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The importance of N-glycosylation on β_3 integrin ligand binding and conformational regulation

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N-glycosylations can regulate the adhesive function of integrins. Great variations in both the number and distribution of N-glycosylation sites are found in the 18 α and 8 β integrin subunits. Crystal structures of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ have resolved the precise structural location of each N-glycan site, but the structural consequences of individual N-glycan site on integrin activation remain unclear. By site-directed mutagenesis and structure-guided analyses, we dissected the function of individual N-glycan sites in β_3 integrin activation. We found that the N-glycan site, β_3-N320 at the headpiece and leg domain interface positively regulates $\alpha_{IIb}\beta_3$ but not $\alpha_V\beta_3$ activation. The β_3-N559 N-glycan at the β_3-I-EGF3 and α_{IIb}-calf-1 domain interface, and the β_3-N654 N-glycan at the β_3-β-tail and α_{IIb}-calf-2 domain interface positively regulate the activation of both $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins. In contrast, removal of the β_3-N371 N-glycan near the β_3 hybrid and I-EGF3 interface, or the β_3-N452 N-glycan at the I-EGF1 domain rendered β_3 integrin more active than the wild type. We identified one unique N-glycan at the βI domain of β_1 subunit that negatively regulates $\alpha_5\beta_1$ activation. Our study suggests that the bulky N-glycans influence the large-scale conformational rearrangement by potentially stabilizing or destabilizing the domain interfaces of integrin.

Glycosylation not only adds extra molecular mass to a protein and helps maintain protein stability, folding, and solubility, but also contributes to another level of structural and functional diversity[1,2]. The attachment of carbohydrate moieties to the amide nitrogen of asparagine (Asn, N) residue, a process named N-linked glycosylation, is one of the most abundant post-translational modifications of protein[2,3]. It has been widely appreciated that protein N-glycans play important roles in many cellular processes such as cell adhesion and migration by modulating the function of cell adhesion molecules including integrins[4–10]. Aberrant N-glycosylations have been observed under pathological conditions such as inflammation and cancer progression and metastasis[4,11–17], underscoring the importance of understanding the molecular function of N-glycans.

Integrins are α/β heterodimeric cell surface glycoproteins that mediate a wide range of biological functions such as development, immune response, and blood clotting[18]. The combination of 18 α and 8 β subunits results in 24 integrin members in human[18] (Fig. 1). Each subunit of integrin contains a large extracellular domain with multiple subdomains, a single transmembrane domain and generally a short cytoplasmic domain. The integrin extracellular domain can be divided into the headpiece and the leg domains (Fig. 1A). Recent structural and functional studies have revealed that integrins can undergo a transition from a bent conformation in the resting state to an extended conformation in the active state as a result of the headpiece extension, headpiece opening and leg domain separation[19]. Such long-range conformational rearrangements are critical for the upregulation of integrin affinity to bind the extracellular ligands[19]. Both α and β integrin subunits are the major carriers of N-glycans (Fig. 1). The importance of integrin N-glycans has been evidenced by the functional effects on integrin expression, cell adhesion, spreading and migration upon the loss or gain of N-glycan sites or the changes in N-glycan contents[8,14,20–23]. Given the large-scale conformational changes of integrin and the bulky N-glycans attached to the moving domains of integrin, it is tempting to speculate that the N-glycans might influence the structural changes and thus the activation of integrins. In line with this possibility, a recent study on EGF receptor (EGFR) demonstrated that the N-glycosylation is critical for the ectodomain conformational rearrangement and

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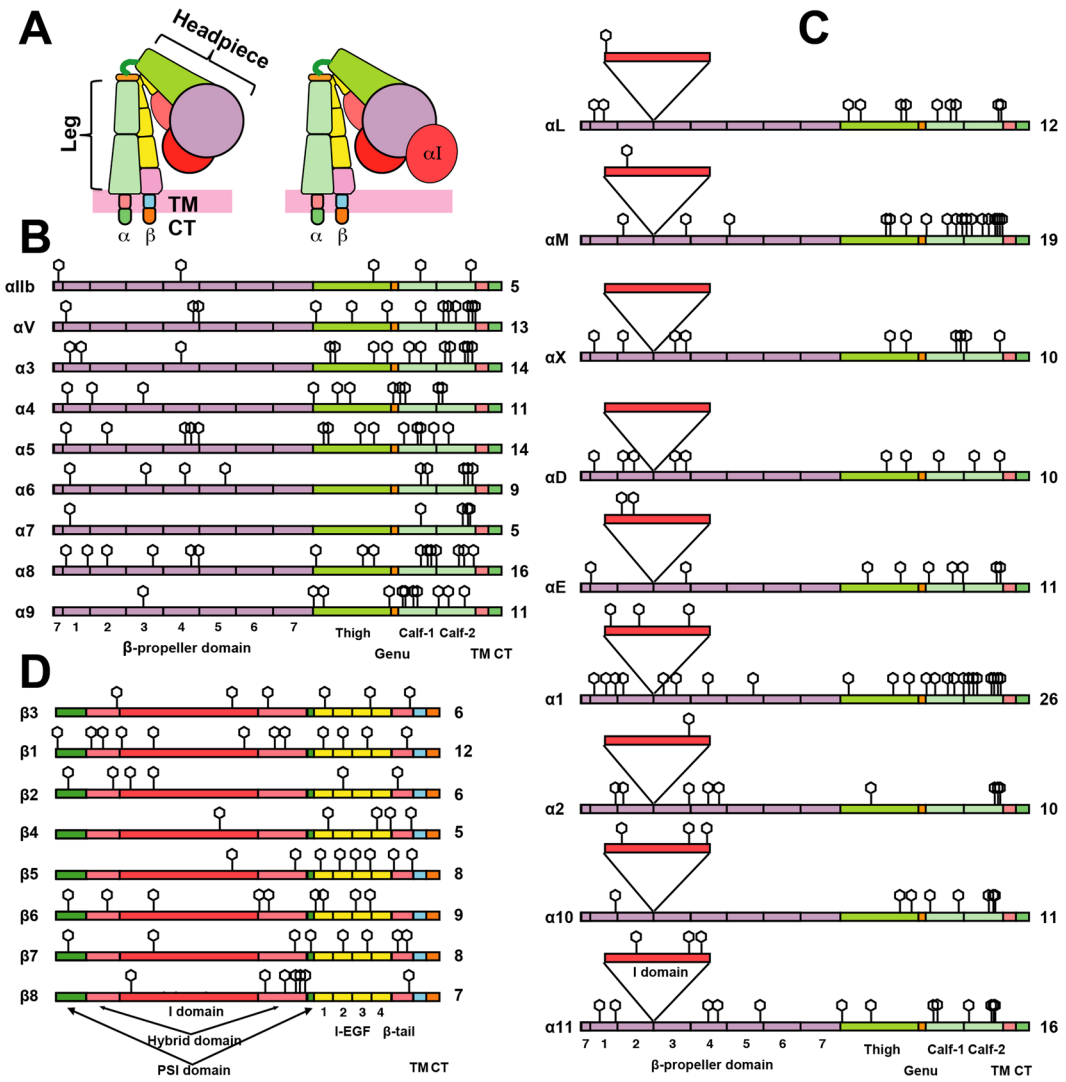

Figure 1. Integrin structure and N-linked glycosylation. **(A)** Cartoon models of integrin in the bent conformation. The domains are color-coded as same as panels **B**–**D**. **(B**,**C)** The distribution of potential N-glycan sites in the integrin α subunits without (**B**) or with (**C**) the αI-domain. The 7 blades of β-propeller domain are labeled. **(D)** The distribution of potential N-glycan sites in the integrin β subunits. The numbers of predicted N-linked glycosylation sites are shown on the right for each integrin subunit.

its orientation relative to the cell membrane[24]. However, how the individual N-glycan regulates integrin conformation and ligand binding has not been well studied.

Among the integrin family, the $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins have been very well characterized both structurally and functionally[25–30]. $\alpha_{IIb}\beta_3$ is essential for platelet-mediated hemostasis and thrombosis[31,32], while $\alpha_V\beta_3$ is important in tumor angiogenesis, metastasis, and inflammation[33,34]. It has been reported that the $\alpha_V\beta_3$ glycosylation differs significantly between primary and metastatic melanoma cells[14]. Although the glycan structures are shaved and only partially resolved in the crystal structures of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$, the precise location of each individual N-glycan site can be readily defined. Many of these N-glycan sites lie in the domain interfaces that will be rearranged or disrupted from the bent to the extended conformational transition during integrin activation. The goal of this study is to investigate the effect of loss of individual N-glycan sites on β_3 integrin activation and relate the function of the N-glycan sites to their structure location. We also extended our study to $\alpha_5\beta_1$ integrin and identified one N-glycan site of β_1 subunit that negatively regulates $\alpha_5\beta_1$ ligand binding.

