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PRMT5 control of cGAS/STING and NLRC5 pathways defines melanoma response to antitumor immunity

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Abstract

Protein arginine methyltransferase 5 (PRMT5) controls diverse cellular processes and is implicated in cancer development and progression. Here, we report an inverse correlation between PRMT5 function and antitumor immunity. PRMT5 inhibition antagonized melanoma growth in immunocompetent but not immunocompromised mice and upregulated an antitumor immune gene signature. PRMT5 methylation of IFI16 (interferon gamma inducible protein 16) and its murine homologue IFI204, which are cGAS (cyclic GMP-AMP synthase)/STING (stimulator of interferon genes) pathway components, attenuated cytosolic-DNA-induced interferon and chemokine production. PRMT5 also inhibited transcription of NLRC5 (NLR Family CARD Domain Containing 5), which regulates genes implicated in MHC1 antigen presentation. Correspondingly, PRMT5 knockdown augmented interferon and chemokine production and increased MHC1 expression. Elevated expression of IFI16/IFI204 and NLRC5 was associated with decreased melanoma growth in murine models and prolonged survival of melanoma patients. Notably, combination of pharmacological (GSK3326595) or genetic (shRNA) inhibition of PRMT5 with immune checkpoint therapy limited growth of murine melanoma tumors (B16F10 and YUMM1.7) and enhanced therapeutic efficacy. Overall our findings provide a rationale to test PRMT5 inhibitors in immunotherapy-based clinical trials as a means to enhance an antitumor immune response.

One Sentence Summary:

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PRMT5 expression in melanoma suppresses inflammation and antigen presentation, suggesting its inhibition could potentiate immunotherapy

INTRODUCTION

Unleashing an antitumor immune response using immune checkpoint inhibitors has revolutionized cancer therapy. Better understanding of immune checkpoint regulatory pathways is now expected to increase the rate of success and efficacy of immune checkpoint therapy (ICT). At present, only a subset of tumor types benefits from ICT, while a notable percentage of patients either fails to respond or acquires resistance to ICT (10–44% objective response rate following ipilimumab, nivolumab, or pembrolizumab treatment in advanced melanoma) (1–4). Thus, understanding tumor-intrinsic mechanisms that underlie either the response to or evasion of ICT should provide tools to overcome intrinsic or adaptive resistance to therapy.

Among issues relevant to responses to ICT are the extent of tumor infiltration by and activation of immune cells, especially CD8 T cells, as well as loss of tumor antigenicity. Activation of oncogenic Wnt/beta-catenin signaling or loss of tumor suppressor PTEN expression hampers CD8 T cell infiltration of tumors and confers resistance to ICT (5, 6). Expression of chemokines such as CXCL9 and CXCL10 or upregulation of the type I interferon response is also regulated by epigenetic factors, including EZH2 (Histone-lysine N-methyltransferase) and LSD1 (Lysine-specific histone demethylase), both of which alter CD8 T cell recruitment to tumors (7, 8). Loss of antigen presentation, a mechanism underlying tumor-intrinsic immune evasion, is associated with resistance to ICT. Homozygous deletion of B2M (beta-2-microglobulin), the beta subunit for all HLA class I complexes, impairs antigen processing and presentation by tumor cells, contributing to resistance to ICT in melanoma and lung cancer (9, 10). These findings support the idea that control of tumor-intrinsic immune suppression, in part through altering the interferon response, chemokine production and antigen presentation, may overcome resistance to ICT.

The epigenetic modifier PRMT5 catalyzes monomethylation and symmetric dimethylation of arginine (Arg, R) residues on histones and non-histone proteins, thereby regulating diverse processes related to oncogenesis, including transcription, RNA splicing, translation and the DNA damage response (11, 12). In lymphomagenesis, the MYC-PRMT5 axis is implicated in maintaining fidelity of pre-mRNA splicing. PRMT5 is also thought to function in removal of introns retained in proliferation genes, supporting growth of glioblastoma tumors, and in control of metastasis in lung cancer (13–15). PRMT5 activity aberrantly decreases in 20–40% of tumors that harbor deletion of *MTAP* (methylthioadenosine phosphorylase), which is often co-deleted with *CDKN2A*, likely due to inhibition by the MTAP substrate 5'-O-methylthioadenosine (MTA). Thus, interestingly, MTAP-deleted tumors are relatively more sensitive to PRMT5 inhibition (16–19).

Several adaptor proteins are reportedly important for PRMT5 activity and substrate selectivity. Among them, WDR77 functions in histone methylation and concomitant transcriptional repression by PRMT5 (20). The adaptor pCln/CLNS1A impacts PRMT5-dependent snRNP (small nuclear ribonucleoproteins) methylation and subsequent splicing

(21), while the adaptor SHARPIN contributes to PRMT5-dependent methylation of SKI, resulting in SOX10 transcriptional activation (19). Thus, expression of PRMT5 adaptor proteins is a critical determinant of which substrate(s) PRMT5 will methylate.

Given that PRMT5, or factors required for its activity, are promising therapeutic targets, especially in MTAP-deleted tumors, small molecule PRMT5 inhibitors have been developed. A SAM non-competitive PRMT5 inhibitor (GSK3326595) reportedly activates the p53 pathway by controlling alternative splicing of MDM4 (22, 23). In addition, two SAM competitive PRMT5 inhibitors (JNJ-64619178 and PF-06939999) are being evaluated in diverse tumor types ([NCT02783300](#), [NCT03573310](#), [NCT03614728](#) and [NCT03854227](#)). Given that PRMT5 is also implicated in embryonic development and hematopoiesis, it likely to exert cell-specific effects by methylation of diverse cellular targets (24, 25).

Here, we identify and characterize a tumor-intrinsic function of PRMT5 in promoting immunosuppression in melanoma. We demonstrate that PRMT5 controls both the cGAS/STING cytosolic DNA sensing pathway as well as MHCI antigen presentation, complementary regulatory modules that define tumor immune evasion and play important roles in the response to ICT.

RESULTS

PRMT5 expression is inversely correlated with an antitumor immune signature.

The finding that SHARPIN interaction with PRMT5 is important for PRMT5 methylation of specific substrates (19) led us to assess SHARPIN expression in cohorts of melanoma tumor specimens. Analysis of melanoma patient datasets revealed that low SHARPIN expression in MTAP-deleted tumors is associated with better survival (fig. S1A and S1B) (19). To identify pathways that may be regulated by SHARPIN, we assessed differentially-expressed genes (DEGs) in cohorts of metastatic melanoma patients specimens. MTAP-deleted tumors harboring low-SHARPIN expression exhibited enrichment of genes associated with immune-related pathways (Th1/Th2, IL-2/Stat5, TNFalpha), relative to those with high SHARPIN expression (fig. S1C and S1D, and table S1). This observation pointed to the possibility that SHARPIN may be involved in the control of immune phenotypes in MTAP-deleted melanoma. Given that both MTAP and SHARPIN positively regulate PRMT5 (19), we asked whether PRMT5 expression levels in melanoma tumors were associated with a particular gene set (Fig. 1A). Notably, low-PRMT5 melanomas exhibited enriched expression of immune-associated genes (Fig. 1B and 1C; fig. S1E, S1F and tables S2, S3), similar to changes seen in MTAP-deleted melanomas with low-SHARPIN expression. Analysis of an independent cohort of melanomas (GSE78220, n = 27) (26) confirmed enrichment of an immune gene signature (allograft rejection and interferon gamma response) in PRMT5-low tumors (fig. S2).

