Integrin α6A splice variant regulates proliferation and the Wnt/β-catenin pathway in human colorectal cancer cells

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The integrin α6 subunit pre-messenger RNA undergoes alternative splicing to generate two different splice variants, named α6A and α6B, having distinct cytoplasmic domains. In the human colonic gland, these splice variants display different patterns of expression suggesting specific functions for each variant. We have previously found an up-regulation of the α6B4 integrin in colon adenocarcinomas as well as an increase in the α6A/α6B ratio, but little is known about the involvement of α6Aβ4 versus α6Bβ4 in this context. The aim of this study was to elucidate the function of the α6Aβ4 integrin in human colorectal cancer (CRC) cells. Expression studies on a panel of primary CRCs confirmed that the up-regulation of the α6B subunit in CRC is a direct consequence of the increase of the α6A variant. To investigate the functional significance of an α6A up-regulation in CRC, we specifically knocked down its expression in well-established CRC cell lines using a small-hairpin RNA approach. Results showed a growth rate reduction in all α6A knockdown CRC cell lines studied. The α6A silencing was also found to be associated with a significant repression of a number of Wnt/β-catenin pathway end points. Moreover, it was accompanied by a reduction in the capacity of these cells to develop tumours in xenografts. Taken together, these results demonstrate that the α6A variant is a pro-proliferative form of the α6 integrin subunit in CRC cells and appears to mediate its effects through the Wnt/β-catenin pathway.

Introduction

The integrin superfamily is composed of the transmembrane receptors responsible for mediating epithelial-basement membrane interactions. Integrins are formed by the heterodimeric association of an α and a β subunit and, to date, 18 α and 8 β subunits have been identified, which can combine to form 24 distinct integrins (1). The existence of multiple splice variants and post-translational modification of most subunits increases the variety of integrins (2). These receptors can mediate intracellular signalling despite their lack of intrinsic kinase activity. Indeed, ligand binding (i.e. laminin, collagen and fibronectin) induces the recruitment of intracellular kinases and adaptor proteins via the cytoplasmic C-terminal domains of either integrin subunit, mediating intracellular signalling to regulate a large spectrum of cell processes including proliferation, adhesion, migration and apoptosis (3,4).

Colorectal cancer (CRC) is the second leading cause of cancer death in North America (5) and accumulating studies confirm an important role for integrin receptors in human colorectal tumourigenesis (6–9). Interestingly, the α6 integrin subunit can heterodimerize with either β1 or β4 to form the α6β1 or α6β4 integrins but in the gut epithelium as well as in CRC, the α6 integrin subunit predominantly associates with β4 (10–12). Moreover, both the α6 and β4 integrin subunits appear to be over-expressed in primary tumours of the human colon (12–14) and CRC cell lines (12,13,15), suggesting an important role for this integrin in CRC progression (7,9). Although the β4 subunit exists as five splice variants, it is the β4A variant subunit that is predominantly expressed in the gut (11). We have previously described a cytosolic variant of β4A resulting from the proteolytic cleavage of the C-terminal domain (cdt), called β4cdt−, that is non-functional for adhesion to laminin and associated with normal intestinal proliferative epithelial cells but it is the wt form, β4cdt+, that is predominantly present in CRC and in all CRC cell lines studied (11,13). To date, signalization from β4 in cancer has been well characterized. For instance, outside-in signalling leads to phosphorylation of the cytoplasmic C-terminal domain of β4, recruitment of SHC/GRB2 and/or IRS1/IRS2 and downstream activation of the MAPK/ERK and PI3K/AKT pathways (16), thus regulating major cell processes involved in tumourigenesis (16–19).

