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1 **Fluoxetine targets an allosteric site in the enterovirus 2C AAA+ ATPase and**  
2 **stabilizes the hexameric complex**

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21

22

23 **Abstract**

24 The enterovirus genus encompasses many clinically important human pathogens such  
25 as poliovirus, coxsackieviruses, echoviruses, numbered enteroviruses and  
26 rhinoviruses. These viruses are the etiological agents of several human diseases,  
27 including hand-foot-and-mouth disease, neonatal sepsis, encephalitis, meningitis,  
28 paralysis and respiratory infections. There is an unmet need for antivirals to treat these  
29 diseases. The non-structural protein 2C is a AAA+ helicase and plays a key role in viral  
30 replication. As such, it is an attractive target for antiviral drug development. Several  
31 repurposing screens with FDA-approved drugs have identified 2C-targeting  
32 compounds such as fluoxetine and dibucaine, but the molecular basis of 2C inhibition  
33 has remained enigmatic. Here we present the 1.5 Å resolution crystal structure of the  
34 soluble fragment of coxsackievirus B3 2C protein in complex with (S)-fluoxetine (SFX),  
35 which reveals a conserved, hydrophobic drug-binding pocket which is distal to the ATP  
36 binding site. To decipher the molecular mechanism of inhibition by fluoxetine and other  
37 2C-targeting compounds, we engineered a soluble, hexameric and ATPase competent  
38 2C protein. Using this system, we show that SFX, dibucaine, HBB and guanidine  
39 hydrochloride inhibit 2C ATPase activity in a dose-dependent manner. Moreover, using  
40 cryo-EM analysis, we demonstrate that SFX and dibucaine lock 2C in a defined  
41 hexameric state, rationalizing their mode of inhibition and allowing us to generate the  
42 first reconstruction of the oligomeric complex. Taken together, these results provide  
43 important structural and mechanistic insights into 2C inhibition and provide a robust  
44 engineering strategy which can be used for structural, functional and drug-screening  
45 analysis of 2C proteins from current or future enteroviruses.

## 46 Introduction

47 Viruses belonging to the genus *Enterovirus*, within the family *Picornaviridae*, are  
48 responsible for a broad range of diseases. This genus contains four clinically relevant  
49 enterovirus (EV) species (EV-A-D) and three rhinovirus species (RV-A-C). Notable  
50 members of these species include polioviruses, coxsackieviruses, echoviruses and  
51 numbered EVs (e.g. EV-A71 and EV-D68). EV-associated diseases include respiratory  
52 infections, hand-foot-and-mouth disease, conjunctivitis, viral myocarditis, pancreatitis,  
53 aseptic meningitis, encephalitis, and acute flaccid paralysis<sup>1</sup>. RVs cause the common  
54 cold but can also trigger the exacerbation of asthma and chronic obstructive pulmonary  
55 disease<sup>2</sup>. While most EV infections are mild and self-limiting, fatal complications may  
56 arise in immunocompromised patients and young children<sup>3</sup>. Outbreaks of EV-A71 and  
57 EV-D68 represent major public health concerns because these viruses are associated  
58 with severe neurological complications<sup>4-6</sup>. Vaccines are only available for poliovirus  
59 and EV-A71, with the latter only approved in China<sup>7,8</sup>. Treatment of other clinically  
60 important EVs is currently limited to supportive care. Given the large numbers of  
61 serotypes (>100 EVs and >200 RVs), the development of a pan-EV and RV vaccine  
62 seems unfeasible. This underscores the urgent clinical need for the development of  
63 broad-spectrum antivirals to treat EV-associated diseases.

64 The EV genome encodes seven non-structural proteins (2A-2C and 3A-3D), which are  
65 involved in replication, and four structural proteins (VP1-VP4) which form the capsid.  
66 Several direct acting agents (DAA) targeting either the capsid, the viral polymerase  
67 (3D<sup>pol</sup>) or the viral protease (3C<sup>pro</sup>) have been identified. While some of these  
68 compounds reached clinical trials, their development was stopped due to limited  
69 efficacy, poor bioavailability or toxicity issues<sup>9</sup>. An alternative target for antiviral drug  
70 discovery is the non-structural protein 2C which exhibits several important hallmarks  
71 of a promising DAA target. 2C is functionally indispensable and plays an essential role  
72 in several steps in the EV life cycle, e.g. viral RNA replication and encapsidation<sup>10</sup>.  
73 Moreover, 2C is highly conserved<sup>11</sup>, raising the possibility for broad-spectrum DAA  
74 development.

75 2C belongs to the helicase superfamily 3 (SF3) and acts as an ATP-dependent RNA  
76 helicase and ATP-independent RNA remodeler<sup>12</sup>. The role of this enzyme in viral  
77 replication likely requires an oligomeric organization, which has already been observed

78 for recombinant 2C proteins<sup>13–15</sup>. Previous studies suggested that the functional  
79 oligomerization state of 2C is hexameric, consistent with other members of the SF3  
80 helicase family<sup>16,17</sup>. Structural and functional characterization of the full-length  
81 oligomeric 2C protein remains challenging due to the N-terminal amphipathic helix  
82 which renders the protein poorly soluble<sup>13–15,18,19</sup>. Recent structures of a soluble,  
83 monomeric fragment of 2C from EV-A71 and PV provided molecular details of the  
84 adenosine triphosphatase (ATPase) domain, a cysteine-rich zinc finger and a carboxyl-  
85 terminal helical domain<sup>16,17</sup>. Nevertheless, a structure of the oligomeric 2C remains  
86 elusive.

87 Given the central role of 2C during viral replication, it is not surprising that several  
88 compounds targeting 2C, such as guanidine hydrochloride (GuaHCl), HBB, MRL-1237  
89 and TBZE-029, have been identified<sup>18,20–22</sup>. Additionally, several drug-repurposing  
90 screens have uncovered FDA-approved drugs such as fluoxetine, dibucaine, pirlindole  
91 and zuclopenthixol as inhibitors of EV-B and EV-D species, but their mode of action is  
92 not yet understood<sup>23,24</sup>. Fluoxetine (Prozac<sup>®</sup>) is a selective serotonin-reuptake inhibitor  
93 that is used clinically for the treatment of major depression and anxiety disorders<sup>25</sup>.  
94 Mode-of-action studies revealed that only the *S*-enantiomer of fluoxetine potently binds  
95 2C and inhibits EV-B and D replication<sup>26,27</sup>. Despite the growing number of identified  
96 2C-targeting compounds, the molecular basis for their antiviral activity remains  
97 unknown<sup>28,29</sup>. Such information would provide an invaluable resource for structure-  
98 based design of potent, pan-enterovirus antivirals with low toxicity.

99 Here, we present the 1.5 Å resolution crystal structure of the soluble fragment of  
100 CV-B3 2C in complex with SFX. The structure reveals a highly conserved hydrophobic  
101 pocket, distal to the ATP binding site, into which the SFX trifluoro-phenoxy moiety  
102 inserts. To functionally validate our structure and study the inhibitory mechanism of  
103 SFX and other 2C targeting compounds, we engineered a CV-B3 2C protein fused to  
104 a heterologous hexamerization domain. The chimeric protein recovered ATPase  
105 activity, allowing us to investigate the inhibitory effect of several antivirals targeting EV  
106 2C, each of which displayed a dose-dependent ATPase inhibition. Moreover,  
107 incubation of our engineered 2C protein with SFX or dibucaine demonstrated that  
108 these drugs stabilize the hexameric complex. This allowed us to capture the first three-  
109 dimensional structure of the 2C hexamer by cryo-EM and suggests an inhibitory

110 mechanism for these drugs. Taken together, these data provide new mechanistic  
111 insights into the mode-of-action of 2C targeting compounds and offer unique tools for  
112 the design and validation of 2C inhibitors.

