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DNA damage induced Pol η recruitment takes place independently of the cell cycle phase

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When DNA is damaged in cells progressing through S phase, replication blockage can be avoided by TLS (Translesion DNA synthesis). This is an auxiliary replication mechanism that relies on the function of specialized polymerases that accomplish DNA damage bypass. Intriguingly, recent evidence has linked TLS polymerases to processes that can also take place outside S phase such as nucleotide excision repair (NER). Here we show that Pol η is recruited to UV-induced DNA lesions in cells outside S phase including cells permanently arrested in G_1. This observation was confirmed by different strategies including global UV irradiation, local UV irradiation and local multi-photon laser irradiation of single nuclei in living cells. The potential connection between Pol η recruitment to DNA lesions outside S phase and NER was further evaluated. Interestingly, the recruitment of Pol η to damage sites outside S phase did not depend on active NER, as UV-induced focus formation occurred normally in XPA, XPG and XPF deficient fibroblasts. Our data reveals that the re-localization of the TLS polymerase Pol η to photo-lesions might be temporally and mechanistically uncoupled from replicative DNA synthesis and from DNA damage processing.

Introduction

Genotoxic insults such as exposure to UV-light provoke the accumulation of different types of DNA lesions[1] and a wide-range cellular response.[2] To maintain cell viability, replication forks that encounter damaged DNA must efficiently bypass these lesions. DNA synthesis across damaged DNA is achieved by specialized DNA polymerases that incorporate nucleotides opposite to damaged bases in a process known as TLS (Translesion DNA Synthesis).[3] TLS must be exquisitely controlled since TLS polymerases are prone to induce mutagenesis due to their permissive active site and their lack of proofreading activity.[4] The existence of regulatory factors that promote an optimal balance between TLS-associated cell survival and TLS-associated mutagenesis is under current investigation.[5]

Most TLS polymerases are members of the Y-family of DNA polymerases which are unique and specifically adapted to achieve lesion bypass. Y-polymerases family members include Pol η, Pol κ, Pol ι and REV1. A B-family member, Pol ζ, is also involved in TLS.[3,4] Pol η can bypass the most abundant UV-induced DNA lesion, CPD (cyclobutane pyrimidine dimer), very accurately.[3,4] In humans, defects in Pol η expression are associated with the cancer prone syndrome XPV (xeroderma pigmentosum variant), characterized by hypersensitivity to UV light.[3,4,6] The bypass of lesions that are bulkier than CPDs may require the sequential recruitment of two TLS polymerases.[7-9] In fact, Pol ι has been shown to bypass 6-4PP (pyrimidine 6-4 pyrimidone photoproducts), a frequent UV-induced DNA lesions, in concert with extender polymerases.[10-12] In line with this, Pol κ and Pol ζ act as extender polymerases after many insults.[9,13,14] Intriguingly, the catalytic activity of REV1 might not be relevant for efficient TLS and, instead, its unique ability to recruit other TLS polymerases to damaged DNA was shown to be central for lesion bypass.[8,15]

The recruitment of Pol η to damaged DNA has been elegantly linked to PCNA ubiquitination.[16-20] Compelling evidence indicates that both events are exclusively linked to S phase.[21,22] This data support a polymerases switch model that restricts TLS function to ongoing DNA replication.[15] According to such model, the blockage of replicative polymerases triggers PCNA ubiquitination at stalled sites. This promotes the recruitment of TLS polymerases that bypass the lesion and allow the continuation of DNA replication.

More recent reports have shown that PCNA ubiquitination is efficiently achieved outside S phase both in mammalian and yeast systems.[23,24] Moreover, REV1 and Pol η are recruited to BrdU negative cells after UV irradiation[25] thus suggesting unidentified functions of TLS polymerases outside S phase. The activity of TLS polymerases in non replicative cells could result from TLS events that are temporally delayed with respect to the progressing replication forks. In line with this, electron microscopy experiments have revealed unfilled DNA gaps left behind the replication forks.[26] This data validates a gap filling model that envisions TLS polymerases sealing gaps containing DNA lesions that result from replication fork re-priming downstream the blocking lesion.[27] The

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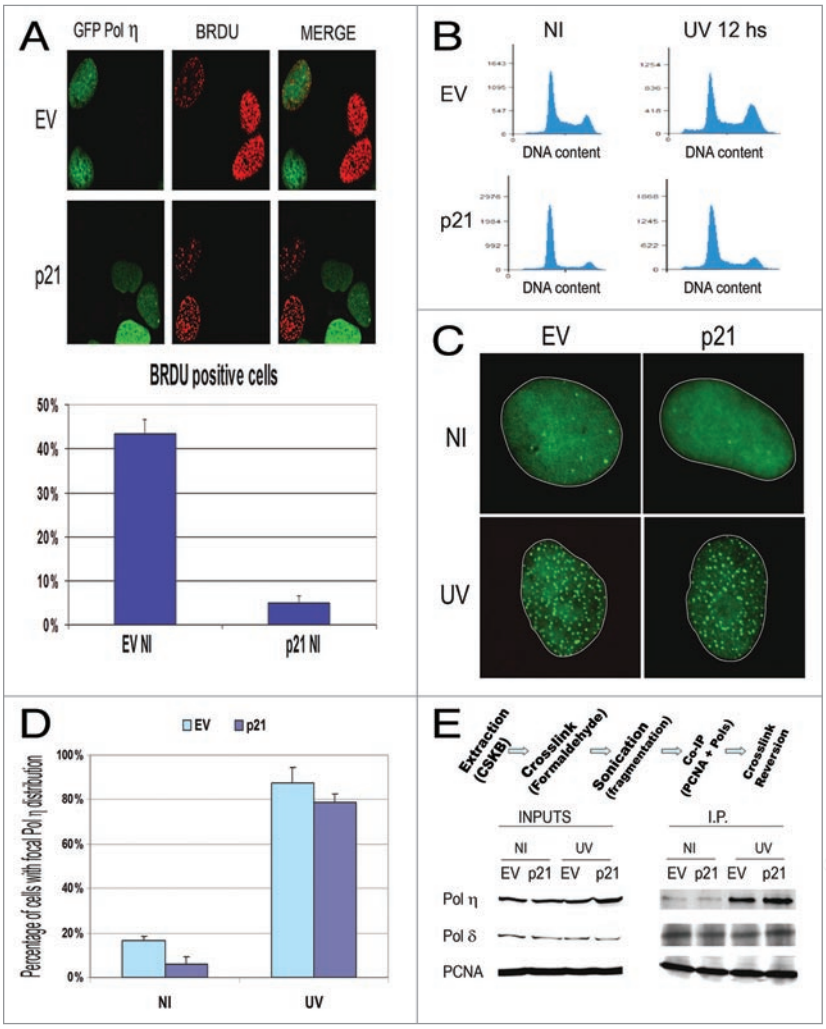

