Higher-order structures of the foot-and-mouth disease virus RNA-dependent RNA polymerase required for genome replication

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Replication of many positive-sense RNA viruses occurs within intracellular membrane-associated compartments. These are thought to provide a favourable environment for replication to occur, concentrating essential viral structural and nonstructural components, as well as protecting these components from host-cell pathogen recognition and innate immune responses. However, the details of the molecular interactions and dynamics within these structures is very limited. One of the key components of the replication machinery is the RNA-dependent RNA polymerase, RdRp. This enzyme has been shown to form higher-order fibrils in vitro. Here, using the RdRp from foot-and-mouth disease virus (termed 3Dpol), we report fibril structures, solved at ~7-9 Å resolution by cryo-EM, revealing multiple conformations of a flexible assembly. Fitting high-resolution coordinates led to the definition of potential intermolecular interactions. We employed mutagenesis using a sub-genomic replicon system to probe the importance of these interactions for replication. We use these data to propose models for the role of higher-order 3Dpol complexes as a dynamic scaffold within which RNA replication can occur.
The replication of genomic material is a key stage of the lifecycle of all viruses. Some of the molecular details of replication are well defined as a result of elegant in vitro analyses. It has also been demonstrated that for many viruses, replication occurs in macromolecular complexes involving both viral and cellular components. However, the molecular details of the architecture of replication complexes remains unknown and thus we do not fully understand how proteins and viral genetic material are associated during the replication process or how dynamic rearrangements of higher-order assemblies may regulate RNA synthesis during viral replication.

The genomes of positive-sense RNA viruses act directly as a messenger RNA and hence are translated immediately after infection. For picornaviruses such as poliovirus (PV), rhinovirus and foot-and-mouth disease virus (FMDV), proteolytic processing of the viral polyprotein ultimately releases the nonstructural proteins involved in genome replication. A key protein is the RNA-dependent RNA polymerase, RdRp, termed 3Dpol. This enzyme (in complex with other viral proteins e.g., 2C, 3A, and 3CD and a replication primer protein termed 3B) produces negative-strand genomic intermediates, from which new positive strands are synthesized.

Biochemical and structural studies have yielded a very good understanding of the stages involved in each step of nucleotide incorporation. In common with other DNA and RNA polymerases, the structure of the picornavirus 3Dpol resembles a cupped right hand, with three defined subdomains, termed thumb, fingers and palm. The finger subdomains are extended in picornaviral 3Dpol structures, when compared to many other polymerases, and contact the tip of the thumb subdomain. This contact is important for maintaining polymerase structure whilst the geometry of the fingers against the thumb form part of the NTP entry channel. The single-stranded template RNA enters 3Dpol through a channel at the face distal to the active site and takes a right-angle turn at the palm active site when the product-template duplex is formed. This emerges from the large front opening. There is, however, no strand separation system identified from polymerase biochemical-structural data alone, but such a mechanism must presumably exist to prevent the formation of a stable RNA duplex.

In contrast to its catalytic role in replication, the non-catalytic roles of 3Dpol are less defined. It forms part of a larger precursor 3CD, and is essential for RNA replication via genomic cyclisation. Previous studies have also suggested that 3Dpol (or larger precursors thereof) has an essential cis-acting role in replication, i.e., a structural function which needs to be provided from the replicating genome, which is distinct from a separate trans-acting role, i.e., the enzymatic function of the polymerase that can be supplied by a separate genome. Dimers and higher-order oligomers of RdRp molecules have been reported in a number of positive-sense RNA viruses. Furthermore, the 3Dpol enzyme of both PV and FMDV has been previously shown to form higher-order fibrillar structures. The PV 3Dpol has also been demonstrated to form planar lattices and higher-order structures have been visualised within infected cells. It has therefore been postulated that the higher-order structures act as a replication scaffold; however, no RNA has been shown within the PV 3Dpol structures and furthermore, these were demonstrated to form in vitro in the absence of RNA. In contrast, the FMDV fibrillar structures were only detected in vitro in the presence of RNA. Indeed, our previous studies showed that primer, RNA template and nucleotides were required for fibrill formation, thus suggesting that they could be part of an active replication complex.

Using immunofluorescent confocal and electron microscopy, FMDV has been shown to dysregulate Golgi and ER-derived membranes in a similar way to PV, but distinct membrane-bound replication complexes comprised of viral RNA, structural and nonstructural proteins, and host-cell proteins have yet to be identified for FMDV. Although both viruses are members of the Picornaviridae, the Aphthovirus FMDV has several unique features that distinguish it from other members of the family including an extended 5′ untranslated region and triplcation of the primer encoding region.

Picornaviruses are responsible for several important human and animal diseases. For example, FMDV, the causative agent of foot-and-mouth disease, is a significant economic problem, such as occurred in the UK in 2001 (where economic losses to farming and tourism were estimated at ~£15 billion at current value). Therefore, a more complete understanding of the composition of the replication machinery is important for therapeutic development. Here, we have trapped the products of a replication assay by crosslinking and thus captured higher-order structures in a range of conformations. Using cryo-electron microscopy, we have calculated the structures of 3Dpol fibrils in 9 conformations at 7–9 Å resolution, with some of these occurring within the same fibril. We also probed the importance of 3Dpol, 3Dpol interfaces within the fibril by mutagenesis and we use these data to build a model of conformational changes in higher-order assemblies of 3Dpol that may be important in replication.

Results

Wild-type 3Dpol can catalyse incorporation of UTP into nascent RNA. We first sought to demonstrate that the recombinant 3Dpol protein was able to bind a synthetic template RNA and was catalytically active. We produced wild-type (WT) 3Dpol in E. coli as previously described, alongside 3Dpol with mutations in the active site (3Dpol-GNN) as a control. To assess the affinity of RNA binding by 3Dpol, a fluorescence polarisation anisotropy (FPA) assay was performed. Samples of 3Dpol WT and 3Dpol GNN were titrated with FITC-labelled 13mer poly-A RNA (Fig. 1a), resulting in Kd values of WT and GNN of 5.83 ± 1.3 and 1.92 ± 0.67 μM, respectively (Fig. 1b). These data showed that both active and inactive enzymes were able to bind RNA in vitro, with the mutant demonstrating significantly higher affinity (p < 0.01).

