Cryo-EM structures reveal high-resolution mechanism of a DNA polymerase sliding clamp loader

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SUMMARY

Sliding clamps are ring-shaped protein complexes that are integral to the DNA replication machinery of all life. Sliding clamps are opened and installed onto DNA by clamp loader AAA+ ATPase complexes. However, how a clamp loader opens and closes the sliding clamp around DNA is still unknown. Here, we describe structures of the *S. cerevisiae* clamp loader Replication Factor C (RFC) bound to its cognate sliding clamp Proliferating Cell Nuclear Antigen (PCNA) en route to successful loading. RFC first binds to PCNA in a dynamic, closed conformation that blocks both ATPase activity and DNA binding. RFC then opens the PCNA ring through a large-scale ‘crab-claw’ expansion of both RFC and PCNA that explains how RFC prefers initial binding of PCNA over DNA. Next, the open RFC:PCNA complex binds DNA and interrogates the primer-template junction using a surprising base-flipping mechanism. Our structures indicate that initial PCNA opening and subsequent closure around DNA do not require ATP hydrolysis, but are driven by binding energy. ATP hydrolysis, which is necessary for RFC release, is triggered by interactions with both PCNA and DNA, explaining RFC’s switch-like ATPase activity. Our work reveals how a AAA+ machine undergoes dramatic conformational changes for achieving binding preference and substrate remodeling.

Keywords: sliding clamp, DNA replication, AAA+, ATPase, clamp loader

INTRODUCTION

In all known cellular life, DNA replication is coordinated by ring-shaped sliding clamp proteins that wrap around DNA to activate DNA polymerases and other factors (Moldovan et al., 2007). Sliding clamps are regulated by their presence on DNA, which in turn is governed by clamp loaders that open the sliding clamp ring and place it onto DNA (Kelch, 2016). The clamp loader of eukaryotes Replication Factor C (RFC) installs the sliding clamp Proliferating Cell Nuclear Antigen (PCNA) in a coordinated and stepwise fashion (Kelch, 2016). First, RFC binds ATP, which is a prerequisite for tight binding to PCNA (Sakato et al., 2012). Next, RFC binds to PCNA, and then opens the PCNA ring. This open ternary complex is now competent to bind to primer-template (p/t) DNA (double-stranded DNA with a single-stranded 5’ overhang). Primer-template binding to the ternary complex triggers ATP hydrolysis in the clamp loader, followed by sliding clamp closure and ultimately release of the clamp loader complex (Chen et al., 2009). Therefore, RFC has two macromolecular substrates, PCNA and p/t-DNA, that must bind sequentially. Yet how clamp loaders achieve this strict sequential order remains unknown.

Clamp loaders are members of the ATPases associated with various cellular activities (AAA+) family (Erzberger and Berger, 2006). Most members of this family function as ringed, homohexameric molecular motors that harvest energy from ATP to translocate substrates through their central pore (Jessop et al.,

2021). However, clamp loaders are not motors but instead are ATP-dependent remodeling switches (Kelch, 2016). In contrast to typical AAA+ ATPases, clamp loaders are heteropentameric with the five different subunits called A-E (Figures 1A&B). Each subunit features a classic AAA+ ATPase module, which holds the ATP sandwiched between the Rossmann fold and Lid domain at the binding interface with the neighboring subunit. The AAA+ modules of every subunit are extended by collar domains, which tightly associate together into a flat disk, enabling dynamic interactions between the five AAA+ modules. In addition to the canonical AAA+ machinery, many clamp loaders contain an A’ domain that bridges the gap between the A and the E subunit. The space between the A’ domain and the AAA+ domain of subunit A is the “A-gate”, (Figure 1C), which serves as the entry site for p/t-DNA binding. It was initially proposed that ATP binding triggers the five AAA+ modules to form a spiral with a symmetrical pitch that matches the geometry of DNA and templates the open clamp (Bowman et al., 2004; Simonetta et al., 05/2009). This symmetric, helical arrangement of the subunits results in a cracked interface between the A and E

Figure 1. Architecture of the eukaryotic clamp loader (RFC) and clamp (PCNA). (A) RFC is composed of five different subunits (named A to E) that each consist of the AAA+ ATPase module and a collar domain. The nucleotide binding site is sandwiched between the N-terminal Rossmann fold domain and the Lid domain of the ATPase module at the subunit interface. The ATPase module and a C-terminal extension of the A subunit called the A'-domain form the A-gate. (B) Domain organization of RFC subunits. (C) Clamp loading begins with binding of ATP to RFC, followed by PCNA binding. How PCNA is opened and DNA binds to the open RFC:PCNA complex is not known. DNA binding triggers ATP hydrolysis, PCNA closure, and RFC ejection.

74 subunits, bridged by the A' domain. As the A' domain stretches
75 away from the A subunit to maintain contact, the A-gate opens
76 and permits p/t-DNA binding (Kelch et al., 2011). However,
77 structures of the human and yeast RFC:PCNA complexes bound
78 to ATP analog show a closed PCNA ring bound to RFC in an
79 autoinhibited state, where the closed A-gate blocks the DNA
80 binding (Bowman et al. 2004; Gaubitz et al. 2020). Additionally,
81 another element called the "E-plug" reaches into RFC's central
82 chamber and sterically occludes DNA binding. This autoinhibited
83 state of RFC bound to closed PCNA is likely the first intermediate
84 in the clamp loading reaction (Gaubitz et al., 2020b; Sakato et al.,
85 02/2012; Thompson et al., 2012).

86 The question remains: How does the clamp loader open the
87 sliding clamp? This is perhaps the most important function of the
88 clamp loader, yet clues as to how this process is achieved remain
89 elusive (Figure 1C). The structure of the T4 phage loader bound to
90 DNA and an open clamp indicated that the clamp adopts a right-
91 handed spiral conformation that matches the helical pitch of DNA
92 (Kelch et al., 2011). However, this structure represents the state
93 after DNA is bound, and does not address how the clamp ring is
94 initially opened. Thus, the structure of a clamp loader bound to an
95 open clamp without DNA has been sought after, as it will
96 illuminate the opening process.

97 RESULTS

98 Structures of RFC:PCNA complexes en route to DNA loading

99 To understand how RFC opens PCNA and subsequently binds
100 DNA, we used single-particle cryo-EM to determine structures of
101 full-length *S. cerevisiae* RFC bound to PCNA and the slowly
102 hydrolyzing ATP analog ATPγS in the presence and absence of
103 primer-template (p/t) DNA. We reconstituted the complex from
104 purified RFC and PCNA subcomplexes that were separately
105 expressed in *E. coli* (Figure S1A). Full-length RFC is functional, as
106 it has the expected ATPase activity profile (McNally et al., 2010)
107 with PCNA and p/t-DNA synergistically activating ATP hydrolysis
108 (Figure 6F).

