Cancer-specific overmethylation of histone H3 lysines is necessary for methionine addiction and malignancy

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Abstract

Methionine addiction is a fundamental and general hallmark of cancer. Methionine addiction results from the overuse of methionine by cancer cells for excess transmethylation reactions. In order to identify excess transmethylation reactions in cancer, we compared the histone H3 lysine methylation status between methionine-addicted cancer cells, normal cells and revertants of methionine-addicted cancer cells which regained methionine independence and lost malignancy. The levels of H3K4me3, H3K9me3 and pan-methyl lysine of histone H3 were elevated in methionine-addicted cancer cells *in vitro* compared to methionine-independent revertants isolated from the cancer cells and to normal cells. Tumorigenicity in nude mice was highly reduced in the methionine-independent revertants compared to the parental cells. The methionine-independent revertants no longer overmethylated pan-methyl lysine of H3, H3K4me3 and H3K9me3. Our previous studies showed that methionine restriction (MR) selectively arrests methionine-addicted cancer cells due to loss of histone H3 lysine methylation, which was stable in normal cells under MR. Our previous and present results suggest that overmethylation of histone H3 lysine is necessary for methionine addiction of cancer, required for the growth of cancer cells *in vitro* and *in vivo,* and necessary for malignancy. Methionine addiction has revealed fundamental molecular changes necessary for malignancy and presents great potential as a pan-cancer therapeutic target.

Signiificance Statement

All cancer cell types are methionine-addicted. Methionine addiction is due to the overuse of methionine by cancer cells for excess transmethylation reactions. In the present study, we showed that the level of histone H3 lysine methylation was elevated in methionine-addicted cancer cells compared to normal fibroblasts and methionine-independent revertants with reduced malignancy that were derived from the methionine-addicted cancer cells. These results suggest that overmethylation of histone H3 lysine is necessary for methionine addiction of cancer and malignancy itself. Methionine addiction has revealed fundamental molecular changes necessary for malignancy and has been shown to be a universal therapeutic target in numerous pre-clinical studies of all major cancer types and has great clinical potential.

Main Text

Introduction

Methionine addiction is a fundamental and general hallmark of cancer and was discovered by one of us (RMH) almost a half century ago (1). Methionine addiction is characterized by a requirement for exogenous methionine for growth by cancer cells even though the methionine-addicted cancer cells synthesize normal or excess amounts of methionine (1). Methionine-addicted cancer cells, require much larger amounts of exogenous methionine than normal cells in order to grow, hence their methionine addiction and dependence (1-4). Methionine restriction (MR) selectively arrests cancer cells in the late-S/G_2 phase of the cell cycle, but not normal cells, where the cancer cells become sensitive to cytotoxic chemotherapy (5-7). Methionine addiction results from the overuse of methionine by cancer cells for excess transmethylation reactions, termed the Hoffman-effect, analogous to the Warburg effect for glucose overuse by cancer cells (8, 9). Methionine addiction is tightly linked to other hallmarks of cancer (10, 11) and was thought possibly the very basis of malignancy itself (12).

Although we have long since known methionine is overused for transmethylation reactions cancer cells (8, 13), we have not, until now, known the fate of at least a significant amount of the excess methyl groups that were transferred. Our previous study showed that histone H3 lysine overmethylation is unstable during MR of methionine-addicted cancer cells and arrests their proliferation. In contrast histone H3 lysine is stable in normal cells in which MR does not arrest their proliferation. These results suggested that histone H3 lysine methylation may be related to methionine addiction.

In the present study, we report that the lysines of histone H3, and specifically histone H3K4me3 and H3K9me3 are over-methylated in methionine-addicted cancer cells, compared to low-malignant methionine-independent revertants derived from high-malignant methionine-addicted the cancer cells and compared to normal human fibroblasts. Our results suggest that histone H3 lysine overmethylation is necessary for methionine addiction, and is required for the growth of the cancer cells *in vitro* and *in vivo*, and thus is necessary for malignancy.

Results

Cancer cells have overmethylation of histone H3 lysines which is depleted by MR while normal methylation of histone H3 lysines in normal cells is stable to MR.