## 113 **Results**

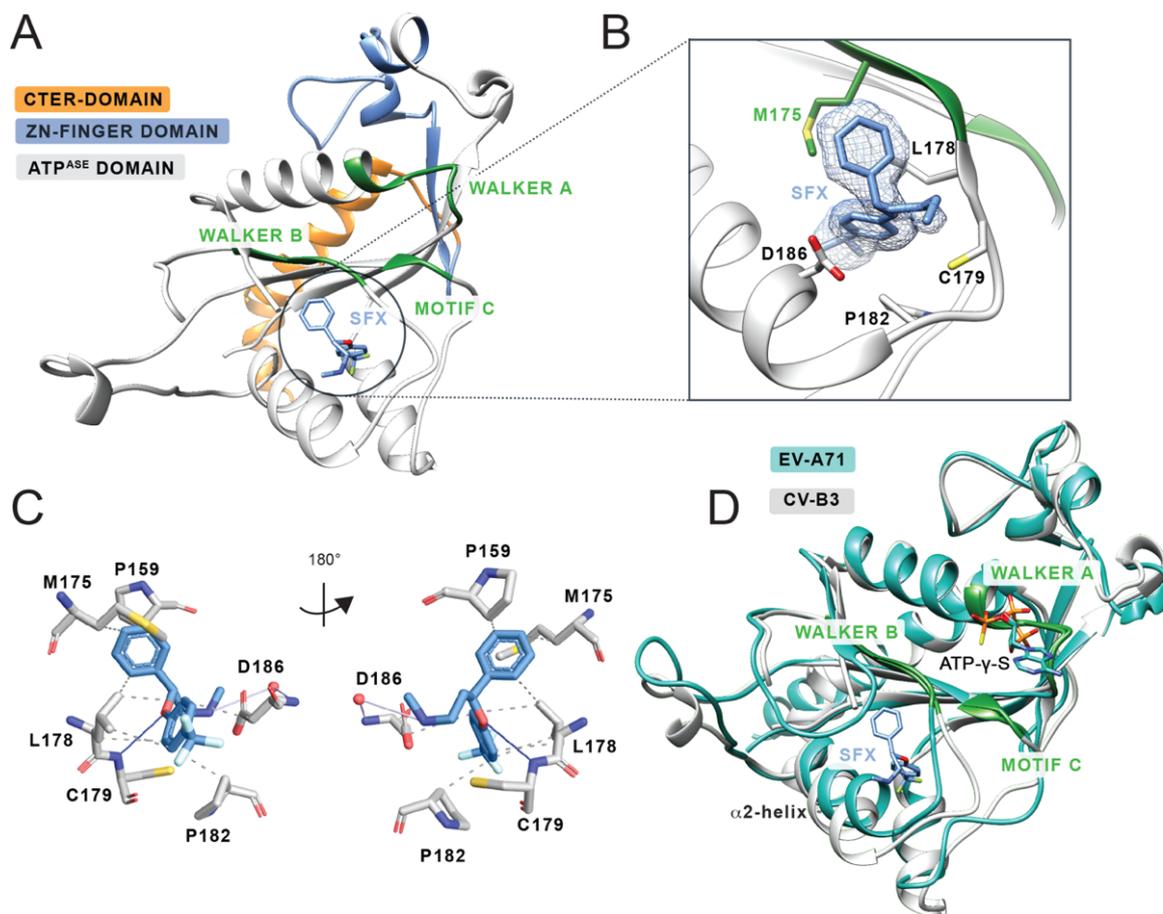
### 114 **Crystal structure of CV-B3 $\Delta$ 116-2C in complex with SFX**

115 Inspired by recent structural reports of 2C protein fragments from EV-A and EV-C  
116 species<sup>16,17</sup>, we attempted a similar truncation strategy to obtain crystals of a  
117 fluoxetine-sensitive EV-B species member, namely CV-B3. Thermal denaturation  
118 experiments showed that SFX increased the melting temperatures ( $T_m + 1.51^\circ\text{C}$ ) of  
119 the CV-B3  $\Delta$ 116-2C, confirming that this truncated protein can be bound by the drug  
120 (Figure S1). To understand how SFX binds to 2C, we attempted to crystallize our  
121 truncated 2C construct in the presence or absence of the drug. While we were able to  
122 obtain a high resolution (1.5 Å) structure of the complex (Table S1), no crystals were  
123 obtained for the apo 2C protein. This suggests that SFX stabilizes the 2C protein in a  
124 conformation prone to crystallization.

125 The overall fold of CV-B3  $\Delta$ 116-2C is similar to the analogous structure from EV-A71  
126 and poliovirus, comprising a Rossmann fold core domain, a zinc finger domain and a  
127 C-terminal helix (Figure 1A and S2). The Walker A motif is located on the loop  
128 connecting  $\beta$ 1 and  $\alpha$ 1, forming a putative phosphate binding loop (P-loop). The Walker  
129 B motif is found between  $\beta$ 3 and  $\alpha$ 2 while the Walker C motif, containing N223, is found  
130 at the tip of  $\beta$ 4<sup>30</sup>. Two large flexible loops located between strands  $\beta$ 2- $\beta$ 3 and  $\alpha$ 2- $\beta$ 4  
131 shield one side of the central sheet. Together, these loops form a hydrophobic cavity  
132 into which the SFX molecule binds (Figure 1A-B and S2). The interaction between SFX  
133 and the CV-B3 2C is mainly mediated by hydrophobic interactions involving the side  
134 chains of residues L157, P159, M175, D176, L178, C179, P182 and D186 (Figure 1B-  
135 C). The complex is also stabilized by a hydrogen bond between the amide group of the  
136 C179 main chain and the hydroxyl group of SFX. Finally, in one of our structures, SFX  
137 interacts via its amino group with the main chain of D186 (Figure 1C).

138 The accessibility of SFX can be associated with two main conformational differences  
139 between the structure of EV-A71 and CV-B3 2C proteins. The first is a  $10^\circ$  tilt of the  $\alpha$ 2  
140 helix (Figure 1D), enlarging the binding site of SFX by moving D186 away from the  
141 methylamine group of SFX. Together with this, the Walker B-carrying loop is positioned

142 away from the binding pocket in the CV-B3  $\Delta$ 116-2C, as illustrated by the 210° rotation  
143 of the side chain of Q180, making the cavity accessible to SFX. In addition, residues  
144 180-182, located downstream of the Walker B motif, participate in main chain hydrogen  
145 bonding with the 224-AGSINA-229 loop, located downstream of motif C (Figure 2A).  
146 This provides direct evidence for crosstalk between the SFX binding site and the  
147 AGSINA loop which is a known hotspot for compound resistance and dependence  
148 mutations<sup>18</sup>.



149

150 **Figure 1. Crystal structure of CV-B3  $\Delta$ 116-2C in complex with SFX.** (A) The C-  
151 terminal part of 2C is highlighted in orange. The Zinc-Finger domain is highlighted in  
152 blue, and the ATPase domain is highlighted in grey. Within the ATPase domain the  
153 catalytic center, comprising Walker A, Walker B and motif C, are highlighted in green.  
154 SFX is located close to the Walker B domain and is colored blue. (B) Zoomed in view  
155 of the SFX binding pocket showing the interacting residues and electron density for the  
156 bound drug. Green residues are part of the Walker B motif. (C) Protein-Ligand  
157 Interaction profile of SFX with the 2C residues. Hydrophobic interactions are shown as  
158 dashed lines (P159, M175, L178, P182 and D186). Hydrogen bonds are shown as blue  
159 lines (C179) and water mediated hydrogen bonds are colored light blue (D186). Figure  
160 generated using PLIP<sup>31</sup>. (D) Overlay of the 2C crystal structure of CV-B3 (grey) with  
161 the 2C crystal structure of EV-A71 (PDB: 5GRB, chain A) (blue) in complex with  
162 ATP- $\gamma$ -S.

### 163 **Mutational analysis of the SFX binding site.**

164 Sequence analysis of the SFX binding site revealed that all residues, except cysteine  
165 at position 179, are conserved in SFX resistant and sensitive EV species (Figures 2A  
166 and S3). To assess the functional consequences of alterations in the SFX binding  
167 residues, we performed mutagenesis of amino acids within, or adjacent to, the drug  
168 binding pocket. L157A, P158A, P159A, M175A, D176A, D176N, L178A, P182A,  
169 D186A and D186N were reverse engineered into a CV-B3 infectious cDNA clone  
170 containing a Renilla luciferase (CV-B3-Rluc) reporter gene. The replication efficiency  
171 of these mutants was then assessed in the presence or absence of 2C inhibitors.  
172 Nearly all substitutions abrogated viral RNA (vRNA) replication. Only P158A mutant,  
173 which is not in direct contact with SFX, was able to replicate. This mutation did not alter  
174 the sensitivity of 2C to SFX or GuaHCl (Figure 2B). We next tested the impact of  
175 introducing less stringent mutations into the SFX binding site. These mutations were  
176 selected using sequence information from other picornaviruses such as hepatitis A  
177 virus and Aichivirus (Figure S3). Surprisingly, even the introduction of chemically  
178 similar amino acids (i.e. L178I and D186E) resulted in no detectable vRNA replication  
179 (Figure S4). Consistently, upon introduction of mutations into a CV-B3 infectious clone  
180 without a reporter gene, no viable viruses were recovered, except for mutation P158A.  
181 Notably, after independent passages of cells transfected with the P159A mutant,  
182 viruses were recovered carrying a second-site compensatory mutation, namely A229V,  
183 which lies in proximity to Pro159. This substitution was previously shown to confer  
184 dependence to several compounds, including GuaHCl, HBB and MRL-1237<sup>18,20,22,32</sup>.  
185 Virus carrying the P159A/A229V double mutation showed a small decrease in  
186 sensitivity to SFX (Figure S5). Taken together, these data demonstrate that residues in  
187 the SFX binding pocket are highly mutationally constrained, presumably to preserve  
188 the function of 2C.

