Atomic resolution dynamics of cohesive interactions in phase-separated Nup98 FG domains

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Cohesive FG domains assemble into a condensed phase forming the selective permeability barrier of nuclear pore complexes. Nanoscopic insight into fundamental cohesive interactions has long been hampered by the sequence heterogeneity of native FG domains. We overcome this challenge by utilizing an engineered perfectly repetitive sequence and a combination of solution and magic angle spinning NMR spectroscopy. We map the dynamics of cohesive interactions in both phase-separated and soluble states at atomic resolution using TROSY for rotational correlation time (TRACT) measurements. We find that FG repeats exhibit nanosecond-range rotational correlation times and remain disordered in both states, although FRAP measurements show slow translation of phase-separated FG domains. NOESY measurements enable the direct detection of contacts involved in cohesive interactions. Finally, increasing salt concentration and temperature enhance phase separation and decrease local mobility of FG repeats. This lower critical solution temperature (LCST) behaviour indicates that cohesive interactions are driven by entropy.
Nuclear pore complexes (NPCs) are equipped with a permeability barrier, which controls nucleocytoplasmic exchange and consequently the macromolecular compositions of the nuclear and cytoplasmic compartments. This barrier must meet apparently contradictory requirements. On the one hand, it acts as a sieve and suppresses the passage of "inert material" as small as ~5 nm in diameter, which prevents an uncontrolled intermixing of nuclear and cytoplasmic contents. At the same time, it allows facilitated passage of nuclear transport receptors (NTRs) that shuttle cargos across the barrier and against concentration gradients. The transported cargos can be as large as newly assembled 60S ribosomal subunits with a 25 nm diameter. Facilitated transport rates can reach ~1000 translocation events or a mass flow of 100 MDa per NPC per second, and NPC passage of a single molecule is usually completed within milliseconds to tens of milliseconds, even for ribosomal subunits. In fact, passage through the NPC barrier can occur so fast that it appears limited only by the diffusion through the bulk buffer and the barrier material behaves like a perfect sink for NTRs.

FG repeat domains are intrinsically disordered protein (IDP) regions anchored to the NPC scaffold containing Phe-Gly (FG) motifs. They are key to transport selectivity. Not only do they bind to NTRs during facilitated translocation but also engage in cohesive intra- and inter-molecular inter-repeat interactions to form the actual permeability barrier. Some cohesive FG repeats were also found to bind to purified constituents of the NPC scaffold, suggesting that the FG repeats could seal the barrier towards the walls of the pore via cohesive interactions.

Spontaneous phase separation of purified cohesive FG domains in an aqueous solvent has been observed before by allowing efficient entry of NTRs and NTR-cargo complexes to partition coefficients of ~1000, while excluding inert macromolecules (lacking favourable interactions with FG motifs) to partition coefficients of ~0.1. The latter sieving effects require the high local FG domain concentration (~200 mg/ml) with properties defined by the macromolecular assembly of FG domain molecules, and an aqueous phase where the FG domain molecules remain rather dilute (concentration < 0.5 mg/ml) and solvated by the aqueous solvent.

The FG phase mirrors the transport selectivity of NPCs very well by allowing efficient entry of NTRs and NTR-cargo complexes to partition coefficients of ~1000, while excluding inert macromolecules (not interacting with FG motifs) to partition coefficients of ~0.1. The latter sieving effects require the high local FG domain concentration (~200 mg/ml) in the FG phase mentioned above. This is in line with the observations that cohesive FG interactions are fundamentally required for NPC transport selectivity. Following entry, cargo-NTR complexes can traverse the FG phase, and cargos can be rapidly released back into the aqueous phase. This suggests that the interactions within the FG phase are dynamic and reversible, even if the FG domains within the FG phase show little to no detectable translational diffusion.

Yeast and vertebrate NPCs contain ~10 different FG domains, which differ widely in the prevalent FG motifs (e.g., GLFG, FSFG, or SLFG), FG motif density, and composition of the inter-FG space. Earlier reconstitution experiments suggested that the Nup98 FG domain is the most critical one for barrier formation. It is enriched in GLFG motifs, has the highest FG-density (1 motif per ~12 residues), and is extremely depleted of charged residues – consistent with the view that FG domains experience water as a poor solvent.

The differences in the sequence and composition of FG domains can impact their biophysical properties. For example, the cohesive fungal FG domains are typically NQ-rich. Magic angle spinning (MAS) NMR analysis of an FG phase assembled from the S. cerevisiae Nsp1 FG domain identified strong cross-polarization (CP) signals for amyloid-like, rigid, inter-chain β-sheets, which originate from the NQ-rich N-terminal subdomain. In addition, the FG domains from the S. cerevisiae Nup98 homologues Nup100 or Nup116 form phases that stain strongly with the amyloid-marker thioflavin-T. In contrast, less NQ-rich FG phases, e.g., Tetrahymena thermophila MacNup98A and Xenopus laevis Nup98 FG domains are essentially thioflavin-negative, and the latter also gives much weaker CP signals for inter-chain β-sheets. In fact, the CP signal is completely lost when Xenopus laevis Nup98 FG domains receive their typical O-GlcNAc modifications. This suggests that there may be different ways of assembling barrier elements but that amyloid-like structures are not fundamental for NPC function. In any case, the key role of FG motifs in cohesive interactions is conserved across species.

How the NPC barrier combines high transport capacity with selectivity is not fully understood. These permeability properties may be explained if cohesive interactions between FG repeats form transient meshes that are smaller than the passive size exclusion limit, while the binding of NTRs to FG motifs transiently opens these meshes, allowing NTRs to "sieve" through partitioning into the barrier. The surface properties of NTRs appear to be optimized for partitioning into the FG phase. This hypothesis provides an explanation for the selectivity of nuclear pores based on the partition coefficient between the FG phase and the cytoplasm/nucleoplasm as well as an explanation for the fast transport rates of NTRs based on the local mobility of the barrier and weak transient interactions between FG motifs and NPC scaffolds.

From another perspective, the selective barrier in NPCs is an early example of biomolecular condensates, amongst many well-characterized systems. These condensates are assembled by phase separation of cellular proteins (and sometimes also nucleic acids) up to a protein concentration of 200–400 mg/ml and some of them exist as membraneless organelles. They are implicated in a wide range of biological events and also pathology. Often, proteins involved in phase separation contain long (>100 residues) intrinsically disordered regions with low complexity sequences and are able to engage in multivalent low-affinity contacts. The underlying interactions that drive phase separation are diverse for different condensates. Many of the well-studied phase-separating IDPs are enriched in both charged and aromatic residues, which were proposed to be involved in electrostatic and/or cation interactions. On the other hand, there are also examples deficient in both charged and aromatic residues, where hydrophobic effects have been implicated. Nup98 FG domains are another extreme in the spectrum: they are highly enriched in aromatic residues (however, exclusively Phe) but deficient in charged residues.

NMR spectroscopy is often the method of choice for obtaining atomic-resolution insights into the dynamics and interactions of IDPs. and has also recently been used to uncover the driving forces of phase separation. However, in the case of Nup98 FG domains, the length of any given native homologue (~500–700 amino acids) and the ~40–50 imperfect repeats within the protein sequence present an obstacle. These sequences remain technically challenging for high-resolution NMR, due to the substantial overlap of resonances and the presence of many similar sub-sequences. Since the overlap of resonances cannot be simply removed, we took the opposite route and utilized a perfect repeat (prf.GLFG$_{22\times12}$) consisting of 52 connected 12 amino-acid peptides GGLFGGNTQPAT, which simplifies the system to its essentials. This allows residue-specific measurements using a combination of solution and MAS NMR spectroscopy, and
provides direct insight into the dynamics of cohesive interactions fundamental to NPC-barrier function.

