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3 PCNA and DNA replication (I)

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Abstract

Replication of the entire genome during S-phase is one of the most important cell cycle events and requires the coordinated activity of a multitude of different enzymes. Proliferating cell nuclear antigen (PCNA) interacts directly and indirectly with most of these proteins and thereby plays a leading part in the coordination of the replication process. PCNA is the processivity factor for the replicative DNA polymerases and the loading platform for additional proteins in DNA replication and repair. In the current review, we present an overview of the diverse PCNA functions in the context of a detailed description of eukaryotic DNA replication. Its essential role in DNA synthesis also makes PCNA a central factor of cell

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cycle control mechanisms. Fluorescent PCNA fusion proteins have been used to label sites of DNA replication and found a wide application as S-phase and cell cycle markers for live cell microscopy. These analyses of the dynamics and progression of DNA replication in living cells have shed new light on current models of S-phase progression.

Introduction

Once upon a time a small nuclear acidic protein was independently identified in proliferating cells with an antibody found in the autoimmune disease systemic lupus erythematosus (SLE) and called "proliferating cell nuclear antigen" (PCNA) [1] as well as by two-dimensional gel electrophoresis as cycling protein and hence termed "cyclin" [2]. A series of studies on growth regulation and cellular transformation ensued. The protein level of the acidic 36 kDa nuclear protein cyclin was found to fluctuate during the cell cycle, in particular during DNA synthesis and the SLE antibody reactive protein was only detected in proliferative or tumor cells but not in quiescence cultures. A few years later, these supposedly different proteins were shown to be one and the same henceforth known as PCNA [3]. Still the story was not to end here and some time later PCNA was found to be identical to the DNA polymerase δ auxiliary factor required for enzyme processivity during the elongation stage of replicative DNA synthesis [4, 5]. PCNA was subsequently shown to share structural and functional similarity to the prokaryotic processivity factors β-subunit of DNA polymerase III and gene 45 product of bacteriophage T4. The crystal structure of human PCNA [6] showed a homotrimeric ring form with a central channel surrounded by basic residues and large enough to fit the DNA. In addition, PCNA is often referred to as the DNA polymerase clamp as it encircles the DNA and thereby tethers the associated polymerase to the template. Its intimate relationship with DNA replication was independently ascertained by cell biological studies, where PCNA was shown to change its subnuclear distribution during S-phase of the cell cycle [7]. Subsequently, PCNA distribution during S-phase was shown to coincide with active sites of DNA synthesis as determined by the incorporation of nucleotides [8, 9]. The role of PCNA in the replication of genetic information is the focus of this chapter.

Loading of PCNA to the replication fork

The first steps after unwinding of the DNA by the replicative helicase are made by the DNA polymerase α/primase complex [10-14] synthesizing a complementary ~12 nucleotide RNA primer, which is then extended with ~20 bases of DNA [15, 16]. The ability to initiate DNA synthesis is a unique feature of the DNA pol α/primase complex. DNA pol α consists of four

