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DOI

[10.1083/jcb.200810035](https://doi.org/10.1083/jcb.200810035)

Publication date

2009

Document Version

Final published version

Published in

Journal of Cell Biology

[Link to publication](#)

Citation for published version (APA):

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<https://doi.org/10.1083/jcb.200810035>

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Heterochromatin protein 1 is recruited to various types of DNA damage

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Heterochromatin protein 1 (HP1) family members are chromatin-associated proteins involved in transcription, replication, and chromatin organization. We show that HP1 isoforms HP1- α , HP1- β , and HP1- γ are recruited to ultraviolet (UV)-induced DNA damage and double-strand breaks (DSBs) in human cells. This response to DNA damage requires the chromo shadow domain of HP1 and is independent of H3K9 trimethylation and proteins that detect UV damage and DSBs. Loss of HP1 results

in high sensitivity to UV light and ionizing radiation in the nematode *Caenorhabditis elegans*, indicating that HP1 proteins are essential components of DNA damage response (DDR) systems. Analysis of single and double HP1 mutants in nematodes suggests that HP1 homologues have both unique and overlapping functions in the DDR. Our results show that HP1 proteins are important for DNA repair and may function to reorganize chromatin in response to damage.

Introduction

DNA can be damaged by various agents, including ionizing radiation (IR) and UV radiation. Cells respond to genotoxic stress by activating DNA damage response (DDR) systems, including DNA repair, cell cycle arrest, senescence, and apoptosis (Bartek and Lukas, 2007). DNA repair pathways each deal with specific types of lesions (Hoeijmakers, 2001). Nucleotide excision repair (NER) removes bulky adducts and UV-induced photoproducts such as cyclobutane pyrimidine dimers and 6-4 photoproducts from the genome, whereas DNA double-strand breaks (DSBs) are removed by homologous recombination or nonhomologous end joining (NHEJ; Hoeijmakers, 2001).

M.S. Luijsterburg and C. Dinant contributed equally to this paper.

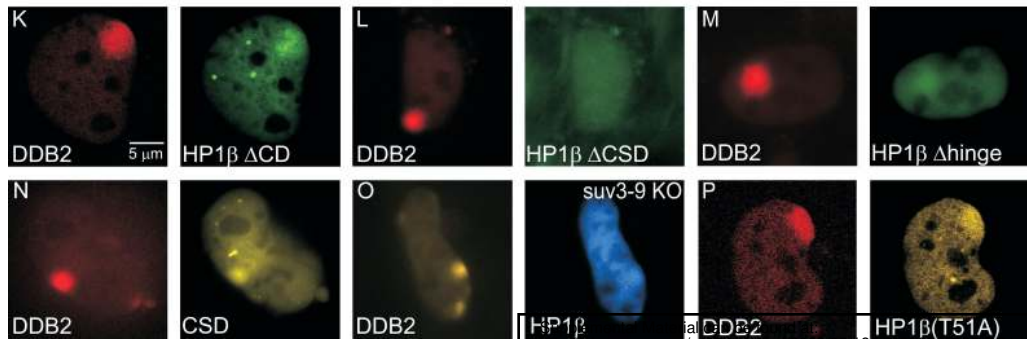
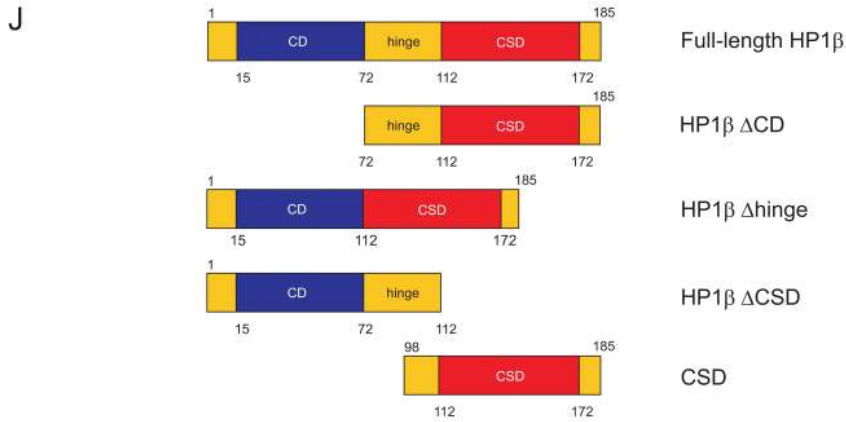
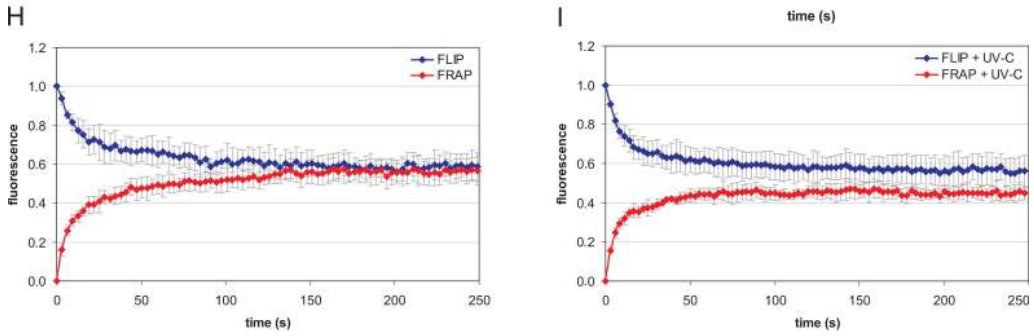
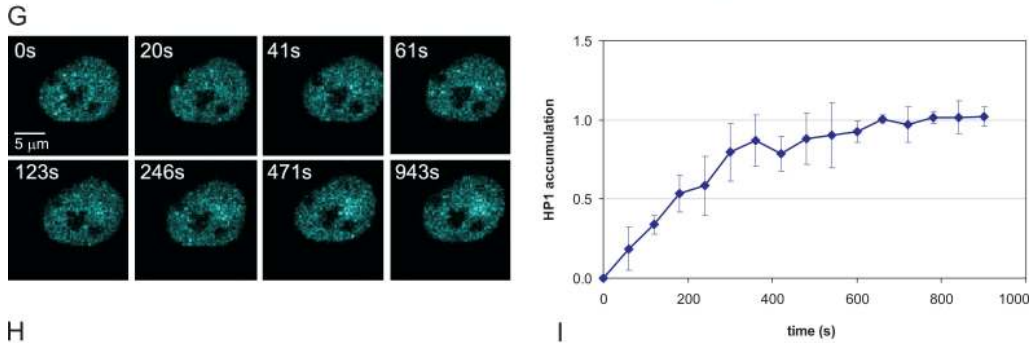
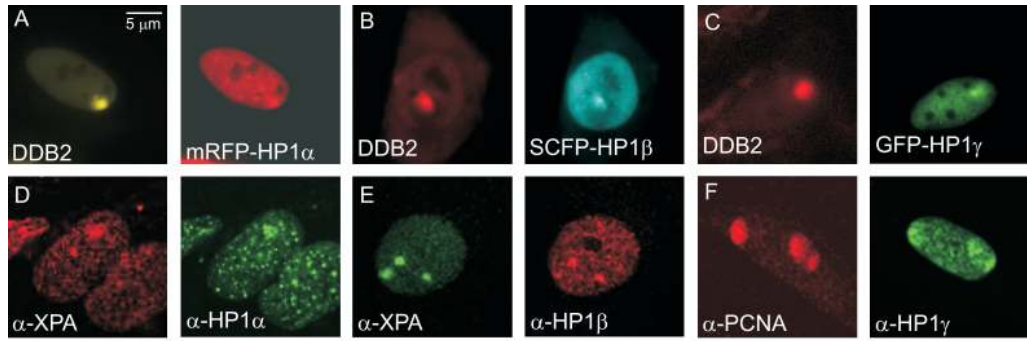
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Abbreviations used in this paper: CD, chromodomain; CSB, Cockayne syndrome protein B; CSD, chromo shadow domain; DDR, DNA damage response; DSB, double-strand break; FLIP, fluorescence loss in photobleaching; FP, fluorescent protein; GGR, global genome NER; HP1, heterochromatin protein 1; IR, ionizing radiation; mRFP, monomeric RFP; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; PCNA, proliferating cell nuclear antigen; SCFP, super cyan FP; TCR, transcription-coupled NER; XP, xeroderma pigmentosum.