Although the majority of α6β4 functions in cancer have been attributed to the β4 subunit, recent evidence suggests that signalization events mediated by the α6 subunit can also regulate the processes involved in tumourigenesis including proliferation and metastasis (20–25). However, the α6 integrin subunit exists as two splice variants, α6A and α6B, generated by the alternative splicing of exon 25, resulting in the formation of two distinct cytoplasmic domains (26). The existence of two variants with distinct C-tails suggests that each may have a specific function in the regulation of cellular processes. In support of this, a study performed using two yeast hybrids has shown that the PDZ domain of each variant can interact with specific intracellular molecules (27,28). Furthermore, other studies have demonstrated that each variant initiates different intracellular signalling events, such as paxillin phosphorylation (29) and RAS-MEK-ERK activation (30). In human tissues, the α6A and α6B variant subunits display distinct patterns of expression (26) as for instance in the skin where α6A is exclusively associated with basal cells. Previous results from our laboratory have shown that in the normal human small intestine, the α6A variant is predominantly associated with proliferative cells in the glands, whereas the α6B variant is mainly localized in quiescent and differentiated cells in the villus epithelium (31). Although also detected in the normal colon, this pattern of expression is lost in primary tumours where α6A becomes ubiquitously expressed in all CRC cells (12), supporting the possibility that inclusion of the α6A subunit into α6β4 integrin generates a pro-proliferative integrin (7). In the context where deregulation of cell proliferation is one of the hallmarks of cancer and that the α6 subunit appears to be involved in the process (20–25), we propose that the pro-proliferative function of α6 is specifically mediated by its α6A splice variant in CRC.

In the present study, we tested this hypothesis using a knockdown approach targeting the mature α6A messenger RNA (mRNA) and found that α6A ablation significantly reduced CRC cell proliferation both in vitro and in xenografts. Furthermore, we also found that this effect was accompanied by a decline in the Wnt/β-catenin signalling pathway.

Materials and methods

Primary antibodies and materials

Primary antibodies used for the detection of the α6A and α6B variants were anti-α6A [western blot (WB): 1/5000, immunofluorescence (IF): 1/1000] (1A10, Millipore, Etobicoke, Ontario) and anti-α6B (WB: 1/500, IF: 1/1000) (6B4, Millipore). These antibodies were originally a generous gift from Dr A.Sonnenberg (Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Other primary antibodies used were anti-integrin β4 (WB: 1/5000, IF: 1/1000) (3E1, Millipore), anti-integrin α6 (IF: 1/1000)

Abbreviations: APC, adenomatous polyposis coli; BrDU, 5-bromo-2-deoxyuridine; CRC, colorectal cancer; IF, immunofluorescence; ISEL, in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; mRNA, messenger RNA; PBS, phosphate-buffered saline; qPCR, quantitative PCR; RM, resection margin; WB, western blot.
GOH3, Millipore), anti-β-actin (WB: 1/75 000) (C4, Millipore), anti-active-β-catenin (WB: 1/2500) (8E7, Millipore) recognizing the dephosphorylated form of β-catenin on Ser37 and Thr41 (sites of GSK3β phosphorylation), anti-β-catenin (WB: 1/2500) (610153, BD Biosciences, Mississauga, Ontario), anti-DVL2 (WB: 1/2500) (30D2, Cell Signaling Technology, Danvers, MA), anti-cytokeratin 18 (WB: 1/1 000 000) (CY-90, Sigma–Aldrich, Oakville, Ontario), anti-histone H1 (WB: 1/1000) (AE-4, Santa Cruz Biotechnology, Santa Cruz, CA), anti-integrin [I (WB: 1/1000) (Mab13, BD Biosciences), anti-GSK3β] (WB: 1/5000) (27C10, Cell Signaling Technology) and anti-H3K27me3 (WB: 1/1000) (07-449, Millipore). The pharmacological inhibitor of GSK3β (SB216763, S3442, Sigma–Aldrich) was used at a final concentration of 20 μM. The protease inhibitor cocktail (P8340) was purchased from Sigma.

Cell culture and generation of CRC cells knocked down for αvβ3 subunit expression

