The Corepressor Silencing Mediator for Retinoid and Thyroid Hormone Receptor Facilitates Cellular Recovery from DNA Double-Strand Breaks

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Abstract

Cells are frequently challenged by DNA double-strand breaks (DSB) that threaten their normal function and survival. In mammalian cells, the repair of DSBs is predominantly mediated by the DNA-dependent protein kinase (DNA-PK) complex. We unexpectedly found that the corepressor silencing mediator for retinoid and thyroid hormone receptor (SMRT) associates with the DNA-PK repair complex. The SMRT/histone deacetylase 3 complex is required for the transcriptional repressive property of the Ku70 subunit of the repair complex. Moreover, SMRT, but not the related Nuclear Receptor Corepressor, is required for cellular recovery from DNA DSBs induced by ionizing radiation or DNA damage–inducing drugs. Thus, the corepressor SMRT plays a novel and critical role in the cellular response to DSBs. (Cancer Res 2006; 66(18): 9316-22)

Introduction

Maintenance of chromosome integrity is a fundamental requirement in all living organisms. Of the various types of DNA damage that arise within cells, DNA double-strand breaks (DSB) are particularly deleterious. Such breaks can be induced by reactive oxygen species, ionizing radiation (IR), and certain anticancer drugs. If not repaired correctly, DSBs can lead to cell death, chromosome translocations, and cancer (1, 2). Nonhomologous end joining (NHEJ), which refers to rejoining the ends of broken DNA with little or no requirement for sequence homology, is the predominant repair mechanism for DSBs in mammalian cells (3, 4).

Major components of the NHEJ pathway are the DNA-dependent protein kinase (DNA-PK) holoenzyme and ligase IV/XRCC4 complex (2, 5). The DNA-PK holoenzyme is composed of three subunits: a catalytic subunit of DNA-PK (DNA-PKcs) and two DNA-binding proteins Ku70 and Ku80 (6, 7). Ku70 and Ku80 bind with high affinity to DNA termini in a sequence-independent fashion (8). It is proposed that Ku70 and Ku80 are the sensor proteins that recognize and bind to DNA termini at the site of DSBs (9, 10). Binding of Ku to DNA leads to the recruitment of DNA-PKcs to form an active DNA-PK holoenzyme (11). Although extensive genetic and biochemical studies have well established the indispensable role of DNA-PK holoenzyme in NHEJ-mediated DSB repair, exactly how DNA-PKcs or Ku70/Ku80 facilitates the DNA repair process in the context of chromatin remains unclear.

The eukaryotic DNA is wound tightly around an octamer of core histones H2A, H2B, H3, and H4 (12) to form nucleosomes, the primary structural unit of chromatin. DNA repair, like other DNA-templated processes, including gene transcription and DNA replication, takes place in the context of chromatin and thus involves modulation of chromatin structure and accessibility. Reversible acetylation of the core histones has been identified as a major regulator of eukaryotic chromatin structure (13, 14). The acetylation of lysine residues in the histone NH_2-terminal tails by histone acetyltransferases (HAT) results in a less restrictive chromatin structure, whereas histone deacetylases (HDAC) remove the acetyl groups from histones, creating a more compacted chromatin structure (13, 15). The acetylation of histones has been shown to correlate well with transcription: nucleosomal histones are often hyperacetylated in transcriptionally active chromatin yet hypoacetylated in silent chromatin (16, 17). HDAC inhibitors, such as valproic acid and trichostatin A, enhance radiosensitivity of human cells (18, 19), indicating a role of histone deacetylation in the repair of DSBs. However, the mechanism by which histone deacetylation is involved in the DNA repair is not well understood.

Silencing mediator for retinoid and thyroid hormone receptor (SMRT) was initially identified as a nuclear receptor corepressor (20, 21). SMRT and the closely related protein Nuclear Receptor Corepressor (N-CoR) are essential for transcriptional repression by unliganded nuclear receptors, such as thyroid receptor and retinoic acid receptor (20, 22, 23). Both SMRT and N-CoR interact directly with multiple HDACs, including HDAC3, HDAC4, HDAC5, and HDAC7 (24–27) and may associate with HDAC1 and HDAC2 via the Sin3 protein under certain conditions (28, 29). In particular, HDAC3 is found in a tight complex with SMRT and N-CoR and its enzymatic activity completely depends on association with the deacetylase activation domain of SMRT or N-CoR (24, 27, 30).