subunits [17], with the polymerase activity localized in the largest subunit (p180) and primase activity shared between the two small subunits (p48 and p58) (reviewed in [18]). The single strand binding heterotrimer replication protein A (RPA) acts as an auxiliary factor or fidelity clamp by stabilizing the pol α/primase complex and reducing the misincorporation efficiency [19]. After the RNA-DNA primer has reached the critical length of 30 nucleotides, replication factor C (RFC) binds to the 3´-OH end of the RNA-DNA primer, inhibits and displaces the DNA pol α/primase complex [20-22]. RFC is the so-called "clamp loader" for PCNA. RFC is a heteropentameric complex consisting of one large (p140) and four smaller subunits (p40, p38, p37, and p36), which share considerable sequence similarity with each other [23] and are conserved in all eukaryotes. The p140 subunit contains two evolutionarily conserved domains. The first is also conserved among prokaryotic ligases and interacts with double-stranded DNA, while the second, conserved between the smaller RFC subunits, forms the interaction surface with PCNA [24, 25]. From the crystal structure of the RFC/PCNA complex it is now evident that the main RFC/PCNA contacts occur through the p140 and the p38 subunit; while p140 interacts extensively with PCNA, p38 only seems to be partially engaged [26]. PCNA itself does not have any DNA-binding activity, but is in a strictly ATP-dependent manner loaded by RFC onto the DNA [27-30]. The RFC catalyzed PCNA loading is the prerequisite for the assembly of pol δ as well as pol ε onto the DNA to form a processive holoenzyme, which then extends the RNA-DNA primer. In the absence of ATP, RFC has a closed two-finger structure also termed U-form. Upon the addition of ATP the RFC pentamer opens up to the so-called C-form. PCNA can be held between the two fingers of the U-form and the structural internal change to the open C-form opens up the PCNA ring so that it can encircle the DNA [31]. To close the PCNA ring around the DNA, ATP hydrolysis is again required [20]. It is not clear, whether RFC dissociates from PCNA after loading or they both form together with pol δ the so-called holoenzyme. Using gelfiltration it was found that RFC dissociated after loading of PCNA and hence the holoenzyme consists only of pol δ and PCNA [32]. Whether RFC can also efficiently unload the PCNA either during or after completion of DNA replication is still under debate. In model loading/unloading systems, human RFC has been shown to unload PCNA from template-primer DNA in an ATP-dependent reaction [33, 34]. The p140 subunit of RFC is exchanged in specific situations by related, RFC-like proteins. If Chl12 (also termed Ctf18), which is involved in sister chromatid cohesion substitutes p140 this modified RFC complex can still upload PCNA [35, 36] but the regulated unloading of PCNA by Ctf18-RFC may serve an important function during the establishment of sister chromatid cohesion [37]. Another RFCp140-like protein, Rad17, preferentially binds primed single-stranded DNA and gapped DNA and is therefore thought to play a role in the

maintenance of genomic stability. A checkpoint sliding clamp-loader model for lesion recognition has been proposed [38].

Loading of the replication polymerases

The initiation complex pol α/primase is not capable of processive DNA synthesis and dissociates from the template DNA after primer synthesis. The processive polymerase for the elongation step of DNA replication is pol δ, which is loaded in a process called "polymerase switch" [21, 22, 39]. The mammalian pol δ is a heterotetramer consisting of one catalytic subunit holding the polymerase and proofreading 3′-5′ exonuclease activity (p125) and three additional subunits p66, p50 and p12 [40-42]. In the absence of PCNA, pol δ is a relatively non-processive polymerase, while together with PCNA as a processivity factor, the activity and processivity of pol δ increases up to 100-fold [5, 43-45]. PCNA is thought to tether pol δ onto the DNA and thereby prevents the dissociation of the polymerase as it extends the primer [46]. Due to their association with the trimeric PCNA ring these pols become highly processive, which justifies the names "polymerase clamp" or "processivity factor for DNA pol" used commonly to refer to PCNA. Structurally, the PCNA clamp is positioned behind the pol δ during DNA synthesis [47].

In addition to pol δ, pol ε might be involved in the processive elongation. Pol ε is also loaded by PCNA [48]. However, pol ε is already very processive in the absence of PCNA, and the precise roles of pol ε and pol δ during the synthesis of the leading and the lagging strand are not yet identified. Genetic and biochemical evidence suggests that lagging strand synthesis is carried out by pol α and pol δ (reviewed in [49]). Immunodepletion analysis suggested that pol δ is essential for lagging strand synthesis and that this function cannot be substituted by pol ε [50]. The human pol ε has four subunits, a large catalytic subunit, p261, and three associated subunits, p59, p12, and p17 [51]. Neutralizing antibodies against human pol ε inhibited the chromosomal replication when microinjected into human fibroblasts [52]. Pol ε is localized to distinct nuclear foci throughout the entire cell cycle. Early in S-phase, pol ε foci did not colocalize with PCNA and newly replicated DNA but were adjacent to each other. However, pol ε foci did colocalize with PCNA foci later in S-phase, suggesting that pol ε might participate with PCNA in DNA replication only late in S-phase. This indicated that pol ε might not be involved in replication but might prepare heterochromatin for replication [53]. Pol ε interacts with proteins of the DNA damage checkpoint machinery [54] and could therefore be involved in checkpoint control of the replicated DNA.

