The ATM Substrate KAP1 Controls DNA Repair in Heterochromatin: Regulation by HP1 Proteins and Serine 473/824 Phosphorylation

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Abstract

The repair of DNA damage in highly compact, transcriptionally silent heterochromatin requires that repair and chromatin packaging machineries be tightly coupled and regulated. KAP1 is a heterochromatin protein and co-repressor that binds to HP1 during gene silencing but is also robustly phosphorylated by Ataxia telangiectasia mutated (ATM) at serine 824 in response to DNA damage. The interplay between HP1-KAP1 binding/ATM phosphorylation during DNA repair is not known. We show that HP1α and unmodified KAP1 are enriched in endogenous heterochromatic loci and at a silent transgene prior to damage. Following damage, γH2AX and pKAP1-s824 rapidly increase and persist at these loci. Cells that lack HP1 fail to form discreet pKAP1-s824 foci after damage but levels are higher and more persistent. KAP1 is phosphorylated at serine 473 in response to DNA damage and its levels are also modulated by HP1. Unlike pKAP1-s824, pKAP1-s473 does not accumulate at damage foci but is diffusely localized in the nucleus. While HP1 association tempers KAP1 phosphorylation, this interaction also slows the resolution of γH2AX foci. Thus, HP1-dependent regulation of KAP1 influences DNA repair in heterochromatin. Mol Cancer Res; 10(3); 401–14. ©2011 AACR.

Introduction

Cells must efficiently repair damaged DNA, which is packaged into chromatin. Phosphoinositide 3-kinase (PI3K) family members, including Ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK), become activated in response to DNA damage and phosphorylate the SQE motif found in many proteins including the core histones that package nucleosomes (1). For example, the ATM kinase phosphorylates histone H2AX at the site of damage, which acts as a scaffold for the assembly of the repair machinery. As most repair factors are highly mobile in the damage, they are thought to require a chromatin environment, including both nucleosomal and higher order chromatin architecture. Chromatin disassembly upon damage and chromatin reassembly after repair must be localized and efficient. Historically, chromatin can be broadly divided into heterochromatin, which is condensed and transcriptionally silent, and euchromatin, which has an open configuration and is associated with actively transcribing genes. On the basis of investigations of repair dynamics, it is thought that the timing and spatial regulation of the repair of these 2 types of chromatin are differentially regulated. Recent work by Noon and colleagues suggests that DNA repair, as measured by the number of H2AX Ser 139 phosphorylated (γH2AX) foci, is bimodal; the population of breaks in euchromatin is repaired within the first 2 to 3 hours following irradiation whereas heterochromatin is repaired later (4).

The H3-trimethyl K9 binding protein (HP1) and KRAB-associated protein 1 (KAP1) are 2 important components of heterochromatin (5, 6). KAP1 is best known as the obligate co-repressor for Krüppel-associated box zinc finger proteins (KRAB-ZFP; ref. 7). KRAB-ZFPs are sequence-specific transcriptional repressors that bind to DNA through their zinc fingers. KAP1 interacts with the KRAB domain and acts as a scaffold for the assembly of gene silencing factors including HP1, the H3-K9 methyltransferase Su(var)3-9 and "Enhancer of zeste" domain protein 1 (SETDB1) and the chromatin remodeling factor Chromodomain helicase DNA-binding protein 3 (CHD3/Mi-2α), which promote transcriptional repression and chromatin condensation (8–11).
HP1 is abundant at sites of facultative and constitutive heterochromatin throughout the nucleus (12). There are 3 isoforms in mammals, HP1α, β, and γ. HP1α and HP1β predominantly localize to heterochromatin, whereas HP1γ has been observed in both euchromatin and heterochromatin (13, 14). HP1 exists as a homodimer and tetramer in vivo, and it is thought that HP1 multimerization contributes to chromatin condensation, which stabilizes constitutive heterochromatic structures including kinetochores, centromeres, and telomeres (15–17).

KAP1 interacts with HP1 through a well-defined peptide motif, PxVxL. Mutagenesis of this motif abolishes the ability of KAP1 to silence transcription (18). In undifferentiated cells, KAP1 is diffuse. Upon differentiation, KAP1 relocates to pericentromeric heterochromatic foci where it colocalizes with HP1. As the KAP1 HP1-binding mutant fails to colocalize with HP1 during differentiation, this supports the idea that its interaction with HP1 is required for its integration into the higher order architecture of heterochromatin (19, 20). Currently, it is not well understood how this interaction is dynamically regulated during the DNA damage response when the repair machinery must gain access to DNA assembled into heterochromatin.

