Published in final edited form as:

Cancer Discov. 2015 March ; 5(3): 288–303. doi:10.1158/2159-8290.CD-14-0625.

PRMT5 is required for lymphomagenesis triggered by multiple oncogenic drivers

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Abstract

Protein arginine transferase 5 (PRMT5) has been implicated as a key modulator of lymphomagenesis. Whether PRMT5 has overt oncogenic function in the context of leukemia/lymphoma and whether it represents a therapeutic target remains to be established. We demonstrate that inactivation of PRMT5 inhibits colony-forming activity by multiple oncogenic-drivers including cyclin D1, c-MYC, NOTCH1 and MLL-AF9. Furthermore, we demonstrate that PRMT5 overexpression specifically cooperates with cyclin D1 to drive lymphomagenesis in a mouse model revealing inherent neoplastic activity. Molecular analysis of lymphomas, revealed that arginine methylation of p53 selectively suppresses expression of crucial proapoptotic and anti-proliferative target genes thereby sustaining tumor cell self-renewal and proliferation and bypassing the need for the acquisition of inactivating p53 mutations. Critically, analysis of human tumor specimen reveal a strong correlation between cyclin D1 overexpression and p53 methylation supporting the biomedical relevance of this pathway.

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The authors declare no conflict of interest

Keywords

Cyclin D1; CDK4; PRMT5; MEP50; Arginine Methylation

INTRODUCTION

Arginine methylation is becoming increasingly appreciated as an important mechanism of post-transcriptional control (1). Proteins targeted by arginine methylation contribute to a variety of cellular processes including transcriptional regulation, chromatin regulation, RNA processing and DNA damage repair (2–4). The type II protein arginine transferase, PRMT5, has been most thoroughly characterized with regard to its function as a histone 3 and 4 methyltransferase. Methylation of H3R8 and H4R3 is associated with transcription repression (5–7). However, PRMT5 also targets multiple soluble proteins, including components of the spliceosome, PIWI proteins (8, 9), RelA (10), EGFR (11), E2f1 (12) and p53 (13), thereby potentially impacting multiple cellular signaling events.

Cyclin D1, together with its binding partners cyclin dependent kinase 4 and 6 (CDK4/6), forms an active complex that promotes cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (14). Aberrant expression and/or regulation of the D cyclins (D1, D2, D3) has been linked to loss of cell cycle control and is considered a driving event in many malignancies. Accumulating evidence has implicated dysregulation of cyclin D1 nuclear export and ubiquitin-dependent degradation during S-phase as key events in the genesis of neoplastic events. Cyclin D1 nuclear export and polyubiquitylation depend upon phosphorylation of a specific threonine residue (Thr-286) (15). The oncogenicity of D1T286A, a constitutively nuclear mutant, has most thoroughly been examined in the context of the Eμ-D1T286A transgenic mouse model (16, 17). In this model, D1T286A expression is targeted to the lymphoid compartment by the immunoglobulin enhancer, thereby providing an expression pattern analogous to that observed in human mantle cell lymphoma (MCL) (18). Analysis of early-stage tumors reveals that nuclear accumulation of D1T286A/CDK4 triggers DNA damage and activation of the ATM/CHK2/p53 checkpoint pathway, which leads to p53-dependent apoptosis (16, 19, 20). A latency period of 4 to 21 months is required for the accumulation of cooperating mutations to counter p53 surveillance before lymphoma can develop (19). The clinical relevance of mutations that specifically disrupt phosphorylation-dependent degradation and nuclear export of cyclin D1 is highlighted by their occurrence in human cancers (21, 22).

p53 is the central regulator of cell fate following numerous stresses including genotoxic stress and oncogene activation (23, 24). The tumor suppressor properties of p53 have been linked to its function as a transcription factor that regulates the expression of target genes linked with cell cycle arrest, apoptosis, senescence and DNA repair (25, 26). Tumor suppressor activities of p53 have also been attributed to its capacity to target Bax at the mitochondria, thereby directly regulating pro-apoptotic functions in a transcription-independent fashion (27). More than 60% of human primary tumors exhibit mutations in the p53 gene (28). In contrast, hematological malignancies exhibit a low frequency of p53

mutation (29, 30), implicating the existence of alternative mechanisms for bypassing p53-dependent tumor suppression.

We provide evidence for a direct link between PRMT5-dependent arginine methylation of p53, reduced expression of pro-apoptotic p53 transcriptional targets and hematologic malignancy. This mechanism is engaged by multiple drivers of hematologic malignancy where it serves as key regulatory event that directly alters promoter engagement by p53, providing a new mechanism by which a p53 modification contributes to neoplastic transformation.

RESULTS

Cyclin D1T286A and PRMT5 cooperatively induce an aggressive T-cell lymphoma/leukemia