Leading and lagging strand synthesis

While the replication of the leading strand by the pol δ holoenzyme is processive and continuous for at least 5-10 kb, the DNA synthesis of the lagging strand continues only until the polymerase encounters the RNA-DNA primer of the previously replicated DNA fragment. These small DNA pieces are about 180-200 bp in size and are called Okazaki fragments. PCNA is supposed to be the central player in this task functioning as a loading platform, which coordinates the proteins involved in the many steps of DNA replication, DNA repair as well as DNA translesion synthesis (TLS) [55, 56]. Among these proteins are Flap endonuclease 1 (Fen1; [57]), DNA Ligase I [58], Dnmt1 [59], DNA Topoisomerase I and IIa [60], p21 [61], cyclin D [62], cyclin A [8], nucleotide excision repair protein XPG [63], mismatch repair proteins MLH1 and MSH2 [64], TLS polymerases (reviewed in [65]) as well as chromatin assembly factor CAF-1 [66] and histone modifier HDAC1 [67]. Among these proteins, p21 might be a control switch from one PCNA-dependent function to another at the DNA replication fork as it interferes with the interactions between PCNA and Fen1, Dnmt1 and DNA Ligase I [58, 59, 68].

Another unsolved problem is derived from the directionality of the polymerases during replication. To achieve identical directionality of the polymerases on the leading and lagging strand, a dimerization of pol δ and a loop back of the lagging strand was proposed [69]. In this way, the coordination of the leading and lagging strand synthesis in establishing an asymmetric replication fork would be simplified. Alternatively association of pol α/primase to one of the two halves of the dimeric pol δ could also occur [70]. Figure 1 shows a schematic representation of one half of the asymmetric replication fork. The synthesis of the lagging strand is very complex, as the replication of each Okazaki fragment has to be initiated and continued with the pol δ holoenzyme. In a process termed maturation of Okazaki fragments the RNA-DNA primer is removed, the DNA gap filled and the DNA fragments sealed. The current model for Okazaki fragment processing suggests that the pol δ holoenzyme polymerizes until it meets the 5′ end of the RNA-DNA primer of the previous Okazaki fragment. Then the holoenzyme invades the fragment and thereby displaces the primer, making the DNA again single-stranded. In this process, PCNA ensures processivity while RPA ensures that this primer displacement process only goes on for 30 nucleotides. RPA, which needs 30 nucleotides to bind efficiently to ssDNA [71, 72] immediately covers the now so-called flap and recruits Dna2, an endonuclease. This would mean that per Okazaki fragment one flap is generated which would then be bound by one RPA trimer. Dna2 possesses DNA unwinding as well as a single stranded endonuclease activity [73, 74], but for the complete removal of the RNA primer, the combined action of the two endonucleases Dna2 and Fen1 is

