

# NFAT promotes carcinoma invasive migration through glypican-6

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Invasive migration of carcinoma cells is a prerequisite for the metastatic dissemination of solid tumours. Numerous mechanisms control the ability of cancer cells to acquire a motile and invasive phenotype, and subsequently degrade and invade the basement membrane. Several genes that are up-regulated in breast carcinoma are responsible for mediating the metastatic cascade. Recent studies have revealed that the NFAT (nuclear factor of activated T-cells) is a transcription factor that is highly expressed in aggressive breast cancer cells and tissues, and mediates invasion through transcriptional induction of pro-invasion and migration genes. In the present paper we demonstrate that NFAT promotes breast carcinoma invasion through induction of GPC (glypican) 6, a cell-surface glycoprotein. NFAT transcriptionally regulates GPC6 induction in breast cancer cells and binds

to three regulatory elements in the GPC6 proximal promoter. Expression of GPC6 in response to NFAT signalling promotes invasive migration, whereas GPC6 silencing with shRNA (small-hairpin RNA) potently blocks this phenotype. The mechanism by which GPC6 promotes invasive migration involves inhibition of canonical  $\beta$ -catenin and Wnt signalling, and up-regulation of non-canonical Wnt5A signalling leading to the activation of JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase). Thus *GPC6* is a novel NFAT target gene in breast cancer cells that promotes invasive migration through Wnt5A signalling.

**Key words:** breast cancer, cancer invasion, glypican, nuclear factor of activated T-cells (NFAT), transcription factor.

## INTRODUCTION

NFAT (nuclear factor of activated T-cells) was originally identified in immune cells as a transcription factor required for the inducible expression of cytokines critical for triggering the immune response [1,2]. Previous studies have revealed that NFAT is ubiquitously expressed and regulates a plethora of transcriptional responses important for cell survival, angiogenesis and cell growth in all cells and tissues [2,3]. Four distinct NFAT family members, NFAT1, NFAT2, NFAT3 and NFAT4, bear considerable sequence similarity and are calcium-responsive [4]. The more distantly related NFAT5 isoform is calcium-insensitive [1]. The mechanisms of NFAT activation comprise one of the best understood calcium-sensitive signalling mechanisms. In resting cells, NFAT is sequestered in a hyperphosphorylated inactive conformation in the cytoplasm and, upon stimulation with ligands that elicit sustained elevation of intracellular calcium, NFAT is dephosphorylated by the serine/threonine phosphatase calcineurin and translocates to the nucleus [5]. A cycle of NFAT relocation to the cytoplasm is mediated by nuclear export kinases including CK1 (casein kinase 1), GSK3 (glycogen synthase kinase 3) and DYRKs (dual-specificity tyrosine-phosphorylated and -regulated kinases) [1]. Classical NFATs typically interact with other transcription factors such as AP-1 (activator protein-1) and GATA proteins to activate transcription [4].

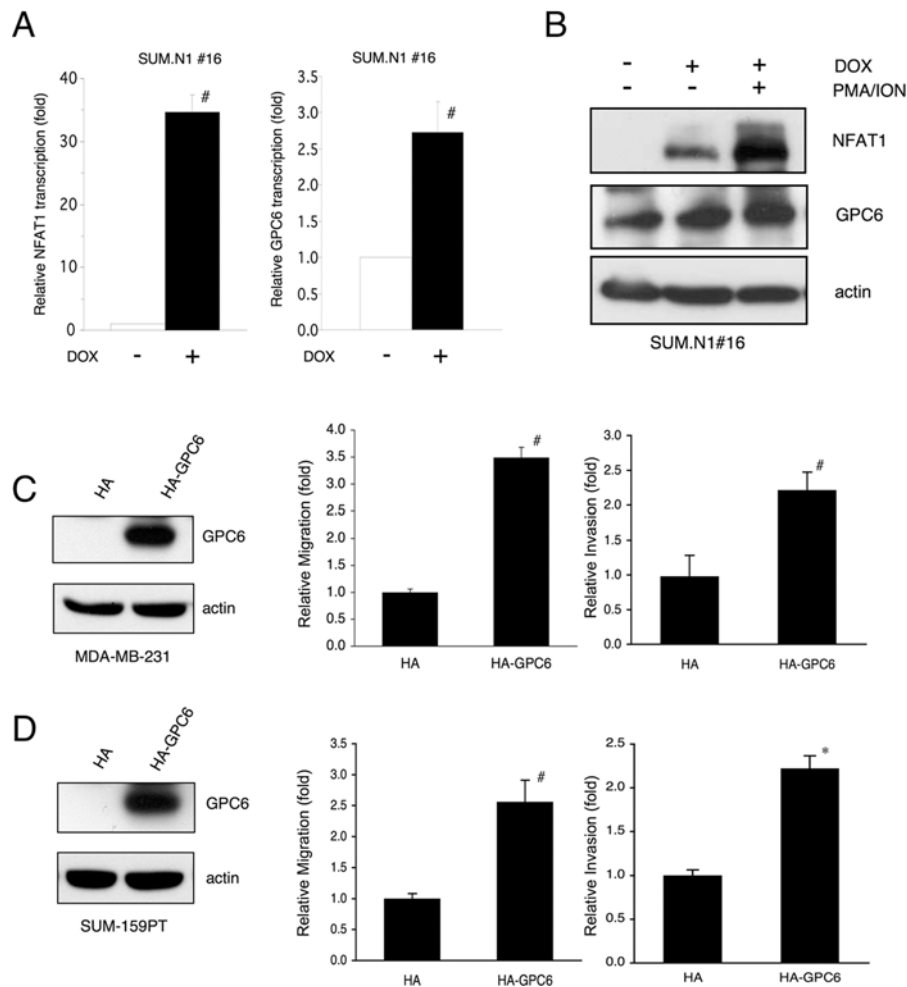
A recent study has demonstrated a role for NFAT signalling in tumorigenesis [3]. Various NFAT isoforms promote cell growth and cellular survival. Isoform-specific functions for NFATs are likely to exist as NFAT1 appears to function as a tumour suppressor, whereas NFAT2 has oncogenic activity [6].

Tumour angiogenesis is a key determinant in the progression of human solid tumours leading to growth, invasive and metastatic dissemination of tumour cells [7,8]. Previous studies have demonstrated a key function for NFAT in both development and tumour angiogenesis. A role for NFAT signalling in angiogenesis is best illustrated in patients with Down's syndrome who have a lower incidence of tumour incidence. One explanation for this finding is the increased expression of *DSCR1* (Down's syndrome critical region 1) and *DYRK1A* that negatively regulate NFAT activity and terminate NFAT-dependent gene transcription [9,10].

