**SUMMARY**

Understanding how to recover fully functional and transcriptionally active chromatin when its integrity has been challenged by genotoxic stress is a critical issue. Here, by investigating how chromatin dynamics regulate transcriptional activity in response to DNA damage in human cells, we identify a pathway involving the histone chaperone histone regulator A (HIRA) to promote transcription restart after UVC damage. Our mechanistic studies reveal that HIRA accumulates at sites of UVC irradiation upon detection of DNA damage prior to repair and deposits newly synthesized H3.3 histones. This local action of HIRA depends on ubiquitylation events associated with damage recognition. Furthermore, we demonstrate that the early and transient function of HIRA in response to DNA damage primes chromatin for later reactivation of transcription. We propose that HIRA-dependent histone deposition serves as a chromatin bookmarking system to facilitate transcription recovery after genotoxic stress.

**INTRODUCTION**

To prevent the deleterious effects of DNA lesions that constantly challenge genome integrity (Hoeijmakers, 2009), cells have developed mechanisms—collectively referred to as the DNA damage response (DDR)—to signal and repair DNA damage (Ciccia and Elledge, 2010; Giglia-Mari et al., 2011; Jackson and Bartek, 2009). Beyond genome stability, the maintenance of a defined organization of the genome into chromatin, with specific patterns of histone variants and their marks (Talbert and Henikoff, 2010), is critical for preserving genome functions and cell identity. Concerning histone H3 variants, as described in mammals, the replicative variant H3.1 is incorporated into chromatin genome-wide in a manner coupled to DNA synthesis during replication and repair. In contrast, the replacement variant H3, while present at telomeric and pericentric heterochromatin, has been strongly associated with actively transcribed genes (Filipescu et al., 2013). However, whether H3.3 deposition drives or simply reflects transcriptional activity is still under debate. Recent data support the idea that histone marking by H3.3 incorporation is required for either the activation or the long-term maintenance of gene expression patterns (reviewed in Skene and Henikoff, 2013; Szenker et al., 2011), illustrating the importance of chromatin integrity in cell fate determination.

DNA damage challenges chromatin integrity by eliciting the destabilization of chromatin structure, followed by restoration of its organization (reviewed in Adam and Polo, 2012; Smerdon, 1991; Soria et al., 2012). In addition, in response to various types of DNA lesions, a local inhibition of transcription ensues, as best characterized for RNA polymerase II (RNAPII)-dependent transcription (Chou et al., 2010; Iacovoni et al., 2010; Moné et al., 2001; Pankotai et al., 2012; Shanbhag et al., 2010; Tornaletti, 2009). Such DNA damage-induced transcription arrest is critical to prevent the production of aberrant transcripts and to avoid interference between transcription and repair machineries (Svejstrup, 2010). Much effort has been devoted to understanding how transcription inhibition is achieved, particularly in response to bulky DNA lesions, which result in RNAPII stalling, followed by DNA damage bypass, RNAPII backtracking, or RNAPII removal by proteasomal degradation (Hanawalt and Spivak, 2008; Tornaletti, 2009). Displacement of stalled RNAPII allows completion of DNA repair and subsequent restart of transcriptional activity in regions that have been silenced in response to DNA damage (Gaillard and Aguilera, 2013). However, what triggers and controls transcription restart once repair is complete is still an open issue. Given the involvement of chromatin marks in regulating gene expression and integrating damage signals (Li et al., 2007; Lujisterburg and van Attikum, 2011), it is tempting to hypothesize that mechanisms involving chromatin marking could contribute to transcription recovery upon DNA damage.

Significant progress has been made in characterizing key players in chromatin dynamics that respond to DNA damage (reviewed in Soria et al., 2012). Among them, histone chaperones, which escort histones upon their synthesis up to their deposition on to DNA (Burgess and Zhang, 2013; De Koning et al., 2007), are of particular interest. Several of these chaperones have been involved in the DDR (reviewed in Avvakumov et al., 2011; Soria et al., 2012), including the H3 variant-specific chaperones...
Transcription Recovery after DNA Repair Involves the Histone Chaperone HIRA

In order to address whether transcription regulation may be connected to chromatin dynamics in response to DNA damage (Figure 1A), we quantified nascent transcripts by 5-ethynyl uridine (EU) labeling after global UV type C (UVC) irradiation in cells depleted of candidate histone chaperones (Figure 1B). Our detailed kinetic analysis of nascent transcript production after UVC irradiation revealed that transcription inhibition and recovery were best achieved at 2 hr and 24 hr postirradiation, respectively (data not shown), which is consistent with previous reports (Jensen and Mullenders, 2010; Nakazawa et al., 2010; Zhang et al., 2012). We thus selected these time points for subsequent analyses. The histone chaperone CAF-1, previously shown to be involved in the cellular response to UVC irradiation (reviewed in Adam and Polo, 2012), did not affect transcription arrest and subsequent recovery after DNA damage as shown by depleting its p60 subunit (Figure 1C; Table S1 [for small interfering RNAs, siRNAs] and Table S2 [for antibodies]). Strikingly, HIRA downregulation, while leaving transcription arrest unaffected, significantly impaired transcription recovery to an extent comparable to that observed in repair-deficient cells (excision-repair cross-complementing 6; sierCC6) (Figure 1C; Figure S1A available online; note that knockdown efficiencies are comparable throughout the experiment). We verified that the inhibitory effect of depleting HIRA on transcription recovery could not be explained by significant alterations in cell cycle progression (Figure S1B). Similar results obtained in U2OS cells (Figure S1D) further indicate that our findings are not limited to a particular cell line. In addition, the defect in transcription restart observed upon HIRA knockdown cannot be due to a general loss of function of the transcription machinery, as attested by the levels and phosphorylation status of RNAPII large subunit, which return to normal 24 hr post-UVC irradiation in HIRA-depleted cells (Figure S1E).

