Unraveling the organization of the FG repeat meshwork that forms the active transport channel of the nuclear pore complex (NPC) is key to understanding the mechanism of nucleocytoplasmic transport. In this paper, we develop a tool to probe the FG repeat network in living cells by modifying FG nucleoporins (Nups) with a binding motif (engineered dynein light chain–interacting domain) that can drag several copies of an interfering protein, Dyn2, into the FG network to plug the pore and stop nucleocytoplasmic transport. Our method allows us to specifically probe FG Nups in vivo, which provides insight into the organization and function of the NPC transport channel.

Introduction

The genetic information of a eukaryotic cell is enclosed by the nuclear envelope, but nuclear pore complexes (NPCs) inserted into the double nuclear membrane mediate nucleocytoplasmic transport of molecules as diverse as proteins, RNAs, and RNPs. The NPC exhibits an overall octagonal structure, 40–60 MD in size, which generates the active transport channel through which nucleocytoplasmic traffic occurs (Rout et al., 2000; Schwartz, 2005). In all eukaryotes, a group of ~30 conserved nucleoporins (Nups), existing in multiple (8, 16, and 32) copies, builds up this huge assembly. A subgroup of these Nups (~30%) contains FG (Phe-Gly)-rich repeat domains, which are thought to form a network of FG repeat filaments that constitute both the permeability barrier and the active transport channel of the NPC (Peters, 2009). Because the FG repeat domains can directly interact with the shuttling transport receptors, it is assumed that these transporters loaded with cargo travel through the NPC by transient low affinity contacts with the numerous FG repeats lining the transport channel (Rexach and Blobel, 1995; Ribbeck and Görlich, 2001). Thus, knowledge of the topological organization of the FG repeat network in the NPC is crucial to gain mechanistic insight into nucleocytoplasmic transport reactions.

Biochemical and biophysical methods revealed that in vitro FG repeat domains are largely unstructured but may form a meshwork via interactions between FG repeats (Denning et al., 2003; Frey et al., 2006; Lim et al., 2007; Patel et al., 2007; Yamada et al., 2010). However, because of their natively unfolded state, the organization of such an FG repeat network within the NPC in the living cells is currently unknown. Hence, several models have been postulated, which predict how the different FG repeat domains are organized and interconnected within the NPC scaffold (Ribbeck and Görlich, 2002; Rout et al., 2003; Lim et al., 2006; Peters, 2009; Yamada et al., 2010). The selective phase model assumes a saturated FG repeat hydrogel formed by the different FG repeat domains that can be overcome by the nuclear transport receptors, which can penetrate through the hydrogel by locally dissolving the FG network (Frey et al., 2006). In another model, the Brownian affinity gating, FG domains are suggested to protrude from the NPC into the cytoplasm and nucleoplasm in a bristlelike fashion (Rout et al., 2000, 2003). Cargo-loaded receptors can bind reversibly to these FG repeats and, thus, have a high probability of entering the central transport channel. The reduction of dimensionality model (for reduction of dimensionality) suggests a coherent hydrophobic FG repeat layer coating the wall of the transport channel and the surface of the nuclear and cytoplasmic filaments. In this case, the cargo–receptor complex binds and diffuses along a two-dimensional surface formed by the FG repeat network.
Figure 1. Recruitment of Dyn2 to eDID-modified FG repeat domains of FG Nups at the NPC can cause a toxic phenotype. (A) Schematic drawing of the FG repeat Nups tested in this study: Nup159 with its natural DID, Nup116, Nsp1, Nup49, Nup57, Nup2, and Nup1 and position of the various FG repeat motifs (FG, GLFG, and FxFG; according to Strawn et al., 2004). Also indicated is the eDID insertion site within the corresponding FG repeat domain after
domains (Peters, 2009). Recently, Yamada et al. (2010) proposed a nonrandomized arrangement of the FG repeat network, organized into two separated zones of traffic within the NPC with different physicochemical properties.

In view of these different models, it is crucial to gain insight into the topological arrangement of FG repeat domains in the NPC scaffold. Hence, we took advantage of a relatively short binding motif (Dyn2 interaction domain) that was inserted into the FG repeat domains of several FG nucleoporins. Upon induction of GAL::DYN2, Dyn2 molecules were recruited into the FG repeat network, thereby interfering with nucleocytoplasmic transport. Our method allows us to analyze the topological arrangement of the different FG repeat domains within the NPC scaffold.

