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***N*-Glycosylation of integrin $\alpha 5$ acts as a switch for EGFR-mediated complex formation of integrin $\alpha 5\beta 1$ to $\alpha 6\beta 4$**

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N-Glycosylation of integrin $\alpha 5\beta 1$ is involved in multiple cell behaviors. We previously reported that the *N*-glycosylations of the calf domain on integrin $\alpha 5$ (S3–5,10–14) are essential for its inhibitory effect on EGFR signaling in regulating cell proliferation. However, the importance of the individual *N*-glycosylation and the underlying mechanisms of inhibition remain unclear. Here, we characterize the S3–5,10–14 mutants in detail and found that the *N*-glycosylation of site-11 (Asn712) is key for cell growth. The restoration of site-11, unlike the other individual sites, significantly suppressed cell growth and EGFR signaling in a manner that was similar to that of wild-type (WT). Mechanistically, this *N*-glycosylation inhibited the response abilities upon EGF stimulation and EGFR dimerization. Interestingly, we found this *N*-glycosylation controlled the EGFR complex formation with integrin $\alpha 5\beta 1$ or $\alpha 6\beta 4$; i.e., the loss of site-11 switched EGFR- $\alpha 5\beta 1$ to EGFR- $\alpha 6\beta 4$, which is well known to promote cellular signaling for cell growth. Moreover, the site-11 *N*-glycan exhibited a more branching structure compared with other sites, which may be required for EGFR- $\alpha 5\beta 1$ formation. Taken together, these data clearly demonstrate that the site-11 *N*-glycosylation on $\alpha 5$ is most important for its inhibitory effect on EGFR signaling, which may provide a novel regulatory mechanism for crosstalks between integrins and EGFR.

Integrins are $\alpha\beta$ heterodimeric transmembrane glycoproteins that link cells to their surroundings such as to the cell-extracellular matrix (ECM) or to other cells¹. In addition to performing a structural role, integrins bidirectionally relay signals, outside-in and inside-out, across the cell membrane^{2,3}, and participate in the growth factor receptors (GFRs) signaling pathways^{4,5}. The crosstalk between epidermal growth factor receptor (EGFR), a well-known GFR, and integrins plays a key role in multiple biological processes in tumors, including cell growth, metastasis, and drug resistance⁶. However, the effect that integrins exert on the regulation of EGFR-mediated signaling seems rather indiscriminate, since a variety of integrins, such as integrin $\alpha 5\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 3$, and $\alpha v\beta 5$ ^{7–10}, have been respectively reported to regulate EGFR signaling, and also the effects of several integrins on EGFR signaling or cell growth are controversial, such as integrin $\alpha 5\beta 1$ ^{11–13} and $\alpha 6\beta 4$ ^{14,15}, which prompted us to explore how integrins regulate EGFR activation in a coordinated manner.

Recently, an accumulating body of evidence has begun revealing mechanisms that describe how integrins and GFRs collaborate at many different levels from gene to post-transcriptional regulation¹⁶. For instance, the mechanisms involved in how integrins regulate EGFR signaling seem to most commonly converge on kinases such as Src family kinases (SFKs)¹⁷, scaffolding proteins such as p130 Crk-associated substrates (p130^{Cas})¹⁸, GTPases-related proteins such as the Ras and Rab family¹⁶, and phosphatases such as T-cell protein tyrosine phosphatase¹⁹, which drives largely around the inner membrane. However, these findings are insufficient to clarify the molecular mechanisms in detail.

In contrast, knowledge is limited regarding the regulatory mechanisms of the outer membrane crosstalks mediated by integrins. In recent years, *N*-glycosylation was believed to be important in this respect, given its roles in regulating cellular signalings, as it provides a set of attractive targets for the diagnosis and treatment of cancer^{20,21}. Several research groups, including ours, have demonstrated that alterations of the *N*-glycan composition on several integrins could regulate their association with other membrane proteins, such as EGFR, urokinase-type

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plasminogen activation receptor, and tetraspanin proteins^{22–24}. In addition, we found that the *N*-glycosylation of a specific domain in the integrin $\alpha 5$ subunit could regulate EGFR signaling and cell proliferation¹¹. Nevertheless, further investigation is needed to fully explore the molecular mechanisms underlying this regulation.

The *N*-glycosylation of integrin $\alpha 5\beta 1$ appears to be very important for its mediation of multiple biological functions including heterodimer formation, cell surface expression, ligand binding, cell adhesion, and migration²⁵. Indeed, complete ablation of *N*-glycosylation on integrin $\alpha 5\beta 1$ by treatment with tunicamycin and *N*-glycosidase F prevents both transport to the cell surface and binding to fibronectin (FN), respectively^{26,27}. Also, changes in *N*-glycan composition of integrin $\alpha 5\beta 1$ could regulate its binding ability to FN and its cell spreading and migration via the overexpression of glycosyltransferase genes such as *N*-acetylglucosaminyltransferase-V (GnT-V), GnT-III, or $\alpha 2,6$ -galactoside sialyltransferase 1 (ST6GAL1)^{28–31}. However, most of the previous studies have examined only total changes by displaying or masking specific glycan epitopes without individual information. In fact, integrin $\alpha 5\beta 1$ is a major carrier of *N*-glycans, particularly the $\alpha 5$ portion that contains 14 potential *N*-glycosylation sites. This complexity raises important questions as to whether individual *N*-glycosylation of $\alpha 5$ plays a specific role in modulating its function. Our previous study revealed that *N*-glycosylation sites 3–5, and site-5 in particular, on the β -propeller domain of $\alpha 5$ is essential for the assembly of $\alpha 5\beta 1$ heterodimer formation and its expression on the cell surface³², which suggests that individual *N*-glycosylation has its own function.

Given the importance of the *N*-glycosylation of integrins in EGFR activation, we considered there might be one or several specific *N*-glycosylation(s) of $\alpha 5$ that regulate EGFR signaling for the control of cell proliferation. To test this hypothesis, we characterized the *N*-glycosylation of the calf domain in $\alpha 5$ in detail and found that *N*-glycosylation of site-11 plays a key role in its inhibitory effect on EGFR function. Furthermore, we found that the site-11 *N*-glycosylation at least partially participates in switching the EGFR complex formation from integrin $\alpha 5\beta 1$ to $\alpha 6\beta 4$. In addition, the specific structures of site-11 *N*-glycan may also be important for the functions, since the site-11 *N*-glycan exhibited more branching structures and the suppression of these branching structures by overexpression of GnT-III significantly decreased the EGFR-integrins complex formation. These findings may provide new insight into the cellular signaling machinery.

Results

The potential *N*-glycosylation of site-11 on the calf domain of integrin $\alpha 5$ is the most important aspect in the development of its inhibitory function. In prior work we identified three *N*-glycosylation sites on the β -propeller domain (S3–5) (Fig. 1a) of the integrin $\alpha 5$ subunit that are essential for $\alpha 5\beta 1$ heterodimerization, cell surface expression, and cell adhesion in CHO-B2 cells³². Based on this, we recently found that *N*-glycosylation of the calf domain, which localizes in the vicinity of the cell membrane of integrin $\alpha 5$ (S3–5,10–14) (Fig. 1a), plays a key role in its inhibitory effect on EGFR signaling¹¹. These findings led to the question of whether there is/are one or several specific *N*-glycosylation(s) of $\alpha 5$ that is needed to regulate the crosstalk between integrin $\alpha 5$ and EGFR.

To test this concept, using the form with *N*-glycosylation sites 3–5 reintroduced, we replaced one-by-one the sites 10–14 within the calf domain, as shown in Fig. 1a (S3–5,10; S3–5,11; S3–5,12; S3–5,13; and, S3–5,14), in the CHO-B2/EGFR cells, which is an integrin $\alpha 5$ deficient but stable overexpression of EGFR cell line we established previously¹¹. As shown in Fig. 1b, in flow cytometry analysis these mutant cells exhibited almost the same expression levels of EGFR and $\alpha 5\beta 1$ on the cell surface by comparison with WT, which suggested that individual *N*-glycosylation of the calf domain has no effect on the expression of EGFR. Meanwhile, these mutants exhibited abilities for cell spreading on fibronectin (FN) that were comparable to those of WT cells (Fig. 1c), which also indicated that these five individual *N*-glycosylation had no significant effect on cell spreading. These results suggest that these re-expressed individual *N*-glycosylation mutants may serve as a reliable cell model that could be used to compare their effects on EGFR signaling and how it regulates cell proliferation.

Next, we compared the cell proliferation ability among these five mutants. As shown in Fig. 1d, cell proliferation was partially decreased in the S3–5,10; S3–5,11; and, S3–5,12 cells, but not in the other two mutants (S3–5,13 and S3–5,14), compared with that in S3–5 cells. However, only S3–5,11 mutants showed significant inhibitory effects on cell proliferation, as the S3–5,10–14 or the WT had. These results suggest that the function of WT integrin $\alpha 5$ on cell proliferation can be duplicated by the S3–5,11 mutant, and that the *N*-glycosylation of site-11 is the most important development for its inhibitory functions.

EGFR-related cellular signaling is strictly controlled by the site-11 *N*-glycosylation of integrin $\alpha 5$.

Given the inhibitory effects of integrin $\alpha 5$ on cell growth via integrin $\alpha 5$ -EGFR complex formation¹¹, we wondered whether the S3–5,11 mutant could also mimic the WT in its inhibition of the EGFR pathway. As shown in Fig. 2a, the aberrant expression levels of phosphorylated EGFR (p-EGFR) and its downstream molecules ERK and AKT were completely revived in the S3–5,11-CHO-B2/EGFR cells, compared with those in WT or S3–5,10–14 cells. Furthermore, the association between EGFR and integrin $\alpha 5$ was greatly increased in the S3–5,11 cells, which was comparable to those in the WT or S3–5,10–14 cells (Fig. 2b). These results clearly demonstrated that the S3–5,11 mutant and WT integrin $\alpha 5$ have comparable effects on EGFR signaling.

