

Overexpression of humbug promotes malignant progression in human gastric cancer cells

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Abstract. Two human gastric cancer cell lines of differing invasive potential, SNU-484 and SNU-638 cells, were examined using subtractive suppression hybridization in a search for any genes associated with metastasis. Of the eight cDNAs identified as being differentially expressed genes, it was determined that *humbug*, which encodes a truncated isoform of aspartyl (asparaginyl) β -hydroxylase (AAH) missing catalytic domain, was overexpressed in highly invasive SNU-638 cells. Expression analysis showed that the mRNA expression level of *humbug* was correlated with invasive potential in various human gastric cancer cell lines. The forced expression of *humbug* to the human gastric cancer cell line AGS increased its anchorage-independent growth in 0.3% agar without affecting cell proliferation. Furthermore, *humbug*-transfected cells migrated more actively and showed an increased invasion rate relative to vector-transfectants or parental AGS *in vitro*. This is the first demonstration that *humbug*, a truncated form of AAH, can be overexpressed during the malignant progression of human gastric cancer cells and that it can function as a metastasis-inducing gene.

Introduction

On a worldwide basis, gastric cancer is one of the most common causes of cancer mortality (1). The treatment of this disease, however, has improved due to early detection followed by surgical resection, emphasizing the need to develop efficient biomarkers to detect gastric cancer as early as possible (2). However, patients with advanced gastric cancer display poor prognosis even if they undergo curative resection, because systemic micrometastasis may have already existed at the time of surgery (3). Mechanisms involved in gastric cancer metastasis are not fully clarified since metastatic progression of

the cancer cells requires the accumulation of altered expression of many different genes and involves multiple steps (4). Although aspects of metastatic progression of gastric cancer remain to be elucidated, several genes have been reported to regulate a positive or negative metastasis of gastric cancer cells (5-7). In the present study, we measured the invasive potential of various gastric cancer cell lines and subsequently performed suppression subtractive hybridization (SSH) to identify metastasis-related genes by comparing differential gene expression profiles between highly invasive SNU-638 and poorly invasive SNU-484 gastric cancer cell lines. Eight different genes were identified as being overexpressed in SNU-638 cells. Of these, we identified *humbug*, an alternative splicing variant of aspartyl (asparaginyl) β -hydroxylase (AAH), as an overexpressed gene in human gastric cancer cells.

In humans, the alternative splicing of aspartyl (asparaginyl) β -hydroxylase (AAH) gene generates at least four transcripts which encode four distinct proteins (8,9); an enzyme aspartyl (asparaginyl) β -hydroxylase, an isoform of aspartyl β -hydroxylase missing the catalytic domain (*humbug*), juncin, and juncate, which are generated by alternative splicing of an ~6 kb mRNA transcript, with exon sharing and exon swapping mainly within the first 13 exons (8,9). *Humbug* mRNA is derived from the first 14 exons of the AAH gene (8-11). *Humbug* can bind calcium and the overexpression of *humbug* increases intracellular calcium levels by promoting its release from intracellular stores and shares a high degree of sequence homology with juncate (8,10).

Herein, we describe the overexpression of *humbug* in highly invasive human gastric cancer cell lines and its effect in enhancing the migration, invasion and anchorage-independent growth of a human gastric cancer cell line AGS *in vitro*. Our results suggest that *humbug* can be overexpressed in human gastric cancer cells and can promote the malignant progression and possibly metastasis of the cells.

Materials and methods

Cell lines. The gastric cancer cell lines SNU-1, -5, -16, -216, -484, -620, -638 and AGS were obtained from the Korean Cell Line Bank of the Cancer Research Center, Seoul National University College of Medicine and maintained in RPMI-1640. The characteristics of these cell lines were described previously (12,13). All media were supplemented with 10% fetal bovine serum, penicillin and streptomycin and all cell

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lines were maintained in a humidified 5% CO₂ atmosphere at 37°C.

Suppression subtractive hybridization (SSH) and differential screening. SSH was performed between SNU-638 and SNU-484 using the PCR-Select™ cDNA subtraction kit (Clontech, Mountain View, CA, USA). SSH and differential screening were performed as described previously (14).

