Published online 15 October 2012 *Nucleic Acids Research, 2012, Vol. 40, No. 22* ***11321–11338***
doi:10.1093/nar/gks947

Suppression and recovery of BRCA1-mediated transcription by HP1γ via modulation of promoter occupancy

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Received November 9, 2011; Revised September 18, 2012; Accepted September 19, 2012

ABSTRACT

Heterochromatin protein 1γ (HP1γ) is a chromatin protein involved in gene silencing. Herein, we show that HP1γ interacts with breast cancer type 1 susceptibility protein (BRCA1) and regulates BRCA1-mediated transcription via modulation of promoter occupancy and histone modification. We used several HP1γ mutants and small interfering RNAs for histone methyltransferases to show that BRCA1–HP1γ interaction, but not methylated histone binding, is important in HP1γ repression of BRCA1-mediated transcription. Time-lapse studies on promoter association and histone methylation after DNA damage revealed that HP1γ accumulates at the promoter before DNA damage, but BRCA1 is recruited at the promoter after the damage while promoter-resident HP1γ is disassembled. Importantly, HP1γ assembly recovers after release from the damage in a BRCA1–HP1γ interaction-dependent manner and targets SUV39H1. HP1γ/SUV39H1 restoration at the promoter results in BRCA1 disassembly and histone methylation, after which transcription repression resumes. We propose that through interaction with BRCA1, HP1γ is guided to the BRCA1 target promoter during recovery and functions in the activation-repression switch and recovery from BRCA1-mediated transcription in response to DNA damage.

INTRODUCTION

In eukaryotic cells, DNA is folded along with histone and non-histone chromosomal proteins to form chromatin. Chromatin dynamics play a critical role in the regulation of transcription, replication, DNA repair and cell cycle progression, and the functional state of chromatin is modulated via epigenetic mechanisms. Covalent modifications of histones and DNA play a role in the regulation of chromatin structure and dynamics. The regulation of the chromatin-modifying factors that generate or remove covalent histone modifications, including acetylation, methylation, phosphorylation, ubiquitination, small ubiquitin-like modifier (SUMO) proteins (SUMOylation) and DNA methylation, are thought to maintain both chromatin integrity and the correct patterns of gene expression (1–5).

One well-characterized histone modification is methylation. The addition and removal of methyl groups has been implicated in both gene activation and repression depending on the site and level of methylation (mono-, di- and trimethylation) (3,6). Specifically, methylation of histone 3 at lysine 9 (H3K9), lysine 20 and lysine 27 has been implicated in transcriptional repression, whereas methylation at lysine 4, lysine 36 and lysine 79 has been implicated in transcriptional activation (7,8). H3K9 exists in mono-, di- and trimethylated forms. The histone methyltransferases (HMTs) G9a and SUV39H1 catalyze the methylation of H3K9, a state that predominates constitutive heterochromatin (9).

In addition to chromatin modification and remodeling, the recruitment of non-histone chromatin proteins is considered important for the regulation of diverse DNA-involved processes, including transcription, replication, DNA repair and chromosome segregation. For example, heterochromatin protein 1 (HP1), a regulatory non-histone protein, is recruited to chromatin through the recognition of methylated H3K9 (H3K9me) (10). HP1, which is named after its association with heterochromatin, functions primarily in the maintenance of structural integrity of chromosomes and the regulation of transcription (11). HP1 genes encode chromosomal proteins that are highly

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conserved from yeast to humans; they interact with chromatin by binding to H3K9me through its conserved N-terminal domain, the chromodomain (11). Yeast has one HP1 (Swi6) (2). Three paralogs, HP1α, HP1β and HP1γ, are found in mammals and flies, and they localize to constitutive or facultative heterochromatin and euchromatin (2). Little is known about the functional differences among these HP1 proteins. All proteins in the HP1 family are adapters that transmit epigenetic status between histones and DNA, resulting in DNA methylation (2). In addition, they have an essential function in the formation of heterochromatin and gene silencing via a mechanistic connection between heterochromatin structure and transcriptional repression (2–5). The loss of HP1γ results in the transcriptional activation of the HIV-1 long terminal repeat (12), and HP1 gene silencing modestly restores tumor necrosis factor-α transcription (13). Although the precise mechanisms through which HP1 regulates gene expression are not fully understood, HP1 seems to link DNA and histones through the recruitment of chromatin modifiers.

BRCA1 (breast cancer type 1 susceptibility protein) is a tumor suppressor involved in maintaining genomic integrity via its diverse functions in DNA damage signaling, DNA repair and transcription. BRCA1 is also implicated in chromatin structural integrity and dynamics via its interaction with and recruitment of chromatin modifiers, which may repair DNA and lead to transcription via regulation of DNA access (14–16). BRCA1 interacts with histone deacetylases (HDACs), histone acetyl transferases, C-terminal-binding protein and its interacting protein, retinoblastoma protein (Rb), RbAp46/48 and the switch/sucrose non-fermentable complex (14). BRCA1 regulates the transcriptional activity of c-myc, JunB, p53, Rb, estrogen receptor, androgen receptor and ZBRK1 (14). Results from several studies have provided evidence of the involvement of BRCA1 in transcription regulation via control of chromatin structure and dynamics in response to DNA damage. Indeed, BRCA1 can activate and suppress the transcription of genes involved in the control of cell cycle progression, apoptosis and proliferation. This evidence supports its role as a transcriptional coregulator that can respond to DNA damage through the formation of transcriptional activator or repressor complexes with chromatin-modifying proteins such as HDACs, p300, SUMO1 and Brahma-related gene 1 subunits (16,17).

In this study, we identified and characterized HP1γ as a novel interacting partner and regulator of BRCA1-mediated transcription in response to DNA damage. We found that HP1γ has a role in the repression of BRCA1-mediated transcription as part of DNA damage recovery via a sequence of recruitment and release events that occurs through HP1γ tethering to BRCA1 at the BRCA1 target promoter. Notably, our data suggest that BRCA1–HP1γ interaction rather than HP1γ binding to H3K9me is important for HP1γ assembly at the BRCA1 target promoter and the resultant transcription repression post-release from DNA damage, because BRCA1 can interact with both wild-type HP1γ and a mutant deficient in H3K9me binding (HP1γV22A), and they can suppress BRCA1-mediated transcription by causing BRCA1 release from the BRCA1 target gene promoter and augmenting histone H3K9 deacetylation and methylation. In contrast, an HP1γ mutant unable to interact with BRCA1 (HP1γW164A) failed to repress BRCA1-mediated transcription. Consistently, HP1γ and HP1γV22A associated with the promoter in HMT-depleted cells that have less H3K9me. Despite the importance of BRCA1–HP1γ interaction for promoter binding and transcriptional repression, the two proteins differ temporally in their assembly/disassembly at the promoter. Using a combination of depletion and overexpression experiments, we linked the repressive function of HP1γ to the modulation of concurrent promoter occupancy and histone modifications. Interestingly, HP1γ recruitment and accumulation of deacetylated and methylated histone at the BRCA1 target promoter were diminished when DNA was damaged. Instead, BRCA1 recruitment and HP1γ release, along with the enhanced transactivation potential of BRCA1, were induced by DNA damage. In contrast, HP1γ assembly was restored, and displacement of BRCA1 began at the promoter during recovery from the BRCA1-mediated transcription after release from DNA damage. Finally, SUV39H1 was targeted to the promoter with elevated histone methylation. Together, our studies provide a chromatin-based connection between promoter occupancy and histone modifications in which HP1γ plays an important role in the regulation of BRCA1-mediated gene expression.

MATERIALS AND METHODS

Plasmid construction

We used yeast 2-hybrid vectors for six overlapping BRCA1 truncated fragments (#1, 1–324; #2, 260–553; #3, 502–802; #4, 758–1064; #5, 1005–1313; #6, 1314–1863) that have been described previously (16). To construct the pLexA- and pB42AD-HP1 family fusion bait and prey vectors, HP1α, HP1β and HP1γ complementary DNAs (cDNAs) were generated via reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA from 293T cells. The recombinant histidine-tagged human HP1γ (His–HP1γ) vector was generated by inserting cDNA containing the entire open reading frame into a pET-28a vector (Millipore, Billerica, MA, USA). The mammalian HP1γ expression vector was generated by inserting the full-length cDNA fragment into pCMV6-SRT (GeneCode, Korea) or pcDNA3-3xFLAG (Invitrogen, Carlsbad, CA, USA). Heterochromatin protein 1γ (HP1γ) mutant derivatives V22A and W164A were generated using a QuikChange Site-directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Double-stranded small interfering RNA (siRNA) for HP1γ, SUV39H1/2 and BRCA1 were generated using a pSUPER vector (18). The siRNA primers were designed to target *HP1γ* (5′-GATCGACGTGTAGTGA ATG-3′), *SUV39H1* (5′-CAGGTGTACAACGTCTTC ATA-3′), *SUV39H2* (5′-AAGCGTTAAGCTGATAAT GTA-3′) and *BRCA1* (16). An *HP1γ* resistant to siRNA knockdown was generated by substituting the CGT

encoding arginine 29 in *HP1γ* to AGA (HP1γR29R) using a QuikChange Site-directed Mutagenesis Kit.

Yeast 2-hybrid assay

Yeast 2-hybrid assays were performed as described previously (16).

Cell culture and transfection

HeLa and 293T cells were grown as described previously (16). Transfections were performed with an Effectene kit (Qiagen, Carlsbad, CA, USA) for expression in mammalian cells and luciferase assays. Hiperfect Transfection Reagent (Qiagen) was used to transfect the siRNAs for *RIZ1*, *SETDB1*, *G9a*, *G9a-like protein-1* (*GLP-1*) and the control (sc-106513, sc-45659, sc-43777, sc-62261 and sc-37007, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Pull-down analysis

His–HP1γ was expressed from pET–HP1γ in *Escherichia coli* BL21 (DE3) cells (Agilent Technologies, Inc.) and purified. The expression and purification of glutathione *S*-transferase (GST) fusion BRCA1 fragment proteins and GST pull-down assays were carried out as described previously (16). His–HP1γ proteins bound to GST–BRCA1 fragments were probed with an anti-HP1γ antibody (05-690, Millipore). In addition, GST pull-down assays were performed using 500 μg nuclear extract from 293T cells to analyse interaction between GST–BRCA1 and endogenous HP1 family proteins. The pull-downed endogenous HP1 proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and binding was analysed via immunoblotting using anti-HP1α, anti-HP1β and anti-HP1γ antibodies (MAB3584, MAB3448 and MAB3450, respectively; Millipore).

Immunoblotting and coimmunoprecipitation

Cell lysates were immunoblotted using anti-BRCA1 (sc-642, Santa Cruz Biotechnology), anti-HP1α, anti-HP1β, anti-HP1γ, anti-SRT (A00725, GeneCode), anti-LexA (06-719, Millipore) and FLAG M2 (F3165, Sigma-Aldrich, Saint Louis, MO, USA) antibodies. For coimmunoprecipitation experiments, cell lysates were subjected to immunoprecipitation, using anti-BRCA1 (sc-6954, Santa Cruz Biotechnology), anti-HP1γ (05-690, Millipore) and anti-FLAG M2 (A2220, Sigma-Aldrich) antibodies. The resulting immunoprecipitates were analysed via immunoblotting with the indicated antibodies.

Transcriptional reporter assay

Reporter assays were carried out as described previously (16). Luciferase activity was standardized against the transfection efficiency for each sample. Each value is the mean ± standard error of the mean for 3–5 experiments ($*P < 0.05$) compared with the reporter alone.

