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## Frequent promoter hypermethylation of *PTPRT* increases STAT3 activation and sensitivity to STAT3 inhibition in head and neck cancer

Noah D. Peyser<sup>1,2</sup>, Maria Freilino<sup>2</sup>, Lin Wang<sup>2</sup>, Yan Zeng<sup>2</sup>, Hua Li<sup>2</sup>, Daniel E. Johnson<sup>1,3</sup>, and Jennifer R. Grandis<sup>4</sup>

<sup>1</sup>Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

<sup>2</sup>Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

<sup>3</sup>Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

<sup>4</sup>Department of Otolaryngology, University of California, San Francisco, San Francisco, CA 94143, USA

### Abstract

STAT3 overactivation is a common event in many cancers, including head and neck squamous cell carcinoma (HNSCC), where STAT3 represents a promising therapeutic target. HNSCC is not characterized by frequent kinase mutations, in contrast to some malignancies where mutational activation of kinases upstream of STAT3 is common. Instead, STAT3 may be activated by loss-of-function (LOF) of negative regulators of STAT3, including by promoter hypermethylation of *PTPRT*. Here we first analyzed The Cancer Genome Atlas (TCGA) data and determined that the *PTPRT* promoter is frequently hypermethylated in several cancers, including HNSCC (60.1% of tumors analyzed) in association with downregulation of *PTPRT* mRNA expression and upregulation of pSTAT3 expression. These findings were confirmed in an independent cohort of HNSCC tumors by methylation-specific PCR (MSP) and immunohistochemistry. We demonstrate that *PTPRT* promoter methylation and gene silencing is reversible in HNSCC cells, leading to *PTPRT*-specific downregulation of pSTAT3 expression. We further show that *PTPRT* promoter methylation is significantly associated with sensitivity to STAT3 inhibition in HNSCC cells, suggesting that *PTPRT* promoter methylation may serve as a predictive biomarker for responsiveness to STAT3 inhibitors in clinical development.

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Corresponding Author: Jennifer R. Grandis, MD, 550 16<sup>th</sup> Street, 6<sup>th</sup> Floor, San Francisco, CA 94143, Phone: 415-514-8084, ; Email: Jennifer.Grandis@ucsf.edu

### Conflict of Interest

The authors declare no conflict of interest.

## Keywords

head and neck cancer; phosphatase; methylation; biomarker

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## Introduction

Head and neck squamous cell carcinoma (HNSCC) is an invasive malignancy with more than 40,000 expected diagnoses and more than 8,000 expected deaths in 2014 in the United States alone.<sup>1</sup> Most cancers, including HNSCC, are characterized by constitutive activation of signal transducer and activator of transcription 3 (STAT3) via phosphorylation of tyrosine 705 (Y705). STAT3 is a transcription factor and potent oncoprotein that activates or maintains many cancer phenotypes including abnormal growth, proliferation, angiogenesis, and invasion as well as evasion of apoptosis and the anti-tumor immune response.<sup>2</sup> While aberrant activation of kinases upstream of STAT3 likely contributes to constitutive activation of STAT3 in cancer, the role of loss-of-function (LOF) of downstream regulators, especially protein tyrosine phosphatases (PTPs), remains incompletely understood.

The PTP superfamily is a large group of enzymes that, in close coordination with tyrosine kinases, tightly regulates diverse signaling pathways by catalyzing the removal of a phosphate group from specific signaling proteins. The largest class of PTPs in the human genome is comprised of the receptor-like PTP (PTPR) family, which contains 21 distinct members. PTPR family members span the membrane once and contain one or two intracellular catalytic domains, as well as a modular extracellular region that typically contains several protein-protein interaction domains. A role for PTPRs in the context of cancer is increasingly apparent, with many members implicated as oncogenes or tumor suppressor genes.<sup>3</sup> We recently reported that members of the PTPR family are frequently somatically mutated across all solid tumors analyzed, including HNSCC, with PTPR type T (PTPRT) representing the single most commonly mutated PTP in HNSCC and across all cancers.<sup>4</sup> Importantly, pSTAT3 is a validated direct substrate of PTPRT, and LOF mutations of PTPRT lead to increased pSTAT3 expression and enhanced HNSCC cell growth/survival.<sup>4,5</sup> As these LOF PTPRT mutations are found in relatively few HNSCC tumors (5.6% of tumors analyzed), we sought to determine if loss of PTPRT expression by aberrant promoter methylation contributes to pSTAT3 overexpression and sensitivity to STAT3 inhibition in HNSCC.

Herein, we report a high frequency of aberrant *PTPRT* promoter hypermethylation in HNSCC and other cancers. We demonstrate that *PTPRT* promoter methylation significantly downregulates *PTPRT* expression, with an associated increase in expression of the PTPRT substrate pSTAT3 in HNSCC. We show that this methylation is reversible, leading to specific downregulation of pSTAT3 in HNSCC cells. Further, we demonstrate a correlation between *PTPRT* promoter methylation and sensitivity to STAT3 inhibition in HNSCC cell lines, suggesting that *PTPRT* methylation may serve as a predictive biomarker of responsiveness to STAT3 inhibitors currently in clinical development.

