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The Human Tumor Antigen PRAME Is a Dominant Repressor of Retinoic Acid Receptor Signaling

Mirjam T. Epping,[1,3] Liming Wang,[1,3,4] Michael J. Edel,[1] Leone Carlée,[1] Maria Hernandez,[2] and René Bernards[1,*]
[1]Division of Molecular Carcinogenesis and Center for Biomedical Genetics
[2]Division of Molecular Genetics
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

Summary

Retinoic acid (RA) induces proliferation arrest, differentiation, and apoptosis, and defects in retinoic acid receptor (RAR) signaling have been implicated in cancer. The human tumor antigen PRAME is overexpressed in a variety of cancers, but its function has remained unclear. We identify here PRAME as a dominant repressor of RAR signaling. PRAME binds to RAR in the presence of RA, preventing ligand-induced receptor activation and target gene transcription through recruitment of Polycomb proteins. PRAME is present at RAR target promoters and inhibits RA-induced differentiation, growth arrest, and apoptosis. Conversely, knockdown of PRAME expression by RNA interference in RA-resistant human melanoma restores RAR signaling and reinstates sensitivity to the antiproliferative effects of RA in vitro and in vivo. Our data suggest that overexpression of PRAME frequently observed in human cancers confers growth or survival advantages by antagonizing RAR signaling.

Introduction

The family of nuclear receptors (NR) consists of ligand-regulated transcription factors that control a wide range of physiological processes in development, differentiation, and homeostasis. Nuclear receptors are composed of a series of conserved domains (named A-F). Their modular structure reveals distinct functional domains, including an N-terminal activation function 1 (AF-1), the DNA binding domain, the hinge region, and a C-terminal ligand binding domain (LBD), which contains a ligand-dependent AF-2 domain (Freedman, 1999). Nuclear receptor function requires receptor dimerization, and based on the mode of dimerization, two subtypes of nuclear receptors can be distinguished. The steroid hormone receptors (class I) form functional homodimers and include estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR). The class II nuclear receptors, such as retinoic acid receptor (RAR), vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), and thyroid hormone receptor (T3R) heterodimerize with a common partner, retinoid X receptor (RXR) (McKenna and O'Malley, 2002).

Several coregulators control the transcriptional activity of nuclear receptors in a ligand-dependent fashion (Xu et al., 1999). Receptors for retinoic acid and thyroid hormone are potent repressors in the absence of ligand, while they function as activators of transcription upon binding of their cognate ligands. Upon ligand binding to these receptors, the conformational change in the LBD induces corepressors to dislodge and coactivators to bind, allowing transactivation (Xu et al., 1999). NCoR and SMRT are corepressors that associate with RAR and T3R in the absence of ligand and mediate transrepression by recruitment of histone deacetylase (HDAC) complexes (Alland et al., 1997; Chen and Evans, 1995; Heinzel et al., 1997; Horlein et al., 1995). In contrast, coactivators including CBP/p300, PCAF, and members of the p160 family (SRC1, TIF-2/GRIP1, and ACTR/RAC3/AIB1) possess intrinsic histone acetyltransferase (HAT) activity and potentiate the transcriptional activity of ligand bound receptors (Anzick et al., 1997; Chen et al., 1997; Hong et al., 1997; Onate et al., 1995; Voegel et al., 1996). The proteins RIP140 and LCoR form a distinct group of coregulators by conferring transcriptional repression to ligand bound nuclear receptors (Cavailles et al., 1995; Fernandes et al., 2003). These ligand-dependent coregulators recruit HDACs to nuclear receptors to attenuate their activity.

Many ligand-dependent modulators of nuclear receptors contain nuclear receptor (NR) boxes, which are leucine-rich motifs with an LXXLL consensus sequence that mediates interactions with the AF-2 domain of the nuclear receptors (Heery et al., 1997; Torchia et al., 1997). These modulators include many coactivators, like SRC1 (Onate et al., 1995), and the corepressors RIP140 (Cavailles et al., 1995) and LCoR (Fernandes et al., 2003).

Signaling through RAR and activation of RAR target genes induce proliferation arrest, differentiation, and apoptosis in a wide variety of cell types. Retinoids have tumor-suppressive activity, and consequently, defects in RAR signaling are implicated in cancers (Altucci and Gronemeyer, 2001; Freemantle et al., 2003). In acute promyelocytic leukemia, translocations of RARα give rise to the PML-RARα and PLZF-RARα chimearic genes, which results in functionally altered receptors that act as constitutive repressors of transcription, thereby preventing cell differentiation (Grignani et al., 1998; He et al., 1998; Lin et al., 1998). Loss of RARβ expression is involved in the progression of a diverse range of solid tumors, including breast and lung carcinomas (Altucci and Gronemeyer, 2001; Freemantle et al., 2003).

We describe here a function of a gene named *PRAME* (*pr*eferentially expressed *a*ntigen in *me*lanoma). PRAME was first described as an antigen in human melanoma, which triggers autologous cytotoxic T cell-mediated immune responses (Ikeda et al., 1997). Interestingly,

*Correspondence: r.bernards@nki.nl
[3]These authors contributed equally to this work.
[4]Present address: Urology Department of Changzheng Hospital, Fengyang Road 415, 200003 Shanghai, P.R. of China.

PRAME expression is retained in the presence of antitumor T cell responses, suggesting that expression of PRAME confers to tumor cells a selective advantage that outweighs the CTL-mediated tumor cell killing (Ikeda et al., 1997). This in turn suggests that *PRAME* overexpression is causally involved in the tumorigenic process. Consistent with this, the frequency of *PRAME* expression in melanoma is 88% in primary melanomas and 95% in metastases (Ikeda et al., 1997). PRAME is not expressed in normal skin and nevi (moles) but is highly expressed in melanomas (Haqq et al., 2005). Furthermore, PRAME is overexpressed in a variety of other human malignancies, including acute and chronic leukemias, medulloblastoma, non-small cell lung carcinoma, head and neck cancer, renal carcinoma, multiple myeloma, and sarcomas (Boon et al., 2003; Ikeda et al., 1997; Oberthuer et al., 2004; van Baren et al., 1998; van't Veer et al., 2002). Importantly, high *PRAME* expression is an independent prognostic marker of poor clinical outcome in breast cancer and neuroblastoma (Oberthuer et al., 2004; van't Veer et al., 2002). High PRAME expression inversely correlates with recurrence-free survival (no metastases) and overall survival in breast cancer (van't Veer et al., 2002). As expression of *PRAME* is low or absent in almost all normal adult tissues except for testis (Ikeda et al., 1997), its specific selection in a variety of tumor types has remained unexplained, as no function for PRAME has been described to date. In the current study, we addressed the function of PRAME and found an unexpected role for PRAME in suppression of retinoic acid signaling.

Results

PRAME Is a Transcriptional Repressor of RAR

To address the function of PRAME in oncogenesis, we searched for conserved motifs and functional domains in the protein. We found that PRAME contains, apart from a nuclear localization signal, seven putative nuclear receptor (NR) boxes, having the LXXLL consensus sequence (Heery et al., 1997; Torchia et al., 1997; Figure 1A). The presence of NR boxes suggested that PRAME could function as a modulator of nuclear receptor signaling. To test if PRAME can act as a transcriptional coactivator or corepressor, a full-length PRAME cDNA was fused to a heterologous DNA binding domain (DBD) of the yeast transcription factor Gal4 and cotransfected with a Gal4-luciferase reporter. Gal4-PRAME caused a dose-dependent inhibition of reporter gene expression, whereas free PRAME had no effect on the Gal4 promoter (data not shown), indicating that PRAME mediates transcriptional repression (Figure 1B). To investigate if PRAME affected transactivation mediated by nuclear receptors, we used luciferase reporter constructs for several class I and class II receptors. These experiments revealed that PRAME inhibited RA-induced activation of a reporter gene driven by retinoic acid-responsive elements (RARE-luciferase) in a concentration-dependent manner (Figure 1C). PRAME cotransfection did not affect estrogen receptor (ER)-driven reporter activation (Figure 1D) nor activation by the other class I nuclear receptors tested, such as the progesterone receptor (PR, Figure 1E) or androgen receptor (data not shown). Moreover, there was no effect of PRAME on ligand-induced transactivation of the class II receptor PPARγ (Figure 1F), indicating that PRAME is not a general inhibitor of class II nuclear receptors. To test if PRAME affected RAR signaling through its heterodimerization partner RXRα, PRAME was cotransfected with RXRα and a RXRα-responsive reporter. Figure 1G shows that PRAME did not affect activation of RXRα by its ligand, 9-cis-RA. To investigate if PRAME can act on all RAR isoforms, we cotransfected PRAME together with RARβ or RARγ and found that PRAME also inhibited signaling through these two RAR isoforms (Figure 1H). These experiments indicate that PRAME is selective in its functional interactions with nuclear receptors and, of the nuclear receptors tested, only interfered with RAR transactivation (Figures 1C–1H). Similarly, PRAME repressed RAR signaling in B16 mouse melanoma cells (Figure S1 available with this article online). However, these data do not exclude the possibility that PRAME can modulate the activity of other nuclear receptors.

