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RESEARCH PAPER

GPX3 promoter methylation predicts platinum sensitivity in colorectal cancer

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ABSTRACT

Epigenetic control of gene expression is a major determinant of tumor phenotype and has been found to influence sensitivity to individual chemotherapeutic agents. Glutathione peroxidase 3 (GPX3, plasma glutathione peroxidase) is a key component of cellular antioxidant regulation and its gene has been reported to be methylated in specific tumor types. GPX3 role in oxidative damage has been associated with sensitivity to platinums in other tumors but its importance in colorectal cancer (CRC) has not been determined. We examined the role of *GPX3* methylation in colorectal carcinoma in determining sensitivity to platinum drugs using primary tumor specimens, cell lines, knockdown cell lines, and tumor cell line xenografts. We find *GPX3* promoter region methylation in approximately one third of CRC samples and *GPX3* methylation leads to reduced GPX3 expression and increased oxaliplatin and cisplatin sensitivity. In contrast, in cell lines with high baseline levels of *GPX3* expression or with the ability to increase *GPX3* expression, platinum resistance is increased. The cisplatin IC_{50} in *GPX3*-methylated cell lines is approximately 6-fold lower than that in *GPX3*-unmethylated lines. Additionally, knockdown cell lines with essentially no *GPX3* expression require N-acetylcysteine to survive in culture underscoring the importance of GPX3 in redox biology. *In vivo, GPX3* methylation predicts tumor xenograft sensitivity to platinum with regression of *GPX3* knockdown xenografts with platinum treatment but continued growth of *GPX3* wild type xenografts in the presence of platinum. These studies demonstrate the importance of *GPX3* for CRC cells resistance to platinums and the potential utility of *GPX3* methylation status as a predictive biomarker for platinum sensitivity in CRC.

Abbreviations: 5FU, 5-fluorouracil; *CHFR*, checkpoint with forkhead and RING finger domains; CRC, colorectal cancer; *GPX3*, glutathione peroxidase 3; *MGMT*, O-6-methylguanine-DNA methyltransferase; MMR, mismatch repair; MSI, microsatellite unstable; MSP, methylation-specific PCR; MSS, microsatellite stable; *MLH1*, mutL homolog 1; qPCR, quantitative real-time PCR

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Introduction

An estimated 50,000 people die from colorectal cancer (CRC) each year in the US.[1] Molecular heterogeneity in CRC is an important factor in determining sensitivity or resistance to individual therapies for metastatic disease, including the association of mutations of *KRAS* with resistance to cetuximab.[2] Epigenetic changes are critical determinants of normal and tumor cellular phenotypes, and represent heritable changes in gene expression not caused by changes in nucleotide sequence.[3] These changes can also be associated with therapeutic sensitivity.[3]

In particular, studies have demonstrated the importance of epigenetic changes in determining tumor resistance or sensitivity to specific chemotherapeutic agents through targeting pathways that are epigenetically dysregulated. Esteller et al. demonstrated that the O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter is hypermethylated in 40% of high grade gliomas and this was associated with tumor regression, increased disease-free survival, and overall survival in these patients when treated with a carmustine based regimen.[4] Subsequent studies have confirmed *MGMT* hypermethylation as a predictor of response to the alkylating agent temozolomide and *MGMT* methylation testing is now available to guide treatment decisions in high grade gliomas.[5-7]

Epigenetic dysfunction is well described in CRC. For example, promoter hypermethylation of the DNA mismatch repair gene *MLH1* leads to microsatellite instability (MSI) in a subset of sporadic CRCs[8] that we have previously reported may predict sensitivity to gemcitabine.[9] In addition, we recently demonstrated that promoter hypermethylation of checkpoint with

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FHA and ring finger (*CHFR*), a mitotic checkpoint gene that causes delayed entry into metaphase under conditions of mitotic stress,[10] is predictive of docetaxel sensitivity in CRC cells.[9] These studies suggest the potential to uncover a subset of patients who may be sensitive to therapies that have not thus far been effective in unselected populations of CRC. However, this same approach might also identify patients with CRC who are more sensitive or resistant to therapies already in clinical practice, and thereby allow better selection among standard therapies. Since the majority of patients with metastatic CRC are treated with a fluoropyrimidine together with irinotecan or oxaliplatin, a way to choose among these later agents would be clinically useful.

Epigenetic silencing of glutathione peroxidase 3 (*GPX3*), a member of a family of selenoproteins with important antioxidant roles,[11] has been reported in a variety of tumor types including gastric, prostate, head and neck, ovarian, and bladder tumors.[12-14] This antioxidant role has led to exploration of *GPX3* as a determinant of chemotherapeutic activities, with evidence for a relationship to platinum agent sensitivity in ovarian clear cell cancers.[15] These compounds, particularly oxaliplatin, are commonly used in the treatment of gastrointestinal malignancies, including CRC, but not all patients respond to these agents, and most patients develop resistance over time.[16-18] An important role for *GPX3* has been shown in CRC development, in that studies of *GPX3* $^{-/-}$ mice develop increased numbers of colonic tumors and a reduction in *GPX3* expression *in vitro* was associated with increased reactive oxygen species (ROS), DNA damage, and apoptosis following oxidative stress.[19] These features would also be expected to alter sensitivity to platinum agents, since, in addition to platinum-DNA adduct formation,[20] these drugs disrupt cellular redox systems as another mechanism of activity.[21] With this important role of GPX3 in managing the oxidative stress response, we hypothesized that loss of expression of *GPX3* is associated with *GPX3* methylation and might predict platinum sensitivity in CRC.

Results

The Cancer Genome Atlas (TCGA) analysis: GPX3 methylation represses gene expression and is associated with MSI status

As a first examination of *GPX3* alterations in colorectal cancer, we queried The Cancer Genome Atlas (TCGA) data for mechanisms of inactivation. To explore genetic alterations, using cBioPortal,[22,23] we found no evidence for mutational inactivation of *GPX3*, a result consistent with the TCGA colorectal publication.[24] Only 3 of 224 sequenced colorectal carcinomas have mutations, and all were missense mutations of neutral or medium predicted functional impact score. We then analyzed *GPX3* expression and alterations in DNA methylation in the promoter region. Five promoter region probes: cg21504918, cg26638444, cg21516478, cg10802379, and cg17820459 all show low level methylation in normal colon tissue and increased methylation in a subset of colorectal tumors. The prevalence of *GPX3* methylation in 292 colorectal cancer TCGA samples is 30% (87 of 292 with β value higher than 0.2, a value higher than all normal tissues). We calculated the Spearman's rank correlation coefficient for *GPX3* promoter DNA methylation with expression, using probe cg26638444, which is the closet to the transcriptional start site. Fig. 1A shows that colorectal cancers with promoter region methylation have lower levels of *GPX3* mRNA expression relative to normal colon and colon tumors without DNA methylation. Since colorectal cancer includes both microsatellite stable (MSS) and instable (MSI), and these phenotypes may be associated with response to chemotherapies including oxaliplatin,[25,26] we examined whether *GPX3* promoter methylation differed between these phenotypes. Indeed, we found that *GPX3* methylation was associated with *MLH1* promoter methylation (Fig. 1B), which results clinically in microsatellite instability (MSI+). This would suggest that if *GPX3* silencing was associated with oxaliplatin sensitivity, this might differ between MSI and MSS patients.

