

## GBP-1 acts as a tumor suppressor in colorectal cancer cells

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**The human guanylate-binding protein 1 (GBP-1) is among the proteins the most highly induced by interferon- $\gamma$  (IFN- $\gamma$ ) in every cell type investigated as yet. *In vivo*, GBP-1 expression is associated with the presence of inflammation and has been observed in autoimmune diseases, inflammatory bowel diseases (IBD) and cancer. In colorectal carcinoma (CRC), the expression of GBP-1 in the desmoplastic stroma has been previously reported to correlate with the presence of an IFN- $\gamma$ -dominated T helper type 1 (Th1) microenvironment and with an increased cancer-related 5-year survival. In the present study, the analysis of GBP-1 expression in a series of 185 CRCs by immunohistochemistry confirmed that GBP-1 is expressed in stroma cells of CRCs and revealed a significantly less frequent expression in tumor cells, which was contradictory with the broad inducibility of GBP-1. Furthermore, three of six CRC cell lines treated with IFN- $\gamma$  were unable to express GBP-1 indicating that colorectal tumor cells tend to downregulate GBP-1. On the contrary, non-transformed colon epithelial cells strongly expressed GBP-1 *in vitro* in presence of IFN- $\gamma$  and *in vivo* in inflammatory bowel diseases. Reconstitution of GBP-1 expression in a negative CRC cell line inhibited cell proliferation, migration and invasion. Using RNA interference, we showed that GBP-1 mediates the antitumorogenic effects of IFN- $\gamma$  in CRC cells. In addition, GBP-1 was able to inhibit tumor growth *in vivo*. Altogether, these results suggested that GBP-1 acts directly as a tumor suppressor in CRC and the loss of GBP-1 expression might indicate tumor evasion from the IFN- $\gamma$ -dominated Th1 immune response.**

### Introduction

Colorectal carcinoma (CRC) is the third most common malignancy and fourth most common cause of cancer mortality worldwide (1). Malignant transformation of CRC has been extensively studied and is based on the concept of multistep carcinogenesis, characterized by the accumulation of mutations and chromosomal rearrangements over time (2). Moreover, tumorigenesis involves multiple host-tumor interactions where molecular and cellular factors of the tumor microenvironment can either promote or inhibit tumor progression (3–6).

**Abbreviations:** CRC, colorectal carcinoma; FBS, fetal bovine serum; GBP-1, guanylate-binding protein 1; IFN- $\gamma$ , interferon- $\gamma$ ; siRNA, small interfering RNA; Th1, T helper type 1.

In CRC, tumor-infiltrating T-cells have been shown to participate in antitumoral responses. Specifically, the presence of T helper type 1 (Th1) cells, cytotoxic and memory T-cells is associated with improved clinical outcome and survival of CRC patients (7–11). Interferon- $\gamma$  (IFN- $\gamma$ ) represents a major mediator of the differentiation and the promotion of the Th1 immune response (12,13). Numerous experiments in mice indicated that IFN- $\gamma$  participates in tumor immunoeediting and exerts antitumorogenic effects on tumor cells (14–19). Furthermore, the presence of a Th1-dominated immune response in CRC correlates with the expression of IFN-target genes and is associated with an angiostatic microenvironment (9,20).

The guanylate-binding protein 1 (GBP-1) is one of the proteins the most highly induced by IFN- $\gamma$  in human eukaryotic cells (21). GBP-1 expression after IFN- $\gamma$  treatment has been documented in a large number of cell lines and primary cells *in vitro* (22,23). GBP-1 belongs to the family of large GTPases, together with dynamin or Mx proteins (24,25). These GTPases are characterized by a nucleotide-dependent oligomerization and a strong cooperative GTPase activity (26–29). In eukaryotic cells, GBP-1 has been shown to exert a mild antiviral and antibacterial activity (30–32). In addition, GBP-1 has been shown to be a robust marker of inflammation and its expression in human tissues has been associated in particular with blood vessels or cells of monocytic origin (20,22). The function of GBP-1 has, therefore, been extensively studied in endothelial cells where it inhibits proliferation, migration and spreading (33–35). In CRC, GBP-1 expression was found to be associated with numerous IFN- $\gamma$  target genes characteristic of an active Th1 immune response (20). A strong expression of GBP-1 has been observed in the tumor stroma and particularly in the blood vessels of about one-third of the tumors in a large cohort of patients and was highly significantly associated with reduced angiogenic activity (20). Accordingly, GBP-1 expression in the stroma of CRC had a significant impact on the course of disease. In addition, GBP-1 was identified as an independent marker of a halved risk of death and highly significantly associated with a prolonged cancer-related 5-year survival of the patients (20). These results were confirmed recently in a comprehensive CRC study of the Cancer Genome Atlas Network (36).

The present study focused on the expression and function of GBP-1, as a major IFN- $\gamma$  target gene, in the tumor cells of CRC.

### Material and methods

#### Tissue samples

This study is based on specimen collected from 185 patients with histologically confirmed CRC having received surgery between 2005 and 2011 in the Department of Surgery of the University Hospital of Erlangen, Germany. This study was approved by the ethics committee of the University Hospital of Erlangen. All participants were informed personally and provided written informed consent for this study. Tissues of tumor were collected from patients undergoing standard surgical procedure for primarily diagnosed CRC. Patients did not receive any tumor-related therapy prior to operation, excluding any therapy-based expression bias. Specimen from patients with ulcerative colitis ( $n = 2$ ) and Crohn's disease ( $n = 3$ ) was collected in the Department of Pathology of the Klinikum Bayreuth, Germany.

#### Immunohistochemistry

Formalin-fixed paraffin-embedded tumor sections (5  $\mu$ m) were dewaxed in xylol (Merck Chemicals, Darmstadt, Germany) and rehydrated in a descending ethanol series (100, 96 and 70%). After antigen retrieval in target retrieval solution, pH 9.00 (TRS9; Dako, Hamburg, Germany), slides were treated with 7.5% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidases. For staining of GBP-1, slides were incubated for 1 h with monoclonal rat anti-GBP-1 (1:300, own laboratory) at room temperature. Subsequently, sections were incubated for 30 min with biotinylated horse antirat IgG, followed by 30 min of incubation with an avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA). The reaction was developed with 0.1% 3,3'-diaminobenzidine hydrochloride (Sigma-Aldrich, Hamburg,

Germany) in 0.03% hydrogen peroxide and counterstained with Mayer's haemalum (VWR International, Ismaning, Germany). After dehydration for 2 min in ethanol (96 and 100%) and 2 min incubation in xylol, sections were mounted in DPX mounting medium (Sigma–Aldrich) and examined by light microscopy (Zeiss Axiovert 200M, Carl Zeiss MicroImaging GmbH, Munich, Germany).