The recruitment of HDACs enables SMRT and N-CoR to modulate the chromatin structure, thus silencing transcription. Although SMRT is most prominently linked with transcriptional repression by nuclear receptors, there is evidence that SMRT plays a role in transcriptional repression by many other transcription factors, such as the leukemogenic fusion protein PLZF (31–34), Notch-binding protein CBF-1 (35), and homeodomain proteins, including Rpx2, Pit-1, and Pbx (36, 37). Here, we show that the SMRT corepressor interacts with the Ku70 subunit of the DNA-PK complex. SMRT, along with HDAC3, is crucial for the inherent repression function of Ku70. Reduction of SMRT, but not N-CoR, in human cells renders cells markedly hypersensitive to IR and DSB-inducing drugs. Thus, the SMRT corepressor plays a novel and unique role in the regulation of DSB repair.

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Materials and Methods

Plasmids. The Flag-SMRT 305 to 669, Flag-SMRT 305 to 559, or Flag-SMRT 506 to 669 constructs have been described previously (38). Full-length human Ku70 and Ku80 were gifts from Dr. Westley Reeves (University of North Carolina, Chapel Hill, NC; ref. 39). Gal4-Ku70 and Gal4-Ku80 constructs were produced by PCR amplification of the full-length Ku70 or Ku80 DNA sequences followed by insertion into pCMX-Gal4-DNA-binding domain (DBD). Gal4-Ku70 plasmids containing Gal4-DBD fusions to Ku70 amino acids 1 to 251, 252 to 406, 407 to 558, 559 to 609, 407 to 505, and 506 to 558 were produced by PCR amplification of the corresponding DNA sequences followed by insertion into pCMX-Gal4-DBD. Control, SMRT, HDAC3, and N-CoR small interfering RNA (siRNA) constructs were described previously (23).

***In vitro* interaction assays.** Baculovirus expression and purification of Flag-SMRT 1 to 891 proteins were carried out as described previously (30). The DNA-PK complex was obtained commercially (Promega, Madison, WI). The DNA-PK complex was incubated with Flag-SMRT proteins bound to anti-Flag agarose beads (Sigma, St. Louis, MO) at 4°C for 2 hours in HERR buffer [50 mmol/L KCl, 20 mmol/L HEPES (pH 7.9), 2 mmol/L EDTA, 0.1% NP40, 10% glycerol, 0.5% nonfat dry milk]. Bound proteins were eluted, resolved by SDS-PAGE, and subjected to immunoblot analysis.

Mammalian cell culture and transient transfection transcription assay. 293T and HeLa cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum and L-glutamine (all from Invitrogen, Carlsbad, CA). Cells were grown at 37°C in 5% CO_2. Cells were transfected with LipofectAMINE (Invitrogen) according to the manufacturer's instructions. At 48 hours after transfection, cells were harvested for immunoprecipitations, luciferase reporter assays, or chromatin immunoprecipitation (ChIP) assays. In luciferase reporter assays, the Gal4 upstream activating sequence (UAS) × 5-SV40 luciferase reporter contains five copies of the Gal4 17-mer binding site. Luciferase assay kit (Promega) was used to determine relative activity of the luciferase gene product. Light units were normalized to a cotransfected β-galactosidase expression plasmid. Fold repression is relative to the Gal4-DBD, and results of duplicate samples are plotted. For transfections coupled with SMRT siRNA knockdown, cells were harvested at 72 hours after transfection to efficiently knockdown SMRT protein. siRNAs were transfected thrice to efficiently knock down HDAC3, and reporter plasmids were cotransfected with HDAC3 siRNA in the last transfection, and 48 hours later, cells were collected for repression assay. siRNAs were transfected twice to efficiently knock down N-CoR. Reporter plasmids were cotransfected with N-CoR siRNA in the second transfection, and 48 hours later, cells were collected for repression assay. The nontargeted siRNA control sequence used was 5′-AGACA-CACGCACTCGTCTC-3′.

