The replication clamp-loading machine at work in the three domains of life

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Abstract | Sliding clamps are ring-shaped proteins that tether DNA polymerases to DNA, which enables the rapid and processive synthesis of both leading and lagging strands at the replication fork. The clamp-loading machinery must repeatedly load sliding-clamp factors onto primed sites at the replication fork. Recent structural and biochemical analyses provide unique insights into how these clamp-loading ATPase machines function to load clamps onto the DNA. Moreover, these studies highlight the evolutionary conservation of the clamp-loading process in the three domains of life.

Replicase
A DNA polymerase with accessory subunits, including a clamp and clamp loader, that replicates chromosomal DNA.

Protomers
Subunits from which a larger structure is built.

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Chromosomal replicases achieve high processivity during DNA synthesis through their direct interaction with a ring-shaped clamp that slides on DNA[1,2]. The sliding-clamp protein is loaded onto DNA by a multiprotein clamp-loader complex that opens and reseals the clamp at the primer–template junction in an ATP-dependent manner[1]. The clamp loader interacts with the surface of the sliding clamp that binds to the DNA polymerase, and it must therefore leave the clamp prior to the use of the clamp by the DNA polymerase (BOX 1). In addition to their role in DNA replication, sliding clamps function with various proteins that are involved in several other cellular processes, including cell-cycle control, DNA repair and apoptotic pathways[3].

Functional and structural analyses indicate that the architecture and mechanism of clamps and clamp loaders are conserved across the three domains of life[1,4–8]. Despite their low level of sequence identity, all sliding clamps, from prokaryotes and eukaryotes alike, form similar planar ring structures with a central channel that is of sufficient width to encircle duplex DNA[9–13] (FIG. 1). Even though the individual clamps exist in different oligomeric states, each has a pseudo six-fold symmetry with six domains forming the complete ring. Likewise, the multiprotein clamp loaders from different organisms are circular heteropentameric complexes with sequence similarities among their subunits[4–6,14]. Biochemical studies indicate that clamp loading is a highly regulated process that involves communication between the different ATPase sites in the clamp-loader complex. Although different clamp loaders share several mechanistic features, there are also significant differences in the way that ATP is used by the various clamp-loading machines. This review provides a summary of our current state of knowledge about the clamp-loading mechanism in the three domains of life.

Prokaryotic clamps and clamp loaders

Escherichia coli **β- and γ-complex.** The *Escherichia coli* chromosomal replicase is a multiprotein assembly that is known as DNA polymerase III (Pol III) holoenzyme[15]. Pol III holoenzyme contains ten different subunits that are organized into three different subcomponents: the Pol III core (comprising three subunits: the α-subunit, the ε-subunit and the θ-subunit), the β-clamp and the γ clamp-loader complex ($\gamma\tau_2\delta\delta'\chi\psi$)[1]. The crystal structure of the β-clamp reveals two identical crescent-shaped protomers that form a ring with a central channel of 35-Å diameter, which is sufficiently wide to accommodate double-stranded DNA (dsDNA)[9] (FIG. 1a). Each β-clamp protomer consists of three domains and dimerizes head-to-tail to generate two structurally distinct 'faces'. One face has several loops, and the C termini protrude from it. In all known clamps, this 'C-terminal face' is the face of the ring that interacts with other proteins, as discussed below.

The γ clamp-loader complex consists of seven subunits ($\gamma\tau_2\delta\delta'\chi\psi$)[16], but the ATPase activity resides only in the γ- and τ-subunits, which are both encoded by the same gene, *dnaX*. The γ-subunit (47 kDa) is truncated by a translational frameshift and is therefore smaller than the full-length τ-subunit (71 kDa)[17–19]. The unique 24-kDa C-terminal extension of the τ-subunit (which is not present in the γ-subunit) binds to DNA B helicase and Pol III core at the replication fork[20–23]. Mixed clamp-loader complexes that contain both γ- and τ-proteins[16], as well as clamp loaders that contain three γ-subunits and no τ-subunits or three τ-subunits and no γ-subunits[24,25], have been characterized. The $\gamma_3\delta\delta'\chi\psi$ complex[24] probably fulfils functions other than replication, such as loading β-clamps onto DNA that is used by repair proteins[1].

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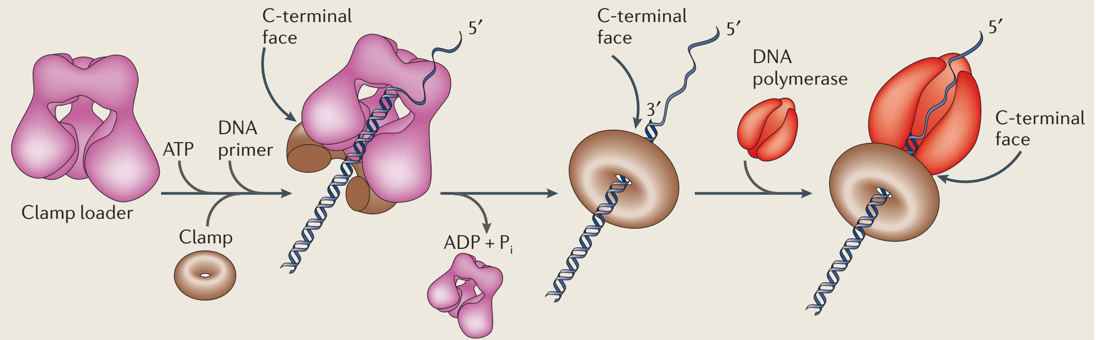
Box 1 | **Clamp loading and the use of sliding clamps in processive DNA synthesis**



The figure represents a generalized mechanism of clamp-loader and clamp–DNA-polymerase action. A multiprotein clamp loader, in the presence of ATP, binds and opens a ring-shaped sliding clamp. In the ATP-bound state, the clamp loader has a high affinity for primer–template junctions with recessed 3′ ends. Binding of DNA stimulates ATP hydrolysis and clamp-loader ejection from DNA, leaving the closed clamp on DNA, properly oriented for use by a replicative DNA polymerase. The clamp loader and DNA polymerase compete for the same C-terminal face of the clamp. Therefore, it is important that the clamp loader leaves the clamp, freeing the clamp for use with other enzymes such as replicative DNA polymerases. Different proteins that are involved in cell-cycle control, DNA replication, DNA repair and the apoptotic pathway bind the clamp. In particular circumstances, two or more proteins can bind to a clamp on DNA at the same time. For example, *Escherichia coli* DNA polymerase III and IV simultaneously bind to the β-clamp for lesion bypass[120,121]. In another example, *Sulfolobus solfataricus* DNA polymerase, flap endonuclease-1 (FEN1) and DNA ligase I bind to proliferating cell nuclear antigen (PCNA) and couple DNA synthesis and Okazaki-fragment maturation[107]. P_i, inorganic phosphate.

