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The many roles of PCNA in eukaryotic DNA replication

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Abstract

Proliferating cell nuclear antigen (PCNA) plays critical roles in many aspects of DNA replication and replication-associated processes, including translesion synthesis, error-free damage bypass, break-induced replication, mismatch repair, and chromatin assembly. Since its discovery, our view of PCNA has evolved from a replication accessory factor to the hub protein in a large protein-protein interaction network that organizes and orchestrates many of the key events at the replication fork. We begin this review article with an overview of the structure and function of PCNA. We discuss the ways its many interacting partners bind and how these interactions are regulated by post-translational modifications such as ubiquitylation and sumoylation. We then explore the many roles of PCNA in normal DNA replication and in replication-coupled DNA damage tolerance and repair processes. We conclude by considering how PCNA can interact physically with so many binding partners to carry out its numerous roles. We propose that there is a large, dynamic network of linked PCNA molecules at and around the replication fork. This network would serve to increase the local concentration of all the proteins necessary for DNA replication and replication-associated processes and to regulate their various activities.

Keywords

Break-induced replication; DNA polymerase; DNA repair; DNA replication; mismatch repair; PCNA; processivity factor; proliferating cell nuclear antigen; sliding clamp; translesion synthesis

1. Introduction

Since its discovery in the late 1970s, our view of proliferating cell nuclear antigen (**PCNA**) and its roles in DNA replication and genome maintenance has expanded considerably. PCNA was originally identified as the target of an autoimmune antibody derived from patients with systemic lupus erythematosus [1]. This protein was later shown to be one produced predominantly in proliferating and transformed cells [2–4]. By the middle of the 1980s, the involvement of PCNA in DNA replication was suggested based on its pattern of staining throughout the cell cycle [5].

Definitive evidence of a role for PCNA in DNA replication came a couple years later with the discovery that PCNA is required for the replication of simian virus 40 *in vitro* [6, 7]. It was soon realized that PCNA was an auxiliary protein for DNA polymerase delta (**pol** δ) that increases its activity by making it more processive [8–10]. PCNA was subsequently shown

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to be an auxiliary factor for DNA polymerase epsilon (**pol** ε) [11–14]. By the early 1990s, the role of PCNA came to be viewed as being the processivity factor of eukaryotic replicative polymerases.

An understanding of how PCNA confers high processivity to DNA polymerases was achieved when the X-ray crystal structure of PCNA was determined [15]. PCNA was shown to be a ring-shaped trimer similar to the structure of the *E. coli* beta clamp determined a couple years earlier [16]. By the middle of the 1990s, it was known that PCNA is loaded onto double-stranded DNA by replication factor C (**RFC**) [17, 18], where the PCNA functions as a sliding clamp that binds and anchors polymerases onto the DNA.

As more and more PCNA interacting partners were identified, it became clear that PCNA is not simply a processivity factor for replicative polymerases. It interacts with and regulates the activities of many proteins involved in Okazaki fragment maturation [19, 20], mismatch repair [21], nucleotide excision repair [22], and translesion synthesis [23–26]. It also interacts with proteins involved in other processes such as cell cycle control [27–29], sister chromatid cohesion [30], epigenetic inheritance [31], and S-phase specific proteolysis [32]. By the early 2000s, PCNA came to be viewed as an important hub protein that is critical for organizing and orchestrating events at the replication fork and other sites of DNA synthesis.

Since the early 2000s, it has become clear that the regulation of several DNA metabolic processes is governed by post-translational modifications of PCNA, most notably ubiquitylation and sumoylation [33, 34]. Ubiquitylation of PCNA promotes translesion synthesis via the recruitment of translesion synthesis polymerases to stalled replication forks [35]. Sumoylation of PCNA inhibits recombination via the recruitment of anti-recombinases to sites of DNA synthesis [36, 37].

In this chapter, we will describe the many roles of PCNA in eukaryotic DNA replication and in replication-associated processes. We will begin by discussing the features of the structure and function of PCNA common to all of its roles. Then we will focus on its roles in normal DNA replication, translesion synthesis and error-free damage bypass, break-induced replication, mismatch repair, and replication-coupled nucleosome assembly.

2. PCNA structure and function

Sliding clamps are proteins that encircle double-stranded DNA and are found in all three domains of life. Although these proteins have different oligomeric states, they all possess a general pseudo-six-fold ring-shaped structure. Bacterial sliding clamps form homodimers, whereas archaeal and eukaryotic sliding clamps form homotrimers and heterotrimers. These sliding clamps function as platforms for recruiting and regulating various enzymes that function in DNA replication and repair, such as polymerases, nucleases, and ligases [38]. Although there is little sequence similarity among the sliding clamps across the domains of life, their striking structural similarity demonstrates the evolutionary importance of having such scaffolds for bringing proteins to sites of DNA synthesis.

Eukaryotic PCNA is a homotrimer with each monomer composed of two similarly folded domains connected by an interdomain-connecting loop [15] (Figure 1A). Domain 1 is

comprised of residues 1 to 117, domain 2 is comprised of residues 135 to 258, and the interdomain-connecting loop is comprised of residues 118 to 134. The six structural domains form a ring with an outer layer of six β-sheets and an inner layer of 12 α-helices that line the central hole of the ring. The central hole is lined with positively charged residues that can form electrostatic interactions with the duplex DNA. The diameter of the hole is ~35 Å, which is wider than the diameter of B-form DNA (~20 Å). Models derived both from X-ray diffraction data [39] and from molecular dynamics simulations [40] provide strong evidence that the DNA is significantly tilted at an angle as it passes through the center of the hole in order to contact the positively charged residues on the α-helices.

Single molecule analysis showed that there are two distinct modes by which PCNA moves along double-stranded DNA [41]. As PCNA diffuses along the DNA, most of time the ring rotates following the helical pitch of the DNA. This rotation ensures that polymerases and other proteins bound to PCNA would be positioned consistently with respect to the DNA helix as the protein complex moves along it. A small fraction of the time, however, PCNA translocates along the DNA without following the helical pitch of the DNA. This mode of movement allows PCNA to more rapidly slide along the DNA. It also allows greater freedom of rotation, which under certain circumstances would allow optimal repositioning of PCNA-bound proteins with respect to the DNA.

The PCNA ring has two faces, which we refer to as the front face and the back face (Figure 1A). The front face points in the direction of DNA synthesis and contains the C-terminus of each monomer as well as the interdomain-connecting loop. The majority of proteins that interact with PCNA do so within a hydrophobic pocket on the front face of PCNA near the interdomain-connecting loop [42–44]. Binding on the front face allows these interacting proteins to access the primer terminus of the replicating DNA. The back face of PCNA, by contrast, points away from the direction of DNA synthesis and contains several extended loops and lysine-164, a site of ubiquitylation and sumoylation [33]. It has been suggested that these post-translational modifications bind specific proteins and hold them in reserve on the back face of PCNA until they are needed [45–47].