Among the PRMT family members, PRMT5, PRMT1, PRMT2, CARM1 and PRMT7 are expressed at relatively high levels in human melanoma specimens (fig. S3A), of which, PRMT5, PRMT1 and CARM1 are co-expressed (fig. S3B). Notably, high PRMT5, PRMT1 and CARM1 expression coincided with lower survival (fig. S3C). Moreover, PRMT5 expression exhibited the strongest inverse correlation with expression of immune response

genes (fig. S3D). Correspondingly, enrichment of an immune pathway signature was also seen in melanomas harboring low-MTAP expression and low PRMT5 activity (fig. S3E), supporting a potential role for PRMT5 in tumor immunity.

PRMT5 inhibition attenuates tumor growth in an immunocompetent murine melanoma model.

To validate *in silico* analyses, PRMT5 function in antitumor immunity was assessed using B16F10 (B16) metastatic murine melanoma cells expressing either scrambled (Scr) or PRMT5-specific shRNA. PRMT5 knockdown (KD) in B16 cells resulted in reduced (83–90%) PRMT5 expression and decreased PRMT5 activity (Fig. 2A). PRMT5-KD did not affect the growth of melanoma cells in culture (Fig. 2B). However, inoculation of these cultures in immunocompetent syngeneic C57BL/6 or in immunocompromised NOD-scid gamma (NSG) mice revealed important differences: in C57BL/6 mice, PRMT5 KD markedly inhibited growth (37.2–62.0% reduction in tumor volume and 28–54% reduction in tumor weight; Fig. 2C, fig. S4A and S4B), phenotypes not seen in B16 cells that were inoculated in the NSG mice (Fig. 2D; fig. S4C). The ability of PRMT5-KD B16 cells to develop tumors in immunocompromised but not immunocompetent mice suggest that PRMT5 inhibition of melanoma growth requires an intact immune system. Along these lines, transfer of B16 tumors from NSG to C57BL/6 mice resulted in growth inhibition of PRMT5-KD but not control (Scr) tumors (Fig. 2E–G; fig. S4D). Both the expression and activity of PRMT5 was attenuated in PRMT5-KD cells (shPRMT5 pools 1 and 2) and tumors (shPRMT5) (Fig. 2E and 2G). Consistent with the observations in B16, PRMT5-KD in YUMM1.7 cells, which are derived from a genetically engineered murine melanoma model (*Braf*^{V600E}/*Pten*^{-/-}/*Cdkn2a*^{-/-}), effectively inhibited tumor growth (52.4% in YUMM1.7; fig. S4E), while having a limited effect on growth of these cells in culture (14.7% in YUMM1.7; fig. S4F and S4G).

To substantiate the phenotypes seen upon KD of PRMT5, we performed gain-of-function studies using YUMMER1.7 cells, derived from UVB-irradiated YUMM1.7 cells, which increased mutation burden and antigenicity (27). Growth of tumors derived from YUMMER1.7 cells is inhibited in C57BL/6 but not Rag^{-/-} mice. Given the enhanced YUMMER1.7 immunogenicity, we asked whether PRMT5 overexpression would reduce their immunogenicity, resulting in increased tumor growth *in vivo*. To do so, we generated YUMMER1.7 cells overexpressing PRMT5 or its adaptor WDR77/MEP50, which is essential for PRMT5 activity (28), or a combination of both (Fig. 2H). YUMMER1.7 cells expressing PRMT5 and WDR77 exhibited elevated expression of PRMT5 protein and correspondingly, increased PRMT5 activity (Fig. 2H). Those cells also showed a moderate growth advantage in culture (relative to EV+EV controls) (Fig. 2I). Notably, *in vivo*, co-expression of both PRMT5 and WDR77 increased tumor growth in immunocompetent mice, relative to mice harboring control YUMMER1.7 tumors (Fig. 2J; fig. S4H). Such gain-of-function phenotypes were restricted to immune-competent animals and not seen in immune-compromised (NSG) mice (Fig. 2K and 2L), suggesting that PRMT5 activity supports tumor growth by limiting antitumor immune responses.

PRMT5 controls melanoma infiltration of immune cell.

Immunocompetent mice harboring tumors derived from PRMT5-KD cells exhibited reduced tumor growth (70 – 75% reduction in tumor weight at day 17) relative to control mice (fig. S4I–J). A markedly higher number of tumor-infiltrating immune cells was found in PRMT5-KD tumors, relative to Scr-expressing control tumors, which included CD45⁺ (3.4-fold increase, left panel), CD3⁺ (5.1-fold), CD4⁺ (4.1-fold) and CD8⁺ (5.1-fold) T cells, as well as natural killer (NK; 4.8-fold) cells, dendritic cells (DCs; 3.7-fold), and macrophages (4.1-fold) (Fig. 3A; fig. S4K). Among increased CD45⁺ cells (12.1-fold, right panel), two immune suppressor cells, MDSC (myeloid-derived suppressor cell, 4.9-fold) and Treg (4.5-fold), were also more abundant in PRMT5-KD tumors compared with Scr control tumors (Fig 3A, right). Thus, the relative abundance of active CD8⁺ T cells (CD44^{hi}CD8⁺) was compared to that of MDSC or Treg. Notably, relative abundance of activated CD8⁺ T (CD44^{hi}CD8⁺) to MDSC (CD11b⁺GR1⁺) or Treg (CD4⁺FOXP3⁺) cells was higher (2.4-fold, $p=0.0246$ for MDSC; 7.0-fold, $p=0.0383$ for Treg) in PRMT5-KD compared with control tumors (Fig. 3B). Consistent with these observations, immunohistochemical analysis of tumors collected at early growth phases (day 12) confirmed increased infiltration of immune cells [CD4⁺ (4.1-fold) and CD8⁺ (5.3-fold)] in tumors harboring PRMT5-KD compared with scrambled shRNA (Fig. 3C).

Conversely, PRMT5-overexpressing YUMMER1.7 tumors (grown in C57BL/6 mice) collected at an early growth phase (day 12) showed decreased immune cell infiltration, relative to control tumors, which included decrease in CD45⁺ (0.46-fold), CD44^{hi}CD4⁺ (0.26-fold), CD44^{hi}CD8⁺ (0.26-fold), natural killer (NK; 0.34-fold) cells, dendritic cells (DCs; 0.32-fold), and macrophages (0.24-fold) (Fig. 3D). To substantiate the possible contribution of key immune infiltrated cell types on the degree of tumor growth, we monitored the growth of PRMT5-KD B16 tumors following depletion of either NK or CD8⁺ T cells. Injection of mice with neutralizing antibodies to NK or CD8⁺ T cells restored the growth of PRMT5-KD tumors, relative to IgG controls (Fig. 3E–H; fig. S4L and S4M). These observations substantiate the control of immune cell infiltration by PRMT5, impacting degree of melanoma growth.

PRMT5 methylates IFI16/IFI204, a SHARPIN-interacting intracellular DNA-sensing protein.

