Mapping inhibitory sites on the RNA polymerase of the 1918 pandemic influenza virus using nanobodies

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Influenza A viruses cause seasonal epidemics and global pandemics, representing a considerable burden to healthcare systems. Central to the replication cycle of influenza viruses is the viral RNA-dependent RNA polymerase which transcribes and replicates the viral RNA genome. The polymerase undergoes conformational rearrangements and interacts with viral and host proteins to perform these functions. Here we determine the structure of the 1918 influenza virus polymerase in transcriptase and replicase conformations using cryo-electron microscopy (cryo-EM). We then structurally and functionally characterise the binding of single-domain nanobodies to the polymerase of the 1918 pandemic influenza virus. Combining these functional and structural data we identify five sites on the polymerase which are sensitive to inhibition by nanobodies. We propose that the binding of nanobodies at these sites either prevents the polymerase from assuming particular functional conformations or interactions with viral or host factors. The polymerase is highly conserved across the influenza A subtypes, suggesting these sites as effective targets for potential influenza antiviral development.

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Influenza A viruses are an important global health concern causing seasonal epidemics and, more rarely, global pandemics. The currently circulating influenza A viruses are thought to be the evolutionary progeny of the virus that caused the 1918–1919 global pandemic, which was responsible for between 50 and 100 million deaths worldwide. The 1918 influenza virus is thought to have jumped from waterfowl into humans and is considered the ‘founder virus’ that has contributed viral genome segments for all subsequent epidemic and pandemic strains. It remains unclear why this virus was so highly pathogenic, but several viral factors as well as secondary bacterial infections have been implicated in the high lethality of the virus.

The genome of influenza A virus is composed of eight different negative-sense RNA segments packaged in the form of viral ribonucleoprotein (vRNP) complexes. Each vRNP consists of oligomeric viral nucleoprotein (NP) and viral RNA (vRNA), which form a large loop arranged as a helical filament, with the viral polymerase bound to the 5' and the 3' ends of the vRNA. The influenza virus RNA-dependent RNA polymerase is a heterotrimeric multifunctional machine composed of polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) subunits, that catalyzes both primer-independent replication (vRNA to complementary RNA (cRNA) and cRNA to vRNA synthesis) and primer-dependent transcription (vRNA to mRNA synthesis) in the nucleus of infected cells. PB1 houses the polymerase active site, whereas PB2 and PA contain, respectively, cap-binding and endonuclease domains required for transcription initiation by cap-snatching. High-resolution structures of the complete viral polymerase from a number of human, avian and bat influenza A viruses, as well as influenza B, C, and D viruses have been determined. These structures have revealed that the polymerase is a highly flexible macromolecule that can assume a variety of distinct conformations, enabling the polymerase to perform both transcription and replication that require different initiation and termination mechanisms. The polymerase genes are among the most highly conserved genes of influenza viruses and as such the polymerase represents an ideal target for antivirals. Current influenza therapeutics target the neuraminidase and polymerase and inhibitors such as baloxavir (Xofluza) and favipiravir have been approved for use in a limited number of countries. However, mutations allow the virus to escape inhibition by these compounds and therefore further antivirals are required. Recently, nanobodies, comprising the variable domain of single-chain camelid antibodies also referred to as VHH, have emerged as novel antiviral agents for both extracellular and intracellular targeting of viral proteins.

In this work, we determine structures of the polymerase from the 1918 pandemic influenza virus and identify sites on the surface of the polymerase that are sensitive to inhibition. Using single-particle analysis (SPA) cryo-EM and X-ray crystallography, we determine the binding location of 20 nanobodies on the RNA polymerase of the 1918 pandemic influenza virus. Functional analysis of the nanobodies shows that a subset of nanobodies strongly inhibits polymerase activity in vitro and in cell culture, and efficiently reduces virus growth. Combining functional and structural data we discover sites of vulnerability on the polymerase and link these to the inhibition of specific polymerase functions, identifying and validating targets for drug discovery.