The proteins that interact with the hydrophobic pocket on the front face of the PCNA ring generally contain one or more PCNA-interacting protein (**PIP**) motifs [48–50] (Figure 1B). PIP motifs are sequences of eight amino acids with a conserved glutamine at position 1, a conserved aliphatic residue (leucine, isoleucine, or methionine) at position 4, and two adjacent, conserved aromatic residues (phenylalanine or tyrosine) at positions 7 and 8. The conserved aromatic side chains bind within the pocket comprised of isoleucine-128 in the interdomain-connecting loop and proline-234 and proline-253 in domain 2. A point that has not been widely appreciated is that the conformation of PCNA changes upon binding a PIP motif. Comparing X-ray crystal structures of PCNA in the presence and absence of a bound PIP motif shows that the backbone of isoleucine-128 moves by ~4 Å and the side chain of this residue moves by ~5 Å. This conformational change is necessary in order to accommodate the aromatic residue in position 8 in the PIP motif.

Recently greater attention has been paid to contacts that PCNA-interacting proteins make with PCNA that occur outside of the canonical PIP motif. For example, the X-ray crystal

structure of full-length flap endonuclease 1 (FEN1) bound to PCNA shows that this is a bipartite interaction [51]. FEN1 has a PIP motif on its C-terminal tail that binds to the front face of PCNA in the usual fashion. In addition, the core domain of FEN1 directly contacts domain 2 of PCNA (Figure 1C). Three different conformations of the PCNA-bound FEN1 are observed in the crystal structure. In one of these states, the active site is oriented away from where the DNA would be positioned. It has been argued that this represents an inactive state of FEN1 and that a large rotation of the core domain of FEN1 about a flexible hinge region is necessary to achieve the active conformation. Thus, these additional contacts not only increase affinity of PCNA-interacting proteins for PCNA, they also can play an important role in regulating these proteins.

Post-translational modifications of PCNA are critical events in the regulation of the DNA metabolic processes in which PCNA participates. These modifications change the binding specificity of PCNA and in some cases act to recruit specific PCNA-interacting proteins to replication forks. The best characterized of these PCNA post-translational modifications are ubiquitylation and sumoylation of lysine-164 [33, 34], and X-ray crystal structures of these modified forms of PCNA have been determined (Figure 1D) [45, 46, 52]. Ubiquitylation of PCNA at this position promotes translesion synthesis by recruiting translesion synthesis polymerases, which themselves contain PIP motifs as well as ubiquitin-binding motifs in their C-terminal tails [35]. Sumoylation of PCNA at this position inhibits recombination by recruiting an anti-recombinase [36, 37], which contains a PIP-like motif that binds on the front face of PCNA as well as a SUMO-binding motif [52].

3. The role of PCNA in normal DNA replication

In eukaryotes, DNA replication is an extraordinarily complex, dynamic, multi-stage process that initiates at origins of replication [53–56]. Before an origin can fire, it must be licensed. Origin licensing occurs during late M phase and early G1 phase, when the pre-replication complex (**pre-RC**) forms at the origin. The pre-RC includes Cdt1, Cdc6, and two hexamers of the Mcm2-7 helicase. Origin firing occurs at the onset of S phase. At this point, the origin is melted by the Mcm2-7 helicases, and the resulting single-stranded DNA is coated by replication protein A (**RPA**). Two replication forks are then assembled at the origin. First, Cdc45 and go-ichi-ni-san (**GINS**) complexes are recruited, which together with the Mcm2-7 hexamers form two Cdc45/Mcm2-7/GINS (**CMG**) complexes, the replicative helicase complexes. Next, other protein factors are recruited to the nascent replication forks, including RFC, PCNA, pol δ, and pol ε. Finally, DNA synthesis begins as the replication forks move away from the origin bidirectionally.

It is critical that each origin fire only once per cell cycle, and PCNA plays an important role in limiting each origin to firing once. Cdt1, a component of the pre-RC is degraded in S-phase in a PCNA-dependent manner [32, 57, 58]. Cdt1 contains a specialized PIP motif called a PIP degron, which contains a threonine at position 5, an aspartate at position 6, and a basic amino acid located four residues following the PIP motif. [58] These specialized PIP motifs bind PCNA with greater affinity than do classical PIP motifs. In the case of Cdt1, this PIP degron is responsible for making the protein a substrate for the E3 ubiquitin ligase $CLR4^{Cdt2}$. When Cdt1 is bound to a PCNA ring that has been loaded on DNA, $CLR4^{Cdt2}$

facilitates the poly-ubiquitylation of Cdt1 leading to its degradation. This ensures that Cdt1 is not available to re-license origins once they have fired.

There is a division of labor among the replicative DNA polymerases at the eukaryotic replication fork. The leading strand is synthesized in a continuous manner by pol ε, and the lagging strand is synthesized in a discontinuous manner by pol δ [59, 60] (Figure 2). Biochemical studies using purified, reconstituted systems have shown that PCNA interacts with and affects DNA synthesis by pol δ and pol ε differently [61]. For example, pol δ binds PCNA with high affinity, whereas pol ε binds PCNA with low affinity. Pol δ alone synthesizes DNA with low processivity, only incorporating up to six nucleotides before dissociating from the DNA template. In the presence of PCNA, the processivity of pol δ increased nearly 100-fold. By contrast, pol ε has a greater intrinsic processivity, incorporating approximately 60 nucleotides before dissociating. The presence of PCNA increases the processivity of pol ε by about six-fold. Overall, on PCNA-primed and RPA-coated single-stranded DNA, pol δ and pol ε have nearly the same processivity, incorporating up to 600 nucleotides per DNA binding event.

The differential interactions between these polymerases and PCNA are partly responsible for selecting the appropriate polymerases for leading and lagging strand synthesis [62]. PCNA strongly favors extension by pol δ over extension by pol ε in competition experiments *in vitro* on RPA-coated single-stranded DNA, a situation analogous to lagging strand DNA synthesis. This preference for pol δ over pol ε is reversed, however, in the presence of the CMG helicase complex [62], a situation analogous to leading strand DNA synthesis. The pol δ-CMG complex synthesizes DNA about five to 10-fold slower than does the pol ε-CMG complex. In addition, the CMG complex selectively utilizes pol ε in competition experiments, and pol ε readily replaces pol δ from an actively extending pol δ-CMG complex. Thus PCNA, together with the CMG complex, maintains the division of labor between pol δ and pol ε at the replication fork.

