Co-translational assembly orchestrates competing biogenesis pathways

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During the co-translational assembly of protein complexes, a fully synthesized subunit engages with the nascent chain of a newly synthesized interaction partner. Such events are thought to contribute to productive assembly, but their exact physiological relevance remains underexplored. Here, we examine structural motifs contained in nucleoporins for their potential to facilitate co-translational assembly. We experimentally test candidate structural motifs and identify several previously unknown co-translational interactions. We demonstrate by selective ribosome profiling that domain invasion motifs of beta-propellers, coiled-coils, and short linear motifs may act as co-translational assembly domains. Such motifs are often contained in proteins that are members of multiple complexes (moonlighters) and engage with closely related paralogs. Surprisingly, moonlighters and paralogs assemble co-translationally in only some but not all of the relevant biogenesis pathways. Our results highlight the regulatory complexity of assembly pathways.
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rotein complexes are a key organizational unit of the proteome. Their modular composition has facilitated the evolution of a very diverse repertoire of folds and corresponding functions. To maintain this very diverse repertoire within the crowded cellular environment poses a logistic burden as the energy gap favoring specific over nonspecific binding decreases with proteome complexity. Therefore, it has been proposed that assembly pathways impose a major restraint on the evolution of protein complexes, whereby duplication events of subunits during divergent evolution may necessitate the diversification of protein interfaces or sophisticated quality control mechanisms to avoid promiscuous binding. Co-translational interactions of nascent polypeptides with their respective binding partner have been discovered for many eukaryotic protein complexes. Homomeric complexes may assemble by co-co assembly in which either nascent chains emerge from consecutive ribosomes of the same mRNA entangled in cis, or alternatively from multiple mRNAs that are clustered by nascent chain interactions in trans. In contrast, many heteromers may rely on co-post assembly, which we will further refer to as co-translational assembly. Here, a soluble, fully synthesized subunit binds to the nascent polypeptide chain of the interactor. Such co-translational assembly events contribute to orphan protein stability and solubility and may be coordinated with the association of assembly chaperones. It has been proposed that they may be beneficial for nascent chain folding or non-promiscuous stoichiometric assembly. It has been hypothesized that they seed assembly pathways when moonlighting interactions are possible. Since co-translational assembly pathways of moonlighters remain largely unexplored, the exact physiological contribution of distinguished co- and/or post-translational assembly pathways remains uncertain.

Nuclear pore complexes (NPC) perforate the nuclear envelope (NE) to facilitate nucleocytoplasmic exchange. They are among the largest, non-polymeric, eukaryotic assemblies and are composed of ~30 different nucleoporins (Nups) that constitute a multi-layered modular architecture of astonishing complexity. Beyond canonical protein interfaces of nucleoporin subcomplexes of up to 10 components, various other types of interactions are crucial for the formation of the higher ordered, eightfold rotational symmetric structure of ~500 nucleoporins in yeast. Those include weak interactions of intrinsically disordered Phenylalanine-Glycine (FG)-rich repeats contained in so-called FG-Nups that function as a velcro and short linear motifs (SLIMs) within so-called linker Nups that facilitate interactions within and across subcomplexes. Furthermore, structured motifs such as beta-propeller complementation and coiled-coil interactions are observed in multiple instances. Interestingly, different Nup subcomplexes that have evolved from each other may contain shared or closely related subunits that assemble promiscuously in vitro. Nevertheless, it remains unclear how such promiscuous interactions are suppressed or discriminated in vivo.

Due to the importance of the NPC as a permeability barrier, faithful assembly imposes a challenge for cells, which is addressed by different pathways depending on the spatiotemporal context. While NPCs are made from pre-existing building blocks during post-mitotic assembly in higher eukaryotes, they are synthesized from scratch during the ubiquitous interphase assembly pathway and Drosophila oogenesis. Interphase assembly is the only known biogenesis pathway in yeast and spatially proceeds from the inside-out at the NE. Although the rough order of subcomplex recruitment to membranes has been resolved, little is known about the early steps of assembly that may occur away and independently from membranes. Besides local and some co-translational events that have been discovered during NPC assembly, it remains unclear which of the above-introduced motifs are subject to such events, in which order they intertwine into the assembly pathways, and how exactly they contribute to faithful assembly.

Here, we elucidate the role of a subset of such motifs for the co-translational de novo assembly of NPC subcomplexes during interphase assembly in Saccharomyces cerevisiae. We show that these events orchestrate complex formation in competitive scenarios.

**Results**

**A framework to investigate co-translational assembly of Nups.** The competition between the specific interactions stabilizing a complex and the far more numerous non-specific promiscuous interactions intensifies with increasing numbers of complex subunits and possible interaction partners. This imposes a severe challenge for the biogenesis of very large complexes with many subunits such as the NPC (Fig. 1a). Co-translational assembly can tolerate higher levels of such competitive binders because it increases the dwell-time of synthesis intermediates at the ribosome in which surfaces that potentially engage in promiscuous interactions are not yet exposed. One would thus predict that cells harness the power of co-translational assembly for the biosynthesis of NPC subcomplexes in the cytosol. Here, we extend our previous theory by including co-translational assembly as a possible assembly enhancer. We derived a mathematical model that captures the essence of this process (Supplementary Note 1), namely how failures in the assembly at intermediate steps accumulate and how this impacts the overall success (Supplementary Fig. 1a, b). This model further reveals that the overall yield of protein complex assembly pathways decreases exponentially with the number of subunits (Supplementary Fig. 1c). It indicates several ways in which co-translational assembly can increase the yield, in particular by capturing intermediates that would otherwise be prone to misassembly or aggregation. In addition, co-translational assembly introduces a hierarchy into the process that is effectively cutting down the number of steps. For small assemblies, this may be an insignificant gain. However, for large assemblies such as the NPC, hierarchical assembly should significantly increase the overall success. Co-translational assembly may also increase the on-rate of a newly synthesized component to a partial complex retained in the vicinity of the ribosome, such that the rate of assembly will be limited by the translation speed instead of the slow diffusion of low abundance species.