Results

Structural characterisation of the 1918 pandemic influenza virus polymerase. We co-expressed the three subunits of the RNA polymerase from influenza A/Brevig Mission/1/1918 (H1N1) virus in insect cells and purified the heterotrimeric complex. In vitro transcription assays showed that the recombinant polymerase was active (Supplementary Fig. 1). Polymerase with added capped RNA primer and vRNA promoter, comprising the 5' and 3' terminal sequences of vRNA, was used for EM grid preparation. We observed monomeric polymerase heterotrimers as well as both dimeric and tetrameric assemblies (Supplementary Fig. 2). The dimeric polymerase species were highly similar to the previously observed dimer implicated in cRNA to vRNA synthesis. However, severe preferential orientation prohibited accurate 3D reconstruction of both dimeric and tetrameric forms. After 2D and initial 3D classification of the monomeric particles a consensus refinement of the particles yielded a high-quality map. Further 3D classification of this map yielded four maps into which models were built, resulting in four distinct polymerase structures. In all four structures the core of the polymerase composed of PB1, the C-terminal domain of PA and the N-terminal one third of PB2 was fully resolved, while the flexible domains including the N-terminal endonuclease domain of PA and the C-terminal two thirds of PB2 showed different arrangements or remained unresolved. To aid in map interpretation the programme deMPHancer was used for low conformational conformations (Supplementary Fig. 3). Both the 5' and 3' vRNA promoters, but no capped RNA primer, was observed in all structures. The 5' promoter was fully ordered forming the hook structure bound by PB1 and PA as observed previously. Only eight bases at the 5' end of the 3' promoter were observed, four of which form a duplex with the 5' promoter while the remaining four are oriented towards the polymerase active site. The seven 3' terminal unresolved sites of the 3' promoter strand have likely entered the active site but remain unresolved.

The most complete structure (class 1), representing ~15% of the particles, shows an almost completely ordered polymerase heterotrimer with only the C-terminal nuclear localisation signal (NLS) domain of PB2 remaining unresolved (Fig. 1a). The overall conformation of the polymerase is similar to the previously observed conformations implicated in transcription although with some differences in the exact location and orientation of the C-terminal domains of PB2, comprising the mid, cap-binding, linker (which together with mid forms a rigid-body domain mid-link), 627 and NLS domains. Specifically, the cap-binding domain is rotated by ~90° relative to the polymerase core with the cap-binding site facing away from the product exit/primer entry channel (Supplementary Fig. 4a). This conformation of the cap-binding domain is stabilised through a correlated rotation of the 627 domain by 26°. The co-rotation of these domains leads to a new interaction between the 424 loop of the cap-binding domain and residues 585–588, 624 and 625 of the 627 domain, instead of the previously observed interaction of the 424 loop with PB1 residues. However, the overall fold of the 627-domain remains the same as observed in previous structures.

As shown previously, the PB2 cap-binding domain can rotate during transcription and here we identify another stable conformation. This conformational flexibility allows the cap-binding domain to probe the environment for capped RNA and capture the cap, orient the uncleaved capped RNA into the PA endonuclease domain and then insert the cleaved capped primer into the polymerase active site through the product exit/primer entry channel. We propose two possible roles for the observed conformation. This cap-binding domain position could represent a conformation where the polymerase is probing the environment, waiting to capture an uncleaved capped RNA still associated with host RNA polymerase IF. Alternatively, this may be an intermediate position that occurs after cleavage of the capped RNA but before the primer enters the polymerase active site. The cap-binding domain moving away from the polymerase core may help it to accommodate primers of variable length,
reported to range between 8 and 15 nucleotides\textsuperscript{18–20}, and orient their 3’ end into the active site.

The class 2a and class 2b structures, representing ~78% of the particles, are similar to the class 1 structure; however, the flexible C-terminal domains of PB2 remain entirely unresolved (Fig. 1b). The two structures (class 2a and class 2b) differ in the orientation of the PA endonuclease and the ordering of the priming loop, a hairpin that protrudes from the PB1 thumb subdomain into the polymerase active site and is involved in the positioning of the template and/or initiating nucleotide in the polymerase active site\textsuperscript{7,15,21}. For class 2a the location and orientation of the PA endonuclease relative to the polymerase core is identical to that in the class 1 structure and very similar to that previously observed in the transcriptase conformations of the polymerase\textsuperscript{2,15}. In the class 2a structure, as in the class 1 structure, the priming loop is fully ordered despite no template RNA being resolved in the active site. In the class 2b structure, the PA endonuclease, together with the C-terminal helices of PB1 and the N-terminal helix of PB2 with which it forms a rigid body, is rotated by 20° relative to the core of the polymerase (Supplementary Fig. 4b). This rotation leads to new interactions between the PA endonuclease and the polymerase core mediated through PA Lys22 interacting with PB1 Thr156, Glu159, and Ser160 (Supplementary Fig. 4b). Interestingly, this rotation correlates with disordering of the priming loop.

