

PRMT5, a Novel TRAIL Receptor-Binding Protein, Inhibits TRAIL-Induced Apoptosis via Nuclear Factor- κ B Activation

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Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and has selective antitumor activity. Although TNF- α -induced intracellular signaling pathways have been well studied, TRAIL signaling is not fully understood. Here, we identified a novel TRAIL receptor-binding protein, protein arginine methyltransferase 5 (PRMT5), as a result of proteomic screening. PRMT5 selectively interacted with death receptor 4 and death receptor 5 but not with TNF receptor 1 or Fas. PRMT5 gene silencing sensitized various cancer cells to TRAIL without affecting TRAIL resistance in nontransformed cells. PRMT5 contributed to TRAIL-induced activation of inhibitor of κ B kinase (IKK) and nuclear factor- κ B (NF- κ B), leading to induction of several NF- κ B target genes. Although IKK inhibition increased sensitivity to both TRAIL and TNF- α , PRMT5 knockdown potentiated TRAIL-mediated cytotoxicity alone. PRMT5 had no effect on TNF- α -mediated NF- κ B signaling. These results show the selectivity of PRMT5 for TRAIL signaling. The PRMT5 small interfering RNA-mediated susceptibility to TRAIL was rescued by ectopic expression of active IKK β , confirming the involvement of PRMT5 in TRAIL resistance by activating the NF- κ B pathway. Collectively, our findings suggest the therapeutic potential of PRMT5 in TRAIL-based cancer treatments. (Mol Cancer Res 2009;7(4):557–69)

Introduction

The tumor necrosis factor (TNF) superfamily plays a critical role in such physiologic and pathologic phenomena as immune system, inflammation, and development through regulation of cellular apoptosis and proliferation (1-3). TNF-

related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, induces apoptosis in a wide variety of tumor cells, including resistant cells to other TNF superfamily members (4, 5). In contrast to TNF and Fas ligand, the cytotoxicity of TRAIL is characterized by selectivity to tumor cells. TRAIL does not kill normal cells, except hepatocytes and several hematopoietic cells, suggesting therapeutic potential for cancer (6–8). Administration of TRAIL suppressed tumor progression, without apparent toxicity, in mouse and in nonhuman primate models (9, 10). TRAIL-based therapies, recombinant TRAIL, and monoclonal antibodies targeting TRAIL receptors have now entered phase I and II clinical trials.

TRAIL binds to cell surface receptors, death receptor 4 (DR4) or death receptor 5 (DR5), triggering their trimerization. The trimerized receptors interact with an adaptor protein Fas-associated death domain (FADD) and then FADD recruits caspase-8 and caspase-10. This signaling complex, consisting of ligand, receptor, FADD, and apical caspases, is named the death-inducing signaling complex (DISC). Caspases are self-activated in DISC and initiate cleavage and activation of downstream effector caspases, resulting in cellular apoptosis (11-13).

TRAIL also binds to decoy receptors that have no ability to transduce apoptosis signaling. Therefore, the decoy receptors compete with DR4/DR5 and diminish TRAIL-mediated cytotoxicity. Certain tumor cells exhibit resistance to TRAIL that is not fully explained by expression levels of DR4/DR5 and decoy receptors. Such TRAIL resistance might be caused by activation of other signaling pathways. TRAIL association with DR4 or DR5 is known to activate the inhibitor of κ B (I κ B) kinase (IKK)/nuclear factor- κ B (NF- κ B), c-Jun NH₂-terminal kinase, and p38 mitogen-activated protein kinase signaling pathways (8, 14). Specifically, NF- κ B is a transcription factor involving various antiapoptotic gene expressions that affect cellular response to TRAIL. NF- κ B is composed of dimers of Rel family members. Classic NF- κ B complexes, typically p65 and p50, are sequestered in the cytoplasm of resting cells by association with the I κ B family proteins. Once the cells are stimulated by TNF superfamily members, I κ B is phosphorylated by the IKK complex containing catalytic subunits IKK α and IKK β and a regulatory subunit IKK γ /NEMO (15-17). This phosphorylation targets I κ B for degradation via the ubiquitin-proteasome pathway and allows nuclear translocation of NF- κ B, leading to transcription of target genes (18, 19). Candidates for NF- κ B-inducible, antiapoptotic proteins include the inhibitor of apoptosis (IAP) proteins (20, 21). Genetic and pharmacologic inhibition of the NF- κ B pathway is well known to enhance TRAIL-induced apoptosis (22, 23).

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Protein arginine methylation is a post-transcriptional covalent modification and is involved in a variety of cellular processes, including signal transduction, RNA processing, and transcriptional regulation (24, 25). Protein arginine methyltransferases (PRMT) transfer the methyl group from *S*-adenosyl-L-methionine to the guanidino nitrogen atoms of an arginine residue. PRMT5 is one of the evolutionarily conserved type II arginine methyltransferases that catalyze monomethylation and symmetric dimethylation (26, 27). Expression of PRMT5 is ubiquitous among various tissues and is elevated in gastric cancer and lymphoma (28, 29). In addition, overexpression of PRMT5 promoted transformation of NIH3T3 cells, suggesting its role in tumor progression (30). The most studied substrate of PRMT5 is nuclear histone, and histone methylation results in transcriptional repression. Because PRMT5 is distributed not only in the nucleus but also in the cytoplasm, cytoplasmic PRMT5 may play some role in tumorigenesis.

In this study, we tried to identify DR4-interacting proteins to clarify new mechanisms of TRAIL-mediated signaling and TRAIL resistance. We found PRMT5 as a novel DR4- and DR5-binding protein that conferred TRAIL resistance on cancer cells. Experiments using small interfering RNA (siRNA) revealed that PRMT5 was associated with NF- κ B activation induced by TRAIL but not by TNF- α . Because expression of active IKK β overcame TRAIL-induced apoptosis in PRMT5-depleted cells, PRMT5 was identified as a new determinant of TRAIL resistance by activating the NF- κ B pathway.

Results

Identification of PRMT5 as a Novel TRAIL Receptor-Binding Protein

To screen proteins in the death receptor-containing complex, lysates of HeLa cells expressing DR4 proteins dually tagged with FLAG and His were applied to anti-FLAG agarose followed by nickel affinity resin. The bound proteins were analyzed by SDS-PAGE and silver staining. We then tried to identify the DR4-binding proteins by liquid chromatography-tandem mass spectrometry after in-gel digestion. As expected, DR4 was detected in a major band (Fig. 1A). Mass spectrometry analysis of another prominent band revealed that it was PRMT5.

We first examined the physical association between exogenous PRMT5 and DR4. As shown in Fig. 1B, FLAG-tagged DR4 proteins were present in precipitates when Myc-tagged PRMT5 was immunoprecipitated with anti-Myc agarose in HCT116 cells. DR5, another TRAIL receptor, was also coimmunoprecipitated with PRMT5 (Fig. 1C). In contrast, other

