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New Histone Incorporation Marks Sites of UV Repair in Human Cells

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SUMMARY

Chromatin organization is compromised during the repair of DNA damage. It remains unknown how and to what extent epigenetic information is preserved in vivo. A central question is whether chromatin reorganization involves recycling of parental histones or new histone incorporation. Here, we devise an approach to follow new histone deposition upon UV irradiation in human cells. We show that new H3.1 histones get incorporated in vivo at repair sites. Remarkably we find that H3.1, which is deposited during S phase, is also incorporated outside of S phase. Histone deposition is dependent on nucleotide excision repair (NER), indicating that it occurs at a postrepair stage. The histone chaperone chromatin assembly factor 1 (CAF-1) is directly involved in the histone deposition process in vivo. We conclude that chromatin restoration after damage cannot rely simply on histone recycling. New histone incorporation at repair sites both challenges epigenetic stability and possibly contributes to damage memory.

INTRODUCTION

Cells are exposed to a variety of genotoxic insults that constantly threaten genome integrity. The cellular response to DNA damage involves specific repair pathways such as nucleotide excision repair (NER), which removes helix-distorting DNA lesions, including UV-induced cyclobutane pyrimidine dimers (CPD) and 6,4 PhotoProducts (6,4-PP) (de Laat et al., 1999; Friedberg, 2001; Reardon and Sancar, 2005). However, in eukaryotic cells, such repair machineries operate on chromatin-embedded DNA substrates (Green and Almouzni, 2002), and DNA folding with histone proteins into chromatin (Kornberg, 1977) poses structural constraints likely to challenge detection and repair of DNA lesions. Furthermore, chromatin organization is a source of epigenetic information, which is not encoded by the DNA sequence but through histone variants, post-translational modifications, and higher-order chromatin structures involving nonhistone proteins (Vaquero et al., 2003). These additional layers of information are important for genome functions. A current challenge is to understand how to integrate chromatin structure within the scheme of DNA repair and how this is associated with maintenance (or loss) of epigenetic information. While molecular machineries that operate at the DNA level for DNA-damage signaling and repair have been characterized in great details, their coordinated action with factors involved in chromatin modulation is still poorly understood.

A current model, which delineates how repair of DNA lesions operates within chromatin, is the "access-repair-restore" model (Green and Almouzni, 2002; Smerdon, 1991). This model proposes that, in a first step, chromatin organization is transiently disrupted to facilitate access of the repair machinery to DNA lesions. A subsequent step is then necessary to restore the preexisting chromatin structure. Much progress has been made in the characterization of factors promoting rearrangements of chromatin structure in the early stages of the DNA-damage response, especially in the context of DNA double-strand breaks (recently reviewed in Peterson and Cote [2004] and van Attikum and Gasser [2005]). Regarding chromatin restoration, histone chaperones (Loyola and Almouzni, 2004) are likely be involved, and, among them, chromatin assembly factor 1 (CAF-1) represents an attractive candidate.

CAF-1 is a conserved nuclear complex consisting of p150, p60 (Kaufman et al., 1995), and p48 subunits (Verreault et al., 1996) in human cells. The recent discovery of its specific association with the replicative histone variant H3.1 in human cells (Tagami et al., 2004) has offered novel insights into a potential role of CAF-1 for the establishment and/or maintenance of histone variant identity in specific chromatin domains. CAF-1 has the unique ability to promote in vitro chromatin assembly in a DNA synthesis-coupled manner on replicating (Smith and Stillman, 1989; Stillman, 1986) and newly repaired DNA (Gaillard et al., 1996). Furthermore, it is recruited to UV-damaged chromatin in vivo (Green and Almouzni, 2003; Martini et al., 1998). Thus, CAF-1 is at the right place to participate in chromatin restoration coupled to repair of UV lesions in a cellular context. However, it remains unknown how restoration of chromatin structure is actually achieved in vivo.

A major unresolved issue relates to histone dynamics within damaged chromatin and to what extent epigenetic information is preserved. It is unclear whether preexisting nucleosomal histones are replaced by new histones within

damaged chromatin or if they are recycled (Figure 1A). Understanding how this cellular process is achieved is crucial to evaluate how stable epigenetic information is when facing genetoxic insults. Indeed, on the one hand, a simple recycling of old histones would ensure the faithful maintenance of epigenetic integrity. On the other hand, incorporating new histones could both challenge this integrity and be used as a marking system of damaged chromatin to monitor postrepair status.

In this paper, we focus on H3.1 histone variant during UV-damage response in human cells to address the issue of chromatin restoration and histone dynamics within damaged chromatin in vivo. We develop a novel approach to visualize de novo incorporation of H3.1 histones at NER sites, and we explore the underlying mechanism, taking advantage of repair-deficient cells, and knocking down candidate histone chaperones. We show that local deposition of new H3.1 histones is dependent on NER proficiency, and our data support a direct involvement of CAF-1 in this process. We can thus exclude a mechanism involving a strict recycling of preexisting histones. We discuss how local incorporation of new histone variants coupled to repair of UV lesions might result in maintenance and/or loss of epigenetic information at UV damage sites as well as contribute to a memory of damage experience.

RESULTS

Visualization of De Novo Incorporation of H3.1 Histones at NER Sites In Vivo

As a first step toward investigation of histone dynamics at repair sites, it was important to set up a system that enabled us to visualize incorporation of new histones into UV-damaged chromatin in vivo. We thus developed a novel assay, based on transient transfection of cells with epitope-tagged histones, which could be easily distinguished from endogenous ones (Figure 1B). For this purpose, we chose the H3.1-HA-Flag variant (referred to as e-H3.1), given its specific association with CAF-1 in vivo (Tagami et al., 2004). Moreover, H3.1 variant is incorporated into chromatin only during S phase, even when constitutively expressed (Ahmad and Henikoff, 2002; Figure S1C), in contrast to H3.3, whose global incorporation into chromatin in a cell-cycle independent manner, obscures the analysis (data not shown). The use of this model system was critical for three reasons. First, the chosen expression vector enables e-H3.1 to be produced at low levels compared to endogenous H3 within the soluble fraction (Figure S1A) throughout the cell cycle (not shown), and e-H3.1 can thus be used as a tracer; this system avoids the cytotoxic effects of histone excess (Gunjan et al., 2005) and introduces minimal bias in the use of exogenous histones by the chromatin assembly machinery. Second, the HA-Flag epitope did not prevent e-H3.1 incorporation into chromatin, as observed on mitotic chromosomes (Figure S1B; Tagami et al., 2004), and this tagged variant is efficiently incorporated into canonical nucleosomes, as previously shown (Tagami et al., 2004); we also checked that the epitope did not interfere with e-H3.1 replication-coupled incorporation into chromatin (Figure S1C), which supports the physiological relevance of following such epitope-tagged histones. Third, 20 hr posttransfection, e-H3.1 histones were expressed at a detectable level in around 40% of the cells (Figure S1B) with limited global incorporation into chromatin, since this required passage through S phase; this system thus enabled us to investigate local incorporation events of soluble e-H3.1.