NFAT signalling also promotes the invasive migration of tumour cells. Both NFAT1 and NFAT5 promote the migration and invasion of breast and colon cancer cells [11]. NFATs probably function to modulate invasion through the induction of promotility and invasion genes. Autotaxin, also known as ENPP2 (exonucleotide pyrophosphatase and phosphodiesterase 2), an enzyme that catalyses the synthesis of LPA (lysophosphatidic acid), is transcriptionally induced by NFAT in breast cancer cells and promotes invasion [12]. Transgenic mice harbouring autotaxin in the mammary epithelium have increased frequency of invasive and metastatic carcinoma [13]. Similarly, NFAT induces the transcription of the *COX2* (cyclo-oxygenase-2) gene in cancer cells thereby enhancing invasive migration [14]. COX-2 is responsible for the synthesis of PGE<sub>2</sub> (prostaglandin E<sub>2</sub>), a potent pro-invasion factor. Thus NFAT signalling in tumour cells induces a transcriptional programme of genes that includes factors that promote invasive migration leading to metastatic dissemination. In the present paper we identify a novel target of NFAT, GPC (glypican) 6, that modulates breast cancer cell invasive migration acting through non-canonical Wnt5a signalling.

Abbreviations used: AP-1, activator protein-1; COX-2, cyclo-oxygenase-2; dox, doxycycline; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; ENPP2, exonucleotide pyrophosphatase and phosphodiesterase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC, glypican; JNK, c-Jun N-terminal kinase; HA, haemagglutinin; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells; PEI, polyethylimine; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; RT, reverse transcription; shRNA, small-hairpin RNA; siRNA, small interfering RNA; TCF, T-cell factor.

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**Figure 1** Activation of NFAT1 enhances GPC6 expression and invasive migration

(A) Total RNA extracted from control or dox-treated SUM.N1#16 cells assayed for NFAT and GPC6 expression by RT-PCR. (B) Protein extracts from untreated cells or cells stimulated with dox (1  $\mu$ g/ml) with or without PMA/ionomycin (ION) (100nM) immunoblotted with anti-HA, anti-GPC6 or anti-actin antibodies. (C) MDA-MB-231 cells transfected with empty vector (HA) or HA-GPC6 and assayed for Transwell migration and Matrigel invasion. GPC6 expression was confirmed by immunoblotting with anti-HA antibodies. (D) Migration and Matrigel invasion of HA-GPC6-transfected SUM-159PT cells. HA-GPC6 expression was revealed by immunoblotting with anti-HA antibody. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. \* $P < 0.05$  and # $P < 0.01$  between the control and transfected cells using an unpaired Student's *t* test.

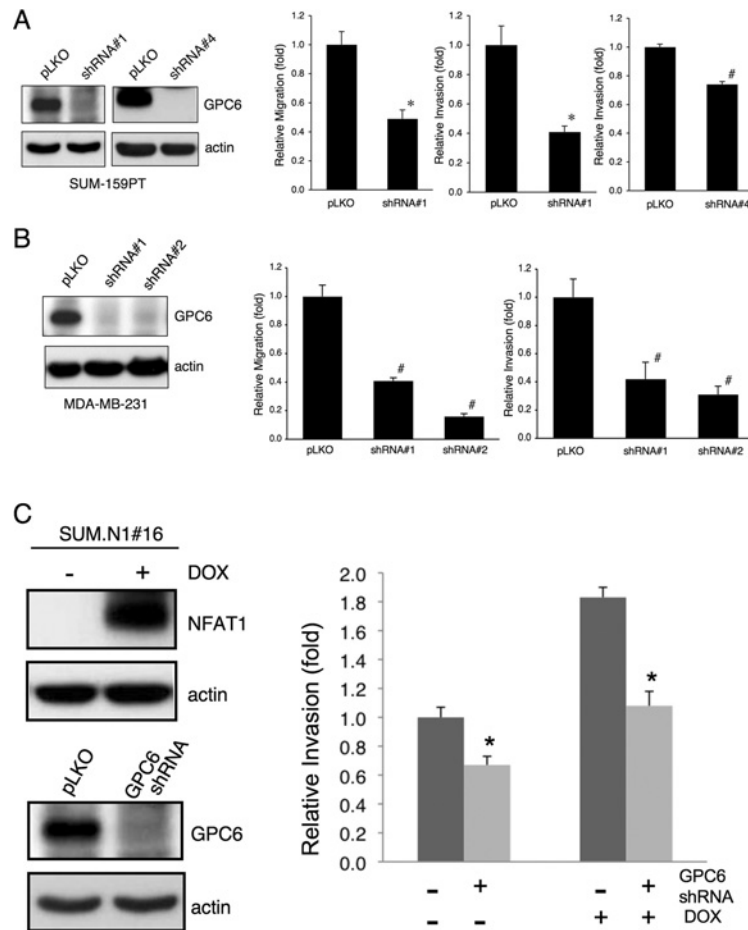
## EXPERIMENTAL

### Cell culture

The human cancer cell lines MDA-MB-231, MCF-7 and HeLa were maintained in DMEM (Dulbecco's modified Eagle's medium) with 1 g/ml glucose, L-glutamine and sodium pyruvate (Mediatech) supplemented with 10% fetal bovine serum (Novo-Tech). The oestrogen-independent breast cancer cell line SUM-159PT was grown in Ham's F-12 medium with L-glutamine (Cambrex) supplemented with 5% fetal bovine serum, 1  $\mu$ g/ml hydrocortisone and 5  $\mu$ g/ml insulin (Sigma-Aldrich). The SUM.N1#16 breast cancer cells with inducible NFAT1 expression have been described previously [14] and were grown in the culture medium for SUM-159PT cells with 20  $\mu$ g/ml blasticidin and 0.1 mg/ml zeocin (Invivogen). Cell lines were verified by multiple methods including DNA barcoding, gene expression and transcriptome analysis, and were kept in culture for less than 6 months after receipt. Expression of NFAT1 was induced with 1  $\mu$ g/ml dox (doxycycline; Clontech) for 16–24 h at 37  $^{\circ}$ C. For NFAT1 activation, breast cancer cells were treated with 50–100 nM PMA (Alexis Biochemicals) and ionomycin (EMD Chemicals) for 16–20 h.

### Plasmids

The HA (haemagglutinin)-NFAT expression vector has been described previously [14]. The complete coding sequence of human GPC6 in pREP4 (gyp6-pREP4) and the human genomic PAC clone 1177C17 that contains the GPC6 promoter were provided by Dr Guido David (VIB Department of Molecular and Developmental Genetics, Leuven, Belgium) [15]. HA-GPC6 was constructed by first cloning an in-frame HA epitope tag between the signal sequence and the coding region of GPC6. The HA-tagged GPC6 was then subcloned into pcDNA3. The human GPC6 proximal promoter from -551 to +2 was prepared by PCR using PAC 1177C17 as DNA template, with forward primer (5'-GTCGCTCGAGTGTATTCTGTATCGAAGGC-3') and reverse primer (5'-GCTGATAAGCTTGGAGACAGTCAGTGGAGG-3'). The amplified DNA was inserted into the XhoI/HindIII sites of the promoter-less luciferase reporter plasmid pGL3-basic (Promega) to generate Pr-551. Other GPC6 promoter constructs were similarly prepared using Pr-551 as a template. To delete the NFAT and AP-1 sites in Pr-551, specific PCR primer pairs with matching restriction enzyme sites at the 5'-ends were used. After amplification using Pr-551 as a template, the open ends of