Results

Distribution of the N-linked glycans on integrins. The potential N-linked glycosylation sites of integrins were predicted based on the presence of the consensus NXT/S sequons (X is any amino acids except proline). As shown in Fig. 1, the putative N-linked glycosylation sites are distributed among almost all the extracellular subdomains of both α and β subunits within the headpiece and leg domains (Fig. 1). Of the 18 human α integrins, half of them contain an extra ligand-binding αI (inserted) domain (Fig. 1A–C). The numbers of N-linked glycosylation sites range from 5 (α_{IIb} and α_7) to 16 (α_8) among the αI-less α-subunits (Fig. 1B), and from 10 (α_2,

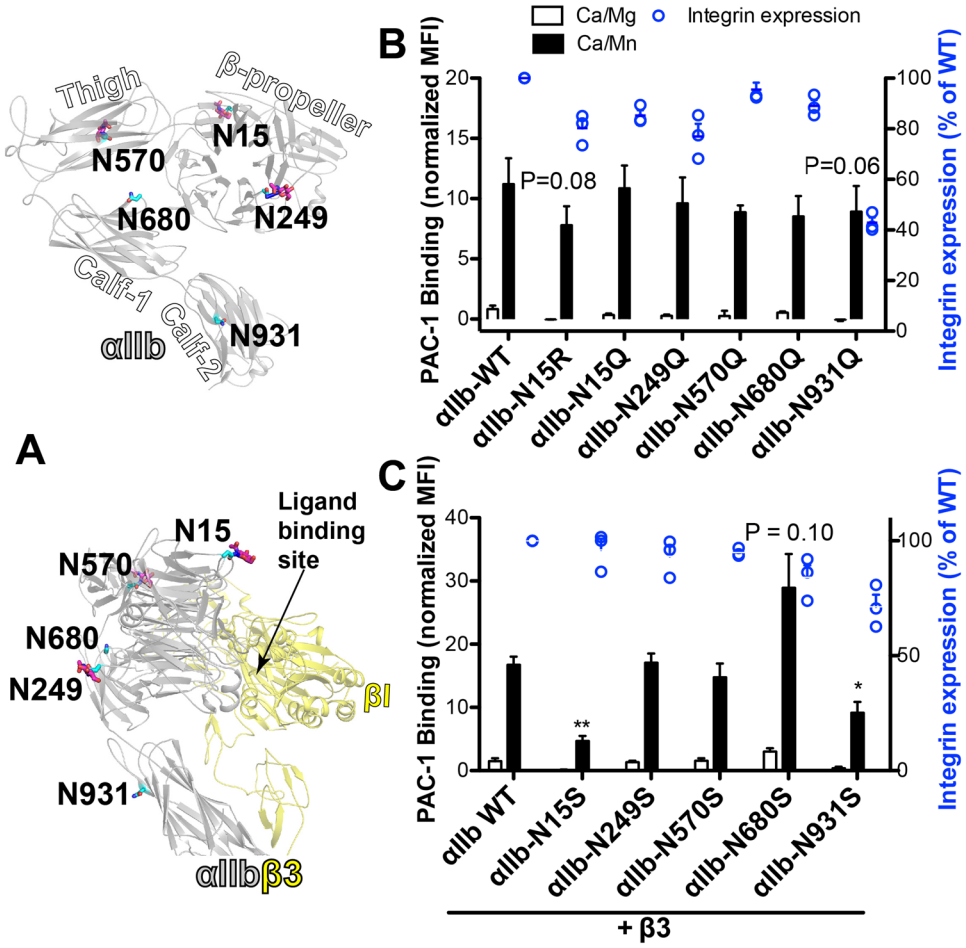

Figure 2. Effect of the individual N-glycan deletion of α_{IIb} subunit on $\alpha_{IIb}\beta_3$ ligand binding. **(A)** Locations of α_{IIb} N-glycan sites in the crystal structure of $\alpha_{IIb}\beta_3$ (PDB code 3FCS) at the bent conformation. Asn residues are shown as sticks with carbons in cyan. N-glycan residues resolved in the crystal structure are shown as sticks with carbons in magenta. Oxygens and nitrogens are red and blue, respectively. It should be noted that the N-glycan residues visualized in the crystal structure are trimmed ones. The native N-glycan chains could be much longer and more complex. **(B,C)** Ligand-mimetic mAb PAC-1 binding of the indicated single mutations of α_{IIb} co-expressed with β_3 in HEK293FT cells in the presence of 1 mM Ca^{2+}/ Mg^{2+} (Ca/Mg) or 0.2 mM Ca^{2+} plus 2 mM Mn^{2+} (Ca/Mn). PAC-1 binding was measured by flow cytometry and presented as mean fluorescence intensity (MFI) normalized to integrin expression (AP3 binding). Data are means ± s.e.m. ($n \geq 3$). Two-tailed t-tests were used to compare the wild type (WT) and the mutants in the Ca/Mn condition. $^*P < 0.05$; $^{**}P < 0.01$.

α_D and α_X) to 26 (α_1) among the αI-containing α-subunits (Fig. 1C). The β-subunits have relatively less N-glycan sites compared with the α-subunits, ranging from 5 (β_4) to 12 (β_1) sites (Fig. 1D). The locations, as well as the numbers of N-glycan sites, vary substantially even within the same subdomains among both α and β subunits. Interestingly, the N-glycan sites are mostly abundant in the leg domains of both α and β subunits, especially in the calf-1 and calf-2 domains of α-subunits (Fig. 1B,C). The α_M and α_1 subunits have the most abundant N-glycans (12 sites) in their calf-1 and -2 domains (Fig.1C). Some of the N-glycan sites, such as the ones adjacent to the transmembrane domains of both α and β subunits are relatively conserved (Fig. 1B–D).

Effect of the loss of individual N-glycan sites of α_{IIb} subunit on $\alpha_{IIb}\beta_3$ integrin expression and ligand binding. $\alpha_{IIb}\beta_3$ integrin has been used as a prototype in understanding integrin structure and function[32]. The α_{IIb} subunit has 5 predicted N-glycan sites: two in the β-propeller domain, one in the thigh domain, one in the calf-1 domain and one in the calf-2 domain (Fig 2A). Three of them have been visualized in the crystal structure (Fig. 2A). We removed the individual N-glycan site of α_{IIb} subunit one by one by the glutamine substitution. Each Asn to Gln mutant of α_{IIb} was co-expressed with wild-type β_3 subunit in HEK293FT cells. The $\alpha_{IIb}\beta_3$ integrin activation was measured by the binding of ligand-mimetic mAb PAC-1 in the physiological Ca^{2+}/ Mg^{2+} condition or in the external integrin activator, Mn^{2+}. Overall, the individual Asn to Gln substitution of α_{IIb} subunit had no significant effect on PAC-1 binding to $\alpha_{IIb}\beta_3$ when activated by Mn^{2+} (Fig. 2B). The α_{IIb}-N931Q mutation slightly reduced PAC-1 binding (Fig. 2B). Compared with the wild-type α_{IIb} subunit, the cell surface

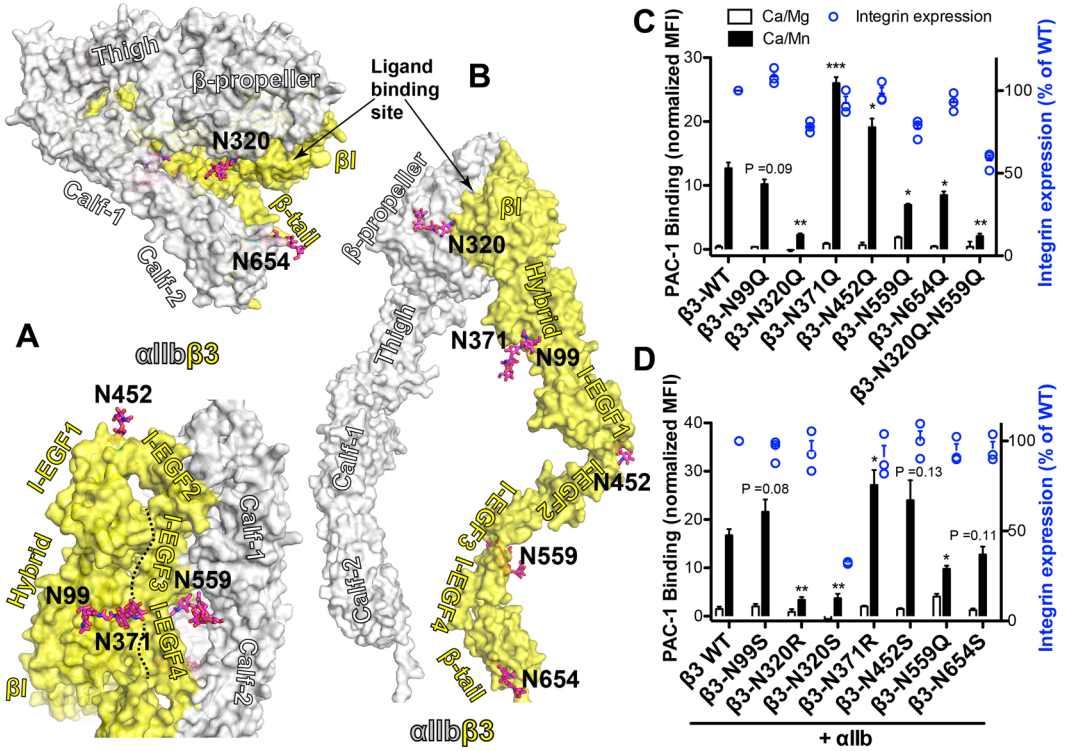

Figure 3. Effect of the individual N-glycan deletion of β_3 subunit on $\alpha_{IIb}\beta_3$ ligand binding. **(A)** Locations of the β_3 N-glycan sites in the crystal structure of $\alpha_{IIb}\beta_3$ (PDB code 3FCS) at the bent conformation shown as the solvent accessible surface in two views. The boundary of the hybrid/I-EGF3-4 domain interface is indicated as a black dotted line. **(B)** Model of the high affinity extended conformation of $\alpha_{IIb}\beta_3$. Asn residues are shown as sticks with carbons in cyan. N-glycan groups resolved in the crystal structure are shown as sticks with carbons, oxygens, and nitrogens in magenta, red, and blue, respectively. **(C,D)** Ligand-mimetic mAb PAC-1 binding of the indicated single mutations of β_3 co-expressed with α_{IIb} in HEK293FT cells in the presence of 1 mM Ca^{2+}/Mg^{2+} (Ca/Mg) or 0.2 mM Ca^{2+} plus 2 mM Mn^{2+} (Ca/Mn). PAC-1 binding was measured by flow cytometry and presented as mean fluorescence intensity (MFI) normalized to integrin expression (AP3 binding). Data are means ± s.e.m. ($n \geq 3$). Two-tailed t-tests were used to compare the wild type (WT) and the mutants in the Ca/Mn condition. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