RNA isolation and RT-PCR

Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen), and cDNA was made using M-MLV reverse transcriptase (M1705, Promega, Madison, WI, USA) according to manufacturer instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays using antibodies specific to trimethylated H3K9 (H3K9me3), acetylated H3K9 (H3K9ac), histone deacetylase 1, HP1γ (07-442, 06-942, 05-614 and 05-690, respectively; Millipore), HP1α, HP1β (MAB3584 and MAB3448, respectively; Millipore), SUV39H1 (2991, Cell Signaling Technology, Danvers, MA), BRCA1 (sc-6954, Santa Cruz Biotechnology), SRT (A00725, GeneCode), FLAG M2 (F3165, Sigma-Aldrich) and mouse and rabbit control serum (sc-2025 and sc-2027, respectively; Santa Cruz Biotechnology) were performed according to manufacturer instructions. DNA representing either 0.1% of the total chromatin sample (input) or 5–10% of the immunoprecipitated DNA was amplified.

Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan). The following primers were used: *SUV39H1* (forward 5′-TTCCGCACG GATGATGGG CG-3′, reverse 5′-TCGTAGATCTGGCCCCGCCG-3′), *SUV39H2* (forward 5′-CCGTCAGACCGCGCCAGT TT-3′, reverse 5′-ACACACCAAGCTCCTCGCGC-3′), *G9a* (forward 5′-GGGAGGAGCTAGGGTTTGAC-3′, reverse 5′-TGTGGTCCGTTCTCATGTGT-3′), *SETDB1* (forward 5′-TTGAAGGCAACCTGGGCC GC -3′, reverse 5′-AGTTCTG TCCCAGCCCGGATT C -3′), *RIZ1* (forward 5′-AGAGAGGACGGCAGCGC CAA-3′, reverse 5′-GTGATGTACGGGGCCGCT GG-3′) and *GLP-1* (forward 5′-CCCCGTTGATGGAA GCAGCCG-3′, reverse 5′-GGCAGCCAGGTGCAAAC ACG-3′). The primers used for ChIP-qPCR were 5′-GC TGGGGTCAAATTGCTGG-3′ and 5′-GCTCGCTCGC TCCCCGGAC-3′ for the *growth arrest and DNA damage inducible gene 45α* (*Gadd45α*) promoter (−127 to +87) and 5′- GTCAACGGATTTGGTCTGTATT-3′ and 5′-AGTC TTCTGGGTGGCAGTGAT-3′ for *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. For quantification, all values were normalized to *β-actin* (for qRT-PCR) or input DNA (for ChIP-qPCR) using the ΔΔCt method with data from three to five independent experiments. All qPCR data were analysed using a TP800 (Takara Bio Inc.).

Competitive protein and DNA binding assays

For competitive BRCA1–HP1γ protein binding assays, coimmunoprecipitation was performed with anti-FLAG antibody using 500 μg nuclear extract from cells transiently expressing FLAG–HP1γ in the presence of 2 or 4 μg wild-type or mutant (with mutated OCT-1 and CAAT-binding sites) *Gadd45α* promoter (−107 to −57) DNA (19) as a competitor. After coimmunoprecipitation,

the quantity of BRCA1 bound to HP1γ was measured via immunoblotting with anti-BRCA1 antibody. BRCA1-*Gadd45α*-promoter binding assays were performed as described previously (16), using nuclear extract from cells transiently expressing either wild-type HP1γ or its mutant derivatives HP1γV22A and HP1γW164A. Either biotin-labeled wild type or mutant *Gadd45α* promoter DNA was incubated with nuclear extract at room temperature in binding buffer (16). After incubation, BRCA1 protein bound to biotin-labeled *Gadd45α* promoter DNA was isolated using streptavidin-agarose (S1638; Sigma-Aldrich) and was detected via immunoblotting with anti-BRCA1 antibody.

RESULTS

BRCA1 interacts with HP1γ

We identified HP1γ as a BRCA1-interacting protein in a yeast 2-hybrid assay using an N-terminal BRCA1 fragment from 1 to 1064, which excluded the transactivation domains at the C-terminus, as a bait. Next, we determined which region(s) of BRCA1 interacted with HP1γ using four overlapping BRCA1-truncated fragments, the N-terminal (amino acids 1–324), internal (260–553) and two central fragments (502–802 and 758–1064) as baits (Figure 1A). HP1γ interacted more strongly with the internal fragment than with the central (758–1064) fragment (Figure 1A). In contrast, the N-terminal and one of the central (502–801) BRCA1 fragments did not interact with HP1γ (Figure 1A). We next examined whether HP1γ associated with the BRCA1 transactivation domains (1005–1313 and 1314–1863) using HP1γ as the bait (Figure 1A). The C-terminal transactivation domain (1005–1863) did not interact with HP1γ (Figure 1A). In addition, we examined whether other two paralogs of the HP1 family, HP1α and HP1β, interacted with BRCA1 using the internal BRCA1 fragment as the bait in a yeast 2-hybrid assay (Supplementary Figure S1A). Unlike HP1γ, HP1α and HP1β could not interact with BRCA1 (Supplementary Figure S1A). Next, we determined which domain of HP1γ interacted with BRCA1 using a yeast 2-hybrid assay with the internal BRCA1 fragment (260–553) as the bait and HP1γ chromodomain and chromoshadow domain fragments as the prey (Supplementary Figure S1B). The chromoshadow domain, but not the chromodomain, of HP1 γ associated with the interacting internal BRCA1 fragment.

We then performed pull-down assays using six GST fusion BRCA1 protein fragments (Figure 1B) immobilized on GST–sepharose beads and recombinant His–HP1γ protein to determine whether BRCA1 interacted directly with HP1γ. Immunoblotting recovered His–HP1γ protein bound to GST–BRCA1 beads at varying levels from each GST–BRCA1 fragment fusion protein. His–HP1γ was bound strongly to one GST–BRCA1 protein (260–553) and bound slightly to another (758–1064) but did not bind to the other four GST–BRCA1 fragment proteins (1–324, 502–802, 1005–1313 and 1314–1863; Figure 1B). This result is consistent with those of the yeast 2-hybrid assay (Figure 1A), and indicates that BRCA1 interacts directly with HP1γ *in vitro* via the internal region (260–553).

Next, we examined whether BRCA1 immunoprecipitates interact with HP1γ immunoprecipitates in mammalian cells. Coimmunoprecipitation experiments were performed using 293 T cell lysates. HP1γ coimmunoprecipitated with BRCA1 and *vice versa* (Figure 1C), indicating that endogenous HP1γ interacts with BRCA1 *in vivo*. In contrast, BRCA1 did not coimmunoprecipitate with HP1α or HP1β (Supplementary Figure S1C). These results are consistent with those of the yeast 2-hybrid assay (Supplementary Figure S1A). Moreover, we performed protein-binding assays using mixtures of six GST–BRCA1 recombinant protein fragments (Figure 1B) and nuclear lysates of 293T cells to determine whether any BRCA1 fragments could interact with endogenous HP1α and HP1β. Immunoblotting with anti-HP1α, HP1β or HP1γ antibodies was used to probe HP1 family proteins bound to GST–BRCA1 beads from each GST–BRCA1 fragment fusion protein. HP1γ bound to GST–BRCA1 #2 and #4 proteins was detected (Supplementary Figure S1D) consistent with the results of the yeast 2-hybrid (Figure 1A) and pull-down (Figure 1B) assays. Unlike HP1γ, endogenous HP1α and HP1β barely interacted with the GST–BRCA1 fragments (Supplementary Figure S1D), a result also consistent with the yeast 2-hybrid (Supplementary Figure S1A) and coimmunoprecipitation (Supplementary Figure S1C) results. Taken together, these findings suggest that BRCA1 interacts directly with HP1γ both *in vitro* and *in vivo*.

HP1γ represses BRCA1-mediated transcription

To understand the role of the BRCA1–HP1γ interaction, we investigated the effect of HP1γ on BRCA1-mediated transcription We evaluated the transcriptional activation activity of BRCA1 by measuring *Gadd45α* messenger RNA (mRNA) expression (Figure 2A) and luciferase reporter activity driven by the *Gadd45α* promoter (Figure 2B), which is regulated by BRCA1 (15,16). BRCA1 alone induced transcription of *Gadd45α* approximately 2.2-fold, whereas HP1γ alone reduced *Gadd45α* transcription to approximately 50% of the basal level in 293T cells (Figure 2A). Moreover, HP1γ together with BRCA1 suppressed BRCA1-induced transcriptional activity from the *Gadd45α* promoter to levels similar to that observed in the presence of HP1γ alone (Figure 2A). Consistent with the *Gadd45α* mRNA result, the results of the luciferase reporter assay showed that HP1γ represses BRCA1-mediated transcription (Figure 2B).

To examine further whether transcriptional repression of the *Gadd45α* gene is a *bona fide* activity of HP1γ and not an artifact of ectopic expression, we investigated the effect of HP1γ through its silencing. We first used RT-PCR to evaluate the total levels of *Gadd45α* transcript after silencing HP1γ. As expected, this level increased dramatically in HP1γ-knockdown cells (Figure 2C). In addition, another BRCA1 target gene transcript ($p21^{CIP1/WAF1}$) was induced in these knockdown cells (Supplementary Figure S2), suggesting a general repressive