Immunoprecipitation. Cells were washed in PBS, resuspended in HERR buffer containing protease inhibitor cocktail (Roche, Nutley, NJ), and then subjected to sonication. Lysates were clarified by centrifugation at 14,000 × *g* for 10 minutes at 4°C. Supernatants were precleared with protein A agarose beads (Invitrogen) for 1 hour at 4°C. Immunoprecipitation was done at 4°C overnight with normal rabbit IgG (Santa Cruz Biotechnology, Santa

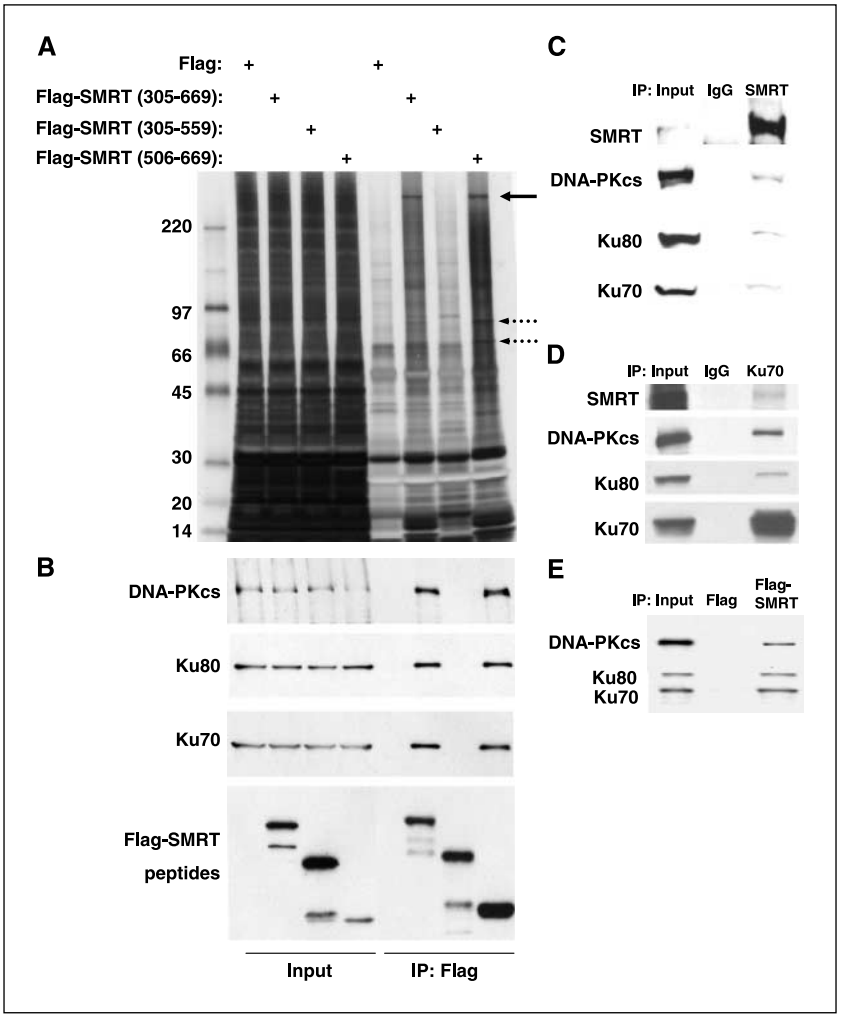

Figure 1. SMRT interacts with the DNA-PK complex. *A* and *B,* SMRT amino acids 506 to 669 interact with the DNA-PK complex in 293T cells. Cells were transfected with pcDNA3-Flag backbone vector, Flag-SMRT 305 to 669, Flag-SMRT 305 to 559, or Flag-SMRT 506 to 669. Whole-cell lysates were immunoprecipitated with anti-Flag agarose beads (*IP:Flag*). Bound proteins were separated by SDS-PAGE. *A, dashed arrows,* detection of novel interacting proteins by silver staining; *solid arrow,* identified as the DNA-PKcs by MALDI-TOF. *B,* immunoblot analysis. Input is 5%. *C,* endogenous DNA-PK complex coprecipitates with SMRT. Immunoprecipitation of 293T cell lysates was done with control rabbit IgG or SMRT antibody followed by Western blot analysis. Input is 1%. *D,* endogenous SMRT coprecipitates with Ku70. Immunoprecipitation of 293T cell lysates was done with control rabbit IgG or Ku70 antibody. Input is 1%. *E,* SMRT polypeptide binds to the DNA-PK complex *in vitro*. Purified recombinant Flag-SMRT 1-891 was immobilized onto anti-Flag agarose beads, incubated with purified DNA-PK complex, and subjected to SDS-PAGE followed by immunoblot. Input is 5%.

