Structural basis of rotavirus RNA chaperone displacement and RNA annealing

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Rotavirus genomes are distributed between 11 distinct RNA molecules, all of which must be selectively copackaged during virus assembly. This likely occurs through sequence-specific RNA interactions facilitated by the RNA chaperone NSP2. Here, we report that NSP2 autocatalyzes its chaperone activity through its C-terminal region (CTR) that promotes RNA–RNA interactions by limiting its helix-unwinding activity. Unexpectedly, structural proteomics data revealed that the CTR does not directly interact with RNA, while accelerating RNA release from NSP2. Cryo-electron microscopy reconstructions of an NSP2–RNA complex reveal a highly conserved acidic patch on the CTR, which is poised toward the bound RNA. Virus replication was abrogated by charge-disrupting mutations within the acidic patch but completely restored by charge-preserving mutations. Mechanistic similarities between NSP2 and the unrelated bacterial RNA chaperone Hfq suggest that accelerating RNA dissociation while promoting intermolecular RNA interactions may be a widespread strategy of RNA chaperone recycling.

RNA chaperones | ribonucleoproteins | genome assembly | rotavirus

Significance

Accurate RNA folding is essential for virus replication. Rotavirus are viruses infecting humans and animals. Rotavirus genome comprises 11 distinct RNAs, and successful replication requires the incorporation of all 11 RNAs into a virion. The RNA chaperone NSP2 binds viral transcripts, regulating their interactions with each other. NSP2 must release RNAs after they base pair prior to their packaging. Using single-molecule fluorescence tools, we dissected the individual steps of the RNA chaperone activity of NSP2. Structural proteomics and cryo-EM studies of the NSP2–RNA complex revealed that NSP2 regulates RNA unfolding and the release of the RNA using its charged C-terminal region. Some aspects of the viral RNA chaperone regulation mirror the conserved autoregulation mechanisms employed by bacterial RNA chaperones.

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Conserved NSP2 CTR Is Required for Efficient RNA Annealing. The CTR of NSP2 consists of a flexible linker (residues 1 through 294) lacking the entire CTR (ΔC) that tethers an α-helix (C-terminal helix, CTH) to NSP2core (i.e., residues 1 through 294, Fig. 1A). The CTH is ampholytic, containing highly conserved, positively (Arg307, Lys308) and negatively charged (Asp306, Asp310, and Glu311) residues. To interrogate the role of the CTR in NSP2 function, we generated NSP2-ΔC (residues 1 through 294) lacking the entire CTR (SI Appendix, Fig. S1). This NSP2-ΔC construct has been previously characterized by others (10, 20) (Fig. 1).

To visualize the CTR conformation in solution, we determined a cryo-EM three-dimensional (3D) reconstruction of full-length NSP2 (henceforth referred to as NSP2) at a global resolution of 3.9 Å (Fig. 1B and SI Appendix, Figs. S2 and S3). As expected, our cryo-EM-derived model of NSP2 revealed an octameric assembly, which was highly similar to previously solved crystal structures of NSP2 (the overall RMSD between equivalent Cα atoms of the refined model presented here and PDB 1L9V is 1.124 Å) (7, 10, 20). Within our density map, the CTH exhibited well-resolved density (local resolutions ranging between 3.6 and 4.0 Å (SI Appendix, Fig. S2)). Due to intrinsic flexibility, the linker region was poorly resolved (SI Appendix, Fig. S3). We also determined a 3D reconstruction of NSP2-ΔC using negative-stain EM which confirmed that NSP2-ΔC remains octameric, demonstrating that the CTR does not play a role in the assembly of NSP2 into functional octamers (SI Appendix, Fig. S1).

Next, we investigated the role of the CTR in the RNA annealing activity of NSP2 using a fluorescence cross-correlation based RNA–RNA interaction assay (19). We chose RNA transcripts S6 and S11, representing RV gene segments 6 and 11, as these have been previously shown to form stable RNA–RNA contacts in the presence of NSP2 (19). In brief, fluorescently labeled transcripts (RNAs S6 and S11) were coincubated in the absence or presence of either NSP2 or NSP2-ΔC, and intermolecular interactions were then quantitated in solution by measuring the cross-correlation function (CCF) amplitudes (21). While a zero CCF amplitude is indicative of noninteracting RNAs, increasing yields of intermolecular interactions result in proportionally higher, nonzero CCF amplitudes (21).

Coincubation of S6 and S11 transcripts alone resulted in a near-zero CCF amplitude indicating that they do not spontaneously interact (Fig. 2B and SI Appendix, Fig. S3). In contrast, addition of NSP2 to an equimolar mixture of these two RNAs produced a high CCF amplitude, indicative of intermolecular RNA duplex formation (Fig. 2B). This observation is in agreement with the known role of NSP2 as an RNA chaperone, facilitating the remodelling and annealing of structured RNAs (19).

