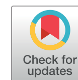

A CRAF/glutathione-S-transferase P1 complex sustains autocrine growth of cancers with *KRAS* and *BRAF* mutations

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The Ras/RAF/MEK/ERK pathway is an essential signaling cascade for various refractory cancers, such as those with mutant *KRAS* (m*KRAS*) and *BRAF* (m*BRAF*). However, there are unsolved ambiguities underlying mechanisms for this growth signaling thereby creating therapeutic complications. This study shows that a vital component of the pathway CRAF is directly impacted by an end product of the cascade, glutathione transferases (GST) P1 (GSTP1), driving a previously unrecognized autocrine cycle that sustains proliferation of m*KRAS* and m*BRAF* cancer cells, independent of oncogenic stimuli. The CRAF interaction with GSTP1 occurs at its N-terminal regulatory domain, CR1 motif, resulting in its stabilization, enhanced dimerization, and augmented catalytic activity. Consistent with the autocrine cycle scheme, silencing GSTP1 brought about significant suppression of proliferation of m*KRAS* and m*BRAF* cells in vitro and suppressed tumorigenesis of the xenografted m*KRAS* tumor in vivo. GSTP1 knockout mice showed significantly impaired carcinogenesis of m*KRAS* colon cancer. Consequently, hindering the autocrine loop by targeting CRAF/GSTP1 interactions should provide innovative therapeutic modalities for these cancers.

mKRAS and mBRAF cancers | autocrine growth cycle | CRAF/GSTP1 complex | refractory cancers

The Ras/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signaling pathway transduces an essential signal for cell growth and for apoptosis prevention. Thus, gain-of-function mutations of the components in this pathway, such as m*KRAS* and m*BRAF*, cause common human cancers including pancreatic, colon, lung cancers, and malignant melanomas. Efforts to develop effective therapeutic small molecule inhibitors against m*KRAS* cancers, however, have been largely unsuccessful due to its undruggable nature (1–3).

Regarding m*BRAF* cancers which share the common RAF/MEK/ERK cascade with m*KRAS* cancers, although certain kinase-specific inhibitors have been developed, they are hampered by vulnerability to drug resistance of which the underlying mechanisms are variable (4, 5). Moreover, the use of some RAF inhibitors causes paradoxical effects, such as unexpected growth of m*KRAS* cells (6) and the occurrence of cutaneous toxicities including squamous cell carcinomas and keratoachanthomas (7). To overcome therapeutic hurdles of directly targeting oncogenic factors, such as m*KRAS* and m*BRAF*, attempts have been made to inhibit downstream signal components, such as MEK or ERK. Although these yielded some promising outcomes in clinical studies in both m*KRAS* and m*BRAF* cancers (8), ERK-dependent feedback effects which, instead, caused growth enhancement were often experienced (9).

A recently developed BRAF inhibitor with high specificity PLX8394, which efficiently blocks activity of a downstream signal component ERK, has been shown to evade innate and acquired resistance as revealed by in vitro and in vivo studies (10). However, evaluation of clinical therapeutic outcomes or long-term results remain elusive. More recently, combination strategies linking inhibition of BRAF, ERK, and EGFR have demonstrated some clinical benefits and have been shown to overcome RAF inhibitor induced paradoxical activation of MAP kinases (11, 12). In these trials as well, targeting signal components, in particular, multiple components, despite promising antitumor effects, contend with inevitable toxicities in normal tissues (13). Thus, a theoretically desirable treatment strategy to overcome the obstacles may involve manipulation of factor(s) distinct from the signal components but that positively modulate the MAP kinase cascade via bystander effects and are selectively expressed or closely associated with the oncogenic features of these cancers. We posit that an autocrine signal loop formed by interactions of GSTP1 with CRAF can be a paradigm for this strategy.

GSTP1 was clinically implicated for its use as a tumor marker (14) and in detoxification of certain types of anticancer drugs (15). Growth promoting activity of GSTP1 on cancer cells, based on the finding that silencing GSTP1 or GSTP inhibitors

Significance

A strategy to overcome therapeutic obstacles of m*KRAS* and m*BRAF* cancers is devised based on the finding, here, that the RAF/MEK/ERK cascade is by-passed by an autocrine signal loop established by interaction of CRAF with GSTP1. The interaction evokes stabilization of CRAF from proteosomal degradation and facilitation of RAF-dimer formation. Thus, blocking CRAF/GSTP1 interactions should generate additive antiproliferative effects.

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suppressed their growth, have also been reported, although details of molecular mechanisms underlying the growth promoting activity was not exploited (16). We have previously demonstrated contemporaneous positivity of KRAS mutations and GSTP1 expression in specimens of human colon cancers, adenomas, and aberrant crypt foci (ACF) and confirmed a close correlation between levels of GSTP1 mRNA and mutation status of KRAS in those tissues (17). We also showed that GSTP1 was induced by v-Kras transfection in KRAS wild-type (WT) colon-cancer cells (17). Based on these earlier observations, a previously unexpected pivotal role of GSTP1 in the growth of refractory cancers, such as those with m*KRAS* and m*BRAF* which transduce RAF/MEK/ERK signaling is unveiled here. In these cells, GSTP1 as an end product of RAF/MEK/ERK signaling, interacts with CRAF, forming an autocrine signal loop to impede its proteasomal degradation and to enhance its dimer formation and enzyme activity.

Accordingly, in this study, we propose an autocrine growth signaling formed by CRAF/GSTP1 interactions in m*KRAS* and m*BRAF* cancers and suggest an approach to overcome certain types of therapeutic refractoriness, such as insufficient efficacy of targeting oncogenic stimuli by hindering this autocrine signal, namely, the CRAF/GSTP1 complex.

Results

Correlations between GSTP1 Expression and *KRAS* or *BRAF* Mutations. Relationships between KRAS mutations and expression of GST isoenzymes were determined. Among the isoenzymes tested in WT *KRAS* (WT*KRAS*) and m*KRAS* cells, only GSTP1 correlated with *KRAS* mutations (*SI Appendix*, Fig. S1*A*). The study in a wider variety of malignant cell lines which included those with m*KRAS* or m*BRAF* and those without these mutations disclosed that GSTP1 expression was well defined in cells with mutations, whereas it was not detected in cells with WT*KRAS*/WT*BRAF* (*SI Appendix*, Fig. S1*B*).

Immunohistochemical quantification of GSTP1 expression in human colon-cancer specimens (*SI Appendix*, Fig. S1 *C* and *D*) revealed compatible results with the cancer cell lines, showing uniformly higher levels in m*KRAS* compared with those in WT*KRAS* specimens. To elucidate a possible cause-and-effect relationship between m*KRAS* and GSTP1 expression, the m*KRAS* gene (KRAS12V) was transduced to WT*KRAS* HepG2 and MCF7 cells, and clear induction of GSTP1 was confirmed (*SI Appendix*, Fig. S1*E*). Furthermore, based on our previous premise that expression of GSTP1 is controlled by the AP-1 transcription factor (17), the c-FOS transcription factor which is directly induced by RAF/MEK/ERK signaling (18) was silenced, resulting in significant suppression of GSTP1 in m*KRAS* M7609 and A 549 cells (*SI Appendix*, Fig. S1*F*).

Suppression of Proliferation of m*KRAS* Cells by Small Interfering RNA GSTP1. To determine whether GSTP1 somehow contributes to the growth of m*KRAS* cancers, small interfering RNA (siRNA) GSTP1 (siGSTP1) was initially employed. Three siRNAs with different sequences were found to be comparable for their silencing capacity of GSTP1 and growth suppressive effects on a m*KRAS* cell line (*SI Appendix*, Fig. S2 *A* and *B*).

