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 Rossman 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.

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 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.

106 structures of labile complexes (Gerlach et al. 2018; Yoo et al. 2018; 127
107 Gaubitz et al. 2020). Mass spectrometry of the DNA-free sample 128
108 reveals that most cross-links are intra-molecular and map to the 129
109 unresolved N- and C-termini of RFC1, with only a few detectable inter- 130
110 molecular cross-links between RFC subunits (Figure 1 – Supplement 131
111 1B; Table 2). No significant cross-links were observed between RFC 132
112 and PCNA.

113 We imaged the RFC:PCNA complex with and without p/t-DNA using 134
114 a 300 kV Titan Krios microscope (Figure 1 – Supplements 1C,D, 2A,B, 135
115 3A,B; Table 3). 3D classification results in four well-defined 136
116 reconstructions from the DNA-free sample, with overall resolutions 137
117 ranging between 3.8 and 4.0 Å (Figure 1 – Supplement 2D). The 138
118 dataset of the DNA-containing sample yielded several well defined 139
119 classes, with overall resolutions ranging between 3.3 and 3.5 Å (Figure 140
120 1 – Supplement 3C, Table 3). We focused on classes in which all 141
121 subunits of RFC and PCNA are visible, although the N- and C-terminal 142
122 regions of the A subunit lack clear density. The quality of the cryo-EM 143
123 reconstructions readily permitted model building using the crystal 144
124 structure as a template (Bowman et al., 2004; Figure 1 – Supplement 145
125 1C, 1D; Table 3).

126

The initial complex of RFC:PCNA is dynamic

131 Three of the classes from the DNA-free sample are of RFC bound to 132
133 closed PCNA in different conformational states (Figure 2). Overall, 134
135 these structures resemble the previous yeast RFC:PCNA crystal 136
137 structure and our recent cryo-EM structure of human RFC 138
139 (hRFC):PCNA (Figure 2 – Supplement 1A; Bowman et al. 2004; Gaubitz 140
141 et al. 2020). The PCNA ring is closed with only the A, B, and C subunits 142
143 of RFC contacting PCNA (Figure 2B). The interaction area between 144
145 clamp loader and clamp averages ~1940 Å² across the three states. 146
147 The nucleotide density in each of the four active sites are most 148
149 consistent with the presence of ATPyS, although the density for the γ - 150
151 phosphate analog in the D subunit is somewhat ambiguous due to low 152
153 local resolution throughout this subunit. Nonetheless, the ATPase 154
155 sites of the B, C, and D subunits are in an inactive state (Figure 2 – 156
157 Supplement 1B), with the AAA+ spiral in the overtwisted state 158
159 observed in the hRFC structure and the previous yeast RFC crystal 160
161 structure (Figure 2 – Supplement 1C; Bowman et al. 2004; Gaubitz et 162
163 al. 2020). Therefore, all three of these structures represent 164

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 Cα 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.

148 autoinhibited states of RFC (termed Autoinhibited₁, Autoinhibited₂,

149 and Autoinhibited₃). Because the Autoinhibited₁, ₂, and ₃ states likely 183 different observed cryo-EM class averages do not represent particles
150 represent ATP-saturated configurations, we place these 184 in discrete states, but rather snapshots along a continuum of motion.
151 conformational states early in the clamp loading reaction. 185 Thus, the autoinhibited state of RFC is conformationally

152 The subunits in the AAA+ spiral have a different tilt in each of the 186 heterogeneous, with the dominant motions driving RFC toward PCNA.
153 Autoinhibited states, thereby slightly altering the intersubunit 187 We propose these motions represent an early phase of the transition
154 interactions (Figure 2 – Supplement 1C). For instance, the 188 toward opening of the PCNA ring.

155 Autoinhibited₃ state exhibits a slightly cracked A-gate (but not open
156 enough for DNA to pass through), whereas the A-gate is closed in the
157 Autoinhibited₁ and ₂ states (Figure 2A, Figure 2 – Supplement 2A). 189 **PCNA opening is coupled to large-scale expansion of RFC**

158 Further, the AAA+ modules of subunits C-D change their position into
159 a more symmetric alignment with overlapping rotation axes relative to
160 Autoinhibited₁ and ₂ (Figure 2 – Supplement 1C). On the other hand,
161 the PCNA ring tilts ~19° relative to the RFC-D in the Autoinhibited₂
162 state relative to the Autoinhibited₁ and ₃ states (Figure 2A, Figure 2 –
163 Supplement 2A).

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.

164 Despite these differences, the three Autoinhibited structures are
165 very similar, and so we asked if these conformations represent distinct
166 intermediates or if they are snapshots along a continuum of
167 conformations. Therefore, we characterized the particles that
168 contribute to the Autoinhibited states using multi-body refinement
169 (Figure 2 – Supplement 2B-I), a computational tool that allows
170 modeling of macromolecular motion (Nakane et al., 2018). To
171 examine motion between clamp and clamp loader, we defined RFC
172 and PCNA as two independent rigid bodies (Figure 2 – Supplement 2
173 B-E). This analysis revealed that the dominant motion is rocking of
174 PCNA towards RFC, with the linker between the ATPase and collar
175 domains serving as a hinge (Figure 2C, Video 1). Other motions include
176 swiveling of the RFC spiral with RFC-D getting closer to PCNA (Figure
177 2 – Supplement 2E, Video 2). These results are not dependent on the
178 particular mask used, as similar motions are observed using different
179 masking strategies (Figure 2 – Supplement 2F-I). Principal component
180 analysis of the multi-body conformers revealed a unimodal
181 distribution of particles along their eigenvalue (Figure 2D, Figure 2 –
182 Supplement 2 D,H). This unimodal distribution indicates that the three

190 Each of the two cryo-EM datasets revealed a class of RFC bound to
191 open PCNA with no DNA bound (Figure 1 – Supplement 2D, 3C). To our
192 knowledge, these are the first high-resolution structures of a clamp
193 loader bound to an open clamp prior to DNA binding. Both
194 reconstructions are highly similar (overall C_α RMSD is 0.74 Å, map to
195 map correlation coefficient is ~0.85) and we refer to these structures
196 as Open₁ and Open₂ (Figure 3 – Supplement 1A-D). PCNA forms a
197 right-handed spiral with a ~20 Å opening that is wide enough for
198 dsDNA to enter (Figures 3A, B). The PCNA ring opens primarily
199 through in-plane rather than out-of-plane motions (in-plane ~19 Å and
200 out-of-plane ~10 Å for Open₂, Figure 3C). Each of the subunits of
201 PCNA twists outward and towards RFC, with the largest distortion in
202 subunit II (Figure 3 – Supplement 1F).

203 PCNA opens at the A-gate of RFC, disrupting the interaction
204 between the first and third subunits of the PCNA ring (termed PCNA-I
205 and PCNA-III, hereafter). The open PCNA ring is directly held by all five
206 subunits of RFC, burying ~3800 Å² of surface area, an approximate
207 ~1860 Å² increase over that of the Autoinhibited states (Figures 3B,
208 Figure 3 – Supplement 2A,B). The RFC-C subunit shifts downward to
209 interact much more tightly with PCNA-II, while PCNA also forms new
210 interactions with RFC-D, RFC-E, and the A' domain of RFC-A (Figure 3
211 – Supplement 2C,D). The overall interface is characterized by an
212 alternating pattern of strong and weak interactions (strong: RFC-A, -C,
213 & -E; weak: RFC-B, -D, & A'). The strong interactions are with the main
214 partner binding pocket of PCNA, using a binding region that
215 resembles a common motif for PCNA-interacting partners. Of these
216 strong interfaces, RFC-A is the most substantial and RFC-E weakest;
217 RFC-A contains a true PCNA interaction motif, while RFC-C and RFC-
218 E's motifs are increasingly degenerate. It is likely that the stronger
219 interactions at the "bottom" of the spiral allows the clamp loader to
220 toggle between the closed and open states of PCNA without releasing
221 RFC.

222 The AAA+ modules of RFC adopt a right-handed spiral whose
223 periodicity matches that of the six contact sites on PCNA. The
224 symmetry of the ATPase spiral can be visualized by the near perfect
225 alignment of the rotation axes that relate adjacent AAA+ subunits
226 (Figures 3F-G). The interfaces between adjacent AAA+ modules
227 become tighter, bringing the catalytic arginine finger residue closer to
228 the neighboring ATPase site and potentiating ATP hydrolysis. This
229 observation explains the modest boost in ATP activity upon PCNA
230 binding (Johnson et al., 2006; Figure 6F). However, similar to the
231 Autoinhibited structures, all four active sites remain bound to ATP
232 analog (Figure 3 – Supplement 1E). Therefore, while opening is
233 necessary to promote ATP hydrolysis by properly positioning the
234 *trans*-acting arginine finger residues across the inter-subunit interface,
235 ATP hydrolysis is not necessary to drive the conformational change
236 from Autoinhibited to Open and opening is likely not sufficient to
237 stimulate ATP hydrolysis on its own.