Given that PRMT5 adaptor proteins play a critical role in substrate selectivity, we asked whether the expression of a particular PRMT5 adaptor(s) was linked with antitumor immunity. Gene Set Enrichment Analysis (GSEA) of metastatic melanoma specimens focused on low-PRMT5 specimens (excluding PRMT5 as a variable; Fig 4A; fig S5A), identified an inverse correlation between expression of several adaptors and the degree of anti-tumor immunity (Fig. 4B; fig. S5B). Among those, low level of SHARPIN expression exhibited a particularly significant correlation with the enrichment of immune gene signatures (Fig. 4B). Given that SHARPIN may serve as an intermediate between PRMT5 and its substrates, we set to identify SHARPIN-binding protein(s). To this end LC/MS/MS analysis was performed on SHARPIN interacting proteins, in the human melanoma WM115 cell line (homozygous *MTAP* deletion and sensitive to SHARPIN KD; (19). Among several SHARPIN-bound proteins that could be linked with antitumor immunity (table S4), was IFI16, a component of the intracellular DNA-sensing cGAS-STING complex. IFI16 contains

a DNA-binding hematopoietic interferon-inducible nuclear protein (HIN) domain (29, 30) and is implicated in controlling p53 transcriptional activity (31), in regulating cell cycle by binding to the retinoblastoma (Rb) protein (32), in anti-microbial immunity by sensing cytosolic DNA (29), and in inflammasome assembly through its interaction with cGAS and STING (23, 33). Interaction of IFI16, or its murine homologue IFI204, with SHARPIN was confirmed in series of IP reactions (Fig. 4C and 4D; fig. S6A–C). Degree of interaction between IFI16 and SHARPIN was enhanced in A375 melanoma cells (which harbor intact *MTAP* expression and higher PRMT5 activity) upon PRMT5 inhibition (using the pharmacological PRMT5 inhibitor, EPZ015666) (fig. S6C), pointing to the possibility that PRMT5 activity may limit IFI16 binding. To substantiate this observation, we assessed changes in IFI16 methylation following PRMT5 inhibition. A375 melanoma cells treated with the PRMT5 inhibitor (EPZ015666), exhibited a 50% decrease in IFI16 methylation, compared with 15% decrease in WM115 cells, which are *MTAP*-deleted and thus have lower basal level of PRMT5 activity (Fig. 4E; fig. S6D). Likewise, IFI204, the murine IFI16 homologue, exhibited reduced methylation (by 57%) in EPZ015666-treated B16 cells (Fig. 4F), which coincided with a stronger interaction with SHARPIN, as seen in the A375 cells (Fig. 4D). Search for consensus RG motifs which harbor arginine residues that could be methylated (19, 34, 35) identified Arg12 located within the N-terminal PYRIN (protein-protein interaction) domain, and Arg538, located in the C-terminal HIN (DNA-binding) domain in the murine IFI204 protein (fig. S6E). Mutating either Arg12, Arg538 or both in IFI204 allowed us to monitor possible changes in Arg methylation by PRMT5. Mutation of either Arg12Ala (R12A) or Arg538Ala (R538A) reduced the degree of IFI204 methylation whereas mutation of both residues further lowered the extent of IFI204 methylation, suggesting that both residues are PRMT5 methylation sites (Fig. 4G). To substantiate these observations, we performed *in vitro* methylation assays with IFI204 proteins. V5-tagged IFI204 proteins (WT, R12A, R538A or R12A/R538A) were immunopurified from HEK293T cells and subjected to *in vitro* methylation reaction using recombinant PRMT5/WDR77 (Fig. 4H; fig. S6F). While IFI204 WT protein was methylated by PRMT5/WDR77, this methylation was no longer seen in the presence of PRMT5i (fig. S6F). Notably, immunopurified WT IFI204, but not a mutant form lacking either Arg12, Arg538 or both, was methylated *in vitro* by recombinant PRMT5 (Fig. 4H).

IFI16/IFI204 methylation attenuates dsDNA-induced TBK1-IRF3 activation and interferon and chemokine production.

IFI16/IFI204 binding to intracellular dsDNA induces expression of *Ifnb1* and the chemokines *Ccl5*, and *Cxcl10* (23, 29, 33). Given that PRMT5 methylates IFI16/IFI204, we asked whether that modification regulated IFI204-dependent IFN β and chemokine induction. Attenuating PRMT5 expression (by shPRMT5) or activity (by EPZ015666) in B16 cells increased expression of *Ifnb1*, *Ccl5* and *Cxcl10* following stimulation with 70 base-pair dsDNA [referred to as V70-mer; (29)] (Fig. 5A and 5B). Conversely, PRMT5 overexpression decreased dsDNA-stimulated expression of all three genes both in B16 and YUMMER1.7 cells (Fig. 5C; fig. S7A). These observations suggest that PRMT5-dependent methylation of IFI204 limits dsDNA stimulation of IFN β and chemokines. Since sensing of dsDNA prompts the cooperation of IFI16/IFI204 with the cGAS-STING pathway to activate TBK1-IRF3 signaling and interferon production (23, 33), we assessed whether PRMT5

methylation of IFI16/IFI204 would impact cGAS-STING signaling. Overexpression of SHARPIN in B16 cells attenuated intracellular dsDNA-mediated activation of TBK1-IRF3 signaling and subsequent *Ifnb1*, *Ccl5* and *Cxcl10* expression (fig. S7B and S7C). Conversely, genetic inhibition of PRMT5 in B16 cells augmented dsDNA-induced activation of TBK1-IRF3 as reflected by levels of STING phosphorylation, dimerization and polymerization [(33, 36); Fig. 5D–F]. Similarly, pharmacological inhibition of PRMT5 in B16 melanoma cells activated TBK1-IRF3 signaling (Fig. 5G). These observations were further substantiated by the finding that ectopic expression of PRMT5/WDR77 in B16 or YUMMER1.7 cells effectively decreased TBK1-IRF3 activation (Fig. 5H; fig. S7D). To further substantiate the importance of IFI204 methylation, which is part of the cGAS-STING complex, we monitored changes in TBK1-IRF3 signaling following DNA stimulation. Ectopically expressed IFI204 in B16 cells activated TBK1-IRF3 signaling (fig. S7E) and increased the expression of *Ifnb1* and *Ccl5* (fig. S7F), following dsDNA treatment, compared to cells that expressed control plasmid. Notably, expression of methylation-defective R12A IFI204 (IFI204Mt1), but not R538A (IFI204Mt2), further increased TBK1-IRF3-mediated expression of downstream genes, relative to changes seen following expression of wildtype (WT) IFI204 (Fig. 5I). Consistent with these observations, IFI204Mt1 expression, but not that of IFI204Mt2, increased STING dimerization and polymerization following dsDNA-stimuli (Fig. 5J; fig. S7G), suggesting a critical role of IFI204 methylation on Arg12 in the dsDNA-stimulated STING pathway activation. In agreement, siRNA-mediated STING-KD markedly reduced, albeit not completely, activation of *Ifnb1*, *Ccl5* and *Cxcl10* seen after PRMT5-downregulation (Fig. 5K and fig. S7H). Of note, changes in PRMT5 expression did not alter cGAS or STING expression (fig. S7I), suggesting that PRMT5 limits the activation but not the expression cGAS/STING pathway components.

The observation that PRMT5 controls cGAS/STING pathway activation by dsDNA led us to explore whether PRMT5 might also regulate RIG-I/TLR3-mediated activation of a type I interferon response following dsRNA stimuli (37). B16 melanoma cells treated with the RIG-I/TLR3 agonist poly(I:C) induced *Ifnb1* and chemokine expression, which was significantly enhanced upon PRMT5 KD (fig. S7J). Consistent with these observations, B16 melanoma cells overexpressing IFI204Mt1, but not IFI204Mt2, exhibited increased induction of *Ifnb1* and chemokine expression by poly (I:C) relative to levels seen in WT cells (fig. S7K). These observations reveal an unexpected role for PRMT5/IFI16 in dsRNA-induced activation of type I interferon response and suggest that PRMT5 controls dsDNA-induced STING-dependent and dsRNA-induced activation of the type I interferon response.

