A complex between phosphatidylinositol 4-kinase IIα and integrin α3β1 is required for *N*-glycan sialylation in cancer cells

Received for publication, August 19, 2018, and in revised form, January 8, 2019 Published, Papers in Press, January 18, 2019, DOI 10.1074/jbc.RA118.005208

Tomoya Isaji[‡1], Sanghun Im[‡1], Akihiko Kameyama[§], Yuqin Wang[¶], Tomohiko Fukuda[‡], and Jianguo Gu[‡2]

From the [‡]Division of Regulatory Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai Miyagi 981-8558, Japan, the [§]Department of Life Science and Biotechnology, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, and the [¶]Department of Pharmacology, Pharmacy College, Nantong University, Nantong, Jiangsu 226001, China

Edited by Gerald W. Hart

Aberrant *N*-glycan sialylation of glycoproteins is closely associated with malignant phenotypes of cancer cells and metastatic potential, which includes cell adhesion, migration, and growth. Recently, phosphatidylinositol 4-kinase IIα (PI4KIIα), which is localized to the trans-Golgi network, was identified as a regulator of Golgi phosphoprotein 3 (GOLPH3) and of vesicle transport in the Golgi apparatus. GOLPH3 is a target of PI4KIIα and helps anchor sialyltransferases and thereby regulates sialylation of cell surface receptors. However, how PI4KIIα-mediated sialyation of cell surface proteins is regulated remains unclear. In this study, using several cell lines, CRISPR/Cas9-based gene knockout and short hairpin RNA–mediated silencing, RT-PCR, lentivirus-mediated overexpression, and immunoblotting methods, we confirmed that PI4KIIα knockdown suppresses the sialylation of *N*-glycans on the cell surface, in Akt phosphorylation and activation, and integrin α3-mediated cell migration of MDA-MB-231 breast cancer cells. Interestingly, both integrin α3β1 and PI4KIIα co-localized to the trans-Golgi network, where they physically interacted with each other, and PI4KIIα specifically associated with integrin α3 but not α5. Furthermore, overexpression of both integrin α3β1 and PI4KIIα induced hypersialylation. Conversely, integrin α3 knockout significantly inhibited the sialylation of membrane proteins, such as the epidermal growth factor receptor, as well as in total cell lysates. These findings suggest that the malignant phenotype of cancer cells is affected by a sialylation mechanism that is regulated by a complex between PI4KIIα and integrin α3β1.

Alteration of the sialylation of glycoproteins has often been observed in several types of malignant tumors, such as those found in breast, ovary, and colorectal cancers (1–3). Sialylation is linked via either an α2,3 or an α2,6 bond to Gal/GalNAc and via an α2,8 bond to sialic acid in glycoproteins through a group of sialyltransferases. The up-regulation of cell surface sialic acid is thought to control cell phenotypes such as cell adhesion, migration, immune response, apoptosis, and cell epithelial–mesenchymal transition (EMT).[3] Sialylation is also believed to be essential for the differentiation potential of human mesenchymal stem cells (4, 5). The sialylation levels of glycoproteins on the cell surface are mainly determined by sialyltransferase, sialidase, and substrate expression (6, 7). Some transcription factors are critical for transcriptional activation of sialytransferases in cancer cells. For example, the expression level of α2,6-galactoside sialyltransferase 1 (ST6GAL1) is up-regulated by the *RAS* oncogene and increases the α2,6 sialylation of β1 integrins, which promotes integrin-mediated cell migration, adhesion, and cell proliferation (8, 9).

As described above, sialylation levels are mainly dependent on their gene expression levels, whereas other mechanisms for regulation are usually neglected. However, Popoff and co-workers (10) and our group (11) independently reported that Golgi phosphoprotein 3 (GOLPH3), which has been identified as an oncogenic protein and is increased in several human solid tumors (12), could anchor sialyltransferases to regulate sialylation on cell surface receptors without regulating the gene expression levels of sialyltransferases. In particular, suppression of GOLPH3 attenuated the levels of cellular sialylation and integrin-dependent cell migration. Furthermore, tumor formation was significantly reduced in mice implanted with GOLPH3 shRNA-expressing cells (11–13). GOLPH3 has multiple cellular functions in vesicle trafficking and in support of Golgi apparatus structure, which has specific affinity for phosphatidylinositol 4 (PI4P) (14, 15). PI4P is mainly catalyzed by phosphatidylinositol 4-kinase IIα (PI4KIIα), which is localized to the trans-Golgi network (TGN) (16). Given the importance of

This work was supported in part by Grants-in-Aid for Scientific Research 15H04354 (to J. G.) and JP17H03808 (to A. K.) from the Japan Society for the Promotion of Science, National Natural Science Foundation of China Grant 31670807, and Grant-in-Aid for Scientific Research on Innovative Areas 18H04868 (to J. G.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors declare that they have no conflicts of interest with the contents of this article.

[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed. Tel.: 81-22-727-0216; Fax: 81-22-727-007; E-mail: jgu@tohoku-mpu.ac.jp.

[3] The abbreviations used are: EMT, epithelial–mesenchymal transition; shRNA, short hairpin RNA; PI4P, phosphatidylinositol 4-phosphate; PI4K, phosphatidylinositol 4-kinase; TGN, trans-Golgi network; ECM, extracellular matrix; EGF, epidermal growth factor; PH, pleckstrin homology; DOX, doxycycline; KD, knockdown; SNA, *Sambucus nigra* agglutinin; WGA, wheat germ agglutinin; PA, pyridilaminated; SSA, *Sambucus sieboldiana* agglutinin; MAM, *Maackia amurensis* mitogen; ConA, concanavalin A; KO, knockout; EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; cDNA, complementary DNA; mRFP, monomeric red fluorescent protein; SALSA, sialic acid linkage–specific alkylamidation; Ctrl, control.

GOLPH3 in sialylation and cell functions, it is plausible that PI4P expression at the TGN may influence sialylation.

Integrins are heterodimeric cell surface adhesion receptors and major carriers of sialyation. The interaction between integrin and the extracellular matrix (ECM) is essential for cell adhesion, migration, viability, and proliferation (17, 18). In fact, glycosylation is a key regulator that plays important roles in modulating integrin functions. For example, integrin α5β1 binding to fibronectin and integrin-mediated cell spreading and migration are modulated by overexpression of glycosyltransferase genes such as *N*-acetylglucosaminyltransferase III and V or ST6GAL1 (19–21). In addition, sialylated *N*-glycans on the membrane-proximal domain of integrin β1 play crucial roles in integrin activation and in complex formation between integrin and EGF receptors and syndecan-4 to regulate cell migration and proliferation (22).

In contrast to the regulation of integrin functions from the extracellular domain, it is well known that integrin function is regulated by its association with cytoplasmic molecules such as focal adhesion kinase and phosphatidylinositol 3-kinase (23, 24). Other studies have detected PI4K activity in the immune complex with integrin β1, suggesting that integrin might also regulate the biosynthesis of PI4P (25–27). Mammalian PI4Ks are classified as types II and III (28). PI4KIIα plays important roles in clathrin-dependent molecular sorting and associates with TGN membranes (29–31), whereas PI4KIIIβ is enriched in the cis-medial Golgi in breast cancer cells (32).

In this study, to further understand the underlying mechanism for GOLPH3 expression on sialylation and cell functions, we investigated the effects of PI4KIIα, which is one of the regulators of GOLPH3 in breast cancer MDA-MB-231 cells. We found that the sialylation on integrins, Akt phosphorylation, and integrin α3-mediated cell migration all were significantly inhibited in PI4KIIα knockdown cells. It was interesting that overexpression of both PI4KIIα and integrin α3 greatly increased sialylation. Conversely, knockout of integrin α3 significantly inhibited sialylation in membrane proteins. These findings suggest a novel mechanism for sialylation, which suggests a new concept for the regulation of glycosylation in cell biology.

Results

We recently reported that the expression of GOLPH3 upregulated cell surface sialylation and cell migration (11). However, the molecular mechanism of posttranslational modification of sialylation on the cell surface remains unclear. Considering that GOLPH3 exhibits a highly selective affinity for PI4P that is similar to the canonical pleckstrin homology (PH) domain in the TGN (33), we hypothesized that the expression of PI4P in the TGN could affect sialylation and cell properties. PI4P is produced mainly by PI4Ks, which are classified as type II and III according to their sensitivity to inhibitors (30). PI4KIIIβ is enriched in early Golgi compartments, whereas PI4KIIα is localized mainly in the TGN and in the endosome (16), where sialyltransferases also are localized (34). Furthermore, PI4KIIα is a dominant PI4K in mammalian cells (35). Therefore, we chose PI4KIIα for functional analysis, and established a line of doxycycline (DOX)–controlled PI4KIIα silencing in MDA-MB-231 breast cancer cells.

Knockdown of PI4KIIα affected the sialylation of N-glycans

First, we verified the expression levels of PI4KIIα and sialyltransferases (ST3GAL3, ST3GAL4, and ST6GAL1) that involve major sialylated *N*-glycans (36). RT-PCR analysis showed that the expression levels of PI4KIIα mRNA were significantly decreased in knockdown cells, whereas the expression levels of ST6GAL1 for α2,6 sialylation and those of ST3GAL3 and ST3GAL4 for α2,3 sialylation were all similar in both cells (Fig. 1*A*). Second, we verified the expression levels and the distributions of PI4P in KD cells. The PH domain of four-phosphate adaptor protein 1 (FAPP1), which preferentially recognizes PI4P, is known for its use as a monitor for PI4P distribution (37). In control cells, PI4P was localized near the medial Golgi marker GM130, as expected (Fig. 1*B*). In contrast, PI4P staining was significantly decreased in knockdown (KD) cells (Fig. 1*B*). These data confirmed that PI4KIIα is mainly involved in the synthesis and localization of PI4P in the TGN, which agrees with previous reports (35).

Next, we tested whether decreased PI4P affects sialylation expression on the cell surface. As shown in Fig. 1*C*, *top panels*, the intensities of the staining in KD cells with *Sambucus nigra* agglutinin (SNA) lectin, which preferentially recognizes α2,6 sialylation of glycoproteins, was weaker than that in control cells. However, the intensities of staining with WGA lectin, which preferentially recognizes GlcNAc residues and hybrid-type and lactohexose *N*-glycans (38), were almost equal in both cells (Fig. 1*C*, *bottom panels*). These data suggested that the expression levels of sialylated glycans were decreased in KD cells. Furthermore, the decrease was confirmed by quantitative analysis using HPLC. Total pyridylaminated (PA) *N*-glycans of cells were examined via anion exchange chromatography, and the ratios of sialylated *N*-glycans *versus* total *N*-glycans were calculated. The expression levels of sialylated *N*-glycans were significantly suppressed in KD cells (Fig. 1*D*). A similar change in α2,6 sialylation on specific membrane proteins such as integrin β1 was also consistently observed (Fig. 1*E*) using *Sambucus sieboldiana* agglutinin (SSA)–agarose. These data suggest that the expression levels of PI4P in the TGN are important for sialylation.

