I-branching *N*-acetylglucosaminyltransferase regulates prostate cancer invasiveness by enhancing α5β1 integrin signaling

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Abbreviations

PCa	prostate cancer
TM	tunicamycin
PPMP	DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol hydrochloride
BAG	benzyl-α-GalNAc

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ABSTRACT

Cell surface carbohydrates are important for cell migration and invasion of prostate cancer (PCa). Accordingly, the I-branching *N*-acetylglucosaminyltransferase (GCNT2) converts linear i-antigen to I-branching glycan, and its expression is associated with breast cancer progression. In the present study, we identified relationships between GCNT2 expression and clinicopathological parameters in patients with PCa. Paraffin-embedded PCa specimens were immunohistochemically tested for GCNT2 expression, and the roles of GCNT2 in PCa progression were investigated using cell lines with high GCNT2 expression and low GCNT2 expression. GCNT2-positive cells were significantly lesser in organ-confined disease than in that with extra-capsular extensions, and GCNT2-negative tumors were associated with significantly better prostate-specific antigen (PSA)-free survival compared with GCNT2-positive tumors. Subsequent functional studies revealed that knockdown of GCNT2 expression in PCa cell lines significantly inhibited cell migration and invasion. GCNT2 regulated the expression of cell surface I-antigen on the *O*-glycan and glycolipid. Moreover, I-antigen-bearing glycolipids were subject to α5β1 integrin–fibronectin mediated protein kinase B (AKT) phosphorylation. In conclusion, GCNT2 expression is closely associated with invasive potential of PCa.

INTRODUCTION

Cell surface carbohydrates reportedly play significant roles in glycoprotein function and in tumor cell proliferation and invasion.[1-3] It is also widely accepted that invasion and metastasis of tumor cells from primary lesion correlates with poor prognosis in several epithelial cancers, including in prostate cancer (PCa).[4]

PCa is the most common malignancy in men and the second leading cause of cancer-related death in the United States and Europe.[5, 6] Its incidence is rapidly increasing in the Asia–Pacific region,[7] and the associated clinical issues have attracted global research interest. Whereas primary prostate tumors are moderately sensitive to androgens, metastatic prostate cancers acquire adaptive hormone independency after androgen deprivation therapy.[8] Although androgen-independent prostate cancers exhibited changes in cell surface carbohydrate structures,[9] the mechanisms related to aberrant glycan with prostate cancer metastasis are yet unclear.

I-branching *N*-acetylglucosaminyltransferase (GCNT2) [10-12] synthesizes I-branched polylactosamine chains (I-antigen) by catalyzing the transfer of *N*-acetylglucosamine (GlcNAc) from uridine diphosphate (UDP)-GlcNAc with a galactose β1-6 linkage of linear lactosamine chains (i-antigens; Fig. 1A). The presentation of these i/I antigens changes dramatically during human development.[13] The i–I antigen conversion reportedly increases the presentation of polylactosamine chains and their functional terminal structures sialyl Lewis X and sialyl Lewis A.[14] In addition, branched

polylactosamine chains have increased affinity for specific lectins.[15] In cancer cells, mannosyl (alpha-1,6-)-glycoprotein beta-1,6-*N*-acetyl-glucosaminyltransferase (MGAT5)-mediated polylactosamine chain branching resulted in increased PCa cell invasion.[16] Moreover, GCNT1-mediated core2 branching of *O*-glycan increased testicular tumor invasion and PCa aggressiveness.[17, 18] However, few previous studies have investigated the relationship between GCNT2 and PCa invasiveness and metastasis, despite the potential for formation of branching glycans. Recently, the expression of GCNT2 was closely associated with the malignant potential of breast cancers.[19] Thus, we investigated associations between GCNT2 expression and clinical features in patients with PCa.

In this study, we demonstrated that GCNT2 expression in PCa specimens from radical prostatectomy procedures was associated with PCa aggressiveness. In addition, knockdown of GCNT2 expression in PCa cells significantly decreased cell migration and invasion. We also demonstrated that GCNT2-expressing PCa cells presented I-antigen carrying *O*-glycans and glycolipids on the cell surface. Moreover, I-antigen-expressing PCa cells had increased α5β1 integrin-mediated protein kinase B (AKT) phosphorylation and migration. Collectively, the present data indicate important the roles of GCNT2 in the progression and invasion of PCa cells.

Materials and methods

Materials

The *O*-glycosylation inhibitor benzyl-α-GalNAc (BAG) was purchased from Sigma–Aldrich (St Louis, MO, USA). The *N*-glycosylation inhibitor tunicamycin (TM) was purchased from Wako Pure Chemical Company (Osaka, Japan). The glucosylceramide synthetase inhibitor DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol hydrochloride (PPMP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies used in this study were listed in Doc. S1.

Cells

The human PCa cell line LNCaP, DU145 and PC3 were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI1640 medium (Wako, Osaka, Japan) containing 100-U/mL penicillin and 100 μg/mL streptomycin with 10% FBS (Hyclone, UT, USA). Immortalized RWPE-1 prostate epithelial cells were also purchased from the American Type Culture Collection. RWPE-1 was maintained in keratinocyte serum-free medium (Life technologies, Carlsbad, CA, USA). DU145-derived GCNT2 knockdown cell lines were established by transfection of 4 different GCNT2siRNA vectors (Doc.S1). All cells were analyzed by STR analysis (BEX co., Ltd., Tokyo, Japan).

Immunohistochemical analysis of PCa specimens

Between 2005 and 2011, 156 PCa patients were treated with radical prostatectomy at the Department of Urology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan. Staging and grading of tumors and patient follow-up were previously described.[20] In brief, Postoperative PSA

133 levels were considered to be increased (PSA recurrence) if they were ≥ 0.2
134 ng/mL during two consecutive visits in a 1-month interval. Time zero was
135 defined as the day of surgery. Patients with constantly detectable PSA levels
136 (< 0.001 ng/mL) after surgery were recorded as recurrences at time zero.
137 Follow-up intervals were calculated from the date of surgery to the last
138 recorded follow-up (median, 47.9 months; range, 26.7-79.6 months).[20] All
139 tumor specimens after radical prostatectomy from these patients were
140 formalin-fixed and embedded in paraffin. Deparaffinized specimens were
141 incubated with the rabbit anti-human GCNT2 polyclonal Ab (1:200,
142 HPA026776; Sigma) followed by incubation with HRP-conjugated goat
143 anti-mouse/rabbit IgG antibody (Dako, Tokyo, Japan). Based on the staining
144 status of Golgi apparatus, specimens with 10% or more positive cancer cells
145 were judged as GCNT2 positive. Informed consent was obtained from all
146 patients prior to participation in this study. The ethics committee of Hirosaki
147 University approved the study protocol, and the study was performed in
148 accordance with the ethical standards of the Declaration of Helsinki.
149 *Determination of GCNT2 expression in prostate cancer cell lines using*
150 *quantitative real-time PCR*
151 Total RNA was isolated using ISOGEN II reagent, and then qPCR
152 analyses were performed using GeneAce SYBR® qPCR Mix α No ROX
153 (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions.
154 Primer sets are listed in Table S1. Gene expression levels were normalized to
155 that of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

156 *Cell proliferation, migration and invasion assays*

157 *In vitro* cell proliferation assays were performed as described

158 previously.[17] *In vitro* cell migration and invasion assays were performed

159 using Boyden chambers as described previously.[18] The numbers of cells on

160 the lower side were counted in triplicate using a Hybrid Cell Counting

161 System (Keyence, Osaka, Japan).

162 *Flow cytometry*

163 Cells (1×10^5) were incubated with or without primary antibody in 100

164 µL of 1% BSA–PBS for 30 min on ice followed by the incubation with

165 fluorescence-conjugated isotype-specific secondary antibodies.