The ability of the protein to incorporate [α-32P] UTP into RNA, was assessed as described utilising a poly-A template and an oligo-d(T) primer, supplemented with a 5:1 ratio of UTP to [α-32P] UTP. After incubation at 30°C, polymerase activity was assessed by dot-blot and quantified by scintillation counting (Fig. 2a, b). Normalised scintillation counts quantifying the dot-blot samples incubated for 30 min (Fig. 2b) show a 25-fold greater incorporation of [α-32P] UTP for WT compared to the enzymatically inactive 3Dpol-GNN.

Formation of higher-order complexes. We have previously demonstrated the formation of higher-order fibrillar complexes of 3Dpol by negative stain transmission electron microscopy (TEM). To investigate the assembly of the fibrils, we employed glutaraldehyde crosslinking both to trap higher-order oligomers and stabilise the fibrils. Samples of recombinant 3Dpol were incubated at 30 °C in the presence of glutaraldehyde and the RNA template-primer used for the activity assay described above. At time intervals, the reaction was stopped by the addition of Laemmli buffer. The samples were resolved by gradient SDS-PAGE and 3Dpol WT or 3Dpol GNN multimers detected by anti-3Dpol immunoblot (Fig. 3a, c, respectively) and quantified by densitometry (Fig. 3b, d, respectively). An increase in oligomer formation over time was evident, which seemed to correlate predominantly to the addition of dimers. Higher-order oligomer
The binding of FITC-labelled 13mer poly-A RNA to recombinant 3Dpol was calculated as average anisotropy after 30 min incubation (Fig. 1a). This analysis showed that 3Dpol-WT was able to form large structures in the presence of glutaraldehyde, similar to those we reported previously (Fig. 4a). Larger structures produced by 3Dpol-GNN appeared as protein aggregates (Fig. 4b), similar to those occasionally found in samples of 3Dpol-WT incubated in the absence of RNA, suggesting that the faster oligomerisation of 3Dpol-GNN (shown in Fig. 3) may result in less ordered assembly. The fibrils were susceptible to RNase treatment (Fig. S1), in agreement with previous data. We have previously reported the X-ray structure of a 3Dpol bearing a mutation in the RNA exit channel. We found this mutant to also be deficient in fibril formation, consistent with a deficiency in RNA binding (Fig. S1). The availability of fixed, stable 3Dpol-WT fibrils led us to investigate their structure by cryo-EM.

To this end, 3Dpol-WT fibrils were produced as above, prepared for cryo-EM and imaged on a Thermo-Fisher Titan Krios cryo-electron microscope. Fibril sections were picked and subjected to 2D class-averaging, which appeared to show variation in fibril diameters (Fig. 4c). Measurements from 1D projections of these 2D classes along the fibril axis confirmed this, showing a bimodal distribution of diameters that ranged between ~20 nm and ~23 nm. The modal diameters were 21.3 nm and 22.6 nm, indicating the presence of at least two forms of fibril (Fig. S2). These values correlate well with the size of fibrils previously measured from negative stain TEM images.

Helical reconstruction with 3D classification was performed, separating the dataset into fifteen self-similar classes. In this analysis, nine classes gave reconstructions that exhibited sharp density. These were individually refined leading to the definition of fibril reconstructions with diameters that reflected the bimodal distribution seen in the 2D class averages. Of these nine conformations, two were defined as 'broad', B1 and B2, corresponding to the 2D classes having a modal diameter of 22.6 nm. Seven reconstructions were classed as 'narrow'; N3-N9, corresponding to the larger group of fibrils having a modal diameter of 21.3 nm. The distinction between broad and narrow fibrils was made based on the helical symmetry of each group (see below). Reconstructions ranged in resolution between 7.3 Å and 9.5 Å (Fig. 4d-g, Fig. S3, Table 1, Movie S1).

Each reconstruction was readily interpreted by rigid-body fitting of the crystal structure for FMDV 3Dpol (PDB ID 2EC0), revealing that all the solved fibril conformations are right-handed 2-start helical assemblies, each comprising a lattice of repeating 3Dpol dimers (Fig. 4h, i). Dimers are rotationally symmetric about their interface and each of the two protofilaments is a ribbon of 3Dpol dimers that is therefore antiparallel. The nine conformations that were resolved through our analysis show that variation in fibril diameter is a consequence of the two protofilaments sliding and flexing relative to each other (Movie S1).

Among these reconstructions, four distinct forms of fibril were identified. The presence of a bimodal distribution of fibril diameters reflects the presence of two helical symmetries. Broad fibrils were found to be 2-start helices having C2 symmetry about the helix axis. Protofilaments had between 9 and 9.2 dimeric subunits per helix turn. Narrow fibrils were not C2 symmetric and exhibited 2-start helical symmetry with between 8.4 and 8.6 3Dpol dimers per helix turn in each protofilament ribbon. A notable difference between
the broad and narrow classes of fibrils was that narrow fibrils could also be described by a left-handed 1-start helix of 3Dpol dimers having a twist of \( \sim -159° \) and an axial rise of 15–17 Å while the twofold symmetric, broad-fibrils could not (Table 1).

Within the narrow and broad classes, fibrils were found to form either tightly packed or open conformations. N3–N8 were tightly packed fibrils in which flexing of protofibrils resulted in a progressive tightening of packing from N3 to N8, with N8 being the most densely packed of the narrow fibril forms. N9 shows a rather different assembly with a longer axial rise and, although the fibril is narrower, a more open structure with large gaps between protofibrils. Similarly, B1 and B2 showed tight and open forms respectively, with B2 being narrower than B1 and having a longer axial rise per subunit. Three-dimensional classification did not distinguish more subtle variations for broad fibrils (similar to those seen in the tightly packed narrow fibril classes (N3–N8)), possibly a consequence of fewer broad fibrils being present in the dataset. In both N9 and B2 (the open fibril conformations) the spaces between protofibrils contained noisy density that was not accounted for by fitting the atomic model for 3Dpol, we postulate that this may be RNA (see below). It is also important to note that the RNA entry and exit sites are not occluded (except by this putative RNA density).