Figure 1. Pol η efficiently assembled into UV induced foci in G_1 arrested cell populations. (A) U2OS cells were transfected with GFP-pol η and p21 or control plasmid (empty vector, EV) and processed 24 hours later. BrdU (10 μM) was added 30 min before fixation and was revealed with specific antibodies to BrdU. The percentage of GFP-pol η cells that incorporated BrdU was determined in two independent experiments (lower panel). Transfection efficiency in U20S cell is above 60%. Co-transfection efficiency (p21 + GFP-Pol η) was monitored by parallel staining with anti p21 antibodies (always above 95%). At least 200 transfected nuclei/sample were counted. (B) U2OS cells were transfected with GFP and p21 or control plasmid (EV) and 24 hours later were UV irradiated (20 J/m^2) as described in the Methods section. The cell cycle profile of the transfected population was determined before irradiation (NI-Not Irradiated) and 12 hours after UV irradiation (UV 12 hrs). (C) U2OS cells were transfected as in A and 24 hours later UV irradiated (20 J/m^2). Cells were fixed 6 hours after UV irradiation and the sub-nuclear distribution of pol η were determined by confocal microscopy. (D) Quantification of three independent experiments as performed in (C). At least 200 transfected nuclei/sample were counted. (E) U2OS cells were transfected as in (A) and UV irradiated (20 J/m^2) 24 hours after transfection. 4 hours later the chromatin bound fraction was crosslinked, sonicated and PCNA was immunoprecipitated (IP) as described in the methods section. PCNA, GFP-pol η and pol δ were detected utilizing specific antibodies. Right: PCNA immunoprecipitations (IP); Left: an aliquot of the PCNA bound fraction used for the PCNA IPs (Inputs).

gap filling pathway might not be restricted to S-phase and also occurs in G_1 and G_2/M phases.[4]

Other functions of TLS polymerases that might take place outside S phase have also been reported recently.[28] Pol κ has been implicated in nucleotide excision repair, a DNA process that takes place in all the phases of the cell cycle.[29] Pol ζ, on the other hand, is associated with DNA interstrand crosslink repair during G_1.[30]

By studying the sub-nuclear localization of Pol η into permanently arrested cells, we show here that Pol η is efficiently recruited to damaged-DNA outside S phase. This was confirmed using local UV irradiation and in vivo multi-photon laser (MPL) induced DNA damage. Interestingly, no defects in Pol η recruitment to damaged-DNA were observed in different NER deficient cell lines. Strikingly, Pol η recruitment outside S phase was not always accompanied by PCNA accumulation into damaged DNA sites. Nevertheless, Pol η accumulation was totally dependent on the integrity of its PCNA-interacting domain indicating that at least the initial recruitment of Pol η outside S phase requires PCNA, but that a subsequent accumulation that consolidates visible Pol η foci might occur in a PCNA-independent manner. Our data reveals that focal organization of the TLS polymerase Pol η at sites of DNA damage might result from signals that are independent of DNA replication and damage recognition and/or processing.

Results

UV irradiation of permanently arrested cells triggers efficient Pol η focal organization. Using different cellular models, two independent groups have reported that the percentage of cells with Pol η organization into focal structures is higher than 80%, an efficiency that is significantly elevated compared to the percentage of cells in S phase in non-synchronized, cycling populations.[31,32] Moreover, two previous reports provided evidence indicating that Pol η recruitment to damaged DNA could indeed take place outside S phase.[25,33] In this work we evaluated Pol η nuclear distribution in U2OS cells permanently arrested in G_1 phase. We transiently expressed the cyclin kinase inhibitor p21,[23,33] which provokes accumulation of cells in G_1 (**Fig. 1B**) and efficiently blocks DNA replication (**Fig. 1A**). UV irradiation did not significantly alter the cell cycle profile of G_1 arrested cells within the time window of the experiment (**Fig. 1B**). Under these experimental conditions we observed a very efficient reorganization of Pol η into nuclear foci in cells arrested in G_1 (**Fig. 1C**). The percentage of cells with detectable Pol η foci in G_1 enriched populations was similar to non arrested control samples (**Fig. 1D**). Moreover, in asynchronous populations (EV), the percentage of cells with detectable Pol η foci formation (approximately 90%) was much higher than the percentage of BrdU positive cells (approximately 40%—compare **Fig. 1A and D**) which suggested that also in cycling cells that are transiting G_1 or G_2/M

phases Pol η reorganizes into foci after UV irradiation. To test if PCNA and Pol η interaction could be favoured by UV irradiation also in G_1 we immunoprecipitated PCNA from the chromatin bound fraction following the experimental design shown in **Figure 1E**. As expected,[33,34] the interaction between PCNA and Pol η increased after UV irradiation. G_1 accumulation did not correlate with any reduction in the level of UV-induced PCNA/Pol η interaction (**Fig. 1E**). Taken together, these data show that Pol η increases its interaction with PCNA and organizes into foci after UV exposure of non cycling cells.

