DNA Double-strand Breaks Lead to Activation of Hypermethylated in Cancer 1 (HIC1) by SUMOylation to Regulate DNA Repair*

Received for publication, September 22, 2012, and in revised form, February 14, 2013. Published, JBC Papers in Press, February 15, 2013, DOI 10.1074/jbc.M112.421610

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Background: The tumor suppressor gene HIC1 (hypermethylated in cancer 1) encodes a transcriptional repressor SUMOylated at Lys-314.

Results: DNA damage favors SUMOylation of HIC1 and its interaction with MTA1 to regulate the DNA repair process.

Conclusion: Our results demonstrate that HIC1 is implicated in the DNA damage response.

Significance: Our work could help explain a mechanism whereby HIC1 loss contributes to tumorigenesis.

HIC1 (hypermethylated in cancer 1) is a tumor suppressor gene frequently epigenetically silenced in human cancers. HIC1 encodes a transcriptional repressor involved in the regulation of growth control and DNA damage response. We previously demonstrated that HIC1 can be either acetylated or SUMOylated on lysine 314. This deacetylation/SUMOylation switch is governed by an unusual complex made up of SIRT1 and HDAC4 which deacetylates and thereby favors SUMOylation of HIC1 by a mechanism not yet fully deciphered. This switch regulates the interaction of HIC1 with MTA1, a component of the NuRD complex and potentiates the repressor activity of HIC1. Here, we show that HIC1 silencing in human fibroblasts impacts the repair of DNA double-strand breaks whereas ectopic expression of wild-type HIC1, but not of nonsumoylatable mutants, leads to a reduced number of γH2AX foci induced by etoposide treatment. In this way, we demonstrate that DNA damage leads to (i) an enhanced HDAC4/Ubc9 interaction, (ii) the activation of SIRT1 by SUMOylation (Lys-734), and (iii) the SUMO-dependent recruitment of HDAC4 by SIRT1 which permits the deacetylation/SUMOylation switch of HIC1. Finally, we show that this increase of HIC1 SUMOylation favors the HIC1/MTA1 interaction, thus demonstrating that HIC1 regulates DNA repair in a SUMO-dependent way. Therefore, epigenetic HIC1 inactivation, which is an early step in tumorigenesis, could contribute to the accumulation of DNA mutations through impaired DNA repair and thus favor tumorigenesis.

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Hypermethylated in Cancer 1 (HIC1)3 is a tumor suppressor gene located at 17p13.3, a chromosomal region frequently hypermethylated or deleted in numerous cancers (1). HIC1 is widely expressed in healthy tissues whereas HIC1 is epigenetically silenced in many human cancers (1, 2). The HIC1 protein is a transcriptional repressor that is composed of three main functional domains: a BTB/POZ protein-protein interaction domain (broad complex, tramtrack and bric à brac/pox viruses and zinc finger) in the N-terminal part of the protein (4), a central region, and a C-terminal domain containing five Krüppel-like C2H2 zinc fingers which allow the specific binding of the protein to HIC1-responsive elements (HiRE, GGCA consensus) (3). The HIC1 central region contains two short phylogenetically conserved motifs: (i) GLDLSKK, allowing the recruitment of the co-repressor CtBP (C-terminal binding protein) (5, 6) and (ii) MK314HEP, whose lysine is competitively acetylated or SUMOylated (7). SUMOylation is a post-translational modification which consists of the covalent attachment of one or several SUMO (small ubiquitin-related modifier) proteins on lysine residues of the target protein. SUMO conjugation is a multistep process (8–10). First, SUMO proteins are synthesized as precursors that must be processed by the SUMO-activating enzyme (E1) to become mature forms. Then, E1 transfers activated SUMO to Ubc9, the SUMO-conjugating enzyme (E2). SUMO is finally transferred from Ubc9 to the substrate with the assistance of one among several SUMO-protein ligases (E3s) that contribute to substrate specificity. Contrary to E1 and E2, which are unique, several E3s have been identified including some members of the PIAS (protein inhibitor of activated STAT) family, the nuclear pore protein RanBP2, the polycomb protein Pc2, and several class II histone deacetylases (HDACs) that enhance SUMOylation independently of their deacetylase activities (8–10). As is the case for