#### Cells, plasmids and reagents

The CRC cell lines DLD-1, HT29, WiDr, SW480 and T84 were purchased from ATCC, whereas HCT116 cells were a gift from B.Malfroy (Institut Curie, Paris, France). DLD-1 cells were cultured in RPMI 1640 (PAA, Pasching, Austria) supplemented with 2 mM glutamine (PAA) and 10% (v/v) fetal bovine serum (FBS, PAA). WiDr and SW480 cells were cultured in Dulbecco's modified Eagle's medium (PAA) with 2 mM glutamine and 10% FBS. HCT116 and HT29 cells were cultured in McCoy's medium (PAA) with 2 mM glutamine and 10% FBS. T84 cells were cultured in Dulbecco's modified Eagle's medium/Ham's medium with 2 mM glutamine and 5% FBS. Cells were maintained at 37°C with 5–8.5% CO<sub>2</sub> and 95% humidity. Human colon epithelial cells (HCEC) were a gift from P. Steinberg (Hannover, Germany). Human colon epithelial cells are human adult colon cells immortalized by transfection of a vector expressing the SV40 large T antigen and were cultivated on collagen-coated plates in SO-10500 cell culture medium (PAN biotech, Aidenbach, Germany) as described previously (37). Two millimolar glutamine, 1 nM dexamethasone (Sigma–Aldrich), 100 nM retinoic acid (Sigma–Aldrich), 38 µg/ml vitamin C and 30 µg/ml bovine pituitary extract (Lonza, Cologne, Germany) were added prior to use. The plasmids pMCV1.4(–) and pMCV2.2(–), which contains a gentamycin resistance cassette, were obtained from Mologen (Berlin, Germany). A Flag-tag sequence (F) was cloned into the pMCV1.4(–) or pMCV2.2(–) plasmids using EcoRV/EcoRI restriction sites. The sequence of GBP-1 was inserted in-frame into pMCV1.4-Flag or pMCV2.2-Flag using the EcoRI restriction site (NCBI accession number: GBP-1, NM\_002053.2). When indicated, cells were treated with 100 U/ml of IFN-γ, 200 U/ml of interleukin 1-beta (IL-1β) or 300 U/ml of tumor necrosis factor-alpha (TNF-α) (all from Roche Applied Science, Mannheim, Germany).

#### Transfections

DLD-1 WiDr, HT29 and T84 cells were transfected with the plasmids pMCV2.2-Flag-GBP-1 (stable cell lines) or pMCV1.4-Flag-GBP-1 (transient transfections) using Lipofectamine™ 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. For the generation of stable DLD-1 cell lines, transfected cells were selected by addition of G418 (500 µg/ml, PAA) and three independent single cell clones were expanded. As a negative control, DLD-1 cells were transfected with pMCV2.2 and selected as a population of cells.

#### RNA interference

RNA silencing was performed in WiDr cells by reverse transfection using Lipofectamine™ RNAiMAX in OptiMEM® medium (both Invitrogen). Transfection was performed in four-well Lab-Tek chamberslides or six-well cell culture plates (Nunc™) at a cell density of  $1.4 \times 10^4$  cells/cm<sup>2</sup> and a final concentration of small interfering RNA (siRNA) of 16 nM. The following siRNAs were used: GBP-1 Stealth Select RNAi™ siRNA (HSS104020), Stealth RNAi™ GAPDH Positive Control Duplexes and Stealth RNAi™ Negative Control Duplexes (Medium GC) (all purchased from Invitrogen/Molecular Probes). Treatment with IFN-γ (100 U/ml) occurred 6–8 h after transfection.

#### Western blot analysis

Proteins extracted from cells were quantified by applying a modified Lowry assay (Bio-Rad DC protein assay, Bio-Rad Laboratories GmbH, Munich, Germany) using bovine serum albumin (Promega GmbH, Mannheim, Germany) as a reference standard. Equal amounts of protein were then separated under reducing conditions in 10% sodium dodecyl sulfate–polyacrylamide gels, transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare GmbH, Freiburg, Germany) and analyzed by western blot as described previously (22). Detection of GBP-1 was performed using a rat anti-GBP-1 monoclonal antibody (clone 1B1, 1:500) and a sheep anti-rat-horse-radish peroxidase antibody (Dako) as secondary antibody (1:5000). Protein detection was performed using the enhanced chemiluminescence western blot detection system (ECL, GE Healthcare) and Rx-films (Fuji, Tokyo, Japan).

#### Proliferation assay

A mixture (1:1:1) of the three single cell clones of DLD-1 cells stably transfected with pMCV2.2-F-GBP-1 (DLD-1-GBP-1), and a population of cells transfected with the control vector pMCV2.2 (DLD-1-CV), were seeded in triplicates in 24-well plates (Nunc™, Thermo Fisher Scientific, Bonn, Germany) at a density of  $3 \times 10^3$  cells/well. Alternatively, WiDr cells were seeded at a density of  $2.5 \times 10^4$  cells/well in case of transfection with siRNA

and  $1 \times 10^4$  cells/well in case of plasmid transient transfection. HT29 and T84 cells were seeded at a density of  $2 \times 10^4$  cells/well before transient transfection. In case of siRNA transfection, cells were treated with IFN-γ (100 U/ml) 8 h after transfection or left untreated. Medium was renewed every 2 days and cells were incubated up to 168 h. Every 24 h, cells from three wells per group were harvested and total cell numbers (cells/well) were determined using a CASY®TT cell counter (Schärfe System GmbH, Reutlingen, Germany). The cell number determined 4 h after seeding or at the time of transfection was used for normalization.

#### 5-Ethynyl-2'-deoxyuridine incorporation assay

Cells in the phase of active DNA synthesis were detected using the Click-iT® EdU Imaging Kits (Invitrogen/Molecular Probes) according to the protocol provided by the manufacturer. Briefly,  $2.5 \times 10^4$  cells were seeded in the wells of Lab-Tek chamberslides (Nunc™). Cells were transfected as indicated with siRNA or plasmid 24 h after seeding. Six hours after siRNA transfection, cells were treated with IFN-γ (100 U/ml) or left untreated. 5-Ethynyl-2'-deoxyuridine (EdU, 10 µM) was added for 2 h to the cells, 48 h following treatment with IFN-γ or 24 h after plasmid transfection. Cells were subsequently washed, fixed with 100% ethanol at 4°C overnight. Then, the Click-iT® reaction cocktail containing AlexaFluor® 555-azide was added to the cells for 30 min. GBP-1 was detected using a rat monoclonal antibody (clone 1B1, 1:100, hybridoma supernatant). After washing, cells were incubated for 1 h at room temperature with an AlexaFluor®488-conjugated goat antirabbit IgG secondary antibody (diluted 1:500) (Invitrogen/Molecular Probes). Nuclei were counterstained with Hoechst 33342 (1:2000 in water, Invitrogen/Molecular probes) for 30 min at room temperature. Coverslips were mounted in fluorescence mounting medium (Dako, Hamburg, Germany). Fluorescence was visualized using a TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) using a  $\times 20$  magnification. Pictures were obtained using the LAS AF software (Leica Microsystems).

#### Detection of apoptosis

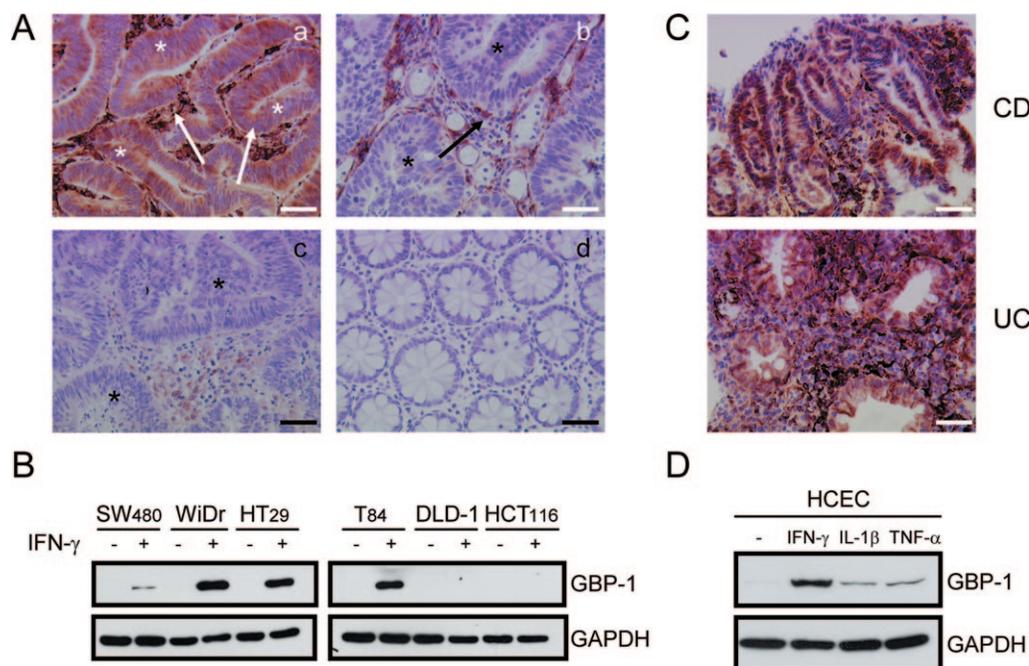
Cells were seeded in the bottom of a six-well plate (Nunc™), transfected and/or treated with IFN-γ as indicated. Apoptosis was detected 24, 48 and 72 h after treatment using the FITC Annexin V Apoptosis Detection kit I (BD Biosciences, Heidelberg, Germany) according to the instructions provided by the manufacturer. Flow cytometry was performed using a FACSCalibur cytometer equipped with the CellQuestPro software (BD Biosciences).

