent binding sites were identified located at the N- and C-term of the protein both depending on the presence of the RRM domain (Fig. 1B in (Vollmer et al, 2012)).

7.4.1. The membrane interaction of Nup53 depends on dimerization and is essential for nuclear assembly

The RRM domain of Nup53 causes homodimerization, but is unable to bind RNA (Handa et al, 2006). Two amino acid changes (F172E/W203E) were introduced that render the RRM domain monomeric. The monomeric state of the mutant was verified by size exclusion chromatography followed by MALLS (Fig. 2A (Vollmer et al, 2012)). The dimerization is also detectable *in vivo* as differentially tagged Nup53 proteins from co-transfected HeLa cells are co-purified by immunoprecipitation against either of the two tags. In vivo this dimerization is abolished by the RRM mutant (F172E/W203E) as well (Fig. 1B (Vollmer et al, 2012)). Testing the RRM mutant in context of the full length protein in the flotation assay revealed a nearly 80% reduction in membrane interaction (Fig. 1C (Vollmer et al, 2012)). To assess the impact of this reduced membrane binding on nuclear assembly and NE formation, endogenous Nup53 was depleted from *Xenopus laevis* egg extracts. The specificity of the depletion was confirmed by western blot analysis of the interacting proteins

and other members of the Nup93 subcomplex (Fig. 2D (Vollmer et al, 2012)). As expected (Hawryluk-Gara et al, 2008), NPC assembly as well as NE formation was blocked in the absence of Nup53, but was re-enabled after adding back the recombinant Nup53 protein. This rescue was not possible with the RRM mutant that is impaired in dimerization as well as membrane binding (Fig. 1E & F (Vollmer et al, 2012)). This effect was not caused by a loss of protein-protein interactions due to the mutations in the RRM domain as verified by GST pulldowns (Fig. S1 (Vollmer et al, 2012)).

7.4.2. Membrane binding is mediated by two binding sites

As described in 7.4. two membrane binding sites were located at the N- and C-term of Nup53. Further truncations narrowed down the responsible regions to an N-terminal fragment (aa 93-267) and a hydrophobic patch at the very C-term mainly depending on the last 8 aas. Interestingly, the N-terminal fragment features two positively charged residues that seem to mediate the binding as charge changing mutations (R105E/K106E) strongly reduce its membrane interaction (Figure 3A (Vollmer et al, 2012)). The positive charge of these residues can also be masked by phosphorylation. Nup53 is one of several proteins that are specifically phosphorylated during mitosis (Stukenberg et al, 1997). By analysis of Nup53 from *Xenopus laevis* egg extracts, taken from different cell cycle stages, different phosphorylation sites could be identified by mass spec analysis (Fig. S3 (Vollmer et al, 2012)). Interestingly, residues specifically phosphorylated in mitosis were also mapped in close proximity to the N-terminal membrane binding region (Tab. S2 (Vollmer et al, 2012)). Two conserved residues S94 and T100 could be phosphorylated *in vitro* by CyclinB/CDK1 which caused a reduction in membrane binding (~50%) similar to the introduction of two phospho-mimicking mutations S94E/T100E (Fig. 3B (Vollmer et al, 2012)). In the context of the full length protein, introduction of negatively charged residues in the N-term or truncations of the C-term reduces the membrane interaction respectively. The combination of mutation and truncation, results in an additive effect which further reduces membrane binding (Fig. 3C (Vollmer et al, 2012)). Also in this case a possible loss of the protein interaction capability was tested for the mutations and truncations. The N-terminal charge and phospho-mimicking mutations (R105E/K106E; S94E/T100E) do not interfere with the interaction to Nup93. For the C-terminal truncations a loss of binding with the

interacting proteins Nup155 and Ndc1 is not seen after removal of the last residue. Further truncations missing the last 8 aas can still interact with Nup155, but not with Ndc1 (Fig. S1 (Vollmer et al, 2012)). Both membrane binding sites individually depend on dimerization. Fragments containing one of the membrane interacting regions combined with mutations in the RRM domain (F172E/W203E), causing monomerization, exhibit only minor membrane interaction ability (Fig. S5 (Vollmer et al, 2012)).

7.4.3. One functional membrane binding site of Nup53 is sufficient to promote post-mitotic nuclear assembly

The information gained from the mapping of the membrane interacting sites was then used to further study the contribution of Nup53s membrane binding activity to NPC and NE assembly. To ascertain that the block in NPC and NE formation caused by mutation of the RRM domain was due to the inability of membrane interaction, endogenous Nup53 was again depleted from *Xenopus laevis* egg extracts. Then different constructs impaired in one or both membrane binding sites by mutation and / or truncation were added back to the reaction. This also allowed assessing the contribution of the individual membrane interaction sites to NPC and NE formation. Interestingly, the constructs that were impaired in one membrane binding site were all sufficient to compensate the depletion of endogenous Nup53. It is noteworthy that the localization of all members of the Nup93 complex is not altered in this situation (Fig. 4D (Vollmer et al, 2012)). This is also true for other proteins of the NPC, like Nup88, Nup58 or members of the Nup107 complex. The transmembrane protein Ndc1, which is a direct interaction partner of Nup53 (Fig. S2 (Vollmer et al, 2012); (Eisenhardt et al, 2014; Mansfeld et al, 2006; Onischenko et al, 2009)), is also properly located even when truncations of Nup53 were used for addback that are not able to interact anymore (Fig. S1 (Vollmer et al, 2012)). In contrast, NE and NPC formation was not possible when endogenous Nup53 was replaced by constructs which had both membrane binding sites impaired. In this situation no closed NE was formed and other members of the Nup93 complex were not recruited to the assembling nuclei (Fig. 4A&B (Vollmer et al, 2012)). Taken together this suggests that after mitosis, either one membrane binding site of Nup53 is sufficient to support NPC assembly.

7.4.4. Interphasic nuclear assembly depends on the C-termial binding site of Nup53

During the cell cycle there are two time phases when NPCs are assembled. At the end of mitosis many NPCs are formed in parallel during the reformation of the NE. In addition also during interphase NPCs can be inserted into the closed NE. This process is different in terms of dynamics and probably also in requirements (see sections 3.4.4.2.3. & 3.4.4.2.4.). Therefore the necessity of Nup53s membrane binding ability for interphasic NPC assembly was tested. For this, nuclei were pre-assembled from *Xenopus laevis* egg extracts that were depleted of endogenous Nup53 and supplemented with recombinant constructs which lacked one functional membrane interaction site. In this situation post-mitotic NPC assembly is possible (Fig. 4A (Vollmer et al, 2012)). After the closed NE was established, the reactions were supplemented with extracts again depleted of Nup53 but also FG-nucleoporins that form the diffusion barrier. NPCs formed in this situation are therefore unable to exclude the influx of proteins into the nucleus. This can be visualized by the addition of fluorescently tagged dextran. When recombinant full length Nup53 is applied in this experimental setup, a fluorescent signal is detectable inside the formed nuclei, demonstrating the presence of open pores that were formed after the NE was established. Hence recombinant full length Nup53 supports the formation of NPCs in interphase. Interestingly, only constructs that feature a functional C-terminal membrane binding site are able to substitute for the wild type protein in this situation (Fig. 5A&B (Vollmer et al, 2012)). These results were verified by determining the total number of NPCs formed after depletion and addback of Nup53. Nuclei formed in extracts that were supplemented with wildtype Nup53 or contructs with an impaired N-terminal membrane binding site showed similar numbers of NPCs. The addition of a construct that lacked a functional C-terminal membrane interaction site after depletion resulted in ~50% less NPCs compared to the situation after adding back the wild type Nup53. NPC assembly in interphase can be specifically blocked by addition of an excess of importin beta after the NE is formed (D'Angelo et al, 2006). Therefore nuclei were formed in Nup53 depleted extracts that were supplemented with recombinant wildtype Nup53 or constructs thereof with one functional membrane binding site respectively. After the addition of importin beta the number of NPCs is similar in all three situations as interphasic assembly is equally blocked. These numbers resembles the one obtained after addback of the Nup53 fragment that lacks the C-terminal membrane interaction site (Fig. 5B (Vollmer et al, 2012)).

7.4.5. Nup53 can deform membranes

One explanation why a membrane interaction of Nup53 is necessary for NPC assembly could be that Nup53 by its membrane binding supports the formation of the nuclear pore membrane. For interphasic nuclear pore assembly the two membranes of the NE have to be fused (Fig. 3B (Schooley et al, 2012)). This process could be aided by local deformation by peripheral membrane proteins like Nup53. For proteins like EHD2, which are involved in trafficking, a membrane deformation was demonstrated *in vitro* by tubulation of liposomes that were incubated with the purified protein (Daumke et al, 2007). To test if Nup53 has the ability to induce membrane curvature, recombinant fragments of Nup53 containing both membrane binding sites were incubated with liposomes and subsequently prepared for and analysed by transmission electron microscopy. Indeed, similar to EHD2 which was used as a positive control Nup53 changed the morphology of membrane liposomes to tubules (Fig. 6 (Vollmer et al, 2012)). To find out if the individual membrane interaction sites contribute equally to this function, fragments with only one functional binding site were tested. Interestingly, only fragments containing the full C-terminal binding site induced liposome tubulation, an effect that was abolished with C-terminally truncated fragments. As fragments lacking the complete N-terminal domain preceding the RRM domain also induce this morphological change, demonstrates the minor role the N-terminal binding side plays in this process. In contrast, also for the formation of membrane tubules, the RRM domain is necessary, although not sufficient. This is evident as a C-terminal fragment that contains the RRM mutations and therefore renders this fragment monomeric is not able to deform liposomes into tubules (Fig. 6A (Vollmer et al, 2012)). The importance of this function is also reflected in the fact, that both yeast homologues can not only interact with membranes (Fig. 1A (Vollmer et al, 2012)), but also deform membranes (Fig. 6B (Vollmer et al, 2012)). Hence this function seems to be evolutionary conserved.

8. Discussion

In the following paragraph I will discuss the findings of my work about Nup53 and put it in relation to the present understanding about the formation of the NE and NPC. This will be done with particular emphasis on the relation of Nup53 to the protein network of the Nup93 complex as well as the nuclear pore biogenesis which is tightly coupled to the NE formation.

In my work I was able to demonstrate the membrane binding ability of vertebrate Nup53. This binding is mediated by two independent sites that interact via different modes with the membrane. Furthermore this membrane interaction is necessary for the formation of NPCs after mitosis as well as during interphase. The finding that Nup53 also has the ability to deform membranes suggests that it plays an active role in the formation and maintenance of the highly curved pore membrane.

8.1. Studying membrane binding *in vitro*

Although NPCs are tightly associated with the underlying nuclear membrane, only a subset of Nups is embedded in the pore membrane by transmembrane domains (Figure 1). Nevertheless recent years have shown that several soluble Nups have the ability to transiently interact with membranes (Drin et al, 2007; Patel & Rexach, 2008). However the function of these membrane interactions and their implication in nuclear pore formation is not understood. The use of *in vitro* systems permits the verification of membrane interactions in the absence of interacting partners. This is a valuable feature in particular for the investigation of Nups as these proteins are usually engaged in a tightly interconnected protein network. The use of recombinant proteins in the liposome flotation assay allowed the investigation of the membrane interaction of two nucleoporins that are both part of the structural protein complexes of NPCs. These are Nup133, which is a part of the Nup107-160 complex (Drin et al, 2007) and Nup53 (Fig. 1 (Vollmer et al, 2012)) which is part of the Nup93 complex. Several different parameters in the assay system can be manipulated to specify the membrane binding mechanism of the investigated proteins. Furthermore the membrane interaction of proteins can be tested in a quantitative manner by the use of fluorescently labelled lipids that allow the comparison of individual samples coupled with the detection of protein amounts by SDS-PAGE followed by western blotting (Bigay & Antonny, 2005). This makes this assay a powerful tool to assess

membrane binding strength differences between different proteins or conditions. Different lipid species can be used to form the vesicles used in this assay to test for proteins that are able to directly interact with phospholipids or that show a preference for a specific membrane composition ((Boucrot et al, 2012); Fig. S7 (Vollmer et al, 2012)). Membrane binding is in many cases sensitive for membrane curvature. By the use of differently sized liposomes which can be generated by extrusion, a preference for higher or lower curvature can be investigated ((Drin et al, 2007); Fig. S4 (Vollmer et al, 2012)). These different membrane biding preferences of proteins can also be verified by introduction of specific point mutations. This proved valuable to demonstrate the presence of an amphipathic helix in an unstructured loop region of Nup133 (Drin et al, 2007) or the charge dependence for the N-terminal membrane binding site of Nup53 (Fig. 3 (Vollmer et al, 2012)). This gives an insight into the molecular function of membrane interaction of different nucleoporins. As a bioinformatic analysis detected putative amphipathic helices in several Nups from yeast and human (Drin et al, 2007), it will be interesting to investigate their potential for membrane binding and the influence of this feature for NPC assembly and function.

The nuclear assembly assay featuring *Xenopus laevis* egg extracts is the ideal test system to assess the contribution of membrane binding for NPC and NE assembly. As the reaction of nuclear assembly occurs *in vitro* in a cell-free system (reviewed in Gant & Wilson, 1997), individual proteins can be completely removed or replaced. In the context of living cells these manipulations are often lethal and therefore difficult to investigate. This allowed the identification of essential proteins that are involved in NPC and NE assembly like proteins of the Nup93 complex (Vollmer & Antonin, 2014). Nup53 was previously shown to be necessary for NPC assembly in the course nuclei formation *in vitro* (Hawryluk-Gara et al, 2008). The identification of the membrane binding and deforming sites by the use of artificial membrane vesicles, allowed the creation of constructs that are specifically impaired in these functions (Fig. 3&6 (Vollmer et al, 2012)). Applied in the nuclear assembly assay this showed for the first time the importance of the membrane binding and deforming function of one member of the NPC (Figure 2; Fig. 4&5 (Vollmer et al, 2012)) for NPC assembly.

The combination of these two *in vitro* systems will help to identify other peripheral membrane proteins that are part of the NPC or the NE. The further elucidation of the

molecular function in membrane binding and the application in the nuclear assembly system will shed light on their contribution for NPC and NE assembly.

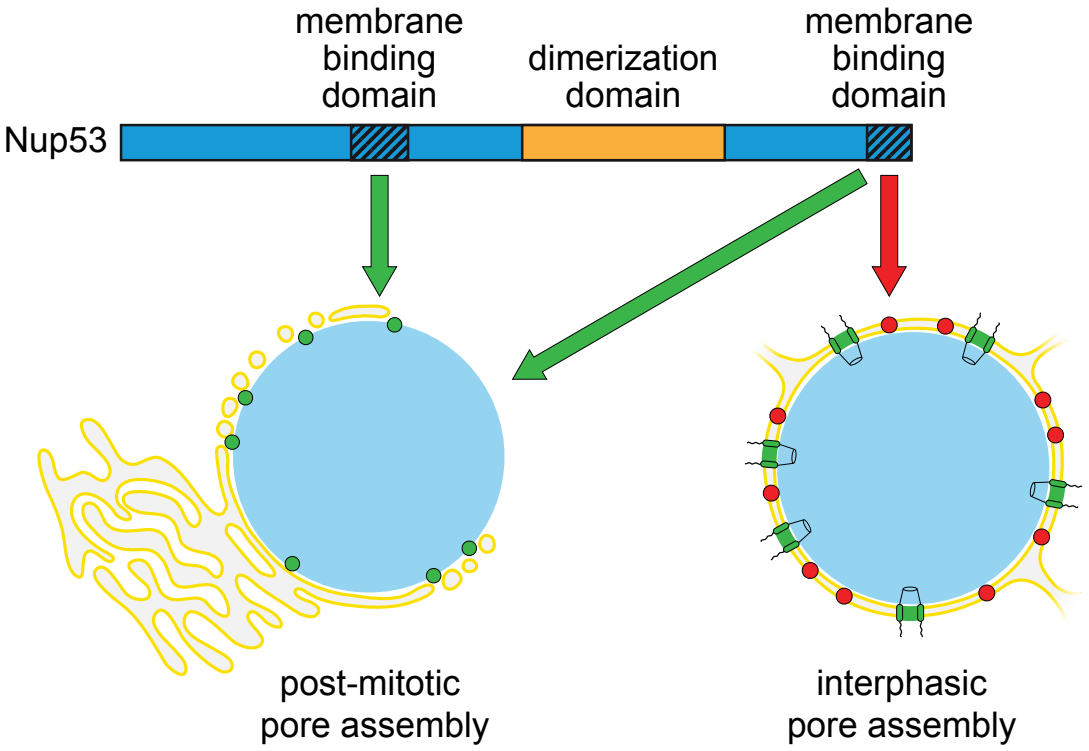

Figure 2 Nup53's role of membrane interaction in the two modes of NPC assembly

For post-mitotic NPC assembly either of the two membrane binding sites of Nup53 is sufficient to stabilize the membrane of the reforming NE (green arrows). For the specific mode of interphasic pore assembly, the C-terminal membrane binding site is required as it induces membrane curvature that is necessary for de-novo formation of nuclear pores (red arrow). The Nup53 protein domain structure is depicted in blue. The RRM domain, necessary for dimerization and membrane interaction, is highlighted in orange.

8.2. Nup53 is essential for nuclear assembly in metazoa

The essential function of Nup53 in metazoa was first demonstrated by depletion in *Caenorhabditis elegans*. RNAi mediated knockdown of this protein caused a complete block in nuclear formation after mitosis (Galy et al, 2003). This is consistent with data obtained from Nup53 depleted HeLa cells that exhibited severe defects in nuclear morphology and decreased levels of other Nup93 complex members (Hawryluk-Gara et al, 2005). The reduced localization of these proteins at the nuclear rim could be caused by a defect in NPC assembly. In deed nuclear assembly reactions using *Xenopus laevis* egg extracts revealed a block in post-mitotic NPC and NE formation after depletion of Nup53 (Hawryluk-Gara et al, 2008) which is

consistent with the data presented here (Fig. 2 (Vollmer et al, 2012)). Membrane vesicles attached to the chromatin surface are unable to fuse in order to form the two membrane layers of the NE ((Hawryluk-Gara et al, 2008); Fig. 2 (Vollmer et al, 2012)). Although Nup53 is conserved among all eukaryotic super-groups (Neumann et al, 2010), its essential function for NPC assembly is not. In *Saccharomyces cerevisiae* double deletion of both Nup53 homologues Nup53 and Nup59 does not result in lethality, although these cells show defects in growth (Marelli et al, 1998). Therefore the necessary function of vertebrate Nup53 seems to be dispensable in this organism. An explanation could be that interacting proteins act redundantly. In accordance with this is the observation that Nup53/Nup59 upon deletion of binding partners becomes essential (Marelli et al, 1998; Miao et al, 2006; Onischenko et al, 2009). Nup170 can interact with Pom152 (Aitchison et al, 1995; Makio et al, 2009) similar to the interaction seen between vertebrate Nup155 and Pom121 (Mitchell et al, 2010; Yavuz et al, 2010). Pom152 forms a tight interaction with the other transmembrane nucleoporins Ndc1 and Pom34 thereby bridging the connection between Ndc1 and Nup170 which would otherwise be done by Nup53/Nup59 (Onischenko et al, 2009). Although Pom121 and Ndc1 are co-enriched in the same vesicles in *Xenopus laevis* egg extracts (Antonin et al, 2005; Mansfeld et al, 2006) a direct interaction is not known. This is further supported by the observation, that Pom121 is also recruited to the chromatin upon Ndc1 depletion (Eisenhardt et al, 2014). As a result Nup53 would connect Ndc1 and Pom121 via Nup155, a function which could be bypassed in yeast and organisms that have no clear Nup53 homologue (DeGrasse et al, 2009; Neumann et al, 2010) by a direct interaction between the transmembrane nucleoporins.

Nup53 forms interactions with Nup155 and Nup93 in vertebrates (Fig. S2 (Vollmer et al, 2012); (Eisenhardt et al, 2014; Hawryluk-Gara et al, 2008; Hawryluk-Gara et al, 2005; Mansfeld et al, 2006; Sachdev et al, 2012) and these interactions are also found with the yeast homologues (Amlacher et al, 2011; Fahrenkrog et al, 2000; Lusk et al, 2002). As these three proteins are conserved in all five eukaryotic super-groups suggests an early evolutionary origin and the presence in the last eukaryotic common ancestor (Mans et al, 2004; Neumann et al, 2010). This subcomplex in connection to the transmembrane nucleoporins Pom121 and Ndc1 seems to play a pivotal role in the formation of the NPC structure as similar phenotypes are observed after individual depletion of the proteins from *Xenopus laevis* egg extracts (Fig. 2 (Vollmer

et al, 2012); (Antonin et al, 2005; Eisenhardt et al, 2014; Franz et al, 2005; Grandi et al, 1997; Hawryluk-Gara et al, 2008; Mansfeld et al, 2006; Sachdev et al, 2012)). Therefore all of these factors are required for the formation of the NE and NPCs and follow a strict order of recruitment. Interestingly, the recruitment of membrane vesicles is preceded by the binding of the Nup107-160 complex (Walther et al, 2003a) and depletion of this complex specifically prevents the formation of NPCs resulting in a closed NE without NPCs (Antonin et al, 2005; Walther et al, 2003a). Although Pom121 is not necessary for the fusion per se, it was speculated that a checkpoint mechanism blocks this process in the absence of Pom121 (Antonin et al, 2005). This seems to be true not only for Pom121, but also for the other transmembrane nucleoporin Ndc1 as well as the aforementioned components of the Nup93 complex. This underlines the important function of these proteins in the formation of the NE and the NPC. Ndc1 as well as the Nup93 complex are all located at the inner ring of the NPC forming together with the Nup107-160 complex the structural backbone of the NPC (Fig.1 (Vollmer & Antonin, 2014)). The assembly of the Nup93 complex at the nuclear pore takes place by a gradual recruitment of the individual members (Fig. 2 (Vollmer & Antonin, 2014)). The depletion of Nup53 from *Xenopus laevis* egg extracts revealed that none of the interacting proteins of the Nup93 complex is present at the chromatin surface (Fig. 4D (Vollmer et al, 2012)). Therefore Nup53 is the first member located at the nuclear membrane which recruits the other proteins of the Nup93 complex. This is in accordance with the observation that Nup53 is essential for the recruitment of Nup155 in *Caenorhabditis elegans* (Rodenas et al, 2009). The localisation of Nup53 is accomplished either via direct membrane binding or by an interaction with Ndc1 which is already present on the chromatin (Fig. 4D (Vollmer et al, 2012)). As constructs lacking either a functional N- or C-terminal membrane binding site are able to localize to the forming NE indicate that the membrane interaction of one site is sufficient for nuclear assembly after mitosis (Fig. 4 (Vollmer et al, 2012)). Interestingly, adding back a fragment of Nup53 that cannot interact with Ndc1 (Fig. S1 (Vollmer et al, 2012)) still permitted nuclear assembly (Fig. 4A&D (Vollmer et al, 2012)), suggesting that this interaction is dispensable. Recent data from our laboratory further characterized the Nup53 - Ndc1 interaction. By carefully dissecting the C-terminal membrane and Ndc1 binding sites revealed that the interaction with Ndc1 is indeed not necessary for the recruitment of Nup53 to the reforming NE (Eisenhardt et al, 2014). Nevertheless, the interaction is

required for nuclear assembly as Ndc1 seems to regulate the membrane deforming function of Nup53. This becomes evident as a construct of Nup53 lacking the Ndc1 interaction site that still deforms membranes is not able to restore NE and NPC formation (Eisenhardt et al, 2014). Nup53 and Nup155 located at the reforming NE, provide the binding platform for the recruitment of Nup93. This is evident as after depletion of Nup93, both proteins are present although at reduced levels on the membrane vesicles (Sachdev et al, 2012). The last two members of the Nup93 complex Nup188 and Nup205 are bound in a mutual exclusive fashion by Nup93 (Amlacher et al, 2011; Theerthagiri et al, 2010). As these interactions are stable during mitosis these proteins are recruited in two separate complexes Nup93-Nup188 and Nup93-Nup205 (Sachdev et al, 2012; Theerthagiri et al, 2010). It is therefore still not clear how the exact stoichiometry of the Nup93 complex looks like and if all Nup93 complexes in NPCs are similarly composed (Vollmer & Antonin, 2014). Subsequently part of the FG-Nups can then be anchored in the centre of the pore as Nup93 is also the key determinant for the recruitment of the Nup62 complex (Sachdev et al, 2012). Probably during this stepwise assembly the membrane vesicles are fused and the forming pore membrane is stabilized by the structural backbone of the NPC (Fig. 5 (Schooley et al, 2012)).

8.3. Nup53 as an interaction hub at the nuclear membrane

Nup53 is engaged in direct interactions with the transmembrane nucleoporin Ndc1 and the soluble components of the Nup93 complex Nup155 and Nup93 (Fig. S1 (Vollmer et al, 2012); (Eisenhardt et al, 2014; Hawryluk-Gara et al, 2008; Sachdev et al, 2012)). These interactions seem evolutionary conserved as they are also found in yeast (Amlacher et al, 2011; Fahrenkrog et al, 2000; Marelli et al, 1998; Onischenko et al, 2009). Nevertheless some of these interactions have been found to be dispensable for NPC assembly, like the interaction of Nup53 and Nup93 (Hawryluk-Gara et al, 2008) as well as Nup53 and NDC1 (Fig.4 (Vollmer et al, 2012); (Hawryluk-Gara et al, 2008)). For the Nup53 – Nup93 interaction, fragments of Nup53 lacking the Nup93 interaction domain were able to substitute the wildtype protein after depletion of endogenous Nup53 in nuclear assembly reactions using *Xenopus laevis* egg extracts. A block in NPC formation was only observed with fragments that are incapable of interacting with Nup155 (Hawryluk-Gara et al, 2008). This is only in part in accordance with the data presented here. Indeed, all fragments that were able to

rescue the Nup53 depletion phenotype were also able to interact with Nup155 (Fig. S1 & 4 (Vollmer et al, 2012)). The necessity of the interaction with Nup155 was further confirmed by a detailed analysis, showing that Nup53 point mutants incapable of Nup155 interaction were not able to substitute for wildtype Nup53 after depletion (Eisenhardt et al, 2014). Nup53 binds to Nup93 via an N-terminal region (Fig. S1 (Vollmer et al, 2012); (Hawryluk-Gara et al, 2008)) and previous results showed that a construct of Nup53 lacking this region is able to support NPC assembly after Nup53 depletion (Hawryluk-Gara et al, 2008). This could not be confirmed using several different Nup53 fragments deficient in Nup93 binding underlining the importance of this interaction (Fig. S6 (Vollmer et al, 2012)). These contradictory observations could be a result of different Nup53 depletion efficiencies as residual amounts of Nup53 were sometimes found in the prepared floated membranes employed in the nuclear assembly reaction which were discarded. Also the interaction of Nup53 to Ndc1, other than first suspected, is essential. Although not necessary for the recruitment to the reforming nucleus (Fig. S1&4 (Vollmer et al, 2012)), the regulation of the membrane interaction of Nup53 by Ndc1 seems to be crucial (see section 8.2. and (Eisenhardt et al, 2014)). Although not impeccably shown for the Nup53-Nup93 interaction, all known protein interactions mediated by Nup53 seem to be essential for the formation of the NPC and the NE after mitosis in vertebrates. Nup53 serves as an interaction point for the assembly of the Nup93 complex and as a crucial link to anchor this complex at the pore membrane (Fig. 5 (Schooley et al, 2012) & Fig. 2 (Vollmer & Antonin, 2014)).

8.4. Nup53 is a membrane binding protein

In addition to the essential protein interactions mediated at the nuclear pore by Nup53, it can also bind directly to the pore membrane. The two yeast homologues Nup53 and Nup59 were previously suspected to be peripheral membrane proteins due to an amphipathic helix that was predicted to be located at C-term (Marelli et al, 2001; Patel & Rexach, 2008)). As the last residues which are supposed to form this helix are highly conserved suggests that also the membrane binding function is evolutionary preserved. Indeed, not only the yeast homologues Nup53 and Nup59 but also vertebrate Nup53 from *Xenopus laevis* are able to directly interact with membranes (Fig. 1 (Vollmer et al, 2012); (Patel & Rexach, 2008)). Unexpectedly, this interaction is not only executed by the C-terminal region, but also by a region located

at the N-terminus (Fig.1 (Vollmer et al, 2012)). This is apparent as C- and N-terminal truncations are still able to interact with liposomes (Fig. 1&3 (Vollmer et al, 2012)).

8.4.1. The two membrane binding sites act via different interaction mechanisms

Surprisingly, the two membrane binding sites seem to act via different mechanisms. The N-terminal membrane binding site was narrowed down to a region that contained a patch of positively charged aas (Fig. 1&3 (Vollmer et al, 2012)). Charge inversion by mutating the specific residues to glutamate decreased the membrane binding of the minimal N-terminal fragment (Fig. 3 (Vollmer et al, 2012)). This demonstrates the dependence of the membrane interaction on these charged residues. Hence, these residues have to be exposed on the surface of the protein in order to interact with negatively charged lipid head groups of the interacting membrane. A similar mechanism is seen with proteins of the BAR-domain family (see section 3.5.). Proteins like amphiphysin or arfaptin have multiple, positively charged residues on their membrane interacting surface which permit the binding to negatively charged membranes (Casal et al, 2006; Peter et al, 2004). Also for amphiphysin charge switch mutations in the interaction surface results in a decreased membrane binding *in vitro* and *in vivo* (Peter et al, 2004). Nup53s membrane interaction could also be mediated by a phospholipid binding domain. Some of these domains like the PH or PX domains that specifically bind phosphoinositides (Lemmon, 2008) are found in some BAR domain proteins (Peter et al, 2004). As Nup53 binds to liposomes made from different lipid species, including vesicles prepared from pure 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Fig. S7 (Vollmer et al, 2012) and unpublished observation), argues against this mode of interaction.

Deletion of the hydrophobic patch at the C-term of Nup53 resulted in a significant decrease in membrane binding (Fig. 3 (Vollmer et al, 2012)). This is in accordance with the observation that the yeast homolog Nup53 loses its ability to bind liposomes when the last 15 aa are deleted (Patel & Rexach, 2008). Already truncation of the last hydrophobic residue, a conserved tryptophan, has a negative effect on the membrane interaction of the C-terminal membrane binding domain (Fig. 3A (Vollmer et al, 2012)). Therefore the C-terminal membrane interaction is probably mediated via hydrophobic interactions. If the C-term of Nup53 is formed into an amphipathic helix, as proposed for Nup53/Nup59 from *Saccharomyces cerevisiae* (Marelli et al, 2001), the hydrophobic residues located on one helix side could be inserted into one

membrane leaflet (Campelo et al, 2008; Graham & Kozlov, 2010). This insertion would produce a local membrane deformation which is verified by the ability of vertebrate Nup53 and the two yeast homologues Nup53/Nup59 to tubulate liposomes (Fig. 6 (Vollmer et al, 2012)). Similar processes have been demonstrated for proteins like endophilin or epsin (Boucrot et al, 2012; Farsad et al, 2001) and the small GTPase Sar1 (Lee et al, 2005). Interestingly, the amphipathic helix of the latter protein shows some sequence similarity to the C-term of yeast Nup53/Nup59 (Patel & Rexach, 2008) further supporting the assumption that this region actually forms an amphipathic helix. The fact that these domains often only form upon membrane binding and are therefore unstructured in solution (Gallop et al, 2006) could also explain the occurrence of high amounts of C-terminally truncated fragments upon expression of full length Nup53, as these regions are often prone to degradation (Fig. 1 (Vollmer et al, 2012)).

8.4.2. Regulation of Nup53's membrane binding function

Nup53 from *Saccharomyces cerevisiae* was shown to be phosphorylated (Marelli et al, 1998) and vertebrate Nup53 was also identified as a mitotic phosphoprotein (Stukenberg et al, 1997) and by mass spectrometric analysis several mitosis specific phosphorylation sites were identified (Tab. S1 (Vollmer et al, 2012)). Interestingly, two of these residues are in close proximity to the region that was identified as N-terminal membrane binding site. The introduction of phosphomimetic mutations as well as *in vitro* phosphorylation similarly decreased the membrane binding ability of the N-term of Nup53 (Fig. 3 (Vollmer et al, 2012)). This indicates that the membrane interaction of Nup53 is, at least in part, regulated in a cell cycle dependent manner. The fact that Nup53 is a target of CDK1 (Blethrow et al, 2008; Lusk et al, 2007b) and that a subset of the identified mitosis specific phosphorylation sites were CDK1 consensus sites (Tab. S2 (Vollmer et al, 2012)) supports this idea. The specific phosphorylation and hence the introduction of negative charge to the protein surface could lower the interaction with the NE, which could be necessary for either NE breakdown or the supply of unbound Nup53 for the next round of NPC assembly after mitosis. In addition the C-terminal membrane binding site seems to be functionally regulated by Ndc1. This is illustrated by the fact that Nup53 which is not able to interact with Ndc1 but with the membrane is not able to support NPC assembly. This is not just a cause of an impaired Nup53 recruitment, as it properly

localizes to the reforming NE although at lower levels (Eisenhardt et al, 2014). Ndc1 could be necessary to regulate the membrane deforming function of Nup53 by direct interaction. This could also be the reason for the close proximity of the Ndc1 and membrane binding sites on Nup53 (Eisenhardt et al, 2014). This demonstrates another advantage of the flotation test system that allows the investigation of factors that influence membrane binding of peripheral membrane proteins in a quantitative way (Fig. 3 (Vollmer et al, 2012)).

8.4.3. Nup53 dimerization is a prerequisite for membrane binding and deformation

Nup53 contains another highly conserved region located at the centre of the protein. This domain adopts the characteristic fold of an RNA recognition motif, but instead of RNA interaction it allows the formation of homodimers via hydrophobic interactions ((Handa et al, 2006); Fig. 2B (Vollmer et al, 2012)). As all fragments lacking the RRM domain were tested negative for membrane binding (Fig. 1B (Vollmer et al, 2012)), the contribution of dimerization for membrane interaction was tested by introduction of two aa changes that prevented the self-interaction of Nup53 *in vitro* and *in vivo* (Fig. 2A&B (Vollmer et al, 2012)). Indeed the membrane interaction was reduced, not only in context of the full length protein (Fig. 2C (Vollmer et al, 2012)), but also for the two individual membrane binding sites (Fig. S5 (Vollmer et al, 2012)). The dimerization could increase the overall avidity of the protein for membranes. Interestingly, proteins of the BAR-domain family also form homodimers which helps to induce membrane curvature probably by imposing the concave dimer surface onto the membrane (Gallop et al, 2006; Masuda et al, 2006; McMahon & Gallop, 2005). This is probably aided by the insertion of peripheral amphipathic helices into the outer membrane leaflet (Bhatia et al, 2009). Although Nup53 has no structural relation to BAR domains, it has similar features including the formation of dimers, the membrane binding by hydrophobic and charge interaction and the induction of membrane curvature (Peter et al, 2004; Qualmann et al, 2011). The latter function depends for both proteins on dimerization (Fig. 6 (Vollmer et al, 2012); (Peter et al, 2004)), but other than for BAR domain proteins (Henne et al, 2007), for Nup53 the dimerization is also essential for membrane binding (Fig. 2&S5 (Vollmer et al, 2012)). Furthermore, many BAR domain proteins also act as curvature sensor which is demonstrated by a higher affinity for smaller vesicles (Bhatia et al, 2009). For Nup53 a similar function was not detected. In fact the use of differently sized liposomes

showed a minor preference for bigger liposomes, probably mediated by the N-terminal binding region (Fig. S4 (Vollmer et al, 2012)). In terms of membrane topology this would agree with the fact, that the membrane of the NE forms, at least in interphase, a comparatively flat membrane surface in contrast to the highly curved bud necks of forming vesicles where some BAR domain proteins are recruited to (Qualmann et al, 2011).

8.4.4. The role of Nup53's membrane interaction during NPC assembly

8.4.4.1. Implications in NE and NPC reformation after mitosis

Vertebrate Nup53 is an essential component for NE and NPC assembly after mitosis ((Hawryluk-Gara et al, 2008); Fig. 2E&F (Vollmer et al, 2012)). Previously it was assumed that the main function of Nup53 was the binding of Nup155 at the reforming Nup93 complex (Hawryluk-Gara et al, 2008).The verification of Nup53's direct membrane binding describes another function for this protein (Vollmer et al, 2012). But this feature is only operational upon dimerization (Fig. 2C (Vollmer et al, 2012)). When endogenous Nup53 was substituted by mutants unable to form dimers in nuclear assembly reactions NPC and NE formation is blocked, probably by the incapability to interact with membranes (Fig. 2D&E (Vollmer et al, 2012)). Another explanation could be the slightly reduced interaction with Nup155 detected in GST-pulldown experiments (Fig. S1 (Vollmer et al, 2012)). The fact that fragments and constructs of Nup53 lacking a functional Nup155 interaction site cannot substitute the wildtype protein further emphasize the importance of this interaction (Eisenhardt et al, 2014; Hawryluk-Gara et al, 2008). Recent data also demonstrated that C-terminal Nup53 fragments comprising the mapped Nup155 interaction domain, but lacking the RRM domain were not interacting with Nup155 (Eisenhardt et al, 2014). This observation suggests that the RRM domain is involved in the formation of the interaction domain. Another possible explanation for the necessary function of dimerization could be that the self-interaction of Nup53 increases the Nup155 concentration on the membrane which in turn could be necessary for nuclear pore and hence NPC assembly. A similar effect is seen with amphiphysin dimers that have a higher avidity to its interaction partner dynamin than the monomeric protein (McMahon & Gallop, 2005). For this, it would be interesting to further elucidate the molecular function of Nup155 in addition to its essential role in NPC assembly. The

identification of the two individual membrane binding sites in Nup53 and the assignment of the responsible aas (Fig. 1&3 (Vollmer et al, 2012)), allowed the determination of the respective contribution for NPC assembly (Figure 2). Interestingly, constructs of Nup53 containing only one functional membrane binding site are sufficient to support post-mitotic NE and NPC assembly after depletion of endogenous Nup53 (Fig. 4 (Vollmer et al, 2012)). This is also observed for fragments that lack the C-terminal membrane and Ndc1 binding site (Fig. 4 (Vollmer et al, 2012)). As described in section (8.4.2.) the Ndc1 interaction is only dispensable when also the membrane deformation ability of Nup53 is impaired (Eisenhardt et al, 2014). In contrast, when constructs of Nup53 are used that do not have a functional membrane binding site, NPC assembly is blocked (Fig. 4 (Vollmer et al, 2012)). Nup53 is a crucial member of the Nup93 complex (Hawryluk-Gara et al, 2008; Rodenas et al, 2009; Vollmer & Antonin, 2014) as it localizes early to the reforming NE (Fig. 4 (Vollmer et al, 2012)). One essential function in this situation is probably also the recruitment of the interacting proteins Nup155 and Nup93 (Vollmer & Antonin, 2014). The many different interactions mediated by Nup53 make it an essential anchor point at the reforming NE membrane in the stepwise assembly of NPCs after mitosis (Fig. 2 (Vollmer & Antonin, 2014); Fig. 5 (Schooley et al, 2012)). In the course of post-mitotic nuclear assembly the membranes of the NE are reformed from the ER network (reviewed in Schooley et al, 2012). It is still not completely clear if this chromatin enclosure by the NE membranes is performed in form of membrane tubes or sheets from ER cisternae (Fig. 2&3A (Schooley et al, 2012) & see section 3.3.). In both cases the edges of the membrane sheets and tubules exhibit a strong positive membrane curvature (Fig. 7 (Vollmer et al, 2012)) similar to the vesicles that are employed in the nuclear assembly assay featuring *Xenopus laevis* egg extracts. This positive curvature has to be maintained as it is also present in the final doughnut shaped membrane topology of the pore membrane (Fig. 7 (Vollmer et al, 2012)). Nup53 could in this situation, by a tight interaction with the membrane stabilize this conformation and in parallel recruit the interacting partners that form the Nup93 complex and hence the structural backbone of the NPC (Fig. 5 (Schooley et al, 2012)). As constructs impaired in one of the two membrane binding site are in both cases able to support NPC formation in this mode of assembly, either binding site seems to be sufficient to stabilize the membrane (Figure 2 left panel). In accordance ER membrane bending proteins of the reticulon family

are localized to membrane regions of high curvature during NE assembly and are implicated to play a role in the subsequent integration of the ER membrane into the NE (Kiseleva et al, 2007).The involvement of these proteins reflects the importance of membrane curvature regulation during NE reformation. In the final structure the positive curvature along the membrane in the pore is accompanied by a negative curvature in the plane of the NE membrane (Fig. 7 (Vollmer et al, 2012); (Antonin & Mattaj, 2005)) and may therefore be at least in part obliterated as this conformation probably minimizes the elastic energy of the membrane topology, similar to the situation in interconnected ER membrane sheets (Terasaki et al, 2013).

8.4.4.2. The role of Nup53 during interphasic NPC assembly

Nuclear pore complex assembly takes place in two distinct stages of the cell cycle. One time point is after mitosis when the chromatin has been segregated and is enclosed by the re-forming NE membranes (reviewed in Schooley et al, 2012; Vollmer & Antonin, 2014)). In addition NPC assembly is also happening in interphase. During this phase of the cell cycle NPC numbers in cells increase at least twofold (Maul et al, 1971). As the chromatin is surrounded by the two membranes of the NE, new NPCs have to be assembled de-novo by fusion of the outer and inner nuclear membrane ((reviewed in Doucet & Hetzer, 2010); Fig. 3B (Schooley et al, 2012) & see section 3.4.4.2.4.). The components necessary for this process are only beginning to emerge, but Nup53 as a membrane binding protein would be a likely candidate to be involved in the formation and the subsequent stabilization of the nuclear pore. Using the different Nup53 constructs containing only one functional membrane interaction site demonstrated, that the C-terminal membrane binding site is crucial for NPC assembly during interphase (Fig. 5 (Vollmer et al, 2012); Figure 2). As Nup53 has the ability to deform membranes by interaction with its C-terminal domain (Fig. 6 (Vollmer et al, 2012)) it could be assumed that this function is essential for the formation of nuclear pores. The intrinsic membrane interaction that is mediated via the dimerization of the RRM domain could be necessary for an initial binding, followed by a tighter interaction that leads to the insertion of the C-terminal hydrophobic residues resulting in the observed deformation of liposomes (Fig. 6 (Vollmer et al, 2012)). This is consistent with the fact, that the single membrane binding sites loose a significant amount of their interaction potential when impaired in dimerization (Fig. S5 (Vollmer et al, 2012)). As the removal of the last residue already

obliterates tubulation emphasizes the importance of this hydrophobic region. A fact also manifested in the high conservation of this domain from yeast to vertebrata (Neumann et al, 2010). The curvature induction at the NE membrane by Nup53 would approximate the two membranes and help in the following fusion (Fig. 7 (Vollmer et al, 2012)). As yeast undergoes closed mitosis (see section 3.2.1.), NPC insertion is only possible via membrane fusion. In accordance with this the membrane deforming function of Nup53 is evolutionary conserved (Fig. 6 (Vollmer et al, 2012)) and overexpression of Nup53 in *Saccharomyces cerevisiae* results in the formation of intranuclear membrane stacks (Marelli et al, 2001). Nevertheless this function is probably redundant as deletion of both Nup53 homologues is not lethal (Marelli et al, 1998; Onischenko et al, 2009). Interestingly, also in this mode of NPC assembly, in yeast as well as in vertebrates, the ER membrane bending reticulons seem to be involved (Dawson et al, 2009). In this situation they could, similar to Nup53, generate membrane curvature that leads to a fusion of the inner and outer nuclear membrane (Doucet & Hetzer, 2010). Except the reticulons also Nup133, a member of the Nup107-160 complex (Figure 1), Sun1 and Pom121 are implicated to play a role in the interphasic NPC assembly (Doucet et al, 2010; Talamas & Hetzer, 2011). In fact Nup133 is the only other metazoan soluble nucleoporin that is known to directly interact with membranes. An exposed loop of the beta propeller domain of this protein has the ability to form an amphipathic helix that can interact with highly curved membrane regions (Drin et al, 2007). Sun1 is a transmembrane protein of the inner nuclear membrane and part of the LINC complex that connects the NE to the cytoskeleton (Crisp et al, 2006). During interphase Sun1 localizes to NPCs and is believed to be involved in NPC assembly by interacting with the transmembrane nucleoporin Pom121 (Talamas & Hetzer, 2011). In addition the latter can interact with Nup160 which is another member of the Nup107-160 complex (Mitchell et al, 2010) and is supposed to mediate the membrane fusion for pore formation (Doucet & Hetzer, 2010). But if these proteins play an active role in nuclear pore formation is not completely clear. Pom121 has no homology to other fusion proteins and is also not evolutionary conserved (Neumann et al, 2010). The finding that the Nup107-160 complex is not involved in formation of the early intermediates of interphase NPC assembly (Talamas & Hetzer, 2011) is in accordance with the fact that the amphipathic helix formed by Nup133 is a curvature sensing and not inducing domain (Drin et al, 2007). This region probably directs the protein and the associated

complex to highly bent membrane regions of newly formed nuclear pores. This is in line with the observation that *in vitro* a fragment of Nup133 containing the membrane binding domain has a higher affinity to smaller vesicles (Drin et al, 2007) and is unable to tubulate liposomes (Fig. 6 (Vollmer et al, 2012)). An additional function of the Nup107-160 complex could be the stabilization of the membrane surface during or after the formation of the nuclear pore. The solved structures of several members of the Nup107-160 complex revealed evidence of a co-evolution and common ancestry with vesicle coat proteins (Brohawn et al, 2008; Schwartz, 2013). The recently determined arrangement of the Nup107-160 complex in the NPC shows a head to tail multimerization (Bui et al, 2013) forming two rings sitting on the outer boundary of the nuclear pore (Figure 1). These rings are on both sides approximately 10 nm elevated from the membrane only touching the membrane surface at one point per Y-complex (Beck & Glavy, 2014). This membrane interaction could be established via a direct membrane binding of Nup133. Another possibility would be that this demonstrates the interaction of Nup160 with Pom121 (Mitchell et al, 2010). Similar to COP mediated vesicle formation the membrane interaction would be mediated by adaptor proteins (D'Arcangelo et al, 2013). In addition other proteins that help sculpting the membrane are necessary as the lattice formed by the COP complex is not able to deform membranes by itself (Kirchhausen, 2000). Interestingly, an analogous mechanism could be possible for the yeast Nic96 complex. As also Nic96 shares the overall architecture of the ancestral element that links it to vesicle coat proteins and the two homologues Nup157/170 share a common ancestry with Nup133 one could draw a functional parallel. In this case Nup53/Nup59 would be involved in the formation of the curved membrane in close connection to the transmembrane nucleoporins Ndc1, Pom152 and Pom34 and the subsequent anchorage of the lattice formed by Nup157/170 and Nic96 that stabilizes the highly bent pore membrane (Rexach, 2009).

In summary, the membrane interaction of Nup53 plays an important role in both modes of NPC assembly. During post-mitotic NPC assembly the membrane binding of Nup53 probably helps to stabilize the membrane curvature of the reforming NE by either one of the membrane interaction sites. However in interphase, de-novo formation of NPCs into the intact NE depends on the membrane deformation of the C-terminal binding site that probably helps in approximation and fusion of the two membranes (Fig. 7 (Vollmer et al, 2012)). The discovery of other proteins involved in

these final fusion steps and the investigation of the precise molecular details that lead to the formation of the nuclear pore will be an interesting task for future studies.

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10. Abbreviations list

aa	amino acid
C-terminus	carboxyl-terminus
FG	phenylalanine glycine dipeptide
GST	glutathione S-transferase
INM	inner nuclear membrane
kDa	kilo Dalton
Mda	mega Dalton
N-terminus	amino-terminus
NE	nuclear envelope
nm	nano meter
NPC	nuclear pore complex
Nup	nucleoporin
ONM	outer nuclear membrane

11. Appendix

Original published articles and submitted manuscripts, which are included in this thesis.

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Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly

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Nuclear pore complexes (NPCs) fuse the two membranes of the nuclear envelope (NE) to a pore, connecting cytoplasm and nucleoplasm and allowing exchange of macromolecules between these compartments. Most NPC proteins do not contain integral membrane domains and thus it is largely unclear how NPCs are embedded and anchored in the NE. Here, we show that the evolutionary conserved nuclear pore protein Nup53 binds independently of other proteins to membranes, a property that is crucial for NPC assembly and conserved between yeast and vertebrates. The vertebrate protein comprises two membrane binding sites, of which the C-terminal domain has membrane deforming capabilities, and is specifically required for *de novo* NPC assembly and insertion into the intact NE during interphase. Dimerization of Nup53 contributes to its membrane interaction and is crucial for its function in NPC assembly.