Coincubation of S6 and S11 in the presence of NSP2-ΔC resulted in a reduced CCF amplitude (Fig. 2B), indicating that...
NSP2-ΔC has a reduced RNA-annealing activity relative to full-length NSP2. This is in agreement with our previous observation that NSP2-ΔC has reduced capacity to promote interactions between RV RNAs (19). Our combined data confirms that the CTR plays a role in the RNA chaperone function of NSP2 irrespective of the RNA substrates chosen.

The NSP2 CTR Reduces the RNA-Unwinding Activity but Does Not Directly Interact with RNA. As the ability of NSP2 to unfold and remodel RNA structures is a prerequisite for its RNA-annealing activity (4), we next investigated the role of the CTR in RNA helix destabilization. We used single-molecule Förster Resonance Energy Transfer (smFRET) to directly compare the abilities of NSP2 and NSP2-ΔC to unwind an RNA stem-loop labeled at the 5′ and 3′ termini with donor and acceptor dyes (Atto532 and Atto647N) (Fig. 2C and SI Appendix, Fig. S4).

In the absence of either protein, the stem-loop alone adopts a folded conformation, resulting in a single, high-FRET population ($E_{FRET} = 0.95$) (Fig. 2D). Incubation with NSP2 produces two distinct FRET populations, corresponding to fully folded ($E_{FRET} = 0.95$) and unfolded ($E_{FRET} = 0.05$) RNA states. No intermediate FRET populations (corresponding to partially unwound stem-loop conformations) were observed, in agreement with previous observations of NSP2-mediated RNA-unwinding (4).

We then measured the ability of NSP2-ΔC to unwind this RNA stem-loop. Surprisingly, in the presence of NSP2-ΔC, the stem-loop was predominantly unfolded ($E_{FRET} = 0.05$) (Fig. 2D). Furthermore, we did not observe differences in binding of either NSP2 or NSP2-ΔC to both folded and unfolded RNA conformations (Fig. 2E). These data demonstrate that NSP2-ΔC has enhanced RNA unfolding activity compared to its full-length counterpart. This result is somewhat paradoxical: while NSP2-ΔC is more efficient at destabilizing RNA structure (Fig. 2D), it is approximately half as efficient at promoting the annealing of structured RNAs as NSP2 (Fig. 2B).

To deduce whether the CTR directly interacts with RNA, we used a combination of structural proteomics techniques (Fig. 3). We performed hydrogen–deuterium exchange-mass spectrometry (HDX-MS) experiments to map regions of NSP2 that become protected from deuterium exchange when bound. We observed significant protection from exchange for peptides that predominantly mapped to deep grooves present on the surface of NSP2, indicating that this is the major RNA-binding site of NSP2 (Fig. 3A and B and SI Appendix, Fig. S5).

We further corroborated the location of RNA-binding sites on NSP2 using UV crosslinking with RBDmap (22, 23). In addition to HDX-MS data, RBDmap identified RNA-linked peptides that map to these surface-exposed RNA-binding grooves (Fig. 3C and
positively charged residues, most notably R68 (Fig. 4). We observed interactions between NSP2 and the RNA in the structure. However, we were able to visualize residue-specific nature of this density prevented us from modeling the ssRNA into occupied, focused map in order to visualize NSP2 then computed a difference map between NSP2 and the RNA−RBDmap (Fig. 3). Multiple regions of NSP2 except the CTR (green box) are protected by bound RNA. (C) Normalized occurrence of the RNA-interacting residues determined using UV crosslinking (identified by RBDmap). (D) RBDmap-identified RNA-binding peptides mapped onto the surface of NSP2 octamer (Left) and its monomer (Right). Structures are colored according to frequency of crosslink occurrence. No RNA:peptide cross-links were identified on the CTR (green box).

Cryo-EM Visualization of NSP2−RNA Interactions. To understand the molecular basis of RNA binding by NSP2, we determined a cryo-EM reconstruction of an NSP2−RNP complex at a global resolution of 3.1 Å (Fig. 4A and SI Appendix, Figs. S1 and S2). While the cryo-EM density corresponding to NSP2 was well resolved, there was no density that could be attributed to the RNA in the high-resolution postprocessed NSP2−RNP map (Fig. 4A). This is likely due to the heterogeneity and intrinsic flexibility of NSP2-bound unstructured single-stranded RNA. Despite this, an additional feature localized to RNA binding sites identified by HDX-MS and RBDmap (Fig. 3) was present in 5-Å low-pass filtered (LPF) maps (Fig. 4B). Notably, such a density feature was not present in 5-Å LPF NSP2 apoprotein maps (Fig. 4C). We attribute this density to NSP2-bound RNA.

To improve visualization of the RNA density, we performed focused classification using C4 symmetry-expanded data with a mask applied to a single RNA-binding face of NSP2 (24–26). The resulting 3D reconstructions readily classified into four dominant populations, three of which had poor RNA occupancy (each class with 26% of input particles), while a single 3D class average (22% of input particles) exhibited improved RNA density (SI Appendix, Fig. S2). Due to the reasons outlined above, the diffuse nature of this density prevented us from modeling the ssRNA into the structure. However, we were able to visualize residue-specific NSP2−RNA contacts (Fig. 4D).