PRMT5 regulates antigen presentation by controlling NLRC5 expression.

To search for PRMT5-regulated genes that may affect tumor immune responses, we surveyed both the TCGA metastatic melanoma dataset (n = 368) and the Cancer Cell Line Encyclopedia (CCLE) (38) (n = 58). Of co-regulated genes (155 genes from TCGA and 135 from CCLE) (fig. S8A), nine were common to both datasets, of which one – the transcriptional activator NLRC5 – had been implicated in regulating MHC class I gene expression (39, 40). NLRC5, along with B2M, HLA-A, -B, and -C, and PSMB9, are implicated in antigen presentation, as predicted by IPA analysis [log (p-value) = -13.9; Fig.

1B]. NLRC5 expression was inversely correlated with PRMT5 expression in both CCLE ($r = -0.516$, $p < 0.0001$) and TCGA ($r = -0.3158$, $p < 0.0001$) datasets (Fig. 6A–C; fig. S8B). Increased NLRC5 expression is also reported in lung cancer cells subjected to PRMT5 KD (14), consistent with our observations (fig. S8C). Given that downregulation of proteins functioning in MHCI-mediated antigen presentation can underlie immune evasion by cancer cells, we asked whether PRMT5 activity inhibited NLRC5 expression and/or altered its regulation of genes implicated in immune evasion (39). Genetic (shRNA) or pharmacological inhibition of PRMT5 (using MTA) increased basal *Nlrc5* expression and that of *Nlrc5* target genes implicated in antigen presentation (MHCI) and processing (such as *Tap1*, *B2m*, and *Psmb9*; Fig. 6D and 6E). In contrast, ectopic expression of PRMT5/WDR77 in B16 or YUMMER1.7 melanomas decreased expression of NLRC5 and its target genes (Fig. 6F; fig. S8D). In agreement with earlier reports (38,40), overexpression of NLRC5 or interferon-gamma stimuli, which induces NLRC5 expression, increased expression of PSMB9 and surface expression of MHCI (H-2K^b) in B16 cells (fig. S8E–G). Importantly, PRMT5 KD in B16 cells increased NLRC5 and PSMB9 expression following interferon gamma treatment (Fig. 6G), with a concomitant increase in surface MHCI expression (Fig. 6H). PRMT5 loss did not alter expression of the IFN- γ receptor (fig. S8H), suggesting that changes were independent of receptor expression (39). Overall, these findings suggest that PRMT5 controls MHCI expression and antigen presentation *via* its effect on NLRC5 transcription.

IFI204 and NLRC5 expression inhibits *in vivo* growth of mouse melanoma tumors.

Given that PRMT5 suppresses IFI204 activity and NLRC5 expression, we asked whether expression of a methylation mutant form of IFI204 or expression of NLRC5 would phenocopy PRMT5 depletion. To do so, we established B16 mouse melanoma cells expressing methylation defective IFI204 (IFI204Mt1), murine NLRC5, or both (Fig. 7A). Ectopic expression of NLRC5 alone, but not of IFI204Mt1 alone, significantly inhibited tumor growth in mice. However, the degree of tumor growth suppression *in vivo* was enhanced in melanoma expressing both NLRC5 and IFI204Mt1 relative to NLRC5 alone, supporting the idea that when combined, both pathways mediate antitumor immunity, as seen upon PRMT5 inhibition (Fig. 7B). When cells were grown in culture, however, we did not observe growth suppression of lines ectopically expressing IFI204Mt1 and/or NLRC5, consistent with lack of changes observed for PRMT5 inhibition, and consistent with the notion that *in vivo* these factors function in tumor immune recognition (Fig. 7C). Of note, immunohistochemical analysis of tumors (collected at day 12) showed increased infiltration of immune cells [CD4⁺ (2.13-fold) and CD8⁺ (2.80-fold)] in tumors expressing NLRC5 and IFI204Mt1 relative to control tumors (fig. S9A). Moreover, expression of PRMT1, PRMT5 and PRMT7 decreased in cells expressing IFI204Mt1 plus NLRC5, suggesting a possible feed-forward mechanism limiting PRMT5 or other PRMTs activity (fig. S9B).

Expression of IFI16 and NLRC5 is associated with prolonged patient survival.

We next asked whether IFI16 and NLRC5 expression are linked with melanoma patient survival. Analysis of 200 melanoma specimens [IFI16- or NLRC5-low (n=100) or IFI16- or NLRC5-high (n=100)] in the TCGA dataset (n=368 metastatic melanomas) revealed a significantly prolonged survival of melanoma patients whose tumors exhibit higher

expression of either IFI16 ($p=0.0257$) or NLRC5 ($p<0.0001$) (Fig. 7D and 7E). Correspondingly, higher expression of IFI16 or NLRC5 correlated with enrichment of an immune gene signature (fig. S9C and S9D), supporting the notion that IFI16 and NLRC5 play an important role in controlling the antitumor immune response.

PRMT5 inhibition enhances immune checkpoint therapy in a murine melanoma model.

Our findings provide the basis for a model highlighting the role of PRMT5 as a suppressor of antitumor immune response, which is achieved by limiting infiltration/activation of immune cells and tumor cell recognition by immune cells (Fig. 8A). Consistent with this model, PRMT5-KD tumors expressed elevated levels of *Ifnb1*, *Ccl5* and *Cxcl10* (Fig. 8B) and higher levels of *Pd-1*(*Cd274*), an immune check-point ligand, relative to control tumors (Fig. 8C). Since enhancing the immune response to so-called “cold” tumors could augment ICT effectiveness (41–43), we asked whether PRMT5 inhibition would augment ICT effectiveness. PRMT5 KD (shPRMT5+IgG) significantly attenuated B16 tumor growth in 6 of 8 mice compared with 1 of 8 in the control group (Scr+IgG) ($p=0.0406$, Fisher’s exact test) (Fig. 8D, upper panel).

When combined with anti-PD1 therapy PRMT5-KD (shPRMT5 + anti-PD1) led to a significant suppression of tumor growth (8 responders out of 8), compared with anti-PD1 treatment alone (Scr + anti-PD1) (0 responders out of 8; $p=0.0002$), an effect that was not achieved upon PRMT5-KD alone (6 responders out of 8; $p=0.4667$) (Fig. 8D, upper panel). However the long-term survival of mice was significantly better when treated with combination of shPRMT5 (PRMT5-KD) + anti-PD1 therapy (median survival = 27 days) relative to mice treated with anti-PD1 alone (median survival = 17.5 days) or PRMT5-KD alone (median survival = 20 days) (Fig. 8D, lower panel; fig. S10A). These finding points to the improved therapeutic efficacy following the combination of PRMT5-KD with anti-PD1 therapy.

