Supplementary Information for

A CRAF/glutathione-S-transferase P1 complex sustains autocrine growth of cancers with KRAS and BRAF mutations

Yoshiro Niitsu, Yasushi Sato, Kunihiro Takanashi, Tsuyoshi Hayashi, Naoko Kubo-Birukawa, Fumiko Shimizu, Naoki Fujitani, Rai Shimoyama, Takehiro Kukitsu, Wataru Kurata, Yasuyuki Tashiro, and Irving Listowsky

Yoshiro Niitsu
Email: y_niitu@shonankamakura.or.jp

This PDF file includes:

Supplementary text
  o Materials and Methods
  o Figures S1 to S9
  o Tables S1
  o SI References
Supplementary Information Text

Materials and Methods

Cell lines
All cell lines were maintained in a 5% CO2 incubator at 37°C. Cell lines used in this study indicated in Table S1.

Immunohistochemistry
Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method with labeled streptavidin-biotin (LSAB, Dako). In brief, sections from paraffin-embedded colorectal cancer tissues were deparaffinized in xylene and heated in an antigen retrieval buffer at 95 °C for 15 min in a water bath. Subsequently the sections were incubated with GSTP1 antibody (ab138491, Abcam) as a primary antibody, and incubated with secondary biotinylated antibody from an LSAB+ peroxidase kit (Dako). They were then incubated with streptavidin-horseradish peroxidase (HRP) conjugate and visualized with DAB chromogen. All human samples were obtained from 18 patients who provided written informed consent before surgery. To evaluate the immune-ostained area with DAB objectively, we performed computer-assisted analysis using WinROOF image-processing software (Mitani Corp, Tokyo, Japan) (1). Three regions of interest (ROIs) were randomly set in each specimen. The area that stained brown were extracted using two distinct macroinstructions composed of algorithms for color extraction based on red-green-blue (RBG) and hue-luminosity-saturation (HLS) parameters, and then staining value was calculated.

Gstp1p2 null mice and treatment with azoxymethane
Gstp1p2 null (Gstp1p2<sup>−/−</sup>) and wild-type (Gstp1p2<sup>+/+</sup>) mouse lines, on a mixed 129xMF1 background, were generated and maintained as reported previously (2). Female mice at 6 weeks of age were treated intraperitoneally with azoxymethane (AOM) at a dose of 10 mg/kg once a week for 2 weeks to induce colorectal carcinogenesis.

Identification of ACF and polyps
Mice were sacrificed at week 8 after treatment with AOM (10 mg/kg i.p.) for evaluation of ACF and at week 16 for evaluation of polyps (n = 6 female mice per treatment groups). After laparotomy, the entire colons were resected and filled with 10% neutral buffered formalin and then opened longitudinally from the anus to the cecum. Each was fixed flat between sheets of filter paper in 10% neutral buffered formalin and then stained with 0.2% methylene blue in saline and ACF and polyps were scored under a stereomicroscope.

Two-step PCR-RFLP analysis for Kras mutations
Two-step PCR-RFLP analysis for Kras mutation was performed as previously described (3).

TaqMan real time RT-PCR for Gstp mRNA
The resected colon was filled with RNA later (Takara), opened longitudinally along the mesenterium, and immersed in RNA later for more than 30 minutes. It was then stained with 0.2% methylene blue solution and ACF and polyps were observed under the stereomicroscope. After ACF, normally-appearing epithelia and polyps were excised in 2 x 2 mm specimens under stereoscope, RNA was extracted by the acid guanidium phenol chloroform (AGPC) method and used for synthesis of cDNA templates with reverse transcriptase. Primers for murine Gstp were: 5’-ccggtgcctacgctg3’ and 5’-ccgattcagtgctgtaga-3’. The TaqMan probe was: 5’-cgtctcactcttgac3’. RNA was quantified by real-time PCR with ABI PRISM 7700 Sequence Detection System (Thermo fisher scientific). TaqMan rodent GAPDH control reagents (Thermo
fishter scientific) were used as the control template. The Gstp and Gapdh relative messages to the calibrator ([Gstp] c and [Gapdh] c, respectively) were quantified by measuring the threshold cycle and by using a standard curve. The final relative mRNA expression level was expressed as the ratio of [Gstp] c / [Gapdh] c.

**Soft agar colony assays**
Cells (1 x 10^6 cells) transduced with lentiviruses encoding pLVCTH-TRKRAB GSTP1 shRNA or control shRNA were suspended in a top layer of complete medium containing 0.33% soft agar and 1 μg/ml of doxycycline hyclate and plated on a bottom layer of complete medium containing 0.5% soft agar and 1 μg/ml of doxycycline hyclate in 60 mm dishes. After 2 to 3 weeks, colonies greater than 100 μm in diameter were counted.

**Tumorigenesis assays**
All experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the Animal Ethics Committees of the Sapporo Medical University. Mice were housed in barrier environments and provided with water and food as approved by the committees. Tumor cells (2 x 10^6 cells) transduced with lentiviruses encoding pLVCTH-TRKRAB GSTP1 shRNA or pLVCTH-TRKRAB control shRNA in 50% matrigel-basement membrane matrix were injected subcutaneously into 6-week-old female NOD-SCID mice (Charles River) (n = 3 to 6 per treatment groups). Mice were provided drinking water containing 2 g/l doxycycline hyclate with 5% sucrose from day 5 following injection. Tumor volume was measured weekly by caliper and calculated with the formula: Length × Width^2 × 0.5. Data represent the mean ± SD of three to six independent experiments. Tumor tissues were collected and snap-frozen in liquid nitrogen for western blotting.

