The abundance of metabolites related to protein methylation correlates with the metastatic capacity of human melanoma xenografts

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Metabolic reprogramming is a major factor in transformation, and particular metabolic phenotypes correlate with oncogeneotype, tumor progression, and metastasis. By profiling metabolites in 17 patient-derived xenograft melanoma models, we identified durable metabolomic signatures that correlate with biological features of the tumors. BRAF mutant tumors had metabolomic and metabolic flux features of enhanced glycolysis compared to BRAF wild-type tumors. Tumors that metastasized efficiently from their primary sites had elevated levels of metabolites related to protein methylation, including trimethyllysine (TML). TML levels correlated with histone H3 trimethylation at Lys4 and Lys27, and methylation at these sites was also enhanced in efficiently metastasizing tumors. Erasing either of these marks by genetically or pharmacologically silencing the histone methyltransferase SETDB1 or EZH2 had no effect on primary tumor growth but reduced cellular invasiveness and metastatic spread. Thus, metabolite profiling can uncover targetable epigenetic requirements for the metastasis of human melanoma cells.

INTRODUCTION
Melanoma represents less than 5% of skin cancers but results in the vast majority of deaths. Although localized early-stage melanoma is often curable by surgical resection, the prognosis for patients with late-stage, metastatic melanoma is poor (1). Therapeutic agents such as dacarbazine, interleukin-2 (IL-2), and/or interferon-α (IFN-α) have little effect on metastatic melanoma, in part because melanoma cells reprogram proliferation and survival pathways during disease progression (2–4). In the past 5 years, several new drugs have been approved for the treatment of advanced melanoma. These include immunotherapies, oncolytic virus therapies, and therapies targeting signaling pathways activated by BRAF V600 mutations (5–7). In particular, targeting the programmed death-1 (PD-1) receptor alone or in combination with inhibitors of cytotoxic T lymphocyte–associated antigen–4 (CTLA-4) improves response rate and progression-free survival in melanoma (8–10). Nevertheless, numerous mechanisms exist to promote therapeutic resistance, and many patients with advanced melanoma ultimately succumb to the disease (11–13). Thus, a substantial unmet clinical need persists in melanoma, particularly in the context of metastatic disease.

A key challenge in studying metastasis is the paucity of models that faithfully recapitulate the molecular determinants of cancer cell dissemination in humans. The metastatic behavior of melanomas in nonobese diabetic/severe combined immunodeficient IL-2 receptor-γ chain null (NSG) mice was shown to correlate with metastasis in the human donors (14). In these models, stage IIIIB/C tumors that progressed rapidly to form distant metastases in patients also efficiently metastasized from the subcutaneous site of implantation to distant organs in the mice. In contrast, stage-matched tumors that did not form distant metastases in patients inefficiently formed macrometastases in mice or not at all. Because metastatic propensity was maintained during multiple passages in mice, the models provide an opportunity to identify cell-autonomous features that predict and promote metastasis in human melanoma.

Reprogrammed metabolic pathways in tumors facilitate the growth of malignant cells (15, 16) and support cell survival in the face of stressors present in the tumor microenvironment (17, 18). Although genomic profiling has been performed on melanoma patients (19, 20), little is known about the metabolic heterogeneity characterizing individual melanomas. Given that metabolism reports a wide array of biological properties, we reasoned that metabolomic profiling might reveal features that differentiate tumors with a high propensity to form distant macrometastases from those without. These features might then provide insights into therapeutic approaches to suppress metastasis. Evidence suggests that metastasis depends on a suite of reprogrammed metabolic activities. For example, loss of anchorage is accompanied by oxidative stress that limits cell viability, unless antioxidant pathways are induced (21, 22). In melanoma, we previously used a subset of the patient-derived xenograft (PDX) lines described above to demonstrate that oxidative stress in circulating tumor cells imposes a major bottleneck on metastasis (23). Providing antioxidants to tumor-bearing mice promoted metastasis, whereas inhibiting antioxidant pathways suppressed it. These findings indicate that melanoma PDX lines can be used to identify tumor-intrinsic properties, including metabolic properties, promoting metastasis.

Here, we used metabolomics in a large panel of melanoma PDX lines to identify metabolic features in primary tumors that correlated with metastatic potential. We observed elevation of several metabolites involved in protein methylation, including trimethyllysine (TML) and dimethylarginine (DMA), in tumors with high metastatic potential. Further analysis revealed that these signatures reflected a particular set of histone methylation marks in highly metastatic tumors and that reversing these marks suppressed metastatic spread without affecting the growth of the primary tumor.
RESULTS
Patient-derived melanoma xenografts have durable metabolomic signatures predicting metabolic features of BRAF mutant tumors
To establish an informative metabolomic workflow in melanoma PDX lines, we first assessed metabolite levels in two PDX lines in which tumors were allowed to grow to various sizes in the subcutaneous space. In both lines, the impact of tumor size on metabolomics was more prominent in small tumors such that tumors with diameters of 0.5 or 1.0 cm were readily distinguishable through unsupervised clustering (fig. S1). However, it was no longer possible to distinguish tumors with diameters of 1.5 cm from those with diameters of 2.0 cm. We therefore performed subsequent experiments in tumors measuring 1.5 to 2 cm, reasoning that metabolic features in these tumors would be less susceptible to the effect of tumor size. We implanted 17 PDX lines into three to four NSG mice per line, giving rise to 62 PDX tumors (Fig. 1A). Among the 17 lines were 9 efficiently metastasizing melanomas (H-met), 6 inefficiently metastasizing melanomas (L-met), and 2 melanomas with intermediate metastatic capacity. The efficient metastasizers formed distant metastases in patients and readily formed macrometastases in NSG mice, whereas the inefficient metastasizers did not form distant metastases in patients and metastasized more slowly in mice (14). Each tumor was sectioned into three fragments, yielding a total of 186 tumor samples for metabolomic analysis. We then used liquid chromatography–tandem mass spectrometry (LC-MS/MS) to determine the relative quantities of 131 metabolites extracted from each specimen. Remarkably, hierarchical clustering revealed that each tumor fragment was most closely related to all other fragments descended from the same human tumor, even when the tumors arose in different mice (Fig. 1A). This demonstrates that each PDX line has a durable metabolomic phenotype that can be tracked from mouse to mouse.