Moreover, we selected the HeLa cells that express relatively high levels of endogenous integrin $\alpha 5$ and EGFR, in order to further assess whether the phenomenon described above is common to other mammalian cells. The previously established $\alpha 5$ -knock-out (KO) HeLa cells¹¹ were respectively reconstituted by either the S3–5,11 or D11 $\alpha 5$ mutant via deletion of the *N*-glycosylation of site-11 only through replacing the Asn712 with Asp (Fig. 3a). Therefore, the significance of the potential site-11 *N*-glycosylation of $\alpha 5$ was further confirmed by using the S3–5,11 and D11 HeLa cells. By comparison with the WT cells, both the S3–5,11 and D11 mutant cells exhibited similar expression levels of EGFR and $\alpha 5\beta 1$ on cell surfaces analyzed by flow cytometry (Fig. 3b), as well as comparable abilities for cell spreading on FN (Fig. 3c). Consistent with the data obtained from CHO-B2/EGFR cells, the inhibitory effect on cell proliferation was also observed in the S3–5,11-HeLa cells, when compared with

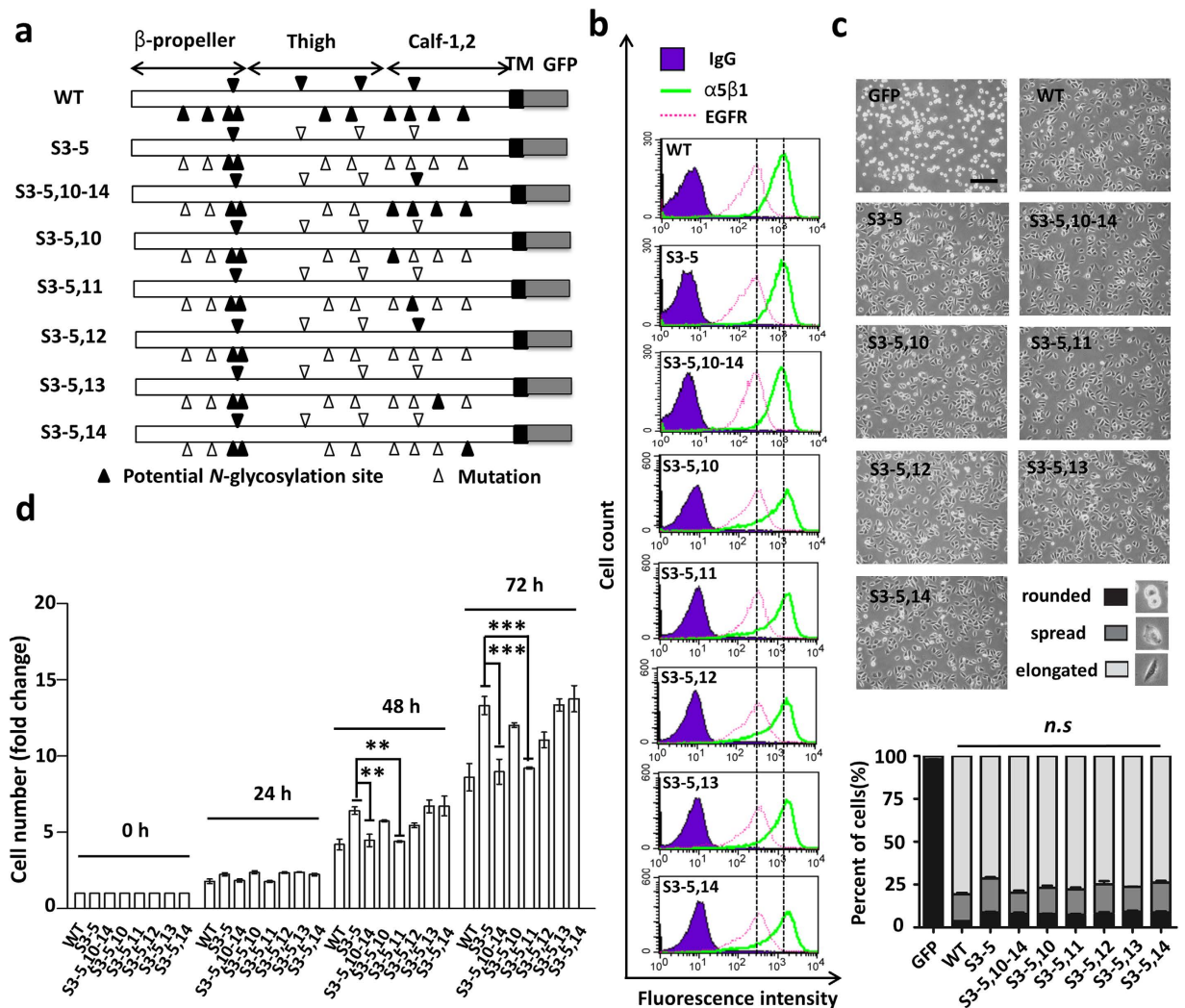


Figure 1. Effects of various unglycosylated mutant $\alpha 5$ subunits expression on cell growth in CHO-B2/EGFR cells. **(a)** Schematic diagram of potential N-glycosylation sites on the WT and mutational integrin $\alpha 5$ subunits (WT; S3-5; S3-5,10-14; S3-5,10; S3-5,11; S3-5,12; S3-5,13 and, S3-5,14). Putative N-glycosylation sites (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q) are indicated by solid triangles, and point mutations are indicated by hollow triangles. **(b)** The integrin $\alpha 5$ mutant cells expressed equal $\alpha 5\beta 1$ and EGFR levels on the cell surface, compared with the WT cells. The stable cell lines were established as described under the “Methods” section. The expression levels of both $\alpha 5\beta 1$ and EGFR were analyzed by flow cytometry. The IgG was used as a control. **(c)** The $\alpha 5$ mutant cells exhibited abilities for cell spreading that were comparable to those of WT. Cells were detached and then replated on the FN-coated dishes. After incubation for 20 min, cells were fixed and the representative images were taken. The percentages of rounded, spread, and elongated cells were statistically analyzed (right bottom panel, $n = 9$). Scale bar, 120 μm . **(d)** The S3-5,11 cells exhibited inhibitory ability for cell growth that was comparable to that of the S3-5,10-14 versions. After starvation for 24 h, cells were supplied with DMEM containing 10% FBS, and then cell numbers were counted and statistically analyzed at indicated times ($n = 3$ individual experiments). All values are reported as the means \pm S.E. (error bars), Student’s *t*-test; *n.s.*, not significant ($p > 0.05$); ** $p < 0.01$; *** $p < 0.001$.

the S3-5 or KO cells (Fig. 3d). It is noteworthy that the inhibitory effect of $\alpha 5$ on cell growth was largely cancelled in the D11 cells, as exhibited by the S3-5 mutants (Fig. 3d). Furthermore, the phosphorylation levels of EGFR and the activations of ERK and AKT were significantly increased in the D11 cells (Fig. 3e), compared with those in the WT or S3-5,11 cells. These results clearly showed that the function of WT and S3-5 mutant integrin $\alpha 5$ on the EGFR of cell proliferation could be duplicated by the S3-5,11 and D11 mutants, respectively, which further highlighted the importance of site-11 N-glycosylation in the regulation of EGFR signaling. Therefore, we employed the S3-5,11 and D11 mutant HeLa cells in subsequent experiments to further clarify the underlying molecular mechanism.

