Functional characterization of the nucleoporins Nup50 and Nup155 in postmitotic nuclear pore complex assembly

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2017

Tag der mündlichen Prüfung:	Dienstag, 19 September 2017
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Abbreviations

µm	micrometer
A. nidulans	*Aspergillus nidulans*
BAF	Barrier-to-Autointegration factor
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BRG1	Brahma-related gene 1
C. elegans	*Caenorhabditis elegans*
C. thermophilum	*Chaetomium thermophilum*
D. discoideum	*Dictyostelium discoideum*
D. rerio	*Danio rerio*
DAPI	4',6-Diamidino-2-phenylindole
DiIC18	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine Perchlorate
DMP	Dimethyl Pimelimidate
DNA	Deoxiribonucleic acid
E. coli	*Escherichia coli*
eGFP	Enhanced green fluorescent protein
EM	Electron microscopy
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FG	Phenylalanine glycine
gp210	Glycoprotein 210
GST	Glutathione-S-transferase
hCG	human chorionic gonadotropin
Impα	Importin α
INM	Inner nuclear membrane
IP-MS	Immunoprecipitation-mass spectrometry
kDa	KiloDalton
Lap2β	Lamin-associated polypeptide 2β

LINC	Linker of nucleoskeleton and cytoskeleton
MEL28/ELYS	Maternal effect lethal/embryonic large molecule derived from yolk sac
mRNA	Messenger RNA
MS	Mass spectrometry
NDC1	Nuclear division cycle 1
NE	Nuclear envelope
NEBD	Nuclear envelope breakdown
NET	Nuclear envelope transmembrane proteins
NLS	Nuclear localization signal
NPC	Nuclear pore complex
Nup	Nucleoporin
ONM	Outer nuclear membrane
POM121	Pore membrane protein 121
Ran	Ras-related nuclear protein
RanBP	Ran binding protein
RNA	Ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-page	Sodium dodecylsulfate – polyacrylamide gel
SUMO	Small ubiquitin-like modifier
TEV	Tobacco etch virus
VRK-1	Vaccinia-related kinase 1
X. laevis/ Xenopus	*Xenopus laevis*

Acknowledgements

In the course of my PhD I have been blessed with many beautiful relationships. This page in my thesis will never be enough to describe how grateful I am to every person that I will mention, and to those whom limited space will not allow me to.

Thank you, Wolfram. No other supervisor could have introduced me into science as you did, with your guidance. I learned from you how much hard work and passion go into every experiment and every line of thinking. You taught me how to ask the right questions and how to get the correct answers, never leaving intellectual honesty and scientific integrity behind. I am honoured to have been able to gain my PhD in your lab.

I have a debt of gratitude towards my thesis advisors Dr. Fulvia Bono and Prof. Ana Garcia-Saez, who supported my PhD with their advice and guidance to the end. I am happy to have the chance to thank Christian Liebig and Aurora Panzera with their support with the light microscopy facility and the Proteome Center of Tübingen in the person of Prof. Boris Macek for support with IP-MS experiments. I would like to thank Nadine for the wonderful maintainance of the frog facility. As a member of the IMPRS “From Molecules to Organisms” PhD program, I have greatly benefitted from the precious work of Sarah Danes, our coordinator.

I am deeply grateful to Allana, who has been an example for me, in and outside of the lab. She is one of the best friends anyone can only hope to meet once in a lifetime. She gave me a shelter in the darkest time of my stay in Tübingen and I will never forget that.

I am grateful to all of the people that in the years preceded and followed me in the Antonin lab: Allana, Ben, Dani, Franzi, Hideki, Katharina, Karin, Micha, Mario, Marion, Nadja, Natalia, Nathalie, Ruchika, Susanne, Nathanael, Rabia. I wish I had the chance to work with Ada as well. I like to think that we have been the kind of lab where we could learn from each other, and be teacher of each other in return. Frog tricks, lab results, cake recipes, nucleoporins, coffee making, frustrations, chromatin, graduation hats making, presenting tips, publishing, and GUVs, there are few topics we haven´t touched.

I am grateful to Dani for his delightful comments to our cakes and another lot of things – but a thing is a thing, another thing is another thing.

The Antonin lab has worked for years in a remarkable environment; being a member of the FML has given me the chance to be inserted in a scientific melting pot of different research

lines. I have found in the FML a beating heart full of talented scientists and friends. Alex, David, Enni, Hannes, Jelena, JP, Luciano, Marek, Maria, Maria L., Michelle, Sarah, Stan, Stefano, to you goes my gratitude. The FriKos; the Christmas parties; the time in tissue culture; the FML kitchen transformed in a career advice desk; I am grateful for all of this.

Also, Tamia, Claudia, Helena, Fabio, Fritz, Milos, thank you.

Liam, to me you are an anchor, and the sea at the same time. I am proud of us.

The fact that I am here writing in the first place would have never occurred if I weren't a member of the best, most imperfect, messiest families in the world. Mum, dad: grazie. Thanks to Giulia, to which this thesis is dedicated - theses always come in three. I have been blesses in the highest possible way with the gift of three people that remind me every day how "there is always a little part of the sky you can raise your head to".

Contents

Summary

The nucleus encloses the genetic material of the cell. Contact and exchange between the nuclear and cytoplasmic compartments are tightly regulated by nuclear pore complexes (NPCs), huge multimeric protein complexes that span the double nuclear envelope. These must ensure specific and timely regulated access to the nucleus to all the factors required for its correct development and maintenance. In order to be able to do so, NPCs must form in a tightly regulated fashion, producing transport-competent complexes as a result. The formation of the pores occurs in two different stages of the cell cycle, during interphase and after mitosis. During these stages, the pores are likely assembled by distinct pathways, due to the differences existing in morphology at these moments of the life of the cell. A deeper understanding of the mechanism of interaction of factors involved in NPC formation, which is far to be complete, will contribute to unravel one of the biggest, most complex cellular events.

In the course of my PhD, I analyzed interactions and ordered recruitment of nucleoporins. *In vitro* nuclear assembly using *Xenopus* egg extracts revealed that a small component of NPCs, nucleoporin Nup50, is required at the early stages of postmitotic NPC assembly. Nup50 is a small nucleoplasmic nucleoporin known to be required for nuclear import. I have identified two distinct sites on the protein that are important for its postmitotic function, each of them dispensable if the other is functional, but required if the other is mutated or compromised. Interestingly, Nup50 binds the NPC assembly initiator MEL28/ELYS; in spite of this, I proved that their interaction is not required for postmitotic NPC assembly, and that they probably perform redundant functions.

Additionally, I have studied the role of the domains of nucleoporin Nup155. Nup155 is a membrane binding protein required for postmitotic NPC assembly at a later step than Nup50. It contains an N-terminal domain, which arranges as a β-propeller fold in its tertiary structure, and a C-terminal domain, which folds as a α-solenoid. Depletion of Nup155 causes failure by *Xenopus* egg extracts to form nuclear pore complexes and a closed nuclear envelope *in vitro*. Nup155 contains a membrane-binding region within its N-terminal. *In vitro* nuclear assembly using *Xenopus* egg extracts show that this membrane binding activity is required at the end of mitosis, as a mutant of Nup155 full length construct affecting the residues involved in membrane binding was not able to replace the endogenous protein in Nup155 depleted extracts to a comparable level with the Nup155 wildtype. Moreover, addback of an N-terminal truncation of Nup155, containing the membrane binding domain, was able to restore nuclear

envelope, but not nuclear pore complex assembly. The work further indicates that Nup155 is found in a closed conformation in solution. Its binding site for Nup53 is found in the N-terminal, but it is partially blocked by its C-terminal, that prevents arranging of the optimal surface contact. Nup93 is required for the conformational change to happen: when Nup93 binds Nup53, the contact surface between Nup53 and Nup155 is optimized and, in this context, Nup93 can localize to the nascent pore and further recruits the Nup-62 complex.

Zusammenfassung

Der Zellkern umschließt das genetische Material der Zelle. Kontakt und Austausch zwischen den nuklearen und zytoplasmatischen Kompartimenten werden durch Kernporenkomplexe (NPCs) vermittelt. NPCs sind riesige multimere Proteinkomplexe, die in die Kernhülle integriert sind. Dafür müssen sich NPCs präzise und wohl-geordnet zu transportkompetenten Komplexe aufbauen. Die Bildung der Poren erfolgt in zwei verschiedenen Stadien des Zellzyklus: in der Interphase und nach der Mitose. Während diesen Stadien werden die Poren wahrscheinlich aufgrund morphologischer Unterschiede der Zellarchitektur auf unterschiedlichen Wegen zusammengebaut. Ein tieferes Verständnis des Mechanismus der Interaktion von NPC-bildenden Faktoren könnte dazu beitragen, einen der größten und komplexesten zellulären Vorgänge zu entschlüsseln.

Im Laufe meiner Promotion analysierte ich Interaktionen und die geordnete Rekrutierung von Kernporenproteinen. *In-vitro*-Zusammenbau des Zellkerns unter Verwendung von Xenopus-Eiextrakten ergab, dass eine Komponente der NPCs, das Kernporenprotein Nup50, in den frühen Stadien der postmitotischen NPC-Bildung erforderlich ist. Nup50 ist ein kleines nukleoplasmatisches Kernporenprotein, von dem bekannt ist, dass es für den Kernimport erforderlich ist. Ich habe zwei verschiedene Stellen in Nup50 identifiziert, die zusammen für die postmitotische Funktion wichtig sind. Beide Stellen sind entbehrlich, wenn die andere funktional ist, aber erforderlich, wenn die jeweils andere Stelle mutiert oder kompromittiert ist. Ich konnte außerdem zeigen, dass Nup50 den Initiator des NPC-Aufbaus, MEL28 / ELYS, bindet, aber diese Interaktion nicht für die postmitotische NPC-Bildung erforderlich ist.

Darüber hinaus habe ich die Rolle der Domänen des Kernporenproteins Nup155 erforscht. Nup155 ist ein membranbindendes Protein, das für die postmitotische NPC-Bildung in einem späteren Schritt als Nup50 erforderlich ist. Es enthält eine N-terminale Domäne, die als β-Propeller in ihrer Tertiärstruktur angeordnet ist, und eine C-terminale Domäne, die sich als α-Solenoid faltet. Durch Depletion von Nup155 können Xenopus-Eiextrakte *in vitro* nicht mehr Kernporenkomplexe und eine geschlossenen Kernhülle bilden. Nup155 enthält eine membranbindende Region innenhalb ihres N-Terminals. *In-Vitro*-Zellkernrekonstitutionen unter Verwendung von Xenopus-Eiextrakten zeigen, dass diese Membranbindungsaktivität am Ende der Mitose erforderlich ist, da eine Mutante des Nup155 Proteins, die nicht mehr an Membranen binden kann, nicht in der Lage war, das endogene Protein in Nup155-depletierten

Extrakten zu ersetzen. Allerdings war das Hinzufügen von Nup155, welches am C-Terminus gekürzt wurde, in der Lage, die Kernhülle wiederherzustellen, aber nicht den Kernporenkomplex zu bilden. Diese Arbeit zeigt weiter, dass Nup155 in Lösung in einer geschlossenen Konformation gefunden werden kann. Die Bindungsstelle für Nup53 befindet sich am N-Terminus, ist aber teilweise durch den C-Terminus blockiert, was den optimalen Oberflächenkontakt der beiden Proteine verhindert. Nup93 ist erforderlich für die Konformationsänderung: Wenn Nup93 Nup53 bindet, wird die Kontaktfläche zwischen Nup53 und Nup155 optimiert und in diesem Zusammenhang kann sich Nup93 an die naszierenden Pore lokalisieren und den Nup-62-Komplex rekrutieren.

1. The evolutionary rise of the nucleus

The nucleus represents the most remarkable difference in cellular organization between eukaryotic and prokaryotic organisms. The establishment of a physical barrier between the cytoplasm and the genetic material offered, together with the new possibilities of regulation, a new set of challenges for the newborn eukaryotic organisms. These include: the uncoupling of mRNA transcription from protein translation; the export of the mRNA, which from that moment on was required to be exported outside of the nucleus; the need for an efficient, yet accurate set of mechanisms allowing import of the newly extra-nuclear synthesized proteins for a variety of processes – from DNA replication and silencing, to transcription and gene regulation.

2. Structure of the nucleus

Aside from the genetic material, that defines the nucleus itself, other structures contribute to its formation in animals. This organelle is additional composed by: the nuclear envelope that surrounds the chromatin, the nuclear pore complexes (NPCs), embedded in the NE and bridge cytoplasm and nucleoplasm, the nuclear lamina, a filamentous network that underlies the NE and contributes to the plasticity and shape of the nucleus, and the LINC, a protein complex found between the lamina and the outer nuclear membrane (ONM), spanning the inner nuclear membrane (INM), and that is involved in many nuclear functions, from structural support to stress response. A graphical representation of the described components of the nucleus is depicted in Figure 1. Chromatin itself can be found as euchromatin, less condensed, transcriptionally active (reviewed in (Allis and Jenuwein, 2016)) or heterochromatin, a more compact, inactive state, often brought in proximity of the nuclear periphery (Barrales et al., 2016; Gartenberg et al., 2004; Imai et al., 2000; Taddei et al., 2004).

2.1 The nuclear envelope

The nuclear envelope (NE) encloses chromatin during interphase of the cell cycle. It is composed by two membranes, each formed by a lipid bilayer. The outer one, closer to the cytoplasm, is defined outer nuclear membrane (ONM), and its membranes extend to the endoplasmic reticulum (ER), with which it is contiguous (see review (Voeltz et al., 2002)). The second double membrane is the inner nuclear membrane (INM), that can be defined as a

specialized compartment of the ER, as it contains a set of specific proteins (reviewed in (Schirmer and Gerace, 2005)).

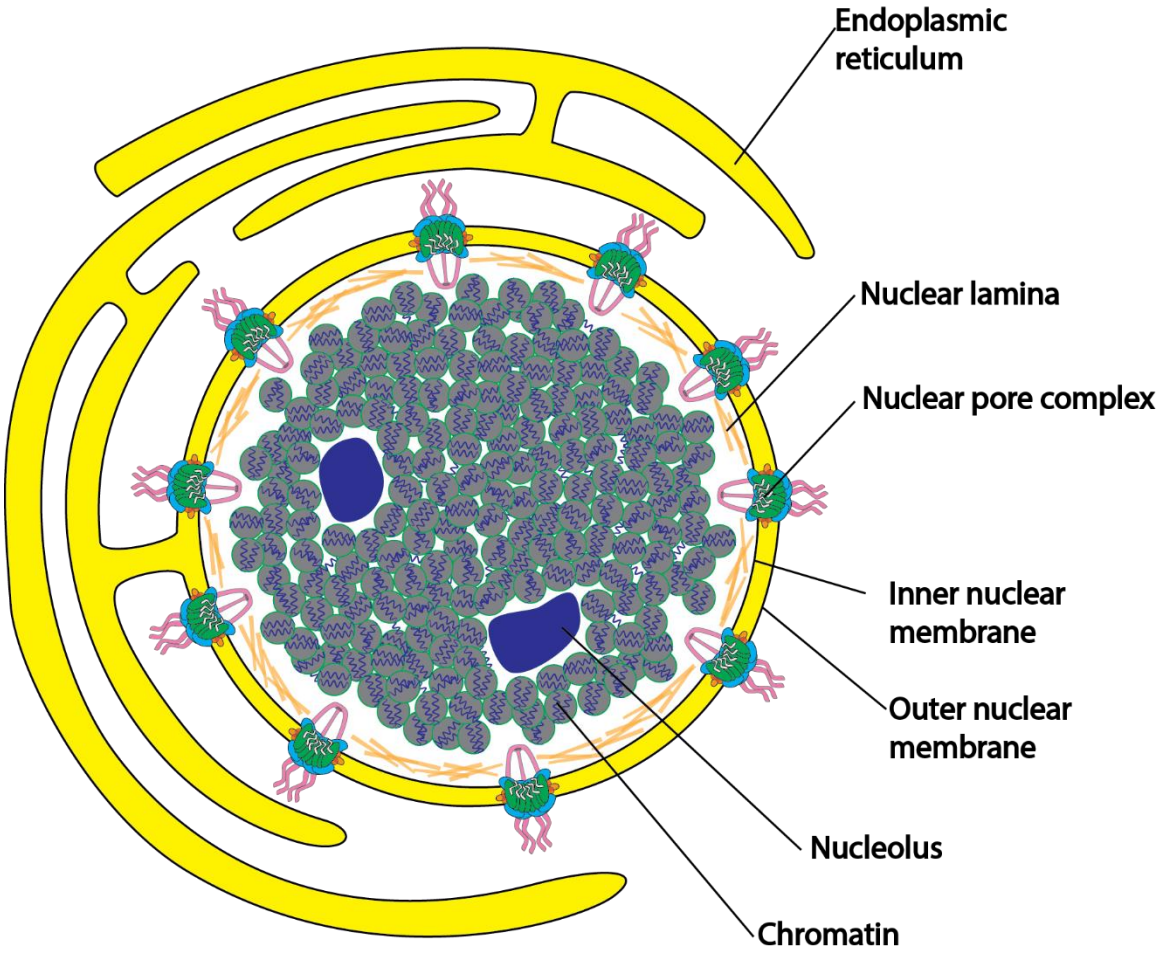


Figure 1. **The nucleus of a eukaryotic cell features distinct traits.** The genetic material, composed by DNA, is wrapped around histone proteins. About 1,7 turns of helical DNA and a histone octamer together form a nucleosome, the core unit of chromatin. Additional scaffolding proteins, in varying arrangements and ratio, contribute to the creation of either euchromatin or heterochromatin, respectively an active and inactive chromatin form, that occupy specific domain within the nucleus (for simplicity not shown in figure). Within chromatin, specialized loci arrange in morphologically distinct regions named nucleoli, that preside at ribosomal biogenesis. Chromatin is enclosed by the nuclear envelope (NE), containing a double membrane. The outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum (ER) and can be considered *de facto* a specialized subregion of the ER. The inner nuclear membrane (INM) contains a unique set of transmembrane proteins that are recruited *in loco* during postmitotic reassembly of the nucleus. The two membranes merge in sites where nuclear pore complexes (NPCs), the gateways of the nucleus, are embedded. Nuclear lamina, composed by lamin proteins, is a fibrous layer sandwiched between the NE and chromatin. The lamina is responsible for a plethora of functions, from tension resistance to signal transduction, and contributes to nuclear migration in specific cells.

Many of these are integral membrane proteins and they overall take the name of NETs (Nuclear Envelope Transmembrane proteins). Many NETs are able to interact with DNA or with chromatin and chromatin associated proteins (Lap2β, lamin-associated polypeptide 2β, MAN1) (Collas et al., 1996; Foisner and Gerace, 1993; Furukawa et al., 1997; Liu et al., 2003; Pyrpasopoulou et al., 1996; Ye et al., 1997). An example is Barrier-to-Autointegration Factor (BAF), a crucial chromatin-binding protein that act as a connection between the INM, the lamina and chromatin (reviewed in (Margalit et al., 2007)), as it is capable to interact with the INM protein LEM domain containing 4 (LEM4). By means of these components, the INM also makes contact with the nuclear lamina (Figure 2).

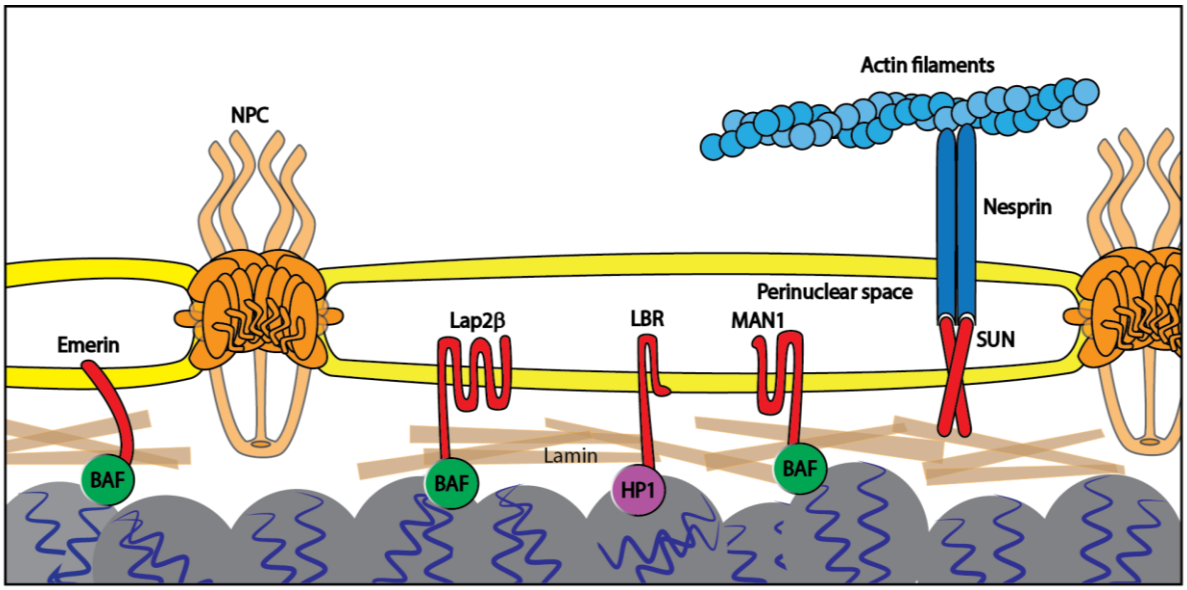


Figure 2. Organization of the nuclear envelope. Proper interplay of the various components in the nuclear environment is key to ensure nuclear integrity, and completion of nuclear functions. Many proteins connect the nuclear lamina to the chromatin: among these, HP1 and Barrier-to-Autointegration factor (BAF), that ensure physical connection, translating into efficient functional communication between these structures. The integral membrane proteins LEM-domain containing 4 (LEM4), emerin, Lap2β span the INM and, by means of lamina- and BAF-binding domains, these ensure tight cross-association of NE, lamina and chromatin. In the perinuclear space, the area enclosed between the INM and the ONM, transmembrane protein SUN associates with KASH domain-containing proteins (in the figure, Nesprin) to form the LINC complex, connecting the nucleoskeletal lamina with the cytoskeleton (in the figure, actin filaments).

NE not only surrounds the genetic material, but interacts with it; in their complexity, these interactions contribute to the regulation of chromatin in multiple ways (Pombo and Dillon, 2015). For instance, silenced chromatin has been found to localize at the nuclear periphery (Barrales

et al., 2016; Kourmouli et al., 2000), and in particular, they do so in a tissue and developmental specific way (reviewed (Van de Vosse et al., 2011)). The role of NET in chromatin regulation is reviewed in (Zuleger et al., 2011).

2.2 The nuclear lamina

A filamentous layer called nuclear lamina, organized as a network of fibrous proteins called lamins, surrounds the chromatin periphery and lies under the INM, with which it is connected via the NETs and the LINC complex. Three lamin genes have been identified, LMNA, LMNB1, LMNB2; they encode for three main isoforms of lamins, lamin A, B, and C, which isoforms differ after alternative splicing events (Goldman et al., 2002). Lamins belonging to group B are the bulk components of the lamina and, as such, they are found on most metazoan cells; lamins belonging to the A and C groups are mainly developmentally expressed and tissue-specific (Stewart and Burke, 1987; Worman et al., 1988). Lamina plays a variety of roles in nuclear biology, ranging from the maintenance of nuclear integrity, to nuclear migration, signal transduction and stress response (Dobrzynska et al., 2016; Gruenbaum et al., 2003; Swift and Dischер, 2014).

2.3 The nuclear pore complex

This approximately 120 megaDalton-sized macromolecular protein complex spans the double nuclear envelope forming an aqueous channel within the envelope. The nuclear pore complex (NPC) is therefore the gatekeeper of the nucleus, the mean by which mRNA, proteins, RNA-protein complexes are shuttled in a controlled manner between in and outside of the nucleus. In a fully formed, functional vertebrate nucleus, the NPC is found from a few hundred to a few thousand copies, depending on the organism, cell type and on the cell cycle stage (Beck et al., 2011; Maeshima et al., 2010; Winey et al., 1997). A single NPC is organized in three superposed rings, accessorized with cytoplasmic filaments and nucleoplasmic basket: the cytoplasmic ring, the nucleoplasmic ring, and the inner ring. The proteins that compose these rings are named nucleoporins or Nups, organized in subcomplexes arranged in an 8-fold symmetrical fashion around the pore, although 9-fold and 10-fold symmetrical NPCs have also been described (Hinshaw and Milligan, 2003). As already mentioned, the roughly 450 nucleoporins that are found in each pore do not arrange in these rings as single proteins, but most of them rather organize in discrete subcomplexes that act as a biochemical minimal units (Matsuoka et al., 1999). Three main subcomplexes can be identified in the NPC: the Nup107-160 complex, alternatively named Y complex, includes in metazoans nucleoporins Nup107,

Nup160, Nup133, Nup85, Nup96, Nup43, Nup37, Sec13 and Seh1 (Boehmer et al., 2003; Harel et al., 2003; Walther et al., 2003a). The terminology Nup93-complex was first used to indicate the Nup93-Nup205 dimer, but it is intended today as the subcomplex containing Nup93, the paralogues Nup205 and Nup188 (see (Andersen et al., 2013)), Nup53 and Nup155 (Vollmer and Antonin, 2014) that is part of the inner ring. Finally, the Nup62-complex, composed by Nup62, Nup98, Nup58/45, Nup54 and RAE1 is the main constituent of the central channel of the pore, responsible for the selectivity barrier that allows or hinders diffusion of solutes above a threshold of about 40 kDa/9 nm (Bonner, 1978; Paine, 1975).

The cytoplasmic and nucleoplasmic rings have a composition not dissimilar from each other, with the exception of MEL28/ELYS, that is exclusively found on the nucleoplasmic ring (Bui et al., 2013). The cytoplasmic and nucleoplasmic rings feature multiple copies of the Y-complexes organized in a head-to-toe superposition (Bui et al., 2013; von Appen et al., 2015), that is connected to the inner ring by bridging copies of Nup155 . This inner ring, composed by members of the Nup93 complex, is responsible for the connection of the NPC to the double nuclear envelope (NE) by contacting to the transmembrane Nups, POM121 (pore membrane protein), gp210 and NDC1 (nuclear division cycle 1), and by close proximity of Nup53 and Nup155 to the NE itself. In fact, Nup53 and Nup155 contain hydrophobic chains that mediate their contact with membrane without including transmembrane domains in their sequences (von Appen et al., 2015).

Two more structures, the nuclear basket and the cytoplasmic filaments, must associate to the the NE-embedded three ring core to complete the nuclear pore complex. Their proper attachment is required for the full functionality of the pore. The cytoplasmic filaments protrude from the NPC to the cytoplasm and are composed by nucleoporins Nup358, Nup214 and Nup88. On the contrary, Nup50, Nup153, Tpr, Nup98 and Rae1, exclusively found on the nucleoplasmic side, are the members of the nuclear basket. Nevertheless, the historical use of terminology “basket” may be inaccurate: there is no evidence that the contacts between the nuclear basket proteins indeed create the shape of a basket. In fact, the nuclear basket components, in particular Nup50 and Tpr, have been proven to have the lowest residential time on NPC among nucleoporins (Niepel et al., 2013; Rabut et al., 2004).

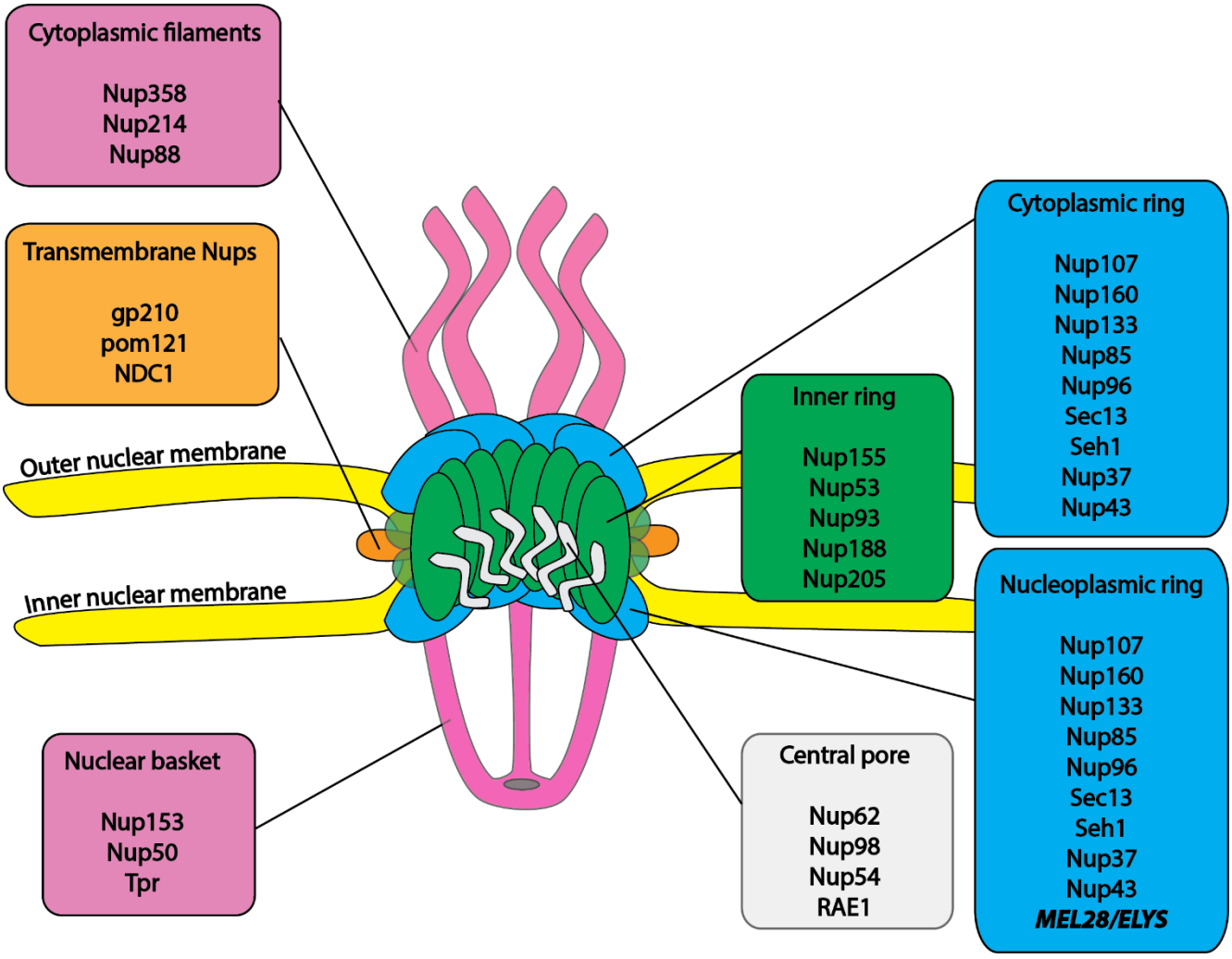


Figure 3. The NPC is composed by 3 stacked rings. Cytoplasmic and nucleoplasmic rings are formed by 16 repeated units each of the Nup107-160 complex, with the addition of MEL28/ELYS only present on the nucleoplasmic ring. Nucleoporins Nup107, Nup160, Nup133, Nup85, Nup37, Nup43, Nup96, Sec13 and SEH1 complete the complex. Between the cytoplasmic and the nucleoplasmic rings, the inner ring complex, formed by Nup155, Nup53, Nup93, Nup205 and Nup188 contacts the NE. The inner ring constitutes the scaffold on which the central channel, composed by Nup62, Nup58, and Nup88, anchors to the whole pore.
On the cytoplasmic side, Nup214, Nup358 and RAE1 protrude from the pore and are responsible for contact with the nuclear export machinery. Nup153, Nup50, and Tpr, positioned on the nucleoplasmic side, take altogether the name of nuclear basket and support nuclear import proteins in their activity.

In all likelihood, this feature is due to necessities of nuclear import shuttling, that requires the players of the cycle to be constantly recycled and available. These two structures, the filaments and the basket, complete the NPC and pose a challenge as they add further levels of complexity when the moment comes, in which the NPC needs to assemble.

Furthermore, the description of the role of NPC in the cell was extended in the course of the last century from gatekeeper of the nucleus to include nowadays genome organizators, chromatin silencers, gene expression regulators (Chatel and Fahrenkrog, 2012). Taken singularly, Nups have been proven to function far beyond the role of members of the NPC, when it became clear that some of them could regulate processes such as gene expression, mitotic regulation and progression, cell differentiation and development (Smitherman et al., 2000).

3. Nucleocytoplasmic transport and the nuclear import cycle

The NPC is responsible for regulated transport of macromolecules of size larger that 40 kDa in and outside of the nucleus. Smaller solutes are generally capable of diffusion through the central channel, with the important exception of steroid hormones, which instead, being cholesterol derivatives, freely diffuse across biomembranes (Chen and Farese, 1999). The best known transport pathway across the NPC is the import pathway, which is rendered possible thanks to a gradient concentration of RanGTP across the NE. This pathway requires proteins termed karyopherins as shuttles, and the components of the nuclear basket are involved in the shuttling process. Proteins that are capable of being imported, named cargos, usually contain motif(s) referred to as nuclear localization sequences (NLSs), that can either be canonical or non-conventional (Freitas and Cunha, 2009), although NLS-independent import phenomena have been reported (Fagotto et al., 1998; Wagstaff and Jans, 2009). NLS mediates the binding of the cargo to Importins. The presence of an NLS *per se*, though, is not sufficient to explain the binding of importins. Biochemical import experiments that make use of chimeric constructs suggest that the tridimensional structure of the protein domain including – and surrounding – the NLS in the cargo critically contributes to the efficiency of importin binding, and, in turn, to its import (Friedrich et al., 2006). Once the importin-cargo complex has formed, interaction with the central channel of the NPC, and the Nup62-complex that constitutes its walls, takes place. The mechanics of these interactions are not yet fully understood, although two main models attempting to describe this process have been proposed, the virtual gating model and the hydrogel/selective phase model, reviewed in (Yang, 2011). Once inside the nucleus, the importin-cargo complex dissociates thanks to the presence of high intranuclear concentrations of Ran-GTP, which bind to Impβ and dissociates it from the cargo.

The gradient of RanGTP across the NE is maintained thanks to two different enzymatic activities localized exclusively on either sides of the nucleus. RanGEF (Ran guanine nucleotide exchange factor), a chromatin binding protein, is able to exchange GDP with GTP on Ran, therefore maintaining an intranuclear high concentration of RanGTP where it is needed for import complex dissociation. RanGAP (Ran GTPase activating protein), together with its partners RanBP1 and RanBP2 (Ran binding proteins 1 and 2), are found on the cytoplasm and stimulates the GTPase activity of Ran.

The small nucleoporin Nup50 is another critical contributor, as it is required for efficient recycle of import proteins and avoidance of futile cycles. Nup50 acts as an agonist to the cargo and competes for Impα binding by means of a small region including its N-terminal (Matsuura and Stewart, 2005). Impα will then release the cargo in the nucleoplasm by exchanging it with Nup50. Impα and Impβ are subsequently recycled to the cytoplasm to execute the next import cycle.

4. Evolution of mitosis

Many lower eukaryotes enter mitosis following a pathway that allows the separation of genetic material to occur within the intact nucleus (Sazer et al., 2014). In these organisms, the most studied of which is the yeast *Saccharomyces cerevisiae*, the mitotic spindle, responsible for generation of the forces that pull sister chromatids apart, is able to polymerize and degrade in a timely manner inside the nucleus. As the mitotic events do not require the nucleus to lose its discrete identity, such mitosis is referred to as closed mitosis. On the contrary, open mitosis features organized breakage of the nuclear envelope, event known as nuclear envelope breakdown (NEBD), which is accompanied by step-by-step disassembly of the NPCs. In other organisms, like the fungus *Apergillus nidulans*, mitotic pathways falls in between these situations, as NE remains intact, but NPCs undergo a partial disassembly (Osmani et al., 2006). In starfish oocytes (Lenart et al., 2003) and in HeLa cells (Dultz et al., 2008), triggering event of NPC disassembly is phosphorylation of Nup98 and consequent detachment of the nucleoporin from the pore structure, event that also constitutes the rate limiting step of the NPC disassembly process (Laurell et al., 2011). Following loss of NPCs and, therefore, of the nuclear barrier, the NE begins to be retracted in the ER, coadjuvated by diffusion of INM proteins across membranes toward the ER compartment. Lamina becomes fenestrated and is

progressively disassembled as lamins are phosphorylated by mitotic kinases. These processes are started by intervention of the mitotic kinase machinery, that includes the CDK1-cyclinB complex, Aurora B kinase, proteins of the NIMA family, and others (for review see (Guttinger et al., 2009)). Nuclear disassembly is required in open mitosis in order for the mitotic spindle, assembled in the cytosol, to be able to access and segregate the genetic material, localized in the nucleus. This new mitotic organization rendered chromatid separation a much better regulated, more error-proof task for the cell, but added a further level of complexity: the need for re-establishment of the nuclear barrier after the nuclear envelope breakdown. Mitotic events tear the nucleus apart and events such as chromatin decondensation and nuclear envelope reassembly reestablish the identity and functionality of the interphasic nucleus. Equally, new NPCs must reassemble at the end of mitosis, to promptly re-establish the permeable nuclear barrier required for a plethora of cellular functions.

5. Nuclear envelope assembly

After being disrupted during NEBD, at the end of mitosis the NE reforms on the decondensing chromatin and by that reestablishes the barrier between the nucleoplasm and the cytoplasm. Nuclear membranes start approaching chromatin in late anaphase. The driving force for this process is constituted by directed migration of INM transmembrane membrane proteins towards chromatin (Anderson et al., 2009). Many INM proteins, among which the aforementioned LBR, emerin, LAP2β, MAN1 associate with chromatin, either directly or by binding with intermediate proteins HP1 or BAF (see Section 2.1). Diverse mechanisms concur to regulate the re-association of nuclear membranes with chromatin and the reestablishment of the NE: these include postmitotic dephosphorylation on INM proteins, regulation of the chromatin proteins BAF and HP1, the Ran system (described in Section 8), and, likely, histone modifications. Many transmembrane INM proteins, including gp210, LBR, LAP2β, emerin, MAN1, NDC1 and POM121 are phosphorylated at the onset of mitosis, which is thought to contribute to the disassembly of the NE and inhibit their re-association with chromatin (Dephoure et al., 2008). Conversely, dephosphorylation of these proteins during mitotic exit could potentially release the inhibition.

An example in support of this mechanism is the recruitment of LBR to chromatin at the end of mitosis. LBR binding to chromatin is prevented *in vitro* by phosphorylation of a specific serine residue in human cells, and LBR dephosphorylation was found responsible of the timing of ER

membrane recruitment of LBR itself. Phosphorylation of the same protein by the serine/arginine-rich protein-specific kinase SRPK1 is required for its association with chromatin *in vitro* (Sellis et al., 2012).

Another example of how phosphorylation/dephosphorylation regulates membrane and INM protein recruitments is given by two chromatin-associated proteins, HP1 and BAF, that link chromosome decondensation and NE formation. Chromatin recruitment of HP1 requires the dephosphorylation of S10H3 and is promoted by H3K9 methylation. HP1 chromatin recruitment in anaphase could cooperate in the association of LBR with chromatin during mitotic exit, in addition to the fact that LBR can also interact directly with DNA, histones and other chromatin-associated proteins (see Section 8). The inner nuclear membrane LEM4 recruits the phosphatase PP2A and at the same time inhibits kinase VRK-1, allowing dephosphorylation of their target BAF, which is then able to recruit further integral membrane proteins like emerin and LAP2β to chromatin during mitotic exit.

The simple recruitment of membranes to chromatin would be incomplete for reestablishment of nucleocytoplasmic compartmentalization. Completion of proper NE reassembly requires fusion of the membranes at the sites where different segments of the newly recruited membranes meet. As the NE and the ER are morphologically similar and physically connected, it is not possible to exclude that NE fusion employs the same machinery and enzymatic activities as the bulk ER. Importantly, two recent studies indicate that components of the ESCRT-III (endosomal sorting complex required for transport) are crucial for NE reformation (Olmos et al., 2015; Vietri et al., 2015). Depletion of members of the ESCRT-III complex results in failure of the NE sealing and consequent leaky nuclei. The ESCRT-III complex was previously known to participate in membrane fusion-related events like HIV virus egress, cytokinesis, vesicle formation into multivesicular bodies (Garrus et al., 2001; Guizetti et al., 2011; Wollert and Hurley, 2010). When involved in NE reassembly, it has been implicated in the closure of final gaps that remain after nuclear membranes have enclosed the chromatin almost completely.

6. Nuclear pore complex assembly

Postmitotic NPC assembly can take place by two distinct pathways, which differ in timing and mechanism. NPCs assemble after mitosis, when the NE is about to reform after NEBD and the nucleocytoplasmic barrier must be re-established. Therefore, in late anaphase, many NPCs

form quickly at the same pace. Importantly, this NPC assembly pathway happens concomitantly with, not subsequently to, the NE reformation and chromatin decondensation (Schellhaus et al., 2016), suggesting that the post mitotic nuclear assembly is a harmonic, inclusive process (Dultz et al., 2008). The most important, best studied steps of postmitotic nucleoporin recruitment are represented in Figure 4.

On the other hand, by the time the cell enters G1, the NE is intact again and NPC formation must overcome the challenge of locally disrupting the NE. This process is started by Nup153, and additionally requires POM121 (Doucet and Hetzer, 2010; Dultz and Ellenberg, 2010) and Nup53 (Vollmer et al., 2012). Nup153 acts as seeding point for the first nucleoporin subcomplex to be recruited, Nup107-160 (Vollmer et al., 2015), whereas in postmitotic nuclear assembly the Nup107-160 recruitment is mediated by Mel28/ELYS (Franz et al., 2007), that is not required in interphase (Doucet et al., 2010). Interphasic NPC assembly is about one order of magnitude slower (Schooley et al., 2012), requires different seeding proteins, and leads to an NPC density per nucleus about doubled per cell cycle (Dultz and Ellenberg, 2010; Maul et al., 1972) before the next mitotic entry, variably with the organism and cell type.

It is common understanding that the assembly of the postmitotic pore, on which I focus here, proceeds in a step-by-step fashion. Nucleoporins join the pore in a precise order, and interact with each other to modify the available surface to act as scaffold for further binding of the next components. These will in turn constitute the scaffolding base that will allow localization of more Nups on the growing pore.

The finding that the NPC starts to assemble rather on the decondensing chromatin than on the reforming nuclear envelope has been a revolution in the field. By identifying MEL28/ELYS as such chromatin-bound seeding point, a major step was taken towards our understanding of NPC postmitotic assembly. The subsequent steps of the ordered recruitment of the Nups, in the form of subcomplexes or “building blocks”, have been extensively analyzed in different experimental systems, ranging from fixed cells, live imaging of different cell types and organisms, to *in vitro* nuclear assembly in *Xenopus* egg extracts (see Section 9).

Following recruitment of the Nup107-160 complex by MEL28/ELYS, we now know that transmembrane Nups (gp210, POM121, Ndc1) and membrane binding Nups Nup53 and Nup155 associate next. Nup53 is recruited earliest, and its activity is modulated by contact with NDC1, allowing colocalization of Nup155 (Eisenhardt et al., 2014a). Only after Nup155 is positioned, Nup93 is able to bind Nup53, which in turn generates the optimal scaffold for further

recruitment of other members of the Nup93- complex, Nup188 and Nup205, and the Nup62-complex. Regulation of Nup53 by NDC1 (Eisenhardt et al., 2014a) and of Nup93 by Nup155 (discussed in Results and Discussion, Section II.4), constitute a representative example of the level of complexity of the assembly coordination. It would be limiting to view NPC assembly as a consecutive recruitment of building blocks, when in fact it takes advantage of the molecular communication within a protein network that takes place at different levels. Nup98 is recruited in a similar time frame to Nup93 (Dultz et al., 2008).

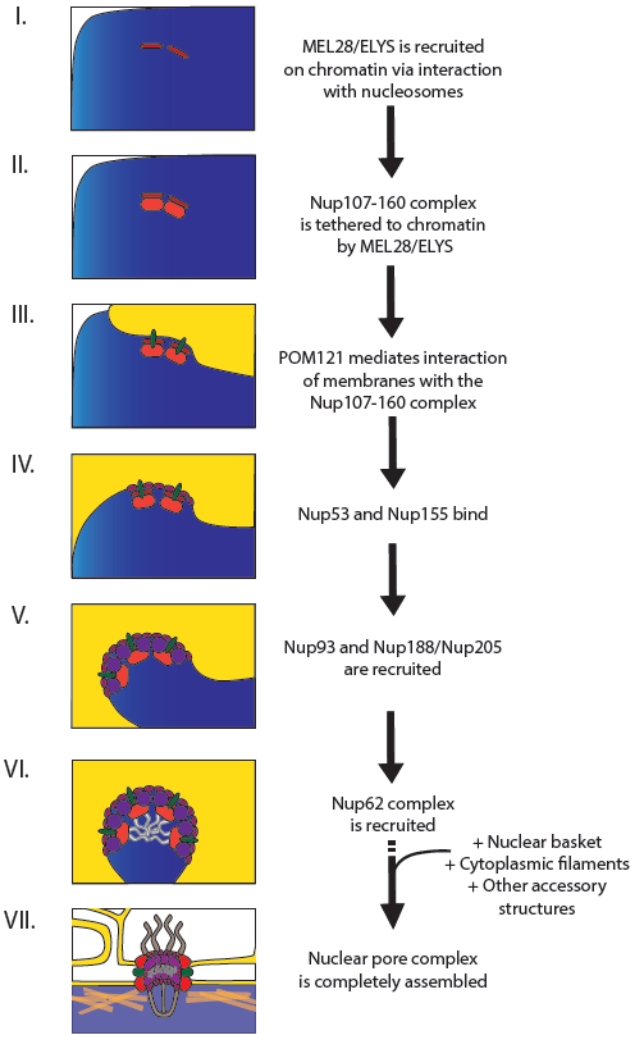


Figure 4. Nucleoporins are recruited in a systematic, ordered fashion for postmitotic NPC assembly.
MEL28/ELYS initiates NPC assembly on the chromatin by binding activated nucleosomes. Once there, it is able to recruit the Nup107-160 complex, which establishes connection with the membranes of the NE via the transmembrane nucleoporin POM121. Nup53 is recruited in sequence, followed by Nup155. Both bind the nuclear envelope and are required for subsequent incorporation of Nup93. Nup93 recruits in turns Nup205 and Nup188. In parallel, Nup93 acts as a scaffold for the recruitment of the Nup62 complex, that takes place in the center of the assembling pore. Nup153, Nup50 and Tpr, as member of the nuclear basket, and cytoplasmic filaments components Nup358 and Nup214 join to complete the final structure of the pore.
(Figure from (Schellhaus et al., 2016)).

Cytoplasmic filaments via Nup358, and the nuclear basket mainly via Nup153, join the core pore at the end of the process, completing the full structure of the NPC. The precise steps that result in assembly of filaments and basket are altogether less well defined.

7. Chromatin is an active player of NPC assembly

The finding that chromatin plays an active and central scaffolding role in the early steps of NPC assembly led researchers in the field to focus on chromatin binding factors which possess the capability to recruit nucleoporins on chromatin templates. Chromatin is in its most compacted state during anaphase entry (Mora-Bermudez et al., 2007) and is therefore still in a high level of compaction when NE and NPC assembly commences in late anaphase (Puhka et al., 2007). A recent systematic screen for scaffolding nucleoporins has attributed to Nup153, Nup50, and Nup133 the capability of recruiting other nucleoporins on a lacO array (Schwartz et al., 2015). As mentioned, MEL28/ELYS, a DNA binding protein found on the decondensing chromatin, was the first chromatin associated factor identified. As seeding factor of postmitotic NPC assembly, it is able to recruit the Nup107-Nup160 complex (Franz et al., 2007). Although MEL28/ELYS is experimentally able to bind DNA directly by an AT-hook motif, recent experiments proved how its role in nuclear assembly rather requires it to binding DNA wrapped in histones in the form of nucleosomes, supporting additional evidence of the crucial role of chromatin as a coordinator of nuclear reformation after mitosis (Inoue and Zhang, 2014; Zierhut et al., 2014). Furthermore, the recruitment of MEL28/ELYS on decondensing chromatin, and therefore NPC assembly, is not widespread, but it is limited to a specific area, the so called “non-core” region. This area includes chromatin lateral to the position of the mitotic spindle, in opposition to the proximal and distal areas named “core” region. Although it is not yet known what causes MEL28/ELYS to land in this specific position, the existence of activated, or competent, regions of chromatin with respect to postmitotic events can be considered one of the proof of the key role of chromatin in nuclear organization at the end of mitosis.

The attachment of MEL28/ELYS itself to chromatin is object of regulation. A requirement for chromatin to be primed prior to NPC attachment was identified. The enzymatic activity of the lysine demethylase LSD1, which is known to remove a methyl group from di- and mono-methylated histones, is required for this (Schooley et al., 2015). Depletion of LSD1 or impairment of its enzymatic activity (by mutation or use of chemical inhibitors) led to malfunction in both NPC and NE formation, reduced the levels of MEL28/ELYS in chromatin and, in turn, blocked NE and NPC assembly at the end of mitosis. The increasing number of experimental observations according to which chromatin requires modifiers to acts a seeding base opens a new path to our understanding of chromatin regulation at the end of mitosis. We know that the

interplay between chromatin and nuclear envelope during mitosis extends beyond mere proximity, as it is likely that each other regulation, in mitosis but not only, involves more players.

8. Coordination of NE and NPC assembly

As the pore complexes must be inserted in the double membrane layer, NPC assembly had been thought for a long time to start on the reforming nuclear envelope. In the past years, thought, chromatin has been found to have a direct role in the early steps of postmitotic assembly, as proofs of direct association between nucleoporins and chromatin increased considerably (see Sections 6 and 7). *In vitro*, membrane fusion is a pre-requisite for NPC assembly, as treatment of the assembling nuclei with the alkylating agent NEM block NPC assembly by inhibiting membrane fusion. Instead, the calcium chelator BAPTA was able to inhibit the NE fusion transiently, whereas its most prominent effect was a complete block of NPC assembly, establishing that the former is a prerequisite for the latter (Macaulay and Forbes, 1996). Connection of the nascent pore to the membranes is now known to occur at a later step, just before association of Nup53 to the pore (Mansfeld et al., 2006). Transmembrane nucleoporins NDC1 and POM121, which migration together with other INM proteins drive NE formation, are recruited on chromatin by redundant mechanisms. Binding to dephosphorylated Barrier-to-Autointegration factor (see Section 2.1) and direct binding to chromatin (Ulbert et al., 2006) both contribute to their postmitotic recruitment, *de facto* linking NE and NPC assembly. Transmembrane nucleoporins NDC1 is not dispensable at the end of mitosis, but is still a matter of debate whether POM121 is required as well (Antonin and Mattaj, 2005; Doucet et al., 2010; Shaulov et al., 2011). Indeed, proteins that bind membrane without containing transmembrane domains, and are found proximally to the NPC-NE contact site, are also reportedly necessary, probably to stabilize the locally curved conformation of the envelope. Among these, Nup53, which binds and stabilizes membranes by two different motifs (Vollmer et al., 2012), and Nup155, that contains a membrane binding motiv (von Appen et al., 2015).

Mitotic onset is characterized by a generalized heavily phosphorylated state of the cell proteome (Dephoure et al., 2008). It has been suggested that the reversal of this situation by mitotic phosphatases could promote interactions between nucleoporins at the end of mitosis. Nevertheless, the redundancy of phosphorylation events and the wide range of functions that kinases and phosphatases catalyze in mitotic entry, progression and exit increases the complexity of the experimental setup and results in the lack of direct evidence in support of this

model. In addition to phosphorylation, other posttranslational modification may also contribute to postmitotic assembly.

Ran is a key regulator of spatial organization of NPC assembly as it is responsible for proper chromatin-directed recruitment of nucleoporins. As discussed in Section 3, high concentrations of RanGTP are generated near the chromatin. At the end of mitosis, nucleoporins are bound to importins via their NLS, which renders them unavailable for NPC assembly. By binding to Impβ, RanGTP supports the release of these Nups in the vicinity of chromatin, and renders them free to associate to forming NPCs. When the RanGTP gradient is disturbed, ectopic, aberrant formation of NPCs in ER membrane stacks distal from the NE is observed, resulting in the structures known as *annulate lamellae* (Walther et al., 2003b).

Nucleoporins susceptible of this regulation include MEL28/ELYS and the Nup107-160 complex, as they are chromatin binding proteins involved in early step of NPC assembly and they reportedly interact with karyopherins (Rotem et al., 2009). Moreover, many nucleoporins bind kariopherins to facilitate their own nuclear import, as they are synthesized in the cytoplasm and must be imported to allow interphasic pore assembly.

In addition, it is possible that the Ran/importin system described above also regulates the recruitment of INM proteins to postmitotic chromatin described in Section 5.

9. Experimental system to approach postmitotic nuclear assembly *in vitro* using *Xenopus* egg extracts

Studying nuclear assembly in the complexity of cellular environments has been a challenge due to simultaneous remodeling events taking place in a spatially confined cellular area. In addition, several of the factors involved in the nuclear assembly events are required for more than just one pathway and, given the importance of tight regulation and control of the cell cycle, the functions of the related factors are sometime partially redundant, hiding the relevance of the single contributors in the wider design of the multiple mitotic pathways. These obstacles have been partially overcome when the first *in vitro* system was developed (Lohka and Masui, 1983). Cytoplasmic extracts derived from *Xenopus* eggs proved to be able to support the generation of a fully formed nucleus from a sperm chromatin template, enclosed by a double envelope and competent for nucleocytoplasmic transport. This *in vitro* system present the enormous advantage of being suited to separate the diverse reactions that concur to nuclear

assembly, and it has been widely used over the years study them: among these, spindle assembly (Maresca and Heald, 2006), nuclear envelope breakdown (Galy et al., 2008) and reformation (Lohka, 1998), NPC postmitotic (Theerthagiri et al., 2010) and interphasic *de novo* pore assembly (Vollmer et al., 2015), nuclear transport (Chan and Forbes, 2006), chromatin decondensation (Magalska et al., 2014).

Egg deposition is induced artificially by injecting the female frogs with hCG (human chorionic gonadotropin). Eggs are collected and dejellinated to remove the protective layer that surrounds them, arrested in metaphase of meiosis II and activated by simulating fertilization with the addition of calcium ionophore, which allows them to proceed to the next step of the cell cycle. Given that during early development the eggs physiologically undergo multiple fast rounds of mitotic duplications, in which protein expression is temporarily inhibited, the resulting cytosol is especially rich in protein content.

To generate interphasic cytosolic extracts, eggs are crushed by three subsequent ultracentrifugation steps (Figure 5A). After the last round of centrifugation, both cytosolic and membrane fractions are required for *in vitro* nuclear assembly**.**

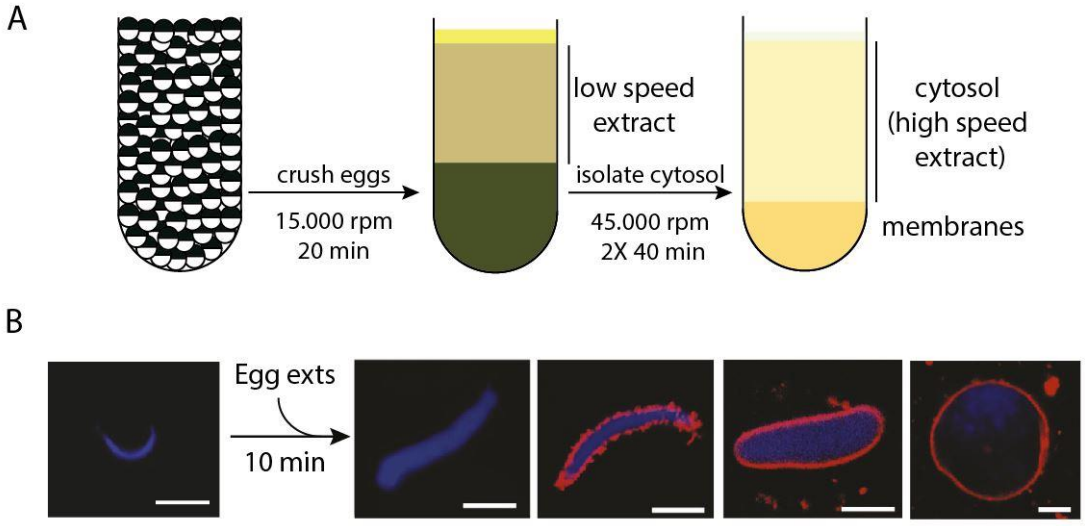


Figure 5: Functional nuclei can be reconstituted in vitro using a cell-free assay based on *Xenopus laevis* egg extracts. (A) Three consecutive centrifugation steps allow fractionation of the eggs, resulting in the generation of cytosolic extracts and total crude membrane fractions. (B) Incubating egg extracts with sperm chromatin, cytosol-free floated membranes and an energy mix regeneration system allows the extracts to promote formation of a fully formed nucleus, in which chromatin is decondensed and enclosed by a double nuclear envelopes, which includes transport competent NPCs.

If required, the cell cycle stage of the extracts can be switched to mitotic, which has been crucial to study early mitotic events as, for instance, spindle assembly (Yokoyama et al., 2014).

Whereas cytosol can be either purified from membranes residues or used immediately, crude membranes must be further fractionated to remove cytosolic contaminants.

The sperm DNA is not wrapped around the classic histone octamer, but rather around a multimer formed by a specific combination of histone variants, the protamines, and canonical histones H3 and H4. Incubation with the cytosolic egg extracts catalyzes the exchange of these sperm nucleosomes with the canonical histone octamers containing H2A and H2B by nucleoplasmin, an abundant protein in extracts (Dilworth et al., 1987; Philpott and Leno, 1992; Philpott et al., 1991) (Figure 5 panel B, first step). Adding an enzymatic energy regeneration mix and floated membranes (Figure 5 panel B, following steps) results in nuclear formation, including chromatin decondensation, nuclear envelope and NPC formation. Nuclear envelope formation is easily monitored by addition to the reaction of the fluorescent membrane dye DiIC18 (1,1'-Dioctadecyl-3,3,3',3'- Tetramethylindocarbocyanine Perchlorate), while NPCs can be detected in immunofluorescence staining by means of the mAB414 antibody, which recognizes a subset of nucleoporins (including Nup62, Nup153, Nup214 and Nup358) and therefore is an efficient indicator of the presence of NPCs.

The preparation of interphase cytosolic extracts, for simplicity indicated from now on as extracts, including the nuclear assembly reaction protocol, is described in detail in (Eisenhardt et al., 2014b).

Aim of the thesis project

The nucleus carries the genetic material of the cell, and the NPCs must ensure precise access to the nucleus to all the factors required for its correct development and maintenance. In addition, NPCs must regulate the nuclear intake of proteins that have been translated in the cytosol and must interact with nucleosomes and DNA at the different stages of cell cycle. In order to do so, NPCs must form in a tightly regulated fashion, producing as a result transport-competent complexes. The formation of the pores occurs in two different stages of the cell cycle, during interphase and after mitosis. During these stages, the pores are likely assembled by distinct paths, due to the differences existing in morphology at these moments of the life of a cell. A deeper understanding of the mechanism of interaction of factors involved in NPC formation, which is far to be complete, will contribute to give clarity to of the biggest, most complex cellular events.

In the course of my PhD, I aimed to increase our current knowledge about postmitotic NPC assembly by shedding lights on mechanisms that regulate interactions and ordered recruitment of nucleoporins. In particular, I have focused on two different steps of the assembly path. In the first part of my thesis, I will discuss my work on nucleoporin Nup50, that original work from the Antonin lab has found to be required at the beginning of the postmitotic NPC assembly. I have identified two distinct sites required for NPC assembly, each dispensable if the other is functional. Secondly, I will introduce my findings on nucleoporin Nup155, that is required later in the assembly process. My work led to the discovery of mechanistic insights about the formation and functionality of the inner ring complex, and on the regulation of interaction between nucleoporins Nup53, Nup155 and Nup93.

Nup50 is a small nucleoplasmic nucleoporin known to be required for nuclear import of NLS-containing cargos. It is largely unstructured, with the exception of a few double helices which existence can be predicted using bioinformatics tools. I confirmed previous data from the Antonin lab according to which Nup50 is required at the beginning of mitosis, and I identified two distinct sites on the protein that are important for this, each of them dispensable if the other is functional, but required if the other is mutated or compromised. Interestingly, Nup50 binds the NPC assembly initiator MEL28/ELYS; in spite of this, I proved that their interaction is not required for postmitotic NPC assembly, and that they probably perform redundant functions.

Nup155 is a membrane binding protein required for postmitotic NPC assembly. It contains an N-terminal domain, which arranges as a β-propeller fold in its tertiary structure, and a C-

terminal domain, which folds as an α-solenoid. Nup155 must bind Nup53 for proper NPC localization; therefore, its binding follows the action of Nup50 in the postmitotic assembly (refer to Figure 4). Previous work performed in the lab (von Appen et al., 2015) identified a membrane-binding region within the N-terminal of Nup155. I wondered whether this membrane binding region of Nup155 was required for NPC assembly, and if so, if it was the sole region required, or if there was any other feature by means of which Nup155 contributes to the pore assembly, that was separated from membrane fusion. It is acknowledged that the Nup53-Nup155 interaction is required to recruit Nup93, and in turn the Nup62 complex (Eisenhardt et al., 2014a; Sachdev et al., 2012). Direct interaction of Nup93 and Nup155 had not proven to be possible, hence the question mark on the requirement for Nup155 in the whole for NPC assembly. My work established that Nup155 is found in a closed conformation in solution. Its binding site for Nup53 is found in the N-terminal, but it is partially blocked by its C-terminal, that prevents the optimal surface contact from taking place. Nup93 is required for the conformational change to happen: when Nup93 binds Nup53, the contact surface between Nup53 and Nup155 is optimized and, in spite of the fact that Nup93 is required for this, it happens without direct contact between Nup93 and Nup155, being Nup53 the main scaffolding element.

List of publications included in the thesis

Submitted papers

Direct membrane interaction of and a self-inhibitory interaction within nucleoporin Nup155 are required for nuclear pore complex assembly

De Magistris, P., Tatarek-Nossol, M., Dewor, M., Antonin, W. (submitted)

Accepted papers

Nuclear reformation at the end of mitosis

Schellhaus, K.*, **De Magistris, P**.*, and Antonin, W.

Journal of Molecular Biology (2016), **428(10)**, 1962-85

The lysine demethylase LSD1 is required for nuclear envelope formation at the end of mitosis

Schooley, A., Moreno-Andrés, D., **De Magistris, P.**, Vollmer, B., Antonin, W.

Journal of Cell Science (2015), **128(18)**, 3466-77

Nup153 recruits the Nup107-160 complex to the inner nuclear membrane for interphasic nuclear pore complex assembly

Vollmer, B., Lorenz, M., Moreno-Andrés, D., Bodenhöfer, M., **De Magistris, P**., Astrinidis, S.A., Schooley, A., Flötenmeyer, M., Leptihn. S., Antonin, W.

Developmental Cell (2015), **33**, 717-728

Personal contribution to shared authorship in publications

Direct membrane interaction of and a self-inhibitory interaction within nucleoporin Nup155 are required for nuclear pore complex assembly
De Magistris, P., Tatarek-Nossol, M., Dewor, M., Antonin, W. (submitted)

As first author of this publication, I designed nuclear assembly experiments under the supervision of Wolfram Antonin. I prepared the extracts, the floated membranes and all reagents used in the *in vitro* nuclear assembly experiments using mRNA addback in Figure 1. I optimized and performed expression, purification, and SUMO tag cleavage of the Nup155 N-terminal constructs used in the nuclear assembly experiments; these, reported in Figure 2 and 3 of the paper, were designed, optimized, performed and analyzed by me. Marianna Tatarek-Nossol and Manfred Dewor purified and expressed GST-tagged proteins, and performed the related pulldowns. I developed the mechanistic model and wrote the paper together with Wolfram Antonin.