To further assure the target sequence specificity of siGSTP1, we carried out an experiment in PANC-1 cells transfected with siGSTP1-resistant complementary DNA (cDNA) which encodes the same amino acids as those of the authentic GSTP1 protein but has distinct codons from the natural GSTP1 gene with substitutions of four nucleotides in the siGSTP1 target sequence. To distinguish between GSTP1 proteins expressed by transfected cDNAs from endogenous GSTP1 proteins by Western blotting, the cDNAs were tagged by FLAG so that GSTP1 proteins expressed by the transfected cDNAs migrated at higher molecular weights relative to the endogenous GSTP1 (*SI Appendix*, Fig. S2*C*). As expected, no suppressive effects of siGSTP1 on the GSTP1 protein expressed by siGSTP1-resistant cDNA were observed while significant suppression of GSTP1 protein expressed by authentic GSTP1 cDNA and endogenous GSTP1 by siGSTP1 was evident (*SI Appendix*, Fig. S2*C*). These results verified the target specificity of siGSTP1 used in the present study. Transfection of siGSTP1 to the m*KRAS* cells at three different dosages (10, 25, and 50 nM) evoked clear dose-dependent growth suppression (*SI Appendix*, Fig. S2*D*), and this siGSTP1 exhibited significant growth suppressive effects on all 10 m*KRAS* cell lines examined (Fig. 1*A*). The mechanisms of growth suppression were then analyzed by dye exclusion assay for cell viability (*SI Appendix*, Fig. S2*E*), by FACS for cell cycle (*SI Appendix*, Fig. S2*F*), and by Western blotting for apoptosis (*SI Appendix*, Fig. S2*G*).

These analyses indicated that siGSTP1 treatment evoked transition of the S-phase cells into the G1 or sub-G1 phase and 20–40% of the cell population underwent cell death due to apoptosis (*SI Appendix*, Fig. S2*F*). Nonspecific inhibitory effects of the siGSTP1 on cell growth were ruled out because it did not cause any growth suppression wt*KRAS* cells (Fig. 1*A*). The growth promoting activity of GSTP1 was further confirmed by transfection of GSTP1 (AxCAhGSTP1) which resulted in significant growth enhancement of WT*KRAS* Hep G2 cells (Fig. 1*B*).

Effects of Short Hairpin RNA GSTP1 on Tumorigenesis of Xenografted m*KRAS* Cells. To substantiate the above in vitro results, in vivo effects of GSTP1 silencing on tumorigenesis were verified. Since short hairpin RNA (shRNA) expressing m*KRAS* cells are unlikely to survive under the continuous suppressive pressure of GSTP1 silencing on growth, we employed an inducible shRNA GSTP1 approach using a green fluorescence protein (GFP)-doxycycline (Dox) system.

The m*KRAS* cells were transduced with lentiviral vector designed to express shGSTP1 by triggering with Dox, and underwent limiting dilution yielding bright GFP positive clones upon Dox addition. Colony formation of cloned m*KRAS* cells was markedly suppressed by addition of Dox to agar gel whereas colonies of WT*KRAS* cells were not suppressed at all by Dox (*SI Appendix*, Fig. S3*A*). Severe combined immunodeficient (SCID) mice inoculated with these cells showed marked reduction of the tumor growth (Fig. 1*C* and *SI Appendix*, Fig. S3*B*) and suppression of GSTP1 expression (*SI Appendix*, Fig. S3*C*) upon addition of Dox to their drinking water, whereas tumor growth of WT*KRAS* cells were not affected by Dox addition at all (Fig. 1*C* and *SI Appendix*, Fig. S3 *B* and *C*).

Impaired Colon Carcinogenesis Induced by Azoxymethane in Gstp1p2 Null Mice. To support the outcome of gene-silencing experiments described above, a gene-deletion approach utilizing *Gstp* null mice (Gstp1p2$^{-/-}$ mice) was employed. The m*KRAS* relevant colon carcinogenesis was induced by administration of azoxymethane (AOM) to these mice and their littermates (Fig. 2*A*). The incidence of *KRAS* mutations in the precancerous lesions, ACF (19), and colon-cancer tissues of WT mice treated with AOM were nearly 70% (Fig. 2*B*).

Gstp expression levels in ACF and colon cancer of littermate mice treated with AOM were significantly elevated as compared to those in their normal colon epithelium (Fig. 2 *C*, *D*, and *E*). By contrast, the number of ACF and colon-cancer lesions in Gstp1p2 $^{-/-}$ mice after AOM treatment was appreciably lower than those in littermate mice (Fig. 2 *F* and *G*). These results confirmed a pivotal role of GSTP1 in m*KRAS* colon carcinogenesis, consistent with suppressed proliferation and tumorigenesis of m*KRAS* cells, revealed by GSTP1 silencing.