expression of $\alpha_{IIb}\beta_3$ was decreased up to 20% among the α_{IIb} N15Q, N249Q, N570Q and N680Q mutations. However, the α_{IIb}-N931Q mutation dramatically decreased the cell surface expression to more than 50% (Fig. 2B). We also performed the Asn to Ser mutation, given most of the α_{IIb} N-glycan sites locate at a loop region and the serine residue is potentially modified by O-linked glycans. Interestingly, when co-expressed with wild-type β_3 in HEK293FT cells, the α_{IIb}-N15S and the α_{IIb}-N931S mutations significantly reduced Mn^{2+}-induced PAC-1 binding (Fig. 2C). There is an increase of PAC-1 binding with the α_{IIb}-N680S mutation, although it is not statistically significant (Fig. 2C). The α_{IIb}-N249S and α_{IIb}-N570S mutations had no effect on PAC-1 binding (Fig. 2C). The Ser substitutions, especially the α_{IIb}-N931S mutation, had less effect on the $\alpha_{IIb}\beta_3$ cell surface expression than the Gln substitutions (Fig. 2B,C). Overall, individual deletion of the α_{IIb} N-glycans had little or no effect on the Mn^{2+}-induced ligand binding of $\alpha_{IIb}\beta_3$ integrin. Of note, the decreased ligand binding by the α_{IIb}-N15S mutation is due to the Ser substitution not the loss of N-glycan since the α_{IIb}-N15Q and α_{IIb}-N15R mutation had no such obvious effect (Fig. 2B).

Effect of the loss of each individual N-glycan site of β_3 subunit on $\alpha_{IIb}\beta_3$ integrin expression and ligand binding. The β_3 subunit has 6 N-linked glycan sites: one in the βI domain, two in the hybrid domain, one in the I-EGF1 domain, one in the I-EGF3 domain and one in the β-tail domain (Fig. 3A), all of which have been resolved in the crystal structure (Fig. 3A). All these N-glycans can move with their attached subdomains during the extension of β_3 integrin (Fig. 3B). Similar to the α_{IIb} Asn to Gln mutations, most of the β_3 Asn to Gln single mutations had little effect on the cell surface expression of $\alpha_{IIb}\beta_3$ in HEK293FT cells (Fig. 3C). Remarkably, the β_3 N320Q, N559Q and N654Q mutations, located at the βI, I-EGF3, and β-tail domains, respectively, all significantly reduced the Mn^{2+}-induced PAC-1 binding (Fig. 3A–C). In contrast, both the N371Q and N452Q mutations, located at the hybrid and I-EGF1 domains, respectively, significantly enhanced the Mn^{2+}-induced PAC-1 binding (Fig. 3A–C). The β_3-N320Q mutation in the βI domain had the most dramatic negative effect on PAC-1 binding among all the mutations. However, the combined mutation β_3-N320Q-N559Q did not further decrease PAC-1 binding, but only further reduced the cell surface expression of $\alpha_{IIb}\beta_3$ (Fig. 3C).

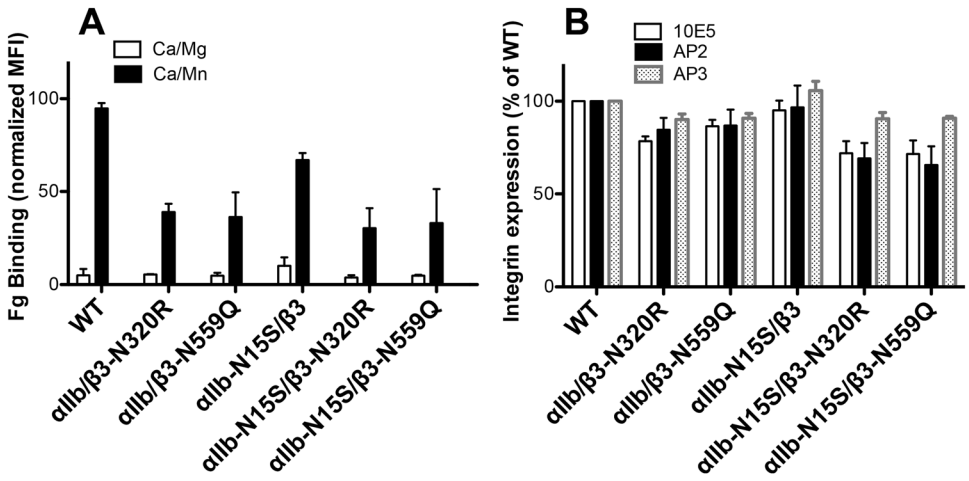

Figure 4. N-glycan deletions decreased $\alpha_{IIb}\beta_3$ integrin activation from outside the cell. **(A)** Fibrinogen (Fg) binding to HEK293FT cells transfected with the indicated $\alpha_{IIb}\beta_3$ constructs. Ligand binding was done in the presence of 1 mM Ca^{2+}/Mg^{2+} (Ca/Mg) or 0.2 mM Ca^{2+} plus 2 mM Mn^{2+} (Ca/Mn). **(B)** Cell surface expression of $\alpha_{IIb}\beta_3$ integrin constructs reported by anti-α_{IIb} mAb 10E5, anti-$\alpha_{IIb}\beta_3$ complex-specific mAb AP2, and anti-β_3 mAb AP3. The ligand or mAb binding was measured by flow cytometry. Data are means ± s.e.m. ($n \geq 3$).

To test whether the mutational effect on the $\alpha_{IIb}\beta_3$ PAC-1 binding is specific to the glutamine substitution, we also mutated the Asn to either Ser or Arg. As seen with the β_3-N320Q mutation, both the β_3 N320R and N320S mutations remarkably reduced the Mn^{2+}-induced PAC-1 binding, although the β_3-N320S mutation also dramatically reduced the cell surface expression of $\alpha_{IIb}\beta_3$ (Fig. 3D). In addition, the β_3 N371R and N452S mutations also increased PAC-1 binding, while the β_3-N654S mutation decreased PAC-1 binding (Fig. 3D). The β_3 N99Q and N99S mutations had no significant effect on Mn^{2+}-induced $\alpha_{IIb}\beta_3$ PAC-1 binding (Fig. 3C,D).

We next tested the effect of selected N-glycan mutations of α_{IIb} and β_3 subunits on the binding of the physiological ligand human fibrinogen (Fg). Consistent with the PAC-1 binding assay, the β_3-N320R, β_3-N559Q and α_{IIb}-N15S all decreased the Mn^{2+}-induced Fg binding to $\alpha_{IIb}\beta_3$ expressed in HEK293FT cells (Fig. 4A). However, the combined mutations, α_{IIb}-N15S/β_3-N320R and α_{IIb}-N15S/β_3-N559Q did not further reduce Fg binding (Fig. 4A). When measured by the anti-α_{IIb} mAb 10E5, the anti-$\alpha_{IIb}\beta_3$ complex-specific mAb AP2, and the anti-β_3 mAb AP3, these N-glycan mutations showed little effect on the cell surface expression of $\alpha_{IIb}\beta_3$ integrin (Fig. 4B), suggesting that these N-glycans may not affect the α_{IIb} and β_3 heterodimerization. Taken together, these data demonstrate the importance of individual N-glycan sites in regulating $\alpha_{IIb}\beta_3$ ligand binding.