We surveyed the known structural repertoire of nucleoporins for domains that could potentially engage in co-translational interactions because they (i) are small interaction motifs found in linker Nups; (ii) complement the fold of another nucleoporin; or (iii) are shared between multiple complexes and thus could be promiscuous interactors (Fig. 1b). This concerns either the competitive binding of Nups for the same binding domains within the NPC or the integration of Nups into distant, functionally unrelated complexes. To elucidate how co-translational association of Nups contributes to the faithful assembly of the NPC, we experimentally validated these motifs in a hypothesis-driven approach.

We first generated a library of C-terminally Twin-StrepII tagged Nups using a scar-free cloning technique in S. cerevisiae. Scar-free cloning preserves the endogenous 3‘ untranslated region (3’UTR) of a messenger RNA and avoids changes that may affect mRNA fate and translation. We used these strains for affinity purification of the respective StrepII-tagged baits (Supplementary Fig. 2a) and analyzed the co-enriched mRNAs by quantitative real-time PCR adapting previously established methods. Below we refer to this
method as RIP-qPCR (Supplementary Fig. 2b). As a positive control, we reproduced the known co-translational interaction of fatty acid synthase subunit Fas1 with nascent Fas2, represented by enrichment of \( \text{fas2-mRNA} \), which was above our signal threshold of 1.5. This interaction was sensitive to the translation-specific inhibitor puromycin, which causes dissociation of the nascent chain and thereby enables the dissection of co-translational interactions from cryptic RNA binding activity35.

Inverse tagging of Fas2 instead of Fas1 did not enrich for either mRNA, as expected (Supplementary Fig. 2c–e).

**Sequential assembly of central transport Nups.** Using the above standards for the experimental validation, we tested the potential co-translational interactions of various full-length Nups that engage with linker Nups. First, we investigated the co-translational landscape of Nic96 which contains several motifs...
to connect the inner ring to the nuclear envelope and the central transport Nup (CTN) trimer (Fig. 1c)\textsuperscript{13,15,36}. Our RIP-qPCR data indicate a co-translational interaction of all three components of the CTN with the nascent chain of Nic96 (Fig. 1d). We did not observe inverse mRNA enrichment for any subunit of the CTN in Nic96 RIP-qPCR experiments (Supplementary Fig. 3a) or co-translational interactions with Nup192 or Nup53 (Fig. 1d).

To investigate if the nascent IM-1 domain of Nic96 co-translationally binds to Nsp1 and Nup57, we turned to selective ribosome profiling (SeRP), as previously described\textsuperscript{4} (Supplementary Figs. 4 and 5a). This method relies on the comparison of ribosome-protected mRNA footprints from a total translome to those that are affinity-purified using a specific bait protein that may engage in interactions with nascent chains. Thereby the onset of the co-translational interaction within a given open reading frame (ORF) is revealed. As a positive control, we reproduced the co-translational onset of the interaction of full-length Fas1 with the nascent chain of Fas2. We observed an enrichment of footprints within the fas2-transcript precisely at the previously reported onset\textsuperscript{4} at the Fas2 assembly domain (Supplementary Figs. 5b, c, 6).

The selective ribosome profiles generated from Nsp1- and Nup57-IPs for nic96-mRNA show simultaneous binding properties to the nascent chain at codon 130. Considering a 30–40 amino acid offset of nascent chain accessibility due to the ribosome exit tunnel\textsuperscript{37}, the measured onset coincides with the exposure of the IM-1 motif of Nic96 from the exit tunnel (Fig. 1e). The synchronous onset further underscores the notion that only fully assembled CTN trimer associates with Nic96\textsuperscript{15,20,38}. SeRP analysis further indicated that Nic96 does not engage with nascent chains of the other known interactors (Supplementary Figs. 6 and 7a).

Independent of Nic96, Nup53 and Nup192 can also directly associate with one another via an N1 interaction site of Nup53\textsuperscript{39} (Fig. 1c). However, we did not detect co-translational assembly of Nup53 with Nup192 or vice versa (Fig. 1f). Nup53 further binds to Nup170/157 with the so-called C-motif\textsuperscript{36}. Interactions with both, Nup157 and Nup170 were reported in vitro\textsuperscript{40}, but integrative modelling highlights the physiological relevance of the interaction with Nup170\textsuperscript{21,41}. We found that Nup53 co-translationally binds to nascent Nup170 but not Nup157 (Fig. 1f).

In our RIP-qPCR experiment, Nup170 and Nup157 did display significant signal reduction for nup53-mRNA upon puromycin treatment. However, the small effect size (<1.5) and additional SeRP experiments (Supplementary Fig. 6) do not support that this reduction was due to co-translational engagement. It is interesting to note that co-translational assembly events are detected only on the physiologically relevant pathway.

Differential co-translational assembly events govern paralogous assembly pathways. We next examined the paralogous linker proteins, Nup100, Nup116, and Nup145N. Interactions of the three linker Nups with Nup192 and Nup170/157 are mediated by the A- and B-motifs\textsuperscript{42} (Fig. 1c). The N-terminal GLEBS domain of Nup116 binds to Gle2 but is absent in Nup100 or Nup145N\textsuperscript{42,43} (Fig. 2a). Indeed, we found that Gle2 binds co-translationally to the nascent chain of Nup116 but not Nup145N or Nup100 (Fig. 2b). We did not detect any additional co-translational events using Nup53, Nup157, Nup170, and Nup192 as baits (Fig. 2c, d, Supplementary Fig. 3b). The interaction of Nup157 with nascent Nup145N was only slightly below the threshold of 1.5-fold enrichment. Validation using SeRP did not detect any ribosome footprint enrichment for nup145-mRNA in a Nup157-SeRP experiment (Supplementary Fig. 6).

