Nup116p and Nup100p are interchangeable through a conserved motif which constitutes a docking site for the mRNA transport factor Gle2p

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Nup116p and Nup100p are highly related yeast GLFG nucleoporins, but only Nup116p is stoichiometrically bound to Gle2p, a previously identified mRNA export factor. A short Gle2p-binding sequence within Nup116p (GLEBS; residues 110–166) is sufficient and necessary to anchor Gle2p at the nuclear pores, whereas the carboxy-terminal domain of Nup116p mediates its own nuclear pore complex (NPC) association. The GLEBS is evolutionarily conserved and found in rat/*Xenopus* Nup98 and an uncharacterized *Caenorhabditis elegans* ORF, but is absent from Nup100p. When the GLEBS is deleted from Nup116p, Gle2p dissociates from the nuclear envelope and clusters of herniated nuclear pores form. When the GLEBS is inserted into Nup100p, Nup100p–GLEBS complements both the thermosensitive and NPC-herniated phenotype of nup116[–] cells, and Gle2p is retargeted concomitantly to the NPCs. Thus, the *in vivo* function of Gle2p is strictly coupled to the short GLEBS within Nup116p which links this putative mRNA transport factor to the nuclear pores.
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Introduction

Nuclear pore complexes (NPC) are large, intricate macromolecular structures present in all eukaryotic cells which mediate the transport of macromolecules between the nucleus and cytoplasm (for review see Nigg, 1997). Of the estimated 50–100 different proteins which constitute the NPC, about 30 are known to date, most of them identified by biochemical and genetic studies in the yeast *Saccharomyces cerevisiae* (for review see Doye and Hurt, 1997). Although the search for novel nucleoporins in yeast will soon reach its conclusion, very little is known about physical interactions between nucleoporins and how they are targeted at and assembled into the NPCs. This challenging task cannot be solved solely by genetic methods, which do not distinguish between physical contact and functional interaction; this requires biochemical analysis, *in vitro* reconstitution and ultimately structural analysis of the NPC and its substructures. Biochemical purification of nucleoporins has already defined subcomplexes of the NPC, but often nucleoporins are not found in a stable

physical association with other NPC constituents, which suggests that many of the interactions within the NPC are less resistant to biochemical purification or are transient.

Of the stable subcomplexes identified in yeast, the Nsp1p complex has been studied extensively in the past. It consists of a heterotrimeric core of Nsp1p, Nup49p and Nup57p, to which Nic96p is peripherally attached (Grandi *et al.*, 1995b). The Nsp1p complex resembles the higher eukaryotic p62–p58–p54 (Nup62–Nup58–Nup54) complex which, however, lacks a Nic96p counterpart (Dabauvalle *et al.*, 1990; Finlay *et al.*, 1991; Guan *et al.*, 1995; Hu *et al.*, 1996). The p62 complex is essential for nuclear protein import (Finlay *et al.*, 1991) and becomes localized at the central gated channel on both sides of the nuclear pore (Guan *et al.*, 1995). Thus this complex is likely to be involved in the translocation step through the pore channel. Recently, both human and *Xenopus* Nic96p homologues were identified and localized to the nuclear baskets and central gated channel (Grandi *et al.*, 1997). Interestingly, Nsp1p is found in another complex with Nup82p which is involved in mRNA export (Grandi *et al.*, 1995a). *Xenopus* p62 is also detected in a less abundant second complex with the putative oncogenic nucleoporin Nup214/CAN (Macaulay *et al.*, 1995). Human Nup214/CAN forms a complex with two other proteins, Nup88 and CRM1 (Fornerod *et al.*, 1997).

Another stable yeast nucleoporin subcomplex, the Nup84p complex, is essential for NPC biogenesis and is composed of Nup84p, Nup145pC, Nup120p, Nup85p, Seh1p and Sec13p (Siniossoglou *et al.*, 1996; Teixeira *et al.*, 1997). In addition, some components of the Nup84p complex such as Nup85p (Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996), Nup120p (Aitchison *et al.*, 1995; Heath *et al.*, 1995) and Nup145pC (Dockendorff *et al.*, 1997; Emtage *et al.*, 1997; Teixeira *et al.*, 1997) are also linked to the mRNA transport machinery. Accordingly, a *nup85* disruption mutant allele was used to isolate a novel mRNA export factor Mex67p (Segref *et al.*, 1997). Several other physical interactions between yeast nucleoporins have been reported, but these components are often associated only in low, i.e. substoichiometric amounts. Examples include interactions between Nic96p and Nup188p (Nehrbass *et al.*, 1996), Nup188p and Pom152p (Zabel *et al.*, 1996) and Nup170p and Nup1p (Kenna *et al.*, 1996).

Nucleoporins are not only integrated into the NPC, but also interact with nuclear transport factors; these interactions can be either biochemically stable (e.g. Srp1p/Nup1p, Srp1p/Nup2p) (Belanger *et al.*, 1994) or dynamic and labile. Therefore, solution binding assays have been developed to identify such transient interactions (Radu *et al.*, 1995; Rexach and Blobel, 1995). Alternatively, the two-hybrid assay has been used to detect weaker protein–protein interactions among the various nucleoporins and nuclear transport factors (Iovine *et al.*, 1995; Stutz *et al.*, 1995).

Among the yeast nucleoporins carrying GLFG-repeat sequences, Nup116p performs a unique role. Although Nup116p is not essential for cell growth, a *NUP116* null mutant grows slowly at permissive temperatures and cell growth stops at 37°C (Wente *et al.*, 1992; C.Wimmer, Thesis, 1994). Strikingly, this temperature-sensitive *NUP116* mutant forms a nuclear envelope seal over the yeast NPC thereby affecting the nucleocytoplasmic traffic of poly(A)⁺ RNA (Wente and Blobel, 1993). Nup116p is highly related to Nup100p (Wente *et al.*, 1992) and the cleaved amino-terminal domain of Nup145p (Fabre *et al.*, 1994; Wente and Blobel, 1994; Teixeira *et al.*, 1997). They share a repetitive GLFG domain followed by a carboxy-terminal domain including a NRM (nucleoporin RNA binding motif), shown to bind homopolymeric RNA *in vitro* (Fabre *et al.*, 1994). By using deletion mutagenesis, Iovine *et al.* (1995) demonstrated a role for the GLFG region of Nup116p in NPC function and nucleocytoplasmic transport. The coupling of Nup116p to the nuclear transport machinery is possibly generated by a physical interaction between the GLFG region of Nup116p and the nuclear import factor Kap95p (Iovine *et al.*, 1995; Iovine and Wente, 1997). Accordingly, it was suggested that Nup116p and also Nup100p, are involved in the recycling of the import factor Kap95p (Iovine and Wente, 1997).

