Systematic analysis of barrier-forming FG hydrogels from *Xenopus* nuclear pore complexes

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Nuclear pore complexes (NPCs) control the traffic between cell nucleus and cytoplasm. While facilitating translocation of nuclear transport receptors (NTRs) and NTR-cargo complexes, they suppress passive passage of macromolecules \( \geq 30 \text{kDa} \). Previously, we reconstituted the NPC barrier as hydrogels comprising *S. cerevisiae* FG domains. We now studied FG domains from 10 *Xenopus* nucleoporins and found that all of them form hydrogels. Related domains with low FG motif density also substantially contribute to the NPC’s hydrogel mass. We characterized all these hydrogels and observed the strictest sieving effect for the Nup98-derived hydrogel. It fully blocks entry of GFP-sized inert objects, permits facilitated entry of the small NTR NTF2, but arrests importin β-type NTRs at its surface. O-GlcNAc modification of the Nup98 FG domain prevented this arrest and allowed also large NTR-cargo complexes to enter. Solid-state NMR spectroscopy revealed that the O-GlcNAc-modified Nup98 gel lacks amyloid-like β-structures that dominate the rigid regions in the *S. cerevisiae* Nsp1 FG hydrogel. This suggests that FG hydrogels can assemble through different structural principles and yet acquire the same NPC-like permeability.

The EMBO Journal (2013) 32, 204–218. doi:10.1038/emboj.2012.302; Published online 30 November 2012
Subject Categories: membranes & transport; proteins
Keywords: exportin; FG hydrogel; importin; nuclear pore complex; O-glycosylation

Introduction

Nuclear pore complexes (NPCs) connect the nuclear interior with the cytoplasm and control the exchange between the two compartments. They are built from nucleoporins (Nups; reviewed in Brohawn et al., 2009 and Hetzer and Wente, 2009) and are equipped with a sieve-like barrier that is freely permeable for small molecules, but becomes increasingly restrictive as inert mobile species approach or exceed 5 nm in diameter (Mohr et al., 2009). This limit corresponds to a mass of \( \approx 30 \text{kDa} \) for spherical proteins. Nuclear transport receptors (NTRs) can overcome this size limit and transfer even very large cargoes across NPCs. Such ‘facilitated translocation’ is used to supply nuclei with proteins and the cytoplasm with nuclear products such as ribosomes or mRNAs.

Importin β-type receptors represent the largest class of NTRs and include importins as well as exportins (reviewed in Fried and Kutay, 2003). The RanGTP gradient model (Görlich et al., 1996; Izaurralde et al., 1997) provides an explanation for the directionality of the corresponding transport pathways: Importins recruit cargo at low RanGTP levels in the cytoplasm, release their cargo upon RanGTP-binding into the nucleus, and return RanGTP-bound to the cytoplasm. There, GTP hydrolysis triggers dissociation of the importin·RanGTP complex and allows the importin to bind a next cargo molecule. A prototypical example is importin β itself (Impβ; Chi et al., 1995; Görlich et al., 1995; Imamoto et al., 1995). It binds import cargo either directly or through an adapter such as importin α (Impα).

Exportins recruit cargoes inside nuclei together with RanGTP (reviewed by Güttler and Görlich, 2011). Examples are CAS/exportin 2, which exports Impz, or CRM1 that exports many substrates, including ribosomal subunits. Another NTR category is exemplified by the homodimeric RanGDP importer NTF2, which is structurally unrelated to Impβ (Bullock et al., 1996; Ribbeck et al., 1998).

Facilitated translocation is remarkably efficient. A single NPC can accommodate \( \approx 1000 \) transport events per second (Ribbeck and Görlich, 2001) and needs as little as \( \approx 10 \text{ ms} \) to translocate an Impβ·cargo complex from the cytoplasmic to the nuclear side of the pore (Yang et al., 2004; Kubitscheck et al., 2005). These numbers imply that NPCs are able to translocate numerous objects simultaneously. Facilitated translocation is not directly coupled to an input of metabolic energy (Schwoebel et al., 1998; Englmeier et al., 1999; Ribbeck et al., 1999). Instead, it is primarily based on a higher permeability of NPCs for NTRs as compared to inert molecules; and it is the cooperation of importins or exportins with the RanGTPase system that renders NPCs into active and highly efficient cargo pumps (Görlich et al., 2003).

Non-globular nucleoporin FG domains are central for the function of the NPC barrier (Hurt, 1988; Strawn et al., 2004; Patel et al., 2007; Hülsmann et al., 2012). Their name derives from the frequent occurrence of Phenylalanine-Glycine (FG) dipeptides. They are typically several 100 residues long and comprise up to 50 FG, FxFG or GLFG motifs separated by spacers of rather variable sequence. For the sake of simplicity, these motifs are here collectively referred to as FG motifs. Metazoan NPCs contain several FG domains with multiple serine and threonine residues being modified with single O-linked β-N-acetylglucosamine (O-GlcNAc) moieties (Finlay et al., 1987; Hanover et al., 1987; Holt et al., 1987; Onischenko et al., 2005). The function of this modification of FG domains is still unknown.
FG motifs bind NTRs during facilitated translocation (Iovine et al., 1995; Rexach and Blobel, 1995; Bayliss et al., 1999; Morrison et al., 2003). How the FG-NTR interaction promotes NPC passage is, however, surprisingly difficult to answer. In fact, one would expect that a mere binding causes retention of NTRs and therefore slows down their passage. Also, such a simple binding cannot explain how inert material is excluded from passage.

To resolve this paradox, several models have been proposed. The ‘virtual gate’ model (Rout et al., 2003) assumes that brushes of FG domains repel inert material and that NTRs overcome this barrier by binding to the domains. The ‘selective phase’ model (Ribbeck and Görlich, 2001) attributes an additional essential property to the barrier-forming FG domains, namely cohesiveness. It assumes that cohesive FG domains multivalently interact with each other and form a sieve-like hydrogel, the selective phase. The mesh size sets an upper size limit for unhindered NPC passage of inert material. According to the model, binding of an NTR to an FG motif disengages the corresponding repeat-repeat contact (Ribbeck and Görlich, 2001; Kustanovich and Rabin, 2004). NTRs can thus ‘melt’ through a dense FG hydrogel. The model is supported by the observations that the yeast S. cerevisiae (sc) contains several highly cohesive FG domains (Frey et al., 2006; Frey and Görlich, 2007, 2009; Patel et al., 2007; Yamada et al., 2010), and that the FG domains from scNspl, the fused FG domains from scNup49p and scNup57p, or the human Nup153 FG domain indeed form FG hydrogels that display permeability properties very similar to authentic NPCs (Frey et al., 2006; Frey and Görlich, 2007, 2009; Milles and Lemke, 2011). That is, they exclude inert macromolecules >5 nm, but allow an up to 20 000 times faster influx of the same macromolecule bound to an NTR. The rates of intragel diffusion are also consistent with the observed NPC transit times being in the 10 ms range (Yang et al., 2004; Kubitscheck et al., 2005).