Movie S1 shows morphing between fibril conformations, this is intended to illustrate the differences in these structures rather than model any conformational changes that may occur. To determine whether the fibril classes solved in our analysis represented discrete structures or continuously varying helices, we traced fibril image sections from each of the reconstructions back to their original micrograph (Fig. 5). We found that the narrow fibrils that contributed images to reconstructions N3–N8 varied between these forms along their length, suggesting that these reconstructions represent snapshots of a continuously flexing assembly. Fibrils were seen to switch conformation between tight and open forms. In most cases, changes in fibril conformation were marked by visible constrictions and the different forms presented as discrete regions of longer fibrils, rather than the continuous variation seen for the narrow/tight classes. Overall, these data suggest that narrow and broad forms were distinct from one another. Fibrils that appear to show both broad and narrow forms along their length were, however, noted and in these images pronounced discontinuities/constrictions were observed.

**Inter- and intradimer interactions.** As noted above, an inspection of our reconstructed density maps along with rigid-body fitting of high-resolution coordinates for 3Dpol indicated that fibrils each comprise two protofibrils that are ribbons of dimers. Analysis of contacts and clashes between 3Dpol monomers in our models confirms this. These data are also consistent with fibril formation by dimer addition as shown in Fig. 3. In all conformations, the dimer interfaces and dimer-dimer interfaces are similar, while different interfaces between protofibrils were seen in tight and open forms (Figs. 5a and 6a–e; Movie S2; Table S1). To describe the interactions between monomers within...
fibris we use a notation incorporating the ‘palm’, ‘fingers’, and ‘thumb’ subdomain nomenclature, while the relative spatial organisation of monomers within the helix are denoted as follows. Where $n$ represents a monomer and then $n'$ is the twofold symmetry-related monomer within a single dimeric subunit (Fig. S4). Here, $n + 1$ represents the next monomer, on the opposite protofilament (and $n' + 1$ its partner within a dimer). For conformations N3-N9 the transformation from $n$ to $n + 1$ and all subsequent increments is defined by the left-handed 1-start helical parameters: a rotation of $-158^\circ$ and an axial rise of $15-17$ Å. For the C2 symmetric broad fibrils, which do not show 1-start helical symmetry, the transformation from $n$ to $n + 1$ (and

Fig. 4 Structure of 3Dpol fibrils. Negative stain TEM of a sample from a polymerase activity assay in the presence of glutaraldehyde after 30-minute incubation. a 3Dpol-WT forms visible fibrils, b 3Dpol-GNN does not show any distinct fibril structure formation. Scale bar 100 nm. c 2D classification and averaging of fibril section images from cryo-EM data shows variation in fibril diameter. d g Helical reconstruction led to the calculation of density maps for nine classes of fibril at 7.3–9.5 Å resolution, four representative maps are illustrated here coloured according to radius (see key below panel g). d The broadest fibril reconstruction; B1 had a diameter of 22.3 nm, C2 symmetry, a helical twist of 39° and an axial rise per subunit of 27 Å. e Conformation B2 has a more open structure, is also C2 symmetric and has a diameter of 21.7 nm, twist of 40° and rise of 31 Å. f N3 is a narrow ($\varnothing = 21.5$ nm) and tightly packed fibril which may also be described by a 1-start helical symmetry with a twist of $-158.8^\circ$ and axial rise of 15 Å. g N9 is a narrow, open fibril structure having a diameter of 20 nm, twist of $-158.6^\circ$ and axial rise of 17.4 Å. h Fitting of the X-ray structure for 3Dpol-WT in complex with template primer RNA and ATP (PDB: 2ECO) into each density map shows that these fibrils are two-start helical lattices comprising protofilaments of 3Dpol dimers. i Protofilament one is coloured violet/thistle, while protofilament two is coloured teal/aquamarine, two shades are used in each protofilament to highlight how 3Dpol monomers form ribbons of dimers.
Table 1 Table to show imaging and reconstruction statistics for each of the fibril reconstructions.

|         | B1 (EMDB-12093) | B2 (EMDB-12094) | N3 (EMDB-12096) | N4 (EMDB-12097) | N5 (EMDB-12098) |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Data collection and processing** |                |                 |                 |                 |                 |
| Magnification  | 75,000          | 75,000          | 75,000          | 75,000          | 75,000          |
| Voltage (kV)  | 300             | 300             | 300             | 300             | 300             |
| Electron exposure (e-/Å²) | 108             | 108             | 108             | 108             | 108             |
| Defocus range (μm) | -1.5-2.0        | -1.5-2.0        | -1.5-2.0        | -1.5-2.0        | -1.5-2.0        |
| Pixel size (Å) | 2.13            | 2.13            | 2.13            | 2.13            | 2.13            |
| Fibril diameter | 22.3 nm         | 21.7 nm         | 21.5 nm         | 21.3 nm         | 20.9 nm         |
| Symmetry   | C2              | C2              | C1              | C1              | C1              |
| Helical twist | 39.003°         | 40.025°         | -158.874°       | -159.002°       | -158.879°       |
| Rise per subunit | 26.9991 Å       | 31.2392 Å       | 15.0274 Å       | 15.0837 Å       | 15.0192 Å       |
| Initial particle images (no.) | 266,263         | 266,263         | 266,263         | 266,263         | 266,263         |
| Final particle images (no.) | 38,886          | 6,903           | 15,167          | 26,032          | 10,714          |
| Map resolution (Å) | 7.3             | 9.5             | 8.5             | 7.7             | 7.6             |
| FSC threshold | 0.143           | 0.143           | 0.143           | 0.143           | 0.143           |
| Map resolution range (Å) | Min: 6.1        | Min: 6.1        | Min: 6.6        | Min: 6.5        | Min: 6.4        |
| (From Relion localres) | Max: 15.8       | Max: 23.7       | Max: 17.2       | Max: 18.3       | Max: 18.3       |
| Mean: 8.3 | Std: 2.8        | Std: 1.7        | Std: 2.0        | Std: 8.8        | Std: 2.0        |