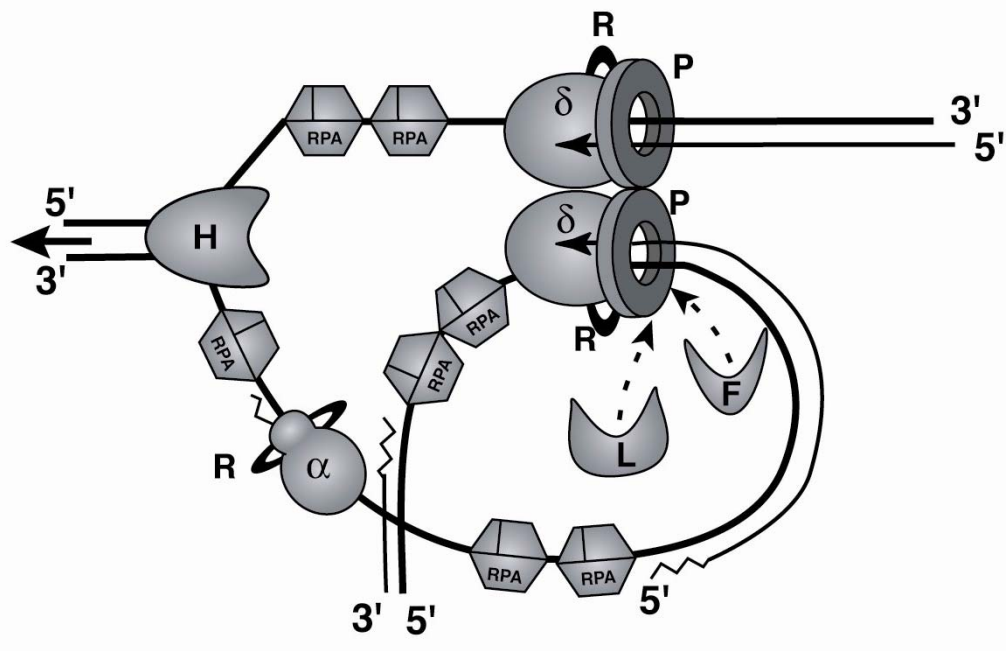

Figure 1. The mammalian DNA replication fork. Schematic illustration of one half of the mammalian replication fork depicting the major proteins involved in the process of DNA replication. The helicase (H) unwinds the DNA and the ssDNA is covered by RPA. The DNA polymerase α/primase complex (α) synthesizes a RNA-DNA primer (ΛΛΛ). The clamp loader RFC (R) loads the clamp PCNA (P) that then recruits DNA polymerase δ (δ). At the lagging strand the Okazaki fragments are then processed by Fen1 (F) and DNA Ligase I (L).

needed [75, 76]. Dna2 cleaves the RNA containing portion of the RNA-DNA primer. Fen1, which is loaded by interaction with PCNA, processes the remaining DNA flap product while DNA Ligase I seals the resulting nick in the DNA (reviewed in [77]).

As there are only three binding sites in the interdomain connecting loop per PCNA ring available, the timing, order and duration of each binding is very important [78]. A 'toolbelt' model has been proposed, in which the PCNA trimer can simultaneously bind three different replication proteins [65]. The crystal structure of human DNA Ligase I has recently been solved and shows DNA Ligase I encircling the DNA while sealing the nicked DNA fragments. This would mean that DNA Ligase I would effectively mask all three protein binding sites on the PCNA trimer, thereby excluding other proteins from binding the clamp [79]. From several structural studies on the conformational change within the Fen1 protein during the actual flap cleavage it has been concluded that Fen1 also encircles the DNA [80-82]. The structure of the full-length Fen1 and PCNA complex showed that – in contrast to DNA Ligase I - each subunit of the PCNA trimer is bound to a single Fen1 molecule [83].

This would mean that instead of Fen1 and DNA Ligase I both binding PCNA simultaneously during Okazaki fragment maturation, Fen1 would need to be dislodged from PCNA in order for DNA Ligase I to interact with the clamp [84]. These findings might suggest that for enzymatic action only one protein is bound to the PCNA trimer at a time. This is in agreement with the results of immunoprecipitation studies, suggesting that different pools of PCNA associated to RF exist. For example, binding of pol δ and DNA Ligase I was mutually exclusive [85]. A model of the replication and maturation of Okazaki fragments is shown in Figure 2.