Northern blotting. Northern blotting was performed as previously described (14). To detect mRNA expression of the subtracted genes, inserts were excised from the pCR2.1 vector by *EcoR* I digestion and used for probes. To detect *AAH* and *humbug*, a 714 bp *Sma* I-*Xba* I fragment in an open reading frame of *humbug* was used as a probe. Following hybridization, the membrane was washed and exposed to film with an intensifying screen at -80°C.

Construction of humbug expression vectors and transfection. To obtain the *humbug* cDNA, polymerase chain reaction (PCR) was performed with SNU-638 cDNA as a template and Advantage Polymerase Mix (Clontech). Specific primers for *humbug* were designed based on the previous sequence (Genbank accession number NM_032466); sense 5'-GCAATGGCCAGCGTAAGAAT-3' and antisense 5'-GAAGCTTTAAGTATCTGGTGG-3'. After an initial denaturation step at 95°C for 2 min, *humbug* was amplified for 30 cycles of 30 sec at 94°C; 1 min at 58°C; 3 min at 72°C. Amino-terminally FLAG-tagged *humbug* expression vectors were generated by cloning *humbug* cDNA into pCMV-Tag 2 (Stratagene, La Jolla, CA, USA) vector in frame. All sequences were verified by DNA sequencing. AGS cells were transfected with the pCMV Tag 2-FLAG-*humbug* using the Lipofectamine Plus (Invitrogen). Forty-eight hours after transfection, the medium was changed with the culture medium supplemented with 500 µg/ml of G418 for selection of stable transfectants.

Western blotting. Western blotting was performed as previously described (14). In brief, cells were washed with ice-cold PBS three times and extracted in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 µM phenylmethyl sulfonyl fluoride, 1 mg/ml leupeptin, 150 mM NaCl). Proteins were separated onto SDS-PAGE and transferred a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked and then incubated for 2 h with anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO, USA). After washing, the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK). To show the equal loading of protein, the membrane was stripped and reprobed with anti-tubulin antibody (InnoGenex, San Ramon, CA, USA).

Cell migration and invasion assay. A migration and invasion assay was performed as previously described (14,15). In brief, the ability of cells to migrate through non-coated (migration) or invade through 10 µg Matrigel-coated filters (invasion) was measured with a modified Boyden chamber (Corning Costar,

Cambridge, MA, USA); 8 µm pore size. Cells were seeded at a density of 1x10⁴ cells/100 µl/well. After incubation for 12 h (migration) or 24 h (invasion) at 37°C in 5% CO₂, the cells that had not penetrated the filter were completely wiped out with cotton swabs and the cells that had migrated to the lower surface of the filter were fixed, stained and counted in 5 randomly selected microscopic fields (x100) per filter. Three independent experiments were performed in triplicate and the data were represented as the average of the three independent experiments with the standard error of the average indicated. Statistical comparison was made using one-way ANOVA. P-values <0.01 were considered very significant.

Anchorage-independent growth in 0.3% agar. Six-well culture plates were covered with a layer of 0.5% agar in a medium supplemented with 20% FBS to prevent the attachment of the cells to the plastic substratum. Cell suspensions (5,000 cells/well) of the control and *humbug*-transfected cells were prepared in 0.3% agar and poured into 6-well plates. The plates were incubated at 37°C in a humid atmosphere of 5% CO₂ for 3 weeks until colonies appeared. The colonies were stained with crystal violet and counted. Colony assays were performed in triplicate three times and results are reported as a mean of three experiments.

Proliferation assay. The cells grown in RPMI-1640 supplemented with 10% FBS were placed in a 24-well plate at a concentration of 1x10⁴/ml cells per well. Following incubation for the indicated time, the viable cells were counted with a hemacytometer after trypan blue staining. Results were calculated as the mean ± SD of triplicate cultures.