109 To prevent particle denaturation during sample preparation for
110 cryo-EM, we cross-linked DNA-free and DNA-bound complexes
111 using the amine-reactive crosslinker
112 bis(sulfosuccinimidyl)suberate (BS3). Mild cross-linking is
113 frequently used to obtain high-resolution cryo-EM structures of
114 labile complexes (Gerlach et al. 2018; Yoo et al. 2018; Gaubitz et
115 al. 2020). Mass spectrometry of the DNA-free sample reveals that
116 most cross-links are intra-molecular and map to the unresolved

Figure 2. The Autoinhibited state is dynamic. (A) Cryo-EM maps of the three Autoinhibited conformations of the RFC:PCNA complex. PCNA tilts closer relative to RFC in Autoinhibited2. The subunit arrangement of the AAA+ module of Autoinhibited3 is changed slightly, which leads to a crack in the A-gate. (B) Top view on the contact sites of PCNA with RFC in the autoinhibited conformation. (C) Principal Component analysis of all Autoinhibited particles reveals a rocking motion of PCNA relative to RFC. The C_{α} displacement of Principal Component 1 (PC1) is indicated by arrows, scaled down by a factor of 2. (D) Principal Component analysis reveals a range of motions within the initial RFC:PCNA complex. Amplitude histogram of the first principal component (PC1) reveals a unimodal distribution of particles, suggesting that this state consists of related particles in continuous motion.

117 N- and C-termini of RFC1, with only a few detectable inter- 138 **The initial complex of RFC:PCNA is dynamic**
118 molecular cross-links between RFC subunits (Figure S1B; Table 139 Three of the classes from the DNA-free sample are of RFC bound
119 S1). No significant cross-links were observed between RFC and 140 to closed PCNA in different conformational states (Figure 2A).
120 PCNA. 141 Overall, these structures resemble the previous yeast RFC:PCNA
121 We imaged the RFC:PCNA complex with and without p/t-DNA 142 crystal structure and our recent cryo-EM structure of human RFC
122 using a 300 kV Titan Krios microscope (Figures S1C, S1D, S2A, 143 (hRFC):PCNA (Figure S4A; [Bowman et al. 2004](#); [Gaubitz et al.](#)
123 S2B, S3A, S3B; Table S2). 3D classification results in four well- 144 [2020](#)). The PCNA ring is closed with only the A, B, and C subunits
124 defined reconstructions from the DNA-free sample, with overall 145 of RFC contacting PCNA (Figure 2B). The interaction area
125 resolutions ranging between 3.8 and 4.0 Å (Figure S2D). The 146 between clamp loader and clamp averages ~1940 Å² across the
126 dataset of the DNA-containing sample yielded several well 147 three states. The nucleotide density in each of the four active sites
127 defined classes, with overall resolutions ranging between 3.3 and 148 are most consistent with the presence of ATPyS, although the
128 3.5 Å (Figure S3C). We focused on classes in which all subunits 149 density for the γ -phosphate analog in the D subunit is somewhat
129 of RFC and PCNA are visible, although the N- and C-terminal 150 ambiguous due to low local resolution throughout this subunit.
130 regions of the A subunit lack clear density. The quality of the cryo- 151 Nonetheless, the ATPase sites of the B, C, and D subunits are in
131 EM reconstructions readily permitted model building using the 152 an inactive state (Figure S4B), with the AAA+ spiral in the
132 crystal structure as a template (Bowman et al., 2004; Figures S1C, 153 overtwisted state observed in the hRFC structure and the
133 S1D; Table S2). 154 previous yeast RFC crystal structure (Figure S4C; [Bowman et al.](#)
134 155 [2004](#); [Gaubitz et al. 2020](#)). Therefore, all three of these structures
135 156 represent autoinhibited states of RFC (termed Autoinhibited1,
136
137

Figure 3: RFC undergoes a large conformational change to open PCNA. (A) Cryo-EM map of RFC bound to an open PCNA ring. **(B)** PCNA is held open by contacts with all five subunits of RFC. **(C)** The Ca displacement from closed to open PCNA is indicated by arrows, scaled up by a factor of four. **(D)** The AAA+ modules widen from the Autoinhibited state (gray) to an open spiral conformation. **(E)** Top view of the AAA+ spiral shows that the E-plug and A-gate block access to RFC's central DNA binding chamber in the Autoinhibited conformation but retract in the open conformation. RFC opens wide enough for DNA to directly enter the central chamber. **(F)** Top view of the Rossmann fold arrangement in the Autoinhibited conformation. The rotation axes that relate neighboring subunits are shown in different colors and are skewed, indicating asymmetric rotations which lead to gaps between the subunits. **(G)** The rotation axes overlay in the Open2 state of RFC, indicating a symmetric arrangement of the AAA+ spiral. Symmetrization closes the gaps, and results in an increased interaction area between neighboring subunits.

157 Autoinhibited2, and Autoinhibited3). Because the Autoinhibited1, 172
158 2, and 3 states likely represent ATP-saturated configurations, we 173
159 place these conformational states early in the clamp loading 174
160 reaction.

161 The subunits in the AAA+ spiral have a different tilt in each of 176
162 the Autoinhibited states, thereby slightly altering the intersubunit 177
163 interactions (Figure S4C). For instance, the Autoinhibited3 state 178
164 exhibits a slightly cracked A-gate (but not open enough for DNA 179
165 to pass through), whereas the A-gate is closed in the 180
166 Autoinhibited1 and 2 states (Figure 2A). Further, the AAA+ 181
167 modules of subunits C-D change their position into a more 182
168 symmetric alignment with overlapping rotation axes relative to 183
169 Autoinhibited1 and 2 (Figure S4C). On the other hand, the PCNA 184
170 ring tilts ~19° relative to the RFC-D in the Autoinhibited2 state 185
171 relative to the Autoinhibited1 and 3 states (Figure 2A). 186

Despite these differences, the three Autoinhibited structures 172
173 are very similar, and so we asked if these conformations represent 174
175 distinct intermediates or if they are snapshots along a continuum 176
177 of conformations. Therefore we characterized the particles that 178
179 contribute to the Autoinhibited states using multi-body refinement 180
181 (Figure S5), a computational tool that allows modeling of 182
183 macromolecular motion (Nakane et al., 2018). To examine motion 184
185 between clamp and clamp loader, we defined RFC and PCNA as 186
187 two independent rigid bodies (Figure S5A-S5D). This analysis 188
189 revealed that the dominant motion is rocking of PCNA towards 190
191 RFC, with the linker between the ATPase and collar domains 192
193 serving as a hinge (Figure 2C, Video S1). Other motions include 194
195 swiveling of the RFC spiral with RFC-D getting closer to PCNA 196
197 (Figure S5D, Video S2). These results are not dependent on the 198
199 particular mask used, as similar motions are observed using 200

Figure 4: A fold-switch mechanism for opening a pore in the Open state of RFC:PCNA. (A) Helix 4 of the RFC-A subunit in Autoinhibited1 is shown in purple. (B) In the open conformation, Lid Helix 4 is displaced and partially unravels, whereby the packing arrangement of the hydrophobic core of the lid domain in RFC-A changes. Ile536 and Leu549 move ~13 Å and ~22 Å from their original position and a pore is formed between the RFC-A and RFC-B subunits.