189

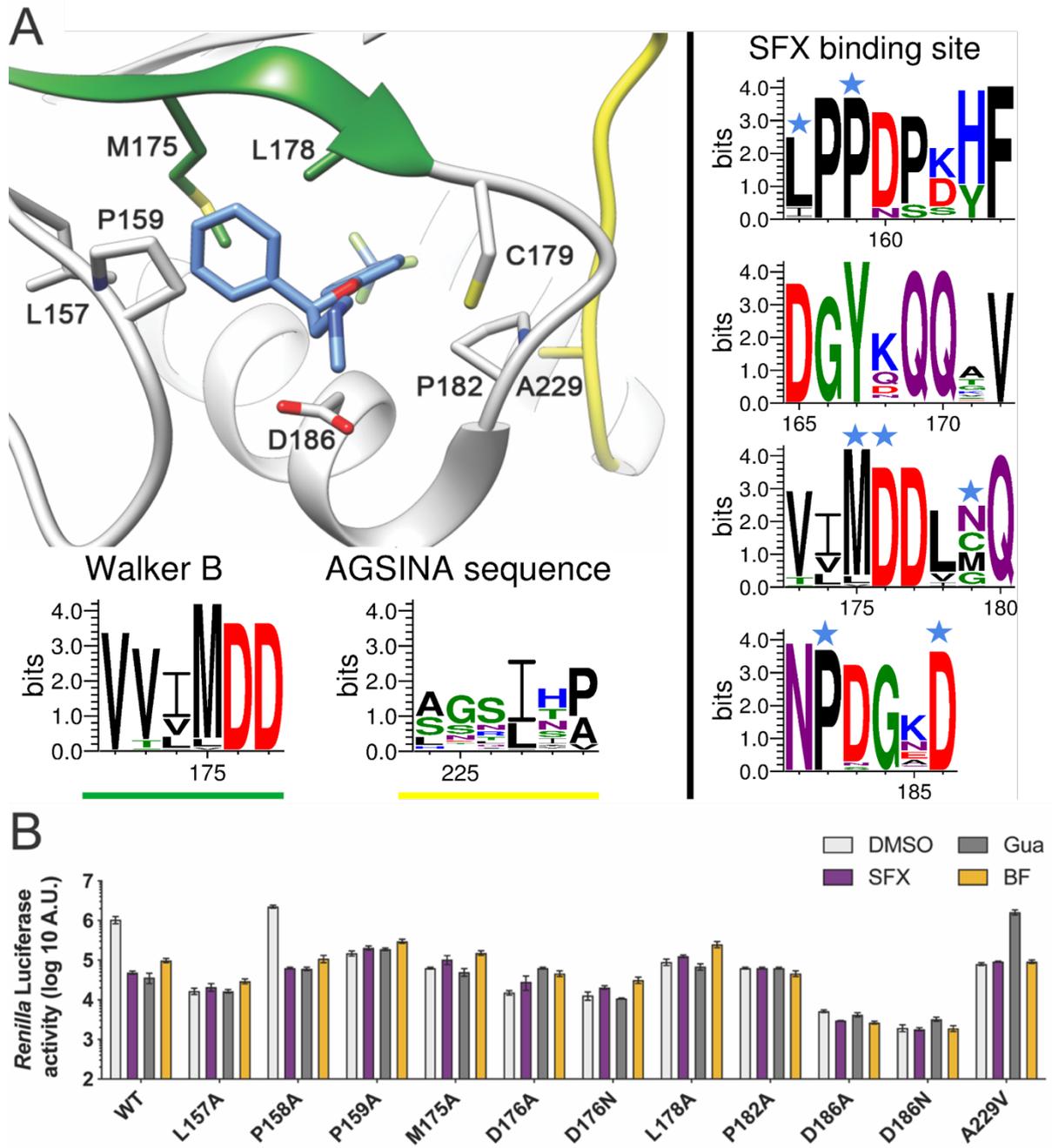
190 To gain more insight into the binding pocket of SFX, we raised resistant CV-B3 via a  
191 clonal selection procedure as described previously<sup>29</sup>. Several SFX-resistant viruses  
192 were obtained, containing single (I227V), double (I227V/A229V) or triple  
193 (A224V/I227V/A229V) mutations in the 224-AGSINA-229 loop (Figure S6). Mutations  
194 in this loop have been previously identified in CV-B3 resistant to 2C-targeting  
195 compounds<sup>18,33</sup>. In addition, we previously demonstrated that substitutions C179Y,  
196 C179F and F190L also provide resistance to SFX<sup>29</sup>. Notably, these latter mutations, as

197 well as 224-AGSINA-229 loop mutations, provide cross-resistance against dibucaine  
198 (Fig S7). Our crystal structure of CV-B3  $\Delta$ 116-2C in complex with SFX now allows us  
199 to cluster these mutations into two groups, those directly involved in SFX binding site  
200 (i.e. C179F, C179Y and F190L), and those in the 224-AGSINA-229 loop located  
201 downstream of the Walker C motif, which do not directly interact with SFX (Figure 2).  
202 The exact role of the 224-AGSINA-229 loop in conferring drug resistance, or  
203 dependence, is difficult to assess. However, its contribution to stabilization of the  
204 Walker B-containing loop in an “open conformation”, suitable for SFX binding,  
205 corroborates this motif as a credible hotspot for resistance mutations. To further  
206 demonstrate that this loop plays an important role in fluoxetine sensitivity, we  
207 transplanted the 224-AGSINA-229 loop from CV-B3 2C into the 2C protein of the EV-  
208 A71 BrCr strain, which contains 224-ASNIIV-229 and is insensitive to SFX, despite the  
209 SFX binding residues being conserved. Indeed, EV-A71 harboring the 224-AGSINA-  
210 229 loop gained sensitivity towards SFX, providing evidence that the 224-AGSINA-229  
211 loop contributes to drug sensitivity (Figure S8).

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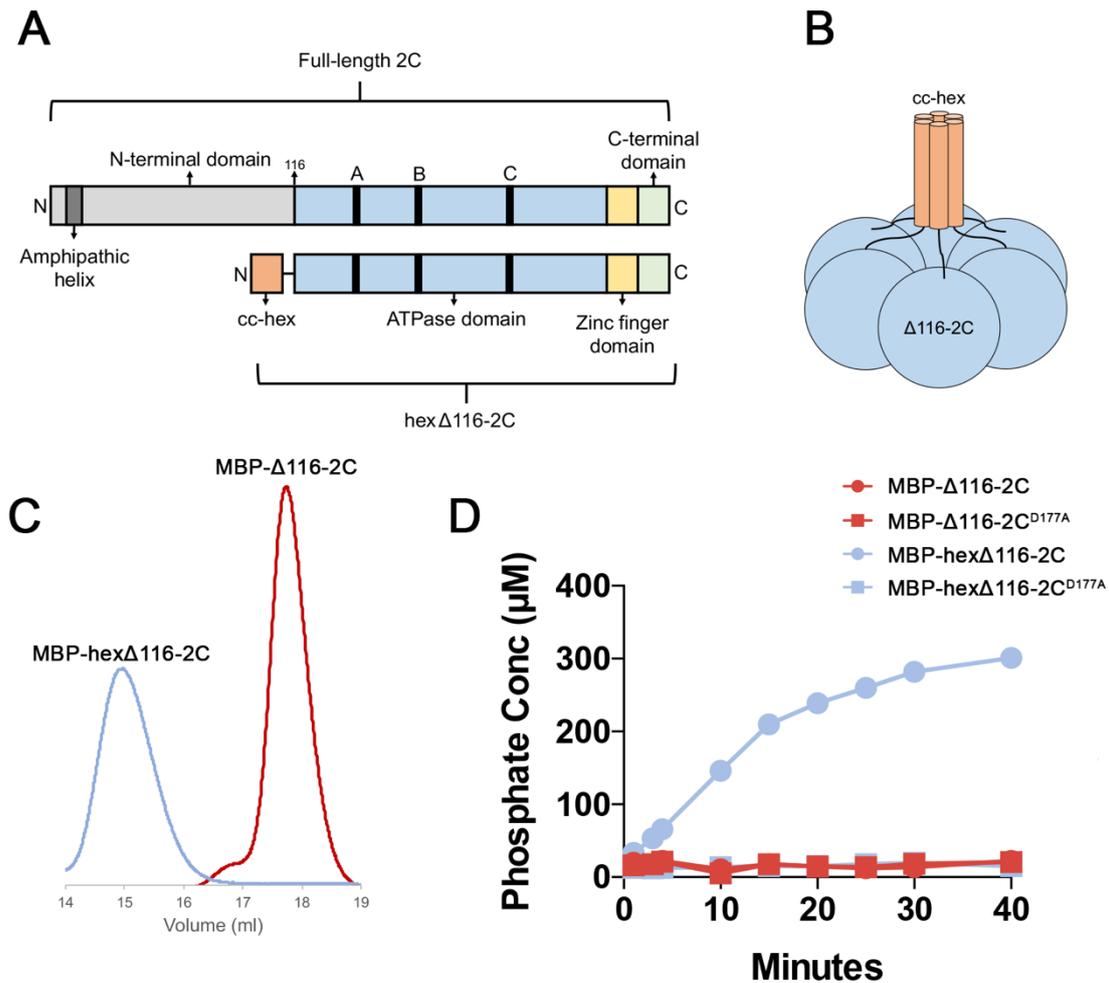
215

216 **Figure 2. Mutational analysis of the SFX binding site in CV-B3 2C.** (A) Schematic  
 217 structure of CV-B3 2C highlighting the residues involved in SFX binding. The Weblogo  
 218 represents the conservation of amino acids based on an alignment of the 2C protein  
 219 from EVs which cause disease in humans. Virus sequences which were used for the  
 220 alignment can be found in Supplement Part XYZ. The blue stars on top of the amino  
 221 acid residues indicate an interaction with the ligand SFX. (B) The residues which  
 222 interact with SFX were introduced into an infectious CV-B3 cDNA clone containing the  
 223 *Renilla* luciferase reporter gene upstream of the capsid coding region (Rluc-CV-B3). In  
 224 vitro transcribed RNA was transfected into cells and Renilla luciferase was used as a  
 225 sensitive and quantitative read out for viral RNA replication.

