

1 **TITLE: Interplay of macromolecular interactions during assembly of human DNA**
2 **polymerase δ holoenzymes and initiation of DNA synthesis**

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20 **ABSTRACT**

21 In humans, DNA polymerase δ (Pol δ) holoenzymes, comprised of Pol δ and the processivity
22 sliding clamp, proliferating cell nuclear antigen (PCNA), carry out DNA synthesis during
23 lagging strand DNA replication, initiation of leading strand DNA replication, and the major
24 DNA damage repair and tolerance pathways. Pol δ holoenzymes are assembled at
25 primer/template (P/T) junctions and initiate DNA synthesis in a coordinated process involving
26 the major single strand DNA-binding protein complex, replication protein A (RPA), the
27 processivity sliding clamp loader, replication factor C (RFC), PCNA, and Pol δ . Each of these
28 factors interact uniquely with a P/T junction and most directly engage one another. Currently, the
29 interplay between these macromolecular interactions is largely unknown. In the present study,
30 novel Förster Resonance Energy Transfer (FRET) assays reveal that dynamic interactions of
31 RPA with a P/T junction during assembly of a Pol δ holoenzyme and initiation of DNA synthesis
32 maintain RPA at a P/T junction and accommodate RFC, PCNA, and Pol δ , maximizing the
33 efficiency of each process. Collectively, these studies significantly advance our understanding of
34 human DNA replication and DNA repair.

35
36 In humans, DNA polymerase δ (Pol δ) holoenzymes, comprised of Pol δ and the processivity
37 sliding clamp, proliferating cell nuclear antigen (PCNA), carry out DNA synthesis during
38 lagging strand DNA replication, the initiation of leading strand DNA replication, and the major
39 DNA damage repair and tolerance pathways¹⁻¹¹. In each of these DNA synthesis pathways, Pol δ
40 holoenzymes are assembled at nascent primer/template (P/T) junctions and initiate DNA
41 synthesis in a complex process that requires the spatially and temporally coordinated actions of
42 many cellular factors that all interact with nascent P/T junctions; namely, the major single strand
43 DNA (ssDNA)-binding protein complex, replication protein A (RPA), the processivity sliding
44 clamp loader complex, replication factor C (RFC), PCNA, and Pol δ (**Figure 1A**)¹²⁻²². Each of

45 these protein–DNA interactions with P/T junctions are unique (**Figure 1B**) and transpire in the
46 context of the other protein–DNA interactions that assemble and disassemble over time.
47 Furthermore, many of these protein–DNA interactions significantly overlap, implying mutual
48 exclusivity and innate competition. Finally, nearly all of the aforementioned cellular factors
49 (RPA, RFC, PCNA, Pol δ) directly engage one another through protein–protein interactions
50 (**Figure 1C**). Currently, the interplay between the macromolecular interactions involved in the
51 assembly of Pol δ holoenzymes and subsequent initiation of DNA synthesis is unknown, greatly
52 limiting our fundamental understanding of human DNA replication and DNA damage repair. In
53 the present study, we design and utilize a novel and efficient Förster Resonance Energy Transfer
54 (FRET) assay that directly and continuously monitors the interactions of PCNA and RPA with
55 P/T junctions as Pol δ holoenzymes are assembled and initiate DNA synthesis. Results reveal
56 that dynamic interactions of RPA subunits with a P/T junction during assembly of a Pol δ
57 holoenzyme and initiation of DNA synthesis both maintain RPA at the P/T junction and
58 accommodate RFC, PCNA, and Pol δ , maximizing the efficiency of each process.

59

60 **Results**

61 *RPA OBA maintains contacts with a P/T junction throughout loading of the resident PCNA.*

62 The ssDNA immediately adjacent to a nascent P/T junction is initially engaged by an RPA
63 heterotrimer in an orientation-specific manner, as depicted in **Figure 1A**. In short,
64 oligonucleotide binding (OB) fold A (OBA) of the RPA1 subunit aligns closer to the 5' end of
65 the ssDNA sequence and OBD of the RPA2 subunit aligns closer to the 3' end such that OBD
66 and OBC directly contact the 3' terminus of the primer strand¹²⁻¹⁹. Next, RFC loads PCNA onto a
67 nascent P/T junction that is engaged by RPA. Specifically, RFC, in complex with ATP, engages
68 the “front face” of a free PCNA clamp in solution, opens the sliding clamp, and the
69 RFC•ATP•“Open” PCNA complex, referred to herein as the loading complex, engages a nascent
70 P/T junction such that the “front face” of the PCNA clamp is oriented towards the 3' terminus of
71 the primer from which DNA synthesis initiates. Loading complexes may be targeted to P/T
72 junctions engaged by RPA through direct protein•protein interactions between subunits 1 – 3 of
73 the RFC complex and the RPA1 subunit of the RPA complex (**Figure 1C**)^{23,24}. Upon engaging a
74 nascent P/T junction, the loading complex adopts an activated conformation in which ATP
75 hydrolysis by RFC is optimized. Herein, the activated conformation of the loading complex is
76 referred to as the activated loading complex. ATP hydrolysis by RFC within the activated
77 loading complex simultaneously closes PCNA around the DNA and the closed (i.e., loaded)
78 PCNA is subsequently released onto the double stranded DNA (dsDNA) region of the nascent
79 P/T junction. The resultant RFC•ADP complex then releases into solution, and this step is rate-
80 limited by dissociation of the RFC•ADP complex from the RPA1 subunit of the resident RPA
81 ^{20,21,23,25}. After completion of PCNA loading, the resident RPA stabilizes the loaded PCNA
82 at/near the nascent P/T junction by prohibiting RFC-catalyzed unloading of PCNA and diffusion
83 of PCNA along the adjacent ssDNA²⁵.

84 RPA maintains contact(s) with a nascent P/T junction throughout loading of the resident
85 PCNA^{25,26}. Given the significant overlap in the DNA footprints of RPA and the activated
86 complex on nascent P/T junctions (**Figure 1B**), it is unclear how this is possible. Specifically,
87 both RPA and RFC (as part of the activated loading complex) directly engage the 3' terminus of
88 the primer strand and at least 13 nucleotides (nt) of the template ssDNA strand immediately
89 downstream of a nascent P/T junction^{12-19,24}. To address this long-standing enigma, we design

90 and utilize novel FRET assays to directly and continuously monitor RPA-DNA interactions
91 throughout the PCNA loading process described above. Initially, we focus on the interaction(s)
92 of RPA OBA with nascent P/T junctions during association and activation of the loading
93 complex.

94 To establish and monitor interactions of loading complexes with P/T junctions, we utilized a
95 previously characterized Cy3, Cy5 FRET pair comprised of a Cy3-labeled P/T DNA substrate
96 (5' ddPCy3/T, **Figure S1**) and Cy5-labeled PCNA (Cy5-PCNA)^{22,25,27}. The primer strand of the
97 5' ddPCy3/T DNA substrate contains a Cy3 donor near the 5' end and is terminated at the 5' end
98 with biotin and at the 3' end with a dideoxynucleotide (ddC). When pre-bound to NeutrAvidin,
99 biotin attached to the 5' end of a primer strand prevents loaded PCNA from diffusing off the
100 dsDNA end of the substrate. For consistency, NeutrAvidin is included in all experiments in the
101 present study that utilize 5' biotin-terminated primers. A 3' dideoxy-terminated primer cannot be
102 extended by a DNA polymerase and is implemented here in consideration of subsequent
103 experiments in the present study that utilize human DNA polymerase δ (pol δ). The Cy5-labeled
104 PCNA homotrimer contains a single Cy5 acceptor at amino acid position 107 of a PCNA
105 monomer. When encircling a P/T junction, either as part of a loading complex or as loaded
106 PCNA, the Cy5 acceptor on the “back face” of the PCNA homotrimer is oriented towards the
107 Cy3 donor on the 5' ddPCy3/T DNA substrate, yielding a FRET. Experiments are performed with
108 adenosine 5'-O-(3-thio) triphosphate (ATP γ S), an ATP analog in which one of the non-bridging
109 oxygens of the γ -phosphoryl group is substituted for sulfur. This substitution severely inhibits
110 hydrolysis of ATP by human RFC and, hence, stalls the PCNA loading pathway at activation of
111 the loading complex^{21,28}.

112 For a Cy3, Cy5 FRET pair, such as those described above, the gain and loss of FRET are
113 directly indicated only by simultaneous, anti-correlated changes in the fluorescence emission
114 intensities of the Cy3 donor and Cy5 acceptor. Specifically, appearance of (or increase in) FRET
115 is indicated by a decrease (i.e., quenching) of Cy3 donor fluorescence emission intensity (I_{563})
116 together with a concomitant increase in sensitized Cy5 acceptor fluorescence emission intensity
117 (I_{665}). Likewise, disappearance of (or decrease in) FRET is indicated by a decrease in sensitized
118 I_{665} together with a concomitant increase (i.e., de-quenching) of I_{563} . Therefore, to directly and
119 continuously monitor change in FRET over time, we utilized a spectrofluorometer that monitors
120 I_{563} and I_{665} essentially simultaneously ($\Delta t = 0.235$ ms) and permits successive additions of
121 components to a given reaction mixture. The approximate FRET efficiency, E_{FRET} , is then
122 calculated for each I_{563} , I_{665} pair over time. To analyze binding and activation of loading
123 complexes on P/T junctions via FRET, the 5' ddPCy3/T DNA substrate is first pre-bound with
124 NeutrAvidin and native RPA and I_{563} and I_{665} are monitored over time (**Figure 2A**). Then,
125 loading complex pre-formed with RFC, Cy5-PCNA and ATP γ S is added to the reaction mixture,
126 the resultant solution is mixed, and the fluorescence emission intensity recording is resumed.
127 Under the conditions of the assay, the loading complex rapidly engages the P/T junction and then
128 the PCNA loading pathway stalls prior to ATP hydrolysis. Thus, an increase in E_{FRET}
129 encompasses all kinetic steps along the PCNA loading pathway up to but not including
130 hydrolysis of ATP by RFC within the activated loading complex^{21,25,28}.

131 Upon addition of the loading complex, I_{665} rapidly increases concomitantly with a rapid
132 decrease in I_{563} after which I_{665} continues to increase slowly while I_{563} continues to decrease
133 slowly (**Figure 2B, Top**). These synchronized, anti-correlated changes in I_{563} and I_{665} are
134 indicative of the appearance and increase FRET (**Figure 2B, Bottom**). As observed in **Figure**
135 **2C**, E_{FRET} traces increase to values significantly above the E_{FRET} traces predicted for no

136 interaction between the loading complex (with Cy5-PCNA and ATP γ S) and 5' ddPCy3/T DNA.
137 The increase in E_{FRET} is comprised of two phases (i.e., biphasic) with a lower limit for the
138 observed rate constant of the first phase of $k_{\text{obs inc},1} \geq 0.275 \pm 0.019 \text{ s}^{-1}$ and an observed rate
139 constant of the second phase of $k_{\text{obs inc},2} = 3.59 \pm 0.01 (\times 10^{-4} \text{ s}^{-1})$. A lower limit of $k_{\text{obs inc},1}$ is
140 indicated due to the kinetic fitting of relatively few time points within its lifetime. The biphasic
141 behavior and kinetic variables observed in the present study agree with that observed in a
142 previous report that analyzed PCNA loading under similar conditions by monitoring sensitized
143 Cy5 acceptor fluorescence emission intensity (I_{665}) via stopped flow²⁵. Next, we repeated these
144 assays utilizing native PCNA and an alternative FRET pair to monitor interactions of RPA OBA
145 with P/T junctions (**Figure 2D**). The Cy3-labeled P/T DNA substrate (ddP/5'TCy3 DNA, **Figure**
146 **S1**) is identical to 5' ddPCy3/T DNA substrate described above except that the Cy3 donor is
147 located towards the 5' end of the template strand, rather than the 5' end of the primer strand. The
148 Cy5-labeled RPA (RPA-OBA-Cy5) contains a Cy5 acceptor on OBA of the RPA1 subunit that
149 faces the Cy3 donor of ddP/5'TCy3 when engaged with the P/T junction (**Figure 2D**, **Figures S4**
150 **– S6**)²⁹. RPA-OBA-Cy5 fully supports RFC-catalyzed loading of PCNA onto P/T junctions
151 (**Figure S7** and **Table S1**).

152 The ddP/5'TCy3 DNA substrate is first pre-bound with NeutrAvidin and RPA-OBA-Cy5 and
153 I_{563} and I_{665} are monitored over time (**Figure 2D**). Here, a significant, constant E_{FRET} is observed
154 prior to the addition of RFC in any form due to the stable interaction of RPA-OBA-Cy5 with
155 ddP/5'TCy3 DNA (**Figures S4 – S6**). Next, loading complex pre-formed with RFC, native
156 PCNA, and ATP γ S is added, and the fluorescence emission intensities are monitored over time.
157 Upon addition of the loading complex, both I_{665} and I_{563} rapidly and very slightly decrease then
158 very slowly decrease over time (**Figure 2E**, *Top*). These synchronized, correlated changes in the
159 fluorescence emission intensities are not attributable to FRET. Thus, any apparent change in the
160 E_{FRET} values observed after the addition of the pre-formed loading complex is due to nonspecific
161 effects³⁰. For the example E_{FRET} trajectory depicted in **Figure 2E** (*Bottom*), the E_{FRET} values
162 observed prior to the addition of the loading complex are maintained after addition of the pre-
163 formed loading complex. For the averaged E_{FRET} trajectory depicted in **Figure 2F**, the E_{FRET}
164 traces observed prior to the addition of the loading complex persist and are maintained at the
165 significantly elevated level above the E_{FRET} traces predicted for no interaction between
166 ddP/5'TCy3 DNA and RPA-OBA-Cy5. This indicates that during binding and activation of the
167 loading complex at a P/T junction the distance/orientation of RPA OBA relative to the 5' end of
168 the template strand does not change. Thus, the interaction(s) of RPA OBA with a P/T junction
169 are not affected by interactions of activated complexes with the RPA1 subunit of RPA or any
170 kinetic step along the PCNA loading pathway up to but not including hydrolysis of ATP by RFC
171 within the activated loading complex. Next, we performed similar assays with ATP (**Figure 3**) to
172 investigate the interaction(s) of RPA OBA with a P/T junction during the remainder of the
173 PCNA loading pathway.

174 First, interactions of RFC and PCNA with P/T junctions were monitored. The 5' ddPCy3/T
175 DNA substrate is pre-bound with NeutrAvidin and native RPA. Then, Cy5-PCNA is added and
176 I_{563} and I_{665} are monitored over time (**Figure 3A**). Here, a low, constant E_{FRET} is observed prior
177 to the addition of RFC in any form due to the presence of both the Cy3 donor and the Cy5
178 acceptor. E_{FRET} values observed during this period are a true experimental baseline signal
179 representing the absence of any interactions between Cy5-PCNA and the 5' ddPCy3/T
180 DNA•RPA complex. Next, pre-formed RFC•ATP complex is added, and the fluorescence
181 emission intensities are monitored. Here, RFC•ATP must first bind a “free” PCNA in solution in

182 order for loading to proceed. Under the conditions of the assay, PCNA loading is stoichiometric
183 and biphasic²⁵; first, all steps up to and including release of the closed PCNA ring on the P/T
184 junction are rate-limited by a kinetic step along the PCNA loading pathway that occurs prior to
185 and much slower than binding of the loading complex to the P/T junction; second, the loaded
186 PCNA repositions relatively slowly on the P/T junction concurrent with release of the RFC•ADP
187 complex into solution via its dissociation from the RPA1 subunit of the resident RPA engaged
188 with the P/T junction. Thus, the appearance and increase in E_{FRET} towards maximal values
189 encompasses all kinetic steps along the PCNA loading pathway and reflects; 1) a relatively fast
190 kinetic step(s) ($k_{\text{obs inc,1}}$) along the PCNA loading pathway that occurs prior to and much slower
191 than binding of the loading complex to the P/T junction and; 2) a relatively slower release of the
192 RFC•ADP complex into solution via its dissociation from the resident RPA engaged with the
193 P/T junction ($k_{\text{obs inc,2}}$)²⁵.

194 Upon addition of RFC•ATP, the observed changes in I_{665} and I_{563} are synchronized and anti-
195 correlated (**Figure 3B, Top**), indicating the appearance and increase in FRET (**Figure 3B,**
196 **Bottom**). As observed in **Figure 3C**, E_{FRET} traces rapidly increase to values significantly above
197 the E_{FRET} values observed for no interaction between Cy5-PCNA and the 5' ddPCy3/T•RPA
198 complex. As expected, the rapid increase in E_{FRET} observed upon addition of RFC is biphasic
199 with observed rate constants of $k_{\text{obs inc,1}} = 5.29 \pm 0.14$ ($\times 10^{-2} \text{ s}^{-1}$) and $k_{\text{obs inc,2}} = 1.72 \pm 0.02$ ($\times 10^{-2}$
200 s^{-1}) (**Table S1**). Importantly, $k_{\text{obs inc,2}}$ agrees very well with the values reported in a previous
201 study that analyzed PCNA loading under similar conditions by monitoring sensitized Cy5
202 acceptor fluorescence emission intensity (I_{665}) via stopped flow. As discussed above, $k_{\text{obs inc,2}}$
203 reports on release of the RFC•ADP complex into solution via its dissociation from the RPA1
204 subunit of the resident RPA engaged with the P/T junction. This kinetic step occurs throughout
205 the time-dependent increase in E_{FRET} observed in **Figure 3C** and accounts for ~30 – 60% of the
206 E_{FRET} signal at any point in time, based on fits of the kinetic data.

207 Next, we repeated these assays utilizing ddP/5'TCy3 DNA substrate, RPA-OBA-Cy5, and
208 PCNA (**Figure 3D**) to monitor the interaction(s) of RPA OBA with P/T junctions. Upon addition
209 of RFC•ATP, neither I_{665} nor I_{563} change over time (**Figure 3E, Top**). These persistent I_{563} and
210 I_{665} values indicate that the observed E_{FRET} values observed prior to the addition of the
211 RFC•ATP complex do not change upon its subsequent addition (**Figure 3E, Bottom**). As
212 observed in **Figure 3F**, the E_{FRET} traces observed prior to the addition of the RFC•ATP complex
213 persist and are maintained at the significantly elevated level above the E_{FRET} traces predicted for
214 no interaction between ddP/5'TCy3 DNA and RPA-OBA-Cy5. This indicates that the
215 distance/orientation of RPA OBA relative to the 5' end of the template strand does not change as
216 the RFC•ADP complex engages and releases from the RPA1 subunit. Altogether, the results
217 presented in **Figures 2** and **3** indicate that the distance/orientation of RPA OBA relative to the 5'
218 end of the template strand does not change throughout the PCNA loading pathway. Thus, the
219 interaction(s) of RPA OBA with a nascent P/T junction is maintained throughout loading of the
220 resident PCNA and unaffected by interactions of RFC complexes (loading complexes,
221 RFC•ADP) with the P/T junction and the RPA1 subunit of the resident RPA. Next, we focused
222 on the interaction(s) of RPA OBD with nascent P/T junctions during loading of the resident
223 PCNA.

224
225 *RPA OBD is transiently displaced from the primer terminus of a P/T junction during loading of*
226 *the resident PCNA.*

227 To establish and monitor interactions of loading complexes with P/T junctions, we utilized
228 the aforementioned Cy5-PCNA and a Cy3-labeled P/T DNA substrate (5'PCy3/T, **Figure S1**)
229 substrate in which the primer strand contains a Cy3 donor near the 5' end and is also terminated
230 at the 5' end with biotin; the 3' terminus of the primer is not modified. As expected, experiments
231 performed with ATP γ S (**Figure 4A – C**) yielded nearly identical results to those observed for the
232 5'ddPCy3/T DNA substrate under identical conditions (**Figure 2A – C**). Specifically, the
233 increase in E_{FRET} is biphasic with a lower limit for the observed rate constant of the first phase of
234 $k_{\text{obs inc},1} \geq 0.336 \pm 0.039 \text{ s}^{-1}$ and an observed rate constant of the second phase of $k_{\text{obs inc},2} = 7.98 \pm$
235 $0.01 \text{ (} \times 10^{-4} \text{ s}^{-1}\text{)}$ (**Figure 4C**). Next, we repeated these assays utilizing native PCNA and an
236 alternative FRET pair to monitor interactions of RPA OBD with P/T junctions (**Figure 4D**). The
237 Cy3-labeled P/T DNA substrate (3'PCy3/T DNA, **Figure S1**) is identical to the 5'PCy3/T DNA
238 substrate described above except that the Cy3 donor is located at the 3' terminus of the primer
239 strand, rather than near 5' end of the primer strand. The Cy5-labeled RPA (Cy5-OBD-RPA)
240 contains a Cy5 acceptor on OBD of the RPA2 subunit that faces the Cy3 donor of the 3'PCy3/T
241 when engaged with the P/T junction (**Supplemental Information, Figures S4 – S6**)²⁹. RFC-
242 catalyzed loading of PCNA onto P/T junctions is fully supported by Cy5-OBD-RPA (**Figure S8,**
243 **Table S1**) and on the 3'PCy3/T DNA substrate (**Figure S9 – S10, Table S1**). The latter results
244 agree with a recent report that revealed that *S. cerevisiae* RFC does not discriminate against
245 nucleotides at the 3' terminus of the primer strand during clamp loading³¹.

246 The 3'PCy3/T DNA substrate is first pre-bound with NeutrAvidin and Cy5-OBD-RPA and
247 I_{563} and I_{665} are monitored over time (**Figure 4D**). A significant, constant E_{FRET} is observed prior
248 to the addition of RFC in any form due to the stable interaction of Cy5-OBD-RPA with
249 3'PCy3/T DNA (**Figures S4 – S6**). Upon addition of the loading complex (RFC•ATP γ S•Cy5-
250 PCNA), I_{665} rapidly decreases concomitantly with a rapid increase in I_{563} after which I_{665}
251 continues to decrease slowly while I_{563} continues to increase slowly (**Figure 4E, Top**). These
252 synchronized, anti-correlated changes in I_{563} and I_{665} are indicative of a decrease in FRET
253 (**Figure 4E, Bottom**). The decrease in E_{FRET} observed in **Figure 4F** is comprised of three phases
254 with observed rate constants of $k_{\text{obs inc},1} \geq 0.165 \pm 0.024 \text{ s}^{-1}$, $k_{\text{obs inc},2} = 8.98 \pm 0.79 \text{ (} \times 10^{-3} \text{ s}^{-1}\text{)}$, and
255 $k_{\text{obs inc},3} = 7.89 \pm 0.04 \text{ (} \times 10^{-4} \text{ s}^{-1}\text{)}$. $k_{\text{obs inc},3}$, which accounts for $67.4 \pm 2.50 \%$ of the E_{FRET} decrease
256 in **Figure 4F**, is nearly identical to $k_{\text{obs},2}$ for the E_{FRET} increase observed in **Figure 4C**.

257 Interestingly, and in contrast to those observed in **Figure 2C** and **2F**, the E_{FRET} behaviors
258 observed in **Figures 4C** and **4F** with ATP γ S are anti-correlated and occur with very similar
259 kinetics; E_{FRET} between PCNA and DNA increases (**Figure 4C**), indicating binding of the
260 loading complex to the P/T junction and adoption of the activated conformation; E_{FRET} between
261 RPA OBD and DNA decreases, indicating that the distance between RPA OBD and the 3'
262 terminus of the primer strand increases. Together, this suggests that during PCNA loading,
263 RPAOBD releases from the 3' terminus of the primer strand to accommodate binding and
264 activation of the loading complex at the nascent P/T junction. Next, we performed similar assays
265 with ATP (**Figure 5**) to investigate the interaction(s) of RPA OBD with a P/T junction during the
266 remainder of the PCNA loading pathway.

267 First, interactions of RFC and PCNA with P/T junctions were monitored (**Figure 5A – C**).
268 The 5'PCy3/T DNA substrate is pre-bound with NeutrAvidin and native RPA. Then, Cy5-PCNA
269 is added and I_{563} and I_{665} are monitored over time (**Figure 5A**). Here, a low, constant E_{FRET} is
270 observed prior to the addition of RFC in any form due to the presence of both the Cy3 donor and
271 the Cy5 acceptor. E_{FRET} values observed during this period represent a true experimental baseline

272 signal representing the complete absence of interactions between Cy5-PCNA and the 5'PCy3/T
273 DNA•RPA complex. Next, a pre-formed RFC•ATP complex is added, and the fluorescence
274 emission intensities are monitored over time. Under the conditions of the assay, the appearance
275 and increase in E_{FRET} towards maximal values encompasses all kinetic steps along the PCNA
276 loading pathway and reflects; 1) a relatively fast kinetic step(s) ($k_{\text{obs inc},1}$) along the PCNA
277 loading pathway that occurs prior to and much slower than binding of the loading complex to the
278 P/T junction and; 2) a relatively slower release of the RFC•ADP complex into solution via its
279 dissociation from the RPA1 subunit of the resident RPA complex engaged with the P/T junction
280 ($k_{\text{obs inc},2}$)²⁵.