166 *Immunocytochemical analysis of I-antigen presentation on PCa cells*

167 Cells were cultured to 80% confluence on glass slides and were treated

168 with 0.6% DMSO, 2 mM BAG, 0.2 µg/mL TM, and 20 µg/mL PPMP on glass

169 slides for 48 h. Immunocytochemical analysis was performed as described

170 previously.[21]

171 *Fibronectin adhesion and spreading assays*

172 Adhesion assays and spreading assays were performed using

173 fibronectin-coated dishes as previously described.[22]

174 *Immunoprecipitation*

175 α5β1 Integrin heterodimer complex levels were determined in lysates

176 from DU145-derived cells. Cells were incubated with 2 µg/mL rabbit

177 monoclonal anti-α5 integrin antibody (EPR7854, Abcam, Cambridge, MA,

178 USA) or mouse monoclonal anti-β1 integrin antibody (P5D2, Abcam) and

were then incubated with protein G Dynabeads (Life Technologies). Immune complexes were eluted from Dynabeads using 3× Laemmli SDS-PAGE sample buffer.

Western blotting

Total cell lysates were prepared using 1% Igepal CA-630 (Sigma, St. Louis, MO, USA) containing protease inhibitor cocktail (Roche, Basel Schweiz). Briefly, samples were separated using 4%–15% SDS-PAGE gradient gels (Bio-Rad, Hercules, CA, USA) and were then transferred onto PVDF membranes. Western blotting analysis was performed using specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. After incubation with secondary antibodies, all samples were enzymatically visualized using Novex® ECL Chemiluminescent Substrate Reagent Kits (Life Technologies) and a ChemiDoc™ XRS+ System (Bio-Rad).

Focal adhesion kinase and protein kinase B stimulation on fibronectin

DU145-derived cell lines were cultured in the absence of serum for 48 h and were then detached using an enzyme-free cell dissociation solution (Millipore). Subsequently, 1×10^5 cells were seeded on 20 µg/mL fibronectin coated 6-well plates. After incubation for 5 min, 10 min, and 20 min, cells were washed once in PBS and were lysed using 1% Igepal CA-630 solution containing protease inhibitor cocktail and PhosStop (Roche).

Inhibition assays

Cells were pretreated with 20 µg/mL anti-α5 integrin antibody (NKI-SAM-1), 10 µg/mL of anti-ß1 integrin antibody (P5D2), or 20 µg/mL of

corresponding control isotype antibodies at on ice for 30 min and migration and fibronectin stimulation assays were performed.

Cells were treated with the AKT inhibitor VIII (10 μM; Cayman Chemical Company, Ann Arbor, MI, USA) or with DMSO, and migration assays were performed. In separate experiments, cells were cultured with the BAG (2 mM), PPMP (20 μg/mL), or DMSO for 48 h and were then subjected to migration and fibronectin stimulation assays. In RGD peptide blocking assay, cells were pretreated with 100, 200, 400, 800 μM of RGD peptide (sc-201176; Santa Cruz) or vehicle control at on ice for 30 min and fibronectin stimulation assays were performed.

Statistical analysis

Associations of GCNT2 status with clinical and histopathological parameters were analyzed using chi-squared tests. PSA-free survival was evaluated using Kaplan–Meier curves, and differences between groups were assessed using the log-rank test. All statistical analyses were performed using SPSS 21.0 software (SPSS, Chicago, IL, USA). Multivariate analysis of in this study used Cox proportional hazards regression analysis to test the association of GCNT2 status with other clinical and pathological parameters, including patients age, initial PSA, clinical stage, biopsy gleason score (GS), post-operation GS, pathological stage, margin status and perineural invasion for the prediction of PSA recurrence.

Results

GCNT2 expression in PCa positively correlates with cancer invasion and PSA recurrence

To confirm that GCNT2 expression correlates with PCa aggressiveness, expression levels of three isoforms of GCNT2 were determined in PCa cell lines using qPCR. A transcript variant (isoform A) of *GCNT2* was the major isoform expressed in PCa cell lines. Whereas high expression of *GCNT2* was observed in the highly invasive PCa cell lines DU145 and PC3, low-level expression of *GCNT2* was observed in the poorly invasive LNCaP cell line (Fig. 1B). This result suggested that the high expression of *GCNT2* correlates with invasive characteristics in PCa cell lines. To evaluate the role of GCNT2 in PCa aggressiveness, PCa specimens were immunohistochemically analyzed using a rabbit anti-GCNT2 polyclonal antibody. In these experiments, GCNT2 expression was detected in a partially healthy prostate gland and was highly expressed in some PCa cells (Fig. 1C). No significant differences in clinical parameters were observed between GCNT2-postive and GCNT2-negative PCa specimens from 156 patients (Table S2). However, >80% of tumor specimens had extraprostatic extensions (pT3 and pT4) that expressed GCNT2 in accordance with pathological parameters (Table S3), and GCNT2-positive patients were at significantly higher risk of PSA recurrence after radical prostatectomy (Fig. 1D). Moreover, nodal metastatic PCa cells also expressed GCNT2 (Figure S1). According to multivariate analyses, PSA levels, margin status, and GCNT2 expression in tumors were independent risk factors for PSA recurrence

248 (Table 1). These results indicate that GCNT2 expression correlates with PCa
249 invasion and progression.
250 *GCNT2 regulates PCa cell migration and invasion*
251 To investigate the role of GCNT2 expression in PCa cells, we established
252 GCNT2 knockdown DU145 cell lines. In subsequently qPCR analyses and
253 western blotting, clone 3 and clone 4 showed >70% inhibition of GCNT2
254 expression (Fig.2A and 2B). Although GCNT2 knockdown significantly
255 inhibited cell proliferation at day three in clone 3 (DU145GCNT2KD3), total
256 cell numbers at day seven did not differ between siControl (DU145NC),
257 DU145GCNT2KD3, and clone 4 (DU145GCNT2KD4) cells (Fig. 2C). These
258 results suggest that GCNT2 expression is not critical for cell proliferation *in*
259 *vitro*. In subsequent experiments, the effects of GCNT2 expression were
260 examined using migration and invasion assays in DU145NC, GCNT2KD3,
261 and GCNT2KD4 cells using a Transwell system. In comparisons with
262 DU145NC cells, migration and invasion was strongly inhibited in GCNT2
263 knockdown cell lines (Figs. 2D and 2E). In further experiments, *GCNT2*
264 expression was transiently inhibited using siRNA transfection in PC3 cells
265 and resulted in decreased invasion potential (Figure S2A). Moreover, wound
266 healing assays showed significantly decreased surface coverage rates in
267 GCNT2 knockdown cell lines compared with that in DU145NC cells (Figure
268 S2B). In a previous study, high expression of GCNT2 was associated with the
269 epithelial-mesenchymal transition (EMT) and accelerated cell invasion in
270 breast cancers.[19] In agreement, comparisons of the present DU145NC and

DU145GCNT2KD4 cell lines with PC3 cells revealed similar patterns of EMT marker expression (Figure S2C), suggesting that GCNT2 regulates migration and invasion without stimulating the EMT in PCa cells.

