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FG-Rich Repeats of Nuclear Pore Proteins Form a Three-Dimensional Meshwork with Hydrogel-Like Properties

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Nuclear pore complexes permit rapid passage of cargoes bound to nuclear transport receptors, but otherwise suppress nucleocytoplasmic fluxes of inert macromolecules ≥30 kilodaltons. To explain this selectivity, a sieve structure of the permeability barrier has been proposed that is created through reversible cross-linking between Phe and Gly (FG)–rich nucleoporin repeats. According to this model, nuclear transport receptors overcome the size limit of the sieve and catalyze their own nuclear pore-passage by a competitive disruption of adjacent inter-repeat contacts, which transiently opens adjoining meshes. Here, we found that phenylalanine-mediated inter-repeat interactions indeed cross-link FG-repeat domains into elastic and reversible hydrogels. Furthermore, we obtained evidence that such hydrogel formation is required for viability in yeast.

Cell nuclei import all their proteins from the cytoplasm and, in return, supply the cytoplasm with nuclear products such as ribosomes, mRNAs, or transfer RNAs. Because interphase nuclei are enclosed by the nuclear envelope, all exchange between the compartments proceeds through the embedded nuclear pore complexes (NPCs). NPCs are equipped with a permeability barrier (*1*, *2*) against uncontrolled nucleocytoplasmic fluxes, which otherwise would have detrimental consequences, e.g., for the ordered course of gene expression (*1*). Objects larger than ~30 to 40 kD are restricted or even excluded from passing the barrier unless they are bound by cognate nuclear transport receptors (NTRs) (*1*, *3*, *4*). Because the actual NPC passage of cargo-NTR complexes is a reversible process, unidirectional cargo transport requires active cargo release from NTRs in the destination compartment.

Facilitated NPC passage requires interactions of NTRs with so-called FG repeats (*5*–*9*) that are found in numerous nuclear pore proteins (*10*), which suggests that the permeability barrier contains or is even built of these repeats [discussed in (*11*, *12*)]. Such repeat domains have up to 50 repeat units (*10*), appear intrinsically unfolded (*13*), and contain short clusters of hydrophobic amino acids [such as FSFG or GLFG in single-letter amino acid code (*14*)] separated by hydrophilic spacers.

Various models have been proposed to explain how these repeat domains create the permeability barrier. The "virtual gating" (*15*) and the "oily spaghetti" (*2*) models suggest that the FG-repeat domains repel molecules that should be excluded from passage. The "selective-phase" model (*11*, *12*) is based on the consideration that a simple binding of NTRs to such repeats would only delay their NPC passage. It assumes that the barrier is a sievelike structure, whereby meshes form through hydrophobic interactions between the FG-repeats' hydrophobic clusters, while the mesh size sets the size limit for passive exclusion. According to this model, NTRs can overcome this size restriction because their binding to hydrophobic clusters competes the noncovalent inter-repeat cross-links and, thereby, transiently opens adjacent meshes of the sieve (see fig. S1).

The models differ foremost in the question of whether the permeability barrier is tightened by inter-repeat interactions or not. If no interactions occurred, aqueous solutions of FG-repeat domains should behave like (viscous) fluids (Fig. 1, right inset). In contrast, if cross-linking prevented a free sliding between the linear polymers, elastic hydrogels should form (Fig. 1, left inset). Provided the cross-links remain intact, such a gel would respond to mechanical forces only by transient deformation and would readopt its original shape once the strain is relieved. We decided to test these predictions for the N-terminal "fsFG"-repeat domain of the essential yeast nucleoporin Nsp1p (*16*), which comprises 18 regular FSFG-repeats and 16 less regular FG-repeats (fig. S2).

The starting point for gelling a polymer into a homogeneous hydrogel is typically a homogeneous aqueous solution. Agarose or gelatin, for example, is first dissolved in hot water, and the respective gels are obtained after cooling. We found that, in our case, the solid-to-gel transition was best triggered by a pH shift. At high pH, the negative net charge of the Nsp1 fsFG-repeat domain is apparently repulsive enough to counteract gelling. Strikingly, however, when alkaline solutions with ≥ 8 mg/ml fsFG repeats were brought to physiological pH (where the net charge of the repeat units is close to zero), transparent and, hence, homogeneous gels formed (*17*). These gels were elastic enough to retain their shape when pushed out of a silicone tubing (Fig. 1). A gel of 26 mg/ml had an elasticity of 1000 to 2000 Pa, measured as Young's modulus by atomic force microscopy, which is comparable to a 0.4% agarose gel.

