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Spatiotemporal Dynamics of Regulatory Protein Recruitment at DNA Damage Sites

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Abstract Mammalian cells are constantly threatened by multiple types of DNA lesions arising from various sources like irradiation, environmental agents, replication errors or by-products of the normal cellular metabolism. If not readily detected and repaired these lesions can lead to cell death or to the transformation of cells giving rise to life-threatening diseases like cancer. Multiple specialized repair pathways have evolved to preserve the genetic integrity of a cell. The increasing number of DNA damage sensors, checkpoint regulators, and repair factors identified in the numerous interconnected repair pathways raises the question of how DNA repair is coordinated. In the last decade, various methods have been developed that allow the induction of DNA lesions and subsequent real-time analysis of repair factor assembly at DNA repair sites in living cells. This combination of biophysical and molecular cell biology methods has yielded interesting new insights into the order and kinetics of protein recruitment and identified regulatory sequences and selective loading platforms for the efficient restoration of the genetic and epigenetic integrity of mammalian cells. J. Cell. Biochem. 104: 1562–1569, 2008. © 2008 Wiley-Liss, Inc.

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DNA lesions arising from environmental and endogenous sources induce a variety of cellular responses including cell cycle arrest, DNA repair, senescence and apoptosis [Hoeijmakers, 2001; Friedberg, 2003]. The DNA damage response is a multistep process involving lesion detection, checkpoint activation, processing of repair intermediates, and finally restoration of the genetic and epigenetic information [Harper and Elledge, 2007]. The immediate and faithful detection of DNA lesions is central to cellular survival and is mediated by DNA damage sensors. Although various DNA damage sensors have been identified in recent years, several important issues remain to be resolved. For example, it is still unclear how DNA damage sensors and repair factors gain access to their respective substrates within the context of chromatin. DNA lesions might be detected through either continuous scanning of the genome via sliding along the DNA, as has been suggested for proteins involved in the mismatch repair (MMR) pathway, or by high affinity binding and transient immobilization of freely diffusing proteins in a distributive manner, so-called assembly on the spot. It has also been proposed that instead of being directly sensed, DNA lesions might rather be indirectly detected through changes in chromatin topology [Bakkenist and Kastan, 2003]. Once a DNA lesion has been successfully detected, it has to be handed over to specific repair factors, which then restore the genetic information. Repair factors could be recruited to the site of lesion by the DNA damage sensors themselves, by recognizing the DNA lesion directly, or by both ways. The multiple activities involved in repair of each type of DNA damage form the so-called repairosome. In addition to their biochemical reconstitution in vitro, recent studies have focused on the coordination of such enzymatic complexes in living cells, testing the existence of such holocomplexes in the cell or rather their

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sequential assembly and or disassembly at repair sites [Politi et al., 2005]. Finally, after the genetic information has been successfully restored, the epigenetic information including methylation patterns and chromatin states has to be re-established. The increasing number of proteins identified in the various DNA repair pathways, raises the question of how these proteins are coordinated in time and space to ensure avid and efficient removal of harmful DNA lesions. In this article we aim to highlight recent findings shedding light on the coordination of DNA repair in living cells.

STUDYING DNA REPAIR IN LIVING CELLS

Pioneering work using mainly in vitro experiments gave detailed insights into the biochemical mechanisms and composition of the various DNA repair pathways. However, the identification of more and more proteins being involved in the various steps of DNA repair, as well as the emerging interconnection between different DNA repair pathways, requires studying the spatiotemporal coordination of DNA repair in living cells. In recent years, several methods have been introduced that allow DNA lesion induction and subsequent real-time analysis of the DNA damage response in living cells [Lukas et al., 2005].

Using ionizing radiation (IR) in combination with fluorescence photobleaching (FRAP) analysis it has been shown that DNA double strand break (DSB) repair factors rapidly diffuse throughout the nucleus until they encounter a break and become transiently immobilized [Essers et al., 2002]. A similar observation has been made for proteins involved in the nucleotide excision repair (NER) pathway [Houtsmuller et al., 1999]. These results indicate that DNA repair is not mediated by binding of a preassembled repair holocomplex, but is rather coordinated by the sequential recruitment of specific repair factors to DNA damage sites.

The disadvantage of using IR is that DNA lesions are scattered randomly throughout the genome. Furthermore, it is not possible to visualize the real-time accumulation of repair proteins and IR induced foci are hardly distinguishable from other nuclear foci like replication sites. Recently, some of these drawbacks have been circumvented by using focal irradiation with charged particles or heavy ions, which allows specific induction of DSBs along the ion or particle track [Jakob et al., 2003; Aten et al., 2004; Hauptner et al., 2006].