Interaction of PRAME with RARα In Vitro and In Vivo

The presence of putative NR boxes in PRAME suggests that PRAME can form direct physical complexes with nuclear receptors to modulate receptor function (Heery et al., 1997; Torchia et al., 1997). Interactions of PRAME with nuclear receptors were demonstrated in GST pull-down experiments using GST fusions of the LBDs of RARα, RXRα, and ERα and in vitro-translated PRAME. The C terminus of PRAME (416–509) associated with GST-RARα-LBD in the presence of ligand but did not bind to GST-RXRα-LBD and GST-ERα (Figure 2A). Concurrently, heterodimerization between GST-RXRα and RARα was shown and ERα was bound to GST-ERα in the presence of estradiol, indicating proper folding of the expressed proteins. Conversely, a GST-PRAME (416–509) fusion protein interacted with in vitro-translated RARα only in the presence of all-trans-RA, but no RXRα was bound to this fragment of PRAME in the presence or absence of its ligand 9-cis-RA (Figure 2B).

Interaction of PRAME with RAR was further probed by mammalian two-hybrid analysis. We used VP16 transactivation domain (TAD) fusion proteins with RARα-LBD or RXRα-LBD together with a Gal4-DBD-PRAME (416–509) and a Gal4-luciferase reporter gene as a readout for interaction (Figure 2C). Association of Gal4-PRAME (416–509) with VP16-RARα was apparent from the two-hybrid signal when both fusion proteins were coexpressed in the presence of ligand, but there was no reporter activation by either construct alone (Figure 2D). Consistent with the GST pull-down assay, no interaction was found between Gal4-PRAME (416–509) and VP16-RXRα, again indicating that the C terminus of PRAME interacts with the LBD of RARα but not with the LBD of RXRα (Figure 2D). Moreover, coexpression of Gal4-PRAME (416–509) and VP16-RXRα resulted in activation of transcription only in the presence of both RARα and RA, demonstrating a requirement for RAR in the effects mediated by PRAME (Figure 2D). In a mammalian two-hybrid assay, Gal4-RARα interacted with VP16-RXRα in the presence of PRAME, reflecting that receptor heterodimerization was not hampered by PRAME in vivo (Figure S1). Together, these data sug-

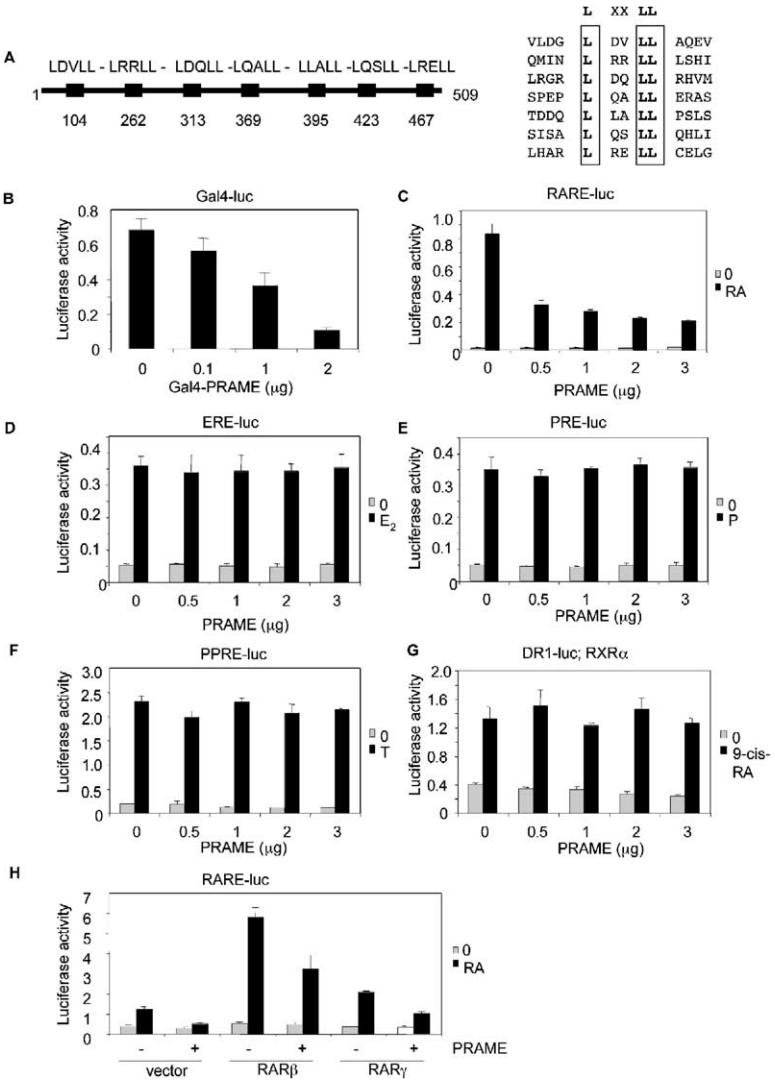

Figure 1. PRAME Is a Repressor of RAR Signaling

(A) Schematic representation of PRAME. Amino acid residue numbers of the seven putative NR boxes (LXXLL motifs) and adjacent amino acids are indicated.

(B) PRAME confers transcriptional repression to a reporter construct. A Gal4-PRAME fusion protein inhibits transcription from a Gal4-luciferase reporter in HEK293 cells.

(C–G) PRAME represses RAR-dependent transactivation. The effects of PRAME on nuclear receptor transactivation were tested in cotransfection experiments using expression vectors for the receptors and luciferase reporters for the nuclear receptors RARα (RARE-luc, where RARE is for RA-responsive element), estrogen receptor (ERE-luc), progesterone receptor (PRE-luc), PPARγ (PPRE-luc), and RXRα (DR1-luc). Cells were treated with 1 μM of hormones (RA, all-trans RA; E_2, estradiol; P, progesterone; T, Troglitazone; 9-cis-RA) for 24 hr prior to measurements.

(H) PRAME represses signaling through RARβ and RARγ. MCF7 cells were cotransfected with expression vectors for RARβ, RARγ, and a specific shRNA to repress endogenous RARα to measure the effects of PRAME on RARβ and RARγ signaling.

gest that the association between RAR and PRAME is the result of a direct and ligand-dependent physical interaction.

To ask if PRAME binds RAR in vivo, we stably expressed a TAP-tagged (Rigaut et al., 1999) PRAME in mouse embryonic fibroblasts (MEFs) to levels comparable to those of endogenous PRAME in the human melanoma cell lines SK23 and A375 (Figure 3A). Endogenous RARα coimmunoprecipitated with TAP-PRAME, indicating that PRAME and RARα form a complex at protein concentrations seen in human tumor cell lines (Figure 3B). Formation of the PRAME-RAR complex in vivo was also dependent on the presence of RA, showing that PRAME, like other proteins that harbor NR box motifs, forms a ligand-regulated complex with RAR (Figure 3C).

