

Induction of Heparanase-1 Expression by Mutant B-Raf Kinase: Role of GA Binding Protein in Heparanase-1 Promoter Activation¹

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Abstract

Heparanase-1 (HPR1), an endoglycosidase that specifically degrades heparan sulfate (HS) proteoglycans, is over-expressed in a variety of malignancies. Our present study sought to determine whether oncogene *BRAF* and *RAS* mutations lead to increased HPR1 expression. Reverse transcription–polymerase chain reaction analysis revealed that *HPR1* gene expression was increased in HEK293 cells transiently transfected with a mutant *BRAF* or *RAS* gene. Flow cytometric analysis revealed that B-Raf activation led to loss of the cell surface HS, which could be blocked by two HPR1 inhibitors: heparin and PI-88. Cotransfection of a *BRAF* or *RAS* mutant gene with HPR1 promoter–driven luciferase reporters increased luciferase reporter gene expression in HEK293 cells. Knockdown of *BRAF* expression in a BRAF-mutated KAT-10 tumor cell line led to the suppression of *HPR1* gene expression, subsequently leading to increased cell surface HS levels. Truncational and mutational analyses of the HPR1 promoter revealed that the Ets-relevant elements in the HPR1 promoter were critical for BRAF activation–induced HPR1 expression. Luciferase reporter gene expression driven by a four-copy GA binding protein (GABP) binding site was significantly lower in BRAF siRNA–transfected KAT-10 cells than in the control siRNA–transfected cells. We further showed that BRAF knockdown led to suppression of the expression of the GABPβ, an Ets family transcription factor involved in regulating HPR1 promoter activity. Taken together, our study suggests that B-Raf kinase activation plays an important role in regulating HPR1 expression. Increased HPR1 expression may contribute to the aggressive behavior of BRAF-mutated cancer.

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Introduction

Heparanase-1 (HPR1) is an endoglycosidase that specifically degrades heparan sulfate (HS) proteoglycans (HSPGs) [1–4]. HSPGs are heavily present on the cell surface and in the extracellular matrix (ECM) and the basement membrane (BM). HPR1 is overexpressed in a variety of malignancies [1–4]. Breakdown of HSPGs in the BM and ECM leads to the release of many growth factors such as fibroblast growth factor and vascular endothelial growth factor that are trapped in the tumor stroma. These growth factors can promote tumor angiogenesis by stimulating endothelial cell proliferation and migration. In addition, breakdown of the BM and ECM allows tumor cells to invade locally or metastasize to a distant site. Recent studies have shown that HPR1 exerts its many biologic functions independent of its enzymatic activity. For example, HPR1 can enhance cell adhesion [5,6], induce vascular endothelial growth factor expression [7], induce tumor and endothelial cell migra-

tion, and induce Akt, p38, and Src phosphorylation [7,8]. HPR1 can induce epidermal growth factor (EGF) receptor phosphorylation and stimulate tumor cell proliferation and growth in an enzymatic activity–independent manner [9]. A conservative, hydrophobic C-terminus domain of HPR1 has been recently identified to mediate these diverse biologic functions [10,11]. HPR1 C-terminus functions as a ligand to

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bind two potential unknown receptors (a 130- and a 170-kDa protein) to activate the phosphatidylinositol 3-kinase pathway [11]. HPR1 may exert its tumor-promoting effect independent of its enzymatic activity.

Molecular mechanisms of HPR1 overexpression in a variety of cancers remain poorly understood. We and others have previously characterized the HPR1 promoter [12,13]. Sequence analysis revealed that the TATA-less, GC-rich promoter of the *HPR1* gene belongs to the family of housekeeping genes. Three Sp1 sites and four Ets relevant elements (ERE) for two GA binding protein (GABP) binding sites (Figure 7) are located in a 0.3-kb proximal promoter region [13]. GABP and Sp1/Sp3 are two transcription factors that regulate HPR1 basal promoter activity [13]. Later studies demonstrated that Egr-1 is involved in HPR1 gene expression in T cells stimulated by PMA plus ionomycin and in tumor cells [14–16]. Recent studies suggest that increased HPR1 expression in bladder and prostate cancers is largely mediated by HPR1 promoter hypomethylation and Egr-1 overexpression and hyperactivation [16,17] (Figure 7). Because tumor suppressor p53 can negatively regulate *HPR1* gene expression [18], p53 gene mutation may also contribute to increased HPR1 expression in a variety of tumors. It is not clear whether oncogene mutation and activation can lead to increased HPR1 expression.

BRAF is an oncogene that is frequently mutated in a variety of malignancies, with the highest frequencies in melanomas and thyroid cancers [19]. We have previously characterized *HPR1* expression and *BRAF* gene mutation in thyroid cancer [20,21]. Interestingly, we found that HPR1 is expressed at relatively low levels in WRO82 and KAT-18 cells, two thyroid tumor cell lines with wild-type BRAF, compared with that in several *BRAF* mutant tumor cell lines. We hypothesize that *BRAF* and *RAS* mutations may be responsible for increased *HPR1* expression in cancer. Here we report that *HPR1* gene expression was induced in the cells transfected with mutant *BRAF* and *RAS* genes and that knockdown of *BRAF* led to decreased *HPR1* expression. GABP, an Ets family transcription factor, was responsible for *BRAF* mutation-induced *HPR1* expression.

Materials and Methods

Plasmids and Cell Lines

pEF6 empty vector, and the vector encoding a wild-type BRAF (pEF6/BRAF), mutant V600E BRAF (pEF6/BRAF^{V600E}), or mutant G12V HRAS (pEF6/HRAS^{G12V}), have been previously described [22]. Luciferase reporter constructs driven by various truncated or mutated HPR1 promoters (Figure 5A) and by a four-copy of the GABP binding site derived from human *FAS* gene have been previously described [13,23]. KAT-10 cells transfected with a small interference (si)RNA vector IMG800 or the vector encoding a *BRAF* siRNA have been previously reported [24]. KAT-10 cells were previously considered a tumor cell line of thyroid origin but were recently verified as identical to HT29, a human colon cancer cell line [25]. KAT-10 cells were grown in complete RPMI 1640 medium containing 10% fetal bovine serum and 500 µg/ml G418. HEK293, a human kidney embryonic epithelial cell line, was purchased from the American Tissue Culture Collection (Manassas, VA). HEK293 cells were grown in minimum essential medium containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and HEPES buffer.

