The Role of *Saccharomyces cerevisiae* PCNA-DNA and RFC-DNA Contacts in Clamp Assembly on DNA

By
Yayan Zhou
Faculty Advisor: Dr. Manju M. Hingorani

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Abstract

The multi-protein clamp loader complex loads circular clamp proteins onto DNA where they serve as mobile tethers to increase DNA polymerase processivity, and coordinate the functions of many other DNA metabolic proteins. The clamp loading reaction involves multiple steps that occur in two stages: (1) ATP binding enables the clamp loader to bind and open the clamp, and then bind DNA; (2) DNA binding triggers rapid ATP hydrolysis by the clamp loader leading to clamp closure on DNA and complex dissociation. Crystal structures of these proteins show a large number of cationic residues in the clamp and clamp loader that directly contact DNA. In order to investigate the role of these contacts in the clamp loading mechanism, we examined single arginine/lysine mutants of the *Saccharomyces cerevisiae* clamp, Proliferating Cell Nuclear Antigen (PCNA) and clamp loader, Replication Factor C (RFC) by transient kinetics.

The study of PCNA shows that loss of even a single cationic residue can alter the rates of all DNA-linked steps in the loading reaction, as well as movement of PCNA on DNA. These results explain an earlier finding that each of the nine arginines and lysines in human PCNA is essential for polymerase δ processivity. Mutations in the N-terminal domain have greater impact than in the C-terminal domain of PCNA, indicating a positional asymmetry in PCNA-DNA contacts that can influence its functions on DNA.

The study of RFC also shows that removal even one of many cationic residues can alter the rates of DNA-linked steps in the reaction. In addition, we tested
the hypothesis that one DNA contact in each RFC subunit can control ATP hydrolysis. Analysis of specific alanine mutants suggest that Arg101 in RFC-D plays a primary role in controlling ATPase activity and Arg88 in RFC-C is important for allowing PCNA-DNA release to end the clamp loading reaction.
Table of Contents

Acknowledgement ............................................................................................................... I

Abstract ............................................................................................................................ II

Table of Contents .............................................................................................................. III

Chapter I  Introduction to DNA replication ................................................................. 1

1.1 Brief introduction to the biochemistry of DNA replication .............................. 1

1.2 Eukaryote replication machinery ........................................................................ 6

1.3 Clamps and clamp loaders processivity factors in DNA replication .......... 13

1.4 *S. cerevisiae* PCNA clamp and RFC clamp loader ........................................ 22

Chapter II  Impact of Individual PCNA-DNA Contacts on Clamp Loading and

Function on DNA ............................................................................................................. 36

2.1 Introduction ............................................................................................................. 36

2.2 Materials and Methods ....................................................................................... 44

  2.2.1 Protein, DNA, and other reagents ................................................................. 44

  2.2.2 Tetramethyl rhodamine-DNA preparation .................................................... 46

  2.2.3 Equilibrium binding of RFC (-/+ PCNA) to ptdNA .................................. 49

  2.2.4 Equilibrium measurement on FRET efficiency of PCNA opening .......... 51

  2.2.5 Kinetics measurements of DNA binding to PCNA•RFC•ATP complex... 52

  2.2.6 Kinetics measurements of PCNA opening and closing ............................ 53

  2.2.7 Kinetics measurements of Pi release ............................................................ 54

2.3 Results .................................................................................................................... 57

  2.3.1 Identify the DNA interacting residues in PCNA ....................................... 57
2.3.2 Effect of mutating inner rim cationic residues on PCNA opening by RFC 57
2.3.3 ptDNA binding and release are influenced by PCNA inner rim cationic residues .......................................................................................................................... 62
2.3.4 PCNA closure around ptDNA is influenced by inner rim cationic residues .......................................................................................................................... 72
2.3.5 Phosphate release toward the end of the PCNA loading cycle is also influenced by inner rim cationic residues .......................................................... 77
2.3.6 A kinetic model of the impact of PCNA-ptDNA contacts during and after clamp loading .................................................................................. 83
2.4 Discussion ......................................................................................................... 94

Chapter III Investigating the Role of RFC-DNA Contacts in the PCNA Clamp Loading Pathway ........................................................................................................... 101
3.1 Introduction ..................................................................................................... 101
3.2 Materials and Methods .................................................................................. 109
3.2.1 Protein, DNA, and other reagents ............................................................ 109
3.2.2 Equilibrium binding affinity measurement of wild type or mutant RFC-DNA interaction .......................................................................................... 111
3.2.3 Kinetics measurement of wild type or mutant RFC-DNA interaction in the absence/presence of PCNA ............................................................................... 112
3.2.4 Steady state ATPase activity measurement by wild type and mutant RFC .................................................................................................................. 113
3.2.5 Kinetics of Pi release assays by wild type and mutant RFC ............... 115
3.3 Results

3.3.1 Effect of mutating single residues in *S. cerevisiae* to alanine on equilibrium binding to DNA

3.3.2 Kinetic measurements of DNA binding/release

3.3.3 Steady state ATPase activities of wild type and mutant RFC in the presence of PCNA

3.3.4 Kinetics measurements of Pi release by wild type and mutant RFC

3.4 Discussion

Appendix A. Buffers

Appendix B. Oligonucleotide Purification by Electrophoresis

Appendix C. Protein Overexpression, Purification and Labeling

C.1 *S. cerevisiae* wild type PCNA, PCNA<sub>WC</sub>, PCNA<sub>FC</sub>, R14A, K20A, R80A, K146A, R149A, and K217A mutants purification

C.2 *S. cerevisiae* wild type RFC, RFC-BR84A, RFC-CR88A and RFC-DR101A purification

C.3 *S. cerevisiae* wild type PCNA, PCNA<sub>WC</sub>, PCNA<sub>FC</sub>, R14A, K20A, R80A, K146A, R149A, and K217A mutants labeling with AEDANS

C.4 Preparation of a Pi probe, MDCC-PBP

C.5 Labeling *E. coli* PBP-A197C with MDCC

References
Chapter I  Introduction to DNA replication

1.1 Brief introduction to the biochemistry of DNA replication

High-fidelity DNA replication is vital for maintaining the integrity of the genetic material in all forms of cellular life. New DNA strands are formed by DNA polymerase-catalyzed extension of a primer strand complementary to a template strand in a 5’ to 3’ direction. During this process, a multiprotein complex is responsible for the unwinding of parental DNA and the synthesis of each of the two DNA strands. The impressive speed and processivity with which the replisome duplicates DNA are a result of a set of tightly coordinated interactions between the replication proteins. The process of DNA replication can be considered in three stages: initiation, elongation, and termination, as described below and the main proteins involved in these stages are listed in Table 1.1.

Initiation: The initiation of replication takes place at a particular sequence in the parental DNA duplex, designated the origin, which is recognized by initiator protein (Arthur Kornberg 1992). Once bound to the origin of replication, initiator proteins recruit DNA helicase to the site. DNA helicase protein, often in the form of a circular hexamer, catalyzes duplex DNA unwinding to expose the template strands. The helicase uses ATP to translocate on DNA and destabilizes the hydrogen bonding between base pairs resulting in strand separation (Singleton, Dillingham et al. 2007). Ahead of the unwinding replication fork, topoisomerase activity prevents excess
supercoiling and tangling of DNA (Ishimi, Sugasawa et al. 1992). The single strands of DNA are stabilized by single strand DNA binding proteins that melt the secondary structures in DNA and thus facilitate DNA polymerase activity (Chase and Williams 1986).

**Elongation:** Synthesis of the new DNA strands occurs as a result of collaboration between the synthetic capacities of multiple polymerases. Two types of polymerases are required: primases and replicative DNA polymerases. The primase synthesizes short lengths of RNA or RNA-DNA and provides a free 3’OH primer end to the DNA polymerase for elongation during DNA replication. According to the studies of DNA replication in model organisms such as *E.coli* and in bacteriophage T4, DNA polymerase catalyzes processive DNA elongation simultaneously of both leading and lagging strands (Stukenberg and O'Donnell 1995; Berdis and Benkovic 1998). Despite the large sequence diversity between different DNA polymerases, there is a striking similarity in their structures, as well as catalytic mechanism they employ for DNA synthesis. The central feature of all the known polymerase structures comprises three subdomains, referred to as the fingers, palm, and thumb, that form a partially open right hand-like shape. The fingers subdomain makes contact with the single-stranded template strand; part of the fingers subdomain, along with the palm subdomain, binds the incoming substrate dNTP pairing with the template base, and the thumb subdomain interacts with the newly formed DNA helix (Joyce and Steitz 1994; Baker and Bell 1998). The basic steps in the DNA replication mechanism are: the template nucleotide is displayed for base pairing with the
incoming nucleotide triphosphate in the palm domain; binding of the correct dNTP induces conformational changes in which the fingers rotate towards the palm and the new base pair is positioned correctly in the catalytic site; the nucleotidyl transfer reaction occurs, the fingers relax into the open conformation, and the polymerase moves forward on the template to begin the next reaction cycle (Hingorani and O’Donnell 2004; Garg and Burgers 2005; Beard WA 2006; Showalter AK, Lamarche BJ et al. 2006).

The nucleotidyl transfer reaction proceeds by nucleophilic attack by the 3’ hydroxyl of the primer terminus on the α-phosphate of the incoming dNTP, followed by release of pyrophosphate. Due to the nature of nucleotide incorporation chemistry, DNA polymerases work unidirectionally to extend the primer from 5’ to 3’. And since the two DNA strands are in antiparallel orientation in the double helix, DNA synthesis occurs continuously on the leading strand and discontinuously on the lagging strand in short Okazaki fragments, which are ~ 1000 bp in prokaryotes and ~ 200 bp in eukaryotes (Tsurimoto, Melendy et al. 1990; Chastain, Makhov et al. 2000).

At an incredible speed of about 1000 nucleotides incorporated into DNA per second in *E.coli* and 100 nucleotides per second in eukaryotes during lagging strand synthesis (Tsurimoto, Melendy et al. 1990; Chastain, Makhov et al. 2000), the replicative DNA polymerase has to balance rapid movement on the template with the necessity to remain attached to it. This process is helped by the accessory sliding clamp and clamp loader proteins. The sliding clamp is a ring-shaped protein that encircles DNA and binds to the polymerase, tethering it to DNA throughout
elongation (Stukenberg, Studwell-Vaughan et al. 1991). Then clamp loader is multi-protein complex that assembles the clamp onto DNA in a reaction powered by ATP binding and hydrolysis. Without the clamp and clamp loader, the processivity of DNA polymerase decreases about 1000 fold, from several thousand nucleotide elongated per DNA binding event to only about ten or so nucleotides, hence these proteins are known as processivity factors that are critical for chromosomal DNA replication (O'Donnell and Studwell 1990).

**Termination:** The Okazaki fragments must be processed into a continuous DNA strand by three steps: first, RNA primers are removed by Pol I in prokaryotes and Flap endonuclease 1 (FEN-1) in eukaryotes and archaea. Then the gaps between fragments are processed by polymerase. Last, a ligase closes the nick between consecutive Okazaki fragments, by forming phosphodiester bonds between their 3’-OH and 5’ phosphate groups, to complete DNA replication (Arthur Kornberg 1992).

Along the years, the DNA replication machinery has been studied extensively in the archaeal, bacterial and eukaryotic system. In the next section, the eukaryote DNA replication machinery will be described in greater detail.
Table 1.1  Major components of the replisome in three domains of life

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Eukaryote</th>
<th>Archaea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initiator protein</strong></td>
<td>DnaA</td>
<td>ORC, Cdc6</td>
<td>ORC, Cdc6</td>
</tr>
<tr>
<td><strong>Helicase</strong></td>
<td>DnaB</td>
<td>MCM</td>
<td>MCM</td>
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<tr>
<td>single-strand binding protein</td>
<td>SSB</td>
<td>RPA</td>
<td>SSB</td>
</tr>
<tr>
<td><strong>clamp</strong></td>
<td>β</td>
<td>PCNA</td>
<td>PCNA</td>
</tr>
<tr>
<td><strong>clamp loader</strong></td>
<td>γ complex</td>
<td>RFC</td>
<td>RFC</td>
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<tr>
<td><strong>primer synthesizer</strong></td>
<td>DnaG</td>
<td>Pol primase/α</td>
<td>primase</td>
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<td><strong>Leading strand polymerase</strong></td>
<td>Pol III</td>
<td>Pol ε</td>
<td>Pol B</td>
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<tr>
<td><strong>Lagging strand polymerase</strong></td>
<td>Pol III</td>
<td>Pol δ</td>
<td>Pol D</td>
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<tr>
<td>lagging strand flap removal protein</td>
<td>Pol I</td>
<td>FEN1</td>
<td>FEN1</td>
</tr>
<tr>
<td>lagging strand gap refill protein</td>
<td>Pol I</td>
<td>Pol δ</td>
<td>Pol D</td>
</tr>
<tr>
<td>lagging strand gap seal protein</td>
<td>Ligase I</td>
<td>Ligase I</td>
<td>Ligase I</td>
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</table>
1.2 Eukaryote replication machinery

In eukaryotic cells, around 40 –50 distinct conserved proteins are required for chromosome replication. Here I present a brief overview of current knowledge on the main events involved in eukaryotic DNA replication and the corresponding components at the replication fork (Fig. 1.1 and 1.2).

**Initiation:** Eukaryotic chromosomal replication starts at various origins in the genome (MacAlpine DM 2005). Origins are bound by the origin recognition complex (ORC), a widely conserved six-subunit protein complex (Bell and Stillman 1992). During the G1 phase of the cell cycle, ORC is bound by another protein – cell division cycle 6 (Cdc6), which then recruits chromatin licensing and DNA replication factor 1 (Cdt1) to promote DNA unwinding by helicase – mini chromosome maintenance (MCM) loading onto DNA. ORC, Cdc6, Cdt1 and MCM form the pre–replicative complex (pre–RC) which initiates DNA melting at the inception of S-phase (Bielinsky and Gerbi 2001; Bell and Dutta 2002; Mendez and Stillman 2003; Scholefield, Veening et al. 2011). MCM is a ring shaped heterohexameric complex (Mcm 2–7 complex) which forms the core of the replicative helicase – the molecular motor that uses ATP binding and hydrolysis to fuel the unwinding of double-stranded DNA (dsDNA) at the replication fork. Activation of MCM helicase activity to facilitate DNA unwinding is a complicated and highly regulated process that requires MCM to associate with two additional factors, cell division cycle 45 (Cdc45) and GINS, to form the Cdc45–MCM–GINS (CMG) complex (Moyer, Lewis et al. 2006; Ilves, Petojevic et al. 2010; Costa, Ilves et al. 2011). After the dsDNA is unwound by
MCM, the single-stranded DNA (ssDNA) is produced and coated rapidly by replication protein A (RPA) to protect ssDNA and prevent dsDNA formation (Oakley and Patrick 2010).

**Elongation:** Initiation of eukaryotic DNA synthesis in the S phase depends on the primase–Pol α complex, a multi-protein complex endowed with polymerase and primase activity. The primase–Pol α complex assembles the RNA–DNA primers required by DNA synthesis on the lagging and leading strand (Waga and Stillman 1994). During primer synthesis, the primase subunits synthesize de novo an oligomer of 7–12 ribonucleotides in length, which undergoes limited extension with deoxyribonucleotides by Pol α. After RNA–DNA primer is formed, two distinct replicative polymerases, polymerase ε, and polymerase δ, replicate the leading and lagging strands. Current data suggest that pol ε catalyzes leading strand synthesis and Pol δ catalyzes lagging strand synthesis, primarily (Kunkel and Burgers 2008; Nick McElhinny, Gordenin et al. 2008). DNA polymerases tend to dissociate from DNA after synthesizing only a few nucleotides. Therefore, in addition to polymerase there are two key accessory proteins, a circular clamp and a clamp loader complex, that function to keep the polymerase on DNA and increase its processivity. In eukaryotes, the processivity factors of replicative polymerases are Proliferating Cell Nuclear Antigen (PCNA clamp) and Replication Factor C (RFC clamp loader complex). PCNA is loaded on to the primer–template junction by RFC and used as a tether to hold polymerase during DNA synthesis (Tsurimoto and Stillman 1991). In the next section, the general structure and function features of clamps and clamp loaders,
specifically of *S. cerevisiae* clamp PCNA and clamp loader RFC will be described in greater detail.

**Termination:** Unlike leading-strand DNA, the lagging-strand DNA is synthesized discontinuously as the replication fork moves in the opposite direction to the polymerase. At the end of an Okasaki fragment, a flap of the RNA–DNA primer is created by displacement synthesis by Pol δ at the junction between an old and a new Okazaki fragment. FEN-1, which is a 5’ to 3’ exonuclease, is responsible for removing the flap to create a gap (Kao, Veeraraghavan et al. 2004). Pol δ fills the gap, leaving a nick which is sealed by DNA ligase I (Fig. 1.1).
Figure 1.1 Major steps occurring during eukaryotic DNA replication. (A), duplex DNA is unwound by MCM helicase and new DNA is replicated on both strands, separately. (B), DNA synthesis on leading and lagging strand in detail. Both strands start from the RNA and DNA primer synthesized by primase–pol α and then are extended by pol δ. While leading strand DNA is synthesized continuously, lagging strand DNA is synthesized discontinuously as the replication fork moves in the opposite direction to the polymerase. To form a continuous piece of DNA on lagging strand, the RNA primer, and possibly the DNA laid down by pol α, must be removed by FEN1, with DNA being subsequently ligated to seal the nick and complete Okazaki fragment maturation.
Figure 1.1 Major steps occurring during eukaryotic DNA replication

A

MCM helicase
unwinding double strand

5’
3’
5’
3’

New DNA synthesis on both strands

3’
5’
3’
5’

leading strand

5’
3’

lagging strand

5’
3’

B

DNA synthesis on leading strand

5’
3’
5’
3’

5’
3’
5’
3’

DNA synthesis on lagging strand

5’
3’
5’
3’

previous
Okazaki fragment

Primer
RNA
5’ = = = 3’
3’

Template
5’

Primase for RNA primer

Polδ
DNA
3’

Polα
DNA
3’

Polδ
DNA
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Figure 1.2 Main components of eukaryotic DNA replisome proteins at replication fork. The main proteins in replication fork are shown in this diagram:

MCM helicase (orange) unwind the duplex DNA which is followed by stabilization and protection of the single-stranded DNA product by RPA (dark blue); primers are synthesized by the primase and Pol α (green); synthesis of the complementary strand is carried out by Pol ε DNA polymerases (light blue) in leading strand and Pol δ DNA polymerases (purple) in lagging strand, accompanied by the processivity factors – a circular sliding clamp PCNA (yellow), which is loaded on DNA in an ATP-fueled reaction by clamp loader RFC (RFC-1:red; RFC-4: green; RFC-3: dark blue; RFC-2: purple RFC-5: light blue).
Figure 1.2 Main components of eukaryotic DNA replisome proteins at replication fork
1.3 Clamps and clamp loaders processivity factors in DNA replication

In all cases examined thus far, replicative DNA polymerases by themselves tend to dissociate from DNA after incorporating 10-30 nucleotides with the growing polymer. The processivity of thousands of nucleotides incorporated into DNA by polymerase without dissociation from the primer-template (Arthur Kornberg 1992), is achieved by attachment of the polymerase to a ring-shaped “sliding clamp” protein. This clamp encircles duplex DNA within its central cavity, allowing the polymerase to move rapidly along DNA while remaining topologically bound to it (Stukenberg, Studwell-Vaughan et al. 1991). Assembly of the clamp on DNA requires another accessory multi-protein complex, the clamp loader, which can catalyze loading of the circular clamps onto primed sites on template DNA using ATP.

Through the entire spectrum of organisms that utilize sliding clamps, the overall structure of these proteins is remarkably conserved, despite very low sequence similarity between them. Crystallographic studies of sliding clamps from different organisms reveal that clamps comprise two or three identical subunits which are arranged in head-to-tail fashion to form a ring with asymmetrical front and back “faces”, and a diameter large enough to encircle duplex DNA (Fig. 1.3). Despite the difference in oligomeric state, they all contain six domains, and each domain has a similar chain folding pattern (Kong, Onrust et al. 1992; Krishna, Fenyo et al. 1994; Moarefi, Jeruzalmi et al. 2000; Matsumiya, Ishino et al. 2001). Many clamp-binding proteins interact with hydrophobic pockets on the front face, and recent data indicate
that a clamp can bind more than one protein at the same time (Querol-Audi, Yan et al. 2012). In addition to their processivity function in DNA elongation, clamps participate in other DNA processes including Okazaki fragment processing, DNA repair reactions, as well as in cellular processes such as cell cycle regulation, cell cycle arrest, and commitment to apoptosis (Warbrick; Paunesku, Mittal et al. 2001; Indiani, McInerney et al. 2005). Thus, clamps serve as mobile platforms on DNA for a wide variety of proteins and enzymes that work on DNA.

The interior of the clamp protein is positively charged, and until recently it was assumed that DNA passes straight through the clamp without any interactions with specific residues. However, MD simulations of human PCNA with DNA (Fig. 1.4A) (Ivanov, Chapados et al. 2006), crystal structure of E. coli β bound to DNA (Fig. 1.4B) (Georgescu, Kim et al. 2008), and EM single particle analysis of P. furiosus PCNA with DNA ligase and DNA (Mayanagi, Kiyonari et al. 2009), indicate that DNA passes through the clamp at a pronounced angle. These findings suggest interactions between the DNA phosphodiester backbone and positively charged residues lining the inside of the clamp, which may play a role in clamp function. For example, it has been proposed that tilting of DNA may favor one protein binding the clamp over another, thus facilitating ordered switching between two or more proteins that act on DNA (Georgescu, Kim et al. 2008; Mayanagi, Kiyonari et al. 2009). It is also possible that the contacts modulate the rate of diffusion of clamps along DNA and thus influence clamp location and function on DNA (Laurence, Kwon et al. 2008).
Figure 1.3 Circular sliding clamps from various organisms. The crystal structures of several sliding clamp proteins reveal a circular six α/β fold arrangement with a remarkably similar three-dimensional overall fold. The sliding clamp proteins are almost always composed of three subunits (bacteriophage T4, bacteriophage RB69, S. cerevisiae PCNA and human PCNA) or two identical subunits (E. coli) (image adapted from (Hingorani and O'Donnell 2000)).
Figure 1.4 Interaction between clamp and DNA. MD simulation of human PCNA bound to duplex DNA complex (A) and crystal structure of *E.coli* β and primer-template DNA complex (B) reveal the relative tilted relationship between clamp and DNA, and indicate interactions between the nucleic acid backbone and the inner positively charged surface of the sliding clamp (image adapted from (Ivanov, Chapados et al. 2006; Georgescu, Kim et al. 2008)).
Clamps are loaded onto primer-template DNA by clamp loaders, which are multi-subunit protein complexes that bind a clamp and DNA and form a topological link between the two in a reaction using ATP. Clamp loader subunits belong to the AAA+ family of proteins (ATPases Associated with a variety of cellular Activities). Unlike other AAA+ ATPases, which are typically hexameric, all clamp loaders are composed of five subunits in a circular arrangement, with a gap between the first and the fifth subunit, at the position of the missing sixth subunit (Fig. 1.6). Each subunit has three domains: (1) C-terminal domain that interacts with other subunits in the complex to form a closed ring at the top of the complex and (2) two N-terminal domains that form an ATPase module for ATP binding and ATP hydrolysis. The module contain the P loop, also known as Walker A motif for ATP binding, Walker B motif for Mg\(^{2+}\) binding, the ATP binding sensor in the form of an arginine finger (SRC motif) helps stabilize the transition state, and the catalytic glutamate residue (Yao and O'Donnell 2012). The ATP sites are located at subunit interfaces, with the ATP binding pocket from one subunit and the SRC motif from the adjacent subunit. The subunits form a claw shaped structure with an N-terminal base for binding the clamp and a central chamber for binding DNA. Even though all clamp loaders have five essential subunits, the protein stoichiometry is different in clamp loaders from different branches of life. Three major variants are listed below:

(1) The bacterial clamp loader is formed from three essential subunits (δ, δ', and the τ /γ proteins), and two accessory subunits (χ and ψ proteins) that are not necessary for the clamp loading process. The crystal structure of *E. coli* γδδ’
complex was solved in 2001 (Fig. 1.6 A) (Jeruzalmi, O'Donnell et al. 2001). It reveals the five subunits of *E. coli* $\gamma_3\delta_3'$ complex arranged in a circular fashion with a partial gap between $\delta$ and $\delta'$. The three $\gamma$ subunits are adjacent to one another, flanked on one side by $\delta$ and the other by $\delta'$. The “claw shaped” complex is held together through inter-subunit interactions among the C-terminal domains, which form a closed collar, while the N-terminal domains are open in a C-shape resembling a typical AAA+ family hexamer lacking one subunit. Crystallography and biochemical studies of $\gamma$ complex have shown that one ATP molecule binds at $\delta'/\gamma$ interface, and two more bind at the two $\gamma/\gamma$ interfaces (Xiao, Naktinis et al. 1995). Since the three $\gamma$ subunits are the only subunits that bind ATP, they have been termed the “motor” of the complex. The $\delta$ subunit is called the “wrench” because it is the main $\beta$ clamp-interacting subunit, and it can open the dimer interface by itself. $\delta'$ has been termed the 'stator'. A stator is the stationary part of a machine against which other parts move. (Jeruzalmi, O'Donnell et al. 2001).

(2) The bacteriophage and archaeal clamp loaders are both composed of two proteins each with one unique protein occupying the A position (gp62 in T4 bacteriophage and RFC-l in archaea) and identical ATPase subunits at the B, C, D and E positions (the gp44 protein in T4 bacteriophage and RFC-s in archaea) (Fig. 1.6C) (Jarvis, Paul et al. 1989; Cann and Ishino 1999; Miyata, Suzuki et al. 2005; Kelch, Makino et al. 2011). While the archaeal clamp loader contains an active ATPase at the A position (Seybert and Wigley 2004), the A subunit in the T4 bacteriophage clamp loader does not have an AAA+ fold (Kelch, Makino et al. 2011).
Both E subunits from archaeal and T4 bacteriophage are not capable of ATP hydrolysis due to lack of an SRC motif contribution from A subunits.

(3) Eukaryotic clamp loaders are made up of five different subunits. The first eukaryotic clamp loader, human RFC, was isolated in 1988, initially identified as a required component of the in vitro SV40 genome replication system (Fairman, Prelich et al. 1988). Electron microscopy studies of human RFC in the presence of ATPγS, reported in 2000, showed the five subunits arranged in a ring with a wider opening on the N-terminal side of the complex (Shiomi, Usukura et al. 2000). The crystal structure of S. cerevisiae RFC-PCNA in the presence of ATPγS confirmed the ring-like arrangement of the C-terminal domains of RFC subunits and the open ring arrangement of the N-terminal domains (Fig. 1.6B) (Bowman, O'Donnell et al. 2004). In the next section, we will describe the S. cerevisiae RFC clamp loader and its partner, the PCNA clamp, in details.

The clamp assembly mechanism appears to be broadly conserved through evolution and involves multiple steps. ATP binding to the clamp loader, which enables it to bind and open the clamp, resulting in a clamp loader•ATP•clamp\textsubscript{open} intermediate ready for entry of DNA into the clamp. DNA binding commits the clamp loader to ATP hydrolysis, followed by clamp closure and clamp•DNA release from complex (Williams, Snyder et al. 2004; Kelch, Makino et al. 2012; Sakato, Zhou et al. 2012).
Figure 1.6 Composition of clamp loaders from the different branches of life. (A), bacterial clamp loaders consist of three different proteins: δ, δ', and γ protein. The δ protein is at the A position, three copies of the ATPase subunits γ are at the B, C and D positions and δ' protein at the E position. (B), eukaryotic clamp loaders consist of five different proteins, RFC-1 through RFC-5. RFC-1 lies at the A position, RFC-4 at B, RFC-3 at C, RFC-2 at D and RFC-5 at the E position. Eukaryotic A subunits contain an additional domain A' that bridges the gap between the A and E positions. (C), bacteriophage clamp loaders consist of two different proteins. The gp62 protein, which lacks an AAA+ module but has an A' domain similar to that of eukaryotic clamp loaders, lies at the A position. The ATPase gp44 protein lies at the B, C, D, and E positions. Archaeal clamp loaders have a similar composition (image adapted from (Kelch, Makino et al. 2012)).
Figure 1.6 Composition of clamp loaders from the different branches of life

A. *E. coli* clamp loader

B. *S. cerevisiae* clamp loader

C. T4 clamp loader
1.4 S. cerevisiae PCNA clamp and RFC clamp loader

*S. cerevisiae* PCNA, is composed of three monomers and each monomer has two β-α-β-β-β domains, referred to as N and C terminal domain, that are connected through a long, flexible linker called the interdomain connector loop (IDCL). The three monomers assemble in a head to tail manner with N terminal domain of one monomer interacting with C terminal domain of an adjacent monomer to form a ring with distinct front and back faces (Fig. 1.7 A and B) (Krishna, Kong et al. 1994).