PRAME Interacts with RAR through a Nuclear Receptor Box Motif

The presence of NR boxes in PRAME suggests that the interaction between PRAME and RARα takes place via one or more of these motifs. To test if the NR boxes of PRAME are required for binding to RAR and inhibition of RAR signaling, point mutations were introduced in each of the seven LXXLL motifs in PRAME by changing conserved leucine (L) residues into valines (V). The resulting PRAME mutants were named after the respective NR boxes that were mutated. One additional mutant was made in which all seven LXXLL motifs were mutated, referred to as PRAME-ΔLXXLL. Six out of seven PRAME single NR box mutants inhibited RAR signaling to a similar extent as wild-type PRAME, except for the PRAME-LREVV mutant (Figure 3E). This mutant was as defective in repressing RAR signaling as the PRAME mutant in which all seven NR boxes were mutated (Figure 3E). Consistent with this observation, endogenous RARα failed to coimmunoprecipitate with a TAP-PRAME-LREVV mutant protein (Figure 3D). Gal4-PRAME-LREVV inhibited reporter activity to a similar extent as Gal4-PRAME, indicating that repression was not affected by the LREVV mutation (Figure S1). In a mammalian two-hybrid assay, Gal4-PRAME (416–509) containing the wild-type LRELL NR box interacted with VP16-RARα, but introduction of the LREVV mutation in

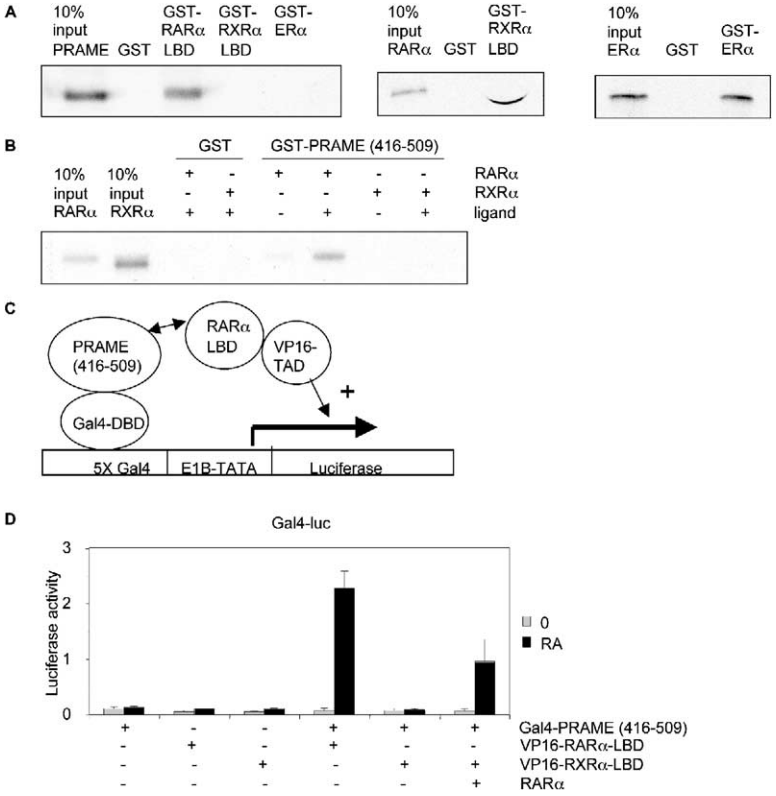

Figure 2. Direct Interaction of PRAME with RARα

(A) In vitro binding of PRAME to RARα. GST pull-down assay showing the interaction of in vitro-translated ^{35}S-labeled PRAME (416–509) with GST-RARα-LBD but not GST-RXRα-LBD and GST-ERα (left panel) in the presence of ligand (1 μM all-trans-RA, 9-cis-RA, or estradiol, respectively). The same protein preparations were used to demonstrate interactions between GST-RXRα-LBD and in vitro-translated RARα (middle panel) and GST-ERα and in vitro-translated ERα (right panel) in the presence of ligand (1 μM all-trans-RA or estradiol, respectively).

(B) Ligand-dependent interaction of PRAME and RARα. GST pull-down assay showing a ligand-dependent interaction of in vitro-translated ^{35}S-labeled RARα but not RXRα with GST-PRAME (416–509). Ligand (1 μM) was all-trans-RA for RARα and 9-cis-RA for RXRα.

(C) Schematic representation of the mammalian two-hybrid assays in Figures 2D and 3F (DBD, DNA binding domain; LBD, ligand binding domain; TAD, transactivation domain).

(D) Mammalian two-hybrid assay for binding of PRAME to RARα. Coexpression of Gal4-PRAME (416–509) with VP16-RARα-LBD but not VP16-RXRα-LBD results in activation of a Gal4-driven luciferase reporter in the presence of ligand (1 μM all-trans-RA and 9-cis-RA).

PRAME disrupted the association (Figure 3F). Taken together, these data suggest that an intact LRELL motif in PRAME is required for binding to RARα and repression of RAR signaling.

PRAME Inhibits RA-Induced Differentiation, Proliferation Arrest, and Apoptosis

RA induces proliferation arrest, differentiation, and apoptosis in many cell types, including F9 mouse embryonic carcinoma cells (Strickland and Mahdavi, 1978). We asked if PRAME expression affected the RA-induced differentiation of F9 cells toward parietal endoderm. F9 cells were stably transfected with PRAME, PRAME-LREVV, or control vector and differentiated in 10^{-7}M RA. In the absence of RA, the morphology of all transfected cells was the same as that of parental F9 cells (Figure 4Aa–4Ac). F9 cells expressing PRAME were resistant to RA-induced morphological differentiation, whereas PRAME-LREVV and vector control transfected cells underwent differentiation (Figure 4Ad–4Af). Undifferentiated F9 cells express Stage-Specific Embryonic Antigen-1 (SSEA-1), which is a marker of stem cells and embryonic carcinoma cells (Solter and Knowles, 1978). F9 cells were stained for SSEA-1 before and after treatment with RA. In agreement with earlier studies (Solter and Knowles, 1978), we found that SSEA-1 expression was lost upon differentiation. Vector-control and PRAME-LREVV F9 cells completely lost SSEA-1 expression, whereas a significant fraction of PRAME F9 cells retained expression of the marker (Figure S2). Parietal endoderm-like F9 cells show coordinated expression and secretion of basement-membrane components, including laminin-1 and collagen IV (Strickland et al., 1980). Conditioned medium of F9 cells contained laminin-1 only after RA treatment, but PRAME-expressing F9 cells secreted less laminin-1 than vector and PRAME-LREVV F9 cells (Figure S2). F9 cells expressing PRAME showed enhanced proliferation compared to PRAME-LREVV and vector controls in standard culture medium (which contains trace amounts of RA), and this difference was more pronounced when the culture medium was supplemented with exogenous RA (Figure 4C). The *RARβ* and the CDK-inhibitor *p21^{cip1}* genes are RA inducible and contain RA-responsive elements in their respective promoters (de The et al., 1990; Liu et al., 1996). Consistent with this, endogenous RARβ and p21 protein expression levels are induced by RA in vector control and PRAME-LREVV-transfected F9 cells but not in PRAME-transfected F9 derivatives (Figure 4B). Furthermore, PRAME inhibited RA-induced activation of a RARβ2 promoter-luciferase reporter (R140-luc) but did not affect a RARβ2 reporter with a mutated RARE (M3M7-luc) (Figure 4D). PRAME-LREVV failed to repress the RARβ2 promoter reporter, consistent with a requirement for binding of PRAME to RARα through an intact LRELL motif to repress RARα transactivation (Figure 4D). Similarly, a p21 promoter-luciferase reporter was activated by RA, and this activation was suppressed by PRAME, but not by PRAME-LREVV expression (Figure 4E). Apart from differentiating, a fraction of F9 cells treated with RA dies by apoptosis (Atencia et al., 1994). PRAME expression in F9 cells conferred resistance to RA-induced apoptosis as activated, cleaved caspase 3 was apparent in vector controls but not in PRAME transfectants (Figure 4F). However, apoptosis was induced by UV irradiation in both PRAME and vector control F9 cells, indicat-