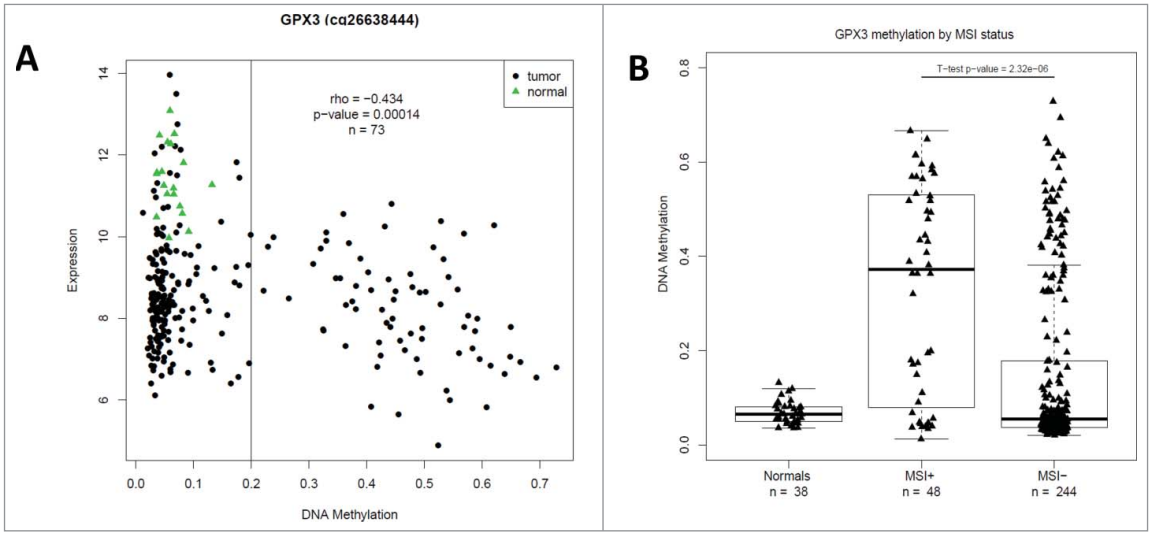

Figure 1. A: *GPX3* promoter methylation and expression are inversely correlated in primary colorectal cancer. TCGA dot plot demonstrating the relationship between *GPX3* methylation (β value on X axis) at cg2663844 located in the promoter region CpG island and expression (y axis) in TCGA CRC samples. Note, all normal colon tissues (triangles) express high levels of GPX3 and are unmethylated (low β value), while a subset of colon cancers have β values above normal (> 0.2) and have reduced expression (Spearman's correlation $= -0.43$). B: GPX3 promoter methylation correlates with MSI phenotype. TCGA dot plot at cg2663844 demonstrating the association between *GPX3* methylation (Y axis) and MSI phenotype (by *MLH1* promoter methylation status) (X axis) in TCGA colon samples. Normal tissue at left, Microsatellite (MSI+) positive samples with higher median methylation (β value) compare with microsatellite stable (MSI-), T-test *P* value $= 2.32e{-}06$.

GPX3 expression correlates with methylation status in primary xenografts specimens

We examined colorectal cancer patient-derived tumor xenografts, established as previously described,[27,28] for *GPX3* promoter methylation status and expression. The use of xenografts allows the remaining normal tissue present in the tumor to not contaminate expression or methylation analysis. Methylation specific PCR (MSP) was performed on DNA isolated from patient xenograft tissue and assessed for *GPX3* promoter methylation. *GPX3* was methylated at a frequency similar to primary tumors (3/12) (Fig. 2A). As we had observed in TCGA tumors, *GPX3* methylation correlates with markedly reduced or absent expression by qPCR (Fig. 2B), with complete loss of expression in xenografts 10 and 24. Unmethylated xenografts expressed varying amounts of GPX3 mRNA, but was statistically higher than the methylated xenografts ($P = 0.01$, two-tailed Student T-test).

Varying sensitivity to platinum drugs among CRC cell lines correlates with GPX3 methylation status

To examine the relationship between *GPX3* expression and platinum sensitivity, we used MTS assays to establish CRC cell lines sensitivity to cisplatin. These lines differed in platinum sensitivity, and these differences correlated with *GPX3* methylation status and *GPX3* mRNA expression (Fig. 3A and B). Methylated and partially methylated cell lines with low level or absent *GPX3* expression, have cisplatin IC_{50} from 2.5 to 6 micromolar, while unmethylated cell lines with higher *GPX3* expression are relatively resistant (IC_{50}: 15–50 micromolar). We also tested sensitivity to oxaliplatin and found similar patterns in terms of oxaliplatin sensitivity and *GPX3* methylation (Fig. 3A). Together, the data indicate a relationship between *GPX3* promoter methylation, decreased *GPX3* expression, and increased platinum sensitivity.

Chronic azacitidine treatment alters CRC cell lines sensitivity to oxaliplatin

The demethylating agent 5-azacitidine can be used to re-express silenced genes and reverse the silenced phenotype.[8] However, the acute cytotoxic effects of this drug can complicate determinations of changes in sensitivity to other chemotherapies, including the platinum agents which we wished to study. To avoid this confounding issue, we used long-term azacitidine treatment at lower doses to achieve gradual gene demethylation without a large degree of cell death. For these studies, Colo205 (*GPX3* unmethylated), SW480 (*GPX3* partially methylated), and HCT116 (*GPX3* methylated) were chronically-exposed to azacitidine and were examined using oxaliplatin MTS assays. We hypothesized that *GPX3* demethylation in HCT116 would result in greater resistance to oxaliplatin, while the unmethylated cell line Colo205 basally expressing *GPX3* would experience no change in sensitivity. We also examined the partially methylated cell line SW480, which has lower expression. Rather than showing no change in sensitivity, treatment of SW480 and Colo205 with azacitidine resulted in increased cell death in the presence of oxaliplatin compared with their non-azacitidine-treated counterparts (Fig. 4A). When GPX3 expression analysis was compared by qPCR (Fig. 4A), performed on cells harvested