PCNA was originally characterized as a DNA sliding clamp for replicative DNA polymerases and as an essential component of the eukaryotic chromosomal DNA replisome. Subsequent studies, however, have revealed its striking ability to interact with multiple proteins that are involved in several metabolic pathways, including Okazaki fragment processing, DNA repair, translesion DNA synthesis, DNA methylation, chromatin remodeling and cell cycle regulation (Maga and Hubscher 2003). Both faces of PCNA are involved in protein-protein interactions. The front face of PCNA, also named as C terminal face, contains the IDCL and a hydrophobic pocket that interacts with the PCNA-binding motif (PIP box) found in many PCNA-binding proteins (Hingorani and O'Donnell 2000; Maga and Hubscher 2003), such as DNA ligase I, Fen1, P21 and polymerase δ. The structure of PCNA bound to clamp loader RFC is shown as an example in Fig. 1.7C. The canonical PIP motif contains eight amino acid residues: Q-xx-(h)-x-x-(a)-(a), where (h) represents residues with moderately hydrophobic side chains (e.g. L, I, M), (a) represents residues with highly hydrophobic, aromatic side chains (e.g. F, Y) and (x) is any
residue (Warbrick 2006). The role of the back face (N terminal face) of PCNA is currently less clear. The back face is emerging as a site of PCNA post-translational modification such as mono-ubiquitylation, poly-ubiquitylation, and SUMOylation (Hoege, Pfander et al. 2002; Stelter and Ulrich 2003; Papouli, Chen et al. 2005).

Structure of PCNA bound to SUMO is shown as an example in Fig. 1.7D (Freudenthal, Brogie et al. 2011). These post-translational modifications are thought to recruit protein factors to replication forks and help hold them in reserve until they are needed on the front face of PCNA (Freudenthal, Brogie et al. 2011).

The PCNA ring has a diameter of about 80 Å. The central hole in the ring has a diameter of about 35 Å. While the overall electrostatic potential of PCNA is negative, the inner surface is positively charged due to the presence of lysine and arginine residues on the α helices lining the inside of the ring. These localized positive charges facilitate the passage of the negatively charged DNA through the central hole. In 2006, an MD analysis of human PCNA•DNA complex identified dynamic interactions occurring asymmetrically between DNA and conserved arginine/lysine residues inside the ring; note: human PCNA shares high sequence and structure identity as S. cerevisiae PCNA (Ivanov, Chapados et al. 2006). These contacts positioned DNA closer to two subunits of the PCNA homotrimer and tilted it by 20°. This finding is consistent with the observation from a crystal structure of E. coli β-DNA complex solved in 2008 that DNA is titled at 22° as it passes through the β ring (Georgescu, Kim et al. 2008). In 2010, a model of S. cerevisiae PCNA bound to DNA derived from X-ray diffraction data revealed an sharpen tilt angle at ~ 40°.
(McNally, Bowman et al. 2010). A more recent EM and MD analysis of human FEN1•PCNA•DNA complex showed that DNA passes through the clamp at a 17° and into the FEN1 active site (Querol-Audi, Yan et al. 2012). In this structure also, the duplex interacts asymmetrically with human PCNA. Based on these data, it has been proposed that this specific yet flexible positioning and orientation of the clamp on DNA may enable it to specifically position and stimulate the catalytic activities of different proteins partners on DNA. In the chapter II, I will describe my study on how the contacts between *S. cerevisiae* PCNA and DNA play the important role in the clamp loading pathway.
Figure 1.7 Structure of *S. cerevisiae* clamp PCNA alone and in complex with protein partners. (A) and (B), PCNA trimer is shown from the front (A) and the side (B) with individual PCNA subunits colored red, yellow and blue. The IDCL, back face and front face, which are found to interact with various proteins, are indicated. (C), structure of PCNA bound to RFC. RFC-A (red) (rest of the RFC subunits are shown in grey) binds the hydrophobic pocket and IDCL of PCNA on the front face though the PIP box. The hydrophobic residues in the PIP box are shown in green (Bowman, O'Donnell et al. 2004). (D), structure of PCNA bound to SUMO. SUMO (magenta) binds PCNA on the back face (Freudenthal, Brogie et al. 2011).
Figure 1.7 Structure of \textit{S. cerevisiae} clamp PCNA alone and in complex with protein partners

A. Front view of PCNA

B. Side view of PCNA

C. Complex of PCNA and RFC

D. Complex of PCNA and SUMO
PCNA is quite stable in its closed form, with a half-life of about 24 min on DNA (Yao, Turner et al. 1996), therefore a clamp loader is required to open the ring at the interface and assemble it around DNA. In 1992, the \textit{S. cerevisiae} Replication Factor C protein complex (RFC) was identified as a clamp loader because of its primer-template DNA binding activity, DNA stimulated ATPase activity, and its ability to stimulate the activity of DNA polymerase (Fien and Stillman 1992). RFC is made up of five different subunits, RFC-A, RFC-B, RFC-C, RFC-D, and RFC-E (also named as RFC-1, RFC-4, RFC-3, RFC-2, and RFC-5, respectively). With the exception of the large RFC-A subunit about 95 kDa, the RFC-B, RFC-C, RFC-D, and RFC-E subunits are approximately similar size at ~ 37 - 40 kDa.

Each subunit of RFC has three domains (domains I-III) (Fig. 1.8A, RFC-D is shown as an example). The N-terminal domains I and II of are AAA+ ATPase modules. The C-terminal domains III pack tightly as a collar region to hold the pentamer complex together (Fig. 1.8B) (Bowman, O'Donnell et al. 2004). In addition to these three domains, RFC-A has the additional domains at both the N and C terminal. The removal of the N-terminal domain (residues 1–275 in \textit{S. cerevisiae}) of RFC-A does not significantly affect clamp loading activity compared to the full-length wild-type protein, therefore, this domain is not considered essential for clamp loading (Uhlmann, Cai et al. 1997). It should be noted that pre-steady state analysis of RFC ATPase activity suggests that the N-terminal truncated RFC complex is not fully active compared full length RFC (Sakato, Zhou et al. 2012). The C-terminal domain (residues 660–861 in \textit{S. cerevisiae}) has not yet been characterized genetically or
biochemically. RFC-A does not have an SRC motif. The Walker B motif in RFC-E is
degenerated. RFC-B, RFC-C, and RFC-D have intact SRC motifs, Walker A and
Walker B motifs therefore, the RFC complex has four catalytic ATPase sites located
at the RFC-E/D (D site), RFC-D/C (C site), RFC-C/B (B site), and RFC-B/A (A site)
subunit interfaces (Fig. 1.8C, RFC-C site is shown as an example). From a recent
RFC mutational study in our laboratory, it was found that the central subunit RFC-C
serves as a critical swivel point in the clamp loader which controls conformational
changes that result in PCNA opening and loading on DNA, and that ATP hydrolysis
by RFC-A subunit is not essential for PCNA clamp loading (Sakato, O'Donnell et al.
2012).
**Figure 1.8 Structure of *S. cerevisiae* clamp loader RFC.** (A), three-domain architecture of RFC-D. (B), five subunits of RFC complex (color code: RFC-A, red; RFC-B, green; RFC-C, blue; RFC-D purple; RFC-E cyan). (C), ATPase site in the RFC-D: RFC-C interface (C site). ATPγS is shown in orange. P loop (Walker A motif), catalytic glutamate in RFC-C, and arginine finger from RFC-D are indicated in green (Bowman, O'Donnell et al. 2004).

A. Domain I-III from RFC-D domain

B. RFC five subunits complex

C. ATPase site in RFC-D:RFC-C interface (C site)
Our laboratory recently reported pre-steady state measurements of several steps in the reaction catalyzed by *S. cerevisiae* RFC and presented a comprehensive kinetic model based on global analysis of the data (Fig. 1.9). Highlights of the reaction mechanism are: (1) ATP binding to RFC initiates slow activation of the clamp loader, enabling it to open PCNA (at 2 s\(^{-1}\)) and bind ptDNA. (2) Rapid binding of ptDNA accelerates formation of the RFC•ATP•PCNA\textsubscript{open}•ptDNA complex, which catalyzes a burst of ATP hydrolysis (at ~25-45 s\(^{-1}\)). (3) Another slow step in the reaction follows ATP hydrolysis and is associated with PCNA closure around ptDNA (8 s\(^{-1}\)). Dissociation of PCNA•ptDNA and Pi release from RFC leads to catalytic turnover; note: ADP release rate has not yet been measured, hence this step is not included in the model (Sakato, Zhou et al. 2012).

The RFC•ATP\(\gamma\)S•PCNA crystal structure (Fig. 1.6B) shows that the subunits of RFC, are arranged in a right handed, spiral shape in this complex. In the structure, the clamp is closed and in contact with the base of the RFC-A, RFC-B, and RFC-C subunits, while RFC-D and RFC-E are suspended above the plane of the clamp. Based on this structure, it was proposed that the closed-PCNA ring bound by RFC was in a stable conformation, and the clamp would open just before ATP hydrolysis and complex dissociation (Bowman, O'Donnell et al. 2004). It is also possible the closed PCNA may be due to crystal packing forces, instability of an open-ring complex, or the arginine to glutamine mutation in the four SRC motifs inhibit PCNA opening. In a later MD-derived model, the open clamp is in an out-of-plane right-handed spiral conformation and in contact with all RFC subunits,
including RFC-D and RFC-E, which are in a matching spiral conformation (Tainer, McCammon et al. 2010). Based on kinetic studies in our laboratory and the MD structure, it was proposed that as RFC binds ATP it begins transition to conformation active and the interaction with PCNA accelerates cooperative structural changes in both proteins that result in additional ATP binding and stabilization of the complex in a spiral, open-clamp conformation.

Currently, there is no crystal structure available of an RFC, PCNA with DNA and nucleotide complex. However, since clamp loaders and clamps are all conserved in all domains of the life, the structures of *E. coli* γ complex•DNA•ADPBeF$_3$ complex and bacteriophage T4 gp44/62•ADPBeF$_3$•gp45•ptDNA complex (Fig. 1.6C) provide structure information of important clues about structural transitions in RFC leading to a PCNA, ATP and ptDNA-bound complex on the brink of ATP hydrolysis (Simonetta, Kazmirski et al. 2009; Kelch, Makino et al. 2011). In both *E. coli* and T4 complex, the ATPase modules of the clamp loaders are in a highly symmetric helical arrangement such that the inside of the protein chamber complements the geometry of the double helix, and ATPase sites are catalytically competent, with the arginine finger poised to stimulate ATP hydrolysis (coordinating BeF$_3$ in this case). In the RFC•ATPγS•PCNA$_{closed}$ structure discussed earlier, only RFC-A and RFC-C ATP-binding sites appeared near catalytic competency, whereas the nucleotides in RFC-B and RFC-D sites were loosely coordinated. Thus, it appears that binding of ptDNA in the central chamber
drives further adjustment of inter-subunit contacts, bringing all catalytic sites on line for ATP hydrolysis.

Based on the structure data and our kinetic model described above, the following changes in protein conformation during the clamp loading reaction which appear significant were proposed: (1) Nucleotide-free RFC exists in a relaxed conformation with relatively weak inter-subunit interactions between the AAA+ ATPase modules. ATP binding causes the first allosteric transition that result in stronger intersubunit interactions and ordering around a central helical axis (Bowman, O'Donnell et al. 2004). (2) This structural rearrangement causes RFC-A, RFC-B, and RFC-C subunits to present a favorable surface for electrostatic interactions with PCNA. (3) Contact between PCNA and RFC is accompanied by another transition, in which twisting of antiparallel β-strands at a PCNA intersubunit interface (Adelman, Chodera et al. 2010; Tainer, McCammon et al. 2010) and corresponding adjustment of RFC stabilize the complex into a spiral with open PCNA. RFC in complex with PCNA can bind additional ATP and both proteins present a positively charged surface to match the helical pitch of double-stranded DNA. (4) Electrostatic interactions between RFC and DNA drive another transition, in which all the interfacial nucleotide-binding sites tighten coordination of ATP and catalytic residues are set for hydrolysis (Chen, Levin et al. 2009). (5) ATP hydrolysis initiates the next cascade of conformational changes that alter the network of contacts between RFC subunits, between RFC and ptDNA, and between RFC and PCNA, allowing clamp closure around ptDNA. (7) Release of ATP hydrolysis products resets RFC to a nucleotide-
free conformation with lower affinity for PCNA and ptDNA. The sequence of conformational changes noted above, highlighted by ATP-binding-led stabilization of the RFC•PCNA<sub>open</sub> complex and ATP-hydrolysis-led destabilization of the RFC•PCNA<sub>open</sub>•ptDNA complex, provides a basic mechanistic explanation of how ATPase activity drives clamp loading onto DNA. There are still several questions remaining about mechanistic details of the clamp loading reaction. In the next two chapters I describe my research on the role of PCNA-DNA and RFC-DNA contacts in the clamp loading mechanism.
Figure 1.9 A model pathway for RFC-catalyzed PCNA loading on ptDNA. The schematic depicts key steps in the clamp loading reaction determined thus far. (I) Binding of two to three ATP molecules to RFC is followed by (II) RFC interaction with PCNA, additional binding of one to two ATP molecules (Chen, Levin et al. 2009), and slow activation steps involving conformational changes in RFC and PCNA that (III) lead to a stable open-clamp complex. (IV) ptDNA binds rapidly and with high affinity to activated RFC•ATP•PCNA complexes, (V) triggering ATP hydrolysis. (VI) Next, another slow step involving conformational changes in RFC and PCNA leads to closure of the clamp around ptDNA, (VII) release of products (P_i, PCNA•ptDNA), and catalytic turnover (ADP release has not been measured thus far). Slow steps are indicated by orange arrows. Proposed partitioning of PCNA between loading-active and loading-inactive forms is also shown (Tainer, McCammon et al. 2010).
**Rationale and outline for PhD dissertation research**

The processivity factor for DNA replication, a circular sliding clamp, is assembled around a primed DNA template by a clamp loader complex in a reaction fueled by ATP binding and hydrolysis. The utilization of clamp and clamp loader accessory proteins to increase the processivity of the DNA polymerases occurs in all living organisms and is essential for DNA replication. My interest has been in *S. cerevisiae* clamp PCNA, clamp loader RFC and in understanding how they work on DNA. The main goals of my research were to:

1. Investigate the role of individual PCNA-DNA contacts in the clamp loading mechanism by transient kinetics coupled with fluorescence spectroscopy and kinetic modeling, in order to determine how PCNA-DNA contacts regulate and coordinate the functions of its partner clamp loader RFC and influence its function on DNA.

2. Investigate the role of individual RFC-DNA contacts in the clamp loading mechanism, specifically to understand how DNA binding to RFC triggers rapid ATP hydrolysis.
Chapter II  Impact of Individual PCNA-DNA Contacts on Clamp Loading and Function on DNA

2.1 Introduction

Sliding clamps encircle DNA and are best known for making DNA replication processive by limiting polymerase dissociation from the primer template junction (Johnson and O'Donnell 2005). Clamps also tether and coordinate exchanges of many other proteins at different sites on DNA, including proteins responsible for DNA replication, repair, recombination, and cell cycle control; for example, PCNA helps coordinate switching of replicative and translesion bypass polymerases at sites of DNA damage (Maga and Hubscher 2003; Moldovan, Pfander et al. 2007). All clamps have the same basic structure with six $\alpha/\beta$ domains distributed across two or three subunits in a planar ring (e.g., *E.coli* $\beta$ and human PCNA, respectively) (Hingorani and O'Donnell 2000). The $\beta$ sheets line the outer rim and form interfaces between subunits; the $\alpha$ helices line the inner rim and present positively charged residues to complement the negatively charged DNA backbone (Fig. 2.1D). The clamp is loaded onto DNA by the clamp loader, a five-subunit complex of AAA+ family proteins that couple ATP binding and hydrolysis to mechanical work (Jeruzalmi, O'Donnell et al. 2001; Bowman, O'Donnell et al. 2004; Iyer, Leipe et al. 2004; Neuwald 2005; Kelch, Makino et al. 2011). Structural and mechanistic analyses of clamp loaders such as *E. coli* $\gamma$ complex (Jeruzalmi, O'Donnell et al. 2001; Bloom...
2006), bacteriophage T4 gp44/62 (Pietroni and von Hippel 2008; Kelch, Makino et al. 2011), and \textit{S. cerevisiae} RFC (Bowman, O'Donnell et al. 2004; Zhuang, Yoder et al. 2006; Chen, Levin et al. 2009; Sakato, Zhou et al. 2012) among others, have shown that ATP binding enables the clamp loader to bind and open the clamp and bind ptDNA, and ATP hydrolysis leads to the release of the clamp-ptDNA product (Fig. 2.1A), which can then be used by DNA polymerase and other proteins. In recent pre-steady state kinetic studies of \textit{S. cerevisiae} RFC and PCNA, we explicitly measured the rates of individual steps in the reaction, including PCNA opening/closing, ptDNA binding/release, ATP hydrolysis, and phosphate (P$_i$) release (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012). The data revealed a pair of slow events that bookend the loading reaction: the first occurs after ATP binding to RFC (RFC activation) and involves conformational changes that enable PCNA opening and ptDNA binding to form an RFC•ATP•PCNA$_{\text{open}}$•ptDNA intermediate primed for ATP hydrolysis; the second occurs after ATP hydrolysis (RFC deactivation) and involves conformational changes that enable PCNA closure and release of the PCNA-ptDNA product.

On ATP binding, the AAA+ modules in the clamp loader subunits begin rearranging toward a right-handed spiral conformation (Bowman, O'Donnell et al. 2004; Sakato, Zhou et al. 2012). PCNA accelerates this activation process (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012) as it interacts with the base of RFC and is held open at one subunit interface (Fig. 2.1B, a molecular dynamics generated model of \textit{S. cerevisiae} RFC bound to open PCNA) (Tainer, McCammon et al. 2010). In the clamp loader•ATP•clamp$_{\text{open}}$ complex, the protein subunits are twisted in a spiral such
that the inner surface of the chamber complements the double helix geometry (Fig. 2.1C, bacteriophage T4 gp44/62•ADP•BeF₃•gp45•ptDNA complex (Kelch, Makino et al. 2011). Structural data indicate that the clamp does not open wide enough to allow direct entry of the duplex portion of ptDNA (Zhuang, Yoder et al. 2006; Kelch, Makino et al. 2011). Kinetic data indicate that short linear ptDNA can bind closed or partially open RFC•ATP•PCNA complex and stimulate PCNA opening (Miyata, Suzuki et al. 2005; Tainer, McCammon et al. 2010; Sakato, Zhou et al. 2012). These findings support a model in which the single-stranded template enters through a small gap between the clamp subunits first, and then ptDNA threads upward through the full protein complex. Cationic residues in both the clamp loader and clamp present sites for ionic interactions with the duplex, and the template exits near the top through a gap between the clamp loader subunits (Simonetta, Kazmirski et al. 2009; Kelch, Makino et al. 2011). ATP binding to at least the three central AAA+ modules is necessary for rapid binding of ptDNA (Sakato, O'Donnell et al. 2012). In turn, interaction with duplex DNA appears essential for the modules to achieve the final symmetric spiral conformation set for ATP hydrolysis (Simonetta, Kazmirski et al. 2009; Kelch, Makino et al. 2011). Consistent with these structural features, kinetic data show that ATP binding to RFC initiates and PCNA binding accelerates formation of the spiral complex, but only DNA binding (ptDNA or linear duplex) triggers a rapid burst of ATP hydrolysis (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012). Primer-template DNA has the same effect on E. coli γ complex (Williams, Snyder et al. 2004). ATP hydrolysis is followed by clamp closure and clamp-ptDNA,
ADP, and Pi release (Williams, Snyder et al. 2004; Sakato, Zhou et al. 2012). The exact nature and sequence of all the events following ATP hydrolysis remain under investigation.

Although ATP binding and hydrolysis initiate the assembly and disassembly phases of the reaction, respectively, interactions with ptDNA accelerate key steps: PCNA opening, ATP hydrolysis, and subsequent product release. Conserved cationic residues inside the *S. cerevisiae* RFC and *E. coli* γ complex that contact DNA are known to be essential for clamp loading (Goedken, Kazmirska et al. 2005; Yao, Johnson et al. 2006). Many conserved cationic residues line the inner rim of clamps as well (e.g., 9 arginines and lysines per human or *S. cerevisiae* PCNA monomer, i.e., 27 per clamp; Fig. 2.1D), but any functional significance of these potential contacts with DNA is less well understood. In the crystal structure of *E. coli* β•ptDNA complex, the duplex is tilted within the ring and the backbone interacts with specific residues (Georgescu, Kim et al. 2008). Mutation of R24 and Q149 disrupts loading of β onto ptDNA, indicating that these direct interactions can influence clamp function. MD analysis and a crystal structure of the *S. cerevisiae* PCNA•ptDNA complex also indicate that the duplex is tilted and contacts specific residues in the ring (Ivanov, Chapados et al. 2006; McNally, Bowman et al. 2010). Individually mutating the arginines and lysines in human PCNA to alanine showed that each one of the nine is required for processive DNA replication by polymerase δ (Fukuda, Morioka et al. 1995). The mutations suppressed initiation of processive DNA synthesis, implying a defect in PCNA loading by RFC. However, the mutations apparently had little effect
on stimulation of RFC•PCNA ATPase activity by ptDNA, leaving open the question of how loss of particular PCNA-DNA contacts affects clamp loading and function on DNA (Fukuda, Morioka et al. 1995). A more recent study of the same mutants in *S. cerevisiae* PCNA (Fig. 2.1D) also detected no significant reduction in ptDNA-stimulated RFC ATPase activity; indeed the rate was only 2-fold lower with a quadruple mutant than with wild type PCNA (K20A/K77A/R80A/R149A—12 of 27 arginines/lysines in the ring eliminated (McNally, Bowman et al. 2010). Thus the earlier finding that loss of even one cationic residue per PCNA monomer ablates processive DNA replication remained unexplained (Fukuda, Morioka et al. 1995).

The effect of ptDNA on the steady state RFC ATPase rate ($k_{cat}$) is not an appropriate measure of the potential effects of DNA binding mutations in PCNA. A major reason is that the effect of ptDNA on $k_{cat}$ is similar in the presence or absence of PCNA, rendering any effect of PCNA mutations undetectable ($k_{cat}$ for RFC = 0.02 s$^{-1}$, RFC•PCNA = 0.05 s$^{-1}$, RFC•ptDNA = 0.7 s$^{-1}$, RFC•PCNA•ptDNA = 0.8 s$^{-1}$). In contrast, stark differences are revealed in the ATPase mechanism with and without PCNA under pre-steady state conditions (Chen, Levin et al. 2009). Another reason is that rate-limiting steps that determine the $k_{cat}$ are not fully known for any clamp loader. Clamp opening and closure are two known slow events (Sakato, Zhou et al. 2012), but the kinetics of ADP release are unknown. Thus, the $k_{cat}$ cannot reveal which steps, if any, in the clamp loading mechanism are altered by disruption of PCNA contacts with DNA. It has been speculated that clamp-DNA interactions may facilitate positioning of ptDNA inside the clamp loader•clamp complex, induce clamp
closure around ptDNA, and possibly influence clamp interactions with target proteins (and protein switching) by altering the relative location and tilt angle of DNA within the ring (Georgescu, Kim et al. 2008; Querol-Audi, Yan et al. 2012). Given the potential functional importance of DNA binding residues within clamps, we examined six single arginine/lysine mutants of *S. cerevisiae* PCNA for effects on individual events constituting the clamp loading reaction. The results demonstrate that the loss of even one cationic residue can alter DNA binding-linked steps in the reaction mechanism as well as movement of PCNA on DNA.
Figure 2.1 Clamp loading reaction, intermediate complex structures, and potential contacts between PCNA and DNA. (A), minimal clamp loading pathway showing the reactants, a key RFC•PCNA<sub>open</sub>•ptDNA intermediate formed after ATP binding, and the products, including topologically linked PCNA-ptDNA. (B), MD model of *S. cerevisiae* RFC bound to ATPγS and open PCNA (Bowman, O'Donnell et al. 2004). (C), crystal structure of bacteriophage T4 gp44/62 clamp loader bound to ADP-BeF<sub>3</sub>, an open gp45 clamp, and ptDNA (Kelch, Makino et al. 2011). (D), positions of the nine arginines and lysines on α helices lining the inside of a PCNA monomer (gray).
Figure 2.1 Clamp loading reaction, intermediate complex structures, and potential contacts between PCNA and DNA
2.2 Materials and Methods

2.2.1 Protein, DNA, and other reagents

PCNA point mutation (R14A, K20A, R80A, K146A, R149A and K217A) was introduced by QuikChange site-directed mutagenesis kit into a PCNA<sub>WC</sub> construct, in which four native cysteines (C22/C30/C62/C81) were replaced with serine and F185W, K107C were made to introduce the FRET pairs as described in (Zhuang, Yoder et al. 2006). The primers designed for generating PCNA point mutations are: R14A, 5’-GAA GAA GCA TCC CTT TTC AAG GCA ATA ATT GAT GGT TTC AAA G-3’ (sense), 5’-CTT TGA AAC CAT CA A TTA TTG CCT TGA AAA GGG ATG CTT CTG C-3’ (antisense); K20A, 5’-GAA TAA TTG ATG GTT TCG CAG ATA GTG TCC AGT TGG-3’ (sense), 5’-CCA ACT GGA CAC TAT CTG CGA AAC CAT CAA TTA TTC-3’ (antisense); R80A, 5’-CCT CAC TAA GTA AAA TCC TAG CTA GTG GTA ACA ACA CCG ATA C-3’ (sense), 5’-GTA TCG FTF TTF TTA CCA CTA GCT AFF ATT TTA CTT AGT GAG G-3’ (antisense); K146A, 5’-CCA TCT GCA ATT GTT CGT GAC TTG TCC C-3’ (sense), 5’-GGG ACA AFT CAC GAA CAA TTF CAG AGA ATT CFF AAG ATG G-3’ (antisense); R149A, 5’-CCG AAT TCT CTA AAA TTG TTG CTG ACT TGT CCC AAT TGA GTG-3’ (sense), 5’-CAA TCA ATT GGG ACA AGT CAG CAA CAA TTT TAG AGA ATT CGG-3’ (antisense); K217A 5’-GCT AAA TAT TTA TTG GAC ATC ATT GCG GGC TCC CTT TCT GAAT AGA-3’ (sense), 5’-CTC TAT CAG AAA AGG AGC CCG CAA TGA TGT CCA ATA AAT ATT TAGC-3’ (antisense). The PCR cycles were set based on the length of the plasmids
and the nature of the mutation: 1 cycle at 95 °C for 1 min; 18 cycles with the following succession: 95 °C for 30 s, at 58 °C for 1 min, at 68 °C for 6 min 30 s; ending with 1 cycle at 68 °C for 8 min and cooling down to 4 °C.

Overexpression, purification of wild type and mutant *S. cerevisiae* PCNA<sub>WC</sub>, *S. cerevisiae* RFC complex, labeling of wild type and mutant *S. cerevisiae* PCNA<sub>WC</sub>, as well as Phosphate-binding protein (PBP), and PBP labeling with MDCC (N-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide, Molecular Probes, Inc) are described in Appendix C.

Short DNAs were synthesized by Integrated DNA Technologies (IDT) and their sequences are as follows: 65-mer (template for ptDNA), 5’-TAG TTA GAA CCT AAG CAT ATT AGT AGC CAT GTG AAT CAG TAT GGT TCC TAT CTG CTG AAG GAA AT-3’; 40-mer (primer for recessed 3’-OH ptDNA junction), 5’-ATT TCC TTC AGC AGA TAG GAA CCA TAC TGA TTC ACA TGG C-3’.

DNAs were purified by 19% (40-mer) or 17% (65-mer) denaturing polyacrylamide gel electrophoresis (Appendix B). Primer-template DNAs (1:1.1 primer to template) were prepared by incubating the two complementary strands (100 mM NaCl, 20 mM Tris-HCl, pH 8.0) at 90 °C in a water bath for 3 min and then allowing the solution to cool slowly to 25 °C over several hours. The DNAs were checked by non-denaturing PAGE to confirm >95% ptDNA prior to use in experiments. TAMRA (5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester) dye, with a succinimidyl ester group that can form a covalent amide bond with either a 3’ or 5’ amine-modified DNA, was used to label DNA for fluorescence-based DNA binding experiments as
described in Sections 2.2.4, Chapter II. TAMRA-40-mer, which has the same sequence as the 40-mer primer above, was first synthesized by IDT with a 3’-amino linker and then labeled with TAMRA as described in Section 2.2.2, Chapter II. TAMRA-ptDNA was prepared by annealing 3’-TAMRA-40-mer with the 65-mer (1:1.1 labeled: unlabeled DNA) as described above.