**Results and discussion**

**Phase separation of Nup98 FG domains exhibit entropy-driven LCST behaviour.** The engineered perfectly repetitive sequence, prf.GLFG_{52×12}, captures several evolutionarily conserved features of native Nup98 FG domains. These include the overall amino-acid composition, the lack of charged residues, and the number and density of FG motifs. We previously showed that prf.GLFG_{52×12} phase separates like native Nup98 FG domains, including its parental FG domain, the wild-type *Tetrahymena thermophila* MacNup98A (Mac98A) FG domain (666 residues) (for sequences see Supplementary Note 1) and that the assembled phases showed similar permutation selectivity. Both the prf.GLFG_{52×12} and Mac98A FG phases are simple systems that, unlike their animal orthologues, do not rely on obligatory O-GlcNAc modification. In addition, they are both thioavin-T-negative, suggesting that they do not form amyloid-like structures.

We previously used polyhistidine-tagged versions of the FG domains. For the purpose of performing high-resolution NMR measurements in this study, we made a construct of polyhistidine-SUMO-prf.GLFG_{52×12}, such that the unwanted histidine-tag could be removed by protease digestion during purification (Methods). As shown in Fig. 1a, a 20 μM (~1.2 mg/ml) solution of the histidine-tag-free prf.GLFG_{52×12} self-assembled into an FG phase, in the form of scattered particles (“FG particles”) at 21 °C. We found the FG domain concentration within the assembled FG phase to be ~4.5 mM (260 mg/ml), which corresponds to 230 mM (translational) diffusion coefficient of ~0.06 μm²/s, however, this is still ~600 times slower than that of its soluble state (~40 μm²/s, from DLS measurement). We previously showed that this difference (when compared to the Mac98A FG domain) is due to the lack of the 44-residue GLEBS domain (binding site for the mRNA export mediator Gle2p) in prf.GLFG_{52×12}, which also contributes to cohesive interactions, and suggested that the overall strength of cohesion could affect the translational mobility of molecules and viscosity of the FG phase material.

Next, we investigated the rotational diffusion of individual amino-acid residues, which is fundamental for understanding the permeability behaviour of FG phases. Since in NPCs the ends of FG domains are anchored to the NPC scaffold, and the FG domains are thus translationally immobile, the permeability of the barrier must depend on local dynamics. Therefore, we analysed the FG phases by NMR spectroscopy, measuring rotational dynamics of the FG domains. We expressed and purified isotope-labelled prf.GLFG_{52×12} and the parental wild-type Mac98A FG domain from *E. coli* cultured in a modified M9 medium (Supplementary Table 2), as described in Methods.

We found that solution NMR measurements of the FG phases of both the Mac98A FG domain and prf.GLFG_{52×12} were not possible due to the severe line broadening caused by the magnetic susceptibility inhomogeneities in the viscous FG phases. Instead, we analysed the samples by MAS NMR spectroscopy, measuring rotational dynamics of the FG domains. We observed a concave curve, indicating a lower critical solution temperature (LCST) phase behaviour, in which phase separations are often driven by hydrophobic interactions i.e., unfavourable entropy of mixing a polymer and the solvent, which increases with increasing temperature. In this case, the water content of LCST-type condensed phases is predicted to be dependent on temperature: higher temperature increases the influence of entropy and since water molecules are restricted in their motion next to a hydrophobic group, water leaves the polymer (known as ‘syneresis’). Indeed, we observed this phenomenon for the FG phase by concentrating the gel-like phase and filling up a glass tube by centrifugation (Fig. 1). We found that the macroscopic FG phase shrunk readily by ~50% in volume when the temperature was increased from 25 °C to 65 °C, indicating that water left the FG phase at higher temperature. Furthermore, we also found that the transition temperature decreased with increasing salt concentration (Fig. 1f), confirming the strong role of hydrophobic interactions. Accordingly, low salt concentrations disfavour cohesive interactions. From the above, conditions (temperature/ [NaCl]/ [FG domain]) for phase separation of prf.GLFG_{52×12} as well as conditions where prf.GLFG_{52×12} remains soluble (c < c_{critical}, and thus only the aqueous phase exists) can be derived from the phase diagram.

**Phase-separated Mac98A FG domain and prf.GLFG_{52×12} show low translational but high rotational mobility.** We analysed the microscopic dynamics of FG phases by fluorescence recovery after photobleaching (FRAP) experiments (Fig. 2a), which reveal the translational mobility of the whole molecules by correlating the diffusive recovery rate of a bleached region to a diffusion coefficient. To this end, we coupled the FG domains with the most weakly “FG-phile” fluorescent dye identified, Atto488, incorporated them into assembled FG phases, and performed photobleaching on the FG phases. The FG phase assembled from wild-type Mac98A FG domain showed essentially no recovery after bleaching even after an hour, meaning that phase-separated FG domains are translationally rather immobile. prf.GLFG_{52×12}, however, showed mobility to an estimated (translational) diffusion coefficient of ~0.06 μm²/s, however, this is still ~600 times slower than that of its soluble state (~40 μm²/s, from DLS measurement). We previously showed that this difference (when compared to the Mac98A FG domain) is due to the lack of the 44-residue GLEBS domain (binding site for the mRNA export mediator Gle2p) in prf.GLFG_{52×12}, which also contributes to cohesive interactions, and suggested that the overall strength of cohesion could affect the translational mobility of molecules and viscosity of the FG phase material.
**Fig. 1 Phase separation of Nup98 FG domains shows a lower critical solution temperature (LCST) behaviour.**

- **a** Both the wild-type Mac98A FG domain and an engineered perfectly repetitive sequence, prf.GLFG$_{52 \times 12}$, phase separate to near-spherical particles following purification and dilution out of 2 M guanidine hydrochloride with an aqueous buffer (Methods), as shown by microscopy (top: phase-contrast microscopy images, below: confocal laser scanning microscopy images). Both FG phases exhibit NPC-like transport selectivity: they allow the partition of a nuclear transport receptor NTF2 (labelled with Alexa Fluor 488, green) to a partition coefficient of ~2000, but exclude the inert probe mCherry protein (partition coefficient < 0.05) (red).

- **b** Sequence of prf.GLFG$_{52 \times 12}$ containing 52 perfect repeats of a 12-mer peptide.

- **c** A dilution of prf.GLFG$_{52 \times 12}$ (15 μM at 400 mM NaCl) at 4 °C does not display phase separation, however when warmed up to 37 °C, it turns turbid readily, indicating phase separation. The process is reversible by cooling back to 4 °C. **d** 20 μM dilutions of prf.GLFG$_{52 \times 12}$ prepared at room temperature and centrifuged at different temperatures. SDS samples of the obtained pellets (FG phase) and supernatants (aqueous phase) are shown on SDS-PAGE, followed by Coomassie blue staining. Critical concentrations for individual conditions are taken as the concentrations of the supernatants. Full scans of gels with molecular weight markers are provided in the Source Data file. *At 7 °C, 100 μM of prf.GLFG$_{52 \times 12}$ is required to determine the critical concentration.**

- **e** A dilution of prf.GLFG$_{52 \times 12}$ analysed by Dynamic Light Scattering (DLS), with the temperature increased from 2 to 40 °C (red). A sharp increase in hydrodynamic radii (from ~5 nm, expected for monomers, to final radii of ~1000 nm, expected for the multimeric FG particles) indicates phase separation at a transition temperature ($T_p$) ~16 °C. As in c, the phase separation is reversible by cooling (blue).