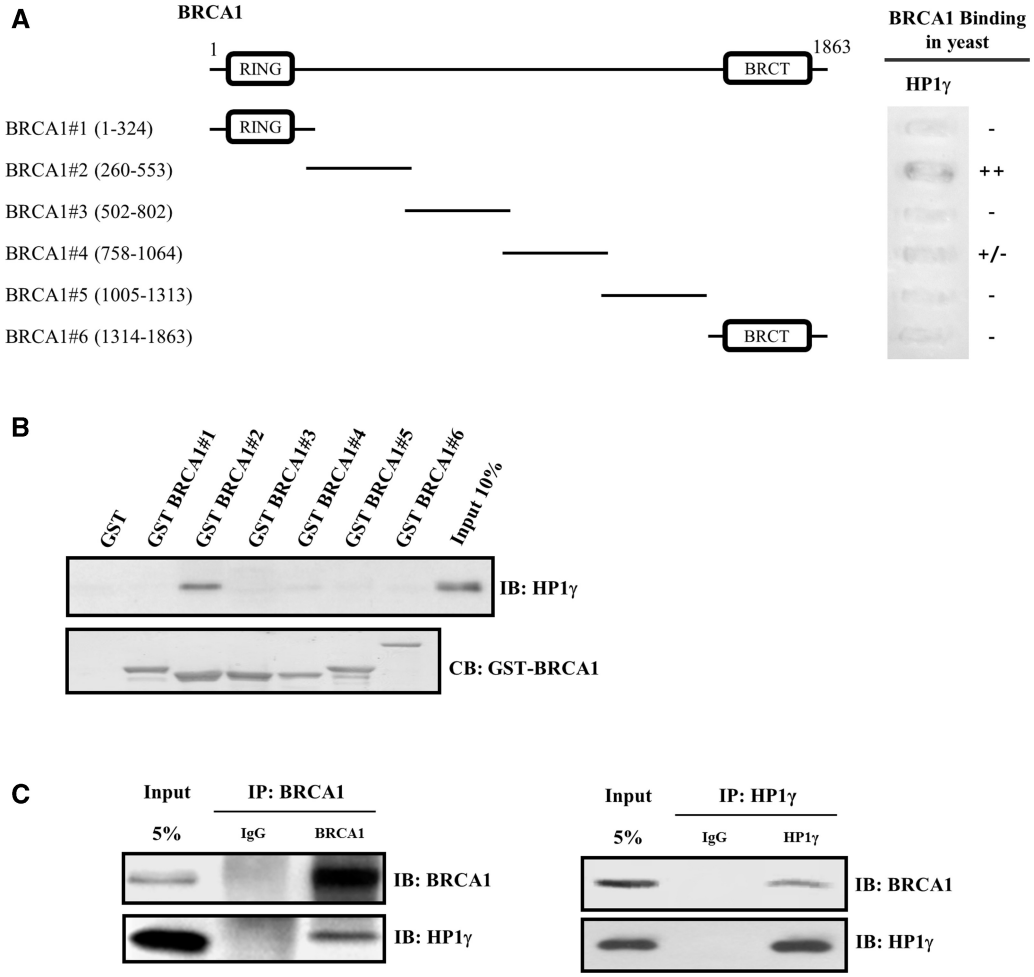

Figure 1. BRCA1 associates with HP1γ *in vitro* and *in vivo*. (**A**) Schematic representation of BRCA1 constructs and the interaction of BRCA1 with HP1γ in a yeast 2-hybrid system. The really interesting new gene domain (RING) and BRCA-1 C-terminal domains (BRCT) are indicated. The numbers indicate the amino acid residues of the respective BRCA1 fragments. Yeast cells transformed with 2-hybrid plasmids were grown under inducing conditions to activate the reporter gene. The streaks represent yeast cells co-transformed with pLexA–BRCA1 (1–324, 260–553, 502–802 and 758–1064) and pB42AD–HP1γ. The capability of the BRCA1 transactivation domains to interact with HP1γ was measured after co-transformation of yeast cells with pB42AD–BRCA1 (1005–1313 and 1314–1864) and pLexA–HP1γ. The BRCA1 internal domain (260–553) interacted with HP1γ. (**B**) *In vitro* interaction of BRCA1 with HP1γ. The six GST–BRCA1 (1–324, 260–553, 502–802, 758–1064, 1005–1313 and 1314–1864, indicated as #1, #2, #3, #4, #5 and #6, respectively) and GST proteins were immobilized on GST-sepharose beads and incubated with the His–HP1γ proteins. His–HP1γ proteins bound to immobilized GST–BRCA1 proteins were analysed via immunoblotting with an anti-HP1γ antibody (upper). Input 10% HP1γ proteins were used. An equivalent amount of GST-BRCA1 protein was used for immobilization (Coomassie blue [CB] staining, lower). (**C**) *In vivo* interaction of BRCA1 with HP1γ. Extracts of 293T cells were immunoprecipitated with anti-BRCA1 (IP: BRCA1) and anti-HP1γ (IP: HP1γ) antibodies. Coimmunoprecipitated HP1γ or BRCA1 protein was immunoblotted with the indicated antibodies. (IgG: immunoglobulin G).

effect of HP1γ on the transcription of BRCA1 target genes. Next, we measured *Gadd45α*-promoter-driven reporter activity after HP1γ silencing (Figure 2D). *Gadd45α* promoter-driven transcription increased approximately 1.7-fold when the level of endogenous HP1γ was reduced (Figure 2D). These luciferase reporter assay results (Figure 2D) are consistent with the RT-PCR results (Figure 2C), suggesting that HP1γ can physiologically regulate BRCA1-mediated *Gadd45α* transcription.

Repression of BRCA1-mediated transcription by HP1γ is dependent on BRCA1–HP1γ interaction

We next examined whether the BRCA1–HP1γ interaction is functionally linked to BRCA1-mediated transcription repression by HP1γ. We used the HP1γ mutant HP1γW164A, which is deficient in binding to the PXVXL motif (HP1 binding motif) (20,21) but competent in binding to H3K9me, and tested whether the HP1γW164A mutant could interact with BRCA1 (Figure 3A and B). We first used a coimmunoprecipitation assay to confirm that HP1γW164 interacts with H3K9me and HP1β but not with the HP1-binding p150 subunit of chromatin assembly factor-1 (CAF-1) (22) and that HP1γV22A interacts with CAF-1 p150 and HP1β but not H3K9me (21). The assay revealed that BRCA1 immunoprecipitated with the wild-type HP1γ but not HP1γW164 (Figure 3B), suggesting that HP1γW164A does not interact with BRCA1. In addition, we examined whether the HP1γV22A mutant, which cannot

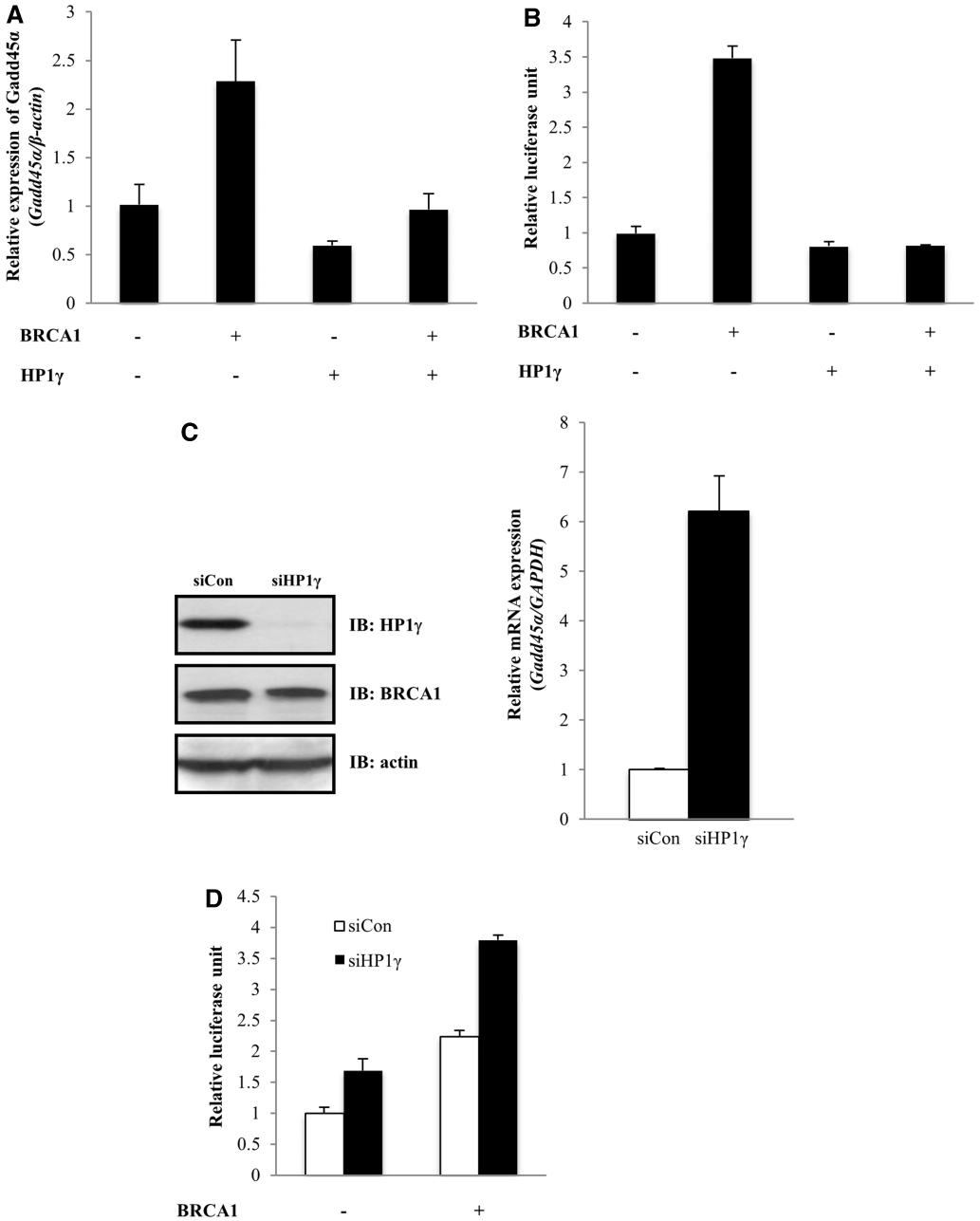

Figure 2. HP1γ represses BRCA1-induced transcriptional activity from the growth arrest and DNA damage inducible gene 45α (*Gadd45α*) promoter. (**A**) The amount of *Gadd45α* mRNA was measured with RT-PCR in the presence of ectopically overexpressed BRCA1 or HP1γ as indicated in 293T cells. Quantitative RT-PCR was performed using specific primers for *Gadd45α* and *β-actin* mRNA. The amount of PCR product generated from *Gadd45α* mRNA in mock cells was arbitrarily set to 1. (**B**) The BRCA1-mediated induction of reporter gene transcription from the *Gadd45α* promoter was monitored in 293T cells in the presence and absence of BRCA1 or HP1γ as indicated. The luciferase activity of the reporter gene alone was arbitrarily set to 1. (**C**) The expression of the *Gadd45α* transcript was determined using quantitative real-time RT-PCR in 293T cells transfected with the indicated plasmids in the absence or presence of siRNA against HP1γ (siRNA control [siCon] and siHP1γ, respectively). Immunoblots showed a reduction in the target protein HP1γ and constant expression of the other proteins (BRCA1 and actin) using indicated antibodies. (**D**) *Gadd45α*-luciferase reporter gene activity was measured in 293T cells transfected with plasmids as indicated in the absence or presence of siRNA against HP1γ in the presence (+) or absence (−) of BRCA1. Induction of the *Gadd45α* promoter by BRCA1 relative to that of the reporter alone was measured with (black) or without (white) HP1γ silencing.

bind to H3K9me (21), can interact with BRCA1. In contrast to HP1γW164A, HP1γV22A interacted with BRCA1 in a manner similar to that with wild-type HP1γ in the coimmunoprecipitation experiment (Figure 3B).

Next, we measured the level of *Gadd45α* mRNA in the presence of wild-type HP1γ or HP1γ mutants (HP1γW164A or HP1γV22A). Although the wild-type HP1γ and the mutants barely affected the level of *Gadd45α* mRNA in the absence of exogenously expressed