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* This work was supported by the CNRS, the “Association pour la Recherche sur le Cancer” (ARC), and the “Ligue Nationale Contre le Cancer,” Comité du Pas de Calais.
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3 The abbreviations used are: HIC1, hypermethylated in cancer 1; ATM, ataxia telangiectasia mutated; DDR, DNA damage response; DSB, double-strand break; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; PIAS, protein inhibitor of activated STAT; RIPA, radioimmunoprecipitation assay; SUMO, small ubiquitin-related modifier.
MEF2 (myocyte enhancer factor 2) transcription factors (11, 12), we previously showed that the deacetylation/SUMOylation switch of HIC1 is orchestrated by a complex containing two deacetylases belonging to different functional classes: SIRT1 (a class III HDAC) that deacetylates HIC1 and HDAC4 (a class II HDAC) that favors its SUMOylation, probably via an E3 ligase activity (7). We also demonstrated that SUMOylation of HIC1 is essential for its activity because its abolition diminishes its transcriptional repression potential (7) and the recruitment of the NuRD complex on HIC1 target genes (13). Despite its functional importance, the stimuli that induce the SUMOylation of HIC1 are still not known. Here, we hypothesized that it could be induced by DNA damage. Indeed, 11 direct target genes of HIC1 have been described supporting a role for the tumor suppressor in development (14), cell cycle regulation (13), cell migration/invasion (15, 16), and in the DNA damage response (DDR) (17), a process regulated at various levels by SUMOylation (18, 19). The most prominent evidence supporting a role of HIC1 in the DDR came from Chen et al. (20), who demonstrated that Hic1 knock-out murine embryonic fibroblasts (Hic1−/−) were more resistant to etoposide treatment than wild-type mouse embryonic fibroblasts. Conversely, these authors found that infection of MCF7 cells with an adenoviral vector expressing HIC1 brings about apoptosis upon etoposide treatment whereas cells infected with a control vector are resistant to the drug, demonstrating that HIC1 is essential to etoposide-induced apoptosis. Moreover, HIC1 is a direct target gene of p53 (1, 21, 22), and HIC1, in part through the repression of SIRT1 (which deacetylates and inactivates p53), regulates the p53-dependent apoptotic DDR (20). In the present study, we demonstrate that down-regulation of HIC1 by RNAi in human fibroblasts treated with etoposide impacts DNA repair. Conversely, ectopic expression of wild-type HIC1 but not of non-SUMOylatable mutants leads to a reduced number of γH2AX foci supporting a role of HIC1 in the regulation of DNA repair in a SUMO-dependent manner. In accordance with this latter observation, we demonstrate that etoposide, bleomycin, or UV-induced DNA double-strand breaks (DSBs) lead to an increase of HIC1 modification by SUMO2 in an ATM (ataxia telangiectasia) mutated-dependent way. This increase of HIC1 SUMOylation is correlated with an increase in its interaction with MTA1. Enhanced SUMOylation of HIC1 requires the prior activation of SIRT1 by SUMOylation on lysine 734 and the subsequent deacetylation of HIC1. Our results also show that DNA DSBs induce the formation of a SIRT1-SUMO1/HDAC4/Ubc9 complex that spaws the deacetylation/SUMOylation switch of HIC1, thus reinforcing the hypothesis that HDAC4 can play the role of SUMO E3 ligase.

**Experimental Procedures**

**Plasmids and Chemicals**

The full-length FLAG-HIC1 and K314R and E316A point mutants as well as the HA-tagged BTB-CR-G4 and BTB-CR-G4 K314R have been described previously (7). The pTL1-HIC1 expression vector has been described previously (13). The psG5-HDAC4, the His-SUMO1, and His-SUMO2 expression vectors were a kind gift from Jacob S. Seeler (Pasteur Institute, Paris, France). The expression vector for SENP2 was kindly provided by Ron Hay (University of Dundee, Scotland). The plasmid encoding MTA1 was kindly provided by Rakesh Kumar (M. D. Anderson Cancer Center, Houston, TX), Jean-Luc Baert (Institute of Interdisciplinary Research, Villeneuve d’Ascq, France) kindly provided the expression vector for FLAG-tagged Ubc9. The expression vectors encoding SIRT1 wild-type and K734R point mutant were a kind gift of Wenlong Bai (University of South Florida College of medicine, Tampa, FL).

Etoposide and bleomycin were purchased from Sigma-Aldrich. The ATM kinase inhibitor KU-55933 was purchased from Santa Cruz Biotechnology.

**Cell Culture and Transfection**

HEK293T, COS-7, and BI-tert cells were maintained in Dulbecco modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and nonessential amino acids and gentamycin. H1299 cells were grown in RPMI 1640 medium (Invitrogen) supplemented with nonessential amino acids, glutamine, 10% fetal calf serum, and gentamycin. Cells were cultured at 37 °C in water-saturated 5% CO2 atmosphere. Cells were transfected in OptiMEM (Invitrogen) by the PEI (Euromedex) method as described previously (5) in 100-mm dishes with 2.5 μg of DNA. Cells were transfected for 6 h and then incubated in fresh complete medium.

