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Trafficking to uncharted territory of the nuclear envelope

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Abstract

The nuclear envelope (NE) in eukaryotic cells serves as the physical barrier between the nucleus and cytoplasm. Until recently, mechanisms for establishing the composition of the inner nuclear membrane (INM) remained uncharted. Current findings uncover multiple pathways for trafficking of integral and peripheral INM proteins. A major route for INM protein transport occurs through the nuclear pore complexes (NPCs) with additional requirements for nuclear localization sequences, transport receptors, and Ran-GTP. Studies also reveal a putative NPC-independent vesicular pathway for NE trafficking. INM perturbations lead to changes in nuclear physiology highlighting the potential human disease impacts of continued NE discoveries.

Introduction

Linking structure to function is a critical goal for understanding the physiological impacts of the nuclear envelope (NE) in eukaryotic cells. At the most basic level, the NE double lipid bilayers provide a physical barrier dividing the cytoplasm and nucleus. The inner nuclear membrane (INM) and outer nuclear membrane (ONM) fuse at discrete sites to form pores that perforate the NE. The structures anchored in these pores, nuclear pore complexes (NPCs), form transport channels for the diffusion of small molecules and the selective trafficking of macromolecules greater than ~40 kDa [1,2]. As such, the NE and NPCs are fundamentally responsible for the compartmentalization of the nucleus and cytoplasm and the resulting separation of function.

Beyond serving as a physical barrier with selective transport channels, the NE harbors multiple critical cellular activities. The NE provides a scaffold for the organization of chromatin into selective zones of heterochromatin and euchromatin, and a platform for genomic transcription and repair [3,4]. In addition, the NE bridges cytoskeletal communications to the chromatin for signaling events [5] and in yeast the NE anchors the cell division machinery [6,7]. These NE functions are inherently dependent on establishing novel INM and ONM protein and lipid compositions.

With the ONM being continuous with the endoplasmic reticulum (ER), mechanisms that set up the ONM composition are considered synonymous with those for the ER. In contrast, the INM harbors a unique protein composition and requires specific trafficking mechanisms [8,9]. The proteins of the INM are synthesized in the cytoplasm, the cytoplasmic ER, or the ONM and must then be localized to the INM. For example, the INM is associated with the nuclear lamins, intermediate filament proteins that are translated in the cytoplasm, imported into the nucleus, and assembled into a lamina network at the INM. Lamin functions are

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topics of intense investigation and extensively reviewed elsewhere [10,11]. Importantly, efforts directed towards defining the INM protein composition estimate as many as ~80 proteins reside at the INM [12]. Common structural domains categorize subsets of INM proteins into proteins families; for example, the LEM, SUN and KASH-domain families [10,13]. The LEM-domain family of proteins contributes to chromatin organization, whereas the SUN and KASH-domain families together are components of a complex that links the nucleoskeleton to the cytoskeleton (the LINC complex). However, the majority of INM proteins lack functional characterization [14].

Taken together, the physical complexity of the INM suggests a significant uncharted territory for NE functions and highlights the importance of understanding INM trafficking mechanisms. We summarize here new insights into how INM composition is established and provide a perspective on how proper trafficking and INM composition impacts the NE environment and nuclear shape and size. We further review intriguing links between the INM and viral life cycles that suggest the potential discovery of novel routes to the INM.

Section I: connections between NPCs and the INM

To date, trafficking of proteins from the cytoplasmic compartment to the INM is thought to occur exclusively through NPCs. Significant work has advanced insights into NPC architecture and transport mechanisms (as reviewed in [15,16]). In total, each NPC comprises >400 individual nucleoporin (Nup) polypeptides that derive from approximately 30 different types of Nups [17]. The Nups associate into discrete subcomplexes that are present in 8-fold radial and bilateral symmetry along the respective central NPC axis and the NE plane [17]. The resulting structural building blocks include an inner ring, an outer ring, a linker complex, and pore membrane proteins (Poms) as diagramed in Figure 1a. Nearly one-third of the Nups share a common unstructured domain with multiple phenylalanine–glycine (FG) repeats separated by characteristic space sequences [18]. These FG domains fill or line the central NPC channel [17], coincidentally forming the basis of the NPC permeability barrier and facilitating NPC translocation via direct FG interactions with transport receptors [19-22]. On each respective NPC face, asymmetric filamentous structures (cytoplasmic fibrils, nuclear basket) extend from the core structure and harbor functions that help define transport directionality [2,15].