The levels of histone H3 lysine methylation were elevated in methionine-addicted cancer cells compared to normal fibroblasts. We evaluated the levels of histone H3 lysine methylation in methionine-addicted cancer cells and methionine-independent normal cells after MR for 96 hours. MR by recombinant methioninase (rMETase) decreased the level of histone H3 lysine methylation (Fig. 1A) and decreased the levels of H3K4me3 and H3K9me3 in cancer cells while arresting their proliferation (Fig. 1B) (14). In contrast, MR did not affect the levels of histone H3 methylation in normal fibroblasts and did not arrest their proliferation (14). These results demonstrate that MR selectively suppressed overmethylation of histone H3 lysine in cancer cells while selectively arresting their proliferation, both in contrast to normal cells

Overmethylation of histone H3 does not occur in methionine-independent revertants derived from methionine-addicted cancer cells.

To compare the methionine addiction of parental cancer cells and methionine-independent revertants derived from the parental cells, we first evaluated their cell proliferation kinetics under MR. The proliferation of parental methionine-addicted cancer cells arrested within 48-72 hours of MR. In contrast, methionine-independent revertants were able to proliferate under MR, similar to normal cells indicating the revertants have lost their methionine addiction (Fig. 2A) (10, 11, 13, 15).

We then compared the methylation status of histone H3 lysine in methionine-independent revertants and their methioninie-addicted parental cells. Parental cancer cells and methionine-independent revertants were cultured in normal medium for 96 hours and histones were extracted. The levels of H3K4me3 and H3K9me3 were decreased in methionine-independent revertants, even without MR, compared to parental methionine-addicted cells (Fig. 2B). The level of pan-methyl lysine of histone H3 was also decreased in methionine-independent revertants (Fig. 2C). These results indicate that the decreased use of methionine for histone H3 lysine methylation was the basis of the methionine-independence of the revertants and their ability to revert, freeing them from methionine addiction.

Methionine-independent revertants lose malignancy.

To compare the malignancy of parental methionine-addicted cancer cells and methionine-independent revertants derived from them, tumorigenicity of the parental and revertant cells was compared in subcutaneous xenograft mouse models. Methionine-addicted parental cancer cells (HCT 116 and H460) and their methionine-independent revertants (HCT 116-R and H460-R, respectively) were compared for their ability to form tumors in nude mice. Only half of 10 mice formed tumors in HCT 116-R compared to all mice with HCT 116 after injection of 1×10^6 cells (Fig. 3A). The mean tumor volume was significantly lower in HCT 116-R tumors than HCT 116 tumors ($P = 0.0085$). Eight of ten mice injected with 5×10^5 parental HCT 116 cells formed tumors and no mice injected with 5×10^5 HCT 116-R cells formed tumors (Fig. 3B). The H460-R revertants formed no tumors in all 5 nude mice when 2.5×10^5 cells were injected compared to the H460 parents which formed tumors in all 5 mice after injection of the same amount of cells (Fig.3C). Immunohistochemistry staining showed that the number of Ki-67-positive cells was significantly lower in the HCT 116-R tumors compared to the parental HCT 116 tumors ($n = 4$, $P < 0.0001$) (Fig. 3D, E).

Immunoblotting showed that the levels of pan-methyl lysine of H3, H3K4me3 and H3K9me3 were much lower in HCT 116-R tumors than in HCT 116 tumors (Fig. 3F, G). These results indicated that malignancy was decreased in methionine-independent revertants compared to parental methionine-addicted cancer cells, due to the decrease in histone H3 lysine methylation. The results of the present study and our previous study (14) indicate that overmethylation of lysines in histone H3 is necessary for both methionine addiction of cancer cells and their malignancy.

Discussion

Methionine dependence of cancer has been known since 1959 when Sugimura showed that tumors in rats slowed their growth when methionine was removed from their diet compared to when other amino acids were removed from the diet (16). It was not until the early 1970s that methionine dependence was found in cultured cancer cells (17, 18) and it was initially claimed that methionine dependence was due to reduced ability of the cancer cells to synthesize methionine from homocysteine (18). At that time in 1976 (1), one of us (RMH) demonstrated that cultured methionine-dependent cancer cells made normal or more-than-normal amounts of methionine from homocysteine, but still required exogenous methionine in order to proliferate (1), first demonstrating that cancer cells were methionine addicted. The requirement for exogenous methionine by methionine-addicted cancer cells was later confirmed by Tisdale in 1984 (19) and in 2019 by Tam's group (20).