- **f** DLS analysis repeated with different concentrations of prf.GLFG$_{52 \times 12}$ and NaCl to determine $T_p$, which is plotted against prf.GLFG$_{52 \times 12}$ concentration. Data are plotted as mean values ± standard deviations (S.D.s) of three replicates. **g** Photographs of 30 mg of phase-separated prf.GLFG$_{52 \times 12}$ after centrifugation into a volume of ~150 μl at 25 °C, heated to 65 °C for 1 min, then cooled down on ice for 15 min. The red arrow marks the boundary between the condensed and the aqueous phase before heating.
selectively stem from rigid parts of a given sample. The reason for this is that in cross-polarization, magnetization is transferred from $^1$H to $^{13}$C atoms through strong, orientation-dependent dipolar interactions. If the peptide chain exhibits fast (ns timescale) isotropic motion, these interactions are averaged, and magnetization transfer can only take place through much weaker through-bond scalar couplings\textsuperscript{74}, resulting in a much weaker CP signal. Interestingly, we found that both the wild-type Mac98A FG domain as well as prf.GLFG\textsubscript{52}$\times$12 exhibit low CP intensity throughout the sequence (direct excitation $^{13}$C signals are over 10-fold stronger than the CP signals), indicating that both sequences lack regions with low mobility. Moreover, both exhibit similar liquid-like spectral characteristics, e.g., narrow $\sim$70–80 Hz linewidths even at low MAS frequency and low-power decoupling. These indicate high local rotational mobility of both FG domains in FG phases on a nanosecond timescale despite their low translational mobility inferred from the FRAP experiments.

This finding has important implications, namely that the FG phases show a non-Brownian behaviour, with uncoupled translational and local rotational motion. Even though a molecule of FG domain is translationally rather immobile, the amino-acid residues can remain locally dynamic. This behaviour is similar to what was observed in various organic polymers (e.g., styrene-butadiene rubber\textsuperscript{75}), where cross-links between polymer chains hinder translation, while locally the polymer chain displays solution-like mobility. Similarly, in these cases, the scale of observation determines whether liquid- or gel-like\textsuperscript{76} properties prevail. We expect native FG domains in NPCs, where the ends of FG domains are anchored to the wall of the NPC, to also exhibit such non-Brownian behaviour.

MAS NMR is also sensitive to amyloid-like backbone interactions, which reduce mobility and thus increase CP intensity\textsuperscript{33,77}. In contrast to Mac98A and prf.GLFG\textsubscript{52}$\times$12 for which amyloid-like backbone interactions were not detected\textsuperscript{26,27}, an alternative perfectly repetitive sequence engineered previously, which lacks proline ("Pro-free prf.GLFG\textsubscript{52}$\times$12\" in Fig. 2b), stains very strongly with the amyloid-marker thioflavin-T\textsuperscript{27}. The FG phase assembled from this sequence exhibits broad lines and a much higher CP signal, confirming the presence of amyloid-like structures in this type of FG phase.

**Fig. 2 Phase-separated Nup98 FG domains show different behaviours at different scales.**

**a** Recovery after bleaching of FG phases assembled from Mac98A FG domain or prf.GLFG\textsubscript{52}$\times$12 spiked with 2.5% Atto488-coupled FG domains (of the same species). Bleached area: manually defined to be circular with a diameter of $\sim$3 μm. Fluorescence recovery over time is shown (recovery is normalized to 1 for a complete recovery). For prf.GLFG\textsubscript{52}$\times$12 the translational diffusion coefficient is shown, as derived from fitting the dataset to a theoretical exponential recovery curve (Methods). The Mac98A FG domain is essentially immobile. Although a slight increase of signal inside the bleached area was observed e.g., at 1800 s and 3600 s, this is likely due to self-recovery of the bleached fluorophore molecules but not due to diffusive recovery, as a change of postbleach profile\textsuperscript{33} is not observed.

**b** 1D $^{13}$C spectra of FG phases assembled from Mac98A FG domain (top), prf.GLFG\textsubscript{52}$\times$12 (middle), and a thioflavin-T-positive, proline-free variant of prf.GLFG\textsubscript{52}$\times$12: (GGGLFGATNSQ)\textsubscript{52} (bottom), obtained by MAS NMR (at 36 °C, 20 kHz MAS). In red, the signal from direct excitation (showing all $^{13}$C atoms), in blue, the cross-polarization (CP) spectrum (selective towards rigid parts of the samples). All spectra were obtained by averaging 1024 scans.

**Simplification of sequence improves spectral resolution and enables sequence-specific assignment.** Due to the liquid-like spectral characteristics of both Mac98A and prf.GLFG\textsubscript{52}$\times$12 FG phases at ambient temperatures, we adapted multidimensional solution NMR pulse sequences for use in proton-detected MAS NMR probes to derive precise residue-specific information on local dynamics. This combination of MAS and solution NMR is especially advantageous for FG phases, as the use of MAS eliminates line broadening, while scalar coupling-based solution NMR pulse sequences exploit long transverse relaxation times stemming from the inherent mobility of the peptide chain, yielding optimal sensitivity.
We first assessed spectral resolution in 2-dimensional amide nitrogen \(^{15}\text{N}\) and proton \(^{1}\text{H}\) \(^{15}\text{N}-^{1}\text{H}\) HSQC\text{SSMAS} spectra, where different peaks reflect the different chemical environments experienced by the NH groups. Scalar couplings were used for magnetization transfer, as dipolar couplings are averaged out by the fast (ns timescale) isotropic motion of the peptide chain. Unsurprisingly, we found that the spectrum of the Mac98A FG domain suffered from severe signal overlap due to the >600 non-equivalent amide groups in the sequence\(^{22}\). The spectrum of prf.GLFG\(_{52}\times 12\), however, displayed overlap due to the >600 non-equivalent amide groups in the motion of the peptide chain. Unsurprisingly, we found that the couplings are averaged out by the fast (ns timescale) isotropic chemical environments experienced by the NH groups.

We then focused on prf.GLFG\(_{52}\times 12\) for detailed measurements of dynamics and interactions. The narrow linewidths in the \(^{15}\text{N}-^{1}\text{H}\) HSQC\text{SSMAS} spectra (\(^{15}\text{N}: <0.19\) ppm, \(^{1}\text{H}: <0.051\) ppm), and the only slightly narrower linewidths in the TROSY spectrum (\(^{15}\text{N}: <0.14\), \(^{1}\text{H}: <0.046\) ppm) confirmed the inherent mobility of the peptide chain, while the narrow chemical shift dispersion of the proton resonances indicates intrinsic disorder of both sequences (wt Mac98A and prf.GLFG\(_{52}\times 12\)) in the FG phases. To confirm this, we compared the \(^{15}\text{N}-^{1}\text{H}\) correlation spectra of prf.GLFG\(_{52}\times 12\) with spectra obtained under strong denaturing conditions (i.e. in the presence of high concentrations of guanidine hydrochloride or hexanediol, which impede phase separation\(^{29}\)) (Supplementary Fig. 3). The similar proton chemical shift dispersion indicates intrinsic disorder under both conditions. This is consistent with other proteins that undergo phase separation in that the protein can remain disordered in both the aqueous phase and in biomolecular condensates\(^{58,59,62,63,78}\).

Three-dimensional HNCA and HN(CO)CA spectra correlating N and H\(_{N}\) with the Ca of the same and the neighboring residue, respectively, were implemented as typical for solution samples, correlating the nuclei via scalar couplings. These two spectra were sufficient to unambiguously assign the 11 observed backbone amide peaks in the \(^{15}\text{N}-^{1}\text{H}\) HSQC\text{SSMAS} spectrum to the 11 non-proline residues of prf.GLFG\(_{52}\times 12\). This sequence assignment is the foundation for further residue-specific measurements, and also implies that the different repeats are indistinguishable by NMR (Fig. 3b and Supplementary Fig. 4).