**DNA constructs**
pcDNA3.1-FLAG-CRAF was kindly provided from the 4th department of Medicine Sapporo Medical University. CRAF cDNA was cloned into pcDNA3.1-V5-His vector (Thermo fisher scientific). pcDNA3.1hRAF384 was generated by cloning Kozak-ATG-(amino acid 56-184 of RAF1)-STOP to pcDNA3.1 at EcoRI and NotI sites. pCMV6-Entry, pCMV6-Myc-DDK-BRAF, pCMV6-Myc-DDK-GSTP1 were purchased from OriGene. To generate pCMV6-Myc-DDK-CRAFΔN (amino acids 193-647) and BRAFΔN (amino acids 149-765), CRAFΔN and BRAFΔN cDNAs were inserted between AsISI and MluI sites of pCMV6-Entry. AxCaHGSTP1 adenoviral vector was obtained from RIKEN DNA bank. AxCAt2 was purchased from Takara. pBabe-puro-KRAS12V, pBabe-puro, pLVTHM and pLVCT-TRKRAB were obtained from Addgene. To generate shRNA expressing vectors, double-strand oligos were cloned into pLMTHM and shRNA expression region was inserted into the Fspl/Mscl restriction sites of pLVCT-TRKRAB. The sequences targeted by GSTP1 shRNA and control shRNA were obtained from The RNAi Consortium; GSTP1 shRNA: ctgcaatacctctccatctgagggagatgtgtttcagcttagtccc-3’. pCMV-CRAF-FLAG-LgBiT and pCMV-SmBit-CRAF-FLAG were constructed by insertion of CRAF-FLAG sequence from pcDNA3.1-FLAG-CRAF to pBiT1.1-CTK/LgBiT and pBiT2.1-N[TK/SmBiT] Vector (Promega), and exchange of promoter TK into CMV. Transfection of these cDNAs were conducted in the presence of lipofectamine 3000.

**siGSTP1 resistant GSTP1 constructs and siRNAs**
Site directed mutagenesis of GSTP1 constructs was generated by overlapping PCR-based strategy and validated by sequencing. For 1st PCR, pcDNA3.1+/C-(K)-DYK vector inserted with the GSTP1 gene (FLAG-tagged to C-terminal, GenScript) was used as a template. Overlap extension PCR was performed with a set of T7 primers: 5’-TAATACGACTCACTATAGG -3’, annealing upstream of the GSTP1 gene and GSTP1-4bpMute-R primers: 5’-gagatctgccacaataaaagttttttcccccttg -3’, and GSTP1-4bpMute-F primers: 5’-ctgtgctccacagggggaaaaaccccttgagg -3’ and BGH Reverse primers: 5’-tagaagggcacagtcaggagg -3’ annealing downstream of the GSTP1 gene, respectively.
Construct of degradation mutant CRAF (phosphor-Serine 621 substituted with Aspartic acid) plasmid
The gene encoding CRAF was amplified by PCR with PrimeSTAR HS DNA polymerase (Takara) using pBABE-puro-CRAF (Addgene) as the template with a forward primer: aaaaagaattcgccaccatggagcacatacagggag and a reverse primer: ttttgcggccgcctacttgtcatcgtcatccttgtagtcgaagacaggcagcctcg. The amplified fragment was digested with EcoRI, and NotI, and ligated into the expression vector pcDNA3.1(+) vector (Addgene). To generate degradation resistant mutant-CRAF fragment, the CRAF expression plasmid was amplified by PCR with PrimeSTAR HS DNA polymerase (Takara) using mutant preparation primer for 5': forward _taatacgactcactataggg / reverse _caaggatggctcgtcagcgctccggttg; Mutant preparation primer for 3': forward _caaccggagcgctgacgagccatccttg/ reverse _tagaaggcacagtcgagg and subcloned into the EcoRI and NotI sites of pcDNA3.1(+) vector (Addgene).

Small interfering RNA (siRNA) transfections
Transfection of siRNA was performed using Lipofectamine RNAiMAX (Thermo fisher scientific) according to manufacturer's instructions. When cells were 20-30% confluent in normal medium without antibiotics, they were transfected with 50 nM of siRNAs in Opti-MEM I for 5 h and again cultured in normal medium without antibiotics. The siRNAs used as follows; GSTP1-A: #2292 (Thermo fisher scientific), GSTP1-B: #2385 (Thermo fisher scientific), GSTP1-C: #s194476 (Thermo fisher scientific), KRAS-A: s7939 (Thermo fisher scientific), KRAS-B: s7938 (Thermo fisher scientific), KRAS-C: HSS105871 (Thermo fisher scientific), KRAS-D: HSS105872 (Thermo fisher scientific), RAF1: #5894, (Bioneer, Daejeon, South Korea), JNK siRNA I: #6232 (Cell Signaling Technology), control: AllStars negative control siRNA (Qiagen). The target sequence of c-FOS siRNA used was 5'-uaucugagaucuauauauuu-3' (4). The transfected cells were used for either cell proliferation assay, immunoblotting, co-immunoprecipitation, BiLC assay, RT-PCR, cell viability assay, or flow cytometry.

Cell proliferation assays
For cell proliferation following siRNA transfection, cells were seeded on 100 mm dishes in normal medium without antibiotics and transfected with 50 nM of siRNA in Opti-MEM I for 5 h using Lipofectamine RNAiMAX. After transfection, cells were cultured in normal medium without antibiotics for 2 h, and then subcultured to 60 mm dishes. The total number of cells were counted for 5 days. For GSTP1 overexpression studies, HepG2 cells were transduced with AxCAhGSTP1 (RIKEN DNA bank), recombinant adenovirus expressing human GSTP1 and AxCAwt (Takara), control adenovirus at 10 MOI and seeded on 60mm dishes. The total number of cells were counted for 5 days. All assays were performed in triplicate.

Immunoblotting
Cells were lysed in lysis buffer (1% NP-40, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, complete mini (Roche) and Phos STOP (Roche)) on ice for 30 min and lysates were obtained by centrifugation at 17000 x g for 15 min at 4 °C. In some experiments, anti-CRAF antibodies or anti-BRAF antibodies were applied to immune-precipitate each corresponding protein. Protein was separated by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with primary antibodies and detected with goat secondary antibodies directed against rabbit or mouse IgGs. Signals were visualized by ECL or ECL prime western blotting detection system (GE healthcare). The antibodies used are listed in Table S1.