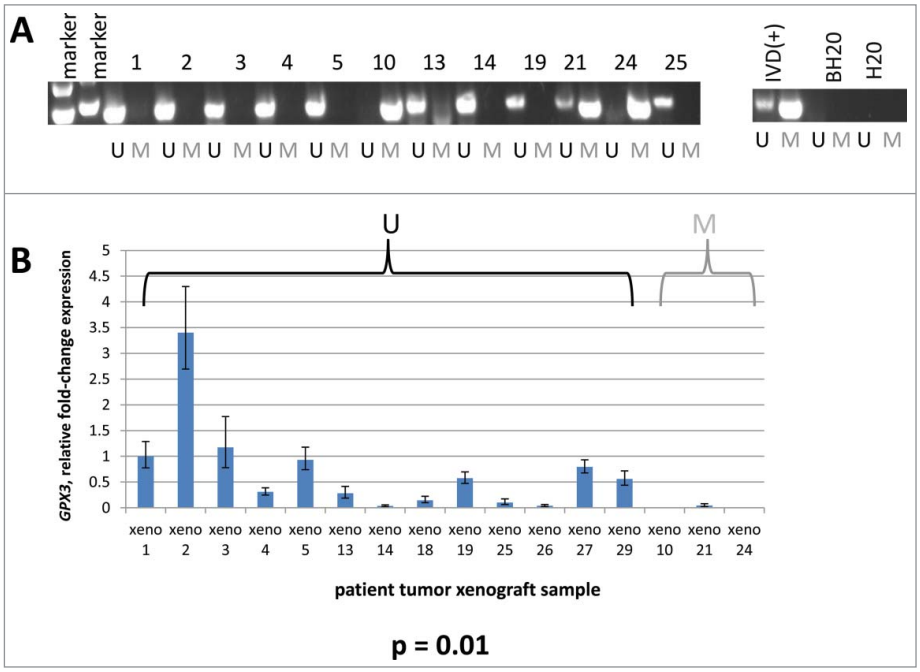

Figure 2. A: *GPX3* expression correlates with methylation status in primary xenograft specimens. *GPX3* msp on gDNA isolated from patient-derived CRC xenograft tissue. PCR products were run on an agarose gel for visualization. IVD: *in-vitro* methylated lymphocyte DNA, BH2O: bisulfite-treated distilled water, H2O: untreated distilled water. The IVD, BH20, and H2O lanes are from the same gel as the other samples but are from a different row. MSP for xenograft #18 not shown on this gel because the gel was run separately due to timing of DNA preparation. However, it demonstrated that xenograft #18 was unmethylated. DNA methylation of GPX3 promoter region is observed in xenografts 10, 21, and 24. B: *GPX3* expression correlates with methylation status in primary xenograft specimens. *GPX3* expression by qPCR on colorectal cancer patient tumor xenograft samples. Methylation status (U: unmethylated, M: methylated) as determined by MSP indicated above the bars from panel A. Xenografts with methylation (10, 21, and 24) have reduced expression compared with unmethylated xenografts (*P-value* calculated using a two-tailed Student's T-test, $P = 0.01$). Samples were run in triplicate and each sample was normalized to GAPDH expression. Error bars indicate standard deviation.

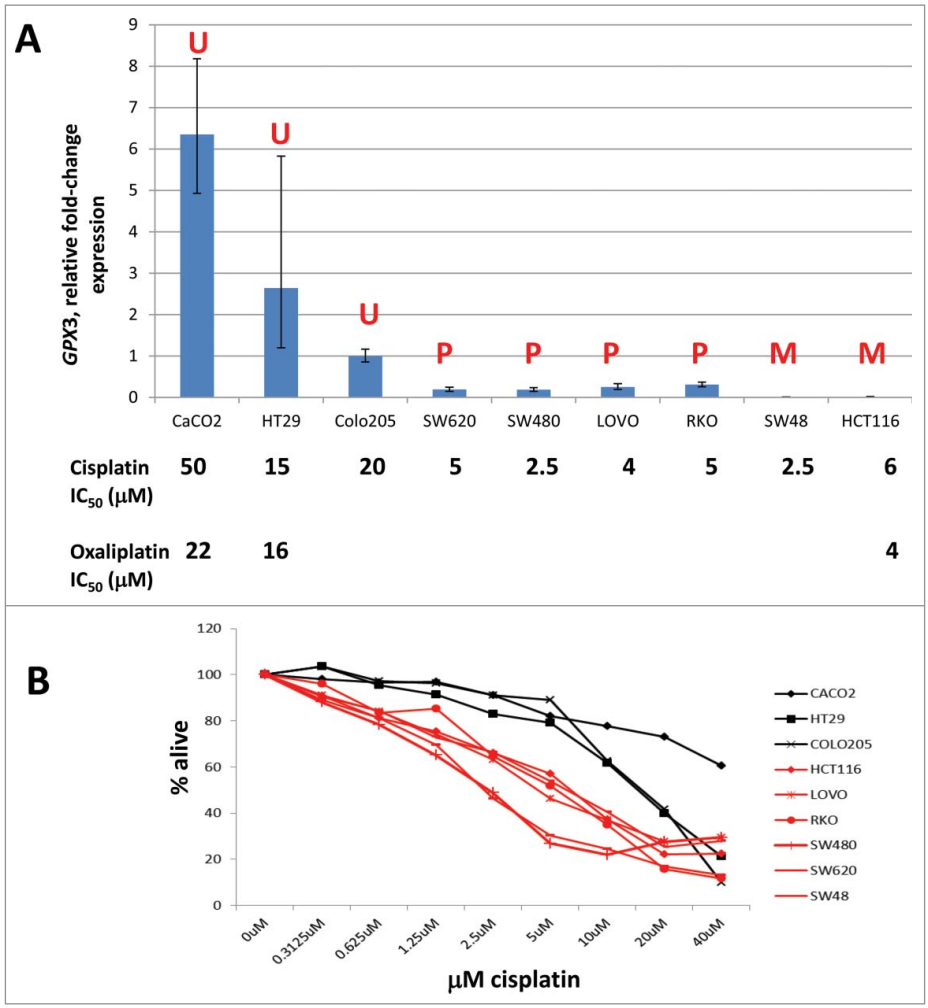

Figure 3. A: Platinum IC_{50} associates with *GPX3* expression and the presence of DNA methylation in CRC cell lines. qPCR indicating relative *GPX3* mRNA levels for 9 CRC cell lines. Samples were run in triplicate and each sample was normalized to its own *gapdh* expression. For comparison between cell lines, Colo205 expression was set to 1. M and P denote *GPX3* promoter methylation or partial methylation and U denotes samples with an unmethylated *GPX3* promoter, as determined in our laboratory. Note, cell lines with partial methylation have repressed levels of *GPX3* expression compare with unmethylated cell lines, and *GPX3* expression is absent in SW48 and HCT116 with complete methylation. Cisplatin and oxaliplatin IC_{50} values are shown beneath the cell line names and were determined by MTS cell proliferation assays. Methylated and partially methylated cell lines have cisplatin IC_{50}: 2.5–6 micromolar, while unmethylated cell lines with high *GPX3* expression are relatively resistant (IC_{50}: 15–50 micromolar). Error bars indicate standard deviation. B: Cisplatin sensitivities among CRC cell lines. Y axis indicates percentage alive based on MTS assays with increasing cisplatin concentration (x axis), used to generate IC_{50} values in Fig. 3A. Black color line indicates cell lines with unmethylated *GPX3*, red/light color line indicates cell lines with methylated or partially-methylated *GPX3*.

prior to the addition of oxaliplatin, we found that *GPX3* expression was not significantly changed in SW480 and Colo205 with chronic azacitidine exposure (minimal increase in SW480 and no change in Colo205). We wondered whether epigenetic changes at other loci caused by azacitidine treatment might be reducing sensitivity to oxaliplatin independent of *GPX3* status. We examined the pro-apoptotic gene *APAF1*, which has been reported to be epigenetically repressed,[29] and found that expression of this gene increased in both SW480 and Colo205, following azacitidine treatment (data not shown). An increase in *APAF1* expression following chronic azacitidine treatment in SW480 and Colo205, could partially explain increased cell death in these cell lines in that the net impact of having no significant increase in *GPX3* expression combined with an increase in *APAF1* expression was increased cell death. In contrast, in HCT116, *GPX3* expression was increased with azacitidine treatment and, despite a concomitant increase in *APAF1* expression (data not shown), HCT116 was more resistant to oxaliplatin (Fig. 4A). We confirmed that the change in expression was due to DNA demethylation, using MSP performed on the same cell samples harvested prior to oxaliplatin treatment (Fig. 4B). This suggests that in HCT116, a demethylation of *GPX3* and a corresponding increase in *GPX3* expression contributed to increased resistance to and growth in oxaliplatin despite changes in *APAF1* expression that would have contributed to cell death.