Nuclear reformation at the end of mitosis
Schellhaus, K.*, **De Magistris, P**.*, and Antonin, W.
Journal of Molecular Biology (2016), **428(10)**, 1962-85

As a co-first author, I wrote this review together with Katharina Schellhaus and Wolfram Antonin. We agreed on the contents, chose the main focus of the article and established the organization of the paper. I wrote the sections “The nuclear envelope emerges from the mitotic ER”, “establishing a nuclear envelope domain, “Nuclear envelope sealing”, “Building NPCs into the nuclear envelope”, “Regulating NPC assembly at the end of mitosis”, “Lamina and LINC complex reassembly”. All authors designed the figures; I generated Figure 3, Figure 4 and Figure 5, whereas Katharina Schellhaus prepared Figure 1 and Figure 2. Wolfram Antonin previsedover the preparation of the review.

The lysine demethylase LSD1 is required for nuclear envelope formation at the end of mitosis

Schooley, A., Moreno-Andrés, D., **De Magistris, P.**, Vollmer, B., Antonin, W.
Journal of Cell Science (2015), **128(18)**, 3466-77

As co-author of this publication, I cooperated to the generation of the *in vitro* data by preparing the high speed interphasic extracts used in the nuclear assembly experiments, and contributed to the editing of the paper. Allana Schooley designed experiments, performed nuclear assemblies, chromatin binding and immunofluorescence data, together with their analysis, and wrote the paper. Daniel Moreno-Andrés contributed with the cell experiments and developed the CellCog method, that he also applied to perform the related data analysis. Benjamin Vollmer expressed and purified the LSD1 contructs for addback experiments. Wolfram Antonin supervised the study, performed nuclear assembly experiments, and wrote the paper.

Nup153 recruits the Nup107-160 complex to the inner nuclear membrane for interphasic nuclear pore complex assembly

Vollmer, B., Lorenz, M., Moreno-Andrés, D., Bodenhöfer, M., **De Magistris, P**., Astrinidis, S.A., Schooley, A., Flötenmeyer, M., Leptihn. S., Antonin, W.
Developmental Cell (2015), **33**, 717-728

As co-author of this publication, I supported the generation of the *in vitro* data by preparing the high speed interphasic extracts and the floated membranes used in nuclear assembly experiments, which were executed by Wolfram Antonin and Susanne Astrinidis, and I contributed to the editing of the manuscript. Benjamin Vollmer designed experiments, optimized, expressed and purified proteins constructs used in the study, performed liposome floatation experiments, and wrote the paper. Mona Bodenhöfer contributed to protein purification. Michael Lorenz performed and analyzed GUV experiments, performed WGA-based experiments and related NPC quantification. The quantification strategy was developed by Allana Schooley, which also contributed to generation of the *in vitro* data and the editing of the paper. Daniel Moreno-Andrés performed the live cell imaging experiments and related data analysis. Mathias Flötenmeyer performed EM and Sebastian Leptihn measured of light scattering of liposomes. Wolfram Antonin supervised the study and wrote the paper.

RESULTS AND DISCUSSION

Part I: Nup50 in nuclear assembly

I. 1. Nup50 is required at the end of mitosis for NPC assembly

Nup50 is a small nucleoporin (50 kDa) which is known to be member of the nuclear basket and which role has been so far exclusively linked to the nuclear import pathway (described in Section 3). Nup50 can be roughly divided in three domains: (i) an N-terminal part (N-region) that is capable of binding Impα, MEL28/ELYS, and Nup153, and that also retains dimerization capability when isolated; (ii) a central phenylalanine-glycine (FG) rich domain (M-region) responsible for Impβ binding; this region contains several of these FG repeats, contributing to the FG mesh of the pore, a unique hydrogel-like structure which is part of the nuclear transport barrier; and (iii) a C-terminal region (R-region) necessary for RanGTP interaction. When incubating protein-A-Sepharose beads coupled to anti-Nup50xl antibodies with *Xenopus* extracts to remove Nup50 (Figure 6A), the protein can be efficiently depleted without significantly affecting other related proteins, with the exception of MEL28/ELYS (Figure 6B), which levels are reduced to about 10-20% of the mock levels.

When Nup50 depleted extracts are used in *in vitro* nuclear assembly reaction described in Section 9, nuclei formed in these conditions exhibit a closed nuclear envelope but NPC formation is impaired (Figure 7). Addback of recombinant Nup50, expressed and purified in *E. coli*, is able to restore the depletion phenotype, demonstrating that it is specifically due to absence of Nup50 and not to undesired codepletion of other factors: hence, Nup50 is indeed required for NPC formation.

In order to insert Nup50 in a molecular context and identify novel interactions with other NPC and nuclear assembly related proteins, mass spectrometry analysis of immunoprecipitations in *Xenopus* egg extract was performed in the lab, with the support of the Proteome center in Tübingen. This allowed identification of many of the diverse factors interacting with Nup50 (data not shown), some of which are involved in both nuclear import and nuclear pore complex formation. Among the former, described and extensively characterized in the context of the nuclear import pathway (Bayliss et al., 2000; Makise et al., 2012; Matsuura and Stewart, 2005) Importin α-β (Impα-β), RanGTP can be enumerated, while the latter interestingly included MEL28/ELYS, the seeding protein that starts NPC postmitotic assembly on chromatin.

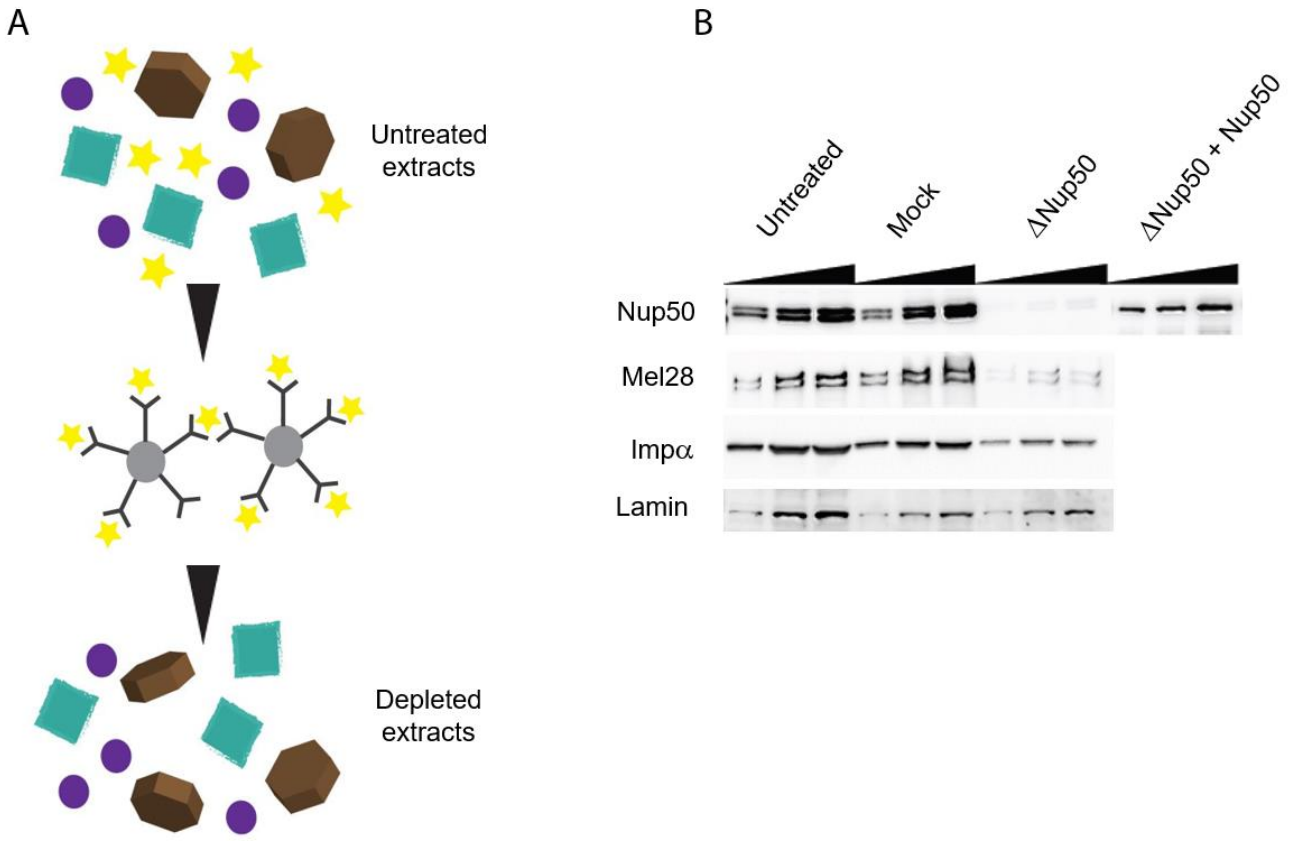
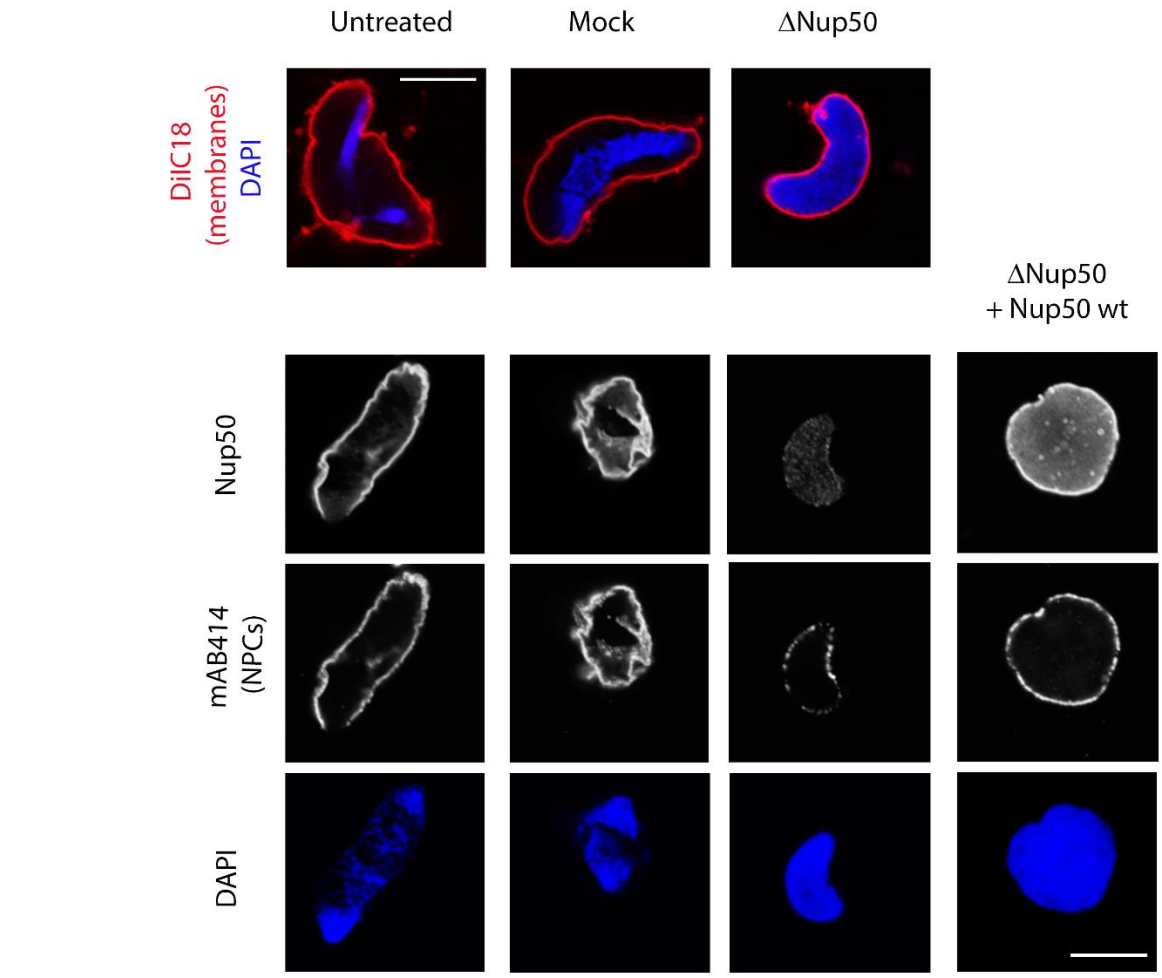


Figure 6: Nup50 can be efficiently depleted from extracts. Anti-Nup50 antibodies can be coupled with Protein-A-Sepharose beads by crosslinking with 10 mM DMP to achieve efficient and specific removal of Nup50 from *Xenopus* egg extracts (panel A). With the exception of MEL28/ELYS (described in I.1), depletion of Nup50 does not importantly reduce the level of other proteins in extracts (panel B).

The MS analysis also pointed out presence of components of the Nup107/Nup160 complex (Nup107, Nup160, Sec13, Seh1A), microtubule interacting proteins like cytoplasmic dynein, and chromatin remodelers, in particular members of the SWI/SNF complex BRG1 and SMARCC1. Nup50 also binds nucleoporin Nup153, which in turns is important for Nup50 localization on the nuclear rim (Makise et al., 2012), but is not relevant for postmitotic NPC formation (Hase and Cordes, 2003).



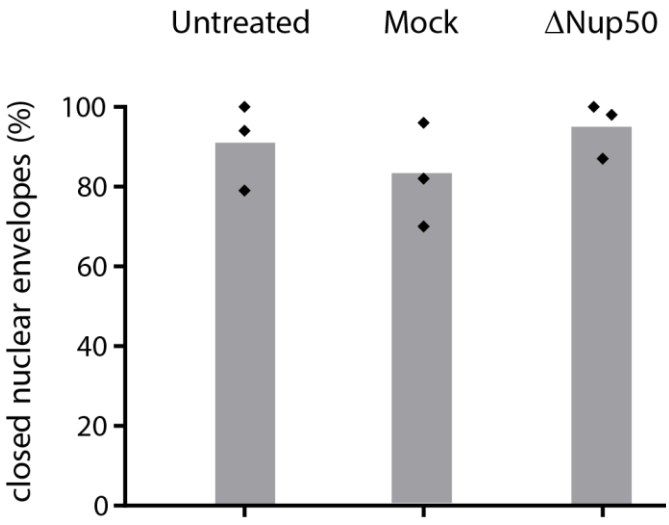


Figure 7: Nup50 is required for NPC assembly at the end of mitosis. When added to sperm chromatin DNA (blue), membranes and an energy mix system, both untreated and mock depleted *Xenopus* egg extract (first and second column) are able to form both a closed envelope (red) and NPCs (mAB414, third row), as quantified from 3 independent experiments (individual data points are shown). Nup50 is located on the nuclear rim (second row). Depletion of Nup50 from the extract blocks NPC formation (third column), despite a closed nuclear envelope has formed around chromatin. Addback of exogenous Nup50 restores capability of NPC formation *in vitro* (fourth column).

In fact, depletion of Nup153 does not affect the overall core structure organization of NPC nor its assembly after mitosis ((Hase and Cordes, 2003) and my data, see Figure 13). This observation suggests that Nup50 is rather important as an assembly cofactor than as a structural component of the nuclear basket and it addresses the possibility that its transport and assembly roles might rely on distinct moieties of the protein.

Interaction with MEL28/ELYS appeared more interesting in this respect. MEL28/ELYS knockout is lethal in mice embryo development (Smitherman et al., 2000) and its depletion from *Xenopus* egg extract in nuclear assembly results in a similar phenotype as described above for Nup50, with nuclei enclosed by a double nuclear envelope devoid of NPCs (Franz et al., 2007). The same study determined that, as well as Nup50, it localizes on chromatin shortly after mitotic exit. Addback of Nup50 after its depletion from extracts (Figure 7) excludes that MEL28/ELYS codepletion is responsible for the observed phenotype. This observation stands for the hypothesis that together with the consolidated, well known role of MEL28/ELYS effect in post mitotic NPC assembly, a second, independent yet indispensable Nup50-dependent pathway takes place. In fact, I will demonstrate here that Nup50 and MEL28/ELYS, although both necessary for assembly, are independently relevant for the process. The MEL28/ELYS-Nup50 interaction is nonetheless significant in the assembly context and as part of my PhD studies I have investigated how MEL28/ELYS contributes to Nup50 positioning on chromatin for NPC assembly (discussed in Section I.7).

I. 2. Nup50 binds chromatin

Unpublished work from the Antonin lab shows that in live cell imaging experiments Nup50 shows ability to directly bind chromatin and is found on it at early stages of nuclear reformation after mitosis in HeLa cells. This finding is especially relevant when describing an NPC assembly factor like Nup50, as the key event of post mitotic NPC assembly initiation is the recruitment of early factors, like MEL28/ELYS, on chromatin.

I have investigated this feature by chromatin binding assay. This is performed by incubating chromatin from sperm heads in the presence of membrane-free *Xenopus* egg extracts and the protein of interest expressed with an N-terminal eGFP tag and purified from *E. coli*. By visualizing direct fluorescence of eGFP, exogenous Nup50 can be detected on chromatin. Endogenous Nup50 can be also detected in the same conditions by immunofluorescence on chromatin substrates that had been incubated in extract for 10 minutes.

My experiments in this direction prove that Nup50 indeed binds to chromatin *in vitro* (Figure 8). In addition, I sought to determine whether this interaction occurs at the level of histones, DNA, or other chromatin scaffold elements. To do so, I employed heat inactivated extracts in

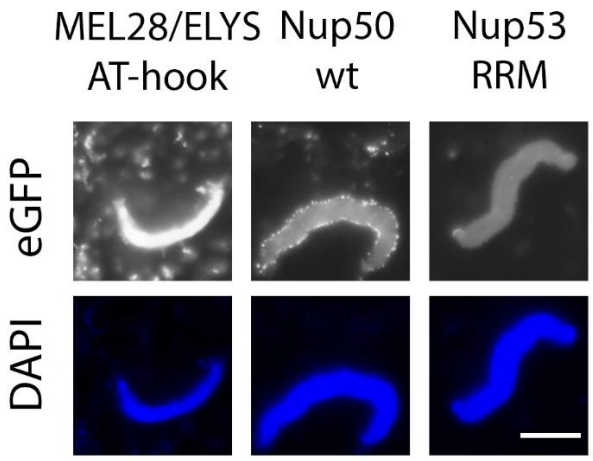


Fig. 8: Nup50 indirectly binds chromatin. Exogenous eGFP-Nup50 forms a punctated pattern on substrate sperm chromatin when incubated for 10 minutes together with sperm and *Xenopus* laevis egg extracts. In comparison, the DNA binding AT-hook sequence on MEL28/ELYS stains sperm homogenously. RRM domain on Nup53, used here as a negative control, does not detectably bind chromatin in the conditions tested. Bar, 10 µm

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the essay, which had been brought to 80°C and centrifuged for removal of all heat sensitive components. This extracts are only competent for primary decondensation of sperm heads, that is, nucleoplasmin-mediated exchange of sperm specific histones with classic histones (see Section 9). In this conditions, exogenous Nup50 is not detected on chromatin, whereas a MEL28/ELYS AT-hook - eGFP construct, which includes MEL28/ELYS direct DNA binding site, is found on sperm (Figure 9 panel C).

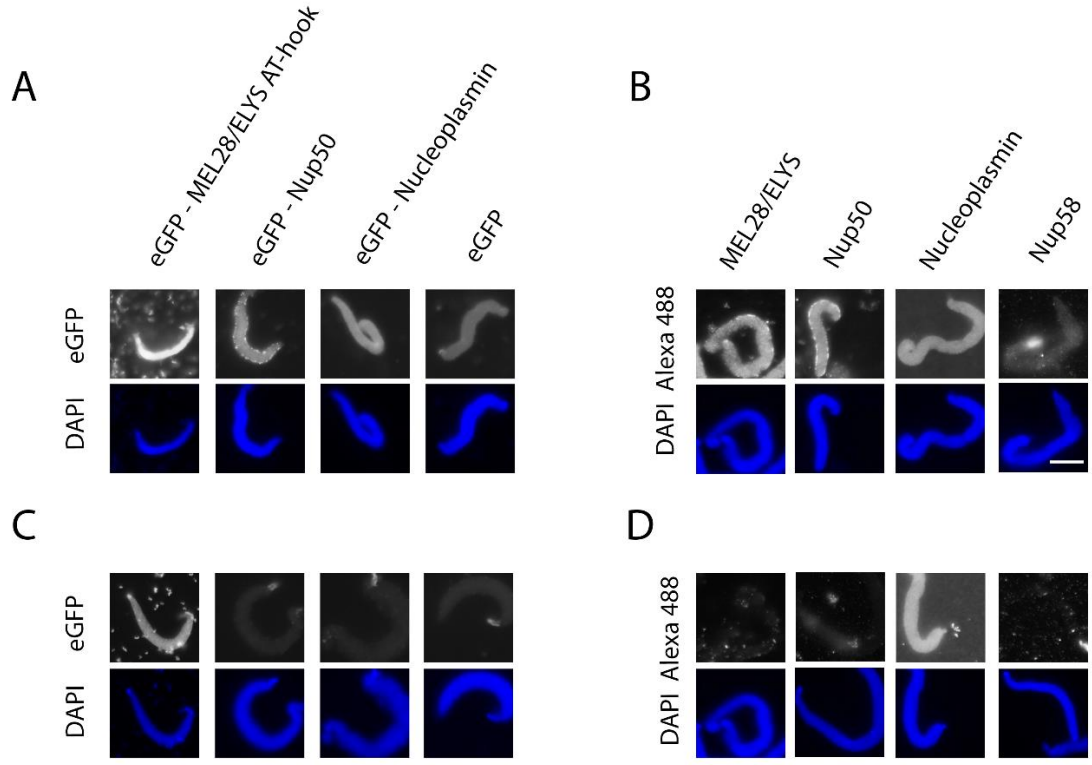

Figure 9. Nup50 binds chromatin. Exogenous eGFP-Nup50 can be detected on chromatin when incubated for 10 minutes together with sperm and *Xenopus laevis* egg extracts (panel A). Immunofluorescence using an anti-Nup50 antibody on chromatin incubated with extracts reveals a similar localization for endogenous Nup50 (panel B). If heat inactivated extract is employed, the fluorescent signal drops, in contrast to MEL28/ELYS chromatin binding fragment (panel C), which binding is known to be direct. Heat inactivation process removes protein content from extracts but the heat resistant nucleoplasmin (panel D). Bar, 10 µm

Therefore, my data indicate that Nup50 binds to chromatin and that this binding is not mediated by direct contact with histones or DNA, but must be due to a third, yet unknown factor. In fact, when heat inactivated extract is used in the essay, nor endogenous Nup50 (Figure 9 panel D) nor eGFP-Nup50 (Figure 9 panel C) are detectable on chromatin.

I. 3. Nup50 binds MEL28/ELYS and Nup107

Immunoprecipitation using anti-Nup50 antibodies were performed in the lab, followed by mass-spectrometry experiments by the proteomic facility in Tübingen. Such experiments identified a set of interactors of Nup50: some of them were known and confirmed previous literature knowledge: among these, nucleoporin Nup153, Impα, Impβ. These interactions have already been identified in the context of nuclear import. Importantly, the MS identified several novel

interactors, never reported before and potentially interesting in the context of nuclear assembly: among these, the Y-complex components Nup107, Nup160, the chromatin binding nucleoporin Mel28/ELYS, cytoplasmic dynein and the chromatin remodelers BRG1 and SMARCC1, components of the SWI/SNF complex (Kadam and Emerson, 2003; Trotter and Archer, 2008). In order to confirm these interactions, immunoprecipitations were repeated in the lab and the eluate used in Western blot to confirm interaction by use of a specific antibody.

I. 5. Nup50 is still functional when mutations for Impα, Mel28, Nup153, FG repeats are introduced

In order to find out which of the Nup50 interaction partners are involved in the assembly function, I have expressed and purified Nup50 mutants to test for impairment in one, or more than one, of these interactions. I performed pulldown experiments, using GST fusions of wild type as well as point mutant fragments of Nup50 to test for effective impairment in binding to interactors by using egg extract. Then, among the mutants designed, I have selected Nup50 mutants defective for each interaction I was interested in investigating. In the case of MEL28/ELYS and Nup153, I selected two different mutants of Nup50, Nup50 D178A and Nup50 W159A. Nup50 D178A was chosen as preliminary transfection experiments showed that this mutant had lost capability of attaching to rim in interphase HeLa cells, implying loss of interaction with Nup153, responsible for its rim localization (Hase and Cordes, 2003). On the other hand, Nup50 W159A showed complete loss of MEL28/ELYS binding with respect to wild type Nup50 in pulldown experiments using *Xenopus* egg extracts (data not shown). This latter mutant also completely abolishes interaction with Nup153; in fact, the two binding sites for Nup153 and MEL28/ELYS are situated in close proximity within residues 144-191 in the central moiety of Nup50 and it has not been possible to experimentally separate them. As it is known that Nup153 is not required in postmitotic NPC assembly, this loss of interaction with Nup153 is unlikely to interfere with the analysis of the results.

Once the mutants have been characterized as described, I have employed them in addback experiments after depleting endogenous Nup50 from *Xenopus* extracts in nuclear assembly. I have employed Nup50 mutants in the full length context that are defective for Impα binding (Nup50 K3ER4DR38AR45D), MEL28/Nup153 binding (Nup50 W159A), a mutant lacking the FG repeats, and a mutant defective for rim localization in interphase (Nup50 D178A).

Interestingly, all these mutants are capable to reconstitute NPCs in our system, a clear indication that none of these residues are required for NPC formation *in vitro*.

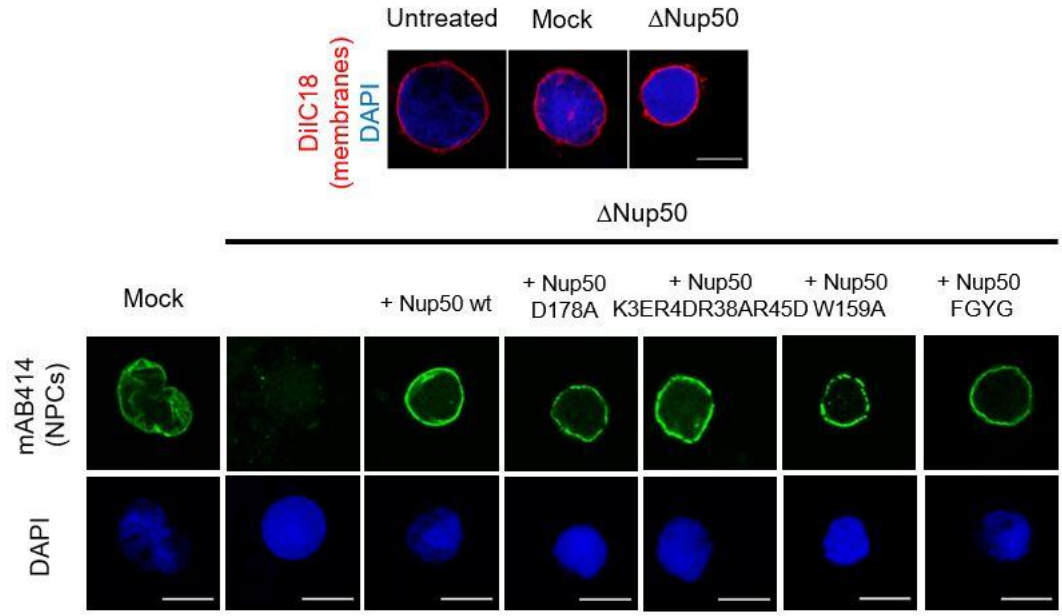

Figure 10: Nup50 mutations affecting the FG repeats (Nup50 FGYG) of Nup50 or its binding to Nup153 (Nup50 D178A), MEL28/ELYS (Nup50 W159A), Impα (Nup50 K3ER4DR38AR45D) do not impair nuclear pore complex assembly, thereby excluding that these interactions concur to Nup50 activity in nuclear reformation. Bar 10 µm

In parallel, I attempted to obtain information about the minimal region of Nup50 capable of retaining chromatin binding. I started preliminary experiments displayed in Fig. 11 to confirm that the mutants I had so far employed in nuclear assembly localize on chromatin.

Interestingly, I found that an eGFP-tagged Nup50 mutant (K3ER4DR38AR45D), which had been described in literature as incapable of displacement of NLS cargo from Impα, stops Nup50 from localizing to chromatin.

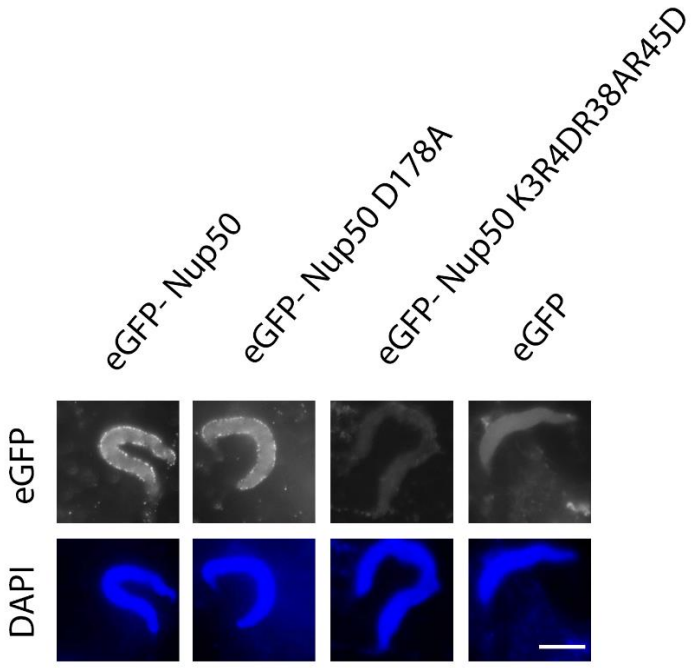

Figure 11: Mutation of Nup50 affecting the binding to Impα (Nup50 K3ER4DR38AR45D) in chromatin binding assay negatively impacts binding to chromatin. Bar 10 µm.

Therefore, these four residues seem to be not only required for Impα binding, but also involved in chromatin binding. Nonetheless, this mutant was able to rescue the depletion phenotype *in vitro* (see Figure 10). This suggests that chromatin binding mediated by N-terminal moiety of Nup50 is not required for NPC assembly after mitosis. This introduces the interesting possibility that Nup50 does not need to localize on chromatin to perform its activity. This would be surprising in light of the fact that early stages of assembly require attachment of recruited factors to nucleosomes.

I. 7. MEL28/ELYS cooperates to bring Nup50 to chromatin

After excluding that Nup50 localization on chromatin mediated by its N-terminal is relevant for assembly, I wondered whether other interactions could contribute to bring endogenous Nup50 to chromatin. MEL28/ELYS could be responsible for this role, taken into account multiple evidences of their direct interactions. In fact, in time course nuclear assembly experiments, MEL28/ELYS is present on chromatin already at t=0, as early as Nup50 (Franz et al 2007 and Figure 22). To verify this hypothesis, I proceeded to deplete MEL28/ELYS from extracts and analyzed localization of endogenous Nup50 in chromatin binding experiments, to detect potential variations in its localization. In addition, I depleted Nup50 and used the depleted

extracts to verify localization of the undepleted MEL28/ELYS in the extracts (Figure 12 panel A, see also Figure 6 panel B). Results are summarized in Figure 12.

Residual levels of Nup50 can be always visualized on chromatin even after it has been depleted from extracts (Figure 12, panel B). Therefore, it is striking to see that depletion of MEL28/ELYS indeed decreased Nup50 signal. Unlike what occurs to MEL28/ELYS in Nup50 depleted extracts, this finding cannot simply be explained by codepletion (Figure 12 panel A). I inferred that MEL28/ELYS may contribute to, if not be responsible for, Nup50 chromatin binding. If so, it is worth pointing out that exogenous eGFP Nup50 K3ER4DR38AR45D, which contains a functional MEL28/binding site, would be expected to be found on chromatin to a certain extent. This could be either explained by a different behavior in this respect between the exogenous e-GFP tagged Nup50 (Figure 11) and the endogenous protein in extracts; alternatively, the difference in the sensitivity between immunofluorescence and direct eGFP fluorescence detection could account for that.

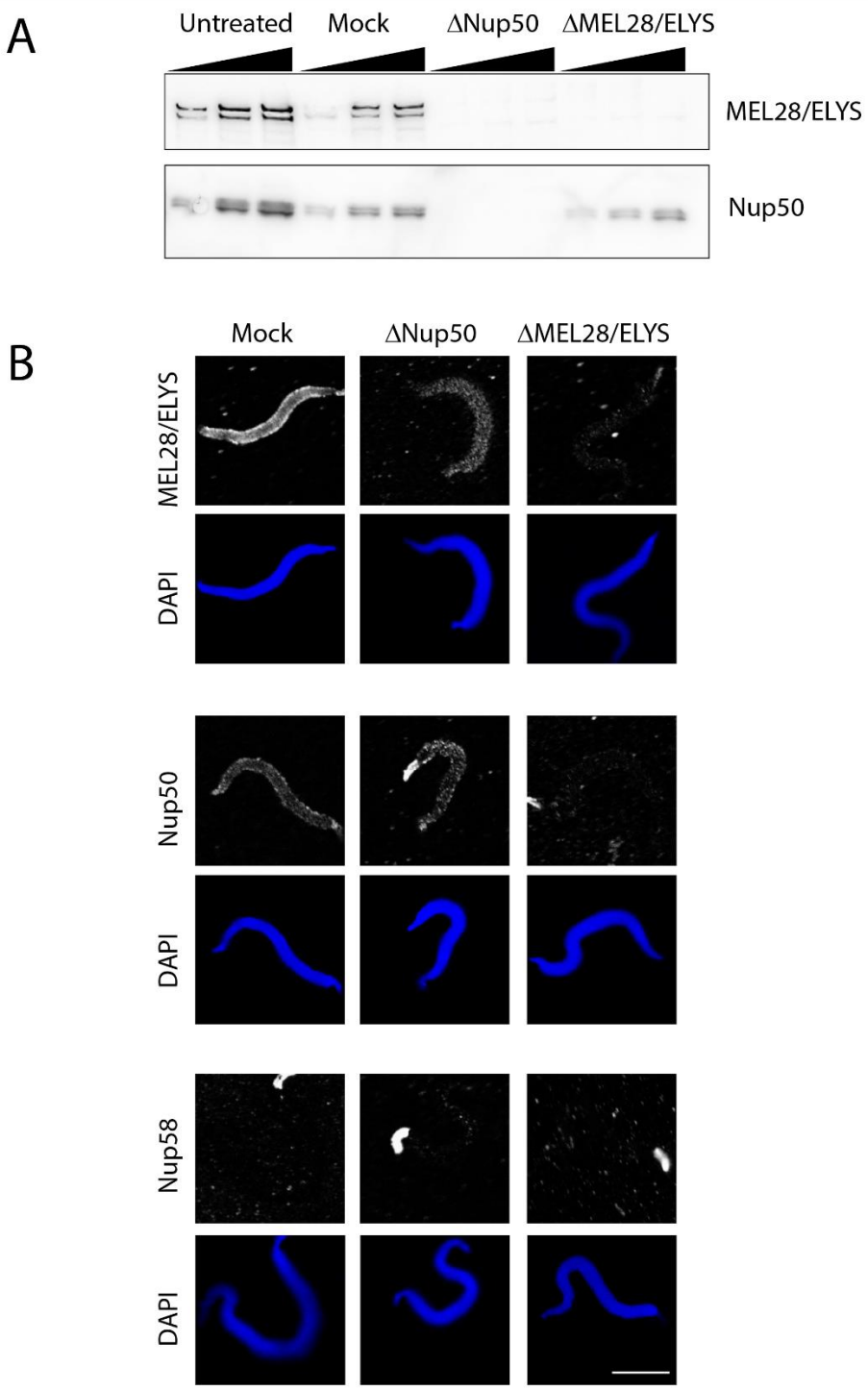

Figure 12: MEL28/ELYS contributes to Nup50 localization on chromatin.
Both endogenous MEL28/ELYS and Nup50 localize on chromatin in mock depleted extracts. Nup58 is used as a negative control. Residual levels of Nup50 are detectable even when it has been depleted from extracts (upper panel), whereas decrease in MEL28/ELYS in the same extracts, in the middle panel, is due to codepletion (as from panel A, also see Figure 6 panel B). Bar, 10 µm.

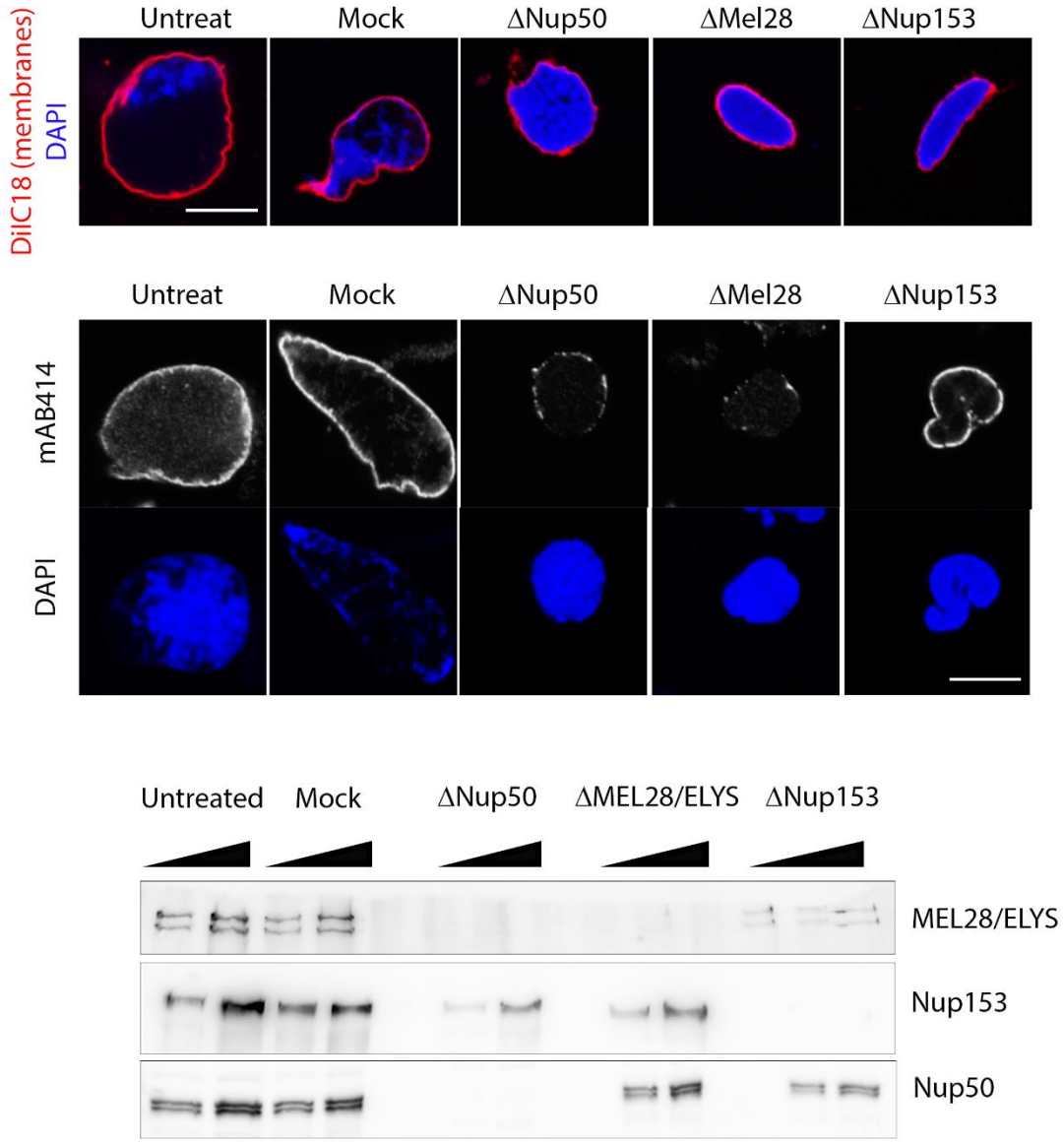

Figure 13: Nup50 and Mel28, but not Nup153, are required for NPC formation.
Nuclei embedded by closed nuclear envelopes (indicated by membrane staining DiIC18, in red), but devoid of NPCs (indicated by mAB414 signal, in greyscale), form *in vitro* when extracts depleted of Nup50 or MEL28/ELYS are used (see also Figure 7). Depletion efficiency is confirmed by Western blotting. On the contrary, depletion of Nup153 has no effect on nuclear formation. Bar 10 µm.

I could observe similar results when using Nup50, Nup153 or MEL28/ELYS depleted extracts in nuclear assembly. Depletion of Nup50 reduced the detectable levels of MEL28/ELYS on chromatin, as discussed, likely because of codepletion, but it also impedes that Nup153

localizes to the rim (Figure 14). Depletion of MEL28/ELYS also reduces the amount of Nup50 and Nup153 on chromatin. As from literature, depletion of Nup153 has no effect on postmitotic nuclear assembly, not on deposition of either MEL28/ELYS or Nup50 to the nuclear rim.

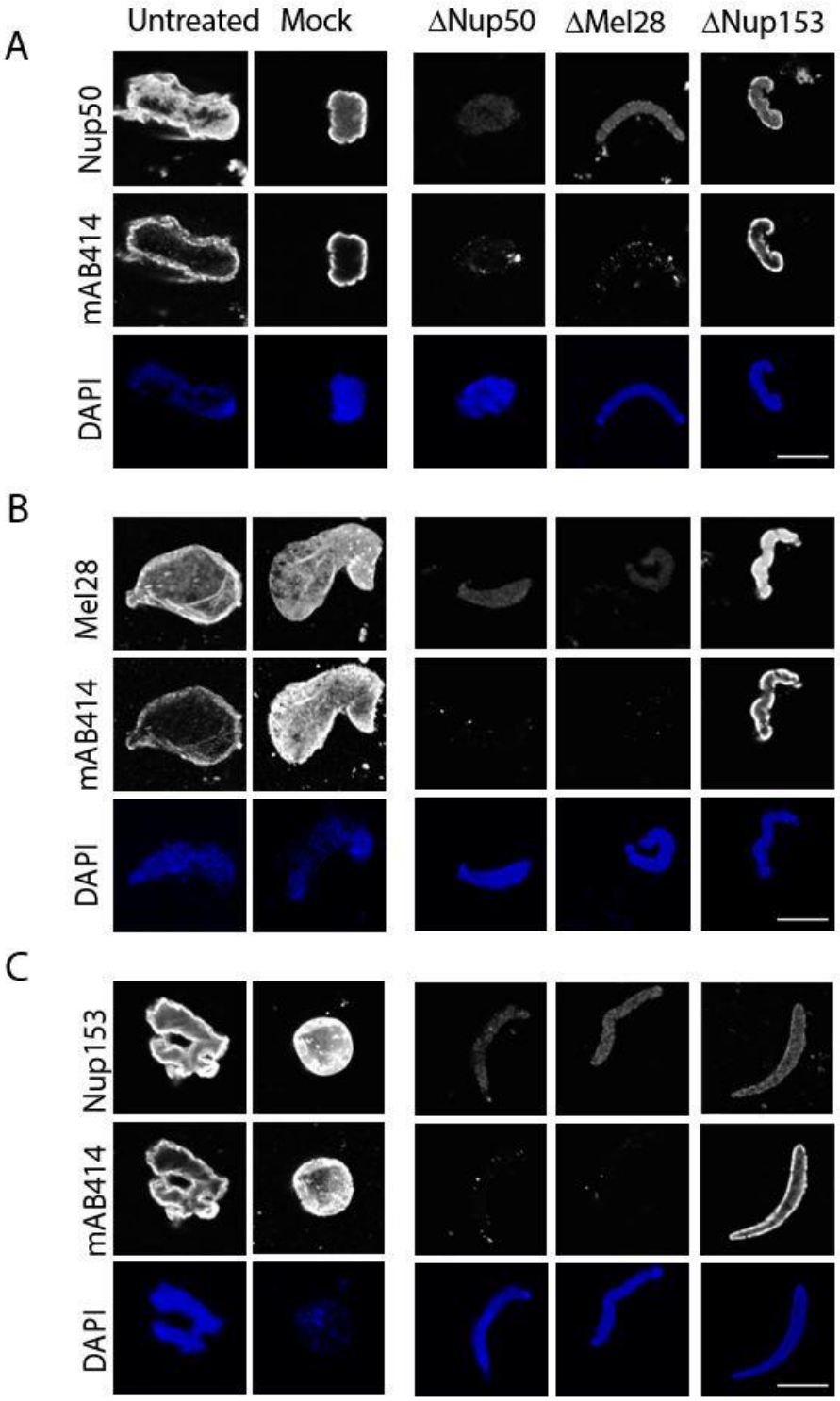

Figure 14:

Extracts depleted of Nup50 or MEL28/ELYS are not able to support NPC assembly, whereas Nup153 is not required for this process.

Upon depletion of Nup50, the protein is absent from chromatin, and so is MEL28/ELYS, which is codepleted (see Western Blot in Figure 13). Nup50 does not localize on chromatin when MEL28/ELYS is depleted, independently from codepletion (see Figure 12).

Bar 10 µm.

When analyzing the effect of each of the three depletions on the specific localization of each on the three nucleoporins, we can observe that, as expected, Nup50 is reduced on chromatin upon MEL28/ELYS depletion (Figure 14, panel A), which does not occur if Nup153 is depleted. MEL28/ELYS is codepleted and therefore its fluorescent signal reduced, upon Nup50 depletion (Figure 14 panel B), whereas levels of Nup153 on chromatin suffer from either Nup50 or MEL28/ELYS depletion (Figure 14 panel C).

These experiments confirm our current knowledge about the interplay between chromatin binding proteins Nup50 and MEL28/ELYS and nucleoporin Nup153, with respect to their activity in nuclear assembly. Nup153 is not required for complex reassembly after mitosis, and its depletion does not negatively impact neither Nup50 nor MEL28/ELYS. Nup50 partially codepletes MEL28/ELYS to levels that are still sufficient to restore pore formation following Nup50 addback (see Figure 7). MEL28/ELYS contributes to bring Nup50 to chromatin, as its level are reduced, although partially, upon MEL28/ELYS depletion both in chromatin binding (Figure 12) and nuclear assembly (Figure 14).

I. 8. Double mutant of Nup50 does not support NPC assembly, but is able of proper folding

Nup50 localizes on chromatin in a way that relies on its N-terminal domain, but the MEL28/ELYS binding domain additionally contributes to this. As it is likely that Nup50 must be found on chromatin to effectively perform its function, I speculated that MEL28/ELYS binding could be an additional way for Nup50 to be recruited locally and, therefore, ensure its correct positioning in the appropriate timeframe for NPC assembly. I wondered whether impairing MEL28/ELYS chromatin-mediated indirect binding, and the N-terminal chromatin direct binding at the same time, by combining the mutations of Nup50 already tested (Figure 10) in one unique double mutant (Nup50 K3ER4DR38AR45DW159A, Figure 15 panel A), could produce a different outcome from the previous experiment when this full-length protein is used in addback. In fact, this mutant does not rescue NPC assembly (Figure 15 panel B) as predicted. Moreover, the double mutant version of Nup50 does not localize to chromatin (Figure 16). When Nup50 is depleted from extracts, nuclei assembled in such extracts may show residual Nup50 staining, clearly distinguishable from rim staining that characterize a properly assembled nucleus (compare mock nuclei and ΔNup50 nuclei) and that is also visible in chromatin binding assay

(Figure 12). This residual staining is absent in the addback, indicating that Nup50 K3ER4DR38AR45DW159A does not attach to chromatin.

Loss of function of the double mutant could nevertheless be explained by incorrect folding of Nup50 during purification, due to accumulation of mutations in its sequence. In order to exclude this, I performed pulldowns using anti-GFP beads incubated with HeLa cell extracts, obtained from cells that had been transfected with an eGFP Nup50xl mutants of interest.

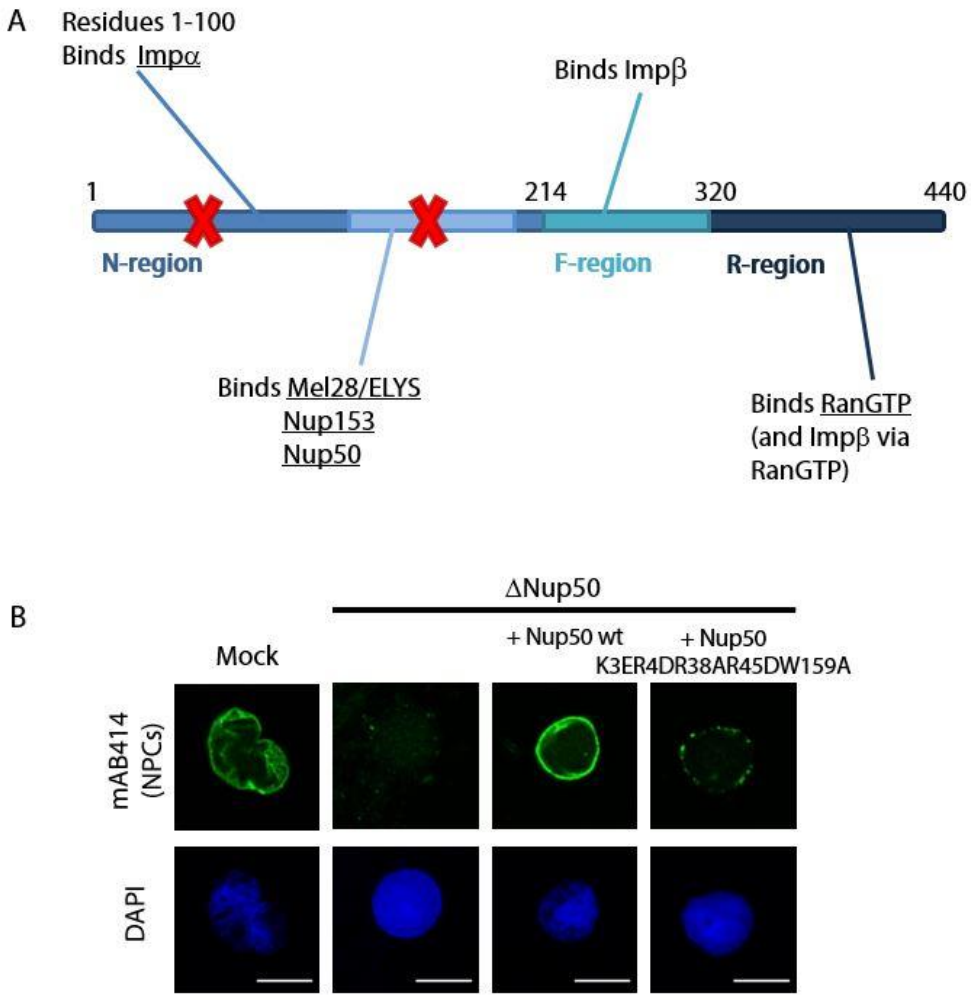

Figure 15: A Nup50 double mutant impaired in Impα and Mel28 binding is not able to rescue NPC assembly.

When Nup50 depleted extracts are supplemented with Nup50 mutant version impaired in MEL28/ELYS and Impα binding, NPC assembly is impaired, as signaled from loss of mAB414 signal compared to mock depleted nuclei and nuclei assembled in the presence of Nup50 wild type. Bar, 10 µm.

These experiments revealed that Nup50 K3ER4DR38AR45DW159A keeps dimerization capabilities, as its eGFP-tagged version efficiently pulls down endogenous human Nup50 contained in HeLa cell extracts. In all likelihood, the double mutant retains its folding and other

unaffected interactions; loss of mAB414 staining in depleted extracts supplemented with the double mutant (Figure 15) is rather the result of a real loss of function than an undesired misfolding effect.

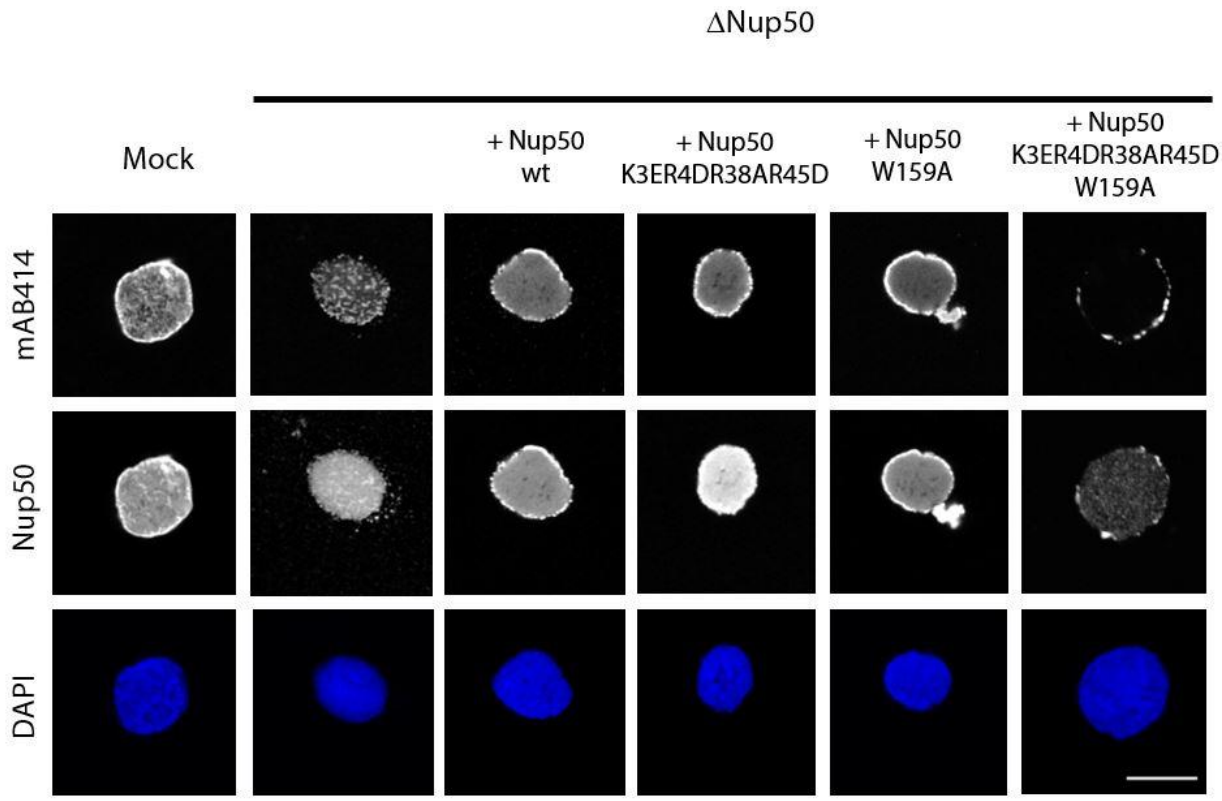

Figure 16: The Nup50 double mutant impaired in Impα and Mel28 binding is not able to localize on chromatin. When Nup50 depleted extracts are supplemented with Nup50 mutant version impaired in MEL28/ELYS and Impα binding, NPC assembly is impaired, as signaled from loss of mAB414 signal compared to mock depleted nuclei and nuclei assembled in the presence of Nup50 wild type (first row), see also Figure 15. In these nuclei, Nup50 cannot localize on chromatin (second row). Bar, 10 µm).

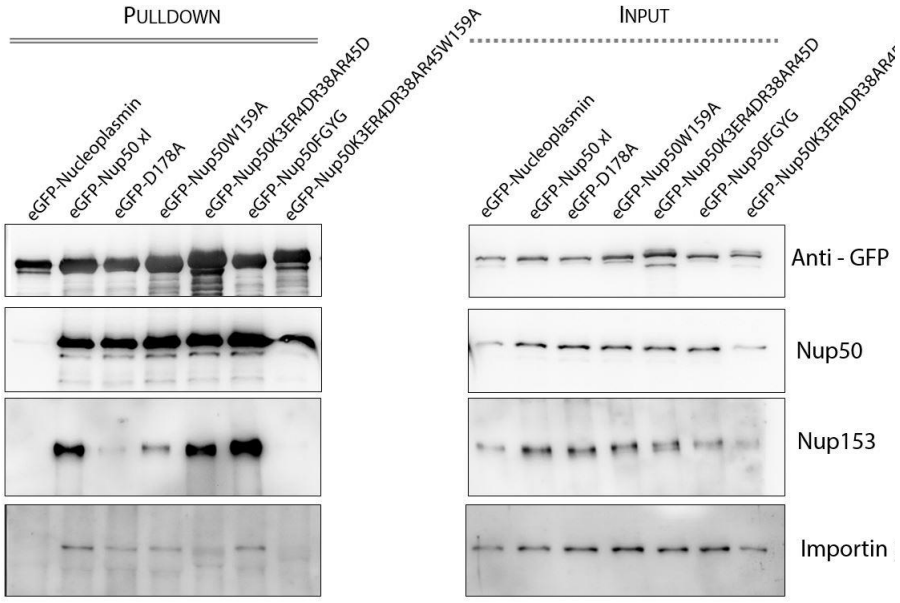

Figure 17: The Nup50 double mutant impaired in Impα and Mel28 binding retains dimerization capability. eGFP pulldown in HeLa cells were transfected with the indicated constructs. Start input levels (on the right) are comparable, nevertheless the double mutant Nup50 K3ER4DR38AR45DW159 A strikingly retains dimerization capability, indicating correct folding of the protein.

I. 9. The N-terminal and middle fragment of Nup50 still work in assembly

Either one of two regions of Nup50, located in the N-terminal domain and in the middle moiety of the protein, seem to be required for NPC assembly. To prove that each of the region is indeed sufficient, I expressed and purified from *E. coli* recombinant Nup50 fragments corresponding to the wild type Nup50 regions that were mutated in the full length context to be used in addback. These experiments revealed that both fragments are functional in NPC assembly (Figure 18), as testified by mAB414 signal, which is restored after either protein was added to the depleted extracts. In the case of the Nup50 144-191, mAB414 signal is found of the rim, but produces an irregular, uneven pattern on it.

This is likely due to a block in nuclear import given by the absence of the N-terminal domain, responsible for binding Impα. As this binding facilitates release of cargos from the import complex, kinetics of import, which are key to ensure further NPC assembly after restoration of import barrier are altered. Alterations of import then undermines capability of the newly formed nuclei to assemble new pores.

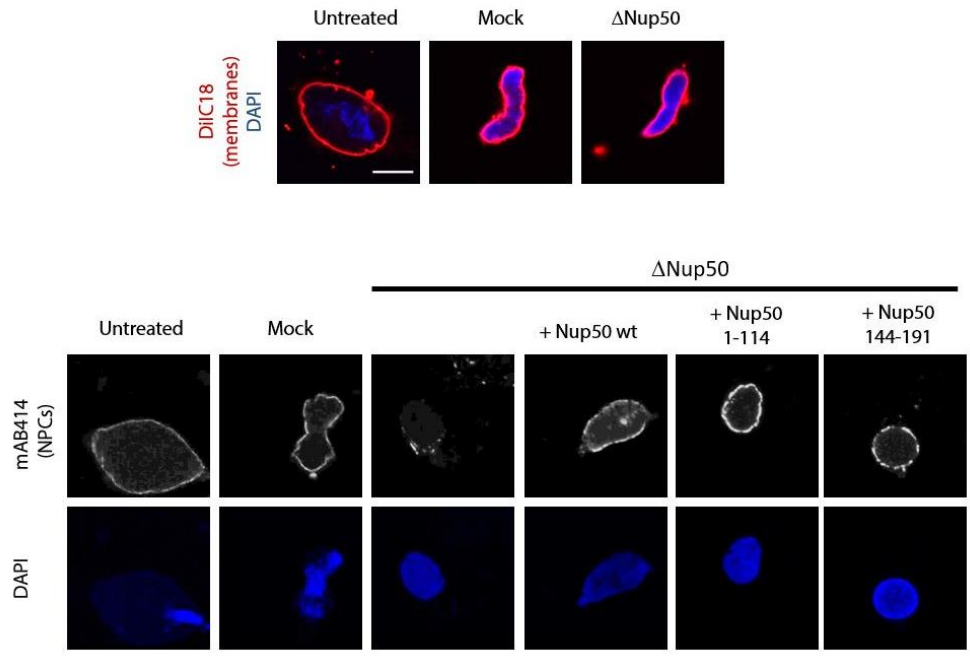

Figure 18: Fragments of Nup50, containing either its chromatin binding domain or its Mel28 binding domain, are able to support NPC assembly. When Nup50 depleted extracts are supplemented with Nup50 fragments corresponding to the N-terminal chromatin binding domain (Nup50 1-141) or the minimal MEL28/ELYS binding fragment (Nup50 144-191), NPC assembly is not dissimilar to mock depleted nuclei and nuclei assembled in the presence of Nup50 wild type, as indication that they are still functional in NPC assembly. Bar, 10 µm).

In conclusion, I have identified a form of Nup50 that was not functional in assembly, and I have excluded that the functional defect was due to a fault in experimental protein purification, or to its misfolding. Two different domains of Nup50, therefore, can independently contribute to its role in nuclear assembly; I have determined that these newly identified functional, localized on different domains, can work in assembly independently of each other, each being equired and sufficient in the absence of the other.

I. 10. Nup50 interacts with a member of the chromatin-remodeling complex SWI/SNF

As mentioned in Section I.1., IP-MS identified in the ATP-dependent chromatin remodeler complex SWI/SNF BRG1 and SMARCC1 two potential interactors. SWI/SNF contains either one of the two mutually exclusive catalytic subunits, BRG1 or BRM, and a variety of other regulatory subunits, some of which are conserved in evolution (reviewed in (Tang et al., 2010)). SWI/SNF has been implicated in several processes ranging from development, differentiation,

spermatogenesis, gene expression and cancer (Bourgo et al., 2009; Marathe et al., 2017; Nasipak et al., 2015; Serber et al., 2016; Wagner et al., 2017; Wu et al., 2017), and undergoes phosphorylation in mitosis (Sif et al., 1998), but it has been never directly linked to chromatin decondensation or remodeling at the end of mitosis so far. I aimed to confirm the interaction biochemically by repeating the IP in *Xenopus* extracts and performing Western Blot analysis using anti-BRG1xl antibody, a kind gift from the Rupp lab, and anti-SMARCC1 commercially available antibody from Thermofisher (PA5-30174).

The BRG1 antibody pulled down BRG1 and the interactor SMARCC1 from the extracts, but not Nup50 (Figure 19). Out of the two antibodies against Nup50 that I used in the pulldown, both efficiently bound Nup50. Nevertheless, none retrieved BRG1 bound to Nup50, and in only one case a faint band corresponding to SMARCC1 could be observed (Figure 19). The presence of this weak band may suggest that an interaction potentially exists between Nup50 and SMARCC1, either very weak, or tightly regulated, to be hardly distinguishable by immunoprecipitation.

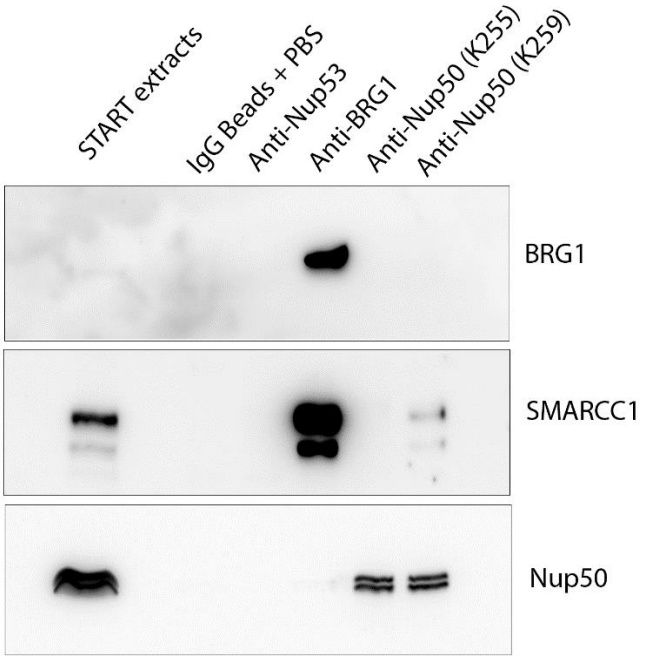

Figure 19: Nup50 interacts weakly with SMARCC1. IP-WB shows a weak interaction between Nup50 and SMARCC1, a regulatory subunit of the SWI/SNF remodeling complex. Anti-BRG1 antibody efficiently pulls down BRG1 and its cofactor SMARCC1. Anti-Nup50 antibody K259, but not K255, pulls down Nup50 and a small amount of SMARCC1, but no interaction with BRG1 is detectable.