Effect of the loss of individual N-glycan sites on $\alpha_{IIb}\beta_3$ activation from inside the cell. Integrin activation can be triggered from both outside and inside the cell, namely outside-in and inside-out signaling[35]. Having found that the loss of individual N-glycan sites can exert either negative or positive effect on $\alpha_{IIb}\beta_3$ activation induced by Mn^{2+} from outside the cell, we asked whether it has the similar effect on integrin inside-out activation, in which the signals are initiated from the cytoplasmic domain and transmitted to the ligand-binding site through large-scale conformational changes. The active mutations in the cytoplasmic domains such as the α_{IIb}-R995A and α_{IIb}-F993A or the overexpression of talin-1 head (TH) domain have been used to mimic integrin inside-out activation[36–38]. When co-expressed with the active α_{IIb}-R995A mutation, the β_3 N99Q, N320Q, N559Q and N654Q mutations all significantly reduced the constitutive PAC-1 binding to $\alpha_{IIb}\beta_3$ (Fig. 5A), while the β_3 N371Q and N452Q mutations significantly enhanced PAC-1 binding (Fig. 5A). The α_{IIb}-F993A mutation rendered $\alpha_{IIb}\beta_3$ more active than the α_{IIb}-R995A mutation did (Fig. 5A,B). Likewise, the β_3 N320R and N559Q mutations significantly reduced α_{IIb}-F993A-mediated $\alpha_{IIb}\beta_3$ activation (Fig. 5B). The β_3-N371R mutation did not further enhance the α_{IIb}-F993A-mediated PAC-1 binding probably because the PAC-1 binding already reached the maximal level (Fig. 5B). Interestingly, the β_3-N559Q mutation in the I-EGF3 domain exerted the most profound defect on $\alpha_{IIb}\beta_3$ inside-out activation (Fig. 5A,B).

We next tested the effect of the loss of single N-glycan sites on TH-induced $\alpha_{IIb}\beta_3$ activation. We performed this assay in the presence of α_{IIb}-R995A or β_3-D723A mutation, which has been shown to greatly enhance the TH-induced $\alpha_{IIb}\beta_3$ activation[39]. Consistent with the data above, the β_3 N320R and N559Q mutations significantly reduced, while the β_3 N371R mutation slightly increased TH-induced PAC-1 binding (Fig. 5C). As seen above, the β_3-N559Q had a more remarkable effect than the β_3-N320R mutation on TH-mediated $\alpha_{IIb}\beta_3$ activation (Fig. 5C). These data demonstrate that the N-glycans can exert both negative and positive effect on $\alpha_{IIb}\beta_3$ inside-out activation. Consistent with the Mn^{2+}-induced PAC-1 binding, the α_{IIb}-N15S mutation reduced TH-mediated $\alpha_{IIb}\beta_3$ activation (Fig. 5D). In contrast, the α_{IIb}-N249S mutation increased TH-mediated $\alpha_{IIb}\beta_3$ activation (Fig. 5D). However, it should be noted again that the negative effect of α_{IIb}-N15S mutation might not be directly due to the loss of N-glycan.

Effect of N-glycan deletions on $\alpha_{IIb}\beta_3$ integrin conformational change. The integrin affinity for ligand binding is tuned by the long-range conformational changes during integrin activation[19]. The ligand

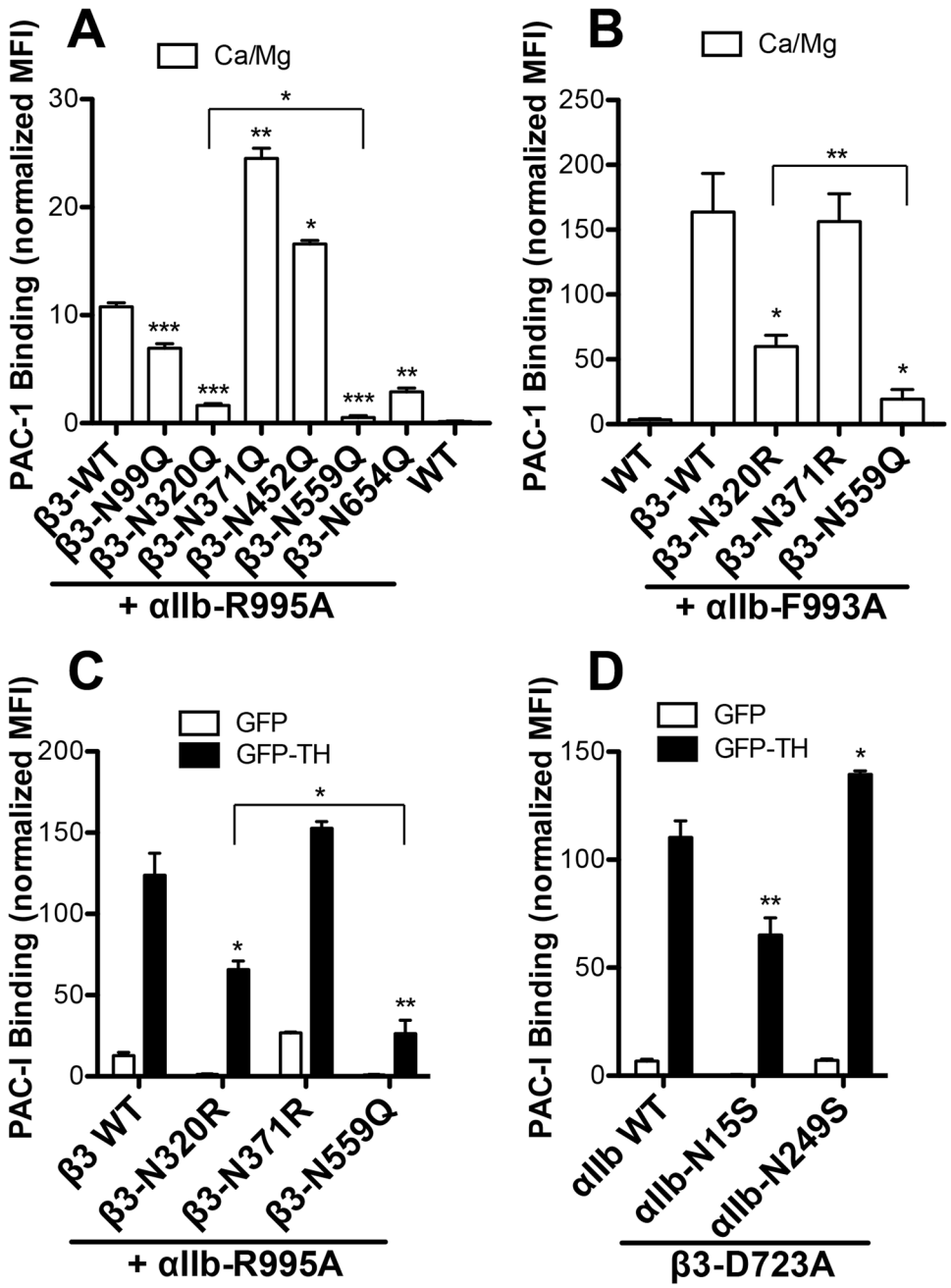

Figure 5. N-glycan deletions decreased $\alpha_{IIb}\beta_3$ integrin activation from inside the cell. (**A**,**B**) PAC-1 binding of HEK293FT cells transfected with the indicated β_3 constructs and the α_{IIb}-R995A or α_{IIb}-F993A mutant. (**C**,**D**) PAC-1 binding of the $\alpha_{IIb}\beta_3$ constructs co-expressed with EGFP or EGFP-Talin1-head (TH) in the HEK293FT cells. The binding was done in the presence of 1 mM Ca^{2+}/Mg^{2+} (Ca/Mg) and measured by flow cytometry. Data are means ± s.e.m. ($n \geq 3$). Two-tailed t-tests were used to compare the wild type (WT) and the mutants in the same condition. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

binding to the headpiece induces integrin ectodomain extension from the outside-in direction. On the other hand, activators from inside the cell also induce integrin conformational rearrangement, resulting in the affinity increase for the extracellular ligands. Since our data show that some of the N-glycans affect ligand binding of $\alpha_{IIb}\beta_3$ integrin, which requires integrin conformational changes, it is tempting to speculate that the N-linked glycans might exert their effect through regulating the conformations of integrin. To test this possibility, we used two conformation-specific mAbs, 319.4 and 370.3, to report the active conformations of β_3 and α_{IIb}, respectively. Eptifibatide (Ept), a high-affinity ligand-mimetic inhibitor that binds both the resting and active $\alpha_{IIb}\beta_3$, was used as a ligand to induce integrin conformational change from outside. Ept induced the binding of both 319.4 and 370.3 mAbs to $\alpha_{IIb}\beta_3$ (Fig. 6). The β_3 N320R and N559Q mutations significantly reduced the Ept-induced binding of both mAbs to $\alpha_{IIb}\beta_3$ (Fig. 6A,B), while the β_3-N371R mutation significantly enhanced the Ept-induced

