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COMMENTARY

Perforating the nuclear boundary – how nuclear pore complexes assemble

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ABSTRACT

The nucleus is enclosed by the nuclear envelope, a double membrane which creates a selective barrier between the cytoplasm and the nuclear interior. Its barrier and transport characteristics are determined by nuclear pore complexes (NPCs) that are embedded within the nuclear envelope, and control molecular exchange between the cytoplasm and nucleoplasm. In this Commentary, we discuss the biogenesis of these huge protein assemblies from approximately one thousand individual proteins. We will summarize current knowledge about distinct assembly modes in animal cells that are characteristic for different cell cycle phases and their regulation.

KEY WORDS: Annulate lamellae, Nuclear envelope, Nuclear pore complex, Nuclear transport

Introduction

Envelopment of the genetic material by the nuclear envelope is a hallmark of eukaryotic cells. By spatially and temporally separating nuclear transcription and RNA processing, as well as cytosolic translation, the nuclear envelope allows eukaryotes to achieve a level of regulation in gene expression that is unprecedented in prokaryotes. However, the separation of the nuclear genome from the cytosolic protein synthesis machinery comes at a price. It requires transport gates that guarantee selective passage of proteins, RNAs, RNA–protein complexes and metabolites. This is achieved by nuclear pore complexes (NPCs), which act as the gatekeepers of the nuclear envelope. In contrast to other transport gates, such as ion channels, metabolite translocators or transporters for polypeptides, which span the respective membrane to form an aqueous channel within a hydrophobic lipid bilayer, NPCs breach the barrier of the nuclear envelope differently: they deform the two membranes of the nuclear envelope to create pores with a diameter of 100 nm into which these complexes are inserted. Accordingly, in most cells NPCs are the largest protein assemblies known, with a total mass of 125 MDa in vertebrates. Here, we will discuss how these huge complexes assemble and integrate into the double-membrane structure of the nuclear envelope at different phases of the cell cycle, with a focus on animal cells.

NPC architecture

Despite their huge size, NPCs are formed by only about thirty different proteins, nucleoporins or Nups, that are – due to the eightfold symmetry of NPCs – present in eight, sixteen, 32 or more copies

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(Alber et al., 2007; Ori et al., 2013). Functionally, nucleoporins can be roughly divided into three groups. First, transmembrane nucleoporins anchor the NPC in the pore membrane. In metazoa, three transmembrane nucleoporins have been identified: POM121, GP210 (also known as NUP210) and NDC1. Members of the second group of nucleoporins belong to the symmetric structural scaffold of the NPC. Finally, largely unstructured nucleoporins containing a high number of phenylalanine-glycine (FG) repeats form the permeability barrier that is essential for nucleocytoplasmic transport.

The NPC structural scaffold is formed by a stack of three rings (Fig. 1): the nucleoplasmic and cytoplasmic rings, and the inner ring (for a review, see Grossman et al., 2012). This arrangement and the nucleoporins creating these structures are similarly found in yeast (Hoelz et al., 2011; Stuwe et al., 2015; Lin et al., 2016). However, here, we will primarily focus our discussion on vertebrate NPCs. Both the cytoplasmic and the nucleoplasmic rings are predominantly formed by multiple copies of an evolutionarily highly conserved complex, the Nup107–Nup160 complex which is, due to its overall shape, also referred to as the Y-complex. In vertebrates, the Y-complex consists of ten nucleoporins, some of which show structural similarities to vesicle coats (Devos et al., 2004; Mans et al., 2004; Brohawn et al., 2008). This complex is thought to stabilize the highly curved pore membrane. The precise arrangement of Y-complex molecules within the cytoplasmic and nucleoplasmic rings is a subject of active research (for a review, see Hoelz et al., 2016). Recent electron microscopic tomographic reconstructions of NPCs suggest that both the cytoplasmic and the nucleoplasmic rings each consist of two concentric rings of eight Y-complexes that are arranged in a head-to-tail manner, resulting in 32 copies of the Y-complex per NPC (Bui et al., 2013; von Appen et al., 2015).

Embedded between the nucleoplasmic and cytoplasmic rings is the inner ring of the structural scaffold. This inner ring is mainly formed by Nup93 complexes, which consist of Nup93, Nup155, Nup53 (also referred to as Nup35) and the orthologs Nup205 or Nup188 (for a review, see Vollmer and Antonin, 2014). New insights into the inner ring architecture were recently achieved by docking of crystal structures of individual nucleoporins or respective subcomplexes into the electron tomogram of the human NPC (Kosinski et al., 2016). Similar to the Y-complex, the Nup93 complex forms four eight-membered stacked rings resulting in 32 copies of the complex per NPC. The NPC inner ring represents the link between the pore membrane and the permeability barrier formed by FG-repeat nucleoporins: Nup93 positions the Nup62 complex, which forms a large part of the central transport channel of the NPC (Sachdev et al., 2012; Chug et al., 2015). Attached to this generally symmetric NPC structure are the nuclear basket and cytoplasmic filaments, asymmetric extensions that extend into their respective compartments.
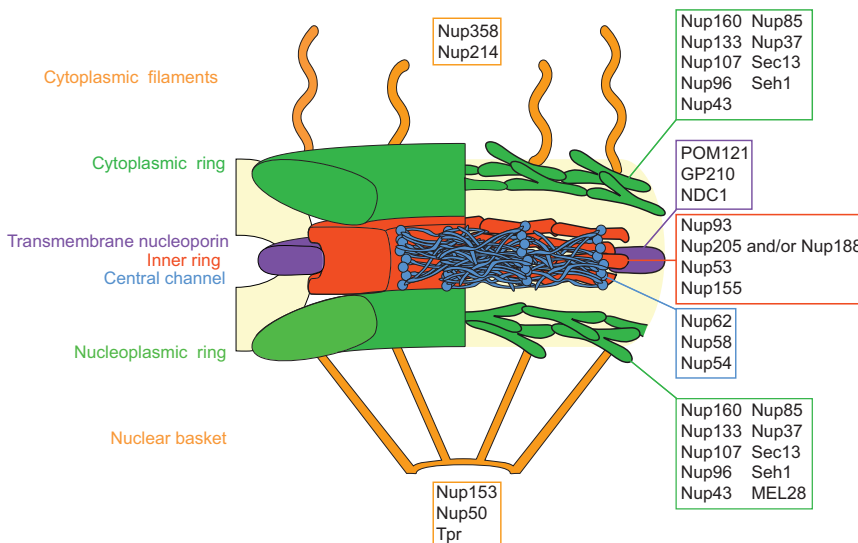
NPCs are embedded into the nuclear envelope at sites where inner and outer nuclear membranes are fused. Membrane association is