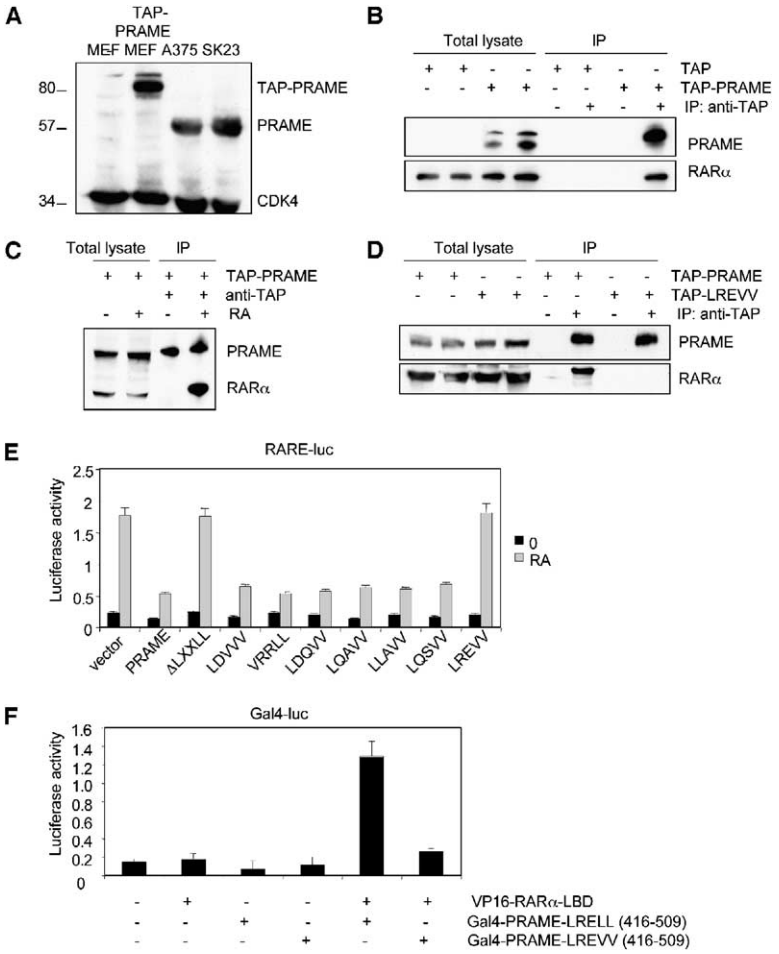

Figure 3. PRAME Interacts with RAR In Vivo through an LXXLL Motif

(A) Comparison of TAP-PRAME expression levels in MEFs to endogenous PRAME protein levels in human melanoma cell lines A375 and SK23. Cell extracts were analyzed by Western blotting using antibodies against PRAME and CDK4.

(B) Association of PRAME and RARα in MEFs. TAP and TAP-PRAME were immunoprecipitated (IP) (using IgG beads, indicated as anti-TAP) and immunocomplexes were analyzed by Western blotting with anti-PRAME and anti-RARα antibodies.

(C) Ligand-dependent binding of PRAME to RARα. TAP-PRAME was immunoprecipitated as in (B) in the absence or presence of RA.

(D) Immunoprecipitation of RARα and PRAME or PRAME-LREVV. TAP-PRAME and TAP-PRAME-LREVV were immunoprecipitated as in (B) and precipitates were immunoblotted for PRAME and RARα.

(E) Effects of PRAME NR box mutants on RAR signaling. MEFs were transfected with RARE-luc and PRAME or PRAME NR box mutants (see text for description) and treated with RA. In each PRAME mutant, one LXXLL motif was changed, except for PRAME-ΔLXXLL, which contained mutations in all seven NR boxes.

(F) Mammalian two-hybrid assay for binding of PRAME or PRAME-LREVV to RARα. The following constructs: Gal4-PRAME-LRELL (416–509) or Gal4-PRAME-LREVV (416–509) and VP16-RARα-LBD were coexpressed in the presence of RA.

ing that PRAME is not a general inhibitor of apoptosis (Figure 4F). Apoptosis was further confirmed by positive Annexin V staining in dying cells (Figure S2). We conclude that PRAME expression confers resistance to RA-induced proliferation arrest, differentiation, and apoptosis by repressing the expression of bona fide endogenous RAR target genes.

PRAME Interacts with Polycomb Group EZH2 to Repress RAR Signaling

Transcriptional repression often involves recruitment of protein complexes harboring one or more *h*istone *d*e*ac*etylases (HDACs) (Kouzarides, 1999). To address the mechanism of repression by PRAME, we asked if repression by PRAME is inhibited by treatment of cells with the HDAC inhibitor trichostatin A (TSA). Figure 5A shows that TSA did not affect transcriptional repression by PRAME, indicating that repression is mostly HDAC independent, whereas repression by BS69, an HDAC-dependent repressor (Masselink and Bernards, 2000), was attenuated by TSA treatment (Figure 5A). Instead, we considered polycomb group (PcG) proteins as candidates for transcriptional repression by PRAME for two reasons. First, PcG proteins act in large complexes that are involved in the initiation and maintenance of heritable gene silencing, and PcG silencing is mostly HDAC independent (reviewed in Jacobs and van Lohuizen, 2002). Second, gene expression profiling of a series of human Wilms' tumors shows that *PRAME* expression closely parallels expression of *EZH2* (M. Eilers, personal communication), a PcG protein associated with malignant progression in breast and prostate cancer (Kleer et al., 2003; Varambally et al., 2002). To test if a complex containing PRAME and EZH2 exists, human HEK293 cells were transfected with expression plasmids for both proteins. In a subsequent coimmunoprecipitation experiment, Myc-tagged EZH2 was found as a binding partner of Flag-tagged PRAME, suggesting that the proteins do interact, whereas no immunoprecipitation of either protein was observed by control IgG (Figure 5C). To test the functional relevance of the PRAME-EZH2 complex in repression by PRAME, endogenous EZH2 was inhibited using RNA interference. The sequence-specific short hairpin RNA (shRNA) vector pRS-EZH2 reduced endogenous EZH2 protein levels after transfection into human cells but did not affect the endogenous levels of another PcG protein EED (Figure 5D). EZH2 knockdown inhibited transcriptional repression by the Gal4-PRAME fusion protein, suggesting that PRAME requires this PcG protein to confer repression (Figure 5B).

To test the functional relevance of endogenous PRAME and EZH2 in repression of RAR signaling, endogenous PRAME and EZH2 were inhibited in A375 melanoma

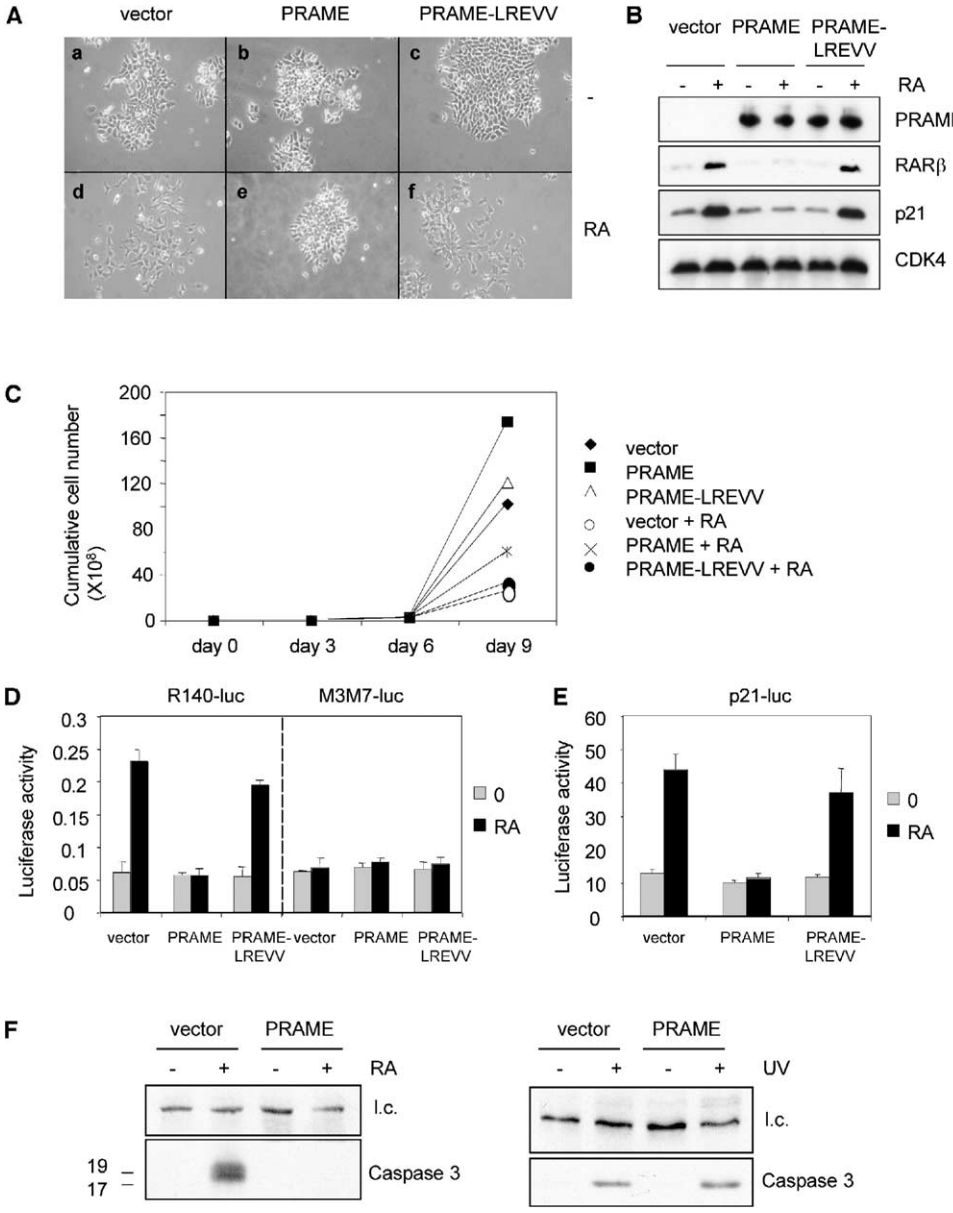

Figure 4. PRAME Is a Repressor of RA-Induced Differentiation, Growth Arrest, and Apoptosis

(A) Effects of PRAME expression on differentiation of F9 embryonic carcinoma cells. F9 cells were stably transfected with vector, PRAME, or PRAME-LREVV (a–c) and differentiated in 10^{-7} M RA (d–f, 200× magnification).