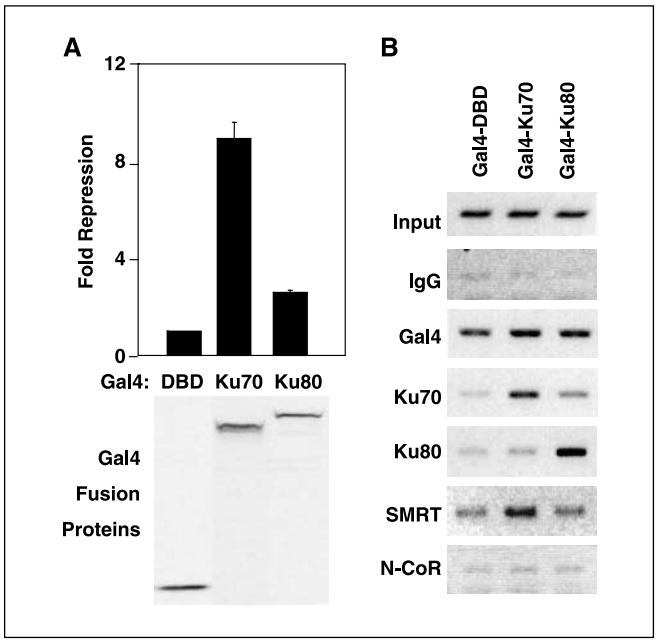

Figure 2. Ku70 represses the transcription of a reporter gene and recruits SMRT to the target DNA region. *A,* the Gal4 UAS × 5-SV40 luciferase reporter was transiently cotransfected with either Gal4-DBD, Gal4-Ku70, or Gal4-Ku80 into 293T cells. Fold repression was measured relative to Gal4-DBD alone at 24 hours after transfection. *Bottom,* equal expression of Gal4-fused proteins. *B,* ChIP analysis of the experiment as in (*A*).

Cruz, CA) or SMRT antibody (PA1-842, Affinity BioReagents, Golden, CO). Samples were then incubated with 25 μL protein A agarose beads for 1 hour. In the Flag-fused protein immunoprecipitation, anti-Flag agarose beads were incubated with cell lysates at 4°C for 4 hours. Immunoprecipitates were washed eight times with HERR buffer. Bound proteins were subjected to SDS-PAGE.

Immunoblot analysis. Immunoblotting with chemiluminescent detection was done as described (38). Antibodies, including mouse DNA-PKcs, Ku80, Ku70 (all from Santa Cruz Biotechnology), mouse SMRT (Abcam, Cambridge, United Kingdom), rabbit HDAC2 (Santa Cruz Biotechnology), rabbit HDAC3 (Abcam), Flag-HRP (Sigma), and Gal-HRP (Santa Cruz Biotechnology) were used.

ChIP assays. ChIP assays were done as described previously (23, 38). Samples were immunoprecipitated with rabbit IgG or polyclonal antibodies recognizing Gal4-DBD (Santa Cruz Biotechnology), SMRT (PA1-842), and N-CoR (25). Anti-Ku70 and anti-Ku80 agarose beads (Santa Cruz Biotechnology) were used for Ku70 and Ku80 immunoprecipitation. Samples were analyzed by conventional PCR using primers specific for UAS × 5-SV40 luciferase reporter: 5′-TGTATCTTATGGTACTGTAACTG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′.

Clonogenic survival assays. The assays were done as described previously (40). Briefly, HeLa cells were transfected with control, SMRT, or N-CoR siRNA constructs. At 24 hours after transfections, each group of cells was divided into six plates of 60 mm. Forty-eight hours later, cells were treated with 1 Gy IR or incubated with 2 μg/mL phleomycin, 0.0015% methyl methanesulfonate (MMS), or 2 mmol/L hydroxyurea for 2 hours. The drug-treated cells were washed with PBS thrice. All cells were harvested and counted. Cells (200, 400, or 1,000) were reseeded on the 100-mm plates for colony formation. After 10 days, cell colonies were fixed with ethanol and stained with crystal violet, and colony numbers were assessed.

Results

SMRT interacts with the DNA-PK complex *in vivo* and *in vitro*. We noted previously that a short region of SMRT (amino acids 506-669), when overexpressed in 293T cells, reproducibly coprecipitates a protein of high molecular weight (Fig. 1*A, solid arrow*). This unknown protein was initially suspected to be SMRT