226

227 **Engineering an enzymatically active 2C hexamer to study the mechanism of**  
228 **action of SFX and other inhibitors.**

229 The 2C protein contains three canonical motifs required for nucleotide triphosphate  
230 binding and hydrolysis, Walker A, Walker B and motif C<sup>11</sup>. Previous experiments  
231 demonstrated that SFX has little effect on the ATPase activity of the truncated,  
232 monomeric 2C protein of CV-B3<sup>33</sup>. However, the optimal activity of SF3 helicases  
233 requires their ATPase domains to be arranged in a hexameric complex<sup>30,34,35</sup>.  
234 Therefore, studies of truncated 2C proteins should be complemented with biochemical  
235 analysis of the enzymatically active hexameric complex. It has been demonstrated  
236 previously that the N-terminal domain of 2C is important for oligomerization and  
237 ATPase activity, but also renders the protein poorly soluble<sup>13,14,19</sup>. With this in mind, we  
238 attempted to purify the MBP-tagged full-length CV-B3 2C, which resulted in a  
239 heterogenous protein preparation as shown by the size exclusion chromatography gel  
240 filtration profile. This indicated that a mixture of high molecular weight 2C complexes  
241 were present, including a putative hexamer peak (Figure S9). As an alternative  
242 strategy, we sought to uncouple CV-B3 2C from the unfavorable biochemical properties  
243 of the N-terminal amphipathic helix but retain the ability to form hexamers. Fusion of N  
244 or C-terminal ‘assistant hexamer’ proteins is an established method to study AAA+  
245 ATPases<sup>36–38</sup>. Based on the proposed hexamer model described for EV-A71 and PV  
246 2C<sup>16,17</sup>, we hypothesized that we could fuse a hexameric parallel coiled-coil, CC-hex-  
247 D24<sup>39</sup>, to the N-terminus of CV-B3  $\Delta$ 116-2C via a 10 amino acid linker sequence  
248 (Figure 3A-B). This would, in principle, enhance the local concentration of the 2C  
249 protein and correctly orientate the molecules in a manner reminiscent of membrane  
250 attachment, normally facilitated by the N-terminal amphipathic helix<sup>40</sup>. The small size  
251 of the CC-hex coiled-coil (32 amino acids) also permits the additional attachment of a  
252 cleavable His-MBP tag to promote solubility and facilitate affinity purification of the  
253 protein (Figure S10). As expected, the hex $\Delta$ 116-2C elutes as a single peak which  
254 corresponds to a hexamer whereas  $\Delta$ 116-2C is exclusively monomeric (Figure 3C).  
255 We next employed the malachite green assay to assess the ATPase activity of our  
256 hex $\Delta$ 116-2C, which demonstrated that the engineered protein is enzymatically active.  
257 In contrast, the monomeric protein and catalytic dead D177A mutant showed little to  
258 no ATPase activity (Figure 3D and S11). Altogether, the data show that the  
259 hexamerization is necessary to promote the 2C-dependent ATPase activity, offering a  
260 technological platform to screen or validate 2C ATPase inhibitors.



261

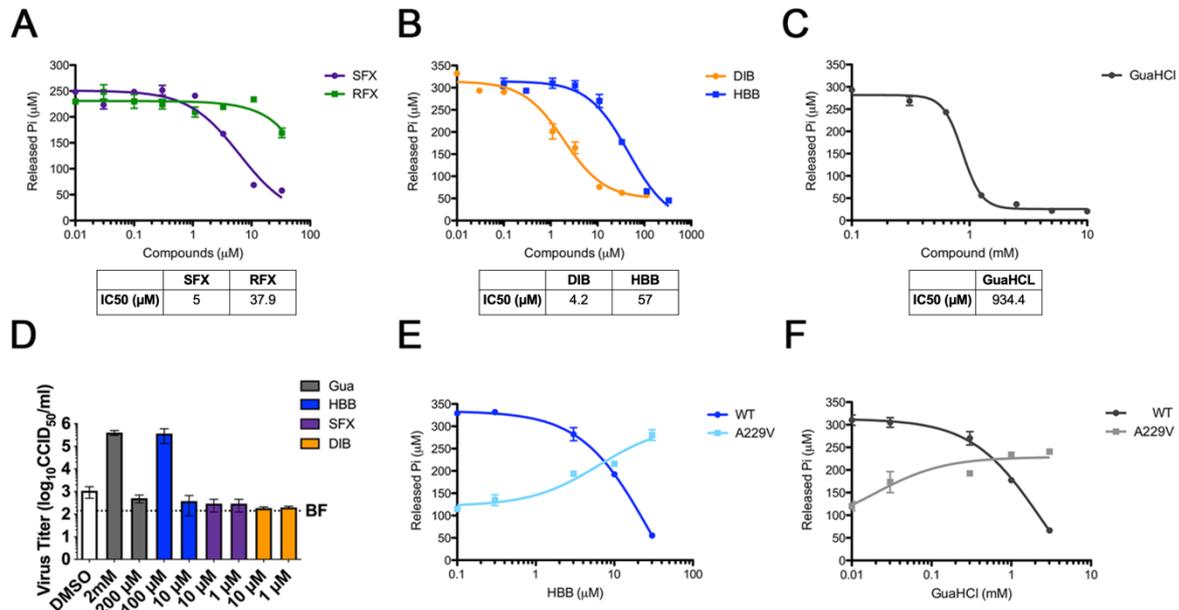
262 **Figure 3. Engineering a soluble, enzymatically active 2C hexamer.** (A) Linear  
 263 representation of the full-length CV-B3 2C protein (top) and the engineered hexΔ116-  
 264 2C construct (bottom). The full-length 2C protein comprises an N-terminal domain (light  
 265 grey), ATPase domain (blue), Walker A, B and C motifs (black), zinc finger domain  
 266 (yellow) and C-terminal extension (green). In hexΔ116-2C (residues 116-329), the N-  
 267 terminal domain, which contains the amphipathic helix (dark grey), is replaced by the  
 268 32-residue cc-hex coiled coil sequence (orange). (B) Schematic representation of the  
 269 hexΔ116-2C construct. (C) Migration profile of the MBP tagged monomeric Δ116-2C  
 270 and hexΔ116-2C by size-exclusion chromatography. (D) Comparison of the ATPase  
 271 activity for the WT hexΔ116-2C, the hexΔ116-2C D177A mutant, WT Δ116-2C and  
 272 Δ116-2C D177A mutant.

273

274 Next, we investigated the effect of the fluoxetine enantiomers - SFX and RFX - on 2C-  
 275 dependent ATP hydrolysis (Figure 4A). We showed that SFX inhibits ATPase activity  
 276 in a dose dependent manner with an EC50 value of ~5 μM, in the same concentration  
 277 range as the dissociation equilibrium constant (Kd of 9.5 μM) obtained in a binding  
 278 assay<sup>26</sup>. Consistent with the binding assay, RFX was markedly less efficient at

279 inhibiting ATPase activity. We also tested the inhibitory effect of dibucaine, HBB and  
 280 GuaHCl, known or suspected to target the 2C protein<sup>19,21,24</sup>. All of the compounds  
 281 reduced the ATPase activity of 2C in a dose-dependent manner, underscoring the link  
 282 between inhibition of ATP hydrolysis and antiviral effect (Figure 4B-C).

283



284

285 **Figure 4. 2C inhibitors reduce the ATPase activity of 2C and have antiviral effect.**  
 286 (A-C) Effects of SFX and RFX (A), dibucaine and [2-(alpha-hydroxybenzyl)-  
 287 benzimidazole] (HBB) (B), or guanidium hydrochloride (GuaHCl) (C) on 2C-dependent  
 288 ATPase activity. (D) Replication of CV-B3 containing the A229V 2C mutation in the  
 289 presence of GuaHCL, HBB, SFX or DIB (or a non-related replication inhibitor, BF,  
 290 which targets a host lipid kinase). At 8 hours post-infection, cells were freeze-thawed  
 291 and virus titers of lysates were determined. (E, F) Comparison of ATPase activity of  
 292 WT 2C and the A229V mutant in the presence of HBB (E) and GuaHCL (F).