Considering that these results may reflect a general role of HIRA associated to any disruption of transcription independent of DNA damage, we examined the effect of HIRA depletion on transcription in undamaged cells treated with 5,6-dichlorobenzimidazole 1-[β-D-ribofuranoside (DRB), a reversible transcription inhibitor (Sensaude, 2011). Remarkably, in this setting, HIRA downregulation did not affect transcription recovery (Figure S1F). Thus, the capacity of HIRA to promote transcription restart after DNA damage is not a general response to transcription inhibition, and it may reflect a direct role of HIRA in UVC damage repair. To address this possibility, we first tested the ability of HIRA-depleted cells to survive UVC damage by performing clonogenic assays. Thus, we demonstrated that HIRA knockdown did not sensitize cells to UVC damage (Figure S1G). We then examined more specifically the repair capacity of HIRA-depleted cells by measuring the removal kinetics of cyclobutane pyrimidine dimers (CPDs), the major UVC-induced DNA photo-products (Figure 2A). These photolesions are known to be excised with slow kinetics (Ehmann et al., 1978); therefore, we focused our analysis on late time points after UVC (24–34 hr). Consistent with the fact that HIRA-depleted cells are not hypersensitive to UVC damage, we found that these cells did not display any significant delay in UVC damage removal when exposed to global irradiation (Figure 2A). In line with these findings, the recruitment of the nucleotide excision repair factor Xeroderma Pigmentosum, complementation group B (XPB) to sites of local UVC irradiation was not affected by HIRA depletion, and DNA repair synthesis revealed by 5-ethyl-2'-deoxyuridine (EdU) incorporation was similar to control (Figure 2B). Thus, these results show that HIRA-depleted cells do not display overall defects in UV damage repair. Similar results were obtained upon CAF-1 and HIRA codepletion (data not shown), thus discarding the hypothesis of a functional redundancy between CAF-1 and HIRA in UVC damage repair. Collectively, our data demonstrate that the histone chaperone HIRA plays a critical role in transcription recovery upon UVC damage and does so without significantly affecting the repair process per se.

HIRA Accumulation in Damaged Chromatin Regions

To determine whether HIRA contribution to transcription recovery after UVC irradiation involves a local action of this chaperone at damage sites, we investigated the recruitment of HIRA to damaged chromatin. As previously demonstrated (Green and Almouzni, 2003; Polo et al., 2006), CAF-1 p60 and p150 subunits accumulated in regions of local UVC irradiation (Figure 2C). Remarkably, we could detect all known subunits of the HIRA complex—HIRA, ubinuclein 1 (UBN1) and calcineurin-binding protein 1 (CABIN1) (Amin et al., 2012)—enriched in damaged chromatin regions (Figure 2C). By contrast, in our cell line model, we did not observe any detectable recruitment of other known H3 histone chaperones (Burgess and Zhang, 2013), including death-domain associated protein (DAXX), nuclear autoantigenic sperm protein (NASP), and antisilencing function 1 (ASF1).
HIRA recruitment to DNA damage was observed in the vast majority of damaged cells, both in tumoral cells and primary fibroblasts (Figure 2C; Figure S2A). It occurred regardless of cell cycle stage and in a UVC dose-dependent manner (Figures S2B and S2C). Individual depletion of HIRA complex subunits enabled us to establish that accumulation of the HIRA subunit in damaged chromatin regions occurred independently of UBN1 but was favored in the presence of CABIN1 (Figure S2D). Thus, besides the active role of UBN1 in stimulating the deposition activity of the HIRA subunit (Ray-Gallet et al., 2011), we describe a function for CABIN1 in stabilizing HIRA at sites of DNA lesions.

Taken together, these data put forward the histone chaperone complex HIRA as a UVC damage response factor that accumulates locally in DNA damaged chromatin regions.

**HIRA-Mediated Accumulation of New H3.3 Histones in Damaged Chromatin Regions**

Because the HIRA complex promotes de novo deposition of the H3.3 variant into chromatin (reviewed in Szenker et al., 2011), we examined new H3.3 histone deposition in damaged chromatin regions. For this, we combined local UVC irradiation with specific tracking of newly synthesized histones. We took advantage of the SNAP-tag technology to fluorescently label new histones...
using U2OS cells engineered to stably express SNAP-tagged histone H3 variants (Dunleavy et al., 2011) (see Figure 3A and Figure S3 for a characterization of the cell lines). Thus, we revealed a local enrichment of newly synthesized H3.3 in UVC-damaged chromatin, as observed in U2OS cells (Figure 3B), but also in HeLa cells stably expressing SNAP-tagged H3.3 (data not shown). Similar to HIRA recruitment, new H3.3 histones accumulated in UVC-damaged regions in the vast majority of damaged cells and throughout interphase (Figures 3B and S2B). Furthermore, we reproduced these findings by monitoring real-time accumulation of new H3.3 at sites of UVC laser micro-irradiation (Figure S2F). Although we also detected new H3.1 histone accumulation both at repair sites and replication foci, consistent with previous results (Polo et al., 2006; Ray-Gallet et al., 2011), we did not observe any detectable accumulation of newly synthesized CenH3, a centromeric histone variant (Figure 3B). These data highlight that not all new histone H3 variants are equally mobilized in response to DNA damage.

In order to characterize the molecular determinants of new H3.3 accumulation in UVC-damaged regions, we analyzed the effect of depleting candidate histone H3 chaperones. We observed a significant reduction of H3.3 de novo accumulation in damaged chromatin regions in cells depleted of HIRA complex subunits, with the most severe effect occurring on depletion of the HIRA subunit itself (Figure 3C), as confirmed with an independent set of siRNAs (Figure S4A). This is consistent with the central role of this protein in the overall stability of the complex, as shown by the reduced levels of the other subunits in HIRA-depleted cells (Figure 3C; Ray-Gallet et al., 2011). In addition, expression of an siRNA-resistant HIRA construct restored the accumulation of new H3.3 histones in UVC-damaged chromatin regions (Figure 3D). These results establish that the defective accumulation of H3.3 in damaged chromatin is a direct and specific consequence of HIRA loss of function. We also found some defect in H3.1 deposition in damaged chromatin regions on depletion of HIRA, indicating a more general impact of this chaperone on chromatin integrity at damage sites (Figure S4B). In contrast to HIRA subunits, depletion of any of the other chaperones tested, including ASF1, CAF-1, and the H3.3-specific chaperone DAXX, did not affect new H3.3 accumulation in damaged regions (Figure 3C; data not shown). We verified that the inhibitory effect of depleting HIRA complex subunits on new H3.3 accumulation could not be explained by alterations in the cell cycle profiles or marked changes in the total levels of new H3.3 (Figure S4C and S4D). It is interesting that new H3.3 accumulation in damaged chromatin remained undetectable 8 hr after local UVC irradiation in HIRA-depleted cells, indicative of an impaired rather than a delayed process (Figure S4E).