Remarkably, the gels remained stable and unaltered in appearance when the temperature was raised to 95°C. Apparently, the increased strength of hydrophobic interactions at increased temperature (*18*) can compensate for the increase of gel-destabilizing thermal motions. The gels, however, were readily dissolved by chaotropic agents such as 6 M guanidinium chloride; this result suggested that gel formation originated indeed from noncovalent, reversible interchain connections.

We next mutated each of the 55 phenylalanines (F) within the FG context to serines (S) (fig. S2). If hydrophobic cross-linking between fsFG repeats occurred, then it should be abolished in this mutant. Indeed, this F→S mutated repeat domain showed no signs of gelling and remained liquid in aqueous solution (Fig. 1), even when the concentration of repeats was raised to 100 mg gel/ml. Thus,

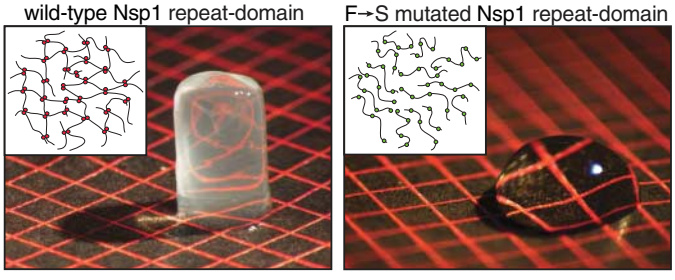

Fig. 1. FG repeats can form an elastic hydrogel in aqueous solution. (**Left**) An aqueous solution with 26 mg/ml wild-type fsFG-repeat domain from Nsp1p (400 μM) was filled into a silicon tubing, where it completed gelling. The formed gel was pushed out of the tubing by gentle pressure, placed onto a patterned support (1 square = 1.4 mm), and photographed. Note that the pattern shows clearly through this transparent gel. Inset illustrates how interactions between the hydrophobic clusters (shown in red) cross-link the repeat domains into a hydrogel. (**Right**) The F→S mutated repeat domain (see fig. S2) remained liquid after identical treatment.

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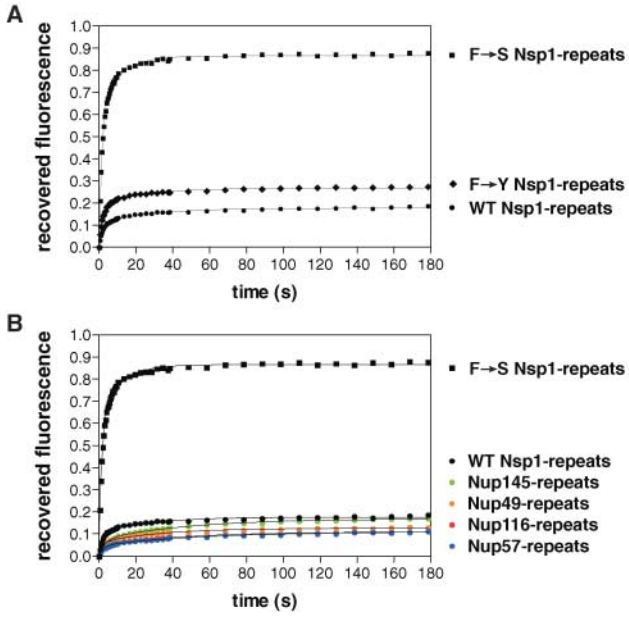

Fig. 2. (**A**) The wild-type Nsp1-repeat hydrogel interacts strongly with the wild-type and F→Y mutated repeat domain, but not with the F→S mutated version. A hydrogel with 400 μM unlabeled wild-type Nsp1 fsFG repeats and 1 μM of indicated fluorescently labeled repeat domains was formed as described in Fig. 1. The mobility of the labeled repeats was determined by FRAP. This method measures the diffusion-driven exchange of bleached molecules with unbleached ones. Photobleached regions of wild-type or F→Y mutated repeats recovered only a small fraction of their initial fluorescence; these repeats were essentially immobile and firmly incorporated into the gel. The F→S mutated version, in contrast, diffused freely inside the gel and recovered fluorescence almost completely. (**B**) GLFG repeats coassemble with Nsp1 fsFG repeats into a hydrogel. Hydrogels with 400 μM unlabeled wild-type Nsp1 fsFG-repeat domain and 1 μM of the indicated fluorescently labeled nucleoporin repeat domain were prepared and analyzed by FRAP. The GLFG repeats from Nup49p, Nup57p, Nup116p, or Nup145p were essentially immobile, which indicated strong heterotypic interactions with the fsFG repeats from Nsp1p.