Analysis of the scNspl FG domain revealed that hydrophobic as well as hydrophilic interactions contribute to inter repeat cohesion (Frey et al., 2006; Ader et al., 2010). Mutating the hydrophobic residues (which are mainly phenylalanines) to serines abolishes gel formation. The N-terminal part of the domain (residues 1–175) is extremely cohesive and comprises inter FG spacers that are very rich in Asn, Gln and Thr. Solid-state NMR (ssNMR) revealed that these spacers engage in interchain β-sheets (Ader et al., 2010)—similar to those found in NO2-rich amyloids (Eanes and Glenner, 1968; Balbirnie et al., 2001). NO2-rich spacers characterize also the very cohesive GLFG domains from S. cerevisiae Nup100p or Nup116p (Yamada et al., 2010; Halfmann et al., 2012). The C-terminal part of the scNspl FG domain (residues 274–601) is rather non-cohesive, apparently because the inter FG spacers are dominated by charged residues that counteract inter FG cohesion (Ader et al., 2010; Yamada et al., 2010). It is, however, adhesive in the sense that it (weakly) binds to and greatly improves the selectivity of the NO2-rich scNspl1–175 FG hydrogel (Ader et al., 2010).

If inter FG repeat cohesion was generally relevant for NPC function, then also eukaryotes other than S. cerevisiae should rely on this principle. We decided to test this prediction exhaustively for the Nups from the frog Xenopus and noticed similarities but also remarkable differences to yeast. Xenopus lacks NO2-rich inter FG spacers; nevertheless, we found all major FG domains to be cohesive and to form FG hydrogels.

We also characterized additional nucleoporin ‘FG-like’ domains representing low complexity sequences that are related to FG domains but contain only few or no FG motifs. We observed that they form hydrogels as well and might therefore substantially contribute to the NPC’s cohesive mass. Of all gels analysed, the O-GlcNAc-modified Nup98 FG hydrogel was the most selective, that is, it discriminated best between inert macromolecules and the tested spectrum of NTRs and NTR–cargo complexes. ssNMR revealed that this type of gel is devoid of β-structures, which is in striking contrast to the already-mentioned scNspl FG hydrogel. This suggests that FG hydrogel-based barriers can assemble through different structural principles and yet gain a very similar, NPC-like, selectivity.

**Results**

**All Xenopus FG domains are cohesive and form FG hydrogels**

Amyloid-like, intermolecular β-sheets between NO2-rich sequences are dominating structural elements in the hydrogel formed by the FG domain from S. cerevisiae Nsp1p (Ader et al., 2010; Petri et al., 2012). This coincides with a high NO2 content (≈27%; Table I) of the most cohesive part of this domain (scNspl1–175) and of other highly cohesive yeast FG domains, such as those from scNup100p or scNup116p (Ader et al., 2010; Yamada et al., 2010; Halfmann et al., 2012). This could suggest that NO2-rich inter FG spacers are required for any hydrogel-based NPC permeability barrier. The NO2 content of Xenopus FG domains is, however, low (average: 9.6%, range: 5.8–12%; Table I) and close to the average of globular proteins from the PDB database (7.7%). This raised the questions if Xenopus FG domains are cohesive and if Xenopus NPCs actually rely on FG hydrogels for maintaining their permeability barriers.

To address this issue, we analysed a comprehensive set of Xenopus FG domains (Supplementary Figure S1). We cloned and expressed FG domains from Nup358, Nup214, Nup153, Nup98, Nup62, Nup58, Nup54, Nup50, Pom121 and CG1 in Escherichia coli, purified them under conditions that prevent any premature cohesion, and concentrated them by lyophilization. We then dissolved the proteins in an aqueous buffer at a concentration of ≈200 mg/ml, which approximates the expected FG repeat concentration in authentic NPCs. Strikingly, none of those protein solutions remained liquid. Instead, all FG domains formed gels (see Figures 1A, 2B and 3A for a selection of gel photographs). In the case of the Pom121 and Nup62 FG domains, the solutions jellified so rapidly that we could not dissolve the lyophilized proteins completely. We therefore retarded gelation by the addition of 0.5–2 M guanidinium hydrochloride.

**Xenopus FG hydrogels deviate from the paradigm of NO2-rich amyloids**

Of all the Xenopus FG domains, the one derived from Nup98 has still the highest NO2 content (12%). We observed efficient hydrogel formation, however, also with a mutant lacking Asn and Gln altogether (Nup98 FG NO2 ≈ S; Figure 1A). It thus seems that Xenopus FG domains can form gels without the hydrophilic interaction potential of NO2-rich inter FG spacers. In this respect, it is remarkable that Xenopus FG domains are considerably more hydrophobic than the FG domains from yeast scNsplp or scNup100p (Table I). Thus, while inter FG
cohesion in *S. cerevisiae* relies on both, hydrophobic and hydrophilic interactions (Ader et al., 2010), hydrogel formation in vertebrates appears to have a stronger hydrophobic basis (see also below). It is also remarkable that even the most highly charged *Xenopus* FG domains, such as the combined Nup358 FG domains with 25% charged residues, formed a hydrogel (Table I and Figure 1A and C).

The Nup98 FG domain forms the FG hydrogel with the strictest sieving effect

In a next step, we explored the permeability properties of all 10 *Xenopus* FG hydrogels. Specifically, we tested their ability to restrict the passage of inert molecules and to allow facilitated translocation of NTRs. For that, we formed the gels on microscope slides, equilibrated them in assay buffer, added fluorescent probes to the buffer reservoir, and measured the entry of these probes into the hydrogels by confocal laser-scanning microscopy. The first set of probes included mCherry (Shaner et al., 2004) as an E26 kDa inert molecule and Alexa488-labelled NTF2 as a small (29 kDa) NTR (Figure 1B).

This probing revealed the Nup98 gel as the most selective barrier. The gel fully excluded mCherry, that is, the gel/buffer partition coefficient for mCherry was essentially zero. The Nup98 gel is thus more restrictive than authentic NPCs, which allow GFP-sized molecules to pass at clearly detectable