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Introduction

The defining feature of the eukaryotic cell is the compartmentalization of genetic material inside the nucleus. The spatial and temporal separation of transcription and translation has enabled eukaryotes to achieve a level of regulatory complexity that is unprecedented in prokaryotes. This is accomplished by the nuclear envelope (NE), which serves as the physical barrier between the cytoplasm and the nucleoplasm. Nuclear pore complexes (NPCs) are the exclusive gateways in the NE allowing diffusion of small substances and regulated trafficking of macromolecules up to a size of 15 nm for ribosomal subunits or even 50 nm for Balbiani ring particles (for review, see Wente and Rout, 2010; Hoelz *et al*, 2011; Bilokapic and Schwartz, 2012). NPCs form large pores in the NE, having a diameter of ~130 nm. Unlike other transport channels, NPCs span two lipid bilayers, at sites where the outer and inner membranes of the NE are fused. Therefore, NPCs are assumed to play an important role in deforming these membranes to form a pore as well as in stabilizing this highly curved pore membrane (Antonin *et al*, 2008).

Given the structural complexity and extraordinary transport capabilities of NPCs, it is quite surprising that these huge macromolecular assemblies of 40–60 MDa are only composed of ~30 different proteins. These nucleoporins (Nups) can be roughly categorized into those forming the structure of the pore and those mediating transport through the NPC. The latter class is characterized by a high number of FG repeats. Two evolutionary conserved subcomplexes form the major part of the structural scaffold. The Nup107–160 complex in metazoa, or the corresponding Nup84 complex in yeast, is the best characterized of these owing to an extensive set of genetic, biochemical and structural data. Computational and structural analyses suggest that this complex is related to vesicle coats (Devos *et al*, 2004; Mans *et al*, 2004; Brohawn *et al*, 2008). It is possible that these proteins form a coat-like assembly stabilizing the curved pore membrane of the NPC, which is analogous to clathrin or COPI and II during vesicle formation (Field *et al*, 2011; Onischenko and Weis, 2011). Notably, neither clathrin nor COP coats interact directly with the lipid bilayers. They are rather linked to the deformed membrane via adaptor and integral proteins (McMahon and Mills, 2004). Although three integral membrane proteins are known in the vertebrate NPC, it is unclear how a possible link between the Nup107–160 complex and the membrane is established.

The second major structural and evolutionarily conserved subcomplex of the NPC is the metazoan Nup93 complex, Nic96 in yeast, which might serve as a link to the pore membrane. In vertebrates, it is composed of five nucleoporins: Nup205, Nup188, Nup155, Nup93 and Nup53. Nup53, also referred to as Nup35 (Cronshaw *et al*, 2002), interacts with Nup93 and Nup155 (Hawryluk-Gara *et al*, 2005), corresponding to Nup170 and Nic96 in yeast (Marelli *et al*, 1998; Fahrenkrog *et al*, 2000). Nup155, Nup93 and Nup53 are each indispensable for NPC formation in vertebrates (Franz *et al*, 2005; Hawryluk-Gara *et al*, 2008; Mitchell *et al*, 2010; Sachdev *et al*, 2012). Interestingly, Nup53 and its corresponding yeast homologues Nup53p and Nup59p interact with the transmembrane nucleoporin NDC1, thereby potentially linking the NPC to the pore membrane (Mansfeld *et al*, 2006; Onischenko *et al*, 2009). Although NDC1 is essential in both vertebrates and yeast (Winey *et al*, 1993; West *et al*, 1998; Mansfeld *et al*, 2006; Stavru *et al*, 2006) it is not found in all eukaryotes (Mans *et al*, 2004; DeGrasse *et al*, 2009; Neumann *et al*, 2010), suggesting that in

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some organisms NPCs can form in the absence of a Nup53–NDC1 interaction. Indeed, *Aspergillus nidulans* is viable in the absence of all three known transmembrane nucleoporins (Liu *et al*, 2009). This suggests that there are alternative modes of interaction between the nucleoporins and the pore membrane.

Here, we show that Nup53 binds membranes directly and independently of other proteins. It possesses two membrane interaction regions, which are important for NPC assembly. Although either site is sufficient for NPC assembly at the end of mitosis, the C-terminal membrane binding site of Nup53 is specifically required for NPC assembly during interphase, probably because of its membrane deforming capabilities.

Results

Nup53 is a membrane binding protein

Overexpression of Nup53 in yeast causes expansion of the NE (Marelli *et al*, 2001). Similar membrane proliferation phenotypes have been observed upon overexpression of nuclear membrane binding proteins, such as lamin B (Prufert *et al*, 2004). Yeast Nup53 contains a C-terminal region predicted to form an amphipathic helix (Marelli *et al*, 2001; Patel and Rexach, 2008), which could serve as a membrane binding module. However, Nup53 interacts with the integral pore membrane protein NDC1 in both yeast and metazoa (Mansfeld *et al*, 2006; Onischenko *et al*, 2009) and thus might be linked to the membrane via this interaction. We therefore tested whether Nup53 is able to interact with membranes independently of other proteins.

To assay for membrane binding, we generated liposomes with an average radius of ~150 nm. These liposomes were incubated with different, recombinantly expressed nucleoporins and floated through sucrose cushions. Liposome binding proteins were recovered after centrifugation from the top fraction (Figure 1A). A Nup133 membrane binding fragment (Drin *et al*, 2007) was used as a positive control and found in the liposome containing top fraction (Figure 1A). Similarly, Xenopus Nup53 was found in the top fraction, indicating membrane interaction. In contrast, a fragment of the FG repeat-containing nucleoporin Nup98 did not bind liposomes. Thus, Xenopus Nup53 binds directly to

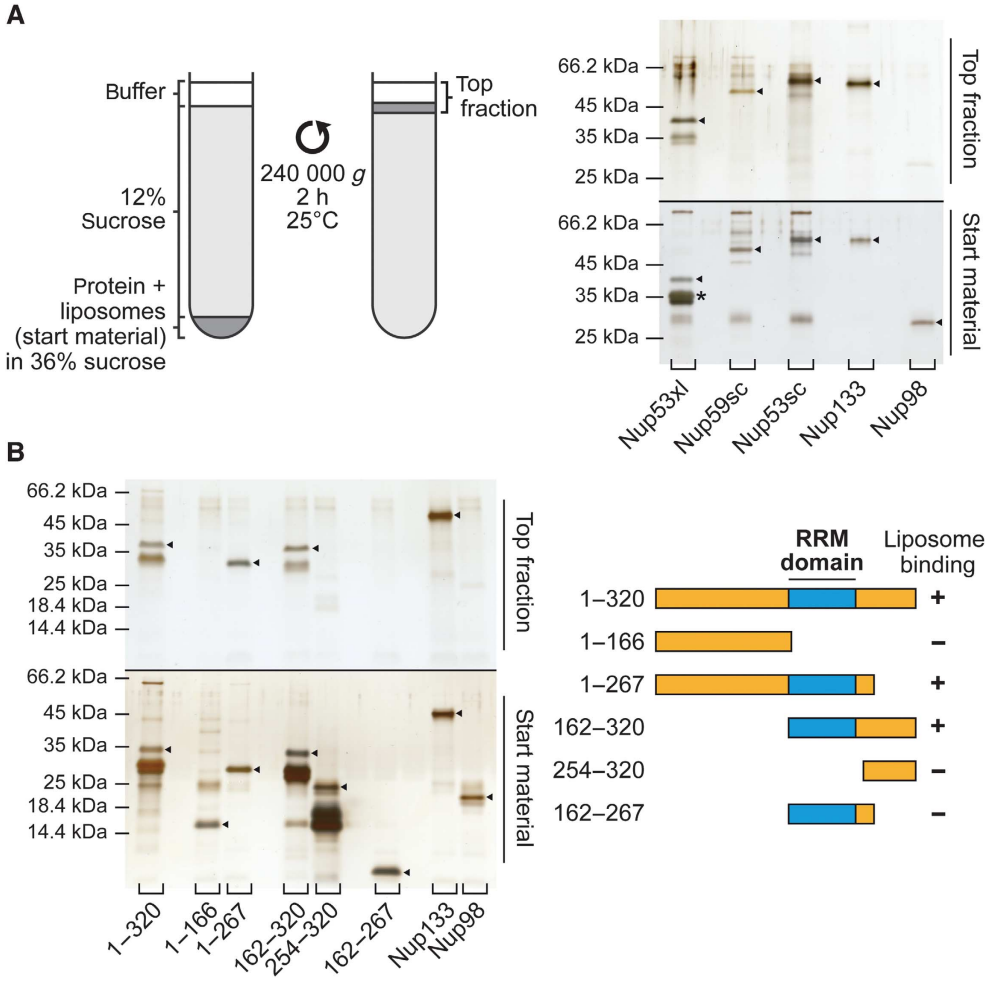

Figure 1 Nup53 directly binds membranes. (**A**) 3 μM recombinant Xenopus Nup53 (Nup53xl), the two yeast orthologues Nup59sc and Nup53sc as well as fragments of Nup133 and Nup98 as positive and negative controls, respectively, were incubated with 6 mg/ml fluorescently labelled liposomes prepared from *E. coli* polar lipids and floated through a sucrose gradient as indicated on the left. Top fractions of the gradient, as well as 3% of the starting material, were analysed by SDS–PAGE and silver staining. Please note that Nup53 is sensitive to C-terminal degradation (*) and that the full-length protein significantly enriched in the liposome bound fraction. (**B**) Full-length (1–320) and different fragments of Xenopus Nup53 as well as fragments of Nup133 and Nup98 were analysed for liposome binding as in (**A**). Only fragments comprising the RRM domain (indicated in blue in the schematic representation) bound liposomes.

membranes independently of other interacting proteins. To determine whether this feature is conserved during evolution, we tested the two yeast homologues Nup53p and Nup59p, which both bound liposomes (Figure 1A).

We next sought to define the regions of Xenopus Nup53 important for its membrane interaction. Nup53 can be roughly divided into three parts: the N-terminus (amino acid (aa) 1–166), a middle region (aa 166–267) comprising a conserved RNA recognition motif (RRM) domain and the C-terminus (aa 267–320) (Figure 1B). We generated different N- and C-terminal truncations of Nup53 and tested them for liposome binding (Figure 1B). While full-length Nup53 (aa 1–320) bound to liposomes, the N-terminal region of the protein (aa 1–166) showed no binding. Extending this fragment by 100 aa to include the RRM domain rendered the protein capable of membrane binding (aa 1–267). The C-terminal half of Nup53 (aa 162–320), which included the RRM domain, also interacted with liposomes. However, a fragment consisting of only the C-terminal region of Nup53 but lacking the RRM domain (aa 254–320) could not bind liposomes. Surprisingly, a fragment comprising only the RRM domain (aa 162–267) did not bind liposomes showing that the RRM domain is necessary but not sufficient for Nup53 membrane binding.

Nup53 dimerization is necessary for membrane binding and NPC formation

As the RRM domain is crucial for Nup53 membrane interaction we investigated the function of this domain. The crystal structure of the mouse domain suggests that it acts as a dimerization rather than an RNA binding module (Handa *et al*, 2006). We designed a mutant of this domain by exchanging two amino acids (F172E/W203E) in the dimerization surface. Size exclusion chromatography in combination with multi-angle laser light scattering revealed that the resulting fragment was monomeric (Figure 2A).

To confirm that the dimerization occurs also *in vivo*, we performed co-transfection experiments in HeLa cells using HA- and myc-tagged Xenopus Nup53. Either α-HA or α-myc antibodies immunoprecipitated both HA- and myc-tagged wild-type Nup53 indicating that the proteins formed a complex (Figure 2B, lanes 5 and 10). If cells were transfected with either construct separately before they were mixed for protein extraction, then no co-immunoprecipitation was observed (Figure 2B, lanes 9 and 14) demonstrating that complex formation cannot occur under the conditions of the immunoprecipitation. Co-transfections of RRM mutants as well as RRM mutants and wild-type protein did not result in complex formation (Figure 2B, lanes 6–8 and 11–13) indicating that the F172E/W203E exchange inhibited dimerization/oligomerization.

Next, we tested the effect of these mutations on membrane binding. In the context of the full-length protein, these mutations decreased liposome binding by 70% (Figure 2C) suggesting that the dimerization of Nup53 is important for its membrane interaction.

As Nup53 is essential for postmitotic NPC formation (Hawryluk-Gara *et al*, 2008) we examined the relevance of Nup53 membrane binding for this. We employed *Xenopus laevis* egg extracts to study nuclear reformation *in vitro* (Lohka, 1998). With antibodies against Nup53 we depleted the endogenous protein without co-depletion of other nucleoporins including the other members of the Nup93 complex: Nup93, Nup155, Nup205 and Nup188 (Figure 2D). These depleted extracts were incubated with sperm heads serving as chromatin template. In the absence of Nup53, NPC formation was blocked (Figure 2E) as reported (Hawryluk-Gara *et al*, 2008). This was indicated by the absence of immunofluorescent signal on the chromatin surface for mab414, an antibody recognizing several nucleoporins that represent a major subfraction of the NPC. Addition of recombinantly expressed wild-type Nup53 to the depleted extracts at levels similar to the endogenous protein (Figure 2D) restored NPC formation. The recombinant protein was integrated into NPCs as indicated by immunostaining with a Nup53 antibody. In contrast, the dimerization and membrane binding defective Nup53 mutant (1–320 F172E/W203E) was unable to substitute for the endogenous protein in NPC formation (Figure 2E).

Individual depletion of several nucleoporins essential for NPC assembly from Xenopus egg extracts also blocks formation of a closed NE. These nucleoporins include POM121, NDC1, Nup155, Nup93 (Antonin *et al*, 2005; Franz *et al*, 2005; Mansfeld *et al*, 2006; Sachdev *et al*, 2012) and Nup53 (Hawryluk-Gara *et al*, 2008). Upon Nup53 depletion, membrane vesicles bound to the chromatin surface but did not fuse to form a closed NE (Figure 2F; Hawryluk-Gara *et al*, 2008). This phenotype was rescued by the wild-type Nup53 protein, but not by the dimerization defective mutant. Together with the liposome-binding assay, this suggests that Nup53 membrane binding could be important for NPC assembly and formation of a closed NE. However, we cannot exclude that the RRM mutant also affects interaction of Nup53 with other nucleoporins. Indeed, in GST pull-down assays we observed a slight reduction of Nup93, Nup205 and Nup155 binding to Nup53 in the context of the RRM mutant as compared to the wild-type protein (Supplementary Figure S1A). In contrast, NDC1 binding to Nup53 was unaffected by the dimerization mutant (Supplementary Figure S1B).

Nup53 possesses two membrane binding regions

These results reveal a crucial role for Nup53 dimerization via its RRM domain in NE reformation. However, the RRM domain alone did not bind to membranes. We tested different Nup53 truncations for liposome binding to map the membrane interaction sites (Figure 3A; Supplementary Figure S2 shows the purity of all recombinant proteins used in the different liposome experiments). An N-terminal fragment of Nup53 including the RRM domain (aa 1–267) bound to liposomes with only a slightly decreased binding efficiency, 83% of the levels of the full-length protein. Further truncations from the N-terminus revealed a minimal membrane binding region between aa 93 and 107 as indicated by a four-fold decrease in liposome binding upon removal of these 15 amino acids. This region comprises a patch of basic residues, which as in other membrane binding proteins might be important for the interaction with negatively charged lipids. Indeed, changing two residues to negatively charged residues (R105E/K106E) abolished membrane binding (Figure 3A, 83% reduction as compared to the 93–267 fragment).

Interestingly, the N-terminal membrane binding region of Nup53 contains one of two regions that were differentially phosphorylated depending on the cell-cycle stage (Supplementary Figure S3; Supplementary Table S1). Two

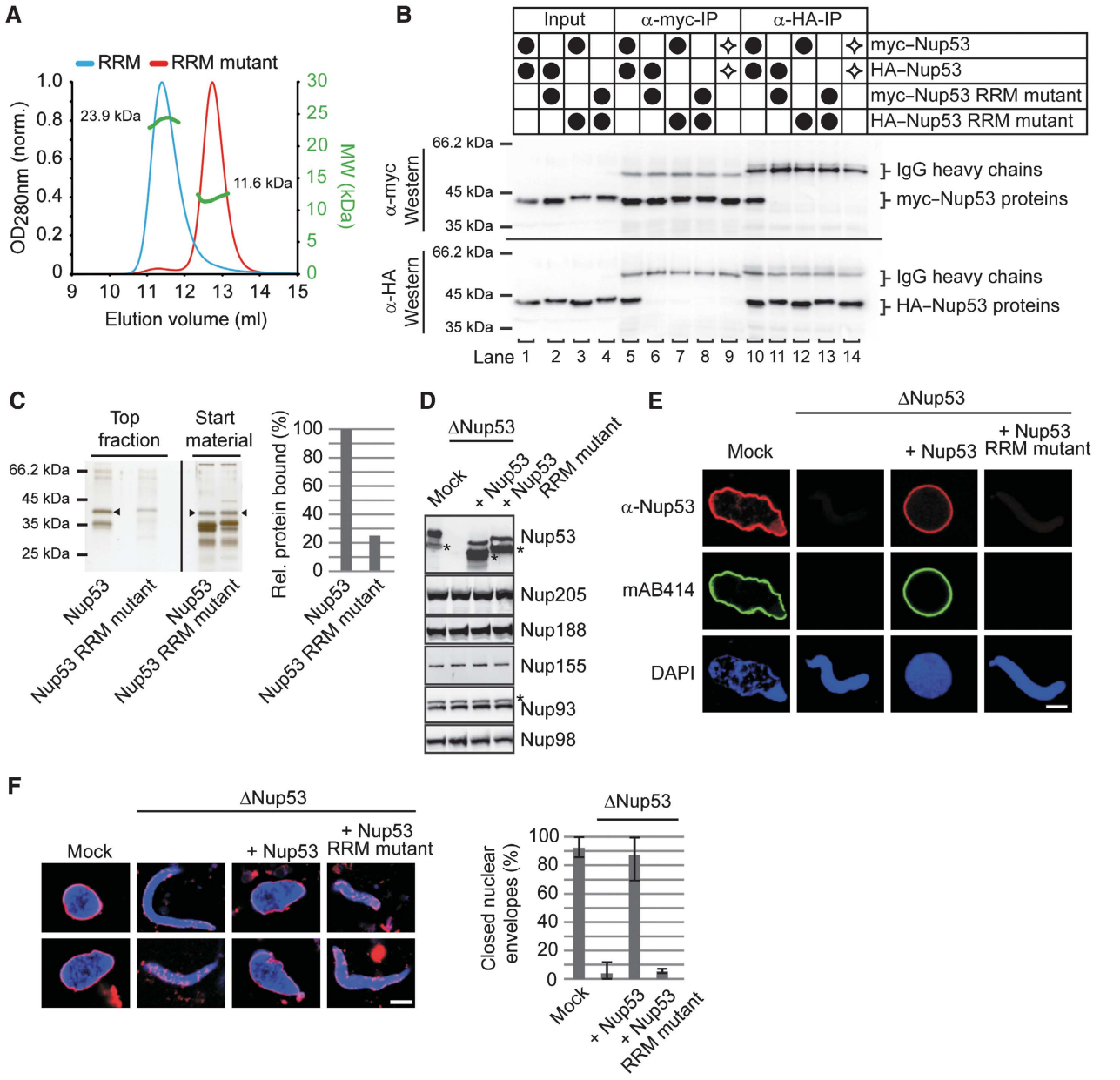

Figure 2 Dimerization of the RRM domain is essential for Nup53 membrane binding and nuclear pore complex formation. (**A**) Size exclusion chromatography on a Superdex75 10/300 GL column followed by multi-angle static laser light scattering of the Xenopus Nup53 RRM domain and the F172E/W203E mutant, which rendered the domain monomeric. The green dots relate to the secondary axis and show the molecular weight of the eluting particles. (**B**) HeLa cells were co-transfected with myc- and HA-tagged Xenopus Nup53 wild-type protein and/or dimerization mutant as indicated (●). Proteins were immunoprecipitated from cell lysates with α-myc or α-HA antibodies, and analysed by western blotting. Ten per cent of the start materials are loaded as input. To exclude complex formation after cell lysis, extracts from single transfected myc–Nup53 and HA–Nup53 cell batches were mixed and processed for immunoprecipitation (✧). Under these conditions, no co-precipitation was observed. (**C**) Full-length Xenopus Nup53 and the respective F172E/W203E mutant (RRM mutant) were analysed for liposome binding as in Figure 1. The right panel shows the quantitation of liposome binding analysed by western blotting and normalized to the levels of the wild-type protein (one out of two independent experiments). (**D**) Western blot analysis of mock, Nup53-depleted (ΔNup53) and Nup53-depleted extracts supplemented with recombinant wild-type protein (Nup53) or the dimerization mutant (Nup53 RRM mutant), respectively. Recombinant proteins were added to approximate endogenous Nup53 levels (judged by the full-length protein, please note for both endogenous and recombinant Nup53 proteins C-terminal degradation products (*)). The recombinant Nup53 migrated slightly faster than the endogenous protein due to absence of eukaryotic post-translational modifications. The Nup93 antibody recognizes a slightly slower migrating cross-reactivity by western blotting (*). (**E**) Nuclei were assembled in mock, Nup53-depleted extracts or Nup53-depleted extracts supplemented with wild-type protein (Nup53) or the dimerization mutant for 120 min, fixed with 4% PFA and analysed with Nup53 antibodies (red) and mAb414 (green). Chromatin was stained with DAPI. Bar: 10 μm. (**F**) Nuclei were assembled as in (**E**), fixed with 2% PFA and 0.5% glutaraldehyde and analysed for chromatin (blue: DAPI) and membrane staining (red: DiIC18, bar: 10 μm). Right panel shows the quantitation of chromatin substrates with a closed nuclear envelope (averages of three independent experiments with >300 randomly chosen chromatin substrates per sample, error bars represent the range).

amino acids identified, Serine 94 and Threonine 100, are phosphorylated during mitosis, most likely by cdk 1 as they possess a consensus site for this kinase (Blethrow *et al*, 2008). To investigate the impact of this modification on membrane binding, we generated mutants mimicking the phosphorylated (S94E/T100E) and the unphosphorylated

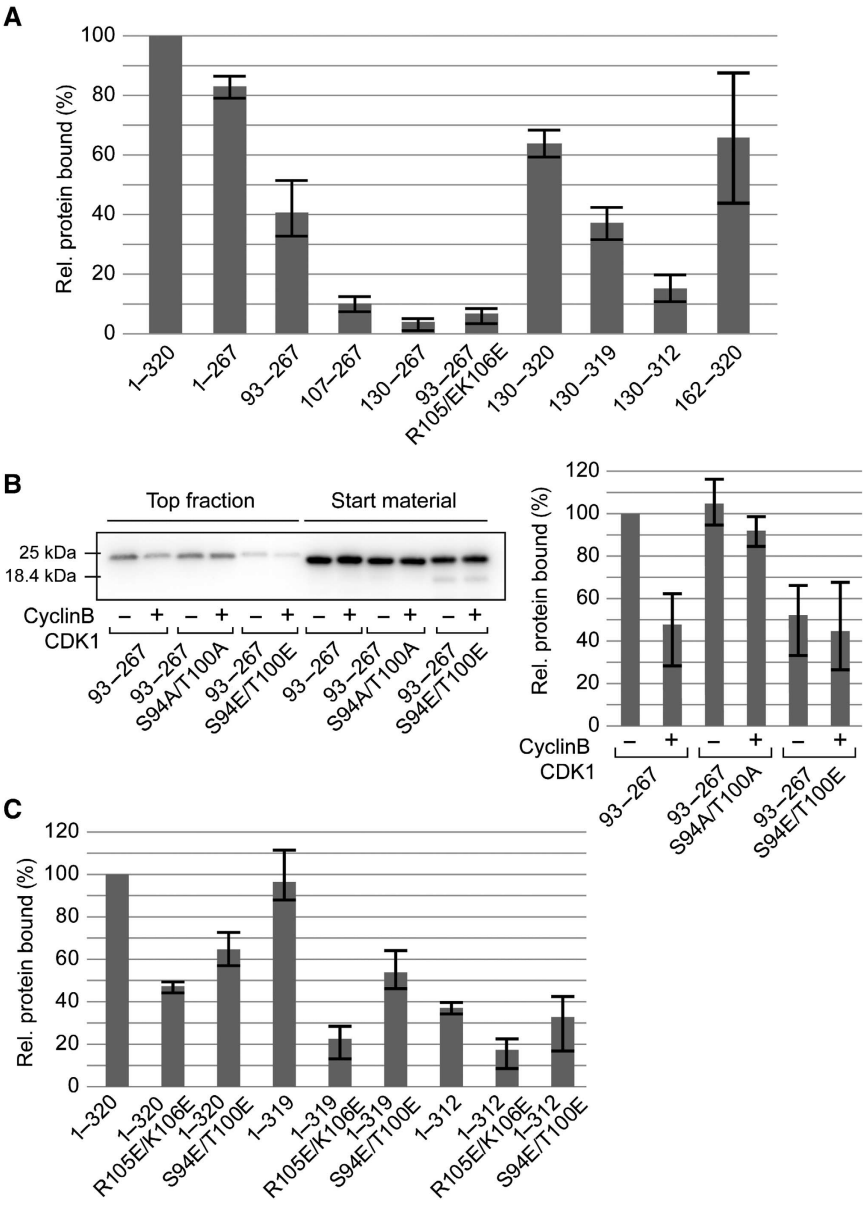

Figure 3 Nup53 possesses two independent membrane binding regions. (**A**) Full-length protein (1–320) and different fragments of Xenopus Nup53 were quantitatively analysed for liposome binding as in Figure 2B (normalized to the full-length protein, three independent experiments). (**B**) A fragment comprising the first membrane binding region and the RRM domain (93–267) as well as a phosphomimetic (93–267 S94E/T100E) and a non-phosphorylatable mutant (93–267 S94A/T100A) was treated with CyclinB/CDK1. Samples were tested for liposome binding as in Figure 1A and analysed by western blotting (left panel) and quantified (right panel: two independent experiments, normalized to liposome binding of wild-type fragment without CDK1 pretreatment). (**C**) Mutants/truncations affecting the N- (1–320 R105E/K106E, 1–320 S94E/T100E), the C-terminal (1–319, 1–312) as well as both (1–319 R105E/K106E, 1–319 S94E/T100E, 1–312 R105E/K106E, 1–312 S94E/T100E) membrane binding sites of Nup53 were quantitatively assayed for liposome binding in the context of full-length protein (normalized to wild-type protein (1–320), average of three independent experiments, error bars represent the range).

(S94A/T100A) state of the protein. The phosphomimetic mutant was impaired in liposome binding (Figure 3B, reduced by 50% compared to the 93–267 fragment) while the S94A/T100A control bound to liposomes with efficiency comparable to the wild type. Furthermore, *in vitro* phosphorylation by CyclinB/CDK1 reduced liposome binding of the wild-type protein by 50%, levels similar to the phosphomimetic mutant (S94E/T100E), but did not affect the S94A/T100A mutant, suggesting that mitotic phosphorylation regulates the membrane binding of Nup53.

The C-terminal part of Nup53 also interacts with membranes, an activity that requires the presence of the RRM domain (Figure 1B). Fragments comprising both regions showed efficient binding to liposomes (aa 130–320 and 162–320) (Figure 3A). The second membrane binding region was mapped to the absolute C-terminus of Nup53 as deletion of the last amino acid reduced liposome binding by 42% (aa 130–319) and removal of the last eight amino acids abolished liposome binding (aa 130–312).

These data suggest that Nup53 possesses two independent membrane binding sites. Consistently, in the context of the full-length protein mutations in the N-terminal site (R105E/K106E as well as S94E/T100E) reduced liposome binding by 50 and 40% (Figure 3C). Deletion of the

C-terminal amino acid had a less prominent effect, but removal of the last eight amino acids reduced liposome binding by 60%. The combination of mutations and truncations affecting both binding sites showed an additive effect supporting the view that each site is individually capable of membrane binding. Our data also suggest that the N-terminal membrane interaction is mediated via a pair of basic amino acids. The N-terminal binding site is additionally dependent on membrane curvature, the fragment binding less efficiently to more highly curved membranes (Supplementary Figure S4). Conversely, the C-terminal membrane binding site is less sensitive to membrane curvature and seems to largely depend on the last amino acid, a hydrophobic tryptophan. This could indicate that the two membrane interaction sites operate via different binding mechanisms. In both cases, the dimerization of Nup53 via the RRM domain is important: mutations in the individual membrane binding fragments (93–267 F172E/W203E and 130–320 F172E/W203E) rendering the RRM domain monomeric reduced their membrane interaction (Supplementary Figure S5).

Nup53 membrane binding is necessary for NPC formation

The Nup53 mutant defective in RRM dimerization, which showed reduced membrane binding, was unable to substitute for the endogenous protein in nuclear assembly (Figure 2). We therefore analysed the contribution of each of the membrane interaction sites to NPC assembly by substituting endogenous Nup53 with constructs defective in the N-terminal membrane interaction site, lacking the C-terminal membrane interaction site, or comprising a combination of both deficient sites (Figure 4A). Surprisingly, mutants of the N-terminal membrane binding site (1–320 R105E/K106E and 1–320 S94E/T100E) supported NPC formation as indicated by mAB414 staining (Figure 4A). They also supported formation of a closed NE (Figure 4A and B). Correspondingly, mutations in the N-terminal binding region did not alter the NE localization of any other nucleoporins (Figure 4D). These nucleoporins include members of the Nup93 complex (Nup93, Nup188, Nup205 and Nup155) as well as the transmembrane nucleoporins NDC1 and POM121. Accordingly, interactions of these mutants with Nup93 and Nup205, which bind the N-terminal part of Nup53 (Fahrenkrog *et al*, 2000; Hawryluk-Gara *et al*, 2008), were unaffected as shown by GST pull downs (Supplementary Figure S1A).

The fragment lacking the C-terminal tryptophan (1–319) also supported NPC assembly and formation of a closed NE. Deletion of this tryptophan did not interfere with Nup53 binding to NDC1 or Nup155 (Supplementary Figure S1B), two binding partners interacting with the C-terminal region. The truncation lacking the last eight C-terminal amino acids (1–312) also allowed for NPC assembly and formation of a closed NE. All tested nucleoporins were located at the NE in these nuclei (Figure 4D). Notably, this truncation did not bind to NDC1 (Supplementary Figure S1B), supporting the view that the Nup53–NDC1 interaction is not required for postmitotic NPC formation (Hawryluk-Gara *et al*, 2008). These observations suggest that a single Nup53 membrane binding region is sufficient for NPC assembly at the end of mitosis.

In contrast to the Nup53 mutants and truncations that abrogate one membrane binding region, mutants affecting both membrane binding sites (1–319 R105E/K106E, 1–319 S94E/T100E, 1–312 R105E/K106E and 1–312 S94E/T100E) did not support NPC assembly and NE reformation (Figure 4A). Under these conditions, MEL28 (also referred to as ELYS) as well as Nup107, which both bind early to chromatin during the NPC assembly process (Galy *et al*, 2006; Rasala *et al*, 2006; Franz *et al*, 2007), as well as the transmembrane nucleoporins, NDC1 and POM121, were detected on the chromatin (Figure 4D). In contrast, Nup205, Nup188, Nup155 and Nup93 were not recruited. However, this lack of recruitment as well as the block in NPC assembly is unlikely to be caused by disrupting Nup53 binding to these interaction partners as the mutations introduced did not affect binding to Nup93, Nup205 and Nup155 in GST pull downs (Supplementary Figure S1A). It is also unlikely that the loss of the Nup53–NDC1 interaction caused this phenotype as the Nup53 fragment 1–312 still allowed for postmitotic NPC assembly but was not able to bind NDC1 (Supplementary Figure S1B). Nup58 representing an FG-containing nucleoporin of the central channel as well as the peripheral nucleoporin Nup88 was also absent on chromatin in the presence of a Nup53 construct that lacked both membrane binding regions (1–312 R105E/K106E) (Figure 4D). These data support the conclusion that the direct Nup53 membrane interaction is important for postmitotic NPC formation.

Interphasic NPC assembly requires the C-terminal membrane binding site of Nup53

Metazoan NPC assembly occurs in two different stages of the cell cycle: at the end of mitosis when NPCs assemble concomitantly with the reforming NE and during interphase when new NPCs are assembled and integrated into the intact NE (Antonin *et al*, 2008; Doucet and Hetzer, 2010). Recent data suggest that there are different requirements for these two possible modes of NPC assembly (Doucet *et al*, 2010). The *in vitro* nuclear assemblies described up to now reflect the situation at the end of mitosis. We therefore assayed whether the mutants that support postmitotic NPC assembly also support NPC formation during interphase. In an experimental set-up developed by the Hetzer laboratory (Dawson *et al*, 2009), nuclei with newly integrated NPCs can be visualized by an influx of dextrans when nucleoporins forming the permeability barrier of newly formed NPCs are depleted. Under these conditions, mutants defective in the N-terminal membrane binding site of Nup53 (1–320 R105E/K106E and 1–320 S94E/T100E) supported interphasic NPC formation (Figure 5A). Conversely, Nup53 truncations affecting the C-terminal membrane binding site did not substitute for the wild-type protein in this mode of NPC assembly. This indicates that, in contrast to postmitotic NPC assembly where both membrane binding regions individually support NPC formation, only the C-terminal membrane binding site of Nup53 is required for interphasic NPC formation.

Interestingly, the Nup53 truncation lacking the C-terminal tryptophan (1–319) did not support interphasic NPC assembly in the dextran influx assay despite the fact that membrane interaction is only slightly reduced (Figure 3C). We confirmed these findings in an independent assay directly counting NPCs identified by mAB414 immunostaining (D'Angelo *et al*, 2006; Theerthagiri *et al*, 2010). NPC numbers were determined on nuclei where NPCs assembled in the postmitotic and interphasic mode and nuclei where interphasic

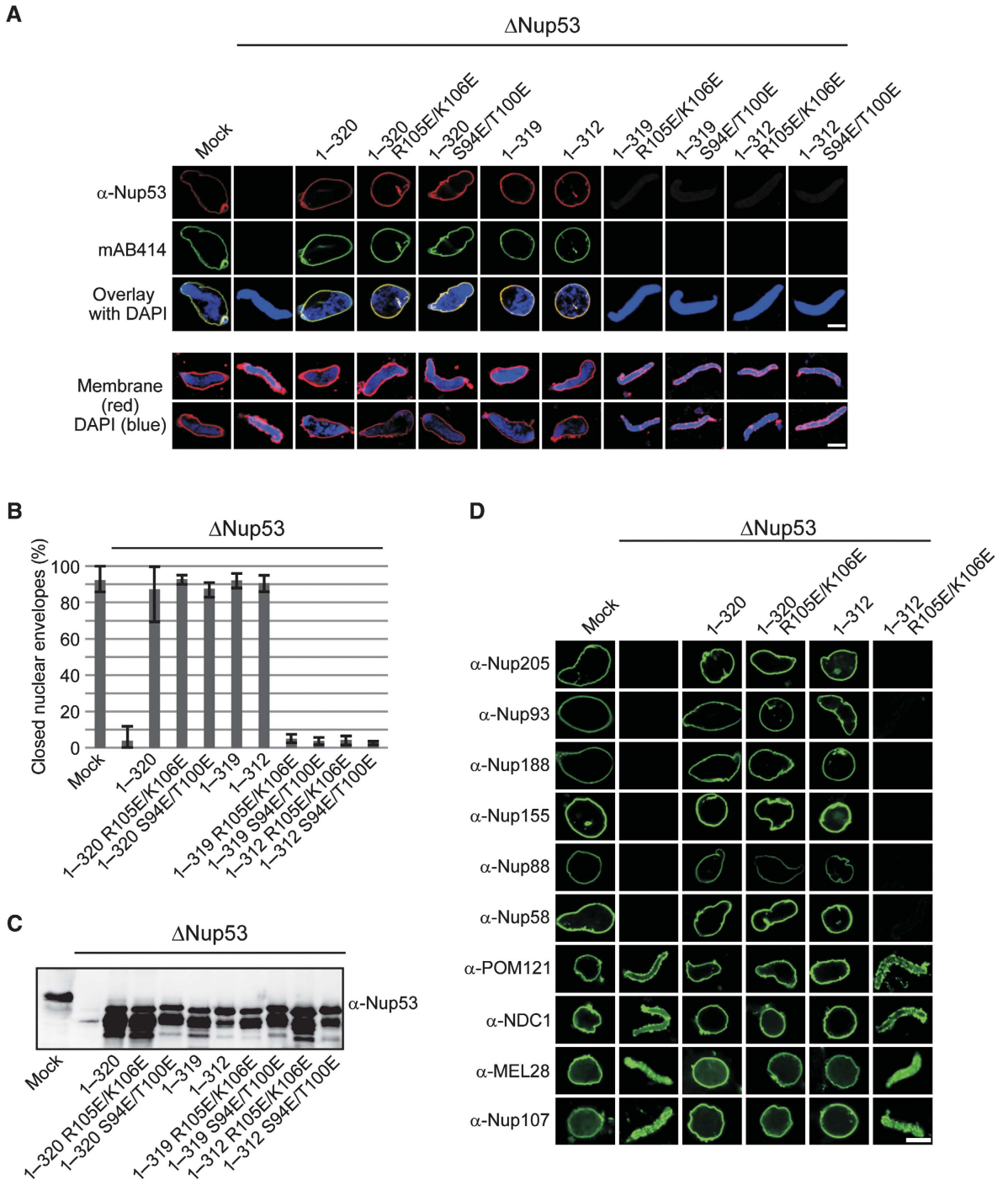

Figure 4 Either of the two membrane binding regions of Nup53 is sufficient for postmitotic NPC assembly. (**A**) Nuclei were assembled in mock, Nup53-depleted extracts or Nup53-depleted extracts supplemented with wild-type Nup53 (1–320), constructs featuring mutations in the N-terminal membrane binding site (1–320 R105E/K106E, 1–320 S94E/T100E), constructs lacking the C-terminal membrane binding site (1–319, 1–312) or both (1–319 R105E/K106E, 1–319 S94E/T100E, 1–312 R105E/K106E, 1–312 S94E/T100E), respectively. Samples were fixed after 120 min with 4% PFA and analysed with Nup53 antibodies (red) and mAb414 (green, upper panel) or with 2% PFA and 0.5% glutaraldehyde and analysed for chromatin (blue: DAPI) and membrane staining (red: DiIC18, lower panel). Bars: 10 μm. (**B**) Quantitation of chromatin substrates with a closed NE was done as in Figure 2F. (**C**) Western blot analysis of extracts used in (**A**) showing the re-addition of the recombinant proteins to approximately endogenous Nup53 levels. (**D**) Nuclei were assembled as in (**A**), fixed with 4% PFA and analysed with respective antibodies. Bar: 10 μm.

NPC formation was specifically blocked by addition of 2 μM importin β (D'Angelo *et al*, 2006; Theerthagiri *et al*, 2010). The NPC numbers of nuclei assembled for 120 min in the presence of recombinant wild-type Nup53 as well as the Nup53 mutant defective in the N-terminal membrane binding site (1–320 R105E/K106E) were reduced by importin β addition after 50 min, i.e., when a closed NE with intact NPC was formed (Figure 5B). In contrast, the Nup53 truncation lacking the C-terminal tryptophan showed after 120 min a lower number of NPCs, which was not sensitive to importin β addition, indicating that in this condition interphasic NPC assembly did not occur.

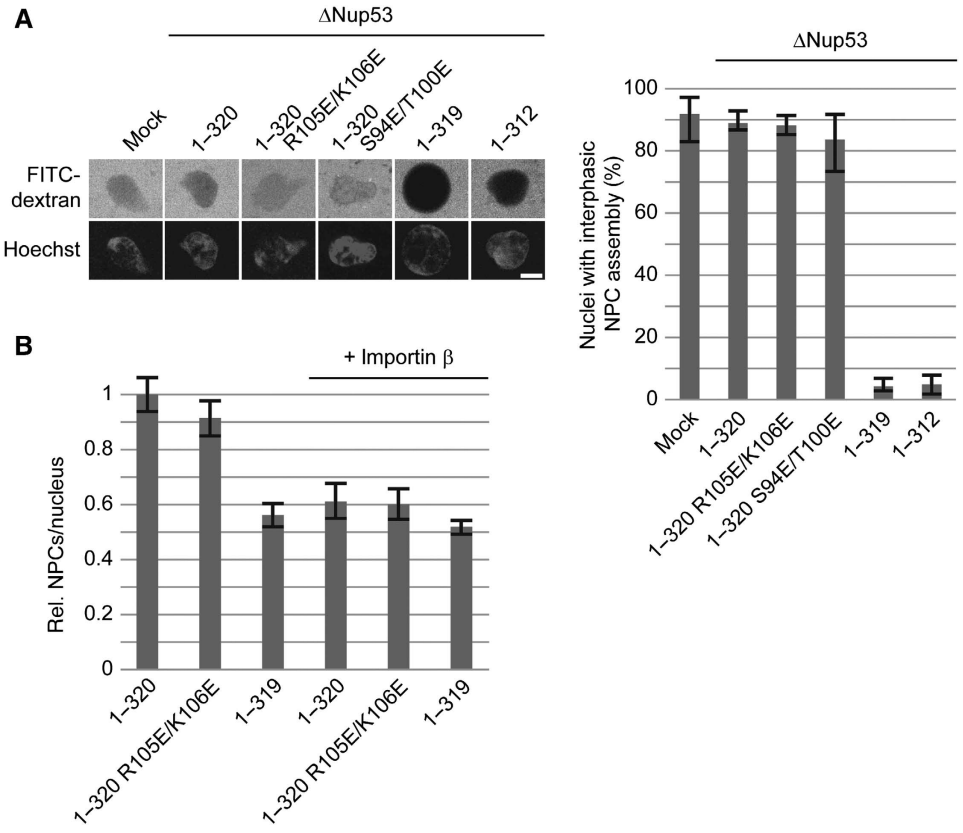

Figure 5 The C-terminal Nup53 membrane binding site is essential for interphasic nuclear pore complex (NPC) formation. (**A**) Nuclei were preassembled in mock or Nup53-depleted extracts supplemented with wild-type full-length protein (1–320), constructs with a mutated N-terminal membrane binding site (1–320 R105E/K106E, 1–320 S94E/T100E), or constructs lacking the C-terminal membrane interaction site (1–319, 1–312), respectively. After 90 min, the samples were supplemented with cytosol depleted of Nup53 and FG-containing nucleoporins. After 60 min, FITC-labelled 70-kDa dextran and Hoechst was added. Bar: 10 μm. The right panel shows the quantitation of three independent experiments with >300 randomly chosen chromatin substrates per sample. Error bars represent the range. (**B**) Nuclei were assembled in Nup53-depleted extracts supplemented with wild-type full-length protein (1–320), a construct with a mutated N-terminal membrane binding site (1–320 R105E/K106E), or a construct lacking the last C-terminal amino acid (1–319), respectively, for 120 min. Where indicated, *de novo* NPC assembly was blocked by the addition of 2 μM importin β after 50 min and nuclei were further incubated for 70 min. For each construct, total NPC numbers per nucleus identified by mAB414 immunofluorescence were quantified from 20 nuclei in 2 independent experiments and normalized to the wild-type full-length protein. Error bars represent the s.e. of the mean.

Nup53 can deform membranes

Many membrane binding proteins induce membrane deformation. Such a function is of special interest in the context of NPC formation as NPCs are integrated in the NE at sites where both nuclear membranes are deformed and fused to a pore. We therefore analysed the morphology of Nup53-bound liposomes using electron microcopy. As reported (Daumke *et al*, 2007), tubulation of liposomes is induced by the EH domain-containing protein EHD2 (Figure 6A). Interestingly, a Nup53 fragment containing both membrane binding sites (93–320) strongly induced liposome tubulation indicating a membrane deformation capability. A shorter fragment containing only the N-terminal membrane binding site and including the RRM domain (93–267) did not induce membrane tubulation, despite the fact that it was able to bind liposomes (Figure 3A). In contrast, a fragment comprising the RRM domain and the full C-terminus of Nup53 (130–320) strongly induced membrane tubulation indicating that the second, C-terminal membrane binding domain deforms membranes. Accordingly, fragments lacking the last eight residues (130–312), and therefore the second membrane binding site or only the last C-terminal tryptophan (130–319) did not cause membrane tubulation. Consistently, fragments mutated in the first, or N-terminal, membrane binding region of Nup53 (93–320 R105E/K106E and 93–320 S94E/T100E) induced membrane deformation. Efficient membrane binding of Nup53 depends not only on the individual membrane binding domains but also on dimerization (Figure 2C; Supplementary Figure S5). To determine the importance of the dimerization of Nup53 for membrane tubulation the RRM mutants of fragments that contained either both (93–320 RRM mutant) or only the C-terminal binding site (130–320 RRM mutant) were tested. Neither of the two fragments induced membrane tubulation emphasizing that membrane interaction is indeed required for this phenotype. The membrane binding fragment of Nup133 did not induce detectable tubulation, emphasizing that membrane binding alone does not account for membrane deformation.

Similar to Xenopus Nup53 both yeast isoforms—Nup53p and Nup59p—induced membrane tubulation (Figure 6B). This indicates that, in addition to membrane binding (Figure 1A), the membrane bending activity of Nup53 is conserved during evolution which suggests an important role for Nup53-induced membrane deformation in NPC formation and/or function.

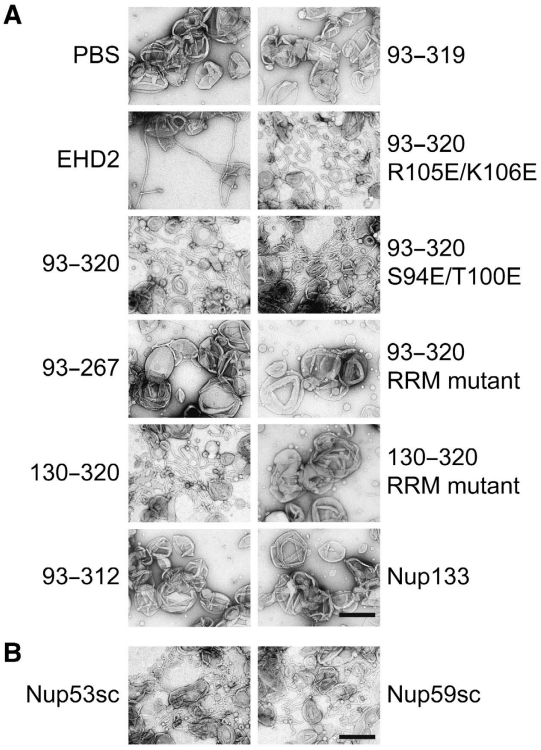

Figure 6 The C-terminus of Nup53 binds and deforms membranes. (**A**) Folch fraction I liposomes were incubated where indicated with 3 μM recombinant Nup53 fragments containing both (93–320), the N-terminal (93–267) or C-terminal (130–320) membrane binding sites including the RRM domain as well as fragments and mutants where the C-terminal (93–312, 93–319), the N-terminal (93–320 R105E/K106E, 93–320 S94E/T100E) membrane interaction site or the dimerization (93–320 RRM mutant and 130–320 RRM mutant) is compromised. The liposome deforming protein EHD2 (aa 1–543) and a fragment of Nup133 were used as positive and negative control, respectively. (**B**) 3 μM recombinant yeast Nup53 and Nup59 protein was incubated with liposomes and analysed. Bars: 400 nm.

Discussion

Here, we show that Nup53 binds membranes directly and independently of other proteins. We demonstrate that dimerization of the protein via its RRM domain is necessary for membrane interaction and identify two separate membrane binding regions within the protein. Binding of Nup53 to membranes is important for NPC assembly. Although either of the two membrane interaction regions is sufficient for postmitotic NPC formation, NPC assembly in interphase specifically requires the C-terminal membrane binding site, probably because of its capacity to induce membrane deformation.