Knockdown of PI4KIIα significantly inhibited cell migration and Akt phosphorylation

Alterations in *N*-glycosylation affect many cellular events involved in cellular signaling and cell migration. Here we examined the effects of PI4KIIα KD on cell migration and Akt activation. As shown in Fig. 2*A*, cell migration on laminin-332 in a Transwell assay was significantly reduced in KD cells compared with control cells. The expression levels of integrin α3β1, a major receptor for laminin-332 and α5β1 on the cell surface were similar in both cells (Fig. 2*B*). Activation of Akt is required for integrin-mediated cell migration (39). As shown in Fig. 2*C*, the level of phosphorylated Akt was apparently decreased in KD cells compared with control cells. These data suggest that PI4P in the TGN plays an important role in Akt activation and in integrin α3–mediated cell migration.

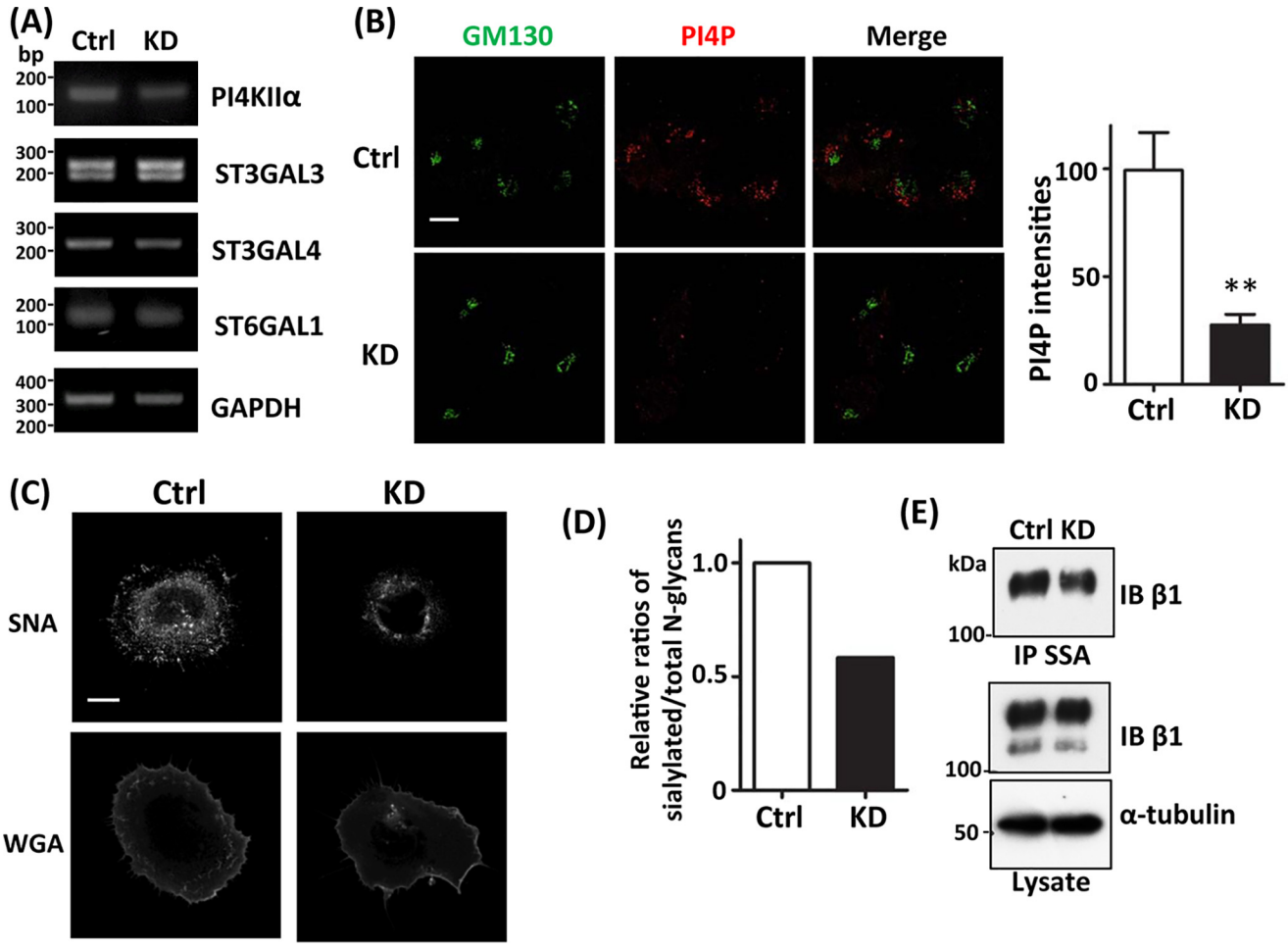

Figure 1. Effects of PI4KIIα gene knockdown on the sialylation of *N*-glycans. *A*, RT-PCR using total RNA extracted from the control (Ctrl) and PI4KIIα KD of MDA-MB-231 cells were carried out to examine the expression levels of PI4KIIα and sialyltransferases that involve sialylated *N*-glycans. The expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *ST3GAL*, β-galactoside α2,3-sialyltransferase. *B*, the amounts and distribution of PI4P in KD and Ctrl cells were examined by expressing mRFP-FAPP, which specifically binds to PI4P. These cells were also immunostained with anti-GM130 antibody, which is a medial Golgi marker. The immunostaining intensities for PI4P were quantified using ImageJ software, averaged by cell numbers, and normalized to the control values (percent). Values represent the means ± S.E. ($n = 10$). **, $p < 0.01$ (Welch's *t* test). *Scale bar* = 10 μm. *C*, Cells were incubated with biotin-conjugated SNA or WGA and then fixed and visualized by Alexa Fluor–streptavidin conjugates. *Scale bar* = 20 μm. *D*, preparation of PA oligosaccharides and calculation of ratios of sialylated *N*-glycans *versus* total *N*-glycans as described under "Experimental procedures." Data were normalized to the control. *E*, equal amounts of cell lysates from Ctrl and KD cells were precipitated by SSA-agarose (preferentially recognizes the α2,6 sialylation) and probed with anti-β1 antibody (*top panel*). The expression levels of integrin β1 in total cell lysates were verified by Western blotting with anti-β1 antibody (*center panel*). α-Tubulin was used as a loading control. KD and Ctrl cells refer to transfectants with DOX-inducible knockdown of PI4KIIα cultured with or without DOX, respectively. *IB*, immunoblot.

Complex formation between integrin α3 and PI4KIIα is important for sialylation

As described above, PI4KIIα regulated sialylation and the integrin α3–mediated phenotype. Therefore, we examined the interaction between PI4KIIα and integrin α3 in the Golgi apparatus. First, we used immunostaining to assess the intracellular distributions of PI4KIIα and α3. Immunostaining showed that PI4KIIα was localized in the vicinity of GM130 and that it was extensively co-localized with integrin α3 (Fig. 3*A*). Notably, co-immunoprecipitation with anti-integrin β1 showed that it was α3, rather than α5, that interacted with PI4KIIα in 293T cell stable expression of PI4KIIα and/or either integrin α3 or α5 (Fig. 3*B*). The interaction between PI4KIIα and α3 was further reciprocally confirmed by immunoprecipitation with either anti-α3 antibody (Fig. 3*C*) or anti-GFP antibody (Fig. 3*D*).

Next we wondered whether this interaction was required for efficient sialylation. To address this question, we precipitated those cell lysates with SSA or *Maackia amurensis* mitogen (MAM) agarose, which preferentially recognize α2,6-sialylated and α2,3-sialylated *N*-glycans, respectively, and then performed Western blotting against integrin β1. Interestingly, both sialylated *N*-glycans were up-regulated on integrin β1 in cells that expressed both PI4KIIα and integrin α3 but not in those that expressed PI4KIIα and α5 (Fig. 3*E*, *top* and *center panels*). In contrast to SSA or MAM lectin blotting, ConA lectin blots showed a similar level in both integrin α3- and α5-expressing cells (Fig. 3*E*, *bottom panel*). ConA lectin equally recognized both mature and immature forms of integrin β1 in α3-expressing cells but only the most immature form of β1 in α5-expressing cells. These data further suggest the importance of α3 for sialylation. Data obtained from flow cytometry analysis via MAM lectin consistently supported this observation, although SSA lectin showed no significant changes in these cells (Fig. 3*F*). Therefore, these results clearly suggest that intracellular com-

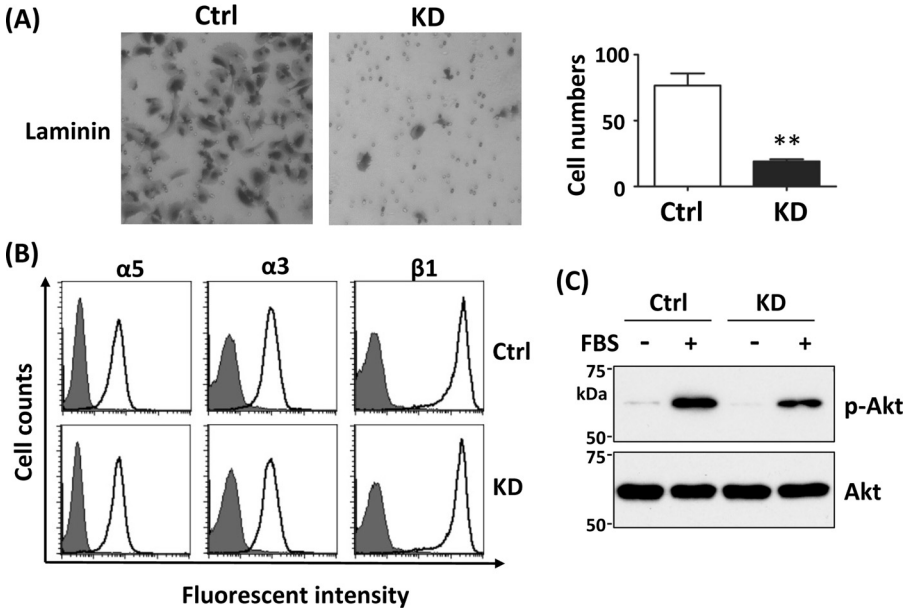

Figure 2. Comparison of cell migration and cellular signaling between Ctrl and KD cells. *A*, cell migration toward laminin-332 was determined by a Transwell assay as described under "Experimental procedures." The quantitative data were obtained by counting the cell number of five random fields. The *p* values were calculated using Welch's *t* test. Values represent the means ± S.E. **, $p < 0.01$. *B*, comparison of the expression levels of α3β1 and α5β1 integrins on the cell surface between Ctrl and KD cells. Both Ctrl and PI4KIIα KD cells were incubated with (*bold line*) or without (*gray shadow*) antibodies against integrin α3, α5, or β1 and subjected to FACS analysis. *C*, comparison of Akt activation upon FBS stimulation between Ctrl and KD cells. After starvation, cells were supplied with fresh medium with (+) or without (−) 3% FBS. The cell lysates were then analyzed by Western blotting with anti-p-Akt or total Akt antibody.

plex formation between PI4KIIα and α3 regulates the biosynthesis of sialylated *N*-glycans.