On the leading strand, only one PCNA ring needs to be loaded, and this occurs when the origin fires and the replication fork is assembled. On the lagging strand, by contrast, one PCNA ring needs to be loaded for each Okazaki fragment. An Okazaki fragment is initiated by DNA polymerase alpha (**pol** α), which has an associated primase that synthesizes a short RNA primer [63, 64]. Pol α then extends this RNA primer by ten to twenty nucleotides of DNA. Next, PCNA is loaded onto DNA by RFC [17, 18, 65]. RFC binds the primer-template junction synthesized by pol α and catalyzes the loading of PCNA in an ATP-dependent manner. RFC binds to the front of the PCNA ring and loads it with the front of the ring facing toward the 3′ end of the primer strand [66]. This ensures that polymerases and other PCNA-interacting enzymes will have access to the primer terminus. Once PCNA is loaded onto the primer strand, pol δ is recruited to synthesize the remainder of the Okazaki fragment.

PCNA also orchestrates the events on the lagging strand during the maturation of each Okazaki fragment. This process occurs when pol δ encounters the 5′ end of the previous Okazaki fragment. Pol δ displaces the 5′ end of the fragment containing the RNA primer and a segment of the DNA, creating a flap. Flap endonuclease 1 (FEN1) contains a PIP motif

and is recruited to the maturing Okazaki fragment via its interaction with PCNA. FEN1 catalyzes cleavage of the flap structure to create a nicked duplex, an activity that is stimulated in the presence of PCNA [67]. DNA ligase I also contains a PIP motif and is recruited via its interaction with PCNA. DNA ligase I catalyzes the sealing of the nick, an activity that is also stimulated by PCNA [68]. Exactly how these sequential enzymatic activities are coordinated by PCNA remains an important unanswered question.

In the yeast system, PCNA is sumoylated during S-phase on lysine-164 by the complex of the E2 SUMO conjugating enzyme Ubc9 and the E3 SUMO ligase Siz1 [33, 34]. It has been estimated that the majority of DNA-bound PCNA is sumoylated during normal DNA replication [69]. Sumoylation of PCNA inhibits recombination by recruiting an anti-recombinase Srs2 [36, 37], which contains a PIP-like motif that binds on the front face of PCNA as well as SUMO-binding motif [52]. Srs2 acts by disrupting the formation of Rad51 nucleoprotein filaments [70, 71], an active species in homologous recombination. PCNA is also sumoylated to a lesser extent on lysine-127, which is located in the interdomain-connecting loop, although the biological significance of this modification is unknown.

4. The role of PCNA in translesion synthesis

DNA damage causes replication forks to stall, because classical DNA polymerases, such as pol δ and pol ε, are unable to efficiently incorporate deoxynucleotides opposite damaged DNA templates. Without a means of overcoming these replication blocks, replication forks collapse resulting in DNA strand breaks, chromosomal rearrangements, and cell death. Translesion synthesis is one of the means by which damaged DNA is bypassed during DNA replication. During translesion synthesis, one or more non-classical DNA polymerases, such as DNA polymerase zeta (**pol** ζ), DNA polymerase eta (**pol** η), DNA polymerase iota (**pol** ι), DNA polymerase kappa (**pol** κ), and Rev1, are recruited to stalled replication forks [72–78]. The mechanisms of these non-classical polymerases differ from those of their classical counterparts so that they are able to incorporate deoxynucleotides opposite damaged DNA with high efficiency. In addition, these non-classical polymerases incorporate nucleotides with very low fidelity, so translesion synthesis is generally error-prone.

When DNA is damaged, a single ubiquitin moiety is attached to lysine-164 on one or more of the PCNA subunits by the complex of the E2 ubiquitin conjugating enzyme Rad6 and the E3 ubiquitin ligase Rad18 [33]. The resulting ubiquitin-modified PCNA (**Ub-PCNA**) plays important regulatory roles in translesion synthesis. It acts as a hub protein for recruiting the non-classical polymerases to stalled replication forks. Many non-classical polymerases, including pol η, pol κ, and pol ι, possess tandem ubiquitin-binding motifs and PIP motifs [35]. These motifs are important for allowing non-classical polymerases to preferentially interact with Ub-PCNA over unmodified PCNA and to co-localize with Ub-PCNA in cells [35, 79–83].

Structural and computational studies have shown that the ubiquitin moiety of Ub-PCNA is dynamic, yet predominantly occupies preferred orientations on the back and on the side of the PCNA ring [45, 84, 85]. This would allow Ub-PCNA to regulate the access of non-classical polymerases to the primer terminus by altering the orientation of its attached

ubiquitin. For example, non-classical polymerases can be held in reserve without affecting the activity of enzymes bound on the front face of the PCNA ring when the ubiquitin moiety is on the back of the PCNA ring. Non-classical polymerases can gain access to the primer-terminus when the ubiquitin moves to the side of the PCNA ring.

In addition to recruiting non-classical polymerases to stalled replication forks, PCNA and Ub-PCNA both regulate the catalytic activity of non-classical polymerases. The catalytic efficiencies of pol η, pol κ, and pol ι are increased in the presence of PCNA [23–26]. In the case of pol η and the non-classical polymerase Rev1, the catalytic efficiency of nucleotide incorporation is increased more in the presence of UbPCNA than in the presence of unmodified PCNA [45, 86, 87]. By contrast, although unmodified PCNA does stimulate the activity of pol ζ, Ub-PCNA does not stimulate it to a greater extent [86].

Two separation-of-function mutations were identified in yeast PCNA that block translesion synthesis in cells [88, 89] (Figure 3). Both of these substitutions (G178S and E113G) are of residues in the β strands that constitute the subunit interface, and the X-ray crystal structures of both mutant proteins reveal perturbations that reduce the number of hydrogen bonds between these strands [90, 91]. The mutant PCNA trimers are less stable than wild-type PCNA, and they are unable to stimulate the catalytic activity of non-classical polymerases [87]. There are two possible explanations for the inability of these mutant proteins to stimulate these polymerases. First, decreased PCNA trimer stability may lead to breathing (i.e., transient opening and closing of the ring) that disrupts the activity of non-classical polymerases to a greater extent than it does other PCNA-interacting enzymes. Second, the PCNA subunit interface may serve as an additional point of contact for non-classical polymerases. Further studies are necessary to distinguish between these possible scenarios.

5. The role of PCNA in error-free damage bypass

In addition to translesion synthesis, which is mutagenic, another pathway for circumventing DNA lesions in the template strand during DNA replication is error-free damage bypass. The detailed mechanism of error-free damage bypass has yet to be elucidated, but it is believed to involve a template-switching event whereby the replicative DNA polymerases moves to the newly synthesized sister strand and uses it as a template [92, 93]. The model for how this template-switching event occurs is that a fork-remodeling enzyme catalyzes regression of the stalled replication fork (Figure 4). In yeast, this is carried out by the Rad5 helicase, and in mammals this is carried out by the Rad5 homologs HLTF and SHPRH. Fork regression leads to the formation of a “chicken foot” intermediate in which the primer terminus of the nascent strand is paired with the newly synthesized sister strand. Extension of the nascent strand to the 5! end of the sister strand and subsequent restoration of the replication fork results in bypass of the DNA damage and resumption of normal DNA replication.