The central channel of the NPC is estimated to be ~50 nm in diameter based on cryo-electron tomography experiments [23]. In addition, eight peripheral channels of ~9 nm diameter might exist between the NE and NPC substructures [24•]. These peripheral channels are predicted to structurally accommodate the cytoplasmic domains of integral INM proteins and allow for maintained membrane insertion while integral INM proteins traverse the NPC pore membrane (Figure 1a).

There is increasing evidence for roles of INM protein localization in mediating new NPC assembly into an intact NE (as reviewed in [25,26]). For example, the integral membrane protein mPom121 is selectively targeted to the INM before its localization to the pore membrane at steady state, and this INM localization step is required for NPC assembly [27-31]. Other INM proteins impacting NPC assembly are the LEM-domain proteins in *Saccharomyces cerevisiae* (sc) scHeh1/Heh2 and the metazoan (m) SUN-domain protein mSUN1 [29,32]. These INM proteins potentially have roles in the generation and stabilization of membrane curvature required for INM and ONM fusion during nuclear pore formation. On the basis of these studies, it is clear that INM proteins are both trafficked through the NPC and play active roles in NPC assembly. This suggests a paradoxical chicken and egg scenario in terms of which comes first. It is also intriguing to consider that

such coupled dependence for INM protein localization and NPC biogenesis might contribute to the unknown mechanism by which NPC number per nucleus is determined.

Section II: NPC-dependent trafficking mechanisms for INM proteins

Three basic classes of proteins are selectively targeted to the INM: peripheral INM proteins anchored through protein–protein interactions, peripheral INM proteins that associate with the INM outer leaflet via amphipathic helices or post-translational modifications, and integral INM proteins. For peripheral INM proteins, localization from the cytoplasm to the nucleus is governed by the same paradigms as that for soluble nuclear proteins (as reviewed in [8,33,34]), followed by INM association once in the nucleus (Figure 2a). These peripheral INM proteins harbor short amino acid spans termed nuclear localization sequences (NLSs), which are recognized by nuclear import receptors known as karyopherins (Kaps) or importins. There are 14 Kap family members in *S. cerevisiae*, and over 20 in metazoan cells [35], each of which interacts with a distinct NLS or nuclear export sequence (NES) encoded in different cargo [36]. Kap-mediated mechanisms for import require direct Kap binding both to the cargo NLS and to the FG-Nups [37]. Kap binding to the FG-Nups mediates docking at the cytoplasmic filaments and translocation through the central NPC channel. Models of the precise mechanism for this FG-dependent Kap movement are reviewed extensively elsewhere [3,18,38]. Importantly, directional release of the import cargo in the nucleus is mediated by binding of the small GTPase Ran (Ran-GTP) to the Kap [34,39].

For integral INM proteins, a convergence of efforts has uncovered key molecular targeting requirements. Integral INM proteins are composed of lumenal, transmembrane and cytosolic domains, all of which must be moved from the rough ER/ONM through the pore membrane of the NPCs. Early reports proposed both active and passive transport mechanisms for integral INM proteins [40,41]. The most comprehensive study to date measured NE dynamics for 15 distinct integral INM proteins using fluorescence-recovery after-photobleaching (FRAP) and photoactivation assays [42•]. These mobility-based assays reveal a full range of integral INM protein dynamics supporting distinct mechanisms of integral INM localization. For one class, the integral INM protein dynamics agree with a lateral diffusion-retention mechanism (Figure 2c), whereas other integral INM proteins require active transport mechanisms with differential requirements for ATP, Ran GTPase activity (Figure 2b), and NPC components (Figure 1b). The ATP requirement for integral INM protein transport remains less defined with speculations of ER licensing steps and/or ATP-dependent restructuring of the NPC [41,43]. We focus here on recent studies with mechanistic insights for several integral INM protein trafficking pathways.