Pol η is recruited to CPDs in all phases of the cell cycle. Our initial observations indicated that Pol η was able to efficiently accumulate into damaged sites outside S phase. To further test this hypothesis we performed detailed time course experiments combining BrdU incorporation with the analysis of Pol η distribution. This analysis provided three crucial observations. First, in the absence of damage induction most of the cells with focal Pol η scored positive for BrdU incorporation, indicating that they were transiting through S-phase (**Fig. 2A**). Second, 1 hour after of UV irradiation, when the amount of cells with Pol η focal pattern doubled, they were also mainly BrdU positive, indicating that in S-phase cells Pol η focal organization occurred with faster kinetics (**Fig. 2A**). Third, at later time points after UV irradiation (2 to 4 hours), the vast majority of the cells showed Pol η focal formation. This suggested that, although with a delayed kinetics, cells outside S-Phase accumulated Pol η as efficiently as S-phase cells (see **Fig. 2A** and **Suppl. Fig. 1** for images).

Induction of local UV damage is a useful tool to study the accumulation of DNA repair factors. This is accomplished by shielding cell monolayer's with polycarbonate filters containing small micro-pores[35,36] combined with antibodies specific for CPDs. This technique provides two substantial advantages when compared to the use of global UV irradiation. First, it facilitates discriminating damaged induced Pol η foci from previously assembled ones. Second, it allows unequivocal detection of Pol η recruitment to damaged DNA in a shorter time window (1 hour) when compared with global irradiation (**Fig. 2B**). When we performed experiments using local UV damage in asynchronous cells populations we observed that the percentage of cells that showed Pol η recruitment to damaged-DNA sites ($CPDs^+$) was higher than 80% in only one hour (**Fig. 2C**). This reinforces the notion that Pol η foci form outside S phase since it represents a proportion of the population that is much higher than the cells transiting S-phase (45% or less—**Fig. 1A**).

Taken together, the data presented in this section indicates that the S-phase independent recruitment of Pol η can take place efficiently in asynchronous populations and that this accumulation is not a consequence of p21 artificial expression.

Pol η recruitment to damaged DNA sites outside S-phase is detected in living cells. Induction of DNA damage with a MPL (multi-photon laser) combined with time lapse imaging allows the study of local recruitment of proteins to damaged DNA sites in vivo. Since MPL-induced DNA damage mimics UV irradiation,[37] we checked whether Pol η was recruited to that type of local DNA lesions (**Fig. 3B**). In fact, it was interesting to observe that a low laser exposure (see Method section) promoted a very

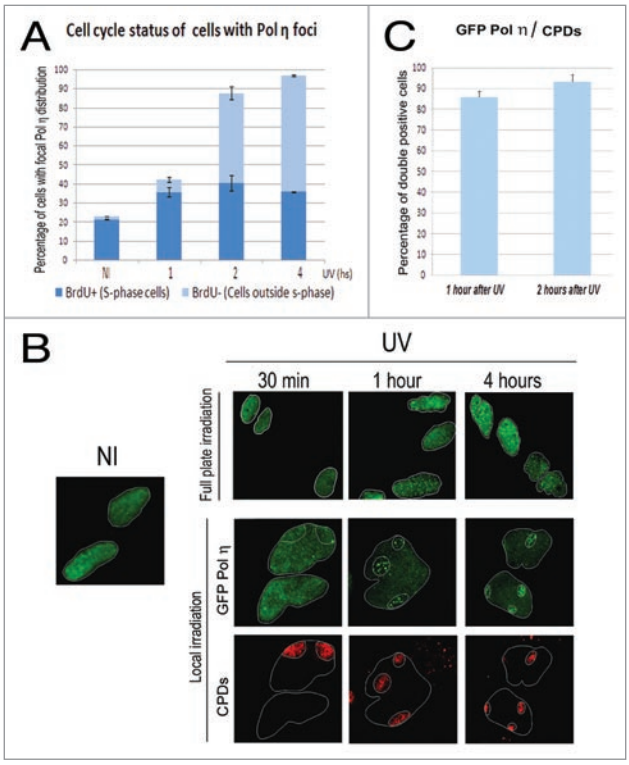

Figure 2. Pol η is recruited to CPDs in all phases of the cell cycle. (A) U2OS cells were transfected with GFP-pol η and were irradiated 24 hours later. BrdU (25 μM) was added 30 minutes before fixation and was revealed with specific antibodies to BrdU. The percentage of GFP-pol η cells that assemble in foci is shown on the y axis. The composition of each bar on the x axis is divided between the cells with positive and negative BrdU staining during the time course of the experiment. At least 200 transfected nuclei per sample were counted. (B) U20S cells were transfected with GFP-pol η. 24 hours later global irradiation of monolayers or local irradiation though polycarbonate filters were performed. CPD specific antibodies were used to detect the irradiated spots when local UV irradiation was utilized. A representative field for each time point is shown in each case. (C) U2OS cells were transfected with GFP-pol η, UV irradiated using polycarbonate filters and fixed 24 hours later. CPDs were revealed with specific antibodies. The percentage of recruitment of GFP-pol η to CPDs was calculated at the indicated time points. At least 200 transfected nuclei per sample were counted.