To directly assess the potential of PRMT5 to drive neoplastic growth, we chose to first assess whether PRMT5 would cooperate with a cancer-derived allele of cyclin D1 to drive lymphomagenesis; this strategy was fueled by previous reports of PRMT5 overexpression in cyclin D1-driven malignancy (5). Initially, 5-FU-treated bone marrow HSPCs transduced with retroviral supernatants encoding PRMT5 and cyclin D1T286A were injected into lethally irradiated, syngeneic C57BL/6 mice. Surprisingly, recipient mice reconstituted with HSPCs overexpressing only D1T286A developed fatal pancytopenia with a remarkable reduction in the white blood cells, red blood cells and platelet counts by 2-weeks post reconstitution (Fig S1A; Fig 1A). The spleen and thymus of D1T286A reconstituted mice exhibited significant atrophy (Fig S1B). These results indicated failure of bone marrow reconstitution by D1T286A. However, all animals transplanted with cells co-expressing D1T286A and PRMT5 survived hematopoietic failure and succumbed to leukemia/lymphoma by 170 days with a median survival age of 147 days (Fig 1A). Macroscopic examination of tumor-burdened mice revealed thymic, splenic and liver involvement; involvement of peripheral blood leukocytosis and increased blast circulation in bone marrow was also readily apparent (Fig 1B–D). Histologic analyses revealed extensive infiltration of lymphoblastoid cells within liver, spleen, thymus, lung and kidney and almost complete effacement of the normal tissue architecture (Fig 1E). D1T286A/PRMT5 chimeric mice (n=7) exhibited accumulation of CD4⁺ lymphocytes in the bone marrow and spleen (Fig 1F–G). Tumor cells were GFP⁺/NGFR⁺ demonstrating maintenance of transgenes (Fig 1F). The tumors analyzed were CD3⁺TCR Vβ⁺ CD4⁺ CD8[−] (Fig S2A and primarily CD25^{neg} CD69^{neg} Fig S2B) consistent with their identity as mature T cells. T-cell clonality was further assessed through both immunophenotypic analysis and PCR-based analysis of the T-cell receptor Vβ repertoire (TCR- Vβ -R) (Table S1; Fig S1D). Whereas CD4⁺ T cells from a wild type mouse used a variety of Vβ chain as expected, those from the tumor-bearing mice did not exhibit outgrowth of a monoclonal TCR Vβ clone suggesting the tumors are oligoclonal. However, because these results could reflect technical issues pertaining to antibody selectivity, we further addressed the suggested oligoclonal nature of tumors. The clonality of the TCR repertoires of 22 individual Vβ gene families (from Vβ 1–20, with the subfamilies Vβ 8.1, 8.2 and 8.3) was assessed by a PCR amplification assay. An oligoclonal pattern was observed in all tumors derived from D1T286A+PRMT5 mice (Fig S1D). In addition, the CD4⁺ tumor cells have phenotypes of memory T cells (CD44^{high}CD62L^{low},

Fig S2C). Interestingly, PRMT5 alone was not sufficient for transformation (Fig 1A; Fig S1C). The generation of mitotic spreads from dispersed tumors and normal lymphocytes revealed chromosomal gains ($>40N$) and increased chromatid breaks associated specifically with the tumor (Fig S2D–E) demonstrating that co-expression of PRMT5 had not reduced DNA damage associated with D1T286A expression (5).

To ensure the phenotype reflected neoplastic growth, cells from the bone marrow of primary leukemia/lymphoma burdened mice were transplanted into sub-lethally irradiated secondary and tertiary recipients. All secondary recipients receiving more than 1×10^5 cells succumbed to CD4⁺ leukemia/lymphoma with an average latency of 62 days (1×10^6 , brown) and 79 days (1×10^5 , black) (Fig S2F). Notably, 1×10^4 cells were sufficient following secondary transplantation for disease manifestation, albeit with reduced penetrance (60%) and longer latency (15–20 weeks) (blue, Fig S2F). Tertiary recipients died rapidly between 18–27 days (red, Fig S2F). The immunophenotype of the leukemia/lymphoma cells in secondary recipients analogous with that of primary disease, with most cells retaining high expression of CD4 (Fig S2G). In addition, the tumor cells were Lin[−], c-Kit⁺ and Sca-1⁺ (Fig S2H). Collectively, this work demonstrates that PRMT5 can function as a driver oncogene in the context of nuclear cyclin D1.

MEP50 phosphorylation is required for D1T286A-dependent neoplastic transformation

Cyclin D1T286A-dependent regulation of PRMT5 reflects phosphorylation of MEP50 on Thr-5 (5). If MEP50 phosphorylation serves as the point of integration for D1T286A, then inhibition of MEP50 phosphorylation should inhibit tumorigenesis. Since endogenous MEP50 levels remain stable after D1T286A and PRMT5 transduction (Fig 1H), we co-expressed either wild type MEP50 or MEP50T5A (an alanine mutation previously shown to make PRMT5/MEP50 complexes refractory to regulation by D1/CDK4 (5) with D1T286A/PRMT5. D1T286A/PRMT5/wtMEP50 mice developed CD4⁺ leukemia/lymphoma with complete penetrance and reduced latency relative to D1T286A/PRMT5 (Fig 2A–C; log rank test $p=0.01$). In striking contrast, mice reconstituted with D1T286A/PRMT5/MEP50T5A died within 2 weeks of transplant (Fig 2A) similar to that observed with D1T286A alone. This again likely reflects hematopoietic failure, as survival can be supported with normal bone marrow. Mice reconstituted without sorting (under these conditions 70% of cells were normal BM) did not die and survive within the observation period. FACS revealed that TA +MEP50T5A+PRMT5 cells are eliminated by 4 weeks post transplantation (Fig 2D). These results support a model wherein MEP50-phosphorylation serves as the point of integration of cyclin D1 with PRMT5 to drive neoplastic growth.

PRMT5 is required for leukemia/lymphoma driven by multiple oncogenes

Given the critical function of PRMT5 in D1-dependent lymphomagenesis, we ascertained whether PRMT5 contributes to neoplastic outgrowth triggered by other oncogenic drivers such as NOTCH 1 (intercellular domain, ICN), c-MYC and MLL-AF9. Ectopic expression of PRMT5 failed to accelerate disease, increase the penetrance of leukemia/lymphoma or alter the phenotype of tumors driven by the ICN domain of NOTCH1, c-MYC, or MLL-AF9 (Fig 3A, Fig S3A–D). However, upon examination of PRMT5 levels in tumors versus

normal splenic lymphocytes, we noted a significant increase in endogenous PRMT5 levels that greatly exceeded that achieved by retroviral transduction (Fig 3B; Fig S3E).