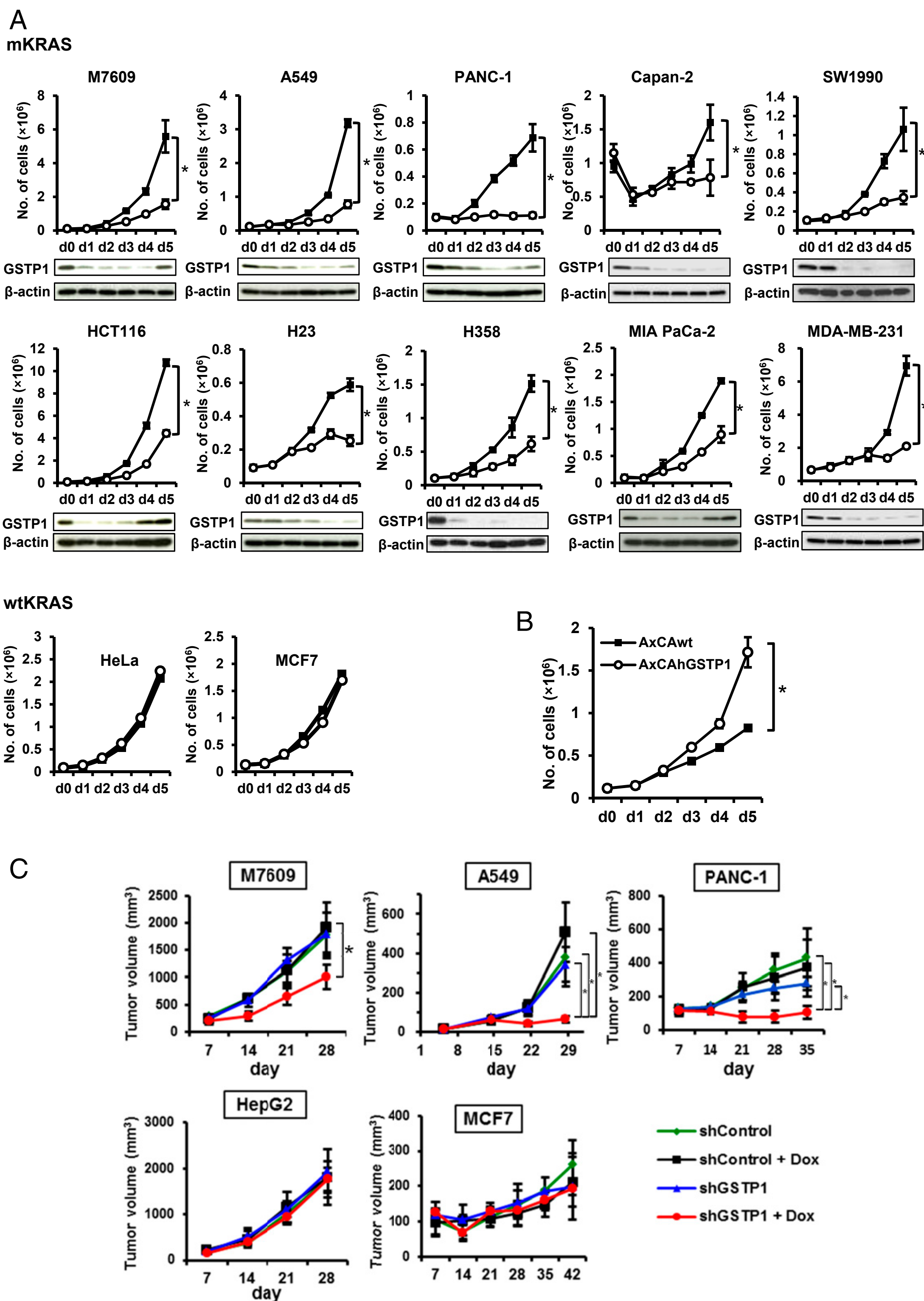

Fig. 1. Effects of GSTP1 silencing or forced expression on growth of m*KRAS* or WT*KRAS* cancer cells. (*A*) Growth curves of m*KRAS* cancer cells treated with siGSTP1. Cell number of m*KRAS* cancer cells was counted for 5 d after siGSTP1 transfection. Data represent the mean ± SD of three independent experiments. Open circles represent cells treated with siGSTP1 and closed squares, siRNA control (siControl). $^*P < 0.05$ by Student *t* test. (*B*) Enhanced proliferation of WT*KRAS* cancer cells by forced expression of GSTP1. HepG2 cells were infected with 10 multiplicity of infection (MOI) of AxCAhGSTP1 and AxCAwt, and cell numbers were counted for 5 d after infection. Data represent the mean ± SD of three independent experiments. $^*P < 0.05$ by Student *t* test. (*C*) Significant suppression of tumorigenesis of m*KRAS* cancer cells but not that of WT*KRAS* cancer cells by GSTP1 silencing. Cells expressing Dox-inducible shGSTP1 were inoculated subcutaneously into nonobese diabetic/SCID mice and subjected to Dox treatment (0.2 g/L). $^*P < 0.05$ by Student *t* test.

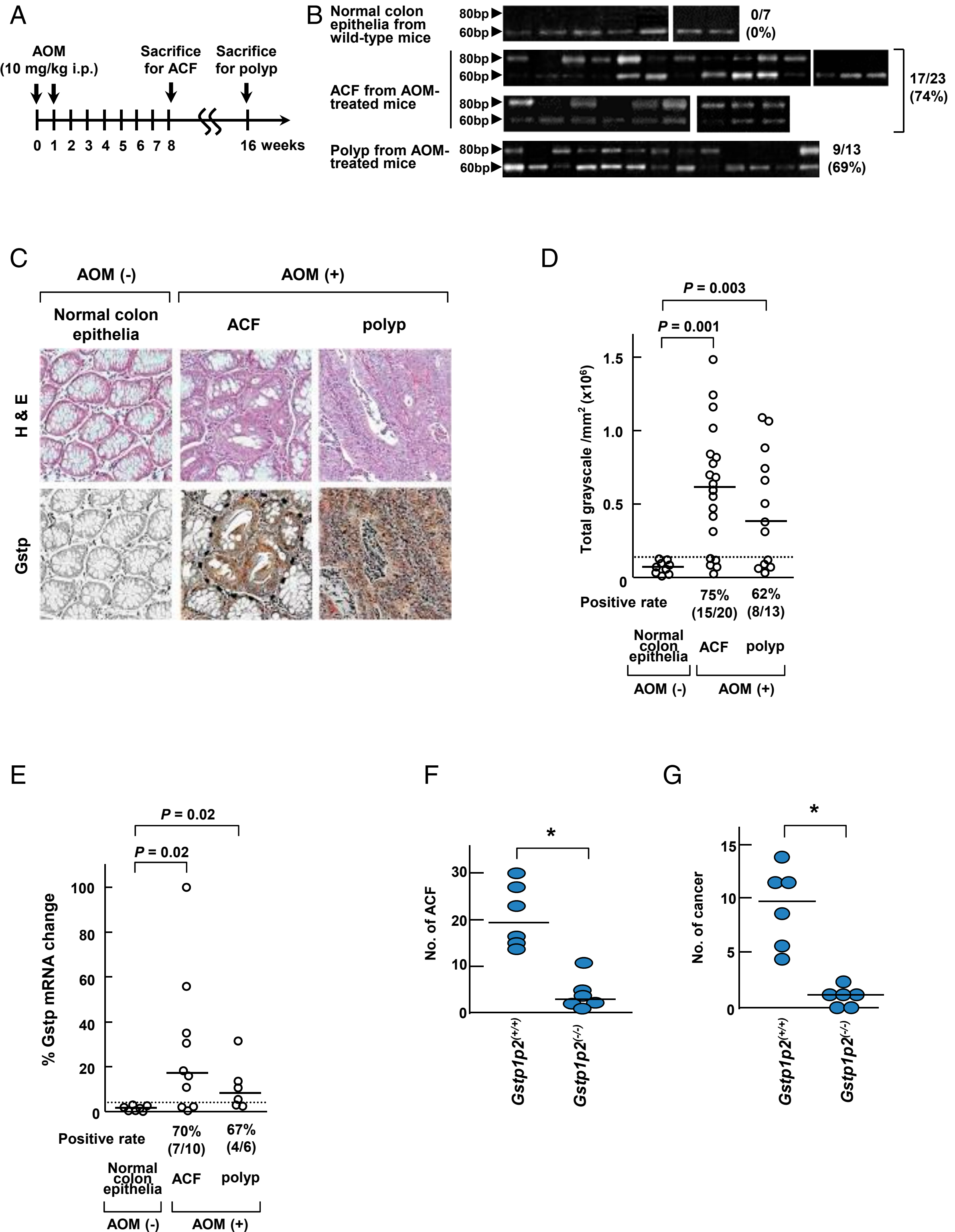

Fig. 2. Suppressive effects of Gstp1p2 gene deletion on the formation of ACF and colon cancers in AOM-treated mice. (*A*) Protocol for AOM treatment. Gstp1p2 null (Gstp1p2[$^{-/-}$]) and WT (Gstp1p2[$^{+/+}$]) mice were injected with AOM (10 mg/kg intraperitoneally), and the number of ACF and cancer nodules were counted at 8 and 16 wk, respectively. (*B*) Two-step PCR-restriction fragment length polymorphism (RFLP) for m*KRAS* in ACF and colon-cancer tissues of *Gstp* null mice treated with AOM and normal colon epithelia of littermate mice. Portions of ACF and cancer in tissues were microlaser dissected and subjected to PCR-RFLP. (*C* and *D*) Immunohistochemical staining for Gstp of ACF and colon-cancer tissues from AOM-treated mice and those of normal colon epithelia from littermate mice. Staining intensity of Gstp in tissues (*C*) was quantified by grayscale image analysis (*D*). The dotted line indicated the cutoff level for normal grayscale level as defined mean ± 2 SD (i.e., 0.13). (*E*) % Gstp mRNA change in ACF and colon-cancer tissues from AOM-treated mice and littermate mice. Portions of ACF and cancer in tissues were microlaser dissected and subjected to mRNA analysis. Gstp mRNA levels were assessed by the method described in *Materials and Methods*. The dotted line indicated the cutoff level for the normal Gstp mRNA level as defined mean ± 2 SD (i.e., 4.2). (*F* and *G*) Effects of Gstp1p2 knockout on the formation of ACF (*F*) and colon polyps (*G*) in AOM-treated mice. *$P < 0.01$; by Mann–Whitney *u* test.

c-jun N-Terminal Kinase Is Irrelevant for GSTP1 Effects on m*KRAS* Cell Growth. Since GSTP1 has long been proposed to affect cancer cell growth by interaction with c-jun N-terminal kinase (JNK) (20), effects of a JNK inhibitor SP600125 on growth of m*KRAS* cells which were treated with siGSTP1 or siControl were determined. With 5–10 μM of SP600125 which caused substantial inhibition of p-JNK (Thr183/Tyr185) (*SI Appendix*, Fig. S4*A*), cell numbers and cell death were not affected at all, whereas only when the cells were treated with both siGSTP1 and the inhibitor, marked growth suppression was observed (*SI Appendix*, Fig. S4 *B* and *C*). Furthermore, siRNA JNK did not affect the growth of m*KRAS* cells (*SI Appendix*, Fig. S4*D*), indicating that growth promotion of m*KRAS* cells by GSTP1 occurs independently from activation of JNK.