Expression of integrin α3 is important for efficient sialylation

To further verify the importance of complex formation between PI4KIIα and integrin α3 for cellular sialylation, we used the CRISPR/Cas9 system to construct an integrin α3 knockout (KO) MDA-MB-231 cell line. Flow cytometric analysis revealed a KO efficiency of more than 95% (Fig. 4*A*), which also was confirmed by Western blotting with anti-integrin α3 (Fig. 4*B*). Notably, the band mobility of integrin β1 on SDS-PAGE in KO cells was faster than in the control cells, suggesting that integrin α3 deficiency leads to an accumulation of the immature β1 form without sialylation. It is well known that integrin α3 is important for laminin-mediated cell adhesion, migration, and several forms of cellular signaling (40–42). Consistent with previous reports (42, 43), expression of α3β1 regulated cell morphology and that of the actin cytoskeleton by promoting lamellipodium and filopodium formation on laminin-332, a specific ligand for integrin α3, whereas lack of α3 blocked their formation (Fig. 4*C*). There were no significant differences between two cells spread on collagen, which is a specific ligand for integrin α1 and α2. Furthermore, cell spreading and migration on laminin-332 were inhibited in KO cells compared with control cells (Fig. 4, *D* and *E*).

Next, we compared total sialylated *N*-glycan levels between KO and control cells, which were quantified using HPLC. Consistent with the data from PI4KIIα KD cells, the ratios of sialylated *N*-glycans *versus* total *N*-glycans were greatly suppressed in KO cells (Fig. 5*A*). Because *N*-glycans of integrin β1 could be directly influenced by α3 knockout (Fig. 4*B*, *center panel*), here we also analyzed sialylation on the EGF receptor, which has been reported to affect receptor activation (44). As expected, sialylation of EGF receptors obtained from KO cells was also decreased compared with control cells (Fig. 5*B*). To investigate whether the phenomenon also occurred in other cell lines, we examined HeLa cells. The knockout efficiency of α3 was assessed via flow cytometry analysis (Fig. 5*C*). Interestingly, a similar tendency of sialylation decline was observed in α3 KO HeLa cells compared with control cells (Fig. 5*D*). To thoroughly examine the glycosylation states caused by knockout of integrin α3 or knockdown of PI4KIIα, we utilized MS-based approaches to identify the *N*-glycan and *O*-glycan structures (Fig. 6, *A* and *B*). Consistently, hyposialylation in *N*-glycans was observed by attenuation of either integrin α3 or PI4KIIα (Fig. 6*A*), which further suggests the importance of this pathway in the biosynthesis of sialylated *N*-glycans. In contrast to *N*-glycans, there were no significant changes in the sialylation of *O*-glycans among these cells (Fig. 6*B*). It was also notable that a decrease in α2,3 sialylation in *N*-glycans could slightly increase α2,6 sialylation in total cell lysates of α3 KO and PI4KIIα KD cells (Fig. 6*A*), which seems contrary to the observation for integrin β1 (Fig. 3*E*). Further study is needed to fully elucidate the mechanism. Nevertheless, these data suggest that complex formation of α3 and PI4KIIα could act as a novel regulator for the sialylation of *N*-glycans.

Discussion

In this study, we found that PI4KIIα expression played an important role in α3β1 integrin-mediated cell migration, cellular signaling, and the expression of sialylation; PI4KIIα could specifically associate with α3 but not α5, and complex formation between PI4KIIα and α3 enhanced sialylation; and deletion of integrin α3 significantly inhibited not only cell adhesion and migration but also sialylation. These observations are the first to directly demonstrate a novel regulatory mechanism for sia-

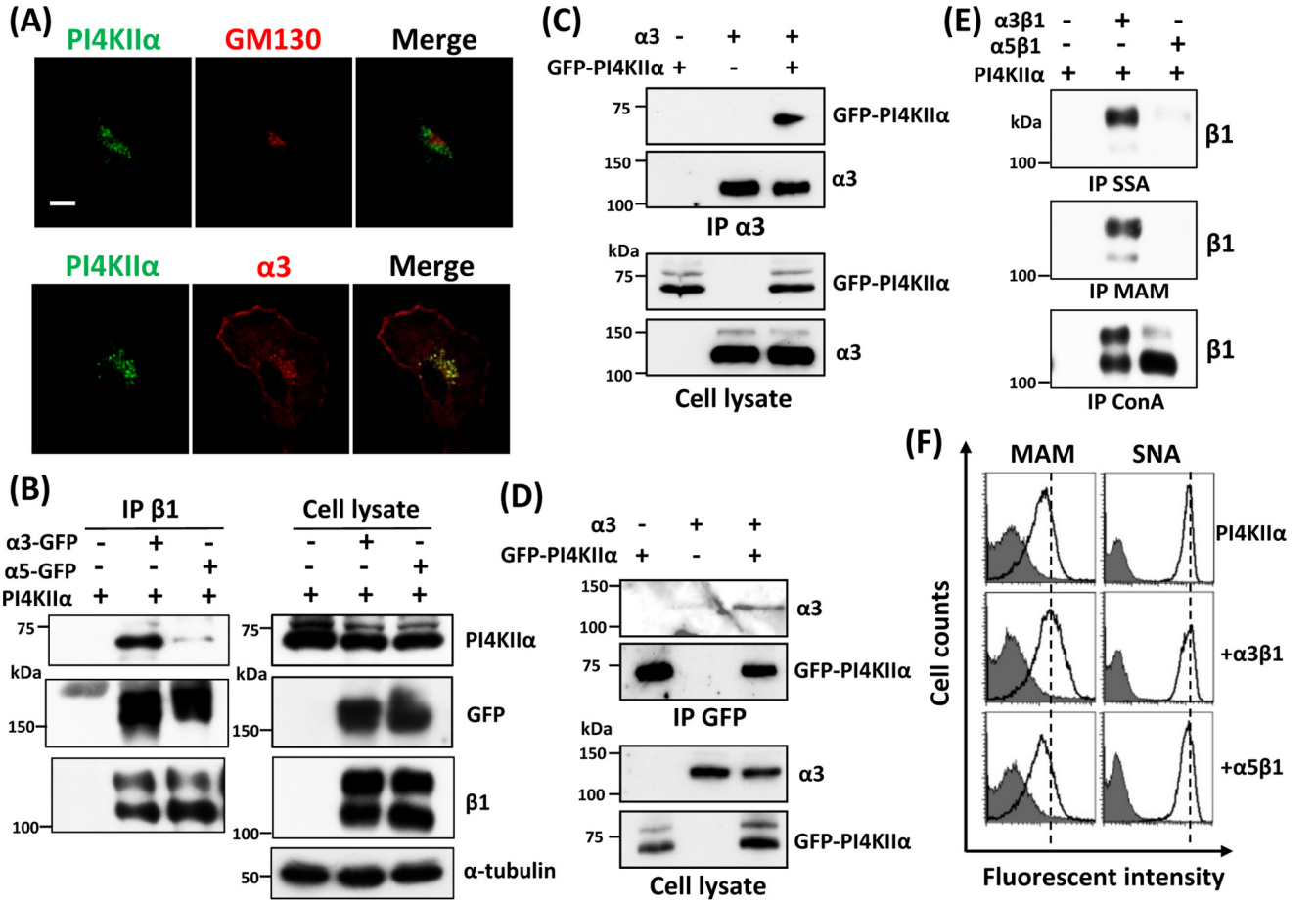

Figure 3. Interaction between integrin α3β1 and PI4KIIα and the effect on sialylation. *A*, MDA-MB-231 cells overexpressed with GFP-PI4KIIα were fixed and then stained with either anti-GM130 (*top panel*) or anti-integrin α3 (*bottom panel*) antibody, followed by detection using anti-mouse Alexa 647 conjugates. *Scale bar* = 20 μm. *B*, three overexpressed 293T cells were established by lentivirus infection: PI4KIIα alone; PI4KIIα, GFP-tagged α3 and β1; and PI4KIIα, GFP-tagged α5 and β1 integrin. Equal amounts of cell lysates were immunoprecipitated (*IP*) with anti-integrin β1 antibody, followed by detection with the indicated antibodies. The cell lysates were used as input to show similar expression levels of PI4KIIα, integrin α3β1, or α5β1 among these cells. *C* and *D*, reciprocal immunoprecipitation was performed using either anti-α3 antibody (*C*) or anti-GFP for tagged PI4KIIα (*D*) using cell lysates from 293T cells stably overexpressed with or without GFP tagged PI4KIIα, which were further transiently transfected with or without α3 and β1 integrin. The cell lysates were used as input to show similar expression levels of integrin α3 and GFP-PI4KIIα. *E*, equal amounts of cell lysates were precipitated with either SSA-, MAM-, or ConA-conjugated agarose, followed by detection with antibody against integrin β1. *F*, 293T cells expressing the indicated genes were incubated with (*bold line*) or without (*gray shading*) biotin-conjugated MAM or SNA and subjected to FACS analysis.

lylation, which may also partially explain the previous observation that GOLPH3 is a special regulator in the sialylation of *N*-glycans and a part of the signaling events that could influence mTOR signaling and tumor progression (11).

Many cancers are associated with sialylated structures such as sialyl Tn, sialyl Lewis antigen (sLe), α2,6-sialylated lactosamine, polysialic acid, and gangliosides (45–48). The altered expression of these structures in cancer cells could be the result of multiple mechanisms. Loss of expression or excessive expression of certain sialyltransferases is frequently observed. For example, up-regulated expression of α2,3 and α2,6 sialyltransferases has been observed in many cancers, such as colon cancer, breast cancer, liver cancer, cervical cancer, choriocarcinomas, acute myeloid leukemias, and some malignancies of the brain (49), and this type of change can affect the structures and functions of some important target *N*-glycoproteins such as integrin β1(50), EGFR (44), and platelet endothelial cell adhesion molecule (51).