NUP116 is linked genetically to several nucleoporins including *NSP1* (Wimmer *et al.*, 1992), *NUP100* (Wente *et al.*, 1992), *NUP145* (Fabre *et al.*, 1994), *NUP84* (S.Siniosoglou, unpublished results) and *GLE1* (Murphy and Wente, 1996). The reasons for these observed genetic interactions are not clear, since no physical contacts between Nup116p and the above-mentioned NPC proteins are known so far. To gain insight into how Nup116p interacts physically with components of the NPC, we affinity-purified Nup116p and interacting proteins. One isolated protein was Gle2p which forms a stable complex with Nup116p. This interaction is crucial for the formation of normal nuclear pores. *GLE2* was recently identified in a synthetic-lethal screen with a *nup100* null allele and shown to have both a structural role at the NPC and a functional role in nuclear poly(A)⁺ RNA export (Murphy *et al.*, 1996). Gle2p shows significant homology with the *Schizosaccharomyces pombe* mRNA export factor Rae1 (Brown *et al.*, 1995) and human mRNA binding protein mrnp 41 which localizes to both the nucleus and the cytoplasm (Kraemer and Blobel, 1997). We found that a short and conserved sequence of 50–60 amino acids within Nup116p serves as a docking site bringing Gle2p to the pores. This interaction is crucial for the *in vivo* function of Gle2p.

Results

Affinity-purification of ProtA-tagged Nup116p reveals complex formation with Gle2p

In order to find components which interact physically with Nup116p and Nup100p, two highly related GLFG nucleoporins in yeast (Wente *et al.*, 1992; see also Figure 1A), both proteins were tagged at their amino-terminal ends using IgG-binding sequences that had been derived from *Staphylococcus aureus* protein A and affinity-purified under non-denaturing conditions. The ProtA–Nup116p and ProtA–Nup100p fusion proteins were both

functional, since they were able to complement the thermosensitive phenotype of the *nup116::URA3* disruption mutant and the synthetic lethality of the *gle2::HIS3/nup100::LEU2* double disruption mutant, respectively. When affinity-purified ProtA–Nup116p and ProtA–Nup100p were analysed by SDS–PAGE, the corresponding fusion proteins could be seen as distinct, Coomassie-stainable bands (Figure 1A). In the case of ProtA–Nup116p, a prominent band migrating at 40 kDa was visible, which was completely absent in the ProtA–Nup100p eluate (Figure 1A).

Based on MALDI mass mapping followed by database searches (see Materials and methods), the Nup116p-associated protein was identified as a hypothetical yeast protein of 40.5 kDa (365 amino acids; DDBJ/EMBL/GenBank accession number YER107C or P40066) whose gene is located on chromosome V. Five unmodified tryptic peptides matched the ORF (sequence coverage 10.4%) and four more peptides matched when taking methionine oxidation and cys-acrylamidation into account. Because of the relatively low sequence coverage, an additional sequencing experiment was performed on the unseparated peptide mixture. The peptides QSLLVATAER and IWDVQNGVPQGR were identified unambiguously by nanoelectrospray tandem mass spectrometry, followed by database searches with peptide sequence tags (see Materials and methods), confirming the identification by MALDI peptide mapping. During the course of this work, the gene for this 40.5 kDa protein was found in a synthetic-lethal screen with a *nup100* null allele and was named *GLE2* (Murphy *et al.*, 1996). Gle2p has related proteins with significant homology in other organisms including the *S.pombe* poly(A)⁺ RNA export protein Rae1 (Brown *et al.*, 1995; DDBJ/EMBL/GenBank accession number U14951) and two hypothetical proteins from human (DDBJ/EMBL/GenBank accession number U84720) and *Caenorhabditis elegans* (DDBJ/EMBL/GenBank accession number Z80216). Bub3p, a *S.cerevisiae* protein which also shows significant homology to Gle2p, is involved in cell cycle check point control (Guénette *et al.*, 1995; DDBJ/EMBL/GenBank accession number YOR026W). All four proteins belong to the superfamily of WD-repeat-containing proteins (Neer *et al.*, 1994).

To discover whether Nup116p can be co-isolated upon purification of Gle2p, Gle2p was tagged at its carboxy-terminal end with ProtA and expressed in a strain which carries the *gle2::HIS3* gene disruption (see Materials and methods). These *gle2*[−] cells are already impaired in growth at the permissive temperature (e.g. 30°C), and stop cell growth at 37°C (data not shown; see also Murphy *et al.*, 1996). Furthermore, *gle2*[−] cells exhibit a significantly increased cell size at the restrictive temperature, a phenotype which is also observed for *nup116* mutant cells (data not shown). The Gle2p–ProtA construct was functional since it was able to complement the thermosensitive phenotype of the *gle2::HIS3* disruption mutant (data not shown). Similar to ProtA–Nup116p, Gle2p–ProtA was efficiently affinity-purified from a whole cell lysate, yielding Coomassie-stainable amounts (Figure 1B). While Nup116p (plus its degradation products) co-purified very efficiently with Gle2p–ProtA, Nup100p did not, as revealed by Western blotting using anti-GLFG antibodies

Fig. 1. Nup116p forms a complex with an evolutionarily conserved protein Gle2p. **(A)** Affinity-purification of ProtA-Nup116p and ProtA-Nup100p. Whole cell lysates derived from yeast strains expressing the indicated ProtA-fusion proteins were applied to IgG-Sepharose columns and the eluates analysed on a SDS-10% polyacrylamide gel followed by Coomassie staining. The positions of the ProtA fusion proteins are indicated. The prominent band around 40 kDa co-purifying with ProtA-Nup116p (indicated by an arrow) was analysed using mass spectrometry as described in Materials and methods. A molecular weight standard was also separated on the gel ranging from 20–120 kDa with a stepwise 10 kDa increase and an additional 200 kDa marker protein. GLFG, GLFG repeat domain; NRM, nucleoporin RNA binding motif. **(B)** Affinity-purification of Gle2p-ProtA by IgG-Sepharose chromatography. Fractions of a total cell extract derived from a *gle2⁻* strain complemented by Gle2p-ProtA (Hom), the insoluble pellet (IP), the soluble supernatant (Sup), the column flow-through (Flow), washes I (lysis buffer) and II (pH 5) (Wash I, II) and the pH 3.4 eluate (Eluate) were analysed by SDS-PAGE followed by Coomassie staining and Western blotting using anti-IgG antibodies (anti-ProtA) and anti-GLFG antibodies. The positions of the Gle2p-ProtA fusion protein and a co-purifying protein of ~120 kDa, which was identified as Nup116p, are indicated.

(Figure 1B). Taken together, these data show that Nup116p and Gle2p form a complex in yeast.

Kap95p was reported to interact with the GLFG-region of Nup116p (Iovine *et al.*, 1995), however we could not obtain positive evidence that Kap95p co-purifies with ProtA-Nup116p, since no band corresponding to Kap95p was seen in the ProtA-Nup116p eluate (Figure 1A). Western blot analysis of the ProtA-Nup116p and ProtA-Gle2p eluate using anti-Kap95p antibodies did not reveal Kap95p in association with Nup116p (data not shown).