An elegant approach to specifically induce DSBs at defined subnuclear sites is the introduction of rare restriction sites into the genome followed by conditional expression of the respective endonuclease. This method was first developed in yeast [Melo et al., 2001; Lisby et al., 2003] but has also been adapted in mammalian cells [Jasin, 1996; Soutoglou et al., 2007]. DSBs can even be followed over time in vivo by flanking the restriction sites with *tet* or *lac* operator cassettes and expression of fluorescently tagged Tet- and/or Lac-binding fusion proteins [Lisby et al., 2003; Soutoglou et al., 2007]. However, the considerable long lag time between induction of the endonuclease and cutting (up to 30 min) does not allow precise kinetic measurements of repair factor assembly at DNA breaks.

In recent years, lasers used in confocal microscopy or microdissection devices have been adapted by various groups to introduce DNA lesions at preselected subnuclear sites in living cells. These microbeam laser techniques are based on the presensitization of DNA with low levels of halogenated thymidine analogs and/or DNA intercalating dyes (e.g., Hoechst 33258), which render the DNA hypersensitive to light within the UVA spectral range. Microirradiation with a UV laser leads to a photochemical reaction that is sufficient to induce various DNA lesions including single strand breaks (SSBs) and DSBs. In addition to SSBs and DSBs, other typical UVA DNA lesions, like thymine dimers, are introduced. On the one hand, this mixture of different DNA lesion types offers the possibility to compare the recruitment of proteins involved in different DNA repair pathways side by side within a single cell. On the other hand, studying the cellular response to one particular type of DNA damage is difficult with the microirradiation approach. To eliminate these problems some groups used laser microirradiation without sensitization [Kim et al., 2002; Lan et al., 2004]. The drawback is that it requires much higher laser energy and can lead to damage of overall cellular structures.

A combination of the described laser microirradiation systems with live cell microscopy and fluorescently tagged fusion proteins, allows studying the recruitment kinetics of DNA repair factors in living cells (Fig. 1).