281 Upon addition of RFC•ATP, the observed changes in I_{665} and I_{563} are synchronized and anti-
282 correlated (**Figure 5B, Top**), indicating the appearance and increase in FRET (**Figure 5B,**
283 **Bottom**). As observed in **Figure 5C**, E_{FRET} traces rapidly increase to values significantly above
284 the E_{FRET} values observed for no interaction between Cy5-PCNA and the 5'PCy3/T•RPA
285 complex. As expected, the rapid increase in E_{FRET} observed upon addition of RFC is biphasic
286 with observed rate constants of $k_{\text{obs inc},1} = 4.34 \pm 0.18$ ($\times 10^{-2} \text{ s}^{-1}$) and $k_{\text{obs inc},2} = 1.78 \pm 0.04$ ($\times 10^{-2}$
287 s^{-1}) (**Table S1**). Each of the observed rate constants agree very well with the values observed in
288 **Figure 3C**. As discussed above, $k_{\text{obs inc},2}$ reports on release of the RFC•ADP complex into
289 solution via its dissociation from the RPA1 subunit of the resident RPA engaged with the P/T
290 junction. This kinetic step occurs throughout the time-dependent increase in E_{FRET} observed in
291 **Figure 5C** and accounts for ~30 – 60% of the E_{FRET} signal at any point in time, based on fits of
292 the kinetic data. Release of the RFC•ADP complex into solution immediately follows hydrolysis
293 of ATP by RFC within the activated loading complex (and concomitant closure of the sliding
294 clamp ring) or release of the closed PCNA ring from the RFC•ADP complex²⁵. Next, we
295 repeated these assays utilizing the 3'PCy3/T DNA substrate, Cy5-OBD-RPA, and PCNA
296 (**Figure 5D**) to monitor the interaction(s) of RPA OBD with P/T junctions.

297 A significant, constant E_{FRET} is observed prior to the addition of RFC in any form due to the
298 stable interaction of Cy5-OBD-RPA with 3'PCy3/T DNA (**Figure 5E, Top, Figures S4 – S6**).
299 After addition of RFC•ATP complex, RPA OBD subsequently releases from the 3' terminus of
300 the primer strand to accommodate binding and activation of the loading complex at the P/T
301 junction (**Figure 4**). Under the conditions of the assay depicted in **Figure 5D**, kinetic steps along
302 the PCNA loading pathway from binding of the loading complex to the P/T junction up to and
303 including release of the closed PCNA ring on the DNA are kinetically invisible; only release of
304 RFC•ADP into solution (via its dissociation from the RPA1 subunit of the resident RPA) is
305 visible. This raises at least three scenarios for E_{FRET} traces observed after the addition of the
306 RFC•ATP complex. First, if RPA OBD interactions are not subsequently re-established with the
307 3' terminus of the primer, E_{FRET} will instantaneously decrease upon addition of the RFC•ATP
308 complex and then remain at a reduced level. Second, if RPA OBD interactions are re-established
309 with the 3' terminus of the primer prior to and much faster than release of RFC•ADP into
310 solution, then a change in E_{FRET} will not be observed upon addition of the RFC•ATP complex.
311 Third, if RPA OBD interactions are re-established concomitantly with or subsequent to release of
312 RFC•ADP into solution, E_{FRET} will instantaneously decrease upon addition of the RFC•ATP
313 complex and then increase over time to E_{FRET} values observed prior to the addition of the
314 RFC•ATP complex.

315 Upon addition of RFC•ATP, the slight changes in I_{665} and I_{563} are synchronized and
316 correlated (**Figure 5E, Top**). This behavior is due to nonspecific effects³⁰. For the example E_{FRET}

317 trajectory depicted in **Figure 5E (Bottom)**, E_{FRET} values observed prior to the addition of the
318 RFC•ATP complex are maintained after addition of the RFC•ATP loading complex. For the
319 averaged E_{FRET} trajectory depicted in **Figure 5F**, the E_{FRET} traces observed prior to the addition
320 of the RFC•ATP complex persist and are maintained at the significantly elevated level above the
321 E_{FRET} traces predicted for no interaction between 3'PCy3/T DNA and Cy5-OBD-RPA. This
322 agrees with the second scenario described above where RPA OBD interactions are re-established
323 with the 3' terminus of the primer prior to and much faster than release of RFC•ADP into
324 solution via its dissociation from the RPA1 subunit of the resident RPA engaged with the P/T
325 junction.

326

327 *Initiation of DNA synthesis by a Pol δ holoenzyme alters the orientation of PCNA encircling a*
328 *P/T junction.*

329 In the next step of human Pol δ holoenzyme assembly, Pol δ engages the “front face” of
330 PCNA encircling a P/T junction, forming a holoenzyme, and subsequently initiates DNA
331 synthesis (**Figure 1A**). Currently, the interplay between the macromolecular interactions of Pol
332 δ , PCNA, and RPA at nascent P/T junctions are unknown. In the present study, we utilize FRET
333 assays to analyze these macromolecular interactions. Initially, we focus on interactions of PCNA
334 with P/T junctions during formation of the Pol δ holoenzyme and subsequent initiation of DNA
335 synthesis (**Figure 6A**).

336 The 5'ddPCy3/T DNA substrate is pre-bound with NeutrAvidin and native RPA. Then, Cy5-
337 PCNA is added, and I_{563} and I_{665} are monitored over time. Next, pre-formed RFC•ATP is added,
338 and the fluorescence emission intensities are monitored over time until PCNA loading is
339 complete. Finally, dGTP is added together with Pol δ at a stoichiometric ratio of Pol δ to DNA
340 and PCNA (i.e., DNA:PCNA:Pol δ = 1:1:1) and the fluorescence emission intensities are
341 monitored over time. Under these conditions, Pol δ is stabilized in the initiation state for DNA
342 synthesis where Pol δ engages PCNA encircling the P/T junction, the P/T junction, and aligns an
343 incoming dGTP at the 3' terminus of the primer strand in a correct base pair (bp) with the
344 template nucleotide (C) immediately 5' of the P/T junction^{24,25,32}. Both extension of the primer
345 (via Pol δ DNA polymerase activity) and degradation of the primer (via Pol δ exonuclease
346 activity) are prohibited due to the utilization of a 3' dideoxy-terminated primer and exonuclease-
347 deficient Pol δ ²⁵.

348 Upon addition of RFC•ATP, the observed changes in I_{665} and I_{563} are synchronized and anti-
349 correlated (**Figure 6B, Top**), indicating the appearance and increase in FRET (**Figure 6B,**
350 *Bottom*). As expected, E_{FRET} traces observed in **Figure 6C** rapidly increase in a biphasic manner
351 upon addition of RFC•ATP and plateau at values significantly above the E_{FRET} traces observed
352 for no interaction between Cy5-PCNA and 5'ddPCy3/T DNA. At the plateau, a PCNA is
353 assembled onto each P/T junction and is rapidly and randomly diffusing along the dsDNA region
354 ($D = 2.24 \times 10^7 \text{ bp}^2/\text{s}$)³³. The biotin/NeutrAvidin complex at the 5' terminus of the template
355 strands prevent diffusion of loaded PCNA off the dsDNA end of the P/T DNA substrate. The
356 resident RPA engaged at the P/T junction prohibits diffusion of PCNA along the adjacent ssDNA
357 as well as RFC-catalyzed unloading of PCNA²⁵. In the current experimental setup, rapid
358 diffusion of loaded PCNA along the dsDNA region is kinetically invisible and, hence, E_{FRET}
359 values observed at the plateau report on the average position of loaded PCNA relative to the 5'
360 end of the primer strand. Upon addition of stoichiometric Pol δ , I_{665} rapidly increases
361 concomitantly with a rapid decrease in I_{563} , after which both fluorescence emission intensities

362 stabilize and persist over time (**Figure 6B, Top**). These synchronized, anti-correlated changes in
363 I_{563} and I_{665} are indicative of a further increase in FRET (**Figure 6B, Bottom**). As observed in
364 **Figure 6C**, upon addition of Pol δ , E_{FRET} traces rapidly increase to values significantly above the
365 E_{FRET} values observed for the 5' ddPCy3/T•RPA•Cy5-PCNA complex (i.e., loaded Cy5-PCNA).
366 The rapid increase in E_{FRET} observed upon addition of Pol δ is comprised of two phases (i.e.,
367 biphasic) with an observed rate constant for the slower phase ($k_{\text{obs inc},2}$) of $8.41 \times 10^{-3} \pm 0.19$ (\times
368 10^{-3} s^{-1}). The overall E_{FRET} increase is due to a further reduction in the distance between the Cy3
369 donor near the 5' end of the primer strand and the Cy5 acceptor on the “back” face of the loaded
370 PCNA ring. Next, we further characterized the enhanced FRET state observed upon addition of
371 Pol δ by monitoring the “% FRET Change” (depicted in **Figure 6C**) under various conditions
372 (**Figure 6D**).

373 For reference, the % FRET change observed in **Figure 6C** is reported in **Figure 6D** (+
374 dGTP, 1X Pol δ). Doubling the concentration of Pol δ in the presence of dGTP (+ dGTP, 2X Pol
375 δ) did not affect the % FRET Change, indicating that the enhanced FRET state is saturated at
376 stoichiometric Pol δ . Next, dGTP was omitted. Under these conditions, Pol δ saturates PCNA
377 encircling P/T junctions to form Pol δ holoenzymes but engages P/T junctions with dramatically
378 low affinity, if at all^{21,22,24,27,34,35}. Inclusion of only Pol δ (- dGTP, + 1X Pol δ) did not yield a %
379 FRET Change and this behavior persisted at increasing concentrations of Pol δ (up to a
380 DNA:PCNA:Pol δ ratio of 1:1:4). This indicates that the enhanced FRET state requires dGTP.
381 Furthermore, these results reveal that Pol δ alone does not displace RPA from a P/T junction
382 upon engaging the resident PCNA, as follows. A large protein, such as Pol δ , simply binding to
383 PCNA encircling a P/T junction decreases the diffusion constant only 2.1-fold³³. Thus, in the
384 absence of dNTPs, if Pol δ displaced RPA from the 5' ddPCy3/T DNA upon engaging the
385 resident Cy5-PCNA, the resultant Cy5-PCNA•Pol δ complex would rapidly diffuse off the
386 ssDNA end of the 5' ddPCy3/T DNA simultaneously with RPA displacement. This would lead to
387 a rapid decrease in E_{FRET} (rate-limited by displacement of RPA) and a subsequent, relatively
388 slow increase in E_{FRET} as free Cy5-PCNA is reloaded by RFC. However, a % FRET Change is
389 not observed in **Figure 6D** when only Pol δ is included, indicating that loaded PCNA persists on
390 P/T DNA throughout Pol δ holoenzyme formation and, hence, RPA maintains contact(s) with the
391 respective P/T junction throughout Pol δ holoenzyme formation. This is directly investigated in
392 **Figure 7** below.

393 Inclusion of only dGTP (+ dGTP, - Pol δ) led to a slight decrease in FRET due to the
394 inhibition of PCNA re-loading by RFC (**Figure S11 - S13**)³⁶. Thus, the enhanced FRET state
395 requires Pol δ . Altogether, the results in **Figure 6D** indicate that formation of the enhanced
396 FRET state following RFC-catalyzed loading of PCNA requires both dGTP and Pol δ . Hence,
397 the enhanced FRET state represents the initiation state for DNA synthesis. Furthermore, these
398 results reveal that formation of the initiation state for DNA synthesis alters the
399 orientation/distance of the closed PCNA ring relative to the P/T junction. Next, we investigated
400 the interactions of RPA with a P/T junction during formation of a Pol δ holoenzyme and
401 subsequent initiation of DNA synthesis.

402
403 *RPA remains engaged with a P/T junction during formation of a Pol δ holoenzyme and initiation*
404 *of DNA synthesis.*

405 Human Pol δ interacts (albeit with unknown affinity) with the RPA1 subunit of an RPA
406 complex that is engaged with a P/T junction (**Figure 1C**) and, hence, these interactions may

407 initially target Pol δ to P/T junctions where it captures a diffusing, loaded PCNA^{23,37}. However,
408 during initiation of DNA synthesis from a nascent P/T junction, the DNA footprints of RPA and
409 Pol δ on a P/T junction significantly overlap (**Figure 1B**)^{12-18,24,32}. To investigate the interplay
410 between the macromolecular interactions of Pol δ and RPA at a nascent P/T junction, we
411 performed FRET assays similar to those described above in **Figure 6** to monitor interactions of
412 RPA OBA with a P/T junction during formation of a Pol δ holoenzyme and initiation of DNA
413 synthesis (**Figure 7**).

414 The ddP/5'TCy3 DNA substrate is pre-bound with NeutrAvidin and RPA-OBA-Cy5 (**Figure**
415 **7A**). Then, PCNA is added followed by pre-formed RFC•ATP complex. After completion of
416 RFC-catalyzed loading of PCNA, I_{563} and I_{665} are monitored over time. Here, a significant,
417 constant E_{FRET} is observed prior to the addition of Pol δ due to the stable interaction of RPA-
418 OBA-Cy5 with ddP/5'TCy3 DNA (**Figures 2, 3, and S4 – S6**). Next, Pol δ alone is added at a 2-
419 fold excess to DNA and PCNA to ensure all loaded PCNA is engaged in a Pol δ holoenzyme and
420 the fluorescence emission intensities are monitored over time. Finally, to demonstrate RPA
421 occupancy of the P/T junction following Pol δ holoenzyme formation, a large excess of
422 poly(dT)₇₀ ssDNA is added, and the fluorescence emission intensities are monitored over time.
423 Human RPA has exceptionally high affinity for ssDNA at physiological ionic strength but can
424 undergo facilitated exchange due to the dynamic ssDNA-binding interactions of its individual
425 OB folds, enabling RPA to exchange between ssDNA sequences when free ssDNA is present in
426 solution (**Figure S14**)^{29,38-40}. The ssDNA binding affinity of human RPA is highest for poly(dT)
427 and each poly(dT)₇₀ accommodates at least two RPA complexes^{12-14,41}. Hence, poly(dT)₇₀ serves
428 as an effective trap to release RPA-OBA-Cy5 from the ddP/5'TCy3 DNA substrate via facilitated
429 exchange and prohibit re-binding.

430 Upon addition of Pol δ alone, the slight changes in I_{665} and I_{563} are synchronized and
431 correlated (**Figure 7B, Top**). For the example E_{FRET} trajectory depicted in **Figure 7B (Bottom)**,
432 the E_{FRET} values observed after addition of Pol δ increase very slightly and then slowly and
433 minimally decrease over time, mirroring the synchronized, correlated changes observed for I_{665}
434 and I_{563} over time (**Figure 7B, Top**). This apparent time-dependent change in the E_{FRET} values
435 observed after the addition of Pol δ is due to nonspecific effects and not attributable to changes
436 in the distance between the cyanine labels³⁰. Upon addition of poly(dT)₇₀, the observed changes
437 in I_{665} and I_{563} are synchronized and anti-correlated (**Figure 7B, Top**), indicating a decrease in
438 FRET (**Figure 7B, Bottom**). For the averaged E_{FRET} trajectory in **Figure 7C**, the E_{FRET} values
439 observed prior to the addition of Pol δ increase very slightly after addition of Pol δ , and then
440 slowly and minimally decrease. These time-dependent changes in E_{FRET} are due to indirect
441 effects on the fluorescence emission intensities of Cy3 and Cy5 (**Figure 7B, Top**). Regardless,
442 E_{FRET} values observed after addition of Pol δ remain within experimental error of the E_{FRET}
443 values observed prior to the addition of Pol δ and also significantly elevated above the E_{FRET}
444 trace predicted for no interaction between ddP/5'TCy3 DNA and Cy5-OBD-RPA. Upon addition
445 of excess poly(dT)₇₀, E_{FRET} rapidly decreases over time to the E_{FRET} trace predicted for no
446 interaction between ddP/5'TCy3 DNA and RPA-OBA-Cy5, indicating release of RPA-OBA-Cy5
447 from ddP/5'TCy3 via facilitated exchange with the ssDNA trap. Together, this suggests that
448 nearly all ($\geq 85.1 \pm 11.6\%$, based on the observed E_{FRET} changes), if not all, RPA maintains
449 contact(s) with a P/T junction throughout formation of the resident Pol δ holoenzyme, which
450 may include direct protein•protein interactions with Pol δ . This agrees with the results discussed
451 above for **Figure 6D**.

452 Next, we repeated the experiments described in **Figure 7A – C** by adding dGTP
453 simultaneously with a 2-fold excess of Pol δ compared to DNA and PCNA (**Figure 7D – F**).
454 Here, excess polymerase ensures all loaded PCNA is engaged with Pol δ in the initiation state for
455 DNA synthesis (**Figure 6D**). Upon addition of Pol δ and dGTP, the slight changes in I_{665} and I_{563}
456 are synchronized and correlated (**Figure 7E, Top**). This behavior is due to nonspecific effects³⁰.
457 For the example E_{FRET} trajectory depicted in **Figures 7E (Bottom)** and the averaged E_{FRET}
458 trajectory depicted in **Figures 7F**, the E_{FRET} values observed prior to the addition of Pol δ and
459 dGTP slowly and minimally decrease after the addition of Pol δ and dGTP due to indirect effects
460 on the fluorescence emission intensities of Cy3 and Cy5 (**Figure 7E, Top**). E_{FRET} values
461 observed after addition of Pol δ and dGTP remain within experimental error of the E_{FRET} values
462 observed prior to the addition of Pol δ and dGTP and also significantly elevated above the E_{FRET}
463 trace predicted for no interaction between ddP/5'TCy3 DNA and Cy5-OBD-RPA. Upon addition
464 of excess poly(dT)₇₀, E_{FRET} rapidly decreases over time to the E_{FRET} trace predicted for no
465 interaction between ddP/5'TCy3 DNA and RPA-OBA-Cy5, indicating release of RPA-OBA-Cy5
466 from ddP/5'TCy3 via facilitated exchange with the ssDNA trap. Together, this indicates that
467 nearly all ($\geq 77.3 \pm 5.7\%$ based on the observed changes in E_{FRET}), if not all, RPA maintains
468 contact(s) with a P/T junction during initiation of DNA synthesis by the resident Pol δ
469 holoenzyme.

470

471 Discussion

472 Human Pol δ holoenzymes are assembled at primer/template (P/T) junctions and initiate
473 DNA synthesis in a complex process that requires the spatially and temporally coordinated
474 actions of RPA, RFC, PCNA, and Pol δ . Each of these factors interact uniquely with a P/T
475 junction and most directly engage one another. Currently, the interplay between these
476 macromolecular interactions during Pol δ holoenzyme assembly and initiation of DNA synthesis
477 is largely unknown. In the present study, we designed and utilized novel FRET assays to monitor
478 these macromolecular interactions using recombinant human proteins. Together with previous
479 work from our lab and others, the results from the present study provide the first complete
480 description of human Pol δ holoenzyme assembly and initiation of DNA synthesis (**Figure 8**).

481 The ssDNA immediately adjacent to a nascent P/T junction is initially engaged by an RPA
482 heterotrimer in an orientation-specific manner (**Figure 1**). Specifically, 30 – 33 nt of ssDNA are
483 engaged with a defined 5'→3' polarity where OBA of the RPA1 subunit aligns closer to the 5'
484 end of the ssDNA sequence and OBD of the RPA2 subunit aligns closer to the 3' end such that
485 OBD and OBC directly contact the 3' terminus of the primer strand. RPA remains engaged with
486 the P/T DNA with exceptionally high affinity but exists in microscopically dissociated states due
487 to the dynamic ssDNA-binding interactions of its individual OB folds. In particular, both OBA
488 and OBD have been directly observed to rapidly bind to and dissociate from ssDNA while RPA
489 remains engaged with ssDNA¹²⁻¹⁹. Next, RFC, in complex with ATP, engages the “front face” of
490 a free PCNA in solution, opens the sliding clamp, and the resultant loading complex engages a
491 nascent P/T junction such that the “front face” of PCNA is oriented towards the 3' terminus of
492 the primer from which DNA synthesis initiates (**Figure 8, Step 1**). Loading complexes may be
493 targeted to P/T junctions engaged by RPA through direct protein•protein interactions between
494 subunits 1 – 3 of the RFC complex and the RPA1 subunit of the RPA complex (**Figure 1C**)^{23,24}.
495 Such targeting, if it occurs, does not observably affect the interaction(s) of RPA OBA with a P/T
496 junction (**Figures 2D - F**). Upon engaging a nascent P/T junction, the loading complex adopts an

497 activated conformation in which ATP hydrolysis by RFC is optimized (**Figures 2A - C** and **4A -**
498 **C**). During association and activation of the loading complex, RPA OBD is released from the 3'
499 terminus of the primer strand to accommodate binding and activation of the loading complex at
500 the nascent P/T junction (**Figure 4D - F**) but RPA remains engaged with the P/T DNA through
501 the persistent interaction(s) of at least RPA OBA with the template strand downstream of the P/T
502 junction (**Figure 3D - F**). Given the dynamics of RPA•ssDNA complexes discussed above, we
503 posit that a loading complex captures a P/T junction that is exposed during microscopic
504 dissociation of RPA OBD, and likely RPA OBC, from the 3' terminus of the primer strand and
505 the template ssDNA immediately 5' of the P/T junction. Microscopic dissociation of RPA OBA
506 and other OB folds downstream of the nascent P/T junction do not manifest to microscopic
507 dissociation of RPA.

508 ATP hydrolysis by RFC within the activated loading complex simultaneously closes PCNA
509 around the DNA and the closed (i.e., loaded) PCNA is subsequently released onto the dsDNA
510 region of the nascent P/T junction (**Figure 8, Step 2**). RPA engaged with a nascent P/T junction
511 promotes release of closed PCNA from RFC•ADP either directly or indirectly via stimulation of
512 ATP hydrolysis by RFC within the activated loading complex (and simultaneous closure of the
513 PCNA clamp)²⁵. Concomitant with closure of PCNA or release of loaded PCNA, the resultant
514 RFC•ADP complex vacates the P/T junction, transferring to the RPA1 subunit, and the
515 interactions of RPA OBD are re-established with the 3' terminus of the primer strand (**Figure 5D**
516 **- F**). The RFC•ADP complex subsequently releases into solution (**Figure 8, Step 3**) and this
517 step is rate-limited by dissociation of the RFC•ADP complex from the RPA1 subunit of the
518 resident RPA engaged with the nascent P/T junction (**Figures 3A - C** and **5A - C**)^{20,21,23,25}. RFC
519 then exchanges ADP for ATP (**Figure 8, Step 4**) and is once again competent for PCNA loading.

520 Loaded PCNA rapidly and randomly diffuses ($D = 2.24 \times 10^7$ bp²/s) along the dsDNA region
521 immediately 5' of the P/T junction³³. Diffusion of loaded PCNA away from a P/T junction is
522 restricted by physical blocks, i.e., “protein roadblocks,” such as sub-nucleosomes and high-
523 affinity transcription factors that rapidly re-assemble on or rebind to nascent DNA generated
524 during DNA replication⁴²⁻⁴⁴. This is mimicked in the current experimental setup where
525 biotin/NeutrAvidin complexes at the 5' termini of the template strands prevent diffusion of
526 loaded PCNA off the dsDNA end of the P/T DNA substrates. The resident RPA engaged at the
527 P/T junction prohibits diffusion of PCNA along the adjacent ssDNA as well as RFC-catalyzed
528 unloading of PCNA²⁵. Recent *in vivo* evidence suggests that enzyme-catalyzed unloading of
529 PCNA from a P/T junction will not occur until the primer is completely extended and ligated to
530 the downstream duplex region⁴⁵. In the absence of catalyzed unloading of PCNA and significant
531 translocation of loaded PCNA, the only pathway for dissociation of loaded PCNA from a nascent
532 P/T junction is through spontaneous opening of the PCNA ring, which is dramatically slow [k_{open}
533 $= 1.25 \pm 0.32$ ($\times 10^{-3}$) s⁻¹]⁴⁶, ~20-fold slower than the observed rate constant RFC-catalyzed
534 loading of “free” PCNA onto a P/T junction in the presence of ATP (**Figure 3, 5, S3, S3 - S9,**
535 **and Table 1**). Thus, upon dissociation of PCNA from a nascent P/T junction via spontaneous
536 opening of the PCNA ring (**Figure 8, Step 5**), RFC utilizes ATP to instantly reload PCNA back
537 onto the nascent P/T junction (**Figure 8, Steps 1 - 3**) such that the loss of loaded PCNA from a
538 nascent P/T junction is very transient and essentially not observed (**Figure S11**)^{21,25}.