The significant increase in endogenous PRMT5 highlighted a potential requirement for increased PRMT5 activity downstream of NOTCH1, c-MYC, and MLL-AF9. To specifically assess a potential requisite role for PRMT5 in the context of these oncogenic drivers, we utilized either a dominant negative PRMT5 allele (PRMT5^{DN}) or an shRNA validated as *Prmt5*-specific (5). *Prmt5* knockdown and PRMT5^{DN} overexpression were confirmed in transduced HSPCs prior to transplantation into irradiated mice (Fig 3C–D). Surprisingly, *Prmt5* knockdown and PRMT5^{DN} overexpression were without effect on the latency of disease (Fig 3A). However, upon western analysis, we noted that c-MYC and ICN driven tumors had restored PRMT5 expression in cells transduced with sh*Prmt5* (Fig 3C; note PRMT5 expression post transplantation). Likewise in tumors driven by cells transduced with myc-tagged PRMT5^{DN}, PRMT5 expression was undetectable (Fig 3D). Similar results were observed with MLL-AF9 (Fig S3F). These results demonstrate strong selection to maintain PRMT5 expression. To independently assess the requisite role for PRMT5 in neoplastic growth driven by c-MYC and ICN, we evaluated the impact of PRMT5^{DN} expression on colony expansion and HSC renewal in methylcellulose. Consistent with a requisite functional role in c-MYC and ICN driven tumor cell proliferation and survival, PRMT5^{DN} significantly inhibited colony formation during serial passage (Fig 3E). Similar results were observed when PRMT5^{DN} was co-expressed with MLL-AF9 (Fig S3G). Collectively, these results suggest that PRMT5 plays a critical role in supporting neoplastic transformation in this setting.

The PRMT5 methyltransferase inhibits Cyclin D1T286A induced apoptosis in a MEP50 phosphorylation-dependent manner

The strong cooperative generation of a leukemic phenotype in mice transplanted with HSPC co-expressing D1T286A and PRMT5 was in stark contrast to mice transplanted with HSPC expressing only D1T286A, wherein all mice died within the first 14 days of transplantation (Fig 1A). This result prompted us to carry out a more extensive analysis of the mechanistic contribution of PRMT5 to D1T286A driven disease. Tumorigenesis driven by constitutively nuclear cyclin D1 mutants is opposed by p53-dependent apoptosis (16). While apoptosis can be overcome through genetic ablation of p53 in mice (19), wild type p53 is retained in 70% of human MCL cases (31). Sequencing of *Tp53* in lymphomas that arise in Eμ-D1T286A single transgenic mice reveals retention of wild-type *Tp53* in 40% of resultant tumors (16), suggesting the existence of uncharacterized mechanisms that permit the bypass of p53-dependent tumor suppression. To gain insights into the regulation of p53 in cyclin D1-driven neoplasia, we generated bone marrow chimeras using HSPC derived from p53^{+/+} or p53^{-/-} mice. HSPC were transduced with retroviruses expressing wild-type cyclin D1 or D1T286A. In contrast to mice reconstituted with p53^{+/+} HSPCs expressing D1T286A, which die by 14 days post reconstitution (Fig 1A; Fig 4A), use of p53^{-/-} donors expressing D1T286A permitted hematopoietic reconstitution (Fig 4A–B). To determine whether this reflected graft failure, we examined the contribution of D1T286A-expressing p53^{-/-} HSPCs to recipient bone marrow in a competitive setting. We transplanted a mixture of transduced (GFP⁺) and control HSPCs at around 1:1 ratio into recipient mice and analyzed donor

contribution to bone marrow cells one month later. Donor p53^{+/+} cells transduced with cyclin D1 contributed to ~55% of bone marrow cells one month post transplant, whereas p53^{+/+} cells transduced with D1T286A contributed to only 5% of recipient bone marrow cells (Fig 4B). Therefore D1T286A impairs the engraftment of HSPCs, and this is dependent on the presence of p53. Previous studies showed that expression of D1T286A causes p53-dependent apoptosis (19). Consistent with this, transduction of wild-type HSPCs with a retrovirus encoding D1T286A triggered extensive p53-dependent apoptosis, while infection with an equivalent MOI of virus encoding wild-type cyclin D1 was without effect (Fig 4C).

Mice transplanted with p53^{-/-} HSPCs that express D1T286A developed aggressive and widespread lymphomas involving the lung, liver, kidney, spleen, thymus and bone marrow, with a significantly reduced latency compared to recipients reconstituted with p53^{-/-} HSPCs (p=0.003 by log rank test; Fig 4D; Fig S4A–E;). Mice reconstituted with p53^{-/-} HSPCs expressing cyclin D1 developed malignancies not significantly different from those transplanted with p53^{-/-} HSPCs in latency, frequency and molecular phenotype (p = 0.36, Fig 4D; Fig S4A), demonstrating that wild-type D1 provided no overt oncogenic activity even on the p53^{-/-} background. Malignancies were not observed in mice transplanted with p53^{+/+} or p53^{+/+} cells expressing D1 (Fig 4D). Critically, mice transplanted with syngeneic HSPCs co-overexpressing a dominant negative allele of p53 (p53DN) and nuclear D1T286A developed CD4⁺ lymphoma by 180 days (Fig 4E; Fig S4F). Taken together, these data suggest that p53 inactivation contributes to sustained growth in the presence of D1T286A, thereby facilitating tumorigenesis.

The synergism of PRMT5 and D1T286A in cells harboring wild type p53 prompted us to ascertain whether PRMT5 attenuated p53-dependent apoptosis. Co-infection of HSPCs with viruses encoding PRMT5 and D1T286A reduced apoptosis of D1T286A-expressing cells to a similar degree as a dominant p53DN (Fig 5A). Expression of D1T286A and PRMT5 was confirmed by western blot and expression of the IRES-linked GFP and mCherry markers (Fig S5A–B). Expression of a catalytically inactive PRMT5, PRMT5⁽³²⁾, failed to protect D1T286A HSPCs from death (Fig 5A), thereby demonstrating the requirement for PRMT5 methyltransferase function.

These findings support a model wherein PRMT5 inactivates p53, thereby promoting survival of D1T286A-expressing cells. To further interrogate this model, the ability of bone marrow derived HSPCs to form colonies in methylcellulose was determined (Fig 5B). Both PRMT5 and p53DN increased the number of colonies generated by D1T286A expressing cells through 5 rounds of serial replating. These results demonstrate that increased PRMT5 methyltransferase activity is as effective as dominant negative p53 with regard to inhibiting D1T286A triggered apoptosis and increasing self-renewal and cell transformation by D1T286A *in vitro* (Fig 5B) or *in vivo* (Fig 1A).