The CRC cell lines Caco-2/15 and T84 (polared) as well as HT29 and DLD-1 (non-polared) were obtained from the American Type Culture Collection (www.ATCC.org) and cultured as described (11–13). All cells were grown in an atmosphere of 95% air and 5% CO₂ at 37°C. Colon cancer cells were plated at 60% confluence 24 h prior to infection with lentiviruses prepared with MISSION® shRNA (Sigma–Aldrich) plasmids for the human αvA integrin containing the shRNA sequence: 5′-CCG GCC TTT GGA CGT AAAG GGA GAA ACT CGT TCT TCT TCT AGT CAA AAG GTT TTT G-3′. The nega

ive control shRNA (sequence: 5′-CCG GCC TTT CAA CGT TCT TCT TCT AGT CAA AAG GTT TTT G-3′) was co-transfected with TOPflash, 4B staining, cells were fixed in MeOH and EtOH, and centrifuged at 3000 r.p.m. for 5 min at 4°C. Supernatants were removed and cells were resuspended in low salt buffer (20 mmol/l Tris–HCl (pH 7.4) and both primary and secondary antibodies were diluted in 5% BSA/PBS. For αvA staining, cells were fixed in MeOH and ETOH, respectively. Non-specific sites were blocked for 1 h at room temperature in a 2% bovine serum albumin solution in PBS (pH 7.4) and both primary and secondary antibodies were diluted in 2% bovine serum albumin–PBS (pH 7.4). Cells were treated with a 0.2% Triton X-100 solution for 5 min prior to anti

body incubation. Primary antibodies were detected with Alexa Fluor 488 or 594 goat anti-mouse secondary antibodies (Invitrogen, A11017, A11032) and Alexa Fluor 488 goat anti-rat secondary antibody (Invitrogen, A11006).

Transfections and luciferase assays

TOPflash and FOPflash reporter plasmids (Millipore) were transfected into CRC cell lines with Effectene transfection reagent (Qiagen) using the manufacturer’s instructions. Firely and renilla luciferase activities were measured using the dual luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, each cell line was plated at 5×10³ cells/well in 12-well plates. Cells were co-transfected with TOPflash or FOPflash reporter plasmids and the renilla luciferase expression plasmid (Promega) and treated with dimethyl sulfoxide or SB216763 for 48 h. Data were obtained by calculating the ratio of firefly/renilla luciferase expressions for the TOPflash and FOPflash reporter plasmids. The FOPflash ratio was subtracted from the TOPflash ratio. Data represent three separate experiments performed in triplicate.

Human colorectal tissues

Samples of 97 CRC and paired normal tissues (at least 10 cm from the tumour) were obtained from patients undergoing surgical resection without prior neoadjuvant therapy. Tissues were obtained after patients’ written informed consent, according to a protocol approved by the Institutional Human Subject Review Board of the Centre Hospitalier Universitaire de Sherbrooke. Diagnoses, staging and grading were performed by the pathologists of the Department of Pathology of the Centre Hospitalier Universitaire de Sherbrooke.

RNA extraction, reverse transcription–polymerase chain reaction and quantitative PCR

RNA extraction and reverse transcription were performed as described previously (31). For competitive PCR, conditions and primers used to co-amplify αvA and αvB have been described previously (12,13,33). αvA was amplified using a PrimeTime assay (IDT, Coralville, IA) composed of primers with the sequences 5′-GATCCCTTACAGCAGTGTAGCGG and 5′-AAGAGAGGCTTACCTCTGAGTAC and a double-quenched hydrolysis probe containing the 5′ fluorophore FAM and the sequence 5′-TGC TAC TZA ZEN GAC AGT GGG ATC TTG ATG and 5′-GAG CGG AGT CAA CCA CAT CC. qPCR was performed according to the manufacturer’s instructions. Data were obtained from patients undergoing surgical resection without prior neoadjuvant therapy. Tissues were obtained after patients’ written informed consent, according to a protocol approved by the Institutional Human Subject Review Board of the Centre Hospitalier Universitaire de Sherbrooke. Diagnoses, staging and grading were performed by the pathologists of the Department of Pathology of the Centre Hospitalier Universitaire de Sherbrooke.