Methionine-independent revertants isolated from methionine-addicted cancer cells revealed that, among such revertants, were those that did not increase their rate of synthesis of methionine from homocysteine, further indicating that decreased methionine synthetic capacity was not the basis of methionine dependence. Then we found that despite the large amounts of endogenous methionine made by cancer cells, under restriction of exogenous methionine, the cancer cells had very low levels of free methionine, low levels of S-adenosylmethionine (SAM) and a low ratio of SAM to Sadenosylhomocysteine (SAH) (2-4). This was explained next when we observed that all cancer cells tested had elevated rates of transmethylation compared to normal cells which used excess amounts of methionine and SAM and depleted their cellular pools (8). We then understood in 1984 (8) that methionine addiction was due to overuse of methionine for transmethylation. This was confirmed by Judde et al. when it was demonstrated that overall transmethylation was strongly reduced in methionine-independent revertants derived from the methionine-addicted cancer cells (12, 13). The phenomenon of methionine-addicted cancer cells was later termed the Hoffman-effect (9).

Tam's group in 2019 (20), confirmed our results of 43 years previously (1) of high methionine flux in tumor-initiating cancer cells resulting in a requirement for exogenous methionine for their proliferation. Tam et al. showed abundant levels of tri-methyl histone H3 in tumor-initiating cells, compared to cancer cells which were not tumor-initiating cells (20). However, the levels of tri-methyl histone H3 in normal cells were not measured by Tam et al. Tam's group showed that MR eliminated tumor-initiating cells (20), similar to our observation 40 years earlier that MR eliminated clonogenic cancer cells (5). Mentch et al. showed that tri-methyl histone H3 was unstable in cancer cells under MR but did not examine the stability of tri-methyl histone H3 in normal cells under MR (21). Our recent previous study also demonstrated that histone H3K4me3 and H3K9me3 were unstable in cancer cells under MR; and we showed for the first time H3K4me3 and H3K9me3 were stable in normal cells under MR (14), which explains why cancer cells arrest under MR and normal cells do not. And most importantly, the present study shows that pan-histone H3 lysine, H3K4me3 and H3K9me3 are not over methylated in normal cells or in methionine-independent revertants that have decreased malignancy and lost methionine addiction, and can proliferate under MR. The present study and our recent study (14) indicate that methionine-addicted cancer cells require histone H3 overmethylation in order to grow *in vitro* and to form tumors and for their malignancy.

The present study showing overmethylated lysines of histone H3, including H3K4me3 and H3K9me3, explains at least in part the fate of an important set of methyl-groups in cancer cells and why cancer cells are methionine addicted and not normal or revertant cells. Most importantly, pan-methyl lysine of histone H3 and marks H3K4me3 and H3K9me3 are not overmethylated in normal cells and low-malignancy methionine-independent revertants derived from methionine-addicted cancer cells. The early methionine-independent revertants isolated in 1978-1979 (10, 15) and also revertants of triple negative breast cancer cells (11), had reduced clonogenicity in agar, a surrogate maker of malignancy and cancer stemness, giving the first hints that methionine addiction was necessary for malignancy. Our present results therefore indicate overmethylation of pan-lysines of histone H3 and marks H3K4me3 and H3K9me3 is necessary for malignancy.

All tested cancer types are methionine addicted as shown in vitro, even if they can grow in homocysteine in place of methionine (22, 23), and tumors in vivo have been universally shown to be sensitive to MR, either by a methionine-free diet or methioninase treatment (23-30). These results indicate that methionine addiction, due to histone H3 lysine overmethylation, is a general hallmark of cancer. Our results also suggest that methionine addiction is a fundamental hallmark of cancer, as it is necessary for malignancy.