We next subjected B16 and YUMM1.7 melanoma models to combined therapy, consisting of anti-PD1 antibodies and the PRMT5 inhibitor GSK3326595. Dose of the PRMT5 inhibitor (GSK3326595; 40 mg/kg) was based on an earlier report (22) and confirmed in the B16 model (fig. S10B). Notably, treatment with GSK3326595 plus anti-PD1 antibodies augmented the anti-tumor response, reflected in reduced tumor size in both B16 (Fig. 8E; fig. S10C and S10D) and YUMM1.7 (Fig. 8F) tumor models relative to anti-PD1 therapy alone. It is noteworthy that tumor growth inhibition seen in both B16 or YUMM1.7 models following treatment with anti-PD1 antibody or PRMT5i alone was limited (1–2 responders; Fig. 8E and 8F), along the expected response of “cold” tumors. Changes in tumor burden, which was monitored at different time points in the course of tumor development, revealed a greater response rate to the combination therapy (57.1–85.7%), than that seen in mice undergoing either monotherapy [anti-PD1 antibody (12.5–33.3%) or PRMT5i (14.3–66.7%)] (Fig. 8E–F; fig. S10C–D; table S5). Consistent with the limited response observed following PRMT5i monotherapy, notable changes were not observed in immune cell infiltration or activation (fig. S10E–G). Importantly, the effective inhibition in tumor growth observed following combination of PRMT5i with anti-PD1 antibody was abolished upon the administration of neutralizing antibodies to deplete CD8⁺ cells (Fig. 8G; fig. S10H). These

observations confirm that CD8⁺ cells mediate anti-tumor immunity elicited by the combination therapy (PRMT5 inhibition with anti-PD1 therapy). Unlike anti-PD1 antibody therapy, administration of anti-CTLA4 antibody did not augment the effect of PRMT5i, compared to control or either treatment alone (fig. S10I and S10J), pointing to a select set of immune checkpoint components that are regulated by PRMT5i.

DISCUSSION

Understanding mechanisms that underlie tumor cell recognition by the immune system is expected to greatly increase the effectiveness of antitumor immunity therapies. Here we report that tumor-intrinsic PRMT5 activity antagonizes the immune response by regulating antigen presentation/processing and production of IFN and chemokines. We show that PRMT5-catalyzed methylation of IFI16/IFI204 represses activation of the intracellular DNA-induced cGAS/STING pathway and inhibits TBK1/IRF3 signaling and IFN and chemokine production. Only one of the two putative methylation sites on IFI16/204 (R12) impacted STING/cGAS signaling, as reflected by enhanced TBK1/IRF3 activation and concomitant IFN and chemokine production, implying that the second (R538) site functions in an independent, yet unknown activity. IFI16 methylation did not alter its subcellular distribution, as has been reported for IFI16 acetylation (44), nor did it alter IFI16/IFI204 stability. As Arg methylation is located within the N-terminal PYRIN domain of IFI16/IFI204, which is implicated in STING-mediated activation of the IFN β promoter (23, 33), we cannot exclude the possibility that such methylation may alter STING complex formation and activity. This last possibility is supported by observations that mutating Arg12 (IFI204Mt1, but not Arg538 in the HIN (DNA binding) domain (IFI204Mt2), is sufficient to enhance dsDNA stimulation of STING activation. Accordingly, STING dimerization and subsequent activation of IRF3 and p65 are impaired in *Staphylococcus*-infected lung tissues in IFI204 knockout mice (45). Thus, further understanding of mechanisms underlying the effects of arginine methylation of IFI16/IFI204 on STING is needed. Unexpectedly, our studies also revealed the importance of IFI204 methylation in IFN and chemokine activation by dsRNA stimuli, consistent with an earlier report of the role of IFI16 in dsRNA-induced signaling (46). In all, our findings establish that IFI16/IFI204 methylation by PRMT5 suppresses dsDNA or dsRNA activation of STING or RIG-I/TLR pathways, respectively, with a concomitant effect on type I IFN and chemokine expression.

We also show that PRMT5 negatively regulates NLRC5 transcription, which then limits antigen processing and presentation, an independent hallmark of tumor evasion of immune surveillance (47). Notably, single cell sequencing of malignant cells from melanoma patients identified MHCI antigen presentation genes as factors downregulated in association with T cell exclusion and, possibly, resistance to ICT (48). Correspondingly, elevated NLRC5 expression enhanced cell surface expression of MHCI and more potently inhibited tumor growth (49). As an epigenetic regulator, PRMT5 may control NLRC5 expression by changing its access to the transcriptional machinery or via methylation of DNA binding proteins, both mechanisms that control NLRC5 transcription (50). Likewise, PRMT5 control of histone 4 arginine methylation could facilitate DNA methyltransferase DNMT3A recruitment and subsequent DNA methylation (51). Given the importance of the antigen processing/presentation pathway to ICT resistance, further understanding of mechanisms

underlying NLRC5 expression in cancer is imperative to establish more durable therapies. Notably, higher expression of IFI16 and NLRC5 coincides with prolonged survival of human melanoma patients.

The finding that tumors lacking PRMT5 exhibit increased expression of both the type I IFN and of the immune checkpoint ligand, Pd-11 (Cd274), provided a rationale for evaluating the effect of anti-PD1 therapy in tumors subjected to PRMT5 inhibition. Indeed, our data clearly demonstrate enhanced efficiency of anti-PD1 therapy on “cold” non-responsive tumors when combined with either genetic or pharmacological inhibition of PRMT5. These findings address an unmet clinical need, namely, the ability to apply combination of PRMT5 inhibition and anti-PD1 therapy to “cold” tumors, which are not responsive to PD1 therapy.

Recent studies report suppression of Tregs and CD8⁺ T cells following treatment with PRMT5 inhibitors (EPZ016666 and DST-437). Notably, administration of the PRMT5 inhibitor (GSK3326595; 40 mg/kg, QD) alone was not sufficient to attenuate tumor growth, nor was it capable of increasing immune cell infiltration, consistent with the limited overall changes we have observed in protein methylation. This is in contrast to the clear changes seen in each of these parameters upon genetic KD of PRMT5 in tumors. These observations suggest that optimization of doses and formulation is necessary to improve therapeutic effects of pharmacological PRMT5 inhibitors. The effect of PRMT5 inhibitors on immune system components, as on stromal and other microenvironmental niches, should thus be monitored following different formulation, dosing and frequency schedules. Nonetheless and significantly, combination of either genetic or pharmacological PRMT5 inhibitor with anti-PD1 therapy led – in both cases – to effective inhibition of melanoma growth, which was CD8⁺ T cell dependent. Although our studies focus on PRMT5 inhibition, the possible inclusion of inhibitors that target multiple PRMT family members may prove effective, as was recently reported (52, 53).

Overall, our findings establish that PRMT5 controls important tumor intrinsic regulatory axes for antigen presentation and cGAS/STING activation, which underlie tumor immune evasion. PRMT5 inhibition may thus enhance antitumor immune responses and provide an opportunity to mitigate a “cold” tumor’s resistance to immune checkpoint therapy.

MATERIALS AND METHODS

Study Design

The objectives of the present study were to: (i) determine the effect of PRMT5 on control of the anti-tumor immune response, (ii) define relevant underlying molecular mechanisms, and (iii) evaluate therapeutic efficacy of PRMT5 inhibition alone or in combination with immune therapy *in vivo*. Our study relied on analyses of human melanoma databases, *in vitro* analyses of signal transduction and gene expression pathways for the type I IFN proinflammatory response and antigen processing/presentation, and *in vivo* animal studies monitoring melanoma growth and response to therapies. We evaluated PRMT5 immune suppressive function in syngeneic murine models of melanoma, using less-immunogenic B16 and YUMM1.7 cells for loss of function studies and immunogenic YUMMER1.7 cells for gain of function studies. Genetic inactivation of PRMT5 was restricted to use of

lentiviral shRNAs (multiple) in studies of both cultured cells and *in vivo*, since total ablation of PRMT5 using CRISPR/CAS9 approaches results in complete lethality (fig S11). We thus complemented our studies using a first-in-class pharmacological inhibitor for PRMT5, EPZ015666, which provided independent confirmation to the genetic-based inhibition studies.