238 In order to rupture the PCNA ring, the AAA+ spiral of RFC widens, 256 **Structures of the RFC:PCNA complex bound to primer-template**
 239 opening the A-gate. RFC opens using a large hinge motion, pivoting 257 **DNA**
 240 around the B-C and C-D subunit interfaces (Figure 3D, 3E; Video 3). 258 To reveal how RFC:PCNA binds and responds to DNA, we analyzed
 241 The RFC-E subunit uses its E-plug to bind PCNA, which pulls the A' 259 two classes that contain DNA-bound RFC:PCNA. One class shows
 242 domain and E-plug up to 45 Å away from the AAA+ module. This 260 PCNA in an open lock-washer shape, and the other has PCNA in a
 243 reveals a large opening of the A-gate (at its most narrow, the A-gate is 261 closed conformation. Therefore, we term these two states DNA^{PCNA-open}
 244 approximately 20 Å wide) (Figure 3E). p/t-DNA can therefore directly 262 ^{open} and DNA^{PCNA-closed}, respectively (Figures 5A-5C). Both classes
 245 enter the open RFC:PCNA complex. 263 contain clear density for p/t-DNA: 18 base pairs of duplex DNA are
 246 Opening of the A-gate separates the RFC-A Lid and collar domains, 264 bound inside the central chambers of RFC and PCNA, and 6
 247 inducing a fold-switching transition in the Lid domain. The majority of 265 nucleotides of the ssDNA template extend through the A-gate,
 248 the last helix of the Lid (Helix α₄; residues 541-546) unravels into a taut 266 preventing its closure. The AAA+ spiral of RFC tracks the minor groove
 249 β-strand conformation (Figures 4A, 4B). The remaining residues in 267 of dsDNA using a suite of residues that are conserved across all known
 250 helix α₄ (residues 536-542) shift forward, causing a major change in 268 clamp loaders to match the helical symmetry of DNA (Kelch et al.,
 251 the core packing of the Lid domain. This "sliding spring" motion leads 269 2011; Simonetta et al., 05/2009).
 252 to a ~11 Å helix displacement, whereby some residues, such as Leu 270 The E-plug beta-hairpin slots into the major groove of the duplex
 253 549, move up to 22 Å from their original position. The stretching of the 271 region of p/t-DNA (Figure 5D). Conserved basic residues at the tip of
 254 RFC-A Lid opens a new pore between the A and B subunits (Figure 272 the E-plug interact directly with both the template and primer strands.
 255 4B). We discuss the role of this pore in the next section. 273 Therefore, the E-plug provides a mechanism for the RFC AAA+ spiral
 274 to recognize both strands of DNA, unlike the clamp loaders from *E.*
 275 *coli* and T4 phage, whose AAA+ spirals only interact with the template
 276 strand (Kelch et al., 2011; Simonetta et al., 05/2009). Moreover, this

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 structure shows that the E-plug changes its role from blocking DNA 295 similar to that seen for the structure of the T4 phage clamp bound to
 278 binding (in the three Autoinhibited states) to one in which it directly 296 clamp loader and p/t-DNA (Kelch et al., 2011).
 279 supports DNA binding. This explains the non-intuitive effect on DNA 297 The DNA^{PCNA-closed} structure has a closed PCNA ring that is distorted
 280 binding we observed previously, where hRFC variants with a mutated 298 from planarity. Upon closure, PCNA loses its interaction with the RFC-
 281 E-plug bind DNA with equivalent affinity as WT-hRFC (Gaubitz et al., 299 E subunit, but retains its interfaces with the other four RFC subunits
 282 2020b). 300 (Figures 5E, 5F). The distortion of the PCNA ring is most prevalent in

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 ATPyS 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 ATPyS. (F) ATPase activity of the "separation pin" mutants. The ATP hydrolysis rate of the RFC-W638G variant is significantly reduced compared to wild type in the presence of PCNA and DNA (p value from one-way ANOVA test: **** P ≤ 0.0001).

283 In DNA^{PCNA-open}, both RFC and PCNA broadly resemble the 301 subunit III, which puckers to maintain its interaction with the RFC-D
 284 conformations seen in Open1 and Open2. The RFC A-gate is open, 302 subunit (Figure 5 – Supplement 1A,B). Interestingly, the interaction
 285 with all 5 subunits gripping PCNA in an open lock-washer shape. 303 between DNA and PCNA becomes more extensive upon PCNA closure
 286 However, both RFC and PCNA constrict relative to the Open1 and 304 (~50 Å² vs 250 Å²). Conserved basic residues lining the inner pore of
 287 Open2 structures (Figures 5G, 5H and Figure 5 – Supplement 1A,B). 305 PCNA also interact directly with the duplex, as has been hypothesized
 288 RFC constricts modestly, pivoting the E, D, and C subunits around a 306 previously (Liu et al., 2017; McNally et al., 2010). We propose that
 289 hinge at the B-C interface. PCNA constricts ~12 Å upon DNA binding, 307 these interactions help to drive the closure of PCNA around DNA.
 290 with most of this constriction occurring in subunit III of PCNA (Figure 5 308 DNA^{PCNA-open} and DNA^{PCNA-closed}, just like the other states described
 291 – Supplement 1A). Subunit III of PCNA is held by the RFC-D and RFC-E 309 herein, are in the fully ATP-bound state: ATPyS in the active sites of
 292 subunits, although RFC-E grips PCNA less tightly in DNA^{PCNA-open} 310 the A, B, C, and D subunits, and ADP in the nucleotide-binding site of
 293 (~3800 Å² total RFC-PCNA interaction area for the Open1&2 structures 311 the E subunit (Figure 5 – Supplement 1C). Therefore, these structures
 294 vs ~3400 Å² for DNA^{PCNA-open}). Overall, the PCNA conformation is 312 represent reaction intermediates following DNA binding but
 313 preceding ATP hydrolysis. Upon binding DNA, the AAA+ spiral

314 constricts (Figure 5H), primarily due to a hinge-like motion at the 372 interface between RFC-C and RFC-B. The AAA+ spiral constricts 373 around an axis coincident with the DNA axis. Subsequent PCNA 374 closure further exaggerates the constriction of the RFC AAA+ spiral 375 (Figure 5I). Despite these movements, the position of the arginine 376 finger within the ATPase active site does not change substantially 377 (Figure 5 – Supplement 2). Thus, DNA binding likely stimulates ATP 378 hydrolysis through another mode of action. One such proposed mode 379 is repositioning a conserved arginine known as the switch residue, 380 which in turn would activate the Walker B glutamate (Kelch et al., 381 2011, 2012). However, we find that this residue is not in the position 382 that was previously predicted to stimulate hydrolysis. Despite this, the 383 active sites appear to be in the fully active state, with all of the 384 catalytic machinery poised to hydrolyze ATP. We discuss the 385 ramifications of this observation on the allosteric activation of RFC 386 below.

387 RFC flips the 3' base of the primer strand

388 Unexpectedly, we observe that the 3' nucleotide of the primer strand 389 is melted in both DNA-bound RFC:PCNA structures, with the base 390 flipped away from the rest of the duplex (Figure 6A, 6B). The base pair 391 is disrupted by a "separation pin" at the base of the RFC-A collar 392 domain that wedges between the DNA strands (Figure 6B). The indole 393 ring of Trp638 replaces the flipped 3' base to maintain stacking 394 interactions. The 3' nucleotide is repositioned inside the pore formed 395 by the unraveling of the RFC-A Lid domain upon opening of the A- 396 gate; this site is closed in the Autoinhibited state (Figure 4). The 397 flipped base stacks against the phenyl ring of Phe582. These residues 398 are conserved in eukaryotic clamp loaders but are absent in bacterial, 399 archaeal or phage clamp loaders (Figure 6 – Supplement 5A). 400 To characterize base flipping, we measured binding of DNA 401 substrates containing the adenine analog 2-aminopurine (2AP). 402 Fluorescence of 2AP is dependent on base-pairing (Frey et al., 1995; 403 Jean and Hall, 2001): fluorescence is low when 2AP is base paired, but 404 high in the free state. To monitor melting, we placed 2AP either at the 405 3' end of the primer strand ($AP_{p=1}$) or at the corresponding site in the 406 template strand ($AP_{t=1}$). Importantly, we find a dramatic increase in 407 2AP fluorescence that is dependent on addition of RFC, PCNA and 408 ATP analog (Figures 6C, Figure 6 – Supplement 1A-C). The increase in 409 2AP fluorescence is not observed in the presence of ADP, which does 410 not support DNA binding (Kelch et al., 2011; McNally et al., 2010).

411 Placement of 2AP at the $p=2$ or $p=3$ position of the primer yields 412 diminished fluorescence, suggesting that only the 3' base is flipped 413 (Figure 6 – Supplement 1B). Therefore, our 2AP experiments validate 414 that RFC- and PCNA-dependent 3' end melting occurs in solution. The 415 human clamp loader, which has a similar separation pin as yeast RFC 416 (Gaubitz et al., 2020), greatly enhances 2AP fluorescence. However, 417 the *E. coli* clamp loader, which binds p/t-DNA but does not melt the 418 primer strand (Simonetta et al., 05/2009), does not alter fluorescence 419 (Figure 6D). Thus melting of the 3' nucleotide is a conserved activity 420 of eukaryotic clamp loaders, but is likely not used by bacterial clamp 421 loaders.

422 To determine the mechanism and role of primer melting, we 423 modified the p/t-DNA and/or key residues in the separation pin and 424 assessed their effects on base flipping, ATP hydrolysis, and DNA 425 affinity. The W638G and F582A variants have attenuated base-flipping 426 as measured by 2AP fluorescence (Figures 6E, Figure 6 – Supplement 427 1C). However, DNA-dependent ATP hydrolysis is minimally affected,

particularly in the F582A variant, whose ATPase rate and apparent affinity for DNA are similar to WT (Figures 6F and Figure 6 – Supplement 1D,E). These results indicate that base flipping requires the separation pin, but base flipping is not required for DNA binding or ATPase activation.