**Co-immunoprecipitation and Immunoprecipitation Assays**

*In Transiently Transfected Cells—* 48 h after transfection, cells were rinsed in cold phosphate-buffered saline (PBS) and lysed in cold IPH buffer (50 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, protease inhibitor mixture (Roche Applied Science)) for co-immunoprecipitation or in RIPA buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) for immunoprecipitation. Cell lysates were cleared by centrifugation (14,000 rpm, 4 °C, 15 min). The supernatants were precleared by incubation with 20 μl of protein A/G-Sepharose beads (Amersham Biosciences) (1 h, 4 °C) and then incubated overnight with 2 μg of antibody. Then, 20 μl of protein A/G beads were added for 30 min. The beads were washed four times with IPH buffer for co-immunoprecipitation. For immunoprecipitation four washes were performed, respectively, with RIPA buffer, RIPA/TNE (v/v) (TNE: 10 mM Tris (pH 7.4), NaCl 150 mM, EDTA 1 mM), TNE/NaCl 0.5%, and TNE alone. Bound proteins were eluted by boiling in Laemmli sample buffer. In the case of immunoprecipitation of SUMOylated HIC1, 10 mM N-ethylmaleimide was added to the lysis and washing buffers to prevent de-sumoylation.

*In Normal BI-Tert Fibroblasts—* To detect the interaction between endogenous HIC1 and MTA1, nuclear proteins enrichment was first performed as described previously (23). Briefly 2 × 105 BI-Tert cells were lysed for 30 min on a rotator at 4 °C in buffer A (50 mM Tris-HCl (pH 7.5), 1 mM DTT (dithiothreitol), 20 mM NaF, 0.5% Triton X-100 supplemented with 1× protease inhibitor mixture (Roche Applied Science)). After centrifugation (1800 × g, 4 °C, 10 min), pellets were washed twice with buffer A and resuspended in IPH buffer. Co-immu-
noprecipitation assays were then performed as described above.

**Western Blotting and Antibodies**

To detect SUMOylated form of proteins, cellular extracts were prepared by directly lysing cells in Laemmli sample buffer as described previously (24). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). After 1 h of blocking in PBS (PBS with 5% defatted powdered milk), the membranes were incubated overnight at 4 °C with specific primary antibodies in PBSTM (PBS with 0.1% Tween) and washed three times with PBS (PBS with 0.1% Nonidet P-40). The membranes were next incubated for 1 h at room temperature with secondary antibodies coupled to peroxidase (Amersham Biosciences) in PBSM, washed three times in PBSN, and revealed by chemiluminescence.

The anti-HIC1 2563 and 2247 antibodies have been described previously (5, 23). Rabbit polyclonal anti SUMO2/3 (Sigma-Aldrich, anti-SUMO), anti-FLAG antibody (M2) was purchased from Sigma-Aldrich, anti-γH2AX from Abcam, and anti-acetylated H3 from Cell Signaling. All the others antibodies used in this study were from Santa Cruz Biotechnology.

**Small Interfering RNA**

BJ-tert fibroblasts were reverse-transfected with RNAiMax according to the manufacturer’s instructions using either a 10 nM concentration of a pool of four small interfering RNAs targeting HIC1 (si HIC1 pool) (H1C1 siGENOME SMART Pool M-006532-01; Dharmacon), four individual HIC1 siRNAs (si HIC1 #1, #3, #4, and #7) (siGENOME set of 4 upgrade, Human HIC1 (3090) MU-006532-01-0010; Dharmacon), or a scrambled control sequence (si Ctrl) as described previously (15). HEK 293T cells were reverse-transfected with interferin according to manufacturer’s instructions using 10 nM small interfering RNA targeting SIRT1 (SIRT1 siGENOME SMART Pool M-003540-01; Dharmacon), or a scrambled control sequence (si Ctrl). 24 h after siRNA transfection, cells were transfected with FLAG-HIC1 and treated with etoposide 24 h later.

**Immunofluorescence Staining**

Cells cultured on coverslips were washed with PBS, fixed for 20 min in cold 3% (v/v) paraformaldehyde, permeabilized in 0.1% Triton X-100 for 5 min, saturated for 30 min in 300 μl of PBS containing 10% (v/v) goat serum, and incubated for 30 min with the anti-HIC1 antibody. The anti-γH2AX was diluted 1:500 in PBS containing 10% (v/v) goat serum and incubated in the dark for 30 min with FITC and Texas Red-labeled secondary antibodies (GE Healthcare) diluted 1:200 in PBS containing 10% (v/v) goat serum. Between each stage, the cells were washed three times for 5 min in PBS. Nuclei were stained with 10 μg/ml Hoechst 33342 for 10 min. The cells were then placed inverted on a drop of Immuno-Fluore Mounting Medium (ICN) on a slide. The slides were stored in the dark at 4 °C and visualized under fluorescence using an Axiosimager Z1 apotome (Zeiss).