Mechanism for Growth Promotion of m*KRAS* Cells by GSTP1 in Relation to Their Signaling Pathway. The relevance of the growth promoting activity of GSTP1 to the signaling cascade of the m*KRAS* cells was considered. By treatment with siGSTP1, while most MAPK relevant components including KRAS-GTP, KRAS, phosphorylated BRAF, BRAF, ARAF, and COT1 were unchanged (*SI Appendix*, Fig. S5 *A*–*C*), but only the CRAF protein was appreciably reduced without reduction of mRNA (Fig. 3*A* and *SI Appendix*, Fig. S5*D*) but with concomitant suppression of phosphorylated CRAF, phosphorylated MEK, and phosphorylated ERK (Fig. 3*A*), suggesting that GSTP1 targets CRAF, leading to the activation of downstream signal components.

Consistent with these results, p-CRAF, CRAF, and p-ERK were reduced in shRNA transfected m*KRAS* cells upon Dox treatment as well as in fibroblasts from GSTP-gene-deleted Gstp1p2$^{-/-}$ mice (Fig. 3 *B* and *C*). These observations were further supported by results showing that transduction of GSTP1 to WT*KRAS* cells brought about enhanced expression of p-CRAF (Ser338), CRAF, p-MEK1/2 (Ser217/Ser221), and p-ERK1/2 (Thr202/Tyr204) (Fig. 3*D*).

Direct Binding of GSTP1 at the Regulatory Domain of CRAF, Promotes the Growth of m*KRAS* KRAS Cells. The possibility that GSTP1 interacts with CRAF and prevents its degradation was explored. As revealed by immunoprecipitation/immunoblotting methods, GSTP1 was coprecipitated with both p-CRAF (Ser338) and CRAF in all three cell lines examined whereas coprecipitation of GSTP1 with BRAF was not detected (Fig. 4*A*).

To directly substantiate the GSTP1/CRAF interaction, biolayer interferometry analysis (BLItz assays) using recombinant CRAF, BRAF, and GSTP1 was performed. Binding of GSTP1 to CRAF (Fig. 4 *B*, *Upper*) with relatively high affinity (Fig. 4*B* lower histogram) was noted, yet no binding to BRAF was detected. This assay also showed that S alkylation of Cys residues of CRAF did not affect the binding to GSTP1 (Fig. 4*B*), suggesting no direct involvement of cysteine residues or disulfide bridges in the interaction.

As a plausible explanation for the observation that, despite the conserved amino acid sequences of CRAF and BRAF, both known to play an essential role forming dimers in m*KRAS* signaling, GSTP1 binds only to CRAF. The presence of a flexible Gly-, Ala-Ser rich N-terminal extension of BRAF not in CRAF was presumed to sterically interfere with the binding of GSTP1 to BRAF, whereas CRAF, which lacks this extension can accommodate GSTP1. This postulate was confirmed by pull-down assays which demonstrated that an N-terminal deletion mutant of BRAF accommodated GSTP1 in a manner comparable to that of intact CRAF (Fig. 4*C*). The finding that the regulatory domain, CR1-deleted mutant of CRAF no longer bound to GSTP1, suggests that the binding region for GSTP1 is in the CR1 region (Fig. 4*C*).

Stabilization, Dimerization, and Activation of CRAF by GSTP1 in m*KRAS* Cells. To explore potential mechanisms by which binding of GSTP1 enhances CRAF levels, effects on CRAF turnover and its ubiquitination status in m*KRAS* cells were examined by immunoblotting. CRAF ubiquitination was, indeed, increased after siGSTP1 treatment of the cells (Fig. 5*A*). Moreover, treatment of the cells with a proteasome inhibitor MG132 brought about restoration of reduced p-CRAF (Ser338) and CRAF (Fig. 5*B*), indicating that, by binding of GSTP1, ubiquitination and proteasomal degradation of CRAF is impeded.

Since RAF dimers are considered to be the active forms to transduce the signal (21), efforts were then made to determine whether GSTP1 participates in dimer formation of CRAFs which were visualized by double transfection of FLAG-tagged and V5-tagged CRAF followed by immunoprecipitation with anti-FLAG antibodies and, subsequently, immunoblotting with anti-V5 antibodies. When these cells were treated with siGSTP1, the intensity of the V5-CRAF band was significantly reduced as compared to that of the FLAG-CRAF band (Fig. 5 *C*, *Upper* and lower histogram), indicating that the effect of siGSTP1 to reduce dimerized V5-CRAF to FLAG-CRAF was more marked than that to reduce FLAG-CRAF itself by destabilization. To corroborate the GSTP1-dependent dimerization, bimolecular luminescence complementation (BiLC) assays were additionally performed. The luminescence intensity of CRAF-FLAG-LgBit/SmBit-CRAF-FLAG was dramatically suppressed to below 20% of its value by siGSTP1 (*SI Appendix*, Fig. S6*A*) whereas expression of each protein revealed by Western blotting was reduced to only 60% after siGSTP1 treatment (*SI Appendix*, Fig. S6*B*). Furthermore, introduction of the GSTP1 plasmid into WT*KRAS* cells clearly augmented CRAF dimer formation as indicated by more increased V5-CRAF than FLAG-CRAF (Fig. 5*D*), confirming the role of GSTP1 in CRAF dimer formation.

Since CRAF/BRAF heterodimers have been shown to be active in m*KRAS* cells (22), the effect of siGSTP1 on this heterodimer formation was also analyzed. Silencing of GSTP1 in the m*KRAS* cells, which were doubly transfected with V5-CRAF and FLAG-BRAF resulted in significantly more reduction of FLAG-BRAF than V5-CRAF (Fig. 5*E*). These results suggest that binding of GSTP to CRAF is sufficient to induce its heterodimerization with BRAF which is without bound GSTP1.

To determine whether the catalytic activity of FLAG-CRAF immunity precipitated from WT*KRAS* cells, transfected with FLAG-CRAF, is enhanced by addition of GSTP1, in vitro kinase assays targeting MEK1 were performed. The results explicitly confirmed that phosphorylation of MEK1 by FLAG-CRAF was, indeed, enhanced in the presence of GSTP1 relative to that in its absence (Fig. 5*F*). When a similar experiment was performed with FLAG-CRAF and FLAG-BRAF immunities precipitated from the WT*KRAS* cells, augmentation of MEK1 phosphorylation by GSTP1 was also confirmed (Fig. 5*G*).

Furthermore, to elucidate the role of GSTP1 activity in stabilization against proteasomal degradation versus dimerization of CRAF, we examined the effect of forced expression of degradation-resistant mutant CRAF on growth suppression by siGSTP1 in m*KRAS* PANC-1 cells. GSTP1 played an almost equal role in protection against proteasomal degradation and in facilitation of dimerization (*SI Appendix*, Fig. S7).

Autocrine Loop of CRAF/MEK/ERK Generated by GSTP1 in Both m*KRAS* and m*BRAF* Cells. The findings described above prompted us to postulate that the function of GSTP1 in signaling pathways of m*KRAS* cells is the facilitation of the CRAF/MEK/ERK cascade in an autocrine loop, paralleling the stimuli of m*KRAS*. This postulate was verified by an experiment applying combinations of siRNAs KRAS (siKRAS) and siGSTP1. Four different siRNAs against KRAS were equally effective in silencing the KRAS