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Fig. 1. General organization principle of nuclear pore complexes. Simplified schematic representation of the different structural elements of NPCs with assignment of the respective nucleoporins. The cytoplasmic and nucleoplasmic rings are shown in green, each mostly formed by 16 copies of the Y-complex, arranged in two eight-membered rings. The inner ring, predominantly formed by 32 copies of the Nup93 complex is shown in red. Transmembrane nucleoporins are depicted in violet, and the cytoplasmic filaments and nuclear basket structure are in orange. Attached to the inner ring are Nup62 complexes (depicted in blue), which form a cohesive meshwork within the central channel through their FG-repeat domains. Not indicated is the position of Nup98, a FG-repeat-containing nucleoporin important for the transport and exclusion function of NPCs; its position in the NPC is less defined, but it might be part of the inner ring. Similarly, Aladin (also known as AAAS), Gle1, Rae1 and Npl1 (also known as hCG1 and NUPL2) have been omitted.

mediated by each of the three scaffold rings. In the case of the cytoplasmic and nucleoplasmic rings, the Y-complex members Nup160 and Nup133 contain amphipathic helixes, which can facilitate membrane binding (Drin et al., 2007; Doucet et al., 2010; Kim et al., 2014; von Appen et al., 2015). For the inner ring, Nup53 and Nup155, both components of the Nup93-complex, can directly interact with the pore membrane (Vollmer et al., 2012; von Appen et al., 2015) and additionally interact with the transmembrane nucleoporins NDC1 and POM121 (Mansfeld et al., 2006; Mitchell et al., 2010; Eisenhardt et al., 2014).
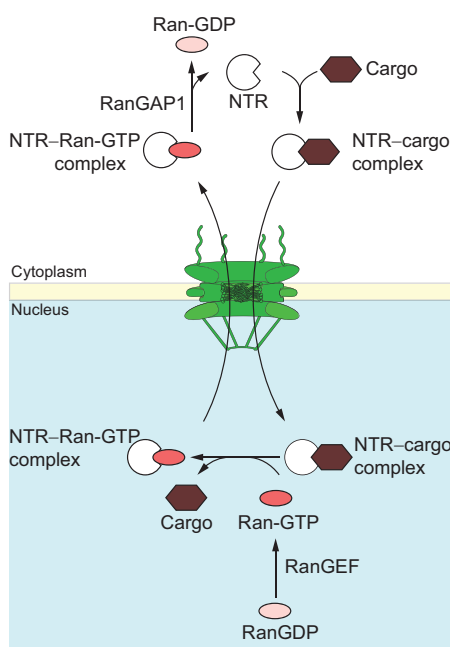
Nucleocytoplasmic transport

NPCs function as selective gates through the nuclear envelope and allow the passage of molecules in two modes: passive diffusion, which is only effective for molecules smaller than 5 nm, and facilitated translocation (reviewed in Gorlich and Kutay, 1999).
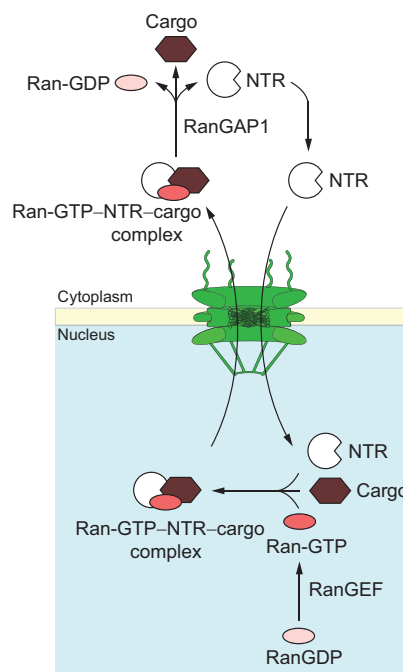
Facilitated translocation requires nuclear transport receptors (NTRs, also called karyopherins), which shuttle between the cytoplasm and the nuclear interior, binding cargo on one side of the nuclear envelope and delivering it to the other (Fig. 2). Thereby, NTRs mediate cargo translocation through the permeability barrier of NPCs. Regarding the direction of transport, nuclear transport receptors can be classified as importins or exportins, although this categorization is not absolute as some NTRs mediate transport in both directions. Importins recognize cargo proteins bearing nuclear localization signals (NLSs) and enable their passage from the cytoplasm into the nucleus. In contrast, exportins bind cargo with nuclear export signals (NESs) in the nucleus and translocate them into the cytoplasm.