The C-terminal autoproteolytic domains (APD) of Nup145N, Nup100 and Nup116 bind to the cytoplasmic filament protein Nup82 in vitro\textsuperscript{13,44}. However, integrative\textsuperscript{21} and in situ structural analysis\textsuperscript{45} stresses the physiological relevance of the interaction with Nup116. Our RIP-qPCR data reveal that Gle2, Nup116 and Nup82 form a hierarchical co-translational assembly chain (Fig. 2e, Supplementary Fig. 3c) that may determine Nup116-specificity for the cytoplasmic filaments. Taken together, our data indicate that some but not all motifs contained in linker Nups engage in co-translational interactions. These co-translational association events may help to specify the paralogous assembly pathways (Fig. 2f).

Domain invasion motifs can act as co-translational assembly domains. Inspired by the above findings, we wondered whether co-translational events may also specify assembly pathways for moonlighters that are members of multiple protein complexes. Two members of the Nup84-subcomplex, Seh1 and Sec13, are incomplete beta-propellers lacking one blade, that interact with the WD40 domain invasion motifs (DIM) of Nup85 and Nup145C, respectively\textsuperscript{18,46}. Seh1 and Sec13 both share moonlighting functions in the Seh1-associated (Sea) complex (Supplementary Fig. 7b)\textsuperscript{47,48}. We first investigated the domain invasion of Nup85 into the incomplete beta-propeller of Seh1 (Fig. 3a). RIP-qPCR analysis revealed that Seh1 enriched for the nup85-mRNA, but not the scc4-mRNA, in a translation-dependent manner (Fig. 3b). To address if the co-translational
association of full-length Seh1 with the nascent chain of Nup85 is indeed mediated by the domain invasion motif at its N-terminus, we turned to SeRP. In line with our RIP-qPCR experiments, SeRP of Seh1-IPs identified an enrichment of footprints within the nup85-ORF (Fig. 3c). We analyzed the translatome-wide data but did not identify similarly strong enrichment patterns of footprints for any other gene (Supplementary Figs. 6 and 7c–f). This analysis suggests that the members of the Sea-complex, which are known to interact with Seh1 but that remain to be structurally analyzed at high resolution, do not mimic assembly intermediates of Nup85. Surprisingly, the onset of the co-translational interaction of Seh1 with nascent Nup85 does not coincide with the emergence of the domain invasion motif (residue 44–101) from the exit channel of the ribosomes. It rather maps to alpha-helices and their connecting loops at positions 405–544. Although distant in sequence, these helices are located right beneath the domain invasion motif in the structure of the Seh1-Nup85 heterodimer. One may speculate that similar to the Sec13-Sec31 heterodimer, interactions of Seh1 with the helical bundle may restrict the flexibility of the alpha-solenoid (Fig. 3d) (see discussion).

Sec13 is part of the COPII coatomer complex and the nuclear pore and the Sea-complex where it binds the domain invasion motifs of at least three of the four known interactors. To investigate the role of Sec13 during the assembly of the respective complexes, we purified Sec13, Sec31, Nup145C, and the Sea-complex protein Mtc5 and analyzed the co-eluted mRNAs by qPCR (Fig. 4a). We found that Sec31 can co-translationally engage with its own mRNA. The IP against Sec13 enriched for sec31-mRNA; however, puromycin treatment only weakly perturbed this interaction. We do not find any other purified component enriched for sec13-mRNA.

We further analyzed the co-translational interaction network of Sec13 and Sec31 using SeRP (Fig. 4b). The resulting data were in line with our targeted RIP-qPCR approach. Sec13 showed an onset at codon 437 within the sec31-ORF (Fig. 4b, c). In contrast, the interaction of Sec31 with nascent Sec31 shows an onset that is shifted to the dimerizing interface at codon 680 (Fig. 4b, d). Sec13 furthermore engages co-translationally with Sec16. This onset is shifted downstream of the DIM to helices within the alpha-solenoid that are located beneath the beta-propeller prior to codon 1367 (Fig. 4b, e), similar...
to the mode of binding observed in Seh1-Nup85 (Fig. 3c, d). The superimposed structures of the insertion blades of Sec16 and Sec31 show only subtle differences in their fold (Fig. 4f). Surprisingly, the known interaction partner within the NPC, Nup145C, did only show very weak signal and no clearly defined onset (Supplementary Fig. 7g). Taken together, these findings illustrate that interaction motifs may co-translationally engage with some but not necessarily all of their interaction partners and that the exact onset of co-translationally interactions may adapt in a versatile way to protein folding.

Coiled coils of the NPC assemble co-translationally. The CTN-subcomplex is densely packed to the equatorial plane of the central channel of the inner ring. It consists of the Nsp1, Nup57, and Nup49 proteins. They contain N-terminal FG-rich intrinsically disordered domains that interact with nuclear transport receptors53. Each of the three members further contains a coiled-coil domain that hetero-trimerizes thus forming the scaffold of the subcomplex (Fig. 5a). To elucidate if and how co-translationally assembly events within the CTN-subcomplex contribute to the organization of the assembly pathway, we first studied the co-translationally landscape by RIP-qPCR. Our results suggest that Nsp1 co-translationally binds to Nup57, but not Nup49 (Fig. 5b). This is in line with previous in vitro analysis showing that Nsp1 cannot directly recruit Nup49 and hence Nup57 acts as an organizer subunit of the trimeric coiled-coil38. To specify the onset of co-translationally entanglement of Nsp1 with nascent Nup57 we used SeRP (Fig. 5c). These experiments highlighted that the minimal requirement for co-translationally interactions is the initial coiled-coil segment 1 (CCS1) that forms a rather long rod-shaped stretch. Neither RIP-qPCR nor selective ribosome profiling experiments detected a translation-dependent interaction of Nsp1 or Nup57 with the nup49-mRNA (Fig. 5b, c and Supplementary Figs. 6 and 7h) suggesting that Nup49 is added to the complex post-translationally.