rapid accumulation of Pol η to damaged DNA (**Fig. 3A**—a more detailed time course is shown in **Suppl. Fig. 2A**). From the experiments described in **Figure 2** and **Supplementary Figure 1** it can be assumed that cells with focal Pol η without damage induction are likely to be transiting S-phase (although cells with diffuse Pol η could be both inside or outside S-phase). Thus, we decided to test whether MPL induced DNA damage promoted Pol η re-localization into the irradiated area in cells with initial diffuse or focal Pol η patterns. In both cases, efficient re-localization of Pol η to discrete focal structures within the irradiated spots was observed (**Suppl. Fig. 2B**).

To confirm the recruitment of Pol η to G_1/G_2 cells it was still imperative to monitor the cell cycle status of the irradiated cells before and throughout the whole time window of the experiment. To do so, we combined MPL damage of living cells with the expression of a fluorescent PCNA chimera which acts as a

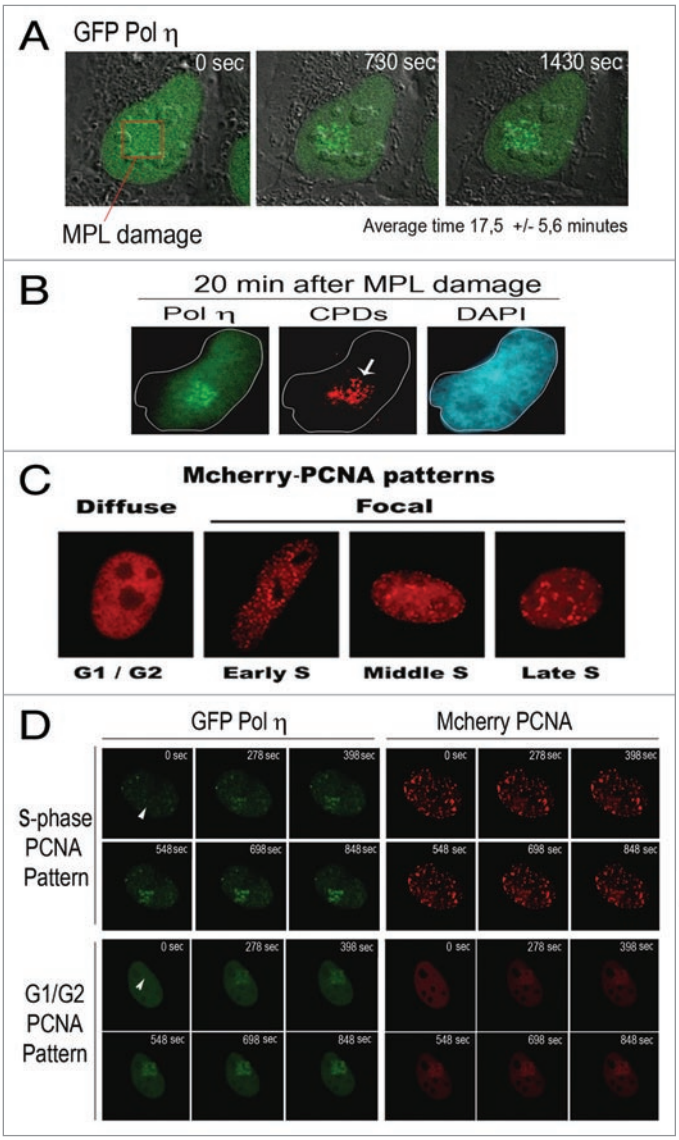

Figure 3. Pol η recruitment to DNA lesions in living cells take place in all phases of the cell cycle. (A) U2OS cells were transfected with GFP-pol η. 24 hours after transfection. A sub-nuclear fraction of cells expressing GFP-pol η were subjected to local MPL irradiation as described in Methods. Images were obtained in 30 second intervals. The first image corresponds to the sample before irradiation. The next two images correspond to the time of detectable accumulation of Pol η within the irradiated area. The average time for detection of Pol η focal accumulation is shown. (B) U2OS cells transfected with GFP-pol η were irradiation with the MPL and fixed after 20 min or 4 hours. CPDs were revealed with specific antibodies. DAPI staining was used to visualize the nuclei. (C) U2OS cells were transfected with mCherry-PCNA. Representative nuclei with the expected patterns of PCNA distribution for each cell cycle stage are shown. (D) U2OS cells were cotransfected with mCherry-PCNA and GFP-pol η. 24 hours after transfection cells were irradiation with a MPL as described in (A). Images were obtained in 30 second intervals. The MPL irradiated area was detected by both PCNA and pol η accumulation. This analysis was performed in a total of 30 cells that were collected during 3 independent experiments.

reliable cell cycle marker. For this task we modified a previously reported mCherry-PCNA chimera.[38,39] In order to guarantee correct localization and function of mCherry-PCNA on transient transfection experiments, we cloned a NLS of SV40 to the N-terminus of the fluorescent protein and introduced a flexible linker peptide separating the fluorescent protein sequence from the PCNA sequence.[40] The distribution patterns of the chimeric protein obtained (**Fig. 3C**) coincided with the previously reported patterns described for GFP-PCNA.[41] A diffused nuclear distribution was observed in G_1/G_2 phases of the cell cycle, while characteristic focal patterns were observed in early, middle and late S phase (**Fig. 3C**).