Knockdown of GPX3 by shRNA decreases GPX3 expression in CaCO2, HT29, and HCT116 and increases platinum sensitivity in cell lines with basal GPX3 expression

Because of the confounding effects of azacitidine in testing the specific role of GPX3 in platinum sensitivity, we created GPX3 shRNA knockdown constructs in CaCO2 (high baseline GPX3 expression), HT29 (moderate baseline expression GPX3), and HCT116 (no or minimal baseline GPX3

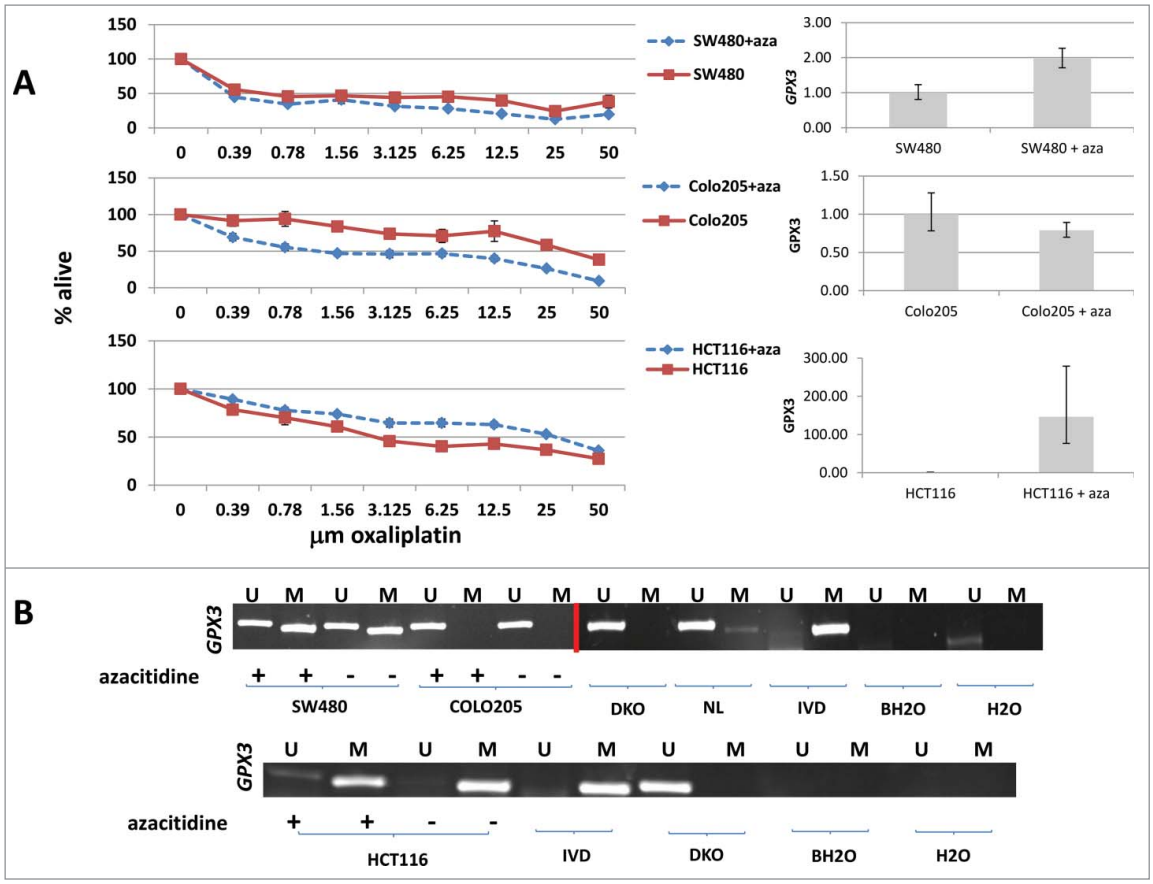

Figure 4. Chronically azacitidine-treated SW480 and Colo205 are more sensitive to oxaliplatin but chronically azacitidine-treated HCT116 is more resistant to oxaliplatin. A: MTS assays indicating increased sensitivity to oxaliplatin after treatment of SW480 and Colo205 with azacitidine and decreased sensitivity to oxaliplatin after treatment of HCT116 with azacitidine. Next to each cell lines' MTS assay are the *GPX3* expression data as determined by qPCR. The degree of increased oxaliplatin resistance in azacitidine-treated HCT116 varies with the extent of *GPX3* demethylation and re-expression. qPCR samples were run in triplicate and each sample was normalized to its own *gapdh* expression. MTS error bars indicate SEM and qPCR error bars indicate standard deviation. B: MSP data demonstrating no detectable demethylation of *GPX3* in either SW480 or Colo205 but detectable demethylation of *GPX3* in HCT116 after treatment with azacitidine. DKO (DNMT double knockout HCT116 cells) and NL (normal lymphocytes) serve as negative controls. The red line indicates that the controls for SW480 and Colo205 were on a 2nd gel (run at the same time) due to space constraints.

expression). Fig. 5A demonstrates the knockdown constructs in CaCO2 cells and the essentially complete repression of *GPX3* with construct #1 in CaCO2. Consistent with GPX3 role in redox biology, CaCO2 cells, with the near complete loss of expression in knockdown construct #1, required the antioxidant N-acetylcysteine (NAC) in the culture media for growth beyond 1–2 weeks. *GPX3* qPCR (Fig. 5B) confirms markedly reduced GPX3 expression at the RNA level in the knockdown CaCO2 and HT29 lines, and although barely detectable in comparison to the other cell lines, minimal HCT116 expression is further decreased in HCT116 #1 cells. The knockdown cell lines do not grow significantly slower than their wild type counterparts (data not shown). MTS assays performed to assess the impact of GPX3 on oxaliplatin sensitivity and representative plots are shown in Fig. 5. Knockdown of *GPX3* in CaCO2 sensitizes these cells to oxaliplatin in both knockdown #1 and #3. There appears to be no impact of knocking down GPX3 in HCT116, consistent with the essentially absent expression in wild type HCT116 at baseline. While to a more limited extent than seen in CaCO2, knocking down GPX3 in HT29 still increases sensitivity to oxaliplatin. We observed no compensatory changes in *GPX1* and *GPX2* expression in the GPX3 knockdown cells (data not shown), suggesting that GPX3 is the key glutathione peroxidase protein associated with sensitivity to oxaliplatin.