In response to DNA damage, P3K family members, such as ATM, phosphorylate KAP1 at serine 824 and this modification colocalizes with γH2AX foci at damage sites (21–23). The hyperacetylation of Mre11, Rad50 and Nbs1 (MRN) complex by tumor suppressor p53-binding protein 1 (53BP1) helps retain activated ATM at sites of damage residing in heterochromatin that require more time to repair. Without the recruitment of 53BP1, pKAP1 foci are not evident at these sites and DNA repair is less efficient. ATM-mediated recombination occurred with approximately 90% efficiency to generate KAP1+/− MEFs. Lentiviruses expressing wild-type and Mut2-mutant KAP1 were used to reconstitute KAP1 knockout MEFs.

Materials and Methods

Cell lines

Early-passage mouse embryonic fibroblasts (MEF) were transduced with a self-excisable, CRE-expressing lentiviral vector (CRE LV). PCR evaluation revealed that CRE-mediated recombination occurred with approximately 90% efficiency to generate KAP1+/− MEFs. Lentiviruses expressing wild-type and Mut2-mutant KAP1 were used to reconstitute KAP1 knockout MEFs.

Short hairpins for HP1α, HP1β, HP1γ, and a scrambled sequence were expressed from a doxycycline-regulatable TRIPZ vector containing puromycin resistance (1 μg/mL) and red fluorescent protein. To obtain HP1 knockout cells, H1299 cells were sequentially infected with the TRIPZ-HP1 targeting lentiviruses. To obtain the vector control, H1299 cells were infected with a TRIPZ-scrambled sequence lentivirus. High red fluorescent protein–expressing cells sorted by fluorescence-activated cell sorter (FACS) were grown in doxycycline (1 μg/mL) for 3 days to diminish HP1 expression.

GFP-tagged short hairpins for KAP1 were expressed from a retroviral backbone and high GFP-expressing U2OS cells were isolated via FACS. A clone with the lowest expression of KAP1 was obtained by serial dilution. The GFP-tagged vector lacking short hairpin RNA (shRNA) was used to create the U2OS-GFP cell line. KAP1 was restored through the stable expression of FLAG-tagged KAP1 maintained under Zeocin (200 μg/mL) selection (pcDNA3.1 vector).

Immunofluorescence

The method for immunostaining U2OS, MEFs, and H1299 cells is described in White and colleagues (22). The following method was used for the KAP1, pKAP1, and for all HP1 antibody immunostaining of the NIH3T3 2/4 cells. For preextraction, cells grown on glass coverslips were washed once in PBS. Cells were then washed with CSK buffer (10 mmol/L PIPES, pH 7.0, 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonylfluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin; Sigma-Aldrich) for 3 minutes. CSK buffer containing 0.5% Triton X-100 was then added for 5 minutes. The cells were then washed with CSK buffer for 3 minutes, followed by 2 washes in PBS. Preextracted cells were fixed in 4% formaldehyde in PBS for 10 minutes and then blocked with Signal Enhancer solution (Invitrogen) as described.
For all U2OS, MEFs, and H1299 samples, coverslips were mounted in Fluoromount-G (Southern Biotech). For the NIH3T3 2/4 samples, coverslips were mounted in antifade fluorescence mounting medium (26).

**Imaging**

Images of the NIH3T3 2/4 cells were acquired using a Leica DMI 6000 B inverted automated microscope with HCX PL APO 100×/1.40–0.70 oil objective lens using a 457/488/514 nm 30 mW Argon-Ion laser for yellow fluorescent protein (YFP), a 561 nm/25 mW diode laser for mCherry, and a 442 nm/70 mW diode laser for cyan fluorescent protein (CFP) imaging. A Z-drive controlled by COMPIX SimplePCI software was used to collect image stacks using a Yokogawa CSU-10 real-time spinning disk confocal attachment with Nipkow and microlens disks. Image stacks were taken (0.4 μm increments) using Hamamatsu ORCA-AG camera (1 × 1 binning; 1,344 × 1,024 pixels). One image from the Z stack is represented in the figure. Image contrast adjustments were conducted using COMPIX SimplePCI and Adobe Photoshop software.

Images of all other cell lines were acquired using a Leica DMRB E-32 confocal laser scanning microscope with an inverted platform using a 100× objective lens with no additional zoom. A 488 helium/neon, a 568 argon/krypton, and a 633 helium/neon laser were used to obtain all confocal images.

**Image analysis**

Colocalization analyses were done using SimplePCI software. Two-dimensional intensity profiles across the transsection site were also obtained for some images. The signal intensity of γH2AX immunofluorescence was analyzed using SimplePCI software. The nuclei of irradiated cells taken at 40× magnification were identified by imaging the Hoechst stain. A circle was manually drawn around each nucleus, and the intensity of γH2AX signals were quantified from 50 cells for each time point using the "Hole Total nucleus, and the intensity of the Hoechst stain. A circle was manually drawn around each quantified with the parental cell acting as an internal control (26).

Fifty parental and reconstituted KAP1 knockdown cells were mixed 50:50 with the reconstituted cells which reconstituted KAP1 knockdown cells. Here, parental U2OS (Invitrogen).