Identification of the Gle2p-binding sequence (GLEBS) within Nup116p

To identify the Gle2p-binding domain within Nup116p, several Nup116p deletion mutants tagged with ProtA were created and expressed in yeast. Following affinity-purification, the mutant proteins were analysed using SDS-PAGE and Coomassie staining (Figure 2A). Strikingly, a short sequence of 56 amino acids present in the amino-terminal part of Nup116p (ranging from residue 110 to 166) contains all the information necessary to bind Gle2p as efficiently as was observed with full-length Nup116p. Accordingly, Gle2p co-purified stoichiometrically with ProtA-Nup116p(110–166). In contrast, the ProtA-tagged GLFG domain or the carboxy-terminal domain of Nup116p did not interact with Gle2p. Since the short Gle2p-binding sequence (GLEBS) is not present within Nup100p and Nup145p, it explains at the molecular level why these two

GLFG nucleoporins do not associate with Gle2p. However, when the GLEBS was compared with the sequences in the data library, highly related amino acid stretches of similar length were found in the rat Nup98 and a *C.elegans* Nup98 homologue (Figure 2A, upper panel; see also Discussion).

To determine whether the Nup116p deletion constructs are functional *in vivo*, we tested their ability to complement the thermosensitive growth defect of *nup116⁻* cells at 37°C (Figure 2B). The Nup116p mutant lacking a short part of its amino-terminal FG-repeat domain [Nup116p(Δ 1–58)] was functional at 37°C. Nup116p mutants expressing only the carboxy-terminal domain, the GLFG domain or the Gle2p-binding sequence [Nup116p(110–166)] did not complement at all whereas those expressing both the amino-terminal and GLFG domains [Nup116p(N+GLFG)] complemented very poorly.

The GLEBS within Nup116p is required for the association of Gle2p with the nuclear pores

To study the intracellular targeting of Gle2p and its assembly with Nup116p, Gle2p was tagged using green fluorescent protein (GFP) at its carboxy-terminal end and expressed in a strain which contained the *gle2::HIS3* gene disruption (see Materials and methods). Gle2p-GFP exhibited a ring-like and punctate nuclear envelope staining typical for a nuclear pore association; small

Fig. 2. Identification of the Gle2p-binding sequence (GLEBS) within Nup116p. **(A)** Upper panel: schematic drawing of the domain organization of Nup116p and Nup100p and the various Nup116p deletion constructs (1–6). The yeast (Sc) Nup116p sequence between amino acids 115 and 166, which precedes the GLFG domain and is shown in dark grey, is evolutionarily conserved and compared to Nup98 from rat (rat) and *C.elegans* (cel). Lower panel: affinity-purification of the various ProtA–Nup116p deletion constructs from yeast strains. The eluates derived from the IgG–Sepharose columns (1–6) were analysed on SDS–polyacrylamide gels followed by Coomassie staining. The positions of the ProtA fusion proteins are indicated by closed squares. The prominent Gle2p band is indicated by arrows. Left gel: SDS–8% PAGE; Right gel: SDS–12% PAGE; Hom, whole cell homogenate; Std, molecular weight standard ranging from 20–120 kDa with a stepwise 10 kDa increase and an additional 200 kDa marker protein. 1–6, eluates from the indicated ProtA–Nup116p deletion constructs. **(B)** Growth properties of strains expressing different *nup116* mutant alleles. Growth of *nup116*[−] cells transformed with the indicated plasmid constructs at 23 and 37°C. Precultures were diluted and equivalent amounts of cells (diluted in 10^{−1} steps) were spotted onto plates. They were incubated for 3 days. For description of strains, see also Figure 3A.

amounts of this construct were also found in the cytoplasm and nucleoplasm (Figure 3A). In *nup133*[−] cells, where NPCs are known to concentrate into clusters, Gle2p–GFP localized in bright spots found at the nuclear envelope. This shows that Gle2p is associated physically with NPCs. Strikingly, in *nup116*[−] cells, Gle2p–GFP did not target the nuclear pores, as no nuclear envelope staining was observed (Figure 3A). In this mutant, Gle2p–GFP may be mislocalized to the cytoplasm, but this is difficult to assess, since no significant increase in cytoplasmic staining was observed. However, Gle2p–GFP was still expressed

at normal levels in *nup116*[−] cells, as demonstrated by Western blotting using anti-GFP antibodies (Figure 3A, insert), suggesting that Gle2p–GFP is not degraded in *nup116*[−] cells. Conversely, Gle2p is not required to target Nup116p to the NPC, because GFP–Nup116p stains the nuclear envelope of *gle2*[−] cells in a punctate manner (Figure 3A). Together these data suggest that Nup116p mediates the NPC association of Gle2p.

To discover whether the GLEBS within Nup116p is sufficient and necessary for *in vivo* targeting of Gle2p, a construct was generated which encoded a ProtA- or GFP-

Fig. 3. Intracellular location of Gle2p-GFP and GFP-Nup116p in *gle2*⁻ and *nup116*⁻ cells. (A) Fluorescence microscopy of living cells expressing Gle2p-GFP and GFP-Nup116p. The *GLE2-GFP* and *GFP-NUP116* constructs were present on *LEU2*- and *TRP1*-containing single copy number plasmids, respectively. These plasmids were selected for by growing cells (strain *gle2*⁻, *nup116*⁻ and *nup133*⁻) in SDC-Leu or SDC-Trp media at 23°C before viewing immobilized cells in a Zeiss fluorescence microscope. Insert: Western blot analysis of whole cell lysates derived from wild-type (wt), *nup116*⁻ and *gle2*⁻ cells, all expressing Gle2p-GFP. The same amount of protein was loaded on each lane and the blot was probed with anti-GFP and anti-hexokinase antibodies. (B) Fluorescence microscopy of living *nup116*⁻ cells expressing GFP-Nup116p, GFP-Nup116p(ΔGLEBS) and GFP-Nup116p-C (carboxy-terminal domain; residue 706–1113) was performed as described in Figure 3A; *nup116*⁻ cells transformed with ProtA-Nup116(ΔGLEBS) were analysed for the location of Gle2p-GFP.

tagged form of Nup116p that was missing amino acid residues 110–166 [Nup116p(ΔGLEBS); Figure 4A]. When ProtA-Nup116p(ΔGLEBS) was expressed in a *nup116*⁻ strain, no complementation of the thermosensitive phenotype was observed at 37°C (Figure 4B), despite GFP-Nup116p(ΔGLEBS) being targeted to the nuclear pores (Figure 3B). Thus, the GLEBS of Nup116p which binds

Gle2p performs a crucial role in the living cell. When analysed using a fluorescence microscope, Gle2p-GFP was not localized at the NPCs in Nup116p(ΔGLEBS) cells, as revealed by the loss of the ring-like and punctate nuclear envelope labelling (Figure 3B). This shows that the GLEBS within Nup116p is required for *in vivo* targeting of Gle2p to the NPCs. As expected, upon purification of