Phenotypes seen in melanoma cells subjected to PRMT5 knockdown were confirmed using pharmacological PRMT5 inhibitors as well as through analysis of PRMT gain of function in overexpression assays. Animal care and related procedures followed institutional guidelines and was conducted with approval of the Institutional Animal Care and Use Committee of Sanford Burnham Prebys Medical Discovery Institute. Animal cohort sizes were designed to detect differences in treatment effects at 80% power (α error rate = 0.05), with the exception of studies conducted to assess immune phenotypes. Mice with unexpected and severe skin atopic dermatitis were excluded. All experiments were conducted 2–3 times, except for tumor studies in which specific immune cells were depleted; in those analyses' cohort size was sufficient to support the statistical power stated above. Each experiment consisted of 3–4 technical replicates. Sample identity for tumor studies were blinded to the investigator who grafted them into mice.

Cell Culture and Treatment

Human and murine melanoma cells [B16F10, purchased from ATCC; YUMM1.7 and YUMMER1.7, obtained from Yale University (27); A375 and WM115, obtained from the Wistar Institute (54)]; and HEK293T cells, from ATCC] were maintained in DMEM (Hyclone) containing 10% fetal bovine serum (Omega Scientific and PEAK serum) plus penicillin/streptomycin (10000 U/ml, Thermo scientific) in 5% CO₂ at 37°C. Stably-transduced cells were maintained with appropriate antibiotics, including puromycin (InvivoGen, 1 µg/ml) and blasticidin (InvivoGen, 10 µg/ml). Cells were maintained in growth phase and did not exceed 80% confluency. Cells were stimulated by treatment with (i) interferon gamma (R&D Systems), (ii) by transfection with LMW (low molecular weight)/HMW (high molecular weight) poly(I:C) (InvivoGen, 250 ng/ml) or by (iii) transfection of vaccinia virus dsDNA V70mer (500 ng/ml for detecting Ifnb1/chemokine expression and 1.5 µg/ml for detecting TBK1/IRF3 activation). V70mer was prepared by annealing the complementing oligonucleotides 5'-CCATCAGAAAGAGGTTTAATATTTTGTGAGACCATCGGGGCCGCGCTCCCCCGCG AGGCCGCCGGCG-3' (29).

Animal Experiments

All animal experiments were conducted with approval of the Institutional Animal Care and Use Committee of Sanford Burnham Prebys Medical Discovery Institute (AUF#18-044). The murine melanoma lines B16F10, YUMM1.7, and YUMMER1.7 were injected subcutaneously (2.0×10^5 cells of B16F10 or YUMM1.7; 4.0×10^5 cells of YUMMER1.7) into the lower right flank of 6–8-week-old male C57BL/6 (B16F10, YUMM1.7, YUMMER1.7) or Nod-Scid-Gamma (NSG) (B16F10, YUMMER1.7) mice. To induce transduced inducible shPRMT5, doxycycline (10 mg/ml, Fisher Bioreagents) was prepared in methylcellulose solution (0.5 % hydroxylmethylcellulose, 0.2% Tween80) and

administered to mice (0.2 ml, oral gavage, QD)(55). Tumor sizes were monitored using calipers. At indicated time points, tumors were collected, weighed and assessed for immune phenotypes using flow cytometry or immunofluorescence. To assess efficacy of immune checkpoint antibodies, mice were grafted with B16F10 or YUMM1.7 (2.0×10^5 cells, s.c.) cells and treated with 200 μ g control IgG [rat IgG2a; BE0089 (BioXcell)], anti-CD152 (CTLA-4) [9H10 (BE0131, BioXcell)] or anti-CD279 (PD1) [RMP1-14 (BE0146, BioXcell)]. Antibodies were injected (i.p.) 3 – 5 times (every 3 days starting from the indicated date). The PRMT5 inhibitor GSK3326595 (Chemitec) was prepared in methylcellulose solution and administered to mice (40 mg/kg, oral gavage, QD). To deplete NK or CD8⁺ cells, mice were treated with anti-NK1.1 antibody [PK136 (BE0036, BioXcell)] or anti-CD8⁺ antibody [2.43 (BE0061, BioXcell), respectively; controls were treated with 200 μ g IgG [rat IgG2b (BE0090, BioXcell)]. Antibodies were injected (i.p.) every 3 days starting one day prior to tumor cell inoculation. The efficiency of depletion was assessed using flow cytometry of blood samples collected at day 8 after tumor inoculation. To assess percent survival of animals, mice bearing tumors exceeding 2,000 mm³ were defined as “dead”.

Gene Set Enrichment (GSEA) and Ingenuity Pathway (IPA) Analyses

GSEA was performed using a GSEA Desktop Application downloaded from software.broadinstitute.org/gsea/. Gene expression (RNA-seq) data from specimens from human melanoma patients obtained from the TCGA (The Cancer Genome Atlas) or GEO (Gene Expression Omnibus) databases was used to identify genes differentially-expressed between patient groups with characteristics of interest (low/high expression of PRMT5 or MTAP). Curated sets of hallmark (50 gene sets) (49) and C5 GO (5917 gene sets) genes from the Molecular Signature Database (MSigDB v6.1) served as input. High-ranked gene sets from the analysis were presented along with the enrichment plot, NES (normalized enrichment score), nominal p value, FDR-q value and a heatmap of the corresponding gene set. For heatmap plots, FPKM values for PRMT5 low (n = 100) and high (n = 100) were obtained from TCGA-SKCM samples for each gene set (Hallmark and GO) were log₂ transformed (FPKM + 0.1) and subsequently converted to row (gene) z-scores using *scale()* function in R. Appropriate numbers of clusters (K) were determined by plotting the “within sum of squares” (a metric denoting dissimilarity among the members of a cluster) versus different values of K. Heatmaps of gene z-scores were plotted using ComplexHeatmap version 2.3.2 (56) by applying k-means clustering (K = 5 in both cases) and 100 k-means runs to get a consensus clustering. Genes associated with immune signature are indicated on the heatmaps. All of the above analyses were performed in R version 3.6.1. For IPA (Qiagen Inc), differentially expressed genes with an unpaired t-test p value of <0.05 and a fold-difference of >2.0 between low and high groups were analyzed using core analysis. High-ranked canonical pathways were presented along with p values (right-tailed Fisher’s exact test), ratio (coverage of pathway), and Z score with pathway directionality (filled blue bars).

Statistical Analysis

Statistical analyses were performed using Prism software (version 7.00, GraphPad). For comparison of means of two groups with normal (or approximately normal) distributions, an unpaired t-test was applied. In multiple t test between two groups, adjusted p values were

computed with Holm-Sidak method. To compare means between >2 groups, we used one-way analysis of variance (ANOVA) with multiple comparison corrections (Dunnnett's or Tukey's test). For animal experiments, we used two-way ANOVA (time and treatment) with Tukey's multiple comparison test. For Kaplan-Meier plots to compare overall survival, we used a log-rank test to determine significance of differences between groups. To evaluate response to therapy in a mouse model, we used Fisher's exact test (version 7.00, GraphPad). For that analysis, we defined a tumor with volume <50 % of control tumors as "responding to treatment". For all analyses, a difference with $p < 0.05$ was considered significant, unless specified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Melanoma specimens with low PRMT5 expression show an enriched immune gene signature.