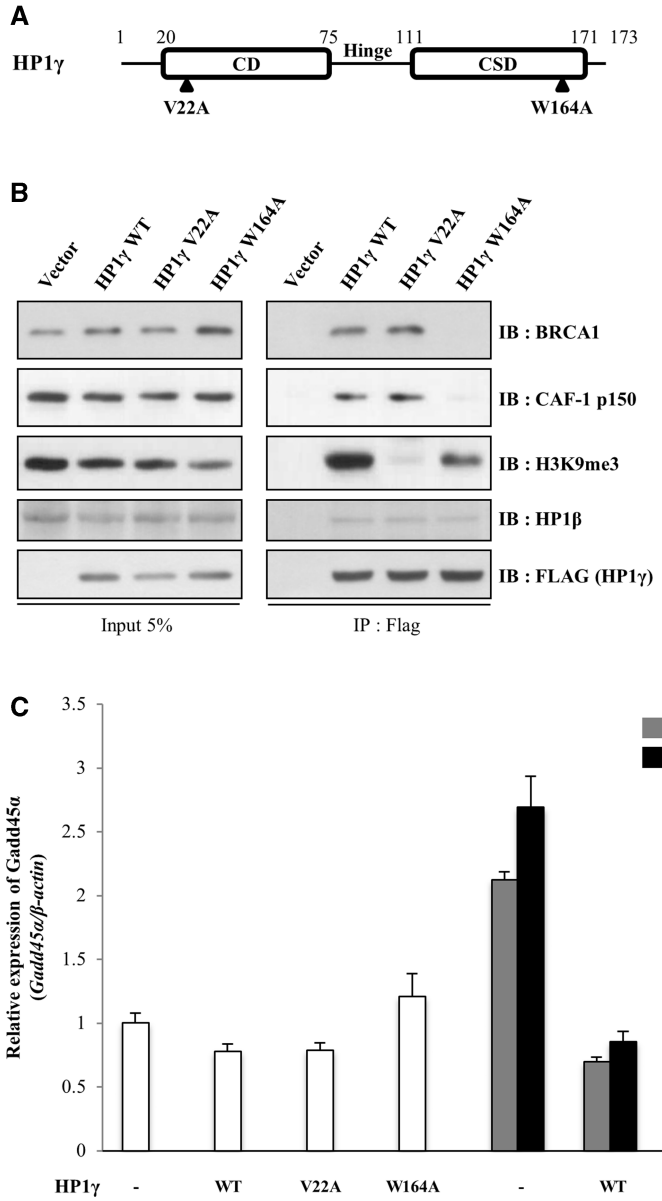

Figure 3. Binding of HP1γ to methylated histone 3 at lysine 9 (H3K9me) is dispensable for BRCA1-mediated transcription repression by HP1γ. (**A**) Schematic diagram of HP1γ wild type and mutant constructs (CD, chromodomain; CSD, chromoshadow domain): wild-type HP1γ (amino acids residues 1–173), V22A (HP1γ with V22A substitution) and W164A (HP1γ with W164A substitution). (**B**) HP1γ and HP1γV22A bind to BRCA1, whereas HP1γW164A cannot. HP1γ and HP1γW164A bind to H3K9me3, whereas HP1γV22A does not. A coimmunoprecipitation assay was performed using cellular extracts from 293T cells ectopically expressing FLAG-tagged HP1γ, HP1γV22A and HP1γW164A. After immunoprecipitation with anti-FLAG antibody, bound endogenous BRCA1, chromatin assembly factor-1 (CAF-1) p150, HP1β and H3K9me3 proteins were detected via immunoblotting using indicated antibodies. (**C**) BRCA1-interacting HP1γ and HP1γV22A can repress BRCA1-induced transcription of *Gadd45α* mRNA via ectopic expression of BRCA1 (grey) or etoposide (black). Total cellular RNA was isolated from 293T cells transiently transfected with expression plasmids as indicated. Expression of *Gadd45α* and *β-actin* transcripts was analysed using RT-PCR. Relative expression of *Gadd45α* was normalized to *β-actin* expression, and expression of a non-transfected mock sample (control) was arbitrarily set to 1.

BRCA1, both HP1γ and HP1γV22A suppressed BRCA1-induced *Gadd45α* transcription through overexpression of BRCA1 or etoposide treatment (Figure 3C). In contrast, the BRCA1 non-interaction mutant HP1γW164A had little effect on BRCA1-induced *Gadd45α* transcription (Figure 3C). Together, these data suggest that BRCA1–HP1γ interaction may be important in the suppression of BRCA1-mediated transcriptional activity by HP1γ and that HP1γ binding to H3K9me is not required for its suppressive effect on BRCA1-induced *Gadd45α* transcription.

HP1γ is targeted to the BRCA1 target promoter in a histone-methylation-independent manner

To understand the molecular mechanism of HP1γ repression of BRCA1-induced transcription, we examined the recruitment of HP1γ at the *Gadd45α* promoter using a ChIP assay. HP1γ was associated with the *Gadd45α* promoter (Figure 4A and Supplementary Figure S3), indicating that it is recruited to the promoter *in vivo*. In contrast, HP1α and HP1β were barely recruited. Together

with the *Gadd45α* promoter transcription and reporter assay results, these ChIP findings suggest that HP1γ can suppress BRCA1-mediated transcriptional activity via association with the *Gadd45α* promoter.

To understand better whether BRCA1–HP1γ interaction plays a role in recruiting HP1γ to the *Gadd45α* promoter, we tested the recruitment of HP1γ mutants (BRCA1-interacting mutant HP1γV22A and non-interacting mutant HP1γW164A) to the promoter. In a manner similar to that observed with the wild-type HP1γ, HP1γV22A assembled at the promoter. By contrast, the association of HP1γW164A with the *Gadd45α* promoter was significantly weaker than that of the wild-type HP1γ or HP1γV22A (Figure 4A).

HP1γV22A, which is BRCA1 interactive but H3K9me-binding deficient, was recruited to the *Gadd45α* promoter (Figure 4A) and repressed BRCA1-activated *Gadd45α* transcription (Figure 3C). Therefore, we wondered whether HP1γ binding to H3K9me was, perhaps, not required for the recruitment of HP1γ to the promoter. To test this hypothesis, we first examined whether the knockdown of HMTs, including SUV39H1/2 (suppressor of variegation 3–9 homology 1/2) (23), G9a (24), SETDB1 (25), RIZ1 (26) and GLP-1 (24), reduced the level of H3K9me at the *Gadd45α* promoter. Although knockdown of SETDB1, RIZ1 and GLP-1 had little effect, knockdown of SUV39H1/2 (Figure 4C) and G9a (Supplementary Figure S4B) reduced H3K9me to approximately 20% and 60%, respectively, the level before knockdown. H3K9me in the concomitant SUV39H1/2 and G9a knockdown cells was reduced to a level similar to that observed in the single SUV39H1/2 knockdown cells (Supplementary Figure S4C). According to these results, we used SUV39H1/2 knockdown (Figure 4B) to deplete H3K9me (Figure 4C) and thereby block the enrichment of H3K9me at the *Gadd45α* promoter. In SUV39H1/2-depleted cells, the recruitment of BRCA1-interacting wild-type HP1γ and HP1γV22A to the *Gadd45α* promoter was unaltered (Figure 4A). These data reveal that HP1γ and HP1γV22A are targeted to the promoter independently of H3K9me.

HP1γ induces disassembly of BRCA1 at the BRCA1 target *Gadd45α* promoter

Although HP1γ physically interacts with BRCA1 (Figure 1), HP1γ and BRCA1 have opposite functions in transcriptional regulation (Figures 2 and 3). Therefore, we examined whether HP1γ regulates the promoter occupancy of BRCA1 by investigating whether HP1γ affects BRCA1 association with the *Gadd45α* promoter using ChIP with an anti-BRCA1 antibody in HP1γ-overexpressing (Figure 5A) and HP1γ-depleted cells (Figure 5B). Although we detected strong HP1γ accumulation at the promoter in HP1γ-overexpressing cells, this ectopic expression knocked BRCA1 off the *Gadd45α* promoter (Figure 5A). In HP1γ-depleted cells, the level of HP1γ at the *Gadd45α* promoter was lower than that before HP1γ depletion, as expected (Figure 5B). In contrast, HP1γ depletion induced recruitment of BRCA1 to the *Gadd45α* promoter. These results, together with HP1γ ectopic expression data, suggest that HP1γ recruitment can occur simultaneously with disassociation of BRCA1 from the *Gadd45α* promoter.

Despite the interaction of BRCA1 and HP1γ (Figure 1), their opposite assembly/disassembly at the promoter may occur because HP1γ-interacting regions of BRCA1 (#2 [260–553] and #4 [758–1064]; Figure 1) overlap in its DNA binding region (452–1079 amino acids) (27). We questioned whether HP1γ competes with BRCA1 target DNA for binding to BRCA1. To answer this question, we used a competitive BRCA1–HP1γ binding assay in the presence of BRCA1 target *Gadd45α* promoter DNA (−107 to −57). BRCA1 bound to the *Gadd45α* promoter containing the two OCT-1 binding sites and CAAT box that are necessary for its binding (19). Using nuclear extracts from cells ectopically expressing FLAG-tagged HP1γ, we performed coimmunoprecipitation with anti-FLAG antibody after adding *Gadd45α* promoter DNA. The level of BRCA1 protein bound to HP1γ reduced gradually as the amount of wild-type *Gadd45α* promoter DNA increased (19). In contrast, the level of BRCA1 protein bound to HP1γ was unaltered in the presence of mutant *Gadd45α* promoter DNA, to which BRCA1 cannot bind (Figure 5C) (19). These competitive coimmunoprecipitation assays revealed that *Gadd45α* promoter DNA competes with HP1γ binding to BRCA1.

We also performed a BRCA1-*Gadd45α* promoter DNA binding assay (Figure 5D), which showed that the level of BRCA1 pulled down with biotin-labeled *Gadd45α* promoter DNA was significantly reduced in the presence of ectopically expressed HP1γ or HP1γV22A. However, the non-interacting HP1 mutant HP1γW164A was unable to reduce the level of BRCA1 bound to *Gadd45α* promoter DNA (Figure 5D and E). Together, the competitive coimmunoprecipitation data and the results of the DNA binding assay indicate that HP1γ inhibits BRCA1 binding to DNA by interacting with BRCA1 (Figure 5D and E), possibly leading to the disassembly of BRCA1 from *Gadd45α* promoter DNA.

In addition to inhibiting the DNA binding of BRCA1, HP1γ promotes the formation of heterochromatin by interacting with histone-modifying enzymes (4–8), and the resulting heterochromatin context can facilitate BRCA1 disassembly. Therefore, we investigated the effect of HP1γ on histone modification at the *Gadd45α* promoter using ChIP with anti-acetyl and anti-methyl H3K9 antibodies. Ectopic expression of HP1γ (∼48%) alone reduced acetylation of H3K9ac at the *Gadd45α*-promoter (Figure 5F). Furthermore, we investigated whether HP1γ could modulate histone methylation status at the *Gadd45α* promoter. HP1γ increased the methylation of H3K9me (∼1.9 fold, Figure 5F). We also investigated the level of acetyl and methyl H3K9 at the promoters of the BRCA1-non-target *erbB2* and *actin* genes in cells expressing HP1γ. HP1γ had little effect on histone modification at these non-target promoters, indicating that the histone modification induced by HP1γ could be promoter specific but not general (Supplementary Figure S5). Taken together with the promoter association results, the ChIP results suggest that HP1γ is recruited to the *Gadd45α* promoter and