I. 11. BRG1 is not required in NPC assembly

Even if BRG1 and Nup50 do not interact directly in *Xenopus* extracts, the possibility of a regulatory interaction between Nup50 and SMARCC1, a subunit of the SWI/SNF complex, may be indicative of a remodeling activity brought on chromatin by Nup50 at the end of mitosis.

I sought to determine whether this interaction is indeed required in the context of postmitotic nuclear assembly. In order to establish this, I performed BRG1 immunodepletion from *Xenopus* egg extracts using magnetic Dynabeads coupled to mouse anti-BRG1xl antibodies. BRG1 can be efficiently depleted from extracts after two rounds of depletion (Figure 20, panel A).

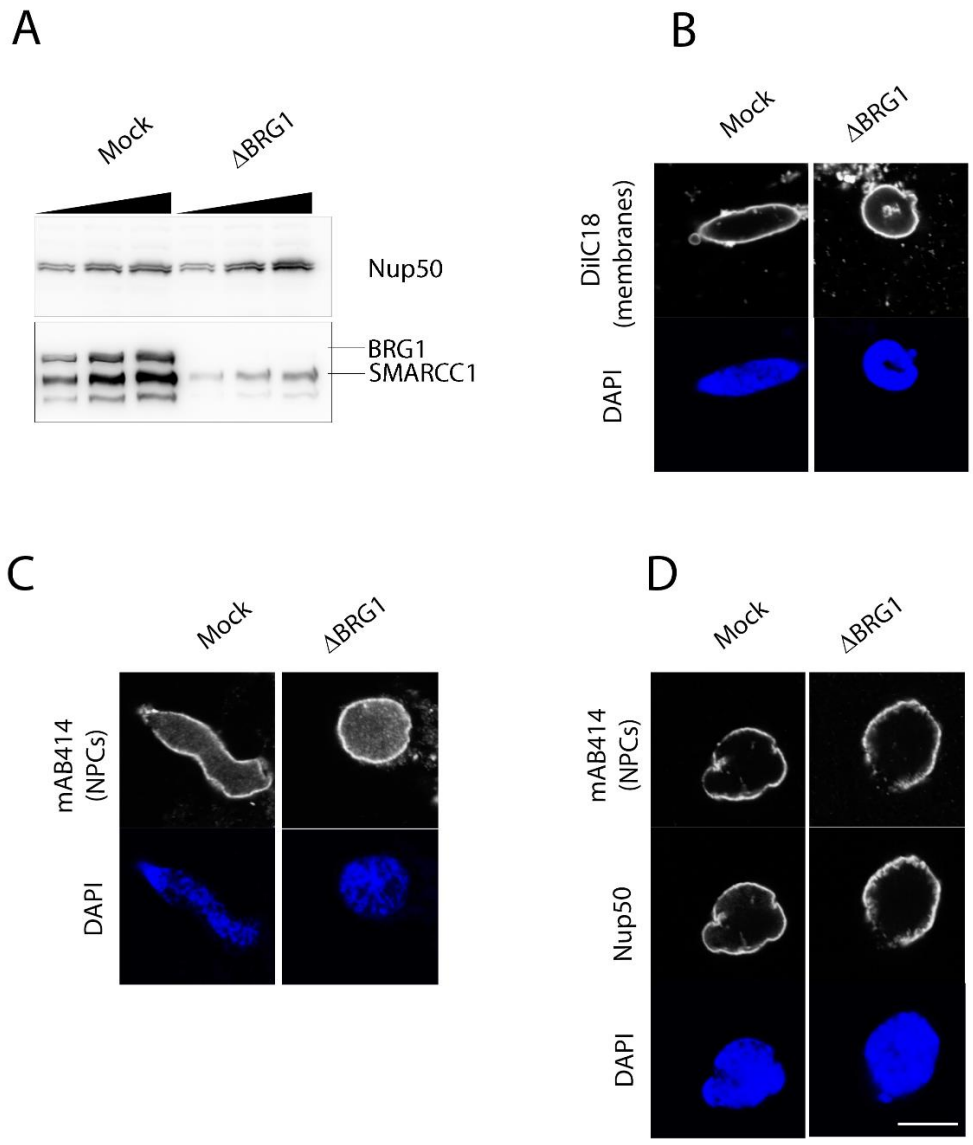

Figure 20: BRG1 is not required for nuclear assembly at the end of mitosis. BRG1 was depleted from Xenopus extracts without affecting Nup50 levels and with only a partial depletion of its interactor SMARCC1 (Panel A).
Nuclei assembled in BRG1 depleted extracts do not exhibit defects in NE assembly (Panel B) nor in NPC assembly (Panel C and D), as DiIC18 and mAB414 signals, respectively, are comparable to mock depleted nuclei. Nup50 localization on the rim did not vary in the absence of BRG1 (Panel D). Bar 10 µm.

When nuclei were assembled in BRG1 depleted extracts, no defects in nuclear assembly could be detected, and such nuclei resembled nuclei assembled in mock extracts when membranes (Figure 20 panel B), NPCs, and Nup50 stainings (Figure 20 panels C and D, respectively) are taken into account. As a conclusion, I was able to exclude that BRG1 catalytic activity in the SWI/SNF complex is required at the end of mitosis. The interaction between Nup50 and

SMARCC1 is nonetheless interesting and potentially relevant during interphase. Chromatin associated to NPCs in functional nuclei is, as a tendency, in the form of euchromatin, an open and transcriptionally active conformation, as described in introduction, Section 2. Nup50 is reportedly the nucleoporin with the lowest residential time at the NPC (Rabut et al., 2004) and it is found at transcriptionally active loci during interphase; this localization is mediated by its N-terminal domain (Buchwalter et al., 2014).

In the light of this information, it can be speculated that Nup50 supports transcription by either recruiting, activating, modulating or enhancing SWI/SNF catalytic activity on such loci; alternatively, it is possible that SMARCC1, and therefore BRG1, are moved to the pore when Nup50 is attached to the nuclear basket, recruiting this remodeler at the nuclear periphery. In conclusion, I was able to confirm the novel interaction between Nup50 and BRG1, and I established that it is not required for postmitotic nuclear assembly. The characterization and specific function of this interaction remains a fascinating open question.

I. 12. Dynein is required in the *in vitro* nuclear assembly

After IP-MS performed using anti-Nup50 antibodies, together with BRG1 and other proteins mentioned above, dynein emerged in the list of potential interactors of Nup50. Cytoplasmic dynein is a microtubule motor responsible for intracellular transport of cargos toward the minus end of microtubules (Chowdhury et al., 2015). In order to establish whether dynein is required in nuclear assembly, I proceeded to sequester it from the *Xenopus* extract, and used these extracts in nuclear assembly. This has been done before by adding dynactin/p50, an interactor of dynein that is also a member of the motor complex on the MT, at increasing concentrations (Hara and Merten, 2015).

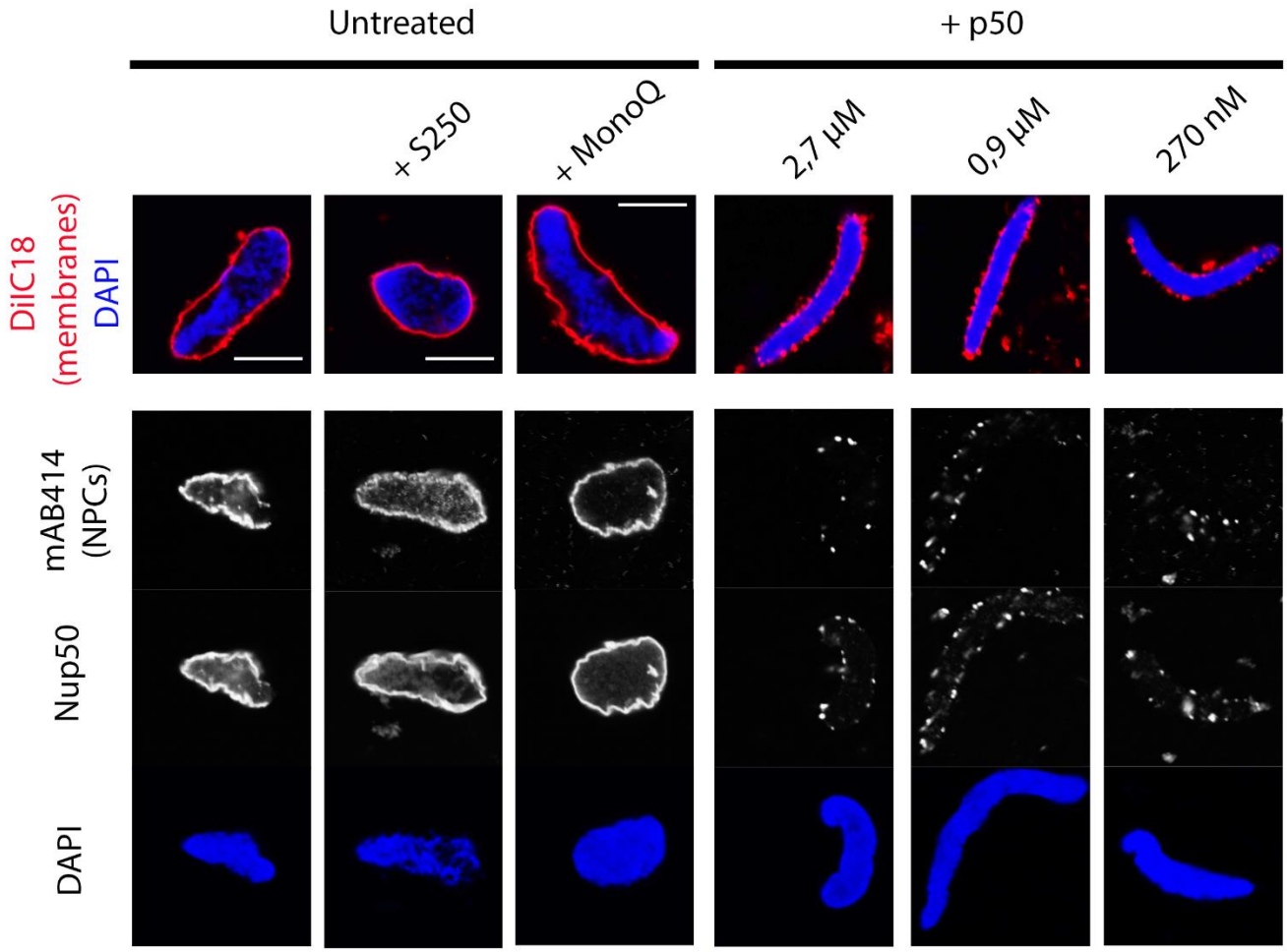

Figure 21. Dynein is required for NE assembly at the end of mitosis.
Nuclei in which dynein is sequestered from extracts using p50 are incapable proper NE assembly compared to control nuclei. Additionally, NPCs cannot form in such extracts. Nevertheless, Nup50 can localize on chromatin. Bar 10 µm.

Control reaction was performed in pure extracts, in the presence of the p50 purification buffer (MonoQ) or S250 extracts buffer. Nuclei assembled in the presence of dynactin/p50, in which dynein is sequestered, exhibit an important block of NE assembly (Figure 21), as quantified from two independent experiments (Figure 22).

This evidence point towards a novel role of dynein in nuclear assembly, that could either be due to its well-known MT motor function, or to an unidentified activity at the end of mitosis. Importantly, this phenotype is dramatically different from the Nup50 depletion phenotype, in which NE formation is not blocked (compare DiIC18 staining in Figure 21 and Figure 7). It is likely that the action of dynein precedes the action of Nup50 in assembly.

If the effects of the two proteins are linked, my hypothesis would address more rather resemble discrete, consecutive steps on the nuclear assembly pathway, than a cooperative action of Nup50 and dynein at the same stage of the pathway.

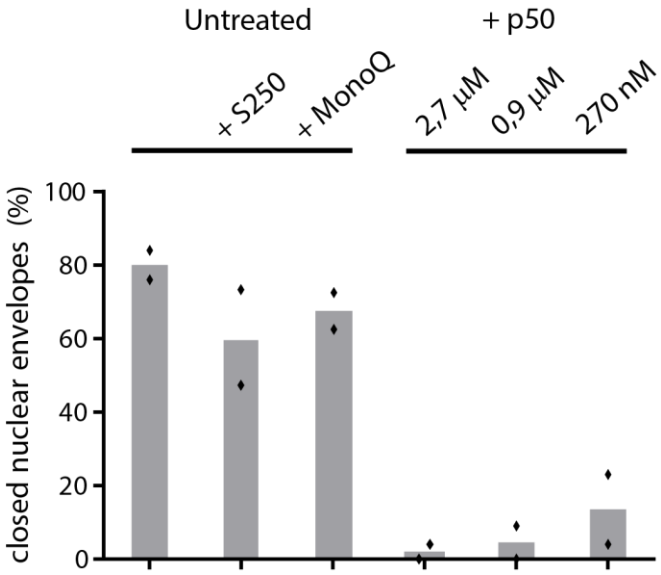

Figure 22. Inhibition of dynein results in block of NE assembly at the end of mitosis.
Quantification of nuclei surrounded by closed envelopes in either control or p50-treated extracts, in which dynein is sequestered from extracts and inactivates, revealed impairment in NE assembly. Mean with individual data points from two experiments are indicated.

I. 13. Tubulin pattern in in vitro nuclear assembly

I have so far identified cytoplasmic dynein as a Nup50 interactor, and have determined that dynein is indeed required at the end of mitosis. These results led me to wonder whether a potential role for its action in NE assembly was linked to its well-described function in MT transport, or rather to a different activity of dynein.

Postmitotic nuclear assembly occurs in a very dynamic moment of the cell cycle. As chromatin decondenses, nuclear envelope surrounds it and seals, while NPCs are formed within its double membranes. Concomitantly, mitotic spindle must be disrupted: surprisingly, very little data describing coordination between nuclear assembly and spindle disassembly are available. During mitosis, ER must be excluded from the spindle region and from chromatin (Anderson and Hetzer, 2008; Puhka et al., 2007) to allow proper contact between the latter. At the onset of anaphase, when the nucleus begins its reformation, several studies support the idea that microtubules are generally not required for nuclear envelope reassembly (Dreier and Rapoport, 2000; Wang et al., 2013), and one in particular reports that microtubules polymerization is blocked in the vicinity of the decondensing chromatin (Xue et al., 2013).

In order to understand whether dynein is required as a MT motor, or as performer of an unknown function, I aimed to describe the pattern of tubulin on sperm chromatin during *in vitro* nuclear assembly reactions. Therefore, I performed time course *in vitro* nuclear assembly and stopped the reactions by fixation at increasing time points (0h, 5 min, 30 min, 60 min, 2h); such assemblies were fixed at room temperature to avoid microtubule depolymerization. These nuclei were either assembled in the presence of the membrane stain DiIC18 or stained after assembly with an anti-αtubulin antibody (Sigma, T6199) and mAB414 antibody. Such experiments would allow me to detect a pattern of tubulin on chromatin and to correlate it to the well-known pattern of membranes and NPC formation on the decondensing chromatin.
Figure 22 shows how such time courses did not highlight a clearly recognizable tubulin pattern on the forming nuclei. Tubulin localized on the sperm periphery after 10 minutes. Nevertheless, it is possible that this localization is due to unspecific binding of the antibody to the sperm tip, which is highly charged and therefore likely to be non-specifically targeted by the anti-tubulin antibody. I was next determined to verify the effect of interference (enhancement and, on the contrary, disruption) of microtubule polymerization in the proximity of the nuclei and study the consequences of these events on nuclear assembly.

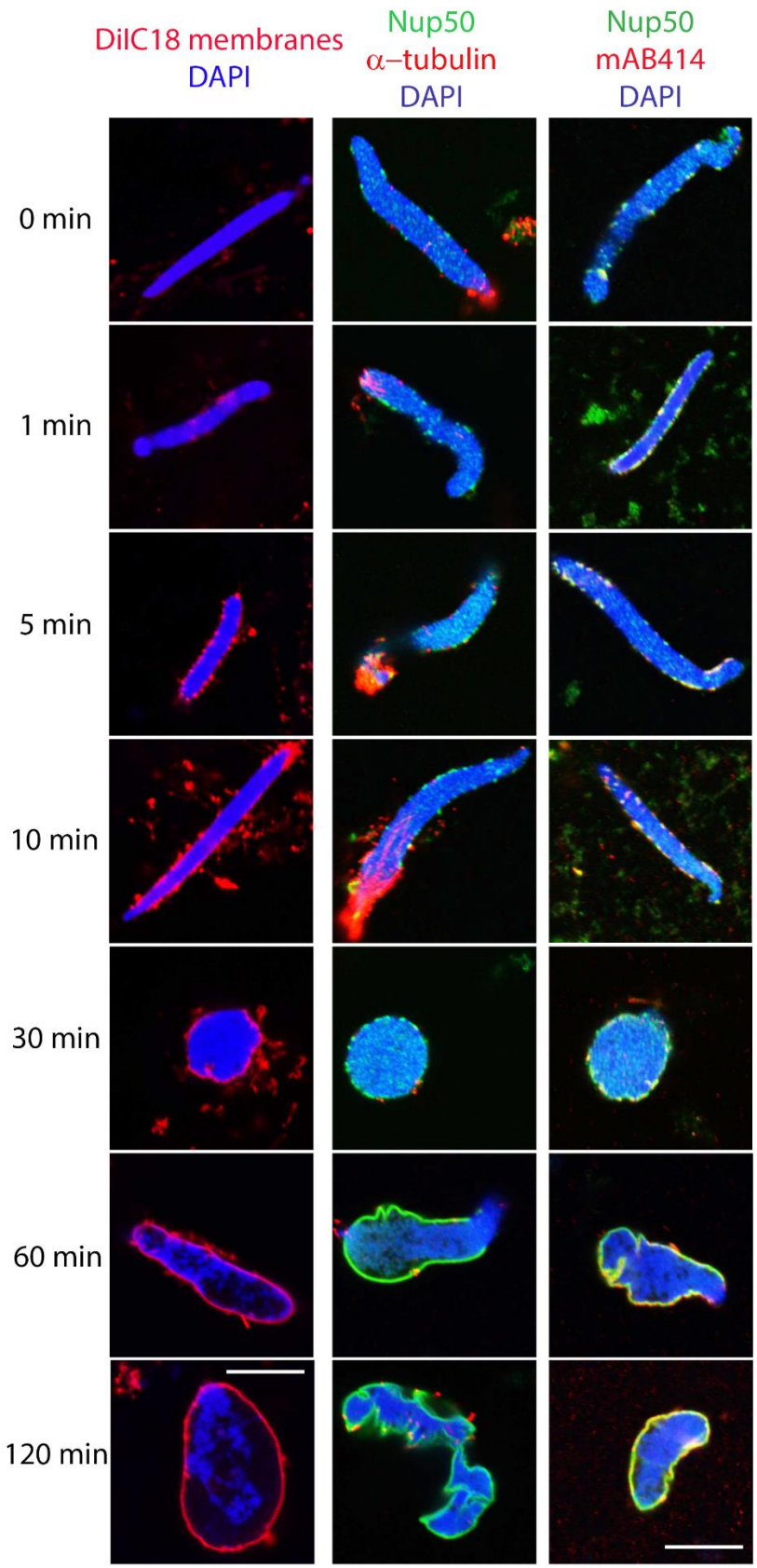

Figure 22: Tubulin pattern on *in vitro* assembled nuclei. Nuclei were assembled in the presence of extracts, sperm chromatin and energy mix, with either unlabeled or DilC18-labelled membranes. After the indicated time points, reactions were stopped by fixation at room temperature and immunofluorescence performed on unlabeled nuclei for detection of Nup50, mAB414 and tubulin. Bar 10 µM.

In order to answer this question, I proceeded with a series of experiments in which I either disrupted MT polymerization by means of addition of 20 μM nocodazole or stabilized polymerized microtubules by adding 10 μM taxol. I then compared such nuclei with nuclei assembled in untreated extracts. After 2h, extracts treated in either nocodazole or taxol were able to fully support nuclear formation. Such nuclei were morphologically indistinguishable from control nuclei (Figure 23), when both membranes (with DiIC18) and NPCs (with mAB414) are examined. This experiment suggests that MTs are not required for postmitotic nuclear assembly and confirms previous literature evidences according to which NE and NPC reformation do not need MT polymerization. Therefore, the activity of dynein at the end of mitosis must be independent from its minus end-directed MT transport. My work provides evidences of a novel player involved in postmitotic nuclear assembly and addresses future research towards a previously undescribed activity of cytoplasmic dynein.

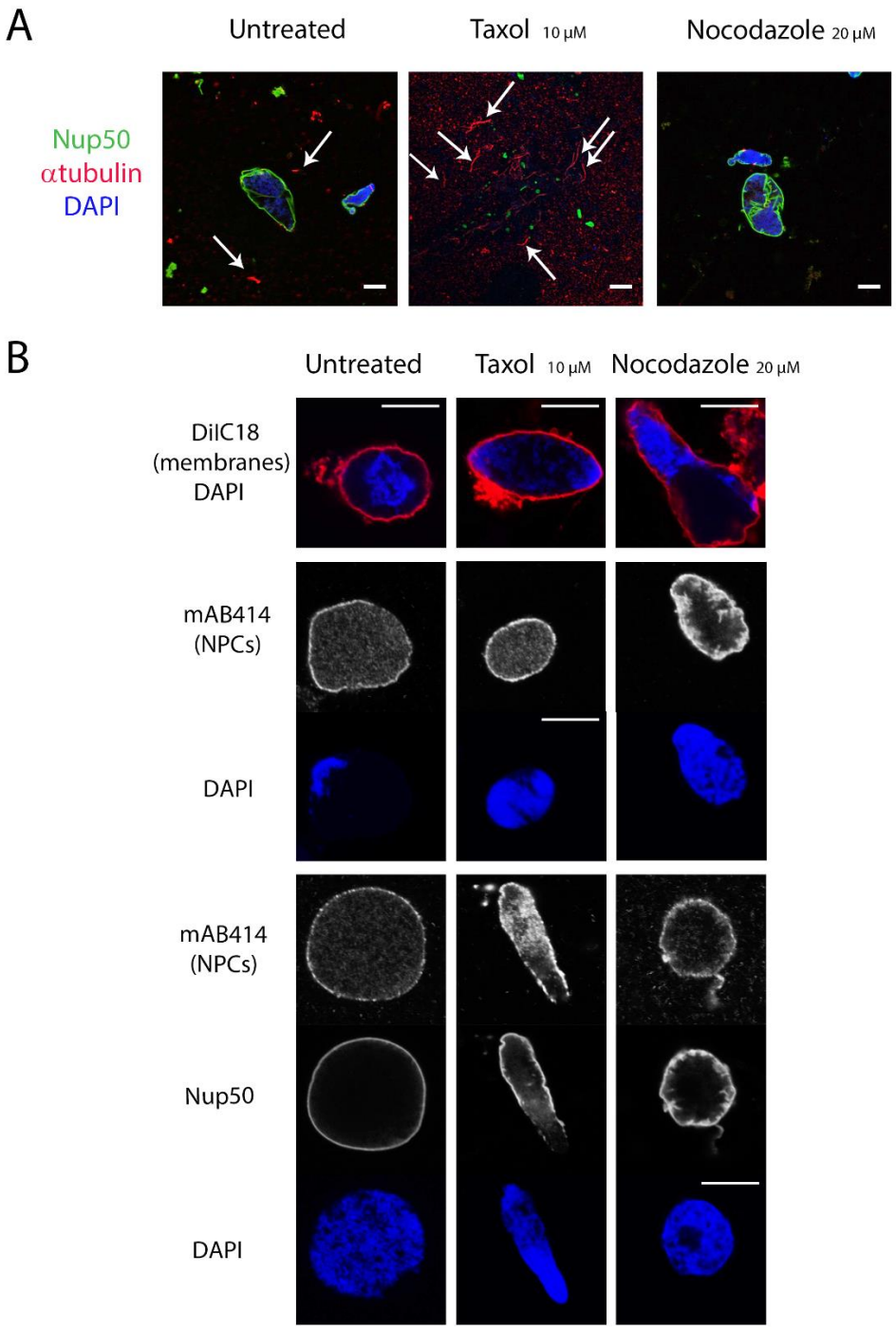

Figure 23. Microtubules are not required for, nor inhibiting, *in vitro* reconstitution of nuclear assembly in *Xenopus* egg extracts. Egg extracts have been treated with either the microtubules stabilizer taxol and microtubule polymerization inhibitor nocodazole. Such extracts were used for *in vitro* nuclear assembly reaction. Microtubule polymerization increased in the reaction were extracts treated with taxol were used, and diminished in extracts treated with nocodazole with respect to nuclei treated with S250 buffer (panel A, see arrows). Nuclei formed in these conditions were unaffected by treatment (panel B): nuclear envelopes formed similarly to mock depleted extracts, mAB414 antibody stained the rim not dissimilarly from control nuclei; Nup50 pattern was comparable to control. Bar 10 µm.

I. 14. Nup50 binds Nup107-160 complex independently of MEL28/ELYS and of Nup153

When listing the Nup50 interactors revealed by the IP-MS experiments, I excluded Nup107-160 as direct interactors at first as they are already known to bind MEL28/ELYS, by which the complex is tethered on chromatin (Franz et al., 2007). Therefore, at first I classified the Nup50-Nup107 interaction as indirect because bridged by MEL28/ELYS. Nevertheless, when depleting Nup50 from *Xenopus* egg extracts, I could observe a codepletion of Nup107 that did not occur when depleting MEL28/ELYS (Figure 24), suggesting that Nup107 interacts with Nup50 by means of a different region than the one that mediates MEL28/ELYS binding.

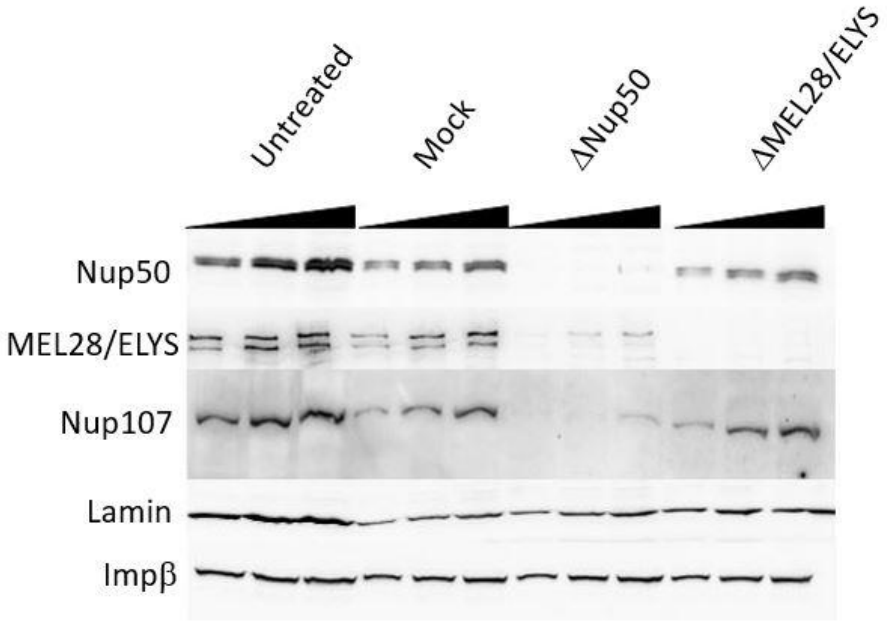

Figure 24: Depletion of Nup50, but not of MEL28/ELYS, codeplete Nup107, pointing toward a direct interaction of Nup50 with the Nup107-160 complex.

To confirm their interaction in vitro, I proceeded to perform pulldown experiments in *Xenopus* egg extracts that had previously been depleted of both MEL28/ELYS and Nup153 (Figure 25 panel A), both common interactors of Nup50 and Nup107-160 (Franz et al., 2007; Hase and Cordes, 2003; Vollmer et al., 2015). I used either mock-depleted or MEL28/ELYS-Nup153 depleted extracts as prey using antibodies against anti-Nup50 and anti- Nup133, a member of the Nup107-160 complex.

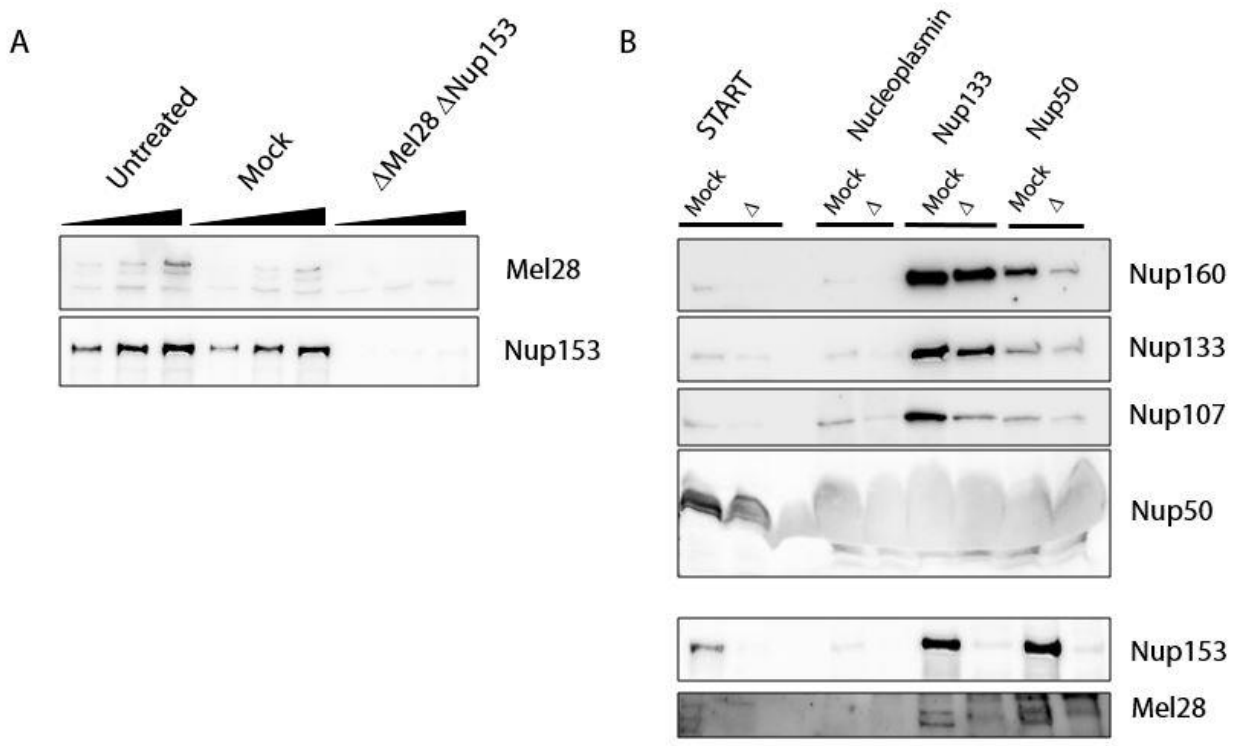

Figure 24: Immunoprecipitation experiments using either mock-depleted or MEL28/ELYS-Nup153 depleted extracts reveal that, in the absence of their common interactors (depletion is shown in panel A), Nup50 retains binding to members of the Nup107-160 complex Nup107, Nup133 and Nup160 (panel B, Δ), although reduced when compared to the interaction that they maintain in the presence of the depleted proteins (panel B, Mock). It was not possible to infer the binding of Nup133 to Nup50, as the protein migrates in a region of the SDS gel very close to the heavy chains of the immunoglobulins employed for the pulldown, which are much more abundant and interfere with the anti-Nup50 antibody.

These experiments highlight how, in the absence of their common interactors MEL28/ELYS and Nup153 (Δ), Nup50 retains binding to members of the Nup107-160 complex Nup107, Nup133 and Nup160, although reduced when compared to the interaction that they establish in the presence of the depleted proteins (Mock). Depletion efficiency, as from panel A, is further confirmed when MEL28/ELYS and Nup153 are detected after immunoprecipitation: both anti-Nup50 and anti-Nup133 antibodies can pull down MEL28/ELYS and Nup153 from the mock-depleted extracts, but not from the extracts that has been depleted of these two proteins.
These results raise the interesting possibility that Nup50 may act as a complementary scaffold for recruitment of the Nup107-160 complex for nuclear assembly, in addition to the well-known mechanism according to which this complex is brought on the chromatin by MEL28/ELYS at the end of mitosis. This hypothesis is rather tempting, as the binding sites of members of the

Nup107-160 complex on Nup50 are probably not mapped on the same domain of the binding site for MEL28/ELYS, as the codepletion data suggests (Figure 24). This would explain why both fragments of Nup50, 1-114 and 144-191, are able to rescue the phenotype: the former might bind the Nup107-160 complex directly or by means of so far unidentified mediators, whereas the latter could promote Nup107-160 complex recruitment on chromatin by means of interactions bridged by MEL28/ELYS. Such an interaction network would find an explanation for its redundancy in the striking importance of paced, correct NPC reformation, that must be critically coordinated spatially and temporally to the whole nuclear reformation at the end of mitosis.

Part II: Nup155 in nuclear assembly

II. 1. Membrane binding of Nup155 is required at the end of mitosis

Nup155 is a membrane binding protein, member of the inner ring complex, required for postmitotic NPC assembly (Franz et al., 2005). Nup155 from vertebrate, *C. thermophilum* and from its two *S. cerevisiae* homologues, Nup170 and Nup157, are composed by an N-terminal domain, which organizes as a β-propeller, and a C-terminal domain, which folds as an α-solenoid. During postmitotic assembly, Nup155 is tethered on the pore by Nup53, which interaction is conserved in evolution (Amlacher et al., 2011; Fahrenkrog et al., 2000; Lusk et al., 2002). In *C. elegans*, *Drosophila* and mouse, Nup155 is an essential gene (Franz et al., 2005; Galy et al., 2003; Kiger et al., 1999), most likely because of its central function in NPC assembly. In *S. cerevisiae,* deletion of both Nup155 homologous Nup170 and Nup157 is lethal (Aitchison et al., 1995) because these proteins, similar in structure, are also essential for NPC assembly in yeast. New evidences suggest, although, that they might perform functions distinct from each other (Makio et al., 2009).

Previous work from the Antonin lab identified a region on the N-terminal β-propeller of nucleoporin Nup155 that is buried into the pore membrane in cryo-EM structure of the human NPC and is required for membrane binding of liposomes *in vitro* (von Appen et al., 2015). Another non-transmembrane, membrane binding nucleoporin, Nup53, had been found to act in nuclear assembly by binding and bending membranes locally, to accompany the double NE and accompany its bending to assume the most favourable topology for proper NPC assembly (Vollmer et al., 2012). Indeed, modeling of the yeast Nup53 and Nup170 into the human tomographic reconstruction suggests that both protein share this mode of membrane interaction (Lin et al., 2016). A similar role for the membrane binding domain of Nup155 could be hypothesized: therefore, I took advantage of two different membrane binding domain mutant versions of Nup155, characterized in (von Appen et al., 2015). One lacks the whole membrane binding region (Nup155 Δ258-267), whereas in the second mutant the first residue of the region is mutated (Nup155 L258D): both reportedly lost their membrane binding capability. When mRNA encoding either Nup155 mutant was added to Nup155 depleted extracts in nuclear assembly experiments, extracts was able to support expression of the protein (Figure 1A, (De Magistris et al., submitted)); nonetheless, extracts in which either version of Nup155 was present were incapable to support proper nuclear assembly. These nuclei were surrounded by

membrane vesicles which failed to fuse in a closed double envelope and lacked NPCs, as clearly indicated from the DiIC18 and the mAB414 staining, respectively (Figure 1B and 1C, (De Magistris et al., submitted)). These experiments confirm literature data about the requirement of Nup155 in nuclear assembly and prove that membrane binding of Nup155 makes the protein indispensable, mediated by a 10-residues long region in its N-terminal domain.

II. 2. Nup155 N-terminal domain rescues NE assembly but is not sufficient to support NPC assembly

Postmitotic nuclear assembly requires Nup155 to be able to bind membranes; this function relies on a short region that includes 10 amino acids of a loop that protrudes from the N-terminal β-propeller. Nevertheless, I wondered if membrane binding was not only necessary, but also sufficient for nuclear assembly, or if there are other functions performed by the C-terminal α-solenoid of Nup155 that must take part in the assembly process. In order to answer this question, I proceeded to add an N-terminal fragment of Nup155, that includes the complete propeller sequence, to Nup155 depleted extracts, and experimentally verify whether this could be able to replace the full length protein. I performed this addback with a protein fragment instead of an mRNA, which was done as described in Result section II.1. Protein addback was required as quantification of the mRNA expressed protein was impossible: in fact, the antibody used in Figure 1A of (De Magistris et al., submitted) was raised against the full length protein, rendering impossible any comparison between the concentration of the fragments and the Nup155 contained in the mock. Protein was expressed in, and purified from, *E. coli*, then its concentration titrated for nuclear assembly.

Interestingly, upon quantification of three independent experiments, I was able to observe a rescue of nuclear envelope formation, but not of NPC assembly (Figure 2A and 2B, (De Magistris et al., submitted)), as indicated by the much reduced mAB414 staining of the nuclei assembled in depleted extracts compared to mock extracts. This suggests that membrane binding capability is required and sufficient for NE assembly, but that NPC assembly requires a yet unidentified activity that can only be carried out in the presence of the C-terminal domain of the protein.

II. 3. Nup155 N-terminal domain supports assembly of a minimal core pore

Nuclei depleted of Nup155, that have been rescued with the N-terminal domain of the protein, exhibit mostly closed envelopes but no assembled pores, as staining with mAB414 highlighted a dotted pattern on this nuclei, which are mostly very small. This resembles the phenotype that was observed with the depletion of other nucleoporins from extracts, like MEL28/ELYS and Nup50 ((Franz et al., 2005), this work) and with the phenotype observed from Sachdev et al. when Nup93 depleted extracts were supplemented with a C-terminal fragment of the protein. As Nup155 is known to be recruited later that MEL28/ELYS and Nup50 for NPC assembly, I decided to investigate which nucleoporins could be found on chromatin in nuclei depleted of Nup155 and rescued with the N-terminal fragment of the protein. As expected, I could detect MEL28/ELYS and Nup107 (Figure 3A, (De Magistris et al., submitted)), as well as Nup50 (data not shown) on the chromatin. Interestingly, I also found that Nup53 was present on these nuclei, in accordance with the fact that it can localize to chromatin even when its Nup155 binding site is disrupted (Eisenhardt et al., 2014a). It is not surprising that recruitment of Nup153 is not impaired, as it could be brought in loco by the Nup107/160 complex, with which it interacts (Krull et al., 2004; Vollmer et al., 2015); this could explain the residual mAB414 staining on nuclei in Figure 2A, as Nup153 is one of the Nups recognized by this monoclonal antibody.

It came to my surprise that Nup93 was absent from these nuclei, as Nup93 is reportedly required for NE formation. Resulting from this, members of the Nup93-complex Nup205 and Nup188, as well as the central channel component Nup62, were also missing. Consistently with Nup62 being absent from the pore, nuclei depleted of Nup155 were unable to import a GFP-tagged Impα-β dependent substrate (Figure 3C, (De Magistris et al., submitted)), confirming, together with the mAB414 staining, that integrity of the pore and its channeling functions are compromised.

Previous Nup93 depletion experiments had highlighted that Nup93 easily codepletes the other complex components Nup205 and Nup188 (Sachdev et al., 2012). Therefore, it had never been possible to determine whether these Nups can be additionally recruited by means of interactions with different pore proteins. Recent work in the thermophile fungus *Chaetomium thermophilum* provided new data about the interactions that ctNup188, ctNup192 (homologous of Nup205) are able to establish in the pore with ctNup170, the Nup155 homologous (Lin et al., 2016). As a consequence, it was possible to hypothesize that Nup188 or Nup205 could

potentially be recruited when Nup93 is not available. My work excluded these additional interactions are sufficient in NPC assembly, and that Nup93 is indeed the anchor that brings Nup188 and Nup205 on the pore.

Moreover, the N-terminal of Nup93 has already been proven to be required for the recruitment of the Nup62 complex; immunofluorescence data confirm that Nup155 depleted nuclei, supplemented with the N-terminal fragment, are lacking Nup93, therefore also Nup62.
Importantly, Western Blot analysis of the depleted extracts proves that the nucleoporins failing to be recruited (Nup93, Nup62, Nup205, Nup188) are not codepleted with Nup155, but rather that their absence from chromatin reflects a functional defect of assembly (Figure 3B, (De Magistris et al., submitted)).

II. 4. Nup155 N- and C-terminal domains interact

The fact that these nuclei are lacking Nup93 but are surrounded by a fully formed envelope is apparently in contrast with data according to which Nup93 itself is required for NE assembly (Sachdev et al., 2012). In the light of the finding that Nup155 C-terminal and N-terminal interact in *S. Cerevisiae* (Flemming et al., 2009), and that this interaction negatively regulates Nup170, it was possible to hypothesize that the C-terminal of Nup155 acts to potentially inhibit nuclear envelope formation, an inhibition that might be released by binding of Nup93. In order to test this, a series of GST pulldowns were performed using a GST-tagged Nup53 version as prey and Nup155 N- (aa 1-589) and C-terminal (aa 589-1344 and aa 504-1388) domains N-tagged with the solubility tag SUMO as preys. These pulldowns allowed to determine that the β-propeller and β-propeller of *Xenopus* Nup155 do interact (Figure 4A, (De Magistris et al., submitted),). Interestingly, in the presence of increasing concentrations of the C-terminal α-solenoid, the interaction of the N-terminal β-propeller of Nup155 with Nup53 is diminished up to complete abolition (Figure 4B, (De Magistris et al., submitted)). The nuclei assembled in Nup155 depleted extracts supplemented with the N-terminal β-propeller domain of Nup155 (aa 1-589) resemble nuclei that form when full length Nup93 is substituted by its C-terminal domain (Sachdev et al., 2012). The NPCs in such nuclei contain most of the structural backbone of the NPC including the Nup107-160 complex, Nup53 and Nup155, but do not include the Nup62-complex, the main component of the central transport channel, responsible for nuclear import. This suggested the interesting idea that C-terminus of Nup93 could overcome the negative

regulation of the Nup155 C-terminus in the formation of the structural backbone of NPCs. The previous experiment was slightly modified with the use of full length Nup53, that contains the Nup93 binding domain (Lin et al., 2016; Sachdev et al., 2012). As in Figure 4B (De Magistris et al., submitted), Nup155 C-terminal reduced the interaction between Nup155 N-terminal and Nup53, but this effect is overcome in the presence of the C-terminal domain of Nup93 (Figure 5, (De Magistris et al., submitted)), that retains capability of binding Nup53 (Sachdev et al., 2012).

It is worth noticing how, in the nuclei assembled in Nup155 depleted extracts in which the N-terminal domain of Nup155 replaces the full-length protein (shown in Figure 3A, (De Magistris et al., submitted)), Nup93 is not recruited on chromatin. One possibility is that, in order to support formation of the structural core of the NPC, Nup93 must undergo structural rearrangements which require its interaction with the C-terminus of Nup155. Alternatively, the binding surface offered by Nup53 might be slightly different in the absence of the C-terminal of Nup155, which is then insufficient for proper recruitment of Nup93. Or yet, in the absence of the auto-inhibitory function of the C-terminus of Nup155, Nup93 could take part in a protein interaction network of inner ring components of the NPC that, in absence of effective tethering, could occur ectopically, and that becomes manifest in the absence of Nup93 on chromatin.
In summary, these data prove that Nup155 must bind membranes to support proper NE and NPC assembly at the end of mitosis. N- and C- terminal regions of Nup155 are capable of interaction, and this interaction weakens the binding of Nup155 N-terminal to Nup53, which might constitute a regulated point during nuclear pore complex formation. The presence of Nup93, which is bound by Nup53, could induce a conformational change in Nup53 that optimizes the binding surface to a level that overcomes the self-inhibition of Nup155 and strengthens the Nup155-Nup53 interaction, supporting recruitment of Nup93 on chromatin and further recruitment of NPC components.

II. 5 The release of Nup155 inhibition may constitute a novel checkpoint of nuclear assembly

In the light of the new data presented in this thesis, I propose the following model for postmitotic NPC assembly.

In a similar timeframe to MEL28/ELYS, Nup50 lands on chromatin early in the assembly pathway, recruited both by MEL28/ELYS via its middle region (Nup50 144-191) and via its

chromatin binding region on the N-terminal domain (Nup50 1-114). To effectively support NPC formation, either moiety is required and sufficient. As its depletion impairs the NPC assembly pathway at a very early step, is possible to speculate that (I) Nup50 cooperates with Mel28 in the recruitment of Nup107-160, (II) that it promotes structural rearrangements and further recruitment of more nucleoporins, or (III) that it recruits enzymatic activities that modify and enable the chromatin, or the pre-pore scaffold, to further recruitment. Next, contact with membranes is established, thanks to the transmembrane proteins POM121, NDC1 (Eisenhardt et al., 2014a), and to both membrane binding sites of Nup53 (Vollmer et al., 2012).

Once Nup53 is recruited, Nup155 is able to contact the forming pore via its N-terminal, which is, at this state, bound to the Nup155 C-terminal in a self-inhibiting conformation. By its membrane binding domain, the N-terminal of Nup155 is critically required to bring nuclear envelope enclosure to completion. The C-terminal of Nup93 binds Nup53 (Sachdev et al., 2012) and triggers structural conformation that changes the conformation of the network of protein composed by Nup155, Nup53 and Nup93 to an open, active state. It is unlikely that Nup93 acts directly on Nup155, as these proteins do not interact in vitro (Eisenhardt et al., 2014a; Sachdev et al., 2012). Nup93 recruits in turn Nup188 and Nup205, to form the Nup93-complex, and the central channel Nup62-complex, which require the N-terminal moiety of Nup93 (Sachdev et al., 2012).

In conclusion, my work identifies in Nup50 a novel player of nuclear pore complex assembly at the end of mitosis. I have discovered that two discrete domains of Nup50 are either required and sufficient for its function. Here I provide evidence of the redundant fashion by which Nup50 can be brought to chromatin via these domains, sign of the critical importance of the action performed by Nup50 in nuclear pore assembly at the end of mitosis.

Moreover, the original data presented in this thesis introduce the membrane binding domain of Nup155 as a new regulatory function by which this nucleoporin acts in nuclear envelope formation at the end of mitosis. Additionally, my work establishes that a self-inhibitory interaction within Nup155 is responsible for regulated recruitment of nucleoporins Nup93 and Nup62, to achieve efficiently orchestrated nuclear pore complex assembly. This auto-regulation likely constitutes an additional checkpoint for fine tuning of nucleoporin recruitment to the pore core.

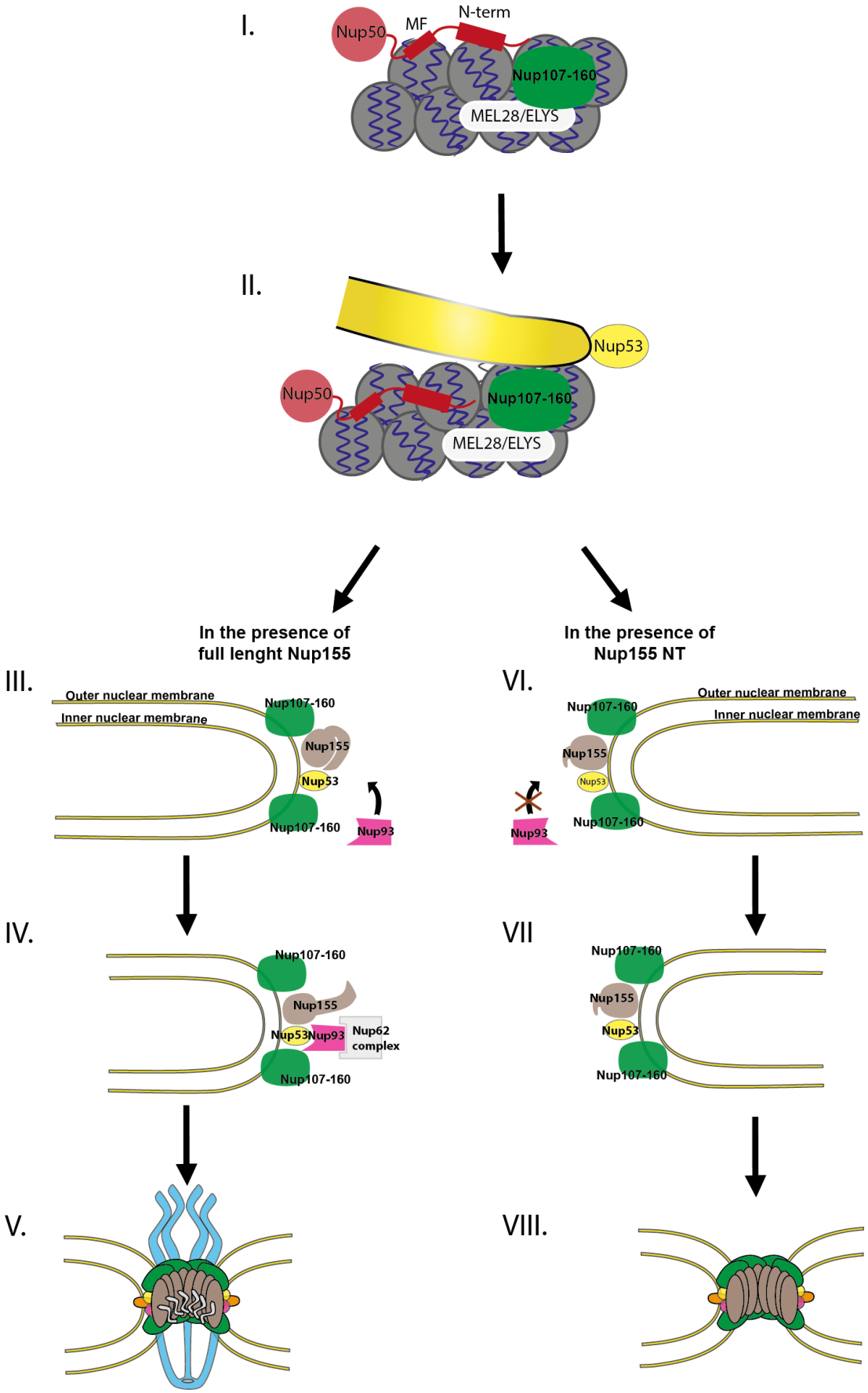
I.
Nup50
MF
N-term
Nup107-160
MEL28/ELYS
II.
Nup53
Nup50
Nup107-160
MEL28/ELYS
In the presence of
full lenght Nup155
III.
Outer nuclear membrane
Inner nuclear membrane
Nup107-160
Nup155
Nup53
Nup107-160
Nup93
IV.
Nup107-160
Nup155
Nup53
Nup93
Nup62
complex
Nup107-160
V.
In the presence of
Nup155 NT
VI.
Nup107-160
Outer nuclear membrane
Inner nuclear membrane
Nup155
Nup53
Nup107-160
Nup93
VII
Nup107-160
Nup155
Nup53
Nup107-160
VIII.

Figure 26. Model of NPC postmitotic assembly.
(I.) MEL28/ELYS (white) localizes on chromatin, recruiting the Nup107-160 complex (green) in loco. Nup50 (red) acts on chromatin in the same time frame. (II.) Next, contact with membranes is established, thanks to the transmembrane proteins POM121, NDC1 (not shown for simplicity) and to Nup53 (yellow). Nup53 is recruited to the nascent pore, contributing to membrane recruitment and curvature stabilization. Nup155 (grey) is recruited via its N-terminus: in the presence of full-length Nup155, (III.) the membrane binding domain supports NE formation. Nup93 (magenta) is recruited. (IV.) Further members of the Nup93 complex are recruited (not shown in figure). Nup62 (light grey) is brought to the center of the pore, to complete the final structure that will eventually be import competent (V.). If only the N-terminal domain of Nup155 is present, (VI.) the membrane binding domain can support NE formation, as it is not inhibited by self-inhibition by the C-terminal domain. Nup93 cannot be recruited. (VII.) In absence of the C-terminal domain of Nup155, the Nup53-Nup155 bond is stable. Nup93 is not required to remove the self-inhibition, but the Nup155-Nup53 dimer alone cannot support further recruitment of the other members of the Nup93 complex nor of Nup62 complex. As a result, (VIII.) the NPCs are assembled without the Nup93- and the Nup62- complexes.

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Appendix

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Review

Nuclear Reformation at the End of Mitosis

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http://dx.doi.org/10.1016/j.jmb.2015.09.016
Edited by R. W. Kriwacki

Abstract

Cells have developed highly sophisticated ways to accurately pass on their genetic information to the daughter cells. In animal cells, which undergo open mitosis, the nuclear envelope breaks down at the beginning of mitosis and the chromatin massively condenses to be captured and segregated by the mitotic spindle. These events have to be reverted in order to allow the reformation of a nucleus competent for DNA transcription and replication, as well as all other nuclear processes occurring in interphase. Here, we summarize our current knowledge of how, in animal cells, the highly compacted mitotic chromosomes are decondensed at the end of mitosis and how a nuclear envelope, including functional nuclear pore complexes, reassembles around these decondensing chromosomes.

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 (Fig. 1). Nuclear pore complexes (NPCs) are the gateways of the NE allowing diffusion of small substances and regulated trafficking of macromolecules up to a size of 50 nm (for review, see Ref. [1]). The NE consists of two membranes that are separated by the perinuclear space. The inner nuclear membrane (INM) is connected to the outer nuclear membrane (ONM) via the pore membrane, points of fusion where NPCs reside. In addition, the ONM is connected to the membrane network of the endoplasmic reticulum (ER) (Fig. 1). Thus, INM and ONM form a continuum with the ER and can be considered as subcompartments of the latter. However, the INM is additionally characterized by a distinct protein composition. Integral membrane proteins of the INM interact at multiple sites with chromatin and the nuclear lamina, which forms a tight proteinous network underlying and stabilizing the NE.

For cell division, the genetic material needs to be passed on to the two emerging daughter cells. After the DNA is replicated in S-phase, it must be physically separated by the mitotic spindle, a microtubule-based structure assembled from predominantly cytoplasmic components. In order to allow microtubule contact to chromosomes, different strategies have evolved (for review, see Ref. [2]): many eukaryotes, including yeasts, employ closed or semiclosed mitosis, during which tubulin and microtubule-associated proteins are imported into the nucleus and an intranuclear spindle assembles. In contrast, metazoan cells divide by open or semiopen mitosis (Fig. 2). In this mode, the NE is at least partially disassembled during prophase to allow microtubules access to the chromatin. Concomitantly, the chromatin becomes increasingly condensed and individualized. During metaphase, chromosomes align at the metaphase plate. Once the spindle assembly checkpoint is satisfied due to proper kinetochore–microtubule attachment and tension, chromosomes are segregated in anaphase to the two emerging daughter cells by the mitotic spindle. In late anaphase and telophase, the nucleus starts to reform. We will discuss here nuclear

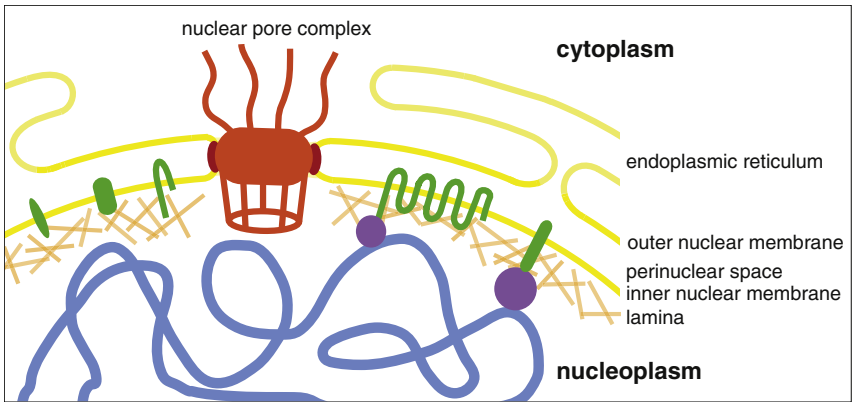

Fig. 1. The metazoan interphase nuclear envelope. The nuclear envelope is formed by two membranes, the inner and outer nuclear membranes that enclose the perinuclear space and that are continuous with the lumen of the ER. Embedded in the nuclear envelope are nuclear pore complexes (red) that shape the two nuclear membranes to a pore. The is defined by a specific set of integral membrane proteins (green) that interact with chromatin (blue), chromatin-associated proteins (violet) and the lamina (beige), a protein meshwork of lamins stabilizing the nuclear envelope.

reformation at the end of mitosis in animal cells, with an emphasis on chromatin decondensation and the reassembly of a functional NE and pore complexes. Comprehensive overviews on how other nuclear structures including nucleoli reform are given in other recent reviews (e.g., see Ref. [3]).

In the last years, progress in answering the relevant questions concerning nuclear reformation has been made both by life cell imaging, mostly in mammalian tissue culture cells (e.g., see Refs. [4–6]) but also in *Caenorhabditis elegans* (for review, see Ref. [7]), and by detailed biochemical analysis. The latter often relies on the use of egg extracts from *Xenopus laevis* that have been extremely instrumental to reconstitute and functionally dissect complicated cellular reactions in a test tube. Often, sperm DNA is used as a chromatin template, around which, after its decompaction, a closed NE including pore complexes is formed. This process occurs naturally when a sperm enters the egg. Although nuclear reassembly in dividing cells has, as far as we know, much in common with this pronuclear assembly, especially, for example, in terms of NPC assembly, some aspects might be adapted to the specific needs of early embryogenesis. In addition, some peculiarities of the cell-free system are attributed to the preparation method. For example, during breakage of the eggs, the ER network fragments and forms vesicles. These vesicles bind to chromatin and fuse to a closed NE upon chromatin incubation in egg extracts, which has been misinterpreted as proof for the existence of membrane vesicles as source of the NE during nuclear reformation at the end of mitosis (for discussion, see Ref. [8]). In this review, we will also attempt to point out where results from *in vitro* experiments should be taken with a grain of skepticism and would benefit from confirmation in living cells.

Mitotic Exit Regulation

Processes initiating the entry of mitosis (Fig. 2) such as NE breakdown, spindle assembly and chromosome alignment are driven by various mitotic kinases, most importantly the cyclin-dependent kinase 1 (CDK1)/cyclin B complex and members of the Aurora and Polo-like kinase (PLK) families (reviewed in Ref. [9]). In addition to its regulation via phosphorylation and dephosphorylation, CDK activation requires binding of cyclins (reviewed in Ref. [10]). In contrast, PLK1 is mainly regulated by its targeting to diverse proteins at different cellular sites throughout the cell cycle that have been primed before by phosphorylation (reviewed in Ref. [11]). Similarly, the localization of Aurora B kinase is tightly regulated during mitotic progression (reviewed in Refs. [12] and [13]). Aurora B is the catalytic subunit of the chromosomal passenger complex (CPC) that additionally consists of the targeting subunits Borealin and Survivin, as well as the bridging subunit INCENP (inner centromere protein). The targeting subunits regulate CPC's localization and translocate the complex from the chromosome arms to the inner centromeric chromatin in early mitosis, where it controls proper attachment of the spindle to the kinetochores. Subsequently, in anaphase, the CPC localizes to the spindle midzone, where it is involved in its stabilization and in cytokinesis.

Mitotic kinases phosphorylate various substrates including nucleoporins (i.e., NPC proteins, Nups), lamins and histones, and they cause a global hyperphosphorylated mitotic state in the cell (e.g., see Ref. [14]). Once the spindle is properly bipolarly attached to the kinetochores, the spindle assembly checkpoint is satisfied and in turn stops inhibiting the anaphase promoting complex (APC) activity (reviewed in Ref. [15]). The active APC, in a complex

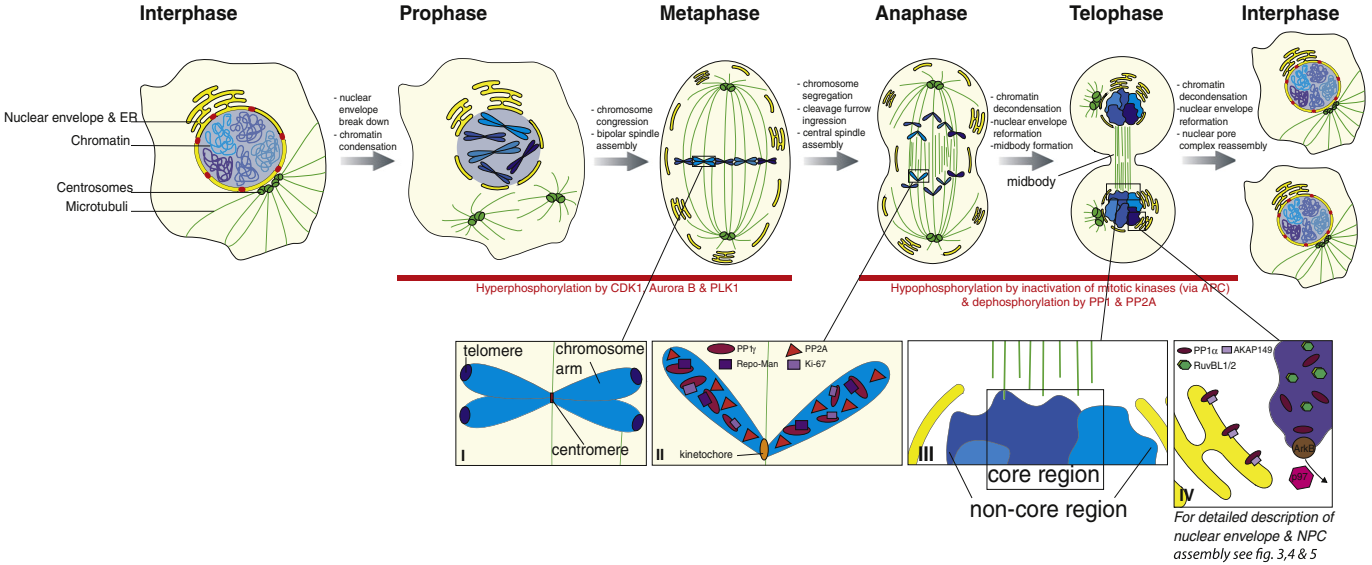

Fig. 2. Mitosis and nuclear reorganization. In interphase, the nuclear envelope encloses the chromatin. When cells enter mitosis, the nuclear envelope breaks down, characterized by nuclear pore complex (red) disassembly and absorption of the nuclear membrane into the ER, microtubule rearrangement and chromatin condensation. In the further mitotic progression, a bipolar spindle assembles and the condensed chromosomes congress to the metaphase plate. All these processes are driven by high activity of mitotic kinases characteristic for the first half of mitosis. The second half of mitosis is characterized by a global decrease in phosphorylation activity both by inactivation of mitotic kinases and by activation of phosphatases. This is required for chromatin decondensation, nuclear envelope and nuclear pore complex reassembly.

with its cofactor CDC20, ubiquitinylates cyclin B and securin and primes them for their proteasomal degradation. The degradation of securin releases the protease separase that is now free to cleave a subunit of the cohesin complex allowing sister chromatid separation. Cyclin B degradation, on the other hand, causes an inactivation of CDK1 that results in the transition from metaphase to anaphase, which is a point of no return. Later in anaphase, the decreased CDK1 activity allows APC to assemble with a different cofactor, CDH1 (CDC20 homolog 1), which broadens its substrate specificity to, for example, Aurora B, PLK1 and its earlier cofactor CDC20. The inactivation and/or degradation of mitotic kinases are necessary but not sufficient to induce mitotic exit. The manifold phosphorylations, previously introduced by mitotic kinases, need to be reversed from their targets to allow for mitotic progression including chromosome segregation, spindle elongation, cytokinesis and reestablishment of the interphasic nucleus. Thus, the ratio of kinase to phosphatase activity defines the shift from early to late mitotic events.

