A Novel Saccharomyces cerevisiae FG Nucleoporin Mutant Collection for Use in Nuclear Pore Complex Functional Experiments

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The nuclear pore complex (NPC) is the essential, conserved, selective portal for nucleocytoplasmic transport in eukaryotic cells. By controlling transport across the NPC and maintaining the separation of transcription and translation machinery, intricate levels of gene regulation are supported in both single and multicellular eukaryotic organisms (reviewed in Raices and D’Angelo 2012). The 60–120 MDa NPC complex is built from multiple copies of a conserved set of ~30 nuclear pore proteins (nucleoporins, Nups; reviewed in Field et al. 2014). Nups are organized into subcomplexes that assemble to generate a transport channel across the nuclear envelope (NE) with nuclear basket and cytoplasmic filament structures extending from the NE (Figure 1). Diverse technologies have been used to enhance our understanding of how structural Nups interact to build the NPC scaffold (Alber et al. 2007; Field et al. 2014; Chug et al. 2015; Stuwe et al. 2015). However, despite extensive study using a variety of approaches, questions remain regarding how the NPC forms a barrier to nonspecific transport of large macromolecules (>40 kDa) while at the same time facilitating specific import and export of molecules against concentration gradients (Rout et al. 2000; Yamada et al. 2010; Hulsmann et al. 2012; Lim et al. 2015). Importantly, the combined use of in vivo and in vitro experimental approaches is critical to fully unravel the mechanisms for nuclear transport and to define discrete Nup functions in a cell.

The FG Nups (11 members in Saccharomyces cerevisiae and humans) are the class of NPC proteins that both generate the NPC permeability barrier and provide binding sites for facilitated transport (Hulsmann et al. 2012; Lord et al. 2015; reviewed in Terry and Wente 2009). Each FG Nup contains an unstructured domain with multiple phenylalanine-glycine (FG) repeat motifs separated by ~10–20 spacer residues comprised mostly of polar amino acids. The 11 FG Nups are characterized by different types of FG repeat motifs (classified into FG, glycine-leucine-phenylalanine-glycine, GLFG; or phenylalanine-any-phenylalanine-glycine, FxFG domains; reviewed in Rout and Wente 1994). Unless specified, in this report, we use the terminology “FG” generically to refer to the entire family of FG, FxFG, and GLFG Nups or when referring to multiple FG domains in a subcomplex. In each FG Nup, structured region(s) flank the FG domain to allow interaction with scaffold Nups. These structural domains effectively anchor FG Nups at discrete NPC sites, either symmetrically in the channel or asymmetrically at the cytoplasmic or nuclear face (Figure 1; Rout et al. 2000). Furthermore, a trio of highly conserved symmetric FG Nups (in S. cerevisiae: Nup1, Nup49, and Nup57; in vertebrates: Nup62, Nup58/Nup45, and Nup54) forms a subcomplex through interactions of their coiled-coil structural domains (Chug et al. 2015; Stuwe et al. 2015).
The unstructured FG domains are modeled to extend into the NPC transport channel (reviewed in Terry and Wente 2009; Kabachinski and Schwartz 2015). For facilitated movement through the NPC, specialized transport receptors bind both the cargo and the FG repeats of FG domains, allowing entry into and through the FG domain network (reviewed in Field et al. 2014; Kabachinski and Schwartz 2015). Directionality of transport is mediated by additional soluble factors found at the NPC faces, or in the nucleus or cytoplasm (reviewed in Kabachinski and Schwartz 2015). With regard to roles in inhibiting the diffusion of macromolecules, the vertebrate GLFG Nup98 is critically important (Hulsmann et al. 2012) and the S. cerevisiae orthologs Nup116 and Nup100 also contribute to the permeability barrier (Lord et al. 2015). Thus, due to their bifunctional role in inhibiting diffusion of molecules and providing binding sites for transport receptors, FG domains constitute the fundamental basis for selective nucleocytoplasmic trafficking.