The $\gamma_3\delta\delta'$ complex, which lacks χ- and ψ-subunits, is referred to as the 'minimal' clamp loader, as the γ-, δ- and δ′-subunits are necessary and sufficient to place the β-clamp on a primed DNA template[24]. The γ-, δ- and δ′-subunits are members of the AAA+ family of ATPases (BOX 2). The χ- and ψ-subunits are not required for clamp loading[24] and the structure of the χψ complex shows no structural similarities to the other clamp-loader subunits[26]. However, the χψ subassembly binds to single-stranded-DNA-binding protein (SSB) and destabilizes the primase–SSB contact, which leads to the dissociation of primase and the assembly of the clamp onto new RNA-primed sites[27,28].

The crystal structure of the $\gamma_3\delta\delta'$ complex reveals that the five subunits are arranged in a circular fashion[6] (FIG. 2a). Each subunit consists of three domains (FIG. 2b). The C-terminal domains of the five subunits pack with one another to form a tight uninterrupted circular 'collar' that holds the pentamer together (FIG. 2a, left). The two N-terminal domains in each subunit are comprised of the AAA+ homology region. These AAA+ homology domains are arranged in a right-handed helix, as described in more detail below[5,29]. The asymmetric circle that is formed by the $\gamma_3\delta\delta'$ pentamer contains a gap between the AAA+ domains of the δ- and δ′-subunits (FIG. 2a, middle and right). This gap, the heteropentamer composition and the helical arrangement of AAA+ domains distinguish clamp-loader complexes from most of the other AAA+ ATPases, which are often circular homohexamers with no gaps[30,31] (BOX 2). Perhaps during the evolution of the clamp loaders, a 'sixth' subunit was displaced to create the gap. This gap is needed for DNA-strand passage, as illustrated in FIG. 2b.

AAA+ family of ATPases
(ATPases associated with a variety of cellular activities). A large family of functionally divergent proteins that have a region of homology around the ATP sites. They are often used to remodel other macromolecules.

Primase
A specialized RNA polymerase that synthesizes short RNA primers to initiate DNA synthesis by DNA polymerases.

Mechanistic insights into* E. coli *clamp loading. The clamp-loading mechanism can be described on the basis of biochemical studies. The δ-subunit of the clamp loader is capable of binding and opening the β-clamp dimer on its own, but it is blocked from binding the β-clamp by the other subunits of the clamp-loader complex[7,32]. The clamp-loader complex does not bind the β-clamp until the ATP-binding sites are occupied. ATP binding drives a conformational change that allows the clamp loader to bind and open the clamp[33]. Although the δ-subunit forms a strong attachment to the β-clamp and drives clamp opening, other subunits of the complex are also known to bind the clamp[1]. The clamp-loader–β-clamp complex then binds primed DNA, which stimulates hydrolysis of ATP[34]. This, in turn, results in the closing of the clamp around the DNA and the ejection of the clamp loader from the β-clamp and the DNA[34–36]. The clamp has two distinct faces (FIG. 1a) and must be positioned on the primed site such that the correct face is properly oriented to functionally interact with the DNA polymerase[9,32]. So, the γ-complex must use the 3′ terminus as a guidepost to locate and orient the clamp at a primed site.

Insight into how the δ-subunit opens the β-clamp is provided by the structure of the δ-subunit bound to a monomeric mutant of the β-clamp[7]. This structure shows two distinct points of contact between the δ-subunit and the β-clamp, which are located on opposite ends of the same α4 helix in the N-terminal domain of the δ-subunit (FIG. 2c). One end of the α4 helix of the δ-subunit partially unfolds and binds a hydrophobic pocket on the surface of the β-clamp. The opposite end of the α4 helix seems to exert a force on the dimeric interface. The result is a distortion at one interface of the clamp, which

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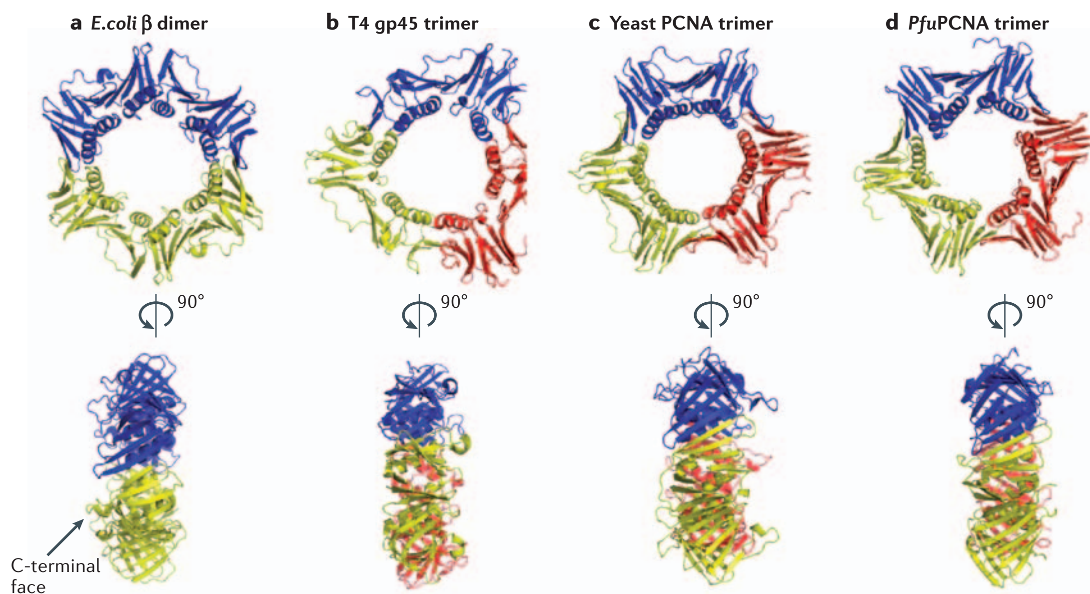

Figure 1 | **Sliding clamps of different organisms.** Crystal structure of (**a**) the *Escherichia coli* β-complex (Protein Data Bank (PDB) code 2POL), (**b**) gp45 of bacteriophage T4 (PDB code 1CZD), (**c**) *Saccharomyces cerevisiae* proliferating cell nuclear antigen (PCNA; PDB code 1PLQ) and (**d**) *Pyrococcus furiosus* PCNA (PDB code 1GE8). Sliding clamps have similar architectures, comprising six domains arranged in a circle. The *E. coli* β-complex is a dimer (three domains per monomer), whereas T4 gp45 and PCNA are trimers (two domains per monomer). The rings have a continuous layer of antiparallel sheets all around them, and 12 α-helices line the central cavity (except in T4 gp45). The chain-folding topologies of each domain are essentially the same, although one β-sheet and α-helix are disrupted in each protomer of the T4 clamp, which probably contributes to its lower stability. The protomers are arranged head-to-tail in all clamps, which results in structurally distinctive 'faces'. The face from which the C-terminal domains protrude is the interactive surface for other proteins (for example, clamp loaders, DNA polymerases and repair proteins).

is inconsistent with clamp closure and is presumed to hold the β-clamp in an open conformation[7,37].

