

# PCNA acts as a stationary loading platform for transiently interacting Okazaki fragment maturation proteins

Anje Sporbert<sup>1</sup>, Petra Domaing<sup>1</sup>, Heinrich Leonhardt<sup>1,2</sup> and M. Cristina Cardoso<sup>1,\*</sup>

<sup>1</sup>Max Delbrueck Center for Molecular Medicine, 13125 Berlin, Germany and <sup>2</sup>Department of Biology II, Ludwig Maximilians University of Munich, 82152 Planegg-Martinsried, Germany

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## ABSTRACT

In DNA replication, the leading strand is synthesized continuously, but lagging strand synthesis requires the complex, discontinuous synthesis of Okazaki fragments, and their subsequent joining. We have used a combination of *in situ* extraction and dual color photobleaching to compare the dynamic properties of three proteins essential for lagging strand synthesis: the polymerase clamp proliferating cell nuclear antigen (PCNA) and two proteins that bind to it, DNA Ligase I and Fen1. All three proteins are localized at replication foci (RF), but in contrast to PCNA, Ligase and Fen1 were readily extracted. Dual photobleaching combined with time overlays revealed a rapid exchange of Ligase and Fen1 at RF, which is consistent with *de novo* loading at every Okazaki fragment, while the slow recovery of PCNA mostly occurred at adjacent, newly assembled RF. These data indicate that PCNA works as a stationary loading platform that is reused for multiple Okazaki fragments, while PCNA binding proteins only transiently associate and are not stable components of the replication machinery.

## INTRODUCTION

Eukaryotic DNA replication takes place at microscopically visible sites in the nucleus called replication foci (RF), which change throughout S-phase (1). Each RF consists of a cluster of replicons and their assembled replisomes, i.e. all factors needed to replicate a replicon unit. These factors include DNA polymerases (pol), a polymerase clamp [proliferating cell nuclear antigen (PCNA)] nucleases [Flap endonuclease 1 (Fen1)] and a DNA Ligase [DNA Ligase I (Ligase)] (2). While the leading strand synthesis is continuous, the lagging strand is

discontinuously synthesized in short pieces (Okazaki fragments), which need to be processed and joined [reviewed in (3)]. The processivity of the elongating polymerase is enhanced through binding to the sliding clamp PCNA. The latter forms a homotrimeric ring around the DNA and, in addition to binding pol  $\delta$ , it is proposed to recruit Okazaki fragment maturation proteins, such as Ligase (4) and Fen1 (5,6) via a PCNA-binding motif. Fen1 is involved in the removal of the RNA–DNA primer, while Ligase is required to seal the remaining nick between adjacent Okazaki fragments [reviewed in (7)]. Many other proteins have been reported to bind to PCNA; raising questions about how binding is coordinated and sterical hindrance avoided during replication [reviewed in (8)]. In photobleaching studies in living cells, we found an unexpected slow exchange of PCNA at RF, leading us to propose that it could be reused over several Okazaki fragments during lagging strand synthesis *in vivo* (9). To study the dynamics of lagging strand synthesis, we have now used dual color photobleaching and *in situ* extractions to directly compare the distribution and dynamics of PCNA with PCNA-binding replication factors (Ligase and Fen1) involved in Okazaki fragment maturation.

## MATERIALS AND METHODS

### Cell culture, plasmids and transfection

Mouse C2C12 myoblast cells were used for all experiments unless otherwise stated and transiently transfected by the calcium phosphate–DNA coprecipitation method as described previously (10) with equal amounts of plasmid DNA for cotransfections. C2C12 cells stably expressing either GFP-PCNAL2 [human PCNA (1)] or GFP-Ligase [human DNA Ligase I (10)] were established and grown as described previously (1). A GFP–FEN1 fusion protein was constructed by fusing an N-terminal SV40 nuclear localization signal followed by enhanced GFP and a 15 amino acid linker sequence to the mouse FEN1 ORF, isolated from a mouse embryo

\*To whom correspondence should be addressed. Tel: +49 30 94172273; Fax: +49 30 94172336; Email: cardoso@mdc-berlin.de

cDNA library (Clontech) by PCR and confirmed by DNA sequencing. mRFP1 fusion proteins were obtained by replacing enhanced GFP in previously described PCNA and Ligase plasmids by the mRFP1 coding sequence (11). The structure of the chimeric proteins, as well as their characterization (expression and localization in mammalian cells) is shown in Supplementary Figure S1 and in refs (1,9,10).