From these results, we conclude that newly synthesized H3.3 histones accumulate in damaged chromatin regions in a HIRA-dependent manner. Thus, we identify a pathway for restoring chromatin structure after genotoxic stress.

New H3.3 Accumulation on Detection of DNA Damage
Because the mechanism of HIRA recruitment to chromatin was still elusive, we wondered how HIRA could be targeted to damaged chromatin regions to mediate H3.3 deposition and which parameters could control this key event. Given that H3.3 deposition by the HIRA complex has been linked to RNAPII (Julien et al., 2012; Placek et al., 2009; Ray-Gallet et al., 2011; Szenker et al., 2011; Yang et al., 2011), we first tested the effect of inhibiting transcription. However, cell treatment with RNAPII inhibitors such as α-amanitin or DRB did not impair HIRA accumulation at sites of DNA damage (Figure S2G). Thus, HIRA recruitment to damaged chromatin regions is not transcription dependent, arguing that HIRA is targeted to DNA damage regardless of the transcription status of the region before damage infliction. To explore whether a more specific connection with the DDR could be involved, we depleted candidate factors of the nucleotide excision repair (NER) pathway, which removes UVC damage (reviewed in Nouspikel, 2009) (Figure 4A). Although depletion of the endonuclease xeroderma pigmentosum group G (XPG) abrogated CAF-1 recruitment to UVC damage because it impaired repair synthesis (Green and Almouzni, 2003), HIRA accumulation still occurred in damaged chromatin regions (Figure 4A). These data indicate that HIRA recruitment is not coupled to late repair steps. However, depletion of early NER factors involved in the recognition of UVC damage, such as Cullin4A (CUL4A) and DNA damage binding proteins 1 and 2 (DDB1 and DDB2, respectively) (Nouspikel, 2009), markedly impaired HIRA recruitment to damaged chromatin and de novo H3.3 accumulation in damaged regions without affecting HIRA total levels and H3.3 production (Figure 4B; Figure S5A and S5B for a second set of siRNAs). Although HIRA targeting to DNA damage could involve an association—direct or indirect—between HIRA complex subunits and these early NER proteins, as recently reported for CABIN1 (Choi et al., 2013), it may also require the E3 ubiquitin ligase activity of DDB1-CUL4A-containing complexes (reviewed in Nouspikel, 2011; Figure S6A).

Consistent with this possibility, by preventing all ubiquitylation reactions using RNA interference against ubiquitin, we inhibited both HIRA and new H3.3 accumulation in UVC-damaged chromatin without any significant effect on HIRA total levels (Figure 4C). To strengthen these data, we perturbed ubiquitin-dependent signaling by proteasome inhibition (Figure S6B). This treatment depletes the cellular pool of free ubiquitin (Dantuma et al., 2006), thus preventing de novo ubiquitylation reactions taking place at damage sites (Figure S6C; Mailand et al., 2007). Consistent with a major role for such ubiquitylation reactions in controlling HIRA-mediated H3.3 deposition in damaged chromatin regions, treatment of cells with proteasome inhibitors—MG132 or epoxomicin—severely impaired the accumulation of HIRA and new H3.3 at sites of UVC damage (Figure S6B; and data not shown). Of note, this inhibition occurred even though CUL4A was still recruited to damaged chromatin (Figure S6D; Ishii et al., 2010). Furthermore, the expression of a dominant-negative form of CUL4A, proficient for UVC damage binding and deficient for ubiquitylation, inhibited HIRA recruitment to damaged chromatin (Figure 4D). These results establish that the ubiquitylation activity of the DDB1-CUL4A complex, rather than its presence at damage sites, is critical for stimulating HIRA and new H3.3 accumulation. Remarkably, once recruited, the HIRA complex remains...
Figure 2. HIRA Function in the DNA Damage Response

(A) Repair activity analyzed by immunofluorescence against CPDs at different time points postglobal UVC irradiation (as depicted on the left) in HeLa cells treated with the indicated siRNAs (siLUC, control). siRNA efficiencies are shown on the western blot panel.

(B) Recruitment of the repair protein XPB and repair synthesis (EdU incorporation) at DNA damage (CPD) analyzed by immunofluorescence 30 min and 4 hr after local UVC irradiation, respectively, in U2OS cells treated with the indicated siRNAs. Local UVC damage is induced by irradiating cells with a UVC lamp through a

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only transiently enriched in damaged chromatin. By exploring the molecular determinants of HIRA dissociation from damaged chromatin, we also shed light on a possible mechanism for HIRA recruitment. Indeed, we observed that HIRA release from UVC-damaged chromatin is impaired in repair-deficient cells (siXPG) and significantly delayed on H3.3 depletion (Figure S3C). These results show that completion of DNA repair and new histone deposition are both required to displace HIRA from damaged chromatin, suggesting that this chaperone is recruited to nucleosome-free damaged DNA.

Collectively, these data demonstrate that HIRA-mediated accumulation of new H3.3 is an early response to DNA damage, dependent on ubiquitylation events mediated by the DDB2-DDB1-CUL4A complex, an early NER factor involved in DNA damage detection. Furthermore, these findings highlight that completion of DNA repair is not a pre-requisite for new histone deposition into damaged chromatin but is critical for HIRA dissociation from UVC-damaged regions.

**HIRA Priming Role for Transcription Recovery**

To further explore the functional relationships between the histone chaperone HIRA and transcription regulation in response to DNA damage, we dissected the relative kinetics of HIRA accumulation and transcription recovery in damaged chromatin regions.

Consistent with its dependency on early repair steps, we detected HIRA recruitment to UVC-damaged chromatin within minutes after damage, reaching a maximum 40 min postirradiation. Furthermore, HIRA recruitment preceded the accumulation of the histone chaperone CAF-1 to DNA lesions (Figure 5A). Interestingly and consistent with a histone chaperone function, we found that HIRA targeting to damaged chromatin was transient, becoming undetectable 5 hr postdamage induction (Figures 5A and 5B). In contrast, the accumulation of new H3.3 at sites of DNA damage remained detectable up to 24 hr postirradiation (Figures 5B and 5C). Thus, we conclude that newly synthesized H3.3 histones stably associate with DNA upon deposition by the HIRA chaperone in damaged chromatin and remain in place at least until repair is complete.