The passage of importins and exportins through NPCs occurs in both direction and the same would, in principle, also be true for importin–cargo and exportin–cargo complexes. Directionality is determined by the small GTPase Ran. Like many small GTPases,

Fig. 2. Nuclear import and export through nuclear pore complexes. An import complex consisting of an NLS-bearing cargo and a nuclear transport receptor (NTR) is formed in the cytoplasm. After translocation through the NPC, Ran-GTP displaces the cargo from the NTR, resulting in nuclear cargo release. This reaction occurs due to the chromatin localization of the Ran guanine nucleotide exchange factor (RanGEF), which is restricted to the nucleus. The NTR–Ran-GTP complex returns to the cytoplasm through the NPC where the Ran GTPase-activating protein (RanGAP1) stimulates GTP hydrolysis, releasing the NTR for another import cycle. Nuclear export cycles require the formation of a trimeric cargo–NTR–Ran-GTP complex in the nucleus. After NPC passage, this complex dissociates due to Ran-GTP hydrolysis, releasing the cargo into the cytoplasm.

Ran requires auxiliary factors to accomplish its GDP–GTP cycle (Fig. 2). The guanine nucleotide exchange factor for Ran (RanGEF), RCC1, is a chromatin-binding protein and therefore restricts exchange of GDP to GTP to the nucleus, resulting in a high nuclear Ran-GTP concentration. Likewise, the GTP hydrolysis of Ran is spatially constrained to the cytoplasm as its GTPase-activating protein (RanGAP1) is primarily bound to the cytoplasmic filaments of NPCs. The remaining fraction is soluble in the cytoplasm, and therefore, in the cytoplasm, Ran is predominantly present in its GDP-bound form.

After translocation of the importin–cargo complex into the nucleus, Ran – in its GTP-bound state – can displace the cargo protein from importin, resulting in cargo release. The newly formed importin–Ran-GTP complex can pass through NPCs into the cytoplasm where it dissociates, due to GTP hydrolysis, and the released importin can function in the next import cycle. The export of a cargo from the nucleus requires, instead, the formation of a trimeric complex consisting of the cargo, the exportin and Ran-GTP in the nucleoplasm. After translocation through the NPC, this complex dissociates once it reaches the cytoplasm due to GTP hydrolysis of Ran. With the exception of mRNA export (for a review, see Natalizio and Wente, 2013), which we do not discuss here, the directionality of nucleocytoplasmic transport is thus established through the Ran-GTP–Ran-GDP gradient across the nuclear envelope.

NPC assembly

Multiple copies of thirty different nucleoporins coordinately assemble into an NPC, which ultimately consists of approximately one thousand individual proteins. In general, two mechanistically different NPC assembly pathways can be distinguished: mitotic and interphase NPC assembly (Doucet et al., 2010; Dultz and Ellenberg, 2010). Mitotic assembly of NPCs occurs only in cells with an open mitosis, during which the nuclear envelope, including all NPCs, disassembles (for a review, see Guttinger et al., 2009). At the end of mitosis, large numbers of NPCs are reassembled rapidly and simultaneously with the reformation of the nuclear envelope (Dultz et al., 2008). In contrast, interphase NPC assembly increases the number of NPCs in the intact nuclear envelope during the course of interphase. In comparison to mitotic NPC assembly, interphase NPC assembly occurs rather sporadically and with much slower kinetics: whereas reassembly of all NPCs during telophase occurs within 10 min in mammalian tissue culture cells (Dultz et al., 2008), interphase assembly of individual NPCs shows a high variability in assembly kinetics, which can last several hours (Dultz and Ellenberg, 2010).

Mitotic NPC assembly – a coordinated reformation of NPCs and the nuclear envelope barrier

At the beginning of mitosis, the nuclear envelope – along with integral membrane proteins – is absorbed into the mitotic endoplasmic reticulum (ER) membrane network (Ellenberg et al., 1997), and simultaneously, NPCs are disassembled. In late anaphase and telophase, the mitotic ER membranes are reorganized and the nuclear envelope reforms and encloses the genome. The segregation of the nuclear envelope membrane from the bulk ER is mediated by the ability of inner nuclear membrane proteins to bind chromatin or chromatin-associated proteins (Ulbert et al., 2006; Anderson et al., 2009). The formation of a closed nuclear envelope additionally requires membrane fusion, and the SNARE machinery, as well as atlastins – GTPases involved in ER membrane fusion – contribute to this process (Baur et al., 2007; Wang et al., 2013). Beyond that, two recent studies have reported a crucial function of endosomal sorting

complex required for transport (ESCRT)-III components for nuclear envelope closure (Olmos et al., 2015; Vietri et al., 2015). In late anaphase, the ESCRT-III complex transiently localizes to the nuclear envelope at places where gaps remain in this barrier. Here, ESCRT-III colocalizes with the microtubule-degrading enzyme spastin at points where microtubules and the reforming nuclear envelope intersect, thereby coordinating spindle disassembly and nuclear envelope sealing (Vietri et al., 2015). In addition to its role in nuclear envelope sealing during mitotic exit, ESCRT-III has been recently found to repair transient nuclear envelope openings in interphase in migrating mammalian cells to prevent leakage in and out of the nucleus and to prevent DNA damage (Denais et al., 2016; Raab et al., 2016).

Two models for mitotic NPC assembly have been proposed (for a review, see Schooley et al., 2012). According to the insertion model, NPCs are reassembled into an intact nuclear envelope (Macaulay and Forbes, 1996; Fichtman et al., 2010; Lu et al., 2011). Thus, NPC formation requires the fusion of the inner and outer nuclear membranes to allow NPC integration. A second, so-called enclosure model suggests that NPC assembly starts before the nuclear envelope encases the chromatin (Burke and Ellenberg, 2002; Walther et al., 2003a; Antonin et al., 2008; Dultz et al., 2008). In this model, the emerging NPCs are surrounded by the growing nuclear envelope membranes. In this scenario, no fusion between the outer and the inner nuclear membrane is required to allow for NPC assembly. Hence, in contrast to the insertion model, the enclosure model predicts that mitotic NPC assembly does not depend on a yet-to-be identified fusion machinery between the outer and inner nuclear membrane.