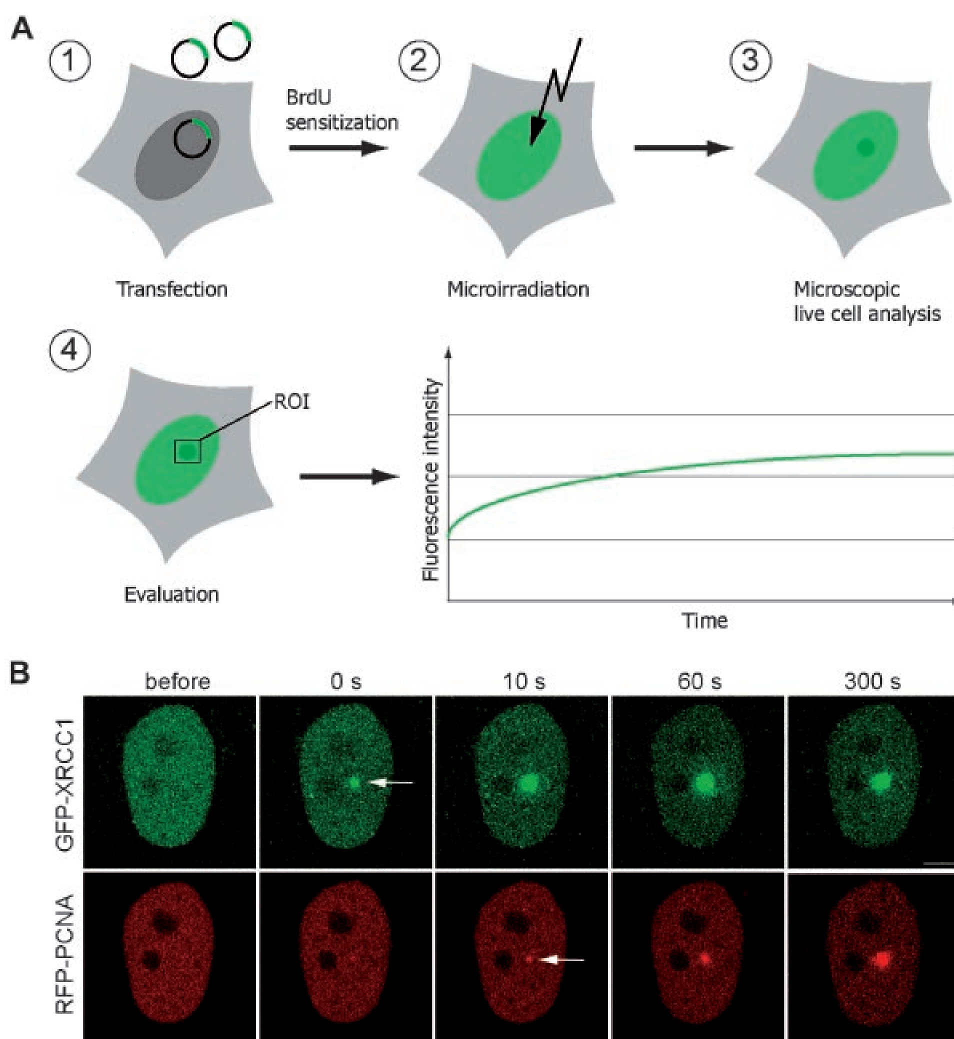

Fig. 1. Studying DNA repair in living cells. **A**: Cells are transfected with expression constructs (circles) coding for fluorescently tagged fusion proteins and sensitized by incubation in medium containing BrdU and/or Hoechst for 24–48 h (1). Microirradiation is performed with a confocal laser (2) and the accumulation of fluorescently labeled proteins at DNA damage sites is monitored in real-time (3). After measuring and normalizing the fluorescence intensity at the microirradiated site (ROI), the recruitment kinetics is plotted as a graph (4). **B**: Analysis of protein recruitment to laser-induced DNA damage sites exemplary illustrated for GFP-XRCC1 and RFP-PCNA in a mammalian cell.

COORDINATION OF DNA REPAIR IN LIVING CELLS

DNA repair requires the coordinated recruitment of multiple enzyme activities to ensure efficient repair of DNA lesions. Re-synthesis of long stretches of DNA in various repair pathways requires stable complex formation for processivity, but this may limit the ability of the repair machinery to respond to later changes like subsequent DNA damages.

So-called loading platforms are considered to play a pivotal role in DNA repair by locally concentrating and coordinating repair factors at sites of DNA damage. Loading platforms are characterized as proteins with no intrinsic enzymatic activity and the ability to interact with numerous proteins through highly conserved binding motifs. The two repair factors X-ray cross complementing factor 1 (XRCC1) and proliferating cell nuclear antigen (PCNA) both are considered to act as central loading platforms in DNA replication and repair [Warbrick, 2000; Caldecott, 2003; Maga and Hubscher, 2003; Moldovan et al., 2007].

PCNA forms a homotrimeric ring around the DNA, which at the same time allows stable association with and sliding along the DNA

double helix. Because of this unique property PCNA is often referred to as a "sliding clamp" being capable of mediating interactions of various proteins with DNA in a sequence-independent manner. Apart from being a central component of the replication machinery, PCNA is also involved in various repair pathways including NER [Shivji et al., 1992], base excision repair (BER) [Gary et al., 1999; Levin et al., 2000], MMR [Johnson et al., 1996; Umar et al., 1996; Jiricny, 2006], and repair of DSBs [Holmes and Haber, 1999; Dorazi et al., 2006]. In addition, PCNA is implicated in the coordination of postreplicative processes such as cytosine methylation and chromatin assembly [Chuang et al., 1997; Moggs et al., 2000]. Most of the PCNA-interacting proteins bind to a common site on PCNA through a conserved PCNA-binding domain (PBD). As more and more PCNA-interacting proteins are identified the question arises, of how binding is coordinated and sterical hindrance avoided in various processes such as DNA replication and repair.

Post-translational modifications such as ubiquitinylation and sumoylation have been shown to target PCNA to different repair pathways [Hoege et al., 2002; Matunis, 2002; Solomon et al., 2004; Moldovan et al., 2007]. In response to DNA damage or stalled replication forks, PCNA is ubiquitylated at the conserved lysine (K) residue 164. While monoubiquitylation of PCNA triggers the error-prone repair of DNA lesions through recruitment of translesion polymerases, polyubiquitylation of PCNA results in error-free bypass of DNA lesions [Hoege et al., 2002]. In contrast to ubiquitylation, which promotes either error-free or error-prone repair, sumoylation of PCNA at the same lysine residue seems to repress DNA repair [Pfander et al., 2005]. The exact mechanisms or factors initiating the ubiquitylation or sumoylation of PCNA thus determining which road PCNA will finally take are still unclear.