|         | N6 (EMDB-12099) | N7 (EMDB-12100) | N8 (EMDB-12101) | N9 (EMDB-12102) |
|---------|-----------------|-----------------|-----------------|-----------------|
| **Data collection and processing** |                |                 |                 |                 |
| Magnification  | 75,000          | 75,000          | 75,000          | 75,000          |
| Voltage (kV)  | 300             | 300             | 300             | 300             |
| Electron exposure (e-/Å²) | 108             | 108             | 108             | 108             |
| Defocus range (μm) | -1.5-2.0        | -1.5-2.0        | -1.5-2.0        | -1.5-2.0        |
| Pixel size (Å) | 2.13            | 2.13            | 2.13            | 2.13            |
| Fibril diameter | 20.9 nm         | 20.7 nm         | 20.7 nm         | 20 nm           |
| Symmetry   | C1              | C1              | C1              | C1              |
| Helical twist | -158.778°       | -158.811°       | -158.545°       | -158.581°       |
| Rise per subunit | 14.9352 Å       | 15.1039 Å       | 14.948 Å        | 17.3913 Å       |
| Initial particle images (no.) | 266,263         | 266,263         | 266,263         | 266,263         |
| Final particle images (no.) | 14,427          | 29,141          | 18,808          | 16,461          |
| Map resolution (Å) | 7.8             | 7.3             | 7.5             | 7.3             |
| FSC threshold | 0.143           | 0.143           | 0.143           | 0.143           |
| Map resolution range (Å) | Min: 6.1        | Min: 6.0        | Min: 6.5        | Min: 6.0        |
| (From Relion localres) | Max: 17.8       | Max: 16.0       | Max: 16.6       | Max: 18.8       |
| Mean: 8.8 | Std: 1.8        | Std: 1.6        | Std: 8.4        | Std: 8.8        |
subsequent odd-numbered monomers) is simply a rotation of $-180^\circ$. The transformation from $n+1$ to $n+2$ (and all subsequent even-numbered monomers) is a rotation of $\sim-140^\circ$ and an axial rise of $\sim27$–$31\,\text{Å}$. Under this notation, for all filament conformations, $n+2, 4, 6$ etc. are successive monomers on the same protofilament as $n$, while $n+1, 3, 5$ etc. are on the other. The dimer interface is between thumb subdomains, predominantly at the C-terminal helix $\alpha_{16}$ (residues 453–467) of $n$ and $n'$ (Fig. 6b). Dimers repeat along each protofilament ribbon with the primary interaction being between the thumb/fingertips of $n$ and the palm sub-subdomain of $n+2$ (and between the palm subdomain of $n'$ and thumb/fingertips of $n'+2$; Fig. 6c). Interactions between protofilaments in each conformation are also twofold rotationally symmetric owing to the antiparallel nature of each protofilament. The interactions are not, however, conserved between fibril forms. In tight conformations, the contact interface is mainly within a loop at amino acid residues 9-17, between $n$ and $n'+9$ or $n'+7$ for B1 and N3–N8, respectively (Fig. 6d). In conformation N8, there is an additional point of contact between $n$ and $n'+5$ between V4 in strand $\beta1$ and K65 in the N-terminal loop of the palm subdomain. In open fibrils, the contacts/clashes between protofilaments are between $n$ and $n'+7$ for B2, and $n$ and $n'+5$ for N9. In both cases $\beta1$ strands in each monomer come together, possibly forming an extended antiparallel $\beta$-sheet (Fig. 6f).

Comparison of our structures for fibrils formed of FMDV 3Dpol with previously published oligomeric forms of PV 3Dpol shows that these molecules pack differently. X-ray crystallography studies have shown that PV 3Dpol molecules form functional dimers via a strong interaction termed ‘interface 1’ between the C-terminal helix in the thumb subdomain, and the back face of the palm subdomain. Furthermore, PV helical polymerase lattices form much larger (50 nm) fibrils, helical reconstructions of which have shown a 6-start helix of protofilaments formed of interface 1 dimers. However, it should be noted that the PV 3Dpol was untagged, whereas the FMDV 3Dpol used here was C-terminally his tagged. 470 residues (of 476 excluding the tag) were modelled. Although density extends beyond this, it is not possible to determine the position of the tag.

Effect of 3Dpol mutations on replication. The cryo-EM data suggested that the fibril protofilaments are assembled via two main intermolecular interactions, a thumb-to-thumb interaction involving residues Y394, G395, T396, F398, P436, Y455, R456, S457, Y459, L460, V463, N464, V466, C467, D469, and A470, and a fingers/thumb-to-palm interaction (involving residues A22, P23, V25, L73, R76, H322, Y323, E324, G325, D329, T330, Y346, D347, A415, R416, R417, I420, P445, L449, and F450). It should be noted that these contact residues were identified by the fitting of high-resolution data into an intermediate resolution map, thus...
no account is taken for potential rearrangement of sidechains upon polymerisation. Helical reconstruction of FMDV 3Dpol fibrils revealed considerable conformational flexibility. Many attempts were made to achieve sufficient resolution as to allow ab initio atomic modelling, these were not however successful.

The third interaction site (between protofibrils) is highly variable. Our fitting experiments led to the definition of contact interfaces for tight forms comprising four residues. The considerable flexing of protofibrils argues for a weak interaction at this site. The open forms show a more intriguing potential interaction —formation of an extended antiparallel β-sheet via hydrogen bonding between residues 4–8. Mutagenesis to target this interface would require disruption of secondary structure. For these reasons, we chose to focus our mutagenesis studies on those interactions that support protofibril formation.

To understand whether the intermolecular interactions above are important for viral replication we employed a replicon system36,37. We hypothesised that if the polymerase intermolecular interactions described here are biologically important, disruption of these interactions would impair function, resulting in reduced replicon replication. We therefore selected several residues from both the thumb-to-thumb interface (Y394, F398, R456, Y459, L460, V463, and C467) and the fingers/thumb-to-palm interface (P23, V25, E324, Y346, D347, I420, P445, L449) for mutation. Selecting residues at random from both interfaces prevented biasing the outcome by focusing the mutagenesis on specific secondary structures or amino acid physical properties; however, it should be noted that all of the residues are conserved between FMDV isolates. Each of the selected residues was individually mutated to alanine in the context of a FMDV replicon in which the structural proteins were replaced by GFP. Measuring GFP expression therefore allows the replication competency of each mutation to be assessed. From each mutant replicon, RNA was generated by T7 transcription and transfected into BHK-21 cells. Replicons with 3Dpol-WT and 3Dpol-GNN were included as controls, the latter for the level of translation from the input RNA. RNA replication was monitored by GFP fluorescence, with replication shown as the number of GFP positive cells at 8 h post-transfection, analysed according to our established protocols37 (Fig. 7a).