The last structure (class 3), representing ~7% of particles, shows a polymerase with a fully ordered core, including the priming loop, and the PB2 cap-binding and mid-link domains, but lacks the PA endonuclease and the PB2 627 and NLS domains (Fig. 1c). The resolved PB2 domains are arranged in a conformation similar to that observed in crystal structures of the apo human H3N2 and avian H5N1 influenza A virus and influenza C virus polymerases as well as cRNA-bound influenza B virus polymerase and, more recently, the cryo-EM structure of the influenza C virus polymerase dimer in complex with host acidic nuclear phosphoprotein 32A (ANP32A), described as a replicase (Supplementary Fig. 4c)\textsuperscript{7,8,17,22}. It is tempting to speculate that, in solution, in the absence of ANP32A and polymerase dimer formation there is nothing against which the PB2 627 domain could pack and so it remains flexible.

Taken together, our characterisation of the polymerase of the 1918 pandemic influenza virus has yielded a number of important observations. The polymerase is highly dynamic with multiple oligomers and conformations present in a single sample. We observe the flexible PB2 C-terminal domains in conformations described for both the transcriptase and replicase conformations, while most molecules show no ordered PB2 C-terminal domains. No vRNA template is observed in the polymerase active site; however, we are able to resolve the priming loop in most structures. These data suggest the ordering of the priming loop is uncoupled from the ordering of RNA in the polymerase active site and is perhaps related to the position of the PA endonuclease domain. We were able to resolve the flexible C-terminal domains of PB2 in a new arrangement, similar to that described for the transcriptase\textsuperscript{7,15}. Specifically, we resolved the PB2 cap-binding domain in a new orientation that we propose is important for capturing an uncleaved capped RNA or orienting the cleaved capped RNA primer into the active site. Interestingly, no capped RNA was observed although the reasons for this remain unclear.

We envisage that these structural insights hold true for all influenza A virus polymerases as there is a very high degree of sequence homology among the A strains (Supplementary Fig. 5a–c), although we cannot exclude the possibility that some of these conformations are due to unknown characteristics of the 1918 influenza polymerase.

**Fig. 1 Cryo-EM structures of the 1918 polymerase in three conformations.** Monomeric conformations of the 1918 polymerase in full transcriptase (class 1) (a), partial transcriptase (class 2a) (b), or replicase conformations (class 3) (c).

**Structural characterisation of the 1918 influenza virus polymerase in complex with nanobodies.** A panel of 24 nanobodies (Nb8189–Nb8212) was generated against recombinantly expressed and purified polymerase of an H5N1 influenza A virus as described previously\textsuperscript{7}. Using cryo-EM we determined the structure of 17 polymerase-nanobody complexes at resolutions between 4.5 and 6.7 Å; this is sufficient resolution to accurately position each nanobody bound to the polymerase heterotrimer (Supplementary Fig. 6, Supplementary Table 1). In addition to the core of the polymerase which was resolved in all structures, the PA endonuclease was resolved in the majority of structures (excluding Nb8189, Nb8190, Nb8192, Nb8196, Nb8202), while the C-terminal domains of PB2 remained unresolved in all structures. The nanobody binding sites were spread across the surface of the polymerase with related nanobodies binding at the same site (Supplementary Fig. 7a, b).

The most populous binding site, termed site 1, is on the PA endonuclease with five nanobodies (Nb8198, Nb8199, Nb8200, Nb8203, Nb8209) that bind at an interface formed by the short helix α1 (residues 1–10) and the long helix α6 (170–185) (Fig. 2a). On the PA linker (site 2), that links the N-terminal PA...
endonuclease domain and the PA C-terminal domain and wraps around PB1, we observe three nanobodies; Nb8202 and Nb8210 bind identically at residues 220–240 and Nb8204 binds at residues 250–265. Nb8207 binds to a unique position, termed site 3, at the product exit channel contacting both PB1 and the N-terminal region of PB2. The PA C-terminal domain is the binding site for eight nanobodies which cluster into four groups (sites 4a–d) across the surface of the domain. Nb8192 and Nb8205 bind to PA residues 426–445 (site 4a) with Nb8205 also making a minor contact with the PB1. Three nanobodies (Nb8189, Nb8190, Nb8201) bind at an interface formed by PA residues 615–630 and PB1 residues 10–15 (site 4b). Nb8191 binds to the PB1 N-terminus and PA residues 405–420, 531–534, 551–557, and 624–626 (site 4c). Finally, Nb8206 binds uniquely to PA residues 309–321 and 555–558 (site 4d).