After local UV irradiation of transfected cells (20 hr posttransfection), we monitored local concentrations of detergent-resistant e-H3.1 at UV damage sites (Figure 1C). At early time points after irradiation (5 min), while we could detect XPC as a marker of NER, we could not observe any local concentration of e-H3.1 (Figure 1C). However, 30 min to 1 hr post-UV irradiation, we detected local recruitment of e-H3.1 to UV damage sites and repair patches, as detected by CPD immunostaining and BiodU incorporation (Figure 1C). This recruitment occurs together with CAF-1 in a substantial fraction of transfected HeLa cells (65%, 9% ± standard deviation [SD], data obtained from three independent experiments) (Figure 1C) as well as in MCF7 cells (not shown). Importantly, we also obtained similar results in normal diploid fibroblasts (64%, 4% ± SD, data obtained from two independent experiments) (Figure S2), which ruled out that this phenomenon was a peculiarity of tumor cells. Noteworthy, given that the local accumulation of e-H3.1 was observed in a substantial fraction of transfected cells, it was unlikely to be restricted to cells with high transgene expression levels nor to specific chromatin regions. Furthermore, the local concentration of e-H3.1 at UV-damage sites was observed at UV doses down to 25 J/m^2 (Figure S3). Thus, this phenomenon is not restricted to high UV doses, which strengthens the physiological relevance of our observations. We also verified that e-H3.1 detergent-resistant fraction truly corresponded to chromatin bound histones since e-H3.1 staining was lost upon DNase treatment (Figure 1D). Finally, e-H3.1 retention at late time points post UV (6 hr), while the histone chaperone CAF-1 already detached from chromatin (Figure 1C), favors an incorporation of these histones into chromatin. Taken together, our findings show that soluble H3.1 histones can be incorporated de novo into chromatin at sites of UV damage.

H3.1 Incorporation at UV-Damage Sites Occurs Outside S phase in an NER-Dependent Manner

To gain mechanistic insight into how H3.1 histones are deposited at UV-damage sites, we sought to characterize associated factors and cellular processes. First, to exclude the possibility that the observed deposition of H3.1 was taking place at replication foci, we selected cells outside S phase using PCNA staining as a reference, and we also used a different filter set for local UV irradiation (8 μm versus 3 μm pore filters). This resulted in recruitment of e-H3.1 to larger nuclear areas, which were unambiguously related to the UV treatment and distinguished from

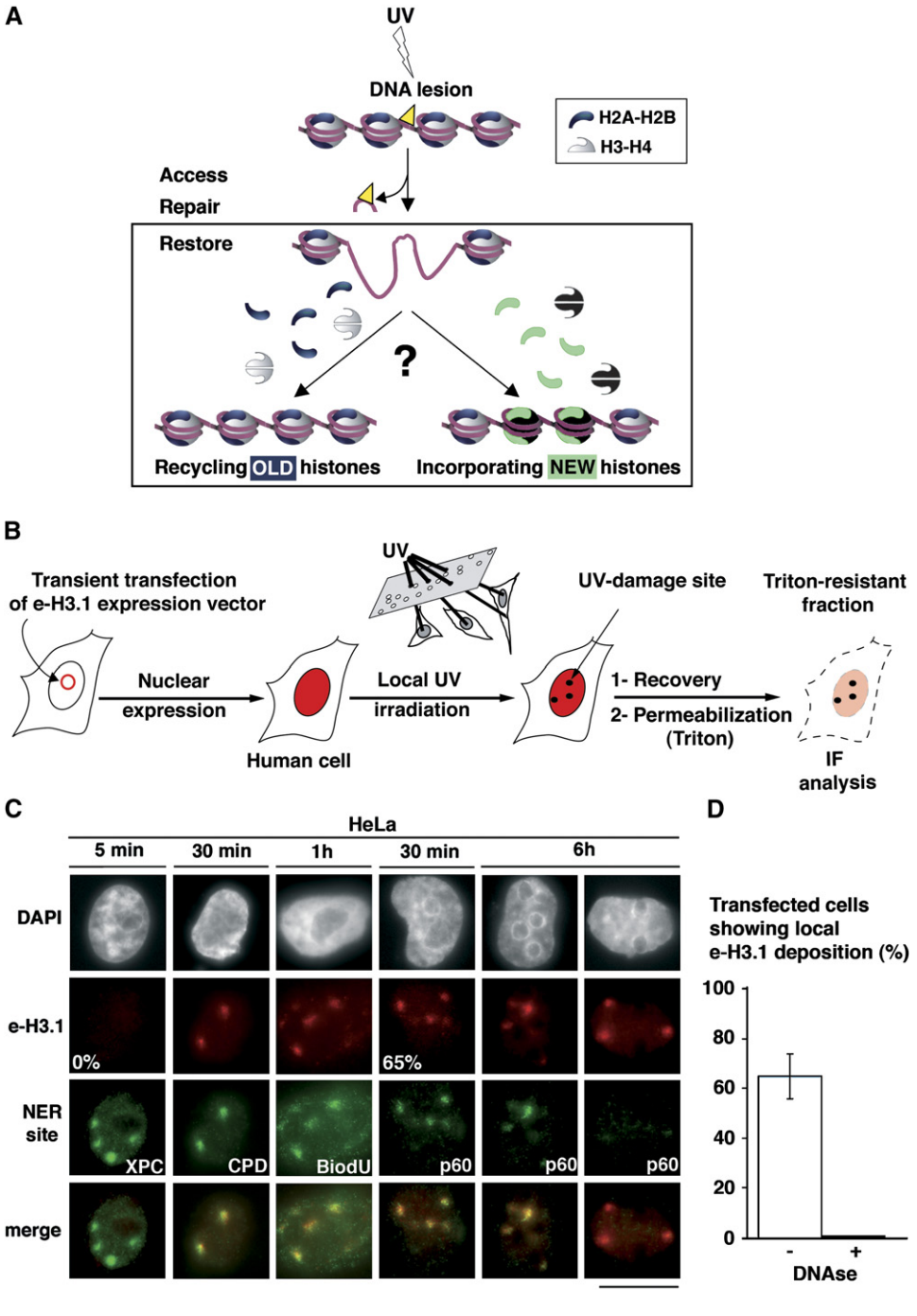


Figure 1. De Novo Incorporation of H3.1 Variant at UV-Damage Sites In Vivo

(A) Restoring chromatin structure after repair of DNA damage: two working hypotheses. Old histones can be recycled and/or replaced by new histones, which may differ from the preexisting ones in terms of histone variants and posttranslational modifications, thus challenging the stability of epigenetic information.

(B) Experimental scheme.

(C) Immunolocalization of chromatin bound e-H3.1 (anti-HA antibody) in e-H3.1-transfected HeLa cells at the indicated time points after local UV irradiation. UV-damage sites are visualized by CPD immunodetection, BiodU incorporation (repair synthesis), and local recruitment of the NER factor XPC or CAF-1 p60. Scale bar, 10 μm.

(D) Plot representing the effect of DNase treatment on the local incorporation of e-H3.1 following local UV irradiation (mean ± SD, 30 min postirradiation in HeLa cells).