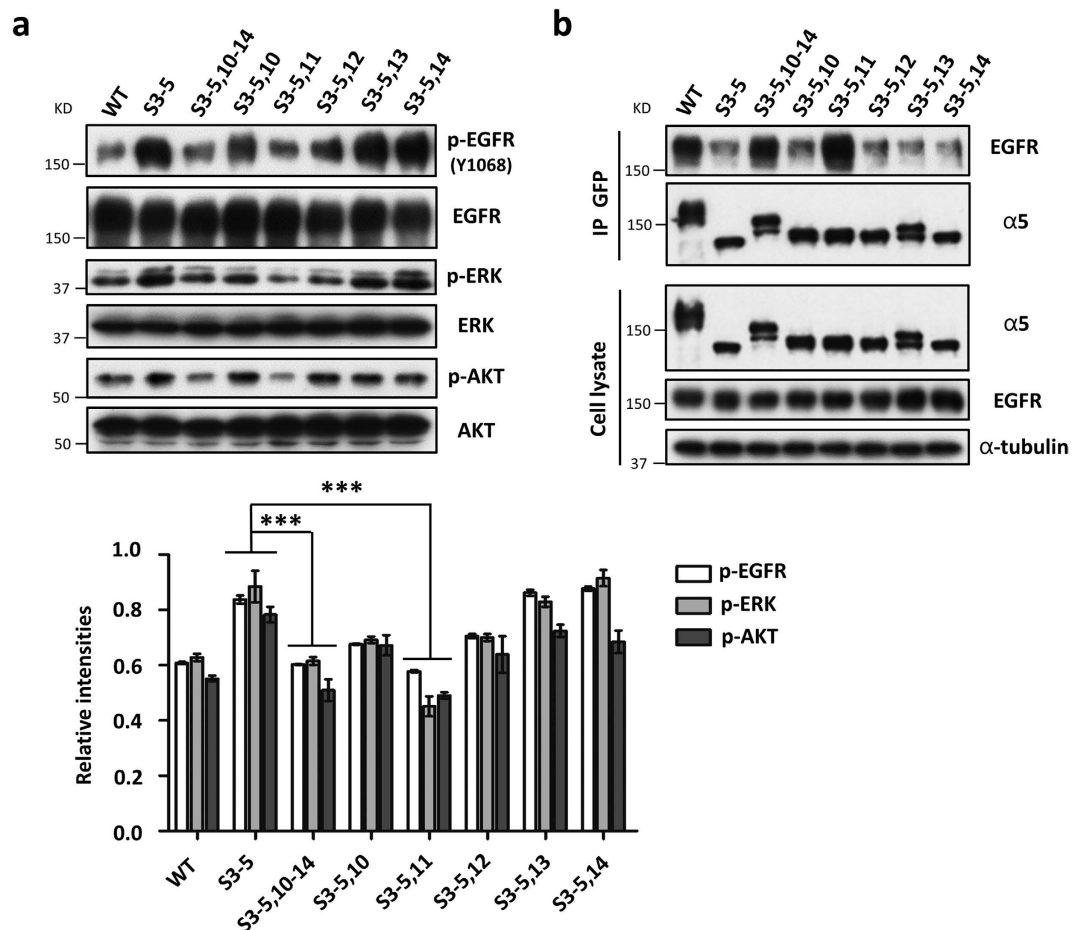


Figure 2. Comparison of the levels of phosphorylated EGFR, related cellular signaling, and $\alpha 5$ -EGFR complex formation among various unglycosylated $\alpha 5$ mutants in CHO-B2/EGFR cells. (a) The expression levels of phospho-EGFR and its downstream signaling were down-regulated in the S3-5,11 cells. Cell lysates from the indicated cells were subjected to western blot (WB) with indicated antibodies (top panel). The relative ratios (phospho-EGFR, phospho-ERK, and phospho-AKT versus EGFR, ERK, and AKT, respectively) were statistically analyzed (bottom panel, $n = 3$ individual experiments). (b) The interaction between integrin $\alpha 5$ and EGFR was increased in the S3-5,11 cells. The indicated cell extracts were immunoprecipitated (IP) with anti-GFP agarose, followed by anti-EGFR and $\alpha 5$ antibodies for WB (top panels). The whole cell extracts were also subjected to WB using indicated antibodies where α -tubulin was used to check equal loading (bottom panels; as an input). All values are reported as the means \pm S.E. (error bars), Student's t -test; *** $p < 0.001$.

Site-11 N-glycosylation inhibits EGFR dimerization in HeLa cells. As described above, the site-11 N-glycosylation of integrin $\alpha 5$ plays a crucial role in the inhibition of EGFR signaling. Next, we explored the underlying mechanisms involved in this phenomenon. Considering that the expression levels of EGFR (or EGF) and the response ability to EGF are important controlling factors for EGFR activation³³, we next asked whether the secretion ability of EGF in D11 cells differed from others, since the site-11 N-glycosylation did not affect the expression level of EGFR on the cell surface, as shown in Fig. 3b. The ELISA data showed similar expression levels of EGF secreted into the condition media among the WT, D11 and S3-5,11 cells (Fig. 4a), indicating that the increased EGFR signaling in D11 cells was not due to the EGF secretion step, so we assessed the effects of EGF in these cells. In contrast to WT or S3-5,11, the responses to EGF stimulation, as reflected by the expression levels of p-EGFR, p-ERK and p-AKT, were significantly increased in the D11 cells in a dose-dependent manner (Fig. 4b). These results suggest that EGFR activation can be suppressed in the presence of the N-glycosylation of site-11 in $\alpha 5$.

Given the evidence that the dimerization of EGFR is an initial and essential event in EGF-induced signal transduction³⁴, we compared EGFR dimerization in these three cell lines to further study the mechanism involved in site-11 N-glycosylation-mediated EGFR inhibition. As shown in Fig. 4c, upon EGF treatment, more EGFR dimers were visualized in D11 cells compared with either the WT or S3-5,11 cells. Collectively, these data suggested that the site-11 N-glycosylation of integrin $\alpha 5$ suppresses the dimerization of EGFR, resulting in an inhibition of EGFR-mediated signaling.

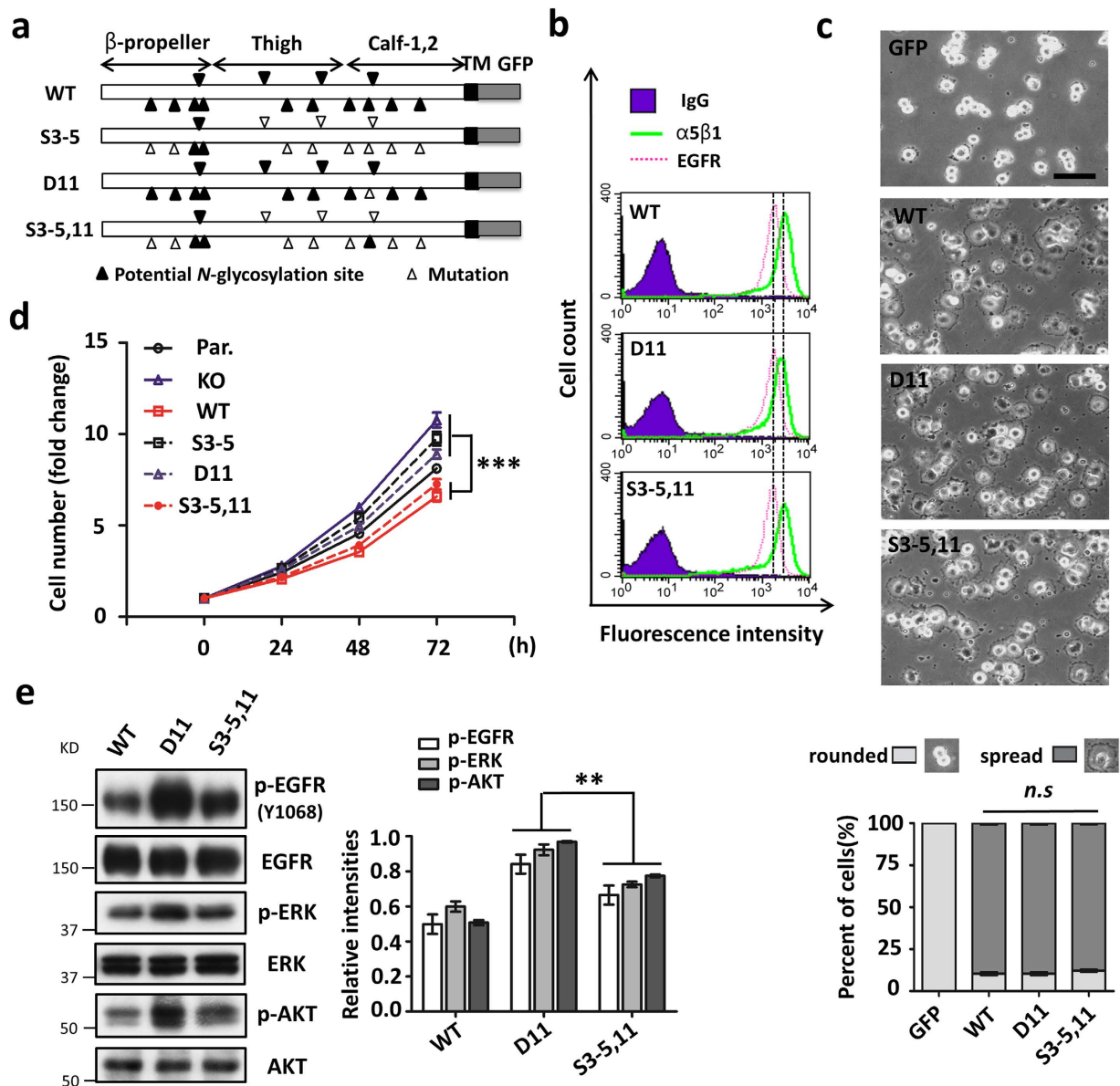


Figure 3. The site-11 N-glycosylation of integrin $\alpha 5$ plays a key role in mediating its growth-inhibitory function in HeLa cells. (a) Schematic diagram of a potential N-glycosylation mutational integrin $\alpha 5$ subunit (WT; S3-5; D11; and S3-5,11). (b,c) Comparison of expression levels of $\alpha 5\beta 1$ and EGFR on the cell surface (b) and cell spreading (c) among the WT; D11; and S3-5,11 cells. The stable rescued-HeLa cell lines were established as described under the “Methods” section. The expression levels of both $\alpha 5\beta 1$ and EGFR on the cell surface were analyzed via flow cytometry (b). The cell-spreading abilities of these cells were analyzed, as described in Fig. 1c ($n = 3$ individual experiments). The percentages of rounded and spread cells were statistically analyzed (c, bottom panel, $n = 9$). Scale bar, 120 μm . (d) The site-11 N-glycosylation suppressed cell growth. The abilities for cell growth were compared among the parent (Par.); integrin $\alpha 5$ -knock-out (KO); WT; S3-5; D11; and S3-5,11 HeLa cells, as described in Fig. 1d ($n = 3$ individual experiments). (e) The expression levels of phospho-EGFR and its downstream signaling were significantly increased in the D11, but not in the S3-5,11 cells compared with the WT. Left panel, WB pattern; right panel, quantitative analysis ($n = 3$ individual experiments). All values are reported as the means \pm S.E. (error bars), Student’s *t*-test; n.s, not significant ($p > 0.05$); ** $p < 0.01$; *** $p < 0.001$).

Loss of the N-glycosylation of site-11 switches the EGFR complex formation from $\alpha 5\beta 1$ to $\alpha 6\beta 4$. Next, we wondered how the site-11 N-glycosylation participates in the inhibition of EGFR signaling. Considering the notion that integrin $\alpha 5$ -mediated EGFR- $\alpha 5$ complex formation can restrict EGFR signaling¹¹, we checked the effects of the site-11 N-glycosylation of EGFR- $\alpha 5$ complex formation via immunoprecipitation experimentation. Reciprocal immunoprecipitates with anti-GFP agarose or anti-EGFR antibody showed that the interaction between integrin $\alpha 5$ and EGFR was significantly decreased in D11 cells, by comparison with the WT or S3-5,11 cells (Fig. 5a), indicating that site-11 N-glycosylation plays a key role in EGFR-integrin $\alpha 5\beta 1$ complex formation.