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Results

**Correlation between total α6 subunit and α6A variant expression in CRC**

We have shown previously that α6B/α6A integrin subunit ratios were significantly reduced in a relatively small set of human CRC samples at the transcript level (12). In an attempt to extend these observations, the mRNA levels of total α6 as well as the individual α6A and α6B variants were analyzed by qPCR in 97 CRCs and their corresponding resection margins (RMs). The level of total α6 subunit mRNA was found to be increased in CRC samples compared with RMs by more than 2-fold, as well as that of α6A, whereas the level of α6B remained stable (Figure 1A). Moreover, a close correlation was observed between the levels of α6 and α6A mRNA (P ≤ 0.0001, Pearson r = 0.588) in human CRC (Figure 1B). When each sample was analyzed individually, the expression of α6A in CRC compared with corresponding RMs was found to be increased in 69 patients, similar in 16 patients and reduced in 12 patients. Taking into consideration that significant levels of α6A are expressed in the crypt of the normal colonic mucosa of the RM, these results confirm that a large proportion of CRC cells express significant levels of α6A. Furthermore, up-regulation of α6A was observed for all tumour stages or grades (Supplementary Figure 1, available at Carcinogenesis Online) although the presence of somatic mutations of adenomatous polyposis coli (APC) had no impact on α6A expression (data not shown). Taken together, these data confirm a sustained up-regulation of the α6A splice variant in human CRC.

**Specific α6A knockdown in CRC cells**

To investigate the involvement of the α6A variant in CRC cell behaviour, four well-characterized CRC cell lines—Caco-2/15, DLD-1, T84 and HT29 cells—were infected with a shRNA targeting the α6A variant. The α6A shRNA was designed in order to recognize a unique sequence of the short exon 25 (1199b) specific to the α6A mRNA transcript. Specificity of knockdown of α6A was first confirmed by competitive PCR using primers that amplify both variants of the α6 integrin subunit in human CRC. 

**α6A knockdown does not affect the intracellular localization of α6B**

In order to verify the localization of α6B in shα6A cells, we first used IF staining on T84 cells, which display hemidesmosomes. Co-staining using a rat anti-α6 antibody and a mouse anti-β4 antibody showed that the α6 integrin subunits co-localize with the β4 subunit in both shctl and shα6A cells (Supplementary Figure 2A–C, available at Carcinogenesis Online and shα6A cells (Supplementary Figure 2D–F, available at Carcinogenesis Online) with a typical punctuated hemidesmosome-like staining pattern. As expected, the relative intensity of staining for levels of α6B (Figure 2B). WB analysis of α6A and α6B performed to extend our observations at the protein level confirmed the specific abolition of the α6A variant, without significantly affecting α6B in all CRC cell lines (Figure 2C).

**α6A knockdown leads to a decrease in α6B mRNA levels**

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both integrin subunits was lower in shα6A cells. Furthermore, using α6 variant-specific antibodies, a significant reduction of the α6A staining observed in shctl cells (Supplementary Figure 2G, available at Carcinogenesis Online) was noted in shα6A cells (Supplementary Figure 2H, available at Carcinogenesis Online), whereas the α6B staining remained comparable between shctl and shα6A cells.
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(Supplementary Figure 21 and J, available at Carcinogenesis Online). These results suggested that abolition of α6A does not alter the distribution of α6B. In order to confirm this observation, cell subfractionation was performed on shctl and shα6A DLD-1 cells, which do not display organized hemidesmosomes. WB analysis confirmed that the membrane localization of α6B was not altered by the abolition of α6A expression (Supplementary Figure 3, F2, available at Carcinogenesis Online). As expected, a decrease in the β4 integrin was observed relative to the β1 integrin. Taken together, these two sets of observations indicate that α6A knockdown has no significant effect on α6B expression and localization.

α6A regulates cell proliferation

As the α6 integrin subunit was reported to be involved in cell proliferation (20,21), we further investigated if this function could be attributed to the α6A splice variant. Therefore, the involvement of the α6A variant in cancer cell growth was first assessed by establishing a growth curve using CRC cell populations knocked down for α6A, but expressing α6B. In vitro. As shown in Figure 3A, a significant reduction in cell number was observed throughout the culture beginning as early as 2 days post-seeding for T84, HT29 and DLD-1 shα6A cells in comparison with shctl cells and at 4 days for Caco-2/15 shα6A cells. Overall, abolition of the α6A subunit led to a significant and sustained reduction of the growth rate in all CRC cells tested.