To test for correlations between metabolomic features and actionable aspects of tumor biology, we first compared profiles between BRAF wild-type (WT) and V600E mutant (Mut) tumors. In an unsupervised analysis, the metabolomic profiles of these two genotypes partially overlapped (Fig. 1B). A supervised analysis revealed that metabolites discriminating between the two genotypes included intermediates from the tricarboxylic acid (TCA) cycle, arginine biosynthesis, polyamine metabolism, and nucleotide biosynthesis (table S1 and fig. S2A). The ratio between citrate, the entry metabolite in the TCA cycle, and the distal glycolytic intermediate pyruvate was decreased in BRAF Mut tumors (Fig. 1C). To examine whether fluxes through glycolysis and the TCA cycle were altered in BRAF Mut tumors, mice bearing either BRAF WT or Mut tumors were infused with [U-13C]glucose using a protocol that achieved steady-state glucose labeling in the plasma (Fig. 1D) and consistent labeling of glucose in tumor tissue regardless of genotype (fig. S2B). Normalized to the enrichment of glucose in these tumors, products of anaerobic glucose metabolism (phosphoenolpyruvate, pyruvate, lactate, and alanine) were much more enriched in BRAF Mut than in BRAF WT tumors (Fig. 1E), consistent with the previously reported role of BRAF in regulating glycolysis (24, 25). Despite the higher enrichment in glycolytic intermediates, there was no difference in labeling of TCA cycle intermediates between the genotypes (Fig. 1E), perhaps indicating a bottleneck to glucose oxidation pathways.

Efficiently metastasizing tumors have abundant methylation-related metabolites and high levels of histone H3 methylation
We next performed supervised analyses [partial least-squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) analysis; Fig. 2, A and B] to identify metabolic differences between H-met and L-met tumors. Note that, in these PDX models, BRAF status alone does not predict metastatic capacity (14). Metabolomics revealed that H-met tumors had increased abundance of several metabolites related to methylation (Fig. 2B and table S3). Among these metabolites, TML and DMA were increased in H-met primary tumors (Fig. 2C), although these metabolites were not significantly altered in macrometastases arising from the H-met tumors (Fig. 2D). TML and DMA are produced from the degradation of methylated proteins rather than from the de novo methylation of free amino acids (26, 27). Thus, we reasoned that their elevation could indicate enhanced abundance of methylated proteins in the H-met tumors. We therefore examined the abundance of trimethylation marks on histone H3, which is among the most abundant trimethylated proteins (28, 29). On average, trimethylated histone H3 was more abundant in H-met melanomas (Fig. 2, E and F), and its abundance correlated positively with free TML (Fig. 2G).

Histone H3 contains several lysine residues that may be trimethylated to activate or silence gene expression (30). Trimethylation of Lys7 and Lys27 (H3K9me3 and H3K27me3), both of which are associated with gene silencing, tended to be elevated in H-met tumors, whereas H3K4, H3K36, and H3K79 trimethylation were not significantly different (Fig. 2H; quantitation is shown in fig. S3). The expression of H3K9me3 and H3K27me3 in distant metastases was comparable to their corresponding primary tumors in most H-met tumor lines examined (fig. S4). Among multiple methyltransferases, SETDB1 and SUV39H1 contribute to trimethylation on H3K9, whereas EZH1 and EZH2 contribute to trimethylation on H3K27 (29). Expression of SETDB1 and EZH2 correlated with the abundance of H3K9 and H3K27 trimethylation, respectively, whereas the methyltransferases SUV39H1 and EZH1 were not as well correlated (fig. S5). We focused our subsequent analyses on SETDB1 and EZH2.

Down-regulation of H3K9me3 and H3K27me3 suppresses tumor cell invasion in vitro
To establish an ex vivo system to study the effects of manipulating H3K9me3 and H3K27me3, we generated primary cultures from three H-met and three L-met tumor lines and examined their behavior in transwell assays. All three lines derived from H-met tumors displayed high levels of invasion relative to the L-met lines (fig. S6). Silencing either SETDB1 or EZH2 using short hairpin RNAs (shRNAs) in H-met M481 cells reduced the amount of H3K9me3 and H3K27me3, respectively (Fig. 3, A and B) and reduced the abundance of TML (Fig. 3, C and D). These findings demonstrate that SETDB1 and EZH2 establish the histone methylation marks that discriminate H-met from L-met tumors, and that these two methyltransferases contribute to the high TML levels in cells from H-met tumors, thus providing a link between the metabolomic signatures and the epigenetic state. Suppressing either methyltransferase reduced the invasive capacity compared to control cells (Fig. 3, E and F).