The heterochromatin protein 1 (HP1) isoforms HP1- α , HP1- β , and HP1- γ are versatile epigenetic regulators with functions in chromatin organization, transcription regulation, and DNA replication (Maison and Almouzni, 2004). Recent studies suggested that HP1 may respond to chromosomal breaks in heterochromatin (Ayoub et al., 2008; Goodarzi et al., 2008). HP1 proteins bind to histone H3 that is methylated at lysine 9 (H3K9me) via their N-terminal chromodomain (CD; Jacobs and Khorasanizadeh, 2002). Additionally, HP1 interacts with a large number of nuclear proteins via its C-terminal chromo shadow domain (CSD), including epigenetic regulators and chromatin remodelling complexes (Nielsen et al., 2002; Fuks et al., 2003; Eskeland et al., 2007).

In this study, we show that all three HP1 proteins are recruited to UV-induced lesions and DSBs in living human cells.

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This recruitment of HP1 depends on the CSD but not on H3K9 methylation or on the repair systems that remove these lesion types. Loss of HP1 proteins renders the nematode *Caenorhabditis elegans* highly sensitive to UV and ionizing irradiation. Our data suggest that HP1 homologues have both distinct and overlapping essential functions in the DDR.

Results and discussion

HP1 proteins are recruited to UV lesions by the CSD

To study whether HP1 proteins respond to UV-induced DNA damage, we locally damaged nuclei of cultured cells with UV-C light either using a UV-C laser (266 nm; Dinant et al., 2007) or by irradiation with a UV-C lamp (254 nm) through a polycarbonate mask (Moné et al., 2001). These methods recruit NER proteins but not the DSB repair proteins NBS1 (Fig. S1 A), Rad50, Rad54, and Ku80 (Houtsmuller et al., 1999; Dinant et al., 2007). At UV-irradiated sites, we observed recruitment of all three HP1 isoforms expressed as fluorescent protein (FP)-tagged fusion proteins (monomeric RFP [mRFP]-HP1- α , super cyan FP [SCFP]-HP1- β , and EGFP-HP1- γ) in human cells (Fig. 1, A–C) and mouse cells (not depicted). The FPs (CFP, YFP, and GFP) alone do not accumulate at locally damaged sites, indicating that accumulation is HP1 dependent (Fig. S1, F–I). Fluorescent immunolabeling with HP1- β -specific antibodies showed that GFP-HP1- β is expressed at $\sim 20\%$ of the level of endogenous HP1- β (Fig. S1, D and E). Importantly, endogenous HP1- α , HP1- β , and HP1- γ accumulate at local UV damage in primary human fibroblasts (Fig. 1, D–F). Comparing the same cells before and after local UV irradiation (Fig. S1, B and C) showed that SCFP-HP1- β accumulated at damaged sites with a $t_{1/2}$ of 180 s after local UV irradiation (Fig. 1 G), which is comparable to the binding kinetics of XPA (xeroderma pigmentosum [XP] group A) at DNA repair sites (Fig. S1 J). To further investigate the binding of HP1 at lesions, we performed photobleaching experiments on mouse cells that express EGFP-HP1- β and that were globally UV-C irradiated with 25 J/m^2 . These experiments confirmed that a small but significant fraction of EGFP-HP1- β is immobilized in UV-irradiated cells on a time scale of several minutes but not in nondamaged control cells (Fig. 1, H and I). Global UV irradiation did not result in visible changes in the nuclear distribution of HP1 (Fig. S2 A) nor did it result in immobilization of GFP-NLS (Fig. S1, K and L).

HP1 proteins contain three distinct domains: the N-terminal CD, the C-terminal CSD, and the hinge region that separates the

CD from the CSD. Deletion mutants of HP1- β lacking the CD, CSD, or hinge (Fig. 1 J) were tagged with EGFP and tested for recruitment to UV-irradiated regions. Interestingly, UV lesions triggered binding of EGFP-HP1- β (Δ CD) and EGFP-HP1- β (Δ hinge), but EGFP-HP1- β (Δ CSD) failed to accumulate (Fig. 1, K–M). To test whether the CSD (amino acids 98–185 of HP1- β) is not only necessary but also sufficient for recruitment, we fused this domain to EYFP and found that EYFP-CSD is indeed recruited to sites of local UV irradiation (Fig. 1 N). Our results show that HP1 recruitment to damaged sites is independent of the CD, suggesting that recruitment of HP1 is independent of H3K9 trimethylation (H3K9me₃). To verify that HP1 recruitment does not require H3K9me₃, we examined accumulation of HP1 in MEFs deficient for Suv39h1 and Suv39h2 (Peters et al., 2001). After UV irradiation, SCFP-HP1- β accumulated at sites of damage, confirming that binding of HP1 requires neither the CD nor H3K9me₃ (Fig. 1 O). In agreement, we did not detect recruitment of EYFP-tagged H3K9 methyltransferase Suv39h1 (not depicted) nor did we detect increased H3K9me₃ at sites of UV lesions (Fig. S2 B). A recent study showed that HP1 is phosphorylated at residue T51 (in the CD) in response to chromosomal breaks, which was suggested to initiate the DDR (Ayoub et al., 2008). We show that HP1- β lacking the T51 phosphorylation site (YFP-HP1- β^{T51A}) is recruited to sites of UV-induced lesions (Fig. 1 P). Together, our results indicate that HP1 proteins are recruited to UV-induced DNA damage through their CSD independently of the CD and of trimethylation at H3K9.