293

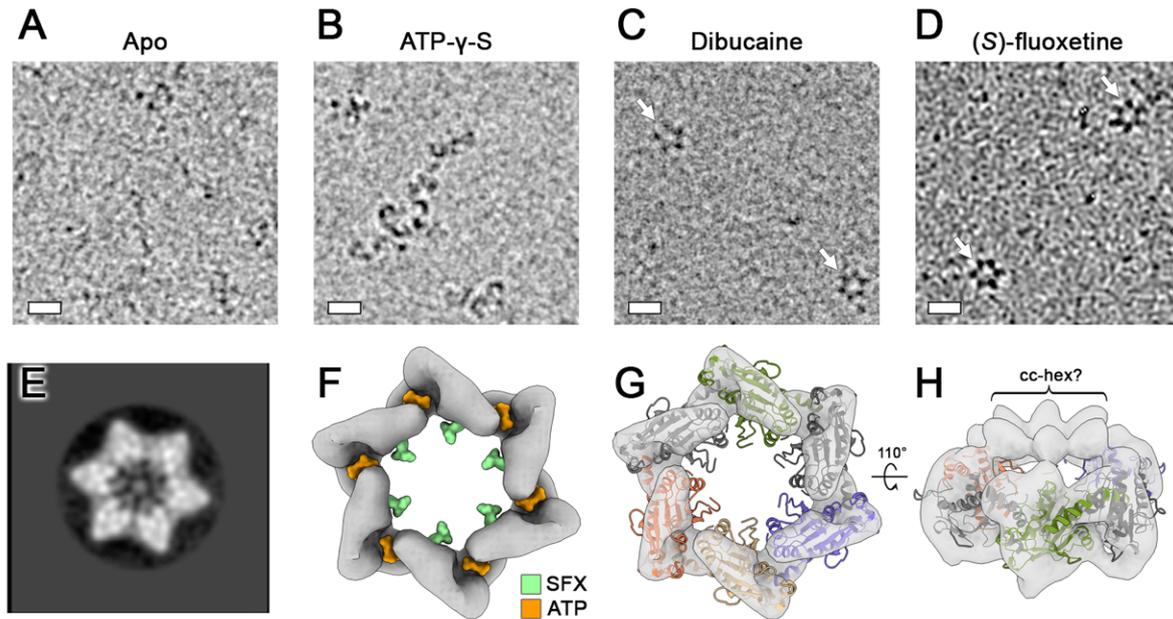
294 It is known that besides drug-resistance, 2C targeting compounds can induce drug-  
 295 dependence mutations. In the latter scenario, the presence of the compound is  
 296 required for efficient enterovirus replication<sup>18,20,32,35</sup>. The 2C mutant, A229V, has been  
 297 shown to confer dependence on GuaHCl for efficient CV-B3 replication<sup>18</sup>, but the  
 298 underlying mechanism is unknown. Upon testing replication of CV-B3 carrying the  
 299 A229V 2C mutation, we observed efficient replication in the presence of GuaHCl and  
 300 HBB, but not SFX and dibucaine (Figure 4D). When the A229V mutant was introduced  
 301 into our hex $\Delta$ 116-2C construct, we observed reduced specific ATPase activity  
 302 compared to the WT protein (Figure 4E). Remarkably, the level of ATPase activity

303 observed with the WT 2C protein is fully and partly recovered in the presence of HBB  
304 and GuaHCl, respectively (Figure 4E-F). These data reveal that the A229V mutation  
305 negatively impacts the ATPase activity of 2C and that GuaHCl and HBB can restore  
306 the enzymatic activity.

307

### 308 **SFX and dibucaine stabilize the 2C hexamer**

309 Having demonstrated that SFX and dibucaine can inhibit the 2C-dependent ATPase  
310 activity, we next wanted to determine how these compounds affect the quaternary  
311 structure of 2C. Cryo-electron microscopy analysis of our untagged engineered protein  
312 (Figure S12), in the absence of any nucleotide or inhibitor, did not reveal any distinct  
313 oligomeric structures (Figure 4A). When the non-hydrolysable ATP analogue,  
314 ATP- $\gamma$ -S, was added, 'hook-shaped' oligomers were apparent (Figure 4B). When  
315 hex $\Delta$ 116-2C was incubated with SFX or dibucaine, well-defined hexamers were  
316 discernable (Figure 4C-D). Single-particle analysis of SFX bound hexamers produced  
317 distinct star-shaped 2D class averages, some of which contained an electron dense  
318 feature in the central channel (Figure 4E). Efforts to determine the cryo-EM structure  
319 of the SFX-bound 2C hexamer were hampered by strong preferred orientation, low  
320 abundance, and instability of the particles. Nevertheless, a  $\sim$ 12 Å resolution  
321 reconstruction of the complex was determined by employing a 30° stage tilt during data  
322 collection (Figure 4F and S13-14). The cryo-EM map corresponds to  $\sim$ 140 kDa of  
323 ordered molecular weight and has a diameter of  $\sim$ 110 Å, consistent with the dimensions  
324 of the EV-A71 2C hexamer model proposed previously<sup>16</sup>. Moreover, a cleft is present  
325 between the 2C protomers, which correlates with the expected location of the ATP  
326 binding pocket (Figure 4G). We next generated a hexameric model of the SFX-bound  
327 CV-B3 2C, based on the crystal structure of the JC polyomavirus large T antigen<sup>41</sup>, as  
328 described by Guan et al<sup>16</sup>, and fitted this as a rigid-body into our cryo-EM map. The  
329 overall agreement between the cryo-EM density and the hexameric model of CV-B3  
330 2C supports the proposed mechanism of C-terminus mediated oligomerization.  
331 Indeed, in our crystal structures, the C-terminal helix ( $\alpha$ 6,  $\alpha$ 7) which appear to be  
332 broken in a "door knob" fashion into two helices  $\alpha$ 6 and  $\alpha$ 7, due to crystal packing, and  
333  $\alpha$ 7 helix is in intensive contact with the Zinc binding domain of a symmetrical chain  
334 (Figure S15).



335

336 **Figure 5. SFX and dibucaine stabilize the 2C hexamer.** (A) Cryo-electron  
337 micrographs of apo, (B) ATP-γ-S incubated, (C) dibucaine incubated or (D) SFX  
338 incubated hexΔ116-2C (scale bar = 10 nm). (E) Representative 2D class average from  
339 untilted micrographs of SFX incubated hexΔ116-2C. Weak density can be observed  
340 within the central of the 2C hexamer. (F) Surface representation of the 12 Å resolution  
341 cryo-EM map of hexΔ116-2C in complex with SFX, generated from 30° tilted cryo-EM  
342 data. The map is shown at a contour level of 0.0661 and the binding positions of SFX  
343 (green) and ATP (orange) are superposed. (G) As shown in (F) with a hexameric 2C  
344 model, generated using the crystal structure of the monomer, overlaid. For clarity, pore-  
345 loop residues 85-100 are omitted. (H) A 110° rotated view of the model shown in (G),  
346 with the map shown at a contour level of 0.059. Putative density for the linker and cc-  
347 hex is indicated.

348

349 Taken together, these results indicate that SFX or dibucaine binding locks 2C in a  
350 hexameric state which does not permit ATP hydrolysis and, by extension, the  
351 associated functions such as helicase activity. Consistent with our cryo-EM  
352 observations, when size exclusion chromatography is performed on hexΔ116-2C in the  
353 presence of SFX, the complex exhibits an increased retention time, indicative of a  
354 reduced hydrodynamic radius (Figure S16A). In contrast, the presence of SFX did not  
355 greatly alter the elution profile of the monomeric Δ116-2C protein, except for a slightly  
356 more pronounced left shoulder peak, indicative of low-level oligomerization (Figure  
357 S16B). Therefore, it is likely that the SFX-mediated stabilization of the hexamer  
358 requires not only a high local concentration of protein, but also their correct orientation  
359 with respect to one another.

360

## 361 Discussion

362 Enteroviruses are globally prevalent pathogens responsible for many diseases. There  
363 is an unmet need for broad-spectrum therapeutics to treat EV infections. The EV 2C  
364 protein is an attractive target for direct-acting antivirals, given that it is a highly  
365 conserved and functionally indispensable protein, undertaking several pleiotropic  
366 functions during the viral lifecycle including membrane rearrangement, RNA unwinding  
367 and genome encapsidation<sup>10</sup>. Over the last decades, several structurally disparate  
368 compounds targeting 2C, such as the FDA-approved drugs fluoxetine and dibucaine,  
369 amongst others, were identified<sup>23,24</sup>. Subsequently, a number of resistance mutations  
370 were obtained to gain insights into their mode of action<sup>33</sup>. However, the binding sites  
371 and the mode of action remained elusive, hampering the rational-based improvement  
372 of the compounds. It has often been hypothesized that 2C inhibitors bind directly in the  
373 catalytic site. One study suggested that dibucaine analogues target the ATP binding  
374 site<sup>42</sup>, but no direct evidence supporting this hypothesis was presented. Recently, we  
375 obtained evidence for an allosteric binding site based on CV-B3, EV-A71 and EV-D68  
376 mutants that were raised against a novel and highly potent 2C inhibitor<sup>29</sup>. Viruses  
377 resistant to this inhibitor contained mutations in, or adjacent to, the  $\alpha$ 2 helix, which is  
378 distal to the catalytic site. Here, we provide a high-resolution crystal structure of the  
379 CV-B3 2C protein in complex with SFX. This crystal structure revealed a hydrophobic  
380 binding pocket that accommodated the ligand between the  $\alpha$ 2 helix and the Walker B  
381 domain. Our data prove that SFX indeed targets an allosteric site on 2C that is clearly  
382 distinct from the catalytic site. The obtained structure not only provides valuable  
383 insights into the mode of action of SFX but also represents a novel tool for structure-  
384 based drug design of highly potent 2C inhibitors.