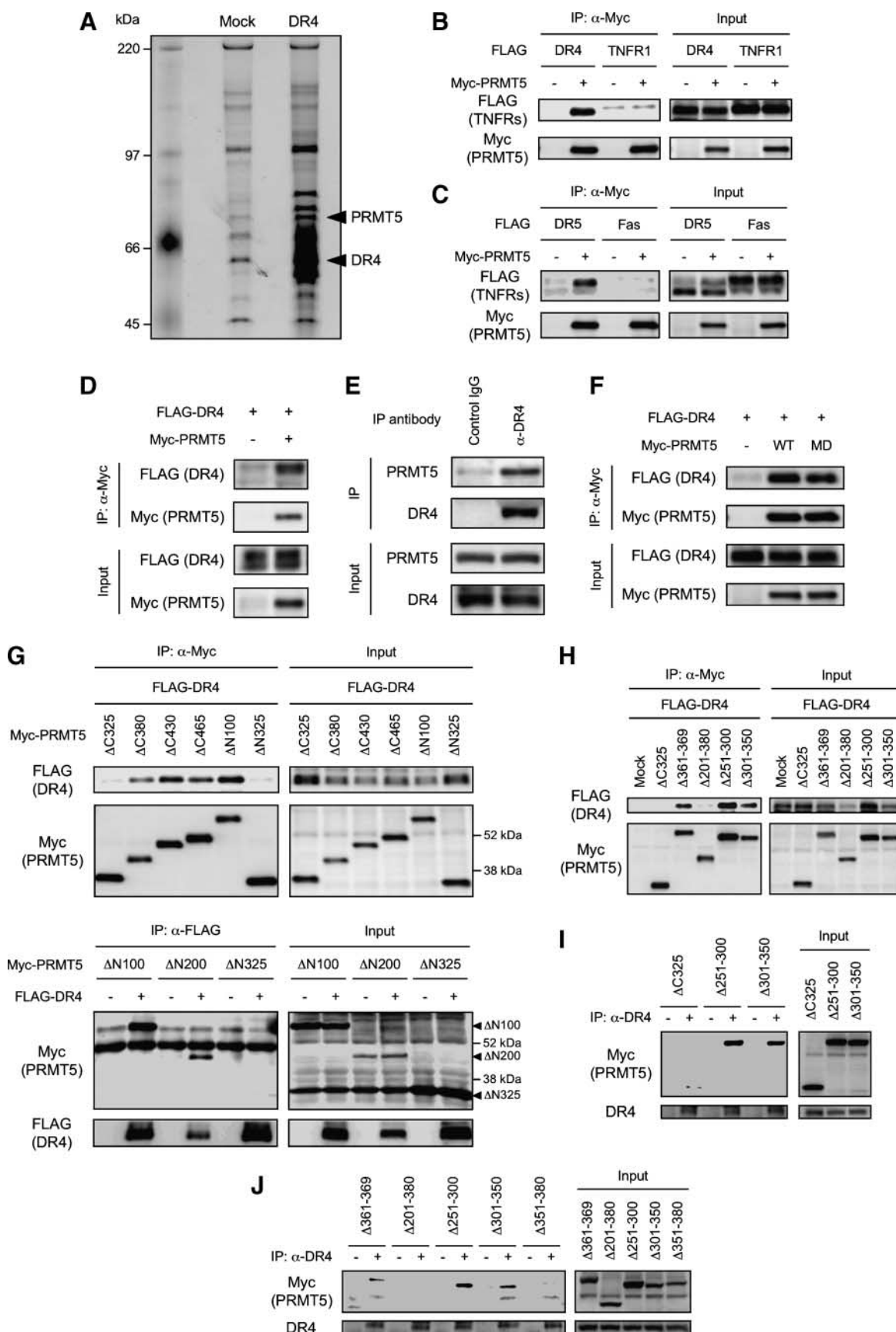
members of the TNF receptor (TNFR) superfamily, TNFR1 and Fas, failed to form complexes with PRMT5 (Fig. 1B and C). HeLa cells used in the screening also showed interaction of PRMT5 with DR4 (Fig. 1D). The binding between PRMT5 and DR4 was confirmed at endogenous levels in HCT116 and HeLa cells (Fig. 1E; Supplementary Fig. S1A). Then, we examined the requirement of methyltransferase activity of PRMT5 for interaction with DR4 using a methyltransferase-dead mutant (MD-PRMT5; ref. 27). Wild-type (WT) and MD-PRMT5 bound identically to DR4 (Fig. 1F), indicating that the methyltransferase activity of PRMT5 is dispensable for its DR4-binding ability.

To determine a critical region in PRMT5 for DR4 association, we constructed several deletion mutants of PRMT5. Full-length DR4 bound to COOH-terminally deleted Δ C380 (amino acids 1-379)-PRMT5, Δ C430 (amino acids 1-429)-PRMT5, and Δ C465 (amino acids 1-464)-PRMT5 but not to Δ C325 (amino acids 1-324)-PRMT5 (Fig. 1G). We found that DR4 formed complexes with NH₂-terminally deleted Δ N100 (amino acids 101-637)-PRMT5 and Δ N200 (amino acids 201-637)-PRMT5 but not with Δ N325 (amino acids 326-637)-PRMT5 (Fig. 1G). These results indicate that the region around amino acids 200 to 380 in PRMT5 is critical for binding to DR4. Furthermore, we used internal deletion mutants lacking parts of amino acids 200 to 380 for fine mapping of DR4-binding region (Fig. 1H-J). The Δ 361-369 mutant lacking methyltransferase domain I (amino acids 361-369) could interact with DR4. Δ 251-300 and Δ 301-350, but not Δ 201-380 or Δ 351-380, bound to DR4. These mutants showed consistent binding properties to exogenous and endogenous DR4. Because neither Δ C325 nor Δ N325 were shown to interact with DR4 in Fig. 1G, we concluded that both amino acids 201 to 250 and 351 to 380 in PRMT5 are required for DR4 binding. Unfortunately, the Δ 201-250-PRMT5 protein was not expressed in cells. The intracellular domain of DR4 and DR4 lacking its death domain also bound to PRMT5, suggesting that the intracellular domain except death domain is involved in PRMT5 binding (data not shown).

PRMT5 Controls TRAIL Sensitivity in Cancer Cells

To determine the involvement of PRMT5 in tumoricidal activity of TRAIL, we designed siRNA targeting *PRMT5*. The siRNAs (*PRMT5*-1 and *PRMT5*-3) used in the experiment effectively reduced *PRMT5* protein expression (Fig. 2A). Knockdown of *PRMT5* dramatically attenuated cell viability in TRAIL-treated HeLa cells compared with untreated cells (Fig. 2B). Under pan-caspase inhibitor (Z-VAD)-treated conditions, the decrease in cell viability was completely overcome.

FIGURE 1. Identification of PRMT5 as a binding partner of DR4/DR5 but not of other TNFR superfamily members. **A.** HeLa cells were transfected with pFLAG-CMV-5c vectors encoding none (Mock) or DR4 in the presence of 10 μ mol/L Z-VAD. Both FLAG- and His-tagged DR4 proteins were sequentially purified by anti-FLAG agarose and nickel nitrilotriacetic acid agarose. Purified proteins were electrophoresed and silver stained. HCT116 (**B** and **C**) and HeLa (**D**) cells were transfected with pFLAG-CMV-5c vectors encoding DR4, TNFR1, DR5, or Fas together with pcDNA3-Myc vectors encoding none (-) or PRMT5 (+) in the presence of 10 μ mol/L Z-VAD. Myc-tagged PRMT5 proteins were immunoprecipitated with anti-Myc agarose (α -Myc). **E.** HCT116 cell lysates were incubated with protein G Sepharose that had been conjugated with control rabbit IgG (Control IgG) or an anti-DR4 antibody (α -DR4). **F.** HCT116 cells were transfected with pcDNA3-Myc vectors encoding none (-), WT-PRMT5 (WT), or MD-PRMT5 (MD) together with pFLAG-CMV-5c-DR4 in the presence of 10 μ mol/L Z-VAD. Myc-tagged PRMT5 proteins were immunoprecipitated. **G** and **H.** HCT116 cells were transfected with pFLAG-CMV-5c vectors encoding none (-) or DR4 (+) together with the indicated PRMT5 deletion mutants in pcDNA3-Myc vectors in the presence of 10 μ mol/L Z-VAD. Myc-tagged PRMT5 (**G, top**) or FLAG-tagged DR4 (**G, bottom**, and **H**) proteins were immunoprecipitated. **I** and **J.** HCT116 cells were transfected with the indicated PRMT5 deletion mutants in pcDNA3-Myc vectors. Cell lysates were incubated with protein G Sepharose that had been conjugated with control rabbit IgG (-) or an anti-DR4 antibody (+). Immunoprecipitated proteins (*IP*) and cell lysates (*Input*) were immunoblotted with the indicated antibodies (**B-J**).