Results

In an effort to understand the invasive and metastatic progression of gastric cancer, we have used SSH to generate a profile of genes overexpressed in the highly invasive gastric cancer cell line. SSH was performed between SNU-638 (highly invasive phenotype) and SNU-484 (poorly invasive phenotype) human gastric cancer cell lines (15). To identify invasion-achievable genes, SNU-638 cDNA was used as a tester. About 230 subtracted cDNA clones were obtained after subtraction, and then, differential screening revealed that 35 cDNA clones were overexpressed in SNU-638 as compared to SNU-484 cells. After sequencing and Northern blot analyses, eight different genes were finally identified as overexpressed in SNU-638 cells (Table I). Of these, the expression of met proto-oncogene (*MET*), calcium binding protein S100A6 (*S100A6*), translationally controlled tumor protein 1 (*TPT1*) and urokinase-type plasminogen activator (*PLAU*) have already been known to be associated with enhanced malignancy of various types of human cancers including gastric cancer (16-21). To further confirm the expression of these candidate genes, we selected six genes and analyzed the expression by Northern blot analysis in several human gastric cancer cell lines (Figs. 1 and 2A), which had been established from primary tumors (SNU-1 and SNU-484) or secondary tumor sites (SNU-5, -16, -216, -620 and -638). Notably, we identified the *AAH* transcript variant 3 (*humbug*) as an overexpressed gene in the cell lines

Clone	Sequence identity	Accession number	Redundancy	Expression ^a
G1	<i>Met</i>	NM_000245	5	>10
G2	<i>S100A6</i>	NM_014624	14	>10
G3	<i>FTH1</i>	NM_002032	4	ND ^b
G4	<i>TPT1</i>	NM_003295	4	5.6
G5	<i>AKAP12</i>	NM_005100	3	>10
G6	<i>PLAU</i>	NM_002658	3	8.5
G7	<i>UBE2E1</i>	NM_182666	1	7.8
G8	<i>Humbug</i>	NM_032466	1	>10

^aRelative expression was determined by densitometry as compared with SNU-484 cells after normalization to β -actin based on Northern blot analysis. The values represent fold expression compared to SNU-484 cells. ^bND, Not determined.

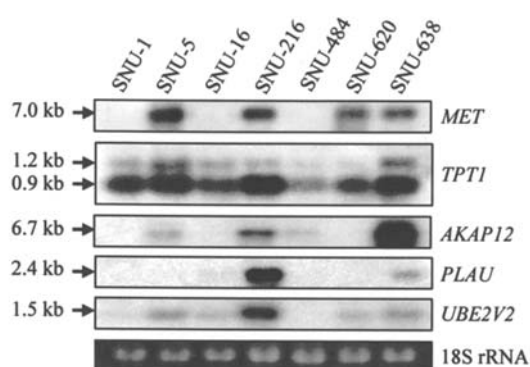


Figure 1. Expression of the selected genes in various human gastric cancer cell lines. Northern blot was performed to determine the mRNA expression level of *MET*, *TPT1*, *AKAP12*, *PLAU* and *UBE2V2* from the indicated human gastric cancer cell lines. To represent an equal loading of non-degraded RNA, 18S rRNA stained with ethidium bromide is shown at the bottom of the panel.

established from secondary tumor sites such as SNU-5, -16, -216 and -638 cells (Table I and Fig. 2). Therefore, we further investigated a possible role of humbug in metastatic progression of gastric cancer cells.

A homology search of clone G8 revealed the perfect match with AAH transcript variant 3 (accession no. NM_032466, corresponding position of clone 8: 1866 to 2284 bp), which encodes an isoform of AAH missing the catalytic domain. We compared the expression profiles between AAH and *humbug* by Northern blot analysis in various human gastric cancer cell lines (Fig. 2). When clone G8 was used as a probe (Fig. 2A), only a 2.9 kb transcript corresponding to *humbug* was detected (9). When a specific cDNA probe for recognizing both AAH and *humbug* was used (Fig. 2B), two transcripts, which encode AAH (4.3 kb transcript) and *humbug* (2.9 kb transcript), respectively, were detected (9). These results suggest that AAH and *humbug* were overexpressed in highly invasive gastric cancer cell lines.