(B) PRAME inhibits RA-induced gene expression. Cells as in (A) were analyzed by Western blotting for PRAME, RARβ, p21, and CDK4 (loading control).

(C) Effects of PRAME on F9 cell proliferation arrest induced by RA. Proliferation curve according to the 3T3 protocol of F9 cells as in (A) in the absence and presence of RA.

(D and E) PRAME represses transcription from the RARβ and p21 promoters. (D) MEFs were cotransfected with a RARβ2-promoter-luciferase (R140-luc) or a RARβ2-luciferase with a mutated RARE (M3M7-luc) and either PRAME, PRAME-LREVV, or empty vector and treated with RA. (E) B16 melanoma cells were transfected with a p21-promoter luciferase construct and either PRAME, PRAME-LREVV, or empty vector and treated with RA.

(F) PRAME inhibits RA-induced apoptosis. F9 cells with PRAME or empty vector transfectants were immunoblotted for cleaved caspase 3. A doublet of 17, 19 kDa caspase 3 fragments is visible after treatment of vector control cells with RA. F9 cells were irradiated with UV light (100 J/m^2) and were immunoblotted for cleaved caspase 3 10 hr after irradiation. Cleaved caspase 3 is visible as a 17 kDa band in lysates of cells after treatment with UV light (l.c., loading control).

cells (which express high levels of PRAME, see below) using specific shRNA vectors, pRS-PRAME (Figure 6B) and pRS-EZH2 (Figure 5D), respectively (Brummelkamp et al., 2002a). After knockdown, the responsiveness of the cells to RA treatment was tested using a RARE-luciferase or RARβ-luciferase reporter gene. Knock-

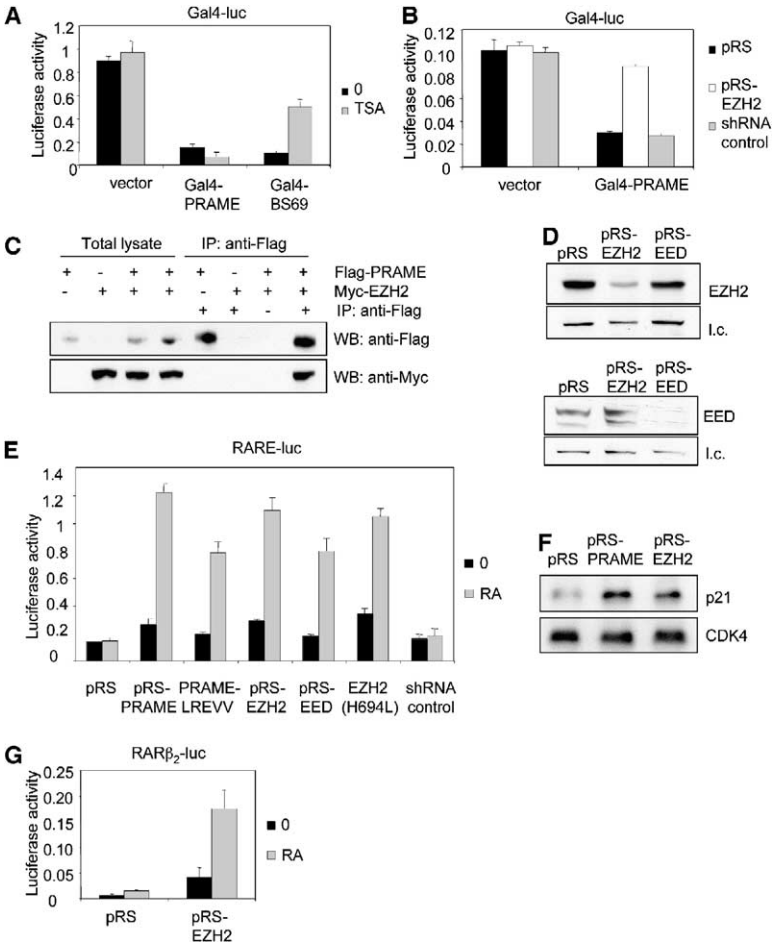

Figure 5. PRAME Interacts with PcG Proteins

(A) Transcriptional repression by PRAME is insensitive to HDAC inhibitors. HEK293 cells were transfected with Gal4-PRAME or Gal4-BS69 fusions and a Gal4-luciferase reporter and treated with 1 μM TSA.

(B) Transcriptional repression by PRAME is mediated by EZH2. HEK293 cells were co-transfected with Gal4-PRAME, a Gal4-luciferase reporter, and pRS-EZH2 or pRS-p21, which was used as a shRNA control vector.

(C) PRAME associates with EZH2. HEK293 cells were transfected with Flag-PRAME and Myc-EZH2 as indicated and immunoprecipitated (IP) using anti-Flag antibodies. Immunoprecipitates were analyzed by Western blotting (WB) using anti-Flag and anti-Myc antibodies.

(D) Specific RNAi vectors efficiently decrease the expression levels of PcG genes *EZH2* and *EED*. A375 cells were transfected with shRNA vectors against *EZH2* and *EED* (pRS-EZH2 and pRS-EED), and protein levels were analyzed by Western blotting. Two isoforms of EED are visible.

(E) Interference with PcG proteins enhances RAR signaling. A375 cells were transfected with the RARE-luciferase reporter and with the indicated constructs. pRS-PRAME is a shRNA vector to reduce endogenous PRAME levels in A375 cells (see Figure 6B) and pRS-p21 was used as a shRNA control vector.

(F) p21 is induced after EZH2 knockdown. A375 melanoma cells were transfected with pRS-PRAME or pRS-EZH2 in the presence of RA and immunoblotted for p21 and CDK4 (loading control).

(G) Knockdown of EZH2 enhances RARβ$_2$ promoter activity in A375 cells.

down of endogenous PRAME resulted in enhanced RAR signaling, as detailed below, and overexpression of PRAME-LREVV had a similar effect (Figure 5E). Knockdown of EZH2 was effective in restoring RAR signaling (Figures 5E and 5F). EZH2 contains a conserved SET domain, which specifies histone methyl transferase (HMTase) activity (Jenuwein et al., 1998). Ectopic expression of a point mutant EZH2 having a HMTase-deficient SET domain (H694L) (Kuzmichev et al., 2002) also restored RAR signaling, indicating that an intact SET domain in EZH2 is required for repression of RAR signaling by PRAME (Figure 5E). EZH2 acts in a multiprotein PcG complex, named PRC2 (Kuzmichev et al., 2002), that also contains the protein EED (Sewalt et al., 1998). We found that knockdown of EED through RNA interference (Figure 5D) also restored RA responsiveness, suggesting that EED, like EZH2, is required for repression of RAR signaling (Figure 5E). Finally, knockdown of EZH2 restored RA-induced expression of the RA target gene p21^{cip1} and activated the RARβ promoter (Figures 5F and 5G). Together, these data suggest a role for PcG proteins in mediating the inhibition of RAR signaling by PRAME.