Co-immunoprecipitation
For co-immunoprecipitation, cells were transiently transfected with pCMV6-Myc-DDK-CRAF and pcDNA3.1-V5-His-CRAF using FuGENE HD (Promega) or Lipofectamine 3000 (Thermo fisher scientific) for 16 h, lysed in 0.5% NP-40 lysis buffer (0.5% NP-40, 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 10% Glycerol, complete mini and Phos STOP) on ice for 30 min and lysates were obtained by centrifugation at 13000 x g for 10 min at 4°C. Equivalent amounts of protein lysates were incubated with Dynabeads protein A (Thermo fisher scientific)-
conjugated antibodies or anti-FLAG M2 magnetic beads (SIGMA) for 2 h or overnight at 4 °C. Complexes were washed with 0.5% NP-40 lysis buffer four times and analyzed by immunoblotting.

**Bimolecular luminescence complementation (BiLC)**

CRAF dimer formation was analyzed by NanoBit protein: protein interaction system (Promega) according to manufacturer’s instructions. A549 cells (6.0 x 10⁵) were transfected with 24 nM siRNA using Lipofectamine RNAiMAX on 60 mm dishes just after seeding, cultured for 24 h and co-transfected with pCMV-CRAF-FLAG-LgBit and pCMV-SmBit-CRAF-FLAG using Lipofectamine 3000. After transfection of plasmids, cells were trypsinized at 24 h and 1 x 10⁴ cells were collected in a new tube. After centrifugation and re-suspension in 40 μl of Opti-MEM I, cells were transferred to 384 well plates (white, Thermo fisher scientific). Just after addition of 10 μl of Nano-Glo Live Cell substrate/buffer (Promega), intensity of chemiluminescence from complemented luminescence protein via CRAF homodimerization was measured by ChemiDoc Touch (Bio-Rad) and quantified by ImageJ software. All assays were performed in triplicate. To confirm the expression levels of CRAF-FLAG-LgBit and SmBit-CRAF-FLAG, the remaining cells after trypsinization were lysed, and analyzed by immunoblotting.

**RT-PCR**

Total RNA was extracted from cells using RNeasy mini kit (Qiagen) and cDNA was synthesized using SuperScript first-strand synthesis system for RT-PCR (Thermo fisher scientific) according to manufacturer’s instructions. Subsequently, PCR was performed with Ex Taq (Takara) according to manufacturer’s instructions: pre-incubation for 1 min at 94 °C, 30 cycles consisting of denaturation for 1 min at 94 °C, annealing for 30 sec at 60 °C and extension for 1 min at 72 °C, followed by a final extension for 5 min at 72 °C. Primers were used as follows; GSTP1 forward: 5'-acctccgctgcaaatacatc-3'; GSTP1 reverse: 5'-ggctaggacctgatgct-3'; CRAF forward: 5'-tttctgcatgtctccct-3'; CRAF reverse: 5'-acttgggtcatctgtctca-3'; GAPDH forward: 5'-gagtcaacggatttggtcgt-3'; GAPDH reverse: 5'-ttgattttggagggatctcg-3'. PCR products were identified by 2% (w/v) agarose gels and visualized by ethidium bromide staining.

**Real time RT-PCR**

Reverse transcription was carried out using SuperScript first-strand synthesis system for RT-PCR (Thermo fisher scientific) according to manufacturer’s instructions. Real time PCR was performed with power SYBR green PCR master mix (Thermo fisher scientific) using GSTP1 primer sets. Analysis was performed using the Applied Biosystems 7500 Real-Time PCR system.

**Cell viability assays**

After siRNA transfection, cells were cultured for 5 days and harvested. The percentages of dead and viable cells were analyzed by trypan blue exclusion. All assays were performed in triplicate.

**Flow cytometry**

For cell cycle analyses, cells were harvested at day 3 after siRNA transfection, fixed with 80% (v/v) ice-cold ethanol and then incubated for 2 h at -20 °C. After washing with PBS, cells were resuspended in 0.5 ml of PI/RNase staining buffer (BD Biosciences) and incubated for 15 min at room temperature. Stained cells were analyzed by FACS Verse flow cytometer (BD).

**Viral infection**

Lentiviral particles were produced by co-transfection with pLVTHM-tTRKRAB constructs together with psPAX2 packaging and pMD2.G envelope plasmid DNA in 293T cells using FuGENE6 according to manufacturer’s instructions. After transfection at 24 h, the cell culture medium was replaced with fresh medium. Lentiviral particles-containing supernatants were harvested at 48 h and 72 h after transfection, filtered on 0.45 μm cellulose acetate membranes and then concentrated using Lenti-X concentrator (Takara) according to manufacturer’s instructions. Virus titers were determined by Lenti-X p24 rapid titer kit (Takara) according to manufacturer’s instructions. Cells were transduced with lentiviruses encoding pLVCTH-tTRKRAB GSTP1 shRNA or pLVCTH-tTRKRAB control shRNA at 50 MOI with 8 μg/ml of polybrene, cultured for 24 h and
selected by limiting dilution in 96 well plates. Cell clones expressing GFP in the presence of doxycycline hyclate (1 μg/ml) were isolated.

**Statistical analyses**
Unless otherwise specified, data were presented as the mean ± S.D of three independent experiments. Statistical analyses were performed using two-tailed unpaired Student's t-test. If compared in two conditions, Mann-Whitney U test were performed. If compared treatment groups to vehicle control, one-way ANOVA with Tukey-Kramer post hoc test was performed. See figure legends for details on types replicate and n values used.
Supplemental Figures

Figure S1

A

|                | wild-type KRAS | mutant KRAS |
|----------------|---------------|-------------|
| HepG2          |               |             |
| HeLa           |               |             |
| MCF7           |               |             |
| M7609          |               |             |
| GSTA1          | X             |             |
| GSTZ1          |               |             |
| GSTT1, 2, 3    | X             |             |
| GSTM1          |               |             |
| GSTP1          |               |             |
| GSTO1          |               |             |
| β-actin        | X             |             |

