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Evolution of a fuzzy ribonucleoprotein complex in viral assembly

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eLife Assessment

This is a **valuable** study that combines biophysical and evolutionary approaches to understand why particular mutations in the SARS-CoV-2 protein N arose during the COVID-19 pandemic. The evidence is **solid** and supports the conclusions.

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Abstract

Previously we showed that the genetic diversity of SARS-CoV-2 nucleocapsid (N) protein explores a wide range of biophysical properties facilitated by non-local impact of point mutations to its intrinsically disordered regions (Nguyen et al., 2024). This includes modulation of self-association, such as the creation of a *de novo* binding interface through the P13L mutation characteristic of Omicron variants. In the present work we focus on the key function of N condensing viral RNA into ribonucleoprotein particles (RNPs) for viral assembly. Lacking high-resolution structural information, biochemical and biophysical approaches have revealed architectural principles of RNPs, which involve cooperative interactions of several protein-protein and protein-RNA interfaces, initiated through oligomerization of conserved transient helices in the central disordered linker of N. Here we study the impact of defining N-protein mutations in variants of concern on RNP formation, using biophysical tools, a virus-like particle assay, and reverse genetics experiments. We find convergent evolution in repeated, independent introduction of amino acid substitutions strengthening existing binding interfaces, compensating for other substitutions that promote viral replication but decrease RNP stability. Furthermore, we show that the P13L mutation of Omicron variants enhances RNP assembly and increases viral fitness. Overall, our data reveal RNP complexes to be highly variable not only in sequence and conformations, but also in thermodynamic and kinetic stability, with its pleomorphism affecting basic architectural principles. We hypothesize that the formation of polydisperse, fuzzy N-RNA clusters with multiple distributed weak binding interfaces optimizes reversible RNA condensation, while supporting host adaptation and allowing for a large sequence space to be explored.

Introduction

Intrinsically disordered proteins are ubiquitous and play key role in many dynamic cellular processes, including cellular signaling, transcriptional regulation, as well as spatio-temporal organization (Dyson and Wright, 2005 [DOI](#); Holehouse and Kragelund, 2024 [DOI](#)). Intrinsic disorder is particularly prevalent in proteins of RNA viruses, for reasons deeply connected to evolution in presence of their high mutation rates and quasispecies nature (Brown et al., 2011 [DOI](#); Gupta and Uversky, 2024 [DOI](#); Tokuriki et al., 2009 [DOI](#); Xue et al., 2014 [DOI](#)).

On a physicochemical level, disorder supports multi-functionality through modulation of the conformational ensemble dependent on ligands and post-translational modifications (Botova et al., 2024 [DOI](#); Carlson et al., 2022 [DOI](#), 2020 [DOI](#); Jack et al., 2021 [DOI](#); Ranganathan et al., 2023 [DOI](#); Savastano et al., 2020 [DOI](#); Syed et al., 2024 [DOI](#)) and the local environment (Nesmelova et al., 2019 [DOI](#); Roden et al., 2022 [DOI](#)), the possibility of transient folding (Alderson et al., 2023 [DOI](#); Bessa et al., 2022 [DOI](#); Zachrdla et al., 2022 [DOI](#); Zhao et al., 2022 [DOI](#)), and the propensity for protein clustering and liquid-liquid phase separation (LLPS) (Alberti et al., 2019 [DOI](#); Brocca et al., 2020 [DOI](#); Perdikari et al., 2020 [DOI](#); Ranganathan and Shakhnovich, 2020 [DOI](#); Savastano et al., 2020 [DOI](#); Zachrdla et al., 2022 [DOI](#)). Disorder also permits a significant degree of sequence variation, as many functionally important features are not dependent on the specific sequence but are encoded in non-local biophysical properties (Alston et al., 2023 [DOI](#); Nguyen et al., 2024 [DOI](#); Zarin et al., 2021 [DOI](#)). Opposite to the achievement of mutational tolerance through high stability, such as exhibited, for example, by thermostable enzymes, mutational tolerance can be achieved in loosely packed and disordered proteins by lowering the potentially deleterious effect of mutations on stability (Tokuriki et al., 2009 [DOI](#); Tokuriki and Tawfik, 2009 [DOI](#)). In addition, disorder and flexibility magnifies potential impact of mutations on conformation and biophysical properties (Nguyen et al., 2024 [DOI](#)). Furthermore, the high degree of sequence variability supports the exploration of short linear interaction motifs (SLiMs) in intrinsically disordered regions for interfacing with a large variety of eukaryotic regulatory and signaling processes, thus favoring adaptability and evolvability on the level of the host-virus interface (Davey et al., 2011 [DOI](#); Duro et al., 2015 [DOI](#); Hagai et al., 2014 [DOI](#); Schuck and Zhao, 2023 [DOI](#)).

In recent years it has become apparent that assemblies of disordered proteins frequently retain significant disorder and conformational flexibility of their interaction partners, generating “fuzzy” complexes (Longhi et al., 2017 [DOI](#); Tokuriki et al., 2009 [DOI](#); Tompa and Fuxreiter, 2008 [DOI](#)). The potential functional advantages of intrinsic disorder in protein interactions include leveraging of weak binding interfaces through allovalency and the adaptability to multiple binding partners (Longhi et al., 2017 [DOI](#); Olsen et al., 2017 [DOI](#)). In fuzzy complexes the total binding energy is distributed into multiple distinct ultra-weak interaction sites (Olsen et al., 2017 [DOI](#)). Similar to individual RNA virus proteins with loose or absent structure, maintaining disorder and a spatial distribution of low-energy interactions in the protein complexes may increase the tolerance for mutations and improve evolvability of protein complexes.

The unprecedented worldwide sequencing effort of SARS-CoV-2 genomes during its rapid evolution in humans provides a unique opportunity to examine these concepts. The genomic database now exceeds in size that of any other virus by orders of magnitude (Elbe and Buckland-Merrett, 2017 [DOI](#); Rochman et al., 2022 [DOI](#)), providing the basis for phylogenetic analyses and for monitoring the emergence of variants of concern and their geographic spread (Hadfield et al., 2018 [DOI](#)). In addition, it exhaustively samples the mutational landscapes of amino acids that can occupy any position of the viral proteins, which reflects their biophysical constraints (Bloom and Neher, 2023 [DOI](#); Nguyen et al., 2024 [DOI](#); Zhao et al., 2022 [DOI](#)). For example, the SARS-CoV-2 nucleocapsid (N)-protein – the most abundant viral protein in infected cells and the focus of the present work – has 419 positions, 86% of which can be assumed by on average 4-5 different amino acids, and up to 12 in positions in the three intrinsically disordered regions (Zhao et al., 2022 [DOI](#)) (Figure 1A [DOI](#)), evidently without fatally compromising dozens of reported N-protein functions (Wu et al., 2023 [DOI](#)). Observed N-protein mutations can modulate basic biophysical properties including its oligomeric state, thermodynamic stability of its two folded domains, LLPS propensity, charge

distributions, and secondary structure content (Nguyen et al., 2024; Zhao et al., 2022), as well as its interactions with RNA (Cubuk et al., 2024; Dhamotharan et al., 2024), kinases (Johnson et al., 2022; Syed et al., 2024) and other host proteins through altered SLiMs (Li et al., 2025; Ren et al., 2024; Schuck and Zhao, 2023; Tugaeva et al., 2023).

In the present work we study the eponymous function of N-protein, which is the spatial condensation of the long genomic RNA (gRNA) into ribonucleoprotein particles (RNPs) for viral assembly. N-protein has two folded domains (the nucleic acid binding domain and the C-terminal dimerization domain, NTD and CTD, respectively), and three intrinsically disordered regions that include the linker between NTD and CTD, and the N-terminal and C-terminal extensions, N-arm and C-arm, respectively (Figure 1A) (Chang et al., 2006). The intrinsically disordered regions amount to $\approx 45\%$ of total residues, which renders N-protein highly flexible with a radius of gyration fluctuating from 5 nm to >8 nm (Różycki and Boura, 2022). RNPs are ≈ 15 nm diameter particles composed of ≈ 10 -15 N-proteins that bind stretches of gRNA (Klein et al., 2020; Yao et al., 2020). 30-40 RNPs are distributed like beads on a string in the ≈ 100 nm virion (Klein et al., 2020; Yao et al., 2020). RNPs appear highly heterogeneous in electron microscopy (EM) (Carlson et al., 2022; Landeras-Bueno et al., 2025; Yao et al., 2020), and as of now, a high-resolution structure has not been determined. The Morgan laboratory has described an *in vitro* model for the assembly of RNPs, where N-protein in the presence of stem-loops of the 5'-UTR RNA readily forms polymorphic ribonucleoprotein complexes that match in size, symmetry, and RNA content what would be expected for the assembled RNPs in virions (Carlson et al., 2022, 2020). In conjunction with biophysical experiments and point mutations exposing essential binding interfaces, this has allowed us recently to develop a coarse-grained structural model of RNPs (Zhao et al., 2024). To examine how architecture and energetics of RNP assemblies can be impacted by N-protein mutations we study a panel of N-proteins derived from ancestral Wuhan-Hu-1 and different variants of concern, including Alpha, Delta, Lambda, and Omicron (see Table 1), in biophysical experiments, VLP assays, and mutant virus. Specifically, we ask how the RNP size distribution and life-time is modulated by: (1) the novel binding interface created by the P13L mutation of Omicron; (2) enhancements of other weak self-association interfaces through G215C of Delta and G214C of Lambda; (3) the ubiquitous R203K/G204R double mutation of Alpha, Lambda, and Omicron. We also test whether the P13L mutation improves viral fitness, similar to G215C and R203K/G204R. The results are discussed in the framework of fuzzy complexes and molecular evolution of N in the course of viral adaptation to the human host. Understanding the salient features of the binding interfaces in viral assembly and their evolution expands our foundation for the design of therapeutics such as assembly inhibitors.

Results

Essential protein-protein interfaces in RNP assembly

Based on biophysical experiments of protein-protein and protein-NA interfaces of full-length (FL) N-protein and domain constructs, in combination with the *in vitro* RNP assembly assay (Carlson et al., 2022, 2020) we have recently proposed a coarse-grained structural model of RNPs that satisfies all known binding interfaces. These include at least two known sites of protein-protein and two for protein-nucleic acid interactions distributed throughout most of the protein (Zhao et al., 2024) (Figure 1B/C). N-proteins are constitutive dimers with K_D in the low nM range by virtue of domain-swapped beta hairpins in the CTD (Chang et al., 2006; Cubuk et al., 2025; Yu et al., 2005; Zhao et al., 2021; Zinzula et al., 2021). N dimers exhibit only ultra-weak further self-association with ≈ 1 mM K_D (Yu et al., 2005; Zhao et al., 2022, 2021) (although nucleic acid impurities can artificially scaffold N-protein into higher oligomers (Carlson et al., 2020; Tarczewska et al., 2021)). However, higher-order assembly is initiated by the occupation of the nucleic acid binding site in the NTD, which allosterically promotes a helical conformation of the leucine-rich sequence (LRS) in the intrinsically disordered linker, allowing LRS helices to form promiscuous coiled-coil oligomers stabilized through hydrophobic interactions with low μ M K_D (Zhao et al., 2023). As demonstrated with the LRS point mutant N:L222P that abrogates these transient helices, LRS oligomerization forms the basis for oligomerization of N-protein dimers into

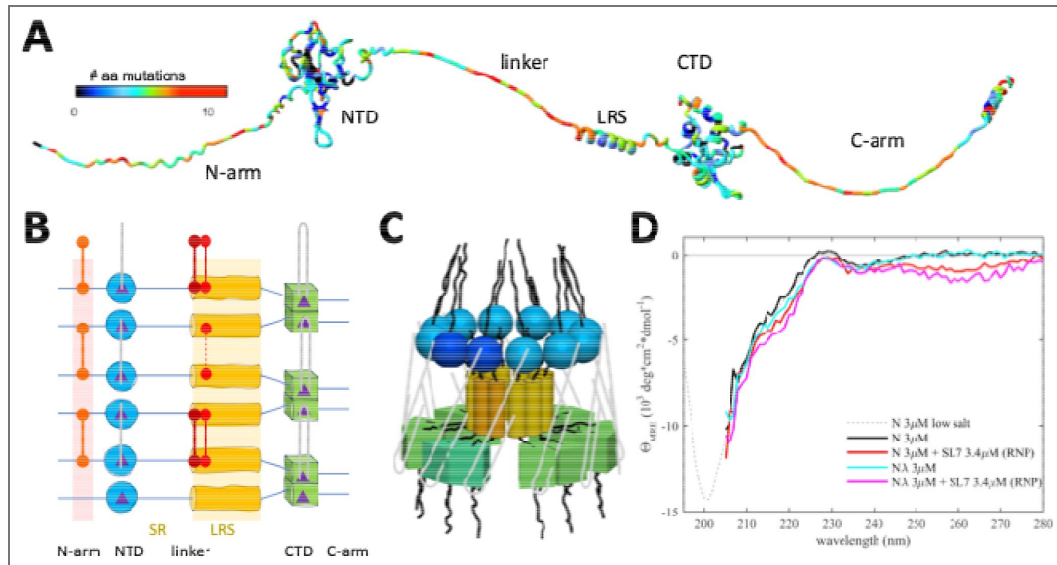


Figure 1. Basic organization of N-protein and RNPs.

N-protein (1-419) has two folded domains, NTD (45-180) and CTD (248-363), and three intrinsically disordered regions including the N-arm (1-44), the central linker (181-247) and the C-arm (364-419). (A) Displayed is an AF2 structure where the disordered N-arm, linker, and C-arm are artificially stretched for clarity. The residues are color-coded according to the number of different amino acids that have been observed at this position in the mutational landscape replacing the Wuhan-Hu-1 sequence. The bioinformatic analysis was carried out as described, previously (Zhao et al., 2023), updated to August 26, 2024, using a threshold of >5 genomes for each mutation. (B) Schematic of protein-protein and protein/RNA interfaces in RNP assembly. The nucleic acid binding domain at the N-terminus (NTD) is indicated in blue, the LRS in yellow, and the dimerization domain (CTD) in green. Regions of self-association are indicated by shaded backgrounds. The linker is subdivided in a serine and arginine-rich region (180-205, SR) and a L-rich region (206-247, LRS). LRS can transiently fold into helices that create a hydrophobic patch for promiscuous self-association (indicated as yellow pattern). For clarity the cartoon only shows 3 neighboring N-protein dimers, although higher-order oligomers assemble in RNPs. Nucleic acid binding sites (purple triangles) preferentially bind single-stranded RNA at the NTD (grey lines), and double stranded RNA at the two sites per CTD dimer, with the ability to cross-link neighboring dimers potentially in various configurations. New inter-dimer interactions evolved in variants of concern are indicated by red connectors, including the promotion of beta-sheet oligomerization through the N:P13L mutation in the N-arm (as in Omicron and Lambda variants), and the introduction of cysteines at the base of the LRS helices in N:G214C (as in Lambda variants) and N:G215C (as in Delta variants). (C) Three-dimensional cartoon of the circular organization of N-protein domains in RNPs, with one dimer shaded slightly darker to highlight the dimeric building blocks. For clarity, subunit sizes are not drawn to scale. Alternate arrangements are depicted in Supplementary Figure S1. (D) CD spectra of N-protein in the presence of SL7 under near physiological salt conditions leading to majority assembly of RNPs. Spectra are corrected for free SL7 contributions. Shown are spectra of ancestral N-protein alone (black), in the presence of SL7 forming RNPs (red), N_λ alone (cyan) and in the presence of SL7 forming RNPs (magenta). Spectra are truncated at < 205 nm due to limited buffer transparency. For comparison the dotted line shows a previously published CD spectrum of ancestral N-protein in low-salt buffer that permits measurement at shorter wavelengths (Nguyen et al., 2024). Triplicate scans yield average standard deviations of 0.13 (N), 0.17 (N+SL7), 0.16 (N_λ), and 0.21 (N_λ +SL7) 103 deg cm²/dmol, respectively, with non-overlapping confidence bands for the different species, for example, between 215-220 nm.

designation	N-protein mutations	in set of defining VOC mutations ⁽¹⁾	predominant oligomeric state at low μ M concentrations	reaction boundary <i>s</i> -value in RNP assay (S)	MP final average Mw in 500-1500 kDa range (kDa)	best-fit mass increase from 0.3 to 3 μ M (kDa)	best-fit effective RNP life-time (sec)
N (ancestral)	none	Wuhan-Hu-1	dimer	19.7	614	63.8	66.3
N:P13L	P13L	λ , o (all)					
N: Δ 31-33	Δ 31-33	o (all)					
N:P13L/ Δ 31-33	P13L, Δ 31-33	o (all)	dimer	20.2	625	118.2	43.6
N:R203K/G204R	R203K, G204R	α , γ , λ , ζ , and o (except XEC)	dimer	19.5	596	70.3	54.6
N _o	P13L, Δ 31-33, R203K, G204R	o (except XEC)	dimer	20.5	617	77.2	58.5
N:G215C	G215C (reduced)	δ (all 21J)	dimer/tetramer	20.4	619	47.8	231
N:G215C*	G215C (oxidized)	δ (all 21J)	tetramer	20.8	649	56.1	41.8
N _{λ}	P13L, R203K, G204R, G214C (reduced)	λ	dimer/tetramer	21.0	669	20.0	67.4
N _{λ} *	P13L, R203K, G204R, G214C (oxidized)	λ	tetramer	21.5	660	51.6	98.2
N:R203M	R203M	δ , κ					
N ₂₁₀₋₄₁₉ *	Δ 1-209	o (not XEC)					

⁽¹⁾ Referring to the most common mutations in the variants of concern, excluding sporadic spontaneous reversions or other variations.

Table 1. Overview of N-protein mutant species studied

RNPs, for example, dodecamers as hexamers of dimers. These oligomers are stabilized by multi-site interactions with double-stranded RNA simultaneously in two sites of the CTD dimer, and by binding preferentially single-stranded RNA to each of the NTDs (Cubuk et al., 2024; Iserman et al., 2020; Korn et al., 2023; Roden et al., 2022), with both RNA interactions allowing inter-dimer interactions that crosslink N-protein dimers within the RNP (Figure 1C) (Zhao et al., 2024). Additional stabilizing protein-protein interfaces may exist in the C-arm (Carlson et al., 2022; Q. Ye et al., 2020), although we have been unable to detect self-association of the C-arm by analytical ultracentrifugation up to low mM concentrations (Zhao et al., 2023).

As suggested in the cartoon of Figure 1C, this supports the hypothesis of a three-dimensional arrangement with a central LRS oligomer with symmetry properties and dimensions similar to low resolution EM images of model RNPs (Carlson et al., 2022, 2020) and cryo-ET of RNPs in virions (Klein et al., 2020; Yao et al., 2020). It should be noted, however, that the arrangement sketched in Figure 1C is not unique and other subunit orientations could be envisioned that satisfy all constraints from experimentally observed binding interfaces, including different oligomers and anti-parallel subunits as illustrated in Supplementary Figure S1. Extending previous ColabFold structural predictions that show multiple N-protein dimers self-assembled via the LRS coiled-coils (Zhao et al., 2023), we attempted the AlphaFold modeling of RNPs combining multiple N dimers with SL7 RNA ligands, mimicking our biophysical assembly model. Current AlphaFold restrictions limit the prediction to pentamers of N-protein dimers with 10 copies of SL7 RNA. While only inconsistent results were obtained – which is not surprising given the large intrinsically disordered regions exceed the predictive power of AlphaFold – some models did produce an overall RNP organization similar to Figure 1C, suggesting such an arrangement is at least sterically reasonable with regard to possible N-protein subunit orientations in an RNP (Supplementary Figure S2).

Using the *in vitro* RNP assembly model of N-protein in mixtures with stem-loop RNA SL7 we measured secondary structure content in circular dichroism (CD) experiments (Figure 1D). While a significant structural change in the RNA when bound to N-protein can be deduced in the near UV, after correction for RNA contributions, in the far-UV the CD spectrum of the RNP mixtures is very similar to that of N-protein alone. A small gain in helicity can be discerned for RNP assembly mixtures at ≈ 220 nm, consistent with the expected coil transition in the LRS stabilizing the RNP. Similarly, small increases in helicity can be discerned for LRS helix-stabilizing cysteine mutants. It is noteworthy that the CD spectrum of the RNP mixture appears to be dominated by disordered chains. Although their signature minimum ellipticity at ≈ 200 nm cannot be observed directly in the high salt buffer required for RNP assembly, the data are quantitatively consistent in their strongly decreasing slope from 205 – 210 nm with the previously measured disordered signature of N-protein in low-salt buffer (without RNP formation). This result is consistent with the absence of significant structure formation in the RNP, and with N-protein intrinsically disordered regions retaining most of their disorder outside the LRS.

As is depicted in the diagram Figure 1B, assembly of approximately 6 N-protein dimers and ≈ 500 bp of RNA requires simultaneous binding at multiple protein-protein and protein-RNA interfaces. Many of these interactions are weak, but they are multivalent and act cooperatively (Zhao et al., 2024). This feature may aid in dynamic assembly and disassembly, and shape the ensemble of complex states to efficiently populate functioning RNPs. As we describe in the following, mutations of N-protein have led to diverse mechanisms modulating and promoting RNP formation through their effect on protein-protein interfaces relevant to RNP stability.