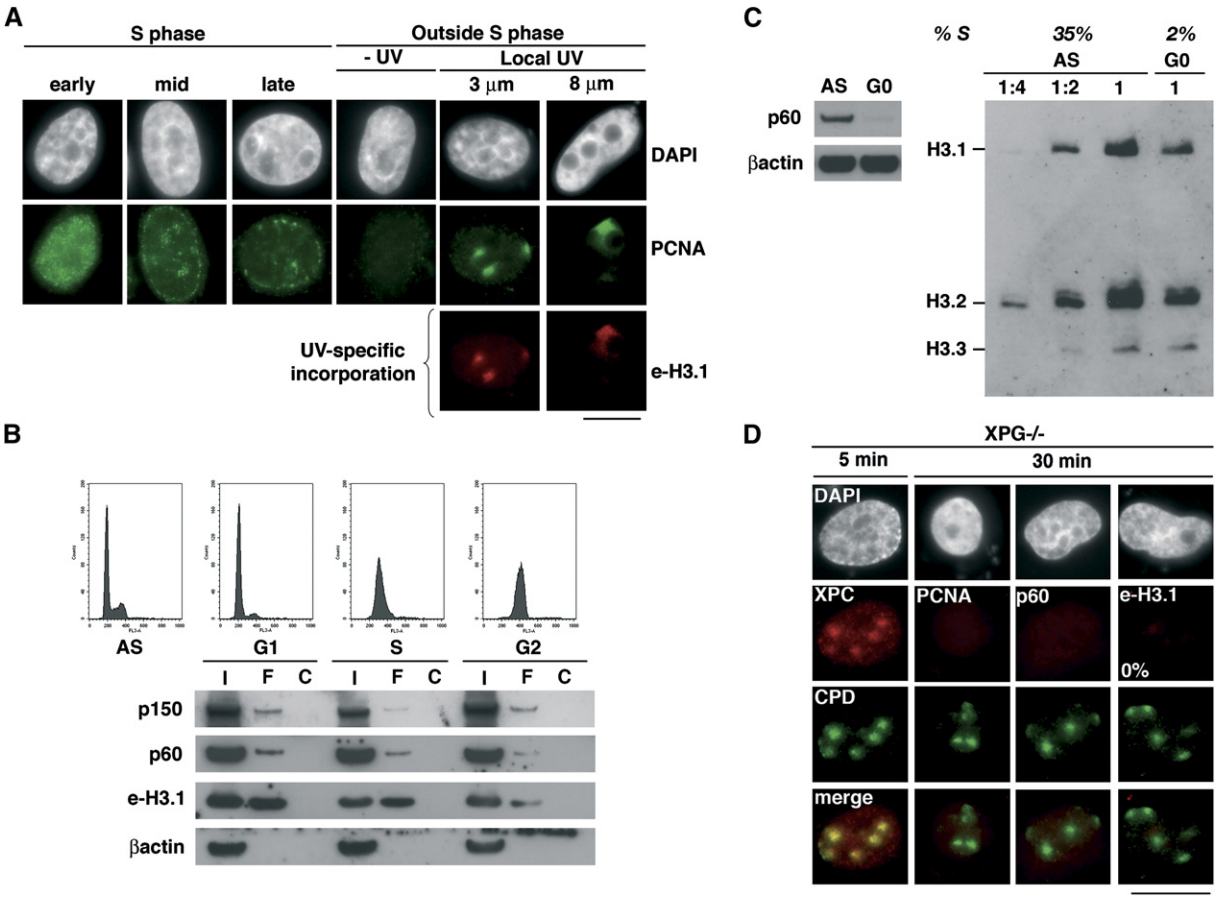


Figure 2. De Novo Incorporation of H3.1 Variant at UV-Damage Sites Occurs outside S Phase in an NER-Dependent Manner

(A) Immunodetection of chromatin bound (detergent-resistant) PCNA in HeLa cells discriminates between cells in S phase and outside S phase. Nuclei of cells in S phase show a global staining pattern (early-, mid- or late-replication profiles), whereas cells outside S phase either show no staining (-UV: undamaged cells) or local staining at UV-damage sites (Local UV). Similar staining profiles are obtained for CAF-1 p150 and p60 subunits (not shown). Local UV irradiation is performed using 3 and 8 μm pore filters. Chromatin bound e-H3.1 colocalizes with PCNA at sites of local UV irradiation in both cases.

(B) Association of soluble e-H3.1 with CAF-1 outside S phase. HeLa cells stably expressing e-H3.1 were synchronized by a double thymidine block in G1, S, and G2 phases as controlled by flow cytometry using asynchronous cells (AS) as a reference (upper panel). Cell extracts containing soluble histones were prepared and subjected to immunoprecipitation using anti-Flag M2 antibody (SIGMA) (F) or Sepharose CL-4B (SIGMA) as a negative control (C), before western blot analysis of the immunoprecipitation products (lower panel). I: 10% of input.

(C) Amount of endogenous H3.1 in the soluble fraction outside S phase. Asynchronous (AS) and G0-arrested MCF7 cells were lysed to prepare cell extracts containing soluble histones. The efficiency of G0 arrest was verified by CAF-1 downregulation (left panel) (Polo et al., 2004). Several dilutions of the samples (as indicated) were used for specific detection of H3 variants by Triton-acid-urea gel analysis (right panel).

(D) Immunolocalization of chromatin bound XPC, PCNA, CAF-1 p60, and e-H3.1 at the indicated time points after local UV irradiation in XPG$^{-/-}$ cells. UV-damage sites are visualized by costaining for CPDs. The percentages indicate the fraction of transfected cells showing local recruitment of e-H3.1 at UV-damage sites. Scale bars, 10 μm.

replication foci (Figure 2A), arguing that local deposition of e-H3.1 was UV-specific and not restricted to S phase.

To support the physiological relevance of these findings, we carefully examined the status of H3.1 outside S phase. First, we tested by coimmunoprecipitation the association of soluble e-H3.1 with the histone chaperone CAF-1, an association so far described only in S phase or in asynchronous cell populations (Groth et al., 2005; Tagami et al., 2004). We found that soluble e-H3.1 readily associates with CAF-1 outside S phase, as efficiently as in S phase (Figure 2B). Second, we analyzed the status of endogenous histones. A Triton-acid-urea gel analysis enabled us to distinguish histone variants based on migration properties (Zweidler, 1978). Using extracts from cycling and quiescent cells, we could thus assess the amount of endogenous H3.1 within the soluble fraction outside S phase. Only a 2- to 3-fold decrease in soluble H3.1 was observed in quiescent cells compared to cycling cells (Figure 2C). Given the proportion of S phase cells in each population (35% in cycling cells versus 2% in G0 arrested cells, as determined by BrdU immunodetection), this difference in H3.1 cannot be explained by a strict S

phase contribution. Therefore, a significant fraction of soluble H3.1 can be available for deposition outside S phase.

Furthermore, the UV specificity of new H3.1 deposition prompted us to consider that repair of UV lesions might be necessary for this mechanism to occur. We thus analyzed local incorporation of e-H3.1 at UV-damage sites in NER-deficient cells. XPG$^{-/-}$ fibroblasts are defective in late steps of the NER process (Cleaver, 2005) and thus display local recruitment of the early NER factor XPC but not of the polymerase accessory factor proliferating cell nuclear antigen (PCNA), which is involved in later steps of the NER pathway (Figure 2D). The transfection efficiency of e-H3.1 in XPG$^{-/-}$ cells was comparable to HeLa cells (Figure S1B) and WI38 fibroblasts (Figure S2). Furthermore, XPG$^{-/-}$ fibroblasts displayed global (i.e., replication-associated) incorporation of e-H3.1 (Figure S1B and data not shown). However, we could not detect local incorporation of e-H3.1 at UV-damage sites in the NER-deficient cells (Figure 2D), which indicates that this process is dependent on NER proficiency and occurs at a late stage in the damage response, after repair of UV lesions.

CAF-1-Dependent Incorporation of H3.1 at UV-Damage Sites

In order to investigate the molecular mechanism of H3.1 deposition at UV-damage sites, we focused on specific histone chaperones. Given that H3.1 readily associates with CAF-1 outside S phase (Figure 2B), and that XPG$^{-/-}$ cells are unable to recruit CAF-1 at UV-damage sites (Figure 2D; Green and Almouzni [2003]), the above data suggest a requirement for CAF-1 in H3.1 deposition at sites of UV lesions. Thus, to explore a direct dependence on CAF-1, we targeted the CAF-1 p60 subunit by RNA interference (RNAi) in HeLa cells, prior to transient transfection with the e-H3.1 expression vector. Importantly, CAF-1 p60 knockdown did not significantly affect subsequent transfection with e-H3.1 (Figure 3A). Remarkably, it did not interfere with the recruitment of the largest subunit of CAF-1 (p150) to UV-damage sites (Figure 3B). However, this knockdown severely impaired e-H3.1 incorporation into chromatin at UV-damage sites, as observed 30 min (Figure 3B) as well as 6 hr postirradiation (not shown). We obtained similar results when using two different p60-specific siRNAs, which ruled out the possibility of an off-target effect. Moreover, RNAi targeting of the chromatin assembly factor *hi*stone *r*egulator *A* (HIRA), which is involved in H3.3 deposition independently of DNA synthesis in vitro (Tagami et al., 2004), did not prevent local incorporation of e-H3.1 (Figure S4). From these results we conclude that de novo incorporation of e-H3.1 occurs locally at UV-damage sites in an NER and CAF-1 dependent manner.