GCNT2 catalyzes the formation of I-antigens on O-glycosylated proteins and glycolipids of PCa cell membranes

Interactions between cells and the extracellular matrix (ECM) have been shown to regulate cell motility.[23] Moreover, cell surface glycan modifications have reported biological functions during adhesion to the ECM and selectins and inhibits NK cell cytotoxicity.[24, 25] Thus, we investigated the effects of GCNT2 on cell surface glycans, and confirmed that GCNT2 converts i-antigen to I-antigen on cell surface carbohydrate structures (Fig. 1A). Specifically, GCNT2-expressing PCa cell lines showed pronounced cell surface presentation of I-antigen (Figure S3A and 3B). In contrast, GCNT2 knockdown cells had limited I-antigen presentation on cell surfaces (Figs. 3A and 3B). GCNT2 reportedly acts on *O*-glycans, *N*-glycans, and glycolipids to form GlcNAc-Gal branches (Fig. 1A).[12] Accordingly, after treatment of DU145NC cells with the inhibitors BAG or PPMP, significantly decreased I-antigen expression was observed. Moreover, co-treatment with BAG and PPMP led to greater inhibition of I-antigen presentation than individual treatments (Fig. 3D). In contrast, treatment with TM did not decrease I-antigen presentation on cell surfaces (Figs. 3C and 3D). Treatment with these inhibitors decreased each glycan on the cell surface (Figure S4), suggesting that I-antigens were formed on *O*-glycan and glycolipid molecules

294 on PCa cell surfaces.

295 *I-antigen enhances α5β1 integrin signaling*

296 Integrin is a well-known heterodimeric receptor of the ECM and has
297 reported roles in cell adhesion.[26] The major stromal ECM motility factor
298 fibronectin has been shown to interact with the integrin α5β1 heterodimer in
299 a glycan-structure dependent manner.[27, 28] In the present experiments, α5
300 integrin and β1 integrin were expressed at similar levels in DU145NC and
301 GCNT2 knockdown cells (Fig. 3E). Thus, to confirm the role of I-antigen in
302 cell–ECM interactions, adhesion and spreading assays were performed using
303 DU145 cell lines on fibronectin-coated plates. Although adhesion on
304 fibronectin did not differ between DU145NC and DU145GCNT2KD4 cells,
305 significantly fewer DU145GCNT2KD4 cells demonstrated spreading activity
306 (Figs. 4A and 4B). Integrin–ECM interactions that mediate outside–inside
307 signals play important roles in cell spreading and migration.[29, 30] Moreover,
308 abnormal heterodimeric forms of these molecules have been shown to inhibit
309 integrin-mediated signaling.[31, 32] However, heterodimeric forms of α5β1
310 integrin did not differ between GCNT2 knockdown and DU145NC cells (Fig.
311 4C). In previous studies, FAK and phosphoinositide 3-kinase/protein kinase
312 B (PI3K/AKT) are reported downstream targets of integrin-mediated
313 signaling.[29, 32, 33] Although FAK phosphorylation at tyrosine 397 (p-Y397)
314 was not affected by GCNT2 expression on fibronectin-coated plates, AKT
315 phosphorylation at serine 473 (p-S473) was significantly less in
316 DU145GCNT2KD4 cells than in DU145NC cells (Figs. 4D). Moreover, AKT

317 p-S473 was inhibited by antibody blocking and RGD peptide of the
318 fibronectin–integrin interaction (Fig. 4E and 4F), suggesting that I-antigens
319 enhance integrin mediated PI3K/AKT signaling.

320 *I-antigens support α5β1integrin–fibronectin induced cell migration*

321 To demonstrate the roles of I-antigens, cell migration assays were
322 performed after treatments of DU145 cells with various inhibitors. In these
323 experiments, function-blocking antibodies against α5 integrin and β1
324 integrin strongly inhibited DU145NC and DU145GCNT2KD4 cell migration
325 (Fig. 5A). Moreover, the AKT phosphorylation inhibitor had high efficacy in
326 DU145 cells without causing cytotoxicity (Figures S5A and S5B), although
327 numbers of migrant cells were significantly fewer among DU145NC cells in
328 which AKT is strongly activated than in DU145GCNT2KD4 cells that
329 express AKT p-S473 at low levels (Fig. 5B). Because I-antigens are carried on
330 *O*-glycan and glycolipids, we determined which glycan is more important for
331 AKT p-S473 and cell migration. Although BAG treated DU145NC cells had
332 significantly reduced migration potential (Fig. 5D), AKT p-S473 did not
333 differ between vehicle control and BAG-treated cells (Fig. 5C). However,
334 after PPMP treatments, DU145NC cells showed strongly inhibited AKT
335 p-S473 and cell migration (Figs. 5E and 5F). In addition, cell viability was
336 >90% after BAG and PPMP treatments (Figures S5C and S5D), indicating
337 that *O*-glycans carrying I-antigens support cell migration, and that
338 glycolipids carrying I-antigens play important roles in integrin–
339 fibronectin-mediated PI3K/AKT activation and cell migration.

Discussion

This study demonstrates that highly metastatic PCa cell lines express *GCNT2* at high levels. On the basis of immunohistochemical analyses of radical prostatectomy specimens, GCNT2 expression on PCa cell surfaces closely correlated with extra-capsular extensions of PCa. It is also noteworthy that patients with GCNT2-negative PCa exhibited better PSA-free survival compared with patients with GCNT2-positive tumors (Fig. 1). Moreover, multivariate analysis revealed that GCNT2 is an independent predictor for PSA recurrence of PCa (Table 1). Subsequent experiments also indicated strong correlations between GCNT2 expression and malignant potential of PCa.

