SPOTLIGHT

FG-nucleoporins caught in the act of liquid–liquid phase separation

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The interior of nuclear pore complexes (NPCs) is densely filled with FG-nucleoporins that form a permeability barrier of a still-obscure nature. Celetti et al. (2019. J. Cell Biol. https://doi.org/10.1083/jcb.201907157) now reveal that FG-nucleoporins can undergo liquid–liquid phase separation and form liquid droplets that mimic permeability barrier properties of intact NPCs.

NPCs regulate the busy bidirectional traffic of macromolecules between the nucleus and cytoplasm. The volume of traffic is enormous, with each nuclear pore complex (NPC) transporting up to 1,000 macromolecules per second in both directions (1). Transport occurs through the central channel of the NPC, which is densely packed with nucleoporins containing intrinsically disordered phenylalanine and glycine repeats (FG-Nups; 2). FG-Nups form the permeability barrier of the NPC; i.e., they prevent the flux of inert macromolecules and only allow passage of larger cargoes that are bound to a nuclear transport receptor (NTR, also called karyopherin or importin/exportin). NTRs interact with FG motifs of central channel FG-Nups through multiple low-affinity binding motifs and thus rapidly cross the NPC, along with their bound cargo (3). In contrast, macromolecules that are unable to interact with FG-Nups in this fashion are prevented from crossing the NPC. For many decades, scientists have puzzled over the structure of the FG-Nup permeability barrier. However, due to the disordered nature of the FG repeat domains, it remains unresolved.

Much of our knowledge of the nature of the permeability barrier comes from in vitro reconstitution experiments that have examined purified FG-Nup repeat domains in the test tube (Fig. 1). It has been known for more than 10 years that FG repeat domains phase separate into solid "hydrogels" (Fig. 1 A). Importantly, these gels fully reproduce the permeability barrier properties of NPCs; i.e., they exclude inert macromolecules and are rapidly penetrated by NTR–cargo complexes.

Yet, is the formation of a mechanically stable hydrogel really required to establish a permeability barrier?

In this issue, a study by Celetti et al. (4) calls our attention to an alternative liquid state of FG-Nups. The authors point to the complexity of molecular aging processes that have recently been observed for several disease-linked RNA-binding proteins (RBPs), e.g., Fused in sarcoma (FUS). FUS undergoes liquid–liquid phase separation (LLPS) and forms liquid-like protein droplets, but then further matures into a solid state in vitro (5). Celetti and colleagues propose that the liquid-like nature of FG-Nups may have been elusive in conventional benchtop/coverslip procedures because it takes several minutes until the formed condensates can be studied under the microscope (4; Fig. 1 A).

To capture the very early phase of FG-Nup phase separation, the team designed a microfluidic device that allowed them to rapidly dilute a highly concentrated stock solution of denatured FG-Nup49 repeat domain within milliseconds into physiological buffer, thus triggering its phase separation. The clever device is also equipped with an optical interrogation region in which the sample flows along a snake-like channel for several minutes and can be observed on an inverted fluorescence microscope. By these means, they could catch phase separation of FG-Nup49 "in flagrante" and saw that the initial FG-Nup condensates were liquid-like droplets (Fig. 1 B). The FG-Nup49 drops displayed properties that are typical for the liquid state; i.e., they coalesced, deformed, and showed fast recovery in FRAP

experiments. Over extended periods of device operation, the droplets indeed appeared to "age" and solidify, eventually leading to clogging of the microfluidic device. These observations provide compelling evidence that the solid hydrogel state of FG-Nups, observed a long time ago in benchtop experiments (6, 7), is preceded by a liquid droplet state and is the result of an in vitro aging phenomenon.

Importantly, Celetti and colleagues put the liquid Nup droplets to the test and examined whether they have NPC-like permeability barrier properties, similar to their solid counterparts (4). To do so, they designed their microfluidic device with a second mixing region, through which they could introduce labeled-cargo molecules and NTRs and observe whether these penetrate the Nup droplets or not. Sure enough, they found that the liquid FG-Nup49 drops were rapidly penetrated by cargo–NTR complexes, but only if the correct NLS and cognate NTR were present. Interestingly, this behavior was not only seen for "normal size" cargo proteins (30–80 kD), but also for a very large model cargo, the recombinant capsid from MS2 bacteriophage; when covered with NLSs and importinα/β, this gigantic cargo was able to accumulate in FG-Nup drops, but in the absence of NLSs and NTR it remained excluded. Small inert proteins lacking an NLS, e.g., mCherry-His$_{12}$ or mCherry, also did not penetrate the FG-Nup49 droplets and either accumulated on the droplet surface in a rim-like pattern or were homogenously excluded. Collectively, these experiments demonstrate that liquid Nup49 droplets perfectly mimic the NPC