Luciferase Reporter Gene Expression

HEK293 cells seeded in a 24-well plate were transfected with pGL3/basic, a 0.7-kb or 3.5-kb HPR1 promoter-driven luciferase reporter gene

plus pEF6 empty vector, pEF6/BRAF, pEF6/BRAF^{V600E}, and pEF6/HRAS^{G12V} by using FuGENE6 (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions (Figure 1B). An internal control plasmid, pCMV/SPORT, which encodes a β-galactosidase gene, was included in the transfection mixture. Control or BRAF siRNA-transfected KAT-10 cells were similarly transfected with the luciferase reporter gene driven by a 0.3-, 0.7-, or 3.5-kb HPR1 promoter (pGL3/HPR-0.3, pGL3/HPR1-0.7, or pGL3/HPR-3.5; Figure 4), by a 0.3-kb HPR1 promoter with various mutated ERE sites (Figure 5, A and B), or by a four-copy GABP binding site (Figure 5C). Luciferase reporter gene without a HPR1 promoter (pGL3/Basic) was included as a negative control. After incubating for 48 hours, cells were harvested and analyzed for luciferase activity by using the luciferin substrate and reading in a TECAN plate reader (Phenix Research Products, Hayward, CA). The relative light unit in each sample was normalized against the β-galactosidase activity measured by a colorimetric assay as previously reported [26]. The means ± SDs of the data in triplicate from one experiment are presented. The experiment was repeated at least once with similar results.

Flow Cytometric Analysis

HEK293 cells were seeded in six-well plates. On 40% confluence, the cells were transfected with pEF6 empty vector, pEF6/BRAF, pEF6/BRAF^{V600E}, and pEF6/HRAS^{G12V}. After incubating for 24 hours, the cells were left untreated or treated with two HPR1 inhibitors, heparin or PI-88 (50 µg/ml). After incubating for another 24 hours, the cells were harvested and used for the preparation of single cell suspensions. Single cell suspensions of HEK293 and KAT-10 cells were incubated with an anti-HS mAb (0.5 µg/sample; clone HepSS; Seikagaku Corp, Chuo-ku, Tokyo, Japan) or mouse immunoglobulin M (IgM) as an isotype control for 30 minutes at 4°C, followed by incubation with fluorescein isothiocyanate-labeled goat antimouse IgM (5 µl/sample) for 30 minutes at 4°C. To increase the fluorescence intensity, the cells were further stained with a fluorescein isothiocyanate-labeled rabbit anti-goat IgG (5 µl/sample) for 30 minutes at 4°C. Cell surface HS levels were analyzed by fluorescence-activated cell sorter (FACS) in a Becton Dickinson flow cytometer.

HPR1 Activity Assay

HPR1 activity in cell lysates was measured by using a novel ELISA as previously described [27–30]. HPR1 activity in cell lysates was calculated based on a standard curve of serially diluted purified platelet HPR1 (starting at 1:200) [31] at a concentration of 1 µl of HPR1 with the activity of degrading 0.133 µg of heparan sulfate per hour at 37°C in HPR1 buffer. HPR1 activity was designated as per 100 U capable of degrading 1 ng of heparan sulfate at 37°C per hour in HPR1 buffer.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from control or BRAF siRNA-transfected KAT-10 cells and HEK293 cells transfected with the pEF6 empty vector, pEF6/BRAF, pEF6/BRAF^{V600E}, and pEF6/HRAS^{G12V} using TRIzol (Invitrogen, Camarillo, CA) and quantified in a spectrophotometer by ultraviolet absorption. Complementary DNA was synthesized by using AMV reverse transcriptase with 500 ng of total RNA and oligo(dT) priming. Polymerase chain reaction (PCR) was conducted by using Taq DNA polymerase (Invitrogen) with a pair of primers listed in Table 1. The PCR conditions for amplifying *HPR1* and *GAPDH* are an initial denaturation of 2 minutes at 94°C, followed by 32 cycles of denaturation for 45 seconds at 94°C, annealing for 1 minute at 55°C, and extension for

Table 1. Primers Used to Amplify HPR1, BRAF, GABP α , GABP β , GAPDH, and 18S rRNA.

Gene	Size (bp)	Forward	Reverse
HPR1	587	5'-TTCGATCCCAAGAAGG-AATCAAC-3'	5'GTAGTGTATGCCATGTAACCTGAAT-C-3'
GAPDH	527	5'TGAAGGTCGGAGTCAACGGATTGGTC-3'	5'ATGGACTGTGGTCATGAGTCCCTCCACG-3'
HPR1	86	5'-GCTAGAGCTCTCGACTCTC-3'	5'-GCAGGCTTCGAGCGCAGC-3'
BRAF	199	5'-TTGAACACCACCAATACCA-3'	5'-GTCTCGTTGCCAAATTGAT-3'
GABP α	116	5'-ACGATGGGGACATGATTGT-3'	5'-TTCTGTGACCAACGGTTCA-3'
GABP β	121	5'-GTGTGAGCAGAGATGCCAGA-3'	5'-TCCTTTGCATTGACATCAGC-3'
18S rRNA	101	5'-ATGCTCTTAGCTGAGTGCCCG-3'	5'-ATTCTAGCTGCGGTATCCAGG-3'

1 minute at 72°C. PCR products were separated on a 1.5% agarose gel by electrophoresis and visualized by ethidium bromide staining. HPR1, BRAF, and GABP α and β expressions in KAT-10 cells were also quantified by real-time reverse transcription (RT)-PCR with primers listed in Table 1. Complementary DNA was synthesized as above used for amplifying these genes in triplicate after a 1:10 dilution. PCR in a 25- μ l volume was conducted by using cyber green mixture and ran initially at 50°C for 2 minutes and at 95°C for 10 minutes followed by 40 cycles of (95°C for 15 seconds and 60°C for 1 minute). PCR was analyzed on Applied Biosystems 7300 (Foster City, CA). The relative C_t value was calculated according to the standard formula $2^{\Delta\Delta C_t}$ ($\Delta/\Delta C_t = \Delta C_t$ value of sample - ΔC_t value of control). ΔC_t value of each sample is the C_t value for BRAF siRNA-transfected cells normalized to 18S ribosomal RNA (rRNA), whereas ΔC_t value of the control is the C_t value for control siRNA-transfected cells normalized to 18S rRNA. The relative expression of these genes from one representative of two or more independent experiments with similar results is presented.