Next, we compared the relative kinetics of HIRA-dependent H3.3 deposition and transcription regulation at sites of local UVC irradiation. When HIRA was enriched at DNA damage (1 hr postdamage induction), we observed a local inhibition of transcription at these locations (Figure 5C), consistent with previous observations (Moné et al., 2001). Remarkably, transcription was still inhibited 5 hr after damage, when HIRA was already released from damaged chromatin regions. It was only long after HIRA release from damaged chromatin that transcriptional activity recovered (20–24 hr; Figure 5C). Thus, although HIRA determines transcription restart after damage, its enrichment in damaged chromatin is not concomitant with transcription recovery. These results imply that HIRA is necessary, but not sufficient, to allow reactivation of transcription. Indeed, transcription does not restart after UVC irradiation in repair-deficient XPG-depleted cells (Figure S1C), even if HIRA is efficiently recruited to damaged regions in these cells (Figure 4A). Thus, HIRA-independent DNA damage repair events occur in the meantime to allow reactivation of transcription.

Together, these data establish that HIRA acts early and transiently at sites of DNA damage, prior to repair completion and transcription recovery. Based on these findings, we propose that HIRA recruitment to damaged regions acts as a priming event making damaged chromatin prone for transcription restart once repair is complete.

**DISCUSSION**

Our study defines a histone deposition pathway that primes chromatin in response to DNA damage allowing transcription recovery after repair (Figure 6). This pathway involves early targeting of the histone chaperone HIRA to DNA lesions, where it promotes de novo deposition of H3.3 histones and renders chromatin prone for later reactivation of transcription. In light of these data, we propose a concept of chromatin bookmarking by histone deposition early on during the DDR that licenses the chromatin substrate for transcription.

**HIRA Targeting to Damaged Chromatin and New H3.3 Deposition**

Although the yeast orthologs of the HIRA complex protect cells against genotoxic agents (Anderson et al., 2009; Kapitzky et al., 2010), the actual role of this chaperone in response to DNA damage is unknown, and in other eukaryotes, the contribution of HIRA to the DDR remains elusive. Here, we characterize a transcription-associated function for HIRA in the DDR. Our observations indicate that, contrary to its yeast counterparts, human HIRA is not required for genome stability per se but instead contributes to transcription restart after repair. Furthermore, HIRA exerts a local action in damaged chromatin regions by depositing newly synthesized H3.3 histones. In this setting, HIRA recruitment is not driven by the transcriptional activity but by the presence of DNA lesions. These findings have broad implications for our understanding of H3.3 deposition that, at least in this instance, does not simply occur as a consequence of transcription. Considering that another set of new histones is deposited by CAF-1 during the late repair steps (Polo et al., 2006), the impact of newly deposited histones on the chromatin landscape at damage sites is particularly intriguing. This is a critical issue given that new soluble histones show a distinct pattern of modifications when compared to nucleosomal histones (Loyola et al., 2006). How much and for how long newly incorporated histones stay in damaged chromatin regions will thus determine the extent to which the original information conveyed by chromatin is ultimately modified, which will be the matter of future investigations.
Figure 3. The HIRA Complex Promotes New H3.3 Accumulation in Damaged Chromatin Regions

(A) Scheme of the assay for monitoring accumulation of newly synthesized histones at UVC damage by fluorescence microscopy on cultured human cells stably expressing SNAP-tagged histones. Pre-existing SNAP-tagged histones are quenched with a nonfluorescent substrate (block) so that only the histones neo-synthetized during the chase period are labeled with the red fluorescent substrate tetramethylrhodamine (TMR)-star during the pulse step. Local UVC damage is induced as in Figure 2B and cells are fixed 1 hr later.

(B) Accumulation of new H3.1 and H3.3, but not CenH3 variant, in UV-damaged regions analyzed by fluorescence microscopy in U2OS cells. CAF-1 p150 subunit immunodetection labels replication and repair foci. As positive controls, we detect new H3.1 at replication foci and new CenH3 at centromeres in early G1 cells.

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In our study, we revealed clearly distinct functions for two chromatin assemblers, HIRA and CAF-1, in response to UVC damage, as only HIRA is critical to facilitate transcription recovery after DNA repair. Another important feature that distinguishes HIRA from CAF-1 is the timing of targeting to damaged chromatin. Our mechanistic studies reveal that HIRA recruitment is a very early and transient response that depends on DNA damage detection by DDB1-CUL4A-containing complexes. These complexes promote ubiquitylation of various substrates in response to DNA damage, including NER factors and histones (Nouspikel, 2011). Ubiquitylation of the HIRA complex itself is an attractive hypothesis to explain how HIRA is recruited to damaged chromatin. However, a recent systems-wide analysis did not identify ubiquitylation changes on HIRA or its associated proteins UBN1 and CABIN1 after UVC damage in human cells (Povlsen et al., 2012). Another hypothesis that should be explored in future studies is that the HIRA complex could possibly recognize one or several of the numerous ubiquitylation targets of DDB1-CUL4A at damage sites. However, in light of recent work in human cells and flies showing that HIRA can directly bind DNA (Ray-Gallet et al., 2011) and is recruited to nucleosome-free regions (Ray-Gallet et al., 2011; Schneiderman et al., 2012), it is tempting to speculate that the ubiquitylation activity of DDB1-CUL4A-containing complexes, which is thought to increase DNA accessibility at damage sites (Nouspikel, 2011), could expose naked DNA stretches, thereby triggering HIRA recruitment. Consistent with such a model where HIRA would be recruited to histone-free DNA, we showed that histone deposition promotes HIRA displacement from UV-damaged chromatin.