188 component analysis of the multi-body conformers revealed a
189 unimodal distribution of particles along their eigenvalue (Figures
190 2D, S5C, S5G). This unimodal distribution indicates that the three
191 different observed cryo-EM class averages do not represent
192 particles in discrete states, but rather snapshots along a
193 continuum of motion. Thus, the autoinhibited state of RFC is
194 conformationally heterogeneous, with the dominant motions
195 driving RFC toward PCNA. We propose these motions represent
196 an early phase of the transition toward opening of the PCNA ring.

197 PCNA opening is coupled to large-scale expansion of RFC

198 Each of the two cryo-EM datasets revealed a class of RFC bound
199 to open PCNA with no DNA bound (Figures S2D, S3C). To our
200 knowledge, these are the first high-resolution structures of a
201 clamp loader bound to an open clamp prior to DNA binding. Both
202 reconstructions are highly similar (overall C_α RMSD is 0.74 Å, map
203 to map correlation coefficient is ~0.85, Figures S6A-S6D) and we
204 refer to these structures as Open1 and Open2. PCNA forms a
205 right-handed spiral with a ~20 Å opening that is wide enough for
206 dsDNA to enter (Figures 3A, B). The PCNA ring opens primarily
207 through in-plane rather than out-of-plane motions (in-plane ~19 Å
208 and out-of-plane ~10 Å for Open2, Figure 3C). Each of the
209 subunits of PCNA twists outward and towards RFC, with the
210 largest distortion in subunit II (Figure S6F).

211 PCNA opens at the A-gate of RFC, disrupting the interaction
212 between the first and third subunits of the PCNA ring (termed
213 PCNA-I and PCNA-III, hereafter). The open PCNA ring is directly
214 held by all five subunits of RFC, burying ~3800 Å² of surface area,
215 an approximate ~1860 Å² increase over that of the Autoinhibited
216 states (Figures 3B, S7A, S7B). The RFC-C subunit shifts
217 downward to interact much more tightly with PCNA-II, while
218 PCNA also forms new interactions with RFC-D, RFC-E, and the

219 A' domain of RFC-A (Figures S7C, D). The overall interface is
220 characterized by an alternating pattern of strong and weak
221 interactions (strong: RFC-A, -C, & -E; weak: RFC-B, -D, & A'). The
222 strong interactions are with the main partner binding pocket of
223 PCNA, using a binding region that resembles a common motif for
224 PCNA-interacting partners. Of these strong interfaces, RFC-A is
225 the most substantial and RFC-E weakest; RFC-A contains a true
226 PCNA interaction motif, while RFC-C and RFC-E's motifs are
227 increasingly degenerate. It is likely that the stronger interactions
228 at the "bottom" of the spiral allows the clamp loader to toggle
229 between the closed and open states of PCNA without releasing
230 RFC.

231 The AAA+ modules of RFC adopt a right-handed spiral whose
232 periodicity matches that of the six contact sites on PCNA. The
233 symmetry of the ATPase spiral can be visualized by the near
234 perfect alignment of the rotation axes that relate adjacent AAA+
235 subunits (Figures 3F-G). The interfaces between adjacent AAA+
236 modules also become tighter, bringing the catalytic arginine finger
237 residue closer to the neighboring ATPase site. This observation
238 explains the modest boost in ATP activity upon PCNA binding
239 (Johnson et al., 2006; Figure 6F). Similar to the Autoinhibited
240 structures, all four active sites are bound to ATP analog (Figure
241 S6E). Therefore, ATP hydrolysis is not necessary to drive the
242 conformational change from Autoinhibited to Open.

243 In order to rupture the PCNA ring, the AAA+ spiral of RFC
244 widens, opening the A-gate. RFC opens using a large hinge
245 motion, pivoting around the B-C and C-D subunit interfaces
246 (Figure 3D, 3E; Video S3). The RFC-E subunit uses its E-plug to
247 bind PCNA, which pulls the A' domain and E-plug up to 45 Å away
248 from the AAA+ module. This reveals a large opening of the A-gate
249 (at its most narrow, the A-gate is approximately 20 Å wide) (Figure
250 3E). p/t-DNA can therefore directly enter the open RFC:PCNA
251 complex.

252 Opening of the A-gate separates the RFC-A Lid and collar
253 domains, inducing a fold-switching transition in the Lid domain.
254 The majority of the last helix of the Lid (Helix α4; residues 541-
255 546) unravels into a taut β-strand conformation (Figures 4A, 4B).
256 The remaining residues in helix α4 (residues 536-542) shift
257 forward, causing a major change in the core packing of the Lid
258 domain. This "sliding spring" motion leads to a ~11 Å helix
259 displacement, whereby some residues, such as Leu 549, move up
260 to 22 Å from their original position. The stretching of the RFC-A
261 Lid opens a new pore between the A and B subunits (Figure 4B).
262 We discuss the role of this pore in the next section.

263 Structures of the RFC:PCNA complex bound to primer- 264 template DNA

265 To reveal how RFC:PCNA binds and responds to DNA, we
266 analyzed two classes that contain DNA-bound RFC:PCNA. One
267 class shows PCNA in an open lock-washer shape, and the other
268 has PCNA in a closed conformation. Therefore, we term these two
269 states DNA^{PCNA-open} and DNA^{PCNA-closed}, respectively (Figures 5A-
270 5C). Both classes contain clear density for p/t-DNA: 18 base pairs
271 of duplex DNA are bound inside the central chambers of RFC and
272 PCNA, and 6 nucleotides of the ssDNA template extend through
273 the A-gate, preventing its closure. The AAA+ spiral of RFC tracks
274 the minor groove of dsDNA using a suite of residues that are
275 conserved across all known clamp loaders to match the helical
276 symmetry of DNA (Kelch et al., 2011; Simonetta et al., 05/2009).

Figure 5: Structures of RFC:PCNA bound to DNA. (A) Schematic representation of the structure of RFC:PCNA bound to primer-template (p/t)-DNA. (B) Cryo-EM map of RFC:PCNA bound to p/t-DNA and open PCNA (termed DNA^{PCNA-open}). (C) Cryo-EM map of RFC:PCNA bound to p/t-DNA with closed PCNA (termed DNA^{PCNA-closed}). (D) The E-plug inserts into the major groove and interacts with both strands of the p/t-DNA. (E) Top view of contact sites of RFC with PCNA. PCNA is held open by contacts with all five subunits in DNA^{PCNA-open}. (F) In DNA^{PCNA-closed}, the interaction between RFC-E and PCNA-III is lost. (G) Overview of structure of Open2. (H) Top view of the AAA+ spiral of DNA^{PCNA-open}. Displacement vectors between Open2 and DNA^{PCNA-open} are indicated by arrows, scaled up by a factor of two. The AAA+ spiral constricts around DNA. (I) The AAA+ spiral of DNA^{PCNA-closed}. Displacement vectors between DNA^{PCNA-open} and DNA^{PCNA-closed} indicate that the AAA+ spiral constricts further around DNA, leading to changes in ATPase sites.