A novel self-association interface through transient β -sheets enabled by the P13L mutation

In early epidemiological analyses, the N:P13L mutation has been identified as the most important driver for N-protein fitness, and it has become an obligatory mutation of all Omicron variants (Obermeyer et al., 2022; Oulas et al., 2021). In our recent survey of biophysical effects of N-protein mutations relative to the ancestral protein, we observed a distinct ability of the N-arm mutant peptide N₁₋₄₃:P13L to form large assemblies at $\approx$ mM concentrations (which is not seen

with the ancestral peptide), while full-length N:P13L exhibited enhancement of LLPS (Nguyen et al., 2024), both indicative of weak interactions. Furthermore, after prolonged storage of P13L N-arm peptide solutions at $\approx$ mM concentrations at 4 °C, an increase in the solution viscosity was observed (Nguyen et al., 2024). In the present work we studied these effects of the P13L mutation in more detail. As shown in Figure 2A, negative-stained EM images show the formation of fibrils of N-arm peptides of Omicron variant N₁₋₄₃:P13L, Δ 31-33. Similarly, fibril formation was observed for N₁₋₄₃:P13L N-arm peptide lacking the deletion Δ 31-33, but not in controls with ancestral N-arm, the ancestral N-arm carrying only the deletion N₁₋₄₃: Δ 31-33, or the disordered ancestral C-arm (Supplementary Figure S3). Thus, the N-arm mutation P13L is responsible for the formation of fibrils in N-arm peptides after prolonged storage. Some of these N-arm fibrils exhibit a twisted morphology with width of $\approx$ 5 nm (Figure 2A), in some instances exhibiting patterns of strand breaks. Such fibrils are frequently encountered in proteins that can stack β -sheets, such as in amyloids (Paravastu et al., 2008). While we have not observed fibril formation in the context of full-length N, and have no evidence such fibrils are physiologically relevant, their occurrence in solutions of truncated N-arm peptide nonetheless demonstrates the introduction of ordered N-arm self-association interfaces in conformations of P13L mutants.

Based on NMR studies of the N-arm, Zachrdla et al. previously reported a propensity for residues 13-19 to transiently populate extended/ β -structure-like conformations (Zachrdla et al., 2022), which we hypothesized may be strengthened through the P13L mutation. To test the formation of β -sheet structure in N:P13L we carried out CD experiments of ancestral N-arm N₁₋₄₃, and mutants carrying the P13L mutation, or Δ 31-33, or both in the Omicron variant N₁₋₄₃:P13L, Δ 31-33 (Figure 2B). While the ancestral N-arm at $\approx$ 1 mM ($\approx$ 4.6 mg/ml) concentrations exhibits CD spectra with a minimum at $\approx$ 200 nm typical of disordered conformations (black), the Omicron N-arm has a significantly higher structure content (magenta), consistent with β -sheets, as revealed by the strong negative mean residue ellipticity in the 210-220 nm range. Interestingly, diluting the 1 mM sample (solid) to a concentration of 0.4 mM (dashed) reveals a large shift in the far-UV spectra from positive to negative ellipticity at $\approx$ 200 nm, as well as a shift in the minimum to lower wavelengths, both indicative of a significant increase of disorder upon dilution. This is consistent with the stabilization of β -sheets in a reversible, strongly cooperative self-association process with an effective K_D in the high μ M to low mM range. Dissecting the origin of the increased β -sheet content, it is apparent that the majority of the effect arises from the P13L mutation (red) alone, with a minor contribution by Δ 31-33.

Finally, confirming the interpretation of the EM images and the CD data, as well as the β -structure propensity reported from NMR data (Zachrdla et al., 2022), the structural prediction of N₁₀₋₂₀:P13L in ColabFold displayed oligomers with stacking β -sheets of residues 12-18 with typical hydrogen bond patterns (Figure 2C), whereas ancestral peptides did not lead to well-organized structures (Supplementary Figure S4). Analogous predictions of N-arms with two other frequent mutations, P13S and P13T, did not lead to β -sheet structures as in P13L.

While this self-association interface in the P13L N-arm is weak and its direct observation in biophysical experiments requires mM concentrations, which far exceed average intracellular concentration of N, such weak interactions can become highly relevant physiologically when high local concentrations are prevailing, for example, when the disordered extension is pre-concentrated while tethered within macromolecular assemblies as in the RNP, or in macromolecular condensates.

Enhanced oligomerization of the leucine-rich sequence through cysteine mutations

The protein-protein interaction interfaces driving LRS self-association have been studied in great detail in biophysical experiments and MD simulations for the ancestral molecule and several mutations in the mutational landscape (Zhao et al., 2022). A key feature is a pattern of hydrophobic residues that can combine to form a transient helix creating a hydrophobic surface stretching from $\approx$ 222 to 234 on one side of the helix. We have previously discovered the self-association of the LRS after analysis of the mutational landscape (Zhao et al., 2022) and reports

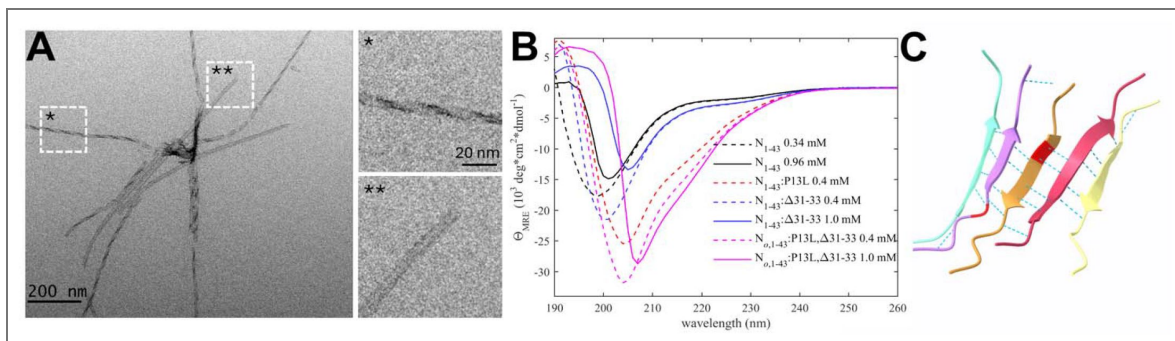


Figure 2. The P13L mutation creates self-association interfaces in the N-arm through stabilization of β -sheets.

(A) Electron micrograph of negatively stained omicron N-arm $N_{o,1-43}:P13L,\Delta31-33$ after equilibration at 10 μM in 20 mM HEPES, 150 mM NaCl, pH 7.50. The magnified regions are examples of twisted (*) and straight (**) fibrils. **(B)** CD spectra of $N_{1-43}:P13L$ (red), $N_{1-43}:\Delta31-33$ (blue), and Omicron $N_{o,1-43}:P13L,\Delta31-33$ (magenta) at 0.4 mM (dashed) and 1.0 mM (solid), in comparison with ancestral N-arm (black). **(C)** Subset of ColabFold prediction of multimers of $N_{10-20}:P13L$ highlighting hydrogen bonds. The P13L residue is highlighted in red in the middle peptide.

of a conspicuous defining mutation N:G215C in Delta variants that correlated with its rise in 2021 among clades with identical spike mutations (Marchitelli et al., 2021 [↗](#); Stern et al., 2021 [↗](#); Zhao et al., 2022 [↗](#)). As deduced from MD simulations, the cysteine at position 215 is located at the base (N-terminal end) of the transient helix and, through its lower flexibility than the glycine, serves to redirect the adjacent upstream disordered residues such that helices are more prone to form stabilizing coiled-coil interactions (Zhao et al., 2023 [↗](#), 2022 [↗](#)). As shown experimentally by sedimentation velocity analytical ultracentrifugation (SV-AUC) in reducing conditions, this enhances non-covalent self-association of LRS peptides as well as full-length N dimers by 2-3 orders of magnitude (Zhao et al., 2023 [↗](#), 2022 [↗](#)). Covalent disulfide bonds in the LRS in non-reducing conditions were found to further promote LRS oligomerization. However, there is no conclusive data yet whether covalent bonds in the LRS occur *in vivo*, or any G215C effect is entirely non-covalent due to the significant strengthening of LRS helix oligomerization (see Discussion). In any event, the G215C mutation leads to enhanced assembly in a VLP assay (Zhao et al., 2024 [↗](#)), and as shown in reverse genetics experiments, *in vivo* confers a significant replication advantage and an altered virion morphology (Kubinski et al., 2024 [↗](#)).

Here we studied a mutation of G214, which in the mutational landscape exhibits a similar mutation pattern as G215. In particular, we focus on an independent introduction of a cysteine in the LRS that occurred in the Lambda variant, prevalent in South America in 2020-2021 (Wink et al., 2022 [↗](#)), with the defining N-protein mutations P13L, R203K/G204R, and G214C (Table 1 [↗](#)). (We will adopt a nomenclature where the complete set of defining mutations of a variant will be referred to by its Greek letter, i.e., N:P13L/R203K/G204R/G214C is N_λ , and analogously the set of Omicron mutations N:P13L/Δ31-33/R203K/G204R are referred to as N_θ ; see Table 1 [↗](#)). The effect of the G214C mutation is unknown. Due to the close proximity of 214 and 215, we asked whether it enhances LRS self-association similarly to G215C, and to this end first synthesized a LRS peptide comprising N₂₁₀₋₂₄₆:G214C. Unexpectedly, unlike the chemically identical N₂₁₀₋₂₄₆:G215C peptide, it exhibited low solubility. This prohibited its characterization at sufficiently high concentrations for study of self-association by SV-AUC, which required in excess of ≈0.4 mM for N₂₁₀₋₂₄₆:G215C peptides to display oligomers. However, dynamic light scattering (DLS) revealed the presence of N₂₁₀₋₂₄₆:G214C complexes with hydrodynamic radii ranging from 6 to 40 nm (in comparison to 1-2 nm for N₂₁₀₋₂₄₆:G215C (Zhao et al., 2022 [↗](#))) in reducing conditions, and slightly larger in non-reducing conditions (Supplementary Figure S5 [↗](#)). For N₂₁₀₋₂₄₆:G214C a cumulant analysis results in radii of 8.8 nm and 10.6 nm and polydispersity indices of 0.40 and 0.35 for reducing and non-reducing conditions, respectively. This shows that while G214C also strengthens the LRS self-association interface, it exhibits different properties compared to G215C.

To gain more insight in the different behavior of the cysteine LRS mutants we carried out MD simulations of monomeric and trimeric LRS peptides N₂₁₀₋₂₄₆. As shown in Figure 3 [↗](#), both cysteine mutants extend the helix by stabilizing the flexible GG motif into a well-defined α-helix turn. Importantly, however, the sulfhydryl groups in position 214 vs 215 assume different orientations relative to the hydrophobic patch serving as the oligomerization interface. Under reducing conditions, this has the potential to also cause variation in physicochemical properties of resulting oligomers, e.g., through repositioning of the adjacent glutamate E216 and resulting changes in the surface electrostatic potential, and to thereby alter the oligomerization scheme (Supplementary Figure S6 [↗](#)). It can be expected that under oxidative conditions the differences between 214C and 215C are exacerbated due to their different relative orientation of the hydrophobic interface of the helix and the sulfhydryl groups, leading to different oligomeric states and different phase separation properties.

Impact of enhanced self-association interfaces on the oligomerization of full-length N

In principle, since N-protein is a constitutive dimer coupled in the CTD, binding interfaces in the LRS and N-arm may form intra-dimer bridges simply further stabilizing the dimeric state. Indeed, coarse-grained molecular simulations of N-protein dimers have revealed large conformational fluctuations where the LRS, for example, can make rare intra-dimer contacts (Różycki and Boura,

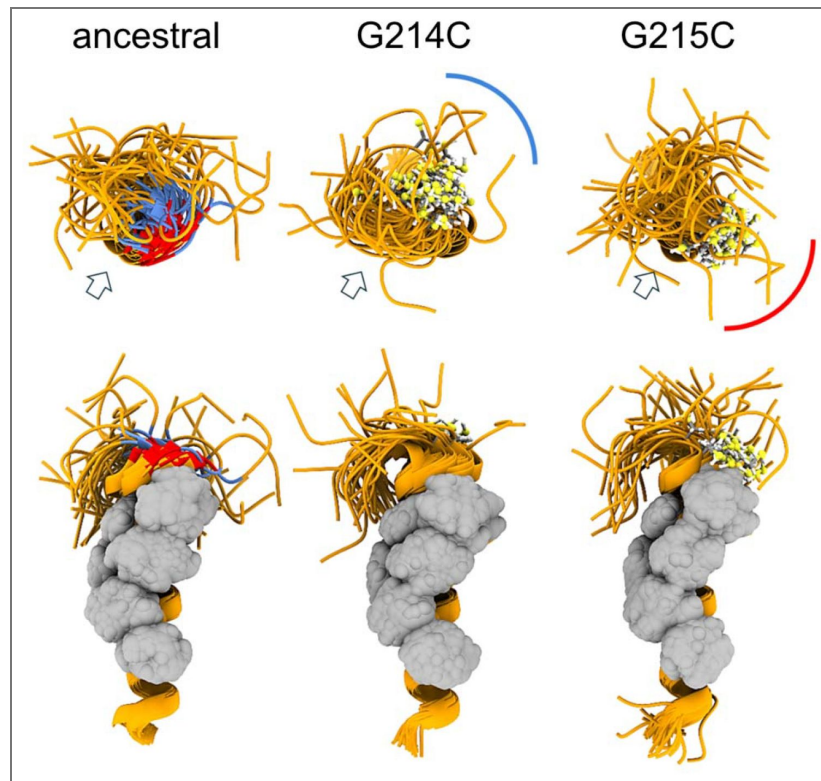


Figure 3. MD simulation of ancestral LRS and comparison with 214C and 215C mutants of Delta and Lambda variants.

Snapshots at equal time intervals (4 ns) taken from the 200-ns MD simulations of the ancestral peptide N₂₁₀₋₂₄₆ and single-point G → C mutants (all monomers were oriented by overlaying the helical region and displayed in orthosteric view). The upper row shows a view from the N-terminus side (with the helix axes perpendicular to the plane of the figure), while the lower row presents a side view (with axes on the plane). For clarity, the disordered C-terminal segment (residues 236-246) has been removed. The glycine and cysteine residues at positions 214 and 215 (colored blue and red, respectively, in the ancestral peptide and rendered as ball-and-stick in the mutants) restructure the flexible backbone around the GG motif into a well-defined α -helix turn, directing the sulfhydryl group in specific orientations (illustrated schematically by the blue and red arches). These orientations can be quantified relative to the Leu-rich central region (indicated by arrows and rendered as gray van der Waals spheres), which forms the hydrophobic interfaces of the oligomers (Zhao et al., 2023). Under reducing conditions, this reorientation of the N-terminus relative to the helix can influence helix binding during the early stages of oligomerization or alter the conformation and physicochemical properties of the resulting oligomers, as illustrated in Supplementary Figure S6.

2022). On the other hand, given the large flexibility of the disordered chain, the same interfaces may lead to higher oligomers if they establish one or two inter-dimer bridges across different dimers, generating different classes of tetramers. We would expect a concentration-dependent probability of such inter-dimer contacts for N-protein in solution.

Experimentally, in the absence of nucleic acid ligands, ancestral N-protein at low μM concentrations is essentially dimeric with only hints of reversible higher-order oligomers (Yu et al., 2005; Zhao et al., 2021). With the strengthened LRS helix stability through the G215C mutation, in reducing conditions, we observe reversible tetramerization with a K_D of 1.0 (0.7 – 1.5) μM (Figure 4A), consistent with previous work (Zhao et al., 2022). We made analogous observations by SV-AUC for N_λ (containing the G214C mutation) in reducing conditions, demonstrating it similarly can form reversible inter-dimer bonds (Figure 4A). Clearly, strengthened LRS helices can alter the N-protein oligomeric state by crosslinking dimers into tetramers.

To study the potential impact of disulfide bonds on N-protein oligomeric state, we prepared oxidized full-length N:G215C and N_λ by extensively dialyzing the reduced protein in TCEP-free buffer while purging air through the dialysate for gentle passive oxygenation. This oxidized sample is referred to as N:G215C* and N_λ^* . Using a DTNB assay we assessed the percentage of free sulfhydryl groups to be $\approx 30\%$ for both N:G215C* and N_λ^* , respectively. Since the LRS mutation provides the sole cysteine in N-protein, we concluded that the majority of N:G215C* and N_λ^* is disulfide-linked in the LRS, which was confirmed by non-reducing SDS-PAGE (Supplementary Figure S7). Due to the non-covalent dimerization in the CTD as well as non-covalent tetramerization via LRS interfaces (see above), these samples may be complex mixtures of dimers and/or higher oligomers with different patterns of covalent and non-covalent intra- and inter-dimer LRS interactions. Using SV-AUC we established that close to half of both the N:G215C* and N_λ^* sample is tetrameric at low μM concentrations (Figure 4A). Because oxidation significantly increases the tetramer population, we conclude that one or two covalent inter-dimer bonds can form that enhance N-protein oligomerization.

In contrast to the modulation of the coiled-coil LRS interfaces, the *de novo* creation of the N-arm self-association interface through beta-sheet interactions enabled by P13L cannot be readily observed in full-length N-protein at low μM concentrations. Similar to the ancestral LRS interface, it provides only ultra-weak binding energies that require mM concentrations to significantly populate oligomers. This is fully consistent with the previous observation by SV-AUC that neither N:P13L, Δ 31-33 nor N_0 with the full set of Omicron mutations show any significant higher-order self-association at low μM concentrations, whereas at high local concentrations – as observed in phase-separated droplets – they can modulate and cooperatively enhance self-association processes (Nguyen et al., 2024). (In fact, P13L can substitute for the LRS promoting LLPS, as observed in the rescue of LLPS by N:P13L, Δ 31-33/L222P mutants whereas N:L222P LRS-abrogating mutants are deficient in LLPS.) Another process that increases the local concentration of N-arm chains is the tetramerization of full-length N-protein. As described earlier, occupancy of the NA-binding site in the NTD allosterically promotes self-assembly of the LRS into higher oligomers (Zhao et al., 2021). We hypothesized that these oligomers may be cooperatively stabilized by additional N-arm interactions in P13L mutants.

To this end, we carried out SV-AUC and DLS experiments of 2 μM ancestral N, and the full-length N-arm mutant N:P13L, Δ 31-33 in the presence of a short oligonucleotide T_{10} that occupies the NTD binding site but is too short for scaffolding multiple N-protein dimers (Zhao et al., 2021) (Figure 4B). Low salt buffer (10 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2PO_4 , 1.8 mM KH_2PO_4 , pH 7.4) in this experiment ensures maximal occupancy of the NTD binding site for nucleic acid by 10 μM T_{10} , which under these conditions has a K_D below 0.1 μM (Zhao et al., 2021). In a control experiment by SV-AUC we measured the affinity of the T_{10} oligonucleotide for ancestral N and N:P13L, Δ 31-33 and observed no significant difference in moderate ionic strength (Supplementary Figure S8). Ancestral N protein in the absence of oligonucleotide sediments at ≈ 4.0 S, reflecting its dimeric state afforded by the CTD dimerization domain (Zhao et al., 2021). When LRS oligomerization is abrogated through introduction of a L222P mutation, binding of T_{10} to the N-protein dimer still

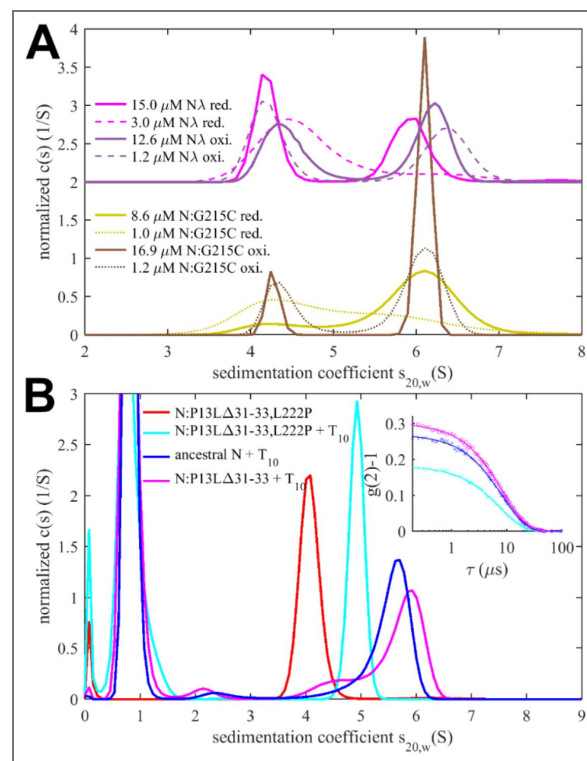


Figure 4. Impact of mutations in self-association interfaces on oligomeric state.

(A) Sedimentation coefficient distributions of cysteine mutants N:G215C (reduced in yellow) and N:G215C* (oxidized in brown), as well as reduced N λ (reduced in magenta) and N λ * (oxidized in violet), with N λ data offset by 2. For each sample data were acquired at high (solid lines) and low (dashed lines) concentration. **(B)** Sedimentation coefficient distributions of 2 μ M N:P13L Δ 31-33, ancestral N, and N:P13L Δ 31-33,L222P in the presence of 10 μ M T₁₀ in low salt buffer. The inset shows DLS autocorrelation data of the same samples (symbols) and single-species fits (lines).

induces a conformational change and increases its *s*-value to ≈ 4.9 S (Zhao et al., 2023 [↗](#)). With the native LRS in the ancestral N this T_{10} -ligated state allows LRS oligomerization, which can be observed through the formation of a reaction boundary in SV-AUC with an *s*-value of ≈ 5.6 S, reflective of a mixture of dimers and tetramers in rapid exchange relative to the time-scale of sedimentation (Schuck, 2010 [↗](#); Zhao et al., 2023 [↗](#)). As a control, we reproduced this previously reported result (Figure 4B [↗](#), blue). Introduction of the N-arm mutations N:P13L, Δ 31-33 causes a further increase of the *s*-value of the reaction boundary to ≈ 5.8 S, indicating an increase in the tetramer stability (Figure 4B [↗](#), magenta). This stabilization of the tetramer is corroborated independently by an increase in the average hydrodynamic radius of N:P13L, Δ 31-33 in mixture with T_{10} (5.51 nm) relative to ancestral N (5.37 nm) or the LRS mutant N:P13L, Δ 31-33,L222P (4.84 nm) (Figure 4B inset [↗](#)). Thus, the N-arm mutation clearly strengthens inter-dimer interactions, even though the added binding energy is too weak to produce detectable tetramer populations at micromolar concentrations by itself. In principle, allosteric interactions between the distant disordered N-arm and the LRS in the disordered linker might exist that cause enhanced tetramerization of N:P13L, Δ 31-33 with occupied NA-site in the NTD. However, a more parsimonious explanation is that the additional self-association interface in the N-arm created by P13L makes inter-dimer contacts that add to the separate oligomerization of the LRS helices in stabilizing tetramers.