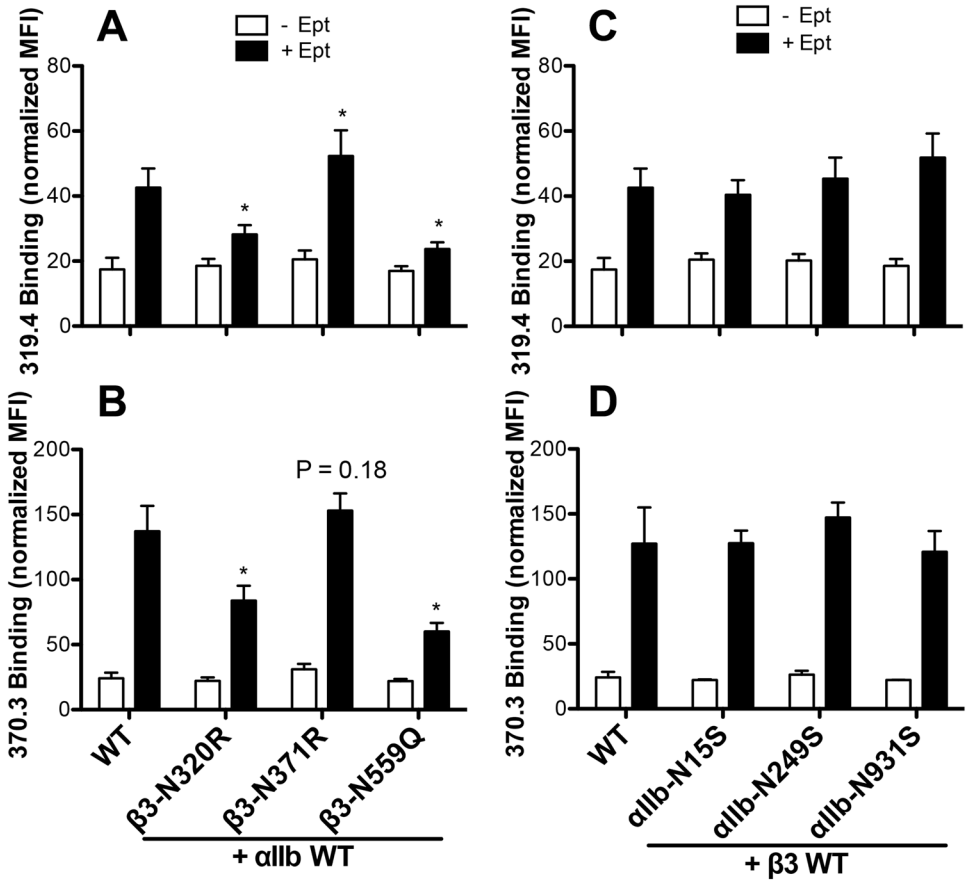

Figure 6. Effect of N-glycan deletions on ligand-induced conformational change of $\alpha_{IIb}\beta_3$ integrin. (**A**–**D**) The binding of the active conformation-specific anti-β_3 mAb 319.4 (**A**,**C**) and the anti-α_{IIb} mAb 370.3 (**B**,**D**) to the indicated $\alpha_{IIb}\beta_3$ constructs expressed in HEK293FT cells in the absence or presence of ligand-mimetic drug eptifibatide (Ept) plus 1 mM Ca^{2+}/Mg^{2+}. The mAb binding was measured by flow cytometry and presented as a normalized MFI to integrin expression (AP3 binding). Data are means ± s.e.m. (n = 3). Two-tailed t-tests were used to compare the wild type (WT) and the mutants in the same condition. *$P < 0.05$.

mAb 319.4 but not 370.3 binding (Fig. 6A,B). However, there was no obvious effect of the α_{IIb} N15S, N249S and N931S mutations on the Ept-induced mAb binding (Fig. 6C,D), although the N15S and N931S mutations reduced the Mn^{2+}-mediated PAC-1 binding (Fig. 2C). These data indicate that individual N-glycans can affect the ligand-induced conformational rearrangement of $\alpha_{IIb}\beta_3$ integrin.

To test the effect of N-glycans on the conformational change of $\alpha_{IIb}\beta_3$ integrin upon inside-out activation, we used the active cytoplasmic mutations β_3-K716A[40,41] and α_{IIb}-R993A. These mutations constitutively induced the binding of mAbs 319.4 and 370.3 to $\alpha_{IIb}\beta_3$ (Fig. 7), indicating the conformational changes of integrin from the inside-out direction. The presence of α_{IIb} N15S, N249S and N931S mutations did not affect the β_3-K716A-mediated binding of anti-β_3 mAb 319.4 (Fig. 7A). However, the α_{IIb} N15S and N931S but not N249S mutations reduced the β_3-K716A-mediated binding of anti-α_{IIb} mAb 370.3 (Fig. 7B). In contrast, the β_3 N320R and N559Q mutations reduced the α_{IIb}-R993A-mediated binding of both 319.4 (Fig. 7C) and 370.3 (Fig. 7D) mAbs. The β_3-N371R mutation had no obvious effect on the α_{IIb}-R993A-mediated binding of both mAbs (Fig. 7C,D) probably because the α_{IIb}-R993A mutation already induced the maximum level of $\alpha_{IIb}\beta_3$ activation as shown in the PAC-1 binding assay (Fig. 5B). These data demonstrate the importance of N-glycans in integrin conformational rearrangement during inside-out activation.

Effect of N-glycan deletions on the activation of $\alpha_V\beta_3$ integrin. The β_3 subunit also forms a heterodimer with α_V subunit. Changes in the complexity of N-linked glycosylation have been observed in $\alpha_V\beta_3$ integrins during the metastatic progression of tumor cells[14]. Having established the functional role of the individual N-glycan sites in $\alpha_{IIb}\beta_3$ integrin activation, we further studied the effect of N-glycan deletions on the function of $\alpha_V\beta_3$ integrin. The attachments of N-glycans have been confirmed in the $\alpha_V\beta_3$ crystal structure for most of the N-glycan sites (Fig. 8A,B). Human fibronectin (Fn) was used as a physiological ligand for $\alpha_V\beta_3$. To avoid the effect from the $\alpha_5\beta_1$ integrin, which is the major Fn receptor, we used the HEK293FT cells with both endogenous α_5 and β_1 subunits being knocked out by the CRISPR/Cas9 technology. Consistent with the $\alpha_{IIb}\beta_3$ integrin, the β_3-N559Q and the β_3-N654Q/S mutation reduced, while the β_3-N371Q/R mutation increased Mn^{2+}-mediated Fn binding to $\alpha_V\beta_3$ integrin (Fig. 8C). However, the β_3-N99Q/S, β_3-N452Q/S, and even the β_3-N320Q/R mutation had no

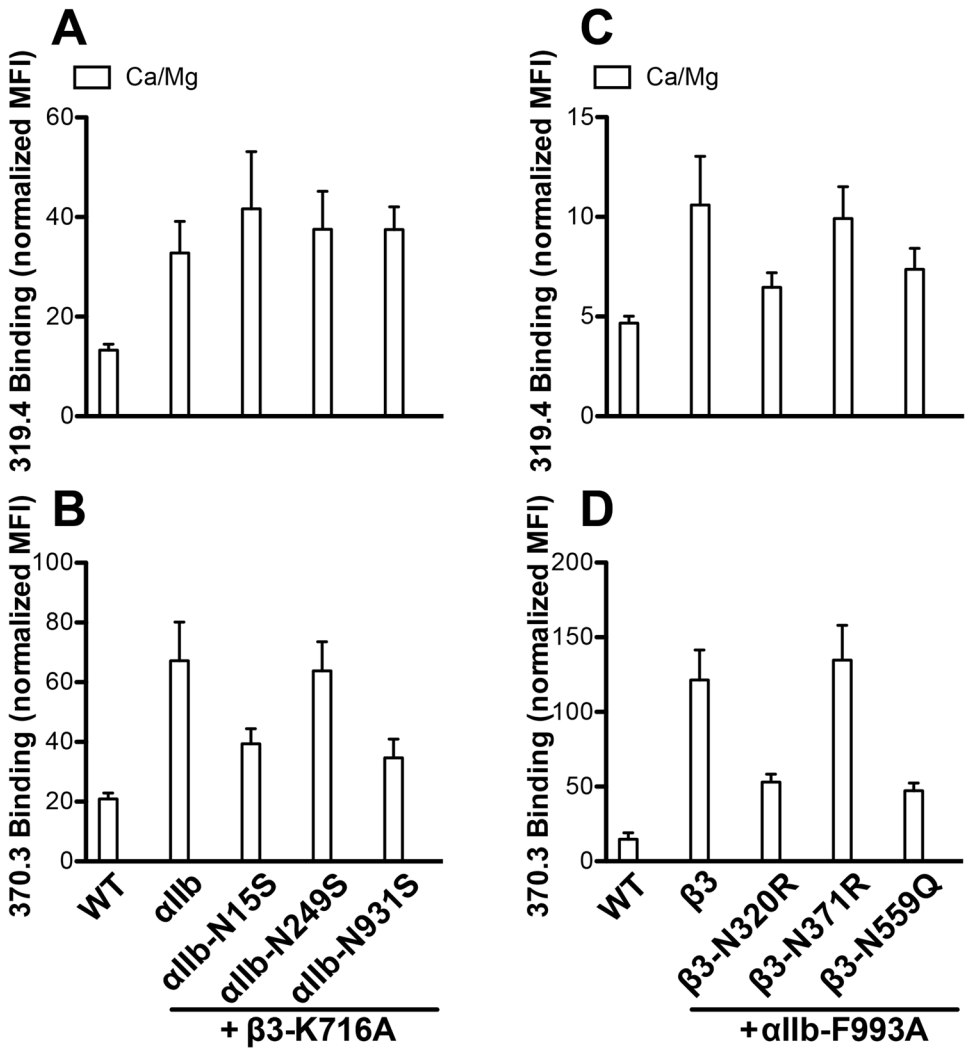

Figure 7. Effect of N-glycan deletions on the $\alpha_{IIb}\beta_3$ conformational change induced by inside-out activation. (**A**,**B**) Binding of the anti-β_3 mAb 319.4 or anti-α_{IIb} mAb 370.3 to the HEK293FT cells transfected with the α_{IIb} WT or the glycan mutants and the active β_3-K716A mutant, which mimics integrin inside-out activation. **(C**,**D)** Binding of the anti-β_3 mAb 319.4 or anti-α_{IIb} mAb 370.3 to the HEK293FT cells transfected with the β_3 WT or the glycan mutants and the active α_{IIb}-F993A mutant, which mimics integrin inside-out activation. Data are means ± s.e.m. (n = 3).

obvious effect on $\alpha_V\beta_3$ Fn binding (Fig. 8C). When co-expressed with the activating α_V-GAAKR mutation, which mimics $\alpha_V\beta_3$ inside-out activation[42], the β_3-N559Q but not β_3-N320R mutation remarkably dampened Fn binding (Fig. 8D). This is also in contrast with the $\alpha_{IIb}\beta_3$ integrin, in which both β_3-N559Q and β_3-N320R mutations greatly reduced the inside-out activation of $\alpha_{IIb}\beta_3$ (Fig. 5B). These data indicate that certain individual N-glycans of β_3 subunit may exert different effects on the function of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins.