ATP and ATPγS were purchased from Sigma-Aldrich. 7-MEG (7-methylguanosine) was purchased from R.I. Chemicals, Inc. PNPase (purine-nucleoside phosphorylase), Proteinase inhibitor cocktail, Phosphorus Standard Solution, 0.65 mM from Sigma-Aldrich. IsoPropyl-1-Thio-β-D-Galactopyranoside (IPTG) was purchased from Gold Biotechnology Inc. and MDCC from Invitrogen. All buffer compositions are listed in Appendix A.

### 2.2.2 Tetramethyl rhodamine-DNA preparation

DNA Labeling with TAMRA: first, 0.1 M sodium tetraborate buffer (pH 8.5) was prepared just before use (0.1520 g Borax powder was added to 4 ml ddH2O in a 15 ml falcon tube and pH was lowered to 8.5 by adding ~220 µl 1 M HCl). ~800 µg TAMRA dye was weighed in amber tube and 80 µl dimethyl sulfoxide (DMSO) was added and vortexed well. Then 913 µg DNA and 300 µl 0.1 M sodium tetraborate were added to total 400µl per reaction. Cap was parafilmed to cover the tube with foil and placed on the roller drum at room temperature overnight.

Butanol extraction: water-saturated butanol was prepared. 5 ml ddH2O was mixed with 5 ml 2-butanol in a 15 ml falcon tube. The mixture was vortexed at max
for 30 s and centrifuged at speed setting #4 for 2 min in the cold room for twice.

Water saturated butanol was the top layer. 400 µl DNA reaction mixture was split into two 1.5 ml clear tubes (each 200 µl). 600 µl water saturated butanol was add to each tube, vortexed and centrifuged at 13,000 rpm for 2 min at room temperature. Top layer that contains unreacted TAMRA dye in butanol was removed to a 15 ml falcon tube with a 1 ml pipette for twice. It is not necessary to remove all of top layer during first two extractions. Be careful not to remove any of the bottom layers, which contain DNA. Top layer in a 15 ml falcon tube was saved and discard later. For the last extraction, butanol was removed as much as possible with a 200 µl pipette when it was close to the bottom layer. Bottom layer was transferred to two 1.5 ml clear tubes and a small hole was poked in each tube cap. Samples were dried in a speed vacuum the samples for 1 hour without heat until the butanol was gone (no smell). Don’t forget to cover the speed vacuum with foil. Don’t shake the sample tubes because of the hole on the cap.

P6 gel-filtration chromatography: ~1 tsp of P6 gel filtration resin was mixed in 50 ml ddH₂O and kept in cold room overnight. A 5 ml P6 column was prepared in a plastic and disposable 10 ml BioRad column and equilibrated with 50 ml buffer P6A (buffer P6A:10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA). When buffer P6A level drops to the gel surface, ~ 400 µl DNA_{TAMRA} solution was layered gently and evenly by using a 200 µl pipette. Allow DNA_{TAMRA} solution to completely enter resin and 1 ml buffer P6A was evenly layered multiple times on top of column with a 1 ml pipette. At the same time, fractions was collected in 1.5 ml capless tubes, 8 drops
(~300 µl) per tube, until the first pink peak that contains TAMRA labeled DNA was done and a more intense second peak that contains free TAMRA was coming through. Total was about 12~15 tubes. Absorbance of all first peak fractions at A$_{260}$ and A$_{555}$ (dilute 1 µl in 1 ml ddH$_2$O) were measured in quartz cuvettes. 5~7 tubes of peak fractions that A$_{260} > 3 \times$ A$_{555}$ (total volume is 1~1.5 ml) were pooled. The pool was split into “not low adhesion” tubes at ~300 µl (400 µl was the maximum). 3 x volume 100 % ethanol and 0.1 x volume 3M NaAc at pH 5.2 were added for ethanol precipitation in -80 ºC overnight. The column was kept in the hood and disposed later.

Denaturing polyacrylamide gel electrophoresis purification of DNA$_{TAMRA}$: DNA$_{TAMRA}$ was centrifuged at 13,000 rpm for 30 min at cold room. Supernatant was removed and dried in the hood. 0.75 mm thickness was used to run 19% PAGE gel to further purify DNA$_{TAMRA}$. Band was visualized using a handheld UV lamp. The fluorescent pink DNA$_{TAMRA}$ band was excised and be careful not to cut the unlabeled DNA bands. The pink band into small pieces was cut and distributed into four 1.5 ml tubes. 400µl elution buffer (50 mM Tris-HCl pH7.5, 50 mM NaCl, 1mM EDTA) was added to each tube. Tube caps were parafilmed, the tubes were covered with foil and placed on roller drum overnight at room temperature. The tubes were centrifuged at 13,000 rpm for 5 min. Supernatant was collected using “not low adhesion” 1.5 ml tubes with gel loading tip as first pick. Another 400 µl elution buffer was added and shaked on the roller drum for 4~5 hours and the supernatant was collected as second pick.
DNA\textsubscript{TAMRA} was concentrated and concentration was measured: the speed vacuum (no heat and use foil) was used on first and second pick DNA\textsubscript{TAMRA} for 3~4 hours to reduce the volume to about half. The solutions were combined and “not low adhesion” 1.5 ml tubes were used to ethanol precipitate overnight. DNA\textsubscript{TAMRA} was centrifuged at 13,000 rpm for 30 min at 4 °C, supernatant was removed and DNA\textsubscript{TAMRA} was dried in the hood. The pellet was resuspended in 1x TE buffer (10 mM Tris-HCl pH7.5 and 1mM EDTA) for a final concentration of ~300 µM. Assume 80% DNA retention from P6 column.

Final DNA\textsubscript{TAMRA} concentration measurement: 1 µl DNA\textsubscript{TAMRA} was diluted in 1 ml ddH2O in quartz cuvette and measured at $A_{260}$. Repeat at least 3 times and take the average reading. For DNA\textsubscript{TAMRA}, $E' = E_{\text{DNA}} + 0.34 \times E_{\text{TAMRA}555}$, $E_{\text{DNA}}$ is the DNA extinction coefficient and $E_{\text{TAMRA}555}$ is the extinction coefficient of TAMRA at 555 nm, which is 65,000L/mol•cm. For final DNA\textsubscript{TAMRA} concentrations, DNA\textsubscript{TAMRA} molar concentration = $(A_{260} \times \text{dilution factor}) / E'$. DNA\textsubscript{TAMRA} was stored as smaller aliquots (usually 30 µl) at -80 °C. The yield was above 50%.

**2.2.3 Equilibrium binding of RFC (-/+ PCNA) to ptDNA**

DNA binding was monitored by fluorescence anisotropy of ptDNA\textsubscript{TAMRA} labeled at the 3′ primer end of 40-mer in equilibrium conditions on a FluoroMax-3 fluorometer (Horiba Jobin-Yvon). The fluorometer was turned on about 20 min prior to usage in order to warm up the lamp.
4 nM ptDNA_{TAMRA} was titrated with 0–50 nM RFC in the absence or presence of PCNA_{WC} or mutants (2:1 [PCNA_{WC}]:[RFC] ratio) in Buffer A containing 0.1 mM ATPγS and 0.05 mg/ml bovine serum albumin (BSA) at 25 °C (Primer-template DNA_{TAMRA} was prepared by annealing a 65-mer template with a 45-mer primer labeled with TAMRA at the 3’ end, as described in Section 2.2.1 and 2.2.2, Chapter II). The samples were excited with vertically polarized light at λ_{EX} = 555 nm, and the anisotropy was calculated from vertically (IVV) and horizontally (IVH) polarized emission intensities at λ_{EM} = 580 nm (r = (I_{VV}−GIVH)/(I_{VV} + 2GIVH), where G is the grating correction factor).

Anisotropy values, corresponding to the fraction of bound DNA, were plotted against RFC concentrations and the isotherm was fitted with a quadratic equation to yield $K_d$:

$$r_{[D\cdot M]} = r_0 + (r_{max}−r_0)\{(K_d+[D_t]+[M_t])−[(K_d+[D_t]+[M_t])^2−4[D_t][M_t]]^{1/2}/2[D_t]\}$$

where $r_{[D\cdot M]}$ is the anisotropy at equilibrium, $r_0$ is anisotropy in the absence of protein and $r_{max}$ is maximal anisotropy, and $D_t$ and $M_t$ are total molar concentrations of DNA and RFC complex, respectively. The data were fit by non-linear regression using KaleidaGraph (Synergy Software).
2.2.4 Equilibrium measurement on FRET efficiency of PCNA opening

PCNA opening/closing was monitored by FRET under steady state conditions on a FluoroMax-3 fluorometer (Horiba Jobin-Yvon) at 25 °C in Buffer A. In all experiments, the reactant concentrations were, 0.25 µM PCNA\textsubscript{WC\textsuperscript{AEDANS}} or PCNA\textsubscript{FC\textsuperscript{AEDANS}} (lacking donor W185), in the absence and presence of 0.6 µM RFC, 0.25 µM ptDNA, and 0.5 mM ATP in Buffer A at 25 °C. The samples were excited at 290 nm or 336 nm and were collected and the fluorescence intensities at 495 nm were obtained from emission scans (450-550 nm). The signal was converted to FRET efficiency by using equation:

\[ E_T = \left( \frac{I_{AD}}{I_A} - 1 \right) \left( \frac{\varepsilon_A}{\varepsilon_D} \right) \]

\( E_T \) is FRET efficiency, and \( I_{AD} \) and \( I_A \) are fluorescence intensities of AEDANS acceptor in the presence (PCNA\textsubscript{WC\textsuperscript{AEDANS}}) or absence (PCNA\textsubscript{FC\textsuperscript{AEDANS}}) of the tryptophan donor in PCNA at \( \lambda_{\text{EX}} = 290 \) nm. \( \varepsilon_A \) (1800 M\(^{-1}\) cm\(^{-1}\)) and \( \varepsilon_D \) (4100 M\(^{-1}\) cm\(^{-1}\)) are the extinction coefficients of AEDANS and tryptophan at 290 nm. A small contribution by RFC tryptophan residues to the FRET signal (inter-protein FRET) was first corrected as described previously (Zhuang, Yoder et al. 2006). Normalized fluorescence data were calculated for PCNA\textsubscript{WC\textsuperscript{AEDANS}} \([F_{AD} (290)]\) and PCNA-FC\textsubscript{AEDANS} \([F_A (290) \text{ and } F_A (336)]\) by dividing background-subtracted kinetic traces measured in the presence of RFC by those measured in the absence of RFC. \( F_{AD} (290) \) is related to \( I_{AD} + X \), \( F_A (290) \) is related to \( I_A + X \), and \( F_A (336) \) is related to \( I_A \), where \( X \) is the amount of inter-protein FRET. \( F_{AD} (290), F_A (290), \) and \( F_A (336) \) were multiplied by 3.12, 1, and 1, respectively. Relative fluorescence
intensities of $\text{PCNA}_{\text{WC}}^{\text{AEDANS}}$ at $\lambda_{\text{EX}} = 290$ nm, $\text{PCNA}_{\text{FC}}^{\text{AEDANS}}$ at $\lambda_{\text{EX}} = 290$ nm, and $\text{PCNA}_{\text{FC}}^{\text{AEDANS}}$ at $\lambda_{\text{EX}} = 336$ nm, were measured on fluorometer to obtain corresponding $I_{\text{AD}} + X$, $I_A + X$, and $I_A$ values. By subtracting $I_A$ from $I_A + X$, we obtained $X$ and consequently, $I_{\text{AD}}$. These corrected $I_{\text{AD}}$ and $I_A$ values were used to calculate $E_T$.

2.2.5 Kinetics measurements of DNA binding to PCNA•RFC•ATP complex

PCNA•RFC•ATP complex interactions with ptDNA were measured at $25^\circ$C in Buffer A containing 0.05 mg/ml BSA, on a KinTek SF-2001 Stopped-Flow instrument (KinTek Corp., Austin, TX). Prime-template DNA$_{\text{TAMRA}}$ was excited at 535 nm and fluorescence emission was measured over time at $> 550$ nm using a 550 nm long-pass cut-off filter (Newport Corporation). Primer-template DNA$_{\text{TAMRA}}$ is the same sequence as in Section 2.2.3, Chapter II. Three-syringe experiments were performed by pre-incubating 0.3 µM RFC, 1.2 µM PCNA$_{\text{WC}}$ or mutants from syringe A and 1.5 mM ATP from syringe B for $\Delta t = 0.02, 0.5, 2$ and 3 second (s) before addition of 0.12 µM TAMRA-ptDNA from syringe C (final ratio: 1:1:1; final concentrations of reactants are: 0.4 µM PCNA$_{\text{WC}}$ or mutants, 0.1 µM RFC, 500 µM ATP and 0.04 µM ptDNA$_{\text{TAMRA}}$). Three or more kinetic traces of 1000 data points over the reaction time were averaged in each experiment to generate raw data and baseline fluorescence from free ptDNA$_{\text{TAMRA}}$ was divided to generate the corrected data. The data were fit to a double exponential function $FL = A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t})$ for initial estimation of rate constants, where $FL$ is the normalized fluorescence, $A_1$
and $A_2$ are the amplitudes for up and down phase, $k_1$ and $k_2$ are rate constants for up and down phase, respectively, and $t$ is reaction time.

### 2.2.6 Kinetics measurements of PCNA opening and closing

Kinetics of PCNA opening/closing was measured on a KinTek SF-2001 Stopped-Flow instrument at 25 °C in Buffer A. PCNA$_{WC}^{AEDANS}$ or PCNA$_{FC}^{AEDANS}$ was excited at 290 nm or 336 nm and fluorescence emission was measured over time at > 450 nm using a 450 nm long-pass cut-off filter (Newport Corporation). In a two-syringe experiments measuring PCNA opening, 0.5µM PCNA$_{WC}^{AEDANS}$ or PCNA$_{FC}^{AEDANS}$ or mutants, with 1.2 µM RFC from syringe A was mixed rapidly with 1.0mM ATP and flowed in the observation cell (final concentrations according to a 1:1 ratio: 0.5 µM PCNA$_{WC}^{AEDANS}$ or PCNA$_{FC}^{AEDANS}$ or mutants, 0.6 µM RFC and 500 µM ATP). In a three-syringe experiment measuring PCNA closing, 0.75µM PCNA$_{WC}^{AEDANS}$ or PCNA$_{FC}^{AEDANS}$ or mutants, 1.8 µM RFC from syringe A and 1.5 mM ATP from syringe B were pre-incubated for $\Delta t = 0.02$, 0.5 and 2 s prior to addition of 0.75 µM ptDNA (final ratio: 1:1:1; final concentrations of reactants are: 0.5 µM PCNA$_{WC}^{AEDANS}$ or PCNA$_{FC}^{AEDANS}$ or mutants, 0.6 µM RFC, 500 µM ATP and 0.75µM ptDNA). Three or more kinetic traces (1000 data points each) were averaged, and the signal was converted to FRET efficiency, as described in Section 2.2.4, Chapter II. The data were fit to single exponential functions FRET efficiency=
A(1 - e^{-kt}) for initial estimation of rate constants, where A is the amplitude, k is rate constant, and t is reaction time.

2.2.7 Kinetics measurements of Pi release

ATP hydrolysis activity of RFC was measured using PBP (Phosphate-binding protein) labeled with MDCC (N-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide), which reports on Phosphate (Pi) release following ATP hydrolysis, as MDCC-PBP fluorescence increases upon binding Pi (Brune, Hunter et al. 1994; Jeong, Kim et al. 2002). MDCC-PBP was excited at 425 nm and fluorescence emission over time at > 450 nm was measured using a 450 nm long-pass cut-off filter (Newport Corporation). The experiments were performed on an SF-2001 Stopped-flow instrument in Buffer A plus 0.3 unit/ml PNPase (Sigma-Aldrich, St. Louis, MO) and 0.2 mM 7-methylguanosine (R. I. Chemical Inc., Orange, CA) at 25 °C, which were used as a phosphate mop to remove Pi contamination prior to the reaction. In the double mixing experiments, 3 µM PCNAWC or mutants, 1.5 µM RFC from syringe A and 1.5 mM ATP from syringe B were pre-incubated for Δt = 0.02, 0.5 and 2 s prior to addition of 7.5 µM ptDNA (final ratio: 1:1:1; final concentrations of reactants are: 0.5 µM RFC, 500 µM ATP, 1 µM PCNAWC or mutants, 2.5 µM ptDNA, and 10 µM MDCC-PBP). In order to generate baselines for double mixing experiment data, control experiments were conducted in which 3 µM PCNAWC or mutants, 1.5 µM RFC from syringe A and 1.5 mM ATP from syringe B were pre-incubated for Δt = 0.02, 0.5 and 2 s prior to addition of only 30 µM MDCC-
PBP from syringe C (final ratio: 1:1:1; final concentrations of reactants are: 1 µM PCNA$_{WC}$ or mutants, 0.5 µM RFC, 500 µM ATP and 10 µM MDCC-PBP). One thousand data points were collected over the reaction time, which was usually 1 s and data were obtained by averaging at least 3-4 traces. Baselines were subtracted from corresponding raw data to eliminate background fluorescence as well as fluorescence increase due to pre-incubation of RFC and PCNA$_{WC}$ with ATP in the delay line.

A Pi calibration curve relating the PBP-MDCC fluorescence signal to Pi concentration was generated prior to each experiment (Jeong, Kim et al. 2002). Pi stock was from Phosphorus Standard Solution (as KH$_2$PO$_4$, 650 µM) (Sigma-Aldrich). For generating a Pi calibration curve for a double mixing experiments, various concentrations of Pi (0, 3, 6 and 9 µM) in Buffer A from syringe A and only Buffer A plus 0.3 unit/ml PNPase and 0.2 mM 7-methylguanosine from syringe B were mixed in delay line for 0.02 s prior to addition of MDCC-PBP (30 µM) from syringe C; note, Buffer A in Pi solution (syringe A) should not contain PNPase and 7-methylguanosine Final concentrations for both experiments are 0, 1, 2 and 3 µM Pi and 10 µM MDCC-PBP. One thousand data points were collected over 0.2 s reaction time and data were obtained by averaging at least 3-4 traces. The base line fluorescence from free MDCC-PBP was subtracted from the saturating fluorescence of MDCC-PBP in the presence of Pi to calculate the fluorescence increase upon MDCC-PBP binding to Pi. This value was plotted against the corresponding Pi concentration and fit to a linear equation. The slope was used to convert the
fluorescence signal from MDCC-PBP binding to Pi released from RFC to Pi concentration. The baseline corrected data from the reactions were divided by the slope from the Pi calibration curve to determine the molar amount of Pi released during the reaction and then fit to a burst equation, \([\text{Pi}] = A(1 - e^{kt}) + Vt\), where \(A\) and \(k\) are burst amplitude and rate constant, respectively, \(V\) is the velocity of the linear phase, and \(t\) is reaction time or a linear equation. Steady state ATPase rate constant \((k_{\text{cat}})\) is obtained by normalizing \(V\) to the enzyme concentration, which is \(3\times[\text{RFC}]\) (each RFC complex is estimated to contain 3 active ATPase sites).
2.3 Results

2.3.1 Identify the DNA interacting residues in PCNA

The side chains of nine arginines and lysines on the inner surface of the PCNA monomer are shown in Fig 2.1D. Residues Lys13, Arg14, Lys20, Lys77, and Arg80 are located in the N-terminal domain I, whereas Lys146, Arg149, Lys210, and Lys217 are located in the C-terminal domain II of PCNA. A subset of six residues distributed across the entire surface (R14, K20, R80, K146, R149, and K217) were chosen and mutated into alanine, and the effects on key steps in the PCNA loading reaction catalyzed by RFC were measured. All the point mutations were made in PCNA\(_{WC}\), in which the four naturally occurring cysteines are modified to serine and F185 and K107 are modified to tryptophan and cysteine, respectively, for FRET based analysis of PCNA opening and closure as described below. Importantly, previous study showed that both the unlabeled and AEDANS-labeled PCNA mutants retain full activity in stimulating the RFC ATPase, as compared with wild-type PCNA, indicating that neither the mutations nor the site-specific labeling of PCNA with AEDANS disrupted the interactions between PCNA and RFC/DNA (Zhuang, Yoder et al. 2006).

2.3.2 Effect of mutating inner rim cationic residues on PCNA opening by RFC

The structural integrity of the PCNA\(_{WC}\) mutants was assessed by measuring FRET across the subunit interface for closed and open clamps (alone and with RFC and ATP, respectively) (Table 2.1). The FRET signal between W185 and AEDANS-
labeled C107 is high when PCNA_{WC}^{AEDANS} is closed and low when the clamp is open. The signal was converted to FRET efficiency ($E_T$) using data from parallel experiments with tryptophan-free PCNA_{FC}^{AEDANS}, and correcting for a small level of inter-protein FRET due to RFC tryptophan, as described in Section 2.2.4, Chapter II. $E_T$ for PCNA_{WC} alone is 0.95, as reported previously (Zhuang, Yoder et al. 2006), and ranges from 0.92 – 0.98 for the mutant clamps, indicating they are also closed in the absence of RFC. PCNA_{WC} $E_T$ drops to 0.74 in the presence of RFC and ATP, on formation of RFC•ATP•PCNA_{open} complex (Table. 2.1). Domain I mutants, PCNA_{WC}-R14A, PCNA_{WC}-K20A, PCNA_{WC}-R80A exhibit similar reduction in $E_T$ with RFC and ATP, indicating these mutant clamps are opened as well. $E_T$ for domain II mutants, PCNA_{WC}-K146, PCNA_{WC}-R149A and PCNA_{WC}-K217A, also drops with RFC and ATP, but PCNA_{WC}-K217A $E_T$ remains quite high compared to PCNA_{WC}, suggesting some perturbation of its interaction with RFC and/or opening, which are unrelated to its interaction with ptDNA.

The kinetics of PCNA opening were measured next in order to determine the rate and extent to which RFC can open these clamps independently of ptDNA. In stopped-flow experiments a solution of RFC and PCNA_{WC}^{AEDANS} was mixed rapidly with a solution of ATP, and the decrease in FRET as a consequence of PCNA opening was measured over time; note: the order of mixing does not affect the PCNA opening rate (Sakato, Zhou et al. 2012). The kinetic trace for PCNA_{WC}^{AEDANS} (Fig. 2.2A) fit empirically to a single exponential yielded $k_{open} = 2.3 \text{ s}^{-1}$, consistent with previous reports of PCNA opening rates (Zhuang, Yoder et al. 2006; Thompson,
Marzahn et al. 2011). Clamps containing domain I mutations R14A, K20A or R80A all showed the same opening kinetics as PCNAWC (Fig. 2.2A). Opening rates for clamps containing domain II mutations K146A or R149A were comparable to PCNAWC (Fig. 2.2B), although the extent of opening was lower for PCNAWC-K146A, consistent with the steady state data (Table 2.1). PCNAWC-K217A, however, exhibited defects both in the rate and extent of opening (Fig. 2.2B, Table 2.1); thus, this mutant was not included in analysis of subsequent ptDNA binding related steps in the reaction.
**Figure 2.2 Kinetics of PCNA opening.** PCNA clamp opening is measured by change in FRET between tryptophan donor and AEDANS acceptor. Rapid mixing of RFC and PCNA\textsubscript{WC}\textsuperscript{AEDANS} with ATP results in lower FRET efficiency as PCNA opens. (A), data are shown for PCNA\textsubscript{WC} and domain I mutants PCNA\textsubscript{WC}-R14A, PCNA\textsubscript{WC}-K20A, and PCNA\textsubscript{WC}-R80A. An exponential fit of the PCNA\textsubscript{WC} trace yields $k_{\text{open}} = 2.3$ s\textsuperscript{-1}. (B), data are shown for PCNA\textsubscript{WC} and domain II mutants PCNA\textsubscript{WC}-K146A, PCNA\textsubscript{WC}-R149A, and PCNA\textsubscript{WC}-K217A. FRET efficiency and opening rates of PCNA\textsubscript{WC}\textsuperscript{AEDANS} and mutants are listed in Table 2.1. All mutants showed the similar opening kinetics as PCNA\textsubscript{WC} except K217 exhibited defects both in the rate and extent of opening.

Final reactant concentrations were 0.6 µM RFC, 0.25 µM PCNA\textsubscript{WC}\textsuperscript{AEDANS} or mutants, and 0.5 mM ATP.
Figure 2.2 Kinetics of PCNA opening.

**Table 2.1 FRET efficiency and opening rates of PCNA\textsubscript{WC}^{AEDANS} and mutants**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$E_T$ (PCNA)</th>
<th>$E_T$ (PCNA+RFC+ATP)</th>
<th>Opening Rate (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>0.95</td>
<td>0.74</td>
<td>2.3</td>
</tr>
<tr>
<td>PCNA-R14A</td>
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<td>PCNA-K217A</td>
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<td>0.84</td>
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2.3.3 ptDNA binding and release are influenced by PCNA inner rim cationic residues

ATPγS binding to RFC leads to formation of stable RFC•ATPγS•ptDNA and RFC•ATPγS•PCNA\textsubscript{open}•ptDNA complexes. We measured formation of these complexes by fluorescence anisotropy of a 40/65-nt ptDNA substrate labeled with TAMRA dye at the 3’ primer end. Titration of ptDNA\textsubscript{TAMRA} with RFC lead to increase in TAMRA anisotropy and the binding isotherm yielded a $K_d$ of 6.5 nM (Fig. 2.3); the data were fit to a quadratic equation corrected for 1.8-fold higher fluorescence intensity of bound versus free ptDNA\textsubscript{TAMRA} (Lundblad, Laurance et al. 1996). The same experiment performed in the presence of PCNA\textsubscript{WC} or PCNA\textsubscript{WC-R80A} mutant yielded similar $K_d$ values of 2.7 nM and 2.2 nM, respectively (Fig. 2.3) (Sakato, Zhou et al. 2012). ATPγS-bound RFC has high affinity for ptDNA in the absence or presence of PCNA; thus, $K_d$ measurements with PCNA mutants are unlikely to report any significant differences unless the mutations severely impact interaction between ptDNA and RFC as well.