It is possible that the histone H3 lysine methylation marks function as genetic switches (31) that turn on or off large number of genes necessary for malignant transformation. For example, we and other investigators have previously reported that MR increased TNF-related apoptosis-induced ligand receptor-2 (TRAIL-R2) expression in cancer cells and enhanced the efficacy of TRAIL-R2 targeted therapy (32, 33). These and other phenotypic changes associated with malignancy, such as aneuploidy (34), may result from the global genomic changes due to histone H3 overmethylation in cancer (12). This will be investigated in further studies. The reprogramming of methionine metabolism (35) in cancer may be initiated by activated oncogenes as suggested by Vanhamme and Szpirer and Sacco et al. (36, 37) where they showed that activated *H-ras* or *K-ras* oncogenes, when transfected into normal cells, caused them to become methionine dependent. It has also been shown that viral infection of normal cells induces methionine addiction (38). The reprogramming of methionine metabolism resulting in methionine addiction, histone H3 lysine overmethylation and malignancy may involve overexpression of specific histone H3 lysine methyltransferases (39). This will be investigated in future studies.

Also important is the finding by Breillout et al. that as cells become more malignant they become more methionine addicted (40). Another important observation is from PET imaging of cancer in patients which consistently shows that [^{11}C] methionine gives a much stronger PET signal than [^{18}F] deoxyglucose in head-to-head comparisons, demonstrating that cancers are methionine addicted in patients and that the Hoffman-effect is stronger than Warburg-effect (41, 42). Other molecules, such as various RNAs, may also be overmethylated in cancer cells (43, 44). Further experiments are necessary to account for all the increased transferred methyl groups in cancers.

In summary, our results suggest that overmethylation of histone H3 lysine is necessary for methionine addiction and necessary for malignancy of cancer cells. Methionine addiction is being widely recognized (45) and may provide a universal target for methionine-restriction cancer therapy, such as with methioninase, since methionine addiction is a general and fundamental hallmark of cancer.

Materials and Methods

Cell culture

The H460 human lang cancer cell line, HCT 116 human colon cancer cell line, MIA PaCa-2 human pancreatic cancer cell line and Hs27 human normal foreskin fibroblasts were used in the present study. All cell types were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin.

Recombinant methioninase production

Recombinant L-methionine α-deamino-γ-mercapto-methane lyase (rMETase) is a 172-kDa molecular homotetrameric PLP enzyme (46). The rMETase gene was cloned in *E.coli*. Fermentation of rMETase-recombinant *E. coli* and purification of rMETase were performed as described (47).

Establishment of methionine-independent revertant cancer cells

The H460, HCT 116 and MIA PaCa-2 parental cell lines were cultured in medium with 1 U/ml of rMETase for more than one month as a first selection and survivaing cells were isolated. These surviving cells were cultured in medium with 5 U/ml rMETase for more than 2 weeks as a second selection and surviving cells were isolated. The surviving cells isolated after the second selection were termed H460-R, HCT 116-R and MIA PaCa-2-R cells, respectively.

Immunoblotting

Extraction of histone from cells and tumors and immunoblotting were performed as described (14). Anti-H3K4me3 antibody (1:1,000, #9751, Cell Signaling Technology, Danvers, MA, USA); anti-H3K9me3 antibody (1:1,000, #13969, Cell Signaling Technology); or anti-pan methyl lysine antibody (1:2,000, ab7315, Abcam, Cambridge, UK) for detection of pan methyl lysine of histone H3 were used as the primary antibody. Total histone H3 was used as the internal loading control (1:2,000, #4499, Cell Signaling Technology). Horseradish-peroxidase-conjugated anti-rabbit IgG (1:20,000, SA00001-2, Proteintech, Rosemont, IL, USA) was used for secondary antibody. Immunoreactive proteins were visualized with Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The UVP ChemStudio (Analytik Jena US LLC, Upland, CA, USA) was used to detect the signals. The signals of H3K4me3, H3K9me3 and pan methyl lysine of histone H3 were normalized to the total histone H3 signal before relative quantification.

Efficacy of MR effected by rMETase on cell proliferation

Cells were cultured in 96-well plates (1×10^3 cells/well) in normal DMEM overnight. The next day, the medium was changed to normal DMEM or DMEM with rMETase (1 U/ml). Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) after 0, 24, 48, 72 and 96 hours of medium change. Experiments were performed three times, each in triplicate.