Using mCherry-PCNA as a cell cycle marker we were able to study the effect of the cell cycle on the dynamics of GFP-pol η recruitment to the damaged-DNA area. A time lapse sequence is shown in **Figure 3D**. The mCherry-PCNA focal pattern of the cell in the upper panel revealed that it was transiting S-phase at the time of MPL irradiation while the diffuse mCherry-PCNA distribution of the cell in the lower panel demonstrated that it was in G_1 or G_2 phase at the time of irradiation. The initial image shows the distribution of both proteins before damage and the white arrows indicate the site of laser damage. Panels 2 to 5 show the timing of Pol η recruitment to the locally irradiated area. The final shot shows the distribution of both proteins at the end of the experiment. Pol η recruitment to MPL damaged DNA was similar in both cases indicating that it was independent of the cell cycle. A more detailed time course combined with phase contrast is shown in **Supplementary Figure 3**. Pol η was efficiently recruited to damaged-DNA in all cells without exception including cells co-expressing p21, mCherry-PCNA and GFP Pol η (not shown) which reinforces the fact that focal Pol η organization was observed in G_1/G_2 cells. Taken together these data and the experiments presented in the previous sections demonstrate that Pol η can be recruited to DNA lesions independently of the cell cycle status.

Functional NER is not required for Pol η recruitment to UV-induced lesions. The participation of Pol κ and ζ in NER after UV irradiation was recently reported.[29,30] To test whether Pol η is recruited to UV lesions before or after processing of the DNA by NER factors, we tested the efficiency of Pol η foci formation in cells defective in NER due to deficiencies in XPA, XPG or XPF expression. During the time window of the experiment, cell cycle distribution was not strongly affected by UV irradiation in all cell lines used, although a detectable increase in the $SubG_1$ fraction was observed in some XP cell lines, presumably due to its defects on DNA repair (**Fig. 4A**). The level of basal Pol η organization into foci was also different between the cells lines (**Fig. 4C**). However, UV-induced organization of Pol η into foci was observed in all cell lines without exception (**Fig. 4B**). We confirmed our observation using serum starved G_1 synchronized cells. To do so we employed hamster CHO9 cells and derivatives defective in XPG expression which efficiently accumulate in G_1 after serum starvation (**Fig. 4D**). Although the percentage of cells with detectable Pol η focal organization in CHO9 cells was lower when compared to human cells, no defects in Pol η foci formation were observed when XPG deficient derivatives were compared to control cells (**Fig. 4E**). Taken together, these data reveals that Pol η recruitment to damaged DNA does not require active NER.