Western Blot

KAT-10 cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 5 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated on ice for 30 minutes and then spun down at 15,000 rpm for 15 minutes at 4°C. After electrophoresis and transfer to nitrocellulose membranes, B-Raf, Sp1, Sp3, Egr-1, phospho-ERK, ERK, and GABP α and β were detected by their specific antibodies followed by horseradish peroxidase-conjugated goat antimouse or rabbit IgG and SuperSignal Western Pico enhanced chemiluminescence substrate (Pierce Chemical Co, Rockford, IL). These antibodies include rabbit anti-B-Raf, Sp1, Sp3, Egr-1 IgG (Santa Cruz Biotechnology, Inc, San Diego, CA), rabbit anti-phospho ERK and ERK IgG (Cell Signaling Technology, Danvers, MA), and rabbit anti-GABP α and β antisera (kindly provided by Dr. U. A. Rapp, University of Würzburg, Würzburg, Germany).

Immunofluorescence Staining of GABP α and β

KAT-10 cells grown on coverslips were washed three times with cold PBS and fixed with methanol at -20°C for 5 minutes. Coverslips were blocked with 5% normal goat serum for 30 minutes at room temperature. GABP α and β were detected by rabbit anti-GABP α or β antisera, followed by fluorescein-conjugated goat antirabbit IgG. The coverslips were mounted with 50% glycerin in PBS containing antifade reagent 1,4-diazabicyclo[2.2.2]octane (25 mg/ml) and 4,6-diamidino-2-phenylindole (0.5 mg/ml; Sigma Chemical Co, St Louis, MO). GABP α or β expression was examined under a fluorescence microscope. The pictures were taken with a digital camera attached in an Olympus BX41TF fluorescence microscope (Leeds Precision Instruments, Inc, Minneapolis, MN).

Statistical Analysis

The differences in the expression of HPR1, BRAF, GABP α or β , and HPR1 activity were statistically analyzed by using an unpaired Student t test. $P < .05$ was considered statistically significant.

Results

Induction of HPR1 Expression by Activation of B-Raf Kinase

We first tested if RAS and BRAF gene mutations led to increased HPR1 gene expression. HPR1 messenger RNA (mRNA) levels in HEK293 cells transiently transfected with the pEF6 empty vector or the vector encoding wild-type BRAF, BRAF^{V600E}, or RAS^{G12V} were analyzed by semiquantitative RT-PCR. As shown in Figure 1A, HPR1 mRNA was undetectable in HEK293 cells transfected with the pEF6 empty vector or the vector encoding wild-type BRAF but was induced in the cells transfected with a constitutively active BRAF^{V600E} mutant or HRAS^{G12V} genes. A 0.7-kb HPR1 promoter-driven luciferase reporter gene expression was dramatically increased in HEK293 cells cotransfected with pEF6/BRAF^{V600E} or pEF6/HRAS^{G12V} but not in the cells cotransfected with pEF6 or pEF6/BRAF (Figure 1B). Expression of a 3.5-kb HPR1 promoter-driven luciferase reporter gene was also slightly increased in HEK293 cells cotransfected with the pEF6 encoding wild-type BRAF but was dramatically increased in the cells cotransfected by pEF6/BRAF^{V600E} and pEF6/HRAS^{G12V}. Induction of HPR1 promoter-driven luciferase expression by HRAS^{V12} was less potent than BRAF^{V600E} mutant, although the former is much more potent in activating the MAP kinase pathway [22].

B-Raf Kinase Activation Leads to Accelerated Cell Surface HS Degradation

We next tested whether increased HPR1 expression by mutant BRAF or HRAS leads to the degradation of HSPG. HEK293 cells were transiently transfected with pEF6, pEF6/BRAF, pEF6/BRAF^{V600E}, or pEF6/HRAS^{V12}. As shown in Figure 2A, the cell surface HS expression was abrogated in the cells transfected with mutant BRAF or RAS gene but not in those transfected with the pEF6 empty vector or pEF6/BRAF (Figure 2A). Two HPR1 inhibitors, PI-88 or heparin (50 μ g/ml each), were able to restore the cell surface HS levels of HEK293 cells transfected with pEF6/BRAF^{V600E} or pEF6/HRAS^{G12V} (Figure 2B). Similar results were obtained in 10/56A cells, immortalized human glomerular epithelial cells (data not shown). These observations suggest that down-regulation of HS expression in transfected cells is due to increased degradation of HS by HPR1.

Inhibition of HPR1 Gene Expression in a BRAF-Suppressed Tumor Cell Line

To verify that B-Raf kinase activation plays a critical role in inducing HPR1 gene expression, we tested if BRAF knockdown leads to decreased

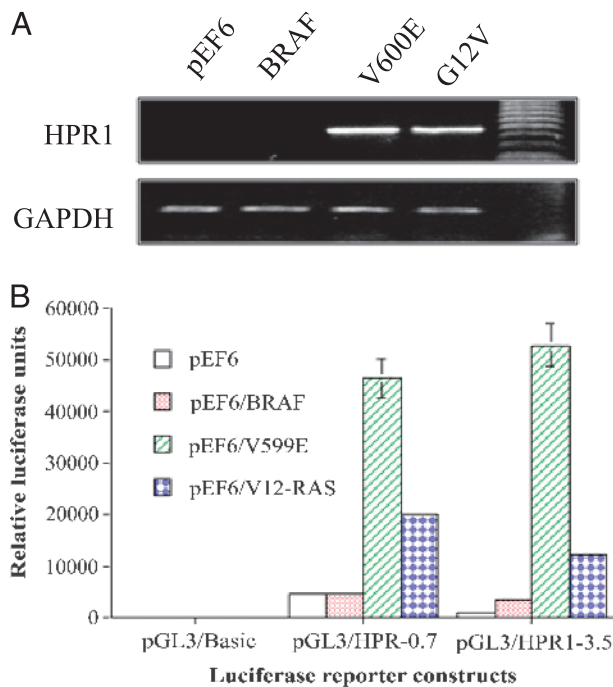


Figure 1. B-Raf kinase activation induces HPR1 expression. (A) Induction of *HPR1* gene expression. HEK293 cells were transiently transfected with the pEF6 empty vector or the vector encoding wild-type BRAF, BRAF^{V600E}, or HRAS^{G12V}. Total RNA was extracted and analyzed for *HPR1* mRNA expression by RT-PCR. (B) Induction of HPR1 promoter-driven luciferase reporter gene expression. HEK293 cells were cotransfected with the empty vector or the vector encoding wild-type BRAF, BRAF^{V600E}, or HRAS^{G12V} plus control luciferase reporter or the luciferase reporter gene driven by a 0.7- or 3.5-kb HPR1 promoter. pCMV/SPORT, which encodes a β -galactosidase gene, was included as an internal control. After incubating for 48 hours, the cells were harvested and analyzed for luciferase activity. The relative luciferase activity was calculated. One representative example in triplicate from three experiments with similar results is presented.