## Results

### Frequent *PTPRT* promoter hypermethylation leads to decreased *PTPRT* mRNA expression

To assess aberrant *PTPRT* promoter methylation in HNSCC, we analyzed TCGA data derived from the Illumina HumanMethylation450 platform. We first determined which CG dinucleotide methylation event was most negatively correlated with *PTPRT* mRNA expression (Figure 1A). We then defined aberrant hypermethylation as a fractional methylation level (beta value) at least three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue samples and found that 60.1% (256/426 tumors analyzed) of HNSCC tumors were hypermethylated (Figure 1B). By this stringent measure, hypermethylated tumors exhibit significantly decreased *PTPRT* mRNA expression levels as determined by RNA-Seq (Figure 1C), suggesting the validity of the above definition and that *PTPRT* hypermethylation has the expected biologic effect. In contrast, copy number alterations of the *PTPRT* gene are relatively infrequent and are not significantly associated with altered *PTPRT* mRNA expression (Supplemental Figure 1). As human papilloma virus (HPV) infection is an etiologic and prognostic factor in a subset of HNSCC, we sought to determine if *PTPRT* promoter hypermethylation is associated with HPV status and observed no significant association ( $P = 1.00$ , Fisher's exact test; *PTPRT* promoter hypermethylation in 21/36 [58.3%] HPV-positive tumors versus 145/243 [59.7%] HPV-negative tumors), suggesting that HPV infection is not a driver of *PTPRT* promoter methylation.<sup>6</sup>

In order to validate TCGA findings in an independent HNSCC human cohort, we performed methylation-specific PCR (MSP) on 45 formalin-fixed, paraffin-embedded oral squamous cell cancers with primers directed at the promoter region of *PTPRT* (representative analysis in Figure 2A). Using this semi-quantitative analysis, a similar high frequency of methylation was observed in this cohort (71.1%, 32/45 tumors analyzed; Figure 2B), further suggesting that *PTPRT* promoter methylation represents a common mechanism of *PTPRT* downregulation in HNSCC.

### The *PTPRT* promoter is frequently hypermethylated across human cancers

Further analysis of TCGA data reveals that the *PTPRT* promoter is frequently hypermethylated across a broad array of cancer types when hypermethylation is defined as a fractional methylation level (beta value) at least three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue samples. The highest incidence of *PTPRT* promoter hypermethylation occurs in colon adenocarcinoma (78.7%, 289/367 tumors analyzed), while HNSCC exhibits the second highest incidence (60.1%) (Figure 3A). Of the cancers analyzed, four exhibit significant downregulation of *PTPRT* mRNA in hypermethylated tumors (HNSCC, colon adenocarcinoma, lung adenocarcinoma, and breast invasive carcinoma;  $P < 0.05$ ), suggesting a functional role for aberrant *PTPRT* promoter methylation across several cancer types (Figure 3B).

### *PTPRT* expression is inversely associated with STAT3 activation in HNSCC

Additional analysis of TCGA data indicates that *PTPRT* mRNA expression is inversely correlated with pSTAT3 expression as determined by reverse-phase protein array (RPPA)

(Figure 4A). Since pSTAT3 is known to be a direct substrate of PTPRT, we hypothesized that *PTPRT* promoter methylation may contribute to increased STAT3 activation.<sup>5</sup> Indeed, representative immunohistochemical staining of an independent cohort of HNSCC tumors demonstrated that those with *PTPRT* promoter methylation, as determined by MSP, express elevated levels of pSTAT3 relative to tumors without *PTPRT* methylation (Figure 4B). Together, these findings suggest that *PTPRT* promoter methylation may be a frequent mechanism that contributes to STAT3 activation in HNSCC.

### ***PTPRT* promoter methylation is reversible, leading to *PTPRT*-specific pSTAT3 downregulation**

To further investigate the association between *PTPRT* promoter methylation and STAT3 activation, we selected a HNSCC cell line (Cal27) that exhibits nearly complete methylation as determined by MSP. Treatment of Cal27 cells with 5-azacytidine (5-aza), a non-specific DNA methyltransferase (DNMT) inhibitor, led to an increase in the unmethylated signal as determined by MSP (Figure 5A), concurrent with overexpression of endogenous wild-type *PTPRT* and downregulation of pSTAT3 expression (Figures 5B and 5C). Transfection of Cal27 cells with shRNA directed against *PTPRT* (shPTPRT) following 5-aza treatment also resulted in an increase in the unmethylated signal (Figure 5A), but the expression of *PTPRT* was significantly downregulated relative to 5-aza treatment alone. Further, pSTAT3 expression remained unaffected under these conditions (Figures 5B and 5C), suggesting that 5-aza-mediated downregulation of pSTAT3 is dependent upon demethylation and re-expression of *PTPRT* in these cells. These results provide mechanistic evidence that *PTPRT* promoter methylation contributes directly to STAT3 activation in HNSCC by downregulation of *PTPRT* expression.