Previous studies have shown that AAH is overexpressed in hepatocellular carcinoma, cholangiocarcinoma and neuroblastoma and induces malignant transformation and cell motility (22-26). Therefore, we examined whether humbug,

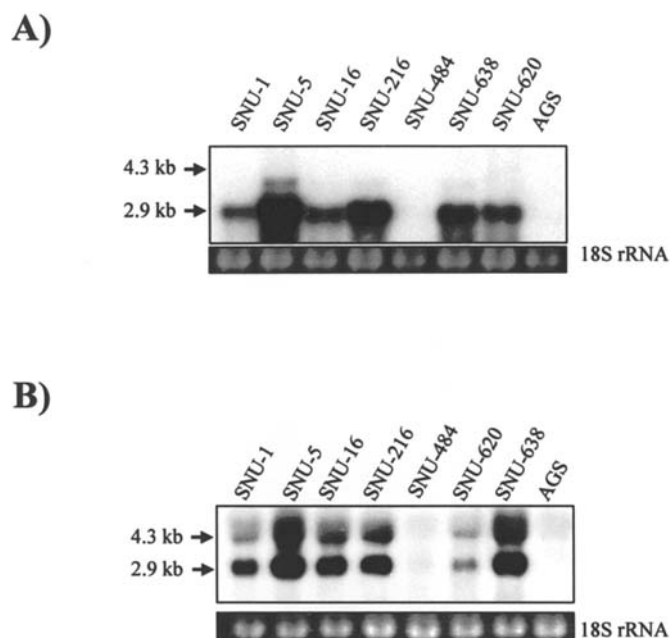


Figure 2. Expression of *humbug* and *AAH* in various human gastric cancer cell lines. Northern blot was performed to determine the mRNA expression level of *humbug* (A) and *AAH* (B) from the indicated human gastric cancer cell lines. To represent an equal loading of non-degraded RNA, 18S rRNA stained with ethidium bromide is shown at the bottom of the panel.

which is a truncated form of aspartyl β -hydroxylase missing the catalytic domain, modulates the malignant transformation of gastric cancer. To test this, AGS cells, which barely express *humbug* (Fig. 2A), were transfected with a FLAG-tagged *humbug* expression vector or with the vector alone. After selection in G418, the pooled population of G418-resistant *humbug*-transfected and empty vector-transfected AGS cells were obtained and designated AGS/hum and AGS/neo, respectively. To rule out the clonal variation of AGS cells, we used AGS/hum and AGS/neo without clonal selection for further experiments. The protein expression of exogenous *humbug* in AGS/hum cells was confirmed by Western blot analysis using an anti-FLAG monoclonal antibody (Fig. 3A). The exogenous *humbug* was detected as a