277 The E-plug beta-hairpin slots into the major groove of the 289 bind DNA with equivalent affinity as WT-hRFC (Gaubitz et al.,
278 duplex region of p/t-DNA (Figure 5D). Conserved basic residues 290 2020b).
279 at the tip of the E-plug interact directly with both the template and 291 In DNA^{PCNA-open}, both RFC and PCNA broadly resemble the
280 primer strands. Therefore, the E-plug provides a mechanism for 292 conformations seen in Open1 and Open2. The RFC A-gate is
281 the RFC AAA+ spiral to recognize both strands of DNA, unlike the 293 open, with all 5 subunits gripping PCNA in an open lock-washer
282 clamp loaders from *E. coli* and T4 phage, whose AAA+ spirals only 294 shape. However, both RFC and PCNA constrict relative to the
283 interact with the template strand (Kelch et al., 2011; Simonetta et 295 Open1 and Open2 structures (Figures 5G, 5H and Figure S8A).
284 al., 05/2009). Moreover, this structure shows that the E-plug 296 RFC constricts modestly, pivoting the E, D, and C subunits
285 changes its role from blocking DNA binding (in the three 297 around a hinge at the B-C interface. PCNA constricts ~12 Å upon
286 Autoinhibited states) to one in which it directly supports DNA 298 DNA binding, with most of this constriction occurring in subunit III
287 binding. This explains the non-intuitive effect on DNA binding we 299 of PCNA (Figure S8A). Subunit III of PCNA is held by the RFC-D
288 observed previously, where hRFC variants with a mutated E-plug 300 and RFC-E subunits, although RFC-E grips PCNA less tightly in
301 DNA^{PCNA-open} (~3800 Å² total RFC-PCNA interaction area for the

Figure 6: RFC separates the 3' end of the primer strand. (A) The last nucleotide in the primer strand is separated from the duplex. **(B)** The collar of RFC-A contains a “separation pin” with two critical residues (Trp638 and Phe582) that stabilize the flipping of the 3' primer nucleotide into the pore between RFC-A and RFC-B. The cryo-EM map is shown in red mesh. **(C)** The primer strand of p/t-DNA contains 3' nucleotide with a 2-aminopurine (2AP) base, an adenine analog that reports on base-pairing and base-stacking. 2AP fluorescence increases in the presence of ATPγS and RFC:PCNA to a higher level than in the unpaired 2-aminopurine-labeled primer strand. **(D)** The human RFC:PCNA complex also induces an increase in 2AP fluorescence emission, whereas the *E. coli* clamp loader, which does not flip the 3' end of the primer (Simonetta et al., 05/2009), does not increase 2AP fluorescence. **(E)** Mutation of Phe582 and Trp638 reduces 2-AP fluorescence in the presence of ATPγS. **(F)** ATPase activity of the “separation pin” mutants.

302 Open1&2 structures vs ~3400 Å² for DNA^{PCNA-open}). Overall, the 323 subunit (Figure S8C). Therefore, these structures represent
303 PCNA conformation is similar to that seen for the structure of the 324 reaction intermediates following DNA binding but preceding ATP
304 T4 phage clamp bound to clamp loader and p/t-DNA (Kelch et al., 325 hydrolysis. Upon binding DNA, the AAA+ spiral constricts (Figure
305 2011). 326 5H), primarily due to a hinge-like motion at the interface between

The DNA^{PCNA-closed} structure has a closed PCNA ring that is 327 distorted from planarity. Upon closure, PCNA loses its interaction 328 with the RFC-E subunit, but retains its interfaces with the other 329 four RFC subunits (Figures 5E, 5F). The distortion of the PCNA 330 ring is most prevalent in subunit III, which puckers to maintain its 331 interaction with the RFC-D subunit (Figure S8A). Interestingly, the 332 interaction between DNA and PCNA becomes more extensive 333 upon PCNA closure (~50 Å² vs 250 Å²). Conserved basic residues 334 lining the inner pore of PCNA also interact directly with the duplex, 335 as has been hypothesized previously (Liu et al., 2017; McNally et 336 al., 2010). We propose that these interactions help to drive the 337 closure of PCNA around DNA. 338

RFC-C and RFC-B. The AAA+ spiral constricts around an axis 327 coincident with the DNA axis. Subsequent PCNA closure further 328 exaggerates the constriction of the RFC AAA+ spiral (Figure 5I). 329 This motion brings adjacent AAA+ modules into close contact, 330 enabling the active sites to fully assemble. The close apposition 331 of neighboring subunits brings each of the arginine finger residues 332 in close contact with the gamma-phosphate of ATP, which is 333 necessary for catalytic activity (Johnson et al., 2006). However, a 334 conserved arginine known as the switch residue is not in the 335 position that was previously predicted to be required for activation 336 of the Walker B glutamate (Kelch et al., 2011, 2012). Despite this, 337 the active sites appear to be in the fully active state, with all of the

318 DNA binding and PCNA closure also induce constriction of the 339 catalytic machinery poised to hydrolyze ATP. We discuss the
319 RFC AAA+ spiral, potentiating ATPase activity. DNA^{PCNA-open} and 340 ramifications of this observation on the allosteric activation of RFC
320 DNA^{PCNA-closed}, just like the other states described herein, are in the 341 below.

321 fully ATP-bound state: ATPyS in the active sites of the A, B, C, 342

322 and D subunits, and ADP in the nucleotide-binding site of the E 343 **RFC flips the 3' base of the primer strand**

Unexpectedly, we observe that the 3' nucleotide of the primer strand is melted in both DNA-bound RFC:PCNA structures, with the base flipped away from the rest of the duplex (Figure 6A, 6B). The base pair is disrupted by a "separation pin" at the base of the RFC-A collar domain that wedges between the DNA strands (Figure 6B). The indole ring of Trp638 replaces the flipped 3' base to maintain stacking interactions. The 3' nucleotide is repositioned inside the pore formed by the unraveling of the RFC-A Lid domain upon opening of the A-gate; this site is closed in the Autoinhibited state (Figure 4). The flipped base stacks against the phenyl ring of Phe582. These residues are conserved in eukaryotic clamp loaders but are absent in bacterial, archaeal or phage clamp loaders (Figure S9 E).