Ran-GTP dependent integral INM protein transport

As noted above, Kaps specifically recognize NLSs to mediate import of soluble cargo. Curiously, the majority of integral INM proteins analyzed to date have putative NLSs. In *S. cerevisiae*, NLSs in scHeh1 and scHeh2 facilitate INM localization through scKap60-Kap95 [44] (Figure 2b). These studies suggest a specific function for scKap60-Kap95 and a distinct pathway for integral INM protein transit (Figure 2b-I), as other scKap-NLSs pairs did not mediate INM localization of scHeh2 [44]. Similarly, mSUN2 contains a NLS that plays a role in import and specifically interacts with the scKap60/Kap95 orthologs, mImp-α/Imp-β [45•]. Interestingly, the most recent studies of scHeh1 and scHeh2 identified an intrinsically disordered (ID) linker domain between the transmembrane and NLS domains [46•]. As shown in Figure 2b-II, these long ID linker domains enable the cytosolic NLS domains that are bound to scKap60-Kap95 to span from the pore membrane region to the FG Nups in the central NPC channel [46•]. Other INM integral membrane proteins contain similar ID regions, suggesting that a shared mechanism exists [46•]. As with soluble protein import,

following NPC translocation, directional release of the integral membrane protein at the INM is facilitated by Ran-GTP binding to the Kap (Figure 2a and b) [39,47].

Diffusion-retention for integral INM protein localization

An early model for INM trafficking invoked passive diffusion of proteins through the pore membrane and INM retention by binding to nucleoplasmic proteins [40,48]. Substantial support for this mechanism comes from analyses of integral INM proteins that associate with the lamins in metazoans [42•,49]. The scMps3, a SUN-domain containing protein, provides additional evidence for selective localization of INM proteins that lack intrinsic active transport mechanisms [50•]. It is reported that interactions of scMps3 with the histone variant, scH2A.Z, mediate INM targeting. Thus, a soluble nuclear protein with an NLS can effectively piggyback an integral INM protein from the ONM to the INM. It will be important to see if this simple, yet surprising, trafficking mechanism is utilized across species.

Multiple mechanisms within single integral INM proteins

Single integral INM proteins can require multiple mechanisms for localization, as revealed by studies of SUN family members, mSUN2 and mUNC-84. For mSUN2, targeting requires three distinct domains [45•]. Two of these domains, the NLS and SUN domain, are sufficient for INM trafficking when transferred to heterologous proteins [45•]. However, single deletion of each domain indicates that neither alone is necessary [45•]. Interestingly, the remaining mSUN2 targeting domain is necessary but not sufficient, and functions in retrograde transport of integral INM proteins from the Golgi to the ER-NE network [45•]. This Golgi retrieval signal is common to many integral INM proteins and could represent the undefined ATP-dependent INM trafficking class [43]. mUNC-84 also requires multiple signals for INM trafficking: a NLS, a NE localization signal, and the transmembrane domain [51•]. Together, these studies highlight the expanding diversity of mechanisms for INM transport.

Section III: links between NPC structure and INM trafficking

Many molecular aspects of integral INM transit remain to be further characterized. The central FG-Nups likely bind scKap60/Kap95 for integral INM proteins with long ID linker domains. In support of this, deletion of FG-domains from the Nup57, Nup100, and Nup145N FG-Nups results in less efficient trafficking of an scHeh1-derived reporter to the INM [46•] (Figure 2b-I). Additional evidence also indicates roles for structural NPC regions flanking the NE. The Pom and inner ring Nups (scPom152, scNup170, scNup188, mNup188 and mNup35) are selectively required in integral INM protein trafficking [42•,44,52,53•] (Figure 1b). These membrane proximal NPC subcomplexes are proposed to serve structural roles in forming the peripheral channels between the pore membrane and the NPC (Figure 1a) [8,9]. However, it is not clear whether these Pom/Nups are required strictly for their structural roles or if they also facilitate transport independent of the FG-Nups. Early work on INM trafficking proposed a size restriction for the cytosolic regions of integral INM proteins with experimentally determined limits of <60–70 kDa [40,41,42•]. This size restriction is potentially linked to the physical restrictions of the proposed peripheral NPC channels. However, with the recent discovery of the role for ID domains, reporters with cytosolic domains as large as 174 kDa have been shown to traffic to the INM [46•]. Continued characterization of endogenous integral INM proteins will be critical in resolving the physiological constraints on this trafficking pathway.