Although the detailed mechanism of error-free damage bypass is not yet known, it is clear that PCNA plays a central regulatory role in this process. As was the case in translesion synthesis, the initiating event in error-free damage bypass is the attachment of a single ubiquitin moiety to lysine-164 on one or more of the PCNA subunits by Rad6 and Rad18 to form Ub-PCNA [33]. Next, the complex of the E2 ubiquitin conjugating enzymes Ubc13/

Mms2 and the E3 ubiquitin ligase Rad5 are recruited to Ub-PCNA. (Rad5 functions in error-free damage bypass both as an E3 ubiquitin ligase and as a fork-remodeling helicase.) This results in the formation of lysine-63 linked poly-ubiquitin chains on PCNA [33].

The attachment of lysine-63 linked poly-ubiquitin chains to PCNA is required for error-free damage bypass, but it is unknown how it facilitates the template-switching process. Recent studies are providing some interesting clues. The presence of these poly-ubiquitin chains on PCNA decreases the formation of complexes between pol δ and PCNA, which prevents normal DNA replication from occurring [94]. It also decreases the ability of non-classical pol η to bypass DNA lesions, which prevents translesion synthesis. This latter effect is not due to decreased binding of pol η, but rather may be due to pol η being trapped in a non-productive complex by the poly-ubiquitin chains. Thus error-free DNA damage bypass may be facilitated by the poly-ubiquitylation of PCNA inhibiting the alternative pathways.

Two separation-of-function mutations were identified in yeast PCNA that block error-free damage bypass [95] (Figure 3). Cells producing the E104A/D105A or the D256A/E257A PCNA mutant proteins are more sensitive to ultraviolet radiation. Glutamate-104 and aspartate-105 are located on a loop immediately adjacent to the subunit interface, and aspartate-256 and glutamate-257 are located at the C-terminus of the protein. Cells producing the quadruple mutant protein, which has all four of these amino acid substitutions, are more sensitive to ultraviolet radiation than those expressing the other two mutant proteins. In these cells, error-free damage bypass is blocked, but error-prone translesion synthesis is unaffected. This latter point suggests that the ability of this mutant protein to be ubiquitylated is not compromised, but this has not been confirmed experimentally. The structural changes in PCNA caused by these substitutions are unknown, and the mechanistic basis for the mutant protein's inability to support error-free damage bypass is not yet understood.

Error-free damage bypass is an active area of research, and several interesting questions remain regarding the role of PCNA in this process. First, does the poly-ubiquitylation of PCNA signal for the recruitment of other protein factors necessary for fork remodeling? Recently, the genome maintenance factor Mgs1 has been suggested as a potential downstream effector of ubiquitylated PCNA that may play a role in this process [96]. Second, once the chicken foot intermediate is formed, is another PCNA ring loaded onto the middle branch of this structure? If so, this would nicely explain how pol δ is recruited to this new primer-template to carry out extension to the end of the newly synthesized sister strand.

6. The role of PCNA in break-induced replication

If translesion synthesis and error-free damage bypass fail to allow the resumption of DNA replication, several other pathways may be used to restart the stalled replication fork. These pathways generally involve the use of the recombination machinery and are beyond the scope of this review; these pathways are described in detail elsewhere [97–99]. However, one such pathway, break-induced replication, does warrant attention. This is because PCNA is required for break-induced replication, and separation-of-function mutations in PCNA

have been identified that block break-induced replication without significantly affecting normal replication and other recombination-dependent replication restart pathways [100].

Break-induced replication is used to repair one-sided double strand breaks (Figure 5) [99, 101–104]. One-sided breaks are formed during DNA replication in several ways. They are formed when a replication fork encounters a single-stranded nick in the template strand. They are also formed when the chicken foot intermediate of a stalled replication fork, which is actually a Holliday junction, is cleaved by a junction-specific endonuclease. Break-induced replication proceeds by processing of the one-sided break in order to form a Rad51 nucleoprotein filament. Strand exchange occurs with the intact sister duplex resulting in the formation of a D-loop, a key recombination intermediate. DNA synthesis starts from the 3! end of the invading strand, and this will become the leading strand of a re-established replication fork. The lagging strand is likely initiated subsequently, and replication proceeds until the end of the replicon or the end of the chromosome. Break-induced replication requires all of the proteins needed for normal DNA replication, except those specific for formation of the pre-RC complex [100].

Two separation-of-function mutations were identified in yeast PCNA that block break-induced replication in cells [100] (Figure 3). Unlike other separation-of-function mutations in PCNA, both of these substitutions (F248A/F249A and R80A) are dominant. This means that only one mutant subunit in a PCNA trimer is sufficient to inhibit break-induced replication. The F248A/F249A substitution is in a β strand within the cleft formed by the two domains of the same monomer. It is near the binding site for PIP motifs on PCNA-interacting proteins, but is likely not close enough to directly contact these motifs. The R80A substitution is on an extended loop near the subunit interface. It is not known how these amino acid substitutions interfere with break-induced replication. These same amino acid substitutions, however, suppress the cold sensitivity of yeast lacking the non-essential Pol32 subunit of pol δ, a subunit that is also essential for break-induced replication [100]. Moreover the decreases in break-induced replication caused by these amino acid substitutions in PCNA are epistatic with the decrease in break-induced replication caused by the absence of the Pol32 subunit of pol δ [100]. This suggests that these two mutant forms of PCNA interact with pol δ in an aberrant manner that interferes with the ability of its Pol32 subunit to perform its essential role in break-induced replication, whatever that may be.

7. The role of PCNA in mismatch repair

Replicative polymerases make errors when synthesizing DNA that can lead to base-base mismatches or short insertions and deletions. These errors are recognized and corrected by mismatch repair [105–111]. The first step of mismatch repair is recognition of the mismatches or insertions/deletions in the newly synthesized DNA. This involves either the MutSα heterodimer composed of Msh2 and Msh6 or the MutSβ heterodimer composed of Msh2 and Msh3. These mismatch recognition complexes have partially overlapping specificities with MutSα preferring base-base mismatches and small insertions/deletions and with MutSβ preferring larger insertions/deletions. The next step of mismatch repair is excision of the mismatch and surrounding DNA from the newly synthesized strand. This requires the MutLα heterodimer composed of Mlh1 and Pms1 in yeast (PMS2 in humans)

and the exonuclease ExoI. The final step of mismatch repair is synthesis of new DNA by pol δ to fill the gap.