Our results support the view that Nup53 is crucial for postmitotic NPC assembly in Xenopus egg extracts (Hawryluk-Gara *et al*, 2008). Depletion of Nup53 blocks NPC assembly and the formation of a closed NE. This phenotype is rescued by the addition of recombinant Nup53, confirming the specificity of the depletion (Hawryluk-Gara *et al*, 2008; Figures 2 and 4). In agreement with the cell-free data, RNAi-mediated depletion of Nup53 in HeLa cells results in severe nuclear morphology defects and reduced levels of Nup93, Nup205 and Nup155 at the nuclear rim, suggestive of defects in NPC assembly (Hawryluk-Gara *et al*, 2005). In *C. elegans*, RNAi knockdown of Nup53 as well as a deletion within the protein blocks postmitotic nuclear reformation and results in embryonic lethality (Galy *et al*, 2003; Rodenas *et al*, 2009), suggesting that Nup53 function is conserved in metazoa. Notably, Nup53 is found in all eukaryotic supergroups indicating that it is part of the NPC in the last common ancestor of eukaryotes (Neumann *et al*, 2010). However, its absence in some eukaryotic organisms shows that its loss can be compensated (DeGrasse *et al*, 2009; Neumann *et al*, 2010). Double deletion of both *S. cerevisiae* orthologues, Nup53p and Nup59p, is viable (Marelli *et al*, 1998; Onischenko *et al*, 2009). However, these strains exhibit growth defects and Nup53 becomes essential when interacting nucleoporins, including integral membrane proteins, are deleted (Marelli *et al*, 1998; Miao *et al*, 2006; Onischenko *et al*, 2009).

NPC assembly is a highly ordered process. In metazoa the NE and NPCs break down and reform during each round of mitosis. Postmitotic reassembly occurs on the decondensing chromatin. The earliest step involves the recruitment of the Nup107–160 subcomplex to the chromatin surface by MEL28/ELYS (Galy *et al*, 2006; Rasala *et al*, 2006; Franz *et al*, 2007), a DNA-binding protein that acts as a seeding point for NPC assembly. Membranes are subsequently recruited to chromatin causing an enrichment of NE/NPC-specific membrane proteins, including the transmembrane nucleoporins POM121 and NDC1 (Antonin *et al*, 2005; Mansfeld *et al*, 2006; Anderson *et al*, 2009). The order of these initial steps has been defined using both *in vitro* experiments and live-cell imaging (Dultz *et al*, 2008); however, less is known about the order of nucleoporin assembly following these events. MEL28 and Nup107 as well as POM121 and NDC1 containing membranes are detectable on the chromatin in Nup53-depleted nuclear assemblies (Figure 4D). The same pattern was seen in Nup93-depleted extracts (Sachdev *et al*, 2012). Our results suggest that Nup53, which is part of the Nup93 complex, is a key determinant for the recruitment of the other members of this complex. In the absence of Nup53, the chromatin recruitment of Nup155, Nup205, Nup188 and Nup93 was impaired (Figure 4D). Similarly, *C. elegans* Nup53 is necessary for the efficient accumulation of Nup155 and Nup58 but not Nup107 at the NE (Rodenas *et al*, 2009). This is also supported by live-cell imaging experiments in HeLa cells, which capture the recruitment of Nup58 slightly after Nup93 (Dultz *et al*, 2008). Accordingly, we have found that upon depletion of the two Nup93 containing subcomplexes, Nup93–Nup188 and Nup93–205, the two other members of the complex, Nup155 and Nup53, are still detectable, albeit at reduced levels on the assembling NPCs (Sachdev *et al*, 2012). Recruitment of the Nup62 complex to the chromatin template is prevented in the absence of both Nup53 (see lack of a Nup58 immunostaining, which is a constituent of the Nup62 complex, in Figure 4D) and Nup93 (Sachdev *et al*, 2012) consistent with the notion that Nup93 is a key determinant in recruiting the Nup62 complex during vertebrate NPC assembly at the end of mitosis (Sachdev *et al*, 2012). Taken together, these data suggest that after the binding of the Nup107–160 complex and nuclear membranes to the chromatin surface, Nup53 recruitment is the next decisive step in NPC assembly. Nup93 (Nup93–Nup188/Nup93–Nup205) binding and the subsequent recruitment of the Nup62 complex follow.

Nup53 interacts with a number of other nucleoporins, including NDC1, Nup155 and Nup93 (Lusk *et al*, 2002;

Hawryluk-Gara *et al*, 2005, 2008; Mansfeld *et al*, 2006; Sachdev *et al*, 2012) a feature that is conserved in yeast (Fahrenkrog *et al*, 2000; Onischenko *et al*, 2009; Amlacher *et al*, 2011). As previously reported (Hawryluk-Gara *et al*, 2008), the interaction of Nup53 with NDC1 is not necessary for postmitotic NPC assembly in Xenopus egg extracts (Figure 4A). A possible explanation might be that Nup53 can interact directly with membranes and that one of its two membrane binding regions is sufficient for postmitotic NPC formation. In addition, Nup53 interaction with other nucleoporins such as Nup155, which in turn binds POM121 (Mitchell *et al*, 2010; Yavuz *et al*, 2010) could be a possible mechanism linking Nup53 to the pore membrane which might compensate for the loss of the direct Nup53–NDC1 interaction.

The interaction of Nup53 with Nup155 is thought to be important for NPC assembly. A previous study found that after depleting Nup53 from Xenopus egg extracts, only fragments capable of binding to Nup155 allow for NPC formation (Hawryluk-Gara *et al*, 2008). However, in this case all fragments that rescued the NE/NPC assembly defect included the RRM domain and all fragments defective in NPC assembly and the Nup155 interaction lacked the intact RRM domain. Similarly, a deletion in *C. elegans* Nup53 blocked NE assembly also impaired the RRM domain (Rodenas *et al*, 2009). We demonstrate here that the RRM domain is important for Nup53 dimerization and in turn for membrane binding. Therefore, we currently cannot rule out that the primary cause for the previously described defects was a loss of the Nup53 membrane interaction.

Nup93 binds directly Nup53 (Hawryluk-Gara *et al*, 2005; Sachdev *et al*, 2012) and the interaction domain resides in the N-terminal half of Nup53 (Hawryluk-Gara *et al*, 2008). This interaction was previously considered to be dispensable for NPC assembly as a fragment lacking the N-terminal region as well as the C-terminal 26 amino acids replaced endogenous Nup53 in nuclear assemblies in Xenopus egg extracts (Hawryluk-Gara *et al*, 2008). This is quite surprising in the light of the results presented here, as this fragment also lacked both membrane binding regions identified in this study and does not allow for Nup93 recruitment which is an essential factor for postmitotic NPC assembly (Sachdev *et al*, 2012). Using a number of different Nup53 fragments that lacked the Nup93 binding region we were not able to replace endogenous Nup53 in NPC assembly (Supplementary Figure S6). Currently, we cannot rule out that this discrepancy is due to different Nup53 depletion efficiencies. In fact, we found that a small percentage of floated membrane preparations used in the nuclear assembly reactions contained minor amounts of Nup53, and we were careful to exclude these from our experiments.

Nup53 is also known as mitotic phosphoprotein of 44 kDa (Stukenberg *et al*, 1997). Indeed, Nup53 from mitotic extracts migrates significantly slower in SDS–PAGE compared to Nup53 from interphasic extracts (Supplementary Figure S3A). We identified a number of mitosis-specific phosphorylation sites on Nup53, of which a subset were consensus sites for CDK1. These findings are consistent with the fact that Nup53 has been identified as a CDK1 target in both yeast and humans (Lusk *et al*, 2007; Blethrow *et al*, 2008). The N-terminal membrane binding region of Nup53 is phosphorylated during mitosis. Phosphomimetic mutations and *in vitro* phosphorylation experiments suggest that CDK1-mediated phosphorylation renders this region incompetent for membrane interaction (Figure 3B). It is therefore possible that this mitotic phosphorylation weakens the interaction of Nup53 with the pore membrane to facilitate NPC disassembly during prophase.

Proteins without integral membrane regions can associate with cellular membranes by a variety of mechanisms (Cho and Stahelin, 2005). First, they can be covalently attached to a lipid moiety. However, we have no indication that Nup53 is modified in such a way. Second, peripheral membrane proteins are recruited to the lipid bilayer by specific protein–lipid interactions that involve binding to particular lipid head groups. In this regard, lipid arrays performed to date have not demonstrated an affinity of Nup53 for any specific lipid (unpublished observation). Furthermore, recombinant Nup53 binds to liposomes prepared from different lipid sources like pure DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), a lipid mixture mimicking the ER/NE lipid composition (Franke *et al*, 1970; Supplementary Figure S7) or folch fraction I (unpublished observation). Third, some proteins are recruited to membranes via electrostatic interactions. As a fraction of the lipid head groups are negatively charged this involves positively charged clusters on the protein surface. Indeed, we found that the first membrane binding region of Nup53 contains such a cluster. Replacing these positive with negative residues as well as introducing negative charge by phosphorylation rendered this site incapable of membrane interaction (Figure 3A and B). Finally, peripheral membrane proteins can associate with lipid bilayers via hydrophobic regions. It has been suggested that the C-terminal region of Nup53 contains an amphipathic helix, which could serve as a hydrophobic module (Marelli *et al*, 2001; Patel and Rexach, 2008). In fact, the C-terminus of Nup53 contains a membrane binding region and deleting the last eight amino acids abolished its membrane interaction. Deletion of the C-terminal tryptophan only slightly reduced membrane binding of Nup53 in the full-length protein (Figure 3C), but inhibited interphasic NPC assembly (Figure 5) suggesting that this residue fulfills an important additional function. Indeed, the insertion of hydrophobic parts of a protein into a membrane is one of several mechanisms by which proteins can deform membranes (McMahon and Gallop, 2005). Our data suggest that the C-terminal membrane binding region and especially the last tryptophan of Nup53 fulfills this task (Figure 6A). Interestingly, *in vivo* intranuclear tubular membranes are induced upon overexpression of yeast Nup53p which is dependent on its C-terminus (Marelli *et al*, 2001). Thus, Nup53 might not only have an important function in binding to the pore membrane in turn promoting the recruitment of other nucleoporins, especially members of the Nup93 complex, but may additionally function to deform the NE membrane into a pore. In the latter instance, the insertion of the Nup53 C-terminus into the hydrophobic phase of the membrane would result in displacement of lipid head groups and reorientation of the hydrophobic lipid side chains, bending the lipid bilayer into a convex shape and inducing membrane curvature necessary to form and stabilize the pore (Antonin *et al*, 2008). The doughnut-like shape of the pore requires likewise stabilization of a concave curvature in the plane of the pore in addition to the convex curved membrane

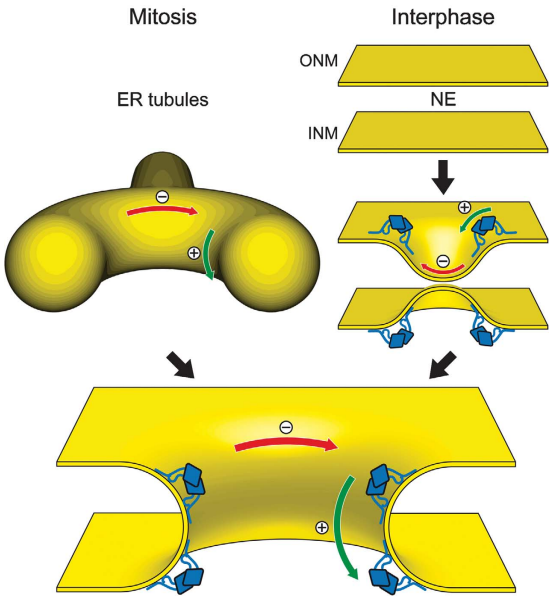

Figure 7 The role of Nup53 in NPC assembly. Schematic drawing of the postmitotic and interphasic modes of nuclear pore assembly focused on the membrane interacting function of Nup53. For the sake of clarity other membrane associated and integral proteins, including nucleoporins, participating in this process are omitted. Left pathway: after mitosis, outgrowing ER tubules (yellow) surround assembling NPCs providing negative/concave (red) and positive/convex (green) curvature which is stabilized by membrane binding Nup53 dimers (blue) for pore formation. Right pathway: in interphase, the intact nuclear envelope membranes (yellow) are deformed by the C-terminal membrane binding site of Nup53 introducing a convex membrane curvature for the approximation and following fusion of the two membranes leading to pore formation.

connection between outer and inner nuclear membrane (see Figure 7). This curvature might be induced and stabilized by a number of at least partially redundant mechanisms, such as formation of a coat-like structure by the Nup107–160 complex (Devos *et al*, 2004; Mans *et al*, 2004; Brohawn *et al*, 2008) or oligomerization of pore membrane proteins, although it is not clear how the different proteins contribute to the different modes of membrane bending. Similarly, in the context of the protein interaction network of an assembled NPC the N-terminal membrane binding region of Nup53, in addition to membrane interaction, might induce and/or stabilize curved membranes. Indeed, Nup53 is only functional when it dimerizes probably because this increases the avidity of the Nup53 membrane interaction. In addition, all the factors might also impose geometrical constrains to the membranes supporting a pore structure.

Although either one of the two membrane binding regions of Nup53 is sufficient for postmitotic NPC assembly and stability, the C-terminal site is specifically required for NPC assembly during interphase. It is a matter of debate whether NPC assembly during these different cell-cycle phases occurs by distinct mechanisms (Doucet *et al*, 2010; Dultz and Ellenberg, 2010; Lu *et al*, 2011). At the end of mitosis, NPC assembly occurs concomitantly with formation of a closed NE. It is possible that this mode of NPC formation does not require fusion between the outer and inner nuclear membrane to form a nuclear pore, in contrast to interphasic NPC assembly. Postmitotic pore assembly could rather arise by the enclosement of the assembling NPCs on the chromatin surface by an outgrowing ER network. In this scenario, Nup53 would stabilize the membrane curvature provided by the ER tubules rather than induce membrane deformation (see Figure 7, left pathway). The different requirements for Nup53 membrane binding regions in postmitotic and interphasic NPC assembly support the view of two different mechanistic pathways. A loss of the membrane deforming capability of Nup53 in postmitotic NPC assembly and NPC stability might be compensated by other factors such as the Nup107–160 complex or integral membrane proteins. However, during metazoan interphasic NPC assembly Nup53-mediated membrane deformation might be crucial for the initial approximation and/or fusion of both membrane layers (see Figure 7, right pathway). Interestingly, ER bending proteins of the reticulon family that induce convex membrane curvature (Hu *et al*, 2008) were shown to be important for NPC assembly into the intact NE both in yeast and in vertebrates (Dawson *et al*, 2009). Currently, it is unknown whether these proteins do also contribute to postmitotic NPC assembly. As their effect on ER membrane reorganization at the end of mitosis is a prerequisite for NE reformation (Anderson and Hetzer, 2008) it is difficult to separate these two functions. Finally, how the fusion of outer and inner nuclear membranes is achieved is largely unclear but our results suggest that Nup53, importantly its C-terminal membrane binding region, is critical for this process.

Materials and methods

Antibodies against POM121 and GP210 (Antonin *et al*, 2005), NDC1 (Mansfeld *et al*, 2006), Nup155 (Franz *et al*, 2005), MEL28/ELYS (Franz *et al*, 2007), Nup107 (Walther *et al*, 2003), Nup53 and Nup58 (Sachdev *et al*, 2012) as well as Nup188, Nup205, Nup98 and Nup53 (Theerthagiri *et al*, 2010) have been described. mAB414 and Nup88 antibodies were from Babco or BD Bioscience, respectively. For quantitation of Nup53 liposomes binding the antibody was affinity purified with a fragment comprising the RRM domain as this domain is included in all tested fragments (please see Supplementary Table S2 for list of all DNA constructs used in this study).

Nuclear assemblies

Nuclear assemblies and immunofluorescence (Theerthagiri *et al*, 2010), generation of affinity resins, sperm heads and floated membranes (Franz *et al*, 2005) as well as prelabelled membranes (Antonin *et al*, 2005) were done as described. Interphasic NPC assembly using dextran influx was monitored as described (Dawson *et al*, 2009) except that mock or Nup53-depleted extracts were incubated with 1.5 vol WGA-Agarose (Sigma) for 40 min. Counting of NPCs was performed on mAB414-labelled nuclei as described (Theerthagiri *et al*, 2010).

Protein expression and purification

Constructs for full-length Xenopus Nup53 and fragments, *S. cerevisiae* Nup53 and Nup59 were generated from a synthetic DNA optimized for codon usage in *E. coli* (Geneart, see Supplementary data) and cloned into a modified pET28a vector with a yeast SUMO solubility tag followed by a TEV site upstream of the Nup53 fragments. Proteins were expressed in *E. coli*, purified using Ni-agarose, His_6 and SUMO tags were cleaved by TEV protease, concentrated using VIVASPIN columns (Sartorius) and purified by gel filtration (Superdex200 10/300 GL or Superdex200 PC 3.2/30, GE Healthcare) either in PBS for liposome binding experiments or in sucrose buffer (Theerthagiri *et al*, 2010) for nuclear assemblies, respectively. Nup53 fragments aa 162–320 and aa 254–320 were purified by size exclusion chromatography without removal of the tags to retain stability. Fragments of Xenopus Nup98 (aa 676-863) as well as human Nup133 (aa 67–514) (Berke *et al*, 2004) were expressed from modified pET28a vectors with a His_6-NusA or

His_6 tag, which was cleaved of by thrombin or precision protease, respectively.

Liposome generation and flotation

E. coli polar lipid extract with 0.2 mol% 18:1–12:0 NBD-PE (1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphoethanolamine) (Avanti polar lipids) were dissolved in ethanol at 45°C. To form liposomes, the mixture was diluted 10-fold into PBS resulting in a final lipid concentration of 6.7 mg/ml while gently agitating. Liposomes were passed 21 × through Nuclepore Track-Etched Membranes (Whatman) with defined pore sizes (50, 100, 200, 400, 800 nm) at 45°C using the Avanti Mini-Extruder. To remove ethanol, liposomes were dialysed against PBS using Spectra/Por 2 dialysis tubing (MWCO 12-14 kDa). Liposome sizes were determined by light scattering using the AvidNano W130i. For quantitation of liposome binding, fluorescence intensity of the protein/liposome mixture and the top fraction was determined using a Molecular Imager VersaDoc MP 4000 Imaging System and ImageJ.

Folch fraction I lipids (Sigma) dissolved in chloroform were dried on a rotary evaporator and overnight under vacuum. PBS buffer was gently added to result in a final lipid concentration of 10 mg/ml. After 2 h of incubation at 37°C to allow spontaneous liposome formation the flask was agitated to dissolve residual lipids. After 10 cycles of freeze/thawing, liposomes were diluted 10-fold in PBS and extruded as described before.

Immunoprecipitation

Xenopus Nup53 as well as the RRM dimerization mutant (F172E/W203E) was cloned with N-terminal myc or HA tag, respectively, into a pSI vector (Promega). HeLa cells were transfected using Fugene 6 (Roche) following manufacturer's instructions, harvested 24 h post transfection and solubilized in 1% Triton X-100 in PBS supplemented with protease inhibitors (2 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 0.1 mg/ml AEBSF final concentration) for 10 min at 4°C. After centrifugation for 10 min at 15 000 *g* samples were diluted five-fold in PBS and employed for immunoprecipitation using α-myc or α-HA antibodies (Roche).

Miscellaneous

For *in vitro* phosphorylation, 3 μM proteins were incubated with 0.33 U/μl CDK1-CyclinB (NEB), 1 mM ATP, 10 mM $MgCl_2$ and 1 mM EDTA in PBS for 1 h at 30°C.

For liposome tubulation copper grids filmed with pioloform and carbon-coated were glow discharged before usage. Proteins were incubated with 1 mg/ml folch fraction I liposomes for 7 min on grids, washed with buffer (10 mM Hepes, 150 mM NaCl, 4.5 mM KCl) and stained with 2% UAc for 2 min and examined on a FEI Technai spirit 120 kV microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: BV and WA designed and performed experiments and wrote the manuscript. AS quantified interphasic NPC assembly, RS and NE performed pull-down experiments, AMS performed the size determinations of the RRM domains. CS cloned constructs and prepared *Xenopus laevis* extracts. JM and BM performed mass spec analysis, UG performed measurements of different liposome sizes.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Information

The direct interaction of Nup53 with nuclear membranes enables nuclear pore complex assembly

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Supplementary information contains:

Supplementary Figures S1–S7 & Table S1–S2

Supplementary Methods

Supplementary References

Supplementary Figure S1

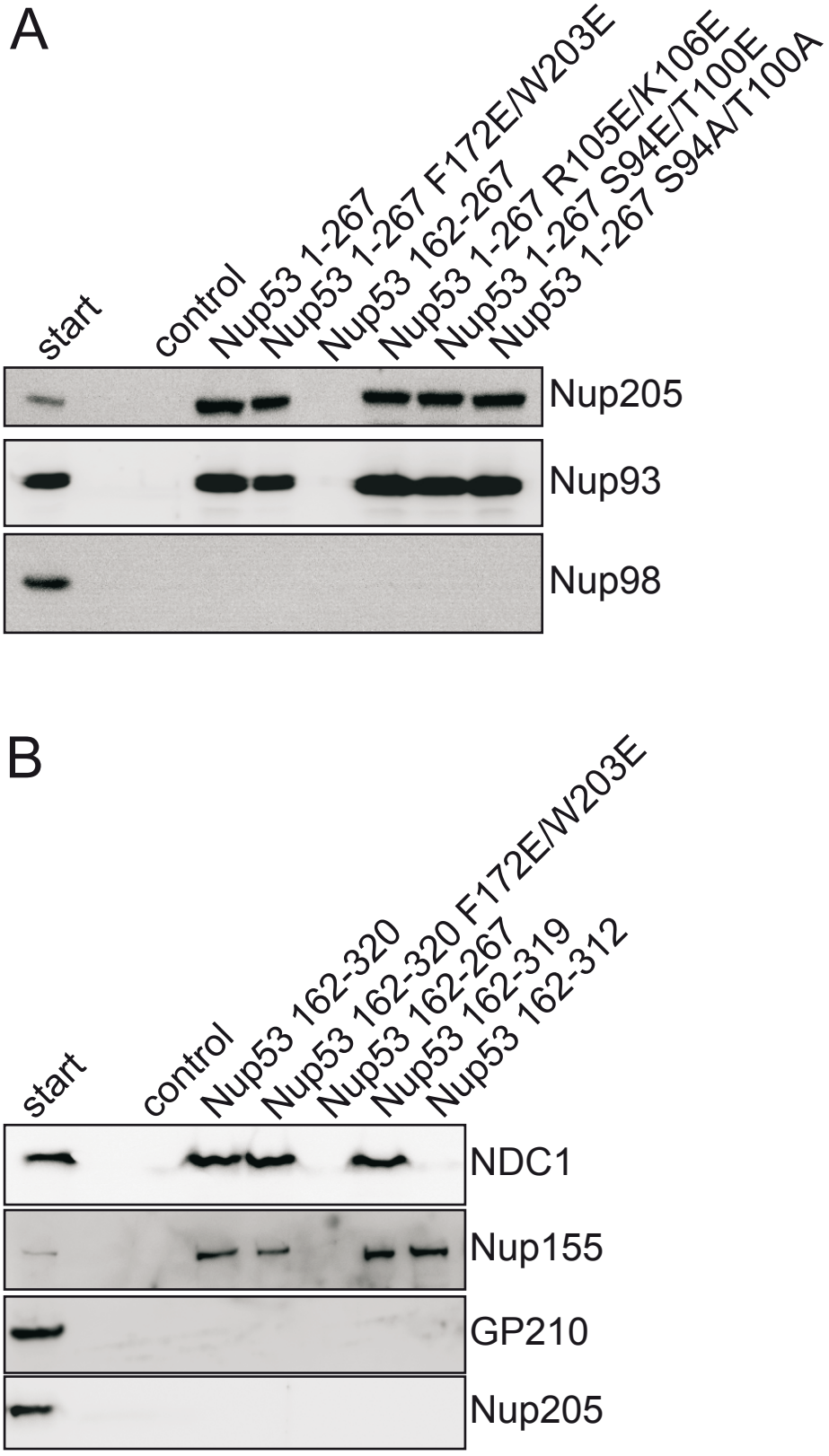


Figure S1

Nup53 has different binding sites for membrane and protein interaction.

(A) GST fusions of an N-terminal fragment of Xenopus Nup53 as well as Xenopus Nup98 (aa 487-634) (control) were incubated with cytosol from Xenopus egg extracts. Eluates were analyzed by western blotting with antibodies against the nucleoporins Nup205 and Nup93, known to bind this region, as well as Nup98 as a negative control. Please note that introducing amino acid changes causing the monomerization of Nup53 (F172E/W203E) also negatively influenced the interaction with Nup205 and Nup93. In contrast, mutations that inactivate the N-terminal membrane binding region (R105E/K106E and S94E/T100E) as well as the S94A/T100A control mutation did not interfere with Nup205 and Nup93 binding.

(B) GST fusions Xenopus Nup98 (aa 487-634) (control), the C-terminal fragment of Xenopus Nup53 (162-320), the RRM mutant (F172E/W203E) and C-terminal truncations were incubated with cytosol (for detection of Nup155 and Nup205) or Triton X-100 solubilized membranes (for NDC1 and GP210 detection) from Xenopus egg extracts. Eluates were analyzed by western blotting with antibodies against the nucleoporins Nup155 and NDC1, known to bind this region as well as Nup205 and GP210 as negative controls. Please note that mutation of the RRM domain (F172E/W203E) did not influence the interaction with NDC1 but results in a decreased binding to Nup155. The C-terminal truncations weakening the C-terminal membrane binding region (162-319 and 162-312) did not interfere with Nup155 binding and the 162-319 fragment was still able to interact with NDC1.

Supplementary Figure S2

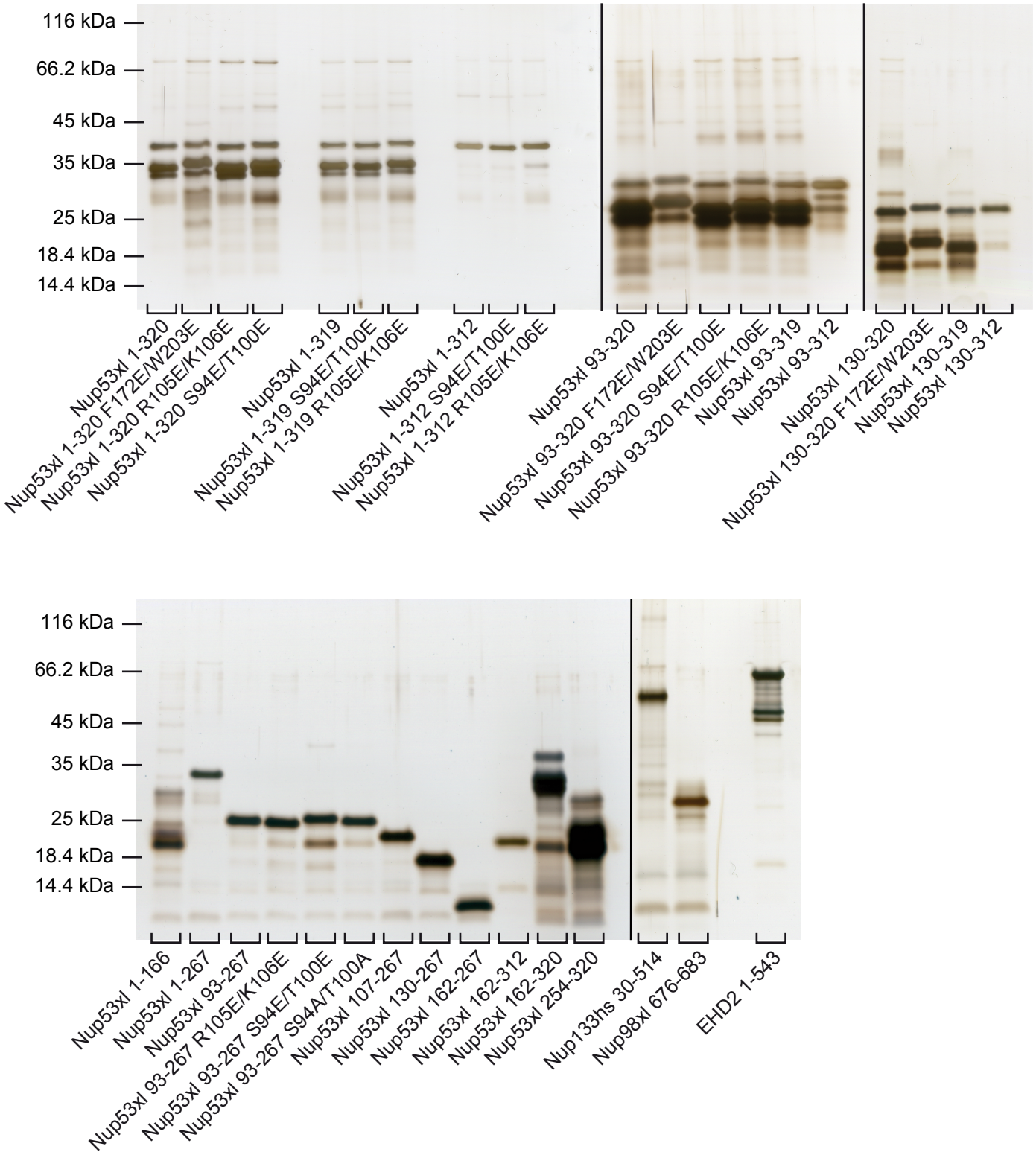
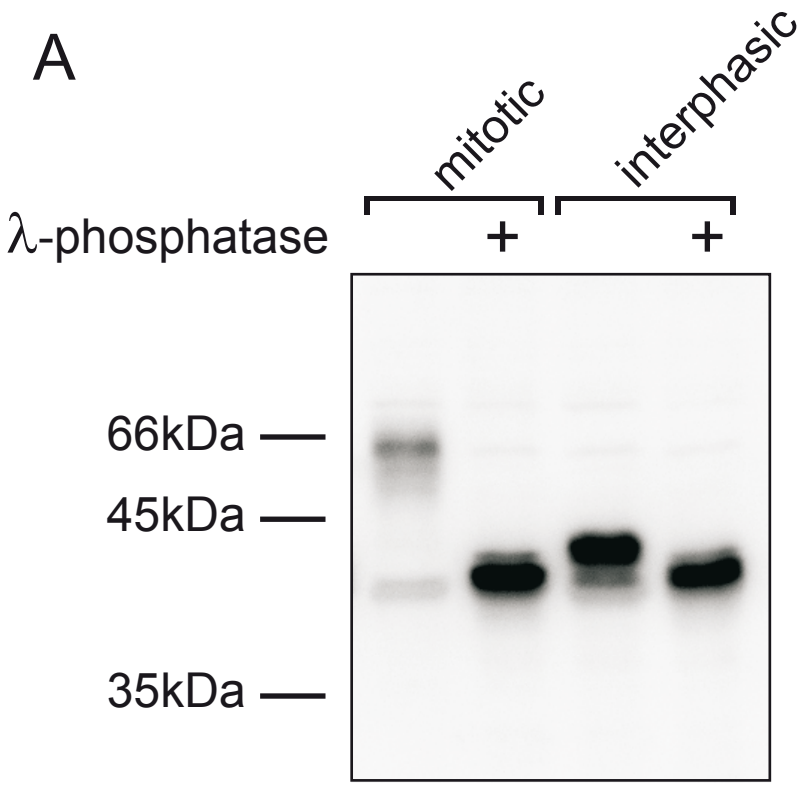


Figure S2

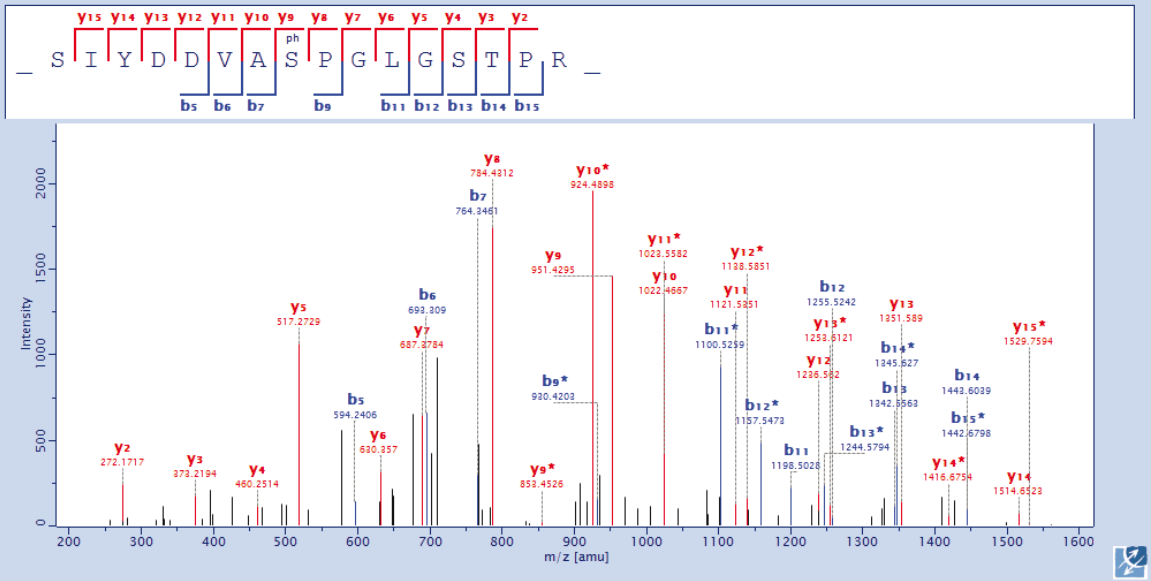
Proteins were separated on Tricine-SDS-PAGE Schägger gels (Schagger & von Jagow, 1987) followed by silver staining.

Supplementary Figure S3



B

Protein IDs: gi_4584796;gi_148232142;gi_15987768;gi_nup53a;gi_nup53b;gi_32450351
Scannumber 8537
Source: 20101220_JM_CO_0221_R7_mitotic_01
Method: CID; ITMS



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Mascot Score:	100.54
Intensity:	13664197

general information

Annotation:	14 of 16 => 88 %
Intensity Coverage:	63 %
Protein Localisation:	89 ... 104

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NaN	479.2136404	4	D	13	1253.6120554	0.0177054
0.310625	594.2405835	5	D	12	1138.5851123	0.0339649
0.1467521	693.3089974	6	V	11	1023.5581693	0.2597628
0.0844919	764.3461112	7	A	10	924.4897554	0.0853789
NaN	931.3444706	8	S	9	853.4526416	0.0651807
0.1978867	930.4203384	9	P	8	784.4311782	0.1097764
NaN	1085.4186981	10	G	7	687.3784143	0.0730627
0.1684698	1100.5258661	11	L	6	630.3569506	0.1070997
0.0851164	1157.5473299	12	G	5	517.2728866	0.1069351
0.0762814	1244.5793583	13	S	4	460.2514229	0.0053902
0.1205219	1345.6270367	14	T	3	373.2193945	0.0125464
0.1888518	1442.6798006	15	P	2	272.171716	0.0410398
NaN	NaN	16	R	1	175.1189522	NaN

Protein IDs: gi_4584796;gi_148232142;gi_15987768;gi_nup53a;gi_nup53b;gi_32450351
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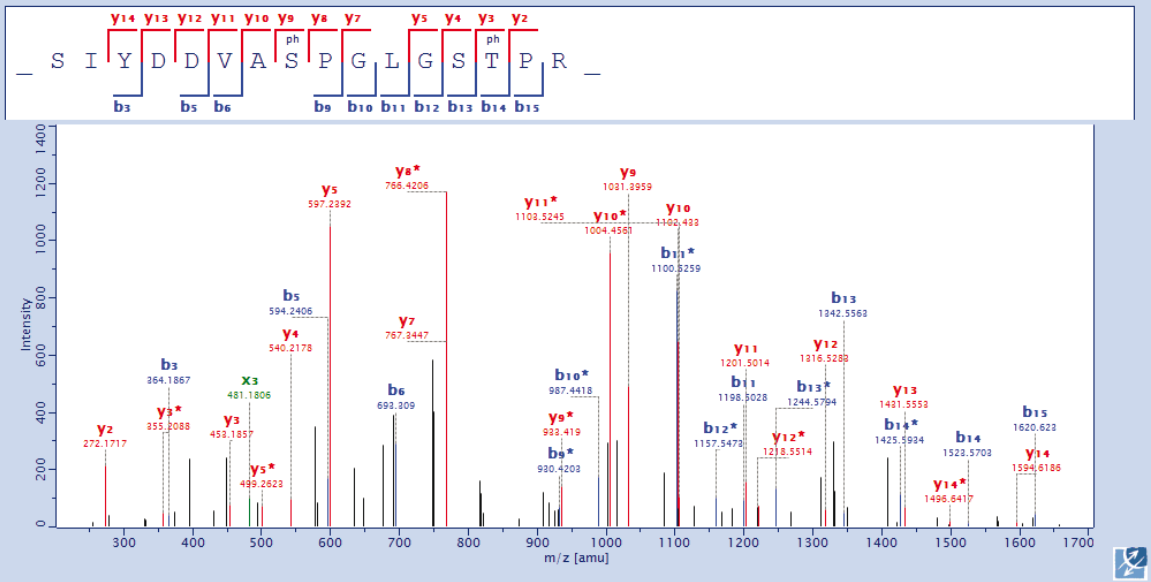
Method: CID; ITMS



parent information

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Charge:	2+
Mass Error [ppm]:	0.13197
PTM Score:	238.2
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Mascot Score:	51.05
Intensity:	18521420

general information

Annotation:	13 of 16 => 81 %
Intensity Coverage:	55 %
Protein Localisation:	89 ... 104

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0.0780403	693.308997	6	V	11	1227.4806609	NaN	1103.5245	0.2493034
NaN	764.3461112	7	A	10	1128.412247	NaN	1004.45609	0.0672901
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0.2336739	987.441802	10	G	7	793.3240099	NaN	767.344745	0.0776179
0.0236689	1100.52587	11	L	6	736.3025462	NaN	710.3232816	NaN
0.1385832	1157.54733	12	G	5	623.2184822	NaN	499.262322	0.0559618
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0.0542153	1425.59337	14	T	3	481.18064	0.1698237	355.20883	0.0389732
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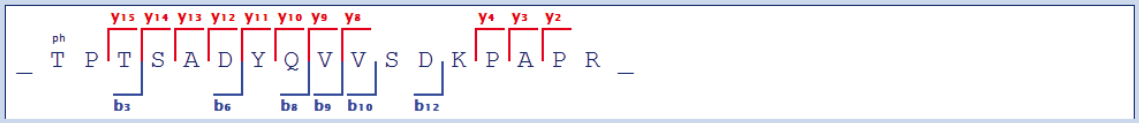
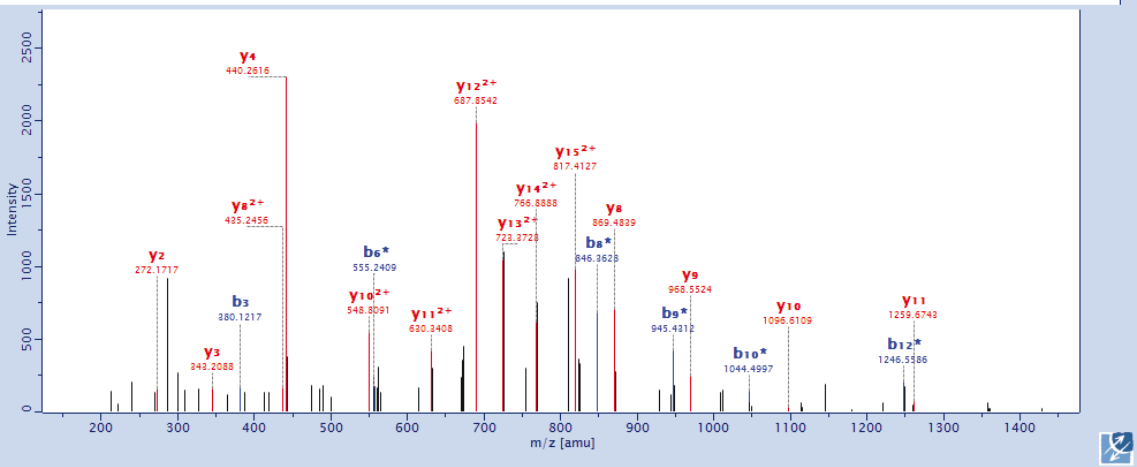
Protein IDs: gi_4584796;gi_148232142;gi_15987768;gi_nup53a;gi_nup53b
Scannumber 5067
Source: 20101126_CO_JM_0221WoAn_R5_IP_mitotic_01
Method: CID; ITMS





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general information

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NaN	538.1908705	5	A	13	1445.7383188	NaN	**723.372798**	**0.0150096**
0.1898076	**555.240917**	6	D	12	1374.701205	NaN	**687.854241**	**0.315559**
NaN	816.281142	7	Y	11	**1259.67426**	**0.1249323**	**630.340769**	**0.0778099**
0.1566467	**846.362824**	8	Q	10	**1096.61093**	**0.0732137**	**548.809105**	**0.0020371**
0.0182254	**945.431237**	9	V	9	**968.552356**	**0.1522949**	968.552356	NaN
0.1110664	**1044.49965**	10	V	8	**869.483942**	**0.0255184**	**435.245609**	**0.0565758**
NaN	1229.5085758	11	S	7	770.4155281	NaN	770.4155281	NaN
0.2735886	**1246.55862**	12	D	6	683.3834997	NaN	683.3834997	NaN
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Protein IDs: gi_4584796;gi_148232142;gi_15987768;gi_nup53a;gi_nup53b
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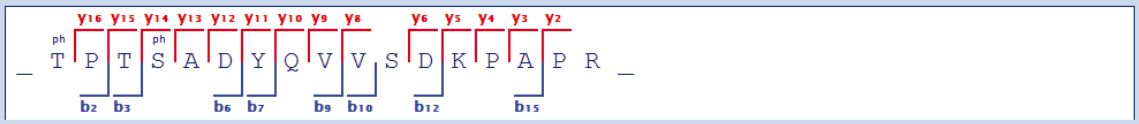

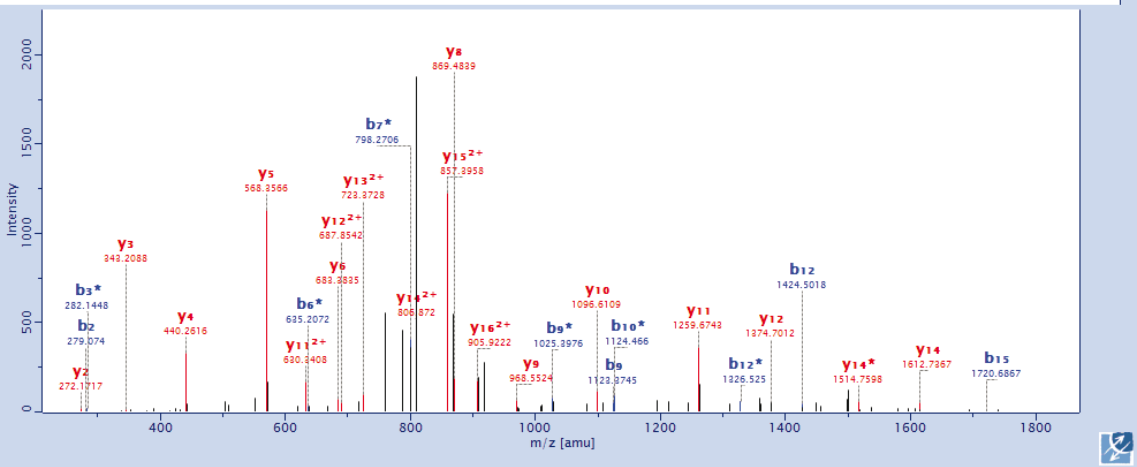

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Intensity:	723052.1

general information

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NaN	547.1200877	4	S	14	**1514.75978**	**0.204207**	**806.871977**	**0.2981887**
NaN	618.1572015	5	A	13	1445.7383188	NaN	**723.372798**	**0.4335988**
0.0729639	**635.207248**	6	D	12	**1374.70121**	**0.0788243**	**687.854241**	**0.1524731**
0.03962	**798.270577**	7	Y	11	**1259.67426**	**0.0252142**	**630.340769**	**0.1820579**
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NaN	NaN	17	R	1	175.1189522	NaN	175.1189522	NaN

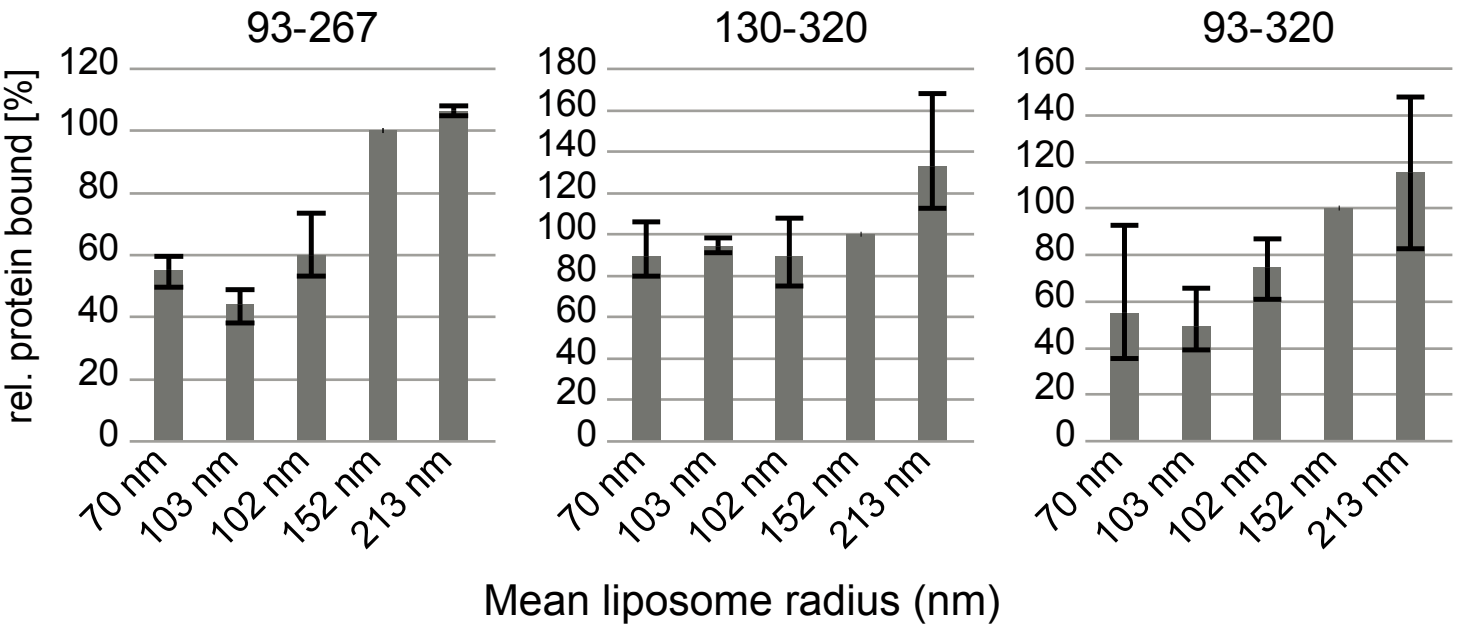
Figure S3

(A) Nup53 is phosphorylated in interphase and mitosis

4 µl of mitotic (CSF arrested) or interphasic Xenopus egg extracts were diluted in 100 µl of phosphatase buffer (NEB) and incubated where indicated with 400 U λ-phosphatase for 30 min at 30°C. Samples were analyzed by 12% SDS-PAGE and Western blotting using the Xenopus Nup53 antibody. Please note the different shifting of mitotic and interphasic Nup53 after phosphatase treatment indicating that Nup53 is a phosphoprotein throughout the cell cycle but hyperphosphorylated during mitosis.