Acute oxaliplatin exposure induces GPX3 expression in wild type CaCO2 but not in knockdown CaCo2 lines

While DNA methylation resulted in diminished expression in primary colorectal tumors (Fig. 1A), primary colorectal xenografts (Fig. 2) and colorectal cancer cell lines (Fig. 3), we noted that some primary tumors and xenografts had relatively low expression without the presence of DNA methylation and complete silencing. Since one of the functions of GPX3 may be to reduce ROS induced damage, we questioned whether induction of *GPX3* expression would be in the cellular response to acute exposure to DNA damage. To test this hypothesis, CaCO2 cells were exposed to oxaliplatin for 48 h and examined for *GPX3* expression. Both wild type and scramble CaCO2 acutely increase *GPX3* expression in response to oxaliplatin (Fig. 5D), suggesting the importance of *GPX3* expression in oxidative damage response. However, as expected, CaCO2 #1 and

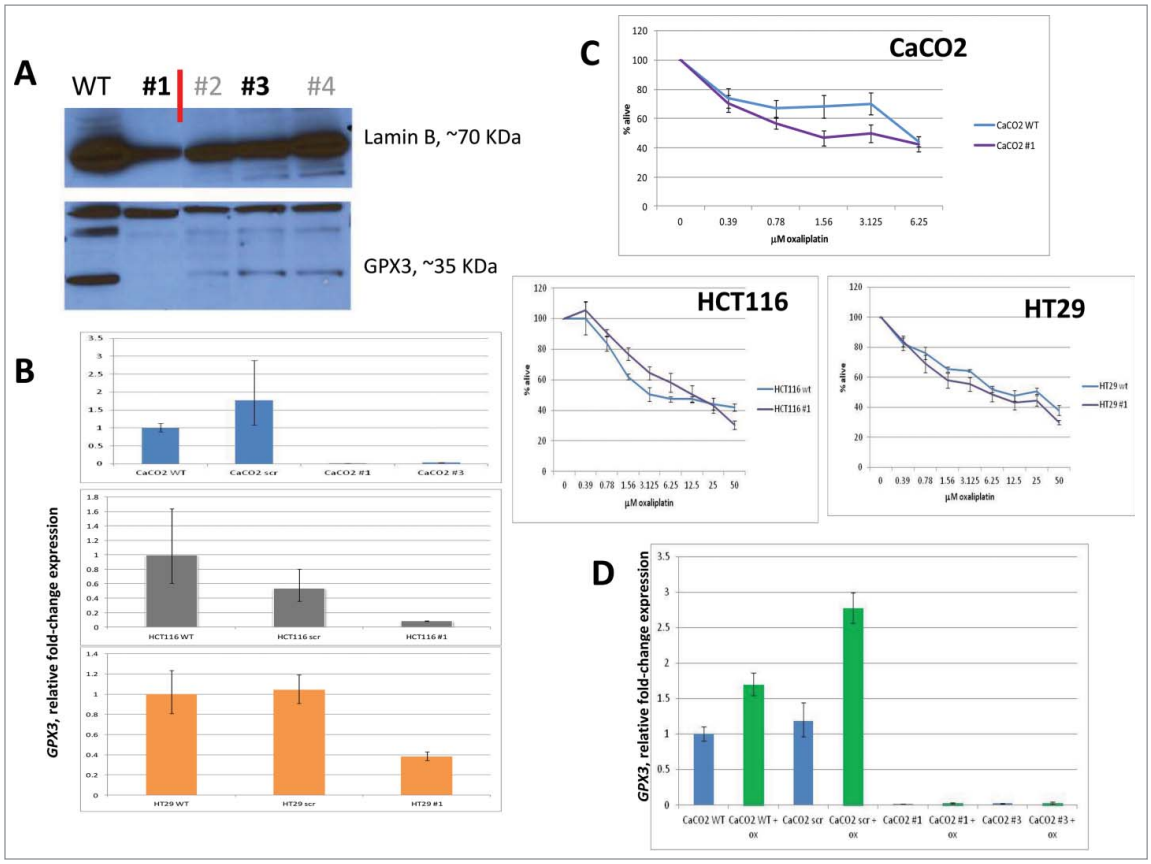

Figure 5. A: shRNA knockdowns of *GPX3* in CaCO2. Western blot demonstrating absent or reduced GPX3 protein expression after lentiviral infection of CaCO2 with different (numbered) shRNA constructs. Constructs #1 and #3 (in bold) were used in subsequent experiments with construct #1 used to knock down expression in HCT116 and HT29 as well. The vertical red line denotes a gap in the gel where a 5th knockdown construct was run but then cropped from the image because no staining was present in that lane even for the Lamin B control. WT: wild type. B: GPX3 qPCR confirms markedly reduced *GPX3* expression in the knockdown CaCO2, HCT116, and HT29 lines. Representative *GPX3* qPCR data demonstrating significantly reduced *GPX3* expression in the knockdown CRC lines. Samples were run in triplicate and each sample was normalized to *GAPDH* expression. Each line was normalized to its baseline *GPX3* expression to show relative change. SCR indicates scramble construct. Error bars indicate standard deviation. C: shRNA oxaliplatin MTS assays demonstrating a minimal increase in the sensitivity of CaCO2 GPX3 knockdown lines to oxaliplatin. Error bars indicate SEM. D: Oxaliplatin exposure induces GPX3 after acute exposure. Acute oxaliplatin exposure induces *GPX3* expression in wild type CaCO2 but not in knockdown CaCO2 lines. CaCO2 cells were plated and then grown in oxaliplatin (3.13 uM final) for 48 h before harvesting for RNA isolation, cDNA synthesis, and qPCR to assess *GPX3* expression. The oxaliplatin concentration used was based on prior IC_{50} measurements. For comparison between the different lines, CaCO2 WT *GPX3* fold change was set to 1. Ox: oxaliplatin. Error bars indicate standard deviation.

CaCO2 #3 knockdown lines are unable to significantly increase *GPX3* expression in response to oxaliplatin due to the shRNA inhibition. In contrast, the *GPX3*-methylated cell line HCT116, when acutely challenged with oxaliplatin as above, resulted in continued absence of *GPX3* expression after oxaliplatin exposure (data not shown), suggesting that epigenetic silencing prevented transcription of this gene and thus precluded the normal response of *GPX3* increased expression in response to damage produced by oxaliplatin.

GPX3 expression is induced during the development of oxaliplatin resistance in chronically-exposed HCT116 and HT29

We next questioned whether long-term treatment could alter *GPX3* expression. To do so, we treated two colorectal cell lines with varying levels of GPX3 expression, either completely silent (HCT116) or unmethylated and expressed (HT29), with increasing concentrations of oxaliplatin over many weeks. The resulting oxaliplatin-resistant HCT116 and HT29 cells were examined for *GPX3* expression (Fig. 6), and both HCT116 and HT29 expressed significantly increased levels of *GPX3* compare with untreated parental controls. While the levels of *GPX3* expression in HCT116 remain low relative to HT29, there was an approximate 3.5-fold induction. This suggests that the resistance to oxaliplatin occurs at least in part due through increasing *GPX3* expression.