### ***PTPRT* promoter methylation is associated with increased sensitivity to STAT3 inhibition in HNSCC cells**

To evaluate whether *PTPRT* promoter methylation may serve as a predictive biomarker for STAT3 targeted therapies, we first determined EC<sub>50</sub> values for selective STAT3 inhibitors in a panel of 8 HNSCC cell lines (Figures 6A and 6B) that exhibit varying levels of *PTPRT* promoter methylation as determined by MSP (summarized in Figures 6C and 6D). Figures 6E and 6F illustrate that *PTPRT* promoter methylation and sensitivity to Stattic (a STAT3 SH2 domain inhibitor) or JSI-124 (a JAK/STAT3 pathway inhibitor) are significantly correlated ( $P < 0.05$ ), indicating that HNSCC tumors that exhibit *PTPRT* promoter hypermethylation may be most sensitive to treatment with STAT3 inhibitors. A similar trend was observed with the JAK2 inhibitor LY2784544, though this observation was not statistically significant (Supplemental Figure 2). Notably, we observe no direct correlation between *PTPRT* promoter methylation and pSTAT3 expression in this cell line panel, suggesting that high pSTAT3 expression *per se* may not be predictive of sensitivity to STAT3 inhibitors. Thus, inhibition of STAT3 directly rather than through upstream kinases may be more efficacious in tumors with *PTPRT* promoter hypermethylation.

We next sought to determine whether *PTPRT* methylation may serve as a predictive biomarker of sensitivity to STAT3 inhibition in a heterotopic tumorigraft model of HNSCC. We inoculated 16 nude mice with  $3 \times 10^6$  BICR 18 cells (which exhibit total *PTPRT*

unmethylation) on one flank, and  $3 \times 10^6$  UMSSC 1 cells (which exhibit near total *PTPRT* methylation) on the other flank. After 10 days, the unmethylated cells had not formed any tumors, while the methylated cells had formed palpable masses in 15/16 mice (mean volume =  $100.3 \text{ mm}^3$ ). As BICR 18 cells are the only HNSCC cells identified to date with total (or near total) *PTPRT* unmethylation, we are therefore unable to determine the relative sensitivities of unmethylated versus methylated HNSCC cells *in vivo*. Nevertheless, starting on day 10 post-inoculation, 8 mice were randomized to receive 50 mg/kg Stattic by oral gavage five times per week, with serial tumor measurements performed three times per week. After 9 days of treatment, a large and statistically significant ( $P < 0.01$ ) reduction in tumor volume was observed in the Stattic treatment arm relative to the no treatment group (Supplemental Figure 3), confirming that a *PTPRT*-methylated HNSCC heterotopic tumorgraft is indeed sensitive to STAT3 inhibition *in vivo*.

## Discussion

The survival rate for HNSCC patients has remained stagnant in recent decades despite advances in the understanding of the biological underpinnings of this disease and improved therapeutic strategies. In 2006, the Food and Drug Administration approved cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), for the treatment of HNSCC patients.<sup>7</sup> Unfortunately, cetuximab has produced limited success in the clinic, at least in part due to the lack of predictive biomarkers, including EGFR expression or copy number gain.<sup>8</sup> There is therefore a need for the identification of both novel targets for pharmacologic inhibition and biomarkers for sensitivity to emerging therapeutics. STAT3 is a promising target for pharmacologic inhibition, and STAT3 inhibitors are being tested in early phase clinical trials.<sup>2</sup> We previously reported that STAT3 activation contributed to cetuximab resistance in HNSCC preclinical models and tumors from patients treated on cetuximab-containing protocols.<sup>9</sup> It is increasingly apparent that most molecular targeted agents will be most effective in subgroups of patients identified by specific genomic/transcriptomic/proteomic characteristics. The identification of biomarkers that identify those individuals most likely to benefit from these agents would allow the design of more scientifically rational clinical trials.

Here we report that the *PTPRT* promoter is frequently hypermethylated in HNSCC and other cancers in association with decreased *PTPRT* expression. A high rate of *PTPRT* promoter hypermethylation has also been reported in sporadic colorectal cancer, where nearly all tumors analyzed were methylated in contrast to no observed methylation in matched normal tissues, as well as in hepatocellular carcinoma, where the *PTPRT* methylation levels progressively increase from adjacent tissue to tumor.<sup>10, 11</sup> Our present findings indicate that *PTPRT* promoter methylation may represent a common event across many cancer types, suggesting that the development of a therapeutic strategy informed by this event may be of wide clinical benefit.

To date, the functional signaling consequences downstream of *PTPRT* promoter methylation have not been described, and as such, no strategies to mitigate the effects of this event have been proposed. Here we demonstrate that *PTPRT* promoter methylation is associated with a decrease in *PTPRT* mRNA expression, which in turn is associated with an increase in

expression of the *PTPRT* substrate pSTAT3. We further evaluated *PTPRT* promoter methylation in an independent cohort of HNSCC and observed a similar frequency to that observed by TCGA, thus confirming the high incidence of this event. We also confirm that *PTPRT* methylation significantly contributes to pSTAT3 upregulation in HNSCC cells. Together, these studies suggest that HNSCC cells and tumors that exhibit high levels of *PTPRT* promoter methylation may be more dependent on STAT3 signaling for growth and survival, thus offering the opportunity to exploit *PTPRT* methylation as a predictive biomarker of sensitivity to STAT3 inhibition. Importantly, we have established an association between *PTPRT* promoter methylation and sensitivity to inhibitors of the STAT3 signaling pathway. These studies therefore suggest that screening for *PTPRT* promoter methylation may ultimately allow the identification of patients who are most likely to benefit from treatment with STAT3 targeting agents and provide a rationale for designing future clinical trials that select for patients with a high degree of *PTPRT* promoter methylation.