Of the fifteen amino acids mutated only five: F398A, R456A, Y459A, L460A, and C467A, significantly reduced FMDV replicon efficiency compared to the WT replicon. All of these residues were located within the thumb-to-thumb interaction (coloured dark magenta, Fig. 7b). All the other mutations tested showed levels of replication equivalent to a WT replicon (coloured orange Fig. 7b, c). This included two mutations (V463 A and V466A) and all of the mutations made at the fingers/thumb-to-palm interface. Overall, these data suggest that residues involved in dimer formation are important for FMDV replication, potentially through allowing the formation of intermolecular 3Dpol-3Dpol interactions within the FMDV replication complex.

Unassigned density within 3D reconstructions may represent RNA. Within each of our fibril reconstructions, density within
the active site of individual 3Dpol molecules was visible, however, this was very weak in comparison to the protein density. Nonetheless, the data suggest the presence of retained RNA at low occupancy within fibrils (Fig. 8, Movie S3). In the open conformations (reconstructions B2 and N9) there is an additional unassigned density that extends between two 3Dpol molecules, bridging an opening in the fibril between protofilaments. The density is poorly resolved but unambiguously present, we suggest that this may represent RNA entering or extruded from the active sites.

Discussion
The RNA-dependent RNA polymerase, 3Dpol, was one of the first proteins to be characterised both in PV and in FMDV and is the key enzyme in viral replication. Both PV and FMDV 3Dpol have been shown to form higher-order structures in vitro in the form of fibrils that have been postulated to be part of viral replication complexes. It would be expected that replication complexes would be dynamic structures, and although there are structural data available for the PV fibrils these are at relatively
low resolution, did not include any RNA and were thus unable to give insight into any dynamics\textsuperscript{30,31}. Here, we report multiple conformations of the fibrils formed by FMDV 3DPol. Some assemblies exhibited multiple conformations along their length. Furthermore, we report the presence of additional density in fibril reconstructions that we attribute to RNA. We probed the interfaces by mutagenesis and are using these data to propose a model for the potential role of conformational changes in the fibril during genome replication.

We have here demonstrated that the recombinant 3DPol protein was able to bind RNA and was catalytically active, this led to the formation of fibrils. In contrast, two mutants that were deficient in replication did not form ordered products. We analysed the structures of the assembled fibril products of a polymerase activity assay after crosslinking. This provided a total of 9 different 3DPol reconstructions, all of which are at higher resolution and distinct from the previously reported structures of PV fibrils, in terms of both dimensions and the orientation of molecules within the helix. The dimerisation interface of the FMDV 3DPol molecules is novel and distinct from the dimerisation of PV 3DPol molecules. The formation of fibrils by dimer addition is also supported by the cross-linking data presented here. Mutations that affected the ability of the polymerase to transcribe replicon RNA were introduced within the dimerisation thumb-thumb interface. This contact region has been implicated in the formation of the PV fibrils and shown to be necessary in the production of virus, despite being distant from the active site\textsuperscript{41}. In PV, a model has been described whereby RNA would travel through the active site between polymerase molecules; however, RNA was not detected\textsuperscript{25,30}. In support of this model, density corresponding to RNA was detected in the FMDV fibril structures presented here. This density appears in the active site of all our reconstructions and is also seen linking individual molecules in the open fibril conformations.

The formation of a classical intracellular, membrane-associated replication complex for FMDV has yet to be demonstrated within infected cells. One could speculate, in the context of a cellular replication complex, that the polymerase molecules could coat newly synthesised negative-sense intermediates and prevent the formation of double-stranded RNA, a potent innate immune response trigger. Avoiding double-stranded RNA formation would also allow for a more energetically-favourable replication process. By coating RNA by the addition of dimers, helical fibrils similar to those described here could be generated. The concept of 3DPol acting in a similar way to the N protein of negative-sense viruses or the coat protein of helical viruses such as tobacco mosaic virus is intriguing.

The structures described here could alternatively represent trapped conformers of the active replication machinery within the functional complex. The different fibril conformations observed could represent different stages of replication. Although there is some density for RNA in the active site of 3DPol molecules within the fibrils, a model where RNA is looping out from the body of a dynamic fibril would be consistent with the relatively weak RNA density seen overall and with the observation that the fibrils are sensitive to RNaseA. However, it is also tempting to speculate that the density seen between 3DPol molecules in the open N9 and B2 conformations correlates with newly synthesised RNA being extruded from the active site.

The 9 fibril structures described here are broadly representative of 4 types: narrow, broad, tight and open. Data for the narrow fibrils were consistent with these being a continuous, flexing assembly, with the broad fibrils a separate, distinct class. Given the breadth of fibril morphology shown here, it is therefore possible that both models described above are correct. Some of the fibrils could form by the simple addition and dissociation of 3DPol subunits and act to coat newly formed RNA transcripts. Others could form part of a dynamic replication complex. Different pools of 3DPol molecules involved in replication would be consistent with the models we proposed as a result of complementation experiments\textsuperscript{19}. Correlative light-electron microscopy studies in replicon-containing cells are underway in order to further elucidate the formation and dynamics of complexes within cells and to establish if this and other nonstructural proteins, such as 3CD, 3A and 3B, are able to interact with the FMDV 3DPol fibrils.

### Methods

**Expression and purification of His-tagged 3DPol**

**WT C-terminal His-tagged 3DPol** was expressed from vector pET 28a (kind gift from Esteban Domingo, Centro De Biologia Molecular Severo Ochoa, Madrid, Spain)\textsuperscript{32} in Codon plus (+) BL21 using methods described previously\textsuperscript{15}. For lysis, the cells were suspended in 20 mM Tris-HCl [pH 8] lysis buffer with 20 mg/ml lysozyme before sonication as described previously\textsuperscript{15}. Lysates were clarified and the supernatant filtered through 0.22 µm filters and subjected to nickel affinity chromatography using 1 ml HisTrap HP columns (GE Healthcare). Elution was carried out via a 25–500 mM imidazole gradient in 50 mM Tris [pH 8], 500 mM NaCl buffer. Fractions containing the protein were pooled, dialyzed and stored in aliquots at -20 °C as described previously\textsuperscript{15}. A replication-deficient enzyme (termed GNN which includes the active site mutations D338N and D339N)\textsuperscript{16} were expressed and purified in the same way and the mutations confirmed by mass spectrometry.