Mutation effects on RNP assembly and stability

As the assembly of RNPs requires the concerted effect of several binding interfaces, we asked whether the enhanced LRS coiled-coil stability and the novel N-arm self-association interface impact the RNP stability. To examine this experimentally we carried out *in vitro* RNP assembly experiments using the assay developed previously by the Morgan laboratory (Carlson et al., 2022 [↗](#), 2020 [↗](#)). As mentioned above, it is based on the observation that mixtures of N-protein with stem-loop RNA from the viral 5'-UTR at low μ M concentrations readily form polymorphic ribonucleoprotein complexes that match in size, symmetry, and RNA content what would be expected for the assembled RNPs in virions (Carlson et al., 2022 [↗](#), 2020 [↗](#)). The use of stem-loop SL7 as RNA substrate helps to minimize structural polydispersity arising from variable secondary structure elements. We embarked on the experimental roadmap introduced previously (Zhao et al., 2024 [↗](#)) consisting of SV-AUC experiments that hydrodynamically resolve RNPs in dynamic assembly equilibrium in solution as fast-sedimenting reaction boundaries (Schuck, 2010 [↗](#); Schuck and Zhao, 2017 [↗](#)), in combination with complementary mass photometry (MP) experiments that can resolve populations of different protein/RNA complexes and their dissociation products at sub- μ M concentrations through interferometric sizing of single-molecule surface adsorption events (Wu and Piszczek, 2021 [↗](#)).

Sedimentation coefficient distributions of ancestral and different mutant full-length N at 3 μ M in the presence of 3.4 μ M SL7 are shown in Figure 5A [↗](#). For reference, under these conditions the RNPs of the ancestral N-protein form a reaction boundary with a weighted average sedimentation velocity of 19.7 S (black). Under otherwise identical conditions, significantly faster sedimentation can be discerned for N:P13L, Δ 31-33 (20.2 S, red). Faster sedimentation can reflect an increase in size of the complexes, and/or increased populations and lifetimes of complexes in dynamic equilibrium, i.e., higher affinity and stability. By contrast, N:R203K/G204R exhibits a slightly lower *s*-value of 19.5 S (blue). Both R203K/G204R and P13L, Δ 31-33 combine in the set of defining Omicron mutations, N_o , which produces RNP boundaries at 20.5 S (cyan). It appears the N-arm mutations can more than compensate for the loss of RNP stability through the R203K/G204R mutation in the linker. The introduction of the LRS cysteine in N:G215C is nearly as effective, with an *s*-value of 20.4 S (orange). Finally, the largest increase in sedimentation velocity can be discerned for the combination of P13L/R203K/G204R with the LRS enhancing cysteine G214C in N_λ with 21.0 S (magenta) (see Table 1 [↗](#)).

When these samples are diluted and equilibrated at tenfold lower concentration, the RNPs are largely dissociated, as may be discerned from the reduced amplitude of the rapidly sedimenting peak and significant population of species with *s*-values between 7 and 18 S (Figure 5B [↗](#)). This

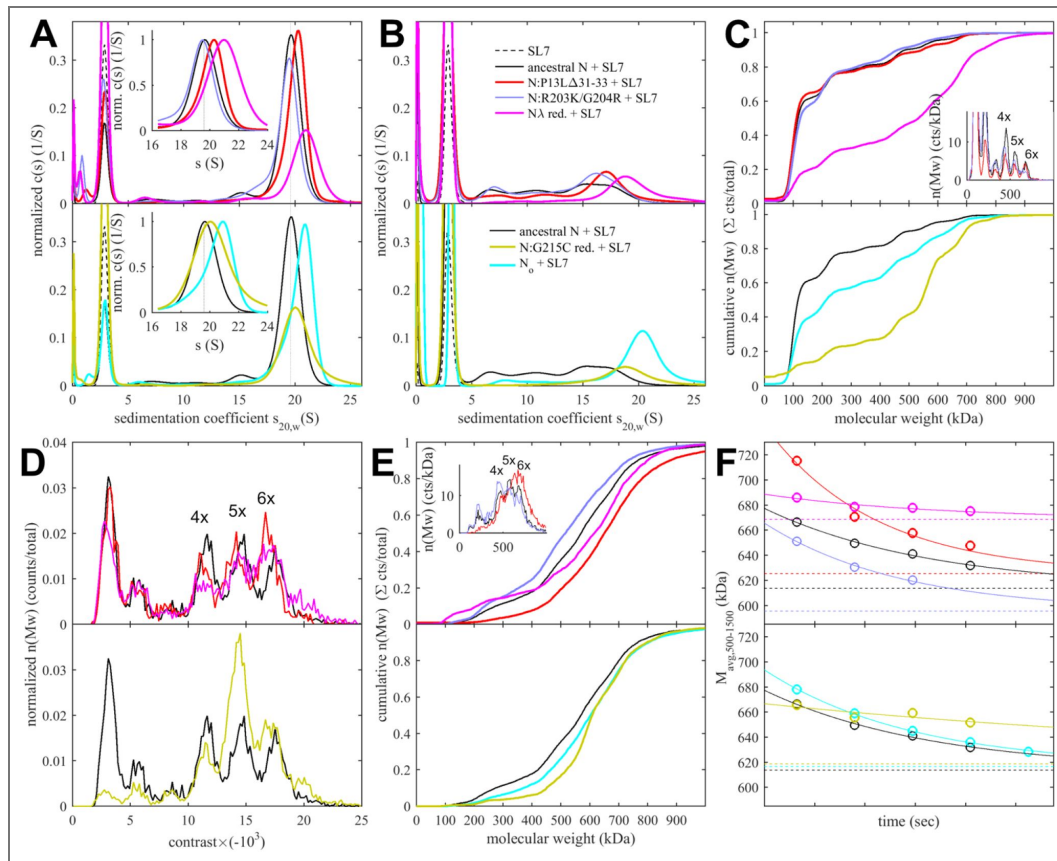


Figure 5. Size distributions and stability of ancestral and mutant RNPs.

Show are SV (A,B) and MP data (C-F) for mixtures of N-protein with stem-loop RNA SL7 in molar ratio of 1(N):1.15(SL7) at high concentration of 3 μ M (A,D) or low concentration of 0.3 μ M (B,C,E,F) in reducing buffer conditions. All panels use the same color scheme for N-protein: ancestral (black), N:P13L Δ 31-33 (red), N:G215C (orange), N λ (cyan), N:R203K/G204R (blue), N λ (magenta). All panels are subdivided in two plots for clarity, with each showing ancestral trace in black for comparison. **(A)** Sedimentation coefficient distributions of mixtures equilibrated at high concentration, with reaction boundary peaks magnified in the inset. For reference, the ancestral RNP s-value is drawn as dotted vertical line. Absorbance data are recorded at 260 nm and are weighted by SL7 content of sedimenting species. Higher reaction boundary s-values signify greater affinity or lifetime of the mutant RNPs. **(B)** Sedimentation coefficient distributions of the same samples as in (A), tenfold diluted and equilibrated, highlight dissociation of most RNPs into a range of intermediate size complexes. **(C)** MP experiments of equilibrated 0.3 μ M mixtures. The measured number distributions are presented as cumulative distributions, which display higher percentages of large species as shifts to the right. Most samples are largely dissociated into dimers, with remaining peaks corresponding to populations of dimer to hexamers of N₂/SL7₂ subunits (as highlighted in the differential distributions in the inset). As an example for the resolution of distinct species, the inset shows the differential distribution (histogram) for N:R203K/G204R (blue), ancestral N (black), and N:P13L Δ 31-33 (red), with the peak labels indicating the number of N-dimer/2SL7 subunits. **(D)** Mass distributions acquired in stopped-flow configuration applied to 3 μ M mixtures. Larger (negative) contrasts correspond to higher molecular weights, with major peaks corresponding to species containing 1, 4, 5, and 6 N₂/SL7₂ subunits. **(E)** For kinetic experiments, mass distributions were acquired in different time intervals after tenfold dilution of 3 μ M mixtures, here showing data collected from 3 sec to 23 sec. **(F)** Number-average molecular weights of assembled RNPs between 500 and 1500 kDa observed in consecutive 20 sec data acquisition intervals after tenfold dilution of 3 μ M mixtures (circles). The dashed horizontal lines are number-averages determined from the equilibrated 0.3 μ M mixtures in **(E)**. The solid lines are a best-fit single exponentials constrained to decay to the measured equilibrium values, yielding RNP lifetimes listed in Table 1 [↗](#).

highlights the cooperative oligomerization of the N-dimer/2SL7 subunits observed previously (Zhao et al., 2024). Interestingly, the only partial dissociation of N_{α} , N:G215C, and N_{λ} RNPs suggests the existence of a subpopulation with higher stability. By contrast, the sedimentation coefficient distribution of RNPs from 0.3 μ M N:P13L, Δ 31-33 is more similar to that of the ancestral N-protein and N:R203K/G204R, suggesting the augmented RNP population caused by combining these mutations is kinetically not as stable and the contributions from N-arm interfaces are more transient.

Side-by-side to the SV experiments, MP measurements were carried out on the same samples to shed more light on the mass distribution of the RNP particles. For discrimination of individual surface adsorption events on the coverslip, the sample concentrations cannot exceed 0.3 μ M protein with 0.34 μ M SL7. As shown in Figure 5C, this results in a ladder of oligomers of N-dimer/2SL7 subunits ranging from $\approx$ 120 kDa (a single N-dimer/2SL7 subunit) to $\approx$ 700 kDa (a hexamer of subunits) (Carlson et al., 2022; Zhao et al., 2024). While this is analogous to the solution data in Figure 5B, for the detailed comparison of SV and MP distributions their different signal weights should be considered: where MP counts individual particles producing a number distribution, SV records a signal proportional to mass, and therefore is strongly skewed toward larger particles compared to MP. Also, in a trade-off between resolution and susceptibility to systematic errors, there is a potential for chemical properties to bias the surface adsorption, and for some adventitious experimental variation originating from the glass substrates. Nonetheless, we obtained results qualitatively consistent with the SV-AUC data, where N:P13L is largely dissociated like ancestral N-protein and N:R203K/G204R at 0.3 μ M; and at the same time, N_{α} , N:G215C, and N_{λ} RNPs retain more of the oligomeric state. Interestingly, N:G215C shows the least fully dissociated dimer and retains more of a pentameric species, which is in contrast to the other cysteine-containing mutant N_{λ} (Figure 5C).

The ladder of oligomeric subunits resolved in MP poses the question whether the largely assembled state at 3 μ M is uniform. Under these conditions SV-AUC shows a single RNP reaction boundary, but it is relatively broad, which would be equally consistent with a reaction boundary from a single complex in rapid association/dissociation exchange on the time-scale of sedimentation (< 1000 sec) (Schuck and Zhao, 2017), as with a polydisperse mixture of several unresolved large oligomers. In order to gain more insight in the mass distribution under these 3 μ M conditions, for some constructs we employed a microfluidic accessory device for the MP instrument allowing rapid dilution with a dead time of < 0.1 sec to minimize RNP dissociation. The resulting data in Figure 5D again exhibit a ladder of oligomeric peaks, where the majority of N-protein assembled in a heterogeneous mixture of RNPs between tetramer and hexamer of subunits. Thus, the dissociation products after dilution in Figure 5C do not seem to originate from a single assembled complex. Furthermore, we observe characteristic differences in the oligomeric distribution between different constructs. Similar to the equilibrated lower concentration conditions, relative to ancestral N, the hexamer population is augmented for N:P13L, Δ 31-33, N:G215C, and N_{λ} . Notably there is again a prominent pentamer population for N:G215C.

Since the disassembly of RNPs after viral entry is another critical step in the viral life cycle, we aimed to probe the kinetic stability of the RNP complexes. To this end, we applied a modified pipettor-based sample application protocol where rapid dilution of the 3 μ M mixture was followed by several consecutive 20 sec periods of data acquisition. The data from the acquisition immediately following the dilution (acquired between 3 – 23 sec) is shown in Figure 5E. It provides mass distributions showing substantial assembly into heterogeneous populations of RNPs. Qualitatively consistent with the 3 μ M mixtures in SV-AUC, the N:R203K/G204R has a clear destabilizing effect on the RNP, whereas all mutants with enhanced binding interfaces exhibit higher populations of larger RNPs, with the greatest enhancement for N:P13L, Δ 31-33. In order to focus on the kinetics of RNP dissociation we calculated the number average molecular weight of RNPs, which is plotted as a function of decay time in Figure 5F and empirically fitted as a single exponential decay attaining the separately measured equilibrium value measured at 0.3 μ M (for distributions see Figure 5C, for number averages Table 1). While N:P13L, Δ 31-33 produces the

largest increase in RNP molecular weight between 0.3 μM and 3 μM , these RNPs have the shortest lifetime ($\tau = 44$ sec vs 66 sec for the ancestral N), suggesting the added H-bond interactions in the N-arm to be rapidly reversible. On the other hand, RNPs of N:G215C have a lower average mass (being dominated by a pentameric oligomer of subunits), but show an increased kinetic stability ($\tau = 231$ sec). RNPs of N_λ carrying both an LRS cysteine and the N-arm mutation are simultaneously of higher average molecular weight and persist most upon dilution (with a best-fit RNP equilibrium level of 669 kDa). (Figure 5F [↗](#)).

Even though it is still unclear whether disulfide bonds of N cysteine mutants form *in vivo*, we were curious about the impact of disulfide-linked oligomers of the cysteine mutants on their RNP structure and stability in our biophysical assembly model, and carried out analogous experiments with the substantially oxidized protein preparations depicted above in Figure 4A [↗](#). As shown in Figure 6A [↗](#), sedimentation coefficient distributions of 3 μM oxidized N:G215C* and oxidized N_λ * (carrying 214C) in assembly mixtures with SL7 show faster reaction boundaries when oxidized as compared to their reduced form, with 20.8 S vs 20.4 S for N:G215C, and 21.5 S vs 21.0 S for N_λ . Upon dilution the RNPs of oxidized N:G215C* protein dissociate similar as those of reduced N:G215C. For oxidized N_λ RNPs dissociation is less, but still significant. The molecular weight distributions in MP measured in the first 20 sec after rapid dilution of the 3 μM stock (Figure 6B [↗](#)) show largely assembled mixtures of tetramers, pentamers, and hexamers of the N-dimer/2SL7 subunits, with a large increase in the population of hexameric RNPs in oxidized vs reduced forms. The tail of even higher molecular weight species is enhanced, pointing to subpopulations of heptameric species especially for oxidized N_λ RNPs. Interestingly, the preference of pentameric RNPs formed by reduced N:G215C is absent after oxidation. N_λ * exhibits the largest population of hexamers observed in any sample, in agreement with the highest *s-value* of the reaction boundary in SV. As indicated by the time-course of RNP dissociation after tenfold dilution (Figure 6C [↗](#)), oxidation enhances the average molecular weight of the RNPs but not their lifetime. We conclude that disulfide-linked N-protein tetramers can incorporate into and aid in the formation of RNPs, modulating their preferred oligomeric state, and that cumulative effect of the N-arm mutation P13L and the LRS cysteine persists for disulfide-linked protein.

Virus-like particle formation and infectivity

We asked whether the observed modulation of RNP size and stability by N mutations impacts the formation and infectivity of virus-like particles (VLPs) (Syed et al., 2021 [↗](#); Zhao et al., 2024 [↗](#)). In this assay, producer 293T cells are co-transfected with plasmids for the four structural proteins S, E, M, and N of SARS-CoV-2, alongside a plasmid containing the viral packaging sequence T20 (nt 20080 – 22222, located near the 3' end of ORF1ab) with a luciferase reporter gene, with a total length of 4,127 nt. This leads to assembly of VLPs, which are collected from the supernatant and applied to receiver cells transfected with entry factors ACE2 and TMPRSS2. Infected receiver cells then express the luciferase reporter and luminescence is measured, as an indicator for the combined efficiency of protein expression, VLP assembly in the producer cells, and entry into the receiver cells (Syed et al., 2021 [↗](#)).

First, a control experiment was carried out with the N:L222P mutant previously shown to abrogate LRS oligomerization and RNP formation. Similar to a second control without N-protein plasmid, N:L222P produced very little luminescence relative to ancestral N (Figure 7 [↗](#)). A positive control was N:R203M (green), which was previously shown to significantly enhance the signal of the VLP assay (Syed et al., 2021 [↗](#)), and did so in the present VLP experiments. Similarly consistent with previous reports (Syed et al., 2021 [↗](#); Wu et al., 2021 [↗](#)), R203K/G204R (blue) led to increased VLP signals. Of the mutations related to N-protein binding interfaces examined in the present work, neither P13L (red), nor $\Delta 31-33$ (light red), nor G215C (yellow) alone led to significant enhancement; only G214C (orange) produced a small but statistically significant enhancement. However, combination of the latter with P13L in P13L/G214C (brown) increased the gain, and further incorporation of the R203K/G204R double mutation to produce the full set of N_λ mutations (purple) substantially increased the measured luminescence. A similar observation was made for the combination of P13L/ $\Delta 31-33$ /R203K/G204R constituting the full set of N_0 mutations (magenta). As

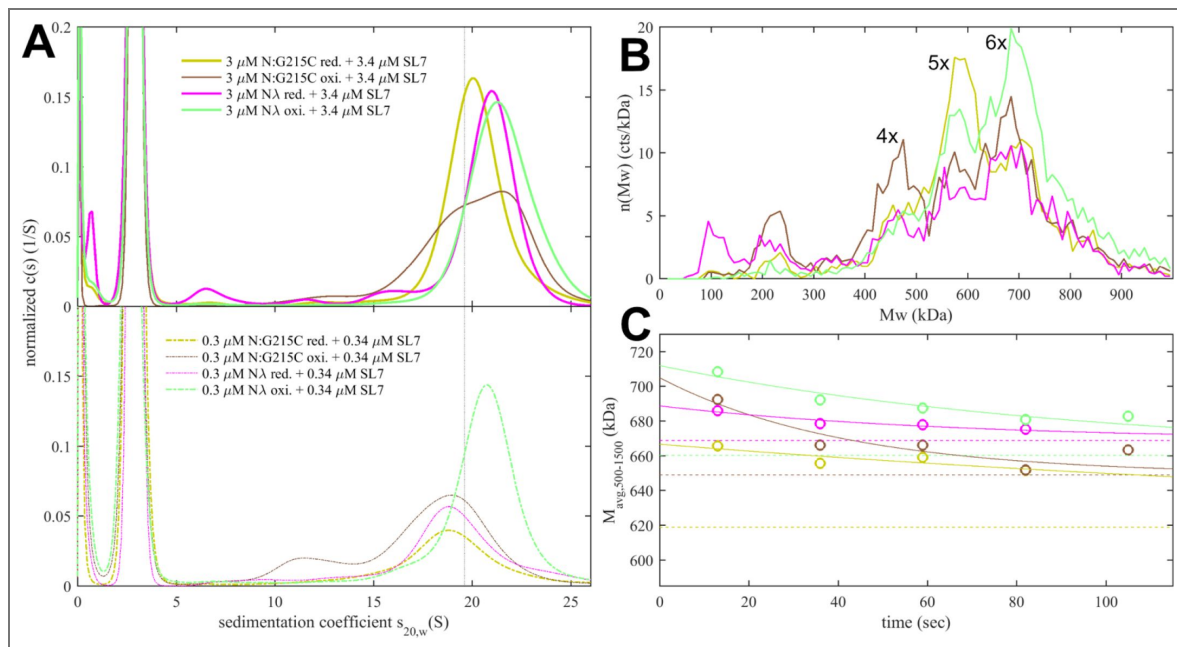


Figure 6. Impact of LRS disulfide bonds on the size and stability of RNPs.

N-protein with cysteine mutations in the LRS were oxidized to form disulfide-linked oligomers (as shown in Figure 4A) and mixed with stem-loop RNA SL7 in molar ratio of 1(N):1.15(SL7). Shown are data for oxidized N:G215C* (brown) and oxidized N λ * (green), and for comparison, reduced N:G215C (yellow) and reduced N λ (magenta). **(A)** Sedimentation coefficient distributions at 3 μM (upper panel) and 0.3 μM (lower panel) protein. **(B)** Molecular weight distributions in MP experiments of the same mixtures rapidly diluted to 0.3 μM protein, acquired from 3 – 23 sec after dilution, with peak labels reflecting the multiples of N dimer/2SL7 subunits. **(C)** Time-course of number-average RNP molecular weights between 500 and 1,500 kDa (circles), determined from rapid dilution experiments in (B) for consecutive 20 sec data acquisition intervals. The solid lines are best-fit single-exponential decays constrained to attain the separately measured equilibrium values at 0.3 μM protein (dashed lines), with lifetimes listed in Table 1.

expanded on in the **Discussion**, the failure to observe enhancement by P13L alone may be related to limitations of the VLP assay in sensitivity, including the restriction to a single round of infection, and protein expression levels.

P13L mutation in N-protein promotes SARS-CoV-2 replication in cell lines

Finally, we characterized the fitness of P13L mutation by introducing it into a recombinant SARS-CoV-2 reporter virus expressing an mCherry gene fused to N via a P2A linker (C. Ye et al., 2020). We infected Vero-TMPRSS2 and A549-ACE2 cells at an MOI of 0.01 with Wuhan-Hu-1 (ancestral) and mutant viruses, and measured mCherry fluorescence as well as viral release into the supernatant at indicated time points post-infection. This revealed that the P13L mutant exhibited a stronger mCherry signal throughout the course of infection (Figure 8A,B) and generated more progeny virus strain in both cell lines (Figure 8C). These findings are consistent with P13L providing a fitness advantage over ancestral virus.