To date, S. cerevisiae is an important model system for investigating FG domain function in vivo, and diverse mutant construction approaches have been developed over the nearly three decades of study. Analyses of strains generated with entire genes deleted found that some FG Nup encoding genes are individually essential (Hurt 1988; Davis and Fink 1990; Wente et al. 1992; Del Priore et al. 1997). Thus, for functional studies with full gene deletions, analysis is limited to non-essential genes. Early studies also used plasmid-based expression of \( \Delta FG \) alleles to complement lethal chromosomal null mutants, and demonstrated that most individual FG domains can be removed with no loss in cell viability (Nehrbass et al. 1990; Grandi et al. 1995; Iovine et al. 1995; Del Priore et al. 1997). Indeed, most plasmid-based individual \( \Delta FG \) strains with only the FG domain absent have minimal growth and transport defects (reviewed in Terry and Wente 2009). Given such functional redundancy within the NPC, to analyze FG domain function, multiple combined deletions of sequences encoding different FG domains must be included within a given strain. However, with 11 FG Nups, the availability of auxotrophic markers to maintain multiple plasmids, each encoding individual NUP genes, has limited analysis using such a strategy to only a few Nups within one strain.

To overcome these limitations, we originally developed a collection of S. cerevisiae mutants wherein NUP genes, with only the sequence...
Although the chromosomal ΔFG mutant strains have been instrumental in NPC functional analysis, they have several important caveats. First, chromosomal deletions preclude easy modification of genes in comparison to plasmid-based expression. Second, the remaining epitope and loxP tags result in nonspecific defects in some of the higher-order multiple ΔFG mutant strains. For instance, we previously reported that the lethality of T7-loxP-nup1ΔFxG myc-loxP-nup2ΔFxG myc-loxP-nup60ΔFG HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup49ΔFG is rescued by plasmid-based expression of untagged nup49ΔFG (Terry and Wente 2007). Therefore, our goal in this study was to generate a new collection of ΔFG mutants which (1) avoid indirect effects from epitope or loxP tagging during strain construction, (2) allow straightforward future mutational analysis of the sequences encoding individual domains, and (3) enable functional analysis of the resulting mutants.

We report here a new approach based on chromosomal null alleles complemented by plasmid-based expression of ΔFG nups, wherein each plasmid encodes multiple FG Nups that are colocated in specialized roles during transport, but it is unknown what attributes (FG type, spacer sequence, location within the NPC) lead to these particular functions.

**RESULTS AND DISCUSSION**

Our efforts focused on deleting the FG domains of the conserved Nsp1-Nup49-Nup57 subcomplex in combination with deletion of FG domains from the nuclear (Nup1, Nup2, and Nup60) and cytoplasmic faces (Nup42, and Nup159) of the NPC can all be deleted without loss of viability. Although harboring severe growth defects, these deletions result in a new GLFG domain-only NPC. This collection will be of use to the community and set the stage for future experiments further probing of FG domain function in vivo.
endogenous FG NUP gene in the presence of a plasmid expressing the corresponding wild type (WT) FG NUP, and (2) shuffling the WT FG NUP plasmid for respective ΔFG nup constructs. Plasmids were engineered to allow expression of multiple FG NUP genes with their respective endogenous 5′ and 3′ UTRs (Figure 2A). Importantly, this plasmid-based expression strategy should not alter Nup stoichiometry within the NPC, because sequence encoding the anchoring structured domains is still present in ΔFG nup constructs.

The specific combinations of FG NUP or ΔFG nup genes cloned into a single expression plasmid was chosen based on the Nups, common physical association in NPC subcomplexes and/or NPC substructural localization (Figure 1 and Figure 2A). One set of plasmids harbored the three genes encoding the three FG Nups of the symmetric Nsp1 subcomplex: Nsp1, Nup49, and Nup57 (Grandi et al. 1995) (designated as NSP1/NUP49/NUP57, or nsp1/nup49/nup57ΔFG when lacking the FG domains). A second set contained genes encoding the two cytoplasmic-oriented FG Nups: Nup159 and Nup42 (designated as C-WT, or CΔFG when lacking the FG domains), and a third, the three nuclear-oriented FG Nups: Nup1, Nup2, and Nup60 (designated as N-WT, or ΔNFG when lacking the FG domains).