Fig. 4. The GLEBS of Nup116p is functional when inserted into Nup100p. **(A)** Schematic drawing of Nup116p, Nup116p(ΔGLEBS), Nup100p and Nup100p which harbours the GLEBS within its GLFG domain (Nup100p-GLEBS) and affinity-purification of the indicated ProtA fusion proteins. Whole cell extracts from yeast strains expressing the indicated ProtA fusion proteins were applied onto IgG-Sepharose columns and the eluates analysed on a SDS-10% polyacrylamide gel followed by Coomassie staining. The positions of the ProtA fusion proteins and Gle2p are indicated. **(B)** Deletion of the GLEBS from Nup116p causes a thermosensitive growth phenotype which is complemented by Nup100p carrying the Gle2p-binding sequence. The nup116⁻ strains expressing the indicated constructs were grown at 23, 33 and 37°C. Precultures were diluted in 10⁻¹ steps and equivalent amounts of cells were dropped on the plates and grown for 3 days. **(C)** Location of Gle2p-GFP in double-disrupted nup116⁻/gle2⁻ cells expressing either ProtA-Nup100-GLEBS or ProtA-Nup100. Living cells were viewed in the fluorescence microscope as described in Figure 3A. **(D)** The GLEBS within Nup116p can also function, albeit less efficiently, if moved to the carboxy-terminal end. The nup116⁻ and nup116⁻/gle2⁻ cells expressing the Nup116p/C-GLEBS (and the other indicated constructs) were grown at 23, 33 and 37°C for 3 days (left panel) or viewed in the fluorescence microscope (right panel) as described in Figure 3A.

ProtA-Nup116p(ΔGLEBS), no Gle2p was found to be associated physically (Figure 4A, lane 2).

The carboxy-terminal domain of Nup116p contains signals for NPC targeting

Since Nup116p(ΔGLEBS) still localizes to the NPCs, whereas the GLFG-domain alone does not (Iovine *et al.*, 1995), we tested whether the carboxy-terminal domain of Nup116p could associate with the nuclear pores. For this purpose, the carboxy-terminal domain of Nup116p was

tagged with GFP and the corresponding construct GFP-Nup116p-C expressed in nup116⁻ cells. As seen in Figure 3B, Nup116p-C contains NPC-targeting signals (GFP-Nup116p-C). GFP-Nup116p-C was also detected in the cytoplasm and nucleoplasm, particularly in those cells with high levels of GFP expression. This may indicate that the GLFG-region somehow contributes to an efficient interaction between Nup116p and the NPC. However, no interaction between the GFP-tagged Nup116p (59-168) and the NPC was observed (data not shown).

Nup100p with inserted GLEBS can complement the nup116⁻ mutant

No synthetic lethal interaction between nup116⁻ and gle2⁻ cells was observed (data not shown; see also Murphy *et al.*, 1996). This is somehow puzzling in the light of the biochemical data shown above. Since Nup116p functions in targeting Gle2p to the nuclear pores, it may be possible to transfer the GLEBS from Nup116p to Nup100p, thereby targeting Gle2p to the pores in a nup116⁻ strain. Therefore, a GFP- or ProtA-tagged construct of Nup100p was made where the Nup116p-derived GLEBS was inserted into the GLFG domain (Figure 4A). When Nup100p–GLEBS was expressed in the nup116⁻ cells, it was able to complement the slow growth phenotype of this strain efficiently at 33°C and, although less efficiently than Nup116p, it was also able to complement the thermosensitive growth defect at 37°C (Figure 4B). In contrast, Nup100p alone was not able to complement the nup116 disruption mutant (Figure 4B; see also Iovine *et al.*, 1995). Accordingly, Gle2p–GFP targets the NPC in nup116⁻ cells when ProtA–Nup100p–GLEBS, but not ProtA–Nup100p, is co-expressed (Figure 4C). Furthermore, Gle2p can be co-isolated with ProtA–Nup100p–GLEBS using IgG–Sephadex chromatography (Figure 4A). These data show that targeting of Gle2p to the NPCs is one of the crucial functions of Nup116p, a role mediated by the GLEBS (see Discussion).

In order to test whether the Gle2p-binding sequence can function at another position within Nup116p, the GLEBS was moved from its amino-terminal location to the carboxy-terminal end (see Materials and methods). This construct could efficiently complement the slow growth phenotype of the nup116 disruption mutant at 33°C, but was less efficient at complementing the thermosensitive growth defect at 37°C (Figure 4D). This inefficient complementation at 37°C may be due to a certain inaccessibility of the GLEBS for Gle2p if attached to the carboxy-terminal end of Nup116p, because the carboxy-terminal domain of Nup116p interacts with other proteins. Nevertheless, Nup116p/C–GLEBS is targeted to the nuclear envelope in nup116⁻/gle2⁻ cells (Figure 4D).

Furthermore, the GLEBS was also fused to two other nucleoporins, Nup49p (which contains GLFG repeat sequences) and Seh1p (a subunit of the Nup84p complex). In both cases, association of Nup40p with Nup49p and the Nup84p complex was observed, but no complementation of the thermosensitive phenotype of the nup116 disruption mutant at either 33 or 37°C was seen (data not shown). This shows that Gle2p has to be targeted to the correct location at the NPC (probably in the neighbourhood of Nup116p/Nup100p) in order to fulfil its important function in the cell.

Analysis of mRNA export and nuclear envelope morphology in nup116 and gle2 mutants

The disruption mutants of *NUP116* and *GLE2* were reported to be impaired in poly(A)⁺ RNA export (Wente and Blobel, 1993; Murphy *et al.*, 1996). We sought to analyse the onset and extent of nuclear RNA export inhibition in our nup116 and gle2 disruption strains in comparison to the thermosensitive mex67-5 cells (Segref *et al.*, 1997). Whereas after 30 min at the non-permissive temperature, the mex67-5 mutant reveals a massive intra-

nuclear accumulation of poly(A)⁺ RNA in 100% of the cells, nup116 and gle2 disruption strains do not show such a dramatic phenotype, although a perinuclear accumulation of poly(A)⁺ RNA can sometimes be seen in gle2⁻ cells. Only after 2 h incubation at the non-permissive temperature, do nup116⁻ cells begin to show an accumulation of intranuclear poly(A)⁺ RNA (Figure 5). On the contrary, gle2⁻ and nup116⁻ mutant cells exhibit a distinct cytoplasmic poly(A)⁺ RNA labelling at the restrictive temperature, which disappears in mex67-5 cells (Figure 5). It was reported that upon accumulation of intranuclear mRNA *rat/mtr* export mutants lose their cytoplasmic poly(A)⁺ RNA signal due to the high RNA turnover in the cytoplasm (Amberg *et al.*, 1992; Kadowaki *et al.*, 1992; Gorsch *et al.*, 1995).