### Cell extracts and western blotting

For extraction experiments (Figure 2), C2C12 cells doubly transfected with GFP-Ligase and GFP-PCNA were extracted with a buffer (50 mM Tris-HCl, pH 8, 120 mM NaCl and 0.5% NP40) containing protease inhibitors by freezing and thawing three times followed by a 30 min incubation on ice. After centrifugation, equal amounts of the cell extract and the remaining cell pellet were analyzed on a 12 or 8% SDS-PAGE followed by western blotting. Amounts of endogenous PCNA and Ligase as well as the respective GFP fusion were detected with anti-Ligase (10) or anti-PCNA (clone PC10, Dako) antibodies.

### Immunofluorescence, *in situ* DNA replication assay and salt extraction

Endogenous PCNA and Ligase or incorporated BrdU (10 min pulse labeling with 100  $\mu$ M BrdU; Sigma) were detected simultaneously after formaldehyde fixation for 10 min followed by ice-cold methanol for 3 min as described previously (9). Salt extractions were performed as described previously (9). For the triple immunostaining (Figure 2B), the following primary antibodies were used: mouse monoclonal anti-PCNA antibody (clone PC10, Dako), rabbit affinity purified anti-DNA Ligase I antibody (10) and a rat monoclonal anti-BrdU antibody (clone BU1/75, SeraLab). Live cell extractions were performed in chambered glass coverslips (Labtek, Nunc) by direct treatments on the microscope stage at room temperature while acquiring images.

### Fluorescence microscopy and photobleaching of living cells

Confocal images (350 nm optical slices) were acquired with a laser scanning microscope LSM510 Meta using a 63 $\times$  NA 1.4 Planapochromat oil immersion objective (Zeiss). GFP/FITC, AlexaFluor568/mRFP and Cy5 were excited sequentially at 488, 543 or 633 nm and detected with a 500–530 nm band pass and 560 or 650 nm long pass filters, respectively, to prevent crosstalk. For live cell microscopy, cells were kept in a FCS2 live cell microscopy chamber (Bioptechs) at 37°C as described (1). Laser power for observation was typically 1–5% (488 nm, 25 mW) and 10–15% (543 nm, 1 mW). Images were acquired in 12 bit mode. Care was taken to prevent over- or under-exposed image pixels and to use identical microscope parameters within one experiment. After acquisition of prebleach images, a part of the nucleus was bleached by either increasing the laser power to 100% at increased magnification for 12–15 s [Fluorescence Depletion by Photobleaching (FDP) experiments] or by scanning only a selected region of interest (ROI, 30  $\times$  30 pixel) for 0.49 s with 100% laser power (dual color FRAP). Thereafter, postbleach images with the initial settings were acquired.

### Image analysis and data processing

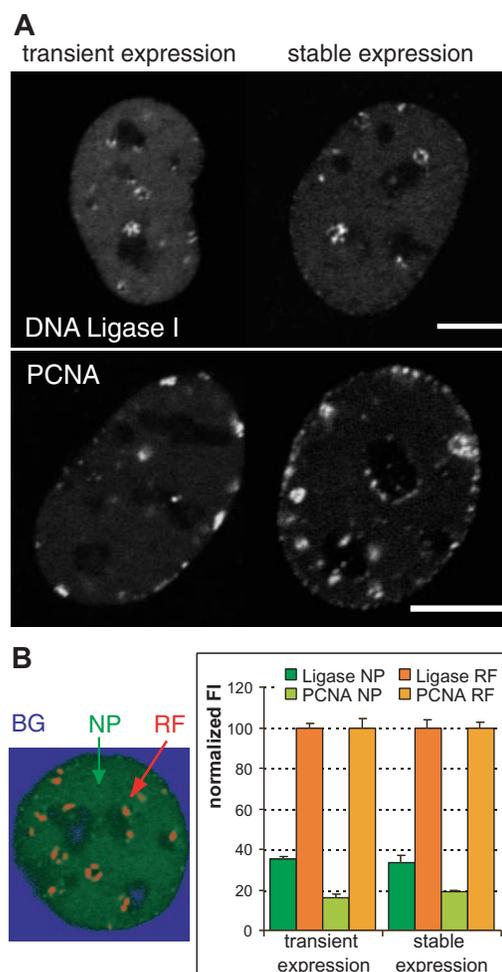
For nuclear distribution analysis of PCNA and Ligase fusions (Figures 1 and 2), mean fluorescence intensities (FIs) of pixels were calculated in each image of a z-stack after thresholding to select only pixels representing nucleoplasmic fluorescence or RF (LSM 510 software). Mean FI of the nucleoplasmic pool (NP) was related to mean FI of RF of each cell.