385 Several structural features are associated with the accessibility of SFX to bind the 2C  
386 protein of CV-B3 but not other enteroviruses. One structural key determinant allowing  
387 SFX to access the binding pocket is the 224-AGSINA-229 loop which is also a hot spot  
388 of resistance mutations. This is supported by our finding that exchange of the 224-  
389 ASNIIV-229 loop of the SFX insensitive EV-A71 BrCr with the 224-AGSINA-229 loop  
390 implemented a SFX sensitive phenotype to the resulting chimeric EV-A71 virus.  
391 Clearly, the 224-229 loop is not the only determinant for SFX sensitivity since we  
392 discovered previously that several clinical isolates of EV-A71 without changes in the  
393 224-ASNIIV-229 are sensitive to SFX. Unfortunately, and despite extensive efforts, an

394 apo-structure of CV-B3 is not available to elucidate the structural changes which occur  
395 upon SFX binding. Comparison of the SFX-bound CV-B3 2C to that of EV-A71 (apo  
396 and ATP- $\gamma$ -S-bound) and PV (apo) shows that the  $\alpha$ 2-helix is tilted 10° away from the  
397 SFX binding site in CV-B3 2C, which creates the solvent accessible volume required  
398 for drug binding. Similarly, the Walker B loop of CV-B3 2C is positioned further away  
399 from the SFX binding site compared to EV-A71 and PV 2C, which further contributes  
400 to formation of the hydrophobic pocket required to accommodate SFX. This suggests  
401 that, in solution, 2C exhibits a high degree of conformational plasticity and that SFX  
402 bind to and stabilizes, an “open conformation” of 2C. Ultimately, an apo structure of  
403 CV-B3 2C is required to confirm this hypothesis.

404 The crystal structure of the 2C monomer in complex with SFX is not sufficient to  
405 decipher its mode of action as this should take into account the expected hexameric  
406 nature of AAA+ proteins. Several lines of evidence suggest that the catalytically active  
407 form of 2C is composed of higher oligomeric structures<sup>12,13,35</sup>. Production of 2C without  
408 its N-terminal helical domain yields monomeric protein that has little, if any, ATPase  
409 activity<sup>33</sup>. To overcome the biochemical limitations of 2C and to study the mode of  
410 action of 2C-targeting antivirals, we engineered an ATP hydrolyzing, hexameric form  
411 of the 2C ATPase domain. The resulting construct produced homogenous oligomers  
412 that displayed robust ATPase activity compared to the inactive monomeric form of 2C  
413 (Figure 3). Using the engineered protein, we demonstrate that the FDA approved drugs  
414 fluoxetine, dibucaine as well as GuaHCl and HBB inhibit the ATPase activity in a dose-  
415 dependent manner. Thus, our hexameric 2C construct represents a novel tool for *in*  
416 *vitro* screening of 2C-targeting ATPase inhibitors. This tool might be of particular  
417 interest to identify potential 2C inhibitors for difficult-to-culture enteroviruses such as  
418 the RV-C species members<sup>43</sup>. Besides this, this platform can provide mechanistic  
419 insights into the mode of action of specific mutations, as shown here for the drug-  
420 dependent A229V mutation which we revealed to impair ATPase activity in the  
421 absence of inhibitors, but to stimulate ATPase activity in the presence of GuaHCl and  
422 HBB.

423 A hexameric structure of 2C has been long sought-after in the picornavirus field but  
424 has remained elusive due to the unfavorable biochemical properties of the full-length  
425 protein, especially its amphipathic N-terminal  $\alpha$ -helix. Therefore, cryo-EM analysis of  
426 CV-B3 2C protein, incubated with SFX, provides the first 3D reconstruction of a

427 hexameric complex, and represents a significant step towards understanding this  
428 enigmatic protein. Although artificially generated by the addition of an hexamerization  
429 domain, the recovery of the ATPase activity and the binding of SFX unambiguously  
430 confirm the relevance of the construct. Previous studies have shown that the 2C protein  
431 of foot-and-mouth disease virus (FMDV), a member of the *Aphthovirus* genus within  
432 the picornaviridae family, employs a coordinated ATP hydrolysis mechanism. Using  
433 negative stain analysis, the same study demonstrated that FMDV 2C(34-318),  
434 containing the motif C mutation N207A (which interferes with ATP hydrolysis but not  
435 binding), could form hexamers in the presence of ATP and RNA<sup>14</sup>. It is likely that  
436 enterovirus 2C proteins must undergo conformational changes during ATP hydrolysis,  
437 RNA binding and unwinding, to drive their helicase function. Our biochemical and cryo-  
438 EM analysis suggests that SFX and dibucaine binding prevents propagation of these  
439 conformational changes by locking 2C in a defined hexameric state. A similar  
440 mechanism was shown for a small molecule inhibitor of the human AAA+ ATPase,  
441 p97<sup>44</sup>. The allosteric p97 inhibitor binds at the interface of two adjacent AAA domains  
442 and prevents the conformational changes that are required for ATPase activity of p97.  
443 As SFX does not bind to a 2C-2C interface, it's likely that drug binding induces  
444 conformational changes within the monomer that translate to stabilization of the  
445 hexamer. As shown for several AAA+ ATPases, cryo-EM provides insights that are not  
446 available from crystallographic analysis alone<sup>44-46</sup>. In the case of 2C, the observed  
447 stabilizing effect of SFX, observed in solution, complements the high-resolution  
448 information obtained by crystallography and provides insights into the allosteric  
449 inhibitory mechanism of 2C targeting compounds. Our findings emphasize the  
450 importance of using an integrative structural biology approach to fully understand the  
451 function and mechanisms of inhibition of 2C. Previous studies have shown that purified,  
452 full-length poliovirus 2C cannot be uncoupled from its solubility tag, and contains a  
453 mixture of oligomeric species – both of which would hamper structural analysis<sup>13</sup>.  
454 Similarly, an N-terminally extended 2C protein of echovirus 30 was shown to form  
455 heterogenous ring-shaped structures<sup>15</sup>. Here we demonstrate that replacement of the  
456 N-terminal region of 2C with a hexamerization domain can produce a homogenous  
457 complex amenable to cryo-EM. Going forward, our engineering strategy should  
458 facilitate further structural and functional studies of oligomeric 2C proteins, and their  
459 interaction with inhibitors, viral RNA, and other viral/host factors.

## 460 **Material and methods**

### 461 **Cells and reagents**

462 Buffalo Green Monkey (BGM) cells (purchased from European Cell Culture Collection  
463 [ECACC]) and HeLa R19 cells (American Type Culture Collection [ATCC]) were  
464 cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Switzerland)  
465 supplemented with 10% (vol/vol) fetal calf serum (FCS; Lonza). All cell lines were  
466 grown at 37 °C in 5% CO<sub>2</sub>. Medium was refreshed every 2–4 days, and cells were  
467 passaged at >80% confluence with the use of PBS and trypsin-EDTA (0.05%) for up  
468 to 10 passages. The cell lines were routinely tested for mycoplasma contamination.  
469 SFX, RFX, GuaHCl and dibucaine were purchased from Sigma Aldrich. HBB was  
470 provided by Prof. Andrea Brancale (Cardiff University). GuaHCl and ATP- $\gamma$ -S ((Sigma  
471 Aldrich) were dissolved in water at 2 M and 20 mM stock concentration, respectively.  
472 All other compounds were dissolved in DMSO at 10 mM stock concentration.

### 473 **Cloning and construct design**

474 For crystallography experiments, the coding sequence of CV-B3 2C (amino acids 117  
475 to 329) was cloned into the expression vector pmCox 20A as previously described<sup>47</sup>.  
476 For ATPase assays and cryo-EM experiments, the coding sequence of CV-B3 2C  
477 (amino acids 116 to 329) was inserted into pET-28b plasmid. The hex $\Delta$ 116-2C  
478 construct was produced by inserting a 32-residue codon optimized cc-hex coding  
479 sequence followed by a linker (resulting peptide:  
480 GELKAI AQELKAI AKELKAI AWELKAI AQGAG; linker: GSGSYFQSNA Genscript) at  
481 the 5' of the CV-B3 2C coding sequence. For both constructs, a 3C protease cleavable  
482 N-terminal His<sub>6</sub>-MBP tag was included to facilitate expression and purification. An  
483 overview of the hex $\Delta$ 116-2C construct is shown in Figure S10. The D177A and A229V  
484 variants were produced by site directed mutagenesis using the  $\Delta$ 116-2C and hex $\Delta$ 116-  
485 2C plasmids as a template.