Moreover, we analysed the ultrastructural organization of the nuclear envelope in nup116 and gle2 mutant cells, since it was reported earlier that nup116Δ and gle2Δ strains show clusters of herniated NPCs at the restrictive temperature (Wente and Blobel, 1993; Murphy *et al.*, 1996). The same striking structural abnormality can be seen in our nup116⁻ (Figure 6, 1 and 2) and gle2⁻ cells (data not shown). Remarkably, Nup116(ΔGLEBS) cells shifted for 5 h to 37°C also develop extended clusters of herniated NPCs, whereas spindle and nucleolar morphology were not altered (Figure 6, 3–6). However, nup116⁻ and Nup116(ΔGLEBS) cells still contain normal nuclear pores in the non-herniated areas of the nuclear envelope (Figure 6, arrow heads). When nup116⁻ cells are complemented by full-length ProtA–Nup116p, no NPC herniations are seen (data not shown). Finally, the aberrant NPC herniation phenotype seen in almost all of the nup116⁻ cells largely disappeared when nup116⁻ cells were complemented by Nup100p–GLEBS (Table I; see also Figure 4B).

Discussion

In this work, we show that Gle2p, a protein previously implicated in NPC structure and mRNA export, associates with the nuclear pores via a short sequence within Nup116p, called the Gle2p-binding sequence (GLEBS). The GLEBS is 50–60 amino acids long and placed between the amino-terminally located degenerate FG-repeat region and the central GLFG repeat domain of Nup116p (see also Wimmer *et al.*, 1992), it is sufficient and necessary to bind Gle2p at the NPC. It has been reported independently that Gle2p is associated with Nup116p (Iovine and Wente, 1997). The GLEBS may assume a particular structure as suggested by the regular alteration between charged and hydrophobic residues in the second half (see also Figure 2). The fact that the GLEBS can be taken out of one protein and inserted functionally into another protein points to an autonomous domain. Furthermore, the GLEBS is highly conserved during evolution and found in higher eukaryotic nuclear pore proteins related to Nup116p. In the case of rat Nup98, the GLEBS sequence is inserted within the GLFG repeat domain showing that its positioning within the nucleoporin is flexible (Figure 7; see also Radu *et al.*, 1995). An uncharacterized *C.elegans* ORF showing homology with vertebrate Nup98 also contains the conserved GLEBS (data not shown). However, this putative *C.elegans* nucleoporin appears to be more

Fig. 5. Analysis of poly(A)⁺ RNA export in *gle2*⁻, *nup116*⁻ and *mex67-5* cells. The intracellular location of poly(A)⁺ RNA in *nup116*⁻, *gle2*⁻ and *mex67-5* ts cells was analysed as described in Materials and methods. Cells were either grown at 23°C or transferred to 37°C for 30 min or 2 h, respectively, before being processed for *in situ* hybridization using a Cy3-labelled oligo(dT) probe (left-hand pictures of a row). DNA was visualized by Hoechst (right-hand pictures of a row).

Fig. 6. Electron microscopy of *nup116* mutant cells. Thin section electron microscopy of *nup116*⁻ cells (1, 2) and *Nup116p*(Δ GLEBS) (3–6) cells grown for 5 h at 37°C. Areas of extensive herniations within the nuclear envelope are shown. In non-herniated areas, normal nuclear pores (indicated by arrow heads) can also be seen. Bars: 0.5 μ m.

related to *Nup145p* since it contains a large carboxy-terminal extension with some similarity to the yeast *Nup145p* carboxy-terminal domain (Figure 7). This suggests that the GLEBS is able to jump back and forth during evolution within NRM-containing GLFG nucleoporins, an

idea which is also supported by our observation that the GLEBS can be taken out from *Nup116p* and functionally inserted into *Nup100p*. We therefore conclude that the conserved GLEBS within NRM-containing GLFG nucleoporins serves as a docking site for attaching target members

Table I. Analysis of the 'NPC herniation' phenotype in *nup116⁻* cells complemented by Nup100-GLEBS

	<i>nup116⁻</i> cells + Nup100-GLEBS	<i>nup116⁻</i> cells + Nup100
Number of inspected cells	200	149
Number of nuclei seen	107	100
Number of nuclei with herniations	56 (52%)	95 (95%)
Number of nuclei without herniations	51 (48%)	5 (5%)

The *nup116⁻* strains transformed with either pRS314-GFP-Nup100-GLEBS or pRS314-GFP-Nup100 were grown for 5 h at 37°C, before they were processed for thin sectioning electron microscopy.

Fig. 7. The evolutionarily conserved GLEBS motif within GLFG nucleoporins. (Upper panel) domain organization of yeast and higher eukaryotic GLFG nucleoporins. Within the five yeast GLFG nucleoporins, only Nup116p contains a GLEBS (Gle2p-binding sequence). A *C.elegans* Nup98 homologue (DDBJ/EMBL/GenBank accession number U50193) contains a large carboxy-terminal domain with homology to the yeast Nup145p carboxy-terminal domain. A partial *S.pombe* cDNA clone (D89145) also exhibits homology to the carboxy-terminal domains of yeast Nup145p and the *C.elegans* Nup98 homologue. The proteolytic cleavage site within Nup145p (Teixeira *et al.*, 1997) is indicated. (Lower panel) Nup116p is drawn as a multi-domain protein which binds to Gle2p via its GLEBS and to importin/karyopherin β -like transport factors (called β -Kaps) via the GLFG region. The carboxy-terminal domain may interact with NPC components yet to be identified.

of the Rae1/Gle2p protein family to the nuclear pores. This represents an additional mechanism for bringing a distinct group of conserved proteins with a potential role in mRNA export to the nuclear pores. Examples of how nucleocytoplasmic transport factors can associate with nuclear pores are importin/karyopherin β , which docks to the FXFG/GLFG repeat domains of nucleoporins (Radu *et al.*, 1995; Rexach and Blobel, 1995) and Ran which may bind to the Ran-binding domains within Nup358 and Nup2 (Dingwall *et al.*, 1995; Wu *et al.*, 1995).

In this context it is worth mentioning that the defects seen in reconstituted nuclei that have been depleted of vertebrate Nup98 (Powers *et al.*, 1995), and in *Xenopus* oocytes microinjected with anti-Nup98 antibodies (Powers *et al.*, 1997) may be also attributed to a co-depletion or altered binding of a corresponding vertebrate Gle2p homologue. Furthermore, chromosomal translocations resulting in the fusion of the GLFG nucleoporin Nup98 to transcriptional regulators and creating myeloid leukaemia may also affect the intracellular location of an attached Gle2p homologue, thereby causing aberrant nucleocytoplasmic transport (Borrow *et al.*, 1996; Nakamura *et al.*, 1996).

Besides its GLEBS, Nup116p has other functionally important regions (Figure 7). Previous work has demonstrated that Nup116p may interact via its GLFG domain with the nuclear import factor Kap95p (Iovine *et al.*, 1995) and it has been suggested that Nup116p is involved in recycling Kap95p into the cytoplasm (Iovine and Wenthe, 1997). Furthermore, the carboxy-terminal domain of Nup116p is linked genetically to the GLFG nucleoporins Nup145p-N and Nup100p, and has been shown to bind to homopolymeric RNA *in vitro* (Fabre *et al.*, 1994).