(B) Fragmentation mass spectra of Xenopus Nup53 peptides carrying mitotic specific phosphorylations

Supplementary Figure S4



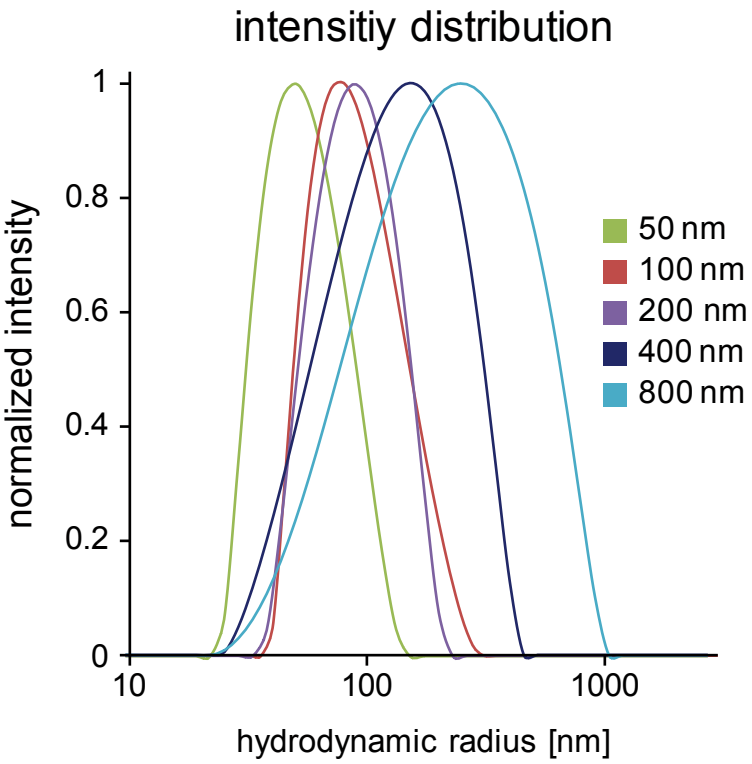


Figure S4

The two Nup53 membrane binding regions show different sensitivity to membrane curvature. Fragments comprising the N-terminal (93-267), C-terminal (130-320) or both (93-320) membrane binding sites, respectively, including the RRM domains were incubated with differently sized liposomes as indicated by the determined mean radii. Liposome binding was quantified as in Figure 2C. Whereas fragments which include the N-terminal membrane binding site (93-267 and 93-320) showed a significantly reduced binding to smaller liposome diameters and thus to higher membrane curvature this effect was not seen for the C-terminal membrane binding site (130-320). The averages of three independent experiments, normalized to the binding of the respective fragments to 150 nm liposomes, are shown. Error bars represent the range. Liposome radii were determined by light scattering after extrusion through membranes of different pore size as indicated for the different measurements. The lower panel shows one exemplary measurement done to determine the average radius of the respective preparation. Please note the rather similar average radii of liposomes prepared using 100 nm and 200 nm membranes.

Supplementary Figure S5

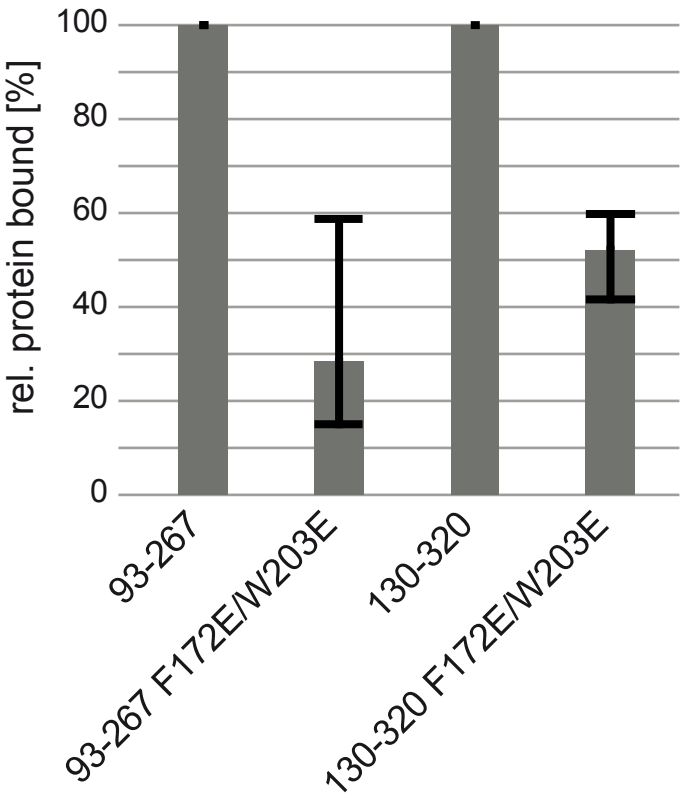


Figure S5

Both Nup53 membrane binding regions require dimerization by the RRM domain.

Nup53 fragments containing the first (93-267) or second (130-320) membrane binding region including the RRM domain were quantitatively assayed for liposome binding as in Figure 2C. Whereas fragments containing the wild type RRM domain bound to liposomes, introduction of two amino acid changes (F172E/W203E), which render the RRM domain incapable of dimerization, reduced liposome binding for both fragments. The averages of three independent experiments, normalized to liposome binding of the wild type protein, are shown. Error bars represent the range.

Supplementary Figure S6

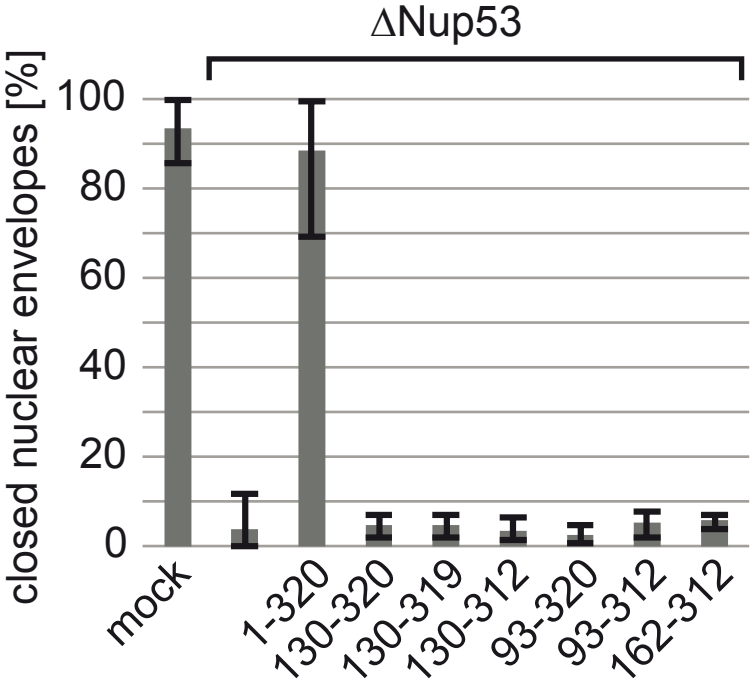


Figure S6

The interaction of Nup53 to Nup93 is necessary for nuclear envelope formation.

Nuclei were assembled in mock, Nup53 depleted extracts or Nup53 depleted extracts supplemented with wild type protein (1-320) or various fragments of Nup53 for 120 min, fixed with 2% PFA and 0.5% glutaraldehyde and analyzed for chromatin and membrane staining. Shown is the quantitation of chromatin substrates with a closed nuclear envelope as done in Figure 2F. Please note that all fragments lacking the N-terminal region of Nup53 necessary for Nup93 interaction (Figure S1A) and especially fragment 162-312 which has the ability to interact with Nup155 (Figure S1B) did not support nuclear envelope formation.

Supplementary Figure S7

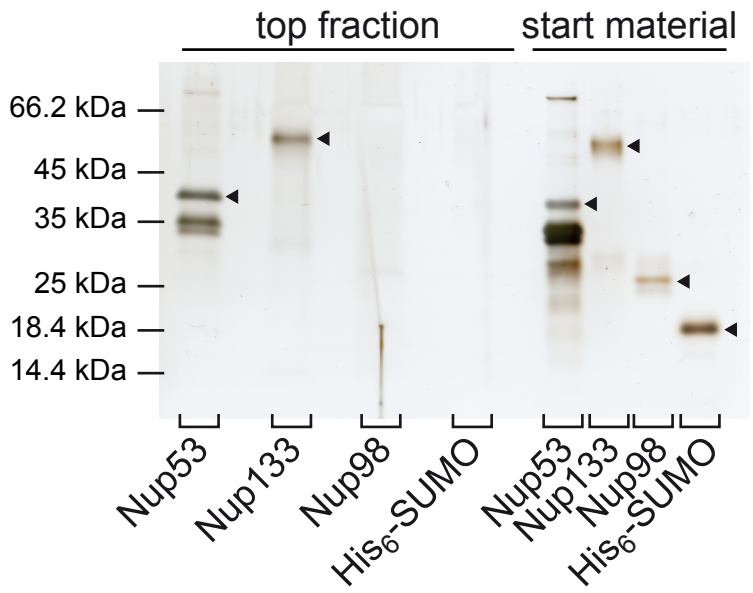


Figure S7

Recombinant Nup53 binds to liposomes mimicking the ER / nuclear envelope lipid composition

3 µM recombinant Xenopus Nup53 (Nup53) a fragment of Nup133 (aa 67-514) as positive control and Nup98 (aa 676-863) and His_6-tagged SUMO as negative controls, were incubated with 6 mg/ml fluorescently labeled liposomes prepared from a lipid mixture mimicking the ER/nuclear envelope lipid composition (see materials and methods). Flotation was done as described in Figure 1A.

Table S1: Peptides and phosphorylation sites identified in Xenopus Nup53 by mass spectrometric analysis

phosphopeptide	amino acid
PSAGAQFLPGFLLGDIPTPV**T**PQPR	T46
SPLH**S**GG**S**PPQPVLPTHK	S60, S64, S67
SPLHSGG**S**PPQPVLPTHK	S67
SIYDDVA**S**PGLGSTPR	S94
SIYDDVA**S**PGLGS**T**PR	S94, T100
MASFSVLHTPLSGAIPS**S**PAVFSPATIGQSR	S124
MASFSVLHTPLSGAIPSSPAVF**S**PATIGQSR	S131
V**S**TPSVSSVFTPPVK	S249
VS**T**PSVSSVFTPPVK	T250
VSTP**S**VSSVFTPPVK	S252
VSTP**S**VSSVF**T**PPVK	S252, T258
VSTPSVSSVF**T**PPVK	T258
SIRTPTQSVGTPR	S263
SIR**T**PTQSVGTPR	T266
SIRTPTQ**S**VGTPR	S270
TPTQSVG**T**PR	T273
TPTSADYQVVSDKPAPR	T288
TPT**S**ADYQVVSDKPAPR	T288, S291

Phosphorylation sites mapped in XenopusNup53 (genebank accession JQ747515) after immunoprecipitation from mitotic or interphase Xenopus egg extracts. Phosphorylation sites are indicated in red. Position T100, T288 and S291 were phosphorylated on Nup53 isolated from mitotic, but not interphasic extracts.

Table S2: DNA constructs used in this study

Constructs
pET28a SUMO Nup53xl 1-166
pET28a SUMO Nup53xl 1-267
pET28a SUMO Nup53xl 1-312
pET28a SUMO Nup53xl 1-312 S94E/T100E
pET28a SUMO Nup53xl 1-312 R105E/K106E
pET28a SUMO Nup53xl 1-319
pET28a SUMO Nup53xl 1-319 S94E/T100E
pET28a SUMO Nup53xl 1-319 R105E/K106E
pET28a SUMO Nup53xl 1-320
pET28a SUMO Nup53xl 1-320 S94E/T100E
pET28a SUMO Nup53xl 1-320 R105E/K106E
pET28a SUMO Nup53xl 1-320 F172E/W203E
pET28a SUMO Nup53xl 93-267
pET28a SUMO Nup53xl 93-267 S94A/T100A
pET28a SUMO Nup53xl 93-267 S94E/T100E
pET28a SUMO Nup53xl 93-267 R105E/K106E
pET28a SUMO Nup53xl 93-267 F172E/W203E
pET28a SUMO Nup53xl 93-312
pET28a SUMO Nup53xl 93-319
pET28a SUMO Nup53xl 93-320
pET28a SUMO Nup53xl 93-320 S94E/T100E
pET28a SUMO Nup53xl 93-320 R105E/K106E
pET28a SUMO Nup53xl 93-320 F172E/W203E
pET28a SUMO Nup53xl 107-267
pET28a SUMO Nup53xl 130-267
pET28a SUMO Nup53xl 130-312
pET28a SUMO Nup53xl 130-319
pET28a SUMO Nup53xl 130-320
pET28a SUMO Nup53xl 130-320 F172E/W203E
pET28a SUMO Nup53xl 162-267
pET28a SUMO Nup53xl 162-267 F172E/W203E
pET28a SUMO Nup53xl 162-312
pET28a SUMO Nup53xl 162-320
pET28a SUMO Nup53xl 254-320
pET28a SUMO Nup59sc 1-528
pET28a SUMO Nup53sc 1-475
pET28a PP Nup133hs 30-514
pET28a NusA Nup98xl 676-863
pET28a GST Nup53xl 1-267
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pET28a GST Nup53xl 1-267 R105E/K106E
pET28a GST Nup53xl 1-267 F172E/W203E
pET28a GST Nup53xl 162-267
pET28a GST Nup53xl 162-312
pET28a GST Nup53xl 162-319
pET28a GST Nup53xl 162-320
pET28a GST Nup53xl 162-320 F172E/W203E
pET28a GST Nup98xl 487-634

Constructs
pSI HA Nup53xl
pSI HA Nup53xl F172E/W203E
pSI myc Nup53xl
pSI myc Nup53xl F172E/W203E

Supplementary Methods

Pulldown experiments

Fragments used for the GST pulldown experiments were cloned into a modified pET28a vector with GST tag followed by a recognition site for TEV protease and purified via the N-terminal His_6 tag. 60 µl GSH–Sepharose (GE Healthcare) were incubated with 300 µg of the respective bait proteins, washed and blocked with 5% BSA in PBS. Beads were incubated with cytosol from Xenopus egg extracts (diluted 1:1 with PBS, and cleared by centrifugation for 30 min at 100,000 rpm in a TLA110 rotor (Beckman Coulter) for 2 h and washed six times with PBS. Bound proteins were eluted by cleavage with TEV protease (0.5 mg/ml) for 1 h at RT and analyzed by SDS-PAGE and Western blotting. For detection of NDC1 and GP210, 5 mg of membranes from Xenopus egg extracts (Antonin et al, 2005) were solubilized in 5 ml 50 mM Phosphate buffer pH 7.4, 500 mM NaCl, 1% Triton X-100 and protease inhibitors (Roche), instead of cytosol and the first four washes with PBS were in the presence of 0.1% Triton X-100.

Mass Spectrometry

2 ml interphasic (Hartl et al, 1994) or (CSF arrested) mitotic (Murray, 1991) Xenopus egg extracts were diluted with 1.2 ml wash buffer (10 mM HEPES, 50 mM KCl, 2.5 mM $MgCl_2$ pH 7.4), cleared by centrifugation for 10 min at 100,000 rpm in a TLA110 rotor and incubated with 50 µl Protein A Sepharose (GE Healthcare), to which affinity purified Nup53 antibodies were bound and crosslinked with 10 mM dimethylpimelimidate (Pierce). After 1h incubation the sepharose was washed 10 times with wash buffer. Proteins were eluted with SDS sample buffer (without DTT) and separated by SDS-PAGE. Gel sections from 30-45 kDa were excised and proteins were in-gel digested by trypsin. The resulting peptide mixtures were measured on an LTQ-Orbitrap XL and processed by MaxQuant software as described (Borchert et al, 2010). Multistage activation was enabled in all MS measurements.

Generation of liposomes

A mixture of lipids resembling the ER/nuclear envelope composition (Franke et al, 1970) (60 mol % phosphatidylcholine, 19.8 mol % phosphatidylethanolamine, 10 mol % phosphatidylinositol, 5 mol % cholesterol, 2.5 mol % sphingomyelin, 2.5 mol % phosphatidylserine 0.2 mol % 18:1-12:0 NBD-PE all Avanti polar lipids) dissolved in chloroform were dried on a rotary evaporator and overnight under vacuum. PBS buffer was gently added to result in a final lipid concentration of 6 mg/ml. After 2 h of incubation at

37°C to allow spontaneous liposome formation the flask was agitated to dissolve residual lipids. After ten cycles of freeze/thawing liposomes were extruded as described before.

DNA sequence of Xenopus laevis Nup53 optimized for expression in E. coli

ATGATGGCAGCAGCATTTAGCATGGAACCGATGGGTGCAGAACCGATGGCACTG
GGTAGCCCGACCAGCCCGAAACCGAGTGCCGGTGCACAGTTTCTGCCTGGTTTTC
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TATGGAAGTTCGTAGTCCGCTGCATAGCGGTGGTAGTCCTCCGCAGCCGGTTCTG
CCGACCCATAAAGATAAAAGCGGTGCACCTCCGGTTCGTAGCATTTATGATGATG
TTGCAAGTCCGGGTCTGGGTAGCACACCGCGTAATACCCGTAAAATGGCAAGCTT
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GTTTTTAGTCCGGCAACCATTGGTCAGAGCCGTAAAACCACCCTGAGTCCGGCAC
AGATGGACCCGTTTTATACCCAGGGTGATGCACTGACCAGTGATGATCAGCTGGA
TGATACCTGGGTTACCGTTTTTGGTTTTCCGCAGGCAAGCGCAAGCTATATTCTGC
TGCAGTTTGCACAGTATGGCAATATTATTAAACATGTGATGAGCAATAATGGCAA
TTGGATGCATATTCAGTATCAGAGCAAACTGCAGGCACGTAAAGCACTGAGCAA
AGATGGTCGTATTTTTGGTGAAAGCATTATGATTGGTGTGAAACCGTGCATTGAT
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TTTACACCTCCGGTTAAAAGCATTCGTACCCCGACCCAGAGCGTTGGTACACCGC
GTGCAGCAAGCATGCGTCCGCTGGCAGCAACCTATCGCACCCCGACCAGCGCAG
ATTATCAGGTTGTTAGCGATAAACCGGCACCGCGTAAAGATGAAAGCATTGTTAG
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DNA sequence of Saccharomyces cerevisiae NUP53 optimized for expression in E. coli

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TATTAAACGTAGCAATGGTCAGAGCCTGAGTCCGGTTCGTAGCGATAGCGCAGAT
GTTCCGGCATTTAGCAATAGCAATGGCTTTAATAATGTGACCTTTGGCAGCAAAA
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GCCTGGAAATTAAAGATGGTCGTAGCCTGTTTCTGCGTAATCGTGGTAAAATTCA
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GATCTGTAGTGA

DNA sequence of Saccharomyces cerevisiae NUP59 optimized for expression in E. coli

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CACCAATAACATTAGCCAGCATGGTCAGGGCAATAATCCGAGCTGGGTTAATAAT
CCGAAAAAACGTTTTACACCGCATACCGTTATTCGTCGTAAAACCACCAAACAGA
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GCGCAGCCAATAATGATATTAATAGCCTGCTGAGCAACTTTAATGATATTCCTCC
GAGCGTTACCCTGCAGGATTGGCAGCGTGAAGATGAATTTGGTAGCATTCCGAGC
CTGACCACCCAGTTTGTTACCGATAAATATACCGCCAAAAAAACCAATCGCAGCG
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CCTTTCGCATTTTTCATAATCATGATACCGGCTGTGATGAAAATGATAGCACCGTG
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CCGATTTTTACAGGCGAAAGCTGGGTTAAACTGACCTATAATAGCCCGAGCAGCG
CACTGCGTGCACTGCAAGAAAATGGTACAATTTTTCGTGGTAGCCTGATTGGTTG
TATTCCGTATAGCAAAAATGCCGTTGAACAGCTGGCAGGTTGCAAAATTGATAAT
GTGGATGATATTGGCGAATTTAATGTGAGCATGTATCAGAATAGCAGTACCAGCA
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GCGCGAAGATGATAATACACCGGCAGGTCATGCAGGCAATCCGACCAATATTAG
CAGCCCGATTGTTGCAAATAGCCCGAATAAACGTCTGGATGTGATTGATGGTAAA
CTGCCGTTTATGCAGAATGCAGGTCCGAATAGCAATATTCCGAATCTGCTGCGTA
ATCTGGAAAGCAAAATGCGTCAGCAAGAAGCAAAATATCGTAATAATGAACCGG
CAGGCTTTACCCATAAACTGAGCAATTGGCTGTTTGGTTGGAATGATCTGTAGTG
A

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Structure and properties of the esterase from non-LTR retrotransposons suggest a role for lipids in retrotransposition

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ABSTRACT

Non-LTR retrotransposons are mobile genetic elements and play a major role in eukaryotic genome evolution and disease. Similar to retroviruses they encode a reverse transcriptase, but their genomic integration mechanism is fundamentally different, and they lack homologs of the retroviral nucleocapsid-forming protein Gag. Instead, their first open reading frames encode distinct multi-domain proteins (ORF1ps) presumed to package the retrotransposon-encoded RNA into ribonucleoprotein particles (RNPs). The mechanistic roles of ORF1ps are poorly understood, particularly of ORF1ps that appear to harbor an enzymatic function in the form of an SGNH-type lipolytic acetylesterase. We determined the crystal structures of the coiled coil and esterase domains of the ORF1p from the *Danio rerio* ZfL2-1 element. We demonstrate a dimerization of the coiled coil and a hydrolytic activity of the esterase. Furthermore, the esterase binds negatively charged phospholipids and liposomes, but not oligo-(A) RNA. Unexpectedly, the esterase can split into two dynamic half-domains, suited to engulf long fatty acid substrates extending from the active site. These properties indicate a role for lipids and membranes in non-LTR retrotransposition. We speculate that Gag-like membrane targeting properties of ORF1ps could play a role in RNP assembly and in membrane-dependent transport or localization processes.

INTRODUCTION

Non-LTR retrotransposons (retrotransposons like the human LINE-1 element that do not contain long terminal repeats, LTRs) represent a major evolutionary force acting on the structure and composition of eukaryotic genomes (1–5). However, despite their significance for evolution and disease, non-LTR retrotransposons are poorly understood on a mechanistic level, especially if compared with LTR retrotransposons and retroviruses (5–7). Whereas all those retroelements propagate in a 'copy-and-paste' fashion via an RNA intermediate, their mechanisms of reverse transcription and genome integration are fundamentally different. Most strikingly, for LTR retrotransposons and retroviruses the reverse transcription takes place in the cytoplasm, in the context of virus-like particles (VLPs) that have a regular scaffold formed by the Gag protein (5–7). Among those, HIV Gag from the human immunodeficiency virus (HIV) is studied best and described as a self-associating multidomain protein that combines both RNA packaging and membrane binding functions (8). In contrast, for non-LTR retrotransposons the reverse transcription takes place in the nucleus and is directly coupled to genomic integration by target-primed reverse transcription (9,10). Furthermore, non-LTR retrotransposons lack homologs of the Gag protein and do not seem to have viral relatives that would allow a horizontal transfer across cell boundaries (5–7).

Instead of Gag, non-LTR retrotransposons frequently encode a distinct multidomain protein named ORF1p (11–19), which is translated from the first open reading frame (ORF1, Figure 1). A typical ORF1p usually contains one or two RNA recognition motif (RRM) domains (18), and it forms multimers, which is often

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indicated by the presence of a coiled coil domain (20–23). The RRM domains are thought to mediate RNA binding (18), leading to the formation of RNP retrotransposition intermediates that contain both ORF1p and ORF2p (harboring the reverse transcriptase function), but that lack the regular shape of VLPs (24,25). The RRM domains occur in the context of two distinct classes of ORF1p architecture (18). These are represented in Figure 1B by the LINE-1 ORF1p from the human LINE-1 element (22), and by the Jockey ORF1p from the *Drosophila melanogaster* Jockey element (12). Proteins that belong to the class of LINE-1-like ORF1ps trimerize (21) and can be identified via their distinct RRM domain (18). Crystal structures of such trimers reveal a highly complex architecture, remotely similar to membrane fusion proteins such as HIV-Env/gp41 or the influenza hemagglutinine-esterase (22). Proteins that belong to the class of Jockey-like ORF1ps are characterized by one or two RRM domains immediately followed by one or more CCHC zinc knuckles (18). These are similar to the zinc knuckles in the nucleocapsid domain of HIV Gag (11,12) and thought to cooperate with the RRM domains in the interaction with RNA (18). Intriguingly, some ORF1ps appear to have additional functionality that goes beyond self-association and RNP formation. This is indicated by sequence analyses that suggest the presence of an esterase domain (15), classified as a lipolytic acetylhydrolase of the SGNH family (26,27) (Figure 1).

To learn whether the esterase domain is more than a mere molecular fossil and to learn about the potential functions of esterase-encoding ORF1ps in non-LTR retrotransposition, we took a structure-based approach and characterized the ORF1p of the ZfL2-1 non-LTR retrotransposon from zebrafish, *Danio rerio* (28). In a HeLa cell-based assay (29), the ORF1p was reported to enhance retrotransposition of the ZfL2-1 element, but it is not essential in these cells (28,30). Furthermore, the protein was shown to self-associate and was suggested to interact with RNA (23), although it lacks an apparent RNA binding domain. We defined the boundaries of two functional domains and determined their crystal structures. The first structure is that of an N-terminal coiled coil domain that we show to cause a dimerization of the molecule, an assembly mode that is clearly distinct from the trimers formed by the LINE-1 ORF1p (21,22). The second structure is that of the esterase domain, demonstrating the three-dimensional conservation and an unexpected disposition to accommodate long fatty acid chains. We also demonstrate that the esterase is enzymatically active and binds to negatively charged liposomes. Together, these findings define a third class of ORF1p architecture and suggest that lipids may play a role in non-LTR retrotransposition.

MATERIALS AND METHODS

Sample preparation

DNA sequences for the expression of the N-terminal construct comprising the coiled coil domain, ZfL2-1_CC (M15-T91, Figure 2A and B), as well as for the esterase domain, ZfL2-1_ES (D136-I302, Figure 2A and C), were amplified by polymerase chain reaction (PCR) from a chemically synthesized and codon-optimized DNA template (Invitrogen), encoding the complete ORF1 protein of the ZfL2-1 element (Uniprot ID Q3LG57) (28). Mutations were generated using QuikChange site-directed mutagenesis PCR (Stratagene). Proteins were expressed from a pETM41P (EMBL) plasmid in *Escherichia coli* BL21-Star cells at 20°C overnight. Purification included maltose binding protein (MBP) and heparin affinity steps followed by size-exclusion chromatography (SEC) into storage buffer (10 mM Tris/HCl, pH = 7.5, 150 mM NaCl).

Multimerization and RNA binding

Analytical SEC and multi-angle laser light scattering (MALLS) experiments were done in reaction buffer (20 mM Tris/HCl, pH = 7.5, 250 mM NaCl, 5 mM $MgCl_2$) on a Superdex 75 (10/300 GL) column that was mounted on an ÄKTA Purifier-10 (GE Healthcare) and followed online by miniDAWN TREOS and Optilab rEX instruments (Wyatt Technologies). The relative contributions of protein and RNA to the total ultraviolet absorption were calculated at each wavelength (simultaneously monitored at 230, 260 and 280 nm) assuming for each substance a constant ratio of its extinction coefficients at 230 and 280 nm (31).

Activity assay

Hydrolysis of *p*-nitrophenol (pNP) esters was monitored in reaction buffer at 25°C on a Tecan Infinite F200 spectrophotometer by an absorption increase at 405 nm for released pNP ($\varepsilon = 17\,800\,l\ mol^{-1}\,cm^{-1}$). Initial velocities were plotted as a function of substrate concentration to determine kinetic parameters, K_M and k_{cat}. For specific activity, 1 U equals a substrate turnover of 1 µmol min^{-1}. To test for peptide deacetylation we used an histone deacetylase fluorimetric assay kit (Enzo Life Sciences) according to the manufacturer's instructions.

Lipid overlay and liposome binding assays

Membrane lipid strips (Echelon Biosciences) contained single lipid species spotted on a hydrophobic membrane (100 pmol per spot) and were incubated with proteins overnight [3× phosphate buffered saline (PBS), 0.3% Tween, 3% bovine serum albumin]. The esterase was used as MBP-His-fusion protein and detected by a specific primary antibody (anti-His, Sigma), followed by a horseradish peroxidase-conjugated secondary antibody.

Liposomes (d = 0.30 µm) were prepared according to the manufacturer's protocol from polar lipid extract (Avanti Polar Lipids). Protein and liposomes were mixed as described (32), incubated and brought to a sucrose concentration of 32% in a centrifugation tube (2.5–5 µM protein, 150 µl). This was overlaid in two steps by a 14% sucrose cushion and topped by a layer of 1 × PBS. Following a centrifugation step, protein from the top fraction (300 µl) was precipitated and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Crystallization and data collection

For crystallization in sitting drops (0.4 over 80 µl reservoir), sample in reaction buffer was mixed 1:1 with reservoir. For the coiled coil domain (Figure 2B), a single crystal was found after 4 months over a reservoir of 0.2 M Na-thiocyanate and 20% (w/v) polyethylene glycol (PEG) 3350. The crystal was flash-frozen in liquid nitrogen, from reservoir solution supplemented with 20% glycerol. For the esterase domain (Figure 2C), crystals grew over 0.1 M Na-Hepes, pH = 7.0, 0.5% (v/v) Jeffamine and 1.1 M Na-malonate. A heavy atom derivative was obtained by an overnight incubation in reservoir supplemented with 2 mM potassium dicyanoaurate. Crystals were flash-frozen from reservoir solution. Diffraction data were collected on beamline PXII of the Swiss Light Source, Villigen, Switzerland, and diffraction images were processed using XDS (33).

Crystal structure solution, refinement and modeling

The structure of the coiled coil domain was solved by molecular replacement using MOLREP (34) from within the CCP4 package (35) and a polyalanine model of an anti-parallel coiled coil as a search model, derived from PDB-ID 1A92 (36). The structure of the esterase domain was solved by single isomorphous replacement with anomalous scattering (SIRAS). SHELX C/D/E was used to identify the sites for phasing and for an initial auto-building of the structure (37,38). Both structures were then (re-)built automatically also to remove any potential model bias in the case of the coiled coil domain, using ARP/wARP (39) and BUCCANEER (40). The model was finished manually in COOT (41), alternating with rounds of refinement using PHENIX (42). Final refinement rounds were done in PHENIX, refining TLS parameters in addition to individual B-factors and including hydrogens. Stereochemical properties were analyzed with MOLPROBITY (43). Modeling of the rotated L281 and of the enclosed palmitate in the context of the closed esterase monomer (ZfES_BA, connecting residues M135-L199 from chain B with residues R200-I302 from chain A) was also achieved in COOT followed by energy minimization in PHENIX. Cavity volumes were extracted using the Voss Volume Voxelator (44) using inner and outer probe radii of 1.2 and 5.0 Å, respectively. Figures were generated in PyMOL (http://pymol.org/) using the APBS plug-in (45) to visualize electrostatic surface potentials.

RESULTS

Esterases are found in distinct clades of non-LTR retrotransposons and share specific properties

Non-LTR retrotansposons (Figure 1A) are thought to be acquired primarily by vertical transmission and were originally grouped into clades of distinct domain composition that date back to the Precambrian era (7). For the classification of newly identified non-LTR retrotransposons, consensus sequences are deposited in RepBase (46) and placed into their respective clade according to sequence alignments of the reverse transcriptase (17).

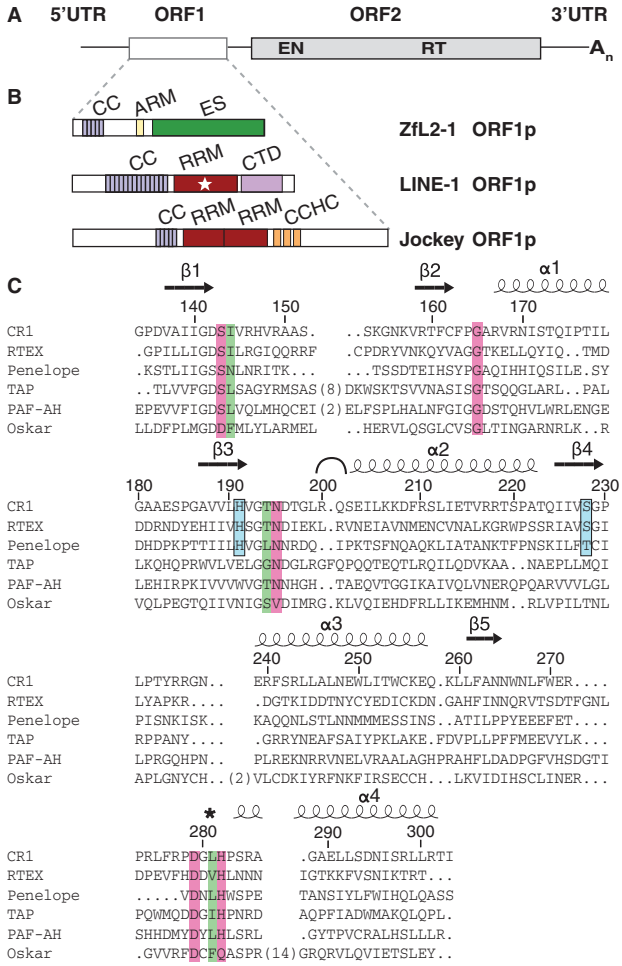

Figure 1. The esterase encoded by non-LTR retrotransposons. (**A**) General organization of an RNA from a non-LTR retrotransposon, encoding a first, accessory ORF1 and a second, catalytic ORF2 that harbors both endonuclease (EN) and reverse transcriptase (RT) functions required for target-primed reverse transcription. (**B**) Class representatives for ORF1ps and their domain architecture. The three structural classes are illustrated by ORF1ps from the *Danio rerio* ZfL2-1 element, the human LINE-1 element and the *Drosophila melanogaster* Jockey element. The unique SGNH-type esterase, ES, is highlighted in green. Coiled coil domains promoting self-association (CC, gray blue) are indicated as well as proposed RNA binding elements: RRM (star) and CTD indicate LINE-1-like RRM and C-terminal domains. RRM and CCHC indicate other RRM domains and Gag-like CCHC zinc knuckles. ARM indicates arginine-rich peptide motifs. (**C**) Structure-based sequence alignment of the ZfL2-1 esterase. Esterases from non-LTR retrotransposons are aligned with crystallized SGNH hydrolases (TAP and PAF-AH, Supplementary Figure S2) and with a non-catalytic SGNH protein (Oskar). Transposon-encoded esterases are from different clades (CR1, RTEX, Penelope) and animal phyla (chordates, cnidarians, sponges). Numbers and secondary structures are from the ZfL2-1 esterase, representing the CR1 clade. An arc indicates the hinge around R200, and an asterisk denotes the remodeled L281 (Figure 3F). Catalytic residues are boxed in magenta, positions of gating residues in green and transposon-specific positions in cyan. See Supplementary Figure S1 for additional details and accession numbers.

We retrieved ORF1p sequences containing an SGNH esterase from RepBase and aligned them with SGNH proteins of known structure (Figure 1C and Supplementary Figure S1). The alignment reveals that

the esterase is not limited to the CR1 clade, where it was originally identified (15), but can also be found in members of the RTE clade, RTEX (17) and even in Penelope-like elements, the most deeply branched clade of non-LTR retrotransposons (19). Also, esterase-containing non-LTR retrotransposons are not limited to certain species, but are distributed over many animal phyla.

Most importantly, despite the low sequence identity, the residues constituting the active site have been strongly conserved. This suggests a beneficial role of esterase structure and enzyme function for the propagation of the respective non-LTR retrotransposons. Moreover, the alignment reveals additional positions (H191, S228) that are conserved only among the transposon-encoded esterases, pointing at specialized transposon-specific properties and a monophyletic origin (Figure 1C and Supplementary Figure S1), despite the scattered presence of the esterase in different clades and species.

Esterase-containing ORF1ps can form multimers but lack structural RNA binding domains

Further analysis of the esterase-containing ORF1ps reveals that the esterase domain is generally preceded by sequences predicted to form coiled coils and connected to these sequences by poorly conserved linkers (Supplementary Table S1). The presence of coiled coil domains indicates these proteins to form multimeric assemblies, as observed for many previously characterized ORF1ps (20–23). Interestingly, none of the esterase-containing ORF1ps in RepBase included RRM domains or any other structural domain known to bind RNA. It hence appears that such a domain combination never occurred in the past or that it bears no selective advantage, for example, because it is functionally redundant or even mutually exclusive. In either case, the observation raises the question how the esterase-containing ORF1ps could interact with RNA and become part of stable RNPs.

We therefore decided to express and purify the ZfL2-1 ORF1p (28) and to characterize its properties *in vitro* (Figure 2). We obtained soluble material for an N-terminal construct comprising the coiled coil domain and for the C-terminal esterase domain (Figure 2A–C). In analytical SEC, the N-terminal construct indeed shows self-interaction (23) and we find it to exclusively form dimers, whereas the esterase domain remains monomeric at concentrations up to 75 µM (Figure 2B and C). Furthermore, the two constructs did not interact with each other and failed to form stable and separable complexes with single-stranded oligo-$(A)_{27}$ RNA (Figure 2D and E) under conditions previously used for the LINE-1 ORF1p (22).

Consequently, the separated domains and the highly positive charge of the esterase alone (pI = 10.8) are not sufficient for a general activity to bind single-stranded RNA. However, we needed to exclude the arginine-rich motif (ARM, Figure 1B) between amino acids G98 and T114 of the ZfL2-1 ORF1p to obtain soluble and non-aggregating protein constructs. Such motifs are frequently found in members of the CR1 clade (Figure 2A and Supplementary Table S1), and it has been described that an N-terminal fragment of the ZfL2-1 ORF1p that includes the ARM can be photocrosslinked to RNA *in vitro* and that it helps to remodel nucleic acid structures (23). We therefore speculate that the esterase-containing ORF1ps could bind their RNA messengers not via structural protein domains, but rather via positively charged peptides that target specific structural RNA elements, as described for the λ-N peptide (47).

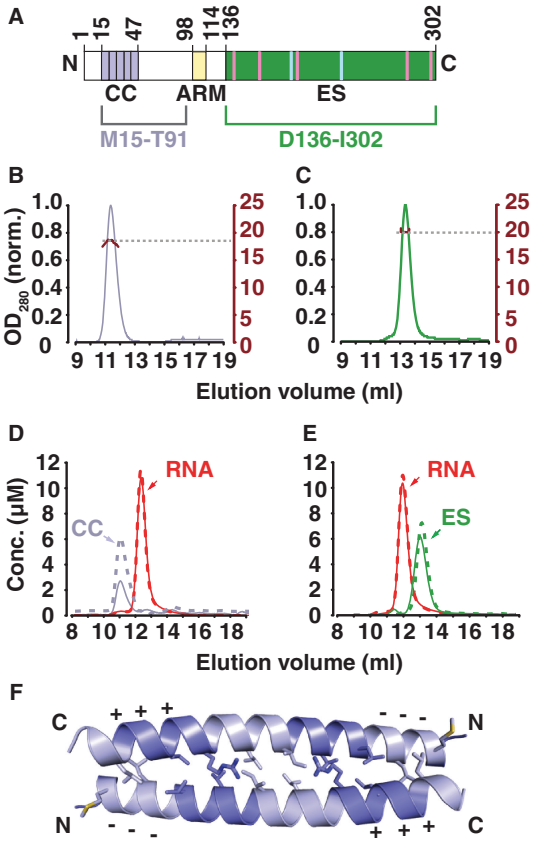

Figure 2. Multimerization and RNA binding properties of the ZfL2-1 ORF1p. (**A**) Domain structure of the ZfL2-1 ORF1p. Domain boundaries (Figure 1B) are numbered, and conserved positions (Figure 1C) are indicated. The soluble and non-aggregating protein fragments used in this work exclude the arginine-rich motif (ARM) and are outlined by brackets. (**B** and **C**) SEC coupled to static MALLS. The N-terminal construct comprising the coiled coil domain (B, residues M15-T91, M_r = 9 kDa) elutes as a non-spherical dimer with a hydrodynamic radius (r_H) of 27 Å and a mass (M_r) of 18 kDa. The isolated esterase domain (C, residues D136–I302, M_r = 19 kDa) elutes as a spherical monomer with a hydrodynamic radius (r_H) of 11 Å and a mass (M_r) of 19 kDa. (**D** and **E**) Quantitative SEC. Proteins and RNAs were either injected separately (dotted lines) or as mixtures (solid lines). Neither the N-terminal construct (D), nor the isolated esterase domain (E) forms stable complexes with oligo-$(A)_{27}$ RNA (red). (**F**) Crystal structure of the coiled coil domain (M15-P47). Heptad repeats within the antiparallel dimer are shaded alternatingly and charge compensation between acidic (−−−) and basic (+++) heptads is indicated. Side chains in hydrophobic positions a and d of each heptad repeat are drawn as sticks.

The coiled coil domain of the ZfL2-1 ORF1p crystallizes as an antiparallel dimer

A proteolytically stable fragment of the N-terminal construct gave crystals that contained two copies of an

antiparallel homodimeric coiled coil per asymmetric unit, and the structure was refined to an R_{free} of 21.5% at 1.55 Å resolution (Supplementary Table S2). Each α-helix comprises five heptad repeats, confining the coiled coil to the region between M15 and P47 as we would predict from the sequence. Because of the charge compensation between the highly acidic N-terminal heptads and the highly basic C-terminal heptads, we presume a preference for the antiparallel orientation also in the absence of crystal packing constraints (Figure 2F), an arrangement that is clearly distinct from the parallel trimeric coiled coil formed by the LINE-1 ORF1p (22). Whereas coiled coil domains and their ability to multimerize are hence frequently present among ORF1ps, the resulting assemblies can differ significantly in terms of structure and likely do not always have a common evolutionary origin.

The esterase domain of the ZfL2-1 ORF1p crystallizes as two dynamic half-domains

The structure of the ZfL2-1 esterase (Figure 3) was solved by SIRAS and refined at 2.5 Å resolution to an R_{free} of 21.0% (Supplementary Table S3). As expected for an SGNH hydrolase, the protein adopts a flavodoxin-like fold with a central sheet of five parallel β-strands sandwiched by α-helices on both sides (Figure 3A and B). The ZfL2-1 esterase is highly similar to well-characterized SGNH hydrolases (Supplementary Figure S2). These include thioesterase I/protease I/lysophospholipase L1 (TAP) (48) from *Escherichia coli* that can remove 1-acyl groups from lysophospholipids, and cytosolic platelate activating factor acetylhydrolase (PAF-AH) (49) from bovine brain that can remove the 2-acetyl group from PAF and has been implicated in lissencephaly (50). Compared with TAP and PAF-AH, the identity and position of the catalytic residues are highly conserved in ZfL2-1. These residues include D279, H282 and S143, which form the catalytic triad, as well as N195 and G165 that line the oxyanion hole (Figure 3C and Supplementary Figure S2A–D).

The crystal packing of the ZfL2-1 esterase is of particular interest because the protein crystallized as a domain-swapped dimer with three molecules (i.e. 1.5 dimers) per asymmetric unit (Figure 3B). As a consequence, each esterase domain in the crystal is composed of two fragments; an N-terminal half (residues 135–199) with ββαβ topology that is complemented by a C-terminal half (residues 200–302) with αβαβα topology from the neighboring molecule. Because the linker between the two halves is short and close to the 2-fold axis of the dimer, a minimal rearrangement of residues R200 and Q201 is sufficient to reconnect the two chains and to obtain a model for the monomer (hinge, Figure 3A).

Although in solution we only detect the monomeric form, the observed crystal packing demonstrates that the esterase can apparently split open into two halves at least transiently, disengaging β-strands β3 and β4 of the central β-sheet and tearing apart the network of hydrogen bonds in the active site (Figure 3C). These dynamics are facilitated by the fact that the core of the esterase is not entirely hydrophobic and even contains enclosed water-filled cavities that require structural rearrangements to gain access to the solvent (Figure 3E and F). Most interestingly, the hydrogen bonds between the two half-domains also include an interaction between the transposon-specific residues H191 and S228 deep inside the core of the esterase (Figure 3C). This indicates that the composition of two reversibly separable half-domains is likely conserved among transposon-encoded esterases and has functional significance.

The ZfL2-1 esterase shows hydrolytic activity

In the crystal of the ZfL2-1 esterase, residues I144, T194 and L281 confine the size of the active site and separate it from the internal cavities (Figure 3E). Based on the comparison with the structure of PAF-AH, these residues are thought to limit the fatty acid moiety of the ester substrate to short carbon chains, whereas the alcoholic moiety of the ester substrate faces the solvent and probably contributes only marginally to substrate specificity (Supplementary Figure S2B and D). Therefore, we used pNP acetate as an ester substrate analog and monitored hydrolysis by absorption change. As expected from the structure, we find the ZfL2-1 esterase to be enzymatically active (Figure 3D). The activity crucially depends on the active site of the enzyme because individual point mutations of the catalytic residues (S143A/Y/Q, H282S) completely abolish hydrolysis. In addition, activity is blocked by a S228A/H191F double mutation of the two transposon-specific residues (Figure 1C and 3C), indicating the internal network of hydrogen bonds to be relevant also for the catalysis. However, the catalytic turnover, k_{cat}, and hence the specific activity of the ZfL2-1 esterase domain is significantly lower than reported for most other SGNH hydrolases (0.92 U mg^{-1}, as compared with 345 U mg^{-1} for TAP) (27), suggesting that it is not optimized for catalytic turnover. Furthermore, because many SGNH hydrolases have a rather broad range of substrate specificity (27), we also tested for phosphoesterase and peptidase activities. However, we failed to detect any activity, neither with pNP phosphate as a substrate, nor using a standard kit for the deacetylation of histone-derived peptides. Finally, regarding the length of the fatty acid chain, pNP esters such as pNP butyrate are hydrolyzed as well (estimated catalytic efficiency, $k_{cat}/K_M = 590\,s^{-1}M^{-1}$), but a quantitative analysis of pNP esters with even longer fatty acid chains is precluded by their limited solubility.

Long fatty acid chains could reach and fill the space between the half-domains

A comparison of the three independent protein copies in the crystal reveals that the side chains of I144 and L281 are flexible and can adopt alternative conformations, suggesting that they could act as gating residues between the active site and the internal cavities (Supplementary Figure S2E and F). Indeed, a simple rotation of L281 would cause the gate to open up and connect the internal cavities to the surrounding solvent. As a consequence, the volume accessible to the fatty acid would increase from ~50 to 270 $Å^3$ and hence would allow the accommodation of longer fatty

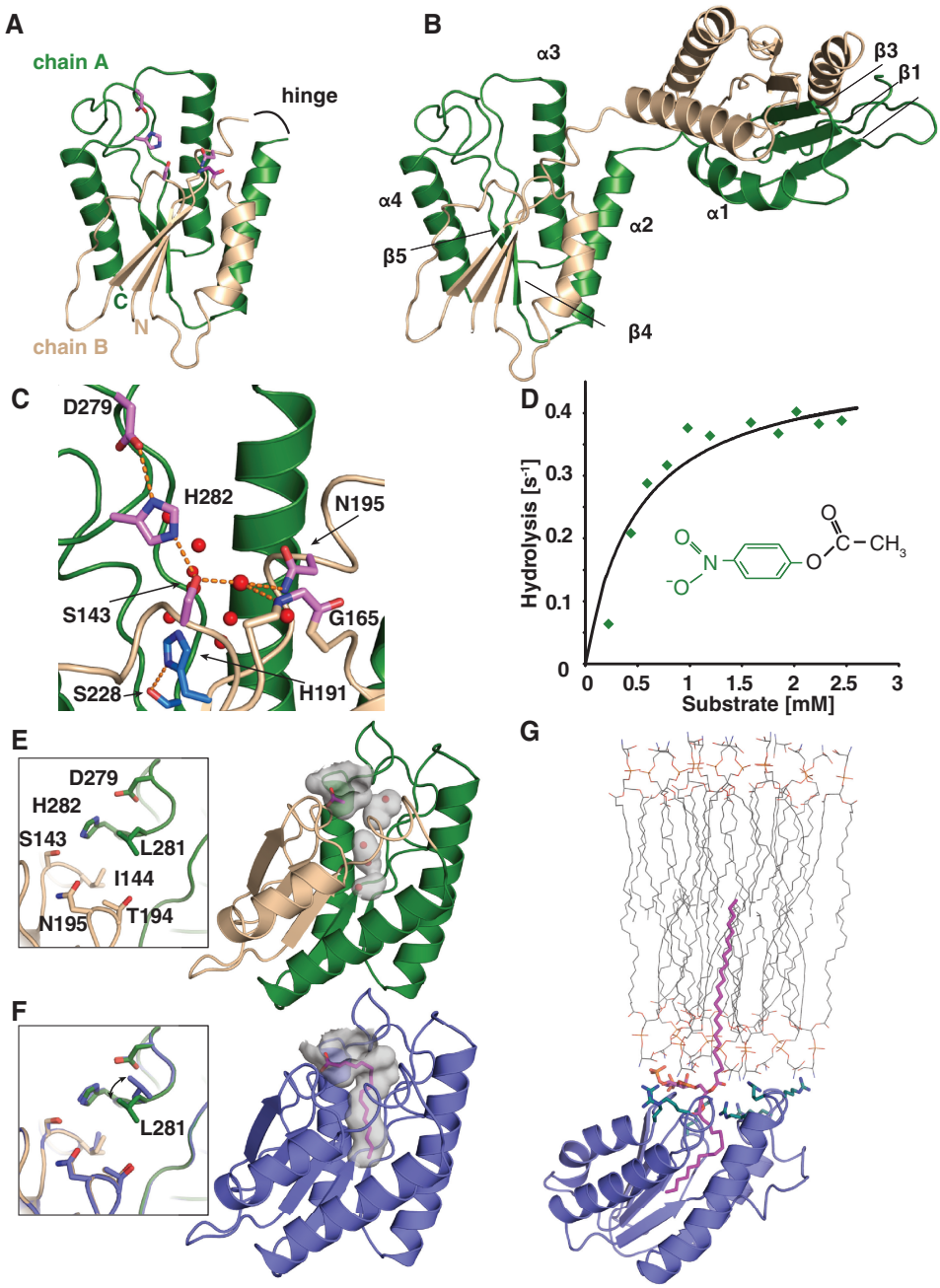

Figure 3. Crystal structure and activity of the ZfL2-1 esterase. (**A** and **B**) Structural overview. The esterase consists of two half-domains (green and wheat) connected by a flexible hinge (A) that allows it to crystallize as a domain-swapped dimer (B). Secondary structure elements are indicated, and conserved catalytic residues are shown as sticks (magenta). (**C**) Details of the active site. Transposon-specific residues are included as sticks (cyan), waters are shown as red spheres and hydrogen bonds as dotted lines. (**D**) Enzymatic activity. Representative Michaelis–Menton curve using pNP actetate (inset) as a substrate. $K_M = 0.43 \pm 0.10\,mM$, $k_{cat} = 0.32 \pm 0.07\,s^{-1}$, $k_{cat}/K_M = 740 \pm 240\,s^{-1}M^{-1}$; standard error from six independent experiments. (**E**) Energy-minimized monomer (gate closed). The active site is marked by a superimposed acetate (magenta sticks, from Supplementary Figure S2D). Internal, water-filled cavities and the active site are shown as inverted surfaces. They are separated by the gating residues I144, T194 and L281 (inset). (**F**) Energy-minimized monomer (gate opened) in presence of a palmitate. The deep invagination accommodating the palmitate (magenta sticks) results from a simple rotation of L281 that opens the gate. Placement of the palmitate was guided by the enclosed waters and superimposed octanoic acid (from Supplementary Figure S2B). The inset shows an overlay of the closed and opened gate. (**G**) Model for Gag-like membrane binding via a sequestered phospholipid [adapted from (52)]. Arginines and lysines are shown as blue sticks and thought to cause an electrostatic attraction. The sequestered palmitate is as in (F).

acid chains such as the ones found in membrane phospholipids (Figure 3E and F).