The clamp must open at least 20 Å to accommodate passage of duplex DNA into the central channel. Comparison of the β-dimer with the β-monomer (from the δ–β structure) shows that the dimeric β-ring is under spring tension in the closed state. Interaction with the δ-subunit disrupts one interface and allows the spring tension between the β-domains to relax, producing a gap for DNA-strand passage[7]. The strong interactions between β-monomers probably maintain this tension until the δ-subunit distorts one of them.

The crystal structure of the minimal *E. coli* clamp loader ($\gamma_3\delta\delta'$) has also been determined in the presence of the poorly hydrolysable ATP analogue ATPγS[38]. Three of the five subunits of the minimal clamp loader are functional ATPases (γ), whereas the other two subunits (δ and δ′) do not bind ATP[39]. The ATP sites of the γ clamp-loader complex are located at the interfaces between subunits (BOX 2). Most of the contacts to each bound nucleotide are formed within one subunit, although the catalytic site requires a conserved arginine residue from a neighbouring subunit. This arginine residue, which is known as an 'arginine finger', is located in a conserved serine–arginine–cysteine (SRC) motif that is present in all known clamp loaders. The structure of the nucleotide-free $\gamma_3\delta\delta'$ complex shows that two of the ATP-binding sites are open (site-1 and site-3), whereas one is sealed shut (site-2) by the close juxtaposition of the adjacent subunit[6]. Interestingly, only two nucleotides are bound in the structure of the $\gamma_3\delta\delta'$ complex in the presence of ATPγS (in site-1 and site-3), whereas site-2 remains empty, and the structure has changed little compared with unliganded $\gamma_3\delta\delta'$ (REF. 38). This indicates that the binding of a third ATP molecule at site-2 is required to promote a structural rearrangement that results in an interaction between the clamp and the clamp loader. Studies of γ-complexes with mutated ATP sites indicate that arginine fingers at different ATP sites serve important and distinct functions. ATP still binds to a γ-complex that lacks arginine fingers, but the resulting ATP–γ-complex binds neither the β-clamp nor DNA. The arginine finger in site-1 is needed to attain a conformation of the clamp loader that binds to the β-clamp, and the arginine fingers of site-2 and site-3 are needed to enable binding to DNA[40–42]. The three ATP molecules then undergo ordered hydrolysis, resulting in the closure of the β-clamp around DNA and the ejection of the γ-complex from the β-clamp and DNA.

At the replication fork, the asymmetric structure of the *E. coli* clamp loader imposes distinct properties onto the two identical Pol III cores, which make them competent for replicating the leading or lagging stand[43,44]. The two τ-subunits of the clamp loader crosslink two Pol III cores that replicate both strands[22]. The single clamp loader within the Pol III holoenzyme assembles β-clamps onto DNA for both leading and lagging stands[23]. During fork progression, the lagging-strand polymerase hops

SRC motif
A three-residue motif (serine–arginine–cysteine) that is conserved in clamp-loading subunits and that contains a catalytic arginine residue that is involved in ATP hydrolysis.

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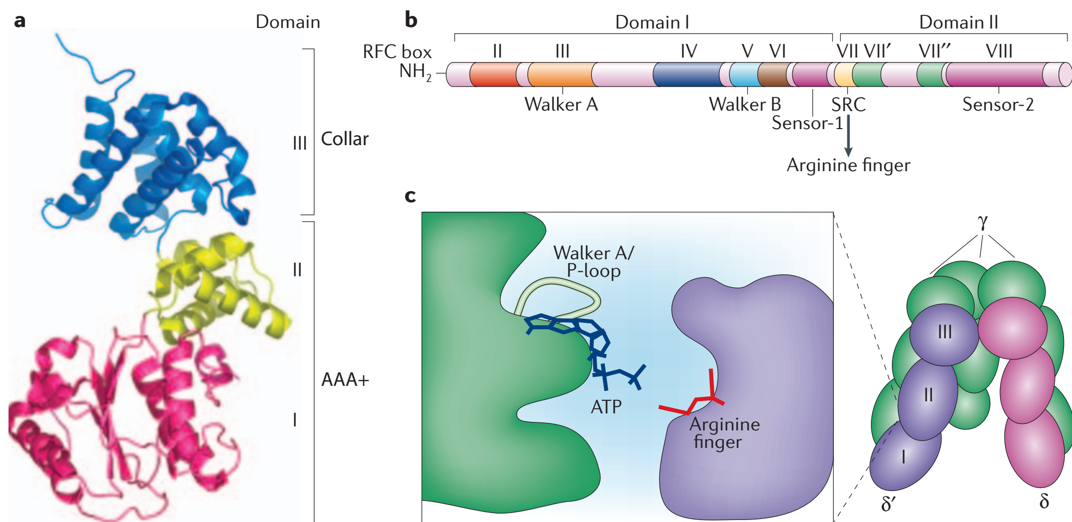
Box 2 | **Clamp-loader subunits are AAA+ proteins**



The AAA+ family includes chaperone-like proteins with a conserved ATP-binding module that is defined by two domains (for example, domains I and II in the γ-subunit of the *Escherichia coli* clamp loader; see figure, part **a**)[122–125]. On the basis of sequence alignments and structural information, seven major classes of AAA+ proteins have been defined. Most of them form closed hexameric assemblies, except class-1 and class-2, which are characterized by open-ring complexes (FIG. 2a and FIG. 4a). Clamp loaders belong to class-1. In the γ-subunit of the *E. coli* clamp loader, the sequences that define the AAA+ family encompass the two N-terminal domains (part **a**). The N-terminal domain I comprises sequences from boxes II–VI, whereas the sequences within the remaining four boxes (boxes VII, VII′, VII″ and VIII) are contained within domain II (see figure, part **b**).

In AAA+ proteins, the nucleotide-binding pocket, which is common in other nucleoside 5′-triphosphatases (NTPases), lies at the apex of three adjacent, parallel β-strands in a compact αβα fold referred to as the AAA+ core, or the RecA-like arrangement. The AAA+ core contains the Walker A (also known as the P-loop) and Walker B ATP-binding motifs. Distinguishing features of AAA+ proteins are box VII and two other nucleotide-interacting motifs, termed sensor-1 and sensor-2 (REF. 126). The box VII motif contains a highly conserved arginine residue that is thought to function as the 'arginine finger' in GTPase-activating proteins[127].

In AAA+ oligomers, ATP-binding and ATP-hydrolysis events occur at the interface of neighbouring subunits and drive conformational changes within the AAA+ assembly that direct translocation or remodelling of the target substrates. The major component of the ATP-binding site is located on one subunit, although other residues that are essential for catalysis are located on the adjacent subunit. Most commonly, this takes the form of an arginine finger. In clamp loaders, the arginine finger is located in a conserved SRC motif in box VII. A bipartite ATP site is shown (see figure, part **c**) in which the δ-subunit of *E. coli* clamp loader (purple) contributes the arginine finger to ATP bound to the γ-subunit (green). The location of this interfacial ATP site within the clamp-loader pentamer ($\gamma_3\delta\delta'$) is indicated on the right. RFC, replication factor C.

from one β-clamp to another upon the completion of each Okazaki fragment, leaving the clamps behind on the DNA[23]. The γ-complex, which can load and unload clamps from DNA, assists in the dissociation of the β-clamp during ongoing fork movement[32]. The presence of the τ-subunit protects the β-clamp in the leading strand from being removed by the γ-complex and stimulates processive DNA synthesis[45]. The τ-subunit also contributes to processivity on the leading strand by interacting directly with DnaB helicase and stimulating its catalytic efficiency for the unwinding of dsDNA[22,23].