(A) PRMT5 expression in metastatic melanoma specimens based on TCGA datasets. Inset shows comparison between low (blue box) and high (red box) PRMT5 expression cohorts (n=368). (B) Top-ranked pathways predicted using the Ingenuity Pathway Analysis (IPA) based on differentially-expressed genes (DEGs) in specimens exhibiting either low or high PRMT5 expression. Blue bars indicate pathways likely inhibited in the PRMT5-high group. (C) Representative immune gene sets enriched in GSEA of DEGs from melanoma specimens with low or high PRMT5. The top 14 genes for each gene set are shown in respective heatmaps. Heatmaps of genes in the enriched gene sets indicating clustering of immune response genes. Columns represent PRMT5 low (n = 100, gray bar) and high (n = 100, yellow bar) TCGA-SKCM samples whereas rows represent genes. Normalized expression levels of the genes in each gene sets were converted to $\log_2(\text{FPKM} + 0.1)$ and subsequently transformed to z-scores. The gene sets were k-means clustered (K = 5),

choosing the K by plotting the “within sum of squares” (a metric denoting dissimilarity among the members of a cluster) versus different values of K. Genes associated with immune response are indicated on the heatmaps.

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Figure 2. Attenuation of melanoma growth following PRMT5 inhibition requires intact host immunity.

(A) Western blot analysis showing PRMT5 expression (upper) and activity (middle) in protein extracts prepared from B16 murine melanoma cells transduced with scrambled (Scr) or PRMT5-specific hairpin shRNAs (shPRMT5-1 or shPRMT5-2) and probed with indicated antibodies. β -actin served as a loading control (lower). Hereafter, “S.exp” and “L.exp” represent short and long exposure, respectively. “SDME-RG” indicates anti-symmetric dimethyl arginine antibody (Cell Signaling). (B) Growth in culture of B16 cells stably expressing shPRMT5 or Scr control (n=5 for each group). (C) Volume of control and PRMT5-KD B16 tumors (Scr; n=8, shPRMT5-1; n=8, shPRMT5-2; n=7) grafted (s.c., 0.2 million cells) into immunocompetent C57BL/6 mice and measured at indicated time points. (D) Volume of control and PRMT5-KD B16 tumors (Scr; n=5, shPRMT5; n=6) grafted (s.c., 0.2 million cells) into immunocompromised NSG mice and measured at indicated time points. (E) Western blot analysis of PRMT5 expression (upper) and activity (middle) in

extracts of tumor cells cultured from indicated tumor pools (Scr-pool, cells from 5 Scrambled-KD tumors; shPRMT5 pools 1 and 2, cells from 3 shPRMT5-KD tumors each). β -actin served as a loading control (lower). **(F)** Control or shPRMT5-KD tumor cells isolated and pooled from tumors grown in NSG mice (panel D) were re-grafted into syngeneic immunocompetent mice (Scr; n=5, shPRMT5; n=6) and assessed at indicated time points. **(G)** Western blot analysis of PRMT5 expression (upper) and activity (lower) in tumors generated as in panel F, using indicated antibodies. GAPDH served as a loading control (middle). **(H)** Western blot analysis of PRMT5 expression and activity in YUMMER1.7 cells expressing indicated expression vectors. GAPDH served as a loading control. **(I)** Growth of YUMMER1.7 cells in culture following transfection with control (EV +EV, n=5) or PRMT5+WDR77 constructs (n=5). (Protein analysis is shown in panel H.) **(J-K)** Volume of control and PRMT5+WDR77-overexpressing YUMMER1.7 cell tumors (Scr; n=8, PRMT5+WDR77; n=8) grafted (s.c., 0.4 million cells) into C57BL6 (J) or NSG (K) mice and measured at indicated time points. **(L)** Western blot analysis of PRMT5 expression and activity in tumors generated as in panel K, using indicated antibodies. GAPDH served as a loading control. Data are presented as means $\pm$ s.d. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001; “ns” not significant.

Figure 3. PRMT5 expression decreases invasion by tumor infiltrating leukocytes (TILs). (A) Immune phenotyping performed using flow cytometry using the indicated cell surface markers on Scr- and shPRMT5-transduced B16 tumors, collected at day 17. Two independent experiments are presented. (B) Ratio of abundance was calculated by dividing number of activated CD8 T cells (CD44^{hi}CD8⁺) by that of MDSC (CD11b⁺GR1⁺) or regulatory T cells (CD4⁺FOXP3⁺). (C) Infiltration of CD4⁺ and CD8⁺ immune cells into B16 tumors 12 days after grafting into C57BL/6 mice, as evaluated by immunohistochemistry (left). Quantification of infiltrated immune cells in Scr-KD (n=5) and shPRMT5-KD (n=3) tumors was performed using Image J. Data are presented as means $\pm$ sem. (D) Immune phenotyping performed using flow cytometry and indicated cell surface markers in YUMMER1.7 tumors expressing control (EV+EV, n=7) or PRMT5+WDR77 constructs (n=4). Tumors were collected at day 12. (E-H) B16 cells stably expressing control (Scr) or PRMT5 shRNA (shPRMT5) were grafted into C57BL/6 mice (n=8)

administered control (IgG) or neutralizing antibodies against either NK1.1 (200 µg/mouse panels E and F) or CD8 (200 µg/mouse panels G and H) every three days starting one day prior to tumor inoculation. Shown are tumor volumes (E and G) and percent survival (F and H). Data are presented as means $\pm$ s.d., unless specified. *, **, *** and **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

Figure 4. PRMT5 methylates the cGAS complex component IFI16/IFI204.

(A) Melanoma patient specimens expressing comparable PRMT5 levels were grouped based on low or high levels of PRMT5 adaptor proteins (namely, SHARPIN, WDR77, RIOK1, COPRS, CLNS1A, and MEN1). DEGs were analyzed using GSEA. (B) Heat map depicting normalized enrichment score (NES) and q value of false discovery rate (FDR-q) for PRMT5 adaptor proteins in immune-associated hallmark gene sets. (C) Immunoprecipitation (IP) followed by immunoblotting (IB) of WM115 cell lysates (1.2 mg) with indicated antibodies. (D) B16 cells were treated with vehicle (DMSO) or PRMT5 inhibitor (PRMT5i; EPZ015666, 10 μ M) for 48 h. IP followed by IB of B16 cells lysates (1.5 mg) was performed with the indicated antibodies. SYM10 indicates anti-symmetric dimethyl arginine antibody (Millipore). (E–F) A375 (E) or B16 (F) cells treated with vehicle or a PRMT5 inhibitor (EPZ015666, 10 μ M) as above before lysates [A375 (1.0 mg), B16 (2.5 mg)] were prepared and subjected to IP followed by immunoblotting with indicated antibodies. (G) B16 cells

stably expressing indicated constructs were treated 24 h with DMSO or PRMT5i (EPZ015666, 10 μ M) before lysates were IP'd with V5 antibody and immunoblotted with indicated antibodies. WT: IFI204 WT; Mt1, Mt2 and Mt1/2: IFI204 mutants R12A, R538A or RR12/538AA, respectively; EV: empty vector. **(H)** *In vitro* methylation assay of WT or mutant IFI204 proteins (200 ng) purified from HEK293T cell lysates, with or without recombinant active PRMT5 plus WDR77 (500 ng) proteins. Proteins were visualized using PonceauS and InstantBlue staining (lower panels) and subjected to autoradiography (upper). Histone 4 served as a positive control.