B

|                | mutant KRAS | wild-type KRAS | mutant BRAF |
|----------------|-------------|---------------|-------------|
| M7609          |             |               |             |
| HCT116         |             |               |             |
| A549           |             |               |             |
| H358           |             |               |             |
| H23            |             |               |             |
| PANc-1         |             |               |             |
| MIA PaCa-2     |             |               |             |
| Capan-2        |             |               |             |
| SW1990         |             |               |             |
| MDA-MB-231     |             |               |             |
| HeLa           |             |               |             |
| MCF7           |             |               |             |
| HepG2          |             |               |             |
| A375           |             |               |             |
| Malme-3M       |             |               |             |
| A2058          |             |               |             |
| SK-MEL-28      |             |               |             |
| GSTP1          | X           |               |             |
| β-actin        | X           |               |             |
C  mutant KRAS

| case 1 | case 2 | case 3 | case 4 | case 5 | case 6 |
|--------|--------|--------|--------|--------|--------|
|        |        |        |        |        |        |
| case 7 | case 8 | case 9 | case 10 | case 11 | case 12 |
|        |        |        |        |        |        |
| case 13 | case 14 | case 15 | case 16 | case 17 | case 18 |
|        |        |        |        |        |        |

wild-type KRAS

| case 1 | case 2 | case 3 | case 4 | case 5 | case 6 |
|--------|--------|--------|--------|--------|--------|
|        |        |        |        |        |        |
| case 7 | case 8 | case 9 | case 10 | case 11 | case 12 |
|        |        |        |        |        |        |
| case 13 | case 14 | case 15 | case 16 | case 17 | case 18 |
|        |        |        |        |        |        |

D

E

F

HepG2  MCF7

| KRAS-GTP | KRAS | GSTP1 | β-actin |
|----------|------|-------|---------|
| WT       | WT   | WT    | WT      |
| KRAS12V  | KRAS12V | GSTP1 | β-actin |

M7609  A549

| c-FOS | GSTP1 | GAPDH |
|-------|-------|-------|
| siControl  1  0.49 | siFOS   1  0.36 |
| siControl  1  0.49 | siFOS   1  0.58 |
Fig. S1. Expression of GST isozymes in wild type and mutant KRAS cells, and GSTP1 specific expression in mutant KRAS and BRAF cells.

(A) Immunoblot analysis of GSTP1 expression in mutant and wild-type KRAS cancer cells. Note that GSTP1 was detected in all three mutant KRAS cells and not in three wild-type KRAS cells whereas expression of other GST isoenzymes varied without correlations to KRAS mutations amongst all cell lines examined. (B) Immunoblot analysis of GSTP1 expression in cancer cells with mutant KRAS, mutant BRAF and wild-type KRAS/BRAF. All fourteen cells with mutant KRAS or mutant BRAF were positive for GSTP1 expression whereas three wild-type KRAS/BRAF cells were negative for GSTP1 expression. (C) Immunohistochemical staining for GSTP1 in human colon cancer tissues classified genetically for mutant and wild-type KRAS. (D) Binary image analysis of GSTP1 expression in human colon cancer tissues described in (C). The mean intensity of stained GSTP1 in mutant KRAS specimens were significantly higher than those in wild-type KRAS specimens. *P < 0.01; by Mann-Whitney U test. (E) Induction of GSTP1 in wild-type KRAS cancer cells by transduction of KRAS12V. Cell lysates were analyzed by immunoblotting for GSTP1, KRAS and KRAS-GTP which was pulled down with the RAS binding domain of CRAF. (F) Downregulation of GSTP1 expression by siRNA c-FOS (siFOS) in mutant KRAS cancer cells. GSTP1 of cell lysates at day 5 of transfection was analyzed by immunoblotting and its relative expression intensity was normalized based on GAPDH.
Figure S2

A

siControl
siGSTP1-A
siGSTP1-B
siGSTP1-C

GSTP1
β-actin

B

No. of cells (×10^6)

d0
d1
d2
d3
d4
d5

10nM
50nM

si Control
si GSTP1-A
si GSTP1-B
si GSTP1-C

C

GSTP1
β-actin

Exogeneous GSTP1

Endogeneous GSTP1

Relative Intensity

D

No. of cells (×10^6)

10nM
25nM
50nM

si Control
si GSTP1-A
si GSTP1-B
si GSTP1-C

E

M7609
A549
PANC-1

Viable cells
Dead cells

F

M7609
A549
PANC-1

p-BAD (S136)
BAD
Capase-9
Cleaved Caspase-9
Capase-7
Cleaved Caspase-7
Cleaved Caspase-3
PARP
Cleaved PARP
GSTP1
β-actin

G

M7609
A549
PANC-1

siControl
si GSTP1
si GSTP1
si GSTP1

mock
siControl
mock
si GSTP1
GSTP1
siControl
GSTP1
si GSTP1
pseudo
siControl
pseudo
si GSTP1

0.0
0.5
1.0
1.5
2.0

Relative Intensity

0.0
0.5
1.0
1.5
2.0

β-actin

mock
siControl
mock
si GSTP1
GSTP1
siControl
GSTP1
si GSTP1
pseudo
siControl
pseudo
si GSTP1
Fig. S2. Similar silencing and growth suppressive effects by three siGSTP1s having distinct sequences.
(A) Similar silencing activities of siGSTP1-A, siGSTP1-B and siGSTP1-C with each distinct sequence (refer to method) on GSTP1 expression in M7609 cells as revealed by immunoblot analysis. NT, no treatment. (B) Similar suppressive activities of siGSTP1s described above on proliferation of mutant KRAS cell, M7609. Data represent the mean ± SD of three independent experiments. (C) Proof of target sequence specificity of siGSTP1-B using siGSTP1-resistant cDNA. SDS-electrophoretic patterns of GSTP1 bands on western blotting, representing similar results from three independent experiments in PANC-1 cells. In lane 1 (siControl) and 2 (siGSTP1) in which PANC-1 cells were transfected with mock plasmids, only lower molecular weight GSTP1 bands representing endogenous GSTP1 were observed and silencing effects of siGSTP1 was noted. In lane 3 (siControl) and 4 (siGSTP1) in which the cells were transfected with natural GSTP1 genes, both higher molecular weight GSTP1 representing the gene products and endogenous lower molecular weight GSTP1 were clearly suppressed by siGSTP1 as compared to those of siControl. In lane 5 (siControl) and 6 (siGSTP1) in which the cells were transfected with siGSTP1-resistant cDNA (pseudo), expression of GSTP1 proteins was unaffected by siGSTP1 as evidenced by the same density high molecular weight band (lane 6) as that of siControl (lane 5), whereas the lower molecular band of endogenous GSTP1 in the same sample preparation (lane 6) was clearly suppressed by siGSTP1 as compared to that of siControl (lane 5). These results confirmed that siGSTP1 in this sample preparation was active. Histogram of SDS band-density of GSTP1 in three rescue experiments. Statistical significance (P values) was calculated by two-tailed Student’s t-test. (*< 0.05; ** <0.01). (D) Clear dose dependency in growth suppression by siGSTP1-B selected for subsequent experiments. (E) Proportion of non-viable cells in populations of mutant KRAS cells treated with siGSTP1 as assessed by dye exclusion assays. M7609, A549 and PANC-1 cell lines, derived from human cancers with high frequency of KRAS mutation (cancers of colon, lung and pancreas respectively), were employed. Data represent the mean ± SD of three independent experiments. (F) Cell cycle analysis of siGSTP1-transfected M7690, A549 and PANC-1 cells by flow cytometry. Note that siGSTP1 caused a decrease of S-phase populations with concomitant accumulation of sub-G1 apoptotic populations. (G) Induction of apoptosis-relevant signaling in M7609, A549 and PANC-1 cells by treatment with siGSTP1. Suppression of p-BAD (Ser136)/BAD, and increments of cleaved caspase-9, 7, 3 and cleaved PARP were evident as revealed by immunoblotting. β-actin was used as an internal control.
Figure S3