**Results and discussion**

**Design of a tool to probe FG repeat Nups in vivo**

We sought to develop a tool for probing individual FG repeat nucleoporins in vivo to gain insight into the organization and function of the FG repeat network. Previously, we showed that under physiological conditions, Dyn2 is recruited to Nup159, a subunit of the Nup82–Nsp1–Nup159 complex located at the cytoplasmic pore filaments (Stelter et al., 2007). Specifically, Dyn2 is bound to a Dyn2 interaction motif (dynemin light chain–interacting domain [DID]) presented between the FG and coiled-coil domain of Nup159, generating a 20-nm elongated structure that could contribute to the formation of the cytoplasmic pore filaments (Fig. 1A). Moreover, the Nup82 complex dimerizes via the Nup159DID in a Dyn2-dependent manner, which facilitates NPC assembly. Dyn2 forms only a small 20-kD dimer and, when deleted or overexpressed, in a wild-type background, does not significantly influence growth or nucleocytoplasmic transport. These characteristics, therefore, make Dyn2, which should overcome the permeability barrier of the NPC, a promising tool to probe the FG repeat network in vivo. Hence, we inserted an engineered DID (eDID) composed of six consecutive Dyn2 binding motifs (derived from Pac11; see also Flemming et al., 2010) into the FG repeat domain of different FG Nups to test for consequences in NPC function. The selected FG Nups were Nup159, Nsp1, Nup49, Nup57, Nup2, Nup1, and Nup116 (Fig. 1A and Fig. S1A), which are either representatives of a discrete topological location in the NPC or are part of distinct NPC modules. Subsequently, we tested whether these eDID-FG Nups can recruit dynein light chain (Dyn2) upon GAL-DYN2 induction.

**Probing eDID-labeled FG repeat domains with Dyn2**

To find out whether eDID insertion interferes with the corresponding Nup function, the eDID-modified FG domain was integrated at the corresponding chromosomal NUP locus (see Materials and methods) and tested for expression and complementation in the absence of chromosomal DYN2 or upon pGAL-DYN2 induction (Fig. 1B). We inserted the eDID roughly in the middle of a FG repeat domain to be sufficiently away from the interaction motifs of the tested nucleoporins (e.g., coiled-coil domain of Nsp1, Nup49, and Nup159; Fig. 1A). Moreover, the insertion in the midst of the FG repeat domain could allow for a maximal penetration of the eDID into the FG repeat network as part of the NPC transport channel. For control reasons, we also shifted the eDID toward the beginning of the FG repeats within Nsp1 (i.e., Nsp1-eDID(2)) or toward the coiled-coil domain of Nup159 (i.e., Nup159-eDID(2)). All these modified FG Nups tolerated the eDID insertion without an apparent growth inhibition (Fig. 1B, glucose). However, we cannot exclude that the eDID insertion into the FG repeat domains can cause synergistic effects when combined with other Nup mutants.

Strikingly, induction of GAL::DYN2 expression induced a lethal or extremely slow growth phenotype in several of the strains that harbored such an eDID-modified FG Nup. Specifically, Dyn2 expression was lethal to cells expressing Nsp1-eDID, Nup49-eDID, Nup57-eDID, Nup2-eDID, and Nup1-eDID but was not toxic in cells expressing Nup116-eDID or Nup159ΔDID-eDID (Fig. 1B, galactose). The growth inhibition (and also the mRNA transport defects; Fig. 2 and Fig. S3B) was not always fully complemented by coexpression of the respective wild-type Nup (Fig. S3). These data suggest some dominant effect of these Nup-eDID constructs with respect to growth and/or nucleocytoplasmic transport.