Bacteriophage T4 gp45 and gp44/62. The replicase of bacteriophage T4 and its close relative RB69 each consist of the gp43 DNA polymerase, the gp45 sliding clamp and the gp44/62 clamp loader (reviewed in REFS 46–48). The T4 gp45 sliding clamp is a homotrimeric ring with inner and outer diameters that are similar to those of the β-clamp[10,49,50]. The gp45 monomer consists of only two domains, but the trimer forms a six-domain ring like the β-clamp. The crystal structure shows that all three interfaces of the gp45 monomers are fully closed[10] (FIG. 1b and FIG. 3a, left). However, solution FRET studies indicate that the gp45 clamp exists in an open state[51] (FIG. 3a, right).

The gp44/62 clamp loader contains four gp44 subunits and one gp62 subunit[50]. The gp44 subunit is homologous to the *E. coli* γ- and δ′-subunits. However, the gp62 subunit is similar to the δ-subunit of the clamp loader as it lacks both the SRC motif and the Walker A motif (also known as a P-loop) and its sequence has diverged from gp44. Although the arrangements of the gp44 and gp62 subunits are not known, they are presumed to form a circle that is similar to the *E. coli* γ-complex (FIG. 3b). The gp44/62 clamp loader has a very low intrinsic ATPase activity, but the presence of both the clamp and primed DNA stimulates its activity[52,53]. The gp44 subunits contain an ATP-binding site each and also seem to be responsible for the main contacts with DNA[52,54]. However, the gp62 subunit is required to achieve stimulation of the ATPase activity of the gp44/62

FRET
(Fluorescence resonance energy transfer). The transfer of energy from a light-activated fluorophore to a second fluorophore that has a longer excitation wavelength. The efficiency of energy transfer depends on the distance between fluorophores.

Walker A and B motifs
These motifs are found in most ATP-binding proteins. The Walker A region, also known as a P-loop, binds ATP, whereas the Walker B region binds Mg^{2+}. Both are required for ATP hydrolysis.

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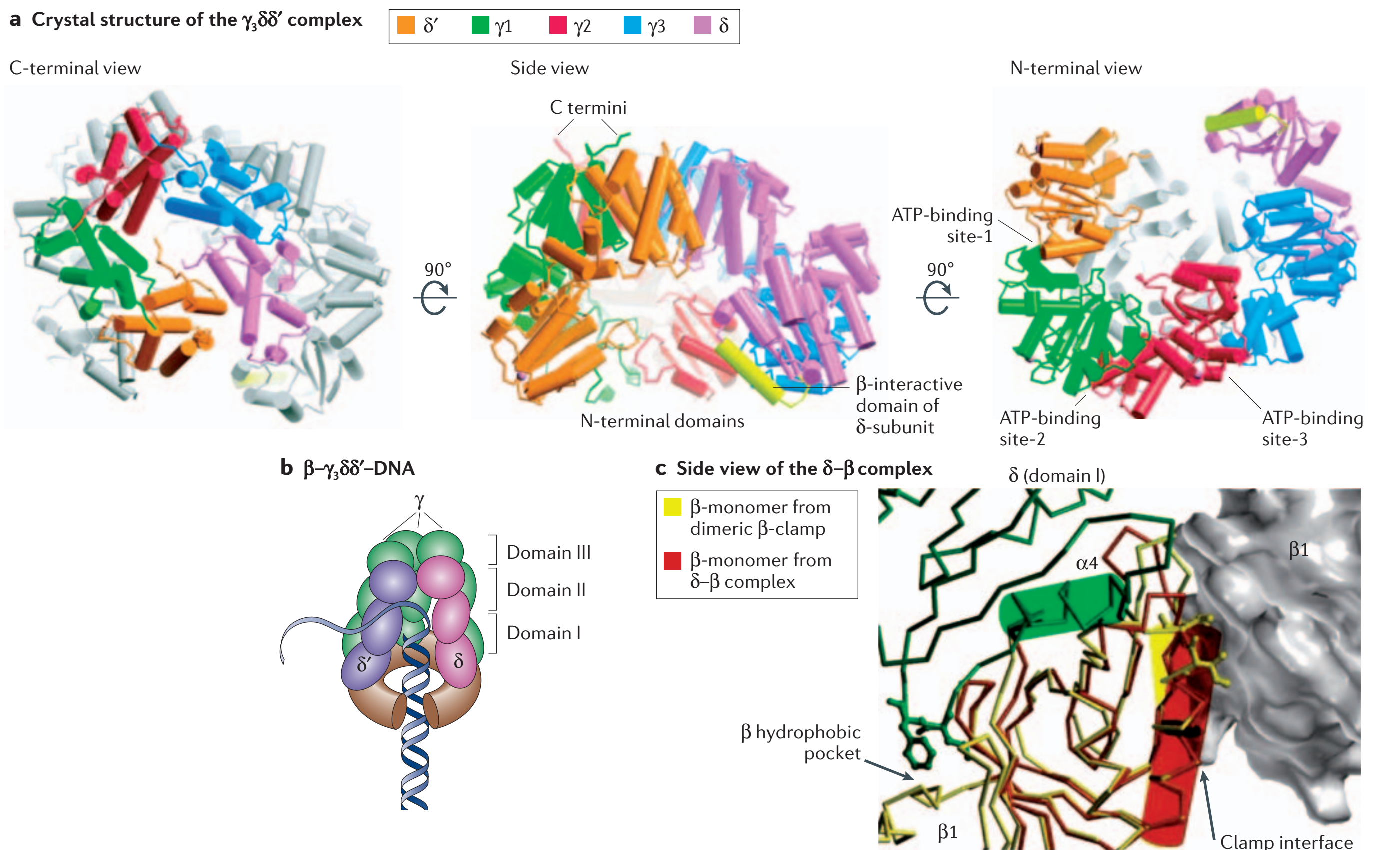