### Table I Composition of FG and FG-like domains from *Xenopus* and *S. cerevisiae*

| Repeat domain | Residues | FG dipeptides per 100 residues | % N+Q | % T | % D+E+K+R | Mean hydrophobicity |
|---------------|----------|--------------------------------|-------|-----|-----------|--------------------|
| *S. c.* Nsp1p FG | 1-175 | 6.8 | 26.7 | 15.9 | 2.3 | 0.15 |
| | 274-601 | 5.2 | 6.7 | 4.6 | 34.8 | –0.06 |
| *S. c.* Nup100p FG | 2-580 | 7.3 | 27.3 | 10.2 | 4.1 | 0.19 |
| *X.l.* Nup214 FG | 1615-2033 | 8.8 | 8.8 | 9.5 | 2.4 | 0.36 |
| FG-like-1 | 443-690 | 0.8 | 8.9 | 10.1 | 6.9 | 0.40 |
| FG-like-2 | 1220-1614 | 0.8 | 6.6 | 12.9 | 7.8 | 0.37 |
| *X.l.* Nup153 FG | 885-1525 | 5.0 | 8.4 | 10.9 | 9.7 | 0.30 |
| 885-1127 | 4.5 | 5.4 | 11.9 | 21.0 | 0.20 |
| 1128-1525 | 5.3 | 10.3 | 10.3 | 2.5 | 0.36 |
| *X.l.* Nup62 FG | 2-352 | 3.1 | 7.1 | 17.8 | 1.7 | 0.41 |
| *X.l.* Nup58 FG | 2-72, 511-598 | 6.9 | 7.5 | 15.0 | 4.4 | 0.34 |
| FG&FG-like | 2-259, 511-598 | 3.2 | 8.9 | 15.6 | 2.9 | 0.40 |
| *X.l.* Nup54 FG | 2-94 | 10.7 | 6.5 | 28.0 | 2.2 | 0.41 |
| FG&FG-like | 2-139 | 7.3 | 11.5 | 25.4 | 1.5 | 0.40 |
| *X.l.* Nup98 FG | 1-485 | 9.6 | 12.0 | 20.4 | 2.3 | 0.37 |
| *X.l.* Pom121 FG | 571-1050 | 1.7 | 10.0 | 15.0 | 7.9 | 0.37 |
| *X.l.* Nup50 FG | 68-285 | 3.7 | 7.4 | 9.8 | 12.9 | 0.29 |
| *X.l.* CG1 FG | 257-411 | 5.8 | 5.8 | 11.6 | 5.2 | 0.40 |
| *X.t.* Nup358 FG | Combined | 2.5 | 10.0 | 7.0 | 25.0 | 0.22 |
| | 1625-1837 | 2.4 | 12.2 | 6.1 | 24.4 | 0.19 |
| | 1967-2119 | 0.7 | 7.2 | 9.2 | 30.1 | 0.16 |
| | 2315-2431 | 3.4 | 8.6 | 6.8 | 19.7 | 0.30 |
| | 2572-2725 | 2.0 | 4.6 | 4.6 | 27.3 | 0.20 |
| PDB average | | 0.3 | 7.7 | 5.5 | 22.8 | 0.37 |

*S. c.*, *Saccharomyces cerevisiae*; *X. l.*, *Xenopus laevis*; *X. t.*, *Xenopus tropicalis*. The mean hydrophobicity was calculated according to the scale of Fauchere and Pliska (1983). The scale is based on partitioning of N-acetyl-amino-acid amides between 1-octanol and water at neutral pH; it ranges between +2.25 for polyW and –1.01 for polyR. The combined Nup358 FG domain is a fusion of nine FG subdomains comprising following residues: 1095–1 180, 1307–1345, 1374–1469, 1504–1528, 1558–1595, 1625–1837, 1967–21 19, 2315–2431 and 2572–2725.
rates (Mohr et al., 2009). Nevertheless, NTF2 was able to enter the Nup98 gel efficiently, reaching within 30 min a partition coefficient of nearly 1000. Importantly, NTF2 did not remain stuck at the gel surface, but moved deep into the gel, on average 10 μm during 30 min incubation. At such diffusion rate, it would take $E_{50}$ ms to cross a 50-nm thick NPC barrier. This is $E_{10}$-fold slower than expected for passing an authentic NPCs (Kubitscheck et al., 2005), but consistent with the observation that the Nup98 FG hydrogel forms an extraordinary tight barrier (see below).

Except for the Nup50 gel, we found that all other Xenopus FG hydrogels allowed a similarly efficient NTF2 influx (Figure 1C). These gels differed, however, clearly from the Nup98 FG (wt) hydrogel measured after 30 min by confocal laser-scanning microscopy. The far-red labelled hydrogel was detected after excitation at 633 nm, mCherry at 561 nm and Alexa488-labelled NTF2 at 488 nm. Arrow illustrates direction of influx. Concentration profiles of mCherry and NTF2 across the buffer/gel boundary are also shown. For normalization, free NTF2 and mCherry concentrations in the buffer had been set to 1. (C) Two-hour-influx of NTF2, mCherry and tCherry into 10 different Xenopus FG hydrogels (for sequences of used domains, see Supplementary Figure S1 and Supplementary Table S1). False-coloured fluorescent signals illustrate partitioning of mobile species between buffer and gels. Note that the isolated FG domains of Nup54 and Nup58 show non-specific binding to tCherry (see also Figure 4).
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Figure 2 Selectivity properties of Nup214 hydrogels. (A) Domain organization of Xenopus laevis Nup214. Red strokes represent FG motifs. Nup214 contains a canonical FG domain (‘FG’) as well as two FG-like domains. (B) Photographs of indicated hydrogels. (C) Simultaneous influx of NTF2 and mCherry or of Impl-IBB-GFP and tCherry into indicated hydrogels (2 h time points). Permeation assays were performed as in Figure 1. Note the deviating scaling of NTF2 and Imp-GFP signals for the FG-like hydrogels. (D) Influx of RanGTP-GFP-CRM1-NES export complex into Nup214 hydrogels as compared to Nup358 FG hydrogels (6 h time points). Influx of free GFP-CRM1 and of a CAS export complex is shown as controls.

hydrogels even showed an enrichment of our (otherwise) inert probes, indicating a propensity for non-specific binding. This illustrates an interesting point, namely that it is not trivial that a gel, which comprises unfolded and rather hydrophobic domains, is well passivated against non-selective interactions with other proteins. We will come back to this important issue later on. Before, however, we will describe the FG hydrogels derived from Nup214, Nup358, Nup153, the Nup62-54-58 complex, and Nup98 in greater detail.

Nup214 contains three gel-forming FG subdomains

Nup214 is localized to the cytoplasmic side of NPCs, where it forms a subcomplex with Nup62 and Nup88 (Kraemer et al., 1994; Bastos et al., 1997; Fornerod et al., 1997). Nup214 comprises two globular domains: an N-terminal beta-propeller binding the mRNA-export factor DDX19/Dbp5 (Weirich et al., 2004; von Moeller et al., 2009) as well as a coiled-coil domain, which presumably accounts for the interaction with Nup62 and Nup88, and which anchors the complex to the scaffold of the NPC (Bernad et al., 2006). Nup214 contains at its C-terminus the already-mentioned canonical FG domain, which comprises 419 residues, features a high density of FG motifs (one motif per 12 residues; Figure 2A; Supplementary Figure S1; Table I), and displays a similar degree of sequence conservation as the beta-propeller or the central coil-coiled region. As mentioned above, this canonical FG domain forms an FG hydrogel (Figures 1C and 2B).