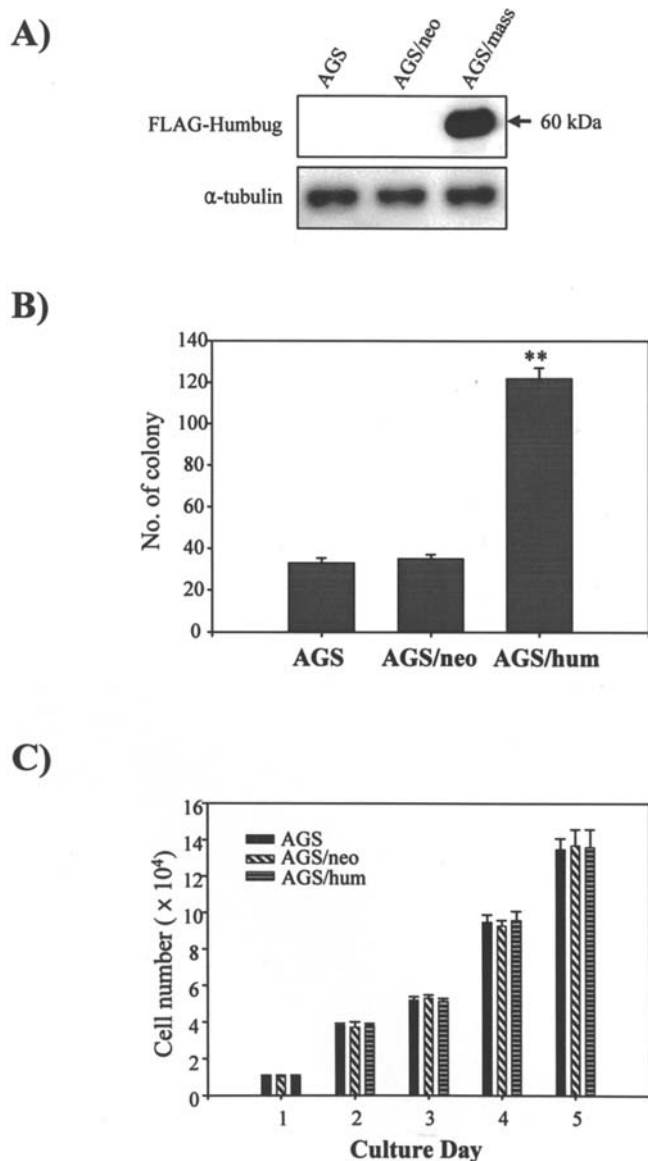


Figure 3. Humbug stimulates anchorage-independent growth of AGS cells. (A) Expression of the humbug protein by stably transfected AGS cells as shown by Western blotting. AGS cells were stably transfected with either the empty pCMV-Tag 2A vector (AGS/neo) or the humbug expression vector (AGS/mass), respectively. Cell lysates (50 μ g) were immunoblotted with an anti-FLAG M2 monoclonal antibody. The bottom represents α -tubulin to show the equal loading of cell lysates. (B) Effect of humbug overexpression on colony formation of AGS cells in 0.3% agar. (C) Effect of humbug overexpression on the proliferation of AGS cells. Data represent (average \pm S.E) of three independent experiments performed in triplicate; ** p <0.01 vs. vector-transfectants (one-way ANOVA).

single band at 60 kDa in AGS/mass, though not in AGS/neo and the parental. The size of humbug observed in Western blot is consistent with the previous report (9). We examined whether humbug affected the ability of anchorage-independent growth of AGS cells. The ability of *humbug*-transfectants to exhibit anchorage-independent cell growth was examined using a colony formation assay in 0.3% agar. AGS/hum cells showed increased anchorage-independent growth as compared to parental AGS and AGS/neo cells (Fig. 3B). The extent of colony formation in 0.3% agar was 3.5-fold higher for AGS/hum as compared with the AGS/neo (p <0.01, one-way ANOVA). The mean \pm S.E. of colony formed per well for

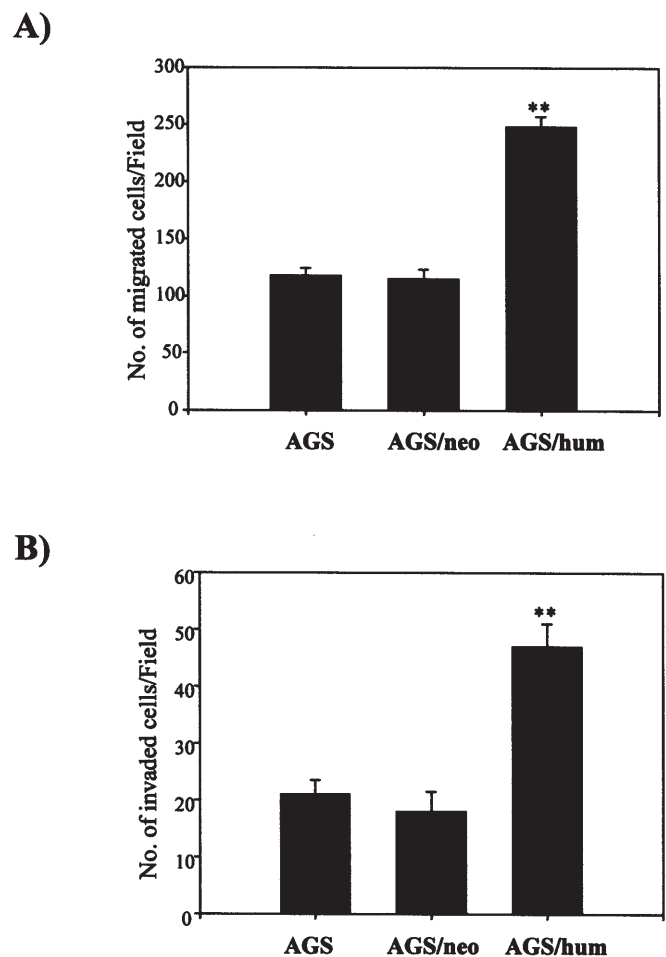


Figure 4. Humbug stimulates cell migration and invasion in AGS cells. Cells that migrated (A) or invaded (B) through the pores in the filter were fixed, stained and counted in five random fields visualized by microscopy ($\times 100$). Data represent (average \pm S.E) three independent experiments performed in triplicate; ** p <0.01 vs. vector-transfectants (one-way ANOVA).