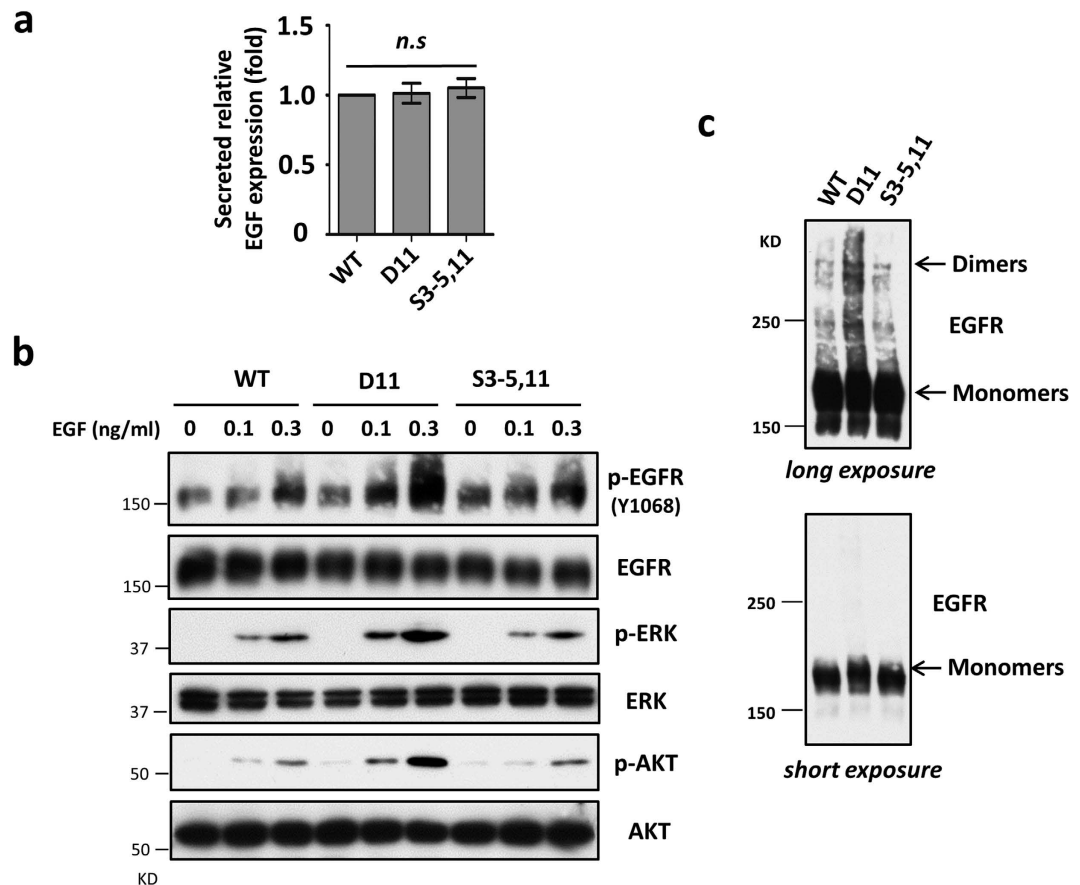


Figure 4. The site-11 *N*-glycosylation of integrin $\alpha 5$ inhibits EGFR dimerization and responses upon EGF stimulation in HeLa cells. **(a)** The WT; S3-5,11; and D11 cells exhibited secretion ability identical to that of EGF. EGF concentrations in the condition media were analyzed by EGF ELISA as described under the “Methods” section. The EGF concentrations in the D11 and S3-5,11 mutants were normalized to WT cells ($n = 3$ individual experiments). All values are the means \pm S.E. (error bars), Student’s *t*-test; n.s, not significant ($p > 0.05$). **(b)** The responses to EGF were decreased in S3-5,11, but not D11 cells, compared with WT. After starvation for 24 h, cells were treated with EGF at the indicated concentrations for 5 min. WB analysis was performed with indicated antibodies. **(c)** The EGFR dimerization abilities were decreased in the WT and S3-5,11 cells, compared with the D11 cells. After starvation for 24 h, cells were treated with EGF (0.1 ng/ml) for 5 min and subsequently cross-linked using 5 mM Sulfo-EGS as a chemical cross-linker on ice for 2 h and stopped with 10 mM Tris for 15 min. Cell lysates from those cells were subjected to WB to detect EGFR monomers and dimers under long exposure (top panels) and short exposure (bottom panels) conditions, respectively. EGFR monomers were used as a loading control.

Given the fact that the crosstalk between other integrins and EGFR is also important for EGFR signaling^{4,5,35,36}, we also detected an association between EGFR and other integrins, including integrin $\beta 4$ and $\alpha 3$, which are highly expressed in HeLa cells. We initially detected the expression levels of integrin $\beta 4$ and $\alpha 3$ in these cell lines. As shown in Fig. 5a,b, these cell lines exhibited the same expression levels of integrin $\beta 4$ and $\alpha 3$ in the whole-cell lysates (Fig. 5a, lower panels) and on the cell surface, as analyzed by flow cytometry (Fig. 5b), respectively, suggesting that the site-11 *N*-glycosylation had no effect on the expressions of integrin $\beta 4$ and $\alpha 3$. Interestingly, the interaction between EGFR and integrin $\beta 4$, but not $\alpha 3$, was significantly increased in the D11 cells compared with that in WT or S3-5,11 cells, which was opposite to the patterns observed in the case of $\alpha 5$ (Fig. 5a). These results indicate that the *N*-glycosylation of site-11 in integrin $\alpha 5$ mediated its association with EGFR, which may inhibit the EGFR-integrin $\beta 4$ complex formation that is known to promote EGFR-mediated signaling^{36,37}.

Integrin $\alpha 5$ and $\beta 4$ competitively associate with EGFR to regulate its signaling in controlling cell proliferation via the site-11 *N*-glycosylation of $\alpha 5$. Based on the opposite complex formation patterns of EGFR- $\alpha 5$ and EGFR- $\beta 4$ in the mutants described above, we hypothesized that the binding of EGFR to integrin $\beta 4$ may be suppressed by the competition from $\alpha 5$ expression. To test this idea, we initially overexpressed the GFP-tagged integrin $\alpha 5$ in the parent HeLa cells and then detected the effect on the EGFR-integrin $\beta 4$ complex formation. As shown in Fig. 6a, the interaction between integrin $\beta 4$ and EGFR was greatly increased in the $\alpha 5$ -KO cells, but was decreased in the $\alpha 5$ overexpression cells, which confirmed our hypothesis. Consistently, when cells proliferated on FN-coated dishes in the WT cells, the interaction between $\alpha 5$ and EGFR was increased,