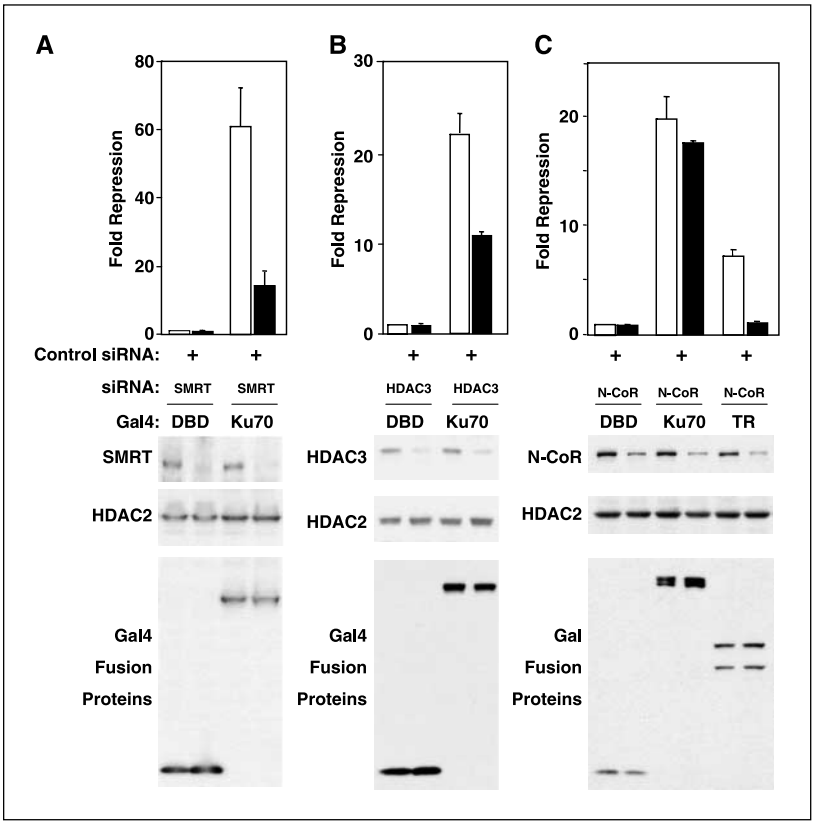

Figure 3. Knockdown of SMRT or HDAC3, but not N-CoR, decreases the transcriptional repression by Ku70. *A,* siRNA knockdown of SMRT followed by transient transfection transcription assay in 293T cells. Cells were harvested at 72 hours after transfection to efficiently knock down SMRT protein. *Bottom,* SMRT knockdown and equal expression of Gal4-fused proteins were verified by immunoblot analysis, in which HDAC2 was a loading control. *B,* effect of siRNA knockdown of HDAC3. *C,* effect of siRNA knockdown of N-CoR. Gal4-TR was included as a positive control for the effect of N-CoR knockdown.