A

| Cell Line | M7609 | A549 | PANC-1 | HepG2 | MCF7 |
|-----------|-------|------|--------|-------|------|
| shControl + Dox | | | | | |
| shGSTP1 + Dox | | | | | |

No. of colonies

| No. of colonies | 250 | 200 | 150 | 100 | 50 | 0 |
|----------------|-----|-----|-----|-----|----|---|
| shControl + Dox | N.S. | ** | ** | N.S. | ** | * |
| shGSTP1 + Dox | | | | | | |

B

| Tumor | M7609 | A549 | PANC-1 | HepG2 | MCF7 |
|-------|-------|------|--------|-------|------|
| shControl | | | | | |
| shGSTP1 | | | | | |

Dox

- - + - - + - - +

C

| Tumor | M7609pLVCTH | A549pLVCTH | MCF7pLVCTH |
|-------|-------------|-------------|-------------|
| GSTP1 | | | |
| β-actin | | | |

| Tumor | M7609pLVCTH | PANC-1pLVCTH | HepG2pLVCTH |
|-------|-------------|-------------|-------------|
| GSTP1 | | | |
| β-actin | | | |
Fig. S3. Effects of GSTP1 silencing induced by Dox on colony formation and tumorigenesis of shGSTP1 transfected mKRAS and wild KRAS cancer cells. (A) Suppression of soft agar colony formation by induction of siGSTP1 with Dox in shGSTP1 transfected mKRAS cancer cells. Upper panel represent histogram of colony number in each agar well. Lower pictures demonstrate suppressed colonies as revealed by dots in agar wells where siGSTP1 is induced by addition of Dox. (B) Pictures of excised tumor nodules from xenografted mice. Note that nodule-size of all three shGSTP1 transfected tumors with mutated KRAS was apparently smaller than that of shControl group. (C) Western blotting of GSTP1 in excised tumors. Note that upon Dox administration, GSTP1 expression was appreciably suppressed in shGSTP1 transfected mutant KRAS tumors. N.S, not significant. Statistical significance (P values) was calculated using the paired two-tailed t test (*< 0.05; **<0.01).
Figure S4

A

|            | M7609 | A549 | PANC-1 |
|------------|-------|------|--------|
| siControl  |       |      |        |
| siGSTP1    |       |      |        |
| 0.01% DMSO|       |      |        |
| 5μM JNK    |       |      |        |
| 0.02% DMSO|       |      |        |
| 10μM JNK   |       |      |        |
| p-JNK (T183/Y185) |     |      |        |
| JNK        |       |      |        |
| p-c-JUN (Ser63) |     |      |        |
| c-JUN      |       |      |        |
| GSTP1      |       |      |        |
| β-actin    |       |      |        |

B

|            | M7609 | A549 | PANC-1 |
|------------|-------|------|--------|
| siControl  |       |      |        |
| siGSTP1    |       |      |        |
| 0.01% DMSO|       |      |        |
| 5μM JNK    |       |      |        |
| 0.02% DMSO|       |      |        |
| 10μM JNK   |       |      |        |
| No. of cells (x 10^5) |       |      |        |

C

|            | M7609 | A549 | PANC-1 |
|------------|-------|------|--------|
| siControl  |       |      |        |
| siGSTP1    |       |      |        |
| 0.01% DMSO|       |      |        |
| 5μM JNK    |       |      |        |
| 0.02% DMSO|       |      |        |
| 10μM JNK   |       |      |        |
| Cell death (%) |       |      |        |

D

|            | PANC-1 | A549 |
|------------|--------|------|
| siControl  |        |      |
| siJNK      |        |      |
| JNK        |        |      |
| β-actin    |        |      |

N.S.
Fig. S4. Irrelevancy of JNK for siGSTP1 induced growth suppression of mutant KRAS cancer cells.

(A) Suppressive effects of JNK inhibitor (JNKi) (SP600125) on phosphorylation of p-JNK in mutant KRAS cells. After siGSTP1 transfection, M7609, A549 and PANC-1 cells were treated with SP600125 for 24 h at day2, lysed at day 3 and analyzed by immunoblotting. (B) JNK inhibitor is irrelevant but GSTP1 relevant proliferation of mutant KRAS cells. After siGSTP1 transfection, cells were treated with SP600125 from day 2 and cell numbers were counted at day 4. Data represent the mean ± SD of three independent experiments. N.S, not significant; by two-tailed Student’s t-test. (C) JNK inhibitor irrelevant but GSTP1 relevant viability of mutant KRAS cells. After siGSTP1 transfection, cells were treated with SP600125 from day 2, and dye exclusion assays were performed at day 4. Data represent the mean ± SD of three independent experiments by two-tailed Student’s t-test; N.S, not significant. (D) No significant difference in growth between siJNK treated and siControl treated mKRAS cells though expression of JNK revealed by western blotting was clearly suppressed by siJNK. Data represent the mean ± SD of three independent experiments by two-tailed Student’s t-test; N.S, not significant.
Figure S5