Intriguingly, the evolutionary related cytoplasmic filament subcomplex that functions as a platform for mRNA export54, is predicted to form a similar hetero-trimeric arrangement as the aforementioned CTN trimer (Fig. 5d)21,55. It consists of Nup159, Nup82, and yet again Nsp1, whereby Nup57 and Nup82 compete for the same binding site within the Nsp1 coiled-coil region22. We wondered if the cytoplasmic filaments are constructed by a similar assembly pathway. Surprisingly, RIP-qPCR and SeRP experiments targeting Nup82 and Nup159 showed no signal (Fig. 5e, f, compare to 5b, 5c). However, we found that Nup159 co-translationally engages with nascent Nup82 (Fig. 5e), suggesting that Nsp1 is rather added post-translationally to the subcomplex. The inverse order of Nsp1 incorporation that we observed for both subcomplexes may help to specify their respective assembly pathway. Although the assembly pathways for CTN- and CF-subcomplexes are distinct, the domain architecture of the C-terminal coiled-coil segments of
Nup57 and Nup82 are structurally similar (Fig. 6a). A notable exception is a small alpha-beta domain unique to Nup57; upstream of the CCS1 that is expanded to a ferredoxin-like domain in vertebrates\textsuperscript{16,20}. Deletion of this domain in Nup57 led to non-stoichiometric CTN-subcomplexes during in vitro reconstitution\textsuperscript{16}. We set out to test if the alpha-beta domain is required for co-translational interactions of Nsp1 with nascent Nup57 in vivo. We deleted the alpha-beta domain by scar-free cloning and further designed two chimeric mutants in which we substituted the CCS1 of Nup57, with either the entire CCS1 of Nup82 thereby removing the alpha-beta domain, or a truncated CCS1 of Nup82 thereby maintaining the alpha-beta domain (Fig. 6a). Removal of the
alpha-beta domain did not impact thermo-sensitivity of the strain implying that overall fitness remained unaffected (Fig. 6b). Contrary, the CCS1 chimeric mutants show mild defects upon the removal of a plasmid-encoded nup57 rescue copy suggesting a fundamental role of CCS1 in faithful CTN formation (Fig. 6b). We used quantitative mass spectrometry to assess complex composition in Nsp1 pull-downs from Nup57 wildtype and mutant strains. CTN components were mildly increased in binding to Nsp1 in the Nup57 alpha-beta deletion strain, while the Nup159-subcomplex remained largely unaffected. In contrast, the CCS1 chimeric mutants effectively decreased the fraction of co-enriched CTN components indicating compromised assembly (Fig. 6c and Supplementary Figs. 8a, b). Although alpha-beta-domain deletion mildly perturbed the integrity of the CTN, it did not impair the co-translational assembly of Nsp1 - Nup57 or CTN - Nic96 (Fig. 6d). In contrast, CCS1 chimeric mutants resulted in the loss of co-translational enrichment of nup57- and nic96-mRNA in a Nsp1 RIP-qPCR experiment. Surprisingly, the chimeric mutants display a previously undetected co-translational enrichment for nup82-mRNA (Fig. 6d). The reason why the Nup82 CCS1 is not sufficient for binding of Nsp1 when duplicated in the Nup57-ORF might be explained by the SeRP data that were obtained under wildtype conditions (Fig. 5f). It showed a slight enrichment that was C-terminally shifted with respect to nup57-ORF and located subsequent to the CCS3 of Nup82, which was not included in the Nup57 chimeric mutant. We thus speculate that the Nsp1 interaction with nascent Nup82 would normally be suppressed due to higher affinity of Nsp1 to Nup57 CCS1 but becomes relevant once effective binding to Nup57 is impaired (Fig. 5g and Supplementary Fig. 8c).

Discussion

Our hypothesis-driven approach has identified many co-translational assembly events that are relevant throughout all stages of NPC biogenesis with local hotspots at the subcomplexes utilizing Nsp1 (Fig. 6e). Our qPCR experiments are in line with many, though not all interaction pairs reported by a recent screen. Specifically, we identified translation-dependent interactions of Seh1—nup85-mRNA, CTN—nic96 mRNA, and Nsp1—nup57-mRNA, while we obtained negative data for Nup192 - nup100-mRNA and Nup157—nup145-mRNA. Differences may be attributed to the experimental design, in particular the use of translation-specific inhibitors such as cycloheximide and puromycin, the biochemical conditions, the design of scarlessly cloned yeast strains and SeRP analysis.

We found that moonlighting proteins that are part of multiple complexes may co-translationally engage in some but not necessarily all alternative assembly pathways. This is exemplified by Seh1 that does co-translationally interact with Nup85 but not with the Sea-complex, Sec13 that co-translationally interacts with Sec31 and Sec16 but not Nup145C or Mtc5, as well as Nsp1 that does co-translationally interact with Nup57 but not with Nup82, unless binding to Nup57 is perturbed. Remarkably, the paralogous Nup159- and CTN-subcomplexes contain the most co-translational interactions. These data suggest that co-translational assembly may be used to organize assembly pathways in a hierarchical manner when multiple outcomes are possible. An interesting aspect is that the domain invasion motifs of Nup85 and Sec16 are not sufficient for the co-translational interactions with Seh1 and Sec13, respectively, but require the trunk of the supporting alpha-helical domains. One may speculate that Seh1 rigidifies the alpha-solenoid of Nup85 (Fig. 3d). This could be beneficial (i) to promote the interaction with Nup120,49,56 and/or (ii) to induce membrane curvature at the nuclear envelope. This is exemplified by the Sec13-Sec31 heterodimer. Here, an increase of rigidity was associated with the induction of membrane curvature of COPII vesicles and therefore might be reminiscent to the inside-out extrusion of the nuclear envelope in interphase assembly57.