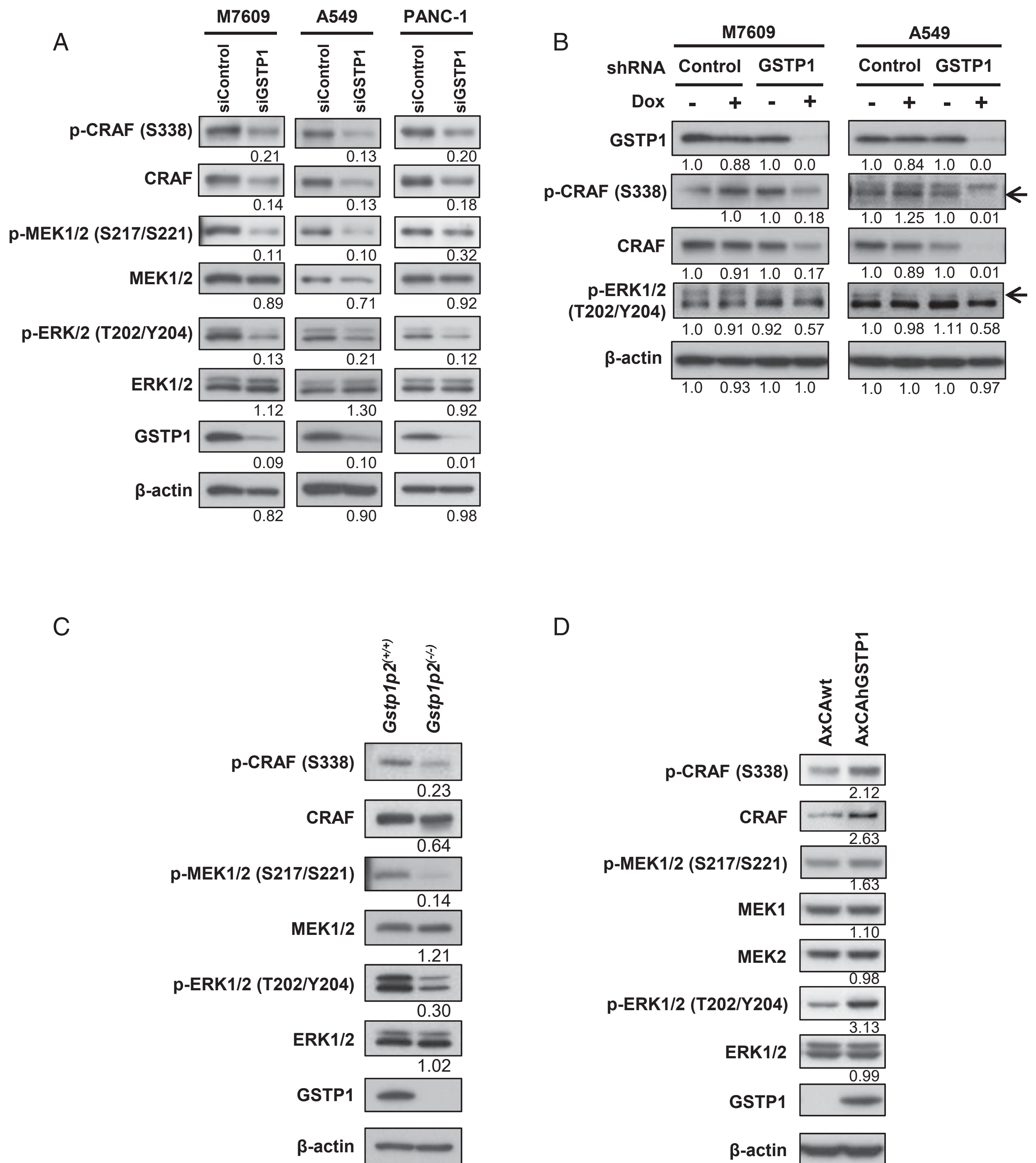

Fig. 3. Attenuation of CRAF/MEK/ERK signaling by GSTP1 silencing in m*KRAS* cancer cells and by forced expression of GSTP1 in wt*KRAS* cancer cells. (*A*) Decreased phosphorylation of CRAF/MEK/ERK and CRAF protein in siGSTP1 treated m*KRAS* in the indicated cancer cells. Cells were transfected with siGSTP1 then lysed at day 3. Lysates were analyzed by immunoblotting for each signal component using corresponding antibodies. (*B*) Suppressed p-CRAF, CRAF, and p-ERK in shGSTP1 transfected m*KRAS* cells treated with Dox. GFP expressing cloned M7609 and A549 cells (refer to *Materials and Methods* for details) were treated with Dox and lysed at day 2. The lysate underwent immunoblotting for GSTP1, p-CRAF, CRAF, p-ERK, and β-actin with each corresponding antibody. (*C*) Impaired CRAF/MEK/ERK signaling in skin fibroblasts from Gstp1p2 null mice. Lysates of skin fibroblasts from Gstp1p2 null mice were analyzed by immunoblotting for each signal component using corresponding antibodies. (*D*) Enhancement of CRAF/MEK/ERK signaling in wt*KRAS* cancer cells by forced expression of GSTP1. HeLa cells were infected with 10 MOI of AxCAhGSTP1, recombinant adenovirus expressing GSTP1 and AxCAwt, control adenovirus for 1 h, and lysed at day 2 of the infection. Lysates were analyzed by immunoblotting for each signal component using corresponding antibodies. Densitometry analysis indicates relative protein levels from one representative of three independent experiments.