To confirm that the decrease in cell number was the result of a specific reduction in cell proliferation, all cell lines were subjected to BrdU incorporation and ISEL assays. A significant reduction in cells entering S-phase was revealed by BrdU incorporation for the four shα6A cell lines relative to their corresponding shctl cells (Figure 3B), while at the same time, ISEL experiments showed that the apoptotic index was negligible in all colorectal cell lines (Figure 3C). These results confirm the pro-proliferative function of the α6A variant on human CRC cells.

α6A variant knockdown reduces tumour growth in xenografts

The capacity of α6A knockout cells to form tumours in vivo was next evaluated by subcutaneous injection of nude mice with T84, HT29 and DLD-1 cells. Caco-2/15 cells were not included in this assay because of the long latency period required to observe tumour formation in nude mice with this cell line. Interestingly, we found that the latency period for the detection of palpable tumours was significantly delayed for T84/shα6A cells compared with T84/shctl (36 days versus 12 days) (Figure 4A), whereas this was not so for HT29 and DLD-1 cells. However, abolition of α6A in T84 and HT29 strongly diminished their growth capacity as tumours in nude mice (Figure 4A and B), resulting in a significant reduction of the tumour weight at the time of the killing (Figure 4C and D). The decrease in proliferation rate observed in DLD-1 shα6A cells in vitro was not transposed into a significant reduction in tumour growth and weight (data not shown).

However, histological haematoxylin and eosin analysis showed that DLD-1 shctl xenograft tumours displayed large necrosis/oedema regions, a feature not observed in DLD-1 shα6A xenograft tumours (Figure 4E). This observation could explain the lack of difference in tumour size development observed, despite the decrease in proliferation in DLD-1 shα6A cells. On the other hand, no histological difference was observed between shctl and shα6A xenograft tumours from T84 and HT29 cells (Figure 4E). As shown in Figure 4F, qPCR analysis of α6A and α6B in tumours confirmed that the α6A knockdown is retained in HT29 and DLD-1 and tends to be retained in T84 xenografts even after 50 days (P < 0.01 for HT29 and DLD-1; P < 0.08 for T84). Taken together, these results demonstrate that the α6A variant can regulate tumour growth in at least a subset of CRC cell lines in xenografts, confirming the pro-proliferative effect of the α6A variant.

α6A knockdown regulates the Wnt/β-catenin pathway

The Wnt/β-catenin pathway is one of the most important regulators of cell proliferation in various systems and is often strongly deregulated in human CRC (35,36), thus suggesting a possible link between α6A expression and Wnt/β-catenin activity in CRC cells. Wnt/β-catenin activity was first evaluated by determining the level of GSK3β phosphorylation of β-catenin at ser37/thr41 using an active β-catenin antibody. WB analyses of whole cell lysates showed a statistically significant decrease in the levels of active β-catenin in all shα6A cell lines relative to total β-catenin compared with shctl cells (Figure 5A). This overall decrease was also reflected in WB analyses of nuclear extracts, where β-catenin was reduced in α6A knockdown Caco-2/15, DLD-1 and T84 (Figure 5B). These results suggest that in these CRC cells, depletion of the α6A integrin variant interferes with the Wnt/β-catenin pathway by enhancing the phosphorylation of β-catenin by GSK3β, targeting it to proteasome degradation and consequently reducing its accumulation in the nucleus.

To evaluate the functional significance of reduced β-catenin levels, the activity of the Wnt/β-catenin pathway was further analyzed by the luciferase assay using a responsive β-catenin/TCF4/LEF reporter plasmid (TOPflash). As shown in Figure 5C, a sharp decrease in TOPflash activity was observed in Caco-2/15, DLD-1 and T84 shα6A cells compared with shctl. In our hands, HT29 cells displayed a below-detection level of TOPflash activity compared with the other cell lines.