Recent studies have suggested that activation of the EMT programs serves as a major mechanism for generating cancer stem cells (52). Interestingly, high expression of ST6GAL1 has been correlated with human-induced pluripotent stem cells and cancer stem cells, indicating that sialylation may be involved in maintaining some aspects of stem cell behavior (4, 5, 53). In fact, ST6GAL1 expression is required for transforming growth factor β–induced EMT. Knockdown of ST6GAL1 prevented a transforming growth factor β–induced increase in cell migration (54).

Given the accumulating evidence of the importance of sialylation in cancer progression, much attention has been paid to elucidating the regulatory mechanisms of its expression. The expression of sialylation on a tumor cell surface can be modulated at different levels (49). The most frequently observed mechanism is modulation of transcription. For example, ST6GAL1 expression is induced by the *ras* oncogene in NIH3T3 cells via its promoter (8). Beyond its promoter activity, in this study, we clearly showed that sialylation could also be regulated by complex formation of PI4KIIα and integrin α3β1 on a posttranscriptional level. In fact, the PI4P-binding Golgi protein GOLPH3, which functions in secretory trafficking in

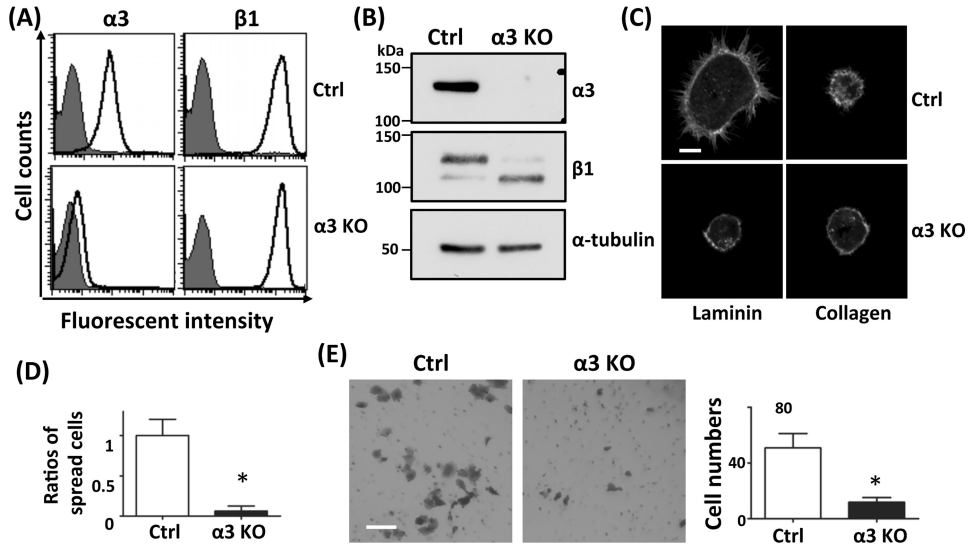

Figure 4. Effects of integrin α3 deficiency on cell spreading and migration. Establishment of integrin α3 KO MDA-MB-231 cells using the CRISPR/Cas9 system is described under "Experimental procedures." The efficiency of α3 KO was assessed via flow cytometric analysis (*A*) and Western blotting (*B*). *A*, Both α3 KO and Ctrl cells were stained with (*bold line*) or without (*gray shading*) anti-α3 or β1 integrin antibody and subjected to FACS analysis. *B*, the same amount of cell lysate was subjected to Western blotting to detect the indicated antibodies. *C*, cells were replated on coverslips coated with the indicated ECM, followed by incubation for 30 min, and were then fixed and stained with Alexa Fluor–conjugated phalloidin. Representative images are shown. *Scale bar* = 10 μm. *D*, cells were detached and then replated on laminin 332-coated dishes. After incubation for 15 min, the cells were fixed. The ratios of cell spreading were calculated. Cell spreading was defined as a cell spread of more than 0.025 μm^2. Values represent the means ± S.E. ($n = 5$). *, $p < 0.05$ (Welch's *t* test). *E*, cell migration toward laminin-332 was determined using a Transwell assay. *Scale bar* = 100 μm. The quantitative data were obtained from three independent experiments. Values represent the means ± S.E. ($n = 6$). *, $p < 0.05$ (Welch's *t* test).

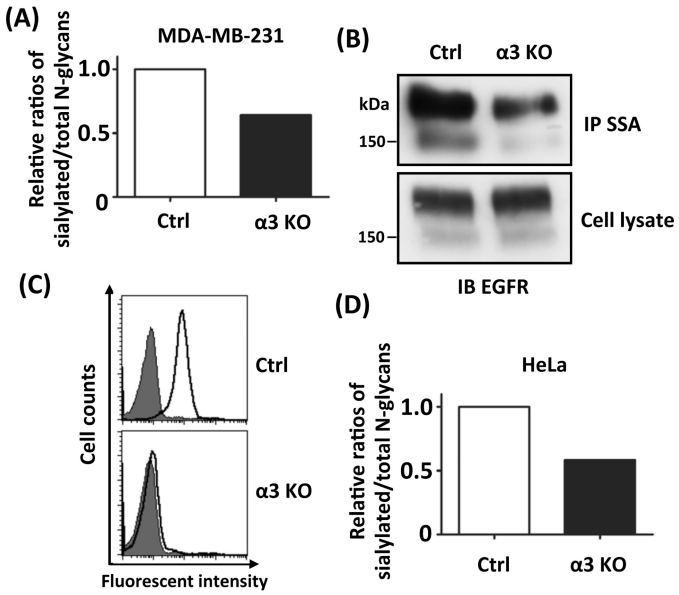

Figure 5. Deficiency of integrin α3 decreased sialylation of *N*-glycans in different cell lines. *A*, *N*-glycans released from both Ctrl and α3 KO MDA-MB-231 cells were pyridylaminated and verified as populations of sialylated *N*-glycans. The relative ratios of sialylated *N*-glycans *versus* total *N*-glycans were calculated by subtracting the neutral portions from the total and then dividing by the total. The control data were set as 1. *B*, equal amounts of cell lysates for control and α3 knockout cells were precipitated via SSA-agarose and probed with anti-EGFR antibody (*top panel*). The same cell lysates were also probed with anti-EGFR antibody as a loading control (*bottom panel*). *IP*, immunoprecipitation. *C*, α3 KO of HeLa cells was established using the CRISPR/Cas9 system and verified via flow cytometric analysis. *D*, the ratios of sialylated *N*-glycans were analyzed as described in *A*.

the Golgi, is also important for the localization of several glycosyltransferases (10, 55, 56). Our previous study showed that knockdown of GOLPH3 leads to down-regulation of both α2,3 and α2,6 sialylation but has no effect on the transcription of sialyltransferases. Therefore, regulation of biosynthetic glycan is not only dependent on the expression levels of glycosyltransferases but also on those of substrates, chaperones, and on the environment of the Golgi apparatus.

Considering that PI4P is a relatively abundant phosphoinositide that is required for the maintenance and function of the Golgi apparatus, which includes intracellular trafficking, it is plausible that PI4K could be involved in membrane transport from the trans-Golgi network to the plasma membrane. In fact, PI4KIIα is known to associate with several cellular receptors such as EGFR, E-cadherin, LDL receptor-related protein, and the Fas receptor (57–60). In addition, alteration of the *N*-glycosylation of such receptors also regulates the functions of these receptors. For example, *N*-glycosylation is required for EGFR trafficking and has an effect on its endocytosis (61, 62). *N*-glycans of the LRP ectodomain also regulate the conformation and bending angle of the receptor (63). Cell surface sialylation protects Fas ligand–induced apoptosis by modification of the Fas ligand receptor (64), which could partially explain why enhancement of apoptosis has been observed in PI4KIIα knockdown cells (60). Thus, the various phenotypes associated with PI4P and membrane proteins might be partially due to alteration of glycosylation.

The expression level of integrin β1 is negatively correlated with the survival rates of patients with invasive breast cancer (65, 66), particularly α3β1 expression in breast carcinoma associated with metastasis (41). During tumor progression, PI4KIIα is also significantly up-regulated along with tumor growth compared with corresponding normal tissue (67). Given the importance of sialylation as described here, it is reasonable to expect up-regulation of both α3β1 and PI4KIIα in cancer cells.