The \(^{13}\text{C}-^{1}\text{H}\) correlation spectrum (\(^{13}\text{C}-^{1}\text{H}\) HSQC\text{SSMAS}) of prf.GLFG\(_{52}\times 12\) (Fig. 3c) reflects the chemical environments experienced by the CH groups, including those in the Phe aromatic sidechain. Three peaks were observed in the aromatic region of the spectrum at 2:2:1 intensity, corresponding to the three non-equivalent CH groups in the aromatic sidechains, showing that the chemical shifts of the Phe residues are averaged faster than the us-timescale. The narrow lines observed in the \(^{13}\text{C}-^{1}\text{H}\) HSQC\text{SSMAS} spectrum, even for the aromatic sidechain, further indicate that the motion of the peptide chain occurs on a nanosecond timescale, fast enough to isotropically average even strong orientation-dependent interactions such as dipolar couplings.

**Effect of phase separation, temperature and salt concentration on local dynamics of the peptide chain points to the importance of hydrophobic interactions in cohesive behaviour.** After analysing the FG domains in the FG phases, we were interested in comparing the NMR spectra of prf.GLFG\(_{52}\times 12\) in the FG phase and in the complementary aqueous phase (HSQC\text{SSMAS} and HSQC, respectively), which may show residues involved in phase separation. We had measured \(^{15}\text{N}-^{1}\text{H}\) and \(^{13}\text{C}-^{1}\text{H}\) correlation spectra for the former without any difficulty, as the protein concentration in the FG phase is in the millimolar range (Supplementary Fig. 1), providing strong signal intensities. For the latter, we prepared a sample in the soluble state based on
To further investigate the origin of these chemical shift perturbations, we assessed local mobility by measuring rotational correlation times in both states of prf.GLFG$_{7\times12}$. For this purpose, we applied the $^{15}$N-$^1$H-TRACT pulse sequence, which allows the measurement of exact rotational correlation times ($\tau_c$) for Brownian particles by eliminating the effect of chemical exchange. For the FG phase, an assumption of Brownian-like local dynamics, with an exponential rotational correlation function can still be used to assess qualitative changes in dynamics. Note that these qualitative changes could be interpreted in the context of different motional models, including anisotropic rotational diffusion, however, this would require extensive further relaxation measurements. We observed high, nanosecond timescale mobility in the soluble state (i.e., short $\tau_c$-values, Fig. 4). The profile of $\tau_c$-values is rather flat and all residues exhibit roughly the same value (2-2.5 ns at 24 °C). In contrast, in the FG phase, not only is $\tau_c$~3-fold longer on average, but also the profile shows two distinct maxima. The maximum at the residues around P10 (Q9 and A11) can be explained by proline’s fixed $\phi$ angle ($\phi \approx -60^\circ$), which limits the number of possible conformations in the five-membered pyrrolidine ring, and introduces rigidity into the protein backbone. Proline also restricts the conformations available to the neighbouring residues, especially the preceding one, and this also appears in their increased $\tau_c$-values. The other maximum appears at residues F4 and L3. The residues were previously shown to be crucial for phase separation behaviour and thus both hydrophobic and $\pi-\pi$ interactions of the aromatic rings likely contribute to cohesive interactions. Such interactions are consistent with the increased $\tau_c$ of the peptide chain at this position.

Since all repeats are identical, FG repeats could cohesively interact with each other within the same molecule even in the soluble state, where intramolecular cohesive interactions are too weak to drive molecular assembly. Notably, with a ~5 nm hydrodynamic radius as determined by DLS measurements, the local concentration of FG motifs in the FG domain in the aqueous phase could also reach ~170 mM, similar to the concentrations of FG motifs in FG phase. Indeed, other cohesive FG repeats were previously shown to interact with each other within the same molecule even in the soluble state, thereby disfavouring the formation of intramolecular interactions by decreasing the probability of interacting with intramolecular cohesive regions. The nearly perfect overlap of the $^{15}$N-$^1$H HSQC spectra of the prf.GLFG$_{7\times12}$ fragments with the full-length protein eliminated the need for sequence assignment (Fig. 4a). Average $\tau_c$-values measured for both the prf.GLFG$_{52\times12}$ (aqueous state) and the prf.GLFG$_{7\times12}$ fragments at 24 °C are significantly shorter than predicted by Stokes’ law for spherical molecules (prf.GLFG$_{52\times12}$: 1.4 ns vs. 22.3 ns, prf.GLFG$_{7\times12}$: 0.7 ns vs. 4.0 ns, assuming a hydration layer of 3.2 Å), showing that neither of the molecules tumbles as a whole. We found that prf.GLFG$_{7\times12}$ exhibited ~2-fold shorter $\tau_c$ than aqueous phase prf.GLFG$_{52\times12}$ (Fig. 4b). However, this difference could not only stem from a reduction of cohesive interactions, but in part also from the reduction of the overall correlation time due to the ~7 times shorter peptide. We thus took a complementary approach to disfavour the intramolecular cohesive interactions by lowering the salt concentration, suggested by what we found earlier in Fig. 1f. Indeed, we found that correlation times were reduced for both aqueous phase prf.GLFG$_{52\times12}$ and prf.GLFG$_{7\times12}$ in the absence of NaCl (Fig. 4c), suggesting that part of the changes in rotational dynamics resulted from changes in intramolecular cohesive interactions. Since the average $\tau_c$ for the prf.GLFG$_{7\times12}$ is still shorter by 30% compared to that of prf.GLFG$_{52\times12}$, the overall size of the two peptides also contributes to the difference in rotational correlation time as predicted.

Given the observations of temperature-dependent phase separation, we also investigated the effect of temperature on local mobility by measuring rotational correlation times at 36 °C in both the aqueous phase and the FG phase. $\tau_c$ of the full-length protein in the aqueous state could not be determined at 36 °C due to the extremely low, ~1 μM critical concentration at this temperature. For prf.GLFG$_{7\times12}$, as expected, we observed that $\tau_c$-values dropped by half upon an increase of 12 °C, indicating increased mobility at higher temperatures. Strikingly, however, the peptide chain of the full-length prf.GLFG$_{22\times12}$ in the FG phase became less mobile: $\tau_c$-values increase almost twofold with the 12 °C increase in temperature (on average from 6 ns to 11 ns). The shape of the profile also changed, with L3 becoming less mobile relative to F4 at the higher temperature. These observations point to the role of hydrophobic interactions in the FG phase.

Moreover, consistent with Fig. 1f, we found that higher NaCl concentration also lowered critical concentrations and drove phase separation of prf.GLFG$_{52\times12}$ more to completion (Fig. 5a). Furthermore, we found that both microscopic and local dynamics of the FG phase of prf.GLFG$_{52\times12}$ are also dependent on salt concentration. FRAP experiments suggest ~3-fold slower translational diffusion (Fig. 5b) upon an increase in NaCl concentration from 150 mM to 600 mM. We also measured residue-specific $\tau_c$ in the FG phase at different NaCl concentrations (Fig. 5c). The overall pattern of $\tau_c$-values with respect to residue number remains the same at each NaCl concentration, with maxima at L3-F4, and Q9-A11, and an increase in salt concentration results in an increased $\tau_c$ for each residue. The relative increase in $\tau_c$ is similar throughout the sequence, suggesting that not only the interacting hydrophobic residues, but the whole chain becomes less mobile at higher salt concentrations. The increase in phase separation propensity and the slowing of the dynamics of FG phase (due to stronger inter- and intramolecular interactions) at higher salt concentrations indicates once again the importance of hydrophobic interactions in cohesive interactions/phase separation of the FG domains.