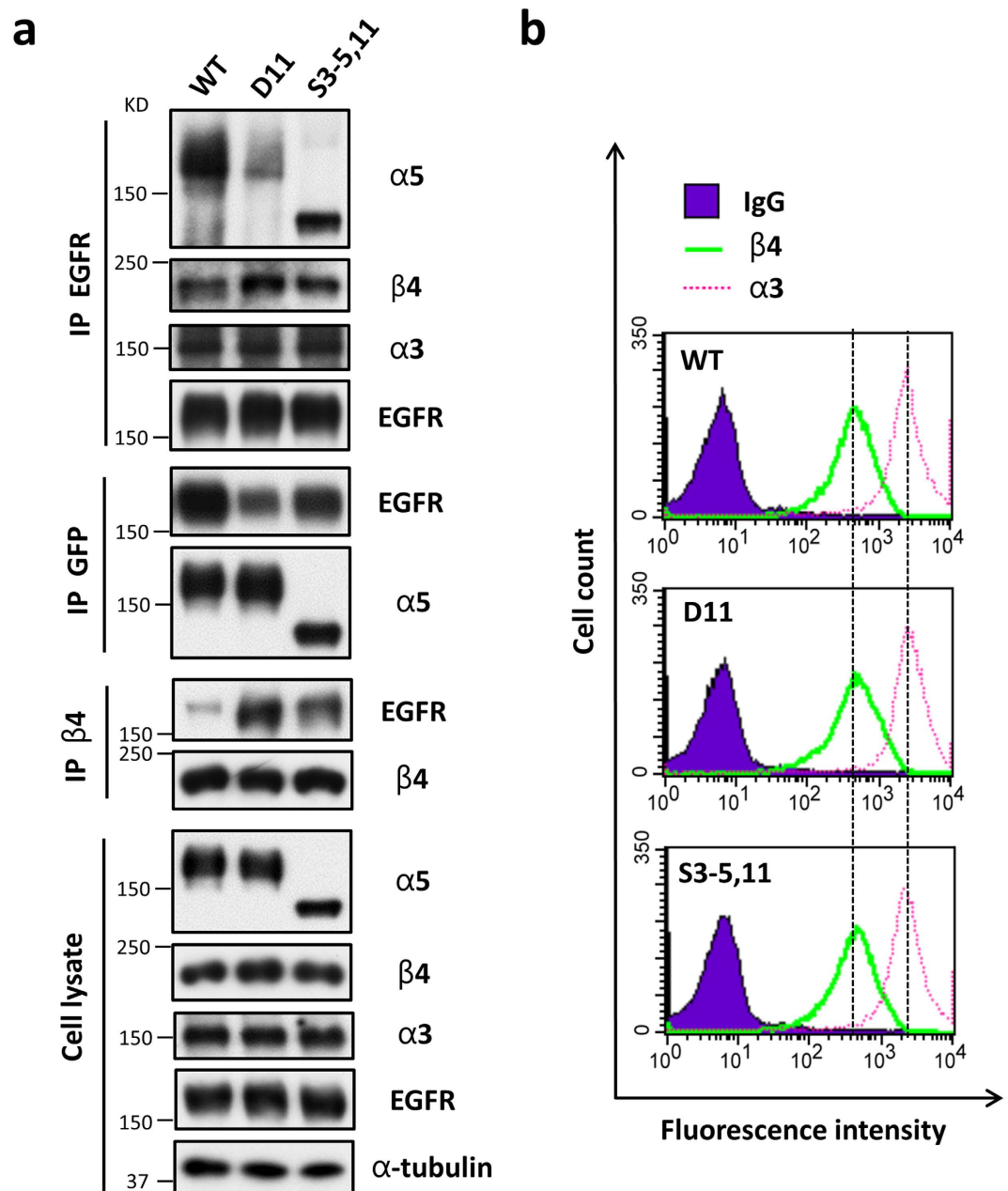


Figure 5. The site-11 *N*-glycosylation of integrin $\alpha 5$ inhibits the EGFR-integrin $\beta 4$ complex formation in HeLa cells. **(a)** Comparison of complex formations between EGFR- $\alpha 5$ and EGFR- $\beta 4$ among the WT; D11; and S3-5,11 cells. The indicated HeLa cell extracts were IP with anti-EGFR antibody (*top IP panels*), anti-GFP agarose (*middle IP panels*), or anti-integrin $\beta 4$ antibody (*bottom IP panels*), and then subjected to WB, reciprocally followed by anti-integrin $\alpha 5$, $\beta 4$, $\alpha 3$, or EGFR antibodies for detection. The whole cell extracts were also subjected to WB as an “input” using the indicated antibodies (*bottom panels*). **(b)** The WT; D11; and S3-5,11 cells exhibit the same expression level of integrin $\beta 4$ and $\alpha 3$ on the cell surface. The expression levels of both integrins $\beta 4$ and $\alpha 3$ were analyzed by flow cytometry. The IgG was used as a control.

but the EGFR-integrin $\beta 4$ association was accordingly decreased (Fig. 6b). In contrast with the changes observed in the WT cells, the associations of EGFR-integrin $\alpha 5$ and EGFR-integrin $\beta 4$ were, to some extent, increased and decreased, respectively, in the D11 cells upon FN treatment (Fig. 6b). Importantly, the association between EGFR-integrin $\beta 4$ was significantly increased in the D11 cells, compared with the WT. These data indicate that integrins $\alpha 5$ and $\beta 4$ competitively associate with EGFR via the *N*-glycosylation of site-11 in $\alpha 5$.

We then investigated whether the EGFR signaling for cell proliferation was also regulated in the interplay between the EGFR-integrin $\alpha 5$ and EGFR- $\beta 4$ complex formation. The EGFR-related cellular signaling and cell proliferation abilities were examined in WT and D11 cells grown on dishes coated with either FN or laminin-332 (LN-332), which is a specific ligand for integrin $\alpha 6\beta 4$. In the WT cells, the phosphorylation levels of EGFR and ERK (Fig. 6c), as well as cell proliferation (Fig. 6d), were significantly decreased upon FN treatment, but these

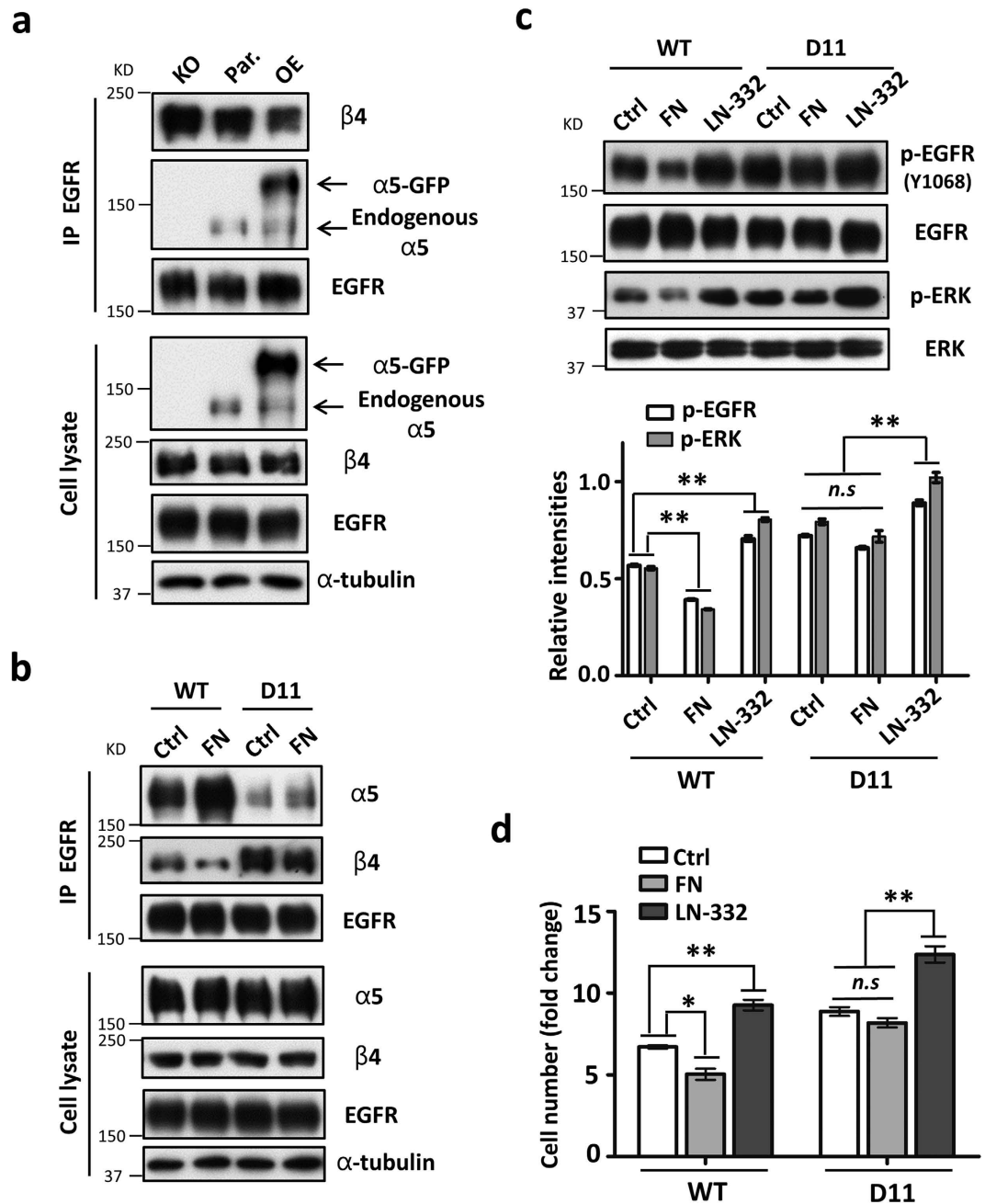


Figure 6. Integrin $\alpha 5$ and $\beta 4$ competitively associated with EGFR and regulated its-related cellular signaling via site-11 N-glycosylation of $\alpha 5$. (a) The competitive relationship between integrins $\alpha 5$ and $\beta 4$ to interact with EGFR in HeLa cells. The integrin $\alpha 5$ (GFP-tagged)-overexpressed HeLa stable cell line (OE) was established as described under the “Methods” section. The extracts from integrin $\alpha 5$ -knock-out (KO), parent (Par.), and OE HeLa cells were IP with anti-EGFR antibody followed by anti-integrin $\alpha 5$, $\beta 4$, and EGFR antibodies for WB (top panels). (b) The interaction patterns between EGFR and integrins $\beta 4$ or $\alpha 5$ in the WT and D11 HeLa cells spread on FN. The indicated cells were cultured on dishes pre-coated with or without 10 $\mu\text{g}/\text{ml}$ FN for 24 h. The resultant cell lysates (as an input; bottom panels) were directly blotted with indicated antibodies or IP with anti-EGFR antibody (top panels) and then blotted with anti-integrin $\alpha 5$, $\beta 4$, and EGFR antibodies. (c,d) Comparison of the phospho-EGFR and phospho-ERK expressions (c) and cell proliferation abilities (d) between the WT and D11 cells spread on FN or laminin-332 (LN-332). The WT and D11 cells were cultured on dishes pre-coated with or without 10 $\mu\text{g}/\text{ml}$ FN or 1 $\mu\text{g}/\text{ml}$ LN-332 for 24 h. The resultant cell lysates were subjected to WB with indicated antibodies (c, top panels), the relative ratios were statistically analyzed (c, bottom panels, $n = 3$ individual experiments). The analysis of cell growth was performed as described in the legend to Fig.1d, the related cell numbers at 72 h were counted and statistically analyzed (d, $n = 3$ individual experiments). All values are reported as the means \pm S.E. (error bars), Student’s *t*-test; n.s, not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$.

were significantly increased under LN-332 treatment. Compared with WT, the D11 cells expressed higher levels of EGFR signaling (Fig. 6c) and cell proliferation (Fig. 6d) upon their respective treatments. Of note, the D11 cells cultured on FN-coated dishes, showed no significant decreases in either EGFR signaling or cell proliferation, while the cells cultured on LN-332-coated dishes still exhibited significant increases in both EGFR signaling and cell proliferation, compared with those in the cells without coating. These data strongly suggested that site-11 *N*-glycosylation plays a crucial role in the EGFR-mediated cellular signaling in controlling cell proliferation. Collectively, these results clearly demonstrated that the *N*-glycosylation of site-11 on integrin $\alpha 5$ is the most important factor for the regulation of EGFR signaling through its complex formation with integrin $\alpha 5$ or $\beta 4$.