**Figure 2** Silencing GPC6 expression reduces invasive migration

SUM-159PT (A) and MDA-MB-231 (B) cells transfected with GPC6-specific shRNA in pLKO and assayed for migration and Matrigel invasion. GPC6 silencing by shRNA was confirmed by immunoblotting. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. \* $P < 0.05$  and # $P < 0.01$  between the control and transfected cells using an unpaired Student's *t* test. (C) SUM.N1#16 cells transfected with empty knockdown vector pLKO or GPC6-specific shRNA#1. Invasion of the transfected cells in Matrigel was determined *in vitro* with or without adding dox to induce exogenous NFAT expression. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. \* $P < 0.05$  between the cells transfected with pLKO or GPC6 shRNA in pLKO, with or without adding dox using an unpaired Student's *t* test. NFAT activation and GPC6 silencing were confirmed by immunoblotting.

the PCR fragments obtained were digested with the appropriate restriction enzymes and ligated. The triple mutant construct was similarly generated by PCR using the mutant Pr-551 plasmid as a DNA template. Sequences of all constructs were confirmed by DNA sequencing.

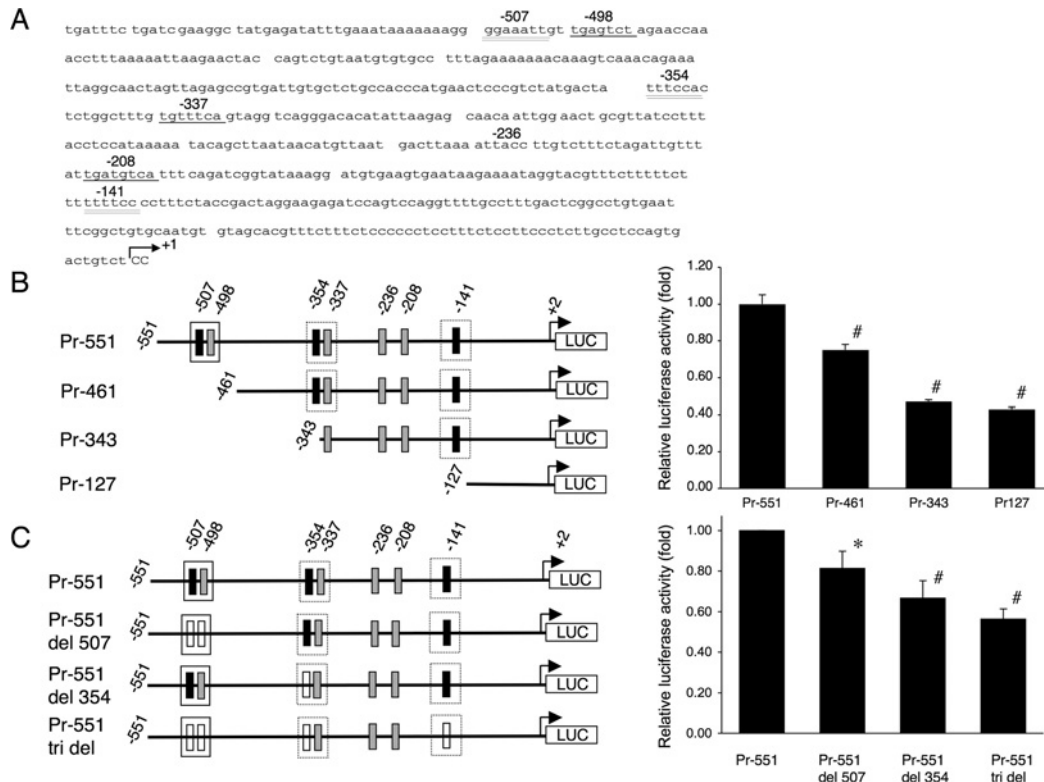
GPC6 shRNA (small-hairpin RNA) was generated by cloning the annealed oligonucleotides into the EcoRI/AgeI site of the pLKO.1-puro lentiviral vector. The sequences of the oligonucleotides were: shRNA#1, 5'-CCGGAAGCCAGATACTTGCCCTGAGCTCGAGTCTCAGGCAAGTATCTGGCTTTTTTTG-3' and 5'-AATTCAAAAAAGCCAGATACTTGCCCTGAGACTCGAGTCTCAGGCAAGTATCTGGCTT-3'; shRNA#2, 5'-CCGGAATCTGTCCTCAGGAATATACCTCGAGGTATATTCCTGAGGACAGATTTTTTTG-3' and 5'-AATTCAAAAAATCTGTCC-TCAGGAATATACCTCGAGGTATATTCCTGAGGACAGATT-3'; and shRNA#4, 5'-CCGGCACAGCAAAGCCAGATACTTCTCGAAGTATCTGGCTTTGCTGTGCTTTTTG-3' and 5'-AATTCAAAAAAGCACAGCAAAGCCAGATACTTCTCGAAGTATCTGGCTTTGCTGTGCT-3'. Wnt5A (S14873) and Wnt5B (S37481) siRNAs (small interfering RNAs) were obtained from Applied Biosystems. The tetracycline-regulated pLKO shRNA vector has been described previously [16], and was used to generate tet-pLKO NFAT1 shRNA, based on sequences published previously [14].

### Immunoblotting

Cell lysates were prepared for immunoblotting as described previously [14]. The anti-HA antibody was purified in-house from the 12CA5 hybridoma. Anti- $\beta$ -actin antibody was from Sigma-Aldrich. Monoclonal anti-GPC6 antibody was from LifeSpan BioSciences. Antibodies specific to Wnt5A/B, total Akt, and phospho-Akt (Ser<sup>473</sup>) were from Santa Cruz Biotechnology. Antibodies specific against JNK (c-Jun N-terminal kinase), phospho-JNK, p38 MAPK (mitogen-activated protein kinase) and phospho-p38 MAPK were from Cell Signaling Technology.

### Real-time RT (reverse transcription)-PCR

Total RNA extracted from cultured cells using TRIzol<sup>®</sup> (Invitrogen) was reverse-transcribed into cDNA using Taqman reverse transcriptase and oligo(dT)<sub>16</sub> (Roche). Quantitative real-time PCR was performed in an ABI Prism 7700 sequence detector using SYBR Green PCR master mix (Applied Biosystems). The PCR consists of a polymerase-activating step of 95 °C for 10 min, followed by 40 cycles of a two-step cycling programme (95 °C for 15 s; 60 °C for 1 min) for the detection of NFAT1, GPC6 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or a