Nup214 contains two additional domains with a predicted non-globular structure, namely a 248 residues long stretch connecting beta-propeller and coiled-coil as well as a 395-
residue long region preceding the canonical FG domain (Figure 2A). They only show a low level of inter species sequence conservation. Their low complexity amino-acid composition resembles canonical FG domains, but their FG motif density is very low (one motif per \( \approx 125 \) residues, Table I). Nevertheless, these two ‘FG-like’ domains also formed hydrogels (Figure 2B). This implies that—assuming a copy number of eight per NPC—Nup214 alone may contribute nearly 1 MDa of hydrogel mass to an NPC.

**The Nup214 FG hydrogel selectively stalls CRM1 export complexes**

NFT2 and the Imp\(\beta\)-IBB-GFP complex (\( \approx 120 \) kDa) entered all three Nup214-derived gels rapidly (Figure 2C). The canonical FG domain (which has the highest FG density) displayed, however, slower intragel diffusion and stronger NTR accumulation near the buffer-gel boundary. The diffusion of NFT2 and the Imp\(\beta\)-cargo complex inside Nup214 FG-like gels was faster than in other *Xenopus* FG hydrogels tested. Remarkably, Nup214 FG-like-2 hydrogel suppressed influx of tCherry even more efficiently than the canonical domain, resulting in a gel/buffer partition coefficient for tCherry being as low as 0.2.

It is known that loading of an FG hydrogel with NTRs counteracts the entry of inert macromolecules (Frey and Görlich, 2009). This effect is probably based on volume exclusion and on NTRs introducing additional cross-links into the gel. This effect was also clearly evident for the canonical Nup214 FG hydrogel, but not for the FG-like ones, which probably reflects the fact that the canonical FG domain retains NTRs more strongly (Figure 2C, left column).

Previous reports indicated that Nup214 acts as a terminal binding site for CRM1 export complexes (Hutten and Kehlenbach, 2006). The interaction between such a RanGTP-CRM1-NESS export complex with the gel of the canonical Nup214 FG domain was indeed remarkable. The complex initially bound very rapidly to the gel, but showed hardly any movement away from the entry site (Figure 2D). This ‘traffic jam’ eventually also blocked further influx of complexes into the gel. The stalling at the buffer-gel boundary was very specific and was observed neither for free CRM1 nor for the RanGTP-CAS-Impz export complex (Figure 2D). The selective retardation of a RanGTP-CRM1-cargo complex depended also on the choice of hydrogel (Supplementary Figure S2). In fact, the only other FG hydrogel that showed a similar effect was the one derived from Nup358/RanBP2, though retention of the RanGTP-CRM1-cargo complex was here not as strict (Figure 2D). Given that Nup358 and Nup214 probably are neighbours on the farthest cytoplasmic side of the NPC (Bernad et al., 2004), this is perhaps not just a pure coincidence.

The stalling indicates a very slow release of the NTR complex from specialized FG binding sites. In authentic NPCs, this could serve a substrate-channelling mechanism, where RanGTP-CRM1-cargo complexes are directly presented to the nearby Nup358/RanBP2-RanGAP system until GTP hydrolysis has occurred and the cargo has been released from the exportin into the cytoplasm. Such substrate channelling could improve the efficiency of these transport cycles, because it would prevent a backflow of RanGTP-CRM1-cargo complexes to the nucleus, and thus make export irreversible even before GTP hydrolysis has occurred. Also, this mechanism could allow cargo-free CRM1 to return to the nucleus without an intervening release into the bulk cytoplasm.

**Hydrogels derived from highly charged FG domains**

The Nup358 FG hydrogel is remarkable for its high content of charged residues (Table I), which is a feature that negatively correlates with the gel-forming propensity of *S. cerevisiae* FG

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**Figure 3** FG subdomains of *Xenopus tropicalis* Nup153 form highly selective hydrogels. (A) Photographs of indicated gels. (B) Permeability properties of Nup153-derived FG hydrogels were analysed as in Figure 2C (2 h time points).
domains (Ader et al., 2010; Yamada et al., 2010). The FG content of Nup358 is scattered over nine individual subdomains (Supplementary Figure S1), and it was striking to observe that all four tested individual subdomains (representing the longest and the most charged FG subdomains) as well as the combination of all nine FG subdomains formed hydrogels (Figure 1A). Moreover, the gel derived from the combined Nup358 FG domains excluded even our smallest inert permeation probe (mCherry) rather efficiently (Figure 1C), which points to an efficient formation of a small-meshed barrier.

Nup153 is asymmetrically localized to the nuclear side of NPCs (Sukegawa and Blobel, 1993). Its FG domain (Nup153\textsuperscript{885–1525}) comprises a total of 641 residues and 32 FG motifs. It can be divided into two parts of rather different amino-acid composition. Nup153\textsuperscript{1128–1525} contains only 2.5\% charged residues, while Nup153\textsuperscript{885–1127} contains 21\% (Table I).

Nevertheless, we observed that the complete Nup153 FG domain and both FG subdomains formed hydrogels (Figure 3A) that efficiently excluded the tetrameric Cherry marker and showed an NPC-like permeability (Figure 3B). These results suggest that the apparent anti-cohesion effect of the charged residues might be compensated by unusually hydrophobic FG motifs (such as FLFG or FIFG), but possibly also by the formation of salt bridges between acidic and basic side chains.

**Synergy between the gel-forming domains of the Nup62·54·58 complex**

The Nup62·54·58 subcomplex (Finlay et al., 1991; Guan et al., 1995) is localized in the central part of NPCs (Grote et al., 1995). *Xenopus laevis* Nup62, Nup54 and Nup58 contain N-terminal FG domains, Nup58 in addition also C-terminal FG repeats. Nup54 and Nup58 further comprise FG-like domains that flank the FG domains; these resemble in amino-acid composition a canonical FG domain, but lack bona fide FG motifs (Figure 4A; Supplementary Figure S1). Nevertheless, we found by binding assays and peptide arrays that these FG-like domains interact with NTRs to a significant extent, possibly through alternative hydrophobic motifs (Supplementary Figure S3). We regard them therefore as further examples of FG-like domains.

The analysis of hydrogels, which include only the canonical Nup54 or Nup58 FG domains, revealed a surprisingly high permeability for and considerable non-specific binding of mCherry and tCherry (Figure 1C). Gels that combined each of the FG and FG-like domains from either Nup54 or Nup58 showed a reduced but still persisting non-specific binding of inert probes (Figure 4B; Supplementary Figure S4). The Nup62 FG gel was already more selective and clearly suppressed entry of mCherry and tCherry, resulting in partition coefficients of 0.7 and 0.6, respectively.

As a next step, we fused FG and FG-like domains of all three Nups in tandem, expecting the corresponding composite gel to behave like an average of the three individual domains. We, however, observed a striking synergy between these domains. The fused domains now formed a gel that completely excluded tCherry and efficiently suppressed entry of mCherry (partition coefficient \(\approx 0.2\); Figure 4B). It is also remarkable that the exquisite barrier performance was lost when the FG-like domains of Nup54\textsuperscript{95–139} and Nup58\textsuperscript{23–259} had been omitted from the fusion.