To demonstrate that eDID-labeled FG repeat domains can recruit Dyn2 to the NPC, we analyzed cells expressing GFP-labeled Dyn2 by fluorescence microscopy. For this purpose, we deleted the endogenous DID from Nup159 so that we could monitor NPC targeting of Dyn2-GFP mediated by another eDID-modified FG Nup. As anticipated, Dyn2-GFP was not detected at the nuclear envelope in a NUP159ΔDID strain (see also Stelter et al., 2007) but exhibited a punctate nuclear envelope labeling characteristic of an NPC localization when the...
Figure 2. Recruitment of Dyn2 to eDID-modified FG repeat Nups can cause inhibition of nuclear import and export processes. (A) Analysis of nuclear mRNA export after 2-h galactose induction of Dyn2 expression in the indicated eDID-FG repeat NUP strains harboring an empty GAL plasmid (left) or pGAL-DYN2 (right). Poly(A)+ RNA was analyzed by in situ hybridization with a Cy3-labeled oligo d[T] probe, and DNA was stained with DAPI. (B) Time
eDID was inserted into the FG repeat domains of Nsp1, Nup159, Nup49, Nup1, and Nup2 (Fig. 1C). Nup116-eDID also recruited Dyn2 to the NPC but less efficiently than the other constructs (Fig. 1C).

To follow Dyn2 recruitment to the eDID-modified Nups biochemically, we performed affinity purification. First, we focused on Nsp1, which is part of two NPC modules: (1) the Nsp1–Nup82–Nup159 complex located at the cytoplasmic pore filaments and (2) the Nsp1–Nup49–Nup57–Nic96 complex present in the central pore channel (Grandi et al., 1993, 1995; Hurwitz and Blobel, 1995; Fahrenkrog et al., 1998). To demonstrate that Dyn2 can reach not only the peripheral but also the central NPC module, we affinity purified tandem affinity purification (TAP)–tagged Nup57 from the NSP1-eDID strain under conditions of GAL::DYN2 repression or expression. Affinity-purified Nup57 containing Nsp1-eDID, Nic96, and Nup49 was coenriched in Dyn2, suggesting that dynemin light chain can penetrate into the central pore channel that contains the Nsp1–Nup57–Nup49 complex (Fig. 1D). In addition to these biochemical data obtained from the Nsp1-eDID cells, we affinity purified the other eDID-modified Nups (either directly via the TAP tag or via an interacting TAP-tagged Nup). This analysis revealed a significant Dyn2 recruitment after 3 h of Dyn2 induction, independent of whether the eDID-Nup was sensitive to Dyn2 expression or not (Fig. S1C). To determine whether Dyn2 can dimerize the eDID-modified Nups, we performed coexpression experiments of GFP-Nup82 and GFP-Nic96, in the presence or absence of Dyn2, in NUP82-TAP NUP159-eDID and NIC96-TAP NUP49-eDID strains, respectively. Affinity purification of the TAP-tagged proteins revealed a certain degree of Dyn2-dependent dimerization of these complexes (Fig. S1D). Collectively, these data suggest that all of the tested eDID-labeled bait proteins significantly coenriched Dyn2, independent of whether the eDID-FG repeat construct caused inhibition of mRNA export or not. Thus, Dyn2 gains access to the eDID-modified Nups assembled into the NPC scaffold.

Recruitment of Dyn2 to eDID-FG repeat domains blocks nucleocytoplasmic transport

Next, we wanted to find out why cell growth stops when Dyn2 is recruited to a distinct group of eDID-modified FG Nups. We hypothesized that this could be a result of a blockage of nucleocytoplasmic transport caused by corrupting the FG repeat network in the active transport channel with Dyn2 molecules. Strikingly, nuclear mRNA export was massively inhibited after an induction of pGAL-DYN2 expression in the nonviable NSP1-eDID, NSP1-eDID(2), NUP49-eDID, NUP57-eDID, NUP1-eDID, and NUP2-eDID cells but not in the viable NUP116-eDID, NUP159ΔDID-eDID, and NUP159ΔDID-eDID(2) cells (Fig. 2A). The onset of this defect is very fast and could already be well observed after a 45-min shift to galactose medium (note that it requires ~20–30 min to initiate GAL promoter-induced protein expression) and, hence, is indicative of a direct transport inhibition rather than an NPC assembly defect (Fig. 2B). Both, poly(A)+ RNA as well as specific mRNAs (e.g., actin and SSA1 heat shock mRNAs) that accumulate in the nucleus tend to cluster in foci that are close to the nuclear envelope (Fig. 2, A and B; and Fig. 3).

We performed EM to reveal the perinuclear poly(A)+ accumulation at the ultrastructural level. In this case, a network of electron-dense particles, which could correspond to accumulated mRNA RNPs and/or a mixture of several RNA/RNP/protein aggregates, were often lining the inner nuclear membrane, with several particles in close proximity to NPCs (Fig. 3). We interpret these data to suggest that upon recruitment of Dyn2 molecules to eDID-modified FG repeat Nups, the NPC transport channel becomes blocked, thus explaining the observed perinuclear accumulation of RNP/protein aggregates.