Next, we treated H-met cell lines with the general methyltransferase inhibitor 3-deazaneplanocin A (DZNep), which reduced both H3K9me3 and H3K27me3 methylation marks in M481 cells (Fig. 4A). In three cell lines derived from H-met tumors, pretreatment with DZNep for 6 days reduced the percentage of invasive cells in a transwell assay (Fig. 4, B and C). We also examined the effects of the EZH2 selective inhibitors EPZ6438 and GSK126. These drugs reduced H3K27me3 abundance and invasion in transwell assays in M481, M632, M205, and UT10 H-met cells (Fig. 4, D to F, and fig. S7). GSK126 inhibits EZH2 by competitively binding the S-adenosylmethionine (SAM)–binding site on its
Fig. 1. Metabolomic profiling of patient-derived melanoma xenografts. (A) Metabolomic workflow for melanoma PDX lines. The heatmap shows unsupervised clustering of metabolomic data from all fragments. Names (for example, M610) and BRAF mutation status are indicated for each PDX line, and metastatic capacity is indicated by color (H-met, high metastatic capacity; L-met, low metastatic capacity; I-met, intermediate phenotype). In the heatmap, columns represent tumor fragments, and rows represent metabolites. The coloring is based on a log2 scale of relative metabolite abundance. (B) Principal components analysis (PCA; unsupervised clustering) of metabolomic signatures of all fragments, with each fragment color-coded according to BRAF status. The t1 and t2 values represent the scores of each sample in principal components 1 and 2, respectively. (C) Citrate-to-pyruvate ratio in BRAF WT and BRAF Mut tumors. For (B) and (C), n = 96 BRAF WT fragments and 90 BRAF Mut fragments. TIC, total ion current. (D) 13C enrichment in plasma glucose in PDX-bearing mice infused with [U-13C]glucose. (E) Fractional abundance of selected mass isotopologs in tumor fragments after infusion with [U-13C]glucose. For each tumor line, n = 3 to 4. For each tumor, three fragments were harvested for analysis. Data from complete isotopolog analysis of all metabolites are shown in table S2. All data are means ± SEM. Statistical significance was assessed using two-way analyses of variance (ANOVAs) followed by Sidak’s tests for multiple comparisons or unpaired Student’s t test. ****P < 0.0001.
Fig. 2. Altered histone trimethylation in H-met melanomas. (A) PLS-DA (supervised clustering) of metabolomic features of H-met and L-met tumors. The $t_1$ and $t_2$ values represent the scores of each sample in principal components 1 and 2, respectively. (B) VIP analysis of metabolites discriminating between H-met and L-met tumors. Methylation-related metabolites are shown in red. The columns to the right indicate whether the abundance of each metabolite is enhanced (red) or reduced (green) in each tumor class. ADP, adenosine diphosphate. (C) Normalized TML and DMA abundance in fragments from six L-met and nine H-met melanoma tumor lines. $n = 66$ L-met fragments and 96 H-met fragments. (D) TML and DMA levels in primary tumors and metastases. $n = 15$ fragments from five individual H-met tumors and 12 fragments from metastases in the same mice. ns, not significant. (E) Abundance of trimethylated histone H3 in H-met and L-met tumors assessed by Western blot. (F) Densitometric quantification of trimethylated histone H3 bands in (E). (G) Correlation between trimethylated histone H3 and free TML in melanoma tumors. TML abundance was obtained from previous LC-MS/MS–based metabolomic analysis. (H) Western blot analysis of histone H3 trimethylation on several distinct lysine residues. All data are means ± SEM. Statistical significance was assessed using unpaired Student’s $t$ tests. *$P < 0.05$ and ***$P < 0.001$. 