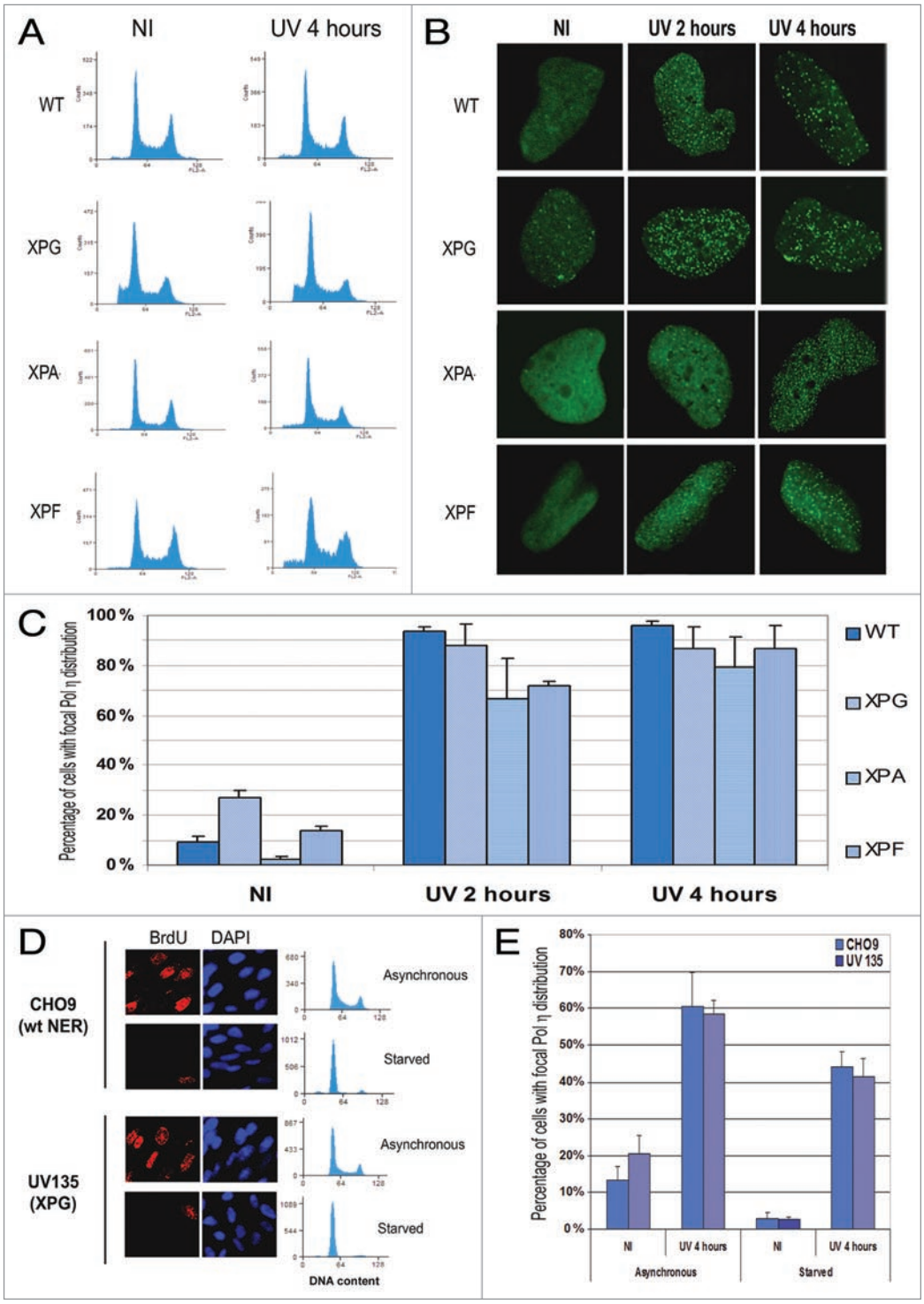

Figure 4. The activation of NER is not required for Pol η recruitment to damaged DNA outside S phase. (A) The indicated cell lines were transfected with GFP-pol η, and 24 hours later UV irradiated (20 J/m^2) when indicated. After fixation, cell cycle profiles were obtained from untreated or UV-treated populations. (B) U2OS cells were transfected with GFP-pol η, UV irradiated when indicated (20 J/m^2) and fixed. The sub-nuclear distribution of Pol η was determined utilizing confocal microscopy. (C) The percentage of cells with detectable Pol η organization into foci was determined in two independent experiments. At least 200 transfected nuclei/sample were counted. (D) CHO wt (CHO9) cells and XPG defective derivatives (UV 135) were synchronized in G_1 by serum deprivation. BrdU was added 30 min before fixation. BrdU incorporation was revealed using specific antibodies. Parallel samples were also fixed and subjected to flow cytometry. (E) Quantification of the percentage of cells with detectable Pol η foci. At least 200 nuclei/sample were analyzed.

The data on **Figures 4A and C** show that XPG deficient cells exhibit no detectable defect in the recruitment of Pol η to damaged DNA. This was surprising since the recruitment of PCNA and Pol δ to DNA lesions requires the correct function