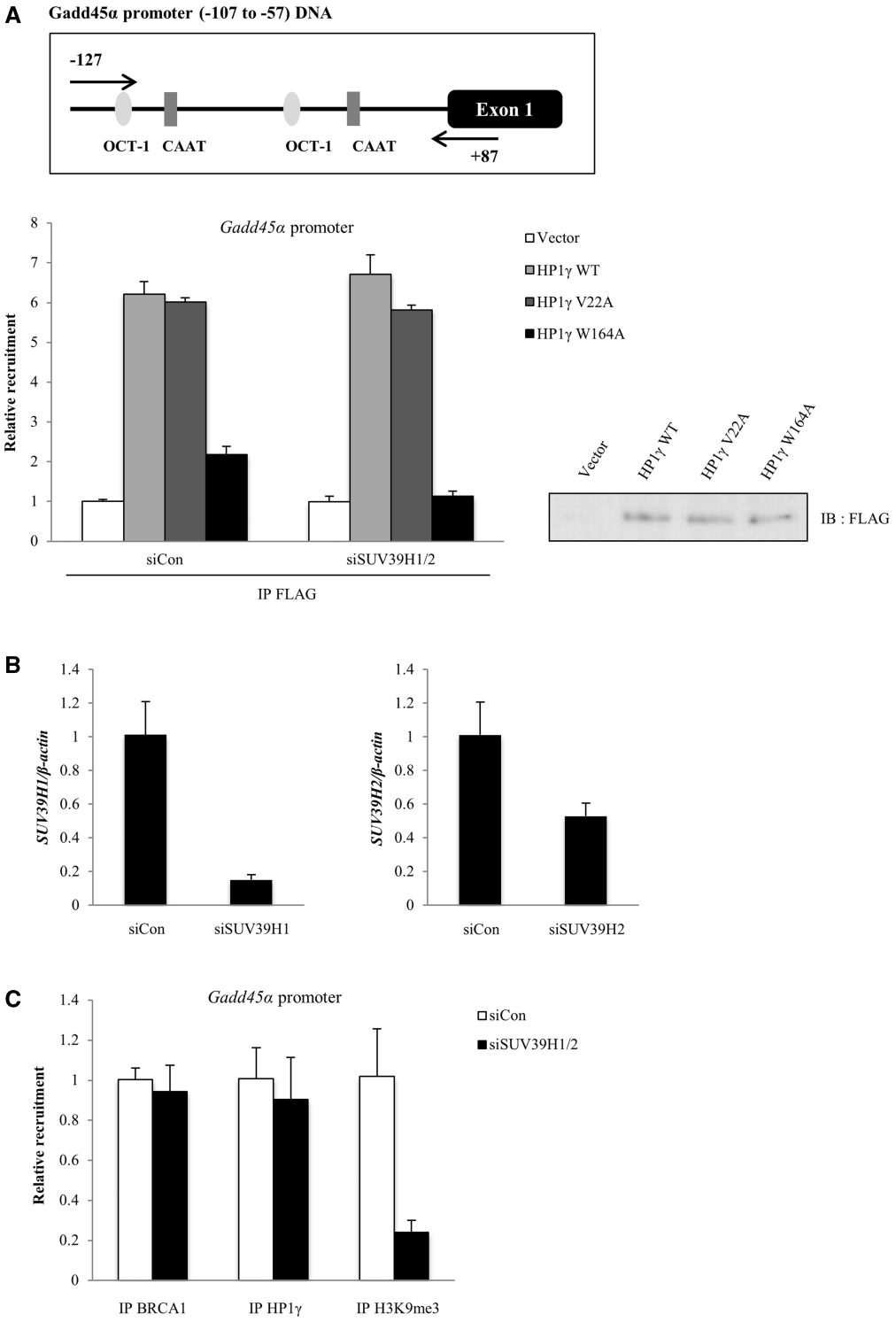

Figure 4. BRCA1–HP1γ interaction is important for targeting HP1γ to the *Gadd45α* promoter. (**A**) BRCA1 interacting HP1γ and HP1γV22A can associate with the *Gadd45α* promoter. ChIP analysis of the *Gadd45α* promoter in 293T cells expressing FLAG-tagged HP1γ, HP1γV22A or HP1γW164A, in the presence (siSUV39H1/2) or absence (siCon) of siRNA specific to SUV39H1 and SUV39H2, was performed using anti-FLAG antibody. The DNA precipitated in the immunocomplexes was amplified by quantitative real-time PCR (qPCR) using primers specific to the *Gadd45α* promoter (−127 to +87) and normalized to input DNA. Immunoblot for ectopically expressed FLAG–HP1γWT, V22A and W164A was performed with anti-FLAG antibody (IB: FLAG) to ensure that all levels are equivalent. (**B**) Depletion of SUV39H1 or SUV39H2 by siRNA specific to SUV39H1 or SUV39H2. To confirm depletion of SUV39H1/2 by specific siRNAs, qPCR was performed. After transfection of 293T cells with pSUPER plasmids generating siRNAs against *SUV39H1* or *SUV39H2*, *SUV39H1* and *SUV39H2* mRNA levels were measured and normalized to *β-actin* mRNA expression. (**C**) Association of BRCA1 and HP1γ with the *Gadd45α* promoter in SUV39H1/2-knockdown cells. Comparable levels of BRCA1 and HP1γ associated with the *Gadd45α* promoter were observed regardless of the depletion of SVU39H1/2. Depletion of SUV39H1/2 reduced the level of H3K9me at the *Gadd45α* promoter. ChIP analysis of the *Gadd45α* promoter in 293T cells transiently transfected with HA-BRCA1 or siSUV39H1/2 was performed using anti-sera specific to the indicated proteins.

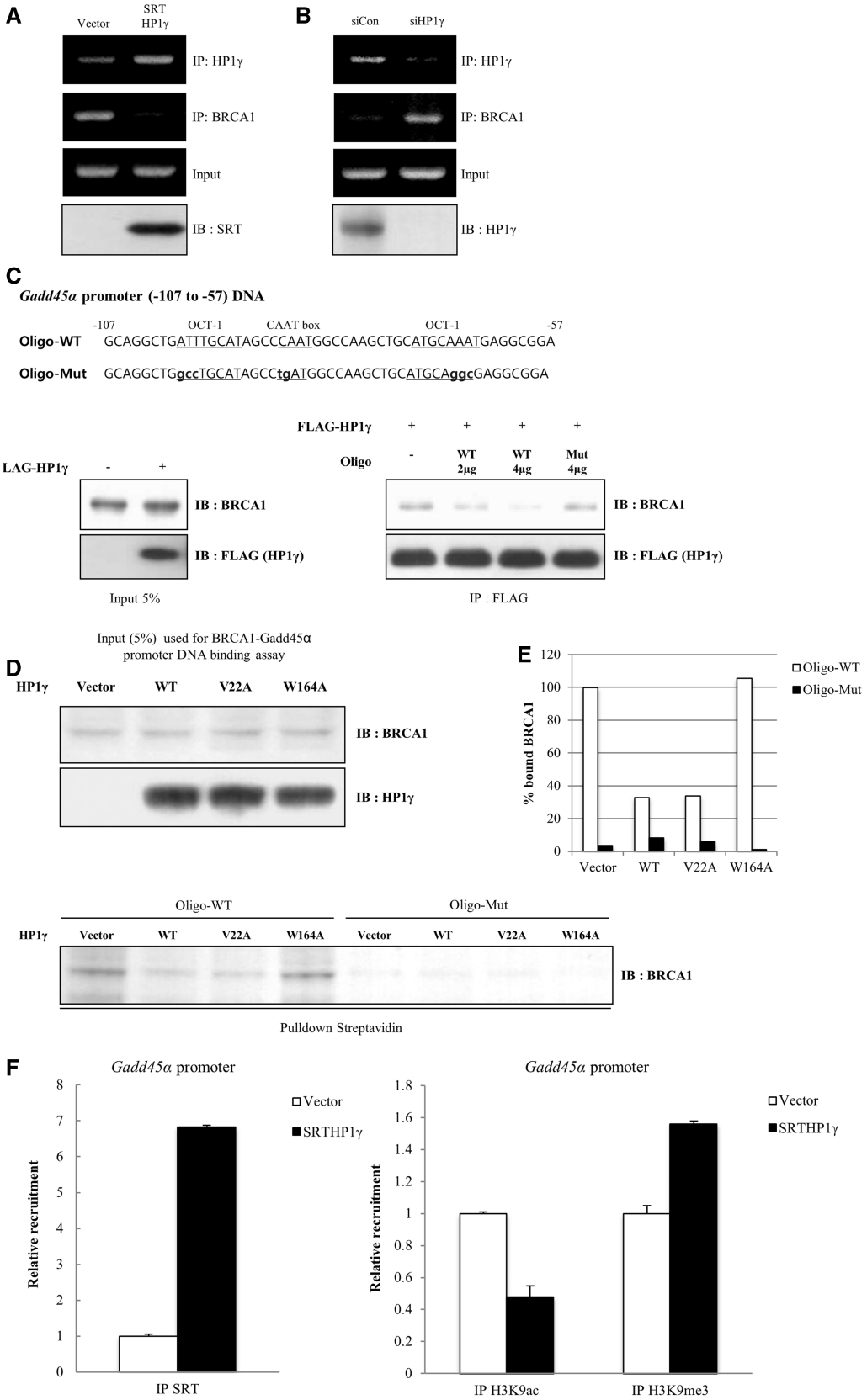

Figure 5. HP1γ regulates the promoter occupancy of BRCA1 at the BRCA1 target *Gadd45α* promoter. (**A**) and (**B**) HP1γ induced disassembly of BRCA1 at the *Gadd45α* promoter. ChIP analyses of the endogenous *Gadd45α* promoter in 293T cells transfected with a plasmid expressing either HP1γ (A) or siRNA against HP1γ (B) were performed with each indicated antibody. The DNA that precipitated in the immunocomplexes was amplified with PCR using primers specific to the *Gadd45α* promoter. (**C**) HP1γ competes with *Gadd45α* DNA for binding to BRCA1. The sequences

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then promotes disassembly of BRCA1 at least partially via inhibition of BRCA1 binding to DNA and increased histone deacetylation and methylation.

Temporally dynamic modulation of HP1γ promoter occupancy is mediated by the BRCA1–HP1γ interaction

Despite the importance of the BRCA1–HP1γ interaction in transcriptional repression and the targeting of HP1γ to the *Gadd45α* promoter, our ChIP data suggested opposite assembly/disassembly of BRCA1 and HP1γ at the promoter. These contradictions led us to speculate about the temporal inconsistency between the association of BRCA1 and HP1γ with the promoter. Therefore, we examined the temporal changes in promoter occupancy under physiological conditions by monitoring the assembly and disassembly of BRCA1 and HP1γ over time (during DNA damage, at release from DNA damage and post-release; Figure 6A). *Gadd45α* transcription is activated by BRCA1 after DNA damage. BRCA1 recruitment to the *Gadd45α* promoter rapidly increased approximately 2.5-fold in etoposide-treated cells compared with recruitment in the non-treated cells (Figure 6A), suggesting that DNA damage induces occupancy of BRCA1 at the promoter. In the presence of etoposide, the level of H3K9ac also increased (Figure 6A), indicating that DNA damage induces histone acetylation at the *Gadd45α* promoter. In contrast, HP1γ at the promoter decreased slightly after DNA damage, and histone methylation at H3K9 declined steadily. These data suggest that DNA damage results in enhanced BRCA1 recruitment, increased H3K9ac levels and reduced HP1γ association and H3K9me at the *Gadd45α* promoter.

After release from etoposide treatment, a temporary transition occurred with a sequence of assembly/disassembly events at the *Gadd45α* promoter. Notably, release from etoposide treatment caused BRCA1 disassembly from the promoter (Figure 6A). In contrast, HP1γ and H3K9me accumulated at the *Gadd45α* promoter after release from DNA damage until 6 h post-release. Six hours after release, the increased levels of HP1γ and H3K9me were consistently maintained. Similarly, SUV39H1 assembly was observed at the promoter after release and accumulated until 12 h post-release (Figure 6B). In HP1γ-depleted cells, SUV39H1 recruitment was absent after release from DNA damage, suggesting that SUV39H1 recruitment is dependent on HP1γ (Figure 6B). Intriguingly, a time course of HP1γ and SUV39H1 recruitment post-release and the dependency of SUV39H1 recruitment on HP1γ provide evidence that SUV39H1 can be recruited after HP1γ assembly at the promoter. From these promoter occupancy data before and after release from DNA damage, we can speculate that a sequence of assembly/disassembly events (simultaneous BRCA1 disassembly and HP1γ assembly and then SUV39H1 assembly) at the transition time point (the switch from transcriptional activation to repression) occurs at this promoter. However, DNA damage and its release had little effect on the *GAPDH* control promoter (Supplementary Figure S6). These data revealed that temporal discrepancy can be observed *in vivo*, and the dramatic transition between recruitment/release of BRCA1 and HP1γ at the *Gadd45α* promoter indeed reflects the switch from transcriptional activation to inactivation.

We next examined whether BRCA1–HP1γ interaction is important for the temporal transition from assembly/disassembly of BRCA1 and concurrent disassembly/assembly of HP1γ after release from DNA damage (Figure 6A). To confirm that the targeting of HP1γ to the promoter occurs independently of H3K9me, we measured HP1γ recruitment at the promoter in SUV39H1/2-depleted cells after release from etoposide treatment. HP1γ recruitment was induced approximately 6 h after release regardless of SUV39H1/2 depletion, suggesting that H3K9me is not required for HP1γ recruitment at the transition (Figure 6C). Therefore, HP1γ recruitment independent of H3K9me together with HP1γ-dependent SUV39H1 recruitment further support the possibility that HP1γ associates with the promoter in the absence of H3K9me3, and SUV39H1 is then recruited at the promoter, resulting in the generation of *de novo* promoter repression through the HP1γ–SUV39H1 interaction.