**Immunoprecipitation of exogenously expressed proteins**

Plasmids expressing MYC and FLAG-tagged genes were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). N-Ethylmaleimide (NEM)-based whole cell extracts were prepared as described in the work of Ivanov and colleagues (27). For immunoprecipitation, 250 μg of extracts were diluted 1:10 in NEM extraction Buffer and then incubated for 3 hours with FLAG Ab-conjugated beads (Sigma) at 4°C. Beads were washed 3 times in NEM extraction buffer. Immunoprecipitated proteins were boiled and resolved on a precast gel (Invitrogen).

Following treatment with 100 nmol/L neocarzinostatin (NCS) for 1 hour, proteins were isolated from reconstituted U2OS KAP1 knockdown cells using the NEM extraction method previously described. For immunoprecipitation, 500 μg of extract was immunoprecipitated as described in the work of Ivanov and colleagues (27).

**Preparation and Western blot analysis of protein extracts**

Cells were washed twice in PBS, then lysed in NP40 extraction buffer containing 50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 2 mmol/L dithiothreitol (DTT), 1× Phosphatase Inhibitor Cocktail (Sigma), and 1× Protease Inhibitor Cocktail (Roche). When indicated, nuclear extracts were prepared as described in the work of Klonova and colleagues (28) with 1% Phosphatase Inhibitor Cocktail 1 (Sigma) added to the lysis buffers.

The anti-KAP1 (C-terminal epitope, amino acids 618–835) and anti-pKAP1-Ser 824 rabbit polyclonal antibodies were generated by the Rauscher laboratory (22). The anti-pKAP1-Ser 473 rabbit polyclonal antibody was generated by the Lee laboratory (25). The anti-H1pα, HP1β, and HP1γ rabbit polyclonal antibodies were kind gifts from David C. Schulz. The anti-actin rabbit polyclonal antibody (20–33, Sigma) and the mouse monoclonal antibodies anti-FLAG (F3165, Sigma), anti-γH2AX-Ser 139 (JBW301, Upstate), and anti-MYC (13–2500, Zymed Laboratories) were purchased.

**Chromatin fractionation**

A total of 1 × 10⁶ cells were plated onto 10-cm plates in duplicate and incubated overnight. Cells were scraped and washed twice with PBS. One replicate was lysed with 100 μL of SDS loading buffer and briefly sonicated. The other replicate was fractionated as described in the work of Mendez and Stillman (29). To extract KAP1 from the NP fraction in Fig. 6C, nuclear extraction buffer containing 500 mmol/L NaCl was substituted in the final extraction step (28).

**Statistical analyses**

For comparative analyses between the control and experimental samples, Student unpaired 2-tailed t tests were conducted using online GraphPad software (http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD).

**Results**

We first evaluated the localization pattern of KAP1 in human U2OS cells over the course of the cellular response to ionizing radiation (IR). The line in the scanning diagram was arbitrarily drawn and represents a cross-section of the immunostained nucleus. At 5 and 30 minutes postirradiation, pKAP1-s824 and γH2AX appear to be in euchromatin as their foci are numerous, highly dispersed, and only partially
overlap with each other as determined by the line scanning diagrams of factor intensity levels (Fig. 1A, panels 6 and 9). In contrast, at 1.5 hour and later, most of the γH2AX and pKAP1-s824 foci completely colocalize (Fig. 1A, panels 12, 15, and 18). Nine hours post-IR, repair appears to be complete as only a few damage sites are seen, similar in number to those in undamaged proliferating cells (Fig. 1A, panels 3 and 21).

Figure 1. A, immunohistochemical detection of pKAP1-s824 and γH2AX-s139 in untreated and irradiated U2OS cells. B, immunohistochemical detection of pKAP1-s824 and HP1α in irradiated U2OS cells. Treated cells were irradiated with 9 Gy IR. Line scans represent the colocalization of proteins within each image. Scale bars (μm, in white). ‘+’ denotes the end of the line in the images (odd numbered) and the scanning diagram (x axis).
Because HP1α-rich heterochromatic regions do not have a distinct staining pattern in human cells (Fig. 1B), we examined γH2AX and pKAP1-s824 in MEFs in which HP1α enrichments can be clearly seen at chromocenters (20). Six hours after irradiation, γH2AX colocalizes in foci with both HP1α and pKAP1-s824, strongly suggesting that at the later time points of the DNA damage response, γH2AX and pKAP1-s824 are enriched in heterochromatin (Supplementary Fig. S1A and S1B).