Next, we studied the effect of removal of each individual N-glycan site in α_V subunit on the activation of $\alpha_V\beta_3$ integrin. α_V subunit has 13 potential N-glycan sites, 10 of which have been confirmed in the crystal structure (Fig. 8A). As shown in Fig. 8E, compared with the wild-type $\alpha_V\beta_3$, most of the individual N-glycan deletions by the N to Q substitutions had no obvious effect either on the cell surface expression or the Fn binding of $\alpha_V\beta_3$ (Fig. 8E). Among all the mutations, only the α_V-N458Q and the α_V-N943Q moderately increased Mn^{2+}-induced Fn binding to $\alpha_V\beta_3$ (Fig. 8E), and only the α_V-N943Q and the α_V-N950Q mutations mildly reduced the cell surface expression of $\alpha_V\beta_3$ (Fig. 8E). We also did the Ser substitutions for each N-glycan site of α_V subunit, but no obvious effect was observed on Mn^{2+}-induced $\alpha_V\beta_3$ Fn binding (Data not shown).

One N-glycan site in the βI domain of β_1 subunit negatively regulates $\alpha_5\beta_1$ integrin activation. Among the integrin β subunits, β_1 subunit has the most abundant putative N-glycan sites (Fig. 1D). When paired with α_5 subunit, the $\alpha_5\beta_1$ heterodimer has 26 potential N-glycan sites. The function of N-glycans in the $\alpha_5\beta_1$ complex formation and cell surface expression or in $\alpha_5\beta_1$-mediated cell adhesion, migration, and interaction with other cell surface receptors has been studied using mutagenesis approach[8]. By comparing the locations of the putative N-glycan sites among the integrin β subunit (Fig. 1D), we found that the β_1 subunit has

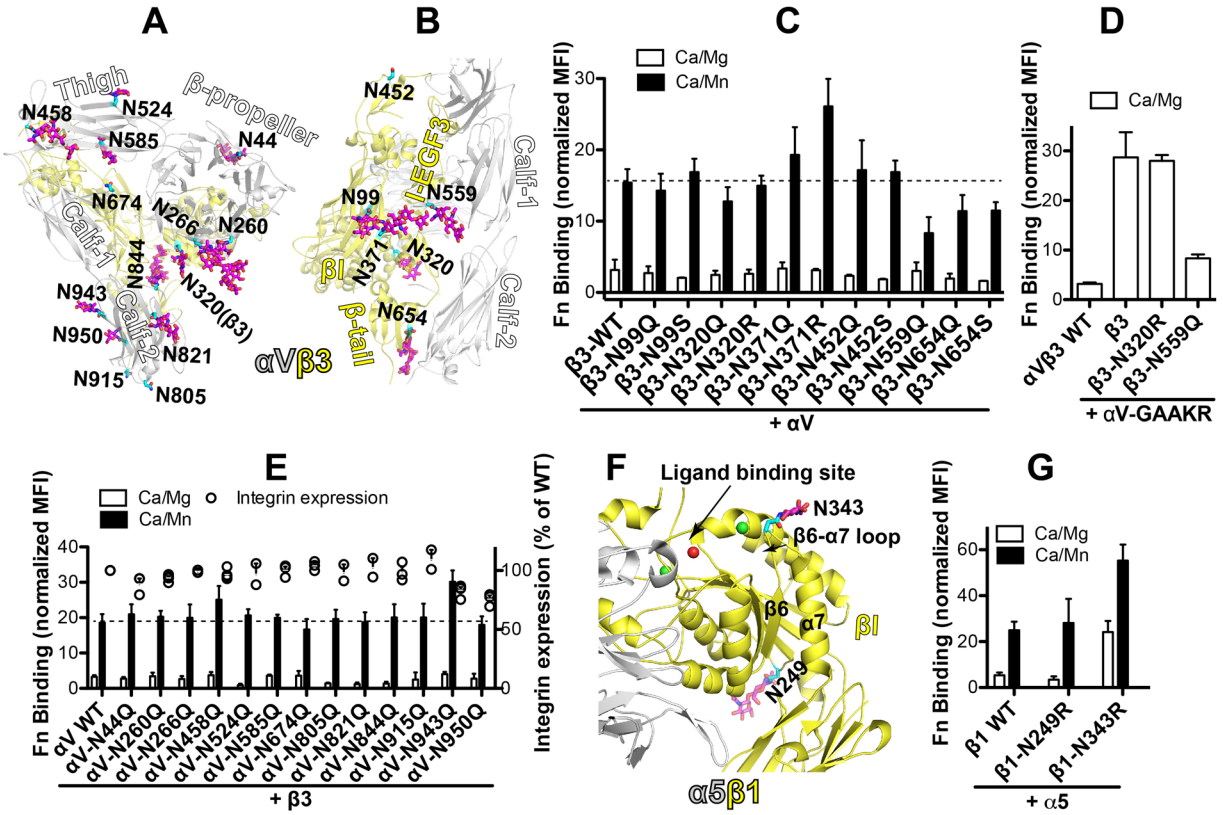

Figure 8. Effect of N-glycan deletions on $\alpha_V\beta_3$ and $\alpha_5\beta_1$ ligand binding. **(A)** Locations of α_V N-glycan sites in the crystal structure of $\alpha_V\beta_3$ (PDB code 4G1E). **(B)** Locations of β_3 N-glycan sites in the crystal structure of $\alpha_V\beta_3$ (PDB code 4G1E). Asn residues are shown as sticks with carbons in cyan. N-glycan residues resolved in the crystal structure are shown as sticks with carbons in magenta. Oxygens and nitrogens are red and blue, respectively. **(C)** Fibronectin (Fn) binding of HEK293FT-$\alpha_5\beta_1$-KO cells transfected with the β_3 WT or the glycan mutants and α_V WT. **(D)** Fn binding of HEK293FT-$\alpha_5\beta_1$-KO cells transfected with the indicated β_3 constructs and the α_V-GAAKR mutant that mimics integrin inside-out activation. **(E)** Fn binding of HEK293FT-$\alpha_5\beta_1$-KO cells transfected with the α_V WT or the glycan mutants and β_3 WT. **(F)** Locations of selected N-glycans at the βI domain of β_1 integrin in the crystal structure of $\alpha_5\beta_1$ headpiece (PDB code 4WJK). Asn and glycans are shown as sticks. Color codes are the same as panels A and B. Metal ions at the ligand-binding site are shown as spheres. **(G)** Fn binding of HEK293FT-$\alpha_5\beta_1$-KO cells transfected with the β_1 WT or the selected glycan mutants and α_5 WT. Fn binding was done in the presence of 1 mM Ca^{2+}/Mg^{2+} (Ca/Mg) or 0.2 mM Ca^{2+} plus 2 mM Mn^{2+} (Ca/Mn). Data are means ± s.e.m. (n = 3).

an N-glycan site of β_1-N343 uniquely residing at the β6-α7 loop of βI domain, which has been determined in the $\alpha_5\beta_1$ headpiece crystal structure (Fig. 8F). A hallmark structural change of the βI domain during integrin activation is the downward movement of the β6-α7 loop and the α7-helix[27,30]. Therefore, we hypothesized that the unique N-glycan of β_1-N343 at the β6-α7 loop might play a role in regulating $\alpha_5\beta_1$ activation. When co-expressed with the wild-type α_5 subunit in the HEK293FT-$\alpha_5\beta_1$-KO cells, the β_1-N343R mutation greatly enhanced the Fn binding to $\alpha_5\beta_1$ both in Ca/Mg and Ca/Mn conditions (Fig. 8G). As a control, the β_1-N249R mutation distal to the ligand-binding site (Fig. 8F) had no effect on $\alpha_5\beta_1$ Fn binding (Fig. 8G). Thus, the loss of the N-glycan at the β_1 β6-α7 loop facilitates $\alpha_5\beta_1$ integrin activation, indicating a negative regulation by this unique N-glycan of β_1 subunit.