Animal studies

4-6 weeks old athymic *nu/nu* female mice (AntiCancer Inc, San Diego, CA, USA), were used in this study. All mice were kept in a barrier facility on a high efficacy particulate air (HEPA)-filtered rack under standard conditions of 12 h light/dark cycles. Animal studies were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specially approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Evaluation of *in vivo* tumorgenicity in a subcutaneous mouse model

Two different doses of HCT 116 and HCT 116-R cells (5×10^5 or 1×10^6 cells / 100 μl each) were injected subcutaneously into the flanks of nude mice. Each group comprised ten mice. H460 and H460-R (2.5×10^5 cells / 100 μl) were also injected subcutaneously to five mice in each group. The mice with HCT 116 or HCT 116-R tumors were sacrificed on day 28 and tumor volume was measured at termination.

H & E staining and immunohistochemistry

Hematoxylin and eosin (H&E) staining and immunohistochemical staining (IHC) were performed as described (33). Rabbit polyclonal anti-Ki-67 antibody (1:16,000, 27309-1-AP, Proteintech) was used as a cell proliferation marker. For immunohistological evaluation, two investigators (K.H. and

Y.A.) selected the five most abundant regions of each tissue and counted Ki-67-positive cells (magnification, 400×) in each of the five regions.

Statistical analyses

All statistical analyses were performed with JMP PRO ver. 15.0.0 (SAS Institute, Cary, NC, USA). Mann-Whitney's U test was used to compare between groups for animal studies. Bar graphs show the mean, and error bars express standard error of the mean. A probability value of $P < 0.05$ was defined as statistically significant.

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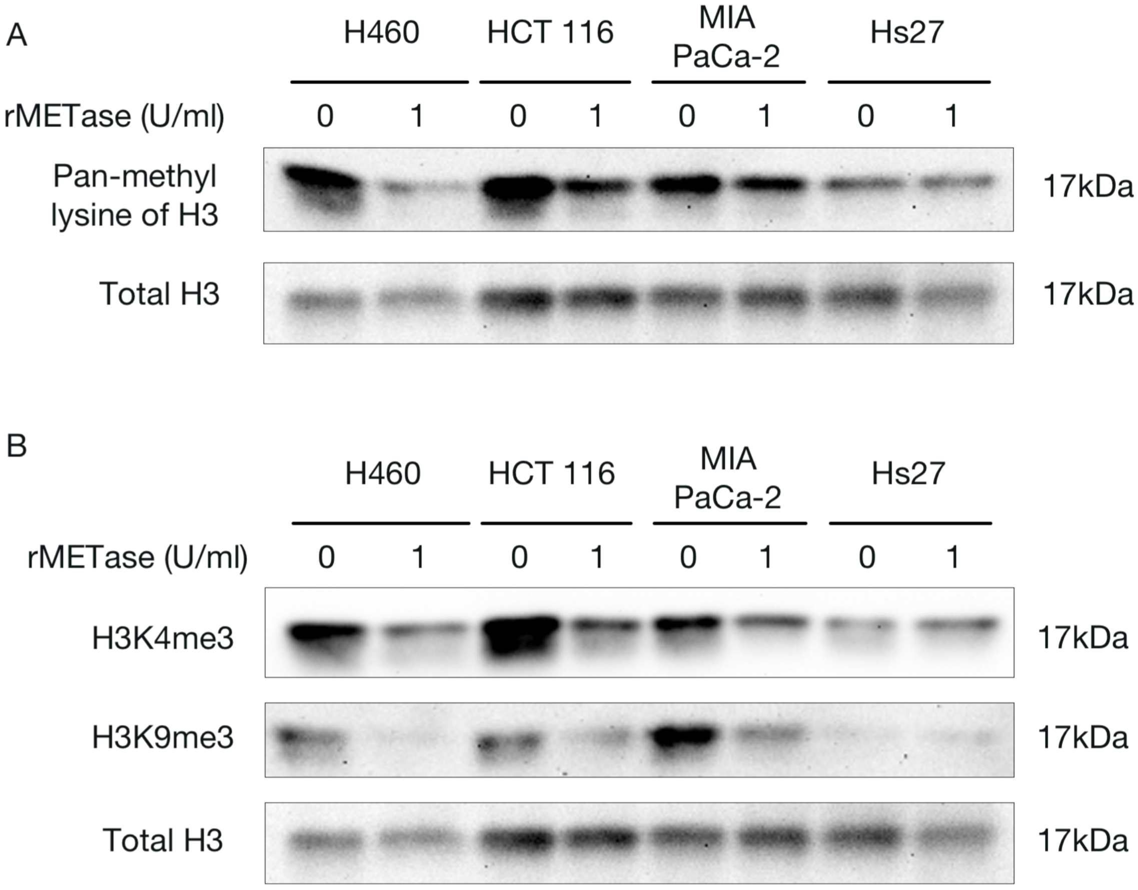
Figures and Tables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H460
HCT 116
MIA PaCa-2
Hs27
rMETase (U/ml)
0
1
0
1
0
1
0
1
H3K4me3
17kDa
H3K9me3
17kDa
Total H3
17kDa