Discussion

Rapid evolution of protein binding interfaces has frequently been observed in viral protein complexes, notably in the virus-host interface, including viral surface glycoproteins as well as ribonuclear proteins and non-structural proteins, with fitness advantages being accomplished, for example, through reshaping the binding interfaces, modulating protein structural dynamics, or altering physicochemical properties (Barozi et al., 2022; Evseev and Magor, 2021; Focosi et al., 2024; Planchais et al., 2022; Rochman et al., 2022). Also, entirely new interactions can arise through the viral mimicry of eukaryotic short linear motifs as a result of frequent mutations in the viral protein intrinsically disordered regions, which can greatly augment the virus-host interface (Davey et al., 2015; Schuck and Zhao, 2023). The mutations we have studied here are of a different category, impacting the interactions among viral proteins that enhance viral multi-protein complexes. Previous examples include the intra-host diversity of polymerase subunit interfaces in H5N1 influenza viruses (Welkers et al., 2019). While such mutations are not directly targeted towards the host, they may still contribute to host adaptation, or be balancing other mutation effects in an epistatic network, conceivably involving modulation of local effective protein concentrations (P. Li et al., 2023). Irrespective of their complete context, they can provide valuable insights in viral protein mechanisms.

Specifically, we have described three different mutations of SARS-CoV-2 N-protein that, in convergent evolution, strengthen the formation of RNPs and enhance viral assembly. N:G214C, N:G215C, and N:P13L have been independently introduced (as highlighted in the phylogenetic trees Supplementary Figure S9-11 generated in Nextstrain (Hadfield et al., 2018)), and persisted in the defining set of mutations in their respective variants of concern (Lambda, Delta, and Omicron, respectively). We have shown here that N:P13L confers a fitness advantage in cell lines, and similarly, N:G215C was shown by Kubinski *et al.* (Kubinski et al., 2024) to impart improved viral growth. This correlates well with our results studying their molecular mechanisms.

For both of the cysteine mutants, molecular dynamics simulations and biophysical studies show how cysteines augment self-association interfaces by extending and redirecting the transiently formed helical coiled-coils in the intrinsically disordered LRS, which play a central role in the assembly of RNPs. By contrast, for N:P13L, unexpectedly, the evolution of RNP stability goes beyond modulation of a previously existing binding interface, and instead we observe the *de novo* formation of an additional dynamic self-association interface in the distant disordered N-arm through the stabilization and stacking of transient β -sheets, that we hypothesize cooperatively contributes to the stability of RNPs. Even though the solution affinity of the N-arm P13L interface is ultra-weak, the average local concentration of N-arm chains across the RNP volume (in a back-

Figure 7. Mutation effect on packaging and cell entry in a VLP assay.

Error bars are standard deviations from $n = 4$. Stars indicate significance ($P > 0.95$) of a two-sided Kolmogorov-Smirnov test comparing the control ancestral measurements with mutants.

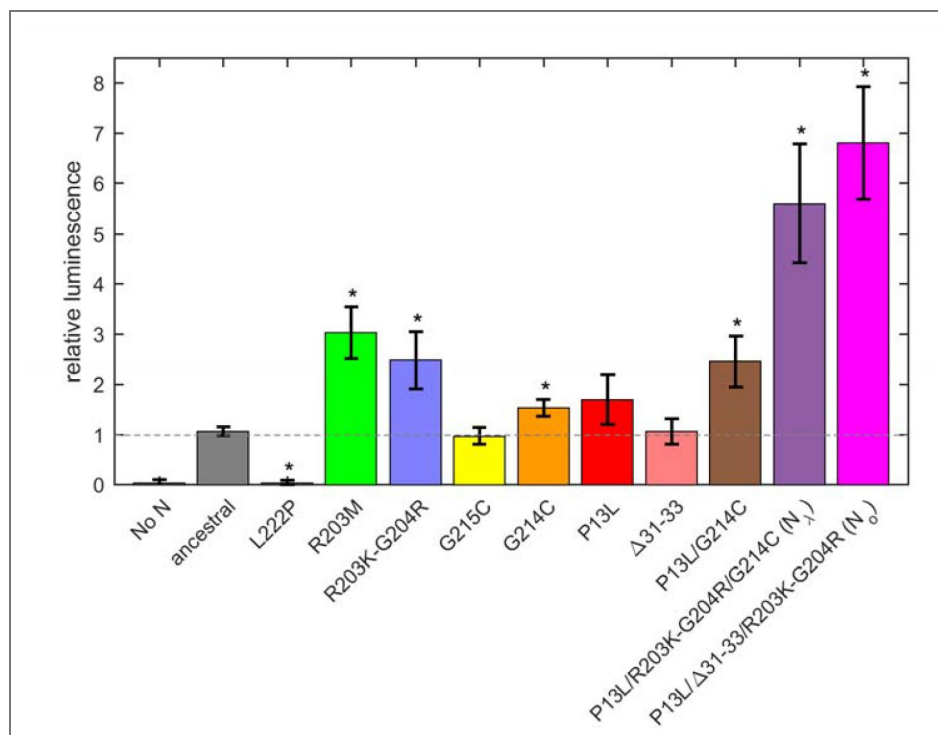
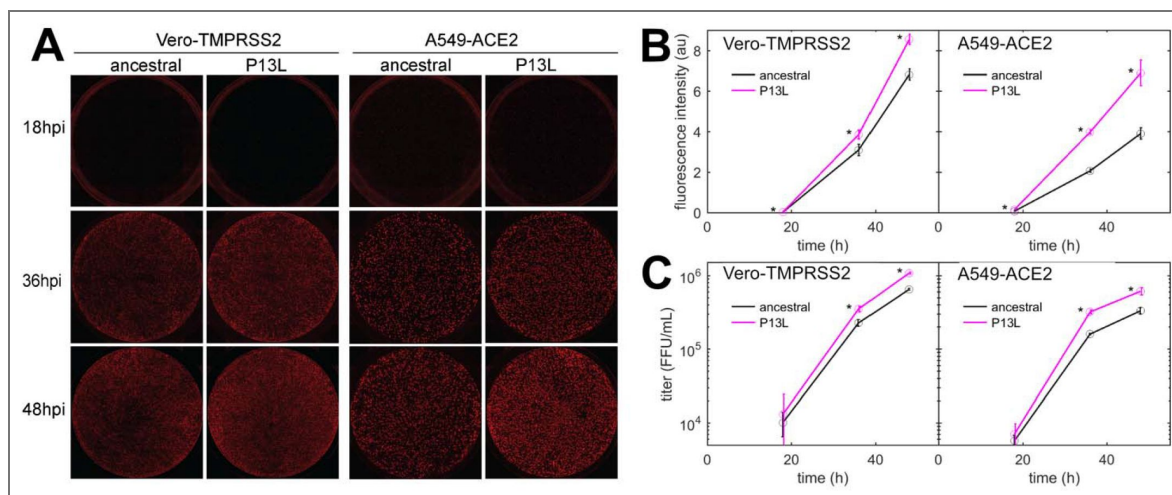


Figure 8. Replication kinetics of recombinant SARS-CoV-2 reporter viruses in cell lines.

(A) Representative images of Vero-TMPRSS2 and A549-ACE2 cells infected with SARS-CoV-2 P13L or WT at different time points post-infection. (B) Quantification of fluorescence intensity from P13L and WT virus infections shown in (A). (C) Viral titers in the supernatant from infected cells. Error bars are standard deviations ($n = 3$), and stars indicate significant differences on a $P=0.95$ confidence level.



of-the-envelope calculation assuming a ≈ 14 nm cube (Klein et al., 2020) with a dodecameric N cluster) is ≈ 7.4 mM, such that disordered N-arm peptides could well create populations of N-arm clusters stabilizing RNPs through this interface.

However, besides the RNP-stabilizing mutants we have also observed unexpected RNP destabilization by the ubiquitous R203K/G204R double mutation, which may be caused by the introduction of additional charges close to the self-association interface in the LRS. In our experiments, this destabilization is more than compensated for by the P13L mutation. (Another scenario where ultra-weak interactions can have a critical impact is in molecular condensates. We previously reported the suppression of LLPS by the R203K/G204R mutation, which is rescued by the additional P13L/ Δ 31-33 mutation (Nguyen et al., 2024). This is consistent with compensatory weak stabilizing and destabilizing impacts of weak interactions on the RNP observed here.)

We arrive at a picture of SARS-CoV-2 RNPs that is far from structurally well defined, matching the concept of fuzzy complexes (Wu and Fuxreiter, 2016). On a molecular level, large portions of the SARS-CoV-2 N-protein (the N-arm, C-arm, and linker) are intrinsically disordered and highly flexible (Cubuk et al., 2021; Różycki and Boura, 2022), which persists in the presence of bound nucleic acid (Cubuk et al., 2024; Guseva et al., 2021; Schiavina et al., 2022). It appears that conformational freedom is also retained to a significant degree in the RNPs. This flexibility could be advantageous for accommodating various RNA secondary structures (Carlson et al., 2022; Landeras-Bueno et al., 2025), and favorably balance the energetic cost of RNP disassembly that is required immediately after viral entry. Also, this serves to accommodate significant sequence variation (Davey et al., 2011; Duro et al., 2015; Schuck and Zhao, 2023). SARS-CoV-2 RNPs appear highly heterogeneous in EM (Carlson et al., 2022; Landeras-Bueno et al., 2025; Yao et al., 2020), and this is reflected in the polymorphic oligomeric states of RNP species we observe in SV-AUC and MP, that we believe is driven by promiscuous self-association or clustering of transient LRS helices (Zhao et al., 2022). Extending previously described characteristics of fuzziness in protein complexes (Duro et al., 2015; Fuxreiter, 2018; Tompa and Fuxreiter, 2008), plasticity seems to involve even basic architectural principles, considering not only the emergence of new distant stabilizing interfaces such as described here in the N-arm, but also the possibility of RNP assembly of truncated $N_{210-419}^*$ lacking one of the major nucleic acid binding interfaces (Adly et al., 2023; Bouhaddou et al., 2023; Mears et al., 2025; Mulloy et al., 2025; Syed et al., 2024) (see below).

Unfortunately, this intrinsic heterogeneity poses significant methodological hurdles. Nonetheless, salient structural features and assembly principles may be derived from constraints of known binding interfaces and oligomeric states of the RNP and its subunits, as observed in SV-AUC and MP. While the arrangement sketched in Figure 1C satisfies these requirements, alternate less symmetrical configurations can be conceived that seem at least equally likely and may coexist in polydisperse mixtures of RNPs. For example, there is no evidence to exclude the possibility of anti-parallel LRS helices pointing the folded nucleic acid-binding domains in different relative orientations, or of mixed co-assemblies with $N_{210-419}^*$ subunits lacking the NTD (Supplementary Figure S1). Uniformity of N-protein/RNA clusters may not be relevant for adequate gRNA condensation.

Beyond the structural model, to study the effect of a larger number of N-protein mutations derived from variants of concern side-by-side in the context of virus assembly, we have carried out experiments using a VLP assay (Syed et al., 2021) (Figure 7). In these experiments, all four structural proteins are transfected into 293T cells to package a reporter RNA into VLPs and their infection of receiver cells can be compared. While this assay has been widely used for rapid assessment of spike protein and N variants (Syed et al., 2021), it has limitations due to the addition of non-genomic RNA and the lack of double membrane vesicles from which gRNA emerges through the NSP3/NSP4 pore complex potentially poised for packaging (Bessa et al., 2022; Ke et al., 2024; Ni et al., 2023). It should also be recognized that the results do not directly reflect the relative efficiency of RNP assembly only, since protein expression levels, their localization, and their posttranslational modifications are not controlled for. Susceptibility for such factors might be exacerbated with mutations that modulate weak protein interactions. For

example, as shown previously (Syed et al., 2024 [\[1\]](#); Zhao et al., 2024 [\[2\]](#)), a GSK3 inhibitor inhibiting N-protein phosphorylation significantly enhances VLP formation and eliminates the advantage provided for by the N:G215C mutation relative to the ancestral N – presumably due to an increase in assembly-competent, non-phosphorylated N-protein erasing an affinity advantage. A similar process may be underlying the absent or marginal improvement in VLP readout from the cysteine LRS mutants and P13L at the achieved transfection level in the present work, and the enhanced signal from R203K/G204R and R203M (the latter being consistent with previous reports (Li et al., 2025 [\[3\]](#); Syed et al., 2021 [\[4\]](#))) modulating protein phosphorylation. Nonetheless, mirroring the results of the biophysical *in vitro* experiments, the addition of RNP-stabilizing P13L and G214C mutations on top of R203K/G204R led to a significantly larger VLP signal.

The VLP assay may be limited in sensitivity to mutation effects due to its restriction to a single round of infection. To avoid this and other potential limitations of the VLP assay for the study of viral packaging, for the key mutation N:P13L we carried out reverse genetics experiments. These showed the sole N:P13L mutation significantly increases viral fitness (Figure 8 [\[5\]](#)).

Regarding the cysteine mutations that have been repeatedly introduced in the LRS prior to the rise of the Omicron variants of concern, it is an open question whether they lead to covalent bonds *in vivo* or *in the* VLP assay. While examples of disulfide-linked viral nucleocapsid proteins have been reported (Kubinski et al., 2024 [\[6\]](#); Prokudina et al., 2004 [\[7\]](#); Wootton and Yoo, 2003 [\[8\]](#)), a methodological difficulty in their detection is artifactual disulfide bond formation post-lysis of infected cells (Kubinski et al., 2024 [\[6\]](#); Wootton and Yoo, 2003 [\[8\]](#)). However, our results clearly show that a major effect of the cysteines already arises in reduced conditions without any covalent bonds, through extension of the LRS helices, and concomitant redirection of the disordered N-terminal sequence. While oxidized tetrameric N-proteins of N:G214C and N:G215C can be incorporated into RNPs, the covalent bonds provided only marginally improved RNP stability. Interestingly, the introduction of cysteines imposes preferences of RNP oligomeric states dependent on oxidation state, consistent with our MD simulations highlighting the impact of cysteine orientation of 214C *versus* 215C relative to the hydrophobic surface of the LRS helices. Overall, considering potentially detrimental structural constraints from covalent bonds on LRS clusters seeding RNPs, energetic penalties on RNP disassembly, as well as the required monomeric state of the LRS helix for interaction with the NSP3 Ubl domain (Bessa et al., 2022 [\[9\]](#)), at present it is unclear to what extent the formation of disulfide linkages between LRS helices would be beneficial or detrimental in the viral life cycle.

Recent work by the Soranno laboratory has identified an additional function of the disordered N-arm in transiently interacting with the NTD (Cubuk et al., 2021 [\[10\]](#)) and dynamically enhancing the affinity of the NTD for RNA (Cubuk et al., 2024 [\[11\]](#)). Using single-molecule Förster Resonance Energy Transfer (smFRET) a fourfold modulatory effect of the P13L/Δ31-33 mutation on the NTD RNA binding affinity was observed in N-arm-NTD constructs. Through MD simulations the reduced NTD affinity for RNA was attributed to the N-arm Δ31-33 deletion (Cubuk et al., 2024 [\[11\]](#)). Superficially, this may seem in slight conflict with our results of similar T_{10} affinity of full-length ancestral N with and without the P13L/Δ31-33 mutation, but results were obtained in different buffer conditions (50 mM TRIS, pH 7.4 in (Cubuk et al., 2024 [\[11\]](#)) *versus* 20 mM HEPES, 150 mM NaCl, pH 7.5 in the present work). In any event, RNA binding of NTD and stabilization of the RNP are different processes; any modulation of N-arm contributions to NTD-RNA interactions through Omicron N-arm mutations Δ31-33 may coexist and be over-compensated for by N-arm self-association interfaces through P13L modulating RNP subunit interactions in the high local N-arm density of the RNP.

The double mutant R203K/G204R arose early in the pandemic and was adopted in several variants of concern (including Alpha, Gamma, Lambda, Zeta, and Omicron BA.1) with the triple nucleotide changes G28881A, G28882A, and G28883C (Mears et al., 2025 [\[12\]](#); Syed et al., 2024 [\[13\]](#)) (Supplementary Figure S12 [\[14\]](#)). As mentioned above, on the protein level N:R203K/G204R has been shown to alter phosphorylation (though in different ways in *in vitro* VLP or *in vivo* reverse genetics experiments) (Johnson et al., 2022 [\[15\]](#); Syed et al., 2024 [\[13\]](#); Yun et al., 2022 [\[16\]](#)); and phosphorylation in turn reduces nucleic acid binding and promotes viral replication as opposed to

assembly functions (Botova et al., 2024 [↗](#); Bouhaddou et al., 2023 [↗](#); Carlson et al., 2020 [↗](#); Syed et al., 2024 [↗](#)). Adding to such a switch, in the present work we observed the loss of RNP stability of N:R203K/G204R relative to the ancestral N, extending the previous observation of reduced LLPS propensity of N:R203K/G204R (Nguyen et al., 2024 [↗](#)). Simultaneously, on the RNA level the N:R203K/G204R mutations also lead to the new formation of a TRS sequence ACGAAC underlying the expression of N₂₁₀₋₄₁₉* in virus-infected cells (though not expected to occur with N:R203K/G204R in the VLP assay lacking the viral RNA-dependent RNA polymerase). It has been hypothesized that N₂₁₀₋₄₁₉* confers increased viral fitness through the suppression of the host anti-viral response (Mears et al., 2025 [↗](#); Mulloy et al., 2025 [↗](#)), and that it can assist RNP formation (Bouhaddou et al., 2023 [↗](#); Syed et al., 2024 [↗](#)). However, the contribution of N₂₁₀₋₄₁₉* to assembly is still unclear: although it is remarkably capable of forming RNPs *in vitro* and VLP assays (Adly et al., 2023 [↗](#); Bouhaddou et al., 2023 [↗](#); Syed et al., 2024 [↗](#)), in infected cells and virions N₂₁₀₋₄₁₉* has been detected only as a minority species (Mears et al., 2025 [↗](#); Mulloy et al., 2025 [↗](#)). Also, the recent major Omicron XEC variant (Scarpa et al., 2025 [↗](#)) (which had close to 60% global frequency at the beginning of 2025; Supplementary Figure S12 [↗](#)) exhibits a fourth consecutive nucleotide change G28884C that maintains a similar RG mutation forming R203K/G204P but ablates the canonical TRS sequence, such that continued expression of N₂₁₀₋₄₁₉* in XEC is in question. We propose that an alternative or additional mechanism to retain viral assembly functions may be presented by the accompanying P13L mutation, which our data suggest can more than restore loss of RNP stability in the combination of RG mutations with P13L. This combination occurs in N₀ and all Omicron variants so far, and was even further stabilized with a cysteine in the LRS in N_λ.

In conclusion, it has been proposed that mutations in SARS-CoV-2 N protein that affect viral assembly can impact infectivity and fitness (Bouhaddou et al., 2023 [↗](#); Syed et al., 2024 [↗](#); Wu et al., 2021 [↗](#); Zhao et al., 2022 [↗](#)). We believe the observed modulations of the RNP assembly and stability studied here highlight a key mechanism for this. Although effects on fitness of viruses carrying N mutations are most likely multi-factorial, they have been observed in reverse genetics tissue culture experiments previously for N:R203K/G204R (Johnson et al., 2022 [↗](#); Mears et al., 2025 [↗](#); Wu et al., 2021 [↗](#)), N:G215C (Kubinski et al., 2024 [↗](#)), and in the present work for N:P13L. On the other hand, the rise of new variants of concern was usually dominated by their spike protein mutations (with the exception of 21I replacement by 21J which has identical spike mutations but acquired N:G215C (Marchitelli et al., 2021 [↗](#); Stern et al., 2021 [↗](#); Zhao et al., 2022 [↗](#)) in the rise of Delta variant), and in many cases N mutations of previously dominant variants were completely replaced by another set of N mutations (dramatically exemplified in the displacement of Delta by Omicron variants). This reinforces the view that these N mutations are secondary to alterations in the immune landscape and transmissibility as the primary driver of evolution (Markov et al., 2023 [↗](#)). Nonetheless, the remarkable plasticity of RNPs offers multiple avenues to modulate stability and to compensate for potentially RNP-destabilizing effects of mutations that are beneficial in other ways. In convergent evolution, this has been a constant theme of N protein mutations throughout the SARS-CoV-2 pandemics up until to date. We hypothesize that the ‘fuzziness’ and pleomorphic ability of RNP assembly, with its variable distribution of overall binding energy into several different weak or ultra-weak protein interfaces, and the poor structural definition ranging from flexible chain configurations to polydisperse oligomeric states, provides an evolutionary advantage of orchestrated disorder to promote epistatic interactions and facilitate host adaptation.

Methods

Protein expression and purification

Full-length N-protein of the wild type and mutant SARS-CoV-2 were expressed and purified as described previously (Nguyen et al., 2024 [↗](#); Zhao et al., 2024 [↗](#)). Briefly, One Shot BL21(DE3)pLysS E.coli (Thermo Fisher Scientific, Carlsbad, CA) was transformed using a pET29a(+) plasmid vector, which contains a kanamycin-resistant gene and the gene encoding the N-protein of interest preceded by 6xHis tag followed by a Tobacco Etch Virus (TEV) cleavage site at the N-

terminus. After cell lysis, the protein was purified by Ni^{2+} affinity chromatography, where on-column unfolding by urea and refolding steps were carried out to remove protein-bound bacterial nucleic acid (Carlson et al., 2020 [DOI](#)). After tag cleavage by TEV protease, 6xHis tag removal was verified through another round of affinity chromatography, and/or via mass spectrometry. Cleaved protein was subjected to a final size exclusion chromatography followed by dialysis into working buffer (20 mM HEPES, 75 mM NaCl, pH 7.50, supplemented with 1 mM TCEP for cysteine containing proteins, unless otherwise mentioned). Protein purity was validated by SDS-PAGE and the absence of nucleic acid was confirmed by an absorbance ratio 260/280 of ≈ 0.50 -0.55. Final protein concentration was determined by UV-Vis spectrophotometry or by refractive index detected SV-AUC.