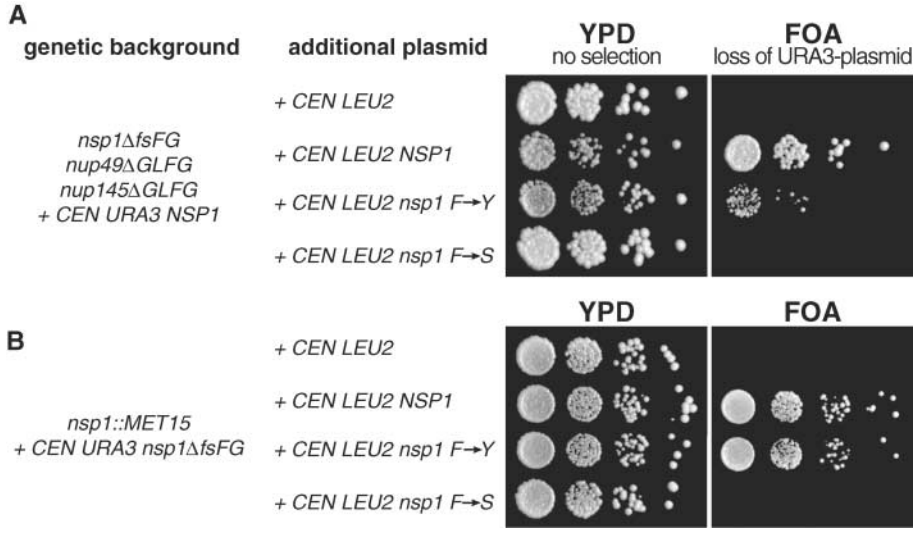

Fig. 3. In vivo effects of F→S and F→Y mutated FG-repeat domains. (**A**) A yeast strain lacking the GLFG-repeat regions of Nup49p and Nup145p, as well as the fsFG-repeat region of Nsp1p, was kept viable by expressing full-length Nsp1p from a low-copy *ARS/CEN* (*CEN*) plasmid harboring a *URA3* marker. The strain was subsequently transformed with indicated *CEN* plasmids carrying *LEU2* as a selectable marker and cultivated for several generations in synthetic complete (SC) medium lacking leucine. To select for cells that lost the original *CEN URA3* expression plasmid for wild-type Nsp1p, equal numbers of cells were plated in 10-fold serial dilutions on plates containing 5-fluoroorotic acid (FOA), which is toxic in the presence of a functional *URA3* gene. The total number of viable cells was determined in parallel by plating cells by the same serial dilution on full medium (YPD). The empty *CEN LEU2* plasmid used as the negative control did not support growth on FOA, which showed that the fsFG repeats of Nsp1p are essential in this genetic background (*19*, *20*). Cells were viable on FOA if the remaining *CEN LEU2* plasmid encoded wild-type Nsp1p. Cells expressing Nsp1p with F→Y mutated repeats were also viable on FOA, but showed a growth defect. Expression of Nsp1p with F→S mutated repeats did not allow for growth on FOA. (**B**) A yeast strain with a genomic deletion of *NSP1* was complemented by a *CEN URA3* plasmid that allowed expression of Nsp1p without fsFG repeats (nsp1ΔfsFG). The FOA test showed that this *URA3* plasmid could be lost, provided wild-type Nsp1p or Nsp1p with F→Y mutated repeats was expressed from the *CEN LEU2* plasmid. The strain expressing Nsp1p with F→S mutated repeats was not viable on FOA, i.e., it died upon loss of the nsp1ΔfsFG-encoding *URA3* plasmid.

inter-repeat contacts between phenylalanines caused the gelling of the wild-type Nsp1 fsFG-repeat domain.

By fluorescence recovery after photobleaching (FRAP), we next established that a fluorescently labeled wild-type Nsp1 fsFG-repeat domain was nearly immobile within the wild-type fsFG-repeat hydrogel (Fig. 2A). In contrast, a fluorescently labeled F→S mutated repeat domain showed no interaction and diffused freely within the wild-type gel (Fig. 2A), which confirmed that the phenyl groups act as mediators of the interactions.