## Materials and Methods

### Genomic and Proteomic Data and Analysis

TCGA methylation and RNA-Seq data were obtained through R via the CGDS-R package or through the TCGA data matrix (<https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>). Reverse-phase protein array data were obtained from The Cancer Proteome Atlas ([http://app1.bioinformatics.mdanderson.org/tcga/\\_design/basic/index.html](http://app1.bioinformatics.mdanderson.org/tcga/_design/basic/index.html)). Statistical calculations were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA).

### Methylation-Specific PCR (MSP)

Two-millimeter diameter cores were obtained from formalin-fixed, paraffin-embedded HNSCC tumors under the auspices of a protocol approved by the Institutional Review Board of the University of Pittsburgh. Tumor DNA was isolated with the QIAamp DNA FFPE Tissue Kit, while the QIAamp DNA Mini Kit was used for isolation of DNA from cell lines, both according to the manufacturer's instructions (Qiagen, Hilden, Germany). Bisulfite conversion of 1 µg of DNA per sample was performed using the EpiTect® Bisulfite Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). MSP primers were designed using MethPrimer software.<sup>12</sup> MSP was performed with the EpiTect® MSP Kit (Qiagen, Hilden, Germany). After reaction completion, products were analyzed by gel electrophoresis on a 2.5% agarose gel. Densitometry was performed using ImageJ and the fractional methylation (beta value) for a particular tumor or cell line was calculated as (methylation signal)/(methylation signal + unmethylation signal).

### Cell Culture

Cal27 and Detroit 562 cells were obtained from ATCC (Manassas, VA). 686LN cells were obtained from Georgia Chen at MD Anderson Cancer Center (Houston, TX). BICR 18 and PE/CA-PJ49 cells were obtained from Sigma-Aldrich (St. Louis, MO). UMSSC cell lines were obtained from Thomas E. Cary at the University of Michigan (Ann Arbor, MI). HSC-2 cells were obtained from Hideo Niwa at Nihon University (Tokyo, Japan). Cal27, Detroit 562, HSC-2, UMSSC 47, and UMSSC 22A were maintained in DMEM (Corning, Corning, NY) containing 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento,



CA). UMSCC 1 were maintained in DMEM containing 10% FBS and 0.4 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO). BICR 18 were maintained DMEM containing 10% FBS, 0.4 µg/mL hydrocortisone, and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). 686LN were maintained in DMEM/F12 (Life Technologies, Carlsbad, CA) containing 10% FBS. PE/CA-PJ49 were maintained in Iscove's DMEM (Corning, Corning, NY) containing 10% FBS and 2 mM L-glutamine. All cells were genotypically verified using the AmpFSTR Identifier PCR Amplification Kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA) and maintained at 37°C and 5% CO<sub>2</sub>.

### 5-azacytidine (5-aza) and shRNA Treatment

Cal27 cells were plated at 250,000 cells per well on 6-well plates and incubated overnight. Medium was then replaced with complete medium containing 1 µM 5-aza (or 50% acetic acid in water as vehicle). 24 hours later, cells were transfected with 2 µg of shRNA targeted toward *PTPRT* (shPTPRT) or non-targeted scrambled sequence (shScr) with 6 µL of FuGENE® HD (Promega, Fitchburg, WI) in 200 µL of Opti-MEM® (Life Technologies, Carlsbad, CA) added directly to medium containing 5-aza (or vehicle). Cells were incubated for an additional 24 hours before DNA, RNA, and protein were harvested for analysis.

### Immunoblotting

Primary antibodies for pSTAT3 and STAT3 were obtained from Cell Signaling Technology (Boston, MA) and for β-tubulin from Abcam (Cambridge, MA). Secondary antibodies were obtained from BioRad (Hercules, MA). Densitometry was performed using ImageJ software.

### Drug Treatment

Cells were plated on 48-well plates at a density of 6800 cells per well and incubated overnight before treatment with increasing concentrations of Stattic (Sigma-Aldrich, St. Louis, MO), JSI-124 (Calbiochem, San Diego, CA), LY2784544 (Eli Lilly and Company, Indianapolis, IN), or DMSO alone as vehicle control in triplicate in the appropriate complete medium. MTT assays were performed after 72 hrs and data were analyzed using GraphPad Prism 5 (GraphPad, La Jolla, CA).