The importance of site-11 *N*-glycan structures in the EGFR- $\alpha 5$ complex formation. The question regarding *N*-glycosylation of site-11 on integrin $\alpha 5$ is why this is the most important for its-mediated EGFR signaling of cell proliferation. We considered whether the structure of site-11 *N*-glycan is different from other sites. Given the fact that GnT-III is an important glycosyltransferase that catalyzes the addition of the bisecting GlcNAc on *N*-glycan and plays critical roles in determining the structure of *N*-glycans since the bisecting GlcNAc suppresses further processing and elongation of *N*-glycans to form branching structures³⁸, we compared the bisecting GlcNAc structure patterns on S3-5,10; S3-5,11; S3-5,12; S3-5,13; and S3-5,14 $\alpha 5$ mutants. These $\alpha 5$ mutants were immunoprecipitated with anti-GFP antibody and then *N*-glycans were detected using E4-PHA and DSA lectin, which specifically recognizes bisecting GlcNAc and branched *N*-glycans, respectively. Interestingly, the E4-PHA reactivities of the $\alpha 5$ subunits (the upper bands), but not the $\beta 1$ subunit (the lower bands), were much weaker in S3-5,11 cells, as with the S3-5 cells, than those in other mutant cells (Fig. 7a). Conversely, the stronger reactivities of DSA on $\alpha 5$ were observed in the S3-5,11 cells (Fig. 7a). It is worth mentioning that although we detected no reactivity of DSA on the $\beta 1$ subunit, which is consistent with previous study³⁹, this may nonetheless suggest other important information. Consistently, reciprocal immunoprecipitates with E4-PHA agarose and L4-PHA agarose, which selectively recognizes $\beta 1,6$ -GlcNAc branching structures catalyzed by GnT-V, showed that the expression levels of GnT-III-modified- and GnT-V-modified- $\alpha 5$ were significantly decreased and increased in S3-5,11 cells, respectively (Fig. 7a). These results indicate that the site-11 *N*-glycan of $\alpha 5$ carries fewer bisecting GlcNAc structures, however, more branching structures compared with the other four *N*-glycosylation sites on the calf domain.

Therefore, we wondered whether an increase in the bisecting GlcNAc on the site-11 *N*-glycan of $\alpha 5$ decreases the EGFR-integrins complex formation. The GnT-III-overexpressed WT, S3-5, and S3-5,11 HeLa cells were established. The branching structures, as reflected by DSA lectin blot, were suppressed in the GnT-III-overexpressed cells (Fig. 7b, the lower panels), and this further highlights the competitive relationship between GnT-III and GnT-V. As expected, the reciprocal immunoprecipitation showed that the interactions of EGFR and $\alpha 5$ were decreased in both the GnT-III-overexpressed WT and S3-5,11 cells (Fig. 7b, the upper panels). The association of EGFR and $\alpha 5$ showed a marginal decrease after overexpression of GnT-III in S3-5 cells, which may suggest that other factors such as *N*-glycans of EGFR are also involved in the complex formation. It is noteworthy that the EGFR- $\beta 4$ complex formation was not changed in the WT or S3-5,11 cells, but were significantly decreased in S3-5 cells after GnT-III overexpression (Fig. 7b). One explanation for these observations is that the EGFR- $\beta 4$ complex formation can also be suppressed by bisecting GlcNAc structures²², which may neutralize the competitive role between $\alpha 5$ and $\beta 4$ to associate with EGFR as described above (Fig. 6), particularly in the WT and S3-5,11 cells. Taken together, these results strongly suggest that the site-11 *N*-glycan of $\alpha 5$ carries many more branching structures, which may promote the EGFR- $\alpha 5$ complex formation and suppress EGFR signaling for cell proliferation.

Discussion

In the present study, we intensively studied the effect that *N*-glycosylation of the calf domain in integrin $\alpha 5$ subunit exerted on EGFR signaling in controlling cell proliferation. We found that *N*-glycan of site-11, which contains abundant branching structures, is the most important aspect of its inhibitory effect on EGFR activation. The molecular mechanisms for the inhibition can be mainly ascribed to two aspects. One is that site-11 *N*-glycosylation promoted the interaction of integrin $\alpha 5\beta 1$ -EGFR, which may interfere with EGFR endocytosis, as described previously¹¹; the other is that this *N*-glycosylation suppressed the EGFR complex formation with integrin $\alpha 6\beta 4$, which is a novel regulatory mechanism for cellular signaling (Fig. 8).

Integrins are thought to crosstalk with EGFR, but an extensive amount of research has been focused on the role of individual integrin subunits and on the results of the activation/inactivation of the EGFR signaling. Previously, we identified integrin $\alpha 5$ as a tumor suppressor via the negative regulation of EGFR signaling¹¹. In the present study, we expanded on these observations and found that integrin $\alpha 6\beta 4$ is involved in the inhibitory effect of $\alpha 5$ on cell proliferation, because integrin $\alpha 5\beta 1$ and $\alpha 6\beta 4$ exhibited a mutual competition in the EGFR signaling for cell proliferation. In fact, integrin $\alpha 6\beta 4$ is widely thought to amplify RTK signaling, including EGFR and ErbB2, either via direct promotion of the Src family kinase-dependent phosphorylation of the P loop in the catalytic site of the RTK and RTK-bound substrates such as Gab1^{14,40}, or by indirect stimulation of the autocrine RTK pathways⁷. Consistently, our results also showed that the EGFR-related cellular signaling and cell proliferation were enhanced with increases in the EGFR- $\beta 4$ complex formation in the null site-11 *N*-glycosylation mutant. Although the interaction patterns between integrin $\alpha 3$ and EGFR showed no changes among those mutants, we could not exclude the possibility of EGFR signaling from other integrins such as $\alpha 1\beta 1$ ¹⁹ and $\alpha v\beta 3$ ⁴¹. Similar to the competition between integrins $\alpha 5\beta 1$ and $\alpha 6\beta 4$, the functional competition between integrins $\alpha M\beta 2$ and $\alpha 5\beta 1$ or integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ was also observed in controlling either leukocyte migration⁴² or trafficking⁴³, respectively. In addition, the interplay between integrins and tetraspanins is known to play a crucial role in cell

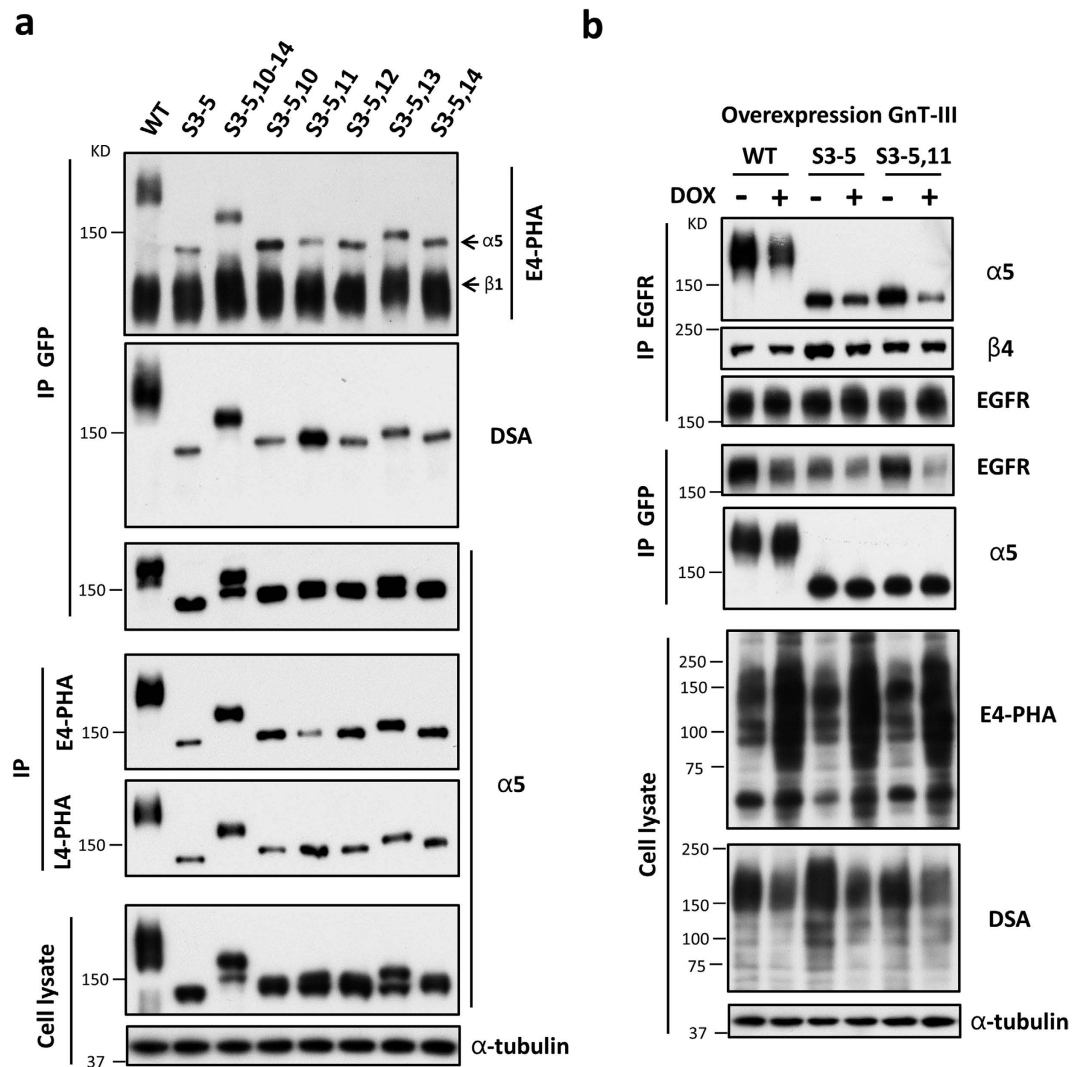


Figure 7. Comparison of *N*-glycosylation patterns on various unglycosylated $\alpha 5$ mutants by lectin blots and the effects of GnT-III overexpression on EGFR- $\alpha 5$ complex formation. (a) The cell lysates from WT and unglycosylated mutant CHO-B2/EGFR cells (as an input; *bottom panels*) were directly blotted with indicated antibodies or IP with anti-GFP agarose (*top panels*), E4-PHA-agarose or L4-PHA-agarose (*middle panels*), and then subjected to WB, reciprocally followed by E4-PHA and DSA lectins or anti-integrin $\alpha 5$ antibody for detection. (b) Comparison the effects of GnT-III on EGFR- $\alpha 5$ and EGFR- $\beta 4$ complex formation in WT, S3-5, and S3-5, 11 HeLa cells. The doxycycline (DOX)-inducible GnT-III overexpression stable cell lines were established as described under the “Methods” section. The indicated cells were cultured in the presence (+) or absence (–) with 1 μ g/ml doxycycline for 72 h, and the cell extracts were IP with anti-EGFR antibody (*top panels*) or anti-GFP agarose (*middle panels*), and then subjected to WB, reciprocally followed by anti-integrin $\alpha 5$, $\beta 4$, or EGFR antibodies for detection. The whole cell extracts were also subjected to WB as an “input” using anti- α -tubulin antibody or E4-PHA and DSA lectins (*bottom panels*).

physiology⁴⁴. All these data indicate that the functions of multiple integrins on EGFR are not redundant, and it highlights the importance of the cooperation between individual integrins in cell biology.