MEP50, being the direct substrate of D1T286A/CDK4, should be required for inhibition of apoptosis. Expression of wild type MEP50 decreased D1T286A-dependent apoptosis, as did co-expression of MEP50 and PRMT5. In contrast, MEP50T5A failed to inhibit D1T286A-induced apoptosis (Fig 5C). Consistent with this, expression of MEP50T5A inhibited the PRMT5-dependent increase in serial replating ability (Fig 5D). These data are consistent

with the impact of MEP50T5A on D1T286A/PRMT5 driven lymphoma (See Fig 2A) and suggest a model where D1T286A-dependent activation of PRMT5, via MEP50 phosphorylation, is necessary for inhibition of p53-dependent apoptosis.

Cyclin D1T286A/CDK4 promotes PRMT5-dependent methylation of p53

PRMT5/MEP50-dependent inhibition of apoptosis in D1T286A expressing cells implies that tumors driven by D1T286A/PRMT5 should maintain wild-type p53. Indeed, DNA sequencing of D1T286A/PRMT5 expressing tumors revealed intact, wild type p53 in 12 of 12 tumors analyzed. To mechanistically interrogate D1T286A/PRMT5 regulation of p53, we determined assessed the ability of D1T286A phosphorylated MEP50/PRMT5 to methylate p53. Wild type p53 or a mutant p53 harboring arginine to lysine mutations positions corresponding to reported sites of PRMT5 methylation: (333, 335, 337, denoted p53RK(13); were utilized as substrates. PRMT5/MEP50 catalyzed methylation of wild type p53, while methylation was abrogated in the p53RK mutant (Fig S6A). Catalytically dead PRMT5 also failed to methylate p53 (Fig S6B). To assess whether D1T286A-dependent phosphorylation of PRMT5/MEP50 induced p53 methylation, immunopurified PRMT5/MEP50 complexes were mixed with purified, active cyclin D1T286A/CDK4 kinase and ATP in CDK4 kinase buffer. PRMT5/MEP50 complexes were then washed extensively and mixed with recombinant p53 and ³H-SAM. Similar to published results using a histone H4 substrate (5), D1T286A/CDK4 kinase triggered a significant increase in PRMT5-dependent methylation of p53 (Fig S6C). To confirm this reflects phosphorylation of MEP50 on Thr-5, we performed an analogous experiment, using PRMT5/MEP50, PRMT5/MEP50T5A or PRMT5/MEP50T5D complexes (MEP50T5D is a phosphomimetic mutant in which threonine 5 was mutated to asparagine (D) to mimic constitutive phosphorylation) (Fig S6D). While D1T286A/CDK4 increased catalysis by PRMT5/MEP50, the MEP50T5A complexes retained only basal activity. By contrast, PRMT5/MEP50T5D complexes exhibited activity equivalent to phosphorylated wild-type PRMT5/MEP50 complexes and were refractory to further activation.

To independently address D1T286A-enhanced PRMT5 activity towards p53, we generated a p53-me2 specific antibody that specifically recognizes p53 symmetrically methylated on arginines 333, 335, 337 (Fig S5C–D). Using this antibody, elevated methyl p53 levels were noted following expression of D1T286A (indicated with double arrows) in NIH3T3 cells that harbor wild-type p53 compared to that in untransfected cells (single arrows) (Fig S6E). We also noted that co-transfection of PRMT5 with D1T286A (triple arrows compared to double arrows) resulted in a strong increase in p53-me2 staining (Fig S6F). Importantly, p53-me2 and PRMT5 were nuclear demonstrating that methylation of p53 did not trigger nuclear exclusion (Fig S6E–F).

D1T286A–dependent activation of PRMT5/MEP50 inhibits p53-dependent induction of pro-apoptotic genes

Tumor suppression by p53 can reflect either transcription-dependent (nuclear) activities and/or transcription-independent (cytoplasmic) activities (27, 33). No differential targeting of p53 to the cytoplasm was observed suggesting a transcription-dependent impact (Fig S6E–F). PRMT5-dependent methylation of p53 occurs within the p53 oligomerization

domain suggesting a direct influence on p53 DNA binding and transcriptional output. To test this, we assessed the gene expression profiles of PRMT5, D1T286A or D1T286A +PRMT5 in HSCPs using a quantitative PCR array containing 84 p53 target genes. D1T286A expression triggered varying induction of p53 target genes, including factors involved in apoptosis, cell cycle and DNA repair (Fig 6A). PRMT5 expression alone had little or no impact on expression of these genes. In contrast, co-expression of PRMT5 with D1T286A antagonized induction of a majority of the genes induced by D1T286A alone (Fig 6A). We independently confirmed that D1T286A expression triggered strong increases in mRNAs encoding *Apaf1*, *Bax*, *Pmaip1* (*Noxa*), *Casp9*, and *Gadd45a* (Fig 6B). We also noted increased expression of *Bbc3* (*Puma*), which was not represented on the array (Fig 6B). Importantly, co-expression of PRMT5 with D1T286A reduced expression of these pro-apoptotic genes. Decreased expression did not reflect the absence of D1T286A (Fig 1H; Fig S5A). We also noted a strong increase in D1T286A-dependent increase in *Cdkn1a* expression, which was also antagonized by PRMT5 (Fig 6B).

Chromatin immunoprecipitation (ChIP) was performed using bone marrow cells isolated from 5-FU treated C57BL/6 mice transduced with D1T286A, PRMT5 or D1T286A +PRMT5 to address p53 recruitment. Expression of D1T286A resulted in a significant increase in p53 occupancy on all promoters tested (Fig 6C). While expression of PRMT5 alone failed to influence p53 occupancy, co-expression with D1T286A significantly reduced p53 occupancy on *Cdkn1a*, *Apaf1*, and *Bax* promoters, but had no statistically significant effect on p53 occupancy on the *Pmaip1* promoter (Fig 6C).