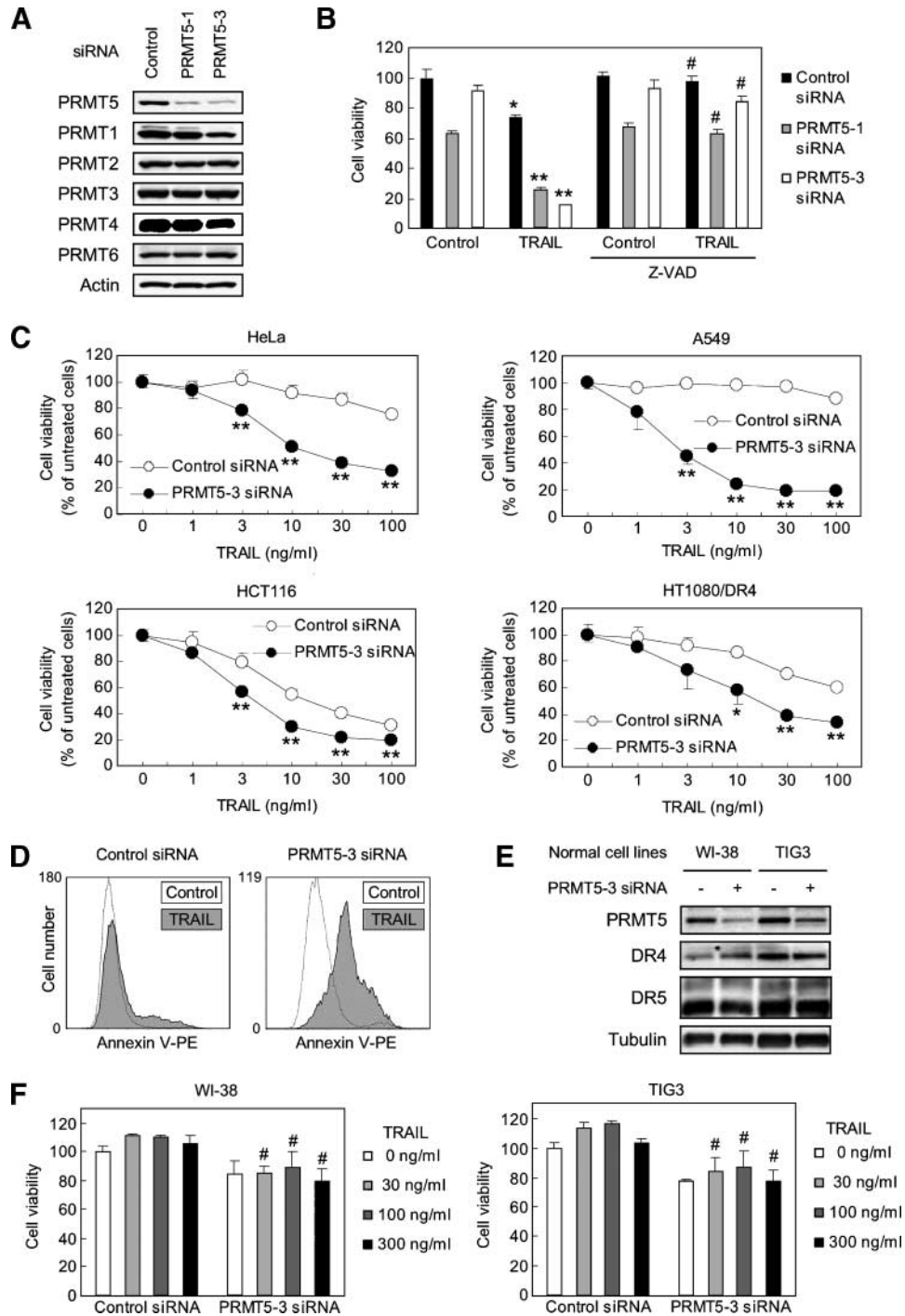


FIGURE 2. Knockdown of PRMT5 potentiates TRAIL-induced apoptosis in cancer cells. **A.** HeLa cells were transfected with control, PRMT5-1, or PRMT5-3 siRNA. Cell lysates were immunoblotted with the indicated antibodies. **B.** HeLa cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL in the presence or absence of 10 μmol/L Z-VAD for 24 h. Cell viability was evaluated using the MTS assay in a percentage of the control siRNA-transfected cells without TRAIL treatment. *, $P = 0.0016$; **, $P < 0.00005$, compared with TRAIL-untreated and the same siRNA-transfected cells; #, $P > 0.05$, compared with TRAIL-untreated and the same siRNA- and Z-VAD-treated cells. **C.** HeLa, A549, HCT116, and HT1080/DR4 cells were transfected with the indicated siRNA and then treated with the indicated concentrations of TRAIL. Cell viability was evaluated using the MTS assay in a percentage of the corresponding cells without TRAIL treatment. *, $P < 0.05$; **, $P < 0.01$, compared with control siRNA-transfected and the same concentration of TRAIL-treated cells. **D.** HeLa cells were transfected with control (left) or PRMT5-3 (right) siRNA and then treated with a vehicle (open areas) or 100 ng/mL TRAIL (closed areas) for 24 h. Cells were stained with Annexin V-phycoerythrin, and the fluorescence was analyzed using a flow cytometer. **E.** WI-38 and TIG3 fibroblast cells were transfected with the indicated siRNA. Cell lysates were immunoblotted with the indicated antibodies. **F.** WI-38 (left) and TIG3 (right) cells were transfected with the indicated siRNA and then treated with the indicated concentrations of TRAIL. Cell viability was evaluated using MTS assay. #, $P > 0.05$, compared with TRAIL-untreated and PRMT5-3 siRNA-transfected cells. Bars, SD of triplicate experiments (**B**, **C**, and **F**).

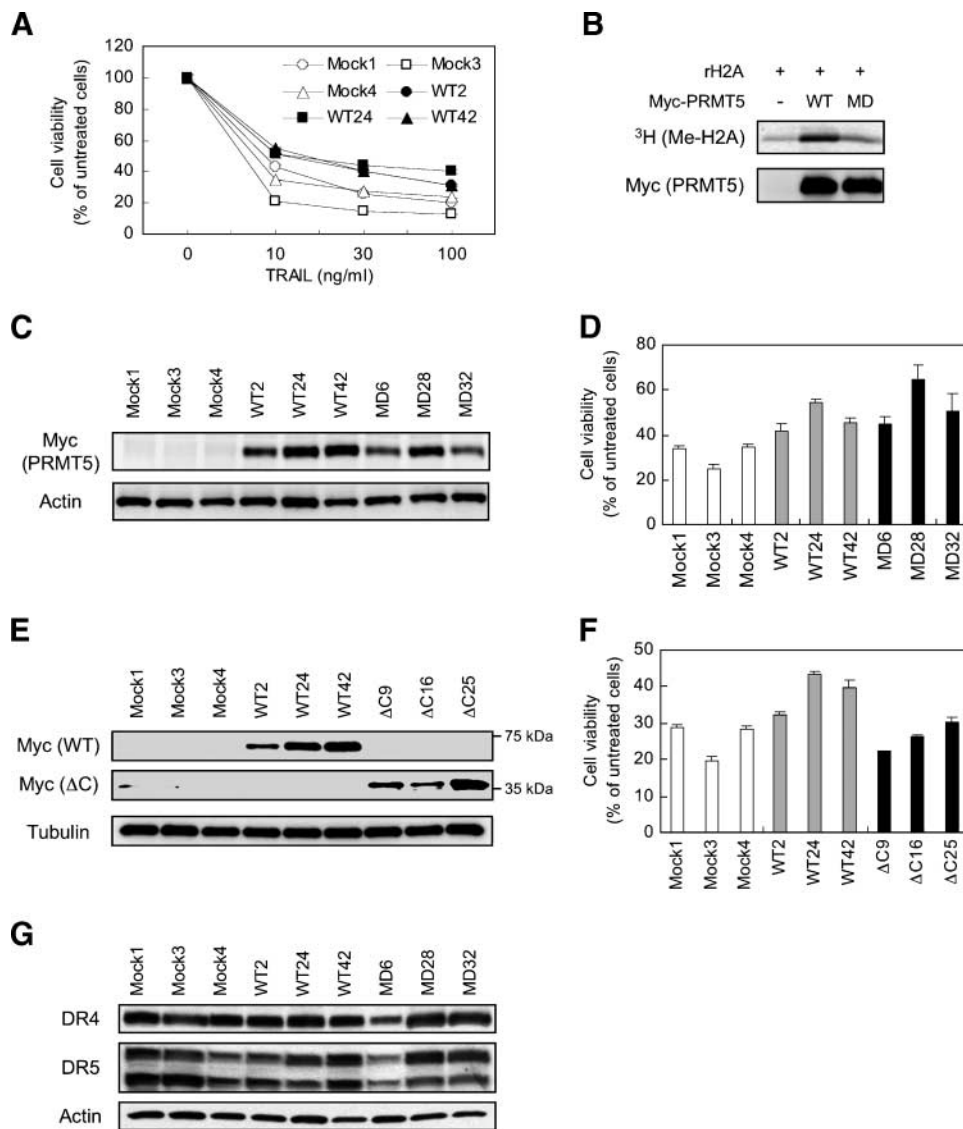


FIGURE 3. PRMT5 confers TRAIL resistance independent of its methyltransferase activity. **A**, **D**, and **F**. HCT116 clones stably transfected with pcDNA3-Myc (Mock1, Mock3, and Mock4), pcDNA3-Myc-WT-PRMT5 (WT2, WT24, and WT42), pcDNA3-Myc-MD-PRMT5 (MD6, MD28, and MD32), or pcDNA3-Myc- Δ C325-PRMT5 (Δ C9, Δ C16, and Δ C25) were treated with the indicated concentrations of TRAIL (**A**) or 30 ng/mL TRAIL (**D** and **F**). Cell viability was evaluated using the MTS assay in a percentage of the corresponding cells without TRAIL treatment. Bars, SD of multiple experiments [**A** and **F** ($n = 3$) and **D** ($n = 5$)]. **B**. HeLa cells were transiently transfected with pcDNA3-Myc vectors encoding none (-), WT-PRMT5 (WT), or MD-PRMT5 (MD). Myc-tagged proteins were immunoprecipitated and subjected to an *in vitro* methyltransferase assay using recombinant H2A (rH2A) as a substrate (*top*). Immunoprecipitated proteins were immunoblotted with an antibody to Myc tag (*bottom*). **C**, **E**, and **G**. Cell lysates of the stable transfectants were immunoblotted with the indicated antibodies.