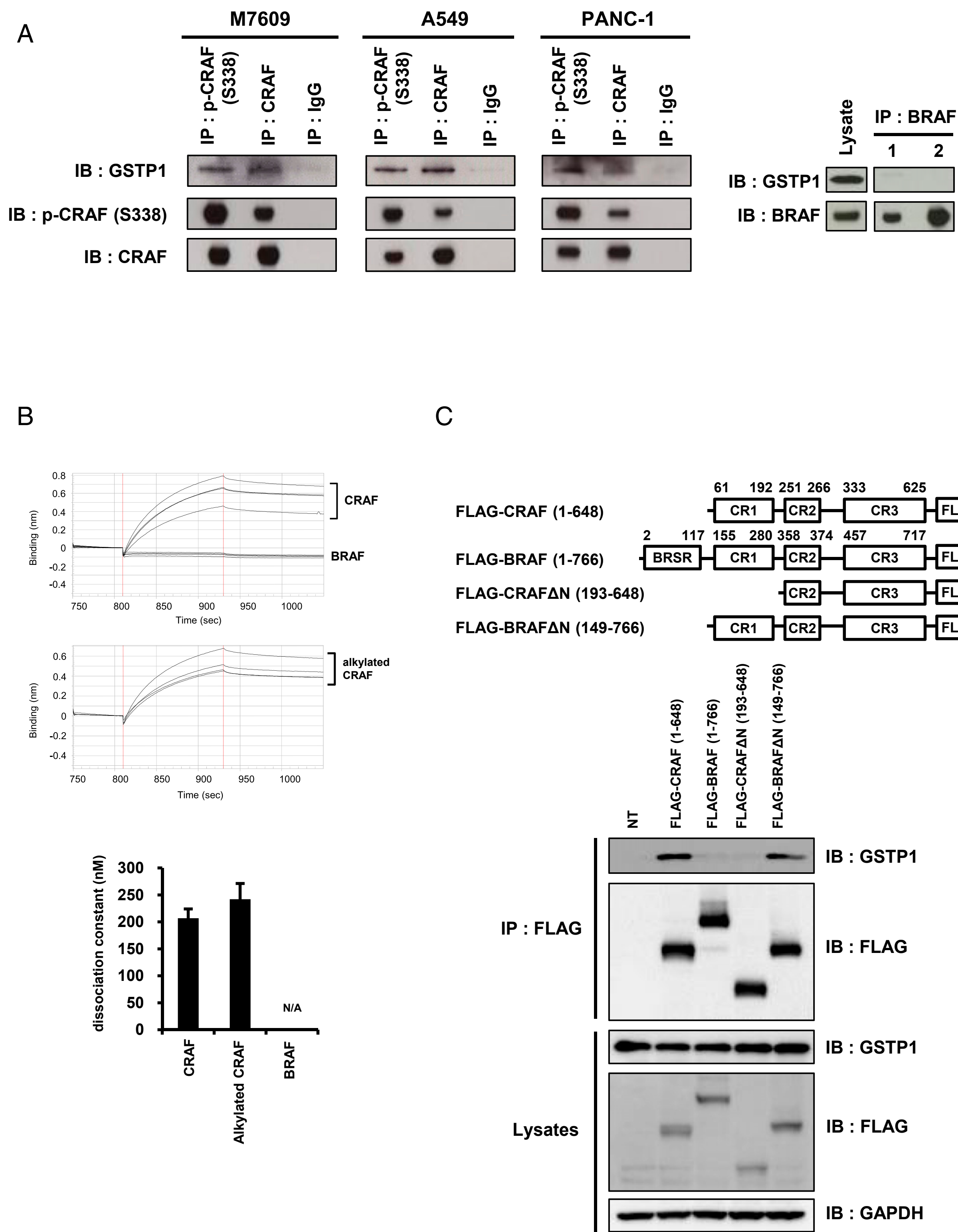

Fig. 4. Binding of GSTP1 to CRAF at its N-terminal domain in m*KRAS* cells. (*A*) Coimmunoprecipitation of GSTP1 with p-CRAF (Ser338) and CRAF but not with BRAF in KRAS mutant cancer cells. GSTP1 in lysates of m*KRAS* cells was coimmunoprecipitated with p-CRAF (Ser338) and CRAF antibodies and subjected to its immunoblotting. Normal immunoglobulin G (IgG) was used as a negative control for immunoprecipitation. Coimmunoprecipitation of GSTP1 with BRAF was carried out with two different anti-BRAF antibodies, lanes 1 and 2, in M7609 cells. (*B*) Kinetic analysis of the interaction between GSTP1 and CRAF and BRAF and alkylated CRAF by biolaser interferometry. Experiments for GSTP1 binding to each target protein were triplicated as shown in upper graphic figures. Dissociation constants for each binding based on the curves in *Upper* are shown in the lower histogram panel. (*C*) Coimmunoprecipitation of GSTP1 with FLAG-CRAF, FLAG-BRAF, FLAG-CRAF deleted of N-terminal CR1 motif (CRAFΔN), and FLAG-BRAF deleted of BRSR motif (BRAFΔN) from the lysate of m*KRAS* cells transfected with each corresponding plasmid. The schematic construct of each plasmid is shown in *Upper*. GSTP1 was pulled down with anti-FLAG antibodies from the lysate of m*KRAS* cancer cells which were transfected with each expression plasmid, cultured for 48 h, and subjected to Western blotting. *Lower* represents results of Western blotting. CR1, conserved regions 1; BRSR and BRAF specific N-terminal region.

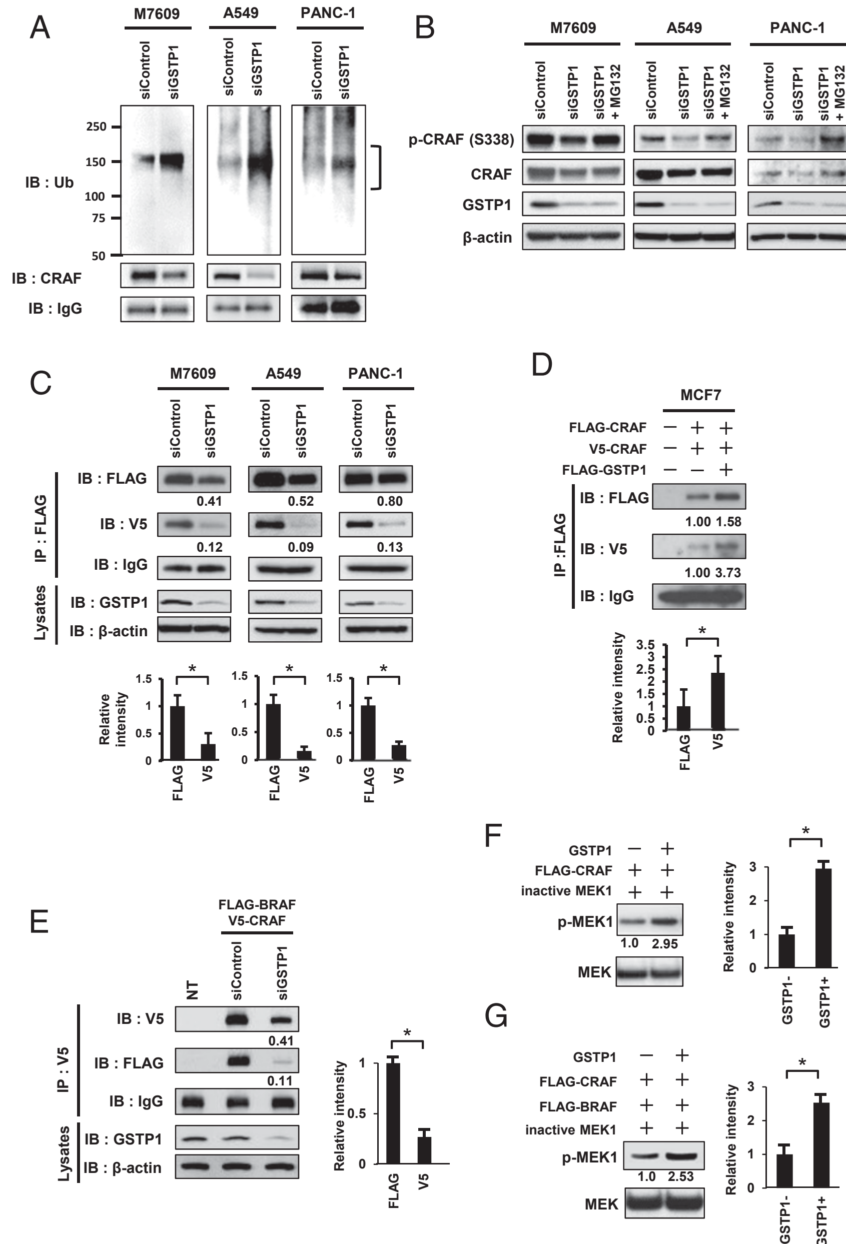

Fig. 5. Increased proteasomal degradation and decreased dimer formation of CRAF in siGSTP1 treated m*KRAS* cancer cells, and enhancement of kinase activity of CRAF by addition of GSTP1 in vitro. (*A*) Increased ubiquitination of CRAF by siGSTP1 silencing in m*KRAS* cancer cells. Ubiquitination of CRAF was evidenced by Western blotting using anti-ubiquitin antibodies. Equal loading of immunoprecipitates was confirmed by immunoblotting of the IgG heavy chain. (*B*) Recovery of CRAF by MG132 in siGSTP1 treated m*KRAS* cancer cells. After siGSTP1 transfection, cells were treated with 5 μM of MG132 for 4 h and lysed. Lysates were analyzed by immunoblotting for p-CRAF and CRAF. (*C*) Decreased CRAF homodimers in siGSTP1-treated m*KRAS* cancer cells. After transfection of siGSTP1 or siControl, cells were cotransfected with FLAG-CRAF and V5-CRAF, cultured for 48 h, and lysed. Lysates were immunoprecipitated with the FLAG antibody and subjected to immunoblotting with the V5 antibody. Difference in band intensity for V5-CRAF and FLAG-CRAF in siGSTP1-treated cells was calculated on the data of three independent experiments and was expressed in a histogram after normalizing their intensities to those in siControl treated cells. *$P < 0.01$ by repeated one-way ANOVA. (*D*) Increased CRAF homodimer in GSTP1 transduced WT*KRAS* cancer cells. After cotransfection with FLAG-tagged CRAF and V5-tagged CRAF, MCF7 cells were cultured for 24 h, transfected with FLAG-GSTP1 or control vector, and lysed. Lysates were immunoprecipitated with the FLAG antibody and subjected to immunoblotting using corresponding antibodies. Difference in band intensity for V5-CRAF and FLAG-CRAF was statistically analyzed as described in *C*. *$P < 0.01$ by repeated one-way ANOVA. (*E*) Decreased CRAF/BRAF heterodimer in siGSTP1 transfected m*KRAS* cancer cells. After siGSTP1 transfection, M7609 cells were cotransfected with V5-tagged CRAF and FLAG-tagged BRAF and lysed. Lysates were immunoprecipitated with the V5 antibody and subjected to immunoblotting with the V5-CRAF and FLAG antibodies. Statistical analysis for the difference in band intensity of V5-CRAF and FLAG-BRAF was carried out similarly as *C* and *D*. *$P < 0.01$ by repeated one-way ANOVA. (*F*) Activation of CRAF kinase activity by GSTP1. HeLa cells were transfected with FLAG-CRAF, and 48 h later, FLAG-CRAF proteins immunopurified were incubated with GSTP1 in kinase reaction mixture containing inactive MEK1, ATP, and Mg2+. Kinase activity of CRAF was analyzed by immunoblotting of p-MEK1 (Ser217). Relative intensities of GSTP1(+) bands to those of GSTP1(−) in three independent experiments were expressed in the right histogram. *$P < 0.01$ by repeated one-way ANOVA. (*G*) Activation of CRAF/BRAF kinase activity by GSTP1. FLAG-CRAF and FLAG-BRAF proteins immunopurified from HeLa cells were incubated with GSTP1 in kinase reaction mixture, and kinase activity was analyzed by immunoblotting of p-MEK1 (Ser217). Relative intensities of GSTP1(+) bands to those of GSTP1(−) in three independent experiments were expressed in the right histogram. *$P < 0.01$ by repeated one-way ANOVA.