539 In the next step of Pol δ holoenzyme assembly, Pol δ engages the “front face” of loaded
540 PCNA encircling a P/T junction, forming a holoenzyme (**Figure 8, Step 6**). Human Pol δ is
541 comprised of four subunits, three of which contain PCNA-binding motifs and simultaneously
542 bind all subunits within a given PCNA homotrimer. This multivalent interaction leads to a

543 significantly high affinity for PCNA encircling P/T junctions ($K_D < 10 \text{ nM}$)³⁴. In the absence of
544 dNTPs (i.e., no DNA synthesis), Pol δ has dramatically low affinity, if any, for P/T junctions
545 ^{22,24,25,27,34}. Thus, it is likely that Pol δ directly engages a loaded PCNA that is rapidly and
546 randomly diffusing along the dsDNA region of a nascent P/T junction. However, human Pol δ
547 interacts (albeit with unknown affinity) with the RPA1 subunit of an RPA complex that is
548 engaged with a P/T junction (**Figure 1C**). Thus, it is possible that these interactions initially
549 target Pol δ to a nascent P/T junction where it captures a diffusing, loaded PCNA^{23,37}. Such
550 targeting, if it occurs, as well as formation of a Pol δ holoenzyme do not displace the resident
551 RPA from nascent P/T junction nor observably affect the interaction(s) of RPA OBA with a P/T
552 junction (**Figure 6D** and **7A-C**).

553 Finally, an assembled Pol δ holoenzyme engages a nascent P/T junction and an incoming
554 dNTP and aligns the incoming dNTP at the 3' terminus of the primer strand in a correct bp with
555 the template nucleotide immediately 5' of the P/T junction (**Figure 8, Step 7**)^{24,25,32}. The results
556 presented in **Figure 6** indicate that formation of the initiation state for DNA synthesis alters the
557 orientation/distance of the closed PCNA ring relative to the P/T junction. Specifically, the
558 distance between the 5' end of the primer strand and the “back face” of the loaded PCNA ring is
559 decreased. In the initiation state for DNA synthesis, Pol δ directly engages 12 – 14 bp of the
560 dsDNA region immediately upstream of the nascent P/T junction and PCNA encircles the next
561 10 – 12 bp directly upstream^{24,32}. Hence, the aforementioned decrease in distance is likely due to
562 the Pol δ •PCNA interaction maintaining loaded PCNA in closer proximity to the 5' end of the
563 primer strand. The results presented in **Figure 7D – F** indicate that during initiation of DNA
564 synthesis by the resident Pol δ holoenzyme the interaction of RPA OBA is not observably
565 affected and nearly all, if not all, RPA maintains contact(s) with a nascent P/T junction. In the
566 initiation state for DNA synthesis, Pol δ directly engages the 3' terminus of the primer strand and
567 4 - 6 nt of the template ssDNA strand immediately downstream of a nascent P/T junction and
568 these protein•DNA interactions significantly overlap with those of the resident RPA complex
569 (**Figure 1B**)^{12-18,24,32}. This situation is similar to that of the overlap of the activated loading
570 complex and the RPA complex at nascent P/T junction where RPA OBD transiently releases
571 from the 3' terminus of the primer strand to accommodate binding and activation of the loading
572 complex (**Figure 8, Step 1**). Thus, we postulate RPA OBD is released from the 3' terminus of the
573 primer strand to accommodate initiation of DNA synthesis by the assembled Pol δ holoenzyme.
574 This hypothesis is currently being investigated.

575 Altogether, the results from the present study reveal that dynamic interactions of RPA
576 subunits with a nascent P/T junction during assembly of a Pol δ holoenzyme and initiation of
577 DNA synthesis both maintain RPA at a P/T junction and accommodate RFC, PCNA, and Pol δ .
578 Furthermore, as discussed above, the only pathway for dissociation of loaded PCNA from a
579 nascent P/T junction during these processes is through spontaneous opening of the PCNA ring,
580 which is dramatically slow and essentially not observed. Hence, the coordinated actions of RPA,
581 RFC, Pol δ and protein roadblocks maximize the efficiency of PCNA utilization throughout
582 these processes. As Pol δ holoenzymes also carry out DNA synthesis during the major DNA
583 repair and tolerance pathways, the current studies provide critical insights and direction for
584 future studies on the DNA synthesis steps of long patch base excision repair, nucleotide excision
585 repair, break-induced repair, mismatch DNA repair, translesion DNA synthesis and homology-
586 dependent recombination^{2-6,11}.

587

588 **Methods**

589 **Oligonucleotides**

590 Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) or Bio-
591 Synthesis (Lewisville, TX) and purified on denaturing polyacrylamide gels. The concentrations
592 of unlabeled DNAs were determined from the absorbance at 260 nm using the calculated
593 extinction coefficients. The concentrations of Cy5-labeled DNAs were determined from the
594 extinction coefficient at 650 nm for Cy5 ($\epsilon_{650} = 250,000 \text{ M}^{-1}\text{cm}^{-1}$). Concentrations of Cy3-
595 labeled DNAs were determined from the extinction coefficient at 550 nm for Cy3 ($\epsilon_{550} = 136,000$
596 $\text{M}^{-1}\text{cm}^{-1}$). For annealing two ssDNAs (as depicted in **Figure S1**), the primer and corresponding
597 complementary template strands were mixed in equimolar amounts in 1X Annealing Buffer (10
598 mM TrisHCl, pH 8.0, 100 mM NaCl, 1 mM EDTA), heated to 95 °C for 5 minutes, and allowed
599 to slowly cool to room temperature.

600 **Recombinant Human Proteins**

601 Human RPA, Cy5-PCNA, exonuclease-deficient Pol δ (referred to herein as simply Pol δ) and
602 RFC were obtained as previously described^{21,47}. The concentration of active RPA was
603 determined via a FRET-based activity assay as described previously⁴⁸. Human RPA containing a
604 Cy5 label at either residue 101 of the RPA2 subunit (Cy5-OBDRPA) or residue 211 of the
605 RPA1 subunit (RPA-OBA-Cy5) was obtained essentially as described for *S. cerevisiae* RPA²⁹.
606 Residue 101 of the RPA2 subunit and residue 211 of the RPA1 subunit reside in the OB-folds D
607 and A of the RPA heterotrimeric complex, respectively. The concentration of active Cy5-labeled
608 RPA was determined by a FRET-based assay that is described in detail in the **Supplementary**
609 **Information**.

610 **Ensemble FRET Measurements**

611 All experiments were performed at room temperature ($23 \pm 2 \text{ }^\circ\text{C}$) and, unless indicated
612 otherwise, in 1X $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl_2 , 5 mM
613 CaCl_2) supplemented with 1 mM DTT, 1 mM ATP, and the ionic strength was adjusted to
614 physiological (200 mM) by the addition of appropriate amounts of KCl. Ca^{2+} is included to
615 account for experimental conditions in future studies and the presence of Ca^{2+} does not affect the
616 amount of RPA that binds to ssDNA (**Figure S6**) nor the amount of PCNA loaded onto DNA by
617 RFC (**Figure S4A and B** and **Table S1**). All experiments were performed in a 16.100F-Q-
618 10/Z15 sub-micro fluorometer cell (Starna Cells) and monitored in a Horiba Scientific Duetta-
619 Bio fluorescence/absorbance spectrometer. Reaction solutions are excited at 514 nm and the
620 fluorescence emission intensities (I) are monitored essentially simultaneously at 563 nm (I_{563})
621 and 665 nm (I_{665}) over time, recording I every 0.17 s. The acquisition rate of the instrument is
622 510,000 nm/min. Thus, for a given recording, the time between the acquisition of I_{563} and I_{665} for
623 each time point is negligible (0.235 ms). For all FRET experiments, excitation and emission slit
624 widths are 10 nm. For any recording of the fluorescence emission intensities (I_{665} and I_{563}), the
625 approximate FRET efficiency is estimated from the equation $E_{\text{FRET}} = \frac{I_{665}}{I_{665} + I_{563}}$. All recorded
626 fluorescence emission intensities are corrected by a respective dilution factor and all time
627 courses are adjusted for the time between the addition of each component and the fluorescence
628 emission intensity recording ($\Delta t \leq 10 \text{ s}$). For each experiment below, the final concentrations of
629 all reaction components are indicated. The concentrations of all nucleotides (ATP, $\text{ATP}\gamma\text{S}$,
630 dGTP) in all experimental solutions described below are each 1.0 mM and, hence, this
631 concentration is maintained for each nucleotide upon mixing.

632 For PCNA loading experiments in the presence of ATP γ S, a Cy3-labeled P/T DNA (20 nM,
633 **Figure S1**), NeutrAvidin (80 nM), and ATP γ S are pre-incubated with RPA (25 nM heterotrimer,
634 wild type, RPA-OBA-Cy5 or Cy5-OBD-RPA) and the resultant solution is transferred to a
635 fluorometer cell, and the cell is placed in the instrument. Fluorescence emission intensities (I_{665}
636 and I_{563}) are monitored over time until both signals stabilize for at least 1 min. Within this stable
637 region, E_{FRET} values are calculated from the observed fluorescence emission intensities (I_{665} and
638 I_{563}) and averaged to obtain the E_{FRET} value observed prior to addition of the loading complex.
639 Finally, a loading complex pre-formed with PCNA (20 nM homotrimer, Cy5-PCNA or PCNA),
640 RFC (20 nM heteropentamer) and ATP γ S is added, the resultant solution is mixed via pipetting,
641 and the fluorescence emission intensities (I_{665} and I_{563}) are monitored over time, beginning 10 s
642 after the addition of loading complex (i.e., $\Delta t \leq 10$ s).

643 For PCNA loading experiments in the presence of ATP, a Cy3-labeled P/T DNA (20 nM,
644 **Figure S1**), NeutrAvidin (80 nM), and ATP are pre-incubated with RPA (25 nM heterotrimer,
645 wild type, RPA-OBA-Cy5 or Cy5-OBD-RPA). Then, PCNA (20 nM homotrimer, Cy5-PCNA or
646 PCNA) is added, the resultant solution is transferred to a fluorometer cell, and the cell is placed
647 in the instrument. Fluorescence emission intensities (I_{665} and I_{563}) are monitored over time until
648 both signals stabilize for at least 1 min. Within this stable region, E_{FRET} values are calculated
649 from the observed fluorescence emission intensities (I_{665} and I_{563}) and averaged to obtain the
650 E_{FRET} value observed prior to addition of the RFC•ATP. Finally, a pre-formed RFC•ATP
651 complex (20 nM RFC heteropentamer) is added, the resultant solution is mixed via pipetting, and
652 the fluorescence emission intensities (I_{665} and I_{563}) are monitored over time, beginning 10 s after
653 the addition of loading complex (i.e., $\Delta t \leq 10$ s).

654 For Pol δ holoenzyme formation and initiation of DNA synthesis experiments with Cy5-
655 PCNA, a solution containing a 5'ddPCy3/T DNA (20 nM, **Figure S1**), NeutrAvidin (80 nM),
656 and ATP is pre-incubated with RPA (25 nM heterotrimer). Then, Cy5-PCNA (20 nM
657 homotrimer) is added, the resultant solution is transferred to a fluorometer cell, and the cell is
658 placed in the instrument. Fluorescence emission intensities (I_{665} and I_{563}) are monitored over time
659 until both signals stabilize for at least 1 min. Within this stable region, E_{FRET} values are
660 calculated from the observed fluorescence emission intensities (I_{665} and I_{563}) and averaged to
661 obtain the E_{FRET} value observed prior to addition of the RFC•ATP. Next, a pre-formed
662 RFC•ATP complex (20 nM RFC heteropentamer) is added, the resultant solution is mixed via
663 pipetting, and the fluorescence emission intensities (I_{665} and I_{563}) are monitored over time,
664 beginning 10 s after the addition of loading complex (i.e., $\Delta t \leq 10$ s). Fluorescence emission
665 intensities (I_{665} and I_{563}) are monitored over time until both signals stabilize for at least 1 min.
666 Within this stable region, E_{FRET} values are calculated from the observed fluorescence emission
667 intensities (I_{665} and I_{563}) and averaged to obtain the E_{FRET} value observed prior to addition of Pol
668 δ . Finally, Pol δ (20 – 80 nM Pol δ heterotetramer, \pm dGTP) is added, the resultant solution is
669 mixed by pipetting, and fluorescence emission intensities (I_{665} and I_{563}) are monitored beginning
670 ≤ 10 s after the addition of Pol δ (\pm dGTP).

671 For Pol δ holoenzyme formation and initiation of DNA synthesis experiments with PCNA, a
672 solution containing ddP/5'TCy3 DNA (20 nM, **Figure S1**), NeutrAvidin (80 nM), and ATP is
673 pre-incubated with RPA-OBA-Cy5 (25 nM heterotrimer). Then, PCNA (20 nM homotrimer) is
674 added, followed by pre-formed RFC•ATP complex (20 nM RFC heteropentamer). The resultant
675 solution is pre-incubated for the duration of RFC-catalyzed loading of PCNA (≥ 5 min),
676 transferred to a fluorometer cell, and the cell is placed in the instrument. Fluorescence emission

677 intensities (I_{665} and I_{563}) are monitored over time until both signals stabilize for at least 1 min.
678 Within this stable region, E_{FRET} values are calculated from the observed fluorescence emission
679 intensities (I_{665} and I_{563}) and averaged to obtain the E_{FRET} value observed prior to addition of the
680 Pol δ . Next, Pol δ (40 nM Pol δ heterotetramer, \pm dGTP) is added, the resultant solution is mixed
681 via pipetting, and the fluorescence emission intensities (I_{665} and I_{563}) are monitored over time,
682 beginning 10 s after the addition of Pol δ (\pm dGTP, $\Delta t \leq 10$ s). Fluorescence emission intensities
683 (I_{665} and I_{563}) are monitored over time until both signals stabilize for at least 1 min. Within this
684 stable region, E_{FRET} values are calculated from the observed fluorescence emission intensities
685 (I_{665} and I_{563}) and averaged to obtain the E_{FRET} value observed after the addition of Pol δ . Finally,
686 poly(dT)₇₀ (413 nM) is added, the resultant solution is mixed by pipetting, and fluorescence
687 emission intensities (I_{665} and I_{563}) are monitored beginning ≤ 10 s after the addition of
688 poly(dT)₇₀.

689

690 SUPPLEMENTAL INFORMATION

691 Supplemental Information includes Supplementary Results, Supplementary Methods,
692 Supplemental Figures S1 – S14, and a Supplemental Table (Table S1).

693

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700 CONFLICTS OF INTEREST

701 The authors declare that they have no conflicts of interest with the contents of this article.¹

702

703 AUTHOR CONTRIBUTIONS

704 J.L.N., K.G.P., R.L.D., S.P., S.K., and V.K. expressed, purified, and characterized all proteins.
705 J.L.N. and L.O.R. performed the experiments. M.H. designed the experiments. M.H. and E.A.
706 analyzed the data and wrote the paper.

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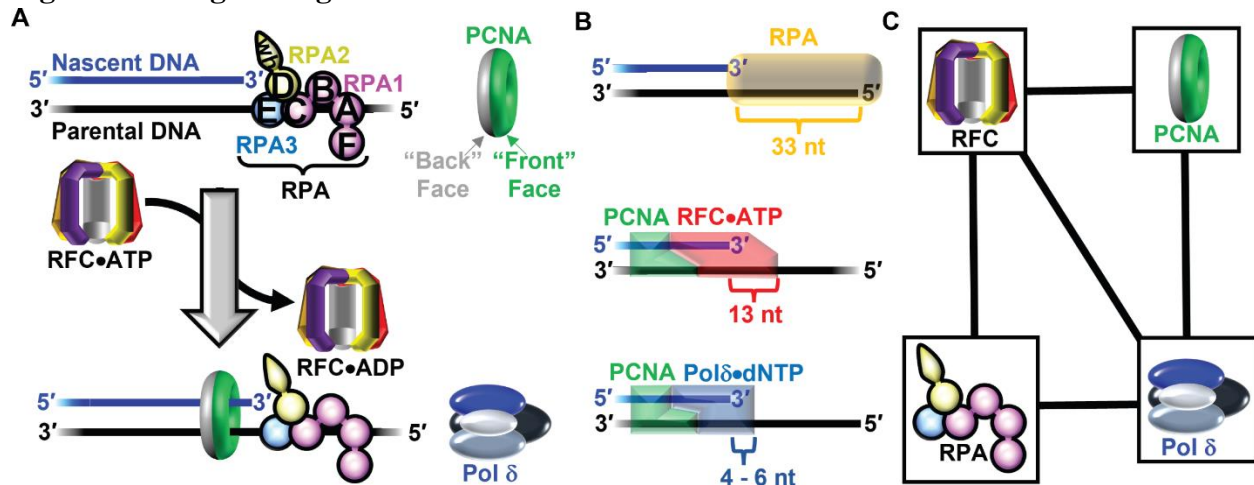
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840 **Figures and Figure Legends**



841 **Figure 1.** Assembly and macromolecular interactions of human Pol δ holoenzymes. (A)

842 Assembly of human Pol δ holoenzymes. The subunits of human RPA are color-coded (RPA1 in

843 pink, RPA2 in yellow, and RPA3 in blue) and depicted to illustrate the OB-folds (A – E) and

844 winged-helix (wh) domain. A nascent P/T junction is pre-engaged by RPA in an orientation-

845 specific manner (as depicted). First, RFC utilizes ATP to load PCNA onto a nascent P/T junction

846 in an orientation-specific manner such that the “front face” of the clamp is oriented towards the

847 3’ terminus of the primer from which DNA synthesis initiates. Next, Pol δ engages the “front

848 face” of the PCNA sliding clamp encircling the nascent P/T junction, forming a holoenzyme, and

849 subsequently initiates DNA synthesis (not shown). (B) Isolated protein•DNA interactions during

850 assembly of the Pol δ holoenzyme. Shown in *Top*, *Middle*, and *Bottom* are the DNA footprints of the

851 RPA complex, the activated RFC•ATP•“Open” PCNA complex (i.e., activated loading

852 complex), and the Pol δ holoenzyme, respectively. For each, the respective DNA region

853 protected (i.e., DNA footprint) is depicted as a given protein complex (shown in color as a

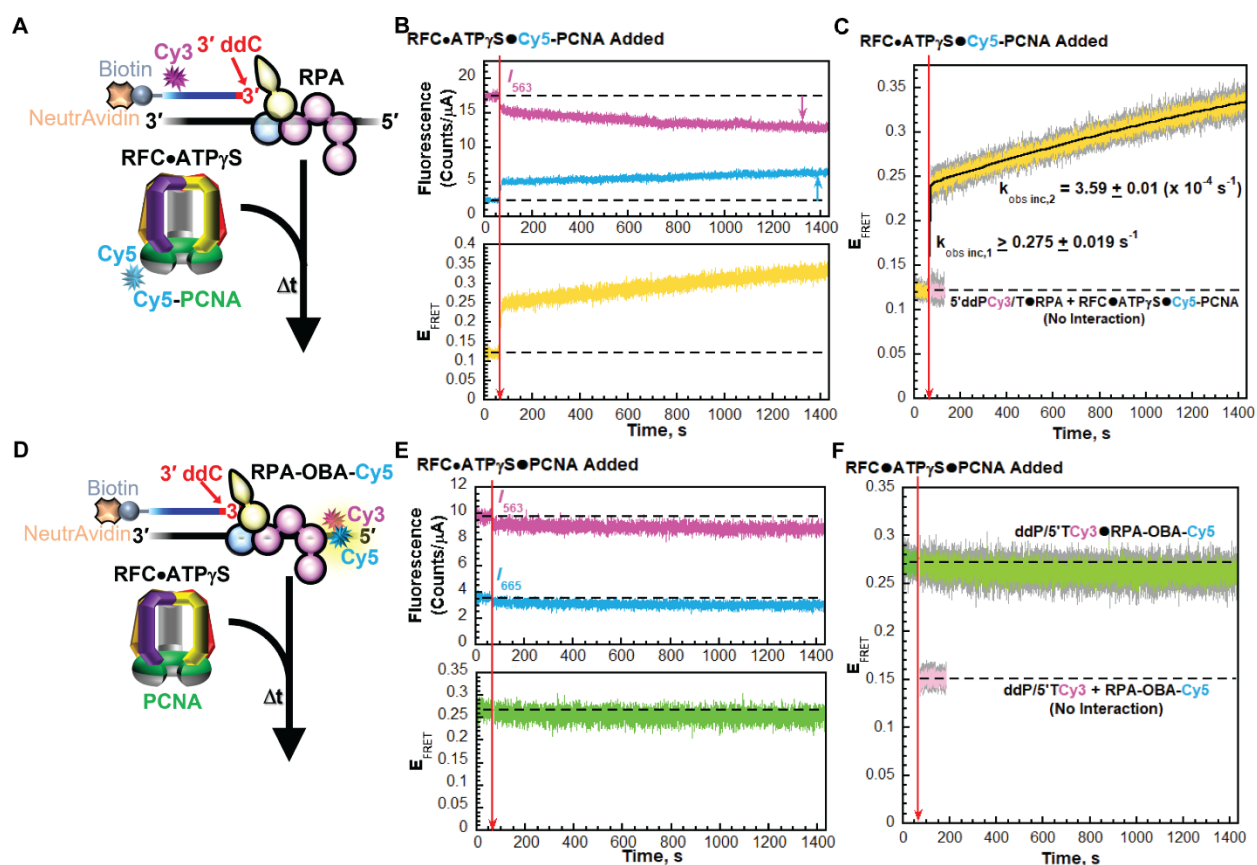
854 simple cartoon shape) overlaid on a P/T junction comprised of a 29 bp duplex region and a 33 nt

855 ssDNA overhang. The protected ssDNA region is indicated for each. (C) Protein•protein

856 interactions of the protein complexes involved in Pol δ holoenzyme assembly. Direct interactions

857 are indicated by a solid black line connecting two respective protein complexes.