Five nanobodies, Nb8193, Nb8194, Nb8195, Nb8197, and Nb8208, were unresolved by cryo-EM, and as the C-terminal domains of PB2 are disordered in our datasets, we suspected that these nanobodies may bind to this region. To test this hypothesis, we purified proteins comprising either the cap-binding and mid-link domains (cap-mid-link, residues 250–538) or the 627 and NLS domains (627-NLS, residues 538–759). Four of the nanobodies bound to cap-mid-link causing a peak shift in size exclusion chromatography; only Nb8195 did not bind (Supplementary Fig. 7c). Crystallisation of the cap-mid-link-nanobody complexes yielded crystals that diffracted to resolutions of 1.7 Å for the Nb8193 complex and 1.9 Å for the Nb8194 complex (Fig. 2b–d, Supplementary Table 2). Nanobodies Nb8193 and Nb8194 form site 5a, each interacting with the cap-binding domain residues Arg389, Asp390, and Arg482. Nb8193 forms additional contacts with Arg264 from the mid domain and Thr524 from the linker region. We also determined the structure of Nb8208 bound to the cap-binding domain at a resolution of 3.1 Å, a product of in-drop proteolysis from crystallisation screens set up with the complete cap-mid-link construct. Nb8208 binds to site 5b interacting with residues Glu391, Asp466, Thr468, and Glu472 (Fig. 2d). The cap-mid-link-Nb8197 complex failed to produce diffraction quality crystals but, based on the similarity of its complementarity-determining regions (CDRs), Nb8197 likely binds at a similar position as Nb8193 and Nb8194. We have not determined the structures of Nb8211 and Nb8212 which, based on their similarity to Nb8200 and Nb8198 (Supplementary Fig. 7a, b), respectively, are expected to bind to the PA endonuclease.

Nanobodies inhibit polymerase activity in vitro and viral growth in cell culture. To assess the inhibitory effect of nanobodies on polymerase activity, we performed an in vitro polymerase activity assay using recombinant RNP complexes derived from the 1918 pandemic influenza virus. β-globin mRNA was added to the vRNPs to facilitate primer-dependent transcription, in the presence of an excess of purified nanobody. These assays showed that a subset of nanobodies significantly inhibit...
transcription and/or vRNA to cRNA replication with Nb8207 being particularly potent (Fig. 3a, Supplementary Fig. 8a). Interestingly, we observe different potencies for nanobodies that bind at similar locations, for example Nb8189 and Nb8190 which bind the same site on the C-terminal domain of PA; this is likely due to differences in the affinities of each nanobody for a given site. To investigate the effect of nanobodies on polymerase activity in cellular environment, we used a minireplicon luciferase-reporter assay, in which each nanobody was expressed along with polymerase subunits and NP from the 1918 pandemic influenza virus and a negative-sense vRNA encoding luciferase (Fig. 3b). This assay revealed that most nanobodies, including the Nb8189Nb8190Nb8191Nb8192Nb8193Nb8194Nb8195Nb8196Nb8197Nb8198Nb8199Nb8200Nb8201Nb8202Nb8203Nb8204Nb8205Nb8206Nb8207Nb8208Nb8209Nb8210Nb8211 Nb8212 No polymerase No Nb

0.0 0.5 1.0 1.5
Relative RNA levels

N8189 N8190 N8191 N8192 N8193 N8194 N8195 N8196 N8197 N8198 N8199 N8200 N8201 N8202 N8203 N8204 N8205 N8206 N8207 N8208 N8209 N8210 N8211 N8212 No polymerase No Nb

0.0 0.5 1.0 1.5
Relative luciferase intensity (Log10)

N8189 N8190 N8191 N8192 N8193 N8194 N8195 N8196 N8197 N8198 N8199 N8200 N8201 N8202 N8203 N8204 N8205 N8206 N8207 N8208 N8209 N8210 N8211 N8212 No polymerase No Nb