PCNA is required for all three steps of mismatch repair [21, 112–115]. It plays an essential role in recognizing the mismatch by interacting with MutSα to form an active mismatch recognition complex [113]. Mutations in MutSα that disrupt its interaction with PCNA lead to a loss of mismatch repair in cells. PCNA plays an important role in excising the mismatch and surrounding DNA from the newly synthesized strand by binding MutLα and activating its latent endonuclease activity [114] and by binding ExoI and regulating its exonuclease activity [115]. Finally, PCNA plays a critical role in repair synthesis by interacting with pol δ.

Two separation-of-function mutations in PCNA (C22Y and C81R) have been identified that inhibit mismatch repair in yeast [113] (Figure 3). The crystal structures of these mutant proteins showed distinct structural alterations caused by these two substitutions [116]. Cysteine-22 is in a loop adjacent to one of the α-helices lining the central hole of the PCNA ring, and the C22Y mutation causes a significant shifting of several of these α-helices. Cysteine-81 is in an extended loop near the subunit interface, and the C81R mutation induces a change in the conformation of this loop leading to a slight destabilization of the PCNA trimer. Both of these mutant proteins bind MutSα, but the ternary complex formed by these mutant proteins, MutSα and DNA is abnormally large. Although the exact nature of these aberrant complexes is unknown, it is likely that this is responsible for the defect in mismatch repair. Recently, additional mutations in PCNA that block mismatch repair were identified. Three of these mutations were clustered around cysteine-22, and seven were near the subunit interface destabilizing the PCNA trimer [117]. These mutant PCNA proteins likely have the same structural perturbations as noted for the C81R and the C22Y mutant proteins, respectively.

Arguably the most important unanswered question about mismatch repair in eukaryotes is how the newly synthesized strand and the template strand are distinguished so that only the newly synthesized strand is excised. The close coupling of the mismatch repair machinery and the replication machinery may allow strand discrimination to be achieved through several means. Nicks in the newly synthesized strand such as those occurring between Okazaki fragments on the lagging strand or those resulting from RNase H2-catalyzed removal of ribonucleotides are important for strand discrimination [118, 119]. Because PCNA is loaded at the replication forks in a precise orientation with the front of the ring facing the direction of DNA synthesis, it may play an important role in strand discrimination. Evidence for this comes from the fact that not only does PCNA activate the latent exonuclease activity of MutLα, it also makes it specific for nicking the newly synthesized strand [120].

8. The role of PCNA in replication-coupled nucleosome assembly

Immediately following DNA replication, nucleosomes must be assembled on the newly synthesized daughter duplexes behind the replication fork. In transcriptionally silent, heterochromatic regions of the genome including the centromeric and telomeric regions of

chromosomes, this is carried out in part by chromatin association factor-1 (CAF-1). CAF-1 is a heterotrimer comprised of Cac1, Cac2, and Cac3 subunits. It functions as a histone H3-H4 chaperone that catalyzes the deposition of nucleosomes onto newly synthesized DNA during S phase. The Cac1 subunit of CAF-1 contains a PIP motif that mediates its interaction with PCNA.

Genetic studies in yeast showed that PCNA plays an essential role in CAF-1-mediated nucleosome assembly [31]. Three separation-of-function mutations in PCNA (D41A/D42A, R61A/D63A, and L126A/I128A) were identified that were defective in gene silencing at telomeric regions and mating-type loci. Aspartate-41 and aspartate-42 are close to the hydrophobic pocket on PCNA where PIP motifs bind, and leucine-126 and isoleucine-128 are on the interdomain connector loop near this binding pocket. Arginine-61 and glutamate-63, by contrast, are on a loop on the back face of the PCNA ring. Although it is not known what structural alterations in PCNA are caused by these substitutions, the mutant proteins are all defective in binding CAF-1 *in vitro* [31]. This suggests that the interaction with PCNA acts to target CAF-1 to newly synthesized DNA.

9. Putting it all together

One of the most important, unanswered questions regarding PCNA's role in DNA replication is how does it interact with and regulate the activity of so many binding partners. One widely discussed idea is that PCNA can function as a "tool belt" by binding several partners simultaneously. Because PCNA is a trimer, it can potentially bind up to three PIP-motif-containing proteins at the same time. Although there is so far no direct evidence that eukaryotic PCNA forms such tool belts, archaeal PCNA has been shown to form tool belts. PCNA from the thermophile *S. solfataricus* P2 is a heterotrimer that can simultaneously bind the DNA polymerase, the flap endonuclease and the DNA ligase [121]. Given this, it is very likely that PCNA tool belts are also formed in eukaryotes. Even if tool belts form, however, there are still far too many proteins that need to interact with PCNA than can be accommodated by the PCNA rings loaded on the leading strand and lagging strand at the replication fork.

A possible answer to this question emerges when one considers a number of seemingly disparate facts. First, it has been widely appreciated that replication occurs in eukaryotic cells in discrete regions of the nuclei known as replication foci or replication factories [122]. Immunofluorescent imaging of PCNA shows that in early S phase, there are roughly 1,200 replication factories, each containing as few as two replication forks, in the nuclei of human cells [123]. These replication factories are 150 nm in diameter on average and contain multiple PCNA molecules. Second, many PCNA-interacting proteins have their PIP motifs within intrinsically disordered regions often near their N-termini or C-termini [124]. This allows these proteins to bind PCNA via flexible tethers without their folded domains remaining in the immediate vicinity of PCNA or being otherwise geometrically constrained. Third, some PCNA-interacting proteins contain multiple PIP motifs often within the same flexible tethers [125]. Multiple PIP motifs in the same protein would allow such a protein to simultaneously interact with two or more different PCNA rings thereby linking them together.

Taken together, these observations suggest that there is a large, dynamic network of linked PCNA molecules at and around the replication fork (Figure 6). Some of the PCNA molecules will be loaded on the DNA, perhaps one per Okazaki fragment on the lagging strand. Others will not be loaded on the DNA, but linked to the DNA by a flexible meshwork of protein-protein interactions. Overall, a single replication factory with one or two replication forks at its core may contain hundreds of PCNA molecules and hundreds of PCNA-binding proteins surrounding the forks, all linked through a network of protein-protein interactions. Such a network would serve to increase the local concentration of all the proteins necessary for normal replication, translesion synthesis, error-free damage bypass, break-induced replication, mismatch repair, and chromatin assembly and regulate their various activities during DNA replication.