NOE reveals spatial proximities between aromatic-aliphatic and aliphatic-aliphatic hydrophobic groups. In order to observe hydrophobic interactions in the FG phase, we recorded a $^{13}$C-filtered NOESY spectrum (Fig. 6), in which cross peaks are a direct indicator of spatial proximity between protons. In general, since the nuclear Overhauser effect (NOE) is proportional to $r^{-6}$, only protons closer than 5.5 Å are expected to give a NOESY cross peak. This means that protons within the same or in neighbouring residues will usually show cross peaks, as the length...
of a residue is 3.4–4 Å. These cross peaks do not necessarily indicate informative residue–residue interactions. Similarly, peaks appearing on the diagonal of the spectrum primarily stem from magnetization not transferred during the mixing period, which obscures contacts between equivalent groups, e.g., Phe–Phe interactions. However, after the assignment of all proton resonances of prf.GLFG52 × 12, we could identify several interesting contacts between non-neighbouring residues.

Although in our case quantitatively correlating NOE cross peak intensities to distance restraints is problematic with such a dynamic system, the observed cross peak pattern points to hydrophobic groups being in close proximity within the FG phase. Considering that some neighbouring protons show no correlations at all (e.g. N7 and F4 Hβ1,2 and their neighbours), even cross peaks between neighbouring residues can be taken as an indication of increased proximity (e.g. L3 Hδ1,2–F4 aromatic protons, in agreement with previously observed Leu–Phe contacts in the Nsp1 FG phase). Remarkably, we observed several cross peaks between protons from non-neighbouring residues (marked by arrows in Fig. 6), most prominently between the aromatic sidechain of F4 and the methyl groups of T8/T12, and also between purely aliphatic groups, such as T8/T12 methyl groups and the P10 β-methylene group. The relatively long mixing time of 100 ms has been used in investigations of other phase-separated systems as well. As mentioned above, a limitation of the current method is that it does not allow for observing contacts between equivalent groups, therefore, proximity between Phe–Phe sidechains was ambiguous in our dataset, even though they are likely present in the system.

Mutagenesis experiments suggest that sequence hydrophobicity determines phase separation behaviour. Finally, observations from NMR studies guided us to the following series
Phase-separation at [prf.GLFG_{52}] = 10 μM

[NaCl] (mM)

| 75 | 150 | 200 | 300 | 600 |
|----|-----|-----|-----|-----|
| Pellet | | | | |
| Supernatant | | | | |

Critical conc. (μM)

7.2 5.3 3.9 2.8 0.5

Fig. 5 Effects of salt concentration on the properties of the FG phase. a Analysis of phase separation by centrifugation as described in Fig. 1d with varying [NaCl] at 27 °C. Full scans of gels with molecular weight markers are provided in the Source Data file. b FG phase assembled from prf.GLFG_{52}×12 containing 2.5% Atto488-coupled prf.GLFG_{52}×12 molecules photobleached in 150 or 600 mM NaCl and the fluorescence recovery after photobleaching. Top: representative images showing the recovery of Atto488 signal (bleached area: manually defined to be circular with a diameter of ~3 μm). Bottom: Signal recovery normalized to 1 for a complete recovery plotted against time post bleaching. Fits (solid lines) to the exponential fit function to five replicates are shown. The translational diffusion coefficients (mean ± S.D.) derived from the fits are shown in the box. c Residue-specific rotational correlation times (τc) of prf.GLFG_{52}×12 in FG phase as determined from the [15N,H]-TRACT experiment, shown as a function of [NaCl]. τc values are plotted as calculated from the best fit values of Rg and Rg from a single measurement. Error bars are shown as ± S.D.s. S.D.s were obtained from the exponential fits of the transverse relaxation rates Rg and Rg, and the Monte Carlo method (N = 10000) was then used to estimate the S.D.s of τc. The temperature was set to 36 °C for all measurements. Due to the absence of H^{15}N, no TRACT data is available for Pro residues.

of mutagenesis experiments. As a basis for these, we chose the previously engineered FG domain variant ‘GLFG_{52}×12’27, which contains 52 GLFG motifs connected by non-identical eight-residue spacers. This variant is as cohesive as the Mac98A FG domain. Unlike prf.GLFG_{52}×12, whose amino-acid composition and locations are restricted by the repetitive nature, GLFG_{52}×12 offers more flexibility in variant design (Fig. 7a and Supplementary Note 1). Here, GLFG_{52}×12 showed a critical concentration of 0.1 μM under our assay condition (25 °C, [NaCl] = 150 mM).

First, we mutated each Phe to Ala (GLAG_{52}×12), as it had previously been shown that Phe residues are crucial for phase separation19,20,23,33,34. Indeed, we observed essentially a complete loss of phase separation behaviour, even up to 100 μM (GXFG/Val_{52}×12), indicating that the system is highly sensitive to changes in hydrophobicity. These results indicate that not only Phe but also Leu residues are required for cohesive behaviour, even in the presence of all Phe^{10,33}.

Next, to prove our hypothesis, that it is the overall hydrophobicity of the sequence that determines the cohesive behaviour, we constructed a mutant (GLLG_{52}×12), where all Phe were mutated to Leu. This variant showed no cohesive behaviour, its critical concentration being above 100 μM. However, this loss in cohesiveness could be caused by the fact that the Leu sidechain contains fewer hydrophobic carbons than that of phenylalanine.

To compensate for the F→L substitution, we produced a Phe-to-Leu variant, GLLG/L_{52}×12, where not only was the Phe sidechain replaced by less hydrophobic Ala (GAFG_{52}×12). Remarkably, this sequence was also rather non-cohesive and behaved similarly to GLAG_{52}×12.

Native Nup98 FG domains have most Leu adjacent to Phe in the form of GLFG motifs or SLFG motifs, but Leu residues are also present in the spacers. We asked if the vicinity of Leu and Phe is required for phase separation and thus we swapped the positions of each Leu with a residue in the middle of the adjacent spacer in GLFG_{52}×12. The corresponding variant, GXFG/L_{52}×12 remained cohesive and exhibited only a slightly higher critical concentration (0.8 μM) than GLFG_{52}×12. Interestingly, replacing all Leu residues in the spacers with Val, which is only slightly less hydrophobic, already increased the critical concentration ten-fold to 8 μM (GXFG/Val_{52}×12), indicating that the system is highly sensitive to changes in hydrophobicity. These results indicate that not only Phe but also Leu residues are required for cohesive behaviour, even in the presence of all Phe^{10,33}.

We also measured the intra-phase diffusion of GLFG_{52}×12, GXFG_{52}×12, and GAFG_{52}×12 by labelling them with Atto488, incorporating them into the FG phase assembled from prf.GLFG_{52}×12 and performing FRAP experiments (Fig. 7b, c). Both Atto488-labelled GLFG_{52}×12 and GXFG/L_{52}×12 partitioned
well into the prf.GLFG52 × 12 phase (partition coeff. > 1000) and showed diffusion rates of ~0.05–0.07 μm²/s. These values are similar to the self-diffusion rate of prf.GLFG52 × 12 (Figs. 2a and 5b). However, GAFG52 × 12 not only partitioned very weakly into the prf.GLFG52 × 12 phase (partition coeff. = 4), but also showed >2 times faster diffusion compared to the above. GLLG/LL52 × 12 partitioned readily into the prf.GLFG52 × 12 phase, but at a lower partition coefficient (110) compared to that of GLFG52 × 12 or GXFG/LL52 × 12, while exhibiting diffusion behaviour similar to the Phe-containing sequences. Thus, we concluded that strength of cohesive interactions, as well as intra-phase dynamics are dependent on hydrophobicity contributed by both Phe and Leu.