It is worth mentioning that integrin $\alpha 5\beta 1$ exerts controversial effects both on EGFR regulation^{11,13} and cell proliferation¹² ranging from stimulatory to inhibitory in different cell types, which is similar to the effects of integrin $\alpha 6\beta 4$ on cell growth^{14,15}. The information concerning the competition between $\alpha 5\beta 1$ and $\alpha 6\beta 4$ in the way they bind to EGFR, and how that might constitute an overwhelming effect may be one possible explanation for these controversies. In agreement with this hypothesis, our previous data showed that in the MDA-MB-231 and HeLa cells, which express relatively low and high levels of endogenous integrin $\alpha 6\beta 4$, respectively, the inhibition effect of integrin $\alpha 5\beta 1$ on EGFR was attenuated in MDA-MB-231 cells compared with HeLa cells¹¹. Although additional analyses is required to fully address the roles of integrin $\alpha 5\beta 1$ in EGFR inactivation in different cells, our data constitute a reasonable argument that the $\alpha 5\beta 1$ -mediated inhibition of EGFR is at least partially dependent on $\alpha 6\beta 4$.

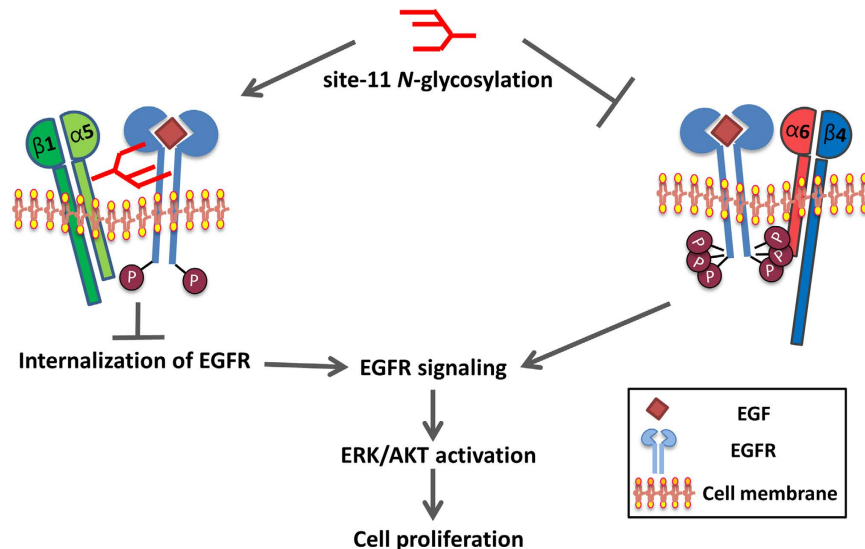


Figure 8. The proposed mechanism for the regulation of EGFR signaling and cell proliferation by the site-11 *N*-glycosylation of integrin $\alpha 5$. The site-11 *N*-glycosylation of integrin $\alpha 5$, which contains abundant branching structures, can serve as a “switch”, which can turn on the EGFR-integrin $\alpha 5\beta 1$ complex formation to restrict the EGFR internalization as described previously¹¹, resulting in an inhibition of EGFR-related cellular signaling for cell proliferation (left panel). Meanwhile, the *N*-glycosylation can also switch off the EGFR-integrin $\alpha 6\beta 4$ complex formation due to a mutual competition as described above, which is well known to lead to an activation of EGFR and its downstream signaling (right panel).

Of note, we further expanded our previous observations on the *N*-glycosylation of integrin $\alpha 5$ ^{11,32} and identified the site-11 *N*-glycosylation of the calf domain in $\alpha 5$ as the most important development in the interplay between integrin $\alpha 5\beta 1$ and $\alpha 6\beta 4$ binding with EGFR, which highlights the importance of the individual *N*-glycosylation of $\alpha 5$. Similarly, the importance of individual *N*-glycosylation has also been addressed in other integrin subunits such as $\beta 1$ ⁴⁵ or $\alpha 3$ ⁴⁶. In addition, a recent study demonstrated how *N*-glycosites and site-specific glycoforms of some secreted proteins in drug-resistant gastric cells are distinctly different from those in the parental versions⁴⁷, which indirectly further highlighted the significance of individual *N*-glycosylations. Considering these results, it is reasonable to speculate that an individual *N*-glycosylation site of glycoproteins may exhibit unique biological functions.

The next issue concerned the question of how *N*-glycosylation mediates the $\alpha 5$ -EGFR association. We previously demonstrated that the *N*-glycosylation of integrin $\alpha 5$ is important for the $\alpha 5$ -ganglioside GM1-EGFR complex formation and its localization in lipid rafts¹¹, indicating the significance of the gangliosides in their interaction. Similar to integrin $\alpha 5$, we previously also identified that *N*-glycosylation of integrin $\beta 4$ is essential for the EGFR- $\beta 4$ complex formation and localization in lipid rafts⁴⁸. Additionally, gangliosides GM3 and GD3, can interact with the *N*-glycans of EGFR, the so-called cis-carbohydrate to carbohydrate interaction⁴⁹. Therefore, it is reasonable to speculate that some glycolipids may act as a “linker” in the lipid raft for interaction between the *N*-glycosylation of $\alpha 5$ and the *N*-glycan from EGFR. Considering the complexity of the lipid rafts, other compositions such as cholesterol and galectins also can be factors linking $\alpha 5$ and EGFR. In fact, galectin-3 could mediate the association between integrin $\beta 4$ and EGFR in a GnT-III-dependent manner²². Consistently, here we identified that introduction of the bisecting GlcNAc structure modified by GnT-III suppressed the $\alpha 5$ -EGFR complex formation, which further indicates that the galectin-3 may also mediate the EGFR- $\alpha 5$ association. Taken together, these data provide evidence for the indirect binding of integrins and EGFR in lipid rafts via specific *N*-glycan structures of integrins. Therefore, it is plausible that the localization patterns of the $\alpha 5$ -EGFR and $\beta 4$ -EGFR complexes in lipid rafts may also be controlled by site-11 *N*-glycosylation. In addition to these indirect interactions, we could not exclude the possibility that an unknown lectin-like domain may exist on EGFR, since the lectin domain on integrin $\alpha M\beta 2$ is known to play an important role in its association with GlcNAc on the non-reducing terminus of the sugar chains on platelets⁵⁰.

Finally, it is important to further understand why site-11 *N*-glycosylation is the most suitable for the integrin $\alpha 5$ -EGFR complex formation of the five putative *N*-glycosylation sites on the calf domain. Although the underlying molecular mechanisms remain unclear, we feel justified in making three speculations based on our observations in the present study. First, the site-11 *N*-glycosylation may localize at a key functional region of $\alpha 5$, which can regulate integrin activation. In fact, introducing an *N*-glycosylation site into integrin $\beta 3$ or $\beta 1$ is known to open the hybrid-1-like domain interface of either $\alpha v\beta 3$ or $\alpha 5\beta 1$, and increase the ligand-binding affinity⁵¹. In addition, based on molecular modeling, the *N*-glycans surrounding the RGD-binding pocket on integrin $\alpha 5$ are believed to be essential for its binding to FN⁵², which highlights the importance of the *N*-glycosylation of integrins. Second, the site-11 *N*-glycosylation of $\alpha 5$ may exhibit an appropriate distance to interact with other molecules such as gangliosides GM1 and GM3, which also contributes to the $\alpha 5$ -EGFR complex formation described above. Third, the site-11 *N*-glycosylation may exhibit a unique glycan epitope that promotes $\alpha 5$ -EGFR complex formation,

such as the abundant branching structures observed on site-11 *N*-glycan in this study. Similarly, we previously found that the site-4 *N*-glycan on integrin $\alpha 5$ was specifically modified by GnT-III, which plays a key role in its mediation of cell spreading and migration³⁹. Furthermore, the site-heterogeneity of *N*-glycans has also been observed on several other proteins, such as immunoglobulin family proteins⁵³. Collectively, these results suggest that both the position and structure of *N*-glycan may play a crucial role in controlling its mediated biological functions. Further studies are required to confirm these ideas.

In summary, this study was particularly focused on the *N*-glycosylations on the calf domain of the integrin $\alpha 5$ subunit. One *N*-glycosylation of site-11 was identified as an “on-and-off” switch that controls EGFR binding to integrin $\alpha 5\beta 1$ and $\alpha 6\beta 4$, which may provide a new perspective on the interplay between integrins and GFRs. In addition, we also highlighted the suppressive effects of GnT-III on regulating the site-11 *N*-glycan-mediated EGFR- $\alpha 5$ complex formation, which may be a plausible molecular mechanism involved in GnT-III-mediated tumorigenesis in several tumors such as human hepatomas, glioma, and ovarian cancers^{54–56}.