Knockdown of PRAME Restores Sensitivity to RA

Human melanomas often have defects in RAR signaling (Demary et al., 2001; van der Leede et al., 1993), and PRAME is expressed in nearly all melanomas (Ikeda et al., 1997) (Figure 6A), raising the possibility that PRAME expression is responsible for their resistance to RA. To test this, we inhibited PRAME expression in human melanoma through RNA interference. Transfection of A375 melanoma cells with a PRAME-specific shRNA vector (pRS-PRAME) caused a significant decrease in levels of endogenous PRAME protein (Figure 6B). Knockdown of PRAME enhanced RAR signaling in A375, FM6, and SK23 human melanoma cells, which are all relatively insensitive to RA (Figure 6C). Similarly, knockdown of PRAME increased the response to RA in human breast cancer cells MCF7 and 1.6.2. (Figure 6D). Together, these data support the notion that PRAME expression confers resistance to RA.

To assess the effect of PRAME knockdown on cell proliferation, we generated stable derivatives of A375 melanoma having shRNA-mediated knockdown of PRAME expression (Figure 6F). These A375-PRAME^{KD} cells were cultured for 15 days according to the 3T3 protocol in the presence and absence of exogenous RA (Figure 6E). PRAME^{KD} cells showed decreased proliferation rates in standard culture medium (having trace amounts of RA) compared to vector control A375 cells; the decreased rates were more pronounced when cultured in the presence of supplemented RA (Figure 6E). Consistent with the notion that PRAME knockdown re-

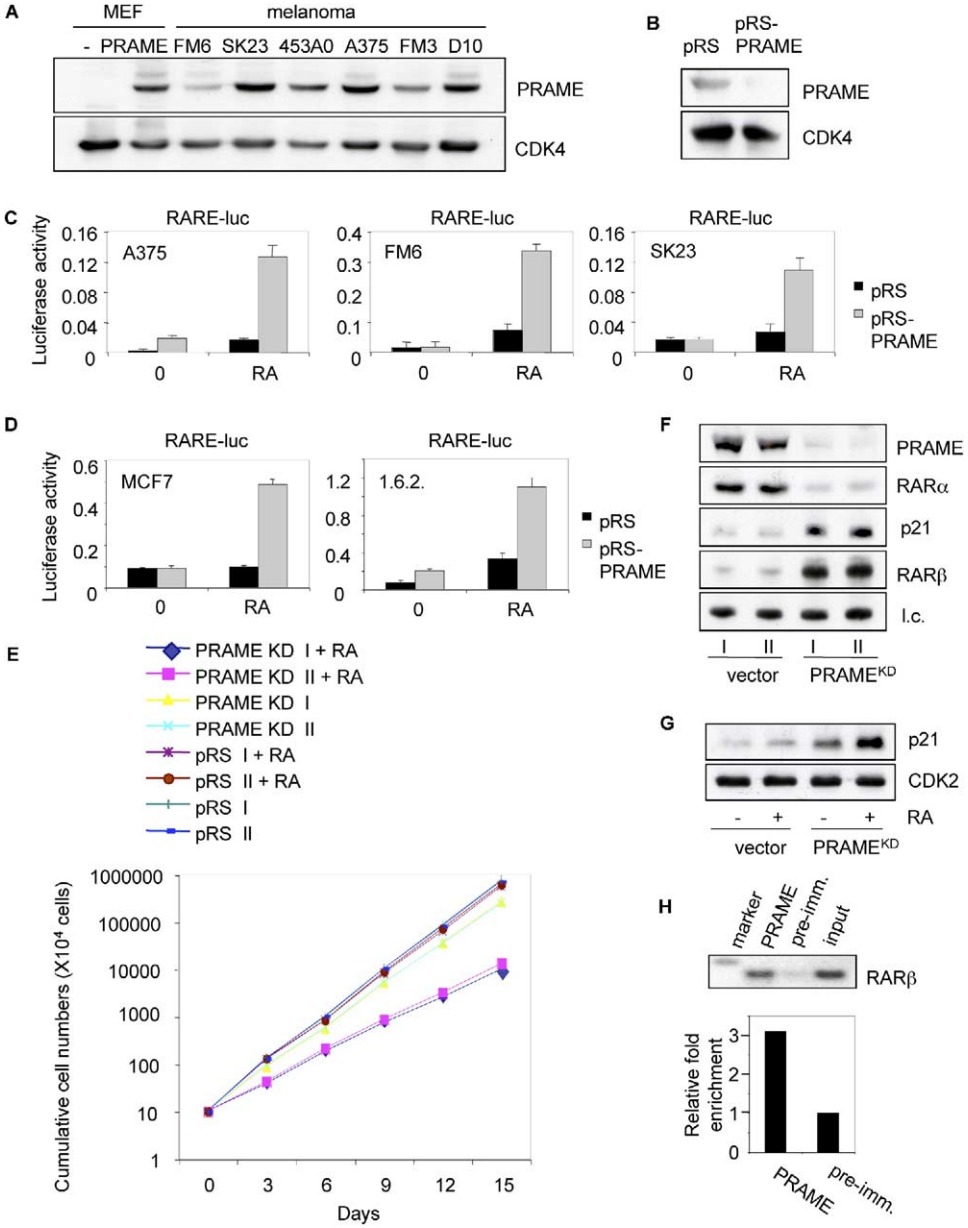

Figure 6. PRAME Knockdown Restores RAR Signaling

(A) PRAME is expressed in human melanoma cells. Western analysis of endogenous PRAME in extracts from human melanoma cells lines. MEFs transfected with PRAME were used as a positive control.

(B) A *PRAME* RNAi plasmid efficiently knocked down PRAME expression. A375 melanoma cells were transfected with a specific shRNA vector, pRS-PRAME, and were compared to cells transfected with empty vector (pRS) by immunoblotting of cell extracts for PRAME and CDK4 (loading control).

(C and D) PRAME knockdown by RNAi results in enhanced RAR signaling. (C) A375, FM6, and SK23 human melanoma cells were cotransfected with the RARE-luciferase reporter and either pRS-PRAME or empty vector (pRS) and treated with RA.

(D) MCF7 and 1.6.2. human breast cancer cells were transfected and treated as in (C).

(E) *PRAME* RNAi restores sensitivity to RA in melanoma. A375 cells were stably transfected with pRS-PRAME to generate A375-PRAME^{KD} cells. Proliferation curve according to the 3T3 protocol of A375-PRAME^{KD} cells and vector control cells in the absence and presence of RA.

(F) Derepression of RAR target genes after PRAME knockdown. A375-PRAME^{KD} cells and control A375 cells were analyzed by Western blotting for protein levels of PRAME, RARα, p21, and RARβ (l.c., loading control).

(G) RA induces p21 in A375-PRAME^{KD} cells. Cells as in (F) were treated with RA and immunoblotted for p21 and CDK2.

(H) PRAME is located at the RARβ promoter. Chromatin immunoprecipitation (ChIP) analysis demonstrates the promoter occupancy by endogenous PRAME on the RARβ promoter in A375 melanoma cells treated with RA. Thirty-five cycles of PCR amplification were used to show promoter occupancy (upper panel). Quantitative PCR showed specific enrichment for the RARβ promoter in the PRAME immunoprecipitate compared to preimmune serum after normalization to GAPDH, which was used as an internal ChIP control (lower panel).

stores RAR signaling, the known RA target genes *RARβ* and *p21^{CIP1}* (de The et al., 1990; Liu et al., 1996) were significantly upregulated in PRAME^{KD} cells (Figure 6F). Induction of these genes was further enhanced by exposure to exogenous RA (Figure 6G and data not shown).

RARα is degraded by the proteasome following treatment of cells with RA (Zhu et al., 1999), and in agreement with the notion that RAR signaling is activated in PRAME^{KD} cells, RARα protein levels were decreased in PRAME^{KD} cells (Figure 6F).

The specific targeting of *PRAME* mRNA by shRNA-mediated RNAi was further investigated by introduction of a PRAME silent mutant, indicated as PRAME^{M}, which contains three silent point mutations in the 21-mer RNAi target sequence. Hence, this mutant is insensitive to pRS-PRAME (Figure S3). Cotransfection of PRAME^{M} restored the insensitivity of A375 cells to RAR signaling in the presence of pRS-PRAME (Figure S3). Introduction of PRAME^{M} into PRAME^{KD} cells rescued the growth defects in these cells and partially restored their resistance to RA-mediated growth arrest (Figure S3). In addition, the levels of RARβ and p21 proteins in these cells were suppressed compared to PRAME^{KD} cells, consistent with a role for PRAME^{M} in inhibition of RAR target gene expression in PRAME^{KD} cells (Figure S3). We conclude that the observed effects of the pRS-PRAME vector are due to specific effects on the intended target and cannot be explained by off-target effects of the shRNA vector.