How does Nup116p achieve its steady state NPC location? We have shown that signals for NPC targeting are located in the carboxy-terminal domain of Nup116p. The GLFG domain may contribute to NPC targeting indirectly via interactions with transport factors (see above), but this domain alone cannot confer a stable NPC association (Iovine *et al.*, 1995). We speculate that the GLFG nucleoporins Nup100p and the cleaved Nup145p-N, which have related carboxy-terminal domains, use a similar mechanism for NPC targeting/association. It will be interesting to find out whether a common receptor (or structure) exists at the NPC to which the conserved carboxy-terminal domains of Nup116p, Nup100p and Nup145p-N bind (see also Figure 7). The two remaining GLFG nucleoporins in yeast, Nup49p and Nup57p, use a different strategy for NPC location, i.e. they employ their unique coiled carboxy-terminal domains to assemble into a complex with Nsp1p and Nic96p (Grandi *et al.*, 1995b). It is not clear whether Nup116p is permanently or dynamically bound to the NPCs. A dual location both at the NPCs and in the nucleoplasm has been reported for Nup98 (Powers *et al.*, 1995). If Nup116p shuttles between the nucleus and cytoplasm, Gle2p may also do so by 'piggy-backing' to its shuttling receptor.

Nup100p, which harbours the GLEBS of Nup116p, can be used as a vehicle to target Gle2p to the nuclear pores. This Nup100p-GLEBS construct can complement the thermosensitive growth phenotype of *nup116⁻* cells at

Table II. Yeast strains

Strain	Genotype	Source or reference
RS453	<i>Mata/a,ade2,his3/his3,leu2,trp1/trp1,ura3/ura3</i>	Segref <i>et al.</i> (1997)
nup133 ⁻	<i>Matα,ade2,his3,leu2,trp1,ura3,nup133::HIS3</i>	Segref <i>et al.</i> (1997)
mex67-5	<i>Mata,ade2,his3,leu2,trp1,ura3,mex67::HIS3</i> (pUN100-LEU2-mex67-5)	Segref <i>et al.</i> (1997)
nup116 ⁻	<i>Matα,ade2,his3,leu2,trp1,ura3,nup116::URA3</i>	Fabre <i>et al.</i> (1994)
gle2 ⁻	<i>Mata or α,ade2,his3,leu2,trp1,ura3,gle2::HIS3</i>	this study
gle2 ⁻ /nup100 ⁻	<i>Mata,ade2,his3,leu2,trp1,ura3,nup100::HIS3,gle2::HIS3</i> (pURA3-NUP100)	this study
gle2 ⁻ /nup116 ⁻	<i>Mata,ade2,his3,leu2,trp1,ura3,nup116::URA3,gle2::HIS3</i>	this study
GFP-NUP116	<i>Matα,ade2,his3,leu2,trp1,ura3,nup116::URA3</i> (pRS414-TRP1-GFP-NUP116)	this study
ProtA-NUP11	<i>Matα,ade2,his3,leu2,trp1,ura3,nup116::URA3</i> (pRS414-TRP1-ProtA-NUP116)	this study
GLE2-GFP	<i>Mata,ade2,his3,leu2,trp1,ura3,gle2::HIS3</i> (pUN100-LEU2-GLE2-GFP)	this study
GLE2-ProtA	<i>Mata,ade2,his3,leu2,trp1,ura3,gle2::HIS3</i> (pUN100-LEU2-GLE2-ProtA)	this study
GFP-Nup100-GLEBS	<i>Matα,ade2,his3,leu2,trp1,ura3,nup116::URA3</i> (pRS314-TRP1-GFP-NUP100-GLEBS)	this study
ProtA-Nup100-GLEBS	<i>Matα,ade2,his3,leu2,trp1,ura3,nup116::URA3</i> (pRS314-TRP1-ProtA-NUP100-GLEBS)	this study
GLE2-GFP/NUP100-GLEBS	<i>Mata,ade2,his3,leu2,trp1,ura3,nup116::URA3,gle2::HIS3</i> (pRS314-TRP1-ProtA-NUP100-GLEBS,pUN100-LEU2-GLE2-GFP)	this study
GLE2-GFP/NUP100	<i>Mata,ade2,his3,leu2,trp1,ura3,nup116::URA3,gle2::HIS3</i> (pRS314-TRP1-ProtA-NUP100,pUN100-LEU2-GLE2-GFP)	this study

37°C, although it is less efficient than Nup116p. Therefore, Nup100p is able to take over the essential Nup116p functions provided that it carries a GLEBS. Nup116p and Nup100p are therefore interchangeable through the short GLEBS and accordingly Gle2p does not necessarily need physical contact with Nup116p to perform its essential role, but it does require targeting to the nuclear pores (or alternatively shuttling). According to our data, Gle2p seems to require contact with one of the two GLFG Nups, Nup116p or Nup100p, in order to perform its *in vivo* function. Attaching the GLEBS to another nucleoporin such as Nup49p or Seh1p did not allow complementation of the nup116 disruption mutant.

It is possible that *in vivo* Nup100p is also a member of the Nup116p–Gle2p complex, but dissociates during biochemical purification. In this context it should be mentioned that *nup100* and *gle2* disruption alleles cause synthetic lethality (note that *nup100*⁻ cells are not impaired in cell growth at any physiological temperatures), whereas the combination of *nup116* and *gle2* disruption alleles gives viable cells (data not shown; see also Murphy *et al.*, 1996). Therefore, compared with Nup116p, Nup100p performs an additional role, perhaps via its carboxy-terminal domain, which overlaps with the function of Gle2p.

Strikingly, both *nup116* and *gle2* mutant cells exhibit a unique morphological abnormality within the double nuclear membrane, called the ‘NPC herniation’ phenotype (Wente and Blobel, 1993; Murphy *et al.*, 1996). This is not as distinct or developed in other nucleoporin mutants that reveal different perturbations such as NPC clustering, grape-like NPC arrangements, nuclear envelope protrusions and blisters (for overview see Murphy *et al.*, 1996). Our data explain for the first time why *gle2* and *nup116* mutants exhibit basically the same unusual nuclear envel-

ope abnormalities. It is the short GLEBS within Nup116p which makes the link between the two proteins. If Gle2p is displaced from the pores by deleting the GLEBS from Nup116p, strong NPC herniations with a membrane seal over the pores are the consequence (see also Figure 6). Thus, the NPC herniation phenotype in *nup116* mutants can be attributed to a lack of the GLEBS and the concomitant dissociation of Gle2p from the pores. Wente and co-workers found that Gle2p is involved in mRNA export (Murphy *et al.*, 1996) and the same is true for *S.pombe* Rae1, which is highly related to Gle2p (Brown *et al.*, 1995). Although we were not able to see a strong nuclear poly(A)⁺ RNA export defect in *gle2*⁻ cells as compared to *mex67-5* cells, it is possible that poly(A)⁺ RNA, which tends to accumulate at the nuclear periphery in *gle2-1* cells (Murphy *et al.*, 1996), is less stable and thus more rapidly degraded.