### Animals

Mice were used in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were housed in a facility certified by the American Association for the Accreditation of Laboratory Animal Care. 5–6 week old Foxn1 nude mice were purchased from Harlan Laboratories (Indianapolis, IN). 16 female mice were inoculated with 3×10<sup>6</sup> BICR 18 cells on the left flank, and 3×10<sup>6</sup> UMSCC 1 cells on the right flank. After 10 days of growth, UMSCC 1 cells had formed palpable tumors in 15/16 mice, while BICR 18 had formed none. At this time, 8 mice were randomized to receive 50 mg/kg Stattic in 1% Tween-80 in PBS by oral gavage five times per week as previously reported in an ovarian cancer model.<sup>13</sup> 7 mice received no treatment. Tumor sizes were blindly measured by caliper in two dimensions and volumes calculated as (large measurement) × (small measurement)<sup>2</sup>. Mice were sacrificed after measurements were taken on day 9 of treatment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

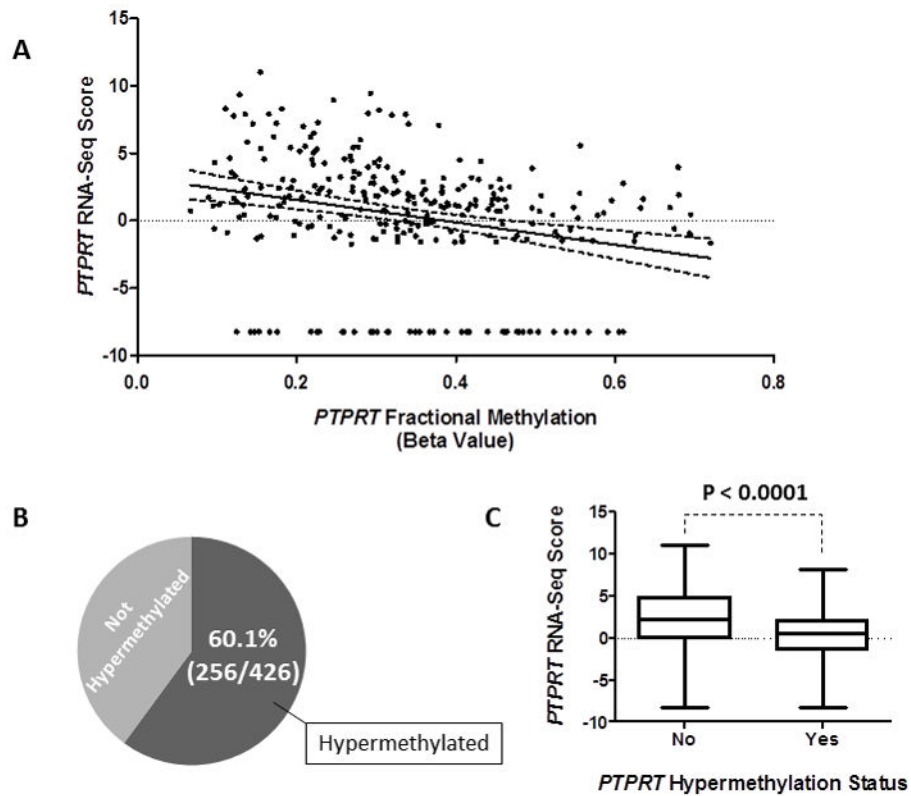
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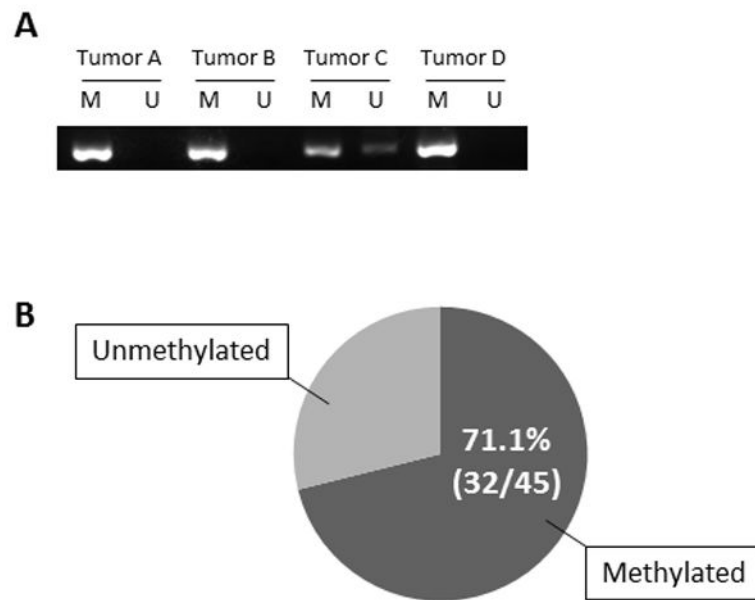
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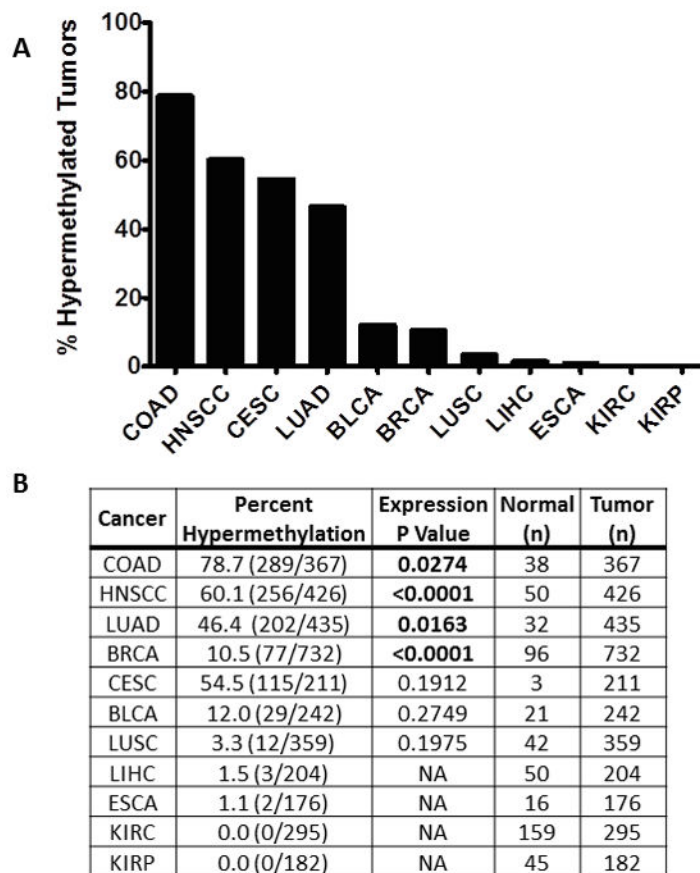
**Figure 1. Frequent *PTPRT* promoter hypermethylation is associated with downregulation of *PTPRT* mRNA in HNSCC tumors**