Independent of whether mitotic NPC assembly follows the insertion or enclosure mode, it is generally agreed to be initiated by association of the nucleoporin MEL28 (also known as ELYS or AHCTF1) with the decondensing chromatin before nuclear envelope reformation (Galy et al., 2006; Rasala et al., 2006; Franz et al., 2007). Although MEL28 can bind to DNA through its AT-hooks *in vitro* (Rasala et al., 2008) recent *in vivo* data indicates that the AT-hooks are, at least in *Caenorhabditis elegans*, not essential for chromatin localization (Gomez-Saldivar et al., 2016). In addition, the presence of histones appears to be crucial for the function of the protein in NPC assembly (Inoue and Zhang, 2014). MEL28 recruits the Y-complex to NPC assembly sites and probably is a part of the nucleoplasmic ring structure within NPCs (von Appen et al., 2015). Subsequently, the transmembrane nucleoporins POM121 and NDC1 join the assembling complex and establish contact with membranes (Rasala et al., 2008). Next, the second structural scaffold complex, the Nup93 complex, is assembled into the forming NPCs (Dultz et al., 2008). In contrast to the Y-complex, which is recruited as a whole, the Nup93 complex is not incorporated as a pre-assembled complex, but as individual subunits, starting with the membrane- and NDC1-binding nucleoporin Nup53 (Vollmer et al., 2012; Eisenhardt et al., 2014). Nup53 recruits Nup155 and Nup93, which is in complex with one of the two paralogs Nup188 or Nup205 (Hawryluk-Gara et al., 2005; Theerthagiri et al., 2010; Sachdev et al., 2012; Eisenhardt et al., 2014). Nup93 also recruits, through its N-terminal coiled-coil domain, the Nup62 complex, which consists of the FG-repeat-containing nucleoporins Nup62, Nup58 and Nup54 (Sachdev et al., 2012; Chug et al., 2015), which forms a large part of the hydrophobic meshwork within the central NPC. At the same time, Nup98, another FG-repeat-containing nucleoporin that is also crucial for the exclusion and transport properties of the NPC (Laurell et al., 2011; Hulsmann et al., 2012), is integrated into the structure (Dultz et al., 2008). The yeast and fungi homologs of

Nup98 bind through short linear motifs to the respective Nup155 and Nup205 homologs (Fischer et al., 2015), and it is likely that similar interactions play a role in vertebrate NPC assembly. Subsequently, the asymmetric NPC components of the nuclear basket and the cytosolic filaments join the complex, but the precise order is unknown.

Whereas the early events of mitotic NPC assembly, such as MEL28-mediated chromatin recruitment of the Y-complex or the order of assembly within the Nup93 complex, are rather well understood, we still lack a clear picture of the assembly choreography of the NPC as a whole; for instance, we do not know whether assembly proceeds sequentially from the nucleoplasmic, the inner and to the cytoplasmic ring structure. In another extreme, yet less likely, scenario one might envision a sequential assembly of eight individual NPC columns each spanning the total NPC height, following the octagonal symmetry of NPCs. Advances in high-resolution microscopy will conceivably help to resolve this.

Interphase NPC assembly – sporadic and slow integration of new NPCs into the nuclear envelope

Whereas mitotic NPC assembly ensures rapid regeneration of thousands of NPCs within the reforming nuclear envelope in telophase, thus re-establishing transport competence of the nucleus within minutes (Dultz et al., 2008), NPCs also continue to be integrated into the nuclear envelope in interphase. Integration of NPCs into the interphase nuclear envelope approximately doubles the number of NPCs in preparation for the next cell division, or possibly in response to changes in cellular requirements during cell differentiation or changes in metabolic activity (Maul et al., 1971; Doucet et al., 2010; Dultz and Ellenberg, 2010). In organisms with a closed mitosis, such as the yeast *Saccharomyces cerevisiae*, it represents the only mode of NPC assembly (Winey et al., 1997).

A recent study using correlative live-cell imaging with high-resolution electron tomography and super-resolution microscopy has shed light on the assembly process (Otsuka et al., 2016): dome-shaped invaginations form at the inner nuclear membranes, which grow in diameter and depth until they fuse with the outer nuclear membrane (Fig. 3). This requires extensive membrane deformation, which might be achieved by various mechanisms, such as the scaffolding functions of curved membrane-binding proteins, or insertion of amphipathic helixes or wedge-shaped membrane domains into the lipid leaflet (Antonin et al., 2008; Rothballer

and Kutay, 2013). Indeed, the Y-complex shows similarity to vesicle coats (Devos et al., 2004; Mans et al., 2004; Brohawn et al., 2008), and a number of nucleoporins possess amphipathic helixes through which they bind and deform membranes (Drin et al., 2007; Vollmer et al., 2012; von Appen et al., 2015). A specific function in interphase NPC assembly has been assigned for three such proteins: Nup53, Nup133 and Nup153. Although Nup53 that lacks its amphipathic helix is functional in mitotic NPC assembly, its membrane-binding and -bending helix is essential for interphase NPC assembly (Vollmer et al., 2012). Similarly, the amphipathic helix of Nup133 has been shown to be required for interphase, but not mitotic, NPC assembly (Doucet et al., 2010). In the case of Nup153, its amphipathic helix mediates its interaction with the inner nuclear membrane during interphase NPC assembly, and mutation of this membrane-binding region, which prevents membrane association, inhibits interphase but not mitotic NPC assembly (Vollmer et al., 2015). Interestingly, membrane bending is apparently not a crucial function of the amphipathic helix in Nup153, as this motif can be replaced by a transmembrane region that does not deform membranes. Rather, Nup153 directs its binding partner, the Y-complex, to NPC assembly sites at the inner nuclear membrane. Of note, in mitotic NPC assembly, as discussed above, recruitment of the Y-complex is executed by MEL28, which directs the Y-complex to the decondensing chromatin. Consistent with this division of function, MEL28 is essential for mitotic but not interphase NPC assembly, whereas Nup153 is crucially required for interphase but not mitotic NPC assembly (Doucet et al., 2010; Vollmer et al., 2015). Two nuclear basket nucleoporins in *S. cerevisiae*, Nup1 and Nup60, contain an N-terminal amphipathic helix, which binds the inner nuclear membrane and might play a similar role to Nup153 in yeast NPC assembly (Meszaros et al., 2015).