By classic mating and sporulation, we first generated a triple deletion strain in which the endogenous chromosomal locus encoding each of the Nsp1-Nup49-Nup57 complex members was deleted in the presence of single WT plasmids. The individual plasmids were then exchanged for a NSP1/NUP49/NUP57 plasmid in the nsp1Δ nup49Δ nup57Δ triple mutant, which was subsequently exchanged for an nsp1/nup49/nup57ΔFG plasmid (Figure 3 and Table 1). We analyzed growth of the resulting nsp1/nup49/nup57ΔFG mutant with the simultaneous deletion of all three of the FG domains in the Nsp1 complex was viable with no noted growth defects at the temperatures tested. This result was consistent with previous genetic analysis of the genes encoding this complex (Fabre et al. 2004), indicating that the reported lethality with the Cre-loxP approach was likely due to tag-specific effects (Strawn et al. 2004).

Using the nsp1Δ nup49Δ nup57Δ triple mutant as a starting point, the sequences encoding the FG domains on the nuclear and cytoplasmic face of the NPC were subsequently deleted (Figure 3 and Table 1). Because higher order gene deletions are difficult to generate and track by mating and sporulation, we adopted an iterative approach in haploid strains where the endogenous FG NUP gene was deleted by a floxed SpHIS cassette followed by recombination of the SpHIS sequence by expression of Cre recombinase (Figure 2B). This approach permitted deletion of multiple genes within one strain without losing availability of auxotrophic markers. In order to accommodate available markers, the asymmetric NUP genes were deleted in the presence of NΔFG and CΔFG plasmids with NSP1/NUP49/NUP57 covering the nsp1Δ nup49Δ nup57Δ deletions (Figure 3). We reasoned that this approach would not select for off-target effects because we previously observed that absence of all five asymmetric FG domains results in minimal growth defects (Terry and Wente 2007). Once the nuclear and cytoplasmic FG Nups were deleted, the WT NSP1/NUP49/NUP57 plasmid was exchanged for the nsp1/nup49/nup57ΔFG plasmid.

By serial dilution and analysis of growth on YPD, we observed that absence of FG domains from both the Nsp1-Nup49-Nup57 subcomplex and the nuclear face had growth defects at all temperatures tested (NΔFG nsp1/nup49/nup57ΔFG). In contrast, the absence of the FG domains both the Nsp1-Nup49-Nup57 subcomplex and the cytoplasmic face resulted in only mildly impacted growth (CΔFG nsp1/nup49/
Deletion of all asymmetric FG domains in combination with nsp1/nup49/nup57ΔFG resulted in a viable strain with drastic growth defects (Figure 4A). To quantitatively analyze growth of all strains, liquid culture growth analysis was conducted at 23°C (Figure 4B). Whereas most strains had doubling times of 2 to 3 hr, NΔFG nsp1/nup49/nup57ΔFG had a doubling time of 6.1 hr; CΔFG nsp1/nup49/nup57ΔFG, 3.4 hr; and NΔFG CΔFG nsp1/nup49/nup57ΔFG 10.3 hr.

To assess whether NPCs are assembled in these ΔFG strains, indirect immunofluorescence microscopy was performed using an antibody raised against the carboxy-terminal (non FG) domain of Nup116. Nup116 is an FG Nup that localizes to cytoplasmic foci when NPC assembly is perturbed (Ryan and Wente 2002), and the vertebrate ortholog, Nup98, associates with the nuclear envelope only after scaffold Nups are recruited following mitosis (Dultz et al. 2008). Therefore, Nup116 localization to the NE rim is a marker for proper NPC assembly. In all the ΔFG strains tested, anti-Nup116 signal was located at the nuclear rim surrounding the nuclear DAPI signal, suggesting that NPC assembly was not notably altered in the mutants (Figure 4C).
### Table 2 Plasmid table