Therefore we examined the interaction in finer detail by measuring the kinetics of ptDNA association after ATP binding to RFC and dissociation after ATP hydrolysis. We have shown previously that increase in fluorescence reports ptDNA\textsubscript{TAMRA} binding to RFC (−/+ PCNA) and decrease reports dissociation of the complex after ATP hydrolysis (Sakato, Zhou et al. 2012). Sequential mixing experiments were performed in which RFC and PCNA\textsubscript{WC} were pre-incubated with ATP for varying times ($\Delta t = 0.02, 0.5$ and 2s) and then mixed with ptDNA\textsuperscript{TAMRA}, and
the signal was monitored over time; note: due to slow activation of RFC after ATP binding, a pre-incubation period of at least 2 s is required for maximal ptDNA binding and ATP hydrolysis (Fig. 2.4A, Fig. 2.8A) (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012). Fig. 2.5 shows the kinetic traces at Δt = 2 s overlaid for all PCNA. The increased signal in PCNA<sub>WC</sub> reports ptDNA binding to RFC•ATP•PCNA complex at 14 s<sup>-1</sup> under these conditions (bimolecular binding constant estimate ~ 1 x 108 M<sup>-1</sup> s<sup>-1</sup>). Following ATP hydrolysis, the decrease in signal at 4 s<sup>-1</sup> reports ptDNA•PCNA (and/or ptDNA) release from RFC. The signal levels out higher than that for free ptDNA in steady state as the short linear substrate slips out from PCNA and is bound again by excess protein. The clamps containing domain I mutations, R14A, K20A or R80A, exhibit slightly slower ptDNA binding compared to PCNA<sub>WC</sub> and distinctly different release kinetics, resulting in higher levels of ptDNA-bound complex in steady state (Fig. 2.4A). Clamps containing domain II mutations, K146A or R149A, exhibit similar ptDNA binding as PCNA<sub>WC</sub> and slightly different release kinetics (Fig. 2.4B).
Figure 2.3 Primer-template DNA binding affinity to different complex. DNA binding was measured by the change in fluorescence anisotropy of ptDNA_{TAMRA} (labeled at the 3’ primer end) on titration with RFC in the presence of non-hydrolyzable ATPγS, in the absence (black) or presence (green) of PCNA_{WC} or PCNA_{WC-R80A} (red). The apparent binding affinity of RFC for ptDNA is slightly weaker in the absence of PCNA ($K_d = 6.5$ nM), but similar in the presence of PCNA_{WC} ($K_d = 2.7$ nM) or PCNA_{WC-R80A} ($K_d = 2.2$ nM).
Figure 2.4 Kinetics of ptDNA binding and release. Interaction of DNA with RFC-ATP-PCNA is measured by change in fluorescence intensity of TAMRA-labeled ptDNA. (A), data are shown for PCNA\textsubscript{WC} and domain I mutants PCNA\textsubscript{WC}-R14A, PCNA\textsubscript{WC}-K20A, and PCNA\textsubscript{WC}-R80A. (B), data are shown for PCNA\textsubscript{WC} and domain II mutants PCNA\textsubscript{WC}-K146A and PCNA\textsubscript{WC}-R149A. Pre-incubation of RFC and PCNA\textsubscript{WC} with ATP (\(\Delta t = 0.02–2\) s) followed by the addition of ptDNA\textsubscript{TAMRA} results in rise (ptDNA binding) and then fall (complex dissociation) of the fluorescence signal. Final reactant concentrations were 0.1 \(\mu\)M RFC, 0.4 \(\mu\)M PCNA\textsubscript{WC} or mutants, 0.04 \(\mu\)M ptDNA\textsubscript{TAMRA}, and 0.5 mM ATP.
Figure 2.4A Kinetics of ptDNA binding and release with PCNA\textsubscript{WC} and domain I mutants
Figure 2.4B Kinetics of ptDNA binding and release with PCNA<sub>WC</sub> and domain II mutants

![Kinetics of ptDNA binding and release with PCNA<sub>WC</sub> and domain II mutants](image)
Figure 2.5 Overlay of kinetics of ptDNA binding and release with PCNA_{WC} and mutants at Δt = 2 s. (A), data are shown for PCNA_{WC} and domain I mutants. Double exponential fit of the PCNA_{WC} trace yields $k_{\text{up}} = \sim 14 \text{ s}^{-1}$ and $k_{\text{down}} = \sim 3.9 \text{ s}^{-1}$. (B), data are shown for PCNA_{WC} and domain II mutants. PCNA domain I mutations exhibit slightly slower ptDNA binding compared to PCNA_{WC} and distinctly different release kinetics, while PCNA domain II mutations exhibit similar ptDNA binding as PCNA_{WC} and slightly different release kinetics. Final reactant concentrations were 0.1 μM RFC, 0.4 μM PCNA_{WC} or mutants, 0.04 μM ptDNA_{TAMRA}, and 0.5 mM ATP.
The data were globally fit to a highly simplified model of ptDNA binding/release using KinTek Explorer (Johnson, Simpson et al. 2009) (Table 2.2), in order to obtain rough estimates of key rate constants. The model contained four steps: (i) $k_1$ – slow RFC activation after binding ATP and PCNA opening (assumed irreversible because PCNA opening is comparable with ATP and ATPγS, indicating minimal PCNA closure prior to ATP hydrolysis) (Zhuang, Yoder et al. 2006), (ii) $k_2/k_2$ – high affinity interaction of ptDNA with the ATP-bound protein complex ($K_d = 2$ nM) (Sakato, Zhou et al. 2012), (iii) $k_3$ – ATP hydrolysis, RFC deactivation and PCNA closure (assumed irreversible after ATP hydrolysis), and (iv) $k_4$ – release of ptDNA (assumed irreversible after ATP hydrolysis) to complete the catalytic cycle. All rate constants were allowed to float during fitting of the PCNA\textsubscript{WC} data, and the relative change in signal for free:bound ptDNA was set at 1:1.8 based on the fluorescence intensity of ptDNA\textsubscript{TAMRA} alone and bound to RFC•ATPγS•PCNA (Sakato, Zhou et al. 2012). Table 2.2 lists the rate constants for all mutants. With PCNA\textsubscript{WC}, the RFC activation step occurs at an apparent rate of 8.9 s\(^{-1}\), ptDNA binding at 1 x 108 M\(^{-1}\) s\(^{-1}\), RFC deactivation at 5.6 s\(^{-1}\) and ptDNA release at 0.5 s\(^{-1}\). The mutant data were fit in the same manner as PCNA\textsubscript{WC} except the ptDNA-independent RFC activation step was fixed at 8.9 s\(^{-1}\). All the domain I mutants exhibit $\geq 2$-fold differences in the rates of one or more ptDNA-associated steps in the reaction. The K20A and R80A mutants exhibit slower ptDNA binding ($k_2$), R14A and R80A mutants exhibit slower RFC deactivation ($k_3$), and all three domain I mutants R14A, K20A, R80A exhibit much faster ptDNA release than PCNA\textsubscript{WC} ($k_4$).
In case of domain II mutants, all the best fit rate constants for K146A are almost the same as for PCNA_{WC} while R149A exhibits somewhat slower RFC deactivation and faster ptDNA release than PCNA_{WC}. The ptDNA binding/release data were analyzed further by fitting to a more comprehensive model of the clamp loading mechanism (along with all other kinetic data), as described later.
Table 2.2 A minimal model of ptDNA binding/release during the clamp loading reaction.

![Diagram of the clamp loading reaction](image)

<table>
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<th>parameters</th>
<th>PCNA_{WC}</th>
<th>R14A</th>
<th>K20A</th>
<th>R80A</th>
<th>K146A</th>
<th>R149A</th>
<th>Event</th>
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<tbody>
<tr>
<td>$^a k_1 (s^{-1})$</td>
<td>$8.9 \pm 0.3$</td>
<td>8.9</td>
<td>8.9</td>
<td>8.9</td>
<td>8.9</td>
<td>8.9</td>
<td>RFC activation, PCNA opening</td>
</tr>
<tr>
<td>$^b k_2 (uM^{-1} s^{-1})$</td>
<td>97 ± 3 0.2 ± 0.007 2</td>
<td>80 ± 2 0.2 ± 0.007 2</td>
<td>37 ± 0.7 0.07 ± 0.001 2</td>
<td>57 ± 1.5 0.11 ± 0.003 2</td>
<td>75 ± 1.6 0.15 ± 0.003 2</td>
<td>77 ± 2.3 0.15 ± 0.005 2</td>
<td>ptDNA binding</td>
</tr>
<tr>
<td>$^c k_2 (s^{-1})$</td>
<td>5.6 ± 3 1.8 ± 0.05</td>
<td>3.5 ± 0.04</td>
<td>2.2 ± 0.05</td>
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<td>2.8 ± 0.08</td>
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<tr>
<td>$K_{d2} (nM)$</td>
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<td>5.5 ± 0.2 3.3 ± 0.09</td>
<td>1.1 ± 0.06</td>
<td>1.5 ± 0.04</td>
<td>ptDNA releasing</td>
<td></td>
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</tr>
</tbody>
</table>

$^a$ Value obtained for PCNA_{WC} was fixed for mutants during global fitting analysis.
$^b$ Standard error values are shown for parameters allowed to float during the fitting.
$^c$ Label specifics parameters that were linked during the fitting.
2.3.4 PCNA closure around ptDNA is influenced by inner rim cationic residues

As noted earlier, ptDNA binding triggers ATP hydrolysis, which in turn leads to PCNA closure and complex disassembly. The ptDNA binding/release kinetics indicate that single arginine and lysine mutations in PCNA can disrupt post-ATP hydrolysis step(s) in the reaction. We investigated whether that includes PCNA closure around ptDNA. Sequential mixing experiments were performed in which RFC and PCNA\textsubscript{WC\textsuperscript{AEDANS}} were pre-incubated with ATP for varying times (\(\Delta t = 0.02, 0.5\) and 2 s) then mixed with ptDNA, and the tryptophan-AEDANS FRET signal was monitored over time; note: at short \(\Delta t\) both FRET decrease (PCNA\textsubscript{WC\textsuperscript{AEDANS}} opening after ATP binding) and subsequent increase (PCNA\textsubscript{WC\textsuperscript{AEDANS}} closing after ATP hydrolysis) are observed, whereas at longer \(\Delta t\) PCNA\textsubscript{WC\textsuperscript{AEDANS}} is fully opened and only the closing phase is observed (Fig. 2.6A) (Sakato, Zhou et al. 2012). Fig. 2.7 shows data at \(\Delta t = 2\) s for all clamps, and an exponential fit of the PCNA\textsubscript{WC\textsuperscript{AEDANS}} trace yields an apparent rate of 8 s\(^{-1}\) for closure. All the mutant clamps close slightly slower than PCNA\textsubscript{WC}, although PCNA\textsubscript{WC-K20A} and PCNA\textsubscript{WC-R80A} are again the worst affected with > 2-fold decrease in the closing rate. These data were analyzed further by fitting to a comprehensive model of the clamp loading mechanism as described later.
Figure 2.6 Kinetics of PCNA closure around ptDNA. Pre-incubation of RFC and PCNA$_{WC}^{\text{AEDANS}}$ with ATP ($\Delta t = 0.02–2$ s) followed by addition of ptDNA results in FRET increase as ATP is hydrolyzed, and the clamp closes around DNA. (A), data are shown for PCNA$_{WC}$ and domain I mutants PCNA$_{WC}$-R14A, PCNA$_{WC}$-K20A, and PCNA$_{WC}$-R80A. (B), data are shown for PCNA$_{WC}$ and domain II mutants PCNA$_{WC}$-K146A and PCNA$_{WC}$-R149A. Final reactant concentrations were 0.6 $\mu$M RFC, 0.25 $\mu$M PCNA$_{WC}^{\text{AEDANS}}$ or mutants, 0.25 $\mu$M ptDNA, and 0.5 mM ATP.
Figure 2.6A Kinetics of PCNA closure around ptDNA with PCNA_{wc} and domain I mutants
Figure 2.6B Kinetics of PCNA closure around ptDNA with PCNA_{WC} and domain II mutants
Figure 2.7 Overlay of kinetics of PCNA closure around ptDNA with PCNA\textsuperscript{WC} and mutants at $\Delta t = 2$ s. (A), data are shown for PCNA\textsuperscript{WC} and domain I mutants. Exponential fit of the PCNA\textsuperscript{WC} trace yields $k_{\text{close}} = 8$ s$^{-1}$. (B), data are shown for PCNA\textsuperscript{WC} and domain II mutants. All the mutant clamps close slightly slower than PCNA\textsuperscript{WC}. PCNA\textsuperscript{WC}-K20A and PCNA\textsuperscript{WC}-R80A exhibit > 2-fold decrease in the closing rate. Final reactant concentrations were 0.6 $\mu$M RFC, 0.25 $\mu$M PCNA\textsuperscript{WC}\textsuperscript{AEDANS} or mutants, 0.25 $\mu$M ptDNA, and 0.5 mM ATP.
2.3.5 Phosphate release toward the end of the PCNA loading cycle is also influenced by inner rim cationic residues

Previous studies of the RFC mechanism indicate that a burst of Pi release occurs after PCNA closure around ptDNA and is associated with PCNA•ptDNA release from RFC (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012). If this model is correct, the Pi release kinetics should reflect the defects in clamp closure and complex disassembly caused by the mutations. We measured Pi release in the first catalytic turnover using a fluorescent reporter MDCC-PBP that binds free Pi rapidly and with high affinity ($k_{on} = 1.4 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and $K_d = 0.1 \mu\text{M}$) (Brune, Hunter et al. 1998). Sequential mixing experiments were performed in which RFC and PCNA WC were pre-incubated with ATP for varying times ($\Delta t = 0.02, 0.5$ and $2 \text{ s})$ and then mixed with ptDNA and MDCC-PBP, and the fluorescence signal was monitored over time; note: due to slow activation of RFC after ATP binding, a pre-incubation period of at least $2 \text{ s}$ is required for maximal ATP hydrolysis and Pi release (Fig. 2.8A). Fig. 2.9 shows data at $\Delta t = 2 \text{ s}$ for all the clamps, and an exponential + linear fit of the PCNA WC trace yields a burst rate $k_{\text{PiRelease}} = 5 \text{ s}^{-1}$ (following a short lag) and $k_{\text{cat}} = 0.8 \text{ s}^{-1}$ ($1.2 \mu\text{M}^{-1} \text{s}^{-1}/3x[\text{RFC}] \mu\text{M}$ (3 RFC subunits hydrolyze ATP in the burst phase).

The clamps containing domain I mutations, R14A, K20A, R80A, all show lower burst rate and amplitude compared with PCNA WC, indicating that the mechanistic defect carries through to the final step(s) of the clamp loading reaction (Fig. 2.9A).

Nevertheless, the apparent steady state ATPase rate of RFC with these mutants is almost the same as with PCNA WC (e.g., $0.6 \text{ s}^{-1}$ for PCNA WC-R80A), consistent with
previous reports (McNally, Bowman et al. 2010), confirming that the ATPase $k_{cat}$ is unsuitable as a primary measure of changes in the PCNA loading mechanism. Also, consistent with the generally milder impact of clamp II domain mutations, K146A, R149A, on earlier steps in the reaction, Pi release with these mutants is comparable with PCNA$_{WC}$, although PCNA$_{WC}$-R149A exhibits a slightly lower burst rate and amplitude (Fig. 2.9B). These data were analyzed further by fitting to a comprehensive model of the clamp loading mechanism (along with all other kinetic data) as described below.
**Figure 2.8 Kinetics of P\textsubscript{i} release following ATP hydrolysis.** RFC-catalyzed P\textsubscript{i} release is measured by change in fluorescence intensity of MDCC-labeled PBP when it binds free P\textsubscript{i}. Pre-incubation of RFC and PCNA\textsubscript{WC} with ATP (Δt = 0.02–2 s) followed by addition of ptDNA results in a burst ATP hydrolysis and P\textsubscript{i} release followed by a linear steady state phase. (A), data are shown for PCNA\textsubscript{WC} and domain I mutants PCNA\textsubscript{WC}-R14A, PCNA\textsubscript{WC}-K20A, and PCNA\textsubscript{WC}-R80A. (B), data are shown for PCNA\textsubscript{WC} and domain II mutants PCNA\textsubscript{WC}-K146A and PCNA\textsubscript{WC}-R149A. Final reactant concentrations were 0.5 µM RFC, 1 µM PCNA\textsubscript{WC} or mutants, 2.5 µM ptDNA, 0.5 mM ATP, and 10 µM MDCC-PBP.
Figure 2.8A Kinetics of P_i release following ATP hydrolysis with PCNA_{wc} and domain I mutants
Figure 2.8B Kinetics of $P_i$ release following ATP hydrolysis with PCNA\textsubscript{wc} and domain II mutants.
Figure 2.9 Overlay of kinetics of \( P_i \) release following ATP hydrolysis with PCNA\(_{WC} \) and mutants at \( \Delta t = 2 \) s. (A), data are shown for PCNA\(_{WC} \) and domain I mutants. An exponential + linear fit of the PCNA\(_{WC} \) trace yields \( k_{P_i \text{ Release}} = \sim 5 \) s\(^{-1} \) and \( k_{\text{cat}} = 0.8 \) s\(^{-1} \) (slope/3×[RFC]). (B), data are shown for PCNA\(_{WC} \) and domain II mutants. All domain I mutants show lower burst rate and amplitude compared with PCNA\(_{WC} \), while domain II mutant K146A is comparable with PCNA\(_{WC} \) and R149A exhibits slightly lower than PCNA\(_{WC} \) in burst rate and amplitude. Final reactant concentrations were 0.5 μM RFC, 1 μM PCNA\(_{WC} \) or mutants, 2.5 μM ptDNA, 0.5 mM ATP, and 10 μM MDCC-PBP.
2.3.6 A kinetic model of the impact of PCNA-ptDNA contacts during and after clamp loading

In order to determine the rate constants of transient events in the reaction all the experimental data were fit simultaneously to a current model of the PCNA loading mechanism using KinTek Explorer (Sakato, Zhou et al. 2012). The following is a description of the analysis for PCNA\textsubscript{WC} and PCNA\textsubscript{WC-R80A}, a representative of the domain I mutants that exhibit the most significant defects (data shown in Fig. 2.10; kinetic scheme shown in Fig. 2.11A). Fig. 2.10A and 10B provide a visual summary of the sequence of events following RFC incubation with ATP and PCNA\textsubscript{WC} or PCNA\textsubscript{WC-R80A}, respectively. The experimental data and the fits to each data set at $\Delta t = 0.02, 0.5, 2$ s with PCNA\textsubscript{WC} and PCNA\textsubscript{WC-R80A} are shown for ptDNA binding/release (Fig. 2.10C, 2.10D), PCNA opening/closing (Fig. 2.10E, 2.10F) and Pi release (Fig. 2.10G, 2.10H), respectively. The fits are shown as black lines overlaying the data and the corresponding best-fit parameters are listed in Table 2.3.

The model begins with ATP binding to RFC with a bimolecular rate constant fixed at $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($k_1$) and dissociation rate at 100 s$^{-1}$ ($k_1^{-1}$). We assumed a fast $k_1$ since the ATPase rates are independent of ATP concentration in our experiments (500 $\mu$M ATP), and $k_1^{-1}$ was based on the measured affinity of RFC for ATP$\gamma$S ($K_d \sim 1\mu$M) (Gomes, Schmidt et al. 2001; Chen, Levin et al. 2009; Sakato, O'Donnell et al. 2012). The same parameters were fixed for mutant PCNA, since this step is independent of interaction with ptDNA. The next step is PCNA binding to RFC (since PCNA accelerates ATP-induced RFC activation), and the rate was fixed at the reported value...
of $2 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($k_2$) (Thompson, Marzahn et al. 2011). The dissociation rate was fixed at 10 s$^{-1}$ for a $K_d$ of 0.05 µM (assuming that the $K_d$ prior to activation is similar to that in the absence of ATP, reported as 0.04 µM) (Thompson, Marzahn et al. 2011). The same parameters were fixed for mutant PCNA. The next step is ATP-induced activation of the complex. $k_3$ and $k_\text{-3}$ were allowed to float during data fitting, but linked to maintain an equilibrium constant of 0.02 ($1/K_3 = k_\text{-3}/k_3$), resulting in a net $K_d$ of 1 nM ($K_d \times 1/K_3$), the reported value for the activated *RFC•ATP•PCNA complex (Thompson, Marzahn et al. 2012) (Fig. 2.11A; * denotes ATP-activated RFC). The fit yielded $k_3 = 13 \text{ s}^{-1}$ and $k_\text{-3} = 0.26 \text{ s}^{-1}$ for PCNA$_\text{WC}$ and identical values for PCNA$_\text{WC}$-R80A. The next step, PCNA opening, was linked to RFC activation during fitting and yielded $k_4 = 3.2 \text{ s}^{-1}$ for both PCNA$_\text{WC}$ and PCNA$_\text{WC}$-R80A. Thus the net rate constant is $2.5 \text{ s}^{-1}$ for *RFC•ATP•PCNA$_\text{open}$ complex formation [$k_\text{net} = k_3 k_4/(k_3 + k_4 + k_\text{-3})$], similar to the measured PCNA opening rate (2.3 s$^{-1}$ in Fig. 2.2A; 2.2 s$^{-1}$ in (Zhuang, Yoder et al. 2006)). The reverse rate ($k_\text{-4}$) was set to zero since fitting yielded a very low value and *RFC•ATP•PCNA$_\text{open}$ complex is stable until after ATP hydrolysis (note: total FRET efficiency $E_T$ was set to 0.95 for closed PCNA and 0.75 for open PCNA for both PCNA$_\text{WC}$ and PCNA$_\text{WC}$-R80A during data fitting; Table 2.1, Fig. 2.2).

We have shown previously that ATP-activated RFC binds ptDNA rapidly without PCNA or with closed, partially open or open PCNA (Sakato, Zhou et al. 2012). With PCNA$_\text{WC}$, data fitting yielded rate constants of $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($k_5$) and $1.2 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($k_6$) for ptDNA binding to *RFC•ATP•PCNA and
*RFC•ATP•PCNA\textsubscript{open} complexes, respectively (Fig. 2.10C; Table 2.3), consistent with the 14 s\(^{-1}\) rate measured at 0.1 µM RFC (Fig. 2.5A). At this stage a striking difference is observed between PCNA\textsubscript{WC} and PCNA\textsubscript{WC-R80A}. ptDNA binds

*RFC•ATP•PCNA\textsubscript{WC-R80A} complex at 0.17 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_5\)) and

*RFC•ATP•PCNA\textsubscript{WC-R80A}\textsubscript{open} complex at 0.4 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_6\)), which are 6-fold and 3-fold slower rates, respectively, than observed for PCNA\textsubscript{WC} (Fig. 2.10D; Table 2.3). ptDNA binding rates were similarly slow for the other domain I mutants—0.22 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_5\)) and 0.44 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_6\)) for PCNA\textsubscript{WC-R14A}, and 0.15 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_5\)) and 0.23 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_6\)) for PCNA\textsubscript{WC-K20A}. Primer-template DNA dissociation rates, \(k_5\) and \(k_6\), were linked to corresponding binding rates to maintain the measured \(K_d\) of ~ 2 nM (Fig. 2.3). The relative change in signal for free:bound ptDNA was set at 1:1.8 during data fitting for both clamps, based on the fluorescence intensity of ptDNA\textsubscript{TAMRA} alone and bound to RFC•ATP\gamma S•PCNA complexes with PCNA\textsubscript{WC} and the mutants. Consistent with the milder effect of domain II mutations, ptDNA binding rates were 1.2 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_5\)) and 0.9 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_6\)) for PCNA\textsubscript{WC-K146A} (comparable to PCNA\textsubscript{WC}), and 0.5 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_5\)) and 0.7 \(x\) 108 M\(^{-1}\) s\(^{-1}\) (\(k_6\)) for PCNA\textsubscript{WC-R149A} (intermediate between PCNA\textsubscript{WC} and domain I mutants). At this point, the assembly phase of the reaction is complete. The mutant data reveal that eliminating a single PCNA-DNA contact in domain I (three contacts per clamp) can decelerate formation of the critical *RFC•ATP•PCNA\textsubscript{open}•ptDNA complex, particularly by disrupting ptDNA binding-induced stimulation of PCNA.
The next step in the reaction, ATP hydrolysis, was set at an irreversible rate of 25 s\(^{-1}\) \((k_7)\), in keeping with the experimentally measured range of 20 – 50 s\(^{-1}\) for PCNA (Chen, Levin et al. 2009) (allowing \(k_7\) to float during data fitting yields a rate of 17 – 30 s\(^{-1}\)). After ATP hydrolysis, a slow step in the reaction limits Pi release, as indicated by the lag phase in the data (Fig. 2.9A). PCNA closure occurs during this lag, as reported by increase in PCNA\(^{AEDANS}\) FRET (Fig. 2.10A). Subsequent complex dissociation (PCNA•ptDNA release), as reported by decrease in ptDNA\(^{TAMRA}\) fluorescence, appears concurrent with the burst of Pi release (Fig. 2.10A). Thus the model includes PCNA closing after ATP hydrolysis, and the fit yields a rate of 5.1 s\(^{-1}\) \((k_8)\) for PCNA\(_{WC}\) (Fig. 2.10E; Table 2.3). PCNA\(_{WC}\)-R80A closure around ptDNA is slightly slower at 2.8 s\(^{-1}\), suggesting that this step is also affected by loss of a PCNA-DNA contact (Fig. 2.10F; Table 2.3). Clamp closure rates are 3 s\(^{-1}\) and 3.3 s\(^{-1}\) for the other domain I mutants, PCNA\(_{WC}\)-R14A and PCNA\(_{WC}\)-K20A, respectively. Note that these rates are consistent with the RFC deactivation/clamp closure rates obtained from the simple model of ptDNA binding/release (Table 2.2). In case of domain II mutants the clamp closure rates were 5.1 s\(^{-1}\) for PCNA\(_{WC}\)-K146A (comparable to PCNA\(_{WC}\)) and 4.1 s\(^{-1}\) for PCNA\(_{WC}\)-R149A (intermediate between PCNA\(_{WC}\) and domain I mutants). The remaining product dissociation steps, PCNA•ptDNA release \((k_9)\) and Pi release \((k_{10})\), were fixed at a high rate of 500 s\(^{-1}\) in the model for both clamps since these events could simply be limited by the preceding slow closure of PCNA (allowing these parameters to float during data fitting yielded rate constants \(> 200\) s\(^{-1}\)). Thus, for PCNA\(_{WC}\), relatively
rapid clamp closure (Fig. 2.10E) is followed by correspondingly rapid Pi release (Fig. 2.10G) and PCNA•ptDNA release (Fig. 2.10C), whereas for PCNA<sub>WC-R80A</sub>, relatively slow clamp closure (Fig. 2.10F) is followed by correspondingly slow Pi release (Fig. 2.10H) and PCNA•ptDNA release (Fig. 2.10D). ADP release rates are completely unknown, hence this step is not included in the model.

The catalytic cycle is now complete, but two additional events were included in the model to account for experimental conditions and data. A PCNA•ptDNA dissociation step; i.e., PCNA slipping off linear ptDNA, was added to allow recycling of these substrates in the steady state phase of the reaction. The fitting yields a dissociation rate of 0.7 s<sup>-1</sup> (k<sub>11</sub>) for PCNA<sub>WC</sub> (the reverse rate, k<sub>-11</sub> was set to zero since there is no measure of closed PCNA slipping onto linear DNA). Interestingly, the dissociation rate for PCNA<sub>WC-R80A</sub> was 2.5-fold higher at 1.8 s<sup>-1</sup>, which suggests that loss of a PCNA-DNA contact enables the mutant to move on ptDNA and slip off faster than wild type PCNA. Faster ptDNA release enables re-binding by excess RFC in the reaction, contributing to more ptDNA bound with PCNA<sub>WC-R80A</sub> compared to PCNA<sub>WC</sub> in steady state (Fig. 2.10C, 2.10D). The dissociation rate was faster (1.5 s<sup>-1</sup>) for PCNA<sub>WC-R14A</sub> and PCNA<sub>WC-K20A</sub> as well. In case of domain II mutants, the dissociation rates were 1 s<sup>-1</sup> for PCNA<sub>WC-K146A</sub> and 0.8 s<sup>-1</sup> for PCNA<sub>WC-R149A</sub>, closer to the rate with PCNA<sub>WC</sub>. Finally, PCNA isomerization was included in the model to better fit a second slow phase observed in PCNA opening/closing kinetics. We have proposed previously that the clamp partitions between different conformations that bind RFC with high or low affinity (PCNA and **PCNA in Fig.
2.11A, respectively). The fit yields similar rate constants of 21 s\(^{-1}\) \(k_{12}\) and 0.9 s\(^{-1}\) \(k_{12}\) for PCNA\(_{WC}\) and 17 s\(^{-1}\) \(k_{12}\) and 0.3 s\(^{-1}\) \(-k_{12}\) for PCNA\(_{WC}\)-R80A. Inclusion of this step is speculative; however, evidence from MD simulations indicates that clamps transiently access multiple conformations, one or more of which may be bound preferentially by the clamp loader (Adelman, Chodera et al. 2010; Tainer, McCammon et al. 2010). Finally, a scaling factor representing the number of ATPase sites per RFC complex was allowed to float during data fitting and indicated a burst of 2.7 ATP molecules hydrolyzed rapidly in one catalytic turnover. This value is consistent with previous kinetic and structural studies of various clamp loaders indicating that 3 ATPase active sites are necessary and sufficient for loading a clamp onto DNA (Schmidt, Gomes et al. 2001; Williams, Snyder et al. 2004; Kelch, Makino et al. 2011; Sakato, O'Donnell et al. 2012; Sakato, Zhou et al. 2012).
Figure 2.10 Global fitting of kinetic data to a minimal mechanism of PCNA loading. Overlay of kinetic data for (A) PCNA$_{WC}$ and (B) PCNA$_{WC}$-R80A mutant from DNA binding/release, PCNA opening/closing, and Pi release experiments provides a visual of rapid ptDNA binding to RFC•ATP•PCNA complex (formed during pre-incubation, $\Delta t = 2$ s), which triggers ATP hydrolysis (not shown), PCNA closure, Pi release, PCNA•ptDNA dissociation and catalytic turnover. Kinetic data measured at $\Delta t = 0.02$, 0.5 and 2 s for (C, D) ptDNA binding/release, (E, F) PCNA opening/closing, and (G, H) Pi release for PCNA$_{WC}$ and PCNA$_{WC}$-R80A, respectively, were all fit simultaneously to the model described in Fig. 2.11 and Table 2.3. The black lines are simulations generated by the model based on parameters listed in Table 2.3.
Figure 2.10 Global fitting of kinetic data to a minimal mechanism of PCNA loading.
Table 2.3. Best-fit parameters for the PCNA loading mechanism

<table>
<thead>
<tr>
<th>Parameters (units)</th>
<th>PCNA&lt;sub&gt;WC&lt;/sub&gt;</th>
<th>PCNA&lt;sub&gt;WC-R80A&lt;/sub&gt;</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (µM&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;) / $k_{-1}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>100 / 100</td>
<td>100 / 100</td>
<td>ATP binding / dissociation</td>
</tr>
<tr>
<td>$K_{d1}$ (µM)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$k_2$ (µM&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;) / $k_{-2}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>200 / 10</td>
<td>200 / 10</td>
<td>PCNA binding / dissociation</td>
</tr>
<tr>
<td>$K_{d2}$ (µM)</td>
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<td>0.05</td>
<td></td>
</tr>
<tr>
<td>$^{a} k_3$ (s&lt;sup&gt;-1&lt;/sup&gt;) / $^{a} k_{-3}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>13 ± 0.2 / 0.26 ± 0.004</td>
<td>13 ± 0.6 / 0.26 ± 0.01</td>
<td>RFC activation</td>
</tr>
<tr>
<td>$^{a} k_4$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.2 ± 0.05</td>
<td>3.2 ± 0.15</td>
<td>PCNA opening</td>
</tr>
<tr>
<td>$^{b} k_3$ (µM&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;) / $^{b} k_{-3}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>105 ± 4 / 0.2 ± 0.008</td>
<td>17.5 ± 1.3 / 0.03 ± 0.002</td>
<td>RFC•ATP•PCNA ptDNA binding / dissociation</td>
</tr>
<tr>
<td>$^{c} k_6$ (µM&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;) / $^{c} k_{-6}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>117 ± 4 / 0.2 ± 0.008</td>
<td>40 ± 0.7 / 0.08 ± 0.001</td>
<td>RFC•ATP•PCNA&lt;sub&gt;open&lt;/sub&gt; ptDNA binding / dissociation</td>
</tr>
<tr>
<td>$^{c} K_{d5}$ or $^{c} K_{d6}$ (µM)</td>
<td>0.002</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>$k_7$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>ATP hydrolysis</td>
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<td>$k_8$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.1 ± 0.05</td>
<td>2.8 ± 0.04</td>
<td>RFC deactivation and PCNA closure</td>
</tr>
<tr>
<td>$k_9$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>500</td>
<td>500</td>
<td>PCNA•ptDNA release from RFC</td>
</tr>
<tr>
<td>$k_{10}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>500</td>
<td>Pi release</td>
</tr>
<tr>
<td>$k_{11}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>1.8 ± 0.08</td>
<td>PCNA•ptDNA dissociation</td>
</tr>
<tr>
<td>$^{d} k_{12}$ (s&lt;sup&gt;-1&lt;/sup&gt;) / $^{d} k_{-12}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>21 ± 0.6 / 0.9 ± 0.02</td>
<td>17 ± 0.9 / 0.32 ± 0.02</td>
<td>PCNA / PCNA** equilibrium</td>
</tr>
<tr>
<td>N</td>
<td>2.7 ± 0.01</td>
<td>2.3 ± 0.03</td>
<td>Number of ATP hydrolyzed rapidly per RFC</td>
</tr>
</tbody>
</table>

<sup>a, b, c, d</sup> Each label specifies parameters that were linked during global fitting analysis.