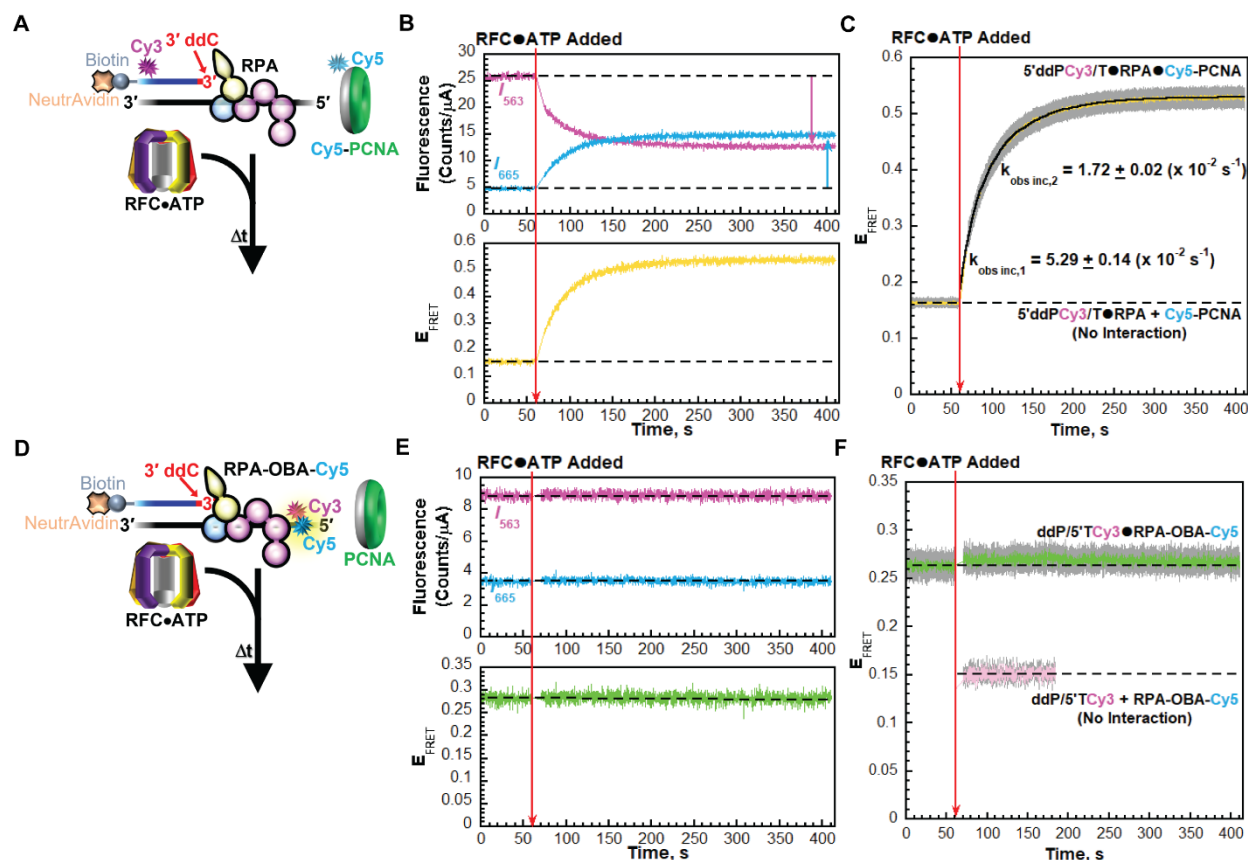
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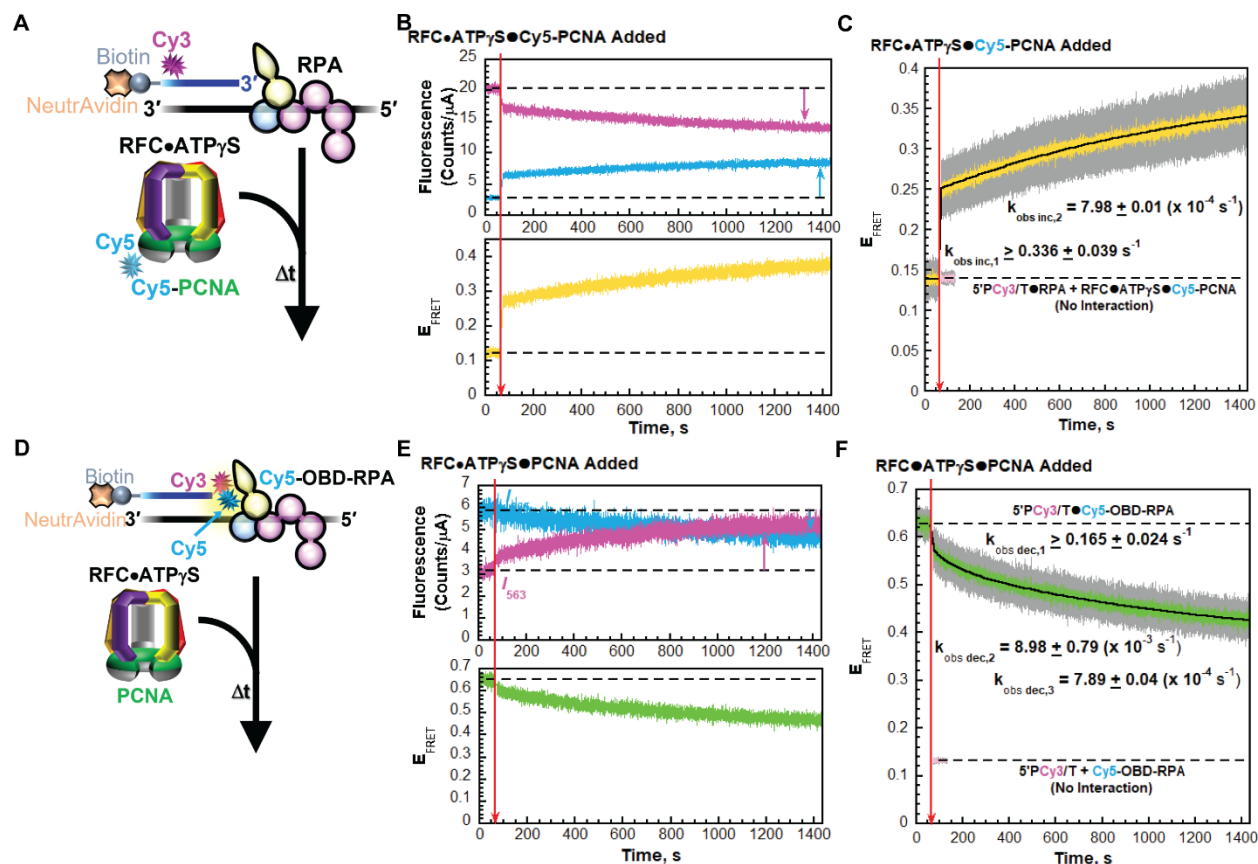
872 **Figure 2.** Interplay of RPA OBA and RFC interactions with nascent P/T junctions during PCNA
 873 loading in the presence of ATP γ S. (A - C) Monitoring interactions of a loading complex with a
 874 P/T junction engaged by RPA. (A) Schematic representation of the FRET experiment performed
 875 with 5' ddPCy3/T (+ NeutrAvidin), RPA, and loading complex pre-formed with RFC, ATP γ S,
 876 and Cy5-PCNA. (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The
 877 time at which the loading complex is added is indicated by a red arrow. Changes in I_{563} and I_{665}
 878 are indicated by magenta and cyan arrows, respectively. For observation, the I_{563} , I_{665} , and E_{FRET}
 879 values observed prior to the addition of the loading complex are fit to flat lines that are
 880 extrapolated to the axis limits. (C) FRET data. Each E_{FRET} trace is the mean of at least three
 881 independent traces with the S.E.M. shown in grey. The time at which the loading complex is
 882 added is indicated by a red arrow. The E_{FRET} trace observed after the addition of the loading
 883 complex is fit to a double exponential rise and the observed rate constants are reported in the
 884 graph. The predicted E_{FRET} trace (pink) for no interaction between 5' ddPCy3/T•RPA complex
 885 and the loading complex is fit to a flat line. (D - F) Monitoring RPA OBA interactions with a
 886 P/T junction that is engaged by a loading complex. (D) Schematic representation of the
 887 experiment. Reactions were carried out exactly as described for panel A above except that
 888 ddP/5' TCy3 DNA, RPA-OBA-Cy5, and PCNA were utilized. (E) Sample time trajectory of I_{563}
 889 and I_{665} (Top) and their E_{FRET} (Bottom) are plotted as described for panel B above. (F) FRET
 890 data. Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M. shown in
 891 grey. Data is plotted as described for panel C above. The E_{FRET} trace observed prior to the

892 addition of the loading complex is fit to a flat line that is extrapolated to the axis limits. The
893 predicted E_{FRET} trace (pink) for no interaction between RPA-OBA-Cy5 and the ddP/5'TCy3
894 DNA is fit to a flat line.

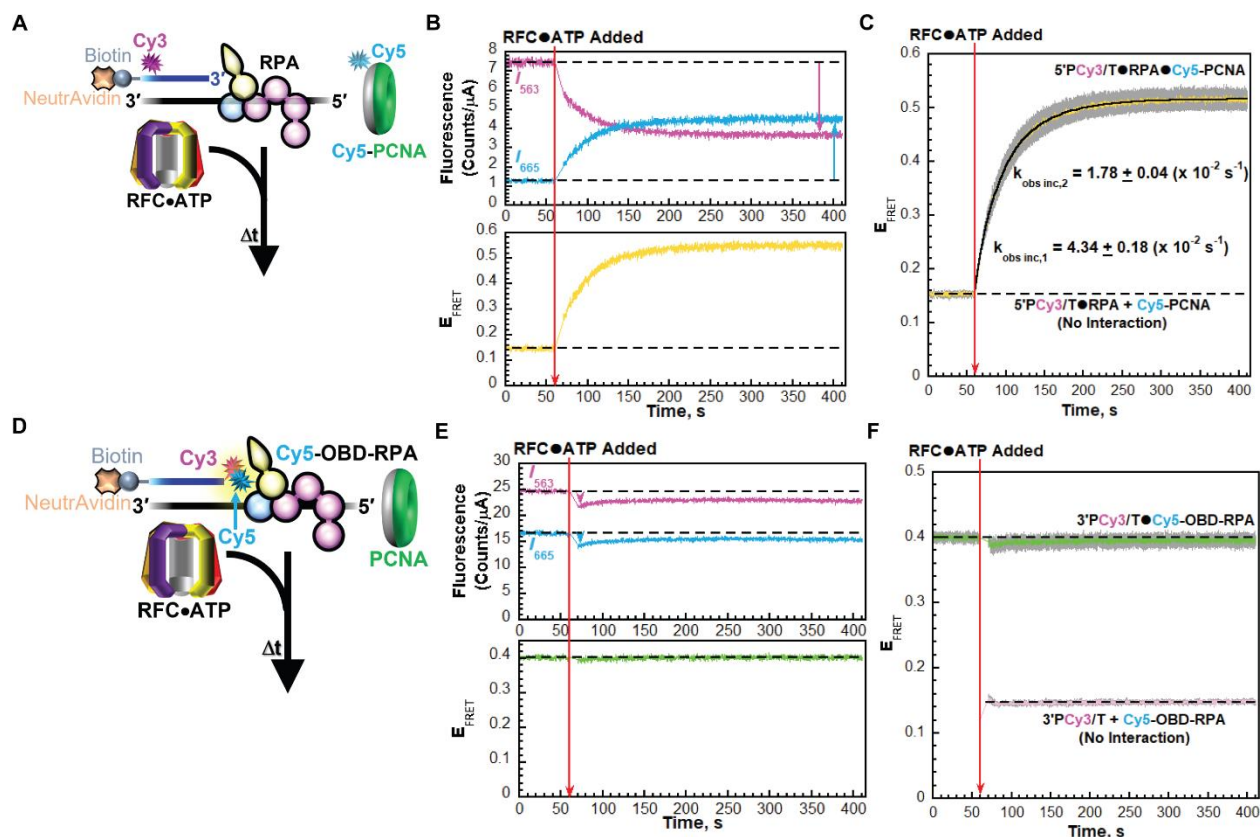
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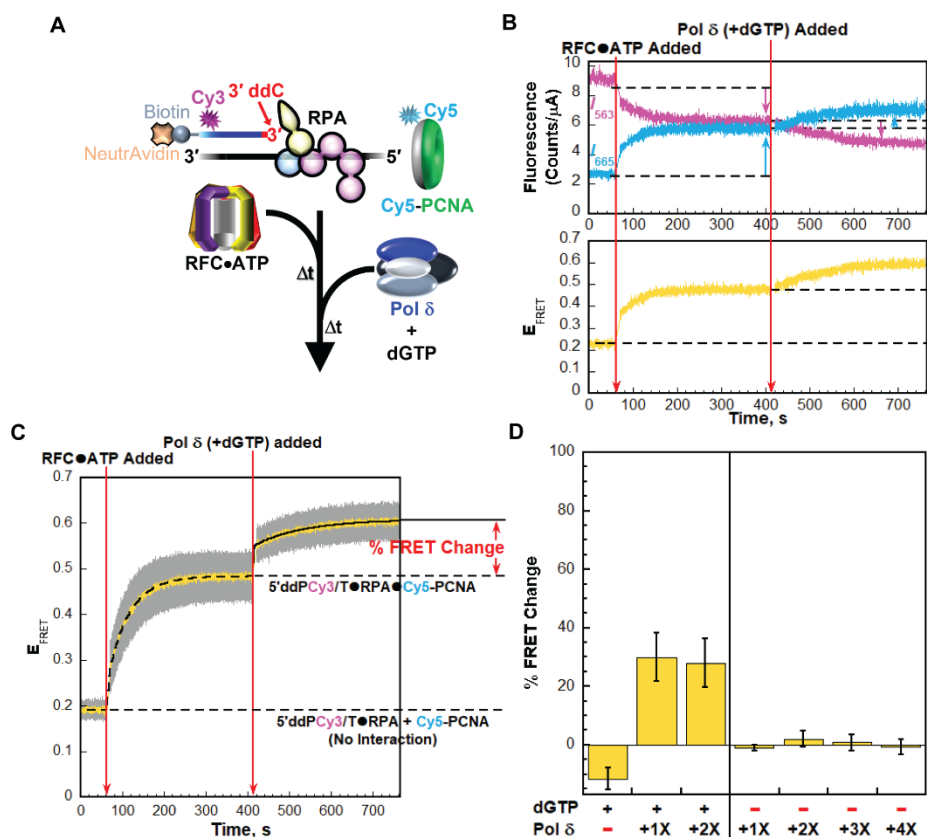
923 **Figure 3.** Interplay of RPA OBA and RFC interactions with nascent P/T junctions during PCNA
 924 loading in the presence of ATP. (A - C) Monitoring interactions of RFC and PCNA with a P/T
 925 junction engaged by RPA. (A) Schematic representation of the FRET experiment performed with
 926 5'ddPCy3/T (+ NeutrAvidin), RPA, Cy5-PCNA and a pre-formed RFC•ATP complex. (B)
 927 Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The time at which the
 928 RFC•ATP complex is added is indicated by a red arrow. Changes in I_{563} and I_{665} are indicated by
 929 magenta and cyan arrows, respectively. For observation, the I_{563} , I_{665} , and E_{FRET} values observed
 930 prior to the addition of the RFC•ATP complex are fit to flat lines that are extrapolated to the axis
 931 limits. (C) FRET data. Each E_{FRET} trace is the mean of at least three independent traces with the
 932 S.E.M. shown in grey. The time at which the RFC•ATP complex is added is indicated by a red
 933 arrow. The E_{FRET} trace observed prior to the addition of the RFC•ATP complex represents the
 934 complete absence of interactions between the 5'ddPCy3/T•RPA complex and Cy5-PCNA and is
 935 fit to a flat line that is extrapolated to the axis limits. The E_{FRET} trace observed after the addition
 936 of the RFC•ATP complex is fit to a double exponential rise and the observed rate constants are
 937 reported in the graph as well as in **Table S1**. (D - F) Monitoring RPA OBA interactions with a
 938 P/T junction that is engaged by RFC. (D) Schematic representation of the experiment. Reactions
 939 were carried out exactly as described in panel A except with ddP/5'TCy3 DNA, RPA-OBA-Cy5,
 940 and PCNA. (E) Sample time trajectory of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom) are plotted
 941 as described in panel B. (F) FRET data. Each E_{FRET} trace is the mean of at least three
 942 independent traces with the S.E.M. shown in grey. Data is plotted as described in panel C. The
 943 E_{FRET} trace observed prior to the addition of the RFC•ATP complex is fit to a flat line that is
 944 extrapolated to the axis limits. The predicted E_{FRET} trace (pink) for no interaction between RPA-
 945 OBA-Cy5 and the ddP/5'TCy3 DNA is fit to a flat line.



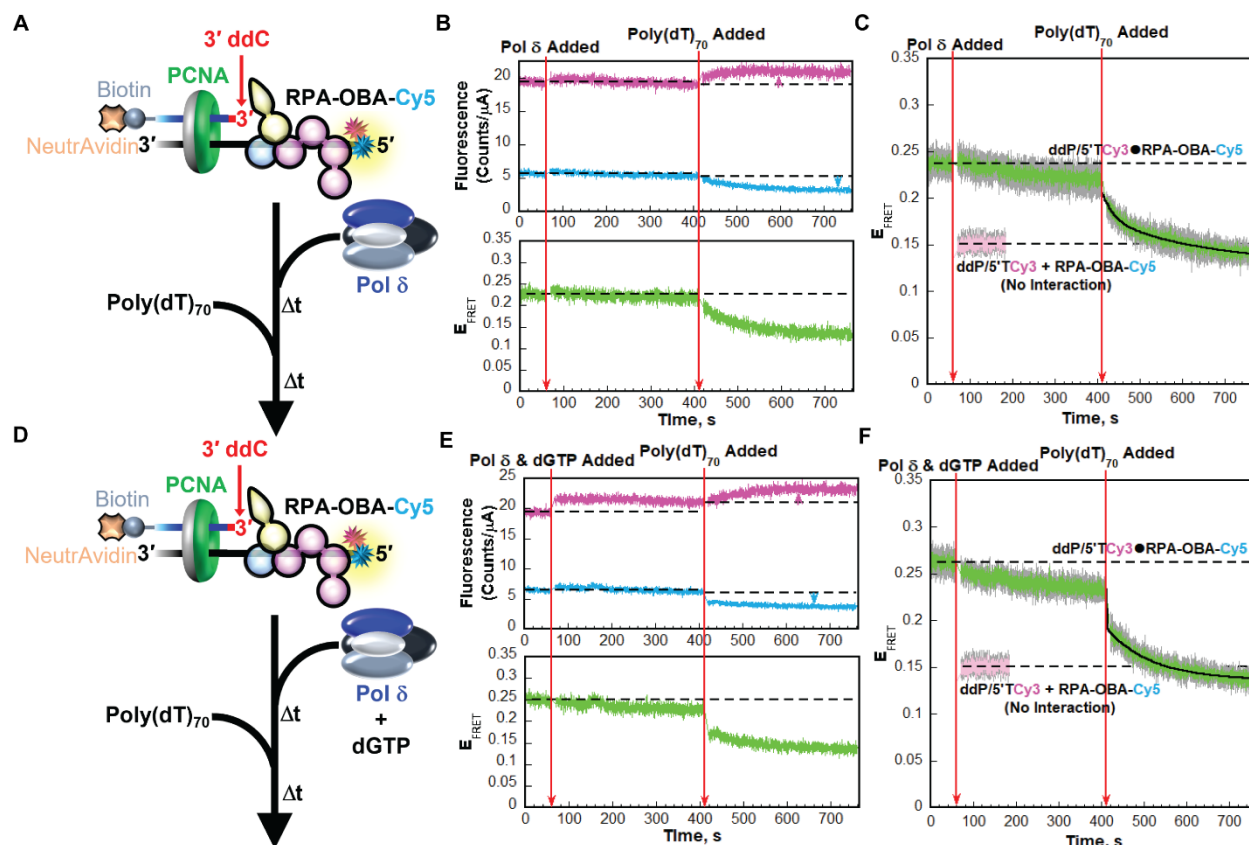
946 **Figure 4.** Interplay of RPA OBD and RFC interactions with nascent P/T junctions during PCNA
 947 loading in the presence of ATP γ S. (A - C) Monitoring interactions of a loading complex with a
 948 P/T junction engaged by RPA. (A) Schematic representation of the FRET experiment performed
 949 with 5'PCy3/T (+ NeutrAvidin), RPA, and loading complex pre-formed with RFC, ATP γ S, and
 950 Cy5-PCNA. (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The
 951 time at which the loading complex is added is indicated by a red arrow. Changes in I_{563} and I_{665}
 952 are indicated by magenta and cyan arrows, respectively. For observation, the I_{563} , I_{665} , and E_{FRET}
 953 values observed prior to the addition of the loading complex are fit to flat lines that are
 954 extrapolated to the axis limits. (C) FRET data. Each E_{FRET} trace is the mean of at least three
 955 independent traces with the S.E.M. shown in grey. The time at which the loading complex is
 956 added is indicated by a red arrow. The E_{FRET} trace observed after the addition of the loading
 957 complex is fit to a double exponential rise and the observed rate constants are reported in the
 958 graph. The predicted E_{FRET} trace (pink) for no interaction between 5'PCy3/T•RPA complex and
 959 the loading complex is fit to a flat line. (D - F) Monitoring RPA OBD interactions with a P/T
 960 junction that is engaged by a loading complex. (D) Schematic representation of the experiment.
 961 Reactions carried out exactly as described for panel A above except with P/5'TCy3 DNA,
 962 Cy5-OBD-RPA, and PCNA. (E) Sample time trajectory of I_{563} and I_{665} (Top) and their E_{FRET}
 963 (Bottom) are plotted as described for panel B above. (F) FRET data. Each E_{FRET} trace is the mean
 964 of at least three independent traces with the S.E.M. shown in grey. Data is plotted as described
 965 for panel C above. The E_{FRET} trace observed prior to the addition of the loading complex is fit
 966 to a flat line that is extrapolated to the axis limits. The predicted E_{FRET} trace (pink) for no
 967 interaction between Cy5-OBD-RPA and the P/5'TCy3 DNA is fit to a flat line.



968 **Figure 5.** Interplay of RPA OBD and RFC interactions with nascent P/T junctions during PCNA
 969 loading in the presence of ATP. (A - C) Monitoring interactions of RFC and PCNA with a P/T
 970 junction engaged by RPA. (A) Schematic representation of the FRET experiment performed with
 971 5'PCy3/T (+ NeutrAvidin), RPA, Cy5-PCNA and a pre-formed RFC•ATP complex. (B) Sample
 972 time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The time at which the RFC•ATP
 973 complex is added is indicated by a red arrow. Changes in I_{563} and I_{665} are indicated by magenta
 974 and cyan arrows, respectively. For observation, the I_{563} , I_{665} , and E_{FRET} values observed prior to
 975 the addition of the RFC•ATP complex are fit to flat lines that are extrapolated to the axis limits.
 976 (C) FRET data. Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M.
 977 shown in grey. The time at which the RFC•ATP complex is added is indicated by a red arrow.
 978 The E_{FRET} trace observed prior to the addition of the RFC•ATP complex represents the complete
 979 absence of interactions between the 5'PCy3/T•RPA complex and Cy5-PCNA and is fit to flat
 980 line that is extrapolated to the axis limits. The E_{FRET} trace observed after the addition of the
 981 RFC•ATP complex is fit to a double exponential rise and the observed rate constants are
 982 reported in the graph as well as in **Table S1**. (D - F) Monitoring RPA OBD interactions with a
 983 P/T junction that is engaged by RFC. (D) Schematic representation of the experiment. Reactions
 984 were carried out exactly as described in panel A except with 3'PCy3/T DNA, Cy5-OBD-RPA,
 985 and PCNA. (E) Sample time trajectory of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom) are plotted
 986 as described in panel B. (F) FRET data. Each E_{FRET} trace is the mean of at least three
 987 independent traces with the S.E.M. shown in grey. Data is plotted as described in panel C. The
 988 E_{FRET} trace observed prior to the addition of the RFC•ATP complex is fit to a flat line that is
 989 extrapolated to the axis limits. The predicted E_{FRET} trace (pink) for no interaction between Cy5-
 990 OBD-RPA and the 3'PCy3/T DNA is fit to a flat line.

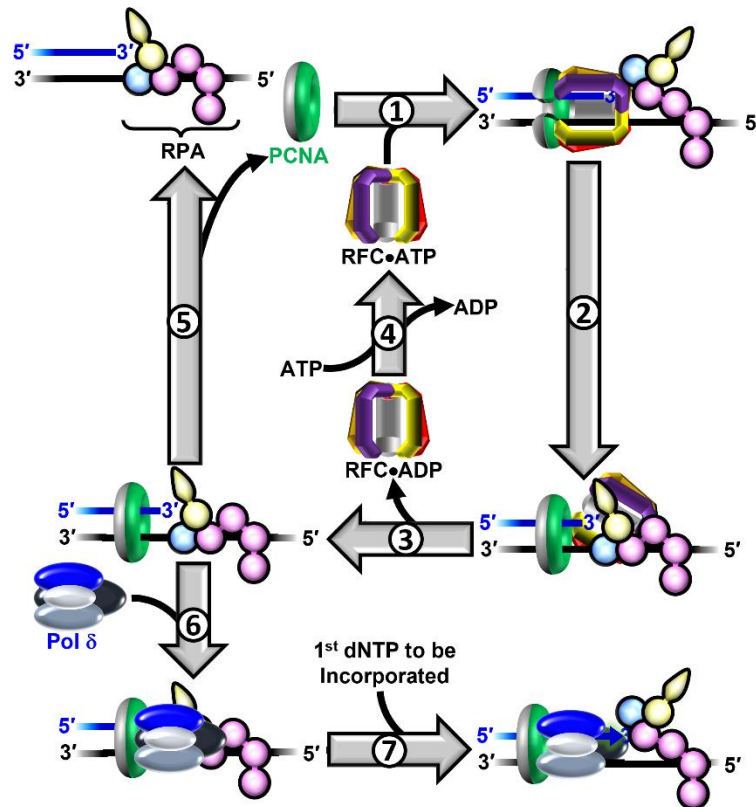


991 **Figure 6.** Formation of a Pol δ holoenzyme at a P/T junction engaged by RPA. (A) Schematic
 992 representation of the FRET experiment performed with 5' ddPCy3/T (+ NeutrAvidin), RPA, Cy5-
 993 PCNA, RFC, ATP, and Pol δ together with dGTP. (B) Sample time trajectories of I_{563} and I_{665}
 994 (*Top*) and their E_{FRET} (*Bottom*). The times at which the RFC•ATP complex and Pol δ (+ dGTP)
 995 are added are indicated by red arrows. For observation, the emission intensity traces and E_{FRET}
 996 values observed in the absence of RFC are each fit to dashed flat lines that are extrapolated.
 997 Also, dashed flat lines are drawn to highlight I values and E_{FRET} values observed at equilibrium
 998 for PCNA loading. Changes in I_{563} and I_{665} observed after each addition are indicated by magenta
 999 and cyan arrows, respectively. (C) FRET data. Each E_{FRET} trace is the mean of at least three
 1000 independent traces with the S.E.M. shown in grey. The times at which the RFC•ATP complex
 1001 and Pol δ (+ dGTP) are added are indicated by red arrows. The E_{FRET} trace observed prior to the
 1002 addition of the RFC•ATP complex is fit to a dashed flat line that is extrapolated to the axis limits
 1003 to depict the average E_{FRET} value for no interaction between Cy5-PCNA and the
 1004 5' ddPCy3/T•RPA complex. The E_{FRET} trace observed after the addition of the RFC•ATP
 1005 complex is fit to a dashed double exponential rise that is extrapolated to the axis limits to depict
 1006 the average E_{FRET} value for complete loading of Cy5-PCNA onto the Cy3-labeled P/T DNA
 1007 substrate (i.e., the 5' ddPCy3/T•RPA•Cy5-PCNA complex). The E_{FRET} traces observed after the
 1008 addition of Pol δ (+ dGTP) is fit to a double exponential rise. The % FRET Change observed
 1009 after the addition of Pol δ (+ dGTP) is depicted in red. (D) Characterization of the % FRET
 1010 Change observed. FRET experiments were repeated in the presence/absence of dGTP and with
 1011 varying concentrations of Pol δ (0 – 100 nM heterotetramer) and the % FRET Change observed
 1012 upon the ultimate addition (either dGTP, Pol δ + dGTP, or Pol δ) was measured. Each column is
 1013 the mean of at least three independent replicates with the S.E.M. shown in black.