**RESULTS**

**HIC1 Regulates the DNA Repair Efficiency and/or Kinetics in a SUMO-dependent Manner—DNA DSBs rapidly induce phosphorylation of the histone H2AX (henceforth called γH2AX) by the ATM kinase at the site of DNA breaks to allow the recruitment of DNA repair proteins (25, 26). To investigate the role of HIC1 in the DDR further, we inactivated HIC1 by siRNA in BJ-tert fibroblasts (15). For that cells were transfected with either a pool of four siRNAs targeting HIC1 (si HIC1 pool) or with two different individual siRNAs (si HIC1 #1 or #3). As assessed by quantitative RT-PCR analyses (data not shown) and by Western blotting, use of the si HIC1 pool or of the si HIC1 #3 resulted in the complete loss of the protein expression whereas the si HIC1 #1 was not efficient (Fig. 1A). To induce DSBs, cells were first exposed to 20 μM etoposide for 1 h. In these experimental conditions no cell death was observed at this stage (data not shown) (20). Then cells were washed and reincubated in fresh medium for 4, 6, 8, or 16 h to monitor the DNA repair process (Fig. 1A). We first examined whether HIC1 knockdown affected the formation of γH2AX foci using immunofluorescent staining with a phospho-H2AX (Ser-139) antibody (Fig. 1A). One hour after etoposide treatment, we found a similar high rate of γH2AX foci in both control (si Ctrl and si HIC1 #1) and in HIC1 knocked-down cells (si HIC1 pool and si HIC1 #3), demonstrating that HIC1 did not prevent the formation of γH2AX foci as previously suggested (20). No differences among the four conditions were seen 4 h after etoposide removal, but salient differences occurred from 6 h. Indeed, approximately 50% of the control cells (si Ctrl) or of the cells transfected with the nonefficient siRNA (si HIC1 #1) still possessed a high rate of γH2AX foci 6 h after etoposide removal, testifying to an efficient repair of the damaged DNA whereas 69% of the cells transfected with the si HIC1 pool and 77% of the cells transfected with the efficient siRNA (si HIC1 #3) showed many DNA breaks. 16 h after etoposide removal only 22% of the control cells still have a high rate of γH2AX foci against approximately 40% for the HIC1 knocked-down cells. These results suggest that knockdown of HIC1 impairs the DNA repair process. To test this hypothesis we next evaluated the DNA repair capacity.
SUMO-HIC1 Controls DNA Repair

**FIGURE 1.** HIC1 takes part in the regulation of DNA repair process in a SUMO-dependent way. A and B, BJ-tert fibroblasts expressing endogenous HIC1 were transfected either with nontarget siRNA control (si Ctrl), either with a pool of four siRNAs targeting HIC1 (si HIC1 pool) or with four individual HIC1 siRNAs called si HIC1 #1, #3, #4, and #17. A, 48 h after transfection BJ-tert cells were incubated with 20 μM etoposide for 1 h, then the drug was removed, and cells were incubated in fresh medium for 4, 6, 8, or 16 h. γH2AX foci were observed using indirect immunofluorescence. The histogram shows the average percentage ± S.D. of cells with a tail moment >2 (*, p < 0.05; **, p < 0.01). RNA interference efficiency was assessed in nontreated control cells (si Ctrl) and the si HIC1 #1 transfected cells. Moreover, 16 h after etoposide removal whereas 100% of the control cells have repaired their DNA, DSB are still present for 5% of the three individual si HIC1 cells. Taken together, these results suggest that HIC1 regulates the efficiency and/or kinetics of DNA repair.

Next, we addressed the importance of HIC1 SUMOylation to this process. To that end, COS-7 cells were transfected with wild-type HIC1 or the K314R mutant (which is neither SUMOylatable nor acetylatable) or the E316A mutant (which remains acetylatable but not SUMOylatable) (7) and incubated with etoposide for 16 h (Fig. 1C). We observed that ectopic expression of wild-type HIC1 leads to a reduced number of γH2AX foci compared with the nontransfected cells. In fact, 66% of NT cells possess >20 γH2AX foci after 16 h in etoposide-containing medium whereas only 23.5% of WT HIC1-expressing cells have a high number of γH2AX foci. However, approximately half of the cells transfected with the K314R or the E316A mutant still have >20 γH2AX foci (52.5% for the K314R-transfected cells and 45.5% for the E316A-transfected cells). Taken together, these results demonstrate that HIC1 takes part in the regulation of the DNA repair process in a SUMO-dependent way. This prompted us to investigate whether SUMOylation of HIC1 is induced by etoposide treatment.