Figure 4 Permeability properties of hydrogels derived from the *Xenopus laevis* Nup62 complex. (A) Domain organizations of Nup54, Nup58 and Nup62. (B) Hydrogels obtained from indicated nucleoporin fragments or domain fusions were probed with NTF2, mCherry or tCherry as in Figure 1B (30 min time points). Please note that the Nup54 and Nup58 hydrogels here also include the FG-like domains, which had been omitted in Figure 1C.
A pure Nup98 FG hydrogel is too restrictive for passage of large NTRs

Nup98 shows by post-embedding immuno-EM a very central localization within the central translocation channel of the NPC (Krull et al., 2004). Thus, it would be ideally positioned to control passage through the pore, and indeed, Nup98 is essential for maintaining a functional permeability barrier in Xenopus NPCs (Hülsmann et al., 2012). It probably occurs in three copies per asymmetric unit, two being anchored through Nup96 to the Nup107–160 subcomplexes (Vasu et al., 2001; Hodel et al., 2002), and one associating with the Nup214·Nup98·Nup62 subcomplex (Griffis et al., 2003). Xenopus Nup98 has an N-terminal FG domain comprising 485 residues (Supplementary Figure S1) with an embedded GLEBS domain that interacts with the mRNA export mediator Gle2p/Rae1 (Pritchard et al., 2013^4^).

The Nup98 FG domain has the highest density of FG motives of all Xenopus FG domains, namely one motif per \( \approx 10 \) residues (Table I). The derived Nup98 FG hydrogel appeared an ideally selective barrier when probed with NTF2 and mCherry (Figure 1B): It completely blocked entry of mCherry, but allowed an at least 100 000-fold faster influx of NTF2. Closer inspection revealed, however, that the extreme tightness towards inert probes had an unexpected side effect, namely that all tested importins and exportins bound only to the gel surface and failed to visibly penetrate into the gel (shown for Imp\( \beta \)-cargo complexes in Figure 5C). Such over-tight barrier in authentic NPCs would impede NTR-mediated nuclear transport of small proteins and even more so the transit of large (e.g., ribosome-sized) cargoes. We therefore considered several mechanisms that might confer a higher permeability to a Nup98 FG gel-based barrier.

A first possibility is that the local FG domain concentration of the in vitro-formed hydrogel is higher than in authentic NPCs, and so lowering the concentration should increase the permeability. The FG domains of, for example, scNsp1p or Nup214 form homogeneous gels over a wide range of concentrations (10–200 mg/ml). The Nup98 FG domain also forms a homogeneous gel at 200 mg/ml. When we attempted, however, to lower the concentration of the Nup98 FG domain, we observed a striking phase separation into a protein-rich and a protein-poor phase. The microscopic appearance of the resulting gels then resembled a ‘holey Swiss cheese’ (Figure 5A). It should be noted that shrinking of the gel drop was evident also for a starting concentration of 200 mg/ml, though in this case, shrinking reduced the volume of the entire gel drop and did not disintegrate the gel. The super-tight Nup98 gel therefore had a protein concentration probably exceeding 200 mg/ml.

The effect can be explained by the concept of a saturated FG hydrogel (Frey and Görlich, 2007), which assumes that all cohesive units of an FG domain can find sufficiently close binding partners only when their concentration exceeds a certain saturation limit. Undersaturated gels should contain a significant share of unpaired cohesive units and hence have a larger mesh size than the equivalent saturated gel. This
state appears, however, instable in an undersaturated Nup98 gel. Here, it appears that the number of cohesive interactions is maximized by shrinking the gel volume until the saturation limit is reached. This shrinking then also leaves protein-poor areas behind (either by an increased buffer volume or as ‘holes in the cheese’). This effect qualifies the Nup98 FG domain as ‘highly cohesive’. The effect precluded analysing the permeability properties of a macroscopic, undersaturated Nup98 gel. It is well possible, however, that the anchoring of the domain to the rigid scaffold of an NPC would counteract such phase separation.

**The Nup98 hydrogels respond to O-GlcNAc modifications**

Another consideration is that native Nup98 is heavily modified by O-linked β-N-acetylglucosamines (O-GlcNAc; Powers et al., 1995), which are introduced by the O-GlcNAc transferase (OGT) that transfers GlcNAc moieties from UDP-GlcNAc to serines and threonines of numerous target proteins (Haltiwanger et al., 1990; Lubas and Hanover, 2000). To study the effects of this modification, we established a preparative method of enzymatically modifying the Nup98 FG domain. The reaction resulted in an electrophoretic size shift of ≈9 kDa (Figure 5D, lanes 2 and 3). Mass spectrometry revealed 46 potential modification sites, namely 29 threonines and 17 serines that occurred in clusters of up to 5 consecutively modified residues (Figure 5B). Otherwise, the modifications were distributed over the entire length of the FG domain. The *in vitro* reconstituted glycosylation reaction can be considered as a formal proof for Nup98 being an OGT substrate.

O-GlcNAc modification drastically changed the permeability properties of the Nup98 hydrogel. The glycosylated hydrogel allowed rapid entry and intragel diffusion not only of NTF2, but also of larger NTR cargo complexes, such as Impβ-IBB-GFP (≈120 kDa) or an ≈480 kDa tetramerized Impβ-IBB-zsGreen complex (Figure 5C). Concomitantly, the permeability towards mCherry increased. Nevertheless, the O-GlcNAc-modified Nup98 gel fully blocked entry of tCherry (Figure 5C). The combination of all these criteria qualifies this gel as the most selective of all the hydrogels derived from *Xenopus* FG domains.

Direct binding assays indicated that the O-GlcNAc modification slightly decreased the strength of the NTR-FG interaction (Figure 5D). Nevertheless, we observed that the O-GlcNAc-modified Nup98 gels absorbed NTRs far more efficiently than the non-modified Nup98 gel. This once again indicates that NTRs experience difficulties in breaking inter repeat contacts of the non-glycosylated gel and therefore enter such gel less efficiently.

**The O-GlcNAc-modified Nup98 FG hydrogel reproduces sensitivity towards the NPC inhibitor WGA**

Wheat germ agglutinin (WGA) is a tetravalent lectin recognizing GlcNAc moieties (Nagata and Burger, 1974). It binds O-GlcNAc-modified Nups (Finlay et al., 1987; Hanover et al., 1987; Holt et al., 1987) and blocks passage through vertebrate NPCs (Finlay et al., 1987). Initially, it was thought to be a selective inhibitor of facilitated translocation. Subsequent studies, however, revealed that the extent of inhibition does not depend on the mode of NPC passage, but on the size of the translocating species (Mohr et al., 2009). WGA blocks NPC passage of typical Impβ-cargo complexes (mass >100 kDa), but also strongly inhibits facilitated translocation of NTF2 or passive passage of GFP (mass ≈30 kDa).