To characterize base flipping, we measured binding of DNA substrates containing the adenine analog 2-aminopurine (2AP). Fluorescence of 2AP is dependent on base-pairing (Frey et al., 1995; Jean and Hall, 2001): fluorescence is low when 2AP is base paired, but high in the free state. To monitor melting, we placed 2AP either at the 3' end of the primer strand ($AP_{p=1}$) or at the corresponding site in the template strand ($AP_{t=1}$). Importantly, we find a dramatic increase in 2AP fluorescence that is dependent on addition of RFC, PCNA and ATP analog (Figures 6C, S9A-C). The increase in 2AP fluorescence is not observed in the presence of ADP, which does not support DNA binding (Kelch et al., 2011; McNally et al., 2010). Placement of 2AP at the $p=2$ or $p=3$ position of the primer yields diminished fluorescence, suggesting that only the 3' base is flipped (Figure S9B). Therefore, our 2AP experiments validate that RFC- and PCNA-dependent 3' end melting occurs in solution. The human clamp loader, which has a similar separation pin as yeast RFC (Gaubitz et al., 2020b), greatly enhances 2AP fluorescence. However, the *E. coli* clamp loader, which binds p/t-DNA but does not melt the primer strand (Simonetta et al., 05/2009), does not alter fluorescence (Figure 6D). Thus melting of the 3' nucleotide is a conserved activity of eukaryotic clamp loaders, but is likely not used by bacterial clamp loaders.

To determine the mechanism and role of primer melting, we modified the p/t-DNA and/or key residues in the separation pin and assessed their effects on base flipping, ATP hydrolysis, and DNA affinity. The W638G and F582A variants have attenuated base-flipping as measured by 2AP fluorescence (Figures 6E, S9C). However, DNA-dependent ATP hydrolysis is minimally affected, particularly in the F582A variant, whose ATPase rate and apparent affinity for DNA are unchanged from WT (Figures 6F and S9D). These results indicate that base flipping requires the "separation pin", but base flipping is not required for DNA binding or ATPase activation.

DISCUSSION

Defining the clamp loading reaction in high-resolution

We have determined a series of structures that provide a high-resolution view of the clamp loading process. Our structures correspond to numerous reaction intermediates, allowing us to order the structures into a coherent description of the clamp loading reaction prior to ATP hydrolysis (Figure 7 and Video S4). The Autoinhibited states represent the transient encounter complex that forms early in the clamp loading process before ring opening. The Open1&2 states represent the stable intermediate

state in which PCNA is opened but DNA has yet to bind. The DNA^{PCNA-open} structure contains p/t-DNA and an open clamp, which is the transient intermediate following DNA binding (Liu et al., 2017; Marzahn et al., 2014; Sakato et al., 02/2012). Finally, the DNA^{PCNA-closed} structure represents the stable intermediate that forms prior to ATP hydrolysis (Marzahn et al., 2014; Sakato et al., 2012). Therefore, our structures delineate the conformational states that span the entire clamp opening and closing process, the central reaction of the clamp loading cycle.

A crab-claw mechanism for opening the sliding clamp

Our structures show that RFC is in a constricted, auto-inhibited conformation upon initial binding to PCNA. This state is highly dynamic, and we captured some of the conformational heterogeneity using multi-body refinement. The primary mode of motion pivots PCNA relative to RFC, such that PCNA approaches the D- and E-subunits of RFC. We speculate that this motion is on-pathway toward a direct interaction between PCNA and all five RFC subunits, facilitating the opening of the PCNA ring. Thus, the dynamics of the Autoinhibited complex are important for the opening of PCNA. Future studies will investigate this possibility.

To open PCNA, our structures show that RFC uses the previously hypothesized "crab-claw" mechanism (Jeruzalmi et al., 2001a, 2001b; O'Donnell et al.). This contradicts the previous suggestion that the *E. coli* clamp loader opens the ring with limited conformational changes in the clamp loader (Goedken et al., 2004; Kelch, 2016). In this "limited change" model, ATP binding places the encounter complex in a conformation that "templates" the open clamp. However, our structures preclude this model for RFC because we observe large conformational changes in the clamp loader upon opening the PCNA ring. Furthermore, the Autoinhibited state of RFC can not template an open PCNA conformation. One possible reason for the discrepancy between the two studies is that different model systems were used; bacterial clamp loaders lack the A' domain that constricts the AAA+ spiral of the yeast clamp loader. Without the A' domain, the bacterial clamp loaders may be free to adopt a conformation that can template the open clamp prior to clamp binding.

The crab-claw motion that we observe is primarily driven by a hinge-like motion that pivots about the RFC-C subunit. This motion allows the A' domain and E, D, and C subunits to grip PCNA tightly, which is impossible in the Autoinhibited state. Kinetic characterization of RFC variants has predicted a hinge role for this region (Sakato et al., 2012), highlighting this subunit's importance in clamp loading. The crab-claw conformational change is remarkable because it requires a fold-switching event in the Lid domain of the RFC-A subunit (Figure 4A, 4B). At a minimum, this would require that helix-4 of the RFC-A Lid to unfold and refold into a new position. The fact that clamp opening is relatively fast (Liu et al., 2017) and does not require ATP hydrolysis indicates that these conformational rearrangements must have a relatively low energy barrier despite the large-scale motion. How the RFC:PCNA complex couples these motions becomes an important question for future studies.

Why use a "crab-claw" mechanism? We envision two non-mutually exclusive hypotheses. First, we hypothesize that this mechanism allows RFC to bind each of its macromolecular substrates (PCNA and p/t-DNA) in the proper order to ensure efficient clamp loading and to avoid futile cycles of ATP

Figure 7: Clamp loading by RFC. Initial binding of RFC to PCNA places the complex in an Autoinhibited state, whereby closed PCNA and the E-plug preclude DNA binding, and an overtightened AAA+ helix inhibits ATPase activity. The Autoinhibited state is dynamic, rocking PCNA relative to RFC as captured by multibody refinement. Upon complete binding to PCNA, RFC uses the crab-claw mechanism to simultaneously open both PCNA and the A-gate, providing an entryway for p/t-DNA. p/t-DNA then binds directly through the A-gate and open PCNA, which are wide enough to accommodate dsDNA entry. The 3' end of the primer is flipped into the pore that is formed between RFC-A and RFC-B. PCNA closes to form additional contacts with DNA, partially detaching from RFC at the E subunit. Finally, ATPase activity and inorganic phosphate release eject RFC, leaving PCNA bound to p/t-DNA in the correct orientation.

460 hydrolysis. For proper clamp loading, RFC must bind PCNA first, 492 into the AAA+ spiral of the T4 and *E. coli* clamp loaders than in
461 because initial binding of p/t-DNA would sterically hinder binding 493 RFC (Figure S10A). Therefore, the large swing in the proportional
462 of the PCNA ring. Therefore, RFC has evolved high affinity for 494 interaction area is the net result of additional interactions from
463 PCNA and only binds p/t-DNA with high affinity after it has bound 495 RFC-A and less from the remaining subunits.