However, it is difficult to imagine how the carbon chain would thread through the gate and displace the internal water molecules. We therefore suggest that it enters laterally and gets engulfed between the two half-domains when they move apart, turning around the described hinge at R200–Q201 as proposed above for the formation

of the domain-swapped dimer (Figure 3A and B). This binding mechanism should be clearly facilitated in the context of the full-length protein, where two esterase domains are closely tethered together by the coiled coil. It would probably help to access phospholipid substrates at the surface of cellular membranes by 'interfacial activation' (51), resulting in membrane binding topologies that are comparable with the one proposed for the interaction of the matrix (MA) domain of HIV Gag with phosphatidylinositol 4,5-bisphosphate (52). Similar to the MA-domain, the putative membrane-facing surface of the ZfL2-1 esterase is rather flat and displays a striking accumulation of positively charged arginine and lysine residues (Figure 3G).

The ZfL2-1 esterase binds negatively charged phospholipids and liposomes

To test whether the ZfL2-1 esterase is able to gain access to phospholipids at membrane surfaces independently of other proteins, we first did an assay with commercially available membrane lipid strips, where proteins that bind prespotted lipids on a solid support are detected by antibody staining (Figure 4). Under stringent salt conditions the ZfL2-1 esterase exclusively binds a selection of negatively charged phospholipids, including phosphatidic acid and phosphatidyl-inositol phosphates (Figure 4A).

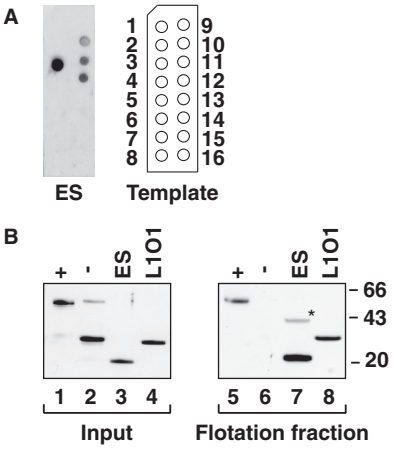

Figure 4. Interaction of the ZfL2-1 esterase with phospholipids and liposomes. (**A**) Lipid overlay assay. An MBP-His-tagged version of the ZfL2-1 esterase (ES) was incubated with membrane lipid strips under stringent salt conditions (3× PBS), and bound protein was detected by immunostaining. The positions of the spotted lipids are as follows: 1, triglyceride; 2, diacylglycerol; 3, phosphatidic acid; 4, phosphatidylserine; 5, phosphatidylethanolamine; 6, phosphatidylcholine; 7, phosphatidylglycerol; 8, cardiolipin; 9, phosphatidylinositol (PI); 10, PI-[4]-phosphate (PI[4]P); 11, PI[4,5]P_2; 12, PI[3,4,5]P_3; 13, cholesterol; 14, sphingomyelin; 15, [3]-sulfogalactosylceramide; 16, blank. The esterase preferentially interacts with phosphatidic acid and the three phosphatidylinositol phosphates. MBP-His alone or GST did not show any signal. (**B**) Flotation experiment using liposomes. Silver-stained gels show input samples (20%, left panel) and samples recovered from the floating liposomes (right panel). Lanes 1, 5: (+), positive control, Nup133 [residues 67–514, (32)], a nucleoporin. Lanes 2, 6: (−) negative control, GST. Lanes 3, 7: ES, ZfL2-1 esterase (residues 136–302). Lanes 4, 8: L1O1, LINE-1 ORF1p [residues 104–337, (22)]. The asterisk denotes a weak ES dimer in the flotation fraction, a likely gel separation artifact.

This assay was followed by an established liposome flotation assay with liposomes prepared from polar lipid extracts [Supplementary Figure S3, (32)]. Here, we find the ZfL2-1 esterase to migrate through a sucrose gradient together with the liposomes, similarly to a truncated nucleoporin, Nup133, that served as a positive control, and in contrast to glutathione-S-transferase (GST), which served as a negative control (Figure 4B). Importantly, liposome binding depends on the integrity of the protein structure because heat-denatured ZfL2-1 esterase no longer co-purifies with the floating liposomes. However, neither our active site point mutations nor the H191F/S228A double mutation prevented liposome binding (Supplementary Figure S3B and C), suggesting that initial membrane surface binding as tested in the liposome flotation assay is primarily driven by protein surface charge (Supplementary Figure S3D) and does not require the sequestration of a fatty acid chain.

Together, these experiments suggest that the esterase domain can indeed target the ZfL2-1 ORF1p to negatively charged membranes in the cell, suggesting a role for membranes in the propagation of non-LTR retrotransposons. Intriguingly, this role may even extend beyond the esterase-encoding elements because a truncated version of the trimeric human LINE-1 ORF1p (22) that starts in the middle of the coiled coil domain (Figure 1B) also co-purifies with liposomes in our assay (Figure 4B).

DISCUSSION

The detailed analysis of the ZfL2-1 ORF1p and the comparison with its relatives from other non-LTR retrotransposons establish the esterase-containing ORF1ps as a separate architectural class that lacks defined RNA binding domains (Figure 1). Nevertheless, ZfL2-1-like ORF1ps show functional analogy with LINE-1-like and Jockey-like ORF1ps in RNP assembly because of their self-association via coiled coil domains and because of their ability to facilitate structural rearrangements of nucleic acids *in vitro* (20,23,53). Additional support for a functional analogy among structurally diverse ORF1ps comes from the fact that retrotransposons from within a given clade [as defined by closely related reverse transcriptases (17)] can harbor ORF1ps from different architectural classes (18). This observation not only hints at similar functions of the diverse ORF1ps but also at an apparent 'exchange' of ORF1ps between otherwise closely related non-LTR retrotransposons. Such an 'exchange' could take place by recombination or mutual cross-insertion of retrotransposons within a common host, or, alternatively, by a consecutive loss and gain of domains. The latter possibility is facilitated by the fact that the loss of transposon-encoded protein domains does not necessarily lead to the extinction of the respective non-LTR retrotransposon because the lost function might still be available 'in trans', i.e. from another retrotransposon or from the host. Indeed, this idea has been promoted previously for the apparent loss and gain of the RNaseH domain in non-LTR retrotransposons and retroviruses (54,55), as it can also reconcile the apparently monophyletic origin of protein domains such as the

esterase and RNaseH with the scattered distribution across clades and species.

The precise role of the ZfL2-1 ORF1p in retrotransposition is still difficult to address. Although the ZfL2-1 element has been shown to retrotranspose in human HeLa cells (28), its ORF1p is dispensable in this assay (30), and therefore HeLa cells are not expected to reveal ZfL2-1 ORF1p-specific functions. In the context of the natural zebrafish host, however, where the ZfL2-1 element needs to retrotranspose in germ line cells to be passed on to the next generation, the ORF1p may be an important factor. Indeed, the presence of the esterase and the conservation of its transposon-specific properties strongly suggest additional functions for ORF1ps in non-LTR retrotransposition that go beyond their known properties in RNP assembly. The crystal structure of the ZfL2-1 esterase and the positive activity assay demonstrate that the respective domains in related ORF1ps are *bona fide* SGNH hydrolases, and are therefore unique among ORF1ps in harboring an enzymatic function. SGNH hydrolases represent a large enzyme family comprising several thousand members from all domains of life, including viruses. However, even for well-characterized members, such as PAF-AH, the physiological substrates and functions largely remain obscure (26,27). The primary targets are presumably carboxyesters, but some family members also hydrolyze thioesters and even isopeptide bonds (27,48). Most intriguingly, the active sites of certain SGNH proteins have dual binding and enzymatic functions (56) or have entirely lost their hydrolytic activity, such as the homologs of PAF-AH in insects (57) or the Oskar protein (58) in *Drosophila* germ cell formation (Figure 1C and Supplementary Figure S1). One can therefore imagine several mechanisms how an SGNH protein could affect retrotransposition. These include the deacetylation of carbohydrate substrates, lipid messengers or regulatory proteins (50,56,59). Another attractive possibility that requires access to the internal cavities is the reversal of protein palmitoylation controlling membrane association of the target (60). However, there are no known protein targets so far, and we failed to detect activity in a histone deacetylation assay.

Considering the common properties of ORF1ps in the formation of RNPs, and based on our lipid binding assays, we therefore favor a role of the esterase domain in membrane targeting, combined with a potentially regulated phospholipase activity. In this scenario, initial targeting of membrane surfaces would be initiated by a charge-mediated interaction, and would be consolidated by the sequestration of an acyl chain in the space between the two dynamic half-domains, resulting in a membrane binding topology similar to the one proposed for the MA-domain of HIV Gag [Figure 3G, (52)]. Subsequent steps of the retrotransposition cycle could then be regulated by a controlled substrate hydrolysis, releasing both surface-assembled RNPs and lysophospholipid, i.e. phospholipids that lack one of their long fatty acid chains. This release only requires a single round of substrate hydrolysis, consistent with the observation that the ZfL2-1 esterase is apparently not optimized for rapid enzymatic turnover. Lysophospholipids can influence the spontaneous curvature and bending elasticity of phospholipid membranes and have been implicated in the formation of membrane vesicles and other specialized membrane structures (61–63). As an additional consequence of the described process, the esterase-containing ORF1ps could therefore not only drive RNP assembly on membrane surfaces, but could also help to include RNPs in membrane vesicles capable of transferring them across membranes (64,65). On a speculative note, such vesicles might even be implicated in the rare instances where non-LTR retrotransposons like the BovB element transfer horizontally between species (66,67). BovB is a member of the RTE clade that, in contrast to RTEX, encodes only rudimentary ORF1ps or lacks them altogether (68).

The suggested mode for the esterase to associate with membranes is clearly inspired by the HIV Gag protein, which uses membranes to self-assemble into retroviral capsids (8). Furthermore, there is a growing body of data from positive-strand RNA viruses that use virally encoded proteins to assemble RNA-replication complexes at specifically induced membrane structures (69). These parallels between retroviruses and positive-strand RNA viruses raise the question how non-LTR retrotranspons fit into the picture and whether the functional analogy that is emerging between ORF1ps could be extended to membrane-related functions. Therefore, we believe that it is worth further exploring the role of membranes in non-LTR retrotransposition, not only in the context of ZfL2-1-like ORF1ps, but also in the context of the trimeric LINE-1-like ORF1ps with their structural similarity to membrane fusion proteins (22), and in the context of the Jockey-like ORF1ps with their Gag-like CCHC knuckles. The respective studies shall provide important mutual insight into the mechanisms of non-LTR retrotransposition, in particular into hitherto unexplored processes such as the regulation and localization of RNP assembly or into the roles of membranes in RNP transport.

ACCESSION NUMBERS

The atomic coordinates and structure factors for the ZfL2-1 ORF1p coiled coil domain and for the ZfL2-1 ORF1p esterase were deposited at the protein data bank (PDB) under ID codes 4c1a and 4c1b, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online including [70,71].

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Structure and properties of the esterase from non-LTR retrotransposons suggest a role for lipids in retrotransposition

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- SUPPLEMENTARY INFORMATION -

Table S1

Domain boundaries

Name	CC-start [a, b]	CC-end [a, b]	ARM-start [a, c]	ARM-end [a, c]	ES-start [a, d]	ES-end [a, d]
CR1-1_DR	M027	K082	G124	T140	D162	T327
CR1-16_DR	n.a.	n.a.	G047	S063	K109	N273
CR1-3_DR	E063	P097	E133	R149	R223	H379
CR1_1a_XT	L073	M120	V132	E148	Q203	E368
CR1-2_XT	T035	L076	D095	G111	Q166	E331
CR1_AC_1	n.a.	n.a.	n.a.	n.a.	Q068	R232
CR1-X1_Pass	F063	R111	E144	P160	R217	R381
CR1-J2_Pass	T077	S118	G150	V166	R220	R384
CR1-L1_Tgu	S043	L091	G116	S132	R187	R351
CR1-K1_Tgu	P104	L152	G181	S132	R252	R461
RTEX-3_NV	N148	K261	n.a.	n.a.	P371	L538
RTEX-2_NV	N151	T264	n.a.	n.a.	P354	L524
RTEX-1_NV	Y151	E261	n.a.	n.a.	N369	G540
RTEX-14_BF	V266	G429	n.a.	n.a.	E496	A664
RTEX-3_BF	Q292	T448	n.a.	n.a.	E529	P697
RTEX-11_BF	D306	K436	n.a.	n.a.	E505	I673
RTEX-5_BF	T276	S382	n.a.	n.a.	Q469	S638
RTEX-2_BF	D356	S411	n.a.	n.a.	D525	S692
RTEX-1_BF	n.a.	n.a.	n.a.	n.a.	V001	P163
RTEX-10_BF	E331	V462	n.a.	n.a.	R550	K722
RTEX-4_BF	N332	C439	n.a.	n.a.	R526	D697
RTEX-1_SK	I284	A447	n.a.	n.a.	Q481	P640
Neptune1_Ren	n.d.	n.d.	n.a.	n.a.	S383	S538
BRIDGE1_FR	n.d.	n.d.	n.a.	n.a.	K242	T399

n.a. = not applicable, n.d. = not detectable

a, amino acid numbers are counted from the last preceding stop codon

b, positions of coiled coil domains (CC) determined according to COILS (70)

c, positions of arginine-rich motifs (ARM) determined by local sequence alignment of elements from the CR1 clade alone

d, positions of esterase domains (ES) determined from the sequence alignment in Supplementary Figure S1

Table S2

Data collection and refinement statistics for the ZfL2-1 ORF1p coiled coil domain

Dataset	**native**	
Data collection		
Wavelength, Å	0.97138	
Resolution Range, Å	37 - 1.55	
Space Group	$P2_1$	
Unit Cell		
dimensions (a / b / c), Å	23.7 / 52.4 / 51.8	
angles (α / β / γ), °	90 / 99.8 / 90	
R_{merge}, %	5.5	(47.4)*
Completeness, %	95.6	(93.3)*
Multiplicity	2.5	(2.5)*
I / σ(I)	10.3	(2.0)*
Refinement		
R_{work}, %	18.6	
R_{free}, %	21.5	
Number of reflections	17443	
Number of molecules per asymmetric unit		
protein molecules	4	
atoms (excluding water)	1143	
water molecules	116	
ligand atoms	30	
Average B-factor (isotropic), $Å^2$	25.8	
Ramachandran plot		
most favored regions, %	100	
disallowed regions, %	0	
R.m.s.d. from ideal geometry		
bond lengths, Å	0.013	
bond angles, °	1.39	

* Values in parentheses correspond to those in the outer resolution shell (1.59 Å - 1.55 Å)

Table S3

Data collection and refinement statistics for the ZfL2-1 ORF1p esterase

Dataset	**native**		**$KAu(CN)_2$**	
Data collection				
Wavelength, Å	1.0000		1.0397	
Resolution Range, Å	46 - 2.50		47 - 2.58	
Space Group	$I4_1$		$I4_1$	
Unit Cell				
dimensions (a / b / c), Å	111.3 / 111.3 / 115.7		110.9 / 110.9 / 116.8	
angles (α / β / γ), °	90 / 90 / 90		90 / 90 / 90	
R_{merge}, %	6.6	(66.3)*	8.6	(57.2)*
Completeness, %	100	(100)*	99.4	(93.6)*
Completeness (anomalous), %			96.6	(89.9)*
Multiplicity	6.9	(7.1)*	6.4	(6.4)*
Multiplicity (anomalous)			3.4	(3.4)*
I / σ(I)	19.1	(3.0)*	13.3	(2.6)*
Refinement				
R_{work}, %	16.7			
R_{free}, %	21.0			
Number of reflections	24346			
Number of molecules per asymmetric unit				
protein molecules	3			
atoms (excluding water)	4025			
water molecules	74			
Average B-factor (isotropic), $Å^2$	51.6			
Ramachandran plot				
most favored regions, %	96.1			
disallowed regions, %	0.0			
R.m.s.d. from ideal geometry				
bond lengths, Å	0.011			
bond angles, °	1.18			

* Values in parentheses correspond to those in the outer resolution shell (2.56 Å - 2.50 Å)

Figure S1

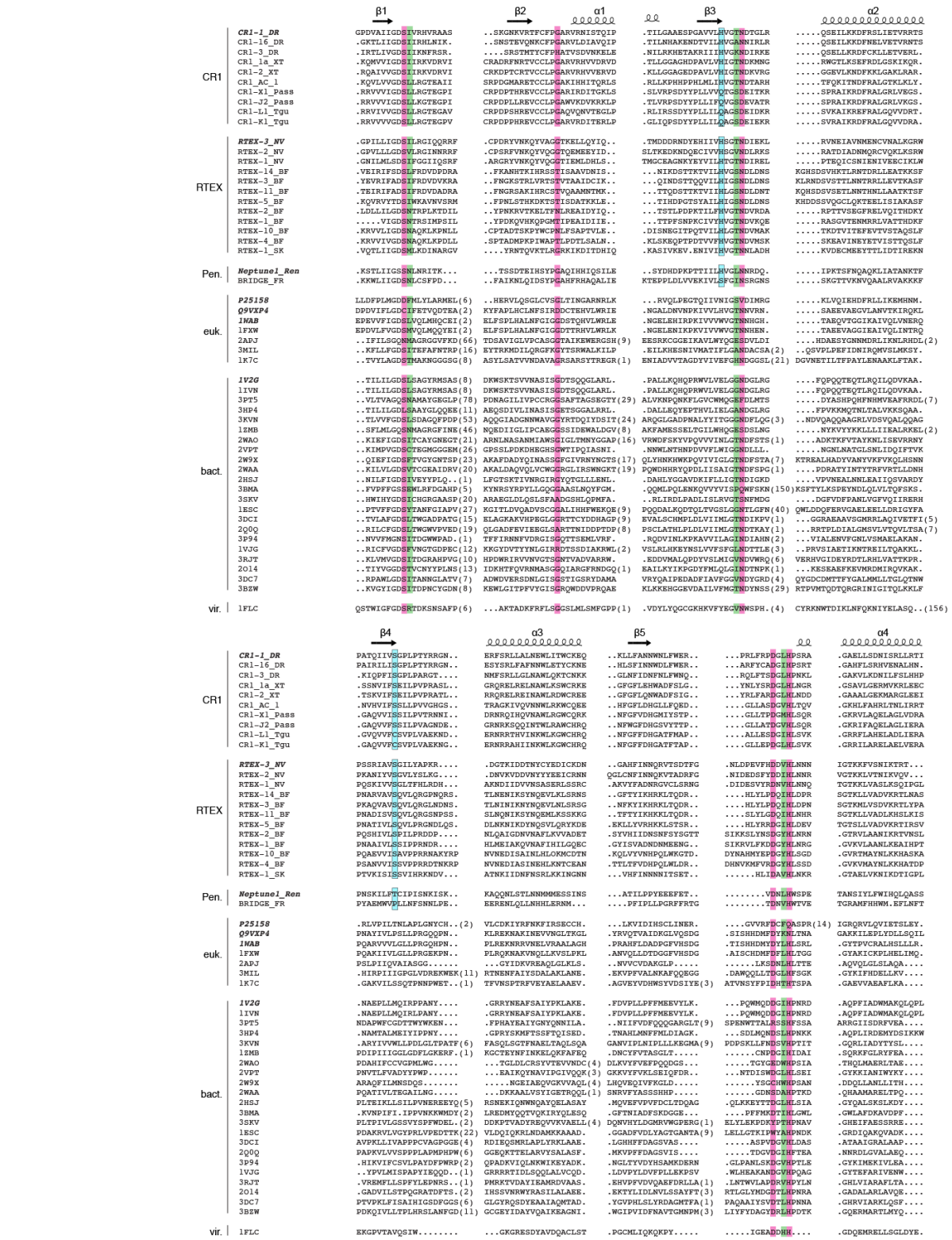

Figure S1. Extended structure-based sequence alignment of esterases from non-LTR retrotransposons with other SGNH proteins.

Esterases from non-LTR retrotransposons are listed by their RepBase ID (46), crystallized SGNH proteins by their PDB ID, and other, non-catalytic SGNH proteins by their Uniprot ID. Non-LTR retrotransposon clades are indicated: CR1, RTEX, and Penelope (Pen.). Animal phyla include chordates (BF, *Branchiostoma floridae*; DR, *Danio rerio*; FR, *Takifugu rubripes*; XT, *Xenopus tropicales*; AC, *Anolis carolinensis*; Pass, *Passeriformes*; Tgu, *Estrildidae*), hemichordates (SK, *Saccoglossus kowalevskii*), cnidarians (NV, *Nematostella vectensis*), and sponges (Ren, *Amphimedon queenslandica*). The other SGNH proteins are grouped according to their origin as eukaryotic (euk.), bacterial (bact.), and viral (vir.). Identifiers of sequences used in Figure 1C are highlighted in bold italics: CR1, CR1-1_DR (ZfL2-1); RTEX, RTEX-3_NV; Penelope, Neptune1_Ren; TAP, 1V2G (*Escherichia coli*); PAF-AH, 1WAB (*Bos taurus*); Oskar, P25158 (*Drosophila melanogaster*). Additional, non-catalytic SGNH proteins are PAF-AH homologs from insects (57), represented by the sequence from *Drosophila melanogaster* (Q9VXP4). The secondary structure assignment corresponds to the crystal structure of the ZfL2-1 esterase. Longer insertions are deleted, including the large insertion between the two half-domains of the viral esterases (number of residues in brackets). Catalytic residues are boxed in magenta, positions of gating residues in green, and transposon-specific positions in cyan.

Figure S2

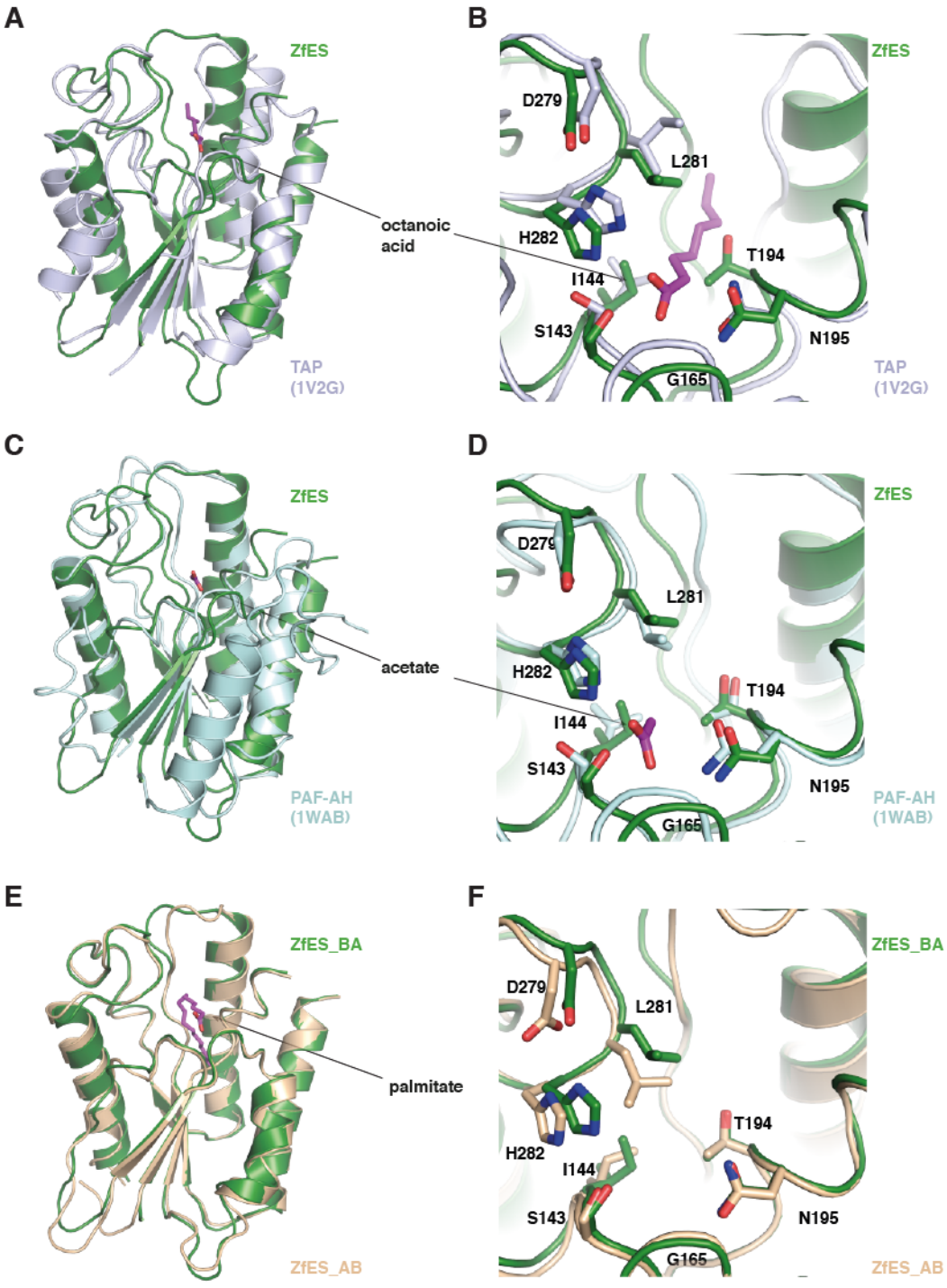
A
ZfES
octanoic acid
TAP (1V2G)
B
ZfES
D279
L281
H282
T194
I144
S143
N195
G165
TAP (1V2G)
C
ZfES
acetate
PAF-AH (1WAB)
D
ZfES
D279
L281
H282
T194
I144
S143
N195
G165
PAF-AH (1WAB)
E
ZfES_BA
palmitate
ZfES_AB
F
ZfES_BA
D279
L281
T194
H282
I144
S143
N195
G165
ZfES_AB

Figure S2. Structural comparison of the ZfL2-1 esterase

(**A, B**) Superposition of the ZfL2-1 esterase (ZfES, green) with the TAP protein from *Escherichia coli* (PDB ID: 1V2G) (48). The overview (A) results from a structure-based DALI search that produced a Z-score of 16.8 and an r.m.s.d. value of 2.6 Å over 178 C_α atoms. The details of the active site (B) reveal the structural conservation of the catalytic residues and the accommodation of an octanoic acid (magenta) in TAP together with the orientation of the gating residues.

(**C, D**) Superposition of the ZfL2-1 esterase (ZfES, green) with the PAF-AH protein from bovine brain cells (PDB ID: 1WAB) (49) The overview (C) results from a structure-based DALI search that produced a Z-score of 19.6 and an r.m.s.d. value of 2.0 Å over 212 C_α atoms. The details of the active site (D) reveal the structural conservation of the catalytic residues and the accommodation of an acetic acid (magenta) in PAF-AH together with the orientation of the gating residues.

(**E, F**) Superposition of two crystallographically independent monomers of the ZfL2-1 esterase (ZfES_BA from Figure 3A, green, and ZfES_AB from the same domain-swapped dimer, Figure 3B). The overview (E) reveals regions of conformational plasticity and corresponds to an r.m.s.d. value of 1.22 Å over 167 C_α atoms. The modeled palmitate (Figure 3F, magenta) is included for orientation. The details of the active site (F) illustrate its flexibility, in particular of the gating residues I144 and L281.

Residues are shown as sticks with oxygens in red and nitrogens in blue.

Figure S3

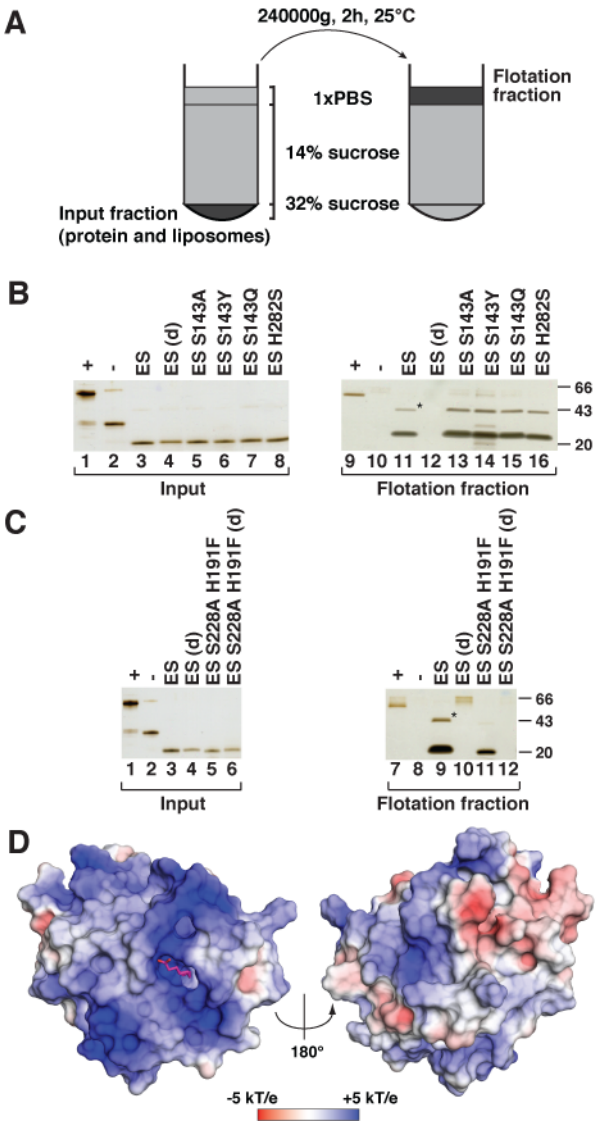
A
240000g, 2h, 25°C
Flotation fraction
1xPBS
14% sucrose
32% sucrose
Input fraction
(protein and liposomes)
B
+
-
ES
ES (d)
ES S143A
ES S143Y
ES S143Q
ES H282S
1 2 3 4 5 6 7 8
Input
9 10 11 12 13 14 15 16
Flotation fraction
66
43
20
C
ES
ES (d)
ES S228A H191F
ES S228A H191F (d)
1 2 3 4 5 6
Input
7 8 9 10 11 12
Flotation fraction
D
180°
-5 kT/e
+5 kT/e

Figure S3. Liposome flotation assay

(**A**) Scheme of the flotation assay (32,71). Protein and liposomes are gently mixed and agitated (30 min, 25°C, 600 rpm), and brought to a sucrose concentration of 32 % in a centrifugation tube (d = 11 mm, 1 x PBS, 2.5 - 5 µM protein, 150 µl). This input fraction is overlaid in two steps by a 14 % sucrose cushion (2 x 850 µl) and topped by a layer of 1 x PBS (300 µl). Following a centrifugation step (2 h, 25° C, 240000 g), protein from the top, flotation fraction (300 µl) contains liposomes that rose through the sucrose gradient, together with potentially attached lipid binding proteins.

(**B, C**) Characterization of the liposome binding properties of the ZfL2-1 esterase. Silver-stained gels show input samples (left panel, 20 %) and samples recovered from the floating liposomes (right panel). Positive (+) controls (Nup133, residues 67-514) and negative (-) controls (GST) are as in Figure 4B. Single point mutations of catalytic residues do not affect liposome binding. Compare lanes 3, 5, 6, 7, 8 with lanes 11, 13, 14, 15, 16 in panel (B). Similarly, a double mutation of the transposon-specific positions does not significantly affect liposome binding either. Compare lanes 3, 5 with lanes 9, 11 in panel (C). However, the interaction with liposomes depends on the structural integrity of the ZfL2-1 esterase and not only on its charge, because heat-denaturation (d) abolishes the interaction. Compare lanes 3, 4 with lanes 11, 12 in panel (B) as well as lanes 3, 4 with 9, 10 and lanes 5, 6 with lanes 11, 12 in panel (C). The asterisk denotes a weak ES dimer in the flotation fraction, a likely gel separation artifact.

(**D**) Electrostatic potential mapped onto the molecular surface of the ZfL2-1 esterase. The modeled palmitate (Figure 3F, magenta) is included for orientation. Potentials are contoured from -5 kT/e (red) to +5 kT/e (blue). Left; top view of the putative membrane binding surface. Right; bottom view. For a side view see Figure 3G.

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48. Lo, Y.C., Lin, S.C., Shaw, J.F. and Liaw, Y.C. (2005) Substrate specificities of Escherichia coli thioesterase I/protease I/lysophospholipase L1 are governed by its switch loop movement. *Biochemistry*, **44**, 1971-1979.

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Chromosoma
DOI 10.1007/s00412 012 0388 3

REVIEW

Building a nuclear envelope at the end of mitosis: coordinating membrane reorganization, nuclear pore complex assembly, and chromatin de-condensation

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Abstract The metazoan nucleus is disassembled and re-built at every mitotic cell division. The nuclear envelope, including nuclear pore complexes, breaks down at the beginning of mitosis to accommodate the capture of massively condensed chromosomes by the spindle apparatus. At the end of mitosis, a nuclear envelope is newly formed around each set of segregating and de-condensing chromatin. We review the current understanding of the membrane restructuring events involved in the formation of the nuclear membrane sheets of the envelope, the mechanisms governing nuclear pore complex assembly and integration in the nascent nuclear membranes, and the regulated coordination of these events with chromatin de-condensation.

Introduction

A functional nucleus relies on the precise structural organization of its genome and the existence of an intact boundary that separates nuclear and cytoplasmic activities, the nuclear envelope (NE). These features are repeatedly established in the mitotically dividing cells of animals. While many lower eukaryotes employ closed or semi-closed mitosis, during which the NE remains at least partially intact (De Souza and Osmani 2009), the onset of mitosis in metazoan cells is marked by dramatic changes to nuclear architecture. Open mitosis requires the complete disassembly of the NE in order to form the mitotic spindle on condensed chromosomes. The consequence of this disassembly is the need to re-build the NE each time the cell divides.

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The NE is composed of two concentric bilayers surrounding the chromatin: the outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum (ER), and the inner nuclear membrane (INM), separated from the ONM by a lumenal space (Fig. 1). These membranes are fused at sites of nuclear pore complex (NPC) integration. NPCs are large protein complexes that contribute to the diffusion barrier of the NE and act as a regulatory gateway for the bidirectional exchange of proteins, RNA, and ribonucleoprotein complexes between the nucleus and the cytoplasm (for review, see Wente and Rout 2010). While the outer membrane is biochemically and functionally similar to the ER, the inner membrane is distinctly characterized by a specific set of integral membrane proteins that establish connections to chromatin and, in metazoan cells, to the overlying nuclear lamina. The lamina is a meshwork of nucleus-specific intermediate filaments called lamins, which maintain the shape and mechanical stability of the nucleus (for review, see Gruenbaum et al. 2005; Shimi et al. 2010). The lamina is also indirectly connected to the cytoplasmic cytoskeleton via linker of nucleoskeleton and cytoskeleton (LINC) complexes that span the NE lumen (for review, see Burke 2012; Starr and Fridolfsson 2010).

Building a nucleus at the end of mitosis involves the complete reconstruction of nuclear membrane sheets and macromolecular NPCs on two sets of de-condensing chromosomes. Here, we review our current understanding of vertebrate NE reassembly as a coordinated process of membrane restructuring, NPC assembly, and chromatin de-condensation.

Re-organizing the mitotic ER

The NE is a distinct domain of the ER, owing to direct and indirect interactions between NE-specific proteins and chromatin. During mitosis, these proteins are released from the

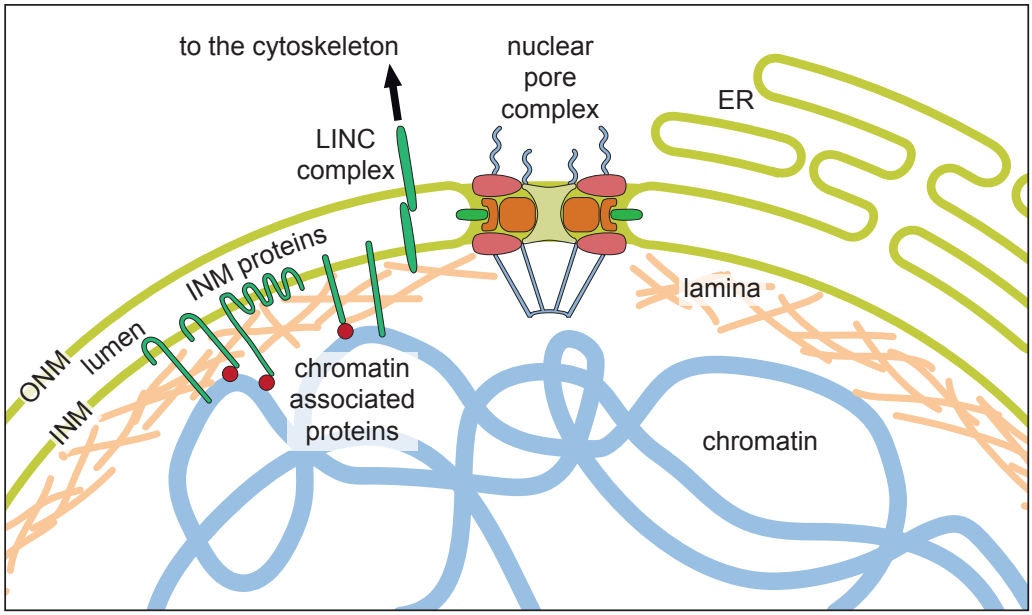

Fig. 1 The vertebrate nuclear envelope. The two membrane sheets of the nuclear envelope are separated by a lumenal space and are contin uous with the bulk endoplasmic reticulum (*ER*) network. The outer nuclear membrane (*ONM*) and the inner nuclear membrane (*INM*) are fused at nuclear pores, where nuclear pore complexes are integrated to regulate bidirectional transport between the cytoplasm and the nucleoplasm. The INM is distinctly characterized by a set of integral membrane proteins that connect the nuclear envelope to chromatin by interacting directly or indirectly via chromatin associated proteins and the nuclear lamina. The nuclear lamina is additionally connected to the cytoplasmic cytoskeleton by the interaction of LINC complex proteins of the ONM and INM across the NE lumen

disassembled lamina and the underlying chromatin, resulting in their redistribution throughout the ER and thus the absorption of the NE membranes in the ER network (Daigle et al. 2001; Ellenberg et al. 1997; Yang et al. 1997). At the end of mitosis, the dramatic reorganization of the mitotic ER gives rise to a new NE forming around each mass of segregating chromatin. The architectural starting point for this ER re-structuring is, however, a matter of debate. In addition to the NE, the entire ER network undergoes significant morphological changes during mitosis. According to two contradictory models, the interphase system of ER sheets and tubules is transformed into either a tubular ER or sheet-like network during mitosis.

The mitotic ER has been observed as an exclusively tubular network (Puhka et al. 2007) and in vitro experiments suggest that an intact tubular ER is required for post-mitotic NE formation (Anderson and Hetzer 2007). This network is recruited via tubule ends that make first contact to the chromatin substrate and become immobilized (Fig. 2a). Subsequent flattening and lateral expansion of membranes on the chromatin surface is proposed to give rise to inner and ONM sheets. In further support of this model, ER tubules have been found to surround post-mitotic chromatin in vivo (Anderson and Hetzer 2008a). Overexpression of reticulons, proteins that shape the ER into tubules, delays NE formation while the depletion of reticulons by siRNA accelerates the formation of a closed NE. These experiments suggest that ER reshaping events, specifically those promoting membrane sheet formation from tubules, are crucial for NE assembly.

Recent live cell imaging and electron microscope tomography studies have provided evidence that NE re-assembly rather initiates from a cisternal, or sheet-like, mitotic ER (Lu et al. 2011) (Fig. 2b). During mitosis, the ER was found to consist almost entirely of extended cisternae, with the exception a few tubules contacting the mitotic spindle (Lu et al. 2009). Cisternal mitotic ER has also been observed in 3D reconstructions of light microscopy sections from *Caenorhabditis elegans* embryos (Poteryaev et al. 2005). The conservation of this ER structure in different cell types and organisms suggests that a sheet-like network could be a general feature of mitotic cells. NE assembly from extended cisternae is initiated by contact between ER sheets and chromatin (Lu et al. 2011). As membrane sheets enclose the chromatin they are organized into a NE-specific domain.

The organization of the interphase ER network varies between cell types and differentiation states (Voeltz et al. 2002). Similarly, the relative abundance of ER sheets and tubules is not the same in all mitotic cells (Puhka et al. 2012). Observations of entirely tubular or cisternal networks might therefore reflect extreme examples on a spectrum of possible mitotic ER arrangements. Assuming that the predominance of mitotic ER sheets and tubules varies between cell types, the question becomes: What is the morphology of the ER that contacts chromatin and gives rise to the sheets of the NE? The transformation of ER tubules into membrane sheets on the chromatin has not been directly visualized (Anderson and Hetzer 2008a). Reticulon-positive membrane tubules have been recorded around the post-mitotic chromatin mass

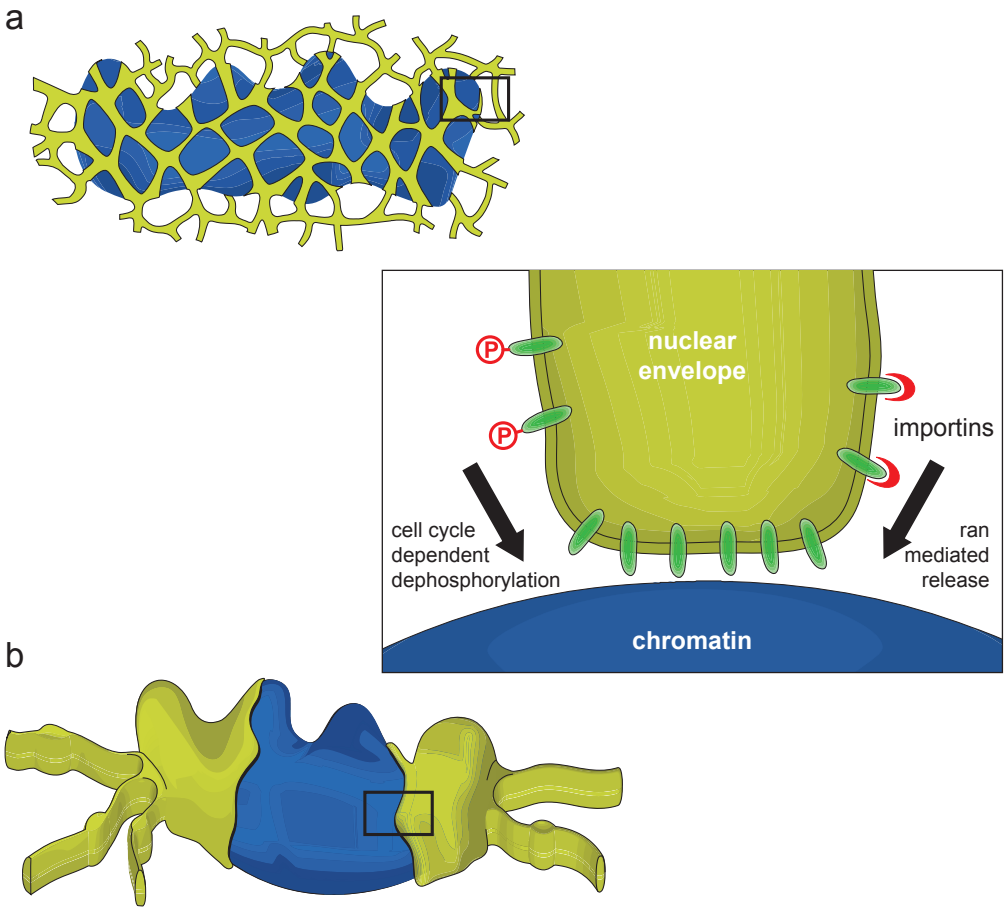

Fig. 2 The nuclear envelope is constructed by the re organization of the mitotic ER on the chromatin. Two models have been proposed to explain nuclear envelope formation based on the predominant organization of the ER during mitosis. In the first model (**a**), a tubular ER network contacts chromatin via tubule ends, which flatten and expand on the chromatin surface to give rise to nuclear envelope sheets. Alter natively, ER derived membrane sheets initiate nuclear envelope formation by associating laterally with the chromatin mass and spreading around it (**b**). In both cases, the regulated recruitment of membrane pro teins of the INM (*inset*, see "Regulating the recruitment of nuclear envelope membranes to chromatin") mediates the accu mulation of nuclear envelope specific membranes and thus the establishment of this distinct ER subdomain

in live cells but in this case the tubules dynamically contact chromatin and do not directly contribute to the NE (Lu and Kirchhausen 2012). It therefore seems likely that the conversion of tubules to cisternal sheets is a prerequisite for the stable association of future NE membranes with chromatin.

Regardless of whether it is initiated by the outgrowth of ER tubules or from cisternal ER sheets, the complete enclosure of chromatin by the NE requires membrane fusion (Fig. 3a). As a subdomain of the ER, it is plausible that the NE employs the ER membrane fusion machinery to achieve this task. Many of the cellular membrane fusion events are mediated by the assembly of SNARE complexes (fo review Jahn and Scheller 2006). Indeed, NE assembly requires NSF and α-SNAP (Baur et al. 2007), fusion factors that activate SNARE proteins (Jahn and Scheller 2006). Integral membrane GTPases of the ER, called atlastins, were recently found to mediate fusion between ER tubules (Hu et al. 2009; Orso et al. 2009). It will be interesting to see if and when atlastins are involved in fusion events necessary for NE reformation. It is currently unknown whether atlastins and the SNAREs involved in ER fusion, such as syntaxin 18 (Hatsuzawa et al. 2000), act cooperatively to form and maintain the membrane network of the ER or whether they mediate distinct fusion events on different types of membranes. Both machineries mediate the approximation and fusion of ER membranes across a cytoplasmic space (Fig. 3a) and are therefore localized to the cytoplasmic side of the respective membranes or in the cytoplasm. The cytoplasmic membrane fusion events required to re-form the NE should be distinguished from the fusion required for NPC assembly into an intact NE, which occurs during interphase and possibly during post-mitotic nuclear formation (Fig. 3b). The nature and localization of the machinery required for fusion between the inner and ONMs during pore insertion have not been identified but might be non-cytoplasmic.