We also evaluated BRCA1 disassembly post-release from DNA damage after the replenishment of siRNA-resistant wild-type HP1γ or either of the HP1γV22 and HP1γW164A mutants in endogenous HP1γ-depleted cells. The BRCA1-interaction-competent HP1γ proteins (wild type and HP1γV22A) rapidly reduced the level of BRCA1 assembled at the promoter after release from

Figure 5. Continued
of Oligo-WT and Olig-Mut in the human *Gadd45α* promoter (−107 to −57) are shown. BRCA1 binds to the OCT-1 and CAAT box (underlined) in *Gadd45α* promoter DNA. Coimmunoprecipitation was performed with anti-FLAG antibody using nuclear extract from cells transiently expressing FLAG–HP1γ in the presence (wild-type [Oligo-WT] or mutant [Oligo-Mut]) or absence (−) of competitor *Gadd45α* promoter DNA (−107 to −57). Increasing amounts of *Gadd45α* promoter DNA (Oligo-WT or Olig-Mut) were added to the coimmunoprecipitation reaction mixture. Equivalent amounts of FLAG–HP1γ were immunoprecipitated with anti-FLAG antibody (IB: FLAG[HP1γ]). After coimmunoprecipitation (IP: FLAG), the amount of BRCA1 bound to HP1γ was measured via immunoblotting with anti-BRCA1 antibody (IB: BRCA1). (**D**) BRCA1-interacting HP1γ and HP1γV22A interfere with BRCA1-*Gadd45α* promoter binding. The binding of BRCA1 to *Gadd45α* promoter DNA was evaluated with a DNA binding assay using nuclear extracts from cells transiently expressing either wild type (WT) or its mutant derivatives V22A and W164A HP1γ. BRCA1 protein bound to biotin-labeled *Gadd45α* promoter DNA (−107 to −57) was isolated with streptavidin-agarose and was detected via immunoblotting with anti-BRCA1 antibody. (**E**) The relative amount of BRCA1 protein bound to the *Gadd45α* promoter DNA in the immunoblot in (D) was analysed using Image J software. (**F**) HP1γ induces deacetylation and methylation of H3K9 at the *Gadd45α* promoter. Expression of SRT–HP1γ induced recruitment of HP1γ to the *Gadd45α* promoter (left). ChIP analysis of the *Gadd45α* promoter in 293T cells expressing HP1γ was performed using anti-sera specific to the indicated proteins. HP1γ reduced the level of acetylated H3K9 (H3K9ac) and enhanced the level of H3K9me at the *Gadd45α* promoter. ChIP analysis of the *Gadd45α* promoter in 293T cells was performed in the presence (black) or absence (white) of SRT–HP1γ using anti-sera specific to H3K9ac (IP: H3K9ac) or H3K9me3 (IP: H3K9me3).

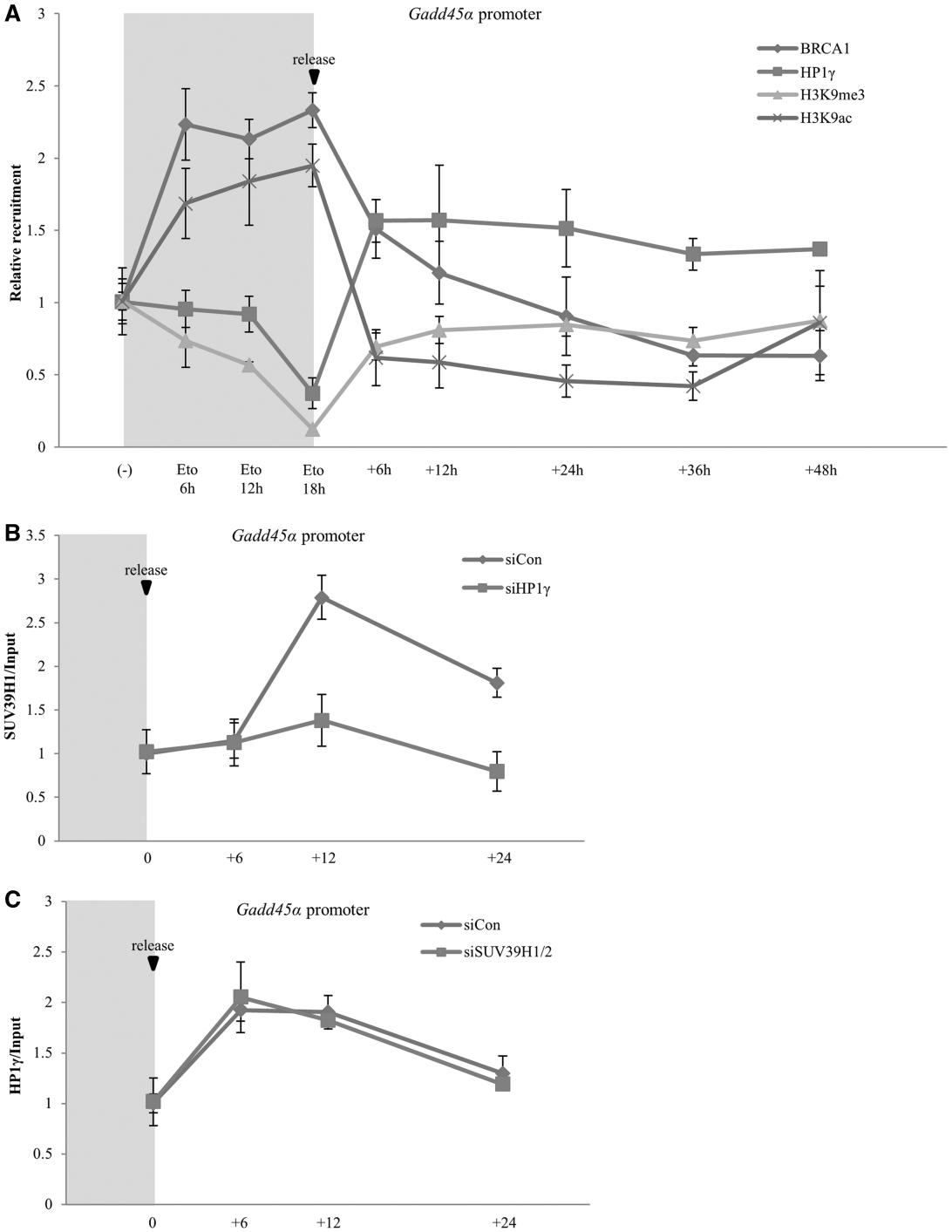

Figure 6. Dynamic promoter occupancy at the *Gadd45α* promoter after DNA damage, release from DNA damage and post-release. (**A**) ChIP analysis of the *Gadd45α* promoter in 293T cells was performed using anti-sera specific to the indicated proteins (anti-BRCA1, anti-HP1γ, anti-H3K9ac and anti-H3K9me) at the indicated time points. Cells were treated with etoposide for 18 h and then released from the treatment. For ChIP analysis, cells were harvested before (−) and after etoposide treatment (Eto 6h, Eto 12h and Eto 18h; indicated by the shadow box). In addition, the cells were harvested after release from etoposide treatment (+6, +12, +24, +36 and +48 h). After the treatment (−), 293T cells were released form etoposide treatment (Eto 18 h, indicated by the arrowhead). The level of each protein associated with the *Gadd45α* promoter was measured with qPCR. The relative amount of immunoprecipitated DNA bound to each protein was normalized to input DNA. (**B**) and (**C**) ChIP analyses of the *Gadd45α* promoter were performed using each indicated antibody (anti-SUV39H1 [B] or anti-HP1γ [C]), as described in (A) after transfection of 293T cells with siRNA against HP1γ (B) or SUV39H1/2 (C). Cells were harvested at the indicated time points after release from etoposide treatment (+6, +12 and +24 h). Etoposide treatment for 18 h is indicated by the shadow box. (**D**) and (**E**) ChIP analysis of the *Gadd45α* promoter was performed using anti-BRCA1 (D) or anti-FLAG (E) antibodies as described in (B) after transfection of 293T cells with siRNA against endogenous HP1γ (siHP1γ) and FLAG-tagged wild-type or mutant HP1γ resistant to siRNA (resistant wild-type HP1γ [Res HP1γWT], resistant HP1γV22A [Res HP1γV22A] and resistant HP1γW164A [Res HP1γW164A]). Immunoblot was performed with anti-HP1γ antibody (IB: HP1γ), to ensure depleting endogenous HP1γ and then replenishing siRNA-resistant wild-type HP1γ or either of the HP1γV22 and HP1γW164A mutants in endogenous HP1γ-depleted cells.

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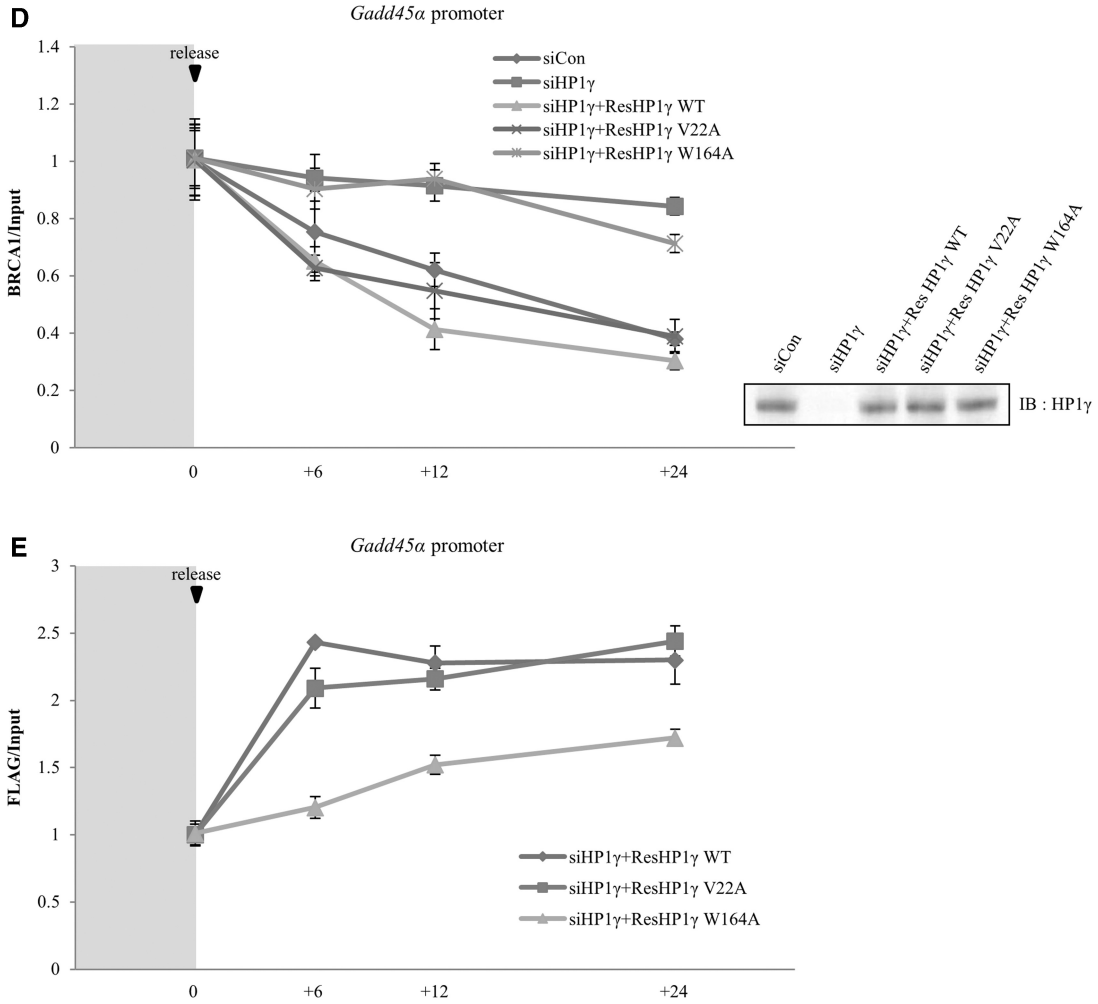

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etoposide treatment. However, BRCA1-interaction-incompetent HP1γW164A caused only moderate BRCA1 disassembly (Figure 6D). These HP1γ silencing and replenishing experiments underscore the notion that prompt BRCA1 disassembly at the transition depends on HP1γ targeting to the promoter mediated by BRCA1–HP1γ interaction.