464 PCNA (Cai et al., 1998; Shiomi et al., 2000). The crab-claw 496 This proportionally large interaction area suggests RFC-A as
465 mechanism for PCNA opening can explain this hierarchy of 497 the subunit primarily responsible for recognizing DNA. This finding
466 binding, as the auto-inhibited state blocks the DNA binding site 498 provides an attractive explanation for how alternative clamp
467 ((Gaubitz et al., 2020b) and Figure 3E). The crab-claw mechanism 499 loaders specifically recognize different DNA structures. RFC-like
468 ensures that RFC's DNA-binding chamber only becomes 500 Complexes or RLCs are only found in eukaryotes and share four
469 accessible once the PCNA ring is open. Our second hypothesis is 501 of RFC's five subunits (RFC-B through RFC-E); each RLC
470 that the crab-claw mechanism enables complex modes of clamp 502 contains a unique A subunit (Majka and Burgers, 2004). We
471 loader regulation. Clamp loader activity could be inhibited by 503 hypothesize that the diminished role of the B, C, D, and E subunits
472 binding partners or post-translational modifications that favor the 504 in DNA recognition allows the A-subunit to assume the role of
473 Autoinhibited state. There are numerous RFC binding partners 505 specifically binding unique structures of DNA. In support of this
474 and post-translational modifications that remain unexplored, and 506 hypothesis, bacterial and phage clamp loaders do not have
475 thus are candidates for playing regulatory roles (Dephoure et al., 507 alternative forms that recognize different DNA structures, and
476 2008; Kim and Brill, 2001; Ochoa et al., 2020; Olsen et al., 2010; 508 their clamp loaders have substantially more contact between DNA
477 Tomida et al., 2008; Wang et al., 2012, 2013).

478

479 **RFC-A subunit drives DNA recognition**

480 To illuminate how RFC recognizes DNA, we measured the relative 512 subunits raises the question of how the pivot point at the C
481 contribution of each RFC subunit to DNA binding. We find that 513 subunit contributes to the activity of RLC complexes. Finally,
482 RFC-A accounts for ~64% of the buried surface area between 514 these findings raise the intriguing possibility of engineering RLCs
483 RFC and DNA. This contrasts with T4 and *E. coli* clamp loaders, 515 with novel specificity and activity.
484 where the A subunits account for ~36% of the binding interface 516 Following this reasoning, we hypothesize that RFC flips the 3'
485 (Figure S10B). Much of this proportional increase arises from 517 nucleotide to specifically recognize the recessed 3' end of p/t-
486 additional interactions between RFC-A and DNA through the 518 DNA. We observe flipping of the 3' nucleotide in both the DNA^{PCNA-}
487 separation pin and the flipped 3' nucleotide. Furthermore, we find 519 open and DNA^{PCNA-closed} structures, indicating that flipping occurs
488 that B, C, D, and E subunits of RFC interact with DNA significantly 520 before ring closure. This observation can explain the "DNA
489 less (~760 Å²) than the comparable subunits of T4 and *E. coli* 521 repositioning transition" that occurs quickly (t_{1/2} ~ 35 ms) after
490 (~1125 Å²). The decrease in DNA interaction from the B, C, D, and 522 initial DNA binding, but before clamp closure (Liu et al., 2017). We
491 E subunits is due to the p/t-DNA duplex region inserting deeper

propose that this transition is the flipping of the 3' nucleotide. Future experiments will investigate this possibility in more detail.

Forces driving clamp loading

Our structures delineate a conformational pathway that illustrates much of the clamp loading reaction. We reveal how: 1) RFC initially binds PCNA, 2) how PCNA is opened, 3) how DNA is bound, and 4) how PCNA closes around DNA. This unprecedented view into the mechanism of clamp loading allows us to hypothesize on the forces that drive this reaction towards the loading of PCNA. We use the interaction areas between and within PCNA, RFC, and p/t-DNA to approximate these forces. PCNA is opened through a large conformational change in both PCNA and RFC. In solution the open form is the predominant state (Zhuang et al., 2006), so it is important to understand what interactions drive this opening. Upon opening, PCNA loses the entire interface between subunits I and III. However, the open PCNA ring increases its interaction area with RFC by contacting all five subunits. Moreover, the crab claw motion of RFC results in tighter association between adjacent AAA+ modules. Altogether, the opening of PCNA and RFC result in an increased interaction area of $\sim 4000 \text{ \AA}^2$ (Figure 3F). We propose that this is the driving force for stabilizing the open form of PCNA.

Once open, p/t-DNA enters the PCNA:RFC complex through the A-gate. The A-gate is wide enough for dsDNA to directly enter into the RFC:PCNA inner chamber. This finding is in direct contradiction of the "filter-and-slide" model for DNA binding that posited that the opening is large enough for only ssDNA to enter such that the clamp loader filtered out dsDNA to accelerate the search for a p/t-junction (Kelch, 2016; Kelch et al., 2011, 2012). The filter-and-slide model was primarily predicated on crystal structures of the T4 phage clamp loader and on FRET data that suggested that initial binding of DNA does not constrict the open clamp (Kelch et al., 2011; Zhuang et al., 2006). While it remains a possibility that other clamp loaders use a filter-and-slide mechanism, our structures clearly indicate that yeast RFC uses the much more simple direct binding model.

Once DNA is bound, PCNA must close around the ring before ejection of the RFC complex (Liu et al., 2017; Marzahn et al., 2014; Sakato et al., 02/2012), which we observe in the transition from DNA^{PCNA-open} and DNA^{PCNA-closed} structures. Both structures show no evidence of ATP hydrolysis, suggesting that PCNA can close before ATP hydrolyzes. In contrast, it had been previously hypothesized that ATP hydrolysis occurs before clamp closure (Sakato et al., 02/2012). While DNA^{PCNA-open} and DNA^{PCNA-closed} have similar overall interaction areas, PCNA interacts with DNA much more intimately in the DNA^{PCNA-closed} structure, with direct contact to several conserved basic residues lining the PCNA inner pore. Lys20, Arg80, and Arg147 in particular show close interaction with the PCNA ring. These residues have been independently identified as critical for efficient DNA binding, ATP hydrolysis and clamp loading (McNally et al., 2010; Zhou and Hingorani, 2012). Therefore, PCNA is an allosteric effector in its own loading.

The final step in clamp loading is the hydrolysis of ATP and the ejection of the clamp loader. Our structures further illuminate how ATP hydrolysis is synergistically activated by PCNA and p/t-DNA. While our reconstructions do not have definitive density for unambiguous localization of side chains in the active sites across all the structures, we observe that the interactions between adjacent AAA+ modules become tighter upon binding both PCNA and p/t-DNA. These tight interactions are critical because much of the catalytic machinery for ATP hydrolysis is provided *in trans* by neighboring subunits (Johnson et al., 2006). However, two observations stand out as surprising. First, the AAA+ spiral is actually less symmetric once p/t-DNA binds (Figure S8B). This was unexpected because DNA had been thought to be the driving force for symmetrizing the AAA+ spiral (Kelch et al., 2011; Simonetta et al., 05/2009), and this symmetry had been thought to favor ATP hydrolysis. But the RFC:PCNA complex (corresponding to the the Open1 and Open2 structures) has ~ 5 -10-fold lower ATPase activity than when both PCNA and DNA are bound (Figure 6F, Chen et al., 5/2009; Gomes et al., 2001; McNally et al., 2010; Sakato et al., 02/2012). The second surprising observation is that a set of conserved arginines (termed the arginine switch residues) within the core of the AAA+ module are not in direct contact with DNA. The arginine switch residues had been hypothesized to flip outward to grip DNA, thereby releasing the catalytic glutamate to activate ATP hydrolysis (Kelch et al., 2011). The lack of flipping of the arginine switch residues in response to DNA binding argues that the proposed arginine switch mechanism is not critical for sensing and responding to DNA binding. Our observations are in agreement with previous studies that found that the arginine switch residues of RFC do not likely play a direct role in activating ATP hydrolysis, but are important for the synergistic activation by both PCNA binding and DNA binding (Liu et al., 2017). Further studies will be necessary to reveal how RFC integrates binding of both PCNA and p/t-DNA to achieve full activation.