Figure 2 | **The *Escherichia coli* γ clamp-loader complex. a** | Crystal structure of the *Escherichia coli* $\gamma_3\delta\delta'$ complex (Protein Data Bank (PDB) code 1JR3). Left-hand diagram: view looking down on the C-terminal domains that form an uninterrupted circular collar. Middle diagram: side view of $\gamma_3\delta\delta'$. The gap between the N-terminal domains of the δ- and the δ′-subunits is facing the viewer. The β-interactive helix in domain I of the δ-subunit is shown in yellow. The β-clamp docks onto domain I of the different subunits (not shown). Right-hand diagram: view looking at the N-terminal face of $\gamma_3\delta\delta'$. The gap between the AAA+ domains of the δ- and the δ′-subunits is apparent. Adapted with permission from REF. 6 © Elsevier. **b** | Cartoon of $\gamma_3\delta\delta'$ binding to DNA and the β-clamp. Each subunit is composed of three domains. The N-terminal domain I binds to the β-clamp, thereby opening it. Primed template fits through the gap between the δ- and δ′-subunits and the open clamp. **c** | The structure of δ-subunit domain I bound to a β-clamp monomer (PDB code 1JQL). Helix α4 of the δ-subunit contacts the β-clamp in two places: a hydrophobic pocket and a loop at the interface. The N-terminal domain of the δ-subunit is shown in green. The structure of the β-clamp monomer (β1) is in yellow for the dimeric form of the clamp, and in red for the β-clamp monomer in the δ–β complex. The grey surface represents a second β-clamp monomer modelled into the δ–β structure. Adapted with permission from REF. 7 © Elsevier.

complex by the gp45 clamp[54]. The four gp44 subunits bind four ATP molecules, which are then hydrolysed during the assembly of the holoenzyme on DNA[53,55]. Given the fact that the gp62 subunit lacks a SRC motif, it seems likely that the gp44 subunit that is adjacent to gp62 will not be able to hydrolyse ATP, and therefore the gp44/62 clamp loader might only have three competent ATP-hydrolysis sites. In this case, one of the three ATP sites might reload and hydrolyse a second ATP molecule in order to achieve the hydrolysis of four ATPs during the clamp-loading process.

Several studies employing a diversity of fluorescent techniques have been used to examine clamp loading and the assembly of the T4 gp43 DNA polymerase with the clamp on DNA[56–61]. These investigations, as well as photocrosslinking experiments[62], show that the binding of gp44/62–4ATP to gp45 is accompanied by the hydrolysis of two molecules of ATP, which seems to open the clamp to accommodate duplex DNA[60,61] (FIG. 3b). Comparison of the gp44/62–gp45 complex in the presence and absence of nucleotides indicates that ATP binding induces a conformational rearrangement of the subunits[57,62]. Opening of the clamp is not observed when a non-hydrolysable ATP analogue, such as ADP, ATPγS or ADP–Al_3F, is used in place of ATP, which indicates that it is the hydrolysis, and not the binding of ATP, that leads to clamp opening[51,63]. Interestingly, gp62 is involved in binding DNA[64,65]. Upon the association of the gp44/62–gp45 binary complex with DNA, further ATP hydrolysis occurs and the open gp45 clamp converts to an out-of-plane left-handed helical conformation[56,66] (FIG. 3b). Clamp opening and closing events are intimately tied to ATP hydrolysis, but only partially triggered by it[66]. So, it seems that nucleotide hydrolysis mainly powers conformational changes in gp44/62 that prepare it for interaction, first with gp45 and then with DNA.

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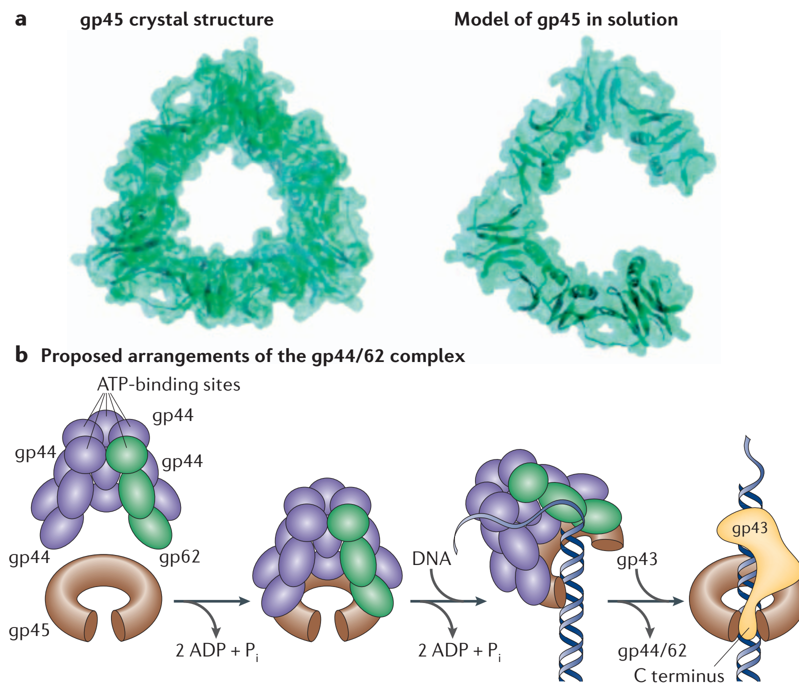

Figure 3 | **The gp45 clamp and the gp44/62 clamp loader of bacteriophage T4.** **a** | Ribbon and surface diagrams of gp45 (Protein Data Bank (PDB) code 1CZD). The crystal structure of gp45 (left) is a closed clamp. On the right is a model of the solution structure of gp45 in the open form. Adapted with permission from REF. 51 © American Chemical Society. **b** | The clamp-loading mechanism of bacteriophage T4. The gp44/62 clamp loader (comprising four gp44 subunits (purple) and one gp62 subunit (green)) hydrolyses two molecules of ATP to bind and stabilize the open gp45 clamp. DNA binding to the gp44/62–gp45 complex results in further ATP hydrolysis, which is followed by the departure of the clamp loader and the association of the C-terminal tail of the gp43 DNA polymerase that is 'jammed' into the middle of one gp45-clamp interface.

Once clamp loading is complete, the clamp loader is ejected and T4 gp43 DNA polymerase associates with gp45 and closes it to an in-plane conformation in an ATP-independent process[48,59,60,67]. The T4 DNA polymerase is reported to interact with the clamp by inserting its C terminus into one of the gp45 interfaces[60], as shown in FIG. 3b. As in the *E. coli* system, both the gp44/62 clamp loader and the gp43 polymerase bind to the C-terminal face of gp45, and the clamp loader must therefore detach from the clamp to make room for the polymerase–clamp interaction[68,69].

The finding that the gp45 clamp spontaneously opens in solution raises the question of why a clamp loader is needed in this system[51]. Indeed, the gp45 clamp has been shown to self-load onto DNA, provided that a macromolecular crowding agent is present[70]. Presumably, the clamp loader orients the clamp on DNA for interaction with the polymerase.

Eukaryotic PCNA and RFC

The eukaryotic replication factor C (RFC) clamp loader assembles the proliferating cell nuclear antigen (PCNA) clamp onto primed DNA for function with the replicative DNA polymerase-δ and -ε[2,71,72]. Eukaryotic PCNA and the *E. coli* β-clamp form very similar ring-shaped structures, except that each PCNA monomer consists of two domains and trimerizes to form a six-domain ring[12] (FIG. 1c). Each PCNA domain has the same chain-folding topology that is observed in the domains of the β-clamp. Like the β-clamp, the PCNA protomers are arranged head-to-tail to create two distinct 'faces' of the ring and, as found in *E. coli*, the eukaryotic clamp loader and polymerase compete for binding to the same face of the PCNA ring[73,74].