Recent work has also demonstrated that E2f1, analogous to p53, can be methylated by PRMT5 (34); here methylation appears to destabilize E2f1 thereby reducing its transcriptional activity and ultimately expression of pro-apoptotic genes. Most of the genes induced by D1T286A are targets of both E2f1 and p53(12, 34). To determine whether E2f1 might differentially engage promoters cooperatively with p53 following D1T286A expression, we performed ChIP using an E2F1-specific antibody. D1T286A transduction resulted in small but significant recruitment of E2F1 to the *Apaf-1*, and *Cdkn1a* promoter regions; recruitment to the *Cdkn1a* promoter was sensitive to PRMT5 (Fig S7A). Strikingly D1T286A triggered a much stronger induction of E2f1 recruitment to the *Pmaip1* promoter and this was entirely reversed by PRMT5 (Fig S7A) suggesting that E2f1 likely contributes more to *Pmaip1* expression. No enrichment of E2F1 was observed at the *Bax* promoter (Fig S7A).

PRMT5-dependent dimethylation of histone H4 arginine 3 (H4R3) is associated with transcriptional repression (5–7). We determined whether direct histone methylation might also contribute to PRMT5-mediated gene silencing of *Apaf-1*, *Cdkn1a*, *Bax* and *Pmaip1* by performing ChIP with an antibody specific for dimethylated histone H4R3 and using primers specific to the proximal promoter regions (~500bp upstream of the first coding exon). Cyclin D1T286A and PRMT5 co-expression resulted in increased methylation of H4R3 at both the *Apaf1* and *Pmaip1* promoters (Fig S7B). These data suggest that *Apaf1* and *Pmaip1* suppression reflects coordinated repressive histone modification and reduced occupancy of E2f1 and p53 all of which are PRMT5/D1T286A dependent.

Elevated p53 arginine methylation in human cancer

If arginine methylation antagonizes p53 activity in response to expression of oncogenic D1 alleles, tumors should exhibit increased p53me2. We utilized the antibody reactive against p53 dimethylated on arginines 333,335,337 (Fig S5C–D) to assess p53me2 status in tumors. Immunoblot of tumor lysates revealed a marked induction of p53me2 in the D1T286A/PRMT5 but not D1T286A/p53DN lymphoid tumors (Fig 7A). These data support a mechanism wherein survival of D1T286A expressing cells is driven by and may require PRMT5-dependent methylation of p53. We also examined p53me2 in murine lymphomas driven by either ICN or c- MYC. Increased arginine p53me2 was noted in all ICN and c- MYC driven tumors examined (Fig 7B–C).

We next examined p53 arginine methylation status in primary human cancers wherein D1 is a driver. Immunohistochemical staining of lymph nodes sections from human MCL patients revealed a marked increase of p53me2 relative to normal lymph node (Fig 7D). Increased staining was readily apparent in 6 of 8 primary MCL specimens. PRMT5 overexpression (Fig S8A) and increased symmetrical dimethylation histone H4R3 (Fig S8B), a marker for PRMT5 methyltransferase activity were also noted in these samples. Forty percent of esophageal squamous cell carcinoma (ESCC) is associated with nuclear cyclin D1 as a driver (35). Immunohistochemistry in tissue microarrays (TMAs) containing paired tumor and adjacent non-neoplastic clinical specimen revealed 44% of the ESCCs exhibited concurrent high p53me2 and high nuclear D1 (Fig 7E); 46% simultaneously exhibited high expression of PRMT5 and high nuclear D1 (Fig S8C–D). Finally, we also assessed p53me2 status in a panel of T-cell leukemia/lymphoma-derived cell lines relative to normal peripheral lymphocytes. Arginine methylation of p53 was readily detected in cancer cell lines with wild type p53, while cell lines harboring mutant p53 exhibited low to undetectable arginine methylation (Fig 7F). These data support the utilization of PRMT5-dependent methylation of p53 as an alternative to the acquisition of inactivating p53 mutations.

DISCUSSION

PRMT5 levels are frequently high in human lymphoid cancers and it is thought to contribute directly to manifestation of the malignancy. Although high levels of PRMT5 are associated with increased proliferation (36), mechanistic insights into its contribution will be critical to determine its potential as a therapeutic target. We have assessed the capacity of PRMT5 to regulate lymphomagenesis triggered by four distinct oncogenic drivers: Cyclin D1, c- MYC, NOTCH1 (ICN), and MLL-AF9. Notably, overexpression of PRMT5 was found to cooperate with an oncogenic allele of D1 (D1T286A). Intriguingly, co-expression of PRMT5 failed to notably increase the penetrance or decrease onset of disease triggered by any of the other three driver oncogenes. However, expression of a PRMT5 dominant negative allele or knockdown of endogenous PRMT5 significantly inhibited the ability of all three to induce neoplastic growth in the colony-forming assay. While seemingly a paradox, this likely reflects the capacity of c- MYC, NOTCH and MLL-AF9 to potently induce high levels of endogenous PRMT5 expression thereby abrogating the need for co-expression. Collectively, these data identify PRMT5 as a point of convergence during lymphomagenesis.

PRMT5 antagonizes p53-dependent tumor suppression

While cyclin D1 has been considered a driver oncogene since its identification as the PRAD1 oncogene in parathyroid adenoma and the BCL1 oncogene in mantle cell lymphoma (37, 38), we have only recently gained insights to the molecular underpinnings whereby it triggers a neoplastic switch. Current evidence suggests that failure to inactivate the nuclear D1/CDK4 kinase during S-phase, dysregulates the “once and only once” regulation of DNA replication initiation, a direct consequence of Cdt1 stabilization (19). Activation of PRMT5 is a key to this phenotype in that it generates repressive histone marks within the *Cul4A/B* promoter. The loss of Cul4A/B ultimately leads to overexpression of Cdt1, a key component of the replication licensing machinery (39). This is in turn sensed by the DSB checkpoint effector ATM (20) that phosphorylates and activates p53.