Consistent with a plugged pore channel, we observe inhibition of other nucleocytoplasmic transport processes in the toxic Nsp1-eDID, Nup49-eDID, Nup57-eDID, and Nup1-eDID strains after GAL::DYN2 induction. A defect in ribosomal 60S and 40S subunit export in these arrested cells was indicated by nuclear accumulation of the large subunit reporter Rpl25-GFP (Fig. S2A) and appearance of the 20S preribosomal RNA (pre-rRNA) intermediate in the nucleoplasm, respectively (Fig. S2B). Interestingly, NUP2-eDID exhibited a pronounced mRNA transport defect but less of a mislocation of the ITS1 pre-rRNA (Fig. 2A and Fig. S2B). Moreover, nuclear protein import was impaired, as revealed by the inability of the Pho4-GFP reporter to enter the nucleus in the NSP1-eDID strain expressing Dyn2 (Fig. 2C). Collectively, recruitment of Dyn2 to the FG repeat domains of several Nups induces inhibition of the major nucleocytoplasmic transport pathways.

Relocation of eDID-modified FG repeat domains to distinct NPC topological positions changes sensitivity toward Dyn2 expression

The data so far did not allow discrimination between whether the FG repeat domain modified with Dyn2 blocks nucleocytoplasmic transport because of the type of FG repeats or the topological location within the NPC scaffold. To distinguish between these possibilities, we sought to change the localization of eDID-FG repeat domains by genetic engineering. Hence, we replaced the FG repeat domain of Nup159 (putative “nontoxic” location) or Nup57 (putative “toxic” location) with the eDID-FG domain of Nsp1 (Fig. 4A). Both chimera, Nup57-eDID-FG Nup1 and Nup159ΔDID-eDID-FG Nup1, were
Figure 3. Perinuclear accumulation of material in cells expressing Nsp1-eDID with bound Dyn2. (A) Perinuclear accumulation of poly(A)^+ RNA in the NSP1-eDID cells harboring the chromosomal integrated GAL::DYN2 after shift from raffinose to galactose medium that was induced (2 h in galactose) and uninduced (0 h in galactose). The indicated RNA was detected by in situ hybridization using appropriate Cy3-labeled RNA probes. DNA was stained with DAPI. (B) Analysis of nuclear export of specific mRNAs encoding actin and SSA1 proteins in the NSP1-eDID dyn2Δ strain harboring the integrated GAL::DYN2 after shift from raffinose to galactose medium. 0 h in galactose (uninduced); 2 h in galactose (induced). The indicated RNA was detected by in situ hybridization using appropriate Cy3-labeled RNA probes. DNA was stained with DAPI. For the detection of SSA1 mRNA, cells were shifted to 37°C for 30 min before cells were fixed and further processed. (C) Transmission EM of thin-sectioned yeast cells expressing the eDID-modified FG repeat domain of Nsp1 with bound Dyn2. (top) Overview micrographs of representative cells are shown derived from strains dyn2Δ + GAL::DYN2 and NSP1-eDID dyn2Δ + GAL::DYN2 in an uninduced and Dyn2-induced condition. (bottom) Higher magnification EM micrographs showing strain NSP1-eDID dyn2Δ + GAL::DYN2 in the Dyn2-induced condition. NPC, nuclear pore complex (filled arrowhead); NE, nuclear envelope; N, nucleus; C, cytoplasm. White arrowheads indicate electron-dense particles accumulating in the perinuclear region with some particles close to an NPC. Bars: (A and B) 5 µm; (C) 250 nm.
Figure 4. **Transplantation of the eDID-FG repeat domain from Nsp1 and Nup1 onto Nup159 causes a loss of toxicity toward Dyn2 expression.** (A) Schematic drawing of the involved FG repeat Nups, the transplantation of the indicated eDID-labeled FG-repeat domain onto Nup57, and the eDID-labeled FG-Nsp1 or FG-Nup1 repeat domain onto Nup159. C-domain, C-terminal domain. (B) To analyze the effect of Dyn2 expression, strains were transformed with an empty plasmid or pGAL-DYN2. The indicated cells were plated on SDC (glucose) and SGC (galactose) plates, and growth was analyzed after 2 and 3 d, respectively. (C) Poly(A)+ RNA export was analyzed after 3 h Dyn2 induction by in situ hybridization using a Cy3-labeled oligo d(T) probe. (D) Subcellular localization of pGAL-DYN2-GFP was analyzed in the NUP159ΔDID-eDID-FG_{Nsp1} Δdyn2 and NUP159ΔDID-eDID-FG_{Nup1} Δdyn2 strain after 30-min galactose induction. Bars, 5 µm.
functional and complemented the respective null mutants nup57Δ and nup159Δ (Fig. 4 B). Interestingly, the eDID-FG\textsubscript{Nup1} attached to Nup57 still yielded a robust growth and mRNA export defect upon Dyn2 expression. However, when the eDID-FG\textsubscript{Nup1} or the eDID-FG\textsubscript{Nup1} was transplanted onto Nup159, neither defective mRNA export nor growth inhibition was observed (Fig. 4, B and C; and Fig. S1 E). Affinity purification of Nup82-TAP from strain \textit{NUP159\textunderscore DID\textunderscore eDID\textunderscore FG\textsubscript{Nup1} showed that Dyn2 was recruited to this NPC module of the cytoplasmic pore filaments upon GAL:\textunderscore DY2 expression (not depicted), which could also be confirmed by GFP\textunderscore Dyn2 localization experiments (Fig. 4 D). These data indicate separate roles of the unique Nsp1 FG repeat domain, which is part of two distinct NPC subcomplexes. Accordingly, the peripheral FG repeat domains of Nup159 and Nsp1 as part of the Nup82 complex may protrude into the cytoplasm to be used for recruitment of nuclear import receptors or termination of nuclear mRNA export (Stelter et al., 2007). Whereas the chemically identical other Nsp1 FG repeat protruding from the Nsp1–Nup49–Nup57 complex is crucially involved in lining the central transport channel to generate the permeability barrier.