**Figure 3** NFAT regulates the transcription of GPC6

(A) Nucleotide sequence of the human GPC6 putative promoter region from  $-551$  to  $+2$ . The transcriptional start site is indicated by an arrow and is assigned  $+1$ . The potential NFAT-binding sites are double underlined and putative AP-1 sites are single underlined. The numbers indicate the positions of the *cis*-regulatory elements relative to the transcriptional start site. (B) Schematic representation of the GPC6 deletion constructs. The filled boxes and stippled boxes represent putative NFAT- and AP-1-binding sites respectively. The results of luciferase assays are depicted on the right. (C) Schematic representation of alterations in the human GPC6 promoter region from  $-551$  to  $+2$ . Deletions of the NFAT- and AP-1-putative binding sites are denoted by open boxes in the respective mutant constructs. Luciferase signals from the various constructs are shown on the right. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. \* $P < 0.05$  and # $P < 0.01$  between the Pr-551 and the promoter construct being examined using an unpaired Student's *t* test.

three-step cycling program ( $95^{\circ}\text{C}$  for 15 s;  $56^{\circ}\text{C}$  for 30 s;  $72^{\circ}\text{C}$  for 45 s) Wnt5A. For NFAT1, the primers were 5'-CGGAGTCC-AAGGTTGTGTTCA-3' (forward) and 5'-TGTGGCTGACTT-CGTTTCCTC-3' (reverse). For GPC6, the primers were 5'-CC-TCTGGGGCTGCTGCTC-3' (forward) and 5'-GTTCCC-CTGCGATCTCCTGGTAG-3' (reverse). For Wnt5A, the primers were 5'-AATAACCCTGTTTCAGATGTCA-3' (forward) and 5'-TACTGCATGTGGTCTCCTGATA-3' (reverse). For GAPDH, the primers were 5'-GCAAAATCCATGGCACCGT-3' (forward) and 5'-TCGCCCACTTGATTTTGG-3' (reverse).

### Transfection and lentiviral shRNA infection

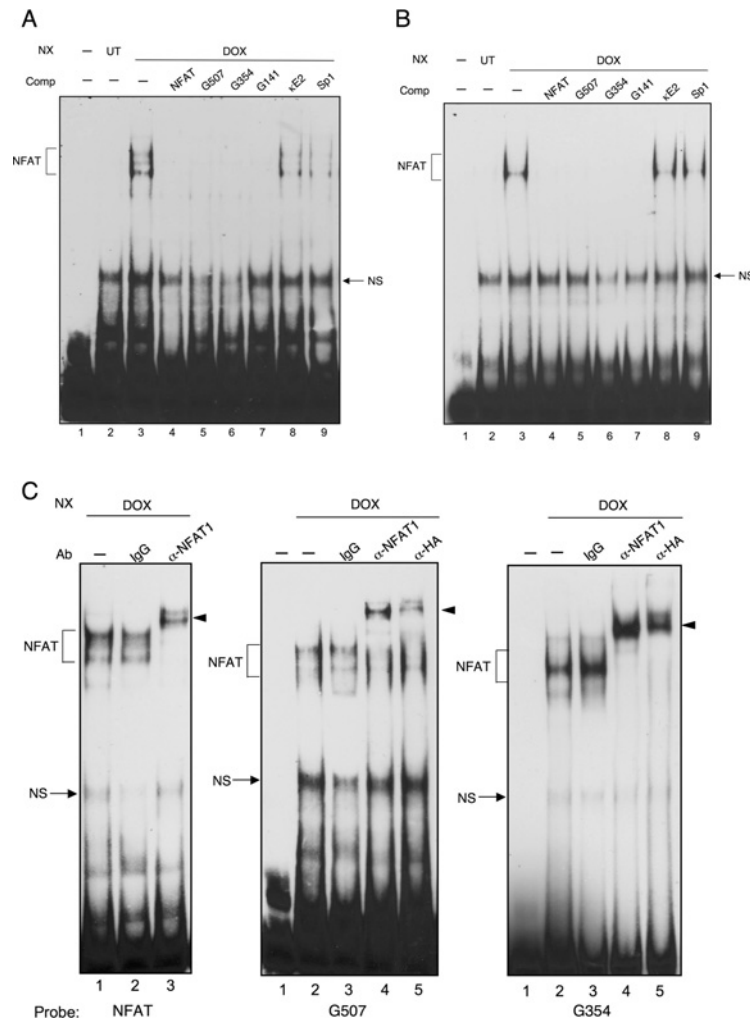
All cell lines except MDA-MB-231 were transfected using PEI (polyethyleimine) from Polysciences. Cells were 80–90% confluent at the time of transfection and were washed three times with sterile PBS. PEI and DNA in the ratio of 3:1 (PEI/DNA) in OptiMEM medium (Invitrogen) were vortex-mixed for 10 s and incubated at room temperature ( $20^{\circ}\text{C}$ ) for 15 min. The DNA complexes formed were added to the cells in growth medium and incubated for 3–4 h at  $37^{\circ}\text{C}$ . The transfection solution was then replaced with fresh growth medium and the cells were grown at  $37^{\circ}\text{C}$  overnight before being harvested for protein lysates, RNA or luciferase assays. MDA-MB-231 cells were transfected using Lipofectamine<sup>TM</sup> 2000 (Invitrogen). Lentiviruses for gene silencing experiments were prepared as described previously [14].

### Luciferase assays

Luciferase assays were performed using Promega's luciferase assay system as described previously [14]. The TOPflash TCF (T-cell factor) reporter plasmid and S37A mutant  $\beta$ -catenin were from Upstate Biotechnology.

### EMSA (electrophoretic mobility-shift assay)

Nuclear protein extracts from untreated or dox-treated SUM.N1#16 cells were prepared using homogenization and high-salt buffer as described previously [17]. Doubled-stranded oligonucleotides were labelled with biotin-11-dUTP using the Biotin 3' End DNA Labeling Kit from Pierce. Nuclear extracts ( $5$ – $10\ \mu\text{g}$ ) were pre-incubated on ice for 30 min in a binding buffer containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 5% glycerol and  $1\ \mu\text{g}$  of poly(dI-dC)·(dI-dC). The protein–DNA complexes were resolved on native PAGE (4% gel) in  $0.5 \times$  TBE (22.5 mM Tris/borate and 0.5 mM EDTA, pH 8.5) at  $4^{\circ}\text{C}$ , and transferred on to Hybond-N<sup>+</sup> membranes (Amersham). For competition mobility-shift or supershift assays, nuclear extracts were pre-incubated with unlabelled competitor oligonucleotides (100-fold molar excess) or antibodies ( $2\ \mu\text{g}$ ) prior to addition of biotinylated probes. The sequences of the sense strands of the double-stranded oligonucleotides used in the present study were as follows: NFAT, 5'-GATCTAAGGA-GGAAAACTGTTTCATGGATC-3'; G507, 5'-GATCTAAG-GGGAAATTGTTGAGTCTAGAACC-3'; G354, 5'-CTATGA-CTATTCCACTCTGGCTTTGTGTTTCAGTAGG-3'; G141,