We built an atomic model of NSP2 into the sharpened map and then computed a difference map between NSP2 and the RNA-occupied, focused map in order to visualize NSP2−RNA contacts. Significant positive density was localized in the basic groove of NSP2 (Fig. 4), consistent with the binding site identified through HDX and RBDmap (Fig. 3). We observed interactions between positively charged residues, most notably R68 (Fig. 4D). Adjacent to this contact are K58, K59, and R60, of which K59 and R60 are directly oriented toward the RNA density (Fig. 4D, Inset). The importance of these residues for RNA capture by NSP2 is strongly supported by previous biochemical studies that identified a number of solvent-exposed lysine and arginine residues (K37, K38, K58, K59, R60, and R68) that span the periphery of the NSP2 octamer (SI Appendix, Fig. S6) and contribute to RNA binding (27).

Furthermore, the identified residues are localized to an unstructured loop within the RNA-binding groove, allowing promiscuous and flexible accommodation of alternative RNA structures with near-identical affinities by NSP2, consistent with previous reports (4, 28). Together with our HDX and RBDmap results, our cryo-EM reconstruction reveals a number of electrostatic contacts that provide a plausible molecular basis for nonspecific NSP2−RNA interactions (Fig. 4 D, Inset). In addition, our EM reconstruction has revealed a number of other residues (R240, K286, and F290) that likely contribute to RNA binding, also identified by HDX and RBDmap (SI Appendix, Figs. S5 and S6). These residues may also participate in nonspecific RNA contacts via electrostatic interactions, hydrogen bonding, and π−π stacking, consistent with a significant nonelectrostatic contribution to the overall free energy of RNA binding to NSP2.

Conserved Acidic Patch within the CTR Promotes RNA Dissociation. Within the cryo-EM density map, the CTRs are poised below the RNA, while making limited contacts with the observed RNA density (Fig. 4D). This suggests that the CTR may play a role in promoting RNA dissociation from NSP2. To investigate this, we performed binding kinetics measurements using surface plasmon resonance (SPR) (Fig. 5A and B). Association rate constants (Ka) remain largely consistent across a range of concentrations of both NSP2 and NSP2−AC (NSP2−AC binds (1.5 ± 0.4)-fold faster than NSP2) (SI Appendix, Table S1). However, NSP2−AC exhibited a (3.2 ± 0.3)-fold slower dissociation rate than NSP2, suggesting a role for the CTR in the displacement of bound RNA (Fig. 5A and SI Appendix, Table S1).

Close examination of our cryo-EM-derived model revealed a conserved acidic patch within the CTR (Fig. 4E). This is in contrast to other clusters of surface-exposed acidic residues on NSP2 that show a low degree of conservation (SI Appendix, Fig. S6). The acidic patch of the CTR is presented directly underneath the density attributed to bound RNA (Fig. 4D), potentially promoting...
RNA displacement from NSP2. Such displacement could be achieved either via direct competition with RNA-binding residues or by providing a negatively charged environment that accelerates RNA dissociation from NSP2 through charge repulsion. Therefore, to further investigate RNA displacement from NSP2, we used RNA competition assays (Fig. 5 C and D). We performed titrations of unlabeled RNA into preformed RNP complexes containing fluorescently labeled RNA to understand the differences in RNA exchange and chaperone recycling between NSP2 and NSP2-ΔC. Using fluorescence anisotropy, we estimated the degree of competition as the concentration of competitor RNA required to displace 50% of prebound RNA from either NSP2 or NSP2-ΔC complexes (IC_{50}). We determined IC_{50} values of 208 ± 11 nM and 890 ± 160 nM for NSP2 and NSP2-ΔC, respectively, confirming that NSP2-ΔC undergoes approximately fourfold reduced RNA exchange, consistent with its approximately threefold slower rate of dissociation from RNA (Fig. 5 A and B).

We then investigated whether the CTR promotes RNA dissociation from NSP2 through directly competing with RNA for binding to basic, RNA-binding residues on the NSP2 core. To achieve this, we measured RNA binding by NSP2-ΔC in the presence of saturating amounts of a synthetic peptide matching the sequence of the CTR. We confirmed that the peptide in isolation was structured and able to directly bind to NSP2-ΔC octamers when added in trans (SI Appendix, Fig. S7). No dissociation of RNA from NSP2-ΔC was observed in the presence of 20-fold molar excess of the CTR peptide over NSP2-ΔC (Fig. 5D). Furthermore, no RNA binding was observed upon incubation with 10 μM CTR peptide (i.e., 400-fold excess), indicating that the CTR does not bind RNA. This suggests that while the CTR is required for RNA displacement from NSP2, this does not occur through direct competition. However, from these data we cannot definitively rule out a model whereby the CTR promotes dissociation in cis (i.e., as part of the intact NSP2 octamer) via increasing the effective concentration of the CTR in proximity to bound RNA in a displacement reaction.