If the glycosylated Nup98 gel would make up the selectivity filter of NPCs, then one would expect that the gel itself shows a similar sensitivity towards WGA. In line with this predic-
Structural characterization of Nup98 FG hydrogels

As a next step, we analysed uniformly $^{13}$C,$^{15}$N-labelled variants of the Nup98 gel by several ssNMR methods (Andronesi et al., 2005). Through-space magnetization transfer (cross-polarization; CP) allows probing of very rigid regions that are stable for at least several milliseconds. Through-bond magnetization (Insensitive Nuclei Enhanced by Polarization Transfer; INEPT) detects highly mobile regions with motions in the nanoseconds regime. The two techniques are less sensitive for protein segments moving in a time frame of microseconds to milliseconds. However, this gap can be closed by direct excitation, which detects signals independently of mobility.

The previously characterized scNsp1 FG hydrogel yielded strong CP signals that can be attributed to the highly cohesive scNsp1$^{11–175}$ FG subdomain (forming the rigid parts of the gel) as well as strong INEPT signals that predominantly stem from the non-cohesive and highly charged scNsp1$^{1274–601}$ FG subdomain (Ader et al., 2010; Figure 7A and B). Analysis of the non-glycosylated Nup98 gel revealed very strong CP signals and very low INEPT signals. This is consistent with our observation that this type of gel is so rigid that importin β-type NTRs fail to enter. The almost complete lack of INEPT-visible mobile elements further suggests that the Nup98 FG domain is cohesive along its entire sequence. This assumption is supported by two-dimensional $^{13}$C,$^{13}$C experiments (Figure 7C), indicating that all amino acid-specific cross-peaks detected by direct excitation also appear in the CP spectrum. It therefore appears that all NMR-resolved parts of the Nup98 FG domain reside in low mobility regions of the gel.

Based on these spectra, we conducted a qualitative analysis of secondary structure using secondary chemical-shift information (Ader et al., 2010). This analysis suggested that the rigid components of non-glycosylated Nup98 gel are not per se associated with the formation of β-strand structures as previously seen for the most rigid (NQ-rich amyloid-like) regions in scNsp1 FG hydrogel. This notion is based on the facts that the relevant cross-peaks in the Nup98 FG hydrogel are broader (Figure 7C, see below) and extend into spectral regions typically not associated with β-strands (Luca et al., 2001).

Figure 7 Solid-state NMR analysis of scNsp1- and Nup98-derived FG hydrogels. (A) 1D cross-polarization (CP) spectra probing very rigid parts of the scNsp1 FG hydrogel (black curve), a non-glycosylated Nup98 FG hydrogel (blue) and a O-GlcNAc-modified Nup98 gel (red). (B) INEPT spectra probing highly mobile regions of the same gels (colour coding as in A). (C) 2D CP spectra probing very rigid parts of the scNsp1 gel (black) and the non-glycosylated Nup98 FG hydrogel (blue). Indicated correlations reflect inter-atomic polarization transfer among aliphatic carbon positions (denoted by α-γ) for selected residue types. Resonance frequencies encode information about the identity of residues and their backbone conformations. (D) 2D direct excitation spectra comparing non-glycosylated (blue) and O-GlcNAc-modified (red) Nup98 FG hydrogels. Resonance peaks indicating β-strand conformation are labelled in green, random coil conformations are coloured in mustard yellow.
Upon glycosylation, CP signal intensities of the Nup98 gel dropped dramatically (Figure 7A), illustrating that the O-GlcNAc modification makes the gel less rigid. Interestingly, we observed only a tiny concomitant increase in the INEPT signal (Figure 7B), suggesting that the glycosylated gel was still largely devoid of fully mobile regions and that the O-GlcNAc-modified Nup98 FG domain remained cohesive along its entire sequence.

2D data sets of the glycosylated Nup98 gel could only be obtained using direct excitation, which omits the preparatory CP step and which is therefore rather insensitive towards protein mobility. The spectra then showed the expected appearance of signals specific for O-GlcNAc-modified threonines and serines (in Figure 7D annotated with ‘glyc’). Their intensities are consistent with the degree of modification measured by mass spectrometry. The fact that these glycosylation-specific signals produced the strongest cross-correlations in 2D spectra following direct excitation suggests that the modifying groups do not just prevent intragel contacts. Instead, they appear to engage in novel interactions that limit their mobility to a microseconds to milliseconds regime that is still largely devoid of fully mobile regions and that the O-GlcNAc-modified Nup98 FG domain remained cohesive along its entire sequence.

Surprisingly, correlations compatible with β-sheet arrangements (Figure 7D, labelled in green) were completely absent from the O-GlcNAc-modified Nup98 gel. Instead, we observed correlations (Figure 7D, mustard yellow) that are typically observed in unstructured protein loop regions (also known as random coil correlations; Luca et al., 2001). This is in striking contrast to the interchain β-sheet structures that dominate inter FG repeat contacts of the highly cohesive NQ-rich FG domains from the yeast S. cerevisiae (Ader et al., 2010). This points to a fully unanticipated conclusion, namely that FG hydrogels can assemble through different structural principles and yet acquire the same NPC-typical permeability.

**Discussion**

The NPC permeability barrier is a central module of the nuclear transport machinery. It suppresses uncontrolled intermixing of nuclear and cytoplasmic contents, but permits rapid passage of NTR-cargo complexes. Directed active nuclear transport requires the barrier to prevent a backflux of cargoes from their destination compartments. Furthermore, importin- and exportin-mediated transport cycles are driven by tightly coupled RanGTPase cycles, which, again, rely on the NPC barrier for retaining free RanGTP inside the nuclear compartment.

**The principle of inter FG repeat cohesion is evolutionary conserved**

We previously proposed the selective phase model to explain NPC barrier function (Ribbeck and Görlich, 2001). It assumes a non-covalent cross-linking of barrier-forming FG domains into a 3D sieve excluding large inert molecules, but allowing NTRs to transiently open adjacent meshes and thus to pass the barrier. If FG hydrogel formation was fundamental for nuclear transport selectivity, then one should expect evolutionary conservation of this principle. Indeed, it is now evident that not only yeast contains cohesive FG domains (Frey and Görlich, 2009; Yamada et al., 2010), but also Xenopus, that is, an organism separated by ≈1 billion years of evolution. We now tested 10 FG domains as well as several FG-like domains from Xenopus and found them all proficient in hydrogel formation. Together, these domains account for ≈1/7 of the so far attributable mass of the NPC proper. Considering that the Xenopus nucleoporins contain additional domains with predicted non-globular structure (e.g., in Pom121, Ndc1 or Nup88), this number might be even higher. We would assume that hydrogel structures within the NPC not only participate in the permeability barrier, but also function as an adaptive glue between certain NPC subcomplexes.

