Cancer-specific overmethylation of histone H3 lysines is linked with methionine addiction and malignancy

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Abstract

Methionine addiction is a fundamental and general hallmark of cancer and is an area of current intense interest. Methionine addiction results from the overuse of methionine by cancer cells for excess transmethylation reactions. In order to identify excess transmethylation reactions in cancer and further understand the basis of methionine addiction, we compared the histone H3 lysine methylation status between methionine-addicted cancer cells, normal cells and rare revertants of methionine-addicted cancer cells which regained methionine independence and lost malignancy. The levels of H3K4me3, H3K9me3 and H3K36me3 were strongly elevated in methionine-addicted cancer cells in vitro compared to methionine-independent revertants isolated from the cancer cells. Tumorigenicity and experimental metastatic potential in nude mice were highly reduced in the methionine-independent revertants compared to the parental cells. 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 linked with methionine addiction of cancer, required for the growth of cancer cells in vitro and in vivo, and necessary for malignancy as the histone lysine overmethylation disappears when the methionine-addicted cells revert to methionine independence and reduced malignancy. Methionine addiction has revealed fundamental molecular changes necessary for malignancy and presents great potential as a pan-cancer therapeutic target.
Introduction

Methionine addiction is a fundamental and general hallmark of cancer and is an area of current intense interest \(^1\text{-}^5\). 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 \(^6\). Methionine-addicted cancer cells, require much larger amounts of exogenous methionine than normal cells in order to grow, hence their methionine addiction and dependence \(^6\text{-}^9\). Methionine restriction (MR) by either methionine-free medium \(^10\text{-}^11\) or by a low-methionine diet \(^12\) or by methioninase \(^13\) selectively arrests cancer cells in the late S/G\(_2\) phase of the cell cycle, but not normal cells or methionine-independent revertants, where the cancer cells become sensitive to cytotoxic chemotherapy \(^11\text{-}^13,^14\). 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 \(^15\text{-}^16\). Methionine addiction is tightly linked to other hallmarks of cancer \(^17\text{-}^18\) and was thought possibly the very basis of malignancy itself \(^19\).

Although we have long since known methionine is overused for transmethylation reactions cancer cells \(^15\text{-}^20\), we have poorly understood the fate of at least a significant amount of the excess methyl groups that were transferred. Our previous studies have shown that histone H3 lysine marks are overmethylated in cancer cells compared to normal cells and that the 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 under MR, which unlike cancer cells 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 \textit{in vitro} and \textit{in vivo}, and thus is necessary for malignancy.

\textbf{Materials and Methods}

\textbf{Cell culture}

The H460 human lung cancer cell line, HCT 116 human colon cancer cell line, MIA PaCa-2 human pancreatic cancer cell line and Hs27 human normal foreskin fibroblast cell line were used in the present study. HCT 116 cells were stably transduced to express green fluorescent protein (GFP) as previously described \textsuperscript{21} and used for an \textit{in vivo} experimental metastatic study. All cell lines 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.

\textbf{Recombinant methioninase production}

Recombinant L-methionine \(\alpha\)-deamino-\(\gamma\)-mercapto-methane lyase (rMETase) is a 172-kDa molecular homotetrameric PLP enzyme \textsuperscript{22}. The rMETase gene was cloned in \textit{E.coli}. Fermentation of rMETase-recombinant \textit{E. coli} and purification of rMETase were performed as described \textsuperscript{23}.

\textbf{Selection and 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 surviving 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.

**rMETase activity assay in vitro**

Medium with 1 U/ml of rMETase were incubated and the methionine levels in the medium were measured with an HPLC (Hitachi L-6200A Intelligent pump; Hitachi, Ltd., Tokyo, Japan) before and after added rMETase as described previously.²⁴

**Efficacy of MR, effected by rMETase, on cell proliferation**

Cells were cultured in 96-well plates (1 × 10³ 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. The medium after 96 hours of culture was collected and the methionine level in the medium was measured with HPLC. Experiments were performed three times, each in triplicate.

**Efficacy of rMETase on viability of methionine-addicted parental cancer-cells and methionine-independent revertant**

Cells were cultured in 96-well plates (1 × 10³ cells/well) in normal DMEM overnight. The next day, cells were treated with rMETase between 0.1 U/ml and 6.4 U/ml. Cell viability was measured after 96 h of treatment using Cell Counting Kit-8 (Dojindo).
Sensitivity curves and IC50 values were calculated as described previously.

**Immunoblotting**

Extraction of histone from cells and tumors and immunoblotting were performed as described. Anti-H3K4me3 antibody (1:1,000, #9751, Cell Signaling Technology, Danvers, MA, USA); anti-H3K9me3 antibody (1:1,000, #13969, Cell Signaling Technology); anti-H3K27me3 antibody (1:1,000, #9733, Cell Signaling Technology); anti-H3K36me3 antibody (1:1,000, #4909, Cell Signaling Technology); anti-H3K79me3 antibody (1:1,000, #74073, 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 primary antibodies. 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, H3K27me3, H3K36me3, H3K79me3 and pan methyl lysine of histone H3 were normalized to the total histone H3 signal for quantification.