N peptides were purchased from ABI Scientific (Sterling, VA), purified by HPLC, examined by matrix-assisted laser desorption/ionization for purity and identity, and lyophilized. The oligonucleotide T₁₀ and stem-loop RNA SL7 were purchased from Integrated DNA Technologies (Skokie, IL) and purified by HPLC and lyophilized by the vendor. After reconstitution, SL7 was subject to thermal denaturation at 95 °C for 2 min followed by gradual cooling to room temperature over 1–2 h. For sequences of oligonucleotides and peptides see [Supplementary Table S1](#) [DOI](#).

The 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) (catalog #22582) and Cysteine-HCl (catalog #44889) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Free thiols in the protein samples were quantified using the Ellman's assay by following the standard protocol from the vendor. Briefly, free thiols react with DTNB, resulting in a measurable yellow-colored product, TNB. The quantity of sulfhydryl groups was calculated by the absorbance of the sample using the molar extinction coefficient of TNB at 412 nm ($14,150 \text{ M}^{-1}\text{cm}^{-1}$). The same samples were analyzed by SDS-PAGE without the addition of a reducing agent. The relative intensities of the monomer and dimer bands reflect the amounts of the reduced and oxidized forms, respectively.

Structure prediction and MD simulations

In the studies of LRS peptides, the initial structures of the monomer and oligomers of the ancestral N₂₁₀₋₂₄₆ ([Supplementary Table S1](#) [DOI](#)) were predicted using AlphaFold3 (AF3). The predicted conformations agreed with those reported in (Zhao et al., 2023 [DOI](#), 2022 [DOI](#)), with the oligomers showing a parallel, left-handed coiled-coil signature. Point mutations were introduced by replacing the corresponding glycine residue with cysteine. Graphics were created using ChimeraX.

MD simulations were performed for monomers and trimers of the ancestral peptides N₂₁₀₋₂₄₆ and two mutant sequences (G214C and G215C), following the protocol described in (Zhao et al., 2023 [DOI](#)). In each case, the initial structure was the top-ranked AF3 model. Each simulation was extended for 250 ns after thermal equilibration under experimental conditions (T = 20°C, P = 1 atm, pH 7.5, and 75 mM NaCl) using the isothermal-isobaric ensemble as implemented in NAMD. The all-atom representation of the CHARMM (param36) force field was used. Structural stabilization of the helical regions was observed within a few nanoseconds, and data were analyzed over the last 200 ns of the simulations.

Structure of the N-arm oligomers was predicted using ColabFold (Mirdita et al., 2022 [DOI](#)), assembling 20 copies of ancestral N₁₀₋₂₀, N₁₀₋₂₀:P13L, N₁₀₋₂₀:P13S, or N₁₀₋₂₀:P13T. Structures were analyzed and displayed using ChimeraX (Pettersen et al., 2021 [DOI](#)).

Electron microscopy

Carbon coated 200 mesh copper TEM grids were glow discharged for ≈ 15 seconds. Next, 4 μL of the sample solution was deposited onto the grids and incubated for 2 minutes. After incubation, excess sample solution was removed by gently touching the edge of the grids with filter paper. A large drop of distilled water was then placed on the grids for 1 minute, followed by removal of the water by contacting the grid edge with filter paper. This rinsing step was repeated three times. The grids were then stained with 5 μL of 1% uranyl acetate (UA) solution for 20 seconds. Any excess UA was removed by touching the edge of the grids with filter paper and then left to air dry. Finally, the

grids were examined with a FEI Tecnai12 Transmission Electron Microscope (FEI, Hillsboro, Oregon), operating at 120 keV beam energy. TEM images are captured using a high-speed, high-resolution Gatan Rio 3k x 3k CMOS camera (Gatan, Warrendale, PA).

Sedimentation velocity analytical ultracentrifugation

SV-AUC experiments were conducted in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Indianapolis, IN) using standard protocols as previously described (Schuck et al., 2015 [↗](#)). AUC cell assemblies filled with samples using 12- or 3-mm charcoal-filled Epon double-sector centerpieces with sapphire windows were loaded into An-50 or An-60 rotors and temperature equilibrated in the rotor chamber at 20 °C for 2-3 hrs. Subsequently, radial scans were acquired with Rayleigh interference optics and absorbance optics at 260 nm and/or 280 nm. Calibration factors for the instrument were determined according to previously published methods (Ghirlando et al., 2013 [↗](#)). Sedimentation boundary data were analyzed using sedimentation coefficient distribution c(s) model in the software SEDFIT (Schuck, 2016 [↗](#)) (<https://sedfitsedphat.nibib.nih.gov/software> [↗](#)).

Mass photometry

Mass photometry measurements were performed in a TwoMP instrument (Refeyn, UK) following the standard protocol (Wu and Piszczek, 2021 [↗](#)) unless otherwise mentioned. Samples were loaded in the mini-wells formed by a silicone gasket which was placed on top of a coverslip mounted on the microscope stage. Two configurations of sample loading were used in the current study. For the time-dependent experiments, the samples were first prepared in the Eppendorf tubes prior to MP experiment. Then the working buffer (9 µL) was loaded onto the coverslip for focusing the objective.

Subsequently, the sample was added to the buffer droplet, gently mixed and the measurement was initiated immediately as the 1st time point. The subsequent acquisitions for the same sample continued for specific time intervals. For the samples which were not subject to this dilution/mixing configuration, sample mixtures were equilibrated 2 – 3 hours, focusing was achieved by using the buffer-free option provided by the data acquisition software, and data was collected immediately after sample application. In either configuration, the impact of surface binding on the sample concentration is < 1% and negligible, as described in the [Supplementary Methods S1](#) [↗](#). Calibration of the TwoMP instrument was performed using the two calibrants, Beta-Amylase from Sweet Potato (Sigma A8781) and Thyroglobulin from Bovine Thyroid (Sigma T9145) as recommended by the manufacturer. MP data was acquired with AcquireMP software and the analysis was performed with DiscoverMP software (Refeyn, UK).

Rapid mixing experiments were carried out on a OneMP-MassFluidix HC system (Refeyn Ltd., Oxford, UK). A rapid-dilution microfluidic chip (MP-CON-51001, Refeyn Ltd., Oxford, UK) was connected to the buffer, sample, and waste lines. Sample was injected into the chip at a flow rate of 8 µL/min, while the buffer was flowed at 1100 µL/min, and 1 min videos were recorded for data acquisition.

Circular dichroism spectroscopy

CD spectra were acquired in a Chirascan Q100 (Applied Photophysics, UK) at 20 °C. Measurements were performed in 0.1 mm (peptides) or 1 mm (proteins) pathlength cuvettes with 1 nm steps, and a 1 sec integration time per data point. Each spectrum represents the average of three independent scans with background subtraction applied.

Dynamic light scattering

Dynamic light scattering measurements of the samples were performed in a Prometheus Panta (Nanotemper, Germany) instrument at 20°C. The samples were loaded into capillaries (Nanotemper PR-AC002) and autocorrelation functions (ACFs) were acquired using the 405 nm laser at the detection angle of 140°. The ACFs were analyzed with discrete species models, size-distribution models, and cumulant analysis in SEDFIT (Parker and Lollar, 2021 [↗](#)).

Virus-like particle assay

The plasmids pLVX-EF1alpha-SARS-CoV-2-E-2xStrep-IRES-Puro (#141385), pLVX-EF1alpha-SARS-CoV-2-M-2xStrep-IRES-Puro (#141386), and pLVX-EF1alpha-SARS-CoV-2-N-2xStrep-IRES-Puro (#141391) were obtained from Addgene. Plasmid pLuc-T20 was a kind gift from Jennifer A. Doudna. The plasmid pIRES2-EGFP (Cat # V011106) was purchased from NovoPro. Plasmids encoding ACE2, pcDNA3.1(+)-SARS-CoV-2 WA1-S and pGAGGS-TMPRSS2 were previously described (Shi et al., 2022, 2021). To construct the plasmid pcDNA3.1(+)-N, the N gene was cloned into pcDNA3.1(+) between the BamHI and NotI sites. Mutations in N were generated by QuikChange™ site-directed mutagenesis, and verified by whole plasmid sequencing. Similarly, to prepare plasmid pIRES2-ME, the M gene was first cloned into pIRES2-EGFP between NheI and BamHI sites, followed by the insertion of the E gene into the resulting plasmid between NotI and a PvuI site introduced after the start codon of EGFP.

The SARS-CoV-2 virus-like particles (VLPs) were prepared as previously described (Syed et al., 2021; Zhao et al., 2024) with some modifications. For SC2-VLP production, 0.8×10^6 293T cells (CLS Cat# 305117, RRID:CVCL_1926) per well were plated in a 6-well plate and allowed to grow 24 hours before transfection. Plasmids Cov2-N (1.34), Cov2-M-IRES-E (0.659), Cov2-Spike (0.0032) and Luc-T20 (2.0) at indicated mass ratios for a total of 4 µg of DNA were diluted in 250 µL Opti-MEM reduced serum medium (Gibco cat# 31985062) at room temperature. 12 µL of TransIT®-293 Transfection Reagent (Mirus Bio, cat# MIR 2704) equilibrated to room temperature was added to each sample, and immediately vortexed. The transfection mixtures were incubated for 25 min at room temperature and then added dropwise to 293T cells after media change with 2 mL of DMEM containing fetal bovine serum and penicillin/streptomycin. Media was changed after 24 h of transfection and at 48 h post-transfection, VLP containing supernatant was collected and filtered using a 0.22 µm syringe filter. Cells in each well were rinsed with 1 mL of PBS and then lysed directly in the wells with 300 µL of NuPAGE™ LDS Sample Buffer (Invitrogen, cat# NP0008) containing HALT protease inhibitors (Thermo Scientific, cat# 87786) and 10 mM DTT.

For the luciferase assay, the wells in 12-well plates were pre-treated with 1 mL poly-D-Lysine (Thermo Fisher Scientific, cat# A3890401) for 30 min, which was removed, washed with PBS once, and finally the plates were airdried. In each well of these pre-treated plates, 1.5×10^5 receiver cells (293T cells transfected with the plasmids encoding ACE2 and pGAGGS-TMPRSS2 at a mass ratio of 1:1 using the TransIT®-293 transfection reagent) were plated. Next day, the media was replaced, and the cells infected with 250 µL of supernatant from the producer cells. After 24 h, the media was removed, and cells were rinsed with PBS and lysed in 150 µL passive lysis buffer (Luciferase Assay System, Promega, cat# E1500) for 15 min at room temperature with gentle rocking. 20 µL of each lysate was transferred to an opaque black 96-well plate in triplicate, and 50 µL of reconstituted luciferase assay buffer was added and mixed with each lysate. Luminescence was measured immediately after mixing using a TECAN plate reader.

Generation of recombinant SARS-CoV-2

The generation and use of recombinant SARS-CoV-2 (rSARS-CoV-2) viruses in tissue culture at biosafety level 3 were approved by NIH Institutional Biosafety (IBC) and the Dual Use Research of Concern Institutional Review Entity (DURC-IRE) Committees (IBC approved case number: RD-22-XI-11).

We generated rSARS-CoV-2 viruses using a bacterial artificial chromosome (BAC)-based SARS-CoV-2 reverse genetics system (C. Ye et al., 2020), with detailed construction and recovery procedures described in our previous study (T. Li et al., 2023). Specifically, we fused the mCherry gene with the N gene via a 2A linker and created an intermediate plasmid, pUC57-NEM, containing a portion of the pBAC-SARS-CoV-2 genome digested with BamHI and SalI restriction enzymes. The P13L mutation was introduced via site-directed mutagenesis on the pUC57-NEM plasmid. PCR amplification was performed to generate fragments containing the NEM genes, which were then

assembled with the BamHI/SalI-digested larger fragment using the NEBuilder® HiFi DNA Assembly Master Mix. A furin cleavage site mutation (R685S) served as the backbone for introducing mutations in the N protein.

Plasmids were purified using the QIAGEN Plasmid Maxi Kit. Confluent BHK21-ACE2 cells (2×10^6 cells/well in 6-well plates, in duplicate, *RRID:CVCL_1914* [↗](#), ATCC) were transfected with 2.5 µg/well of pBAC-SARS-CoV-2 using the TransIT-LT1 transfection reagent. After 6 hours, the medium was replaced with fresh DMEM containing 2% FBS. At 48-72 hours post-transfection, mCherry-positive cells exhibiting signs of viral infection were detached, collected with the supernatant, labeled as P0, and stored at -80°C . The P0 viral stock was centrifuged to remove cell debris and used to infect fresh Vero E6-TMPRSS2 (*RRID:CVCL_0574* [↗](#), ATCC) cells for 48-72 hours. The resulting P1 viral stock was collected, and viral titers were determined following next-generation sequencing (NGS) confirmation of the viral genome sequence.

SARS-CoV-2 infection

Vero E6-TMPRSS2 and A549-hACE2 (*RRID:CVCL_0023* [↗](#), ATCC) cell lines were seeded in 12-well plates at 3×10^5 cells/well. The next day, cells were infected at MOI 0.01 with an inoculation period of 1 hour, followed by a medium change to fresh culture medium. Supernatants were collected, and infected cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at indicated time before removal from the BSL-3 laboratory for fluorescence imaging using Cytation 5 (BioTek). Virus released into the supernatant was titrated using Vero cells via the limiting dilution method. Cell lines were confirmed to be mycoplasma-free using MycoStrip (InvivoGen, rep-mys-50).

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Additional files

[Supplemental Table S1](#) [↗](#)

[Supplemental Figure S1](#) [↗](#)

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References

- Adly AN**, Bi M, Carlson CR, Syed AM, Ciling A, Doudna JA, Cheng Y, Morgan DO (2023) Assembly of SARS-CoV-2 ribonucleosomes by truncated N_s variant of the nucleocapsid protein. *Journal of Biological Chemistry* **299**:105362 <https://doi.org/10.1016/j.jbc.2023.105362>
- Alberti S**, Gladfelter AS, Mittag T. (2019) Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell* **176**:419-434 <https://doi.org/10.1016/j.cell.2018.12.035>
- Alderson TR**, Pritisanac I, Kolarić Đ, Moses AM, Forman-Kay JD (2023) Systematic identification of conditionally folded intrinsically disordered regions by AlphaFold2. *Proceedings of the National Academy of Sciences* **120**:2022.02.18.481080 <https://doi.org/10.1073/pnas.2304302120>
- Alston JJ**, Soranno A, Holehouse AS (2023) Conserved molecular recognition by an intrinsically disordered region in the absence of sequence conservation. *bioRxiv* <https://doi.org/10.1101/2023.08.06.552128>
- Barozi V**, Edkins AL, Tastan Bishop Ö. (2022) Evolutionary progression of collective mutations in Omicron sub-lineages towards efficient RBD-hACE2: Allosteric communications between and within viral and human proteins. *Comput Struct Biotechnol J* **20**:4562-4578 <https://doi.org/10.1016/j.csbj.2022.08.015>
- Bessa LM**, Guseva S, Camacho-Zarco AR, Salvi N, Maurin D, Perez LM, Botova M, Malki A, Nanao M, Jensen MR, *et al.* (2022) The intrinsically disordered SARS-CoV-2 nucleoprotein in dynamic complex with its viral partner nsp3a. *Sci Adv* **8** <https://doi.org/10.1126/sciadv.abm4034>
- Bloom JD**, Neher RA (2023) Fitness effects of mutations to SARS-CoV-2 proteins. *Virus Evol* **9**:2023.01.30.526314 <https://doi.org/10.1093/ve/vead055>
- Botova M**, Camacho-Zarco AR, Tognetti J, Bessa LM, Guseva S, Mikkola E, Salvi N, Maurin D, Herrmann T, Blackledge M. (2024) A specific phosphorylation-dependent conformational switch in SARS-CoV-2 nucleocapsid protein inhibits RNA binding. *Sci Adv* **10**:1-15 <https://doi.org/10.1126/sciadv.aax2323>
- Bouhaddou M**, Reuschl AK, Polacco BJ, Thorne LG, Ummadi MR, Ye C, Rosales R, Pelin A, Batra J, Jang GM, *et al.* (2023) SARS-CoV-2 variants evolve convergent strategies to remodel the host response. *Cell* **186**:4597-4614.e26 <https://doi.org/10.1016/j.cell.2023.08.026>
- Brocca S**, Grandori R, Longhi S, Uversky V. (2020) Liquid-liquid phase separation by intrinsically disordered protein regions of viruses: Roles in viral life cycle and control of virus-host interactions. *Int J Mol Sci* **21**:1-31 <https://doi.org/10.3390/ijms21239045>
- Brown CJ**, Johnson AK, Dunker AK, Daughdrill GW (2011) Evolution and disorder. *Curr Opin Struct Biol* **21**:441-446 <https://doi.org/10.1016/j.sbi.2011.02.005>
- Carlson CR**, Adly AN, Bi M, Howard CJ, Frost A, Cheng Y, Morgan DO (2022) Reconstitution of the SARS-CoV-2 ribonucleosome provides insights into genomic RNA packaging and regulation by phosphorylation. *Journal of Biological Chemistry* **298**:102560

<https://doi.org/10.1016/j.jbc.2022.102560>

Carlson CR, Asfaha JB, Ghent CM, Howard CJ, Hartooni N, Safari M, Frankel AD, Morgan DO (2020) Phosphoregulation of Phase Separation by the SARS-CoV-2 N Protein Suggests a Biophysical Basis for its Dual Functions. *Mol Cell* **80**:1092-1103.e4 <https://doi.org/10.1016/j.molcel.2020.11.025>

Chang CK, Sue SC, Yu TH, Hsieh CM, Tsai CK, Chiang YC, Lee SJ, Hsiao HH, Wu WJ, Chang WL, *et al.* (2006) Modular organization of SARS coronavirus nucleocapsid protein. *J Biomed Sci* **13**:59-72 <https://doi.org/10.1007/s11373-005-9035-9>

Cubuk J, Alston JJ, Incicco JJ, Holehouse AS, Hall KB, Stuchell-Brereton MD, Soranno A. (2024) The disordered N-terminal tail of SARS-CoV-2 Nucleocapsid protein forms a dynamic complex with RNA. *Nucleic Acids Res* **52**:2609-2624 <https://doi.org/10.1093/nar/gkad1215>

Cubuk J, Alston JJ, Incicco JJ, Singh S, Stuchell-Brereton MD, Ward MD, Zimmerman MI, Vithani N, Griffith D, Wagoner JA, *et al.* (2021) The SARS-CoV-2 nucleocapsid protein is dynamic, disordered, and phase separates with RNA. *Nat Commun* **12**:1936 <https://doi.org/10.1038/s41467-021-21953-3>

Cubuk J, Incicco JJ, Hall KB, Holehouse AS, Stuchell-Brereton MD, Soranno A. (2025) The dimerization domain of SARS-CoV-2 nucleocapsid protein is partially disordered and forms a dynamic high-affinity dimer. *Cell Rep Phys Sci* **6**:102695 <https://doi.org/10.1016/j.xcrp.2025.102695>

Davey NE, Cyert MS, Moses AM (2015) Short linear motifs - Ex nihilo evolution of protein regulation Short linear motifs - The unexplored frontier of the eukaryotic proteome. *Cell Communication and Signaling* **13**:9-11 <https://doi.org/10.1186/s12964-015-0120-z>

Davey NE, Travé G, Gibson TJ (2011) How viruses hijack cell regulation. *Trends Biochem Sci* **36**:159-169 <https://doi.org/10.1016/j.tibs.2010.10.002>

Dhamotharan K, Korn SM, Wacker A, Becker MA, Günther S, Schwalbe H, Schlundt A. (2024) A core network in the SARS-CoV-2 nucleocapsid NTD mediates structural integrity and selective RNA-binding. *Nature Communications* **15**:1-16 <https://doi.org/10.1038/s41467-024-55024-0>

Duro N, Miskei M, Fuxreiter M. (2015) Fuzziness endows viral motif-mimicry. *Mol Biosyst* **11**:2821-2829 <https://doi.org/10.1039/c5mb00301f>

Dyson HJ, Wright PE (2005) Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol* **6**:197-208 <https://doi.org/10.1038/nrm1589>

Elbe S, Buckland-Merrett G. (2017) Data, disease and diplomacy: GISAID's innovative contribution to global health. *Global Challenges* **1**:33-46 <https://doi.org/10.1002/gch2.1018>

Evseev D, Magor KE (2021) Molecular Evolution of the Influenza A Virus Non-structural Protein 1 in Interspecies Transmission and Adaptation. *Front Microbiol* **12** <https://doi.org/10.3389/fmicb.2021.693204>

Focosi D, Spezia PG, Maggi F. (2024) Subsequent Waves of Convergent Evolution in SARS-CoV-2 Genes and Proteins. *Vaccines (Basel)* **12**:887 <https://doi.org/10.3390/vaccines12080887>

Fuxreiter M. (2018) Fuzziness in protein interactions—a historical perspective. *J Mol Biol* **430**:2278-2287 <https://doi.org/10.1016/j.jmb.2018.02.015>

Ghirlando R, Balbo A, Piszczek G, Brown PH, Lewis MS, Brautigam CA, Schuck P, Zhao H. (2013) Improving the thermal, radial, and temporal accuracy of the analytical ultracentrifuge through external references. *Anal Biochem* **440**:81-95 <https://doi.org/10.1016/j.ab.2013.05.011>

Gupta MN, Uversky VN (2024) Protein structure–function continuum model: Emerging nexuses between specificity, evolution, and structure. *Protein Science* **33**:1-36 <https://doi.org/10.1002/pro.4968>

Guseva S, Perez LM, Camacho-Zarco A, Bessa LM, Salvi N, Malki A, Maurin D, Blackledge M. (2021) ¹H, ¹³C and ¹⁵N Backbone chemical shift assignments of the n-terminal and central intrinsically disordered domains of SARS-CoV-2 nucleoprotein. *Biomol NMR Assign* **15**:255-260 <https://doi.org/10.1007/s12104-021-10014-x>

- Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P, Bedford T, Neher RA (2018) NextStrain: Real-time tracking of pathogen evolution. *Bioinformatics* **34**:4121-4123 <https://doi.org/10.1093/bioinformatics/bty407>
- Hagai T, Azia A, Babu MM, Andino R. (2014) Use of Host-like Peptide Motifs in Viral Proteins Is a Prevalent Strategy in Host-Virus Interactions. *Cell Rep* **7**:1729-1739 <https://doi.org/10.1016/j.celrep.2014.04.052>
- Holehouse AS, Kragelund BB (2024) The molecular basis for cellular function of intrinsically disordered protein regions. *Nat Rev Mol Cell Biol* **25**:187-211 <https://doi.org/10.1038/s41580-023-00673-0>
- Iserman C, Roden CA, Boerneke MA, Sealfon RSG, McLaughlin GA, Jungreis I, Fritch EJ, Hou YJ, Ekena J, Weidmann CA, *et al.* (2020) Genomic RNA Elements Drive Phase Separation of the SARS-CoV-2 Nucleocapsid. *Mol Cell* **80**:1078-1091 <https://doi.org/10.1016/j.molcel.2020.11.041>
- Jack A, Ferro LS, Trnka MJ, Wehri E, Nadgir A, Nguyenla X, Fox D, Costa K, Stanley S, Schaletzky J, *et al.* (2021) SARS-CoV-2 nucleocapsid protein forms condensates with viral genomic RNA. *PLoS Biol* **19**:e3001425 <https://doi.org/10.1371/journal.pbio.3001425>
- Johnson BA, Zhou Y, Lokugamage KG, Vu MN, Bopp N, Crocquet-Valdes PA, Kalveram B, Schindewolf C, Liu Y, Scharton D, *et al.* (2022) Nucleocapsid mutations in SARS-CoV-2 augment replication and pathogenesis. *PLoS Pathog* **18**:e1010627 <https://doi.org/10.1371/journal.ppat.1010627>
- Ke Z, Zhang H, Wang Y, Jingning Wang, Peng F, Jia Wang, Liu X, Hu H, Li Y. (2024) N-terminus of SARS-CoV-2 nonstructural protein 3 interrupts RNA-driven phase separation of N protein by displacing RNA. *Journal of Biological Chemistry* 107828 <https://doi.org/10.1016/j.jbc.2024.107828>
- Klein S, Cortese M, Winter SL, Wachsmuth-Melm M, Neufeldt CJ, Cerikan B, Stanifer ML, Boulant S, Bartenschlager R, Chlanda P. (2020) SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat Commun* **11**:5885 <https://doi.org/10.1038/s41467-020-19619-7>
- Korn SM, Dhamotharan K, Jeffries CM, Schlundt A. (2023) The preference signature of the SARS-CoV-2 Nucleocapsid NTD for its 5'-genomic RNA elements. *Nat Commun* **14**:3331 <https://doi.org/10.1038/s41467-023-38882-y>
- Kubinski HC, Despres HW, Johnson BA, Schmidt MM, Estes LK, Pekosz A, Crothers JW, Roychoudhury P, Greninger AL, Jerome KR, *et al.* (2024) Variant mutation in SARS-CoV-2 nucleocapsid enhances viral infection via altered genomic encapsidation. *bioRxiv* <https://doi.org/10.1101/2024.03.08.584120>
- Landeras-Bueno S, Hariharan C, Avalos RD, Norris AS, Snyder DT, Hastie KM, Harkins S, Zandonatti M, Rajamanickam RR, Olmedillas E, *et al.* (2025) Structural stabilization of the intrinsically disordered SARS-CoV-2 N by binding to RNA sequences engineered from the viral genome fragment. *Nat Commun* **16** <https://doi.org/10.1038/s41467-025-61861-4>
- Li P, Xue B, Schnicker NJ, L-YR Wong, Meyerholz DK, Perlman S. (2023) Nsp3-N interactions are critical for SARS-CoV-2 fitness and virulence. *Proceedings of the National Academy of Sciences* **120**:2017 <https://doi.org/10.1073/pnas.2305674120>
- Li T, Kang I, Hu Z, Gibbs J, Ye C, Kosik I, Shi G, Holly J, Kosikova M, Compton A, *et al.* (2023) Syncytia Formation Promotes Virus Resistance to Interferon and Neutralizing Antibodies. *bioRxiv* <https://doi.org/10.1101/2023.12.12.571262>
- Li Y, Li M, Xiao H, Liao F, Shen M, Ge W, Ou J, Liu Y, Chen L, Zhao Y, *et al.* (2025) The R203M and D377Y mutations of the nucleocapsid protein promote SARS-CoV-2 infectivity by impairing RIG-I-mediated antiviral signaling. *PLoS Pathog* **21**:1-28 <https://doi.org/10.1371/journal.ppat.1012886>
- Longhi S, Bloyet LM, Gianni S, Gerlier D. (2017) How order and disorder within paramyxoviral nucleoproteins and phosphoproteins orchestrate the molecular interplay of transcription and replication. *Cellular and Molecular Life Sciences* **74**:3091-3118 <https://doi.org/10.1007/s00018-017-2556-3>
- Marchitelli V, Troise C, Vesuviano O, Parisi A, Zooprofilattico I, Bianco A, Zooprofilattico I, Del Sambro L, Zooprofilattico I, Somma R, *et al.* (2021) Evidence for the dependence of the SARS-Cov-2 Delta high diffusivity on the associated N1li: G215C nucleocapsid mutation. *Res Sq*

<https://doi.org/10.21203/rs.3.rs-846225/v1>

Markov P V, Ghafari M, Beer M, Lythgoe K, Simmonds P, Stilianakis NI, Katzourakis A. (2023) The evolution of SARS-CoV-2. *Nat Rev Microbiol* **21**:361-379 <https://doi.org/10.1038/s41579-023-00878-2>

Mears H V, Young GR, Sanderson T, Harvey R, Barrett-Rodger J, Penn R, Cowton V, Furnon W, De Lorenzo G, Crawford M, *et al.* (2025) Emergence of SARS-CoV-2 subgenomic RNAs that enhance viral fitness and immune evasion. *PLoS Biol* **23**:1-29 <https://doi.org/10.1371/journal.pbio.3002982>

Mirdita M, Schütze K, Moriwaiki Y, Heo L, Ovchinnikov S, Steinegger M. (2022) ColabFold: making protein folding accessible to all. *Nat Methods* **19**:679-682 <https://doi.org/10.1038/s41592-022-01488-1>

Mulloy RP, Evseev D, Bui-Marinos MP, Sharlin N, Corcoran JA (2025) A truncated SARS-CoV-2 nucleocapsid protein enhances virus fitness by evading antiviral responses. *bioRxiv* <https://doi.org/10.1101/2025.02.15.638421>

Nesmelova I V., Melnikova DL, Ranjan V, Skirda VD (2019) Translational diffusion of unfolded and intrinsically disordered proteinsProgress. In: *Molecular Biology and Translational Science* Elsevier B.V. pp. 85-108 <https://doi.org/10.1016/bs.pmbts.2019.05.004>

Nguyen A, Zhao H, Myagmarsuren D, Srinivasan S, Wu D, Chen J, Piszczek G, Schuck P. (2024) Modulation of biophysical properties of nucleocapsid protein in the mutant spectrum of SARS-CoV-2. *eLife* **13** <https://doi.org/10.7554/eLife.94836>

Ni X, Han Y, Zhou R, Zhou Y, Lei J. (2023) Structural insights into ribonucleoprotein dissociation by nucleocapsid protein interacting with non-structural protein 3 in SARS-CoV-2. *Commun Biol* **6**:193 <https://doi.org/10.1038/s42003-023-04570-2>

Obermeyer F, Jankowiak M, Barkas N, Schaffner SF, Pyle JD, Yurkovetskiy L, Bosso M, Park DJ, Babadi M, MacInnis BL, *et al.* (2022) Analysis of 6.4 million SARS-CoV-2 genomes identifies mutations associated with fitness. *Science* (1979) **1208**:1-14 <https://doi.org/10.1126/science.abm1208>

Olsen JG, Teilum K, Kragelund BB (2017) Behaviour of intrinsically disordered proteins in protein-protein complexes with an emphasis on fuzziness. *Cellular and Molecular Life Sciences* **74**:3175-3183 <https://doi.org/10.1007/s00018-017-2560-7>

Oulas A, Zanti M, Tomazou M, Zachariou M, Minadakis G, Bourdakou MM, Pavlidis P, Spyrou GM (2021) Generalized linear models provide a measure of virulence for specific mutations in SARS-cov-2 strains. *PLoS One* **16**:1-17 <https://doi.org/10.1371/journal.pone.0238665>

Paravastu AK, Leapman RD, Yau W-M, Tycko R. (2008) Molecular structural basis for polymorphism in Alzheimer's β -amyloid fibrils. *Proceedings of the National Academy of Sciences* **105**:18349-18354 <https://doi.org/10.1073/pnas.0806270105>

Parker ET, Lollar P. (2021) Measurement of the translational diffusion coefficient and hydrodynamic radius of proteins by dynamic light scattering. *Bio Protoc* **11**:e4195 <https://doi.org/10.21769/BioProtoc.4195>

Perdikari TM, Murthy AC, Ryan VH, Watters S, Naik MT, Fawzi NL (2020) SARS-CoV-2 nucleocapsid protein phase-separates with RNA and with human hnRNPs. *EMBO J* **39**:1-35 <https://doi.org/10.15252/embj.2020106478>

Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, Ferrin TE (2021) UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Science* **30**:70-82 <https://doi.org/10.1002/pro.3943>

Planchais C, Reyes-Ruiz A, Lacombe R, Zarantonello A, Lecerf M, Revel M, Roumenina LT, Atanasov BP, Mouquet H, Dimitrov JD (2022) Evolutionary trajectory of receptor binding specificity and promiscuity of the spike protein of SARS-CoV-2. *Protein Science* **31**:1-19 <https://doi.org/10.1002/pro.4447>

Prokudina EN, Semenova NP, Chumakov VM, Rudneva IA (2004) Transient disulfide bonds formation in conformational maturation of influenza virus nucleocapsid protein (NP). *Virus Res* **99**:169-175 <https://doi.org/10.1016/j.virusres.2003.11.008>

- Ranganathan S, Dasmeh P, Furniss S, Shakhnovich E. (2023) Phosphorylation sites are evolutionary checkpoints against liquid-solid transition in protein condensates. *Proc Natl Acad Sci U S A* **120** <https://doi.org/10.1073/pnas.2215828120>
- Ranganathan S, Shakhnovich EI (2020) Dynamic metastable long-living droplets formed by sticker-spacer proteins. *eLife* **9**:1-25 <https://doi.org/10.7554/eLife.56159>
- Ren J, Wang S, Zong Z, Pan T, Liu S, Mao W, Huang H, Yan X, Yang B, He X, *et al.* (2024) TRIM28-mediated nucleocapsid protein SUMOylation enhances SARS-CoV-2 virulence. *Nat Commun* **15** <https://doi.org/10.1038/s41467-023-44502-6>
- Rochman ND, Wolf YI, Koonin E V. (2022) Molecular adaptations during viral epidemics. *EMBO Rep* **23**:1-22 <https://doi.org/10.15252/embr.202255393>
- Roden CA, Dai Y, Giannetti CA, Seim I, Lee M, Sealton R, McLaughlin GA, Boerneke MA, Iserman C, Wey SA, *et al.* (2022) Double-stranded RNA drives SARS-CoV-2 nucleocapsid protein to undergo phase separation at specific temperatures. *Nucleic Acids Res* **50**:8168-8192 <https://doi.org/10.1093/nar/gkac596>
- Różycki B, Boura E. (2022) Conformational ensemble of the full-length SARS-CoV-2 nucleocapsid (N) protein based on molecular simulations and SAXS data. *Biophys Chem* **288**:106843 <https://doi.org/10.1016/j.bpc.2022.106843>
- Savastano A, Ibáñez de Opakua A, Rankovic M, Zweckstetter M. (2020) Nucleocapsid protein of SARS-CoV-2 phase separates into RNA-rich polymerase-containing condensates. *Nat Commun* **11**:6041 <https://doi.org/10.1038/s41467-020-19843-1>
- Scarpa F, Branda F, Ceccarelli G, Romano C, Locci C, Pascale N, Azzena I, Fiori PL, Casu M, Pascarella S, *et al.* (2025) SARS-CoV-2 XEC: A Genome-Based Survey. *Microorganisms* **13** <https://doi.org/10.3390/microorganisms13020253>
- Schiavina M, Pontoriero L, Tagliaferro G, Pierattelli R, Felli IC (2022) The Role of Disordered Regions in Orchestrating the Properties of Multidomain Proteins: The SARS-CoV-2 Nucleocapsid Protein and Its Interaction with Enoxaparin. *Biomolecules* **12**:1302 <https://doi.org/10.3390/biom12091302>
- Schuck P. (2016) *Sedimentation Velocity Analytical Ultracentrifugation: Discrete Species and Size-Distributions of Macromolecules and Particles* Boca Raton, FL: CRC Press.
- Schuck P. (2010) Sedimentation patterns of rapidly reversible protein interactions. *Biophys J* **98**:2005-2013 <https://doi.org/10.1016/j.bpj.2009.12.4336>
- Schuck P, Zhao H. (2023) Diversity of short linear interaction motifs in SARS-CoV-2 nucleocapsid protein. *mBio* **14**:e02388-23 <https://doi.org/10.1128/mbio.02388-23>
- Schuck P, Zhao H. (2017) *Sedimentation Velocity Analytical Ultracentrifugation: Interacting Systems* Boca Raton, FL: CRC Press.
- Schuck P, Zhao H, Brautigam CA, Ghirlando R. (2015) *Basic Principles of Analytical Ultracentrifugation* Boca Raton, FL: CRC Press.
- Shi G, Chiramel AI, Li T, Lai KK, Kenney AD, Zani A, Eddy AC, Majdoul S, Zhang L, Dempsey T, *et al.* (2022) Rapalogs downmodulate intrinsic immunity and promote cell entry of SARS-CoV-2. *Journal of Clinical Investigation* **132**:e160766 <https://doi.org/10.1172/JCI160766>
- Shi G, Kenney AD, Kudryashova E, Zani A, Zhang L, Lai KK, Hall-Stoodley L, Robinson RT, Kudryashov DS, Compton AA, *et al.* (2021) Opposing activities of IFITM proteins in SARS-CoV-2 infection. *EMBO J* **40**:e106501 <https://doi.org/10.15252/embj.2020106501>
- Stern A, Fleishon S, Kustin T, Mandelboim M, Erster O, Mendelson E, Mor O, Zuckerman NS, Bucris D, Sofer D, *et al.* (2021) The unique evolutionary dynamics of the SARS-CoV-2 Delta variant Israel Consortium of SARS-CoV-2 sequencing. *medRxiv*
- Syed AM, Ciling A, Chen IP, Carlson CR, Adly AN, Martin HS, Taha TY, Khalid MM, Price N, Bouhaddou M, *et al.* (2024) SARS-CoV-2 evolution balances conflicting roles of N protein phosphorylation. *PLoS Pathog* **20**:e1012741 <https://doi.org/10.1371/journal.ppat.1012741>

- Syed AM, Taha TY, Tabata T, Chen IP, Ciling A, Khalid MM, Sreekumar B, Chen P-Y, Hayashi JM, Soczek KM, et al. (2021) Rapid assessment of SARS-CoV-2-evolved variants using virus-like particles. *Science* (1979) **374**:1626-1632 <https://doi.org/10.1126/science.abl6184>
- Tarczewska A, Kolonko-Adamska M, Zarębski M, Dobrucki J, Ożyhar A, Greb-Markiewicz B. (2021) The method utilized to purify the SARS-CoV-2 N protein can affect its molecular properties. *Int J Biol Macromol* **188**:391-403 <https://doi.org/10.1016/j.ijbiomac.2021.08.026>
- Tokuriki N, Oldfield CJ, Uversky VN, Berezhovsky IN, Tawfik DS (2009) Do viral proteins possess unique biophysical features?. *Trends Biochem Sci* **34**:53-59 <https://doi.org/10.1016/j.tibs.2008.10.009>
- Tokuriki N, Tawfik DS (2009) Protein Dynamism and Evolvability. *Science* (1979) **324**:203-207 <https://doi.org/10.1126/science.1169375>
- Tompa P, Fuxreiter M. (2008) Fuzzy complexes: polymorphism and structural disorder in protein-protein interactions. *Trends Biochem Sci* **33**:2-8 <https://doi.org/10.1016/j.tibs.2007.10.003>
- Tugaeva K V., Sysoev AA, Kapitonova AA, Smith JLR, Zhu P, Cooley RB, Antson AA, Sluchanko NN (2023) Human 14-3-3 Proteins Site-selectively Bind the Mutational Hotspot Region of SARS-CoV-2 Nucleoprotein Modulating its Phosphoregulation. *J Mol Biol* **435**:167891 <https://doi.org/10.1016/j.jmb.2022.167891>
- Welkers MRA, Pawestri HA, Fonville JM, Sampurno OD, Pater M, Holwerda M, Han AX, Russell CA, Jeeninga RE, Setiawaty V, et al. (2019) Genetic diversity and host adaptation of avian H5N1 influenza viruses during human infection. *Emerg Microbes Infect* **8**:262-271 <https://doi.org/10.1080/22221751.2019.1575700>
- Wink PL, Volpato FCZ, Monteiro FL, Willig JB, Zavascki AP, Barth AL, Martins AF (2022) First identification of SARS-CoV-2 lambda (C.37) variant in Southern Brazil. *Infect Control Hosp Epidemiol* **43**:1996-1997 <https://doi.org/10.1017/ice.2021.390>
- Wootton SK, Yoo D. (2003) Homo-Oligomerization of the Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein and the Role of Disulfide Linkages. *J Virol* **77**:4546-4557 <https://doi.org/10.1128/jvi.77.8.4546-4557.2003>
- Wu D, Piszczek G. (2021) Standard protocol for mass photometry experiments. *European Biophysics Journal* **50**:403-409 <https://doi.org/10.1007/s00249-021-01513-9>
- Wu H, Fuxreiter M. (2016) The structure and dynamics of higher-order assemblies: amyloids, signalosomes, and granules. *Cell* **165**:1055-1066 <https://doi.org/10.1016/j.cell.2016.05.004>
- Wu H, Xing N, Meng K, Fu B, Xue W, Dong P, Tang W, Xiao Y, Liu G, Luo H, et al. (2021) Nucleocapsid mutations R203K/G204R increase the infectivity, fitness, and virulence of SARS-CoV-2. *Cell Host Microbe* **29**:1788-1801.e6 <https://doi.org/10.1016/j.chom.2021.11.005>
- Wu W, Cheng Y, Zhou H, Sun C, Zhang S. (2023) The SARS-CoV-2 nucleocapsid protein: its role in the viral life cycle, structure and functions, and use as a potential target in the development of vaccines and diagnostics. *Virol J* **20**:6 <https://doi.org/10.1186/s12985-023-01968-6>
- Xue B, Blocquel D, Habchi J, Uversky A V., Kurgan L, Uversky VN, Longhi S. (2014) Structural disorder in viral proteins. *Chem Rev* **114**:6880-6911 <https://doi.org/10.1021/cr4005692>
- Yao H, Song Y, Chen Y, Wu N, Xu J, Sun C, Zhang J, Weng T, Zhang Z, Wu Z, et al. (2020) Molecular Architecture of the SARS-CoV-2 Virus. *Cell* **183**:730-738.e13 <https://doi.org/10.1016/j.cell.2020.09.018>
- Ye C, Chiem K, Park JG, Oladunni F, Platt RN, Anderson T, Almazan F, de la Torre JC, Martinez-Sobrido L. (2020) Rescue of SARS-CoV-2 from a single bacterial artificial chromosome. *mBio* **11**:1-10 <https://doi.org/10.1128/mBio.02168-20>
- Ye Q, West AMV, Silletti S, Corbett KD (2020) Architecture and self-assembly of the SARS-CoV-2 nucleocapsid protein. *Protein Science* **29**:1890-1901 <https://doi.org/10.1002/pro.3909>
- Yu IM, Gustafson CLT, Diao J, Burgner JW, Li Z, Zhang J, Chen J. (2005) Recombinant severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein forms a dimer through its C-terminal domain. *Journal of Biological Chemistry* **280**:23280-23286 <https://doi.org/10.1074/jbc.M501015200>

- Yun JS, Song H, Kim NH, Cha SY, Hwang KH, Lee JE, Jeong CH, Song SH, Kim S, Cho ES, *et al.* (2022) Glycogen Synthase Kinase-3 Interaction Domain Enhances Phosphorylation of SARS-CoV-2 Nucleocapsid Protein. *Mol Cells* **45**:911-922 <https://doi.org/10.14348/molcells.2022.0130>
- Zachrdla M, Savastano A, Ibáñez de Opakua A, Cima-Omori M, Zweckstetter M. (2022) Contributions of the N-terminal intrinsically disordered region of the severe acute respiratory syndrome coronavirus 2 nucleocapsid protein to RNA-induced phase separation. *Protein Science* **31**:2003-2005 <https://doi.org/10.1002/pro.4409>
- Zarin T, Strome B, Peng G, Pritišanac I, Forman-Kay JD, Moses AM (2021) Identifying molecular features that are associated with biological function of intrinsically disordered protein regions. *eLife* **10**:1-36 <https://doi.org/10.7554/eLife.60220>
- Zhao H, Nguyen A, Wu D, Li Y, Hassan SA, Chen J, Shroff H, Piszczek G, Schuck P. (2022) Plasticity in structure and assembly of SARS-CoV-2 nucleocapsid protein. *PNAS Nexus* **1**:pgac049 <https://doi.org/10.1093/pnasnexus/pgac049>
- Zhao H, Syed AM, Khalid MM, Nguyen A, Ciling A, Wu D, Yau W, Srinivasan S, Esposito D, Doudna JA, *et al.* (2024) Assembly of SARS-CoV-2 nucleocapsid protein with nucleic acid. *Nucleic Acids Res* **52**:6647-6661 <https://doi.org/10.1093/nar/gkae256>
- Zhao H, Wu D, Hassan SA, Nguyen A, Chen J, Piszczek G, Schuck P. (2023) A conserved oligomerization domain in the disordered linker of coronavirus nucleocapsid proteins. *Sci Adv* **9**:eadg6473 <https://doi.org/10.1126/sciadv.adg6473>
- Zhao H, Wu D, Nguyen A, Li Y, Adão RC, Valkov E, Patterson GH, Piszczek G, Schuck P. (2021) Energetic and structural features of SARS-CoV-2 N-protein co-assemblies with nucleic acids. *iScience* **24**:102523 <https://doi.org/10.1016/j.isci.2021.102523>
- Zinzula L, Basquin J, Bohn S, Beck F, Klumpe S, Pfeifer G, Nagy I, Bracher A, Hartl FU, Baumeister W. (2021) High-resolution structure and biophysical characterization of the nucleocapsid phosphoprotein dimerization domain from the Covid-19 severe acute respiratory syndrome coronavirus 2. *Biochem Biophys Res Commun* **538**:54-62 <https://doi.org/10.1016/j.bbrc.2020.09.131>

Peer reviews

Reviewer #1 (Public review):

Summary:

The authors attempted to clarify the impact of N protein mutations on ribonucleoprotein (RNP) assembly and stability using analytical ultracentrifugation (AUC) and mass photometry (MP). These complementary approaches provide a more comprehensive understanding of the underlying processes. Both SV-AUC and MP results consistently showed enhanced RNP assembly and stability due to N protein mutations.