Comparison with other AAA+ machines

Clamp loaders have long been models for structure and mechanism of AAA+ proteins (Guenther et al., 1997). However, they are unusual in that they are pentameric protein remodeling switches instead of the more typical hexameric rings that act as processive motors (Hanson and Whiteheart, 2005; Kelch, 2016). We note that conformational changes that we observe here in RFC appear to be more dramatic than those typically seen during motor function. This is likely because the constraints imposed by ring closure limits the types of motions that are available. On the other hand, the open nature of the RFC complex is less constrained and so can adopt more dramatic conformational changes. We further note that these types of large conformational changes are more commonplace in other members of the Initiator/Loader class of AAA+ machines. We propose that the open nature of this class provides larger conformational variability that is necessary for the regulation of these switch-like machines.

MATERIALS AND METHODS

Protein Purification

RFC was purified as described previously with minor modifications (Finkelstein et al., 08/2003). pET(11a)-RFC[2+3+4] and pLANT-2/RIL-RFC[1+5] were transformed into BL21(DE3) *E. coli* cells (Millipore). After preculture, transformants were grown in 4 liter of prewarmed terrific broth medium supplemented with 50 $\mu\text{g/mL}$ kanamycin and 100 $\mu\text{g/mL}$ ampicillin at 37 °C and induced with IPTG at an optical density of 0.8. Protein expression was continued at 18 °C for 15 hours. Cells were pelleted and

639 resuspended in 300 ml lysis buffer (30 mM Hepes-NaOH pH 7.5, 694 **Electron Microscopy**

640 250 mM NaCl, 0.25 mM EDTA, 5% glycerol, 2 mM DTT, 2 µg/mL
641 aprotinin, 0.2 µg/mL pepstatin, 2 µg/mL leupeptin, 1 mM PMSF).
642 RFC was purified by chromatography over a 10 mL SP-Sepharose
643 column (80 mL gradient of 300–600 mM NaCl in BufferC) and a
644 10 mL Q-Sepharose column (40 mL gradient of 150–500 mM NaCl
645 in Buffer C, GEHealthcare). Peak fractions of hRFC were pooled
646 and dialyzed overnight into a buffer with 30 mM Hepes-NaOH pH
647 7.5, 250 mM NaCl, 5% glycerol, and 2 mM DTT.

648 PCNA was purified as described previously with modifications
649 (McNally et al., 2010). BL21(DE3) *E. coli* cells were transformed
650 with a pET-28 vector that encodes PCNA with a Precission™
651 protease cleavable N-terminal 6-His tag. After transformation,
652 preculture and induction, 1 liter of cells was grown overnight at 18
653 °C in terrific broth medium supplemented with 50 µg/mL
654 kanamycin. Cells were pelleted and resuspended 30 mM Hepes
655 pH 7.6, 20 mM imidazole, 500 mM NaCl, 10% glycerol, and 5 mM
656 b-mercaptoethanol. The cells were lysed, centrifuged and the
657 filtered lysate was applied to a 5 mL HisTrap FF column
658 (GEHealthcare). The column was washed with a buffer at 1 M
659 NaCl, and subsequently washed with a buffer at a low salt
660 concentration (50 mM NaCl). PCNA was eluted with a step of 50
661 % with 500 mM imidazole. The eluted protein was cleaved with
662 Precission™ protease for 2 hours at room temperature and
663 applied to a 5 mL HiTrap Q HP column (GE Healthcare). Protein
664 was eluted from the Q HP column with a 2M NaCl buffer in a 100
665 mL gradient. Peak fractions were dialyzed against buffer
666 containing 30 mM Tris pH 7.5, 100 mM NaCl, and 2 mM DTT.
667 Purified proteins were concentrated with an Amicon
668 concentration device, aliquoted and frozen in liquid nitrogen for
669 storage at -80 °.

670 **Crosslinking and Mass Spectrometry**

671 RFC and PCNA were mixed in a 1/1 ratio and gel filtered into 1
672 mM TCEP, 200 mM NaCl, 25 mM Hepes-NaOH, pH 7.5, and 4
673 mM MgCl₂. The protein complex was diluted to 3 µM and after the
674 addition of 1 mM ATPyS and 3 min incubation, 1 mM of
675 Bis(sulfosuccinimidyl)suberate (BS3, Thermo Scientific Pierce)
676 was added for crosslinking. For crosslinking of DNA-bound
677 RFC:PCNA, 1 mM ATPyS was added to the protein complex first
678 and incubated for 2 min. 7 µM primer/template DNA was added
679 and incubated for another 1 min. The primer sequence was 5'-
680 GCAGACACTACGAGTACATA-3' and the template sequence
681 was 5'-TTTTTTTTTTTATGTACTCGTAGTGTCTGC-3'.
682 Crosslinking was started with 1 mM BS3, incubated for 15 min at
683 room temperature, and neutralized with Tris-HCl.
684 Sample without DNA was analyzed by mass spectrometry. The
685 sample was reduced, alkylated, and loaded onto SDS-PAGE gel.
686 The gel band corresponding to the crosslinked complex >150kDa
687 was excised, destained, and incubated with trypsin. The digested
688 peptides were extracted and desalted as previously described
689 (Peled et al., 2018) and analyzed with LC-MS coupled to a
690 ThermoFisher Scientific Q Exactive Mass Spectrometer in data
691 dependent mode selecting only precursors of 3. The data was
692 searched against the UniProt database, using Byonic and XlinkX
693 of the Proteome Discoverer 2.3 package.

695 **Negative-Staining EM**

696 100 nM of RFC:PCNA was applied on carbon-coated 400-mesh
697 grids. Excess sample was blotted from the grid surface, the grids
698 were washed twice with 50 mM Hepes pH 7.5 and stained with
699 1% uranyl acetate. RFC:PCNA was imaged on a 120kV Philips
700 CM-120 microscope fitted with a Gatan Orius SC1000 detector.

702 **Cryo-EM sample preparation**

703 Quantifoil R 0.6/1 (DNA dataset) grids were washed with ethyl
704 acetate. Quantifoil and C-flat grids (Electron Microscopy
705 Sciences) were glow discharged with Pelco easiGlow for 60s at
706 25mA (negative polarity). 2.8 - 3 µL sample was applied to grids
707 at 10°C and 95% humidity in a Vitrobot Mark IV (FEI). Samples
708 were blotted with a force of 5 for 5s after a 2 s wait and plunged
709 into liquid ethane.