**DNA DSBs Induce an ATM-dependent Increase of HIC1 SUMOylation and Interaction with MTA1**—To test whether DNA damage can induce HIC1 SUMOylation, HEK 293T cells were transfected with the wild-type FLAG-HIC1, the FLAG-HIC1 K314R, or the FLAG-HIC1 E316A point mutant with or without a vector expressing His-tagged SUMO2 (His-SUMO2). 36 h after transfection, cells were treated with 20 μM etoposide for 16 h. Cells were immediately lysed, and cellular extracts were immunoblotted with anti-FLAG, anti-SUMO2/3, or anti-γH2AX antibodies to detect the occurrence of DSBs (Fig. 2A). No differences in unmodified HIC1 expression and in global SUMOylation level were observed after etoposide treatment. However, we could detect an isofrom of higher molecular mass that increases after DNA damage in wild-type HIC1 transfected of BJ-tert fibroblasts expressing or not HIC1 by performing the neutral comet assay which specifically measures DSB at the level of individual cells (Fig. 1B). For that, BJ-tert fibroblasts were transfected either with si Ctrl, si HIC1 #1 and si HIC1 #3 as described above. To definitively prove that the observed effects were not owed to off-targets effects, cells were transfected with two other supplemental individual siRNA targeting HIC1: si HIC1 #4 and si HIC1 #17 that knock-down the expression of the HIC1 protein with the same efficiency that si HIC1 #3 (Fig. 1B, Western blot). Results showed that the same amounts of DNA fragments were generated from the control and the HIC1 knocked-down cells (Fig. 1B, 0h after etoposide removal). However, 6 h after reincubation in etoposide-free medium, we found that cells transfected with the si HIC1 #3, #4 and #17 clearly exhibited a significantly higher level of unrepaired DNA than the control cells (si Ctrl) and the si HIC1 #1 transfected cells. Moreover, 16 h after etoposide removal whereas 100% of the control cells have repaired their DNA, DSB are still present for 5% of the three individual si HIC1 cells. Taken together, these results suggest that HIC1 regulates the efficiency and/or kinetics of DNA repair.

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cells (compare lanes 2 and 3) but that cannot be observed in the K314R and E316A mutants (lanes 4–7), supporting the conclusion that this isoform is a SUMOylated form of HIC1. Accordingly, this isoform is more highly expressed when cells overexpress SUMO2 in the basal condition and is clearly increased upon etoposide exposure (Fig. 2A, compare lanes 2 and 3 with lanes 9 and 10). Moreover, it can no longer be detected when cells are co-transfected with the previously described HIC1 desumoylase SENP2 (7) (Fig. 2B). To prove definitively that this was a SUMOylated isoform of HIC1, extracts of HEK 293T cells were transfected with wild-type or E316A FLAG-HIC1, treated or not with etoposide, and subjected to reciprocal co-immunoprecipitation analyses with antibodies against FLAG and SUMO2/3 (in the presence of N-ethylmaleimide to prevent de-SUMOylation). Anti-SUMO2/3 co-precipitated FLAG-HIC1 in the basal and, in a more significant way, in the etoposide-treated condition (Fig. 2C, upper panel). Conversely, anti-FLAG co-precipitated endogenous SUMO2/3 in WT FLAG-HIC1-transfected cells but not in cells expressing the E316A mutant (Fig. 2C, lower panel). Such an increase in HIC1 SUMOylation was also demonstrated (i) in H1299 and COS-7 cells after etoposide treatment (data not shown), demonstrating that this phenomenon is not cell type-specific and (ii) in HEK 293T upon UV irradiation and bleomycin treatment (which also leads to DSBs) (Fig. 2, D and E), demonstrating that it is not drug-specific. SUMOylation of HIC1 occurs early in the DDR because we were able to detect it just 5 min after etoposide incubation (Fig. 2F).

Shortly after DSBs occur, the ATM kinase pathway is activated. ATM phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair, or apoptosis (27). To test whether SUMOylation of HIC1...
requires the activation of the ATM pathway, FLAG-HIC1-transfected cells were preincubated with a specific inhibitor of ATM (KU-55933) prior to etoposide treatment (Fig. 2G). Effectiveness of the ATM inhibitor was confirmed by the absence of the autophosphorylation of ATM on serine 1831. Whereas the basal SUMOylation of HIC1 remained unchanged, the enhanced HIC1 SUMOylation normally observed after DSBs formation did not occur in the presence of the ATM inhibitor. Taken together, these results demonstrate that DNA double-strand breaks induce activation of the ATM kinase pathway leading to the specific SUMOylation of HIC1 by SUMO2. We demonstrated previously that SUMOylation favors the repressive capacity of HIC1 (7) in part by potentiating the interaction between HIC1 and MTA1 (13). In accordance with these results, co-immunoprecipitation experiments between HIC1 and MTA1 in transfected HEK 293T cells (Fig. 2, H and J) revealed a stronger interaction between the two proteins after treatment with etoposide. Furthermore, a similar result was obtained in BJ-tert fibroblasts which express endogenous HIC1 and MTA1 proteins (Fig. 2I). Taken together, these results demonstrate that in response to DSBs, SUMOylation of HIC1 increases to favor the recruitment of the NuRD complex by HIC1.