The permeability barrier of NPCs has to fulfill requirements of both high transport capacity and high selectivity. Cohesive interactions are fundamental for the formation of such a barrier, concentrating and assembling the FG repeats into a condensed phase, while the permeation selectivity is observed because mobile species encounter the chemical properties of the FG phase, and beyond a certain size they must overcome the self-affinity, i.e., cohesion, of the FG repeats. To conclude this study, our approach allows the measurement of residue-specific local dynamics, through which we could begin to understand the dynamics of inter-FG repeat cohesive interactions within the FG phase. FG domains in the FG phase exhibit slow translational motion, but fast local rotational motion. This apparent contradiction reflects the biological requirements of the NPC barrier, which needs to be kinetically stable to repel inert macromolecules but dynamic enough to allow fast facilitated passage by NTRs, as suggested before. Although we do not directly measure on/off rates of cohesive FG repeat interactions, the consistent observation of high mobility on a nanosecond timescale and the promiscuous interactions of Phe sidechains suggest that the cohesive FG repeats interact with each other with high on/off rates without a conformational selection mechanism. This may have two consequences: first, even weak NTR-FG repeat interactions could efficiently compete with the attraction between the FG repeats. Second, these interactions during translocation would require low activation energies as the entropic penalty is minimized. Notably, the binding without conformational selection may also extend to the interactions between FG repeats and NTRs. Moreover, in contrast to mutagenesis approaches, we could also directly detect contacts involved in cohesive interactions.

An ultimate goal within the field is understanding all transport-relevant interactions within an FG phase and their dynamics, however, a number of issues remain unsolved. For example, although we showed that phase separation of the studied FG domains is driven mainly by entropy (hydrophobic interactions), the

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**Fig. 6** 13C-filtered 1H-1H NOESY spectrum of the FG phase of prf.GLFG52 × 12. Cross peaks in this spectrum indicate spatial proximity between protons. Arrows mark cross peaks belonging to correlations between non-neighbouring residues and represent unambiguous spatial proximities between hydrophobic groups. Due to the large dynamic range of peaks, the contour levels were adjusted as follows: 1x (green peaks), 2x (red peaks) and 9.5x (dark blue peaks), the latter colour indicating the strongest signals. The applied mixing time was 100 ms and the measurement temperature was 31 °C. Dashed lines guide the eye to the assignments of chemical shifts; in case of overlap all possibilities are listed. The cross peaks indicating contacts involving the aromatic phenylalanine sidechain are boxed, and the skyline projection of the boxed region is shown next to the box.
physical roles of Phe residues are not totally clear. In one possible scenario, Phe residues preferentially interact with each other via π-π stacking or T-stacking interactions and these interactions interplay with hydrophobic interactions contributed by non-specific hydrophobic groups in the system. In another scenario, aromatic groups are just non-specific components of a hydrophobic core, among all other hydrophobic groups. To resolve this question, information on the distances between aromatic groups within the FG phase would be valuable. Moreover, molecular dynamics (MD) simulations could also be useful in obtaining a complete picture of motion in the FG phase, including protein sidechains, and NMR data can then be used to calibrate and validate MD studies.

While FG motif-NTR interactions have been well-studied in solution, these interactions might show unprecedented qualities when occurring within a condensed FG phase, as here we have shown that cohesive interactions and phase separation have an impact on the local FG dynamics. Here we demonstrated at the nanoscopic scale that prf.GLFG52 × 12 recapitulates many of the biophysical properties of native Nup98 FG domains, and is thus an ideal starting point for studying FG motif-NTR interactions. The methodology of applying NMR spectroscopy to measure the atomic-scale biophysical properties of an FG phase, and a perfectly repetitive sequence to simplify NMR analysis could also be extended to the study of interactions between FG domains and NTRs in the future.

Our study provides an example of entropy-driven phase separation. In contrast, enthalpy-driven phase separation shows an upper critical solution temperature (UCST) behaviour (i.e., Fig. 7 influence of hydrophobic residues on the phase separation of FG domains. a Critical concentrations for phase separation were determined on a series of GLFG52 × 12 variants. GLFG52 × 12 is an engineered FG domain variant, which contains 52 GLFG motifs flanked by non-identical sequences (spacers) of equal length. The spacers do not contain Phe and Leu. ‘//’ indicates the swapping of a spacer residue with a residue from the GLFG motif. For a detailed description of the variants, see the main text. For space economy, the N-terminal sequences up to the fourth FG motif are shown, while the C-terminal sequences (~620 residues) follow the same design strategy (see Supplementary Note 1 for complete sequences). Phase separation of the variants were analysed as in Fig. 1d at 25 °C, at [FG domain] = 10 μM, [NaCl] = 150 mM. *The analyses for GAFG52 × 12, GLLG52 × 12 and GLLAG52 × 12 were repeated at [FG domain] = 100 μM, and no significant phase separation was observed (Supplementary Fig. 6). Full scans of gels with molecular weight markers are provided in the Source Data file. b Images showing FG particles of unlabelled prf.GLFG52 × 12 premixed with 2.5, 15, 2.5 and 2.5% (by mole fraction) of Atto488-coupled GLFG52 × 12, GAFG52 × 12, GXFG//L52 × 12, and GLLG//L52 × 12, respectively. Apparent partition coefficients of the Atto488-coupled species into the assembled FG particles are indicated on the left as quantified by measuring the Atto488 signals IN: OUT of the FG particles. The FG particles were photo bleach at 150 mM NaCI as in Fig. 5b. c Signal recovery in the FRAP experiments shown in b normalized to 1 for a complete recovery of the signal and plotted against time post bleaching. Five replicates with different FG particles are shown. The translational diffusion coefficients derived from fitting the recovery (solid lines) to theoretical recovery curves (Methods) are shown next to the recovery curves as mean ± S.D.
higher temperature disfavours the phase separation), e.g., in case of IDPs enriched in both aromatic and charged residues, like DDX48 or hnRNPA1. Given the lack of charged residues, in particular Arg, it is not surprising that we observed a different temperature dependence of phase separation in Nup98 FG domains. It has been demonstrated that the UCST/LCST behaviours of resilin/elastin-like repeat proteins are dependent on the number of charged residues. It is noteworthy that some other FG domains in NPCs, like repeat proteins are dependent on the number of charged residues. DNA sequences of FG domain variants were generated with the assistance of the GenScript and cloned into a bacterial expression vector for overexpression and purification (see below). See Supplementary Table 1 for a complete list of the plasmids and the DNA sequences of FG domain variants.

Methods

DNA sequences of FG domain variants. DNA sequences encoding the FG domain variants were generated with the assistance of the Gene Designer program. Each variant was optimized for codon usage of *E. coli* expression. DNA fragments were synthesized by GenScript and cloned into a bacterial expression vector for overexpression and purification (see below). See Supplementary Table 1 for a complete list of the plasmids and the sources.

Recombinant protein expression and purification

TMacNup98A (Mac98A) FG domain. Procedures were as published previously with modifications for 15N/13C isotope labelling; *E. coli* NER. Cells were transformed with a plasmid, encoding the protein fused to an N-terminal histidine-tag (Supplementary Note 1; Supplementary Table 1), were allowed to grow in a modified M9 medium (supplemented with trace elements) containing the isotopes (Supplementary Table 2) at 37 °C until OD600 reached ~0.8. Expression of the target protein was induced by 0.4 mM IPTG, 30 °C for 16h. Then the protein was extracted, purified by Ni chromatography and lyophilized as previously described. Lyophilized powder was weighed for quantification and stored at −20 °C until use.

Pro-free prf.GLFG52 × 12. Since a histidine-tag may introduce unwanted signals in high-resolution NMR measurements, we designed a construct with a SUMO protease cleavage site46,49 such that the histidine-tag was cleaved after Ni chromatography (as described below), namely His8–ZZ–scSUMO–prof.GLFG52 × 12 (plasmid number: pSNG064, see also Supplementary Table 1). The construct was expressed in *E. coli* cells in the M9 medium as described above. The cells were resuspended in cold 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM DTT. 50 nM His18-tagged-fusions (Supplementary Table 1) and purified as previously described. Pro-free prf.GLFG52 × 12 was expressed as His-tagged-fusions (Supplementary Table 1) and purified by native Ni(II) cleate chromatography, as described previously26,29.