Sec13 and Seh1 also exemplify that the onset of co-translational interactions may adapt in a versatile way to protein folding. In fact, it appears likely that the domain invasion motifs are subject to stronger selection pressure because its binding interfaces in the incomplete beta-propeller are the same for each interactor and therefore only allow for subtle changes. Consequently, other structural features may be evolutionarily more accessible to organize a unique assembly pathway for the interactors of moonlighting proteins. The fact that minor changes in the open binding interfaces in the incomplete beta-propeller of Seh1 and Sec13 contribute to the selection of different binding partners was previously demonstrated by attempts to substitute Sec13 with Seh1 in a Sec31-Seh1 fusion construct within sec13Δ-S. cerevisiae strains. The fusion was lethal, highlighting the idea that the domain invasion blades are tailor-made for their native interactors.

In our study, the CTN-subcomplex nicely emphasizes how co-translational association may orchestrate assembly pathways if promiscuous interactions could form. The physiological CTN composition was controversial and had been addressed using different techniques.25, Our data point to a model where Nsp1 binds the CCS1 domain of nascent Nup57 in a co-translational manner, which is crucial for preventing the formation of the promiscuous Nup49-Nup57-Nup49 trimer. Full length Nup49 is then bound in a post-translational manner. The resulting heterotrimer binds to the IM-1 assembly domain of Nic96, yet again co-translationally. This experimentally determined assembly outline agrees with our theoretical considerations (Supplementary Note 1) that suggest a benefit from the hierarchical organization (Supplementary Fig. 1). It shows that not necessarily all, but at least several of the individual steps of a given assembly pathway may occur co-translationally (Supplementary Fig. 8c). Our chimeric Nup57 mutants interfere with the respective co-translational interaction with Nsp1 in vivo and highlight its benefits. In this case, the perturbation of a co-translational complex biogenesis reduced the efficiency of complex formation (Supplementary Fig. 8b) as well as fitness (Fig. 6b). It resulted in
competitive assembly scenarios (Fig. 6d). The consequent co-translational engagement of Nsp1 with nascent Nup82 interfered with the native linear outline of the respective assembly pathway (Supplementary Fig. 8c). Taken together with the recent mass spectrometric study that has revealed the temporal order of subunit engagement\(^2^9\), our findings provide new insights into NPC biogenesis. It has been previously shown that most of the Nup-subcomplex encoding mRNAs, including those of the CTN complex (Nup62 in Drosophila melanogaster) are dispersed in the cytosol\(^2^7\). The advantage of translation under relatively dilute conditions in the cytosol could be that stoichiometric subcomplexes are formed but higher-
order interactions, such as subcomplex oligomerization, are pre-
vented due to the low protein concentrations. The benefit of co-
translational assembly could be to increase the dwell time of inter-
mediate assembly steps to nevertheless facilitate efficient assem-
by. Hereby, at least four early modules are synthesized separately, the Nup84-, CTN-, Nup159-, and inner ring complexes. Next, subcomplexes are recruited to sites of NPC biogenesis in prox-
imity to membranes where their local concentration is increased and higher-order interactions across subcomplexes, such as ring formation, become kinetically favored. Here, Nup53 and Nup170 that were previously assembled co-transcriptionally seed the recruitment of further components such as Nup188 and Nup19229 presumably already associated with Nup10031. Subse-
quently, additional subcomplexes are recruited, various of which were pre-assembled stoichiometrically in a co-translational manner. The concept of co-translational assembly thus ele-
cally complements present scientific models of NPC biogenesis and explains how promiscuous interactions are avoided in such a very complex macromolecular assembly.

**Methods**

**Yeast strains and growth media.** Strepti-tagged yeast strains were obtained by scar-free homologous recombination using the MX/xb cassette33. Briefly, the MX/xb cassette was amplified with gene-specific overhangs for homologous recombination. PCR products were transformed and positive clones were selected on YPD-high phosphate plates supplemented with 300 µg/mL hygromycin B (ForMedium) and 3 g/l potassium dihydrophosphate (monobasic). To remove the MX/xb cassette, MX4 positive clones were grown in low phosphate YPD to induce endonuclease expression33 and transformed with a codon-optimized twin-
Strepti-tag (5’TTCGAAAAG 3′ and 3’TGG TTCTGGTGGCGGTTCAGGTGGTTCATCTGCTTGGAGTCATCCTCAAA
TTCGAAAAG 3′)29 with respective gene-specific overhangs (primers and gene blocks are listed within the Source Data file). Once transformed, clones were screened on YP galactose plates. The successful gene-target integration of twin-
Strepti-tag was validated by PCR.

For Sec13, the integration of the MX/xb cassette remained unsuccessful unless an additional copy of Sec13 was acquired on equilibrated Streptargin resin (IBA) supplemented with 60 µL of Bio-Rack (IBA) to prevent unspecific binding and 0.1 µL RiboBlock (Invitrogen) to inhibitor RNA degradation. Inactivation of the lyase was achieved by shaking the beads by 4 to 5 hr. Then, beads were subjected to subsequent wash steps. Briefly, beads were centrifuged at 500 g for 4 min. Supernatant was removed and beads were washed 3-4 times with 1 mL of wash buffer A (20 mM Hepes-KOH, pH 7.5, 140 mM KCI, 20 mM MgCl2, 1 mM PMSF, 0.01 % Igepal, and COMPLETE EDTA-free protease inhibitor (Roche), 0.1 mg/mL CHX (Sigma-Aldrich) or 0.01 mg/mL puroymycin (Sigma-Aldrich)). Cells were disrupted under cyrogenic conditions using the CryoMill (Retsch) at 30 Hz for 10 s.