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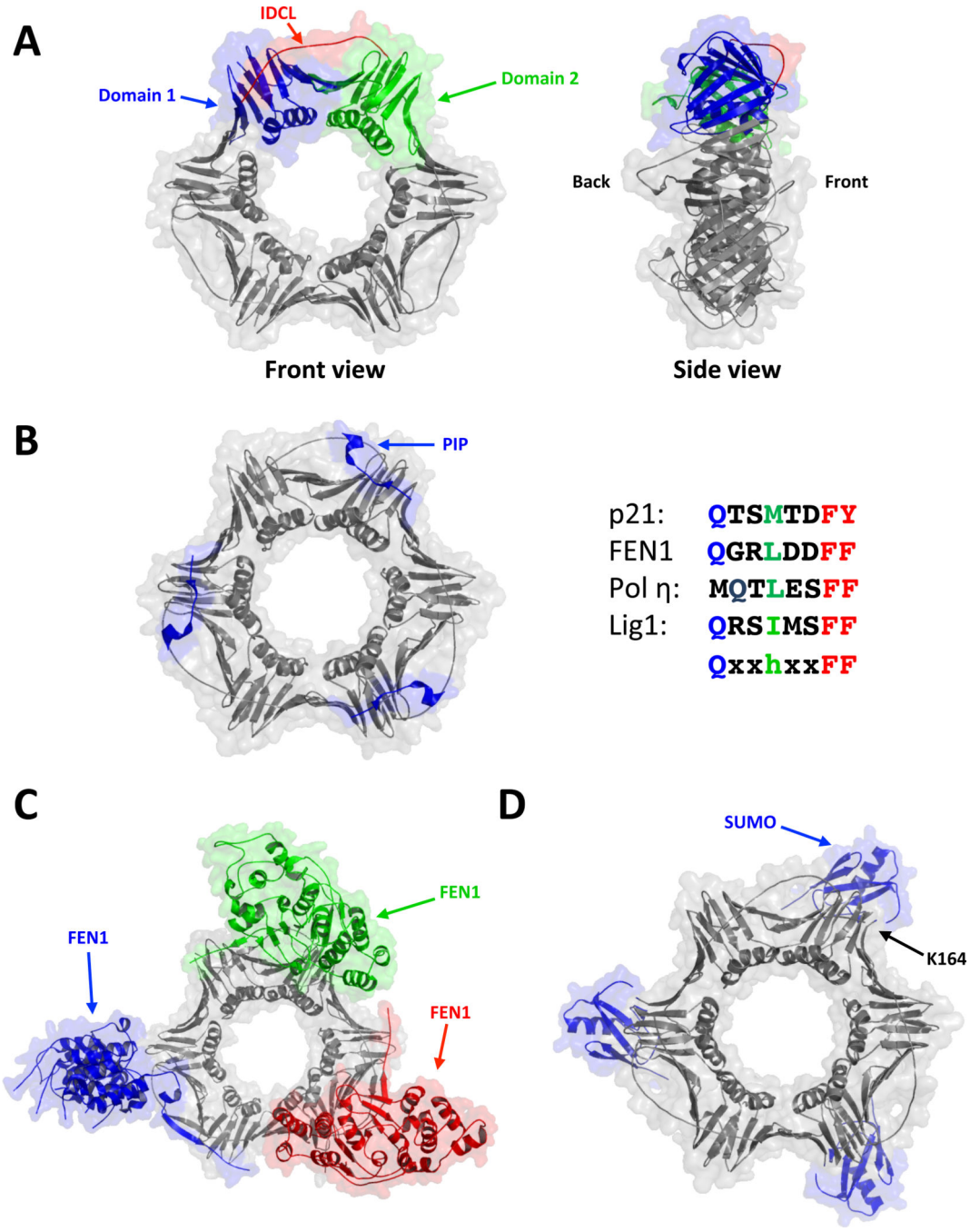

Figure 1. PCNA structure

(**A**) The structure of yeast PCNA (PBD: 1PLQ) [15] is shown from a front view and a side view. Domain 1 is blue, domain 2 is green, and the inter-domain connecting loop (IDCL) is red. (**B**) The structure of yeast PCNA bound to the PIP motif of the Cdc9 DNA ligase (PDB: 2OD8) [126] shown from a front view. The PIP motif is blue. Shown also are the sequences of several PIP motifs. (**C**) The structure of human PCNA bound to three full-length FEN1 proteins (PDB: 1UL1) [51] is shown from a front view. The three FEN1 molecules are blue,

green, and red. (**D**) The structure of yeast SUMO-modified PCNA (PDB: 3PGE) [46] is shown from a front view. The SUMO moieties are blue.

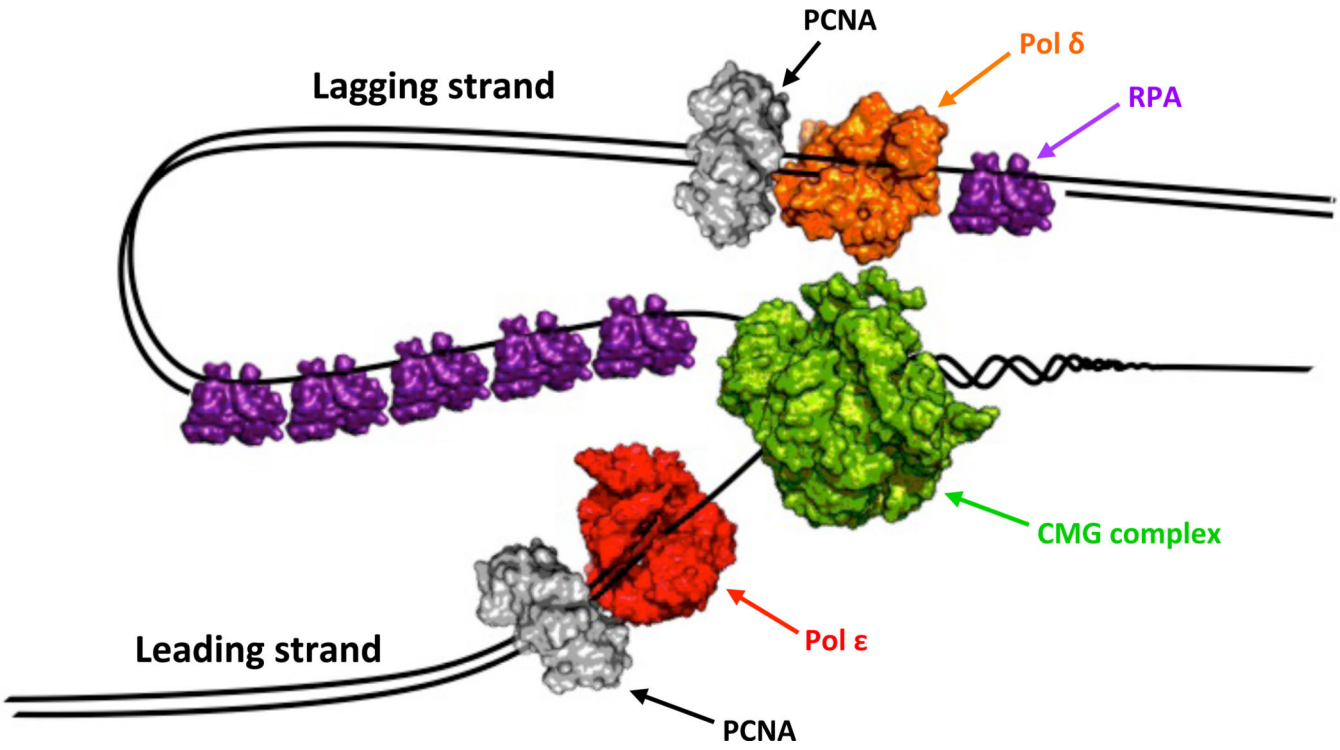

Figure 2. The replication fork

A representation of the replication fork is shown with the leading strand on the bottom and the lagging strand on the top. PCNA is grey, pol ε is red, pol δ is orange, RPA is purple, and the CMG complex is green.