For FRAP analysis, series of images were corrected for cellular movements and focal drift. Selected areas for quantification of mean FI were kept constant for all time points. Background determined outside the nucleus was subtracted from all images. FI of an area was related to the total nuclear fluorescence (T) (12):

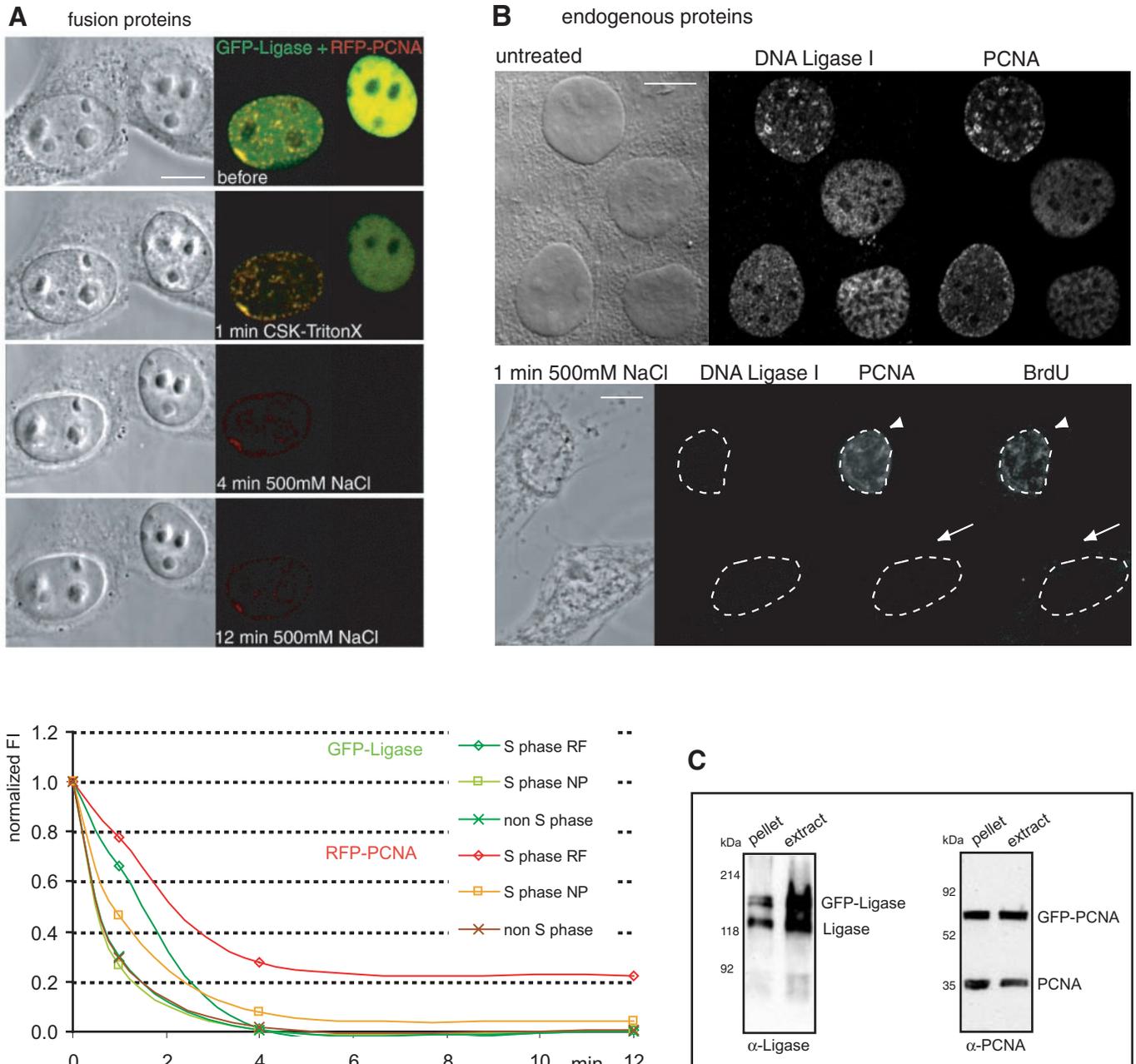
$$\text{relative FI:FI}_r = \frac{(\text{FI}_t \times T_0)}{(\text{FI}_0 \times T_t)}$$

or normalized to the initial prebleach value of the same area:

$$\text{relative FI:FI} = \frac{\text{FI}_t}{\text{FI}_0}$$



**Figure 1.** Ligase has a higher nucleoplasmic fraction than PCNA (A) Confocal images of live C2C12 cells in S-phase transiently or stably expressing GFP-Ligase or GFP-PCNA. (B) Quantification of NP and RF-bound protein (green/red in the false color image) by determining their mean FI ( $n = 5$  cells with 5–7 z slices). Scale bar, 5  $\mu$ m.