In summary, we describe a method that allows probing the different FG repeat–containing Nups in vivo to gain insight into their topological arrangement in the NPC. In the past, the in vivo role of FG repeats for NPC structure and function was studied by deleting large parts of the FG repeat domains in yeast (Nehrbass et al., 1990; Strawn et al., 2004) or by generating mutations (Frey et al., 2006). Our newly developed tool to manipulate FG repeats in vivo depends on the recruitment (theoretically six) Dyn2 homodimers to a specific FG repeat domain that carries a row of Dyn2 binding motifs. Upon binding of Dyn2 to the eDID-FG Nup, the in vivo function of this FG repeat domain could be manipulated in a way that massively blocked nucleocytoplasmic transport. It appears that eDID-carrying FG repeats that belong to Nups of the central transport channel (e.g., Nsp1 and Nup57) or the nuclear basket (e.g., Nup1 and Nup2) cause a massive nucleocytoplasmic transport defect upon Dyn2 binding. However, probing eDID-FG repeats that are physically linked to the cytoplasmic pore filaments (i.e., Nup159) with Dyn2 did not interfere with nucleocytoplasmic transport. For Nup116-eDID, we cannot make a firm conclusion because the eDID insertion partly impaired assembly into the NPC.

We assume that binding of Dyn2 homodimers to eDID\textunderscore modified FG repeat domains generates a bulky structure (the DID\textunderscore Dyn2 assembly forms a ~20-nm-long rod in vitro; see Stelter et al., 2007) that plugs the NPC transport channel and restricts passage of the cargo-loaded receptor (e.g., karyopherins, an mRNA exporter) through the FG repeat network. In addition, Dyn2 homodimers can cross-link neighboring eDID\textunderscore modified FG repeats (also see Stelter et al., 2007), further impairing passage of transport receptors through the FG meshwork. We suggest exploiting this approach further as biochemical (e.g., purification), cell biology (e.g., Förster resonance energy transfer), and genetic tool (e.g., mutant screen) for probing not only the NPC but also other macromolecular machines.