CAF-1 Function Is Necessary after Repair of UV Lesions

The above data provide evidence for a specific involvement of CAF-1 in H3.1 dynamics during UV response. However, it remains unclear whether this relates (1) to a direct requirement for CAF-1 in histone deposition at UV-damage sites in vivo or (2) to CAF-1 impact on UV response through induction of UV-damage signaling or repair. In order to discriminate between these possibilities, we first analyzed UV-damage checkpoint activation upon CAF-1 loss of function using the same conditions as above (i.e., siRNA against CAF-1 p60 in HeLa cells). We monitored cell-cycle distribution by flow cytometry (Figure 4A) and verified that both sip60 and siGFP control cells displayed similar distributions throughout the cell cycle at the time of UV irradiation. Interestingly, both of them accumulated in G2/M upon global UV irradiation, arguing that CAF-1 function was dispensable for UV-induced cell-cycle arrest. We next examined phosphorylation of the checkpoint kinase Chk1, which is part of the signaling pathway activated in response to UV irradiation. Induction of this phosphorylation event was not impaired upon CAF-1 p60 knockdown (Figure 4B). Taken together, these results indicate that CAF-1 function is dispensable for UV-damage checkpoint activation. We thus infer that CAF-1-mediated incorporation of e-H3.1 at UV-damage sites does not reflect a connection with induction of UV-damage signaling.

Alternatively, this could relate to an involvement of CAF-1 before initiation of the repair process, for example if CAF-1 was to act as a histone acceptor during chromatin disruption and/or in a feedback loop to block the NER pathway. We thus examined how CAF-1 loss of function by RNAi in HeLa cells would impact on the sequential steps of the NER process. For this, we targeted both p60 (not shown) and p150 subunits. In the latter case, we performed an extensive study. To circumvent the accumulation of cells in S phase arising from CAF-1 p150 loss of function (Hoek and Stillman, 2003; Quivy et al., 2004; Ye et al., 2003), we used short-term siRNA treatment (48 hr) and selected cells outside S phase by immunofluorescence analysis as described above (Figure 2A). We verified that CAF-1 p150 knockdown did not affect the expression of factors involved in NER, such as PCNA and XPC, nor CAF-1 p60 total protein amount, although it led to a reduction in p60 phosphorylation level (Figure 5A). Importantly, CAF-1 p150 depletion impaired p60 local recruitment to chromatin at NER sites (Figure 5B). In these loss-of-function conditions, we first focused on the earliest step of the repair pathway by following recruitment of XPC factor, which is involved in UV-lesion detection. Contrary to CAF-1 p60, XPC (Figure 5C) was efficiently recruited to UV-damage sites upon CAF-1 loss of function, with the same kinetics and in the same proportion of cells as compared to cells treated with siGFP control. Late steps in the repair pathway were not significantly affected. Indeed, we did not detect a significant change in the rate of UV-lesion removal (6,4-PP or CPD) (Figure 5D), and repair synthesis also occurred efficiently in the absence of CAF-1 p150, as monitored by local PCNA recruitment (Figure 5E) and BiodU incorporation (Figure 5F).

From these data, we conclude that CAF-1 p150 expression and p60 recruitment to chromatin are dispensable for completion of the repair process, from lesion detection up

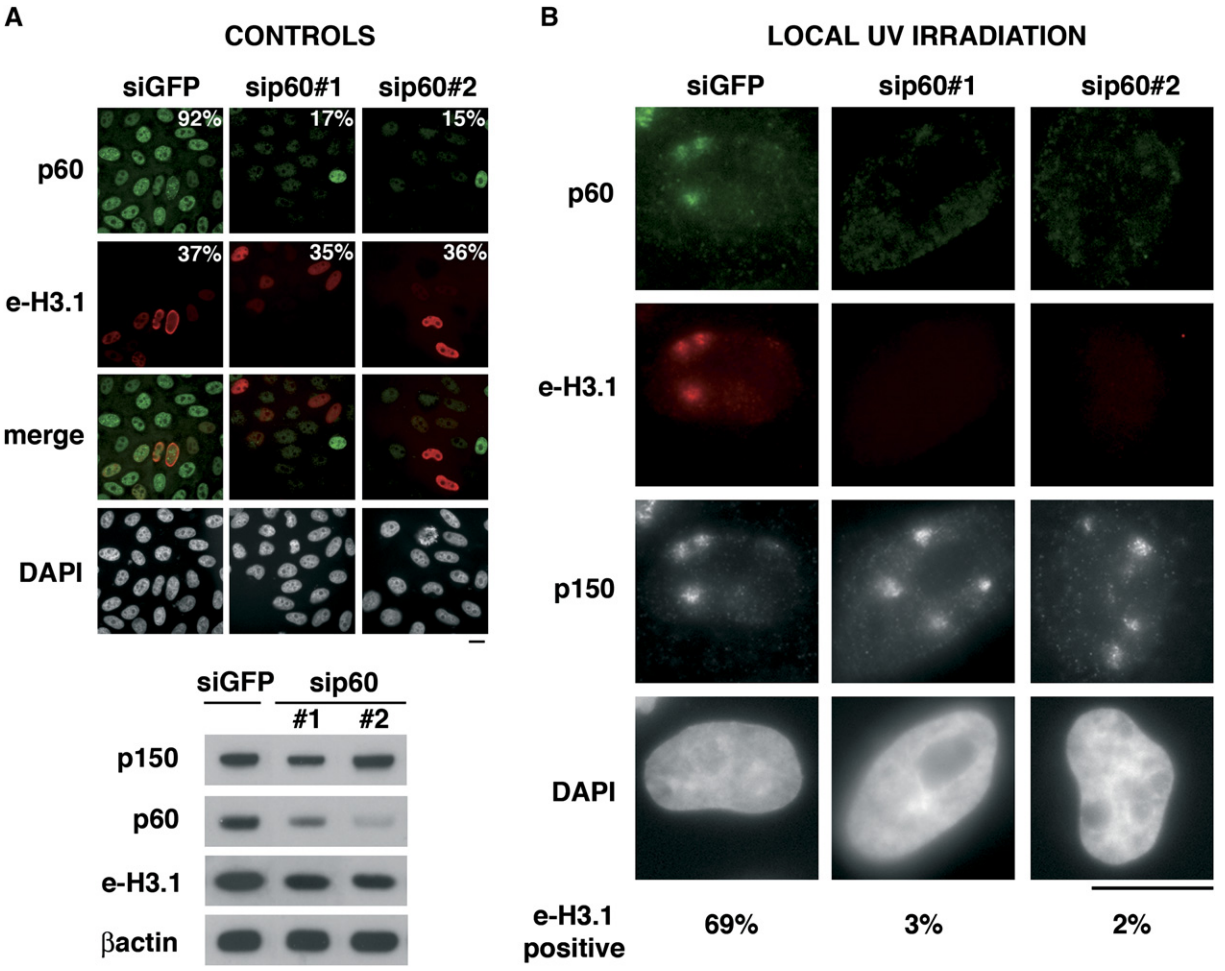


Figure 3. CAF-1 Requirement for H3.1 Incorporation at UV-Damage Sites

(A) Efficiency of p60 siRNA and e-H3.1 transfection analyzed by immunofluorescence (upper panel) and western blot (lower panel) on HeLa cells treated with a control siRNA targeted against GFP (siGFP) or a p60-specific siRNA (sip60#1 or sip60#2) and subsequently transfected with e-H3.1 vector. Percentages of p60 and e-H3.1 expressing cells are indicated.