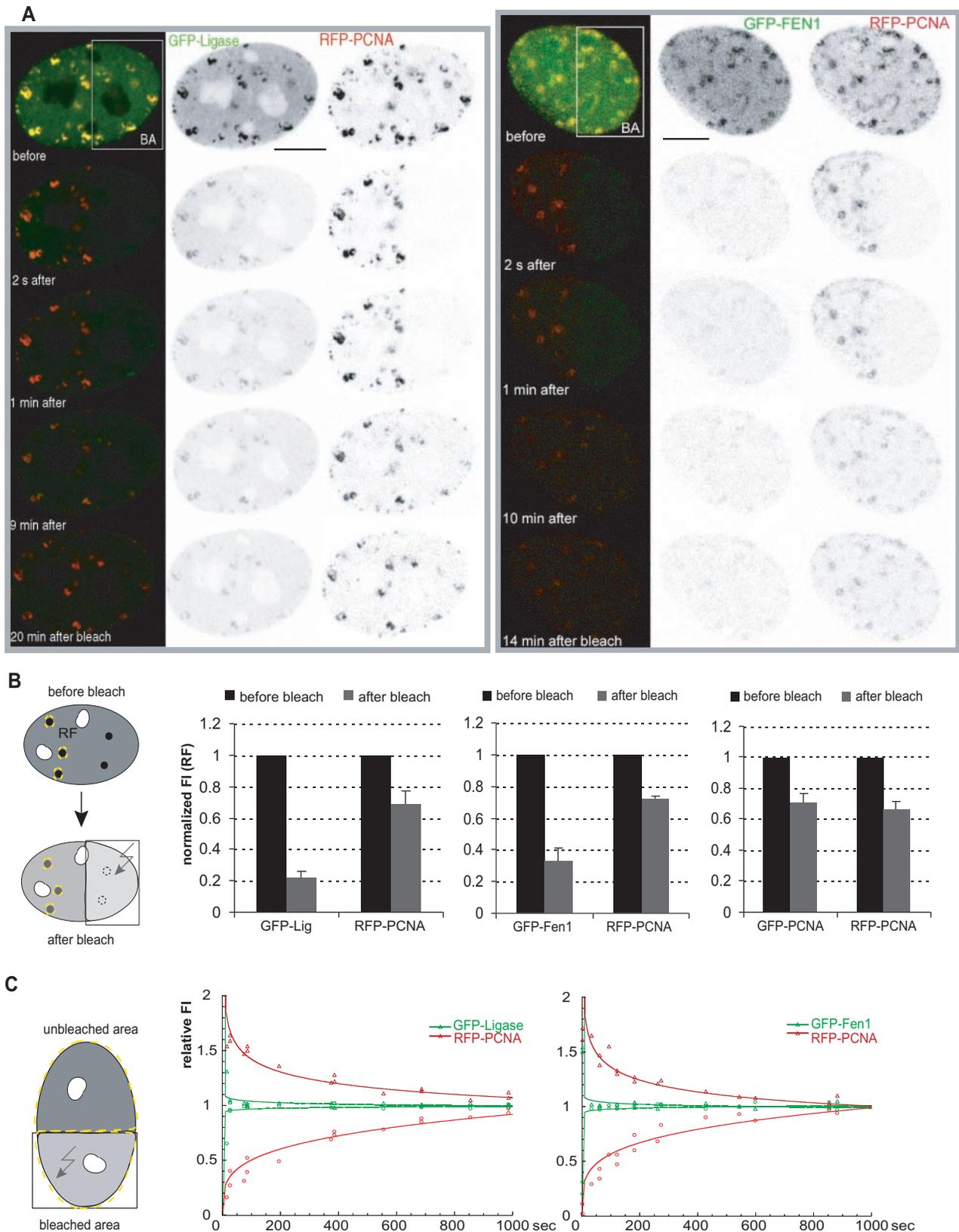
**Figure 2.** Ligase is less strongly associated to replication sites than PCNA (A) Time-lapse *in situ* salt extraction of non-S- and S-phase cells expressing GFP-Ligase and RFP-PCNA showing that GFP-Ligase is largely extracted after short permeabilization with Triton X-100, while RFP-PCNA remains associated at RF for many minutes (for quantification, see diagram). (B) Identical results were obtained for endogenous Ligase and PCNA, which were detected by immunofluorescence. Scale bar, 10  $\mu$ m. (C) C2C12 cells were cotransfected with both GFP-PCNA and GFP-Ligase to ensure the same number of transfected cells and the same percentage of S-phase cells. Cells were permeabilized and extracted, and equal amounts of the cell extract and the insoluble cell pellet were analyzed by SDS-PAGE followed by western blotting. Whereas most of the endogenous Ligase and the respective GFP fusion protein were found in the soluble fraction, under the same conditions, endogenous PCNA and GFP-PCNA were only partially extracted from the pellet and found equally distributed in both fractions. In this direct comparison, PCNA showed a stronger, label-independent association with nuclear structures than Ligase.