We analyzed our atomic model of NSP2 to evaluate the distances between acidic residues within the CTR and the basic, acidic patch (D306, D310, and E311) annotated.

Fig. 4. Cryo-EM structure of the NSP2–RNP complex. (A) A 3.1-Å-resolution reconstruction of the NSP2–RNP complex. (B and C) Cryo-EM maps of NSP2–RNA (B) and NSP2 apoprotein (C) LPF to 5 Å. A cryo-EM density feature (peach) is attributed to bound RNA in the LPF RNP map. Both maps are reconstructed with D4 symmetry. (D) Direct visualization of interactions between NSP2 and RNA using C4 symmetry expansion and focused classification. The positive difference density map corresponding to RNA (peach) is overlaid onto the unsharpened NSP2–RNP complex map determined through symmetry expansion and focused classification (gray, transparent density) and atomic model of NSP2. (Inset) Zoom-in of the CTR positioned relative to RNA density (Top) and RNA-interacting residues (Bottom). (E) The surface electrostatic potential analysis of NSP2. (Inset) Zoom-in of the CTR, with residues within the acidic patch (D306, D310, and E311) annotated.

Fig. 5. The CTR promotes RNA dissociation noncompetitively. (A and B) SPR sensograms of NSP2 (A) and NSP2–ΔC (B) binding to RNA. Although NSP2–ΔC binds RNA with an approximately sixfold higher affinity, this is due to a modest (1.5-fold) increase in K_{diss} and a larger (3.2-fold) decrease in K_{on}. (C) RNA competition assay. The fractional binding of fluorescently labeled RNA was determined by fluorescence anisotropy. Labeled RNA (10 nM) fully bound to NSP2 (orange) or NSP2–ΔC (blue) was titrated with unlabeled RNA of identical sequence to compete for NSP2 binding against labeled RNA. The IC_{50} values for NSP2 and NSP2–ΔC are 208 ± 11 nM and 890 ± 160 nM, respectively. The NSP2–RNP complex undergoes strand exchange more readily than the NSP2–ΔC–RNA complex. (D) RNA binding by NSP2 in the presence of the CTR peptide. CTR peptide (10 μM) was added to preformed NSP2–ΔC–RNA complexes. The CTR peptide does not compete with RNA for binding to NSP2–ΔC. (E) Estimated distances between acidic residues within the CTR and the R86 that interacts with RNA. Note the nearest side chain (E311), which is 18 Å away from R86.
RNA-binding residues localized to flexible loops within the RNA-binding grooves (Fig. 5E and SI Appendix, Fig. S6). The distances (∼10 to 30 Å) between acidic residues within the CTR and the RNA-interacting residues are incongruent with a direct competition model. While R68 was demonstrated to directly interact with RNA (Fig. 4D), it is 18 Å away from acidic residues within the CTR. This further demonstrates that while the CTR promotes dissociation of RNA from NSP2, it does not do so through direct competition for NSP2-core binding (Fig. 5E). Collectively, our data suggest that conserved acidic patches within the CTR promote dissociation of bound RNA from NSP2 via charge repulsion.

**CTR Acidic Patch Is Required for Viral Replication.** To validate our findings, we employed a reverse-genetics approach to rescue recombinant RVs with point mutations within the CTR. We assessed the effects of amino acid substitutions within the CTR on viral replication by attempting recombinant virus rescue (Materials and Methods). Five attempts to rescue a triple-alanine mutant D306A/D310A/E311A (referred to as AAA) were unsuccessful (Fig. 6A), suggesting that these mutations abrogate virus replication. Remarkably, a triple mutant containing charge-preserving mutations D306E/D310E/E311D (referred to as EED) was successfully rescued along with the wild-type virus (Fig. 6A and SI Appendix, Fig. S8). This directly demonstrates the essential role of the CTR acidic patch in RV replication.

During the course of infection, RV assembly occurs in large, cytoplasmic viral replication factories, assembled from NSP2 and NSP5 and containing viral RNAs (2, 16, 29–31). While the C-terminal hexa-histidine–tagged NSP2 (NSP2-6xHis) and the EED mutant were able to support viral replication (Fig. 6A–C), we sought to determine whether these mutants had any effect on replication factory assembly. Remarkably, neither C-terminal 6xHis-tagging of NSP2 nor introduction of the charge-preserving mutations had any noticeable impact on the formation of viroplasms (Fig. 6D) at 6 h postinfection in MA104 cells expressing NSP5-EGFP (Fig. 6D). Single-molecule (sm) fluorescence in situ hybridization (FISH) (Materials and Methods) confirmed that these NSP5-EGFP–tagged inclusions represented functional viroplasms that...
support accumulation of viral RNAs. Importantly, the kinetics of viroplasm formation (e.g., readily observable <6 hpi) were comparable to those observed for wild-type virus replicating in the NSP5-EGFP cell line (31, 32).