Fig. 3. SETDB1 and EZH2 regulate H3 trimethylation and invasion of primary cells derived from H-met PDX lines. (A) Western blot of SETDB1 and H3K9me3 after shRNA knockdown of SETDB1 in H-met M481 melanoma cells. Ctl, control. (B) Western blot of EZH2 and H3K27me3 after shRNA knockdown of EZH2 in H-met M481 melanoma cells. (C and D) Relative abundance of TML in H-met M481 melanoma cells after SETDB1 or EZH2 silencing. KD, knockdown. (E and F) Quantitation of invaded cells in a transwell invasion assay after silencing of SETDB1 (E) or EZH2 (F) in H-met M481 melanoma cells. All data are means ± SEM. Statistical significance was assessed using one-way ANOVA followed by Dunnett's tests for multiple comparisons. *P < 0.05.
Fig. 4. Pharmacological inhibition of H3K9 and H3K27 trimethylation suppresses invasiveness of primary cells derived from H-met PDX lines. (A) Expression of H3K9me3 and H3K27me3 after DZNep pretreatment in cells derived from the H-met melanoma line M481. (B) Invasion of melanoma cells derived from three H-met PDX lines after treatment ex vivo with DZNep. Scale bars, 100 μm. (C) Quantitation of invasion after 6 days of DZNep pretreatment. (D) Abundance of H3K27me3 in H-met M481 cells after 6 days of pretreatment with EPZ6438 or GSK126. (E and F) Quantitation of transwell invasion after 6-day EPZ6438 (E) or GSK126 (F) pretreatment. (G) Western blot of H3K27me3 in H-met cells with or without GSK126 and SAM treatment for 6 days. (H) Quantitation of H-met cell invasion with or without GSK126 and SAM treatment. All data are means ± SEM. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s tests for multiple comparisons. *P < 0.05, **P < 0.01, and ***P < 0.001.
SET domain (31). To test whether this inhibitory effect was reversed by SAM, we added SAM to the culture medium together with GSK126 and examined the effects on M481 cell invasion. SAM restored both the abundance of H3K27me3 and the invasive phenotype of GSK126-treated cells (Fig. 4, G and H), indicating on-target efficacy of the drug.

**Down-regulation of H3K9me3 and H3K27me3 suppresses melanoma metastasis in vivo**

To test whether down-regulation of H3K9me3 or H3K27me3 can suppress melanoma metastasis in vivo, we implanted M481 cells expressing a control shRNA or shRNAs against either SETDB1 or EZH2 into the subcutaneous space of NSG mice. shRNAs targeting SETDB1 or EZH2 resulted in durable in vivo suppression of the methyltransferases and histone trimethylation on Lys9 or Lys27 (Fig. 5, A and B). None of the SETDB1 or EZH2 shRNAs altered tumor size in the subcutaneous space, indicating that these epigenetic modifications are dispensable for primary tumor growth in this model (Fig. 5, C and D). However, suppressing either SETDB1 or EZH2 markedly reduced the metastatic burden (Fig. 5, E and F, and fig. S8) and led to decreased free TML in primary tumors (Fig. 5, G and H), further indicating that histone H3 trimethylation at K9 and K27 provides a major source of free TML in these tumors. Suppression of metastasis without changes in primary tumor growth also occurred when SETDB1 was silenced in UT10, another H-met tumor line (fig. S9).

Next, we pharmacologically blocked H3K27 trimethylation in vivo using GSK126. The drug was administered intraperitoneally to M481-bearing mice, either daily (50 mg/kg) or twice per week (150 mg/kg). Either regimen reduced H3K27me3 in the primary tumors (Fig. 6A). In no case did GSK126 suppress the growth of established subcutaneous tumors (Fig. 6, B and C), but it reduced the metastatic burden, similar to the effects of genetic EZH2 silencing (Fig. 6, D and E). Together, these data demonstrate that elevated H3K9me3 and H3K27me3 contribute to the formation of distant metastases from human melanoma tumors in mice, but not to the growth of primary tumors in the subcutaneous space.

Metastasis requires a complex cascade of events involving local invasion, escape to and survival in the circulation, seeding at a distant site, and ultimately growth to produce a macroscopic tumor. Acquisition of each property may be regulated independently, making it of interest to determine where, along the cascade, specific molecular events exert their effects. Data from these melanoma PDX lines, particularly the analysis of the invasive capacity of H-met–derived primary cultures, suggested that H3K9 and H3K27 trimethylation might participate in proximal events such as invasion. To provide support for this hypothesis in vivo, we bypassed the initial steps of metastasis by injecting M481 cells expressing control or SETDB1-silencing shRNAs into the tail vein of NSG mice. In this experiment, SETDB1 silencing had no effect on metastatic burden or overall survival (Fig. 7, A and B), suggesting that high levels of H3K9 trimethylation are dispensable after the cells reach the circulation. This finding also implies that SETDB1 and EZH2 act at a different step of metastasis in these models than antioxidant pathways, which were demonstrated to support metastasis by counteracting oxidative stress in the circulation and visceral organs (23). We used the metabolomic data to further examine the relationship between antioxidant metabolism and SETDB1/EZH2 function. Cystathionine, a precursor of cysteine and glutathione (GSH), was elevated fourfold in H-met tumors, although levels of GSH itself were not different between H-met and L-met tumors (Fig. 2B and table S3). Furthermore, cystathionine levels were not reduced by silencing either SETDB1 or EZH2, and neither methyltransferase affected GSH levels (fig. S10). Thus, although it is possible that some aspects of antioxidant metabolism are regulated epigenetically, the ability of EZH2 and SETDB1 to promote metastasis in these models does not appear to require significant changes in antioxidant function.

**DISCUSSION**

Metabolic reprogramming is a hallmark of cancer and a potentially powerful indicator of the biochemical impact of a broad array of biological features in malignant tissue (15, 32). Metabolism is highly dynamic and responds to both cell-intrinsic and cell-extrinsic effects. Studying metabolic phenotypes among 17 genetically diverse, human-derived melanomas growing in consistent microenvironments allowed us to assess tumor-intrinsic influences on metabolism. These tumors had highly heterogeneous metabolic signatures, but these signatures were remarkably durable among tissue fragments descended from the same parental tumor, with an excellent consistency from mouse to mouse. The fact that both the metabolic and biological features, particularly metastatic potential, were conserved from mouse to mouse made it possible to identify novel connections between metabolism and melanoma biology. We identified distinct sets of metabolic differences that correlate with two clinically important features of melanoma tumor biology: BRAF mutation status and the capacity to give rise to distant metastases in vivo.