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0.0 0.5 1.0 1.5
Relative luciferase intensity (Log10)
Fig. 3 Effect of nanobodies on 1918 polymerase function and virus growth. a Effect of nanobodies on the activity of recombinant 1918 RNP in the presence of a source of capped primer. Data are mean ± s.e.m. n = 3 independent RNP purifications and reactions. Ordinary one-way ANOVA was used to compare mRNA and cRNA levels in the absence (no Nanobody) and presence of the indicated nanobody. P < 0.05 is considered significant. b Effect of nanobodies on 1918 influenza polymerase activity using a luciferase-reporter minireplicon assay. Data are mean ± s.e.m. n = 3 independent transfections with n = 2 technical replicates. Ordinary one-way ANOVA was used to compare the relative luciferase intensity in the presence and absence of nanobodies. P < 0.05 is considered significant. c Effect of two sets of nanobodies on the replication of a reassortant A/WSN/1933 virus encoding PB1, PB2, PA and NP of A/Brevig Mission/1/1918 (WSN-1918\(^{RNP}\)) virus in HEK293T cells. Data are mean ± s.e.m. n = 3 independent transfections and infections for each set of nanobodies. P values are as follows: for Nb8190 P = 0.0007, P = 0.0067, P = 0.0331 and P = 0.0199, for Nb8207 P = 0.0006, P = 0.0063, P = 0.0301 and P = 0.0185, for Nb8210 P = 0.0084, P = 0.0925, P = 0.3444 and P = 0.0696, for Nb8191 P = 0.1030, P = 0.0009, P = 0.0141 and P < 0.0001, for Nb8192 P = 0.1227, P = 0.0008, P = 0.0136 and P < 0.0001, for Nb8208 P = 0.1179, P = 0.0011, P = 0.0205 and P = 0.0025, and for Nb8210 P = 0.7246, P = 0.9865, P = 0.2269 and P = 0.0340, at 16, 24, 32 and 48 h post infection, respectively. P < 0.05 is considered significant. For quantification of viral RNA levels during the course of infection, see Supplementary Fig. 8b. Source data are provided as a Source Data file.

Discussion

In this study we have solved the structure of the RNA polymerase of the 1918 pandemic influenza virus in four distinct conformations. These conformations show features common with previously determined structures implicated in either transcription or replication. In a conformation characteristic of a transcriptase we observed a novel arrangement of the PB2 cap-binding domain. This finding is in agreement with the idea that this domain is extremely flexible, which allows it to sample the environment for nascent host capped RNA, bind to it and then orient it towards the PA endonuclease for cleavage, followed by positioning of the short capped RNA product into the polymerase active site for transcription initiation. We also observed a correlation between the ordering of the priming loop in the active site and the position of the PA endonuclease, suggesting crosstalk between the two. However, the significance of this observation remains unclear.

In our attempt to map inhibitory sites on the polymerase of the 1918 pandemic influenza virus we have determined the structure of 20 nanobodies bound to the polymerase heterotrimer or a fragment of the PB2 subunit. A combination of in vitro and cell-based polymerase assays and virus growth inhibition assays led to the identification of multiple sites on the polymerase surface that are sensitive to inhibition by nanobodies (Fig. 5a, b). In order to transcribe viral genes and replicate the viral RNA genome the influenza virus RNA polymerase must interact with viral and host factors and adopt at least three distinct conformations to act as a transcriptase, replicase, or encapsidase. We speculate that binding of nanobodies most likely inhibits polymerase function by locking it in a particular conformation, preventing it from
assuming a particular functionally important conformation or inhibits interactions with viral or host factors.

The site bound by Nb8207, one of the most potent nanobodies identified in our study, is located at the product exit channel, formed by PB1 and the N-terminal region of PB2. Binding of a nanobody at this site inhibits RNA production in both transcription and replication without affecting other polymerase activities such as CTD binding (Figs. 4a–d, 5a, b). We speculate that nanobody binding could inhibit RNA exit by blocking the product exit channel or might prevent the polymerase from assuming an active conformation as binding of a nanobody at this site is incompatible with the packing of the C-terminal domains of PB2 in both transcribe and replicate conformations.

The site bound by Nb8208 is located on the cap-binding domain of PB2. This region of the PB2 cap-binding domain packs against the PB1 palm subdomain in the encapsidase conformation of the polymerase as observed in the dimeric polymerase bound by the host protein ANP32A (Fig. 5a, b)22. Nanobody binding at this site severely affected both steps of replication but had no effect on primary transcription, Pol II CTD binding and polymerase activity in vitro in agreement with the idea that binding of Nb8208 affects formation of the ANP32A-bound dimeric polymerase.