<sup>e</sup> Standard error values are shown for parameters allowed to float during data fitting.
**Figure 2.11 A clamp loading model showing the steps influenced by contact between PCNA and DNA.** (A), the schematic depicts each step in the mechanism used for global analysis of the kinetic data. Activated RFC is designated as *RFC*; a separate step shows the equilibrium between loading-active (PCNA) and loading-inactive (**PCNA**) clamp conformations; steps where the reverse rate constants are expected to be very small or are completely unknown are considered irreversible. (B), a cartoon depicting the clamp loading reaction. I–III, binding of two to three ATP molecules to RFC (I) facilitates PCNA and additional ATP binding toward slow formation of active *RFC*-ATP-PCNA (II) and *RFC*-ATP-PCNA\textsubscript{open} complexes (III). IV and V, ptDNA binds both intermediate complexes (IV), leading to an *RFC*-ATP-PCNA\textsubscript{open}-ptDNA complex that is ready for ATP hydrolysis (V). VI and VII, next, another slow step in the reaction designated as RFC deactivation leads to closure of PCNA around DNA (VI) and release of PCNA-ptDNA and P\textsubscript{i} products and catalytic turnover (VII). VIII, PCNA can slip off the linear ptDNA, recycling both substrates in steady state. Slow steps in the mechanism are indicated by orange arrows. Steps IV (ptDNA binding and priming RFC for ATP hydrolysis) and VI (PCNA closure following ATP hydrolysis) are suppressed, and step VIII (PCNA-ptDNA dissociation) is enhanced by mutation of N-terminal domain I cationic residues in PCNA.
Figure 2.11 A clamp loading model showing the steps influenced by contact between PCNA and DNA.

A  Minimal mechanism of RFC-catalyzed PCNA loading on DNA

\[
\begin{align*}
RFC + ATP & \underset{k_{-1}}{\rightleftharpoons} RFC\cdot ATP \underset{k_2}{\longrightarrow} RFC\cdot ATP\cdot PCNA \underset{k_3}{\rightarrow} RFC\cdot ATP\cdot PCNA_{\text{open}} \\
PCNA & \underset{k_4}{\longrightarrow} RFC\cdot ATP\cdot PCNA_{\text{open}} \cdot DNA \quad \underset{k_6}{\longrightarrow} RFC\cdot ATP\cdot PCNA_{\text{open}} \cdot DNA
\end{align*}
\]

B  Effects of altering PCNA-DNA contacts on the clamp loading mechanism
2.4 Discussion

In the *E. coli* β•DNA crystal structure, arginine and lysine residues as well as glutamine and histidine directly contact the DNA backbone, inducing a 22° tilt (Georgescu, Kim et al. 2008). As in the mutational study of human PCNA (Fukuda, Morioka et al. 1995), substitution of β R24 and/or Q149 with alanine inhibited initiation of processive DNA synthesis by pol III; in this case the study also showed that the mutations inhibited loading of β on ptDNA (Georgescu, Kim et al. 2008). The authors speculated that the underlying basis of the defect was disruption of β closure around DNA due to loss of these contacts. Also, as in the MD study of human PCNA (Ivanov, Chapados et al. 2006), the authors posited that asymmetric positioning of DNA within the clamp may enable its switching between proteins bound to different subunits. Structural alignment of the β dimer and *S. cerevisiae* PCNA trimer shows high correspondence among cationic residues that may contact duplex DNA: β Q15 with PCNA K13 and K14; β R24 with PCNA K20; β R73 with PCNA K77 and K210; β R80 with PCNA R80 and K217; β K198 with PCNA K77 and K210; β R205 with PCNA R80 and K217 (Fig. 2.1D). In EM structures of *P. furiosus* Pol B•PCNA•DNA (Mayanagi, Kiyonari et al. 2011) and DNA ligase•PCNA•DNA (Mayanagi, Kiyonari et al. 2009) complexes, the duplex passing through PCNA is tilted by 13° and 16°, respectively, and positioned asymmetrically within the ring. For the latter structure, the authors proposed that contacts between K81, K84 and K209 in one PCNA monomer and DNA help stabilize the orientation (Mayanagi, Kiyonari et al. 2009). According to structural alignment, *P. furiosus* PCNA residues K78, K81 and K209...
correspond to *S. cerevisiae* PCNA K77, R80 and K217, respectively (Fig. 2.1D). The finding that *S. solfataricus* Rad9-Rad1-Hus1 clamp (9-1-1) can bind DNA ligase, DNA polymerase and FEN1 simultaneously (Dionne, Nookala et al. 2003), and a crystal structure of three human flap endonuclease molecules (FEN1) bound to one PCNA clamp (Sakurai, Kitano et al. 2005), lend further credence to the hypothesis that up to three proteins can bind a trimeric clamp and wait their turn to act on DNA. A recent EM and MD analysis of human FEN1-PCNA and FEN1-9-1-1 binary complexes indicates highly flexible linkage between the proteins in the absence of DNA, allowing them to adopt different relative orientations (Querol-Audi, Yan et al. 2012). In the DNA-bound ternary complexes, however, the proteins are stabilized in one orientation with tilted DNA passing through the clamp and into the FEN1 active site. The authors suggested that contacts with PCNA help anchor DNA and, consequently, FEN1 in a conformation that enables catalysis (Querol-Audi, Yan et al. 2012).

Detailed understanding of the mechanism of PCNA loading by RFC enabled us to test whether and how a specific contact between clamp and DNA might influence the actions of another protein that works with the clamp on DNA—in this case the clamp loader. The assessment is complicated by the fact that the other protein also binds DNA. In case of clamp loaders, the central chamber of the 5-subunit complex presents a large DNA binding surface (Fig. 2.1A, 2.1B); hence we did not anticipate substantive reduction in the DNA binding affinity of RFC•ATP•PCNA complex on removal of a single cationic residue in PCNA (Fig.
2.3). The steady state RFC ATPase rate was also not expected to be informative, since the $k_{\text{cat}}$ values are similar with or without PCNA (Chen, Levin et al. 2009) and may be limited by events that are independent of PCNA-DNA interaction. However, with knowledge of kinetic and thermodynamic parameters governing distinct steps in the clamp loading reaction (Sakato, Zhou et al. 2012), we could directly measure any effects of disrupting individual arginine and lysine contacts on events related to DNA binding and release. It was necessary to study single PCNA mutants because of the unresolved question of why loss of any one of the nine cationic residues per human PCNA monomer results in near complete loss of processive DNA replication (Fukuda, Morioka et al. 1995), and whether the functional significance of these individual contacts for DNA polymerase might apply to other PCNA binding proteins as well. *S. cerevisiae* PCNA has nine arginine/lysine residues in the same positions as in human PCNA (Fig. 2.1D). We mutated a subset of six of these to alanine (R14, K20, R80, K146, R149, and K217) and measured the impact on the clamp loading mechanism.

The following is a summary of the clamp loading mechanism based mainly on kinetic and structural analysis of *S. cerevisiae* proteins, supplemented with analogous information about *E. coli* and bacteriophage T4 proteins. Prior to ATP binding, the clamp loader subunits are in a relatively disorganized conformation that does not complement an open clamp or DNA (*E. coli* γ complex structure) (Jeruzalmi, O'Donnell et al. 2001). ATP binding initiates activation of the clamp loader and the AAA+ modules begin to organize in a right-handed spiral (Fig. 2.11B, I) that can
bind a clamp at its base (Fig. 2.11B, II) (S. cerevisiae RFC•ATPγS•PCNA\textsubscript{closed} structure) (Bowman, O'Donnell et al. 2004). The interaction stimulates completion of ATP binding and accelerates clamp loader activation to a conformation wherein all subunits hold the clamp in an open spiral (Fig. 2.11B, III) (S. cerevisiae RFC•ATPγS•PCNA\textsubscript{open} MD structure) (Tainer, McCammon et al. 2010). Primer-template DNA binds the clamp loader•ATP•clamp complex rapidly and with high affinity (Fig. 2.11B, IVa and IVb). Primer-template DNA binding induces and accelerates clamp opening (Sakato, Zhou et al. 2012), implying that the single-stranded template can slip into the complex before the clamp is fully open. Indeed structural data thus far suggest that the gaps in both the clamp loader and clamp may not open wide enough for direct entry of duplex DNA (Kelch, Makino et al. 2011). Once inside, ptDNA can thread upward through the central chamber of the complex. Cationic residues and amino-terminal helix dipoles within the clamp loader track the template strand of an A-form duplex (akin to an RNA primer:DNA template hybrid). The 3’ end of the primer is blocked at the roof of the chamber and the template strand can bend and escape through the gap between the clamp loader subunits (T4 gp44/62•ADP-BeF\textsubscript{3}•gp45•ptDNA and E. coli γ complex•ADP-BeF\textsubscript{3}•ptDNA structures) (Simonetta, Kazmirski et al. 2009; Kelch, Makino et al. 2011). Cationic residues in the open clamp track the minor groove of duplex DNA (T4 gp44/62•ADPBeF\textsubscript{3}•gp45•ptDNA structure) (Kelch, Makino et al. 2011). DNA binding locks the AAA+ modules into catalytically active conformation triggering ATP hydrolysis (Fig. 2.11B, V); S. cerevisiae RFC and E. coli γ complex kinetics
indicate three ATP molecules are hydrolyzed rapidly per catalytic turnover (Williams, Snyder et al. 2004; Sakato, Zhou et al. 2012). ATP hydrolysis breaks the symmetry of the AAA+ spiral (T4 gp44/62•ADPBeF₃• ADP•gp45•ptDNA structure) (Kelch, Makino et al. 2011), causing deactivation of the clamp loader and disrupting its interactions with both ptDNA and the open clamp. As a result, the clamp closes around ptDNA (Fig. 2.11B, VI) and the complex dissociates, releasing the clamp•ptDNA and Pi products (Fig. 2.11B, VII); E. coli γ complex kinetics indicate that Pi is released prior to clamp•ptDNA (Anderson, Thompson et al. 2009). The clamp can slide off the ends of short linear ptDNA used in vitro, and both substrates become available for subsequent catalytic turnovers in the steady state phase (Fig. 2.11B, VIII). Notably, this recycling step provides an indirect measure of clamp movement on DNA. The model also includes an equilibrium between PCNA conformations wherein one form preferentially binds the clamp loader (inclusion of this step yields better fitting of all the kinetic data, and MD studies of human PCNA indicate interchange between multiple conformations) (Tainer, McCammon et al. 2010).

Of the six cationic residues in S. cerevisiae PCNA examined in this study (Fig. 2.1D), R14A, K20A or R80A mutations in the N-terminal domain I slowed binding of ptDNA to RFC•ATP•PCNA (Fig. 2.5A, and Fig. 2.11B, IV). Primer-template DNA binding prior to full PCNA opening was disrupted the most (Table 3.3, Fig. 2.11B, IVa). We interpret this result to mean that after template strand entry, these contacts are important for guiding ptDNA movement upward through the
ptDNA after ATP hydrolysis. The domain I mutations also slowed PCNA closure around from the clamp loader (Fig. 2.7A and Fig. 2.11B, VI). All three mutants display correspondingly slower complex dissociation (Fig. 2.5A) and Pi release (Fig. 2.9A). The third effect of the domain I mutations was to hasten clamp slippage off ptDNA. This result indicates that loss of even one of the contacts (three per homotrimer) can alter the position and movement of the clamp on DNA, which in turn could affect the activities of clamp bound proteins. Thus, for the analogous K14A, K20A and K80A mutants of human PCNA, the loss of processive pol δ activity can be explained by disruption of both clamp loading and position on DNA (Fukuda, Morioka et al. 1995). Of the two *S. cerevisiae* PCNA C terminal domain II mutations examined in this study, mutant K146A exhibited similar clamp loading kinetics as wild type PCNA and the differences were < 2-fold for R149A (Fig. 2.5B, 2.7B, 2.9B). For the analogous human PCNA mutants R146A and R149A, a defect after clamp loading (e.g., in productive interaction with the polymerase) may underlie the loss of processive pol δ activity (Fukuda, Morioka et al. 1995). Contacts between the PCNA N terminal domain I and DNA have greater mechanistic significance, suggesting that positional bias of DNA toward this domain is important for clamp function.

To summarize, we tested a popular hypothesis that direct contacts between circular clamps and DNA are important determinants of clamp position and motion on DNA, which in turn could modulate the activities of clamp-binding proteins at their sites of action on DNA. By measuring the RFC catalyzed PCNA loading
mechanism at high resolution, it was possible to assess the functional significance of individual ionic interactions between the clamp and DNA. The results confirm asymmetric DNA binding within the clamp that modulates the function of a clamp-binding protein and influences clamp movement on DNA. PCNA mutants that can be loaded but exhibit altered dynamics on DNA present useful tools for investigating how clamps regulate and coordinate the functions of other DNA metabolic proteins.
3.1 Introduction

The clamp loading reaction occurs in two main stages: (a) Assembly—on binding ATP, the loader forms a complex containing an open clamp and primer-template (ptDNA), and (b) Disassembly—on ATP hydrolysis, the loader releases the clamp-ptDNA product (Chen, Levin et al. 2009; Sakato, Zhou et al. 2012). Both clamp and clamp loader serve essential functions in DNA replication, repair and recombination, and are therefore conserved through evolution (Yao and O'Donnell 2012). Clamp loaders are composed of five subunits in a circular arrangement, with a gap between the first and the fifth subunit. Each subunit has three domains (domains I-III). The N-terminal domains I and II are AAA+ ATPase modules, which have Walker A and B motifs for ATP binding and hydrolysis as noted in the Introduction chapter (Kelch, Makino et al. 2012; Yao and O'Donnell 2012). Domain I and II are arranged in a right–handed spiral with an overall pitch that closely matches double-stranded DNA. The C-terminal domains III tightly pack into a full circle to hold the pentamer complex together at the top.

The *S. cerevisiae* RFC•ATPγS•PCNA crystal structure shows that the RFC subunits form a chamber and on the inside these subunits present several positive charged residues that are proposed to contact the duplex portion of DNA in ptDNA.
Three of these residues are conserved in clamp loaders from different branches of life (Bowman, O'Donnell et al. 2004). Table 3.1 shows these conserved residues from each subunit of the clamp loader in three major variants: \textit{S.cerevisiae} RFC, \textit{E.coli} γ-complex and bacteriophage T4 gp44/62; note: in \textit{E.coli} γ-complex, δ, γ, γ, γ and δ' subunits correspond to the A, B, C, D and E subunits in \textit{S.cerevisiae} RFC. In T4 gp44/62, gp62 is at the A position and the four identical gp44 subunits are in B, C, D and E position. Further structural details are shown in the Section 1.3, Introduction chapter. The δ subunit in \textit{E.coli} and gp62 in subunit T4 are highly divergent in sequence and structure, therefore we don’t find conserved DNA binding residues in these two subunits. In a previous study, all three conserved positive residues in B, C and D subunits of RFC were mutated to alanine and all three residues in each subunit were only mutated to alanine, and these mutant complexes were found to be defective in DNA binding (Johnson, Yao et al. 2006; Yao, Johnson et al. 2006). However, since these were partial analyses of multiple-residue mutants, the study did not answer how each residue contributes to DNA binding by the RFC clamp loader. The crystal structure of \textit{E. coli} γ complex bound to DNA (γ complex•ADPBeF$_3$•ptDNA) indicates all three residues within the three γ subunits and the δ' subunit contact the DNA backbone and may help organize it in a tight right-handed spiral form to match DNA double helix as shown Fig. 3.1 (Simonetta, Kazmirski et al. 2009). In the absence of DNA, the γ complex is not as well organized into a spiral chamber (Jeruzalmi, O'Donnell et al. 2001). It is not clear if all of the contacts are necessary
for clamp loader organization and DNA binding and if one or more of these may have different roles in the clamp loading reaction.

A recent structure of T4 clamp loader in complex with the clamp and DNA (gp44/62•ADPBeF₃•gp45•ptDNA) also shows tight spiral organization of the gp44/62 subunits and the three conserved residues in contact with the backbone (Kelch, Makino et al. 2011). In addition of this study the authors proposed an allosteric switch mechanism to explain how clamp loader ATPase activity is triggered by binding of ptDNA. By comparing the structure of T4 clamp loader bound to DNA and *S. cerevisiae* clamp loader without DNA (Fig. 3.2) (Bowman, O'Donnell et al. 2004), they hypothesized that the first residue in the sequence among those three conserved DNA binding residues (referred to as “switch residue”) undergoes a conformational change on binding DNA which in turn affects the conformation of a catalytic glutamate residue in the conserved Walker B motif of the ATPase active site. In the absence of DNA (*S. cerevisiae* RFC•ATPγS•PCNA structure), the switch residue interacts with the glutamate and holds it in an inactive conformation (Arg383 in A subunit, Arg84 in B subunit, Arg88 in C subunit and Arg101 in D subunit) (Fig 3.3A). Upon ptDNA binding (T4 gp44/62•ADPBeF₃•gp45•ptDNA structure), the corresponding switch residue (R80 in three gp44 subunits) in T4 interacts with DNA instead and the glutamate is free to coordinate a water molecule for hydrolysis of the γ-phosphate of ATP (Kelch, Makino et al. 2011) (Fig. 3.3B). Similarly, on comparing the *E. coli* clamp loader γ complex structures with and without DNA, the first DNA binding residue (Arg100) in γ subunit is found to be in a different conformation,
bound to DNA in the former case and pointed toward glutamate in the latter case (Jeruzalmi, O'Donnell et al. 2001; Simonetta, Kazmirski et al. 2009); note: E subunit in *S. cerevisiae* RFC, gp62 and gp44 (E position) in T4 gp44/62, and δ and δ’ subunits in *E. coli* are not capable of ATP hydrolysis, so we did not consider switch residues in these subunits in our current study.

Therefore, according to this hypothesis, one particular DNA binding residue in the clamp loader controls switching of the clamp loader from low ATP hydrolysis state for RFC ($k_{cat} = 0.05 \text{ s}^{-1}$) to high ATP hydrolysis state for RFC•DNA ($k_{cat} = 0.6 \text{ s}^{-1}$). However, this very specific and important hypothesis is based on limited crystal structures from different clamp loaders of three different organisms. Therefore, I decided to test this important hypothesis in greater detail. My approach was to first make single mutants of this DNA binding residue in B, C and D subunits in *S. cerevisiae* RFC and examine their affects on DNA binding activity and how they may control RFC ATPase activity in the clamp loading reaction; note: ATP hydrolysis by the A subunit in *S. cerevisiae* RFC is not essential for PCNA clamp loading and the corresponding subunit in *E. coli* and T4 clamp loaders does not bind the duplex portion of ptDNA, therefore, I focused on the RFC-B, RFC-C and RFC-D subunits first.
Table 3.1 Three conserved DNA binding residues within each subunit of clamp loaders from different organisms. The residues showed in bold are proposed to function in the “allosteric switch mechanism” hypothesis for DNA binding-induced ATP hydrolysis by the clamp loader.

<table>
<thead>
<tr>
<th></th>
<th>S. cerevisiae RFC</th>
<th>E. coli γ-complex</th>
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<tr>
<td>A subunit</td>
<td>R383 R434 K462</td>
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<td>N/A</td>
</tr>
<tr>
<td>B subunit</td>
<td>R84 R90 K149</td>
<td>R100 R105 K161</td>
<td>R80 R85 K111</td>
</tr>
<tr>
<td>C subunit</td>
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<td>R100 R105 K161</td>
<td>R80 R85 K111</td>
</tr>
<tr>
<td>D subunit</td>
<td>R101 R107 R175</td>
<td>R100 R105 K161</td>
<td>R80 R85 K111</td>
</tr>
<tr>
<td>E subunit</td>
<td>R106 R114 K182</td>
<td>K85 R94 T121</td>
<td>R80 R85 K111</td>
</tr>
</tbody>
</table>
Figure 3.1 Crystal structure of three $\gamma$ and $\delta'$ subunits (green) from *E. coli* clamp loader in complex with DNA (gold). The three conserved DNA binding residues are labeled in red (first DNA binding residue in sequence), blue (second) and purple (third). From left to right, three $\gamma$ and $\delta'$ subunits are corresponding to the E, D, C and B subunits in eukaryotic clamp loaders; the relative position of five subunits is shown in a cartoon.
Figure 3.2 Crystal structures of clamp loader from *S.cerevisiae* and T4 (clamp and DNA are colored in grey and gold) (image adapted from (Kelch, Makino et al. 2012)).

A. *S.cerevisiae* clamp loader RFC bound with clamp PCNA

B. T4 clamp loader gp44/62 bound with clamp gp45 and DNA
**Figure 3.3 Illustration of “allosteric switch mechanism” hypothesis.** (A), *S. cerevisiae* clamp loader with switch in the “off” position (low ATP hydrolysis). The Walker B catalytic glutamate (orange) is held in an inactive conformation by a conserved basic residue termed the switch residue (Arg 383 in RFC-A; yellow) (B), T4 clamp loader bound to DNA with the switch in the “on” position (high ATP hydrolysis). The switch residue (Lys80 of gp44) releases the Walker B glutamate and binds instead to the DNA phosphate backbone. The catalytic glutamate is now free to access the active site and participate in catalysis (image adapted from (Kelch, Makino et al. 2012)).
3.2 Materials and Methods

3.2.1 Protein, DNA, and other reagents

Alanine mutations were introduced into the Arg 84 of pET16a-RFC4 gene and Arg 101 of pET16a-RFC2 gene, respectively using the QuikChange method (Stratagene); note: RFC-B, RFC-C and RFC-D are also named as RFC-4, RFC-3 and RFC-2. The primers used for mutation generation are: RFC-4$_{R84A}$, 5’- GAA CGC TTC AGA TGA CGC AGG TAT TGA TGT CGT C-3’ (sense), 5’-GAC GAC ATC AAT ACC TGC GTC ATC TGA AGC GTT C-3’; RFC-2$_{R101A}$, 5’- GTT GAA CGC TTC TGA CGA AGC TGG TAT CTC TAT TGT AAG AG-3’ (sense), 5’-CTC TTA CAA TAG AGA TAC CAG CTT CGT CAG AAG CGT TCA AC-3’ (antisense).

The PCR cycles were set based on the length of the plasmids and the nature of the mutation: 1 cycle at 95 °C for 1 min; 18 cycles with the following succession: 95 °C for 50 s, at 60 °C for 5 min, at 68 °C for 7 min 5 s; ending with 1 cycle at 68 °C for 15 min and cooling down to 4 °C.

To construct pET11a-RFC(4$_{R84A}$+3+2), plasmid pET11a-RFC(4+3+2) and plasmid pET16a-RFC4$_{R84A}$ were digested with SacI/AflII. The small fragment containing Arg 84 mutation in pET16a-RFC4$_{R84A}$ was ligated into the large fragment from pET11a-RFC(4+3+2) to form pET11a-RFC(4$_{R84A}$+3+2). To construct pET11a-RFC(4+3+2$_{R101A}$), plasmid pET11a-RFC(4+3+2) and plasmid pET16a-RFC2$_{R101A}$ were digested with ApaI/SnaBI. The small fragment containing Arg 101 mutation in pET16a-RFC2$_{R101A}$ was ligated into the large fragment from pET11a-RFC(4+3+2) to
form pET11a-\textit{RFC}(4+3+2_\textit{R101A}). All the fragments after digestion were gel-purified using gel extraction kit (QIAquick Company) before ligation.

To construct pET11a-\textit{RFC}(4+3_\textit{R88A}+2), alanine mutations were directly introduced into Arg 88 of pET11a-\textit{RFC}(4+3+2). The primers used for mutation generation are: 5’-CAC TGC ATC CGA TGA CGC AGG TAT TGA TGT GGT G-3’ (sense), 5’-CAC CAC ATC AAT ACC TGC GTC ATC GGA TGC ATT C-3’ (antisense). The PCR cycles were set based on the length of the plasmids and the nature of the mutation: 1 cycle at 95 °C for 1 min; 18 cycles with the following succession: 95 °C for 50 s, at 60 °C for 5 min, at 68 °C for 10 min; ending with 1 cycle at 68 °C for 10 min and cooling down to 4 °C. The open reading frames of all the mutated genes were confirmed by DNA sequencing.