Materials and methods

Construction of yeast strains and growth

Chromosomal integration of the eDID\textunderscore Flag\textunderscore LoxP motif [LoxP for subsequent deletion of the selectable marker from the eDID\textunderscore Flag site by Cre recombinase–induced recombination; Fig. S1, amino acid sequence] into the various FG repeat domains of FG Nups was performed in the \textit{dyn2Δ} (MalA try2Δ 1 trp1Δ1 his3Δ1-300 ura3-52 dyn2Δ::loxP) or \textit{NUP159\textunderscore DID\textunderscore dyn2Δ} strain (MalA trp1Δ1 his3Δ1-15 ura3-52 leu2Δ3 ade2Δ1-12 nup159Δ KanMX dyn2Δ KanMX prsA14\textunderscore NUP159\textunderscore DIID). The eDID\textunderscore Flag\textunderscore LoxP construct was cloned into a p\textsc{fado} vector yielding p\textsc{fado}\textunderscore LoxP\textunderscore eDID\textunderscore Flag\textunderscore LoxP\textunderscore HisMX4, which was used as the template for the respective integration PCRs. Integration of the eDID\textunderscore Flag\textunderscore LoxP was performed in the different yeast strains in the presence of a plasmid-based wild-type copy of the corresponding NUP gene to be targeted with eDID\textunderscore Flag. Deletion of the HisMX4 cassette and the resulting in-frame fusion of the eDID\textunderscore Flag with the respective FG domains were performed using Cre recombinase (Baudin et al., 1993). Correct integration of the eDID\textunderscore Flag label at the corresponding NUP locus and expression of the eDID\textunderscore modified FLAG protein was verified by Western blot analysis of whole-cell lysates using anti-Flag antibodies. In these strains, Dyn2 was expressed from a p\textsc{GAL}\textunderscore DY2 plasmid or from a chromosomally integrated GAL\textunderscore DY2. To analyze the GFP\textunderscore Dyn2 localization in the eDID\textunderscore NUP strains, we transformed these strains with a NUP159 shuttle plasmid and deleted the chromosomal NUP159 gene with a HisMX4 cassette. These strains were transformed with p\textsc{RS414\textunderscore NUP159\textunderscore DIID} and shuffled on 5-fluoroorotic acid. Growth was analyzed on YPD (yeast peptone dextrose; glucose), YPR (yeast peptone raffinose), or YPG (yeast peptone galactose) plates as well as on minimal synthetic dextrose complete (SDC) versus synthetic galactose complete (SGC) plates. Additional strains and plasmids used in this study are listed in Table S1 and Table S2.

Analysis of nucleocytoplasmic transport in yeast cells

Analysis of nuclear mRNA export was performed by fluorescence in situ hybridization as previously described (Doye et al., 1994). In brief, cells were grown in SDC\textunderscore leu medium and transferred to YPR medium for 4 h before 2% galactose was added for the indicated time points. Nuclear accumulation of poly(A)\textunderscore RNA or distribution of ITS1\textunderscore containing pre\textunderscore RNAs was determined using Cy3\textunderscore labeled oligo(dT) and 5'C\textunderscore G\textunderscore C\textunderscore A\textunderscore A\textunderscore A\textunderscore C\textunderscore T\textunderscore T\textunderscore C\textunderscore T\textunderscore T\textunderscore A\textunderscore A\textunderscore T\textunderscore A\textunderscore T\textunderscore A\textunderscore T\textunderscore C\textunderscore T\textunderscore 3' probes, respectively. Nuclear accumulation of actin mRNA was analyzed with Cy3\textunderscore labeled actin probe (Strasser and Hurt, 2001). SSA1 mRNA export was analyzed in logarithmically growing cells incubated with 2% galactose for 30 min to induce Dyn2 followed by a further shift to 37°C for 30 min to induce SSA1 expression. SSA1 mRNA was detected using the Cy3\textunderscore labeled DNA probe (Strasser and Hurt, 2001). Nuclear export of the ribosomal 40S subunit was followed by fluorescence in situ hybridization, as previously described, using a Cy3\textunderscore labeled ITS1\textunderscore specific probe for detection of ITS1\textunderscore containing pre\textunderscore RNAs (Pertschy et al., 2009). Export of the ribosomal 60S subunit was monitored using the Rpl25\textunderscore EGFP reporter (Gadal et al., 2001). Cells were grown in SDC\textunderscore leu/ura medium overnight and transferred to YPR medium for 4 h. Dyn2 was induced for the indicated time points, and the Rpl25\textunderscore EGFP, Nop1\textunderscore monomeric RFP (mRFP), and DAPI distributions were examined by fluorescence microscopy using a microscope (Imager Z1; Carl Zeiss) with a 63 or 100x, NA 1.4 Plan Apochromat oil immersion lens (Carl Zeiss) and differential interference contrast, humanized EGFP, DAPI, mRFP, or HEC3 filter sets. Pictures were acquired with a camera (AxioCam MRm; Carl Zeiss) and AxioVision 4.3 software (Carl Zeiss). Analysis of the subcellular Dyn2 location was performed in the \textit{NUP159\textunderscore DID\text underscore dyn2Δ} NUP\textunderscore eDID\textunderscore Dyn2 strains transformed with the plasmid p\textsc{galo\textunderscore DY2\textunderscore Flag\textunderscore GFP}. Cells were induced for 30 min in galactose medium, and expression was subsequently stopped by adding glucose. The GFP\textunderscore Dyn2 localization was analyzed after 1 h by fluorescence microscopy.