gene in m*KRAS* cells, most likely ruling out off-target effects (*SI Appendix*, Fig. S8*A*). Dose-dependent suppression of cell growth was confirmed with siKRAS-A (*SI Appendix*, Fig. S8*B*). When siKRAS and siGSTP1 were cotransfected in these cells, additive growth suppression was observed as compared to that found with each individual siRNA (Fig. 6*A*). These data establish that, once GSTP1 is induced, growth signals may be enabled through this cascade independently of the upstream stimulus of m*KRAS* (scheme, Fig. 6*B*). Comparable functions of GSTP1 were displayed in m*BRAF* cells. In those cells, silencing of GSTP1, which apparently has no direct interaction with m*BRAF*, also caused significant growth suppression (*SI Appendix*, Fig. S8*C*) with impaired CRAF/MEK/ERK signaling (*SI Appendix*, Fig. S8*D*) by itself.

Moreover, in a manner similar to the case of m*KRAS* cells, in the m*BRAF* cells which showed dose-dependent sensitivity to its inhibitor PLX4720 (*SI Appendix*, Fig. S8*E*), additive growth suppression effects of siGSTP1 were observed when m*BRAF* cells were treated with siGSTP1 in combination with PLX4720 (Fig. 6*C*). The growth promoting effects of CRAF/GSTP1 interactions in mBRAF cells, although not as great as those in m*KRAS* cells, were substantiated by growth suppression observed using siCRAF (*SI Appendix*, Fig. S9 *A* and *B*). Thus, in m*BRAF* cells, the autocrine loop of GSTP1 also functions (scheme, Fig. 6*D*).

Discussion

The results reported here are consistent with the view that, in m*KRAS* cancers, there is a direct cause-and-effect relationship between m*KRAS* and GSTP1 expression and that the GSTP1-CRAF interaction perpetuates an autocrine cycle that circumvents the primary growth signal. The fundamental concept is outlined in the scheme (Fig. 6*B*).

Underlying the mechanism for the autocrine growth promotion, there are three indispensable molecular events disclosed in this study: enrichment of CRAF through inhibition of proteasomal degradation, CRAF stabilization effects of GSTP1 facilitation of the RAF dimer (CRAF-CRAF and CRAF-BRAF) formation, and activation of catalytic activity of CRAF. It has been proposed that the N-terminal receptor binding domain (RBD) lobe of CRAF folds over and obstructs the C-terminal catalytic lobe (21). We suggest that activation as well as enhanced dimerization of CRAF occur upon GSTP1 binding to the RBD reported here which prevents this folding and opens dimerization capacity with other RAF family members (23). It is reasonable to propose a similar mechanism as that for dimerization can take place for activation of CRAF by GSTP1 to transduce the signal to the downstream protein MEK. The suggestion that GSTP1 involves both dimer formation and activation of CRAF is consistent with previous reports, indicating that dimerization is a critical step for activation of RAF enzyme activity (21). In this regard, elucidation of the possibility that S-S glutathionylation of cRaf is relevant to the dimerization of activation mechanisms is a future task.

A unique and intriguing characteristic of the proposed autocrine cycle is that, once GSTP1 is expressed, it keeps functioning bypassing mutations of KRAS. This notion was substantiated by the findings that siGSTP1 exerted additive effects to the inhibition of m*KRAS*. Involvement of GSTP1 in the autocrine growth cycle of m*KRAS* cells was verified by effects of gene silencing on both in vitro cell proliferation and tumorigenesis of xenografted cells as well as effects of gene transfection on cell proliferation and of gene deletion on colon carcinogenesis.

With regard to the GSTP1 silencing effects on tumorigenesis, results seemingly consistent with our observations have previously been reported (16, 24, 25). However, those studies neither explored molecular mechanisms of GSTP1 functions in cancer growth nor considered GSTP1 involvement in autocrine signaling. Furthermore, the growth promoting activity of GSTP1 was simply ascribed to inhibition of JNK (16, 24, 25). Moreover, the present study established that inhibition of JNK is unlikely to be a mechanism of growth promotion by GSTP1.

With regard to the colon carcinogenesis in Gstp1p2 null mice, there is an apparent contradiction between our results and those of previous reports. Contrary to the impaired carcinogenesis in the present study, enhanced skin and lung cancers in Gstp1p2 null mice were reported (26, 27). However, in those previous studies, as the authors pointed out, the carcinogens were surmised to be detoxified by Gstp1p2 in WT mice and, thus, less active to induce cancers as compared to those in GSTp1p2 null mice, resulting in relatively increased carcinogenesis in the latter mice. In this regard, it may be reasonable to conclude that, unlike carcinogens in previous reports, our carcinogen AOM used in the present study is not detoxified by GSTp1p2 because treatment with AOM, indeed, induced ACF and colon cancers even in the presence of GSTp1p2 in WT mice, and AOM rather needed GSTp1p2 for its activity to induce m*KRAS* cancers since, in GSTp1p2 null mice, formation of these lesions was significantly impaired. Since, in m*BRAF* cancers, the MEK/ERK cascade which should induce expression of GSTP1 is the primary growth signal, and in the current investigation, GSTP1 was, indeed, expressed in all m*BRAF* cells examined, it is highly likely that the autocrine growth cycle is active in these cancers as well even though BRAF itself does not accommodate GSTP1 binding. Consistent with this assumption, siGSTP1 was effective to suppress the growth of m*BRAF* cells alone and showed additive growth suppression in combination with BRAF inhibitors. Thus, as shown in the scheme in Fig. 6*D*, the bypass of BRAF mutations by linkage of GSTP1 to the CRAF's proliferation promoting function is considered to be, indeed, active in m*BRAF* cells as well.

In a series of previous studies, the strategy of utilizing small molecule inhibitors of GSTP1 enzymatic activity to impede cancer cell growth has been widely sought (28–30). However, those approaches may not necessarily be effective to suppress the GSTP1-CRAF interaction cited here. Recently, Louie et al. showed that silencing and/or inhibition of GSTP1 selectively blocked growth of triple-negative breast cancers (31). This inhibitory effect was attributed to interference with proposed GSTP1 activation of GAPDH that enhances glycolysis in the cancer cells, but the role of GSTP1 in growth promotion via CRAF/MEK/ERK signaling was not considered. In addition, targeting GSTP1 using GSTP1 inhibitors or siGSTP1 described in this study and in some previous reports may engender undesirable adverse effects since GSTP1 is known to interact with several intracellular factors and various biological functions (32, 33). Therefore, strategies to specifically block CRAF/GSTP1 interactions may be more desirable. In this regard, results showing that GSTP1 also bound S-alkylated CRAF (Fig. 4*B*) indicate that the CRAF-GSTP1 binding is likely to be noncovalent and need not involve disulfide bridges or S glutathionylation (33, 34), suggesting potential strategies blocking the CRAF/GSTP1 interactions.