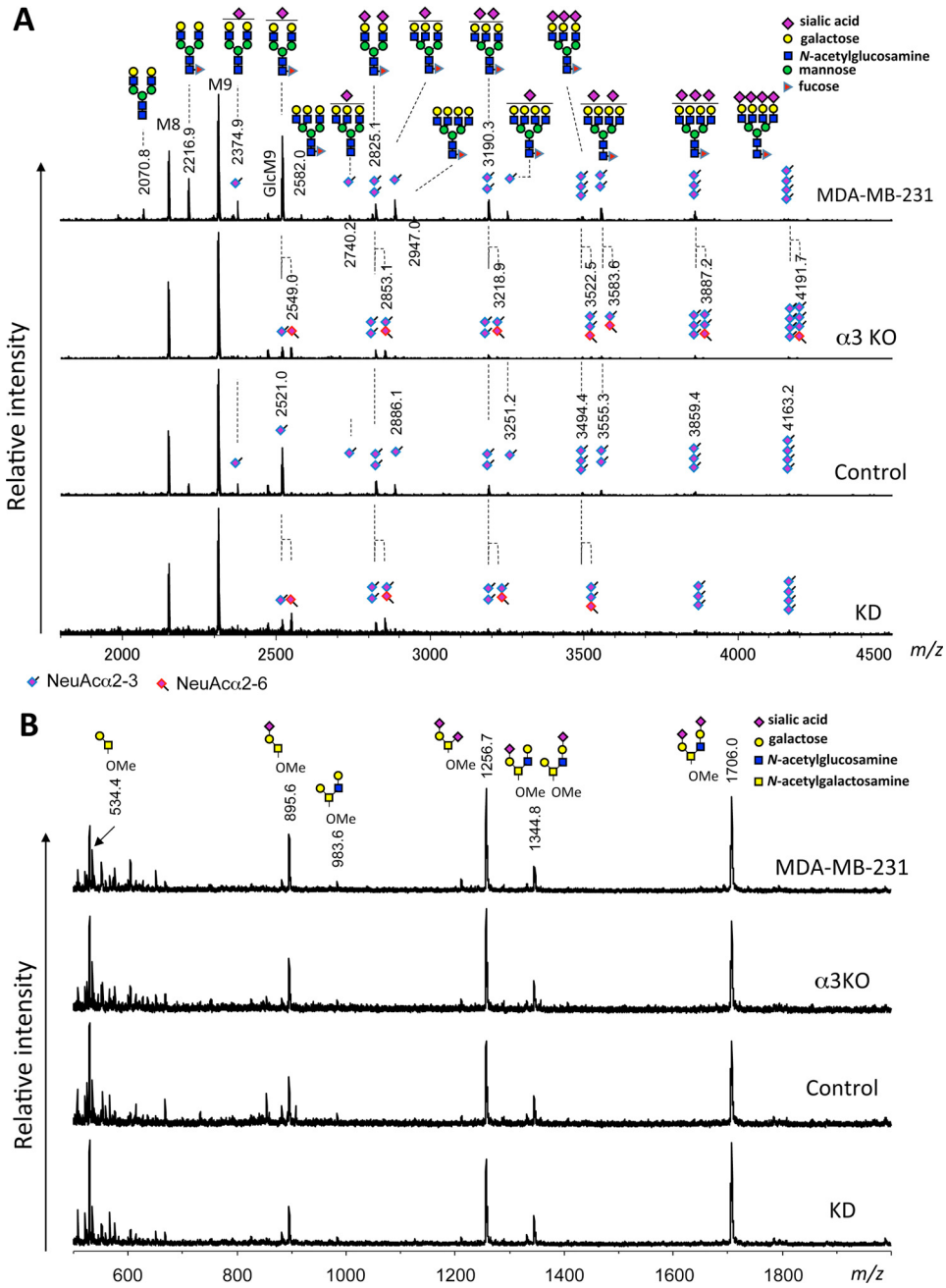

Figure 6. MS spectra of glycans obtained from cultured cells. *A*, MS spectra of *N*-linked glycans. The reducing ends of the *N*-linked glycans were derivatized with aoWR (86). The sialic acids of the *N*-linked glycans were differentially amidated with methylamine (+13.0 Da) for α2,3-sialic acids and isopropylamine (+41.1 Da) for α2,6-sialic acids via the SALSA method (87). MDA-MB-231 and α3 KO cells refer to parent and α3 knockout cells, respectively. KD and control cells of MDA-MB-231 cell line refer to transfectants with DOX-inducible knockdown of PI4KIIα cultured with or without DOX, respectively. *B*, MS spectra of *O*-linked glycans. The *O*-linked glycans were obtained as the corresponding alditols and then permethylated.

These facts raise the question of why the interaction of PI4KIIα with integrin α3β1 but not of α5β1 regulates sialylation. So far, the association underlying the mechanism between α3β1 and PI4KIIα remains unclear. However, the specificity of α3β1 could be due to its interaction with the tetraspanin family, such as CD151, CD63, and CD9. In fact, other studies have reported that integrin α3β1 and the tetraspanin family can interact with type II of PI4K (25–27). In addition, palmitoylation could also be a plausible factor because PI4Ks are proteins with membrane association and activity that are highly dependent on such a modification (68, 69), and palmitoylation also plays an important role in the association between integrin and several molecules, such as tetraspanin and c-Met (70, 71). We assume that the association between PI4KIIα and α3, but α5, might also relate to tetraspanin or palmitoylation.

It is noteworthy that both cell spreading and migration on laminin 332 of integrin α3 KO cells were significantly suppressed (Fig. 4), which further supports the notion that α3β1 is a major receptor of lamin-332 compared with integrin α6β1 and α6β4 in epithelial cells (43, 72). Accumulating evidence shows that integrin α3β1 is important for tumor metastasis in human breast and prostate cancer cell lines (40, 41) and sialy-

lation of adhesive molecules such as integrin β1, which, in turn, contributes to tumorigenesis (73). In this study, α3 deficiency significantly inhibited sialylation, indicating a novel link between sialylation and this integrin, which demonstrated the importance for both forms of functional expression. Although the MS analysis clearly showed that the complex between PI4KIIα and integrin α3 regulates the biosynthesis of sialylation on *N*-glycans but not *O*-glycans, we could not completely exclude other possibilities for the regulation of *N*-glycan structures other than sialylation or gangliosides. In addition, it has been reported that GOLPH3 containing a PI4P binding domain could control Golgi localization of core 2 *N*-acetylglucosaminyltransferase 1 for the biosynthesis of *O*-glycans (74). Thus, further studies are required to explain the underlying mechanism for different influences, such as localization of glycosyltransferase, pH environment (75), and cholesterol homeostasis (76) through this system. Taken together, this study may provide a new concept for the regulation of glycosylation and could suggest insights for the development of cancer treatment.

Experimental procedures

Cell lines and cell culture

The HeLa and 293T cells were obtained from the RIKEN Bioresource Research Center (Japan). The MDA-MB-231 cells were purchased from the ATCC. All cell lines were maintained at 37 °C in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2.

PCR for mRNA expression analysis

Total RNA was prepared with TRIzol (Invitrogen), and 1.0 μg of RNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio Inc.) according to the manufacturer's instructions. PCR primers against ST3GAL3, ST3GAL4, ST6GAL1, and GAPDH have been described previously (54). The following primers were used for PI4KIIα: 5′-CTCCAGCGGAAGCTACTTCG-3′ and 5′-TCCACTTAGGATTAAGATGCCCA-3′.

shRNA-mediated silencing of PI4KIIα in MDA-MB-231 cells

Conditional knockdown of the target gene was achieved using the DOX-inducible CS-RfA-ETBsd lentivirus vector (RIKEN) with minor modifications (11, 54). The following oligonucleotides were inserted into pENTR/H1/TO (Invitrogen): 5′-CACCAGAAGCAGAACCTCTTCCTGATGATATGTGCATCAGGAAGAGGTTCTGCTTCT-3′ and 5′-AAAAAGAAGCAGAACCTCTTCCTGATGCACATATCATCAGGAAGAGGTTCTGCTTCT-3′ (29). The use of LR Clonase allowed the inserted oligo to be transferred to CS-RfA-ETBsd, which is an encoding DOX-dependent transactivator for shRNA expression. The resultant vector was then transfected into 293T cells with packaging plasmids of calcium phosphate for the preparation of viruses. MDA-MB-231 cells were then infected with the obtained viruses and selected for stable integration with 1 μg/ml blasticidin. shRNA-mediated silencing of PI4KIIα was induced by addition of 1 μg/ml DOX for 72 h in the established cell line, and cells cultured in DOX-free medium were used as a control.

Gene introduction using the lentivirus system

The cDNA sequences for the PH domain of human FAPP1, PI4KIIα, and integrin α3 were cloned from HeLa cells and inserted into pENTR vectors (pENTR/D-TOPO cloning kit, Invitrogen). To obtain the *N*-terminal GFP-tagged PI4KIIα, C-terminal mRFP-tagged FAPP1 and a C-terminal GFP-tagged integrin α3, in-fusion enzyme (Clontech) was used with standard PCR protocols. The linkers for GFP-PI4KIIα, mRFP-FAPP1, and α3-GFP were 5′-GGGGS-3′, 5′-KNPPVAT-3′ (37), and 5′-LELKLRILQSTVPRARDPPVAT-3′, respectively. The resultant cDNAs were confirmed by DNA sequencing using an ABI Prism 3130 sequencer (Applied Biosystems Japan Ltd., Tokyo, Japan). The subcloned cDNAs were transferred into CSII-EF-Rfa (11) via LR Clonase (Invitrogen) for lentivirus production. The resultant vectors (CSII-EF-GFP-PI4KIIα/mRFP-FAPP1/α3-GFP) and the previously constructed integrins of either α5-GFP- or β1-overexpressed lentiviral vectors (CSII-EF-α5GFP (77)/β1 (78)) were then transfected into 293T cells with packaging plasmids via calcium phosphate during the preparation of viruses. Either MDA-MB-231 or 293T cells were then infected with the obtained viruses for further experiments.

Cell migration (Boyden chamber assay)

Cell Migration was performed as described previously with minor modifications (11, 79). Each Transwell (BD BioCoat control inserts, 8.0-μm inserts; BD Biosciences) was coated only on the bottom side with 1 μg/ml laminin-332 (Oriental Yeast Co., Ltd.) in PBS containing 0.1% BSA at 4 °C for 24 h and then blocked with 5% BSA in DMEM at 37 °C for 1 h. Cells were trypsinized and suspended in DMEM containing 1% FBS. The suspended cells were centrifuged, and the cell pellets were resuspended in an assay medium (0.1% BSA in DMEM containing 1% FBS) and diluted to 3×10^5 cells/ml; cell viability was confirmed by trypan blue staining. Cell suspensions were then added to the Transwell. Following incubation, the membranes were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet solution (Merck) for 24 h. After washing the Transwells with PBS, cells that migrated to the lower side were counted using a phase-contrast microscope.

Immunostaining

Cells (1×10^5) were plated on glass coverslips (MatTek), precoated with or without ECM for 24 h, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with PBS containing 3% BSA and 0.01% Tween 20 (PBSBT) for 1 h. Cells were then incubated with the indicated primary antibodies (GM130, BD Biosciences, 610822; α3, Millipore, P1B5) at 4 °C overnight, followed by incubation with Alexa Fluor 647–conjugated secondary antibodies (Molecular Probes) for 1 h. For lectin staining, cells on coverslips were incubated with either biotinylated SNA lectin (Vector Laboratories, B-1305) or wheat germ agglutinin (WGA) lectin (J-Oil Mills, J220) at 4 °C for 30 min in DMEM, fixed and blocked with PBSBT for 1 h, followed by incubation with streptavidin-conjugated Alexa Fluor 647 at 4 °C for 1 h in the dark. All samples were mounted using Prolong Diamond Antifade mounting medium (Molecular Probes). Images were acquired by sequential excitation using an Olympus FV1000 laser-scanning confo-

cal microscope with an UPlanSApo ×60/1.35 oil objective operated with F10-ASW version 4.02 software.

Cell spreading and adhesion experiment

Coverslips were coated with 1 μg/ml laminin-332 or 2 mg/ml gelatin in PBS containing 0.1% BSA at 4 °C for 24 h and then blocked with 5% BSA in DMEM at 37% for 1 h. Cells were replated on the coverslips in DMEM with 0.1% BSA. After incubation for 10 min, the areas of adherent cells were measured using ImageJ. We defined cells spread at more than 0.025 μm^2 as adherent cells. After incubation for 30 min, the cells were fixed with 4% paraformaldehyde and stained with phalloidin–Alexa Fluor 647 (Molecular Probes).