Integrin–ECM-mediated signaling is reportedly central to solid tumor locomotion,[29, 30] and integrin function is reportedly regulated by glycan structure.[32] Integrin α5β1 is a well-known fibronectin receptor and its binding function is regulated by *N*-glycan. Moreover, depletion of *N*-glycan from α5β1 integrin by *N*-glycosidase F inhibited α5β1 integrin–fibronectin interactions,[28] and cell motility was positively associated with the formation of MGAT5-mediated GlcNAcβ1-6 branching *N*-glycans.[22] Inhibition of α-mannosidase I by 1-deoxymannojirimycin in fibroblasts led to the formation of high mannose type *N*-glycan, and although immature α5β1 integrin was overexpressed on cell surfaces, the immature form strongly inhibited fibronectin binding affinity.[27] GCNT2 also transformed GlcNAc

363 β1-6 residues to galactose and formed the I-antigen on *N*-glycans (Fig. 1).[12]
364 In the present study, we demonstrated that I-antigens predominantly carried
365 *O*-glycans and glycolipids (Fig. 3), and were not affected by α5β1 integrin
366 heterodimerization and fibronectin binding affinity. Knockdown of GCNT2
367 also led to similar α5β1 integrin expression levels, suggesting that
368 GCNT2-mediated branched-form I-antigen indirectly regulates α5β1 integrin
369 function in PCa cells.
370 Previous studies showed associations of glycolipid with malignant
371 potential of melanomas,[34] breast cancers,[35, 36] and prostate cancers.[37] In
372 accordance, glycolipids were expressed on various cell surfaces, and their
373 glycan structures were modified by several glycosyltransferases. Moreover,
374 these glycolipids regulated not only cell–cell interactions by glycan–glycan
375 interactions [38] but also regulated cell adhesion and migration by
376 glycolipid/tetraspanin or glycolipid/caveola interactions.[39]
377 Glycosphingolipid GM3 (NeuAcα2-3Galβ1-4Glcβ1-Cer) was also
378 predominantly expressed at cell adhesion cites and inhibited cell migration.
379 In a previous study of bladder carcinomas, high expression of GM3 blocked
380 integrin and tetraspanin interactions and inhibited Src kinase signaling.[40]
381 In the present study, GCNT2 had the potential to modify glycolipids, and its
382 inhibition strongly reduced cell migration (Fig. 2). In addition, GCNT2
383 knockdown PCa cells showed strongly reduced α5β1 integrin–
384 fibronectin-mediated AKT phosphorylation (Fig. 4). Previously, AKT
385 phosphorylation was regulated by integrin linked kinase, which is a known

386 downstream target of integrin activated Src kinase.[33, 41] Moreover, effects of
387 AKT activation on proliferation, survival, and migration have been
388 reported [42] and suggest that glycolipid-bearing I-antigens stabilize
389 integrin-mediated signaling and/or integrin/tetraspanin-mediated Src kinase
390 activation. Because inhibition of AKT phosphorylation inhibited PCa cell
391 migration, glycolipid-bearing I-antigens enhance AKT phosphorylation and
392 cell migration.
393 To establish a new diagnostic marker and therapeutic target, Carroll *et al.*
394 immunized mice against the androgen-independent cell line PC3 and
395 established a monoclonal antibody (mAb) against F77.[43] Subsequently, high
396 expression of the F77 antigen was shown in androgen-independent prostate
397 cancer cell lines (PC3 and DU145), which had reduced cell proliferation *in*
398 *vitro* and *in vivo* in the presence of the F77 mAb. In a subsequent study, the
399 F77 antigen was shown to be carried by glycolipids.[44] Moreover, a recent
400 study showed that the F77 mAb recognized blood group H antigen-like
401 glycan structures and GlcNAc β1-6Gal/GalNAc branching structures.[21, 45] In
402 the present study, GCNT2, which catalyzes GlcNAc β1-6Gal branching, was
403 more strongly expressed in androgen-independent cell lines than in an
404 androgen-sensitive cell line (LNCaP). Moreover, I-antigen-expressing PCa
405 cells showed strongly activated AKT. Taken together, these data suggests
406 that glycolipid-carrying I-antigens play important roles in PCa proliferation
407 and migration.
408 Although the mechanisms of GCNT2-mediated cancer progression

remain poorly understood, the present experiments demonstrate that GCNT2-formed I-antigens are predictive of the malignant potential of PCa cells. However, further research is necessary to determine the utility of GCNT2 as a therapeutic target and biomarker for PCa.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1

I-branching *N*-acetylglucosaminyltransferase (GCNT2) expression correlates with prostate cancer (PCa) progression.

(**A**) Biosynthetic pathways for I-antigen. (B) Expression level of GCNT2 was determined using quantitative polymerase chain reaction analysis. (**C**) PCa specimens were stained with an anti-GCNT2 antibody followed by staining with a horseradish peroxidase-conjugated secondary antibody. Counterstaining was performed using hematoxylin and eosin. GCNT2-positive cancer cells are indicated in brown. (**D**) Prostate-specific antigen-free survival periods were compared between GCNT2-positive and GCNT2-negative specimens, and survival analysis was conducted using Kaplan–Meier curves. Scale bars: 200 μm.

Figure 2

I-branching *N*-acetylglucosaminyltransferase (GCNT2) expression controls prostate cancer cell invasion.

(**A**) Messenger RNA expression of GCNT2 was determined in DU145 cells using qPCR. GCNT2 expression levels were normalized to those of human glyceraldehyde-3-phosphate dehydrogenase. Clone 3 and Clone 4 (DU145GCNT2KD3 and DU145GCNT2KD4) showed >70% inhibition of GCNT2 expression. (B) Protein expression of GCNT2 was determined in DU145-derived cells using western blotting. The GCNT2 expression level

592 was lower in DU145GCNT2KD3 and 4 than that in DU145NC. (C) *In vitro*
593 cell proliferation was similar in DU145GCNT2KD3 and DU145GCNT2KD4
594 cells at day seven, and strongly reduced cell migration (D) and invasion (E)
595 was observed in Transwell assays. Assays were conducted in triplicate; *, $p <$
596 0.05
597
598 **Figure 3**
599 I-branching *N*-acetylglucosaminyltransferase (GCNT2) expression regulates
600 I-antigen presentation on prostate cancer cells.
601 Cell surface I-antigen expression was determined using flow cytometry (FC)
602 and immunocytochemistry (IHC) with anti-I antigen human antisera (Ma).
603 (**A**) DU145GCNT2KD3 and DU145GCNT2KD4 showed decreased cell
604 surface I-antigen expression in FC analyses. (B) DU145NC and
605 DU145GCNT2KD4 cells were cultured on glass slides and were stained with
606 anti-I antigen human antisera (Ma) or the human IgM isotype control. The
607 brown color indicates I-antigen expression and the blue color indicates
608 nuclear staining. DU145GCNT2KD4 cells had strongly reduced I-antigen
609 expression. DU145 cells were cultured with benzyl-α-GalNAc (BAG),
610 tunycamycin (TM), or
611 DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol
612 hydrochloride (PPMP) for 48 h. I-antigen expression was determined using
613 IHC (C) and FC (D). I-antigen expression was significantly reduced in
614 BAG-treated cells and PPMP-treated cells. TM-treated cells had no effect on

I-antigen presentation. Co-treatment with BAG and PPMP strongly reduced I-antigen expression compared with either treatment alone. Population comparison was performed using Flowjo softwear. Assays were conducted in triplicate. (E) Integrin expression was determined using FC, and expression of α5 and ß1 integrins was similar in DU145NC and GCNT2 knockdown cell lines. Scale bars: 200 μm.

Figure 4

I-antigen regulates cell spread and α5ß1 integrin–fibronectin interactions that are mediated by AKT phosphorylation in DU145NC and DU145GCNT2KD4 cells.