All mutant RFC proteins were soluble and were purified by the wild type purification procedure (see Appendix C.2). Phosphate-binding protein (PBP) was purified and labeled with MDCC (N-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide, Molecular Probes, Inc.) as described in Appendix C.4 and C.5. Primer-template DNA or ptDNA\textsubscript{TAMRA} was prepared by annealing a 40-mer primer (or 40-mer primer 3’ labeled TAMRA) to a 65-mer template as described in Section 2.2.1 and 2.2.2, Chapter II. 37-mer DNA (5’-ATT TCC TTC AGC AGA TAG GAA CCA TAC TGA TTC ACA T-3’) was synthesized by Integrated DNA Technologies (IDT) and purified by 19% denaturing polyacrylamide gel electrophoresis as described in Appendix B.
3.2.2 Equilibrium binding affinity measurement of wild type or mutant RFC-DNA interaction

Wild type or mutant RFC binding to TAMRA-labeled ptDNA were measured using direct fluorescence of TAMRA on a FluoroMax-3 fluorimeter (Jobin-Yvon-Horiba, Edison, N.J.). Emission scans (565-590 nm) of ptDNA\textsubscript{TAMRA} ($\lambda_{\text{ex}} = 555$ nm; $\lambda_{\text{em}} = 582$ nm) were collected in quartz cuvette at 25 °C. An increasing volume of wild type or mutant RFC stock (3-7 $\mu$M) was titrated into a 250 ul DNA stock containing 1 nM DNA, 100 $\mu$M ATP\textsubscript{γS} at 100 mM NaCl in Buffer A (Appendix A) containing 0.05 mg/ml BSA to make up a 0 - 100 nM final concentration of RFC. An emission scan was performed following each addition of RFC. The peak value of fluorescence intensity obtained from the emission scan was plotted versus the concentration of RFC and the apparent dissociation constant ($K_D$) for the interaction obtained by fitting the data to a quadratic equation for a 1:1 interaction of a ligand with a macromolecule:

$$[D \cdot M] = F_0 + (F_m - F_0) \frac{((K_D + [D_t] + [M_t]) - ((K_D + [D_t] + [M_t])^2 - 4[D_t][M_t])^{1/2})/2[D_t])}{2[D_t]}$$

where D·M is the fraction of bound DNA, $F_0$ is TAMRA fluorescence in the absence of protein and $F_m$ is maximal TAMRA fluorescence, and $D_t$ and $M_t$ are total molar concentrations of DNA and RFC, respectively. The data were fit by non-linear regression using KaleidaGraph (Synergy Software).
3.2.3 Kinetics measurement of wild type or mutant RFC-DNA interaction in the absence/presence of PCNA

Kinetics of primer-template DNA binding/release to wild type and mutant RFC•ATP complex (-/+PCNA) were measured at 100 mM NaCl and 25 °C in Buffer A containing 0.05 mg/ml BSA, on a KinTek SF-2001 Stopped-Flow instrument. Primer-template DNA_TAMRA was excited at 535 nm and fluorescence emission was measured over time at > 550 nm using a 550 nm long-pass cut-off filter (Newport Corporation). Primer-template DNA_TAMRA is the same sequence as in Section 3.2.2, Chapter III. Three-syringe experiments were performed by pre-incubating 0.3 µM wild type or mutant RFC and 1.2 µM PCNA (If present) from syringe A and 1.5 mM ATP from syringe B for ∆t = 1, 3 and 8 s (in the absence of PCNA) or ∆t = 0.02, 0.05 and 3 s (in the presence of PCNA) before addition of 0.12 µM 40/65 nt ptDNA_TAMRA from syringe C (final ratio: 1:1:1; final concentrations of reactants are: 0.1 µM wild type or mutant RFC, 0.4 µM PCNA (if present), 500 µM ATP and 0.04 µM ptDNA_TAMRA). Three or more kinetic traces of 1000 data points over the reaction time were averaged in each experiment to generate raw data and baseline fluorescence from free ptDNA_TAMRA was divided to generate the corrected data. The data were fit to a double exponential function FL = A₁(1 - e⁻ᵏ₁ᵗ) + A₂(1 - e⁻ᵏ₂ᵗ) for initial estimation of rate constants, where FL is the normalized fluorescence, A₁ and A₂ are the amplitudes for up and down phase, k₁ and k₂ are rate constants for up and down phase, respectively, and t is reaction time.
3.2.4 Steady state ATPase activity measurement by wild type and mutant RFC

ATPase activity of RFC was analyzed by malachite green assay. This assay is based on the complex formed between malachite green molybdate and free orthophosphate under acidic conditions, and the formation of the green molybdophosphoric complex could be measured at 655 nm. The list below is the materials to prepare the color reagent:

1. 0.045% (w/v) malachite green hydrochloride (MG): 0.223 g malachite green hydrochloride + 50 ml H₂O.
2. 4.2% (w/v) ammonium molybdate in 4 M HCl (AM): 2.1 g ammonium molybdate + 17 ml 36% HCl + 33 ml H₂O.
3. 34% (w/v) sodium citrate: 22.2 g citric acid + around 20 ml H₂O + 50 ml 6 M NaOH; the solution was stirred in the cold room around 1 hour to cool down to the room temperature; then around 3-5 ml 10 N NaOH was added to lower the pH around 7.0 and the solution was left in the room temperature overnight; the next day, less than 1 ml 10 N NaOH was added into solution to adjust final pH to 8.0 and H₂O was added to make the final volume to 100 ml.
4. 1% (v/v) Triton X-100: 0.5 ml Triton X-100+ 50 ml H₂O.
5. 0.6 M PCA: 0.5ml 70 % (w/v) PCA + 9.5 ml H₂O.

MG:AM was mixed with 3:1 ratio in the falcon tube wrapped with aluminum foil and shaked in the cold room more than 30 min (the mixture could be stored in the 4 °C). Then the mixture was passed through by 0.2 µm tuffyn filter (Paul corporation) (after filtration, the mixture should be used within 5 days). Triton X-100
(stock: 1% (v/v)) was added into the mixture to make the final concentration of 0.17% (after adding Triton X-100, the mixture (MG/AM/TX) should be used within 4 hours).

The ATPase activity reaction was contained 0.5 µM RFC, 1 µM PCNA, 1 mM ATP in Buffer A at 25 °C. The reaction was started when RFC was added and at reaction time 300, 600, 900 and 1200 s, 30 µl reaction aliquot was transferred into another tube contained 30 µl 0.6 M PCA. The quenched reaction (30 µl reaction aliquot + 30 µl 0.6 M PCA) was vortexed at speed 10 for 10 s and left on ice; note, for RFC-D_{R101A} which has high ATPase activity, reaction was quenched at 30, 60, 90 and 120 s. 15 µl reaction aliquot at each time was transferred into another tube which contains 30 µl 0.6 M PCA+15 µl Buffer A. After all the reactions were finished, they were centrifuged at 2,000 rpm for 2 min and 50 µl from each reaction was transferred into 96 well microplate (Costar Company). Then, 8 x 100 µl MG/AM/TX was added into the wells containing 50 µl quenched reactions by using an 8-channel pipette and the samples were quickly swirled by 5 times using pipette tips and incubated for 40 s. Immediately after 40 s incubation, 8 x 12.5 µl 34% sodium citrate was added into the samples by 8-channel pipette to prevent the ATP hydrolysis by the color reagents and quickly swirled by 5 times. After that, the absorbance of the samples were measured by microplate reader (Bio-lab) at 655 nm with 5 s premix; note, once the sodium citrate was added, the sample should be measured between 10 and 20 min.

A Pi calibration curve was generated at the same time of each experiment. Pi stock was from Phosphorus Standard Solution (as KH₂PO₄, 650 µM) (Sigma-
Aldrich). 100 µl 0, 25, 50 and 75 µM (final concentration) of Pi was made in Buffer A. Then, 25 µl aliquot from each concentration was transferred in to microplate contained 25 µl 0.6 M PCA. The rest procedures were same as described above. The absorptions measured at 665 nm was plotted and against the corresponding Pi concentration and fit to a linear equation. The slop was used to calculate the concentration of Pi which is released from RFC catalyzed-ATP hydrolysis.

3.2.5 Kinetics of Pi release assays by wild type and mutant RFC

Kinetics of ATPase activity by wild type and mutant RFC in various conditions were measured by stopped-flow Pi release assays (Section 2.2.7, Chapter II) at 25 ºC in Buffer A plus 0.3 unit/ml PNPase and 0.2 mM 7-methylguanosine. MDCC-PBP was excited at 425 nm and fluorescence emission over time at > 450 nm was measured using a 450 nm long-pass cut-off filter (Newport Corporation). In the two-syringe experiments, 1.0 µM wild type or mutant RFC, 2 µM PCNA (if present) from syringe A was mixed rapidly with 1.0 mM ATP and 20 µM MDCC-PBP (final concentrations according to a 1:1 ratio: 0.5 µM wild type or mutant RFC, 1 µM PCNA (if present), 500 µM ATP and 10 µM MDCC-PBP). In the three-syringe experiments, 1.5 µM wild type or mutant RFC, 3 µM PCNA from syringe A and 1.5 mM ATP from syringe B were pre-incubated for Δt = 0.02, 0.05 and 3 s prior to addition of 7.5 µM ptDNA or ssDNA and 30 µM MDCC-PBP (final ratio: 1:1:1; final
concentrations of reactants are: 0.5 µM wild type and mutant RFC, 1 µM PCNA, 500 µM ATP, 2.5 µM ptDNA or ssDNA, and 10µM MDCC-PBP).
3.3 Results

3.3.1 Effect of mutating single residues in *S. cerevisiae* to alanine on equilibrium binding to DNA

RFC-B<sub>R84</sub>, RFC-C<sub>R88</sub> and RFC-D<sub>R101</sub> were chosen and mutated to alanine individually by site directed mutagenesis. The effects of these mutations were measured first on RFC-DNA binding activity. It has been shown previously RFC binds DNA stably and with high affinity in the presence of ATPγS. I measured formation of RFC•ATPγS•ptDNA complex by the change in fluorescence intensity of a 40/65 ptDNA substrate labeled with TAMRA dye at the 3’ primer end. Titration of ptDNA<sub>TAMRA</sub> with wild type RFC results in about 2.2-fold increase in TAMRA fluorescence and the binding isotherm yields a $K_d$ of $1.8 \pm 0.05$ nM (Fig. 3.4), which is similar to the previous studies (Sakato, Zhou et al. 2012; Zhou and Hingorani 2012). The same experiment was performed with each RFC mutant. In all cases, the signal change is lower for the mutants than for wild type RFC, suggesting that the DNA bound complexes may have slightly different conformations. However, all the RFC mutants exhibits similar $K_d$ values as wild type RFC, which suggests that a single mutation in the DNA binding interface may not have significant impact on binding affinity of RFC to DNA. Since RFC has many positive residues on this interface, it is not surprising that there appears to be little defect in DNA binding as the remaining residues could compensate for the loss of one of them and thus leave the DNA binding affinity unchanged. It should be noted that the equilibrium assay has to be performed with ATPγS, which may not report the same effect on DNA binding as
ATP. Therefore, I decided to examine the interaction between RFC-DNA in more
detail by measuring the kinetics of ptDNA association after ATP binding to RFC and
dissociation after ATP hydrolysis, as described in the next section.
Figure 3.4 Primer-template DNA binding affinity of wild type or mutant RFC in the presence of ATPγS. Fluorescence change of TAMRA-labeled 40/65 nt ptDNA was used to assay its interaction with wild type or mutant RFC in the presence of ATPγS. The mutants have similar $K_d$ as wild type RFC. Final reactant concentrations are 1 nM ptDNA$_{TAMRA}$, 0 – 100 nM RFC and 0.1 mM ATPγS.
3.3.2 Kinetic measurements of DNA binding/release

In order to address how the mutants affect the kinetics of DNA binding to and release from the RFC•ATP complex, sequential mixing experiments were performed in which wild type or mutant RFC proteins were pre-incubated with ATP for various time ($\Delta t = 1, 3$ and $8$ s) and then mixed with ptDNA$_{\text{TAMRA}}$, and the signal was monitored up to $1$ s as shown in Fig 3.5A; note: previous studies have shown that due to slow activation of RFC after ATP binding, a pre-incubation period of about $8$ s is required for maximal ptDNA binding and ATP hydrolysis in the absence of PCNA (Sakato, Zhou et al. 2012). The increase in TAMRA fluorescence intensity reports ptDNA binding to wild type RFC•ATP complex and the bound fraction increases with $\Delta t$ as more active RFC•ATP complex is formed during pre-incubation; note, in my experiments the maximum binding is seen in $\Delta t = 3$ s. Following ATP hydrolysis, the decrease in signal reports ptDNA release from RFC. The signal levels out higher than that for free ptDNA in steady state due to excess RFC in the reaction that can bind the released ptDNA$_{\text{TAMRA}}$. The trace at $\Delta t = 8$ s was fit empirically to a 2-exponential function and yielded $k_{\text{up}} = 14$ s$^{-1}$ which provides an estimate of the apparent bimolecular ptDNA binding constant $k_{\text{on}} \approx 140$ $\mu$M$^{-1}$ s$^{-1}$ at $0.1$ $\mu$M RFC, and $k_{\text{down}} = 9$ s$^{-1}$ indicating rate of DNA release after ATP hydrolysis (These rates are similar to previously reported rates) (Sakato, Zhou et al. 2012).

Both RFC-B$_{R84A}$ and RFC–D$_{R101A}$ mutant exhibit similar ptDNA binding and release kinetics as wild type RFC ($k_{\text{up}} = 14$ s$^{-1}$ and $k_{\text{down}} = 8$ s$^{-1}$ for RFC-B$_{R84A}$; $k_{\text{up}} = 18$ s$^{-1}$ and $k_{\text{down}} = 9$ s$^{-1}$ for RFC-D$_{R101A}$). The fluorescence peak (which we interpret
as the maximum bound fraction) of RFC-B_{R84A} and RFC-D_{R101A} are slightly lower than wild type RFC, which is consistent with the lower signal change observed for RFC-B_{R84A} and RFC-D_{R101A} in the equilibrium DNA binding experiments with ATPγS. RFC-C_{R88A} binds DNA at the similar rate as wild type (k_{up} = 15 s^{-1}) but has distinctly different release kinetics (k_{down} = 2 s^{-1}) and an apparently higher level of ptDNA-bound in steady state. These results are summarized in Fig. 3.6A with kinetic traces at Δt = 8 s overlaid for all RFC complexes. It should be noted that after DNA binding to RFC•ATP complex, the following steps occur at minimum: ATP hydrolysis, RFC deactivation, ADP release, Pi release and DNA release. The apparent slower rate seen in the RFC-C_{R88A} release phase could be due to defects in any of these steps. Currently, in our laboratory, we can measure ATP hydrolysis by $^{32}$P radioactivity assay, Pi release by MDCC-PBP reporter assay and thus determine if these steps contribute to the defect.

The effect of these RFC mutations was also examined on DNA binding/release in the presence of PCNA. The same experimental scheme described above was performed, except RFC and PCNA were pre-incubated with ATP for shorter time (Δt = 0.02, 0.05 and 3 s) before they were mixed with ptDNA_{TAMRA}; note: previous studies have shown that, in the presence of PCNA, RFC and ATP need shorter pre-incubation to achieve maximal ptDNA binding and ATP hydrolysis (Δt = 2 s, at least) (Sakato, Zhou et al. 2012). As shown in Fig 3.5B, the rate of ptDNA binding to ATP-activated wild type RFC is same with or without PCNA (apparent k_{on} ~ 120 μM^{-1} s^{-1} with PCNA and apparent k_{on} ~ 140 μM^{-1} s^{-1} without PCNA), which is
consistent with our previous report (Sakato, Zhou et al. 2012). Although the release rate is slightly lower, \( k_{\text{off}} = 4 \text{ s}^{-1} \) than in the absence of PCNA, this is likely because PCNA closure–related conformational change contributes to the apparent slower release of ptDNA by RFC as reported previously (Sakato, Zhou et al. 2012); note: slower ptDNA release results in higher peak bound fraction in the presence of PCNA.

RFC-B\(_{R84A}\) still exhibits similar ptDNA binding and release kinetics as wild type RFC (\( k_{\text{up}} = 12 \text{ s}^{-1} \) and \( k_{\text{down}} = 4 \text{ s}^{-1} \)). RFC-C\(_{R88A}\) shows a defect in ptDNA release kinetics similar to that observed in the absence of PCNA. RFC-D\(_{R101A}\) exhibits defects in both binding and release, and the maximum bound fraction is less than half that of wild type RFC. Due to the small amplitude change in release phase of RFC-C\(_{R88A}\) and RFC-D\(_{R101A}\) in the presence of PCNA, two-exponential function cannot fit properly. Thus, we don’t report rates of these two mutants here and will try to determine it by fitting to a kinetics model in the future. While RFC-D\(_{R101A}\) shows similar kinetics profile as wild type RFC without PCNA, the severe defects seen in the presence of PCNA suggests that some steps involving PCNA are defective. In the future, I plan to test this hypothesis with PCNA binding and PCNA opening/closure assays that are already available in our laboratory.

It should be also noted that the most recent kinetic model shown in Fig.1.9, Introduction chapter, globally fits data for multiple steps in the clamp loading pathway such as PCNA opening, DNA binding, ATP hydrolysis, DNA release and PCNA closure, and yields rate constant of DNA binding to RFC\( \bullet \)ATP\( \bullet \)PCNA\(_{\text{open}}\) (\( k_{\text{on}} = 50 \mu\text{M}^{-1} \text{ s}^{-1} \)) and the rate of ptDNA \( \bullet \)PCNA release from RFC (\( k_{\text{off}} = 8 \text{ s}^{-1} \)). The
same model will be also applied to wild type and mutant RFC data in the future to generate $k_{on}$ and $k_{off}$ rate constants.
Figure 3.5 Kinetics of primer-template DNA binding/release to wild type or mutant RFC•ATP complex (-/+PCNA). (A) and (B), interaction of DNA with wild type or mutant RFC•ATP in the absence (A) and in the presence (B) of PCNA are measured by change in fluorescence intensity of TAMRA-labeled ptDNA. Pre-incubation of wild type or mutant RFC, PCNA (if present) and with ATP at various times followed by the addition of ptDNA_{TAMRA} results in rise (ptDNA binding) and then fall (ptDNA release) of the fluorescence signal. The DNA binding rate and fraction increased with increasing pre-incubation times and reached maximum values at 3 s (-/+PCNA). Data were fitted with double exponential function to get initial $k_{up}$ and $k_{down}$ rates. Final reactant concentrations were 0.1 µM RFC, 0.4 µM PCNA, 0.04 µM ptDNA_{TAMRA}, and 0.5 mM ATP.
Figure 3.5A Kinetics of primer-template DNA binding/release to wild type or mutant RFC•ATP complex in the absence of PCNA.
Figure 3.5B Kinetics of primer-template DNA binding/release to wild type or mutant RFC•ATP complex in the presence of PCNA

\[ k_{\text{up}} = 12 \text{s}^{-1} \]
\[ k_{\text{down}} = 4 \text{s}^{-1} \]

- wild type RFC
- RFC-B<sub>R84A</sub>
- RFC-C<sub>R88A</sub>
- RFC-D<sub>R101A</sub>
Figure 3.6 Overlay of kinetics of primer-template DNA binding/release to wild type or mutant RFC•ATP complex in the absence (Δt = 8 s) and in the presence (Δt = 3 s) of PCNA. (A), in the absence of PCNA, all mutants (RFC-B$_{R84A}$, blue; RFC-C$_{R88A}$, green; RFC- D$_{R101A}$, purple) bind DNA at similar rate as wild type RFC (red). RFC-C$_{R88A}$ exhibits distinct different kinetics in ptDNA release. (B), in the presence of PCNA, RFC-D$_{R101A}$ exhibits defects in both binding and release DNA. RFC-C$_{R88A}$ still binds and retains DNA at higher level. Final reactant concentrations were 0.1 µM RFC, 0.4 µM PCNA, 0.04 µM ptDNA$_{TAMRA}$, and 0.5 mM ATP.
3.3.3 Steady state ATPase activities of wild type and mutant RFC in the presence of PCNA

Primer–template DNA binding to RFC•ATP•PCNA complex triggers rapid ATP hydrolysis, but the trigger mechanism is still unclear. As noted earlier in the Introduction, the Kuriyan and O'Donnell groups proposed an “allosteric switch mechanism” that a particular DNA binding residue controls this process by affecting the orientation of catalytic glutamates in Walker B motif when it binds DNA. If this hypothesis is correct, the catalytic glutamate in the mutated RFC subunits that are missing this switch residue may assume an active conformation for ATP hydrolysis. Therefore, we might see a high level of ATPase activity by RFC mutants even in the absence of DNA. To test this hypothesis, the steady state ATP hydrolysis rate of RFC mutants without DNA was first measured by malachite green assay in which phosphate (Pi) forms a complex with malachite green molybdate and the absorptions of this complex could be measured by 655 nm as described in Section 3.2.4, Chapter III. RFC-C<sub>R88A</sub> has similar ATPase rate as compared to wild type RFC (k<sub>cat</sub> = 0.05 s<sup>-1</sup>). RFC-B<sub>R84A</sub> has a slightly higher ATPase rate (k<sub>cat</sub> = 0.07 s<sup>-1</sup>) and RFC-D<sub>R101A</sub> has 4-fold higher ATPase rate (k<sub>cat</sub> = 0.22 s<sup>-1</sup>) (Fig 3.7, Table 3.2). Stimulated RFC ATPase activity without DNA is consistent with the “switch” hypothesis, but this occurs only with RFC-D<sub>R101A</sub>, suggesting that the R101 residue in D subunits plays a greater role in controlling RFC ATPase activity compared with analogous R84 and R88 residues in the B and C subunits of RFC.
Figure 3.7 Steady state ATPase activities of wild type and mutant RFC in the presence of PCNA. RFC-B_{R84A} (blue) is slightly higher and RFC-C_{R88A} (green) is similar to wild type RFC (red) in ATPase activity, while RFC-D_{R101A} exhibits significantly higher activity (purple). The $k_{\text{cat}}$ value for each RFC complex is shown in Table 3.2. Final reagent concentrations: 0.5 µM RFC, 0.5 mM ATP, 1 µM PCNA, 0.02% (Weight/Volume) malachite green and 0.6% (Weight/Volume) ammonium molybdate.

Table 3.2 Steady state ATP hydrolysis rate ($k_{\text{cat}}$) of wild type and mutant RFC generated from Fig. 3.7

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<td>wild type</td>
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</tr>
<tr>
<td>RFC-B_{R84A}</td>
<td>0.07 s$^{-1}$</td>
</tr>
<tr>
<td>RFC-C_{R88A}</td>
<td>0.05 s$^{-1}$</td>
</tr>
<tr>
<td>RFC-D_{R101A}</td>
<td>0.22 s$^{-1}$</td>
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</table>
3.3.4 Kinetics measurements of Pi release by wild type and mutant RFC

Steady state measurements only reveal minimal information about the reaction mechanism, dominated by one or more rate limiting steps in the reaction. Therefore, I decided to measure the ATPase kinetics under pre-steady state conditions to determine the effect of RFC mutations on steps other than the rate limiting steps that define \( k_{\text{cat}} \). I chose to measure phosphate (Pi) release kinetics by a fluorescence based assay in which MDCC-labeled PBP binds free Pi rapidly resulting in a large increase in fluorescence intensity as described in Section 2.2.7, Chapter II. In previous kinetic studies we found that the wild type RFC hydrolyses ATP (\(^{32}\)P-ATP radioactivity assay) and releases Pi (MDCC-PBP assay) at 0.02 s\(^{-1}\) alone and at a linear rate of 0.05 s\(^{-1}\) in the presence of PCNA. In the presence of ptDNA, the Pi release rate increases, and RFC shows maximal ATPase activity in the presence of PCNA and ptDNA, after pre-incubation for 3 s with ATP and PCNA. Under this condition, ATP hydrolysis and Pi release show burst kinetics, with the amplitude indicating rapid ATP hydrolysis by three subunits, and followed by the linear steady-state rate \( k_{\text{cat}} = 1.1 \text{ s}^{-1} \) (Fig. 3.8) (Chen, Levin et al. 2009). According to the “switch” hypothesis, by mutating the DNA binding residues, RFC may lose regulation of ATPase activity, hence we might see burst ATPase kinetics even in the absence DNA with these mutants.

A systematic series of experiments was performed to test this idea. First, two-syringe experiments were performed, in which RFC from one syringe of the stopped-flow instrument was mixed rapidly with ATP and MDCC-PBP reporter from
the other syringe, and the reaction was monitored over time (Fig. 3.9A). The fluorescence signal was converted into Pi concentration using a Pi calibration curve and plotted versus time. Pi is released at a linear rate of 0.02 s\(^{-1}\) by RFC-B\(_{R84A}\) and RFC-C\(_{R88A}\), which is comparable to wild type RFC. The RFC-D\(_{R101A}\) mutant releases Pi at a linear rate as well, though it is higher at 0.06 s\(^{-1}\), indicating again that the ATPase activity of RFC-D\(_{R101A}\) is stimulated to some extent over wild type RFC.

Next, we examined how PCNA affects the pre-steady state ATPase kinetics of the mutants. Pi is released at a rate of 0.1 s\(^{-1}\), 0.05 s\(^{-1}\), and 0.23 s\(^{-1}\) by RFC-B\(_{R84A}\), RFC-C\(_{R88A}\) and RFC-D\(_{R101A}\), respectively in the presence of PCNA (Fig. 3.9B) (Table 3.3). All RFC complexes have higher ATPase rates, which is consistent with earlier observation that PCNA enhances RFC-catalyzed ATP hydrolysis activity to a small extent. While the ATPase rate of RFC-C\(_{R88A}\) is similar to wild type RFC, the ATPase rate of RFC-D\(_{R101A}\) is about 4 times higher, again providing some support for the hypothesis that an arginine at this position suppresses RFC ATPase activity. RFC-B\(_{R84A}\) shows a slightly higher rate compared to wild type in the presence of PCNA; note: RFC-B\(_{R84A}\) ATPase rate is same as wild type RFC without PCNA, suggesting that some misregulation of the ATPase activity of this mutant as well.

The \(k_{\text{cat}}\) values from the pre-steady state Pi release measurements with PCNA are similar to the \(k_{\text{cat}}\) values determined earlier under steady state conditions. Specifically, there is no detectable burst activity of RFC mutants in the absence of DNA. This result may mean that the “switch” hypothesis is incorrect because removing the switch residues does not allow burst ATP hydrolysis in the absence of
DNA. Alternatively, one other possibility to consider is that RFC may need to bind DNA to organize the global structure of the ATPase active site, which is a more complex role for DNA in the ATPase mechanism than just setting the position of the catalytic glutamate. The crystal structure of *E. coli* γ complex bound to DNA showed a highly symmetrical arrangement of AAA+ modules of the three γ and δ’ subunit (Fig. 3.10A). This symmetry leads to the near identical packing of adjacent subunits along a spiral. The three interfacial nucleotide-binding sites are overlaid perfectly and organized tightly to bind ATP (Simonetta, Kazmirski et al. 2009). However, in the structure of *E.coli* γ complex alone in the absence of DNA, the five subunits are packed in a much looser arrangement such that the nucleotide-binding sites are poorly organized and the catalytic residues are far away from each other for ATP hydrolysis (Fig 3.10B) (Jeruzalmi, O'Donnell et al. 2001).

Based on the structural analysis, it is possible that the switch residue may locally influence the conformation of the catalytic glutamate, however to trigger the burst of ATP hydrolysis and Pi release, RFC needs to bind DNA and achieve a highly symmetrical global arrangement. Therefore, we next tested whether ptDNA triggers the rapid ATP hydrolysis and Pi release prior to steady state $k_{cat}$ for the RFC mutants. Three-syringe experiments were performed by pre-incubating wild type or mutant RFC (+PCNA) with ATP for various time ($\Delta t = 0.02, 0.05$ and $3s$) and then mixing with ptDNA and MDCC-PBP; note: Pi release in the absence of PCNA will be measured in the future. All the mutants show burst ATPase kinetics, indicating that DNA binding does trigger rapid ATP hydrolysis (Fig. 3.11). These results are
summarized in Fig. 3.12A with kinetic traces at $\Delta t = 3$ s overlaid for all RFC complexes. The data were fitted to an exponential + linear equation as shown in Section 2.2.7, Chapter II which provides an estimate of the rate of Pi release in the burst phase ($k_{\text{Pi release}}$), burst amplitude which indicates the number of ATPase active sites per RFC molecule in the first turn over, and the steady state rate ($k_{\text{cat}}$). All the mutants release Pi slightly slower than wild type RFC in the burst phase. RFC-C$_{R88A}$ shows lowest burst amplitude (0.77 $\mu$M) and this could be related to the severe defect observed in DNA release by this mutant (Fig. 3.6B). RFC-B$_{R84A}$ shows intermediate burst amplitude (0.81 $\mu$M). Since RFC-B$_{R84A}$ has similar DNA binding/release characteristic as wild type RFC, it is possible that some other step in the mechanism related to PCNA is affected in this mutant (for example, PCNA closure after ATP hydrolysis). These steps will be examined in future experiments. RFC-D$_{R101A}$ exhibits slightly lower burst amplitude than wild type RFC (0.94 $\mu$M). It is interesting that this mutant has a significant defect in DNA binding (Fig. 3.6B) but still has almost full burst ATP hydrolysis and Pi release. This result suggests that the catalytic glutamate within RFC-D subunit may be in the “active” state for ATP hydrolysis by default because of loss of regulation by the DNA binding residue R101. As long as there is some contact with DNA to help organize the RFC complex and the ATPase active site structure, the presence of the unsuppressed catalytic glutamate could trigger rapid ATP hydrolysis.