Budding yeast employs a well-studied mitotic exit regulation mechanism, mainly driven by the phosphatase CDC14 (reviewed in Ref. [16]). CDC14 is activated by its release from the nucleolus in early anaphase, induced by the signaling cascade network FEAR (CDC fourteen early anaphase release network) and later sustained by the signaling cascade network MEN (mitotic exit network). However, whereas mitotic entry regulation by the different mitotic kinases is generally conserved in all eukaryotes, CDC14 does not play the prevalent role in mitotic exit regulation in metazoans. Although homologs are found in a large variety of organisms, they seem to have functions unrelated to mitotic exit—however, a role in mitotic exit cannot be excluded either (reviewed in Ref. [17]). Instead, members of the PP1 and PP2A protein phosphatase families seem to be the key players in regulating mitotic exit in metazoans.

The number of catalytic protein phosphatase subunits encoded by the human genome is much smaller as compared to the number of protein kinases. An increased substrate specificity and regulation spectrum is achieved by a large number of regulatory subunits that associate with the catalytic subunits and change their substrate binding capability and localization. PP1 typically forms a heterodimer consisting of one of the four almost identical catalytic subunits, PP1α, PP1β/δ, PP1γ1 or PP1γ2 in mammals and one of several regulatory subunits. PP2A usually forms a heterotrimeric complex consisting of the catalytic subunit PP2Aα or PP2Aβ, the scaffolding subunit PR65 (protein phosphatase 2 regulatory subunit) α or β and one of at least 15 different isoforms of regulatory subunits that belong to the B55, B56, B″ or B‴ families. Although it is controversial whether PP1 and PP2A are direct or indirect antagonists of CDK phosphorylation, both certainly play important roles during mitotic exit.

So far, four different regulatory subunits have been described to be involved in late mitotic functions of PP1: Repo-Man, PNUTS (phosphatase 1 nuclear targeting subunit), Ki-67 and AKAP149 (A-kinase anchoring protein). Repo-Man targets PP1γ to anaphase chromosomes (Fig. 2, inset II) [18] resulting in dephosphorylation of histone H3 at T3, S10 and S28 [5]. Loss of Repo-Man impairs the reversal of these mitotic H3 phosphorylations and causes abnormally shaped nuclei with irregular NEs and cytoplasmic NPC formation [5]. These observations point to a regulatory role of Repo-Man in NPC reassembly in the reforming NE at the end of mitosis, as cytoplasmic NPC formation, so-called *annulate lamellae*, is often seen upon interfering with this process [19,20]. Repo-Man binds and recruits importin β, a key regulator of NE/NPC reassembly (see sections "Establishing a Nuclear Envelope Membrane Domain" and "Regulating NPC Assembly at the End of Mitosis"), to anaphase chromosomes but the molecular details and mechanisms of the nuclear reformation defects seen upon Repo-Man depletion remain to be elucidated.

Another factor that targets PP1γ to anaphase chromosomes, only recently identified, is Ki-67 (Fig. 2, inset II). Ki-67 is part of the perichromosomal layer, a coat of mainly nucleolar proteins and RNAs assembling around mitotic chromatin forming an intersection to the surrounding cytoplasm (reviewed in Ref. [21]). Depletion of Ki-67 reduces PP1γ targeting in anaphase but does not detectably affect NE reformation [22,23]. It remains to be seen whether Ki-67 function is at least in part redundant with Repo-Man and whether codepletion of both targeting subunits aggravates mitotic exit defects.

The targeting subunit PNUTS recruits PP1α to the reforming nucleus—probably via reviving nuclear import—during mitotic exit but later than chromatin recruitment of PP1γ by Repo-Man [18,24]. PNUTS accumulates at the nuclear periphery just before the sealing of the NE and has been suggested to regulate chromatin decondensation [24] but the precise targets of PP1α-mediated dephosphorylation important for this remain to be identified.

The transmembrane-domain-containing protein AKAP149 targets PP1 in a phosphorylation-regulated manner to the reforming NE at the end of mitosis (Fig. 2, inset IV) [25–28]. The PP1 anchoring by AKAP149 is necessary for the dephosphorylation of lamin B resulting in the reformation of the nuclear lamina.

In addition to the recruitment of PP1 catalytic subunits at a specific time point to their site of action, PP1 activity is further controlled via modifications of its regulatory subunit. For example, phosphorylation

of Repo-Man by CDK1/cyclin B prevents PP1 binding, as well as stable chromatin targeting [5,29]. In addition, the catalytic subunit can also be directly regulated. In *Xenopus* egg extracts, protein kinase A phosphorylates the inhibitor 1 upon mitotic entry that, in turn, binds and blocks PP1 [30]. The catalytic subunit of PP1 is further inhibited through direct phosphorylation by CDK1. Low CDK1 levels during mitotic exit are proposed to trigger PP1 to dephosphorylate itself, as well as inhibitor 1 in order to obtain full phosphatase activity. It remains to be seen how conserved this regulation is. Nevertheless, it is conceivable that regulation of PP1 occurs in time, by controlling the activity of the catalytic subunits, and in space, by defining the localization of the complex via its targeting subunits.

In addition to PP1 phosphatases, PP2A complexes have been implicated in mitotic exit regulation. A genome-wide RNAi screen against phosphatases identified the PP2A–B55α complex as key mitotic exit phosphatase [31]. Downregulation of the regulatory subunit B55α delayed exclusively mitotic exit, while the catalytic and scaffolding subunits retarded also mitotic entry, hinting to additional early mitotic functions of these PP2A subunits. The assembly of the holoenzyme is prevented by mitotic phosphorylation of B55α [31] but might be in addition controlled by phosphorylation and methylation of the catalytic subunit [32]. Furthermore, importin β interaction with the PP2A–B55α is suggested to regulate mitotic exit function of the complex via nucleoplasmic/cytoplasmic transport or via importin β's function as a molecular chaperone [31]. In contrast to the latter study in human cells, another PP2A regulatory subunit, B55δ, was implicated in mitotic exit regulation using *Xenopus* egg extracts [33]. Immunodepletion of PP2A–B55δ leads to premature mitotic entry and blocks exit from mitosis. The experiments suggest that this effect is due to direct or indirect dephosphorylation of CDK substrates.

One of the crucial targets for PP2A dephosphorylation might be the small chromatin binding protein BAF (barrier-to-autointegration factor). BAF functions as a bridge between chromatin, lamins and INM proteins (reviewed in Ref. [34]) and is involved in late nuclear mitotic events, for example, the reformation of the NE, further discussed in later paragraphs (see section "Establishing a Nuclear Envelope Membrane Domain"). Aside from its manifold other interaction partners, BAF binds to the INM protein LEM4 (LEM domain containing 4) [35]. Upon mitotic entry, BAF is phosphorylated by VRK1 (the vaccinia-related kinase 1) that leads to its dissociation from chromatin, LEM4 and other INM proteins. This occurs simultaneously with NE breakdown [36,37]. Upon mitotic exit, LEM4 inhibits VRK1 and recruits PP2A. PP2A dephosphorylates BAF and enables its chromatin and INM protein binding capability that is necessary for the nuclear reformation [35] (Fig. 3). Thus, it is conceivable that LEM4 acts as a PP2A regulatory subunit in this case. This, however, is a matter of controversy, as a different study suggests that PP4 is the major BAF phosphatase [38] and thus additional features of BAF regulation during mitotic exit might be involved. Nevertheless, the LEM4–BAF pathway shows that further possibilities of tuning protein phosphatase activities and new regulatory subunits might be involved in mitotic exit control, awaiting their identification and characterization.

In summary, PP1 and PP2A phosphatases have each been independently linked to mitotic exit control (Fig. 2, insets II and IV). However, the relative contributions and respective importance of the two phosphatase families remain controversial. As both are controlled by phosphorylation, it is conceivable that PP1 and PP2A activities interdepend from each other by cross-dephosphorylation or inhibiting the corresponding inactivating kinases. Indeed, a mitotic phosphatase relay system was recently described in fission yeast, where PP1 activation is required for the reactivation of PP2A to coordinate mitotic progression and exit [39]. It will be interesting to see whether similar principles also account for mitotic exit regulation in metazoans.

Chromatin Decondensation

Chromatin structure in the interphase nucleus is not random: instead, chromosomes assemble in specific territories, which are often cell type specific and maintain a relative radial position with respect to the nuclear periphery (reviewed in Refs. [40] and [41]). Within the last years, it has become increasingly clear that this three-dimensional organization plays an important role in regulation of gene expression (reviewed in Ref. [42]). Also, on smaller scales, many local and long-range contacts among genes and other sequence elements that organize the genome exist. A number of controversial models of the interphasic chromatin structure aim explaining genome organization, each based on different microscopy techniques, as well as genomic approaches such as 3-C, 4-C, 5-C and Hi-C (reviewed in Ref. [43]). The first level of compaction is achieved by wrapping the DNA around nucleosomes—consisting of core histone octamers—to form a 10-nm chromatin fiber (referring to the diameter of the fiber). Already the next layer of compaction is disputed: recent results question the existence of the 30-nm fiber for which different models have been proposed based on electron microscopy and *in vitro* assembly studies, and these rather suggest a more dynamic, disordered folding accompanied by nucleosome fluctuations influencing the chromatin accessibility (reviewed in Ref. [44]). The mechanism of formation

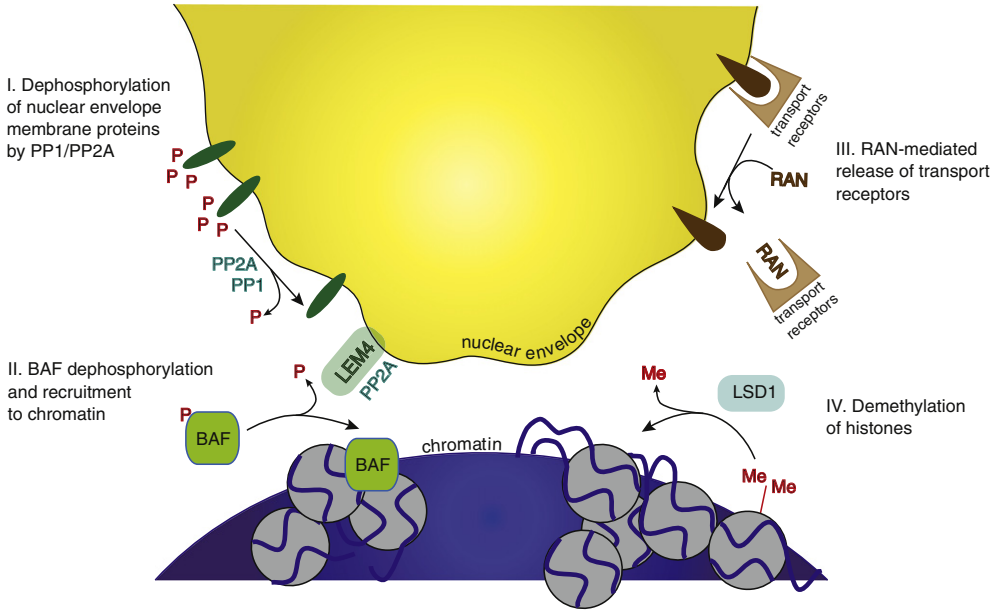

Fig. 3. Regulation of nuclear membrane recruitment to chromatin. Nuclear membrane recruitment to chromatin at the end of mitosis is regulated by dephosphorylation of INM proteins (I) and chromatin proteins (II) such as the LEM4/PP2A-mediated dephosphorylation of BAF. Not only RAN-mediated release of transport receptors (III) but also changes on the chromatin landscape (IV) such as LSD1-mediated histone demethylation might contribute.

of any higher-order organization, although clearly evident from a variety of experiments, remains similarly controversial.

Equally unsecured is our knowledge about the structural organization of mitotic chromosomes, a topic that has been fascinating biologists for decades. Although it was apparent from early days of mitosis research that mitotic chromatin is condensed, estimates about the compaction grade of mitotic chromatin in animal cells compared to its interphase state differ considerably, from 2-fold to 50-fold [45,46]. As for the interphasic chromatin structure, microscopic, biophysical and recently chromosome conformation capture methods led to various models attempting to explain how these structures are organized. These models fall mainly into two broad categories: one class of models proposes that the DNA hierarchically folds into increasingly higher-order structures (e.g., see Refs. [47] and [48]). The second class suggests that mitotic chromatin forms series of loops that are attached to a central chromosome scaffold axis (e.g., see Ref. [49]). Interestingly, recent chromosome conformation capture results suggest that mitotic chromatin indeed consists of loops of various sizes [50]. According to this study, compartmentalization and specific domains of interphasic chromatin are lost during formation of mitotic chromosomes, leading to homogenous mitotic chromosome structures independent of the cell type. In contrast, another recent study suggests that the DNase I sensitivity profile of mitotic and interphasic chromatin is not changed globally [51]. This indicates that the accessibility of chromatin is not altered during the cell cycle, with a few local exceptions such as hypersensitive regions in interphase that indeed lose accessibility to a larger extent than other regions.

Although many potential chromatin condensation factors have been identified, their exact functions often remain controversial. This might be attributed to the fact that mitotic chromatin condensation, in preparation for sister chromatid separation, most likely requires several distinct activities that are due to their contemporaneous and probably interdependent nature hard to distinguish in molecular terms: this includes disentanglement of sister chromatid DNA molecules, compaction of chromatin into the thread-like structure and probably the formation of a longitudinal scaffold axis with a certain rigidity. Condensins and topoisomerase II, both major chromosomal components, are often regarded as key factors in establishing the mitotic chromosome structure. Because of its decatenation activity [52], topoisomerase II is certainly involved in DNA disentanglement [53]. Whether it is in addition required for chromatin compaction is controversial: experiments in fission yeast and *Xenopus* egg extracts have implicated a requirement for topoisomerase IIα in chromatin condensation [54,55], in agreement with pioneering work from the Laemmli laboratory, where the protein was identified as a major nonhistone component of the scaffold axis [56,57] involved in chromatin condensation [58,59]. However, knockdowns of topoisomerase IIα in fly

and human cell lines impair chromosome segregation but do not result in prominent condensation defects [60,61]. Condensins have been identified as chromatin condensation factors in *Xenopus* egg extracts [62,63]. In most eukaryotes, two condensin complexes exist, condensin I and condensin II, which form a ring-like structure including an ATPase subunit. There is universal agreement that condensins are involved in sister chromatid disentanglement [53,64]. Whether these protein complexes actually drive mitotic chromatin compaction has always been controversial (reviewed in Ref. [65]). RNAi data from various organisms suggest that cells lacking condensins are defective in chromosome segregation rather than chromatin condensation [66–68]. Alternatively, a so-far unknown regulator of chromosome architecture was suggested to induce mitotic chromatin formation, later inhibited by the Repo-Man–PP1 complex and replaced by condensins in their possible function of stabilizing mitotic chromosomes [29]. This model results from the observation that conditional knockout of the shared condensins I and II subunit SMC2 causes anaphase chromatin bridges and loss of compact chromosome architecture, which can be rescued by the inhibition of Repo-Man-guided PP1 recruitment to anaphase chromosomes. However, recent experiments show that, at least in meiotic cell divisions in mice, condensins have a crucial role in chromatin thread formation [69] and indicate that condensins might be indeed functioning in both DNA disentanglement and compaction.

Two condensin interacting proteins have been implicated in mitotic chromatin condensation, MCPH1 (microcephalin 1) and KIF4A (kinesin family member 4A) [70,71]. MCPH1 is thought to regulate loading of condensin II on chromatin [70]. MCPH1 mutations lead to premature condensation, delayed decondensation and disturbed metaphase chromatin structure [72]. This seemingly counterintuitive phenotype for a condensation factor loading protein might be explained by an altered ratio of condensins I and II on the chromatin. In line with this, shifting the ratio of condensin I to condensin II complexes affects mitotic chromatin structure in *Xenopus* egg extracts [73], pointing to the possibly of counteracting or at least nonredundant functions of condensins I and II, which also target to chromatin at different time points during mitotic entry [74]. In the case of KIF4, its depletion was suggested to cause hypercondensation [71]. However, recent results indicate that KIF4A depletion rather affects structural integrity of mitotic chromosomes [75]. Therefore, it was proposed that KIF4A and condensins promote lateral chromosome compaction by loop formation, while topoisomerase II promotes axial compaction upon decatenation of the loops.

Histone modifications are crucial regulators of chromatin structure and function, most prominently in remodeling interphasic chromatin in order to stimulate or repress gene expression. Whether changes in chromatin structure induced by histone modifications also play a role in mitotic chromatin compaction and decompaction is arguable. Some posttranslational histone modification patterns depend on the developmental or cell cycle stage (reviewed in Ref. [76]). For example, H3K27 trimethylation shows a different pattern in interphase compared to mitosis, hinting to partial remodeling of the epigenome during mitosis [77]. Striking mitotic marks are phosphorylations of threonine 3 and serine 10 of histone H3. The former is involved in recruiting CPC to the centromere in early mitosis (reviewed in Ref. [13]). The second, widely used as a convenient mitotic mark, was due to its correlation with the compacted chromatin state thought to be involved in or even cause chromatin condensation and conversely dephosphorylation of H3S10 in chromatin decondensation (e.g., see Ref. [78]). However, H3S10 phosphorylation and chromatin condensation can be uncoupled and are thus not essential for each other [79–82]. Thus, H3S10 phosphorylation might be part of another mitotic function, unrelated to chromatin condensation. It is, for example, required for the release of the heterochromatin protein HP1 from chromatin [83] and it is conceivable that it is similarly involved in the detachment and/or recruitment of other chromatin binding factors during mitosis. Newer results obtained by cross-linking experiments in yeast argue again for an involvement of H3S10 phosphorylation in chromatin condensation by recruiting the histone deacetylase Hst2p to this modified site. Hst2p deacetylates H4K16, enabling the interaction of the H4 tail with the neighboring nucleosome leading ultimately to chromatin condensation [84]. However, it remains open whether this mechanism also contributes to mitotic chromatin condensation in metazoans. Yeasts undergo closed mitosis and compact their chromatin to a much smaller extent [85] and thus might use a different, less sophisticated, mechanism. Additionally, the decrease of the distance of two loci on a single chromosome as analyzed by Wilkins *et al.* [84] does not necessarily reflect the condensation of the whole genome.

The highly condensed mitotic chromatin is segregated to the two emerging daughter cells once the spindle assembly checkpoint is satisfied. Maximal compaction of mitotic chromosomes is, however, not attained at metaphase but rather at late anaphase when segregation is almost complete [86]. This compaction is achieved by axial shortening of the chromosome arms in a condensin-independent manner, regulated by Aurora B kinase activity. The late maximal compaction could be necessary to resolve anaphase chromatin bridges by a “pulling-apart” mechanism, or it could serve as a security mechanism shortly before NE reformation to ensure

that all chromosomes are integrated into the reconstituted nucleus. The chromokinesin KIF22 is involved in this maximal axial compaction in late anaphase, although the exact function still needs to be uncovered [87]. Later on, the compacted chromatin decondenses in a yet-ill-defined process.

Chromatin decondensation has been mostly investigated on sperm chromatin after fertilization. The highly compacted sperm DNA decondenses by exchanging the protamines X and Y—sperm-specific histones—to the canonical core histone proteins H2A and H2B. This exchange is executed by the oocyte protein nucleoplasmin in an ATP-independent manner [88,89]. This process differs from decondensation of somatic chromatin at the end of mitosis, as the latter does not involve protamine to histone exchange. Indeed, chromatin decondensation at the end of mitosis does not require nucleoplasmin [82], consistent with the fact that nucleoplasmin is absent from somatic cells [90]. Using a cell-free assay based on *Xenopus* egg extracts and isolated mitotic chromatin from somatic cells, it was shown that mitotic chromatin decondensation requires cellular energy in the form of ATP and GTP [82]. This suggests that chromatin decondensation is an active process and not simply chromatin relaxation caused by the dissociation of chromatin condensation factors. Consistently, in this assay, chromatin decondensation depends on the presence of egg extracts, in contrast to earlier observations where a basal decondensation activity of mitotic chromatin was observed in the presence of only buffer and an ATP-regenerating system [24]. This discrepancy might be explained by a less harsh chromatin isolation procedure used in Landsverk *et al.* [24] that might retain more proteins on the chromatin.

The ATP dependence is, at least in part, explained by the dependence of chromatin decondensation on the AAA^+-ATPase p97 (also known as valosin-containing protein (VCP) in vertebrates and CDC48 in yeast) [91]. p97, in a complex with its cofactors UFD1 (ubiquitin fusion degradation 1) and NPL4 (nuclear protein localization 4), is required for the removal of ubiquitinylated Aurora B kinase from chromatin (Fig. 2, inset IV). It is currently unclear whether Aurora B needs to be removed from chromosomes in order to inhibit its kinase activity toward chromosomal substrates. Alternatively, removal might be necessary in order to function at a different location at this late mitotic state or simply to increase chromatin accessibility for chromatin decondensation factors at specific sites. In HeLa cells, p97-UFD1-NPL4 seems to be directly antagonizing Aurora B activity already at early mitotic stages, which is required for faithful chromosome segregation [92]. Also in this case, the relevant Aurora B targets, whose phosphorylations need to be prevented, are unidentified.

A second ATPase complex formed by RuvBL1 and RuvBL2 (RuvB-like 1/2, also known as Pontin/Tip49 and Reptin/Tip48) is involved in chromatin decondensation at the end of mitosis [82]. RuvBL1 and RuvBL2 are AAA^+ -ATPases that form together a mixed dodecameric complex. They are involved in a variety of cellular processes including snoRNP, telomerase complex and spindle assembly, chromatin remodeling, transcriptional regulation and signal transduction (reviewed in Ref. [93]). The precise function of RuvBL1/2 in chromatin decondensation, like in many other processes, still needs to be uncovered. However, their chromatin enrichment during mitotic exit [82] and their known function as components of different chromatin remodeling complexes in interphase implicate that they might similarly act by restructuring chromatin at the end of mitosis. Certainly, it is also possible that RuvBL1/2 recruits and activates, or removes and inactivates, relevant chromatin decondensation or condensation factors, respectively, to their site of action. Although the recombinant RuvBL1/2 complex rescues the depletion phenotype of these ATPases from *Xenopus* egg extracts regarding chromatin decondensation, the complex alone is not sufficient to drive chromatin decondensation, indicating that other, yet-unknown crucial factors are required [82]. The fact that chromatin decondensation depends on GTP hydrolysis [82] suggests that a GTPase is involved in the process. Although being involved in many mitotic processes [94] including NE and pore complex reformation at the end of mitosis (see sections “Establishing a Nuclear Envelope Membrane Domain” and “Regulating NPC Assembly at the End of Mitosis”), RAN (Ras-related nuclear protein) seems not to be involved, as excess of RAN mutants does not block chromatin decondensation (our unpublished data). Therefore, future studies are needed to identify the GTPase involved in chromatin decondensation and its precise function.

To establish a fully functional interphase nucleus, not only the chromatin needs to decondense but also an NE needs to reform (discussed in detail in section “The Nuclear Envelope Emerges from the Mitotic ER”). Formation of the NE was suggested to contribute to chromatin decondensation, via involvement of the INM proteins SUN1 (Sad1 and UNC84 domain containing 1) and the lamin B receptor (LBR). In the case of LBR, a truncated version of the protein, missing the domain necessary for the chromatin interaction, causes failure of NE assembly and inhibition of chromatin decondensation [95]. However, cells expressing truncated LBR undergo apoptosis in early G1 phase of these daughter cells and the observed condensed phenotype might be attributed to apoptosis that is also characterized by hypercondensed chromatin. SUN1, a member of the LINC (linker of nucleoskeleton and cytoskeleton)

complex, which in interphase connects the cytoplasmic and nuclear cytoskeleton, accumulates in anaphase on the chromosome periphery concomitantly with the initiation of the NE reformation. SUN1 is suggested to target the histone acetylase hALP, which acetylates histones H2B and H4 on several sites, to the NE [96]. siRNA-mediated downregulation of SUN1 leads to delayed decondensation, often accompanied by apoptosis, while a fusion protein consisting of the N-terminus of SUN1 lacking the transmembrane region and full-length hALP induces premature decondensation, even before chromatin segregation. Although it is not clear to which extent the N-terminus of SUN1 enhances hALP chromatin localization, these results suggest that histone modifications are involved in the chromatin condensation–decondensation cycle. To which extent the NE contributes to this remains unclear. *In vitro*, chromatin decondensation and formation of a closed NE can be functionally separated [82]. Here, chromatin can decondense in the absence of NE formation. However, the presence of membranes leads to a further enlargement of the volume occupied by chromatin [82,97]. This is most likely due to the fact that formation of a functional NE including NPCs allows for nuclear import. This increase in nuclear volume is referred to as nuclear swelling or nuclear expansion [89,98]. Whether it only reflects an increase of nuclear volume due to the presence of more nucleoplasmic proteins or indeed a further decompaction of the chromatin remains to be seen.

We still are largely ignorant about the factors involved in chromatin decondensation, as well as their regulation or the exact structural rearrangements occurring, but it is important to consider that mitotic chromatin consists of different structural and functional compartments: one can, at least, distinguish the chromosome arms, the centromeres involved in mitotic spindle attachment via the kinetochores and telomeres that protect the chromosome ends (Fig. 2, inset I). It is most likely too simple to imagine that all these domains decondense via the same mechanism—however, we are far from understanding the differences yet. Chromatin regions that are more densely packed during interphase undergo less compaction/decompaction during mitosis compared to less densely packed areas—although both undergo obvious condensation/decondensation [99]. This is in line with the formation of homogenous mitotic chromosomes [50]. Also, decompaction of the chromatin is not the only requirement for the formation of a properly functional nucleus. The chromatin needs to acquire a highly elaborated structure consisting of different territories and domains with different grades of compaction—often correlating with the transcriptional activity (reviewed in Ref. [40]). Additionally, nuclear bodies—most prominent thereby nucleoli—need to reform, each enriching different protein and RNA factors usually at specific gene loci to fulfill their individual functions in the interphase nucleus (reviewed in Refs. [3] and [100]).

The global interphase chromatin pattern is similar between the mother and daughter cells [101]. Life cell imaging of chromatin decondensation revealed a radial expansion mechanism with little relative rearrangements, meaning that "mitotic chromatin neighbors" also become "interphasic neighbors" [99]. Thus, the interphasic genome structure is already established before NE reformation. How can the cell inherit this specific structure if all mitotic chromosomes show a similar, homogenous structure? The timing of sister chromatid separation—probably defined by the individual amount of centromeric heterochromatin—defines the position of single chromosomes in the reforming nucleus [101]: sister chromatids positioned close to the spindle poles separate earlier than the ones close to the cleavage furrow. It is unlikely that sister chromatid separation timing is the only mechanism to transfer the information necessary to reestablish such a subtle interphasic nuclear structure. Epigenetic memory cannot only be retained by chromatin localization but it is conceivable that chromatin modification scenarios are involved. Among these, retention of transcription factors and other chromatin binding proteins on the chromatin during mitosis—although globally removed as transcription is inhibited during mitosis - or maintenance of some specific posttranslational histone modifications could contribute (reviewed in Ref. [102]). How these events are coordinated with overall chromatin decompaction and NE reformation remains to be seen.

The Nuclear Envelope Emerges from the Mitotic ER

The NE reforms on the decondensing chromatin and reestablishes the barrier between the nucleoplasm and the cytoplasm. The nuclear membranes are continuous with the ER membranes; therefore, the NE can be regarded as a subdomain of the ER. This becomes especially obvious during metazoan mitosis, when the NE breaks down and its membranes merge into, and are, at least on a light--microscopical level, undistinguishable from the bulk ER [103–105]. The morphology of the mitotic ER and thus the starting point for ER restructuring leading to NE reestablishment at the end of mitosis is a matter of debate. Some studies suggest that ER sheets convert into fenestrated sheets and tubules [106–108] whereas others propose that tubules transform into sheets [109–112]. Depending on the model of mitotic ER structure, NE reformation at the end of mitosis is differently envisioned: ER tubules are thought to extend from the ER network, contact the decondensing chromatin, become immobilized and flatten and expand to give rise to INM and ONM

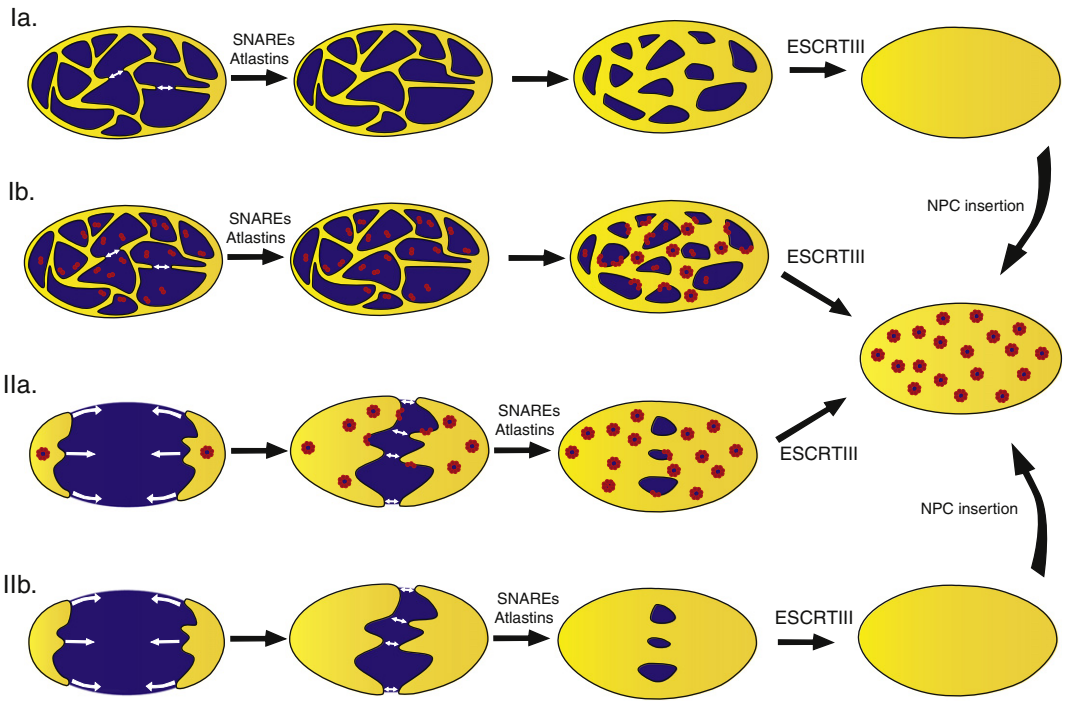

Fig. 4. Nuclear envelope reformation by ER restructuring. The nuclear envelope is formed by reorganization of the mitotic ER. Tubular ER structures are suggested to contact the chromatin, form a network on the surface, flatten and close the remaining holes to form a closed nuclear envelope (Ia and Ib). Alternative models propose that ER membrane sheets contact the chromatin, spread on its surface and enclose it (IIa and IIb). Necessary membrane fusion and sealing events are indicated. Both types of models are compatible with a simultaneous formation of a closed nuclear envelope and NPCs (enclosure models, Ib and IIa) or integration of NPCs in the already sealed nuclear envelope (insertion models, Ia and IIb).

sheets [106] (Fig. 4, Ia and Ib). Alternatively, flat ER membrane sheets would contact and subsequently enclose the chromatin to form the NE [111] (Fig. 4, IIa and IIb). As both interphasic and mitotic ER network morphologies vary considerably between cell types [107,113], it is conceivable that both modes of NE formation exist, depending on the cell type.

The ER interacts with microtubules in interphase and this interconnection undergoes important remodeling during mitosis. The mitotic ER is excluded from the central spindle area and from the chromosomes until the onset of NE reformation in late anaphase [108,114]. In mitosis, the association of ER membranes with microtubules is strongly reduced, and it is very likely that this contributes to ER exclusion from the spindle [112,115,116]. Indeed, mitotic phosphorylation of the integral ER membrane protein STIM1 (stromal interaction molecule 1) abolishes its interaction with microtubules and a STIM1 phosphorylation mutant causes aberrant accumulation of ER membranes within the mitotic spindle in HeLa cells [116]. Microtubule binding of another ER membrane protein, CLIMP-63 (cytoskeleton-linking membrane protein 63), is similarly negatively regulated by mitotic phosphorylation [117,118] and it is conceivable that this modification similarly helps to exclude the ER from the spindle area. In addition, an active mechanism contributes to the clearance of the ER from the central spindle area: the ER membrane proteins REEP3 and REEP4 (receptor expression-enhancing proteins 3 and 4) function as linkers between the ER and microtubules and transport the ER that has entered the spindle area to the spindle poles [4]. Depletion of REEP3/REEP4 causes cytokinesis and chromosome segregation defects, as well as aberrant shaped nuclei in interphase. This highlights the importance of correct mitotic ER morphology and distribution.

Microtubules *per se* seem not to be required for NE reformation [112,119,120] and microtubule formation rather needs to be inhibited close to chromatin [121]. Depletion of DPPA2 (developmental pluripotency associated 2), a chromatin binding and microtubule destabilizing protein, from *Xenopus* egg extracts or the addition of the microtubule stabilizing drug taxol, prevents NE formation. Interestingly, nuclear expansion is inhibited by pervasively depolymerizing microtubules when DPPA2 is delocalized from chromatin and thus ectopically active or when microtubule depolymerizing agents

such as colcemid or nocodazol are added [121,122]. This effect could be due to a reduction of ER membranes around the reassembled nuclei, as ER membranes are required for nuclear membrane expansion to allow nuclear growth [122–124]. Notably, the NE formed *in vitro* in the presence of colcemid and nocodazol reportedly does not contain NPCs [120] and hence the subsequent, expected lack of nuclear import could explain, at least in part, the nuclear growth defect. Consistently, long-term exposure of sublethal concentrations of the microtubule inhibitor colchicine or viniblastine induces formation of *annulate lamellae* in tissue culture cells [125] that also indicates, as previously mentioned, a malfunction in NPC reassembly into the reforming NE at the end of mitosis [19,20]. The lack of NPCs could indicate that microtubules contribute to the proper segregation of nuclear membrane domains from the ER membrane continuum at the end of mitosis, including transmembrane nucleoporins crucially required for NPC formation [126,127]. It remains to be seen whether an NE reformed in the absence of functional microtubules contains typical INM proteins at a comparable level.

Establishing a Nuclear Envelope Membrane Domain

In late anaphase, nuclear membranes start approaching chromatin [103,104,128]. It is thought that integral membrane proteins of the INM and their chromatin binding affinity are the driving force for this process [123,129]. Many INM proteins including LBR [130–132] and the LEM-domain-containing proteins LAP2β (lamin-associated polypeptide 2β) [133,134], MAN1/LEMD3 [135] and emerin [136] associate with chromatin, in the case of LBR by interacting with HP1 [137]. LEM-domain-containing proteins interact with the previously introduced chromatin-associated protein BAF (see section “Mitotic Exit Regulation”). BAF recruits LEM-domain-containing proteins to chromatin during mitotic exit, and the LEM proteins reciprocally modulate the distribution of BAF during interphase [138–140]. In addition to BAF-mediated recruitment, binding of several INM proteins to chromatin, including transmembrane nucleoporin NDC1 (nuclear division cycle 1) and POM121 (pore membrane protein), can occur by a direct DNA binding capability that relies on the presence of basic domains [129]. The rapid recruitment of membranes to chromatin at the onset of anaphase might therefore be explained by the existence of more than one chromatin interaction strategy of INM proteins. These multiple interactions might also explain why individual INM proteins are nonessential for nuclear reassembly *in vivo,* with the exception of LBR, for which opposing results have been reported [95,123].

Binding of both, soluble and membrane proteins, to the chromatin surface at the end of mitosis does not occur uniformly but can be referred to two zones on the chromatin area, called core and noncore chromatin regions (Fig. 2, inset III). The core (or central) region is established on the surfaces proximal and distal to the mitotic spindle, and the noncore region is established on the surfaces lateral to the mitotic spindle. The core region is enriched in A-type lamins but also with emerin and LAP2β, which are recruited locally by BAF [138,141]. Other factors such as lamin B, LBR and nucleoporins localize preferentially on the peripheral noncore region [138,142,143]. Interestingly, the initial steps of NPC formation, that is, the binding of chromatin by the nucleoporin MEL28/ELYS (maternal effect lethal/embryonic large molecule derived from yolk sac) and the recruitment of Nup107-160 complex on the noncore region, control the formation of chromatin subdomains [144], linking NPC assembly to the establishment of chromatin reorganization at the end of mitosis. It is currently unclear which specific features of noncore chromatin render it competent for MEL28/ELYS binding and subsequent NPC assembly.

The reassociation of nuclear membranes with chromatin and the reestablishment of the NE are tightly regulated in time and space. Different mechanisms are involved, including phosphorylation/dephosphorylation cycles on INM proteins, regulation of the chromatin proteins BAF and HP1, presumably the RAN system and potentially also histone modifications (summarized in Fig. 3). Chromatin binding of nuclear membranes is controlled *in vitro* by the counteracting activities of CDK1/cyclin B [145–147], which blocks chromatin association, and protein phosphatases, namely PP1 [146,148], which promote membrane recruitment. A variety of integral NE proteins, including GP210, LBR, LAP2β, emerin, MAN1, NDC1 and POM121 are phosphorylated at the onset of mitosis, which is thought to prevent their association with chromatin and contribute to the disassembly of the NE [14,127,149–151]. Conversely, one would expect that dephosphorylation of these proteins during mitotic exit triggers their chromatin recruitment. Although this is conceivable, in most instances, evidence for a direct contribution of phosphorylation/dephosphorylation cycles in the regulation of chromatin binding and NE dynamics of these proteins is lacking. The best-characterized example is the recruitment of LBR to chromatin at the end of mitosis, which also points out that the regulation might be more complex. LBR binding to chromatin is prevented *in vitro* by phosphorylation of a specific serine residue in an arginine/serine repeat domain [148,152,153]. The timing of ER membrane recruitment of LBR to anaphase chromosomes is controlled by LBR dephosphorylation in human cells [154]. In addition to dephosphorylation in its arginine/serine repeat domain, phosphorylation of LBR by the

serine/arginine-rich protein-specific kinase SRPK1 [152,153,155] is required for its association with chromatin *in vitro*.

Two chromatin-associated proteins, HP1 and BAF, link chromosome decondensation and NE formation. Chromatin recruitment of HP1 requires the dephosphorylation of histone H3 at S10 and is promoted by H3K9 methylation, a characteristic histone modification of heterochromatin [83,156–158]. HP1 chromatin recruitment in anaphase [159] could cooperate in the association of LBR with chromatin during mitotic exit, in addition to the fact that LBR can also interact directly with DNA, histones and other chromatin-associated proteins [160]. The presence of Aurora B on mitotic chromosomes prevents the recruitment of nuclear membranes and by that ensures that the NE does not assemble before successful segregation of the chromatin [91,161]. The putative phosphorylation targets of Aurora B in this process are unknown, but it is possible that serine 10 phosphorylation of histone H3 is involved: this particular phosphorylation, in fact, would prevent HP1 chromatin localization [83]. Chromatin recruitment of BAF during anaphase is crucial for NE reassembly and is regulated by its phosphorylation. BAF phosphorylation by the kinase VRK1 reduces its affinity for chromatin [37], and loss of the BAF-mediated link between chromatin and nuclear membranes contributes to NE disassembly [36]. As discussed above (see section “Mitotic Exit Regulation”), the INM LEM4 is required for BAF dephosphorylation during mitotic exit by recruiting PP2A and by inhibiting VRK1 [35]. Thus, the NE/chromatin interaction via LEM proteins is at least in part regulated by cell-cycle-dependent phosphorylation/dephosphorylation cycles of BAF (Fig. 3).

Cell-cycle-dependent waves of phosphorylation and dephosphorylation can account for the temporal coregulation of mitotic chromosome decondensation, NE formation and NPC assembly. However, NE assembly, as well as NPC assembly, must be restricted to the chromatin. This regulation is thought to be provided by the small GTPase RAN, which in interphase functions in nucleoplasmic/cytoplasmic transport of cargos across the NPC. RAN, in its GTP-bound state that is locally generated in the nucleus, stimulates the release of importin-bound cargo in the nucleoplasm, but it is also required for many mitotic processes. Despite the absence of an NE, chromatin is demarcated by a high concentration of the GTP-bound RAN throughout the cell cycle [162] and GTP-bound RAN-mediated release of importins from a variety of target proteins controls a range of processes, varying from spindle assembly and chromatin segregation to assembly of the nuclear membranes and nuclear pores around chromatin, in later stages of mitosis (for review, see Ref. [94]). It is conceivable that the RAN/importin system, used by the cell to target integral membrane proteins to the NE in interphase [20,163], also regulates the recruitment of INM proteins to post-mitotic chromatin in a similar way (Fig. 3) [164]. Importin β binds LBR during mitosis [95,165] and this inhibitory complex dissociates in the presence of GTP-bound RAN [165]. The importin family might prevent undesired interactions between the positively charged DNA binding domains of INM proteins and chromatin during mitosis. In the case of LBR, the importin β and chromatin binding sites overlap. Indeed, a functional RAN cycle is essential for nuclear assembly *in vitro* [166,167] but whether this is directly via regulation of NE/chromatin interactions remains to be seen.

In parallel with RAN as a spatial marker for chromatin, chromatin modifications during mitotic exit might also contribute to the regulation of nuclear membrane recruitment. In addition to the possible involvement of H3 Ser10 dephosphorylation in regulation of HP1 chromatin localization, the lysine--specific demethylase LSD1 has been implicated in regulation of NE reformation. LSD1 catalyzes the demethylation of monomethylated and dimethylated lysines K4 and K9 of histone H3 tails [168]. Downregulation in HeLa cells extends telophase and affects NE reassembly [97]. *In vitro* experiments suggest that nuclear membrane recruitment to chromatin is impaired upon inhibition or removal of LSD1. Although nonhistone protein targets of LSD1 demethylase activity cannot be excluded, the identification of the histone demethylase LSD1 as an essential regulator of nuclear assembly indicates that cell cycle regulated chromatin state and more precisely histone modifications play a role in controlling nuclear membrane binding on the decondensing chromatin (Fig. 3).

In summary, the reversal of mitosis-specific phosphorylations on nuclear membrane proteins regulates the timing of nuclear membrane recruitment to chromatin; nonetheless, the precise sites of modification have yet to be identified and it is currently not clear how prevalent this mode of regulation is. Changes on the chromatin landscape at the end of mitosis contribute similarly; these include binding of chromatin-associated factors such as BAF and HP1 and probably also changes in the histone modification patterns. Spatial organization by the RAN system might facilitate the binding of nuclear membrane proteins to chromatin by exposing their DNA binding domains proximally to chromatin, but the contribution of such a mechanism has not been proved yet.

Nuclear Envelope Sealing

Complete fusion of the membranes at the newly forming nucleus is required for reestablishment of

nuclear compartmentalization. As the NE and the ER are connected and share similar mechanics, it is possible that NE fusion employs the same machinery and factors as the bulk ER. In *Xenopus* egg extracts, NSF (NEM-sensitive factor) and α-SNAP (soluble NSF adaptor protein), factors of the SNAP receptor (SNARE) activation, are critically required for formation of a closed NE [112,169], suggesting that SNARE-mediated membrane fusion is needed. However, the specific SNARE proteins involved in NE formation still await identification. Formation and maintenance of an ER network additionally requires integral membrane GTPases, atlastins, which mediate fusion between ER tubules [170,171]. *In vitro*, formation of a closed NE is blocked by a dominant negative version of atlastin [112], suggesting an involvement of this GTPase, most likely because the NE formation is initiated in this experimental setup by an ER network formed on the chromatin surface [166]. Given the controversy whether ER sheets or tubules are the membrane structure initiating NE formation, it remains to be seen whether and to which extent atlastins also directly contribute to closed NE formation *in vivo*.

Two recent studies indicate components of the ESCRT-III (endosomal sorting complex required for transport) complex as involved in NE reformation, as depletion of ESCRT-III constituents results in failure to seal the NE and, therefore, in leaky nuclei [172,173]. ESCRT-III is known to participate in constricting the neck of membrane buds or even entire cells, during vesicle formation into multivesicular bodies, HIV virus egress and cytokinesis. During NE reformation, it is suggested to function in a topologically similar event: the closure of final gaps that remain open when NE encloses the chromatin. These gaps might be holes in the NE that remain after membrane flattening and expansion of an ER network [106] if not filled by NPCs (Fig. 4, insets Ia and Ib, see below). Alternatively, the holes could be the ones remaining when sheet-like membranes enclosing the chromatin merge (Fig. 4, insets IIa and IIb) [111].

Although the studies in Refs. [172] and [173] agree on the crucial role of the ESCRT-III complex in NE closure, each adds distinctive insights into ESCRT-III function in nuclear sealing. Vietri and collaborators show that the ESCRT-III complex recruits the microtubule severing ATPase spastin [173]. Spastin is suggested to disassemble spindle microtubules, which would otherwise prevent NE sealing. Olmos and colleagues show that the ATPase p97 recruits the ESCRT-III complex via its adaptor protein UFD1 to function in NE sealing [172]. Interestingly, earlier *in vitro* data suggested that p97 is required for NE reassembly together with UFD1 and another cofactor, NPL4 [174]. p97 depletion impairs formation of a closed NE, although membrane vesicles still bind but fail to fuse to an ER-like network on the chromatin template. This phenotype is difficult to reconcile with sealing of small holes in the reforming NE mediated by the ESCRT-III complex. It rather suggests that p97 is also involved in additional, yet-uncharacterized steps in NE reformation. As sperm chromatin was directly incubated with interphasic extracts in these experiments [174], it is unlikely that the extraction of Aurora B from chromatin mediated by p97, observed on mitotic chromatin [91], accounts for the crucial p97 function also in this experimental system.

During vesicle formation in multivesicular body formation, the ESCRT machinery recognizes ubiquitinylated membrane proteins [175]. The fact that p97, which recognizes ubiquitinylated proteins via its adaptors UFD1 and NPL4, is involved in the pore sealing process [176] points into the same direction. If so, it remains to be seen which NE membrane proteins are crucial ubiquitinylated targets for the ESCRT-III function in NE sealing.

Building NPCs into the Nuclear Envelope

The coordinated reassembly of NPCs begins concomitantly with the reformation of the NE. NPCs form large pores in the envelope with a diameter of approximately 130 nm at the sites where the ONM and INM fuse [177]. Only a few of the roughly 30 different nucleoporins are integral membrane proteins residing in the ER during mitosis. Most nucleoporins are soluble during open mitosis in animals and are recruited from the cytosol to reassemble NPCs during mitotic exit. Two profoundly different modes for NPC reassembly at the end of mitosis have been proposed, insertion or enclosure (see Fig. 4, discussed in Ref. [178]). According to insertion models, NPCs assemble and integrate into the two juxtaposed membrane sheets of an intact NE [111,179,180]. NPC formation would thus follow the formation of a closed NE and requires the fusion of the ONM and INM across the NE lumen. Alternatively, enclosure models propose that NPC reassembly does not occur by insertion into the sealed NE, but it is rather accomplished by the contact and envelopment of the assembling NPCs on the chromatin surface by the outgrowing ER-derived membranes [178,181–183]. Both suggested modes of NE formation (an ER network that forms and flattens on the chromatin surface or outgrowing ER membrane sheets; discussed earlier in the text) are compatible not only with enclosure but also with insertion models (Fig. 4).

The general NPC structure can be regarded as a stack of three rings with cytoplasmic and nucleoplasmic extensions: the outer or 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 midplane of the NE lays the so-called 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 dimensions and masses vary among organisms, this general structural arrangement, including the 8-fold symmetry, is conserved (for review, see Ref. [184]). Understanding the assembly pathway of these huge structures lastly embedded in the two membranes of the NE remains a formidable task. *Xenopus* egg extracts have been extensively employed for delineating the assembly pathway, as individual steps such as initiation, membrane association, termination of the NPC scaffold assembly and establishment of the transport channel can be disconnected and studied separately in this system.

Despite the differences in the models for NPC reassembly at the end of mitosis, it is commonly agreed that the process is initiated on chromatin (Fig. 5) by the nucleoporin MEL28/ELYS [19,185–187]. MEL28/ELYS can bind DNA directly, but recent elegant reconstitution experiments show that its binding to histone-containing chromatin is crucial for NPC assembly [188,189]. MEL28/ELYS acts as a seeding point for NPC formation and recruits the Nup107-160 complex to assembly sites [19]. The Nup107-160 complex is an essential scaffolding component of NPCs and forms the largest part of the cytoplasmic and nucleoplasmic rings [177,190]. *In vitro*, MEL28/ELYS and the Nup107-160 complex can bind to chromatin in the absence of membranes [19,183,186,191]. The first connection between the assembling NPC and nuclear membranes is achieved by the subsequent association of the transmembrane nucleoporin POM121 with the newly forming pores [126], a process likely mediated by binding of POM121 to the Nup107-160 complex [192,193]. It is also likely that NDC1, another transmembrane nucleoporin that is found at forming pores at the same time [127], also contributes to the connection of NPCs to membranes, but the mechanism remains to be established.

The following steps can be ordered starting from the membrane sites of the pore and proceeding toward the center of the pore. First, nucleoporins of the second major structural complex within NPCs, the Nup93 complex, join the assembling pore, presumably forming the majority of the inner ring [6]. The Nup93 complex contains the nucleoporins Nup93, Nup53 and Nup155 and the two orthologues Nup188 and Nup205. In contrast to the Nup107-160 complex, which is recruited as a preassembled complex, the Nup93 complex builds from individual components [124,194–196]. In assembled NPCs, the different components of the complex are present in different numbers ranging from 16 to 48 [197]. The precise arrangement of these nucleoporins, with respect to each other and within the inner ring,

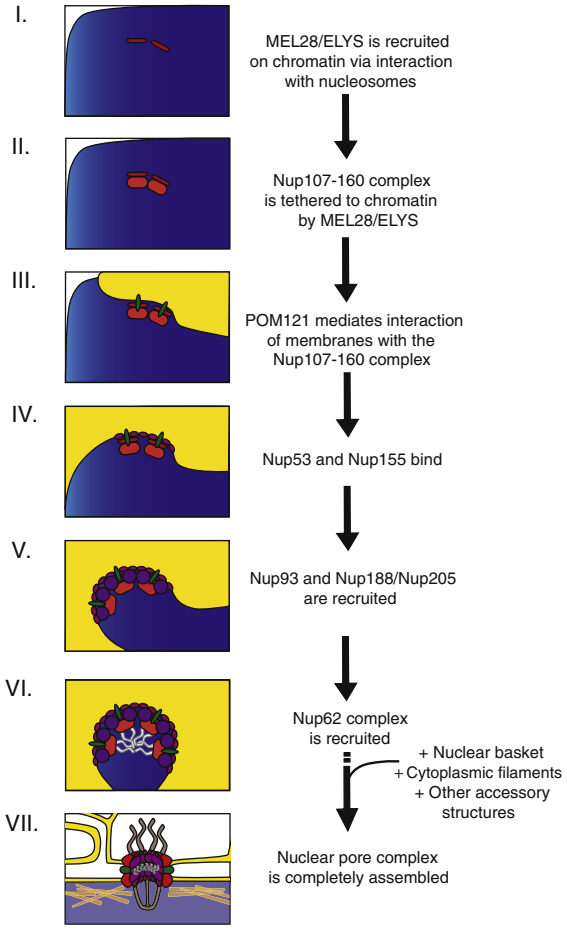

Fig. 5. Ordered NPC assembly at the end of mitosis. The chromatin binding nucleoporin MEL28/ELYS initiates NPC assembly on the chromatin (I) by recruiting the Nup107-160 complex (II), which in turn associates with the nuclear envelope membranes via the transmembrane nucleoporin POM121 (III). The recruitment 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 (IV) and promote the incorporation of Nup93, Nup188 and Nup205 to complete the structural backbone of the NPC (V). The subsequent recruitment of FG-repeat-containing nucleoporins of the Nup62 complex (VI) combined with the previous association Nup98 (data not shown) establishes the central channel, a hydrophobic meshwork that confers the transport properties of the NPC. The fully assembled NPC (VII) 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.

remains to be defined. In the assembly process of the Nup93 complex, Nup53 is the first to associate with the nascent pore, followed by Nup155 [198,199]. Both proteins can directly bind membranes [194,200] and both also interact with the transmembrane nucleoporins NDC1 and POM121

[127,192,193] and therefore constitute a second connection between the NPC and membranes at the pore. Interaction of Nup53 with NDC1 also modulates Nup53's membrane bending activity, which is crucial for successful NPC assembly [198]. This indicates that protein–protein and protein–membrane interactions are not only required for timely recruitment of the different NPC components but also involved in a more sophisticated interplay that we just begin to unravel. Nup93 interacts with Nup53 and is subsequently incorporated [196], together with its binding partners Nup188 and Nup205 [124], to complete the structural backbone of the pore. Nup93, probably together with interactions via Nup205/Nup188, recruits the FG-repeat-containing nucleoporins of the Nup62 complex [196,201]. The Nup62 complex members Nup62, Nup58 and Nup54/45, together with the FG-containing nucleoporin Nup98, form a large part of the hydrophobic meshwork localized in the center of the pore. Nup98 is recruited at the same time as the Nup93 complex [6] by a still-ill-defined mechanism. It is possible that its interaction to the Nup107-160 [202] takes part in Nup98 recruitment.

The formation of peripheral NPC structures, such as the nuclear basket on the nucleoplasmic side and the cytoplasmic filaments, follows the establishment of the structural pore and central channel [6]. On the nuclear side, Nup153 is required for the recruitment of Nup50 and TPR [203–205]. It is likely that Nup153 itself is recruited via its interaction with the Nup107-160 complex [202]. If so, it remains to be seen why Nup153 only interacts with Nup107-160 complexes located in the nuclear ring structure. The order of events in the assembly of the cytoplasmic filaments is less defined, but Nup358 is required for this [206].

Despite significant progresses in delineating the assembly pathway of NPCs, a number of important questions remain. Many nucleoporins are symmetrically distributed within the nucleoplasmic and cytoplasmic rings of the NPC, including the Nup107-160 complex [177], but the timing and details of the mechanism by which the cytoplasmic portion of the NPC assembles remain elusive. Within the nucleoplasmic and cytoplasmic rings, 16 Nup107-160 complexes arrange into two concentric rings of eight units [177]. It is unclear whether these rings assemble simultaneously or whether these are distinguishable events. The Nup107-160 complex interacts with different nucleoporins in the nucleoplasmic and cytoplasmic rings, for example, with MEL28/ELYS in the nucleoplasmic ring and Nup358 in the cytoplasmic ring. What defines these different interaction patterns remains to be elucidated. The same questions apply for Nup98, which is present in 48 copies within the vertebrate NPC [197]. Finally, despite the known octagonal symmetry of the pore, further work is needed to establish whether the numerous copies of each subcomplex are recruited simultaneously around the pore circumference.

Once a closed NE with functional NPCs has reassembled, the nuclei further expand, and they assemble and accommodate more NPCs, a process that continues during all interphase and that is hence referred to as interphase or *de novo* NPC assembly. It is a matter of debate whether NPC reformation at the end of mitosis and interphase NPC assembly follow the overall same pathway. In all likelihood, at least in interphase, NPC assembly follows an insertion pathway as NPC integrates into the already intact NE. Whereas NPC reformation at the end of mitosis critically requires MEL28/ELYS [19,185], it is dispensable for NPC formation occurring in interphase [20]. New evidences suggest that the function of MEL28/ELYS as initiating assembly point is taken over by Nup153 in interphase NPC assembly. Nup153 would then direct the Nup107-160 complex to the INM and pore assembly sites [207]. This functional difference in initiation, on the chromatin at the end of mitosis and on the nuclear membranes during interphase, might be an indication that, in contrast to interphase NPC assembly, NPC reassembly at the end of mitosis follows an enclosure pathway. The need for membrane deforming and/or membrane deformation sensing modules required in some nucleoporins specifically for interphase NPC assembly [20,194,207] is in agreement with this hypothesis. However, a step toward a definitive answer will be made when the fusion machinery required for the fusion of ONM and INM is identified. The prediction for the enclosure model is that NPC reassembly does not depend on this machinery, in contrast to interphase NPC assembly. On the other hand, the insertion model forecasts that both assembly modes depend, in all likelihood, on the same fusion machinery.

Regulating NPC Assembly at the End of Mitosis

As the NE breaks down at the beginning of mitosis, NPCs disassemble into their building blocks, which are, with the exception of the few transmembrane nucleoporins, largely dispersed in the mitotic cytosol as single proteins or in some cases as subcomplexes and do not reassemble into NPCs until mitotic exit. Some nucleoporins have additional functions during mitosis outside of NPC formation, including centrosome positioning, spindle assembly, kinetochore organization, the spindle assembly checkpoint, chromosome segregation and cytokinesis (for review, see Ref. [94]). Several nucleoporins, including members of the Nup107-160 complex, Nup98, Tpr and Nup53, are hyperphosphorylated during mitosis [127,150,208–212] and it has been suggested that this phosphorylation cascade acts as a

general mechanism to keep nucleoporins dissociated, preventing premature NPC reassembly and at the same time allowing for their diverse mitotic functions. For instance, hyperphosphorylation of Nup98 initiates the NPC disassembly at the beginning of mitosis [209]. Conversely, interactions between nucleoporins could be promoted by late mitotic dephosphorylations. However, the fact that the kinases and phosphatases involved perform a variety of functions in mitotic entry, progression and exit (see section "Mitotic Exit Regulation") results in the lack of direct evidence for such a mechanism. Moreover, identifying causative phosphorylation events is complicated by a high degree of redundancy, as exemplified by the Nup98 case: Nup98 is phosphorylated by CDK1 and members of the NIMA-related kinase family at 13 different sites to allow for its dissociation from NPC at the entry of mitosis [209]. In addition, it remains to be seen whether other posttranslational changes on nucleoporins regulate NPC disassembly and reassembly.

NPC reassembly is initiated on, and thus directed to, the chromatin surface by RAN. As discussed for NE reassembly, high concentrations of RAN-GTP generated in the vicinity of the chromatin are supposed to release transport receptors from nucleoporins that, in turn, can interact and assemble to NPCs. Good evidence for this model is the aberrant formation of NPCs in ER membrane stacks distal from the NE, *annulate lamellae*, when the RAN-GTP gradient is disturbed [213]. MEL28/ELYS and the Nup107-160 complex are likely candidates for such a RAN-dependent regulation because they bind transport receptors and associate with chromatin in the early stages of NPC assembly [19,186,191,213]. However, many nucleoporins bind transport receptors to facilitate nuclear transport, and these events are not restricted to FG-repeat-containing regions, as in Nup50 and Nup153 for example [214,215]. In addition, nucleoporins also bind transport receptors to allow their import to the nucleoplasmic side of the pore, where they function in *de novo* NPC assembly during the entire interphase [20,207,216]. Notably, Nup153 membrane interaction capability, which is required for initiating interphase NPC assembly, is regulated *in vitro* by the transport receptor transportin [207]. This opens the possibility that both initiating steps, MEL28/ELYS chromatin recruitment after mitosis and Nup153 INM binding in interphase, are regulated by RAN, consistent with the proposal that interphase NPC assembly is also regulated by this GTPase [217]. However, definitive proof for this model is lacking.

As similarly discussed for the regulation of NE reformation, changes on the chromatin landscape during mitotic exit might regulate NPC reassembly. Despite its DNA binding activity, MEL28/ELYS requires nucleosomes for its proper recruitment to initiate NPC reassembly on the chromatin [188,189]. It is tempting to speculate that histone modifications might contribute to this, especially as MEL28/ELYS distribution is nonhomogenous on the chromatin during mitotic exit, but it first accumulates at the chromosomal noncore region [144]. Furthermore, downregulation of the lysine demethylase LSD1 affects NPC reformation indicated by a high presence of *annulate lamellae* [97], and *in vitro*, MEL28/ELYS chromatin interaction is reduced upon LSD1 depletion. Whether a corresponding increase in methylated histone H3 is causative for the effects and whether the phenotype is primarily an NE defect or an NPC assembly defect remain to be established.

Lamina and LINC Complex Reassembly

In addition to proper NE and NPC formation, nuclear assembly at the end of mitosis must include reestablishment of additional structures. The nuclear lamina is a fibrous structure formed by lamins and located underneath the NE. It is connected to the NE via interactions of lamins with INM proteins and members of the LINC complex. The lamina is depolymerized at the onset of mitosis [218] by the CDK1-mediated phosphorylations of lamins [219,220]. Conversely, lamins are dephosphorylated at the end of mitosis to allow reassembly of this structure. PP1, which is recruited to the NE by AKAP149, removes mitotic phosphorylations from lamin B in telophase [25,221]. Although some association of nuclear lamins is observed during early stages of NE reformation [103], the bulk of nuclear lamins are reassembled into the lamina only after the nuclei have regained competence for nuclear import [26,222]. Arrangements of lamins into the lamina during interphase have been studied intensively, but we still miss a clear picture of how the assembly process occurs (reviewed in Ref. [223]). Similarly, we do not know when the interactions to NPCs are established [103,224], occurring most likely via the lamin-interacting nucleoporin Nup153 [225,226].

The LINC complexes provide physical connection between the backbone scaffold of the intranuclear and extranuclear compartments and play pivotal roles in a vast series of evolutionally divergent tasks, from yeast to mammals (reviewed in Ref. [227]). Since nuclear integrity is lost at the onset of mitosis, it is conceivable that the LINC complexes lose connection with both sides of the NE. Indeed, the LINC complex component SUN1 is phosphorylated by CDK1 at the onset of mitosis that disrupts its lamina interaction [228], whereas binding to KASH domains on the lumenal side is not affected. As the LINC complexes provide mechanical stability to the nucleus, it is possible that torsional stress that tears the nucleus during prophase contributes to release

the LINC. Whether LINC complexes themselves remain intact during mitosis as suggested previously [228] for SUN1 and Nesprin 2 remains to be seen under nonoverexpression conditions and for other LINC complex pairs. Independent of this, at the end of mitosis, the LINC complex needs to reestablish its interaction to the nucleoskeleton and probably also to the cytoskeleton. If the LINC complex disassembles during mitosis, it also needs to reassemble and all these pathways remain to be established.

Conclusion

At the end of the open mitosis in metazoans, the interphase nucleus competent for DNA transcription and replication, pre-RNA processing and many other nuclear functions such as biogenesis of ribosomal subunits need to reestablish. For some processes including NE and pore complex reassembly, we have a detailed knowledge about the steps and factors involved despite the fact that a number of open questions remain. For others such as chromatin decondensation, we are just beginning to identify the factors involved and we are largely ignorant about the molecular mechanisms. In addition, the regulation in time and space of the different processes occurring at the end of mitosis is far from being understood and especially how they are coordinated with each other. It is similarly often unclear but conceivable that malfunctions in the different pathways or their coordination would have implications for human diseases. For example, lamina assembly faults are linked to a variety of human diseases, summarized as laminopathies (for review, see Ref. [229]). It remains to be seen whether this applies also to other processes and will be an interesting avenue for future research.

Acknowledgements

We are thankful to Daniel Moreno-Andrés, Marion Weberruss and Hideki Yokoyama for critical comments on the manuscript. This work was supported by the German Research Foundation and the European Research Council (AN377/3-2 and 309528 CHROMDECON to W.A.), a PhD Fellowship of the Boehringer Ingelheim Fonds to A.K.S. and a PhD Fellowship of the International Max Planck Research School “From Molecules to Organisms” to P.D.M.

Received 31 July 2015;
Received in revised form 17 September 2015;
Accepted 19 September 2015
Available online 28 September 2015

Keywords:
nuclear envelope;
nuclear pore complex;
chromatin decondensation;
mitotic exit

†A.K.S. and P.D.M. contributed equally to this work.