(B) Immunolocalization of chromatin bound CAF-1 p60, p150, and e-H3.1 30 min after local UV irradiation of the same cells. The percentages indicate the fraction of transfected cells showing local e-H3.1 recruitment (e-H3.1 positive). We used Triton-resistant CAF-1 p150 staining as a reference to select cells outside S phase. Scale bars, 10 μm. Data were obtained from at least two independent experiments.

to repair synthesis. Our results thus eliminate the hypothesis of a feedback loop of CAF-1 function on the NER process and the possibility that CAF-1 could act in early steps of the UV-damage response. Our data rather provide new evidence that CAF-1 plays a critical role after repair of UV lesions in the cell and directly stimulates de novo deposition of H3.1 histones at NER sites, a process that is not restricted to S phase cells. Interestingly, we also observed local deposition of e-H3.1 at sites of laser microirradiation (Figure 6), which mainly generates oxidative base damage and DNA breaks. New histone deposition is thus not restricted to the response to UV-C induced pyrimidine dimers, extending the generality of our findings to other types of DNA damage. Getting back to our initial question (Figure 1A), not only do we show that new histones are incorporated at sites of DNA damage in vivo, but we also provide mechanistic insights into the deposition process.

DISCUSSION

In eukaryotes, the cellular response to genotoxic insults involves both repair of DNA lesions and changes at the level of chromatin, including chromatin disruption and subsequent restoration. A long-standing issue with respect to such chromatin dynamics in the restoration step is to determine whether it involves histone recycling and/or deposition of new histones. This is obviously a critical question in the context of reestablishing the epigenetic status of a damaged region. In the present study, we have examined, for the first time, the dynamics of the H3.1 histone variant during UV response in human cells. We show that

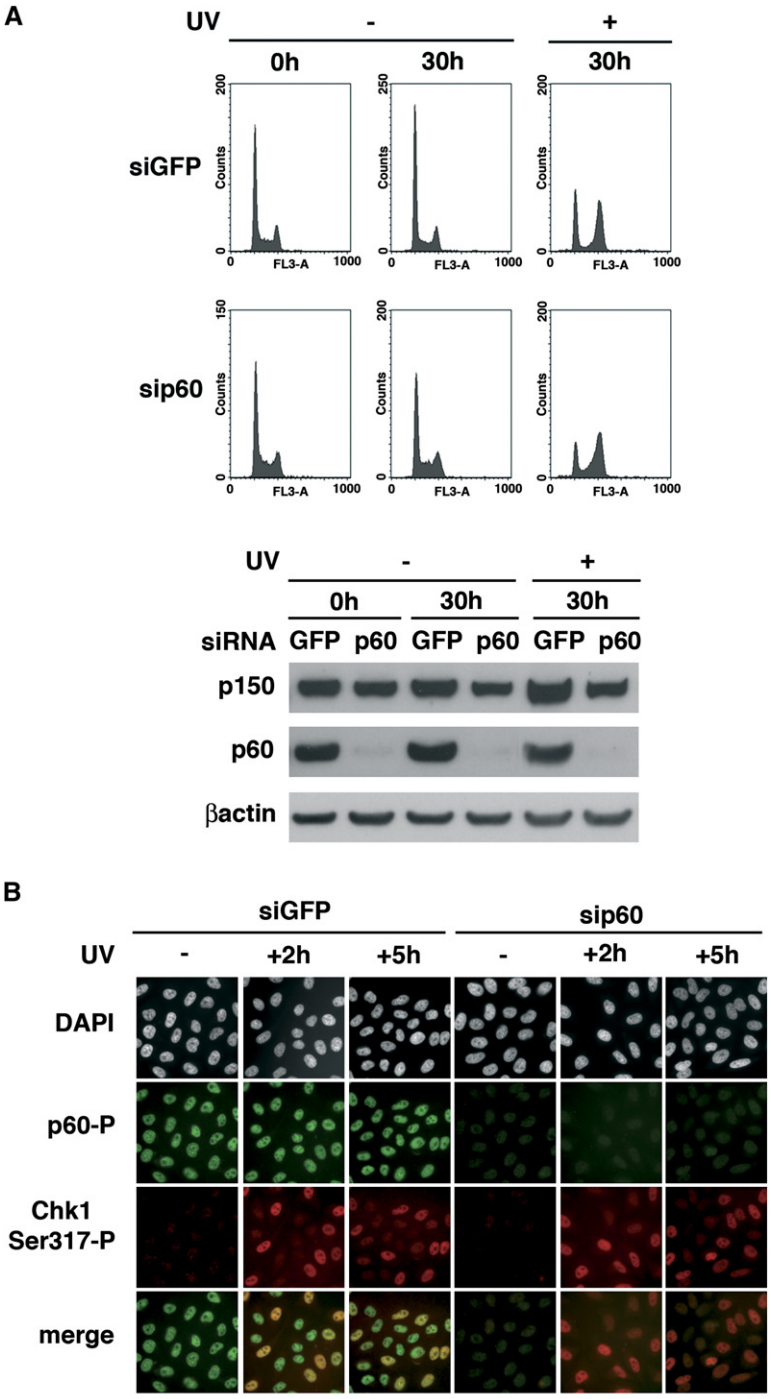


Figure 4. CAF-1 Function Is Dispensable for Induction of UV-Damage Signaling

HeLa cells were treated with a control siRNA targeted against GFP or a p60-specific siRNA before global UV irradiation at 10 J/m^2 (+), using unirradiated cells (–) as a reference.