We hypothesized that the base-flipping mechanism functions to specifically recognize the 3' end of the primer. By flipping the base, the separation pin could potentially act as a quality control mechanism to verify proper status of the primer end. We tested this hypothesis by measuring how WT-RFC and the W638G and F582A variants respond to various nucleic acid architectures. If our hypothesis were true, we would expect that the W638G and F582A variants would lose the ability to discriminate against 'incorrect' nucleic acid substrates. We tested ATPase activity against a series of nucleic acid substrates that include: ssDNA, 3' phosphate, 3' abasic sites, a 3' ribonucleotide, an RNA primer, ssDNA-dsDNA junctions of opposite polarity (i.e. recessed 3' ends, Figure 6 – Supplement 2A). We performed these assays using nucleic acid concentrations at or near the K_d for the various forms of RFC Figure 6 – Supplement 1A, so that any deviations in activity or binding would be observable. However, we observe nearly identical ATPase activity profiles for the variants as we do for WT-RFC (Figure 6 – Supplement 2B-D). Therefore, the biochemical characterization of variants with reduced base-flipping does not support our hypothesis that the separation pin acts to discriminate against incorrect substrates.

To directly assess the physiological role of base-flipping in normal RFC function, we measured growth of yeast strains carrying the WT, W638G, or F582A variants as the only copy of RFC1 (Figure 6 – Supplement 3). We tested yeast growth across a wide variety of DNA damaging treatments: ultraviolet radiation (UV), hydroxyurea (HU) or methylmethane sulfonate (MMS). Because base-flipping is thought to have a strong temperature dependence (Yin et al. 2014), we measured yeast growth over a broad temperature range (18 °C to 37 °C). Surprisingly, we find no obvious growth phenotype across our broad spectrum of conditions (Figure 6 – Supplement 3). Thus, we currently find no obvious role for the separation pin, despite its conservation in RFC complexes across all eukaryotes. Further investigation will be required to discern the functional role, if any, of the base-flipping mechanism of RFC.

422 DISCUSSION

423 Defining the clamp loading reaction in high-resolution

424 We have determined a series of structures that provide a high-resolution view of the clamp loading process. Our structures 425 correspond to numerous reaction intermediates, allowing us to order 426 the structures into a coherent description of the clamp loading 427 reaction prior to ATP hydrolysis (Figure 7 and Video 4). The 428 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 a possible stable intermediate that forms if ATP hydrolysis were stalled for whatever reason (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 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

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.

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 cannot 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

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 hydrolysis. For proper clamp loading, RFC must bind PCNA first, because initial binding of p/t-DNA would sterically hinder binding of the PCNA ring. Therefore, RFC has evolved high affinity for PCNA and only binds p/t-DNA with high affinity after it has bound PCNA (Cai et al., 1998; Shiomi et al., 2000). The crab-claw mechanism for PCNA opening can explain this hierarchy of binding, as the auto-inhibited state blocks the DNA binding site ((Gaubitz et al., 2020) and Figure 3E). The crab-claw mechanism ensures that RFC's DNA-binding chamber only becomes accessible once the PCNA ring is

open. Our second hypothesis is that the crab-claw mechanism enables complex modes of clamp loader regulation. Clamp loader activity could be inhibited by binding partners or post-translational modifications that favor the Autoinhibited state. There are numerous RFC binding partners and post-translational modifications that remain unexplored, and thus are candidates for playing regulatory roles (Dephoure et al., 2008; Kim and Brill, 2001; Ochoa et al., 2020; Olsen et al., 2010; Tomida et al., 2008; Wang et al., 2012, 2013).

RFC-A subunit drives DNA recognition

To illuminate how RFC recognizes DNA, we measured the relative contribution of each RFC subunit to DNA binding. We find that RFC-A accounts for ~64% of the buried surface area between RFC and DNA. This contrasts with T₄ and *E. coli* clamp loaders, where the A subunits account for ~36% of the binding interface (Figure 6 – Supplement 4B). Much of this proportional increase arises from additional interactions between RFC-A and DNA through the separation pin and the flipped 3' nucleotide. Furthermore, we find that B, C, D, and E subunits of RFC interact with DNA significantly less (~760 Å²) than the comparable subunits of T₄ and *E. coli* (~1125 Å²). The decrease in DNA interaction from the B, C, D, and E subunits is due to the p/t-DNA duplex region inserting deeper into the AAA+ spiral of the T₄ and *E. coli* clamp loaders than in RFC (Figure 6 – Supplement 4A). Therefore, the large swing in the proportional interaction area is the net result of additional interactions from RFC-A and less from the remaining subunits.

This proportionally large interaction area suggests RFC-A as the subunit primarily responsible for recognizing DNA. This finding provides an attractive explanation for how alternative clamp loaders specifically recognize different DNA structures. RFC-like Complexes or RLCs are only found in eukaryotes and share four of RFC's five subunits (RFC-B through RFC-E); each RLC contains a unique A subunit (Majka and Burgers, 2004). We hypothesize that the diminished role of the B, C, D, and E subunits in DNA recognition allows the A-subunit to assume the role of specifically binding unique structures of DNA. In support of this hypothesis, bacterial and phage clamp loaders do not have alternative forms that recognize different DNA structures, and their clamp loaders have substantially more contact between DNA and the B, C, D, and E subunits. The more pronounced role of the A subunit in eukaryotic clamp loaders allows for dramatically more plasticity in function. Further, the diminished role of the remaining subunits raises the question of how the pivot point at the C subunit contributes to the activity of RLC complexes. Finally, these findings raise the intriguing possibility of engineering RLCs with novel specificity and activity.

Following this reasoning even further, we hypothesized that RFC flips the 3' nucleotide to specifically recognize the recessed 3' end of p/t-DNA. We observe flipping of the 3' nucleotide in both the DNA^{PCNA-open} and DNA^{PCNA-closed} structures, indicating that flipping can occur before ring closure. This observation can explain the "DNA repositioning transition" that occurs quickly (t_{1/2} ~ 35 ms) after initial DNA binding, but before clamp closure (Liu et al., 2017). We propose that this transition is the flipping of the 3' nucleotide. However, the flipping mechanism does not appear to be used to discriminate between different DNA architectures. The W638G and F582A variants have a similar DNA discrimination profile as WT-RFC, despite having very different base-flipping activity (Figure 6E, Figure 6 – Supplement 2). Moreover, the physiological role of base-flipping is unclear, as yeast carrying these variants have no obvious cellular defects (Figure 6

– Supplement 3). We still hypothesize that there is likely a role for the flipping activity, as the separation pin is conserved across RFC complexes from yeast to humans. Moreover, this separation pin is not found in the related 9-1-1 clamp loader Rad24-RLC (Castaneda et al. 2021; Zheng et al. 2021). A separation pin extension is found in the related loader Ctf18 (Figure 6 – Supplement 5B) but the flipping amino acids are not conserved. (Predictions for or against a separation pin in the final loader subunit Elg1 are weak due to very limited sequence homology between RFC1 and Elg1.) Future experiments will investigate the role of base-flipping 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 ~4000 Å² (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. Rapid kinetics studies showed that ATP hydrolysis precedes clamp closure under normal conditions (Liu et al., 2017; Marzahn et al., 2014; Sakato et al., 02/2012). Taken together, these two points could lead to the conclusion that ATP hydrolysis provides the energy to actively close the clamp after loading DNA. However, we observe this transition from our DNA^{PCNA-open} and DNA^{PCNA-closed} structures, and neither structure shows evidence of ATP hydrolysis, suggesting that PCNA can close before ATP hydrolyzes. To harmonize all of the available data, we must draw a new conclusion, which is that while ATP hydrolysis typically occurs prior to clamp closure, it is not strictly required, and clamp closure can precede hydrolysis if the hydrolysis step becomes rate-limiting, as would likely occur with the slowly hydrolysable ATPγS. It still remains possible that

ATP hydrolysis could make clamp closure *easier*, by weakening interactions between RFC and PCNA/DNA, but in this view clamp closure is still a spontaneous process and does not require harvesting energy from ATP hydrolysis. Therefore, it is possible that ATP hydrolysis can proceed from either DNA^{PCNA-open} and DNA^{PCNA-closed} states, but most commonly from the DNA^{PCNA-open} state.

This raises the question as to how DNA stimulates ATP hydrolysis and subsequent ejection of the clamp loader. We note that the ATPase active sites do not change much from the Open to DNA^{PCNA-open} or DNA^{PCNA-closed} conformations (Figure 5 – Supplemental 2). It is also surprising that the AAA+ modules are already in a symmetrized pose prior to DNA loading, 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. Despite this symmetry, the RFC:PCNA complex (corresponding to 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). This implies that, whereas clamp opening is both necessary and sufficient for symmetrizing the AAA+ modules, this symmetry by itself is not sufficient to stimulate ATP hydrolysis.