AGS, AGS/neo, and AGS/mass was 33 ± 2.4 , 35 ± 2.1 and 122 ± 5.1 , respectively. However, the proliferation of AGS/mass cells was similar to that of parental AGS and AGS/neo (Fig. 3C). Then, we examined whether overexpression of humbug could affect migration and invasion of AGS cells using a Transwell migration assay. AGS/mass cells exhibited increased cell migration through filters as compared to parental AGS and AGS/neo cells (Fig. 4A). The extent of migrated cells was 2.1-fold higher for AGS/mass as compared with the AGS/neo (p <0.01, one-way ANOVA). The mean \pm S.E. of migrated cells per field ($\times 100$) for AGS, AGS/neo and AGS/mass was 118 ± 6.4 , 115 ± 8.1 and 248.4 ± 9.1 , respectively. Similar results were obtained from an invasion assay using Matrigel-coated filters (Fig. 4B). AGS/hum cells showed significantly increased invasiveness over that of the vector-transfected control (p <0.01, one-way ANOVA).

Discussion

In the present study, we found that *humbug* is overexpressed in human gastric cancer cell lines and the level of its expression is correlated with the invasive potential of various gastric cancer cell lines. Furthermore, we provided evidence



SPANDIDOS PUBLICATIONS Effect role of *humbug* in gastric cancer progression by cell invasion and anchorage-independent growth.

Previous studies demonstrated that *AAH* is abundantly expressed in a broad range of malignant neoplasms and transformed cell lines, including those of hepatic, biliary, breast, colon, pulmonary, pancreatic and neural origin (22-26). Overexpression of *AAH* is associated with malignant transformation and increased cell motility in several types of cancer cell lines (22-26). In contrast, the expression level and role of the *humbug* gene in human cancer has not been clarified. A recent report revealed that *humbug* is overexpressed and may be a useful prognostic marker for colon cancer (27). This report showed that expression levels of *humbug* correlate with histological grade and tumor behavior. However, expression of *AAH* was not correlated with tumor grade or survival. Herein, we demonstrated that *humbug*, a truncated form of *AAH* missing the catalytic domain, is overexpressed and may induce the malignant transformation of human gastric cancer cells.

How might *humbug* increase anchorage-independent cell growth and cell motility? The molecular mechanisms by which *AAH* regulates cell motility are uncertain; however, evidence suggests that *AAH* may mediate its effects on cell migration by activating notch signaling pathways. In this regard, functional studies have demonstrated that *AAH* catalyzes post-translational hydroxylation of β -carbons of aspartyl and asparaginyl residues present in EGF-like domains of certain proteins (8,28,29). The consensus sequence for *AAH* hydroxylation is present in the EGF-like domains of notch, notch homologs and the ligand jagged (8). *Humbug* is a truncated form of *AAH* missing catalytic domain of the β -hydroxylase, though in spite of the absence of enzymatic activity, it can increase anchorage-independent growth, invasion and motility of human gastric cancer cells. *Humbug* is derived from the first 14 exons of the *AAH* gene and can bind calcium (8-10,23). Overexpression of *humbug* increases intracellular calcium levels by promoting its release from intracellular stores (10,11). Since calcium levels have a critical role in regulating various signaling pathways, increased *humbug* expression may be important for modulating changes in cell shape, cellular adhesion and migration that mediate tumor progression.

Collectively, our results indicate that overexpression of *humbug* can/may promote cell migration and anchorage-independent growth in human gastric cancer cell lines and suggests a potential role for *humbug* in the processes of tumor cell migration, invasion and possibly metastasis. Further study to evaluate the functions and regulation mechanisms of *humbug* will expand our understanding of the role played by *humbug* in tumor progression and may provide a useful prognostic marker for gastric cancer.

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