Finding the stalled BRCA1 disassembly post-release from DNA damage in the presence of BRCA1-interaction-incompetent HP1γW164A prompted us to test whether the BRCA1–HP1γ interaction is important for the restoration of the HP1γ assembly at the promoter during recovery. To do this, we monitored HP1γ assembly post-release from DNA damage after the replenishment of siRNA-resistant wild-type or mutant HP1γ in endogenous HP1γ-depleted cells. The assembly of the BRCA1-interaction-competent wild-type HP1γ and HP1γV22A occurred promptly post-release from etoposide treatment (Figure 6E). However, the assembly of the BRCA1-interaction-defective HP1γW164A at the promoter was delayed and attenuated post-release compared with the assembly of the wild type and HP1γV22A. These results are consistent with the attenuated disassembly of BRCA1 observed with mutant HP1γW164A lacking BRCA1 interaction, further supporting the importance of the BRCA1–HP1γ interaction in targeting HP1γ to the *Gadd45α* promoter for the restoration of HP1γ assembly and repression (switch from activation back to repression) during recovery post-release from DNA damage.

Transcription of the *Gadd45α* gene is usually repressed, and its transcription is activated/derepressed by BRCA1 via recruitment of BRCA1 to the promoter when DNA damage occurs (16,28). To study the role of BRCA1 in the HP1γ disassembly/assembly at the promoter, we monitored the level of HP1γ and H3K9me throughout the repression-activation-recovery cycle (during DNA damage, at release from DNA damage and post-release) in BRCA1-depleted cells (Figure 7). Before DNA damage, the level of HP1γ accumulated at the promoter in the BRCA1-depleted cells was slightly greater than that in the presence of BRCA1 (control siRNA; Figure 7B), suggesting that BRCA1 is not required for the HP1γ assembly at the BRCA1 target promoter in the absence of DNA damage and that HP1γ can be efficiently recruited and accumulated at the promoter in BRCA1-deficient cells.

HP1γ fall-off from the promoter barely occurred in the etoposide-treated BRCA1 knockdown cells (Figure 7B), possibly because available BRCA1 is insufficient for HP1γ fall-off after DNA damage through competitive occupancy of the promoter DNA. The release from etoposide treatment had little effect on HP1γ recruitment in BRCA1-depleted cells, unlike its rapid recruitment in the presence of BRCA1 (Figure 7B). These results suggest that BRCA1 is required for HP1γ to be disassembled and reassembled at the promoter after DNA damage and post-release for recovery, respectively, because HP1γ rarely falls off and thus its restoration at the promoter is unnecessary post-release.

However, we cannot rule out the possibility that the failure of post-release HP1γ recruitment to the promoter in BRCA1-depleted cells may be a result of BRCA1 deficiency (or deficient HP1γ–BRCA1 interaction) or sufficient HP1γ at the *Gadd45α* promoter (grossly similar to the transcriptionally repressive context) for the recovery, such that HP1γ recruitment is unnecessary for restoration. To address reasons for HP1γ unresponsiveness in the BRCA1-depleted cells after DNA damage, we evaluated disassembly/assembly of the siRNA-resistant wild-type HP1γ and HP1γV22 and HP1γW164A mutants in endogenous HP1γ- and BRCA1-depleted cells (in which BRCA1–HP1γ interaction cannot take place) after DNA damage. The disassembly/assembly of all tested HP1γ was unresponsive to DNA damage and post-release, and the levels at the promoter were similar (Figure 7C). These results suggest that BRCA1 is required for HP1γ responsiveness with a functional consequence, and BRCA1–HP1γ interaction is important for the restoration of HP1γ assembly during post-release recovery.

Etoposide treatment barely induced a decline in H3K9me at the promoter in the BRCA1-depleted cells (Figure 7D). After release from etoposide treatment, increased H3K9me was absent in the BRCA1-depleted cells (Figure 7D). Moreover, a roughly similar level of H3K9ac was observed throughout the damage period and post-release in BRCA1-depleted cells (Figure 7E). These results indicate that BRCA1 is important for transitional H3K9 modification in the active-repressive transcriptional context in response to DNA damage and its release. These results, along with the BRCA1/HP1γ silencing results, led us to conclude that BRCA1–HP1γ interaction plays a role in the recovery of BRCA1-mediated transcription after release from DNA damage (i.e. transition from activation to repression) via restoration of HP1γ assembly.

DISCUSSION

In this study, we report a novel function for HP1γ in the negative regulation of BRCA1-mediated transcription through modulation of promoter occupancy. Based on our data, we propose a model that illustrates the mechanism through which HP1γ regulates BRCA1-mediated transcription (Figure 7F). In a quiescent state, HP1γ and histone-modifying enzymes (HDACs and HMTs such as SUV39H and G9a) reside on the *Gadd45α* promoter to repress transcription (Figure 7Fa, repressed state). After DNA damage, BRCA1 binds to the promoter through its regulatory cis elements, with concurrent HP1γ disassembly and activates transcription (Figure 7Fb, derepressed/active state). When transcription is turned off post-release from DNA damage, HP1γ assembly is restored at the BRCA1 target promoter through its interaction with BRCA1 (Figure 7Fc, HP1γ recruitment). Subsequent BRCA1 disassembly and histone-modification steps, such as HDAC- or HMT-dependent H3K9me, follow during recovery to ensure HP1γ settlement and enrichment (Figure 7Fd, HP1γ enrichment).

H3K9me is dispensable for targeting HP1γ to the *Gadd45α* promoter: Importance of the BRCA1–HP1γ interaction

HP1γ protein contains a chromodomain and chromoshadow domain (Figure 3A). HP1γ, via its chromodomain, recognizes and binds to H3K9me, which is a surrogate heterochromatin marker (29). In addition, HP1γ interacts with a variety of chromatin-binding proteins, including CAF-1 and Polycombs, via its chromoshadow domain (29). In our study, the chromoshadow domain of HP1γ interacted with the internal region of the BRCA1 protein (Figure 1A and Supplementary Figure S1). Surprisingly, the effect of HP1γV22A, which cannot bind to H3K9me, was consistent with that of wild-type HP1γ (Figures 3C and D, 4A, 5D and E and 6D). Therefore, both HP1γ and HP1γV22A compromise the promoter occupancy and activation of transcription by BRCA1. In contrast, HP1γW164A, which cannot bind to BRCA1 but binds to H3K9me3, is unable to modulate the promoter occupancy of BRCA1 and repress BRCA1-mediated transcription, reflecting the importance of the BRCA1–HP1γ interaction. Furthermore, like wild-type HP1γ, HP1γV22A interacts with BRCA1 and associates with the *Gadd45α* promoter. These data exclude the possibility that the binding of HP1γ to the *Gadd45α* promoter is mediated by binding to H3K9me via its methyl-histone binding motif. Our findings revealed that HP1γ could be recruited to the *Gadd45α* promoter regardless of histone methylation status at the promoter. Intriguingly, HP1γV22A induced deacetylation and methylation of histones. These observations support the possibility that the induction of histone deacetylation and methylation is accompanied by HP1γ binding to the promoter, and that histone deacetylation and methylation are not prerequisites for the recruitment of HP1γ to the promoter. The observation that HP1γV22A has a transcriptional suppressor function combined with the underlying molecular mechanism dissected in this study provide insight into the possible sequence of events leading to the downregulation of BRCA1-activated transcription by HP1γ. The likely sequence is as follows: (i) HP1γ resides at the BRCA1 target promoters under quiescent conditions; (ii) BRCA1 is recruited to the BRCA1 target promoter and interacts with the promoter after DNA damage; (iii) BRCA1 induces histone acetylation, leading to the activation or

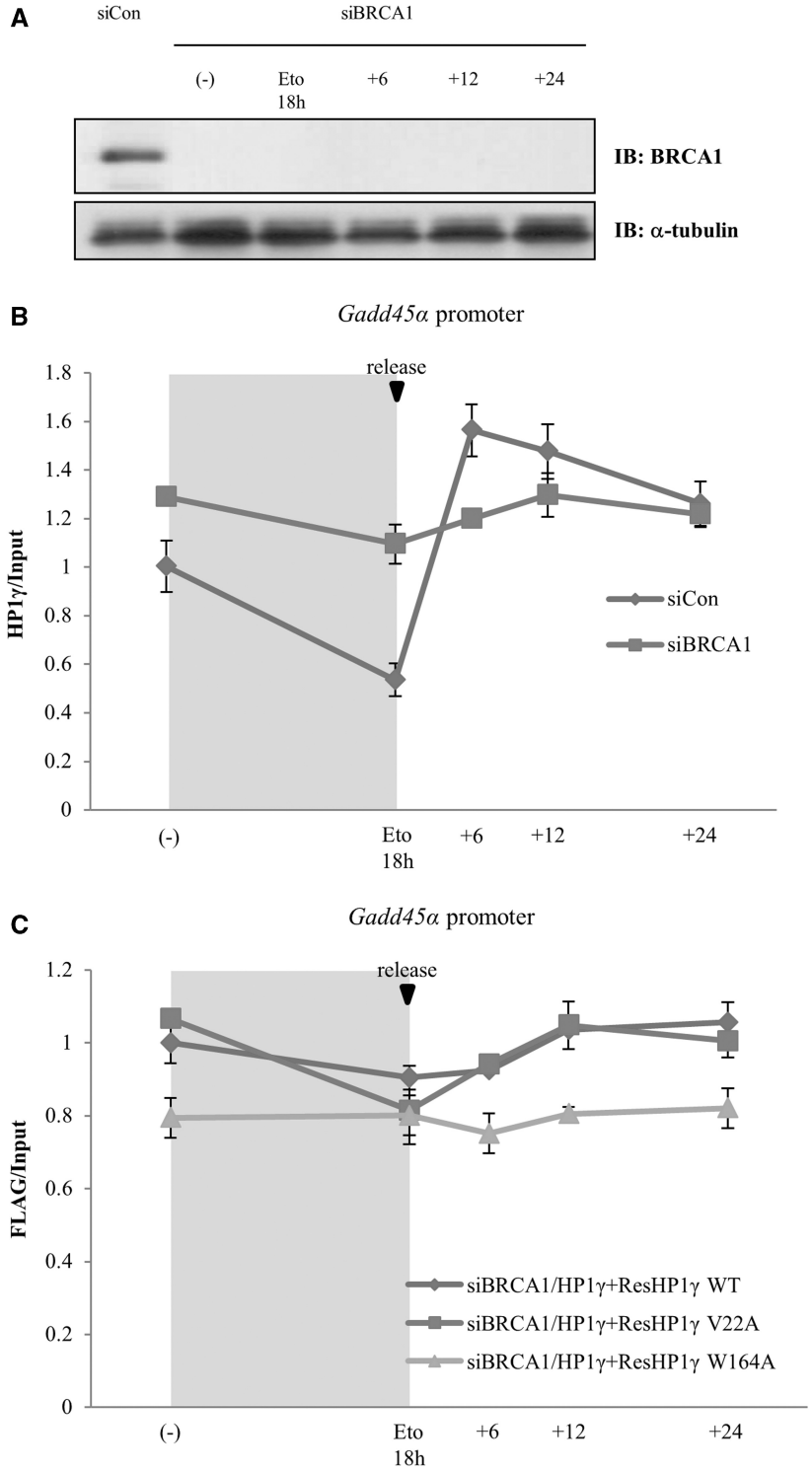

Figure 7. Recruitment of HP1γ at the *Gadd45α* promoter via its interaction with BRCA1, post-release from DNA damage. (**A**) Immunoblots for BRCA1 were performed with anti-BRCA1 antibody using extracts from cells harvested at the indicated time points (before [−] and 18 h after etoposide treatment (Eto 18 h), and post-release from etoposide treatment [+6, +12 and +24 h]) to ensure depletion of endogenous BRCA1 by siBRCA1. The constant amount of α-tubulin protein is shown as a quantitative control for BRCA1 proteins. (**B**) ChIP analyses of the *Gadd45α* promoter were performed using anti-HP1γ antibody after transfection of 293T cells with siRNA against BRCA1 as described in (A). BRCA1-depleted cells were harvested at the indicated time points: before (−), after (Eto 18 h) and at release from etoposide treatment (+6, +12 and +24 h). The recruitment level before etoposide treatment in mock knockdown cells (siCon) was arbitrarily set to 1. Etoposide treatment for 18 h is indicated as the shadow box. (**C**) ChIP analysis of the *Gadd45α* promoter was performed using anti-FLAG antibody throughout the cycle of DNA damage (etoposide treatment for 18 h, release from etoposide [Eto 18 h] and post-release [+6, +12, and +24]) after transfection of 293T cells with siRNAs against BRCA1 (siBRCA1) and endogenous HP1γ (siHP1γ) and FLAG-tagged wild-type or mutant HP1γ resistant to siRNA (Res HP1γWT, Res HP1γV22A, or Res HP1γW164A) as described in Figure 6D.