#### Wound healing assay

Cells were grown on gelatine-coated six-well plates (Nunc™) until confluence. Confluent cell monolayers were scratched using a sterile 1 ml pipette tip. Cells were washed twice with phosphate-buffered saline and cultured for 8 days in RPMI + 10% FBS. Images of the same areas were taken immediately after scratching (0 h) and every 2 days using an Olympus digital camera mounted on an Axiovert 25 microscope (Zeiss). The area of the wounding scratches was evaluated at different time points using the TScratch program ([www.cse-lab.ethz.ch](http://www.cse-lab.ethz.ch)) and expressed as relative percentage of the initial area at 0 h, which was set to 100%. All experiments were performed in triplicates, and one representative experiment of four is shown.

#### Invasion assay

The ability of DLD-1-GBP-1 and DLD-1-CV cells to invade a matrix was compared. To this purpose,  $6 \times 10^4$  cells in serum-free medium were seeded in invasion chambers coated with matrigel (BD BioCoat Matrigel Invasion Chambers, BD biosciences). The invasion chambers were incubated in 24-well plates (BD Falcon) containing either RPMI medium supplemented with 10% FBS as chemoattractant or serum-free RPMI as a negative control. In addition,  $6 \times 10^4$  cells in serum-free medium were seeded in uncoated cell culture inserts (BD biosciences) and allowed to migrate toward RPMI + 10% FBS. Cells were incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Non-invading cells were removed from the upper surface of the inserts using a cotton swab. Invading cells at the lower side of the membrane were then fixed with 10% formalin (Sigma–Aldrich) for 10 min and stained with 0.2% crystal violet for 15 min. After washing, pictures of the membranes were taken using an Olympus digital camera mounted on an Axiovert 25 microscope (Zeiss) and the number of invading cells was determined. The percentage of invading cells was calculated as the ratio of the number of cells invading through the matrigel insert membranes to the number of cells migrating through the control insert membranes. Alternatively, the invasion ability of WiDr cells in presence or absence of IFN-γ and siRNA was investigated. To this purpose,  $7.5 \times 10^4$  WiDr cells were seeded in invasion chambers 24 h after treatment with IFN-γ and further incubated for 48 h at 37°C and 5% CO<sub>2</sub>. The percentage of invading cells in presence or absence of IFN-γ is presented. All experiments were performed in triplicates, and one representative experiment of three is shown.



**Fig. 1.** Distribution of GBP-1 expression in CRC. (A) Immunohistochemical staining of GBP-1 in CRC tissues (a–c) and healthy mucosa (d) of different patients. Areas with tumor cells are labeled with asterisks and GBP-1-positive cells appear in brown (arrows). Examples of GBP-1-positive (a and b) and negative (c and d) tissues are given. In positive tumor samples, stroma cells are always positive and tumor cells are either positive (a) or negative (b). Scale bars = 10  $\mu$ m. (B) Expression of GBP-1 in CRC cell lines. Western blot analysis showed that GBP-1 is not expressed by all CRC cell lines after treatment with IFN- $\gamma$ . Immunohistochemical detection of GAPDH demonstrates that equal concentrations of proteins were blotted onto the membranes. (C) GBP-1 is highly expressed in the colon mucosa of individuals with ulcerative colitis (UC) or Crohn’s disease (CD). Immunohistochemical staining of GBP-1 in CD ( $n = 3$ ) and UC ( $n = 2$ ) tissues showed a strong expression of GBP-1 in colon epithelial cells. Scale bars = 10  $\mu$ m. (D) GBP-1 expression is strongly induced by inflammatory cytokines (IFN- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$ ) in human colon epithelial cells. GAPDH was used as a loading control for the western blot experiments.

*Soft agar assay*

Anchorage-independent growth in soft agar was evaluated in order to compare the *in vitro* tumorigenicity of stably transfected DLD-1 cells and control cells, or of IFN- $\gamma$ -treated and untreated WiDr cells in presence or absence of a siRNA directed against GBP-1 or a control siRNA. First, the wells of a 24-well plates (Nunc™) were coated with a solution of RPMI 1640/10% FBS containing 0.5% agar (Invitrogen/Molecular Probes) for 30 min at 37°C. On the top of this layer,  $3 \times 10^3$  cells (DLD-1, clone mix 1:1:1) or  $5 \times 10^3$  cells (WiDr, 24 h after treatment with IFN- $\gamma$ ) embedded in RPMI 1640/10% FBS/0.25% agar were seeded per well in sextuplates. Cells were allowed to grow for 8 days and then were stained with 0.5 ml of 0.005% of crystal violet in 2% ethanol. The average number of colonies was determined by analyzing three different optical fields per well in six different wells per experimental condition (Zeiss Axiovert 25 M, magnification  $\times 20$ ).

*Tumor mouse model*

DLD-1 cells ( $5 \times 10^6$ ) stably transfected with either pMCV2.2-Flag-GBP-1 (DLD-1-GBP-1, cell clones #2.10, #49 and #74.46, mixed 1:1:1) or the parental vector pMCV2.2 (DLD-1-CV, cell population), respectively, were injected subcutaneously into the pectoral mammary fat pad of female Foxn1<sup>tm</sup> mice (20 animals per experimental condition; Jackson Laboratories, Bar Harbor). All animals were implanted subcutaneously with a microchip transponder (BackHome; Virbac, Vienna, Austria) for individual identification. Tumors were measured three times a week in two dimensions, using a caliper, and the tumor volume was calculated according to the formula  $[l \times w \times w/2]$ . The experiment was stopped 33 days after cell injection, when tumor sizes eventually exceeded 2000 mm<sup>3</sup>. Mice were sacrificed and dissected, and the tumor tissue was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use or was fixed in 10% neutral buffered formalin (Sigma–Aldrich) for histological examination. Animal experiments were conducted according to the national guidelines for the care and use of animal.

*Statistical analysis*

Student’s *t*-tests were performed using the GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA).

**Results**

*GBP-1 is differentially expressed in tumor and stroma cells of CRC*

We investigated the spatial distribution of the expression of the GBP-1 protein in a series of 185 CRC tumors (Supplementary Table 1, available at *Carcinogenesis* Online). In order to avoid area-dependent sampling bias, we refrained from using tissue microarray and instead opted for the use of whole tissue sections. For each tumor, two consecutive sections were prepared and stained using an anti-GBP-1 antibody or without primary antibody as a control (Figure 1A). The control sections of GBP-1-positive tumors were consistently negative. Expression was categorized in negative, low, intermediate and high according to the percentage of positive cells. Overall, GBP-1 expression was detected in 88.1% of the samples but only 35.7% of the tumors exhibited a high expression, i.e. they were positive in more

**Table I.** Expression of GBP-1 in CRC patients ( $n = 185$ ) included in the analysis

	N	%
Stroma cells		
Negative	22	11.9
Low	48	25.9
Intermediate	49	26.5
High	66	35.7
Tumor cells		
Negative <sup>a</sup>	105	56.8
Low	39	21.1
Intermediate	22	11.9
High	19	10.3

<sup>a</sup>Samples with negative expression in tumor cells include tumors with negative expression in stroma cells ( $n = 22$ ) as well as tumors positive in stroma cells ( $n = 83$ ).