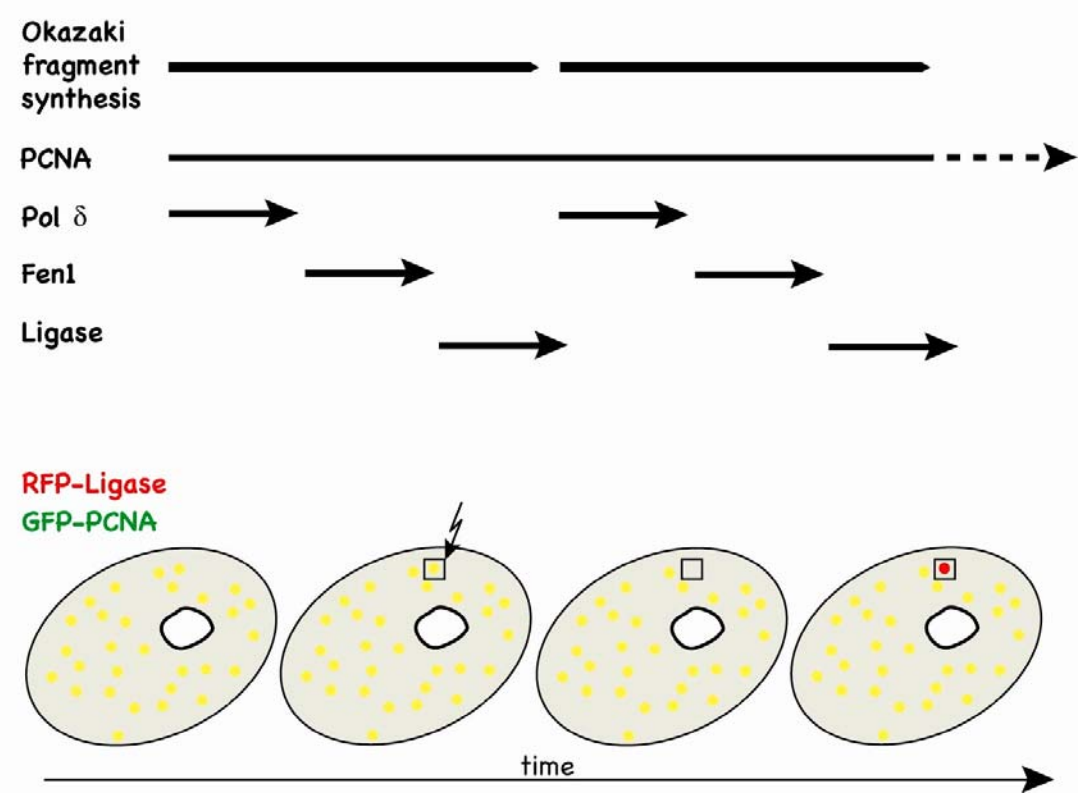

Figure 2. Sequential steps of Okazaki fragment maturation. Due to the polarity of the DNA synthesis reaction, the replication of the lagging strand is discontinuous. Based on data from double photobleaching analysis, it is proposed that PCNA is stably bound and reused for the replication of several Okazaki fragments while polymerase δ and the maturation proteins Fen1 and DNA Ligase I are loaded once per Okazaki fragment. A schematic representation of a double FRAP experiment (photobleached region marked by a square and an arrow indicating the laser beam) on a cell with GFP-PCNA and RFP-Ligase I. Double labelled replication foci (merge of green and red is shown in yellow) exchange Ligase I in a matter of seconds while PCNA does not. This experiment among others indicated the different dynamic properties of proteins involved in DNA replication and the function of PCNA as a stable loading platform for the loading of Okazaki fragment maturation proteins.

The structure of a replication focus and the regulation of PCNA subnuclear distribution

DNA replication occurs at discrete nuclear foci as has been demonstrated by the visualization of the incorporation of modified nucleotides [86-90]. These foci have been termed replication foci (RF), the subnuclear sites of ongoing DNA replication. Morphologically one focus includes many active replisomes consisting of enzymes and auxiliary factors involved in the duplication of the genome at one origin [91-93]. The S-phase has been subdivided according to the temporal-spatial patterns of the RF into three main stages: early, mid and late S-phase [88]. While in early S-phase the RF are small and uniform in size, in mid and late S-phase the size of the microscopically resolved RF is increased albeit not uniformly and their number is reduced. With deconvolution microscopy as well as thin sectioning electron microscopic analysis, these large foci in mid and late S-phase were shown to be composed of many small discrete RF of possible identical size to the ones in early S-phase [94, 95].

As for the incorporation of modified nucleotides, three different patterns of PCNA immunofluorescence staining have been identified in S-phase cells corresponding to the part of the genome being replicated [8, 96-98]. Figure 3 shows the localization of GFP-PCNA in a mouse myoblast cell line during the