The activation of p53 in this context is highly apoptotic (Figures 1–2) (40) and it effectively inhibits tumorigenesis triggered by D1T286A (19). Inactivating mutations in p53 occur at a surprisingly low frequency in animal models of cancer driven by cyclin D1 and even lower frequency in human MCL (31). The retention of wild-type p53 is generally associated with the loss of key effectors such as p19^{ARF} (p14^{ARF} in human cells) or with the overexpression of MDM2 (18, 40–42). Since neither of these mechanisms was observed in D1T286A–driven tumors (16); data herein), we considered alternative mechanisms for bypassing p53 function. Among the potential non-histone targets of PRMT5/MEP50 is p53. Given that arginine methylation was suggested to modify the pro-apoptotic response of p53, we considered whether D1T286A-dependent activation of PRMT5/MEP50 might directly inhibit p53 and p53-dependent cell death thereby precluding the selection for inactivating p53 mutations. Consistent with this hypothesis, co-expression of PRMT5 with D1T286A bypassed p53-dependent apoptosis and resulted in the rapid acquisition of an aggressive CD4⁺ lymphoma that retained wild-type p53. The need for co-expression of PRMT5 in this system likely reflects its very low expression in HSCs relative to progenitor lineages. This is in contrast to the Eμ-D1T286A model where D1T286A expression is targeted to lineage committed IgM⁺/IgD^{low} B-lymphocytes that express PRMT5 (3). In the latter model, PRMT5 levels are at a threshold wherein phosphorylation-dependent increases in its function are sufficient for disease manifestation.

Importantly, we also noted increased PRMT5 expression and p53 methylation in primary human MCL and in esophageal squamous cell carcinoma. The physiological importance of arginines R333, 335 and 337 in the regulation of p53 function is further emphasized by their mutation in Li Fraumeni families that present a wide spectrum of tumors (43, 44).

Similar to D1T286A, we noted increased p53 methylation on arginines targeted by PRMT5 in NOTCH and MLL-AF9 triggered tumors, but not those generated by c-MYC expression, suggesting that PRMT5 may contribute to p53 inactivation in tumors where NOTCH or MLL-AF9 function as drivers, thereby alleviating selection for p53 mutation. In the context of c-MYC, it has already been established that p53 mutation or biallelic deletion of Arf are the primary genetic events for p53 bypass (42). Importantly however, our data suggest that PRMT5 may represent a unique therapeutic target in multiple neoplastic settings. In point of fact, PRMT5 inhibition can induce lymphoma cell death (45); and has also been suggested

as a therapeutic target for glioblastoma (46). Collectively, the data support a model wherein PRMT5 exhibits broad pro-proliferative and pro-survival activities and wherein the precise mode of action by PRMT5 likely reflects genetic and perhaps tissue specificity.

PRMT5/MEP50-mediated methylation modifies p53-dependent transcription

An open question that remains is why do certain oncogenic drivers utilize mechanisms to bypass p53-dependent tumor suppression such as methylation (e.g. cyclin D1), while others select for mutation of p53 (e.g. c-MYC). One possibility is that each mechanism, while reducing p53 function, permits the maintenance of key p53 functions that are important for tumor progression. Data demonstrating that mutant p53 alleles frequently have neoplastic activities and thus do not equate with p53 deletion support this conclusion (28). Likewise, methylated p53, while having significantly reduced transcriptional activity at many gene targets (*Cdkn1a* and *Apaf1*), exhibits less sensitivity at other targets (e.g. *Pmaip1*). A second more applicable consequence of maintenance of wild type p53 might reflect in how tumors respond to therapeutic intervention. With D1 driven tumors such as MCL, inhibition of PRMT5 should not only directly impact many transcriptional programs, but should also permit functional reactivation of wild type p53 and p53-dependent tumor suppressive activities. The development of PRMT5 selective inhibitors will allow further investigation of these concepts in many distinct tumor contexts.

METHODS

Cell Culture

HEK 293T (obtained from and authenticated by American Type Cell Culture) and NIH3T3 cells (a gift from Charles J. Sherr St Jude, authenticated by Southern Blot) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. HSB2, LOUCY, 8402, PF382, TALL-1 (obtained from and authenticated by American Type Cell Culture) cells were maintained in RPMI-1640 containing 10% FBS, 1% Glutamine and 1% Pen/Strep supplemented with 0.05mM 2-mercaptoethanol. MOLT-3, MOLT-4 and CEM cells (obtained from and authenticated by American Type Cell Culture) were maintained in RPMI-1640 containing 10% FBS and 1% Pen/Strep.

Plasmids and Retroviruses

Cyclin D1 and D1T286A were flag-tagged and subcloned into MSCV-IRES-GFP (MigR1). The 6X myc tagged human PRMT5 from pCS2-PRMT5 and myc-tagged human MEP50 from pcDNA3-myc-MEP50 (5) were subcloned into pcDNA3, MSCV-IRES-tNGFR (tNGFR), or MSCV-IRES-mCherry (mCherry subcloned with MigR1 and pCS2-mCherry) vectors. p53R175H (p53DN) was subcloned into tNGFR and mCherry vectors. PRMT5 and p53RK mutants were generated with Quik Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. All clones were sequenced in their entirety. Retroviral supernatants were generated by transient transfection of 293T cells with Lipofectamine.

Bone Marrow Transplantation

All animal experiments were conducted in compliance with Animal Care and Use Committee of University of Pennsylvania and Medical University of South Carolina. Bone marrow transplantation (BMT) experiments were performed as previously described (47). Briefly, bone marrow cells were collected from 6–8-week-old C57BL/6 or B6.129S2-Trp53tm1Tyj/J (p53^{−/−}) (The Jackson Laboratory) mice 4 days after intravenous administration of 5-fluorouracil (5-FU; 150 mg/kg) and retrovirally transduced *ex vivo* in the presence of IL-3, IL-6, and stem cell factor (SCF). Retroviral supernatants with equal titers were used to produce similar transduction efficiencies. GFP⁺ NGFR⁺ cells ($0.5\text{--}1 \times 10^6$) were then injected intravenously into lethally irradiated (900 rads) B6 recipients. Chimeric mice were maintained on antibiotics for 2 weeks.