A) Methylation at the CG dinucleotide denoted cg04541293 significantly correlates with decreased *PTPRT* mRNA expression ( $n = 279$ , Pearson  $r = -0.2670$ ,  $P < 0.0001$ ,  $R^2 = 0.07131$ , 95% confidence interval depicted). B) *PTPRT* promoter hypermethylation (defined as a methylation level greater than three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue samples) was assessed in 426 tumors from TCGA. C) *PTPRT* hypermethylation is significantly associated with downregulation of *PTPRT* mRNA (two-tailed unpaired t test). Whiskers represent minimal or maximal values.



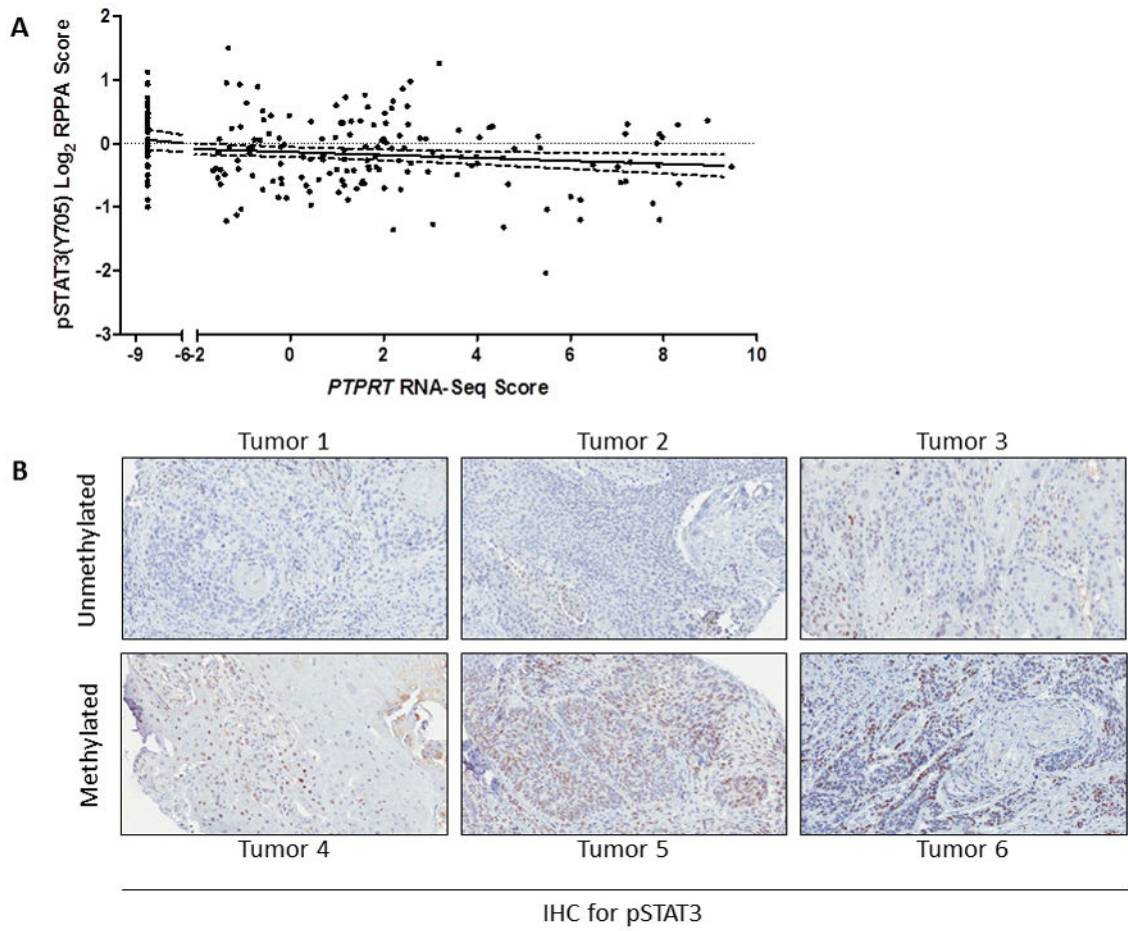
**Figure 2. The *PTPRT* promoter is frequently methylated in an independent cohort of HNSCC tumors**

A) Representative MSP analysis of the *PTPRT* promoter from four HNSCC tumors. M denotes primers amplifying methylated sequences, and U denotes primers amplifying unmethylated sequences. B) Summary of MSP analysis of 45 HNSCC tumors. A tumor is considered methylated when the methylation level is >50% of the total signal.