There remain many possible avenues for DNA to stimulate ATP hydrolysis. In many AAA+ enzymes, it has been shown that certain residues couple ligand binding and ATP hydrolysis by activating the Walker B glutamate residue (Zhang and Wigley 2008). A set of conserved arginines (termed the arginine switch residues) within the core of the AAA+ module were proposed to play this role in clamp loaders (Kelch et al., 2011). The arginine switch residues had been hypothesized to flip outward to grip DNA, thereby releasing the Walker B catalytic glutamate to activate ATP hydrolysis. However, the lack of flipping of the arginine switch residues in response to DNA binding in our structures 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). An alternative route, involving a different arginine residue interacting with the ATPase active site, has recently been proposed for DnaC and extended to RFC (Puri et al. 2021). However, we again do not see structural evidence supporting this mechanism. We cannot rule out these mechanisms (or a combination of the two), as these types of interactions may occur just before hydrolysis and are not readily apparent in stalled structures. A recent study on the T₄ clamp loader suggests that structural rigidity of a “central coupler” that encircles DNA is important for hydrolysis (Subramanian et al. 2021). Thus, tight binding of RFC to DNA could provide rigidity necessary to stimulate ATP hydrolysis.

Lastly, we note that 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 and its role in stimulating ATPase activity

upon DNA binding should not be overlooked. 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.

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (<i>Escherichia coli</i>)	BL21(DE3)	Novagen	69450	Chemically competent cells
recombinant DNA reagent	pET(11a)-RFC[2+3+4] (plasmid)	Finkelstein et al., 2003		Expression plasmid
recombinant DNA reagent	pLANT-2/RIL[1+5] (plasmid)	Finkelstein et al., 2003		Expression plasmid
recombinant DNA reagent	pRS413-RFC1 (plasmid)	This study		Plasmid for yeast expression of Rfc1 from endogenous promotor
Strain, strain background (<i>S. cerevisiae</i>)	BY4743 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 Δrfc1::KanMX4/RFC1 (YOR217W)	Dharmacon™	YSC1055 (22473)	Yeast Heterozygous Collection
software, algorithm	RELION	doi: 10.7554/eLife.42166	Relion 3.0.2	
software, algorithm	cisTEM	doi: 10.7554/eLife.35383	cisTEM-1.0.0-beta	https://cistem.org/software
software, algorithm	Ctffind	doi: 10.1016/j.jsb.2015.08.008	Ctffind 4.1	
software, algorithm	UCSF Chimera	UCSF, doi: 10.1002/jcc.20084		http://plato.cgl.ucsf.edu/chimera/
software, algorithm	ChimeraX	UCSF, doi: 10.1002/pro.3943	ChimeraX-1.2	https://www.cgl.ucsf.edu/chimerax/
software, algorithm	COOT	doi: 10.1107/S0907444910007493	Coot-0.9.4	http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
software, algorithm	Phenix	doi: 10.1107/S0907444909052925	Phenix-dev-3699	https://phenix-online.org

software, algorithm	PyMOL	PyMOL Molecular Graphics System, Schrodinger LLC		https://www.pymol.org/
software, algorithm	GraphPad PRISM	GraphPad	GraphPad PRISM 9.2.1	http://www.graphpad.com/
other	Pyruvate kinase	Calzyme	107A0250	
other	Lactate Dehydrogenase	Worthington Biochemical Cooperation	LS002755	
other	Phosphoenol- pyruvic acid monopotassium salt	Alfa Aesar	B20358	

683 Protein Purification

684 RFC was purified as described previously with minor modifications
685 (Finkelstein et al., 08/2003). pET(11a)-RFC[2+3+4] and pLANT-2/RIL-
686 RFC[1+5] were transformed into BL21(DE3) *E. coli* cells (Millipore).
687 After preculture, transformants were grown in 4 liter of prewarmed
688 terrific broth medium supplemented with 50 µg/mL kanamycin and
689 100 µg/mL ampicillin at 37 °C and induced with IPTG at an optical
690 density of 0.8. Protein expression was continued at 18 °C for 15 hours.
691 Cells were pelleted and resuspended in 300 ml lysis buffer (30 mM
692 Hepes-NaOH pH 7.5, 250 mM NaCl, 0.25 mM EDTA, 5% glycerol, 2
693 mM DTT, 2 µg/mL aprotinin, 0.2 µg/mL pepstatin, 2 µg/mL leupeptin,
694 1 mM PMSF). RFC was purified by chromatography over a 10 mL SP-
695 Sepharose column (80 mL gradient of 300–600 mM NaCl in BufferC)
696 and a 10 mL Q-Sepharose column (40 mL gradient of 150–500 mM
697 NaCl in Buffer C, GEHealthcare). Peak fractions of hRFC were pooled
698 and dialyzed overnight into a buffer with 30 mM Hepes-NaOH pH 7.5,
699 250 mM NaCl, 5% glycerol, and 2 mM DTT.

700 PCNA was purified as described previously with modifications
701 (McNally et al., 2010). BL21(DE3) *E. coli* cells were transformed with a
702 pET-28 vector that encodes PCNA with a Precision™ protease
703 cleavable N-terminal 6-His tag. After transformation, preculture and
704 induction, 1 liter of cells was grown overnight at 18 °C in terrific broth
705 medium supplemented with 50 µg/mL kanamycin. Cells were pelleted
706 and resuspended 30 mM Hepes pH 7.6, 20 mM imidazole, 500 mM
707 NaCl, 10% glycerol, and 5 mM β-mercaptoethanol. The cells were
708 lysed, centrifuged and the filtered lysate was applied to a 5 mL
709 HisTrap FF column (GEHealthcare). The column was washed with a
710 buffer at 1 M NaCl, and subsequently washed with a buffer at a low
711 salt concentration (50 mM NaCl). PCNA was eluted with a step of 50 %
712 with 500 mM imidazole. The eluted protein was cleaved with
713 Precision™ protease for 2 hours at room temperature and applied to
714 a 5 mL HiTrap Q HP column (GE Healthcare). Protein was eluted from
715 the Q HP column with a 2M NaCl buffer in a 100 mL gradient. Peak
716 fractions were dialyzed against buffer containing 30 mM Tris pH 7.5,
717 100 mM NaCl, and 2 mM DTT. Purified proteins were concentrated
718 with an Amicon concentration device, aliquoted and frozen in liquid
719 nitrogen for storage at -80 °.

720 Crosslinking and Mass Spectrometry

721 RFC and PCNA were mixed in a 1/1 ratio and gel filtered into 1 mM
722 TCEP, 200 mM NaCl, 25 mM Hepes-NaOH, pH 7.5, and 4 mM MgCl₂.
723 The protein complex was diluted to 3 µM and after the addition of 1
724 mM ATPγS and 3 min incubation, 1 mM of
725 Bis(sulfosuccinimidyl)suberate (BS₃, Thermo Scientific Pierce) was
726 added for crosslinking. For crosslinking of DNA-bound RFC:PCNA, 1
727 mM ATPγS was added to the protein complex first and incubated for 2
728 min. 7 µM primer/template DNA was added and incubated for another
729 1 min. The primer sequence was 5'-GCAGACACTACGAGTACATA-3'
730 and the template sequence was 5'-
731 TTTT TTTT TTTT ATGTACTCGTAGTGTCTGC-3'. Crosslinking was
732 started with 1 mM BS₃, incubated for 15 min at room temperature,
733 and neutralized with Tris-HCl.

734 Sample without DNA was analyzed by mass spectrometry. The
735 sample was reduced, alkylated, and loaded onto SDS-PAGE gel. The
736 gel band corresponding to the crosslinked complex >150kDa was

737 excised, destained, and incubated with trypsin. The digested peptides
738 were extracted and desalted as previously described (Peled et al.,
739 2018) and analyzed with LC-MS coupled to a ThermoFisher Scientific
740 Q Exactive Mass Spectrometer in data dependent mode selecting only
741 precursors of 3. The data was searched against the UniProt database,
742 using Byonic and XlinkX of the Proteome Discoverer 2.3 package.

743 Electron Microscopy

744 Negative-Staining EM

745 100 nM of RFC:PCNA was applied on carbon-coated 400-mesh grids.
746 Excess sample was blotted from the grid surface, the grids were
747 washed twice with 50 mM Hepes pH 7.5 and stained with 1% uranyl
748 acetate. RFC:PCNA was imaged on a 120 kV Philips CM-120
749 microscope fitted with a Gatan Orius SC1000 detector.

751 Cryo-EM sample preparation

752 Quantifoil R 0.6/1 (DNA dataset) grids were washed with ethyl
753 acetate. Quantifoil and C-flat grids (Electron Microscopy Sciences)
754 were glow discharged with Pelco easiGlow for 60 s at 25 mA
755 (negative polarity). 2.8 - 3 µL sample was applied to grids at 10 °C and
756 95% humidity in a Vitrobot Mark IV (FEI). Samples were blotted with a
757 force of 5 for 5 s after a 2 s wait and plunged into liquid ethane.

759 Cryo-EM data collection

760 RFC:PCNA was imaged on a Titan Krios operated at 300 kV and
761 equipped with an GIF energy filter at 130000 × magnification and a
762 pixel size of 0.53 Å using a K2 Summit detector in super-resolution
763 counting mode. The data was collected in four sessions with a target
764 defocus range of -1.1 to -2.4 and a total exposure of ~49-51 e-/Å² per
765 micrograph averaging 50 frames. Image shift was used to record three
766 images per hole with SerialEM (Mastronarde, 2003). Defective
767 micrographs were discarded leaving a total of 6109 micrographs for
768 processing. RFC:PCNA:DNA was imaged on a Titan Krios operated at
769 300 kV at 81000 × magnification and a pixel size of 0.53 Å with a K3
770 detector in super-resolution mode. A total of 4499 micrographs were
771 collected in one day with a target defocus of -1.2 to -2.3 and a total
772 exposure of ~40 e-/Å² per micrograph averaging 30 frames.