Inhibition of GSK3β rescues Wnt/β-catenin pathway

To further investigate the possible contribution of GSK3β to α6A-mediated β-catenin activation, we evaluated the effect of GSK3β inhibition on the rescue of responsive β-catenin/TCF4/LEF reporter plasmid activity and target gene expression in α6A knocked down T84 cells. As shown in Figure 6, we found that the pharmacological inhibition of GSK3β with SB21673 led to a significant stimulation of TOPflash activity in both shctl and shα6A cells (Figure 6A). Moreover, GSK3β inhibition stimulated TOPflash activity to the same level in both shctl and shα6A cells. To further extend these observations, the effect of α6A knockdown and GSK3β inhibition on Wnt/β-catenin target gene expression was analyzed by qPCR. We chose to investigate LGR5, CCD1 and CCD2, three well-documented target genes of the Wnt/β-catenin pathway in the intestine (37,38). First, shα6A cells were found to display a significant reduction of mRNA levels for LGR5 and CCD2 relative to shctl cells (Figure 6B), confirming the inhibition of Wnt/β-catenin transcriptional activity on these two target genes. GSK3β inhibition in shα6A cells resulted in a significant stimulation of LGR5 and CCD2 mRNA expression (Figure 6B). When both shctl and shα6A cells treated with the GSK3β inhibitor were compared, LGR5 mRNA expression was still significantly lower in shα6A cells than in shctl cells, whereas CCD2 mRNA expression was similar (Figure 6B), suggesting at least a partial rescue. No significant change in CCD1 mRNA expression was observed under these conditions (data not shown). These results suggest a role for the α6A integrin subunit in the control of Wnt/β-catenin activity and some of its target genes through GSK3β, which in turn may regulate CRC proliferation.

Down-regulation of DVL2

The promoting effect of α6A on the expression of the active/nuclear form of β-catenin and the fact that inhibition of GSK3β could rescue the activity of the Wnt/β-catenin pathway suggest that α6A could interfere with a signalling event upstream to GSK3β. Thus, we investigated the possibility that α6A could act through the main GSK3β regulator, DVL2 (39). Interestingly, WB analyses of whole cell lysates revealed that knockdown of α6A led to a significant decrease in protein levels of DVL2 in all four cell lines tested (Figure 6C) although its transcript levels were not affected (Figure 6D), suggesting that integrin α6A regulates the Wnt/β-catenin pathway through DVL2.

Discussion

Over the past several years, there has been an increase in evidence showing that integrin receptors can have important functions in
Fig. 3. Knocked down α6A splice variant decreases cell proliferation. (A) Cell counts over a 3–6 day period after the seeding of Caco-2/15, DLD-1, T84 and HT29 stably expressing shα6A or shctl. Cells were counted at the indicated times. (B) BrdU labelling assay in Caco-2/15, DLD-1, T84 and HT29 shα6A and shctl cells at 2 days post-seeding. (C) ISEL assay in Caco-2/15, DLD-1, T84 and HT29 shα6A and shctl cells at 2 days post-seeding. Cytochalasin D (CD)-treated cells were used as positive control for apoptosis. Statistical analysis between shctl and shα6A: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, t-test, n = 3.
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Fig. 4. Knockdown of α6A variant in human CRC cells inhibits their growth in xenografts. (A and B) Tumour growth (mm³) following subcutaneous injection of 2×10⁶ T84 and HT29 stably expressing shα6A and shctl cells into nude mice. Tumour volumes were determined by external measurement \( V = (d^2 \times D)/2 \).