Abbreviations used:
NPC, nuclear pore complex; INM, inner nuclear membrane; ONM, outer nuclear membrane; ER, endoplasmic reticulum; CPC, chromosomal passenger complex; APC, anaphase promoting complex.

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RESEARCH ARTICLE

The lysine demethylase LSD1 is required for nuclear envelope formation at the end of mitosis

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ABSTRACT

The metazoan nucleus breaks down and reassembles during each cell division. Upon mitotic exit, the successful reestablishment of an interphase nucleus requires the coordinated reorganization of chromatin and formation of a functional nuclear envelope. Here, we report that the histone demethylase LSD1 (also known as KDM1A) plays a crucial role in nuclear assembly at the end of mitosis. Downregulation of LSD1 in cells extends telophase and impairs nuclear pore complex assembly. *In vitro*, LSD1 demethylase activity is required for the recruitment of MEL28 (also known as ELYS and AHCTF1) and nuclear envelope precursor vesicles to chromatin, crucial steps in nuclear reassembly. Accordingly, the formation of a closed nuclear envelope and nuclear pore complex assembly are impaired upon depletion of LSD1 or inhibition of its activity. Our results identify histone demethylation by LSD1 as a new regulatory mechanism linking the chromatin state and nuclear envelope formation at the end of mitosis.

KEY WORDS: Nuclear envelope formation, Nuclear pore complex, Mitotic exit, Lysine (K)-specific demethylase 1A, KDM1A, Histone modification, MEL28, ELYS, POM121, NDC1

INTRODUCTION

The nuclear genome is organized and maintained within the two membranes of the nuclear envelope. The envelope itself is a compartment of the endoplasmic reticulum (ER) and the outer nuclear membrane is continuous with the ER network. The inner nuclear membrane contains a unique set of integral membrane proteins that connect to chromatin and the nuclear lamina. The exchange of molecules between the cytoplasm and the nucleoplasm is mediated by nuclear pore complexes (NPCs), macromolecular protein assemblies that are found at points of fusion between the inner and outer membranes of the nuclear envelope. In metazoa, the nuclear envelope breaks down during mitosis in order to facilitate the capture of highly condensed chromosomes and their segregation by the spindle apparatus. The complex architecture of the interphasic nucleus must therefore be reestablished upon mitotic exit when the nuclear envelope and pore complexes reassemble on the decondensing chromatin (for reviews see Güttinger et al., 2009; Schooley et al., 2012).

Phosphorylation cascades regulate the structural changes to nuclear organization that occur during mitosis, including massive chromatin condensation and nuclear envelope disassembly. At the onset of mitosis, the phosphorylation of lamins (Heald and McKeon, 1990; Peter et al., 1990) and inner nuclear membrane proteins (Foisner and Gerace, 1993; Pyrpasopoulou et al., 1996) initiates the disassembly of the nuclear envelope. The reestablishment of the interphase nucleus at the end of mitosis is coordinated by the reversal of mitotic phosphorylation events due to the inactivation of mitotic kinases and the activation of phosphatases (Wurzenberger and Gerlich, 2011). Dephosphorylation of lamins enables the reassembly of the nuclear lamina (Thompson et al., 1997). Meanwhile dephosphorylation of inner nuclear membrane proteins increases their affinity for chromatin and initiates nuclear membrane recruitment (Pfaller et al., 1991; Ito et al., 2007) and nuclear envelope formation.

Upon nuclear disassembly, the nuclear membranes are absorbed in the mitotic ER and it is currently unclear whether they maintain their identity as a distinct subcompartment within the network during mitosis. Experiments in *Xenopus laevis* egg extracts, which have been used extensively to study nuclear envelope and NPC assembly in cell-free systems (Gant and Wilson, 1997), suggest that indeed they do maintain their identity. The ER derived from egg extract preparations gives rise to distinct vesicle populations that possess different capacities to support nuclear envelope formation. One such population is enriched for the transmembrane nucleoporins POM121 and NDC1, has a high affinity for chromatin, and is part of the initial wave of nuclear membrane recruitment to chromatin during cell-free nuclear assembly (Antonin et al., 2005; Mansfeld et al., 2006). A second vesicle population has a lower affinity for chromatin, is recruited relatively late during nuclear assembly, and is enriched for a third transmembrane nucleoporin, GP210 (also known as NUP210). These distinct vesicle types can also be biochemically separated from a third vesicle pool enriched for typical ER-membrane proteins, which are not strictly essential for nuclear assembly. The physical and functional separation of nuclear membrane vesicle populations implies that there is microscale partitioning of different membrane domains in the mitotic ER. At the end of mitosis, nuclear membranes segregate from the bulk ER and enclose the decondensing chromatin to form a new nuclear envelope. Chromatin binding by integral inner nuclear membrane proteins is thought to be a crucial determinant in this process (Ulbert et al., 2006; Anderson et al., 2009).

Concomitant with the formation of the nuclear envelope and thus the establishment of a diffusion barrier between the cytoplasm and the nuclear interior, NPCs re-assemble to ensure its transport competence (Bodoor et al., 1999; Daigle et al., 2001). Post-mitotic NPC assembly is initiated by the recruitment of a subset of nucleoporins to chromatin and is particularly well characterized due to the faithful reconstitution of these events in *Xenopus* egg extracts. The chromatin-binding nucleoporin MEL28 (also known as ELYS and AHCTF1) initiates NPC

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Received 10 April 2015; Accepted 27 July 2015

Journal of Cell Science (2015) 128, 3466-3477 doi:10.1242/jcs.173013

assembly by recruiting the Nup107–Nup160 complex, a major structural component of the NPC (Harel et al., 2003; Walther et al., 2003b; Rasala et al., 2006, 2008; Franz et al., 2007; Gillespie et al., 2007; Rotem et al., 2009). Membranes are next connected to the assembling NPC by interactions between the transmembrane nucleoporin POM121 and the Nup107–Nup160 complex (Antonin et al., 2005; Mitchell et al., 2010; Yavuz et al., 2010). NDC1 is also likely to be involved at this step (Mansfeld et al., 2006; Stavru et al., 2006) but its function is less defined. Components of the second major structural subunit of the NPC, the Nup93 complex, subsequently assemble stepwise from the membrane building laterally towards the centre of the NPC (Dultz et al., 2008; Sachdev et al., 2012; Vollmer et al., 2012), which allows for the recruitment of the central channel components, the FG-nucleoporins. The final steps of NPC assembly are the addition of peripheral nucleoporins, which form extensions to the cytoplasmic and nucleoplasmic sides of the pore (Bodoor et al., 1999; Hase and Cordes, 2003; Dultz et al., 2008). The small GTPase ran spatially regulates NPC reassembly by promoting the release of transport receptor bound proteins, such as MEL28 (Franz et al., 2007; Rotem et al., 2009), at post-mitotic chromatin (Walther et al., 2003a). Several nucleoporins are also hyper-phosphorylated during mitosis (Laurell et al., 2011) but the extent to which their dephosphorylation regulates post-mitotic NPC formation is currently not clear.

Although the crucial events of post-mitotic nuclear envelope assembly occur on decondensing chromatin, the regulatory mechanisms that coordinate envelope assembly and the chromatin state at that time are not well understood. Here, we identify dynamic demethylation of histone H3 by the Lysine (K) Specific Demethylase, LSD1 (also known as KDM1A), as a new mechanism coordinating the recruitment and assembly of nuclear envelope and NPC components on post-mitotic chromatin. We report that the loss of LSD1 demethylase activity blocks nuclear envelope and pore complex formation *in vitro*, through impaired recruitment of MEL28, and POM121- and NDC1-containing membrane vesicles. We find that downregulation of LSD1 in human cells elongates telophase and results in ectopic NPC assembly outside of the nuclear envelope. Our data imply that LSD1-dependent demethylation of histones is a requirement for proper nuclear assembly at the end of mitosis.

RESULTS

LSD1 demethylase activity is required for cell-free nuclear assembly

In an effort to identify regulatory landmarks of nuclear envelope reassembly at the end of mitosis we screened various classes of chemical inhibitors for their ability to block nuclear assembly *in vitro*. Cell-free nuclear formation can be faithfully recapitulated on a sperm chromatin template using the cytosolic and membrane fractions of *Xenopus laevis* egg extracts induced to mimic the late mitotic state (for a review, see Gant and Wilson, 1997). After initial DNA decondensation, a nuclear envelope forms around the chromatin, which continues to decondense and reorganize, giving rise to a functionally competent nucleus. A closed nuclear envelope can be visualized by the smooth nuclear rim incorporation of the fluorescent lipophilic membrane dye 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC18) (Fig. 1, untreated). NPCs are integrated in the newly formed envelope and can be observed by immunolabelling with mAB414, an antibody that recognizes a subset of FG-repeat-containing nucleoporins (Davis and Blobel, 1986). We identified a group of inhibitors [*N*-methyl-*N*-propargylbenzylamine (pargyline), *trans*-2-phenylcyclopropylamine (2-PCPA), and the 2-PCPA derivative trans-2-(2-benzyloxy-3,5-difluorophenyl)cyclopropylamine hydrochloride (S2101)] targeting the histone lysine demethylase LSD1 that severely impairs nuclear envelope and NPC assembly (Fig. 1).

In order to determine whether LSD1 specifically plays a role in nuclear assembly it was immuno-depleted from *Xenopus* egg extract cytosol using a polyclonal antibody generated against full-length *Xenopus* LSD1 that recognizes both the *Xenopus* and human protein (supplementary material Fig. S1). LSD1 was efficiently removed from the egg cytosol, whereas the levels of other proteins, including the nucleoporins Nup107, Nup62 and MEL28, the nuclear GTPase ran, which is important for nuclear transport, and CoREST (also known as RCOR1), which functions as an LSD1 cofactor in H3K4 demethylation (Shi et al., 2005), were unaffected (Fig. 2A). LSD1 depletion rendered the extracts incompetent for nuclear envelope and NPC assembly (Fig. 2B,C). Addition of purified recombinant *Xenopus* LSD1 rescued the formation of a closed nuclear envelope with integrated NPCs, validating the specificity of the depletion phenotype and confirming the requirement for LSD1 in nuclear assembly. We designed a demethylase-deficient version of LSD1 by

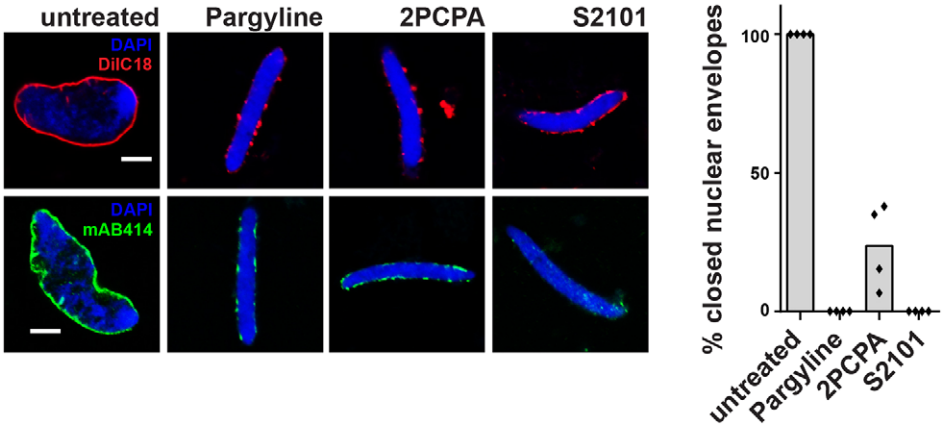

Fig. 1. LSD1 inhibitors block cell-free nuclear assembly. Nuclei assembled on sperm chromatin in *Xenopus* egg extracts for 120 min were fixed and analyzed by confocal microscopy. Where indicated 5 mM pargyline, 2.5 mM 2PCPA or 0.25 mM S2101 was added to assembly reactions at *t*=0. Membranes were pre-labelled with DiIC18 (upper panel, red in overlay), NPCs were immuno-labelled following fixation using mAB414 (lower panel, green), and chromatin was stained with DAPI (blue in overlays). Scale bars: 5 µm. In each experiment, 100 randomly chosen chromatin substrates were scored. The mean percentage of nuclei with closed nuclear envelopes from four independent experiments is shown. Data points from individual experiments are indicated.

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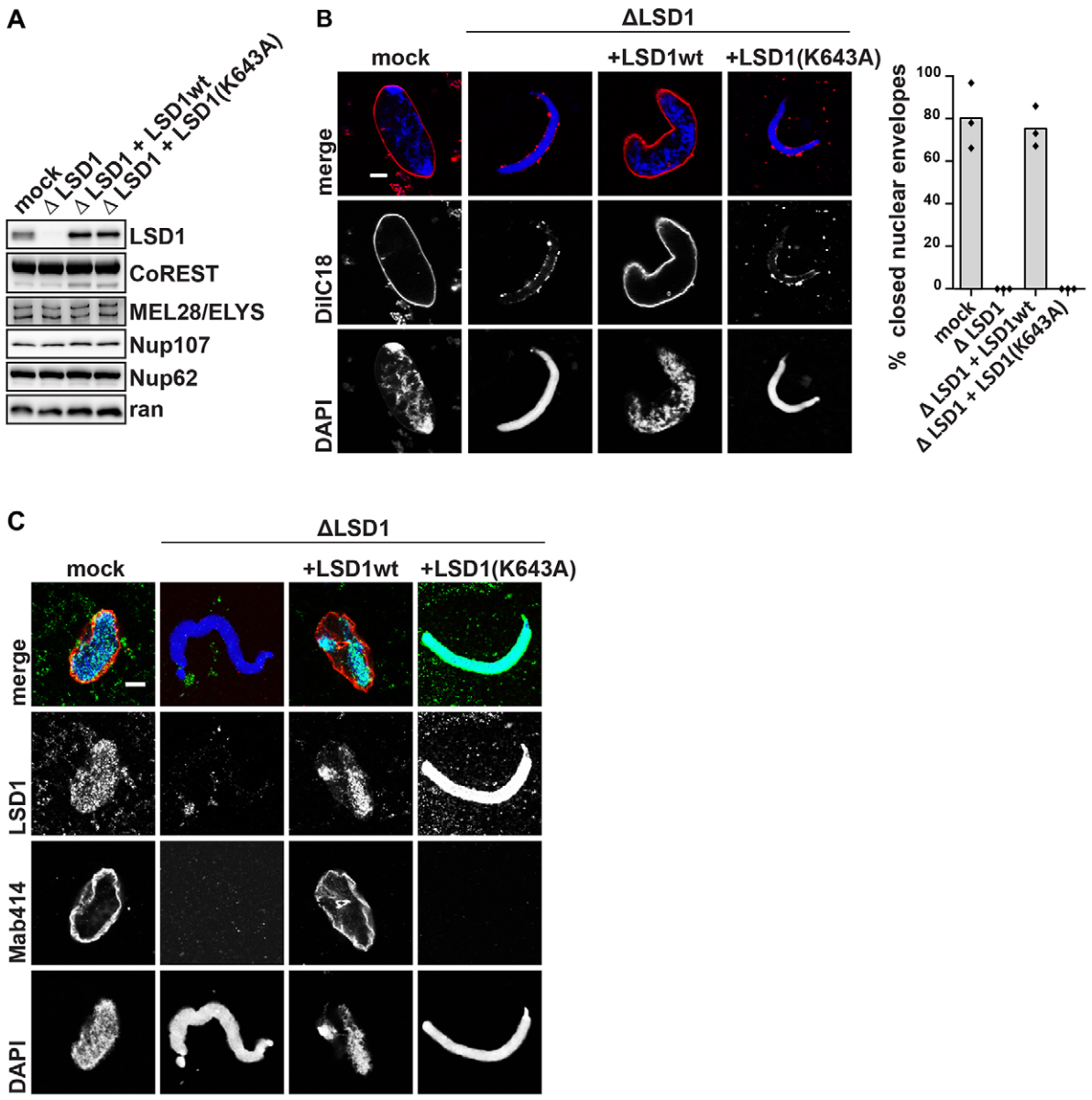

Fig. 2. Cell-free nuclear assembly requires the demethylase activity of LSD1. (A) Western blot analysis of mock-depleted, LSD1-depleted (ΔLSD1), and LSD1-depleted *Xenopus* egg extracts supplemented with either recombinant wild-type LSD1 (LSD1wt) or the catalytically inactive LSD1(K643A) mutant. (B) Confocal microscopy images of fixed nuclei assembled for 120 min in the indicated extract conditions. Membranes were pre-labelled with DiIC18 (red in overlay) and chromatin was stained with DAPI (blue in overlay). The mean percentage of closed nuclear envelopes for 100 randomly chosen chromatin substrates in each of three independent experiments is shown. Data points from individual experiments are indicated. (C) Nuclei assembled for 120 min in the indicated extract conditions were fixed and analyzed for the presence of immunolabelled LSD1 (green in overlay) and NPCs (mAB414, red in overlay) on the chromatin (DAPI, blue in overlay) by confocal microscopy. Scale bars: 5 µm.

mutating a lysine residue in the FAD-binding pocket, based on structural and functional studies of the human homologue (Stavropoulos et al., 2006). This catalytically inactive LSD1 mutant [a lysine residue exchanged to an alanine residue in position 643, LSD1(K643A)] failed to rescue the nuclear assembly defect (Fig. 2B,C), indicating that the demethylase activity of LSD1 is crucial for nuclear envelope and NPC assembly.

Loss of LSD1 in human cells extends telophase and promotes the formation of annulate lamellae

We performed immunofluorescence experiments in HeLa cells and found that LSD1 localized to the nucleus during interphase but was largely excluded from chromatin during mitosis (supplementary material Fig. S2). It could first be seen re-associating with chromatin early in telophase coincident with NPC formation, which was detected by mAB414 staining (supplementary material Fig. S2A,B), and prior to abscission, which was visualized by the loss of midbody-associated α-tubulin between the segregated chromatin masses (supplementary material Fig. S2C) (Guizetti et al., 2011). To assay the importance of LSD1 during mitotic exit in cells, we employed RNA interference (RNAi) and followed mitotic events by live-cell imaging. LSD1 was efficiently depleted by small interfering RNA (siRNA) for up to 72 h in HeLa cells stably expressing histone H2B fused to a mCherry reporter (Fig. 3A; supplementary material Fig. S3A). Cells were imaged over ∼20 h by time-lapse fluorescence microscopy and chromatin features were

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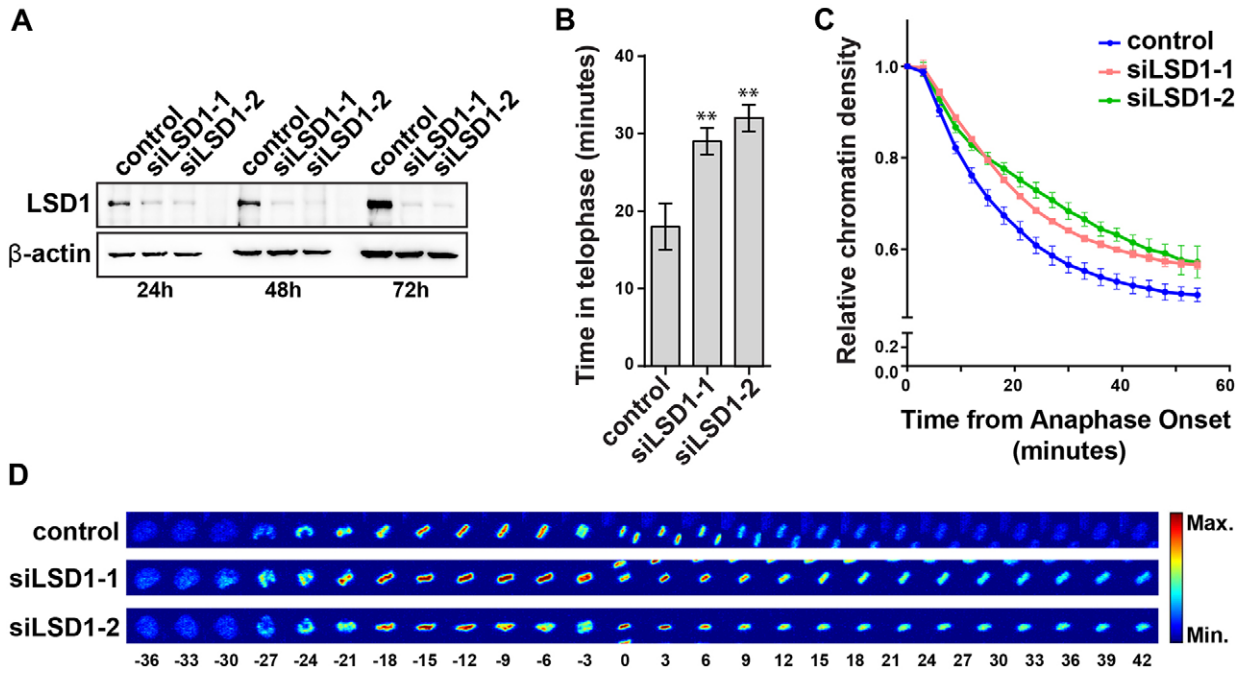

Fig. 3. LSD1 is required for the timely establishment of interphasic chromatin after mitosis in human cells. (A) Western blot analysis of whole-cell lysates from HeLa cells stably expressing H2B–mCherry and transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides as indicated. Lysates were harvested at 24, 48 and 72 h post-transfection. (B) HeLa cells stably expressing H2B–mCherry and transfected with 20 nM siRNA as indicated were subjected to time-lapse microscopy starting 30 h after transfection. Mitotic events were analyzed with CellCognition. The mean of the median time spent in telophase is plotted for more than 100 mitotic events per condition in three independent experiments. Error bars represent s.d. $^{**}P<0.01$ (Student's *t*-test). (C) The average fluorescence intensity of the H2B–mCherry signal was extracted from the CellCognition data acquired in B as an indication of chromatin density. The density was normalized to the first anaphase frame in individual mitotic tracks and the mean relative density for more than 100 mitotic events per condition from three independent experiments is plotted over time. Error bars represent s.d. (D) HeLa cells stably expressing H2B–mCherry and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting at 30 h after transfection. Maximum intensity projections of the mCherry signal from five optical *z* sections are represented as heat maps. Mitotic track positions are normalized to the first anaphase frame.

annotated and tracked during mitotic events using the image analysis software CellCognition (Schmitz and Gerlich, 2009; Held et al., 2010). Based on the morphological annotation of chromatin, LSD1-depleted cells displayed a significant extension in the length of telophase (Fig. 3B). In the absence of LSD1, cells maintained condensed telophasic chromatin for 10–15 min beyond control cells, which spent an average of 18 min in telophase. Accordingly, the progressive decrease in chromatin density that occurs upon mitotic exit, based on the average fluorescence intensity of the H2B–mCherry signal, was delayed and incomplete in LSD1-depleted cells compared to controls (Fig. 3C). This extended duration of chromatin compaction following chromosome segregation could be clearly visualized in individual mitotic tracks (Fig. 3D). Although one LSD1-targeting siRNA significantly extended the duration of metaphase, the timing of other cell cycle stages was not affected by LSD1 depletion (supplementary material Fig. S3B,G) and a longer metaphase was not a prerequisite for extended chromatin compaction after anaphase (see, for example, Fig. 3D). In order to confirm that mitotic exit is delayed in the absence of LSD1, mitotic spindle dynamics were tracked in live cells stably expressing EGFP-tagged α-tubulin and mCherry–H2B. In addition to prolonged telophasic chromatin features (supplementary material Fig. S3E,F), LSD1-depleted cells maintained midbody-associated tubulin for an average of 15–30 min longer than controls, indicating that abscission, the final step in cytokinesis, is delayed (supplementary material Fig. S3I). Taken together, these data suggest that the role of LSD1 during mitosis is specific to the events occurring in telophase. Consistent with this, addition of the LSD1-targeting inhibitors 2-PCPA and S2101 to HeLa cells similarly prolonged telophase (supplementary material Fig. S4A,B).

In addition to extended telophase, the average nuclear volume in unsynchronized populations of LSD1-knockdown cells was significantly reduced compared to control populations (Fig. 4A; supplementary material Fig. S3C). This observation suggests that LSD1 depletion has a lasting effect on chromatin compaction during interphase. In order to further investigate the impact of LSD1 knockdown on nuclear architecture in interphasic cells, we employed immunofluorescent labelling of various nuclear envelope and NPC proteins. Labelling with mAB414 revealed that in addition to NPCs in the nuclear envelope, LSD1-depleted cells (Fig. 4B) or cells treated with chemical inhibitors of LSD1 (supplementary material Fig. S4C) possessed significant cytoplasmic staining for NPCs indicative of annulate lamellae. These arrays of NPCs inserted into membrane stacks of the endoplasmic reticulum (Kessel, 1992) have been found to form when post-mitotic NPC assembly is impaired (Franz et al., 2007). We counted NPCs, based on the mAB414 signal, and found a reduction in their total number at the nuclear envelope when LSD1 was depleted, although NPC density was unaffected (Fig. 4C,D).

Despite the reduction in nuclear volume and the number of NPCs at the nuclear envelope, LSD1-depleted cells were not defective in nuclear lamina assembly, as shown by the smooth rim staining of lamins A and B (Fig. 4E). Similarly, the inner nuclear envelope proteins lamin B receptor (LBR) and emerin did not seem to be affected by LSD1 knockdown and were found at the nuclear envelope. The nucleoporins Nup62, of the central channel, Nup153, from the nucleoplasmic NPC face, and Nup88, from the cytoplasmic face of the NPC, were all found at the nuclear rim in LSD1-depleted cells. Furthermore, MEL28, required for the initial

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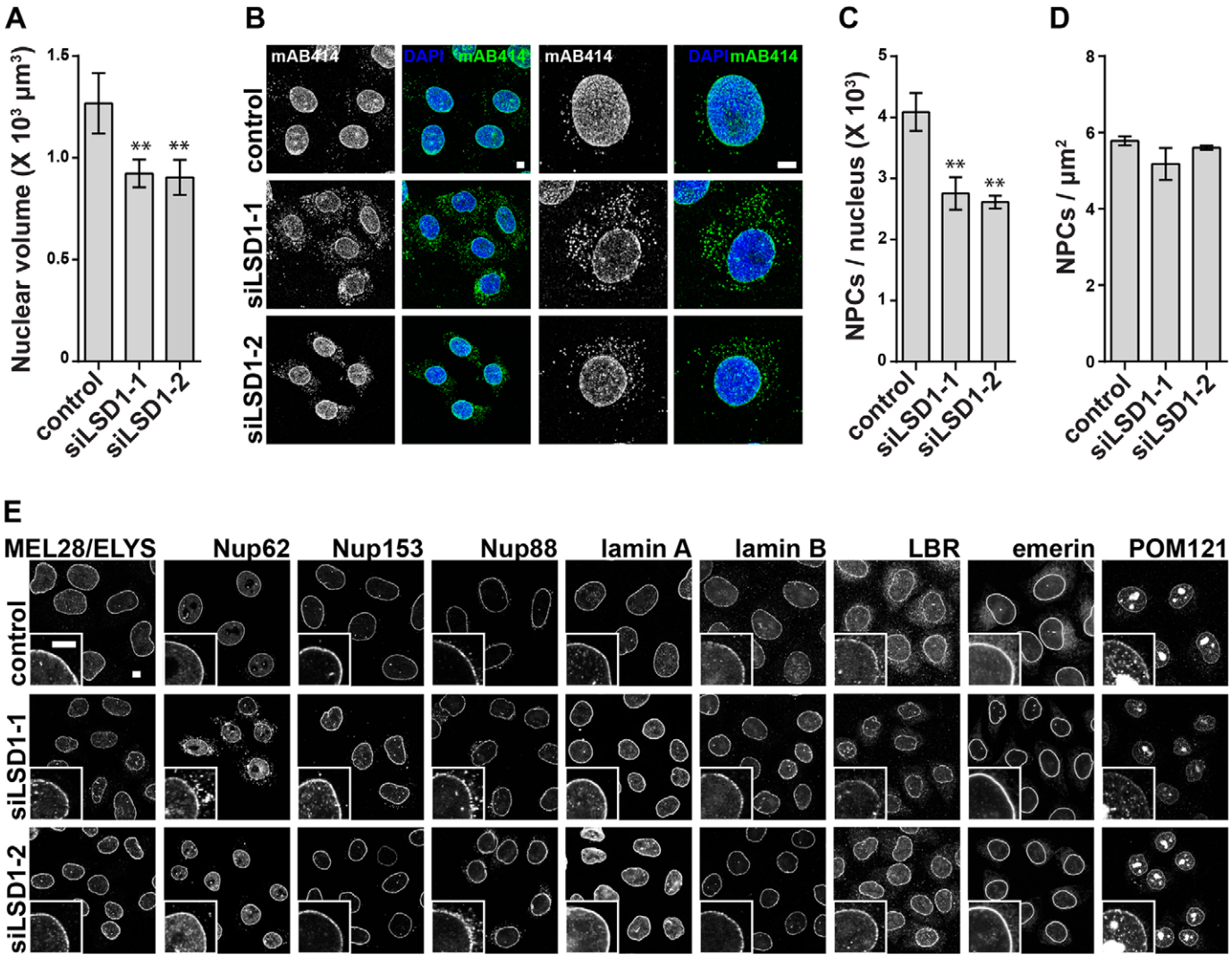

Fig. 4. Loss of LSD1 in human cells affects nuclear volume and NPC assembly. (A) Quantification of nuclear volume based on DAPI staining in HeLa cells transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides and fixed at 48 h post-transfection. The average nuclear volume from more than 80 nuclei per condition in three independent experiments is plotted. Error bars represent s.d. $^{**}P<0.01$ (Student's *t*-test). (B) HeLa cells transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides were fixed at 48 h post-transfection and processed for immunofluorescence. NPCs were immunolabelled using mAB414 (green in overlay) and DNA was stained with DAPI (blue). Maximum intensity projections are shown. (C) Quantification of mAB414-labelled NPCs in HeLa cells transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides and fixed at 48 h post-transfection. The number of NPCs over a specified surface area was counted and was used to calculate the total number per nucleus based on the nuclear volume. The mean number of NPCs per nucleus for 10 nuclei per condition in three independent experiments is plotted. Error bars represent s.d. $^{**}P<0.01$ (Student's *t*-test). (D) Quantification of NPC density in HeLa cells transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides and fixed at 48 h post-transfection. The average number of mAB414-labelled NPCs over a specified surface area was counted and the mean NPC density for 10 nuclei per condition in three independent experiments is plotted. Error bars represent s.d. (E) HeLa cells transfected with 20 nM control, siLSD1-1 or siLSD1-2 siRNA oligonucleotides were fixed at 48 h post-transfection and processed for immunofluorescence. Nuclear envelope proteins were immunolabelled as indicated and representative images are shown. Insets provide a higher magnification view. Scale bars: 5 µm.

seeding of NPC assembly on post-mitotic chromatin, and POM121, a transmembrane nucleoporin, could also be detected at the nuclear envelopes of LSD1-depleted nuclei.

Taken together, these data suggest that at the end of mitosis LSD1 is required to reestablish interphasic nuclear architecture that is conducive to nuclear expansion and NPC assembly.

LSD1 is not essential for chromatin decondensation

Nuclear reformation at the end of mitosis involves the almost simultaneous decondensation of highly compacted mitotic chromatin and assembly of a closed NPC-containing nuclear envelope (Schooley et al., 2012). Although difficult to functionally discern in a cellular context, these two integral mitotic exit processes can be biochemically separated *in vitro*. Given the apparent compaction of chromatin in both cell-free nuclear assemblies (Fig. 1A and Fig. 2B) and in HeLa cells (Fig. 3C,D) when LSD1 function was impaired, we wondered whether LSD1 had a direct role in chromatin decondensation.

The initial decondensation of sperm chromatin, the DNA template employed in our cell-free nuclear assembly assays (Figs 1 and 2), in *Xenopus* egg extracts occurs because of the exchange of sperm-specific protamines by the maternal histones H3 and H4 (Philpott and Leno, 1992). We tested whether this specialized decondensation event requires LSD1 by incubating

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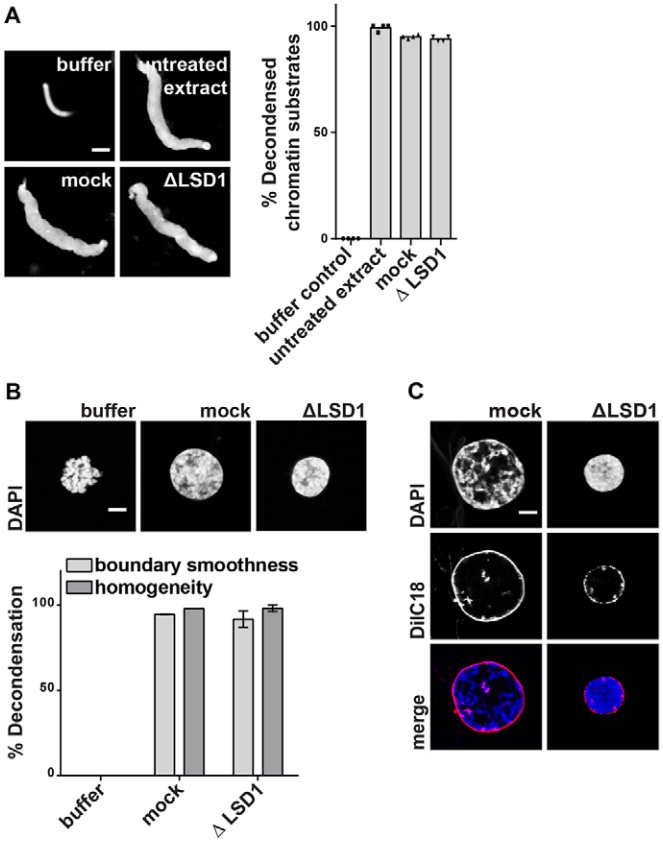

Fig. 5. LSD1 is not required for the membrane-independent decondensation of chromatin *in vitro*. (A) *Xenopus laevis* sperm chromatin heads were incubated in *Xenopus* egg extracts for 10 min, fixed and analysed by confocal microscopy. Chromatin was stained with DAPI. The mean percentage of decondensed chromatin substrates for 100 randomly chosen templates in each of four independent experiments is shown. Data points from individual experiments are indicated. (B) Mitotic chromatin clusters from HeLa cells were incubated with buffer, or mock or LSD1-depleted *Xenopus* egg extracts in the absence of added membranes. After 120 min, samples were fixed, stained with DAPI and analysed by confocal microscopy. The smoothness of the chromatin boundary (light grey) and the homogeneity of DAPI staining (dark grey) were analysed as described previously (Magalska et al., 2014). The mean percentage decondensation is plotted for 10 chromatin substrates per condition in each of three independent experiments. Error bars represent s.d. (C) Mitotic chromatin clusters from HeLa cells were incubated in mock or LSD1-depleted egg extracts in the presence of DiIC18-labelled membranes (red in overlay). Samples were fixed after 120 min, stained with DAPI (blue in overlay), and analysed by confocal microscopy. Scale bars: 5 µm.

sperm chromatin in egg extracts lacking membranes and an energy-regenerating system for 10 min. Under these conditions, histone exchange occurs and results in sperm chromatin decondensation (Fig. 5A, compare buffer to untreated extract) but nuclear assembly cannot proceed. Depletion of LSD1 did not affect sperm chromatin decondensation (Fig. 5A). Thus, the block in cell-free nuclear assembly in the absence of LSD1 activity (Figs 1 and 2) is not due to a defect in sperm DNA de-compaction.

Sperm DNA decondensation is mechanistically distinct from the decondensation of highly compacted chromatin at the end of mitosis, and we have recently established a cell-free assay to specifically analyze the latter process (Magalska et al., 2014). To this end, we examined changes to the topology of mitotic chromatin clusters isolated from HeLa cells when incubated with *Xenopus* egg extracts. In this modified version of the nuclear assembly assay, the highly condensed mitotic chromatin has been found to progressively decondense in a way that is morphologically analogous to the chromatin of cells exiting mitosis. These structural changes can be quantified based on the smoothness of the chromatin boundary and the homogeneity of DAPI staining, both increasing over time as chromatin decondenses. In the absence of membranes, depletion of LSD1 from the egg extracts had an apparently modest effect on chromatin decondensation, which was not quantifiably different based on the parameters described (Fig. 5B).

In the presence of membranes, mitotic chromatin clusters not only decondense but also support the formation of a closed nuclear envelope containing NPCs that are competent for nuclear import (Magalska et al., 2014). Accordingly, the addition of membranes to the extracts results in larger nuclei that accommodate further chromatin decondensation (Philpott et al., 1991; Wright, 1999) also known as secondary decondensation or nuclear swelling (Fig. 5C; supplementary material Fig. S4D, mock and untreated). However, when LSD1 activity was blocked by the addition of chemical inhibitors or its specific immuno-depletion, minimal membrane recruitment occurred and chromatin did not undergo any additional decondensation. Consistent with the nuclear assembly assay on sperm chromatin (Figs 1 and 2), blocking LSD1 activity impaired nuclear envelope formation on the HeLa chromatin substrate. These data suggest that LSD1 is not required for chromatin decondensation, at least the steps that occur *in vitro* in the absence of a closed nuclear envelope, but rather to render post-mitotic chromatin competent for the recruitment and assembly of nuclear envelope components.

LSD1 demethylase activity mediates the recruitment of nuclear envelope membranes and MEL28 to chromatin *in vitro*

To confirm that LSD1 plays a role in the recruitment of nuclear envelope membranes to chromatin we employed DNA-coated magnetic beads (Heald et al., 1996). DNA was chromatinized by incubation with *Xenopus* egg extract cytosol and incubated with the same membrane fractions as used in cell-free nuclear assembly reactions. Chromatinization resulted in the appearance of chromatin-binding proteins, including Ku70 (also known as XRCC6) and LSD1 (Fig. 6A), on the DNA beads. The membranes isolated from egg extracts are a mixture of different nuclear envelope precursor and ER vesicles. These include the early chromatin-binding fraction containing POM121 and NDC1, the late chromatin-binding fraction containing GP210, and a bulk ER vesicle population containing, among many other proteins, reticulon 4 (RTN4) and calnexin. Each of these membrane proteins was recruited to DNA beads chromatinized in mock-treated cytosol. However, when DNA was chromatinized in LSD1-depleted cytosol, we observed a significant reduction in the recruitment of POM121- and NDC1-containing vesicles (to 26% or 41%, respectively, Fig. 6B). The recruitment of both GP210 and ER-marker-containing vesicles (RTN4 and calnexin) was unaffected by LSD1 depletion, suggesting that membrane recruitment was not generally affected. Addition of recombinant wild-type LSD1 but not the demethylase deficient K643A mutant to LSD1-depleted extracts rescued the recruitment of POM121 and NDC1. Importantly, binding of MEL28, which is required for initiation of NPC assembly on the decondensing chromatin, was also reduced upon LSD1 depletion (to 50%) and rescued by the recombinant wild-type protein. The levels of dimethylated H3K4 were elevated by more than twofold upon LSD1 depletion or addback of the catalytically dead K643A mutant, validating H3K4me2 as an LSD1 substrate in

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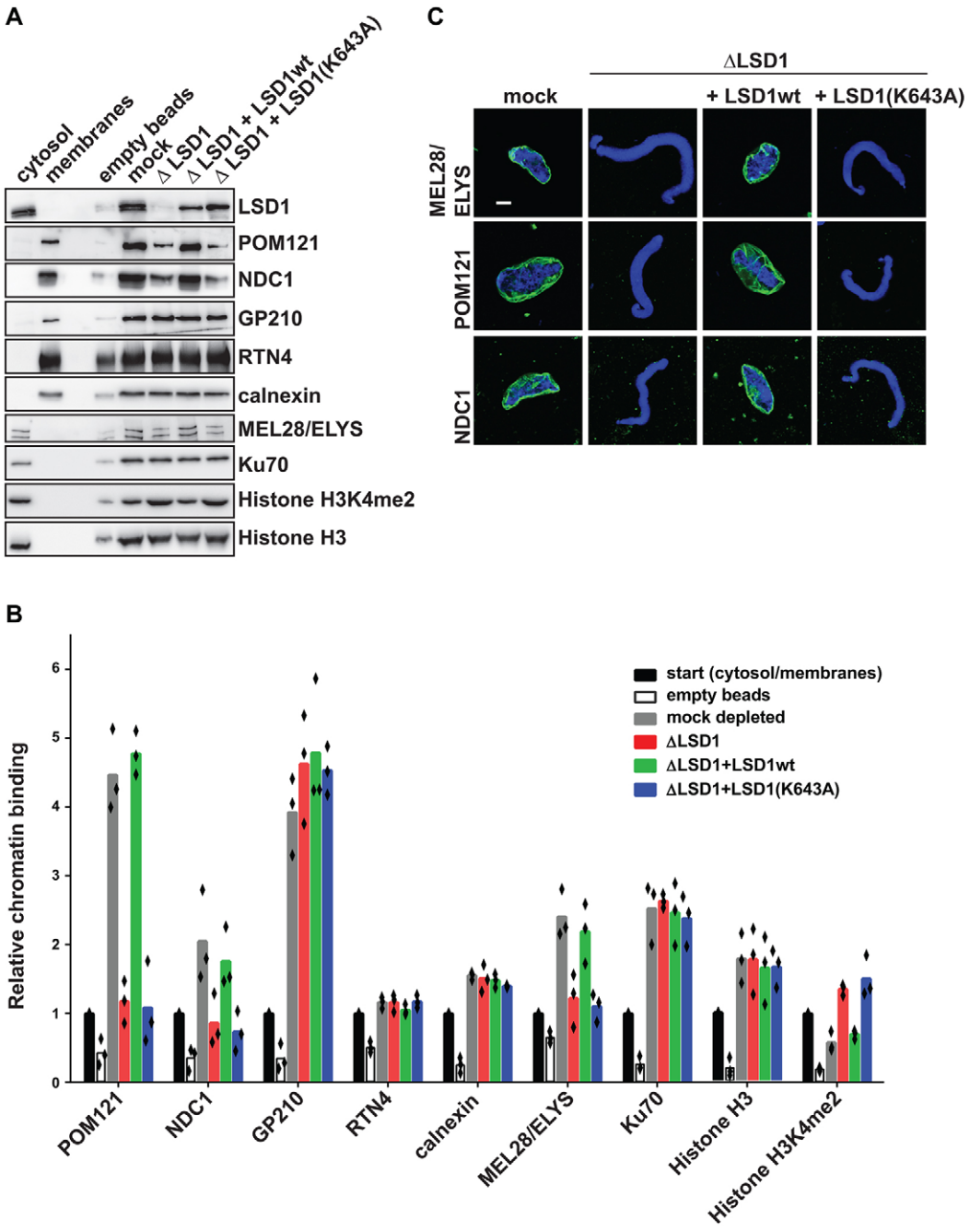

Fig. 6. The recruitment of MEL28, and POM121- and NDC1-containing membrane vesicles to chromatin *in vitro* depends on LSD1. (A) Linearized plasmid DNA immobilized on magnetic beads was chromatinized for 3 h in mock, LSD1-depleted or LSD1-depleted *Xenopus* egg extract cytosol supplemented with either wild-type LSD1 (LSD1wt) or the catalytically inactive LSD1(K643A) mutant, as indicated. After re-isolation, beads were washed and incubated a second time with cytosol treated as before and floatation-purified egg extract membranes. Bead-bound material was re-isolated after 1 h, washed and analysed by western blotting. For comparison, equal amounts (corresponding to the second incubation) of mock-treated extracts and membranes were analysed. Magnetic beads without DNA (empty beads) were used to estimate the background level of binding of extract components to the beads. (B) Quantification of proteins re-isolated with chromatinized DNA beads performed as in A. The mean intensity from three independent experiments normalized to the start material (cytosol or membranes) is plotted. Individual data points are indicated. (C) Nuclei assembled for 120 min in the indicated extracts conditions were fixed and analysed for the presence of MEL28, POM121 and NDC1 (green in overlay) by immunofluorescence and confocal microscopy. Chromatin is stained with DAPI (blue). Scale bar: 5 µm.

Xenopus egg extracts and on chromatinized DNA beads. Consistent with reduced recruitment to DNA beads, nuclei assembled *in vitro* on sperm chromatin in the absence of functional LSD1 lack MEL28, POM121 and NDC1 (Fig. 6C). These data indicate that the efficient recruitment of both MEL28, and POM121- and NDC1-containing nuclear envelope precursor vesicles to chromatin is dependent on LSD1 activity and suggest that LSD1 functions in nuclear assembly by regulating the chromatin-dependent recruitment of nuclear envelope and pore complex proteins at the end of mitosis.

DISCUSSION

The restoration of interphasic nuclear architecture at the end of mitosis requires massive reorganization of the condensed mitotic chromatin and the coordinated reassembly of a functional nuclear envelope. We

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have found that the catalytic activity of LSD1 plays a crucial role in nuclear formation on post-mitotic chromatin. Interfering with the function of LSD1 either by inhibition or by depletion inhibits cell-free nuclear assembly by blocking chromatin recruitment of MEL28, and POM121- and NDC1-containing membranes. In human cells, reduction of LSD1 by siRNA-mediated depletion severely extends telophase and results in significantly smaller nuclei as well as ectopic NPC formation in the cytoplasm. Our data suggest that in addition to dephosphorylation events and the action of the small GTPase Ran (Hetzer et al., 2002), histone demethylation plays a crucial role in post-mitotic nuclear envelope and pore complex formation.

Mitotic histone modifications are crucial both for marking the interphasic transcriptional state of the chromatin (for a review, see Moazed, 2011; Wang and Higgins, 2013) and for chromosome segregation during anaphase (Kawashima et al., 2010; Yamagishi et al., 2010). The most prominent of these modifications is the phosphorylation of histone H3 at T3, T11, S10 and S28 by the kinases Aurora B and haspin. These marks are removed during mitotic exit by the phosphatase PP1, which is recruited to anaphase chromatin by its targeting cofactor RepoMan (also known as CDCA2) (Trinkle-Mulcahy et al., 2006) and mKI67 (Booth et al., 2014; Takagi et al., 2014). Although it has been recently proposed to be upstream of a histone modification cascade that promotes mitotic chromosome condensation (Wilkins et al., 2014), phosphorylation of histone H3 at S10 is dispensable for chromosome condensation (Hsu et al., 2000; MacCallum et al., 2002) and to date there is no evidence to suggest that the reversal of histone phosphorylation events are specifically required for chromatin decondensation at the end of mitosis (Magalska et al., 2014). Instead, chromatin remodelling downstream of histone dephosphorylation has been linked to nuclear envelope assembly by promoting the recruitment of LBR through heterochromatin protein 1β (HP1β) (Ye et al., 1997; Haraguchi et al., 2000; Fischle et al., 2005) and importin-β-bound nucleoporins through RepoMan (Vagnarelli et al., 2011). The results presented here indicate that demethylation of histone H3 plays a comparable role in the regulation of nuclear reassembly.

Our data suggest that LSD1-mediated demethylation is required for the recruitment of MEL28 and nuclear envelope precursor vesicles, enriched for POM121 and NDC1, to chromatin *in vitro*. In the absence of LSD1, MEL28, and POM121- and NDC1-containing vesicles were not efficiently recruited to chromatinized DNA beads nor were they found on sperm chromatin in 2-h nuclear assembly reactions. These defects were rescued by the addition of recombinant catalytically active LSD1 (Fig. 6). Both MEL28, and POM121- and NDC1-containing vesicles are crucial for NPC assembly (Antonin et al., 2005; Mansfeld et al., 2006; Franz et al., 2007). During mitosis, MEL28 is localized to kinetochores (Rasala et al., 2006). Although it is considered the seeding point for NPC assembly on chromatin (Franz et al., 2007; Gillespie et al., 2007), the precise mechanism governing the redistribution of MEL28 on post-mitotic chromatin is currently unknown. Our data suggest that the proper localization of MEL28 at the end of mitosis depends on changes to chromatin that occur downstream of LSD1 activity. As the recruitment of POM121- and NDC1-containing vesicles to chromatin during nuclear assembly relies on the presence of MEL28 (Rasala et al., 2008), it is likely that the block in nuclear envelope precursor vesicle recruitment we observe occurs, at least partially, upstream of MEL28. However, nuclear envelope precursor vesicles crucial to *in vitro* nuclear assembly, including POM121-containing vesicles, have been found to bind to DNA in the absence of MEL28 (Ulbert et al., 2006). Furthermore, a soluble fragment of POM121 can competitively block nuclear assembly *in vitro* without disrupting the recruitment of MEL28, and in turn the Nup107–Nup160 complex, owing to distinct binding sites on chromatin (Shaulov et al., 2011). Finally, nuclei assembled in the absence of MEL28 form pore-free, albeit closed, nuclear envelopes (Franz et al., 2007), which we did not observe upon LSD1 depletion. Taken together, the nuclear assembly defect we observe in the absence of LSD1 activity is most likely the result of a block in the recruitment of multiple factors that interact independently with chromatin in the early stages of mitotic exit.

Reduction of LSD1 by RNAi significantly extended telophase in HeLa cells. The interphasic nuclei were significantly smaller and we observed a reduction in the total number of NPCs assembled at the nuclear envelope. Because NPC density was not affected by the loss of LSD1 (Fig. 4D), we cannot be certain whether the smaller LSD1-depleted nuclei are specifically defective for NPC formation or rather for nuclear envelope membrane expansion. However, substantial annulate lamellae formation, which is ectopic NPC assembly in the cytoplasm, was also found in LSD1-knockdown cells. Annulate lamellae are typically observed when interfering with NPC re-assembly at the end of mitosis, for example upon MEL28 depletion (Walther et al., 2003b; Franz et al., 2007), suggesting that LSD1 activity is upstream of NPC assembly on chromatin. The relatively modest effect on nuclear envelope assembly in cells could be explained by a lower efficiency of LSD1 depletion compared to the *in vitro* experiments. Although we observed a similar phenotype upon chemical blockade of LSD1 activity (supplementary material Fig. S4A–C), these experiments are difficult to interpret because of predominant cell mortality at the effective concentrations. Alternatively, the role of LSD1-mediated demethylation in post-mitotic nuclear assembly might be partially redundant in HeLa cells. Chemical blockade of LSD1 activity has been found to preferentially inhibit the growth of pluripotent cell types in culture but to have very little impact on the growth rate of HeLa cells (Wang et al., 2011).

The *in vitro* assays employed in our study allowed us to separately query the role of LSD1 in chromatin decondensation and nuclear envelope assembly, events that occur simultaneously in cells. Sperm-chromatin-specific decondensation, an event that occurs immediately after fertilization due to the nucleoplasmin-dependent exchange of sperm protamines for maternal histones (Philpott et al., 1991), was not hindered in LSD1-depleted extracts (Fig. 5A). In uninhibited extracts, decondensed sperm chromatin is the substrate for the assembly of a closed nuclear envelope containing NPCs. Once a functional envelope has formed, the import of nuclear proteins leads to an increase in nuclear volume, a process that is also referred to as nuclear swelling or expansion, or secondary decondensation (Philpott et al., 1991; Wright, 1999). A loss of LSD1 activity, either upon immuno-depletion or after chemical inhibition, blocked nuclear envelope and pore complex formation on the sperm chromatin. In the absence of nuclear envelope formation and the establishment of nuclear import, secondary decondensation could not occur and thus the nuclei assembled appeared small and condensed (Figs 1 and 2). Nevertheless, this should be not misinterpreted as a defect in chromatin decondensation. Similarly, blocking LSD1 activity did not significantly impair the decondensation of mitotic chromatin clusters from HeLa cells in the absence of membranes (Fig. 5B; supplementary material Fig. S4D, top panel). Upon addition of membranes, extracts in which LSD1 activity was removed or blocked gave rise to strikingly smaller nuclei compared to relevant controls (Fig. 5C; supplementary material Fig. S4D, bottom panels).

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This size discrepancy can be attributed to a loss of secondary decondensation in the absence of a closed and functional nuclear envelope when LSD1 activity is blocked. Our data therefore indicate that chromatin decondensation per se is not dependent on LSD1. Nonetheless, loss of LSD1 activity resulted in smaller nuclei in both our membrane-free decondensation assay (Fig. 5B; supplementary material Fig. S4D) and in HeLa cells (Fig. 4A; supplementary material Fig. S3C and Fig. S4C), suggesting that LSD1 plays a role in reestablishing interphasic chromatin organization at the end of mitosis. Considering its impact on nuclear envelope formation, we postulate that LSD1 is required to generate post-mitotic chromatin that is competent for proper nuclear envelope and NPC assembly.

LSD1 is a nuclear amine oxidase that catalyses the FAD-dependent demethylation of histone H3 at lysine residues 4 and 9 (Shi et al., 2004, 2005; Forneris et al., 2005). It acts mainly as a transcriptional repressor (Shi et al., 2004, 2005; Lee et al., 2005) by demethylating H3K4me2, a mark of active transcription (reviewed in Black et al., 2012). However, it has also been found to demethylate repressive H3K9me2 marks and activate the ligand-dependent transcription of androgen-receptor-responsive genes (Metzger et al., 2005; Wissmann et al., 2007). Importantly, demethylation of histone H3 by LSD1 is locus specific, although the mechanistic details governing this specificity are currently unknown. Consistent with its repressive role, LSD1 has been implicated in heterochromatin formation and maintenance in *Drosophila* and *S. pombe* (Lan et al., 2007; Rudolph et al., 2007).

In the context of mitotic exit, it is not clear how LSD1-dependent demethylation primes chromatin for nuclear envelope assembly and this will be an interesting avenue for future research. Because the catalytic activity of LSD1, and not simply its presence on the chromatin, was required for cell-free nuclear assembly (Fig. 2), it is unlikely that LSD1 itself acts as a scaffold for the recruitment of nuclear envelope proteins at the end of mitosis. Instead, LSD1-mediated demethylation might result in an accumulation of specific histone marks that facilitate the recruitment and assembly of envelope components. Indeed, we observed a twofold increase in dimethylated H3K4 upon LSD1 depletion or re-addition of the catalytically inactive mutant on chromatin beads (Fig. 6A,B). However, the contribution of other histone modifications such as methylated H3K9, another LSD1 target, should not be excluded. It is equally possible that in modulating chromatin organization downstream of histone H3 demethylation, either globally or at the chromatin surface, LSD1 promotes a state of chromatin that is competent for nuclear envelope assembly. Finally, LSD1 has been found to demethylate other chromatin-associated proteins including p53 and DNMT1 (Huang et al., 2007; Wang et al., 2009) and it is certainly possible that its role in nuclear envelope assembly is due to demethylation of non-histone substrates.

LSD1 was previously found to localize to the mitotic spindle and to function in chromosome segregation in dividing HeLa cells (Lv et al., 2010). In our live-cell imaging experiments, we observed a tendency towards extended metaphase in LSD1-depleted cells (supplementary material Fig. S3B,G), which could be indicative of spindle-related defects in chromosome alignment. However, we did not observe a significant increase in the number of lagging chromosomes or chromosome bridges in the siRNA-treated cells (data not shown). We found that LSD1 was excluded from chromatin starting at prometaphase and re-associated with chromatin during telophase, concomitant with mAB414 staining and, presumably, nuclear envelope formation but prior to abscission (supplementary material Fig. S2). A similar dissociation of LSD1 from mitotic chromatin has also been observed in mouse embryonic stem cells (Nair et al., 2012). LSD1 is phosphorylated during mitosis (Lv et al., 2010), and it is possible that this phosphorylation controls its association with chromatin. Alternatively, dynamic histone modifications could be responsible for the cell-cycle-dependent localization of LSD1. For example, phosphorylation of histone H3 at serine 10 blocks the binding of LSD1 to a synthetic H3 peptide *in vitro* (Forneris et al., 2005).

The mitotic-dependent dissociation of LSD1 from chromatin modulates rapid gene expression changes in embryonic stem cells (Nair et al., 2012). LSD1 has also been found to maintain both the undifferentiated state and proliferative capacity of pluripotent cells, which express relatively high levels of LSD1 (Sun et al., 2010; Adamo et al., 2011; Yang et al., 2011; Nair et al., 2012). Here, we have identified a new transcription-independent role for LSD1 in the reestablishment of nuclear architecture following mitotic cell division. Whether the capacity for LSD1 to support nuclear envelope formation on post-mitotic chromatin plays a role in cancer, particularly in cancer stem cells where its expression is frequently mis-regulated (reviewed in Amente et al., 2013), remains a question for future research.

MATERIALS AND METHODS

Antibodies and chemicals

Commercial antibodies against the following proteins were used for immunofluorescence in HeLa cells: LSD1 (Abcam, ab17721), α-tubulin (Sigma, T6199), Nup62 (BD Biosciences, 610498), Nup153 (Abcam, SA1, ab96462), Nup88 (BD Biosciences, 611896), Lamin A (Abcam, ab26300), Lamin B2 [EPR9701(B), Abcam, ab151735], Lamin B receptor (Epitomics, 1398-1) and emerin (Sigma, HPA000609). The human MEL28 (Franz et al., 2007) and POM121 (Mansfeld et al., 2006) antibodies were kind gifts from Iain Mattaj (EMBL Heidelberg, Germany) and Ulrike Kutay (ETH Zurich, Switzerland), respectively. mAB414 (Covance, MMS-120R) was used for immunofluorescence in HeLa cells and on *in vitro* assembled nuclei. With the exception of the mAB414 (used to detect Nup62), Ku70 (H3H10, Abcam, ab3114) and CoREST (Millipore, 07-455) antibodies, which were employed for western blotting, antibodies raised against *Xenopus* proteins were employed in immunoblotting and immunofluorescence in assays based on *Xenopus* egg extract. Antibodies against *Xenopus laevis* GP210 (Antonin et al., 2005), NDC1 (Mansfeld et al., 2006), POM121 and RTN4 (Vollmer et al., 2015) have been described previously. For the anti-MEL28 antibody, a construct comprising amino acids 2290–2408 was expressed from a pET28a vector and used for antibody production in rabbits. The same was done for calnexin, using a construct comprising amino acids 516–606. The *Xenopus* anti-LSD1 antibody was generated in rabbits using full-length LSD1 expressed from a pET28a vector and used at 1:1000 as serum for western blotting both in *Xenopus* egg and HeLa cell extracts and 1:100 for immunofluorescence on nuclei assembled *in vitro*. For affinity purification of the antiserum in order to generate affinity resins for depletion experiments (see below) recombinant LSD1 was coupled to Affigel 10 (Biorad).

The LSD1 inhibitors Pargyline-HCL (Sigma), 2PCPA-H_2SO_4 (BPS Bioscience) and S2101 (Calbiochem) were diluted in water and stored at −20°C prior to use. DiIC18 and secondary antibodies (Alexa-Fluor-488-conjugated goat anti-rabbit-IgG and Cy3-conjugated goat anti-mouse-IgG antibodies) were obtained from Invitrogen.

Protein expression and purification

The construct for *Xenopus laevis* LSD1 (GenBank accession number KR078281) was generated from synthetic DNA optimized for codon usage in *E. coli* (Geneart). The K643A mutant was generated by mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent). Sequences were cloned into a modified pET28a vector containing an N-terminal yeast SUMO solubility tag followed by a TEV cleavage site. Proteins were expressed in *E. coli* and purified by Ni-affinity chromatography. His_6- and SUMO-tags were cleaved using tabacco etch virus (TEV) protease and proteins were concentrated using VIVASPIN columns (Sartorius) prior to

further purification by size exclusion chromatography (Superdex 200 10/300, GE healthcare) in sucrose buffer (250 mM sucrose, 50 mM KCl, 10 mM Hepes-KOH, 2.5 mM $MgCl_2$).

Cell-free nuclear assembly

Nuclear assemblies and immunofluorescence, including the preparation of high-speed interphasic extracts from *Xenopus laevis* eggs, generation of sperm heads and production of floated labelled and unlabelled membranes were performed as described in detail previously (Eisenhardt et al., 2014). Affinity resins used in depletion experiments were generated by crosslinking either purified unspecific rabbit IgG or affinity purified LSD1 IgG to protein-A–sepharose using 10 mM dimethyl pimelimidate (Thermo Scientific). LSD1 was immunodepleted by incubating the resin with egg cytosol in a ratio of 1:1 and rotating at 4°C for 25 min. Unbound cytosol was used immediately. Fluorescence images were acquired using a confocal microscope [FV1000; Olympus; equipped with a photomultiplier (model R7862; Hamamatsu)] using 405-, 488- and 559-nm laser lines and a 60× NA 1.35 oil immersion objective lens.

Cell-free chromatin decondensation

Isolation of mitotic clusters from HeLa cells, preparation of low-speed interphasic egg extracts and chromatin decondensation reactions were performed as described in detail previously (Magalska et al., 2014). Immunodepletion of LSD1 for chromatin decondensation was performed as for nuclear assembly reactions (above). Chromatin decondensation was quantified from confocal images of DAPI-stained nuclei based on two key parameters described previously (Magalska et al., 2014): roughness of the chromatin boundary (perimeter^2/area) and chromatin heterogeneity (the relative internal area occupied by prominent structures, which were recognized using the Fiji StructureJ plugin http://www.imagescience.org/meijering/). Smoothness and homogeneity were defined as the differences from the maximal roughness and maximal relative area, respectively. A maximum of 20% was adopted for the fully decondensed state in both analyses. The fully condensed state was set to zero.

For sperm DNA decondensation, 20 µl of *Xenopus laevis* egg extract was incubated with 0.3 µl sperm heads (3000 sperm heads per µl) for 10 min. Samples were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde on ice, stained with DAPI and analysed on a Axiovert 200 M fluorescence wide-field microscope (Zeiss). Images for both decondensation assays were acquired using a confocal microscope [FV1000; Olympus; equipped with a photomultiplier (model R7862; Hamamatsu)] using 405- and 559-nm laser lines and a 60× NA 1.35 oil immersion objective lens.

Cell culture experiments

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 500 units/ml penicillin-streptomycin (all from Gibco). HeLa cells stably expressing H2B–mCherry (generated as described in Schmitz et al., 2010) were maintained in DMEM additionally supplemented with 0.5 µg/ml puromycin (Gibco). The H2B–mCherry and tubulin–EGFP cell line (Held et al., 2010) was a kind gift from Daniel Gerlich (IMBA, Vienna) and was maintained in DMEM additionally supplemented with 0.5 µg/ml puromycin (Gibco) and 500 µg/ml G-418 (Geneticin; Life Technologies). The following siRNA oligonucleotides were employed in knockdown experiments: siLSD1-1 (AOF2_2), 5′-ACATCTTACCTTAGTCATCAA-3′, siLSD1-2 (AOF2-6), 5′-AGGCCTAGACATTAAACTGAA-3′, and AllStars negative control siRNA (all from Qiagen). Additionally, the following PP2A oligonucleotides were routinely used as a pool mixed 1:1:1 (Schmitz et al., 2010): 5′-GACCAGGATGTGGACGTCAAA-3′, 5′-CCAGGAUGUGGA-CGUCAAATT-3′ and 5′-UUUGACGUCCACAUCCUGGTC-3′ (Qiagen) (Schmitz et al., 2010). Reverse transfections of 20 nM siRNA were carried out in HeLa cell suspensions using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

HeLa cells were processed for immunofluorescence by fixation with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) on ice for 10 min prior to permeabilization with 2% Triton X-100 in blocking buffer (3% bovine serum albumine and 0.1% Triton X-100 in PBS). Where indicated, cells were permeabilized prior to fixation with 0.1% Triton-X 100 in a 60 mM PIPES, 20 mM HEPES, 10 mM EGTA, 4 mM $MgSO_4$ buffer (pH 7). Immunofluorescence staining was performed as described previously (Eisenhardt et al., 2014). Images were acquired using a confocal microscope (FV1000; Olympus), 405-, 488- and 559-nm laser lines, and a 60× NA 1.35 oil immersion objective lens. For Nup localization, the photomultiplier (model R7862; Hamamatsu) was employed. The 488-nm laser line and a GaAsP detector were employed to image NPCs for quantification. NPCs labelled with mAB414 were counted for a given surface area from maximum intensity projections comprising five 0.25-µm-spaced optical *z*-sections of the nuclear surface using the Fiji 3D Object Counter (http://fiji.sc/3D_Objects_Counter). The total nuclear volume was measured from the DAPI signal of 0.5 µm optical *z*-sections traversing the entire nucleus using the surpass volume function in IMARIS X64 7.6.3.