Since all our attempts to rescue NSP2-AAA mutants were unsuccessful, we also examined whether these mutations disrupted the formation of the NSP2/NSP5-rich viroplasms. As described in Materials and Methods, we transfected an NSP2–AAA-expressing DNA construct into MA104 cells stably expressing NSP5 (29, 30) and analyzed the formation of viroplasm-like structures through immunofluorescence imaging of NSP5 (Fig. 6E). We observed that NSP2-AAA supported the formation of NSP5/NSP2-rich cytoplasmic inclusions morphologically identical to those formed by wild-type virus (31), allowing us to conclude that NSP2-AAA mutant retained its capacity to form viroplasm-like structures. Together, these results further confirm that the conserved charged residues within the acidic patch of the NSP2 CTR are essential for virus replication.

Discussion

Long RNAs adopt an ensemble of diverse stable structures that limit spontaneous RNA–RNA interactions through the sequestration of sequences required for intermolecular base pairing (19, 33–36). This necessitates the action of RNA chaperone proteins to bind and refold RNA structures in order to promote RNA annealing between complementary sequences (37–39).

In order to function as an RNA chaperone, NSP2 must capture, unwind, anneal, and release complementary RNA sequences (19, 40, 41). Previous structural studies have provided static snapshots of crystallographically averaged NSP2–RNA complexes (7, 20, 27). However, due to the highly dynamic nature of the protein–RNA interactions required for its RNA chaperone activity, they have only revealed limited insights into the molecular mechanisms of NSP2. Our recent work (4, 19, 42) indicates that for NSP2–RNP complexes, such heterogeneity arises from poorly defined protein–RNA stoichiometries and the ability of bound RNA to adopt multiple configurations and orientations. To overcome these challenges, we used a combination of single-molecule fluorescence, cryo-EM, structural proteomics, and biophysical assays to decipher the mechanism of NSP2 chaperone function.

Previous work suggests that the CTR of NSP2 is essential for RV replication (17). Using single-molecule fluorescence techniques, we have directly shown that the CTR of NSP2 is important for promoting RNA–RNA interactions. However, we only identified interactions between RNA and basic residues located in flexible loops within the RNA-binding groove of NSP2 but not the CTR. Remarkably, similar RNA recognition mechanisms have been reported in other RNA chaperones including Escherichia coli StpA and HIV-1 NC (43, 44). Collectively, these results highlight the role of the CTR in NSP2 RNA chaperone activity but not RNA binding.

Mechanism of the CTR-Assisted RNA Displacement and Its Role in RNA Matchmaking. We propose a model whereby the RV RNA chaperone NSP2 binds to RNA with high affinity, resulting in RNA structure destabilization (Fig. 7A). Fluorescence correlation spectroscopy (FCS) analysis of high- and low-FRET RNA species points out that full-length NSP2 binds to both the unfolded and folded RNA conformations, priming RNAs for efficient RNA annealing (Fig. 2). These results are fully consistent with our previous single-molecule fluorescence studies of NSP2 (4), confirming that upon binding to NSP2, only two FRET states are observed—a high-FRET state corresponding to a folded RNA stem-loop and a low FRET state that corresponds to an unfolded RNA conformation (Fig. 2 D and E). These results are not compatible with the RNA-sliding mechanism, which would be expected to yield intermediate FRET states, reminiscent of the mechanisms employed by positively charged disordered chaperones that enhance nucleic acid folding via local charge screening (45). Notably, NSP2-mediated RNA-unfolding is distinct from that of the other viral RNA chaperone avian reovirus σNS that was shown to destabilize RNA structures via multiple, dynamic interconverting intermediate states (4, 42). Thus, single-molecule biophysics data reveal that NSP2 is able to capture folded RNA stem-loop structure within a single RNA-binding groove. This result in general relaxation of RNA structure without fraying of the RNA stem termini, so that the RNA structure is sufficiently destabilized that it adopts a completely open conformation. Since NSP2 preferentially binds unstructured RNA (4), it forms a stable RNP complex with an unstructured RNA until intermolecular annealing is achieved. By binding to multiple RNAs concomitantly via surface-exposed grooves (Fig. 7A, cyan) (4, 19, 39) NSP2 octamers act as matchmakers of complementary sequences, promoting intermolecular RNA–RNA interactions. Our single-molecule fluorescence and binding kinetics experiments also indicate that removal of the CTR does not perturb RNA binding but slows RNA release (~3.2-fold increase in koff). Moreover, CTR removal results in a~2.4-fold increase in the RNA-unwinding activity of NSP2–ΔC as well as an approximately twofold decrease in its RNA-annealing activity. Additionally, smFRET data reveal that binding to NSP2–ΔC energetically favors low-FRET (unfolded) RNA conformations, resulting in remodelling of structured RNAs (Fig. 2D). The resulting increased stability of NSP2–ΔC–RNA complexes precludes efficient RNA annealing, yielding kinetically trapped RNP complex intermediates (Fig. 7 B and C). We propose that conserved acidic patches within the ampholytic CTR (Fig. 7D, red) accelerate RNA displacement from NSP2 via charge repulsion, thus enabling RNA chaperone recycling and duplex release. Removal of the ampholytic CTRs in NSP2 variants derived from two distinct RV viruses (strains SA11 and RF) has a similar outcome on RNA chaperone activity in vitro (SI Appendix, Fig. S3), suggesting a conserved role of the CTR in NSP2 function. This model is further supported by our observation that removal of the unstructured region downstream of the ampholytic CTR (Fig. 7A)
does not alter the RNA-unwinding activity of NSP2 ([SI Appendix, Fig. S4]). Indeed, this partial truncation has been previously shown to support viral replication (10). Exctually, we have exploited recent technical advances in reverse genetics of RVs to directly demonstrate the pivotal role of these conserved acidic residues in RV replication (Fig. 6). Moreover, while a single NSP2 octamer can efficiently achieve RNA stem-loop unwinding at subnanomolar concentration, we have previously shown that intermolecular strand-anchoring requires a stoichiometric excess of NSP2 octamers (19). Thus, it is likely that intermolecular annealing takes place between RNAs presented by multiple NSP2 octamers. However, given the current available data, we cannot rule out the possibility of intermolecular annealing within the same RNA-binding groove or via protruding single-stranded sections.