We have identified several inhibitory sites on the C-terminal domain of PA. The site bound by Nb8190 corresponds to a site against which the 627 domain of PB2 is packed in the transcriptase conformation, potentially preventing the polymerase from assuming the transcriptase conformation. Accordingly, Nb8190 inhibited transcription although it had no effect on the binding of the polymerase to Pol II CTD (Fig. 4a). The site bound by Nb8191 overlaps with the binding site of the Pol II CTD23,24 and, indeed, this nanobody was found to inhibit the binding of the viral polymerase to the CTD of Pol II and, consequently, inhibit transcription (Fig. 4a, b). The site bound by Nb8192 overlaps with the polymerase dimer interface that has been identified as important for cRNA to vRNA replication7. Binding at this site would therefore prevent polymerase dimerisation and, in agreement, Nb8192 was found to inhibit RNA replication (Fig. 4c, d), as demonstrated previously for Nb82057 which has a binding site overlapping with that of Nb8192. Although the site bound by Nb8192 has not been shown to contribute to Pol II CTD binding our data suggest that it might play a role as Nb8192 also inhibited Pol II CTD binding and transcription. Overall, our data reinforce previous findings that the PA C-terminal domain plays a central role in multiple functions during RNA synthesis including Pol II CTD binding during transcription and replication.
providing a polymerase dimerisation interface during replication (Fig. 4a–d).

In summary, we have solved the structure of the RNA polymerase of the 1918 pandemic influenza virus. We have also identified sites on the polymerase surface that are sensitive to inhibition by nanobodies and proposed inhibitory mechanisms. These inhibitory sites are highly conserved across the polymerases of influenza A virus strains (Supplementary Figs. 5a–c, 9c) and as such present exciting therapeutic targets for small molecule compounds or inhibitory peptides.

Methods

No statistical methods were used to predetermine sample size. The experiments were not randomised and investigators were not blinded to allocation during experiments and outcome assessment.

Cells. Human embryonic kidney 293T (HEK293T), Madin-Darby Bovine Kidney (MDBK), and S9 insect cells were sourced from the Cell Bank of the Sir William Dunn School of Pathology. HEK293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM) + 10% Fetal Cali Serum (FCS) and S9 cells were maintained in SF-900 II serum-free medium. Cell lines have not been authenticated but tested negative for mycoplasma contamination.

Protein expression and purification. The three polymerase genes from influenza A/Breivig Mission/1/1918 (H1N1) virus were codon optimised for insect cells and synthesised (Synbio Technologies). Genes were then cloned into the Multibac system with protein expression and purification synthesised (Synbio Technologies). Genes were then cloned into the Multibac system with protein expression and purification. Plasmids encoding C-terminally His-tagged nanobodies were tested negative for mycoplasma contamination. Cell lines have not been authenticated but tested negative for mycoplasma contamination. Plasmids encoding C-terminally His-tagged nanobodies were constructed and the constructs digested with restriction enzymes. Recombinant DNA was ligated into the pET22b(+) expression vector (Novagen) and transformed into Escherichia coli BL21 (DE3). The plasmids were transferred into the expression strain E. coli BL21 (DE3) and induced with 1 mM IPTG for protein expression.

Cryo-EM sample preparation. To determine the structure of the polymerase an approach similar to that described by Kouba et al was utilised26. Briefly, polymerase was mixed with a 1.2 molar excess of vRNA promoters (5′ vRNA 5′-AGUAAACAAGGCC-3′, 3′ vRNA 5′-GCCCUGCUUUUGCCUUAUU-3′) with a 3 nucleotide long extension at the 3′ end (italics)) and a 50 molar excess of Baloxavir (Aobious) and left on ice for 10 min. Subsequently a 1.5 molar excess of synthetic capped mRNA primer (5′-m7GpppAm-AUCUAUAAUAG) and MgCl2 to a final concentration of 5 mM were added. To allow limited extension of the product, nucleotides were added to a final concentration of 1 mM CTP, and 1 mM ApCpG (adenosine-5′-[(αβ)-methylene]triphosphate). The reaction mixture was incubated at 20 °C overnight before being applied to a Superdex 200 Increase 10/300 GL column in a buffer containing 20 mM HEPES-NaOH, pH 7.5, 500 mM NaCl at 20 °C. Sequence comparison of the nanobodies was carried out using Clustal O. The data was collected using SERIAME3. Data was processed using the EMAN2 software.