Discussion

The great variation in the number and distribution of N-linked glycosylation sites among integrin subunits add another level of heterogeneity to the very complicated integrin family (Fig. 1). An increasing body of evidence indicates that integrin N-glycans contribute to cell adhesion and migration probably by affecting integrin expression, internalization, and association with other cell surface molecules[5,15,16]. However, a direct connection between integrin N-glycans and activation-dependent ligand binding has been missing. In addition, previous work studied the functional effect of either the overall changes in integrin glycosylation or the combined N-glycan sites such as within the same integrin subdomains[20,43,44], but the function of each individual N-glycan site has not been well documented. In this study, using the structurally well-characterized $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, and $\alpha_5\beta_1$ as model integrins, we found that the loss of certain individual N-glycan site either reduced or enhanced integrin activation reported by the changes in the binding of ligands or active conformation-specific mAbs, indicating that the N-linked glycosylation can exert both positive and negative effects on integrin function.

Among the N-glycan mutations of the α_{IIb} subunit, only the α_{IIb}-N15S mutation largely reduced the Mn^{2+}- and TH-induced $\alpha_{IIb}\beta_3$ activation. It seems that the negative effect on integrin activation is not due to the loss of N-glycan of α_{IIb}-N15 since the α_{IIb}-N15R and α_{IIb}-N15Q mutations didn't have much effect on $\alpha_{IIb}\beta_3$ ligand binding. A previous study showed that the α_{IIb}-N15Q mutation inhibited pro-α_{IIb} maturation, complex formation, and degradation[45], but we didn't see an obvious effect on the cell surface expression of $\alpha_{IIb}\beta_3$ with this mutation. The α_{IIb}-N15 resides at the blade 7 of β-propeller domain close to the interface formed by the α_{IIb} β-propeller and the β_3 βI domain. It is not readily known why the α_{IIb}-N15S mutation affects $\alpha_{IIb}\beta_3$ ligand binding. A similar N-glycan site of α_V integrin, α_V-N44, locates at the blade 1 of α_V β-propeller domain, but its mutation to Gln or Ser had no effect on Mn^{2+}-induced $\alpha_V\beta_3$ Fn binding. Thus, the negative effect of α_{IIb}-N15S on $\alpha_{IIb}\beta_3$ ligand binding should be specific to the Ser residue, probably due to the gain of O-linked glycosylation as indicated by the change of molecular weight of α_{IIb}-subunit (data not shown), which may affect ligand binding directly or indirectly through affecting $\alpha_{IIb}\beta_3$ conformational change. The N-glycans and O-glycans differ in the composition and the formation of the branches within the glycan structures, which determine the interaction with other molecules[12]. Moreover, the N-glycans also vary significantly in length and complexity[12]. Although the complete deletion of α_{IIb}-N15 N-glycan had no effect on $\alpha_{IIb}\beta_3$ ligand binding, the negative effect of α_{IIb}-N15S mutation by the potential gain-of-O-glycan suggests that the structure variations of α_{IIb}-N15 N-glycan may regulate the $\alpha_{IIb}\beta_3$ function, which is clearly worth further investigation.

Remarkably, almost all the β_3 single N-glycan deletion mutations affected the $\alpha_{IIb}\beta_3$ ligand binding induced either by Mn^{2+} or by the activating mutations that mimic integrin inside-out activation. Our structural analysis revealed an interesting pattern of the N-glycan location and its impact on integrin ligand binding. All the three β_3 N-glycans, including N320, N559, and N654, which positively regulate $\alpha_{IIb}\beta_3$ activation, locate at the α_{IIb}-β_3 inter-chain interfaces. The β_3-N320 glycan locates near the interface of α_{IIb} β-propeller and β_3 βI domain and inserts into the interface between the headpiece and the leg domains in the bent conformation of $\alpha_{IIb}\beta_3$ (Fig. 3A). Similarly, the β_3-N559 glycan lodges in the interface of α_{IIb} calf-1/2 and β_3 I-EGF3 domains. The β_3-N654 glycan resides at the interface of α_{IIb} calf-2 and β_3 β-tail domains (Fig. 3A). All of these interfaces are disrupted during integrin activation due to the headpiece extension and leg domain separation (Fig. 3B)[19]. The hydrophilic and bulky glycan groups may destabilize these interfaces at the bent conformation by repulsive interactions and therefore facilitate integrin conformational change. Consequently, deletion of these wedge-like glycans dampens $\alpha_{IIb}\beta_3$ ligand binding and conformational change potentially due to the stabilization of the bent inactive conformation of integrin. This is consistent with the previous studies showing that introducing an artificial N-glycan site into the βI and hybrid domain interface of β_3 subunit[46] or into the thigh and calf-1 domain interface of α_{IIb} subunit[47] renders $\alpha_{IIb}\beta_3$ constitutively active by enforcing integrin extension. In contrast, the β_3 N-glycans, attaching to N371 and N452, which negatively regulate $\alpha_{IIb}\beta_3$ activation, are located close to the intra-chain interfaces of β_3 subunit. Unlike the β_3-N559 glycan that inserts into the domain interface, the β_3-N371 glycan lies on the interface formed by the β_3 hybrid and I-EGF3/4 domains (Fig. 3A), which is disrupted upon the β_3 extension (Fig. 3B). The loss of β_3-N371 glycan increased $\alpha_{IIb}\beta_3$ activation, indicating that this glycan contributes to stabilizing the interface highly possibly by acting like a door bolt. In addition, the loss of the β_3-N99 N-glycan, which is adjacent to the N-glycan of β_3-N371 but farther away from the hybrid/I-EGF interface, slightly decreased $\alpha_{IIb}\beta_3$ activation. The close contacts between β_3-N371 and β_3-N99 N-glycans may influence the conformation of the β_3-N371 N-glycan, which in turn affects the hybrid/I-EGF interface. Upon deletion of the β_3-N99 N-glycan, the conformation of the β_3-N371 N-glycan may further stabilize the hybrid/I-EGF interface at the bent conformation. The β_3-N452 glycan resides at the opposite side of the acute angle formed by the I-EGF1 and I-EGF2 domains (Fig. 3A). This angle opens to almost 180° after integrin extension (Fig. 3B), which places the β_3-N452 glycan at the newly formed I-EGF1/2 domain interface (Fig.3B). As a result, the bulky N-glycan of β_3-N452 may exert repulsive tension to the interface and thus destabilize the extended conformation. Indeed, the removal of β_3-N452 N-glycan facilitated $\alpha_{IIb}\beta_3$ activation. Thus, our data demonstrate a location-specific contribution of individual N-glycan sites in regulating integrin activation largely through affecting integrin conformational changes.

A direct link between integrin N-glycans and the conformational regulation has been missing until a recent study, in which the effect of N-glycans on the conformational equilibria of $\alpha_5\beta_1$ integrin was elegantly investigated[48]. Our data are consistent with their results showing that the complex N-glycans stabilize the extended active conformation relative to the bent resting conformation. It was also proposed that the bulky N-glycans might exert their regulatory roles by crowding or repulsive interactions within the domain interfaces[48], which is in agreement with the structure interpretations of our data. However, only the combined effects of the overall changes of N-glycans on integrin affinity were examined in the $\alpha_5\beta_1$ study and the results might be a mixture of both negative and positive effects, although the positive effect was obviously dominated[48]. Our data show that individual N-glycan sites exert different effects on the β_3 integrin activation, arguing the importance of investigating the functional role of individual N-glycans in integrin activation.

Although $\alpha_V\beta_3$ integrin shares the same β_3 subunit with $\alpha_{IIb}\beta_3$, it is influenced differently by the loss of certain β_3 N-glycan sites. Particularly, the β_3-N320 mutation greatly reduced $\alpha_{IIb}\beta_3$ activation but had no effect on $\alpha_V\beta_3$ activation. Moreover, the α_V-N844 glycan, although buried at the interface between the β_3 βI and β-tail domain in the bent conformation of $\alpha_V\beta_3$ (Fig. 8A), also had no effect on Mn^{2+}-mediated $\alpha_V\beta_3$ Fn binding, consistent with a recent study[29]. The requirement of large-scale conformational changes for $\alpha_V\beta_3$ ligand binding remains controversial[29,49], but an increasing body of data supports the importance of integrin extension and headpiece opening in $\alpha_V\beta_3$ activation[29]. However, since the function of $\alpha_{IIb}\beta_3$ is to mediate platelet aggregation for hemostasis, it requires the activity of $\alpha_{IIb}\beta_3$ to be strictly regulated[31], while the fundamental function of $\alpha_V\beta_3$ is to mediate cell adhesion and migration that are essential for cell survival and proliferation[34], and thus the activation of $\alpha_V\beta_3$ might not be as strictly regulated as $\alpha_{IIb}\beta_3$ integrin. The different effect of β_3 N-glycans on $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ activation might be attributed to the differences in their biological function. Indeed, the differences in the affinity regulation between $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ had been reported previously[50,51].

The natural variants that cause the loss- or gain-of-function due to individual glycosylation mutations have not been reported for β_3 integrins. Nevertheless, a recent study showed that aberrant glycosylation of both α_V and β_3 subunits were observed between primary and metastatic melanoma cells, which modify the integrin-mediated tumor cell migration[14]. However, it is not known which N-glycan site is changed during the tumor cell progression. Moreover, N-glycans can have different types depending on their contents, including the high-mannose, hybrid, and complex N-glycans. It has been shown that the composition of N-glycans such as branching and sialylation regulates the function of N-glycans[2]. Considering the importance of individual N-glycan sites in integrin activation as determined in the present study, it will be of great interest to determine if the heterogeneity of certain individual N-glycans affects their regulatory roles in integrin function. Of the 13 N-glycan sites of α_V subunit, deletion of each individual site had little effect on $\alpha_V\beta_3$ expression and activation. It will be interesting to know if the simultaneous removal of multiple N-glycan sites will affect integrin function as shown in the study of $\alpha_5\beta_1$ integrin[20,44].