1014 **Figure 7.** RPA dynamics during formation of a Pol δ holoenzyme and initiation of DNA
 1015 synthesis. (A) Schematic representation of the FRET experiment performed with 5'ddP/5'TCy3
 1016 (+ NeutrAvidin), RPA-OBA-Cy5, PCNA, RFC, ATP, Pol δ , and poly(dT)₇₀. (B) Sample time
 1017 trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The times at which Pol δ and
 1018 poly(dT)₇₀ are added are indicated by red arrows. For observation, the emission intensity traces
 1019 and E_{FRET} values observed in the absence of Pol δ are each fit to dashed flat lines that are
 1020 extrapolated. Also, dashed flat lines are drawn to highlight the I values and E_{FRET} values
 1021 observed at equilibrium after the addition of Pol δ . Changes in I_{563} and I_{665} observed after the
 1022 addition of poly(dT)₇₀ are indicated by magenta and cyan arrows, respectively. (C) FRET data.
 1023 Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M. shown in grey.
 1024 The times at which the Pol δ and poly(dT)₇₀ are added are indicated by red arrows. The E_{FRET}
 1025 trace observed prior to the addition of the Pol δ is fit to a dashed flat line that is extrapolated
 1026 to the axis limits to depict the average E_{FRET} value for the interaction between RPA-OBA-Cy5 and
 1027 ddP/5'TCy3. The predicted E_{FRET} trace (pink) for no interaction between RPA-OBA-Cy5 and the
 1028 ddP/5'TCy3 DNA is fit to a flat line that is extrapolated to the axis limits. The E_{FRET} trace
 1029 observed after the addition of the poly(dT)₇₀ is fit to a double exponential decay. (D) Schematic
 1030 representation of the FRET experiment performed in the same manner as that depicted in panel
 1031 A except that Pol δ is added simultaneously with dGTP. (E) Sample time trajectories of I_{563} and
 1032 I_{665} (Top) and their E_{FRET} (Bottom) plotted as in panel B. (F) FRET data plotted and analyzed as
 1033 in panel C. Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M.
 1034 shown in grey.

1035
 1036



1037 **Figure 8.** Assembly of the human Pol δ holoenzyme and initiation of DNA synthesis. A nascent
 1038 P/T junction is pre-engaged by RPA in an orientation-specific manner (as depicted) and PCNA
 1039 resides in solution (*Top Left*). **1)** RFC•ATP engages the "front face" of a free PCNA in solution,
 1040 opens the sliding clamp, and the resultant loading complex engages a nascent P/T junction such
 1041 that the "front face" of PCNA is oriented towards the 3' terminus of the primer from which DNA
 1042 synthesis initiates. Upon engaging a nascent P/T junction, the loading complex adopts an
 1043 activated conformation in which ATP hydrolysis by RFC is optimized. RPA OBD is released
 1044 from the 3' terminus of the primer strand to accommodate binding and activation of the loading
 1045 complex at the nascent P/T junction. **2)** ATP hydrolysis by RFC within the activated loading
 1046 complex simultaneously closes PCNA around the DNA and the closed (i.e., loaded) PCNA is
 1047 subsequently released onto the dsDNA region of the nascent P/T junction. Concomitant with
 1048 closure of PCNA or release of loaded PCNA, the resultant RFC•ADP complex vacates the P/T
 1049 junction, transferring to the RPA1 subunit, and the interactions of RPA OBD are re-established
 1050 with the 3' terminus of the primer strand. **3)** The RFC•ADP complex releases into solution via
 1051 dissociation from the RPA1 subunit of the resident RPA engaged with the nascent P/T junction.
 1052 **4)** RFC then exchanges ADP for ATP. **5)** In the absence of catalyzed unloading of PCNA and
 1053 significant translocation of loaded PCNA, the only pathway for dissociation of Pol δ , loaded
 1054 PCNA may dissociate from a nascent P/T junction via spontaneous opening of the PCNA ring. **6)**
 1055 In the presence of Pol δ , the DNA polymerase engages the "front face" of loaded PCNA
 1056 encircling a P/T junction, forming a holoenzyme. **7)** An assembled Pol δ holoenzyme engages a
 1057 nascent P/T junction and an incoming dNTP and aligns the incoming dNTP at the 3' terminus of
 1058 the primer strand in a correct base pair with the template nt immediately 5' of the P/T junction.
 1059

1060 Supplemental Information

1061 TITLE: **Interplay of macromolecular interactions during assembly of human DNA** 1062 **polymerase δ holoenzymes and initiation of DNA synthesis**

1063
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1071 Supplementary Results

1072 *Experimental reaction buffer components do not affect the amount of RPA that binds to ssDNA*
1073 *nor the amount of PCNA loaded onto DNA by RFC.*

1074 Calcium (Ca^{2+}) is included in all experimental reaction buffers in the current study to account
1075 for experimental conditions in future studies. Rigorous control experiments were carried out to
1076 demonstrate that the results presented in the present study are not attributed to indirect effects of
1077 buffer components. We initially focused on RPA-DNA interactions. First, the concentration of
1078 active RPA heterotrimer was calculated as previously described in 1X Mg^{2+} buffer (25 mM
1079 HEPES, pH 7.5, 125 mM KOAc, 10 mM $\text{Mg}(\text{OAc})_2$) supplemented with 1 mM DTT, 1 mM
1080 ATP, and ionic strength adjusted to 200 mM by addition of KOAc¹. The ssDNA substrate
1081 utilized in these assays (poly(dT)₃₀-FRET) accommodates one RPA heterotrimer and is shown in
1082 **Figure S1**. Next, these titrations were repeated in the same experimental reaction buffer except
1083 with amended amounts of RPA heterotrimer that accounted for the calculated concentration of
1084 active RPA heterotrimer (**Figure S2A**) and the data (E_{FRET} values) is plotted as a function of the
1085 ratios of the concentrations of active RPA heterotrimer and DNA (i.e., [RPA]:[DNA], **Figure**
1086 **S2B**). As expected, saturation occurs at a ratio of 1:1 (1.017 ± 0.0545 [RPA]:[DNA]). Finally,
1087 these experiments were repeated with the same preparation of human RPA heterotrimer except in
1088 1X $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer. In this $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer, Ca^{2+} is included in addition to magnesium (Mg^{2+})
1089 and the acetate (OAc^-) from Mg^{2+} buffer is replaced with chloride (Cl^-). As observed in **Figure**
1090 **S2C**, saturation occurs in 1X $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer at ratio (0.938 ± 0.0507 [RPA]:[DNA]) that is
1091 within experimental error of that observed in 1X Mg^{2+} buffer (1.017 ± 0.0545 [RPA]:[DNA],
1092 **Figure S2C**). This indicates that experimental reaction buffer components do not affect the
1093 amount of RPA that binds to ssDNA. Next, we focused on RFC-catalyzed loading of PCNA onto
1094 a P/T junction.

1095 RFC-catalyzed loading of PCNA onto nascent P/T junctions that are engaged with RPA was
1096 monitored exactly as described in **Figure 3** in the main text. Under the conditions of the assay,
1097 PCNA loading is biphasic and stoichiometric^{2,3}. Shown in **Figure S3A – B** is the data observed
1098 in 1X Mg^{2+} buffer. Upon addition of RFC, the observed changes in I_{665} and I_{563} are synchronized
1099 and anti-correlated (**Figure S3A, Top**), indicating the appearance and increase in FRET (**Figure**
1100 **S3A, Bottom**). As observed in **Figure S3B**, E_{FRET} traces rapidly increase to values significantly
1101 above the E_{FRET} values observed for no interaction between Cy5-PCNA and the

1102 5' ddPCy3/T•RPA complex. As expected, the rapid increase in E_{FRET} observed upon addition of
1103 RFC•ATP is biphasic with observed rate constants of $k_{\text{obs inc},1} = 0.122 \pm 0.004 \text{ s}^{-1}$ and $k_{\text{obs inc},2} =$
1104 $1.28 \pm 0.05 \text{ (x } 10^{-2}) \text{ s}^{-1}$ (reported in **Table S1**) and an overall (i.e., total) amplitude of $A_{\text{T}} = 0.351$
1105 ± 0.019 . Importantly, the rate constants observed in **Figure S3B** agree very well with values
1106 reported in a previous study ($k_{\text{obs inc},1} = 0.134 \pm 0.010 \text{ s}^{-1}$ and $k_{\text{obs inc},2} = 2.03 \pm 0.03 \text{ (x } 10^{-2}) \text{ s}^{-1}$)
1107 that analyzed PCNA loading under similar conditions by monitoring sensitized Cy5 acceptor
1108 fluorescence emission intensity (I_{665}) via stopped flow. Under these conditions, $k_{\text{obs inc},1}$ describes
1109 a kinetic step along the PCNA loading pathway that occurs prior to and much slower than
1110 binding of the loading complex to the nascent P/T junction. $k_{\text{obs inc},2}$ describes release of the
1111 RFC•ADP complex into solution via dissociation from RPA engaged with the nascent P/T
1112 junction². The overall amplitude (A_{T}) indicates the overall increase in FRET observed upon
1113 stable loading of a single Cy5-PCNA onto each 5' ddPCy3/T DNA substrate. Shown in **Figure**
1114 **S3C – D** is the data observed in 1X $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer. E_{FRET} traces observed upon addition of
1115 RFC•ATP are also biphasic. Importantly, the overall amplitude observed in **Figure S3D** ($A_{\text{T}} =$
1116 0.368 ± 0.014) is within experimental error of that observed in the alternative buffer ($A_{\text{T}} = 0.351$
1117 ± 0.019 , **Figure S3B**), indicating that experimental reaction buffer components do not affect the
1118 amount of PCNA loaded onto DNA by RFC. $k_{\text{obs},2}$ observed in **Figure S3D** ($k_{\text{obs inc},2} = 1.72 \pm$
1119 $0.02 \text{ (x } 10^{-2}) \text{ s}^{-1}$, **Table S1**) agrees very well with that observed in **Figure S3B** for the alternative
1120 buffer ($k_{\text{obs inc},2} = 2.03 \pm 0.03 \text{ (x } 10^{-2}) \text{ s}^{-1}$, **Table S1**) suggesting that $k_{\text{obs inc},2}$ under these
1121 conditions also represents release of the RFC•ADP complex into solution via dissociation from
1122 RPA engaged with the nascent P/T junction. $k_{\text{obs inc},1}$ observed for each experimental reaction
1123 buffer are within 2-fold of each other. This suggests either that $k_{\text{obs inc},1}$ describes either; 1) the
1124 same kinetic step(s) for each condition and, hence, this step is dependent on the experimental
1125 reaction buffer components; or 2) a distinct step(s) for each condition. Regardless, the identical
1126 total amplitudes (A_{T}) observed in **Figure S3** indicate that both experimental reaction buffers
1127 fully support RFC-catalyzed loading of PCNA onto P/T junctions.

1128
1129 *RPA rapidly engages a nascent P/T junction in a stable interaction.*

1130 To investigate RPA/DNA interactions at P/T junctions, we designed and carried out FRET
1131 assays utilizing Cy3, Cy5 FRET pairs comprised of a Cy3-labeled P/T DNA substrate (**Figure**
1132 **S1**) and a corresponding Cy5-labeled RPA. To monitor interactions of RPA OBA with P/T DNA
1133 we utilized a substrate (**Figure S4A**, ddP/5'TCy3 DNA) with a 3' dideoxy-terminated primer and
1134 a Cy3 donor near the 5' end of the template strand and RPA with a Cy5 acceptor on OBA
1135 (**Figure S4B**, RPA-OBA-Cy5) that faces the Cy3 donor (**Figure S4C**)⁴⁻¹¹. To monitor
1136 interactions of RPA OBD with P/T DNA we utilized a substrate (**Figure S4D**, 3'PCy3/T) with a
1137 Cy3 donor on the 3' terminus of the primer strand and RPA with a Cy5 acceptor on OBD
1138 (**Figure S4E**, Cy5-OBD-RPA) that faces the Cy3 donor (**Figure S4F**)¹². Each Cy3-labeled P/T
1139 junction was tested for stable interaction with the corresponding Cy5-labeled RPA by monitoring
1140 the FRET signals observed at equilibrium after excitation with 514 nm light. Here, Cy5 on RPA
1141 can be excited via FRET from Cy3 on a P/T junction only when the two cyanine labels remain in
1142 close proximity of each other (i.e., $\leq 10 \text{ nm}$), as depicted in **Figures S4C** and **S4F**. This is
1143 indicated by an increase in the fluorescence emission intensity of the Cy5 acceptor at 665 nm
1144 (Cy5 acceptor fluorescence emission maximum, I_{665}) and a concomitant decrease in the
1145 fluorescence emission intensity of the Cy3 donor at 563 nm (Cy3 donor fluorescence emission
1146 maximum, I_{563}). A FRET signal is clearly observed for both Cy3, Cy5 FRET pairs only when
1147 both the respective Cy3-labeled P/T junction and the corresponding Cy5-labeled RPA are present

1148 **(Figure S4G and S4H)**. Collectively, this indicates that RPA engages a P/T junction in a stable
1149 interaction. This equilibrium FRET assay was adapted to determine the concentrations of active
1150 Cy5-labeled RPAs **(Figure S5)**.

1151 To analyze the kinetics of RPA binding to nascent P/T junctions **(Figure S6A)**, I_{563} and I_{665}
1152 are first monitored over time for ddP/5'TCy3 DNA alone. Then, the fluorescence emission
1153 intensity recording is paused, RPA-OBA-Cy5 is added to the reaction mixture, the resultant
1154 solution is mixed, and the fluorescence emission intensity recording is resumed within 10 s of the
1155 addition (i.e., “dead time” ≤ 10 s). Upon addition of RPA-OBA-Cy5, I_{665} rapidly increases
1156 concomitantly with a rapid decrease in I_{563} **(Figure S6B, Top)** after which both fluorescence
1157 emission intensities stabilize and persist over time. These synchronized, anti-correlated changes
1158 in I_{563} and I_{665} are indicative of the appearance and increase in FRET **(Figure S6B, Bottom)**. As
1159 observed in **Figure S6C**, E_{FRET} traces rapidly increase to values significantly above the E_{FRET}
1160 traces predicted for no interaction between RPA-OBA-Cy5 and ddP/5'TCy3 DNA. Furthermore,
1161 the rapid increase in E_{FRET} observed upon addition of RPA-OBA-Cy5 is comprised of at least
1162 two phases (i.e., biphasic) with an observed rate constant for the slower phase ($k_{\text{obs inc},2}$) of $9.45 \times$
1163 $10^{-3} \pm 2.23 \times 10^{-3} \text{ s}^{-1}$. Similar results are observed for the 3'PCy3/T DNA, Cy5-OBD-RPA FRET
1164 pair **(Figure S6D – F)** except that association of the Cy5-labeled OBD of RPA with the Cy3-
1165 labeled P/T junction is completed within the dead time of the experiment (≤ 10 s). This behavior
1166 agrees with previous studies that observed differential kinetics for binding of OBA and OBD to
1167 ssDNA in the context of the complete, heterotrimeric RPA complex¹²⁻¹⁴. Altogether, the results
1168 presented in **Figures S4 and S6** indicate that RPA rapidly engages P/T junctions in a stable
1169 interaction.

1170
1171 *PCNA is loaded onto a P/T junction in 1:1 stoichiometry regardless of the position of the Cy3*
1172 *label.*

1173 In order to investigate the effects of the Cy3 donor located at the 3' primer terminus of the of
1174 the 3'PCy3/T DNA substrate **(Figure S1)** on RFC-catalyzed loading of PCNA, we carried out
1175 PCNA loading experiments under two different conditions. First, we investigated the kinetics of
1176 PCNA loading by carrying out PCNA loading reactions in the presence of ATP exactly as
1177 described in in the main text except with 3'Cy3P/T DNA **(Figure S9A)**. In short, 20 nM
1178 3'PCy3/T DNA (pre-bound with 80 nM NeutrAvidin and 1 mM ATP are pre-incubated with 25
1179 nM wild type RPA. Then, 20 nM Cy5-PCNA is added. PCNA loading is initiated by the addition
1180 of 20 nM pre-formed RFC•ATP complex and monitored via FRET over time **(Figure S9B)**.
1181 Under these conditions the concentrations of RFC, PCNA, and P/T DNA are stoichiometric (i.e.,
1182 1:1:1). As observed in **Figure S9C**, E_{FRET} traces rapidly increase to values significantly above
1183 the E_{FRET} values observed for no interaction between Cy5-PCNA and the 3'PCy3/T•RPA
1184 complex. The rapid increase in E_{FRET} observed upon addition of RFC is biphasic with observed
1185 rate constants of $k_{\text{obs inc},1} = 4.42 \pm 0.10 (\times 10^{-2}) \text{ s}^{-1}$ and $k_{\text{obs inc},2} = 1.59 \pm 0.09 (\times 10^{-2}) \text{ s}^{-1}$ **(Table**
1186 **S1)**. The rate constants observed for the 3'Cy3P/T DNA substrate **(Figure S9C, Table S1)** are
1187 nearly identical to those observed for the 5'Cy3P/T DNA substrate **(Table S1)**, indicating that
1188 the Cy3 label on the 3' terminus of the primer of the 3'Cy3P/T DNA substrate does not affect
1189 kinetics of RFC-catalyzed loading of PCNA onto P/T junctions. The overall total amplitude (A_{T})
1190 observed for the 3'Cy3P/T DNA substrate **(Figure S9C)** is > 2 -fold less than that observed for
1191 the 5'Cy3P/T DNA substrate, likely due to the Cy5 label on PCNA being oriented away from the
1192 Cy3 donor (rather than towards) when Cy5-PCNA is loaded onto the 3'Cy3P/T DNA substrate
1193 by RFC. Next, we investigated the stoichiometry of PCNA loading by characterizing the overall

1194 total amplitudes (A_T) of PCNA loading via a titration of the E_{FRET} signal observed at equilibrium
1195 (i.e., when A_T has been achieved). In short, a Cy3-labeled P/T DNA (55 nM of either 5'Cy3P/T
1196 or 3'Cy3P/T pre-bound with 220 nM NeutrAvidin) is pre-saturated with wild type RPA prior to
1197 the addition of RFC (55 nM). Cy5-PCNA is then titrated in and the equilibrium E_{FRET} is
1198 monitored. As expected for the 5'PCy3/T DNA substrate, E_{FRET} increased linearly with Cy5-
1199 PCNA up to a Cy5-PCNA: Cy3-DNA ratio of 1:1 after which the E_{FRET} values remain constant
1200 (**Figure S10**)^{2,3}. This confirms the validity of the approach and that RFC-catalyzed loading of
1201 PCNA onto the 5'PCy3/T DNA substrate is stoichiometric. For the 3'PCy3/T DNA substrate,
1202 identical behavior is observed. This indicates that the location of the Cy3 label on the primer
1203 strand does not affect the amount of PCNA loaded onto DNA by RFC. Together, the results
1204 presented in **Figures S9** and **S10** demonstrate that the Cy3 donor located at the 3' primer
1205 terminus of the of the 3'PCy3/T DNA substrate has no effect on RFC-catalyzed loading of
1206 PCNA on the resident P/T junction.

1207
1208 *dGTP inhibits RFC-catalyzed re-loading of PCNA onto a P/T junction.*

1209 In the current study, RFC-catalyzed loading of PCNA onto a P/T junction in the presence of
1210 ATP is stoichiometric. After dissociation of the RFC•ADP complex into solution, the loaded
1211 PCNA is left behind on the duplex region of the P/T junction and randomly and rapidly diffuses
1212 along the double-stranded DNA (dsDNA)¹⁵. Recent *in vivo* evidence suggests that enzyme-
1213 catalyzed unloading of PCNA from a P/T junction will not occur until the primer is completely
1214 extended and ligated to the downstream duplex region¹⁶. Physical blocks i.e., “protein
1215 roadblocks,” restrict translocation of PCNA away from the P/T junction via diffusion. Diffusion
1216 of loaded PCNA along the duplex region is restricted by high-affinity DNA-binding proteins,
1217 such as histones and transcription factors, that rapidly bind nascent dsDNA upstream of P/T
1218 junctions during S-phase of the cell cycle when DNA replication occurs. This is emulated in the
1219 current study by the biotin-NeutrAvidin complex at the blunt duplex end of the DNA substrates.
1220 RPA engaged with the ssDNA downstream of nascent P/T junctions prohibits diffusion of loaded
1221 PCNA along ssDNA as well as RFC-catalyzed unloading of PCNA^{2,3}. In the absence of
1222 catalyzed unloading of PCNA and significant translocation of loaded PCNA, the only pathway
1223 for dissociation of loaded PCNA from a nascent P/T junction is through spontaneous opening of
1224 the PCNA ring, which is dramatically slow [$k_{open} = 1.25 \pm 0.32 (x 10^{-3}) s^{-1}$]¹⁷, ~20-fold slower
1225 than the observed rate constant RFC-catalyzed loading of “free” PCNA onto a P/T junction in
1226 the presence of ATP (**Figure 3, 5, S3, S3 – S9**, and **Table 1**). This suggests that, upon
1227 dissociation of PCNA from a nascent P/T junction via spontaneous opening of the PCNA ring,
1228 RFC utilizes ATP to instantly reload PCNA back onto the nascent P/T junction such that the loss
1229 of loaded PCNA from a nascent P/T junction and, hence, E_{FRET} , is not observed^{2,18}. To directly
1230 confirm this, we continuously analyzed DNA-PCNA interactions over time as reaction
1231 conditions progressively evolved (**Figure S11A**). First, the 5'ddPCy3/T DNA substrate is pre-
1232 saturated with native RPA, Cy5-PCNA is added, and I_{563} and I_{665} are monitored over time. Next,
1233 pre-formed RFC•complex is added to the and the fluorescence emission intensities are
1234 monitored over time until PCNA loading is complete. Under the conditions of the assay, PCNA
1235 loading is stoichiometric and biphasic². Finally, unlabeled PCNA is added in excess and the
1236 fluorescence emission intensities are monitored over time. Under these conditions, once loaded
1237 Cy5-PCNA dissociates from the Cy3-labeled P/T junction via spontaneous opening of the PCNA

1238 ring, re-loading of “free” Cy5-PCNA is prohibited due to the 70-fold excess of unlabeled PCNA,
1239 and consequently the observed FRET decreases. Here, the disappearance of FRET is rate-limited
1240 by and directly reports on spontaneous opening of the PCNA ring.

1241 Upon addition of RFC•ATP, I_{665} rapidly increases concomitantly with a rapid decrease in I_{563}
1242 after which both fluorescence emission intensities stabilize and persist over time (**Figure S11B**,
1243 *Top*). These synchronized, anti-correlated changes in I_{563} and I_{665} are indicative of the
1244 appearance and increase in FRET (**Figure S11B**, *Bottom*). As expected, E_{FRET} traces observed in
1245 **Figure S11C** rapidly increase in a biphasic manner upon addition of RFC•ATP and plateau at
1246 values significantly above the E_{FRET} traces observed for no interaction between Cy5-PCNA and
1247 5’ddPCy3/T. At this point, a Cy5-PCNA has been loaded onto each 5’ddPCy3/T DNA and
1248 RFC•ADP has released into solution and exchanged ADP for ATP. Both observed rate constants
1249 for RFC-catalyzed loading of PCNA in the presence of ATP are in excellent agreement with
1250 results from the current study obtained under the same experimental conditions (**Table S1**). k_{obs}
1251 $_{\text{inc}, 1}$ (in **Figure S11C**), which encompasses all kinetic steps along the PCNA loading pathway up
1252 to and including release of loaded PCNA onto P/T DNA, is 4.46 ± 0.29 ($\times 10^{-2}$) s^{-1} . $k_{\text{obs}, \text{inc}, 2}$ (in
1253 **Figure S11C**), which reports on release of RFC•ADP into solution (via its dissociation from the
1254 resident RPA engaged with the P/T junction) is 1.70 ± 0.12 ($\times 10^{-2}$) s^{-1} .