HPR1 gene expression. Knockdown of BRAF expression in a BRAF-mutated KAT-10 cell line by stable transfection of *BRAF* siRNA has been previously reported [24]. RT-PCR analysis revealed that BRAF knockdown reduced *BRAF* and *HPR1* mRNA levels by 60% and 38%, respectively (Figure 3A). *HPR1* enzymatic activity in the cell lysates of BRAF siRNA-transfected KAT-10 cells was reduced by 30% compared with that in control siRNA-transfected cells (Figure 3B). However, the cell surface HS levels were dramatically increased in BRAF siRNA-transfected KAT-10 cells compared with that in control siRNA-transfected KAT-10 cells (Figure 3C). These observations strongly suggest that B-Raf kinase activation leads to increased *HPR1* expression.

HPR1 Promoter Activity Is Decreased in BRAF-Suppressed KAT-10 Cells

Our cotransfection experiment in Figure 1 suggests that mutant *BRAF* or *HRAS* leads to increased *HPR1* promoter activation. To corroborate this observation, we tested whether *HPR1* promoter activity was decreased in BRAF-suppressed KAT-10 cells. The luciferase reporter gene driven by a 0.3-, 0.7-, or 3.5-kb *HPR1* promoter was introduced into control or BRAF siRNA-transfected KAT-10 cells. As shown in Figure 4, luciferase reporter gene expression driven by 0.3-, 0.7-, and 3.5-kb *HPR1* promoter were at least two-fold higher in control than

BRAF siRNA-transfected KAT-10 cells. These observations suggest that down-regulation of *HPR1* gene expression in BRAF-suppressed KAT-10 cells is mediated by suppression of *HPR1* promoter activity.

Identification of a GABP-Binding Site as a Critical cis-Responsive Element Responsible for BRAF-Induced *HPR1* Gene Expression

We and others have previously shown that several transcription factors, mainly Sp1, the Ets family transcription factors, and Egr-1 (Figure 7) play an important role in regulating basal and inducible *HPR1* promoter activity [12–16]. GABP, a member of the Ets family transcription factors, can be activated by B-Raf kinase. We tested whether elimination of the Ets transcription factor binding site in the *HPR1* promoter could abrogate BRAF mutation-induced *HPR1* promoter activity. A panel of *HPR1* promoter constructs with the ERE sites being mutated (Figure 5A) was introduced into control or BRAF siRNA-transfected KAT-10 cells. Consistent with our previous observations [13], mutation of any one of the four ERE sites significantly reduced *HPR1* promoter activity (Figure 5B). Luciferase gene expression driven by a 0.3-kb *HPR1* promoter in control siRNA-transfected KAT-10 cells was about two-fold higher than that in BRAF siRNA-transfected KAT-10 cells. Elimination of any of the four ERE sites in this 0.3-kb *HPR1* promoter led to a comparable *HPR1* promoter activity in control or in BRAF siRNA-transfected KAT-10 cells. These observations suggest that the presence of all ERE sites in the *HPR1* promoter is required for mediating BRAF mutation-induced *HPR1* promoter activation.

To verify that BRAF mutation-induced *HPR1* gene expression is indeed mediated by the GABP binding sites present in the *HPR1* promoter, we tested whether luciferase reporter gene driven by a four-copy GABP binding site is higher in control than in BRAF siRNA-transfected KAT-10 cells. As shown in Figure 5C, there was no difference in the luciferase activity in control and BRAF siRNA-transfected KAT-10 cells transfected with a pGL3/Basic reporter construct. However, luciferase gene expression driven by the 4 \times GABP binding site was almost two-fold higher in control than in BRAF siRNA-transfected KAT-10 cells. This observation strongly suggests that the transcriptional activity of the Ets family transcription factors is reduced in BRAF-suppressed KAT-10 cells.

Down-regulation of GABP β Expression in BRAF-Suppressed KAT-10 Cells

Several Ets family transcription factors are upregulated in various malignancies [32,33], probably because of oncogene mutation and activation. Here we tested whether decreased *HPR1* promoter activity in BRAF-suppressed KAT-10 cells was due to decreased GABP expression or activation. We first conducted immunofluorescence (IF) staining to determine whether there was a difference in GABP nucleus translocation between control and BRAF-suppressed KAT-10 cells. As shown in Figure 6A, both GABP α and β were extensively stained in the cytoplasm and nuclei of control siRNA-transfected KAT-10 cells. The signals for GABP β but not GABP α were much weaker in both the cytoplasm and nucleus in BRAF siRNA-transfected cells than in control siRNA-transfected cells. To verify that GABP β expression was downregulated in BRAF-suppressed KAT-10 cells, Western blot analysis was conducted to compare GABP α and β expression. GABP β was expressed at a significantly lower level in BRAF siRNA-transfected than in control siRNA-transfected KAT-10 cells (Figure 6B). ERK phosphorylation and BRAF expression were dramatically reduced in BRAF siRNA-transfected KAT-10 cells compared with those in control siRNA-transfected