**SIRT1 Activation Is Essential for Etoposide-induced HIC1 SUMOylation**—HIC1 can be SUMOylated either by members of the PIAS family like PIAS1 and PIASxα (which act on a non-modified HIC1 K314) or as a result of the sequential action of SIRT1 (which acts on an acetylated HIC1 K314) and HDAC4 (7). To determine which mechanism takes place to induce the SUMOylation of HIC1 upon etoposide treatment, HEK 293T cells were transfected with FLAG-HIC1 and treated with etoposide for 16 h. Then we performed immunoprecipitation of HIC1 and investigated the level of its acetylated form by Western blotting using an anti-acetyllysine antibody (Fig. 3A). Whereas the total level of HIC1 remained unchanged, acetylated HIC1 was reduced in response to DNA damage, thus inversely mirroring the increase in SUMOylation (Fig. 2) and suggesting the intervention of the SIRT1-HDAC4 complex.

In accordance with this result, down-regulation of SIRT1 expression by RNA interference prevented SUMOylation of HIC1 in response to etoposide (Fig. 3B), demonstrating that the increase of HIC1 K314 SUMOylation required prior deacetylation by SIRT1. Next, we performed a co-immunoprecipitation experiment including HIC1 and SIRT1 in the presence or absence of etoposide (Fig. 3C). Surprisingly, we did not observe any difference in interaction between the proteins in the two conditions. Therefore, we hypothesized that HIC1 and SIRT1 interact together in a constitutive manner but that SIRT1 activity is specifically activated after DSBs to deacetylate HIC1. Yang et al. (28) demonstrated that SIRT1 can be SUMOylated on lysine 734 by SUMO1 and that this SUMOylation increases its deacetylase activity. Therefore, we investigated whether SIRT1 experienced increased SUMOylation in response to DNA damage. To this end, wild-type SIRT1 or the non-SUMOylatable K734R mutant was co-transfected with a vector encoding His-tagged SUMO1. Cells were incubated or not with etoposide, and cell extracts were analyzed by Western blotting using an anti-SIRT1 antibody (Fig. 3D). As with HIC1, we found an increase of a higher molecular mass isoform of SIRT1 upon etoposide treatment that is not observed for the K734R mutant and that occurred rapidly (Fig. 3E) in an ATM-dependent fashion (Fig. 3F). We also observed an enhanced SUMOylation of SIRT1 after UV irradiation and bleomycin incubation (Fig. 3, G and H). Taken together, these results demonstrate that, in response to DSBs, activation of the ATM pathway leads to SIRT1 activation by SUMOylation on lysine 734 to promote the deacetylation of HIC1 on lysine 314.

**Activation of the HIC1 Deacetylation/SUMOylation Switch by DNA Damage Depends on the Formation of a SUMO1-SIRT1/HDAC4/Ubc9 Complex**—We previously showed that the HIC1 deacetylation/SUMOylation switch is orchestrated by a complex containing SIRT1 and HDAC4 in which HDAC4 favors the SUMOylation of HIC1 independent of its deacetylase activity, potentially by acting as an E3 SUMO ligase (7). To confirm this latter hypothesis we performed co-immunoprecipitation assays using transfected HIC1 and FLAG-Ubc9 in HEK 293T in the presence or absence of ectopic HDAC4 (Fig. 4A). As shown by immunoblotting with the anti-FLAG antibody, we were able to detect the HIC1/Ubc9 interaction only in the presence of overexpressed HDAC4, demonstrating that HDAC4 indeed plays the role of an E3 ligase; HDAC4 facilitates the association of the E2-conjugating enzyme Ubc9 with its target HIC1. Moreover, and in accordance with the results presented in this study, we demonstrated an enhanced expression of HDAC4 (Fig. 4, B and C) and an increase in its interaction with Ubc9 (Fig. 4B) and SIRT1 (Fig. 4C) upon etoposide treatment. Next, we chose to test the influence of SIRT1-SUMOylation on its interaction with HDAC4 using co-immunoprecipitation experiments. As shown in Fig. 4D, the SIRT1 K734R mutant interacts with HDAC4 to a lesser extent than the wild-type SIRT1, strongly suggesting that the increase of SIRT1 SUMOylation observed after DSBs leads not only to an increase of SIRT1 deacetylase activity (28), but also to an enhancement of the SIRT1/HDAC4 interaction. On the contrary, and in close agreement with the existence of a constitutive interaction between SIRT1 and HIC1 (independent of the activation of SIRT1), we did not observe any difference in interactions between HIC1/SIRT1 wild-type and HIC1/SIRT1 K734R (Fig. 4E). Taken together, our results demonstrate that DSBs favor the formation of a SIRT1-SUMO1/HDAC4/Ubc9 complex that activates the HIC1 deacetylation/SUMOylation switch (Fig. 5).