HP1 recruitment to UV lesions is independent of NER

Cells from placental mammals are fully dependent on NER for the removal of UV-induced DNA injuries, involving transcription-coupled NER (TCR) and global genome NER (GGR; Hoeijmakers, 2001). UV lesions trigger several chromatin-related events, such as recruitment of CAF-1, incorporation of histone H3.1 (Polo et al., 2006), and ubiquitylation of H2A (Bergink et al., 2006). These events strictly depend on the binding and activity of NER proteins that are involved in recognition and subsequent processing of DNA lesions (Bergink et al., 2006; Polo et al., 2006). To investigate whether HP1 binding is a late or early event in NER, we tested accumulation of HP1- β in repair-deficient XP-A cells that have compromised GGR and TCR. SCFP-HP1- β accumulated in XPA mutant cells after UV irradiation (Fig. 2 A), suggesting that HP1- β binding does not occur after DNA repair is finished. We then considered the possibility that HP1 binding

Figure 1. Recruitment of HP1s to UV damage depends on the CSD. (A–C, right) Damage-induced accumulation of mRFP-HP1- α (A), SCFP-HP1- β (B), and EGFP-HP1- γ (C) in living HeLa or MRC5 cells 30 min after local irradiation at 100 J/m^2 through $5\text{-}\mu\text{m}$ pores. (A–C, left) The site of local DNA damage is indicated by accumulation of FP-tagged DDB2. (D–F, right) Immunolabeling of endogenous HP1- α (D), HP1- β (E), and HP1- γ (F) in locally UV-irradiated confluent human fibroblasts irradiated at 100 J/m^2 through 3- (D and E) or $8\text{-}\mu\text{m}$ pores (F; cells are shown 30 min after irradiation). UV-damaged sites are visualized by local accumulation of XPA (D and E) or PCNA (F). (G) Accumulation of SCFP-HP1- β during the first 15 min after localized UV-C laser damage. Fig. S1 (B and C) shows the predamage distribution of HP1 in the same cell as shown in G and accumulation of DDB2-mCherry to indicate the site of damage. (H and I) Combined FLIP/FRAP analysis on NIH/3T3 cells expressing EGFP-HP1- β . Cells were either mock treated (H) or globally irradiated at 25 J/m^2 (I). Half of a cell nucleus was bleached, and FLIP was measured in the nonbleached half 30 min after UV irradiation (blue line), whereas FRAP was measured in the bleached half (red line). (J) HP1- β deletion mutants. The CSD is indicated in red, the CD in blue, and the hinge in yellow. Numbers represent amino acid positions. (K–N) Nuclear localization of EGFP-HP1- β (Δ CD) (K), EGFP-HP1- β (Δ CSD) (L), EGFP-HP1- β (Δ hinge) (M), and EYFP-CSD (N) in living HeLa or MRC5 cells locally irradiated at 100 J/m^2 through $5\text{-}\mu\text{m}$ pores. (O and P) Recruitment of SCFP-HP1- β in Suv3-9-deficient mouse cells (O) and recruitment of EYFP-HP1- β^{T51A} in MRC5 cells (P). The site of local DNA damage is indicated by accumulation of DDB2-mVenus or DDB2-mCherry, and images were taken 30 min after UV irradiation. Error bars indicate SD.

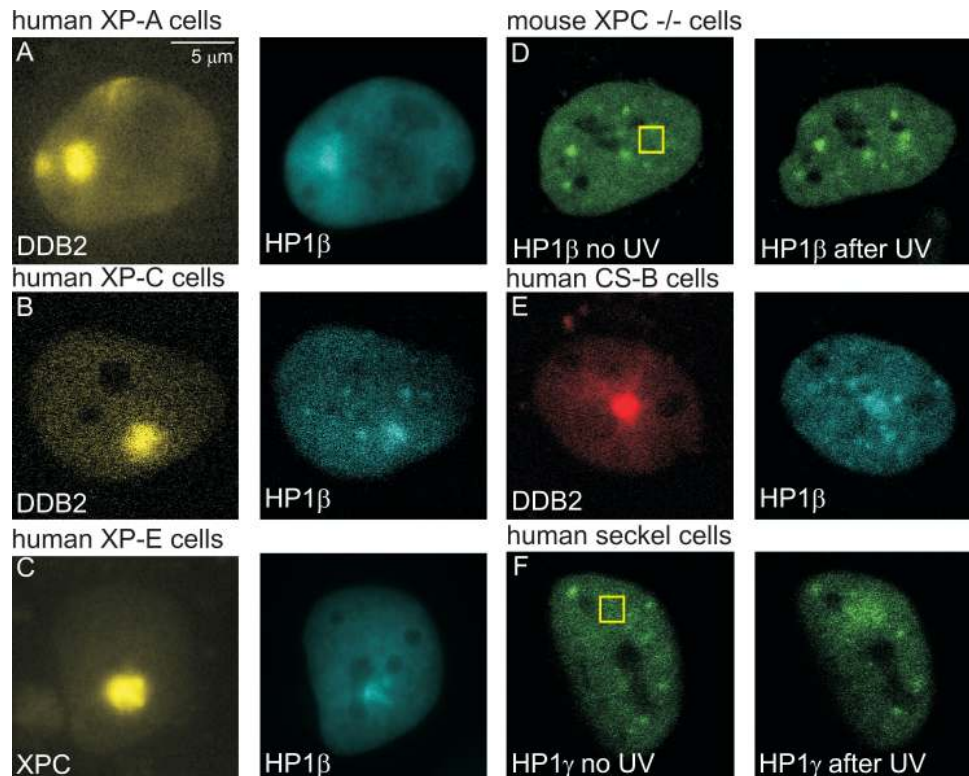


Figure 2. Recruitment of HP1 in NER-deficient cells. (A–F) Damage-induced accumulation of SCFP-HP1- β , EGFP-HP1- β , and EGFP-HP1- γ in human fibroblasts deficient for XPA (A), XPC (B), DDB2 (C), MEFs deficient for XPC (D), human fibroblasts deficient for CSB (E), and human Seckel cells (F), which have severely reduced expression of ATR kinase. Cells were locally UV lamp irradiated at 100 J/m² through 5- μ m pores (A and C) or irradiated using a UV-C laser (B and D–F). The site of local DNA damage is indicated by accumulation of DDB2-mVenus, DDB2-mCherry, XPC-mVenus (A–C), or by a square (D and F). Images were taken 30 min after UV irradiation.

is an early step after damage detection and tested accumulation in DDB2-deficient and XPC-deficient cells. Accumulation of SCFP-HP1- β was observed in both cell types (Fig. 2, B–D). Because binding of repair proteins involved in GGR is dependent on XPC (Volker et al., 2001), these results suggest that HP1 recruitment to damaged sites is independent of the activity of GGR proteins. Damage detection in TCR requires stalled RNA polymerase II, and subsequent recruitment of NER factors depends on the Cockayne syndrome protein B (CSB) protein (Fousteri et al., 2006). Recruitment of HP1 was also observed in CSB-deficient cells, indicating that HP1 binding is not dependent on TCR (Fig. 2 E).

In addition to DNA repair, cells respond to damaged DNA by activating ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad3-related) kinase signaling pathways, resulting in activation of cell cycle checkpoints and phosphorylation of a variety of proteins involved in the DDR (Bartek and Lukas, 2007). To determine whether ATR is required for the recruitment of HP1 to UV lesions, we examined the accumulation of HP1- α , HP1- β , and HP1- γ in Seckel cells, which have severely reduced ATR expression (O’Driscoll et al., 2003). Accumulation of the HP1 isoforms after local UV irradiation was not affected in these cells (Fig. 2 F and not depicted), demonstrating that HP1 recruitment to sites of DNA damage does not require DNA damage-induced signaling mediated by the ATR kinase.