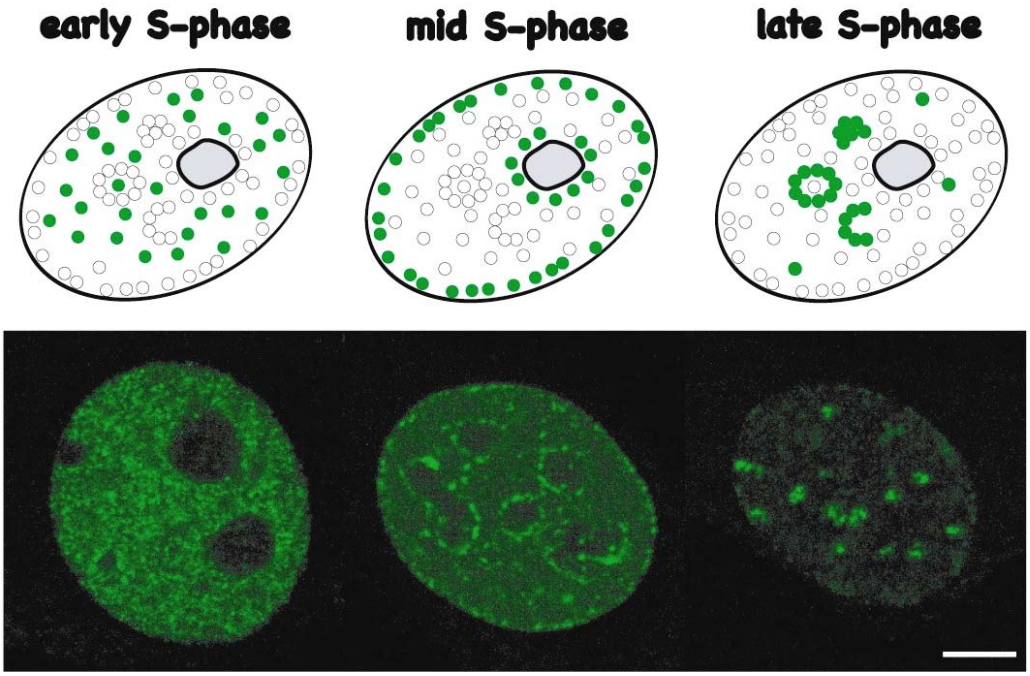

Figure 3. The progression of DNA replication throughout S-phase: the upper panel shows a schematic representation of early, mid and late replication patterns of mammalian cells. In the lower panel corresponding single confocal sections of mouse myoblast cells with GFP-PCNA labelled replication foci are shown. Scale Bar, 5 μm.

different stages of S-phase. At the start of S-phase, PCNA becomes resistant to extraction from nuclei with detergents, for example Triton X-100, while in non-S-phase cells PCNA is easily extractable [7]. The level of PCNA reflects the proliferative state of the cell. Most G0/G1 cells do not express significant amounts of PCNA while there is a clear increase in late G1 and a doubling of the PCNA in S-G2 phase [99]. It had been suggested that phosphorylation of PCNA is responsible for the changes in the nuclear localization [3] but 2D gels showed that PCNA is not phosphorylated or otherwise posttranslationally modified in a way that affects the charge of the protein [100].

Under normal growth conditions SUMO and ubiquitin modification of PCNA have not yet been identified. However, in response to DNA damage the highly conserved residue K164 is mono-ubiquitinated or modified by SUMO [101]. This residue is not involved in the interaction of PCNA with PCNA-binding proteins (reviewed in [102]). SUMO is an ubiquitin-related protein that regulates protein-protein interactions and possibly antagonizes ubiquitination through competition for similar lysine residues in substrates [103]. SUMO modification of PCNA inhibits its ubiquitination and the DNA repair functions. Monoubiquitinated PCNA displays the same replicative functions as unmodified PCNA, but it specifically interacts with the translesion synthesis (TLS) polymerases, needed for replication across DNA lesions [104]. This suggested that ubiquitination increased the functionality of PCNA as a sliding clamp promoting mutagenic DNA replication [105]. It has also been shown that PCNA, which is mutated at K164 can load to replication sites but not to repair sites [106].

Mobility and turnover of replication proteins at RF

Using DNA double pulse-labeling experiments to mark DNA fragments, which are replicated at consecutive time points, it was suggested that the newly replicated DNA gradually moves away from their site of replication [107, 108]. In these experiments though only the replicated DNA was visualized and therefore it was not clear whether only the DNA, only the replication machinery or both entities were moving [108]. Different approaches and techniques have been trying to solve this problem since then. With the usage of GFP-PCNA in living cells, it was found that although the pattern of nuclear RF undergoes defined changes during the progression of S-phase, the individual foci do not show directional movements, merge or divide. In addition, the RFs are heterogeneous in size and lifetime. Furthermore, the assembly and disassembly of RF patterns are gradual and coordinated but asynchronous through S-phase [95]. Time lapse microscopy followed by overlay of the fluorescent PCNA patterns over time revealed that new RF assembled adjacent to previous ones by an indirect mechanism, termed domino effect [109]. Further comparing the localization of labeled nucleotides with GFP-PCNA

revealed that the separation of nascent DNA and the replication machinery was caused by the appearance of GFP-PCNA at RF positions adjacent to previous RF during S-phase progression [109, 110].