Nuclear import of the chromosomal GFP\textunderscore tagged Pho4 was examined, as previously described (Kaffman et al., 1998), in the yeast strains \textit{NSP1\textunderscore eDID\textunderscore dyn2Δ}, \textit{NSP1\textunderscore eDID\textunderscore Gal\textunderscore DY2}, and \textit{dyn2Δ Gal\textunderscore DY2}. Cells were grown in YPD\textunderscore supplemented with 11 mM KH\textsubscript{2}PO\textsubscript{4} (high phosphate) to an OD of ~0.1–0.2 before galactose was added to a final concentration of 2% for an additional 3 h. Finally, cells were centrifuged and washed twice in water and in low phosphate medium (SRC + all medium + 220 µM KH\textsubscript{2}PO\textsubscript{4}) before being resuspended in low phosphate medium followed by a further incubation for 1 h at 30°C. Pho4\textunderscore GFP import was analyzed in the fluorescence microscope.
Affinity purification of TAP-tagged Nups

Affinity purification of TAP-tagged bait proteins was performed as previously described (Puig et al., 1998; Janke et al., 2004). In brief, cells were broken in standard purification buffer containing 150 mM NaCl, 50 mM K(OAc), 20 mM Tris-HCl, 2 mM Mg(OAc), and 0.1% NP-40, pH 7.5, in a mill (PULVERISETTE; FRITSCH), extracts were centrifuged for 10 min at 3,400 g, and the supernatant was further centrifuged at 35,000 g for 20 min. The supernatant was incubated with 300 µl IgG (GE Healthcare) slurry at 4°C for 1 h. IgG beads with bound proteins were washed several times with purification buffer. Tobacco etch virus cleavage was performed at 16°C for 90 min in purification buffer. Tobacco etch virus elutes were supplemented with CaCl2 to a final concentration of 1 mM and incubated with calmodulin beads (GE Healthcare) for 60 min at 4°C. Calmodulin beads with the bound protein complexes were treated with SDS sample buffer and analyzed by SDS-PAGE, Coomassie staining, or Western blotting. Antibodies raised against recombinant Dyn2 were made in rabbit (Stelter et al., 2007). TAP-tagged strains were grown overnight to an OD of 1.5, and galactose was added to a final volume of 2% for an additional 3 h before affinity purification.

Thin-section EM

The yeast cells were fixed with 2% formaldehyde/2% glutaraldehyde for 45 min on ice. After spheroplasting, postfixation with osmium tetroxide, and contrasting en bloc with uranyl acetate, they were dehydrated with a graded series of ethanol and embedded in glycid ether 100–based resin. After polymerization at 60°C, ultrathin sections were cut with an ultramicrotome (Ultracut S; Reichert). They were contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (EM 10 CR; Carl Zeiss) at an acceleration voltage of 60 kV.

Miscellaneous

Integration of the TAP cassette at the NUP57, NUP49, NUP116, NUP60, NUP1, Nic96, or NUP82 chromosomal locus in the indicated yeast strains was performed as previously described (Puig et al., 1998; Janke et al., 2004).

Online supplemental material

Fig. S1 shows Dyn2 recruitment to eID-labeled FG domain Nups. Ribosomal export was analyzed in Fig. S2. Fig. S3 shows analysis of growth and nuclear pore distribution of NUP-eID + Pgal-DYN2 strains transformed with the respective wild-type Nups. Table S1 and Table S2 include yeast strains and plasmids used in this study, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201105042/D11.

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