The overall research design appears well planned, and the experiments were carefully executed.

Strengths:

SV-AUC, performed at higher concentrations (3 μ M), captured the hydrodynamic properties of bulk assembled complexes, while MP provided crucial information on dissociation rates and complex lifetimes at nanomolar concentrations. Together, the methods offered detailed insights into association states and dissociation kinetics across a broad concentration range. This represents a thorough application of solution physicochemistry.

Weaknesses:

Unlike AUC, MP observes only a part of solution. In MP, bound molecules are accumulated on the glass surface (not dissociated) thus concentration in solution should change as time

develops. How does such concentration change impact the result shown here?

Comments on revisions:

The response from the authors is appropriate and reasonable.

<https://doi.org/10.7554/eLife.108922.2.sa3>

Reviewer #2 (Public review):

Summary:

In this manuscript, the authors apply a variety of biophysical and computational techniques to characterize the effects of mutations in the SARS-CoV-2 N protein on the formation of ribonucleoprotein particles (RNPs). They find convergent evolution in multiple repeated independent mutations strengthening binding interfaces, compensating for other mutations that reduce RNP stability but which enhance viral replication.

Strengths:

The authors assay the effects of a variety of mutations found in SARS-CoV-2 variants of concern using a variety of approaches, including biophysical characterization of assembly properties of RNPs, combined with computational prediction of the effects of mutations on molecular structures and interactions. The findings of the paper contribute to our increasing understanding of the principles driving viral self-assembly, and increases the foundation for potential future design of therapeutics such as assembly inhibitors.

Weaknesses:

For the most part, the paper is well-written, the data presented support the claims made, and the arguments made easy to follow. However, I believe that parts of the presentation could be substantially improved. I found portions of the text to be overly long and verbose and likely could be substantially edited; the use of acronyms and initialisms is pervasive, making parts of the exposition laborious to follow; and portions of the figures are too small and difficult to read/understand.

Comments on revisions:

The authors have adequately addressed all of my concerns.

<https://doi.org/10.7554/eLife.108922.2.sa2>

Reviewer #3 (Public review):

Summary:

This manuscript investigates how mutations in the SARS-CoV-2 nucleocapsid protein (N) alter ribonucleoprotein (RNP) assembly, stability, and viral fitness. The authors focus on mutations such as P13L, G214C, G215C combining biophysical assays (SV-AUC, mass photometry, CD spectroscopy, EM), VLP formation, and reverse genetics. They propose that SARS-CoV-2 exploits "fuzzy complex" principles, where distributed weak interfaces in disordered regions allow both stability and plasticity, with measurable consequences for viral replication.

Strengths:

* The paper demonstrates a comprehensive integration of structural biophysics, peptide/protein assays, VLP systems, and reverse genetics.

- * Identification of both de novo (P13L) and stabilizing (G214C/G215C) interfaces provides a mechanistic insight into RNP formation.
- * Strong application of the "fuzzy complex" framework to viral assembly, showing how weak/disordered interactions support evolvability, is a significant conceptual advance in viral capsid assembly.
- * Overall, the study provides a mechanistic context for mutations that have arisen in major SARS-CoV-2 variants (Omicron, Delta, Lambda) and a mechanistic basis for how mutations influence phenotype via altered biomolecular interactions.

Weaknesses:

The weaknesses are shared via detailed comments to follow.

Comments on revisions:

The authors have addressed the criticisms of the original manuscript satisfactorily.

<https://doi.org/10.7554/eLife.108922.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

The authors attempted to clarify the impact of N protein mutations on ribonucleoprotein (RNP) assembly and stability using analytical ultracentrifugation (AUC) and mass photometry (MP). These complementary approaches provide a more comprehensive understanding of the underlying processes. Both SV-AUC and MP results consistently showed enhanced RNP assembly and stability due to N protein mutations.

The overall research design appears well planned, and the experiments were carefully executed.

Strengths:

SV-AUC, performed at higher concentrations (3 μ M), captured the hydrodynamic properties of bulk assembled complexes, while MP provided crucial information on dissociation rates and complex lifetimes at nanomolar concentrations. Together, the methods offered detailed insights into association states and dissociation kinetics across a broad concentration range. This represents a thorough application of solution physicochemistry.

We thank the Reviewer for this positive assessment.

Weaknesses:

Unlike AUC, MP observes only a part of the solution. In MP, bound molecules are accumulated on the glass surface (not dissociated), thus the concentration in solution should change as time develops. How does such concentration change impact the result shown here?

We agree with the Reviewer that the concentration in solution above the surface will change with time; however, the impact of surface adsorption turns out to be negligible. To show this

we have added a calculation as Supplementary Methods that is based on the number of imaged adsorption events, the fraction of imaged area to total surface area, and the initial sample volume and concentration. Under our experimental conditions the reduction is less than 1%, which is well within the range of experimental concentration errors.

This is in line with the observation that surface adsorption of proteins to glass is critical and needs to be prevented when working at picomolar concentrations (Zhao H, Mayer ML, Schuck P. 2014. Analysis of protein interactions with picomolar binding affinity by fluorescence-detected sedimentation velocity. *Anal Chem* 86:3181–3187. doi:10.1021/ac500093m), but is ordinarily negligible when working at the mid nanomolar concentration range. The difference in the MP experiments is that where usually the surface adsorption to glass and plastic is invisible, it is being imaged and quantified in MP. The negligible impact of surface adsorption on solution concentration in typical MP experiments is also in line with the results of several studies that have successfully measured dissociation constants of binding equilibria by MP (Young G et al., *Science* 360 (2018) 432; Wu & Piszczek, *Anal Biochem* 592 (2020) 113575; Solterman et al. *Angewandte Chemie* 59 (2020) 10774) with samples in the 5-50 nM range and similar experimental setup. It should be noted that in the MP experiments no surface functionalization is employed, in contrast to optical biosensors that utilize surface-immobilized ligands and polymeric matrices and thereby enhance the surface binding capacity.

Even though this depletion effect is negligible under ordinary MP conditions, the Reviewer raises a good point and readers may have a similar question with this novel technique. For this reason, we have added in the MP section of the Methods the sentence “In either configuration, the impact of surface binding on the sample concentration is < 1% and negligible, as described in the Supplementary Methods S1.” and added the detailed calculations in the Supplement accordingly. The use of SV as a traditional, orthogonal technique and the observation of consistent results with those of MP should further dispel readers’ methodological concerns in this point.

Reviewer #2 (Public Review):

Summary:

In this manuscript, the authors apply a variety of biophysical and computational techniques to characterize the effects of mutations in the SARS-CoV-2 N protein on the formation of ribonucleoprotein particles (RNPs). They find convergent evolution in multiple repeated independent mutations strengthening binding interfaces, compensating for other mutations that reduce RNP stability but which enhance viral replication.

Strengths:

The authors assay the effects of a variety of mutations found in SARS-CoV-2 variants of concern using a variety of approaches, including biophysical characterization of assembly properties of RNPs, combined with computational prediction of the effects of mutations on molecular structures and interactions. The findings of the paper contribute to our increasing understanding of the principles driving viral self-assembly, and increase the foundation for potential future design of therapeutics such as assembly inhibitors.

Thank you for highlighting the strengths of our paper and the potential impact on future design of therapeutics.

Weaknesses:

For the most part, the paper is well-written, the data presented support the claims made, and the arguments are easy to follow. However, I believe that parts of the presentation could be substantially improved. I found portions of the text to be overly long and verbose and likely could be substantially edited; the use of acronyms and initialisms is pervasive, making parts of the exposition laborious to follow; and portions of the figures are too small and difficult to read/understand.

We are glad the Reviewer concurs the data support our conclusions, and finds the arguments easy to follow. We appreciate the comment that the work was not optimally presented. To address this point, we have identified multiple opportunities to streamline the text without jeopardizing the clarity. We have also rewritten the end of the Introduction.

As recommended, we have reduced and harmonized the use of acronyms and abbreviations throughout the text to improve readability. Specifically, we have now spelled out nucleic acid (NA), intrinsically disordered regions (IDR), full-length (FL), AlphaFold (AF3), and variants of concern (VOC).

Finally, we have improved the presentation of most figures, adding labels and new panels, and increased the label font sizes to facilitate more detailed inspections of the data.

Reviewer #3 (Public Review):

This manuscript investigates how mutations in the SARS-CoV-2 nucleocapsid protein (N) alter ribonucleoprotein (RNP) assembly, stability, and viral fitness. The authors focus on mutations such as P13L, G214C, and G215C, combining biophysical assays (SV-AUC, mass photometry, CD spectroscopy, EM), VLP formation, and reverse genetics. They propose that SARS-CoV-2 exploits "fuzzy complex" principles, where distributed weak interfaces in disordered regions allow both stability and plasticity, with measurable consequences for viral replication.

Strengths:

- (1) The paper demonstrates a comprehensive integration of structural biophysics, peptide/protein assays, VLP systems, and reverse genetics.*
- (2) Identification of both de novo (P13L) and stabilizing (G214C/G215C) interfaces provides a mechanistic insight into RNP formation.*
- (3) Strong application of the "fuzzy complex" framework to viral assembly, showing how weak/disordered interactions support evolvability, is a significant conceptual advance in viral capsid assembly.*
- (4) Overall, the study provides a mechanistic context for mutations that have arisen in major SARS-CoV-2 variants (Omicron, Delta, Lambda) and a mechanistic basis for how mutations influence phenotype via altered biomolecular interactions.*

We are grateful for these comments highlighting this work as a significant conceptual advance.

Weaknesses:

- (1) The arrangement of N dimers around LRS helices is presented in Figure 1C, but the text concedes that "the arrangement sketched in Figure 1C is not unique" (lines 144-146) and that AF3 modeling attempts yielded "only inconsistent results" (line 149).*

The authors should therefore present the models more cautiously as hypotheses instead. Additional alternative arrangements should be included in the Supplementary Information, so the readers do not over-interpret a single schematic model.

We agree that in the absence of high-resolution structures the RNP models are hypothetical, and have now emphasized this in the Results, following the Reviewer's recommendation. To present alternative arrangements that satisfy the biophysical constraints upfront, we have promoted the previous Supplementary Figure 11 showing different models to the first Supplementary Figure, and expanded it with examples of different oligomers. In this way it is referenced early on in the Results and in the legend to Figure 1C. We agree this strengthens the manuscript, as one of the take-home messages is the inherent polydispersity of the RNPs.

The fact that AF3 can only provide inconsistent results will not come as a surprise, given the substantial disordered regions of the complex, and is a drawback of AF3 rather than our structural model. We slightly emphasized this point so as to clarify that the presentation of the AF3-based RNP structure serves solely as supporting evidence that our hypothetical model is sterically reasonable.

The new Results paragraph reads:

“As suggested in the cartoon of Figure 1C, this supports the hypothesis of a three-dimensional arrangement with a central LRS oligomer with symmetry properties and dimensions similar to low resolution EM images of model RNPs (Carlson et al., 2022, 2020) and cryo-ET of RNPs in virions (Klein et al., 2020; Yao et al., 2020). It should be noted, however, that the arrangement sketched in Figure 1C is not unique and other subunit orientations could be envisioned that satisfy all constraints from experimentally observed binding interfaces, including different oligomers and anti-parallel subunits as illustrated in Supplementary Figure S1. Extending previous ColabFold structural predictions that show multiple N-protein dimers self-assembled via the LRS coiled-coils (Zhao et al., 2023), we attempted the AlphaFold modeling of RNPs combining multiple N dimers with SL7 RNA ligands, mimicking our biophysical assembly model. Current AlphaFold restrictions limit the prediction to pentamers of N-protein dimers with 10 copies of SL7 RNA. While only inconsistent results were obtained – which is not surprising given the large intrinsically disordered regions exceed the predictive power of AlphaFold – some models did produce an overall RNP organization similar to Figure 1C, suggesting such an arrangement is at least sterically reasonable with regard to possible N-protein subunit orientations in an RNP (Supplementary Figure S2)”

(2) Negative-stained EM fibrils (Figure 2A) and CD spectra (Figure 2B) are presented to argue that P13L promotes β -sheet self-association. However, the claim could benefit from more orthogonal validation of β -sheet self-association. Additional confirmation via FTIR spectra or ThT fluorescence could be used to further distinguish structured β -sheets from amorphous aggregation.

We completely agree that the application of multiple orthogonal biophysical methods can strengthen the conclusions. In addition to EM fibrils and CD spectra (a classical gold standard technique for protein secondary structure in solution), we already have support from ColabFold modeling, as well as NMR results from the Zweckstetter lab showing the potential for β -sheet-like conformations.

Furthermore, we believe the evidence for the absence of ‘amorphous aggregates’ is very strong, as this would be inconsistent with the long-range order required to create the visibly fibrillar morphology in EM, and amorphous aggregates would be inconsistent with the increased solution viscosity. In this context, it is also highly relevant that the β -sheet-like secondary structure recorded by CD is concentration-dependent and reversible upon dilution. The long-range spatial order of fibrils is consistent with the formation of secondary structure in solution.

In addition, it must be kept in mind that what we see is specific to N-arm peptides carrying the P13L mutation (in EM, CD, and structural prediction) and does not occur in the other two

N-arm peptides (ancestral N-arm and N-arm with deletion of 31-33), linker peptides, or C-arm peptides.

Most importantly, as elaborated in more detail below, we do not claim that fibril formation is physiologically relevant. At the heart of this – in the context of the evolution of fuzzy complexes – is that the P13L mutation creates additional weak protein-protein interactions. Indeed, the assembly of fibrils geometrically requires at least two interfaces for each subunit. These weak interactions are at play physiologically in the context of the disordered RNP particles, and in macromolecular condensates, but not in the formation of fibrils. Therefore, while we appreciate the suggestion for FTIR spectra ThT staining, we are afraid further emphasis on the fibril structure might confuse the reader, and therefore we would rather clarify upfront that these fibrillar assemblies are not thought to form *in vivo* from full-length protein, but merely demonstrate the presence of N-arm self-association interfaces in the model of truncated peptides.

Accordingly, we have amended the Results paragraph reporting the fibrils:

“Thus, the N-arm mutation P13L is responsible for the formation of fibrils in N-arm peptides after prolonged storage. Some of these N-arm fibrils exhibit a twisted morphology with width of »5 nm (Figure 2A), in some instances exhibiting patterns of strand breaks. Such fibrils are frequently encountered in proteins that can stack β -sheets, such as in amyloids (Paravastu et al., 2008). While we have not observed fibril formation in the context of full-length N, and have no evidence such fibrils are physiologically relevant, their occurrence in solutions of truncated N-arm peptide nonetheless demonstrates the introduction of ordered N-arm self-association interfaces in conformations of P13L mutants.”

And more completely summarized experimental evidence prior to describing the ColabFold prediction results (which previously did not include mention of the NMR):

“Finally, confirming the interpretation of the EM images and the CD data, as well as the b-structure propensity reported from NMR data (Zachrdla et al., 2022), the structural prediction of N[10-20]:P13L in ColabFold displayed oligomers with stacking b-sheets ...”

(3) In the main text, the authors alternate between emphasizing non-covalent effects ("a major effect of the cysteines already arises in reduced conditions without any covalent bonds," line 576) and highlighting "oxidized tetrameric N-proteins of N:G214C and N:G215C can be incorporated into RNPs". Therefore, the biological relevance of disulfide redox chemistry in viral assembly in vivo remains unclear. Discussing cellular redox plausibility and whether the authors' oxidizing conditions are meant as a mechanistic stress test rather than physiological mimicry could improve the interpretation of these results.

The paper could benefit if the authors provide a summary figure or table contrasting reduced vs. oxidized conditions for G214C/G215C mutants (self-association, oligomerization state, RNP stability). Explicitly discuss whether disulfides are likely to form in infected cells.

We thank the Reviewer for raising this most interesting point. The reason why the biological relevance of N disulfides remains unclear is simply that this is still unknown, unfortunately. Recently, Kubinski et al. have strongly argued for the formation of disulfides in infected cells, but in our view the evidence remains weak since the majority of disulfide bonds in that work presented as post-lysis artifacts, and it appears the non-covalent effects alone could explain the physiological observations. We aimed for a balanced presentation and wrote in the relevant Results section:

“Covalent disulfide bonds in the LRS in non-reducing conditions were found to further promote LRS oligomerization. However, there is no conclusive data yet whether covalent

bonds in the LRS occur in vivo, or any G215C effect is entirely non-covalent due to the significant strengthening of LRS helix oligomerization (see Discussion).”

Despite the uncertainty regarding physiological disulfide bond formation, we believe it is useful to ask whether covalently crosslinked N dimers would aid or constrain RNP assembly in our biophysical model. We have now better explained this motivation in the Results section describing the RNP experiments:

“Even though it is still unclear whether disulfide bonds of N cysteine mutants form in vivo, we were curious about the impact of disulfide-linked oligomers of the cysteine mutants on their RNP structure and stability in our biophysical assembly model.”

The referenced paragraph from the Discussion reads:

“Regarding the cysteine mutations that have been repeatedly introduced in the LRS prior to the rise of the Omicron VOCs, it is an open question whether they lead to covalent bonds in vivo or in the VLP assay. While examples of disulfide-linked viral nucleocapsid proteins have been reported (Kubinski et al., 2024; Prokudina et al., 2004; Wootton and Yoo, 2003), a methodological difficulty in their detection is artifactual disulfide bond formation post-lysis of infected cells (Kubinski et al., 2024; Wootton and Yoo, 2003). However, our results clearly show that a major effect of the cysteines already arises in reduced conditions without any covalent bonds, through extension of the LRS helices, and concomitant redirection of the disordered N-terminal sequence. While oxidized tetrameric N-proteins of N:G214C and N:G215C can be incorporated into RNPs, the covalent bonds provided only marginally improved RNP stability. Interestingly, the introduction of cysteines imposes preferences of RNP oligomeric states dependent on oxidation state, consistent with our MD simulations highlighting the impact of cysteine orientation of 214C versus 215C relative to the hydrophobic surface of the LRS helices. Overall, considering potentially detrimental structural constraints from covalent bonds on LRS clusters seeding RNPs, energetic penalties on RNP disassembly, as well as the required monomeric state of the LRS helix for interaction with the NSP3 Ubl domain (Bessa et al., 2022), at present it is unclear to what extent the formation of disulfide linkages between LRS helices would be beneficial or detrimental in the viral life cycle.”

We feel that this text addresses the Reviewer’s comment, and that expanding the existing discussion further would conflict with other recommendations to shorten and focus the text.

Finally, we have addressed the valuable suggestion of a new table summarizing the oligomeric state and self-association of the different cysteine mutants by inserting a new column in the existing Table 1 reporting all species’ oligomeric state at low micromolar concentrations. In this way they can be compared at a glance with the other mutants as well. A more detailed comparison of the concentration-dependent size-distribution is provided in Figure 4.

(4) VLP assays (Figure 7) show little enhancement for P13L or G215C alone, whereas Figure 8 shows that P13L provides clear fitness advantages. This discrepancy is acknowledged but not reconciled with any mechanistic or systematic rationale. The authors should consider emphasizing the limitations of VLP assays and the sources of the discrepancy with respect to Figure 8.

We thank the Reviewer for this comment, which highlights a very important point.

For clarification and to improve the cohesion of the manuscript we have inserted a reference to the Discussion after the presentation of the VLP results, which provides a natural transition to the following description of the reverse genetics experiments:

“As expanded on in the Discussion, the failure to observe enhancement by P13L alone may be related to limitations of the VLP assay in sensitivity, including the restriction to a single round of infection, and protein expression levels.”

This references a paragraph in the Discussion about the limitations of the VLP assay in general and the reasons we believe the enhancement by P13L alone was not picked up:

“...While this assay has been widely used for rapid assessment of spike protein and N variants (Syed et al., 2021), it has limitations due to the addition of non-genomic RNA and the lack of double membrane vesicles from which gRNA emerges through the NSP3/NSP4 pore complex potentially poised for packaging (Bessa et al., 2022; Ke et al., 2024; Ni et al., 2023). It should also be recognized that the results do not directly reflect the relative efficiency of RNP assembly only, since protein expression levels, their localization, and their posttranslational modifications are not controlled for. Susceptibility for such factors might be exacerbated with mutations that modulate weak protein interactions. For example, as shown previously (Syed et al., 2024; Zhao et al., 2024), a GSK3 inhibitor inhibiting N-protein phosphorylation significantly enhances VLP formation and eliminates the advantage provided for by the N:G215C mutation relative to the ancestral N – presumably due to an increase in assembly-competent, non-phosphorylated N-protein erasing an affinity advantage. A similar process may be underlying the absent or marginal improvement in VLP readout from the cysteine LRS mutants and P13L at the achieved transfection level in the present work, and the enhanced signal from R203K/G204R and R203M (the latter being consistent with previous reports (Li et al., 2025; Syed et al., 2021)) modulating protein phosphorylation. Nonetheless, mirroring the results of the biophysical in vitro experiments, the addition of RNP-stabilizing P13L and G214C mutations on top of R203K/G204R led to a significantly larger VLP signal.