In addition, HIRA function in the DDR may not be restricted to UVC photoproducts but could also be involved in response to other types of DNA lesions, including DNA breaks, as recently described (Yang et al., 2013). Indeed, in support of this broader function and consistent with a previous study (Adamson et al., 2012), we observed a transient accumulation of HIRA at sites of UVA laser microirradiation (Figure S2E). It is striking that, while HIRA is the predominant histone chaperone mediating de novo H3.3 deposition in damaged chromatin, it is able to deposit new H3.3, which has been associated with transcriptional activation in various contexts and organisms—cross-tolerance in fission yeast, early embryonic development and nuclear reprogramming in Xenopus, muscle differentiation in mouse cells, and response to viral infection in human cells (Chujo et al., 2012; Jullien et al., 2012; Placek et al., 2009; Szenker et al., 2012; Yang et al., 2011). Maintaining a certain level of H3.3 histones, which are prone to exclude repressive proteins and to carry specific sets of posttranslational modifications promoting transcription (reviewed in Szenker et al., 2011), could ensure that repaired chromatin remains a preferred substrate for RNA polymerase. Consistent with this model, we showed that the defect in transcription restart observed upon HIRA knockdown is not due to a loss of function of the transcription machinery. In this context, it will be interesting to explore whether mutations in H3.3 recently identified in pediatric brain tumors (Schwartzentrub et al., 2012; Sturm et al., 2012; Wu et al., 2012) could misregulate the response of HIRA to DNA damage and thereby affect the maintenance of genome function.

In addition, beyond a direct effect on H3.3 deposition, a more general role of HIRA in the maintenance of chromatin integrity, even indirectly by affecting H3.1 deposition, should also be integrated. Furthermore, while HIRA is the predominant histone chaperone mediating de novo H3.3 deposition in damaged chromatin regions in our cell line models, we cannot exclude a possible contribution of other chaperones in distinct cellular contexts such as in neurons, where DAXX-mediated H3.3 deposition controls the expression of genes responsive to neuronal activation (Michod et al., 2012).

Most important, we have identified at the level of chromatin organization a key priming event for transcription restart that occurs early in the DDR, independently of global DNA repair. While our study characterized HIRA-mediated reactivation of transcription after DNA damage on a genome-wide scale, it remains to be determined whether this mechanism applies similarly to gene-rich and noncoding regions as found in heterochromatin. More subtle effects on transcription profiles at specific genomic regions could also be encountered upon milder alterations in histone dynamics, which should be possible to detect using emerging high-throughput technologies for nascent RNA sequencing (Churchman and Weissman, 2011; Core et al., 2008).

In conclusion, we provide an understanding of transcriptional control after DNA damage involving chromatin bookmarking by histone chaperones to license repaired DNA to be transcribed. These findings open up avenues to unravel how genotoxic stress could influence transcriptional programs via seemingly minor changes in chromatin resetting and, thus, have a profound impact on cell fate.

(C and D) Accumulation of new H3.3 in UV-damaged regions requires the HIRA complex. New H3.3 accumulation at DNA damage is revealed in U2OS cells treated with the indicated siRNAs (siLUC, control) (C) or with the indicated combinations of siRNAs and plasmids (D). XPA labels repair sites, and hemagglutinin (HA) immunodetection is used to visualize exogenous HIRA. Efficiencies of siRNA and plasmid transfections are shown on the western blots and on the right immunofluorescence panel. Error bars on the graphs represent SD from three independent experiments. Scale bars, 10 μm. See also Figures S3 and S4.
Figure 4. HIRA-Mediated H3.3 Accumulation Is an Early Response Dependent on Ubiquitylation Events upon DNA Damage Detection

(A) Scheme indicating the early and late NER proteins targeted by RNA interference in subsequent experiments. Recruitment of HIRA and CAF-1 p150 to DNA damage regions marked by XPB is analyzed by immunofluorescence 30 min and 1 hr after local UVC irradiation, respectively, in U2OS cells treated with the indicated siRNAs (siLUC, control).

(B) HIRA and new H3.3 accumulation in UVC-damaged regions analyzed 30 min and 1 hr postlocal UVC irradiation respectively, in U2OS cells treated with the indicated siRNAs. siRNA efficiencies, HIRA, and H3.3 total levels are shown on the western blot panel. The levels of new H3.3 incorporated into chromatin are analyzed by SNAP labeling (bottom panel).

(C) HIRA and new H3.3 accumulation at DNA damage analyzed as in (B).

(D) HIRA accumulation at DNA damage analyzed 30 min postlocal UVC irradiation in U2OS cells transiently transfected with the indicated plasmids. The expression of CUL4A full-length (WT) and truncated mutant (DN) is shown on the western blot panel. Error bars on the graphs represent SD from at least two independent experiments. Scale bars, 10 μm.

See also Figures S5 and S6.
EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment
HeLa and U2OS cells were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (EUROBIO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). BJ primary fibroblasts were grown in the same medium supplemented with 15% fetal calf serum and antibiotics. U2OS cells stably expressing H3.1-, H3.3-, or CenH3-SNAP (Dunleavy et al., 2011) were maintained in the presence of 100 μg/ml G418 (Invitrogen).

RNAPII-dependent transcription was inhibited by incubating cells in the presence of 100 μM DRB for 2 hr or 10 μg/ml α-amanitin for 8 hr (Sigma-Aldrich). For proteasome inhibition, cells were treated with 10 μM MG132 (Merck) in dimethyl sulfoxide (DMSO) for 2 hr. DNA breaks were induced by treating cells with 50 μg/ml phleomycin (Sigma-Aldrich) for 1 hr. UV Irradiation Cells were subjected to global or local UVC irradiation (254 nm), to laser-induced UVC damage (266 nm) and to UVA laser microirradiation (405 nm) as described in the Extended Experimental Procedures.

Flow Cytometry
Cells were fixed in ice-cold 70% ethanol before DNA staining with 50 μg/ml propidium iodide (Sigma-Aldrich) in PBS containing 0.05% Tween and 0.5 mg/ml

Figure 5. HIRA-Mediated H3.3 Deposition Does Not Coincide with Transcription Recovery
(A) Recruitment kinetics of HIRA and CAF-1 p60 to DNA damage analyzed by immunofluorescence at the indicated time points after local UVC irradiation in U2OS cells.
(B) Accumulation kinetics of new H3.3 and its chaperone HIRA at DNA damage monitored at the indicated time points after local UVC irradiation in U2OS cells.
(C) Nascent RNA synthesis (EU labeling) analyzed at the indicated time points after local UVC irradiation (100 J/m²) in U2OS cells in parallel with the accumulation of new H3.3 and its chaperone HIRA at DNA damage. Arrowheads point to sites of irradiation. Error bars on the graphs represent SD from three independent experiments. Scale bars, 10 μm.
RNase A (USB/Affymetrix). DNA content was analyzed by flow cytometry using a C6 flow cytometer (Accuri). Proportion of cells in each step of the cell cycle was estimated using the Dean/Jett/Fox algorithm on FlowJo software (TreeStar).

siRNA and Plasmid Transfection
siRNA purchased from Dharmacon or Eurofins MWG Operon (Table S1) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) following manufacturer’s instructions. The final concentration of siRNA in the culture medium was 30–50 nM. Cells were harvested 24–72 hr posttransfection.