**3DPol RNA-binding by fluorescence polarisation anisotropy**

The ability of FMDV 3DPol to bind to RNA was examined using fluorophore polarisation anisotropy (FPA) adapted from published protocols\textsuperscript{45}. FPA measures the tumbling rate of a fluorescently labelled molecule. After excitation by polarised light, the excited fluorophore emits light with the degree of polarisation inversely proportional to the tumbling rate of the fluorescent molecule in the solution. Therefore, the smaller the molecule, the higher the tumbling rate resulting in the depolarisation of the emitted light as the fluorophore reorientates in solution during the lifetime of the excitation. FPA was undertaken in a black opaque 384-well format (Perkin Elmer). 20 µl of RNA binding buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 0.1% Triton X-100) added to each well followed by addition of purified 3DPol or mutant 3DPol-GNN. 20 µl of 20 mM 3’F-labelled labelled 16mer poly-A (Dharman) (kind gift from Thomas Edwards, University of Leeds) was overlaid into each well and samples incubated at room temperature for 1 h. Control samples that contained no fluorescent RNA to establish background polarisation were included. Polarisation was measured using a Tecan plate reader with a 480-nm excitation filter and 530 nm S and P channel emission filters.

**3DPol Activity Assay**

Activity of purified FMDV 3DPol was assayed by the ability of the protein to incorporate [α-32P] UTP into nascent RNA transcripts, as previously described\textsuperscript{29,34}. The final concentration of 3DPol that was included in the assays was 18.52 µM. Reactions were stopped by the addition of 1.5 µl 500 mM EDTA at the indicated times. For scintillation, 2 µl of each reaction was blotted onto filter paper (Whatman) and allowed to dry prior to being washed as described previously\textsuperscript{14}. The dry filter paper was exposed to film for imaging before the individual dots on the filter paper were excised and scintillated with the addition of 3 ml scintillation fluid (Perkin Elmer). Radioactive counts were measured as scintillation counts per minute.

For glutaraldehyde crosslinking, 3DPol activity assays, substituting [α-32P] UTP with 4 mM UTP were employed. Samples were incubated with 5 mM glutaraldehyde at 30 °C for increasing time periods and reactions stopped by the addition of 30 µl 2X Laemmli buffer. The samples were denatured and analysed by gradient SDS-PAGE (12–4% resolving gels, and 4% stacking gel). Immunoblotting was performed as described previously\textsuperscript{35} with primary rabbit anti-3DPol polyclonal antibody (kind gift from Francisco Sobrino, Centro De Biología Molecular Severo Ochoa, Madrid, Spain) and detected with anti-rabbit HRP (Sigma-Aldrich).

### Transmission electron microscopy (TEM)

Carbon-coated copper grids were prepared by glow discharge at 25 mA for 30 seconds. The activity assay master mix was prepared as described for the crosslinking assay. Samples were incubated for 30 min at 30 °C and then immediately 10 µl was adsorbed onto carbon-coated copper EM grids for 1 min. The excess sample was removed and the grids washed twice with 10 µl dH2O for 5 seconds prior to staining with 10 µl 2% uranyl acetate for 30 min at 30 °C and then immediately 10 µl was adsorbed onto carbon-coated copper EM grids for 30 min at 30 °C. The activity assay master mix was then applied and allowed to dry for 1 min. The grids were prepared by glow discharge at 25 mA for 30 seconds. The activity assay master mix was applied and allowed to dry for 1 min. The grids were then examined using a 1400 equipped with a 0.1 mm objective aperture channel. The images were magnified to 50,000 x magnification.

### Cryo-electron microscopy (Cryo-EM)

Activity assay master mix was prepared as described above and incubated at 30 °C for 30 min. 10 µl of the master mix was loaded on a C-flat (protochips) CFI 121.3-4-C grid (1.2 µm holes) that had been...
prepared by addition of a continuous carbon support film and glow discharged at 25 mA for 1 min. The grids were blotted for 6 seconds at blot force 10, using an FEI Vitrobot Mark IV in chamber conditions of 80%, and 10% relative humidity. They were screened and initial analysis performed using a JEOL 2200 TEM equipped with a Direct Electron DE20 camera, optimised grids were then imaged on a Thermofisher Titan Krios in the Astbury Biostructure Laboratory using a Falcon III detector in integrating mode operated at 40 frames per second. Data were collected at nominal 75,000x magnification, giving a sampling frequency of 1.065 Å/pixel. Two-second exposures were collected at a dose rate of 54 e⁻/Å²/second and a defocus range of ~1.5 to 2 microns.

Image processing. Preliminary analysis of a small dataset collected on a JEOL 2200 was performed using Relion 2.0.3. A single 2D class average was used to generate a starting model using SPIDER. Initial estimates of approximate helical parameters were determined by taking measurements from 2D class average images (twist = 40° and rise per subunit of 29.8 Å). Calculation of an initial helical reconstruction was performed giving rise to an interpretable density map with twist = 42.4° and rise of 30 Å. Inspection of this preliminary map revealed a two-start helical assembly (Fig. S5).

Collective analysis of the initially larger dataset was undertaken at the Astbury BioStructure Laboratory. 5417 micrographs were recorded from which 266,263 images of fibril sections were selected. Motion correction was performed using Motioncor2 with dose-weighting46. CTF estimation was performed using GCTF47. Helical reconstruction was performed using Relion 3.0 followed by 3.148,49.