**FG particles preparation for permeation assays and confocal laser scanning microscopy.** A previously described procedure was applied with minor modifications: 2 μl of a 1 mM (−60 μg/μl) FG domain stock solution (for each, lyophilized powder dissolved in 2 M guanidine hydrochloride) was rapidly diluted with 100 μl (for Mac98A FG domain) or 50 μl (for prf.GLFG52 × 12) assay buffer (20 mM sodium phosphate buffer (NaPi) at pH 6.8, containing 150 mM NaCl, 5 mM DTT). For each, 15 μl of the mixture was mixed with 15 μl peroxidase containing 2 μM Alexa488-labelled NT2F and 10 μM mCherry in assay buffer. Therefore, final concentrations of Mac98A FG domain of prf.GLFG52 × 12 was 1 μM and 20 μM, respectively. A lower assay concentration for Mac98A FG domain was used due to its lower critical concentration. The resulting mixture was placed on collagen-coated micro-slides 18-well (IBIDI, Germany). FG particles were allowed to sediment for 60 min to the bottom of the slide. Then Alexa488 (for tracking NT2F) or mCherry signals were acquired with 488 nm excitation, respectively with a Leica SP5 confocal scanning microscope equipped with a 63× oil immersion objective and hybrid detectors (standard mode, in which nonlinear response of the detector was auto-corrected) at 21 °C. Partition coefficients (taken as signal inside FG phase: signal in buffer) were quantified as previously described27. Leica Application Suite X 3.3.0 was used for data acquisition and quantification.

**Fluorescence recovery after photobleaching (FRAP) and estimation of diffusion coefficients.** For all, 2 μl of unlabelled FG domain stock (1 mM FG domain in 2 M guanidine hydrochloride) was rapidly diluted with 100 μl pre-cooled assay buffer (20 mM NaPi pH 6.8, 150 mM NaCl, 5 mM DTT or NaCl) specified in the figures on ice. At this point phase separation was suppressed by the ice temperature. Then 30 μl of the FG solution was quickly mixed with 1 μl of a given Atto488-labelled FG domain (Supplementary Table 1), which had been adjusted to a concentration of 15 μM, except for GAGSF52 × 12: 90 μM. Therefore, the final protein concentration of unlabelled FG protein was 20 μM; the concentration of Atto488-labelled species was 0.5 μM (Atto488-labelled GAGSF52 × 12: 3 μM). The mixtures were incubated at room temperature to allow phase separation and then transferred to micro-slides for confocal laser scanning microscopy at 21 °C. FG particles (n = 5 typically) of radii r = 4–5 μm were photobleached near the centres at 488 nm. The bleach area was manually defined to a circular region with a radius of 1.5 μm, however, we found that the actual bleached area was always 2–3 μm in radius, due to the diffusion during bleaching and Gaussian blurring of the laser beam. After bleaching, Atto488 signals were acquired at 2 s intervals. Raw data were corrected for photobleaching during acquisition and normalized to the initial signal. The recovery curves were fitted to a simple exponential model as follows:

\[
C(t) = A \left(1 - \exp \left(-\frac{t}{\tau} \right) \right)
\]

where \(C(t)\) is the normalized signal at time \(t\). A is a preexponential scaling factor close to 1, which was used to account for incomplete recovery (due to a small immobile fraction of protein in the sample), and \(\tau\) is the characteristic diffusion time, during which \(1/e\) of the fluorescence recovers. Half-time for recovery (\(1/2\)) is...
Analysis of phase separation by centrifugation (centrifugation assay). In general, each 1 μl of FG domain stock (1 mM protein in 2 M guanidine hydrochloride) containing ~60 μg protein was rapidly diluted with assay buffer (typically 20 mM NaPi pH 6.8, 5 mM DTT, 150 mM NaCl or [NaCl] specified in the figure) to the concentration stated in the figures. After 1 min, the FG phase (insoluble content) was pelleted by centrifugation (21130 g, 30 min, using a temperature-controlled Eppendorf 5424 R centrifuge equipped with an FA-45-24-11 rotor) at the temperature specified in the figure. The equivalent ratio (6%) of the pellet (FG phase) and supernatant was analysed by SDS-PAGE/Comassie blue staining. Critical concentration for phase separation of a given FG domain (or variant) was taken as the concentration that remained in the supernatant, which was estimated with a concentration series loaded onto the same gel. All experiments were repeated three times with similar results, and the representative gel images/critical concentrations are shown.

Data analysis. Analysis of phase separation by centrifugation (centrifugation assay) and calculations.

Analysis of phase separation by Dynamic Light Scattering (DLS). In general, a stock solution of prf.GLFG52 × 12 was rapidly diluted with filtered NaCl solutions containing 20 mM NaPi pH 6.8, as described above, to protein concentrations and NaCl concentrations stated in Fig. 1F. For protein concentrations > 50 μM, lyophilized powder of prf.GLFG52 × 12 was dissolved and serially diluted in the NaCl solutions. 10 μl of each solution was analysed in a closed cuvette using a DynaPro NanoStar DLS instrument (Wyatt Technologies). To acquire temperature-dependent phase separation curves, the temperature was automatically raised by 1 °C per min, typically in the range of 2–40 °C (or as indicated in Fig. 1e, also cooling from 40 to 2 °C). DLS signal was acquired continuously. Phase transition was indicated by a sharp increase in the light scattering intensity, and the transition temperature was rounded up to the nearest 0.1 °C. The Wyatt Technologies Dynamics 7.1.5 software was used for autocorrelation analysis and computation of hydrodynamic radii. Three datasets for each condition were averaged, and standard deviations (S.D.) were shown as error bars in Fig. 1F.

NMR measurements

Sample packing. Formation of FG phases has been observed by hydration of lyophilized protein powder11,19. For solid-state NMR measurements, we used a similar method: roughly 1.1 mg of lyophilized powder of a given FG domain was packed into 1.3 mm rotors. Using excess buffer, samples were hydrated in a tabletop centrifuge (Eppendorf 5418 R, rotor: FA-45-24-11) at 25 °C. The sample was set to 40 °C (or as indicated in Fig. 1e, also cooling from 40 to 2 °C). The temperature was set to 2.7 ms for the H/N transfer, 10 ms for the CO-CA transfer, and 11.2 ms for the CB/CA and CA/N simultaneous transfer. The HNCA spectrum99 was acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h, sampling was identical to the previous experiments and a 3D spectrum79, acquired in 32 h.

Forming homogeneous gels in solution NMR tubes would have been advantageous, as it would have allowed the use of standard solution pulse sequences. This, however, was not possible, as the hydrogels exhibited severe magnetic susceptibility inhomogeneities, which could only be averaged out by magic angle spinning.

General spectral parameters for solid-state measurements. All spectra, except when noted otherwise were recorded in a 1.3 mm MAS probe at 20 kHz MAS spinning frequency in a 600 MHz wide bore Bruker Avance III HD spectrometer. Unless indicated otherwise, the temperature was set to 37 °C at 500 kHz VT gas flow. Typical pulse lengths were 2.5 μs on 1H, 4.2 μs on 13C, and 5.0 μs on 15N. In all experiments, where applicable, water suppression was achieved by a 100 ms MISISIPP pulse train. Decoupling during acquisition periods were 5 kHz TPPM95 on protons, and to 5 kHz WALTZ95 on 15N, and for double-labelled samples, also on 13C. Recycle delays were set to 1.0 s unless noted otherwise. Unless noted otherwise, all spectra were processed using a cosine squared window function using the Bruker Topspin 4.0.8. software package.