Cell adhesion and spreading on fibronectin was examined by (A) harvesting cells after a 30 min culture on fibronectin-coated 96-well plates. DU145NC and DU145GCNT2KD4 cells showed no significant differences in adhesion potential. (B) Cells were harvested from fibronectin-coated glass slides after 30 min of incubation, and spreading cells were visualized using crystal violet solution. Percentages of spreading cells were significantly less in DU145GCNT2KD4 cells than in DU154NC cells. (C) Cell lysates from DU145 cells were immunoprecipitated using anti-integrin α5 or ß1 antibodies, and heterodimers were detected. GCNT2 expression had no effect of heterodimerization of α5ß1 integrin. (D) Focal adhesion kinase (FAK) and protein kinase B (AKT) are downstream targets of integrin signaling. FAK phosphorylation at tyrosine 397 (p-Y397) was similar in DU145NC and

638 DU145GCNT2KD4 after integrin–fibronectin interactions. In contrast,
639 AKT phosphorylation at serine 473 (p-S473) was strongly inhibited by
640 GCNT2 knockdown in DU145 cells. (E) A functional blocking antibody
641 against α5 and ß1 integrins inhibited α5ß1 integrin–fibronectin interactions.
642 Pre-treatment of DU145NC and DU145GCNT2KD4 cells with anti-integrin
643 α5 or ß1 led to lower FAK p-Y397 and AKT p-S473 levels compared those
644 after pre-treatment with IgG isotype control. (F) Pre-treatment of DU145NC
645 cells with RGD peptide (100, 200, 400 or 800 μM) inhibited FAK p-Y397 and
646 AKT p-S473 in a concentration-dependent manner.
647
648 **Figure 5**
649 Glycolipid carrying I-antigens play important roles in integrin-mediated
650 AKT phosphorylation and migration of prostate cancer cells.
651 (A) Functional blocking antibodies against α5 and ß1 integrins significantly
652 inhibited cell migration in DU145NC and DU145GCNT2KD4 cells. (B) AKT
653 phosphorylation at serine 473 (p-S473) was inhibited by the AKT inhibitor
654 VIII (10 mM), and cell migration was inhibited in DU145NC cells but not
655 DU145GCNT2KD4 cells. (C) DU145NC cells were cultured with
656 benzyl-α-GalNAc (BAG),
657 DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol
658 hydrochloride (PPMP) or with dimethyl sulfoxide (DMSO) for 48 h. (C)
659 DU145NC cells were cultured with DMSO or BAG on fibronectin-coated
660 dishes for 20 min. Depletion of *O*-glycan had no effect on AKT p-S473. (D)

BAG treated DU145NC cells had significantly inhibited cell migration. (E) Depletion of glycolipids in DU145NC cells significantly inhibited AKT p-S473 and (F) migration. Migration assays were conducted in triplicate.

Supporting Information

Fig. S1. Nodal metastatic prostate cancer expresses I-branching N-acetylglucosaminyltransferase (GCNT2).

Fig. S2. I-branching *N*-acetylglucosaminyltransferase (GCNT2) expression regulates prostate cancer cell migration and invasion without affecting the epithelial mesenchymal transition.

Fig. S3. I-antigen expression on the cell surface of GCNT2-expressing prostate cancer cell lines.

Fig. S4. Treatment with glycosylation inhibitor decreases presentation of *O*-glycan, *N*-glycan and glycolipid on prostate cancer cell surface.

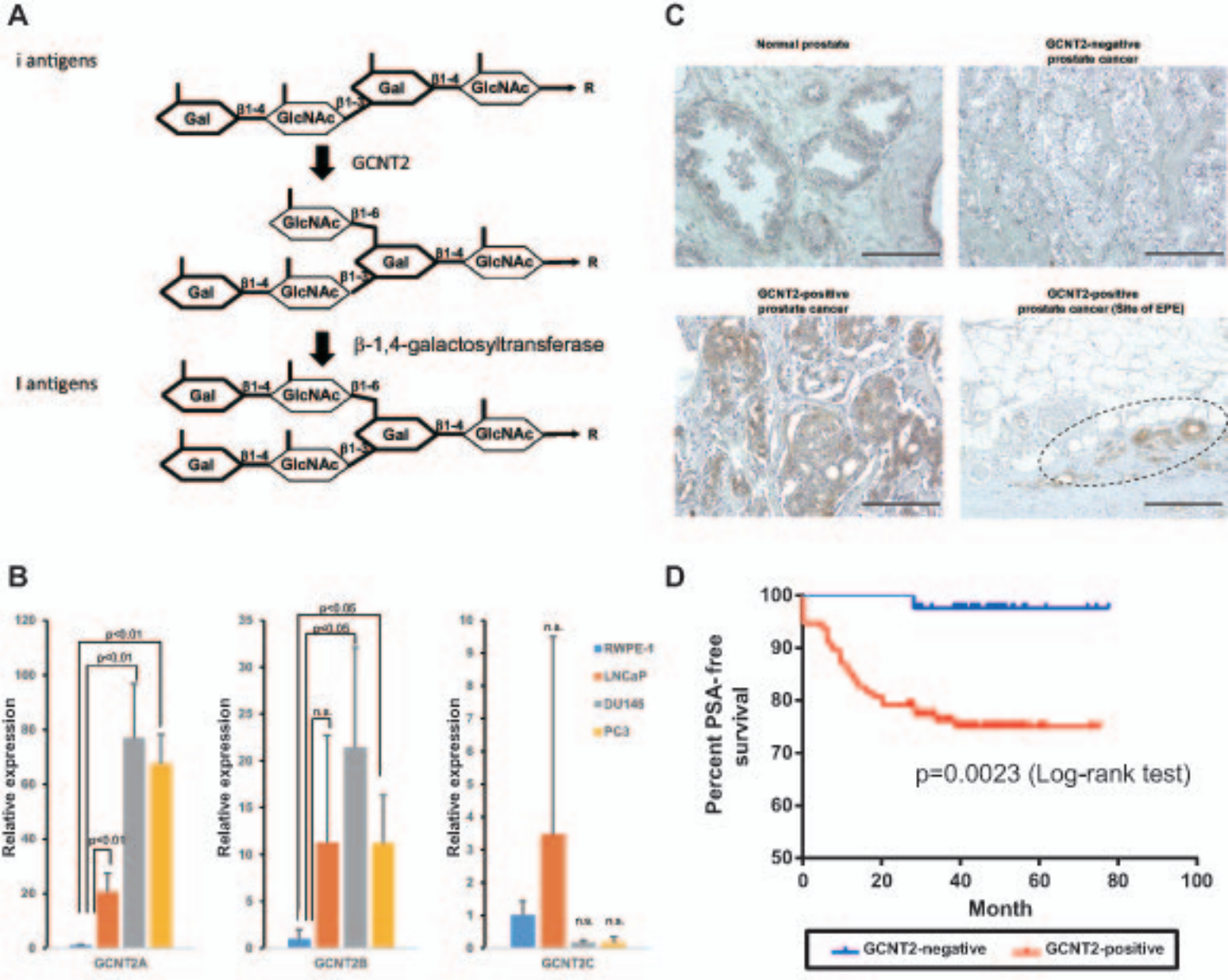
Fig. S5. The AKT inhibitor, the *O*-glycosylation inhibitor, and the glucosylceramide synthetase inhibitor had no effects on cell viability during assays.