To further confirm that PRAME is part of the RAR transcription complex, we examined the recruitment of PRAME to the promoters of endogenous RA-responsive genes. We used A375 melanoma cells, which were cultured in the presence of RA and determined the status of the endogenous transcription complexes present on the RARβ promoter using chromatin immunoprecipitation (ChIP). The presence of this promoter in the chromatin immunoprecipitates was analyzed by quantitative PCR using a specific pair of primers spanning the retinoic acid-responsive region in the RARβ promoter. As shown in Figure 6H, immunoprecipitation of endogenous PRAME resulted in enrichment of the RARβ promoter in the precipitates relative to a preimmune serum from the same rabbit, indicating occupancy by PRAME of this promoter. These data show that PRAME is located at a genuine RAR target gene promoter and that it acts on the chromatin template to block RAR-RXR-dependent gene transcription.

Knockdown of PRAME and Treatment with RA Inhibit Melanoma Growth In Vivo

To examine the role of PRAME in RA responsiveness in vivo, we used a human melanoma xenograft model. Nude mice were subcutaneously transplanted with parental A375 into one flank and A375-PRAME^{KD} cells in the opposite flank and the mice were treated orally daily with either 5 mg/kg RA or vehicle only, while tumor volumes were measured weekly. Tumor growth was severely retarded by RA treatment in PRAME^{KD} melanomas but not in parental A375 melanomas that grew in a different anatomical location in the same mice (Figures 7A and 7B). Together, these data suggest that PRAME functions as a negative regulator of RAR signaling, and its overexpression may contribute to RA unresponsiveness of human melanomas.

Discussion

The human tumor antigen PRAME is frequently overexpressed in human cancer, but its function has remained obscure. In the current study we identify PRAME as a ligand-dependent corepressor of retinoic acid receptor signaling. PRAME interacts only with ligand bound RAR (holo-receptor) through a nuclear receptor (NR) box and attenuates RAR signaling in the presence of agonist in a Polycomb-dependent fashion. In this respect, PRAME is distinct from the major nuclear receptor corepressors identified thus far. For instance, the established nuclear receptor corepressors N-CoR and SMRT interact with apo-receptors in the absence of ligand and their interaction is lost upon ligand binding of the receptor (Xu et al., 1999). PRAME resembles the proteins RIP140 (Cavailles et al., 1995) and LCoR (Fernandes et al., 2003), which also are ligand-dependent corepressors of nuclear receptors. However, LCoR and RIP140 act on a variety of class I and class II nuclear receptors, repress transcription in a HDAC-dependent fashion, are widely expressed in normal tissues, and do not appear to be overexpressed in cancer. In contrast, *PRAME* expression is absent in adult tissues and is selected for during oncogenesis, and PRAME requires Polycomb proteins for repression and appears to act with a considerable degree of specificity on RAR.

We provide several lines of evidence that PRAME functions to negatively regulate cellular responses to retinoids. First, PRAME expression in RA-sensitive F9 mouse embryonic carcinoma cells inhibited the physiological consequences of RA treatment, notably cell-cycle arrest, differentiation, and apoptosis. Consistent with this, PRAME inhibits RA target genes p21 and RARβ, thus interfering with the transcriptional responses to RA. Second, PRAME is frequently overexpressed in human melanomas (Ikeda et al., 1997), which are also frequently resistant to RA (Demary et al., 2001; van der Leede et al., 1993). A causal relationship between these two apparently unrelated observations was revealed by our demonstration that knockdown of PRAME levels in melanoma by RNA interference resulted in restoration of the sensitivity to the antiproliferative effects of RA, in greatly enhanced RAR signaling and induction of RA target genes p21 and RARβ. Importantly, in a mouse xenograft experiment, melanoma cells having PRAME knockdown regained sensitivity to the antiproliferative effects of RA treatment, again highlighting the inverse relationship between PRAME expression and sensitivity to RA in an animal model.

Unlike the established nuclear receptor corepressors, which recruit HDAC complexes of varying composition, repression by PRAME is relatively insensitive to treatment with HDAC inhibitors, suggesting an HDAC-independent mechanism. Our data indicate that PRAME interacts with the PcG protein EZH2 in vivo and that EZH2 is involved in transcriptional repression by PRAME. Significantly, knockdown of EZH2 levels re-

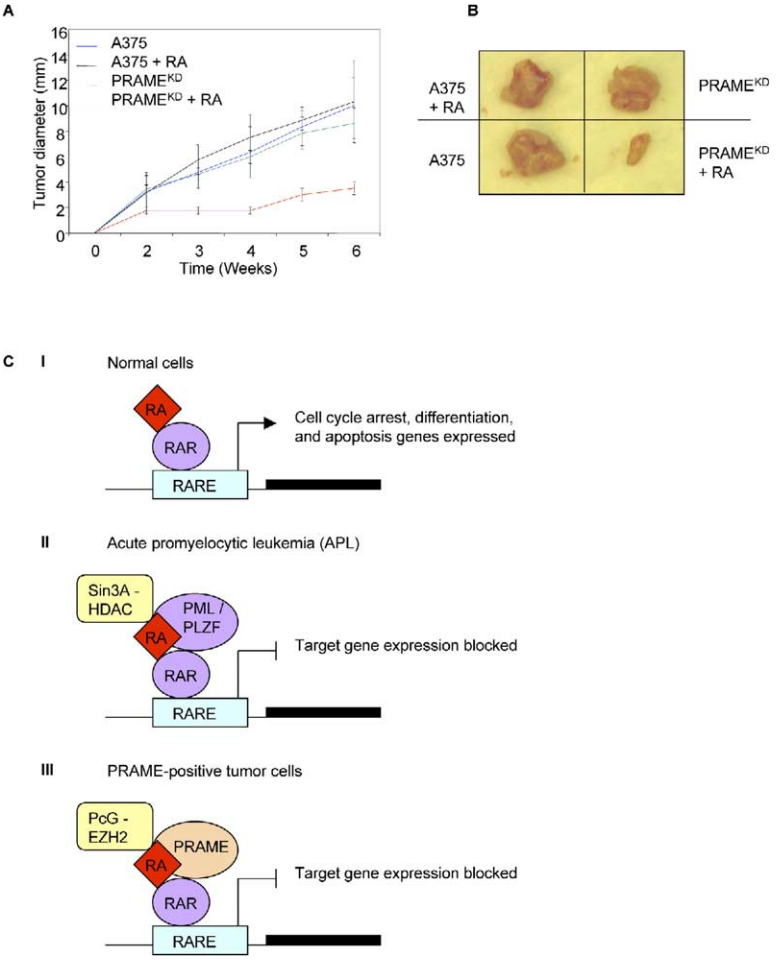

Figure 7. PRAME Knockdown Restores RA-Induced Growth Arrest In Vivo

(A) Xenograft of PRAME RNAi melanoma cells is sensitive to RA treatment. A375-PRAME^{KD} cells and control A375 cells were injected in the right and left flanks of nude mice, respectively, and mice were treated orally with 5 mg/kg RA daily. Tumor growth was measured weekly.

(B) Dissected tumors from the experiment in (A).

(C) Model for PRAME-mediated repression of RAR signaling. (I) In the absence of ligand, RARα is a repressor of gene transcription, but upon binding of RA, target genes are expressed to induce cell-cycle arrest, differentiation, and apoptosis. (II) In promyelocytic leukemia (APL), the PML-RARα and PLZF-RARα translocations result in functionally altered receptors, which are transcriptional repressors under physiologic RA concentrations, preventing myelocyte differentiation. The PML and PLZF parts of the fusion receptors recruit repressor complexes containing HDAC. (III) PRAME binds RAR in the presence of RA to block target gene expression, which requires PcG proteins including EZH2. The resulting constitutive inhibition of RAR signaling resembles the repression as seen in APL (II). RARE: retinoic acid-responsive element.

stored RAR signaling in melanoma cells, suggesting a role for PcG proteins in regulation of the effects of RA through interaction with PRAME. PcG proteins act in large, multimeric complexes and mediate heritable gene silencing through chromatin remodeling (Jacobs and van Lohuizen, 2002). Dysregulation of PcG proteins has been implicated in cancer, and overexpression and amplification of *EZH2* are prognostic for progression to metastatic disease in breast and prostate carcinoma (Kleer et al., 2003; Varambally et al., 2002). Inhibition of RAR transactivation by PcG proteins has been reported in embryonic development, but not in adult tissues. The mouse homolog of the *Drosophila Polycomb* gene, *M33*, is thought to antagonize the RAR pathway and to function in the establishment of the early temporal sequence of *Hox* gene activation in the embryo (Bel-Vialar et al., 2000). From studies in *M33*-deficient mice, it has been proposed that *M33* may play a role in defining access to retinoic acid response elements localized in the regulatory regions of several *Hox* genes (Core et al., 1997).