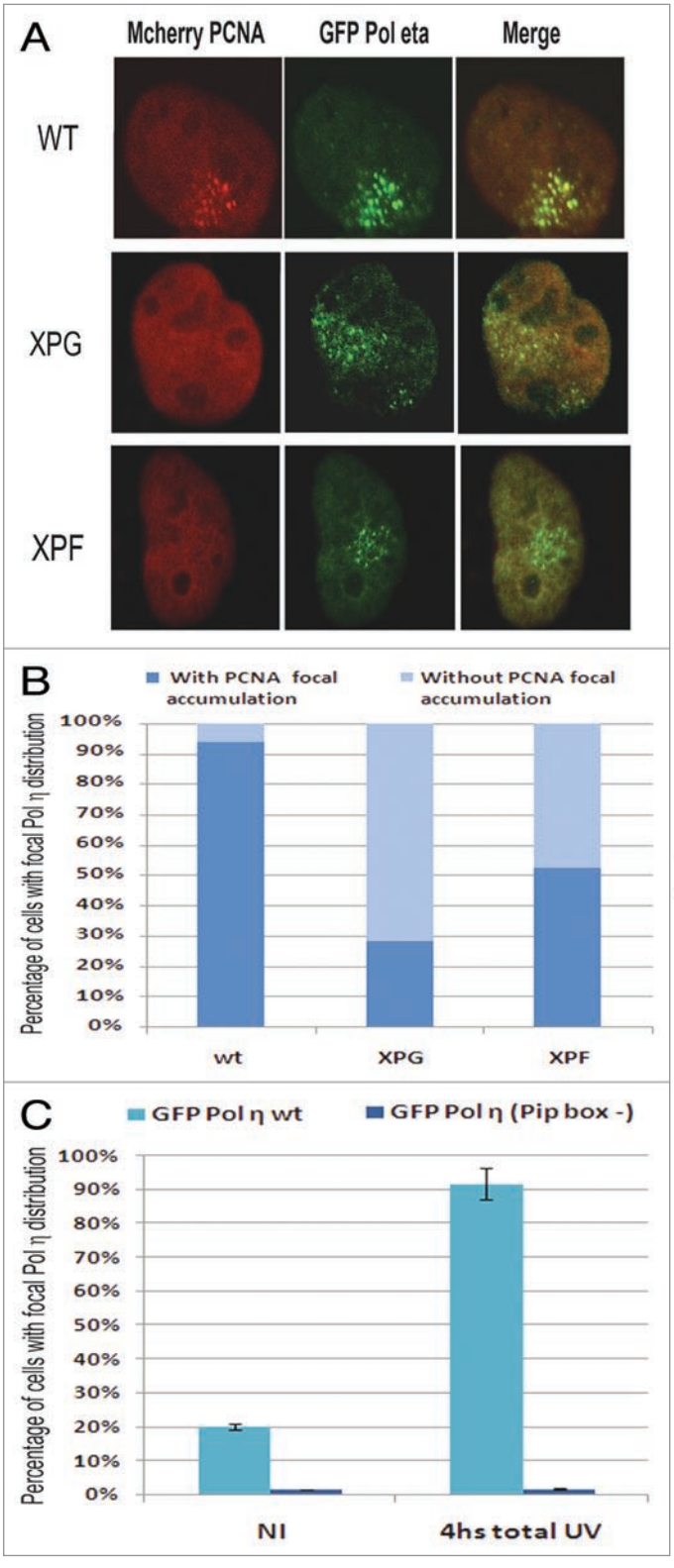

Figure 5. Pol η recruitment to local damage is independent of PCNA in NER deficient cells outside S phase. (A) XPG defective, XPF defective and control samples were transfected with mCherry-PCNA and Pol η. 24 hours later cells were UV irradiated (80 J/m^2) utilizing polycarbonate filters. 2 hours after UV irradiation cells were fixed. The localization of mCherry PCNA and GFP-pol η to lesions was analyzed using confocal microscopy. (B) Cells with diffused mCherry-PCNA distribution (G_1/G_2) were scored for Pol η focal recruitment to local damaged areas. In the population of cells positive for Pol η recruitment the accumulation of focal mCherry-PCNA into the damaged area was also assessed. The composition of each bar on the x-axis is divided between the cells that were positive and negative for focal mCherry-PCNA accumulation. (C) U2OS cells were transfected with GFP-Pol η and GFP-Pol η (Pip box mutant). 24 hours later cells were UV irradiated (40 J/m^2). 4 hours after UV irradiation were fixed and the sub-nuclear distribution of Pol η was determined by confocal microscopy.

of NER proteins.[41-43] In agreement, local UV irradiation experiments demonstrated that the recruitment of mCherry-PCNA to the damaged area in XPG and XPF defective cells outside S phase was impaired (**Fig. 5**). Pol η on the other hand, was recruited to the local lesions even in cells with impaired PCNA accumulation (**Fig. 5**). From this observation it could be hypothesized that outside S-phase Pol η is recruited to damage sites independently of its interaction with PCNA. To test this we construct a Pol η mutant unable to interact with PCNA (Pol η Pip box-) as previously described.[32] Remarkably, this mutant was totally incapable to assemble foci both before and after UV irradiation (**Fig. 5C** and ref. 44), indicating that even in the cases where no efficient PCNA accumulation is achieved into local damaged areas, Pol η still requires its ability to interact with PCNA in order to be recruited. It is uncertain how Pol η accumulates more efficiently than PCNA in the XPG and XPF deficient cell lines but it could be speculated that while the initial recruitment of Pol η might depend on PCNA, the subsequent accumulation might be regulated by a PCNA-independent unidentified pathway.

Discussion

In the past it has been broadly accepted that the focal organization of proteins involved in the DNA damage processing always correlates with functional activity of these proteins.[45] Here we show that the localization of the TLS polymerases Pol η in focal patterns is temporally and mechanistically dissociated from the DNA synthesis associated with TLS and NER. The implications of our findings are discussed bellow.

Pol η foci formation outside S phase and TLS. The gap filing model suggests that Pol η can participate in TLS outside S phase (see Introduction). Therefore, the efficient UV-dependent focal organization of Pol η in cycling cells outside S-phase (reviewed in ref. 25) could reveal Pol η participation in gap filling TLS. On the other hand, our observations obtained using G_1 permanently arrested cells demonstrate that at least in those situations Pol η assembly into focal structures is not triggered by TLS signals, since there is no replicative gap to be filled. In fact, Pol η organization into foci was observed both in U2OS cells transfected with p21 (**Fig. 1**) and starved CHO cells (**Fig. 4**). These data corroborate that UV induced Pol η re-localization could take place in absence of replication forks and replication-associated single stranded DNA gaps.

Pol η foci formation outside S phase and NER. When the association of Pol η focus formation with NER was tested no defects were observed in cells deficient for XPA, XPG and XPF. While our data demonstrates that Pol η can be recruited to damaged DNA

independently of NER activation it does not rule out a possible role of Pol η in NER. Indeed it is tempting to speculate a potential facilitation of Pol η participation in NER as a consequence of the proximity to DNA lesions within focal structures. Since the misincorporation frequency of Pol η on undamaged DNA templates is low,[46] Pol η proximity to DNA lesions might turn it into a suitable DNA polymerase to synthesize short DNA tracks. In fact, Pol η participation in NER might be central for the processing of closely spaced UV lesions on opposite DNA strands. A similar role of Pol ζ has been reported for NER-dependent repair of interstrand DNA crosslinks.[30] In addition, Pol κ participation in NER is suggested to be particularly favoured in non replicative cells[29] which suggests a potential advantage for TLS polymerases aggregation around DNA lesions outside S phase. Taking together, the previous findings indicate that the molecular signals that promote Pol η recruitment to DNA outside S-phase might be independent of DNA repair associated signals. In such scenario, the initial Pol η recruitment to lesions might increase the availability of Pol η in the surroundings of damaged areas which in turn may facilitate the selection of Pol η to resolve or bypass a given DNA lesion.