Figure 1. Methionine restriction decreases the level of pan-methyl lysine of H3, H3K4me3 and H3K9me3 in cancer cells, but not in normal fibroblasts *in vitro*.

(A) Immunoblot of pan-methyl lysine of H3 in lung cancer, colon cancer and pancreatic cancer cells and normal fibroblasts grown in normal medium or medium with rMETase (1 U/ml) for 96 hours. (B) Immunoblot of H3K4me3 and H3K9me3 in lung cancer, colon cancer and pancreatic cancer cells and normal fibroblasts grown in normal medium or medium with rMETase (1 U/ml) for 96 hours.

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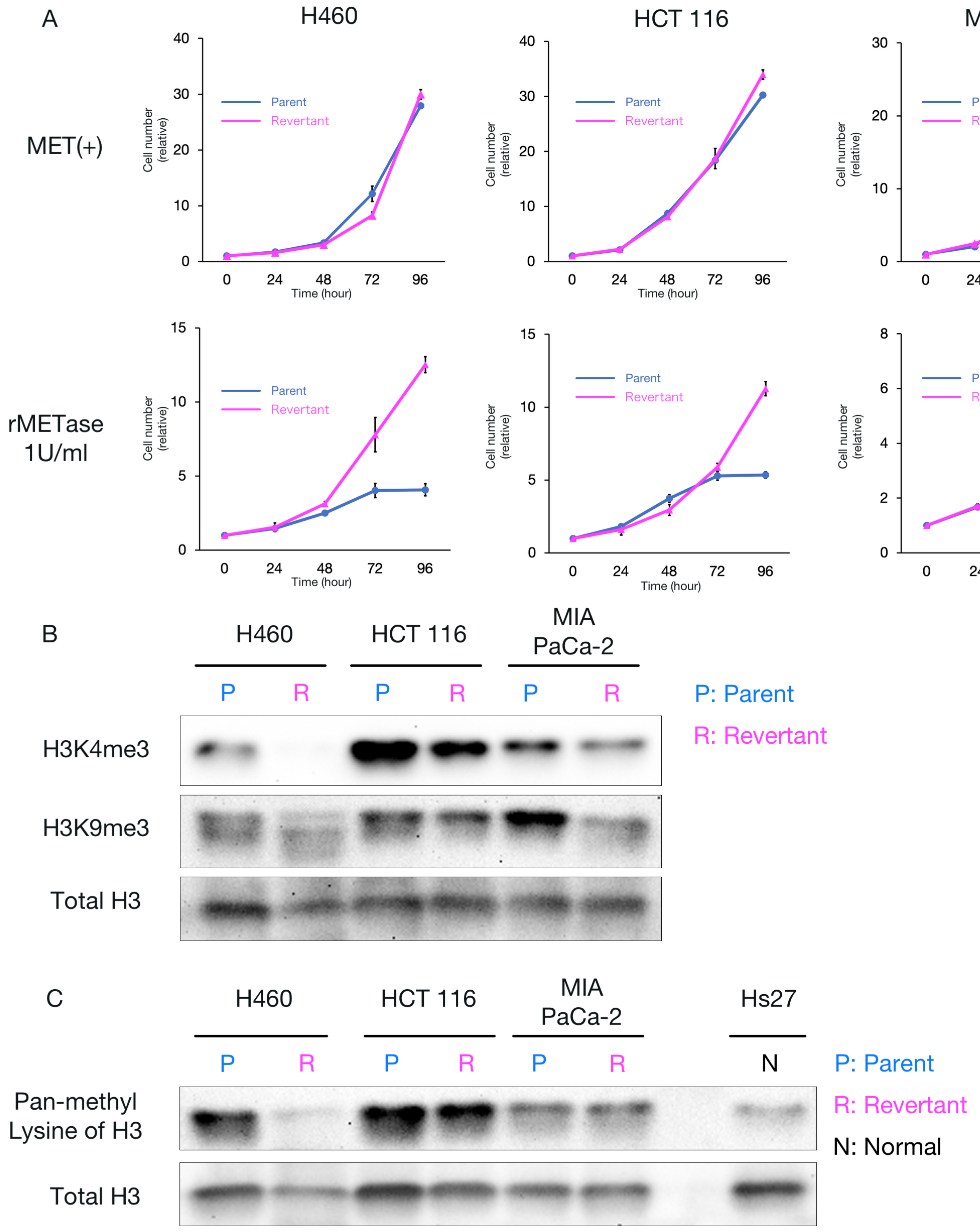