774 Data Processing

775 Micrograph frames were aligned in IMOD (Kremer et al., 1996) with 2x
776 binning, resulting in a pixel size of 1.06 Å/pixel. Initial CTF estimation
777 and particle picking was performed using cisTEM (Grant et al., 2018;
778 Rohou and Grigorieff, 2015). Following particle picking, particles were
779 extracted with a box size of 240 pixels and subjected to 2D
780 classification into 100 classes. Particles from classes with well defined
781 features were selected for processing in Relion (Figure 1 –
782 Supplements 2A,B, 3A,B). Coordinates and combined micrographs
783 were imported into Relion 3.0.2 (Zivanov et al., 2018), CTF parameters
784 were re-estimated with Gctf1.06 (Zhang, 2016) and particles were
785 subjected to several rounds of 3D classification (Figure 1 –
786 Supplements 2D, 3C). For 3D classification of the RFC:PCNA dataset,
787 an ab initio model was generated with cisTEM, down-filtered to 50 Å
788 and used as reference (Figure 1 – Supplement 2C). For 3D classification
789 of the RFC:PCNA:DNA dataset, class Open1 of the RFC:PCNA dataset
790 was down-filtered to 60 Å and used as reference. Selected, well
791 resolved 3D classes were refined with Relion. The cryo-EM density was

792 postprocessed in Relion for estimating the resolution and density 846 performed in the presence of 375 nM DNA, 0.5 μ M RFC, and 2.5 μ M
793 modified with PHENIX for model building and refinement (Terwilliger 847 PCNA. Experiments Figure 6D and E were performed with 150 nM
794 et al., 2020 Table 3). Model information was not used during density 848 DNA, 1 μ M RFC, and 2.5 μ M PCNA.

795 modification.

796

797 **Model Building and Refinement**

798 The crystal structure of yeast RFC bound to PCNA (PDB ID: 1SXJ) was
799 used for initial fitting of Autoinhibited₁. All subunits were split into
800 globular domains and fitted into the cryo-EM density with UCSF
801 Chimera (Pettersen et al., 2004). The model was adjusted in Coot
802 (Emsley and Cowtan, 2004), and real-space iteratively refined with
803 two macrocycles in PHENIX1.17 (Liebschner et al., 2019).
804 Autoinhibited_{2,3} cryo-EM densities were rigid body fit with the refined
805 model of Autoinhibited₁, manually adjusted in Coot and refined.
806 The refined model of Autoinhibited₁ (Figure 1 – Supplement 1C) was
807 fragmented into individual subunit domains and rigid body fitted into
808 the cryo-EM density of Open₂. The resulting model was further
809 flexibly fitted and refined with Namdinator (Kidmose et al., 2019). The
810 resulting model was adjusted in Coot, and refined in PHENIX. The
811 model of Open₂ was used for fitting the Open₁ cryo-EM density. The
812 fitted model was manually adjusted in Coot and refined in PHENIX.
813 The cryo-EM density of DNA^{PCNA-closed} (Figure 1 – Supplement 3C) was
814 fitted using the Autoinhibited₁ model and DNA was modelled in Coot.
815 The resulting model was further flexibly fitted and refined with
816 Namdinator (Kidmose et al., 2019). The model was then adjusted in
817 Coot, and refined in PHENIX. The Namdinator output model of
818 DNA^{PCNA-closed} was used for fitting of the DNA^{PCNA-open} cryo-EM
819 density. The fitted model was manually adjusted in Coot and
820 subjected to refinement in PHENIX. Interface areas were analyzed
821 with the PISA server (Krissinel and Henrick, 2007). UCSF Chimera and
822 Pymol were used for figure generation (DELANO and W. L., 2002;
823 Pettersen et al., 2004).

824 **ATPase Assays**

825 0.3 μ M (Figure 6F) or 0.15 μ M RFC (Figure S9D) was incubated with a
826 master mix (3 U/mL Pyruvate kinase, 3 U/mL Lactate dehydrogenase,
827 1 mM ATP, 670 μ M Phosphoenol pyruvate, 170 μ M NADH, 50 mM Tris
828 (pH 7.5), 0.5 mM TCEP, 5 mM MgCl₂, 200 mM potassium glutamate,
829 40 mM NaCl, 1 μ M PCNA, and annealed primer/template DNA (2 μ M
830 Figure 6F, varying amounts Figure 6 – Supplement 1D). ATPase
831 activity was measured at 25 °C with the 2014 EnVision® Multilabel
832 Plate Reader to detect NAD⁺. Rates were obtained from a linear fit of
833 the slopes using GraphPad Prism. For the ATPase activity
834 measurements shown in Figure 6 – Supplement 2, 0.12 μ M RFC was
835 incubated with 1 μ M PCNA and 0.03 μ M different DNA constructs (as
836 described in Table 4) and the master mix and buffer described above.
837 ATPase activity was measured at room temperature. For each data
838 point three experimental replicates were performed.

839 **2-AP Fluorescence**

840 2-Aminopurine (2AP) fluorescent samples were excited at 315 nm (5
841 mm slit width), and emission was detected at 370 nm (7 mm slit width)
842 with a FluoroMax 4 (Horiba Jobin Yvon Inc). Reactions contained 150 or
843 375 nM annealed DNA (Table 4) and 0.5 or 1 μ M RFC in a buffer with 50
844 mM Hepes-NaOH pH 7.5, 200 mM NaCl, 4 mM MgCl₂, 1 mM TCEP and
845 were carried out at room temperature. Experiments Figure 6C were

849

850 **Plasmid generation**

851 The separation pin variants were introduced with site-directed
852 mutagenesis in either pLANT-2/RIL-RFC[1+5] for protein purification
853 or pRS413-RFC₁ for yeast complementation. pRS413-RFC₁ contains
854 the entire RFC₁ sequence, where RFC₁ is expressed under the control
855 of its own promotor.

856

857 **Yeast strains and spot assay**

858 The genotype of the *S. cerevisiae* strain which was used in this study
859 for transformation with the pRS413 plasmids is described in the Key
860 Resources Table. *S. cerevisiae* culture, transformation, and tetrad
861 dissection, were performed as previously described (Gomes et al.,
862 2000).

863 For the spot assay, yeast grown on SC-His plate at 30°C for 2 days was
864 inoculated into 3ml SC-His media and grown for 3-4 hours to an OD of
865 0.8. Serial 10-fold dilutions of the culture starting from OD of 0.2 were
866 plated as 4 μ l drops onto YPD plates with or without chemical additives
867 (0.01% methyl methanesulfonate (MMS), 100 mM hydroxyurea (HU)).
868 For UV treatment, the spotted yeast was irradiated with 30, or 100
869 J/m² using a UVP UV Crosslinker. The plates were imaged after
870 incubating at 18°C for 7 days, or at 30°C, 37°C for 3 days.

871

872 **ACKNOWLEDGMENTS**

873 The authors thank Drs. C. Xu, KK Song, and K. Lee for assistance with
874 data collection, and Drs. C. Xu, and A. Jecrois for advice on data
875 processing. Additionally, we thank Dr. M. Hingorani for providing us
876 with the RFC expression plasmids. We thank J. Andrade and Dr. B.
877 Ueberheide for analyzing the crosslinked sample with mass
878 spectrometry. We thank members of the Kelch, Royer, and Schiffer
879 labs for helpful discussions. This work was funded by NIGMS (R01-
880 GM127776). C.G. was supported by an Early and Advanced Postdoc
881 Mobility (grant numbers 168972 and 177859) Fellowship of the Swiss
882 National Science Foundation. G.D. was supported by LL2008 project
883 with financial support from the MEYS CR as a part of the ERC CZ
884 program.

885

886 **COMPETING INTERESTS**

887 The authors declare no conflict of interest.

888

889 **VIDEOS**

- 890 **Video 1** RFC Motion along Eigenvalue 1 with masks on PCNA and RFC
891 **Video 2** RFC Motion along Eigenvalue 2 with masks on PCNA and RFC
892 **Video 3** Morph closed to open
893 **Video 4** PCNA loading by RFC

894 **SOURCE DATA**

- 895 **Figure 1 – Source data 1** Image of full RFC and PCNA gel filtration gel.
896 **Figure 6 – Source data 1** 2-aminopurine fluorescence and ATPase data.
897 **Figure 6 – Figure Supplement 1– Source data 1** 2-aminopurine fluorescence and ATPase data.
898 **Figure 6 – Figure Supplement 2– Source data 1** ATPase data.
899

900 **TABLES**

901 **Table 1:** Clamp loader structures previously obtained for the various states in the clamp loading cycle.