**Figure 4** NFAT directly binds to the GPC6 proximal promoter

Oligonucleotides containing NFAT- and AP-1- binding sites in the IL (interleukin)-2 (**A**) and GPC6 (**B**) promoter with a NFAT site at  $-354$  and an AP-1 site at  $-337$ , incubated with nuclear extracts from SUM.N1#16 cells. DNA-protein complexes were resolved and detected by chemiluminescence. Nuclear extracts (NX) used were from untreated (UT) or dox-treated cells. Molar excesses (100-fold) of unlabelled double-stranded oligonucleotides as indicated at the top of the lanes were used as competitors. – signifies no added competitor; NFAT, NFAT-AP1 sites in the IL-2 promoter; G507, GPC6 probe with an NFAT site at  $-507$  and an AP-1 site at  $-498$ ; G354, GPC6 probe with an NFAT site at  $-354$  and an AP-1 site at  $-337$ ; G141, GPC6 probe with an NFAT site at  $-141$ ;  $\kappa$ E2, immunoglobulin  $\kappa$  chain enhancer E box; Sp1, consensus sequence of the transcription factor SP1. The bracket at the left of the blot indicates the protein-DNA complexes formed by NFAT. The arrow at the right-hand side denotes non-specific binding (NS). (**C**) Biotinylated oligonucleotide probes indicated at the bottom incubated with nuclear extracts from dox-treated SUM.N1#16 cells in the presence of non-immune IgG or antibodies specific against NFAT1 or HA. – signifies no IgG or antibody; IgG, 2  $\mu$ g of non-immune IgG;  $\alpha$ -NFAT, 2  $\mu$ g of NFAT1 polyclonal antibody;  $\alpha$ -HA, 2  $\mu$ g of monoclonal HA antibody. The brackets at the left-hand side of the blots indicate the NFAT-DNA complexes, whereas the arrowheads on the right-hand side denote the supershifted DNA-protein complexes.

5'-CTTTTTCTTTTTTCCCCTTCTACCGCATAGGAAGA-GA-3'; and  $\kappa$ E2, 5'-GATCAAGGCAGGTGGCCAGATC-3'. The double-stranded transcription factor SP1 (specificity protein 1) consensus oligonucleotide was purchased from Promega. The anti-NFAT1 antibodies and mouse non-immune IgG used in supershift experiments were from Santa Cruz Biotechnology and Jackson ImmunoResearch Laboratories respectively.

### Migration and invasion assay

The assays were performed using Transwell chambers with 8  $\mu$ m pore filters (Corning) as described previously [14]. For migration assays, the cells were incubated at 37°C for 1–3 h. For invasion assays, the filters were coated with 5–10  $\mu$ g Matrigel (BD Biosciences) and the cells were allowed to invade for 3–20 h.

## RESULTS

### Activation of NFAT stimulates GPC6 expression and promotes invasive migration

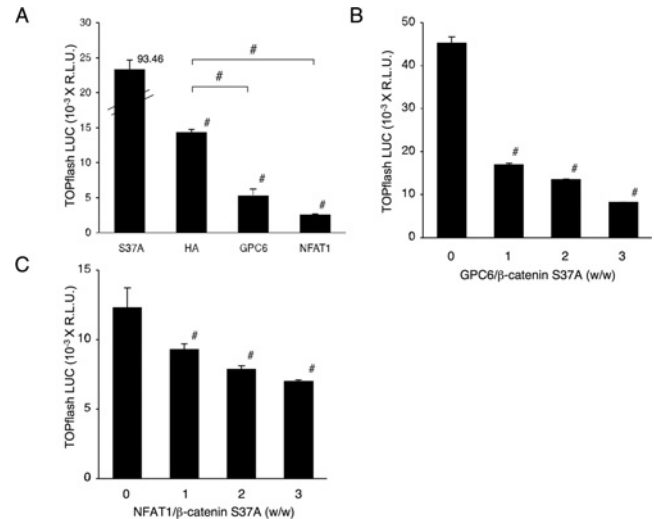
To identify and characterize NFAT target genes that promote carcinoma cell invasive migration, we previously generated SUM-159PT breast cancer cells that inducibly express NFAT1 upon stimulation with dox [14]. Examination of expression profiles by cDNA microarray analysis performed previously revealed that in addition to *COX2* [14], *GPC6* is a distinct gene induced by NFAT1 activation. Transcriptional induction of NFAT was concomitant with a 3-fold induction of GPC6 in SUM.N1#16 cells treated with dox (Figure 1A). Increased GPC6 protein expression was also observed in cells stimulated with PMA and ionomycin to activate NFAT (Figure 1B). Thus GPC6 is induced in breast cancer cells in an NFAT1-dependent manner.

Since NFAT is a pro-migration and invasion transcription factor [11], we next sought to determine whether GPC6 induction by NFAT1 contributes to these phenotypes. Expression of HA-tagged GPC6 significantly enhanced both migration and invasion of MDA-MB-231 and SUM-159-PT cells, compared with the control (Figures 1C and 1D). To confirm the functional role of GPC6 in modulating invasive migration, shRNA was used. Two distinct GPC6 shRNAs quantitatively silenced GPC6 expression compared with the control and significantly reduced cell migration and invasion of SUM-159-PT and MDA-MB-231 cells (Figures 2A and 2B). To provide a cause-and-effect demonstration that induction of GPC6 by NFAT1 promotes invasion, GPC6 shRNA was introduced into SUM.N1#16 cells followed by induction of NFAT by dox. Inducible expression of NFAT1 stimulates invasion as expected, and this was reduced to control levels in the presence of GPC6 shRNA (Figure 2C). Therefore GPC6 is one factor that contributes to the pro-invasion activity of NFAT.

### NFAT regulates GPC6 transcription

To determine whether *GPC6* is a direct NFAT target gene we performed analysis of the GPC6 promoter. The NFAT minimal consensus sequence is GGAAA(A) or TGGAAAGT. NFAT also typically transactivates genes in co-operation with the AP-1 (*fos/jun*) complex. The AP-1 minimal consensus binding sequence is TGASTCA, where S is either a cytosine or guanine. Typically NFAT- and AP-1-binding sites are separated by seven or less nucleotides. Analysis of the GPC6 proximal promoter revealed several NFAT- and AP-1-binding sites upstream of the transcriptional start site (Figure 3A). A series of 5'-deletion mutants of the GPC6 promoter subcloned upstream of a luciferase reporter gene were transfected into SUM-159PT revealing that transcription was significantly reduced by deletion of the NFAT- and AP-1-binding sites at -507 and -498 (Figure 3B, right-hand panel). A further reduction in activity was observed by deletion of the NFAT site at -354, but no difference was seen between Pr-343 and Pr-127. Multiple luciferase reporter constructs were also used in which the NFAT sites and/or AP-1 sites were replaced by unrelated sequences (Figure 3C, left-hand panel). Removal of the NFAT (-507) and AP-1 (-498) sites significantly reduced luciferase activity compared with the intact promoter (Figure 3C, right-hand panel). Interestingly, a more significant reduction in reporter activity was observed when the NFAT site at -354 was replaced. The most significant reduction in luciferase activity was observed with Pr-551 triple mutant construct, in which all the three NFAT sites are mutated.