It should be noted that a significant amount of ATP is already hydrolyzed by RFC-D$_{R101A}$ before it is mixed with DNA (Fig. 3.12B). This is because of the 4-fold
higher ATPase rate of this mutant in the absence of DNA compared to wild type RFC. Thus, about 25% of the RFC-D_{R101A} mutant in the reaction has already hydrolyzed ATP prior to addition of DNA and observation. This condition leads to the jump in Pi release signal at the start of the reaction, as shown in Fig. 3.12B (since MDCC-PBP binds Pi at a very fast rate of 14 μM⁻¹ s⁻¹). When the amount of Pi released from pre-incubation time (0.25 μM) and from the burst phase (0.94 μM) is added together, the total amount of Pi adds up to almost three active ATPase sites per RFC-D_{R101A} complex in one turnover, which is similar to wild type RFC. Therefore, replacing this arginine residue with alanine in RFC-D subunit allows full ATPase activity even without optimal interaction between RFC and DNA. This finding provides evidence that the mutation of this switch residue releases suppression of ATP hydrolysis.
Figure 3.8 Kinetics of Pi release by wild type RFC under different reaction conditions. Pi is released at a rate of 0.02 s\(^{-1}\) when RFC is alone (red), at a rate of 0.05 s\(^{-1}\) in the presence of PCNA (green). A burst phase of Pi release followed by a linear steady rate of 0.7 s\(^{-1}\) is observed when RFC is pre-incubated with ATP for 8 s prior to addition of ptDNA (blue). The burst ATPase activity and steady rate is maximal (\(k_{\text{cat}} = 1.1\) s\(^{-1}\)) when PCNA is included during pre-incubation (purple). Final reagent concentrations: 0.5 \(\mu\)M RFC, 0.5 mM ATP, 1 \(\mu\)M PCNA, 2.5 \(\mu\)M ptDNA, 10 \(\mu\)M MDCC-PBP.
Figure 3.9 Kinetics of Pi release by wild type or mutant RFC (+/+ PCNA). (A), two-syringe stopped-flow experiments measuring pre-steady-state Pi release kinetics show that when wild type or mutant RFC is mixed with ATP in the absence of PCNA, RFC-B_{R84A} (blue) and RFC-C_{R88A} (green) have the same ATPase rate as wild type RFC (red). RFC-D_{R101} (purple) exhibits a higher ATPase rate. (B), in the presence of PCNA, all RFC complexes have higher ATPase rates. While RFC-B_{R84A} is slightly higher and RFC-C_{R88A} is similar to wild type RFC, RFC-D_{R101} has 4-fold higher activity. The $k_{cat}$ value of each RFC complex (+/+ PCNA) is shown in Table 3.3. Final reactant concentrations: 0.5 μM RFC, 1 μM PCNA, 0.5 mM ATP and 10 μM MDCC-PBP.
Figure 3.9 Kinetics of Pi release by wild type and mutant RFC (-/+ PCNA).

A. Pi release of wild type and mutant RFC in the absence of PCNA

B. Pi release of wild type and mutant RFC in the presence of PCNA

Table 3.3 Pi release rate of wild type and mutant RFC (-/+ PCNA) generated from Fig. 3.9

<table>
<thead>
<tr>
<th></th>
<th>RFC+ATP</th>
<th>RFC+PCNA+ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>0.02 s$^{-1}$</td>
<td>0.05 s$^{-1}$</td>
</tr>
<tr>
<td>RFC-B$_{R84A}$</td>
<td>0.02 s$^{-1}$</td>
<td>0.1 s$^{-1}$</td>
</tr>
<tr>
<td>RFC-C$_{R88A}$</td>
<td>0.02 s$^{-1}$</td>
<td>0.05 s$^{-1}$</td>
</tr>
<tr>
<td>RFC-D$_{R101A}$</td>
<td>0.06 s$^{-1}$</td>
<td>0.23 s$^{-1}$</td>
</tr>
</tbody>
</table>
Figure 3.10 Clamp loader needs DNA to organize into “ATP hydrolysis competent” structure. (A), the highly symmetrical spiral ATPase subunits of the clamp loader and DNA duplex from crystal structure of *E. coli* γ complex bound to DNA. The DNA interacting helices are shown in yellow. The three rotation axes that relate the B (γ) subunit to the C (γ) subunit, the C (γ) subunit to the D (γ) subunit, and the D (γ) subunit to the E (δ’) subunit are shown in blue, red and green, respectively. The three axes are nearly coincident with each other and with the axis of the DNA duplex (not shown) (image adapted from (Simonetta, Kazmirski et al. 2009)). (B), the core catalytic residues for ATP hydrolysis in B (γ) subunit from *E. coli* γ complex bound to DNA (green) and *E. coli* γ complex alone (blue). The catalytic residues in the *E. coli* B (γ) complex alone are too far away from ADP•BeF₃ to coordinate for ATP hydrolysis.
Figure 3.10 Clamp loader needs DNA to organize into “ATP hydrolysis competent” structure

A. *E. coli* γ complex in “ATP hydrolysis competent” arrangement

B. Core catalytic residues in γ complex of *E. coli* with/without DNA
Figure 3.11 Kinetics of Pi release with primer-template DNA in the presence of PCNA. Pre-incubation of wild type or mutant RFC, PCNA with ATP (Δt = 0.02, 0.05 and 3 s) followed by ptDNA addition results in a rapid burst of Pi release and a linear steady state. The burst amplitude increases with Δt and reach the maximum at Δt = 3s. Data were fitted to an exponential + linear equation to get initial $k_{\text{Pi release}}$, burst amplitude and $k_{\text{cat}}$. Final reactant concentrations: 0.5 μM RFC, 1 μM PCNA, 2.5 μM ptDNA, 0.5 mM ATP and 10 μM MDCC-PBP.
Figure 3.11 Kinetics of Pi release with primer-template DNA

Note: the burst amplitudes of wild type and mutant RFC are: wild type, 1.1 µM; RFC-B_{R84A}, 0.81 µM; RFC-C_{R88A}, 0.77 µM and RFC-D_{R101A}, 0.94 µM.
Figure 3.12 Overlay of the Pi release kinetics of wild type and mutant RFC with/without primer-template DNA at $\Delta t = 3s$. (A), in the presence of DNA, all mutants (RFC-B$_{R84A}$, blue; RFC-C$_{R88A}$, green; RFC-D$_{R101A}$, purple) exhibit slightly lower burst ATPase activity than wild type RFC (red). (B), in the absence of DNA, a large amount of ATP is hydrolyzed by RFC-D$_{R101A}$ before it mixed with DNA. Final reactant concentrations: 0.5 $\mu$M RFC, 1 $\mu$M PCNA, 2.5 $\mu$M ptDNA, 0.5 mM ATP and 10 $\mu$M MDCC-PBP.
The result with ptDNA shows that binding to this substrate can trigger rapid ATP hydrolysis by the mutants. The lower burst amplitudes, especially for RFC-\textsubscript{B\textsubscript{R84A}} and RFC-\textsubscript{C\textsubscript{R88A}}, suggest that these residues make a significant contribution to appropriate contact between RFC and ptDNA, which organizes the clamp loader into an ATPase-active form. Primer-template DNA binding appears necessary to achieve full activation, even though we do observe some unregulated ATPase activity with RFC-\textsubscript{D\textsubscript{R101A}} and RFC-\textsubscript{B\textsubscript{R84A}} mutants in the absence of DNA, therefore, it is difficult to test the “switch” hypothesis directly.

In a previous study, Siying Chen (Hingorani laboratory) had observed that single-stranded DNA (ssDNA) can also stimulate RFC ATPase activity but with little burst kinetics, suggesting that this partial DNA substrate can help organize the ATPase active sites to some extents as well. I tested this substrate to determine if it can help reveal greater differences between the wild type and mutant RFC complexes. The same experimental scheme as described above was performed, except 37-mer ssDNA was substituted for ptDNA in the reaction. Fig. 3.13 shows that RFC-\textsubscript{D\textsubscript{R101A}} exhibits the highest burst of ATP hydrolysis and Pi release, even higher than wild type RFC. RFC-\textsubscript{B\textsubscript{R84A}} and RFC-\textsubscript{C\textsubscript{R88A}} show almost no burst activity. This result supports the hypothesis that R101 in RFC-D plays a significant role as a switch residue in suppressing ATP hydrolysis in the absence of contact with ptDNA substrate. It also suggests that R101 may be the primary switch - that RFC-D subunit must hydrolyze ATP first in order to enable ATP hydrolysis by the RFC-B and RFC-C subunits.
Figure 3.13 Kinetics of Pi release with single-stranded DNA at $\Delta t = 3$ s. Pre-incubation of wild type or mutant RFC, PCNA with ATP ($\Delta t = 3$ s) followed by ssDNA addition. RFC-D$_{R101A}$ (purple) shows higher burst ATPase activity than wild type RFC (red). RFC-B$_{R84A}$ (blue) and RFC-C$_{R88A}$ (green) exhibit no burst ATPase activity. Final reactant concentrations: 0.5 µM RFC, 1 µM PCNA, 2.5 µM ssDNA, 0.5 mM ATP and 10 µM MDCC-PBP.
3.4 Discussion

The eukaryotic RFC clamp loader comprises five unique subunits that arrange into a chamber with a right-handed spiral shape matching the helical symmetry of DNA. Inside the RFC chamber, three conserved residues in each subunit are proposed to contact DNA. Once an RFC•ATP•PCNA\textsubscript{open} complex is formed, DNA binding to RFC triggers rapid ATP hydrolysis, which leads to closure of the clamp and ejection of the clamp loader from the clamp on DNA. Among the three conserved DNA binding residues in each subunit, the first one has been recently hypothesized to control the clamp loader ATPase activity. In the present study, we tested this hypothesis by individually mutating the first DNA binding residue of RFC-B, RFC-C and RFC-D subunits to determine the role of these residues in binding DNA and triggering ATP hydrolysis.

The central chamber of the clamp loader presents a large DNA binding surface, hence we did not anticipate substantive reduction in the DNA binding affinity of RFC complex on removal of a single cationic residue. However, with knowledge of kinetic and thermodynamic parameters governing distinct steps in the RFC-catalyzed clamp loading reaction (Sakato, Zhou et al. 2012), we could directly measure any effects of disrupting individual RFC-DNA contacts on events related to DNA binding and release in the mechanism. We first measured the kinetics of DNA binding/release by RFC and observed that while the mutants can bind ptDNA rapidly, RFC-C\textsubscript{R88A} has a severe defect in DNA release (Fig. 3.6A). In the clamp loading mechanism, RFC releases DNA after ATP hydrolysis, along with PCNA closure, Pi
release, as well as ADP release (rate unknown). Therefore, it is possible that mutation of the R88 residue disrupts any of these steps, possibly by altering RFC conformation. The other mutants; RFC-B_{R84A} and RFC-D_{R101A} do not show such a severe defect in DNA release. We also investigate how these mutants affected DNA binding/release under clamp loading conditions (+PCNA). Interestingly, RFC-D_{R101A} exhibits significant defects in both binding and release of ptDNA in the presence of PCNA (Fig. 3.6B), even though this is not the case in the absence of PCNA (Fig 3.6A). At this time, we are considering the possibility that mutation of the R101 residue causes conformation changes that affect PCNA binding, opening and closing, and these defects may be reflected in the DNA binding/release kinetics in the presence of PCNA. Future experiments measuring RFC-PCNA interactions, PCNA opening and closing will determine more precisely which step in the reaction is blocked when the R101 contact with DNA is removed. Another possibility is that since this mutant has abnormally high ATPase activity in the presence of PCNA and ATP (Fig 3.9B), there may be a lower fraction of RFC\_ATP\_PCNA_{open} complex in the reaction that can rapidly bind DNA after pre-incubation with ATP. The RFC-C_{R88A} mutant still shows a large defect in DNA release in the presence of PCNA, thus the problem with RFC-DNA interaction observed in the absence of PCNA (Fig. 3.6A) persists during the clamp loading reaction (Fig. 3.6B). This result suggests that proper contact between RFC-C and DNA at this subunit is important for ending the reaction with PCNA\_DNA release from RFC.
As earlier RFC mutational study in our laboratory showed that RFC-C ATP binding activity is key for RFC-ATP•PCNA_{open} assembly and RFC-D contributes significantly to ptDNA release (Sakato, O'Donnell et al. 2012). Moreover, T4 gp44/62•ADPBeF_{3}•ptDNA structure with open gp45 clamp indicates that the main distortion of the clamp is caused by contact with gp44 in the C position. This result suggests that the C subunit is most important for inducing spiral conformation of both clamp and clamp loader (Kelch, Makino et al. 2011). According to all these findings, RFC-C subunit appears to be essential in both complex assembly and disassembly, while RFC-D is important in ATP hydrolysis and complex disassembly.

In all clamp loaders tested thus far, ATPase activity is enhanced greatly upon binding of ptDNA (Berdis and Benkovic 1996; Gomes, Schmidt et al. 2001; Ason, Handayani et al. 2003). The recent crystal structure of the T4 clamp loader (Kelch, Makino et al. 2011) was used to develop the idea of an allosteric switch mechanism to explain how DNA binding could switch on the ATPase activity of the clamp loader subunits. This mechanism could apply not just to clamp loaders, but also to other AAA+ proteins, in which a conserved asparagine can hold the catalytic glutamate in an inactive conformation until the appropriate ligand binds to the protein (Zhang and Wigley 2008). My data show that removal of R101 in RFC-D subunit stimulates the ATPase activity 4-fold higher than wild type RFC in the absence of DNA (Fig. 3.9B), consistent with the switch hypothesis. However, unlike the simple “ATPase on or off” scenario suggested by the switch hypothesis, we found the case is more complicated. Since we did not observe any burst of ATP hydrolysis by RFC-
D$_{R101A}$ mutant in the absence of DNA (Fig. 3.9B), simply removal of this residue is not enough to turn “on” the RFC ATPase, although it may allow local movement of the catalytic glutamate to stimulate some ATP hydrolysis. The structural data indicate there also has to be global organization of the RFC ATPase site structure upon contact with DNA in order to observe full activity. Based on the comparison of *E. coli* clamp loader with and without DNA, it appears that binding of ptDNA in the central chamber drives adjustment of inter-subunit contacts, bringing all catalytic sites into correct conformation (Fig. 3.10). This step is essential, as shown by the burst ATPase activity of all RFC mutants in the presence of ptDNA (Fig. 3.12A). Thus, we propose a modification of the switch hypothesis — that global contacts between multiple DNA binding residues on RFC and ptDNA occur first to organize RFC ATPase modules for ATP hydrolysis, after which, movement of the switch arginine away from the catalytic glutamate, especially, in the RFC-D ATPase site, leads to rapid ATP hydrolysis.

We also tried ssDNA as a substrate to stimulate global reorganization of RFC without direct contact with the switch residues (ssDNA binds at the top of the clamp loader; Fig. 3.2B) (Simonetta, Kazmirski et al. 2009; Kelch, Makino et al. 2011). Preliminary data show that RFC-D$_{R101A}$ exhibits a burst of ATP hydrolysis and Pi release with ssDNA that is much higher than for wild type RFC and the other mutants, supporting the idea that RFC-D may be the first one to start ATP hydrolysis and then the rest of subunits in the clamp loader continue the reaction. It is interesting to note RFC-D is the top most ATPase active subunit in the RFC spiral and is
possible that ATP hydrolysis proceeds in sequence starting from this subunit in the complex. This proposal is different from the one put forward by the Kuriyan’s group after solving the bacteriophage T4 gp44/62•ADPBeF₃•gp45•ptDNA complex structure. In this structure, ADP is bound in the B site and breaks the symmetric spiral of AAA+ modules, while ATP analogue ADPBeF₃ is bound to the C and D sites (Kelch, Makino et al. 2011). The authors proposed that this structure represents a state in which only the first ATPase site, the B site has catalyzed hydrolysis. It is worth noting that this structure may arise due to a crystal artifact, and it is also possible that the mechanism differs in detail between RFC and T4 gp44/62. In future studies I will try more stringent experimental conditions (such as higher NaCl concentration) to find ways to emphasize the differences in DNA binding, ATP hydrolysis and PCNA opening/closing activities between wild type RFC and RFC-DR₁₀₁₅ mutant to fully define the importance of this subunit and R101 in the clamp loading reaction.

Based on all the data thus far, we can propose a preliminary model for the contribution of RFC subunits during PCNA loading on DNA. ATP binding to RFC-C induces conformation change toward the peripheral subunits such that RFC adopts an active spiral form. Contact between RFC-C and PCNA helps twist the clamp open or help stabilize the twisted clamp open out of plane. The opened PCNA clamp contacts all RFC subunits to form the RFC_{active}•ATP•PCNA_{open} complex. DNA binding to this complex further organizes the RFC structure into an “ATP hydrolysis competent state”, in which all the subunit interfacial AAA+ modules tighten coordination of
ATP in the active sites. Then, as R101 switch residue in the RFC-D subunit moves away from the catalytic glutamate due to interaction with the DNA backbone, and ATP hydrolysis occurs. ATP hydrolysis by RFC-D breaks the contact between RFC-D (and perhaps RFC-E) and PCNA, and the clamp start to close. Next, RFC-B and RFC-C subunits hydrolyze ATP leading to disruption of contacts between these two subunits and PCNA, and clamp closure. The relaxation of RFC-C back into the deactivated form appears to be essential to release RFC from PCNA-DNA. This model is somewhat speculative at this time, but future experiments are planned to address in detail.

RFC ATPase activity is very tightly regulated by DNA, suggesting that this coupling has high biological significance. As shown in our most current kinetic model, the clamp loading pathway involves an ordered series of many different steps. ATP hydrolysis is suppressed until the clamp loader binds and opens the clamp and then binds ptDNA. The rapid ATP hydrolysis allows clamp closing on ptDNA and complex dissociation. Thus, ATP binding and hydrolysis control the specificity and directionality of the reaction, allowing clamp loading to occur specifically on ptDNA substrate and minimizing wasteful ATP hydrolysis and futile cycles before the full RFC•ATP•PCNA_{open}•ptDNA complex is formed.
Appendix A. Buffers

Buffer A: 30 mM Hepes-NaOH at pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 2 mM DTT

Buffer B: 20 mM Tris-HCl at pH 7.5, 0.5 mM EDTA pH 8.0, 10% glycerol, 5 mM DTT

Buffer C: 30 mM Hepes-NaOH at pH 7.5, 20 mM NaCl, 10% glycerol

Buffer D: 25 mM Tris-HCl at pH 7.5, 200 mM NaCl, 10% glycerol

Buffer E: 30 mM Hepes-NaOH at pH 7.5, 0.25 mM EDTA pH 8.0, 5% glycerol

1×TBE Buffer: 89 mM Tris-base, 29 mM Boric acid, 2 mM EDTA pH 8.0

Formamide buffer: 45% formamide by volume in 1×TBE Buffer
Appendix B. Oligonucleotide Purification by Electrophoresis

The protocol follows three steps: DNA separation from impurities by electrophoresis in a denaturing urea gel, DNA electro-elution from gel, and ethanol precipitation to concentrate the sample. A 19% gel was used for purification of 40-mer DNA or 37-mer DNA while a 17% gel was used for 65-mer DNA.

Step 1: A 19% gel was prepared by mixing 21 g urea with 5 ml 10 x TBE buffer and 23.75 ml 38:2% Acrylamide:Bis (w/v) and 3.6 ml H₂O (21.25 ml 38:2% Acrylamide:Bis and 6.1 ml H₂O instead for a 17% gel) in an flask with a side arm. The flask was swirled in hot water in a boiler pot for several min, until the urea crystals were dissolved, and then the sidearm was connected to vacuum to degas the solution for few min. Then 30 µl TEMED and 150 µl 10% APS was added over the degassed solution and poured into the gel casting system set with 1.5 mm comb and spacers. The gel was pre-run at 400 V for 45-60 min at room temperature in 1x TBE Buffer.

The DNA sample was prepared by resuspension in 150 µl of formamide buffer (Appendix A) with vigorous vortexing. The sample was loaded in the gel well, which was previously flushed to remove excess urea, along with a marker consisting of 10 µl formamide buffer and a pinch of bromophenol blue powder. The gel was electrophoresed at 400 V until the dye line traveled 4/5 of the gel (about 5-6 hours).
The DNA band was visualized under UV on a fluorescent-background TLC silica plate, and excised in small pieces.

**Step 2:** The electro-eluter chamber was assembled with two BT1 non-porous membranes at both ends to prevent DNA leakage into the outer compartment, and one BT2 porous membrane adjacent to one BT1 membrane, forming a partition. The DNA was eluted from the gel pieces at 100V in 1x TBE Buffer, at 4 °C. The eluted DNA was collected after 12 hours and 15 hours, and its DNA concentration was calculated based on absorbance readings at 260 nm.

**Step 3:** The eluted DNA was transferred in “not low adhesion” eppendorf tubes for ethanol precipitation. The sample was mixed with 3 x volumes of cold absolute ethanol and 0.1x volume of 3 M sodium acetate, pH 5.2, and stored at -80 °C for at least 5 hours. After that, the samples were centrifuged at 14,000 rpm in a microcentrifuge at 4 °C for 30 min (with the lid hinge toward outside). The supernatant was discarded, and the pellet was dried and resuspended in 10 mM Tris-HCl at pH 8.0. The DNA concentration was measured at 260 nm and the sample was stored in eppendorf tubes at -80 °C.
Appendix C. Protein Overexpression, Purification and Labeling

C.1 *S. cerevisiae* wild type PCNA, PCNA<sub>WC</sub>, PCNA<sub>FC</sub>, R14A, K20A, R80A, K146A, R149A, and K217A mutants purification

**Transformation:**

About 20-40 ng of “pET wt PCNA”, “pET PCNA<sub>WC</sub>”, “pET PCNA<sub>FC</sub>”, “pET R14A PCNA”, “pET K20A PCNA<sub>WC</sub>”, “pET R80A PCNA<sub>WC</sub>”, “pET K146A PCNA<sub>WC</sub>”, “pET R149A PCNA<sub>WC</sub>”, and “pET K217A PCNA<sub>WC</sub>” plasmid DNAs were each transformed into 100 µl BL21 (DE3) competent cells following the standard heat shock procedure of 30 min on ice, 90 s at 42 °C, 2 min on ice followed by addition of 200 µl sterile LB media and further incubation at 37 °C for 45 min shaker. The transformed cells were plated on a LB/Amp plate and incubated O/N at 37 °C.

**Growing biomass:**

For 4 Liter culture, 2 x 4 L flasks containing 2 liters of sterile LB media each, 100 µg/ml ampicillin were seeded with one colony from the plate. The colony was resuspended in 1 ml of sterile LB media and 30 µl was put into each flasks. After ~8-9 hours of growth at 37 °C and 200 rpm, the culture reached OD<sub>600</sub> of 0.6. Then it was induced with 0.5 mM IPTG (1 ml of 1 M IPTG) at 25 °C for 6 hours with shaking at 230 rpm. 1 ml/0.5 ml samples were taken before/after induction. The cells
(4 L of culture) were harvested by centrifugation at 5000 rpm for 10 min at 4 °C, and the cell pellets were resuspended in cold Tris Sucrose resuspension buffer (10 mM Tris-HCl, pH 7.5 and 10% sucrose w/v) to make ~ 35 ml final total volume and stored at -80 °C.

**Induction gel:**

The induced and uninduced samples collected during biomass growth were centrifuged at 13,000 rpm for 1 min. The pellet was resuspended in 40 µl ddH₂O and 10 µl of 5x SDS dye by vigorous vortexing, boiling and spinning at 13,000 rpm for 5 min individually. 5 µl from the samples were loaded along with a broad range marker on a 14 % SDS-acrylamide gel.

**Cell lysis:**

The biomass was thawed in a beaker of water placed on ice and lysed in final total ~ 40 ml volume containing 0.3 mg/ml lysozyme, 1 mM of PMSF (PMSF needs to be dissolved in isopropanol), 5 mM DTT, 2 µl/ml protease inhibitor cocktail (Sigma-Aldrich) and cold Tris Sucrose resuspension was added to make up the final volume. As described above, the lysate was stirred for 1 hour at 4 °C, freezed/thawed 3 times (2 min in liquid N₂ followed by thawing at 37°C for ~10 min), then dounced (avoid air bubbles). After that, the protein was separated from cellular debris by centrifugation at 17,000 rpm (SS34 rotor) for 1 hour, at 4 °C. Supernatant was decant to a graduated cylinder and cold Tris Sucrose resuspension buffer containing 5 mM
DTT, 1 mM PMSF and 2 µl/ml protease inhibitor cocktail was added to make the total volume 100 ml.

**Streptomycin Treatment:**

100 ml supernatant after lysis was poured in the 250 ml glass beaker containing a stirring bar in an ice bath and stirred slowly enough not to disturb DNA clumping after adding streptomycin. 9 ml 10 % (w/v) streptomycin was added drop-wise in the lysate at ~1 ml/min and stirred for total 30 min. After that, the protein was separated from DNA by centrifugation at 17,000 rpm for 30 min, at 4°C. Supernatant was decant to a graduated cylinder and cold Tris Sucrose resuspension buffer containing 5 mM DTT, 1 mM PMSF and 2 µl/ml protease inhibitor cocktail was added to make the total volume 100 ml.

**Ammonium Sulfate Fractionation:**

100 ml supernatant after streptomycin treatment was poured in the 250 ml glass beaker containing a stirring bar in an ice bath and stirred slowly. 25 g (NH₄)₂SO₄ (40%) was added in the supernatant slowly, dissolved and stirred for 30 min. After that, the protein was separated by centrifugation at 17,000 rpm for 30 min, at 4°C. Supernatant containing PCNA was decanted to a new 250 ml glass beaker in an ice bath and 25 g (NH₄)₂SO₄ (75%) was added in the supernatant slowly, dissolved and stirred for 30 min. The protein was separated by centrifugation at 17,000 rpm for 30 min, at 4°C. PCNA was fractionated in pellet in this step and was resuspended in 2 x
Buffer B (Appendix A) to make the total volume ~ 25 ml. The protein was dialyzed in a dialysis tubing (MWCO 6 – 8000, 3.3 ml/cm) with 450 ml Buffer B in the cold room overnight to reduce the salt concentration to ~100 mM.

**DEAE column:**

A 20 ml DEAE column was packed, cleaned with 180 ml of ddH₂O and equilibrated with 160 ml Buffer B₉₁₅₀ (Buffer B containing 150 mM KCl) at 1→2 ml/min at room temperature. Column was further equilibrated by 20 ml Buffer A₉₁₅₀ in the cold room. The dialyzed pool of PCNA was mixed with equal amount of volume of 2 x Buffer A₀ and loaded onto the column at 1 ml/min (Total amount loaded: ~ 50 ml). After a washing step with 200 ml of B₉₁₅₀, PCNA was eluted by 200 ml gradient from B₉₁₅₀ - 4₀₀ gradient at 1 ml/min and 2 ml fractions were collected after 10ml delay. 5 µl of the load, flow through, wash and 16 µl even numbered fractions were analyzed on 14% SDS-PAGE gel (PCNAwc is shown in Fig.C.1). PCNA was eluted in the middle of the gradient, and fractions containing PCNA protein of significant amount and purity were pooled. The pooled PCNA could be either dialyzed in a dialysis tubing (MWCO 6 – 8000, 3.3 ml/cm) with 500 ml Buffer B in the cold room overnight or diluted with 2 x Buffer B to reduce the salt concentration to ~ 40 mM and directly loaded into Q column.
**Fast Flow Q Sepharose column:**

A 6ml Q-Fast Flow Sepharose column was washed with ddH$_2$O, packed, and equilibrated with 50 ml buffer B$_{Na100}$ at room temperature. Column was further equilibrated by 10 ml Buffer B$_{Na100}$ in the cold room. Dialyzed or diluted protein was loaded onto the column at 1 ml/ml. After a washing step with 60 ml of Buffer B$_{Na290}$, PCNA was eluted by 60 ml gradient from B$_{Na290-700}$ gradient at 1 ml/min and 1 ml fractions were collected after 10 ml delay. 10 µl of the load, flow through, wash and 16 µl even numbered fractions were analyzed on 14% SDS-PAGE gel (PCNAwc is shown in Fig.C.1). PCNA was eluted in the middle of the gradient, and fractions containing PCNA protein of significant amount and purity were pooled. If PCNA was prepared for DNA binding and Pi release assay, protein was then dialyzed in a dialysis tubing (MWCO 6 – 8000, 3.3 ml/cm) with 500 ml Buffer C in the cold room overnight. If PCNA was for FRET labeling, protein was dialyzed in a dialysis tubing (MWCO 6 – 8000, 3.3 ml/cm) with 500 ml Buffer D in the cold room overnight and then concentrated in Centriplus10 with Buffer D until the concentration reach to > 5 mg/ml ≈ 50 µM trimer. The final concentration of PCNA was measured by Bradford assay using BSA as standard.
Figure C.1 Purification of PCNA\textsubscript{WC} Protein. Samples from DEAE column (upper gel) and Q column (lower gel) were analyzed on 14 % SDS-PAGE (Mr-marker, L-load, FT-Flow through, W-Wash).