(C and D) Weight (g) of tumours from T84 and HT29 shctl and shα6A cells at the time of killing. Statistical analysis between shctl and shα6A: * \( P \leq 0.05 \), ** \( P \leq 0.01 \), t-test, \( n = 4 \). (E) Representative haematoxylin and eosin staining images for T84, HT29 and DLD-1 shctl and shα6A xenograft tumours. Scale bars = 200 μm for main panels and 50 μm for inserts. Hash symbols denote necrosis/oedema regions. (F) qPCR analyses for the expression of α6A and α6B transcript levels in xenograft tumours from T84, HT29 and DLD-1 shctl and shα6A cells. Data are expressed by α6A normalized to α6B levels. ** \( P \leq 0.01 \), § \( P = 0.0783 \), t-test, \( n = 4 \).
Fig. 5. Regulation of the Wnt/β-catenin pathway by the α6A variant subunit. Representative WB and graph of the densiometric analysis for the detection of active β-catenin and total β-catenin in the whole cell extract (A) and β-catenin in nuclear extracts (B). β-Actin served as loading control in cell extracts and histone H1 for nuclear extracts. Statistical analysis between shctl and shα6A: *P ≤ 0.05, **P ≤ 0.01, t-test, n = 3. (C) TOPflash assay of the response of β-catenin/TCF4 promoter activity in the α6A variant knocked down cell lines and their corresponding shctl. Results showed the net luciferase/renilla ratio (Topflash − FOPflash). Statistical analysis between shctl and shα6A: *P ≤ 0.05, **P ≤ 0.01, t-test, n = 3.
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Fig. 6. Regulation of the Wnt/β-catenin pathway. (A and B) Inhibition of GSK3β rescues Wnt/β-catenin activity. (A) Response of β-catenin/TCF4 promotor activity in α6A knocked down T84 cells and controls ± SB216763. Statistical analysis between untreated shctl and shα6A: **P ≤ 0.001; statistical analysis between SB216763-treated shctl and shα6A: **P ≤ 0.001; statistical analysis between versus + SB216763: *P ≤ 0.05, **P ≤ 0.001; ANOVA, n = 3. (B) qPCR analysis for the expression of LGR5 and CCD2 transcript levels in shctl and shα6A cells. Statistical analysis between untreated shctl and shα6A: *P ≤ 0.05, **P ≤ 0.0001, ***P ≤ 0.0001, t-test, n = 3. (C and D) Knockdown of α6A reduces DVL2 protein levels in the four cell lines tested. (C) Representative WB and graph of the densitometric analysis of the detection of DVL2 protein levels in shctl and shα6A cells. Statistical analysis between untreated shctl and shα6A: *P ≤ 0.05, **P ≤ 0.0001, t-test, n = 3. (D) qPCR analysis for the expression of DVL2 transcript levels in shctl and shα6A cells. Data were normalized to RPLPO levels. (E) Working model for the involvement of the α6AP14 integrin in the regulation of the Wnt/β-catenin pathway. (Left) The α6AP14 integrin is over-expressed in CRC. When present, α6A regulates positively DVL2 at the protein level. DVL2, which inhibits GSK3β-mediated β-catenin phosphorylation, enhances β-catenin stability and translocation into the nucleus for the activation of the transcription of specific target genes involved in cell proliferation. (Right) Knockdown of the α6A subunit in CRC cells results in a decrease in DVL2 levels, thus allowing β-catenin phosphorylation by GSK3β. β-Catenin being targeted to the cytoplasm is no longer translocated to the nucleus resulting in repression of the transcription of Wnt/β-catenin-specific target genes. In this context, pharmacological inhibition of GSK3β with SB216763 restores Wnt/β-catenin pathway activity by bypassing the regulation of DVL2 by the α6A integrin subunit. The ‘?’ box denotes a still unknown mechanism by which α6A could be involved in the repression of key proteins regulating DVL2 degradation.

analysis. Data were normalized to RPLPO as reference gene. Statistical analysis between untreated shctl and shα6A: **P ≤ 0.001; statistical analysis between SB216763-treated shctl and shα6A: **P ≤ 0.001; statistical analysis between versus + SB216763: *P ≤ 0.05, **P ≤ 0.001; ANOVA, n = 3. (C and D) Knockdown of α6A reduces DVL2 protein levels in the four cell lines tested. (C) Representative WB and graph of the densitometric analysis of the detection of DVL2 protein levels in shctl and shα6A cells. Statistical analysis between untreated shctl and shα6A: *P ≤ 0.05, **P ≤ 0.0001, ***P ≤ 0.0001, t-test, n = 3. (D) qPCR analysis for the expression of DVL2 transcript levels in shctl and shα6A cells. Data were normalized to RPLPO levels. (E) Working model for the involvement of the α6AP14 integrin in the regulation of the Wnt/β-catenin pathway. (Left) The α6AP14 integrin is over-expressed in CRC. When present, α6A regulates positively DVL2 at the protein level. DVL2, which inhibits GSK3β-mediated β-catenin phosphorylation, enhances β-catenin stability and translocation into the nucleus for the activation of the transcription of specific target genes involved in cell proliferation. (Right) Knockdown of the α6A subunit in CRC cells results in a decrease in DVL2 levels, thus allowing β-catenin phosphorylation by GSK3β. β-Catenin being targeted to the cytoplasm is no longer translocated to the nucleus resulting in repression of the transcription of Wnt/β-catenin-specific target genes. In this context, pharmacological inhibition of GSK3β with SB216763 restores Wnt/β-catenin pathway activity by bypassing the regulation of DVL2 by the α6A integrin subunit. The ‘?’ box denotes a still unknown mechanism by which α6A could be involved in the repression of key proteins regulating DVL2 degradation. Pathways/molecules activated are in black, whereas those inhibited are in grey.
specific cytoplasmic partners. For instance, it has been reported that α6A can interact with MSS4 and α6B with BIN1 via their GFFKR motif (28). MSS4 has been found to act as a regulator of the stress response and apoptosis (42), whereas BIN1 has been characterized as a tumour suppressor through its strong ability to inhibit c-MYC transcriptional activity (43). On the other hand, GIPC, a glut1-binding protein, has been found to bind to the type 1 PDZ domains of both α6A and α6B, the interaction being stronger on α6A (27). Incidentally, an small interfering RNA-targeting GIPC has been found to inhibit pancreatic cancer growth in an orthotopic mouse model (44).