Tumor xenografts from GPX3 knockdown line demonstrate increased sensitivity to cisplatin

To determine the impact of GPX3 on platinum sensitivity *in vivo*, mice implanted with cell line xenografts of CaCO2 wild type and CaCO2 #1 were treated with cisplatin and tumor sizes were monitored (Fig. 7). Untreated wild type and CaCO#1 knockdown xenografts grew similarly. CaCO2 is intrinsically relatively resistant to cisplatin with a high cisplatin IC_{50}; accordingly, cisplatin-treated wild type xenografts had a growth rate decline but not regression compare with untreated wild type xenografts. However, tumor xenografts from *GPX3* knockdown cells were markedly more sensitive to cisplatin compared with their wild type counterparts with essentially no measurable tumor after 2 weeks,

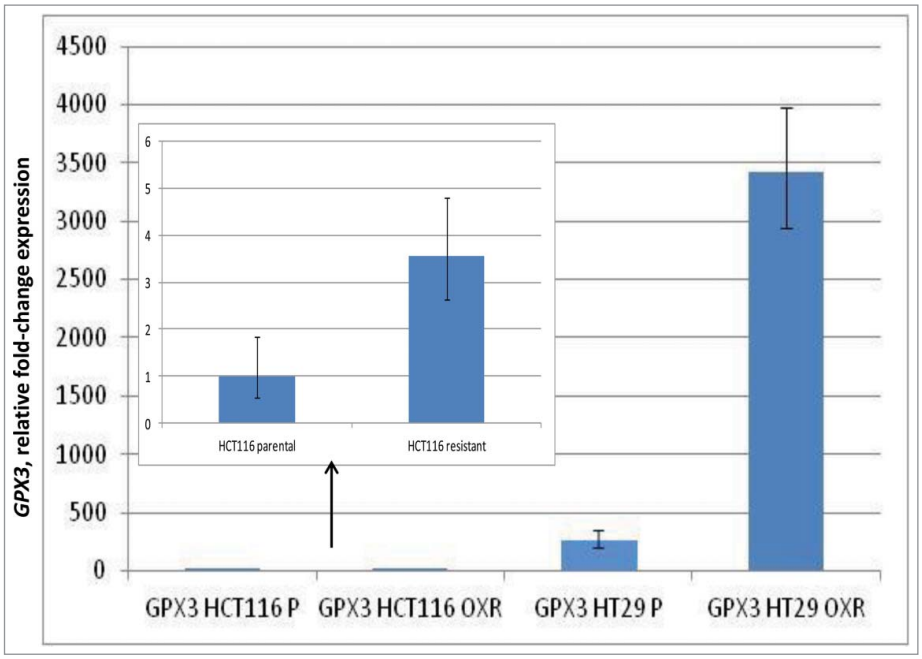

Figure 6. Oxaliplatin exposure induces GPX3 after chronic exposure. *GPX3* expression is induced during the development of oxaliplatin resistance in chronically exposed wild type HCT116 and HT29. HCT116 and HT29 cells were exposed to oxaliplatin over a period of weeks to create oxaliplatin-resistant lines. Cells were then harvested for RNA isolation, cDNA synthesis, and qPCR to assess *GPX3* expression. For comparison between the different lines, HCT116 WT (parental) *GPX3* fold change was set to 1. To better assess the degree of *GPX3* in the HCT116 lines, arrow demonstrates just the HCT116 lines. P: parental, OXR: oxaliplatin resistant. Error bars indicate standard deviation.

and maintained a similar trend beyond until study termination. These data, using xenografted, isogenic cell lines differing only in their expression of *GPX3*, validate the importance of GPX3 for platinum sensitivity.

Discussion

CRC is the 3rd leading cause of cancer deaths among US men and women.[1] In addition to new treatments, molecular-based strategies to optimize current treatments are needed. Epigenetic changes can be used to predict chemosensitivity in tumor tissues to help improve clinical outcomes.

Here we demonstrate that *GPX3* promoter methylation, which is present in one third of TCGA CRC samples and correlates with MSI status, is associated with decreased *GPX3* expression in patient tumor xenografts and in CRC cell lines. We first confirmed methylation status prevalence with primary specimens, choosing primary tumor xenografts to ensure that neoplastic cells were purely human, thus avoiding contamination from normal tissue. Using CRC cell lines, *GPX3* promoter methylation and expression directly correlated with platinum

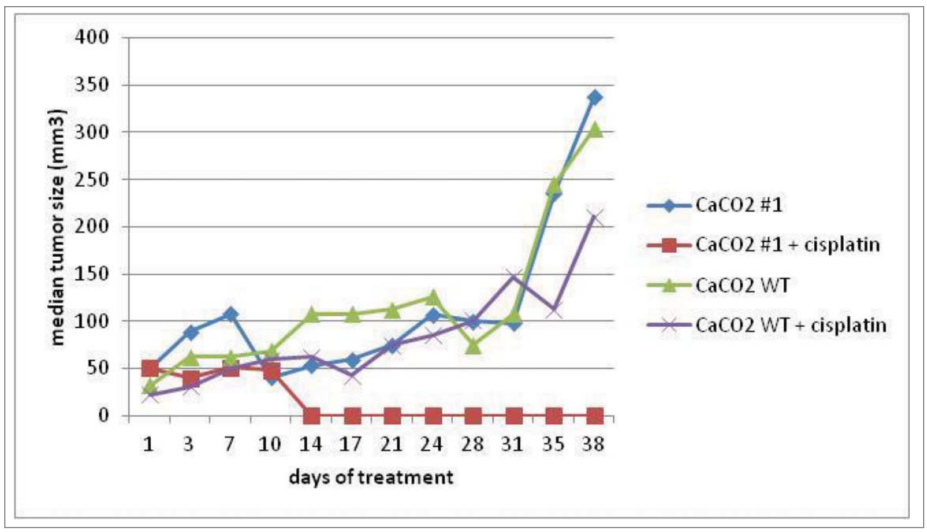

Figure 7. Tumor xenografts from *GPX3* knockdown line demonstrate increased sensitivity to cisplatin. Parental CaCO2 cells and construct #1 knockdown cells were injected into immunodeficient mice. Following initial tumor growth, mice were treated with cisplatin or untreated, as described in methods section. There was no significant effect of *GPX3* knockdown on tumor growth rate in the untreated mice, and no significant growth inhibition in the parental CaCO2 tumors when treated with cisplatin. However, in knockdown cells, cisplatin resulted in tumor regression.

drug sensitivity and, when azacitidine treatment was used to demethylate *GPX3* leading to its re-expression, platinum sensitivity decreased. Thus, in HCT116, azacitidine treatment led to an approximately 100-fold increase in *GPX3* expression and resulted in resistance to oxaliplatin. In contrast, in SW480 and Colo205, when azacitidine treatment did not result in increased *GPX3* expression, subsequent treatment of those cells with oxaliplatin resulted in cell death. These data suggest the functional importance of *GPX3* silencing in cancer cells on sensitivity to platinums.