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as described previously with minor modifications (11, 80). Briefly, cells were gently rinsed three times with PBS at room temperature and solubilized in cold lysis buffer A (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Brij98), which included protease inhibitors. Protein concentrations of lysates were determined via BCA assay (Pierce). The lysates were immunoprecipitated using either anti-GFP-agarose (MBL), anti-integrin α3 antibody (Millipore, P1B5), or anti-integrin β1 (P5D2, DSHB) with Ab-Capcher Protein A-R28 (Protenova, Tokushima, Japan) for 1 h at 4 °C and gentle rotation. After washing with lysis buffer, the immunoprecipitates were subjected to SDS-PAGE. The proteins were probed with anti-integrin α3 (Santa Cruz Biotechnology, sc-6592) or anti-GFP antibody (Rockland, 600-101-215) and then detected using anti-goat IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology) with an Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore). For analysis of the lectin precipitates, we used lysis buffer B (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100). Cell lysates were precipitated with either SSA-agarose (J-Oil Mills, J318), MAM (also called MAL-1)–agarose (J-Oil Mills, J310) or concanavalin A (ConA)–agarose (J-Oil Mills, J303), which specifically recognize either α2,6- and α2,3-sialylation or total *N*-glycans, respectively. The precipitants were detected using either anti-integrin β1 (BD Biosciences, 610468) or anti-EGFR (Cell Signaling Technology, 4267). Antibody agents against Akt (9272) and p-Akt (4060) were purchased from Cell Signaling Technology. α-Tubulin (Sigma, T6199) was used as a loading control.

Flow cytometric analysis

Flow cytometric analysis was performed as described previously with minor modifications (11, 79, 80). Briefly, semiconfluent cells were detached from the culture dishes using trypsin containing 1 mM EDTA. The cells were subsequently stained with or without primary mouse anti-α3 (P1B5), anti-β1 (P5D2), and anti-α5 integrin (Millipore, HA5) in PBS, followed by incubation with Alexa Fluor 647–conjugated secondary antibodies. For staining cells with biotin-conjugated lectins (MAM and SNA), we used PBS buffer containing 0.5 mM $CaCl_2$ and $MgCl_2$. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and Cell Quest Pro software (BD Biosciences).

PA oligosaccharide preparation of N-glycosylation and quantitative analysis of sialylated N-glycans by anion exchange HPLC

To release *N*-glycans from glycoproteins, the lyophilized cell pellets (1–2 mg) were heated with 200 μl of anhydrous hydrazine at 100 °C for 10 h using an oil bath. Removal of hydrazine and acetylation of *N*-glycans were carried out with a graphite carbon column (81). The released *N*-glycans were then 2-pyridylaminated using the Pyridylamination Manual Kit (Takara Bio Inc.), and then the excess 2-aminopyridine was removed via phenol–chloroform extraction (81) with a gel filtration column (82). The prepared PA oligosaccharides treated with or without neuraminidases were subjected to a HPLC system (JASCO) equipped with a TSKgel DEAE-5PW column (7.5 × 75 mm, Tosoh) and analyzed as described previously (19). HPLC chromatogram data were analyzed using chromNAV software (JASCO). The amounts of total and neutral *N*-glycans were calculated based on the peak area of neutral fraction with or without neuraminidase treatment. Relative ratios of sialylated *N*-glycans *versus* total *N*-glycans were calculated by subtracting the neutral portions from the total and then dividing by the total. The data were normalized to the control as 1.

Generation of CRISPR/Cas9-based integrin α3 KO cells

CRISPR/Cas9-based integrin α3 cells were established as described previously (83, 84). Briefly, the single guide RNA-specifying oligo sequences spanning human integrin α3 (forward, 5′-CACCGCATCGGGCACAGCGAGCTCC-3′; reverse, 5′-AAACGGAGCTCGCTGTGCCCGATGC-3) were chosen from the human KO library (85). Then the oligos were cloned into pSpCas9 (BB)-2A-GFP, which was a kind gift from Dr. Feng Zhang (Addgene plasmid ID 48138). The plasmid was transfected into cells according to the manufacturer's instructions (Amaxa Cell Line Nucleofector kitV). After 4 days of transfection, GFP-positive cells were sorted using a FACSAria II (BD Bioscience). Following 10-day culture, α3-negative and GFP-negative cells were sorted, and the procedure was repeated twice. The KO cells were defined by flow cytometry and Western blot analysis as described above.

Mass spectrometry of glycans

Total plasma membrane proteins of cells were prepared using a plasma membrane protein extraction kit (101Bio). For *N*-glycan analysis, a previous report (86) suggests that the pellets are dissolved and denatured and then digested by glycopeptidase F (Takara Bio Inc.). The released *N*-glycans were captured on hydrazide beads (BlotGlyco, 5 mg, Sumitomo Bakelite Co., Ltd.) using a process recommended by the manufacturer. Sialic acids of the *N*-glycans on the beads were then differentially amidated with methylamine for α2,3-sialic acids and isopropylamine for α2,6-sialic acids using the sialic acid linkage–specific alkylamidation (SALSA) method (87). The derivatized *N*-glycans were liberated, labeled with aoWR (a component of BlotGlyco, Sumitomo Bakelite Co., Ltd.) (86), and purified according to the manufacturer's instructions. The obtained glycan solutions were mixed with a 2,5-dihydroxybenzoic acid solution (10 mg/ml in 30% acetonitrile) at a ratio of 1:10. Aliquots (0.5 μl) of the mixed solutions were deposited onto a

MALDI target plate and dried. MS spectra were acquired with a MALDI quadrupole ion trap TOF mass spectrometer (AXIMA-QIT, Shimadzu Corp.). For *O*-glycan analysis, pellets of the membrane fractions were dissolved in a 1% SDS solution. Aliquots (10 μl) of the solution were transferred into 90 μl of 0.6 M sodium borohydride containing 60 mM NaOH and incubated at 45 °C for 16 h. Then the mixtures were neutralized, diluted to 1 ml, and applied onto a solid-phase extraction cartridge (Sep-Pak C18 Vac 1cc, 50 mg, Waters Corp.), and then the cartridge was washed with distilled water (1 ml). The eluents and washings were combined and lyophilized. The obtained residues were dissolved in 1% acetic acid in methanol (100 μl) and evaporated using a centrifuge evaporator. The procedure of dissolution and evaporation was repeated. The obtained residues were permethylated according to previous reports (88). The permethylated glycans were dissolved in a 2,5-dihydroxybenzoic acid solution (20 μl, 10 mg/ml in 30% acetonitrile), and aliquots (0.5 μl) of the mixed solutions were deposited onto a MALDI target plate and dried. MS spectra were acquired with a MALDI-TOF mass spectrometer (Ultraflex, Bruker Daltonik).

Author contributions—T. I., T. F., and J. G. conceptualization; T. I., S. I., A. K., Y. W., and J. G. data curation; T. I., S. I., A. K., Y. W., and J. G. formal analysis; T. I. and J. G. supervision; T. I., S. I., A. K., Y. W., T. F., and J. G. validation; T. I., S. I., A. K., and Y. W. investigation; T. I., S. I., A. K., Y. W., T. F., and J. G. methodology; T. I. and S. I. writing-original draft; T. I., S. I., A. K., Y. W., T. F., and J. G. writing-review and editing; S. I. visualization; A. K. and J. G. funding acquisition.

Acknowledgments—We thank Dr. Takashi Nishikaze for informative suggestions regarding the SALSA method and Dr. Wai Wai Thet Tin for technical assistance with glycan analysis.