During S phase, stalling of replication forks at UV-induced lesions can result in DSBs. To exclude that HP1 accumulation

is caused by replication stress, we determined the cell cycle stage by expressing mCherry–proliferating cell nuclear antigen (PCNA) together with SCFP-HP1- β and DDB2-mVenus in repair-deficient XP-A cells and wild-type MRC5 cells. Recruitment of HP1- β was observed in wild-type (not depicted) and NER-deficient cells (Fig. S2, C and D) in S phase as well as in non-S phase cells, as shown by the distribution of PCNA. Clear accumulation of endogenous HP1- β was observed in G0 cells (Ki67-negative cells) at sites of UV irradiation (Fig. S2 E), showing that HP1 accumulation is not the result of stalled replication.

Together, these results show that HP1 binding to UV-damaged areas occurs in both cycling and quiescent cells and is independent of the activity of preincision GGR proteins, the TCR factor CSB, and ATR kinase.

UV-induced HP1 accumulation persists in the absence of functional NER

To determine whether loss of HP1 accumulation depends on the presence of UV-induced DNA lesions, we measured HP1 accumulation in repair-deficient cells. Binding of HP1- β was observed up to \sim 4 h after local UV irradiation in XP-A cells (Fig. 3 A). Conversely, in XPA-deficient cells that were transiently transfected with mVenus-XPA (to restore the repair capacity), bound HP1- β levels gradually decreased and HP1 accumulation had almost disappeared 4 h after UV irradiation (Fig. 3 B), which is consistent with the rate of DNA repair (van Hoffen et al., 1995). Accordingly, accumulation of YFP-tagged CSD became

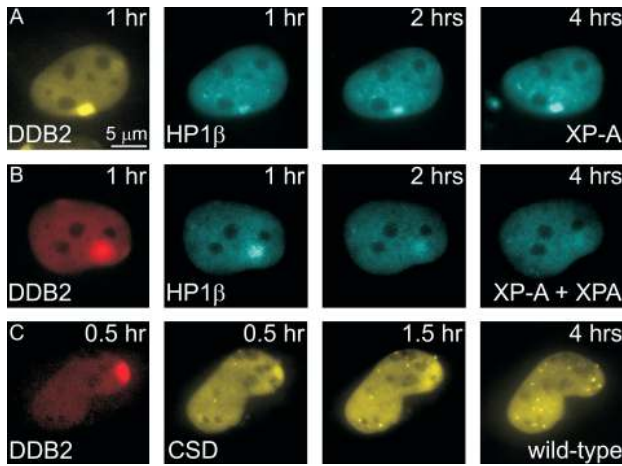


Figure 3. Long-term accumulation of HP1- β in repair-proficient and repair-deficient cells. (A) Repair-deficient XP-A cells were transfected with DDB2-mVenus and SCFP-HP1- β . Cells were irradiated at 100 J/m², and accumulation of HP1- β was monitored for 4 h after UV irradiation. (B) Repair-deficient XP-A cells were transfected with mVenus-XPA (to complement the repair-deficient phenotype), DDB2-mCherry, and SCFP-HP1- β . Cells were irradiated at 100 J/m², and accumulation of HP1- β was monitored for 4 h after UV irradiation. (C) Wild-type (MRC5) cells were transfected with DDB2-mCherry and YFP-CSD, locally irradiated (100 J/m²), and accumulation of the CSD was monitored for 4 h after UV irradiation. The accumulation of DDB2-mVenus or DDB2-mCherry indicates the site of local damage.

undetectable \sim 4 h after irradiation similar to full-length HP1 (Fig. 3 C), suggesting that the UV lesions directly or indirectly create a binding site for the CSD of HP1, resulting in its recruitment to UV lesions.

This is the first example of a protein that is recruited to sites of UV-induced DNA damage independent of any of the known UV damage recognition factors, suggesting that HP1 proteins are involved in the DDR via a novel mechanism that acts in parallel to the well-studied NER repair pathway.

HP1 is recruited to DSBs

To study the response of HP1 to chromosomal breaks, we performed photobleaching experiments on human cells that express EGFP-HP1- β and that were globally irradiated with x rays (5 and 10 Gy). Photobleaching experiments indicated that a small fraction of HP1 became immobile in response to IR (Fig. S3 A), suggesting increased binding of HP1 to chromatin in response to chromosomal breaks. IR did not result in visible changes in the nuclear distribution of HP1 proteins (Fig. S3 B). To confirm immobilization of HP1 at sites of double-strand DNA breaks, we tested HP1 recruitment to locally inflicted DSBs. Cells were irradiated with a dose of \sim 20 Gy of soft x rays (extended UV 20 nm) through a nickel filter containing 5- μ m pores, resulting in local accumulation of γ H2AX. At locally damaged sites, we observed binding of GFP-HP1- β (Fig. 4 A), which is consistent with our fluorescence loss in photobleaching (FLIP) data (Fig. S3 A). To confirm these results, we irradiated cells with α -particles from a radioactive Americium (Am-241) source (Aten et al., 2004; Stap et al., 2008). Irradiation of human U2OS and mouse NIH/3T3 cells with α -particles resulted in linear tracks of γ H2AX. Binding of EGFP-HP1- β in

mouse cells (Fig. 4 B) and endogenous HP1- β in human U2OS cells (Fig. S3 C) colocalized with the linear γ H2AX pattern. In addition, accumulation of GFP-HP1- α and GFP-HP1- γ (Fig. S3, D and E) was observed in MRC5 cells, showing that all HP1 isoforms are recruited to DSBs.