Revisiting the link between proteins accumulation and function. While no artificial effects are associated to Pol η ectopic expression[44,47] the interpretation of data involving fluorescent-PCNA expression should be cautious. In fact, it has recently been reported that the organization of UV-induced foci containing a Pol η mutant defective in ubiquitin binding[48] is mainly dependent on the increased levels of fluorescent PCNA co-expression.[49] While many of our experiments were performed in the context of mCherry-PCNA co-expression, similar experiments were always performed in the absence of ectopic PCNA obtaining identical results. Moreover, it is remarkable that Pol η recruitment to damaged DNA is also observed in the absence of detectable PCNA accumulation to lesions in NER deficient fibroblasts. While more experiments are required to identify the signals that promote Pol η foci formation outside S phase it is interesting to point out that REV1 has both the capacity to associate with Pol η[50-53] and to interact with DNA associated proteins thought its BRCT domain.[54,55] Moreover, REV1 is recruited to damaged DNA outside S phase and this event is independent of PCNA.[56] Therefore, the interaction of Pol η with other TLS polymerases might promote their recruitment and/or their retention in the surroundings of DNA lesions.

Numerous proteins involved in the DNA damage response accumulate into foci at sites of DNA lesions. It is tempting to assume that this localization correlates with functional activity of these proteins. For example, the localization of DNA polymerases in focal patterns at sites of DNA damage can be taken as evidence, both spatial and temporally, of their participation in DNA synthesis associated with DNA repair. However, the data reported herein indicate that Pol η focus formation might not necessarily be a sign of active function. Our findings indicate that Pol η can efficiently regroup into visible structures in the vicinity of DNA lesion independently of the correct and/or complete activation of repair associated signals. Taken together, the experiments performed in this work present new ideas that support a novel "be ready for" model of recruitment of Pol η which has different implications that the current "S-Phase linked" model. These findings will be important to figure out the mechanism of action of permissive DNA polymerases in the future.

Materials and Methods

Cell culture, transfections and UV irradiation. U2OS cells were obtained from ATCC and grown in DMEM (Invitrogen) supplemented with 10% FBS. Healthy skin fibroblast transformed with SV40 (#GM00637) and the SV40-transformed fibroblast XPA #GM04312, XPF #GM08437 and XPG #GM14931 cell lines were purchased from Coriell Repositories. CHO9 cells and UV135 were described previously.[41] Transfections were performed using Lipofectamine 2000 (6 μl/10^6 cells). The p21 expression vector was described previously.[23] GFP-Pol η was a gift of Dr. Lehmann and was described elsewhere.[21] UVC irradiation was delivered with a CL-1000 ultraviolet cross linker equipped with 254 nm tubes (UVP). For full cell irradiation, doses from 10 to 40 J/m^2 were delivered after removal of the culture media. For local irradiation, polycarbonate filters containing multiple 5 μm pores (Millipore #TMTP01300) were positioned in direct contact with cells and subjected to 100 J/m^2 (due to the polycarbonate shielding the actual UV dose that the cell receive is lower as reported in).[57] For detection of replicative DNA synthesis cells were incubated for 30 min in DMEM 10% FBS containing 10 μM BrdU (SIGMA). Serum starvation of CHO was achieved by incubating the cells in serum free media for 2 days.

Multi-photon laser (MPL) treatment. Live cell imaging and MPL treatment were performed at 37°C using a Zeiss Axiovert 100M confocal microscope as previously described.[38] Briefly a Coherent Mira modelocked Ti:Sapphire laser was used at 800 nm utilizing a pulse-length of 200 fs and a repetition rate of 76 MHz. Damage was delivered performing 4 iterations on the selected area using 50% of the laser power and a 4X optical zoom utilizing a PlanApo 63X objective. After the induction of DNA damage cells were followed using time-lapse confocal microscopy. Images settings of 256 x 256 pixels and around 150 milliseconds scanning times were used unless specified.

Cell cycle analysis. Cells were fixed with ice-cold ethanol and samples were resuspended in phosphate-buffered saline (PBS) containing RNase I (50 μg/ml) and propidium iodide (PI) (25 mg/ml, Sigma). Stained samples were subjected to fluorescence activated cell sorting (FACScalibur, Becton Dickinson), and data were analyzed using the Summit 4.3 software (DAKO Cytomation). When indicated, the profiles shown were obtained by gating the GFP positive cells by dual channel FACS analysis.

Immunostaining and microscopy. Cells were plated on 10 mm round coverslips, transfected and fixed. For imaging, cells were fixed in 4% paraformaldehyde-sucrose for 15 min at room temperature, followed by a 10 min incubation with 0.1% Triton as described before.[33] For BrdU staining, prior to immunofluorescence cells were submitted to a denaturizing step with HCl 1.5 M for 4 min in order to expose BrdU epitope for antibody detection.

For CPD staining, fixed samples were subjected to denaturalization with NAOH 0.07 M for 4 min in order to expose CPDs for antibody detection. Blocking was performed overnight in PBS 2% donkey serum (SIGMA). Coverslips were incubated for 1 hour in

primary antibodies: α BrdU (Amersham), α γH2AX (Upstate), α CPD (MBL), αRad51.[58] Secondary anti-mouse-conjugated Cy2/Cy3 antibodies were from Jackson ImmunoResearch. GFP-Pol η and Mcherry PCNA were detected by auto-fluorescence. Nuclei were stained with DAPI (SIGMA). Images were obtained with a Zeiss Axioplan confocal microscope.

Protein analysis. Cells were grown on 10 cm plates, transfected and lysed as described before.[33] For immunoprecipitations of chromatin associated PCNA a previously described protocol was used.[34] Immunoprecipitations were performed using αPCNA PC10 (Santa Cruz). Western blots were performed using α PCNA PC10, α GFP (Santa Cruz), α pol η (Santa Cruz), α pol δ (Abcam) and α actin (SIGMA). Incubation with secondary antibodies (SIGMA) and detection (ECL-Amersham) were performed according to manufacturers' instructions.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/SoriaCC8-20-Sup.pdf

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