At least for the early steps in mitotic NPC assembly, the order of events is well defined. In contrast, initiation of interphase assembly is still a matter of speculation. Super resolution microsocopy of interphase NPC assembly has revealed that Nup107, which is part of the Y-complex that forms the nucleoplasmic and cytoplasmic ring, is found on the assembling NPCs before Nup358 (also known as RANBP2) (Otsuka et al., 2016). Thus, not unexpectedly, the structural NPC core assembles before the cytoplasmic filaments. Interestingly, electron microscopy data indicate that some invaginations at the inner nuclear membrane, the likely intermediates of interphase NPC assembly, are surrounded by an
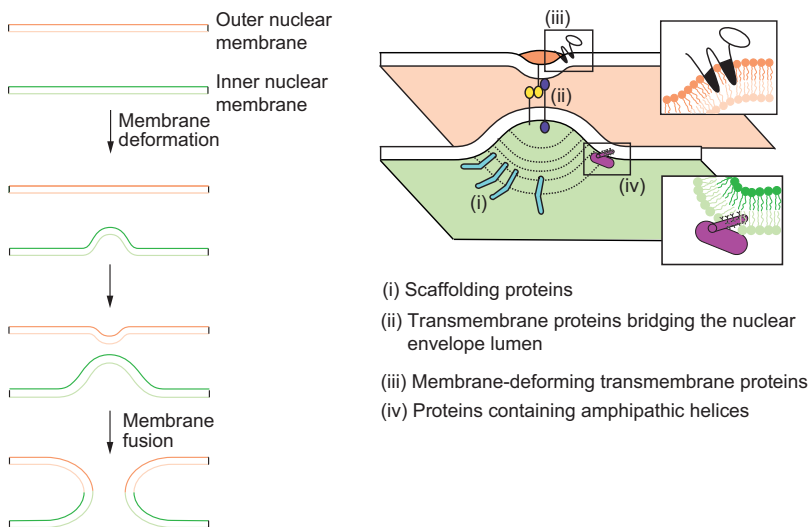

Fig. 3. Membrane remodeling for NPC biogenesis. Insertion of NPCs into the intact nuclear envelope requires pore formation. This process involves membrane apposition, which is achieved by membrane deformation, and fusion of the outer (orange) and inner (green) nuclear membrane. Different classes of membrane-shaping proteins might contribute to this. Membrane-scaffolding proteins and/or complexes (e.g. the Y-complex) could shape the pore membrane by providing a rigid scaffold (i). Amphipathic helixes (iv) could also insert into the lipid bilayer, and curve the membrane (e.g. Nup133, Nup153 and Nup53). Similarly, membrane proteins with a wedge-shaped region (e.g. reticulons, iii) can deform membranes. Integral membrane proteins that form complexes in the lumen between outer and inner nuclear membranes (e.g. the LINC complex formed by SUN proteins and Nesprins), or integral membrane proteins with lipid-binding capabilities could also contribute to membrane approximation and fusion (ii).

Outer nuclear membrane

Inner nuclear membrane

Membrane deformation

Membrane fusion

(i) Scaffolding proteins

(ii) Transmembrane proteins bridging the nuclear envelope lumen

(iii) Membrane-deforming transmembrane proteins

(iv) Proteins containing amphipathic helixes

eightfold rotationally symmetric ring structure (Otsuka et al., 2016). It is highly possible that this represents the assembling nucleoplasmic ring largely formed by the Y-complexes. The binding of Nup153 to the inner nuclear membrane might seed this interphase assembly, as it recruits the Y-complex with its scaffolding and membrane-coating functions. However, as amphipathic helixes cannot only deform membranes, but also preferentially bind to positively curved membranes (i.e. act as sensors of membrane bending), it is similarly possible that the amphipathic helix of Nup153 detects and binds to sites of NPC formation that are already deformed. These deformed sites could be generated by a variety of different proteins (Fig. 3), such as Nup53 and Nup155, membrane-deforming transmembrane proteins, including reticulons, which have been implicated in interphase NPC formation (Dawson et al., 2009), or SUN proteins, which are part of a nuclear-envelope-spanning protein complex, the LINC complex, and so might contribute to the apposition of inner and outer nuclear membranes and thus NPC assembly (Talamas and Hetzer, 2011; for a recent discussion, see Jahed et al., 2016). Furthermore, the transmembrane nucleoporin POM121, which is crucial for interphase NPC assembly (Doucet et al., 2010; Funakoshi et al., 2011), localizes to interphase NPC assembly sites prior to the Y-complex (Doucet et al., 2010; Dultz and Ellenberg, 2010); this is consistent with the idea that Nup153 recognizes pre-established sites for future NPC assembly.

A third way of increasing NPC numbers – inserting double-membrane sheets with preassembled NPCs into the growing nuclear envelope

Whereas interphase NPC assembly is a comparatively slow and sporadic event, rapidly dividing cells have found a way to increase NPC numbers within their quickly expanding nuclear envelope. Recent work proposes that in *Drosophila* blastoderm embryos, cytoplasmic double-membrane structures that are already filled with NPCs, so-called annulate lamellae, insert into the nuclear envelope to keep NPC density constant when these nuclei expand (Hampoelz et al., 2016).