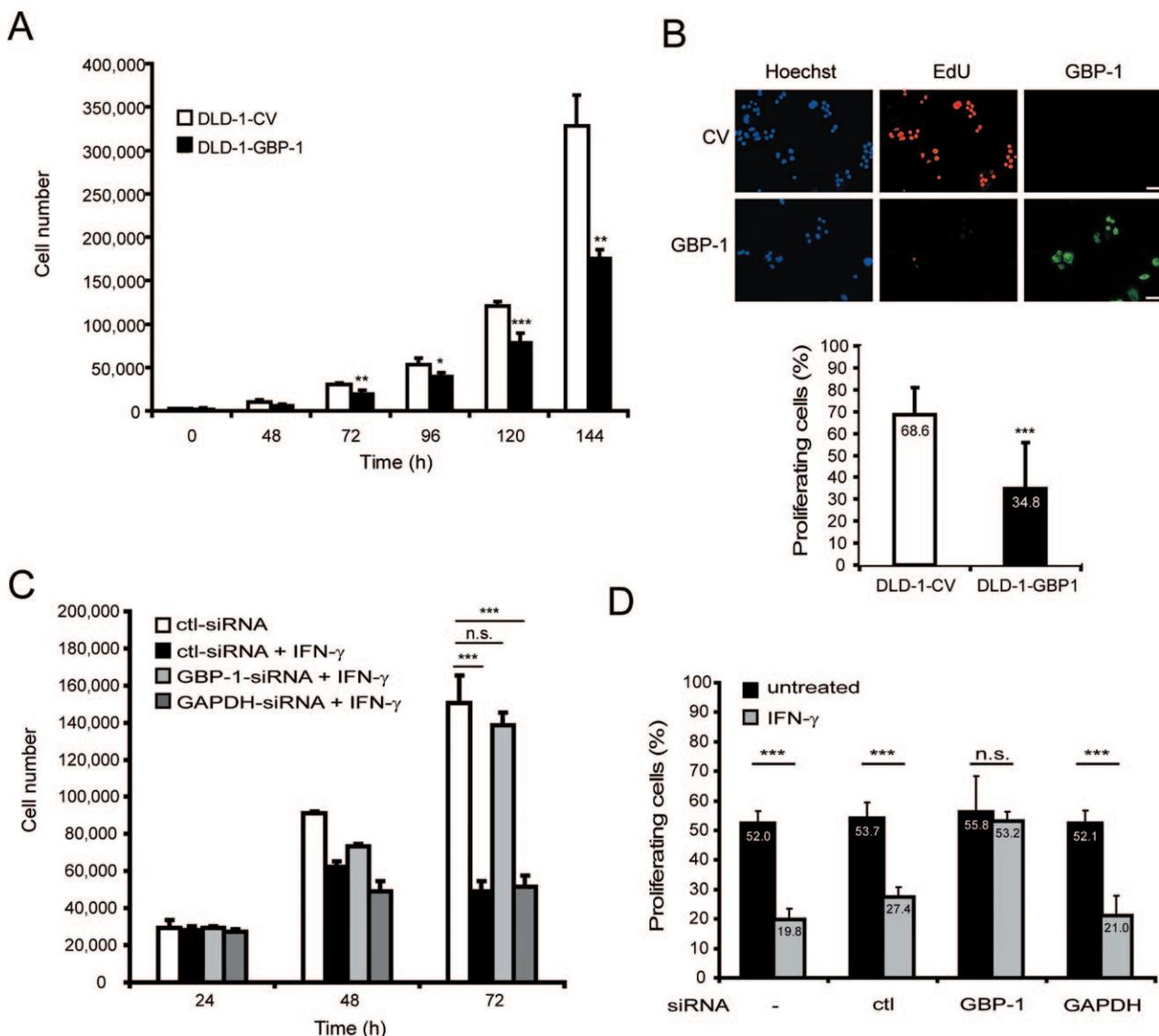
than 75% of the histological section (Table I). The analysis of the spatial distribution of GBP-1 expression revealed that the protein is expressed in the tumor cells only in half (80/163, 49.1%) of the tissues which are positive for GBP-1 in the desmoplastic stroma (Table I). In no case, GBP-1 expression was observed in the tumor cells in absence of a signal in the desmoplastic stroma.

These results suggested that the signal inducing GBP-1 expression may originate from the stroma and that tumor cells of a subgroup of CRC may have become resistant to the inducing factor, which is most likely IFN- $\gamma$ . Accordingly, we investigated the expression of GBP-1 in six CRC cell lines (SW480, WiDr, HCT116, HT29, T84 and DLD-1) in the presence or absence of IFN- $\gamma$  (Figure 1B). None of the cell lines expressed GBP-1 in absence of stimulation. In the presence of IFN- $\gamma$ , GBP-1 was detected in T84, HT29 and WiDr cells, whereas HCT116 and DLD-1 cells did not express the protein, and SW480 cells showed

only a very low expression (Figure 1B). These results are in clear agreement with the *in vivo* situation in CRC. Besides HEK293 cells (38), HCT116 and DLD-1 cells are the only cell lines detected as yet, where GBP-1 expression cannot be induced by IFN- $\gamma$ . On the contrary, a strong GBP-1 signal was generally observed in the colon mucosa of patients with ulcerative colitis and Crohn's disease (Figure 1C). In addition, GBP-1 expression was inducible in non-transformed colon epithelial cells after treatment with IFN- $\gamma$ , interleukin 1-beta or tumor necrosis factor-alpha (Figure 1D). These results suggested that the ability to express GBP-1 may be lost in the tumor cells of CRC.