As a next step, we probed heterotypic interactions between the FXFG and GLFG repeats and found that the GLFG-repeat domains from Nup49p, Nup57p, Nup145p, and Nup116p became firmly incorporated into the Nsp1 fsFG-repeat hydrogel (Fig. 2B). Considering that FG, FXFG, and GLFG repeats account for ~12% of the total NPC mass (*15*), that all repeat types tested so far could engage into inter-repeat interactions (Fig. 1 and Fig. 2B), and that their estimated local concentration at NPCs of ~100 mg/ml (*7*) exceeds the critical concentration for gelling (~8 mg gel per milliliter), we propose that extensive regions of the NPC and possibly the entire permeability barrier are organized in such a hydrogel structure.

Having established that the wild-type Nsp1 repeats formed a hydrogel in vitro, while the F→S mutated repeats did not, we wanted to elucidate the effect of this mutation on NPC function in vivo. Because of redundancy, none of the individual FXFG-repeat or GLFG-repeat domains is essential for NPC function in the yeast *Saccharomyces cerevisiae*, and only combined deletions are lethal (*19*). We thus chose genetic backgrounds where the Nsp1 fsFG-repeat domain is essential for viability (*19*) and found that full-length Nsp1p with an F→S mutated repeat domain ("Nsp1p F→S") was unable to substitute for wild-type Nsp1p (Fig. 3A and fig. S3).

We then repeated the experiment in a wild-type genetic background, where the fsFG repeats of Nsp1p are nonessential (*19*, *20*). Here, expression of the Nsp1 rod domain (which lacks the fsFG-repeat domain) was sufficient to rescue the lethal chromosomal *NSP1* deletion (*20*) (Fig. 3B). As expected, the lethal phenotype of the complete *NSP1* deletion was also rescued by expressing wild-type full-length Nsp1p. In contrast, Nsp1p with F→S mutated repeats failed to complement the *NSP1* deletion (Fig. 3B), even though the mutant protein was expressed, stable (fig. S4), and able to bind its interaction partners Nup49p and Nup57p (*21*) (fig. S5), as well as to assemble into NPCs (fig. S6). Thus, although deletion of the fsFG repeats from Nsp1p was tolerated, replacement of all copies of Nsp1p in NPCs by the F→S mutated version was lethal. In other words, the permeability barrier can tolerate permuting the anchoring points for FG repeats at the NPC

structure (*22*), as well as a significant reduction in mass (*19*), but not a major change toward a more hydrophilic composition.

The F→S mutated Nsp1 repeats failed to form a hydrogel (Figs. 1 and 2) as well as to bind NTRs (Fig. 4A). To distinguish which of these failures might cause the described lethal effect in vivo, we designed a less drastic mutant, where the phenylalanines (F) within the Nsp1 fsFG-repeat domain had been mutated to tyrosines (Y). The F→Y mutated repeats also failed to bind NTRs (Fig. 4A), probably because the additional OH group at the phenyl ring cannot be accommodated into the FXFG binding pockets of the NTRs (*8*, *9*, *23*, *24*). Remarkably, *nsp1* with F→Y mutated repeats fully complemented the *NSP1* deletion in a wild-type background (Fig. 3B) and even showed complementation in several of the genetic backgrounds, where the fsFG repeats of Nsp1p are essential (Fig. 3A and fig. S3). Thus, the F→Y mutation only abolished NTR binding, but apparently left other interactions intact that are essential, but redundant with other repeat domains.

Therefore, we tested the behavior of the F→Y mutant repeats inside the wild-type fsFG-repeat gel and observed by FRAP a low mobility of the F→Y mutant, i.e., a clear binding between F→Y mutant and wild-type Nsp1 repeats (Fig. 2A). Perhaps the underlying interactions are not purely hydrophobic, but involve also "aromatic" interactions: π stacking and certain geometries of T-shape interactions of the π-electron systems could occur, not only between two phenyl rings, but also between a phenyl and the hydroxyphenyl group of tyrosine, or even between two hydroxyphenyl groups. Indeed, the F→Y mutated repeat domain also formed a homotypic hydrogel (Fig. 4B).