To determine whether NFAT directly binds to the GPC6 promoter, EMSAs were performed. An oligonucleotide probe that contains a NFAT/AP-1 composite binding site that binds NFAT proteins in Jurkat T-cells was used [18]. No detectable DNA-protein complex was detected when this probe was incubated with nuclear proteins from untreated cells, probably due to low levels of endogenous nuclear NFAT (Figure 4A, lane 2). In contrast, multiple bands were detected when the probes were allowed to bind to nuclear extracts from dox-treated cells (Figure 4A, lane 3). These complexes are competed by the addition of a 100-fold molar excess of unlabelled probe (Figure 4A, lane 4) or oligonucleotides that contain the NFAT sites in the GPC6 promoter (Figure 4A, lanes 5-7). No competition was observed by adding equivalent amounts of non-specific competitors (Figure 4A, lanes 8 and 9). To investigate whether similar protein-DNA interactions were detected with GPC6 probes, a probe (G354) that contains the NFAT site (-354) and AP-1 site (-337) in the GPC6 promoter was used. Several protein-DNA complexes were detected with



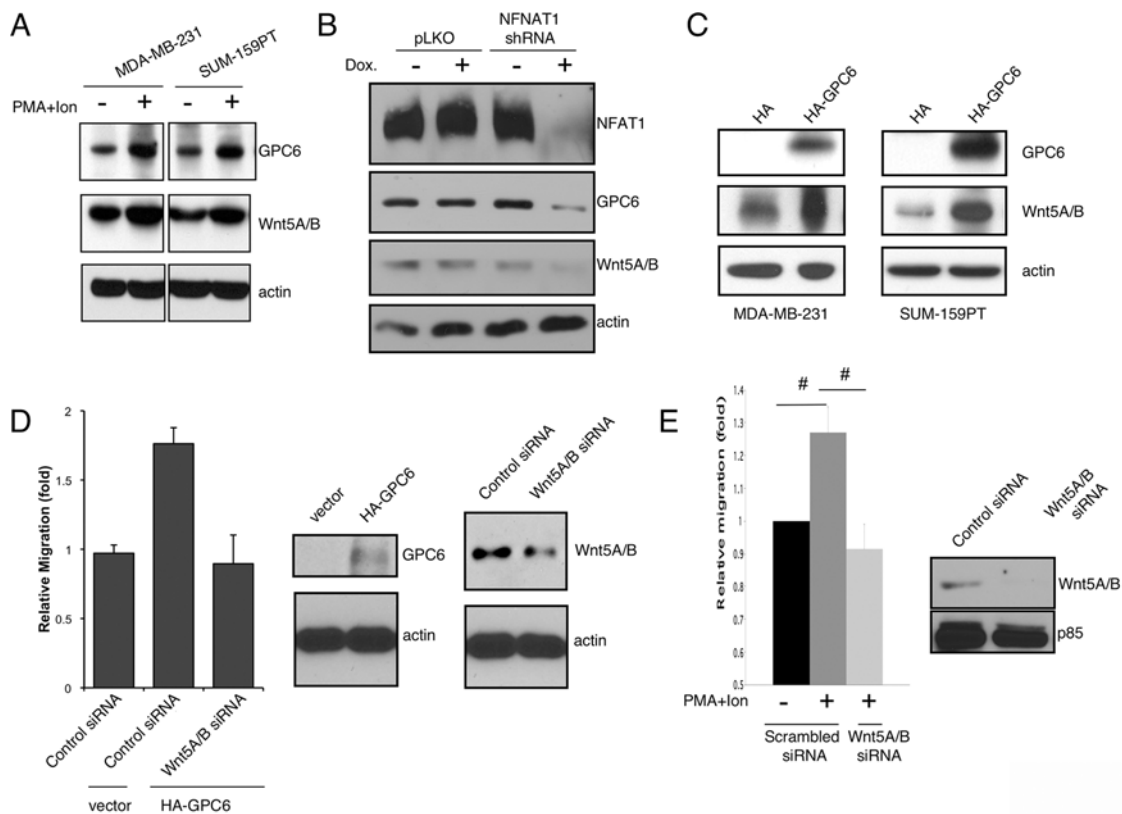
**Figure 5** GPC6 and NFAT1 inhibit canonical Wnt/ $\beta$ -catenin signalling

(A) SUM 159-PT cells transiently transfected with TOPflash and S37A mutant  $\beta$ -catenin, HA, GPC6 or NFAT1 plasmids, along with pCS2-(n)- $\beta$ gal to control for transfection efficiency. Luciferase activities were determined 24 h after transfection. (B) Mutant S37A  $\beta$ -catenin transiently transfected with increasing amounts of GPC6. Luciferase activities from TOPflash were determined 24 h after transfection. (C) Similar to (B), except plasmids encoding NFAT1 were used. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. # $P < 0.01$  in luciferase activities between the S37A and HA, GPC6 and NFAT1 plasmids, between HA control and GPC6 plasmids, and between HA control and NFAT1 plasmids as indicated by the brackets (unpaired Student's *t* test).

nuclear extracts from dox-treated cells, but not untreated cells (Figure 4B, compare lanes 2 and 3). These complexes have a similar electrophoretic mobility as the complexes formed with the NFAT probe (Figure 4A). Again binding was confirmed using specific (Figure 4B, lanes 4-7) and non-specific (Figure 4B, lanes 8 and 9) competitors. Moreover, NFAT was present in these DNA-protein complexes, since a supershift was observed in the presence of the NFAT antibody (Figure 4C, left-hand panel, lane 3), compared with irrelevant IgG (Figure 4C, left-hand panel, lane 2). DNA-protein complexes were also detected with the GPC6 G507 probe from dox-treated SUM.N1#16 cells and were supershifted with anti-NFAT1 or -HA antibodies (Figure 4C, middle panel, lanes 4 and 5). Finally, using a biotinylated GPC6 G354 probe, NFAT binding activity was also observed by a supershift in the presence of NFAT1 or HA antibodies, but not non-immune IgG (Figure 4C, right-hand panel). In summary, NFAT1 induces GPC6 expression by direct binding to several elements on the GPC6 proximal promoter.

### GPC6 and NFAT1 inhibit Wnt/ $\beta$ -catenin and activate Wnt5A signalling

We next evaluated the mechanism by which the NFAT1/GPC6 signalling axis promotes breast cancer cell invasive migration. We focused on Wnt signalling because Wnt proteins are involved in controlling cell migration and are frequently deregulated in human solid tumours. Studies have also shown that GPCs regulate metastasis by modulating Wnt [19,20]. A TOPflash reporter plasmid, which contains two sets of three copies of the TCF-binding site upstream of a TK (thymidine kinase) minimal promoter and a luciferase open reading frame was used to examine the TCF/ $\beta$ -catenin signalling in SUM-159PT cells [21]. A constitutively active S37A  $\beta$ -catenin mutant served as positive control. Robust TOPflash activity was detected in cells expressing S37A  $\beta$ -catenin (Figure 5A). Conversely, cells expressing