Consistent with GPX3 role in cells antioxidant system, when *GPX3* was essentially completely knocked down in CaCO2 (a line which has unmethylated and high expression of *GPX3* at baseline), the cells required the antioxidant NAC to survive. NAC was not needed if there was any residual *GPX3* expression in CaCO2. This reflects the importance of at least a minimal amount of *GPX3* expression for CaCO2 growth and maintenance in cell culture, a ROS-rich environment. We also demonstrate that *GPX3* is upregulated after platinum exposure; although, expectedly, the degree varies with the cells' ability to express *GPX3* at baseline; knockdown lines and methylated lines have blunted or delayed upregulation responses. The azacitidine experiments and the oxaliplatin challenge experiments demonstrate that the capacity to increase *GPX3* expression, not merely baseline *GPX3* expression, is important for mediating platinum resistance. In the acute setting, azacitidine can demethylate and allow re-expression of GPX3; meanwhile, chronic exposure to platinum may allow for demethylation and upregulation of *GPX3* in the absence of azacitidine. When tested in cell line tumor xenografts, the *GPX3* knockdown CaCO2 line was strikingly more sensitive to platinum treatment than its wild type counterpart, again confirming the importance of *GPX3* for platinum resistance.

Although not the only alteration that determines MSI status, *MLH1* methylation leads to an MSI+ phenotype. Interestingly, there is a strong association between MLH1 methylation and *GPX3* methylation. Methylation of *GPX3* is found in 87 of 292 (30%) CRC samples. *GPX3* methylation is seen in 28 of 48 (58%) MLH1 methylated tumors compared with 59 of 244 (24%) MLH1 unmethyated tumors (Fisher test *P*-value = 8.161e-06). Additionally, however, *GPX3* methylation is strongly associated with the CpG island methylator phenotype-high (CIMP-H) phenotype in that 54 of 87 (62%) CIMP-H tumors have *GPX3* methylation while 33 of 205 (16%) CIMP low or negative have *GPX3* methylation (Fisher test *P*-value = 2.143e-14). Whether GPX3 methylation is directly related to *MLH1* silencing through mechanisms related to subsequent microsatellite instability or is associated with the CIMP-H phenotype cannot be determined through these association studies. MLH1 methylation and the resultant MSI+ phenotype also correlates with the CpG island methylator-high phenotype (CIMP-H); as such, it is unclear if MLH1 methylation directly leads to GPX3 methylation, through microsatellite instability, or if GPX3 methylation is a consequence of the CpG island methylator-high phenotype (CIMP-H) in general. Although CIMP is a well recognized phenomenon in colorectal cancer, it was not examined further here for association with platinum sensitivity because a precise definition of CIMP has not been established and published reports of CIMP in CRC use many different panels and criteria for scoring CIMP, including CIMP-negative, CIMP-high and CIMP-low. These issues are described in a review by Hughes et al.[30] Additionally, this study focused on a specific epigenetic change and the potential association of the silencing of *GPX3* to directly alter a phenotype that might be predicted by the change in DNA damage response.

Previous analyses have explored the complex potential association of MSI+ with chemosensitivity and resistance. Early clinical studies suggested that MSI+ tumors were less sensitive to nucleoside analog 5-flurouracil (5FU) than MSI- tumors; of note, 5FU has been the backbone of CRC chemotherapy regimens for the past 3–4 decades. However, with the addition of oxaliplatin to 5FU, now the standard adjuvant regimen, more recent studies demonstrate that MSI+ tumors do benefit from this combination just as MSI- tumors do.[25,26] These data suggest that the platinum compound may be responsible for the sensitivity of MSI+ tumors to the current adjuvant standard of care regimen of 5FU and oxaliplatin. The correlation of GPX3 methylation with the MSI+ phenotype may be one underlying factor for the sensitivity of MSI+ tumors to 5FU combined with oxaliplatin when they are resistant to 5FU alone.

Although the impact of GPX3 methylation on platinum sensitivity could just be a surrogate for other processes, such as global methylation changes impacting drug sensitivity, the magnitude of our *in vivo* data suggests GPX3 is itself affecting the phenotype. Though it is likely not the sole determinant of platinum resistance, and other groups have explored alternative genes (such as *BRCA1* and *ERCC1*, and the association of their methylation with platinum sensitivity),[31] taken together, the GPX3 data presented here demonstrate the importance of GPX3 for platinum resistance in CRC. *GPX3* promoter methylation predicts this relationship and potentially can be used as a predictive biomarker. Interestingly, though a decrease in *GPX3* expression has also been linked to chemosensitivity in other tumors as well,[15] *GPX3* methylation was associated with chemoresistance in head and neck cancer.[12] A potential application is in the choice between first-line oxaliplatin or irinotecan in stage IV CRC. Both oxaliplatin and irinotecan are approved in conjunction with 5-fluorouracil for first-line treatment of patients with metastatic colon cancer.[32] If a patient's tumor demonstrates methylation and silencing of *GPX3*, it may be more likely to respond to oxaliplatin. However, if a patient's tumor has unmethylated (and highly expressed) GPX3, it may be less likely to respond to oxaliplatin and thus the patient may be better served by a regimen not containing platinum but instead containing another agent, such as irinotecan. Thus, the methylation status of *GPX3* could inform treatment strategies and could be tested prospectively in a first-line metastatic trial.

Materials and methods

The Cancer Genome Atlas (TCGA) analysis

We analyzed 292 samples of colorectal carcinoma and 38 adjacent normal samples from the Cancer Genome Atlas project (TCGA)[24] that had both DNA methylation. TCGA used Illumina Infinium HumanMethylation450 BeadChip (Illumina)[33] for DNA methylation profiling. Two hundred and fifty-five of 292 samples have mRNA expression measured using RNAseq for mRNA expression profiling. We calculated the Spearman's rank correlation coefficient for GPX3 promoter DNA

methylation, using probe cg26638444, and mRNA expression. Samples with β value higher than 0.2 were considered as methylated. MSI status of a sample was derived using MLH1 promoter methylation status (probe cg00893636) and threshold 0.2.

Analyses described above as well as plots including boxplots, and scatterplots were performed in R[34] using standard methods and customized routines.

Cell lines and culture conditions

Cell lines (RKO, SW48, LOVO, HCT116, SW480, SW620, COLO205, CACO2 and HT29) were obtained from ATCC. Cells were maintained in McCoys 5A media with 10% fetal bovine serum at 37°C in 5% CO_2.

Methylation specific PCR (MSP)

Genomic DNA was extracted using a DNA extraction kit (Qiagen, # 13,343). The extracted DNA was quantified using a NanoDrop spectrophotometer and up to 1 μg of extracted genomic DNA was used for bisulfite treatment. Bisulfite conversion was done using the EZ DNA Methylation kit (Zymo Research, #D5001). *GPX3* MSP primers are as previously described.[12] PCR was performed as previously described [35] in a 25 μl reaction volume containing RedTaq DNA polymerase (Sigma, #D4309) and template DNA, with 35 cycles at 60°C or 65°C annealing temperature. DKO cells (DNMT double knockout HCT116 cells), normal human peripheral lymphocytes (NL), and bisulfite treated water (BH_2O) serve as negative methylation controls. IVD serves as the positive methylation control.