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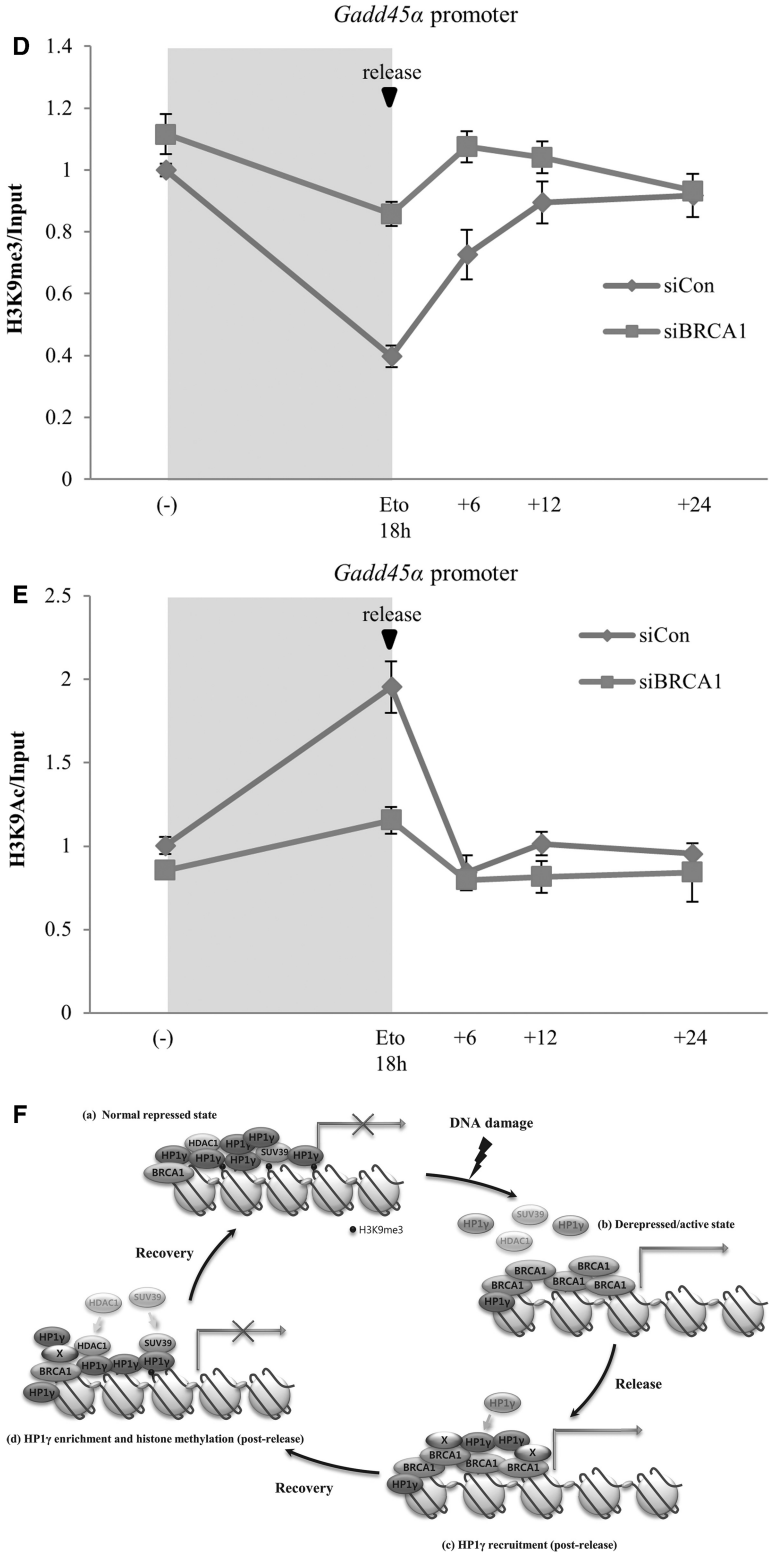

Figure 7. Continued.
(**D**) and (**E**) ChIP analyses of the *Gadd45α* promoter were performed as described in (A) using anti-H3K9me3 (D) and H3K9ac antibodies (E) after transfection of 293T cells with siRNA against BRCA1. The recruitment level before etoposide treatment in mock knockdown cells (siCon) was arbitrarily set to 1. Etoposide treatment for 18 h is indicated as the shadow box. (**F**) Proposed model for HP1γ targeting the *Gadd45α* promoter in an H3K9me-independent but BRCA1 interaction-dependent manner during recovery after release from DNA damage. (**a**) In the quiescent unstressed state, HP1γ and histone-modifying enzymes including histone deacetylases and HMTs are located on the *Gadd45α* promoter to repress transcription. (**b**) When DNA is damaged, BRCA1 is recruited into the *Gadd45α* promoter and activates the transcription. (**c**) and (**d**) After release from DNA damage, HP1γ is recruited at the BRCA1 target promoter via its direct or indirect (such as involving additional protein(s) indicated as X) interaction with BRCA1 accompanied by BRCA1 disassembly and recruitment of histone-modifying enzymes. Finally, the promoter occupancy and chromatin context are restored to quiescence.

repression of BRCA1-mediated transcription; (iv) post-release from DNA damage, HP1γ assembly at the promoter is restored and results in the release of BRCA1 from the promoter and simultaneous recruitment of additional HP1γ during recovery; (v) the level of deacetylated or methylated histones is enhanced via its interactions with HDAC and HMT and (vi) BRCA1-mediated transcription is repressed (Figure 7F). Our results also suggest that BRCA1, a novel HP1γ-interacting partner, mediates HP1γ targeting to chromatin. This hypothesis is supported by experiments showing that the methylated histone-binding motif in HP1γ is not involved in this binding event. However, we could not rule out the possibility that the release of BRCA1 from the promoter and HP1γ restoration during the recovery (Figure 7Fc and d) may depend on an indirect interaction of BRCA1 with HP1γ, because BRCA1 does not contain an obligate HP1 binding motif (PXVXL) and HP1γW164 mutant appears to have reduced promoter occupancy even without DNA damage (Figure 4A and 7C) in comparison with HP1γ or HP1γV22A.

HP1γ can generate a *de novo* repressive promoter without H3K9me: The dual roles of HP1γ as a reader and a regulator for the writer

Methylated histones are mostly present at transcriptionally inactive or repressed regions and target primarily HP1γ proteins to chromatin. However, the view of HP1 acting merely as a passive heterochromatin protein has been challenged by the results in this study—namely that after DNA damage (an event that leads to an active promoter), HP1γ can be targeted to the *Gadd45α* promoter activated by BRCA1 during recovery after DNA damage. Even more striking is the observation that the mutant HP1γV22A induces histone deacetylation and methylation to reach levels similar to that induced by the wild-type HP1γ. Therefore, in addition to its passive maintenance of pre-existing heterochromatin, HP1γ is an active heterochromatin inducer, according to these results. An important factor involved in the targeting of HP1γ to its binding site on chromatin could be its interaction with specific DNA-binding proteins such as transcriptional factors (including activators and repressors) and RNA polymerase II (30) or RNA (31–33). The observations made here indicate that the interaction of HP1γ with BRCA1 can aid in determining the specific targeting and positioning of HP1γ at its cognate binding site, the *Gadd45α* promoter. In addition, the interaction of HP1 with chromatin-associated proteins and chromatin-remodeling proteins (data not shown) can lead to chromatin modification and remodeling after binding. These modification and remodeling events can be bidirectional (i.e. from an active to repressed chromatin structure or the reverse). More important, we found that the presence of HP1γ at the BRCA1-activated promoter led to the repressive chromatin having deacetylated and methylated histones during recovery post-release from DNA damage. This finding, together with the interaction and transcription results, suggests that BRCA1 can target HP1γ to the activated *Gadd45α* promoter.

Next, the associated chromatin-modifying factors, including HDAC, G9a and SUV39H1, which also interact with HP1γ, play a role in chromatin modification via collaborative integration of histone deacetylation and methylation to achieve transcriptional downregulation. Therefore, our results provide a partial explanation for HP1γ targeting to a variety of chromatin structures and its functions in constructing *de novo* heterochromatin from euchromatin or facultative heterochromatin in addition to maintaining previously existing heterochromatin.

In a previous report, we described another BRCA1 binding partner, SUMO1, that affects BRCA1-induced transcription (16). A recent report has suggested that SUMOylation is involved in *de novo* targeting of HP1 before HMTs (33). In the most likely scenario, protein interaction and SUMOylation promote *de novo* targeting of HP1γ to a specific chromatin location, including the *Gadd45α* promoter, and further induce histone modification, eventually switching off BRCA1-activated transcription (from activation to repression).

Dynamics of promoter occupancy for the switch from activation to repression: Relay of BRCA1–HP1γ–SUV39H1–H3K9me3

Based on our data, we are tempted to speculate that the stepwise cycle initiated by BRCA1–HP1γ interaction could be finely adjusted and when promptly tuned, switch the system on and off in response to a variety of environmental and endogenous cues, including DNA damage. We have chased dynamic *Gadd45α* promoter occupancy and histone modifications in combination with knockdown and ectopic expression after release from a DNA damage cue. Given that under these conditions, sequential events occurred collaboratively and concurrently via a multistep mechanism involving BRCA1–HP1γ interaction–dependent targeting, the targeting of HP1γ to the promoter can be considered a recovery step. In this respect, this BRCA1–HP1γ system can be applied to the cellular response to stress and damage under *bona fide* physiological circumstances, perhaps in the formation of promyelocytic leukemia bodies and senescence-associated heterochromatin foci induced by oncogene activation, reactive oxygen species, or ultraviolet radiation. In conclusion, we examined the molecular mechanism underlying BRCA1-mediated transcription regulation by HP1γ and describe a functional interplay between HP1γ and histone modifications whereby HP1γ represses BRCA1-mediated transcription via modulation of promoter occupancy and chromatin context as an epigenetic writer regulator, but not as just a simple reader.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

ACKNOWLEDGEMENTS

The authors thank all members of Lee's laboratory and Paul Dong Rhee for helpful comments.

FUNDING

National R&D Program for Cancer Control, Ministry of Health and Welfare, Republic of Korea [1020210]; National Research Foundation of Korea [2009-0074556, 2010-0018546, 2011-0030830]. Funding for open access charge: National Research Foundation of Korea [2010-0018546].

Conflict of intertest statement. None declared.

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