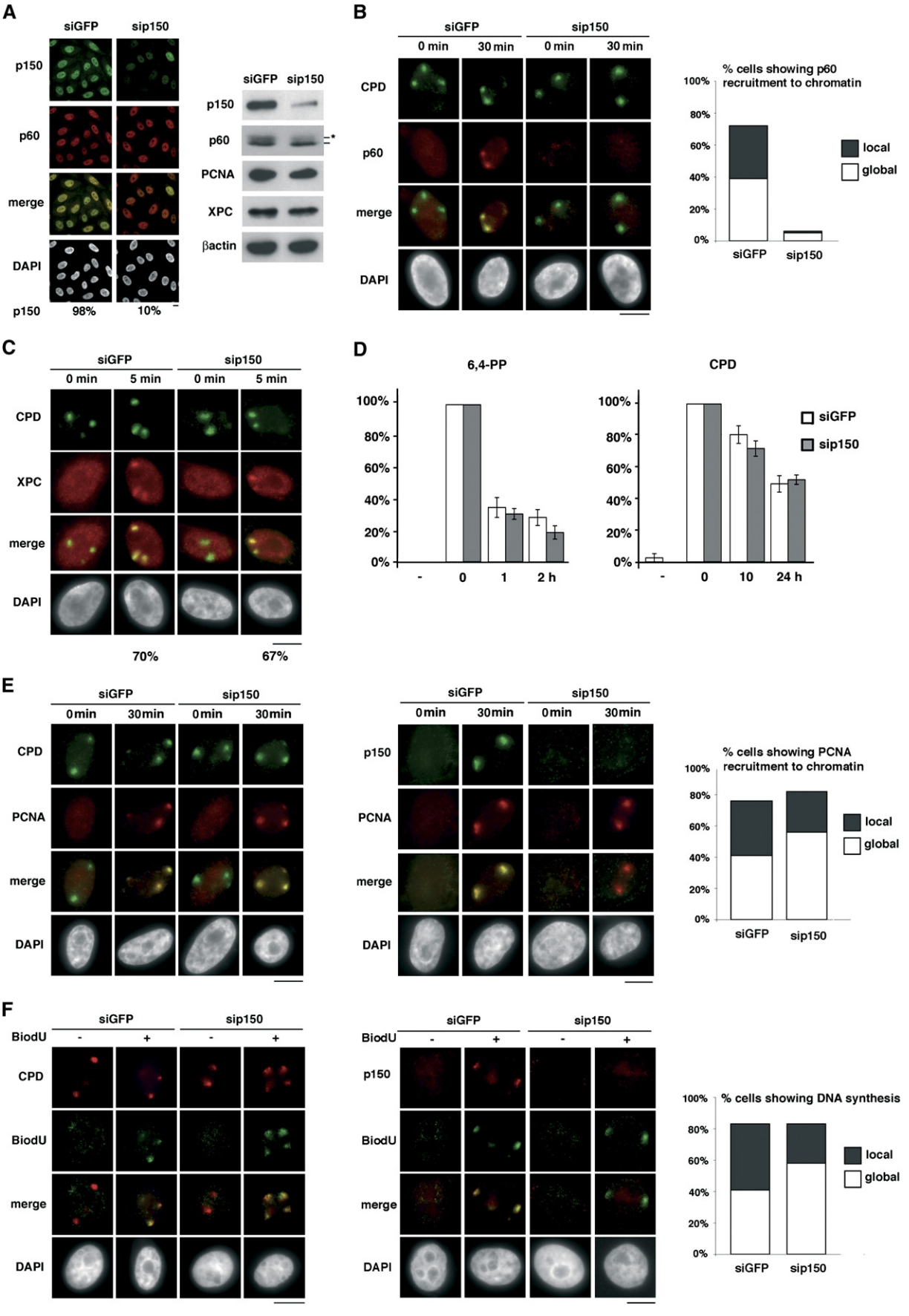
(A) Cell-cycle distribution was analyzed by flow cytometry (upper panel) at the time of UV irradiation (0 hr) and 30 hr afterwards. CAF-1 p60 knockdown was controlled by western blot (lower panel).

(B) Chk1 phosphorylation on Serine 317 was monitored by immunofluorescence 2 hr and 5 hr after UV irradiation. Scale bar, 10 μm.

de novo deposition of H3.1 variants occurs at UV-damage sites in a UV-repair-dependent manner and that the histone chaperone CAF-1 is specifically and directly involved in this process.

Histone Dynamics during Chromatin Restoration at Repair Sites

While incorporation of newly synthesized histones by de novo chromatin assembly is well characterised during DNA replication (Kaufman and Almouzni, 2006), it was unclear whether a similar mechanism would take place during repair, when extensive DNA and histone syntheses do not occur. In particular, NER is characterized by a short patch of repair synthesis of about 30 nucleotides in length (Reardon and Sancar, 2005). A tentative scenario for histone dynamics during the repair response would thus involve recycling of preexisting histones rather than new histone deposition. In this work, however, we show de novo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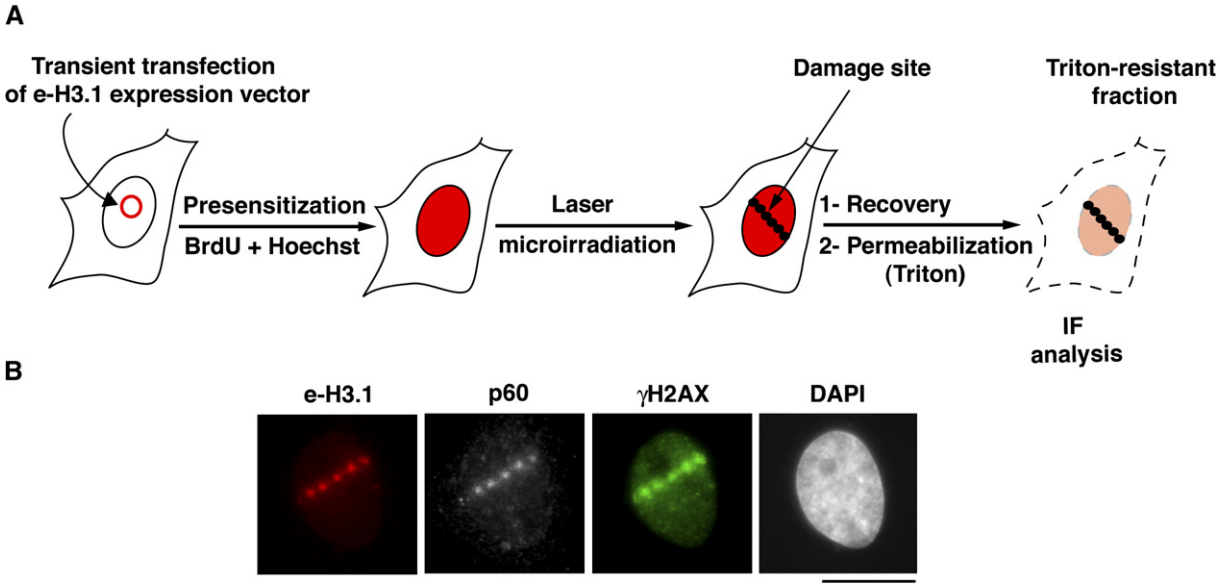

Figure 6. e-H3.1 Deposition at Sites of Laser Microirradiation

(A) Scheme of the experiment.

(B) Immunodetection of e-H3.1 and CAF-1 p60 subunit 30 min after exposure to the laser beam. Sites of microirradiation are visualized by γH2AX staining. Scale bar, 10 μm.

incorporation of H3.1 histones at NER sites in response to local UV irradiation. Given our unselective irradiation approach, incorporation of new H3.1 histones is unlikely to be restricted to specific chromatin regions, in particular to regions initially devoid of nucleosomes such as active ribosomal genes (Conconi et al., 2005). Furthermore, the strong overlap with the damaged chromatin region argues that new histone deposition is not a global response to local UV damage, in marked contrast to p53-dependent chromatin relaxation reported to affect the whole nucleus (Rubbi and Milner, 2003).

Remarkably, our data challenge the idea that H3.1 incorporation into chromatin is restricted to S phase and support a more general coupling of H3.1 deposition to DNA synthesis events. It is noteworthy that the fraction of newly synthesized H3.1 is likely to be limited outside S phase, in light of the tight coupling between H3.1 synthesis and DNA replication (Osley, 1991; Wu et al., 1982). Yet, if we consider the entire pool of histones immediately available for deposition onto DNA, our data suggest that a significant fraction of H3.1 can be available outside S phase. This pool could be sufficient to ensure new H3.1 incorporation in response to low UV doses. However, we do not exclude that high requirements upon exposure to high UV doses may need more histones and that these situations could (1) favor more active histone recycling at damage sites, (2) stimulate new H3.1 synthesis, or (3) inhibit H3.1 degradation.