Figure 2. Methionine-independent revertants are able to proliferate under methionine restriction and have lower levels of H3K4me3, H3K9me3 and pan-methyl lysine of H3 compared to their parental methionine-addicted cancer cells *in vitro*.

(A) Cell proliferation assay of methionine-addicted parental cancer cells and their methionine-independent revertants under methionine restriction. Cells were cultured in normal medium or medium with rMETase (1

U/ml) for 24, 48, 72 and 96 h (mean ± SEM, n = 3). (B) Immunoblot of H3K4me3 and H3K9me3 in methionine-addicted cancer cells and methionine-independent revertants grown in normal medium. (C) Immunoblot of pan-methyl lysine of H3 in methionine-addicted cancer cells, methionine-independent revertants and normal fibroblasts grown in normal medium.

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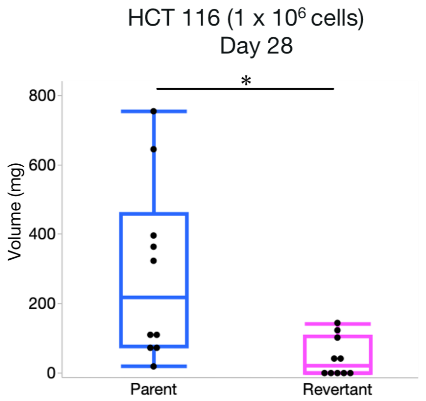
A



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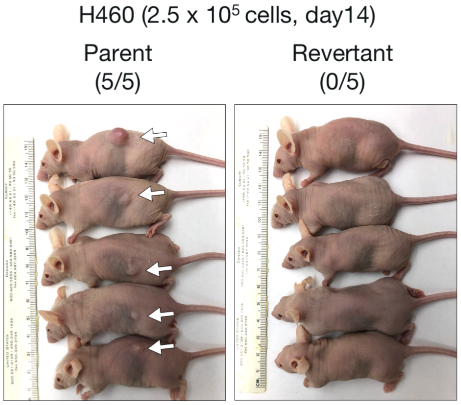
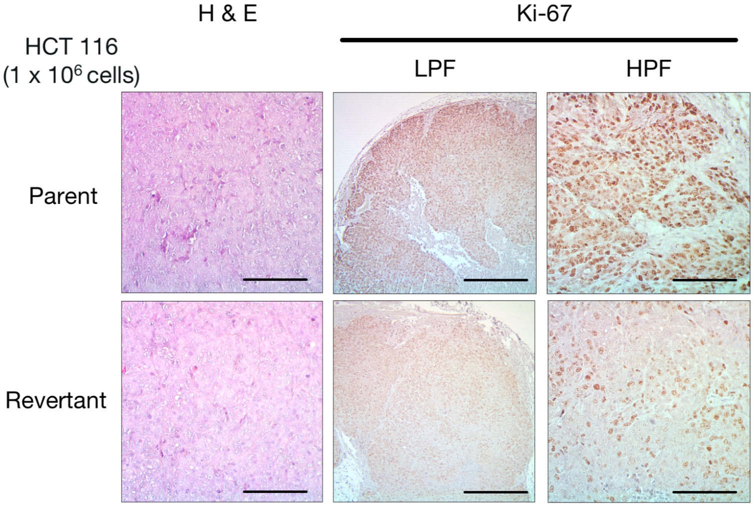