*GBP-1 inhibits proliferation, migration and invasion of CRC cells*

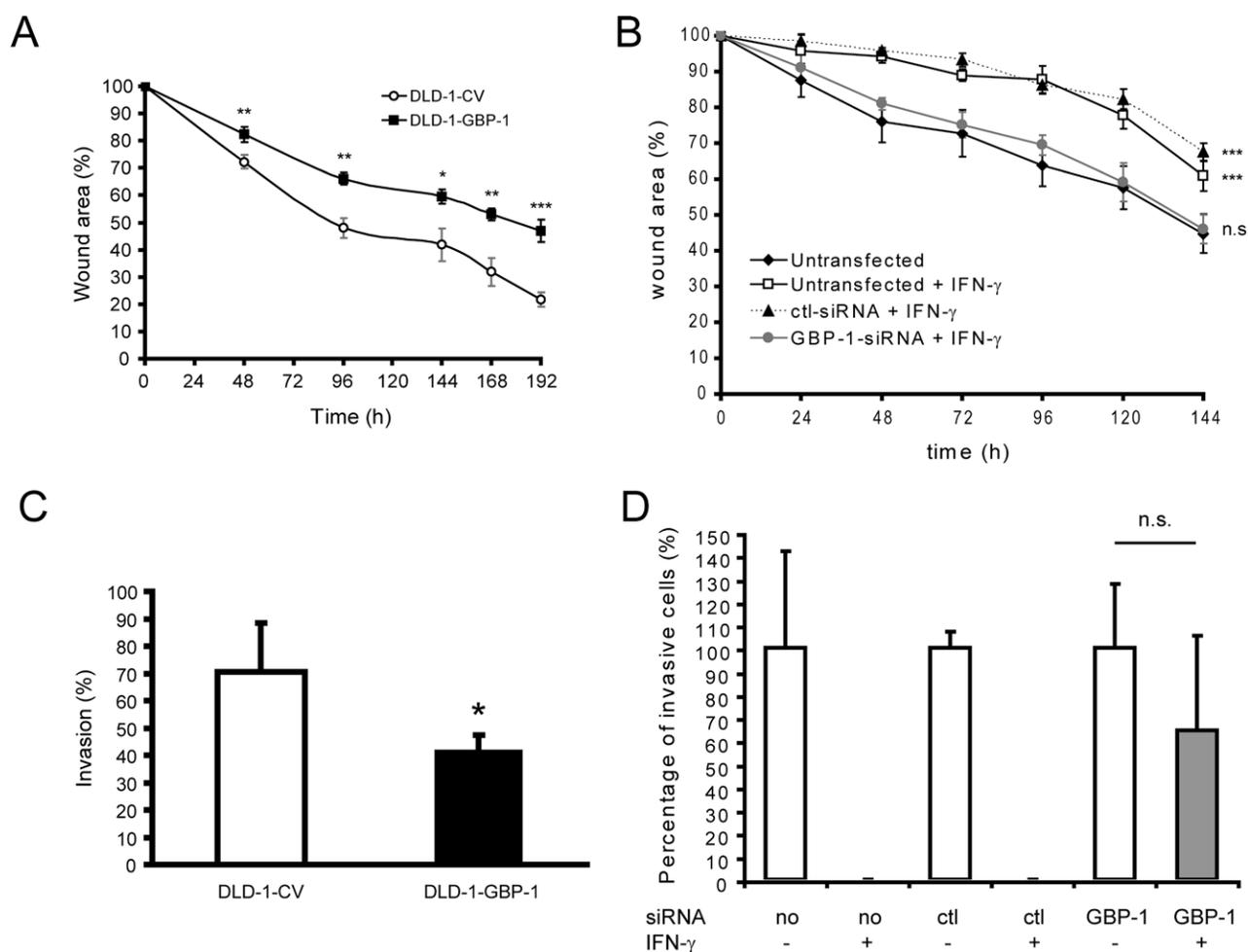
In order to investigate whether GBP-1 expression may cause a growth disadvantage for CRC cells, we reintroduced GBP-1 into DLD-1 cells



**Fig. 2.** GBP-1 inhibits the proliferation of CRC cells. (A) Proliferation assay with DLD-1-GBP-1 (black bars) and DLD-1-CV (white bars) cells was performed in triplicate. Mean cell numbers ( $\pm$ SD) of three independent experiments are indicated. Student's *t*-tests revealed that GBP-1 significantly inhibits the proliferation of DLD-1 cells. (B) Assessment of EdU incorporation in DLD-1-GBP-1 (black bars) and DLD-1-CV (white bars) cells. Proliferating cells having incorporated EdU were visualized after Click-iT reaction with Alexa-Fluor 555 (red); GBP-1-positive cells are displayed in green and the nuclei in blue (scale bars = 75  $\mu$ m). For evaluation, the number of proliferating cells was determined in 10 different optical fields. Mean percentage of proliferating cells ( $\pm$ SD) are indicated. GBP-1-positive cells proliferated significantly less than the control cells (CV) (Student's *t*-test,  $P \leq 0.001$ ). (C) The proliferation of WiDr cells transfected with negative control siRNA (ctl, white bars) was strongly reduced by treatment with IFN- $\gamma$  (100 U/ml, black bars) (Student's *t*-test,  $P \leq 0.001$ ). The inhibition of proliferation was rescued when cells were transfected with GBP-1 siRNA (light gray bars) but not when cells were transfected with GAPDH siRNA (dark gray bars) (Student's *t*-test,  $P \leq 0.001$ ). (D) Assessment of EdU incorporation in WiDr cells in absence (black bars) or in presence (gray bars) of IFN- $\gamma$ . Cells were either untransfected or transfected with negative control siRNA (ctl), GBP-1 siRNA or GAPDH siRNA. Mean percentage of proliferating cells from five different optical fields ( $\pm$ SD) are indicated.

by stable transfection (DLD-1-GBP-1 cells). Subsequently, the proliferation of these cells was compared with control cells stably transfected with the parental vector (DLD-1-CV cells) (for expression control, see [Supplementary Figure 1A](#), available at *Carcinogenesis* Online). A mixture of three independent cell clones was used in order to minimize individual clonal effects. First, the proliferation of DLD-1-CV and DLD-1-GBP-1 cells was compared by cell counting every 24h for 7 days. The proliferation of DLD-1-GBP-1 cells was significantly lower than the proliferation of DLD-1-CV cells (Student's *t*-test after 144h,  $P \leq 0.01$ ) ([Figure 2A](#)). In addition, proliferation was assessed using an EdU incorporation assay. EdU is a nucleoside analog of thymidine, which is incorporated into DNA during active DNA synthesis and is used as an alternative for 5-bromo-2'-deoxyuridine (BrdU). In agreement with the results above, the fraction of proliferating cells having incorporated EdU was significantly lower in DLD-1-GBP-1 cells as compared with DLD-1-CV cells (Student's *t*-test,  $P \leq 0.001$ ) ([Figure 2B](#)).

IFN- $\gamma$  is known to exert antiproliferative effects on various tumor cells including breast cancer, melanoma, CRC and oral squamous carcinoma (39–42). GBP-1 is a major IFN- $\gamma$  target gene and has been shown to mediate the antiproliferative effects of IFN- $\gamma$  in endothelial cells (33). Accordingly, we investigated whether the inhibition of GBP-1 expression might abrogate the antiproliferative effects of IFN- $\gamma$ . WiDr cells were chosen for this purpose since they respond to IFN- $\gamma$  treatment with a robust GBP-1 expression ([Figure 1B](#)). WiDr cells were transfected with GBP-1 siRNA and, as controls, an unrelated negative control siRNA and a GAPDH siRNA. Subsequently, cell numbers were monitored for 72h ([Figure 2C](#)). The expression of GBP-1 and GAPDH was specifically inhibited with the respective siRNA, as demonstrated by western blot ([Supplementary Figure 1B](#), available at *Carcinogenesis* Online). Treatment with IFN- $\gamma$  drastically reduced cell number when cells were transfected with negative control siRNA (ctl) or GAPDH siRNA (Student's *t*-test after 72h,  $P \leq 0.001$ ) ([Figure 2C](#)). However, the inhibitory effect of IFN- $\gamma$  was almost