With PCNA supposed to be the loading platform for the proteins involved in the elongation of DNA replication and in the maturation of Okazaki fragments, the question of the relative mobility and dynamics of the individual proteins involved in these processes arises. In non S-phase cells, PCNA diffuses through the nucleoplasm at a rate comparable to GFP alone, but gets transiently immobilized at RF during S-phase with a very low exchange rate in and out of these structures [109]. For S-phase cells, different models have been discussed for the dynamic behavior of PCNA: i) the PCNA ring stays stably bound during lagging strand synthesis together with a dimeric pol δ; ii) PCNA is recycled within an RF; or iii) a new PCNA ring is loaded for each new Okazaki fragment [109]. In order to clarify that, several points have to be considered. The length of an Okazaki fragment is ~ 180-200 nucleotides. With a replication fork progression in mammalian cells of an average rate of about 1.7 kb per minute [93] that means that it takes 6-7 s for the synthesis of one Okazaki fragment. In one minute about 10 Okazaki fragments would be synthesized, which requires the loading of about 30 PCNA molecules per minute, if a new PCNA ring is loaded at each Okazaki fragment. Estimates in mammalian cells suggest an average of 5 replicons per RF corresponding to 10 replication forks [93, 111], one would expect the loading of 300 PCNA molecules per minute at each replication focus [109].

Using fluorescence recovery after photobleaching (FRAP), PCNA was found stably bound at RFs with a turnover in the order of minutes within the same cells and foci. This suggested that PCNA remained associated with the replication machinery for multiple rounds of Okazaki fragment maturation [109]. This behavior is in contrast to proteins involved in initiation of DNA replication as RPA34, where a very rapid fluorescence recovery at RF within seconds after photobleaching was observed [109]. Older models had suggested that Ligase I or Fen1 and PCNA were loaded onto the replicating DNA as a stable complex with a 1:1 stoichiometry [112] and thus would have to remain associated with replication sites for a similar time period. To directly test the dynamics of PCNA and PCNA binding Okazaki fragment proteins (DNA Ligase I and Fen1), double FRAP experiments were performed with simultaneous measurements of exchange rates of the different proteins. A very fast and complete exchange of DNA Ligase I and Fen1 within a few seconds after the bleach was detected suggesting that these factors and PCNA are independently loaded at the replication fork and stay for different times. The data is consistent with a model, where PCNA stays at one RF throughout the synthesis of several Okazaki fragments, while DNA Ligase I and Fen1 exchange after each fragment [113].

Models of mammalian DNA replication progression

There are approximately $4x10^4$ origins of DNA replication in a mammalian cell nucleus, which have to fire once during each round of replication. This number corresponds to the initiation events on the leading strand, while for the lagging strand - due to the discontinuous synthesis of the Okazaki fragments - there are approximately $2\text{-}3x10^7$ initiation events in mammalian cells [70]. One important and still very unclear feature of DNA replication is how the replication of the genome progresses throughout S-phase. Are these origins of replication activated according to a specific program, which means that every origin "knows" when it will be replicated (clock model)? Or is the progression of replication self propagating whereby after replication is initiated at specific origins it continues by a domino effect-like mode to trigger the replication of their neighboring origins (domino model)? There are several requirements for a program controlling the replication-timing: (i) labels to indicate that origins are early, mid or late, (ii) factors that recognize these labels and activate the origins according to the S-phase stage and (iii) a checkpoint system that ensures that everything is replicated at the right time (reviewed and discussed in [114]). A domino model would require (i) a way to indicate the early replicating origins, (ii) a method of activating the "next-in-line" origins and (iii) a checkpoint system ensuring that everything is replicated only once during S-phase.