Could the Gle2p/Nup116p complex perform additional roles in the cell? Notably, the thermosensitive *rae1-1* mutant shows, besides a poly(A)⁺ RNA export defect, a disorganized actin and tubulin cytoskeleton (Brown *et al.*, 1995). Furthermore, a human protein homologous to Rae1 (designated mrnp 41) is located not only at the pores and in the nucleoplasm, but also on cytoplasmic cytoskeletal elements, most likely the actin and tubulin meshwork (Kraemer and Blobel, 1997). Human mrnp 41 is physically associated with poly(A)⁺ RNA and co-localizes with a major cytoplasmic poly(A)⁺ RNA binding protein in the cell. Thus, mrnp 41 could participate in cytoplasmic transport of mRNPs along cytoskeletal elements (Kraemer and Blobel, 1997). The nuclear envelope distortions seen in *gle2-1* mutant cells therefore could be the consequence of a disturbed interaction between nucleoporins and the cytoskeleton. Alternatively, our findings would also be consistent with a model where Gle2p controls the export

of a subset of mRNAs and/or factors which are required for correct NPC and nuclear envelope biogenesis.

Interestingly, Gle2p shows significant sequence homology with Bub3p which is involved in cell cycle check point control (Brown *et al.*, 1995) and the *NUP116* gene contains a cell cycle (MluI) box in its promoter sequence which is found typically in genes expressed at the G₁/S boundary (Kilmartin *et al.*, 1993). Furthermore, the *rae1-1* ts allele causes, besides its cell cycle independent inhibition of poly(A)⁺ RNA export, a particular vulnerability to loss of Rael function during G₂/M, also pointing to an additional role in cell cycle progression (Brown *et al.*, 1995). Therefore, the evolutionarily conserved Rael proteins may have an extra role in the check point control of nuclear membrane and pore biogenesis.

In summary, a biochemically stable NPC subcomplex has been identified consisting of Gle2p and Nup116p. The Gle2p member in this complex can only perform its crucial function for the cell when it gains physical contact with Nup116p. This requires the presence of an evolutionarily conserved docking sequence within Nup116p. Genetic and biochemical analysis of *GLE2* should shed further light on its exact role, *in vivo*, in NPC structure and nucleocytoplasmic transport.

Materials and methods

Yeast strains and growth, microbiological techniques and plasmids

The yeast strains used in this work are listed in Table II. Cells were grown in minimal SDC or rich YPD medium. Minimal SDC medium/plates contained all amino acids and nutrients except those used for the selection. For counter selection of *URA3*-containing plasmids, 5-fluoroorotic acid (5-FOA; CSM medium, BIO101, La Jolla, CA) was added. Genetic manipulations of yeasts were performed as described by Sherman (1990). The following yeast plasmids were used: pUN100, *ARS/CEN* plasmid with the *LEU2* marker; pRS314 and pRS414, *ARS/CEN* plasmids with the *TRP1* marker; pRS315, *ARS/CEN* plasmid with the *LEU2* marker; pRS316, *ARS/CEN* plasmid with the *URA3* marker.

Generation of NUP116 and NUP100 fusion constructs

All Nup116p and Nup100p constructs, which were tagged amino-terminally with either two IgG-binding domains derived from *S.aureus* Protein A (ProtA) or green fluorescent protein (GFP), were expressed under the control of the *NOP1* promoter. *NUP116* truncation constructs contained authentic 3' non-coding sequences. Expression of all fusion proteins was verified by Western blot analysis, using anti-Protein A or anti-GFP antibodies.

Construct 1: pRS414-ProtA-NUP116(Δ 1–58); the construct was as described in Wimmer *et al.* (1992).

Construct 2: pRS414-ProtA-NUP116(2–1113); in a two-step process, a PCR fragment of 1.6 kb (aa 2–536) was joined to a 0.5 kb *HindIII*–*SpeI* fragment released from 1, thereby generating a 2.1 kb fragment with an authentic *HindIII* site. Subsequently, a triple ligation was performed using plasmid 1 (*SacI*–*SpeI*), a *NOP1* promoter–*ProtA*-cassette (*SacI*–*XhoI*) and the 2.1-kb fragment (*XhoI*–*SpeI*) described above. This full-length construct behaves in a way similar to construct 1.

Construct 3: pRS414-ProtA-NUP116(59–705); construct 1 was cleaved at the unique *SpeI* site, filled in and blunt-end ligated to an oligonucleotide containing stop codons in all possible reading frames. This construct is called NUP116(N+GLFG) in Figure 2.

Construct 4: pRS315-ProtA-NUP116(59–168);

Construct 5: pRS315-ProtA-NUP116(110–166); for constructs 4 and 5, PCR fragments were generated, encoding either amino acids 59–168 or 110–166 from Nup116p and fused in-frame to a *NUP116* DNA fragment containing the stop codon plus 300 bp of the 3' non-coding sequence.

Construct 6: pUN100-ProtA-NUP116(168–705); a 5.0 kb *KpnI*–*XbaI* fragment, encoding the GLFG-domain of Nup116p (residues 168–705) plus 3' non-coding sequences was obtained through several cloning steps from construct 3. This fragment was ligated with a *SacI*–*KpnI* fragment of the *NOP1*-*ProtA* cassette into a *SacI*–*XbaI* digested pUN100 vector. This construct is called NUP116(GLFG) in Figure 2.

Construct 7: pRS414-ProtA-NUP116(706–1113); genomic DNA encoding full-length Nup116p was cut using *SpeI*, blunt-ended and joined in-frame to the *NOP1* promoter–*ProtA* cassette. This construct is called NUP116(C) in Figure 2.

Construct 8: pRS414-ProtA-NUP116(Δ 110–166); construct 1 was cut using *SpeI*–*SacI*, releasing the *NOP1* promoter–*ProtA* cassette together with the part encoding [N+GLFG]. In a two-step ligation, it was replaced by a *NOP1* promoter–*ProtA* cassette with flanking *SacI*–*XhoI* sites and a second DNA construct made of two PCR fragments (*XhoI*–*PvuI* and *PvuI*–*SpeI*) encoding the amino-terminal half of Nup116p, but missing amino acids 110–166. This construct is also called NUP116(Δ GLEBS).

Construct 9: pRS316-ProtA-NUP100 or pRS314-ProtA-NUP100; a 3.2-kb *BamHI*–*XhoI* fragment was released from pSW78 (CEN–*LEU2*–NUP100, kindly provided by S.Wente) and subcloned into pRS316 or pRS314. A *BamHI* fragment consisting of a 0.3 kb PCR fragment encoding the amino-terminal part of Nup100p and the *NOP1* promoter–*ProtA* cassette was inserted at the authentic *BamHI* site.