Figure 5. PRMT5 methylation of IFI204 determines the degree of cGAS/STING pathway activation.

(A–C) B16 cells were: transduced with either scramble (Scr) or Prmt5-specific shRNAs (shPRMT5–1, shPRMT5–2) (A), treated for 24 hr with PRMT5i (EPZ015666, 10 μ M) (B), or subjected to ectopic expression of control (pLX304 and pLenti) or PRMT5 + WDR77 (pLX304-WDR77/pLenti-PRMT5) (C). Following respective treatments, cells were stimulated with dsDNA (transfected V70mer; 500 ng/ml). Six hr later cell lysates were prepared and assayed using qPCR for expression of indicated transcripts. (D–F) Analysis of cGAS/STING complex components by Western blot analysis (D), semi-native-PAGE (E), or BlueNative-PAGE (F) of proteins prepared from B16 cells subjected to PRMT5 KD using corresponding shRNA (as in panel A) followed by stimulation with dsDNA (V70mer; 1.5 μ g/ml) for indicated times. Lower panels show Ponceau S staining (lower panels in E, F) “d” and “m” (panel E) represent “dimer” and “monomer” forms of STING. (G–H) Analysis of cGAS/STING complex components with indicated antibodies using Western blot analysis of

lysates prepared from B16 cells either treated with PRMT5i (as in panel B) or stably expressing PRMT5+WDR77 (as in panel C) following stimulation with dsDNA (transfected V70mer; 1.5 µg/ml) for indicated times. **(I)** B16 cells stably expressing pLX304 (EV), IFI204WT (WT), the IFI204R12A mutant (Mt1) or the IFI204R538A mutant (Mt2) were transfected with V70mer (500 ng/ml) for 6 hr and then assessed for expression of indicated transcripts by qPCR. **(J)** B16 cells stably expressing IFI204 plasmids (as in panel I) were transfected with V70mer (1.5 µg/ml) for indicated times followed by analysis of cell lysates by semi-native-PAGE blotting with indicated antibodies and Ponceau S staining. STING dimer (d) and monomer (m) forms are noted. **(K)** B16 cells transduced with Scr or Prmt5-specific shRNAs were transfected with scrambled control (siCont) or Sting-specific (siSting) siRNAs for 48 hr. Cells were then stimulated 6 h with dsDNA (transfected V70mer; 500 ng/ml) before lysates were prepared for qPCR analysis of indicated transcripts. Western blot inset depicts level of STING expression. Data are presented as means $\pm$ s.d. *, **, *** and **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

Figure 6. PRMT5 negatively regulates NLRC5 to modulate MHC I antigen presentation.

(A) Correlation of PRMT5 expression with that of genes implicated in antigen presentation in melanoma lines (CCLE, cancer cell line encyclopedia datasets, n=58) was evaluated using Pearson's correlation coefficient (plotted on X-axis) and $-\log(p \text{ value})$ (plotted on the Y-axis). Blue line indicates cutoff level for $p < 0.05$. (B) Pearson's correlation of PRMT5 and NLRC5 mRNA expression in melanoma cell lines (CCLE, n=58). (C) Pearson's correlation of PRMT5 and NLRC5 mRNA expression in melanoma patient specimens (TCGA, n=368). (D–F) qPCR analysis of genes implicated in antigen presentation was performed in B16 cells either transduced with Scr or *Prmt5*-specific shRNAs (shPRMT5–1, shPRMT5–2) (D), treated with PRMT5i (MTA, 100 μM for 24 hr) (E), or stably expressing EV or PRMT5+WDR77 (F). (G) Immunoblotting of lysates of B16 cells transduced with scrambled (Scr) or shPRMT5 and treated 24 hr with indicated concentrations (ng/ml) of interferon gamma (IFN γ) using antibodies to indicated proteins. (H) Cell surface MHC I

expression (H-2K^b) in B16 cells subjected to indicated treatments, as assessed by flow cytometry (left). Quantification of mean fluorescence intensity (MFI) (right). Data are presented as means $\pm$ s.d. *, **, *** and **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

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Figure 7. Co-expression of mutant IFI16/IFI204 and NLRC5 inhibits melanoma growth

(A) B16 cells were transduced with EV or expression vectors harboring IFI204Mt1 and/or NLRC5 and then analyzed by Western blotting for indicated proteins (A). (B) Tumor growth was assessed in mice (n=8) grafted with B16 cells established in (A). (C) Growth of B16 cells in culture (established in A) was monitored using ATPlite assay. Data are presented as means $\pm$ s.d. Statistical significance of changes in tumor growth and cell growth were assessed using two-way ANOVA with Tukey's correction and one-way ANOVA with Dunnett's test. (D, E) Left panels show classification of specimens based on low or high levels of IFI16 (D) or NLRC5 (E) expression (based on TCGA, metastatic population of melanoma, n=368). Right panels show overall survival of melanoma patients based on relative expression of IFI16 (D) or NLRC5 (E).

Figure 8. PRMT5 inhibition synergizes with anti-PD1 immune check-point therapy.

(A) Proposed model for PRMT5 control of IFN/chemokine expression and antigen presentation pathways. (B–C) Expression of transcripts encoding indicated IFN/chemokines (B) and immune checkpoint components (C) based on qPCR of tumors transduced with control (Scr-KD, n=7) or PRMT5-KD (shPRMT5, n=7), 17 days after tumor cell inoculation (D) B16 cells transduced with scrambled (Scr) or shPRMT5 were grafted into C57BL/6 mice (n=8) subsequently treated with control IgG or anti-PD1 antibody (200 μ g/mouse at days, 8, 11, 14, 17 and 20). Tumor volume (upper) and percent survival (lower) were assessed at indicated time points. (E) B16 cells were grafted into syngeneic C57BL/6 mice (n=6–8) subsequently treated with PRMT5i (GSK3326595, 40 mg/kg from day 10) and/or anti-PD1 antibody (200 μ g/mouse at days 11, 14 and 17). Tumor volume (upper) and percent survival (lower) were assessed at indicated time points. (F) YUMM1.7 cells were grafted into syngeneic C57BL/6 mice (n=7–8) subsequently treated with PRMT5i (GSK3326595,

40 mg/kg from day 7) and/or anti-PD1 antibody (at days 8, 11, 14 and 17). Tumor volume (upper) and percent survival (lower) were monitored at indicated time points. (G) YUMM1.7 cells were grafted into syngeneic C57BL/6 mice (n=7) subsequently administered anti-CD8⁺ antibody (200 µg/mouse) every three days, starting one day prior to tumor inoculation. As indicated, mice were also administered PRMT5i (GSK3326595, 40 mg/kg from day 8) and/or anti-PD1 antibody at days 9, 12, 15 and 18. Tumor volume (upper) and percent survival (lower) were monitored at indicated time points. For statistical analyses, tumor response was calculated based on tumor volume and percent survival, using Fisher's exact test and a log-rank test, respectively. *, **, *** and **** represent p<0.05, p<0.01, p<0.001 and p<0.0001, respectively.