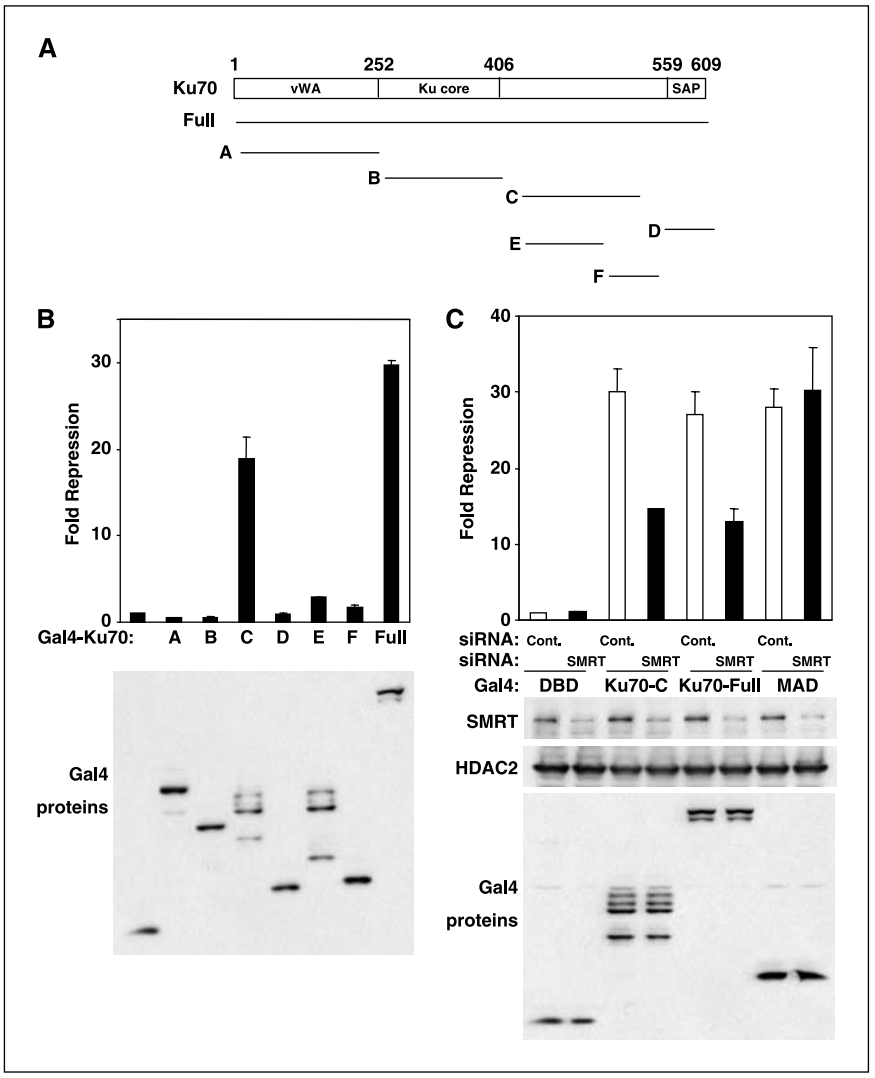

Figure 4. Repression domain of Ku70. *A,* schematic representation of the Ku70 protein and deletion mutants used to map the repression domain of Ku70. Gal4-DBD fusions to Ku70 contained Ku70 amino acids 1 to 609 (*Full*), Ku70 amino acids 1 to 251 (*fragment A*), Ku70 amino 252 to 406 (*fragment B*), Ku70 amino acids 407 to 558 (*fragment C*), Ku70 amino acids 559 to 609 (*fragment D*), Ku70 amino acids 407 to 505 (*fragment E*), and Ku70 amino acids 506 to 558 (*fragment F*). *B,* repression activity of Ku70 domains fused to Gal4-DBD. *Column 1,* Gal-DBD alone. *Bottom,* comparable expression of Gal4-fused proteins. *Fragments C and E,* the lower bands correspond to the molecular weight of the truncated protein; higher molecular weight bands are presumably nonspecific. *C,* effect of SMRT knockdown on the repression function of Ku70-derived polypeptides. *Bottom,* SMRT knockdown and equal expression of Gal4-fused proteins were verified by immunoblot analysis. Gal4-MAD was included as a negative control.

or closely related N-CoR. However, immunoblot analysis excluded this possibility (data not shown). The protein band was isolated and subjected to mass spectrometry sequence analysis. Interestingly, it was identified as DNA-PKcs. Immunoblot analysis confirmed that DNA-PKcs coimmunoprecipitated with SMRT 506 to 669 along with the two other subunits of the DNA-PK complex, Ku70 and Ku80 (Fig. 1*B*); these can also be seen in the silver-stained polyacrylamide gel (Fig. 1*A, dashed arrows*). Notably, another region of SMRT (amino acids 305-559) does not interact with the DNA-PK complex, indicating that the interaction with DNA-PK complex is specific to the region 506 to 669. The interaction of the DNA-PK complex with SMRT was confirmed with endogenous proteins in 293T cells, where the DNA-PK complex coprecipitated with endogenous SMRT (Fig. 1*C*). Reciprocally, endogenous SMRT coprecipitated when DNA-PK complex was immunoprecipitated via the Ku70 subunit. (Fig. 1*D*). The binding of recombinant Flag-SMRT to the purified DNA-PK complex *in vitro* (Fig. 1*E*) suggested that SMRT directly interacts with the DNA-PK complex.

SMRT is specifically recruited by Ku70. The Ku70/Ku80 heterodimer binds to DNA termini at the site of DSBs (8, 10). In addition, Ku proteins are able to bind to specific sites in the promoter region of several genes (41–43), which result in the transcriptional repression of target genes. Because SMRT serves as a corepressor for a variety of transcription factors, we hypothesized that Ku proteins might recruit SMRT to repress the transcription of genes to which it was bound. To test this, full-length Ku70 and Ku80 were fused to the heterologous Gal4-DBD, such that they were recruited to a cotransfected luciferase reporter gene containing multiple Gal4 binding sites. Consistent with previous findings (43), Ku70 strongly repressed transcription in 293T cells, whereas Ku80 was only a weak repressor (Fig. 2*A*). ChIP analysis revealed that endogenous SMRT was specifically recruited by Gal4-Ku70 (Fig. 2*B*). Of note, endogenous N-CoR was not recruited by Ku70 (Fig. 2*B*).