| Vector | Name in Text | Description | Residues Deleted | Plasmid Backbone* | Auxotrophic Marker | ΔFG Restriction Site | Residues Added | Source |
|--------|-------------|-------------|------------------|-------------------|--------------------|---------------------|-----------------|--------|
| pSW222 | NSP1        |             |                  | prRS315           | LEU2               |                     |                 | This study |
| pSW3428| nsp1ΔFx FG  | 179–591     | prRS315          | LEU2              | Nhel               | Ala Ser             | This study     |        |
| pSW3524| nsp1ΔFGA-FxFG| 3–591       | prRS314          | TRP1              | SpeI               | Thr Ser             | This study     |        |
| pSW3444| NUP49       |             | prRS314          | LEU2              |                     |                     |                 | This study |
| pSW3513| NUP49       |             | prRS314          | TRP1              | HI5               |                     | This study     |        |
| pSW3548| NUP49       |             | prRS313          | TRP1              | SpeI               | Thr Ser             | This study     |        |
| pSW3549| nup49ΔGLFG  | 2–223       | prRS314          | TRP1              | Nhel               | Ala Ser             | This study     |        |
| pSW3512| NUP57       |             | prRS36           | URA3              |                     |                     |                 | This study |
| pSW3550| nup57ΔGLFG  | 2–236       | prRS314          | TRP1              | Nhel               | Ala Ser             | This study     |        |
| pSW3521| NSP1, NUP57 |             | prRS36           | URA3              |                     |                     |                 | This study |
| pSW3554| NSP1, NUP49, NUP57 | | prRS36 | URA3 |       |                     |                 | This study |
| pSW3555| NSP1, NUP49, NUP57 | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW3643| NSP1/NUP49/ NUP57 | | prRS314 | TRP1 |       |                     |                 | This study |
| pSW3551| nup49ΔGLFG, nup57ΔGLFG | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW3552| nsp1ΔFGA-FxFG, nup57ΔGLFG | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW3553| nsp1ΔFGA-FxFG, nup49ΔGLFG | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW3644| nsp1ΔFGA-FxFG, nup49ΔGLFG, nup57ΔGLFG | | prRS315 | LEU2 |       |                     |                 | This study |
| pSW3547| nsp1/nup49/ nup57ΔFG | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW3551| nup49ΔGLFG, nup57ΔGLFG | | prRS313 | HIS3 |       |                     |                 | This study |
| pSW812 | NUP1        |             | prRS315          | LEU2              |                     |                     |                 | This study |
| pSW3634| NUP1        |             | prRS314          | TRP1              | HI5               |                     | This study     |        |
| pSW3637| nup1ΔFx FG  | 384–888     | prRS315          | LEU2              | AvrI              | Pro Arg             | This study     |        |
| pSW3635| NUP2        |             | prRS314          | TRP1              | AvrI              | Pro Arg             | This study     |        |
| pSW3638| nup2ΔFx FG  | 189–527     | prRS314          | TRP1              | AvrI              | Pro Arg             | This study     |        |
| pSW3636| NUP60       |             | prRS314          | TRP1              | AvrI              | Pro Arg             | This study     |        |
| pSW3639| nup60ΔFx F  | 397–512     | prRS314          | TRP1              | AvrI              | Pro Arg             | This study     |        |
| pSW3640| NUP1, NUP2, NUP60 | | prRS314 | TRP1 |       |                     |                 | This study |
| pSW3642| N- WT       |             | prRS316          | URA3              |                     |                     |                 | This study |
| pSW3641| NΔFG        |             | prRS315          | LEU2              |                     |                     |                 | This study |
| pSW3801| NUP42       |             | prRS315          | LEU2              |                     |                     |                 | This study |
| pSW3802| NUP42       |             | prRS314          | TRP1              | H 1                  |                     |                 | (Adams et al. 2014) |
| pSW3645| nup42ΔFG    | 4–364       | prRS315          | LEU2              | Xhol              | Leu Glu             | This study     |        |
| pSW3448| nup42ΔFG    | 4–364       | prRS317          | LYS2              | Xhol              | Leu Glu             | This study     |        |
| pSW3657| nup42ΔFG    | 4–364       | prRS314          | TRP1              | Xhol              | Leu Glu             | (Adams et al. 2014) |
| pSW3647| NUP159      |             | prRS314          | TRP1              | AvrI              | Pro Arg             | (Adams et al. 2014) |
| pSW3648| nup159ΔFG   | 464–876     | prRS313          | LYS2              | HI5               |                     | This study     |        |
| pSW3646| NΔFG        |             | prRS317          | LYS2              | HI5               |                     | This study     |        |
| pSW3500| NUP100      |             | prRS313          | TRP1              |                    |                     | This study     |        |
| pSW3501| NUP100      |             | prRS313          | TRP1              |                    |                     | This study     |        |
| pSW3502| Nup100ΔGLFG | 2–570       | prRS313          | HIS3              | SpeI              | Thr Ser             | This study     |        |
| pSW3503| Nup100ΔGLFG | 2–570       | prRS314          | TRP1              | SpeI              | Thr Ser             | This study     |        |
| pSW3504| NUP116      |             | prRS313          | HIS3              |                    |                     | This study     |        |
| pSW3506| NUP145      |             | prRS314          | TRP1              |                    |                     | This study     |        |
| pSW3656| nup145ΔGLFG | 10–209      | prRS314          | TRP1              | Nhel              | Ala Ser             | This study     |        |

* These plasmids contain bacterial resistance (AMP*) and high copy replication (ori) sequences, yeast centromeric (CEN6) and replication (ARS4) sequences, and the indicated yeast auxotrophic marker (Siskorski and Hieter 1989).