Cells were transiently transfected with plasmid DNA (1 µg/ml final) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions 48 hr before subsequent cell treatment. For rescue experiments, cells were concomitantly transfected with siRNA (30 nM final) and plasmid DNA (0.375 µg/ml final) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions 72 hr before subsequent cell treatment. Plasmids are described in the Extended Experimental Procedures.

Colony-Forming Assays
Cells were replated 48 hr after siRNA transfection and exposed to global UVC irradiation the following day. Colonies were stained 12 days later with 0.5% crystal violet/20% ethanol and counted. Results were normalized to plating efficiencies and analyzed by a two-way analysis of variance followed by Bonferroni posttests with Prism 5 software (ns, nonsignificant, “p < 0.05,” “p < 0.01, and “”p < 0.001).”

Cell Extracts and Western Blot
Cell extracts were obtained as described in the Extended Experimental Procedures and run on 4%–20% Mini-PROTEAN TGX gels (Bio-Rad). Proteins of interest were probed using the appropriate primary (Table S2) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch).

Immunofluorescence
Cells were subjected to immunostaining with primary (Table S2) and secondary antibodies conjugated to Alexa-Fluor 488, 594, or 680 (Invitrogen) as described in the Extended Experimental Procedures.

Figure 6. Model for Chromatin Bookmarking by HIRA-Dependent Histone Deposition Allowing Transcription Restart after Repair of DNA Damage
Scheme representing how H3 histone chaperones contribute to restoring nucleosomal organization in response to DNA damage. Early ubiquitylation associated with damage detection targets HIRA to damaged chromatin regions where it deposits new H3.3 histones and promotes subsequent events in chromatin dynamics, including CAF-1-mediated H3.1 deposition. Although not essential for global repair, these HIRA-dependent chromatin changes are key in restoring a proper chromatin organization and in bookmarking damaged chromatin for subsequent transcription restart (“priming” function).

Nascent RNA Labeling
Nascent RNA labeling by EU incorporation was performed with Click-IT RNA Imaging kits (Invitrogen). Refer to the Extended Experimental Procedures for details.

Visualization of DNA Repair Synthesis
EdU was incorporated into cells during 4 hr immediately after local UVC irradiation and revealed using the Click-IT EdU Alexa Fluor 594 Imaging kit (Invitrogen) according to manufacturer’s instructions before CPD labeling by immunofluorescence.

SNAP Labeling of Histones
The SNAP labeling protocol is as described in Bodor et al. (2012). Refer to the Extended Experimental Procedures for details.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.029.

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EXTENDED EXPERIMENTAL PROCEDURES

UV Irradiation

Cells grown on glass coverslips (VWR) were irradiated with UVC (254 nm) using a low-pressure mercury lamp. Conditions were set using a VLX-3W dosimeter (Vilbert-Lourmat). For global irradiation, cells in PBS were exposed to 10 J/m² unless stated otherwise. For local irradiation (Katsumi et al., 2001; Moné et al., 2001), cells were covered with a polycarbonate filter (5 μm pore size, Millipore) and irradiated with 150 J/m² UVC unless stated otherwise. For laser-induction of UVC damage (Dinant et al., 2007), cells were grown on quartz coverslips (SPI supplies) and irradiated for 50 ms using a 2 mW pulsed diode-pumped solid-state laser emitting at 266 nm (repetition rate up to 10 kHz, Rapp OptoElectronics, Hamburg GmbH) directly connected to a Zeiss LSM 700 confocal microscope adapted for UVC transmission with all-quartz optics. The laser was attenuated using a neutral density filter OD1 and focused through a 40x glycerol objective.

For UVA laser micro-irradiation, cells grown on glass coverslips were presensitized with 10 μM 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) for 24 hr at 37 °C. Damage was introduced with a 405 nm laser diode (3 mW) focused through a Plan-Apochromat 63X/1.4 oil objective to yield a spot size of 0.5-1 μm using a LSM710 NLO confocal microscope (Zeiss) and the following laser settings: 38% power, 50 iterations, scan speed 12.6 μsec/pixel.

Plasmids

The pPHA vector and pHIRA-HA expression construct were obtained by replacing EYFP by a HA tag in pEYFP-N1 (Clontech) and pEYFP-N1-HIRA (human HIRA sequence from (Hall et al., 2001) subcloned into pEYFP-N1 and edited by Genescript). The pcDNA3-HA-2UL4A expression vector was purchased from Addgene (#19907; Hu et al., 2008). The pcDNA3-HA-CUL4A DN expression vector (gift from Carlos de Noronha) encodes the first 545 amino acids of human CUL4A, which lack the neddylation site and the interaction domain with RING-box protein 1 (RBX1) (Sarkis et al., 2011) resulting in an inactive form for ubiquitylation. A Nuclear Localization Signal (NLS) from SV40 large T antigen was inserted upstream of the HA tag to compensate for the loss of a NLS due to the carboxy-terminal truncation. Primer sequences (Eurosins MWG Operon) are available upon request. All constructs were verified by direct sequencing.

Cell Extracts and Western Blot

Total extracts were obtained by scraping cells in Laemmli buffer (50 mM Tris HCl pH 6.8, 1.6% SDS (Sodium Dodecyl Sulfate), 8% glycerol, 4% β-mercaptoethanol, 0.0025% bromophenol blue) followed by 5 min denaturation at 95 °C. Cytosolic and nuclear extracts were obtained as previously described (Martini et al., 1998). The chromatin fraction was prepared by addition of benzonase (Novagen) to the pellet after nuclear extraction.

For western blot analysis, extracts were run on 4%–20% Mini-PROTEAN TGX gels (Bio-Rad) in running buffer (200 mM glycine, 25 mM Tris, 0.1% SDS) and transferred onto nitrocellulose membranes (Protran) with a Trans-Blot SD semidry transfer cell (Bio-Rad). Total proteins were revealed by MemCode staining (Pierce). Proteins of interest were probed using the appropriate primary (Table S2) and HRP (Horseradish Peroxidase)-conjugated secondary antibodies (Jackson Immunoresearch), detected using SuperSignal West Pico chemiluminescence substrates (Pierce). For semiquantitative analysis of western blot, serial dilutions of protein samples were loaded in order to obtain comparable signals in the linear range of detection, and signals were analyzed using Image J software.