1255 Upon addition of excess, unlabeled PCNA, the observed changes in I_{665} and I_{563} are
1256 synchronized and anti-correlated (**Figure S11B**, *Top*), indicating a decrease in FRET (**Figure**
1257 **S11B**, *Bottom*). As observed in **Figure S11C**, upon addition of excess unlabeled PCNA, E_{FRET}
1258 traces decrease to E_{FRET} values observed for no interaction between Cy5-PCNA and 5’ddPCy3/T
1259 DNA (i.e., all Cy5-PCNA remaining “free” in solution) indicating that all Cy5-PCNA dissociates
1260 from the 5’ddPCy3/T•RPA complex. Furthermore, the decrease in E_{FRET} observed upon addition
1261 of excess unlabeled PCNA is comprised of a single phase (i.e., monophasic) with an observed
1262 rate constant [$k_{\text{obs}, \text{dec}} = 1.93 \pm 0.01$ ($\times 10^{-3}$) s^{-1}] that is in excellent agreement with the rate
1263 constant for spontaneous opening of the PCNA ring [$k_{\text{open}} = 1.25 \pm 0.32$ ($\times 10^{-3}$) s^{-1}]¹⁷ and is 23.1
1264 ± 1.50 -fold slower than the rate constant for RFC-catalyzed loading of “free” PCNA onto a P/T
1265 junction in the presence of ATP [$k_{\text{obs}, \text{inc}, 1} = 4.46 \pm 0.29$ ($\times 10^{-2}$) s^{-1}]. Altogether, this confirms
1266 that; 1) dissociation of PCNA from nascent P/T junctions is governed entirely by spontaneous
1267 opening of the PCNA ring; and 2) upon dissociation of PCNA from a nascent P/T junction,
1268 RFC•ATP instantly reloads PCNA back onto the nascent P/T junction such that the loss of
1269 loaded PCNA from a nascent P/T junction is not observed. In other words, RFC together with
1270 ATP continuously maintain loaded PCNA on all P/T junctions.

1271 Interestingly, when the experiments described in **Figure S11** and **Figure 6** (in the main text)
1272 were repeated by replacing unlabeled PCNA and Pol δ , respectively with dGTP (1 mM final
1273 concentration, **Figure S12A**), I_{665} decreases slightly over time concomitantly with a slight
1274 increase in I_{563} over time (**Figure S12B**, *Top*). These synchronized, anti-correlated changes in
1275 I_{563} and I_{665} are indicative of a decrease in FRET (**Figure S12B**, *Bottom*). As observed in **Figure**
1276 **S12C**, upon addition of dGTP, E_{FRET} traces very slightly, but reproducibly, decrease (% FRET
1277 Change = -11.7 ± 3.7 %) with an observed rate constant [$k_{\text{obs}, \text{dec}} = 3.75 \pm 0.41$ ($\times 10^{-3}$) s^{-1}] that
1278 agrees with the rate constant observed in **Figure S11C** [$k_{\text{obs}, \text{dec}} = 1.93 \pm 0.01$ ($\times 10^{-3}$) s^{-1}] for
1279 PCNA unloading via spontaneous opening of the PCNA ring. This suggests that; 1) PCNA
1280 dissociates from the P/T junction under these conditions via spontaneous opening of the PCNA
1281 ring; and 2) dGTP (at a concentration of 1 mM) inhibits RFC-catalyzed reloading of PCNA back
1282 onto the P/T junction. Regarding the latter point, previous studies of human RFC observed that

1283 dGTP decreases DNA-dependent nucleotide triphosphate hydrolysis by RFC approximately 4-
1284 fold and significantly tempers PCNA-dependent stimulation of Pol δ -mediated DNA synthesis
1285 by ~8-fold, suggesting that dGTP significantly inhibits loading of PCNA onto P/T junctions by
1286 RFC¹⁹. To directly test this, we repeated PCNA loading assays (described in **Figures 3, 5, S3,**
1287 **and S7 – S9**) in the presence of dGTP (**Figure S13**).

1288 Upon addition of RFC•dGTP, the observed changes in I_{665} and I_{563} are synchronized and
1289 correlated (**Figure 13B, Top**). This behavior is due to nonspecific effects²⁰. For the example
1290 FRET trajectory depicted in **Figure 13B (Bottom)**, E_{FRET} values observed prior to the addition of
1291 the RFC•dGTP complex are maintained after addition of the RFC•dGTP loading complex. For
1292 the averaged E_{FRET} trajectory depicted in **Figure S13C**, the E_{FRET} traces observed prior to the
1293 addition of the RFC•dGTP complex persist and are maintained at the E_{FRET} values observed for
1294 no interaction between Cy5-PCNA and the 5' ddPCy3/T•RPA complex. This indicates that dGTP
1295 (at a concentration of 1 mM) prohibits loading of PCNA onto P/T junctions by RFC under these
1296 conditions and, hence, likely inhibits re-loading of PCNA when it is added (at a concentration of
1297 1 mM) after a significant incubation time (in **Figure S12** and **Figure 6**) where ATP has been
1298 depleted and ADP has increased through spontaneous hydrolysis of ATP and RFC-catalyzed
1299 PCNA loading and re-loading.

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1301 *Human RPA undergoes facilitated exchange with free ssDNA.*

1302 The human RPA complex has exceptionally high affinity for ssDNA at physiological ionic
1303 strength but can undergo facilitated exchange due to the dynamic ssDNA-binding interactions of
1304 its individual OB folds, enabling the human RPA complex to rapidly exchange between free and
1305 ssDNA-bound states when free, high-affinity ssDNA-binding proteins are present in solution. In
1306 this process, human RPA complexes exist in microscopically dissociated states that only undergo
1307 macroscopic dissociation when free, high-affinity ssDNA-binding proteins are available to
1308 occupy the ssDNA that is exposed during the microscopic dissociation events^{12,13,21,22}.
1309 Accordingly, this concentration-dependent RPA turnover should also be observed when free
1310 ssDNA is present in solution. Here, microscopically dissociated states of RPA complexes only
1311 undergo macroscopic dissociation when free ssDNA sequences are available to occupy the OB
1312 folds of RPA that are exposed during the microscopic dissociation events. This behavior is akin
1313 to intersegmental transfer of DNA-binding proteins that contain at least two DNA binding
1314 domains²³. To test this, we analyzed the kinetics of RPA dissociation from nascent P/T junctions
1315 (**Figure S14A**).

1316 First, ddP/5' TCy3 DNA is pre-incubated with RPA-OBA-Cy5 at a ratio of 1:1 and I_{563} and
1317 I_{665} of the resultant mixture are monitored over time. Then, the fluorescence emission intensity
1318 recording is paused, buffer containing varying concentrations of free poly(dT)₇₀ is added to the
1319 reaction mixture, the resultant solution is mixed, and the fluorescence emission intensity
1320 recording is resumed within 10 s of the addition (i.e., “dead time” ≤ 10 s). The ssDNA binding
1321 affinity of human RPA is highest for poly(dT) and each poly(dT)₇₀ accommodates at least two
1322 RPA complexes^{4-6,24}. Hence, poly(dT)₇₀ serves as an effective trap to release RPA-OBA-Cy5
1323 from the ddP/5' TCy3 DNA substrate via facilitated exchange and prohibit re-binding. Upon
1324 addition of 0.25 μM poly(dT)₇₀, the observed changes in I_{665} and I_{563} are synchronized and anti-
1325 correlated (**Figure S14B, Top**), indicating a decrease in FRET (**Figure S14B, Bottom**). As
1326 observed in **Figure S14C**, E_{FRET} traces observed in the presence of 0.25 μM poly(dT)₇₀ rapidly
1327 decrease to the E_{FRET} traces predicted for no interaction between RPA-OBA-Cy5 and
1328 ddP/5' TCy3 DNA. Furthermore, the rapid decrease in E_{FRET} observed upon addition of

1329 poly(dT)₇₀ is comprised of at least two phases (i.e., biphasic) with an observed rate constants of
1330 $k_{\text{obs dec},1} = 9.02 \pm 0.49 \text{ (x}10^{-2}\text{) s}^{-1}$ and $k_{\text{obs dec},2} = 7.09 \pm 0.13 \text{ (x}10^{-3}\text{) s}^{-1}$. As expected, RPA-OBA-
1331 Cy5 remained tightly bound to the ddP/5'TCy3 DNA in mock reactions lacking poly(dT)₇₀
1332 (**Figure S14D**). In contrast, RPA-OBA-Cy5 rapidly and completely dissociated from the
1333 ddP/5'TCy3 DNA at each concentration of poly(dT)₇₀ (**Figure S14D**) and the rates RPA-OBA-
1334 Cy5 dissociation are dependent upon the concentration of poly(dT)₇₀ (**Figure S14E**), indicating a
1335 second order, bimolecular reaction. Altogether, this indicates that human RPA complexes can
1336 undergo concentration-dependent facilitated exchange between ssDNA sequences.

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1338 **Supplementary Methods**

1339 *Determining stoichiometry of RPA:ssDNA interactions via FRET.* The Cy3/Cy5-labeled ssDNA
1340 oligonucleotide (poly(dT)₃₀-FRET) accommodates one RPA heterotrimer and is shown in **Figure**
1341 **S1**²⁵. All experiments were performed at room temperature ($23 \pm 2 \text{ }^\circ\text{C}$) in either 1X Mg²⁺/Ca²⁺
1342 buffer and the ionic strength adjusted to 200 mM by addition of KCl or in 1X Mg²⁺ buffer with
1343 the ionic strength adjusted to 200 mM by addition of KOAc. The excitation and emission slit
1344 widths were set to 5 nm. The poly(dT)₃₀-FRET ssDNA is titrated with increasing concentrations
1345 of RPA. For each RPA addition, fluorescence emission intensities (I_{665} and I_{563}) are monitored
1346 over time until both signals stabilize for at least 1 min. Within this stable region, E_{FRET} values are
1347 calculated from the observed fluorescence emission intensities (I_{665} and I_{563}) and averaged to
1348 obtain the final E_{FRET} value for a given RPA addition. Under the experimental conditions, RPA
1349 binding is stoichiometric and, hence, E_{FRET} increases linearly until the ssDNA is saturated with
1350 Cy5-labeled RPA (i.e., equivalence point)^{12,25}. Data is plotted as a function of the ratio of the
1351 concentrations of active RPA and DNA (i.e., [RPA]:[DNA]) and fit to two segment lines (a
1352 linear regression with a positive slope and a flat line). The equivalence point (RPA per DNA) is
1353 calculated from the intersection of the two segment lines.

1354 *Equilibrium FRET assays to characterize RPA interactions.* One or more of the following
1355 components are pre-equilibrated in a fluorometer cell with 1 mM ATP; Cy3-labeled P/T DNA
1356 (20 nM, **Figure S1**), NeutrAvidin (80 nM), and a Cy5-labeled RPA (25 nM heterotrimer, RPA-
1357 OBA-Cy5 or Cy5-OBD-RPA). The cell is subsequently placed in the instrument, the respective
1358 solution is excited at 514 nm, and fluorescence emission spectra (530 nm – 750 nm) are recorded
1359 (1 spectra/25.9 ms) until the spectra stabilizes for at least 1 min. Spectra observed within this
1360 stable period are averaged to obtain the final spectra for the respective condition.

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1362 *Equilibrium FRET assays to determine the concentration of active human Cy5-labeled RPA.* A
1363 Cy3-labeled Bio-P/T DNA substrate (**Figure S1**) is titrated with increasing concentrations of a
1364 human Cy5-labeled RPA. For each RPA addition, fluorescence emission intensities (I_{665} and I_{563})
1365 are monitored over time until both signals stabilize for at least 1 min. Within this stable region,
1366 E_{FRET} values are calculated from the observed fluorescence emission intensities (I_{665} and I_{563}) and
1367 averaged to obtain the final E_{FRET} value for a given RPA addition. Under the experimental
1368 conditions, RPA binding is stoichiometric and, hence, E_{FRET} increases linearly until the ssDNA is
1369 saturated with Cy5-labeled RPA (i.e., equivalence point)^{12,25}. Data is fit to two segment lines (a
1370 linear regression with a positive slope and a flat line) and the equivalence point is calculated
1371 from the intersection of the two segment lines. The concentration of the active Cy5-labeled RPA

1372 (in μM) is determined by dividing the amount of RPA-binding sites (in pmoles) by the total
1373 volume (in μL) of Cy5-labeled RPA at the equivalence point. Each Cy3-labeled P/T DNA
1374 substrate utilized in the present study accommodates 1 RPA heterotrimer. Hence, for a given
1375 Cy3-labeled P/T DNA, Cy5-labeled RPA FRET pair, the amounts of RPA-binding sites is equal
1376 to the amount of the respective Cy3-labeled P/T DNA.

1377 *Pre-steady state FRET assays to monitor RPA-DNA interactions.* A solution containing a Cy3-
1378 labeled P/T DNA (20 nM, **Figure S1**), NeutrAvidin (80 nM, Thermo Scientific), and ATP (1
1379 mM, Thermo Scientific) is pre-incubated, and the resultant solution is transferred to a
1380 fluorometer cell that is then placed in the instrument. Fluorescence emission intensities (I_{665} and
1381 I_{563}) are monitored over time until both signals stabilize for at least 1 min. Within this stable
1382 region, E_{FRET} values are calculated from the observed fluorescence emission intensities (I_{665} and
1383 I_{563}) and averaged to obtain the E_{FRET} value observed prior to addition of Cy5-labeled RPA.
1384 Then, a Cy5-labeled RPA (25 nM heterotrimer, either Cy5-OBDRPA or RPA-OBA-Cy5) is
1385 added, the resultant solution is mixed by pipetting, and the fluorescence emission intensities (I_{665}
1386 and I_{563}) are monitored over time, beginning 10 s after the addition of the Cy5-labeled RPA (i.e.,
1387 $\Delta t \leq 10$ s). To determine the predicted E_{FRET} trace for a Cy5-labeled RPA remaining completely
1388 disengaged from a Cy3-labeled P/T DNA, these experiments are repeated with Cy5-labeled RPA
1389 alone and with a Cy3-labeled P/T DNA alone and the E_{FRET} is calculated for each time point by

1390 the equation
$$E_{\text{FRET}} = \frac{(I_{665}^{\text{RPA}} + I_{665}^{\text{DNA}})}{(I_{665}^{\text{RPA}} + I_{665}^{\text{DNA}}) + (I_{563}^{\text{RPA}} + I_{563}^{\text{DNA}})}$$

1391 *Equilibrium FRET assays to determine the stoichiometry of RFC-catalyzed loading of PCNA*
1392 *onto P/T junctions.* A Cy3-labeled P/T DNA (55 nM of either 5'Cy3P/T or 3'Cy3P/T pre-bound
1393 with 220 nM NeutrAvidin) is saturated with RPA prior to the addition of RFC (55 nM) and then
1394 Cy5-PCNA is titrated in. After each addition of Cy5-PCNA, E_{FRET} is monitored over time until
1395 the signal stabilizes for at least 1 min. E_{FRET} values observed within this stable period are
1396 averaged to obtain the E_{FRET} value observed for the respective addition of Cy5-PCNA.

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1398 *Pre-steady state FRET assays to monitor PCNA unloading.* A solution containing a 5'ddPCy3/T
1399 DNA (20 nM, **Figure S1**), NeutrAvidin (80 nM), and ATP (1 mM) is pre-incubated with RPA
1400 (25 nM heterotrimer). Then, Cy5-PCNA (20 nM homotrimer) is added, the resultant solution is
1401 transferred to a fluorometer cell, and the cell is placed in the instrument. E_{FRET} is monitored over
1402 time until the signal stabilizes for at least 1 min. E_{FRET} values observed within this stable period
1403 are averaged to obtain the E_{FRET} value observed prior to addition of RFC•ATP. Next, a pre-
1404 formed RFC•ATP complex (20 nM RFC heteropentamer, 1 mM ATP) is added, the resultant
1405 solution is mixed via pipetting, and E_{FRET} is monitored beginning ≤ 10 s after the addition of
1406 RFC (i.e., $\Delta t \leq 10$ s) and continues until the signal stabilizes for at least 1 min. E_{FRET} values
1407 observed within this stable period are averaged to obtain the E_{FRET} value observed prior to
1408 addition of unlabeled PCNA. Finally, unlabeled PCNA (1.4 μM homotrimer) is added, the
1409 resultant solution is mixed by pipetting, and E_{FRET} is monitored beginning ≤ 10 s after the
1410 addition of unlabeled PCNA ($\Delta t \leq 10$ s).

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1412 *Pre-steady state FRET assays to monitor facilitated exchange of RPA between ssDNA sequences.*
1413 A solution containing ddP/5'TCy3 (50 nM, **Figure S1**), NeutrAvidin (200 nM, Thermo

1414 Scientific), and ATP (1 mM, Thermo Scientific), and RPA-OBA-Cy5 (55 nM heterotrimer) is
1415 pre-incubated, and the resultant solution is transferred to a fluorometer cell that is then placed in
1416 the instrument. Fluorescence emission intensities (I_{665} and I_{563}) are monitored over time until
1417 both signals stabilize for at least 1 min. Within this stable region, E_{FRET} values are calculated
1418 from the observed fluorescence emission intensities (I_{665} and I_{563}) and averaged to obtain the
1419 E_{FRET} value observed prior to addition of poly(dT)₇₀. Then, poly(dT)₇₀ (0.0 – 2.25 μM) is added,
1420 the resultant solution is mixed by pipetting, and the fluorescence emission intensities (I_{665} and
1421 I_{563}) are monitored over time, beginning 10 s after the addition of the poly (dT)₇₀ (i.e., $\Delta t \leq 10$ s).

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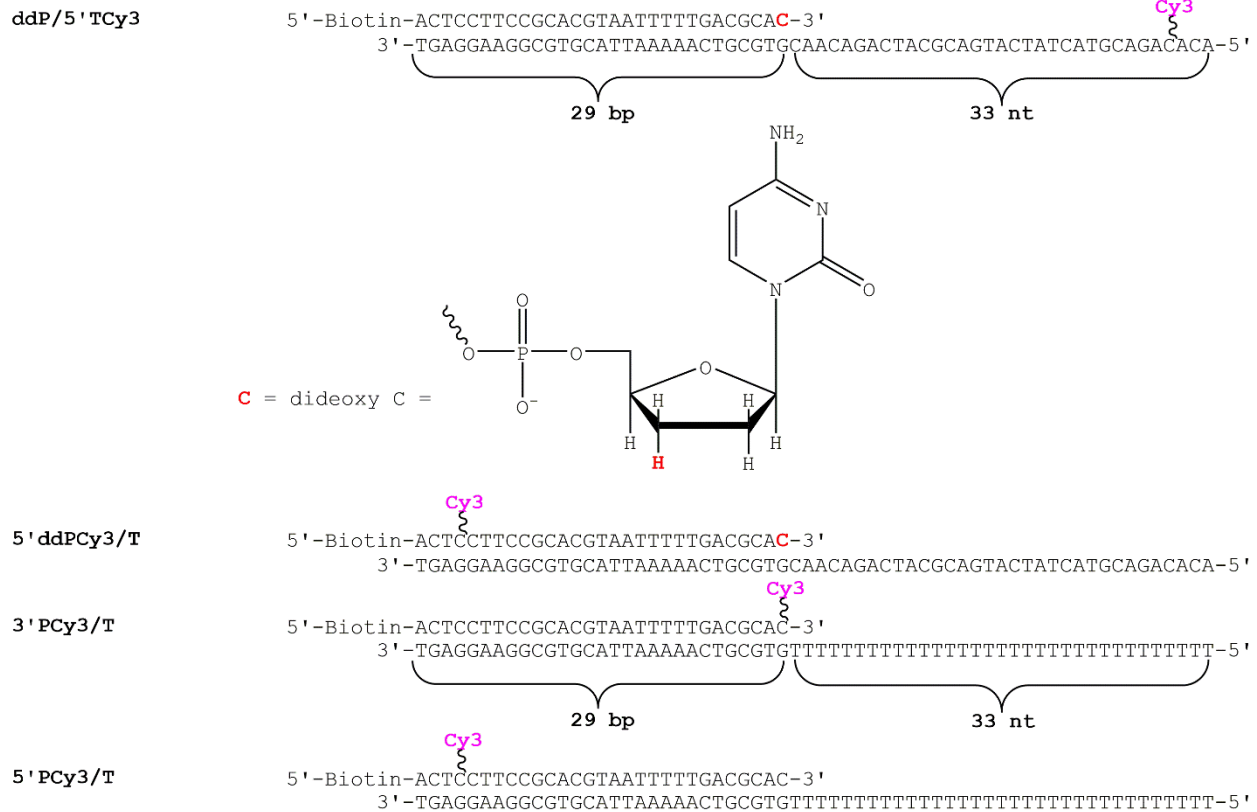
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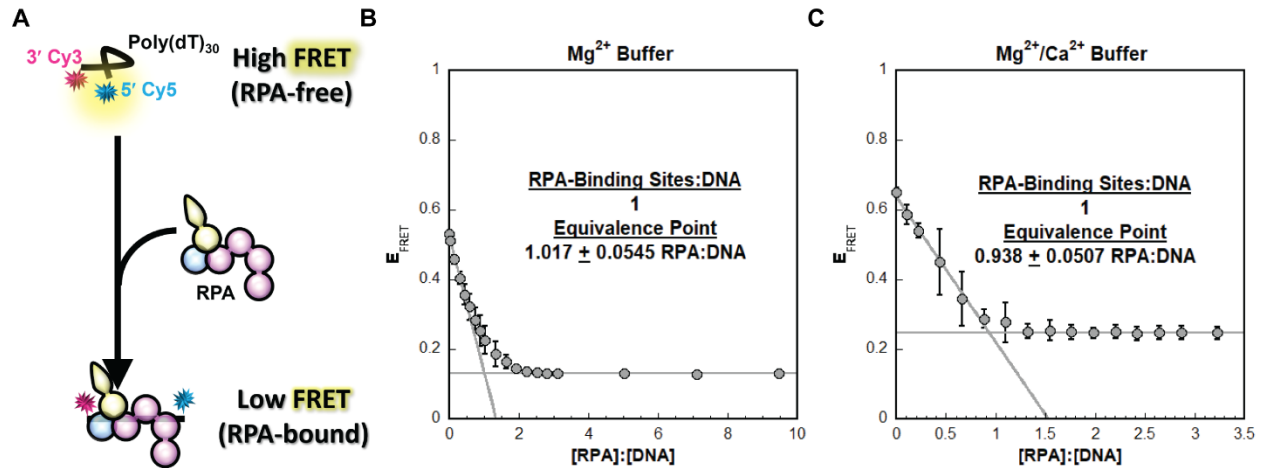
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1431 **Supplemental Figures**



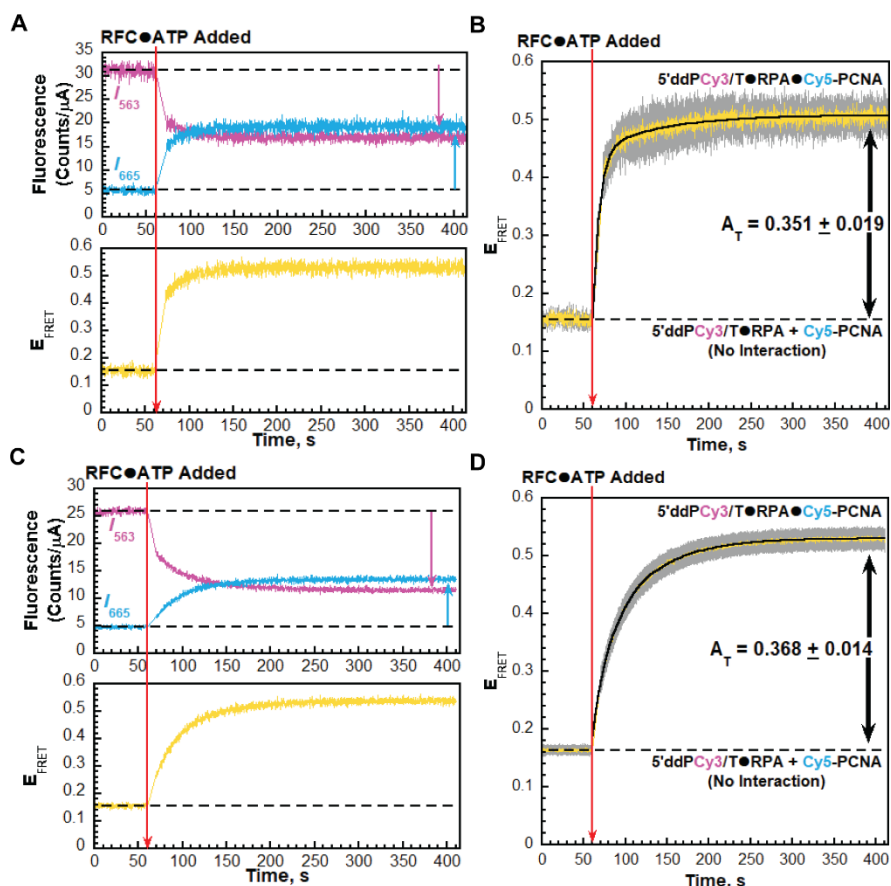
1432 **Figure S1.** DNA substrates utilized in this study. For substrates containing dsDNA regions, the
1433 sequences and lengths (29 bp) of these regions are all identical. When annealed, each substrate
1434 mimics a nascent P/T junction. The size of the dsDNA P/T region (29 bp) is in agreement with
1435 the requirements for assembly of a PCNA ring onto DNA by RFC^{2,3,18}. The ssDNA regions
1436 adjacent to the 3' end of the P/T junctions are 33 nt in length and accommodate one RPA
1437 heterotrimer⁴⁻⁶. RPA prevents loaded PCNA from sliding off the ssDNA end of the substrate².
1438 When pre-bound to NeutrAvidin, the biotin attached to the 5'-end of a primer strand prevents
1439 loaded PCNA from sliding off the dsDNA end of the substrate. ssDNA comprised only of T (i.e.,
1440 poly(dT)_x) is incapable of adapting stable secondary structures²⁶. Primers terminated at the 3'
1441 end with a dideoxy C nucleotide cannot be extended by Pol δ.