Cryo-EM image collection and processing. For the initial structure determination of the polymerase, data was collected on a 300 kV Titan Krios equipped with a K2 Summit (Gatan) camera and a GIF Quantum energy filter at the Oxford Particle Imaging Centre. The data was collected using SERIAME3. Data was processed
on-the-fly using cryoSPARC-Live V2.15. Data were motion corrected and the CTF estimated using patch motion correction and patch CTF estimation, respectively (cryoSPARC). Particles taken from a subset of the dataset were used to generate templates for complete picking using the cryoSPARC template picker. To remove bad particles, 2D classes were generated before particles from good classes were subjected to ab initio model picking using the cryoSPARC template picker. To remove bad particles, 2D classes were generated before particles from good classes were subjected to ab initio model picking using the cryoSPARC template picker. To remove bad particles, 2D classes were generated before particles from good classes were subjected to ab initio model picking using the cryoSPARC template picker. To remove bad particles, 2D classes were generated before particles from good classes were subjected to ab initio model picking using the cryoSPARC template picker. To remove bad particles, 2D classes were generated before particles from good classes were subjected to ab initio model picking using the cryoSPARC template picker.
MgCl₂, 1 mM DTT, and 2U/µl RNasin in a reaction volume of 20 µl. After 4 h incubation at 30 °C, RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. RNA levels were analysed using primer extension as previously described. In brief, RNA was reverse-transcribed using excess 32P-labelled primers specific to positive-sense mRNA and cRNA. Primer extension products derived from mRNA and cRNA can be distinguished by gel electrophoresis. Total RNA and cRNA were digested with RNase-free DNase I, and the digested RNAs were reverse-transcribed in the presence of a primer extension reaction mixture containing 0.5% FCS and L-glutamine. The efficiency of reverse genetics was evaluated by titrating the super-replicon product after transfection.

Luciferase minireplicon assay. Approximately 0.16 × 10⁶ HEK293T cells were transfected with 0.04 µg of each plasmid encoding PB1, PB2, PA, and NP of influenza virus, and virus titres were determined by plaque assay. At each time point, cells were infected with a reassortant in 10⁶ MgCl₂, 1 mM DTT, and 2U of A/Brevig Mission/1918 at a multiplicity of infection of 0.01. Cell culture medium was collected 12, 24, 36 and 48 h after transfection. After transfection, and total cell lysates were obtained by adding 100 µl Luciferase Cell Culture Reagent (Promega) to the cells, followed by vigorous shaking for 10 min. The presence of luciferase enzyme was determined using a luciferase-based detection system following the manufacturer’s instructions (Promega). In brief, 100 µl of Luciferase Assay Reagent (Promega) was added to 20 µl of whole cell lysate in a 96-well opaque plate. The plate was immediately read in microplate reader SpectraMax M3 (Molecular Devices). Two technical replicates were carried out for each replication and mean values were calculated. These mean values for three independent transfections were used in subsequent analysis in Excel (Microsoft) and Prism 8 (GraphPad).

Influenza virus reverse genetics. Recombinant influenza virus was produced by reverse genetics as described previously. In brief, −6 × 10⁶ HEK293T cells, cultured in DMEM with 10% FCS, were transfected with 0.5 µg of each plhW2000 plasmid encoding PB1, PB2, PA, NP of A/Brevig Mission/1918 (H3N2) (EMD-12065), A/Fujian/01/2002 (H3N1) virus. 0.2 µg of plasmid encoding luciferase RNAi vector, and 0.8 µg nanobody plasmid or an empty pcDNA3 vector. Cells were collected 20 h after transfection, and total cell lysates were obtained by adding 100 µl Luciferase Cell Culture Lysis Reagent (Promega) to the cells, followed by vigorous shaking for 10 min. The presence of luciferase enzyme was determined using a luciferase-based detection system following the manufacturer’s instructions (Promega). In brief, 100 µl of Luciferase Assay Reagent (Promega) was added to 20 µl of whole cell lysate in a 96-well opaque plate. The plate was immediately read in microplate reader SpectraMax M3 (Molecular Devices). Two technical replicates were carried out for each replication and mean values were calculated. These mean values for three independent transfections were used in subsequent analysis in Excel (Microsoft) and Prism 8 (GraphPad).

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Western blotting: Western blotting to determine the expression of nanobodies was carried out using a specific goat anti-alpaca polyclonal antibody conjugated to HRP (Jackson ImmunoResearch), while actin was blotted using a rabbit anti-β-actin primary antibody (Sigma-Aldrich) and then goat anti-rabbit secondary antibody conjugated to HRP. Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or Chemiluminescent HRP Substrate (Millipore) were used for detection. Note that the anti-alpaca polyclonal antibody exhibits varying levels of affinity for different nanobodies; hence, band intensities for different nanobodies in western blots are not directly comparable.