**Composite hydrogels from neighbouring FG domains**

We expect the territorial boundaries between individual FG domains to be blurred and FG domains with adjacent anchor points to intermix and to form composite gels. This will certainly apply to FG domains that originate from the same subcomplex, where the contour length of the domains (typically in the 100-nm range) by far exceeds the distance between the respective anchor points (which is presumably just a few nm). Our observations with the Nups62:54:58 subcomplex indicate that such FG domain mixing might be even functionally relevant, because here the composite gel comprising all gel-forming domains displayed a far better selectivity than any of the individual homotypic gels (Figure 4). Extending this, it appears well possible that other FG domains, which form individually only gels with poor exclusion of inert macromolecules (e.g., the Pom121 FG domain), might actually perform well in the context of an authentic NPC.

**NPCs apparently contain several gel layers of distinct mesh sizes**

Our data further suggest that FG hydrogel-containing regions prevail not only in the central channel, but also extend far to the NPC peripheries. Nup358, for example, contains large sequence stretches of high gel-forming propensity. A peptide contained within the gel-forming part was previously localized ≈70 nm in cytoplasmic direction from the NPC mid-plane (Walther et al., 2002). On the other side, Nup153 localizes far to the nuclear side and also contributes a substantial share of gel-forming FG domain mass. In between, we expect FG gels derived from Nups 214, 98, 62, 58 and 54, as well as from Pom121. The long distances between the farthest cytoplasmic and farthest nuclear gel-forming FG domain anchor points suggest that the permeation path across an NPC leads through stratified layers of gels that differ in composition and selectivity. Even within the central channel we cannot expect the barrier-forming gel to be fully homogeneous, because different anchor points and the fact that naturally occurring FG domains do not represent perfectly repeated sequences will impose a compositional bias. Our data further point to a coarser sieving at the periphery and strict sieving at the Nup98-dominated narrowest point of the central channel.

**O-GlcNAc modification as a permeability modifier of FG hydrogel-based barriers**

The permeability of NPCs needs to be balanced. Too loose barriers will favour intermixing of nuclear and cytoplasmic contents and deteriorate the RanGTP gradient, while too tight barriers might block passage of NTRs carrying large cargoes. It is tempting to assume that the yeast S. cerevisiae achieves...
an optimal balance by combining extremely cohesive FG domains (such as those from scNup100p or scNup116p, which are related to NQ-rich amyloids) with non-cohesive ones that are typically highly charged. We now observed that Xenopus (and thus perhaps other metazoan species) can use an additional way of modifying their FG gel permeability, namely by extensive O-GlcNAc modifications of their most tightly interacting FG domains.

As exemplified for the Nup98 hydrogel, glycosylation makes the entire gel more dynamic (to a microseconds to milliseconds regime; Figure 7) and greatly favours the entry of large NTR-cargo complexes (Figure 5C). The attached sugar is very hydrophilic and so it probably improves the water solubility of the hydrogel and allows the gels to retain a higher content of water. Nup62 is another highly glycosylated Nup of Xenopus NPCs (Finlay et al, 1991), and we observed, also in this case, that the modification weakens inter FG repeat cohesion and favours entry of large NTRs into a formed gel (Supplementary Figure S5). The O-GlcNAc modification of Nup98 or Nup62 subcomplex might be beneficial already before an incorporation into NPCs by suppressing precipitation of these otherwise rather aggregation-prone molecules.

An interesting aspect is the reversibility of the O-GlcNAc modification (Gao et al, 2001). Cells might exploit this effect and regulate the permeability of their NPCs through changes in the activities of OGT and/or of the antagonistic glycosidase. Our preliminary data indicate that Nup98 from Xenopus eggs is O-GlcNAc modified to only 50% of the levels reached by excess of OGT, leaving indeed room for an upregulation as well as for a downregulation of the modification.

**Xenopus and S. cerevisiae appear to prefer different modes of inter FG repeat cohesion**

The most cohesive S. cerevisiae FG repeat regions (from scNup100p, scNup116p or the scNsp1 N-terminus) resemble NQ-rich amyloids not only in sequence, but also by the occurrence of interchain β-sheets, which account for the most stable interactions at least within an scNsp1 FG hydrogel (Ader et al, 2010; Yamada et al, 2010; Halfmann et al, 2012). Such β-structures are less prevalent in a non-modified Nup98 FG hydrogel and entirely absent in the fully O-GlcNAc-modified gel (Figure 7D). It thus appears that the bulkiness of the sugars sterically excludes interchain β-sheets. The fact that the glycosylated Nup98 domain still forms a highly selective gel suggests that other intermolecular interactions dominate. One plausible structural element would be analogous to micelles, where hydrophobic residues of FG motifs aggregate to ‘miniaturized hydrophobic cores’ that are interconnected by hydrophilic and possibly glycosylated spacers.

The observation that the Nup98 FG domain has a considerably larger proportion of hydrophobic residues than the NQ-rich yeast FG domains is consistent with this assumption. Taken together, it appears that there are at least two fundamentally different ways of organizing a functional NPC barrier.

Given these striking differences in gel structures, it is remarkable that the NQ-rich FG domains from scNup100p or scNup116p can functionally replace the Nup98 FG domain in reconstituted Xenopus NPCs and thus support facilitated passage of Xenopus NTRs (Hülsmann et al, 2012). Conversely, yeast NTRs can enter the glycosylated Nup98 FG hydrogel in a highly facilitated manner (Supplementary Figure S6). This suggests that both types of inter FG repeat contacts become instable when the engaged FG motifs are bound by a transiting NTR.

**Why do vertebrates not rely on NQ-rich FG domains?** One possible explanation is that such domains not only form reversible hydrogels, but also pose a risk of occasionally forming true amyloid fibres (Halfmann et al, 2012), which might then trigger pathogenic processes. The accumulated risk should be greater for long-lived vertebrates than for a unicellular, rapidly multiplying species.

**Sequence requirements for a selective FG hydrogel**

We found that a wide range of nucleoporin FG- and FG-like domains, which share hardly any sequence identity, is able to form hydrogels. This begs the questions of how diverse the sequence space of gel-forming modules actually can be and what sequence features are critical for a well-performing barrier.

A first requirement is certainly that the sequence is sufficiently hydrophobic to become self-interacting (cohesive). Indeed, NQ-rich FG domains and the Nup98 FG domain become non-cohesive when their hydrophobic residues are mutated to serines (Frey et al, 2006; Hülsmann et al, 2012). For conferring intermolecular interactions, these hydrophobic residues should, however, not be buried by intramolecular interactions; that is, the sequence must not adopt a globular fold. This criterion is probably easy to meet, as only a tiny fraction of random sequences can be expected to yield a globular domain.

Furthermore, heat-shock proteins or the protein degradation machinery must not misrecognize FG domains as molten globules or misfolded proteins. Indeed, typical Hsp70 recognition motifs are more hydrophobic than FG motifs (Rüdiger et al, 2000) and appear strongly underrepresented in FG domains. A maximized distance to Hsp70-recognition motifs could also explain why certain residues, such as Cys, Tyr, or Trp, are significantly underrepresented in FG domains as compared to globular domains.