The lysate was thawed and transferred into 1.5 mL tubes. The crude lysate was cleared at 15,000 x g at 4 °C for 3 min. Afterward, the cleared supernatant was loaded onto equilibrated Streptatin resin (IBA) supplemented with 60 µL of Bio-Lock (IBA) to prevent unspecific binding and 0.1 µL RiboBlock (Invitrogen) to inhibit RNA degradation. The lysate was loaded on the beads by 4 to 5 hr. Then, beads were subjected to subsequent wash steps. Briefly, beads were centrifuged at 500 g for 4 min. Supernatant was removed and beads were washed 3-4 times with 1 mL of wash buffer A (20 mM Hepes-KOH, pH 7.5, 140 mM KCI, 20 mM MgCl2, 0.01 % Igepal and COMPLETE EDTA-free protease inhibitor, 0.1 mg/mL CHX or 0.01 mg/mL puroymycin) for 1 min to end-by-end mixing, followed by two washes (1 min, 4 min) with wash buffer B (20 mM Hepes-KOH, pH 7.5, 140 mM KCI, 20 mM MgCl2, 0.01 % Igepal and COMPLETE EDTA-free protease inhibitor, 0.1 mg/mL CHX or 0.01 mg/mL puroymycin). After the washes, the beads were resuspended in 500 µL of 10 mM Tris-HCl, pH 8.0. Following a 10 min incubation, 20 % of RNA samples were pretreated with 10 µL of pre-warmed phenol-chloroform-isomyl alcohol (PCI, 65 °C, Invitrogen). This mixture was then incubated at 65 °C, 1,200 rpm for 5 min followed by snap cooling on ice for 10 min. Next extractions were centrifuged at 15,000 x g for 10 min and the aqueous phase was again subjected with 750 µL of PCI. This time, the extraction was performed at room temperature and occasional vortexing for 5 min. The centrifugation was repeated. Finally, residual PCI was removed by a diethyl ether wash, and the remaining organic solvent was evaporated in a Speedvac (Eppendorf).

RNA was precipitated by adding 3 mL NaOAc, pH 5.5 to a final concentration of 0.3 M, 2.5 µL Glycoloside (Invitrogen), and equivalent amounts of isopropanol. Precipitates were placed into the –80 °C freezer overnight. Samples were centrifuged at 15,000 x g at 4 °C for 90 min. The resulting pellet was washed in 70 % EtOH, dried in a Speedvac (Eppendorf), and resuspended in 20 µL of 10 mM Tris-HCL, pH 8.0. Typically, precipitations yielded 150–250 ng/µL of RNA.

For reverse transcription reactions, 500 ng of RNA were applied, and cDNA was synthesized according to the manufacturer’s instructions of the VILO Reverse Transcription kit (Invitrogen) including the optional ezDNase step.
Real-time qPCR was conducted using the TaqMan Fast and Advanced Master Mix (Applied Biosystems) according to the manufacturer’s manuscript. FAM-labelled qPCR probes were purchased from Applied Biosystems as specified in Supplementary Table 1. The qPCR was performed using the QuantStudio 5 cycler (Applied Biosystems, 50 °C: 2 min, 95 °C: 2 min; 40 cycles: 95 °C: 0:01 min, 60 °C: 0:20 min). Images were taken every cycle within the annealing/extension step. All qPCR assays were performed in technical triplicates and each experiment was analyzed using the QuantStudio analysis software (v1.5.1). Briefly, quality assessment was performed within the QuantStudio software and if suggested by the software individual technical replicates were omitted. Experiments in which two points of a technical were omitted did not pass our quality control filter. These few cases are also indicated in the Source Data file.

Selective ribosome profiling. The selective ribosome profiling experiments were conducted according to previously published protocols by the Bukau lab.34,58. Each biologically independent replicate was obtained from 800 mL of yeast cultures grown in YPD to an OD(600) of 0.5–0.6 in analogy to the RIP-qPCR experiment. Harvest was performed as previously described for the RIP-qPCR experiments. Subsequently, cells were lysed in 3 mL of ribosome profiling buffer (20 mM HEPES-KOH pH 7.5, 140 mM KCl, 10 mM MgCl2, 1 mM PMSF, 0.01 % IGEPAL, 0.1 mg/ ml CHX, 1 tablet of cOMplete protease inhibitor per 50 mL) using the cryo-mill (30 Hz, 2 min).

Then, the lysate was thawed and centrifuged at 15,000 g at 4 °C for 3 min. The absorbance at 260 nm of a 1:100 dilution was measured to determine the amount of RNase I (Ambion). Here, 20 U of RNase I per A260 was applied to convert.

|野生型 | Nsp1-StrepII (Nup57WT) | Nsp1-StrepII (Nup57Δαβ) |
|---|---|---|
| Nup57 | Nup57 CCS1(Nup82)FL | Nup57 CCS1(Nup82)Δαβ |
| Nup58 | Nup58 CCS1CCS2CCS3 | Nup58 CCS1CCS2CCS3 |
| Nup57Δαβ | Nup57Δαβ CCS1|Nup82FL | Nup57Δαβ CCS1CCS2CCS3 |
| Nup57 CCS | Nup57 CCS1(Nup82)FL | Nup57 CCS1(Nup82)Δαβ |

|野生型 | Nsp1-StrepII | pRS316_nup57:Δαβ |
|---|---|---|
| Nup57 | Nup57 CCS1(Nup82)FL | Nup57 CCS1(Nup82)Δαβ |
| Nup57Δαβ | Nup57Δαβ CCS1|Nup82FL | Nup57Δαβ CCS1CCS2CCS3 |
| Nup57 CCS | Nup57 CCS1(Nup82)FL | Nup57 CCS1(Nup82)Δαβ |

**Selective ribosome profiling.** The selective ribosome profiling experiments were conducted according to previously published protocols by the Bukau lab.34,58. Each biologically independent replicate was obtained from 800 mL of yeast cultures grown in YPD to an OD(600) of 0.5–0.6 in analogy to the RIP-qPCR experiment. Harvest was performed as previously described for the RIP-qPCR experiments. Subsequently, cells were lysed in 3 mL of ribosome profiling buffer (20 mM HEPES-KOH pH 7.5, 140 mM KCl, 10 mM MgCl2, 1 mM PMSF, 0.01 % IGEPAL, 0.1 mg/ml CHX, 1 tablet of cOMplete protease inhibitor per 50 mL) using the cryo-mill (30 Hz, 2 min).