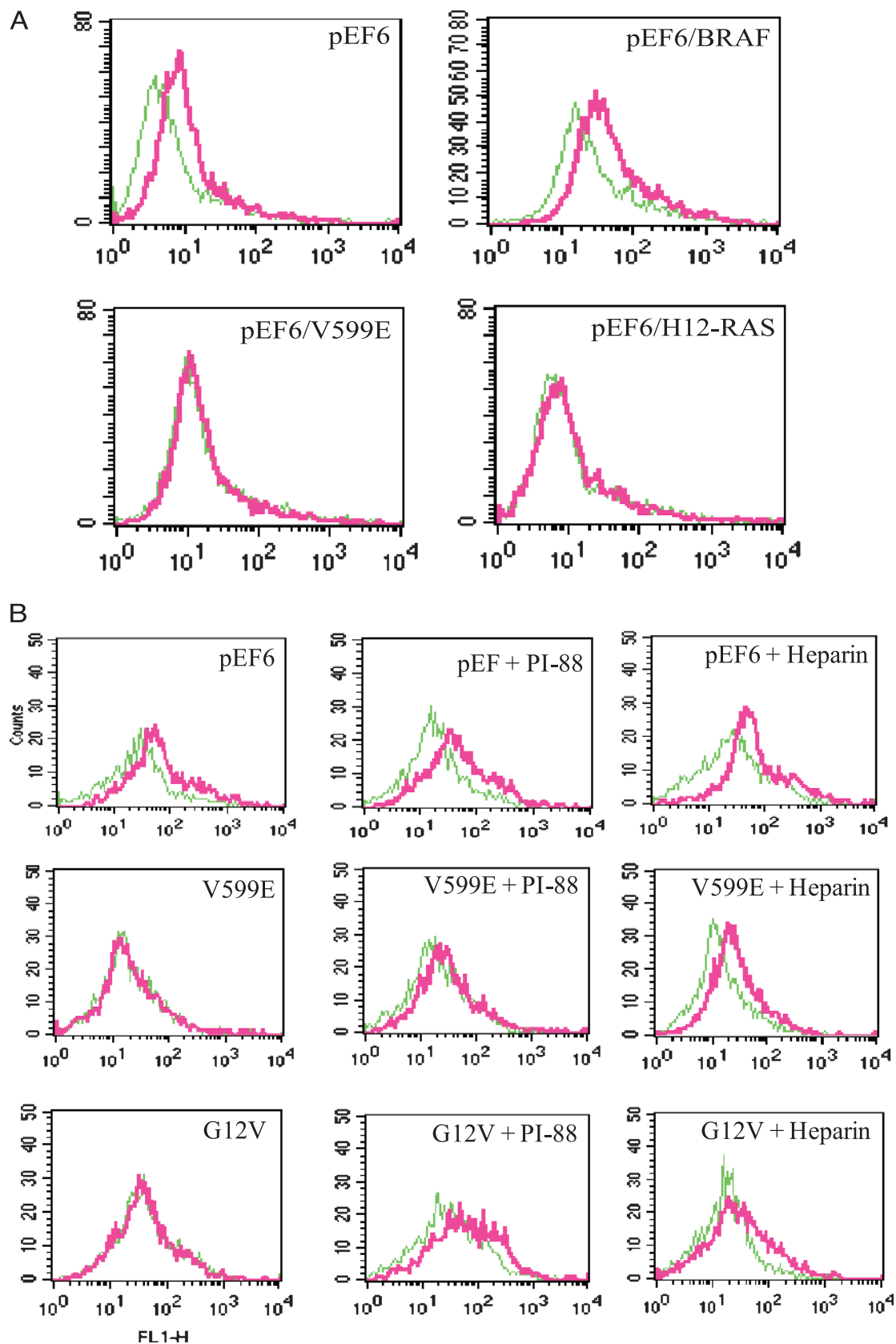


Figure 2. B-Raf kinase activation induces cell surface HS degradation. (A) HEK293 cells transiently transfected with the indicated vectors. After incubating for 48 hours, the cells were harvested and analyzed for cell surface HS levels by FACS. (B) HPR1 inhibitors restored cell surface HS levels. Cells transiently transfected with the empty vector or the vector encoding BRAF^{V600E} or HRAS^{G12V} in the absence or presence of PI-88 or heparin (50 μ g/ml) for 48 hours were analyzed for cell surface HS levels by FACS with anti-HS mAb. Green line indicates IgM control; red line, anti-HS mAb.

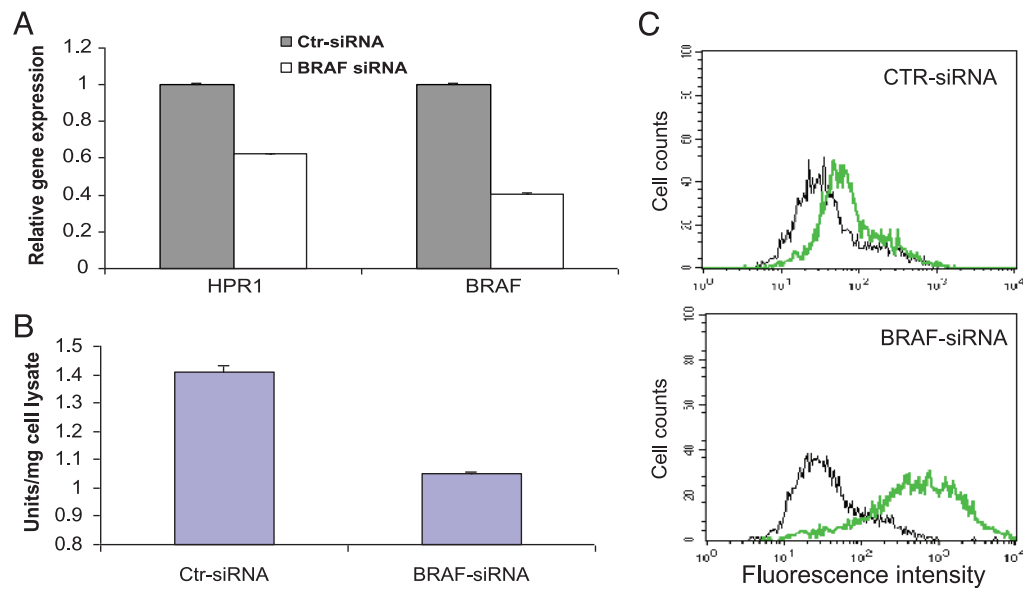


Figure 3. *BRAF* gene knockdown leads to decreased HPR1 expression and increased cell surface HS levels. (A) Real-time RT-PCR analysis of *HPR1* and *BRAF* mRNA. Total RNA was extracted from KAT-10 cells and analyzed for *HPR1* and *BRAF* mRNA by real-time RT-PCR. One of two experiments in triplicate with similar results is shown. (B) HPR1 enzymatic activity. Cell lysates of KAT-10 cells transfected with control or BRAF siRNA were analyzed for HPR1 activity by ELISA. (C) FACS analysis of the cell surface HS levels. Single cell suspensions of KAT-10 cells transfected with control or BRAF siRNA were analyzed for cell surface HS levels by FACS. Black line indicates IgM control; green line, anti-HS mAb.

KAT-10 cells. The levels of Sp1, Sp3, total ERK, and Egr-1 expression were not reduced or were only marginally reduced in BRAF siRNA-transfected KAT-10 cells. To further verify the reduction of GABP β in BRAF siRNA-transfected cells, we conducted real-time RT-PCR and found that GABP β mRNA levels were 30% lower in BRAF siRNA-transfected cells than in control siRNA-transfected KAT-10 cells. However, there was no difference in GABP α mRNA levels between BRAF siRNA- and control siRNA-transfected KAT-10 cells. These observations suggest that suppression of BRAF expression leads to downregulation of GABP β expression, subsequently leading to decreased HPR1 promoter activation and gene expression.