To define KAP1 and HP1 dynamics at a region of heterochromatin during DNA damage, we used a murine cell line, which contains a stably integrated multicopy array of the L-SceI-T transgene integrated at a single locus (ref. 30; Fig. 2A). The transgene contains a single SceI restriction site flanked by arrays of lac operator and tetracycline response element repeats. The transgene array can be visualized by expressing CFP-lac repressor. A single double-strand break site can be induced in the array by expressing a YFP-tagged SceI endonuclease-glucocorticoid receptor fusion protein (YFP-SceI-GR). Treatment of cells with triamcinolone acetonide triggers its translocation into the nucleus and cleavage of the SceI site. Because SceI sequences are not present in murine cells, DNA breaks only occur at the transgene array, which produces a localized site of DNA damage (30). Studies of this and other transgene arrays have shown that they are highly condensed and enriched in heterochromatic modifications and binding proteins, including histone H3 tri-meK9 and HP1, presumably due to mechanisms of repeat-induced silencing (26). Thus, this SceI transgene array is an ideal model for understanding DNA repair dynamics in heterochromatin.

**Figure 2.** A, model depicting the L-SceI-T transgene found in NIH3T3 2/4 cells. B, immunofluorescent confocal microscopy of NIH3T3 2/4 cells cotransfected with plasmids expressing CFP-LacI and YFP-SceI-GR and grown in the presence of triamcinolone acetonide (TA) using antibodies specific for KAP1 or HP1α (B) and pKAP1-s824 or γH2AX-s139 (D). Line scans represent the colocalization of proteins within each image. Scale bars (µm, in white). Line graphs depict the number of CFP-LacI foci that also exhibited positive KAP1 or HP1α staining and pKAP1 or γH2AX staining in transfected NIH3T3 2/4 cells grown in the presence or absence of TA. **x** denotes the end of the line in the images (Merge) and the scanning diagram (x axis).
Figure 3. A, model depicting the Ser 473 and Ser 824 phosphorylation sites on KAP1. The PxVxL HP1-binding motif is underlined. Western blot (WB) analysis of proteins isolated from U2OS cells at various time points following exposure to either 9 Gy IR (B) or 100 nmol/L NCS (C) using antibodies specific for pKAP1-s473, pKAP1-s824, KAP1, and tubulin. D, immunohistochemical detection of pKAP1-s473 and γH2AX in U2OS cells following IR. Line scans represent the colocalization of proteins within each image. *, denotes the end of the line in the images (odd numbered) and the scanning diagram (x axis). Scale bars (μm, in white). E, reconstituted U2OS KAP1 knockdown (KD) cells grown in the presence or absence of 100 nmol/L NCS using antibodies specific for pKAP1-s473, pKAP1-s824, KAP1, and tubulin. Western blot analysis. F, immunoprecipitation (IP) of FLAG-HP1α coexpressed with an MYC-KAP1 in HEK293 cells. The levels of FLAG-HP1α, MYC-KAP1, pKAP1-s824, and pKAP1-s473 were examined via immunoblotting.
Prior to damage, endogenous KAP1 and HP1α are enriched at the array. Both proteins are also enriched and colocalize at chromatocenters which are easily identifiable in murine cells (Fig. 2B and Supplementary Fig. S1C and S1D). Taken together, this indicates that the transgene array is a faithful model for endogenous heterochromatin and that KAP1 and HP1α are enriched at the array prior to the induction of damage.

To define the dynamics of KAP1 and HP1α in response to double-strand breaks (DSB) at the transgene array, we treated the cells with triaminolone acetonide, which translocates the Sce-GR endonuclease into the nucleus so that it can target the Sce site. Introduction of a small number of highly localized DSBs is capable of activating the DNA damage response resulting in the phosphorylation of H2AX and KAP1 at this site (Fig. 2B). The number of cells with KAP1 and HP1α at the transgene array was counted to determine whether they were lost during the damage response. In the absence of damage, KAP1 and HP1α were detected at 98% and 91% of the arrays, respectively. After 9 hours, only about 80% contained KAP1 and HP1 (Fig. 2C). Thus, both were present at both early and late times in DNA repair response.

We next examined the kinetics of KAP1 and H2AX phosphorylation following Sce cleavage of the transgene. Phosphorylated KAP1 and H2AX were enriched on the array (Fig. 2D). In the absence of damage, both pKAP1-s824 and γH2AX were enriched at 28% of the arrays, which defines their background levels in this system. Upon addition of triaminolone acetonide to the Sce-GR-expressing cells, the number of loci enriched for pKAP1-s824 peaked by 1.5 hours at about 82% and remained at about 70% for the rest of the time course. Phosphorylated H2AX levels also rapidly increased at the array after damage and persisted with kinetics similar to pKAP1-s824 (Fig. 2E). Taken together, these data indicate that KAP1 and H2AX are rapidly phosphorylated in response to the induction of Sce-mediated DSBs in heterochromatin. As total KAP1 is enriched at the site prior to damage, this also suggests that it is the heterochromatin-associated population that is phosphorylated in response to damage.