Helical reconstructions were obtained using the associated software for the Helical Reconstruction package, relion_particle_backtrace). Following 2D classification and analysis, a dataset of fibril images that had contributed to sharp class averages was selected. 3D classification was performed using RELION50 with k = 15, leading to the definition of nine classes of fibrils having diameters that reflected the full distribution of diameters identified by measurements of the radial density profiles. Inspection of the resulting reconstructions revealed that maps corresponding to the narrower class of fibril (Ø ~ 20–21.5 nm) could be described as left-handed 1-start helices with twist values of ~158.8° and axial rise values of ~15–17 Å. The broader class of fibrils (Ø ~ 21.7–22.3 nm) were twofold symmetric about the helix axis and were processed as right-handed 2-start helices (twist = ~40°, axial rise = ~27–31 Å) with C2 symmetry imposed. 3D-automated refinement led to the calculation of density maps for each class at resolutions ranging between 7.6 and 10.9 Å. As the reconstructions comprised an asymmetric unit that was a dimer, there was a twofold symmetry present that could not be accommodated within the helical reconstruction process. This was subsequently imposed on half-maps using UCSF Chimera22. Briefly, two copies of each half-map were opened, one was rotated 180° about the x axis (perpendicular to the helix axis), the fit in map function was then used to align the two maps and the density was averaged. Symmetrized half-maps were then imported into Relion for postprocessing analysis, resolution estimates ranged between 7.3 and 9.5 Å. Fibril images corresponding to each reconstruction were traced back to their original micrographs using trin-parts-trace.py (https://github.com/attamatti/relion_particle_backtrace).

Reconstructed density maps were interpreted by docking of published coordinates for the FMDV 3Dpol protein using UCSF Chimera22 and visualised using UCSF Chimerax51.

Replicon plasmids. The FMDV replicon plasmid, pRep-pGFP, along with equivalent constructs containing the replication-defective GNN point mutation to the 3Dpol polymerase, have been described previously37. In this replicon, the capsid protein-coding region has been replaced with a green fluorescent reporter protein. Mutations were introduced into the pRep-pGFP replicon 3Dpol coding region by overlapping PCR site-directed mutagenesis. The sequences of primers are available from the samples (designated as ‘n’) are specified in both the results section and individual figure legends for all of the quantitative data. All values are presented as mean ± SEM with the indicated sample size where applicable. Statistical differences were determined using two-tailed unpaired t test and two-way analysis of variance (ANOVA) where appropriate. Statistics were performed using GraphPad Prism 8.

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

Data availability
CryoEM maps have been deposited in the EM-databank (https://www.ebi.ac.uk/pdbe/emdb) with the accession numbers (B1) EMD-12093, (B2) EMD-12094, (N3) EMD-12096, (N4) EMD-12097, (N5) EMD-12098, (N6) EMD-12099, (N7) EMD-12100, (N8) EMD-12101, (N9) EMD-12102. The raw micrograph movies are deposited in the EMPIAR database with accession number 10062. Excel spreadsheets showing all data values, the calculated averages and errors for Figs. 1a, 1b, 3b, 3d and 7a are provided (Supplementary Data 1). Any remaining information can be obtained from the corresponding author upon reasonable request.

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References
1. Schlegel, A., Giddings, T. H., Ladinsky, M. S. & Kirkegaard, K. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J. Virol. 70, 6576–6588 (1996).
2. Gosert, R. et al. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. 77, 5487–5492 (2003).
3. Novoa, R. R. et al. Virus factories: associations of cell organelles for viral replication and morphogenesis. Biol. Cell 97, 147–172 (2005).
4. Bienz, K., Egger, D., Rasser, Y. & Rossart, W. Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures. Virology 131, 39–48 (1983).
5. Knox, C., Moffat, K., Ali, S., Ryan, M. & Wileman, T. Foot-and-mouth disease virus replication sites form at the nucleus and close to the Golgi apparatus, but exclude marker proteins associated with host membrane compartments. J. Gen. Virol. 86, 687–696 (2005).
6. Monaghan, P., Cook, H., Jackson, T., Ryan, M. & Wileman, T. The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells. J. Gen. Virol. 85, 933–946 (2004).
7. Nayak, A., Goodfellow, I. G. & Beldham, G. J. Factors Required for the Uridylation of the Foot-and-Mouth Disease Virus 3B1, 3B2, and 3B3 Peptides by the RNA-Dependent RNA Polymerase (3DPol) In Vitro. J. Virol. 79, 7698–7706 (2005).
8. Goodfellow, I. G., Polacek, C., Andino, R. & Evans, D. J. The poliovirus 2C cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes. J. Gen. Virol. 84, 2359–2363 (2003).
9. Gamarnik, A. V. & Andino, R. Switch from translation to RNA replication in a positive-stranded RNA virus. Genes Dev. 12, 2293–2304 (1998).
10. Flint, M. & Ryan, M. D. Virus-encoded proteinases of the picornavirus supergroup. J. Gen. Virol. 78, 699–723 (1997).
11. Peersen, O. B. Picornaviral polymerase structure, function, and fidelity modulation. Virus Res 234, 4–20 (2017).
12. Steitz, T. A. A mechanism for all polymerases. Curr. Opin. Struct. Biol. 8, 54–63 (1998).
13. Brautigam, C. A. & Steitz, T. A. Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. Curr. Opin. Struct. Biol. 17, 236–239 (2007).
14. Ferrer-Orta, C., Arias, A., Escamis, C. & Verdaguer, N. A comparison of viral RNA-dependent RNA polymerases. Curr. Opin. Struct. Biol. 16, 27–34 (2006).
15. Ferrer-Orta, C. et al. Structure of foot-and-mouth disease virus RNA-dependent RNA polymerase and its complex with a template-primer RNA. J. Biol. Chem. 279, 47212–47221 (2004).
16. Ferrer-Orta, C. et al. The structure of a protein primer–polymerase complex in the initiation of genome replication. EMBO J. 25, 880–888 (2006).
17. Ferrer-Orta, C., Agudo, R., Domingo, E. & Verdaguer, N. Structural insights into replication initiation and elongation processes by the FMDV RNA-dependent RNA polymerase. *Curr. Opin. Struct. Biol.* 19, 752–758 (2009).

18. Ferrer-Orta, C. et al. Multifunctionality of a Picornavirus Polymerase Domain: Nuclear Localization Signal and Nucleotide Recognition. *J. Virol.* 89, 6848–6859 (2015).

19. Herbod, M. R. et al. Both cis and trans Activities of Foot-and-Mouth Disease Virus 3D Polymerase Are Essential for Viral RNA Replication. *J. Virol.* 90, 6864–6883 (2016).