H460 (2.5 x 10^5 cells, day14)
Parent
(5/5)
Revertant
(0/5)

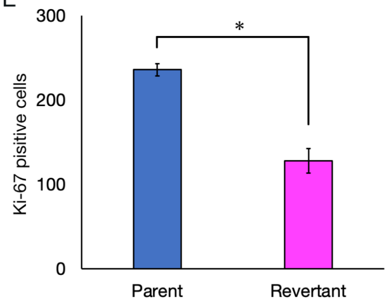
B

		Cell number	
		5 x 10^5	1 x 10^6
HCT 116	Parent	8/10	10/10
	Revertant	0/10	5/10

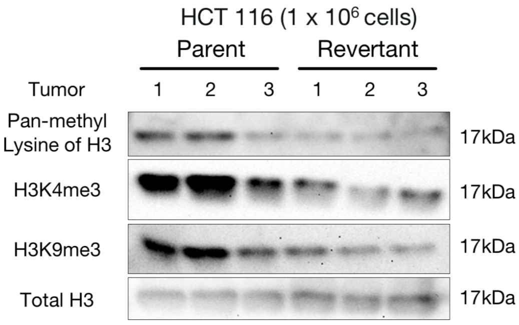
D


H & E
Ki-67
HCT 116
(1 x 10^6 cells)
LPF
HPF
Parent
Revertant

E



F


HCT 116 (1 x 10^6 cells)
Parent
Revertant
Tumor
1
2
3
1
2
3
Pan-methyl Lysine of H3
H3K4me3
H3K9me3
Total H3
17kDa
17kDa
17kDa
17kDa

G

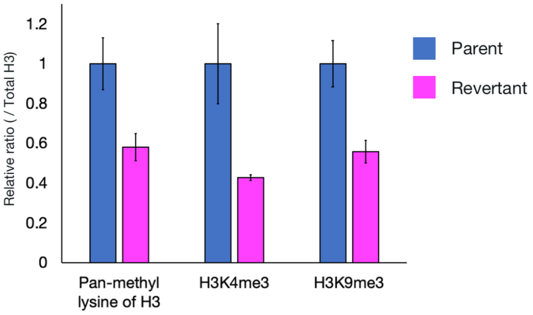


Figure 3. Low-malignancy methionine-independent revertants have lower histone H3 methylation and less tumorigenicity than parental methionine-addicted cancer cells *in vivo*.

(A) Mean tumor volume of HCT 116 and HCT 116-R at 28 days after 1×10^6 cells were injected (n = 10. *, $p < 0.01$). (B) Tumor volume at day 28 of HCT 116 and HCT 116-R. (C) Representative tumor images of H460 and H460-R on 14 days after 2.5×10^5 cells were injected. White arrow: tumors. (D) Representative images of H & E staining and immunohistochemical staining for Ki-67 of HCT 116 and HCT 116-R. LPF: low power field (40×). HPF: high power field (200×). Scale bar: 100 µm (H & E and HPF of Ki-67), 500 µm (LPF of Ki-67). (E) Quantification of the Ki-67 positive cells. Five images were randomly obtained in high power fields (400×) and the positive cells compared (mean ± SEM, n = 4). *, $p < 0.0001$. (F) Immunoblot of pan-methyl lysine of H3, H3K4me3 and H3K9me3 in subcutaneous tumors formed from HCT 116 and HCT 116-R (n = 3). (G) The ratio of pan-methyl lysine of H3, H3K4me3 or H3K9me3 / total H3 in the tumors formed from HCT 116 and HCT 116-R (mean ± SEM, n = 3).

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