RFC structure and composition. RFC consists of five different proteins that are homologous to one another and to the subunits of the *E. coli* clamp loader[14], and, therefore, each RFC subunit belongs to the AAA+ family. In *Saccharomyces cerevisiae*, the five subunits of the clamp loader are referred to as either subunits A–E or 1–5 (REF. 5). The alphabetical nomenclature was proposed on the basis of clamp-loader structures, in which subunits are labelled alphabetically by going around the circular pentamer (FIG. 4a). Four of the RFC subunits share a similar three-domain architecture that is characteristic of the *E. coli* γ-, δ- and δ′-subunits and are referred to as the small subunits of RFC (RFCB, RFCC, RFCD and RFCE or RFC4, RFC3, RFC2 and RFC5, respectively)[5,75]. The RFCA/RFC1 subunit is sometimes referred to as the large subunit, as it contains the three domains of the small subunits along with N- and C-terminal extensions[76]. In both yeast and human RFC, deletion of the N-terminal region of the large subunit, up to the region of homology to the other RFC subunits, is tolerated with no loss of activity[75,77,78]. This N-terminal region binds DNA nonspecifically and its function is unknown.

Other RFC-like subunits have been identified that replace RFCA/RFC1. These subunits include Rad24 (REFS 79,80), Ctf8 and Elg1 (REFS 81–83) in *S. cerevisiae*. The resulting alternative clamp loaders are thought to carry out specialized functions in the DNA-damage-checkpoint response and at sites of sister-chromatid cohesion. In response to DNA damage, human RAD17–RFC (Rad24–RFC in *S. cerevisiae*) loads a unique RAD9–RAD1–HUS1 (Ddc1–Rad17–Mec3 in *S. cerevisiae*) heterotrimeric clamp onto the 5′ end of a primed DNA, the function of which is still unclear[84–87]. In *S. cerevisiae*, both Rad24–RFC and Ctf8–RFC can unload PCNA clamps *in vitro*, but whether this activity is important for their function *in vivo* requires further study[88,89].

Eukaryotic RFC and the *E. coli* γ-complex clamp loaders share many structural and functional similarities, but also have several important differences. RFCA/RFC1 occupies an analogous position to the δ-clamp-opening subunit in the *E. coli* clamp loader and contains conserved clamp-interacting residues[75,90], yet it does not open the clamp like the *E. coli* δ-subunit[89]. The RFCE/RFC5–RFCD/RFC2 complex opens the clamp without other subunits, which leads to the suggestion that RFC opens the PCNA clamp from the opposite side of the clamp interface compared with the *E. coli* γ-complex[89]. Both RFC and the *E. coli* γ-complex require only the binding of ATP or ATPγS to open their respective clamps and place them onto DNA; the clamp then remains open and the clamp loader stays bound to the clamp and DNA[89,91–94]. Similarly to the *E. coli* γ-complex, ATP hydrolysis is needed to eject RFC from the clamp and DNA[95]. Compared with the *E. coli* γ-complex, RFC contains one additional competent

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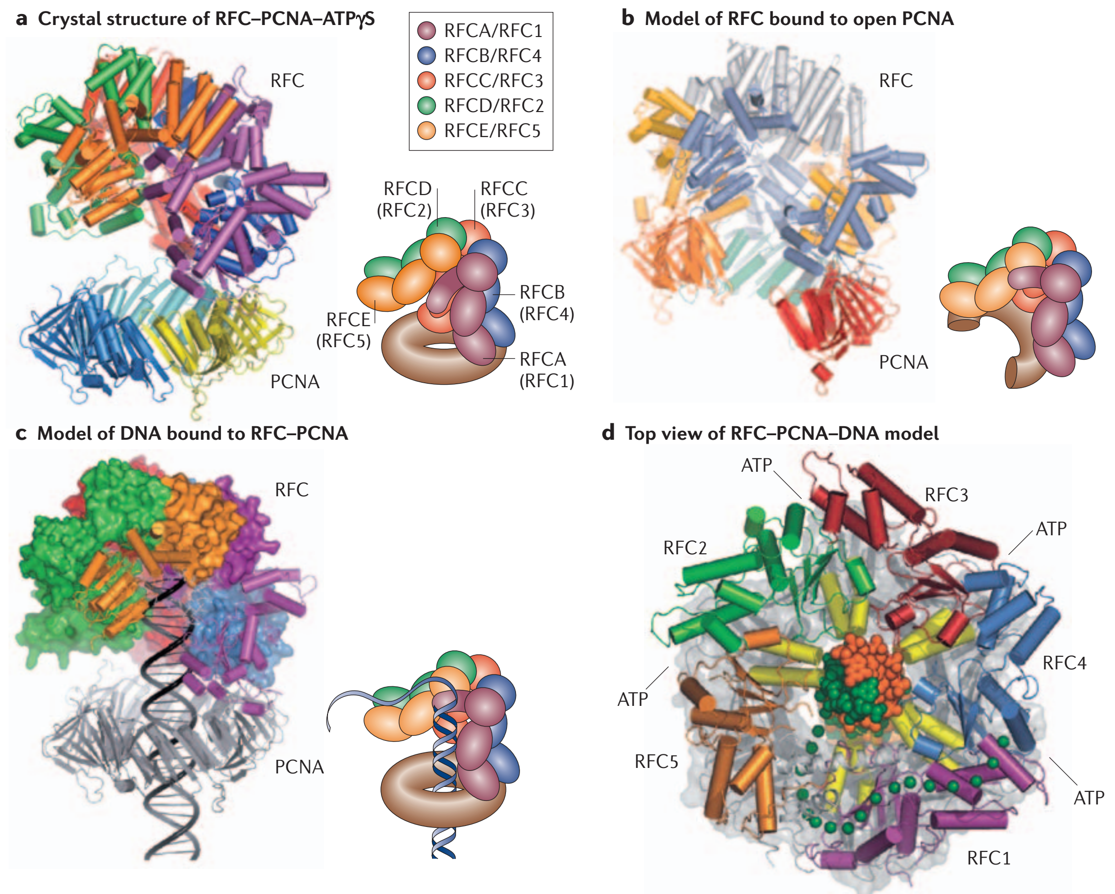

Figure 4 | **The eukaryotic RFC–PCNA binary complex. a** | Shown on the left is the crystal structure of the *Saccharomyces cerevisiae* RFC (replication factor C)–PCNA (proliferating cell nuclear antigen) complex that was solved in the presence of ATPγS (Protein Data Bank (PDB) code 1SXJ; ATPγS is not visible in the structure above). RFCD (also known as RFC2) and RFCE (also known as RFC5) do not contact the closed PCNA clamp. The diagrammatic representation of the RFC–PCNA crystal structure on the right shows the nomenclature for clamp-loader subunits, and PCNA is shown in brown. **b** | Model of RFC–PCNA, which indicates that PCNA might open into a right-handed spiral, thereby docking onto the spiral of AAA+ domains of all five RFC clamp-loader subunits. Adapted with permission from REF. 97 © National Academy of Sciences, USA. **c** | Model of the DNA that is positioned in the RFC–PCNA–ATPγS structure. The AAA+ domains of RFC subunits are arranged in a helix with a pitch that tracks the minor groove of B-form DNA. The C-terminal collar functions as a screw cap that blocks the continued threading of DNA through the structure. The diagram on the right illustrates how single-stranded DNA (ssDNA) at a primed site might bend sharply to exit out the side of RFC. **d** | Top view of the RFC–PCNA–DNA model. The C-terminal collar is removed. The AAA+ domains of the RFC subunits are colour coded as in panel **a**. PCNA is in grey and DNA is green and orange. The α-helices of RFC subunits that track DNA are highlighted in yellow. Each subunit contains two of these α-helices. Green spheres indicate a possible exit path for template ssDNA from the central chamber. Parts **c** and **d** are adapted with permission from REF. 5 © Macmillan Magazines Ltd.