We also examined the effects of PRMT5 knockdown on TRAIL cytotoxicity in HeLa, A549, HCT116, and HT1080 cells that had been stably transfected with DR4 (HT1080/DR4). As shown in Fig. 2C, *PRMT5* gene silencing enhanced sensitivity to TRAIL in all the tested cell lines. Increases in Annexin V reactivity (Fig. 2D) and sub-G₁ population (data not shown) in PRMT5-siRNA transfectants were consistently observed. Then, we examined the role of PRMT5 on TRAIL sensitivity in nontransformed cells. Immunoblot analysis revealed that PRMT5-3 siRNA transfection suppressed PRMT5 expression without consistent effects on DR4 and DR5 expression in normal fibroblast WI-38 and TIG3 cells (Fig. 2E;

Supplementary Fig. S1B). Under this condition, however, knockdown of PRMT5 did not affect their sensitivity to TRAIL, although marginal growth inhibition was observed in PRMT5-3 siRNA transfectants (Fig. 2F). Collectively, these findings indicate that PRMT5 confers TRAIL resistance on cancer cells but not on nontransformed cells.

Next, we established several PRMT5 stably transfected clones using HCT116 cells that highly express DR4 (Supplementary Fig. S1C) and are sensitive to TRAIL (Fig. 2C). The established WT-PRMT5-expressing clones showed moderately higher resistance to TRAIL than mock transfectants (Fig. 3A). Because PRMT5 is an abundant protein, transfection of

PRMT5-expressing plasmids could marginally increase the total expression level of PRMT5 protein (data not shown). That might be the reason why PRMT5 transfection did not dramatically confer TRAIL resistance on HCT116 cells. To examine the requirement of methyltransferase activity, we generated MD-PRMT5-expressing clones. An *in vitro* methyltransferase assay verified diminished methyltransferase activity of MD-PRMT5 proteins (Fig. 3B). PRMT5 expression levels in the selected clones are shown in Fig. 3C. These MD-PRMT5-expressing cells were refractory to TRAIL as well as WT-PRMT5-expressing cells (Fig. 3D). Furthermore, we established stable transfectants expressing the Δ C325-PRMT5 mutant that could not bind to DR4 (Fig. 3E). In contrast to WT-PRMT5-expressing cells, Δ C325-PRMT5-expressing cells were sensitive to TRAIL-like mock transfectants, indicating that the binding between DR4 and PRMT5 is essential for PRMT5-mediated TRAIL resistance (Fig. 3F). Moreover, PRMT5 could not methylate DR4 proteins when the same procedures shown in Fig. 3B were done (data not shown). These results suggest that PRMT5 endows cancer cells with TRAIL resistance in a methyltransferase-independent manner. Expression levels of DR4 and DR5 were not correlated with TRAIL sensitivity, suggesting that PRMT5 expression defines TRAIL sensitivity in the similar cellular contexts (Fig. 3G).

Blockade of TRAIL-Mediated NF- κ B Signaling by PRMT5 Knockdown

To elucidate the mechanisms of PRMT5-regulated TRAIL sensitivity, we first examined cell surface expression of DR4 and DR5 after PRMT5-siRNA transfection in HeLa cells. Flow cytometric analysis revealed that knockdown of PRMT5 did not alter the amount of DR4 or DR5 expressed on the cell membrane (Fig. 4A). We next investigated TRAIL-induced DISC formation using HT1080/DR4 cells. When DR4 was immunoprecipitated under TRAIL-treated conditions, coprecipitated FADD was observed in PRMT5-siRNA transfectants as well as in control siRNA-transfected cells (Fig. 4B), suggesting that PRMT5 did not affect DISC formation with DR4. During the experiment, we found the inhibition of TRAIL-induced I κ B α degradation in PRMT5-siRNA transfectants (Fig. 4B). Time-course analysis of I κ B α expression in TRAIL-treated cells revealed that I κ B α degradation in PRMT5-siRNA transfectants occurred later than that in control siRNA transfectants (Fig. 4C, *left*). On the other hand, PRMT5 siRNA had no effect on TNF- α -induced degradation of I κ B α . Upstream IKK complexes phosphorylate I κ B α when cells were stimulated, leading to degradation of I κ B α (16). The TRAIL-mediated phosphorylation rate of endogenous I κ B α was reduced by PRMT5 gene silencing (Fig. 4C, *right*). To investigate the involvement of IKK-mediated I κ B phosphorylation, we carried out an *in vitro* kinase assay of the endogenous IKK complexes that were immunoprecipitated with an anti-NEMO antibody. We used recombinant I κ B α as a substrate of the assay. Under control siRNA-transfected conditions, kinase activity of TRAIL-treated cells was roughly twice as high as that of vehicle-treated cells (Fig. 4D). PRMT5 knockdown diminished TRAIL-induced, but not TNF- α -induced, IKK activation, indicating that PRMT5 knockdown-reduced IKK activity inhibits I κ B α degradation. PRMT5 siRNA also decreased phosphorylation of NF-

κ B p65 at Ser⁵³⁶ that was known to be phosphorylated by IKK or other kinases and was critical for NF- κ B activation (Fig. 4E).

Reporter assay confirmed that TRAIL-induced NF- κ B activation was attenuated by transfecting PRMT5 siRNA into HT1080/DR4 and HCT116 cells (Fig. 5A). We obtained similar results using HeLa cells (data not shown). PRMT5 knockdown did not affect TNF- α -induced NF- κ B activation (Fig. 5B). To confirm again whether methyltransferase activity was required for TRAIL-induced NF- κ B activation, we used the indirect methyltransferase inhibitor periodate-oxidized adenosine, blocking broad methylation reactions. Pretreatment of cells with periodate-oxidized adenosine hardly repressed NF- κ B reporter activity in TRAIL-stimulated cells (Fig. 5C). This result adds further evidence that PRMT5 acts as a TRAIL resistance factor independent of its methyltransferase activity.

We then checked the transcription of NF- κ B target genes, including the IAP family. Although TRAIL treatment induced expression of *cIAP1* and *cIAP2* mRNA, knockdown of PRMT5 reduced the induction in HCT116 and HT1080/DR4 cells (Fig. 6A and B). We obtained similar results using HeLa and A549 cells (data not shown). TRAIL-induced cIAP2 protein expression was also cancelled by PRMT5 gene silencing (Fig. 6C). Members of the IAP family are well known to block apoptosis by binding to and inhibiting caspases (31, 32). Consistently, PRMT5 siRNA-transfected cells showed increase in TRAIL-induced caspase cleavage (Fig. 6C). These results suggest that PRMT5 regulates TRAIL-induced expression of apoptosis-inhibitory genes, thus blocking apoptosis.

PRMT5 Contributes to TRAIL Resistance by Activating NF- κ B Signaling

To evaluate the importance of the NF- κ B pathway, we measured TRAIL cytotoxicity under NF- κ B-inhibiting conditions. We used the IKK inhibitor BMS-345541 (33, 34). As expected, NF- κ B inhibition by BMS-345541 potentiated cell death induced by TRAIL as well as by TNF- α in HCT116, HT1080/DR4 (Fig. 7A), HeLa, and A549 cells (data not shown). In contrast, siRNA targeting PRMT5 greatly sensitized cells to TRAIL but not to TNF- α (Fig. 7B). This selective sensitization probably results from specific binding of PRMT5 to DR4/DR5 but not to TNFR1 (Fig. 1B) and from specific inhibition of NF- κ B signaling under TRAIL stimulation (Fig. 5). To ascertain whether NF- κ B contributes to TRAIL resistance as a downstream factor of PRMT5, HT1080 cells were transfected with PRMT5 siRNA followed by transfecting active IKK β tagged with GFP and then treated with TRAIL. Although more than half of TRAIL-treated and PRMT5 siRNA-transfected cells underwent apoptosis, active IKK β expression significantly overcame the apoptosis (Fig. 7C). These results indicate that PRMT5 attenuates TRAIL-induced apoptosis via activating NF- κ B signaling.