One recent study examined the effects of depleting Nups in the inner ring NPC structure from *Xenopus* nuclear assembly and import assays [53•]. The absence of mNup188-Nup93

has no apparent effect on the NPC permeability barrier; however, it results in a twofold increase in import rate for integral INM protein reporters [53•]. This change in INM trafficking correlates directly with a threefold increase in the size of the nuclei, whereas NPC assembly and permeability remains unaffected. Intriguingly, nuclei co-depleted of mNup205-Nup93 are similar in size to control nuclei [53•]. Together, this suggests that mNup188 is a major effector of integral INM protein trafficking in *Xenopus* and potentially in other metazoans. In contrast, in *S. cerevisiae*, removal of an inner ring Nup (scNup188, scNup170) does not accelerate, but rather inhibits transport of the INM proteins scHeh1/scHeh2 and scDoa10 [44,52]. Moreover, the cells lacking either scNup170 or scNup188 do not result in significant changes in nuclear size or NE expansion [54,55]. This species-specific effect on INM trafficking could reflect differences in NPC component redundancy wherein the *S. cerevisiae* genome harbors paralogues of multiple Nups. Indeed, in the absence of both scNup170 and scNup157 (the scNup170 paralog), NE projections and invaginations of membrane sheets are observed [56]. The difference could also be due to the distinct experimental approaches, with the yeast experiments only capable of assaying viable genetic deletion mutants versus the *Xenopus* experiment assaying *in vitro* biochemically depleted extracts. Finally, the different physiological consequences on nuclear size and shape could be linked to species-specific differences in INM functions and proteins. For example, *S. cerevisiae* lacks nuclear lamins and INM lamin-associated proteins [57]. Overall, structural disturbances of NPC, notably within the inner ring Nup subcomplex, lead to significant alterations in integral INM protein trafficking.

Section IV: proper INM trafficking requirements in nuclear physiology

As a whole, the cohort of INM proteins act in a number of diverse nuclear functions, many of which have been recently summarized [5,58-64]. These roles include transcriptional activation and silencing, chromatin organization, genomic stability and repair, DNA replication, cell division, nuclear positioning, and linkers between cytoskeleton and nucleoskeleton complexes. As such, one would predict that perturbations in INM trafficking have pleiotropic cellular effects.

In addition to the mutually dependent links between the NPC assembly and INM trafficking, there are also inherent connections between NPCs and proper NE lipid homeostasis and between the NE and chromatin organization. There are well-established roles for INM proteins in lipid homeostasis [58,65•,66-68], with altered expression and localization leading to effects on nuclear shape and NE integrity. In *S. cerevisiae*, two NE-ER integral membrane proteins, scApq12 and scBrr6, aid in NE membrane homeostasis and fluidity. Cells lacking functional scApq12 and scBrr6 accumulate NE sheets, show disturbances in lipid composition, and are defective in nucleocytoplasmic transport [68]. Another INM regulator of lipid biosynthesis is the phosphatidate phosphatase scPah1 [58]. Interestingly, scPah1 INM localization requires the scSpo7/Nem1 activator complex and is mediated through a phosphorylation-regulated amphipathic helix in scPah1 [69]. *S. cerevisiae* cells with mutations in *sc-pah1* or *sc-spo7/nem1* have gross NE expansion [58]. Additionally, nuclear localization of the metazoan ortholog of scPah1, mLipin1, leads to significant impacts on lipid biosynthesis and nuclear shape. When mLipin is localized to the INM, lipid biosynthetic target genes are repressed and the nucleus coincidentally increases in nuclear eccentricity (ratio of horizontal–vertical axes) [70]. Therefore, the INM composition includes several protein regulators of lipid biosynthesis and contributes greatly to maintaining NE morphology.