Clamp loader prior to clamp binding			
Species	Composition	Reference	PDB accession number
<i>E. coli</i>	Clamp loader alone	Jeruzalmi et al., 2001	1JR3
<i>E. coli</i>	Clamp loader, ADP	Kazmirski et al., 2004	1XXI
<i>E. coli</i>	Clamp loader, ATP analog	Kazmirski et al., 2004	1XXH
<i>E. coli</i>	Clamp loader, ATP analog, primer/template DNA	Simonetta et al., 2009	3GLF
Encounter complex of clamp loader bound to the closed clamp			
<i>H. sapiens</i>	Clamp loader bound to the clamp, ATP analog	Gaubitz et al., 2020	6VVO
<i>S. cerevisiae</i>	Clamp loader bound to the closed clamp, ATP analog	Bowman et al., 2004	1SXJ
Clamp loader bound to the clamp and primer/template DNA			
T4 phage	Clamp loader, open clamp, ATP analog, DNA	Kelch et al., 2011	3U6o
T4 phage	Clamp loader, closed clamp, ATP analog, DNA	Kelch et al., 2011	3U5Z
T4 phage	Clamp loader, closed clamp, ATP analog, ADP, DNA	Kelch et al., 2011	3U61

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903

904 **Table 2: List of BS3 Crosslinks**

XlinkX Score	Type	# Crosslink Spectral Matches	Sequence A	Position A	Sequence B	Position B	Protein A	Protein B
58,66	Inter	1	[K]LHLPPGK	100	[K]LAATR	274	RFC4	RFC1
58,64	Inter	3	[K]LELNVVSSPYHLEITPSDM GNDR	82	S[K]TLLNAGVK	385	RFC5	RFC1
56,99	Inter	3	[K]YVNTFMK	285	DIL[K]R	220	RFC2	RFC5
56,47	Inter	1	NQI[K]DFASTR	98	[K]LAATR	274	RFC3	RFC1
52,59	Inter	2	E[K]VKNFAR	109	TME[K]YSK	160	RFC2	RFC5
50,97	Inter	1	NQI[K]DFASTR	98	RPDANSI[K]SR	484	RFC3	RFC1
48,17	Inter	1	GASEALA[K]R	182	[K]IVKER	269	RFC1	RFC5
45,16	Inter	1	YT[K]NTR	139	[K]EEER	267	RFC3	RFC1
41,65	Inter	1	[K]LEEQHNIATK	249	YT[K]NTR	139	RFC1	RFC3
91,6	Intra	3	[K]LEEQHNIATK	249	RPDANSI[K]SR	484	RFC1	RFC1
72,73	Intra	4	EAELLV[K]KEEER	266	[K]LAATR	274	RFC1	RFC1
71,87	Intra	12	QLIAGMPAEGGDGEAAE[K] AR	245	R[K]LEEQHNIATK	249	RFC1	RFC1
71,27	Intra	2	E[K]FKLDPNVIDR	495	[K]LAATR	274	RFC1	RFC1
71,03	Intra	1	F[K]LDPNVIDR	497	[K]LAATR	274	RFC1	RFC1
71,03	Intra	9	[K]TSTPLILICNER	446	S[K]TLLNAGVK	385	RFC1	RFC1
64	Intra	1	EAELLV[K]KEEER	266	S[K]KLAATR	273	RFC1	RFC1
62,71	Intra	1	RPDANSI[K]SR	484	SA[K]YYR	678	RFC1	RFC1
62,2	Intra	2	YAPTNLQOVCGN[K]GSVMK	314	L[K]NWLANWENSKK	321	RFC1	RFC1
61,3	Intra	4	EAELLVK[K]EEERSK	267	[K]LAATR	274	RFC1	RFC1
60,15	Intra	1	FAFACNQSN[K]IIEPLQSR	149	VT[K]NLAQVK	275	RFC4	RFC4
60,15	Intra	3	YS[K]LSDEDVLKR	165	VT[K]NLAQVK	275	RFC4	RFC4
58,98	Intra	1	IPATV[K]SGFTR	767	HAG[K]DGSGVFR	340	RFC1	RFC1
58,55	Intra	4	GASEALA[K]R	182	VT[K]SISSK	190	RFC1	RFC1
57,1	Intra	3	RPDANSI[K]SR	484	[K]EEER	267	RFC1	RFC1
56,99	Intra	1	KLEEQHNIAT[K]EAELLVK	259	[K]EEER	267	RFC1	RFC1
56,99	Intra	1	DNVVREED[K]LWTVK	296	[K]EEER	267	RFC1	RFC1
56,41	Intra	1	[K]YNSMTHPVAIYR	773	LGTSTD[K]IGLR	698	RFC1	RFC1
53,33	Intra	1	Y[K]CVIINEANSLTK	136	L[K]IDVR	69	RFC5	RFC5
52,59	Intra	2	[K]ASSPTVKPASSK	77	[K]TKPSSK	90	RFC1	RFC1
52,59	Intra	2	HAG[K]DGSGVFR	340	GSVM[K]LK	319	RFC1	RFC1
52,59	Intra	2	ASSPTV[K]PASSK	84	[K]TKPSSK	90	RFC1	RFC1
51,79	Intra	2	[K]LEEQHNIATK	249	[K]LAATR	274	RFC1	RFC1
50,97	Intra	1	[K]TATSKPGGSK	845	S[K]TLLNAGVK	385	RFC1	RFC1
50,34	Intra	1	KMPVSNVIDVSETPEGE[K]K	68	LPLPA[K]R	75	RFC1	RFC1
49,59	Intra	4	EKF[K]LDPNVIDR	497	RPDANSI[K]SR	484	RFC1	RFC1
47,92	Intra	1	LGTSTD[K]IGLR	698	[K]LAATR	274	RFC1	RFC1
47,92	Intra	1	S[K]TLLNAGVK	385	[K]LAATR	274	RFC1	RFC1

47,92	Intra	1	GASEALA[K]R	182	[K]LAATR	274	RFC1	RFC1
47,85	Intra	2	SISS[K]TSVVVLGDEAGPK	195	[K]LEEQHNIATK	249	RFC1	RFC1
47,85	Intra	1	[K]YNSMTHPVAIYR	773	[K]TATSKPGGSK	845	RFC1	RFC1
46,57	Intra	4	R[K]LEEQHNIATK	249	GASEALA[K]R	182	RFC1	RFC1
46,35	Intra	1	[K]ASSPTVKPASSK	77	VT[K]SISSK	190	RFC1	RFC1
45,16	Intra	1	YAPTNLQQVCGN[K]GSVMK	314	[K]EEER	267	RFC1	RFC1
45,16	Intra	1	E[K]FKLDPNVIDR	495	[K]EEER	267	RFC1	RFC1
45,16	Intra	2	[K]LEEQHNIATK	249	[K]EEER	267	RFC1	RFC1
44,72	Intra	1	E[K]FKLDPNVIDR	495	RPDANSI[K]SR	484	RFC1	RFC1
44,45	Intra	1	YAPTNLQQVCGN[K]GSVMK	314	[K]LEEQHNIATK	249	RFC1	RFC1
44,14	Intra	2	NLP[K]MRPFDR	462	S[K]TLLNAGVK	385	RFC1	RFC1
44,14	Intra	1	RPDANSI[K]SR	484	GASEALA[K]R	182	RFC1	RFC1
44,12	Intra	1	[K]LEEQHNIATK	249	[K]TKPSSK	90	RFC1	RFC1
43,7	Intra	1	NLP[K]MRPFDR	462	LGTSTD[K]IGLR	698	RFC1	RFC1
43,7	Intra	1	[K]YNSMTHPVAIYR	773	TATS[K]PGGSK	850	RFC1	RFC1
41,98	Intra	2	LGTSTD[K]IGLR	698	RPDANSI[K]SR	484	RFC1	RFC1
41,98	Intra	1	[K]LEEQHNIATK	249	F[K]LDPNVIDR	497	RFC1	RFC1
41,98	Intra	1	HAG[K]DGSGVFR	340	VT[K]SISSK	190	RFC1	RFC1
41,94	Intra	1	NQI[K]DFASTR	98	YT[K]NTR	139	RFC3	RFC3
40,95	Intra	1	NLAQV[K]ESVR	281	IHKLNN[K]A	322	RFC4	RFC4
40,92	Intra	1	KLPLPA[K]R	75	[K]EEER	267	RFC1	RFC1

905

906 **Table 3: Cryo-EM data collection, processing, and model statistics**

Table 3: Cryo-EM data collection, processing, and model statistics

Dataset	NO DNA				DNA		
Magnification	130,000				81,000		
Voltage (keV)	300				300		
Cumulative exposure (e-/Å ²)	49-51				40		
Detector	K2 Summit				K3™		
Pixel size (Å)	1.059				1.06		
Defocus range (µm)	-1.1 to -2.4				-1.2 to -2.3		
Micrographs used (no.)	6109				4499		
Initial particle images (no.)	954,291				1,331,440		
Symmetry	C1						
Class Name	Auto-inhibited ₁	Auto-inhibited ₂	Auto-inhibited ₃	Open ₁	Open ₂	DNA-open	DNA-closed
Final Refined particles (no.)	55,308	68,227	60,036	46,069	63,752	46300	76270
Applied B factor (Å ²)	-100	-159.352	-163.938	-100	-106.457	-105.857	-105.313
Map resolution (Å, FSC 0.143)	3.8	3.9	4.0	4.0	3.5	3.4	3.3
Model-Map CC_mask	0.78	0.77	0.77	0.76	0.78	0.79	0.77
Bond lengths (Å), angles (°)	0.002, 0.585	0.002, 0.561	0.002, 0.558	0.002, 0.574	0.002, 0.542	0.002, 0.518	0.002, 0.523
Ramachandran Outliers, Allowed, Favored	0.00, 3.16, 96.84	0.00, 3.11, 96.89	0.00, 2.89, 97.11	0.00, 3.08, 96.92	0.00, 3.38, 96.62	0.00, 2.23, 97.77	0.00, 2.16, 97.84
Poor rotamers (%), MolProbity score, Clashscore (all atoms)	0.00, 1.68, 9.05	0.00, 1.68, 9.42	0.00, 1.68, 9.95	0.00, 1.67, 9.26	2.01, 1.91, 8.67	1.09, 1.54, 8.44	1.09, 1.55, 9.18
Accession number, EMDB, PDB	25568, 7THJ	25569, 7TIC	25614, 7THV	25615, 7TKU	25753, 7T18	25616, 7T1B	25617, 7T1D