Although oncogenic BRAF does not predict metastatic efficiency in these melanoma PDX models, this mutation has been reported to affect some aspects of cellular metabolism (14, 33). Xenografts derived from melanoma cell lines containing BRAF V600E exhibit enhanced growth in response to dietary ketone bodies, indicating that this mutation imposes functionally significant changes to metabolic preferences in the tumor (34). We therefore tested whether BRAF mutations imposed consistent metabolic differences in live, human-derived melanoma tumors. Our metabolic analysis revealed marked differences between tumors containing and lacking BRAF V600E. Mutant tumors had low ratios of pyruvate to citrate, consistent with altered glucose handling compared to tumors with WT BRAF. This was confirmed by using isotope tracing to assess metabolic flux in vivo. After infusion of [U-13C]glucose to generate steady-state labeling of plasma glucose, BRAF V600E tumors had enhanced labeling of all measured products of anaerobic glucose metabolism, including the glycolytic intermediates lactate and alanine, relative to BRAF WT tumors. Despite higher labeling in glycolytic intermediates in BRAF V600E tumors, labeling in TCA cycle intermediates was not different between the genotypes. This labeling pattern could reflect a preference for anaerobic metabolism and total flux that is maintained in vivo, just as has been reported previously in primary tumors (35). Our in vivo analysis, therefore, demonstrated that transformed melanoma tissues harboring the BRAF V600E mutation have acquired a functional bottleneck between glycolysis and pyruvate oxidation.

Posttranslational modifications of histone proteins, including histone methylation, dynamically alter chromatin structure and function, contributing to epigenetic regulation of gene expression (35). Cancer involves frequent perturbations of the epigenetic landscape, and these changes contribute to tumor initiation and progression (36–41). Histone methylation is particularly important in melanoma, with numerous
Fig. 5. SETDB1 and EZH2 are dispensable for the growth of subcutaneous melanomas but are required for the efficient formation of macrometastases in vivo. (A) Western blot of SETDB1 and H3K9me3 in H-met melanoma M481 PDX lines expressing control or SETDB1-silencing shRNAs. (B) Western blot of EZH2 and H3K27me3 in H-met melanoma M481 PDX lines expressing control or EZH2-silencing shRNAs. (C and D) Growth of subcutaneous M481 tumors expressing control shRNAs or shRNAs targeting SETDB1 or EZH2. (E and F) Metastatic burden based on bioluminescence signal after SETDB1 or EZH2 silencing in H-met melanoma M481. (G and H) Relative abundance of free TML in subcutaneous tumors. Relative abundance of TML was determined by LC-MS/MS and normalized by protein concentration. All data are means ± SEM. Statistical significance was assessed using two-way ANOVAs followed by Sidak’s tests for multiple comparisons (C and D) or one-way ANOVA followed by Dunnett’s tests for multiple comparisons (E and F). *P < 0.05. For each group, n = 8 to 10, including two independent experiments.
reports correlating EZH2 expression with clinical aggression and metastasis in both humans and mice (39, 42, 43). The key EZH2-regulated genes relevant to melanoma metastasis are incompletely characterized but may include the tumor suppressor adenosylmethionine decarboxylase 1 (AMD1), whose suppression by EZH2 in human and murine melanoma cells facilitates metastatic spread (39). SETDB1 is encoded by a gene located on chromosome 1q21, which is recurrently amplified in human melanoma, and SETDB1 expression accelerates tumor formation in a zebrafish model of BRAF Mut melanoma (40, 44). In our PDX models, the epigenetic marks induced by EZH2 and SETDB1, H3K27me3 and H3K9me3, respectively, were associated with intrinsic metastatic capacity. Reducing these marks by genetically or pharmacologically inhibiting the methyltransferases was sufficient to reduce metastasis without influencing subcutaneous tumor growth. This indicates a specific metastasis-promoting role for both EZH2 and SETDB1 in our models.

The overall histone methylation state is sensitive to cellular metabolism, because histone methyltransferase activity depends on the ratio of the methyl donor SAM to the product of the methylation reaction, S-adenosylhomocysteine (SAH) (45). Levels of metabolic precursors for SAM biosynthesis also help couple nutrition status with histone methylation (46–48). Furthermore, histone demethylases of the Jumonji domain-containing family convert α-ketoglutarate (α-KG) to succinate, and the ratio of these two metabolites also influences histone methylation (49). Here, none of these metabolites predicted histone methylation as well as the methylated amino acids TML and DMA, which arise from the degradation of methylated proteins. This was a surprising finding given the large number of methylated proteins, and even more marked in that not all histone methylation marks were enhanced in H-met tumors. However, silencing either EZH2 or SETDB1 was sufficient to reduce free TML levels in H-met cells and tumors. To our knowledge, this is the first evidence that free TML levels