Mammalian cells use homologous recombination or NHEJ to remove DSBs from the genome (Wyman and Kanaar, 2006). The latter pathway is initiated by the KU70/80 dimer, which was shown to interact with HP1- α (Song et al., 2001). To test whether HP1 accumulation at DSBs depends on NHEJ, we irradiated wild-type and KU80-deficient CHO cells with α -particles. Recruitment of endogenous HP1- β was observed in both cell types at all γ H2A.X tracks, showing that HP1 association is independent of NHEJ (Fig. 4 C). These results show that HP1 proteins are recruited to DSBs, which is in contrast to the mobilization of HP1 after DNA damage, as reported recently (Ayoub et al., 2008). In that study, microscopic analysis showed spreading of GFP-HP1- β into a larger area at sites of laser-assisted DNA damage inflicted at heterochromatic sites (Ayoub et al., 2008), which was interpreted as dissociation of HP1- β from heterochromatin. An alternative explanation could be that HP1 does not spread into neighboring chromatin but that the apparent spreading of HP1 actually reflects accumulation of HP1 at sites of DNA damage. To explore this, we have used the same procedure that Ayoub et al. (2008) used to damage DNA locally. Cells were sensitized with Hoechst, and a narrow strip spanning the nucleus was irradiated using a 405-nm laser, resulting in clear accumulation of NBS1-mCherry. At sites marked by NBS1 accumulation, we observed accumulation of GFP-HP1- β (Fig. 4, D and E). The CD and T51 residue (a protein kinase target in HP1) of HP1- β are dispensable for recruitment to these damaged sites (Fig. 4, F and G). Similar to what is observed for UV-induced lesions, we find that the CSD alone is sufficient for binding and that GFP-HP1- β (Δ CSD) does not bind to the damaged DNA sites (Fig. 4, H and I). We could not detect loss or dispersal of HP1 in the damage region before accumulation of HP1. Moreover, monitoring the same cells before and after damage induction showed that HP1 accumulated at higher levels in the locally damaged area compared with the predamage distribution of HP1 (Fig. 4, D and E), showing that the accumulation of HP1- β reflects de novo binding of HP1 molecules at damaged sites. We subsequently measured the binding kinetics of GFP-HP1- β in cells sensitized with BrdU in which a strip spanning the nucleus was irradiated using a 337-nm laser (Fig. 4 J). HP1- β rapidly accumulated at damaged sites with a $t_{1/2}$ of 85 s. The CSD-dependent accumulation of HP1 is markedly different from the recently reported reappearance of HP1 at heterochromatic sites after a transient (5 min) dispersal of HP1 (Ayoub et al., 2008). The latter study showed phosphorylation of HP1 at T51 (T51P) in response to localized laser-assisted DNA damage, IR, and etoposide treatment. Incubation of the CD of HP1- β with CK2 resulted in a weakened interaction with the H3K9me₂ peptide in vitro (Ayoub et al., 2008). However, it remains unclear whether T51-phosphorylated HP1- β has a lowered affinity for chromatin in vivo, as binding of HP1- β to chromatin is influenced by its dimerization with HP1- α , interactions with the H3 histone fold, and an RNA component

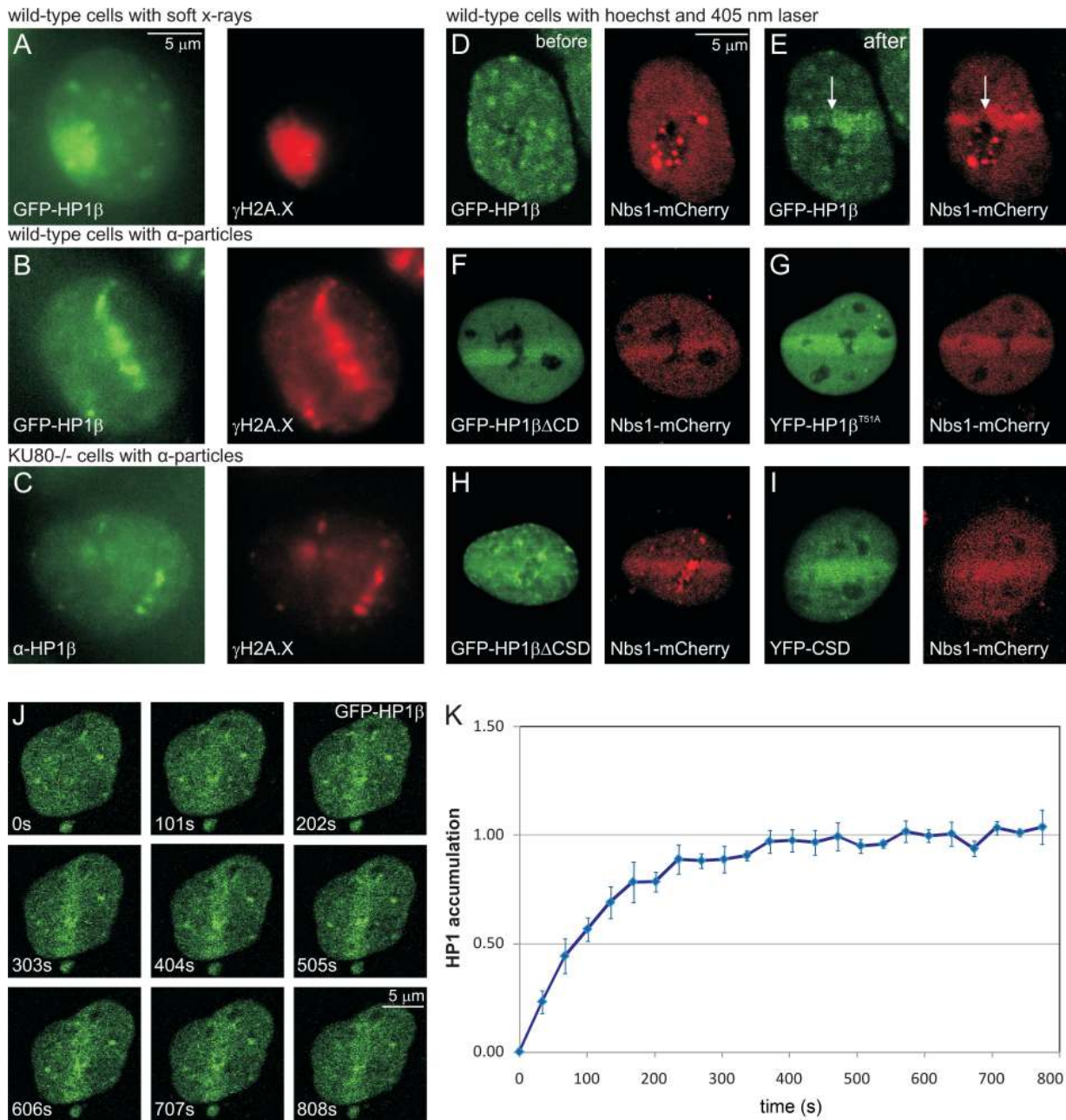


Figure 4. **Recruitment of HP1- β to DSBs.** (A) Mouse cells expressing EGFP-HP1- β (green) were locally irradiated with soft x rays through a nickel mask with pores of 5 μ m and subsequently labeled for γ H2AX (red). (B) Mouse cells expressing EGFP-HP1- β (green) were irradiated with α -particles and subsequently labeled for γ H2AX (red). (C) Hamster cells deficient in Ku80 were irradiated with α -particles and subsequently labeled for endogenous HP1- β (green) and γ H2AX (red). Cells are shown 30 min after irradiation. (D–I) Wild-type U2OS cells expressing various HP1- β fusion proteins were sensitized with 10 μ g/ml Hoechst 33342 for 5 min and locally irradiated (five iterations) in a strip spanning the nucleus using a 405-nm laser at 70% output. GFP-HP1- β before and after laser-assisted damage (the damaged area is indicated by arrows; 5 min; D and E), GFP-HP1- β Δ CD (F), YFP-HP1- β ^{T51A} (G), GFP-HP1- β Δ CSD (H), and YFP-CSD (I). Accumulation of NBS1-mCherry indicates the site of laser-induced DNA damage. (J) GFP-HP1- β accumulation in BrdU-sensitized U2OS cells during the first 800 s after irradiation using a 337-nm laser. (K) Quantification of GFP-HP1- β accumulation as described in J. Error bars indicate SD.