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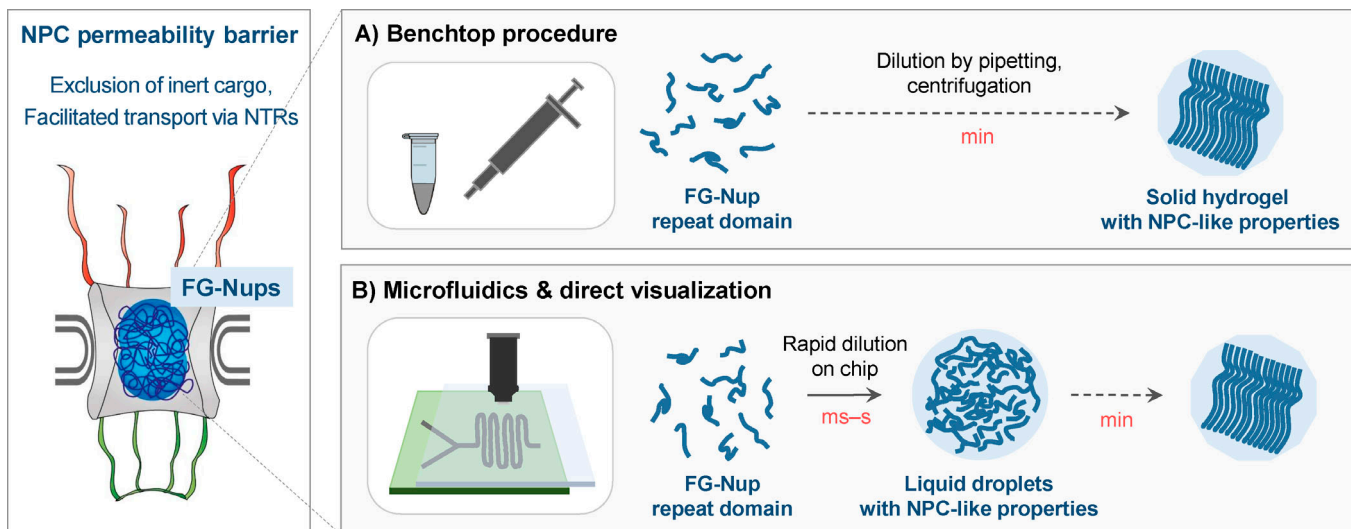
Figure 1. FG-nucleoporins undergo LLPS and form liquid droplets that mimic permeability barrier properties of NPCs. (A) Benchtop procedures have previously shown that purified FG-Nup repeat domains form macroscopic hydrogels in vitro. These solid particles have NPC-like properties; i.e., they exclude inert cargoes and are penetrated by NTR–cargo complexes. **(B)** A new microfluidic device that allows observation of phase-separated FG-Nup condensates immediately after their formation (ms–s timescale) now reveals that FG-Nups initially form fluid droplets before further maturing into a solid hydrogel. Nup droplets already have NPC-like properties, demonstrating that the solid state is not required for the permeability barrier. The NPC cartoon on the left has been reproduced with permission from authors of reference 2 and licensed under BY-NC-ND 4.0.

permeability barrier functions, facilitating transport via NTRs and mediating passive exclusion of inert cargoes. This implies that a stable FG–FG meshwork, as potentially present in FG-Nup hydrogels, is not an essential requirement for forming the permeability barrier. Instead, weak and highly dynamic inter- and intramolecular contacts between FG repeat domains, as present in the liquid state, can already yield NPC-like permeability properties.

The new study raises an important question: if both the liquid and the solid state yield NPC-like permeability properties, which of the two states is the physiological permeability barrier found in the interior of the NPC in vivo? Celetti and colleagues point out that this key issue remains to be determined, as we currently still lack the technologies to directly probe whether the FG-Nups in the nano-sized NPC are liquid-, gel-, or solid-like or a continuum in between (4). The authors note that FG repeat domains of FG-Nups can be >500 amino acids in length, and hence they consider it to be quite possible that a highly dynamic liquid matrix could form even if the FG-Nups are tethered at one end. They further emphasize that a highly dynamic liquid state could explain how cargoes of vastly different sizes, including ribosomal subunits and large viruses, could enter and cross the NPC on a very fast timescale.

Assuming that the liquid state exists in vivo, how is it kept dynamic and liquid-like, considering that in vitro the liquid state is highly transient and rapidly converts to more-solid states? Celetti and colleagues speculate that the heterogeneity of FG-Nups—there are 10 different FG-Nups with distinct disordered regions—could antagonize the solidification process, as previously reported for mixtures of phase-separating and amyloidogenic proteins (8, 9). Second, the authors raise the possibility that the presence of NTRs, which interact with FG residues in the FG repeat domains, may affect the material properties of Nup condensates. Moreover, it is tempting to speculate that posttranslational modifications (PTMs), e.g., O-GlcNAcylation or phosphorylation, could tune the material state of FG-Nup condensates, as PTMs are well-known modulators of intrinsically disordered protein (IDP) interactions and of phase separation (10).

In the future, the new microfluidic device could help to address these interesting hypotheses. The authors propose to implement microrheology on the device, which would allow quantitative measurements of the material properties of the formed droplets over time. The device could also be useful to study the LLPS behavior of other phase-separating proteins that undergo rapid in vitro aging, such as RBPs that form pathological aggregates in neurodegenerative

disorders (5, 11). This promises new insights into the mechanisms that influence and tune the material state of FG-Nups and other IDPs—so stay tuned for more news from the microfluidic device!

Acknowledgments

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