The NΔFG CΔFG nsp1/nup49/nup57ΔFG strain results in a GLFG-only NPC: the GLFG domains of Nup100, Nup116, and Nup145 (paralogous to each other and orthologous to vertebrate Nup98; Ryan and Wente 2000) are the only FG domains remaining. The other two GLFG domains in Nup49 and Nup57 are absent. Considering previous reports that GLFG domains are required for the formation of the NPC permeability barrier (Hulsman et al. 2012; Lord et al. 2013), and that modification of GLFG Nups relaxes the barrier in vitro (Labokha et al. 2013), this strain will be of interest for subsequent studies of NPC transport capacity and nuclear permeability. We have generated plasmids encoding Nup100, Nup116, and Nup145 and FG deletions (Table 2) for use in such analysis.

The plasmid-based expression of ΔFG nups in chromosomal null strains as presented here provides a straightforward way to introduce new sequences, mutations, or deletions into nup genes for analysis of FG Nup function in vivo. We previously assessed FG domain functional complementation using plasmid-based expression of ΔFG nups and swapped FG (SFG) nups (Iovine et al. 1995; Adams et al. 2014; Lord...
et al. 2015). The “swapped” strategy involves replacing the endogenous FG domain with that of another Nup. These studies revealed that FG domains of different Nups have inherently distinct function in vivo, because only select FG domains could functionally replace those tested. It is likely that sequence differences underlie distinct functionality. Indeed, individual domains from different FG Nups have distinct in vitro biochemical and biophysical characteristics (Lim et al. 2007; Yamada et al. 2010; Labokha et al. 2013). The genetic tools generated in this report will allow future investigations to conduct highly detailed tests of what sequences contribute to specialized function during transport and what biophysical and biochemical properties of FG domains contribute to the NPC permeability barrier and selectivity mechanism.

MATERIALS AND METHODS

Yeast strains and growth

Table 1 lists the yeast strains generated in this study. Yeast genetic methods were conducted according to standard procedures (Sherman et al. 1986). Yeast strains were grown in either YPD (2% peptone, 2% dextrose, 1% yeast extract) or selective minimal media (Sherman et al. 1976). Yeast genetic tools generated in this report will allow future investigations to conduct highly detailed tests of what sequences contribute to specialized function during transport and what biophysical and biochemical properties of FG domains contribute to the NPC permeability barrier and selectivity mechanism.

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Plasmid construction

Table 2 lists the plasmid generated in this study. Plasmid cloning was performed according to standard molecular biology strategies, and ΔFG plasmids were generated by amplifying a wild type NUP plasmid to replace the FG domain with a unique restriction site (Figure 2A). Most FG domains were replaced with the restriction sites AvrII, Nhel, and SpeI to generate compatible cohesive ends (with the exception of Xhol for nup42ΔFG). FG domain boundaries were based on Strawn et al. (2004), and indicated in Table 2. Immunoblotting confirmed loss of FxFG and GLFG domains in strains transformed with ΔFG plasmids (data not shown).

Immunofluorescence

Yeast strains were grown to midlog phase (OD600 ~0.5) in YPD medium at 23°C, processed and labeled as in Ho et al. (2000). Briefly, samples were incubated with anti-Nup116-CTD rabbit antibodies (WU600, Iovine et al. 1995) overnight at 4°C. Bound primary antibodies were detected with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes) and samples were stained with 0.1 mg/mL DAPI. Wide-field images were acquired using a microscope (BX50; Olympus) equipped with a motorized stage (Model 990000, Ludl), Olympus 100× NA1.3 UPlanFl objective, and digital charge coupled device camera (Orca-R2; Hamamatsu). Images were processed with ImageJ (NIH).

Data availability

Strains and plasmids are available upon request. Table 1 contains genotypes for each individual strain. Table 2 contains information for each plasmid.

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