The VLP assay may be limited in sensitivity to mutation effects due to its restriction to a single round of infection. To avoid this and other potential limitations of the VLP assay for the study of viral packaging, for the key mutation N:P13L we carried out reverse genetics experiments. These showed the sole N:P13L mutation significantly increases viral fitness (Figure 8).”

(5) Figures 5 and 6 are dense, and the several overlays make it hard to read. The authors should consider picking the most extreme results to make a point in the main Figure 5 and move the other overlays to the Supplementary. Additionally, annotating MP peaks directly with “2×, 4×, 6× subunits” can help non-experts.

We completely agree with the Reviewer – these figures were very dense. To mitigate this problem without having the reader to switch back-and-forth to the supplement, we subdivided the panels of Figure 5 and showed only a subset of curves in each. In this way the data are easier to read while still readily compared. It is a large figure, but it contains the key data for the present work and is therefore worthwhile to have in one place. For the MP histogram data we also have inserted the suggested peak labels. Similarly, we have split Figure 6A into two panels for clarity.

(6) The paper has several names and shorthand notations for the mutants, making it hard to keep up. The authors could include a table that contains mutation keys, with each shorthand (Ancestral, No/No, Nλ, etc.) mapped onto exact N mutations (P13L, Δ31-33, R203K/G204R, G214C/G215C, etc.). They could then use the same glyphs (Latin vs Greek) consistently in text and figure labels.

Yes, we agree this is a problem and we apologize for the confusion. However, it is not possible to refer exclusively to either Latin or Greek terminology, which we feel would be even more detrimental to readability (the former being exhaustively lengthy and the latter being imprecise). But we have used a rational system: If the complete set of mutations of a variant are present, then its Greek letter will be used as an abbreviation, and otherwise we use Latin amino acid/position indicators for individual mutations or combinations thereof.

Unfortunately, previously we inadvertently failed to explicitly mention this, and we are most grateful for the Reviewer to point this out.

We have now rectified this by including upfront the sentence:

“We will adopt a nomenclature where the complete set of defining mutations of a variant will be referred to by its Greek letter, i.e., N:P13L/R203K/G204R/G214C is N_λ , and analogously the set of Omicron mutations N:P13L/ Δ 31-33/R203K/G204R are referred to as N_o ; see Table 1”

This will define the two shorthands N_λ and N_o used. Furthermore, as suggested and pointed to in the text, Table 1 does provide the keys to mutation and variants, including the information in which variant any of the other mutations studied here occur.

(7) The EM fibrils (Figure 2A) and CD spectra (Figure 2B) were collected at mM peptide concentrations. These are far above physiological levels and may encourage non-specific aggregation. Similarly, the authors mention "ultra-weak binding energies that require mM concentrations to significantly populate oligomers". On the other hand, the experiments with full-length protein were performed at concentrations closer to biologically relevant concentrations in the micromolar range. While I appreciate the need to work at high concentrations to detect weak interactions, this raises questions about physiological relevance.

This is indeed an important point to clarify. We agree that much lower nucleocapsid protein concentrations are present in the cytosol on average, and these were used in our RNP assembly experiments. However, there are at least two important physiologically relevant cases where high local N concentrations do occur:

(1) Once assembled in RNPs, the disordered N-terminal extensions are locally at a very high concentration within the volume they can explore while tethered to the NTD. A back-of-the-envelope calculation assuming 12 N-protein subunits confining 12 N-terminal extensions to the volume of a single RNP ($\approx 14 \times 14 \times 14 \text{ nm}^3$ by cryoEM; Klein et al 2020) leads to an effective concentration of 7.4 mM. Obviously the N-arm peptides are not completely free and there will be constraints that would hinder or promote encounter complex probability, but interfaces with mM Kd are clearly strong enough to populate Narm-Narm contacts extending from N-protein in the RNP.

Additionally, any interaction where N-proteins are brought in close proximity could allow weak N-arm interactions to provide additional stability. Besides the RNP, we demonstrate this in our Results for nucleic-acid liganded N tetramers (Figure 4B), but this might similarly occur in complexes with NSP3 or host proteins. Generally, it is quite common that small additional binding energies play important roles in the modulation of multivalent protein complexes.

(2) Within the macromolecular condensate the local concentration will be substantially higher than on average within the infected cell. While we do not know its precise concentration, it is well-established that the sum of many ultra-weak interactions is driving the formation of this dense liquid phase. In our previous eLife paper (Nguyen et al., 2024) we have shown LLPS is suppressed with the R203K/G204R mutation, but it is ‘rescued’ with the additional P13L/ Δ 31-33 mutation of the Omicron variant showing strong LLPS. Similarly, LLPS is suppressed by the LRS mutant L222P, but rescued in conjunction with P13L. This is another biologically relevant scenario where weak interactions are critical.

We have emphasized these points in the revised manuscript as described below.

Specifically:

(a) Could some of the fibril/ β -sheet features attributed to P13L (Figure 2A-C) reflect non-specific aggregation at high concentrations rather than bona fide self-association motifs that could play out in biologically relevant scenarios?

We understand this concern from the experience with proteins that often have limited solubility and tendencies to aggregate, sometimes accompanied by unfolding and driven by hydrophobic interactions, or clustering on the path to LLPS. However, we are struggling to reconcile the picture of non-specific aggregation with the context of our P13L N-arm peptides. The term ‘non-specific aggregation’ implies the idea of amorphous aggregates, which we would contend is inconsistent with the observed geometry of fibrils, which exhibit long-range order. In addition, non-specific aggregation does not lead to increased solution viscosity, which we describe, but fibril formation does. Another connotation of ‘aggregates’ is irreversibility. However, we find the beta-sheet-like conformation seen at 1 mM becomes significantly more disordered when the same sample is diluted to 0.4 mM peptide. This is consistent with a reversible self-association driven by a conformational change toward ordered secondary structure.

To highlight the reversibility, we have clarified the description: “Interestingly, diluting the 1 mM sample (solid) to a concentration of 0.4 mM (dashed) reveals a large shift in the far-UV spectra ... both indicative of a significant increase of disorder upon dilution. This is consistent with the stabilization of β -sheets in a reversible, strongly cooperative self-association process with an effective K_D in the high mM to low mM range.”

We have also inserted a concentration conversion to mg/ml units, which shows even 1 mM of peptides is only ~5 mg/ml, i.e. not excessively high. “While the ancestral N-arm at »1 mM (» 4.6 mg/ml) concentrations exhibits CD spectra with a minimum at »200 nm typical of disordered conformations (black)”

With regard to the question of specificity, we have studied similar N-arm peptides without P13L mutations and with the 31-33 deletion under equivalent conditions. But we observe the reversible self-association, conformational change, and fibril formation only for those containing the P13L mutation, consistent with ColabFold predictions. Neither did we observe fibrils with disordered C-arm peptides.

How these weak self-association motifs in the N-arm can be physiologically relevant in the context of full-length protein modulating the stability of multi-molecular complexes and enhancing LLPS was outlined above, and further clarified in the manuscript as detailed below.

(b) How do the authors justify extrapolating from the mM-range peptide behaviors to the crowded but far lower effective concentrations in cells?

As pointed out above, the key to this question is the local preconcentration as the N-arm peptides are tethered to the rest of protein in the context of flexible multi-molecular assemblies. Another mechanism to consider is the formation of condensates. The response to the next comment will expand on this.

The authors should consider adding a dedicated section (either in Methods or Discussion) justifying the use of high concentrations, with estimation of local concentrations in RNPs and how they compare to the in vitro ranges used here. For concentration-dependent phenomena discussed here, it is vital to ensure that the findings are not artefacts of non-physiological peptide aggregation..

The use of high concentration in biophysical experiments is quite common, for example, in NMR or crystallography, insofar as they elucidate molecular properties. We believe this is obvious; the Reviewer will certainly agree with us, and this does not require further

elaboration. The property observed in this case is the existence of specific, weak protein self-association interfaces in the N-arm.

Our response to the Reviewer's point 7(a) addresses the distinction between artefactual aggregation and self-association of N-arm peptides. The relevance of these weak protein self-association interfaces in the context of the full-length protein is the second underlying question.

As we have previously stated in a dedicated Results paragraph:

“In contrast to the modulation of the coiled-coil LRS interfaces, the de novo creation of the N-arm self-association interface through beta-sheet interactions enabled by P13L cannot be readily observed in full-length N-protein at low M concentrations. Similar to the ancestral LRS interface, it provides only ultra-weak binding energies that require mM concentrations to significantly populate oligomers. This is fully consistent with the previous observation by SV-AUC that neither N:P13L,31-33 nor N₀ with the full set of Omicron mutations show any significant higher-order self-association at low M concentrations, whereas at high local concentrations – as observed in phase-separated droplets – they can modulate and cooperatively enhance self-association processes (Nguyen et al., 2024). (If fact, P13L can substitute for the LRS promoting LLPS, as observed in the rescue of LLPS by N:P13L,31-33/L222P mutants whereas N:L222P LRS-abrogating mutants are deficient in LLPS.) Another process that increases the local concentration of N-arm chains is the tetramerization of full-length N-protein. As described earlier, occupancy of the NA-binding site in the NTD allosterically promotes self-assembly of the LRS into higher oligomers (Zhao et al., 2021). We hypothesized that these oligomers may be cooperatively stabilized by additional N-arm interactions in P13L mutants.”

To state completely unambiguously why weak interfaces are important, we have followed the Reviewer's suggestion and added an additional clarification already earlier, at the end of the P13L Results section:

“While this self-association interface in the P13L N-arm is weak and its direct observation in biophysical experiments requires mM concentrations, which far exceed average intracellular concentration of N, such weak interactions can become highly relevant physiologically when high local concentrations are prevailing, for example, when the disordered extension is pre-concentrated while tethered within macromolecular assemblies as in the RNP, or in macromolecular condensates.”

Furthermore, we have added early in the Discussion:

“Even though the solution affinity of the N-arm P13L interface is ultra-weak, the average local concentration of N-arm chains across the RNP volume (in a back-of-the-envelope calculation assuming a ≈ 14 nm cube (Klein et al., 2020) with a dodecameric N cluster) is ≈ 7.4 mM, such that disordered N-arm peptides could well create populations of N-arm clusters stabilizing RNPs through this interface. However, besides the RNP-stabilizing mutants we have also observed unexpected RNP destabilization by the ubiquitous R203K/G204R double mutation, which may be caused by the introduction of additional charges close to the self-association interface in the LRS. In our experiments, this destabilization is more than compensated for by the P13L mutation. (Another scenario where ultra-weak interactions can have a critical impact is in molecular condensates. We previously reported the suppression of LLPS by the R203K/G204R mutation, which is rescued by the additional P13L/ Δ 31-33 mutation (Nguyen et al., 2024). This is consistent with compensatory weak stabilizing and destabilizing impacts of weak interactions on the RNP observed here.)”

Reviewer #1 (Recommendations for the Authors):

In Figure 1B, it is unclear what the orange lines connecting polypeptides represent, as well as the zig-zag orange lines in the N-arm.

We thank the Reviewer for this comment. We intended this to represent regions of self-association but recognize the patterned background is confusing. We have changed this now to solid-colored backgrounds, and indicated this in the figure legend:

“Regions of self-association are indicated by shaded backgrounds.”

Regarding presentation, in Figure 5 (MP), the relationship between mass and oligomer size should be shown more clearly.

We agree. To this end we have labeled the peaks in the MP histograms in Figure 5 with the oligomeric state of the 2N/2SL7 subunits.

Reviewer #2 (Recommendations for the Authors):

I find the science of the paper to be convincing and compellingly supported.

Thank you for this positive statement.

My primary complaints are with presentation or minor technical questions that, honestly, primarily arise due to my own ignorance and unfamiliarity with some of the techniques employed.

My primary issue is with the figures. I find, generally, the text in axes labels, ticks, and legends to be too small to comfortably read. This is particularly true in the CD spectra and

other data presented in Figures 1D, 2B, 4, 5, 6, and 8.

We agree and have increased the font size of all text and labels of the plots in Figure 1, 2, 4, 5, 6, and 8.

I also found the use of initialisms to be a bit overbearing and inconsistent. For example, the authors repeatedly switch between spelling out "nucleic acid" and the initialism "NA" (which is also never explicitly spelled out in the text). With the already substantial length of the text, my own personal opinion would be to suggest spelling out all initialisms in the interest of making the reading easier.

This is a valid criticism. To improve the readability, we have followed this advice and systematically spelled out “nucleic acid” instead of using “NA”. Similarly, we have now written out full-length instead of the abbreviation FL, and omitted the abbreviation IDR for intrinsically disordered regions, as well as VOC for variant of concern, and AF3 for AlphaFold.

Regarding the reference to mutants, we have now explained upfront the system of Latin and Greek nomenclature we consistently applied.

“We will adopt a nomenclature where the complete set of defining mutations of a variant will be referred to by its Greek letter, i.e., N:P13L/R203K/G204R/G214C is N_{I} , and analogously the set of Omicron mutations N:P13L/ Δ 31-33/R203K/G204R are referred to as N_{O} ; see Table 1”

I found the text to be verbose, bordering on overly so; the Introduction is more than two pages long. The section "Enhanced oligomerization of the leucine-rich sequence through cysteine mutations" has two long paragraphs of introduction before the present results are discussed, et cetera. An (admittedly, very rough) estimation of the length of the paper places it at ~9,000 -10,000 words long, and I think that the presentation might benefit from significant editing and

shortening.

We agree the manuscript is longer than would be desirable, and we generally prefer not to insert mini-introductions into Results sections. On the other hand, in order to make a solid contribution to understanding the big picture of fuzzy complexes in molecular evolution of RNA virus proteins it is indispensable to go into the details of RNP assembly and several of the interfaces. Therefore, we feel the length is in the range that it needs to be without losing clarity. In addition, other Reviewer suggestions to extend the discussion, for example, of limitations of VLP assays and the in vivo state of cysteines, conflict with significant shortening.

In the particular case of the cysteine mutations, cited by the Reviewer, we believe it is important to add detailed background on G215C, because the Results proceed in a comparison of the self-association mode between G215C and G214C. This is of significant interest in the present context not only for the independent introduction of interface-enhancing mutations highlighting the evolution of fuzzy complexes, but also because it illustrates the pleomorphic ability of RNPs.

Nonetheless, we have slightly shortened this text and merged the background into a single paragraph. More generally, we have critically reread the text to remove tangential sentences where possible and to make it more concise.

I have a few more specific comments.

In Figure 1A, I suggest explicitly labeling the location of the LRS, as it comes up repeatedly.

Yes, we thank the Reviewer for this suggestion and have introduced this label in Figure 1A.

In Figure 1B, the legend indicates that the red lines indicate "new inter-dimer interactions." However, these red lines are overlayed on a vertical stripe of red squiggles; it is unclear to me and not explicitly described in the legend what these squiggles are meant to illustrate.

We agree this background was confusing. As mentioned in our Response to Reviewer #1 we have replaced the structured background with a solid background and explained in the figure legend that these areas depict regions of self-association.

On lines 44-45, the authors state, "The IDRs amount to 45%, ..." 45% of what?

Thank you, this was unclear. We have now clarified "The IDRs amount to $\approx 45\%$ of total residues"

In lines 244 - 246, the authors compare the sizes of complexes in reducing versus non-reducing conditions as measured by dynamic light scattering, stating, "However, dynamic light scattering (DLS) revealed the presence of N210-246:G214C complexes with hydrodynamic radii 244 ranging from 6 to 40 nm (in comparison to 1-2 nm for N210-246:G215C(Zhao et al., 2022)) in reducing conditions, and slightly larger in non-reducing conditions (Supplementary Figure S4)." Using this single statistic seems to me to be a less-than-ideal way of characterizing what seems to me to be happening here. In Supplementary Figure 4, it appears to me that what is happening is that in non-reduced conditions, the sample is monodisperse, whereas in reducing conditions, the distribution becomes polydisperse/bimodal, with two clearly separate populations. I feel that this could use a more

thorough description rather than just stating the overall range of particle sizes.

Yes, the Reviewer is correct – it is indeed a good idea to be more precise here. To this end we have carried out cumulant analyses on the autocorrelation functions, as a time-honored method to quantify the polydispersity. Both samples are polydisperse, but more so in reducing conditions. We have now added “For N210-246:G214C a cumulant analysis results in radii of 8.8 nm and 10.6 nm and polydispersity indices of 0.40 and 0.35 for reducing and non-reducing conditions, respectively”

Finally, I have one remaining comment that is a result of my own inexperience with circular dichroism and interpreting the spectra. For me personally, I would appreciate a more thorough description/illustration of the statistics involved in the CD spectra, but perhaps this is not necessary for people who are more familiar with interpreting these kinds of data. For example, in Figure 1D, it is not clear to me what the error bars/confidence intervals for the CD data look like. I see many squiggles, some of which the authors claim are significant (e.g., the differences between ~215 - 230 nm), and others are not worthy of comment. Let's say, for example, that I fit a smoothed spline through these data and then measure the magnitude of the fluctuations from that spline to define/quantify confidence intervals. What does that distribution look like? Or maybe the confidence intervals are so small that all squiggles are significant?

Thank you, this is a good question. As mentioned in the methods section, the CD spectra shown are averages of triplicate scans. Therefore, it is straightforward to extract the standard deviation at each wavelength from the three measurements (although a spline would probably work just as well). The values are what one would expect for the squiggles to be random noise. In the region 215 – 220 nm characteristic for helical secondary structure the standard deviations are small relative to the separation between curves, which indicates that the differences are highly significant. Naturally, the curves do overlap in other spectral regions, which would make a plot including the wavelength-dependent error bars or confidence bands too crowded. Therefore, we have kept the plot of the averaged triplicate scans, but have now provided the average standard deviations for all species in the figure legend and mentioned their significant separation:

“Triplicate scans yield average standard deviations of 0.13 (N), 0.17 (N+SL7), 0.16 (N₁), and 0.21 (N₁ +SL7) 10³ deg cm²/dmol, respectively, with non-overlapping confidence bands for the different species, for example, between 215-220 nm.”

Reviewer #3 (Recommendations for the Authors):

(1) The Discussion reiterates much of the background (mutational tolerance, fuzziness, SLiMs) already covered in the Introduction, diluting focus on the key new findings. The authors should consider shortening and refocusing the discussion on the main contributions in light of existing knowledge of viral assembly.

In the Introduction we have provided background on intrinsically disordered proteins in general and their mutational tolerance, as well as the concept of fuzzy complexes. The first several paragraphs of the Discussion have a different focus, which is protein binding interfaces between viral proteins (obviously key in fuzzy complexes), specifically their modulation and the remarkable de novo introduction of binding interfaces. We believe this deserves emphasis, since this highlights a novel aspect of fuzziness, for the mutant spectrum of RNA viruses to encode a range and of assembly stabilities and architectures.

To reduce redundancy between the end of the Introduction and the beginning of the Discussion, we have shortened the last paragraph of the Introduction and removed its preview of the conclusions, as described in the response to the next comment of the Reviewer (see below).

Unfortunately, the length of the Discussion is dictated in part also by the need to discuss methodological aspects, among them the limitations of VLP assays, and the redox state of the cysteine in the LRS mutants, which were important points recommended by other suggestions of the Reviewers. Similarly, we believe the discussion of other potential functions of Omicron N-arm mutations is warranted, as well as the background of the R203K/G204R double mutation that has attracted significant attention in the field due to its effects on phosphorylation and expression of truncated N species that also form RNPs. Our goal was to integrate the results by us and other laboratories regarding specific mutation effects into a comprehensive picture of molecular evolution of N, which we believe the framework of fuzzy complexes can provide.

(2) The Abstract and early Introduction set a broad stage (IDPs, fuzziness), but don't explicitly state the concrete hypotheses that the experiments test. Please add 2-3 sentences in the Introduction that enumerate testable hypotheses, e.g.:

(a) P13L creates a new N-arm interface that increases RNP stability.

(b) G214C/G215C strengthens LRS oligomerization to stabilize higher-order N assemblies.

We agree the introduction can be improved. However, it seems to us that it cannot be neatly framed in the hypothesis – answer dichotomy, without losing a lot of nuances and without requiring an even longer and more detailed introduction.

One of the main questions is to test whether the framework of fuzzy complexes can be applied to understand molecular evolution of N, and we feel the introduction is already flowing well towards this:

“... In fuzzy complexes the total binding energy is distributed into multiple distinct ultra-weak interaction sites (Olsen et al., 2017). Similar to individual RNA virus proteins with loose or absent structure, maintaining disorder and a spatial distribution of low-energy interactions in the protein complexes may increase the tolerance for mutations and improve evolvability of protein complexes.”

The unprecedented worldwide sequencing effort of SARS-CoV-2 genomes during its rapid evolution in humans provides a unique opportunity to examine these concepts. ...”

To bring this to a more concrete set of questions in the end, we have shortened and rewritten the last paragraph in the Introduction:

“To examine how architecture and energetics of RNP assemblies can be impacted by N-protein mutations we study a panel of N-proteins derived from ancestral Wuhan-Hu-1 and different VOCs, including Alpha, Delta, Lambda, and Omicron (see Table 1), in biophysical experiments, VLP assays, and mutant virus. Specifically, we ask how the RNP size distribution and life-time is modulated by: (1) the novel binding interface created by the P13L mutation of Omicron; (2) enhancements of other weak self-association interfaces through G215C of Delta and G214C of Lambda; (3) the ubiquitous R203K/G204R double mutation of Alpha, Lambda, and Omicron. We also test whether the P13L mutation improves viral fitness, similar to G215C and R203K/G204R. The results are discussed in the framework of fuzzy complexes and molecular evolution of N in the course of viral adaptation to the human host. Understanding the salient features of the binding interfaces in viral assembly and their evolution expands our foundation for the design of therapeutics such as assembly inhibitors.”

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