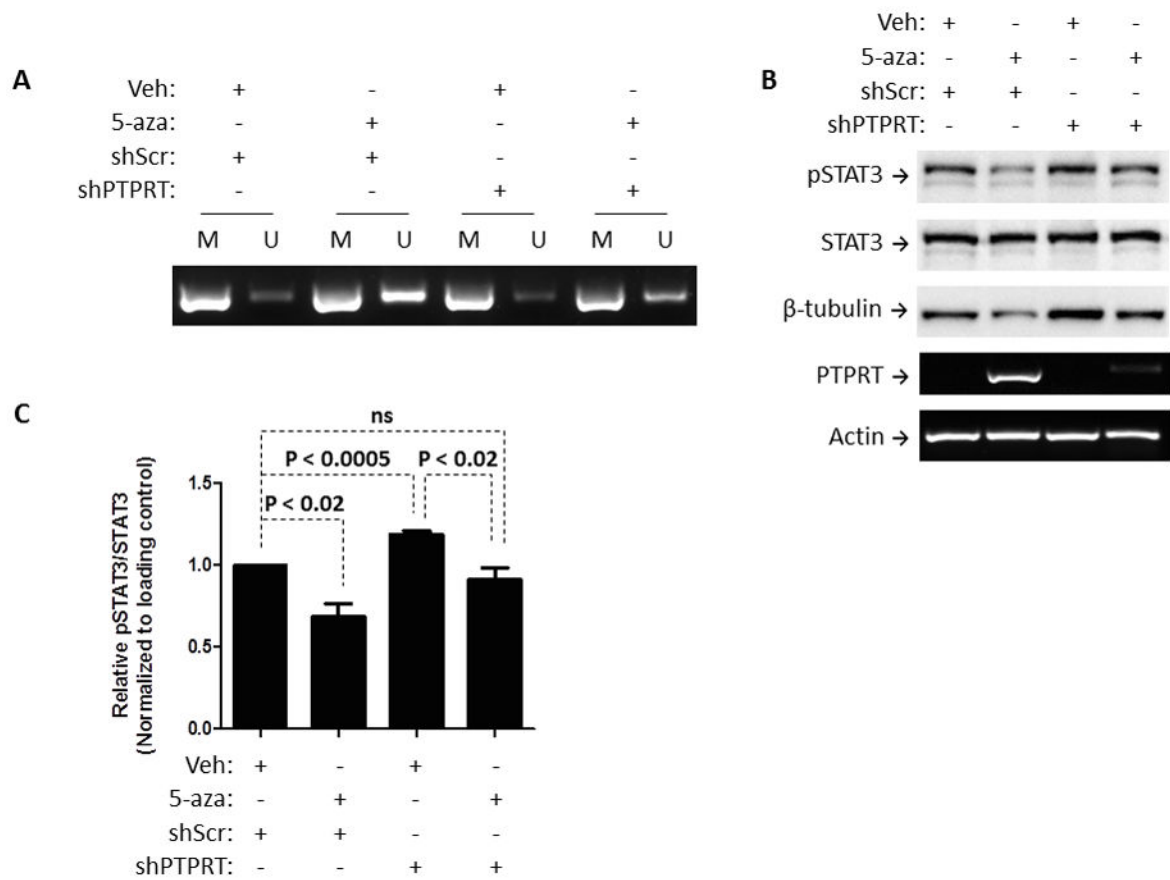
**Figure 3. The *PTPRT* promoter is frequently hypermethylated across cancer types in association with downregulated *PTPRT* mRNA**

COAD, colon adenocarcinoma; HNSCC, head and neck squamous cell carcinoma; LUAD, lung adenocarcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; BLCA, bladder urothelial carcinoma; LUSC, lung squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; ESCA, esophageal carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma. A) The *PTPRT* promoter is frequently hypermethylated in several cancers. B) Table summarizing hypermethylation across cancers. Expression P values represent the results of unpaired two-tailed t tests between hypermethylated and non-hypermethylated tumors, with those in bold denoting significant ( $P < 0.05$ ) downregulation of *PTPRT* mRNA expression in hypermethylated tumors. Normal (n) and Tumor (n) denote the number of organ site-matched normal tissue or number of tumor samples used in this analysis, respectively.