SMRT and HDAC3 are required for transcriptional repression by Ku70. We next addressed the requirement of SMRT and HDAC3 for Ku70-mediated repression. siRNA was used to specifically reduce expression of SMRT or HDAC3 protein in human 293T cells. Remarkably, loss of SMRT almost abolished the repression function of Ku70 (Fig. 3*A*). Ku70 repression was also markedly reduced by the knockdown of HDAC3, a core component of the SMRT corepressor complex (Fig. 3*B*). Consistent with its absence in the previous ChIP

analysis, loss of N-CoR had little effect on the repressive activity of Ku70 (Fig. 3*C*).

SMRT-dependent repression localizes to a unique region of Ku70. A series of Ku70-derived polypeptides (Fig. 4*A*) was fused to the Gal4-DBD to determine the repression domain of Ku70. The NH_2 terminus of Ku70 contains a putative von Willebrand domain, which may be important for protein-protein interactions (2, 44). The central DBD is referred as the "Ku-core" because this region shares high amino acid similarity with Ku80 DBD (44, 45). The COOH-terminal region encompasses a SAF-A/B, acinus, and PIAS (SAP) domain, a putative DNA-binding motif that may be involved in chromosomal organization (44–46). Interestingly, none of these characterized domains (regions A, B, and D) possessed any repression function, whereas a region between the Ku core and SAP domain (region C; amino acids 407-558) showed strong repressive activity (Fig. 4*B*). Further mapping revealed that region C was the minimal repression domain of Ku70, as neither region E nor F (both derived from C) was sufficient to maintain the repression function (Fig. 4*B*). SMRT knockdown markedly decreased the repressive activity of region C (Fig. 4*C*), underscoring the importance of SMRT in Ku70-mediated repression.

Knockdown of SMRT enhances cellular sensitivity to DSBs. Given the well-established role of Ku70 in the repair of DSBs, we next explored the functional effects of SMRT knockdown. HeLa cells were transfected with SMRT or N-CoR siRNA. SMRT or N-CoR protein expression was specifically reduced without any secondary effect on the level of cellular DNA-PKcs or Ku70/Ku80 proteins (Fig. 5*A*). These cells were treated with IR, which causes DSBs and subsequent cell death (47). In control, unirradiated cells, knockdown of SMRT had only a minor effect on survival (Fig. 5*B*). However, reduction of SMRT led to substantial radiosensitization. By contrast, knockdown of N-CoR levels had little effect (Fig. 5*B*).

To further investigate the role of SMRT in facilitating cellular survival after DNA damage, we assessed the effects of SMRT or N-CoR knockdown on cells treated with cytotoxic agents. Cells lacking SMRT, but not N-CoR, were more susceptible to phleomycin, a radiomimetic drug that induces free radical attack on sugar moieties leading to DNA DSBs (Fig. 5*C*; ref. 48). In addition, the presence of SMRT also protected cells from MMS, which creates adducts and apurinic sites leading to DSBs (49). By contrast, SMRT knockdown had no significant effect on survival after treatment with low-dose hydroxyurea, which arrests DNA replication by depleting nucleotide pools without inducing DSBs (Fig. 5*C*; ref. 50). Thus, SMRT is required for the recovery from both IR and chemical-induced DSBs.