Fig. 6. Down-regulation of H3K27me3 by GSK126 treatment suppresses melanoma metastasis in vivo. (A) Western blot of H3K27me3 in H-met M481 tumors after either vehicle or GSK126 treatment using either of the two dosing schedules. (B and C) Growth of subcutaneous H-met M481 tumors in mice treated with vehicle or GSK126 at 50 mg/kg daily (B) or 150 mg/kg twice weekly (C). (D and E) Metastatic disease burden in visceral organs based on total bioluminescence signal after vehicle or GSK126 treatment at 50 mg/kg daily (D) or 150 mg/kg twice weekly (E). Data in (B) and (C) are means ± SEM. Statistical significance was assessed using two-way ANOVAs followed by Sidak’s tests for multiple comparisons (B and C) or unpaired Student’s t tests (D and E), *P < 0.05. For the daily treatment regimen group, n = 4 to 5; for the twice weekly treatment regimen group, n = 14 to 15, including two independent experiments.
correlate with specific histone methylation marks. Our findings do not exclude the possibility that histone marks other than those we examined are also altered in H-met tumors and contribute to high free TML levels and metastasis. Notably, although metabolomics directed us to EZH2 and SETDB1 as regulators of metastasis in these models, the data suggest that differential expression of these methyltransferases, rather than metabolic factors (SAM/SAH ratio, α-KG/succinate ratio, etc.), determined H3K27me3 and H3K9me3 levels. This is consistent with the finding that these specific marks, rather than a global enhancement of all H3 trimethylation, predicted metastatic capacity.

Tumor metastasis requires the acquisition of several distinct biological features, including local invasion and extravasation from the primary tumor, survival in the circulation, and the ability to seed a distant site and ultimately form a new tumor. Each step of the cascade presumably imposes barriers that culminate in the low overall efficiency of metastasis. In principle, therapies aimed at these bottlenecks would protect against metastatic melanoma. The PDX models used here support this idea. Reversible metabolic changes that enable cells to resist oxidative stress in the circulation are essential for metastasis in mice (23). Chronic treatment with antioxidants helped melanoma cells overcome this barrier, enhancing metastatic disease, whereas treatments that increased oxidative stress suppressed metastasis (23). In these same models, we find that silencing EZH2 or SETDB1 does not impair the formation of macrometastases once melanoma cells reach the circulation, suggesting that these methyltransferases act on a more proximal component of the metastatic cascade. This is consistent with the previous observation that the abundance of circulating melanoma cells in NSG mice correlated with metastatic efficiency both in mice and in the donor patients, suggesting that escape from the primary tumor is a functional limitation of metastasis in these models (14). Our new data indicate that the intrinsic invasiveness ex vivo of cells derived from these tumors correlates with metastatic efficiency in vivo and that both activities are suppressed by suppressing trimethylation of H3K9 and H3K27.

Given the importance of epigenetic dysregulation in tumor progression, there is a substantial interest in treating cancer by restoring the “normal” epigenetic landscape of differentiated, nonmalignant cells (50). Drugs that target epigenetic regulators have been applied in the clinic and in genetically modified mice to treat hematologic malignancies including DNA methyltransferase inhibitors, histone deacetylase inhibitors, Janus kinase 1 (JAK1) and JAK2 inhibitors, and mutant isocitrate dehydrogenase inhibitors (51–54). Our findings suggest that the risk of metastatic melanoma can be mitigated with epigenetic modulators targeting distorted histone methylation. A key challenge will be to define the specific genes whose epigenetic regulation promotes melanoma metastasis, as these studies might nominate additional therapeutic strategies with better specificity over genome-wide inhibition of histone methylation.

**MATERIALS AND METHODS**

**Patient-derived melanoma xenografts and isolation of primary cells**

All mouse experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at the University of Texas (UT) Southwestern Medical Center (protocol 2016-101694). Human PDX models were cultivated and passaged, as described (23, 55). Briefly, cell suspensions were first prepared in staining medium [L15 medium containing bovine serum albumin (1 mg/ml), 1% penicillin/streptomycin, and 10 mM Hepes (pH 7.4)] with 25% high-protein Matrigel (product 354248, BD Biosciences). Subcutaneous implantations of 50-μl cell suspension were performed in the right flank of NOD.CB17-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice (UT Southwestern Medical Center). The 50-μl cell suspension usually contained 100 melanoma cells unless otherwise specified. For metastasis experiments, tumor growth was monitored, and the size of the subcutaneous tumors was measured weekly with calipers. At that point, when any tumor in the mouse cohort reached 2 cm in its largest diameter, primary tumors and corresponding metastases were harvested after bioluminescence imaging (BLI) examination (see details below). Intravenous injections were performed by injecting 1000 melanoma cells into the tail vein of NSG mice in a 100-μl staining medium, followed by periodic assessment of bioluminescence. To establish primary cell lines, tumors were cut into small pieces and further dissociated in Sterile Closed-System Tissue Grinders (SKS Science) with collagenase IV (200 U/ml)...
(Worthington) and deoxyribonuclease (DNase) (50 U/ml) for 20 min at 37°C, followed by 0.05% trypsin-EGTA and DNase (100 U/ml) for 2 min at 37°C. Single-cell suspensions were then obtained after the cells were filtered using a 40-µm cell strainer. Isolated primary melanoma cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% Hepes, and 1% penicillin/streptomycin.