ATPase site that is located in RFCA/RFC1, and RFC therefore possesses four ATPase sites. However, the ATPase site of RFCA/RFC1 is not required for clamp-loading activity and its function remains unknown[96].

The RFC–PCNA complex. A crystal structure of the *S. cerevisiae* RFC–PCNA complex has been solved in the presence of ATPγS[5] (FIG. 4a). The AAA+ nucleotide-binding domains of the five RFC subunits, which also contain the PCNA-clamp-interacting elements, are arranged in a spiral, whereas the PCNA ring is planar and closed. As a consequence of the RFC spiral binding the planar closed PCNA, only three RFC subunits contact PCNA (RFCA/RFC1, RFCB/RFC4 and RFCC/RFC3). Recent biochemical studies show that RFCD/RFC2 and RFCE/RFC5 subunits do in fact bind PCNA and are needed to open the clamp[89]. Therefore, it is likely that one or more of the mutations that were introduced for structure analysis might have prevented the formation of the open PCNA–RFC intermediate. Molecular simulations of PCNA indicate that the PCNA clamp opens out-of-plane to form a right-handed helix[97]. A right-handed PCNA helical 'lock washer' nicely docks onto RFC, and probably enables the open PCNA clamp to bind all five RFC subunits[97] as shown in FIG. 4b. A recent FRET study of RFC and PCNA confirms and extends this view[98]. The findings indicate that during clamp loading, PCNA undergoes sequential in-plane opening, then out-of-plane partial closing onto DNA, followed by full closure of the ring[98]. An electron microscopy (EM) reconstruction of

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an archaeal RFC bound to PCNA and DNA also provides experimental support for this model[4] (see below).

Insights into the way that clamp loaders bind to DNA were first obtained from the yeast RFC–PCNA crystal structure. The spiral shape of RFC has an overall pitch that closely matches DNA, and RFC forms a central chamber into which duplex DNA can fit (FIG. 4c). The helices and loops that line the central cavity of RFC are oriented to interact electrostatically with the DNA modelled inside (FIG. 4d). Several basic residues on these helices are conserved in prokaryotic and eukaryotic clamp loaders. Mutation of some of these residues in yeast RFC and in the *E. coli* γ- and δ′-subunits results in a significantly reduced affinity of the mutant clamp loaders for DNA, and supports the proposed location of DNA within the central chamber of the clamp loader[89,99].

The crystal structure of RFC–PCNA also provides an explanation for the recognition of a primed template. The C-terminal domains of the five RFC subunits form a closed cap, and, therefore, DNA in the central chamber must bend out of the gap in the side of RFC. Duplex DNA is too stiff to bend and exit through the gap, whereas the flexible single-stranded DNA (ssDNA) of a primer–template junction might easily bend out through the gap (FIG. 4c). A nicked dsDNA also provides sufficient flexibility to support PCNA loading[100].

Archaeal PCNA and RFC

Archaea, the third domain of life[101], are proposed to possess a DNA-replication apparatus that is similar to that of eukaryotes[102]. Archaea contain a PCNA clamp and an RFC clamp loader that function with B-family DNA polymerases[103,104]. The two crystal structures of an archaeal clamp that have been solved so far are of PCNA in complex with a peptide that was derived from flap endonuclease-1 (FEN1) of *Archaeoglobus furiosus*[105] and of PCNA from *Pyrococcus furiosus* (*Pfu*PCNA)[13]. *Pfu*PCNA forms a homotrimeric ring that is similar to yeast PCNA (FIG. 1c,d). Although the overall structure is very similar to clamps from various organisms, *Pfu*PCNA seems to contain more ion pairs at the interface and fewer intermolecular main-chain hydrogen bonds. The additional ion pairs of *Pfu*PCNA might lead to the instability of the clamp trimer and could account for the self-loading that has been observed during *in vitro* DNA synthesis in the absence of the clamp loader[106]. In Archaea, some PCNAs also exist as heterotrimeric complexes[107].

Archaeal RFC versus other clamp loaders. Archaeal genomes encode small (RFC-s) and large (RFC-l) RFC subunits[108]. The two subunits of archaeal RFC form a complex with a 1:4 (RFC-l:RFC-s) stoichiometry[109–111] with the apparent exception of the *Methanobacterium thermoautotrophicum* clamp loader, for which a heterohexameric composition has been proposed[112]. Both RFC-s and RFC-l contain seven of the eight highly conserved RFC motifs, which are referred to as box II–VIII in yeast and human RFC clamp-loader subunits[14] (BOX 2). Archaeal RFC-s typically shares ~40% sequence identity with the eukaryotic RFC small subunits (yeast RFCB/RFC4, RFCC/RFC3, RFCD/RFC2 and RFCE/RFC5). The RFC-l subunit is more divergent, with ~20% identity to yeast RFCA/RFC1, and it lacks the RFC box I motif[106,108]. RFC-l binds to the clamp in a similar manner to the *E. coli* δ-subunit. The crystal structure of *Pfu*PCNA, complexed with a peptide that contains the PCNA-binding sequence of the RFC-l subunit[113], shows a similar interaction mode to that observed in the complex between the human inhibitor of cyclin-dependent protein kinase p21$^{WAF1/CIP1}$ and PCNA, as well as to the RB69 phage gp45 clamp complexed with a peptide from RB69 DNA polymerase[11,49].

The structures of RFC-s from *P. furiosus* and *Archaeoglobus fulgidus* have been solved[114,115], although RFC-s alone is inactive as a clamp loader. The RFC-s subunits have similar chain folds to the eukaryotic and bacterial clamp-loader subunits. The crescent-shaped small subunit is organized into three domains: domains I and II comprise the canonical AAA+ fold and are responsible for interaction with nucleotides (ADP or ADPNP), whereas domain III forms a five-helix bundle and mediates subunit contacts. The RFC-s of *A. fulgidus* forms hexameric rings[114], whereas *P. furiosus* RFC-s crystallizes as a dimer of trimers[115]. EM studies indicate that *P. furiosus* RFC-s can also form hexameric rings at physiological pH values[116]. In *A. fulgidus* RFC-s, ADP occupies all six sites, whereas in the *P. furiosus* RFC-s structure only four of the six subunits contain ADP. In both RFC-s structures, the conformation of the nucleotide-binding domain is very similar to that observed in the structure of nucleotide-free *E. coli* γ-complexes.