The Wnt pathway has been recognized as the dominant force behind the proliferative activity of the intestinal epithelium both in its physiological state and in CRC (45), thus suggesting a possible relation between the pro-proliferative α6A(β4) integrin and the activation of the Wnt/β-catenin pathway in both normal colonic crypts and in CRC. In normal crypt cells, the Wnt/β-catenin pathway is mainly modulated by the Wnt ligands of the stem cell niche (45). In CRCs, Wnt activation mainly occurs through mutation of the APC gene (35,46), which regulates β-catenin phosphorylation, thus favouring the accumulation of β-catenin in the nucleus, which after binding with T-cell factor results in the transcriptional activation of pro-proliferative target genes (47). However, it has been established that a number of mutations allow the retention of APC function and regulation of the activity of β-catenin in CRC cells despite mutation in APC (35,46,48). In agreement with these findings, herein using CRC cell lines that harbour various APC mutations (46), we demonstrated that abolition of α6A expression has a significant impact on a series of end points used to monitor the Wnt/β-catenin pathway. Indeed, based on the observations that down-regulation of α6A levels results in a reduction of the relative amounts of both active (evaluated as the non-GSK3β phosphorylated form) and nuclear β-catenin, whereas the pharmacological inhibition of GSK3β restored the activity of the Wnt/β-catenin pathway as evaluated by TOPflash assays and the modulation of specific target genes including LGR5 and CDD2, we suggest that α6A interferes with the activity of GSK3β. Interestingly, the expression of DVL2, which is recruited to the Wnt receptor complex upon ligand activation and acts on the activation of Wnt/β-catenin signalling by preventing constitutive proteolytic degradation of β-catenin (39), was significantly diminished at the protein level in α6A knocked down CRC cells, suggesting that the α6Aβ4 integrin acts upstream from GSK3β. The mechanism still remains to be elucidated, but it is noteworthy that selective degradation pathways involving autophagy and prickle-1-dependent proteasomes have been reported previously for DVL. Indeed, during starvation-induced autophagy, the E3 ubiquitin ligase Von Hippel–Lindau protein tumour suppressor (pVHL) was shown to mediate DVL ubiquitination and its recognition by p62/SQSTM1, targeting it for selective autophagy (49). On the other hand, prickle-1, a planar cell polarity protein, can interact directly with DVL to mediate its ubiquitination and subsequent proteasome degradation via its destruction box (D-box) motif (50). Thus, via their regulatory functions on DVL degradation, pVHL and prickle-1 could negatively regulate Wnt/β-catenin activity.

In conclusion, this study identified for the first time a specific cell function for the α6A splice variant. Our results suggest that α6A(β4) regulates cell proliferation and the Wnt/β-catenin pathway through DVL2/ GSK3β (Figure 6E). However, the α6A cytoplasmic-associated protein responsible for this mechanism remains to be elucidated, as well as the molecular mechanisms responsible for the increase of α6A in CRC cells.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: J.-F.G., N.B. and J.-F.B. are inventors of a patented technology related to integrin alpha 6. The other authors disclose no conflicts.

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