711 **Cryo-EM data collection**

712 RFC:PCNA was imaged on a Titan Krios operated at 300 kV and
713 equipped with an GIF energy filter at 130000 × magnification and
714 a pixel size of 0.53 Å using a K2 Summit detector in super-
715 resolution counting mode. The data was collected in four sessions
716 with a target defocus range of -1.1 to -2.4 and a total exposure of
717 ~49-51 e-/Å² per micrograph averaging 50 frames. Image shift
718 was used to record three images per hole with SerialEM
719 (Mastronarde, 2003). Defective micrographs were discarded
720 leaving a total of 6109 micrographs for processing.
721 RFC:PCNA:DNA was imaged on a Titan Krios operated at 300 kV
722 at 81000 × magnification and a pixel size of 0.53 Å with a K3
723 detector in super-resolution mode. A total of 4499 micrographs
724 were collected in one day with a target defocus of -1.2 to -2.3 and
725 a total exposure of ~40 e-/Å² per micrograph averaging 30 frames.

727 **Data Processing**

728 Micrograph frames were aligned in IMOD (Kremer et al., 1996)
729 with 2x binning, resulting in a pixel size of 1.06 Å/pixel. Initial CTF
730 estimation and particle picking was performed using cisTEM
731 (Grant et al., 2018; Rohou and Grigorieff, 2015). Following particle
732 picking, particles were extracted with a box size of 240 pixels and
733 subjected to 2D classification into 100 classes. Particles from
734 classes with well defined features were selected for processing in
735 Relion (Figures S2A,B and S3A,B). Coordinates and combined
736 micrographs were imported into Relion 3.0.2 (Zivanov et al.,
737 2018), CTF parameters were re-estimated with Gctf1.06 (Zhang,
738 2016) and particles were subjected to several rounds of 3D
739 classification (Figures S2D and S3C). For 3D classification of the
740 RFC:PCNA dataset, an ab initio model was generated with
741 cisTEM, down-filtered to 50 Å and used as reference (Figure S2C).
742 For 3D classification of the RFC:PCNA:DNA dataset, class Open1
743 of the RFC:PCNA dataset was down-filtered to 60 Å and used as
744 reference. Selected, well resolved 3D classes were refined with
745 Relion. The cryo-EM density was postprocessed in Relion for
746 estimating the resolution and density modified with PHENIX for
747 model building and refinement (Terwilliger et al., 2020 Table S2).

749 **Model Building and Refinement**

The crystal structure of yeast RFC bound to PCNA (PDB ID: 1SXJ) was used for initial fitting of Autoinhibited1. All subunits were split into globular domains and fitted into the cryo-EM density with UCSF Chimera (Pettersen et al., 2004). The model was adjusted in Coot (Emsley and Cowtan, 2004), and real-space iteratively refined with two macrocycles in PHENIX1.17 (Liebschner et al., 2019). Autoinhibited2,3 cryo-EM densities were rigid body fit with the refined model of Autoinhibited1, manually adjusted in Coot and refined. The refined model of Autoinhibited1 from the DNA dataset (Figure S3C) was fragmented into individual subunit domains and rigid body fitted into the cryo-EM density of Open2. The resulting model was further flexibly fitted and refined with Namdinator (Kidmose et al., 2019). The resulting model was adjusted in Coot and refined in PHENIX. The model of Open2 was used for fitting the Open1 cryo-EM density. The fitted model was manually adjusted in Coot and refined in PHENIX. The cryo-EM density of DNA^{PCNA-closed} (Figure S3C) was fitted using the Autoinhibited1 model and DNA was modelled in Coot. The resulting model was further flexibly fitted and refined with Namdinator (Kidmose et al., 2019). The model was then adjusted in Coot, and refined in PHENIX. The Namdinator output model of DNA^{PCNA-closed} was used for fitting of the DNA^{PCNA-open} cryo-EM density. The fitted model was manually adjusted in Coot and subjected to refinement in PHENIX. Interface areas were analyzed with the PISA server (Krissinel and Henrick, 2007). UCSF Chimera and Pymol were used for figure generation (DELANO and W. L, 2002; Pettersen et al., 2004).

ATPase Assays

0.3 μ M (Figure 6F) or 0.15 μ M RFC (Figure S9D) was incubated with a master mix (3 U/mL Pyruvate kinase, 3 U/mL Lactate dehydrogenase, 1 mM ATP, 670 μ M Phosphoenol pyruvate, 170 μ M NADH, 50 mM Tris (pH 7.5), 0.5 mM TCEP, 5 mM MgCl₂, 200 mM potassium glutamate, 40 mM NaCl), 1 μ M PCNA, and annealed primer/template DNA (2 μ M Figure 6F, varying amounts Figure S9D). ATPase activity was measured at 25 °C with the 2014 EnVision® Multilabel Plate Reader to detect NAD⁺. Rates were obtained from a linear fit of the slopes using GraphPad Prism.

2-AP Fluorescence

2-Aminopurine (2AP) fluorescent samples were excited at 315 nm (5 mm slit width), and emission was detected at 370 nm (7 mm slit width) with a FluoroMax 4 (Horiba Jobin Yvon Inc). Reactions contained 150 or 375 nM annealed DNA and 0.5 or 1 μ M RFC in a buffer with 50 mM Hepes-NaOH pH 7.5, 200 mM NaCl, 4 mM MgCl₂, 1 mM TCEP and were carried out at room temperature. Experiments Figure 6C were performed in the presence of 375 nM DNA, 0.5 μ M RFC, and 2.5 μ M PCNA. Experiments Figure 6D and E were performed with 150 nM DNA, 1 μ M RFC, and 2.5 μ M PCNA.

ACKNOWLEDGMENTS

The authors thank Drs. C. Xu, KK Song, and K. Lee for assistance with data collection, and Drs. C. Xu, and A. Jecrois for advice on data processing. Additionally, we thank Dr. M.

Hingorani for providing us with the RFC expression plasmids. We thank J. Andrade and Dr. B. Ueberheide for analyzing the crosslinked sample with mass spectrometry. We thank members of the Kelch, Royer, and Schiffer labs for helpful discussions. This work was funded by NIGMS (R01-GM127776). C.G. was supported by an Early and Advanced Postdoc Mobility (grant numbers 168972 and 177859) Fellowship of the Swiss National Science Foundation. G.D. was supported by LL2008 project with financial support from the MEYS CR as a part of the ERC CZ program.

AUTHOR CONTRIBUTIONS

C.G. and B.A.K. designed experiments. C.G. performed cryo-EM sample preparations, data collection and processing with the help of G.D. G.D. provided valuable insight into optimization of cryo samples and reconstruction refinement. C.G. & X.L., performed biochemical experiments. C.G., N.P.S., J.A.H. and B.A.K. built and C.G. refined the atomic models. C.G., X.L., J.P., and B.A.K. analyzed the data and wrote the paper with comments from coauthors.

COMPETING INTERESTS

The authors declare no conflict of interest.

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