In a previous study, a phosphomimetic mutant of KAP1, S473E, exhibited diminished HP1 association in vitro suggesting that the phosphorylation of KAP1 at Ser 473 may disrupt its interaction with HP1 (25). To determine whether this KAP1 modification plays a role in regulating HP1-KAP1 association in damaged chromatin, we examined Ser 473 phosphorylation in U2OS cells following irradiation or 100 nmol/L NCS (a radio-mimetic) treatment. High levels of pKAP1-s824 were prevalent within the first hour of exposure. In contrast, phosphorylation of KAP1 at Ser 473 peaked after 2 to 3 hours of treatment with either IR or NCS (Fig. 3B and C) and was not ATM or DNA-PK dependent (Supplementary Fig. S2A).

Because pKAP1-s824 accumulates specifically at sites of DNA damage, we next sought to determine whether pKAP1-s473 also colocalized with γH2AX in damage foci. While pKAP1-s473 was undetectable in untreated U2OS cells, it was detected after irradiation, but its pattern was diffuse (Supplementary Figs. S2B and S3D). It was also not detected at the break site in NIH3T3 2/4 cells (Supplementary Fig. S2C). These data suggest that although KAP1-s473 increases in response to DNA damage, only pKAP1-s824 is enriched at damage foci.

Because Ser 824 phosphorylation precedes Ser 473 phosphorylation in the damage response, we next examined whether pKAP1-s473 was dependent on Ser 824 phosphorylation. To address this question, we used a U2OS cell line stably expressing a KAP1 shRNA to deplete endogenous KAP1 protein (Figs. 3E and 4B). These cells were reconstituted with a FLAG-tagged wild-type KAP1 or the KAP1-SS823/824AA mutant using shRNA-resistant cDNAs. Both serines were mutated to alanine in the KAP1-SS823/824AA construct to avoid the possibility that Ser 823 becomes phosphorylated when Ser 824 is mutated (Fig. 3E). As pKAP1-s473 was detected on immunoprecipitates of KAP1-SS823/824AA, this indicates that these events are not interdependent (Fig. 3F).

We directly tested the effects of Ser 473 phosphorylation on HP1 interaction in vitro. First, we generated biotinylated peptides encompassing the HP1-binding domain (HP1BD) of KAP1 with or without a phosphorylated serine at position 473 (Supplementary Fig. S2D). Then, we tested whether the presence of a phospho- serine at 473 affected the ability of glutathione S-transferase (GST)-tagged HP1 isoforms to precipitate HP1BD peptides in vitro. Western blot analysis of the resulting GST-HP1 pull-down assay revealed little difference in the ability of HP1 to associate with HP1BD peptides with or without the phosphorylated Ser 473 residue (Supplementary Fig. S2E).

Next, we asked whether HP1 associates with pKAP1 in vivo. Similar to U2OS cells, HEK293 cells exhibit a temporal difference in Ser 473 and 824 phosphorylation (Fig. 3B and Supplementary Fig. S2F). Following cotransfection and immunoprecipitation after damage, Western blot analyses revealed that HP1α, β, and γ robustly associate with KAP1 phosphorylated at Ser 473 and Ser 824 (Fig. 3F and Supplementary Fig. S2G and S2H). Taken together, these data suggest that the HP1-KAP1 association is not affected by phosphorylation at these sites.

Overwhelming biochemical and immunocytologic data indicate that KAP1 and HP1 exist as a complex, which plays a critical role in organizing higher order heterochromatin architecture and regulating gene silencing (11, 19, 20, 31). Therefore, we asked whether the HP1-KAP1 interaction also regulates Ser 473 and 824 phosphorylation following DNA damage. To do this, we used a KAP1 construct (KAP1-Mut2) with a mutation in the HP1-binding motif (RV487, 488EE) which abolishes the HP1 interaction (Fig. 4A). KAP1-Mut2 does not colocalize with HP1 at pericentric heterochromatin and shows reduced transcriptional repressor activity (18). As expected, KAP1-Mut2 does not coimmunoprecipitate with HP1α.
with HP1α in untreated or irradiated HEK293 cells (Supplementary Fig. S3A).

To determine whether the HP1 interaction regulates KAP1 phosphorylation during damage, we stably depleted KAP1 in U2OS cells using shRNAs and then reconstituted with shRNA-resistant wild-type KAP1 or the KAP1-Mut2 mutant (Fig. 4B). Wild-type KAP1 was efficiently phosphorylated in response to IR. Interestingly, KAP1-Mut2

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**Figure 4.** A, model depicting full-length wild-type and Mut2 mutant KAP1. Western blot analyses of proteins isolated from (B and C) U2OS, U2OS KAP1 knockdown, and reconstituted knockdown cells post 9 Gy IR using pKAP1-s824, pKAP1-s473, KAP1, and actin antibodies. D and E, parental MEFs, KAP1 knockout MEFs, and reconstituted knockout MEFs post-IR using pKAP1-s824, pKAP1-s473, KAP1, and actin antibodies. F, Western blot analyses of proteins isolated from H1299 vector or HP1 knockdown cells using for KAP1, HP1α, HP1β, HP1γ, and actin antibodies. G, Western blot analyses of proteins isolated from H1299 vector or HP1 knockdown cells post 9 Gy IR using antibodies specific for pKAP1-s824, pKAP1-s473, KAP1, and tubulin (G), or pChk2-t68 and actin (H), or γH2AX-s139 and actin (I).
was also rapidly phosphorylated and the levels of Ser 473 and Ser 824 were higher and persisted longer indicating that the interaction with the HP1s regulates it (Fig. 4C).