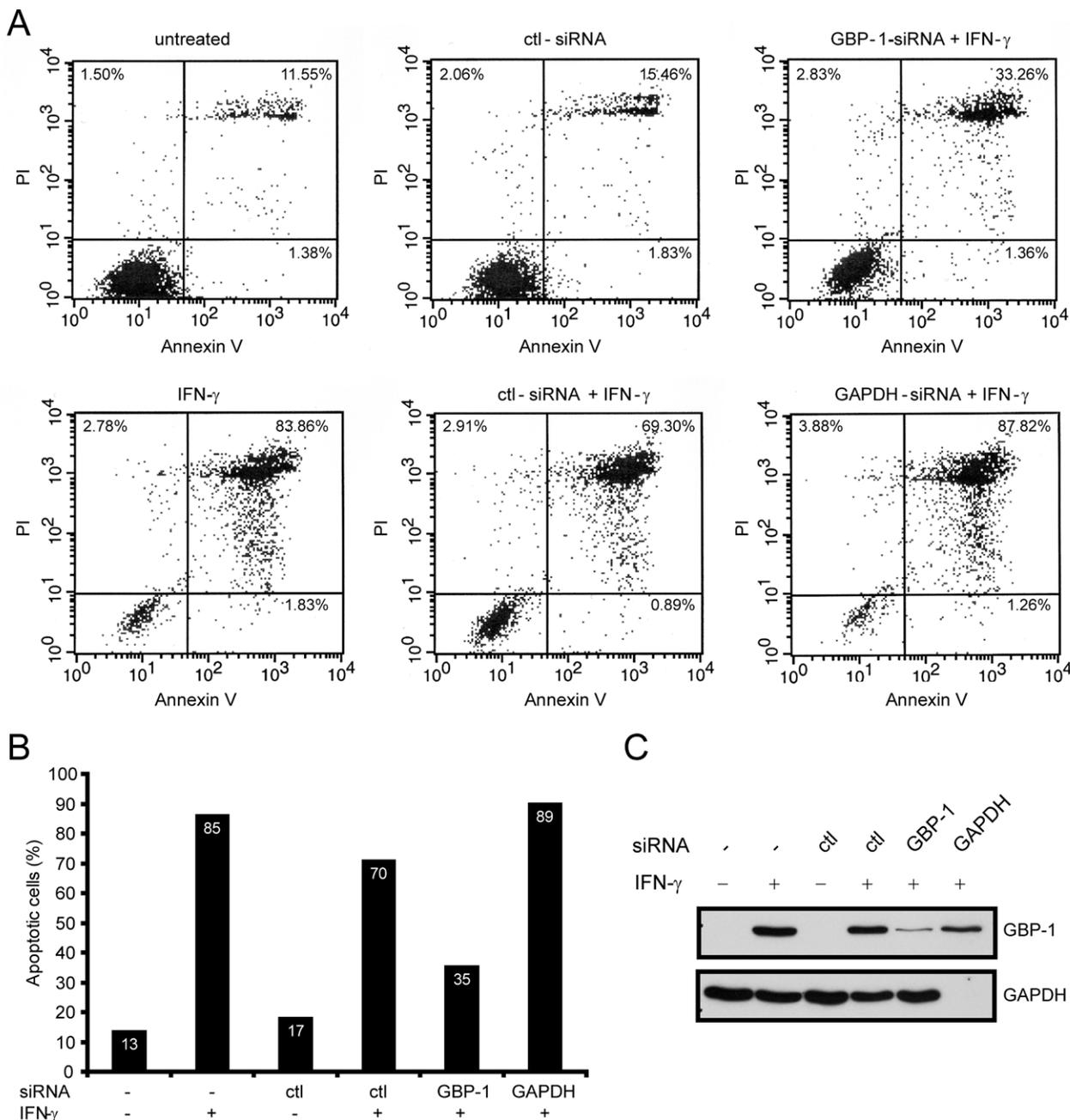


**Fig. 3.** GBP-1 impairs migration and invasion of CRC cell lines. (A) The ability of DLD-1-GBP-1 and DLD-1-CV cells to migrate was investigated using a wound healing assay. *In vitro* scratch wounds were generated by scraping confluent cell monolayers with a sterile pipette tip. Pictures from the same area were acquired immediately after scratching (0h) and every 24h for 8 days. The area of the scratch wounds at different time points was determined relative to the initial area at 0h and is indicated as percentage. Results are means  $\pm$  SD from three replicates. (B) The migration of WiDr cells (black lozenges) was significantly inhibited after treatment with IFN- $\gamma$  (100 U/ml, white squares, Student's *t*-test, 144h,  $P < 0.0001$ ) in a wound healing assay. Silencing of GBP-1 with a siRNA (gray circles) abrogated the effects of IFN- $\gamma$  (Student's *t*-test, 144h,  $P < 0.0001$ ) compared with untreated WiDr (Student's *t*-test, 144h,  $P < 0.0001$ ). Migration was also inhibited in cells transfected with control siRNA (black triangles and dotted line) before treatment with IFN- $\gamma$  compared with untreated WiDr (Student's *t*-test, 144h,  $P < 0.0001$ ). (C) The invasion capability of DLD-1-GBP-1 cells (black bars) through a Matrigel-coated membrane was significantly lower as compared with DLD-1-CV cells (white bars) (Student's *t*-test,  $P \leq 0.05$ ). Invasion was calculated as the ratio of the number of cells invading through the Matrigel-coated membranes to the number of cells migrating through uncoated membranes and is expressed in percents. The mean values ( $\pm$ SD) of three replicates are shown. (D) Treatment with IFN- $\gamma$  (100 U/ml) completely abrogated the invasion capability of WiDr cells (number of invasive cells = 0). This effect was reverted in presence of siRNA against GBP-1 (gray bar) but not in presence of control siRNA. Invasion is expressed in percents of invading cells, with the untreated cells set to 100% for each condition. The mean values ( $\pm$ SEM) of three replicates are shown.

completely abolished in presence of GBP-1 siRNA (Figure 2C). These results could be fully confirmed using EdU incorporation. Forty-eight hours after treatment with IFN- $\gamma$ , cell proliferation rates were approximately halved in WiDr cells and WiDr cells transfected with negative control siRNA (ctl) or GAPDH siRNA (Student's *t*-test,  $P \leq 0.001$ ) (Figure 2D and Supplementary Figure 2A, available at *Carcinogenesis* Online). In contrast, the antiproliferative effect of IFN- $\gamma$  was inhibited in cells transfected with GBP-1 siRNA (Figure 2D and Supplementary Figure 2A, available at *Carcinogenesis* Online). Analyses at the single cell level showed that the transfection of GBP-1

siRNA did not inhibit GBP-1 expression in all WiDr cells in presence of IFN- $\gamma$ . Of note, EdU incorporation was reduced by the half in GBP-1-positive cells as compared with negative cells under these conditions (Student's *t*-test,  $P \leq 0.001$ ) (Supplementary Figure 2B, available at *Carcinogenesis* Online).

In order to investigate whether the expression of GBP-1 alone is able to inhibit the proliferation of CRC tumor cells which are competent to respond to IFN- $\gamma$ , GBP-1 was ectopically expressed in WiDr, HT29 and T84 cells. In each cell lines, the expression of GBP-1 significantly reduced the proliferation (Supplementary Figure 3A-C,



**Fig. 4.** GBP-1 is necessary for IFN- $\gamma$ -induced apoptosis in CRC cell lines. (A) Flow cytometric analysis of Annexin V-FITC/PI double staining: WiDr cells untransfected, transfected with negative control siRNA (ctl-siRNA), GBP-1 siRNA or GAPDH siRNA, were either left untreated or treated with IFN- $\gamma$  (100 U/ml) for 72h. Untreated cells were primarily Annexin V-FITC and PI negative, indicating that they were viable and not undergoing apoptosis. After treatment, four populations of cells were detected: viable cells (Annexin V-FITC/PI negative), dead cells (Annexin V-FITC negative/PI positive), early apoptotic (Annexin V-FITC positive/PI negative) and late apoptotic cells (Annexin V-FITC positive/PI positive). (B) Percentage of Annexin V-FITC positive/PI negative and Annexin V-FITC/PI positive cells per  $10^4$  cells counted in the above experiment (A). (C) The expression of GBP-1 and GAPDH in the cells subjected to flow cytometric analysis was controlled by western blot.

available at *Carcinogenesis* Online). In addition, ectopic expression of GBP-1 reduced the number of proliferating cells in an EdU incorporation assay (Supplementary Figure 3D, available at *Carcinogenesis* Online). This confirmed that GBP-1 is both necessary and sufficient to mediate the antiproliferative effects of IFN- $\gamma$  in CRC cells.