Recently, the kinetics of PCNA recruitment to subnuclear sites of DNA damage in living cells has been analyzed [Solomon et al., 2004; Essers et al., 2005; Mortusewicz and Leonhardt, 2007]. Interestingly, recruitment of PCNA to DNA damage sites is independent of RFC, which is required to load PCNA onto DNA during DNA replication [Hashiguchi et al., 2007]. It has previously been shown that PCNA stably associates with DNA replication sites serving as a loading platform for proteins involved in lagging strand synthesis [Sporbert et al., 2002, 2005]. During DNA repair, PCNA remains stably bound over a long time period at DNA damage sites, whereas several PCNA-interacting proteins (e.g., DNA Ligase I) show a high turnover [Mortusewicz et al., 2006]. These results indicate that PCNA not only serves as a central and stable loading platform during DNA replication, but also coordinates the recruitment of multiple enzymatic activities to DNA repair sites. Accordingly, the maintenance DNA methyltransferase DNMT1, which is known to associate with replication sites through binding to PCNA, is likewise recruited to DNA repair sites by PCNA [Mortusewicz et al., 2005]. We propose that DNMT1 recruitment to repair sites preserves cytosine methylation patterns in the newly synthesized DNA, thus contributing to the restoration of epigenetic information after repair of DNA damage.

The second central loading platform in DNA repair, XRCC1, was first identified in a mutant cell line with a defect in SSB repair and increased sensitivity to alkylating agents and ionizing irradiation, resulting in elevated frequency of spontaneous chromosome aberrations and deletions [Thompson et al., 1982]. XRCC1 was found to interact with various proteins involved in SSB repair and BER including PARP-1, PARP-2 [Masson et al., 1998; Schreiber et al., 2002], DNA polymerase beta [Caldecott et al., 1994; Kubota et al., 1996], and DNA Ligase III [Caldecott et al., 1994; Wei et al., 1995]. Recently, XRCC1 was also found to interact with PCNA, which could facilitate the detection and repair of DNA lesions arising during DNA replication [Fan et al., 2004].

A direct comparison of the recruitment kinetics of the two loading platforms PCNA and XRCC1 revealed different recruitment and binding kinetics, with the immediate and fast recruitment of XRCC1 preceding the slow and continuous recruitment of PCNA. Furthermore, introducing multiple DNA lesions sequentially within a single cell, demonstrated that these different recruitment and binding characteristics have functional consequences for the ability of PCNA and XRCC1 to respond to successive DNA damage events [Mortusewicz and Leonhardt, 2007]. While the avid and transient binding of XRCC1 allows a flexible response to multiple consecutive DNA lesions, the stable binding of the processivity factor

PCNA limits its capacity to respond to successive damage events.

The central role of PCNA and XRCC1 as loading platforms in the coordination of DNA repair pathways became apparent when the recruitment kinetics of the two highly homologous enzymes DNA Ligases I and III was compared [Mortusewicz et al., 2006]. Deletion and mutational analysis revealed that the specific recruitment of DNA Ligases I and III to distinct repair pathways is mediated through interaction with their respective loading platforms (Fig. 2). This specific targeting of repair factors may have evolved to accommodate the particular requirements of different repair pathways, for example, single nucleotide replacement versus synthesis of short stretches of DNA, and thus may enhance the overall efficiency of DNA repair.