For live imaging experiments, HeLa cells expressing H2B–mCherry or both H2B–mCherry and α-tubulin–EGFP were transfected with siRNA oligonucleotides and seeded in eight-well µ-slide chambers (Ibidi). Starting at ~30 h post-transfection, cells were imaged using a Plan-Apochromat 10× NA 0.45 objective and a 561-nm diode laser on a LSM 5 live confocal microscope (Zeiss) equipped with a heating and CO_2 incubation system (Ibidi). ZEN software (Zeiss) was used to acquire images from five 7.5-µm-spaced optical *z*-sections at various *xy* positions every 3 min. Single position *.ome files were generated from the maximum intensity projections in ZEN and converted into image sequences with Fiji software. Segmentation, annotation, classification and tracking of cells progressing through mitosis were performed using the Cecog analyser (http://www.cellcognition.org/software/cecoganalyzer) based on the CellCognition platform (Held et al., 2010).

Chromatin recruitment assay

Coupling of genomic DNA in a linearized plasmid to magnetic M-280 Streptavidin beads (Invitrogen) was as described previously (Ulbert et al., 2006). Once coupled, DNA beads were blocked with 1% lipid-free bovine serum albumin in coupling buffer (2.5% polyvinylalcohol, 1 M NaCl, 50 mM Tris-HCl pH 8.0, 2 mM EDTA), rotating overnight at 4°C. DNA was chromatinized by incubation with high-speed interphasic *Xenopus* egg cytosol (DNA beads:cytosol=1:2.5), shaking at 350 rpm for 3 h at 20°C. Beads were washed once with sucrose buffer and tested for protein recruitment by further incubation with egg cytosol and 10× floated membranes [i.e. tenfold more concentrated than described previously (Eisenhardt et al., 2014)] purified from *Xenopus* eggs (DNA beads:cytosol:membranes=1:0.5:1), shaking at 350 rpm for 1 h at 20°C. After three washes with sucrose buffer, DNA beads were resuspended in Laemmli sample buffer and boiled. Supernatants were subjected to SDS-PAGE and transferred onto nitrocellulose for immunoblotting. Signal intensities were quantified using the Fusion Capt advance software.

Acknowledgements

We thank C. Liebig (Light Microscopy Facility of the MPI for Developmental Biology) for supporting image acquisition and analysis, Alexander Blässle for help with the generation and maintenance of the scripts used to automate the analysis of chromatin decondensation in various assay systems, and Adriana Magalska for helpful discussions.

Competing interests

The authors declare no competing or financial interests.

Author contributions

A.S. and W.A. designed and performed experiments and wrote the manuscript. D.M.-A. performed the live-cell experiments in H2B–mCherry and α-tubulin–EGFP cells and generally supported live-cell experiments and their analysis. B.V. expressed and purified recombinant LSD1. P.D.M. supported the preparation of *Xenopus* egg extracts.

Funding

This work was supported by core funding of the Max Planck Society; and the European Research Council (ERC) [grant number 309528 CHROMDECON to W.A.].

Supplementary material

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.173013/-/DC1

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Supplementary figures

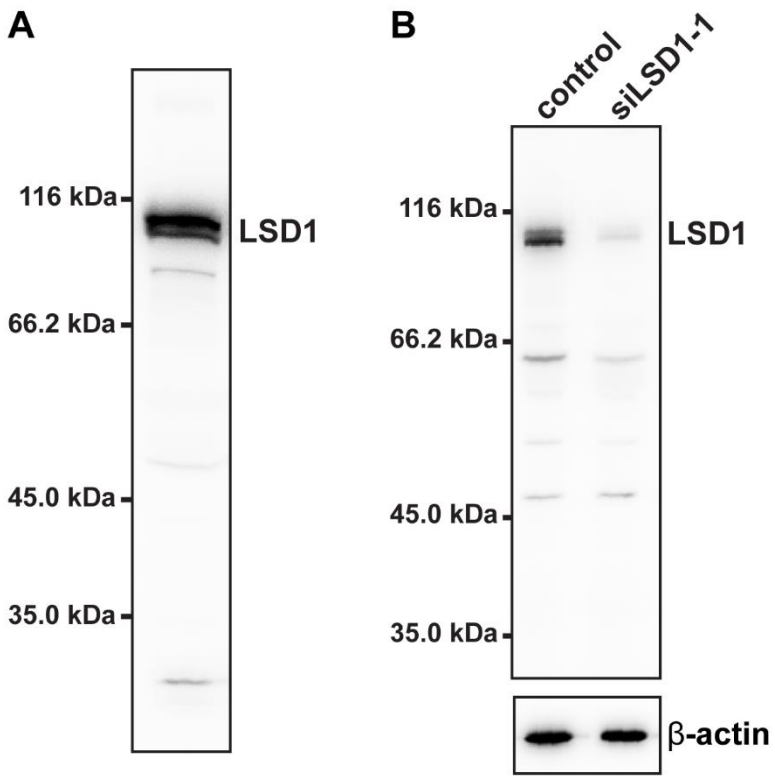


Fig. S1. Characterisation of a new *Xenopus laevis* LSD1 antibody

Antiserum generated in rabbits against the full length *Xenopus laevis* LSD1 protein was tested for specificity in immunoblots of lysates from *Xenopus* egg extracts (A) or HeLa cells (B) subjected to SDS-PAGE. Note the strong reduction of the human LSD1 signal upon RNAi mediated LSD1 downregulation.

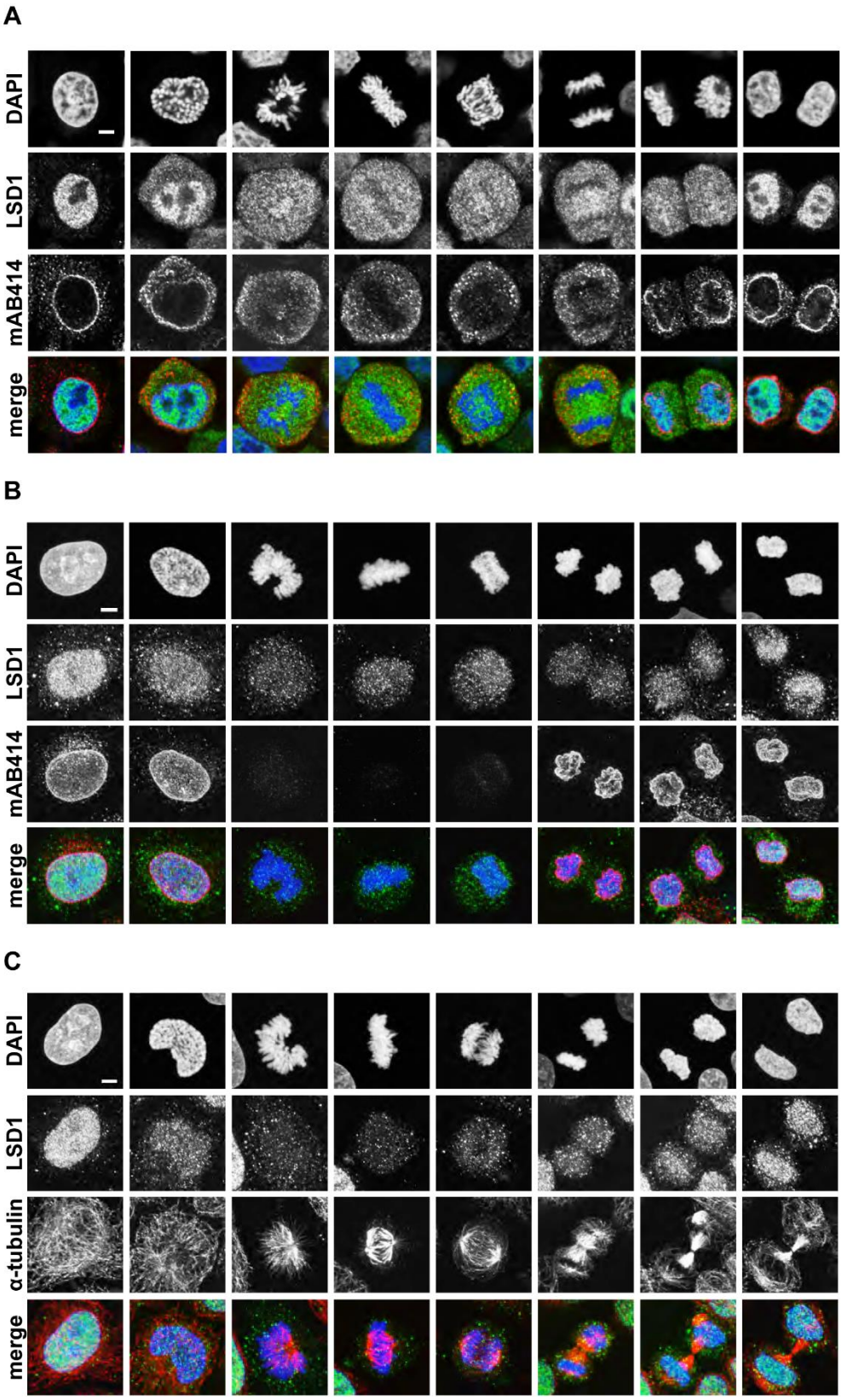


Fig. S2. LSD1 localisation in HeLa cells

(A) The cell cycle-dependent localization of LSD1 in unsynchronised HeLa cells was analyzed by immunofluorescence and confocal microscopy. LSD1 (green in overlay) and NPCs (mAB414, red) were immunolabeled and chromatin was stained with DAPI (blue).

(B) LSD1 (green) and NPCs (mAB414, red) immunolabeled in cells permeabilized with 0.1% Triton-X 100 prior to fixation. Chromatin was stained with DAPI (blue). Maximum intensity projections are shown.

(C) LSD1 (green) and α-tubulin (red) were immunolabeled in cells permeabilized with 0.1% Triton-X 100 prior to fixation. Chromatin was stained with DAPI (blue). Maximum intensity projections are shown.

Scale bars: 5 μm

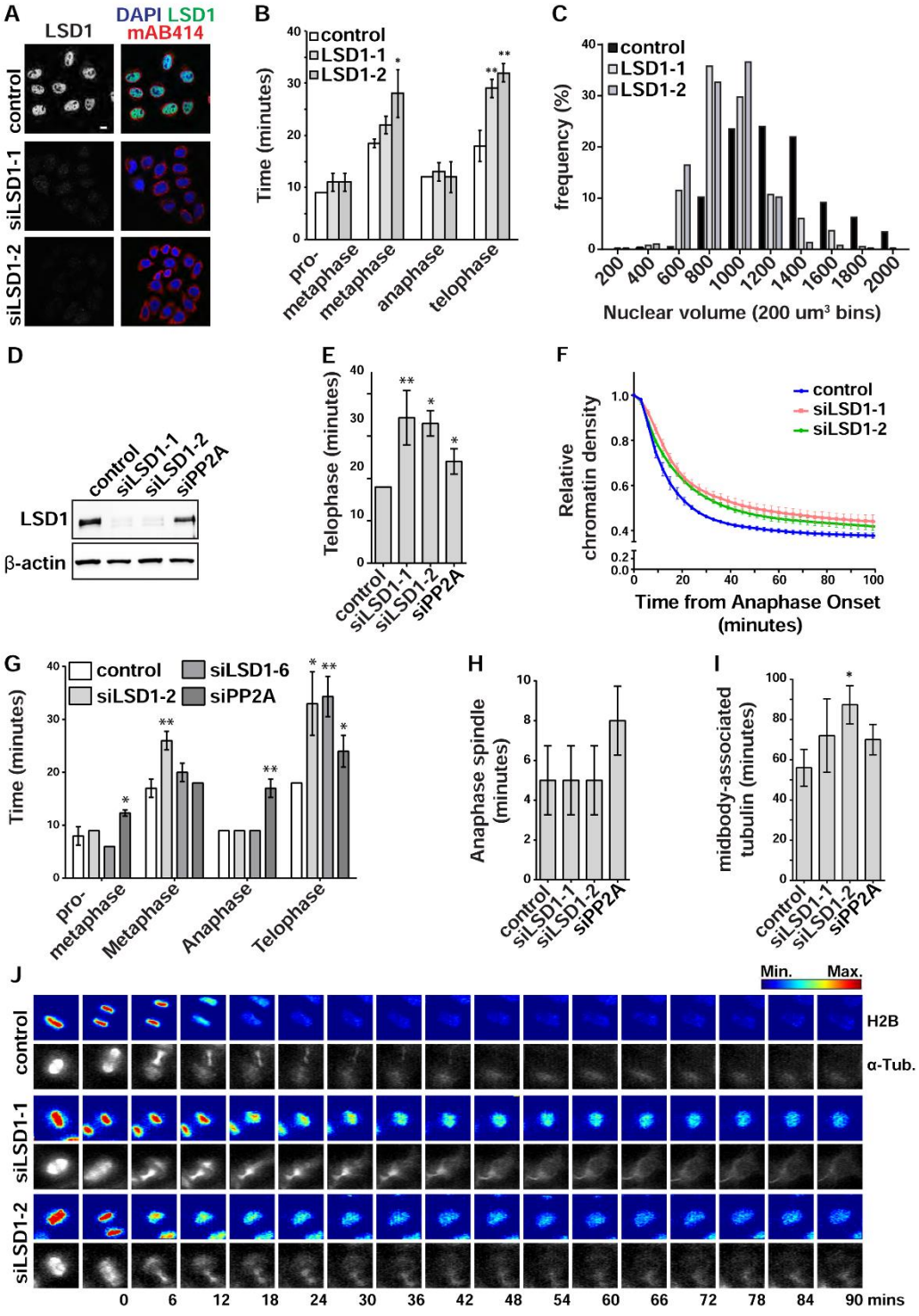


Fig. S3. Further characterization of siRNA mediated downregulation of LSD1 in HeLa cells

(A) HeLa cells transfected with 20 nM control, LSD1-1 or LSD1-2 siRNA oligos were fixed 48h post-transfection and processed for immunofluorescence. LSD1 (top panel, green in overlay) and NPCs (mAB414, red in overlay) were immune-labeled and chromatin was stained with DAPI (blue in overlay). Scale bar: 10 µm

(B) HeLa cells stably expressing H2B-mCherry and transfected with 20 nM siRNA as indicated were subjected to time-lapse microscopy starting 30h post-transfection. Mitotic events were analyzed with CellCognition. The mean of the median time spent in each cell cycle stage indicated is plotted for more than 100 mitotic events per condition in 3 independent experiments. Error bars represent s.d. *P<0.05, Student's t-test.

(C) Quantification of nuclear volume based on DAPI staining in HeLa cells transfected with 20 nM control, LSD1-1 or LSD1-2 siRNA oligos and fixed 48h post-transfection. Nuclear volume measurements from 383 nuclei in 3 independent experiments were pooled for each condition and assigned to 200 μm^3 bins. The nuclear volume frequency for each bin is plotted and the center of each bin is indicated on the x-axis.

(D) Western blot analysis of whole cell lysates from HeLa cells stably expressing H2B-mCherry/α-tubulin-EGFP and transfected with 20 nM control, LSD1-1, LSD1-2, or PP2A siRNA oligos as indicated. Lysates were harvested 48h post-transfection.

(E) HeLa cells stably expressing H2B-mCherry/ α-tubulin-EGFP and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting 30h after transfection. Mitotic events were analyzed with CellCognition. The mean of the median time spent in telophase based on chromatin features is plotted for more than 100 mitotic events per condition in 3 independent experiments. Error bars represent s.d. *P<0.05, **P<0.01, Student's t-test.

(F) The average fluorescence intensity of the H2B-mCherry signal was extracted from the CellCognition data acquired in (B) as an indication of chromatin density. The density was normalized to the first anaphase frame in individual mitotic tracks and the mean relative density for more than 100 mitotic events from three independent experiments is plotted over time. Error bars represent s.d.

(G) HeLa cells stably expressing H2B-mCherry/ α-tubulin-EGFP and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting 30h post-transfection. Mitotic events were analyzed with CellCognition. The mean of the median time spent in each cell cycle stage based on chromatin features is plotted for more than 100 mitotic events per condition in 3 independent experiments. Error bars represent s.d. *P<0.05, **P<0.01, Student's t-test.

(H) HeLa cells stably expressing H2B-mCherry/ α-tubulin-EGFP and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting 30h post-transfection. Mitotic events were analyzed with CellCognition. The mean of the median duration of the anaphase spindle, based on the α-tubulin signal, is plotted for more than 100 mitotic events per condition in 3 independent experiments. Error bars represent s.d.

(I) HeLa cells stably expressing H2B-mCherry/ α-tubulin-EGFP and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting 30h post-transfection. Mitotic events were analyzed with CellCognition. The mean of the median duration of detectable midbody-associated α-tubulin is plotted for more than 100 mitotic events per condition in 3 independent experiments. Error bars represent s.d. *P<0.05, Student's t-test.

(J) HeLa cells stably expressing H2B-mCherry/ α-tubulin-EGFP and transfected with 20 nM siRNA were subjected to time-lapse microscopy starting 30h after transfection. Maximum intensity projections of the mCherry and EGFP signals from five optical z sections are shown. Mitotic tracks are normalized to the first anaphase frame.

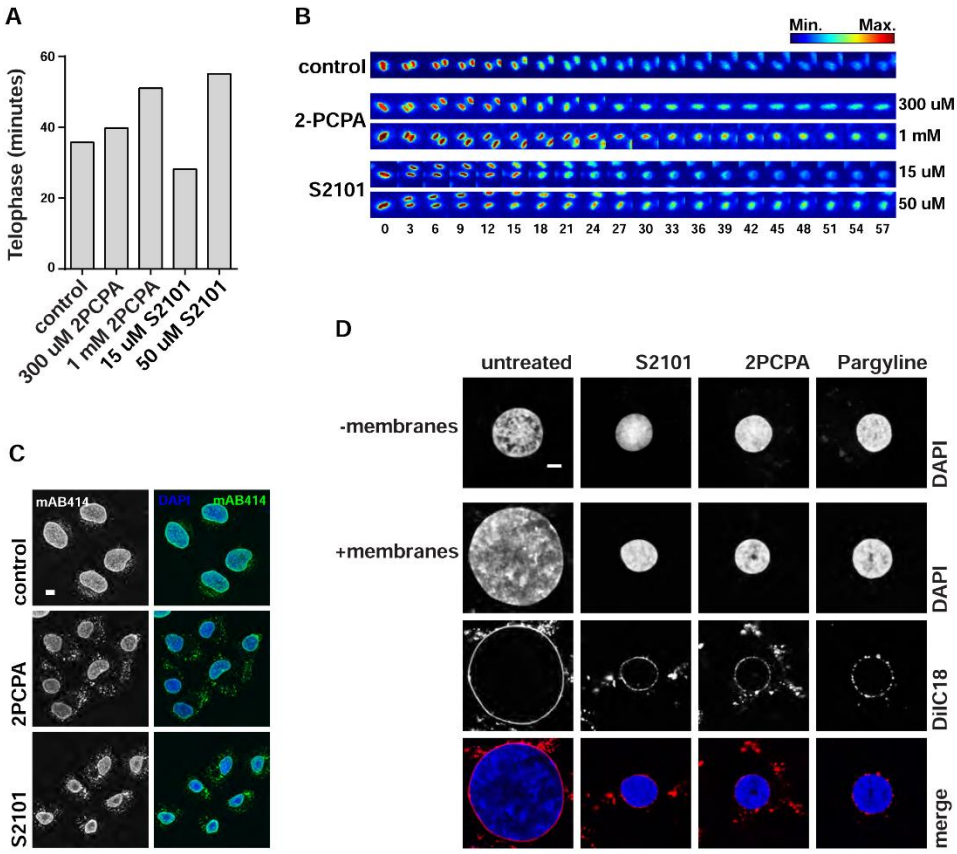


Fig. S4. Chemical inhibition of LSD1 extends the duration of telophase and promotes the formation of annulate lamellae in HeLa cells but does not block membrane-independent chromatin decondensation *in vitro*

(A) HeLa cells stably expressing H2B-mCherry were treated with different concentrations of the LSD1 inhibitors 2-PCPA and S2101 as indicated and immediately subjected to time-lapse microscopy. Mitotic events were analyzed with CellCognition (control: 73 events, 300 µM 2-PCPA: 35 events, 1mM 2-PCPA: 10 events, 15 µM S2101: 62 events, and 50 µM S2101: 22 events). The number of mitotic events analysed was drastically reduced in the presence of LSD1 inhibitors due to cell lethality at the effective concentrations. The median time spent in telophase is plotted and individual data are shown.

(B) HeLa cells stably expressing H2B-mCherry were treated with different concentrations of the LSD1 inhibitors 2-PCPA and S2101 as indicated and immediately subjected to time-lapse microscopy. Representative maximum intensity projections of the mCherry signal from five optical z sections are represented as a heat maps. Mitotic tracks are normalized to the first anaphase frame.

(C) HeLa cells treated with 300 µM 2-PCPA or 15 µM S2101 as indicated for 24h, fixed, and processed for immunofluorescence. NPCs were immuno-labelled using mAB414 (green in overlay) and DNA was stained with DAPI (blue). Maximum intensity projections are shown.

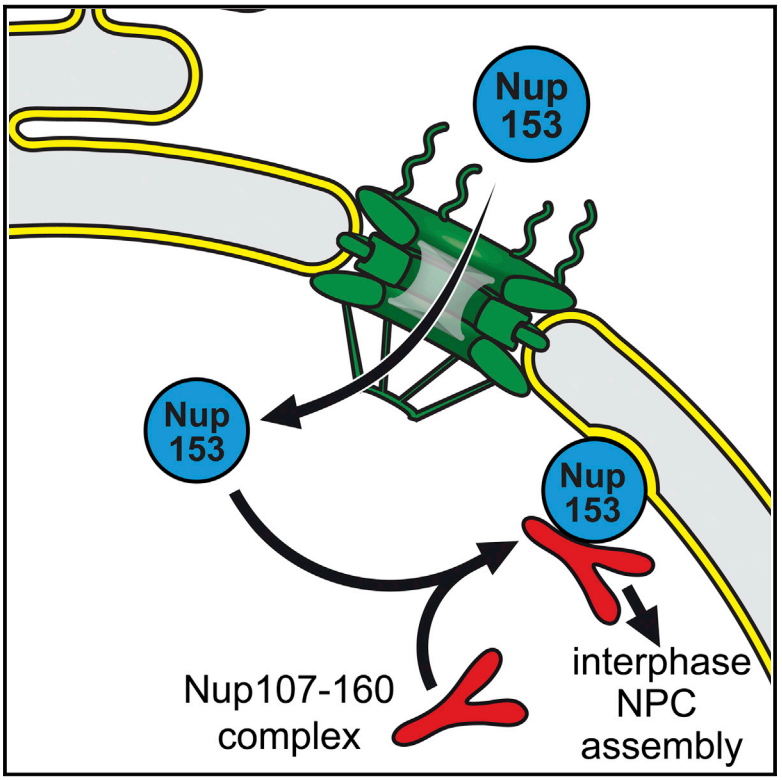
(D) Mitotic chromatin clusters from HeLa cells were incubated with Xenopus egg extracts in the presence or absence of added membranes. Where indicated 5 mM pargyline, 2.5 mM 2PCPA or 0.25 mM S2101 was added to decondensation reactions at t = 0. After 120 min samples were fixed and analyzed by confocal microscopy. Added membranes were pre-labeled with DiIC18 (red in overlay) and chromatin was stained with DAPI (blue in overlay). Scale bar: 5 µm.

Article

Developmental Cell

Nup153 Recruits the Nup107-160 Complex to the Inner Nuclear Membrane for Interphasic Nuclear Pore Complex Assembly

Graphical Abstract



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In Brief

Nuclear pore complexes assemble and integrate into the intact nuclear envelope during interphase. Vollmer et al. show that the nucleoporin Nup153 is critical for this process. It binds to the nuclear membrane and recruits the Nup107-160 complex, an essential structural component of nuclear pore complexes, to newly forming pores.

Highlights

- Nup153 binds synthetic membranes via its N terminus
- Transportin binding regulates Nup153 synthetic membrane interaction
- Nup153 membrane binding is required for interphasic nuclear pore complex assembly
- Nup153 recruits the Nup107-160 complex to the inner nuclear membrane

Vollmer et al., 2015, Developmental Cell *33*, 717–728
June 22, 2015
http://dx.doi.org/10.1016/j.devcel.2015.04.027

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Developmental Cell
Article

Nup153 Recruits the Nup107-160 Complex to the Inner Nuclear Membrane for Interphasic Nuclear Pore Complex Assembly

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http://dx.doi.org/10.1016/j.devcel.2015.04.027

SUMMARY

In metazoa, nuclear pore complexes (NPCs) are assembled from constituent nucleoporins by two distinct mechanisms: in the re-forming nuclear envelope at the end of mitosis and into the intact nuclear envelope during interphase. Here, we show that the nucleoporin Nup153 is required for NPC assembly during interphase but not during mitotic exit. It functions in interphasic NPC formation by binding directly to the inner nuclear membrane via an N-terminal amphipathic helix. This binding facilitates the recruitment of the Nup107-160 complex, a crucial structural component of the NPC, to assembly sites. Our work further suggests that the nuclear transport receptor transportin and the small GTPase Ran regulate the interaction of Nup153 with the membrane and, in this way, direct pore complex assembly to the nuclear envelope during interphase.

INTRODUCTION

Nuclear pore complexes (NPCs) are gatekeepers of the nucleus. They restrict the diffusion of proteins and nucleic acids between the cytosol and nuclear interior and enable tightly controlled transport between these compartments (Wente and Rout, 2010). Nuclear import of soluble proteins is mediated by transport receptors that bind cargo proteins in the cytosol. This interaction massively enhances the passage of otherwise inert cargos through NPCs. In the nucleoplasm, transport receptors are detached from cargos by binding to the small GTPase Ran in its GTP-bound form. The RanGTP-transport receptor complexes then traverse the NPC in the opposite direction. RanGTP hydrolysis at the cytoplasmic side of the NPC frees transport receptors, which are able to act in another round of the cycle.

Despite their enormous size and flexibility with regard to transport substrates, NPCs are composed of only about 30 different proteins, nucleoporins, present in multiple copies (Bui et al., 2013). They can be roughly categorized into structural nucleoporins, which form the scaffold of the pore, and those responsible for the transport and exclusion functions of the NPC. Nucleoporins of the latter class are characterized by a high number of phenylalanine glycine (FG) repeats that form a meshwork within the pore. A stack of three rings forms the NPC scaffold. The middle ring is laterally linked to the pore membrane and connected to the central transport channel formed mostly by the FG nucleoporins. The cytoplasmic and nucleoplasmic rings are connected to cytoplasmic filaments and the nuclear basket structure, respectively.

Most structural nucleoporins are part of one of two evolutionarily conserved subcomplexes within the pore. The Nup93 complex (Nic96 complex in yeast) forms a large part of the inner ring and connects the pore membrane to the central transport channel (Vollmer and Antonin, 2014). The Nup107-160 complex (Nup84 complex in yeast) forms the cytoplasmic and nucleoplasmic rings (Bui et al., 2013) and is, because of its Y shaped structure (Lutzmann et al., 2002), also referred to as Y-complex. This complex is related to vesicle coats and presumably stabilizes the curved pore membrane of the NPC (Brohawn et al., 2008). Connected to the nucleoplasmic ring is the nuclear basket, a fish trap-like structure extending to the nuclear interior. In metazoans, three nucleoporins localize to the basket, Nup153, Nup50, and TPR (Cordes et al., 1997; Guan et al., 2000; Sukegawa and Blobel, 1993), the best characterized being Nup153.

Nup153 possesses a tripartite structure (Ball and Ullman, 2005). The N-terminal region is important for NPC targeting (Bastos et al., 1996; Enarson et al., 1998), most likely because it mediates binding to the Y-complex (Vasu et al., 2001). A central zinc-finger-containing domain interacts with Ran (Nakielny et al., 1999). The C-terminal FG-repeat-containing region provides binding sites for a variety of transport receptors (Moroianu et al., 1997; Nakielny et al., 1999; Shah and Forbes, 1998; Shah et al., 1998). Because of its localization at the nucleoplasmic exit site of NPCs as well as its interactions with transport receptors and Ran, Nup153 is thought to assist in the dissociation of import cargo-transport receptor complexes and thus facilitates nuclear import cycles. Indeed, Nup153 depletion reduces importin α/β mediated import (Ogawa et al., 2012; Walther et al., 2001). Nup153 is also important for mRNA export from

the nucleus (Bastos et al., 1996; Ullman et al., 1999). Although Nup153 is essential in *C. elegans* and HeLa cells (Galy et al., 2003; Harborth et al., 2001), no homologs have been found in yeast species. Nonetheless, yeast nucleoporins (such as Nup1 and Nup60 in *S. cerevisiae*, Nup124 in *S. pombe*) might share functional and sequence features with Nup153 (Cronshaw et al., 2002; Hase and Cordes, 2003; Varadarajan et al., 2005).

While significant progress has been made in understanding how NPCs function in nuclear transport, elucidating the NPC formation pathway remains a formidable task. In metazoa, NPC assembly occurs at two different stages of the cell cycle: at the end of mitosis and during interphase. During mitotic exit, NPC assembly is concomitant with the formation of a closed nuclear envelope (NE). The early steps of postmitotic NPC formation, such as the recruitment of a subset of nucleoporins to the chromatin, are particularly well characterized due to their faithful reconstitution in *Xenopus* egg extracts. Assembly is initiated by MEL28/ELYS, a chromatin binding nucleoporin, which recruits the Y-complex (Franz et al., 2007; Gillespie et al., 2007; Harel et al., 2003; Rasala et al., 2006, 2008; Rotem et al., 2009; Walther et al., 2003a). Interphasic NPC assembly in metazoan is relatively poorly characterized. It takes place under fundamentally different conditions. Whereas large numbers of NPCs form in a short time span during mitotic exit, NPC assembly events in interphase are rare and sporadic (D'Angelo et al., 2006; Dultz and Ellenberg, 2010). Both assembly pathways require the Y-complex as an essential structural component of the NPC (D'Angelo et al., 2006; Doucet et al., 2010; Harel et al., 2003; Walther et al., 2003a). However, while the Y-complex is recruited to the chromatin by MEL28 at the end of mitosis, a feature essential for postmitotic NPC assembly, MEL28 is not required for interphasic NPC assembly (Doucet et al., 2010). It is possible that NPC assembly during interphase is rather initiated at the nuclear membranes (Doucet et al., 2010; Dultz and Ellenberg, 2010; Vollmer et al., 2012), but the precise mechanism by which this occurs has not been defined.

Here, we show that the nuclear basket component Nup153 is required for NPC assembly during interphase but not at the end of mitosis. Nup153 binds the inner nuclear membrane via its N terminus, a feature that is fundamental for its function in interphasic NPC assembly as it recruits the Y-complex to the assembling pores. Transportin regulates the interaction of Nup153 with the membrane and thus might direct interphasic NPC assembly specifically to the NE from inside the nucleus.

RESULTS

Nup153 Interacts via Its N Terminus Directly with Membranes

Nup153 contains a region within its N terminus that directs it to the inner nuclear membrane (Enarson et al., 1998). It is possible that Nup153 is localized to the NE due to interactions with integral inner nuclear membrane proteins or lamins, proteins that underlay and stabilize the NE, but it could also bind directly to the lipid bilayer. To test for a direct membrane interaction we incubated the purified recombinant N-terminal region comprising the first 149 aa of *Xenopus laevis* Nup153 with small unilamellar liposomes with a NE lipid composition (Lorenz et al., 2015). Due to their density, lipid vesicles float up through a sucrose gradient. Membrane binding proteins can be identified in the top fraction together with the liposomes. This is indeed the case for Nup153 (Figure 1A) as well as for a Nup133 fragment previously identified as membrane interacting (Drin et al., 2007). The N-terminal region of Nup153 showed preference for small vesicles with high curvature independent of lipid composition (Figure 1B).

Sequence analysis of the N terminus of Nup153 identified a conserved region among vertebrates that might form an amphipathic helix, as depicted in the helical wheel representation (Figures 1C and 1D). We generated a point mutation (a valine to glutamate exchange in position 50, V50E) that predictably disrupts the hydrophobic surface of the helix. Indeed, the V50E mutation impaired liposome binding in flotation assays (Figures 1A and 1B). To directly visualize the interaction of Nup153 with membranes, we generated giant unilamellar vesicles (GUVs) with sizes up to 50 μm using the NE lipid composition. When the EGFP-tagged N terminus of Nup153 was added to the exterior of these GUVs, it was efficiently recruited to the vesicle membrane indicating a direct membrane interaction (Figure 1E). As a negative control, we employed purified recombinant EGFP, which did not bind the GUV membrane. Importantly, the V50E mutant did not bind to GUVs, confirming that the amphipathic helix is responsible for the membrane binding capacity of Nup153.

The N-terminal fragment of *Xenopus* Nup153 fused to EGFP, when ectopically expressed in HeLa cells, localized to the NE, presumably the inner nuclear membrane (Figures 1F and S1C). Overexpression of this fragment induced NE proliferation (Movie S1), as is typical for nuclear membrane-interacting proteins (Ralle et al., 2004). Membrane proliferation has previously been observed for the overexpression of full-length Nup153 (Bastos et al., 1996). In our experiments, the V50E mutation abolished NE localization and membrane proliferation. Instead, the protein localized to the nucleoplasm, indicating that the mutation is sufficient to prevent the interaction of the N terminus of Nup153 with membranes in cells.

To confirm the direct membrane binding of full-length Nup153, we turned to the human ortholog, due to the low expression yield of the *Xenopus* Nup153. Human Nup153 possesses 42% amino acid identity and 55% similarity with the *Xenopus* protein. The fluorescently labeled human Nup153 efficiently bound GUVs (Figure 1G). The corresponding membrane-binding-deficient mutation (V47E in the human protein) abrogated the membrane interaction.

Nup153 Membrane Binding Is Not Required for NPC Targeting in Mammalian Cells

To understand the functional implication of the direct membrane interaction, we tested whether nuclear membrane targeting of Nup153 is required for its incorporation into NPCs. HeLa cells were transfected with EGFP-tagged full-length human Nup153 as well as the membrane-binding-deficient mutant (V47E). Both proteins localized to the nuclear rim and showed a typical punctate pattern (Daigle et al., 2001) on the NE surface (Figures 2A and S2). The pattern overlaps with mCherry-labeled Nup62 but not lamin B, indicating proper NPC localization. Despite its nuclear rim localization, the V47E mutant exhibited increased nucleoplasmic staining consistent with an abolished direct membrane interaction.

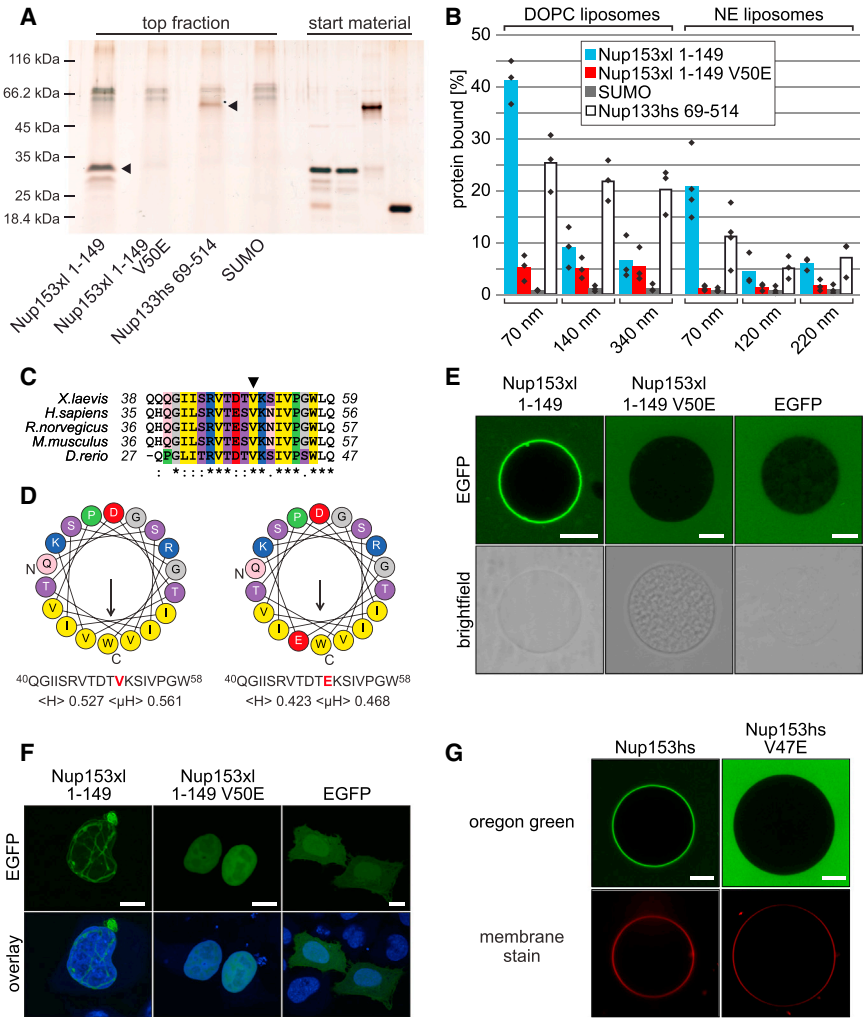

Figure 1. Nup153 Interacts via Its N Terminus Directly with Membranes

(A) 3 μM of the N-terminal domain (aa 1–149) of *Xenopus* Nup153 and the corresponding V50E mutant fragment, as well as a membrane-interacting fragment of Nup133 and SUMO as positive and negative controls, respectively, were incubated with 2.5 mg/ml fluorescently labeled 70 nm NE liposomes and floated through a sucrose gradient. The top gradient fractions and the starting materials were analyzed by SDS-PAGE and silver staining.

(B) Binding of the Nup153 N terminus, the V50E mutant, SUMO, and the Nup133 fragment to DOPC or NE liposomes of different diameters were analyzed by flotation experiments and quantified by western blotting (columns are the average bound quantities of three or four independent experiments; individual data points are indicated).

(C) Sequence alignment of the N-terminal region of vertebrate Nup153.

(D) Amino acid sequence of *Xenopus* Nup153 with the predicted amphipathic helix in a helical wheel representation (generated with HeliQuest (Gautier et al., 2008)). Valine (V) was exchanged to glutamate (E) in the membrane binding mutant, indicated in red. The length of the arrow within the helix is proportional to the mean hydrophobic moment (< μH >), which is also indicated as well as the hydrophobicity (< H >) as calculated by HeliQuest.

(E) NE lipid GUVs were incubated with 500 nM EGFP-tagged Nup153 N terminus, the corresponding V50E mutant, or EGFP alone.

(F) HeLa cells transfected with EGFP-tagged Nup153 N terminus, the V50E mutant, or EGFP. Chromatin is stained with DAPI (blue in overlay).

(G) NE lipid GUVs were incubated with Oregon-green-labeled full-length human Nup153 and the corresponding V47E mutant.

Bars, 10 μm. See also Figure S1 and Movie S1.

Immunoprecipitation from transfected HEK cells demonstrated that the V47E mutation did not impair known interactions, namely, to the Y-complex (Nup160 and Nup107) or other nucleoporins (Nup50 and TPR), to A/C and B-type lamins, or to components of the nuclear import machinery, Ran, importin α and β, and transportin (Figure 2B). Together these results indicate that membrane binding is not required for the assembly of Nup153 into NPCs. Furthermore, the V47E mutation does not disturb the interaction network of Nup153 but rather specifically affects its direct membrane binding.

Nup153 Membrane Interaction Is Not Required for NPC Assembly at the End of Mitosis

We used *Xenopus* egg extracts to assess whether the membrane binding capacity of Nup153 is important for the assembly and function of NPCs. Nup153 can be specifically immuno-depleted from these extracts without affecting the levels of other nucleoporins, including TPR and Nup50 as well as two components of the Y-complex, Nup133, and Nup107, which interact with Nup153 within intact NPCs (Figure 2C). Similarly, the levels of lamin LIII, a *Xenopus* B-type lamin, and components of the nuclear transport machinery (Ran, importin α and β, transportin) were unchanged.

When de-membranated sperm heads are incubated with egg extracts, a NE forms around the decondensing chromatin in a process resembling the reassembly of the nucleus at the end of mitosis (Gant and Wilson, 1997). The NE contains NPCs visualized with the antibody mAB414, which recognizes several FG nucleoporins, as seen in the control (mock) depletion (Figure 2D). When Nup153 was depleted, the protein was absent from the nuclear rim confirming the depletion efficiency. The assembled nuclei contained a closed NE with NPCs that were unevenly distributed. This NPC clustering phenotype upon Nup153 depletion has been previously observed (Walther et al., 2001) and is best visualized by surface rendering of confocal stacks (Figure 2D). Addition of recombinant Nup153 to endogenous levels (see Figure 2C) rescued the NPC clustering phenotype (Figures 2D–2F) demonstrating its specificity. NPC clustering was also rescued by the Nup153 membrane-binding mutant, which indicates that the Nup153 membrane interaction is not required for proper NPC spacing.

Depletion of Nup153 did not affect the localization and regular distribution of lamin B or integral inner nuclear membrane proteins, such as LBR or BC08. Several nucleoporin antibodies including those recognizing the integral pore membrane protein POM121, the Y-complex members Nup133 and Nup107 as well

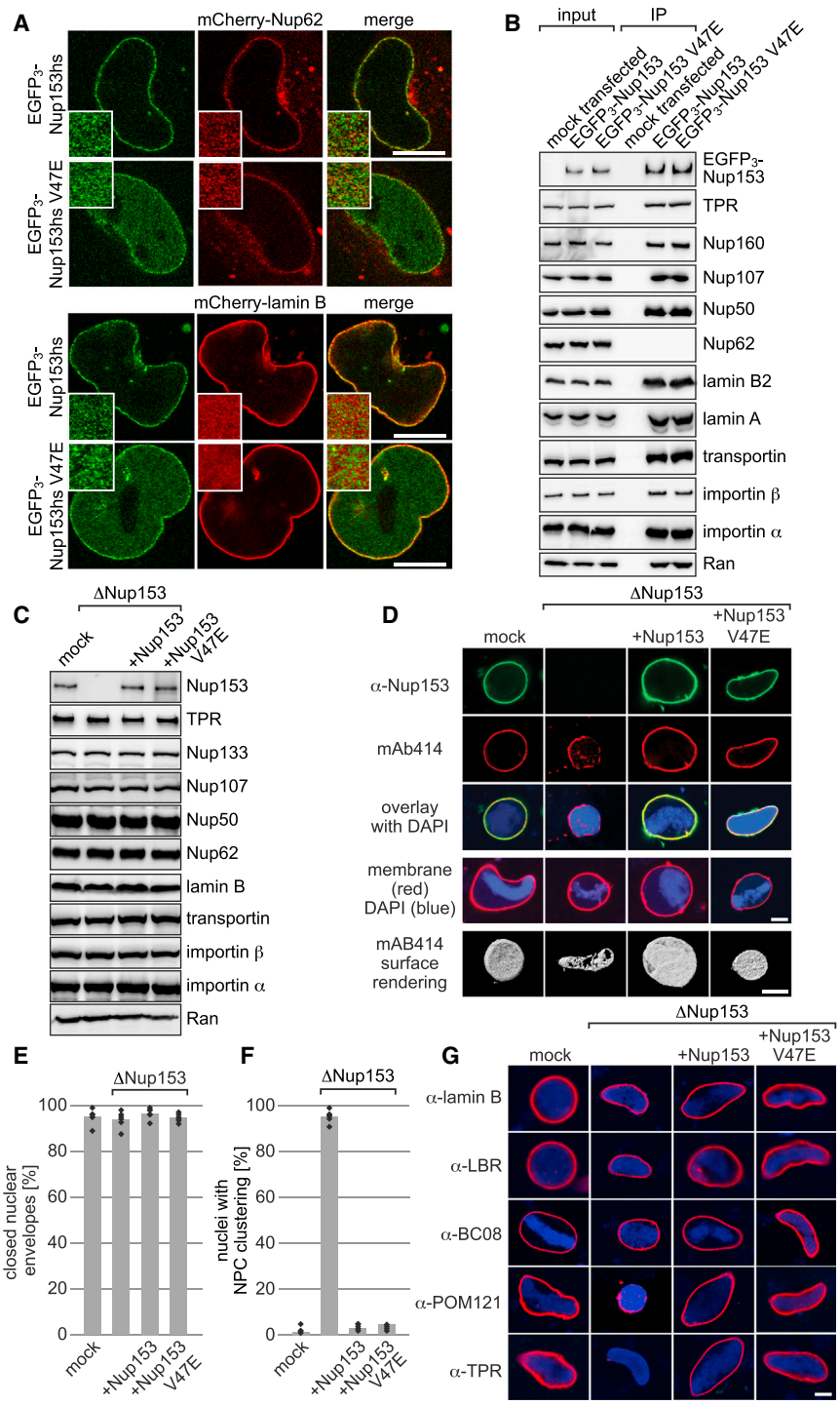

Figure 2. Nup153 Membrane Binding Is Not Required for Its NPC Targeting and NPC Assembly at the End of Mitosis

(A) HeLa cells were co-transfected with triple-EGFP human Nup153 or the corresponding V47E mutant and mCherry-Nup62 or mCherry-lamin B and analyzed by live cell imaging. Insets show the nuclear surface at a 1.5-fold higher magnification.

(B) HEK293 cells were mock transfected, transfected with triple-EGFP human Nup153 or the V47E mutant. Inputs and immunoprecipitates from cell lysates were analyzed by western blotting.

(C) Western blot of mock-depleted, Nup153-depleted (ΔNup153), and Nup153-depleted *Xenopus* egg extracts supplemented with recombinant wild-type Nup153 or the membrane-binding mutant (Nup153 V47E).

(D) Nuclei assembled for 120 min in extracts generated as in (C) were analyzed by immunofluorescence for Nup153 (green) and mAB414 (red). DNA was stained with DAPI (blue) and membranes with DiIC18 (fourth row, red). The fifth row shows the surface rendering of confocal stacks of mAB414-labeled nuclei.

(E) Quantification of chromatin substrates with closed NEs. Columns are the average of six independent experiments with individual data points (each 100 randomly chosen chromatin substrates) indicated.

(F) Quantification of nuclei with NPC clustering identified by mAB414 staining (mean of six independent experiments with individual data points [100 chromatin substrates each] indicated).

(G) Immunofluorescence on nuclei assembled as in (D). Chromatin is stained with DAPI (blue).

Bars, 10 μm. See also Figure S2.

as the Nup93 complex members Nup155 and Nup53 showed a patchy staining (Figures 2G and S2B). These observations demonstrate that the structural backbones of NPCs can assemble in the absence of Nup153. Furthermore, Nup58 as a central transport channel nucleoporin (Figure S2B) is also present at NPCs lacking Nup153. Consistent with previous reports, TPR was not detectable at Nup153-depleted nuclei, as it depends on this interaction for NPC localization (Hase and Cordes, 2003; Walther et al., 2001). Re-addition of recombinant wild-type or membrane-binding-deficient Nup153 rescued not only the NPC clustering phenotype but also TPR recruitment. This is consistent with the notion that the V47E mutation does not interfere with TPR binding (Figure 2B).

Taken together, these results suggest that the membrane binding capability of Nup153 is not crucial for NPC assembly at the end of mitosis. The interaction of Nup153 with membranes is not mandatory for proper NPC spacing, as the V47E mutant also rescues the NPC clustering phenotype. However, we observed that the nuclei assembled in the presence of the V47E mutant were smaller in comparison to the wild-type addback (Figures 2D, 2G, and S2B). Similarly, nuclei lacking Nup153 were smaller than control nuclei.

Nup153 Membrane Interaction Is Not Required for Efficient Nuclear Import

The addition of membranes to sperm DNA decondensed in egg extracts results in a fully closed NE-containing NPCs within

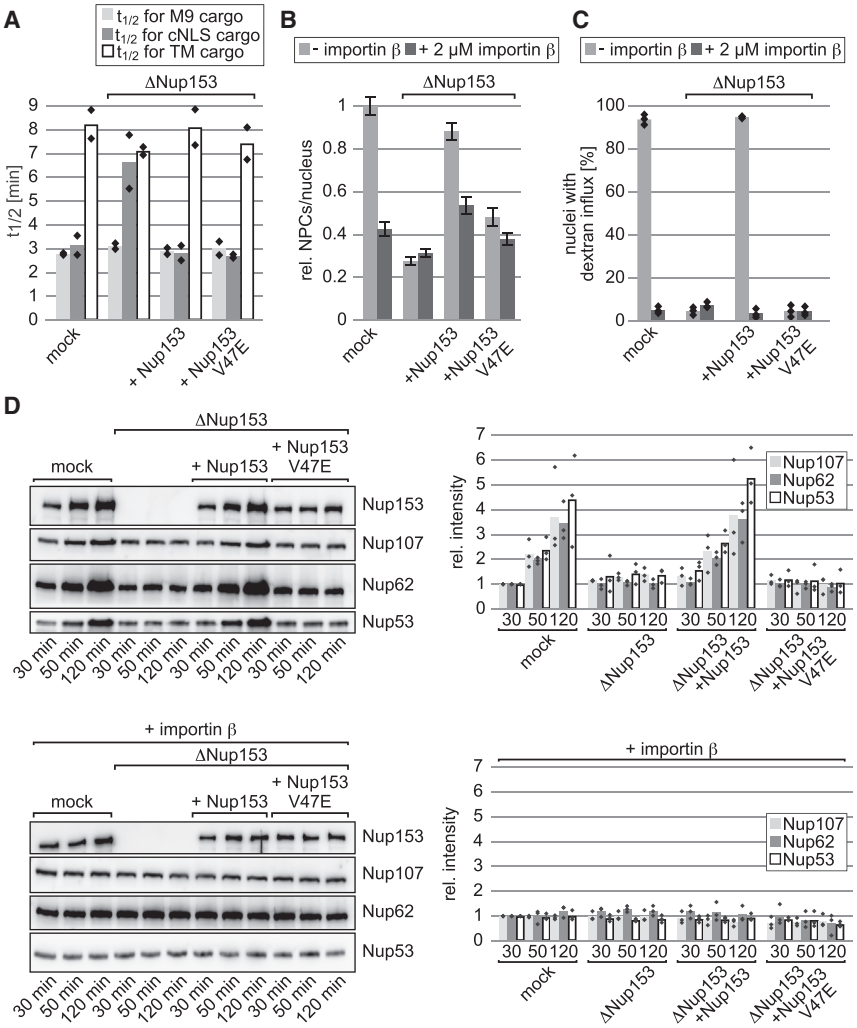

Figure 3. Nup153 Membrane Interaction Is Not Important for Nuclear Import Efficiency, but Rather for Interphasic NPC Assembly

(A) Nuclei were assembled in mock, Nup153-depleted extracts, or Nup153-depleted extracts supplemented with Nup153 or the V47E mutant. After closed NE formation, import rates for soluble M9 and cNLS cargos as well as for a transmembrane (TM) cargo were determined (average of two independent experiments, individual data points are indicated).

(B) Nuclei assembled as in (A) were fixed after 120 min, and NPCs per nucleus were counted based on mAB414 staining. Where indicated, interphasic NPC assembly was blocked by importin β addition after 50 min (average from a total of 50 nuclei in five independent experiments, normalized to the mock control; error bars are SEM).

(C) Nuclei assembled as in (A) for 50 min were supplemented with cytosol depleted of FG nucleoporins in addition to being either mock depleted or Nup153 depleted. After a further 60-min incubation, fluorescein isothiocyanate (FITC)-labeled 70 kDa dextran and Hoechst were added, and the percentage of nuclei with nuclear dextran, i.e., those in which interphasic NPC assembly occurred, was determined (average of three independent experiments, each comprising 100 nuclei; individual data points are indicated).

(D) Nuclei were assembled as in (A) and re-isolated after 30, 50, and 120 min. Nucleoporin levels were determined by quantitative western blotting. Plotted are the averages from three independent experiments, individual data points are indicated. In the lower panel, importin β was added after 30 min to block interphasic NPC assembly.

20 min. After this assembly, which reproduces nuclear reformation at the end of mitosis, the nuclei grow in size—the extent to which depends on the extract quality and an ATP re-generating system—for another 180 min. This nuclear growth requires import of nuclear proteins through NPCs. During this time, new NPCs integrate into the growing NE (D'Angelo et al., 2006) in a process reproducing interphasic NPC assembly, which, in turn, allows for more import and accelerated growth.

Nup153 contributes to the efficiency of nuclear transport cycles (Ogawa et al., 2012; Walther et al., 2001). We therefore tested whether the Nup153 membrane interaction is necessary for its role in nuclear import. We added different nuclear import substrates to nuclei assembled for 30 min in vitro (i.e., 20 min after addition of membranes), which is the time point when a closed NE had formed. Nuclear import rates for soluble cargos can be determined by the time-dependent protection of different import substrates from a cytoplasmic protease as they accumulate in the nucleus (described in Theerthagiri et al., 2010). The translocation of integral membrane proteins from the ER to the inner nuclear membranes can be assessed in a similar way when the reporter is reconstituted into liposomes, which are then added to the assembly reactions and integrated into the ER. Compared to mock reactions, nuclei depleted of Nup153 exhibited reduced import of a soluble import cargo with a classical bipartite nuclear localization signal (cNLS cargo), which is imported in an importin α/β-dependent manner (Figure 3A). In contrast, import of a transportin-dependent cargo (containing an M9 sequence) was not affected by Nup153 depletion. The dependency of the importin α/β import pathway on Nup153 has been previously reported (Walther et al., 2001). Import of the cNLS-containing cargo was rescued by the re-addition of either wild-type or membrane-binding-deficient Nup153. Transport of a transmembrane cargo through NPCs was not affected by Nup153 depletion or the addition of either wild-type or membrane-binding-deficient Nup153. These results demonstrate that the membrane interaction of Nup153 is not important for efficient nuclear import.

Nup153 Is Necessary for Interphasic NPC Assembly

We next tested whether interphasic NPC assembly was affected by Nup153 depletion. Nuclei were assembled for 120 min, and individual NPCs were counted using mAB414 staining (D'Angelo et al., 2006; Vollmer et al., 2012). Addition of 2 μM importin β, which blocks interphasic NPC assembly (D'Angelo et al.,

2006), to nuclei formed under control conditions resulted in a reduction in the number of NPCs per nucleus by approximately 60% when added 50 min after starting the reaction (Figure 3B). Depletion of Nup153 caused a severe reduction in NPC number, which was not further affected by the addition importin β. Re-addition of wild-type Nup153 but not the membrane-binding-deficient V47E mutant rescued the reduced number of NPCs formed to nearly wild-type levels. These data suggest that NPC formation during interphase requires Nup153, specifically in its capacity to bind the NE.

The antibody mAB414 recognizes several FG nucleoporins including Nup153 (Sukegawa and Blobel, 1993). Although we did not employ overall staining intensity as readout for NPC numbers but counted individual NPC-containing spots on the NE, this procedure might be considered as biased due to the loss of a major antigen. Furthermore, counting might also be affected by the NPC clustering observed in Nup153-deficient nuclei. We therefore employed an assay for interphasic NPC assembly that is independent of mAB414 staining. In this setup, interphasic NPC assembly proceeds in the presence of extract depleted of the nucleoporins forming the permeability barrier of the pore. Nuclei with newly integrated NPCs lack this barrier and can be visualized by an influx of fluorescently labeled dextrans (Dawson et al., 2009; Vollmer et al., 2012). It should be noted that each nucleus is counted as either competent or deficient for interphasic NPC assembly. The addition of importin β, for example, completely inhibited interphasic NPC assembly monitored by dextran influx. Dextran influx was blocked in nuclei formed in the absence of Nup153, consistent with a block in interphasic NPC assembly (Figure 3C). Wild-type Nup153 but not the V47E mutant rescued interphasic NPC assembly based on dextran influx (Figure 3C).

As an alternative means to assess the increase in NPCs, we re-isolated the chromatin substrate in the assembly reactions at different time points and quantified the relative abundance of Nup107, Nup62, and Nup53 as members of the Y-complex, Nup62 complex, and Nup93 complex, respectively (Figure 3D). In mock-depleted extracts the levels of these nucleoporins re-isolated with the chromatin increases over time. However, this accumulation is not observed in Nup153-depleted extracts. The addition of wild-type Nup153 but not the V47E mutant rescues the depletion phenotype. Also in this assay, addition of importin β 30 min after initiation of nuclear assembly blocks the time-dependent increase in NPCs on the chromatin indicating that this experimental setup is a valuable readout for NPC assembly in the NE. It thus suggests that the increase in Nup107, Nup62, and Nup53 levels from 50 to 120 min represents interphasic NPC assembly.

Having identified an essential role for Nup153 and specifically its membrane interaction in interphasic NPC assembly, we wondered how Nup153 could function in this process. Two known Nup153 interactors are necessary for interphasic NPC assembly, Ran, and the Y-complex (D'Angelo et al., 2006; Doucet et al., 2010). Ran is most likely required to release import receptors from targets that are crucial for NPC assembly. Meanwhile the Y-complex is a structural component of NPCs. We speculated that Nup153 might localize these crucial components to the inner nuclear membrane.

Nup153 Recruits the Y-Complex to the Inner Nuclear Membrane for Interphasic NPC Assembly

To assess whether Nup153-dependent recruitment of Ran or the Y-complex to the NE is important for interphasic NPC assembly, we generated fusion proteins containing the Nup153 membrane binding domain (MBD, aa 1–149) and either the Y-complex (ycBD, aa 210–338) or the Ran binding domain (RanBD, aa 658–890) of human Nup153 (Figures 4A and S3). When added to Nup153-depleted extracts, the MBD-ycBD fusion resulted in larger nuclei. Conversely, the Nup153 phenotype was not rescued by the addition of either the MBD-RanBD fusion or any construct containing a defective membrane-binding domain (MBD V50E) (Figure 4B). Furthermore, only the MBD-ycBD construct restored interphasic NPC assembly when Nup153 was depleted (Figures 4C–4E). These data suggest that the requirement for Nup153 in interphasic NPC assembly is due to its capacity for directing the Y-complex to the nuclear membranes. To specifically assay interphasic NPC formation and to visualize the Y-complex in this process, we added Y-complex, purified from *Xenopus* egg extracts (see Figure S3) and fluorescently labeled, to pre-assembled nuclei (Figure 4F). The Y-complex foci formed in this way partly overlap with wheat germ agglutinin (WGA) staining, which labels NPCs formed both post-mitotically and after the formation of a closed NE. They can be either newly formed NPCs (overlapping with WGA staining, which recognizes mostly nucleoporins of the central channel) or NPCs that are just emerging (probably lacking a major subfraction of nucleoporins recognized by WGA). The appearance of Y-complex-containing NPCs was blocked by Nup153 depletion, confirming that interphasic Y-complex recruitment depends on Nup153. The addition of importin β also blocked the formation of Y-complex foci, validating the approach as a measure of interphasic NPC assembly. Importantly, addition of Nup153 but not the membrane-binding mutant rescued interphasic NPC assembly in Nup153-depleted nuclei. In agreement with the nuclear assembly data, the MBD-ycBD fusion protein but not the MBD-RanBD construct rescued the formation of Y-complex foci and thus the assembly interphasic NPCs.

Next, we asked whether we could directly recruit the Y-complex to the inner nuclear membrane thereby bypassing the function of Nup153. Nup153 Y-complex binding region or the Ran binding region were each fused N-terminally to EGFP and at the C terminus with the transmembrane protein BC08, yielding EGFP-ycBD-BC08 and EGFP-RanBD-BC08 (Figure 5A). BC08 contains a C-terminal transmembrane region and efficiently targets to the inner nuclear membrane (Theerthagiri et al., 2010). Both constructs were expressed in *E. coli*, purified, and reconstituted into small liposomes. To test the functionality of the fusion proteins, these liposomes were incubated with purified Y-complex or recombinant Ran and floated through a sucrose gradient. Liposomes containing the EGFP-ycBD-BC08 protein efficiently bound the Y-complex but not Ran, and EGFP-RanBD-BC08 liposomes bound Ran but not the Y-complex (Figure 5B). Similarly, when incorporated into GUVs, EGFP-ycBD-BC08 recruited fluorescently labeled Y-complex to the GUV membrane and EGFP-RanBD-BC08 recruited Ran (Figure 5C).

When EGFP-ycBD-BC08 or EGFP-RanBD-BC08-containing liposomes were added to nuclear assembly reactions, they efficiently targeted to the inner nuclear membrane (Figures 5D and

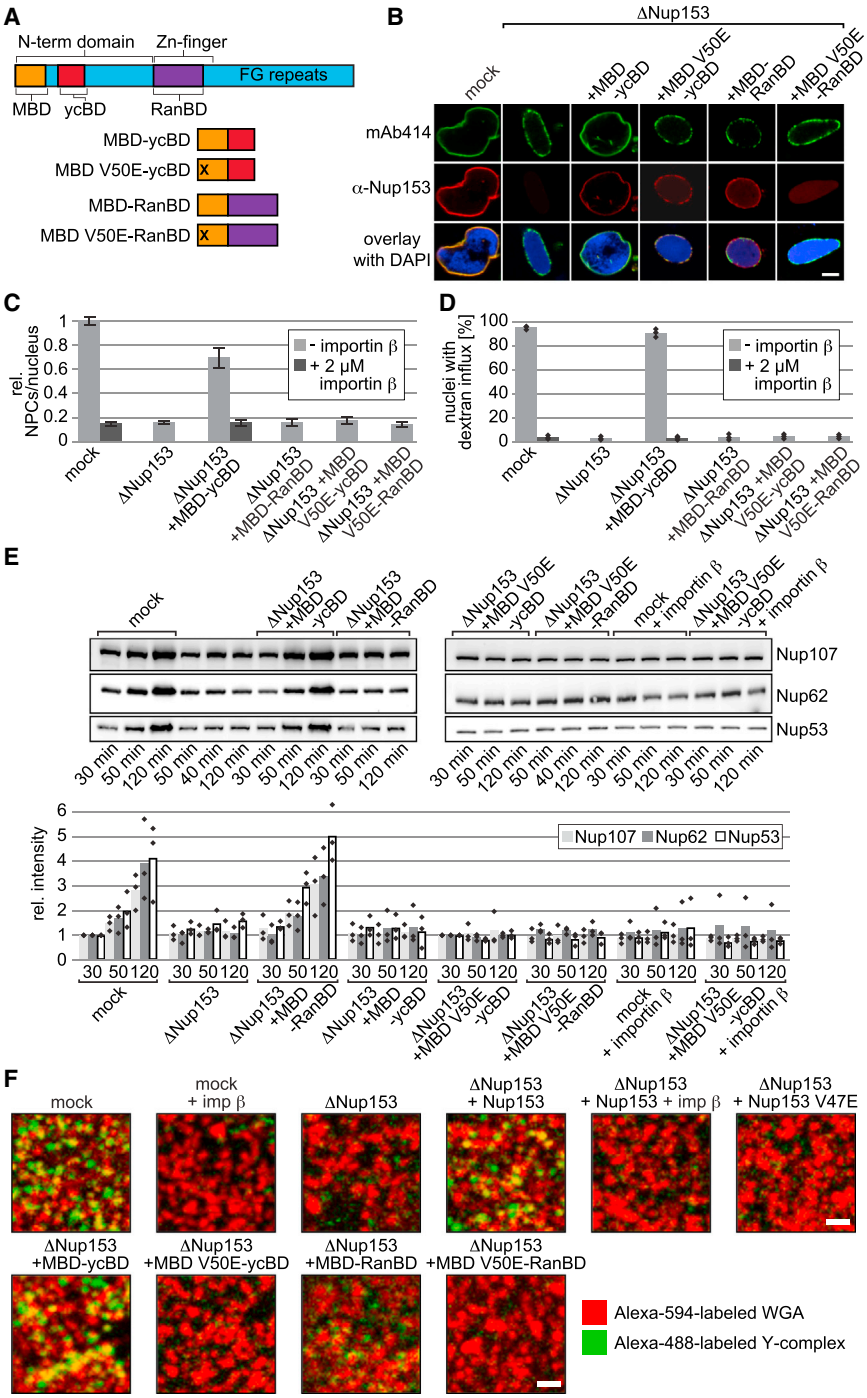

Figure 4. Membrane Recruitment of the Y-Complex Is Important for Interphasic NPC Assembly

(A) Schematic representation of the Nup153 fusion constructs. The Nup153 membrane binding domain (MBD) and the corresponding V50E mutant were fused to the Y-complex (ycBD) and the Ran-interacting region (RanBD).