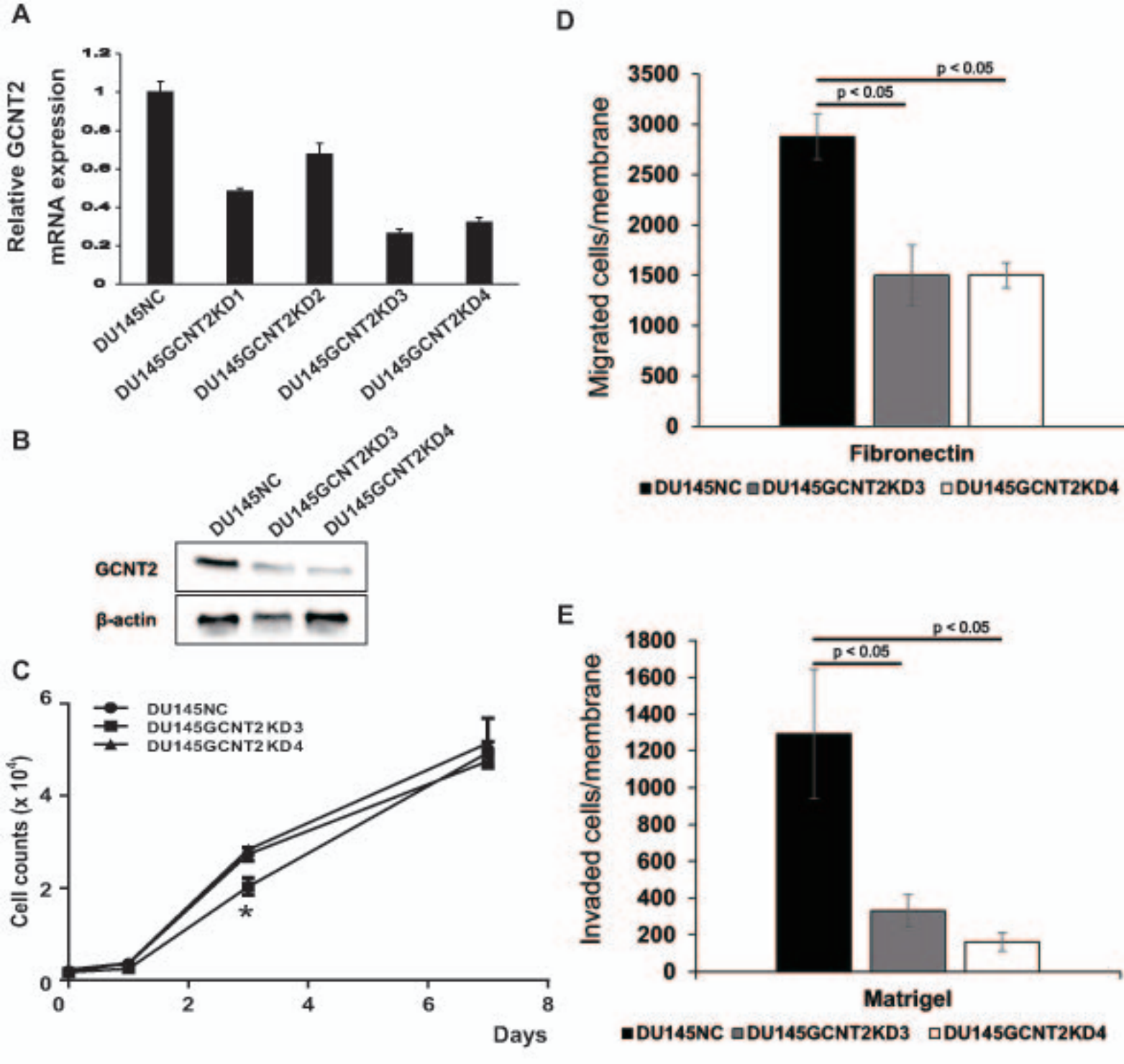
Table S1. Primer list

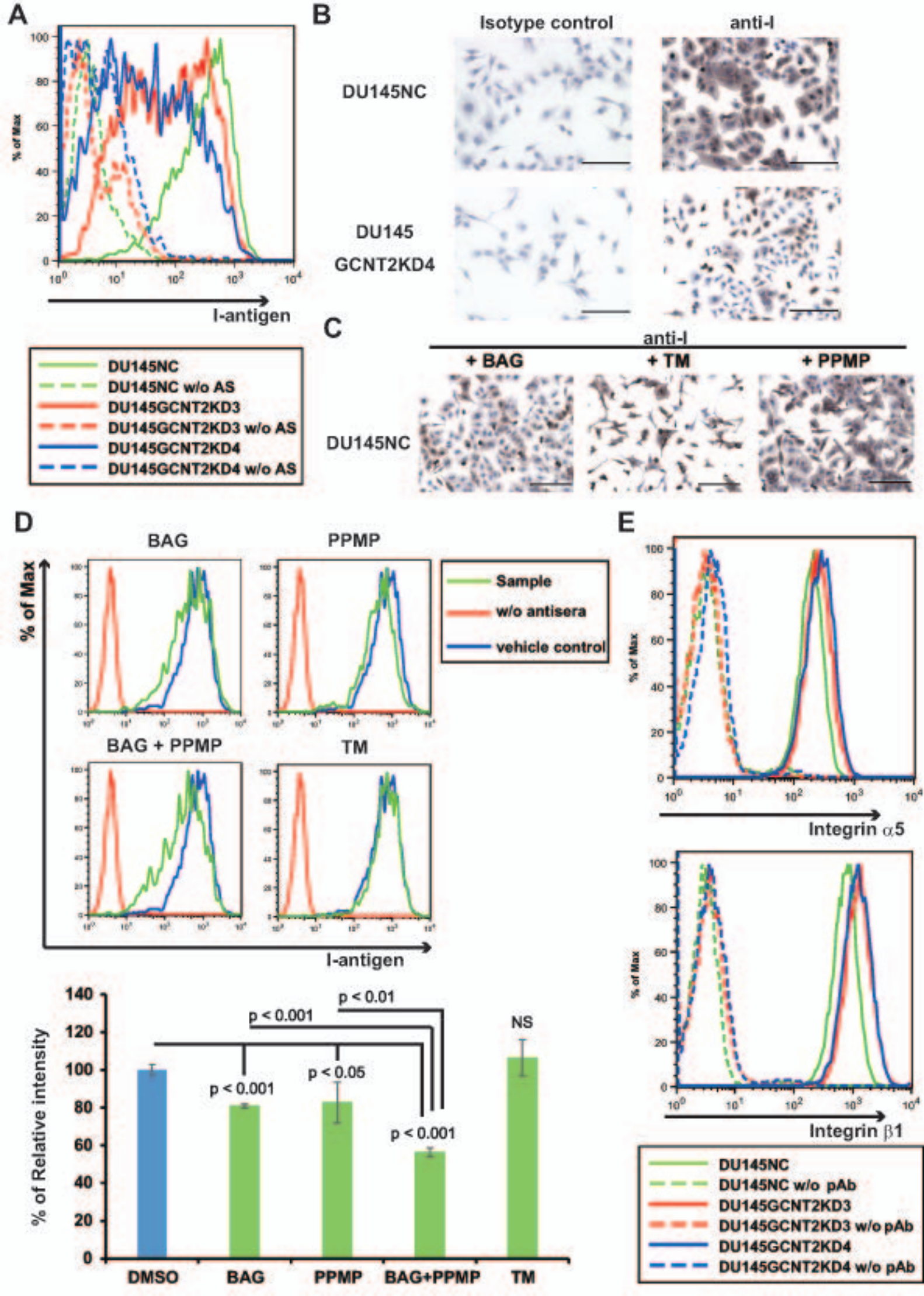
Table S2. I-branching *N*-acetylglucosaminyltransferase status and patient data