The described principle of CTR-assisted RNA dissociation from NSP2 is strikingly similar to that of the bacterial RNA chaperone protein Hfq (46–48). Hfq possesses an unstructured C-terminal domain (CTD) with an acidic tip that drives RNA displacement from the core of Hfq (49). Unlike the Hfq CTD, we do not observe competition between the CTR and RNA for NSP2<sub>ΔC</sub> binding (50, 51). It is important to note that it is possible that the CTR promotes RNA displacement only when tethered to NSP2<sub>ΔC</sub> (e.g., through increased effective concentration of the CTR in proximity to bound RNA). Nevertheless, the CTR modulates the kinetics and thermodynamics of NSP2–RNA complex formation to accomplish RNA chaperone recycling. We propose that this may represent a conserved mechanistic feature of multimeric RNA chaperones that capture RNA with high affinity and require auto-regulation to assist RNA dissociation in order to promote efficient matchmaking.

Materials and Methods

Proteins and RNAs. NSP2 and NSP2–ΔC (RNA strains SA11 and RF) were expressed and purified as described previously (19). RNAs used in this study are listed in [SI Appendix, Table S2]. RNA sequences S5, S6, and S11 were produced and labeled using previously described in vitro transcription and labeling protocols (19). Unstructured 20mer (labeled and unlabeled), unstructured 10mer (labeled), and biotinylated unstructured 10mer RNAs were purchased from Integrated DNA Technologies. Double-labeled ([SI Appendix, Table S2]) stem-loop RNA was purchased from IBA Life Sciences.

Negative-Static EM and Data Processing. For negative-stain grid preparation, 4 μl of sample (at various concentrations ranging from 100 to 500 nM) was incubated on glow-discharged (using PELCO easiGlow), carbon-coated Formvar 300- mesh Cu grid (Agar Scientific) for 90 s prior to blotting, stained twice with 20 μl 2% uranyl acetate (first stain immediately blotted, the second stain incubated for 20 s prior to blotting), and allowed to dry. Micrographs were collected on a FEI Tecnai 12 transmission electron microscope operated at 120 kV and equipped with a Gatan UltraScan 4000 charge-coupled device camera operated at a nominal magnification of 30,000 × (giving a 3.74 Å/pixel sampling on the object level). From 23 micrograph images taken with a nominal defocus of -3 μm, 14,740 particles were picked using template-based autotiling within Relion 3. Multiple rounds of two-dimensional and 3D classification resulted in the selection of a subset of 2,864 particles. These particles were used to determine an ∼22 Å resolution NSP2–ΔC reconstruction with D4 symmetry applied.

Cryo-EM and Data Processing. NSP2 RNP complex was assembled by incubating NSP2 with 40mer RNA. A range of different NSP2:RNA ratios were tested, but ratios above 1 RNA: 2 NSP2 monomer resulted in immediate complex precipitation. A 1 RNA: 2 NSP2 molar ratio gives a stoichiometry of 1 RNA per binding site on NSP2, thereby ensuring saturation of NSP2 with RNA. To establish optimal cryo-EM grid conditions, grids with a range of NSP2 RNP concentrations were made. RNP complex was incubated at room temperature (25 °C) for 30 min prior to application to Quantifoil 1.2/1.3 holey carbon grids and vitrification. At 25 μM RNP complex (∼1 mg/mL), no particles were present in the ice; this was probably due to preferentially resting on the carbon instead of distributing evenly throughout the ice. This was ameliorated by a twofold increase in the RNP concentration (while maintaining a 1 RNA: 2 NSP2 monomer molar ratio), producing a suitable particle distribution within the ice. Further details on sample preparation, data analysis and analysis are given in [SI Appendix, SI Methods].