### 486 **Protein expression and purification**

487 The CV-B3  $\Delta$ 116-2C protein used for crystallogenesis was produced in *Escherichia*  
488 *coli* T7 lq Express pLysS (New England BioLabs). Cells were grown in TB medium  
489 containing 100  $\mu$ g/mL of ampicillin and 34  $\mu$ g/mL chloramphenicol at 37°C. Expression  
490 was induced overnight at 17°C with 0.5 mM isopropyl -thiogalactopyranoside (IPTG).  
491 The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM

492 Tris, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 0.1% Triton) complemented with  
493 0.25 mg/mL lysozyme, 10 µg/mL DNase and 1 mM of PMSF. The bacterial sample  
494 was sonicated and centrifuged at 18000 g for 30 min 4°C. Soluble CV-B3 Δ116-2C  
495 was purified from the supernatant by immobilized metal-affinity chromatography  
496 (IMAC) on a HisTrap column (GE Healthcare) and eluted with a buffer consisting of 50  
497 mM Tris, 300 mM NaCl, 500 mM imidazole pH 8. The eluted protein was subject to a  
498 dialysis in 50 mM Tris pH 8, 400 mM NaCl, 10 mM Imidazole, 1 mM DTT (dithiothreitol),  
499 followed by an overnight cleavage by TEV protease (molar ratio: 1/20), followed by a  
500 second IMAC to remove the tag and uncleaved protein. The recombinant protein was  
501 finally purified by size-exclusion chromatography (SEC) on a HiLoad 16/60 Superdex  
502 75 column (GE Healthcare) in 10 mM Hepes pH 7.5 and 300 mM NaCl.

503

504 For electron microscopy and functional assays, *Escherichia coli* Rosetta 2 (DE3) Cells  
505 (Sigma-Aldrich) were transformed with the plasmids coding for the WT, D177A and  
506 A229V CV-B3 2C proteins and grown in 2xYT medium containing 50 µg/mL of  
507 kanamycin and 30 µg/mL chloramphenicol at 37°C until the OD<sub>600nm</sub> reach 0.3. The  
508 temperature was then reduced to 18°C and when the OD<sub>600nm</sub> reached 0.5, protein  
509 expression was induced with 0.5 mM isopropyl -thiogalactopyranoside (IPTG).  
510 Following expression for 16 hours, the cells were harvested by centrifugation and  
511 resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 5% glycerol,  
512 0.1% Triton, 1 mM MgCl<sub>2</sub>) complemented with 0.25 mg/mL lysozyme, 10 µg/mL DNase  
513 I and 1 EDTA-free protease inhibitor cocktail. The bacterial sample was sonicated and  
514 centrifuged at 25000 g for 45 min at 4°C. The supernatant was passed through a 0.45  
515 µm filter and incubated with 1 mL of Ni-NTA resin at 4°C for 1 hour on a roller. The  
516 beads were washed with wash I buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole,  
517 1 mM MgCl<sub>2</sub>) and wash II buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM  
518 MgCl<sub>2</sub>) and then eluted with a buffer consisting of 50 mM Tris, 300 mM NaCl, 500 mM  
519 imidazole pH 8. For ATPase assays, the eluted proteins were concentrated to ~200 µL  
520 and purified by size-exclusion chromatography on a Superose 6 10/300 GL column  
521 (GE Healthcare) in 10 mM Hepes pH 7.5, 150 mM NaCl and 10 mM MgCl<sub>2</sub>. The protein-  
522 containing fractions were pooled, concentrated to ~4 mg/mL and flash-frozen in 20 µL  
523 aliquots. To remove the N-terminal His<sub>6</sub>-MBP tag for cryo-EM analysis, the Ni-NTA  
524 eluted hexΔ116-2C protein was concentrated to 200 µL, combined with 2 µg of HRV-  
525 3C protease (Sigma) and dialyzed overnight in buffer containing 50 mM Tris (pH 8),

526 200 mM NaCl and 0.5 mM DTT. The cleaved protein was finally purified by size-  
527 exclusion chromatography (SEC) on a Superose 6 10/300 GL column (GE Healthcare)  
528 in 25 mM Tris (pH 8), 300 mM NaCl, 1 mM MgCl<sub>2</sub>. The fractions corresponding to  
529 hexΔ116-2C were pooled, concentrated to ~4 mg/mL and flash-frozen in 10 μL  
530 aliquots.

531

### 532 **Crystallogenes, data collection and structure determination**

533 Structure Screens 1 and 2 and Stura Footprint Screen (Molecular Dimensions Ltd)  
534 were used for initial screening. Trials were assessed in SWISSCI 3 Lens Crystallization  
535 Plates with three wells per reservoir using 400, 300 and 200 nL drops. The drops  
536 contained increasing volumes (100, 200 and 300 nL) of protein solution at a  
537 concentration of 8-9 mg/mL in complex with or without 2.5 mM SFX (from 50 mM stock  
538 in 100% DMSO) and 100 nL mother liquor. Crystal hits were observed only in presence  
539 of SFX, in 0.1M MES pH 6.5, Polyethylene glycol (PEG) 20K 12%. Optimization of  
540 crystal-growth conditions led to the following condition: 0.1M MES pH 5.7 – 6.7, PEG  
541 20K 7.5/15 %. Crystals appeared after 24 h. They were soaked in 25% (v/v) PEG 200  
542 added to the mother liquor and cooled in liquid nitrogen. X-ray diffraction data were  
543 collected at European Synchrotron Radiation Facility, Grenoble (ESRF). The structure  
544 was solved by molecular replacement using Phaser with PV-2C-ΔN-3Mut structure  
545 (PDB code: 5Z3Q, D chain) as the searching model and was refined to a resolution of  
546 1.4 Å. The crystal belongs to a space group of P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, with 1 copy of CV-B3 Δ116-2C  
547 in the asymmetric unit (ASU). In addition, crystals were also obtained in another  
548 crystallization solution containing 0.1M MES pH 6.3 – 6.7, PEG 5 000 MME 23/33 %,  
549 0,2M ammonium sulphate. Crystals in mother liquor were soaked with 0.3 μL of 20 mM  
550 ATP overnight at 20°C. The datasets were collected at Proxima-2A-Synchrotron  
551 SOLEIL. The structure was solved by molecular replacement using Phaser with the  
552 structure of CV-B3 Δ116-2C in complex with SFX as a search model and refined to a  
553 resolution of 1.5 Å. All of the data sets were processed using XDS<sup>48</sup> and scaled  
554 with SCALA (Collaborative Computational Project, Number 4, 1994). The model  
555 building was performed in Coot<sup>49</sup>, and the structures were refined using phenix.refine<sup>50</sup>.  
556 The statistics of data collection, structure refinement and structure validation were  
557 summarized in Table S1. Figures of CV-B3 Δ116-2C structures and structural  
558 alignments were drawn by Chimera<sup>51</sup>.

559

## 560 **Protein stability assay**

561 To assess the folding of CV-B3  $\Delta$ 116-2C and its ability to bind SFX, the thermal stability  
562 of recombinant CV-B3  $\Delta$ 116-2C in presence of SFX was monitored by fluorescence-  
563 based thermal shift assay (TSA) using a Bio-Rad CFX Connect. TSA plates were  
564 prepared by dispensing into each well the 2C protein (final concentration of 15  $\mu$ M in 10  
565 mM Hepes pH 7.5 and 300 mM NaCl) which was mixed with 1  $\mu$ L of SFX (from 20 mM  
566 stock in 100% DMSO, 1 mM final concentration in 4% DMSO) and a SYPRO orange  
567 solution in concentrations recommended by the manufacturer in a final volume of  
568 25  $\mu$ L.

## 569 **Reverse engineering of SFX interaction residues**

570 The CV-B3 mutations in 2C L157A, P158A, P159A, M175A, M175I, M175G, D176A,  
571 D176N, L178A, L178I, P182A, D186A, D186E, D186N were first introduced into the  
572 Rluc-CV-B3 reporter viruses with Q5 site-directed mutagenesis kit (New England  
573 Biolabs, Bioké, Leiden, The Netherlands)<sup>52,53</sup>. The Rluc-CV-B3 reporter viruses contain  
574 a *Renilla* luciferase gene upstream of the capsid coding region which allows a fast and  
575 quantitative read-out for virus replication. Next, a 705 bp fragment containing the  
576 desired mutation was isolated using the enzymes BssHII and XbaI and reintroduced  
577 into the original non-mutagenized Rluc-CV-B3 backbone. After site-directed  
578 mutagenesis the newly generated plasmids were subjected to Sanger sequencing to  
579 confirm the existence of the 2C mutation. The corresponding primers which were used  
580 for site-directed mutagenesis can be found in Table S3. Viral RNA was transcribed *in*  
581 *vitro* using the T7 RiboMAX Express Large Scale RNA production system (Promega,  
582 Leiden, The Netherlands) according to the manufacturer's protocol. In a 96-well, HeLa  
583 R19 cells were transfected with 7,5 ng of viral RNA. One hour after transfection, the  
584 medium was replaced by fresh medium and/or compound containing medium. After 8  
585 hours, cells were lysed and luciferase activity was determined using the Renilla  
586 luciferase Assay System (Promega, Leiden, The Netherlands). Since most of the  
587 mutations abrogated virus replication, we cloned the above-mentioned 2C mutations  
588 into the p53CB3/T7 infectious clone using BssHII and XbaI<sup>53</sup>. Similar to the Rluc-CV-  
589 B3 plasmids, the infectious clones were linearized with MluI and RNA was transcribed  
590 *in vitro*, and 150 ng of viral RNA was transfected into 6-wells containing either HeLa  
591 R19 or BGM cells. All transfections were done in triplicates. Transfection of CV-B3-  
592 2C[A229V] was done in the presence of 1mM GuaHCl. 3 days after transfection, the