While we focus here on H3.1 histones, which specifically associate with CAF-1 in vivo (Tagami et al., 2004), we do not rule out the possibility that other H3 variants could also be incorporated at NER sites. We should stress that the cell-cycle independent incorporation of H3.3 can mask local deposition events and makes this analysis more difficult. However, in a few cells exposed to high UV doses, we could observe a local concentration of e-H3.3 at UV-damage sites (data not shown). In this respect, it is noteworthy that visualization of a local accumulation of

Figure 5. CAF-1 Function Is Dispensable for NER

(A) Immunofluorescence (left) and western blot analyses (right) of HeLa cells 48 hr posttransfection with a control siRNA targeted against GFP (siGFP) or a p150-specific siRNA (sip150). Percentages of p150-expressing cells are indicated below. *: phosphorylated p60.

(B, C, and E) p60, XPC, and PCNA recruitment to UV-damage sites (CPD) analyzed by immunofluorescence at the indicated times after local UV irradiation in siRNA-treated HeLa cells. Percentages of cells showing XPC recruitment to UV lesions are indicated below. Percentages of cells showing global (replication-associated) or local (repair-associated) recruitment of p60 and PCNA to chromatin are plotted on the right.

(D) Time course of UV lesion removal. We analyzed DNA extracted from siRNA- treated and UV-irradiated HeLa cells as described in Experimental Procedures. A plot compiling the results relative to the initial amount of UV damage and corrected for the total amount DNA is presented for 6,4-PPs (left) and CPDs (right) (mean ± SD). −: unirradiated cells.

(F) Repair synthesis at UV damage sites (CPD) revealed by BiodU incorporation in siRNA treated HeLa cells. *In situ* DNA synthesis was performed in the presence (+) or absence (−) of BiodU as a control. Percentages of cells showing global or local BiodU incorporation (DNA synthesis associated with replication and repair, respectively) are plotted on the right. Scale bars, 10 μm.

ectopically expressed H3.3 by immunofluorescence analysis could be achieved so far only upon H3.3 overexpression and/or in response to massive transcription activation (Ahmad and Henikoff, 2002; Janicki et al., 2004; Schwartz and Ahmad, 2005). Thus, incorporation of this variant at damage sites, which is a limited phenomenon detectable under extreme conditions, most likely reflects an unspecific mechanism to fill gaps.

Regardless of which histone variant gets incorporated de novo at repair sites, a prerequisite for such a process is local eviction of nucleosomal histones. Interestingly, a recent study in yeast provides evidence for loss of nucleosomes at a double-strand break (Tsukuda et al., 2005). While direct evidence for nucleosome eviction at NER sites is still a matter of debate (Thoma, 1999), our findings support the view that nucleosomes are initially disassembled to permit incorporation of new histones at UV-damage sites in human cells. The mechanism and extent of histone eviction as well as the fate of evicted histones are still open issues. Furthermore, the incorporation of new histones within damaged chromatin does not preclude partial recycling of old histones. This would be ensured by histone chaperones that would assist their reassembly. In this respect, the histone chaperone antisilencing function 1 (ASF1) is an interesting candidate both as a donor of new histones and as an acceptor for evicted histones, given its documented synergy with CAF-1 in vitro (Mello et al., 2002) and its role in chromatin disassembly in yeast (Adkins and Tyler, 2004; Schwabish and Struhl, 2006) and in buffering excess S phase histones in human cells (Groth et al., 2005). However, due to the multiplicity of ASF1 functions, extensive studies will be required to elucidate its precise role(s) in histone dynamics at damage sites.

CAF-1 Function at Repair Sites

In this report, we also provide insight into the function of CAF-1 during the UV response in vivo by exploring the dynamics of the replicative H3.1 variant. Furthermore, our loss-of-function studies showing that CAF-1 p150 is recruited to damage independently of CAF-1 p60, but not vice versa, support a key role for CAF-1 large subunit as the molecular link to UV damaged chromatin in vivo. This is consistent with the reported interaction of this subunit with PCNA (Moggs et al., 2000; Shibahara and Stillman, 1999), whereby the CAF-1 complex would be targeted to sites of repair synthesis. Interestingly, the p150 subunit can support de novo histone incorporation only in connection with p60. Indeed, the siRNA against p60 still permits p150 recruitment, yet no H3.1 can be deposited (Figure 3). We also noticed that CAF-1 p150 and p60 knockdowns have strinkingly different outcomes. While CAF-1 p150 loss of function results in S phase accumulation followed by checkpoint activation (Hoek and Stillman, 2003; Quivy et al., 2004; Ye et al., 2003), p60 knockdown does not affect cell-cycle distribution (Nabatiyan and Krude [2004]; this study). These observations suggest that, although both subunits are required for CAF-1 function in chromatin assembly, they are also likely to display distinct properties, which are currently under investigation (J.-P. Quivy, A. Gérard, D.R., and G.A., unpublished data).

Importantly, we show for the first time in human cells that CAF-1 is dispensable for repair of UV damage. Similarly, in budding yeast, the NER pathway is functional in CAF-1 mutant strains (Game and Kaufman, 1999), yet these strains display a moderate increase in UV sensitivity (Game and Kaufman, 1999; Kaufman et al., 1997) and show genome instability (Myung et al., 2003). Thus, CAF-1 importance in response to UV damage in vivo is most likely related to its specific contribution downstream of the repair process. A similar function of CAF-1 could also be at work in response to other types of DNA damage (Lewis et al., 2005; Linger and Tyler, 2005; Moggs et al., 2000; Nabatiyan et al., 2006; Okano et al., 2003). Our data, showing that e-H3.1 also accumulates at sites of laser microirradiation, clearly support this possibility. Collectively, our findings underscore a critical role for the histone chaperone CAF-1 in de novo deposition of H3.1 at UV-damage sites, most likely as an H3.1-H4 histone donor that is directly involved in chromatin restoration coupled to NER. This fundamental process should be considered as an integral part of the damage response for our understanding of genetic and epigenetic stability.

Epigenetic Stability Versus Memory of Damage at Repair Sites

While CAF-1-mediated deposition of H3 and H4 histones at repair sites contributes to restoration of a proper chromatin structure, it is only the very first step in de novo chromatin assembly. To fully restore chromatin organization, additional histone and nonhistone proteins are necessary. Furthermore, our results showing that incorporation of new histone occurs raise the important issue of how epigenetic memory can be preserved. In this respect, it will be important to evaluate to which extent the incorporation of new histones can spread from the damage site, given that CAF-1 was shown to stimulate histone deposition in vitro several hundred bp distant from the initial damage (Gaillard et al., 1997). Indeed, large-scale epigenetic changes would have a stronger impact on genome function. Furthermore, we anticipate that modifications associated with newly synthesized histones (Sobel et al., 1995) should be detected at least transiently at NER sites. Given that both histone posttranslational modifications and histone variants can contribute to epigenetic marking (Henikoff and Ahmad, 2005; Turner, 2002), the question that ensues is whether, how, and when original marks will be reestablished. Neighboring chromatin regions that did not undergo extensive rearrangements during repair may then serve as an epigenetic template for newly incorporated histones. Indeed, histone-modifying enzymes can be recruited to specific histone modifications through defined protein modules and thereby allow the spreading of these modifications to neighboring nucleosomes (Jenuwein and Allis, 2001; Turner, 2002). Alternatively, a partial recycling of old histones might contribute to the maintenance of epigenetic information by a semiconservative

mechanism, similar to the one already proposed for DNA replication: this model involves disruption of parental $(H3\text{-}H4)_2$ tetramers into H3-H4 dimers, which can associate with newly deposited ones (Tagami et al., 2004). Conversely, the incorporation of H3.1 variants into a defined chromatin region can also be viewed as an imprint for newly repaired chromatin, as a memory of the damage event. A critical issue is to determine if such an imprint is part of a short-term response, which could impact on damage signaling (maintenance and/or recovery), or if it is a long-term mark. In this respect, it would be interesting to examine the persistence of H3.1 variants at their incorporation sites over several cell cycles. Such a memory of damage could play a role in processes such as radiation-induced genomic instability, which arises in the progeny of the damaged cells after several generations (Little, 2003).