(Nielsen et al., 2001; Maison et al., 2002; Dialynas et al., 2007; Mateos-Langerak et al., 2007). Our results indicate that neither the CD nor the T51 residue are required for the binding of HP1 at sites of DNA damage. It cannot be excluded that phosphorylation of HP1 bound at damaged sites plays a role in phospho-dependent interactions with DDR proteins. Interestingly, HP1 is also recruited to oxidative DNA lesions (unpublished data), indicating that HP1 responds to a variety of DNA lesions in mammalian cells.

Loss of HP1 renders *C. elegans* highly sensitive to UV irradiation and chromosomal breaks

Because loss of all HP1 isoforms in mammalian cells is lethal (Filesi et al., 2002; Schotta et al., 2004), we used the nematode *C. elegans* to test whether HP1 is functionally required for the DDR, as conditional HP1-deficient nematodes are available (Coustham et al., 2006). Nematodes are a very suitable model

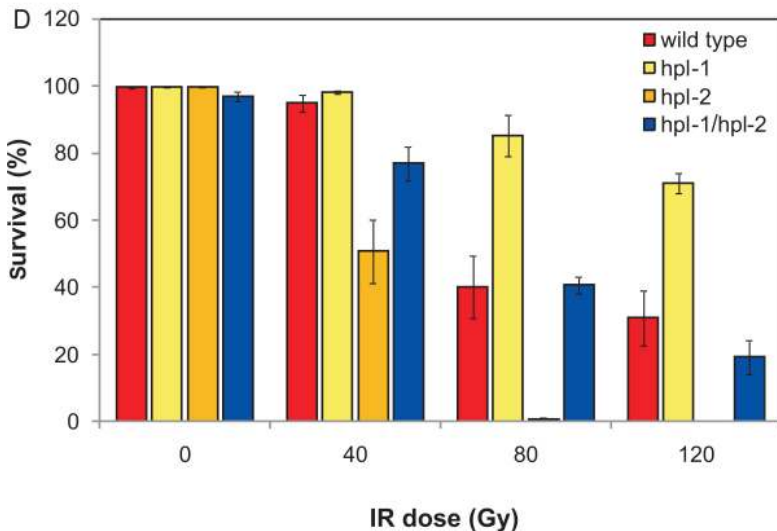
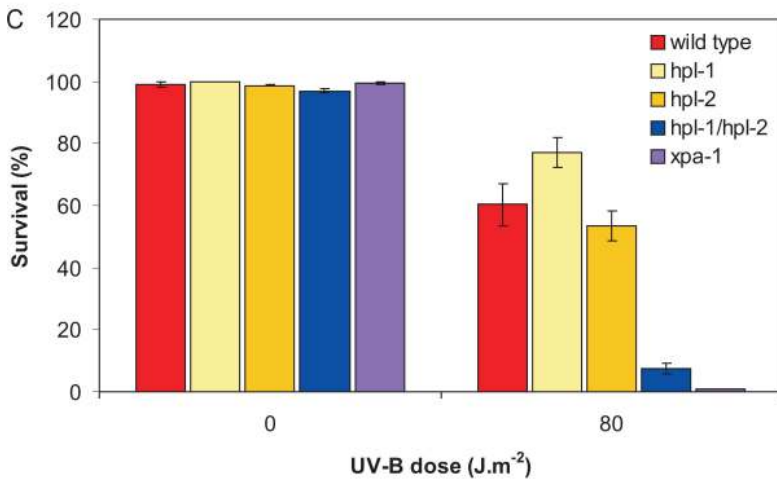
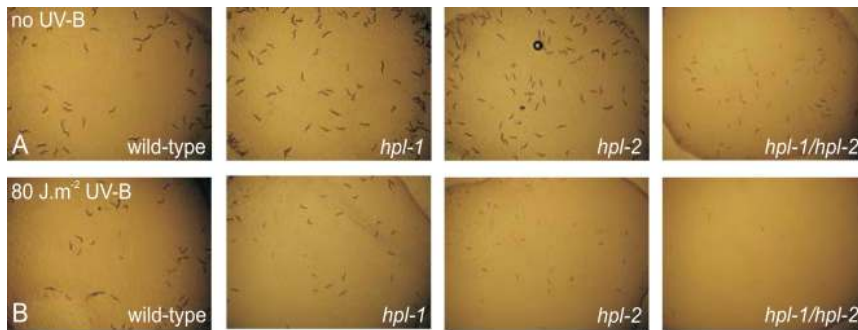


Figure 5. Survival of *C. elegans* HP1 knockout worms upon UV irradiation and IR. (A) Hatching of wild-type, *hpl-1Δ*, *hpl-2^{ts}*, and *hpl-2^{ts}/hpl-1Δ* mutant eggs 8 h after collection. (B) Hatching of wild-type, *hpl-1Δ*, *hpl-2^{ts}*, and *hpl-2^{ts}/hpl-1Δ* mutant eggs 8 h after collection and subsequently irradiated with UV-B at 80 J/m². (C) Quantification of hatching and nonhatching eggs after UV irradiation relative to nonirradiated eggs. In addition to wild-type, *hpl-1Δ*, *hpl-2^{ts}*, and *hpl-2^{ts}/hpl-1Δ* mutant eggs, the survival of *xpa* mutant eggs was also quantified. (D) Quantification of hatching and nonhatching eggs after IR (40–120 Gy) relative to nonirradiated eggs. Bars show the survival of wild-type eggs and of *hpl-1Δ*, *hpl-2^{ts}*, and *hpl-2^{ts}/hpl-1Δ* mutant eggs. Representative assays performed in duplicate or in quintuplicate are shown. For each assay, at least 120 animals were scored. Error bars indicate SD.

system to study the DDR because their response to DNA damage is similar to that of mammals (O’Neil and Rose, 2006; van Haften et al., 2006). Two HP1 homologues (HPL-1 and HPL-2) are present in *C. elegans*. To study sensitivity to DNA damage, we used animals lacking HPL-1 (*hpl-1Δ*) and carrying a temperature-sensitive allele of HPL-2 (*hpl-2^{ts}*), which is expressed at 20°C but not at 25°C (Coustham et al., 2006). Eggs of single- and double-mutant animals were exposed to UV-B radiation (80 J/m²) and transferred to 25°C. Wild-type and NER-deficient *xpa-1*-null eggs (Stergiou et al., 2007) were assayed in parallel (Fig. 5). Exposure to UV-B was used because it penetrates nematodes much better than UV-C light (unpublished data). Irradiation

with UV-B caused an immediate growth arrest in *hpl-2^{ts}/hpl-1Δ* double-mutant worms, similar to NER-deficient *xpa-1* mutant worms. In contrast, single HP1-like protein mutants exhibit comparable UV sensitivity as wild-type worms (Fig. 5, A–C). It should be noted that *hpl-2^{ts}* worms displayed considerably slower growth after UV irradiation, resulting in a smaller size compared with irradiated *hpl-1Δ* worms (Fig. 5 B). An increased UV-sensitive phenotype was also obtained when juvenile *hpl-2^{ts}/hpl-1Δ* worms were irradiated instead of eggs (unpublished data). This indicates that loss of both HP1 proteins renders *C. elegans* highly sensitive to UV irradiation. We subsequently exposed germ cells of single- and double-mutant animals to x rays