Annulate lamellae are cytoplasmic membrane cisternae that are continuous with the ER. Like the nuclear envelope, they might thus be regarded as a subdomain of the ER. Annulate lamellae pore complexes (ALPCs) are inserted into the membranes, but, in contrast to nuclear envelope NPCs, they face on both sides the cytoplasm. ALPCs resemble NPCs structurally, at least on the electron microscopic level, and in terms of protein composition, although some nucleoporins might be absent from ALPCs (Cordes et al., 1996; Miller and Forbes, 2000). Annulate lamellae are found in many cell types, but they are highly abundant in germ cells and early embryonic cells, as well as in cancer and virus-infected cells (for a review, see Kessel, 1992). As annulate lamellae are tightly packed with ALPCs, they have been speculated to serve as storage compartments for NPC components (Spindler and Hemleben, 1982; Cordes et al., 1995); ALPCs might disassemble and their nucleoporins be used for NPC assembly. Interestingly, a recent study on annulate lamellae in *Drosophila* blastoderm embryos suggests an alternative scenario (Hampoelz et al., 2016). In these embryos, cell cycle progression is very rapid and limits the length of interphase to ~10 min. Despite this short time, the nuclei grow rapidly and keep their approximate NPC density in the nuclear envelope constant. Live-cell imaging and electron microscopy analysis has revealed that annulate lamellae align with and integrate into the nuclear envelope (Fig. 4). It has been proposed that the part of the nuclear envelope underlying the annulate lamellae sheet will open and retract, allowing the previous annulate lamellae sheet to
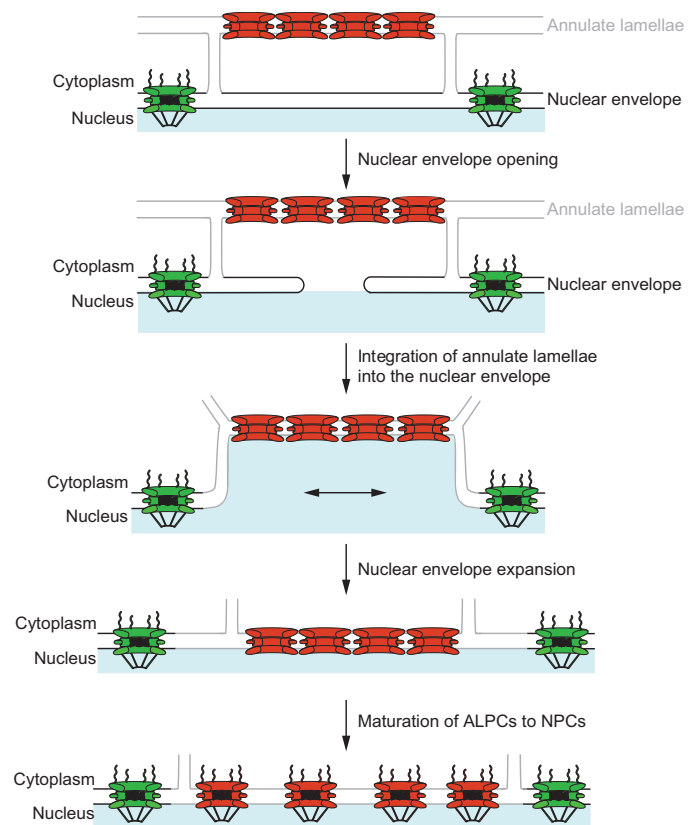
Fig. 4. Model of nuclear expansion by annulate lamellae insertion into the nuclear envelope. An annulate lamellae double membrane is aligned parallel to the nuclear envelope. Opening and local retraction of the nuclear envelope establishes the annulate lamellae as a new nucleoplasmic–cytoplasmic boundary. Maturation of annulate lamellae pore complexes (ALPCs) into NPCs involves recruitment of the central transport channel-forming Nup62 complex as well as the cytoplasmic filament and nuclear basket structure. As subdomains of the ER, both nuclear envelope and annulate lamellae are part of a membrane continuum; membrane remodeling therefore can account for the proposed changes. However, NPCs in the nuclear envelope region that is replaced by the annulate lamellae structure impose a topological challenge, because these NPCs cannot be retracted along with the membranes into the new nuclear envelope. Thus, the replaced nuclear envelope region might be devoid of NPCs, as shown in the schematic drawing, or existing NPCs might be disassembled.

constitute the new nuclear envelope in this region. Accordingly, ALPCs that were part of the inserted annulate lamellae sheet become NPCs, and in this manner, contribute to the rapid increase in NPC numbers during nuclear growth. It is currently unclear how the opening in the nuclear envelope, which will be replaced by the annulate lamellae structure, originates. Any remaining gaps in the nuclear envelope, not sealed in telophase, might be starting points for this process (Hampoelz et al., 2016). Alternatively, mechanical nuclear envelope rupture or disassembly of a NPC might account for this.

Importantly, despite a significant rearrangement of the nuclear envelope during annulate lamellae insertion, the permeability barrier between the cytoplasm and the nucleoplasm remains intact, as fluorescently labeled 155 kDa dextran injected into the cytoplasm is excluded from the nucleus (Hampoelz et al., 2016). It is possible that gaps in the expanding nuclear envelope are rapidly sealed, for example, by the ESCRT III complex as described above. Alternatively, the space between the annulate lamellae sheet and the nuclear envelope could constitute an

already fully enclosed area that is physically separated from the remaining cytoplasm (Fig. 4). As ALPCs contain the nucleoporin Nup98, which comprises a large part of the NPC permeability barrier (Laurell et al., 2011; Hulsmann et al., 2012), it is expected that the transition from ALPCs to NPCs does not impact upon their exclusion properties.