with  $t$  = postbleach images, 0 = prebleach image. Curves describing the FDP datasets (Figure 3 and Supplementary Figure S4) were generated in KaleidaGraph 3.5. Images shown in Figure 4B were generated using the same threshold per channel. Diagrams were generated with Microsoft Excel 5.0 or Origin 5.0. Adobe Photoshop 5.5 was used for other image processing steps (thresholding and overlays) and Adobe Illustrator 8.0 was used to assemble and annotate all figures.

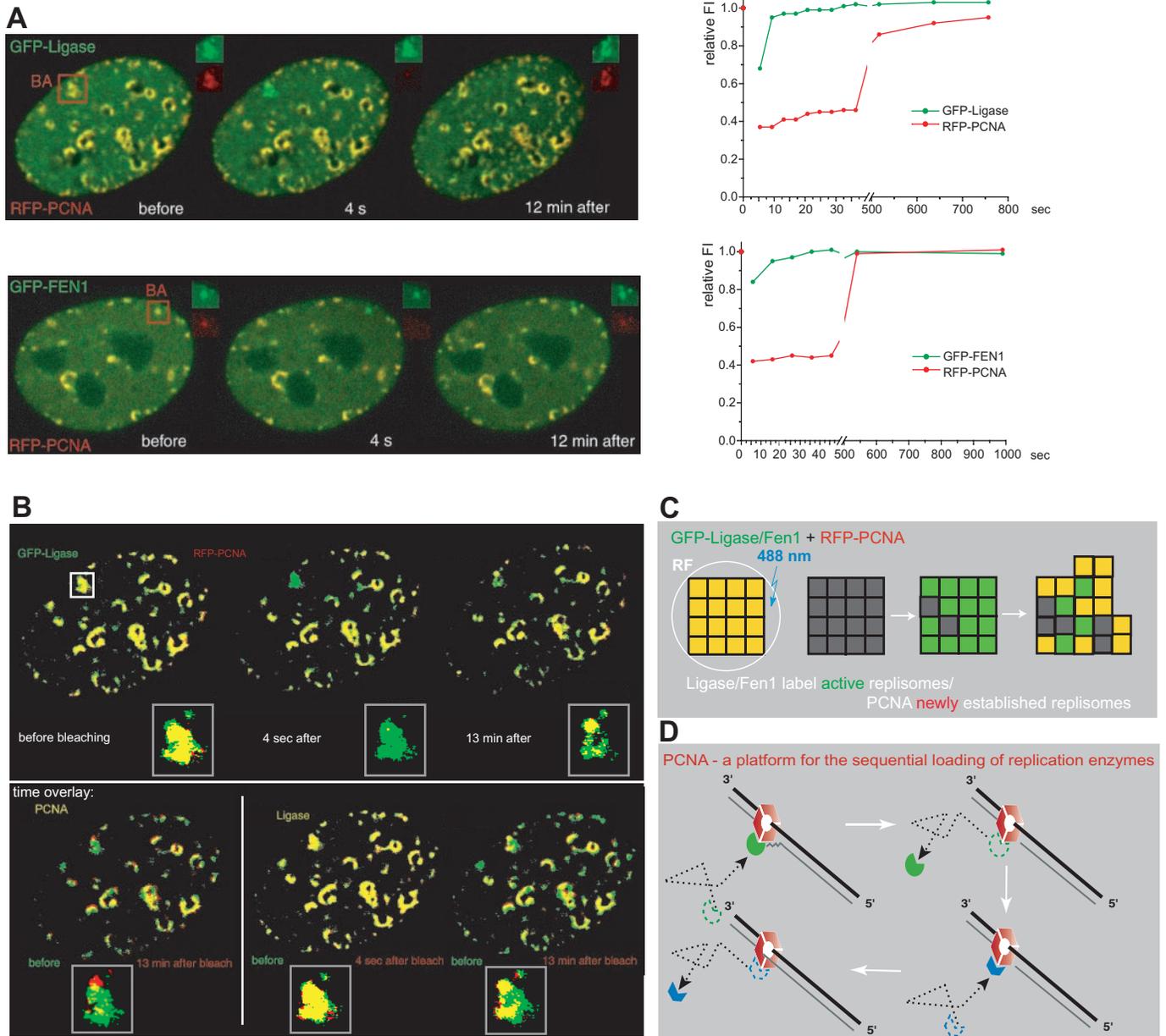
## RESULTS

### Ligase is less tightly associated to RF than PCNA

To study the dynamics of different components of the replication machinery in living mammalian cells, we first compared the distribution of PCNA with the PCNA-binding enzyme Ligase. We used fluorescent fusions of these proteins to GFP or to mRFP1 (for characterization see Supplementary



**Figure 3.** Ligase and Fen1 have a shorter residence time at RF than PCNA (A) Half of the nucleus (BA) was bleached by one single, 12 s bleach pulse in live cells expressing GFP-Ligase or GFP-Fen1 and RFP-PCNA, resulting in an almost complete depletion of Ligase and Fen1 from the unbleached nucleoplasm and RF, in contrast to PCNA. (B) Different residence times of PCNA, Ligase and Fen1 at RF shown by loss of fluorescence from unbleached RF immediately after bleaching (16–20 RF from five cells were analyzed in each case). (C) Fast equilibration of the remaining Ligase and Fen1 between bleached and unbleached half of the nucleus within a few seconds in contrast to a slow equilibration of PCNA over minutes (three cells each). Scale bar, 5  $\mu$ m.



**Figure 4.** Fast reassociation of Ligase and Fen1 at active RF contrasts to slow assembly of PCNA at newly established RF (A) In S-phase cells expressing GFP-Ligase or GFP-Fen1 and RFP-PCNA, one RF was bleached (BA). Ligase and Fen1 showed recovery at the bleached RF within a few seconds, while reappearance of PCNA fluorescence occurred several minutes later (for quantification, see diagrams). (B) Detailed spatial (insets, top panel) and time overlay analyses (insets, lower panel) showing the fast recovery of Ligase (top panel, mid) at previously bleached RF (lower panel, middle). However, the shape of the recovered Ligase focus changes over time (lower panel, right). In contrast, delayed recovery of PCNA occurs only at a small portion of the Ligase-labeled RF (top panel, middle and right) and is mostly adjacent to the bleached RF (lower panel, left). (C) Schematic interpretation of the data with fast exchanging Ligase/Fen1 continuously labeling active and newly assembled replisomes, in contrast with PCNA reassembling mostly at newly established replisomes. (D) Model of PCNA (red) as a stationary loading platform for the sequential loading of replication enzymes (here, represented by Fen1 in green and Ligase in blue) at the lagging strand synthesis. For simplicity, only one replicating lagging strand and one Okazaki fragment are shown. Scale bar, 5  $\mu$ m.