Discussion

The molecular mechanisms of increased HPR1 expression in a variety of malignancies are poorly understood. Increased HPR1 expression in prostate and bladder cancers is mediated by increased Egr-1 expression and HPR1 promoter hypomethylation [16,17]. p53 mutation may also contribute to increased HPR1 expression because wild-type p53 can negatively regulate HPR1 expression [18]. In the present study, we provide several lines of evidence that *RAS* or *BRAF* gene mutations are responsible for increased HPR1 expression: 1) mutant *RAS* or *BRAF* genes were able to directly activate the HPR1 promoter and to induce *HPR1* gene expression; 2) increased HPR1 expression in mutant BRAF or RAS-transfected HEK293 cells led to the loss of cell surface HS, which could be blocked by two HPR1 inhibitors; 3) knockdown of *BRAF* gene expression in *BRAF*-mutated KAT-10 cells led to decreased HPR1 expression and increased cell surface HS levels; and 4) mechanistic studies demonstrated that GABP β , a critical transcription factor involved in regulating *HPR1* gene expression, was downregulated when BRAF expression was suppressed. These observations collectively suggest that increased HPR1 expression in a wide range of tumors could be

caused in part by mutation of oncogenes such as *BRAF*, *RAS*, or their upstream growth factor receptors such as *EGFR* (Figure 7).

The Ets transcription factor family has more than 30 members that bind to the core DNA sequence GGA(A/T). GABP is a unique member of the Ets family because its α and β subunits form a heterodimer complex that often binds tandem ERE sites separated by approximately 10 to 30 bp. A recent genome-wide GABP occupancy study indicates that many GABP binding locations contain two ERE sites [34]. There are two sets of tandem ERE sites within the proximal region of the HPR1 promoter (Figure 7) [13]. Mutation of any one of the ERE sites should eliminate the binding of GABP to one set of tandem ERE sites. In addition, GABP cooperates with its neighboring Sp1, so that disruption of one set of GABP binding site leads to a significant reduction

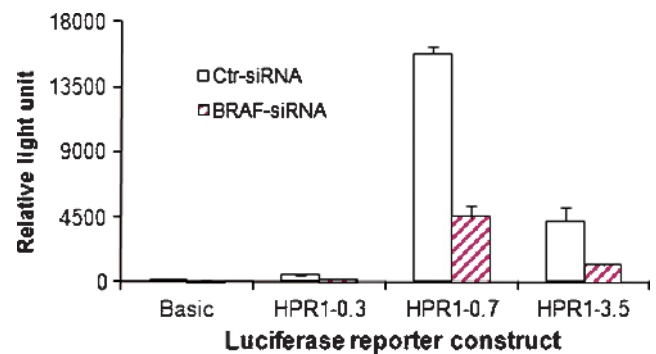


Figure 4. Decreased HPR1 promoter activity in BRAF siRNA-transfected KAT-10 cells. Control or BRAF siRNA-transfected KAT-10 cells were transiently transfected with the luciferase reporter gene expression driven by a 0.3-, 0.7-, or 3.5-kb HPR1 promoter. pGL3/Basic luciferase reporter construct was included as a negative control. After incubating for 48 hours, the cells were harvested and analyzed for luciferase activity.