NPCs are asymmetric structures. Whereas the structural scaffold within the plane of the nuclear envelope is largely symmetrically arranged, different sets of nucleoporins form the asymmetric nucleoplasmic and cytoplasmic extensions (see above, Fig. 1). This raises the question of how the asymmetry of NPCs in the nuclear envelope, required for its transport function, is guaranteed if these complexes are already preassembled in cytoplasmic annulate lamellae? ALPCs, at least in *Drosophila* blastoderm embryos, are composed of only the symmetric part of NPCs (Hampoelz et al., 2016): the outer- and inner-ring comprising structural nucleoporins of the Y-complex and the Nup93 complex, as well as the barrier-forming FG-nucleoporin Nup98. Two exceptions are the cytoplasmic nucleoporin Nup358 and MEL28, which are also found in ALPCs. Despite being part of the cytoplasmic filaments, Nup358 has been reported also to stabilize the cytoplasmic ring structure of the NPC by interacting with the Y-complexes (von Appen et al., 2015). It is possible that MEL28 fulfills a similar function within the nucleoplasmic ring. Only after their integration into the nuclear envelope do ALPCs mature, and the nucleoporins that form the remaining nucleoporins of the cytoplasmic filaments and the nuclear basket structure are integrated, as well as the central channel-forming Nup62 complex (Hampoelz et al., 2016). Thus, it is likely that the transport competence of these complexes is only established once they are part of the nuclear envelope.

Regulation of NPC assembly

The generation of transport gates in the nuclear envelope is expected to be coupled to the metabolic activity of a cell, and hence to the level of nuclear–cytoplasmic transport capacity required. Indeed, there is a general correlation between transport activity and NPC number per nuclear envelope. For instance, the nuclear envelopes of maturing oocytes in amphibians possess millions of NPCs (Cordes et al., 1995), reflecting the high protein synthesis rate in preparation for the rapid cell divisions after fertilization. It is likely that the regulation of NPC numbers occurs at the transcriptional level except for rapidly dividing cells, but the underlying mechanisms are not defined.

In addition to the likely regulation of NPC numbers or their density per nucleus, the assembly process itself is controlled. This has been best studied for NPC reformation at the end of mitosis. Most, if not all, mitotic processes are regulated by phosphorylation–dephosphorylation cycles. Indeed, many nucleoporins, including members of the Y-complex, Nup98 and Nup53, are phosphorylated by mitotic kinases, and hyperphosphorylation of Nup98 at the beginning of mitosis initiates its dissociation from NPCs and triggers NPC disassembly (Macaulay et al., 1995; Favreau et al., 1996; Onischenko et al., 2005; Mansfeld et al., 2006; Glavy et al., 2007; Laurell et al., 2011). Interestingly, many of these phosphorylation sites identified in Nup98 are localized within an unstructured domain that, at least in yeast and fungi, interacts with Nup155 and Nup205 through short linear motifs (Laurell et al., 2011; Fischer et al., 2015). Thus, mitotic phosphorylation might be a general mechanism to keep nucleoporins in a dissociated state. Conversely, dephosphorylation at the end of mitosis might enable interactions between nucleoporins and thus promote NPC assembly (Fig. 5A). In the case of Nup53, mitotic phosphorylation by cyclin-
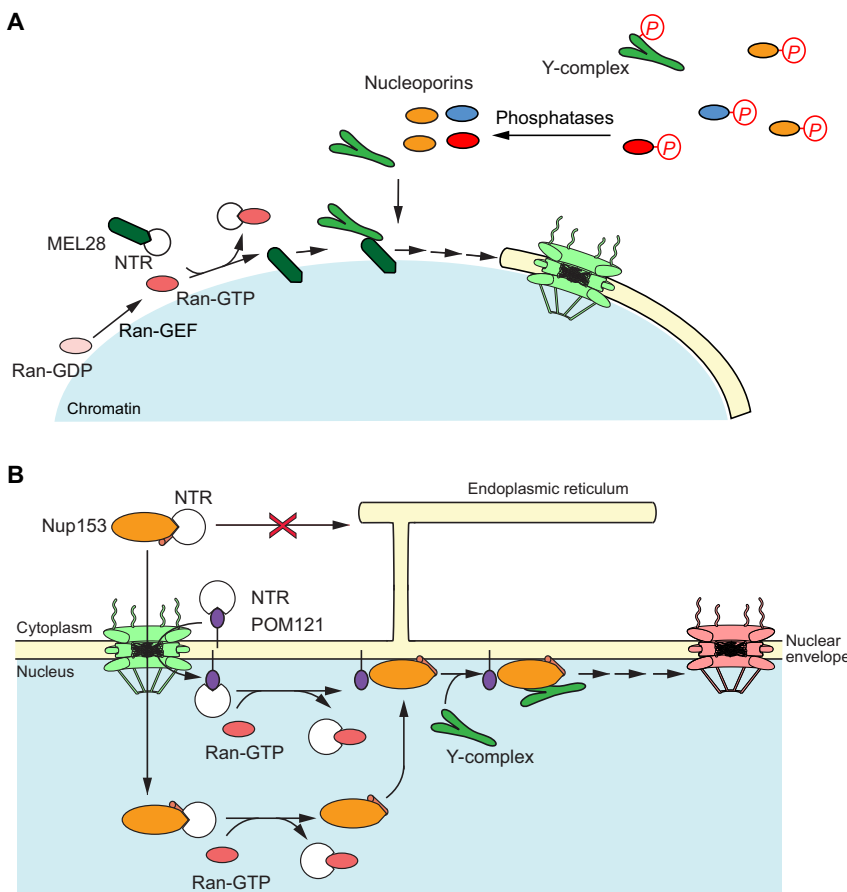
Fig. 5. Regulation of NPC assembly. (A) Mitotic NPC assembly is regulated by Ran and phosphatases. The local generation of Ran-GTP on the chromatin allows release of inhibitory NTRs from nucleoporins (exemplified for MEL28). MEL28 binds to the decondensing chromatin and recruits the Y-complex. Dephosphorylation of nucleoporins enables their interaction and allows NPC assembly. (B) Model for Ran regulation of interphase NPC assembly. NTR binding to Nup153 in the cytoplasm prevents its membrane interaction and mediates its nuclear import. In the nucleus, Ran-GTP-mediated NTR release from Nup153 allows its interaction with the inner nuclear membrane. Here, Nup153 recruits the Y-complex for interphase NPC assembly. POM121 is directed to the inner nuclear membrane by NTR- and Ran-mediated nuclear import.