(B) Nuclei were assembled in mock-depleted, Nup153-depleted, and Nup153-depleted extracts supplemented with the different MBD-fusions. Samples were fixed after 120 min and visualized using mAB414 (red), and Nup153 immunofluorescence. DNA was stained with DAPI (blue in overlay). Please note that the MBD-fusions are detected as the Nup153 antibody is directed against the Nup153 N terminus. Bar, 10 μm.

(C) NPC numbers in nuclei assembled as in (B) were determined using mAB414 staining as in Figure 3B (average from 30 nuclei from three independent experiments).

(D) Nuclei were assembled as in (B) and interphasic NPC assembly was analyzed by dextran influx as in Figure 3C.

(E) Nuclei were assembled as in (B). Chromatin was re-isolated at indicated time points, and nucleoporin levels were determined as in Figure 3D.

(F) Nuclei were assembled in mock-depleted, Nup153-depleted, and Nup153-depleted extracts supplemented with wild-type Nup153, the membrane-binding mutant (Nup153 V47E) or the different MBD fusions. Purified Alexa-488-labeled Y-complex (green) was added after 50 min and after another 60 min Alexa-594-WGA (red) for 10 min. Samples were fixed and the NE analyzed for WGA-positive structures, i.e., NPCs. Where indicated, interphasic NPC assembly was blocked by addition of importin β together with the Y-complex. Bars, 1 μm.

See also Figure S3.

S4C). Interestingly, Nup153 depletion resulted in larger nuclei when EGFP-ycBD-BC08 was incorporated compared to EGFP-RanBD-BC08 nuclei. Most importantly, EGFP-ycBD-BC08 but not EGFP-RanBD-BC08 incorporation into the nuclear membrane restored interphasic NPC assembly when Nup153 was depleted (Figures 5E–5G and S4B). We conclude that recruitment of the Y-complex to the inner nuclear membrane is sufficient to bypass the requirement for full-length Nup153 in interphasic NPC assembly in vitro. Thus, the crucial function of Nup153 in interphasic NPC assembly is to direct the Y-complex to the newly forming NPCs at the NE. This is consistent with the fact that a Nup153 construct lacking the Y-complex binding site cannot substitute Nup153 in interphasic NPC assembly (Figures S4E–S4G).

Annulate Lamellae Formation Depends on Nup153 Membrane Interaction

NPCs can assemble outside the nucleus in the membranes of the ER forming annulate lamellae (AL). AL form in egg extracts in the absence of chromatin, and this process is highly induced upon addition of the constitutively active Ran mutant Q69L, which is blocked in the GTP-bound state (Walther et al., 2003b). We wondered whether this NPC assembly mode also depends on Nup153 and specifically on its membrane targeting capability. Membranes were incubated with control or Nup153-depleted cytosol, re-isolated, and analyzed by western blotting (Figures 6A and 6B). As expected, addition of RanQ69L strongly induced AL formation in control extracts, evidenced by an increased presence of Nup107, Nup62, and Nup53

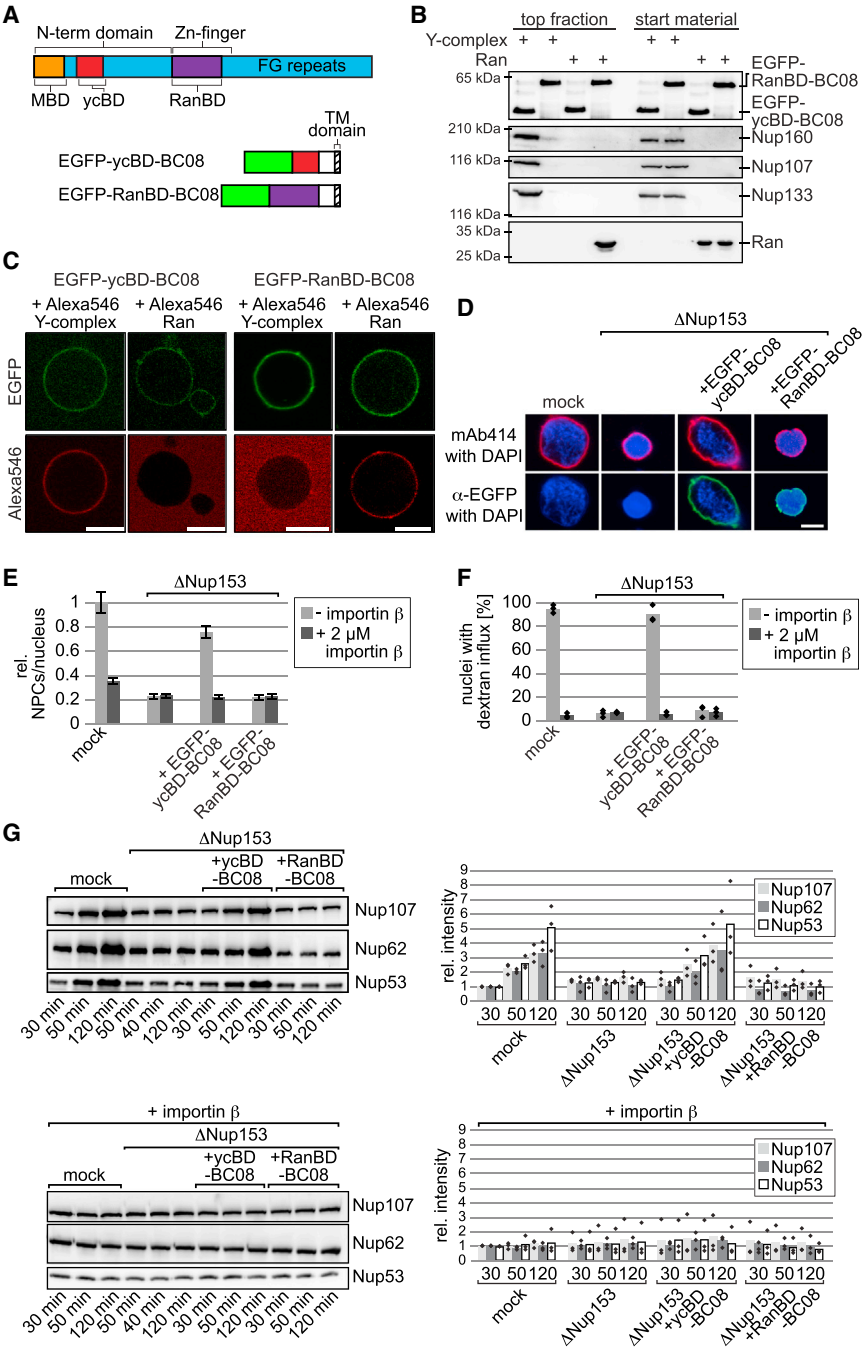

Figure 5. Inner Nuclear Membrane Tethering of the Y-Complex Bypasses the Nup153 Necessity for Interphasic NPC Assembly

(A) Schematic representation of the Nup153 fusion constructs. The Y-complex and the Ran-interacting region, flanked by EGFP (green) and the inner nuclear membrane protein BC08, yield EGFP-ycBD-BC08 or EGFP-RanBD-BC08, respectively.

(B) EGFP-ycBD-BC08 or EGFP-RanBD-BC08 was reconstituted into NE liposomes and incubated with Y-complex or Ran. Start material (50% for the EGFP-ycBD-BC08 or EGFP-RanBD-BC08, 30% for the Y-complex and Ran) and top fractions were analyzed using EGFP, Nup160, Nup133, Nup107, and Ran antibodies.

(C) EGFP-ycBD-BC08 or EGFP-RanBD-BC08 were reconstituted into NE lipid GUVs and membrane recruitment of Alexa-546-labeled Y-complex or Ran was analyzed.

(D) Nuclei were assembled in mock or Nup153-depleted extracts supplemented with empty, EGFP-ycBD-BC08, or EGFP-RanBD-BC08-containing liposomes. Samples were fixed after 120 min and visualized using EGFP (green) and immunofluorescence for mAB414 (red). DNA was stained with DAPI (blue).

(E) NPC numbers in nuclei assembled as in (D) were determined using mAB414 staining as in Figure 3B (average from 30 nuclei from three independent experiments).

(F) Nuclei were assembled as in (D) and interphasic NPC assembly analyzed by dextran influx as in Figure 3C.

(G) Nuclei were assembled as in (D). Chromatin was re-isolated at indicated time points, and nucleoporin levels were determined as in Figure 3D.

Bars, 10 μm. See also Figure S4.

in the re-isolated membrane fraction. The transmembrane pore proteins POM121, GP210, and NDC1 were found in equal quantities, independent of the presence of RanQ69L. Reticulon 4, an ER marker, was used to control for equal membrane re-isolation efficiency. Nup153 depletion severely reduced the quantity of soluble nucleoporins re-isolated with membranes. Furthermore, the addition of RanQ69L did not result in increased re-isolation of the soluble nucleoporins as was seen in mock-depleted extracts, indicating a block in AL formation. Addition of wild-type Nup153, but not the Nup153 mutant defective for direct membrane binding, to depleted extracts rescued AL formation. Analysis of the re-isolated membrane fraction by electron microscopy showed AL in mock and Nup153-depleted extracts supplemented with the wild-type protein but not in depleted extracts supplemented with the V47E mutant (Figure 6C). Thus, these data indicate that Nup153 membrane binding is also required for NPC assembly in the ER.

Transportin Regulates Nup153 Membrane Interaction

Having identified a Nup153 membrane interaction that is crucial for both interphasic NPC assembly and AL formation, we wondered why the protein does not localize to cytoplasmic membranes under normal growth conditions but is rather specifically found at the NE (Figures 1F, S1C, and 2A). The N-terminal region of Nup153 interacts with transportin and mediates its import to the nucleus, a pre-requisite for its incorporation in NPCs (Bastos et al., 1996; Enarson et al., 1998; Nakielny et al., 1999; Shah and Forbes, 1998; Figure S5). Rapid transportin-dependent import of Nup153 might prevent its membrane association outside of the nucleus. We wondered whether transportin interaction could also directly affect Nup153 membrane binding similar to

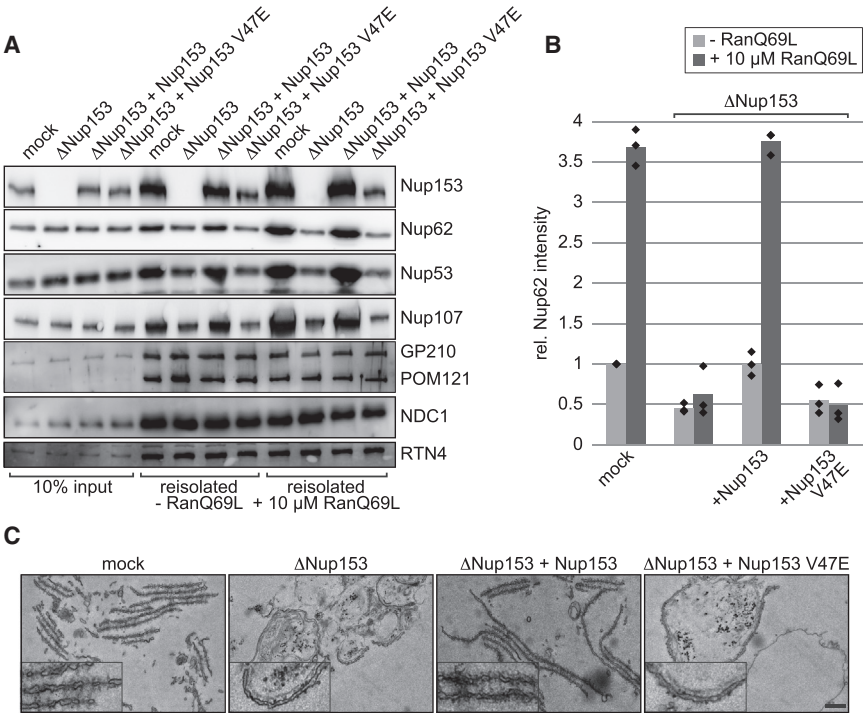

Figure 6. Nup153 Membrane Interaction Is Required for AL Formation

(A) Mock, Nup153-depleted cytosol, or Nup153-depleted cytosol supplemented with Nup153 or the V47E mutant were incubated for 4 hr with membranes and where indicated with RanQ69L. 10% of the start material and the re-isolated membranes were analyzed. Reticulon 4 (RTN4) serves as an ER marker.

(B) Quantification of Nup62 re-isolated with membranes (average intensity value from three independent experiments performed as in A), normalized to mock control in the absence of RanQ69L).

(C) Transmission electron microscopy of re-isolated membranes as in (A) in the presence of 10 μM RanQ69L. Insets show a 3-fold higher magnification. Bar, 500 nm.

Kap123, which regulates membrane interaction of the spindle body component Nbp1p and the C-terminal domain of the transmembrane nucleoporin Pom33p in yeast (Floch et al., 2015; Kupke et al., 2011). To test this, the N terminus of Nup153 was employed in liposome flotation assays after pre-incubation with transportin. The presence of transportin strongly reduced the ability of Nup153 to interact with membranes (Figure 7A). Importin β, a related import receptor that does not bind to this region, had no effect on the membrane binding of Nup153. Addition of RanQ69L, which releases import receptors including transportin from their cargos, reversed the inhibitory effect of transportin. Together, these data indicate that transportin inhibits Nup153 membrane binding and that this block is released by RanGTP. As high RanGTP concentrations are found in the nucleus, it is conceivable that Nup153 can only function as a membrane-interacting protein once it has reached the nucleus.

DISCUSSION

Here, we show that Nup153 can directly interact with membranes via an N-terminal amphipathic helix. This membrane interaction is important for interphasic NPC assembly as well as AL formation. During interphasic NPC assembly, Nup153 recruits the Y-complex, a crucial structural component of newly forming pores, to the inner nuclear membrane. Transportin binding to Nup153 inhibits its membrane interaction presumably by masking the membrane interaction surface of Nup153. Taken together, our results imply a model in which transportin binding to Nup153, following its synthesis in the cytoplasm, would prevent Nup153 from interacting with membranes outside of the nucleus (Figure 7B). After translocation through NPCs, Nup153 is released from transportin due to high nucleoplasmic concentrations of RanGTP. The liberated Nup153 interacts with the inner nuclear membrane and recruits the Y-complex to this locality where it functions in interphasic NPC assembly.

NPC assembly, both at the end of mitosis and in interphase, is regulated by Ran and transport receptors (D'Angelo et al., 2006; Walther et al., 2003b). At the end of mitosis, the chromatin binding nucleoporin MEL28 is a critical Ran-regulated target (Franz et al., 2007; Rotem et al., 2009). Our data suggest that Nup153 acts during interphasic NPC assembly as an important Ran target. In the different assembly modes, MEL28 or Nup153, once liberated from the inhibitory effect of importin β or transportin, could recruit the Y-complex to NPC assembly sites.

The Y-complex as a basic structural component of the NPC is crucial for NPC formation both at the end of mitosis and during interphase (D'Angelo et al., 2006; Doucet et al., 2010; Harel et al., 2003; Walther et al., 2003a). Nup153 is dispensable for NPC assembly at the end of mitosis (Figure 2) as previously observed (Walther et al., 2001). Although NPCs cluster upon Nup153 depletion, the number of NPC assembled by the postmitotic mode are most likely not reduced. Nuclei reisolated after 30 min, at which point most NPCs would have been assembled in the postmitotic mode, do not contain strikingly different quantities of Nup107, Nup62, or Nup53 when Nup153 is depleted (Figure 3D). Furthermore, the fact that nuclear import of both M9 and transmembrane cargoes is not affected at this time point, argues for comparable NPC numbers. At the end of mitosis, NPC assembly is initiated on the decondensing chromatin by MEL28, which recruits the Y-complex (Franz et al., 2007; Gillespie et al., 2007; Rasala et al., 2006; Figure 7B). However, MEL28 has been reported to be dispensable for interphasic NPC assembly (Doucet et al., 2010), most likely because it is initiated at the NE. During interphase, it is Nup153 that acts as the crucial Y-complex recruitment factor at the inner nuclear membrane in a, presumably, chromatin-independent manner. Accordingly, AL formation is Nup153 dependent but does not require MEL28, as it is initiated at the membrane in the absence of chromatin. Loss of MEL28 actually induces AL formation,

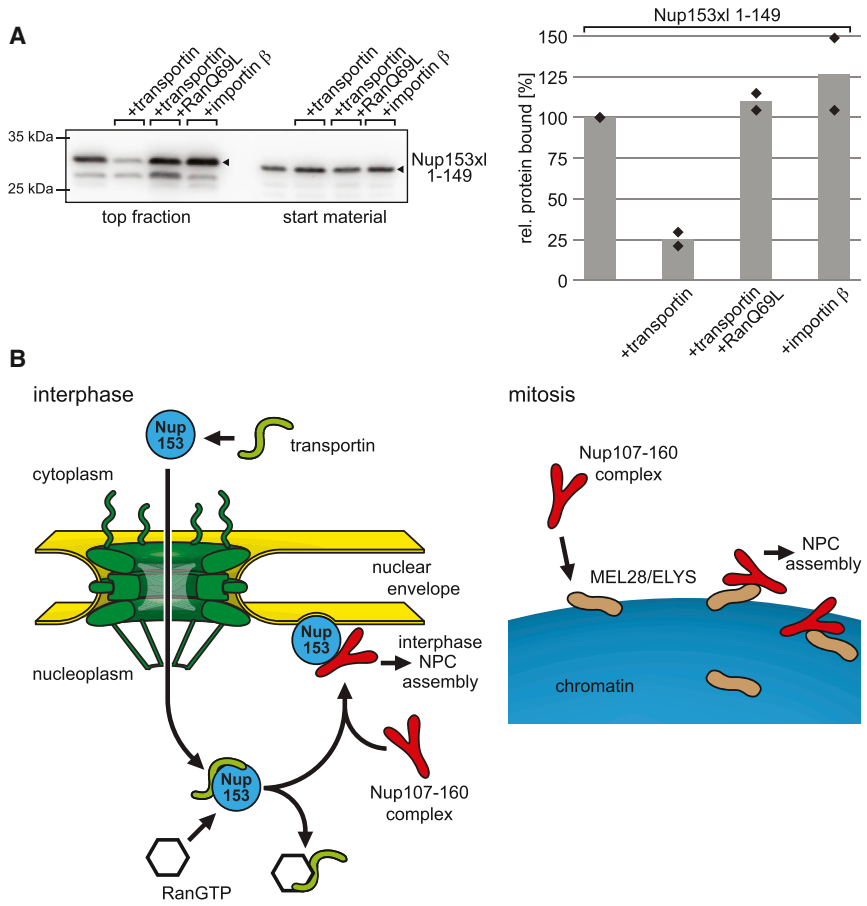

Figure 7. Transportin Regulates Nup153 Membrane Interaction and Might by that Function in Interphasic NPC Assembly

(A) 3 μM of the *Xenopus* Nup153 N terminus was, where indicated, pre-incubated with 5 μM transportin, 5 μM importin β, or 5 μM transportin along with 15 μM RanQ69L. Proteins were incubated with fluorescently labeled NE liposomes and floated through a sucrose gradient. Top gradient fractions and input materials were analyzed by western blot. For quantification from two independent experiments, liposome binding was normalized to the untreated Nup153 N terminus.

(B) Model for transportin and Nup153 function in interphasic NPC assembly (left panel). Transportin binding to Nup153 in the cytoplasm prevents its membrane interaction and mediates its nuclear import. In the nucleus, RanGTP releases transportin from Nup153, which becomes free to interact with the inner nuclear membrane and to recruit the Y-complex for interphasic NPC assembly. In contrast, at the end of mitosis (right panel), MEL28/ELYS recruits the Y-complex to chromatin and NPC assembly sites without an essential contribution from Nup153.

See also Figure S5.

presumably because it prevents postmitotic NPC assembly from being initiated on the chromatin (Franz et al., 2007).

Although Nup133, a component of the Y-complex, possesses an evolutionary conserved amphipathic helix (Drin et al., 2007; Kim et al., 2014), it does not seem to be sufficient to target the Y-complex to the inner nuclear membrane during interphasic NPC assembly. One possible explanation is that the Nup153 and Nup133 membrane interaction motifs need to act together to possess sufficient affinity for the inner nuclear membrane. In addition, other interactions, such as those occurring between the transmembrane nucleoporin POM121 and the Y-complex (Mitchell et al., 2010; Yavuz et al., 2010) might contribute. While a fragment containing this Nup133 motif does bind liposomes (Figure 1A), the assembled Y-complex does not detectably bind liposomes or GUVs (Figures 5B and 5C), which might, however, be explained by the larger vesicle sizes (Figure S4A).

Whether the Nup153 mediated membrane recruitment of the Y-complex is the initial step of interphasic NPC assembly is an open question. Due to the experimental setup of the interphasic NPC formation assay, it is difficult to determine the precise assembly order as it was done for the postmitotic NPC formation pathway (described in Schooley et al., 2012). The amphipathic helix of Nup133 has been proposed to target the Y-complex to the highly curved pore membrane during interphasic NPC assembly (Doucet et al., 2010). The amphipathic helix of Nup153 shows a similar preference for high curvature (Figure 1B) and can induce membrane tubulation in vitro (Figure S1B). However, it is of course difficult to distinguish whether these observations represent a curvature sensing or inducing function. In other words, it is unclear whether Nup153 itself initiates interphasic pore assembly by inducing membrane curvature or whether it binds already curved membranes. If Nup153 only binds highly curved membranes, its recruitment would necessarily be preceded by proteins inducing pore formation, such as Nup53, reticulons, and POM121 (Dawson et al., 2009; Doucet et al., 2010; Dultz and Ellenberg, 2010; Vollmer et al., 2012). Interestingly, the fact that the fusion of Nup153's Y-complex binding region to an inner nuclear membrane protein can replace Nup153 in interphasic NPC assembly suggest that Nup153 induced membrane curving might not be critical for its function in interphasic NPC formation.

In summary, our work identifies a crucial function for Nup153 in interphasic NPC assembly. We provide insight on an interesting mechanistic difference between NPC assembly at the end of mitosis and during interphase. Whereas postmitotic assembly is initiated by the MEL28-mediated recruitment of the Y-complex to chromatin, interphasic NPC formation crucially requires Nup153 to interact with the inner nuclear membrane to localize the Y-complex to nascent assembly sites. It is currently unclear whether Nup153 recognizes a distinct feature at the site of the newly forming NPC, and this will be an interesting avenue for future research.

EXPERIMENTAL PROCEDURES

Nuclear assemblies, immunofluorescence, electron microscopy, generation of affinity resins, sperm heads, and floated unlabeled or DiIC18-labeled

membranes were carried out as described (Eisenhardt et al., 2014). Interphasic NPC assembly using dextran influx was performed as in Vollmer et al. (2012). Nuclear import assays using EGFP-NPM2 (importin α/β-dependent cargo), EGFP-Nplc-M9-M10 (transportin-dependent cargo), and EGFP-LBR (aa 146–258), re-isolation of nuclei for western blotting, as well as NPC counting are described in Theerthagiri et al. (2010). Liposome generation and flotations were done as in Eisenhardt et al. (2014) and Vollmer et al. (2012). Pure lipid and EGFP-ycBD-BC08/EGFP-RanBD-BC08-containing GUVs were generated as described (Lorenz et al., 2015); see also Supplemental Experimental Procedures in the Supplemental Information.

To visualize the Y-complex on newly assembling NPCs, the purified complex was labeled with Alexa 488. 50 min after initiation of the nuclear assembly reaction, 10 μl of the sample was supplemented with 0.25 μl Alexa-488-labeled Y-complex (approximately 0.05 μg/μl). Where indicated, interphasic NPC assembly was inhibited by addition of 2 μM importin β at this time point. After an additional 60 min, 0.15 μl 0.25 μg/μl Alexa-594-labeled WGA (Life Technologies) was added. Samples were fixed after additional 10 min in 2% paraformaldehyde in 80 mM Pipes (pH 6.8), 1 mM $MgCl_2$, and 150 mM sucrose and spun through a sucrose cushion on poly-L-lysine coated coverslips. Samples were imaged with a confocal microscope LSM780 (Zeiss) equipped with an Apochromat 63×/1.40 Oil DIC M27 objective with the following acquisition settings: 24-μm pinhole for track 488 nm and 16-μm pinhole for track 561 nm; scaling X = 0.082 μm, Y = 0.082 μm, Z = 0.236 μm; zoom = 5.0–7.5× and 19 to 45 slices per nucleus. 5 μm square of the best in focus slice at the bottom surface of the nucleus was selected, and the two channels were merged.

For quantifying nucleoporin levels on assembling nuclei, chromatin was isolated following a slightly modified version of the protocol described in (Baur et al., 2007). 20 μl of a nuclear assembly reaction was diluted in 1 ml sperm isolation buffer (20 mM Tris-HCl [pH 7.4], 70 mM KCl, 10 mM EDTA, 2 mM DTT, and 2% polyvinylpyrolidone), and chromatin was recovered by centrifugation through 0.5 ml of a 1.3 M sucrose cushion at 5000 × *g* for 10 min in an Eppendorf cooling centrifuge. After carefully removing the supernatant, the nuclei were re-suspended in 20 μl SDS sample buffer and analyzed by western blotting using α-Nup107, α-Nup53, and mAB414 antibodies (to detect Nup62). To block interphasic NPC assembly, we added 2 μM importin β 30 min after initiation of the assembly reaction (i.e., 20 min after addition of membranes) as postmitotic NPC assembly is usually completed at this time point (i.e., chromatin is by then enclosed by an nuclear envelope).

AL were assembled in 15 μl *Xenopus* egg extract cytosol supplemented with 1.5 μl floated membranes, glycogen, and an energy regenerating system (Eisenhardt et al., 2014). After 4 hr at 20°C, samples were processed for electron microscopy or diluted in 1 ml sucrose buffer. Membranes were pelleted by centrifugation (10 min at 15,000 × *g*), washed, and analyzed by western blotting.

Antibodies, protein expression, and purification as well as the transfection experiments are described in detail in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.04.027.

AUTHOR CONTRIBUTIONS

B.V. and W.A. designed the experiments. B.V. performed liposome floatation experiments, B.V. and M.B. purified proteins, M.L. determined NPC numbers, M.L. and D.M.-A. performed live-cell imaging and WGA-based NPC labeling experiments, P.D.M., S.A.A., and A.S. performed nuclear assembly and related experiments, M.F. performed electron microscopy, S.L. performed light scattering of liposomes, and W.A. wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by core funding of the Max Planck Society and the European Research Council (grant agreement 309528 CHROMDECON to W.A.) and by a PhD Fellowship of the IMPRS "From Molecules to Organisms" to P.D.M.

Received: November 10, 2014
Revised: February 27, 2015
Accepted: April 28, 2015
Published: June 4, 2015

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Developmental Cell

Supplemental Information

Nup153 Recruits the Nup107-160 Complex to the Inner Nuclear Membrane for Interphasic Nuclear Pore Complex Assembly

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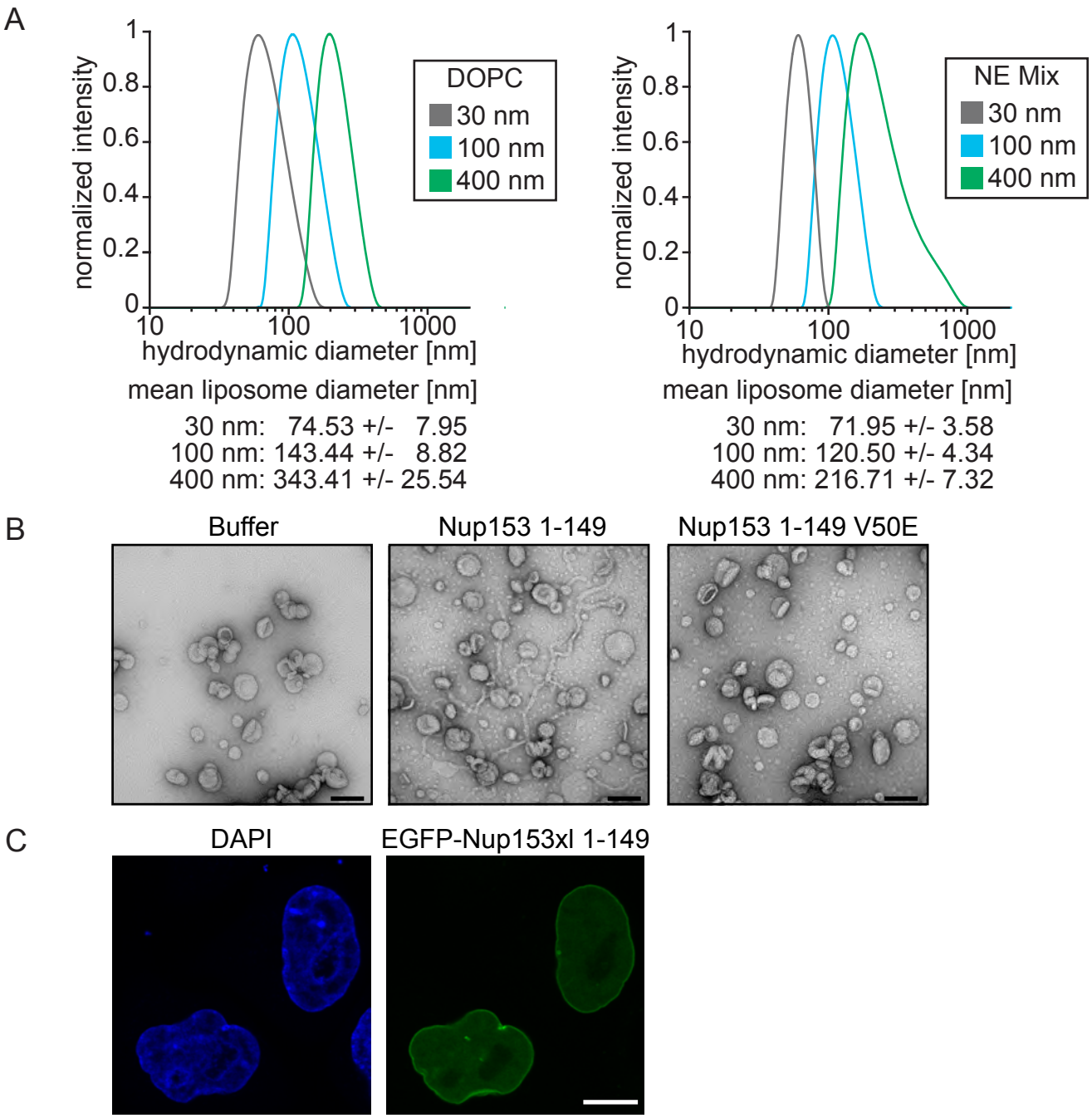


Figure S1, related to Figure 1

(A) Exemplary measurement of the size distribution of DOPC and NE liposomes used in Figure 1A and B as determined by light scattering. The mean diameters of the different sized liposome preparations are indicated below.

(B) The N-terminus of Nup153 can deform membranes: 3 µM *Xenopus* Nup153 N-terminus (aa 1-149) or the corresponding V50E mutant was incubated with 1 mg/ml Folch Fraction I liposomes (average diameter approx. 70 nm). The samples were analyzed on copper grids by transmission electron microscopy on a FEI Technai spirit 120 kV microscope as in (Vollmer et al., 2012). Please note the liposome tubulation in the presence of Nup153 N-terminus, which is not induced by the membrane binding defective V50E mutant or the buffer control. Bar: 100 nm.

(C) EGFP-tagged Nup153 N-terminus localizes to the NE: Images show HeLa cells with lower expression levels of EGFP-tagged Nup153 N-terminus as compared to Figure 1F. DNA was stained with DAPI (blue). Bar: 10 µm.

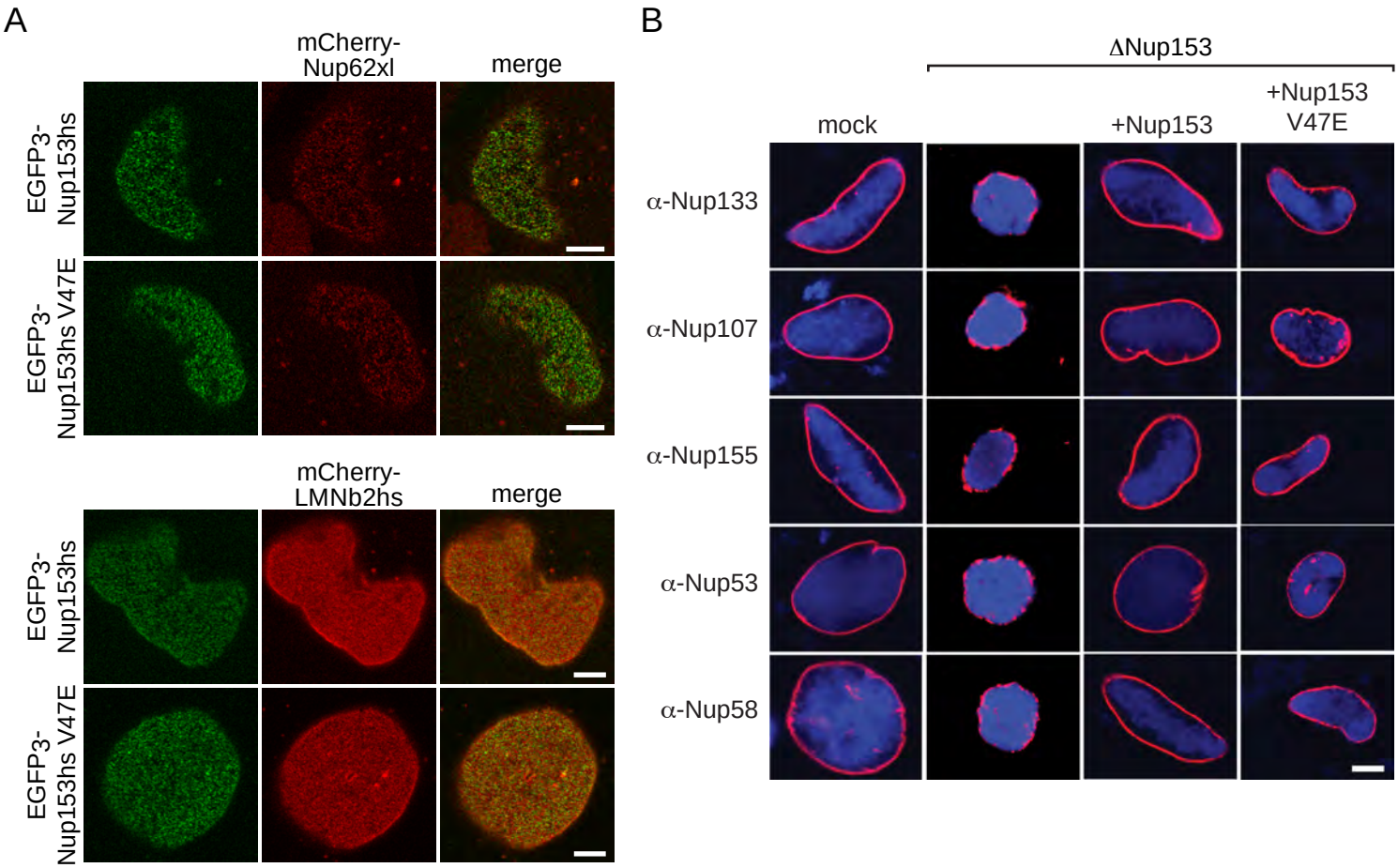


Figure S2, related to Figure 2

(A) Complete images of the nuclear surface of HeLa cells co-transfected with triple-EGFP-tagged human Nup153 or the corresponding V47E mutant and mCherry-Nup62 or mCherry-lamin B which are shown as insets in Figure 2A. Bars: 5 µm.

(B) Nuclei were assembled for 120 min in mock, Nup153 depleted (ΔNup153) or Nup153 depleted extracts supplemented with recombinant wild type protein (Nup153) or the membrane-binding mutant (Nup153 V47E). Samples were analyzed by immunofluorescence for the presence of Nup107 and Nup133 (nucleoporins of the Y-complex), Nup155 and Nup53 (nucleoporins of the Nup93-complex) and Nup58 (a central channel nucleoporin). DNA was stained with DAPI (blue). Bar 10 µm.

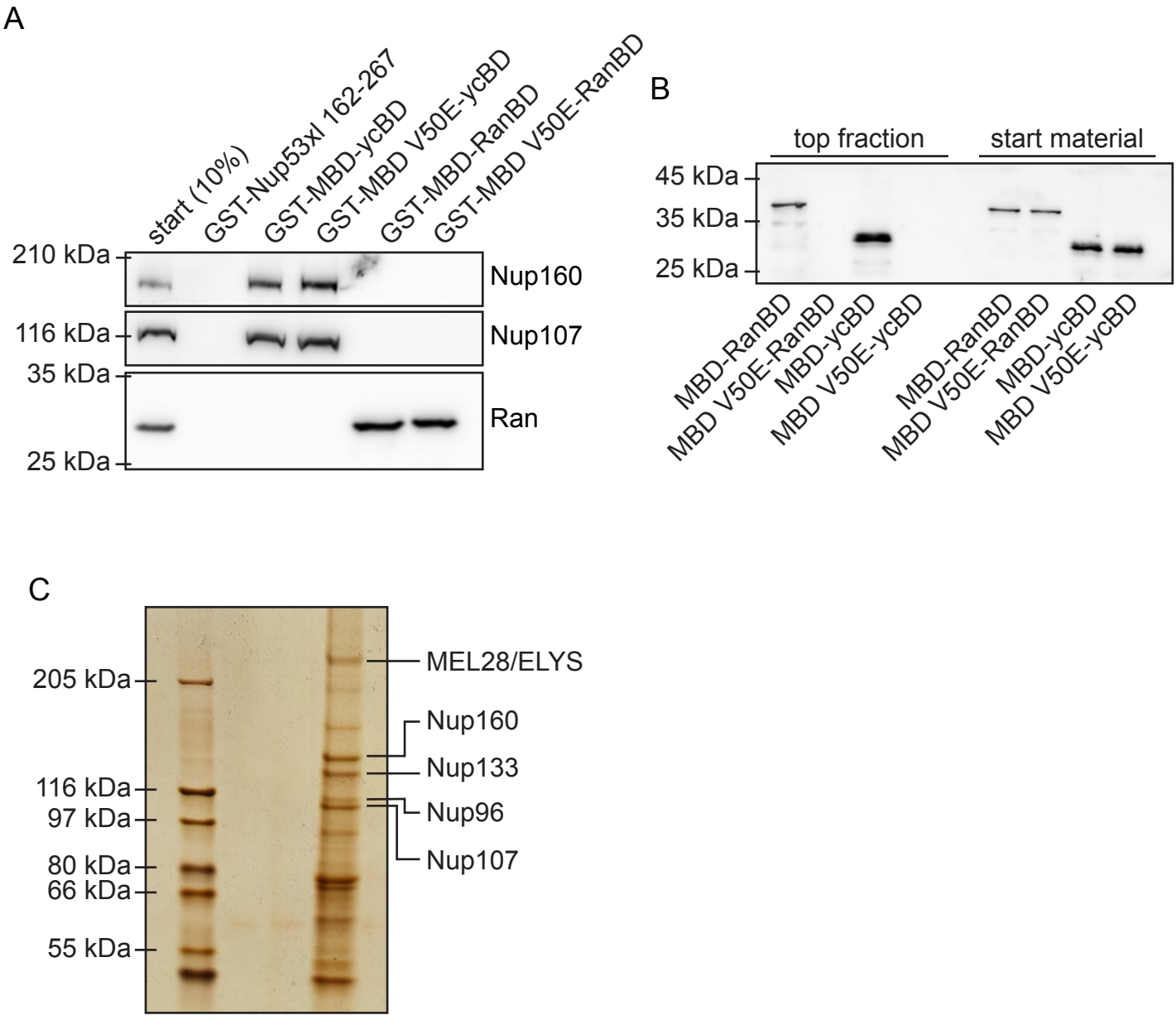
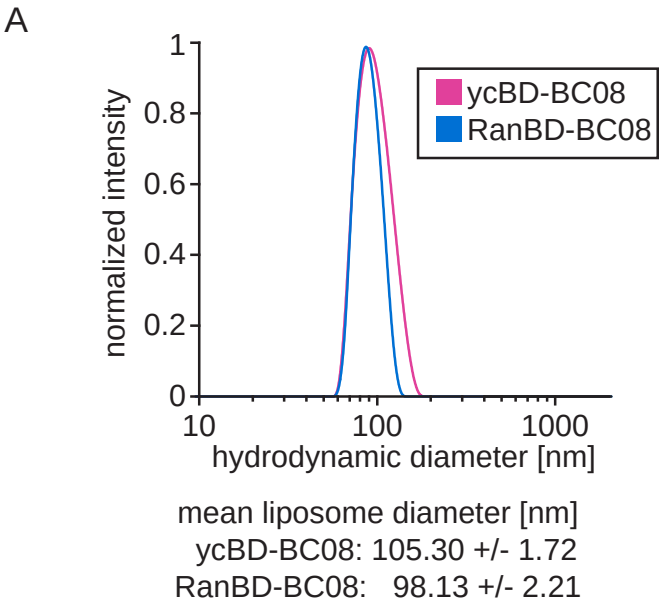


Figure S3, related to Figure 4

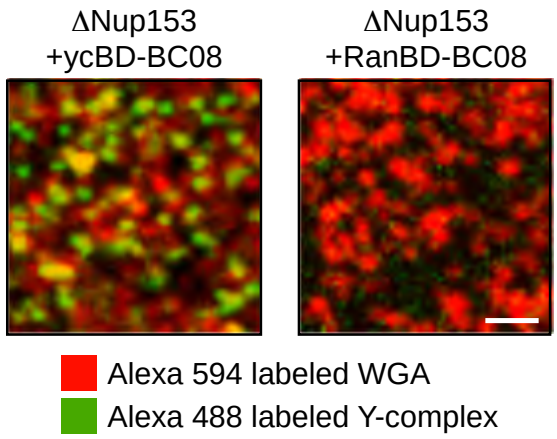
(A) GST-fusions of the RRM domain of *Xenopus* Nup53 (aa 162-267) (Vollmer et al., 2012) as a control and GST-fusions of the MBD-ycBD and MBD-RanBD constructs as well as the corresponding membrane binding mutants (V50E), were incubated with cytosol from *Xenopus* egg extracts. Eluates were analyzed by western blotting with antibodies against Nup160, Nup107 and Ran.

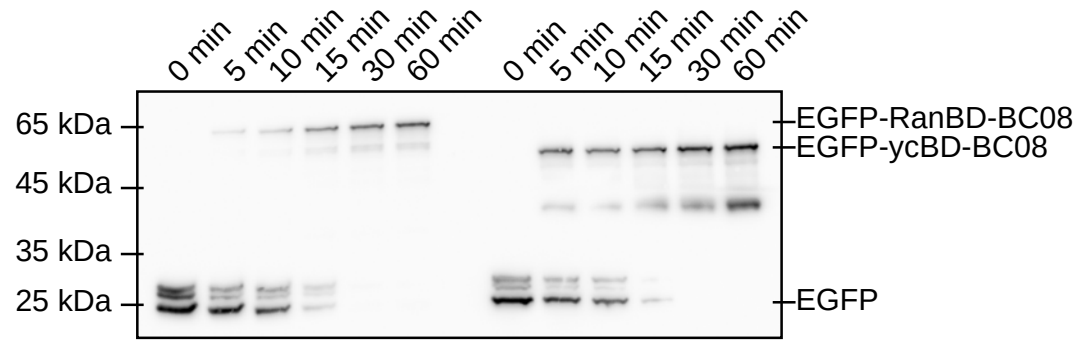
(B) 3 µM of the MBD-ycBD and MBD-RanBD fusion proteins as well as the corresponding membrane binding mutants (V50E) were incubated with fluorescently labeled 70 nm NE liposomes and floated through a sucrose gradient. Top gradient fractions and input materials were analyzed by western blot with the α-Nup153 antibody which is directed against the MBD.

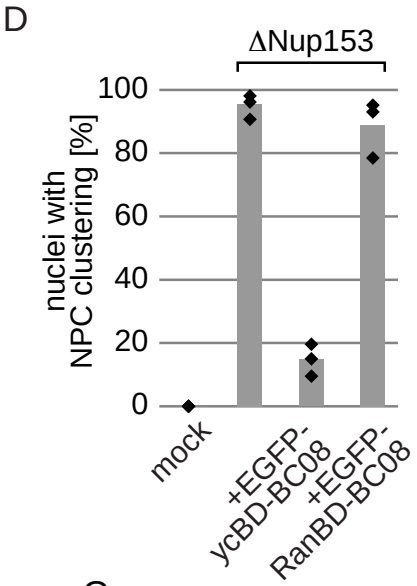
(C) Silverstaining of the purified Y-complex. The Y-complex was purified from *Xenopus* egg extracts using TAP-tagged Nup98 (Walther et al., 2003). After TEV protease elution the complex was separated on a 5%-12% SDS-PAGE.

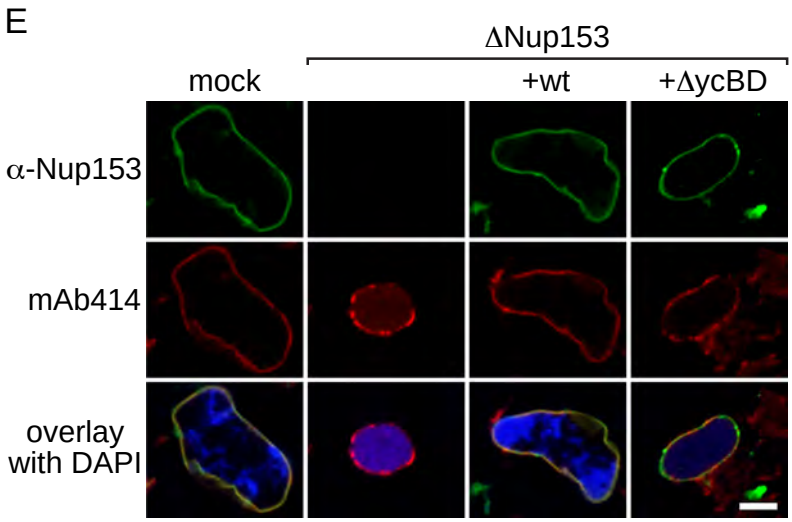


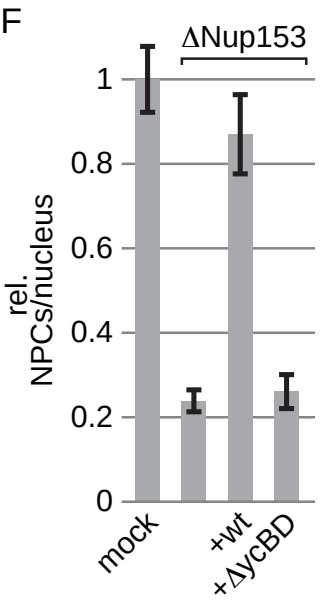
mean liposome diameter [nm]
ycBD-BC08: 105.30 +/- 1.72
RanBD-BC08: 98.13 +/- 2.21











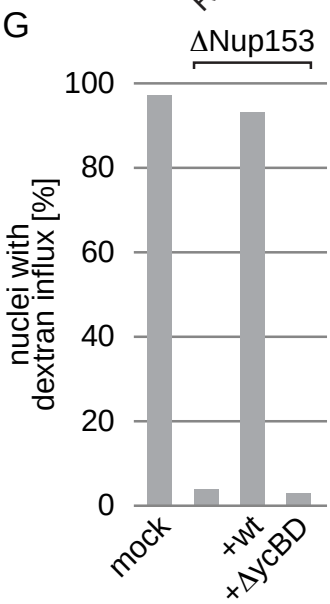


Figure S4, related to Figure 5

(A) Size distribution of EGFP-ycBD-BC08 and EGFP-RanBD-BC08 proteo-liposomes used in Figure 5B and D as determined by light scattering. The mean liposome diameter of the two preparations is indicated.

(B) The ycBD-BC08 fusion recruits the Y-complex to NPC assembly sites. Nuclei were assembled in Nup153 depleted extracts supplemented with ycBD-BC08 or RanBD-BC08 containing liposomes (the EGFP tag was cleaved before liposome reconstitution to avoid interference with the Alexa-488 labeled Y-complex signal). NPCs are visualized with Alexa-594 labeled WGA (red in overlay) and, the purified labeled Y-complex is shown in green. Bar: 1 µm.

(C) The EGFP-RanBD-BC08 and EGFP-ycBD-BC08 fusions translocate to the inner nuclear membrane as determined by a protease protection assay described in (Theerthagiri et al., 2010). Nuclei were assembled in 60 µl *Xenopus* egg extracts. After 50 min, i.e. when a closed NE has formed, proteo-liposomes containing the EGFP-RanBD-BC08 or EGFP-ycBD-BC08 fusions were added. At indicated time points after liposome addition, a 10 µl sample was taken, NusA fused TEV protease was added, and protease cleavage was stopped by addition of SDS sample buffer and boiling. Protease protection of the reporter was analyzed by Western blotting. The position of the un-cleaved (EGFP-RanBD-BC08 and EGFP-ycBD-BC08) and the cleaved EGFP reporter are indicated. Quantification of the EGFP signal reveals a half time of inner nuclear membrane transport of 6.7 min and 7.3 min for EGFP-RanBD-BC08 or EGFP-ycBD-BC08, respectively.

(D) Quantification of nuclei assembled as in Figure 5D with NPC clustering identified by mAB414 staining (three independent experiments with 100 chromatin substrates each). Please note the rescue of the NPC clustering phenotype by the EGFP-ycBD-BC08 construct probably because in this situation newly formed NPCs are inserted in all areas of the nuclear envelope.

(E) Nuclei were assembled for 120 min in mock, Nup153 depleted (ΔNup153) or Nup153 depleted extracts supplemented with recombinant wild type protein (wt) or a Nup153 deletion mutant lacking the Y-complex interaction site (ΔycBD, human Nup153 lacking aa 211-337, see extended experimental procedure section). Samples were analyzed by immunofluorescence with α-Nup153 and mAB414 antibodies. DNA was stained with DAPI (blue). Bar 10 µm

(F) NPC numbers in nuclei assembled as in (B) were determined using mAB414 staining (average from 10 nuclei, normalized to the mock control, error bars are SEM).

(G) Nuclei were assembled as in (B) and interphasic NPC assembly on more than 100 nuclei was analyzed by dextran influx.

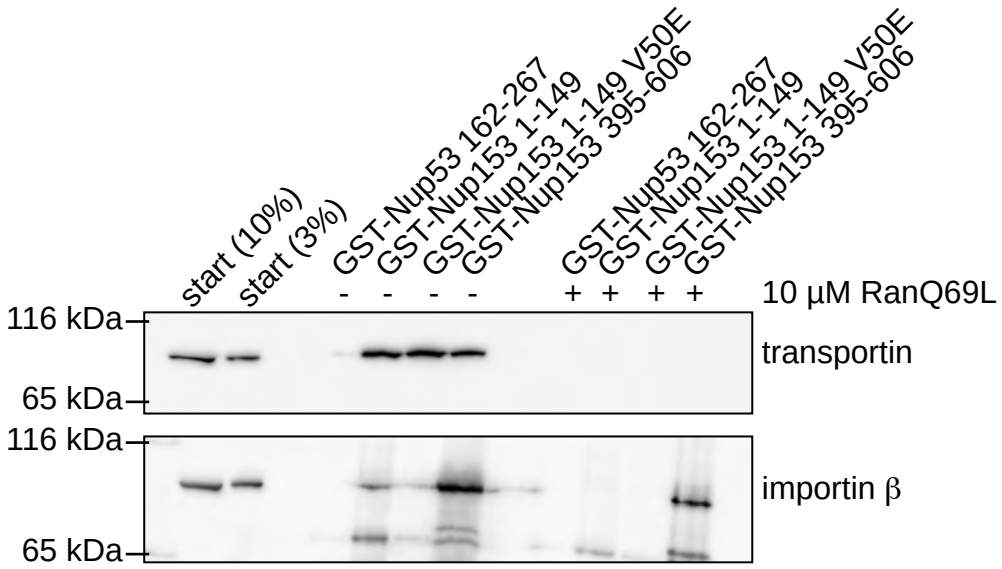


Figure S5, related to Figure 7

GST-fusions of the RRM domain of *Xenopus* Nup53 (aa 162-267) (Vollmer et al., 2012) as a control, of *Xenopus* Nup153 (aa-149), the corresponding membrane binding mutant (V50E), or of a *Xenopus* Nup153 fragment (aa 395-608) that is known to bind Nup50 (Makise et al., 2012), were incubated with cytosol from *Xenopus* egg extracts. Where indicated, 10 µM RanQ69L was added to the incubation. Eluates were analyzed by western blotting with antibodies against transportin and importin β. Please note that transportin binding to the Nup153 N-terminal fragment is not affected by the V50E mutation. The Nup153 aa 395-608 fragment binds transportin, consistent with a previous report that mapped a transportin binding site to a partially overlapping Nup153 fragment (aa 440-720) (Shah and Forbes, 1998)

Supplemental Movie 1

3D reconstruction (generated with IMARIS) of confocal stacked images of a representative HeLa cell transfected with EGFP-Nup153xl 1-149, as shown in Figure 1F.

Supplemental Experimental Procedures

DiIC18 (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate), DiDC18 (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine Perchlorate), fluorescently labeled dextrans, Alexa dyes and secondary antibodies were obtained from Life Technologies, detergents from EMD, and lipids from Avanti Polar Lipids.

Antibodies

Antibodies against Nup107 (Walther et al., 2003), GP210 (Antonin et al., 2005), NDC1 (Mansfeld et al., 2006), Nup160 (Franz et al, 2007), Nup53 and LBR (Theerthagiri et al., 2010) and Nup58 (Sachdev et al., 2012) have been described. mAB414 (Babco), transportin and Ran (558660 and 610341, BD Bioscience), EGFP (11814460001, Roche), as well as TPR, human lamin A and B2 antibodies (ab58344, ab26300, ab151735 abcam) were purchased. Antibodies for POM121, RTN4, Nup153 and Nup133, were generated against recombinant fragments of the respective proteins (*Xenopus* POM121 aa 1-314, RTN4 aa 763-1043 and Nup153 aa 1-149, human Nup133 aa 67-514). Antibodies for Nup155, Nup50, importin β, lamin B (expressed as GFP-laminB3, kind gift from Rebecca Heald) are against the *Xenopus* full-length proteins, *Xenopus* importin α and BC08 antibodies are a kind gift from Iain Mattaj.

Protein expression and purification

Constructs for the *Xenopus* Nup153 N-terminus (aa 1-149) were generated from synthetic DNA optimized for codon usage in *E. coli* (Geneart), human full-length constructs from EGFP3-hNup153 (Rabut et al., 2004) and the corresponding V50E or V47E mutants by mutagenesis using QuikChange site-directed mutagenesis kit (Agilent). The Nup153 N-terminus (wildtype and V50E mutant) was cloned into a modified pET28a vector with a yeast SUMO solubility tag followed by a TEV site or into a modified pET28a vector with EGFP upstream of Nup153. MBD-ycBD and MBD-RanBD fusions as well as the corresponding membrane binding mutants were generated by insertion of human Nup153 fragments (aa 210-338 or aa 658-890) into the above mentioned constructs of the *Xenopus* Nup153 N-terminus (wildtype and V50E mutant) in the SUMO containing pET28a vector.

The Nup153 mutant lacking the Y-complex binding domain (Nup153 ΔycBD, Figure S3) was generated by cloning sequentially the N-terminal 210 aa of human Nup153 and the C-terminal Nup153 portion starting from aa 338 into the SUMO containing pET28a vector. Both fragments are joined by a GGSKLGGS linker.

Proteins were expressed in *E. coli* and purified using Ni-agarose. His_6- and SUMO-tags were cleaved using TEV protease and proteins were concentrated using VIVASPIN columns (Sartorius) and separated by gelfiltration (Superdex200 10/300 GL or Superdex200 PC 3.2/30, GE Healthcare) in HEPES buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1mM DTT). SUMO and EGFP were expressed and purified from the corresponding empty vectors. Human Nup133 (aa 67-514) was generated as described (Vollmer et al., 2012).

Y-complex was purified from *Xenopus* egg extracts using TAP-tagged Nup98 (Walther et al., 2003) and labelled using Alexa Fluor 546 carboxylic acid succinimidyl ester in 200 mM $NaHCO_3$ pH 8.4. Human RanQ69L was expressed from a modified pET28a vector with a His_6-GST tag, which was cleaved of using TEV protease. The protein was separated from the tag after dialysis using Ni-agarose and further purified by gelfiltration (Superdex200 10/300 GL). Purified ranQ69L was labelled using Alexa Fluor 546 C5 maleimide in HEPES buffer. Excess dye was removed by gelfiltration (Superdex200 PC 3.2/30).

EGFP-ycBD-BC08 or EGFP-RanBD-BC08 where generated by insertion of human Nup153 fragments (aa 210-338 or aa 658-890) into an BC08 reporter construct (Theerthagiri et al., 2010) between EGFP and BC08. The corresponding constructs were expressed, purified and reconstituted into small unilamellar liposomes (see below).

Transfection experiments

Plasmids encoding the *Xenopus* Nup153 N-terminus and the corresponding V50E mutant (cloned into a modified pEGFP-C3 vector) were transfected into HeLa cells using Fugene 6 (Roche) following the manufacturer's instructions. After 24 h cells were fixed and analyzed by confocal microscopy. For immunoprecipitations, EGFP3-hNup153 constructs were transfected into HEK293 cells. 24 h post-transfection cells were harvested and lysed in lysis buffer (50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100 supplemented with protease inhibitors (2 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotinin, 0.1 mg/ml AEBSF final concentration) for 30 min at 4°C. After centrifugation for 15 min at 15.000 x g the supernatant was immunoprecipitated with GFP-Trap beads (Chromotek) for 2 h, washed 5x with lysis buffer, 2x with lysis buffer supplemented with 500 mM NaCl, 2x with lysis buffer, and 1x with lysis buffer without Triton X-100 and finally eluted with SDS-sample buffer. Eluates and lysed cells (corresponding to 5% of the eluates) were analyzed. For colocalization experiments (Figure 2A), pEGFP3-Nup153hs or pEGFP3-Nup153hsV47E were co-transfected with mCherry-hLMNB2 (kind gift of Martin Hetzer) or mCherry-Nup62xl using jetPRIME (Polyplus transfection). 24 h after transfection live cells were imaged at 37°C with a confocal microscope LSM780 (Zeiss) equipped with incubation chamber and using an Apochromat 63x/1.40 Oil DIC M27 objective.

GST 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) was incubated with 300 µg of the indicated 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.

Liposome generation and flotation

NE lipid mixture - 60 mol% L-α-phosphatidylcholine, 19.8 mol% L-α-phosphatidylethanolamine, 10 mol% L-α-phosphatidylinositol, 5 mol% Cholesterol, 2.5 mol% Sphingomyelin, 2.5 mol% L-α-phosphatidylserine, 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) or DOPC mixture - 99.8 mol% 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOPC), 0.2 mol% 18:1-12:0 NBD-PE - were dissolved in chloroform to a final concentration of 1 mg/ml. Chloroform was evaporated in a glass vial under a low stream of Argon until an even lipid film formed followed by incubation under vacuum for 1-2 h. Liposomes were formed by gentle addition of HEPES buffer to a final concentration of 5 mg/ml. After 1 h of incubation at 45°C, flask was shaken to dissolve residual lipids. After ten cycles of freeze/thawing liposomes were either snap frozen in liquid nitrogen and stored at -80°C or directly

used. Different sized liposomes were formed by passing liposomes sequentially through Nuclepore Track-Etched Membranes (Whatman) with defined pore sizes (400, 100, 30 nm) at 45°C using the Avanti Mini-Extruder until desired size was reached. For 30 nm, liposomes were incubated in a sonication bath for 5 min before final extrusion. To ensure equal concentrations of different sized liposomes, fluorescence intensity was determined after extrusion using a Molecular Imager VersaDoc MP 4000 Imaging System and ImageJ. Concentrations were adjusted by dilution. Liposome sizes were determined by light scattering using the AvidNano W130i with 10 measurements per sample each for 10 sec and analyzed using the iSize software.
For liposome flotations proteins (6 µM) were mixed 1:1 with liposomes (5 mg/ml) and incubated for 30 min at 25°C. 75 µl of the protein/liposome mixture was brought to 37% sucrose concentration in a total volume of 150 µl. 1.7 ml 12% sucrose cushion in HEPES buffer was overlaid, followed by 300 µl HEPES buffer on top. Samples were spun for 2 h at 55 000 rpm in a TLS-55 rotor (Beckman) at 25°C. Liposomes containing top layers were collected (450 µ l). Fluorescence intensities of the start protein/liposome mixture and the top fraction were determined. Usual liposome recovery rates are 60%. Collected fractions were precipitated by the method described by Wessel & Flügge (Wessel and Flugge, 1984). To compare different samples, pellets were resuspended in normalized volumes of sample buffer according to the determined fluorescence signal. Binding efficiency was determined by Western Blot analysis using the Fusion Capt advance software, comparing band intensities of start materials with collected fractions.

Generation of GUVs and analysis

Detergent solubilized EGFP-ycBD-BC08 and EGFP-RanBD-BC08 were reconstituted in proteo-liposomes via gelfiltration (Eisenhardt et al., 2014). For this, 20 µl of the NE lipid mix without NBD-PE (30 mg/ml in 10% octylglucopyranoside) was mixed with 20 µl of 2 µM 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 room temperature. 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 NE lipid mix (10 mg/ml) and 0.8 nM DiDC18 by electroformation as described (Angelova and Dimitrov, 1986). Equal amounts of the lipid-chloroform solution (25 µg total lipids) were distributed onto two 5 mm x 5 mm platinum gauzes (ALS) and processed as described before. Electroformation time at 10 Hz was reduced to 70 min.
GUVs were visualized in an 8 well glass observation chamber (Chambered #1.0 Borosilicate Coverglass System, Lab-Tek) that 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. Soluble proteins were added to a final concentration of 500 nM. Proteins (25 nM for the purified labelled y-complex) and buffers used for GUV preparation matched the osmotic pressure of the sucrose solution using an osmometer. 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. 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 unit.

Dextran exclusion assay to monitor interphasic NPC formation

Nuclei were assembled by incubating 0.3 µl demembranted sperm heads (10.000/µl)(Gurdon, 1976) with 10 µl mock or depleted cytosol and, where indicated, recombinant proteins or proteo-liposomes. After 10 min, which allows for decondensation of sperm DNA, 0.2 µl floatation purified membranes from egg extracts, 0.2 µl 200 µg/µl glycogen, and 0.2 µl energy mix (50 mM ATP, 50 mM GTP, 500 mM creatine phosphate and 10 mg/ml creatine kinase in sucrose buffer [10 mM HEPES, 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.4]) was added. After additional 40 min, 20 µl of mock or Nup153 depleted extracts, which had been incubated for 20 min with 30 µl WGA-Agarose (Sigma) to remove the permeability barrier forming nucleoporins, were added. Where indicated 2 µM importin β was added to inhibit further interphasic NPC assembly together with the WGA-treated extracts. After additional 50 min, 0.6 µl of a solution containing 5 µg/µl streptavidin and 5 µg/µl biotinylated WGA (both from Life Technologies) was added in order to reduce the diffusion of 70kDa dextrans through intact pores (Dawson). After 10 min, 2.5 µl of a mixture containing 0.25 µg/µl Alexa-488 labelled WGA (Life Technologies) and 0.25 µg/µl Hoechst in sucrose buffer was added. Samples were directly mounted on microscope slides and analysed by fluorescence microscopy. Nuclei, identified by Hoechst staining, that excluded the fluorescent dextran were counted as negative for interphasic NPC assembly.