FG-rich nucleoporin repeats are essential for viability (*18*) and engage in two known kinds of interactions: binding of NTRs (*6*, *25*) and hydrogel formation that arises through inter-repeat contacts (this study). If only the NTR binding, but not the inter-repeat contacts, represented essential functions, then the F→S and the F→Y mutant should both behave as if they were complete loss-of-function mutants. We observed, however, a different scenario, namely that the F→Y mutant could rescue, in certain genetic backgrounds, an otherwise lethal deletion of FG repeats. The simplest explanation for these data are that inter-repeat contacts and, hence, hydrogel formation are also required for NPC function and viability.

How could NTRs cross a permeability barrier containing FG repeats that are F→Y mutated and, hence, unable to bind NTRs? NTRs should still be able to locally dissolve the reversible Phe-Tyr cross-links by binding the "wild-type side." However, they should fail to break Tyr-Tyr contacts, which, in turn, could explain why in some genetic backgrounds the mutation is not tolerated (fig. S3).

An analogous consideration could explain also a puzzling set of previous findings: Even though NTRs are probably all optimized to cross the permeability barrier rapidly, they differ in their affinity for the different types of FG repeats (*26*), and they are differently affected by genetic deletions of individual FG-repeat domains (*19*). NTRs should indeed have problems penetrating homotypic hydrogels of those FG repeats to which these NTRs bind too weakly. Mixed gels formed by heterotypic interactions, however, should pose less of a problem and should constitute a more robust system, simply because an NTR could locally dissolve the hydrophobic contact between two different repeat units by binding to any of them.

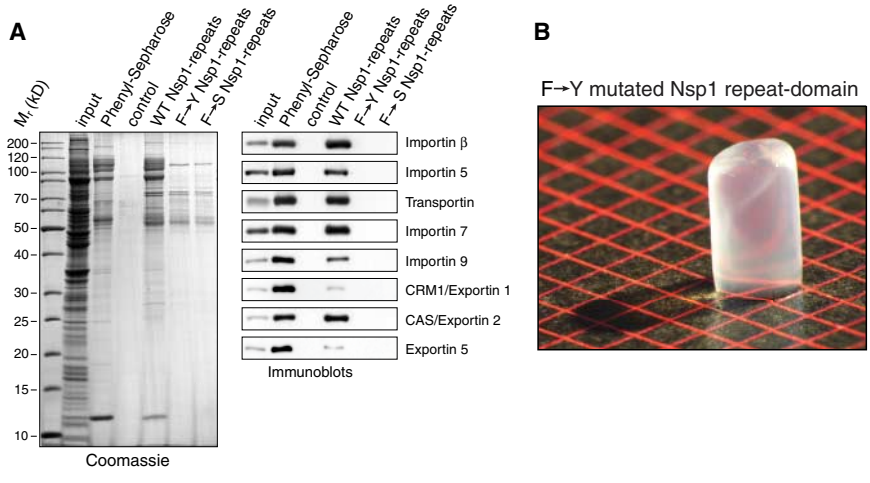

Fig. 4. (**A**) Mutation of the phenylalanines within the Nsp1-repeat domain to serines or tyrosines prevents binding of NTRs. A hypotonic extract from human HeLa cells was bound to indicated forms of immobilized Nsp1-repeat domains. Bound fractions were analyzed by SDS–polyacrylamide electrophoresis (SDS-PAGE), followed by either Coomassie staining or immunoblotting with antibodies specific for indicated importins and exportins. Phenyl-Sepharose served as positive control for NTR binding (*12*). Importins and exportins typically migrate between 90 and 135 kD, NTF2 at 12 kD. Nsp1 repeats were covalently bound via an engineered C-terminal cysteine to iodoacetamide-Sepharose 4B. No protein had been coupled to the negative control beads. Nonreacted iodoacetamide groups had been quenched with β-mercaptoethanol. Further tests revealed that yeast NTRs such as importin β (Kap95p) or Ntf2p behaved as the human ones. (**B**) F→Y mutated Nsp1-repeat domains also form a homotypic hydrogel. F→Y mutated repeat domains (400 μM) were treated as the wild-type repeat domain in Fig. 1; however, in order to avoid oxidation of the tyrosines, the handling was under argon, and β-mercaptoethanol (50 mM) was added as a scavenger. The resulting hydrogel had elastic properties similar to those of the wild-type fsFG-repeat gel, although it was not as transparent and, thus, was less homogeneous.

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14. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid. In FXFG repeats, X is preferentially S, T, G, A, or N.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/314/5800/815/DC1
Materials and Methods
Figs. S1 to S6
References

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