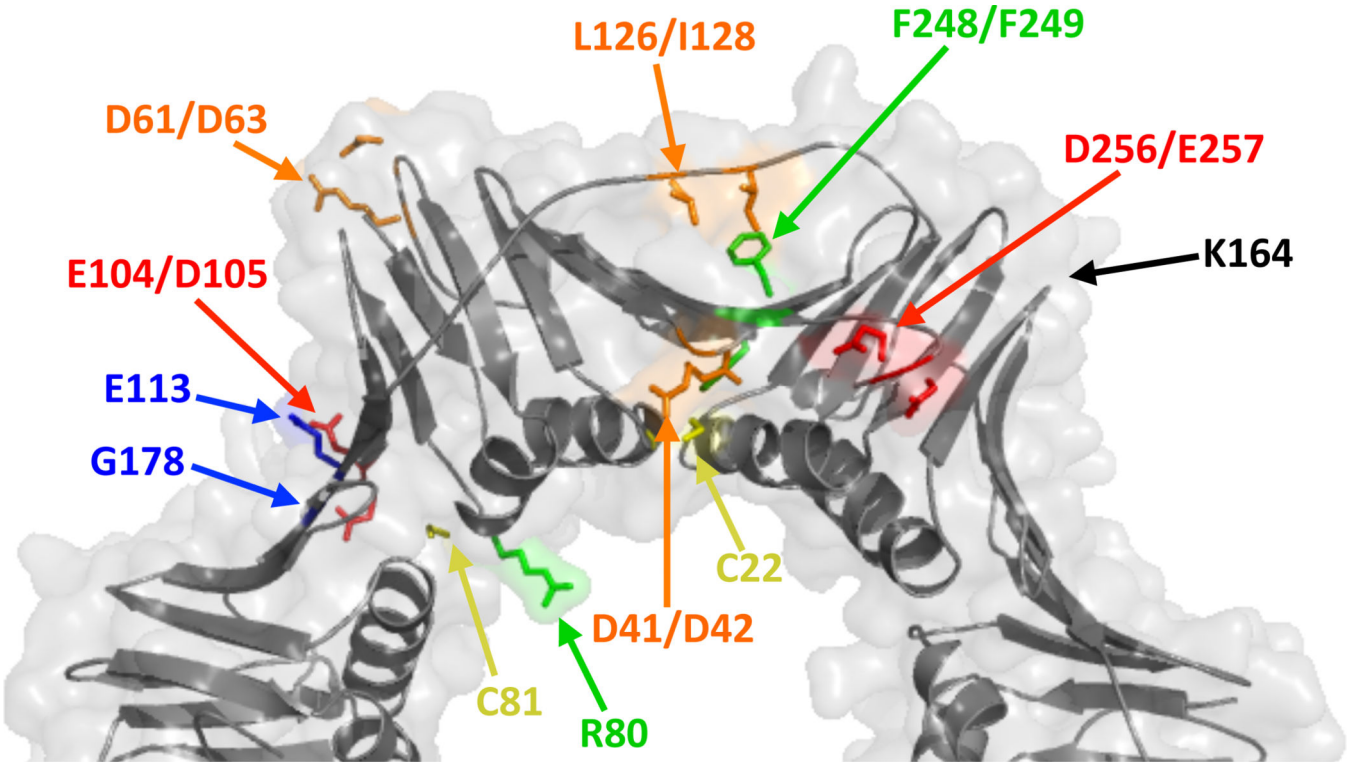

Figure 3. Separation-of-function mutations in PCNA

The locations of the separation-of-function mutations in yeast PCNA are shown from a front view. Mutations affecting translesion synthesis are blue, mutations affecting error-free damage bypass are red, mutations affecting break-induced replication are green, mutations affecting mismatch repair are yellow, and mutations affecting replication-coupled nucleosome assembly are orange.

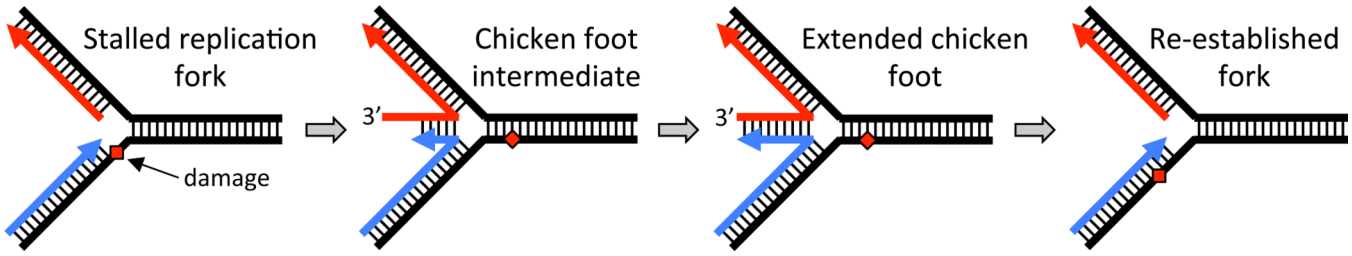

Figure 4. Error-free damage bypass

A schematic of a stalled replication fork is shown. The leading strand is blue, the lagging strand is red, and the location of the DNA damage is indicated by a red square. The stalled replication fork is converted into the chicken foot intermediate, the chicken foot intermediate is extended, and the replication fork is then re-established.