907

908 **Table 4: DNA sequences**

Template name	sequence	Primer name	sequence	Name used in assay
Template30-20-A	TTTTTTTTTAAATGTACTCGTAGTGTCTGC	Primer20-3'-abasic	GCAGACACTACGAGTACAT/3dSp/	p/t-DNA 3'-abasic
		Primer20-3'-T-phosphate	GCAGACACTACGAGTACATT/3Phos/	p/t-DNA 3' PO ₄
		Primer20-3'-T-RNA	rGrCrArGrArCrArCrUrArCrGrArGrUrArCrArUrU	RNA primer/DNA template
		Primer20-3'-riboT	GCAGACACTACGAGTACATrU	p/t-DNA 3' ribo
		Primer20-3'-T	GCAGACACTACGAGTACATT	p/t-DNA
		Primer20-2AP-0	GCAGACACTACGAGTACAT/3 ₂ AmPu/	p/t-AP, p=1
		Primer20-2AP-2	GCAGACACTACGAGTAC/i ₂ AmPr/TA	p/t-AP, p=3
Template30-T-1	TTTTTTTTTTTTGTACTCGTAGTGTCTGC-3'	Primer20-2AP-1	GCAGACACTACGAGTACA/i ₂ AmPr/A	p/t-AP, p=2
Template30-20-2AP	TTTTTTTTT/i ₂ AmPr/ATGTACTCGTAGTGTCTGC-3'	Primer20-3'-T	GCAGACACTACGAGTACATT	p/t-AP, t=1
Template20-5'-A	AATGTACTCGTAGTGCTGC	Primer20-3'-T	GCAGACACTACGAGTACATT	Blunt DNA
		Primer 20-3'-T-10ext	GCAGACACTACGAGTACATTTT TTTTTTTT	3' overhang DNA
Template30-20-A-3'T	AATGTACTCGTAGTGCTGCTTTTTTTTTT	Primer 20-3'-T-10ext	GCAGACACTACGAGTACATTTT TTTTTTTT	3' overhang dumbbell DNA
		polyT 20	TTTTTTTTTTTTTTTTTTTT	ssDNA (poly T)

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910

911 SUPPLEMENTAL FIGURE LEGENDS

912 **Figure 1 – Supplement 1. Characterization and cryo-EM of full-length RFC:PCNA . (A)** SDS-PAGE gel of purified RFC and PCNA
 913 after gel filtration. A fraction with stoichiometric amounts of RFC and PCNA was used for grid preparation. **(B)** Crosslinking of the
 914 RFC:PCNA at a concentration of 1 mM Bissulfosuccinimidyl suberate (BS3) led to the identification of intermolecular and intramolecular
 915 crosslinks in RFC, and are shown in schematic representation. 88% of the crosslinks mapped to RFC-A and no crosslinks in PCNA
 916 were detected, although PCNA was detected in the sample. **(C)** Local resolution of reconstructions (center) and a representative
 917 section of each complex subunit for each reconstruction. **(D)** Fourier shell correlation (FSC) curves for the two halves of the
 918 reconstructions as well as model vs map curves.

919

920 **Figure 1 – Supplement 2. Schematic of yRFC:PCNA cryo-EM processing. (A)** A down-filtered micrograph taken on a Thermo
 921 Fisher Scientific Titan Krios with a Gatan K2 detector is displayed. **(B)** 2D class averages show different side views. **(C)** The 3D
 922 reference for refinement was generated *ab initio* with cisTEM (Grant et al., 2018). **(D)** The *ab initio* model was downfiltered to 50 Å and
 923 used as reference for 3D classification. The first round of classification was performed with the 2x binned particle stack. Further rounds
 924 of classification with the unbinned stack improved the resolution. 3D classification with local angular search further helped to improve
 925 the resolution of the reconstructions representing complexes with closed PCNA (blue).

926

927 **Figure 1 – Supplement 3. Schematic of yRFC:PCNA:DNA cryo-EM processing. (A)** A downfiltered micrograph taken on a Thermo
 928 Fisher Scientific Titan Krios with a Gatan K3 detector is displayed. **(B)** 2D class averages show different side views. **(C)** Class Open1 of
 929 the dataset without DNA was downfiltered to 60 Å and used as reference for 3D classification. The first round of classification was
 930 performed with the 4x binned particle stack and the second round of classification with the 2x binned stack. Further classification with
 931 the unbinned stack with resolution limit and without alignment helped to separate different conformational states and improved the
 932 resolution.

933

934 **Figure 2 – Supplement 1. RFC:PCNA complexes in autoinhibited conformations. (A)** Side views of the atomic models of
 935 Autoinhibited1, the RFC:PCNA crystal structure (Bowman *et al.*, 2004), and of the human RFC:PCNA complex show similarity. **(B)**
 936 Close-up of the nucleotide binding sites. The cryo-EM map is shown in yellow overlaying the atomic model. The catalytically important
 937 Walker A lysine, Walker B glutamates, and *trans*-acting arginine fingers are shown. The arginine fingers are distant within the active
 938 sites of RFC-B,C,D in Autoinhibited1&2 and in RFC-B,D of Autoinhibited3, rendering these active sites inactive. For comparison, the
 939 nucleotide binding sites in the atomic model of the RFC:PCNA crystal structure (PDB 1SXJ) are shown. Here, the SRC motif arginine
 940 fingers were mutated to glutamines. RFC-E is not catalytically competent and has ADP-bound. The A' domain does not donate *trans*-
 941 acting arginine fingers. **(C)** Top views on the AAA+ spiral of the T4 clamp loader, RFC:PCNA crystal structure and Autoinhibited1-3.
 942 The T4 clamp loader, which has DNA bound, is in an active conformation. Here, the rotation axes that relate the subunits are coincident
 943 with each other and the central axis of DNA. In contrast, the symmetry of the AAA+ spiral of RFC in the autoinhibited conformation is
 944 distorted, and the axes are skewed in all these structures.

945

946 **Figure 2 – Supplement 2. Multibody analyses with all 183,571 particles combined from the three Autoinhibited states to**
 947 **investigate the dynamic initial complex of RFC with PCNA. (A)** Superpositions of Autoinhibited 1&2 and 1&3 highlight differences in
 948 the orientation of PCNA towards RFC as well as the opening of the A-gate. **(B)** The two masks used for the first multibody refinement
 949 define PCNA and RFC as separate rigid bodies. **(C)** Principal Component (PC) analysis revealed the two most dominant motions with
 950 RFC and PCNA. **(D)** Amplitude histogram of PC2 is unimodal. **(E)** PC2 reveals a swiveling motion. The C_α displacement is indicated by
 951 modevector generated arrows, scaled down by a factor of 2. **(F)** The two masks used for the second multibody refinement were chosen
 952 to match domain boundaries determined with the ENM DynOmics server (Li et al., 2017) and to capture motion between the A' and the
 953 AAA+ module of the A-gate. **(G)** PC analysis revealed two dominant motions. **(H)** Amplitude histogram of PC1 is unimodal. **(I)** Multibody
 954 refinement 2 also revealed rocking and swiveling as dominant motions.

955

956 **Figure 3 – Supplement 1. Comparison of RFC bound to open PCNA from different datasets. (A)** Top and side view of the cryo-
 957 EM map of RFC bound to open PCNA, which was obtained from the dataset without DNA. **(B)** Top and side view of the cryo-EM map of
 958 RFC bound to open PCNA obtained from the dataset with DNA. **(C)** Overlay of the two models for Open1 and Open2 shows that the
 959 two models strongly resemble each other. **(D)** Open1 superposed to Open2. Open2 is colored by RMSD. **(E)** Close-up of the nucleotide
 960 binding sites in Open1 and in Open2. The cryo-EM map is shown in yellow overlaying the atomic model. Critical catalytic residues are
 961 shown as sticks. All active sites are occupied with ATPγS. **(F)** PCNA intrasubunit distortions that occur for opening. The C_α
 962 displacement is indicated by modevector-generated arrows, scaled up by a factor of four.