NPC counting using mAB414 labeling

Nuclei were assembled by incubating 0.3 µl demembranted sperm heads (10.000/µl) with 10 µl mock or depleted cytosol and, where indicated, recombinant proteins or proteo-liposomes. After 10 min, which allows for decondensation of sperm DNA, 0.2 µl floatation purified membranes from egg extracts, 0.2 µl 200 µg/µl glycogen, and 0.2 µl energy mix (50 mM ATP, 50 mM GTP, 500 mM creatine phosphate and 10 mg/ml creatine kinase in sucrose buffer) was added. After assembly for 120 min, samples were fixed with 4 % paraformaldehyde and 0,5% glutaraldehyde in 80 mM Pipes pH 6.8, 1 mM $MgCl_2$, 150 mM sucrose, spun through a sucrose cushion on Poly-L-Lysine coated coverslips and stained with mAB414 and Alexa-488 labelled α-mouse antibodies and DAPI. Where indicated, further interphasic NPC assembly was inhibited by addition of 2 µM importin β 50 min after initiating the assembly reaction. Although postmitotic NPC assembly is in the assembly reaction usually completed after 30 min (i.e. the time when a closed nuclear envelope has formed, 20 min after addition of membranes) we add also in this assay importin β at the 50 min time point to ensure that only interphasic NPC assembly is affected. The NPCs from 30 - 50 nuclei in each independent experiment were imaged by acquiring stacked images of the envelope surface as well as through entire nuclei. Image recording was performed using an inverted Olympus Fluoview 1000 confocal laser scanning system utilizing an UPlanSApo 60x/1.35 oil objective. DAPI was excited by a 405 nm DPSS laser and emission was collected between 425 to 475 nm. Alexa-488 was excited by an argon ion laser at 488 nm and emission was collected between 500 to 545 nm. The pinhole size was set to 80 µm.

NPCs were counted using Imaris 7.7.1 software (Bitplane Scientific solutions). Individual pores were detected using the semi-automatic spot detection function in surpass mode with the spot detection diameter set to 0.25 µm. The threshold was manually adjusted on the intensity max filter setting in each condition to ensure detection of individual spots. In the same samples, surface area was calculated with the surface function of Imaris based on the absolute intensity of the DAPI signal. NPCs per µm² were calculated, extrapolated to the total surface area of the individual nuclei and plotted as the total number of NPCs per nucleus relative to the mock control. We use this normalization as overall NPCs numbers vary between different experiments due to variability of the egg extract quality.

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Direct membrane binding of and a self-inhibitory interaction within nucleoporin Nup155 are required for nuclear pore complex formation

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Running title

Nup155 must bind membranes and self-inhibit for NPC formation

Keywords

Nuclear envelope, nuclear pore, Nup155, Nup53, Nup93, nuclear assembly

Summary statement

Nuclear pore complexes are huge protein assemblies that integrate into the two membranes of nuclear envelope. We show that membrane binding and an auto-inhibitory self-interaction within the nuclear pore complex component Nup155 are crucial for the assembly pathway of these complexes.

Abstract

Nuclear pore complexes (NPCs) are the gateways through the nuclear envelope. How they form into a structure containing three rings and integrate into the nuclear envelope remains a challenging paradigm for coordinated assembly of macro-complexes. In vertebrates, the cytoplasmic and nucleoplasmic rings of NPCs are mostly formed by multiple copies of the Nup107-Nup160 complex, whereas the central, or inner ring, is composed of Nup53, Nup93, Nup155 and the two paralogues Nup188 and Nup205. Inner ring assembly is only partially understood. Using *in vitro* nuclear assembly reactions, we show that direct pore membrane binding of Nup155 is critical for NPC formation. Replacing full-length Nup155 with its N-terminal β-propeller allows assembly of the outer ring components to the NPC backbone that also contains Nup53. However, further assembly, especially the recruitment of the Nup93- and the Nup62-complexes, is blocked. Self-interaction between the N- and C-terminal domains of Nup155 has an auto-inhibitory function that prevents interaction between the N-terminus of Nup155 with the C-terminal region of Nup53. Nup93 can overcome this block by binding to Nup53, thereby promoting inner ring and NPC formation.

Introduction

Nuclear pore complexes (NPCs) are the essential gatekeepers of the nuclear envelope. They form transport gates in the two double membranes of the nuclear envelope, which restrict diffusion of

macromolecules and, when required, allow highly efficient directed transport of proteins, nucleic acids and RNA-protein complexes between the cytoplasm and the nucleoplasm. In vertebrates, about thirty different proteins, called nucleoporins or Nups, contribute to the structure of the eightfold symmetric NPCs, each in 8, 16 or more copies (Beck and Hurt, 2017; Ori et al., 2013) making up a total mass of about 125 MDa. How more than five hundred individual components assemble into a macro-complex, and how they integrate in the double membranes of the nuclear envelope, is a research question yet to be answered.

Roughly, the different nucleoporins can be categorized into three different classes: transmembrane nucleoporins anchor the NPC in the pore membrane, structural nucleoporins assemble as the scaffold of the pore, and largely unstructured nucleoporins containing FG (phenylalanine-glycine)-repeats form the permeability barrier and transport gate of the pore. The NPC structural scaffold can be viewed as a stack of three rings (for review see (Beck and Hurt, 2017)): a cytoplasmic and a nucleoplasmic ring are both largely formed by an evolutionary conserved subcomplex of the NPC, in vertebrates the Nup107-Nup160-complex or Y-complex (Bui et al., 2013; von Appen et al., 2015). Sandwiched in between these two outer rings is the inner ring, which is mainly formed by multiple copies of the Nup93-complex, which consists of Nup93, Nup155, Nup53 and the two paralogues Nup205 and Nup188 (for review see (Vollmer and Antonin, 2014)). The Nup93-complex links the pore membrane with the central channel. Nup53 and Nup155 interact with the pore membrane directly (Vollmer et al., 2012; von Appen et al., 2015) and via the transmembrane nucleoporins POM121 and NDC1 (Eisenhardt et al., 2014a; Mansfeld et al., 2006; Mitchell et al., 2010). In turn, Nup93 recruits via its N-terminus the Nup62-complex (Chug et al., 2015; Sachdev et al., 2012) which forms a large part of the central transport channel of the NPC.

In vertebrates, NPCs assemble following two distinct assembly pathways, depending on the cell cycle state (for a recent review see (Weberruss and Antonin, 2016)): at the end of mitosis, NPCs reassemble on the decondensing chromatin in parallel with the nuclear envelope. After nuclear envelope assembly is completed, NPCs form *de novo* into the intact nuclear envelope during interphase. Mitotic NPC assembly, best studied in a cell-free system using *Xenopus* egg extracts (Lohka, 1998), is initiated on the chromatin by MEL28/ELYS, which acts as a seeding point for NPC formation (Franz et al., 2007; Galy et al., 2006; Rasala et al., 2006). MEL28/ELYS recruits the Y-complex, a major structural component of the outer rings to the assembling pores. Membrane connection is established next: likely, this happens via the transmembrane nucleoporin POM121 and probably also NDC1 (Antonin et al., 2005; Mansfeld et al., 2006; Rasala et al., 2008), which are the following components to be recruited. The next known assembly step involves components of the Nup93-complex. In contrast to the Y-complex, which at least in *Xenopus* egg extracts is recruited as a preformed unit, components of the Nup93-complex are added in a sequential manner: first, Nup53 binds to assembling NPCs via its membrane binding motif and its interaction with NDC1 (Eisenhardt et al., 2014a; Vollmer et al., 2012). Nup53 recruits Nup155 and Nup93 (Eisenhardt et al., 2014a; Hawryluk-Gara et al., 2005; Sachdev et al., 2012), the latter being in a complex with one of the two paralogues Nup188 or Nup205 (Theerthagiri et al., 2010). Nup93 additionally recruits the Nup62-complex as a major component of the central channel (Chug et al., 2015; Sachdev et al., 2012). The later events in this assembly pathway are less well-defined. Also less clear is which assembly pathway interphase NPC formation takes.

Here, we focus on the conserved NPC component Nup155. Vertebrate Nup155 and its two *S. cerevisiae* homologues, Nup170 and Nup157, each possess an N-terminal β-propeller region (Devos et al., 2004; Lin et al., 2016; Mans et al., 2004; Seo et al., 2013). The C-terminal half of the protein is formed by a

large α-helical domain (Flemming et al., 2009; Lin et al., 2016; Whittle and Schwartz, 2009). In *C. elegans*, drosophila and mouse, Nup155 is an essential gene (Galy et al., 2003; Kamath et al., 2003; Kiger et al., 1999; Zhang et al., 2008), most likely because of its crucial function in NPC assembly (Franz et al., 2005). Consistently, *S. cerevisiae* lacking both Nup155 homologous Nup170 and Nup157 is not viable (Aitchison et al., 1995) because these proteins, similar in structure, are also essential for NPC assembly in yeast, although probably performing distinct functions (Makio et al., 2009). We demonstrate here that direct membrane interaction of vertebrate Nup155 is crucially required for NPC formation. Nup155 mutants or fragments which cannot bind to membranes are not able to replace the endogenous protein in NPC assembly. We further show that the C-terminal region of Nup155 has an auto-inhibitory function: it binds to the N-terminus of Nup155, decreasing the strength of the Nup155-Nup53 interaction to a level that does not support NPC assembly. In the course of mitotic NPC assembly, the auto-inhibitory effect of the C-terminal interaction of Nup155 must be overcome by Nup93 binding to Nup53, triggering conformational changes that allow further progression of NPC assembly.

Results

Direct membrane binding of Nup155 is crucial for NPC assembly

We have previously shown that, in the cryo-EM structure of the human NPC, a loop within Nup155 β-propeller region dips into the pore membrane and is required for membrane binding of the recombinant protein in liposome floatation assays (von Appen et al., 2015). In order to test whether the membrane binding capability of Nup155 has a crucial role in NPC assembly, stability or function, we aimed to replace the Nup155 by protein versions defective in membrane interaction. For this, we depleted Nup155 from *Xenopus laevis* egg extracts using specific antibodies without co-depletion of other nucleoporins including the other members of the Nup93-complex: Nup53, Nup93, Nup188 and Nup205 (Fig. 1A). *Xenopus* egg extracts are widely used to faithfully reconstitute nuclear envelope and NPC assembly *in vitro* when sperm DNA is added as a chromatin template (Eisenhardt et al., 2014b; Lohka, 1998). Whereas in mock depleted extracts a closed nuclear envelope was formed on the chromatin template, indicated by a smooth membrane staining, upon Nup155 depletion membrane vesicles bound to the chromatin surface, but failed to form a closed nuclear envelope (Fig. 1B and 1C) as previously reported (Franz et al., 2005). This is due to a failure of NPC assembly, as observed upon depletion of a number of nucleoporins including Nup93, Nup53, POM121 and NDC1 (Antonin et al., 2005; Grandi et al., 1997; Hawryluk-Gara et al., 2008; Mansfeld et al., 2006; Sachdev et al., 2012; Vollmer et al., 2012). Indeed, NPCs were absent on the chromatin. This was indicated by the strong reduction in mAB414 staining, an antibody which recognized FG-repeat containing nucleoporins (Davis and Blobel, 1986) and is widely used as a general marker for NPC assembly. Similarly, RNAi mediated downregulation of Nup155 reportedly decreased mAB414 staining and NPC numbers in tissue culture cells (Mitchell et al., 2010).

To add back Nup155, we substituted Nup155 depleted extracts with mRNA encoding *Xenopus* Nup155, resulting in expression of the protein. Addition of the mRNA encoding wildtype Nup155 restored nuclear envelope and NPC formation showing the point specificity of the depletion phenotype. Addition of mRNA encoding for Nup155 with a point mutation (L258D) as well as a ten amino acid deletion (Δ258-267), which both are defective in direct membrane binding (von Appen et al., 2015),

did not restore nuclear envelope and NPC formation. This indicates that membrane binding of Nup155 is crucial for *in vitro* NPC assembly.

The Nup155 R385H mutant associated with cardiac disease does not abolish NPC assembly

Interestingly, a point mutation within human Nup155, an arginine to histidine exchange in position 391, has been linked to atrial fibrillation and early sudden cardiac death (Zhang et al., 2008). To test whether this mutation would impact the of Nup155 function in NPC assembly, we generated the corresponding mutation in *Xenopus* Nup155, R385H. Addition of the mRNA encoding this mutant restored nuclear envelope and NPC formation in Nup155 depleted extracts to similar levels as the wildtype Nup155 mRNA (Fig. 1B). This indicates that, at least in the *in vitro* system, the Nup155 R385H mutant is functional in NPC assembly.

The C-terminal α-solenoid region of Nup155 is dispensable for nuclear envelope formation

Nup155 predictably contains two distinct structural regions, similarly to its yeast homologues Nup157 and Nup170 (Flemming et al., 2009; Kosinski et al., 2016; Seo et al., 2013; Whittle and Schwartz, 2009): an N-terminal β-propeller, which interacts with Nup53 and POM121 (Mitchell et al., 2010), and a C-terminal α-solenoid region, which binds the export cofactor Gle1 and the nucleoporin Nup98 (Lin et al., 2016). Interestingly, addition of the N-terminal β-propeller region of Nup155 (aa 1-589) rescued the depletion phenotype of the nuclear envelope assembly (Fig. 2). A closed nuclear envelope, indicated by a smooth membrane staining, formed around the chromatin template. However, these nuclei showed only much reduced mAB414 staining with respect to the mock sample, comparably to the Nup155 depleted nuclei (Fig. 2A). This could indicate that the Nup62-complex was not recruited to the assembling NPCs when the N-terminal β-propeller region of Nup155 replaced the full-length protein. Indeed, when analyzing by immunofluorescence Nup155 depleted nuclei and Nup155 depleted nuclei supplemented with the N-terminal region of Nup155 (aa 1-589) with a variety of antibodies against nucleoporins, we detected on the chromatin MEL28/ELYS as well as Nup107, a member of the Y-complex (Fig. 3A). Nup53 was present on the nuclei in the absence of Nup155, consistently with the notion that it can be recruited to NPCs even if it cannot interact with Nup155 (Eisenhardt et al., 2014a). Nup153, which is part of the nuclear basket structure of the NPC and interacts with the Y-complex (Vasu et al., 2001) was also detected at the NPCs of these nuclei. The weak mAB414 staining observed might have therefore been Nup153, which is also recognized by this antibody (Fig. 3A). Nup93, as well as the Nup62-complex component Nup62, were absent from the assembling pores, which was not due to depletion from the extract (Fig. 3B). Since the Nup62-complex is crucially required for the nuclear transport function of the NPC, these nuclei were unable to import an EGFP-tagged nuclear import substrate, in contrast to the mock control (Fig. 3C). Together, these data suggest that nuclei in which endogenous Nup155 had been replaced by the N-terminal β-propeller fragment form a part of the inner ring components, but lack Nup93 as well as Nup188, Nup205 and the Nup62-complex, which are recruited via Nup93. Since depletion of Nup93 in *Xenopus* egg extracts co-depletes Nup205 and Nup188 (Sachdev et al., 2012), it has been hard to establish whether Nup205/Nup188 can be recruited by additional binding partners: indeed, biochemical analysis in *Chaetomium* identified novel bridge-like interactions between Nup53, Nup98, Nup155 and Nup205

homologues (Lin et al., 2016). Our work confirms that the Nup53-Nup155 interaction and recruitment to the assembling NPCs is not sufficient for proper localization of Nup205 and Nup188 at NPCs at the end of mitosis, and that Nup93 is required for this.

The C-terminus of Nup155 has an autoinhibitory effect on the integration of Nup155 into the network of NPCs protein interaction

The nuclei assembled in Nup155 depleted extracts supplemented with the N-terminal β-propeller domain of Nup155 (aa 1-589) showed a closed nuclear envelope and lacked Nup93 (Fig. 2 and 3). This was surprising in the light of our previous finding that depletion of Nup93 does not allow for closed nuclear envelope formation (Sachdev et al., 2012; Theerthagiri et al., 2010) because in this situation Nup53 and importantly Nup155 were present in extracts. We were therefore wondering whether the C-terminus of Nup155 has a negative, potentially autoinhibitory, influence on nuclear envelope formation, more specifically on the protein interaction network formation necessary for inner pore ring assembly.

We tested this idea by checking whether the N- and C-terminal domains of *Xenopus* Nup155 can interact as shown for the yeast homologue Nup170 (Flemming et al., 2009). GST-pulldowns using the recombinant, GST-tagged Nup155 N-terminal β-propeller region (aa 1-589) and SUMO-tagged C-terminal fragments of Nup155 (aa 589-1344 and aa 504-1388) indeed showed an interaction between both regions (Fig. 4A). Next, we tested whether the C-terminal of Nup155 interferes with the described interaction between the N-terminal of Nup155 and the C-terminal region of Nup53 (Amlacher et al., 2011; Eisenhardt et al., 2014a; Lin et al., 2016). When titrating increasing amounts of the SUMO-tagged C-terminal Nup155 fragment (aa 504-1388), the interaction between a GST-tagged C-terminal Nup53 fragment (aa 162-320) to the SUMO-Nup155 N-terminal β-propeller region (aa 1-589) was weakened (Fig. 4B).

The nuclei assembled in Nup155 depleted extracts supplemented with the N-terminal β-propeller domain of Nup155 (aa 1-589) resembled nuclei we have previously observed when replacing full-length Nup93 by its C-terminal domain (Sachdev et al., 2012). These nuclei contained large parts of the structural backbone of the NPC including the Y-complex, Nup53 and Nup155 but lacked the Nup62-complex, the main component of the central transport channel, required for nuclear import. We therefore speculated that the C-terminus of Nup93 could overcome the inhibitory effect of the Nup155 C-terminus in NPC structural backbone assembly. We tested this hypothesis by performing GST-pulldowns using the N-terminal domain of Nup155 and Nup53 (aa 1-312), which allows for the Nup155 interaction but also includes its N-terminal region, important for Nup93 binding (Lin et al., 2016; Sachdev et al., 2012). As expected, addition of the C-terminus of Nup155 weakened the interaction (Fig. 5). This effect is overcome by addition of the C-terminal region of Nup93 (SUMO-tagged aa 608-820). Interestingly, this region of Nup93 enhances the Nup53-Nup155 interaction, consistently with previous results (Sachdev et al., 2012). Notably, the Nup93- and Nup155-binding regions within Nup53 have been mapped to different regions. Together, these pulldown data indicate that the N- and C-terminal regions of Nup155 can interact in isolation, and that this interaction weakens the binding of Nup155 to Nup53. Binding of Nup93 to the N-terminus of Nup53 could induce a change in Nup53, which overcomes the auto-inhibitory effect of Nup155 C-terminus and strengthens the Nup53-Nup155 interaction.

Discussion

Here, we show that direct interaction of Nup155 with membranes is crucially required for NPC formation. Surprisingly, the N-terminal β-propeller region of Nup155 is, at least *in vitro*, sufficient to rescue the nuclear envelope depletion phenotype of Nup155, indicating that assembly of the structural backbone of the NPC can take place. Furthermore, self-interaction between the N- and C-terminal regions of Nup155 has an auto-inhibitory function, preventing efficient Nup155-Nup53 interaction, which is nonetheless required for further and functional NPC assembly.

Nup155 belongs to a group of nucleoporins such as Nup53, Nup133 and Nup153 (Drin et al., 2007; Vollmer et al., 2015; Vollmer et al., 2012) which can directly interact with the pore membrane without containing a transmembrane region. Direct membrane interaction of both Nup53 and Nup155 is crucial for NPC assembly ((Vollmer et al., 2012) and this study), probably because the membrane interaction motifs also stabilize the highly curved nuclear membranes. Cryoelectron tomographic reconstruction of the human nuclear pore indeed suggests that Nup155 interacts with the pore membrane (von Appen et al., 2015). Modelling of the yeast Nup53 and Nup170 structures into the human tomographic reconstruction proposes that this mode of membrane interaction is conserved for both proteins (Lin et al., 2016). Indeed, also genetic and biochemical studies suggest that Nup170 and Nup157 (Aitchison et al., 1995; Miao et al., 2006; Tcheperegine et al., 1999), as well as Nup53 (Marelli et al., 1998; Marelli et al., 2001; Miao et al., 2006; Onischenko et al., 2009; Tcheperegine et al., 1999), are located close to the pore membrane. In the filamentous fungus *Aspergillus nidulans,* no genes encoding for the homologues of vertebrate Nup53 nor yeast Nup59 have been identified, and NDC1 is non-essential (Osmani et al., 2006). In contrast, Nup170 is encoded by an essential gene *in A. nidulans*, and in the semi-open mitosis in this organism, in which NPCs disassemble partially, Nup170, its interaction partner Gle1 and the Y-complex are the only non-transmembrane proteins which remain at the pore (Osmani et al., 2006). It is likely that *Asperigillus* Nup170 interacts directly with the pore membrane to fulfil its function in NPCs: The corresponding leucine in position 258 is conserved in *A. nidulans*, as well as the hydrophobic nature of the region 258-267 we deleted in our experiments.

In vertebrates, Nup53 interacts with both Nup155 and Nup93 (Eisenhardt et al., 2014a; Hawryluk-Gara et al., 2005), thereby indirectly connecting these two nucleoporins, which do not detectably bind each other (Eisenhardt et al., 2014a; Mitchell et al., 2010; Sachdev et al., 2012). Nup93 in turn interacts with either Nup188 or Nup205 (Theerthagiri et al., 2010) and recruits the Nup62-complex (Chug et al., 2015; Sachdev et al., 2012) to the assembling NPCs (Fig. 6). This interaction network is evolutionary conserved: biochemical work using purified recombinant *Chaetomium thermophilum* nucleoporins established that, in this fungus, Nup170 interacts with the C-terminus of Nup53 (Amlacher et al., 2011; Lin et al., 2016). This dimer then binds a second dimer formed by Nic96 (homologue to metazoan Nup93) and either Nup188 or Nup192 (homologue to metazoan Nup205) in a mutually exclusive way, resulting in formation of either a Nup170/Nup53/Nic96/Nup188 or a Nup170/Nup53/Nic96/Nup192 tetramer (Lin et al., 2016; Stuwe et al., 2014).

Re-addition of the N-terminal β-propeller region of Nup155 rescued the nuclear envelope, but not the NPC assembly phenotype of the Nup155 depletion. We have previously observed a similar phenotype when adding the C-terminal fragment of Nup93 to Nup93 depleted extracts (Sachdev et al., 2012). In that study, the structural backbone of the NPC could form, including the Y-complex as outer ring as well as Nup53 and Nup155 as inner ring components. The C-terminus of Nup93 was suggested to stabilize the Nup53-Nup155 interaction. Given that the N-terminal region of Nup93 is required for

recruitment of the Nup62-complex, which forms a large part of the central channel of the NPC, these components were missing from the nuclei. Similarly to the previously observed phenotype, here we show that Y-complex components and Nup53 were present when the N-terminal of Nup155 was added to Nup155 depleted nuclei. The Nup93-, and thus the Nup62-complexes, were missing. Surprisingly, in comparison to the Nup93 depletion phenotype, which does not support assembly of the NPC structural backbone, the Nup155 fragment allowed this. A possible explanation could be that the C-terminus of Nup155 prevents NPC assembly in the absence of Nup93, probably because it has an inhibitory function which can be overcome by the presence of the C-terminal region of Nup93 (Fig. 6). In line with this hypothesis, overexpression of the C-terminal domain of Nup170 is toxic in *S. cerevisiae,* but toxicity can be rescued by overexpression of the N-terminal domain (Flemming et al., 2009). As the separated N- and C-terminal domains of Nup170 reciprocally interact in yeast two hybrid and pulldown assays, it has been suggested that, in the presence of excess of the C-terminal domain of Nup170, the Nup170-Nup53 dimer does not assemble correctly. Indeed, our pulldown assays indicate that the C-terminus of Nup155 interferes with Nup155 binding to Nup53 by interacting with the N-terminal Nup155 β-propeller domain. Interestingly, the presence of Nup93, more precisely its C-terminal region, can overcome the auto-inhibitory function of Nup155. It can be hypothesized that the auto-inhibition of full length Nup155 constitutes regulated point in the mitotic NPC assembly path: upon binding of Nup93, Nup53 signals that the assembly is proceeding correctly, and the Nup155-Nup53-Nup93-Nup188/205 complex is able to further recruit the Nup62-complex. It remains open why, in the nuclei assembled in depleted extracts in which the N-terminal domain of Nup155 replaced the full-length protein, Nup93 is not detected on the chromatin template. It is possible that during assembly of the structural NPC backbone Nup93 undergoes some structural rearrangements which needs to be supported by the C-terminus of Nup155. Alternatively, in the absence of the auto-inhibitory function of the C-terminus of Nup155, protein interactions between the inner ring components of the NPC could occur ectopically, resulting in a lack of Nup93 on chromatin templates.

A homozygous Nup155 mutation, R391H in humans, has been linked to atrial fibrillation and early sudden cardiac death (Zhang et al., 2008). The R391H mutation was suggested to affect NPC localization of Nup155. Indeed, in a targeting assay, in which Nup155 R391H was ectopically localized to chromatin, its interaction partners Nup53 and POM121 showed less co-recruitment as compared to the wildtype Nup155 control (Schwartz et al., 2015). However, given that Nup155 is essential in *C. elegans*, drosophila and mouse, most likely because of its crucial role in NPC assembly, it seems questionable that organisms are viable without Nup155 being able to localize to assembling NPCs. Indeed, our results suggest that the *Xenopus laevis* protein with the corresponding R385H mutation supports NPC assembly and is localized to the assembled NPCs. Most likely, the homozygous R391H mutation in humans causes the observed pathological phenotype for reasons different from defective NPC assembly. We would rather speculate that the homozygous mutation causes a milder deviation in NPC function affecting most severely heart cells, resulting in the cardiac defects. Indeed, a similar pattern has been observed for Nup93, another essential component of the inner ring module of NPCs: mutations causing a nephrotic phenotype were found in combination where at least one allele would support NPC assembly but caused a defect in NPC transport function (Braun et al., 2016). Patients with Nup93 mutations blocking NPC assembly on both alleles were not found; in all likelihood, this condition is not compatible with life, given the essential function of Nup93.

In conclusion, our work provides evidence for the requirement of the N-terminal membrane binding domain of Nup155 for a proper nuclear envelope and NPC formation. Although required and sufficient

for nuclear envelope formation, this N-terminal domain is not sufficient for proper NPC assembly, as recruitment of Nup93 and members of the Nup62-complexes are not supported after its addback to Nup155 depleted nuclei. Yet, the assembly of a large part of the structural NPC backbone, including the outer ring forming Y-complex, is possible. We demonstrate an auto-inhibitory function of the C-terminal domain of Nup155 in inner pore ring assembly, which is required for assembly of functional NPCs. Finally, mutations of Nup155 associated with pathological condition of atrial fibrillation did not correlate with nuclear assembly defects *in vitro*, therefore pointing at a different malfunction of the protein as responsible for this phenotype. Identifying this malfunction remains an interesting research avenue.

Experimental procedures

Protein expression and purification

GST-fusions of *Xenopus laevis* Nup53 fragments (Vollmer et al., 2012) and the SUMO-tagged *Xenopus laevis* Nup93 fragment (Sachdev et al., 2012) have been described. N-(aa 1-589) and C-terminal (aa 589-1388 and 504-1388) *Xenopus laevis* Nup155 fragments were cloned as codon optimized sequences for expression in *E. coli* into a modified pET28a vector with a yeast SUMO solubility tag followed by a Tobacco Etch Virus (TEV) cleavage site. The fragments were expressed in *E. coli* by autoinduction at 18°C and purified by Ni-affinity chromatography.

For addback experiments to egg extracts, the SUMO-tag was removed from the N-terminal Nup155 fragment by incubating the purified protein with TEV protease. The His6-tagged SUMO moiety and the TEV protease were then removed via further incubation with Ni-affinity beads. Unbound containing tag-cleaved protein was dialyzed against sucrose buffer with high salt (250 mM sucrose, 750 mM KCl, 10 mM Hepes-KOH, 2.5 mM $MgCl_2$).

In vitro nuclear assembly

Preparation of high speed interphase extracts, sperm heads, floated labelled and unlabeled membranes required for *in vitro* nuclear assembly as well as immunofluorescence experiments were carried out as described in (Eisenhardt et al., 2014b). Fluorescence images were acquired using a confocal microscope [FV1000; Olympus; equipped with a photomultiplier (model R7862; Hamamatsu)] using 405-, 488- and 559-nm laser lines and a 60× NA 1.35 oil immersion objective lens. Antibodies against Nup107 (Walther et al., 2003), Nup160 (Franz et al., 2007), MEL28/ELYS (Franz et al., 2007), Nup53 (Theerthagiri et al., 2010), Nup205 (Theerthagiri et al., 2010), Nup188 (Theerthagiri et al., 2010), Nup153 (Vollmer et al., 2015), Nup155 (Franz et al., 2005) have been described. mAB414 and His6 antibodies were purchased from Covance (MMS-120R) and Roche (11922416001), respectively. The antibody against Nup62 was a kind gift from Birthe Fahrenkrog, and was used at a concentration of 1:50.

The anti-Nup155 antibody for depletion was generated in rabbits using full-length Nup155 (see (Vollmer et al., 2015)). Anti-Nup155 beads and mock beads were generated by coupling anti-Nup155 serum and unspecific rabbit IgG, respectively, to protein-A-Sepharose resin (GE Healthcare) with 10 mM dimethyl pimelimidate (Thermo Fisher Scientific). Nup155 and mock immunodepletions for addback of Nup155 fragments were performed by mixing *X. laevis* high speed extracts with beads in 1:0.8 ratio twice rotating at 8°C for 15 minutes each.

For rescue experiments with full length Nup155 and Nup155 mutants, mRNA encoding *Xenopus laevis* Nup155 (GenBank accession NM_001087331.1) was prepared using the mMESSAGE mMachine kit (Life Technologies) and added to extracts to a final concentration of 300 ng/µl. For rescue experiments with the Nup155 N-terminal domain (aa 1-589), the fragment was added to the reactions to a final concentration of 100 nM. To compensate for volume change, the same volume of high salt sucrose buffer was added to the mock reactions.

To test nuclear import, a NLS-2xGFP protein and its nuclear localization sequence (NLS) mutant as negative control, both cloned into the pTrcHisB vector ((Yang et al., 2004), were expressed in *E. coli* and purified by Ni-Affinity and size exclusion (Superdex 200 (Amersham Pharmacia)) chromatography. Proteins were dialyzed to sucrose buffed and added to the assembly reaction in final concentration of 300 nM.

Acknowledgements

We are grateful to Birthe Fahrenkrog for providing the anti-Nup62xl antibody. We thank Katharina Schellhaus, Hideki Yokoyama and Daniel Moreno-Andrés for critical reading of the manuscript.

Competing interest

The authors declare that they have no conflict of interest with the contents of this article.

Author contribution

PDM and WA conceived the study, designed, performed and analyzed nuclear assembly experiments, and wrote the manuscript. MTN and MD expressed and purified GST-tagged and SUMO-tagged proteins and performed related GST pulldown experiments. All authors edited and approved the manuscript.

Funding

This work was supported by funding of the Max Planck Society and the European Research Council (grant agreement 309528 CHROMDECON to WA) and by a PhD Fellowship of the IMPRS “From Molecules to Organisms” to PDM.

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Figure legends

Figure 1: Direct membrane binding of Nup155 is crucial for NPC assembly

(A) Western blot analysis of mock and Nup155 depleted Xenopus egg extracts, with or without addition of Nup155 addback and mutants.

(B) Confocal microscopy images of fixed nuclei assembled for 120 min in mock depleted (mock) and Nup155 depleted (ΔNup155) *Xenopus* egg extracts supplemented with either buffer, wildtype Nup155 mRNA, or mRNA encoding the membrane binding mutants L258D and Δ258-267, as well as the mutant Nup155 R385H causing cardiac disease. Membranes were pre-labelled with DiIC18 (red in overlay) and chromatin was stained with DAPI (blue in overlay). The lower panels show the immunofluorescence staining for Nup155 (green in overlay) and NPCs (mAB414, red in overlay) on the chromatin (DAPI, blue in overlay). Scale bars: 5 μm.

(C) The average percentage of closed nuclear envelopes for 100 randomly chosen chromatin substrates in each of three independent experiments is reported. Data points from individual experiments are indicated.

Figure 2: The Nup155 β-propeller region is sufficient for formation of a closed nuclear envelope

(A) Confocal microscopy images of nuclei assembled for 120 min in mock depleted, Nup155 depleted (ΔNup155), and Nup155 depleted *Xenopus* egg extracts supplemented with 100 nM of the Nup155 1-589 fragment. Membranes have been pre-labelled with DiIC18 (red in overlay) and chromatin was stained with DAPI (blue in overlay). The lower panels show the immunofluorescence analysis for Nup155 (green in overlay) and NPCs (mAB414, red in overlay) on the chromatin (DAPI, blue in overlay). Scale bar: 10 μm.

(B) Average percentage of closed nuclear envelopes for 100 randomly chosen chromatin substrates in each of three independent experiments. Data points from the three individual experiments are indicated.

Figure 3: Nup155 supports assembly of the NPC structural backbone

(A) Nuclei were assembled in mock, Nup155 depleted extracts (ΔNup155) or Nup155 depleted extracts supplemented with Nup155 1-589 for 120 min, fixed and stained with the respective antibodies. Scale bar, 10 μm.

(B) Western blot analysis of mock and Nup155 depleted *Xenopus* egg extracts from the experiment in (A).

(C) Nuclei were assembled as in (A). After 60 min an EGFP-tagged importin α/β dependent nuclear imports substrate was added. Nuclei were fixed after furter 70 min and analyzed by confocal microcopy. Quantitation shows the average of two independent experiments with 100 chromatin substrates analyzed per condition. Data points from the two individual experiments are indicated.

Figure 4: The C-terminal domain of Nup155 weakens the Nup53-Nup155 interaction

(A) GST-fusion constructs of the *Xenopus* Nup53 RRM domain (aa 162-267, control) or a Nup155 N-terminal fragment (aa 1-589) were incubated with the SUMO-tagged Nup53 RRM domain (aa 162-267, control) or two C-terminal Nup155 fragments (aa 589-1388 and aa 504-1388) pulled-down and analyzed by Western blotting. The right panel shows the average of the SUMO-Nup155 preys bound to GST-control or GST-Nup155 N-terminal fragment (aa 1-589) baits from three independent experiments.

(B) GST-fusion constructs of the *Xenopus* Nup53 RRM domain (aa 162-267, control) or a C-terminal fragment (aa 162-320) were incubated with the SUMO-tagged Nup53 RRM domain (aa 162-267, control) or the Nup155 N-terminal fragment (aa 1-589). Where indicated, a SUMO-tagged C-terminal Nup155 fragment (aa 504-1388) was added in a two or six-fold excess over the SUMO-tagged N-terminal Nup155 fragment. The right panel shows the average of the SUMO-Nup155 1-589 prey bound to GST-Nup53 162-320 bait in the absence or presence of a two and six fold excess SUMO-tagged C-terminal Nup155 fragment (aa 504-1388) from three independent experiments.

Eluates and 2% of the input were analyzed by western blotting using an antibody against the His6-tag.

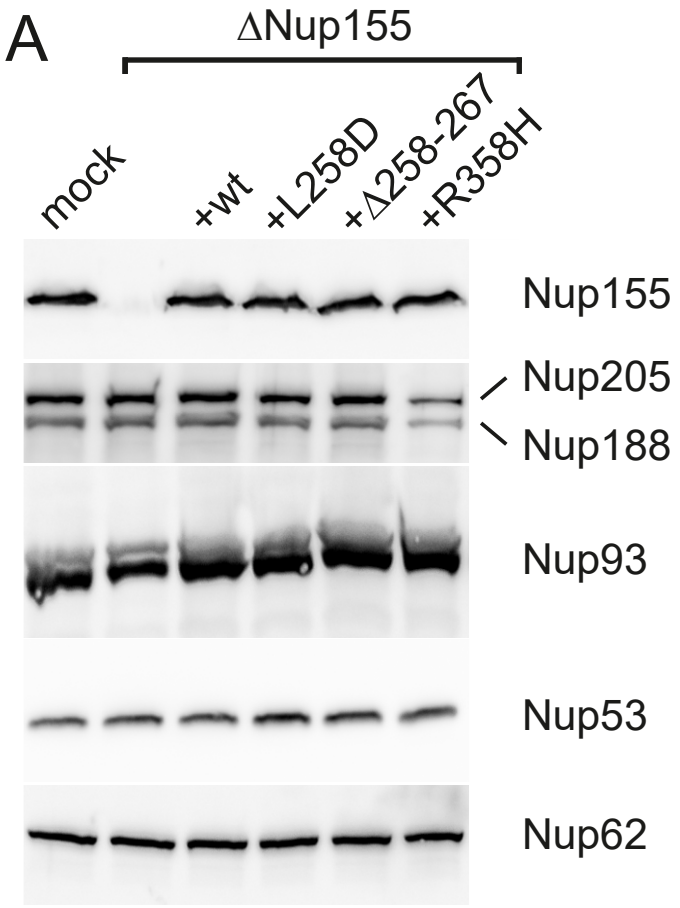
Figure 5: The C-terminal domain of Nup155 weakens the Nup53-Nup155 interaction

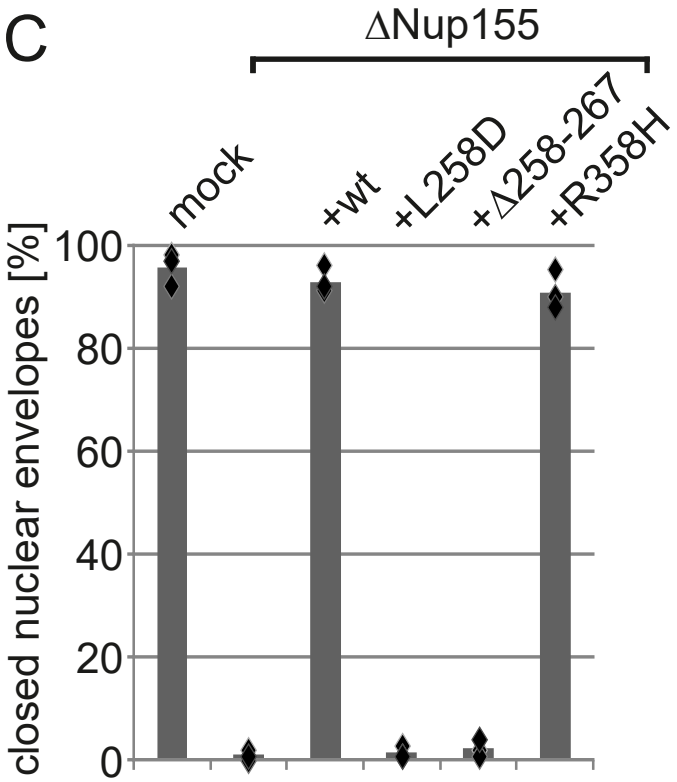
GST-fusion constructs of the *Xenopus* Nup53 RRM domain (aa 162-267, control) or an Nup53 fragment including the Nup93 and Nup155 binding regions (aa 1-312) were incubated with the SUMO-tagged Nup53 RRM domain (aa 162-267, control) or the Nup155 N-terminal fragment (aa 1-589). Where indicated, a SUMO-tagged C-terminal fragments of either Nup155 (aa 504-1388) or Nup93 (aa 608-820) were added in six-fold excess over SUMO-tagged N-terminal Nup155 fragment. The right panel shows the average of the SUMO-Nup155 1-589 prey bound to GST-Nup53 1-312 bait in the absence or presence SUMO-tagged C-terminal Nup155 fragment (aa 504-1388) and SUMO-tagged Nup93 (aa 608-820) from three independent experiments. Eluates and 2% of the input were analyzed by western blotting using an antibody against the His6-tag.

Figure 6: Nup155 is recruited to the pore via Nup53 and requires Nup93 to become competent for NPC assembly

Model for inner pore ring assembly: Nup53 binds to the nascent pore via its pore membrane interaction and contributes to curvature stabilization. Nup155 is recruited in loco via its N-terminal domain. At this step, Nup155 is found in a self-inhibitory conformation, due to the interaction of its N- and C- terminal moieties. Binding of Nup93 to Nup53 stabilizes the Nup155-Nup53 interaction by overcoming the auto-inhibitory effect of the C-terminal of Nup155 on the complex. Nup93 then recruits the Nup62-complex, leading to assembly of transport competent NPCs. The direct and mutually exclusive Nup93 interaction partners Nup188 and Nup205 are not shown.

Figure 1


A
ΔNup155
mock
+wt
+L258D
+Δ258-267
+R358H
Nup155
Nup205
Nup188
Nup93
Nup53
Nup62


C
ΔNup155
mock
+wt
+L258D
+Δ258-267
+R358H
closed nuclear envelopes [%]
100
80
60
40
20
0

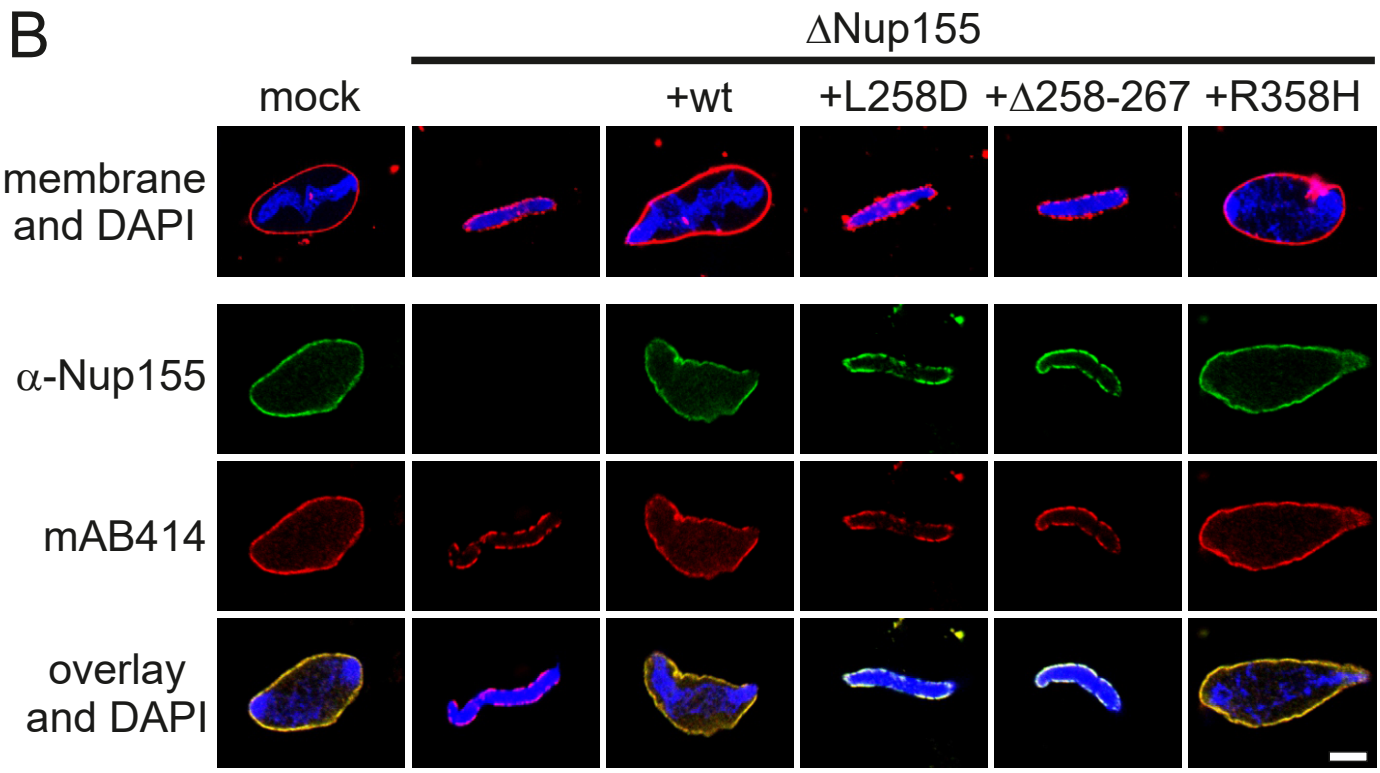

B
ΔNup155
mock
+wt
+L258D
+Δ258-267
+R358H
membrane and DAPI
α-Nup155
mAB414
overlay and DAPI

Figure 2

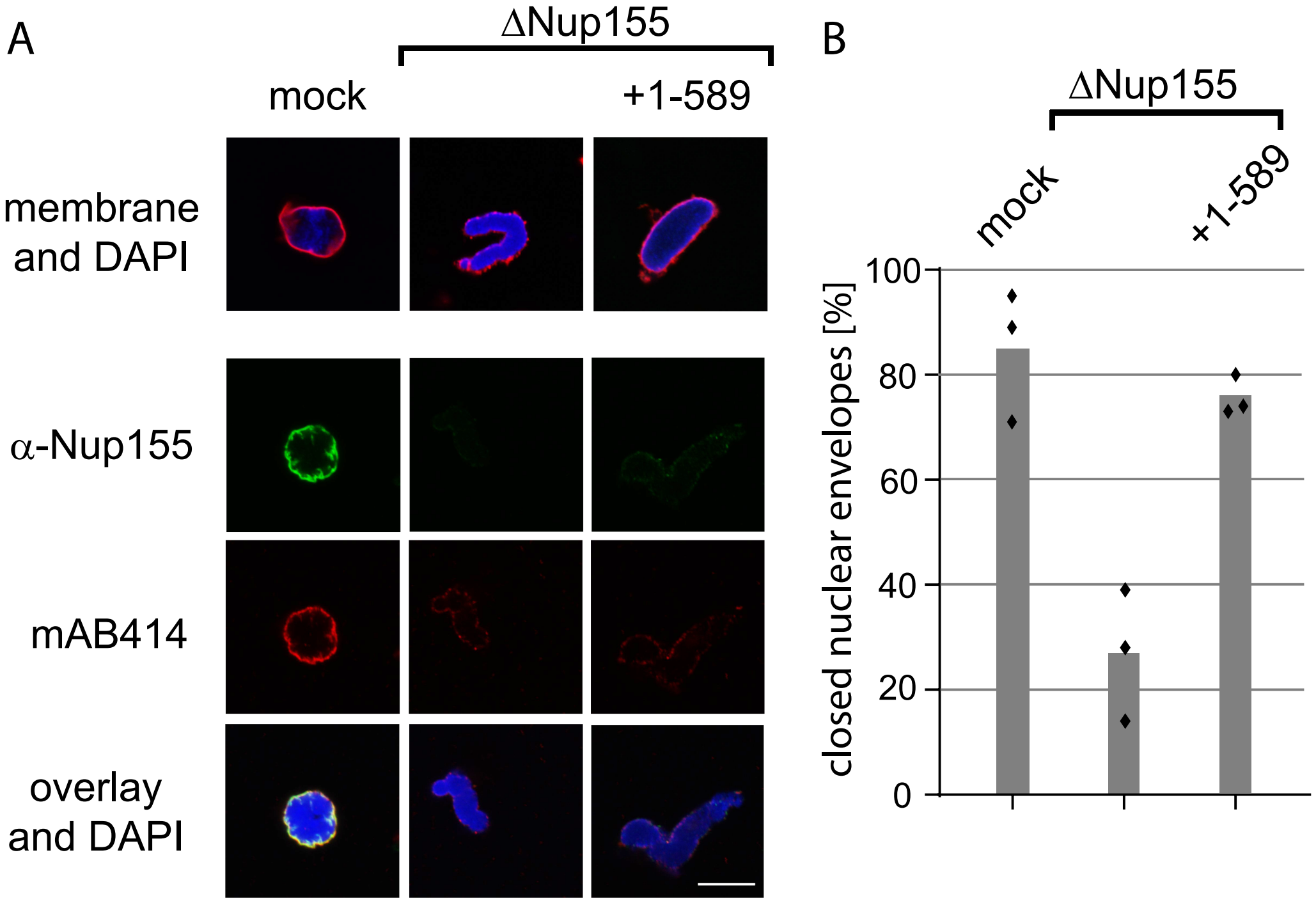

A
ΔNup155
mock
+1-589
membrane and DAPI
α-Nup155
mAB414
overlay and DAPI
B
ΔNup155
mock
+1-589
closed nuclear envelopes [%]
100
80
60
40
20
0

Figure 3

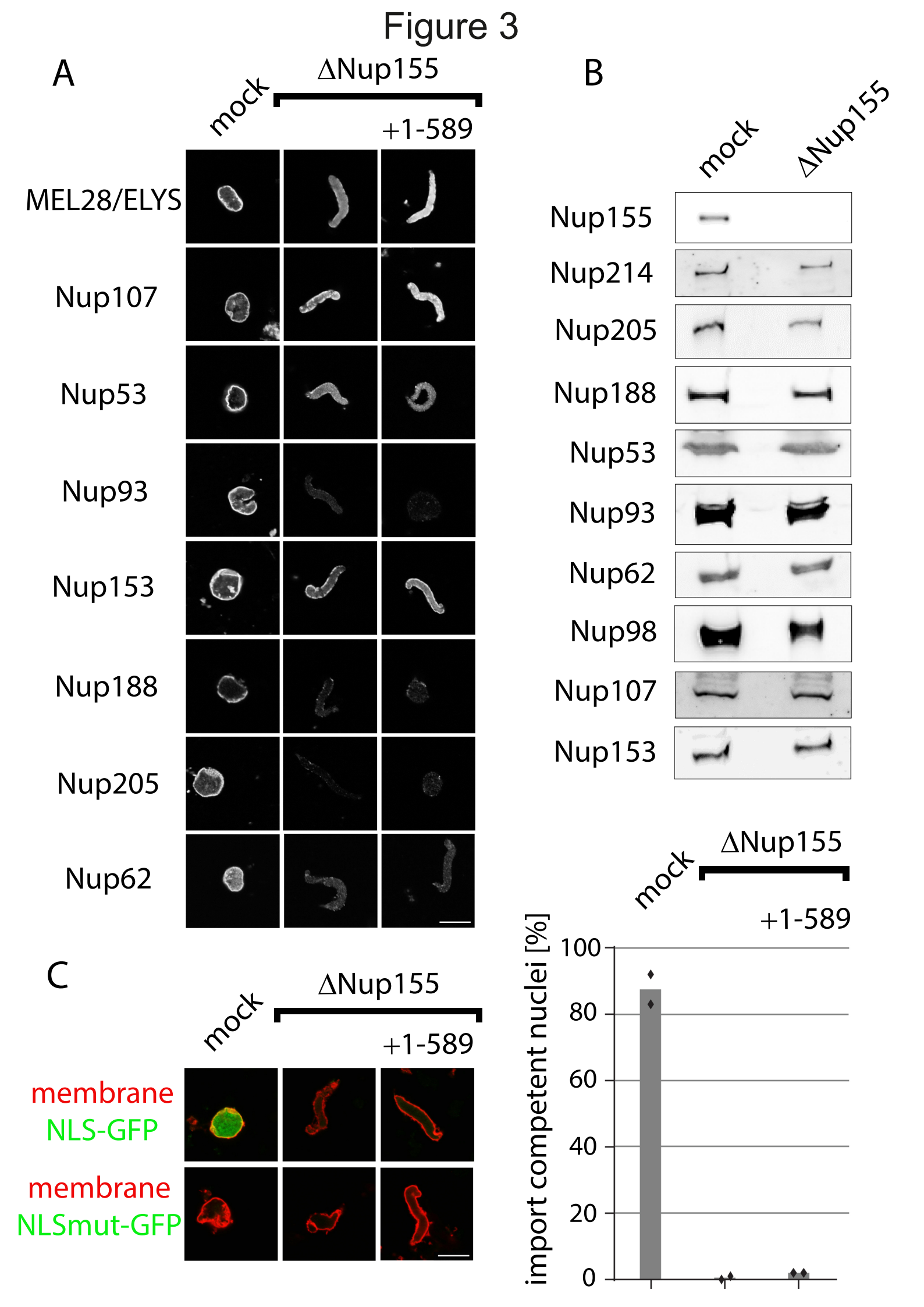

A
mock
ΔNup155
+1-589
MEL28/ELYS
Nup107
Nup53
Nup93
Nup153
Nup188
Nup205
Nup62
B
mock
ΔNup155
Nup155
Nup214
Nup205
Nup188
Nup53
Nup93
Nup62
Nup98
Nup107
Nup153
C
mock
ΔNup155
+1-589
membrane
NLS-GFP
membrane
NLSmut-GFP
mock
ΔNup155
+1-589
import competent nuclei [%]
100
80
60
40
20
0

Figure 4

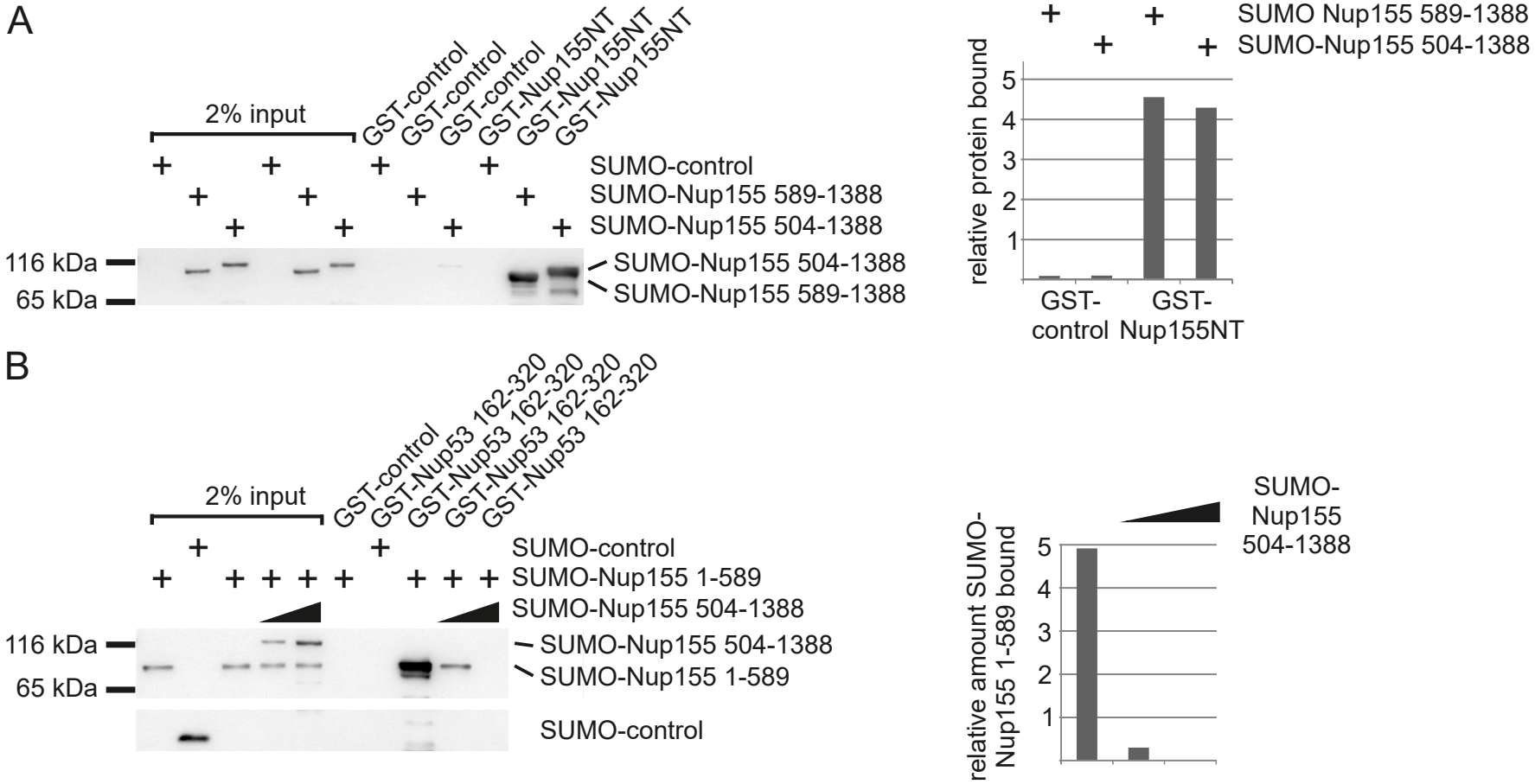
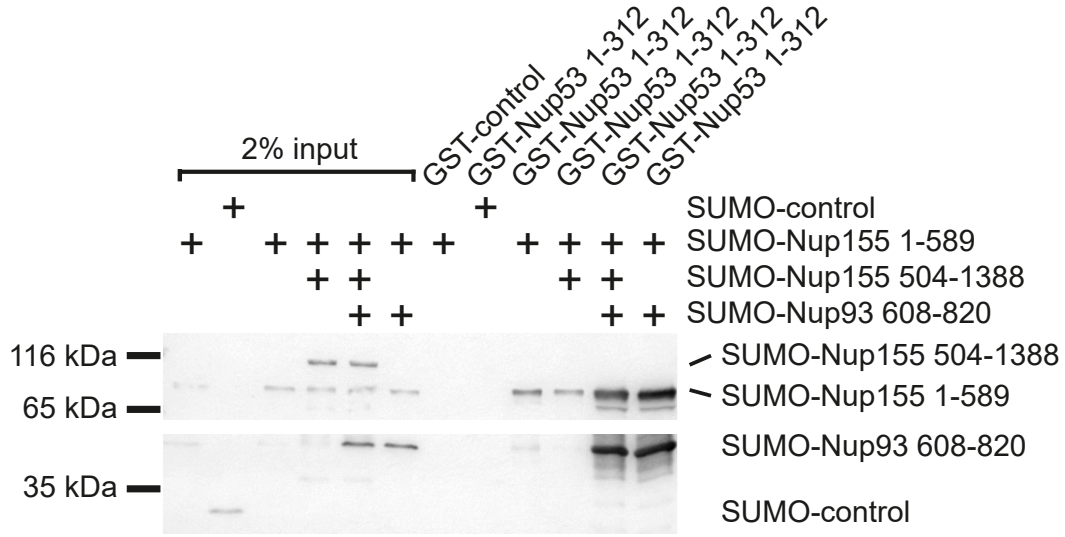

A
2% input
GST-control
GST-control
GST-control
GST-Nup155NT
GST-Nup155NT
GST-Nup155NT
SUMO-control
SUMO-Nup155 589-1388
SUMO-Nup155 504-1388
116 kDa
65 kDa
SUMO-Nup155 504-1388
SUMO-Nup155 589-1388
SUMO Nup155 589-1388
SUMO-Nup155 504-1388
relative protein bound
5
4
3
2
1
GST-control
GST-Nup155NT
B
2% input
GST-control
GST-Nup53 162-320
GST-Nup53 162-320
GST-Nup53 162-320
GST-Nup53 162-320
SUMO-control
SUMO-Nup155 1-589
SUMO-Nup155 504-1388
116 kDa
65 kDa
SUMO-Nup155 504-1388
SUMO-Nup155 1-589
SUMO-control
SUMO-Nup155 504-1388
relative amount SUMO-Nup155 1-589 bound
5
4
3
2
1

Figure 5


2% input
GST-control
GST-Nup53 1-312
GST-Nup53 1-312
GST-Nup53 1-312
GST-Nup53 1-312
GST-Nup53 1-312
SUMO-control
SUMO-Nup155 1-589
SUMO-Nup155 504-1388
SUMO-Nup93 608-820
116 kDa
65 kDa
35 kDa
SUMO-Nup155 504-1388
SUMO-Nup155 1-589
SUMO-Nup93 608-820
SUMO-control

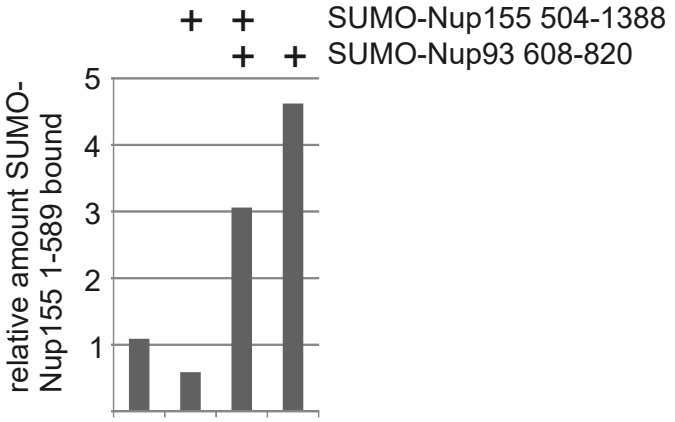
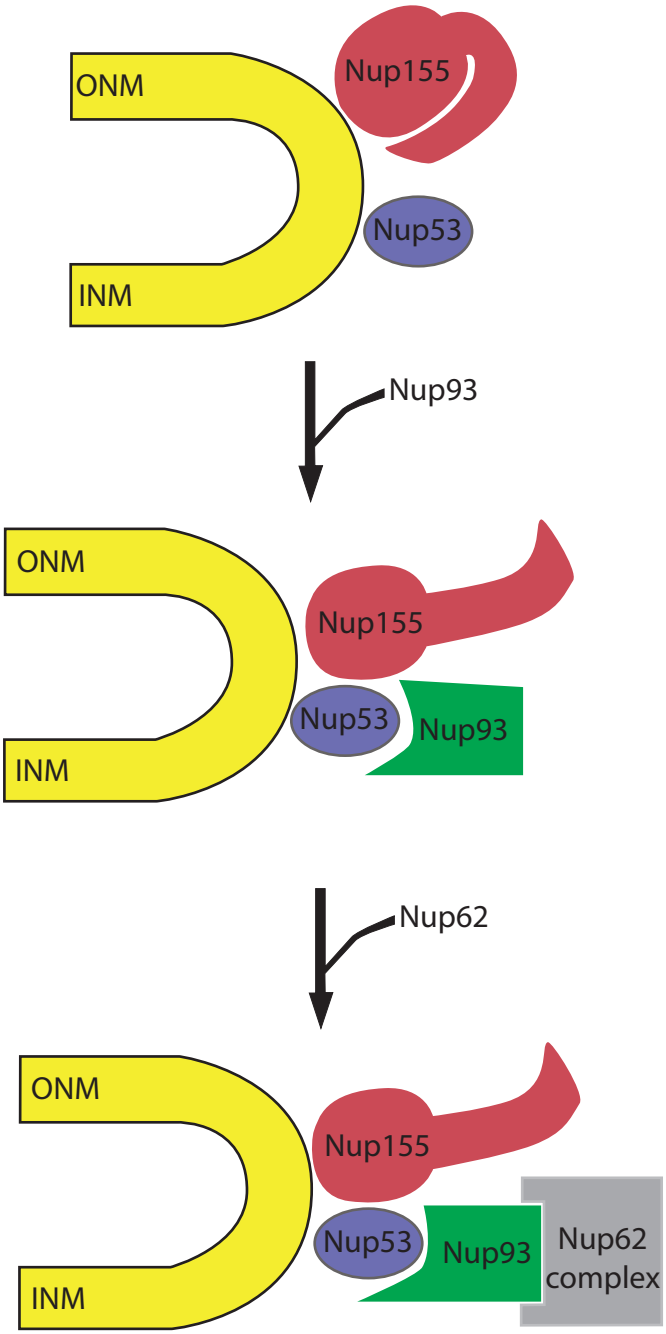

SUMO-Nup155 504-1388
SUMO-Nup93 608-820
relative amount SUMO-Nup155 1-589 bound
5
4
3
2
1

Figure 6


ONM
Nup155
Nup53
INM
Nup93
ONM
Nup155
Nup53
Nup93
INM
Nup62
ONM
Nup155
Nup53
Nup93
Nup62
complex
INM