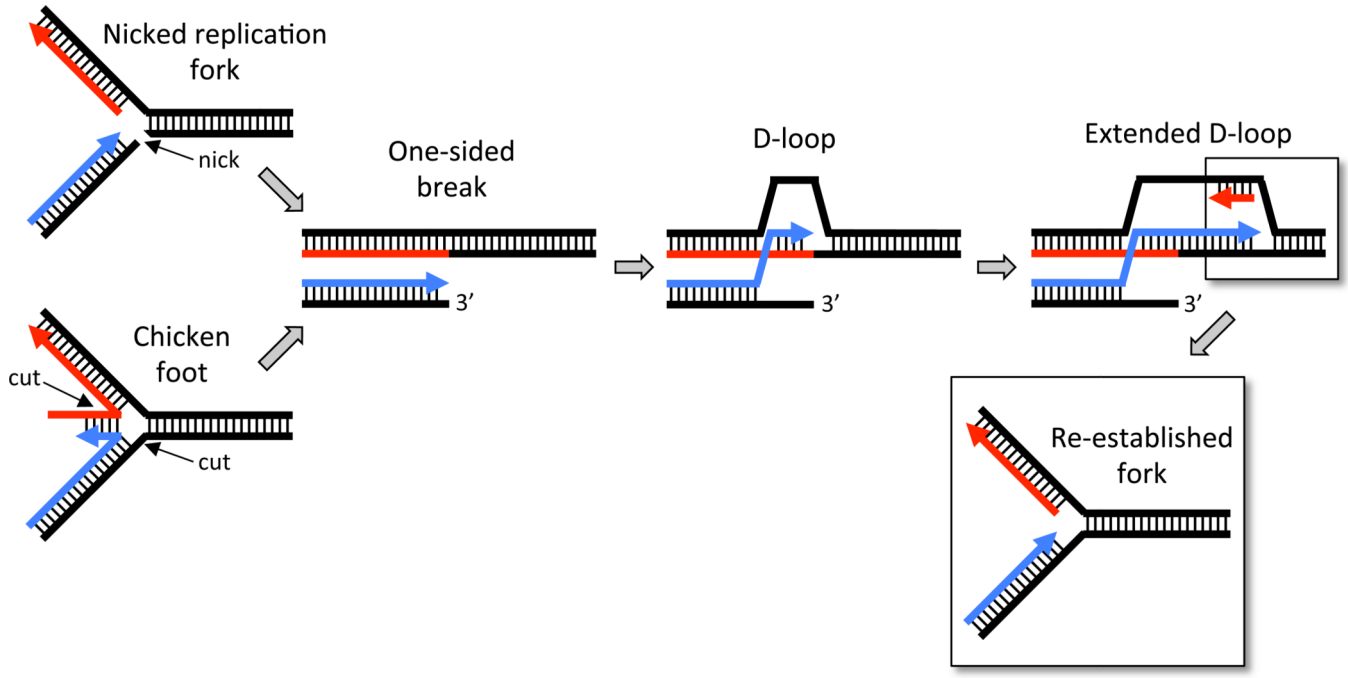

Figure 5. Break-induced replication

A schematic of a replication fork with a nick in the leading strand template is shown. The leading strand is blue, and the lagging strand is red. This gives rise to a one-sided break. A schematic of a chicken foot intermediate is shown. Resolution of this four-way junction by cutting at the indicated sites also gives rise to a one-sided break. The one-sides break is converted into a D-loop, the D-loop is extended, and the replication fork is then re-established.

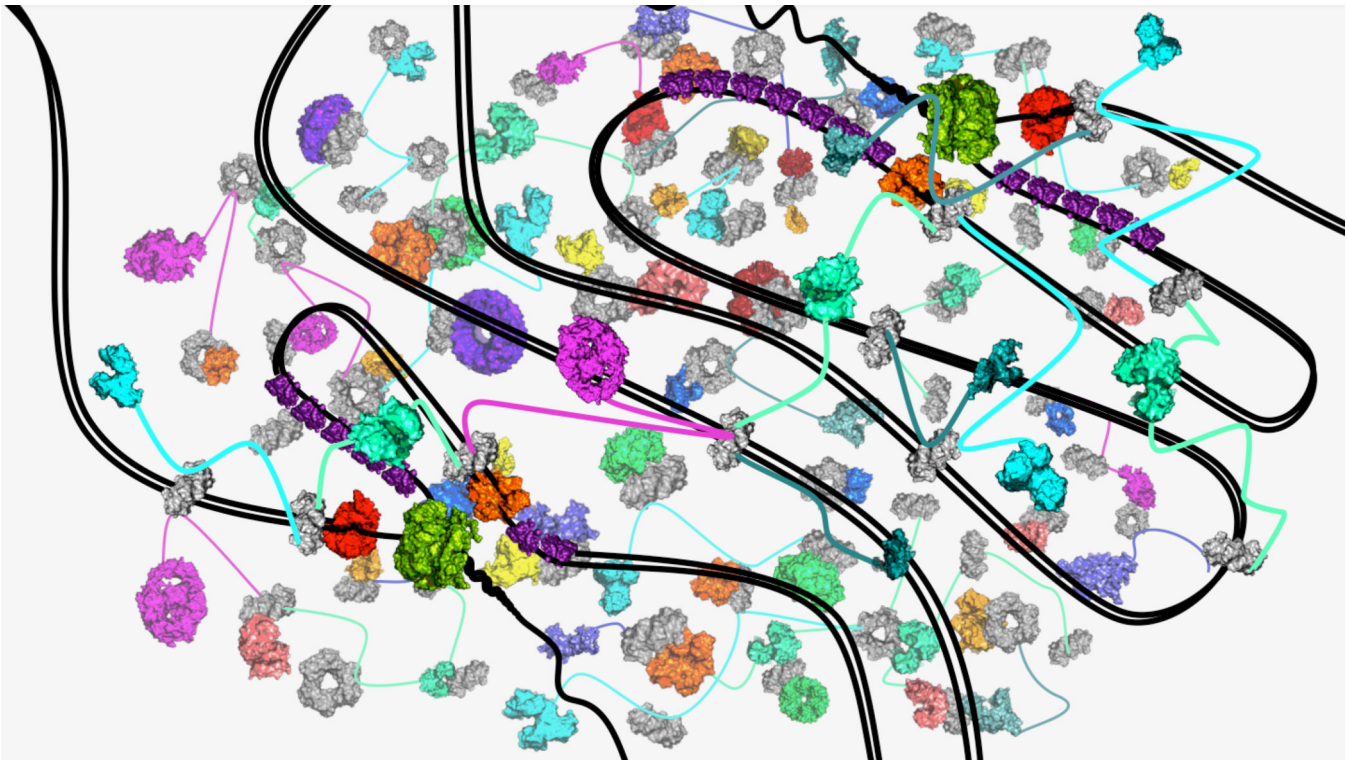

Figure 6. A replication factory

A representation of a single replication factory is shown containing two replication forks drawn to scale. Many PCNA molecules (grey) are shown on the DNA (black lines) as well as off the DNA. These PCNA molecules are linked together by many PCNA-interacting proteins (various colors) to form a large, flexible network surrounding the replication forks. The variously colored lines depict the intrinsically disordered regions of these PCNA-interacting proteins.