MAS NMR 1H spectra with direct excitation and 1H-13C cross-polarization. 13C-detected experiments were recorded on a [13C,15N]-labelled samples, averaging 1024 scans. Cross-polarization (CP) spectra on FG phases assembled from TMACNup98A FG domain and prf.GLFG52 × 12 were recorded at an optimal CP condition of $\omega_1^{\text{p}} = \omega_1^{\text{CP}}$. (average fields of 30 kHz on both nuclei), using an 80–100% ramp on 1H with an optimal length of 13 ms. For proton-detected experiments, the optimal CP condition was found to be 50 kHz on 13C, using a 80–100% ramp, and 68 kHz on 1H (average fields), the length of the CP transfer was set to 4 ms. Relaxation delays were optimized to allow for full signal recovery between scans. Spectra were processed with an exponential window function, applying a line broadening corresponding to half the linewidth (60 Hz, 60 Hz, and 80 Hz, in Fig. 2b, top, middle and bottom, respectively).

MAS NMR 13N-1H and 13C-13H correlation spectra. 1H-13N HSOQC95 (using refocused INEPT blocks to allow for MISSISSIPPI water suppression) and TROSY97 spectra were recorded at 20 kHz MAS spinning frequency at 31 °C in a 600 MHz wide bore Bruker Avance III HD spectrometer. 14N-1H INEPT delays were set to 1.3 ms. Each spectrum averaged four scans per point. Spectra were processed with 100 ms acquisition time in the indirect 15N, and 400 ms in the direct 1H dimension. 13C-1H HSQC95 spectra were recorded with a phase sequence analogous to the 13N-1H HSOQC95 experiment. The 13C-1H HSQC95 experiment in Fig. 3c was recorded on a u-[13C]-labelled sample without 13C-labelling in order to eliminate the effect of one-bond 13C-13C scalar couplings on the gas-phase 13C-1H INEPT delays were set to 1.1 ms. The 13C dimension was sampled to 40 ms. The spectrum used for overlay with a solution spectrum (Supplementary Fig. 5) was measured on a [13C,15N]-labelled sample to make it comparable to the solution spectrum that had to be recorded on an isotopically labelled sample for sensitivity reasons. The cooling gas was set to a temperature of 23 °C at 500 kHz gas flow, which was found to be 31 °C at the sample after calibration. The spectrum was sampled to 50 ms in the 13C dimension, but only 10 ms were used for processing.

For the H(H)+CH NOE experiment the NOE mixing block was added to the 1H-13C-13H HSQC95 block analogously as previously described95. The spectrum was recorded on an 800 MHz Bruker Avance III HD spectrometer in a narrow-bore detection probe. All experimental conditions were identical as described above. The NOE mixing time was set to 100 ms. Assignment of the side chains was accomplished with a similar H(H)+CH pulse sequence on the 600 MHz Avance III HD spectrometer, the 1H-13C-13H mixing correlating nuclei within the same spin system was achieved with a WALTZ-166 scheme with 12.5 μs pulse lengths applied for 9.6 ms.

Determination of rotational correlation times with TRACT (MAS NMR). TRACT experiments95 were recorded using buffers with varying NaCl concentrations (75, 150, 200, 300, or 600 mM NaCl) and at two different temperatures (24 °C and 36 °C) at a NaCl concentration of 150 mM. Residue-specific rotational correlation times (τc) were measured for the peptide chain using pseudo-3D experiments95, where a series of 2D 13N-1H planes were recorded with varying increments for both the α and the β states. The 1H dimension was sampled to 100 ms, the 13N dimension to 40 ms (swEEP widths: 47.1 ppm on 1H and 25.0 ppm on 15N). Each spectrum averaged 32 scans per point. For both states, seven points were sampled in the pseudo-dimension such that the signal in the last increment was ~30% of the original. Each peak in a pseudo-3D spectrum was fitted with an exponential decay using the Bruker Destrip software. Errors of the relaxation rates were estimated as one standard deviation of the fit. τc-values were calculated as described by Lee et al.79. Errors of τc were determined from the errors of the relaxation rates using the Monte Carlo method (10000 runs). Experiments at varying NaCl concentrations were repeated on two independent samples with similar results.

Temperature calibration. Temperature calibration in solid-state probes was achieved using a solution consisting of 80% ethylene glycol and 20% DMSO-d6, filled into a 1.3 mm rotor, which was then spun at 20 kHz and cooled/ heated with the variable temperature gas flow. The exact temperature was determined from the chemical shift difference of the ethylene and the hydroxyl protons.

Solution NMR experiments. Solution samples (‘soluble state’) of prf.GLFG52 × 12 were prepared by dissolving the lyophilized sample [u-[15N]-labelled for [15N,1H]-TRACT experiments, u-[13C,15N]-labelled for [13N,1H] and 13C-13H HSQC spectra] in ice-cold buffer (20 mM NaPi, pH 6.8, 150 mM NaCl, unless otherwise specified) such that its
concentration was 15 μM. This solution was then centrifuged at 25°C in a temperature-controlled tabletop centrifuge at 18000 × g for 1 h. The supernatant (containing ~3 μM prfGLFG12-12) was then quickly transferred to a 3 mm NMR tube. This procedure ensured the absence of phase separation in solution samples, since the critical concentration of prfGLFG12-12 during centrifugation is lower than that at the temperature applied (24°C) during the measurements. Since D₂O is necessary for locking, however, it could influence phase separation behaviour of FG domains, the 3 mm NMR tube was placed in a 5 mm tube containing pure D₂O due to the concentric placement of the tubes, the samples could still be shimmed adequately.

HSQC spectra were recorded on a 700 MHz Bruker Avance III HD spectrometer equipped with a 5 mm CPPTCI H/F-C/N/D Z-GRD cryoprobe. HSQC spectra were sampled to 1 s on H (for processing, only 400 ms were used) and 50 ms on the heteronucleus. The 15N-H HSQC spectra were recorded with the hsqcetgpsi standard Bruker pulse sequence, also using the standard parameter set. [13N, 1H]-TRACT experiments were performed at 400 MHz. The Bruker Neo spectrometer was equipped with a 5 mm PH QXI-H/P-C/N-D-05 cryoprobe. All spectra were recorded as described above on a 700 MHz Bruker Avance III HD spectrometer and 150 mM NaCl concentration ([15N, 1H]-TRACT, 15N-1H HSQC) were recorded as described above on a 700 MHz Bruker Avance Neo spectrometer equipped with a 5 mm CPPTCI H/F-C/N/D Z-GRD cryoprobe. TRACT experiments at 36 °C were measured on a 700 MHz Bruker Avance Neo spectrometer equipped with a 5 mm CPPTCI H/F-C/N/D Z-GRD cryoprobe. All spectra were recorded on a 700 MHz Bruker Avance III HD spectrometer.

Statistics and reproducibility. No statistical method was used to predetermine sample size. No data were excluded from the analyses. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. Experiments shown in Figs. 1a, 1d, 5a, 7a and Supplementary Figs 2a, 2b, 6, 7 were repeated three times on independent samples with similar results. Experiments shown in Figs. 1c, 1g, 2a and 5c were repeated twice on independent samples with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. A source data file is provided with this article. The data that support the findings of this study are provided in the Supplementary Information or Source Data file or are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

Code availability. The Jupyter notebook for fitting TRACT data is provided in Supplementary File 1.

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E.E.N. and S.C.N. planned and conducted all the experiments. C.G., D.G. and L.B.A. conceived the overall concepts of the study. All authors contributed to experiment design, data analysis, interpretation, and manuscript writing.

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