**DISCUSSION**

Post-translational modification of proteins with SUMO has been shown to have a plethora of effects, but the concept that SUMOylation is mainly associated with transcriptional repression has slowly emerged (29). Indeed, many transcription activators including Elk1 and Sp3 have been shown to be negatively regulated by SUMO (30, 31). Several transcription co-regulators like BRCA1 (breast cancer 1) (32, 33), KAP1 (Krüppel-associated box (KRAB) domain-associated protein 1) (34, 35) and NEMO (NF-κb essential modulator) (24, 36) are also SUMOylated. Interestingly, changes in their SUMOylation states occur after genotoxic stress. Interaction of NEMO with the SUMO E3 ligase PIASy has been shown to increase after DNA damage, and SUMO1 modification of NEMO is required...
for activation of NF-κB in response to etoposide and camptothecin (24, 36). Park et al. (32) demonstrated that SUMO1 represses BRCA1-induced transcription of *Gadd45a*, *p21*, and *p27* genes in response to DNA damage, and co-localization of BRCA1, SUMO1, and several PIAS E3 ligases with H2AX foci has been brought to the fore (33). Finally, SUMOylation of the co-repressor KAP1 is required for its interaction with the methyl histone transferase SETDB1 (SET domain bifurcated 1) and with the NuRD complex (37); doxorubicin was shown to decrease KAP1 SUMOylation via its ATM-mediated phosphorylation on serine 824 (34, 35). In accordance with the role of SUMOylation in transcriptional repression and DNA damage response, we have

**FIGURE 3.** Etoposide induces activation of SIRT1 by SUMOylation leading to subsequent deacetylation of HIC1. A, HEK 293T cells were co-transfected with the pTL1-HIC1 vector and FLAG-tagged Ubc9 in the presence or absence of HDAC4. 48 h after transfection, co-immunoprecipitation (IP) of HIC1 was performed using the anti-HIC1 2563 antibody. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting (*WB*) with the indicated antibodies. B and C, HEK 293T cells were co-transfected with the indicated vectors, treated with etoposide, and cell lysates were subjected to co-immunoprecipitation using the anti-FLAG antibodies. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting with the anti-HDAC4 and the anti-SIRT1 antibodies. D, HEK 293T cells were co-transfected with pTL1-HIC1 and either the wild-type SIRT1 or the K734R SIRT1 point mutant. 48 h after transfection cells were lysed, and extracts were submitted to co-immunoprecipitation with the anti-HIC1 2563 antibody. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting with the anti-HIC1 and the anti-SIRT1 antibodies.

**FIGURE 4.** A SUMO1-SIRT1/HDAC4/Ubc9 complex drives the etoposide-induced HIC1 deacetylation/SUMOylation switch. A, HEK 293T cells were co-transfected with the pTL1-HIC1 vector and FLAG-tagged Ubc9 in the presence or absence of HDAC4. 48 h after transfection, co-immunoprecipitation (IP) of HIC1 was performed using the anti-HIC1 2563 antibody. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting (*WB*) with the indicated antibodies. B and C, HEK 293T cells were co-transfected with the indicated vectors, treated with etoposide, and cell lysates were subjected to co-immunoprecipitation using the anti-FLAG antibodies. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting with the anti-HDAC4 and the anti-SIRT1 antibodies. E, HEK 293T cells were co-transfected with pTL1-HIC1 and either the wild-type SIRT1 or the K734R SIRT1 point mutant. 48 h after transfection cells were lysed, and extracts were subjected to co-immunoprecipitation with the anti-HIC1 2563 antibody. The immunoprecipitates as well as 1.25% of the whole cell lysate (*Input*) were then analyzed by Western blotting with the anti-HIC1 and the anti-SIRT1 antibodies.
SUMO-HIC1 Controls DNA Repair

FIGURE 5. Sketch depicting the main findings of this study. Our work demonstrates that DNA DSB formation induced by etoposide, bleomycin, or UV irradiation leads to the deacetylation of HIC1 followed by an increase in its SUMOylation at lysine 314 in an ATM-dependent manner. This deacetylation/SUMOylation switch is allowed by the activation of SIRT1 by SUMOylation (a) that in turn, on the one hand, deacetylates HIC1 and, on the other hand, recruits HDAC4 and Ubc9 close to HIC1 to allow its subsequent SUMOylation. (b). c, in its SUMOylated form HIC1 recruits more efficiently the NuRD complex via enhanced interaction with MTA1 to repress the transcription of some target genes which remain to be identified to favor the DNA repair process.