**Figure 6** Activation of NFAT induces Wnt5A signalling through GPC6

(A) Lysates prepared from the indicated cell lines with or without overnight incubation with PMA and ionomycin, immunoblotted with anti-GPC6, anti-Wnt5A/B or anti-actin antibodies. (B) MDA-MB-231 stable pools harbouring control tet-pLKO or NFAT1 shRNA were induced with dox (100 nM) for 72 h and lysates immunoblotted with anti-NFAT1, anti-GPC6, anti-Wnt5A/B or anti-actin antibodies. (C) MDA-MB-231 or SUM-159PT cells transiently transfected with empty vector (HA) or HA-GPC6. Lysates were immunoblotted with anti-GPC6, anti-Wnt5A/B or anti-actin antibodies. (D) SUM 159-PT cells transiently transfected with empty vector control or HA-GPC6, together with control siRNA or Wnt5A/B siRNA, then assayed for migration. HA-GPC6 expression and Wnt5A/B silencing were confirmed by immunoblotting. (E) SUM-159-PT cells transiently transfected with either scrambled siRNA or Wnt5A/B siRNA were left unstimulated or stimulated with PMA and ionomycin (100 nM) and assayed for migration in Transwell assays. A portion of the lysates were immunoblotted with anti-Wnt5A/B or anti-actin antibodies. # $P < 0.05$  (unpaired Student's  $t$  test). All experiments are representative of at least three independent experiments.

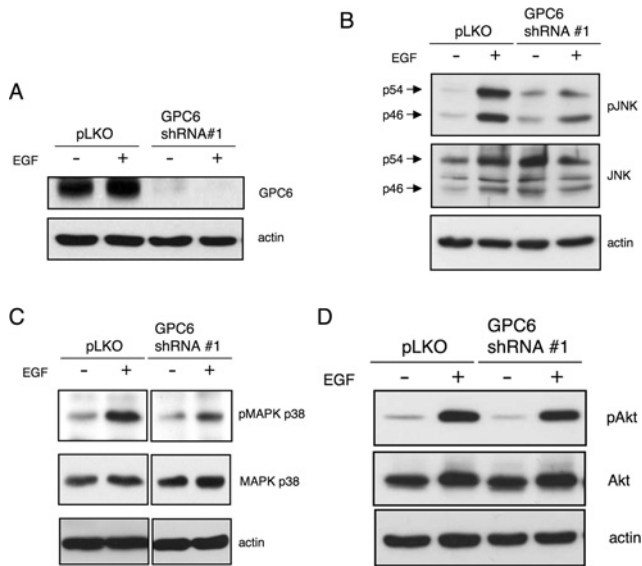
exogenous GPC6 or NFAT1 showed significantly reduced luciferase activity compared with the control cells, indicating GPC6 and NFAT1 inhibit the association of  $\beta$ -catenin with the TCF transcription factors to form transcriptionally active complexes that bind to Wnt target genes. Consistent with this, TOPflash activity induced by S37A  $\beta$ -catenin was significantly diminished by expression of increasing amounts of GPC6 (Figure 5B) or NFAT1 (Figure 5C). These results indicate that NFAT1 and GPC6 inhibit canonical Wnt signalling.

Although certain GPCs inhibit canonical Wnt/ $\beta$ -catenin signalling, they can also enhance non-canonical Wnt [20,22]. Importantly, non-canonical Wnt5A promotes cell migration and invasion [23,24]. To determine whether NFAT1 and GPC6 promote cancer cell invasive migration through non-canonical Wnt5A signalling, we evaluated the induction of Wnt5A by NFAT1 and GPC6. Increased GPC6 expression was detected in two distinct breast cancer cell lines treated with PMA and ionomycin to activate endogenous NFAT, along with a concomitant increase in Wnt5A/B expression (Figure 6A). In addition, inducible silencing of NFAT1 using tetracycline-regulated shRNA (tet-pLKO) led to a significant reduction in GPC6 expression that was concomitant with reduced Wnt5A/B levels (Figure 6B). Moreover, expression of exogenous GPC6 in MDA-MB-231 and SUM-159PT cells led to increased Wnt5A/B expression (Figure 6C). This increase was not due to enhanced

transcription as revealed by quantitative RT-PCR with primers specific for Wnt5A and GPC6 (Supplementary Figure S1A at <http://www.BiochemJ.org/bj/440/bj4400157add.htm>). Moreover, enhanced migration induced by GPC6 expression was completely blocked by Wnt5A/B siRNA (Figure 6D). Consistent with this, induction of NFAT by doxycycline in SUM.N1#16 led to up-regulation of Wnt5A/B protein levels (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/440/bj4400157add.htm>). Finally, stimulation of SUM-159-PT with PMA and ionomycin increased cell migration and this was completely blocked in cells transfected with Wnt5A/B siRNA (Figure 6E). Collectively, these results provide a cause-and-effect demonstration that non-canonical Wnt5 signalling is at least one mechanism by which the NFAT/GPC6 signalling axis promotes cell motility.

#### GPC6 activates JNK and p38 MAPK

Finally, to investigate the signalling mechanisms that function downstream of GPC6 and Wnt5A, we examined the activation of JNK and p38 MAPK, which known to be activated by Wnt5A [25]. Quantitative silencing of GPC6 using shRNA (Figure 7A) attenuated EGF (epidermal growth factor)-stimulated JNK (Figure 7B) and p38 MAPK phosphorylation (Figure 7C). This reduction in JNK and p38 MAPK activation was not due to a



**Figure 7** GPC6 regulates JNK and p38 MAPK signalling

(A–C) SUM-159-PT cells infected with GPC6 shRNA #1 or control empty vector pLKO, serum-starved and then stimulated with EGF for 20 min, and analysed by immunoblotting with anti-GPC6 (A), anti-phospho-JNK (p-JNK) and total JNK (B), anti-phospho-p38 MAPK (pMAPK p38) and total p38 MAPK (C) antibodies. (D) SUM-159PT cells transfected with vector (HA) or HA-GPC6, serum starved and then stimulated with EGF for 20 min, and lysates analysed by immunoblotting for phospho-Akt (pSer<sup>473</sup>) (pAkt) or total Akt. Anti-actin antibody served as control in all cases. Results are representative of at least three independent experiments.

global non-specific reduction of signalling pathways, since GPC6 shRNA does not attenuate EGF-stimulated Akt phosphorylation (Figure 7D).

## DISCUSSION

Although NFAT was originally identified as a transcription factor that mediates the immune response, it is now evident that NFAT isoforms function as key transcriptional regulators in all cells and tissues [5]. Functions ranging from heart valve development, myogenesis, angiogenesis, cartilage formation and neuronal development have all been ascribed to NFAT [1,2]. In addition, a recent study has pointed to a role for NFAT in the regulation of various phenotypes associated with tumorigenesis, including cell growth, survival, endothelial cell signalling and angiogenesis [3]. Our previous studies demonstrated that NFATs are expressed in breast cancer cells and tissues from patients with invasive breast carcinoma, and that NFAT activation promotes invasive migration [11]. To date, two NFAT target genes, *COX-2* and autotaxin (*ENPP2*), have been shown to promote cancer cell invasive migration through the production of PGE<sub>2</sub> and LPA respectively [12,14]. In the present study, we identify *GPC6* as an NFAT-induced gene that also promotes invasive migration.