### A

|         | M7609 siControl | A549 siControl | PANC-1 siControl | M7609 siGSTP1 | A549 siGSTP1 | PANC-1 siGSTP1 |
|---------|-----------------|----------------|------------------|---------------|---------------|---------------|
| KRAS-GTP| 0.98            | 1.32           | 1.01             | 0.96          | 0.91          | 0.98          |
| KRAS    | 0.97            | 0.91           | 0.98             | 0.96          | 0.90          | 0.98          |
| GSTP1   |                 |                |                  |               |               |               |
| β-actin |                 |                |                  |               |               |               |

### B

|         | M7609 siControl | A549 siControl | PANC-1 siControl | M7609 siGSTP1 | A549 siGSTP1 | PANC-1 siGSTP1 |
|---------|-----------------|----------------|------------------|---------------|---------------|---------------|
| p-BRAF (S445) | 1.12           | 1.00           | 0.95             | 0.96          | 0.90          | 0.87          |
| BRAF    | 0.96            | 0.90           | 0.87             |               |               |               |
| GSTP1   |                 |                |                  |               |               |               |
| β-actin |                 |                |                  |               |               |               |

### C

|         | siControl | siGSTP1 |
|---------|-----------|---------|
| GSTP1   |           |         |
| ARAF    |           |         |
| COT1    |           |         |
| β-actin |           |         |

### D

|         | M7609 | A549 | PANC-1 |
|---------|-------|------|--------|
| GSTP1   |       |      |        |
| CRAF    |       |      |        |
| GAPDH   |       |      |        |
Fig. S5. Effect of GSTP1 silencing on KRAS-GTP/KRAS, p-BRAF(Ser445), BRAF, ARAF, and COT1, and CRAF mRNA expression.
M7609, A549 and PANC-1 cells were lysed at day 3 after siGSTP1 transfection for analyses of KRAS-GTP/KRAS (A), p-BRAF (Ser445)/BRAF (B), and ARAF and COT1 in PANC-1 (C) by immunoblotting. KRAS-GTP was pulled-down with antisera against the Ras binding domain of CRAF. GSTP1 silencing showed no apparent effect on expression of these factors. (D) For analyses of CRAF mRNA expression, total mRNAs of M7609, A549 and PANC-1 cells were extracted on day 3 of siGSTP1 transfection and PCR for CRAF mRNA was carried out with its primers. Note that there are no differences in CRAF mRNA levels between siControl and siGSTP1 treated samples.
Figure S6

A

|          | siControl | siGSTP1 |
|----------|-----------|---------|
| CRAF-FLAG-LgBiT | 1.00 | 0.59 |
| SmBiT-CRAF-FLAG | 1.00 | 0.61 |
| IB : FLAG |          |         |
| IB : GSTP1 |          |         |
| IB : β-actin |         |         |

B

| Chemiluminescence | siControl | siGSTP1 |
|-------------------|-----------|---------|

Relative intensity

*
Fig.S6. Effects of GSTP1 knockdown on CRAF homodimers analyzed by BiLC (Bimolecular luminescence complementation) assays. 
(A) After transfection of GSTP1 siRNA, A549 cells were co-transfected with CRAF-FLAG-LgBit and SmBit-CRAF-FLAG at 24 h and BiLC assays were performed at 48 h. Expression levels of CRAF-FLAG-LgBit and SmBit-CRAF-FLAG were analyzed by immunoblotting. Densitometry analysis indicates relative protein levels from one representative of three independent experiments. (B) Intensity of chemiluminescence (upper panel) was quantified by ImageJ software (lower histogram). *P < 0.001; by two-tailed Student’s t-test.
Rescue study using CRAF mutant expressing PANC1 (Proteasomal degradation-resistant CRAF)

Effect of GSTP1 siRNA on the growth of CRAF mutant cells

Each PANC1 polyclonal cell was transfected with 50nM of siRNA (control and GSTP1) on the day following cell seeding (3000 cells/well) in 10% FBS RPMI without antibiotics at 96 well plate. After 72h treatment, cell count was performed.

Data analyses and statistical evaluations were carried out using Microsoft Excel. All of the results are the mean ± standard deviations in quadruplicate. Statistical comparisons between groups were made using two-tailed t-test comparing two variables.

Figure S7

A

| GSTP1 | #1: Control siRNA | #2: GSTP1 siRNA |
|-------|-------------------|-----------------|
| 1.00  | 0.41              | 0.88            |
| 0.39  | 0.99              | 0.57            |

| C-Raf | #1: Control siRNA | #2: GSTP1 siRNA |
|-------|-------------------|-----------------|
| 1.00  | 0.91              | 1.31            |
| 0.95  | 1.21              | 1.24            |

| β-actin | #1: Control siRNA | #2: GSTP1 siRNA |
|---------|-------------------|-----------------|
| 1.00    | 1.10              | 1.04            |
| 1.14    | 1.06              | 1.10            |

#1; Control siRNA
#2; GSTP1 siRNA

B

Figure S7

Mock
Wild type
Mutant
Fig. S7. Effect of GSTP1 siRNA on the growth of mKRAS cells transduced with degradation resistant CRAF mutant (phosphor-Serine 621 substituted with Aspartic acid) gene.

(A) Mock-, wild-, degradation resistant mutant-CRAF plasmid transduced PANC1 cells were treated with 50nM of siRNA (control and GSTP1) on the day following cell seeding (3 x 10^5 cells/well) in 10%-FBS RPMI without antibiotics at 6 well plate. After 48h treatment, cells were lysed in lysis buffer and proteins of interest were analyzed by western blotting. Individual value under image shows the ratio compared with mock/control siRNA. (B) Mock-, wild-, degradation resistant mutant-CRAF plasmid transduced PANC-1 cells were treated with 50nM of siRNA (control and GSTP1) on the day following cell seeding (3 x 10^3 cells/well) in 10%-FBS RPMI without antibiotics at 96 well plate. After 72h treatment, cell count was performed. All of the results are the mean ± standard deviations in quadruplicate. Statistical comparisons between groups were made using two-tailed t-test comparing two variables; NS, not significant.
Figure S8

A

GSTP1

KRAS

β-actin

B

No. of cells (x10^6)

PLX4720 (μM)

0

0.001

0.01

0.1

1

10

100

1000

A375

A2058

SK-MEL-28

Caco-2

No. of cells (x10^6)

PLX4720 (μM)

0

0.001

0.01

0.1

1

10

100

1000

A375

A2058

SK-MEL-28

Caco-2
Fig. S8. Suppressive effect of siKRAS, siGSTP1 and BRAF-inhibitor on growth of mKRAS and mBRAF cancer cells.