KAP1 was previously shown to be phosphorylated at serine 473 during the G2–S boundary (25). To determine whether the differences in pKAP1-s473 of KAP1-WT and Mut2 were due to alterations in the cell cycle, the cell-cycle profiles of reconstituted U2OS KAP1 knockout cells were examined using flow cytometry. As differences were not detected before or after irradiation (Supplementary Fig. S3B), this suggests that the increase in KAP1-Mut2 phosphorylation was not due to cell-cycle changes but due to changes in the interaction with HP1.

To further explore this, we used KAP1 knockout MEF cells. The KAP1 knockout MEFs were infected with lentivirus expressing KAP1-WT, KAP1-Mut2, and vector alone resulting in levels of KAP1 similar to endogenous expression (Fig. 4D). Similar to the U2OS cells, KAP1-Mut2 was more efficiently phosphorylated at both Ser 473 and Ser 824 in the knockout MEFs (Fig. 4E). Together, these results suggest that the interaction of HP1 with KAP1 regulates its DNA damage–dependent phosphorylation.

To further evaluate the role of HP1 in KAP1 Ser 473 and 824 phosphorylation, we used H1299 cells in which all 3 HP1 isoforms (α, β, γ) have been stably knocked down (Fig. 4F). In the HP1 knockout H1299 cells, pKAP1-s473 and s824 levels increase more rapidly and persist over the course of the damage response (Fig. 4G). Thus, the data from the 3 separate systems suggest that the HP1–KAP1 association regulates serine 473 and 824 phosphorylation during the DNA damage response.

Like KAP1, H2AX and Chk2 are also phosphorylated by ATM in response to DNA damage (32, 33). Because HP1 affects KAP1 phosphorylation, we examined whether Chk2 and H2AX phosphorylation levels also increase in the absence of HP1. As Chk2 and H2AX phosphorylation were not enhanced in HP1 knockout cells (Fig. 4H and I), this suggests that HP1 does not regulate the phosphorylation of all ATM substrates.

In a previous report as well as in this study, we showed that pKAP1-s824 colocalizes with γH2AX in discreet foci in response to DNA damage (22). Therefore, we next wanted to investigate the role of HP1 in regulating this organizational pattern. To do this, we used the U2OS KAP1 knockout cells (Figs. 3E and 4B and C) generated by stably expressing KAP1 shRNA as well as GFP. GFP-positive KAP1 knockout cells were mixed in a 50:50 ratio with parental U2OS cells, which served as an internal control (Fig. 5A). In parental U2OS cells, pKAP1-s824 and γH2AX completely colocalized 3 hours after IR treatment. After KAP1 knockdown, γH2AX foci still form, but the pKAP1 signal is no longer detected (Fig. 5A, panel 4, and Supplementary Figs. S3C). In cells reconstituted with KAP1-WT, pKAP1-s824 was again seen in foci, which colocalized with γH2AX (Fig. 5A, panel 8). However, in KAP1-Mut2 reconstituted cells, pKAP1-s824 was both diffuse and more intense (Fig. 5A, panel 12). The increased signal intensity is consistent with the higher levels of Ser 824 phosphorylation detected in Mut2-reconstituted U2OS knockout cells (Fig. 4C). The pKAP1-s824 pattern was also consistent with the MEF knockout cells reconstituted with KAP1-Mut2 (Fig. 5B, panel 12). These results indicate that the interaction of KAP1 with HP1 regulates the organization of pKAP1-s824 into discreet foci after DNA damage.

To evaluate whether KAP1-Mut2 was able to accumulate at sites of damage, we knocked KAP1 down in NIH3T3 2/4 cells and reconstituted them with FLAG-tagged KAP1-WT or Mut2 (Supplementary Fig. S3D). The percentage of NIH3T3 2/4 cells with pKAP1-s824 at the transgene was highest at 1.5 hours following Src-cleavage (Fig. 2E). At this time point, KAP1-WT was enriched at the cleaved transgene whereas KAP1-Mut2 was not (Supplementary Fig. S3E). These data suggest that DNA breaks residing in heterochromatin are not high affinity binding sites that enable KAP1 to accumulate in the absence of HP1 association.