**Mouse 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.

**Comparison of *in vivo* tumorigenicity of methionine-addicted parental cancer cells and methionine-independent revertants 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 PBS each) and 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 tumor were sacrificed on day 28 and tumor volume was measured at termination.

**Comparison of methionine-addicted parental cancer cells and methionine-independent revertants to forma experimental liver-metastasis**

HCT 116-GFP and HCT 116-R-GFP (1 × 10^6 cells / 100 μl PBS) were injected to the spleen of nude mice. Each group comprised three mice. The mice were sacrificed on day 42 and fluorescence intensity was measured at termination with the UVP ChemStudio (Analytik Jena US LLC).

**H & E staining and immunohistochemistry**

Hematoxylin and eosin (H&E) staining and immunohistochemical staining (IHC) were performed as described 26. 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.

Results

Methionine dependence is decreased in methionine-revertant cancer cells.

We first evaluated the activity of rMETase in vitro. The level of methionine in the medium was decreased to 30% rapidly 1 hour after addition of 1 U/ml of rMETase (Fig. 1A). These results show that rMETase rapidly and extensively depletes the medium of methionine.

To compare the methionine requirement of methionine-addicted parental cancer cells and methionine-independent revertants derived from the parental cells, we then evaluated their cell proliferation kinetics under MR effected by rMETase and the IC50 of rMETase. The proliferation of parental methionine-addicted cancer cells arrested within 48-72 hours of MR. In contrast, methionine-independent revertants were able to continuously proliferate under MR, similar to normal cells10,17,18,20 (Fig. 1B). The sensitivity to rMETase was lower in revertant MIA PaCa-2 cells than parental MIA PaCa-2 cells, with the IC50 value was higher in revertant cells than parental cells (1.62 vs 0.67 U/ml, respectively) (Fig.1C). These results indicate that the revertants have lost their methionine addiction. Comparison of methionine use in normal medium which has excess methionine did not show a difference between methionine-addicted cancer cells and methionine-independent revertants over 96 hours (data not shown). Future experiments will compare the two cell types of growth limiting
Overmethylation of histone H3 does not occur in methionine-independent revertants.

We compared the methylation status of histone H3 lysine in methionine-independent revertants and their methionine-addicted parental cells. Parental cancer cells and methionine-independent revertants were cultured in normal medium for 96 hours and histones were extracted. The level of pan-methyl lysine of histone H3 was also strongly decreased in methionine-independent revertants (Fig. 2A) as well as in parental cancer cells under MR (Fig. 2B). The levels of H3K4me3, H3K9me3, H3K27me3, H3K36me3 and H3K79me3 were decreased in methionine-independent revertants, even without MR, compared to parental methionine-addicted cells (Fig. 2C). These results indicate that the decreased use of methionine for histone H3 lysine methylation was, at least in part, 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, the 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. The mean tumor volume was significantly lower in HCT 116-R tumors than HCT 116 tumors after injection of $1 \times 10^6$ cells to nude mice ($P = 0.0085$), and only half of 10 mice formed tumors in HCT 116-R compared to all mice with HCT 116 (Fig. 3A). While 8 out of 10 mice injected with $5 \times 10^5$ parental HCT 116 cells formed tumors, 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).

The metastatic potential of HCT 116-GFP cells and HCT116-R-GFP cells was also compared after spleen injection in nude mice. The parental HCT 116-GFP cells formed significantly more liver metastasis compared to revertant HCT-116-R-GFP cells (p = 0.011)(Fig. 3D).

Immunohistochemistry staining showed that the number of Ki-67-positive cells was significantly lower in the HCT 116-R subcutaneous tumors compared to the parental HCT 116 subcutaneous tumors (n = 4, P < 0.0001) (Fig. 4A, B).

Immunoblotting showed that the levels of pan-methyl lysine of H3, H3K4me3, H3K9me3, H3K27me3, H3K36me3 and H3K79me3 were much lower in HCT 116-R tumors than in HCT 116 tumors (Fig. 4C, D). 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 25 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 27. It was not until the early 1970s that methionine dependence was found in cultured cancer cells 28, 29 and it was initially claimed that methionine dependence was due to reduced ability of the cancer cells to synthesize methionine from homocysteine 29. At that time in 1976 6, 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, 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 and in 2019 by Tam’s group.