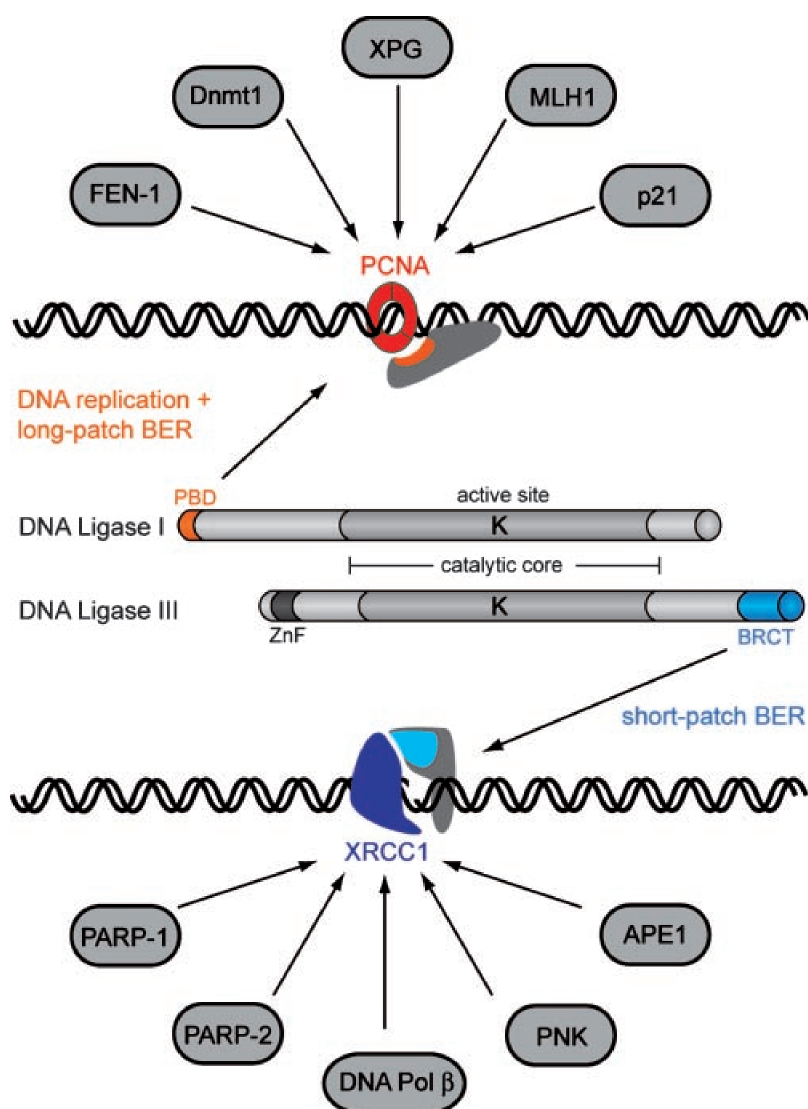

Fig. 2. The loading platforms PCNA and XRCC1 target interacting proteins to different repair pathways. All DNA Ligases use the same catalytic mechanism and show high sequence similarity in the catalytic core. The active site lysine residue (K) in the center of the catalytic domain is directly involved in the ligation reaction. However, DNA Ligases have non-overlapping functions in DNA repair and replication and are not interchangeable. DNA Ligases I and III are targeted to different repair pathways through their regulatory PBD and BRCT domains, which mediate interaction with PCNA and XRCC1, respectively. Other interacting proteins (only selective examples are shown) might be recruited in similar ways to their respective repair substrate. Selective recruitment of specialized proteins by central loading platforms may accommodate the specific requirements of different repair pathways and thereby enhance repair efficiency.

LOADING PLATFORMS AND ORDERLY EXECUTION OF DNA DAMAGE REPAIR

Efficient repair of DNA lesions would also require the orderly loading of factors required at different times during the repair of a specific DNA lesion via their respective loading platforms. How is then binding of numerous factors regulated within the cell in response to genetic insults? It has been previously shown, that different PCNA-interacting proteins have distinct binding affinities for PCNA and subtle changes within the PCNA binding domain can dramatically change the binding affinities of a given protein for PCNA. Thus, binding to PCNA at sites of DNA repair or replication could simply be hierarchically ordered according to the binding affinity of a respective protein for a specific state of PCNA. Consequently, this would result in constant occupation of the PCNA-binding site by the strongest PCNA-binding protein. However, the fact that most PCNA-interacting proteins show a high turnover would also result in a flexible access giving every factor a chance to bind. Sequential binding might then be ordered by the respective function of a given protein in the repair process. Repair factors might thus be constantly recruited to and dissociate from PCNA until they encounter their respective repair substrate within the surrounding chromatin. This would result in the initiation of the appropriate enzymatic reaction, which would generate the preferred substrate for the next factor to bind.

Binding affinities and interactions could further be regulated by posttranslational modifications like phosphorylation. A recent large-scale screen for ATM and ATR substrates identified over 700 proteins involved in various processes including DNA replication, DNA repair, and checkpoint activation as well as proteins involved in pathways previously not directly connected to the DNA damage response, like RNA splicing, the spindle checkpoint and chromatin remodeling [Matsuoka et al., 2007]. In addition, loading platforms themselves might be regulated to respond to altered requirements. Furthermore, it has become clear that ubiquitylation plays a central role in the DNA damage response regulating protein turnover and recruitment of repair factors [Bennett and Harper, 2008]. In fact, mutant PCNA unable to be ubiquitinated was found not to accumulate at repair sites in living cells [Solomon et al., 2004]. How these and other posttranslational modifications contribute to the spatiotemporal coordination of repair factor assembly at DNA damage sites will be a challenge for future studies.

Most current methods used for the real-time analysis of the DNA damage response in living cells have the drawback of introducing a variety of different DNA lesions. More refined and quantitative methods are needed to obtain valuable data for detailed mathematical modeling of the DNA damage response in living cells. This would include the induction of a defined subset of DNA lesions in physiological relevant concentrations. Together with improvements in microscopic imaging methods and single molecule tracking this should provide a detailed insight into the spatiotemporal coordination and regulation of DNA repair in living cells.

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