Immunoﬂuorescence

Cells grown on glass coverslips (VWR) were either fixed directly with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS, or pre-extracted before fixation with 0.5% Triton X-100 in CSK buffer (Cytoskeletal buffer: 10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂). Pre-extraction was required only for visualizing CAF-1 subunit recruitment to damage sites, not for the other chaperones. For CPD staining, DNA was denatured with 0.5 M NaOH for 5 min. Samples were blocked in 5% BSA (Bovine Serum Albumin, Sigma-Aldrich) in PBS supplemented with 0.1% Tween before incubation with primary (Table S2) and secondary antibodies conjugated to Alexa-Fluor 488, 594 or 640 (Invitrogen). Coverslips were mounted in Vectashield medium with DAPI (Vector) and observed with a Leica DML6000 epifluorescence microscope using a 40x or 63x oil objective. Percentages of positively stained cells were obtained by scoring at least 100 cells in each experiment. Statistical analyses using t test or one-way ANOVA followed by Tukey post-tests were performed with Prism 5 software. ns: nonsignificant, *: p < 0.05, **: p < 0.01, ***: p < 0.001.

Nascent RNA Labeling

Nascent RNA labeling was systematically performed 71 hr post-siRNA transfection, and siRNA efficiency was confirmed both at 48 hr (time of the first irradiation) and 72 hr posttransfection (time of fixation). Cells were incubated in DMEM supplemented with 0.5 mM EU for 1 hr, rinsed in cold medium and in PBS before fixation in 2% paraformaldehyde. EU incorporation was revealed with Click-IT RNA Imaging kits (Invitrogen) using either Alexa Fluor® 594 or 647 dyes according to manufacturer’s instructions. Coverslips were mounted in Vectashield medium with DAPI (Vector). The mean EU fluorescence intensity per cell was obtained using ImageJ software by averaging the mean gray value of the EU signal measured in each nucleus. Nuclear segmentation was based on DAPI
staining. Results were analyzed by two-way ANOVA followed by Bonferroni post-tests with Prism 5 software. Statistical analyses of transcription recovery were performed for the final time-point in each experiment.

**SNAP Labeling of Histones**

Pre-existing SNAP-tagged histones were labeled by incubating cells with 2 μM SNAP-cell TMR star (New England Biolabs) for 15 min (pulse) followed by a 30 min-incubation in fresh medium. For specific labeling of newly-synthetized histones, pre-existing histones were first quenched by incubating cells with 10 μM SNAP-cell Block (New England Biolabs) for 30 min followed by a 30 min-wash in fresh medium and a 2 h-chase (if cells were pre-extracted before fixation) or a 30 min-chase in fresh medium (if cells were to be directly fixed afterward, i.e., when SNAP detection was combined with EU or CyclinA labeling). The pulse step was then performed as described above. Cells were irradiated before or after the pulse and then imaged in real time, or processed for immunostaining, or harvested in Laemmli buffer for electrophoresis and in-gel fluorescence analysis with a Typhoon FLA 9000 (GE Healthcare-Life Sciences). The intensities of fluorescent bands corresponding to TMR star-labeled H3.3-SNAP were quantified using ImageJ software and normalized to Coomassie staining. The accumulation of new histones at sites of UVC laser micro-irradiation was quantified using ImageJ software. To correct for overall bleaching of the signal due to repetitive imaging, fluorescence intensities after background substraction were normalized against intensities measured in an undamaged nucleus in the same field.

**SUPPLEMENTAL REFERENCES**


Figure S1. The Histone Chaperone HIRA Is Required for Transcription Recovery after DNA Damage, but Not after Inhibition of Transcription Elongation, Related to Figure 1

(A) Unnormalized data from Figure 1C. Transcriptional activity is measured by EU incorporation in UVC-irradiated HeLa cells treated with the indicated siRNAs (siLUC: control). siRNA efficiencies before (-), 2 hr and 24 hr post-UVC irradiation are controlled on the western blot panel. The moderate transcription defect already observed upon HIRA depletion in undamaged cells was exacerbated upon DNA damage reaching ca. 50% reduction compared to control. Error bars on the graph represent SEM from three independent experiments.

(B) Cell-cycle distribution of HeLa cells treated with the indicated siRNAs analyzed by flow cytometry at the indicated time-points after global UVC irradiation.

(C) Transcriptional activity is measured as in (A) in control (siLUC) versus repair deficient HeLa cells (siXPG) before (-), 2 hr and 16 hr after global UVC irradiation.

(D) Transcriptional activity is measured as in (A) in control (siLUC) versus HIRA deficient U2OS cells before (-), 2 hr and 16 hr after global UVC irradiation. The efficiency of HIRA depletion is controlled on the western blot panel.

(E) Levels and phosphorylation status of RNAPII large subunit analyzed by western blot on total extracts from UVC-irradiated HeLa cells treated with the indicated siRNAs. Black arrowhead: RNAPII hyperphosphorylated form.

(F) Transcriptional activity in DRB-treated HeLa cells transfected with the indicated siRNAs. EU incorporation is measured by fluorescence microscopy in untreated cells (-), during (DRB) and 1 hr after DRB treatment (Recovery) as shown on the experimental scheme. siRNA efficiencies are controlled on the western blot panel. Error bars on the graphs represent SEM from two independent experiments. Scale bars, 10 μm.

(G) Clonogenic survival of HeLa cells treated with the indicated siRNAs in response to UVC irradiation. Error bars on the graph represent SD from three independent experiments.
Figure S2. Characterization of HIRA and H3.3 Accumulation in DNA Damage Regions, Related to Figure 2

(A) Recruitment of HIRA to damaged chromatin regions (marked by XPB) analyzed by immunofluorescence 30 min after local UVC irradiation in the indicated cell lines.

(B) HIRA and new H3.3 accumulation in DNA damage regions analyzed 30 min and 1 hr after local UVC irradiation respectively in U2OS cells costained for Cyclin A to label S and G2 cells.