(40–120 Gy) and determined the survival of eggs. Remarkably, we observed that *hpl-1Δ* animals are more resistant to IR than wild-type animals (Fig. 5 D), suggesting that loss of HPL-1 is beneficial for repair of these types of damages. Conversely, *hpl-2^{ts}* animals were extremely sensitive to IR, showing that HPL-2 is essential for the response to IR. Interestingly, double-mutant worms showed an intermediate phenotype, which is comparable with wild-type worms. These results suggest that HPL-1 and HPL-2 have opposing effects on IR sensitivity. It is tempting to speculate that *hpl-1Δ* animals are more resistant to IR as a result of an altered, possibly more accessible organization of heterochromatin in these animals. This is reminiscent of a recent study on HP1 in mammalian cells in which the total HP1 pool was reduced (Goodarzi et al., 2008). However, our results also indicate that loss of HPL-2 results in strong IR sensitivity, suggesting an essential function in the DDR after chromosomal breaks. In conclusion, it appears that HP1 proteins have partly redundant roles in response to UV damage, whereas they seem to have unique functions in response to IR. This reveals an essential role for the HP1 proteins in response to UV-induced DNA damage and chromosomal breaks, possibly through different mechanisms.

HP1 and the DDR

What is the molecular role of HP1 in the DDR? Our data suggest that HP1 recruitment does not require DNA repair activity because binding of HP1 at sites of UV lesions and DSBs is independent of any of the known damage recognition proteins (Fig. 2 and Fig. 4 C). In TCR, stalled RNA polymerase II initiates NER, which could trigger binding of HP1 proteins (Mateescu et al., 2008). However, HP1 proteins also accumulate at damaged sites in cells in which transcription is blocked with α -amanitin (unpublished data), suggesting that HP1 proteins are recruited through a damage detection system that is different from that for TCR and GGR. HP1 binding depends on its CSD but not the CD or H3K9 trimethylation. It is possible that DNA damage-induced changes in local chromatin structure are recognized by HP1 proteins. In agreement with this, we show that HP1 accumulation persists in repair-deficient cells in which lesions are not removed (Fig. 3 A). ACF1 interacts with the CSD of HP1 and accumulates at UV lesions (Fig. S3, F and G; Eskeland et al., 2007), suggesting that this remodelling factor may cooperate with HP1 to modify chromatin structure in damaged areas. However, HP1 recruitment was still observed in cells depleted for ACF1 (unpublished data). Consistent with an essential role for HP1 in facilitating DNA repair, we found that HP1-deficient nematodes are extremely sensitive to UV-induced DNA damage. HP1 isoforms each distinctly contribute to the sensitivity to UV- and IR-induced DNA damage, suggesting divergent functions for HP1 family members to different types of DNA damage. In support of this idea, neuronal cells derived from HP1- β -deficient mice but not HP1- α -deficient animals display genomic instability (Aucott et al., 2008). Recently, it was shown that replication of pericentromeric heterochromatin in mouse cells depends on binding of the p150 subunit of CAF-1 to the CSD of HP1 (Quivy et al., 2008). In analogy to these findings, we favor a scenario in which HP1 proteins play a role in reorganizing higher order chromatin structure, as recently suggested by Kruhlak et al. (2006) and Solimando et al. (2009),

which is essential for DNA repair. In conclusion, our experiments reveal an intriguing link between HP1 proteins and DNA repair systems.

Materials and methods

Cell lines

Cell lines used in this study were HeLa, U2OS, CHOK1, NIH/3T3, NIH/3T3 EGFP-HP1- β (Mateos-Langerak et al., 2007), VH10 hTERT fibroblasts, ATR-deficient GM18366-hTERT Seckel cells (Bergink et al., 2006), Suv3-9h double-knockout MEFs (provided by T. Jenunien, Research Institute of Molecular Pathology, Vienna, Austria; Peters et al., 2001; Schotta et al., 2004), and KU80-deficient XR-V15B CHO cells (Mari et al., 2006). The NER-deficient SV40-immortalized cell lines were XP4PA (XP-C), XP20S (XP-A), XP12RO (XP-A), XP23PV (XP-E), MEFs XPC^{-/-}, and CS1AN (CS-B). All cell lines were cultured as described previously (Luijsterburg et al., 2007). For immunolocalization experiments, hTERT human fibroblasts were grown to confluency for 10 d. Subsequently, cells were synchronized in G0 by keeping them for a minimum of 5 d in medium supplemented with 0.2% FCS.

DNA constructs

HP1- α and HP1- β cDNA were ligated in frame with mRFP and SCFP3a. EGFP-HP1- β and EGFP-HP1- γ were provided by P. Hemmerich (Fritz Lipmann Institute, Jena, Germany; Schmiedeberg et al., 2004). All constructs were transiently transfected in various cell lines cells using Lipofectamine 2000 (Invitrogen). EGFP-HP1- β was stably expressed in mouse NIH/3T3 cells (Mateos-Langerak et al., 2007), and EGFP-HP1- α and EGFP-HP1- γ were stably expressed in MRC5-SV cells. HP1- β (Δ CD) was tagged with EGFP (Mateos-Langerak et al., 2007). EGFP-HP1- β (Δ CSD) and EGFP-HP1- β (Δ hinge) were provided by T. Misteli (National Cancer Institute, Bethesda, MD; Cheutin et al., 2003), and EYFP-tagged CSD was provided by Y. Hiraoka (Osaka University, Osaka, Japan). HP1- β ^{T51A} was created by overlap PCR and fused to EYFP. XPC and DDB2 were fused to mVenus. In addition, DDB2 was fused to mCherry (Luijsterburg et al., 2007). ACF1-EGFP was provided by P.D. Varga-Weisz (Babraham Institute, Cambridge, England, UK). EYFP-Suv3-9H1 was provided by R.W. Dirks (Leiden University Medical Center, Leiden, Netherlands). NBS1-mCherry was provided by J. Lukas (Institute of Cancer Biology and Centre for Genotoxic Stress Research, Copenhagen, Denmark). The cDNAs for SCFP3a and mVenus were provided by J. Goedhart (University of Amsterdam, Amsterdam, Netherlands), and mCherry and mRFP cDNA were provided by R.Y. Tsien (University of California, San Diego, La Jolla, CA).

UV-C irradiation

UV lamp-induced damage was inflicted using a UV source containing four UV lamps (TUV 9W PL-S; Philips) as described previously (Moné et al., 2004; Luijsterburg et al., 2007) or using a customized UV cross-linker (CL-1000; UVP) containing two UV-C lamps. UV laser-induced damage was inflicted by using a 2-mW pulsed (7.8 kHz) diode-pumped solid-state laser emitting at 266 nm (Rapp Optoelectronic GmbH) as described previously (Dinant et al., 2007).

Irradiation with x rays

We used an x-ray generator (150 kV; 15 mA; dose rate, 2.18 Gy/min; HF160; Pantak) to irradiate cells globally as previously described (Syljuasen et al., 2004).