Collectively, these data provide evidence for the local dynamics of variant histones at UV-damage sites in vivo and put forward CAF-1 as a key player in this process. This fundamental mechanism contributes to the maintenance of chromatin organization along with genome integrity in response to genotoxic insults.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa cells (gift from M. Bornens, Curie Institute, Paris, France), HeLa cells stably transfected with H3.1-Flag-HA (HemAgglutinin) or H3.3-Flag-HA (Tagami et al., 2004), WI38 diploid fibroblasts (LGC Promochem, Molsheim, France), MCF7 cells (gift from O. Delattre, Curie Institute, Paris, France), and $XPG^{-/-}$ cells (XP3BR, gift from A. Sarasin, IGR, Villejuif, France) were grown in Petri dishes (Falcon Plastics, Cockeysville, MD) in the appropriate medium complemented with 10% foetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Company). HeLa, MCF7, and $XPG^{-/-}$ cells were grown in DMEM, and WI38 cells were grown in MEM α medium. MCF7 cells were arrested in G0 by 48 hr treatment with 10 nM ICI182780 (Carroll et al., 2000; Polo et al., 2004).

Cell Irradiation

For UV irradiation, cells were treated with UV-C (254 nm) using a low-pressure mercury lamp, and conditions were set using a VLX-3W dosimeter (Vilbert-Lourmat). Cells were either subjected to global (10 J/m^2) or local UV irradiation (150 J/m^2, using 3μm or 8μm pore filters (Millipore) as described (Gérard et al., 2006; Green and Almouzni, 2003; Mone et al., 2001).

For laser microirradiation, cells were presensitized by incubation with 10 μM BrdU and 1.6 μM Hoechst 33258 before microirradiation with a UV-A laser line (406 nm, 50% output, 10 ms, beam expander 4) focused through a 100× oil objective on a Deltavision RT microscope (Applied Precision) (adapted from Limoli and Ward [1993], Lukas et al. [2003], and Rogakou et al. [1999]).

Flow Cytometry

Cells were fixed in ice-cold 70% ethanol before DNA staining with 50 μg/ml propidium iodide (Sigma Aldrich) in phosphate buffer saline containing 0.5 mg/ml RNase A (Amersham). DNA content was analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson).

Antibodies

Primary antibodies against CAF-1 subunits were anti-p150 (ab7655 Abcam), monoclonal anti-p60 (ab8133 Abcam), and polyclonal anti-p60 characterized in our laboratory (Green and Almouzni, 2003). Monoclonal anti-p60 mAb8133 recognizes only phosphorylated p60, whereas polyclonal anti-p60 recognizes both phosphorylated and unphosphorylated forms. Other primary antibodies were anti HIRA (gift from P. Adams, Fox Chase center, PA), anti-PCNA PC10 (Dako) and FL-261 (Santa Cruz), anti-XPC (gift from F. Hanaoka, Osaka University, Osaka, Japan), anti-CPD (Kamiya Biomedicals), anti-6,4-PP (gift from T. Matsunaga, Kanazawa University, Kanazawa, Japan), anti-γH2AX (JBW301, Upstate), anti-phospho-Chk1 Ser317 (Cell Signaling), anti-H3 (ab7834, Abcam), anti-HA (3F10, Roche), and anti-βactin (AC15, Sigma Chemical Company). Secondary antibodies coupled to Fluorescein IsoThioCyanate, Texas red, Cyanin3, or Horse Radish Peroxidase were purchased from Jackson Laboratory. Highly cross-absorbed anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 680 (Molecular Probes), and anti-rat Cyanin3 antibodies were used for triple labeling.

Immunofluorescence

Immunofluorescence on paraformaldehyde fixed cells, image capture, and processing were performed as described previously (Green and Almouzni, 2003). DNase treatment was carried out as described (Martini et al., 1998). Percentages of positively stained cells were obtained by scoring over 200 cells in at least two independent experiments. To take into account e-H3.1 transfection efficiency, we calculated systematically the ratio between the fraction of cells exhibiting local e-H3.1 accumulation and the fraction of transfected cells. To focus on cells outside S phase, we used either PCNA or CAF-1 Triton-resistant staining, which displays characteristic patterns in S phase nuclei (Figure 2A).

Cell Extracts and Western Blot

Total and Triton-treated cell extracts were made and subjected to western blotting as described (Martini et al., 1998). Cell extracts containing soluble histones were obtained by cell lysis in high salt-extraction buffer (50 mM Tris pH 7.5, 300 mM NaCl, 0.5% NP-40, and protease inhibitors), after centrifugation (14,000 rpm, 15 min, 4°C) to remove insoluble components.

Triton-Acid-Urea Gel Analysis

For specific detection of H3 variants, samples were run on a Triton-acid-urea (TAU) gel (12% polyacrylamide, 6 M urea, 5% acetic acid, 0.4% Triton X-100) overnight at 200 V in 5% acetic acid (Zweidler, 1978). After protein transfer on nitrocellulose membrane (Thiriet and Albert, 1995), H3 variants were detected using anti-H3 antibody.

Analysis of UV-Lesion Removal

Total genomic DNA was extracted from UV-C irradiated cells (10 J/m^2). DNA samples in 0.2 M NaOH were incubated at 37°C for 15 min and boiled and spotted onto Hybond N+ membrane (Amersham) using a Bio-Dot apparatus (Bio-Rad). Two dilutions (1 and 0.5 μg) were spotted for each time point. The membrane was dried at 80°C for 1 hr and blocked with 5% milk before immunodetection of UV lesions (6,4-PPs and CPDs). Total DNA was subsequently visualized on the same membrane by ethidium bromide intercalation. Quantitation was performed using Quantity One analysis software (Bio-Rad).

In Situ DNA Synthesis

Cells were grown on collagen-fibronectin-treated glass coverslips and subjected to local UV irradiation. They were allowed to recover for 20 min postirradiation at 37°C. Biotin-16-deoxyuridine (BiodU) incorporation was performed by in situ run-on on Triton-permeabilized cells for 40 min at 37°C under conditions described for isolated nuclei (Taddei et al., 1999). FITC-conjugated streptavidin (Enzo) and biotinylated anti-streptavidin antibody (Abcys) were used for biotin immunodetection on paraformaldehyde-fixed cells.

siRNA Design and Cell Transfection

siRNA oligonucleotides (MWG-Biotech and Sigma Genosys) were targeted against the following mRNA sequences: CAF-1 p150,

AAGGAGAAGGCGGAGAAGCAG (Quivy et al., 2004); CAF-1 p60#1, AAGCGUGUGGCUUUCAAUGUU; CAF-1 p60#2, AAUCUUGCUCGU CAUACCAAA; green fluorescent protein (GFP), AAGCUGGAGUAC AACUACAAC. siRNAs were chemically synthesized with the Silencer siRNA construction kit (Ambion) according to manufacturer's instructions. SiRNA transfections were performed using oligofectamine reagent (Invitrogen) in Optimem1 medium and standard culture medium lacking antibiotics (Invitrogen). The final concentration of siRNA in the culture medium was 30 nM.

Cells were transiently transfected with H3.1-Flag-HA expression vector pOZ-FH-C, a Moloney Murine Leukemia Virus-derived vector, which allows a low expression level of the transgene (Nakatani and Ogryzko, 2003; Tagami et al., 2004). Transfection was performed using Lipofectamine2000 (Invitrogen) according to manufacturer's instructions 20 hr before subsequent cell treatment.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/3/481/DC1/.

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