**Cell labeling and sorting**

All procedures have been described before (23). All antibody labeling was performed on ice for 20 min, followed by washing and centrifugation. Cells were stained with directly conjugated antibodies to mouse CD45 (30-F11-APC, eBioscience), mouse CD31 (390-APC, BioLegend), Ter119 (TER-119-APC, eBioscience), and human HLA-A, B, C (G46-2.6-BV421, BD Biosciences) to select live human melanoma cells and exclude endothelial and hematopoietic cells. Cells were examined on an LSRFortessa cell analyzer (BD Biosciences) or sorted on a FACSAria II 2.6-BV421, BD Biosciences) to select live human melanoma cells and Ter119 (TER-119-APC, eBioscience), and human HLA-A, B, C (G46-2.6-BV421, BD Biosciences) to select live human melanoma cells and exclude endothelial and hematopoietic cells. Cells were examined on an LSRFortessa cell analyzer (BD Biosciences) or sorted on a FACS Aria cell sorter (Becton Dickinson). SETDB1 or EZH2 knockdown cells were sorted as DsRed and green fluorescent protein (GFP) double-positive cells.

**Analysis of cell invasion**

Invasion assays were carried out by seeding 1 x 10^5 cells on Corning BioCoat Tumor Invasion Systems (351158, Corning). The Corning FluoroBlok 24-Multwell Insert System was used as a cell migration control (354166, Corning). Fetal bovine serum (10%) in RPMI 1640 was used as the chemoattractant. The invaded cells were labeled by Calcein AM fluorescent dye (354216, Corning) after 24-hour incubation at 37°C. Fluorescence of invaded cells was read at a wavelength of 494/517 nm (excitation/emission) (BioTek). The invasion percentage was calculated as [(mean relative fluorescence unit (RFU) of cells invaded through Corning Matrigel Matrix-coated membrane toward chemoattractant)/(mean RFU of cells migrated through uncoated Corning FluoroBlok membrane toward chemoattractant)] × 100. To examine the invasion of drug-treated melanoma cells, the cells were pretreated with DZNep (ApexBio Technology), EZP6438 (Selleck Chemicals), or GSK126 (Selleck Chemicals) for 3 to 6 days before seeding onto the upper chamber of the transwell system. Other experiments used a different BioCoat Tumor Invasion System (354165, Corning) and cell migration control (354578, Corning). For experiments using this system, noninvasive cells on the upper membrane of the insert were removed with Q-tips, and invasive cells attached to the bottom membrane of the insert were stained with crystal violet.

**Lentiviral transduction of human melanoma cells**

All melanoma tumors expressed DsRed and luciferase, as previously described (23). All shRNAs used for knockdown were expressed from either the pGIPZ microRNA-based construct with TurboGFp (GE Dharmacon) or a pGFp-C-shLenti vector (OriGene). For SETDB1, OriGene shRNA clones TL309514A and TL309514AB were used. For EZH2, GE Dharmacon shRNA clones V2LHS_17507 and V2LHS_17509 were used. For virus production, 5 µg of the appropriate plasmid, 1 µg of pMD2G, and 3 µg of psPAX2 were transfected into 293T cells using FuGENE 6 (Promega) according to the manufacturer’s instructions. Replication-incompetent viral supernatants were collected 48 hours after transfection and filtered through a 0.45-µm filter. DsRed and GFP double-positive cells were sorted and placed in culture or implanted into the NSG mice as needed.

**LC-MS/MS metabolomic analysis**

After dissection of subcutaneous and metastatic tumors, the tissue was divided into three fragments and frozen in liquid nitrogen, minimizing the time between euthanasia and flash-freezing the sample. Fragments weighing about 100 mg were homogenized in 80% cold methanol using an electronic tissue disruptor (Qiagen), vortexed, and extracted overnight at −80°C. On the following day, metabolites from 300-µl homogenates were further extracted for 20 min at 4°C in 80% methanol, then centrifuged at 13,000g for 20 min, and dried down in a SpeedVac (Thermo Fisher Scientific). Before analysis, dried metabolites were dissolved in 0.03% formic acid in water and then analyzed using LC-MS/MS composed of a Nexera Ultra High Performance Liquid Chromatograph system (Shimadzu) coupled to an AB Sciex QTRAP 5500 triple quadrupole mass spectrometer. Details of the chromatography conditions and mass spectrometry conditions were as previously reported (56). MultiQuant software (AB Sciex) was used to integrate chromatogram peaks. The intensity of each ion was calculated by normalizing single ion counts versus total ion counts of the entire chromatogram. The data matrix was then exported into the SIMCA-P+TM software (Umetrics) and transformed by mean-centering and Pareto scaling. Both unsupervised and supervised multivariate data analyses (MDAs), including PCA and PLS-DA, were applied to classify the samples (57). Principal components were generated by MDA to represent the major latent variables in the data matrix and were described in a score scatterplot, with VIP scores ≥1 considered as significantly discriminating between the groups (http://umetrics.com/sites/default/files/kb/multivariate_faq.pdf).