FACS

Single cell suspensions prepared from bone marrow and spleen were stained on ice in PBS plus 2% FBS and analyzed on FACSCalibur, FACSVerse, LSRII or FACSARIA (BDBiosciences). Files were analyzed in Flowjo (TreeStar).

Cell and Colony Growth in Methylcellulose

For assessing the total hematopoietic progenitor cell activity, bone marrow was harvest from 5-fluorouracil treated C57BL/6 mice. After red blood cell lysis using ACK lysis buffer (Lonza), 2×10^4 nucleated cells were plated in triplicates into methylcellulose medium (MethoCult 3234, Stem Cell Technologies) supplemented with 50 ng/ml Flt3L, 50 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, and 10 ng/ml IL-7 (Stem cell Technologies). The colony number was counted 7 days after replating.

Immunohistochemistry and Immunofluorescence

Tissue was fixed in 4% buffered formalin and subsequently was dehydrated, paraffin embedded, and sectioned. Tissue sections were immunostained as described previously (19), with meR-p53 at 1: 200 dilution and PRMT5 at 1: 150 dilution as the primary antibody. For immunofluorescence, cells were fixed, blocked and immunostained as described (48).

p53 Signaling Pathway PCR Array

Total RNA was extracted using the RNeasy Micro kit (Qiagen), and used as template to synthesize cDNA (RT² First Strand Kit) for quantitative RT-PCR (qRT-PCR) analysis with the Mouse p53 Signaling Pathway PCR Array (PAMM-027; SuperArray Bioscience Corporation). Primers for *Apaf1*, *Cdkn1a*, *Bax*, *Pmaip1*, *Casp9*, *Gadd45a*, and *Bbc3* were generated according to the RT² qPCR Primer Assay, Qiagen.

Western Blot and Chromatin Immunoprecipitation (CHIP)

Western blot was carried out as previously described (5). Chromatin was prepared using truChIP Low Cell Chromatin Shearing Kit (Covaris) and sheared into 200–700 bp fragments using a Covaris S2 instrument (duty cycle, 2%; intensity, 3; 200 cycles per burst; 4 min). Immunoprecipitation was performed using the IgG, p53 (FL-393), E2F-1 (C-20) and H4R3 (Abcam) antibodies with a Quick Chip Kit (Imgenex, San Diego, CA). Quantification of the

precipitated DNA was determined with qPCR (Qiagen, QuantiTect SYBR Green Mastermix) and normalized with the input genomic DNA. Primers used are: *Apaf-1* (E2F-1) forward, 5'-TAGTTTTGTAGGCACACAGCTCTAAATAGGAG-3', *Apaf-1* (E2F-1) reverse, 5'-CGGATGAGTTTGCTCACACCCTCCACC-3'; *Pmaip1* (E2F-1) forward, 5'-GCCCCAGCAATGGATACGA-3', *Pmaip1* (E2F-1) reverse, 5'-TGCTCAACCCCCAAATTGCT-3'. The other primers are from Qiagen EpiTect ChIP qPCR Primers.

Statistical Analysis

Statistical significance was determined by Student's t test using the Excel software; $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Nancy Speck for advice and technical expertise for BM reconstitution experiments and Zhaorui Lian for technical assistance.

GRANT SUPPORT

This work was supported by CA11360 (JAD) and P01-CA098101 (JAD, AKR, HN, AJP). We thank NIH-P30-DK050306, the Molecular Pathology and Imaging, Molecular Biology and Cell Culture Core Facilities.

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STATEMENT OF SIGNIFICANCE

We have identified and functionally validated a crucial role for PRMT5 for the inhibition of p53-dependent tumor suppression in response to oncogenic insults. The requisite role for PRMT5 in the context of multiple lymphoma/leukemia oncogenic drivers suggests a molecular rationale for therapeutic development.

Figure 1. PRMT5 cooperates with D1T286A to drive T-cell leukemia/lymphoma

(A) Kaplan-Meier survival analysis of mice reconstituted with FACS purified (GFP-NGFR double positive) BM-derived HSC transduced with MigR1 and tNGFR vectors (–), MigR1-cyclin D1 (D1), MigR1-cyclin D1T286A (T286A) and tNGFR-6xmyc-PRMT5 (PRMT5) retroviruses. (B) Representative photographs of involved organs derived from T286A +PRMT5 mice. (C) White blood cell (WBC) counts from the peripheral blood of the indicated genotype; *p<0.05. (D) Wright-Giemsa-stained peripheral blood and bone marrow single cell suspensions from WT and T286A+PRMT5 mice. Arrows indicate white blood

cells in the WT and lymphoma cells in the T286A+PRMT5 mice blood smear. Scale bar, 30 μ m. **(E)** Histology of the spleen, liver, thymus, lung and kidney of tumor burdened mice of the indicated genotype. Scale bar, 1,000 μ m. **(F)** Representative FACS of bone marrow and spleen. **(G)** Quantification of data from F: **p<0.01 **(H)** Western analysis of spleen lysates prepared from the indicated genotype.

Figure 2. MEP50 promotes D1T286A+PRMT5-induced T-cell lymphoma in mice
 (A–C) 5 -FU–treated bone marrow cells were transduced with MigR1-D1T286A (T286A), tNGFR-PRMT5, mCherry-MEP50 or mCherry-MEP50T5A retroviruses as indicated. GFP-NGFR-mCherry triple positive or GFP-NGFR double positive cells were purified and transplanted into recipient mice. (A) Survival depicted by Kaplan-Meier survival analysis. (B) FACS of bone marrow and spleen derived single cell suspensions. (C) Summary of the data in B; **p<0.01. (D) 5 -FU–treated bone marrow cells were transduced with T286A, PRMT5 and MEP50T5A retroviruses and transplanted into recipient mice. Bone marrow

cells were isolated 2 and 4 weeks following bone marrow transplantation (BMT) and GFP-NGFR-mCherry triple positive cells were quantified by FACS. ** $p < 0.01$ compared to percentage of triple positive cells before BMT.