Figure S1), either stably or transiently expressed in mammalian cells. Although Ligase (10,13) and PCNA (14,15) have both been shown to spread uniformly throughout the nucleus in non-S-phase cells and to redistribute to sites of active DNA replication during S-phase, a closer look revealed quantitative differences in their distribution in live (Figure 1A) and fixed (data not shown) S-phase cells. PCNA showed distinct replication structures and a low NP, whereas Ligase displayed a higher NP pool. After a threshold-based

assignment of pixels to NP or RF, mean FI and the ratio between nucleoplasmic and RF-bound fluorescence were determined (Figure 1B). In both stably and transiently transfected cells, the fraction of Ligase in the NP during S-phase was  $\sim$ 2-fold higher as compared with PCNA (Figure 1B). This was also observed in human HeLa cells with different fluorescent fusion proteins and at different expression levels, ruling out a label- or expression-level-related artifact (data not shown).

To investigate whether the differential distribution of PCNA and Ligase reflects different association properties of the two proteins, salt extractions of endogenous PCNA and Ligase and the respective fusion proteins were performed (Figure 2). Live cells coexpressing GFP-Ligase and RFP-PCNA were permeabilized for 1 min directly on the microscope stage followed by extraction with phosphate buffer containing 500 mM NaCl (Figure 2A) for several minutes. In non-S-phase cells, the amount of both GFP-Ligase and RFP-PCNA was already reduced during permeabilization, whereas in S-phase cells primarily nucleoplasmic GFP-Ligase was extracted. The following incubation with 500 mM NaCl rapidly reduced the amount of RF-bound GFP-Ligase further, while RFP-PCNA largely resisted the extraction for >10 min. This indicates that Ligase is far less strongly associated at RF than PCNA. In cells coexpressing GFP-PCNA and RFP-PCNA, both PCNA fusions were synchronously extracted, ruling out a potential effect of the fluorescent label (Supplementary Figure S2). This differential extraction of Ligase and PCNA was also observed for the endogenous non-tagged proteins (Figure 2B). Both proteins were extracted in non-S-phase nuclei (Figure 2B, arrow) while PCNA, in contrast to Ligase, remained associated at RF in S-phase cells (identified by BrdU incorporation; Figure 2B, arrowhead) even after 5 min extraction (data not shown). Similarly, extraction experiments monitored by western blot analysis (Figure 2C) showed that both endogenous Ligase and GFP-Ligase are more readily extracted than PCNA and GFP-PCNA.