963

964

Figure 3 – Supplement 2. RFC interacts with all five subunits to hold PCNA open. (A) Overview of interaction sites of RFC with PCNA in the autoinhibited conformation. The three PCNA subunits and RFC's AAA+ module are shown in a cartoon flattened onto the page. RFC-D and RFC-E are not in contact with PCNA. (B) Cartoon overview of interaction sites of RFC with PCNA in the open conformation. RFC-D and RFC-E now contact PCNA. (C) Close up views of the RFC-PCNA interaction sites in Autoinhibited1 are shown, the rest is omitted for clarity. The contact between PCNA and RFC-A is mediated by a short helix and adjacent hydrophobic and aromatic residues that insert into PCNA's hydrophobic pockets. This conformation is commonly seen in binding partners which contain a PCNA-interacting protein (PIP) motif or derived motifs. (D) Contacts of RFC-A and RFC-B with PCNA do not change significantly upon PCNA opening. The interaction of PCNA with RFC-C becomes more extensive, and RFC-D and RFC-E establish new contacts to PCNA. RFC-C and RFC-E insert into PCNA's hydrophobic pocket but do not employ a PIP motif. The E-plug and A' domain reinforce the interaction with PCNA.

975

Figure 5 – Supplement 1. (A) PCNA constriction in DNA^{PCNA-open} and DNA^{PCNA-closed}. Displacement vectors between Open2 and DNA^{PCNA-open} are shown as green arrows, scaled by a factor of four (left). Displacement vectors between DNA^{PCNA-open} and DNA^{PCNA-closed} are shown as green arrows, scaled by a factor of four (right). Upon DNA binding, the PCNA lock-washer constricts in DNA^{PCNA-open}, due to a motion at the NTD of PCNA-III. PCNA is closed in a puckered conformation in DNA^{PCNA-closed} through constricting motions of PCNA-I and PCNA-III. (B) The overlay of the rotation axes in the open conformation of RFC is indicative for spiral symmetry. The tilt angles show that in the DNA-bound structures (DNA^{PCNA-open} and DNA^{PCNA-closed}), the rotation axes become more tilted upon PCNA closure, indicating that DNA binding and PCNA closure slightly disrupt the symmetric arrangement of the AAA+ spiral. (C) Close-up of the nucleotide binding sites in DNA^{PCNA-open} and DNA^{PCNA-closed}. The cryo-EM map is shown in yellow overlaying the atomic model. Critical catalytic residues are shown as sticks. All active sites are occupied with ATPγS.

985

Figure 5 – Supplement 2. Superposition of the ATPase active sites across conformations. In all cases, the active sites were aligned about their Walker A and Walker B motifs. (A) Comparing the active sites of Closed1 to Open2. (B) Comparing the active sites of Open2 to DNAPCNA-open. (C) Comparing the active sites of DNA^{PCNA-open} to DNA^{PCNA-closed}. The most substantial change occurs upon clamp opening (A), which brings the arginine fingers in the RFC B-D active sites close to the γ-phosphate. This change does not occur in RFC-A, whose hydrolysis is dispensable for clamp loading. After opening, DNA binding (B) and then clamp closure (C) do not change the active sites much, indicating that DNA binding does not stimulate hydrolysis by reorganizing the ATPase binding interface.

992

Figure 6 – Supplement 1. The separation wedge has two critical residues. (A) Fluorescence intensity traces for 375 nM of p/t AP_{p=1} in the presence of RFC:PCNA with and without nucleotide or primer AP_{p=1}. (B) Placement of 2AP at different positions. The fluorescence with p/t AP_{p=1} in the presence of ATPγS and RFC:PCNA increases ~4-fold in relation to the sample without ATPγS, whereas placement of the oligo further away from the 3'-OH end reduces the fluorescence increase to ~2-fold. (C) Results from (Figure 6 B) could be recapitulated using p/t-DNA with 2-AP in the template strand (t=1). The *E. coli* clamp loader, which does not have a separation pin, does not change fluorescence in the presence of ATPγS. (D) ATPase activity of the 'separation pin' mutants. DNA binding affinity and maximum ATP hydrolysis rate is reduced in RFC^{W638G}. (E) The steady-state ATP hydrolysis rate of the RFC-W638G variant is significantly reduced compared to wild type (p value= 0.0005). The RFC-F582A variant binds DNA with similar affinity than wild type (p value = 0.0288). DNA binding affinity for RFC-W638G is slightly reduced (p value <0.0001). Error bars reflect the standard deviation from three replicates. The number above the error bar indicates the mean. The asterisks correspond to p-values from one-way ANOVA tests comparing variants to wild type, 'ns' = not significant.

1004

Figure 6 – Supplement 2. Discrimination of different primer template junctions by RFC. (A) Cartoon depiction of different DNA substrates that were used to probe if the separation pin acts to discriminate different moieties at the primer template junction. (B-D) Similar ATPase activity profiles of the wild type and the two separation pin variants indicate that the separation pin is not critical to discriminate between these DNA substrates. Error bars on these bar graphs reflect the standard deviation from three replicates. The number above the error bar indicates the mean. The asterisks on these graphs correspond to p-values from one-way ANOVA tests comparing different DNA substrates with an unmodified primer template junction (p/t DNA): 'ns' – not significant P > 0.05, *P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

1012

1013 **Figure 6 – Supplement 3. Functional characterization of separation pin mutants in *S. cerevisiae*.** To directly assess the
1014 physiological role of the separation pin using yeast with, we performed spot assays with strains that express either wild type RFC1 or
1015 the F582A or the W638G variant. The two variants and the wild type display similar colony sizes across a variety of conditions,
1016 including varying temperatures, UV radiation, and treatment with hydroxyurea (HU) or methyl Methanesulfonate (MMS), indicating that
1017 there is no striking phenotype with the tested conditions.

1018

1019 **Figure 6 – Supplement 4. Differences in how duplex p/t-DNA is held in the central chamber of clamp loaders. (A)** In the T4 and
1020 *E. coli* clamp loaders, the duplex p/t-DNA extends farther into the central chamber, enabling more substantial contacts with the B-E
1021 subunits compared to RFC. **(B)** Contribution of RFC subunits to DNA binding. RFC-A dominates contact with p/t-DNA when compared
1022 to other clamp loaders.

1023

1024 **Figure 6 – Supplement 5. Conservation of the separation pin. (A)** The separation pin is conserved in RFC1 across eukaryotes.
1025 Sequence alignment shows the conservation of the 'separation pin' among 5 eukaryotic species. The conserved sequences are marked
1026 by blue boxes. The fully conserved residues are in white with a red background, the highly conserved residues are in red, and the less
1027 conserved ones are in black. F582 and W638 are pointed out by the blue arrow. **(B)** A potential separation pin in the Ctf18 clamp
1028 loader. The structure of yeast Ctf18 (blue) predicted by AlphaFold (Jumper *et al.*, 2021, Varadi *et al.*, 2021) is superposed onto collar
1029 domain of the DNA^{PCNA-open} structure of yeast RFC (RFC1 in red, primer strand in yellow, and template strand in orange). Key residues
1030 of the RFC1 separation pin are highlighted (Phe 582, Gln 636, & Trp 638), as well as their predicted counterparts in Ctf18 (Glu427,
1031 Asn477, & Leu481). While the separation pin helical hairpin is likely conserved, the key residues that mediate base-flipping in RFC1 are
1032 not conserved.

1033

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A **B**

6109 micrographs
 ↓ **automatic picking**
 954 291 particles
 ↓
2D classification
 834 099 particles

2D class averages

C

Ab initio Model

D

3D classification

834 099 particles, 4.24 Å/pixel

561 405 particles, 2.12 Å/pixel

336 272 particles, 1.06 Å/pixel

local angular search ↓ 241 793 particles

**refinement
map sharpening**

Autoinhibited2
 68227 particles
 3.9 Å

Autoinhibited1
 55308 particles
 3.8 Å

Autoinhibited3
 60036 particles
 4.0 Å

↓ 123 200 particles, 1.06 Å/pixel

↓ 105 145 particles

**refinement
map sharpening**

Open1
 46069 particles
 4.0 Å

A **B** **C**

RFC-A PIP motif

RFC-A 397 DNMSVVG YF 405

Consensus xQxxΨxxΩΩ

Ψ=M, I, L, V

Ω=F, Y

D

A **B** **C**

● Nucleotide

RFC

- A
- B
- C
- D
- E

A Closed1 (color) to Open2

B Open2 (color) to DNA^{PCNA-open}

C DNA^{PCNA-open} (color) to DNA^{PCNA-closed}

	V_{max} (μ M/s)	K_d app (nM)
WT	0.121 $\pm$ 0.014	8.9 $\pm$ 2.0
F582A	0.131 $\pm$ 0.007	13.8 $\pm$ 0.7
W638G	0.062 $\pm$ 0.005	31.6 $\pm$ 2.4

A **B** **C** **D**

A **B**

Clamp loader interactions with p/t-DNA			
	Interaction Area B, C, D, E subunits (Å ²)	Interaction Area A subunit (Å ²)	Reference
T4Phage	1149	659	Kelch et al 2011
<i>E. coli</i>	1100	619	Simonetta et al 2009
<i>S. cerevisiae</i>	762	1369	this work, DNA ^{PCNA-closed}

A

<i>S. cerevisiae</i>	YFD	DF	DFT	PI	591...634	QLW	SL	LP	LHA	645
<i>C. elegans</i>	FF	DY	GIM	PL	568...609	GS	WK	LL	NEQS	620
<i>D. melanogaster</i>	FF	H	DYS	LAP	714...754	SA	WS	LL	PTQA	765
<i>D. rerio</i>	FF	H	DYS	LAP	876...919	QT	WS	LL	PTQA	930
<i>H. sapiens</i>	FF	H	DYS	IAP	884...927	QN	WS	LL	PAQA	938

B