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

**Data availability** All data are available from the corresponding authors and/or included in the paper or Supplementary Information. Crystallographic coordinates and maps generated in this study have been deposited in the PDB with accession codes 7NFO, 7NFL, and 7NFT for the Nb8193, Nb8194, Nb8208 complexes, respectively. Cryo-EM density maps with the corresponding atomic coordinates have been deposited in the Electron Microscopy Data Bank and the Protein Data Bank with the following accession codes for the 1918 polymerase heterotrimer family 1 (PDB 7NIK, EMDB-12342), class 2a (PDB 7NJA, EMDB-12343), class 2b (PDB 7NHJ, EMDB-12353), class 3 (PDB 7NHD, EMDB-12352). Cryo-EM density maps with the corresponding atomic coordinates have been deposited in the Electron Microscopy Data Bank and the Protein Data Bank with the following accession codes for the complexes between the 1918 polymerase heterotrimer and nanobodies Nb8189 (PDB 7NIK, EMDB-12361), Nb8190 (PDB 7NLU, EMDB-12362), Nb8191 (PDB 7NIR, EMDB-12363), Nb8192 (PDB 7NJS, EMDB-12364), Nb8196 (PDB 7NJS, EMDB-12371), Nb8198 (PDB 7NIJ, EMDB-12372), Nb8199 (PDB 7NIS, EMDB-12373), Nb8200 (PDB 7NJB, EMDB-12375), Nb8201 (PDB 7NKJ, EMDB-12428), Nb8202 (PDB 7NKZ, EMDB-12429), Nb8203 (PDB 7NK4, EMDB-12430), Nb8204 (PDB 7NKK, EMDB-12431), Nb8205 (PDB 7NKS, EMDB-12433), Nb8206 (PDB 7NKA, EMDB-12435), Nb8207 (PDB 7NKC, EMDB-12437), Nb8209 (PDB 7NKL, EMDB-12440), Nb8210 (PDB 7NKR, EMDB-12447). Source data are provided with this paper.

Received: 4 June 2021; Accepted: 21 December 2021; Published online: 11 January 2022

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Acknowledgements
We thank P. Palese and R. Fouchier for plasmids, I. Berger for the Multibac system, and G. G. Brownlee, M. Martinez-Alonso, and members of the Fodor and Grimes laboratories for helpful comments and discussions. We also thank Instruct-ERIC, part of the European Strategy Forum on Research Infrastructures (ESFRI), Instruct-ULTRA (EU H2020 Grant 731005), and the Research Foundation—Flanders (FWO) for support with nanobody discovery. We thank Alison Landquist for the technical assistance during nanobody discovery. This work was supported by Medical Research Council (MRC) programme grant MR/B009945/1 (to E.F.), Wellcome Investigator Awards 200835/Z/16/Z (to J.M.G.), Clarendon Fund and Medical Science Doctoral Training Centre at the University of Oxford (to Z.Z.), MRC studentships (to A.P.W. and I.S.M.); We thank Diamond Light source for access to the MX beamlines (proposal number MX19946). Electron microscopy provision was provided through the OPIC electron microscopy facility, which was founded by a Wellcome JIF award (060208/Z/00/2) and is supported by a Wellcome equipment grant (093305/Z/10/Z). Computation was performed at the Oxford Biomedical Research Computing (BMRC) facility, a joint development between the Wellcome Centre for Human Genetics and the Big Data Institute supported by Health Data Research UK and the NIHR Oxford Biomedical Research Centre. Part of this work was supported by Wellcome administrative support grant (203141/Z16/2).

Author contributions
J.R.K., Z.Z., L.C., H.F., E.F. and J.M.G. conceived and designed the study. J.R.K., L.C., H.F., I.S.M., carried out cloning of recombinant baculoviruses and protein purification. J.R.K and L.C. collected and processed X-ray crystallography and electron microscopy data and built and refined models. Z.Z., A.P.W. and I.S.M. performed functional assays and analysed data. E.F. and I.S.M. designed and generated nanobodies. J.M.G. and E.F. supervised the structural and functional studies, respectively. J.R.K., Z.Z., L.C., H.F., E.F. and J.M.G. wrote the paper with input from all co-authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-27950-w.

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