The ability to migrate and invade tissues is a common feature of tumor cells. GBP-1 has been described to exert antimigratory and anti-invasive effects in endothelial cells, thereby preventing angiogenesis (34,35). On the contrary, GBP-1 has been shown to promote invasion of some glioblastoma cells and oral squamous cell carcinoma cells (43,44). Therefore, we tested the effects of GBP-1 on migration and invasion in CRC cells. Migration was investigated using a wound healing assay (Figure 3A). Migration of stably transfected DLD-1-GBP-1 cells was significantly lower than the migration of DLD-1-CV cells (Student's *t*-test at 192h,  $P \leq 0.001$ ) (Figure 3A). Furthermore, the inhibition of migration induced by IFN- $\gamma$  in WiDr cells was annihilated in the presence of siRNA directed against GBP-1 but not in the presence of a control siRNA (Figure 3B). Hence, GBP-1 mediates the antimigratory effects of IFN- $\gamma$  in CRC cell lines. Next, we compared the invasion capability of DLD-1-GBP-1 and DLD-1-CV cells through a Matrigel matrix (Figure 3C). Invasion was significantly reduced for DLD-1-GBP-1 cells as compared with DLD-1-CV cells (Student's *t*-test,  $P \leq 0.05$ ). Treatment of WiDr cells with IFN- $\gamma$  completely inhibited cell invasion (Figure 3D). This effect was reverted when GBP-1 expression was silenced with siRNA but not in presence of a control siRNA (Figure 3D). This showed that GBP-1 is necessary and sufficient to inhibit both migration and invasion of CRC cell lines.

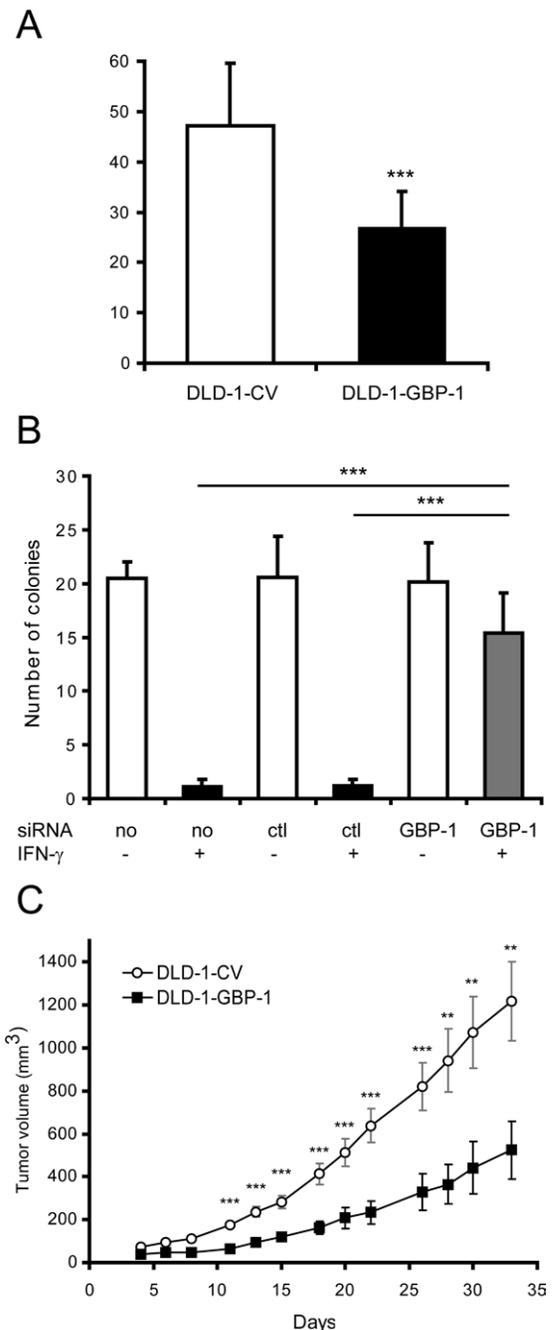
*GBP-1 is required for IFN- $\gamma$ -induced apoptosis in CRC cells*

IFN- $\gamma$  is known to exert proapoptotic effects on cells [for review, see (45)]. In addition, previous studies in endothelial cells indicated that GBP-1 may also modulate apoptosis in interferon-treated cells (46). Accordingly, the antitumorigenic effects of GBP-1 in IFN- $\gamma$ -treated colorectal cancer cells could be partially due to the induction of cell death. Therefore, we investigated the induction of apoptosis by GBP-1 using flow cytometry. DLD-1-GBP-1 and DLD-1-CV cells exhibited similar ratios of early (Annexin V positive) and late (Annexin V/PI positive) apoptotic cells, which were below 20% (Supplementary Figure 4A, available at *Carcinogenesis* Online). Similarly, no difference in the percentage of apoptotic cells was observed between WiDr cells transiently transfected with a GBP-1 expression vector or the respective control vector (Supplementary Figure 4B, available at *Carcinogenesis* Online).

However, treatment of WiDr cells with IFN- $\gamma$  significantly increased the percentage of apoptotic cells up to 85% (Figure 4A and 4B) and induced the expression of GBP-1 (Figure 4C). This remained unchanged when the cells had been previously transfected with negative control siRNA (ctl) or GAPDH siRNA (70 and 89% of apoptotic cells, respectively) (Figure 4A–C). In the presence of GBP-1 siRNA, the induction of GBP-1 expression by IFN- $\gamma$  was almost completely abrogated (Figure 4C) and the rate of apoptosis was reduced to 35%, demonstrating that GBP-1 is required for IFN- $\gamma$ -induced cell death (Figure 4A and 4B). This suggested that GBP-1 is necessary but not sufficient to induce apoptosis in CRC cells.

*GBP-1 exerts antitumorigenic effects in CRC cells in vitro and in vivo*

Finally, we investigated whether GBP-1 expression exerts antitumorigenic effects in CRC cells. First, anchorage-independent growth of DLD-1-GBP-1 and DLD-1-CV cells was compared (Figure 5A). DLD-1-GBP-1 cells developed significantly less clones in 2.5% soft agar than DLD-1-CV cells (Student's *t*-test,  $P \leq 0.0001$ ). Moreover, we showed that treatment with IFN- $\gamma$  inhibited the colony formation of WiDr cells in agar (Figure 5B). In presence of siRNA against GBP-1, but not of control siRNA, anchorage-independent growth of WiDr cells was restored (Figure 5B). These results showed that GBP-1 reduces the tumorigenicity of colorectal tumor cells *in vitro*. In order to investigate the effect of GBP-1 expression on the growth of DLD-1-derived tumors *in vivo*,



**Fig. 5.** GBP-1 exerts antitumorigenic effects in CRC cells. (A) DLD-1-GBP-1 cells developed significantly less colonies in soft agar (2.5%) as control cells (DLD-1-CV) (Student's *t*-test,  $P \leq 0.0001$ ). Mean cell numbers ( $\pm$ SD) of six independent experiments are indicated. One of four experiments with identical outcome is shown. (B) Treatment with IFN- $\gamma$  (100 U/ml) inhibited colony formation of WiDr cells in soft agar. This effect was reverted in presence of siRNA against GBP-1 (gray bar, Student's *t*-test,  $P \leq 0.0001$ ) but not in presence of control siRNA. The number of colonies in IFN- $\gamma$ -treated cells transfected with siRNA against GBP-1 was significantly increased compared with untransfected or ctl-siRNA transfected WiDr (Student's *t*-test, both  $P \leq 0.001$ ). Mean cell numbers ( $\pm$ SD) of 12 independent wells are indicated. One of two experiments with identical outcome is shown. (C) GBP-1 inhibits tumor growth in an immune-deficient mouse model. Tumor growth was monitored in Foxn1<sup>nu</sup> mice injected with  $5 \times 10^6$  DLD-1 cells stably transfected either with pMCV2.2-Flag-GBP-1 (DLD-1-GBP-1, filled square) or the parental vector pMCV2.2 (DLD-1-CV, open circle). Mean tumor volumes ( $\pm$ SEM) of individual animals are indicated. Animals that did not develop a tumor at the end of the experiment or died due to unrelated reasons were excluded. Statistical significance (*P*-values) was determined applying a Student's *t*-test.