We previously showed that an increase this rate of synthesis of methionine from homocysteine was not necessary for reversion to methionine independence, further demonstrates 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 S-adenosylhomocysteine (SAH) compared to normal cells and methionine-independent revertants. 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. We then understood that methionine addiction was due to overuse of methionine for transmethylation. This was confirmed when it was demonstrated that overall transmethylation was strongly reduced in methionine-independent revertants derived from the methionine-addicted cancer cells. The phenomenon of methionine-addicted cancer cells was later termed the Hoffman-effect.

Tam’s group in 2019, confirmed our results of 44 years previously 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. 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, similar to our previous
observation that MR eliminated clonogenic cancer cells. Mentch 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. 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, which explains why cancer cells arrest under MR and normal cells do not.

And most importantly, the present study shows that pan-methylation of histone H3 and all of trimethylated histone H3 marks are not over methylated in methionine-independent revertants that have decreased malignancy and lost methionine addiction, and can proliferate under MR. The present study and our recent study indicate that malignant cells require histone H3 overmethylation in order to grow in vitro and to form tumors and metastasis.

The present study thus shows that pan-overmethylated lysines of histone H3, including H3K4me3 and H3K9me3, explain 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 methionine-independent revertants isolated from SV-40-transformed human fibroblasts and revertants of triple negative breast cancer cells had reduced clonogenicity in agar, a surrogate maker of malignancy, giving the first hints that methionine addiction was necessary for malignancy. Our present results therefore indicate overmethylation of pan-methyl lysine of histone H3 and trimethyl histone H3 marks is necessary for malignancy as indicated by tumorigenicity and metastasis, both of which were reduced in methionine-independent revertants with greatly reduced or eliminated methylation of histone H3 lysine marks.
Although methionine-addicted cancer cells and methionine-independent revertants grew similarly *in vitro* in the presence of methionine, the revertants had greatly reduced malignancy as see by reduced tumorigenicity and metastasis, similarly that non-cancer cells such as fibroblasts do not form tumors *in vivo*\(^3\). All tested cancer types are methionine addicted as shown in vitro, even if they can grow in homocysteine in place of methionine\(^3\)\(^3\),\(^3\)\(^4\), and tumors in vivo have been universally shown to be sensitive to MR, either by a methionine-free diet or methioninase treatment\(^1\)\(^2\),\(^3\)\(^4\)\(^-\)\(^4\)\(^0\). These results indicate that methionine addiction, due at least in part to histone H3 lysine overmethylation, is a general hallmark of cancer. Our results also suggest that methionine addiction is the most fundamental hallmark of cancer, as it is necessary for malignancy.

It is possible that the histone H3 lysine methylation marks function as genetic switches\(^4\)\(^1\) 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\(^2\)\(^6\),\(^4\)\(^2\). These and other phenotypic changes associated with malignancy, such as aneuploidy\(^4\)\(^3\), may result from the global genomic changes due to histone H3 overmethylation in cancer\(^1\)\(^9\). This will be investigated in further studies. The reprogramming of methionine metabolism\(^4\)\(^4\) in cancer may be initiated by activated oncogenes as suggested by Vanhamme and Szpirer and Sacco et al.\(^4\)\(^5\)\(^,\)\(^4\)\(^6\) where they showed that activated *H-ras* or *K-ras* oncogenes, when transfected into normal cells, caused them to become methionine addicted. It has also been shown that viral infection of normal cells induces methionine addiction.\(^4\)\(^7\) The reprogramming of methionine metabolism resulting in methionine addiction, histone H3 lysine overmethylation and malignancy may be a result of overexpression of specific histone H3 lysine methyltransferases\(^4\)\(^8\). Gene expression change between methionine-addicted cancer cells and methionine0independent
revertants will be investigated in future studies using chromatin immunoprecipitation (ChIP).

Also important is the finding by Breillout et al. that as cells become more malignant they become more methionine addicted \(^{32}\). Another important observation is from PET imaging of cancer in patients which consistently shows that \(^{11}\text{C}\) methionine gives a much stronger PET signal than \(^{18}\text{F}\) deoxyglucose in head-to-head comparisons, demonstrating that cancers are methionine addicted in patients and that the Hoffman-effect is stronger than Warburg-effect \(^{49, 50}\). Other molecules, such as various RNAs, may also be overmethylated in cancer cells \(^{51, 52}\). Further experiments are necessary to account for all the transferred methyl groups in cancers.

In summary, our results suggest that overmethylation of histone H3 lysine is linked with methionine addiction and malignancy of cancer cells, and possibly necessary for both fundamental hallmarks of cancer. Methionine addiction is being widely recognized \(^{53}\) and may provide a universal target for methionine-restriction cancer therapy, such as with methioninase which depletes methionine in the tumors rapidly \(^{54}\), since methionine addiction is a general and fundamental hallmark of cancer.

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Figure legends

Figure 1. Methionine dependence is decreased in methionine-revertant cancer cells in vitro.