GPC6 is a member of the GPC family of HSPGs (heparin sulfate proteoglycans) [26]. Using both pharmacological, genetic and shRNA approaches we demonstrate that *GPC6* is a direct NFAT-target gene in breast cancer cells, and that NFAT directly binds to the GPC6 promoter and thereby stimulates transcription. In turn, increased GPC6 expression enhances invasive migration *in vitro* in a manner that requires Wnt5A signalling. GPC6 appears to be both necessary and sufficient at promoting invasive migration, since exogenous expression enhances and GPC6 shRNA decreases both migration and invasion through Matrigel.

A role for GPCs in various phenotypes associated with tumorigenesis is increasingly evident, although the results to date have not provided a unifying model. Studies have shown that GPCs can either potentiate or inhibit growth factor signalling. GPC1 is expressed in breast carcinoma, glioma and pancreatic cancer cells [27–29]. Loss of GPC1 reduces growth, metastasis and tumorigenicity [30,31], and enhances FGF-2 (fibroblast growth factor-2) signalling [27,28]. In pancreatic cancer, GPC1 is required for TGF- $\beta$  (transforming growth factor- $\beta$ ) signalling [31]. Increased GPC3 expression has also been detected in thyroid cancer, hepatocellular carcinoma, melanoma, squamous cell carcinoma of the lungs, Wilms tumour and hepatoblastoma [32–36]. Ectopic expression of GPC3 in breast cancer cells leads to decreased growth and cell invasion, and enhances sensitivity to apoptotic insults [37,38]. However, relatively little is known concerning the expression or functional roles of GPC6 in human solid tumours and phenotypes associated with cancer progression. Reduced expression or loss of function of GPC6 has recently been detected in sporadic retinoblastoma and autosomal-recessive omdysplasia [39,40]. To our knowledge, the present study is the first to identify a functional role for NFAT and GPC6 signalling in carcinoma invasive migration.

GPCs regulate a variety of intracellular signalling mechanisms and, depending on the cellular context, can either stimulate or inhibit signalling. For example, GPC3 inhibits Hedgehog signalling by competing with Patched for Hedgehog binding [41]. In contrast, GPC3 stimulates Wnt by enhancing and/or stabilizing the interaction of Wnt proteins and their receptors [19]. Since non-canonical Wnt signalling has been shown to promote cell migration in an NFAT-dependent manner [42], we evaluated whether GPC6-mediated invasive migration is dependent on Wnt5A. We show that increased GPC6 expression induced by NFAT enhances Wnt5A protein in the three distinct breast cancer cell lines examined, reminiscent of studies showing that GPC3 inhibits canonical Wnt signalling in a manner that depends on the activation of non-canonical Wnt [20,22]. The present study points to Wnt5a functioning downstream of NFAT and GPC6 in modulating invasive migration in breast cancer cells. In a separate study, Wnt5a has been shown to also promote breast cancer cell migration, but in an NFAT-dependent manner, suggesting that Wnt5a may also function upstream and be required for efficient NFAT activation [42]. These opposing observations imply that Wnt5a and NFAT are dependent on each other for efficient signal relay, such that perturbation in one or the other protein results in inhibition of breast cancer phenotypes such as invasive migration. The precise mechanisms by which Wnt5a leads to NFAT activation and in turn how Wnt5a activity is modulated by NFAT remain to be determined.

Increased Wnt5A expression has been detected in melanoma, pancreatic cancer, breast cancer and gastric cancer [24,43,44]. Although we detected expression of Wnt5A in various breast cancer cell lines of distinct origin, it is presently unknown whether Wnt5A expression is increased in aggressive invasive and metastatic carcinomas compared with normal epithelial cells and tissues. Nonetheless, Wnt5A is known to enhance migration and invasion, and studies have also shown that it enhances proliferation and metastasis [23,24,43,45]. Clearly the mechanisms by which Wnt5A promotes cancer progression will depend on the cellular context and whether it functions in a cell-autonomous manner or whether it also functions in the context of the tumour microenvironment. Finally, we also show that GPC6 induction of Wnt5A stimulates the activation of JNK and p38 MAPK. This is reminiscent of GPC3, which also enhances p38 MAPK and JNK activity [20,22]. Although the contribution of either JNK or p38 MAPK to invasive migration in NFAT



and GPC6 signalling is presently unknown, it is known that JNK can be activated by Wnt5A to promote the migration and invasion of breast epithelial and cancer cells [46–48]. Likewise, non-canonical Wnt4A and Wnt5A are capable of activating p38 MAPK [49,50].

In summary, we report the identification of a novel NFAT target gene, *GPC6*, which functions to stimulate the invasive migration of breast cancer cells. Similar to *GPC3*, *GPC6* probably serves as a coreceptor to facilitate and/or stabilize the interaction of Wnts and their receptors to stimulate non-canonical Wnt5A downstream. This in turn will activate secondary downstream pathways that are associated with promoting cell migration and invasion. These observations indicate that NFAT and *GPC6* modulate cell invasion through precisely controlled signalling loops and cross-talks with other signalling pathways.

## AUTHOR CONTRIBUTION

Gary Yiu, Y. Rebecca Chin and Aura Kaunisto designed and performed experiments; Gary Yiu and Alex Tokor conceptualized the study; and Gary Yiu and Alex Tokor wrote the paper.

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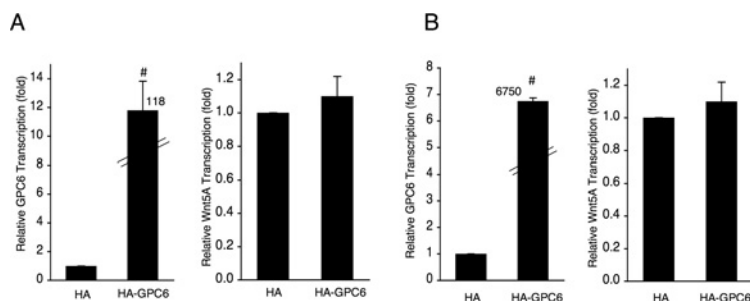
Published as BJ Immediate Publication 26 August 2011, doi:10.1042/BJ20110530

## SUPPLEMENTARY ONLINE DATA

# NFAT promotes carcinoma invasive migration through glypican-6

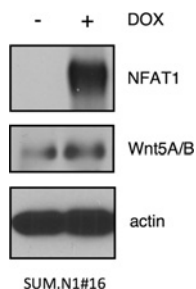
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**Figure S1 Real-time quantitative PCR analysis of GPC6 or Wnt5a expression**

MB-231 (A) and SUM-159PT (B) cells transiently transfected with vector (HA) or HA-GPC6. Results are means  $\pm$  S.D. of triplicate measurements for at least two independent experiments. # $P < 0.01$  (unpaired Student's *t* test).



**Figure S2 Immunoblot analysis**

Protein extracts from untreated SUM.N1#16 cells or cells stimulated with dox (1  $\mu$ g/ml) were immunoblotted with anti-HA, anti-Wnt5A/B or anti-actin antibodies.

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