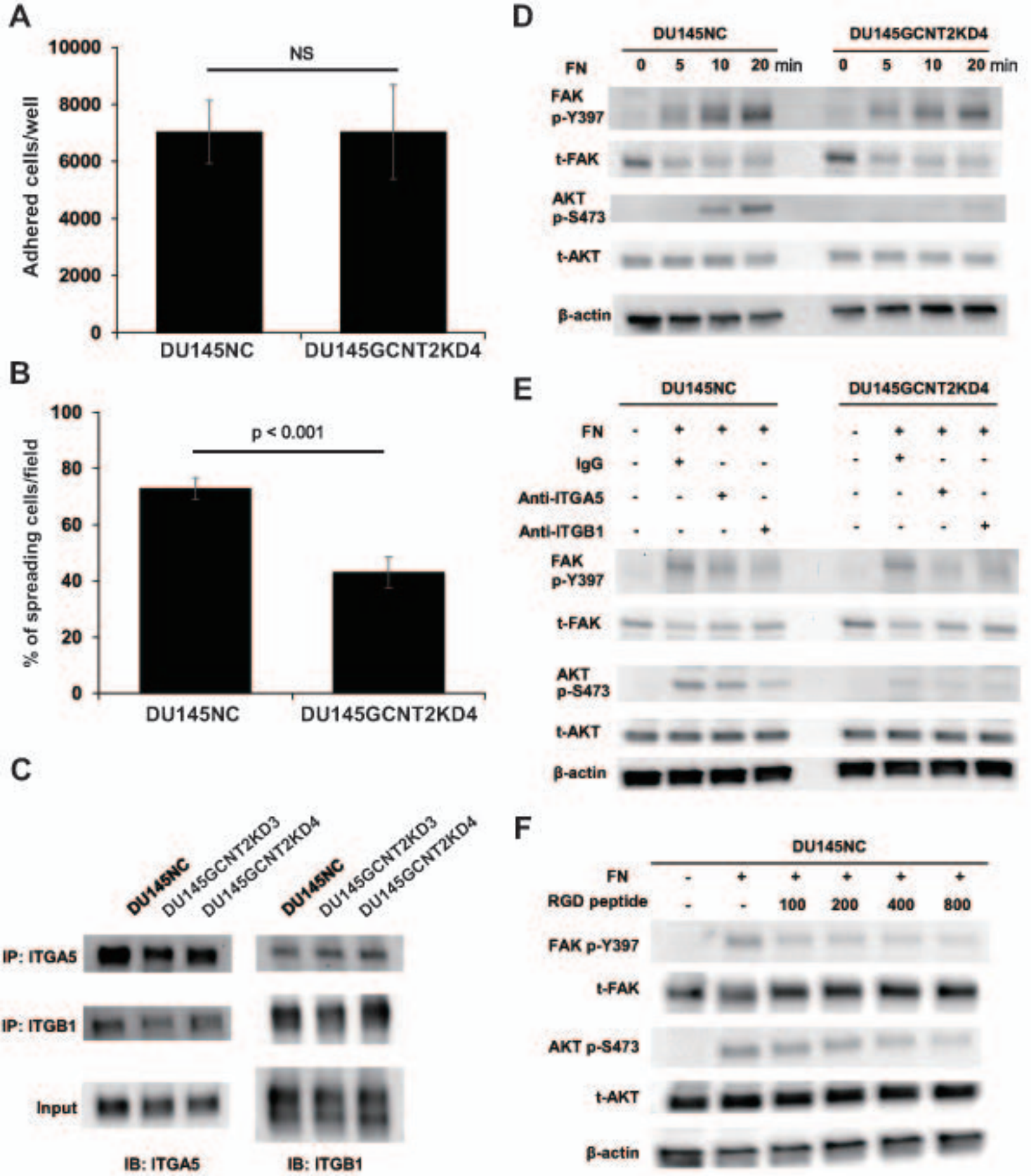
Table S3. I-branching *N*-acetylglucosaminyltransferase status and pathological parameters

Doc. S1. Supplementary Materials and Methods


A
i antigens
Gal
β1-4
GlcNAc
β1-3
Gal
β1-4
GlcNAc
R
GCNT2
GlcNAc
β1-6
Gal
β1-4
GlcNAc
R
β-1,4-galactosyltransferase
I antigens
GlcNAc
β1-6
Gal
β1-4
GlcNAc
R
C
Normal prostate
GCNT2-negative prostate cancer
GCNT2-positive prostate cancer
GCNT2-positive prostate cancer (Site of EPE)
B
Relative expression
p<0.01
n.s.
RWPE-1
LNCaP
DU145
PC3
GCNT2A
GCNT2B
GCNT2C
D
Percent PSA-free survival
p=0.0023 (Log-rank test)
Month
GCNT2-negative
GCNT2-positive


A
Relative GCNT2 mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
DU145NC
DU145GCNT2KD1
DU145GCNT2KD2
DU145GCNT2KD3
DU145GCNT2KD4
B
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4
GCNT2
β-actin
C
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4
Cell counts (x 10⁴)
6
4
2
0
*
0
2
4
6
8
Days
D
Migrated cells/membrane
3500
3000
2500
2000
1500
1000
500
0
p < 0.05
p < 0.05
Fibronectin
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4
E
Invaded cells/membrane
1800
1600
1400
1200
1000
800
600
400
200
0
p < 0.05
p < 0.05
Matrigel
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4


A
% of Max
I-antigen
DU145NC
DU145NC w/o AS
DU145GCNT2KD3
DU145GCNT2KD3 w/o AS
DU145GCNT2KD4
DU145GCNT2KD4 w/o AS
B
Isotype control
anti-I
DU145NC
DU145
GCNT2KD4
C
anti-I
+ BAG
+ TM
+ PPMP
DU145NC
D
BAG
PPMP
BAG + PPMP
TM
% of Max
I-antigen
Sample
w/o antisera
vehicle control
% of Relative intensity
p < 0.001
p < 0.01
p < 0.001
p < 0.05
p < 0.001
NS
DMSO
BAG
PPMP
BAG+PPMP
TM
E
% of Max
Integrin α5
Integrin β1
DU145NC
DU145NC w/o pAb
DU145GCNT2KD3
DU145GCNT2KD3 w/o pAb
DU145GCNT2KD4
DU145GCNT2KD4 w/o pAb