(C and D) Recruitment of HIRA to UVC regions analyzed as in A in U2OS cells exposed to increasing UVC doses (C) or treated with the indicated siRNAs (D). siRNA efficiencies are controlled on the western blot panel.

(E) Recruitment of HIRA to sites of UVA laser micro-irradiation (marked by γH2AX) analyzed by immunofluorescence 15 min after damage with a 405 nm laser in BrdU presensitized U2OS cells.

(F) Real-time accumulation of newly synthesized histones at sites of UVC laser micro-irradiation monitored in H3.3-SNAP U2OS cells as depicted on the scheme. N: cell number.

(G) Recruitment of HIRA to DNA damage regions analyzed as in A in U2OS cells treated with the indicated transcription inhibitors. α-AMan: α-Amanitin. Transcription inhibition is monitored by a reduced EU staining in the nucleoplasm (bottom immunofluorescence panel) and by the degradation of RNAPII large subunit or the loss of its hyperphosphorylated form (arrowhead, western-blot panel). We did not test the effect of DRB or α-Amanitin on new H3.3 deposition as these transcription inhibitors interfere with new histone synthesis. Error bars on the graphs represent SD from at least two independent experiments. Scale bars, 10 μm.
Figure S3. Characterization of U2OS Cell Lines Stably Expressing SNAP-Tagged H3 Variants, Related to Figure 3

(A) Western blot analysis of the expression levels of SNAP-tagged H3.1 (1) and H3.3 (3) proteins on total extracts from the corresponding U2OS cell lines. -: parental U2OS. SNAP-tagged histones represent less than 5% of total endogenous H3.

(B) Cell-cycle profiles of the indicated cell lines analyzed by flow cytometry.

(C and D) Incorporation of H3-SNAP into chromatin revealed by SNAP-tag immunostaining on mitotic chromosomes (C) and by western blot analysis of cytosolic (cy), nuclear (nu), and chromatin fractions (ch) prepared from the indicated cell lines (D).

(E) Visualization of H3-SNAP by fluorescence microscopy in the indicated cell lines after in vivo labeling assays with TMR-star (red) in pulse (P), quench-pulse (QP) or quench-chase-pulse (QCP) experiments combined with SNAP-tag immunostaining (green). The pulse labels all pre-existing H3-SNAP, while the quench-chase-pulse labels only the new H3-SNAP synthesized during the chase step. Quenching efficiency is revealed by omitting the chase.

(F) Incorporation profiles of new H3 variants analyzed by quench-chase-pulse experiments in U2OS cells. New H3.1 deposition is restricted to S phase and colocalizes with replication foci (marked by CAF-1 p150), while new H3.3 are deposited independently of replication. Scale bars, 10 μm.
**Figure S4. HIRA-Dependent H3 Accumulation in DNA-Damaged Chromatin, Related to Figure 3**

(A) HIRA and new H3.3 accumulation at DNA damage analyzed as in Figures S2D and 3C in U2OS cells treated with a second set of siRNAs. siRNA efficiencies are shown on the western blot panel.

(B) New H3.1 accumulation in DNA damage regions (marked by XPA) 1 hr after local UVC irradiation in U2OS cells treated with the indicated siRNAs (siLUC: control). HIRA depletion affects new H3.1 deposition only at DNA damage, not at replication foci in S phase (right cell in each field). Similar results were obtained using siHIRA#2, siHIRA#3 and siHIRA 3′ UTR (data not shown).

(C) Cell-cycle profiles of U2OS cells treated with the indicated siRNAs.

(D) In-gel fluorescence analysis of new H3.3-SNAP levels in total extracts from U2OS cells treated with the indicated siRNAs and subject to in vivo labeling with TMR-star (quench-chase-pulse experiment). Total levels of new H3.3 are normalized to Coomassie staining (loading control).

(E) New H3.3 accumulation in DNA damage regions (marked by XPA) analyzed by immunofluorescence at the indicated time points after local UVC irradiation in U2OS cells treated with the indicated siRNAs. Error bars on the graphs represent SD from two independent experiments. Scale bars, 10 μm.
Figure S5. Regulation of HIRA Dynamics in UVC-Damaged Regions by NER Factors and New H3.3 Histones, Related to Figure 4

(A and B) Recruitment of HIRA to DNA damage regions (marked by XPB) analyzed by immunofluorescence 30 min after local UVC irradiation in U2OS cells treated with the indicated siRNAs (siLUC: control). siRNA efficiencies are shown on the western blot panels. The asterisk indicates a nonspecific band.

(C) HIRA release from UVC damage sites analyzed by immunofluorescence at the indicated time points after local UVC irradiation in U2OS cells treated with the indicated siRNAs. siH3.3 efficiency was controlled by analyzing new H3.3 accumulation in damaged regions (marked by XPA) 1 hr after local UVC irradiation in U2OS cells (right immunofluorescence panel) or by western blot on total extracts. Error bars on the graphs represent SD from two independent experiments. Scale bars, 10 μm.
Figure S6. Ubiquitin-Dependent Regulation of HIRA-Mediated H3.3 Accumulation at DNA Damage Sites, Related to Figure 4

(A) The recruitment of HIRA to DNA damage is not affected by depleting RNF8 (Ring Finger protein 8), another E3 ubiquitin ligase involved in the response to UVC irradiation (Marteijn et al., 2009), arguing for a specific role of DDB1-CUL4A containing complexes. The efficiency of RNF8 depletion is revealed on the right immunofluorescence panel by the lack of 53BP1 foci upon treatment with the radiomimetic agent phleomycin.

(B) HIRA and new H3.3 accumulation in UVC damaged chromatin regions is severely impaired in U2OS cells treated with the proteasome inhibitor MG132, while their association to chromatin anywhere else in the nucleoplasm remains similar to control (DMSO: dimethyl sulfoxide, vehicle).

(C) The efficiency of MG132 treatment is revealed by the lack of ubiquitin conjugates (Ub (FK2)) in UVC damage regions (marked by XPB) as monitored by immunofluorescence with FK2 antibody in U2OS cells.

(D) Recruitment of HIRA and HA-CUL4A to DNA damage analyzed by immunofluorescence 30 min after local UVC irradiation in U2OS cells transiently transfected with HA-CUL4A and treated with MG132 or DMSO. Error bars on the graphs represent SD from two independent experiments. Scale bars, 10 μm.