593 transfected cells were subjected to three freeze-thaw cycles and the lysates were  
594 passaged 3 times on either HeLaR19 or BGM cells, respectively. If CPE was observed  
595 during passaging of the viruses, viral RNA was isolated with the NucleoSpin RNA Virus  
596 kit (Macherey-Nagel, Leiden, The Netherlands) according to the manufacturer's  
597 protocol. The viral RNA was reverse transcribed into cDNA with random hexamer  
598 primers using the TaqMan Reverse Transcription Reagents (Applied Biosystems) and  
599 PCR was performed to isolate the 2C regions with the forwards primer binding in 2B 5'  
600 CTAACCAAATATGTGAGC 3' and the reverse primer binding in 3A 5'  
601 CTCACTGTCTACCGATTTGAG 3'. The same primers were used for Sanger  
602 sequencing of the PCR product. If virus was obtained, virus titers were determined by  
603 endpoint dilution titration and calculated according to the method of Reed and Muench  
604 and expressed as 50% cell culture infective dose (CCID<sub>50</sub>)<sup>54</sup>. The obtained CV-B3-  
605 2C[A229V] virus was titrated in the presence of 2mM GuaHCl.

#### 606 **Single-cycle infection**

607 Confluent HeLa R19 (25.000 cells/ well in a 96-well) cells were infected with virus at a  
608 multiplicity of infection (MOI) of 0.1 or 1 at 37 °C for 30 min. Next, the medium was  
609 removed, and fresh (compound-containing) medium was added to the cells. At the  
610 indicated time points, the medium was discarded, and cells were subjected to three  
611 times freeze thawing to determine the virus titers by endpoint dilution using the  
612 methods of Reed and Muench. In the case of RLuc-CV-B3 infection, cells were lysed  
613 8 hrs post infection to determine the luciferase activity with the *Renilla* luciferase Assay  
614 System (Promega). The CV-B3-2C[A229V] mutant was titrated in the presence of 2mM  
615 GuaHCl.

#### 616 **ATPase Assay**

617 The release of inorganic phosphate during 2C-mediated ATPase hydrolysis was  
618 measured using the Malachite Green Phosphate Assay Kit (Sigma Aldrich,  
619 Zwijndrecht, The Netherlands). Each 50 µL reaction comprised 500 nM recombinant  
620 2C protein and 1 mM ATP in reaction buffer (10 mM HEPES (pH 7.5), 150 mM NaCl  
621 and 10 mM MgCl<sub>2</sub>). The reaction buffer was always prepared fresh and recombinant  
622 2C proteins were thawed immediately prior to use. For testing the inhibitory effect of  
623 2C targeting compounds, the final DMSO concentration in each reaction was 1%.  
624 Samples were incubated for 30 min at 37°C and then diluted 4-fold with water to give

625 a final ATP concentration of 0.25 mM, as per the manufacturer's instructions. To  
626 terminate the enzyme reaction, 80  $\mu$ L of the diluted sample was mixed with 20  $\mu$ L of  
627 Solution AB in a 96-well plate. The plates were incubated for 30 min at room  
628 temperature and absorbance was measured using a microplate reader at OD<sub>630</sub>. To  
629 determine the phosphate concentration in each sample, the OD<sub>630</sub> values were plotted  
630 against a standard curve using GraphPad Prism version 8.

### 631 **Cryo-electron microscopy**

632 Hex $\Delta$ 116-2C (1 mg/mL) was either used directly for grid preparation or first incubated  
633 with 5 mM ATP-y-S, 100  $\mu$ M SFX or 100  $\mu$ M dibucaine (final DMSO concentration of  
634 0.5%), at 4°C for 30 minutes. 3  $\mu$ l of each sample was dispensed on Quantifoil R1.2/1.3  
635 200-mesh grids (Quantifoil Micro Tools GmbH) that had been freshly glow discharged  
636 for 30 seconds at 20 mA using a PELCO easyGlow™ Glow Discharge Cleaning  
637 System (Tedpella). Grids were blotted for five seconds using Whatman No. 1 filter  
638 paper and immediately plunge-frozen into liquid ethane cooled by liquid nitrogen using  
639 a Vitrobot Mark IV plunger (Thermo Fisher Scientific) equilibrated to ~95% relative  
640 humidity, 4°C. Screening of the apo, ATP-y-S incubated and SFX or dibucaine  
641 incubated samples was performed using a 200 kV Talos Arctica (Thermo Fisher  
642 Scientific) equipped with a Gatan K2 Summit direct detector. Subsequently, two data  
643 sets were collected on the SFX incubated sample using a Titan Krios Cryo-TEM  
644 (Thermo Fisher Scientific) operating at 300 keV. For the first data set, movies were  
645 collected using a K2 direct electron detector operating in electron counting mode,  
646 at 165,000 $\times$  magnification corresponding to a calibrated pixel size of 0.842  $\text{\AA}$ /pix over  
647 a defocus range of -1.5 to -2.5  $\mu$ m. 1527 movies were collected using a dose rate of  
648 8.8 e<sup>-</sup>/pix/sec for a total of 4 seconds (40 fractions), resulting in a total exposure of ~50  
649 e<sup>-</sup>/ $\text{\AA}^2$  (1.25 e<sup>-</sup>/ $\text{\AA}^2$ /fraction). To account for the preferred orientation exhibited by the 2C  
650 hexamers, an alpha tilt of +30 degrees was used for the second data collection. Movies  
651 were collected using a K3 direct electron detector operating in super resolution mode,  
652 at 64,000 $\times$  magnification corresponding to a super resolution pixel size of 0.69  $\text{\AA}$ /pix  
653 over a defocus range of -2 to -4  $\mu$ m. 6119 movies were collected using a dose rate of  
654 12.6 e<sup>-</sup>/pix/sec for a total of 2 seconds (37 fractions), resulting in a total exposure of  
655 ~54 e<sup>-</sup>/ $\text{\AA}^2$  (1.45 e<sup>-</sup>/ $\text{\AA}^2$ /fraction). All cryo-EM data were acquired using the EPU 2  
656 software (Thermo Fisher Scientific).

## 657 **Image processing**

658 For the untilted data, collected movie stacks were manually inspected and then  
659 imported in Relion version 3.0.1<sup>55</sup>. Drift and gain correction were performed with  
660 MotionCor2<sup>56</sup>, and GCTF was used to estimate the contrast transfer function for each  
661 micrograph<sup>57</sup>. Approximately 200 particles were picked manually and 2D classified.  
662 The best resulting class was then used as a template for autopicking in Relion,  
663 resulting in 42,206 particles. Fourier binned ( $2 \times 2$ ) particles were extracted in a 240-  
664 pixel box and subjected to a round of 2D classification after which 3616 particles were  
665 retained. Using the 'molmap' command in UCSF chimera, residues 358-628 of the JC  
666 polyomavirus helicase (PDB ID: 5J40<sup>41</sup>) were used to generate a 50 Å resolution  
667 starting model. Particles selected from 2D classification were 3D auto-refined (with C6  
668 symmetry), which produced a highly anisotropic map. Following unsuccessful attempts  
669 to solve the preferred particle orientation with buffer additives, tilted data collection was  
670 employed as described previously<sup>58</sup>. Tilted movies were Fourier binned ( $2 \times 2$ ) during  
671 motion correction with MotionCor2<sup>56</sup>, and goCTF was used to estimate the defocus  
672 gradient of each micrograph<sup>59</sup>. Approximately 1000 particles were picked manually and  
673 2D classified. The best resulting class was then used as a template for autopicking in  
674 Relion, resulting in 180,069 particles. Fourier binned ( $2 \times 2$ ) particles were extracted  
675 in an 80-pixel box and subjected 3D classification, after which 53,854 particles were  
676 selected. After a second round of 3D classification, 11,024 particles were retained. A  
677 final round of no-alignment 3D classification was performed, yielding a final stack of  
678 6856 particles. Subsequent 3D auto-refinement (with C6 symmetry) and post-  
679 processing yielded a map with an estimated resolution of 12 Å, based on the gold-  
680 standard FSC = 0.143 criterion. An ad-hoc negative B-factor of  $-200 \text{ \AA}^2$  was applied  
681 during the final post-processing step. An overview of the data processing pipeline is  
682 shown in Supplementary Figure 13. Figures of the CV-B3 hexΔ116-2C reconstruction  
683 were made in UCSF ChimeraX<sup>60</sup>.

## 684 **Data availability**

685 Atomic coordinates and structure factors for CV-B3 Δ116-2C in complex with SFX are  
686 deposited in the Protein Data Bank under accession codes 6S3A and 6T3W. The EM  
687 density map for the SFX incubated hexΔ116-2C has been deposited to the Electron  
688 Microscopy Data Bank under the accession EMD-12798. All reagents and relevant  
689 data are available from the authors upon request.

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