Quantitative real time PCR (qPCR)

cDNA was prepared from isolated RNA (Qiagen RNeasy kit, # 74,104) using the iScript cDNA synthesis kit (Bio-Rad, # 1,708,890). qPCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad, #1,725,270) and a "My iQ iCycler" thermal cycler (Bio-Rad). PCR was performed in triplicate for each sample: 1 μl of cDNA product, 7.5 μl of SsoAdvanced SYBR Green master mix, 0.6 μl of combined forward and reverse primers (10 μM each), and up to a total volume of 15 μl with sterile water per PCR reaction. PCR was performed with an initial incubation step at 95°C for 3 min followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. These 40 cycles were followed by melting curve analysis. *GPX3* qPCR primers are as previously described.[15] *Apaf-1* primers were designed with the help of the Primer3 program[36]: forward, AACCAGGATGGGTCACCATA and reverse, ACTGAAACC CAATGCACTCC. Each cell line sample was normalized to its own *GAPDH* control using the following primers to amplify *GAPDH* in the samples: forward, GAAGGTCGGAGTCAACG GATTT and reverse, ATGGGTGGAATCATATTGGAAC. Calculations were made using the ΔΔCt method. Primers were synthesized by IDT.

In vitro *cytotoxic assays*

Cytotoxicity of the drugs was determined by MTS assay (Promega, # G5421). Drugs tested were oxaliplatin (Sigma, # O9512) and cisplatin (Sigma, # 479,306). In brief, cells were plated in each well of 96 well plates 24 h prior to the addition of drug. After 24 h, drug was added in increasing concentrations; each drug concentration was done in triplicate and allowed to incubate for two days. After two days, MTS assays were performed per manufacturer instructions. Raw absorbance values were background subtracted with wells containing only media. Cell viability was calculated by the following formula (absorbance of treated well/absorbance of mock*100) for each drug concentration. Cell viability was plotted against drug concentrations and the concentration at which 50% of the cells are viable is taken as IC_{50}.

Cell growth rate assays

To measure cell growth rates, the CellTiter-Glo (Promega, #G7570) system was used. Cells from each line were plated in triplicate on day 1 of the assay. CellTiter reagent was added on day 2 and baseline luminescence measurements were read 60 min after substrate addition. Subsequent measurements were made at 24 h intervals.

Re-expression of GPX3 *in colorectal cancer cell lines*

SW480, COLO205, and HCT116 were maintained as above but with the addition of azacitdine to a final concentration of 500 nM 5-azacitidine (Sigma, # A2385) during twice weekly media changes. This treatment strategy was performed on these 3 cell lines continuously, over a period of months. Untreated cell lines were similarly maintained and passaged. Oxaliplatin cytotoxicity assays were performed using MTS reagent. For these assays, 3,000 (SW480) or 4,000 (COLO205, HCT116) cells were plated per well into 96-well plates 24 h prior to oxaliplatin addition. Each drug concentration was performed in replicates of five. On the day of plating, additional samples of these azacitidine-treated and untreated cells were pelleted, frozen on dry ice, and stored at −80°C for molecular studies. After 48 h of incubation with oxaliplatin, the MTS in vitro cytotoxic assays were performed.

Nucleic acid from the frozen cell pellets corresponding to each MTS assay was isolated using the ZR-Duet DNA/RNA MiniPrep kit (Zymo Research, # D7001) or separately using Qiagen DNA and RNA isolation kits. DNA bisulfite conversion was performed using the EZ DNA Methylation kit and MSP was performed as described above. cDNA was prepared from the isolated RNA using the iScript cDNA synthesis kit and qPCR was performed as described above.

GPX3 *knockdown*

The Sigma MISSION pLKO.1-puro lentiviral shRNA system (Sigma, #NM_0,02084) was used to repress *GPX3* expression in CRC cell lines, following the manufacturer's instructions. The Sigma pLKO clones TRCN0000008678, TRCN0000273647, TRCN0000273648, and TRCN0000273651 correspond to constructs #1, #2, #3, and #4, respectively. A pScramble construct was used as a control vector. The knockdown lines were cultured as above but with the addition of puromycin (Sigma, # P8833) for the duration of the studies (CaCO2 at 8 μg/ml, HT29 at 3 μg/ml,

and HCT116 at 1 μg/ml). CaCO2 infected with construct #1 virus (CaCO2 #1) was not viable beyond approximately 2 weeks. To minimize any toxicity from the transfection itself, the cell lines were infected again with construct #1 virus at a lower titer. Additionally, CaCO2 *GPX3* knockdown line #1 required N-acetylcysteine (NAC) (Sigma, # A9165) to a final concentration of 1 mm for maintenance in culture. CaCO2 #3 was able to be maintained in culture without the addition of NAC, though this knockdown did still have some minimal GPX3 expression detectable. Though these lines were maintained in puromycin +/− NAC, these drugs were not included during growth rate, MTS, or drug-challenge assays. Anti-GPX3 antibody at 1:500 (Abcam,# ab27325) and Anti-Lamin B antibody at 1:2,500 (Santa Cruz, # sc-6,217) were used for western blots.

Acute oxaliplatin challenge experiments

CaCO2 cells were plated and then grown in oxaliplatin (3.13 μM final) for 48 h before harvesting for RNA isolation, cDNA synthesis, and qPCR to assess *GPX3* expression, as above. The oxaliplatin concentration used was based on prior IC_{50} measurements.

Chronic oxaliplatin exposure in HCT116 and HT29

HCT116 and HT29 cells were cultured for weeks in increasing concentrations of oxaliplatin, based on the lines' IC_{50} values to develop oxaliplatin-resistant cell lines. RNA isolation, cDNA synthesis, and qPCR to assess *GPX3* expression were performed, as above.

Xenograft models

Antitumor activity of cisplatin was evaluated in xenograft models. *GPX3* knockdown and wild type CaCO2 cells (2×10^6) in Phosphate Buffered Saline and matrigel in the ratio of 1:1 were injected subcutaneously into the two flanks of homozygous female athymic nude mice (Simonson Laboratories). Five mice were used for each experimental condition. Cisplatin (5 mg/kg) was administered once tumors became palpable. The mice were treated only until the time point when the mock animals were sacrificed due to tumor volume. Length (L) and width (W) of tumors were measured with a caliper until the tumors reached 2 cm. Tumor volume (TV) was calculated with the following formula $TV = 1/2*(\text{Length}*\text{width}^2)$. Matrigel for xenograft studies was obtained from BD PharMingen (#354,234). Cisplatin was obtained from the Johns Hopkins Hospital pharmacy. All small animal experiments described conformed to the guidelines of the Animal Care and Use Committee of Johns Hopkins University. Mice were maintained in accordance with the guidelines of the American Association of Laboratory Animal Care.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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