Genetics screens for mutants with NE structural defects (conducted by monitoring the localization of Nups) have identified requirements for nuclear transport (Ran/Kap), RNA metabolism, chromatin structure, secretion, protein degradation, glycosylphosphatidyl

inositol (GPI) anchoring, and lipid biosynthesis [71-75]. Some of these mutants might directly compromise localization or expression of INM proteins that regulate lipid biosynthesis (e.g. scApq12, scBrr6, and scPah1) and therefore result in the observed NE mutant phenotypes. For example, loss of function mutants in genes encoding components of the scRSC chromatin-remodeling complex show gross NE structural defects [75]. These NE defects are rescued by the addition of a membrane fluidizing agent, benzyl alcohol, and upon inhibition of transcription [75]. Thus, in these RSC mutants, altered transcription of lipid biosynthetic genes or, alternatively, changes in NPC and/or INM protein contacts with chromatin might contribute to the NE phenotype. It has also been shown that *sc-spo7* mutants combined with Golgi trafficking mutants have even more severe defects [59]. Links between Golgi trafficking and NE expansion suggest that the Golgi/ER network might regulate trafficking to the NE. Further evidence for this connection is the requirement of a Golgi retrieval sequence for mSUN2 INM localization [45•]. Within these contexts, it is clear that delicate balance of both localization and activity of INM proteins is key in maintaining appropriate nuclear shape, size and function.

Section V: a potential NPC-independent trafficking pathway for the NE

With the analysis of novel INM proteins, new insights into NE trafficking mechanisms have been gained. For soluble protein transport and RNA export, multiple insights have also come from studies of viral life cycles and nucleocytoplasmic dynamics [76]. Interestingly, recent studies of Herpes Simplex Virus (HSV) trafficking suggest that the virus has pirated cellular factors linked to endogenous INM trafficking to enable its proliferation. Although the NPC channel allows passage of cargo up to ~39 nm in size [77], the HSV capsid diameter is ~125 nm [78]. Thus, HSV gains nuclear access through docking and uncoating at the NPC cytoplasmic face [79]. In contrast, mature capsids exit the nucleus through a different, non-NPC mechanism [80•]. The mature capsids are enveloped into a vesicle from the nuclear face of the INM, and are observed in vesicles in the NE lumen. The capsids in the vesicles are then de-enveloped through membrane fusion with the ONM, resulting in release into the cytoplasm. This process is termed nuclear egress [80•] and requires that HSV capsids initially interface directly with the INM. To do this, the virus exploits endogenous protein kinase-C (PKC) and encodes viral Cdc2-like kinases, which together phosphorylate lamins [81,82]. This leads to local disruption of the lamin network at the INM and allows the capsids to interact with an INM-localized nuclear envelopment complex composed of viral proteins pUL31 and pUL34 [83]. These two viral proteins are targeted to the INM through an INM-targeting domain of pUL34 [84]. Therefore, understanding the INM localization mechanism for pUL34 will potentially identify host INM trafficking targets for HSV therapies.

Remarkably, HSV capsid primary envelopment at the INM results in the appearance of striking membrane vesicles in the NE lumen [80•]. Such a vesicular trafficking pathway through the NE lumen has not been reported for endogenous cellular proteins. In this light, it is intriguing to re-examine known *S. cerevisiae* mutants with defects in NE homeostasis. Indeed, an acetyl coenzyme A carboxylase mutant, *sc-acc1-7-1*, with altered fatty acid biosynthesis shows aberrant NE phenotypes [65•]. These include accumulation of large NE lumenal vesicles, expanded NE lumenal space, and cytoplasmic vesicles adjacent to the NE and NPC [65•]. Additionally, mice lacking either m-torsinA or mLAP1 exhibit NE-lumenal vesicles similar to the sc-acc1-1 (italics) mutant [85•]. The m-torsinA protein is an AAA+ protein with predicted ATPase activity and resides in the ER-NE lumen where it is membrane-associated and interacts with the lumenal domain of the integral INM protein mLAP1 [86]. The m-torsinA knockout mice show neuronal selective phenotypes, whereas mLAP1 knockout show NE-lumenal vesicles across many different cell types [85•]. Thus,

mouse models with mutant and knockout alleles of m-torsinA and mLAP1 provide further insight into a potential vesicular trafficking pathway between the INM and ONM [85•].