Construct 10: pRS314-ProtA-NUP100–GLEBS(110–166); a 0.75-bp *EcoRI* fragment was released from construct 9 and replaced by two PCR generated fragments, the authentic 0.75-bp fragment with the 5' *EcoRI* site changed into a *PstI* site and a 0.15 kb *EcoRI*–*PstI* fragment encoding amino acids 110–166 of Nup116p, thereby generating a hybrid protein where the GLEBS of Nup116p was inserted at amino acid 276 of Nup100p.

Construct 11: pRS315-ProtA-NUP116(Δ GLFG); a PCR fragment encoding amino acids 2–168 was generated and inserted into construct 17 thus giving rise to Nup116p missing residues 169–705. GFP was replaced by ProtA.

Construct 12: pRS414-ProtA-NUP116 (C-GLEBS); construct 8 was cut using *SpeI*, releasing the *NOP1* promoter–*ProtA* cassette together with the part encoding [N+GLFG]. After religating the plasmid, the 3' located *EcoRI*–*XhoI* fragment was replaced by two PCR fragments, thus generating an *ApaI* site prior to the stop codon. A PCR fragment encoding amino acids 110–166 of the GLEBS was inserted at the *ApaI* site. Finally, the *SpeI* fragment which had been removed previously was replaced.

Construct 13: pRS315-ProtA-GLEBS-SEH1;

Construct 14: pRS315-ProtA-GLEBS–NUP49; to generate a hybrid encoding ProtA-tagged GLEBS–Seh1p and GLEBS–Nup49p, construct 5 missing the NUP116 stop codon and the 300 bp of the 3' non-coding sequence was used. A PCR fragment encoding full-length *SEH1* or *NUP49* missing the ATG start codon but containing 300 bp of the 3' non-coding sequence was inserted in frame 3' of the GLEBS coding sequence using an *ApaI* site.

Construct 15: pRS414-GFP-NUP116(2–1113); like construct 2; ProtA was replaced by GFP.

Construct 16: pRS414-GFP-NUP116(Δ 110–166); like construct 8; ProtA was replaced by GFP.

Construct 17: pRS315-GFP-NUP116(706–1113); a PCR fragment was generated and fused to a cassette containing the *NOP1* promoter and sequences encoding GFP.

Construct 18: pRS314-GFP-NUP100; like construct 9; ProtA was replaced by GFP.

Construct 19: pRS314-GFP-NUP100–GLEBS(110–166); like construct 10; ProtA was replaced by GFP.

Cloning and gene disruption of GLE2 and generation of gle2[–]/nup116[–] and gle2[–]/nup100[–] double-disruption mutants

The *GLE2* gene was cloned from yeast genomic DNA by PCR, using two oligonucleotides priming 784 nucleotides downstream and 550 upstream of the *GLE2* ORF. The resulting 2.4 kb PCR product was cloned into a pRS316 vector cut with *EcoRI*–*BamHI* yielding pRS316–

URA3–GLE2. For the ProtA and GFP tagging of Gle2p, a unique *Xba*I site was generated by PCR-mediated mutagenesis, just prior to the *GLE2* stop codon. The DNA sequences encoding either the ProtA or the GFP tag were then introduced in frame as *Xba*I fragments into the carboxy-terminal end of *GLE2* and inserted into pUN100. Both fusion proteins were expressed under the native *GLE2* promoter. For the gene disruption of *GLE2*, an internal *Nde*I–*Nsi*I fragment was removed (from methionine [119] to methionine [339]) and replaced by the *HIS3* marker. The *gle2::HIS3* allele which carried 0.84 kb at the 3' flanking region and 0.56 kb at the 5' flanking region, was excised from the pUN100 vector and the purified fragment *gle2::HIS3* was transformed into the diploid RS453 strain. Transformants were selected on SDC-His plates and analysed for the correct integration at the *GLE2* gene locus (as verified by PCR–Southern). *GLE2/gle2::HIS3* heterozygous diploids were sporulated and tetrads dissected.

The generation of the *nup116::URA3* and *nup100::LEU2* disrupted strains has been described earlier (Fabre *et al.*, 1994). Double-disruption strains *nup116::URA3/gle2::HIS3* and *nup100::LEU2/gle2::HIS3* were generated by mating the corresponding single knock-out mutants, followed by sporulation and tetrad analysis. In the latter case, the pRS316–URA3–NUP100 plasmid was present to complement the synthetic lethal phenotype of the *nup100::LEU2/gle2::HIS3* double disruption mutant.

Mass spectrometric protein identification

The Gle2p was identified using the strategy described previously, consisting of a first screen by peptide mass mapping followed by mass spectrometric sequencing of the unseparated peptide mixture (Shevchenko *et al.*, 1996). The protein band excised from 1-D PAGE was in-gel digested with trypsin. A small aliquot (0.3 ml) of the tryptic peptides resulting from the enzymatic degradation was analysed by Matrix Assisted Laser Desorption Ionization (MALDI). This aliquot was deposited on microcrystalline surfaces made by the fast evaporation method (Vorm *et al.*, 1994) and rinsed twice before analysis. Spectra were calibrated on matrix ion peaks of known mass. The peptide mass maps were searched against a non-redundant protein database (nrdb) maintained by the group of C.Sander (EMBL/EBI). For mass spectrometric sequencing by nanoelectrospray tandem mass spectrometry, gel pieces were further extracted, the resulting peptide mixture dried in a speed vac and redissolved and concentrated in a two-needle assembly as described previously (Wilm and Mann, 1996; Wilm *et al.*, 1996). An API III (Sciex, Toronto, Canada) triple quadrupole mass spectrometer was employed for sequencing. Tandem mass spectra peptide sequence tags (Mann and Wilm, 1994) were used to identify the protein. All database searches were performed using the program PeptideSearch developed by M.Mann.

Expression and localization of GFP-fusion proteins in yeast

To analyse the *in vivo* location of GFP-tagged Nup116p, Nup100p and Gle2p constructs, strains with corresponding disrupted genes expressing the GFP-fusion proteins plus the *ADE2* gene [provided on the plasmid pASZ11; the *ADE2* abolishes the strong vacuolar autofluorescence due to the *ade2* mutation (Segref *et al.*, 1997)] were examined in the fluorescein channel of a Zeiss Axioskop fluorescence microscope. Pictures were taken with a Xillix Microimager camera and processed by digital imaging using the software *Openlab* (Improvision, Coventry, UK).

Miscellaneous

DNA manipulations (restriction analysis, end filling reactions, ligations, PCR amplifications, etc.) were carried out essentially according to Maniatis *et al.* (1982). Preparation of protein extracts, purification of ProtA fusion proteins from yeast, SDS–PAGE, Western blotting, indirect immunofluorescence, samples for electron microscopy and analysis of poly(A)⁺ RNA export were performed as described earlier (Segref *et al.*, 1997).

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