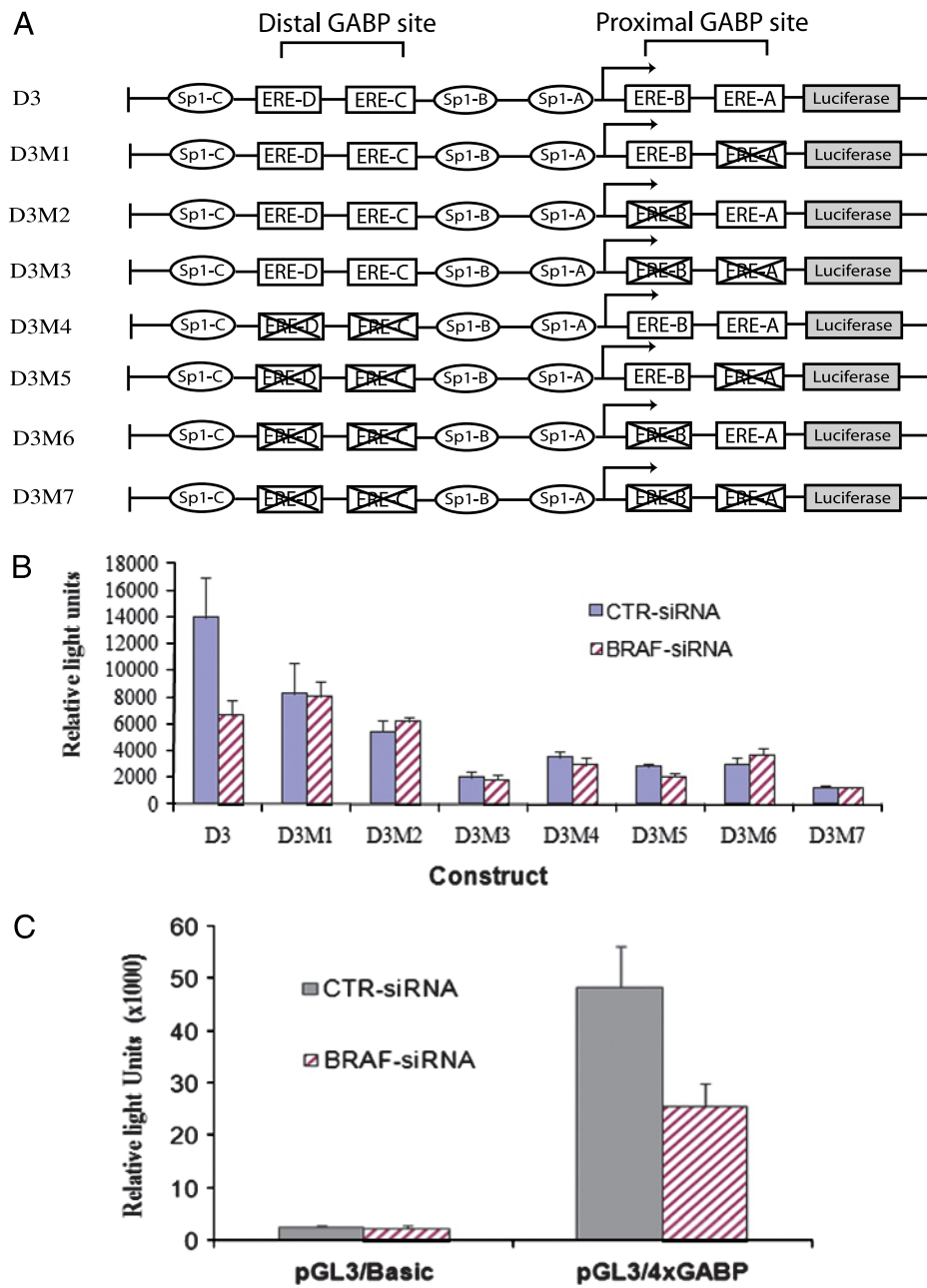
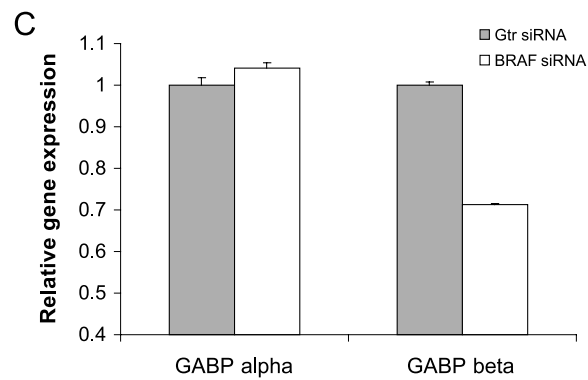
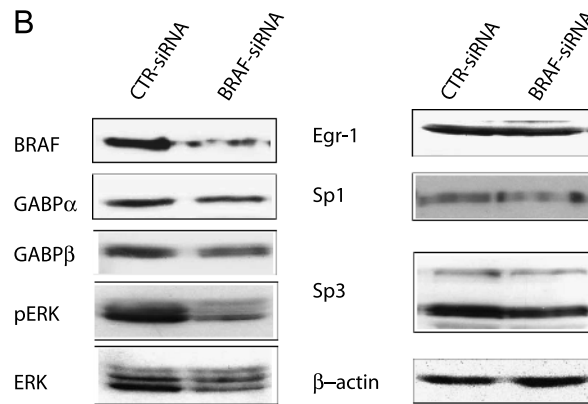
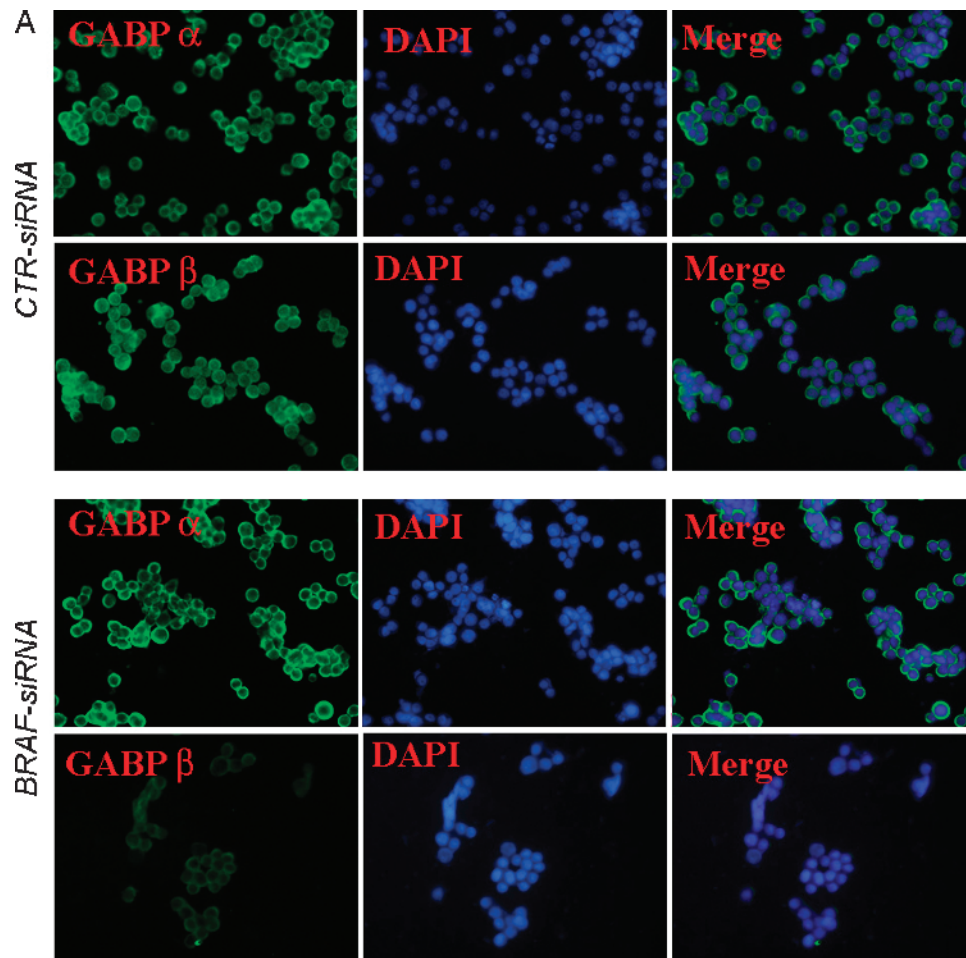


Figure 5. Evidence that the GABP binding site in the HPR1 promoter is critical for BRAF-mediated *HPR1* gene regulation. (A) Schematic illustration of a panel of the HPR1 promoter-driven luciferase reporter constructs. ERE sites marked with a cross sign denote those with altered nucleotides can no longer be bound by Ets transcription factors. (B) Control or BRAF siRNA-transfected KAT-10 cells were transiently transfected with the luciferase reporter constructs as shown in (A) or with 4×GABP binding site-driven luciferase reporter construct (C). After incubating for 48 hours, the cells were harvested and analyzed for luciferase activity.

Figure 6. Down-regulation of GABPβ in BRAF-suppressed KAT-10 cells. (A) IF staining of GABPα and β. Control or BRAF siRNA-transfected KAT-10 cells were stained with an anti-GABPα and β rabbit IgG, followed by staining with fluorescein-conjugated goat antirabbit IgG. A normal rabbit antiserum was included as a negative control. No nonspecific signal was presented (photograph not shown). (B) Western blot analyses of the signaling molecules and transcription factors involved in regulating HPR1 gene expression. Cell lysates from control or BRAF siRNA-transfected KAT-10 cells were analyzed for the expression of several transcription factors and signaling molecules by their specific antibodies. (C) Real-time RT-PCR analyses of GABPα and β expression. Total RNA was extracted from KAT-10 cells transfected with BRAF or control siRNA expression vector and analyzed for GABPα and β by real-time RT-PCR. One of two experiments in triplicate with similar results is shown.