20. Herold, J. & Andino, R. Poliovirus RNA replication requires genome circularization through a protein–protein bridge. *Mol. Cell* 7, 581–591 (2001).

21. Andino, R., Rickles, G. E. & Baltimore, D. A functional ribonucleoprotein complex forms around the 3′ end of poliovirus RNA. *Cell* 63, 369–380 (1990).

22. Spagnuolo, C. A., Morasco, B. I., Smerage, L. E. & Flanagan, J. B. Viral precursor protein P3 and its processed products perform discrete and essential functions in the poliovirus RNA replication complex. *Virology* 485, 492–501 (2015).

23. Hansen, J. L., Long, A. M. & Schultz, S. C. Structure of the RNA-dependent RNA polymerase of poliovirus. *Structure* 5, 1109–1122 (1997).

24. Luo, G. et al. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NSSB) of hepatitis C virus. *J. Virol.* 74, 851–863 (2000).

25. Lyle, J. M., Bullitt, E., Bienz, K. & Kirkegaard, K. Visualization and functional analysis of RNA-dependent RNA polymerase lattices. *Sci. (80-) 296, 2218–2222 (2002).

26. Hashom, M., Nagel, K., Kobel, L., Unge, T. & Rohayem, J. The active form of the norovirus RNA-dependent RNA polymerase is a homodimer with cooperative activity. *J. Gen. Virol.* 90, 281–291 (2009).

27. Chinnaswamy, S., Murali, A., Li, P., Fujisaki, K. & Kao, C. C. Regulation of De Novo-Initiated RNA Synthesis in Hepatitis C Virus RNA-Dependent RNA Polymerase by Intermolecular Interactions. *J. Virol.* 84, 5923–5935 (2010).

28. Spagnuolo, C. A., Bullitt, E., Flanagan, J. B., Kirkegaard, K. Enzymatic and nonenzymatic functions of viral RNA-dependent RNA polymerases within oligomeric arrays. *RNA* 16, 382–393 (2010).

29. Benton, M., Dommer, R., Forrest, S. J. & Stonehouse, N. J. Formation of higher-order foot-and-mouth disease virus 3Dpoly complexes is dependent on elongation activity. *J. Virol.* 86, 2371–2374 (2012).

30. Teller, A. B. et al. Interstitial Contacts in an RNA-Dependent RNA Polymerase Lattice. *J. Mol. Biol.* 412, 737–750 (2011).

31. Wang, J., Lyle, J. M. & Bullitt, E. Surface for Catalysis by Poliovirus RNA-Dependent RNA Polymerase. *J. Mol. Biol.* 425, 2529–2540 (2013).

32. O’Donnell, V. K., Pacheco, J. M., Henry, T. M. & Mason, P. W. Subcellular distribution of the foot-and-mouth disease virus 3A protein in cells infected with viruses encoding wild-type and bovine-attenuated forms of 3A. *Virology* 287, 151–162 (2001).

33. Midgley, R. et al. A role for endolysosomal reticulum exit sites in foot-and-mouth disease virus infection. *J. Gen. Virol.* 94, 2636–2646 (2013).

34. Dee, S. A. et al. Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PLoS One* 13, e0194509 (2018).

35. Thompson, D. et al. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Rev. Sci. Tech. 21, 675–687 (2002).*

36. Tulloch, F. et al. FMDV replicons encoding green fluorescent protein are replication competent. *J. Virol. Methods* 209C, 35–40 (2014).

37. Herod, M. R. et al. Employing Transposon Mutagenesis to Investigate Foot-and-Mouth Disease Virus Replication. *J. Gen. Virol.* 96, 3507–3518 (2015).

38. Baltimore, D. & Franklin, R. M. Preliminary data on a virus-specific enzyme system responsible for the synthesis of viral RNA. *Biochem. Biophys. Res. Commun.* 99, 388–392 (1962).

39. Polatnick, J. & Arlinghaus, R. B. Foot-and-mouth disease virus-induced ribonucleic acid polymerase in baby hamster kidney cells. *Virology* 31, 601–608 (1967).

40. Pata, J. D., Schultz, S. C. & Kirkegaard, K. Functional oligomerization of picornavirus RNA-dependent RNA polymerase. *RNA* 1, 466–477 (1995).

41. Watkins, C. L., Kempf, B. J., Beaucourt, S., Barton, D. J. & Peersen, O. B. Picornaviral polymerase domain exchanges reveal a modular basis for distinct biochemical activities of viral RNA-dependent RNA polymerases. *J. Virol.* 295, 10624–10637 (2020).

42. Arias, A. et al. Mutant viral polymerase in the transition of virus to error catastrophe identifies a critical site for RNA binding. *J. Mol. Biol.* 353, 1021–1032 (2005).

43. Selvaraj, M. et al. The Structure of the Human Respiratory Syncytial Virus M2-1 Protein Bound to the Interaction Domain of the Phosphoprotein P Defines the Orientation of the Complex. *MBio* 9, e01554–18 (2018).

44. Ellingham, M., Bunda, D. H. J., Rowsland, D. J. & Stonehouse, N. J. Selection and characterization of RNA aptamers to the RNA-dependent RNA polymerase from foot-and-mouth disease virus. *RNA* 12, 1970–1979 (2006).

45. Forrest, S. et al. Inhibition of the foot-and-mouth disease virus subgenomic replicon by RNA aptamers. *J. Gen. Virol.* 95, 2649–2657 (2014).

46. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14, 331–332 (2017).

47. Zhang, K. Gete Real-time CTF determination and correction. *J. Struct. Biol.* 193, 1–12 (2016).

48. Scheres, S. H. W. RELION: Implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* 180, 519–530 (2012).

49. Scheres, S. H. & Scheres, S. H. W. Helical reconstruction in RELION. *J. Struct. Biol.* 198, 163–176 (2017).

50. Frank, J. et al. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J. Struct. Biol.* 116, 190–199 (1996).

51. Heymann, J. B. BoSf: image and molecular processing in electron microscopy. *J. Struct. Biol.* 133, 156–169 (2001).

52. Petersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612 (2004).

53. Goddard, T. D. et al. UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci.* 27, 14–25 (2018).

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Competing interests

The authors declare no competing interests.

Additional information

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