(A) rMETase activity assay in vitro. rMETase was added to the medium (1 U/ml) and the methionine levels in the medium were measured at 0 hour, 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 24 hours (n = 3). (B) 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). (C) Sensitivity to rMETase. The parental MIA PaCa-2 cells and its revertant MIA PaCa-2-R cells were cultured for 96 h with various concentrations of rMETase (mean ± SEM, n = 3).

Figure 2. Methionine-independent revertants have lower levels pan-methyl H3 lysine and trimethyl histone H3 marks compared to their parental methionine-addicted cancer cells in vitro.

(A) Immunoblot of pan-methyl lysine of H3 in methionine-addicted cancer cells, methionine-independent revertants and normal fibroblasts grown in normal medium. (B) Immunoblot of pan-methyl H3 lysine in methionine-addicted cancer cells and normal fibroblasts grown in normal medium or medium with rMETase (1 U/ml) for 96 hours. (C) Immunoblot of H3K4me3, H3K9me3, H3K27me3, H3K36me3 and H3K79me3 in
methionine-addicted cancer cells and methionine-independent revertants grown in normal medium.

Figure 3. Low-malignancy methionine-independent revertants have less tumorigenicity and metastatic potential than parental methionine-addicted cancer cells in vivo.

(A) Mean tumor volume of HCT 116 and HCT 116-R at 28 days after $1 \times 10^6$ 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 $2.5 \times 10^5$ cells were injected. White arrow: tumors. (D) Experimental liver metastasis: parent methionine-addicted HCT 116-GFP and methionine-independent revertant HCT 116-R-GFP. Right representative fluorescence image of liver metastasis. Left Fluorescence density (mean ± SEM, $n = 3$).

Figure 4 Low-malignancy methionine-independent revertants have lower Ki-67 and histone H3 methylation than parental methionine-addicted cancer cells in vivo.

(A) Representative images of H & E staining and immunohistochemical staining for Ki-67 of HCT 116 and HCT 116-R subcutaneous tumors. 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). (B) Quantification of the Ki-67 positive cells. Five images were randomly obtained in high power fields (400×) and the positive cells compared (mean ± SEM, $n = 4$). *, $p < 0.0001$. (C)
Immunoblot of pan-methyl H3 lysine and trimethyl histone H3 marks in subcutaneous tumors formed from HCT 116 and HCT 116-R (n = 3). (D) The ratio of pan-methyl H3 lysine or trimethyl histone H3 marks / total H3 in the tumors formed from HCT 116 and HCT 116-R (mean ± SEM, n = 3).
### Figure 2

#### A

|       | H460 | HCT 116 | MIA PaCa-2 | Hs27 |
|-------|------|---------|-------------|------|
| P     | P    | P       | P           | P    |
| R     | R    | R       | R           | N    |

- **Pan-methyl H3 lysine**
- **Total H3**

#### B

|       | H460 | HCT 116 | MIA PaCa-2 | Hs27 |
|-------|------|---------|-------------|------|
| rMETase | 0    | 1       | 1           | 0    |
| Pan-methyl H3 lysine |     |         |             |      |
| Total H3 |     |         |             |      |

#### C

|       | H460 | HCT 116 | MIA PaCa-2 |
|-------|------|---------|------------|
| P     | P    | P       | P          |
| R     | R    | R       | R          |

- **H3K4me3**
- **H3K9me3**
- **H3K27me3**
- **H3K36me3**
- **H3K79me3**
- **Total H3**

- **P**: Parent
- **R**: Revertant
- **N**: Normal
Figure 3

A. HCT 116 (1 x 10^6 cells, s/c)  
   Day 28  
   \[ p < 0.01 \]

B. Subcutaneous  
   | Cell number |
   |-------------|
   | 5 x 10^6    | 1 x 10^6    |
   | Parent      | 8/10        | 10/10       |
   | Revertant   | 0/10        | 5/10        |

C. H460 (2.5 x 10^5 cells, s/c)  
   Day 14  
   Parent (5/5)  
   Revertant (0/5)

D. HCT 116-GFP  
   Liver metastasis after spleen injection (1 x 10^6 cells)  
   Day 42  
   \[ p = 0.011 \]

   Parent  
   Revertant
Figure 4

A

H & E

Ki-67

HCT 116 (1 x 10^6 cells, s/c)

Parent

Revertant

LPF

HPF

B

K-67 positive cells

Parent

Revertant

P < 0.0001

C

HCT 116 (1 x 10^6 cells, s/c)

Tumor

Parent

Revertant

Pan-methyl H3 Lysine

H3K4me3

H3K9me3

H3K27me3

H3K36me3

H3K79me3

Total H3

D

Relative ratio (Total H3)

Pan-methyl H3 Lysine

H3K4me3

H3K9me3

H3K27me3

H3K36me3

H3K79me3