In addition, the FG domains should bind NTRs, a property which is most efficiently conferred by FG motifs (Cushman et al, 2006). FG domains should, however, be selected against non-specific protein interactions that would deteriorate the selectivity of the barrier or even set seeding points for protein aggregation that could irreversibly plug the NPC. Given that accessible hydrophobic residues, which are typical for FG domains, are prone for causing non-selective interactions, this is a rather non-trivial criterion. It is met by as-yet undeciphered rules for the sequence context of FG motifs. We found, however, also instances where distinct FG domains ‘passivate’ each other. The FG domains of Nup54 or Nup98, for example, display a significant non-selective interaction with tCherry, which is fully suppressed in the presence of the Nup62 FG domain (compare Figures 1C and 4B; Supplementary Figure S4). The NQ-rich part of the scNsp1 FG domain also forms a gel with non-selective protein binding, and again, this is suppressed by other FG repeats, namely by the charged region of the same FG domain (Ader et al, 2010). Finally and most importantly for facilitated NPC passage, FG hydrogels should assemble such that NTRs can locally dissolve the meshes. The precise mechanism of this process is still fully obscure. So far, we only have evidence that it
Ni(II) chelate beads were loaded with FG domains from bacterial in vitro glycosylation and lyophilized. FG domains were then rebuffered to 20% acetonitrile chromatography was performed in 6 M Guanidinium hydrochloride. DTT, and snap-frozen after adding 250 mM sucrose. Column equilibrated in 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 2 mM MgCl2) for 16 h at room temperature. Equilibrated in assay buffer (20 mM Tris pH 7.5, 130 mM NaCl, 2 mM DTT, 1% Tween-20). The beads were then resuspended in 20 volumes of 5 μM OTG and 1 mM UDP-GlcNAc (Sigma-Aldrich U4375) and rotated for 16 h at room temperature. Beads were subsequently washed with OTG buffer, and GluHc buffer and finally eluted with 0.5 M imidazole/HCl pH 7.5, 6 M guanidinium hydrochloride, 2 mM DTT. Eluted proteins were buffer exchanged out potentially false-positive glycosylation sites. Modified FG domain was included as a negative control to sort peptides containing the appropriate modifications. The non-modified FG domain was rebuffered to 20% acetonitrile + 0.08% TFA and lyophilized.

In vitro glycosylation
Ni(II) chelate beads were loaded with FG domains from bacterial lysates, washed with GluHc buffer (50 mM Tris–HCl pH 7.5, 6 M guanidinium hydrochloride, 2 mM DTT), and equilibrated in OTG buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 2 mM MgCl2, 2 mM DTT, 1% Tween-20). The beads were then resuspended in 20 volumes of 5 μM OTG and 1 mM UDP-GlcNAc (Sigma-Aldrich U4375) and rotated for 16 h at room temperature. Beads were subsequently washed with OTG buffer, and GluHc buffer and finally eluted with 0.5 M imidazole/HCl pH 7.5, 6 M guanidinium hydrochloride, 2 mM DTT. Eluted proteins were buffer exchanged on a C18 column to 20% acetonitrile + 0.08% TFA and lyophilized.

FG hydrogel formation
Lyophilized FG domains were resuspended at 200 mg/ml in a buffer (see below). Gel formation was allowed for 16 h at room temperature in a humidified chamber. Subsequently, the hydrogels were equilibrated in assay buffer (20 mM Tris pH 7.5, 130 mM NaCl, 2 mM MgCl2) for 16 h at room temperature.

All FG domains studied here formed gels in a physiological buffer, while inert substances were used at 3 M of 13C, 15O and gels were dialyzed against 200 mM Potassium phosphate buffer (pH 7.5) before analysis. ssNMR experiments were conducted using 1H and 3.2 mM triple-resonance (1H,13C,15N) probeheads at static magnetic fields of 11.7 T and 6.4 T (Bruker Biospin). Through-space transfer experiments were performed at 10.92 kHz magic angle spinning and 295 K. Typical proton field strengths for 90° pulses and SPINAL64 (Fung et al., 2000) decoupling ranged between 70 and 83 kHz. (13C,15N) correlation spectra (Figure 7D) were obtained using proton-driven spin diffusion schemes under MIRROR conditions (Scholz et al., 2008) employing mixing times of 40 ms. In the case of 2D ssNMR spectroscopy after direct excitation, mixing times were set to 20 ms (Nup98 FG) and 200 ms (O-GlcNAc-Nup98 FG). 1H magnetization was produced using CP (contact times of 0.5 and 0.6 ms) or direct excitation using 90° 13C pulses at 50 kHz r.f. field strength. Through-bond transfer experiments were performed at 10.92 kHz magic angle spinning and 295 K using an INEPT-TOBBSY scheme (Andronesi et al., 2005). A TOBSY (Baldus and Meier, 1996) mixing time of 6.6 ms and 10 kHz GARP (Shaka et al., 1985) decoupling were employed.

Mass spectrometric mapping of O-GlcNAc modified sites
The enzymatically glycosylated Nup98 FG domains were dissolved at 2 mg/ml in 2 M Urea, 50 mM Tris–HCl pH 7.5, 2 mM DTT and digested with 0.1 mg/ml chymotrypsin for 16 h at 25 °C. Peptides were desalted on a C18 matrix and subjected to β-elimination (2 h at 50°C and pH 12), which converts a modified serine to an α-amino propenoic acid derivative and a modified threonine to an α-amino butenoic acid derivative (Wells et al., 2002). The resulting peptides were analysed without further chemical modifications under standard conditions with an LTQ Orbitrap XL equipped with Agilent nano LC System. Obtained spectra were analysed with Mascot 2.2 and searched for unmodified peptides and peptides containing the appropriate modifications. The non-modified FG domain was included as a negative control to sort out potentially false-positive glycosylation sites.

DBJ/EMBL/GenBank nucleotide sequence accession numbers
The accession numbers are xtNup153: JX993585 and xlnNup54: JX993586.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

Acknowledgements
We wish to thank Jürgen Schülemann and Uwe Pleßmann for excellent technical support; Koray Kirli for the pKK vectors; Maarten Fornerod for a xlnNup214 clone; Irene Böttcher-Gajewski for help in photography; Jennifer Seefeldt for critical reading of the manuscript; and the Max-Planck-Gesellschaft for financial support. The ssNMR work was supported by the Netherlands Organization for Scientific Research (VICI grant 700.10.443 to MB).
and by the European Community’s Seventh Framework Program FP7/2007–2013 under grant agreement no. 211800.

Author contributions: AA conceived, performed and analysed experiments, prepared figures and edited the manuscript; SG performed and analysed ssNMR experiments; SF generated tCherry and supplied reagents; BBH generated and validated initial Xenopus Nup constructs; HU led mass spectrometric analysis; MB led ssNMR analysis; DG supervised the project, conceived and analysed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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