Atomic Model Building. An atomic model of NSP2 published by Jayaram et al. (52) (PDB 1L9Y) was fit into the cryo-EM densities using ChimeraX (53) and subjected to automated flexible fitting and refinement using Namdator (54). The Namdinator model was used for multiple iterative rounds of manual adjustment in Coot (55) and real-space refinement in Phenix (56). Models for NSP2 appropionate and NSP2-RNP were validated using MolProbity (57) as implemented in Phenix.

Protein Sequence Conservation and Surface Electrostatic Analyses. Full-length NSP2-coding sequences of group A RVs were obtained from GenBank ([SI Appendix, Table S4]). Sequences of avian strains and of rearranged RNA segments of mammalian strains were excluded from the analysis. Protein sequence conservation and multiple-sequence alignment (MSA) was performed using the online Consurf (58) server. Output from the Consurf MSA was used to generate a sequence logo using the Weblogo server (59). Maps and models were visualized using ChimeraX (53), and the electrostatic surfaces were determined using the Adaptive Poisson–Boltzmann Solver plugin (60).

smFRET Measurements. SmFRET measurements of freely diffusing, dual-labeled RNA stem-loops in the presence and absence of NSP2 were performed on a home-built confocal microscope, as described previously (4). Briefly, the samples were excited using pulsed interleaved excitation (61) at wavelengths of 630 nm (488-nm laser) and 532 nm (640-nm laser) with typical laser powers of 100 μW (measured before the 60x water immersion objective [Plan Apo IR 60x/1.27 W Nikon, Düsseldorf, Germany]). Fluorescence signal was split between the green and red detection channels using a DualLine Z532/635 beamsplitter (AHF) and the emission spectra filtered using a Brightline 582/75 filter (Semrock) for green detection and HQ700/75 and ET700/75 filters (Chroma) for red detection. Measurements were performed in 8-well chamber slides (Lab-Tek, WWR) in a buffer composed of 1/3 phosphate-buffered saline (PBS, 45 mM NaCl, 3 mM phosphate, and 1 mM KCl), 1 mM Trollox to reduce photobleaching (62), and 0.01% (vol/vol) Tween20 to prevent sticking of the sample to the glass surface. The dual-labeled RNA stem-loop (Attos532/ Attos647N-labeled) was diluted to 25 μM and incubated with 5 nM NSP2 (either full length or the ΔC mutant). Data were analyzed with the open-source software package PAM (63) using the same burst search parameters and correction factors as described in ref. 4. To measure species-selective fluorescence CFs, we defined two subpopulations based on the FRET efficiency: the low-FRET population with E < 0.4 and the high-FRET population with E > 0.6. For each burst, the CF for acceptor photons after acceptor excitation was calculated including photons within a time window of 20 ms.

SPR. A Biacore 3000 was used to analyze the binding kinetics of NSP2 and NSP2–ΔC to S-biotinylated-10mer RNA ([SI Appendix, Table S2]). All experiments were performed in SPR buffer (150 mM NaCl, 25 mM Heps, pH 7.5, and 0.1% Tween-20). RNAs were immobilized on a streptavidin (SA) sensor chip (GE Healthcare) with an analyte surface density of ~20 resonance units (RU). Analyte measurements were performed at 25 °C and a flow rate of 40 μL/min. The chip surface was regenerated between protein injections with a 40 μL 0.05% sodium dodecyl sulfate (SDS) injection. Data were analyzed using BIAevaluation 3.1 software (GE Healthcare). The kinetic parameters were derived assuming a binding stoichiometry of 1:1.

RNA Competition Assay. A total 250 nM NSP2 and NSP2–ΔC (RF) was pre-incubated with 10 nM 20mer AlexaFluor488-labeled RNA ([SI Appendix, Table S2]) in binding buffer (50 mM NaCl, 25 mM Heps pH 7.5). Fluorescence anisotropy measurements were performed in the presence of various concentrations of unlabeled 20mer RNA in low-volume Greiner 384-well plates. Data were recorded at 25 °C in a PHERStar Plus multidetection plate reader (BMG Labtech) equipped with a fluorescence polarization optical module (λ<sub>ex</sub> = 485 nm; λ<sub>em</sub> = 520 nm). The data were normalized and binding curves were fitted in Origin 9.0 using a Hill binding curve resulting in R<sup>2</sup> values of 0.997 and 0.991 for NSP2 and NSP2–ΔC respectively.

CTR Peptide Competition Assay. In order to maximize any potential competition between the CTR peptide and RNA, assays were performed under conditions that favored dissociation of NSP2–ΔC from RNA. The binding assay was performed in PBS buffer (150 mM NaCl, 10 mM potassium phosphate, and 3 mM potassium chloride). A total 25 nM AlexaFluor488-labeled RNA was incubated with 20-fold excess NSP2–ΔC (500 nM, RF strain). After 30 min
at room temperature (~25 °C), the CTR peptide was added in 20-fold excess of NSP2–ΔC (i.e., 10 μM). To investigate direct CTR–RNA interactions, 25 nM 10mer RNA was also co-incubated with 10 μM CTR peptide. Fluorescence anisotropy measurements of RNA alone, RNA–NSP2–ΔC, RNA–NSP2–ΔC–CTR, and RNA:CTR were performed in triplicate as described above for RNA competition assays.