#### **Transient association of Okazaki fragment maturation proteins Ligase and Fen1 at RF in contrast to PCNA**

These differences in subnuclear distribution and binding to RF suggest that PCNA and Ligase turn over at different rates. We performed fluorescence photobleaching analysis to test this hypothesis directly. Since individual RF contains clusters of different numbers of replicons and replisomes activated at different times, it was necessary to avoid experimental designs that would involve comparisons of one RF with another; we accordingly employed a novel protocol that allows a direct comparison of the dynamics of different proteins at the same RF in live cells. We extended the widely used photobleaching technique to a dual color approach by bleaching both GFP and mRFP fusion proteins and observing simultaneously their exchange dynamics. This approach guarantees the same biological environment for both proteins and excludes cell-to-cell and focus-to-focus variations. As described previously (16), we used a modified FRAP approach, bleaching half of the nucleus to observe both dissociation and association properties of a protein, with an extended bleach period to emphasize the dissociation properties. We refer to this experimental setup as Fluorescence Depletion by Photobleaching (FDP) and since we measured two proteins simultaneously, we call this dual color FDP (dcFDP).

Using this approach, we compared the mobility of the Okazaki fragment proteins Ligase and Fen1 with that of PCNA. In live S-phase cells coexpressing GFP-Ligase or GFP-Fen1 and RFP-PCNA (for characterization see Supplementary Figure S1) about half of the nucleus was bleached for 12–15 s (Figure 3A). This extended bleach period not only bleached fluorophores in the selected region but also caused

the depletion of most of the Okazaki fragment proteins Ligase and Fen1 from the unbleached half of the nucleus. In contrast, PCNA was mainly bleached in the selected half of the nucleus. In identical experiments with fixed cells (data not shown), 90–95% of the initial FI of the unbleached half of the nucleus remained after bleaching; this shows that unintended bleaching by stray light was minor. Further controls with cells coexpressing GFP-PCNA and RFP-PCNA ruled out that the observed differences on the dynamics were caused by the fluorescent label (Figure 3B and Supplementary Figure S4A). The FI was quantified to evaluate the amount of PCNA, Ligase and Fen1 fluorescence depleted due to dissociation from RF and diffusion into the bleach area (Figure 3B). While 70–75% of the initial PCNA remained at the original RF, only ~20–30% of the initial Ligase or Fen1 did so (see Figure 3B), indicating a higher dissociation rate of the latter proteins. The equilibration of Ligase and Fen1 between bleached and unbleached half of the nucleus was achieved within seconds after the bleach interval while PCNA slowly redistributed during the following 10–20 min (Figure 3C). Similar results were found in early S-phase cells (Supplementary Figure S3), indicating that the faster turnover of Ligase and Fen1 relative to PCNA at RF is independent of the S-phase stage. The virtually complete depletion of Ligase and Fen1 from the entire S-phase nucleus within the bleach period indicates that these molecules reside only for a very short time (<10 s) at replication sites and have a very high nuclear mobility, reaching every point in the nucleus within seconds and thus being bleached during the extended bleach period. Only a small fraction of PCNA seems to be mobile, while the remainder stays bound for a relatively long period at its site of action. Taken together, the rapid depletion of Ligase and Fen1 from RF compared with the much more stable association of PCNA to RF is consistent with the different extractability found in the *in situ* salt extractions and supports the notion that the higher nucleoplasmic fraction of Ligase and Fen1 indeed reflects a higher turnover and a shorter residence time at RF.