In conclusion, the essential growth signaling CRAF/MER/ERK cascade in cancers, such as those with m*KRAS* and m*BRAF* cells, can be controlled by specific interactions of CRAF with GSTP1 in the promotion of an autocrine growth cycle. Accordingly, prospects for innovative therapeutic approaches to have eventual translational relevance to these cancers may be developed based on the strategy to interfere with the CRAF-GSTP1 interaction.

Materials and Methods

Biolayer Interferometry. Dissociation constants (K_d) between GSTP1 and RAFs (CRAF and BRAF) were determined using the BLItz system (ForteBio) according to the manufacturer's instructions. Biosensor chips with carboxyl groups (amine reactive second-generation biosensor [ForteBio]) were hydrated overnight prior to the experiment in water. Placental GSTP1 (Sigma-Aldrich)

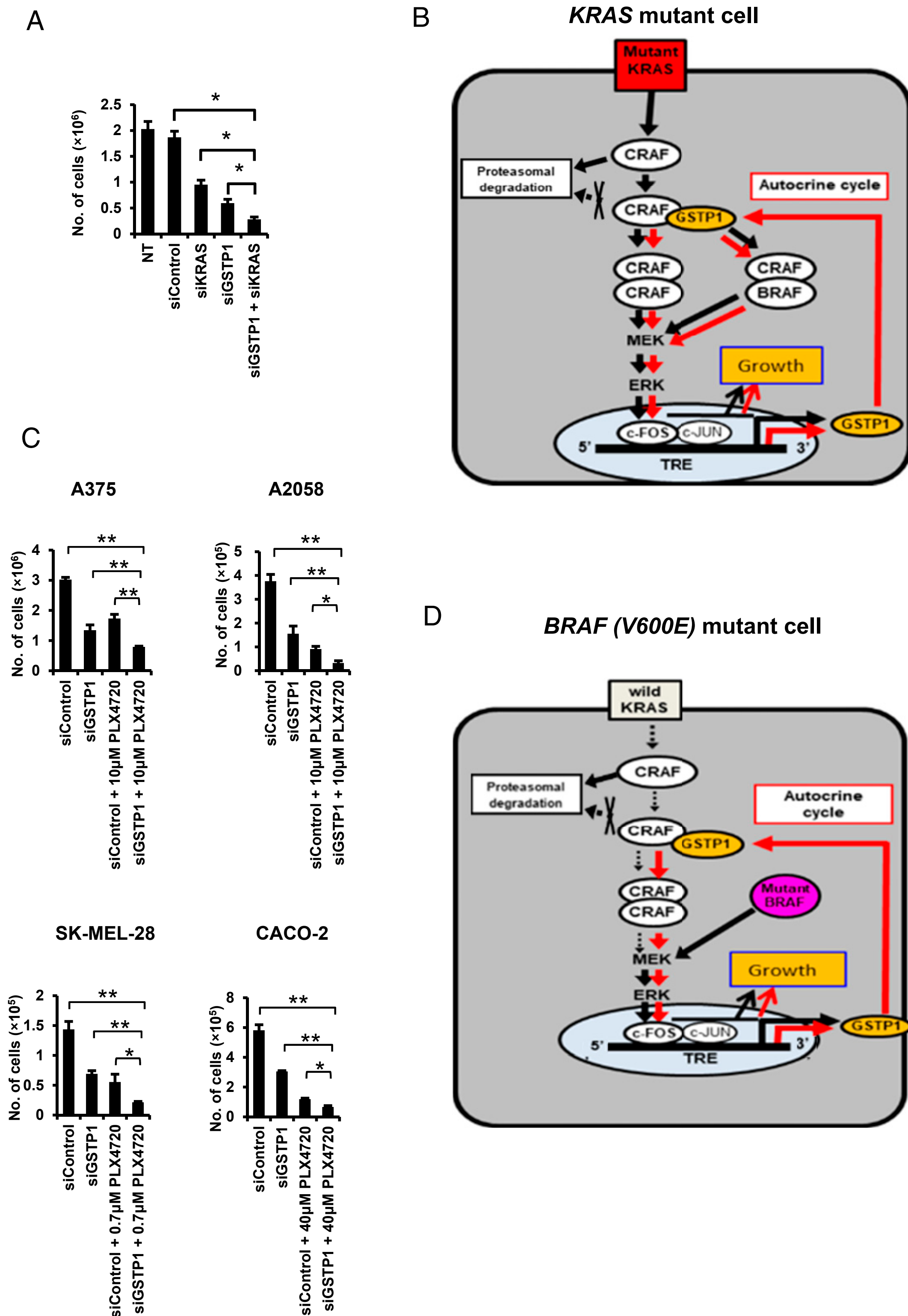

Fig. 6. Additive effects of siGSTP1 on the growth of siKRAS-treated m*KRAS* cancer cells and BRAF inhibitor-treated m*BRAF* cells. (*A*) Additive suppressive effects of siGSTP1 on the growth of siKRAS-treated m*KRAS* cancer cells. At day 2 of 50 nM siGSTP1 transfection, M7609 cells were treated with siKRAS at 10 nM, and cell numbers were counted at day 5. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.01$ by repeated one-way ANOVA. (*B*) Schematic of proposed autocrine activation of CRAF by GSTP1 in m*KRAS* cells. Note that, in m*KRAS* cells, the growth signal from m*KRAS* is augmented by GSTP1, end product of the signal, through interaction with CRAF. (*C*) Additive suppressive effects of siGSTP1 on the growth of inhibitor-treated m*BRAF* cancer cells. m*BRAF* cancer cells were transfected with 50 nM siGSTP1 or siControl, and at day 2 additionally treated with PLX4720 at each indicated concentration. At day 5, cell numbers were counted. Data represent the mean ± SD of three independent experiments. $^{*}< 0.05$; $^{**}P < 0.01$ by repeated one-way ANOVA. (*D*) Schematic of autocrine activation of CRAF by GSTP1 in m*BRAF* cells. Note that in m*BRAF* cells, the autocrine feedback loop is overriding the primary m*BRAF* signal.

was immobilized on the biosensor in acetate buffer (pH 3.0). After immobilization, the redundant carboxyl groups were blocked by ethanolamine (Wako chemicals). Recombinant CRAF (Origene) and BRAF (Origene) were used without dilution in the association step. The buffer (25 mM Tris·HCl, pH 7.3, 100 mM glycine, 10% glycerol) was used for equilibration and dissociation steps, which is same composition as supplied by recombinant CRAF and BRAF solutions. The time course of BLItz experiments for obtaining baseline, immobilization, blocking, equilibration, association, and dissociation were 300, 300, 120, 30, 120, and 120 s, respectively. All assays were performed in triplicate.

In Vitro Kinase Assays. HeLa cells were transfected pcDNA3.1-FLAG-CRAF and pCMV6-Myc-DDK-BRAF (Addgene) using FuGENE6 HD, treated with 50 ng/mL epidermal growth factor (BD Biosciences) for 10 min and lysed with 0.5% Nonidet P-40 lysis buffer on ice for 30 min after 48 h. Protein lysates were clarified by centrifugation at 13,000 × g for 10 min at 4 °C, incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 h at 4 °C, and washed with 0.5% Nonidet P-40 lysis buffer four times. To stabilize GSTP1 with GSH, 1 µg of human placental GSTP1 (Sigma-Aldrich) was preincubated with 2–5 mM glutathione in 0.1 M phosphate buffer, pH 6.5 for 20 min at 37 °C. After washing with assay dilution buffer I (Merck-Millipore), FLAG-CRAF protein and/or FLAG-BRAF was incubated with 1 µg of placental GSTP1 and 1 µg of inactive MEK1, magnesium/ATP mixture (Merck-Millipore) in assay dilution buffer I (Merck-Millipore) for 1 h at 30 °C. Kinase activity was analyzed by immunoblotting for p-MEK1 (Ser217).

Animals. 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.

More detailed information about the *Materials and Methods* of this study are provided in the *SI Appendix*.

Data Availability. All data relevant to this paper are available in the main text and *SI Appendix*.

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