Cellular transformation, tumor development, and cancer progression are affected by many pathways, and RAR signaling is one of the pathways which can be disturbed in malignant disease. The effects of retinoids are tumor suppressive in a wide variety of tissues, where binding of RA to RAR induces a transcriptional response resulting in cell-cycle arrest, differentiation, and apoptosis (Altucci and Gronemeyer, 2001) (Figure 7C). Consistent with this, loss of RARβ expression is correlated with tumor progression in solid tumors, and genetic evidence indicates that RARβ is involved in tumorigenesis. Introduction of exogenous RARβ in several RA-unresponsive tumor cell lines can restore their growth-inhibitory responses to RA (Altucci and Gronemeyer, 2001). Perturbations of RARα function cause promyelocytic leukemia, where the PML-RARα and PLZF-RARα translocation products recruit HDAC/Sin3A-containing repressor complexes, thereby preventing normal RARα target gene expression and promyelocyte differentiation (Grignani et al., 1998; He et al., 1998; Lin et al., 1998; Figure 7C). Our data indicate that PRAME interacts with RAR in a ligand-dependent manner and inhibits RAR-dependent transactivation through interaction with PcG complexes. We propose that *PRAME* expression in tumor cells renders these cells resistant to normal retinoid action (Figure 7C). In this respect, *PRAME* overexpression phenocopies the PML-RARα and PLZF-RARα translocations in that both interfere with RAR signaling under physiological concentrations of RA. Melanoma cells and other tumor cells that overexpress *PRAME* may have a selective advantage over PRAME-negative cells,

which would provide an explanation for why *PRAME* expression is positively selected during oncogenesis, even though its presence elicits a cytotoxic T cell-mediated immune response (Ikeda et al., 1997). Since *PRAME* is a marker of poor outcome in breast cancer and neuroblastoma (Oberthuer et al., 2004; van't Veer et al., 2002), it is likely that *PRAME* expression contributes to tumor progression rather than the early stages of oncogenic transformation. Thus, our data suggest that *PRAME* overexpression represents a novel mechanism by which tumor cells can escape from tumor-suppressive RAR signaling.

Experimental Procedures

Plasmids, Reagents, and Antibodies

The PRAME NR box mutants, the PRAME^{M} silent mutant, and EZH2 (H694L) were made using the QuikChange Site-Mutagenesis kit (Stratagene). pRS-PRAME was generated by ligating synthetic oligonucleotides against the target sequence GGTGCCTGTGATG AATTGTTC into pRETRO-SUPER as described (Brummelkamp et al., 2002b). PRAME^{M} contains three silent mutations in the shRNA target sequence resulting in GGCGCCTGCGACGAATTGTTC. RA-responsive luciferase constructs were kindly provided by Dr. H. Stunnenberg (Nijmegen, The Netherlands) and PcG expression and shRNA vectors were kind gifts of Dr. M. van Lohuizen (Amsterdam, The Netherlands). The RNAi target sequence in EED was AAGCAC TATGTTGGCCATGGA, the target sequence in EZH2 was AAG ACTCTGAATGCAGTTGCT, and the target sequence in RARα was GCCTTGCTTTGTCTGTCAG. PRAME antisera and affinity-purified antibodies were a generous gift from Dr. P. Coulie (Brussels, Belgium) and were generated by immunizing rabbits with peptides FPEPEAAQPMTKKRKVDG (AH-151/serum 440) and CGDRTFYD PEPIL (AH-152/serum 442). The anti-EZH2 mouse monoclonal antiserum was a kind gift from Dr. A. Otte (Amsterdam, The Netherlands). All-trans-retinoic acid (RA), 9-cis-retinoic acid (9-cis-RA), β-estradiol, progesterone, TSA, and Flag (M2) antibody were from Sigma, and Troglitazone was from Alexis Biochemicals. Antibodies against RARα (C-20), RARβ (C-19), p21 (F5), Myc (9E10), CDK4 (C-22), and CDK2 (H-298) were from Santa Cruz Biotechnology, anti-EED was from Transduction laboratories, anti-cleaved caspase-3 (Asp 175) was from Cell Signaling, the SSEA-1 antibody (MC-480) was from R&D Systems, and anti-Laminin-1 was from Monosan (EHS-Laminin, PS040).

Cell Cultures and Transfections

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were carried out using the Lipofectamine^{2000} reagent (Invitrogen), except for MCF7, U2OS, HEK293, and MEFs, which were transfected using calcium phosphate precipitation. Hormone-based reporter assays were done in DMEM supplemented with charcoal-stripped FCS (Hyclone) and steroid hormones were added in phenol red-free DMEM. In reporter assays, 0.5 μg of firefly luciferase reporter, 1 ng CMV-renilla luciferase as an internal control, and 3 μg of the indicated DNA were transfected. Hormones and TSA were added 24 hr after transfection and assays were performed 48 hr after transfection. In RNAi experiments, RA was added 72 hr after transfection and assays were performed 96 hr after transfection. Luciferase activities shown represent ratios of firefly luciferase to renilla luciferase internal control values, and normalized luciferase activities are the average ± SD from three independent transfections. F9 cells were stained by incubation with SSEA-1 antibody (dilution 1:40) and with goat-anti-mouse-FITC conjugated antibody (Zymed, dilution 1:400). For detection of apoptosis, F9 cells were stained with Annexin V-biotin antibody (Boehringer Mannheim, dilution 1:50) and with streptavidin-PE-Cy5 (BD Pharmingen, dilution 1:500) and subjected to flow cytometry analysis.

GST Pull-Down Assays

GST fusion proteins were expressed and purified from the *E. coli* BL21 (DE3) strain. Total bacterial extracts were prepared in NETN lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) supplemented with protease inhibitors (Complete; Roche), and GST fusion proteins were purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). ^{35}S-methionine-labeled proteins were prepared by in vitro transcription/translation using the TNT coupled reticulocyte lysate system (Promega) and equal amounts of GST fusion proteins on glutathione beads were incubated with in vitro translation products in NETN buffer for 2 hr, washed, and fractionated by SDS-PAGE.

Western Blotting and Coimmunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with protease inhibitors (Complete; Roche), and 0.2 nM PMSF and proteins were separated on 8%–14% SDS-PAGE gels. Cell lysates and conditioned media (20 μl) from F9 cells were subjected to SDS-PAGE gels under reducing and nonreducing conditions, respectively. Proteins were transferred to polyvinylidine difluoride membranes (Immobilon-P, Millipore) and Western blots were probed with the indicated antibodies. For coimmunoprecipitations, cells were lysed in ELB buffer (0.25 M NaCl, 0.1% NP-40, 50 mM HEPES, pH 7.3) supplemented with protease inhibitors and PMSF. Lysates were incubated with IgG-coated sepharose beads (Amersham Pharmacia Biotech) to immunoprecipitate TAP-tagged proteins[19], indicated as anti-TAP, or with protein A beads coated with the indicated antibodies, and the precipitates were separated on SDS-PAGE gels. For ChIP analysis, PRAME polyclonal rabbit antisera 440 and 442 were used.

Mouse Tumor Xenografts

Female 5- to 6-week-old athymic BALB-C nude mice (*nu/nu*) were s.c. implanted with 1 × 10^{6} cells bilaterally into the axial regions. Each mouse received A375-PRAME^{KD} cells in its right flank and control A375 cells in its left flank. Mice were randomized into treatment groups and treated with 5 mg/kg RA or vehicle (ethanol in sunflower oil) orally with a 20-gauge intragastric feeding tube daily. The pRS vector which was used to generate A375-PRAME^{KD} cells is a self-inactivating retroviral vector, to prevent re-activation and spreading of virus (Brummelkamp et al., 2002a; Brummelkamp et al., 2002b). The experiment was performed twice, with n = 20 and n = 10 mice, and results were similar in both experiments.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/122/6/835/DC1/.

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