Discussion

Although abnormal cells in living organisms undergo apoptosis to eliminate themselves, most cancer cells have a defect in the ability to induce apoptosis (35). Extrinsic induction of apoptosis in cells with defective intrinsic pathways provides an exciting approach for cancer therapy. Particularly, TRAIL

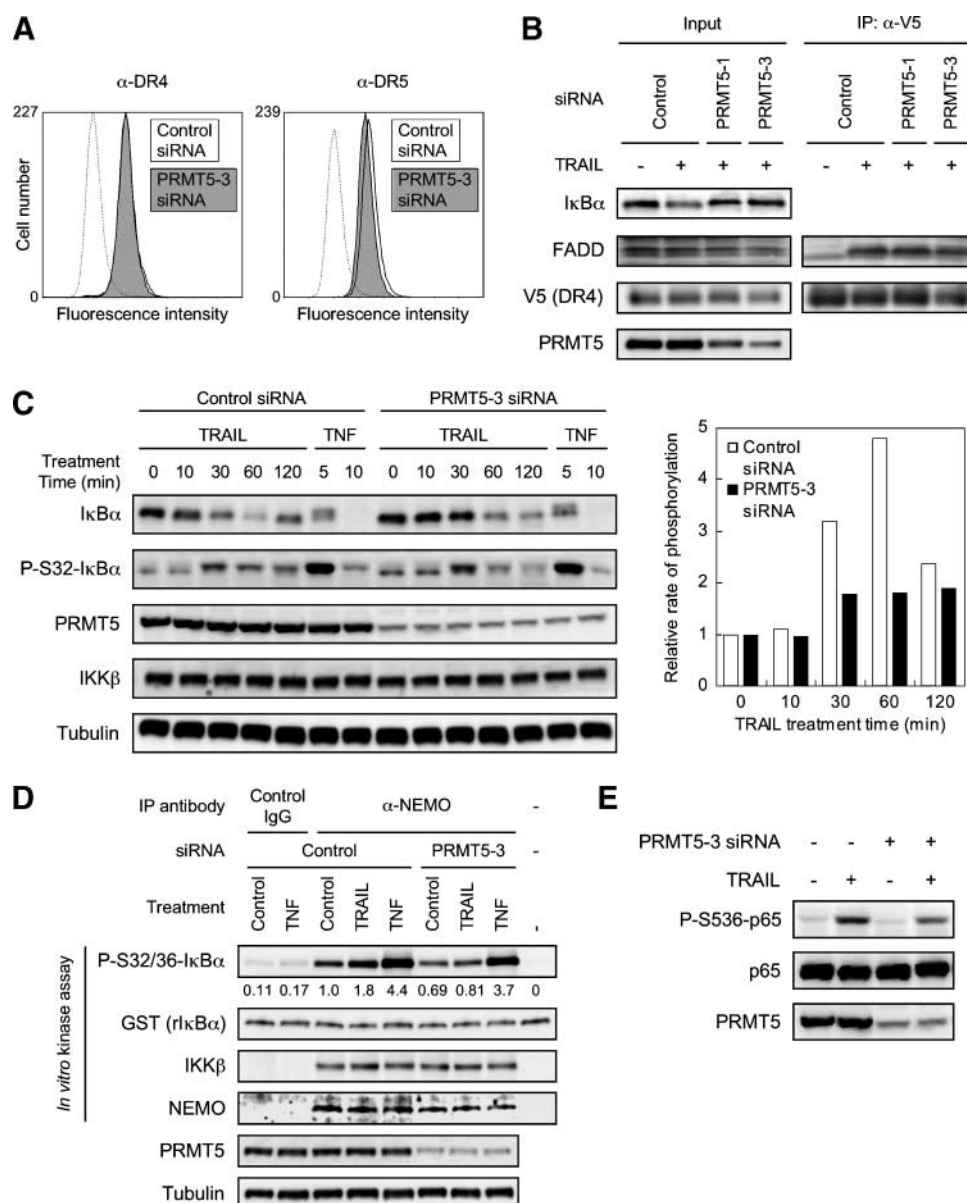


FIGURE 4. Involvement of PRMT5 in TRAIL-induced IKK activation and I κ B degradation. **A.** HeLa cells were transfected with the indicated siRNA and then incubated with control mouse IgG or antibodies to DR4 (*left*) or DR5 (*right*). Fluorescence was analyzed using a flow cytometer. Broken lines, fluorescence of control IgG-treated and control siRNA-treated cells; open and closed areas, control and PRMT5-3 siRNA-treated cells, respectively. **B.** HT1080/DR4 cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL for 30 min. V5-tagged DR4 proteins were immunoprecipitated with anti-V5 agarose. Immunoprecipitated proteins (*IP*) and cell lysates (*Input*) were immunoblotted with the indicated antibodies. **C.** HT1080/DR4 cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL or 20 ng/mL TNF- α for the indicated times. Cell lysates were immunoblotted with the indicated antibodies (*left*). Relative intensities of the phospho-I κ B α bands normalized with total I κ B α bands were quantified (*right*). **D.** HT1080/DR4 cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL for 30 min. Endogenous IKK complexes were immunoprecipitated with anti-NEMO agarose and incubated with GST-tagged recombinant I κ B α (*rI κ B α*) as described in Materials and Methods. Control experiments were done with control rabbit IgG agarose. Reactions (*in vitro* kinase assay) and cell lysates were immunoblotted with the indicated antibodies. Relative intensities of the phospho-I κ B α bands were quantified. **E.** HT1080/DR4 cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL for 30 min. Cell lysates were immunoblotted with the indicated antibodies.

is a remarkable molecule because of its potent death-inducing ability, selectivity to tumor cells, and lack of significant toxicity in normal organs. However, certain cancer cells exhibit resistance to TRAIL, but resolution of that resistance leads to successful outcomes in TRAIL-based therapy.

Using proteomic screening, we identified PRMT5 as a novel component of death receptor complexes. PRMT5 bound to

DR4/DR5 and prevented TRAIL-induced apoptosis. Because knockdown of PRMT5 by siRNA sensitized cancer cells to TRAIL (Fig. 2), PRMT5 is expected to be a potential molecular target for combined therapy with TRAIL. Specifically, down-regulation of PRMT5 did not enhance TRAIL-mediated cytotoxicity in normal cells (Fig. 2F). Therefore, PRMT5 contributes to TRAIL resistance selectively in cancer cells,

suggesting that PRMT5 targeting therapy may have low adverse effects. Because normal fibroblast WI-38 and TIG3 cells express PRMT5 proteins comparably with HCT116 cells (Supplementary Fig. S1D), the invariant resistance to TRAIL in normal cells does not simply result from expression levels of PRMT5. In addition to cancer cell selectivity, three lines of evidence have proven that PRMT5 is selective to TRAIL signaling among TNF superfamily members. First, PRMT5 physically interacts with TRAIL receptors DR4 and DR5 but not with TNFR1 or Fas (Fig. 1B and C). Second, PRMT5 siRNA suppressed TRAIL-induced IKK and NF- κ B activation, although those induced by TNF- α were not affected (Figs. 4 and 5). Third, PRMT5 siRNA-treated cells showed increased TRAIL sensitivity but were still refractory to TNF- α , in contrast to IKK inhibitor-treated cells (Fig. 7). TNF- α appears to be related to many inflammatory diseases such as rheumatoid arthritis and viral hepatitis (36). In this context, PRMT5 might serve as a less toxic target without modulating TNF- α signaling. Furthermore, expression levels of PRMT5 probably define TRAIL sensitivity in the similar cellular contexts (HCT116 clones stably expressing PRMT5; Fig. 3), but those of death receptors do in the different cellular contexts (HCT116 and HeLa cells). HCT116 cells that had higher expression of death receptors than HeLa cells (Supplementary Fig. S1C) showed TRAIL sensitivity compared with HeLa cells when they were transfected with control siRNA (Fig. 2C).

Immunoprecipitation using deletion mutants revealed that PRMT5 associates with DR4 through amino acids 200 to 251 and 351 to 380 (Fig. 1). This region is less conserved among other PRMT family members, except the highly conserved methyltransferase domain I (amino acids 361-369; ref. 24). The conserved GXGXXG motif of domain I in protein methyltransferases is known to be a S-adenosyl-L-methionine binding site and is critical for the catalytic activity (25). In fact, the MD-PRMT5 mutant in which the GAGRG motif (amino acids 365-369) was substituted with GAARG had substantially reduced methyltransferase activity (Fig. 3B). A mutant lacking domain I also bound to DR4 (Fig. 1H and J) as well as MD-PRMT5 (Fig. 1F), suggesting that a region specific to PRMT5 is required for association with DR4. PRMT5 endogenously associated with DR4 in nonstimulated cells (Fig. 1E). Examining the association after TRAIL treatment revealed that PRMT5 bound equally to DR4 regardless of TRAIL stimulation (data not shown). Insulinoma-glucagonoma clone 20 was reported to be constantly associated with DR4 and DR5, such as PRMT5 (37), and was also shown to bind to TNFR1. Insulinoma-glucagonoma clone 20 expression rendered cells more susceptible not only to TRAIL-induced but also to TNF- α -induced apoptosis by increasing recruitment of FADD and caspase-8 to DISC. In this situation, PRMT5 is different from insulinoma-glucagonoma clone 20 in its selectivity to TRAIL signaling and irrelevance on DISC formation.