DEAE column:

Q column:
C.2  *S. cerevisiae* wild type RFC, RFC-BR84A, RFC-CR88A and RFC-DR101A purification

Transformation:

Wild type RFC was overexpressed from two plasmids. RFC1 and RFC5 genes were inserted into pLANT (2b)/RIL-(RFC1+5), which contains kanamycin resistance marker and genes for rare codon tRNAs. RFC2, 3, 4 genes were inserted into pET (11a)-RFC(2+3+4), which contains ampicillin resistance marker. Mutant RFCs were overexpressed as same as wild type except they have mutations in the corresponding positions of plasmid pET (11a)-RFC(2+3+4) as described in Section 3.2.1, Chapter III. 50 ng of each plasmid was transformed into 100 µl BL (DE3) competent cells by standard heat shock procedures as described above, which were plated on a LB/Amp/Kan plate and incubated O/N at 37 °C.

Growing biomass:

For 12 Liter culture, 6 × 4 L flasks containing 2 liters of sterile LB media each, 100 µg/ml ampicillin and 50 µg/ml kanamycin were seeded with one colony from the plate. The colony was resuspended in 680 µl of sterile LB media and 100 µl was aliquoted into the 6 flasks. The cells were grown at 37 °C with vigorous shaking at 230 rpm until the culture reached an $A_{600}$ value of 0.6 (9-10 hours). Next, 0.5 mM IPTG (1 ml of 1 M IPTG) was added to the cultures followed by incubation for 3 hours at 37 °C. 1 ml/0.5 ml samples were taken before/after induction. The cells (from 12 L of culture) were harvested by spinning at 5000 rpm for 10 min at 4 °C,
and the cell pellets were resuspended in cold Hepes Sucrose buffer (10 mM Hepes-NaOH, pH 7.5, 10% (w/v) sucrose) to make ~70 ml final total volume. The biomass should be immediately stored in 2 x 50 ml falcon tubes in -80°C freezer, where it can be kept for weeks-months, and the induction was verified as described in Appendix C1.

Cell lysis:

The biomass was thawed in a beaker of water placed on ice and lysed in final total ~120 ml volume containing 0.3 mg/ml lysozyme, 1 mM of PMSF (PMSF needs to be dissolved in isopropanol), and ~60 ml 2x cold Hepes Sucrose resuspension buffer was added to make up the volume. As described above, the lysate was stirred for 1 hour at 4 °C, freeze/thawed 3 times (2 min in liquid N$_2$ followed by thawing at 37 °C for ~10 min), then dounced, (avoid air bubbles). After that, the proteins were separated from cellular debris by centrifugation at 17,000 rpm (SS34 rotor) for 1 hour, at 4 °C. After that, RFC complex was purified by ion exchange chromatography.

Fast Flow SP-Sepharose column:

A 10 ml SP column was packed, cleaned with 100 ml of ddH$_2$O and equilibrated with 100 ml Buffer $E_{Na250}$ (Buffer E containing 250mM KCl) at 1→2 ml/min at room temperature. Column was further equilibrated by 20 ml Buffer $E_{Na250}$ in the cold room. RFC from supernatant after was loaded onto the column at 1 ml/min (total amount loaded: ~120 ml). After a washing step with 100 ml of Buffer $E_{Na300}$, RFC was eluted by 75 ml gradient from $E_{Na300-600}$ gradient at 1 ml/min and 1 ml
fractions were collected after 10 ml delay. 5 µl of the load, flow through, wash and 16 µl even numbered fractions were analyzed on 10% SDS-PAGE gel (Fig. C.2). Fractions could be left in the cold room overnight and processed in the next day.

**Fast Flow Q Sepharose column:**

A 5 ml Q-Fast Flow Sepharose column was washed with ddH$_2$O, packed, and equilibrated with 50 ml buffer B$_{Na100}$ at room temperature. Column was further equilibrated by 10ml buffer B$_{Na100}$ in the cold room. Fractions from SP column containing equal amount of five complexes of RFC protein was pooled. Conductivity of the pooled RFC was measured (0.1 ml pooled protein + 1.4 ml H$_2$O) and dilution volume was calculated to make the final 100 mM salt. After that, a small amount RFC sample was diluted by Buffer E to final volume 1.2 ml and the conductivity of the diluted RFC was measured (0.5 ml diluted protein +1.0 ml H$_2$O). If the salt concentration was between 110 and 120 mM, then a dilution volume for the large mount RFC sample which could be loaded onto the Q column within 20min at a time was prepared (around 17-18 ml total volume per time). Once Buffer E was added into the RFC pools, samples should be invert gently but quickly for several times, and immediately loaded onto the Q column at 1 ml/min. This step was repeated until all the proteins were loaded (Normally around 3 or 4 times were needed to be done for regular yield: 3-5 mg for 12 liters). After a washing step with 50 ml of Buffer E$_{Na110}$, RFC was eluted by 40 ml gradient from E$_{Na110}$ – 500 gradients at 1 ml/min and 0.8 ml fractions were collected after 5ml delay. 10 µl of the load, flow through, wash and 16
µl even numbered fractions were analyzed on 10% SDS-PAGE gel (Fig. C.2).

Fractions containing equal amount of five complexes of RFC protein were pooled from high to low salt. Conductivities of the pooled proteins were measured. If the salt concentration is $\geq 200$ mM, then the pools were aliquoted and stored in -80 °C. Otherwise, the salt concentration could be adjusted by addition of the neighboring fractions. The concentration of RFC was measured by Bradford assay using BSA as standard.
**Figure C.2 Purification of wild type RFC protein.** Samples from SP column (upper gel) and Q column (lower gel) were analyzed on 10 % SDS-PAGE (Mr-marker, P-pellet, L-load, FT-Flow through, W-Wash).
C.3 *S. cerevisiae* wild type PCNA, PCNAWC, PCNAFC, R14A, K20A, R80A, K146A, R149A, and K217A mutants labeling with AEDANS

**Labeling PCNA with AEDANS:**

PCNA in Buffer D was adjusted to the concentration of 50 µM. TCEP (final concentration: 0.5 mM) was added and incubated on rotator for 10 min at room temperature. Then, ¼ volume of AEDANS (final concentration: 2 mM, AEDANS should be dissolved in DMF) was added and incubated on rotator for 15 min at room temperature and this step was repeated for another 3 times to finish the AEDANS solutions. After that, the reaction tube was moved into cold room and rotated for overnight.

**P-6 gel filtration:**

A P6 column was prepared as described below. 2.5 g of P6 resin (Bio-rad) was weighed and soaked in H₂O for more than 4 hours and then degased. After that, the resin was changed into 20ml degased Buffer D, swirled, settled down for several min and this step was repeated for 3 times. Then the resin was packed and equilibrated with another 20 ml degased Buffer D. PCNA labeling reaction was quenched with 5 mM DTT. The reaction volume was reduced to 400 µl by ultrafiltration using Buffer D pre-socked a Centricon10 spinning at 6000 rpm for 1-1.5 hours. Retentate was transferred to a 1.5 ml tube. Centricon10 unit was rinsed
with 100 µl Buffer D and combined with the saved retentate. During this ultrafiltration step, a certain portion of free dye was simultaneously removed.

The top part from the packed P6 column was removed and the buffer lever was lowered down to the surface of the resin by gravity flow. The labeling reaction was load directly onto the P6 column. Column was run by gravity flow until the reaction completely enters the resin. Buffer D was overlaid and the top part to the column was reassembled. The resin was run by Buffer D and the 300 µl fractions were collected at after 1.5 ml delay. After ~ 20 fractions, 2 µl drops from even-numbered fractions were placed on the saran wrap on the black surface and illuminated by a hand-held UV light at 366 nm. When the fluorescence showed up, the neighboring odd-numbered fractions were placed on the saran wrap too. Once the peak fractions of the labeled protein were determined from fluorescence, they were analyzed on 14% SDS-PAGE gel. Fractions containing PCNA protein of significant amount were pooled, aliquoted and stored in -80°C. The degree of labeling was calculated from the formula:

\[
\frac{A_{336}/\varepsilon_{\text{AEDANS}}}{[\text{PCNA}]}
\]

\(\varepsilon_{\text{AEDANS}} = 5430 \text{ (M}^{-1}\text{.cm}^{-1})\), \(A_{336}\) is the absorbance of labeled PCNA at 336 nm, \([\text{PCNA}]\) is the concentration of PCNA (trimer) measured by Bradford assay.
C.4 Preparation of a Pi probe, MDCC-PBP

Solution preparation:

Autoclave one empty 50 ml flask and 1 x 100 ml LB in a 500 ml flask. Pour 10 ml sterile LB into the autoclaved 50 ml flask. Autoclave 50 ml 60 % (v/v) glycerol. Prepare three of 14% SDS-PAGE gels and keep at 4 °C until use. Make 6 ml 12.5 mg/l (1000x) tetracycline: weigh 75 mg tetracycline and add 50 % ethanol to final volume 6 ml in a 15 ml falcon tube. Aliquot 0.2 ml to an eppendorf tube and wrap the rest solution by aluminum foil and store at - 20 °C. Make 1 L of 4 M NaCl: weigh 233.76 g NaCl and add ddH$_2$O to final volume 1 L. Make 50 ml of 1 M MgCl$_2$: weigh 10.165 g MgCl$_2$-6H$_2$O and add ddH$_2$O to final volume 50 ml. Make 1 L 0.5 M EDTA pH8.0: 186.1 g EDTA·2H$_2$O and add ~ 20 g NaOH and add 800 ml ddH$_2$O, mix vigorously and adjust pH to 8.0 and add ddH$_2$O to final volume 1 L, then autoclave to sterilize. Make 1 L of 1 M Tris-HCl pH 7.8, pH 7.6 and pH 7.5. The pH of Tris buffer must be well-adjusted especially for a Q-Sepharose column, because it is performed at the edge of binding conditions (Tris buffers, pH 7.8 & 7.6, and the theoretical pI of this PBP is 6.91). To get well-adjusted pH of Tris, re-adjust pH after ~ 1-2 hours in room temperature and add ddH$_2$O to final volume. Make sure that you sign up the shaker on the hallway for 8 x 0.5 L cultures during second and third days. Pack 30 ml FFQ-Sepharose column in a XK16 column and wash with plenty ddH$_2$O at ~ 1-5 ml/min at room temperature and equilibrate with ~ 300 ml of 10 mM Tris-HCl, pH 7.8 at 3 ml/min. After equilibration, store column in the cold room.
PBP-Glycerol Stock Preparation:

Streak out a swab of PBP culture on a LB-tetracycline plate (tetracycline concentration is 12.5 mg/L; note: make tetracycline stock in 50 % ethanol and store at – 20 °C covered with aluminum foil) and incubate overnight (~ 16 hours) at 37 °C. Pick one well-isolated and nice-round shaped colony from the plate into 25 ml LB with tetracycline and grow culture to an OD$_{600}$ of 0.4 at 250 rpm in a shaker at 37 °C for ~ 3-5 hours. Place 1.5 ml of the culture in a sterile 2 ml vial (O-ring seal screw cap, sterile, liquid nitrogen fine) with 0.5 ml 60 % glycerol (autoclaved and chilled). Vortex the samples well to ensure even distribution of glycerol and rapidly freeze the vial by immersing it in liquid nitrogen and store at - 80°C.

Day 1:

Thaw out one 2 ml glycerol stock of PBP in a 37 °C water bath, and add the contents to 10 ml of LB-tetracycline (10 µl of 12.5 mg/ml filter sterilized tetracycline, 12.5 mg/L final) in a 100 ml autoclaved flask. Grow the culture at 37 °C for ~ 6 hours with constant shaking at 250 rpm. After 6 hours of growth, transfer the 10 ml culture to 100 ml LB-tetracycline in a 500 ml flask (100 µl 12.5 mg/ml tetracycline stock). Grow the culture at 37 °C, with constant shaking at 250 rpm for ~ 16 hours. Set 8 x 2 L holders in the shaker for tomorrow morning.

Prepare 4 L minimal media (MM) according to the composition below.
Minimal media of 4 L: add 100 mM Hpes (95.32 g), 20 mM KCl (40 ml 2 M stock or 5.96 g), 15 mM (NH$_4$)$_2$SO$_4$ (7.92 g), 1 mM MgCl$_2$ (4 ml 1 M stock), 10 µM FeSO$_4$ (11.12 mg), 1 µg/ml thiamine (4 mg), 0.25 % (v/v) glycerol (10 ml 100 % glycerol), 2
mM KH₂PO₄ (8 ml 1 M stock or 1.09 g), stir in ~ 3.9 L H₂O, adjust pH to 7.5 with 10 N NaOH (~ 20 ml), then fill up to 4 L with ddH₂O. Split the media into 8 x 2 L flasks (500 ml each) and autoclave. Cool the media to room temperature and add ~ 500-600 µl 12.5 mg/ml tetracycline stock (15 mg/L final).

**Day 2:**

Prepare 0.2 M rhamnose (1.82 g of 2 M rhamnose in the desiccator to final 50 ml with ddH₂O). Prepare resuspension buffers (RBs) for tomorrow. Resuspension buffer I (1 L): 10 mM Tris-HCl, pH 7.6 (10 ml 1 M stock), 30 mM NaCl (7.5 ml 4 M stock), 982.5 ml ddH₂O. Resuspension buffer II (100 ml): 33 mM Tris-HCl, pH 7.6 (3.3 ml 1 M stock) and make volume to 100 ml with ddH₂O. Resuspension buffer III (100 ml): 33 mM Tris-HCl, pH 7.6 (3.3 ml 1 M stock), 40 % (w/v) sucrose (40 g), 0.1 mM EDTA (20 µl 0.5 M stock) and make volume to 100 ml with ddH₂O. Resuspension buffer IV (ice cold): 0.5 mM MgCl₂ (100 µl 1 M stock) and make volume to 200 ml with ddH₂O and place in –20 °C freezer for 5 min and store in 4 °C. RB I, II and III can keep in room temperature. Shake 8 x 0.5 L minimal media with tetracycline at 230 rpm on the shaker at 37 °C and pre-warm them for ~ 30-60 min. After 16 hours of growth, add 10 ml of the culture to the pre-warmed minimal media in each flask and incubate at 37 °C with rapid shaking at 230 rpm. Take 1 ml samples of the minimal media before adding the culture for baseline OD measurement, and another 1 ml sample after adding the 10 ml culture for OD measurement at 0 hour time point. Measure OD₆₀₀ of the culture after each hour until the OD₆₀₀ shows a net increase of 0.2 (note: there should have a jump in OD₆₀₀.
between the first and third hour of growth). The increase in OD value over time should be similar to the values shown below: 0 hour: OD$_{600}$ is 0.039 and net change in OD is 0; 1 hour: OD$_{600}$ is 0.065 and net change in OD is 0.026; 2 hours: OD$_{600}$ is 0.151 and net change in OD is 0.112; 3 hours: OD$_{600}$ is 0.256 and net change in OD is 0.217. Sometimes net change will take ~ 6-7 hours to reach. When the net change in OD reaches 0.15 – 0.2, add 5 ml of filter sterilized 200 mM rhamnose (2 mM final) to each flask. Incubate the culture for an additional ~ 16-24 hours at 37 °C with rapid shaking at 230 rpm. The OD$_{600}$ after overnight induction should be ~ 1.5 (the cultures must have a pink color after the overnight incubation). Book and warm up the centrifuge and F10 rotor to room temperature for tomorrow morning.

**Day 3:**

To minimize Pi contamination, avoid glassware throughout purification as much as possible. Perform FFQ-Sepharose column at room temperature. Prepare elution buffer (EB) at room temperature and dialysis buffer (DB) at 4 °C. Elution buffer: 150 ml of 10 mM Tris-HCl pH7.6 with 0 mM NaCl and 150 ml of 10 mM Tris-HCl pH7.6 with 200 mM NaCl. Dialysis buffer: 2 L of 10 mM Tris-HCl pH 7.5.

PBP is exported into the periplasm after expression. To collect the periplasmic fraction, osmotic shock is used. There are several keys to ensure a good yield of PBP protein: culture must be pink colored; for each step of osmotic shock, remove previous buffer nicely and resuspend pellet in next buffer nicely; RBIV must be ice cold. If cell cultures are turned into pink in color, check OD$_{600}$. If OD$_{600}$ is around 1.5, start harvesting cells. Centrifuge the culture at 5,000 rpm for 15 min, and resuspend
the pellet in 650 ml RBI with a spatula at RT and split to two centrifuge bottles. Centrifuge biomass at 5,000 rpm for 15 min at RT, and resuspend the pellet in 350 ml RBI with a spatula and combine into one bottle. Centrifuge again at 5,000 rpm for 20 min at RT and discard carefully the supernatant (the cell pellet tends to be very loose at this point). Resuspend the pellet thoroughly in 100 ml RBII with a spatula, add a stir bar and stir vigorously for 2 min at RT. Cool down the centrifuge and rotor to 4 °C. To the stirring solution, add 100 ml RBIII and continue stirring vigorously for an additional 10 min. Centrifuge at 9,000 rpm for 20 min at 4 °C. During this spin, prepare an ice-bath and move RBIV into a -20 °C freezer 5 min before addition. Discard the supernatant, and rapidly but thoroughly resuspend the pellet in 200 ml ice-cold RBIV with a spatula and gently stir for 15 min in an ice bath. During this step, periplasmic proteins are released. Remove a stirring bar before spin. Save 100 µl of the resuspended solution for the purification gel, and centrifuge the solution at 9,000 rpm for 20 min at 4 °C. Transfer the supernatant into a clean cylinder and it should be pinkish purple. The supernatant now contains PBP; determine the concentration of the supernatant to obtain PBP yield (~ 300-350 mg). Measure absorbance for 10 µl of the supernatant at A_{280} and A_{320}, and calculate the PBP concentration (molecular weight of PBP = 34.45 kD) using the formula: Total PBP (mg) = (A_{280} - A_{320}) / 1.78 mg^{-1}.ml^{-1} x dilution factor (100) x total volume (200 ml). If the yield of PBP protein is much less than 350 mg, repeat the step of adding RBIII and RBIV one more time and measure the concentration again.
Protein Purification with FFQ-Sepharose column:

Perform FFQ-Sepharose column at room temperature and no wash step required. Add ~ 2 ml of 1 M Tris-HCl pH 7.8 (make up final concentration to 10 mM) into ~ 200 ml supernatant and gently but nicely mix. Take 1 ml for a gel sample of loading. Load the supernatant slowly ~ 0.5-1.0 ml/min onto 30 ml FFQ-Sepharose column. Watch the column every 5 min to ensure that there is no leakage. Do not wash the column after loading the protein. Elute bound protein with 300 ml of 10 mM Tris-HCl, pH 7.6: 0 – 200 mM NaCl gradient at 1.5 ml/min and collect ~ 80 fractions of 1.5 ml each. Keep eluting and checking alternative numbered fractions by mixing 1 µl sample with 100 µl Bradford in a microplate until a first peak shows up and passes. This corresponds to PBP and determines which elution fractions have to be examined on gels. Analyze 10 µl of every alternate fraction on 14 % SDS-PAGE (Fig. C.3). After confirming the presence of protein in the elute, wash the column with 200 ml 2 M NaCl. Pool (~ 50-65 ml) and dialyze the fractions containing PBP in a dialysis bag (MWCO 6-8,000, 3.3 ml/cm) overnight against 2 L dialysis buffer (DB) in the cold room.

Day 4:

Transfer the PBP from a dialysis bag to a falcon tube and mix well by gentle inversion. Check PBP concentration: PBP (µM) = A_{280} / 61880 M^{-1}.cm^{-1} x dilution factor x 10^6; PBP (mg/ml) = A_{280} / 1.78 mg^{-1}.ml^{-1} x dilution factor. Freeze at -80 °C until labeling after measuring the concentration. The concentration of the pooled fraction should be ~ 6 mg/ml (150 – 200 mg total).
Figure C.3 Purification of phosphate binding protein (PBP). 10 µl of the sample from alternate fractions was analyzed on 14 % SDS-PAGE (M–marker, L–load). Fractions containing PBP were pooled and labeled with MDCC.
C.5 Labeling *E. coli* PBP-A197C with MDCC

**Preparation:**

Measure the concentration of the protein after dialysis as described earlier. Labeling efficiency is optimum when the concentration of the PBP in the reaction is > 60 mM. Concentrate the protein using an YM-10 centriprep if the concentration of the protein is lower than 60 mM. The 17 ml FFQ column is used for labeling of ~ 130-200 mg PBP. If PBP is less or more than this range the column should be scaled down or up. Pack a 17 ml FFQ column in a XK16 column and wash with plenty ddH$_2$O at ~ 1-5 ml/min and equilibrate with ~ 200 ml washing buffer (10 mM Tris-HCl pH 8.0) at 3 ml/min and keep in the room temperature. Make 100 ml exchange buffer (EB): 20 mM Tric-HCl pH 8.1; 500 ml washing buffer (WB): 10 mM Tris-HCl pH 8.0; 100 ml WB with 50 mM NaCl and keep them in the room temperature. Presoak 2 x Centriplus10 with EB. Prepare two 14 % SDS-PAGE gels and keep at 4 °C. Equilibrate a roller drum to RT before use. Make three main stock reagents are needed. Stock 1:50 mM 7-Methyguanosine (7-MEG): MW: 283.2, dissolve 14.2 mg in 1 ml ddH$_2$O and aliquot 50 µl and store at - 80 °C. Stock 2: 1 U/µl Purine Nucleoside Phosphorylase (PNPase): Read the label of a PNPase bottle and add an appropriate volume (~ 200 ml) ice-cold ddH$_2$O to entire bottle (Sigma). Rinse out all over the inner surface of the bottle by tilting and briefly vortex. Aliquot 50 µl fractions and store at - 80°C as lab stock. Upon thawing each tube at first time, further aliquot 10 µl and keep as personal stock. Stock 3 (perform in the dark): 25 mM MDCC: MW: 383.5, add 520 µl N, N-dimethylformamide (DMF), mix by
vortexing. Rinse out all over the inner surface of the bottle by titling and vortex well. This substance is light sensitive, so turn off all lights before opening the colored bottle. Wrap the MDCC solution bottle with aluminum foil after dissolving, and store at - 20 °C. Estimate how much stock reagents are needed. PNPase (µl) = \{\text{final concentration of PNPase (0.2 U/ml) x total volume of PBP (µl)}\} / 1 \text{ U/µl PNPase stock; 7-MEG (µl) = \{\text{final concentration of 7-MEG (200 µM) / 7-MEG stock concentration (50 x 10}^3 \text{ µM)} \} x \text{total volume of PBP (µl) x 10}^3\}; \text{MDCC (µl) = \{PBP concentration (µM) x 1.5\} / \{MDCC stock concentration (25 x 10}^3 \text{ µM) x total volume of PBP (µl) x 10}^3\}.\}

**MDCC Labeling:**

MDCC is relatively stable under white illumination. Labeling and purification can be carried out in the light. But protect the MDCC stock bottle from light. Thaw PBP and equilibrate to RT. Add PNPase and 7-MEG to the tube and incubate on the roller drum at moderate speed for above 30 min at RT. This mopping is important because apo PBP is more efficiently labeled than the Pi-bound form. In the dark, bring the MDCC stock solution bottle to room temperature, vortex well and add MDCC to the mopped PBP tube. Wrap the tube of the labeling reaction by aluminum foil and incubate on the roller drum at a moderate speed for above 1 hour at RT.
**Buffer exchange (4 hours):**

Split the labeling reaction into 2 x presoaked Centriprep10. Prepare a gel sample of “after labeling”; take 10 ml protein and add 10 ml 2 x no-dye SDS loading buffer. Spin (ss34 rotor) at 4k rpm 10 – 20 °C, 30 min. Transfer the filtrates to a falcon tube, and fill reservoir up to a 15 ml line with the labeling reaction if any left or EB. Every time after spin, record the volumes of the filtrate and buffer. Repeat previous steps as needed. Usually five times is enough to get the 7/8 x volume of the initial buffer replaced. For a last spin, do not add buffer and just concentrate down to 2.5 ml x 2 (Table C.1). Sometimes free MDCC precipitates along with spin steps (white precipitates), but it will be removed by a following filtering. Ultrafiltration removes a certain amount of free MDCC in parallel with exchanging buffer. Transfer the retentates into a falcon tube and filtrate with φ = 0.2 mm filter (low protein binding such as Tuffryn). Rinse the Centriplus units with 10 ml EB, filtrate the EB buffer with the filter used for the retentate, and combine with the previously filtered retentate. Finally, total volume is ~ 15 ml and the 23/24 x volume of the initial buffer is exchanged.

**FFQ column purification:**

Remove the top part from the packed FFQ column. Lower the buffer level down to the surface of the resin by gravity flow. Load the labeling reaction directly onto the Q column (~ 15-17 ml). Run the column by gravity flow until the reaction completely enters the resin. Overlay washing buffer (WB) and reassemble the top part to the
column. Wash at ~ 1.5-3.0 ml/min with 50 ml of WB or until yellow passes. Elute with 150 ml of 0 – 50 mM NaCl gradient in 10 mM Tris-Cl, pH 8.0 at 1.5 ml/min. Collect elution fractions (1.5 ml / 61 drops / tube). Be careful that drop size becomes double soon after the labeled PBP starts to elute. Keep your eyes on a fraction collector during elution. Once elution becomes yellow in color, start to check $A_{280}$ (protein) and $A_{430}$ (MDCC) of alternative numbered fractions by mixing 10 ml protein with 990 ml H$_2$O. Plot these absorbance values along with fraction number in an excel template “PBP_Labeling_Template” (Figure C.4). When the protein peak passes, stop elution. Although yellow-colored fractions could still continue, these contain just free dye (only conjugated MDCC efficiently absorbs 430 nm light). PBP (Pi-free, native condition), $\varepsilon_{280} = 61880$ (M$^{-1}$·cm$^{-1}$), MW 34453.8 and MDCC (conjugated), $\varepsilon_{430} = 46800$ (M$^{-1}$·cm$^{-1}$). Pool fractions containing PBP labeled with MDCC at above 70 %. Sometimes, fractions with extraordinary high labeling follow the labeled PBP peak. Do not take them because these include labeled non-PBP proteins. Check concentration and labeling efficiency of the pooled protein by measuring $A_{260}$ and $A_{430}$. If the concentration is above 100 µM, proceed to aliquot 0.5 µl and store at - 80 °C. Otherwise concentrate protein before storage. To check MDCC labeling quality, run a 14 % SDS-PAGE gel (Figure C.5). The loading volumes of “after labeling” (AL) and “after purification” (AP) samples are adjusted to be same amount of PBP. The FT+W sample in Figure C.5 was prepared from a mixture of flow-through and washing fractions during the FFQ column. Take a picture on a UV illuminator with 365 nm (Figure C.5). Stain gel with CBB and
destain and scan. After labeling, wash the column at 3 ml/min with 3 x column volume of 2 M NaCl or until no yellow color elutes. Transfer the washed resin into a 50 ml falcon tube and store in the cold room.
**Table C.1 PBP labeling buffer exchange chart.**

<table>
<thead>
<tr>
<th>Start volume</th>
<th>Spin</th>
<th>Filtrate</th>
<th>Retentate</th>
<th>Volume ratio of the original buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 15 ml PBP x 2</td>
<td>→ 30 min</td>
<td>Total 12.5 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 6.5 ml PBP x 2</td>
<td>→ 30 min</td>
<td>Total 27.5 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 7.5 ml PBP x 2</td>
<td>→ 30 min</td>
<td>Total 42.5 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 7.5 ml PBP x 2</td>
<td>→ 30 min</td>
<td>Total 56 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 1 ml PBP x 2 +6.5 ml EB x 2</td>
<td>→ 30 min</td>
<td>Total 70 ml</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>+ 7.0 ml EB x 2</td>
<td>→ 30 min</td>
<td>Total 81 ml</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>+ 7.0 ml EB x 2</td>
<td>→ 30 min</td>
<td>Total 95 ml</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>+ 7.0 ml EB x 2</td>
<td>→ 30 min</td>
<td>Total 107 ml</td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>(No addition)</td>
<td>→ 30 min</td>
<td>Total 120 ml</td>
<td>Total 6 ml</td>
<td></td>
</tr>
<tr>
<td>Final total is 17 ml by adding 12 ml EB</td>
<td></td>
<td></td>
<td>1/32</td>
<td></td>
</tr>
</tbody>
</table>
Figure C.4 Labeling efficiency of PBP with MDCC. Absorbance of 10 ml of the sample from the FFQ column fractions was measured at 280 and 430 nm to measure the concentration of PBP and MDCC label respective. Pink line is PBP concentration, blue line is conjugated MDCC concentration and green line is the labeling percentage.
Figure C.5 MDCC labeling quality check with SDS-PAGE gel and UV scan. AL is after labeling sample, AP is after purification sample and FT+W sample was prepared from a mixture of flow-through and washing fractions during the FFQ column. Left is CBB stained gel and right is UV scan in 365 nm.
References