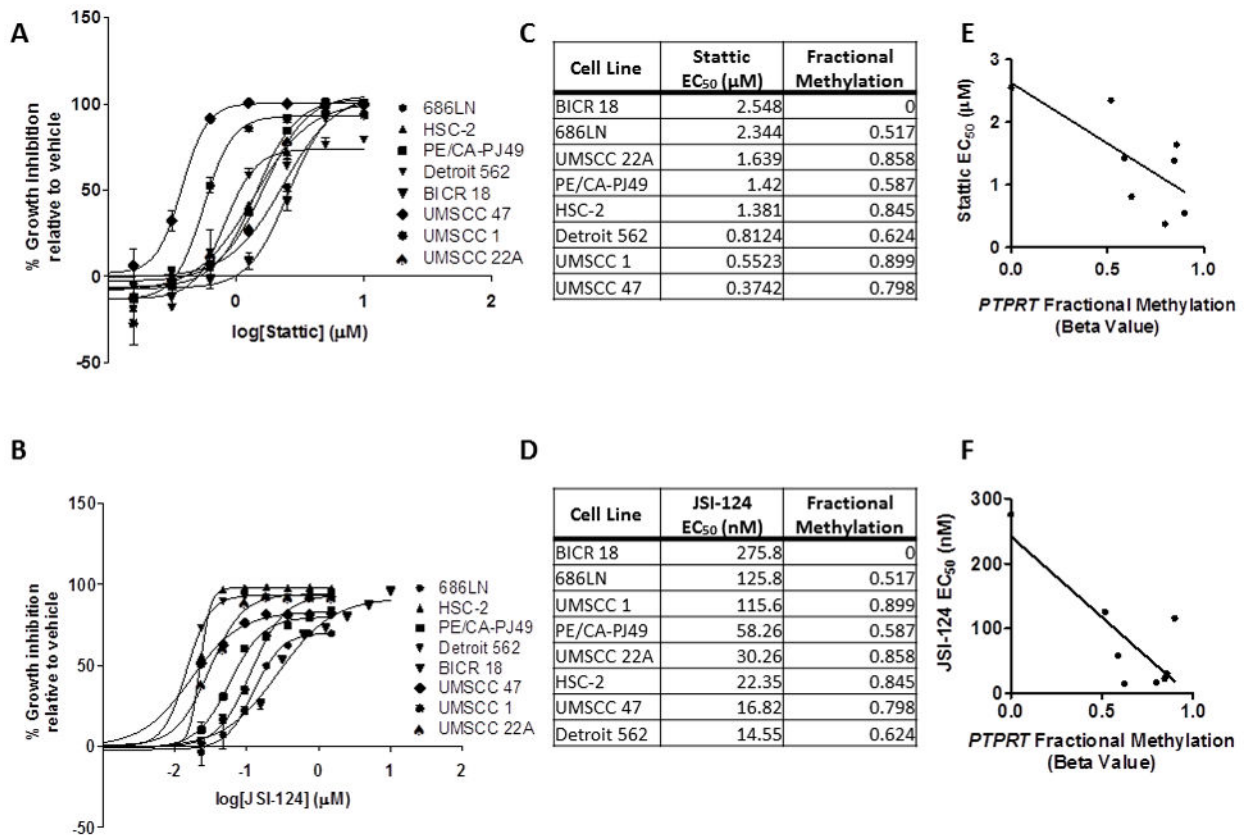
**Figure 4. *PTPRT* promoter methylation and mRNA expression are associated with pSTAT3 expression**

A) *PTPRT* mRNA expression is significantly correlated with pSTAT3 expression (n = 184, Pearson  $r = -0.1958$ ,  $P < 0.008$ ,  $R^2 = 0.03835$ , 95% confidence interval depicted). B) Representative immunohistochemical analysis of pSTAT3 expression in six HNSCC tumors with the indicated *PTPRT* methylation status, performed as previously described.<sup>4</sup>



**Figure 5. *PTPRT* promoter methylation is reduced by 5-azacytidine treatment, leading to *PTPRT*-specific pSTAT3 downregulation in HNSCC cells**

A) MSP analysis of the *PTPRT* promoter in HNSCC cells (Cal27) following treatment with 1  $\mu$ M 5-azacytidine (or vehicle) and shRNA targeted toward *PTPRT* (shPTPRT) or scrambled non-targeting shRNA (shScr). B) Western blot and RT-PCR analysis of Cal27 cells treated as indicated. C) Graphical representation of pSTAT3/STAT3 expression analyzed by Western blot following the indicated treatments (analyzed by unpaired two-tailed t tests, n = 3).



**Figure 6. Increased *PTPRT* promoter methylation is associated with increased sensitivity to STAT3 inhibition**

A,B) Dose-response curves for 8 HNSCC cell lines treated with increasing concentrations of Stattic or JSI-124. MTT assays were performed after 72 hours of treatment. C,D) Tables of EC<sub>50</sub> values and fractional methylation determined by MSP for the cell lines treated in A and B, sorted from least to most sensitive. E,F) The *PTPRT* fractional methylation level of the cells treated in A and B correlates with sensitivity to Stattic (n = 8, Pearson r = -0.7916, P < 0.05, R<sup>2</sup> = 0.5178) and JSI-124 (n = 8, Pearson r = -0.8224, P < 0.02, R<sup>2</sup> = 0.6763).