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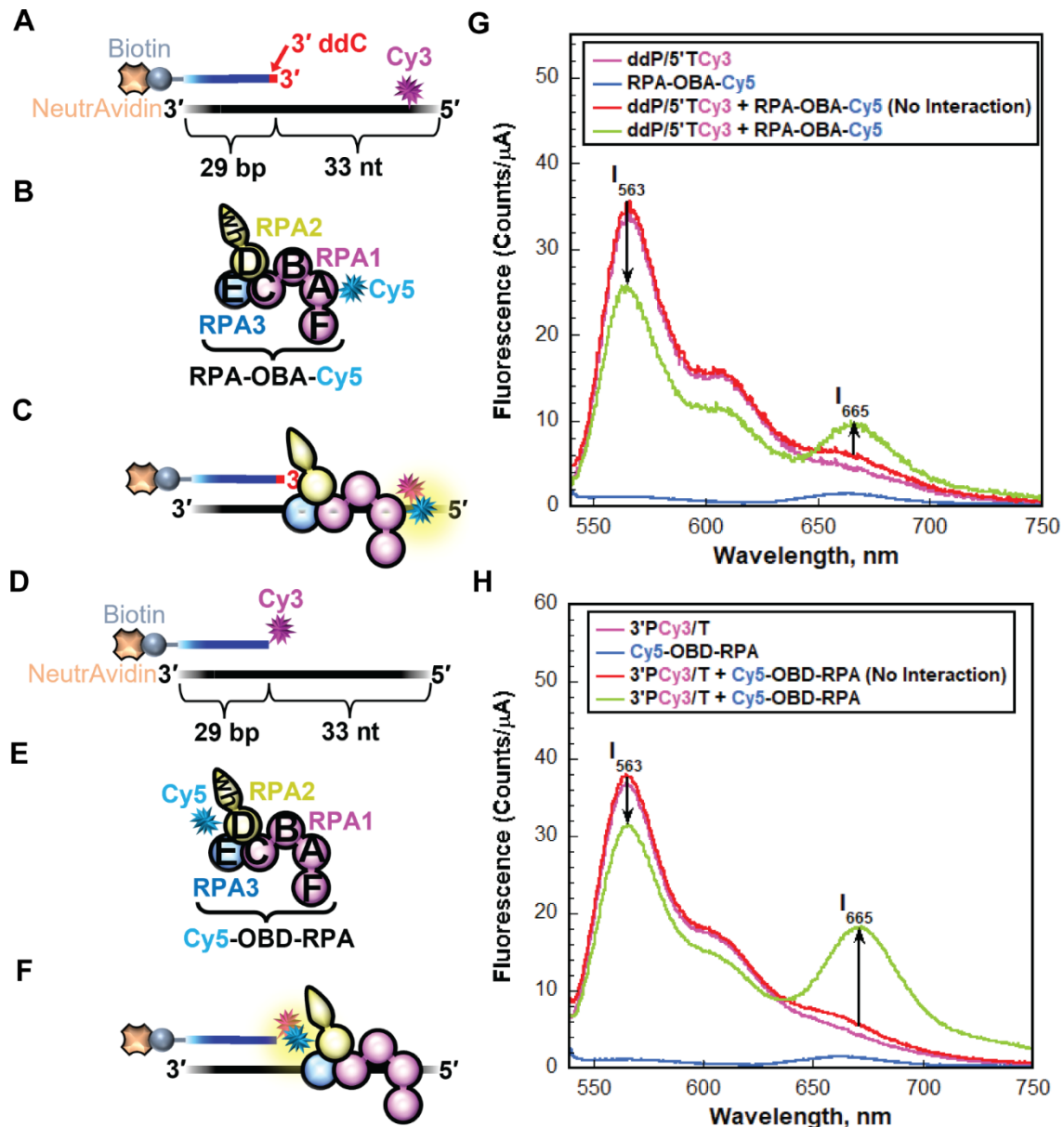
1453 **Figure S2.** Effect of experimental reaction buffers on RPA binding ssDNA. (A) Schematic
1454 representation of the FRET experiment utilizing poly(dT)₃₀ FRET ssDNA and native RPA.
1455 Poly(dT)₃₀ FRET is terminally labeled with a 3' Cy3 (FRET donor) and a 5' Cy5 (FRET
1456 acceptor). In the absence of RPA, free DNA (poly(dT)₃₀-FRET) forms a compact, flexible
1457 structure, bringing the two cyanine fluorophores close together and yielding a high E_{FRET}.
1458 Binding of an RPA stretches the engaged ssDNA and increases its bending 2 – 3 fold^{12,27},
1459 thereby increasing the Cy3, Cy5 distance and reducing E_{FRET}. (B - C) FRET data for RPA
1460 titrations carried out in Mg²⁺ Buffer (panel B) and Mg²⁺/Ca²⁺ Buffer (panel C). For each,
1461 poly(dT)₃₀-FRET (10 nM) is titrated with RPA and E_{FRET} is monitored. The observed E_{FRET} is
1462 plotted as a function of the ratio of concentrations of active RPA and DNA (i.e., [RPA]:[DNA]).
1463 Each data point represents the mean ± S.E.M. of at least three independent measurements. The
1464 equivalence points for each panel are indicated with standard errors of the calculations.

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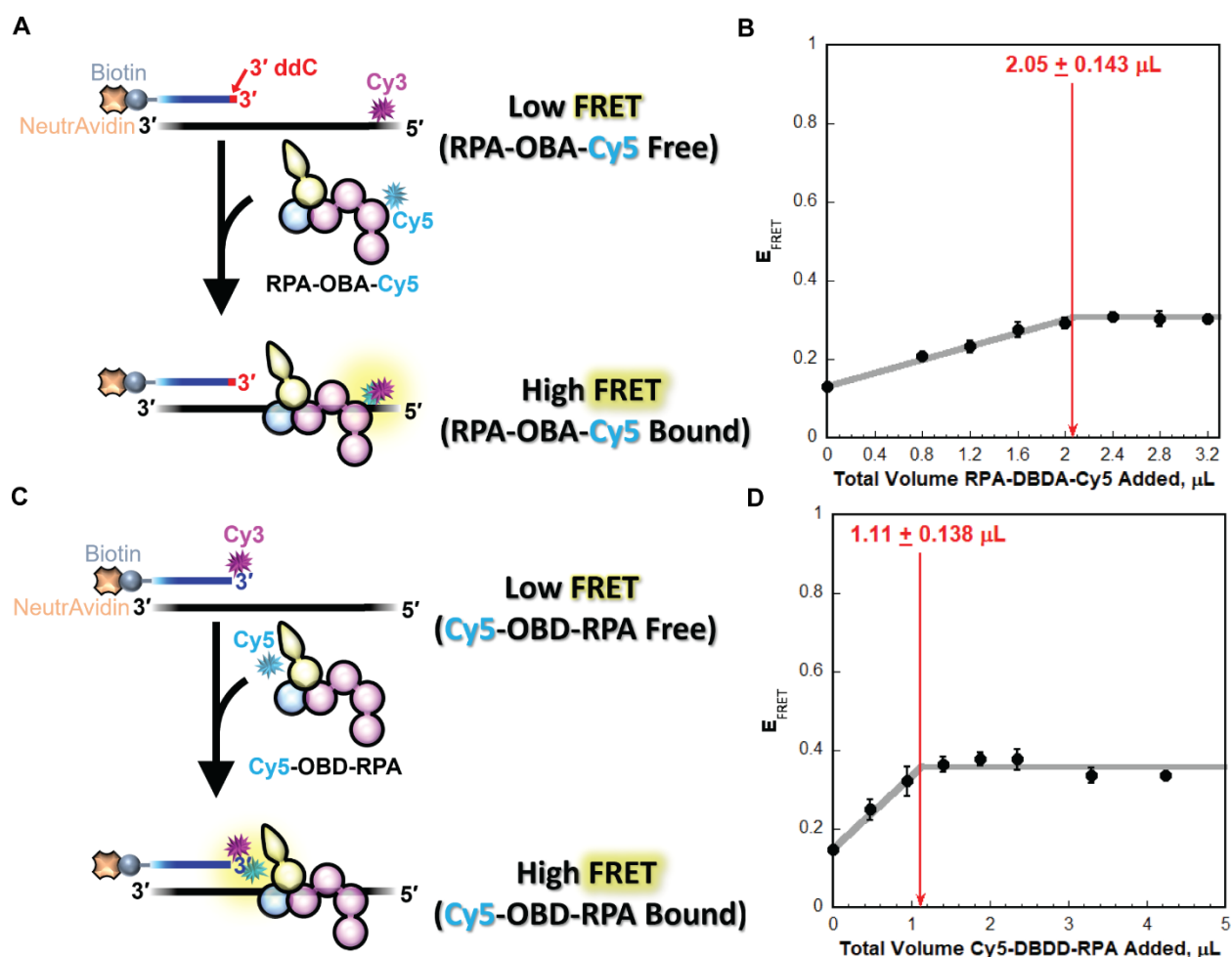


1485 **Figure S3.** Effect of experimental reaction buffers on RFC-catalyzed loading of PCNA onto P/T
 1486 junctions. Experiments are carried out on the 5'ddPcy3/T DNA substrate and the results are
 1487 plotted and analyzed exactly as described in **Figure 3A – C** in the main text. (**A - B**). Data
 1488 observed with native RPA in Mg^{2+} buffer. (**C – D**) Data observed with native RPA in Ca^{2+}/Mg^{2+}
 1489 buffer. Data is from **Figure 3B – C** in the main text. For E_{FRET} traces in panels **B** and **D**, each is
 1490 the mean of at least three independent traces with the S.E.M. shown in grey. E_{FRET} traces
 1491 observed after the addition of RFC are each fit to double exponential rises and the A_T are
 1492 reported in the respective graph as well as in **Table S1** along with other kinetic variables.

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1507 **Figure S4** Monitoring interaction between RPA and a P/T junction by FRET. (A - C) Schematic
 1508 representations of the Cy3-labeled P/T DNA substrate (panel A, ddP/5'TCy3, **Figure S1**) and
 1509 Cy5-labeled RPA (panel B, RPA-OBA-Cy5) utilized to monitor the interaction of RPA OBA
 1510 with a P/T junction (panel C). (D - F) Schematic representations of the Cy3-labeled P/T DNA
 1511 substrate (panel D, 3'PCy3/T, **Figure S1**) and Cy5-labeled RPA (Cy5-OBD-RPA) utilized to
 1512 monitor the interaction of OBD with a P/T junction (panel F). RPA subunits are color-coded and
 1513 depicted as in **Figure 1**. (G - H) Fluorescence emission spectra of RPA-P/T DNA interactions.
 1514 I_{665} and I_{563} are denoted in each spectrum. FRET is indicated by an increase in I_{665} and a
 1515 concomitant decrease in I_{563} , both of which are denoted in each panel by black arrows. For each
 1516 panel, the predicted spectrum for no interaction between the respective Cy5-labeled RPA and the
 1517 corresponding Cy3-labeled P/T DNA is determined by adding the spectrums of the individual
 1518 components. The spectrums obtained for the interactions between RPA-OBA-Cy5 and the
 1519 ddP/5'TCy3 DNA substrate and between Cy5-OBD-RPA and the 3'PCy3/T DNA substrate are
 1520 shown in panels G and H, respectively.

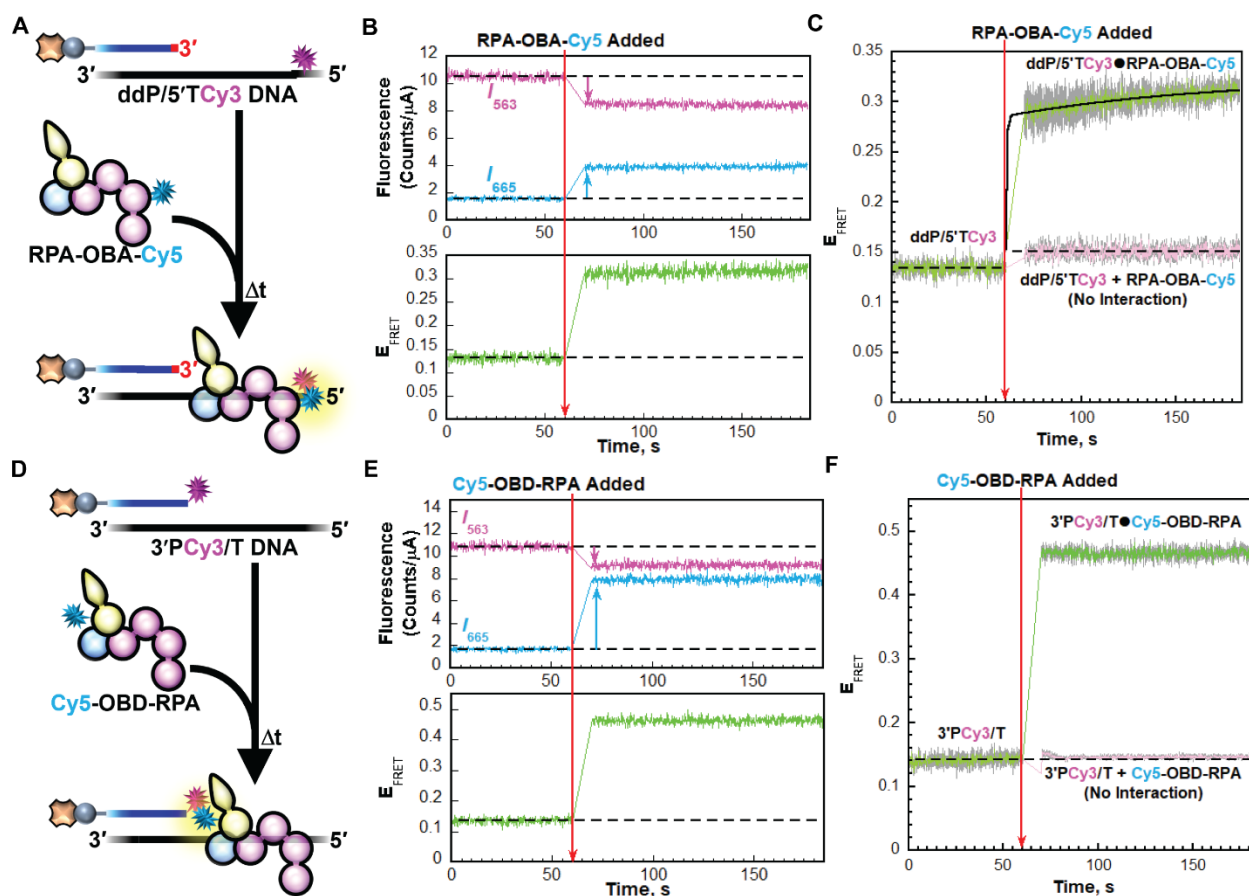


1521 **Figure S5.** Concentration of active Cy5-labeled RPA. (A) Schematic representation of FRET-
 1522 based active site titration of RPA-OBA-Cy5. (B) Titration of ddP/5'Cy3T DNA (3.014 pmole,
 1523 3.014 pmole RPA binding sites) with RPA-OBA-Cy5. Each data point represents the mean \pm
 1524 S.E.M. of at least three independent measurements. The equivalence point is indicated with
 1525 standard error of the calculation. Saturation is reached at approximately 2.05 μL total RPA-
 1526 OBA-Cy5 added, yielding a concentration of 1.47 μM (3.014 pmole RPA binding sites/2.05 μL
 1527 total RPA-OBA-Cy5 added = 1.47 pmole/ μL = 1.47 μM). (C) Schematic representation of
 1528 FRET-based active site titration of Cy5-OBD-RPA. The assay is carried out as described in
 1529 panel A except that the FRET donor is 3'PCy3/T DNA (**Figure S1**) and the FRET acceptor is
 1530 Cy5-OBD-RPA. (D) Titration of 3'PCy3/T DNA (2.43 pmole, 2.43 pmole RPA binding sites)
 1531 with Cy5-OBD-RPA. Each data point represents the mean \pm S.E.M. of at least three independent
 1532 measurements. The equivalence point is indicated with standard error of the calculation.
 1533 Saturation is reached at approximately 1.11 μL total Cy5-OBD-RPA, yielding a concentration of
 1534 2.19 μM (2.43 pmole RPA binding sites/1.11 μL total Cy5-OBD-RPA added = 2.19 pmole/ μL =
 1535 2.19 μM).

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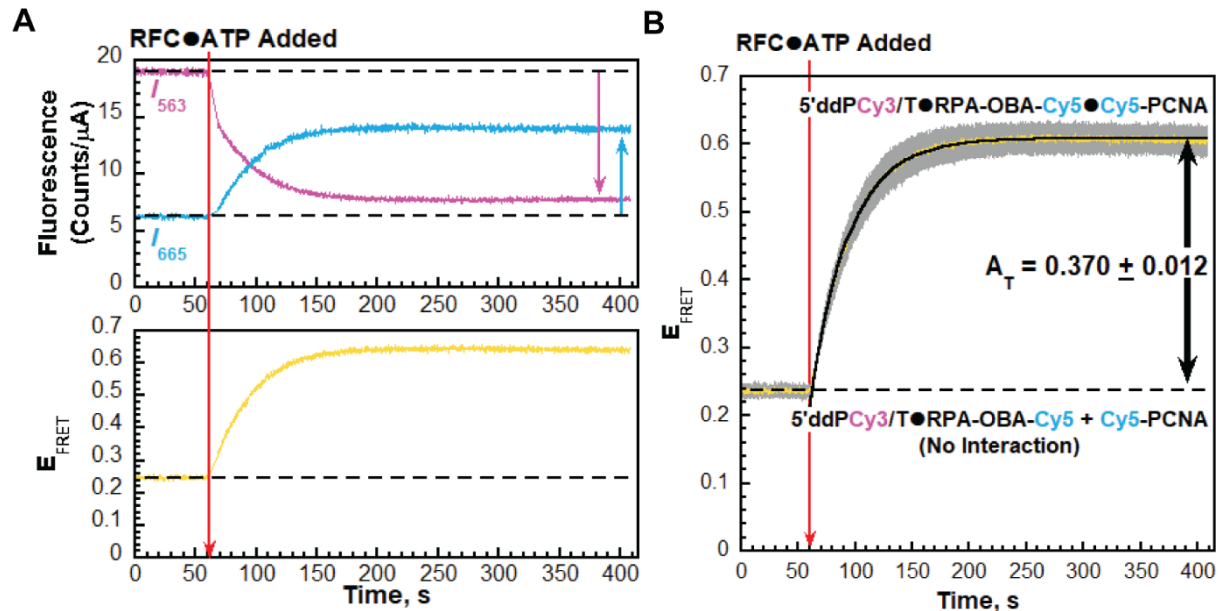
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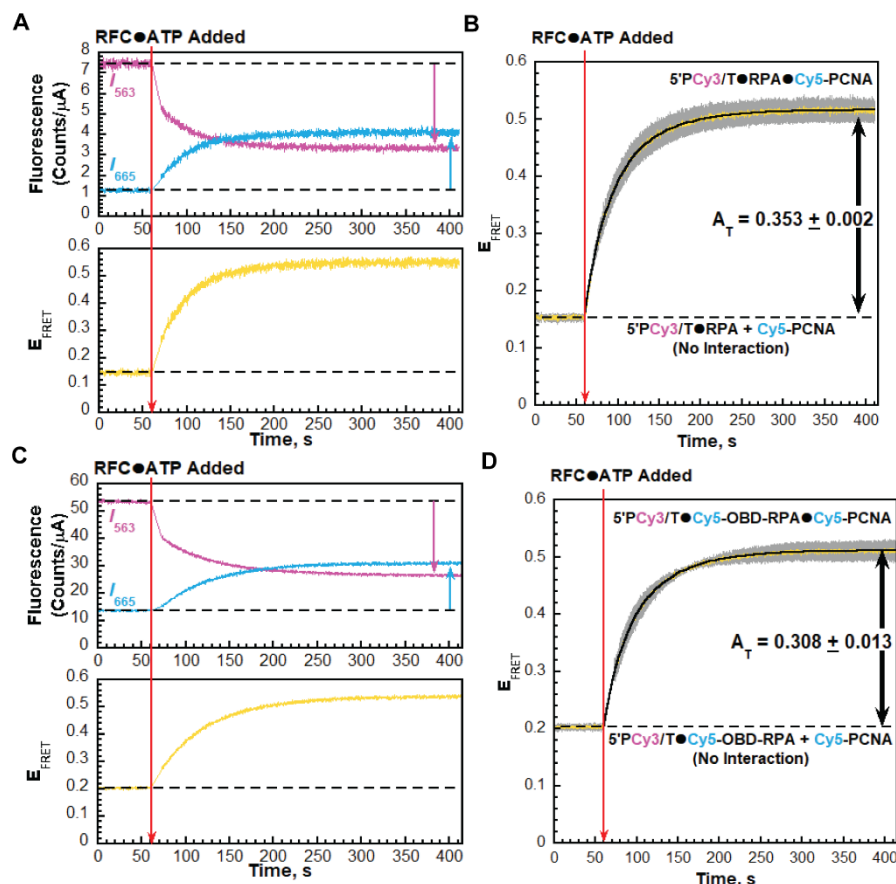
1539 **Figure S6.** RPA engaging a P/T junction. **(A)** Schematic representation of the FRET experiment
 1540 performed with ddP/5'TCy3 DNA (+ NeutrAvidin) and RPA-OBA-Cy5 **(B)** Sample time
 1541 trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The time at which RPA-OBA-Cy5 is
 1542 added is indicated by a red arrow. For observation, the I_{563} , I_{665} , and E_{FRET} values observed prior to
 1543 the addition of RPA-OBA-Cy5 are fit to flat lines that are extrapolated to the axis limits.
 1544 Changes in I_{563} and I_{665} are indicated by magenta and cyan arrows, respectively. **(C)** FRET data.
 1545 Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M. shown in grey.
 1546 The time at which RPA-OBA-Cy5 is added is indicated by a red arrow. The E_{FRET} trace observed
 1547 prior to the addition of RPA-OBA-Cy5 is fit to a flat line. The E_{FRET} observed after the addition
 1548 of RPA-OBA-Cy5 is fit to a double exponential rise and the observed rate constant for the
 1549 second, slower phase ($k_{\text{obs inc},2}$) is reported in the graph. The predicted FRET trace (pink) for no
 1550 interaction between RPA-OBA-Cy5 and the 5'TCy3 DNA is fit to a flat line. **(D – F)**
 1551 Experiments, results, and data analysis carried out for 3'PCy3/T DNA and Cy5-OBD-RPA
 1552 exactly as described in panels A – C except FRET traces observed after the addition of Cy5-
 1553 OBD-RPA were not fit to a kinetic model.