(A) Immunoblot analysis of GSTP1 and KRAS in mutant KRAS cells, M7609, transfected with four types of siKRAS. Note that all siRNAs equally suppressed expression of KRAS but not that of GSTP1, indicating that siRNA against an upstream factor of CRAF with which GSTP1 interacts does not suppress GSTP1. (B) Dose-dependent suppression of proliferation of M7609 cells by siKRAS. Data represent the mean ± SD of three independent experiments. (C) Suppression of proliferation of BRAF (V600E) cells by siGSTP1. Melanoma (A375, A2058 and SK-MEL-28) and colon cancer cells (Caco-2) were transfected with siGSTP1 and cell numbers were counted at day 5. Data represent the mean ± SD of three independent experiments. Data represent the mean ± SD of three independent experiments. *P < 0.05; by two-tailed Student’s t-test. (D) Effects of GSTP1 silencing on CRAF/MEK/ERK pathway of BRAF (V600E) cancer cells. Cell lysates were extracted at day 3 after siGSTP1 transfection and analyzed by immunoblotting as indicated. Note that in all cells, quite similar pattern of signal components as those in siGSTP1 treated mutant KRAS cells were seen, indicating that the autocrine loop is functioning in mBRAF cancer cells as well. Densitometry analysis indicates relative protein levels from one representative of three independent experiments. (E) Dose-dependent suppression of proliferation of BRAF (V600E) cancer cells by its specific inhibitor, PLX4720. Cells were incubated with appropriate concentrations of PLX4720 for 4 days and cell numbers were counted. Data represent the mean ± SD of three independent experiments. Note that all four cancer cells (A375, A2058, SK-MEL-28 and Caco-2) with mutant BRAF (V600E) examined showed dose dependent sensitivity to PLX4720.
Figure S9

A

|          | A375          | SK-MEL-28     | CACO-2       |
|----------|---------------|---------------|--------------|
| siControl | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| siCRAF   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