From these observations of NE vesicles in HSV pathogenesis and in yeast and metazoans mutants, we speculate that an endogenous vesicular trafficking pathway between the INM to the ONM might exist (Figure 2d). The vesicles in NE lumen of the m-torsinA knockouts and the *sc-acc1-7-1* mutant could contain cellular cargo and vesicular trafficking machinery of such a pathway. This pathway would be independent of the NPC-dependent pathways shown in Figure 2a–c. Further, the m-torsins and mLAP1 might co-operate together to mediate NPC-independent vesicular trafficking between the ONM and INM. In support of this hypothesis, over-expression of m-torsinA inhibits HSV production and further results in disrupted localization of integral INM localized pUL34 viral protein from NE to cytoplasmic vesicles [87]. The disturbance in pUL34 localization might stem from defective m-torsinA-dependent trafficking pathway through the NE. Interestingly, the AAA+ protein family includes the NSF ATPase involved in membrane fusion events of the secretory pathway [88]. Thus, this suggests that the torsin protein family may be the missing piece of the puzzle in understanding the ATP-dependent INM trafficking pathway. Torsin orthologs have not been reported in yeast, though to date the ATP-dependent INM transport has only been described in metazoan systems. Future studies in metazoans and yeast will be important to further resolve this putative INM and ONM vesicular trafficking pathway.

Conclusion

There remain many significant questions to be answered and uncharted NE territory to explore. Importantly, understanding how perturbations in INM trafficking and NE composition result in human diseases are only beginning to be resolved. The reports to date of direct patho-physiology implications for altered NE protein function indicate that this is an area ripe for discovery. For example, several recent reviews have documented the clear evidence for devastating human inherited diseases linked to genes encoding lamins and lamin-associated INM proteins [89-91]. Proteomic characterization of the NE proteome across multiple different cell types [12,92,93], paired with continued studies of INM protein targeting and INM protein functions will be needed to contribute to a deeper understanding of the tissue-specific nature of INM disease alleles [89-91]. Expanded analysis of the INM trafficking mechanisms and INM protein function in model systems [94] will allow a further convergence of temporal and spatial requirements for NE-associated proteins from the single cell level to the context of multicellular organism development, cell differentiation, and tissue morphogenesis.