Establishing a NE membrane domain

The NE is rapidly established by the concentration of specific proteins from the mitotic ER network on the decondensing chromatin. In vitro, the recruitment of NE-forming membranes depends on transmembrane proteins (Collas et al. 1996; Newport and Dunphy 1992; Wilson and Newport 1988). Integral proteins of the INM including LBR (Collas et al. 1996; Pyrpasopoulou et al. 1996; Ye and Worman 1994), and the LEM-domain containing proteins Lap2β (Foisner and Gerace 1993; Furukawa et al. 1997), MAN1/LEMD3 (Liu et al. 2003) and emerin (Hirano et al. 2005) bind chromatin. The nucleoplasmic domain of LBR

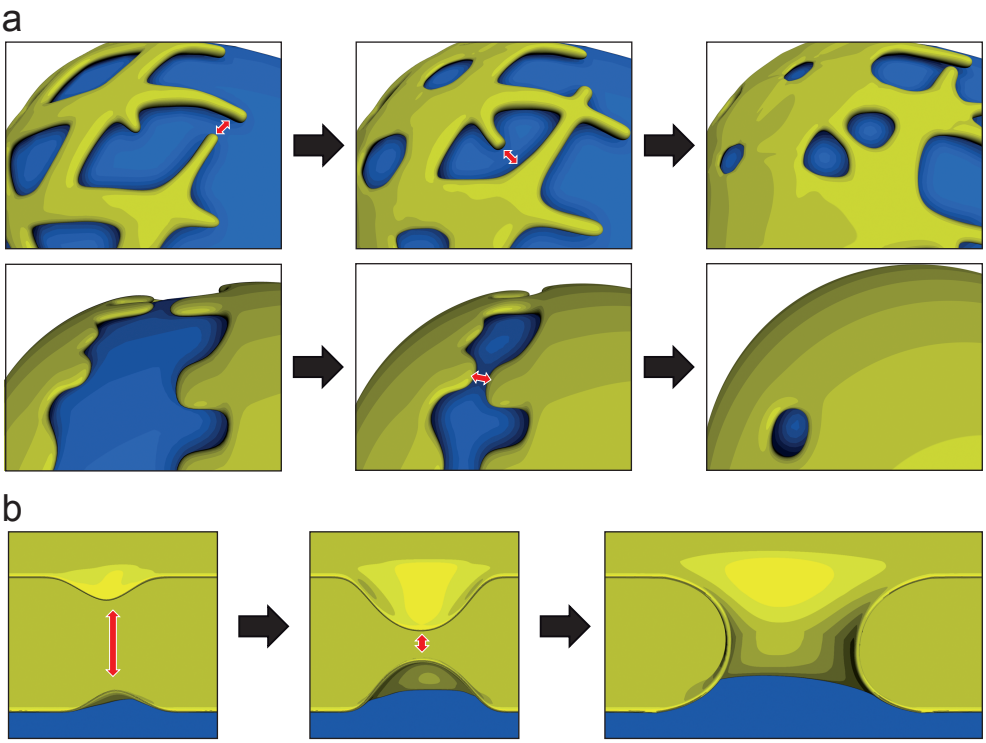

Fig. 3 Membrane fusion is required for nuclear envelope formation. Cytoplasmic fusion between outgrowing ER derived tubules (**a**, *upper*) or sheets (**a**, *lower*) is required for re assembly of a nuclear enve lope around the chromatin mass at the end of mitosis. A second type of fusion between the outer and INMs across the lumenal space is required to create a pore in the intact nuclear envelope (**b**) for the insertion of NPCs during interphase and possibly post mitotically

interacts with heterochromatin-binding protein (HP1) (Ye et al. 1997), while the LEM domain-containing proteins interact with the chromatin-associated protein barrier to autointegration factor (BAF) (see Brachner and Foisner 2011). Several proteins of the INM, as well as the transmembrane nucleoporins NDC1 and POM121, also possess intrinsic DNA-binding capacities based on the presence of basic domains (Ulbert et al. 2006b). The use of multiple chromatin interaction strategies by the INM proteins could at least partially account for the rapid accumulation of membranes on chromatin at the onset of anaphase.

With the exception of LBR, for which contradicting results have been reported (Anderson et al. 2009; Lu et al. 2010), none of the INM proteins are essential for nuclear reassembly in vivo. The depletion of individual INM proteins delays but does not inhibit NE formation in cultured cells and co-depletion of multiple INM proteins or depletion of the chromatin factor BAF, exacerbates the delay (Anderson et al. 2009), suggesting that the chromatin-binding NE proteins could play a redundant role in NE membrane recruitment. Furthermore, removing one INM protein, Lap2β, does not affect the distribution of another, LBR, despite delaying NE formation, implying that the recruitment of various nuclear membrane proteins is not only redundant but also cooperative towards NE assembly.

In addition to chromatin binding by INM proteins, the formation of membrane micro-domains has been proposed to support the segregation of NE membranes from the bulk ER (Mattaj 2004). A number of in vitro experiments in different experimental systems have revealed specific pools of membrane vesicles with the capacity to bind chromatin and give rise to a NE (Antonin et al. 2005; Buendia and Courvalin 1997; Chaudhary and Courvalin 1993; Collas et al. 1996; Ulbert et al. 2006b; Vigers and Lohka 1991; Vollmar et al. 2009). Although these NE membrane populations are likely to originate during the process of their isolation and fractionation when the mitotic ER vesiculates, membrane micro-domains have been found to segregate into distinct vesicles (Simons and Toomre 2000). It is therefore possible that the identification of NE-specific vesicles reflects the existence of micro-domain organization within the seemingly homogeneous mitotic ER.

The existence of NE-specific lipid rafts within the ER is unlikely given the low relative abundance of cholesterol at these membranes. However, the possibility that distinct lipid compositions contribute to functional partitioning at the NE, in analogy to the mitochondria-associated ER membrane (Fujimoto and Hayashi 2011), cannot be excluded. In support of this notion, NE vesicles isolated from sea urchin egg extracts are specifically enriched in phosphoinositides (Larijani et al. 2000), which confer a unique level of fluidity at the membrane (Zhendre et al. 2011). It should be noted that sea urchin pronucleus formation differs significantly from nuclear assembly in vertebrates (Collas 2000) and distinct lipid compositions have not been detected in vertebrate NE membranes to date.

In addition to lipid-mediated domain organization, membrane coating proteins have been proposed to function in micro-domain formation at different endosome compartments (Zerial and McBride 2001). If an analogous strategy

is employed by the NE, lamins could represent attractive candidates for the coating protein component. Several INM proteins interact with lamin B (see Wilson and Foisner 2010 for a comprehensive review), which can be found on mitotic ER-derived membrane vesicles (Chaudhary and Courvalin 1993; Gerace and Blobel 1980). However, despite recent advances in the study of membrane micro-domains (Simons and Gerl 2010), there is no direct evidence for NE subdomain formation in the ER, nor is it clear that such domain organization would impact NE reformation.

Regulating the recruitment of NE membranes to chromatin

Nuclear membranes first re-associate with chromatin during the late stages of anaphase (Daigle et al. 2001; Ellenberg et al. 1997; Robbins and Gonatas 1964). This recruitment can be artificially accelerated in vivo by overexpressing chromatin-binding membrane proteins, or by depleting reticulons to alter ER organization (Anderson et al. 2009). In both cases, premature NE formation interferes with chromosome segregation underlining the importance of robust temporal coordination between chromatin and nuclear membrane dynamics during the cell cycle.

Phosphorylation of nuclear lamins (Heald and McKeon 1990; Peter et al. 1990) and INM proteins (Foisner and Gerace 1993; Pyrpasopoulou et al. 1996) initiates disassembly of the NE at the onset of mitosis. The major driving force of mitotic phosphorylation, cdk1-cyclin B, has been found to inhibit the association of membranes with post-mitotic chromatin in vitro (Newport and Dunphy 1992; Pfaller et al. 1991), likely via one or several downstream kinases (Newport and Dunphy 1992; Vigers and Lohka 1992). If mitotic phosphorylation prevents the association of membranes with chromatin, the process must be reversed at the end of mitosis (Fig. 2, inset). Indeed, membranes isolated from mitotic *Xenopus* egg extracts, containing active cdk1-cyclin B, can be induced to bind chromatin when they are first incubated with interphase cytosol (Ito et al. 2007). This shift in membrane affinity for chromatin is due to the activity of phosphatases, such as PP1 (Ito et al. 2007; Pfaller et al. 1991).

The target of mitotic phosphorylation events that regulate membrane recruitment is on the membranes and not the chromatin (Pfaller et al. 1991). In vitro experiments using protein-free liposomes imply that lipid recruitment to chromatin could be specifically regulated during the cell cycle (Ramos et al. 2006). However, biological membranes are covered with proteins, largely due to mosaics of transmembrane proteins and their interaction partners, with relatively little area of exposed lipids (Dupuy and Engelman 2008; Takamori et al. 2006). Thus although regulation at the lipid surface may be a contributing factor, it is more likely that the cell cycle-dependent recruitment of membranes to chromatin is mediated by the integral nuclear membrane proteins.

Two INM proteins that are recruited quickly following the onset of anaphase, Lapβ and LBR, are phosphorylated during mitosis, preventing their association with chromatin (Foisner and Gerace 1993; Ito et al. 2007; Courvalin et al. 1992). The precise regulation of LBR by mitotic phosphorylation is particularly well studied. In post-mitotic extracts, an arginine-serine repeat-containing region of LBR mediates its recruitment to chromatin (Takano et al. 2002). Phosphorylation of a specific serine residue within this domain prevents LBR binding to chromatin in vitro (Ito et al. 2007; Nikolakaki et al. 1997; Takano et al. 2004) and its de-phosphorylation controls the timing of ER membrane recruitment to anaphase chromosomes in human cells (Tseng and Chen 2011). Given the redundancy of INM protein recruitment, it is likely that other integral NE proteins are regulated similarly. In fact, the pore membrane proteins NDC1, POM121, and GP210 as well as the INM proteins emerin and MAN1 are also phosphorylated during mitosis (Dephoure et al. 2008; Mansfeld et al. 2006; Ellis et al. 1998; Favreau et al. 1996), but the significance of these events with regard to nuclear membrane recruitment is unclear.

Although exit from mitosis is characterized by an overall decrease in phosphorylation, the in vitro association of LBR with chromatin also requires specific phosphorylation events, which are mediated by serine/arginine-rich protein-specific kinase 1 (Nikolakaki et al. 1997; Takano et al. 2002; Dreger et al. 1999). Similarly, Lap2β is phosphorylated within its chromatin-binding region during interphase (Dreger et al. 1999). These observations suggest that a simple model of mitotic phosphorylation and post-mitotic de-phosphorylation cannot account for the precise timing of membrane recruitment to chromatin but rather that multiple site-specific phosphorylation events tune this process.

In addition to cell cycle-dependent phosphorylation events, transport receptors and the GTPase ran may regulate the association of INM proteins with chromatin. Chromatin is demarcated throughout the cell cycle by a high concentration of the GTP-bound ran (Kalab et al. 2002). Ran is best known for its function in nucleo-cytoplasmic transport across the NPC, where it stimulates the release of importin-bound cargo in the nucleus, but it is also required for nuclear assembly in vitro (Hetzer et al. 2000; Zhang and Clarke 2000), where it provides the positional information necessary to specify that nuclear assembly occurs on the decondensing chromatin (for review, see Hetzer et al. 2002). Integral membrane proteins can be targeted to the interphase NE via importins (Doucet et al. 2010; Turgay et al. 2010), and it is possible that the ran-importin system could similarly regulate the recruitment of INM proteins to post-mitotic chromatin (Turgay et al. 2010; Antonin et al. 2011) (Fig. 2, inset). In agreement with this notion, LBR was found to interact with importin β during mitosis (Ma et al.

2007; Lu et al. 2010) and this inhibitory complex could be dissociated in the presence of ranGTP (Ma et al. 2007). The importin β and chromatin-binding sites of LBR overlap, thus it is conceivable that members of the importin family act as molecular chaperones to prevent undesired interactions between the DNA-binding domains of INM proteins and chromatin during mitosis.

It has also been suggested that importins mediate the recruitment of NE membranes to chromatin by bridging the membrane precursors and either ran (Ma et al. 2007) or NLS-containing chromatin proteins (Lu et al. 2012). This model requires a stable interaction between ran and importins, which is difficult to rectify with the ran-dependent dissociation of importin-cargo complex during nuclear import. Furthermore, it is not clear how a non-canonical importin interaction with two binding partners is established. Nonetheless, the contribution of NLS-containing chromatin proteins could represent an important link between post-mitotic chromatin structure and membrane recruitment and warrants further investigation.

In summary, the timing of nuclear membrane recruitment to chromatin is regulated by the reversal of mitosis-specific phosphorylation events on nuclear membrane proteins. With few exceptions, the relevant target proteins, precise sites of modification, and the phosphatases involved have yet to be identified. Spatial organization by the ran system might contribute to nuclear membrane recruitment by exposing DNA-binding domains of membrane proteins in the vicinity of chromatin but the relevance of such a mechanism has not been determined.

Building NPCs in the NE: post-mitotic assembly modes

As the ER membranes are reorganized to accommodate the distinct composition of the NE and enclose the chromatin, the coordinated assembly of NPCs begins. NPCs form large pores in the NE, having a diameter of approximately 100 nm. Unlike membrane transporters, which give rise to channels within a single lipid bilayer, NPCs span two lipid bilayers at sites where the outer and inner membranes of the NE are fused. As a result, only a small sub-fraction of the roughly thirty NPC proteins (nucleoporins) are integral membrane proteins residing in the pore membrane. Most nucleoporins do not possess membrane-spanning domains and are thus recruited from the cytosol to assemble NPCs at the conclusion of open mitosis in animals.

Post-mitotic NPC assembly has been proposed to proceed via two fundamentally different modes: insertion or enclosure. In an insertion model (Fig. 4a), NPCs assemble and integrate into the two juxtaposed membrane sheets of the intact NE (Fichtman et al. 2010; Lu et al. 2011; Macaulay and Forbes 1996). Formation of the pore requires the fusion of the outer and INMs across the lumen of the NE

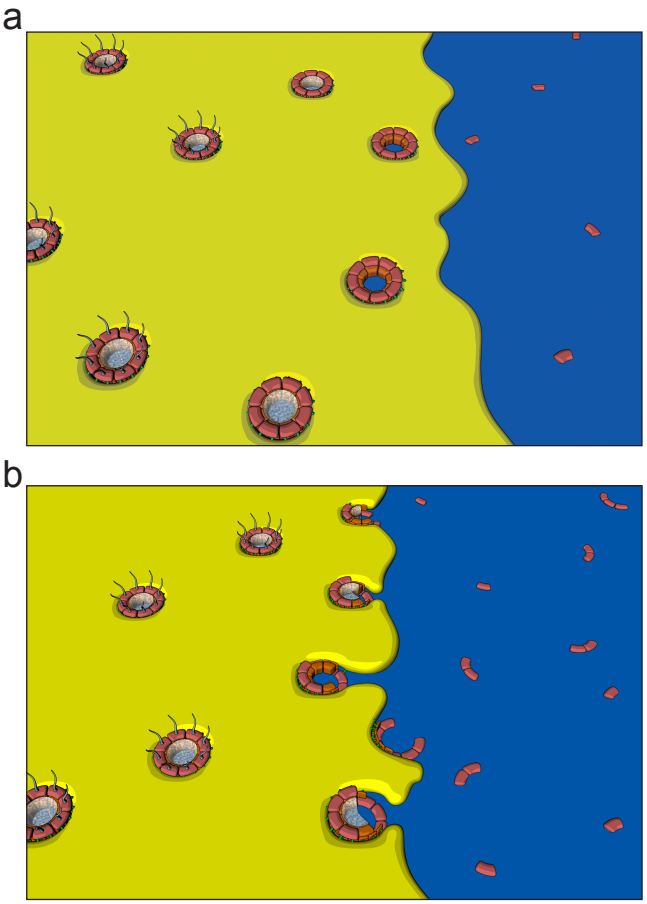

Fig. 4 Post mitotic NPC assembly as envisioned by the insertion and enclosure models. As the cisternal sheets of the nuclear membrane wrap around chromatin, NPC assembly proceeds by either insertion into the locally intact nuclear envelope (**a**) or by enclosure of NPC assembly intermediates by the outgrowing membranes (**b**). In both cases, NPC assembly is initiated by the Mel 28/ELYS dependent recruitment of the Nup107 160 complex to chromatin. Following the initial contact between nuclear membranes and the Nup107 160 complex, additional nucleoporins are incorporated in the assembling NPCs (see also Fig. 5 for details)

(see Fig. 3b). In dividing metazoan cells the number of NPCs roughly doubles during interphase (Dultz and Ellenberg 2010; Maul et al. 1972; Doucet and Hetzer 2010), when NPCs must be formed by insertion into the intact NE. Furthermore, organisms that employ closed mitosis for cell division, such as yeast, can only assemble new NPCs by insertion into the intact nuclear membranes (Rexach 2009; Winey et al. 1997). Thus, an insertion model represents a unifying mechanism for NPC assembly across species and in all stages of the cell cycle.

In contrast to interphase NPC assembly, which occurs as a collection of singular and sporadic events, the post-mitotic assembly of thousands of NPCs in metazoan cells proceeds simultaneously and rapidly, on average one order of magnitude faster, in order to quickly re-establish nuclear compartmentalization (Dultz and Ellenberg 2010; D'Angelo et al.

2006; Dultz et al. 2008). The distinct kinetics of post-mitotic NPC formation could be explained by the use of a mechanistically unique assembly mode. Enclosure models suggest (Antonin et al. 2008; Burke and Ellenberg 2002; Walther et al. 2003a) that post-mitotic NPC assembly following open mitosis does not occur by insertion into intact membrane sheets but is rather completed by the envelopment of the assembling NPCs on the chromatin surface by the outgrowing ER-derived membranes (Fig. 4b). In this case, NPC assembly is initiated by the recruitment of the Nup107-160 complex to chromatin, which has been observed in vitro (Walther et al. 2003a) and in vivo (Dultz et al. 2008; Belgareh et al. 2001). Membranes are subsequently recruited, resulting in the enrichment of NE-specific membrane proteins, including the integral membrane nucleoporins POM121 and NDC1 (Antonin et al. 2005; Mansfeld et al. 2006; Rasala et al. 2008). Kinetic analyses of individual NPC proteins suggest that the ordered recruitment of NE components at the end of mitosis is distinct from interphase pore assembly, where POM121 gradually accumulates prior to the recruitment of the Nup107-160 complex (Doucet et al. 2010; Dultz and Ellenberg 2010). This reversal of recruitment events implies that post-mitotic NPC assembly is initiated on the chromatin, as the enclosure model proposes, while interphase insertion of NPCs begins on the nuclear membranes.

The DNA-binding protein Mel-28/ELYS recruits the Nup107-160 complex and acts as a seeding point for post-mitotic NPC assembly (Franz et al. 2007; Gillespie et al. 2007; Rasala et al. 2008). In agreement with the notion of chromatin-directed NPC assembly at the end of mitosis, Mel-28/ELYS is essential for post-mitotic NPC formation but is dispensable to this end during interphase (Doucet et al. 2010). Conversely, the transmembrane nucleoporin POM121 is proposed to be specifically required for interphase NPC assembly, where it initiates pore formation on the membranes. However, it should be noted that the dispensability of POM121 for the formation of NPCs at the end of mitosis (Doucet et al. 2010) is not a consistent observation in the field (Antonin et al. 2005; Shaulov et al. 2011) and could be attributed to an incomplete depletion, resulting in a small pool of residual POM121 that was sufficient for post-mitotic assembly but completely consumed when nuclei reached interphase.

The existence of membrane intermediates specific to post-mitotic and interphase pore formation can be inferred from differences in the requirement of membrane bending and curvature-sensing proteins. Our recent work demonstrates distinct functions of the membrane-associated nucleoporin Nup53, which are essential for pore formation post-mitotically or during interphase (Vollmer et al. 2012). While either of the two Nup53 membrane-binding sites is sufficient for post-mitotic NPC assembly, interphase assembly specifically requires the second binding site at the C terminus. As the C-terminal-binding site was also found to induce membrane curvature, this could indicate that a unique membrane deformation activity is required for interphase pore assembly. Similarly, ER-bending proteins of the reticulon family that induce convex membrane curvature (Hu et al. 2008) were found to be important for NPC assembly into the intact NE both in yeast and vertebrates (Dawson et al. 2009). It is difficult to ascertain whether reticulons also contribute to post-mitotic NPC assembly because their role in ER membrane reorganization at the end of mitosis is a prerequisite for NE reformation (Anderson and Hetzer 2008a). Interestingly, a membrane curvature sensing domain of the Nup107-160 complex member Nup133 was found to be required for interphase but not post-mitotic assembly (Doucet and Hetzer 2010). It is possible that specific membrane curvature events are required during the insertion of interphase NPCs when the two nuclear membranes approximate and fuse (Fig. 3b). Other modes of pore membrane stabilization might be sufficient at the end of mitosis, when NPCs on the chromatin are enclosed by the outgrowing ER.

The existence of cell cycle-dependent differences in the molecular requirements of NPC formation does not unambiguously prove the use of distinct assembly mechanisms. The specific requirement for Mel-28/ELYS during post-mitotic assembly, for example, could rather reflect a need for the efficient recruitment of NPC components during open mitosis when they cannot be enriched in the nucleus by active NPC-dependent import. Assembly of NPCs into an intact envelope requires the Nup107-160 complex on the nucleoplasmic site of the NE (D'Angelo et al. 2006; Walther et al. 2003a). Thus, regardless of the assembly mode employed, NPC components will need to be enriched on the chromatin at the end of mitosis. Similarly, the unique requirement for proteins inducing membrane curvature during interphase NPC formation does not prove the use of dissimilar assembly mechanisms at different points in the cell cycle although it strongly implies distinct modes.

Nuclear formation can be decelerated in *Xenopus* extracts, which are commonly used to recapitulate post-mitotic NPC assembly, by reducing the temperature of the reaction (Fichtman et al. 2010). Under these conditions, a NE intermediate that possesses a closed NE but no pores or NPCs can be observed, suggesting that post-mitotic NPC assembly proceeds by insertion and requires the fusion of outer and INMs. However, the lower temperature might specifically inhibit or delay the post-mitotic mode of assembly, resulting in an artificial bias towards interphase NPC assembly. Recent live cell imaging experiments suggest that the local generation of NE membranes on chromatin from ER cisternae precedes NPC assembly, which would also implicate an insertion mode for post-mitotic NPC assembly (Lu et al. 2011). However, the precise order of recruitment,

particularly with regard to the small number of nucleoporins that might be sufficient to seed NPC assembly is difficult to ascertain. In order to ultimately resolve this issue, it will be crucial to determine whether the hitherto unknown factors mediating fusion of the outer and INM are equally required for interphase and post-mitotic NPC assembly.

Importantly, while the tubular or cisternal organization of the post-mitotic ER recruited to chromatin would appear to favor enclosure or insertion of NPCs, respectively, these structures are in principle compatible with both assembly modes. Although it is easy to imagine how intermediates of NPC assembly are seeded in the gaps of a tubular ER network and enclosed by the flattening and expansion of those membrane areas (Anderson and Hetzer 2008b; Antonin et al. 2008), an ER network on the chromatin surface might first close the gaps to form a closed NE into which NPCs are assembled according to the insertion model. Similarly, outgrowth of flat ER cisternae could first form a closed NE, at least locally, into which NPCs are inserted (Lu et al. 2011) (Fig. 4a). However, it is also possible that the growing sheets of the cisternae enclose assembling NPC intermediates similar to waves flowing around wooden posts on a beach (Fig. 4b).

Assembling NPCs at the end of mitosis: ordered recruitment of nucleoporins

A single vertebrate NPC has a mass of roughly 60MDa, an approximate diameter of 100 nm and consists of multiple copies of 30 unique nucleoporins, which are arranged to give rise to a cylindrical pore with eightfold rotational symmetry (Brohawn et al. 2009). Nucleoporins can be categorized based on their contribution to either the structural scaffold or the transport properties of the NPC. The latter group consists of nucleoporins with phenylalanine-glycine (FG) repeat sequences that mostly occupy the central channel of the pore and contribute to the diffusion barrier and regulated transport capacity of the NPC (Weis 2007). The construction of this macromolecular structure is accomplished by the sequential recruitment of nucleoporins (Bodoor et al. 1999; Dultz et al. 2008; Haraguchi et al. 2000). Immunofluorescence and live cell imaging in cultured mammalian cells, as well as depletion experiments in *Xenopus* egg extracts, have elucidated the order and interdependence of the important recruitment steps (Fig. 5).

Post-mitotic NPC assembly starts on chromatin, where the DNA-binding nucleoporin Mel-28/ELYS recruits the Nup107-160 complex (Franz et al. 2007; Walther et al. 2003a; Rotem et al. 2009; Rasala et al. 2008; Harel et al. 2003). In vitro, these events can occur in the absence of membranes. The subsequent association of the transmembrane nucleoporin POM121 at the newly forming pores (Antonin et al. 2005) is thought to be mediated by direct binding of POM121 to the Nup107-160 complex (Mitchell et al. 2010; Yavuz et al. 2010) and constitutes the first connection between the assembling NPC and nuclear membranes. The transmembrane nucleoporin NDC1 is also

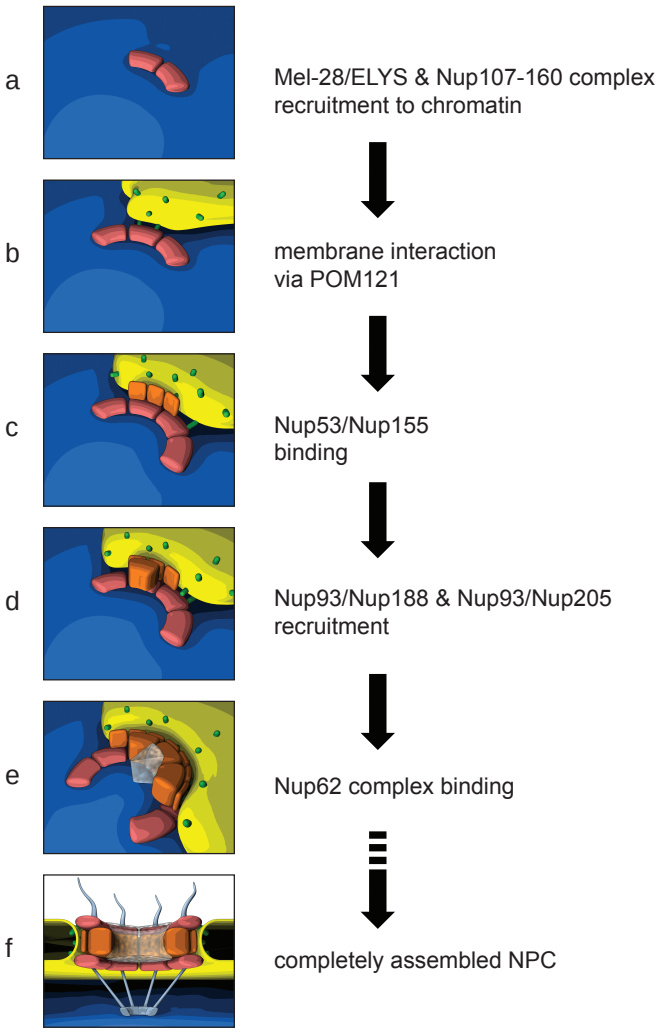

Fig. 5 Model for the ordered assembly of NPCs at the end of mitosis (see text for details and alternative models). The DNA binding nucleoporin Mel 28/ELYS initiates NPC assembly on the chromatin by recruiting the Nup107 160 complex (**a**), which in turn associates with the nuclear envelope membranes via the transmembrane nucleoporin POM121 (**b**). The recruitment of the Nup93 complex is mediated by its membrane associated nucleoporins, Nup53 and Nup155, which interact with integral membrane proteins at the nascent pore membrane (**c**) and promote the incorporation of Nup93, Nup188, and Nup205 to complete the structural backbone of the NPC (**d**). The subsequent recruitment of FG repeat containing nucleoporins of the Nup62 complex (**e**) combined with the previous association Nup98 (not shown) establishes the central channel, a hydrophobic meshwork that confers the transport properties of the NPC. The fully assembled NPC (**f**) consists of multiple copies of the component nucleoporins, which are arranged in octagonal symmetry to create a cylindrical channel. Peripheral structures include the cytoplasmic filaments and the nuclear basket, protruding from opposite faces of the NPC. Initial membrane contact (**b**) is depicted according to the enclosure model. It should be noted that the order of events is the same for both the enclosure and insertion modes of NPC assembly

found at the NE around this time. The steps following membrane recruitment can be ordered in space starting from the membrane and building laterally towards the center of the pore, as was suggested by the protein-protein interaction network of yeast (Rexach 2009). First, the Nup93 complex joins the assembling pore (Dultz et al. 2008). Recent experiments in *Xenopus* egg extracts suggest that the recruitment of the Nup93 complex proceeds by assembly of the individual components rather than by recruitment of the preassembled complex (Sachdev et al. 2012; Theerthagiri et al. 2010; Vollmer et al. 2012). Of these components, Nup53 is the first to associate with the nascent pore, probably followed by Nup155. Both proteins interact with the transmembrane nucleoporins NDC1 and POM121 (Mansfeld et al. 2006; Mitchell et al. 2010; Yavuz et al. 2010) and thus provide a second link between the NPC and membranes at the pore. The capacity for Nup53 to interact directly with membranes may further contribute to the formation or stability of the growing NPC (Vollmer et al. 2012). Nup93 interacts with Nup53 and is consequently incorporated, along with its binding partners Nup188 and Nup205 to complete the structural backbone of the pore. Nup93 subsequently recruits the FG repeat-containing nucleoporins of the Nup62 complex. The FG-containing nucleoporin Nup98 is recruited concomitantly with Nup93 (Dultz et al. 2008) and has recently been found to be key to the establishment of the transport and exclusion properties of the pore (Hulsmann et al. 2012; Laurell et al. 2011). Together, these FG nucleoporins form a substantial part of the hydrophobic meshwork in the center of the pore (Ribbeck and Gorlich 2001).

Open questions remain regarding the construction of a fully assembled NPC. Many nucleoporins, including the Nup107-160 complex, are symmetrically distributed on both the nucleoplasmic and cytoplasmic side of the NPC (Brohawn et al. 2009; Rout et al. 2000; Belgareh et al. 2001) but the timing and mechanistic details regarding assembly of the cytoplasmic portion of the NPC are largely unknown. Similarly, the formation of peripheral NPC structures, such as the nuclear basket and the cytoplasmic filaments, follows the establishment of the structural pore and central channel but the precise order of events is not well defined. Finally, although the complete pore possesses octagonal symmetry, it is not clear whether the numerous copies of each subcomplex are recruited simultaneously. This question is beyond the resolution of current experimental techniques.

Regulating NPC assembly on chromatin at the end of mitosis

Nucleoporins play diverse roles during mitosis (for review, see Chatel and Fahrenkrog 2011) but they do not assemble NPCs until mitotic exit. Multiple nucleoporins, including members of the Nup107-160 complex, Nup98, and Nup53, are phosphorylated by mitotic kinases (Favreau et al. 1996; Glavy et al. 2007; Laurell et al. 2011; Macaulay et al. 1995; Mansfeld et al. 2006; Onischenko et al. 2005), and it is tempting to speculate that mitotic phosphorylation acts as a general mechanism to keep nucleoporins in a dissociated state. Indeed, hyperphosphorylation of Nup98 interferes with its associations at the pore and initiates the disassembly of the NPC at the start of mitosis (Laurell et al. 2011). Conversely, dephosphorylation at the end of mitosis should promote interactions between nucleoporins and thus NPC assembly. In most instances direct evidence for such a mechanism is lacking because the kinases and phosphatases responsible perform a plethora of functions that are essential to mitotic entry, progression, and exit. Furthermore, the identification of decisive phosphorylation events is complicated by a high degree of redundancy. For example, Nup98 is phosphorylated at 13 different sites by cdk1 and members of the NIMA-related kinase family during mitosis (Laurell et al. 2011).

Spatial regulation of NPC assembly on chromatin is provided by the localized concentration of ranGTP (Kalab et al. 2002). The importance of this spatial information is underlined by the aberrant formation of NPCs in ER membrane stacks apart from NE when the ranGTP gradient is disturbed (Walther et al. 2003b). Transport receptors of the importin family bind a large proportion of nucleoporins and have been proposed to regulate the post-mitotic formation of NPCs by blocking the relevant interactions between NPC components (Harel et al. 2003; Walther et al. 2003b). This inhibition is reversed by the ranGTP-dependent release of importin-bound nucleoporins in the vicinity of chromatin, which is required for NPC formation at the NE. MEL-28/ELYS and the Nup107-160 complex represent attractive candidates for such a mode of regulation because they bind transport receptors and associate with chromatin in the early stages of NPC assembly (Walther et al. 2003b; Rasala et al. 2008; Rotem et al. 2009; Franz et al. 2007). However, the functional outcome of transport receptor binding is generally difficult to dissect due to the existence of multiple distinct interaction-dependent activities. Interactions between FG nucleoporins and transportins or importins are required to facilitate transport of cargoes through the NPC, a function that may also extend to other NPC components, such as Nup50 (Lindsay et al. 2002). Several nucleoporins also bind transport receptors in order to be imported to the nucleoplasmic side of the pore, where they contribute to interphase NPC assembly. The integral membrane nucleoporin POM121 is transported in this way (Doucet et al. 2010; Funakoshi et al. 2011) and it is likely that the Nup107-160 complex employs a similar mechanism. Thus, as for the temporal regulation of NPC assembly, challenges still lie ahead in deciphering the molecular mechanisms that control NPC assembly on post-mitotic chromatin.

Unpacking chromatin during mitotic exit

In metazoans, the establishment of an interphase nucleus that is competent for regulated transcription and replication depends on the coordination of chromatin de-condensation and NE formation during mitotic exit. Whereas the molecular mechanisms involved in NE and NPC assembly are beginning to emerge, much less is known about the contemporaneous changes that occur on chromatin at the end of mitosis.

We are only starting to understand the molecular and structural dynamics that determine chromatin organization. This reflects the inherent complications in investigating the complexity of protein-DNA interactions involved in packing DNA molecules into chromatin (Maeshima et al. 2010). As a result, the structural rearrangements and relevant effector molecules that enable the 50-fold compaction of mammalian mitotic chromosomes are far from understood (for review, see Belmont 2006; Ohta et al. 2011).

Chromosomes achieve maximal compaction during anaphase, a feat that requires the chromokinesin KID (Ohsugi et al. 2008) and the mitotic kinase Aurora B at the chromatin (Mora-Bermudez et al. 2007). The de-condensation of mitotic chromosomes during late anaphase of mitosis requires the extraction of polyubiquitinylated Aurora B by the AAA ATPase p97 (Ramadan et al. 2007). The precise consequences of Aurora B inhibition, including the relevant targets of this kinase, and of other p97-dependent activities are not currently understood. In addition to p97, the protein phosphatase PP1 and its nuclear targeting unit PNUTS have been implicated in post-mitotic chromatin de-condensation (Landsverk et al. 2005; Lee et al. 2010). Mitotic exit is generally promoted by the activities of PP1 and PP2A (Wurzenberger and Gerlich 2011) but the molecular targets regulated by these phosphatases, particularly with regard to post-mitotic changes to chromatin structure, are largely unknown.

Coordinating the establishment of the NE and interphase chromatin architecture

Interphase chromatin architecture is not random. Individual chromosomes occupy distinct territories within the 3D organization of the nucleus, which are maintained throughout a lifetime of cell divisions (reviewed in Cremer et al. 2006; Misteli 2007). Furthermore, highly condensed chromatin regions, known as heterochromatin, are predominantly found at the nuclear periphery and have been observed in close proximity to the NE (reviewed in Akhtar and Gasser 2007; Francastel et al. 2000). Proteins of the nuclear lamina, INM, and NPC interact with chromatin during interphase and have been implicated in chromatin organization at the envelope. Our current understanding of how these interactions impact chromatin structure and transcriptional activity are beyond the scope of this review and are discussed thoroughly by others (Capelson et al. 2010; Zuleger et al. 2011; Kubben et al. 2010).

Although many of the interactions between chromatin and proteins at the nuclear periphery are either transient or established following the formation of a closed NE, the broad organization of chromatin in the nucleus must be established as the chromosomes de-condense and could be coupled to NE formation. In support of this hypothesis, common focal points for chromatin condensation and de-condensation have been observed at the nuclear periphery (Hiraoka et al. 1989). Multiple transmembrane proteins of the NE have been found to impact chromatin de-condensation (Korfali et al. 2010; Chi et al. 2007). However, the molecular mechanisms that account for the involvement of NE proteins in chromatin de-condensation have yet to be elucidated.

We are therefore still confronted with the question of how chromatin organization is established at the end of mitosis. A recent study suggests that the peripheral identity of chromatin is maintained throughout the cell cycle (Olins et al. 2011). Peripheral chromatin of both interphase nuclei and mitotic chromosomes, termed epichromatin, can be characterized by presence of a specific nucleosome-based and conformation-dependent epitope. Although the functional significance of epichromatin is currently unclear, it is tempting to speculate that the continuity of peripheral chromatin architecture contributes to the establishment of nuclear organization at the end of mitosis. In this context, epichromatin could provide a scaffold for components of the NE. Phosphatidylserine is associated with histones specifically localized to epichromatin and it might provide a seeding point for nuclear membranes at these defined chromatin regions (Prudovsky et al. 2012). During mitosis, a layer of largely nucleoplasmic proteins and ribonucleoproteins, collectively referred to as perichromatin, associates with non-repetitive DNA sequences at the chromatin periphery (for review, see Hernandez-Verdun and Gautier 1994; Van Hooser et al. 2005), and it is possible that epichromatin also mediates this localization. Importantly, perichromosomal components have been proposed to contribute to the early events of post-mitotic nuclear assembly (Hernandez-Verdun and Gautier 1994).

Structural features of chromatin are often correlated with post-translational modifications to histones. As specific histone phosphorylation and methylation events are reportedly coordinated with the cell cycle (Oki et al. 2007; Markaki et al. 2009), they might contribute to the changes in chromatin structure observed during the cell cycle. However, there is no evidence that histone modifications actually mediate the dramatically altered compaction of chromatin during mitosis. For example, histone H3 phosphorylation at serine 10 is perhaps the most prominent mitotic histone modification but

it is not essential for chromatin condensation in yeast or vertebrates (Hsu et al. 2000; MacCallum et al. 2002). Nonetheless, cell cycle-specific histone modifications could regulate the association of non-histone factors with chromatin.

Two chromatin-binding proteins, HP1 and BAF, provide a link between the de-condensing chromosomes and NE assembly by binding to LBR and LEM domain containing INM proteins, respectively. Interestingly, HP1 binding to chromatin is inhibited by H3 phosphorylation at serine 10 and promoted by methylation at lysine 9 (Fischle et al. 2005; Bannister et al. 2001; Lachner et al. 2001; Hirota et al. 2005). Although LBR can interact directly with histones and with other chromatin-associated proteins (reviewed in Olins et al. 2010), the regulation of HP1 binding by cell cycle-dependent modifications of histone H3 could regulate the post-mitotic association of the INM protein with chromatin. BAF binds to both histone H3 and histone H1 in vitro, which might mediate its interaction with chromatin, but this interaction is not dependent on post-translational histone modifications (Montes de Oca et al. 2005). Instead, BAF has been found to promote the accumulation of interphase histone H3 marks at the end of mitosis (Montes de Oca et al. 2011).

The recruitment of BAF to chromatin occurs in early anaphase and is required for post-mitotic NE assembly (Gorjanacz et al. 2007; Margalit et al. 2005; Segura-Totten et al. 2002; Furukawa et al. 2003). BAF directs the post-mitotic incorporation and interphase distribution of LEM-domain containing proteins, which reciprocally modulate the distribution of BAF during interphase (Haraguchi et al. 2008; Margalit et al. 2007; Ulbert et al. 2006a; Brachner and Foisner 2011). The INM protein LEM4 was recently found to act at the convergence of NE assembly and chromatin structure (Asencio et al. 2012). During mitosis, the association of BAF with chromatin is negatively regulated by vrk1-dependent phosphorylation (Gorjanacz et al. 2007; Nichols et al. 2006), which is reversed by PP2A upon mitotic exit (Asencio et al. 2012). These counteracting events require LEM4 and its interaction with both the kinase and the phosphatase to control BAF-dependent NE assembly on the chromatin. It is currently unclear how the interaction of LEM4 with vrk1 and PP2A is controlled to ensure this regulation. As LEM2 was found to interact with PP1, it will be interesting to determine whether a similar regulatory mechanism is employed and to identify the downstream targets. PP1 has been implicated as a link between chromatin re-organization during mitotic exit and NE reassembly with its regulatory subunit RepoMan (Vagnarelli et al. 2011).

Fluorescence imaging data from human cells indicates that DNA-binding and INM proteins are not recruited uniformly to chromatin at the end of mitosis. From late anaphase until the establishment of an import competent nucleus, the chromatin mass can be divided into two distinct territories. When telophase chromatin is viewed in the axis of the mitotic spindle, the "core" refers the central region at surfaces both proximal and distal to the spindle. Along the same axis, the more peripheral chromatin domain corresponds to "noncore" chromatin. The core region is enriched in A-type lamins and is established by the local accumulation of BAF (Haraguchi et al. 2008). Accordingly, Lap2β and emerin are found at core chromatin in late anaphase before being distributed rather homogeneously at the rim of the completed nucleus (Dabauvalle et al. 1999; Haraguchi et al. 2000). Conversely, LBR is recruited to the noncore region, where nucleoporins and lamin B also accumulate (Chaudhary and Courvalin 1993; Haraguchi et al. 2000, 2008). The DNA-binding nucleoporin MEL-28/ELYS was recently found to control the establishment of these subdomains (Clever et al. 2012), an event that requires the Nup107-160 complex. Thus, the initial stages of NPC formation on chromatin are linked to the establishment of distinct chromatin regions. The importance of these transient chromatin domains in the establishment of a functional nucleus has yet to be determined.

Conclusions

Re-establishing the vertebrate nuclear compartment after mitosis invokes remarkable changes to chromatin structure and ER membrane organization. As chromatin de-condenses, NPCs are assembled and incorporated in the re-forming nuclear membranes to ensure that regulated exchange can occur across the otherwise impermeable nuclear boundary. The construction of a functional nucleus thus requires seamless coordination and multifaceted interactions between membrane, NPC, and chromatin components. Nuclear membranes are segregated from the mitotic ER in anaphase due to interactions between transmembrane proteins destined for the NE with chromatin-associated factors. Assembly of copious NPCs is also initiated on chromatin, but whether NPCs assemble and are inserted into intact NE sheets or are rather enclosed by the re-forming NE remains controversial. Mitotic kinases and phosphatases, along with the activity of the ran system, provide the temporal and spatial cues that control nuclear membrane and NPC protein recruitment and assembly on de-condensing chromatin. The molecular mechanisms underlying the transition of nuclear membranes and NPC components to a post-mitotic state with the capacity to form a NE are beginning to emerge. Comparatively, little is known about the series of structural changes that occur on chromatin during de-condensation and render it competent for the initial recruitment of nuclear membranes and NPC components. The faithful completion of post-mitotic nuclear assembly relies on the coordination

of major NE and chromatin restructuring events as well as the construction of functional NPCs, and it is likely that several mitotic signaling nodes link these processes.

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Review

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The diverse roles of the Nup93/Nic96 complex proteins – structural scaffolds of the nuclear pore complex with additional cellular functions

Abstract: Nuclear pore complexes mediate the transport between the cell nucleoplasm and cytoplasm. These 125 MDa structures are among the largest assemblies found in eukaryotes, built from proteins organized in distinct subcomplexes that act as building blocks during nuclear pore complex biogenesis. In this review, we focus on one of these subcomplexes, the Nup93 complex in metazoa and its yeast counterpart, the Nic96 complex. We discuss its essential function in nuclear pore complex assembly as a linker between the nuclear membrane and the central part of the pore and its various roles in nuclear transport processes and beyond.

Keywords: chromatin segregation; Nic96 complex; nuclear pore complex; nuclear transport; Nup93 complex; spindle assembly checkpoint.

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Introduction

Eukaryotic cells possess a large number and variety of intracellular membranes that define cellular organelles by specific protein compositions and microenvironments. The most prominent organelle is the nucleus, which is already detectable at the level of light microscopy. The nucleus is surrounded by the nuclear envelope, a double membrane barrier that protects the genomic information and separates nuclear processes such as DNA replication and transcription from cytoplasmic ones such as translation. The barrier function of the nuclear envelope demands elaborate transport systems that allow for the controlled exchange between the two separated compartments – the cytosol and nucleoplasm. How transport receptors such as importins and exportins mediate and regulate the transport of proteins and RNAs across the nuclear envelope has been intensively studied and has found its way into cell biology textbooks (for a review see Fried and Kutay, 2003; Tran et al., 2007). Much less is known about the actual transport gates, nuclear pore complexes (NPCs). These huge protein assemblies of around 50 MDa in yeast (Yang et al., 1998; Rout et al., 2000) and around 125 MDa in vertebrates (Reichelt et al., 1990; Ori et al., 2013) reside in the nuclear envelope. Transport gates like ion channels, metabolite translocators or transporters for poly-peptides span the respective membrane to form an aqueous channel within one hydrophobic lipid bilayer. In contrast, NPCs are inserted into the pore formed by two membranes of the nuclear envelope (Figure 1). How the nuclear membranes are shaped to a pore remains mechanistically poorly defined. Furthermore, it is largely unclear to which extent NPC proteins – nucleoporins – are involved in pore formation and stabilization of the highly curved pore membrane (for a review see Antonin, 2009; Rothballer and Kutay, 2013).

Despite their huge size, NPCs are composed of only around 30 different nucleoporins. Because of the eight-fold symmetry of the pore, nucleoporins are present in 8, 16, 32 or more copies per NPC (for a review see Bilokapic and Schwartz, 2012; Grossman et al., 2012; Adams and Wente, 2013). They can be roughly categorized into those forming the scaffold of the pore, the structural nucleoporins, and those responsible for the transport functions of the NPC. The latter contain a high number of phenylalanine glycine (FG) repeats that form a meshwork via cohesive interactions within the pore, which in turn excludes most macromolecules from the passage but allows translocation of import and export receptor-bound targets (for a review see Fried and Kutay, 2003; Wente and Rout, 2010). Thus, these nucleoporins are important for both the exclusion and for the transport capabilities of the pore.

The overall scaffolding structure of the NPC can be seen as a stack of three rings (Figure 1): The outer or

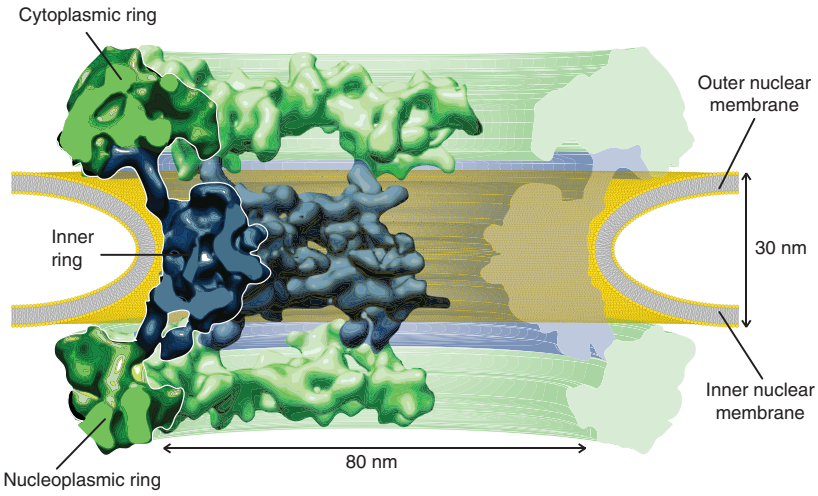

Figure 1 Structural model of the nuclear pore complex.
Nuclear pore complexes localize where the two membranes of the nuclear envelope are fused and form pores. The pore structure is stabilized by protein complexes arranged in concentric rings, which act as a scaffold at the highly bent membrane surface. Only three of the nucleoplasmic, inner and cytoplasmic ring protein complexes making up the repetitive units of the eight-fold symmetry of the nuclear pore complex are shown. Nucleoplasmic and cytoplasmic rings (green) are mainly formed by Nup107/160 (Nup84) complexes. The inner ring (blue) is primarily formed by Nup93/Nic96 complexes. Transmembrane pore proteins and the structures forming the central channel, cytoplasmic filaments and the nucleoplasmic basket are omitted. Adapted from Bui et al. (2013).

cytoplasmic ring is connected to the cytoplasmic filaments whereas the nuclear ring is connected to the nuclear basket. Sandwiched between those two peripheral rings and located in the mid-plane of the nuclear envelope lays the spoke or inner ring. The inner ring is laterally linked to the pore membrane and connected to the central transport channel formed mostly by the FG-repeat-containing nucleoporins. Although NPC dimension and mass vary among organisms this overall structural arrangement, including the eight-fold symmetry, is conserved.