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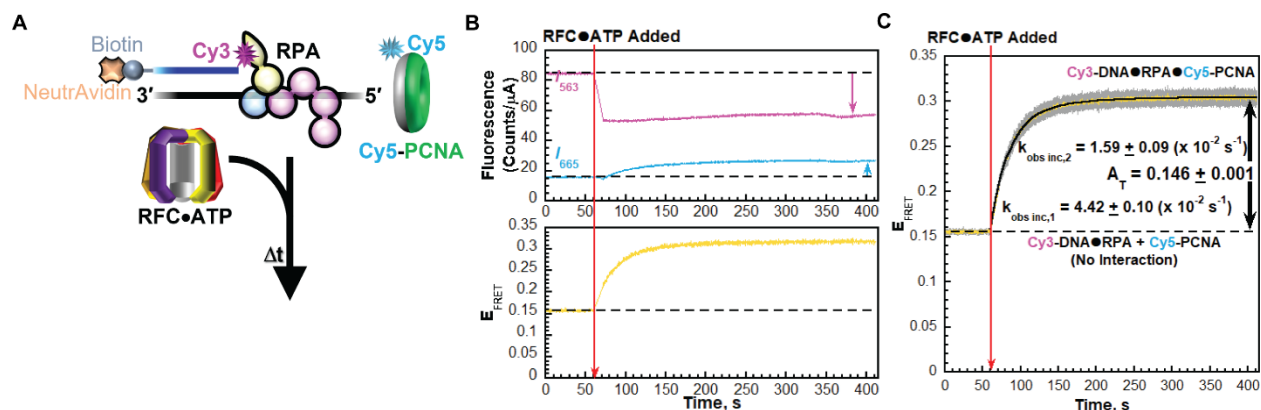
1560 **Figure S7.** Effect of Cy5 labeling of RPA OBA on RFC-catalyzed loading of PCNA onto P/T
1561 junctions. Experiments are carried out with RPA-OBA-Cy5 on the 5'ddPCy3/T DNA substrate
1562 and the results are plotted and analyzed exactly as described in **Figure 3A – C** in the main text.
1563 E_{FRET} trace in panel **B** is the mean of at least three independent traces with the S.E.M. shown in
1564 grey. E_{FRET} trace in panel **B** observed after the addition of RFC is fit to an exponential rise and
1565 the A_T is reported in the graph as well as in **Table S1** along with other kinetic variables. The
1566 total amplitude observed on the 5'ddPCy3/T DNA substrate in $\text{Mg}^{2+}/\text{Ca}^{2+}$ buffer with RPA-
1567 OBA-Cy5 ($A_T = 0.370 \pm 0.012$) is within experimental error of that observed under the same
1568 experimental conditions with native RPA ($A_T = 0.368 \pm 0.014$, **Figure 3C**, **Figure S3D**, **Table**
1569 **S1**). This indicates that RPA-OBA-Cy5 fully supports RFC-catalyzed loading of PCNA onto P/T
1570 junctions.

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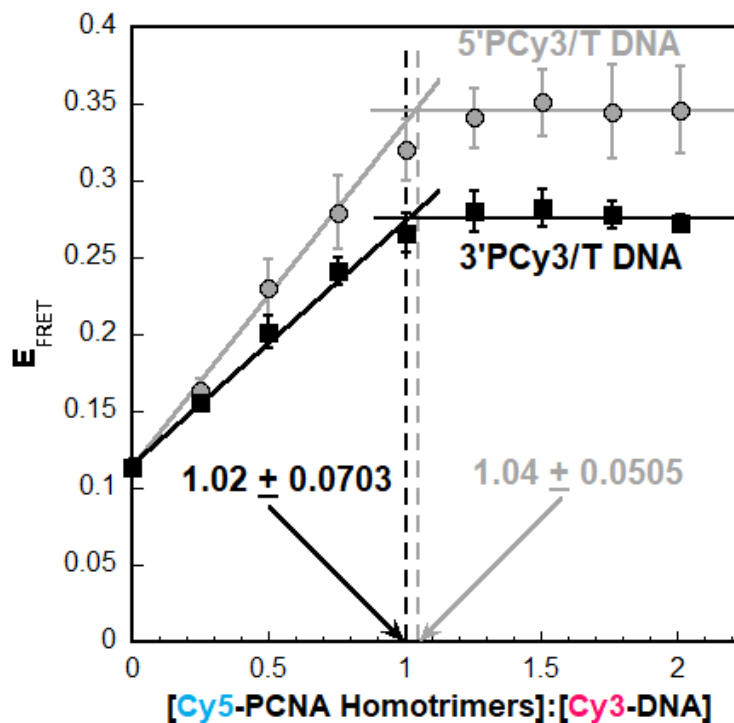
1589 **Figure S8.** Effect of Cy5 label on OBD of RPA on RFC-catalyzed loading of PCNA onto P/T
 1590 junctions. Experiments are carried out on the 5'PCy3/T DNA substrate and the results are plotted
 1591 and analyzed exactly as described in **Figure 5A – C** in the main text. (**A – B**) Data observed with
 1592 native RPA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer (from **Figure 5B – C** in the main text). (**C – D**) Data observed
 1593 with Cy5-OBD-RPA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer. For E_{FRET} traces in panels **B** and **D**, each is the mean
 1594 of at least three independent traces with the S.E.M. shown in grey. E_{FRET} traces observed after
 1595 the addition of RFC are each fit to double exponential rises and the total amplitudes (A_T) are
 1596 reported in the respective graph as well as in **Table S1** (along with other kinetic variables).
 1597 The total amplitude observed with Cy5-OBD-RPA ($A_T = 0.308 \pm 0.013$) agrees very well with
 1598 that observed under the same experimental conditions with native RPA ($A_T = 0.353 \pm 0.002$,
 1599 **Figure 5C, Table S1**). Furthermore, the rate constants [$k_{\text{obs inc},1} = 5.06 \pm 0.18 (x 10^{-2}) \text{ s}^{-1}$, $k_{\text{obs inc},2}$
 1600 $= 1.83 \pm 0.02 (x 10^{-2}) \text{ s}^{-1}$, **Table S1**] observed with Cy5-OBD-RPA are nearly identical to those
 1601 observed under the same experimental conditions with native RPA [$k_{\text{obs inc},1} = 4.34 \pm 0.18 (x 10^{-2})$
 1602 s^{-1} , $k_{\text{obs inc},2} = 1.79 \pm 0.04 (x 10^{-2}) \text{ s}^{-1}$, **Table S1**]. Together, this indicates that RPA-OBA-Cy5
 1603 fully supports RFC-catalyzed loading of PCNA onto P/T junctions.

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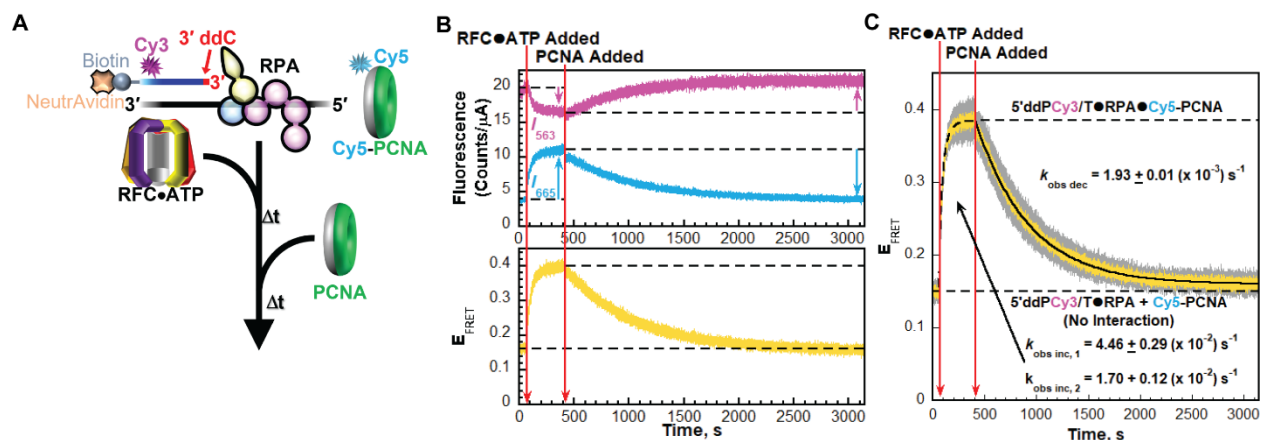
1612 **Figure S9.** Effects of primer Cy3 donor location on the kinetics RFC-catalyzed loading of
 1613 PCNA onto P/T junctions. (A) Schematic representation of the FRET experiment. Reactions
 1614 were carried out exactly as described in 5A in the main text except with 3'Cy3P/T DNA. Note
 1615 the Cy5 label on PCNA will be oriented away from the Cy3 donor on the 3' terminus of the
 1616 primer strand when Cy5-PCNA is loaded onto the 3'Cy3P/T DNA substrate by RFC (B) Sample
 1617 time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The time at which the RFC•ATP
 1618 complex is added is indicated by a red arrow. Changes in I_{563} and I_{665} are indicated by magenta
 1619 and cyan arrows, respectively. For observation, the I_{563} , I_{665} , and E_{FRET} values observed prior to
 1620 the addition of the RFC•ATP complex are fit to flat lines that are extrapolated to the axis limits.
 1621 (C) FRET data. Each E_{FRET} trace is the mean of at least three independent traces with the S.E.M.
 1622 shown in grey. The time at which the RFC•ATP is added is indicated by a red arrow. The E_{FRET}
 1623 trace observed prior to the addition of the RFC•ATP complex represents the complete absence of
 1624 interactions between the 3'PCy3/T•RPA complex and Cy5-PCNA and is fit to a flat line that is
 1625 extrapolated to the axis limits. The E_{FRET} trace observed after the addition of the RFC•ATP
 1626 complex is fit to a double exponential rise and the observed rate constants ($k_{\text{obs inc,1}}$ and $k_{\text{obs inc,2}}$)
 1627 and A_T are reported in the graph as well as in Table S1. The predicted E_{FRET} trace (pink) for no
 1628 interaction between 5P'Cy3/T•RPA complex and the loading complex is fit to a flat line.

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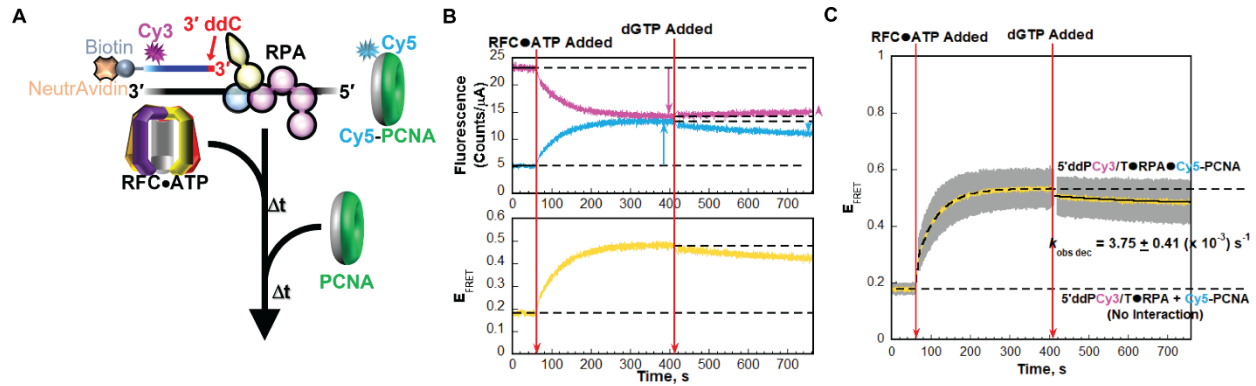
1646 **Figure S10.** Titrations of the steady state FRET signal. Results are plotted as a function of the
1647 $[\text{Cy5-PCNA homotrimer}]:[\text{Cy3-DNA}]$ ratio and each data point represents the mean \pm S.E.M. of
1648 at least three independent measurements. Data observed for the 5'PCy3/T and 3'PCy3/T DNA
1649 substrates is shown in grey and black, respectively. Under the experimental conditions, RFC-
1650 catalyzed loading of PCNA onto the 5'PCy3/T is stoichiometric (**Figure S9**) and, hence, FRET
1651 increases linearly until the DNA is saturated with PCNA (i.e., the equivalence point)^{2,3} at a ratio
1652 of 1 PCNA homotrimer:1 P/T DNA. Data is fit to two segment lines (a linear regression with a
1653 positive slope and a flat line) and the equivalence points (indicated with standard errors of the
1654 calculations) are calculated from the intersection of the two segment lines. Equivalence points
1655 for each P/T DNA substrate are indicated on the graph.

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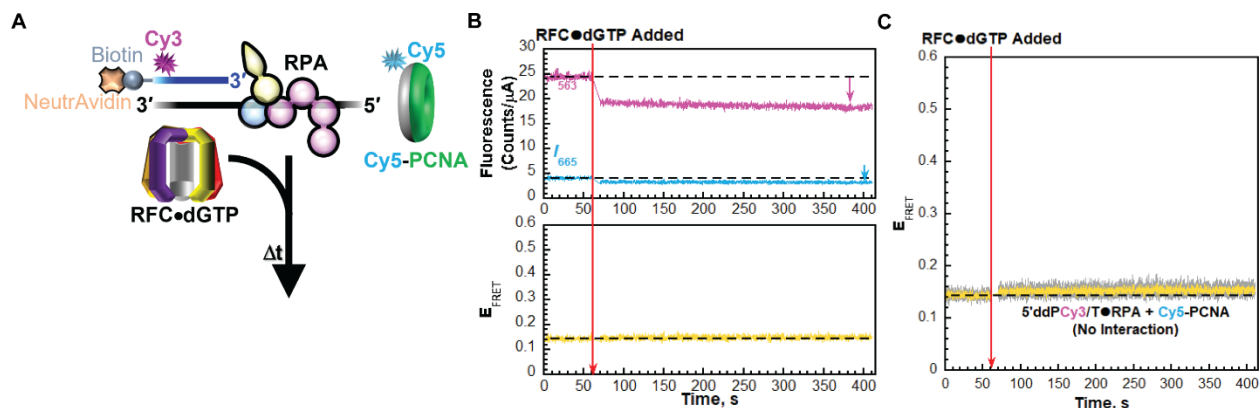
1672 **Figure S11.** Dynamics of PCNA encircling a P/T junction. (A) Schematic representation of the
 1673 FRET experiment performed with 5'ddPCy3/T DNA, RPA, Cy5-PCNA, RFC, ATP, and PCNA.
 1674 (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom). The times at which
 1675 the RFC•ATP complex and unlabeled PCNA are added are indicated by red arrows. For
 1676 observation, the emission intensity traces and E_{FRET} values observed in the absence of RFC are
 1677 each fit to dashed flat lines that are extrapolated. Also, dashed flat lines are drawn to highlight
 1678 the I values and E_{FRET} values observed at equilibrium after the addition of unlabeled PCNA.
 1679 Changes in I_{563} and I_{665} observed after each addition are indicated by magenta and cyan arrows,
 1680 respectively. (C) FRET data. Each E_{FRET} trace is the mean of at least three independent traces
 1681 with the S.E.M. shown in grey. The times at which RFC•ATP and unlabeled PCNA are added
 1682 are indicated by red arrows. The E_{FRET} trace observed prior to the addition of the RFC•ATP
 1683 complex is fit to a dashed flat line that is extrapolated to the axis limits to depict the average
 1684 E_{FRET} value for no interaction between Cy5-PCNA and the 5'ddPCy3/T•RPA complex. The
 1685 E_{FRET} trace observed after the addition of the RFC•ATP complex is fit to a dashed double
 1686 exponential rise that is extrapolated to the axis limits to depict the average E_{FRET} value for
 1687 complete loading of Cy5-PCNA onto the Cy3-labeled P/T DNA substrate (i.e., the
 1688 5'ddPCy3/T•RPA•Cy5-PCNA complex). The rate constants observed for the E_{FRET} increase ($k_{obs\ inc, 1}$
 1689 and $k_{obs\ inc, 2}$) are reported in the figure. The E_{FRET} trace observed after the addition of
 1690 unlabeled PCNA is fit to a single exponential decay and the observed rate constant ($k_{obs\ dec}$)
 1691 is reported in the graph.

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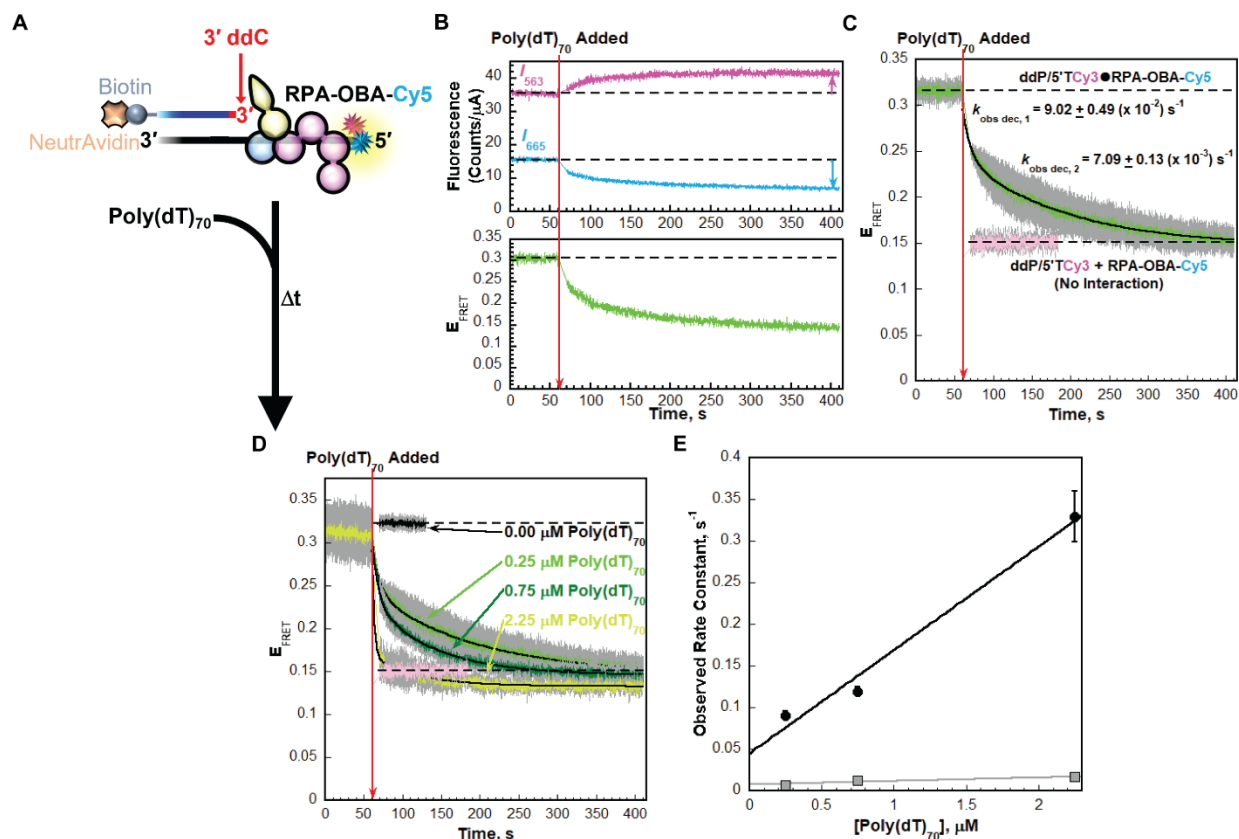
1705 **Figure S12.** Effects of added dGTP on the dynamics of PCNA encircling a P/T junction. (A)
 1706 Schematic representation of the FRET experiment performed with 5'ddPCy3/T DNA, RPA, Cy5-
 1707 PCNA, RFC, ATP, and dGTP. (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET}
 1708 (*Bottom*). The times at which the RFC•ATP complex and dGTP are added are indicated by red
 1709 arrows. For observation, the emission intensity traces and E_{FRET} values observed in the absence
 1710 of RFC are each fit to dashed flat lines that are extrapolated. Also, dashed flat lines are drawn to
 1711 highlight the I values and E_{FRET} values observed at equilibrium after the addition of RFC•ATP.
 1712 Changes in I_{563} and I_{665} observed after each addition are indicated by magenta and cyan
 1713 arrows, respectively. (C) FRET data. Each E_{FRET} trace is the mean of at least three independent
 1714 traces with the S.E.M. shown in grey. The times at which RFC•ATP and dGTP are added are indicated
 1715 by red arrows. The E_{FRET} trace observed prior to the addition of the RFC•ATP complex is fit to a
 1716 dashed flat line that is extrapolated to the axis limits to depict the average E_{FRET} value for no
 1717 interaction between Cy5-PCNA and the 5'ddPCy3/T•RPA complex. The E_{FRET} trace observed
 1718 after the addition of the RFC•ATP complex is fit to a dashed double exponential rise that is
 1719 extrapolated to the axis limits to depict the average E_{FRET} value for complete loading of Cy5-
 1720 PCNA onto the Cy3-labeled P/T DNA substrate (i.e., the 5'ddPCy3/T•RPA•Cy5-PCNA
 1721 complex). The E_{FRET} trace observed after the addition of dGTP is fit to a single exponential
 1722 decay and the observed rate constant ($k_{obs\ dec}$) is reported in the graph.

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1729 **Figure S13.** Effects of dGTP on RFC-catalyzed loading of PCNA onto a P/T junction. (A)
 1730 Schematic representation of the FRET experiment performed with 5' ddPCy3/T DNA, RPA, Cy5-
 1731 PCNA, RFC, and dGTP. (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET}
 1732 (Bottom). The time at which the RFC•dGTP complex is added is indicated by a red arrow. For
 1733 observation, the emission intensity traces and E_{FRET} values observed in the absence of RFC are
 1734 each fit to dashed flat lines that are extrapolated. Changes in I_{563} and I_{665} observed after each
 1735 addition are indicated by magenta and cyan arrows, respectively. (C) FRET data. Each E_{FRET}
 1736 trace is the mean of at least three independent traces with the S.E.M. shown in grey. The times at
 1737 which RFC•dGTP is added is indicated by red arrows. The E_{FRET} trace observed prior to the
 1738 addition of the RFC•dGTP complex is fit to a dashed flat line that is extrapolated to the axis
 1739 limits to depict the average E_{FRET} value for no interaction between Cy5-PCNA and the
 1740 5' ddPCy3/T•RPA complex.

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1764 **Figure S14.** Facilitated exchange of RPA between ssDNA sequences. (A) Schematic
 1765 representation of the FRET experiment performed with ddP/5'TCy3 DNA, RPA-OBA-Cy5,
 1766 ATP, and poly(dT)₇₀. (B) Sample time trajectories of I_{563} and I_{665} (Top) and their E_{FRET} (Bottom)
 1767 observed with 0.235 μM poly(dT)₇₀. The time at which poly(dT)₇₀ is added is indicated by a red
 1768 arrow. For observation, the emission intensity traces and E_{FRET} values observed in the absence
 1769 poly(dT)₇₀ are each fit to dashed flat lines that are extrapolated. Changes in I_{563} and I_{665} observed
 1770 after each addition are indicated by magenta and cyan arrows, respectively. (C) FRET data
 1771 observed in the presence of 0.250 μM poly(dT)₇₀. Each E_{FRET} trace is the mean of three
 1772 independent traces with the S.E.M. shown in grey. The times at which poly(dT)₇₀ is added is
 1773 indicated by a red arrow. The E_{FRET} trace observed prior to the addition of poly(dT)₇₀ is fit to a
 1774 dashed flat line that is extrapolated to the axis limits. The E_{FRET} trace observed after the addition
 1775 of poly(dT)₇₀ is fit to a double exponential decline and the observed rate constants ($k_{\text{obs dec,1}}$ and
 1776 $k_{\text{obs dec,2}}$) are reported in the graph. The predicted E_{FRET} trace (pink) for no interaction between
 1777 RPA-OBA-Cy5 and the ddP/5'TCy3 DNA is fit to a flat line. (D) FRET data observed in the
 1778 presence of increasing concentrations of poly(dT)₇₀. Each E_{FRET} trace is the mean of three
 1779 independent traces with the S.E.M. shown in grey. The times at which poly(dT)₇₀ is added is
 1780 indicated by a red arrow. The E_{FRET} trace observed after the addition of buffer is fit to a flat line.
 1781 The E_{FRET} traces observed after the addition of a non-zero concentration of poly(dT)₇₀ are fit to
 1782 double exponential declines and the observed rate constants ($k_{\text{obs dec,1}}$ and $k_{\text{obs dec,2}}$) are plotted in
 1783 panel E as a function of poly(dT)₇₀ concentration.

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RPA	Wild-type								RPA-OBA-Cy5	Cy5-OBD-RPA		
Cy3 P/T DNA	5'ddPCy3/T				5'PCy3/T		3'PCy3/T		5'ddPCy3/T		5'PCy3/T	
Buffer	Mg ²⁺		Mg ²⁺ /Ca ²⁺		Mg ²⁺ /Ca ²⁺		Mg ²⁺ /Ca ²⁺		Mg ²⁺ /Ca ²⁺		Mg ²⁺ /Ca ²⁺	
Variable	Value	StdErr	Value	StdErr	Value	StdErr	Value	StdErr	Value	StdErr	Value	StdErr
$k_{obs inc, 1r}$ ($\times 10^{-2}$) s ⁻¹	12.2	0.4	5.29	0.14	4.34	0.18	4.42	0.10	Not Observed		5.06	0.18
$k_{obs inc, 2r}$ ($\times 10^{-2}$) s ⁻¹	1.28	0.05	1.72	0.02	1.79	0.04	1.59	0.09	2.98	0.05	1.83	0.02
A_T	0.351	0.019	0.368	0.014	0.353	0.002	0.146	0.001	0.370	0.012	0.308	0.013

1788 **Table S1.** Kinetic variables of RFC-catalyzed loading of PCNA onto a P/T junction.
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