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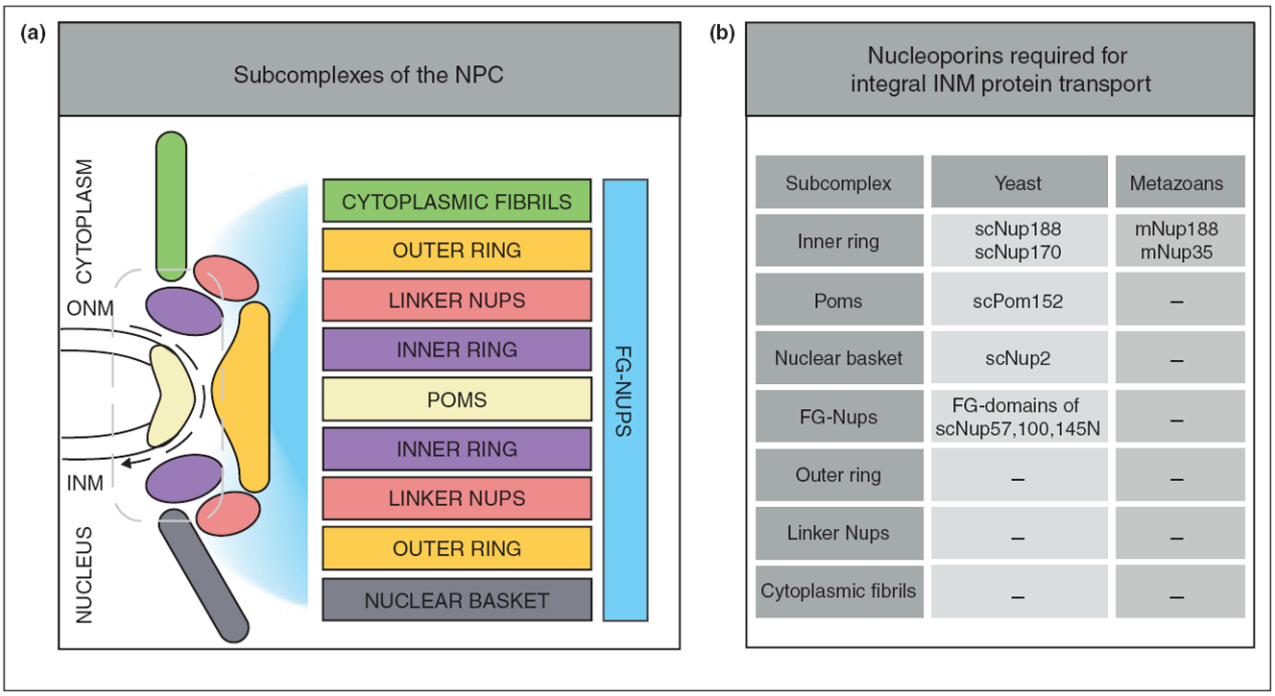

Figure 1. Subcomplexes of NPC and requirements for integral INM protein transport

(a) The ~30 Nups of the NPC assemble into subcomplexes which serve as the building blocks of the NPC [17]. The symmetric subcomplexes include the inner ring (purple), outer ring (yellow), Poms (beige), linker Nups (red) and a central set of FG-Nups (blue). The asymmetric subcomplexes include the cytoplasmic fibrils (green) and nuclear basket (dark gray), which extend into the cytoplasm and nucleus and also harbor FG-Nups. The space between the inner ring subcomplex and Poms represents a putative peripheral channel (gray dashed box) for transit of integral INM proteins (black dashed line). **(b)** Select proteins of the FG-Nup family (scNup100, scNup57, scNup145N), the inner ring (scNup170, scNup188, mNup53, mNup188), the Poms (scPom152) and the nuclear basket (scNup2) have been implicated in integral INM protein transport [42•,44,46•,52].