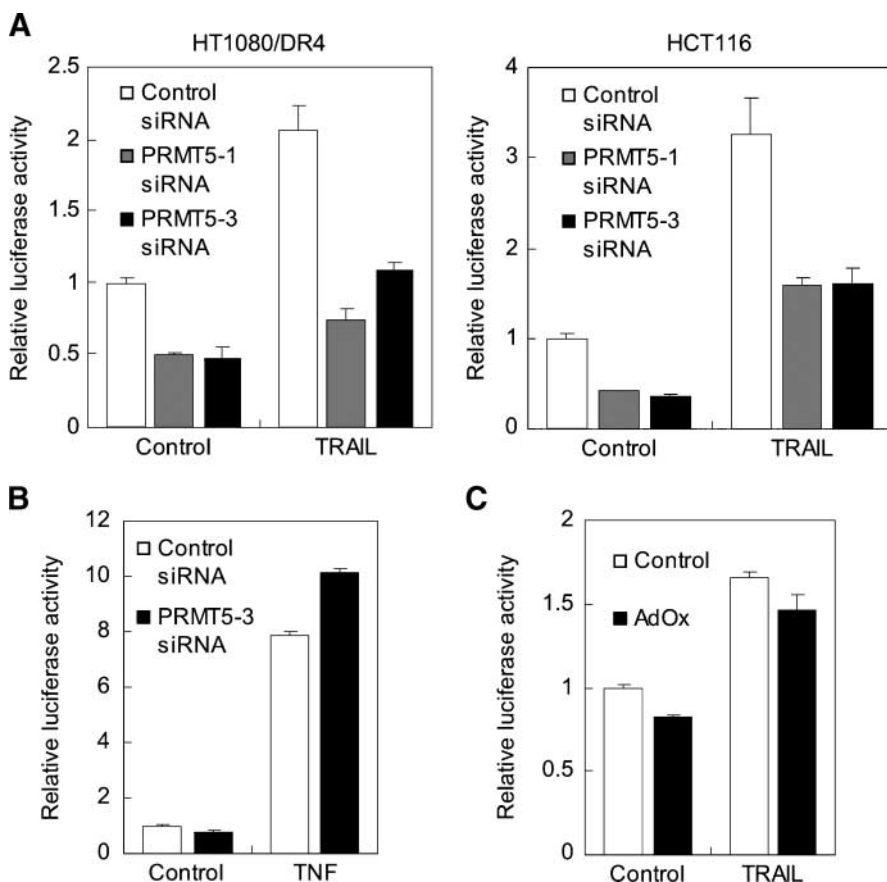


FIGURE 5. TRAIL-induced NF- κ B activation is dependent on PRMT5. HT1080/DR4 (**A**, *left*, and **B**) or HCT116 (**A**, *right*) cells were transfected with the indicated siRNA. After transfection for 24 h, cells were then transfected with pNF- κ B-Luc and pRL-TK plasmids. After a further 24 h incubation, cells were treated with 100 ng/mL TRAIL (**A**) or 20 ng/mL TNF- α (**B**) for 4 h. Luciferase activities were measured as described in Materials and Methods. **C.** HT1080/DR4 cells were transfected with pNF- κ B-Luc and pRL-TK plasmids. Four hours later, cells were treated with the 50 μ M methyltransferase inhibitor periodate-oxidized adenosine (AdOx) for 44 h. Then, cells were treated with 100 ng/mL TRAIL for 4 h. Luciferase activities were measured. Bars, SD of triplicate experiments (**A-C**).

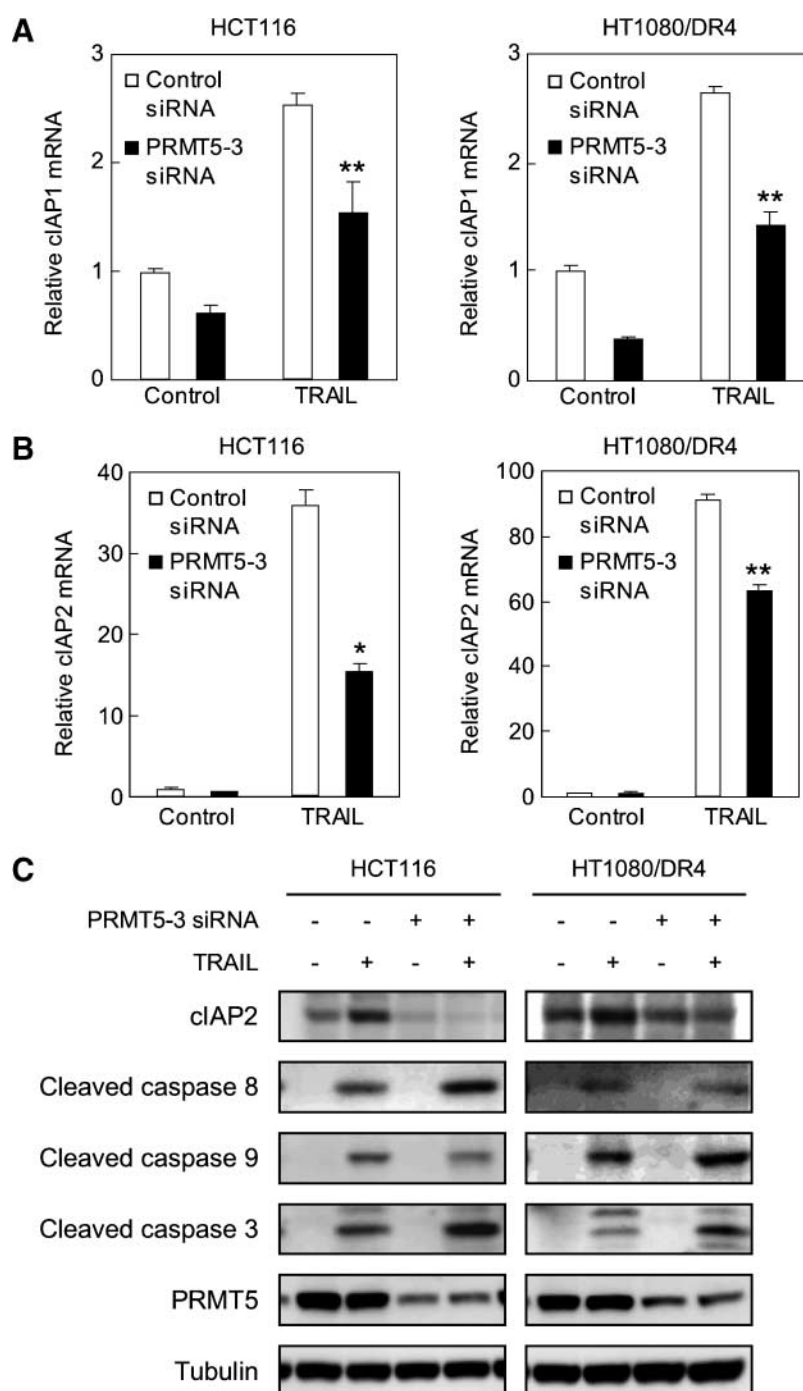


FIGURE 6. PRMT5-mediated transcription of NF- κ B target genes. **A** to **C**. HCT116 (*left*) or HT1080/DR4 (*right*) cells were transfected with the indicated siRNA and then treated with 100 ng/mL TRAIL for 3 h. Total RNA was separated and analyzed using quantitative RT-PCR (**A** and **B**) as described in Materials and Methods. Levels of *cIAP1* (**A**) and *cIAP2* (**B**) mRNA were normalized to the level of *GAPDH* mRNA. *, $P < 0.005$; **, $P < 0.0005$, compared with control siRNA-transfected and TRAIL-treated cells. Bars, SD of triplicate experiments (**A** and **B**). Cell lysates were immunoblotted with the indicated antibodies (**C**).

Protein expression levels of PRMT1 and PRMT4 were reduced by PRMT5-3 to some extent but not by PRMT5-1 siRNA (Fig. 2A; Supplementary Fig. S1E). However, PRMT5-1 siRNA showed identical results to PRMT5-3 siRNA in Figs. 2B, 4B, and 5A. In addition, the DR4-binding domain in PRMT5 does not have homology with other members of PRMT family as mentioned above. The effects of PRMT5 siRNA, therefore, results from the PRMT5 knockdown. We confirmed that both siRNAs had no homology with other PRMT members.

Although methylation of substrates is important for PRMT5 functions, PRMT5 can act in either a methyltransferase-dependent or a methyltransferase-independent manner. As a component of the androgen receptor cofactor complex, PRMT5 enhances androgen receptor-driven transcription independent of its methyltransferase activity (38). In our study, catalytically inactive PRMT5 bound to DR4 and reduced sensitivity to TRAIL (Figs. 1F and 3D). PRMT5 knockdown, but not the methyltransferase inhibitor, suppressed TRAIL-induced NF- κ B transcriptional activity (Fig. 5A and C). Therefore,

PRMT5 has nonenzymatic functions involved in TRAIL signaling.