Most structural nucleoporins are part of one of two evolutionary conserved subcomplexes within the pore, which are defined by genetic and biochemical interactions. These subcomplexes are the metazoan Nup107-160 and Nup93 complexes or the respective Nup84 and Nic96 complexes, in yeast. In metazoa, proteins of both subcomplexes have very high resident times at the NPC and are among the longest living proteins in cells (Rabut et al., 2004; Savas et al., 2012; Toyama et al., 2013). The Nup107-160/Nup84 complex constitutes to a large extent the cytoplasmic and nucleoplasmic rings of NPCs (Alber et al., 2007b; Bui et al., 2013). The nine or seven nucleoporins forming the Nup107-160 complex in vertebrates or the Nup84 complex in yeast, respectively, assemble an overall Y-shaped structure with one copy of each nucleoporin per complex (Lutzmann et al., 2002; Bui et al., 2013; Thierbach et al., 2013). A huge number of structural and biochemical studies have defined the protein interaction network within this subcomplex and have led to a model of how the individual nucleoporins and their subdomains, mostly beta-propellers and large alpha-helical repeat regions (Bilokapic and Schwartz, 2012), give rise to the structure (Brohawn et al., 2008). The precise arrangement of the individual subcomplexes in the pore has been recently determined (Bui et al., 2013), resolving a long-standing debate (Leksa and Schwartz, 2010).

The Nup93/Nic96 complex is part of the inner ring (Krull et al., 2004; Alber et al., 2007b) and comparatively less studied than the Nup107-160/Nup84 complex. However, recent years have seen considerable progress in understanding its interaction network and functions, which we will address in this review. The eponymous protein Nic96 was first identified in *Saccharomyces cerevisiae* in a complex with the FG-repeat containing nucleoporins Nsp1, Nup57 and Nup49 (Grandi et al., 1993) that are involved in nuclear transport functions. Nic96 is not an integral component of the Nsp1-Nup57-Nup49-complex but anchors it to the pore (Schlaich et al., 1997). Thus it is reasonable to group Nic96 with its other interacting proteins, which together form a biochemically well-defined and stable complex (Amlacher et al., 2011). The terminus Nup93-complex was first coined to describe the complex formed by Nup93 and Nup205 in vertebrates (Grandi et al., 1997). The latter identified interacting proteins Nup188 and Nup53 (Miller et al., 2000; Hawryluk-Gara et al., 2005) as well as Nup155 are also regarded as

part of this subcomplex (Tran and Wente, 2006; Wente and Rout, 2010; Bilokapic and Schwartz, 2012; Rothballer and Kutay, 2013). We therefore use the term Nup93/Nic96 complex to refer to the subcomplex containing the nucleoporins Nup53, Nup93, Nup155 Nup188, Nup205 in vertebrates and accordingly the same nucleoporins in *S. cerevisiae*, which in some cases occur as two orthologues because of genome duplication (Table 1).

Similar to proteins of the Nup107-160/Nup84 complex, the structural composition of these nucleoporins is rather simple. Vertebrate Nup53, which is also referred to as Nup35, as well as its two yeast homologues Nup53 and Nup59, contain a central noncanonical RNA recognition motif (RRM) domain that mediates dimerization rather than RNA binding (Handa et al., 2006; Vollmer et al., 2012). The remaining parts of the proteins are not structurally resolved. Vertebrate Nup155 and its two yeast homologues Nup170 and Nup157 each possess an N-terminal seven-bladed beta-propeller (Devos et al., 2004; Mans et al., 2004; Seo et al., 2013). The second half of the protein is an α-helical domain where the first 12 α-helices form a curved, overall C-shaped structure followed by an extended superhelical stack (Lutzmann et al., 2005; Flemming et al., 2009; Whittle and Schwartz, 2009). Nup93 or its yeast counterpart Nic96 contains an N-terminal coiled-coil domain (Grandi et al., 1993, 1997) followed by an α-helical region. This domain is not just a simple α-solenoid fold as first suggested, but forms a straight, elongated rod made by an α-helical stack (Jeudy and Schwartz, 2007; Schrader et al., 2008). The largest two proteins of the Nup93/Nic96 complex, Nup205 (Nup192 in yeast) and Nup188, are composed of α-helical tandem repeats forming an α-solenoid like fold (Andersen et al., 2013; Sampathkumar et al., 2013) and show a similar overall structure (Amlacher et al., 2011, Andersen et al., 2013). They might have originated from a gene duplication (Alber et al., 2007b) and should be regarded as orthologues. This fits well with the observation that both proteins interact with Nup93/Nic96 in a mutually exclusive way (Theerthagiri et al., 2010; Amlacher et al., 2011).

Multiple copies of the individual nucleoporins form the huge structural assembly of the pore complex. In *S. cerevisiae,* Nic96 is present in 32 copies per NPC (Alber et al., 2007b). Similarly, each of the orthologous pairs of Nup157/Nup170, Nup59/Nup53 as well as Nup192/Nup188 make up 32 copies. Therefore a 1:1:1:1 stoichiometry within the yeast Nic96 complex is feasible. This stoichiometry is consistent with the fact that a hetero-tetrameric complex consisting of Nup192, Nup170, Nic96 and Nup53 from the thermophile fungus *Chaetomium thermophilum* has been reconstituted *in vitro* (Amlacher et al., 2011). As vertebrate Nup53, and its yeast homologues Nup59 and Nup53, dimerise, it is likely that the actual stoichiometry of the Nic96 complex *is* 2:2:2:2 with 16 copies of each dimer per NPC. It is unclear whether each octameric Nic96 complex contains one copy of each orthologous pair of Nup157/170, Nup53/59 and Nup192/Nup188 or whether other arrangements exist, giving rise to Nic96 complexes within the NPC of slightly different flavors.

In vertebrates, Nup155 and Nup93 are the most abundant nucleoporins, present in 48 copies per NPC (Ori et al., 2013). Nup205 is present in 32 copies and Nup188 in 16 copies per NPC, also adding up to 48 copies per NPC for these orthologues. Nup53 is found in lower numbers making up 32 copies per NPC. Although, inaccuracies in the challenging task determining absolute protein stochiometries within NPCs should be considered, it seems more likely that in vertebrates not all Nup93 complexes possess a relative 1:1:1:1 stoichiometry. In addition, interactions between nucleoporins and therefore the composition of the Nup93/Nic96 subcomplexes might be dynamic within the assembled NPC. An interesting example is Nup53 in *S. cerevisiae*, which shows a stronger interaction with Nup170 during interphase than in mitosis, but conversely a stronger binding to Nic96 in mitosis compared to interphase (Makhnevych et al., 2003).

In summary, the stoichiometry of the Nup93/Nic96 complex nucleoporins within subcomplexes, in contrast to the well-defined Nup107-160/Nup84 complex, is not entirely understood and it is very possible that not all Nup93/Nic96 subcomplexes within an NPC are identical.

Nuclear pore complex assembly

Little is known about how the different subcomplexes interact with each other and how the huge protein interaction network of the NPC of about 500 different proteins is formed. However, the assembly process of NPCs has revealed insights into the protein interactions relevant for NPC formation, which is most likely also important for the intact NPC.

Metazoan cells undergo open mitosis and thus NPC re-assembly occurs concomitant with nuclear envelope reformation on the segregated and decondensing chromatin [for a review see (Schooley et al., 2012)]. Because thousands of NPCs re-assemble nearly synchronously, the order of events has been analysed in detail for this assembly pathway both by light microscopy and using biochemical assays. In the latter case, *Xenopus* egg extracts have been very useful as it is possible to reconstitute the

Table 1 Nup93/Nic96 complex components and their conservation.

S. cerevisiae	S. pombe	A. nidulans	C. thermophilum	A. thaliana	C. elegans	D. melanogaster	Vertebrata
Nup53/Nup59 (gi: 2497161/ 2498160)	Nup40 (gi: 2542466)	No clear homologue	ctNup53 (NCBI: JF276285)	Nup35/mitotic phosphoprotein N end (MPPN) family protein (gi: 820878)	npp-19 (gi: 174663)	Nup53 (gi: 32851)	Nup53/Nup35 (gi: 129401)
Both not essential, also not in double deletions (Marelli et al. 1998; Fahrenkrog et al. 2000)	Not essential (Chen et al. 2004)		–	–	Essential (Maeda et al. 2001; Ródenas et al. 2009; Galy et al. 2003)	Not essential (Buszczak et al. 2007)	Required for NPC assembly (Hawryluk-Gara et al. 2005; Hawryluk-Gara et al. 2008)
Nic96 (gi: 1171704)	Nup97/npp106 (gi: 2538861/2539077)	An-Nic96 (gi: 67541633)	ctNic96 (NCBI: JF276284)	Nup93 (gi: 818760/824902)	npp-13 (gi: 171774)	Nup93–1/Nup93–2 (gi: 41804/32350)	Nup93 (gi: 9688)
Essential (Grandi et al. 1993)	Nup97 essential (Cho et al. 2007); npp106 not essential (Yoon et al. 1997)	Essential (Osmani et al. 2006)	–	Two isoforms	Essential (Galy et al. 2003)	Isoform 1 essential; isoform 2 not essential (Mummery-Widmer et al. 2009)	Essential in D. rerio (Allende et al. 1996) and required for NPC assembly (Grandi et al. 1997; Sachdev et al. 2012)
Nup157/Nup170 (gi: 731497/586442)	Nup155 (gi: 2543502)	An-Nup170 (gi: 67541136)	ctNup170 (NCBI: JF276286)	Nup155 (gi: 838050)	npp-8 (gi: 176966)	Nup154 (gi: 34527)	Nup155 (gi: 9631)
Individually not essential, only in double deletions (Aitchison et al. 1995)	Not essential (Kim et al. 2010)	Essential (Osmani et al. 2006)	–	–	Essential (Kamath et al. 2003; Galy et al. 2003; Franz et al. 2005)	Essential (Kiger et al. 1999)	Essential in M. musculus (Zhang et al. 2008) and required for NPC assembly (Franz et al. 2005)
Nup188 (gi: 1709216)	Nup184 (gi: 15214122)	An-Nup188 (gi: 259489084)	ctNup188 (NCBI: JF276287)	No clear homologue	No clear homologue	Nup188 (gi: 36397)	Nup188 (gi: 23511)
Not essential (Zabel et al. 1996; Nehrbass et al. 1996)	Not essential (Whalen et al. 1999)	Essential (Osmani et al. 2006)	–			Not essential (Mummery-Widmer et al. 2009)	Not required for NPC assembly (Theerthagiri et al. 2010)
Nup192 (gi: 1352974)	Nup186 (gi: 74583074)	An-Nup192 (gi: 67515511)	ctNup192 (NCBI: JF276288)	Nup205 (gi: 835195)	npp-3 (gi: 174823)	Nup205 (gi: 33002)	Nup205 (gi: 23165)
Essential (Kosova et al. 1999)	Essential (Chen et al. 2004)	Essential (Osmani et al. 2006)	–	–	Essential (Galy et al. 2003)	Not essential (Mummery-Widmer et al. 2009)	Not required for NPC assembly (Theerthagiri et al. 2010)

stepwise process of nuclear reformation including NPC reassembly in a test tube (Gant and Wilson, 1997).

The earliest step of NPC assembly identified is the binding of the nucleoporin Mel-28/ELYS to the decondensing chromatin probably via its AT-hooks. Mel-28/ELYS binds and recruits the Nup107-160 complex to chromatin. Next, nuclear membranes bind to the chromatin via the nucleoplasmic domain of several inner nuclear and pore membrane proteins including the transmembrane nucleoporins Ndc1 and Pom121.

Following Nup107-160, the Nup93 complex is the second major structural subcomplex of the NPC that assembles post-mitotically (Dultz et al., 2008). In contrast to the Nup107-160 complex, the Nup93 complex is not recruited as a complete subcomplex to the pore but rather incorporates the individual components in a stepwise manner (Figure 2). The order of assembly can be understood due to depletion experiments that define which nucleoporins are required for the recruitment of others. Nup53 is first integrated into the assembling NPC via direct membrane interactions (Vollmer et al., 2012). Although it also binds the pore membrane protein Ndc1 (Mansfeld et al., 2006), this interaction is not mandatory for Nup53 recruitment (Hawryluk-Gara et al., 2008; Vollmer et al., 2012; Eisenhardt et al., 2013). The interaction is rather necessary to modulate membrane curvature induced by Nup53 at the pore membrane (Eisenhardt et al., 2013). Via a direct interaction, Nup53 recruits Nup155 (Eisenhardt et al., 2013), which can also bind the transmembrane nucleoporin Pom121 (Mitchell et al., 2010; Yavuz et al., 2010). Although its interaction with Pom121 might contribute to Nup155 localization, *in vitro* experiments suggest that binding to Nup53 is more important. Disrupting this interaction blocks Nup155 recruitment (Eisenhardt et al., 2013). Interestingly, not all eukaryotes possess integral pore membrane proteins (Mans et al., 2004; DeGrasse et al., 2009; Neumann et al., 2010) and in *Aspergillus nidulans* deletion strains lacking all three known pore membrane proteins are viable (Liu et al., 2009). Thus, direct membrane binding of Nup53 and possibly other nucleoporins might contribute to NPC assembly in these cases. The Nup53-Nup155 interaction is stabilized by the binding of the C-terminal domain of Nup93 (Sachdev et al., 2012). Via its N-terminal region Nup93 in turn interacts in a mutually exclusive fashion with Nup188 or Nup205 (Theerthagiri et al., 2010). In addition, the N-terminal coiled-coil region of Nup93 is directly connected to Nup62 (Grandi et al., 1997; Sachdev et al., 2012). This FG-repeat containing nucleoporin is a member of a larger subcomplex forming a major part of the hydrogel meshwork in the centre of the NPC that is crucial for the transport and exclusion functions of the pore. Thus, the stepwise assembly of the Nup93 complex starts at the pore membrane and stretches into the interior of the pore, forming the second large structural subcomplex of the NPC and recruits and anchors the Nup62 complex.

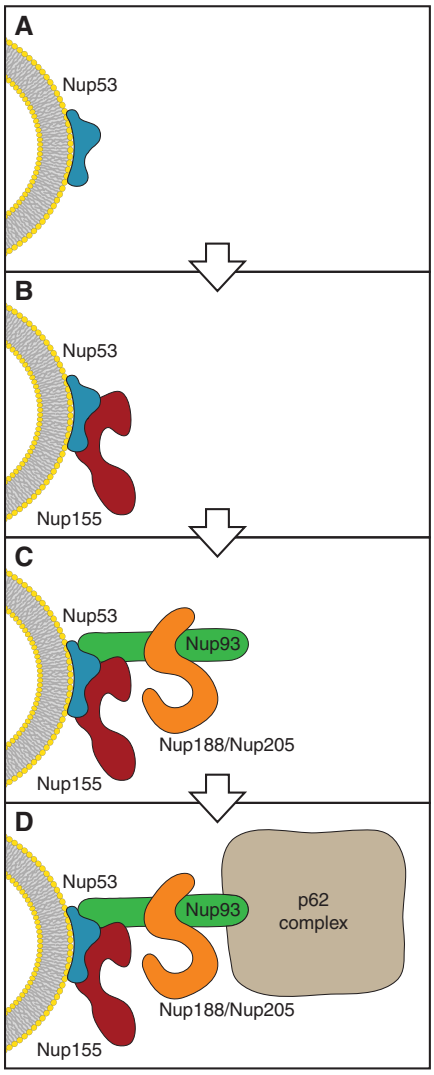

Figure 2 Stepwise recruitment of the Nup93 complex.
(A) During the nuclear pore complex formation, Nup53 (blue) directly binds to the pore membrane (B) and recruits Nup155 (red) to the assembly site. (C) This interaction is further stabilized by the C-terminal domain of Nup93 (green), which binds either Nup188 or Nup205. (D) Via its N-terminal coiled-coil region, Nup93 recruits the Nup62 complex (brown) to the assembling NPC leading to the formation of the central channel. Please note that for the sake of simplicity the stoichiometry within the complex is not represented (see main text for details).

In metazoa, NPCs assemble not only after mitosis but also during interphase by *de novo* insertion into the closed nuclear envelope. In contrast to the mode of post-mitotic assembly, which is initiated on the chromatin by

Mel-28/ELYS, NPC assembly during interphase begins on nuclear membranes (Doucet et al., 2010). The initial recruitment order of the different scaffolding complexes to the assembling NPC is not defined, but as it starts on the lipid bilayer it is possible that the Nup93 complex is part of the initiation process. The membrane interaction of Nup53 and specifically its capability to deform membranes, which is probably critical to approximate the two nuclear membranes for fusion and/or to form the highly curved pore membrane, are required in this process (Vollmer et al., 2012). Apart from the assembly order of the different subcomplexes, the sequence of nucleoporin recruitment within the Nup93 complex is most likely identical during post-mitotic and interphasic NPC formation. Presumably, it follows the biochemically determined interaction progression from the pore membrane inwardly to the central channel. As *S. cerevisia*e undergo closed mitosis, the assembly of NPCs can only occur by insertion into the closed nuclear envelope. Therefore, the conserved and well-defined interaction network of the Nic96 complex in this organism serves as a perfect paradigm for the interphasic mode of assembly. Similar to the situation in metazoan, the transmembrane nucleoporin Ndc1 interacts with Nup53 and its orthologue Nup59 (Uetz et al., 2000). This interaction is mutually exclusive, indicating that Nup53 and Nup59 compete for the same binding site on Ndc1 (Onischenko et al., 2009). Nup53/Nup59 in turn recruits the two Nup155 homologues Nup157 and Nup170 (Marelli et al., 1998; Lusk et al., 2002; Alber et al., 2007a; Onischenko et al., 2009). The link to the central channel is formed by the binding of Nic96 and its interaction partners Nup188/Nup192, which in turn recruits part of the transport channel formed by the Nsp1-Nup49-Nup57 complex, equivalent to the metazoan Nup62 complex (Grandi et al., 1993, 1995; Schlaich et al., 1997; Fahrenkrog et al., 2000). By elegant biochemical work using proteins from a thermophilic fungus, part of this interaction network has been reconstituted *in vitro* (Amlacher et al., 2011). The improved biochemical properties of the relevant proteins allowed for the testing of several direct interactions within the Nic96 complex. Here, as in vertebrates, the binding of Nic96 to Nup188 and Nup192 is mutually exclusive. Furthermore, a direct interaction between Nup192 and Nup53 was demonstrated. This interaction enables the formation of a heterotetrameric complex consisting of Nup192, Nup170, Nic96, and Nup53. Thus, the interplay between the members of the Nup93/Nic96 complex reveals a highly complex interaction network that is evolutionarily conserved as essential for the formation of NPCs.

Although the Nup93/Nic96 complex is a central structural component of the NPC, not all of its nucleoporins are essential or crucial for NPC assembly (Table 1). The only nuclear pore transmembrane protein conserved between yeast and metazoa is Ndc1. Although Nup53 and Nup59 establish the link to Ndc1, they are not essential in *S. cerevisiae* and can be deleted alone or in combination (Marelli et al., 1998; Fahrenkrog et al., 2000). In the fungus *A. nidulans* both proteins are absent (Osmani et al., 2006). In contrast, vertebrate Nup53 is crucial for NPC assembly (Hawryluk-Gara et al., 2005, 2008) because of its direct membrane interaction (Vollmer et al., 2012) and its function in recruiting Nup155 (Eisenhardt et al., 2013). Although yeast Nup53 and Nup59 can similarly bind membranes (Marelli et al., 2001; Patel and Rexach, 2008; Vollmer et al., 2012) and therefore bring its interaction partners Nup157 and Nup170 to the pore membrane, this necessity might be bypassed by the interaction of Nup170 with the integral pore membrane protein Pom152 (Makio et al., 2009). Likewise, the membrane deformation activity of yeast Nup53/Nup59 might be substituted by other proteins. For example, the membrane shaping reticulons are involved in NPC formation but the underlying mechanism remains poorly defined (Dawson et al., 2009). In vertebrates, the membrane curving activity of Nup53 is required for NPC formation in interphase, but not at the end of mitosis (Vollmer et al., 2012). It is conceivable that during mitotic exit the re-growing nuclear envelope encloses assembling NPCs without the need of outer and inner nuclear membrane deformation and fusion (Schooley et al., 2012).

Two other members of Nup93/Nic96 complexes, Nup155 and Nup93, are highly conserved in all eukaryotic phyla (Neumann et al., 2010). The importance of these two proteins is further emphasized by the fact that both are essential in basically all organisms tested so far. In contrast to Nup53/Nup59, yeast Nup157 and Nup170 are essential and cannot be deleted in combination (Aitchison et al., 1995; Kenna et al., 1996). Similarly, Nup170 is also indispensable in *A. nidulans* and mutation in the Nup155 gene in mouse is embryonic lethal (Osmani et al., 2006; Zhang et al., 2008). Down-regulation of both Nup157 and Nup170 in *S. cerevisiae* blocks NPC assembly as does the depletion of Nup155 in *Xenopus laevis* egg extracts (Franz et al., 2005; Makio et al., 2009).

Nic96 is essential in yeast (Grandi et al., 1993; Kim et al., 2010). This observation is consistent with the fact that Nup93 depletion blocks NPC assembly in *X. laevis* egg extracts (Grandi et al., 1997; Sachdev et al., 2012) and mutations in the Nup93 gene in *Danio rerio* is embryonic lethal (Allende et al., 1996). Upon depletion by RNAi in HeLa cells the nuclei develop an aberrant morphology accompanied by reduced cellular levels of its interacting

partners (Hawryluk-Gara et al., 2005) which could also here be caused by a block in NPC formation.

Surprisingly, Nup188 and Nup205 are individually, but also in combination, dispensable for NPC formation *in vitro* (Theerthagiri et al., 2010; Sachdev et al., 2012). Accordingly a Nup188 null mutant in *Schizosaccharomyces pombe* has no effect on viability, and deletion of Nup188 in *S. cerevisiae* has no effect on growth or nuclear morphology (Nehrbass et al., 1996; Zabel et al., 1996; Whalen et al., 1999). In higher plants and in *C. elegans*, no clear Nup188 homologues were found (Galy et al., 2003; Tamura et al., 2010). In contrast, deletion of the Nup188 gene in *A. nidulans* is lethal (Osmani et al., 2006). The paralogue of Nup188, Nup205, plays a critical role in several organisms. In *S. cerevisiae*, *S. pombe*, *C. elegans* and *A. nidulans* the deletion of this protein is lethal (Kosova et al., 1999; Galy et al., 2003; Chen et al., 2004; Osmani et al., 2006). However, Nup205 is not found in all eukaryotic phyla, a fact that could be explained by either losses or gains in different lineages (Neumann et al., 2010).

In summary, some nucleoporins of the Nup93/Nic96 complex such as Nup155 or Nup93 are critical in most if not all species. The loss of other components such as Nup53 or Nup205/Nup188 might be tolerated in some organisms indicating redundancies in the interaction network that is crucial for the assembly and function of NPCs.

Nuclear transport functions

The main functions of NPCs are to exclude substances that should not pass the barrier of the nuclear envelope and at the same time allow for the selective transport of proteins and RNAs. The formation of the barrier that excludes inert substances with a radius larger than 2.5 nm (Mohr et al., 2009) has been mainly attributed to FG-repeat containing nucleoporins (Ribbeck and Gorlich, 2002; Strawn et al., 2004; Hulsmann et al., 2012). However, members of the Nup93/Nic96 complex have also been implicated in this function. Deletions of Nup188 or Nup170 in *S. cerevisiae* increase the passive permeability of the NPC (Shulga et al., 2000; Shulga and Goldfarb, 2003). In *Drosophila* cells and *C. elegans* embryos, RNAi mediated down-regulation of Nup93 and/or Nup205 also increases the permeability barrier of NPCs (Galy et al., 2003; Chen and Xu, 2010; Hachet et al., 2012). It is possible that gaps within the structural part of the NPC are responsible for the elevated permeability in these cases. However, it is more likely that the lack of these nucleoporins decreases the levels of FG-repeat-containing nucleoporins, which in turn affects the permeability barrier. Indeed, yeast Nup170 and metazoan Nup93 are required for efficient recruitment of FG-repeat containing nucleoporins (Kenna et al., 1996; Sachdev et al., 2012). A similar lack of exclusion is observed in nuclei lacking the FG-repeat nucleoporins Nup98 and/or the Nup62 complex (Theerthagiri et al., 2010; Laurell et al., 2011; Hachet et al., 2012; Hulsmann et al., 2012; Sachdev et al., 2012).

Nuclear protein import defects upon deletion of Nup93/Nic96 complex nucleoporins as observed for Nup53, Nup59, Nic96 and Nup192 might similarly be caused by affecting recruitment of FG-repeat containing nucleoporins (Grandi et al., 1995; Marelli et al., 1998; Fahrenkrog et al., 2000; Rodenas et al., 2009; Sampathkumar et al., 2013). Alternatively, as many components of the Nup93/Nic96 complex are crucial for NPC assembly deletion of these nucleoporins could result in a reduced total number of NPCs and thus to a diminished nuclear transport efficiency (Gomez-Ospina et al., 2000; Makio et al., 2009). However, specific contributions of these structural nucleoporins to the transport pathway cannot be excluded. For example, RNAi mediated down-regulation of Nup205 or Nup93 decreases nuclear import of Smad 1 without affecting other nuclear import reporters (Chen and Xu, 2010). In *S. cerevisiae*, Nup53 has been implicated in regulating nuclear import in a cell cycle dependent manner controlled by the spindle assembly checkpoint protein Mad1 (Makhnevych et al., 2003; Cairo et al., 2013). During G2/M phase, structural changes within the Nic96 complex free binding sites on Nup53 for the import receptor Kap121. This sequestering of Kap121 causes a block in Kap121 mediated nuclear import during this stage of the cell cycle and demonstrates how a nucleoporin can inhibit a specific import pathway.

Whereas the cytoplasmic-nucleoplasmic transport of soluble cargos across the nuclear envelope is well studied, comparatively less is known about the passage of membrane proteins through NPCs (Lusk et al., 2007a; Antonin et al., 2011). Integral membrane proteins are synthesised in most, if not all instances, by ribosomes at the rough endoplasmic reticulum. The signal sequences that direct these proteins to the inner nuclear membrane as well as the necessary co-factors are now beginning to emerge (King et al., 2006; Turgay et al., 2010; Zuleger et al., 2011). The nucleoplasmic domain of most membrane proteins that are able to pass the NPC is smaller than 60 kDa (Soullam and Worman, 1993; Ohba et al., 2004). It is thus unlikely that they extend into and cross the FG-repeat domain filled central channel of the NPC. Although reporter transmembrane proteins with artificial extended unfolded linker regions that could span into the

central channel require FG-repeat containing nucleoporins for efficient NPC passage (Meinema et al., 2011), it is questionable whether this reflects a general mechanism. It is unclear whether such long flexible linker domains are present in natural inner nuclear membrane proteins that allow this mode of transport.

It has been suggested that integral membrane proteins move through the pore in proximity to the membrane, possibly via peripheral channels in NPCs (Powell and Burke, 1990; Theerthagiri et al., 2010). Recent cryo-electron tomography of human NPCs confirmed the existence of these channels (Maimon et al., 2012; Bui et al., 2013). They show the smallest constriction at the level of the inner ring, which is formed at least in part by the Nup93/Nic96 complex. Consistent with the idea of transmembrane transport through these channels in close proximity to the membrane, nucleoporins of Nup93/Nic96 complex have been implicated in this process. Deletion of Nup188 or Nup170 in *S. cerevisae* or RNAi mediated down-regulation of Nup155 and Nup53 in human or *Drosophila* cells reduced nuclear localization of several membrane proteins (Deng and Hochstrasser, 2006; King et al., 2006; Mitchell et al., 2010; Zuleger et al., 2011; Busayavalasa et al., 2012). It is unclear whether this is caused by a reduction in nuclear import of the proteins or an increased backflow to the outer nuclear membrane and the endoplasmic reticulum. The latter scenario would be consistent with an increased diameter of the channels in the absence of the respective proteins, a hypothesis that remains to be tested. The opposite effect of a facilitated inner nuclear membrane targeting is observed upon down-regulation of Nup188 or Nup205 in HeLa cells (Mitchell et al., 2010; Antonin et al., 2011). The same is seen in *Xenopus* egg extracts upon Nup188 depletion (Theerthagiri et al., 2010). NPCs lacking Nup188 show an improved passage of inner nuclear membrane proteins through NPCs whereas the transport of soluble cargos through the central pore is not affected. This implies that the presence of Nup205/Nup188 restrains the route of membrane proteins. Structural re-arrangements within the inner ring involving Nup205/Nup188 are possibly required for this passage, which in turn would be facilitated in the absence of either Nup205 or Nup188. Interestingly, the structural analysis of the yeast homologues shows a high degree of shape flexibility of both proteins (Flemming et al., 2012; Andersen et al., 2013; Sampathkumar et al., 2013), which supports the view that nucleoporin re-arrangements within the inner ring of NPCs are possible.

In summary, whereas most defects in nuclear transport and exclusion of soluble proteins connected to nucleoporins of the Nup93/Nic96 complex might be indirectly caused by an accompanied reduction in overall pore density and/or recruitment of certain FG-repeat-containing nucleoporins, there is some evidence that nucleoporins of this complex positively or negatively contribute to the passage of integral membrane proteins through the pore. How they perform this task is currently not understood and represents an interesting avenue for future research. It is particularly unclear whether the seemingly opposite effects on the localization of inner nuclear transmembrane proteins upon interference with Nup93/Nic96 complex nucleoporins is caused by the different nature of reporters, used in the diverse test systems. Alternatively, it could indicate that distinct passage-related features are affected, e.g. in some cases the constricted size of the peripheral channels, in others the flexibility necessary for protein re-arrangements within the inner ring of the NPCs.

Additional functions of Nup93/Nic96 complex proteins

The Nup93/Nic96 complexes constitute a crucial structural part of the NPC, but the nucleoporins forming these subcomplexes are also involved in a growing list of other cellular functions, including mitotic processes. In *C. elegans*, depletion of Nup205 disturbs the synchronous nuclear envelope breakdown of male and female pronuclei in embryos (Hachet et al., 2012) and, similar to depletion of Nup93, affects chromatin condensation (Galy et al., 2003) and spindle orientation (Schetter et al., 2006). Defects in chromatin segregation are also seen upon depletion of Nup53 or Nup155 in *C. elegans* embryos (Franz et al., 2005; Rodenas et al., 2009). In Drosophila, a hypomorphic mutant allele of the Nup155 homologue blocks decondensation of nurse cell chromosomes (Gigliotti et al., 1998). In all instances, an indirect effect of less efficient NPC transport or exclusion functions in interphase cannot be excluded but a more direct role for these nucleoporins is conceivable.

In line with the Nup155 depletion phenotype in *C. elegans*, the yeast homologue Nup170 plays a role in chromosome segregation, probably by maintaining heterochromatic structures in the centromeric regions (Kerscher et al., 2001). Deletion of Nup170 leads to defects in kinetochore integrity and chromosome segregation fidelity but the cells are still viable. Interestingly, overexpression of its orthologue, Nup157, can complement the Nup170 deletion although the defects are not seen when Nup157 is deleted. It is possible that, similarly to the above mentioned case of Nup53 (Makhnevych et al.,

2003), Nup170 plays a regulatory role in the nuclear transport of specific factors necessary for chromosome segregation. Alternatively, Nup170 might affect chromatin structure. Similar to the metazoan Nup155 (Mendjan et al., 2006; Kehat et al., 2011), Nup170 interacts with chromatin-modifying complexes, a feature that is important for the formation of subtelomeric chromatin regions and their localization to the nuclear envelope (Van de Vosse et al., 2013). It is not clear whether these interactions also account for the role of Nup170 in chromosome segregation. Nonetheless, these studies linking yeast Nup170 and vertebrate Nup155 with chromatin remodeling factors puts both proteins on the growing list of nucleoporins involved in genome organization and gene expression [for a review see (Liang and Hetzer, 2011); Van de Vosse et al., 2011)]. Likewise, the Nup170 orthologue Nup157 is involved in transcriptional activation of genes by recruiting them to NPCs (Ahmed et al., 2010).

In humans, Nup155 has been linked to atrial fibrillation, a common form of sustained clinical arrhythmia (Zhang et al., 2008). A homozygous point mutation in Nup155 was identified in an affected family. When overexpressed in human cell lines, the mutated protein does not properly localize to the nuclear rim and affects the permeability of NPCs. However, the link between this defect in NPC function and heart problems is unresolved. It was speculated that defective nuclear transport of Hsp70 mRNA and protein is responsible, but it is unclear why heart cells would be primarily affected. It is also possible that the interaction between Nup155 and the histone deacetylase HDAC4 is causative as this enzyme is involved in transcriptional regulation of a number of cardiac genes (Kehat et al., 2011). Thus, the chromatin regulatory capability of Nup155/Nup157/Nup170 might be the connecting feature of a number of seemingly unrelated cellular processes affected by this nucleoporin.

In *C. elegans*, a role for chromatin recruitment has been shown for Nup93 and Nup205 (Ikegami and Lieb, 2013). These nucleoporins bind tRNA and snoRNA genes downstream of the RNA polymerase III pre-initiation complex and are required for cleavage of the transcripts into mature tRNAs and snoRNAs. Thus, Nup93/Nup205 mediated NPC association might facilitate efficient processing of RNA polymerase III transcripts.

In addition to chromatin interactions, a second major domain where components of the Nup93/Nic96 complex are involved is the spindle assembly checkpoint. Many components of this regulatory network, which inhibits anaphase onset until all chromosome kinetochores are properly connected to spindle microtubules, associate with NPCs during interphase (for review see Wozniak et al., 2010). These include Mad1 and Mad2, which interact with proteins of the Nup93/Nic96 complexes. Nup53, Nup157 and Nup170 in yeast (Iouk et al., 2002; Scott et al., 2005) and Nup53 in vertebrates (Hawryluk-Gara et al., 2005). Although the function of this interaction is unclear it is tempting to speculate that it is a way to sequester both checkpoint proteins during interphase. Interestingly, yeast Nup53 is differentially phosphorylated during the cell cycle and this phosphorylation regulates its ability to interact with Mad1 (Lusk et al., 2007b). Mitotic Nup53 phosphorylation might therefore help to dissociate Mad1 from the NPC, allowing it to associate with kinetochores. In vertebrate cells, depletion of Nup53 leads to decreased cellular levels of Mad1 (Hawryluk-Gara et al., 2005). As these cells do not exhibit spindle assembly checkpoint defects, the pool of residual Mad1 seems to be sufficient to perform its essential role in chromosome segregation.

In HeLa cells, RNAi mediated depletion of Nup188 affects spindle assembly, resulting in chromosome misalignment during metaphase (Itoh et al., 2013). As Nup188 interacts with NuMA, a key factor involved in spindle assembly, this function could help to efficiently recruit NuMA to centrosomes and hence be important for focusing microtubules at centrosomes. It will be interesting whether this function of Nup188 is independent of Nup93 and how the mitotic phosphorylation of Nup188 (Dephoure et al., 2008) influences its mitotic function.

In conclusion, proteins of the Nup93/Nic96 complexes are involved in a growing list of cellular tasks that are not obviously connected to their role in scaffolding the NPC structure and transport function. This includes roles in gene regulation and mitotic processes as observed for many other nucleoporins. The underlying molecular mechanisms are in many instances unclear but their investigation promises interesting and surprising insights in the coming years.

Conclusion and future directions

In summary, considerable progress has been made in the last years in defining the molecular structure and diverse functions of the Nup93/Nic96 complexes. The precise stoichiometry of the nucleoporins within these subcomplexes and in NPCs is still unclear. It also remains to be determined if this stoichiometry applies to all NPCs in different tissues under diverse developmental and environmental conditions. Similarly, the precise structural arrangement of the Nup93/Nic96 complexes within the

NPCs needs to be established. How nucleoporins of these subcomplexes can specifically affect the passage of integral membrane proteins through the pore remains poorly defined. Apart from their classical roles in NPC structure and transport, many members of the Nup93/Nic96 complexes are involved in additional cellular processes. It still remains to be established how the different proteins contribute to these processes, how they are regulated and whether these moonlighting functions are independent of their NPC localization, and their nuclear transport function.

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A single herpesvirus protein mediates vesicle formation in the nuclear envelope

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Abstract

Herpesviruses assemble capsids in the nucleus and egress by unconventional vesicle-mediated trafficking through the nuclear envelope. Capsids bud at the inner nuclear membrane into the nuclear envelope lumen. The resulting intralumenal vesicles fuse with the outer nuclear membrane delivering the capsids to the cytoplasm. Two viral proteins are required for vesicle formation, the tail-anchored pUL34 and its soluble interactor pUL31. Whether cellular proteins are involved is unclear. Using giant unilamellar vesicles we show that pUL31 and pUL34 are sufficient for membrane budding and scission. pUL34 function can be by-passed by membrane-tethering of pUL31 demonstrating that pUL34 is required for pUL31 membrane recruitment but not for membrane remodelling. pUL31 can inwardly deform membranes by oligomerizing on its inner surface to form buds which constrict to vesicles. Thus, in contrast to other cellular vesicle formation processes, a single viral protein mediates all events necessary for membrane budding and abscission.

Introduction

Viruses exploit amazing strategies overcoming various cellular membranes to realize their complex life cycle including cell entry, genome replication, assembly of virus particles as well as their egress. For many viruses that replicate in the cell nucleus a major barrier is the nuclear envelope, a double membrane structure enclosing the chromatin. It consists of the inner nuclear membrane that, in animal cells, is underlaid by a tight lamina meshwork that connects and stabilizes the nuclear envelope, and the outer nuclear membrane, which is continuous with the endoplasmic reticulum (for review see [1,2]). The nuclear envelope is perforated by nuclear pore complexes, huge macromolecular assemblies of up to 125 MDa in vertebrates, which act as selective gateways between the cytoplasm and the nucleus [3]. Nuclear pores are used as entry paths for most viruses that replicate in the nucleus (for review see [4,5]). In many cases this involves an at least partial disassembly of the viral capsid at the cytoplasmic side or core of the nuclear pore complex, since these particles are too large to pass the pore intact.

Whereas many viruses assemble in the cytoplasm, herpesviruses package their newly replicated genomic DNA into capsids of up to 125 nm within the nucleus. Unlike many other DNA viruses herpesviruses do not depend on the breakdown of the nuclear envelope during mitosis for their release and can also replicate in non-dividing cells. Thus, they face the challenge for passing the nuclear envelope a second time, now in the opposite direction. Since herpesvirus capsids are too large for passage through the nuclear pore, they are transported through the nuclear envelope by a vesicle-mediated transport, also described as a "envelopment-deenvelopment" pathway [6]. Capsids bud at the inner nuclear membrane and, thus, acquire a (primary) envelope resulting in a nascent virus located in the lumen between the inner and outer nuclear membranes (for review see [7,8]. Subsequently, the primary envelope fuses with the outer nuclear membrane resulting in translocation of the capsid to the cytoplasm. Final maturation including assembly of tegument and secondary envelopment follows in the cytoplasm and mature virions are released at the plasma membrane.

Egress from the nucleus is a multifaceted process. In all herpesviruses studied it involves the formation of a hetero-dimeric nuclear egress complex consisting of two viral proteins that are conserved throughout the herpesvirus family. One component is a type II transmembrane protein, tail-anchored in the nuclear envelope and termed pUL34 in herpes simplex virus 1 and pseudorabies virus. It interacts with a soluble component, pUL31, and recruits it to the nuclear envelope [9-15]. In the absence of this complex nuclear egress is blocked and capsids accumulate in the nucleoplasm. One function of the pUL31-pUL34 complex is the recruitment of viral and cellular kinases that locally phosphorylate and disrupt the nuclear lamina [15-19], a pre-requisite for access of capsids to the inner nuclear membrane. In addition, the pUL31-pUL34 complex might also contribute to the nuclear membrane restructuring necessary for herpesvirus nuclear egress. Transient or stable transgenic expression of pUL31 and pUL34 is sufficient to drive formation of correctly sized primary envelopes in the perinuclear space between the outer and inner nuclear membrane [10,20]. Thus, pUL31 and pUL34 are the only two viral proteins required for budding and fission of vesicles at the inner nuclear membrane. Whether this process is additionally dependent on the recruitment and function of cellular factors is currently unknown.

Primary envelopment of herpesvirus capsids requires extensive restructuring of the host inner nuclear membrane to allow envelope formation and fission. Similar membrane deformations have

been intensively investigated as they are at the heart of vesicle mediated intracellular trafficking in the secretory and endocytic pathways. Many such processes are directed by complex but conceptually straightforward mechanisms in which coat proteins impose curvature on the cytosolic membrane face, thereby generating buds which ultimately constrict to vesicles. The best-known coat proteins are clathrin and the COP I and COP II complexes, which assemble on the outer side of the membrane and thereby result in the release of vesicles into the cytosol [21]. Membrane deformation in the opposite direction, i.e. away from the cytosol, such as the invagination of the endosomal membrane during formation of multivesicular bodies, or egress of HIV and other enveloped viruses at the plasma membrane, both mediated by the ESCRT machinery, are less frequent [22]. pUL31-pUL34 mediated inner nuclear membrane engulfment, much like the suggested pathway for nuclear egress of large ribonucleoprotein complexes recently identified in drosophila [23] represents one of these exceptional pathways. It involves vesicle budding and scission from the nucleoplasm into the intermembrane space of the nuclear envelope, which is connected and topologically identical to the lumen of the endoplasmic reticulum. The molecular machinery mediating inner nuclear membrane deformation and scission remains largely obscure. It is especially unclear whether it requires proteins within the lumen of the nuclear envelope that could assemble a coat on the outer surface of the nascent vesicles, similar to COPI, COP II and clathrin coats in the cytoplasm.

We have reconstituted the function of Pseudorabies Virus (PrV) pUL31 and pUL34 in a simple membrane system by using giant unilamellar vesicles (GUVs). We show that the two viral proteins are sufficient for budding and fission of membrane vesicles into the lumen of GUVs, a process that is topologically identical to inwardly directed vesicle formation at the inner nuclear membrane during herpesvirus nuclear egress. Artificial membrane recruitment of pUL31 alone results in the same membrane remodelling and generates intra-GUV vesicles. Accordingly, fusion of pUL31 to the transmembrane domain of an inner nuclear membrane protein can substitute for pUL34 in cells and mediate nuclear envelope restructuring. Thus, pUL31 and pUL34 are sufficient for vesicles formation without the need for additional (cellular) proteins. Moreover, we can assign separate functions to pUL31 and pUL34 during herpesvirus nuclear egress: pUL34 recruits pUL31 to the membrane and provides membrane anchorage, whereas pUL31 mediates membrane budding and scission.

Results

pUL31 and pUL34 are sufficient for membrane budding and scission

Viral pUL31 and pUL34 are necessary for nuclear egress of herpesvirus capsids. To investigate their function in detail, we expressed the transmembrane protein pUL34 in *E. coli*, and, after purification, labelled it with the fluorescent dye Alexa 546 before reconstitution into liposomes with a nuclear envelope-like lipid composition ([24,25] , see Methods for detail). Large unilamellar vesicles (GUVs) were generated from these proteo-liposomes. The GUV membrane contained pUL34 as an integral membrane component, which can be detected by its fluorescent label (Fig. 1a). The vast majority of these GUVs were large vesicles with no detectable membrane/vesicle structures in the interior.

When added to pUL34-GUVs, purified recombinant EGFP-pUL31 was efficiently recruited to the membranes (Fig 1b) consistent with a direct pUL31-pUL34 interaction [9,12,14]. In the presence of pUL31 the GUV membrane was deformed and invaginated leading to small intralumenal vesicles inside the GUVs, which contained both, pUL31 and pUL34. This effect was specific for pUL31 as it was not induced by addition of EGFP alone. The same result was observed with untagged pUL31 confirming that the EGFP tag did not cause this effect (Supplementary Fig. S1). As a control we replaced pUL34 by the inner nuclear membrane protein SCL1/BC08 [26]. Like pUL34, SCL1 is a type II transmembrane protein with a single membrane-spanning domain at its C-terminus. Thus, the N-terminal region preceding the transmembrane domain of both pUL34 and SCL1 face the nucleoplasm. EGFP-pUL31 was not recruited to SCL1-GUVs and membrane invaginations were not observed above background levels demonstrating that this process specifically requires pUL31 and pUL34.

Cascade blue labelled neutravidin was used as a fluid phase marker to assess permeability between the bulk solution and the pUL31 induced vesicles. When the fluid phase marker was added together with pUL31 to pUL34-GUVs the label was found in intralumenal vesicles (Fig. 1c). If added 15 min after pUL31 addition, intra-GUV vesicles without fluid phase marker were detected. Thus, the lumina of vesicles are disconnected from the bulk solution indicating membrane scission. This is consistent with the three dimensional reconstitution of an EGFP-pUL31 treated pUL34-GUV which shows highly mobile vesicles distant and apparently detached from the GUV membrane (Fig. 1d). If the fluid phase marker was added to SCL1-GUVs together with EGFP-pUL31 or to pUL34-GUVs in the absence of EGFP-pUL31 (Fig. 1c and Supplementary Fig. S2) no intra-GUV fluorescence was detected consistent with the notion that the pUL31-pUL34 interaction specifically induces vesicle budding into the GUV lumen.

These data show that in a minimal membrane system the two viral proteins pUL31 and pUL34 are sufficient to induce membrane perturbations generating intra-GUV vesicles. The observed restructuring of the limiting membrane (Fig. 1d) is reminiscent of the inwardly directed primary vesicle formation into the lumen of the nuclear envelope during herpesvirus nuclear egress. Thus, this GUV system is a valuable tool to investigate pUL31-pUL34 mediated vesicle formation in detail.

pUL31 mediates membrane budding and scission

Many membrane deforming processes specifically involve integral membrane proteins [27-29]. To assess whether the membrane spanning C-terminus of pUL34 is required for formation of intralumenal vesicles we artificially recruited a pUL34 fragment lacking its transmembrane domain to GUVs (Fig.

2a). To this end, the Alexa 546 labelled soluble domain (aa 1-240) of pUL34 fused to an N-or C-terminal His_6-tag was added to GUVs incorporating the Ni^{2+} chelating lipid Ni-NTA-DGS. GUVs coated with these tethered pUL34 fragments showed no detectable vesicle structures in their interior. When EGFP-pUL31 was additionally added, it was efficiently recruited to the GUV membranes independent of whether pUL34 was attached to the membrane via its N- or C-terminus consistent with the fact that the transmembrane region of pUL34 is not required for pUL31 interaction [9,30]. More importantly, pUL34-mediated pUL31 recruitment was sufficient for intralumenal vesicle formation. This indicates that presence of an authentic transmembrane region is not essential for this process.

Next we tested whether pUL34 plays an active role in the invagination process or whether it is merely required for pUL31 recruitment. When His_6-tagged EGFP-pUL31 was directly bound to Ni-NTA-DGS containing lipid GUVs intralumenal vesicles were efficiently induced (Fig. 2b). Three dimensional reconstitution of a His_6-EGFP-pUL31 treated GUV shows many highly mobile vesicles distant from the GUV membrane indicating that they have been disconnected (Fig. 2c). Thus, artificial membrane tethering of pUL31 is sufficient for induction of membrane invaginations and membrane scission. This effect was specific for pUL31 as a His_6-tagged EGFP, despite efficient recruitment to the GUV membrane, did not induce intra-GUV vesicle formation (Fig. 2b). Thus, the function of pUL34 in this minimal system is to target pUL31 to the membrane.