References

1. Christie, D. R., Shaikh, F. M., Lucas, J. A., 4th, Lucas, J. A., 3rd, and Bellis, S. L. (2008) ST6Gal-I expression in ovarian cancer cells promotes an invasive phenotype by altering integrin glycosylation and function. *J. Ovarian Res.* **1,** 3 CrossRef Medline
2. Lin, S., Kemmner, W., Grigull, S., and Schlag, P. M. (2002) Cell surface α2,6 sialylation affects adhesion of breast carcinoma cells. *Exp. Cell Res.* **276,** 101–110 CrossRef Medline
3. Zhuo, Y., Chammas, R., and Bellis, S. L. (2008) Sialylation of β1 integrins blocks cell adhesion to galectin-3 and protects cells against galectin-3-induced apoptosis. *J. Biol. Chem.* **283,** 22177–22185 CrossRef Medline
4. Hasehira, K., Hirabayashi, J., and Tateno, H. (2017) Structural and quantitative evidence of α2–6-sialylated *N*-glycans as markers of the differentiation potential of human mesenchymal stem cells. *Glycoconj. J.* **34,** 797–806 Medline
5. Wang, Y. C., Stein, J. W., Lynch, C. L., Tran, H. T., Lee, C. Y., Coleman, R., Hatch, A., Antontsev, V. G., Chy, H. S., O'Brien, C. M., Murthy, S. K., Laslett, A. L., Peterson, S. E., and Loring, J. F. (2015) Glycosyltransferase ST6GAL1 contributes to the regulation of pluripotency in human pluripotent stem cells. *Sci. Rep.* **5,** 13317 CrossRef Medline
6. Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664 CrossRef Medline
7. Grabenhorst, E., and Conradt, H. S. (1999) The cytoplasmic, transmembrane, and stem regions of glycosyltransferases specify their *in vivo* functional sublocalization and stability in the Golgi. *J. Biol. Chem.* **274,** 36107–36116 CrossRef Medline
8. Dalziel, M., Dall'Olio, F., Mungul, A., Piller, V., and Piller, F. (2004) Ras oncogene induces beta-galactoside α2,6-sialyltransferase (ST6Gal I) via a RalGEF-mediated signal to its housekeeping promoter. *Eur. J. Biochem.* **271,** 3623–3634 CrossRef Medline
9. Seales, E. C., Jurado, G. A., Singhal, A., and Bellis, S. L. (2003) Ras oncogene directs expression of a differentially sialylated, functionally altered β 1 integrin. *Oncogene* **22,** 7137–7145 CrossRef Medline
10. Eckert, E. S., Reckmann, I., Hellwig, A., Röhling, S., El-Battari, A., Wieland, F. T., and Popoff, V. (2014) Golgi phosphoprotein 3 triggers signal-mediated incorporation of glycosyltransferases into coatomer-coated (COPI) vesicles. *J. Biol. Chem.* **289,** 31319–31329 CrossRef Medline
11. Isaji, T., Im, S., Gu, W., Wang, Y., Hang, Q., Lu, J., Fukuda, T., Hashii, N., Takakura, D., Kawasaki, N., Miyoshi, H., and Gu, J. (2014) An oncogenic protein Golgi phosphoprotein 3 up-regulates cell migration via sialylation. *J. Biol. Chem.* **289,** 20694–20705 CrossRef Medline
12. Scott, K. L., Kabbarah, O., Liang, M. C., Ivanova, E., Anagnostou, V., Wu, J., Dhakal, S., Wu, M., Chen, S., Feinberg, T., Huang, J., Saci, A., Widlund, H. R., Fisher, D. E., Xiao, Y., *et al.* (2009) GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. *Nature* **459,** 1085-U1068 CrossRef Medline
13. Zeng, Z., Lin, H., Zhao, X., Liu, G., Wang, X., Xu, R., Chen, K., Li, J., and Song, L. (2012) Overexpression of GOLPH3 promotes proliferation and tumorigenicity in breast cancer via suppression of the FOXO1 transcription factor. *Clin. Cancer Res.* **18,** 4059–4069 CrossRef Medline
14. Dippold, H. C., Ng, M. M., Farber-Katz, S. E., Lee, S. K., Kerr, M. L., Peterman, M. C., Sim, R., Wiharto, P. A., Galbraith, K. A., Madhavarapu, S., Fuchs, G. J., Meerloo, T., Farquhar, M. G., Zhou, H., and Field, S. J. (2009) GOLPH3 bridges phosphatidylinositol-4-phosphate and actomyosin to stretch and shape the Golgi to promote budding. *Cell* **139,** 337–351 CrossRef Medline
15. Ng, M. M., Dippold, H. C., Buschman, M. D., Noakes, C. J., and Field, S. J. (2013) GOLPH3L antagonizes GOLPH3 to determine Golgi morphology. *Mol. Biol. Cell* **24,** 796–808 CrossRef Medline
16. Clayton, E. L., Minogue, S., and Waugh, M. G. (2013) Mammalian phosphatidylinositol 4-kinases as modulators of membrane trafficking and lipid signaling networks. *Prog. Lipid Res.* **52,** 294–304 CrossRef Medline
17. Park, C. C., Zhang, H., Pallavicini, M., Gray, J. W., Baehner, F., Park, C. J., and Bissell, M. J. (2006) β1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and *in vivo*. *Cancer Res.* **66,** 1526–1535 CrossRef Medline
18. Takada, Y., Ye, X., and Simon, S. (2007) The integrins. *Genome Biol.* **8,** 215 CrossRef Medline
19. Lu, J., Isaji, T., Im, S., Fukuda, T., Kameyama, A., and Gu, J. (2016) Expression of *N*-acetylglucosaminyltransferase III suppresses α2,3-sialylation, and its distinctive functions in cell migration are attributed to α2,6-sialylation levels. *J. Biol. Chem.* **291,** 5708–5720 CrossRef Medline
20. Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K., and Taniguchi, N. (2004) Introduction of bisecting GlcNAc into integrin α5β1 reduces ligand binding and down-regulates cell adhesion and cell migration. *J. Biol. Chem.* **279,** 19747–19754 CrossRef Medline
21. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. (2002) Aberrant *N*-glycosylation of β1 integrin causes reduced α5β1 integrin clustering and stimulates cell migration. *Cancer Res.* **62,** 6837–6845 Medline
22. Hou, S., Hang, Q., Isaji, T., Lu, J., Fukuda, T., and Gu, J. (2016) Importance of membrane-proximal *N*-glycosylation on integrin β1 in its activation and complex formation. *FASEB J.* **30,** 4120–4131 CrossRef Medline
23. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110,** 673–687 CrossRef Medline
24. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **6,** 56–68 CrossRef Medline
25. Yauch, R. L., Berditchevski, F., Harler, M. B., Reichner, J., and Hemler, M. E. (1998) Highly stoichiometric, stable, and specific association of integrin α3β1 with CD151 provides a major link to phosphatidylinositol 4

kinase, and may regulate cell migration. *Mol. Biol. Cell* **9,** 2751–2765 CrossRef Medline
26. Berditchevski, F., Tolias, K. F., Wong, K., Carpenter, C. L., and Hemler, M. E. (1997) A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* **272,** 2595–2598 CrossRef Medline
27. Yauch, R. L., and Hemler, M. E. (2000) Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem. J.* **351,** 629–637 CrossRef Medline
28. Boura, E., and Nencka, R. (2015) Phosphatidylinositol 4-kinases: function, structure, and inhibition. *Exp. Cell Res.* **337,** 136–145 CrossRef Medline
29. Wang, Y. J., Wang, J., Sun, H. Q., Martinez, M., Sun, Y. X., Macia, E., Kirchhausen, T., Albanesi, J. P., Roth, M. G., and Yin, H. L. (2003) Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114,** 299–310 CrossRef Medline
30. D'Angelo, G., Vicinanza, M., Di Campli, A., and De Matteis, M. A. (2008) The multiple roles of PtdIns$_4$P: not just the precursor of PtdIns$_{4,5}$P-2. *J. Cell Sci.* **121,** 1955–1963 CrossRef Medline
31. Weixel, K. M., Blumental-Perry, A., Watkins, S. C., Aridor, M., and Weisz, O. A. (2005) Distinct Golgi populations of phosphatidylinositol 4-phosphate regulated by phosphatidylinositol 4-kinases. *J. Biol. Chem.* **280,** 10501–10508 CrossRef Medline
32. Morrow, A. A., Alipour, M. A., Bridges, D., Yao, Z., Saltiel, A. R., and Lee, J. M. (2014) The lipid kinase PI4KIIIβ is highly expressed in breast tumors and activates Akt in cooperation with Rab11a. *Mol. Cancer Res.* **12,** 1492–1508 CrossRef Medline
33. Moravcevic, K., Oxley, C. L., and Lemmon, M. A. (2012) Conditional peripheral membrane proteins: facing up to limited specificity. *Structure* **20,** 15–27 CrossRef Medline
34. Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E. G., Warren, G., and Nilsson, T. (1995) Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. *J. Cell Sci.* **108,** 1617–1627 Medline
35. Lu, D., Sun, H. Q., Wang, H., Barylko, B., Fukata, Y., Fukata, M., Albanesi, J. P., and Yin, H. L. (2012) Phosphatidylinositol 4-kinase IIα is palmitoylated by Golgi-localized palmitoyltransferases in cholesterol-dependent manner. *J. Biol. Chem.* **287,** 21856–21865 CrossRef Medline
36. Bhide, G. P., and Colley, K. J. (2017) Sialylation of *N*-glycans: mechanism, cellular compartmentalization and function. *Histochem. Cell Biol.* **147,** 149–174 CrossRef Medline
37. Balla, A., Tuymetova, G., Tsiomenko, A., Várnai, P., and Balla, T. (2005) A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-IIIα: studies with the PH domains of the oxysterol binding protein and FAPP1. *Mol. Biol. Cell* **16,** 1282–1295 CrossRef Medline
38. Itakura, Y., Nakamura-Tsuruta, S., Kominami, J., Tateno, H., and Hirabayashi, J. (2017) Sugar-binding profiles of chitin-binding lectins from the hevein family: a comprehensive study. *Int. J. Mol. Sci.* **18**, E1160 CrossRef Medline
39. Grottke, A., Ewald, F., Lange, T., Nörz, D., Herzberger, C., Bach, J., Grabinski, N., Gräser, L., Höppner, F., Nashan, B., Schumacher, U., and Jücker, M. (2016) Downregulation of AKT3 Increases migration and metastasis in triple negative breast cancer cells by upregulating S100A4. *PLoS ONE* **11,** e0146370 CrossRef Medline
40. Mitchell, K., Svenson, K. B., Longmate, W. M., Gkirtzimanaki, K., Sadej, R., Wang, X., Zhao, J., Eliopoulos, A. G., Berditchevski, F., and Dipersio, C. M. (2010) Suppression of Integrin α3β1 in breast cancer cells reduces cyclooxygenase-2 gene expression and inhibits tumorigenesis, invasion, and cross-talk to endothelial cells. *Cancer Res.* **70,** 6359–6367 CrossRef Medline
41. Morini, M., Mottolese, M., Ferrari, N., Ghiorzo, F., Buglioni, S., Mortarini, R., Noonan, D. M., Natali, P. G., and Albini, A. (2000) The α3β1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. *Int. J. Cancer* **87,** 336–342 CrossRef Medline
42. Cagnet, S., Faraldo, M. M., Kreft, M., Sonnenberg, A., Raymond, K., and Glukhova, M. A. (2014) Signaling events mediated by α3β1 integrin are essential for mammary tumorigenesis. *Oncogene* **33,** 4286–4295 CrossRef Medline
43. Dogic, D., Rousselle, P., and Aumailley, M. (1998) Cell adhesion to laminin 1 or 5 induces isoform-specific clustering of integrins and other focal adhesion components. *J. Cell Sci.* **111**, 793–802 Medline
44. Liu, Y. C., Yen, H. Y., Chen, C. Y., Chen, C. H., Cheng, P. F., Juan, Y. H., Chen, C. H., Khoo, K. H., Yu, C. J., Yang, P. C., Hsu, T. L., and Wong, C. H. (2011) Sialylation and fucosylation of epidermal growth factor receptor suppress its dimerization and activation in lung cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 11332–11337 CrossRef Medline
45. Varki, A., Kannagi, R., Toole, B., and Stanley, P. (2015) in *Essentials of Glycobiology* (Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., and Seeberger, P. H., eds.) pp. 597–609, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
46. Brockhausen, I. (2006) Mucin-type *O*-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Rep.* **7,** 599–604 CrossRef Medline
47. Dall'Olio, F., Malagolini, N., Trinchera, M., and Chiricolo, M. (2014) Sialosignaling: sialyltransferases as engines of self-fueling loops in cancer progression. *Biochim. Biophys. Acta* **1840,** 2752–2764 CrossRef Medline
48. Schultz, M. J., Swindall, A. F., and Bellis, S. L. (2012) Regulation of the metastatic cell phenotype by sialylated glycans. *Cancer Metastasis Rev.* **31,** 501–518 CrossRef Medline
49. Lu, J., and Gu, J. (2015) Significance of β-galactoside α2,6 sialyltranferase 1 in cancers. *Molecules* **20,** 7509–7527 CrossRef Medline
50. Woodard-Grice, A. V., McBrayer, A. C., Wakefield, J. K., Zhuo, Y., and Bellis, S. L. (2008) Proteolytic shedding of ST6Gal-I by BACE1 regulates the glycosylation and function of α4β1 integrins. *J. Biol. Chem.* **283,** 26364–26373 CrossRef Medline
51. Kitazume, S., Imamaki, R., Ogawa, K., Komi, Y., Futakawa, S., Kojima, S., Hashimoto, Y., Marth, J. D., Paulson, J. C., and Taniguchi, N. (2010) α2,6-sialic acid on platelet endothelial cell adhesion molecule (PECAM) regulates its homophilic interactions and downstream antiapoptotic signaling. *J. Biol. Chem.* **285,** 6515–6521 CrossRef Medline
52. Ye, X., and Weinberg, R. A. (2015) Epithelial-mesenchymal plasticity: a central regulator of cancer progression. *Trends Cell Biol.* **25,** 675–686 CrossRef Medline
53. Swindall, A. F., Londoño-Joshi, A. I., Schultz, M. J., Fineberg, N., Buchsbaum, D. J., and Bellis, S. L. (2013) ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. *Cancer Res.* **73,** 2368–2378 CrossRef Medline
54. Lu, J., Isaji, T., Im, S., Fukuda, T., Hashii, N., Takakura, D., Kawasaki, N., and Gu, J. (2014) β-Galactoside α2,6-sialyltranferase 1 promotes transforming growth factor β-mediated epithelial-mesenchymal transition. *J. Biol. Chem.* **289,** 34627–34641 CrossRef Medline
55. Schmitz, K. R., Liu, J., Li, S., Setty, T. G., Wood, C. S., Burd, C. G., and Ferguson, K. M. (2008) Golgi localization of glycosyltransferases requires a Vps74p oligomer. *Dev. Cell* **14,** 523–534 CrossRef Medline
56. Pereira, N. A., Pu, H. X., Goh, H., and Song, Z. (2014) Golgi phosphoprotein 3 mediates the Golgi localization and function of protein *O*-linked mannose β-1,2-*N*-acetylglucosaminyltransferase 1. *J. Biol. Chem.* **289,** 14762–14770 CrossRef Medline
57. Minogue, S., Waugh, M. G., De Matteis, M. A., Stephens, D. J., Berditchevski, F., and Hsuan, J. J. (2006) Phosphatidylinositol 4-kinase is required for endosomal trafficking and degradation of the EGF receptor. *J. Cell Sci.* **119,** 571–581 CrossRef Medline
58. Tokuda, E., Itoh, T., Hasegawa, J., Ijuin, T., Takeuchi, Y., Irino, Y., Fukumoto, M., and Takenawa, T. (2014) Phosphatidylinositol 4-phosphate in the Golgi apparatus regulates cell-cell adhesion and invasive cell migration in human breast cancer. *Cancer Res.* **74,** 3054–3066 CrossRef Medline
59. Qin, Y., Li, L., Pan, W., and Wu, D. (2009) Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl. *J. Biol. Chem.* **284,** 22544–22548 CrossRef Medline
60. Chu, K. M., Minogue, S., Hsuan, J. J., and Waugh, M. G. (2010) Differential effects of the phosphatidylinositol 4-kinases, PI4KIIα and PI4KIIIβ, on Akt activation and apoptosis. *Cell Death Dis.* **1,** e106 CrossRef Medline