The association between PRMT5 and tumor has been reported (30). Several genes, including suppressor of tumorigenicity 7, were derepressed in PRMT5 antisense-expressing cells. In contrast, PRMT5-overexpressing cells showed reduced expression of these genes, elevated methylation in histone, and increase in transformation. These findings by Pal et al. suggest that PRMT5 regulates cell proliferation by controlling expression of genes involved in tumor suppression. These in-

vestigators recently revealed that low levels of particular micro-RNA induce PRMT5 translation in human lymphoma (29). PRMT5 proteins were elevated in lymphoma cells over normal B lymphocytes. Decrease in miR-92b and miR-96 augmented PRMT5 translation in lymphoma, leading to increased methylation of histone and repressed expression of suppressor of tumorigenicity 7. Knockdown of PRMT5 inhibited proliferation of lymphoma. miR-96 was, however, independently reported to attenuate TRAIL-induced caspase-3 activation (39). Because caspase-3 and FADD are putative targets of miR-96, miR-96

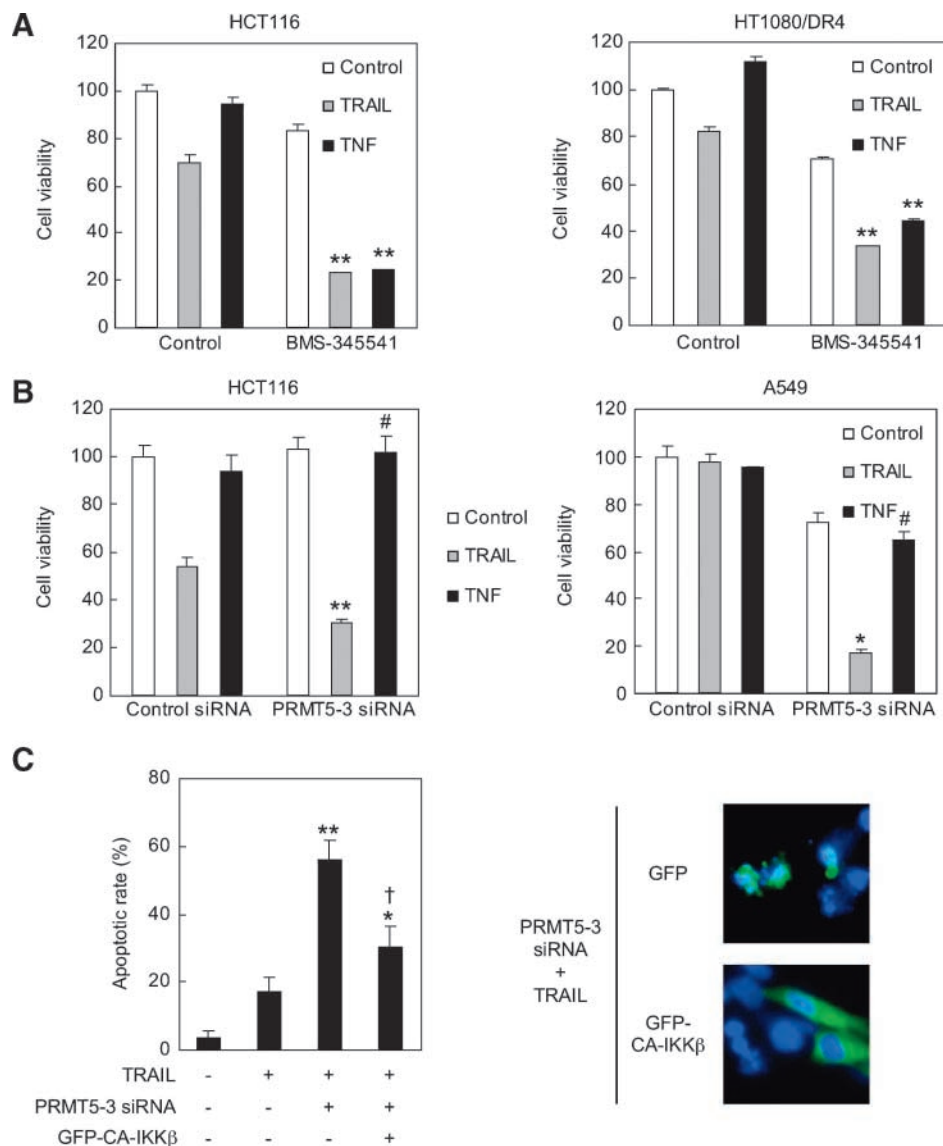


FIGURE 7. PRMT5 contributes to TRAIL resistance via NF- κ B activation. **A.** HCT116 (left) or HT1080/DR4 (right) cells were treated with 10 ng/mL TRAIL or 20 ng/mL TNF- α in the presence or absence of the 10 μ M/L IKK inhibitor BMS-345541. Cell viability was evaluated using the MTS assay. **, $P < 0.001$, compared with TRAIL-untreated or TNF-untreated and BMS-345541-treated cells. **B.** HCT116 (left) or A549 (right) cells were transfected with the indicated siRNA and then treated with 10 ng/mL TRAIL or 100 ng/mL TNF- α . Cell viability was evaluated using the MTS assay. *, $P < 0.05$; **, $P < 0.001$; #, $P > 0.05$, compared with TRAIL or TNF-untreated and PRMT5-3 siRNA-transfected cells. **C.** HT1080 cells were transfected with control (-) or PRMT5-3 (+) siRNA. After transfection for 24 h, cells were also transfected with pEGFP-C1 (-) or pEGFP-C1-CA-IKK β (+). After additional incubation for 24 h, cells were treated with 100 ng/mL TRAIL for 6 h. Cells were fixed and stained with Hoechst 33342. Cellular images were visualized using a fluorescence microscope. The rate that apoptotic cells showed condensed and fragmented nuclei was determined by counting >100 GFP-positive cells (left). Green, GFP proteins; blue, nuclei (right). *, $P = 0.037$; **, $P = 0.00061$, compared with control siRNA-transfected and TRAIL-treated cells, respectively; †, $P = 0.0054$, compared with IKK β -untransfected, PRMT5-3 siRNA-transfected and TRAIL-treated cells. Bars, SD of triplicate experiments (**A-C**).

may reduce caspase-3 activity. These studies indicate difficulty in combining TRAIL with PRMT5 targeting therapy using microRNA.

PRMT5 proteins exist in both cytoplasmic and nuclear compartments of cells. However, most studies have focused on the histone methyltransferase activity of PRMT5. Our finding that PRMT5 functions as a binding partner of the transmembrane receptors sheds light on novel roles of cytoplasmic PRMT5. Some types of testicular tumor showed marked increases in cytoplasmic expression of PRMT5 and the androgen receptor coactivator p44, with a concomitant decrease in nuclear expression of those proteins (40). Similar distributional change of PRMT5 was reported in cells treated with AS1411, an oligonucleotide aptamer targeting nucleolin, which is a substrate for PRMT5 (41). These cells with cytoplasmic distribution of PRMT5 might show altered sensitivity to TRAIL.

Requirement of caspase activation for TRAIL-induced apoptosis has been extensively described (8). However, TRAIL-activated NF- κ B promotes expression of IAPs, which inhibit caspases. We observed that IAP induction diminished with concomitant caspase activation in PRMT5-depleted cells (Fig. 6). Mismatch between messenger and protein levels of cIAP2 suggest that PRMT5 may have an effect on cIAP2 translation. This result suggests that PRMT5-mediated IAP expression is involved in TRAIL-induced apoptosis. Ricci et al. reported that reduction of TRAIL-induced cIAP2 by sorafenib treatment sensitizes cancer cells to TRAIL (20). Sorafenib is a multikinase inhibitor approved for treatment of advanced renal cell carcinoma and reported to potentiate cytotoxicity of TRAIL (42). Because PRMT5 siRNA shows similarity to sorafenib in TRAIL signaling, targeting PRMT5 may have therapeutic potential in combination with TRAIL.

Materials and Methods

Reagents and Cell Culture Conditions

Recombinant human soluble KillerTRAIL, soluble TNF- α , and histone H2A were purchased from Alexis Biochemicals, Wako Pure Chemical, and Upstate, respectively. Z-VAD-FMK was obtained from Peptide Institute. Periodate-oxidized adenosine and BMS-345541 were obtained from Sigma.

Human cervical cancer HeLa and normal lung fibroblast WI-38 and TIG3 cells were cultured in DMEM (Nissui) supplemented with 10% fetal bovine serum (Japan Bioserum). Human colon cancer HCT116, lung cancer A549, and fibrosarcoma HT1080 cells were cultured in RPMI 1640 (Wako) supplemented with 10% fetal bovine serum.

Plasmid Construction

Human *DR4* and *DR5* cDNA were generated by PCR with the MGC clones (ID 3857315 and 3458466, respectively; Open Biosystems) and subcloned into pcDNA3.1-V5/His (Invitrogen) and pFLAG-CMV-5c (Sigma) vectors. Human *TNFR1* and *Fas* cDNA were generated with the HeLa cDNA library (Stratagene) and then were subcloned into a pFLAG-CMV-5c vector.