#### **Ligase and Fen1 continuously associate at active RF while PCNA remains stably bound**

The higher turnover of Ligase and Fen1 at RF compared with PCNA found in the dcFDP experiments supports our previous suggestion that PCNA remains bound over several rounds of Okazaki fragment synthesis (9). To test this further, we performed traditional FRAP experiments with PCNA and the PCNA-binding proteins but simultaneously measured the recovery of two fluorescently labeled proteins at the same RF (Figure 4, dual color FRAP). After bleaching one RF labeled with GFP-Ligase and RFP-PCNA, Ligase recovered within seconds while recovery of PCNA occurred only after a few minutes (Figure 4A, upper panel). Similar observations were made in experiments comparing the recovery of GFP-Fen1 and RFP-PCNA (Figure 4A, lower panel), at other S-phase stages (data not shown) and in cell lines stably expressing GFP-Ligase or GFP-PCNA (Supplementary Figure S4B).

We have shown before that PCNA reassociation occurs predominantly at newly assembled, adjacent RF (9). Since RF change constantly throughout S-phase, we compared the recovery/reassembly sites of Ligase and PCNA with their prebleach sites by time overlay analysis (Figure 4B, lower

panel). We found that PCNA reassociated at only a small part of the Ligase-labeled RF (upper panel, right). The recovered Ligase focus initially resembled the prebleach focus (lower panel, middle) but changed after several minutes (lower panel, right). In contrast, the recovered PCNA fluorescence was found mostly at adjacent sites (lower panel, left), and the remaining minimal overlap with previous sites is likely due to the limited optical resolution. This analysis indicates that the Ligase continuously exchanges at the active RF, while bleached PCNA remains at the bleached focus, and reassembly occurs at newly initiated RF (Figure 4C).

## DISCUSSION

The coordination of leading and lagging strand synthesis is a fundamental but poorly understood task of the DNA replication machinery. Since Okazaki fragments in mammals are ~180–200 bases long, an initiation event must occur  $2\text{--}3 \times 10^7$  times on the lagging strand versus  $\sim 4 \times 10^4$  times (approximate number of origins) on the leading strand (17). The need for efficient and accurate processing and ligation of all Okazaki fragments seems best served by a stable association of the respective, processing enzymes (Ligase, Fen1, etc.) with the replication machinery. These enzymes are proposed to be loaded to replication sites by their association with the PCNA ring (8,18). Several possible models for PCNA dynamics have been discussed previously (9): the PCNA ring might stay stably bound during lagging strand synthesis together with a dimeric polymerase, it might be recycled within the focus or a new ring might be loaded for each Okazaki fragment. It is interesting to note that the prokaryotic counterpart of the PCNA clamp is left behind and a new clamp is loaded at the next Okazaki fragment (19). One can imagine different scenarios for the interaction of PCNA with Ligase or Fen1 at the lagging strand. In one, Ligase or Fen1 and PCNA could be loaded onto replicating DNA as a stable complex with a 1:1 stoichiometry (20), then stay together and thus remain associated with replication sites for a similar time period. In this case, one would predict similar dynamic behavior of PCNA and PCNA-binding proteins in photobleaching experiments. The same kinetics would be expected if PCNA and Ligase/Fen1 are independently recruited but stayed for a similar time, i.e. for the synthesis of one Okazaki fragment at the replication fork. The synthesis of one Okazaki fragment at an average fork progression rate of 1.7 kb per min (21) takes ~6–7 s. Within this time, at least 50% fluorescence recovery should be measured for PCNA assuming the other 50% is stably bound at the leading strand. If no Ligase or Fen1 is at the leading strand, 100% recovery would be expected. The data presented demonstrate a very fast and complete exchange of Ligase/Fen1 within a few seconds in the extraction experiments, which is consistent with them being loaded at every Okazaki fragment. In stark contrast, PCNA is stably bound at replication sites and its turnover is very limited occurring mostly in the order of minutes within the same cells and foci as fast exchange of Ligase/Fen1 takes place. Although part of the same replication machinery and localized at the same nuclear sites, PCNA, Fen1 and Ligase show very different dynamics. This argues strongly for an alternative model, in which Ligase or Fen1 and PCNA are independently loaded

at the replication fork and stay for different times (Figure 4D). An extension of the latter is that PCNA may stay bound throughout the synthesis of several Okazaki fragments, while Ligase and Fen1 exchange after each fragment. Here, one would expect a different residence time for PCNA and Okazaki fragment proteins in the replication complex, which would be visualized as a difference in kinetics in photobleaching experiments. A consequence of this model is that PCNA does not 'bring' Ligase or Fen1 to sites of replication. Instead, PCNA appears to act as a stationary loading platform (Figure 4D) that is reused over multiple Okazaki fragments with PCNA-binding proteins associating transiently and subsequently dissociating rather than being part of one stable, multifunctional, processive replication machinery.

The combination of *in situ* extractions and dual color photobleaching studies described here will also be useful to study the dynamics of other complex biological processes.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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*Conflict of interest statement.* None declared.

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