**In vivo isotope tracing**

In vivo [U-13C]glucose tracing was performed when subcutaneous tumors reached 2 cm in diameter. Before infusions, mice were fasted for 16 hours and then 27-gauge catheters were placed in the lateral tail vein under anesthesia. An aqueous solution of [U-13C]glucose (400 mg/kg) (Cambridge Isotope Laboratories) in 125 µl of saline was infused using a syringe pump as a bolus over 1 min, followed by infusion of [U-13C]glucose (12 mg/kg per min) at a rate of 150 µl/hour for 150 min. Blood samples of 20 µl were obtained periodically to assess fractional enrichment of plasma glucose. At the end of the infusion, animals were euthanized and then tumors were harvested and immediately frozen in liquid nitrogen. Metabolites were extracted as described above. For analysis by gas chromatography–mass spectrometry, myristic acid was added to the supernatant as an internal standard, and the samples were evaporated and derivatized by tert-butyl dimethylsilylation (TBDMS reagent, Thermo Fisher Scientific). Isotopolog distribution calculations were performed as previously described (58).

**In vivo treatment of xenografts with GSK126**

One hundred melanoma cells were injected subcutaneously into the right flanks of NSG mice. When tumors became palpable, the mice were injected intraperitoneally with GSK126 (Selleck Chemicals) at either 50 mg/kg daily or 150 mg/kg twice weekly. Captisol (20%) was used as the vehicle. The preparation of GSK126 solution was previously described (59). Tumor growth was monitored weekly using a caliper. Mice were euthanized when the primary tumor reached 2 cm in size. At the end of the experiment, tumors were immediately frozen in liquid nitrogen, and organs were analyzed for micrometastases and macrometastases by bioluminescence imaging and visual inspection.

**Western blot analysis**

Total protein lysates were prepared in radioimmunoprecipitation assay buffer and quantified using the BCA Protein Assay (Thermo Fisher Scientific). Histones were extracted by an EpiSeeker histone extraction kit (Abcam). Protein was separated on 4 to 20% SDS–polyacrylamide
gel electrophoresis gels, transferred to polyvinylidene difluoride membranes, and probed with antibodies against trimethylated histone H3, β-actin, p62, LC3 (Sigma-Aldrich), H3K4me3, H3K9me3 (Abcam), H3K27me3 (Millipore), H3K36me3, H3K79me3, histone H3, EZH2 (Cell Signaling), SETDB1 (Thermo Fisher Scientific), Atg5 (Cell Signaling), cyclophilin B (Abcam), and SUV39H1 (Santa Cruz Biotechnology). Quantification was performed using ImageJ after normalizing to loading controls.

Bioluminescence imaging
All procedures were previously described (23). Briefly, 100 luciferase-DSRed+ cells were injected into the right flank, and tumor growth was monitored until the diameter approached 2 cm. Mice were then imaged using an IVIS Imaging System 200 Series (Caliper Life Sciences) with Living Image software. An un.injected control mouse was included to establish background luminescence. To perform luminescence imaging, 100 μl of phosphate-buffered saline with d-luciferin monopotassium salt (40 mg/ml) (Gold Biotechnology) was subcutaneously injected. Anesthesia was initiated after about 3 min, followed by imaging after an additional 2 min. The whole body was first imaged, followed by euthanasia, organ harvest, and organ imaging. Exposure times ranged from 10 to 60 s to avoid signal saturation. The bioluminescence was quantified with “region of interest” measurement tools in Living Image software (PerkinElmer). To track tumor metastasis, mice were imaged once a month (23).

Statistical methods
Sample sizes were not predetermined using statistical methods. Variation was illustrated using SE. To assess statistical significance between two groups, a two-tailed Welch’s unequal variance t test was used. When applying ANOVA, we first used the Brown-Forsythe test to determine whether variation was homogeneous among the groups. If the assumption of equal variance was violated, a log2 transformation was applied. If variation did not differ significantly among the experimental treatments, two-tailed Student’s t tests were used. If more than two treatments were being compared, a one-way ANOVA followed by Dunnett’s multiple comparisons test was used. If more than two groups were being compared in an experiment with multiple measurements, a two-way ANOVA followed by Sidak’s multiple comparisons test was used. Criteria for mouse usage were previously described (23). Briefly, for all in vivo experiments, mouse cages were randomized between treatments, but all mice in the same cage received the same treatment. No blinding was used. Both male and female mice were used. In xenograft assays, we injected five 6- to 8-week-old NSG mice per treatment. For longer assays, 10 mice per treatment were used to anticipate non-melanoma-related deaths in this highly immunocompromised strain. Mice dying from opportunistic infections and other causes unrelated to melanoma were excluded from the experiment (23).

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/11/eaao5268/DC1

**REFERENCES AND NOTES**


**fig. 57. Effect of EZH2 inhibition on H-met cell invasiveness.**

**fig. 58. Assessment of metastasis in visceral organs of mice bearing M481 H-met tumors.**

**fig. 59. Examination of SETDB1 knockdown in UT101 tumor line in vivo.**

**fig. 60. Examination of cytostatinone and GSH in M481 primary tumors after SETDB1 or EZH2 knockdown.**


26. J. Leiper, P. Vallance, Biological significance of endogenous methylarginines that inhibit nitric oxide synthases.


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The abundance of metabolites related to protein methylation correlates with the metastatic capacity of human melanoma xenografts

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