To further confirm that KAP1 requires HP1 to form damage foci, we examined pKAP1-s824 foci formation in IR-treated H1299 HP1 knockdown cells (Fig. 5C). In parental cells, pKAP1-s824 and γH2AX colocalize in discrete foci (Fig. 5C, panel 3). In contrast, pKAP1 staining is diffuse in HP1 knockdown cells (Fig. 5C, panel 4). Taken together, these data support the hypothesis that HP1 regulates the nuclear organization of Ser 824 phosphorylation following DNA damage.

We next used cellular fractionation to determine the location of KAP1 before and after DNA damage. Slightly higher levels of KAP1-Mut2 were observed in the chromatin fraction (nuclear pellet, NP) of reconstituted MEF KAP1 knockout and U2OS KAP1 knockdown cells (Fig. 6A and data not shown). In addition, a slightly higher level of KAP1 was found in the chromatin fraction isolated from HP1 knockout cells (Fig. 6B). These data revealed that HP1 is not required for the retention of KAP1 in detergent insoluble chromatin. In irradiated samples, DNA damage did not significantly affect the ability of KAP1 or HP1 to associate with chromatin (Fig. 6A and B). If HP1 association was required for KAP1 to be retained in chromatin following DNA damage, then an increase in soluble (nuclear extract, NE) pKAP1 would be evident. In all 3 cell systems used in this study, equivalent levels of phosphorylated KAP1 were observed in the NP fraction from irradiated cells (Fig. 6A and B and data not shown). These data suggest that HP1 association is not required for KAP1 to be anchored in chromatin in the presence or absence of 9 Gy IR.

We next sought to determine the effect of disrupting the HP1–KAP1 interaction on DNA repair. Formation of γH2AX foci is often used to identify sites of DNA damage (34), and the kinetics of their formation and resolution is a measure of DNA repair efficiency (24, 35). Therefore, we used quantitative image analysis to compare γH2AX staining in cells where the HP1–KAP1 interaction was disrupted by depletion of HP1, depletion of KAP1, or reconstitution with an HP1-binding mutant. At 30 minutes post-IR in H1299
Figure 5. A, detection of pKAP1-s824 and γH2AX-s139 in U2OS cells mixed with reconstituted KAP1 knockdown cells 3 hours post 9 Gy IR. B, detection of pKAP1-s824 and γH2AX-s139 in parental MEFs, KAP1 knockout MEFs, and reconstituted KAP1 KO MEFs 3 hours post 9 Gy IR. C, detection of pKAP1-s824 and γH2AX-s139 in H1299 vectors and HP1 knockdown cells 3 hours post 9 Gy IR. Line scans represent the colocalization of proteins within each image. Scale bars (mmol/L, in white). , denotes the end of the line in the images (odd numbered) and the scanning diagram (x axis).
cells without HP1, the γH2AX signal increased 2-fold over the vector control (P ≥ 0.0001). At 9 hours post-IR, the γH2AX signal intensity in HP1 knockdown cells was 50% less than in the vectors control (P = 0.0079). These data suggest that DSBs are repaired more rapidly when HP1 is not present (Fig. 6C). Similarly, U2OS KAP1 knockdown cells reconstituted with the vector (P = 0.0005) or the KAP1-Mut2 mutant (P = 0.0054) exhibited increased γH2AX staining at 5 minutes post-irradiation compared with the parental cells. These cells also displayed a 30% reduction in γH2AX staining 9 hours after damage (vector, P = 0.0001; KAP1-Mut2, P = 0.0226). Throughout the time course, the γH2AX signal intensity in the KAP1-WT reconstituted cells resembled the parental U2OS cells (Fig. 6D). Taken together, these data suggest that disruption of the HP1–KAP1 interaction promotes the rapid formation and resolution of γH2AX foci in response to DNA damage suggesting that repair is more efficient.

Discussion
The KAP1 corepressor has turned out to be an interesting probe for defining mechanistic interfaces between gene silencing, heterochromatin, and DNA repair. It was previously reported to be phosphorylated at Ser 824 by PI3K...
family kinases, such as ATM, in response to DNA damage suggesting that it is at the interface of these pathways (22, 23). In addition to the Ser 824 site, KAP1 is also phosphorylated at Ser 473 at the G1 to S-phase boundary, which may disrupt its ability to associate with HP1 (25). Here, we report that KAP1 is phosphorylated at Ser 473 in response to DNA damage. While Ser 824 phosphorylation is rapidly induced within the first 30 minutes, phosphorylation of KAP1 at Ser 473 only becomes detectable about 1 hour following damage. In addition, Ser 824 phosphorylation is localized to sites of DSBs, whereas pKAP1-s473 staining is diffuse.