Then, the lysate was thawed and centrifuged at 15,000 g at 4 °C for 3 min. The absorbance at 260 nm of a 1:100 dilution was measured to determine the amount of RNase I (Ambion). Here, 20 U of RNase I per A260 was applied to convert.
polymers into monomers. RNase I was incubated by end-to-end mixing for 20 min at 4 °C. The reaction was quenched by adding 200 U Superscript In (Invitrogen). Then, ribosomes were pelleted using a 25% sucrose cushion (20 mM Hepes-KOH pH 7.5, 150 mM NaCl, 50 mM K(OAc), 2 mM Mg(OAc)2, 1 mM DTT, 5% glycerol, 0.05% (v/v) IGEPAL, 1 mM benzamidine, 1 tablet/50 mL of cOmplete EDTA-free protease inhibitor, and 1 mL/50 mL BioLock (IBA)) in adaptation to Fischer et al. Resuspension was frozen drop-wise in liquid nitrogen and lysed with the cryo-mill (30 Hz, 2 min). The cell lysate was thawed and cell debris was pelleted by ultracentrifugation at 4 °C. The supernatant was applied to 500 µL bed volume of Streptactin sepharose resin and incubated for 1 h on a rolling mixer at 4 °C. The flow-through was collected and the column was washed with 1 vol of Hepes-NB buffer and the elution was repeated with 5 vol of Hepes-NB buffer. The eluted peptides were transferred and solvents were evaporated in a speed vac (Eppendorf). Dried peptides were resolved in 80 µL of aqueous formic acid was added per well and elution was repeated. Finally, 80 µL of HPLC water. 20 µL of these peptides were then subjected for peptide concentration measurement with the Qubit DNA High Sensitivity kit in the Qubit fluorometer.

Analysis of Nsp1-subcomplexes. One liter of yeast culture was set to an OD(600) 0.05 and grown to OD(600) 1.2–1.4 at 30 °C and 130 rpm in baffles flasks. Cells were harvested by centrifugation, washed in ice-cold PBS and resuspended in Hepes-NB (20 mM Hepes-KOH pH 7.5, 150 mM NaCl, 50 mM K(OAc), 2 mM Mg(OAc)2, 1 mM DTT, 5% glycerol, 0.05% (v/v) IGEPAL, 1 mM benzamidine, 1 tablet/50 mL of cOmplete EDTA-free protease inhibitor, and 1 mL/50 mL BioLock (IBA)) in accordance to Fischer et al. Resuspension was frozen drop-wise in liquid nitrogen and lysed using the cryo-mill (30 Hz, 2 min). The cell lysate was thawed and cell debris was pelleted by ultracentrifugation (35,000 × g, 20 min, 4 °C). The supernatant was applied to 500 µL bed volume of Streptactin sepharose resin and incubated for 1 h on a rolling mixer at 4 °C. The resin was washed with 4 × 5 mL of Hepes-NB. Protein was eluted in Hepes-NB supplemented with 20 mM D-desthiobiotin (IBA) in four elution steps (5 × 350 µL and 1 × 500 µL) each time incubating the resin 5 min with the elution buffer. To avoid unnecessary dilution of the elution fractions, the first fraction was omitted. After elution, protein concentration was determined by measuring the absorbance at 280 nm. 500 µL of elution was immediately supplemented with 20 mM TCEP and incubated for 30 min at 37 °C. Next, the pull-downs were subsequently alkylated using 20 mM iodoacetamide (IAA) incubated in the dark for 20 min at room temperature and further processed by adding 12 % aqueous phosphoric acid to obtain a final concentration of 1.2 % of phosphoric acid.

Pre-processed pull-downs were mixed with S-trap binding buffer, transferred to S-trap Protifil plates (Protifil), and treated according to the manufacturer’s protocol. Finally, the protein was converted into peptides using a 1:100 Trypsin: protein ratio by supplementing the corresponding amount of Trypsin in 125 µL of digestion buffer that was added to each condition. Trypsin digest was carried out overnight at 4 °C.

Before elution, 80 µL of digestion buffer was added to each well of the S-trap digestion plate and eluted in an OASIS elution plate (Waters). Next, 80 µL of 0.2% of aqueous formic acid was added per well and elution was repeated. Finally, 80 µL of aqueous acetonitrile (ACN) containing 0.2% formic acid was applied and peptides were recovered. The eluted peptides were transferred and solvents were evaporated in a speed vac (Eppendorf). Dried peptides were resolved in 80 µL of HPLC water. 20 µL of these peptides were then subjected for peptide concentration assays (Thermo Scientific). The remaining peptides were cleaned up using the OASIS desalting plates.

Dried peptides were reconstituted in 5% acetonitrile (ACN) with 0.1% formic acid (FA). Peptides were loaded onto a C4,CoA trapping column (particle size 3 µm, L = 20 mm) and separated on a C18,CoA analytical column (particle size
a nano-HPLC (Dionex U3000 RSLCnano) at a temperature of 55 °C. Transcripts were extracted for 6 runs with a flow rate of 6 μL/min using a loading buffer (100% H2O with 0.05% trifluoroacetic acid). Peptides were separated by a gradient of water (buffer A: 100% H2O and 0.1% FA) and acetonitrile (buffer B: 80% ACN, 20% H2O, and 0.1% FA) with a constant flow rate of 250 nL/min. The gradient went from 4% to 48% buffer B in 90 min. All solvents were LC-MS grade and purchased from Riedel-de Haen/Honeywell (Seelze, Germany).