Human *PRMT5* cDNA was generated with the MGC clone (ID 3833019) and subcloned into a pcDNA3 vector (Invitrogen) with a Myc tag sequence. Substitutions of appropriate codons for the Ala codon GCA or stop codon TAG in the

pcDNA3-Myc-*PRMT5* to generate the MD mutant (G367A), COOH-terminal deletion mutants, and internal deletion mutants were accomplished using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The NH₂-terminal deletion mutants were generated by PCR with *PRMT5* cDNA.

Constitutively active human *IKK β* cDNA (S177E/S181E) in a pEGFP-C1 vector (Takara Bio) and human *I κ B α* cDNA in a pGEX-6p-3 vector (GE Healthcare) have been previously established in our laboratory (43).

siRNA Design

Stealth RNAi oligonucleotides were designed by BLOCK-iT RNAi Designer (Invitrogen). The following siRNA pairs directed to nucleotides 937 to 961 and 1,655 to 1,679 in the open reading frame of *PRMT5* were used: PRMT5-1-sense (973-961) CAGCCACTGATGGACAATCTGGAAT and PRMT5-3-sense (1,655-1,679) CCGGCTACTTTGAGACTGTGCTTTA. Stealth RNAi Negative Control Medium GC Duplex (Invitrogen) was used as negative control. Cells were transfected for 48 or 72 h using Lipofectamine RNAiMAX (Invitrogen).

Transient and Stable Transfection

Cells were transfected with appropriate plasmids for 24 h using Lipofectamine 2000 (Invitrogen) for HeLa cells, FuGENE 6 (Roche Applied Science) for HT1080 cells, or FuGENE HD (Roche) for HCT116 cells. A stable transfectant clone of HT1080 or clones of HCT116 were selected by cultivating them in medium containing 400 or 1,000 μ g/mL G418 (Sigma), respectively.

Purification of Recombinant Proteins

Cultures of BL21 Star *Escherichia coli* (Invitrogen) containing pGEX-6P-3-*I κ B α* were incubated for 2 h with 0.1 mmol/L IPTG (Wako) at 30°C. The cells were solubilized in a lysis buffer [20 mmol/L NaH₂PO₄ (pH 8.0), 150 mmol/L NaCl, 20% glycerol, and 1 mg/mL lysozyme VI (ICN)] using a freeze-thaw cycle. GST-tagged proteins were purified using glutathione Sepharose 4B (GE Healthcare) and eluted with 10 mmol/L glutathione in 50 mmol/L Tris-HCl (pH 8.0), dialyzed with TBS, and concentrated.

Immunoprecipitation and Immunoblot Analysis

Cells were solubilized in the previously described lysis buffer (13) containing 1 mmol/L phenylmethylsulfonyl fluoride, 15 μ g/mL aprotinin, and 1% phosphatase inhibitor cocktails 1 and 2 (Sigma). Tagged proteins were immunoprecipitated with anti-FLAG (M2; Sigma), anti-V5 (V5-10; Sigma), or anti-Myc (9E10; Santa Cruz Biotechnology) agarose. In some experiments, cell lysates were incubated with protein G Sepharose (Zymed) conjugated with control rabbit IgG or an anti-DR4 antibody (H-130; Santa Cruz Biotechnology), control rabbit IgG-conjugated agarose (Santa Cruz Biotechnology), or anti-NEMO-conjugated agarose (FL-419; Santa Cruz Biotechnology).

Then, the immunoprecipitated proteins or cell lysates were electrophoresed and immunoblotted as described previously (44, 45). We used primary antibodies to FLAG tag (M2; Sigma); Myc tag (9E10; Roche); PRMT2 (Aviva Systems Biology); PRMT3 or PRMT5 (Upstate); DR4 or DR5 (CT; ProSci); PRMT6 (Imgenex); α -tubulin (YL1/2; AbD Serotec);

actin (C-2), caspase-3 (H-277), I κ B α (C-21), IKK α / β (H-470), GST tag (B-14), NEMO (FL-419), or p65 (C-20; Santa Cruz Biotechnology); cIAP2 (F30-2285) or FADD (ref. 1; BD Biosciences); V5 tag (Invitrogen); or PRMT1, PRMT4, caspase-8 (1C12), cleaved caspase-9 (Asp³³⁰), phospho-Ser³²-I κ B α , phospho-Ser³²/Ser³⁶-I κ B α (5A5), or phospho-Ser⁵³⁶-NF- κ B p65 (7F1; Cell Signaling Technology).

Proteomic Screening of DR4-Binding Proteins

Cells were solubilized in the lysis buffer. DR4-containing complexes were immunoprecipitated with anti-FLAG agarose and eluted with 200 ng/mL of the 3 \times FLAG peptide (Sigma) in 10 mmol/L imidazole-containing buffer A [0.1% Triton X-100, 50 mmol/L NaH₂PO₄ (pH 8.0), 300 mmol/L NaCl]. The eluates were then applied to nickel nitrilotriacetic acid agarose (Qiagen). After a wash with 20 mmol/L imidazole-containing buffer A, bound proteins were eluted with 250 mmol/L imidazole-containing buffer A without Triton X-100. The purified proteins were subjected to SDS-PAGE and the gel was stained with the SilverQuest Silver Staining Kit (Invitrogen). Liquid chromatography-tandem mass spectrometry analysis was done as described previously (46).

In vitro Methyltransferase Assay

Immunoprecipitated Myc-tagged PRMT5 proteins were reacted with 2 μ g histone H2A with 5 μ L S-adenosyl-L-methionine (37 MBq/mL; GE Healthcare) in 30 μ L PBS for 2 h at 37 $^{\circ}$ C. Subsequently, the reactions were subjected to SDS-PAGE. The ³H-labeled proteins were detected using BAS-TR2040 (Fujifilm) and Typhoon (GE Healthcare).

In vitro Kinase Assay

Endogenous IKK complexes were immunoprecipitated with anti-NEMO antibody-conjugated agarose. The immunoprecipitated proteins were incubated with 0.5 μ g recombinant GST-I κ B α in HEPES-buffered saline containing the 20% magnesium/ATP cocktail (Upstate) for 1 h at 30 $^{\circ}$ C. Reactions were immunoblotted.

Cell Viability and Apoptosis Assay

To assess cell viability, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega) assay was employed. Cells were treated with TRAIL or TNF- α for 24 h. Then, the cells were incubated with MTS for 2 h, and the absorbance was measured at 490 nm, with a reference at 690 nm, using Benchmark Plus (Bio-Rad).

To assess apoptosis, phycoerythrin-conjugated recombinant Annexin V binding was measured using the Annexin V-phycoerythrin apoptosis detection kit I (BD Biosciences). The cells were transfected with siRNA for 48 h, treated with 100 ng/mL TRAIL for 24 h, and stained with Annexin V-phycoerythrin. The fluorescence was analyzed using the Cytomics 500 flow cytometer and Cytomics RXP software (Beckman Coulter).

To visualize apoptotic cells that showed nuclear condensation and fragmentation, HT1080 cells were transfected with control or PRMT5-3 siRNA. The following procedures have been described previously (43).

Reporter Assay

Cells were transfected with various plasmids and a pNF- κ B-Luc plasmid (Takara) together with a phRL-CMV plasmid (Promega) as the internal control. Twenty-four or 48 hours later, the cell extracts were assayed for luciferase activity using the Pikka Gene Dual Kit (Toyo Ink). Luciferase activity was measured using LB 960 Microplate Luminometer Centro (Berthold).

Quantitative Reverse Transcription-PCR

RNA was purified from cells using the RNeasy Mini Kit (Qiagen) and subjected to reverse transcription using SuperScript III (Invitrogen). Real-time PCR was done using LightCycler 480 Probes Master, Universal ProbeLibrary, and LightCycler 480 (Roche). Quantitative PCR Human Reference Total RNA was obtained from Stratagene. Primers for *cIAP1* are GAAAATGCTGACCCACCAAT and GGCAGATT-TAACCACAGGTGTA, and probe sequence is CCTGGAGA. Primers for *cIAP2* are CTTGTCCTTGCTGGTGCAT and AA-GAAGTCGTTTTCTCCTTTGT, and probe sequence is TGGGCAGC. Primers for *GAPDH* are AGCCACATCGCT-CAGACAC and GCCCAATACGACCAAATCC, and probe sequence is TGGGGAAG. Levels of *cIAP1* and *cIAP2* mRNA were normalized to the level of *GAPDH* mRNA.

Detection of Cell Surface Receptors

Cells were labeled with mouse anti-DR4 (HS101; Alexis) or anti-DR5 (HS201; Alexis) antibodies in HBSS supplemented with 14 mmol/L HEPES and 2% fetal bovine serum followed by incubation with Alexa Flour 488-conjugated goat anti-mouse IgG (Invitrogen). The fluorescence was analyzed using Cytomics 500 and Cytomics RXP.

Statistical Analysis

Each *P* value was calculated with Student's or Welch's *t* test after *F* test for equal variances.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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