B

|          | A375          | SK-MEL-28     | CACO-2       |
|----------|---------------|---------------|--------------|
| siControl | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| siCRAF   | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
Fig. S9. Suppressive effect of siCRAF on growth of mKRAS and mBRAF cancer cells.
(A) Apparent CRAF silencing by siCRAF in mBRAF cells as evidenced by Western blotting.
(B) Suppressive effects of siCRAF on growth of mBRAF cancer cells. Melanoma (A375 and SK-MEL-28) and colon cancer cells (Caco-2) were transfected with siCRAF and cell numbers were counted at day 5. Data represent the mean ± SD of three independent experiments. *P < 0.05; by two-tailed Student’s t-test.
Table S1. Key recourses table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-GST3/GSTpi antibody | Abcam | Cat# ab138491 |
| Anti-phospho-Raf-1 (Ser338) mouse mAb | Merck-Millipore | Cat# 05-538 |
| Raf-1 antibody (C-20) rabbit polyclonal | Santa Cruz Biotechnology | Cat# sc-227 |
| Raf-B antibody (F-7) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-5284 |
| Raf-B antibody (F-3) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-55522 |
| GSTT antibody (FL-240) rabbit polyclonal | Santa Cruz Biotechnology | Cat# sc-32938 |
| Ubiquitin antibody (P4D1) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-8017 |
| Phospho-MEK1/2 (Ser217/221) antibody | Cell signaling technology | Cat# 9121 |
| MEK1/2 antibody | Cell signaling technology | Cat# 9122 |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody | Cell signaling technology | Cat# 9101 |
| p44/42 MAPK (Erk1/2) (137F5) rabbit mAb | Cell signaling technology | Cat# 4695 |
| p-B-RAF (Ser445) | Cell signaling technology | Cat# 2696 |
| c-FOS | Cell signaling technology | Cat# 2250 |
| phospho-c-Jun (Ser63) II antibody | Cell signaling technology | Cat# 9261 |
| c-Jun rabbit mAb | Cell signaling technology | Cat# 2361 |
| EGF Receptor (15F8) rabbit mAb | Cell signaling technology | Cat# 4405 |
| A-Raf (D2P9P) rabbit mAb | Cell signaling technology | Cat #75804 |
| Anti-MAP3K8/COT antibody | Abcam | Cat# ab137589 |
| Bad | Cell signaling technology | Cat#9292 |
| phospho-Bad (Ser136) | Cell signaling technology | Cat#4366 |
| Caspase-7 | Cell signaling technology | Cat#9242 |
| Caspase-3 | Cell signaling technology | Cat#9662 |
| Cleaved Caspase-3 (Asp175) | Cell signaling technology | Cat#9664 |
| Caspase-9 | Cell signaling technology | Cat#9502 |
| Cleaved-Caspase-9 | Cell signaling technology | Cat#7237 |
| Chemical Name                                                                 | Supplier                                      | Cat#                  |
|------------------------------------------------------------------------------|-----------------------------------------------|-----------------------|
| Cleaved-Caspase-7                                                            | Cell signaling technology                     | Cat#8438              |
| PARP                                                                         | Cell signaling technology                     | Cat#9542              |
| Cleaved- PARP                                                                | Cell signaling technology                     | Cat#5625              |
| Anti-GST-π                                                                   | MBL                                           | Cat# 312              |
| KRAS                                                                         | Sigma-Aldrich                                 | Cat# WH0003845M1      |
| Anti-GSTZ1 mouse monoclon antibody, clone 1G12                               | Sigma-Aldrich                                 | Cat# WH0002954M1      |
| FLAG                                                                         | Sigma-Aldrich                                 | Cat# F3165            |
| Monoclonal anti-β-actin, clone AC-15                                        | Sigma-Aldrich                                 | Cat# A5441            |
| Mouse monoclonal to GST alpha                                               | Abcam                                         | Cat# ab55129          |
| Rabbit polyclonal to GSTM1                                                  | Abcam                                         | Cat# ab77925          |
| Mouse polyclonal to GST Omega 1                                              | Abcam                                         | Cat# ab88604          |
| Mouse monoclonal (6C5) to GAPDH                                              | Abcam                                         | Cat# ab8245           |
| V5 tag monoclonal antibody                                                   | Thermo fisher scientific                      | Cat# R960-25          |
| Rabbit anti-Human Raf, phosphorylated (Ser338)                               | US biological                                 | Cat# L10031886        |
| Anti-Raf-1 antibody                                                          | Merck-Millipore                               | Cat# 07-396           |
| p-JNK (14.Thr 183/Tyr 185)                                                   | Santa Cruz                                    | Cat# sc-293136        |
| Mouse monoclonal (6C5) to GAPDH                                              | Abcam                                         | Cat# ab8245           |
| **Chemicals and recombinant proteins**                                       |                                               |                       |
| MG132                                                                        | Merck-Millipore                               | Cat# 474790           |
| JNK inhibitor (SP600125)                                                     | Merck-Millipore                               | Cat# 420119           |
| L-Glutathione reduced                                                        | Sigma-Aldrich                                 | Cat# G4251            |
| Hexadimethrine bromide (polybrene)                                          | Sigma-Aldrich                                 | Cat# H9268            |
| doxycycline hyclate                                                          | Sigma-Aldrich                                 | Cat# D9891            |
| iodoacetamide                                                                | Sigma-Aldrich                                 | Cat# I1149            |
| N-ethylmaleimide                                                             | Sigma-Aldrich                                 | Cat# E3876            |
| 50% matrigel-basement membrane matrix                                        | BD Biosciences                                | Cat# 354234           |
| PLX4720                                                                      | Selleck chemicals                             | Cat# S1152            |
| GST from human placenta                                                      | Sigma-Aldrich                                 | Cat# G8642            |
| complete mini                                                                | Roche                                         | Cat# 11836170001      |
| Phos STOP                                                                    | Roche                                         | Cat# 04906837001      |
| **Critical Commercial Assays**                                               |                                               |                       |
| Lipofectamine RNAiMAX transfection reagent                                  | Thermo fisher scientific                      | Cat# 13778150         |
| Lipofectamine 3000 reagent                                                   | Thermo fisher scientific                      | Cat# L3000015         |
| Power SYBR green PCR master mix                                              | Thermo fisher scientific                      | Cat# 4367659          |
| Product Name                                                                 | Supplier       | Catalog Number |
|-----------------------------------------------------------------------------|----------------|----------------|
| RNeasy mini kit                                                             | Qiagen         | Cat# 74104     |
| HiSpeed plasmid midi kit (25)                                               | Qiagen         | Cat# 12643     |
| PrimeSTAR-HS DNA Polymerase                                                 | TAKARA         | Cat# R010A     |
| Raf-1 kinase assay kit, chemiluminescence detection                        | Merck-Millipore| Cat# 17-360    |
| K-RAS activation assay kit                                                 | Cell Biolabs   | Cat# STA-400-K |
| PI/RNase staining buffer                                                    | BD Biosciences | Cat# 550825    |
| LSAB+ peroxidase kit                                                        | Dako           | Cat# K0690     |
| FuGENE HD transfection reagent                                              | Promega        | Cat# E2311     |
| NanoBiT protein:protein interaction system                                  | Promega        | Cat# N2014     |
| In situ cell death detection kit, POD                                       | Roche          | Cat# 11684817910 |
| DAB substrate                                                               | Roche          | Cat# 11718096001 |
| CCK-8 assay kit                                                             | RIKEN          | Cat# CK04      |

**Experimental models: Cell lines**

| Cell Line         | Supplier | Catalog Number |
|-------------------|----------|----------------|
| A549              | RIKEN cell bank | Resource# RCB2095 |
| PANC-1            | RIKEN cell bank | Resource# RCB0098 |
| CACO-2            | RIKEN cell bank | Resource# RCB0988 |
| 293T              | RIKEN cell bank | Resource# RCB2202 |
| MCF7              | Tohoku University Cell Bank | ID: TKG0479 |
| M7609             | Sapporo medical university | NA |
| HeLa              | Sapporo medical university | NA |
| HepG2             | Sapporo medical university | NA |
| Capan-2           | ATCC      | Cat# HTB-80    |
| SW1990            | ATCC      | Cat# CRL-2172  |
| HCT116            | ATCC      | Cat# CCL-247   |
| H23               | ATCC      | Cat# CRL-5800  |
| H358              | ATCC      | Cat# CRL-5807  |
| MIA PaCa-2        | ATCC      | Cat# CRL-1420  |
| MDA-MB-231        | ATCC      | Cat# HTB-26    |
| A375              | ATCC      | Cat# EC8813005 |
| A2058             | ATCC      | Cat# CRL-11147 |
| SK-MEL-28         | ATCC      | Cat# CRL-11147 |
| Plat-A            | Cell Biolabs | Cat# RV-102   |
| DLD-1             | HSRRB     | Cat# JCRB9094  |
| Malme-3M          | ATCC      | Cat# HTB-64    |
SI References

1. M. Hirabaru, et al., Expression of alpha smooth muscle actin in living donor liver transplant recipients. *World J. Gastroenterol.* **20**, 7067–7074 (2014).
2. C. J. Henderson, C. R. Wolf, Disruption of the glutathione transferase Pi class genes. *Methods Enzymol.* (2005) https://doi.org/10.1016/S0076-6879(05)01007-4.
3. K. Miyanishi, et al., Glutathione S-transferase-pi overexpression is closely associated with K-ras mutation during human colon carcinogenesis. *Gastroenterology* **121**, 865–874 (2001).
4. P. Apostolou, et al., AP-1 Gene Expression Levels May Be Correlated with Changes in Gene Expression of Some Stemness Factors in Colon Carcinomas. *J. Signal Transduct.* **2013**, 1–5 (2013).