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Figure 3. PRMT5 is required for lymphoma/leukemia driven by NOTCH 1(ICN) and c-MYC
 5-FU-treated bone marrow cells were transduced with empty MigR1 vector (–) or with vectors encoding the indicated cDNAs and transplanted into recipient mice. (A) Kaplan-Meier survival curves. (B) Western analysis of PRMT5 in ICN1 or c-MYC plus PRMT5 induced lymphoma/leukemia. (C) 5-FU-treated bone marrow cells were transduced with shPRMT5 and sh-control viral supernatants followed by MigR1-c-MYC (MYC) or MigR1-ICN1 (ICN1). Cells were transplanted into recipient mice and bone marrow isolated 2~3 months post-transplantation. Western analysis depicts PRMT5 expression in bone marrow

before BMT and in spleen after BMT. **(D)** 5-FU–treated bone marrow-derived HSPC were transduced with MigR1-c-MYC (MYC) and tNGFR-myc-PRMT5 retrovirus or ICN and myc-PRMT5 as indicated. Western analysis performed as indicated. **(E)** 5-FU–treated bone marrow cells were transduced with MigR1-ICN1 (ICN1) and tNGFR-myc-PRMT5 (left) or MigR1-c-MYC (MYC) and tNGFR-myc-PRMT5 retroviruses (right) and plated into methycellulose media. Colonies were subjected to 5 rounds of serial replating. Colony quantification following each plating is provided. * $p < 0.05$, ** $p < 0.01$.

Figure 4. Cyclin D1T286A triggers p53-dependent apoptosis of BM-derived lymphocytes
5-FU-treated bone marrow cells (p53^{+/+} or p53^{-/-} donor mice) were transduced with either MigR1 empty vector (-), MigR1-cyclin D1wt (D1) or MigR1-cyclin D1T286A (T286A) retroviruses. (A) GFP⁺ cells were sorted and transplanted into irradiated (900 rads) recipient mice. (B) GFP⁺:GFP⁻ (1:1) were transplanted into recipient mice and bone marrow cells were isolated 1 month after transplantation and GFP⁺ cells were quantified by FACS. (C) Cells were transplanted into recipient mice and bone marrow cells were isolated 9 days after transplantation. GFP⁺ cells were gated and the apoptotic cells identified by FACS for Annexin V positive cells. Top: Representative FACS profile. Bottom: Quantification of 3-independent experiments (**p<0.01.). (D) 5-FU-treated bone marrow cells (from p53^{+/+} or p53^{-/-} donor mice) transduced with indicated retroviruses, were sorted for GFP, and transplanted into lethally irradiated recipients. Survival is illustrated by a Kaplan-Meier

analysis. (E) 5-FU–treated bone marrow cells transduced with indicated retroviruses, were sorted for GFP-NGFR double positive populations, and transplanted into lethally irradiated recipients. Survival is illustrated by a Kaplan-Meier analysis.

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Figure 5. PRMT5/MEP50 inhibits Cyclin D1T286A-dependent apoptosis and potentiates colony outgrowth
5-FU-treated bone marrow was transduced as follows: MigR1, tNGFR and mCherry empty vectors (–), MigR1-cyclin D1 (D1), MigR1-cyclin D1T286A (T286A), tNGFR- PRMT5 (PRMT5), tNGFR- PRMT5 (PRMT5Δ), tNGFR- p53DN (p53DN), mCherry-MEP50 (MEP50), mCherry-MEP50T5A (T5A). Cells were isolated for analysis 9 days post-transplantation. (A and C) GFP and mCherry double positive (A) or GFP, NGFR and mCherry triple positive (C) cells were gated and apoptotic cells were identified by a fluorescence-labeled Annexin V. Data are mean ± SD (**p<0.01). (B and D) Double (B) or triple positive cells (D) were purified and plated in methylcellulose cultures. Total colony numbers were scored 7 days after each plating.

Figure 6. Analysis of differential gene expression profiling by PRMT5 and D1T286A

Lin⁻, 5-FU-treated bone marrow cells were transduced with MigR1 and tNGFR vectors (–), cyclin D1T286A (T286A), PRMT5 or D1T286A+ PRMT5 and transplanted into lethally irradiated recipient mice. Nine days post-transplantation, GFP and NGFR double positive bone marrow cells were isolated for the following analyses: (A) Expression profiles of p53 target genes (B) RT-PCR analysis of *Apaf1*, *Cdkn1a*, *Bax*, *Pmaip1*, *Casp9*, *Gadd45a* and *Bbc3*. (C) Detection of effect of PRMT5 and D1T286A on the *Apaf1*, *Cdkn1a*, *Bax* and

Pmaip1 promoters. ChIP was performed using IgG or p53 (FL-393) (blue) antibody;
*p<0.05; **p<0.01.

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Figure 7. p53me2 is increased in mouse lymphoma/leukemia models and primary human cancer (A) Western analysis of meR-p53 in cyclin D1T286A/PRMT5 and cyclin D1T286A/ p53DN- induced lymphoma/leukemia. (B) Western blot of meR-p53 and p53 in ICN1- induced lymphoma/leukemia. Membranes used in figure 3 were re-blotted for p53 or p53me2. The β-Actin blot is provided again for continuity. (C) Western blot of meR-p53 and p53 in c-MYC-induced lymphoma/leukemia. Membranes used in figure 1 and figure 3 were re-blotted for p53 or p53me2. The β-Actin blot is provided again for continuity. (D) Immunohistochemical staining of IgG (top left) and meR-p53 (top right and bottom) in

primary human mantle cell lymphoma samples (specimen number in parentheses). Scale bar, 800 μm . (E) Quantification of Immunohistochemical staining of meR-p53 and cyclin D1 in primary human ESCC samples. Scoring was conducted in a blinded fashion. (F) Western blot of meR-p53 and PRMT5 in normal lymphocytes or 9 human T-ALL cell lines.

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