demonstrated that the transcriptional repressor HIC1 is activated by SUMOylation after DNA double-strand breakage to regulate the DNA repair process. This enhanced SUMOylation requires its prior deacetylation by SIRT1 which constitutively interacts with HIC1 but which is also activated by SUMOylation following DNA damage (Fig. 5). SUMOylation of SIRT1 at lysine 734 was first demonstrated by Yang et al. (28). In this study the authors demonstrated a decrease in SIRT1 SUMOylation after genotoxic stress induced either by H$_2$O$_2$ or UV radiation. They also observed that H1299 or HCT116 cells that ectopically expressed wild-type SIRT1 were less sensitive to UV-induced apoptosis whereas cells overexpressing the K734R mutant incurred a higher rate of apoptosis than parental cells. These authors therefore proposed that SUMOylation of SIRT1 at lysine 734 could represent a molecular switch that tips the balance from survival to cell death in response to DNA damage. We also observed a decrease of SIRT1 SUMOylation after H$_2$O$_2$ incubation that was correlated with a de-SUMOylation of HIC1 (data not shown). However, in our hands, modification of SIRT1 with SUMO-1 increases after UV irradiation in HEK 293T (Fig. 3G). This discrepancy is probably due to the dose of UV used, the differing time of exposure in the two studies, and differences in the sensitivity of cells toward UV irradiation because in our experimental conditions we did not observe significant cell death. Consistent with these observations, one could hypothesize that SUMOylation of SIRT1 acts as a sensor of DNA damage intensity. In the case of repairable damage SUMOylation of SIRT1 increases to allow the deacetylation/SUMOylation switch of HIC1 and DNA repair. On the other hand, if the damage is too extensive, SENP1 desumoylates and inactivates SIRT1 to ensure the acetylation and activation of the proapoptotic substrates p53 and p73. Moreover, HIC1 can bind to the SIRT1 promoter to repress its transcription (13, 20) whereas SIRT1 (38) and HIC1 (1, 21, 22) are both p53 direct target genes, suggesting the existence of a complex equilibrium among the three proteins during the different steps of the DDR. Interestingly, we found that the increased SUMOylation of HIC1 and SIRT1 observed after etoposide treatment both require an activated ATM kinase pathway. So investigation of the putative phospho-SUMOylation of HIC1 and/or SIRT1 by ATM or by the ATM-activated kinase Chk2 will be helpful to further decipher the molecular mechanisms leading to HIC1 activity upon DSBs. Preliminary results obtained from mass spectrometry analyses identified several HIC1 phosphorylation sites, most notably serine 294, which is included in a conserved Ser/Pro consensus site. However, a nonphosphorylatable mutant of this serine (HIC1 S294A) remains SUMOylatable, and an increase of SUMOylation still occurs after treatment of HIC1 S294A mutant bearing cells with etoposide (data not shown).

HDACs are best known as transcriptional co-repressors through deacetylation of histone tails (39), but increasing evidence suggests that some of them can also act as SUMO E3 ligases. This is the case for HDAC2, which favors SUMOylation of eIF4E (40), HDAC7 which promotes modification of PML (promyelocytic leukemia protein) with SUMO1 (41), and HDAC4 which potentiates the SUMOylation of the androgen receptor (42), of MEF2 (11, 12), and of HIC1 (7). In the present study, we showed that HDAC4 is able to interact with Ubc9 as previously reported by Zhao et al. (11), but our work demonstrated for the first time that this interaction is stimulated by DNA damage. As with HIC1, SUMOylation of MEF2 requires its prior deacetylation by SIRT1, but until now the mechanism by which the two deacetylases acted had not been deciphered. Here, we show that SUMOylation of SIRT1 favors its interaction with HDAC4 and that a SIRT1-SUMO-1/HDAC4/Ubc9 complex is loaded on HIC1 to drive the deacetylation/SUMOylation switch (Fig. 5).

Our previous work has also demonstrated that SUMOylation of HIC1 potentiates its repressor function (7) in part by favoring its interaction with MTA1, one of the components of the nucleosome remodeling and deacetylase (NuRD) complexes (13). In accordance with these results, we observed an increased interaction between HIC1 and MTA1 upon etoposide treatment of transfected cells or of normal human fibroblasts endogenously expressing HIC1 and MTA1 (Fig. 2, F–H). Several studies have highlighted the importance of MTA1 in the regulation of the DNA damage response. More particularly, depletion of

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MTA1 in mouse embryonic fibroblasts (MTA1−/−) (43, 44) or silencing of MTA1 by RNAi in U2OS (45) each renders cells hypersensitive to ionizing radiation, demonstrating that MTA1 interferes with the DNA repair process. Interestingly, the mechanisms by which MTA1 regulates DNA repair have been shown to be both p53-dependent, by regulating p53 stability (therefore independent of its role as a co-repressor) (43, 46) and p53-independent by repressing the transcription of the cdk inhibitor p21WAF1 (44).

To summarize, our results demonstrate that HIC1 regulates the DNA repair process in a SUMO-dependent way. Our results suggest a model whereby HIC1 recruits MTA1 to the promoters of SUMO-sensitive target genes that remain to be identified but whose repression is important for proper DNA repair. Identifying these target genes specifically repressed by HIC1/MTA1 upon etoposide treatment by a ChIP-seq approach will be the next step to a better understanding of the exact contribution of HIC1 to the DNA repair process and how epigenetic inactivation of HIC1 could favor tumorigenesis.

Acknowledgments—We thank Drs. Jean-Luc Baert, Wenlong Bai, Ron Hay, Rakesh Kumar, and Jacob S. Seeler for the expression vectors used in this study, and Drs. Brian Rood and Sebastien Pinte and Prof. Tony Lefebvre for critical reading of the manuscript.

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