Irradiation with soft x rays

Cells were plated in custom-made culture dishes containing an ultra-thin Mylar bottom (Aten et al., 2004; Stap et al., 2008). The dishes were placed on a soft x-ray source. We used a modified EG2 electron bombardment evaporation source (VG Scienta), which was fitted with a carbon anode to generate 277-eV photons. For detailed information about this type of source, see Agarwal and Sparrow (1981). The source was operated at 3-kV electron energy and 8-mA electron current. The unit was mounted in a vacuum chamber ($P < 10^{-5}$ Torr) equipped with a Mylar film window (2- μ m thick), which was supported by a stainless steel grid (1-mm maze size) to withstand atmospheric pressure. Cells were irradiated through a metal filter (Stork Veco BV) with pores of 5 μ m to inflict local damage. Cells were irradiated for 6 min, corresponding with a dose of \sim 20 Gy.

α -Particle irradiation

Cells were cultured in carbon-coated Mylar dishes. Cells were irradiated using an Americium (Am-241) source with an activity of 140 kBq at an

angle of 30° with the horizontal plane to obtain long linear arrays of DSBs. The radioactive source just contacts the Mylar as described previously (Stap et al., 2008). Cells were irradiated at room temperature for 0.5 min and subsequently fixed using paraformaldehyde (final concentration 2%).

Laser irradiation after sensitization with Hoechst

U2OS cells expressing EGFP-HP1- β were sensitized with 10 μ g/ml Hoechst 33342 (Invitrogen) for 5 min and locally irradiated (five iterations) in a strip spanning the nucleus using a 405-nm laser at 70% output. NBS1-mCherry was used as a marker for laser-induced damage.

UV-A (337 nm) irradiation after sensitization with BrdU

U2OS cells expressing EGFP-HP1- β were incubated with 10 μ M BrdU one day before irradiation. Cells were irradiated in a strip spanning the nucleus with a 30-Hz 337-nm laser (PALM Laser Technologies) at ~61% laser output. For more details, see Lukas et al. (2003).

Immunofluorescence

Fixed cells were immunolabeled as described previously (Aten et al., 2004; Stap et al., 2008). The following primary antibodies were used: mouse monoclonal antibody against γ H2AX (clone JBW301; Millipore), mouse monoclonal antibody against XPA (clone 12F5; Abcam), rat monoclonal antibody against HP1- β (1:250; provided by P. Singh, Research Center Borstel, Borstel, Germany; Wreggett et al., 1994), and mouse monoclonal antibody against HP1- β (1:500). Secondary antibodies used were goat anti-mouse Cy3, goat anti-rat FITC (Jackson ImmunoResearch Laboratories), and goat anti-mouse Alexa Fluor 546 (Invitrogen). All antibodies were diluted in PBS containing 0.1% Triton-X 100 and 1% FCS. Fluorescence microscopy images were acquired using a microscope (DM RA HC; Leica) equipped with a Plan Apo 100 \times 1.40 oil objective (Carl Zeiss, Inc.) and a cooled charge-coupled device camera (KX1400; Apogee Instruments).

Microscopic analysis

Cells were imaged with a confocal microscope (LSM 510 or LSM 510 META; Carl Zeiss, Inc.) equipped with a 63 \times Ultrafluar 1.2 NA glycerol immersion lens (Carl Zeiss, Inc.) and a 30-mW Argon laser (488 and 514 nm) or with a widefield fluorescence microscope (Axiovert 200M; Carl Zeiss, Inc.) equipped with a 100 \times Plan Apochromat 1.4 NA oil immersion lens (Carl Zeiss, Inc.) and a xenon arc lamp with monochromator (Cairn Research). Images were recorded with a cooled charge-coupled device camera (Coolsnap HQ; Roper Scientific). Both microscopes were equipped with an objective heater, and cells were examined in microscopy medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 20 mM D-glucose, and 20 mM Hepes) at 37°C.

Combined FRAP and FLIP

FRAP and FLIP analyses were performed as described by Hoogstraten et al. (2002) and Zotter et al. (2006). In brief, ~50% of the nuclei of NIH/3T3 cells expressing EGFP-HP1- β were bleached using 100% power of a 488 and 514 laser line. The loss of fluorescence from the nonbleached half (FLIP) and the gain of fluorescence in the bleached half (FRAP) were determined. The data were corrected for background values and normalized to prebleach intensity.

C. elegans UV-B and IR survival assay

The *C. elegans* strains used were Bristol N2 (wild type), *hpl-1(tm1624)*, *hpl-2(tm1489)*, *hpl-2(tm1489)*, *hpl-1(tm1624)* (constructed using PCR to confirm deletions), and *xpa-1(ok698)*. The *hpl-1(tm1624)* and *xpa-1(ok698)* are likely null alleles (Schott et al., 2006; Stergiou et al., 2007), whereas *hpl-2(tm1489)* is a null allele that causes severe growth delay and sterility only at 25°C but not at 20°C (Coustham et al., 2006). Thus, it was possible to test UV and IR sensitivity by growing animals at 20°C and transferring them to 25°C after UV or IR irradiation. To test UV sensitivity, eggs were collected from gravid adult animals by ClNaO/NaOH treatment and placed on culture plates seeded with HT115 bacteria at 20°C. 8 h after egg collection, eggs were irradiated using two FS20 erythral UV-B lamps (Phillips) and transferred to 25°C. To test IR sensitivity, young adult animals were irradiated using a ¹³⁷Cs source and recovered for 4 h after which the animals were allowed to lay eggs for 18 h at 25°C. Survival rate was determined by counting the amount of hatching eggs (surviving animals) and nonhatching, dead eggs. Additional details are available upon request.

Online supplemental material

Fig. S1 shows validation of local UV irradiation and expression of FP-tagged HP1. Fig. S2 shows that HP1 recruitment is independent of H3K9me₃ and the cell cycle. Fig. S3 shows increased binding of HP1

after IR and α -particle irradiation and recruitment of ACF1 to local UV damage. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200810035/DC1>.

We thank Drs. P. Hemmerich, T. Misteli, R.W. Dirks, Y. Hiraoka, R.Y. Tsien, J. Goedhart, P.D. Varga-Veisz, and T. Jenuwein for providing constructs and cell lines and Dr. P. Singh for providing HP1 antibodies. We thank J. Verhoeven and C. van Oven for technical assistance with soft x-ray experiments. We are grateful to the *Caenorhabditis* Genetics Center and the Japanese National Bioresource Project for *hpl-1*, *hpl-2*, and *xpa-1* strains. We also thank Drs. E.M.M. Manders and T.V.V.J. Gadella (Center for Advanced Microscopy, University of Amsterdam, Amsterdam, Netherlands) for support.

This work was supported by ZonMW (grant 912-03-012 to R. van Driel, A.B. Houtsmuller, L.H.F. Mullenders, and W. Vermeulen), the Netherlands Organisation for Scientific Research (grant 2007/09198/ALW/825.07.042 to M.S. Luijsterburg), the Danish National Research Foundation (C. Dinant), the Swedish Research Council (N.P. Dantuma), the Dutch Cancer Society (J. Stap and J.A. Aten), and the Association for International Cancer Research (grant 08-0084 to H. Lans).

Submitted: 6 October 2008

Accepted: 22 April 2009

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