61. Yamabhai, M., and Anderson, R. G. (2002) Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. *J. Biol. Chem.* **277,** 24843–24846 CrossRef Medline
62. Sato, Y., Takahashi, M., Shibukawa, Y., Jain, S. K., Hamaoka, R., Miyagawa, J., Yaginuma, Y., Honke, K., Ishikawa, M., and Taniguchi, N. (2001) Overexpression of *N*-acetylglucosaminyltransferase III enhances the epidermal growth factor-induced phosphorylation of ERK in HeLaS3 cells by up-regulation of the internalization rate of the receptors. *J. Biol. Chem.* **276,** 11956–11962 CrossRef Medline
63. Matoba, K., Mihara, E., Tamura-Kawakami, K., Miyazaki, N., Maeda, S., Hirai, H., Thompson, S., Iwasaki, K., and Takagi, J. (2017) Conformational freedom of the LRP6 ectodomain is regulated by *N*-glycosylation and the binding of the Wnt antagonist Dkk1. *Cell Rep.* **18,** 32–40 CrossRef Medline
64. Swindall, A. F., and Bellis, S. L. (2011) Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells. *J. Biol. Chem.* **286,** 22982–22990 CrossRef Medline
65. Jonjić, N., Lucin, K., Krstulja, M., Iternicka, Z., and Mustać, E. (1993) Expression of β-1 integrins on tumor-cells of invasive ductal breast-carcinoma. *Pathol. Res. Pract.* **189,** 979–984 CrossRef Medline
66. Yao, E. S., Zhang, H., Chen, Y. Y., Lee, B., Chew, K., Moore, D., and Park, C. (2007) Increased β1 integrin is associated with decreased survival in invasive breast cancer. *Cancer Res.* **67,** 659–664 CrossRef Medline
67. Li, J., Lu, Y., Zhang, J., Kang, H., Qin, Z., and Chen, C. (2010) PI4KIIα is a novel regulator of tumor growth by its action on angiogenesis and HIF-1α regulation. *Oncogene* **29,** 2550–2559 CrossRef Medline
68. Zhou, Q., Li, J., Yu, H., Zhai, Y., Gao, Z., Liu, Y., Pang, X., Zhang, L., Schulten, K., Sun, F., and Chen, C. (2014) Molecular insights into the membrane-associated phosphatidylinositol 4-kinase IIα. *Nat. Commun.* **5,** 3552 CrossRef Medline
69. Baumlova, A., Chalupska, D., Różycki, B., Jovic, M., Wisniewski, E., Klima, M., Dubankova, A., Kloer, D. P., Nencka, R., Balla, T., and Boura, E. (2014) The crystal structure of the phosphatidylinositol 4-kinase IIα. *EMBO Rep.* **15,** 1085–1092 CrossRef Medline
70. Yang, X., Kovalenko, O. V., Tang, W., Claas, C., Stipp, C. S., and Hemler, M. E. (2004) Palmitoylation supports assembly and function of integrin-tetraspanin complexes. *J. Cell Biol.* **167,** 1231–1240 CrossRef Medline
71. Coleman, D. T., Gray, A. L., Kridel, S. J., and Cardelli, J. A. (2016) Palmitoylation regulates the intracellular trafficking and stability of c-Met. *Oncotarget* **7,** 32664–32677 Medline
72. Goldfinger, L. E., Hopkinson, S. B., deHart, G. W., Collawn, S., Couchman, J. R., and Jones, J. C. (1999) The α3 laminin subunit, α6β4 and α3 β1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin. *J. Cell Sci.* **112,** 2615–2629 Medline
73. Hedlund, M., Ng, E., Varki, A., and Varki, N. M. (2008) α2–6-linked sialic acids on *N*-glycans modulate carcinoma differentiation *in vivo*. *Cancer Res.* **68,** 388–394 CrossRef Medline
74. Ali, M. F., Chachadi, V. B., Petrosyan, A., and Cheng, P. W. (2012) Golgi phosphoprotein 3 determines cell binding properties under dynamic flow by controlling Golgi localization of core 2 *N*-acetylglucosaminyltransferase 1. *J. Biol. Chem.* **287,** 39564–39577 CrossRef Medline
75. Maeda, Y., Ide, T., Koike, M., Uchiyama, Y., and Kinoshita, T. (2008) GPHR is a novel anion channel critical for acidification and functions of the Golgi apparatus. *Nat. Cell Biol.* **10,** 1135–1145 CrossRef Medline
76. Mesmin, B., Bigay, J., Moser von Filseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013) A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI_4P exchange by the ER-Golgi tether OSBP. *Cell* **155,** 830–843 CrossRef Medline
77. Hang, Q., Isaji, T., Hou, S., Zhou, Y., Fukuda, T., and Gu, J. (2016) N-Glycosylation of integrin α5 acts as a switch for EGFR-mediated complex formation of integrin α5β1 to α6β4. *Sci. Rep.* **6,** 33507 CrossRef Medline
78. Hou, S., Isaji, T., Hang, Q., Im, S., Fukuda, T., and Gu, J. (2016) Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells. *Sci. Rep.* **6,** 18430 CrossRef Medline
79. Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) N-glycosylation of the beta-propeller domain of the integrin α5 subunit is essential for α5β1 heterodimerization, expression on the cell surface, and its biological function. *J. Biol. Chem.* **281,** 33258–33267 CrossRef Medline
80. Sato, Y., Isaji, T., Tajiri, M., Yoshida-Yamamoto, S., Yoshinaka, T., Somehara, T., Fukuda, T., Wada, Y., and Gu, J. (2009) An *N*-glycosylation site on the β-propeller domain of the integrin α5 subunit plays key roles in both its function and site-specific modification by β1,4-*N*-acetylglucosaminyltransferase III. *J. Biol. Chem.* **284,** 11873–11881 CrossRef Medline
81. Tanabe, K., and Ikenaka, K. (2006) In-column removal of hydrazine and *N*-acetylation of oligosaccharides released by hydrazionolysis. *Anal. Biochem.* **348,** 324–326 CrossRef Medline
82. Yanagida, K., Natsuka, S., and Hase, S. (1999) A pyridylamination method aimed at automatic oligosaccharide analysis of *N*-linked sugar chains. *Anal. Biochem.* **274,** 229–234 CrossRef Medline
83. Ran, F. A., Hsu, P. D., Lin, C. Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., Scott, D. A., Inoue, A., Matoba, S., Zhang, Y., and Zhang, F. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154,** 1380–1389 CrossRef Medline
84. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8,** 2281–2308 CrossRef Medline
85. Shalem, O., Sanjana, N. E., Hartenian, E., Shi, X., Scott, D. A., Mikkelson, T., Heckl, D., Ebert, B. L., Root, D. E., Doench, J. G., and Zhang, F. (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343,** 84–87 CrossRef Medline
86. Toyoda, M., Kaji, H., Sawaki, H., Togayachi, A., Angata, T., Narimatsu, H., and Kameyama, A. (2016) Identification and characterization of sulfated glycoproteins from small cell lung carcinoma cells assisted by management of molecular charges. *Glycoconj. J.* **33,** 917–926 CrossRef Medline
87. Nishikaze, T., Tsumoto, H., Sekiya, S., Iwamoto, S., Miura, Y., and Tanaka, K. (2017) Differentiation of sialyl linkage isomers by one-pot sialic acid derivatization for mass spectrometry-based glycan profiling. *Anal. Chem.* **89,** 2353–2360 CrossRef Medline
88. Kameyama, A., Yamakoshi, K., and Watanabe, A. (2019) A rapid separation and characterization of mucins from mouse submandibular glands by supported molecular matrix electrophoresis. *Biochim. Biophys. Acta Proteins Proteom.* **1867,** 76–81 CrossRef Medline