DLD-1-GBP-1 and DLD-1-CV cells were injected into the mammary fat pad of immune-deficient Foxn1<sup>tm</sup> mice (for expression controls, see [Supplementary Figure 5A](#), available at *Carcinogenesis* Online). Tumor growth was monitored for 33 days ([Figure 5C](#)). At the end of the experiment, tumors were resected and the expression of GBP-1 was confirmed by immunohistochemistry ([Supplementary Figure 5B](#), available at *Carcinogenesis* Online). From day 4 on, tumors derived from DLD-GBP-1 cells were significantly smaller than tumors derived from DLD-1-CV (at day 33, Student's *t*-test,  $P \leq 0.01$ ) ([Figure 5C](#)). This experiment was repeated using another immune-deficient mouse strain (NMRI) with identical outcome ([Supplementary material and methods](#) and [Supplementary Figure 5C](#), available at *Carcinogenesis* Online). These data showed that GBP-1 exerts antitumorigenic effects on CRC cells *in vitro* as well as *in vivo*.

## Discussion

GBP-1 is an inducible protein, which is produced in cells stimulated by inflammatory cytokines, IFN- $\gamma$  being the strongest inducer (21–23). IFN- $\gamma$  has been proposed as a major player of antitumor immunity since IFN- $\gamma$ -knock-out mice have been shown to be more prone to tumor development (12,47). In the present study, we investigated the expression of GBP-1 in CRC and focused on the distribution of the protein expression within the tumor. Overall, the percentage of positive tumors observed in our study was 88.1% as compared with 30% positivity reported in a tissue microarray study of 388 tumors described previously by our group. The apparent difference is due to the rating of low positive specimen. In the tissue microarray, samples were graded positive only when more than 33% (at least two of three samples obtained from a tumor) were positive. In the present study, which focused on the expression pattern of a whole tumor section, a positive rating was given when more than 10% of the cells were positive, explaining the higher percentage of positive tumors. In the present cohort, less than half of the tumors expressing GBP-1 in the stroma also expressed the protein in the tumor cells. This suggested that GBP-1 expression had been lost in tumor cells. On the contrary, normal colon epithelial cells of inflammatory diseases such as Crohn's disease or ulcerative colitis produced high levels of GBP-1 [this study and (48)], indicating that epithelial cells are generally able to express GBP-1. The absence of GBP-1 expression in tumor cells might result from a downregulation, due to gene mutation or epigenetic changes, but might also be the consequence of an impaired IFN- $\gamma$  response pathway. Defects in the IFN- $\gamma$  response pathway have been observed in cell lines derived from prostate carcinoma and melanoma or in tissue samples of squamous cell carcinoma (49–52). In addition, the loss of responsiveness to IFN- $\gamma$  in terms of proliferation and HLA expression has been observed in some CRC cell lines (40). In particular, DLD-1 and SW480 cells were resistant to IFN- $\gamma$  treatment, whereas WiDr cells exhibited full sensitivity (40). These results are well in agreement with the patterns of GBP-1 expression observed in the present study. Therefore, we concluded that the absence of GBP-1 expression in the tumor cells of CRC might rather be due to an acquired resistance of tumor cells to IFN- $\gamma$  than to mutation of the *GBP1* gene or epigenetic changes.

Direct antitumor effects are considered as one of the mechanisms contributing to immunosurveillance and tumor rejection by IFN- $\gamma$  (12,45,53). In the present study, we showed that GBP-1 mediates the antitumorigenic effects of IFN- $\gamma$  on CRC tumor cells both *in vitro* and *in vivo*. In particular, the *in vivo* reconstitution of GBP-1 expression in CRC cells induces a strong reduction of tumor size, as also previously observed in another model using mouse mammary carcinoma cells (54). GBP-1 reconstitution experiments as well as siRNA-mediated inhibition of GBP-1 demonstrated that GBP-1 is both, necessary and sufficient, to inhibit of cell proliferation, migration and invasion. These results are in agreement with the well-documented antiproliferative, antimigratory and anti-invasive effects of GBP-1 in endothelial cells (22,33,34). In contrast, GBP-1 has been shown to activate invasion in glioblastoma cells, where its expression can be quite unusually induced by the epidermal growth factor receptor pathway (43), and

in oral squamous carcinoma cell lines, which constitutively express GBP-1 (44). These findings may indicate pathway- or cell type-specific activity of GBP-1.

IFN- $\gamma$  has been shown to exert paradoxical effects on apoptosis (12,45,53). In particular, hematopoietic cells are protected against IFN- $\gamma$ -induced cell death. Epithelial cells and most tumor cells are sensitive to IFN- $\gamma$ -induced apoptosis, which remains however tightly regulated (12,45,53). In the present work, we observed that GBP-1 is required for the proapoptotic effects of IFN- $\gamma$  in CRC cells. However, the effects of GBP-1 on apoptosis and cell death seem to be time and cell dependent. In endothelial cells, the induction of GBP-1 by IFN- $\alpha$  leads to a short-term inhibition of apoptosis followed by an induction of senescence (46). In human CRC cells, GBP-1 was found to exert a protective role against apoptosis after short-term IFN- $\gamma$  treatment (48), whereas we reported here that GBP-1-mediated IFN- $\gamma$ -induced apoptosis after longer incubation times. However, our data showed that GBP-1 alone was not sufficient to induce apoptosis suggesting a role of GBP-1 in the regulation rather than in the execution of IFN- $\gamma$ -induced cell death.

In CRC, the presence of an IFN- $\gamma$ -dominated Th1 adaptive immune response is associated with a better prognosis for the patients (7–9). Furthermore, the expression of GBP-1 correlates with the expression of other IFN- $\gamma$ -target genes and the presence of a Th1-type response in CRC, breast cancer and melanoma metastases (20,55,56). In breast cancer, GBP-1 expression in the stroma is associated with elevated numbers of tumor-infiltrating cells and the absence of tumor relapse (56). In CRC, expression of GBP-1 in association with other interferon-stimulated genes is highly significantly associated with a prolonged cancer-related 5-year survival and represents an independent prognostic marker for a halved risk of death (20). A recent comprehensive study of the Cancer Genome Atlas Network fully confirmed these results (36). Moreover, GBP-1 expression in tissue seems to be related to a transcriptional signature of immune-mediated tissue-specific destruction observed not only in tumors but also in case of infection, allograft rejection or autoimmune diseases (57). This phenomenon has been called 'immunologic constant of rejection' and is characterized notably by the expression of interferon-stimulated genes (58,59). In this framework, our findings indicate that GBP-1 may not only represent a marker for the presence of an IFN- $\gamma$ -dominated immune response in tissues but may be a central execution molecule in immune-mediated tumor destruction.

In conclusion, we showed that GBP-1 induces a decrease of tumorigenicity in CRC tumor cells, characterized by an inhibition of cell proliferation, migration and invasion, suggesting that GBP-1 acts as a tumor suppressor in CRC. Furthermore, the loss of sensitivity to IFN- $\gamma$  observed in several CRC cell lines and the specific loss of GBP-1 expression in CRC tumor cells indicate a possible mechanism of tumor escape from the IFN- $\gamma$ -dominated antitumor immune response.

## Supplementary material

Supplementary materials and methods, Table 1 and Figures 1–5 can be found at <http://carcin.oxfordjournals.org/>

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