Methods

Antibodies and reagents. The experiments were performed using the following antibodies: monoclonal antibodies (mAbs) against integrin $\alpha 5$ (610634) and integrin $\beta 4$ (611233) were obtained from BD Biosciences; the mAbs against human integrin $\alpha 5\beta 1$ (MAB1999) and integrin $\beta 4$ (MAB2059) were from Millipore; the mouse pAbs to EGFR (Sc-120), integrin $\alpha 3$ (Sc-6592), or integrin $\alpha 3$ (Sc-32237) and the rabbit pAbs to integrin $\beta 4$ (Sc-9090) or EGFR (Sc-03) were from Santa Cruz Biotechnology; rabbit mAbs to EGFR (catalog no. 4267), p-EGFR (catalog no. 3777), ERK1/2 (catalog no. 9102), p-ERK1/2 (catalog no. 4370), AKT (catalog no. 9272), and p-AKT (catalog no. 4060) were from Cell Signaling Technology; and, the mAb against α -tubulin was from Sigma. Biotinylated erythroagglutinating phytohemagglutinin (E4-PHA) and Datura stramonium agglutinin (DSA) lectins were obtained from Seikagaku Kogyo Inc. (Tokyo, Japan). Alexa Fluor[®] 647 goat anti-mouse IgG was obtained from Invitrogen (Life Technologies). The peroxidase-conjugate goat against mouse, donkey against goat, and goat against rabbit IgG antibodies were obtained from Chemicon, Santa Cruz Biotechnology, and Cell Signaling Technology, respectively. The fibronectin (FN) and laminin-332 (LN-332) were from sigma and Oriental Yeast Co., Ltd. (Tokyo, Japan), respectively; the EGF (AF-100) was from PeproTech; the control mouse IgG1 was from TONBO biosciences; and the Sulfo-EGS Biotin was from Thermo Fisher Scientific. The agarose-conjugated anti-green fluorescent protein (GFP) antibody (RQ2) and the Streptavidin-conjugated agarose were obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) and Millipore, respectively. The biotinylated-conjugated erythro-agglutinating phytohemagglutinin (E4-PHA)-agarose (J311) and leucoagglutinating phytohemagglutinin (L4-PHA)-agarose (J312) were obtained from J-OILMILLS (Tokyo, Japan). The Quantikine[®] Human EGF Immunoassay kit was obtained from R&D Systems.

Cell lines and cell culture. The 293T (for the lentivirus production) and HeLa cell lines were provided by the RIKEN cell bank (Japan). The stable EGFR overexpressed CHO-B2 cell line (CHO-B2/EGFR) and integrin $\alpha 5$ -KO HeLa cells were previously established in our laboratory¹¹. The other stable cell lines used in this study were established as mentioned below. All cell lines were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), under a humidified atmosphere containing 5% CO₂, except for the virus production.

Integrin $\alpha 5$ and GnT-III expression vectors. The vectors of GFP-tagged-WT; S3–5; and S3–5,10–14 integrin $\alpha 5$ were previously established in our laboratory³². The mutation vectors (S3–5,10; S3–5,11; S3–5,12; S3–5,13; S3–5,14; and, D11) were constructed using a site-directed mutagenesis kit (Takara Bio) according to the manufacturer's instructions. The resultant cDNAs were sequenced to confirm the presence of the desired mutations. We used a Gateway[™] cloning System kit (Thermo Fisher Scientific) to acquire all the expression vectors. Briefly, the LR clonase enzyme was used to transfer the cDNAs of integrin $\alpha 5$ from the entry vectors into CSII-CMV-Rfa (for rescuing the related- $\alpha 5$ in CHO-B2/EGFR cells, kindly provided by Dr. H. Miyoshi, Riken, Tokyo, Japan) and CSII-EF-Rfa (for rescuing the related- $\alpha 5$ in $\alpha 5$ -KO HeLa cells or overexpressing $\alpha 5$ in parent HeLa cells). The doxycycline (DOX)-inducible GnT-III-overexpressing lentiviral vectors (CSIV-TRE-Rfa-CMV-KT-GnT-III) was previously established in our laboratory²⁸.

Virus production and infection. The virus production and infection was performed as described previously¹¹. In brief, the CSII-CMV-Rfa (or CSII-EF-Rfa)-based integrin $\alpha 5$ - and CSIV-TRE-Rfa-CMV-KT-GnT-III- lentivirus vectors were cotransfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T cells. After transfection for 48 h, the lentivirus supernatants were collected. For the integrin $\alpha 5$ -overexpression stable cell lines establishment, the CHO-B2/EGFR, $\alpha 5$ -KO HeLa, or parent HeLa cells were infected with the related CSII-CMV-Rfa or -CSII-EF-Rfa lentivirus. The GFP-positive cells were sorted 3 times using FACS Aria II after infection for 72 h. For the GnT-III-overexpression stable cell line establishment, the infected cells were also selected 3 times by the Kusabira Orange marker using FACS Aria II. The stable cell lines were used in subsequent studies. The expression of GnT-III was induced by addition of 1 μ g/ml DOX, and the cells cultured under DOX-free medium were used as the control.

Western blot (WB) and immunoprecipitation (IP). For WB, the indicated cells were washed with ice-cold PBS and then lysed in the cell lysate (TBS (20 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 1% Triton X-100) with protease and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan) for 30 min. After centrifugation at 1,000 \times g for 10 min, the supernatant was collected and protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific). The protein lysates were resolved by non-reducing SDS-PAGE for integrin $\alpha 5$ or reducing SDS-PAGE for other proteins. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) and detected with indicated primary and secondary antibodies

or with biotinylated lectins as indicated and a Vectastain ABC kit (Vector Laboratories) using an Immobilon Western Chemiluminescent HRP Substrate (Millipore), according to the manufacturer's instructions. For IPs, cells were lysed with 20 mM TBS buffer without detergent by being passed through a 21-G needle, as described previously⁵⁷. Briefly, cells were resuspended in the TBS with protease and phosphatase inhibitors and lysed by being passed through a 21-G needle 30 times. After centrifugation at $1,000 \times g$ for 10 min, the supernatant was collected. The remaining pellet was again syringed and centrifuged, and the second postnuclear supernatant was combined with the first. The supernatants were immunoprecipitated with anti-GFP-agarose, E4-PHA-agarose, L4-PHA-agarose, anti-EGFR (Sc-03), or anti-integrin $\beta 4$ (Sc-9090) antibody and Ab-Capcher Protein A-R28 agarose (Protenova, Tokushima, Japan) for 1 h at 4 °C with rotation, then the immunoprecipitates were washed twice with TBS and subjected to 6% SDS-PAGE.

Cell growth analysis. To assay the cell growth curves, the cells (3×10^4 or 1×10^4) were seeded in FN (10 $\mu\text{g}/\text{ml}$) pre-coated 6-cm dishes or LN-332 (1 $\mu\text{g}/\text{ml}$) pre-coated 24-well plates overnight and then serum-starved for 24 h. After starvation, the cells were supplied with DMEM containing 10% FBS. The photos of the same areas on the cultured dishes were taken at the indicated times (0, 24, 48, and 72 h), and the cell numbers were counted and normalized to those at 0 h for statistical analysis.

Flow cytometry analysis of cells. Flow cytometric analysis was performed as described previously⁴⁵. Briefly, semi-confluent indicated cells were detached from the 10-cm culture dishes and subsequently stained with either the mouse IgG or primary mouse anti- $\alpha 5\beta 1$, anti-EGFR (Sc-120), anti- $\alpha 3$ (Sc-32237), or anti- $\beta 4$ (MAB2059) antibody for 1 h on ice, followed by incubation with Alexa Fluor[®] 647 goat anti-mouse IgG for 1 h. During incubation, the cells were mixed gently every 10 min by flicking. After incubation, cells were washed 3 times with ice-cold PBS, and then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Cell-spreading assay. The cell-spreading assays were performed as described previously with minor modifications³². Briefly, 6-well plates were coated with FN (10 $\mu\text{g}/\text{ml}$) in PBS overnight at 4 °C and then blocked with 1% bovine serum albumin (BSA) in DMEM for 1 h at 37 °C. The indicated cells were detached and suspended in serum-free DMEM with 0.1% BSA at 3×10^4 cells/ml. After replating on the FN-coated dishes for 20 minutes, non-adherent cells were removed by washing with PBS, and the attached cells were fixed with 4% paraformaldehyde in PBS, and representative photos were then taken by phase contrast microscopy. The ratios of the rounded, spread, or elongated cells were statistically analyzed.

ELISA. The indicated cells were grown on 6-well plates for 24 h and then incubated with 0.5 ml serum-free media. After incubation for 72 h, the media was collected and stored at -80°C until assay. The concentration of EGF in each group medium was assayed using a Quantikine[®] Human EGF Immunoassay kit, according to a procedure described by the manufacturer.

Chemical cross-linking of EGFR. To assay the dimerization of EGFR, cells were prestarved for 24 h and then treated with EGF (0.1 ng/ml) for 5 min. After stimulation, cells were immediately washed twice with ice-cold PBS and subsequently cross-linked with 5 mM Sulfo-EGS dissolved in PBS on ice for 2 h and then stopped using 10 mM Tris for 15 min. Finally, the cells were solubilized with lysis buffer and subjected to 5% SDS-PAGE, as described above, to detect both EGFR monomers and dimers. Dimers were visualized as >300 kD bands, in which monomers served as the loading control.

Statistical analysis. Results are reported as the mean \pm S.E. Statistical analyses were performed using a Student's *t* test and GraphPad Prism version 5. Statistical significance was defined as $p < 0.05$ (not significant (*n.s.*), $p > 0.05$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

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Author Contributions

Q.H. performed all the experiments with the help of T.I., T.F., Y.Z., S.H., Q.H. and T.I. constructed the virus expression and the $\alpha 5$ -knock-out vectors. Q.H., T.F., Y.Z. and S.H. did the cell-sorting experiments and constructed the related-stable cell lines. J.G. designed the experiment. Q.H. and J.G. analyzed the data, prepared the figures and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional Information

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