**HDX-MS.** An automated HDX robot (LEAP Technologies, FT Lauderdale, FL) coupled to an Acquity M-Class liquid chromatography and HDX manager (Waters, United Kingdom) was used for all HDX-MS experiments. Differential HDX-MS of NSP2 was performed using NSP2 (10 μM) or preincubated NSP2-RNP complexes (10 μM + 2 μM 20mer RNA, SI Appendix, Table S2). A total 30 μl of protein-containing solution was added to 135 μl deuterated buffer (10 mM potassium phosphate buffer pH 8.0, 82% D2O) and incubated at 4 °C for 0.5, 2, 30, or 120 min. After labeling, HDX was quenched by adding 100 μl of quench buffer (10 mM potassium phosphate, 2 M guanidine hydrochloride, and pH 2.2) to 50 μl of the labeling reaction. A total 50 μl of the quenched sample was passed through immobilized pepsin and aspergillopepsin columns (Affiplor, Mrttin, Czech Republic) connected in series (20 °C), and the peptides were trapped on a VarGuard Precolumn (Acquity UPLC BEH C18 [1.7 μm, 2.1 mm × 5 mm, Waters, United Kingdom] for 3 min. The peptides were separated using a C18 column (75 μm × 150 mm, Waters, United Kingdom) by gradient elution of 0 to 40% (vol/vol) acetonitrile (0.1% vol/vol formic acid) in H2O (0.3% vol/vol formic acid) over 7 min (50 μl/min). Peptides were detected using a Synapt G2S mass spectrometer (Waters, United Kingdom). The mass spectrometer was operated in mobility-assisted data-independent analysis with the dynamic range extension enabled (HDM5+) mode was used to separate peptides prior to collision-induced dissociation (CID) fragmentation in the transfer cell. CID data were used for peptide identification, and uptake quantification was performed at the peptide level (as CID results in deuterium scrambling). Data were analyzed using PLGS (version 3.0.2) and DynamX (64) (version 3.0.0) software (Waters, United Kingdom). Restrictions for peptides in DynamX are as follows: minimum intensity = 1,000, minimum products per amino acid = 0.3, max sequence length = 25, max ppm error = 5, and file threshold = 3. The software Deuterom (65) was used to identify peptides with statistically significant increases/decreases in deuterium uptake (applying a 99% confidence interval) and to prepare Woods plots.

**Native MS.** NSP2 and its C-terminal truncation was extensively dialyzed into 200 mM ammonium acetate, pH 7.6 overnight at 4 °C. CTR peptide was diluted to a final concentration of 100 μM, and then further diluted to the appropriate concentration to achieve a 1:1 protein:peptide stoichiometric ratio. Nano Electro-spray ionisation (NanoESI)–IMS–MS spectra were acquired with an Orbitrap UHMR mass spectrometer (Thermo Fisher Scientific), operated as previously described (4). Data were processed using Xcalibur Qual Browser version 4.0.27.19 and UniDec version 2.7.1.

**UV Cross-linking MS with RBDmap.** A total 10 μM NSP2 was incubated with 5′-A25-S11 RNA in a final volume of 100 μL. NSP2–RNP complexes were incubated at room temperature for 30 min and applied to a single well of a 384-well Nanoimager S with an Olympus 100× oil apochromatic oil immersion objective (NA=1.4). Dye excitation was performed with an ONI laser illumination system at the wavelengths 405 nm (DAPI), 488 nm (eGFP), 561 nm (Alexa Fluor550), and 640 nm (Atto647N), with laser intensities set to 10% (405 nm), 2% (488 nm), 5% (561 nm), and 7% (641 nm). Fluorescent signals were recorded with a sCMOS camera with a pixel size of 0.117 μm. Images were acquired over a field of view of the camera chip resulting in a total imaging region of 50 μm × 80 μm. Exposure times were adjusted accordingly to the signal intensity to avoid pixel saturation. Typical exposure times were 30 ms for all channels. Images were recorded consecutively for each channel, from the lowest to the highest energy excitation wavelength. All images were processed in ImageJ (68), and figures were assembled in CorelDraw2020.

**Data Availability.** Sequencing data are available for rescued recombinant RVs (GenBank IDs: MW074066, MW074067, and MW074068). The following wwPDB accession codes have been assigned to the EM data: NSP2 (PDB 7PKO, EMD-13474), NSP2–RNP complex (7PKP, EMD-13475), and focused 3D class of the RNP (EMD-13475). All other data needed to evaluate the conclusions in the paper are present in the paper and/or SI Appendix.

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