Unlike the γ-complex, the *A. fulgidus* RFC-s complex undergoes substantial conformational changes that are associated with nucleotide binding[38,115,117]. Nucleotide binding leads to a more open and asymmetric complex, and this change might correspond to opening of the PCNA ring by RFC during the clamp-loading reaction. Biochemical analysis of *A. fulgidus* RFC indicates that it binds two molecules of ATP in the absence of other factors, and a maximum of four ATP molecules in the presence of the clamp[118]. Binding of a nucleotide to the large subunit and three of the small subunits is sufficient for clamp loading.

After loading the clamp onto DNA, ATP hydrolysis by RFC-s subunits releases PCNA. By contrast, ATP hydrolysis by the large subunit is required for catalytic recycling of RFC[118]. Mutation of the arginine finger in the small subunits results in a complex that cannot load PCNA[115]. This result is analogous to arginine-finger mutations in the *E. coli* clamp loader[40]. Possible insight into the defect comes from studies of the structure of RFC-s complexed with a non-hydrolysable analogue of ATP, ADPNP[115]. The structure shows that a helix in the N-terminal region is located close to the arginine finger and therefore might control access of this residue to the nucleotide site and thereby regulate ATP hydrolysis. Interestingly, this helix is in RFC box II and is conserved in the other clamp-loader subunits[14]. Analysis of *A. fulgidus* RFC in which this helix in RFC-s is deleted indicates that the ATPase activity of RFC-s is coupled to clamp release, because the mutant RFC releases PCNA prematurely due to unrestrained ATP hydrolysis.

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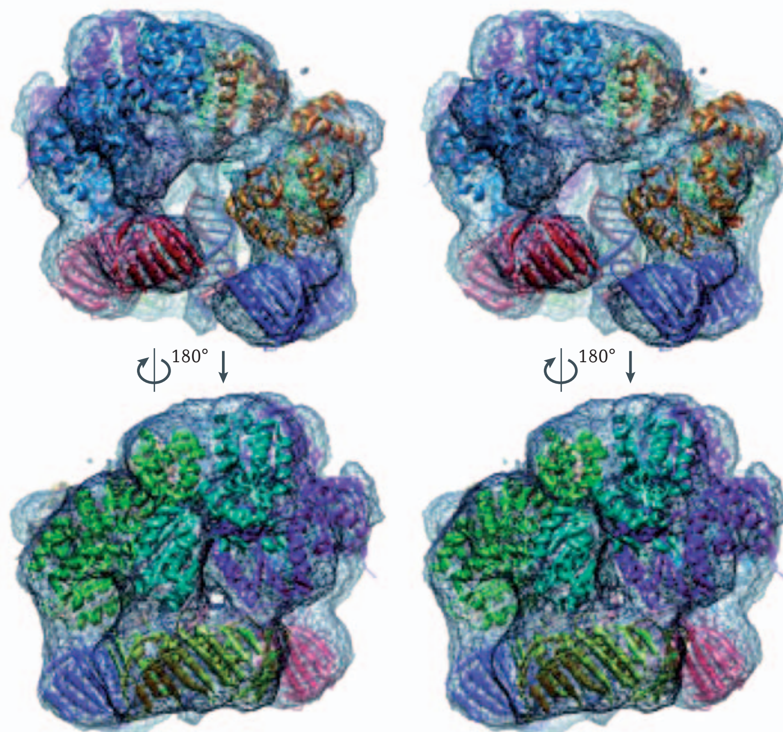

Figure 5 | **The archaeal RFC–PCNA–DNA ternary complex.** Shown is the fitting of atomic models of small replication factor C (RFC-s) subunits and PCNA (proliferating cell nuclear antigen) subunits into the electron microscopy three-dimensional map of the clamp-loading complex. Stereo pair of front (upper) and back (lower) views of the RFC–PCNA–DNA complex. RFC-s subunits are shown in gold, green, cyan, violet and blue. The RFC-s indicated in gold corresponds to the large RFC (RFC-l) subunit. The three PCNA protomers are shown in blue, yellow and red (best visible in the lower part of the bottom two structures). An 11-nucleotide DNA duplex is fitted into the internal chamber of the clamp loader and encircled by PCNA. Adapted with permission from REF. 4 © National Academy of Sciences, USA.

Bacteriophage T4 and archaeal clamp loaders have similar subunit compositions: each contains two different subunits in a 1:4 stoichiometry. In archaea, the single-copy subunit (RFC-l) has an ATPase motif, unlike T4 gp62. Also, the single-copy gp62 subunit in the T4 clamp loader is smaller than the other subunits (gp44 is homologous to RFC-s). Despite these differences, these two clamp loaders bind only four ATPs[53,55,118]. How the archaeal RFC limits the access of ATP to one of its five sites is currently unknown.

Mechanism of clamp loading. Important insights into RFC-mediated PCNA-clamp opening have been derived from a recent EM reconstruction analysis of the *P. furiosus* RFC–PCNA–DNA ternary complex in the presence of ATPγS[4,119] (FIG. 5). The structure shows PCNA in an open helical conformation. The right-handed spiral configuration allows PCNA to dock extensively onto the helical surface of RFC. This result is consistent with molecular dynamics simulations of yeast PCNA, which indicate that PCNA assumes a right-handed spiral conformation when it opens[97]. The EM analysis shows duplex DNA bound within the centre of the RFC–PCNA complex[4]. The 5-Å gap in the out-of-plane, right-handed conformation of the ring is too small to allow the passage of duplex DNA. Therefore, either the ring opens further during clamp loading, or the single-stranded portion of a primed site is threaded through the gap during clamp loading.

Concluding remarks

Clamps and clamp loaders are at the centre of many DNA metabolic pathways such as replication, repair and recombination. In particular, the strategy of using clamps and clamp loaders as a way of achieving high processivity is well conserved across the evolutionary spectrum. Although there are important differences in clamps and clamp loaders between the three domains of life, the striking similarities are far more extensive than the differences. Presumably, the basic architecture and function of clamp–clamp-loader complexes was achieved very early on in evolution. An advantage of well conserved multiprotein machines is the complementarity observed by research from different laboratories that study clamp loaders from diverse organisms. Therefore, studies in *E. coli* and other prokaryotes, bacteriophage T4 and RB69, and various eukaryotes and archaea have led to a comprehensive view of how these important machines function, and also of how they differ. However, many aspects of the clamp-loading mechanism remain poorly characterized. For example, does the clamp loader pry open the interface of a clamp, or capture and stabilize a clamp that opens spontaneously? What are the various ATP-induced conformational changes in the clamp loader that enable it to bind the clamp and to accommodate DNA in the internal chamber? How does the clamp loader sense the 3′ or 5′ directionality of the primed site? How are clamps recycled from DNA after their use in repair and other DNA metabolic pathways? These and many other questions remain for exciting future investigations.

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Competing interests statement
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