Sustained phosphorylation of pKAP1-s824 at DNA breaks in heterochromatin is maintained by the 53BP1-dependent hyperaccumulation of MRN complex that facilitates the retention of activated ATM (4). In this context, KAP1 phosphorylation serves as a marker for sustained ATM activity at an unrepaired site of damage. Here, we show that disruption of organization of KAP1 in chromatin by diminishing HP1 expression or mutating the HP1BD on KAP1 interferes with the ability of pKAP1-s824 to accumulate at sites of DNA damage. Confinement of this modification to DNA breaks seems to be unique to this site, as the pattern of damage-induced phosphorylation at Ser 473 is diffuse.

Initially, the diffuse staining pattern of pKAP1-s824 in both the KAP1-Mut2-reconstituted cells and the HP1 knockdown cells suggested that KAP1 was not chromatin bound in the absence of the HP1 interaction. Because fixation is required to look at the modification, this could not be tested using live cell techniques. Instead, we used cellular fractionation to evaluate its association with chromatin. Surprisingly, these results showed that even in the absence of HP1, KAP1 is chromatin bound. When the extraction was conducted under high salt conditions (500 mmol/L NaCl), KAP1, but not HP1 or H2AX, became extractable. No difference in its extractability was observed in the presence or absence of HP1 (Supplementary Fig. S4). These data suggest that KAP1 is not an integral chromatin component like H2AX and HP1 but that it is associated with chromatin in an HP1-independent manner. Therefore, HP1 is not the only factor retaining it in chromatin. Consistent with this, KAP1 and pKAP1 can associate with the histone subunits H2B, H3.1, and H2AX in addition to chromatin components like 53BP1 and KRAB-ZFPs, like ZNF317 in vivo (Supplementary Fig. S5A and S5E).

Previous reports suggest that KAP1 is released from chromatin in response to DNA damage, which results in chromatin decondensation and increased repair factors accessibility (23). Here, we show that KAP1 is present at a region of heterochromatin prior to damage and that pKAP1-s824 foci form rapidly at the site upon the induction of DSBs. In addition, our results indicate that KAP1 is not depleted from heterochromatin during the damage response suggesting that the population of phosphorylated KAP1 present at break sites is not recruited but present before damage. This is similar to H2AX, which is also an inherent component of chromatin. Damage-induced phosphorylation of H2AX at Ser 139 by ATM spreads from kilo- to megabases on either side of DNA break sites (36, 37). Our data suggest that KAP1, like H2AX, is not recruited to sites of damage, but that resident proteins near a break are coordinately targeted for phosphorylation.

In this study, we also report a new role for HP1 in regulating the phosphorylation of KAP1 at serine 473 and 824. While neither modification affects the ability of KAP1 to associate with HP1 or the chromatin infrastructure, interaction of KAP1 with HP1 either regulates its initial phosphorylation or its dephosphorylation.

Beyond increased phosphorylation, disruption of HP1-KAP1 association promoted a rapid formation and resolution of γH2AX foci (shown by increased γH2AX staining at early time points post-IR and by decreases in the level of γH2AX staining at the 9-hour time point). The temporal shifts in the level of γH2AX signal intensity occurred when HP1–KAP1 interaction was ablated in 3 different ways: stable knockdown of HP1, stable knockdown of KAP1, and the expression of the HP1-binding mutant of KAP1. The efficiency of γH2AX foci resolution in the absence of the KAP1–HP1 interaction is consistent with work by Goodarzi and colleagues (24). Here, our data indicate that HP1 restricts pKAP1-s824 to damage foci and suggest that higher levels of pKAP1-s824 and pKAP1-s473 as well as the spread of pKAP1-s824 beyond γH2AX foci enhance the efficiency of γH2AX foci resolution in cells lacking the HP1–KAP1 interaction.

Signaling by damage response pathways to KAP1 Ser 824 seems to be one critical step for effective DNA repair in heterochromatin. Currently, KAP1 is thought to promote gene silencing in euchromatin and to maintain chromatin compaction in heterochromatin by condensing nucleosomes. The Ser 824 phosphorylation site is located in the extreme carboxy terminus where KAP1 associates with SETDB1 and Mi2α/C/CHD3. Reconstitution experiments with an S824A mutant support the hypothesis that the decondensation of global chromatin in response to stress may result from Ser 824 phosphorylation which disrupts the ability of KAP1 to associate with this silencing machinery (4, 23, 24). However, the gene silencing activity of KAP1 does not diminish in irradiated cells (Supplementary Fig. S5F). Moreover, pKAP1-Ser 473 and pKAP1-Ser 824 can still associate with SETDB1 in vivo following damage (Supplementary Fig. S5G).

These studies begin to provide a portrait of the differences in the repair of DNA in euchromatin and heterochromatin. The bimodal repair kinetics of DSBs observed in euchromatin and heterochromatin make it likely that the outcome of ATM signaling on the regulation of these factors differs according to the transcriptional permissiveness of the chromatin in which they reside.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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HP1 Modulates KAP1 Phosphorylation in Heterochromatin


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References


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