These data suggest that pUL31 membrane recruitment induces vesicle budding and scission supposedly by self-assembly of a coat-like structure on the deforming membrane similar to clathrin or components of the COP I/II or ESCRT machinery [21,22]. To test for membrane induced oligomerization we bound limiting amounts of His_6-pUL31 to Ni-NTA-DGS-GUVs. Under these conditions no intralumenal vesicles were detected (Fig. 3). When adding increasing amounts of EGFP-pUL31 devoid of a His_6-tag the protein was recruited to the membrane and induced, in a concentration dependent manner, intralumenal vesicle formation. EGFP-pUL31 membrane recruitment was specifically mediated by GUV tethered His_6-pUL31 as in the absence of His_6-pUL31 no EGFP-pUL31 membrane labelling or vesicle formation was observed.

pUL31-pUL34 mediated membrane re-sculpting requires cholesterol and sphingomyelin

The GUVs used so far contained a lipid composition resembling the nuclear envelope. We next assessed whether any specific lipid or lipid class in this mixture is required for intralumenal vesicle formation. GUVs were reconstituted with full-length pUL34 in the absence of specific lipid components. EGFP-pUL31 was efficiently recruited to the membrane of pUL34-GUVs independent of the lipid composition (Fig. 4a). In fact, efficient formation of intralumenal vesicles was observed in all instances except when cholesterol or sphingomyelin were absent from the GUV membrane. Also in the absence of both negatively charged lipid classes, phosphatidylserine and phosphatidylinositol, intralumenal vesicles formed efficiently. Identical results were obtained when His_6-tagged EGFP-pUL31 was directly targeted to Ni-NTA-DGS-GUVs (Fig. 4b). These data indicate that cholesterol and sphingomyelin are required for pUL31-induced membrane invagination and scission. Both lipids interact and are crucial for lateral segregation of membrane components leading to membrane subdomain formation such as rafts[31]. However, we did not observe a pUL31-pUL34 induced phase separation of sphingolipid-cholesterol from other membrane components (Supplementary Fig. S3). We rather propose that cholesterol and sphingomyelin are required for intra-GUV vesicle formation as they permit membrane fluidity [32].

Membrane tethering of pUL31 induces nuclear membrane alterations

The *in vitro* experiments using GUVs indicated that the sole function of pUL34 in membrane restructuring is to recruit pUL31 to the membrane. To test the validity of this hypothesis we sought to bypass the need of pUL34 for nuclear membrane remodelling in mammalian cells. When expressed in HeLa cells, EGFP-pUL31 localized to the nucleoplasm (Fig. 5a) as previously reported for the untagged protein [10,30]. mCherry-pUL34 localized to the endoplasmic reticulum and nuclear envelope. Co-transfection of both constructs resulted in an enrichment of pUL34 at the nuclear envelope, presumably the inner nuclear membrane, re-localization of pUL31 to the nuclear envelope and, importantly, the appearance of pUL31-pUL34 containing speckles in close proximity to the nuclear envelope. This phenotype has been previously observed. The detected speckles were found to be local proliferations and alterations of the nuclear envelope with intralumenal vesicles resembling primary enveloped virus particles but lacking the capsids [10].

Similar speckles were observed when pUL31 with C-terminally fused transmembrane domains of pUL34 or SCL1 were transfected in the absence of pUL34 (Fig. 5b). A third chimeric construct encoding pUL31 with an N-terminally fused transmembrane region of the nuclear pore membrane protein POM121 was also tested. Here, the EGFP was localized upstream of the transmembrane region and driven by an ER localization signal into the endoplasmic reticulum/nuclear envelope lumen. Transfection of this construct similarly resulted in a nuclear envelope labelling and speckle formation. Together, these data indicate that also in cells the necessity of pUL34 for pUL31 mediated nuclear membrane re-sculpting can be bypassed by directly tethering pUL31 at its N- or C-terminus to the inner nuclear membrane.

Discussion

We have reconstituted herpesvirus pUL31-pUL34 dependent membrane invagination and scission using a minimal set of protein components and a simple model for the eukaryotic nuclear envelope. In this system, pUL34 is only required to recruit pUL31 to the membrane. Presence of pUL31 at the membrane is sufficient to induce GUV-internal vesicles, a process topologically similar to the formation of primary envelopes. In an analogous manner fusion of pUL31 to a transmembrane domain can bypass the necessity of pUL34 for vesicle formation in the nuclear envelope lumen in cells.

The minimal GUV model system provides several fundamental new insights into the process of herpesvirus nuclear egress. First, it shows that pUL31-pUL34 mediated membrane deformation at the inner nuclear membrane can be uncoupled and is thus functionally disconnected from lamina disassembly. It is formally possible that pUL31-pUL34 regulated lamina dynamics are a driving force for restructuring the inner nuclear membrane, similar to other cytoskeleton assembly and disassembly processes that are linked to membrane shape changes of a variety of organelles [33,34]. However, in our minimal system deformation of the GUV membrane leading to vesicle formation occurs in the absence of lamin proteins. Second, pUL31 and pUL34 have well defined and separate functions in primary envelopment of herpesvirus capsids. pUL31 and pUL34 are both crucial for this process and co-expression of both proteins in uninfected cells induces formation and scission of vesicles from the inner nuclear envelope [10,20]. It could be envisioned that cellular proteins participate in the necessary restructuring of the inner nuclear membrane because a similar process occurs during nuclear export of large ribonucleoprotein particles in drosophila neurons [23]. However, our work shows that cellular proteins are not essential to execute the basic membrane restructuring necessary for nuclear egress including membrane deformation, budding away from the nucleoplasm, and scission to generate vesicles detached from the inner nuclear membrane. Third, pUL31 membrane recruitment is sufficient for membrane remodelling resulting in vesicle formation both *in vitro* and in transfected cells. Thus, this viral protein is the driving force both for membrane budding and scission in herpesvirus nuclear egress which constitutes a new archetype of these non-conventional inwardly directed budding events.

Herpesvirus nuclear egress can be regarded as vesicle-mediated transport through the lumen of the nuclear envelope. In this respect the process has an inverted topology compared to classical vesicular trafficking pathways through the cytosol. The prime factors inducing membrane deformation and scission in vesicular trafficking localize and act on the outer surface of the exvaginated membrane and/or the vesicle. Our results demonstrate that such an outer membrane coat is not required for vesicle formation during herpesvirus egress. Rather, formation of the nuclear egress complex on the emerging inner vesicle surface is sufficient to drive membrane bending and scission. Different mechanisms implicated in membrane remodelling might be envisioned to promote vesicle formation [29]: First, integral membrane proteins can deform membranes. Well known examples are reticulons and caveolins, which oligomerize and possess unusual hydrophobic segments which might form wedge shaped hairpins in the membrane[27,28] and both features can contribute to membrane shaping [29]. However, sequence analysis does not suggest such an unusual topology of the pUL34 transmembrane region and there is no indication for pUL34 oligomerization. Our data rather show that pUL34 is dispensable for membrane restructuring leading to vesicle formation in the nuclear envelope lumen. Furthermore, membrane invaginations can be detected in the GUV system with pUL31 alone indicating that a transmembrane region does not play a compulsory role in the process.

A second widely discussed mechanism that can contribute to membrane deformation and budding is the insertion of amphipathic helixes into the lipid bilayer [25,35,36]. However, neither pUL31 nor pUL34 are predicted to form such helixes. More importantly, helix insertion has to take place into the outer lipid leaflet of the nascent vesicle to increase the outer in relation to the inner surface area. pUL31 is localized in the interior of the vesicle discounting such a mechanism. Similarly, protein crowding generating lateral pressure, which can curve membranes [37], can be excluded for pUL31 mediated membrane budding as this mechanism generates membrane deformation in the opposite direction, i.e. outwardly from the limiting membrane.

In addition to protein driven processes lipid induced changes can restructure membranes. Phase separation can generate membrane curvature and induce budding and scission in simple membrane systems [38,39]. However, the lipid mixture of the nuclear envelope /endoplasmic reticulum does not undergo phase separation [32]. Accordingly, we have no indication for a locally induced phase separation giving rise to the observed invaginations (Supplementary Fig. S3). Changes in lipid composition, especially the localized generation of non-cone shaped lipids or their enrichment such as the unconventional phospholipid lysobisphosphatidic acid on internal vesicles of multivesicular bodies [40] can contribute to membrane curving. However, there is no evidence that pUL31 mediates lipid modifying reactions or has a binding preference for a specific lipid.

Thus, we prefer a model in which pUL31 acts as a scaffolding protein self-assembling at the inner surface of the forming vesicle. This is consistent with the fact that budding sites at the inner nuclear membrane are more electron dense, an observation that implies high protein density [8,10]. This process is reminiscent of the invagination processes mediated by the ESCRT machinery during formation of intra-endosomal vesicles in multi-vesicular body formation. In this process ESCRT-I and ESCRT-II complexes direct membrane budding away from the cytosol, and ESCRT-III cleaves the bud necks from their cytosolic faces [22,41,42]. In a mechanistically related process during HIV egress at the plasma membrane, the viral gag protein assembles and drives initial bud formation, but requires the ESCRT complex components for membrane scission [43,44]. Similar to the gag/ESCRT machinery the nuclear egress complex requires no energy input for both membrane budding and scission (Supplementary Fig. S4). Interestingly, whereas in the case of the gag/ESCRT mediated vesicle formation several proteins are required and the steps of membrane budding and scission are functionally separated, a single protein, namely pUL31, fulfils this task in herpesvirus nuclear egress. This suggests that pUL31 has the intrinsic ability to scaffold and cleave its own bud neck. How this is mechanistically achieved is an interesting avenue for future research.

In summary, our work establishes that pUL34 mediated membrane recruitment of pUL31 drives vesicle budding and constriction. It shows that a single protein, pUL31, is sufficient to induce inwardly directed membrane deformation and scission. It will be interesting to see whether cellular orthologs of herpesvirus pUL31 exist, especially for the process of RNP egress at the nuclear envelope, which is topologically comparable to herpesvirus nuclear egress. Finally, the methods established and employed here to express and reconstitute pUL34 into GUVs are generally applicable for other single and multiple transmembrane-spanning proteins and thus presents a valuable tool for studying these proteins in minimal and well defined membrane systems.

Methods

$DiDC_{18}$ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt), Alexa Fluor 546 carboxylic acid succinimidyl ester and cascade blue labelled neutravidin were obtained from Life Technologies, naphtopyrene form Sigma and detergents from Calbiochem. The nuclear envelope lipid mix consists of 5 mol % cholesterol , 2.5 mol % sphingomyelin, 2.5 mol % Na-phosphatidylserine, 10 mol % Na-phosphatidylinositol, 20 mol % phosphatidylethanolamine, and 60 mol % phosphatidylcholine (all from Avanti polar lipids). In lipid mixtures lacking a specific component the respective lipid was replaced by an equimolar amount of phosphatidylcholine.

Protein expression and purification

Constructs for expression of pseudorabies virus pUL31 and pUL34 were generated from a synthetic DNA optimized for codon usage in *E. coli* (Geneart). pUL31 was expressed from a modified pET28a vector with a His_6-tag and a TEV site followed by an EGFP protein amino terminal of pUL31. The soluble domain of pUL34 (aa 1-240), untagged pUL31 as well as EGFP were expressed from a modified pET28a vector with a His_6-tag followed by a TEV site. For C-terminal His_6-tagging of the soluble domain of pUL34 (aa 1-240), the fragment was expressed from a modified pET28a vector lacking the amino terminal His_6-sequence. All proteins were expressed in *E. coli* BL21de3 and purified using Ni-NTA Agarose. Where applicable, the His_6-tags were cleaved off.

Full-length pUL34 and SCL1 were expressed from a modified pET28a vector with an N-terminal MISTIC (membrane-integrating sequence for translation of integral membrane protein constructs) fragment [45] followed by a thrombin cleavage site and purified as described [46]. Proteins were labelled using Alexa Fluor 546 carboxylic acid succinimidyl ester in 200 mM $NaHCO_3$ pH 8.4 or the same buffer containing 1% (wt/vol) cetyltrimethylammonium bromide for labelling of transmembrane proteins and purified by gel filtration on a Sephadex G50 fine column (GE Healthcare).

Generation of GUVs

Detergent solubilized and labelled SCL1 and pUL34 were reconstituted in proteo-liposomes via gelfiltration. For this, 20 µl of the nuclear envelope lipid mix (30 mg/ml in 10% octylglucopyranoside) was mixed with 20 µl of 2 µM pUL34 or SCL1 protein and 100 µl PBS. The sample was applied to a Sephadex G50 fine filled Econo chromatography column (0.5 × 20 cm, Biorad) to remove the detergent. The formed proteo-liposomes were collected and pelleted for 30 min at 100.000 rpm in a TLA120.2 (Beckman Coulter) rotor at 4°C. The pellet was resuspended in 120 µl 20 mM HEPES pH 7.4, 100 mM KCl, 1 mM DTT. 5 µl of resuspended proteo-liposomes were dried onto two 5 mm x 5 mm platinum gauzes (ALS) under vacuum for at least 1 h at RT. The gauzes were placed in parallel (5 mm distance) into a cuvette (UVette, Eppendorf) and submerged in 259 mM sucrose solution and for 140 min an AC electric field with 10 Hz, 2.2 V was applied followed by 20 min 2 Hz at 42°C.

Lipid GUVs were generated from chloroform dissolved lipid mixes where indicated supplemented with 1 mol % Ni-NTA-DGS (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl], Avanti Polar lipids) and 0.8 nM $DiDC_{18}$ as described [47].

GUV vesicle budding reaction

An 8 well glass observation chamber (Chambered #1.0 Borosilicate Coverglass System, Lab-Tek) was blocked with 5 % (wt/vol) BSA in PBS and washed with PBS. For each reaction 50 µl of freshly prepared GUVs were mixed with 150 µl PBS and placed into a well. Unless indicated otherwise, all soluble proteins were added to a final concentration of 500 nM, cascade blue labelled neutravidine at 3.8 µM. Proteins and buffers used for GUV preparation matched the osmolarity of the sucrose solution. The mixture was incubated for 5 min and imaged immediately at room temperature on an inverted Olympus Fluoview 1000 confocal laser scanning system utilizing an UPlanSApo 60x/1.35 oil objective. The cascade blue labelled neutravidine was excited by a 405 nm DPSS laser and emission was collected between 425 to 475 nm. EGFP and Alexa 546 were excited by an argon ion laser at 488 nm and 515 nm. Emission for EGFP was collected between 500 to 545 nm and between 570 to 625 nm for Alexa 546. DiD was excited by a 635 nm DPSS laser and emission was collected between 655 to 755 nm. The pinhole was set to one airy. 3D reconstructions were generated using Imaris software (version 7.4, Bitplane).

Transfection experiments

pUL31 was cloned into an pEGFPC3 vector (Clontech), pUL34 into the same vector in which the EGFP was replaced by an mCherry protein. pEGFPC3 based vectors or pUL31 chimera with C-terminal transmembrane regions were generated by fusing the transmembrane region of pUL34 (aa 238-262) or *Xenopus laevis* SCL1/BC08 (aa 74-96). pUL31 with an N-terminal transmembrane region was generated by introducing the N-terminus of *Xenopus laevis* POM121 (including the transmembrane region) into a modified pEGFPC3 vector with an signal sequence of rat GP210 upstream of the EGFP [48] (see supplementary information for resulting amino acid sequences). Constructs were transfected into HeLa (S3) cells using Fugene 6 (Promega) and fixed after 24 h with 4% paraformaldehyde containing 1 µg/ml DAPI (4',6-Diamidin-2-phenylindol).

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Figure legends

Fig. 1: The pUL31-pUL34 complex is sufficient to induce intralumenal vesicles

a) Recombinant Alexa-546 labelled pUL34 (red) was reconstituted into giant unilamellar vesicles (GUVs). The GUV membrane was stained with the lipophilic dye $DiDC_{18}$.
b) Alexa-546 labelled pUL34 or the inner nuclear membrane protein SCL1 were reconstituted GUVs (red in overlay). Addition of EGFP-pUL31 (green in overlay) induced formation of intra-GUV vesicles on pUL34 but not SCL1 GUVs. Quantitation shows the mean (+/-SEM) of three independent experiments each including at least 70 GUVs per condition and experiment.
c) Cascade blue labelled neutravidin (fluid phase marker) was added together with EGFP-pUL31 (upper row) or 15 min after EGFP-pUL31 addition to GUVs (lower row) with reconstituted Alexa-546 labelled pUL34 or SCL1.
d) Three dimensional reconstruction of an EGFP-pUL31 (green) treated pUL34-GUV (red).
e) Representative pictures illustrating the sequential steps of the invagination process visualizing EGFP-pUL31 (green) and pUL34 (red).
Scale bars are 5 µm (2 µm in panel d).

Fig. 2: Membrane tethering of pUL31 is sufficient to induce intralumenal vesicles

a) An Alexa-546 labelled N- or C-terminally His_6-tagged pUL34 fragment (upper or lower panel, respectively) comprising the soluble domain (aa 1-240) was bound to 1% Ni-NTA-DGS containing GUVs. Membranes were stained with $DiDC_{18}$. Addition of EGFP-pUL31 induced intra-GUV vesicles.
b) His_6-tagged-EGFP or His_6-tagged-EGFP-pUL31 was directly bound to Ni-NTA-DGS containing GUVs. Membranes were stained with $DiDC_{18}$. Quantitation shows the mean (+/-SEM) of three independent experiments each including at least 80 GUVs per condition and experiment.
c) Three dimensional reconstruction of a His_6-EGFP-pUL31 (green) treated GUV containing 1% Ni-NTA-DGS. Membranes were stained with $DiDC_{18}$ (grey)
d) Representative pictures illustrating the sequential steps of the invagination process visualizing His_6-EGFP-pUL31 on Ni- NTA-DGS containing GUVs.
Scale bars are 5 µm.

Fig. 3: pUL31 self-interacts on membranes

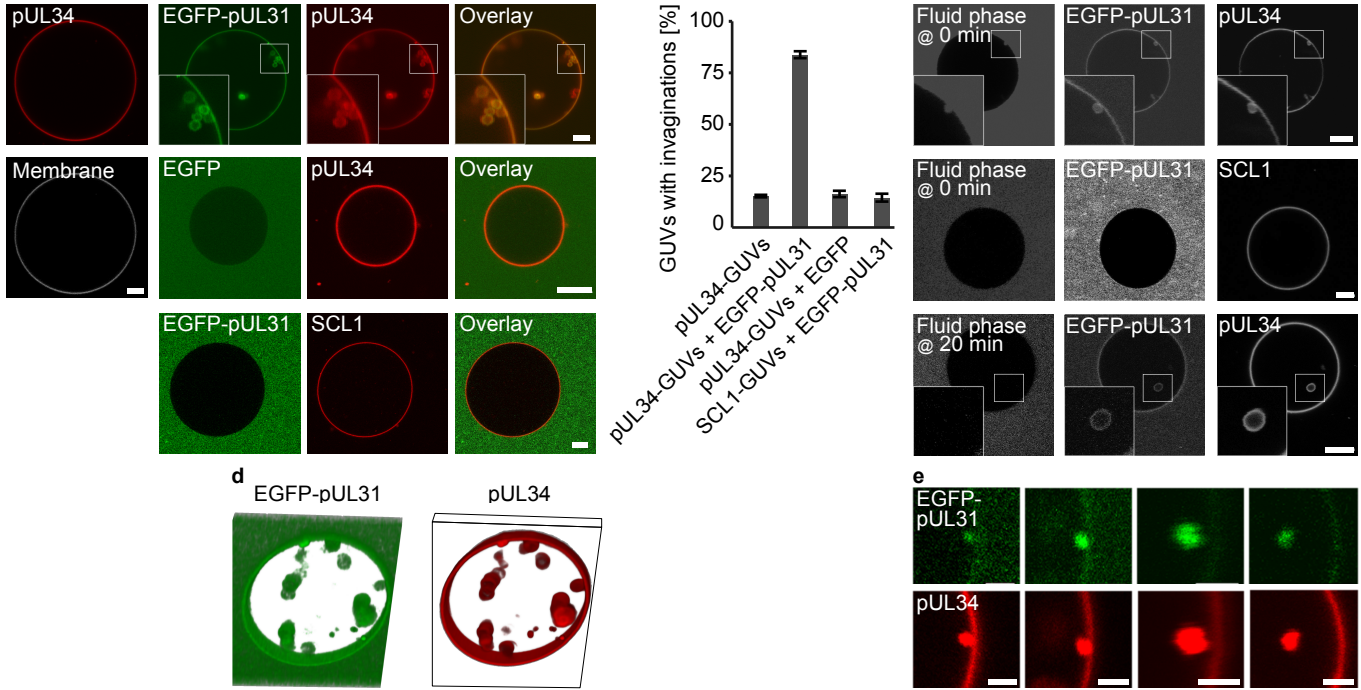
Where indicated 60 nM His_6-tagged-pUL31 was directly bound to Ni-NTA-DGS containing GUVs. Increasing amounts of EGFP-pUL31 was added to the GUVs showing a pUL31 mediated membrane recruitment of EGFP-pUL31 and formation of intra-GUV vesicles. Quantitation shows the mean (+/-SEM) of three independent experiments each including at least 60 GUVs per condition and experiment.

Fig. 4: Cholesterol and sphingomyelin are required for pUL31 mediated vesicle formation

a) Recombinant Alexa-546 labelled pUL34 was reconstituted into GUVs containing the nuclear envelope lipid mix (complete lipid mix) or the same lipid mix lacking either cholesterol (chol), sphingomyelin (SM), the negatively charged phospholipids phosphoinositol (PI), phosphatidylserine (PS) or both (PI+PS). Membrane invaginations were induced and quantified after EGFP-pUL31 addition (mean (+/-SEM) of three independent experiments each including at least 50 GUVs per experiment and condition).
b) His_6-tagged-EGFP-pUL31 was directly bound to Ni-NTA-DGS containing GUVs with the same lipid compositions as in (A) and invaginations were quantified (mean (+/-SEM) of three independent experiments each including at least 60 GUVs per experiment and condition) Scale bars are 5 µm.

Fig. 5: Membrane tethering of pUL31 in cells induces nuclear envelope alterations

a) HeLa cells were transfected with EGFP-pUL31 (green), mCherry-UL34 (red) or both. Co-transfection results in a co-localization of both proteins in speckles in close proximity of the nuclear envelope.
b) Chimeric constructs of EGFP-pUL31 C-terminally fused to the transmembrane region of pUL34 or SCL1 or N-terminally fused to the transmembrane region of POM121 were transfected in HeLa cells.
Chromatin is stained with DAPI (blue), scale bars are 5 µm.


a
pUL34
Membrane
b
EGFP-pUL31
pUL34
Overlay
EGFP
pUL34
Overlay
EGFP-pUL31
SCL1
Overlay
GUVs with invaginations [%]
100
75
50
25
0
pUL34-GUVs
pUL34-GUVs + EGFP-pUL31
pUL34-GUVs + EGFP
SCL1-GUVs + EGFP-pUL31
c
Fluid phase @ 0 min
EGFP-pUL31
pUL34
Fluid phase @ 0 min
EGFP-pUL31
SCL1
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EGFP-pUL31
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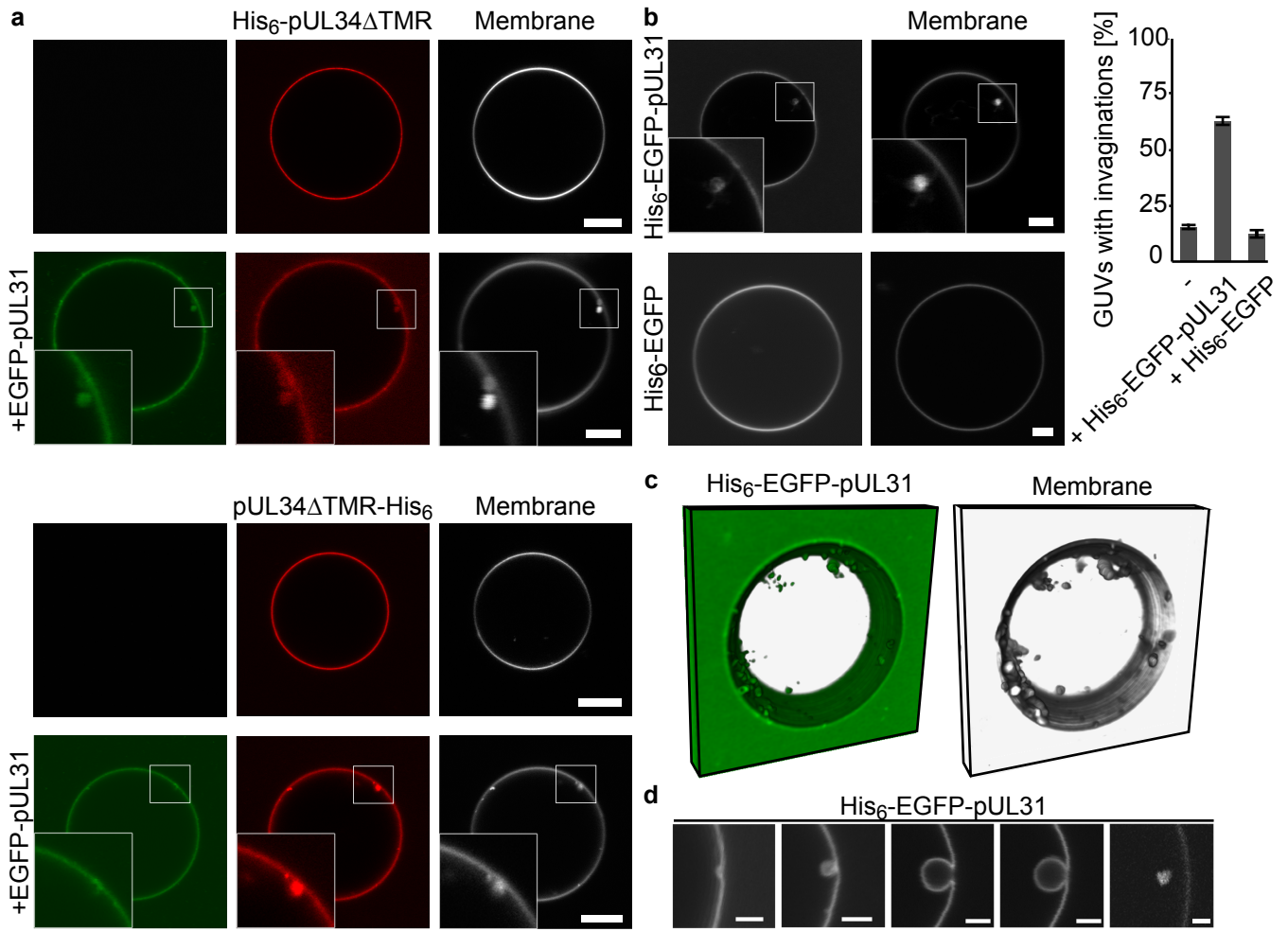
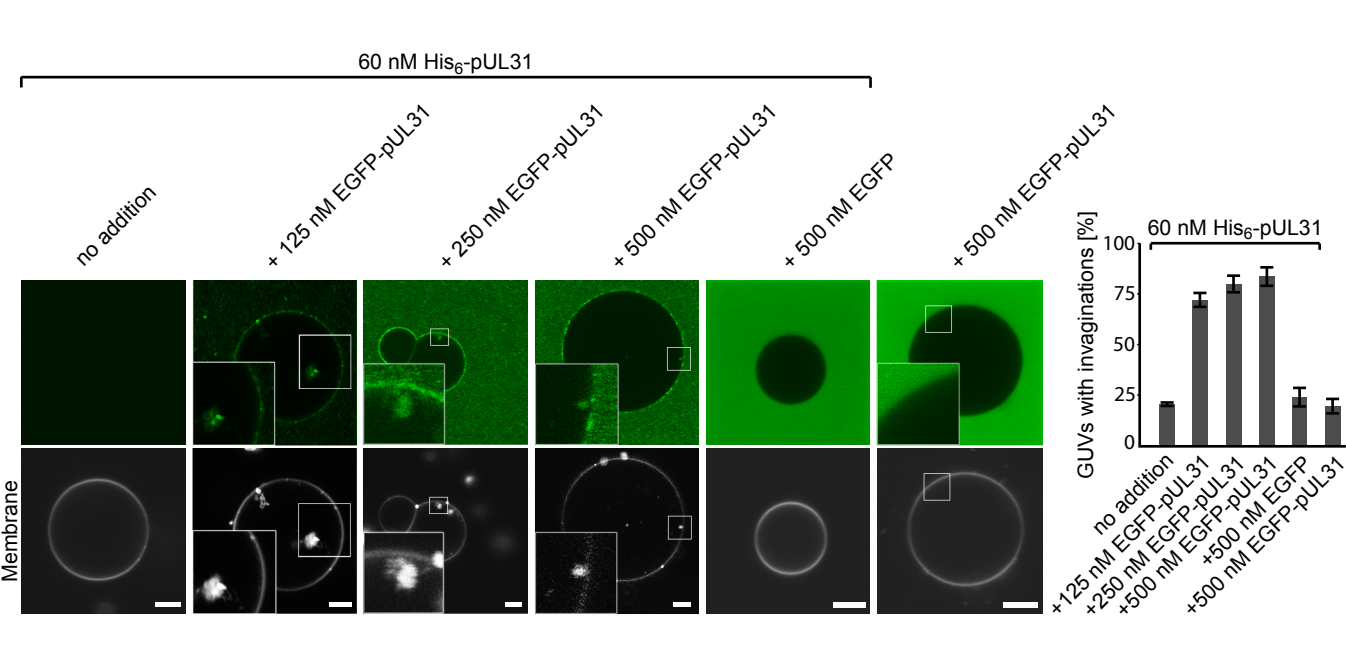
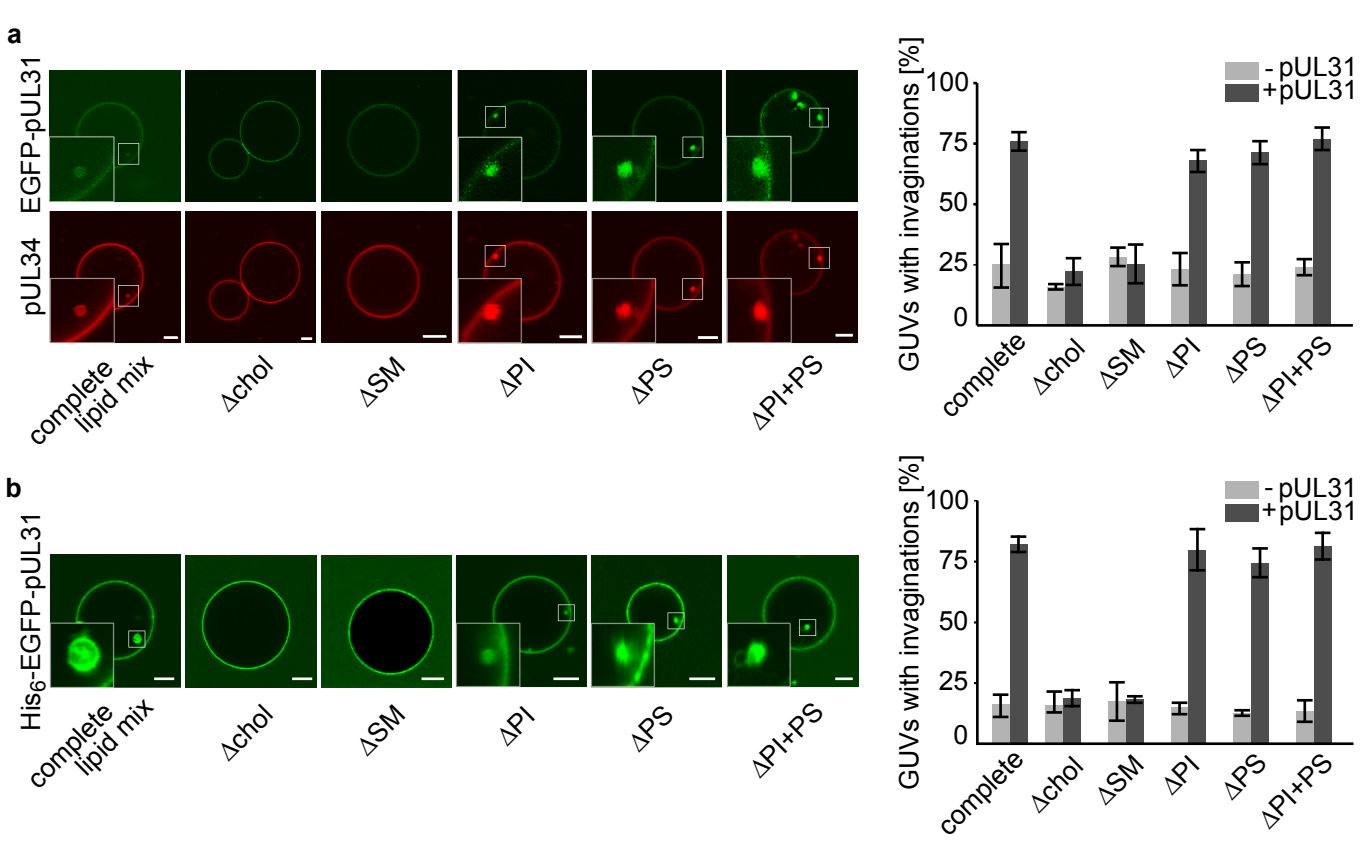
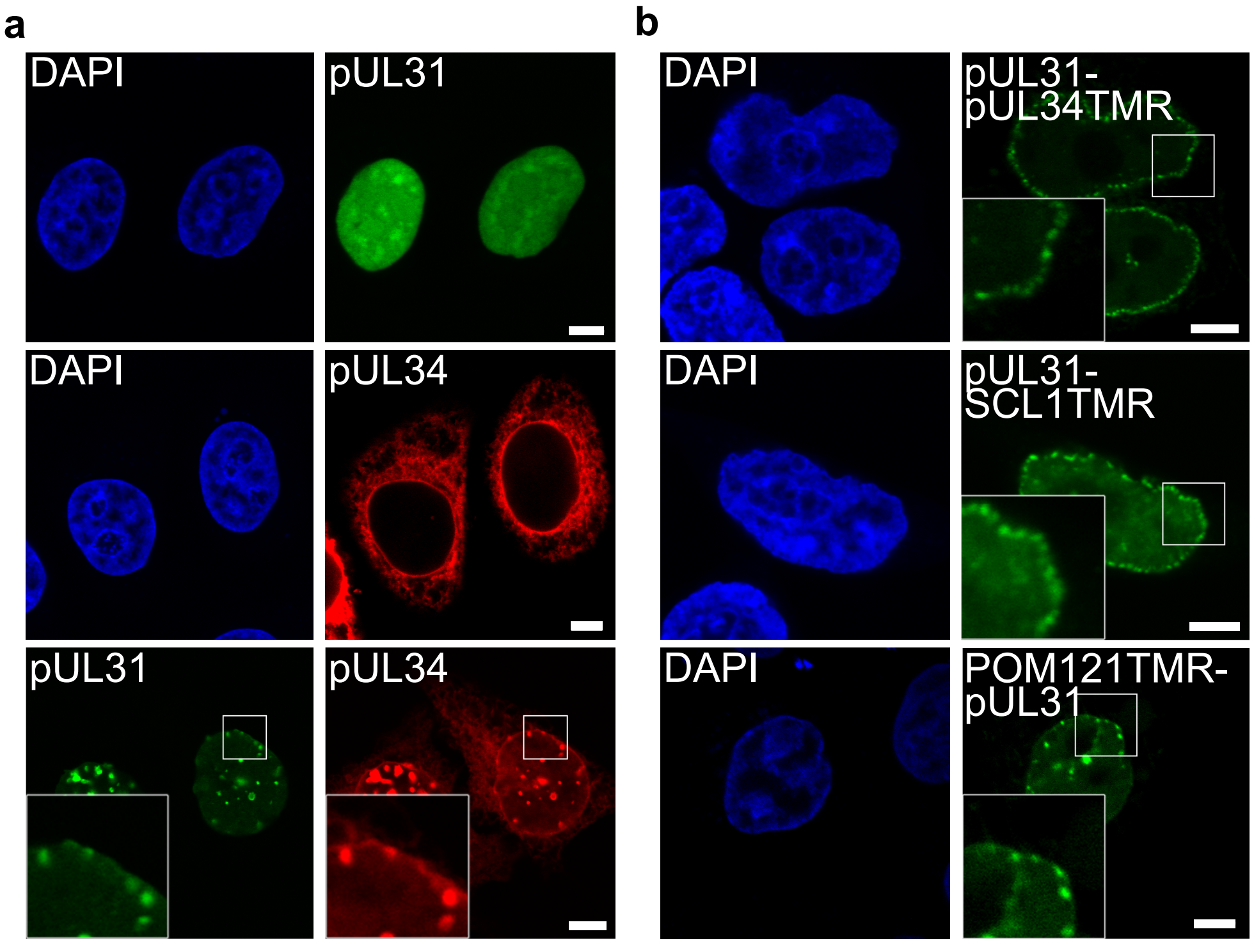
d
EGFP-pUL31
pUL34
e
EGFP-pUL31
pUL34


a
His6-pUL34ΔTMR
Membrane
+EGFP-pUL31
pUL34ΔTMR-His6
Membrane
+EGFP-pUL31
b
Membrane
His6-EGFP-pUL31
His6-EGFP
GUVs with invaginations [%]
100
75
50
25
0
-
+ His6-EGFP-pUL31
+ His6-EGFP
c
His6-EGFP-pUL31
Membrane
d
His6-EGFP-pUL31


60 nM His6-pUL31
no addition
+ 125 nM EGFP-pUL31
+ 250 nM EGFP-pUL31
+ 500 nM EGFP-pUL31
+ 500 nM EGFP
+ 500 nM EGFP-pUL31
Membrane
60 nM His6-pUL31
GUVs with invaginations [%]
100
75
50
25
0
no addition
+125 nM EGFP-pUL31
+250 nM EGFP-pUL31
+500 nM EGFP-pUL31
+500 nM EGFP
+500 nM EGFP-pUL31


a
EGFP-pUL31
pUL34
complete lipid mix
Δchol
ΔSM
ΔPI
ΔPS
ΔPI+PS
GUVs with invaginations [%]
100
75
50
25
0
- pUL31
+pUL31
complete
Δchol
ΔSM
ΔPI
ΔPS
ΔPI+PS
b
His6-EGFP-pUL31
complete lipid mix
Δchol
ΔSM
ΔPI
ΔPS
ΔPI+PS
GUVs with invaginations [%]
100
75
50
25
0
- pUL31
+pUL31
complete
Δchol
ΔSM
ΔPI
ΔPS
ΔPI+PS


a
DAPI
pUL31
DAPI
pUL34
pUL31
pUL34
b
DAPI
pUL31-
pUL34TMR
DAPI
pUL31-
SCL1TMR
DAPI
POM121TMR-
pUL31

Supplementary Information to

A single herpesvirus protein mediates vesicle formation in the nuclear envelope

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pUL31 sequence optimized for E. coli expression

ATGTTTGAACGTCGTCGTCTGCTGCGTCGTAAAAGCAGCGCAGCACGTCGTAAAACCCTGACCCGTGCAGCAC
GTGATCGTTATGCACCGTATTTTGCCTATGCAGCAGCACAGCCGAGTGATGAAGTTACCACCGTTCGTGGTCTG
AGCAATCCGCTGATTAAAACCGCACCGGTTACCCTGCCGTTTGATCTGGGTCAGGCAGTTGCAGATAATTGTCT
GAGCCTGAGCGGTATGGGTTATTATCTGGGTCTGGGTGGTTGTTGTCCGACCTGTGCAGCAGCAGAACCGCGT
CTGGGTCGTAGCGATCGTGCAGCACTGGTTCTGGCCTATGTTCAGCAGCTGAATAGCATTTATGAATATCGTGT
TTTTCTGGCAAGCGTTGCAGCCCGTGATCCGAGCGAACGTGCACTGGAAGAAGTTCTGGCACATCCGGAACTG
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CAGGGTGCACTGATGATGTATGTTGTGTTTCCGGAAAAAAGCGTGCATGTTCATCATCGTGTTCTGGATCGTCT
GCTGGGTGCATGTGCAGGTCATCGTATTGTTGCACATGTTTGGCAGACCATGTTTGTTCTGGTTGTTCGTAAAA
AAGGTGATGGTCGTCCGGCAGATGATGTTCCGGCAGTTAGCGCAAGCGATATTTATTGTAAAATGCGCGATAT
TAGCTTTGATGGTGAACTGCTGCTGGAATATAAACGTCTGTATGCAGCCTTTGAAGATTTTCGTCCTCCGCGTC
CG

pUL34 sequence optimized for E. coli expression

ATGAGCGGCACCCTGGTTCAGCGTCTGAAACTGATTCTGAGCGGTGGTAATCTGCGTTGTAGTGATGGTGAAA
CCGCATGTGATCCGGAACGTCCTCCGACCCGTTGTGTTTTTCAGGTTCATGGTCAGGATGGTAGCAATGATACC
TTTCCGCTGGAATATGTTCTGCGTCTGATGCGTAGCTGGGCACATGTTCCGTGTGATCCGTATGTTCGTGTTCA
GAATACCGGTGTTAGCGTTCTGTTTCAGGGCTTTTTTTTTCGTCCGGCAGATGCACCGCTGGCAGCAATTACCG
CAGAACATAATAATGTTATTCTGGCAAGCACCCATAGCACCGGTATGAGCCTGAGCGCACTGGATGATATTAA
ACGTGCCGGTGGTGTTGATACCCGTCCGCTGCGTGCAATGATGAGCGTTAGCTGTTTTGTTCGTATGCCTCGTG
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ATATCCGCCTCCGGCACGTTTTCCGGCAAGCCTGCTGCATACCCTGCTGGGTCTGCGTCGTCTGGCAGGTTATG
CAGTTGCATGTGTTACCGGTGCACTGGCAATTGTGATTATTCTGAATATGCGC

EGFP-pUL31-UL34TMR construct

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD
HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYI
MADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA
GITLGMDELYKSGLRSMEQKLISEEDLHMFERRRLLRRKSSAARRKTLTRAARDRYAPYFAYAAAQPSDEVTTVRGLS

NPLIKTAPVTLPFDLGQAVADNCLSLSGMGYYLGLGGCCPTCAAAEPRLGRSDRAALVLAYVQQLNSIYEYRVFLAS
VAARDPSERALEEVLAHPELFFAYYVLRDGGLRDVRVLFFEDPDAQGALMMYVVFPEKSVHVHHRVLDRLLGACA
GHRIVAHVWQTMFVLVVRKKGDGRPADDVPAVSASDIYCKMRDISFDGELLLEYKRLYAAFEDFRPPRPLDLRRLA
GYAVACVTGALAIVIILNMR

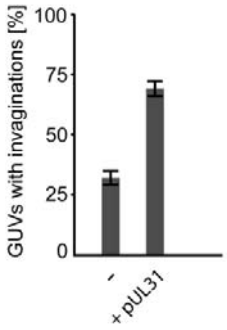
EGFP-pUL31-SCL1TMR construct

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD
HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYI
MADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA
GITLGMDELYKSGLRSMEQKLISEEDLHMFERRRLLRRKSSAARRKTLTRAARDRYAPYFAYAAAQPSDEVTTVRGLS
NPLIKTAPVTLPFDLGQAVADNCLSLSGMGYYLGLGGCCPTCAAAEPRLGRSDRAALVLAYVQQLNSIYEYRVFLAS
VAARDPSERALEEVLAHPELFFAYYVLRDGGLRDVRVLFFEDPDAQGALMMYVVFPEKSVHVHHRVLDRLLGACA
GHRIVAHVWQTMFVLVVRKKGDGRPADDVPAVSASDIYCKMRDISFDGELLLEYKRLYAAFEDFRPPRPLDNRKN
MLLSVAIFLLFALGYYCWTL

EGFP-Pom121TMR-pUL31

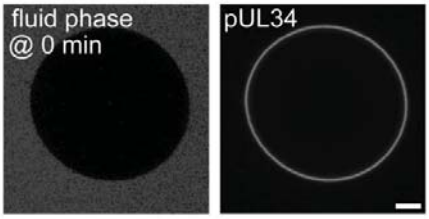
MARASLIQPGLWALLLLQAVGPAVAAKLGGGGGTSRPVATMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGE
GDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA
EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTP
IGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKSGLRSMEQKLISEEDLHMEGGCCWD
LVKRIQPIALPLALSLVTCALAYIYNQLTVAGLLLASCWTYRSSGRRIMFERRRLLRRKSSAARRKTLTRAARDRYAPYF
AYAAAQPSDEVTTVRGLSNPLIKTAPVTLPFDLGQAVADNCLSLSGMGYYLGLGGCCPTCAAAEPRLGRSDRAALVL
AYVQQLNSIYEYRVFLASVAARDPSERALEEVLAHPELFFAYYVLRDGGLRDVRVLFFEDPDAQGALMMYVVFPEKS
VHVHHRVLDRLLGACAGHRIVAHVWQTMFVLVVRKKGDGRPADDVPAVSASDIYCKMRDISFDGELLLEYKRLYA
AFEDFRPPRP

EGFP is indicated in green, pUL31 in red, the respective parts of pUL34, *Xenopus laevis* SCL1 and POM121 in blue, linker regions in black and the gp210 signal sequence in violet.



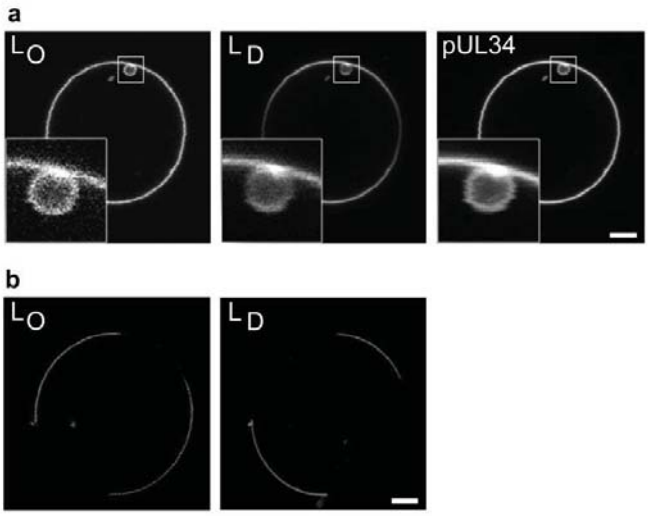
Supplementary Figure S1: pUL31-pUL34 mediated vesicle formation is not mediated by the fluorescent tags

Recombinant unlabelled pUL34 was reconstituted into GUVs stained with $DiDC_{18}$. The presence of intra-GUV vesicles was quantified in the absence or presence of unlabelled pUL31. Quantitation shows the mean (+/-SEM) of three independent experiments each including at least 80 GUVs per experiment and condition.



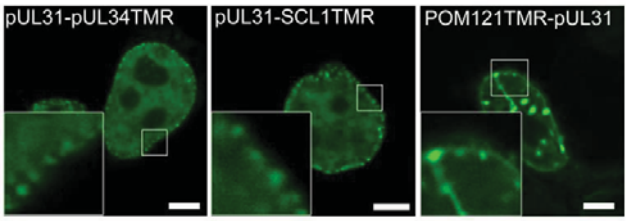
Supplementary Figure S2: pUL34-GUVs show no uptake of a fluid phase marker in the absence of pUL31

Cascade blue labelled neutravidin as fluid phase marker was added in the absence of EGFP-pUL31 to pUL34-GUVs.



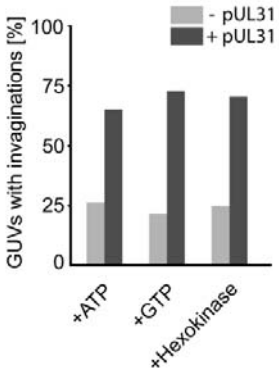
Supplementary Figure S3: pUL31-pUL34 interaction does not induce a phase separation on the GUV membrane

a) UL31 was added to pUL34-GUVs loaded with the membrane dyes naphthopyrene and DiDC18, which label the liquid ordered (LO) or liquid disordered phase (LD), respectively (A). Although we cannot exclude an enrichment of specific lipids in internal vesicles there is no induction and separation of liquid ordered and disordered phases detectable.
b) As a control for the functionality of the membrane dyes their segregation is tested on phase separating membrane GUVs (33 mol % cholesterol, 33 % sphingomyelin, 33 % phosphatidylcholine).



Supplementary Figure S4: Membrane tethering of pUL31 induces nuclear envelope alterations in HEK293 cells

Chimeric constructs of EGFP-pUL31 C-terminally fused to the transmembrane region of pUL34 or SCL1 or N-terminally fused to the transmembrane region of POM121 were transfected in HEK293 cells. Chromatin is stained with DAPI (blue), scale bars are 5 µm.



Supplementary Figure S5: pUL31-pUL34 mediated GUV invaginations do not require cellular energy

EGFP-pUL31 induced formation of internal vesicles on pUL34-GUVS was quantified in the presence of 10 mM ATP, 10 mM GTP or an energy depletion system (135 mM glucose and 10 U/ml hexokinase). For each condition at least 50 GUVs were analysed.