A
Adhered cells/well
10000
8000
6000
4000
2000
0
NS
DU145NC
DU145GCNT2KD4
B
% of spreading cells/field
100
80
60
40
20
0
p < 0.001
DU145NC
DU145GCNT2KD4
C
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4
DU145NC
DU145GCNT2KD3
DU145GCNT2KD4
IP: ITGA5
IP: ITGB1
Input
IB: ITGA5
IB: ITGB1
D
DU145NC
DU145GCNT2KD4
FN
0 5 10 20 min
0 5 10 20 min
FAK p-Y397
t-FAK
AKT p-S473
t-AKT
β-actin
E
DU145NC
DU145GCNT2KD4
FN
IgG
Anti-ITGA5
Anti-ITGB1
FAK p-Y397
t-FAK
AKT p-S473
t-AKT
β-actin
F
DU145NC
FN
RGD peptide
- + + + + +
- - 100 200 400 800
FAK p-Y397
t-FAK
AKT p-S473
t-AKT
β-actin

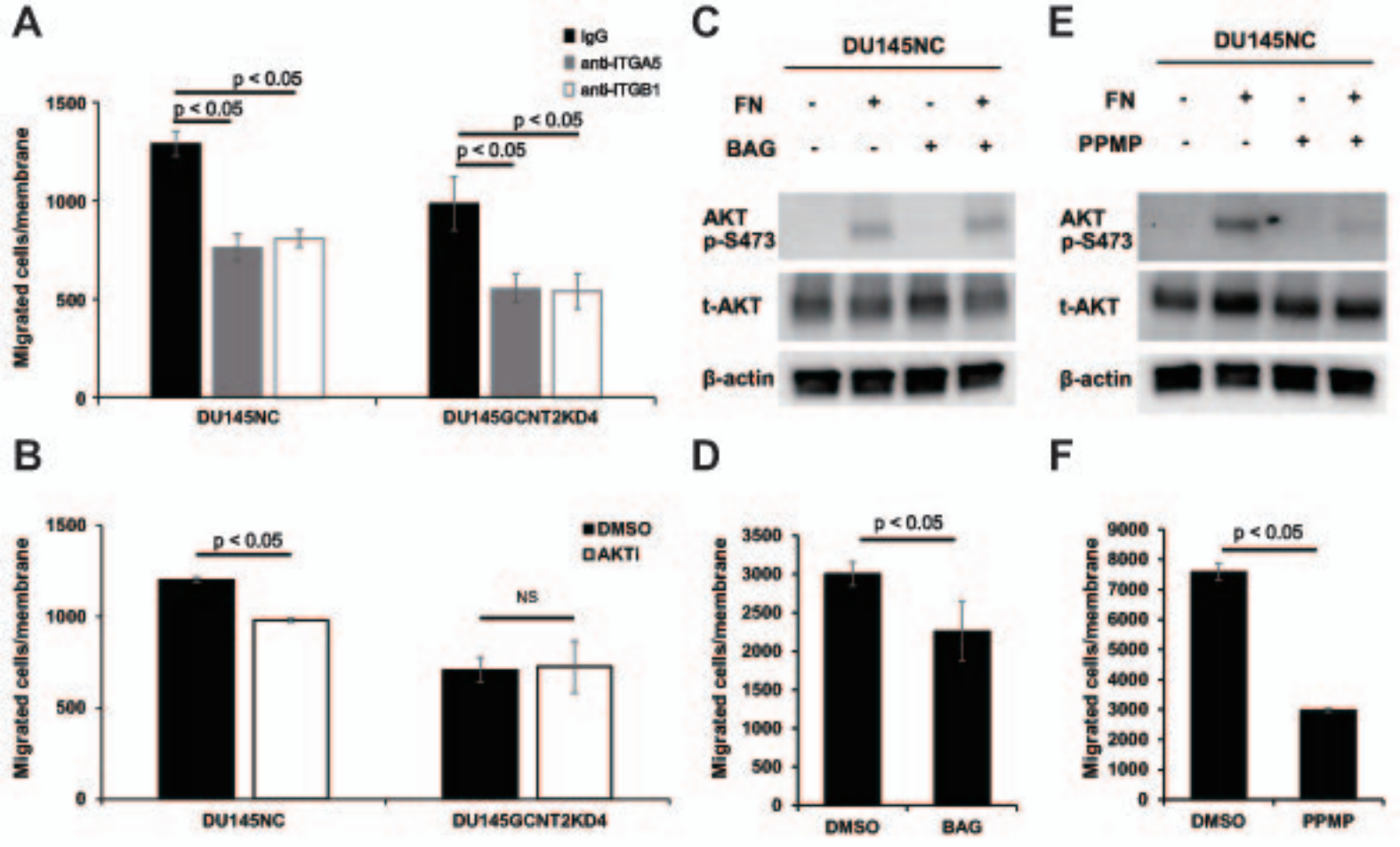

A
IgG
anti-ITGA5
anti-ITGB1
p < 0.05
p < 0.05
p < 0.05
p < 0.05
Migrated cells/membrane
1500
1000
500
0
DU145NC
DU145GCNT2KD4
B
DMSO
AKTi
p < 0.05
NS
Migrated cells/membrane
1500
1000
500
0
DU145NC
DU145GCNT2KD4
C
DU145NC
FN - + - +
BAG - - + +
AKT p-S473
t-AKT
β-actin
D
p < 0.05
Migrated cells/membrane
3500
3000
2500
2000
1500
1000
500
0
DMSO
BAG
E
DU145NC
FN - + - +
PPMP - - + +
AKT p-S473
t-AKT
β-actin
F
p < 0.05
Migrated cells/membrane
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
DMSO
PPMP

Table 1 Cox proportional-hazards model for predicting prostate specific antigen-free survival

Multivariate analysis

	p value	Exp (B)	95.0% CI	
			min	max
Age	**0.759**	**1.012**	**0.940**	**1.089**
iPSA[†]	**0.022**	**1.065**	**1.009**	**1.123**
cT2≤	**0.920**	**1.043**	**0.458**	**2.374**
biopsy GS[‡]	**0.076**	**2.622**	**0.903**	**7.611**
post-Ope GS[‡]	**0.701**	**0.819**	**0.295**	**2.276**
pT3≤[§]	**0.446**	**1.534**	**0.510**	**4.612**
Margin status[¶]	**0.027**	**0.134**	**0.015**	**0.253**
perineural invasion	**0.822**	**1.121**	**0.413**	**3.041**
GCNT2 status	**0.032**	**9.021**	**1.203**	**67.630**

[†]pre-treatment with prostate specific antigen, [‡]Gleason score, [§]extra-capsular extension, [¶]cancer presence at the resected margin, GCNT2; I-branching *N*-acetylglucosaminyltransferase