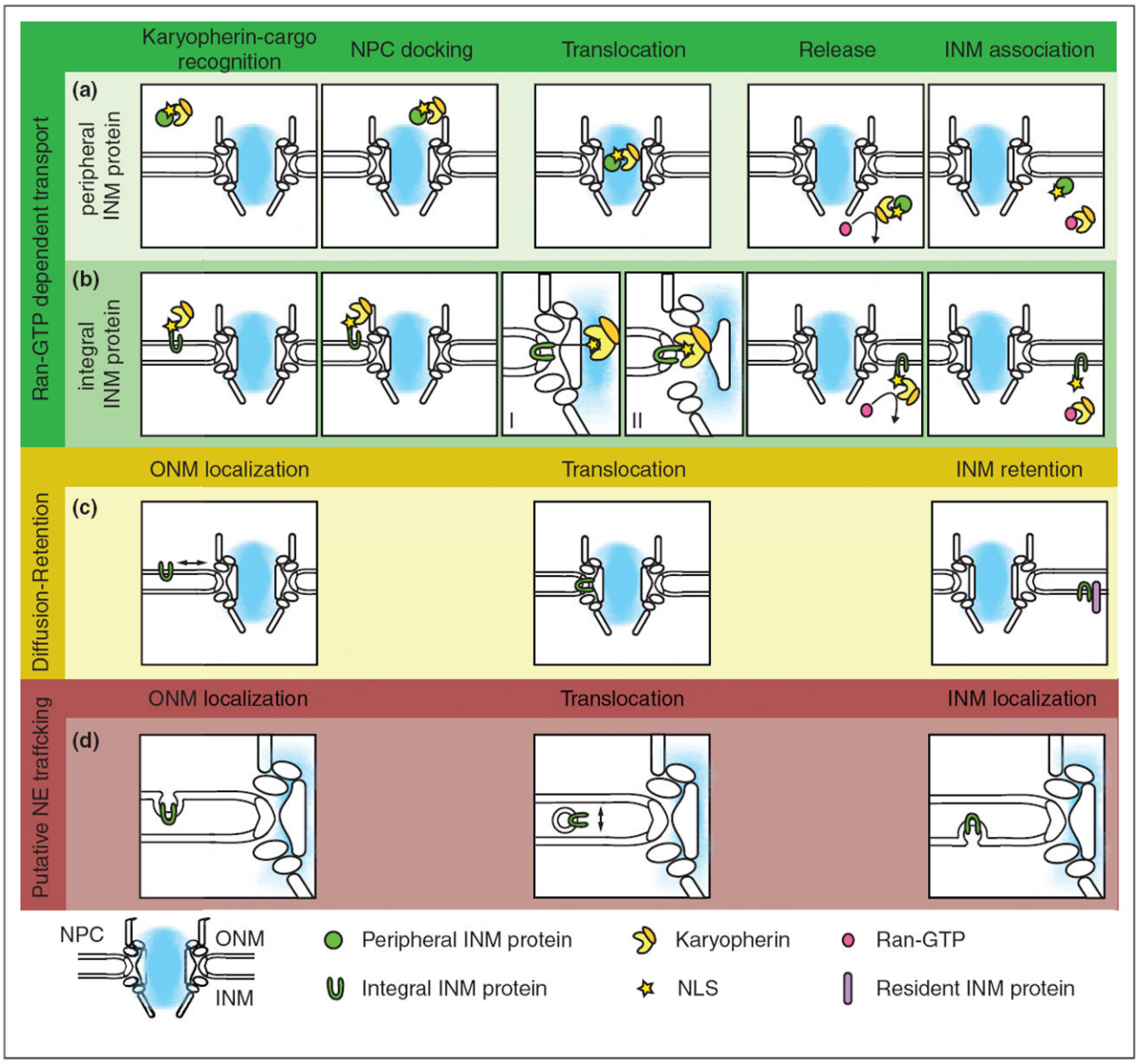

Figure 2. Pathways for the localization of INM proteins

(a, b) The Ran-GTP dependent pathways for peripheral and integral INM localization require an NLS (yellow star), karyopherins (scKap60/Kap95 or mImp-α/β, yellow/orange caps), Ran-GTP (pink circle), and FG-Nups of the NPC channel (blue region). **(a)** Peripheral INM proteins are transported similar to soluble proteins; however, after Ran-GTP mediated release, the peripheral INM proteins anchor to the INM through lipid modifications, amphipathic alpha helices, or protein–protein interactions with integral INM proteins. **(b)** Integral INM proteins remain embedded in the NE throughout translocation. (b-I) The integral INM protein may contain a long intrinsically disordered linker domain allowing for karyopherin transit through the central FG-Nup channel [44,46•]. (b-II) Alternatively, the karyopherins may translocate with structural remodeling of the NPC needed to accommodate the cargo-karyopherin complex. **(c)** A diffusion-retention mechanism contributes the localization of peripheral and integral INM proteins through protein–protein interactions with INM proteins (purple) [42•,50•]. **(d)** A putative NE trafficking pathway

might exist, which utilizes a vesicular trafficking pathway through the NE lumen that is NPC-independent. The illustration represents NE lumenal vesicles as observed during Herpes Simplex Virus nuclear egress [80•], and potential dynamics of vesicles observed in mutant phenotypes with *S. cerevisiae* (*sc-acc1-1-7*) [65•] and metazoans (m-torsinA and LAP1) [85].