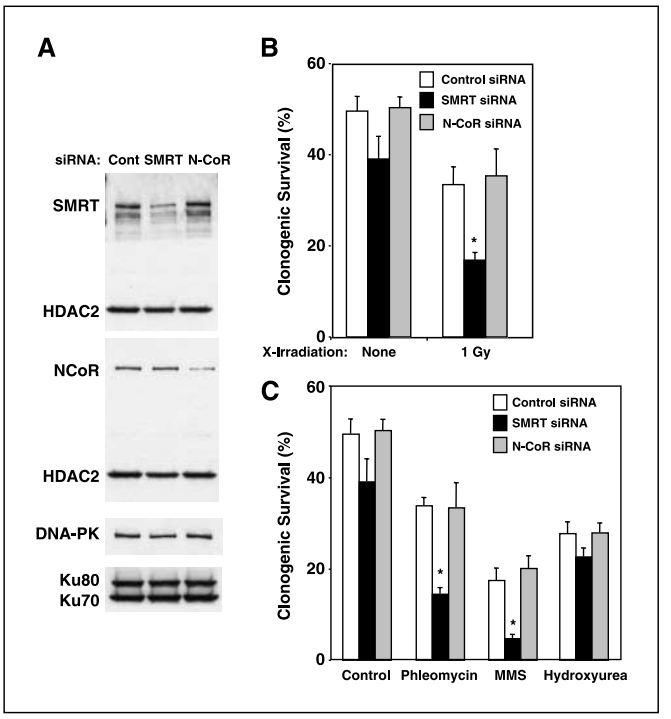

Figure 5. Knockdown of SMRT, but not N-CoR, enhances cell sensitivity to IR and DSB-inducing drugs. HeLa cells with SMRT knockdown or N-CoR knockdown were treated with IR or drugs that induce DSB. *A,* SMRT or N-CoR knockdown was verified by immunoblot using HDAC2 as a loading control. The cellular level of DNA-PKcs, Ku70, and Ku80 protein was not changed after knockdown of SMRT or N-CoR. *B,* effect of IR. *C,* effect of treatment with phleomycin (2 μg/mL), MMS (0.0015%), or hydroxyurea (2 mmol/L) for 2 hours. After treatment, 200, 400, or 1,000 cells were reseeded on the 100-mm dishes for colony formation. The proportion of surviving colonies was determined 10 days later. *, $P < 0.05$ versus control.

Discussion

We have shown that the SMRT corepressor interacts with the Ku70 subunit of the DNA-PK DSB repair complex and is required for its transcriptional repression function. Knockdown of SMRT impaired the ability of cells to recover from DSBs. SMRT and closely related N-CoR have overlapping functions as corepressors for nuclear receptors (51). However, genetic deletion of N-CoR is lethal, showing that SMRT cannot compensate for critical functions of N-CoR (52). Here, conversely, we show that SMRT, but not N-CoR, is uniquely required for Ku70-mediated repression and cellular protection from DNA DSBs.

The Ku70 subunit of the DNA-PK DSB repair complex is known to repress transcription of several genes, including glycophorin B, xanthine oxidoreductase (XOR), and parathyroid hormone-related polypeptide (41–43). Our data suggest that SMRT functions as corepressor for Ku70. Similar to the interaction between nuclear receptors and SMRT, Ku70 has not been detected as a component of core SMRT complexes (24–27). Indeed, the interaction between endogenous SMRT and the DNA-PK complex is relatively weak (Fig. 1*C* and *D*) and may be regulated by DNA.[4] The region implicated in our studies as the repression domain of Ku70 is not conserved in Ku80 and has not been previously assigned a function. This is consistent with the observation that, although Ku70 and Ku80 share similar domain organization, only Ku70 possesses a strong repression function (2, 6). One of the distinct functions of Ku80 is to provide the docking site for DNA-PKcs (53). In addition, Ku80 binds at certain promoters, such as that of the *XOR* gene, and recruits Ku70 to repress transcription (41). Thus, each of the Ku subunits of the DNA-PK complex has unique functions, allowing them to cooperatively respond to a variety of cellular signals.

To the best of our knowledge, the SMRT-HDAC3 corepressor complex is the first example of a mammalian deacetylase complex indicated to involve in cellular recovery from DSBs. In yeast, the class III HDAC Sir2 has been suggested to play a role in NHEJ-mediated DSB repair (54), although this conclusion is complicated by pleiotropic changes in the Sir mutant strains (55). More recently,

[4] C. Palmer, J. Yu, and M. Lazar, preliminary observation.

the class I HDAC Rpd3, in a complex with Sin3, has been shown to play a role in efficient DSB repair by NHEJ in yeast, where histone H4 is hypoacetylated in the vicinity of chromosomal DSB (56).

Paradoxically, HATs have also been implicated in NHEJ pathway. Mammalian Tip60 and its yeast homologue Esa1 are required for DSB repair (57–59); the Esa1-containing NuA4 HAT complex is recruited to a DSB in living yeast (57, 60). One intriguing explanation for the involvement of both HATs and HDACs in DSB repair is that they might cooperate together to dynamically change the chromatin structure to facilitate DSB repair process. Acetylation presumably relaxes the chromatin and allows access of repair proteins to the DNA templates. Histone deacetylation, which is expected to create a compacted local chromatin structure (13, 15), might serve to prevent transcription or DNA replication machinery from interfering with DNA repair complexes. In addition, deacetylase-mediated condensation of the damaged chromatin region might facilitate the juxtaposition and subsequent ligation of the two broken DNA molecules. This function could be particularly important *in vivo*, where damaged DNA ends might otherwise become separated from one another. Thus, the role of SMRT complexes in the function of the DNA-PK complex may contribute to the radiosensitivity noted in human cells treated with HDAC inhibitors (18, 19).

Acknowledgments

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