Eluting peptides were analyzed in data-dependent acquisition mode on a Fusion Lumos mass spectrometer (ThermoFisher Scientific) coupled to the nano-HPLC by a Nano Flex ESI source. MS1 survey scans were acquired over a scan range of 350-1600 m/z with a charge ratio (m/z) in the Orbitrap detector (resolution = 120k, automatic gain control (AGC) = 2e5, and maximum injection time: 50 ms). Sequence information was acquired by a “ddMS” OT HCD” MS2 method with a fixed cycle time of 2 s for MS/MS scans. MS2 scans were generated from the most abundant precursors with a minimum intensity of 3e4 and charge states from two to five. Selected precursors were isolated in the quadrupole using a 1.4 Da window and fragmented using higher-energy C-trap dissociation (HCD) at 30% normalized collision energy. For Orbitrap MS2, an AGC of 1e4 and a maximum injection time of 54 ms were used (resolution = 30k). Dynamic exclusion was set to 30 s with a mass tolerance of 10 parts per million (ppm). Each sample was measured in duplicate LC-MS/MS runs.

MS raw data were processed using the MaxQuant software (v1.6.6.0) with customized parameters for the Andromeda search engine. Spectra were matched to a Saccharomyces cerevisiae database downloaded from UniProtKB (April 2021), a contaminant and decoy database, with a minimum Tryptic peptide length of seven amino acids and a maximum of two missed cleavage sites. Precursor mass tolerance was set to 20 ppm, while fragment ion tolerance was 0.8 Da. All possible modifications (carboxymethylation and oxidation) were included as variable modifications. The Protein N-terminus and oxidation of methionine residues were included as variable modifications. A false discovery rate (FDR) below 1% was applied at protein, peptide, and modification levels. The ‘match between runs’ option was enabled and only proteins identified by at least one unique peptide were considered for further analysis.

All proteomics data (including acquisition and data analysis parameters) associated with this manuscript have been deposited at the ProteomeXchange and only proteins identified by at least one unique peptide were considered for further analysis.

For the SeRP analysis, a Limma-analysis \(^{39}\) of the TE was conducted considering groupings of IP-experiments vs. total RNA-experiments, p-values from respective Student t-tests were adjusted using the Benjamini-Hochberg method.

**Statistics and Reproducibility.** Data in figures was illustrated as mean with corresponding standard deviation (SD) using GraphPad Prism (v9.0.0). Dashed lines in qPCR graphs represent wildtype background levels (no bait) determined for cycloheximide and puromycin, respectively. Significance levels of qPCRs for one mRNA obtained from the same batch under cycloheximide and puromycin treatments was determined by applying a two-sided Student’s t-test for paired samples by assuming a normal distribution of the data unless otherwise stated (ns: \(p > 0.05\); *: \(p < 0.05\); **: \(p < 0.01\); ***: \(p < 0.001\)). Comparison of Nsp1-Strep pull downs in Nup57-wildtype and Nup57-mutant strain background was conducted using a two-sided, unpaired t-test (ns: \(p > 0.05\); *: \(p < 0.05\); **: \(p < 0.01\); ***: \(p < 0.001\)). Co-translational mRNA enrichment was assigned based on the two following parameters: (I) average signal of cycloheximide treated lyse 2.15 and (II) a statistically significant signal decrease upon puromycin treatment. For mass spectrometry, Nup49 and Nup57 and Nup57 and Nup82 were grouped into CTN and cytoplasmic filaments, respectively. Significance levels were calculated in respect to Nsp1 by a two-sided, unpaired Student's t-test (ns: \(p > 0.05\); *: \(p < 0.05\); **: \(p < 0.01\); ***: \(p < 0.001\)).

Western Blot analysis, Coomassie and Silver Staining of the pull-downs shown in Supplementary Fig. 2a, d, 4c and 8a were performed at least twice. The denaturing PAGE for ribosome profiling (Supplementary Fig. 4d) was run once for the respective replicate. Growth assays as shown in Fig. 6b were repeated twice.

Co-translational event density (Fig. 6e) was calculated by dividing the number of co-translational events by the number of proteins per group. Secondary binding and collective binding as previously observed for the translation-dependent interactions of Gle2 - nup82 and CTN - nic96 was considered as one event.

**Strains.** Yeast strains used in this study are listed in Supplementary Table 4.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The underlying RIP-qPCR data, uncropped images (e.g. Western Blots, growth assays), polysome profiles, Limma-results, underlying mass spectrometry data and primers which were generated in this study are provided in the Source Data file. The selective ribosome profiling data used in this study are available in the European Nucleotide Archive database under accession code PRJEB50305. The mass spectrometry data generated in this study have been deposited in the PRIDE database under accession codes PXD030626 and PXD028413.

The previously published structures for 4XMN [https://doi.org/10.2210/pdb4XMN/pdb] (Fig. 3a; Seh1-Nup85 dimer within the Nup84 subcomplex), 4H8Z [https://doi.org/10.2210/pdb4H8Z/pdb] (Fig. 4c, d; COPII poly coat consisting of Sec13-Sec31), JMEK [https://doi.org/10.2210/pdbJMEK/pdb] (Fig. 4e, f; COPII complex) and 5CWS [https://doi.org/10.2210/pdb5CWS/pdb] (Figs. 5a and 6a; Central transport Nup-trimer) are accessible at the Protein Data Bank (PDB). The integrative structure of the cytoskeletal filaments \(^{31}\) is available in the PDB-Dev under accession code PDBDEV_00000105 (Fig. 5d). The electron density map of the NPC \(^{27}\) is deposited in the Electron Microscopy Data Bank under accession code EMDE-10198 (Fig. 1a). Source data are provided with this paper.

**Code availability**

Matlab scripts for analysis and plotting of SeRP data were deposited to Zenodo (https://doi.org/10.5281/zenodo.5887401). The script suite for SeRP \(^{39}\) can be found on Zenodo (https://doi.org/10.5281/zenodo.2602493) and includes the required reference genome files for the coding and non-coding genome of Saccharomyces cerevisiae (R64-1-1). Revisions and Accepted: 11 February 2022; Published: online 09 March 2022
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manuscript. J.B. designed experiments, analyzed data, wrote the manuscript. J.D.L., E.M.S., and K.R.P. supervised the project. G.H. performed modeling and wrote the manuscript. V.B. designed experiments, supervised the project. M.B. conceived the project, designed experiments, analyzed data, supervised the project, and wrote the manuscript.

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