It is generally accepted and directly tested at the genome level that the transcriptionally active domains replicate early while the inactive and highly repetitive parts replicate late ([115-118]; recent progress is reviewed in [119]). This has been recently visualized further with histone methylation (m) on lysine 9 (K9) of histone 3 (H3) during DNA replication: H3K9m1 was largely restricted to early RF, H3K9m2 was the predominant colocalized with mid S-phase RF while H3K9m3 marked the late-replicating RF [120]. RFs, which were marked by pulse-chase with nucleotide analogues were maintained at subsequent cell cycle stages and cell cycles [93, 111, 121, 122] arguing for the maintenance of "replicative units". Synchronized cells pulse-labeled with two different nucleotide analogues at the very beginning of two consecutive S-phases, showed a high degree of colocalization for genomic regions first replicated during S-phase [93, 111]. These results argue for replicative units, which stably maintain their replication timing during S-phase. Epigenetic marks might be involved in controlling replication timing in general and/or determining the early firing origins [123-125]. Further evidence is required to determine whether the later replicating origins are predetermined (clock model) or result from the propagation of the replication "wave" from the earlier sites (domino model) [109, 126].

It is generally assumed that the minichromosome maintenance proteins (Mcm) 2-7 form a complex at origins in G1-phase licensing the DNA for

replication. They are thought to be continuously displaced from the freshly replicated DNA ensuring thereby that every piece of DNA is only replicated once per S-phase (reviewed in [127]). Recently, also PCNA was suggested to play a role in the mechanisms that limit DNA replication to one round per cell cycle. The ubiquitin-mediated destruction of Cdt1, another potential replication-licensing factor, requires the interaction with chromatin-bound PCNA [128-130]. However none of these findings argue exclusively for one model or the other but provide a mechanism in both cases for limiting replication of each genomic region to once per cell cycle.

Combined photobleaching and time overlay analysis of cells stably expressing GFP-PCNA, showed that new RFs assemble de novo at sites directly located next to the previously firing RFs. These results rule out a simple sliding or jumping of the replication elongation machinery to adjacent origin clusters and argues for an indirect mechanism of origin activation consistent with the domino model. This would mean that the activation of the first origin clusters would start a chain reaction leading to the activation of later origin clusters depending on the relative spatial distribution of the genome within the nucleus. This would then create a self-propagating system, maintaining the same temporal order of replication over cell generations provided the same initial sites would be used [109, 113]. This striking adjacency of the replication sites over S-phase is not expected to occur predictably from the clock model (Figure 4). The DNA replication itself has an influence on the chromatin remodeling, as the replication machinery needs access to the entire genome. Furthermore, it has been indirectly demonstrated in vivo that large-scale chromatin decondensation occurs before, or coincident

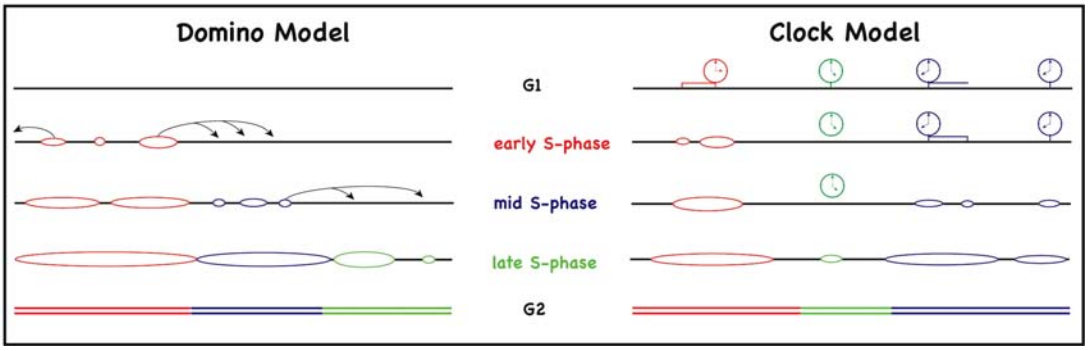

Figure 4. Models for the propagation of DNA replication throughout the genome. Schematic illustration of the two principal models of S-phase progression; the domino model and the clock model. According to the domino model early replication origins are epigenetically determined and their firing leads to the sequential and preferential activation of adjacent replicon arrays. This model predicts that subsequent replication foci form adjacent to previously firing ones. According to the clock model early, mid and late replicating origins are marked specifically and are set to replicate at specific times during S-phase.

with S-phase [131]. CDC45, a protein involved in the initiation of replication promotes, if targeted to specific chromatin sites, a dramatic decondensation of chromatin in the targeted region [132]. Future experiments should test the role of locally induced decondensation in determining the progression of genome replication.

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