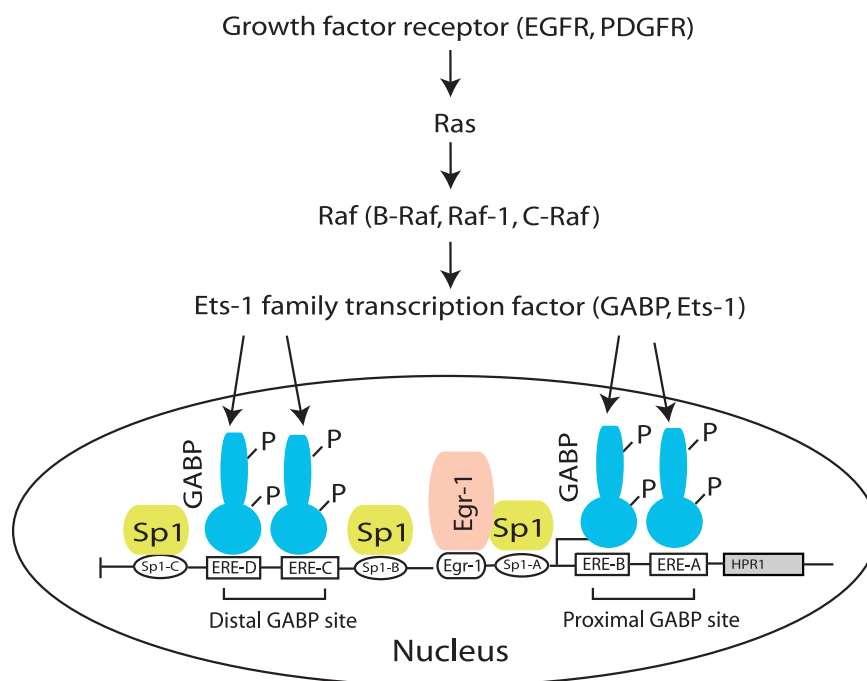


Figure 7. Signaling pathway and transcription factors involved in *HPR1* gene expression in cancer. Mutations and/or overexpression of the growth factor receptor genes such as *EGFR* and its downstream signaling molecules such as *RAS* and *BRAF* genes result in increased expression and/or translocation of the Ets family transcription factors such as GABP and Ets-1. These transcription factors in cooperation with several other transcription factors overexpressed in cancer, such as Sp1 and Egr-1, lead to HPR1 expression in cancer. HPR1 promoter demethylation and p53 mutations also contribute to increased *HPR1* gene expression (not shown).

of HPR1 promoter activity [13]. This may partially explain that, although one set of the GABP binding sites in the HPR1 remains functional, there was no significant difference in luciferase reporter gene expression driven by the HPR1 promoter in control and BRAF siRNA-transfected KAT-10 cells.

GABP is one of the transcription factors that are involved in regulating *HPR1* gene expression [13]. Our present study demonstrates that GABP β expression was downregulated in BRAF-suppressed KAT-10 cells, as analyzed by Western blot and real-time RT-PCR. IF staining revealed that the signals for GABP β expression were reduced in both cytoplasm and nucleus. It is likely that the reduced presence of GABP β in nucleus may not be due to its inability to translocate into nucleus. Nevertheless, because the HPR1 promoter- and 4 \times GABP binding site-driven luciferase reporter gene expression was significantly lower in BRAF siRNA than in control siRNA-transfected KAT-10 cells, this suggests that GABP plays an important role in mediating mutant BRAF-induced HPR1 expression. It should be noted that *BRAF* gene mutation may induce the expression of other Ets family members that also contribute to HPR1 expression. For example, activation of the MAP kinase pathway leads to increased Ets2 expression and phosphorylation, subsequently leading to increased matrix metalloproteinase 9 expression [35–37].

Previous studies have shown that GABP can be phosphorylated by Raf-1 kinase [38,39]. Fromm and Burden [40] showed that GABP α can be phosphorylated at threonine 280 and activated by ERK and JNK kinases in neuregulin-1-stimulated muscle cells. However, a recent study suggests that GABP α phosphorylation is not essential for neuregulin-1-induced acetylcholine receptor expression [41]. Although it is likely that MAP kinase activation due to *BRAF* gene mutation may lead to increased phosphorylation of GABP α , increased GABP β expres-

sion in BRAF-mutated cell lines may play a dominant role in mediating BRAF mutation-induced HPR1 expression.

It has been well documented that several transcription factors of the Ets family, in particular Ets-1, Ets-2, and PEA, are overexpressed in a variety of malignancies such as breast and ovarian cancers. GABP is ubiquitously expressed in normal tissues. GABP plays a critical role in regulating the expression of BRCA1 and prolactin, both of which are involved in tumorigenesis [42–44]. Our present study suggests the possibility that GABP β expression can be upregulated in a variety of cancers due to the activation of the MAP kinase pathway. GABP and Sp1, another housekeeping transcription factor that is upregulated in a variety of cancers [45], can cooperate to induce HPR1 expression in cancer (Figure 7). Egr-1 is another transcription factor involved in regulating *HPR1* gene expression [12–15]. Egr-1 overexpression in cancer may also contribute to increased HPR1 expression (Figure 7). Our present study shows that there was no significant difference in Sp1 and Egr-1 expression in control and BRAF siRNA-transfected KAT-10 cells. In addition, when the GABP binding sites in the HPR1 promoter were mutated, there was no difference in luciferase gene expression in control or BRAF siRNA vector-transfected KAT-10 cells. These observations suggest that Sp1 and Egr-1 do not contribute to mutant B-Raf kinase-mediated increase of HPR1 expression. Because we did not analyze the expression of other Ets family member, it remains unknown if other Ets transcript factors may also be required for mutant BRAF-induced HPR1 expression.

BRAF-mutated thyroid cancers and melanomas tend to be more aggressive than those without BRAF mutation [19,46]. B-Raf kinase activation due to the *BRAF* gene mutations leads to the up-regulation of several molecules that are involved in tumor metastasis [19,46]. For example, BRAF^{V600E} mutation has been shown to regulate melanoma

metastasis by increasing fibronectin expression and by promoting interaction of melanoma cells with neutrophils to facilitate extravasation across the endothelial cell lining as well as enhancing proliferation in the lung microenvironment [47,48]. Inhibition of these processes leads to a decrease of lung metastasis of BRAF-mutated melanoma cells by four- to five-fold [49]. *BRAF* gene mutation also leads to the up-regulation of matrix metalloproteinase expression in thyroid cancer and melanoma [50,51]. Our present study demonstrates that *BRAF* mutation led to up-regulation of HPR1, an endoglycosidase that is involved in tumor angiogenesis and metastases. This observation is consistent with a prior study showing that sorafenib, an inhibitor of B-Raf kinase and other growth factor receptor, blocks vascular development in malignant melanomas in a xenograft model [52]. It is anticipated that sorafenib treatment may lead to a significant decrease in HPR1 expression, subsequently suppressing tumor angiogenesis. These studies collectively suggest that up-regulation of HPR1 and other molecules due to *BRAF* gene mutation may act in concert to promote tumor angiogenesis and metastases.

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