dependent kinase 1 (CDK1) blocks one of its two membrane-binding regions and thus might promote its dissociation from the nuclear membrane, a process which is reversed during mitotic exit (Vollmer et al., 2012). However, direct evidence for such a dissociation–reassociation mechanism is often lacking because the responsible kinases and phosphatases perform pleiotropic mitotic functions and the identification of crucial phosphorylation sites is challenging due to a high degree of redundancy (Laurell et al., 2011). Interestingly, whereas a general decay in mitotic kinase activity is thought to be required for mitotic NPC reassembly, CDK1 activity in interphase appears to be crucial for NPC assembly during this phase of the cell cycle (Maeshima et al., 2010). However, the precise targets of this kinase during interphase NPC assembly are currently unknown.

Dephosphorylation of nucleoporins at the end of mitosis is believed to temporally regulate interactions between nucleoporins as well as nucleoporin binding to the nuclear envelope and by that to allow NPC assembly specifically in telophase. A second layer of regulation controls the correct localization of the site of NPC assembly, achieved by the Ran-GTPase (Fig. 5A). In addition to its function in nuclear transport in interphase, Ran regulates a number of mitotic processes by executing the same functions as during nuclear import–export cycles (for a comprehensive review, see Forbes et al., 2015). In this context, NTRs bind and inhibit key mitotic proteins, including a number of nucleoporins, which blocks relevant interactions between NPC components (Harel et al., 2003; Walther et al., 2003b). Because of the chromatin localization of the RanGEF RCC1, high levels of Ran-GTP are generated on mitotic chromatin, allowing there the release of the inhibitory NTRs from mitotic factors. In the case of nucleoporins, this allows for initiation of NPC assembly at the nuclear envelope with the chromatin-binding nucleoporin MEL28 being one of the relevant regulatory Ran targets (Franz et al., 2007; Rotem et al., 2009). The importance of this spatial information is underscored by the aberrant assembly of ectopic NPC as cytoplasmic annulate lamellae that has been observed upon disruption of the Ran-GTP gradient (Walther et al., 2003b; Franz et al., 2007; Vollmer et al., 2015).

Interestingly, in addition to mitotic NPC assembly, interphase NPC assembly is also regulated by the Ran system (D'Angelo et al., 2006). One explanation for this might be the requirement of Ran-dependent nuclear import of those nucleoporins that participate in NPC assembly on the nuclear side (Fig. 5B). Indeed, the transmembrane nucleoporin POM121 contains a NLS that directs it to the inner nuclear membrane (Doucet et al., 2010; Yavuz et al., 2010; Funakoshi et al., 2011). Another example is Nup153, which has to be imported into the nucleus to bind the inner nuclear membrane and to recruit the Y-complex as discussed above (Vollmer et al., 2015). Interestingly, the membrane-binding motif of Nup153 is masked by its binding to its NTR. This could prevent its binding to membranes in the cytoplasm and so prevent ectopic NPC assembly. Once imported into the nucleus, binding of Ran-GTP to the NTR would release Nup153 and enable its function in NPC assembly. Surprisingly, Ran-GTP has also been proposed to function on the cytoplasmic side of the nuclear envelope in NPC assembly *in vitro* (D'Angelo et al., 2006). It is currently unclear whether cytoplasmic Ran-GTP could indeed contribute to NPC assembly in a cellular context, or whether this finding reflects a non-physiological situation *in vitro* where any steps of NPC assembly that are regulated by Ran could occur on either side of the nuclear envelope.

In vitro, annulate lamellae formation can be induced by artificially increasing the Ran-GTP concentration in the cytoplasm (Walther et al., 2003b). However, because high Ran-GTP levels are restricted to the nucleus (Kalab et al., 2002), it is unlikely that Ran regulates ALPC formation in the cellular context. Nevertheless, phosphorylation and/or dephosphorylation events might regulate ALPC assembly in a similar manner to as in NPC formation. In *Drosophila* syncytial embryos, ALPCs disassemble and reassemble during each mitosis in a CDK1- and phosphatase-driven manner (Onischenko et al., 2005). Currently, it is unclear how the integration of annulate lamella into the nuclear envelope in the *Drosophila* embryos described above is regulated. This pathway appears to be specific to a very short time in the development of *Drosophila* embryos and is not observed in later developmental stages (Hampoelz et al., 2016). Changes in the nuclear envelope itself, such as increased mobility of nuclear envelope components in early embryos, might account for this difference.

Outlook

We have summarized here our current knowledge of NPC assembly throughout the cell cycle. As described above, we have a rather good understanding of the early events of mitotic NPC assembly that starts with the decondensing chromatin, including the regulation of the process. However, the later events of mitotic NPC assembly are less well defined. It remains open whether a rigorous sequential order also exists for the subsequent steps, or whether the multiple redundant protein interactions within the NPC allow for several alternative assembly pathways to take place.

Interphase NPC assembly is comparatively less understood. We lack a clear picture of the assembly order, the key steps and, presumably, a major part of the regulatory network. It is, for example, unclear whether (and, if so, how) pore formation in the nuclear envelope barrier is coordinated with the assembly of NPCs, including their permeability barrier, to avoid uncontrolled pore expansion and major leakage of nuclear material into the cytoplasm.

The recently described integration of annulate lamellae, including their pore complexes, into the nuclear envelope represent a different strategy of increasing NPC numbers in *Drosophila* blastoderm embryos. It will be interesting to see whether similar processes also occur in other physiological conditions and, if so, how they are coordinated.

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