Among the 24 human integrins, the function of N-glycans of $\alpha_5\beta_1$ has been relatively well characterized. The N-glycan sites that are important for $\alpha_5\beta_1$ heterodimer formation and biological function have been determined in both α_5 and β_1 subunits[20,44]. Recent studies have mapped the important biological function to several individual N-glycan sites. For example, the N-glycan sites at the α_5 β-propeller domain are important for $\alpha_5\beta_1$-mediated cell adhesion and migration[21,52]; one of the N-glycans at the α_5 calf-1 domain is important for the complex formation with EGFR and the inhibition of EGFR signaling[53]; the N-glycan at the β_1 β-tail domain is important for β_1 activation and interaction with other cell membrane proteins including syndecan-4 and EGFR[54]; the 3 N-glycan sites at the β_1 βI domain are important for $\alpha_5\beta_1$ complex formation and cell spreading[44]. Furthermore, recent kinetic studies of the correlation between $\alpha_5\beta_1$ affinity and conformation suggest that the complex-type N-glycans of $\alpha_5\beta_1$ help stabilize the high-affinity conformation[48]. In the current study, we identified one N-glycan site at β_1-N343 in the βI domain, which negatively regulates β_1 activation since the removal of this glycan site rendered $\alpha_5\beta_1$ constitutively active. The β_1-N343 glycan may directly regulate the conformational change of βI domain by restraining the movement of the β6-α7 loop, or directly regulate ligand binding due to its proximity to the ligand binding site as proposed based on the structure modeling studies[55,56]. We expect to identify more location-specific functions of individual N-glycan sites in $\alpha_5\beta_1$ and other integrins. These N-glycan sites may work in concert to balance the biological activity of integrin.

Understanding of integrin conformation and affinity regulation has been greatly assisted by the mutagenesis studies. Critical residues and interactions that are important to integrin activation have been identified based on the loss- or gain-of-function mutations[57]. By site-directed mutagenesis, our study provides evidence that individual N-linked carbohydrate residues, depending on their structural locations, regulate integrin activation at least in part through influencing the conformational rearrangement. Exactly how these N-glycan "hotspots" are modified and regulated and how they contribute to integrin affinity and biological function need to be studied in more detail using both structural and cell biology approaches.

Materials and Methods

DNA constructs. DNA constructs of human $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, and EGFP-tagged mouse talin-1-head (EGFP-TH) were as described[42,57,58]. All the mutations were introduced by PCR using PfuTurbo DNA polymerase following the protocol of the QuikChange XL site-directed mutagenesis kit (Agilent Technologies). The introduced mutations were confirmed by DNA sequencing (Retrogen).

Antibodies and protein ligands. PAC-1 (BD Bioscience) is a ligand-mimetic mAb (IgM) specific for activated $\alpha_{IIb}\beta_3$ integrin[59]. AP3 is a conformation-independent anti-β_3 mAb[60] and was conjugated with Alexa Fluor 488 (ThermoFisher Scientific) or Sulfo-NHS-Biotin (ThermoFisher Scientific). 10E5 is an anti-α_{IIb} mAb[27,61]. 319.4 and 370.3 are mAbs that recognize the active conformations of β_3 and α_{IIb}, respectively[57]. Human fibrinogen (Fg) (Enzyme Research Laboratories) and human fibronectin (Fn) (Sigma-Aldrich) were conjugated with Alexa Fluor 647 (ThermoFisher Scientific). PE-labeled MAR4 (BD Bioscience) is a non-functional anti-β_1 integrin mAb. VC5 is anti-α_5 integrin mAb (BD Bioscience). Alexa Fluor 647 conjugated goat anti-mouse IgM and PE-conjugated streptavidin were from ThermoFisher Scientific.

Cell lines. HEK293FT cells (ThermoFisher Scientific) were cultured in DMEM plus 10% FBS at 37 °C with 5% CO_2. The α_5 and β_1 subunits double-knockout HEK293FT (HEK293FT-$\alpha_5\beta_1$-KO) cells were generated by the CRISPR/Cas9 gene editing technology using the α_5 and β_1 CRISPR/Cas9 KO plasmids from Santa Cruz Biotechnology. In brief, cells were transfected with the α_5 KO plasmids for 5–7 days. The α_5-negative cells were selected by single cell sorting after staining with the anti-α_5 mAb VC5. The established α_5-KO cells were transfected with the β_1 KO plasmids and the β_1-negative cells were selected by single cell sorting after staining with the anti-β_1 mAb MAR4. Single cell clones with the lowest expression of both α_5 and β_1 subunits were selected for the following experiments.

Soluble ligand binding assay. PAC-1 and Fg binding of HEK293FT cells transfected with $\alpha_{IIb}\beta_3$ were as described[39,57]. For EGFP-TH-induced ligand binding, HEK293FT cells were co-transfected with integrin constructs and EGFP or EGFP-TH for at least 24 hours. Ligand binding was performed in HBSGB buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 5.5 mM glucose, and 1% BSA) with 5 μg/ml PAC-1 or 50 μg/ml Alexa Fluor 647-labeled Fg in the presence of 10 μM eptifibatide ($\alpha_{IIb}\beta_3$-specific inhibitor) or 1 mM Ca^{2+}/Mg^{2+} (Ca/Mg) or 0.2 mM Ca^{2+} plus 2 mM Mn^{2+} (Ca/Mn) at 25 °C for 30 min. Cells were then washed and incubated on ice for 30 min with the detecting reagents: 10 μg/ml Alexa Fluor 488-labeled AP3 (for Fg binding) or Alexa Fluor 488-labeled AP3 plus Alexa Fluor 647-labeled goat anti-mouse IgM (for PAC-1 binding). For EGFP-TH-induced PAC-1 binding, cells were washed and incubated on ice with biotin-labeled AP3 plus PE-labeled streptavidin

and Alexa Fluor 647-labeled goat anti-mouse IgM. Human Fn binding to $\alpha_V\beta_3$ or $\alpha_5\beta_1$ integrins were performed with HEK293FT-$\alpha_5\beta_1$-KO transfectants. Cells were first incubated with 50 μg/ml Alexa Fluor 647-labeled Fn in the presence of 10 μM cilengitide ($\alpha_V\beta_3$-specific inhibitor), 5 mM EDTA (for $\alpha_5\beta_1$), Ca/Mg or Ca/Mn, and then washed and incubated with 10 μg/ml Alexa Fluor 488-labeled AP3 (for $\alpha_V\beta_3$) or PE-labeled MAR4 (for $\alpha_5\beta_1$). Integrin positive or integrin and EGFP double-positive cells were acquired for calculating the mean fluorescence intensity (MFI) by flow cytometry using Accuri C6 cytometer (BD Biosciences). Ligand binding was presented as normalized MFI, i.e. ligand MFI (after subtracting the ligand MFI in the inhibitor or EDTA condition) as a percentage of integrin MFI. By this calculation, the binding of ligands was normalized to total integrin expression.

Conformation-specific antibody binding. Binding of the active conformation-specific anti-β_3 mAb 319.4 and anti-α_{IIb} mAb 370.3 to the HEK293FT transfectants was performed as described[39,57]. In brief, cells were first incubated with 10 μg/ml biotin-labeled 319.4 or 370.3 in the HBSGB buffer containing 1 mM Ca^{2+}/Mg^{2+} in the absence or presence of 10 μM $\alpha_{IIb}\beta_3$-specific ligand-mimetic inhibitor eptifibatide at 25 °C for 30 mins, and then washed and incubated with 10 μg/ml Alexa fluor 488-labeled AP3 and Alexa fluor 647-labeled streptavidin on ice for 30 min. AP3 positive cells (expressing $\alpha_{IIb}\beta_3$ integrin) were acquired for calculating the MFI by flow cytometry. The 319.4 or 370.3 mAb binding was presented as normalized MFI, i.e. streptavidin MFI as a percentage of AP3 MFI. By this calculation, the binding of conformation-specific mAbs was normalized to total integrin expression.

Statistical analysis. The statistical analysis was performed using the GraphPad Prism software. Two-tailed Student's *t*-test was used to calculate the p values for comparing the two experimental groups, for example, the wild type and the mutant data under the same condition. The assays were repeated independently at least three times for statistical analysis.

Data Availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**, 97–130 (1993).
2. Moremen, K. W., Tiemeyer, M. & Nairn, A. V. Vertebrate protein glycosylation: diversity, synthesis and function. *Nature reviews. Molecular cell biology* **13**, 448–462 (2012).
3. Aebi, M. N-linked protein glycosylation in the ER. *Biochimica et biophysica acta* **1833**, 2430–2437 (2013).
4. Scott, D. W. & Patel, R. P. Endothelial heterogeneity and adhesion molecules N-glycosylation: implications in leukocyte trafficking in inflammation. *Glycobiology* **23**, 622–633 (2013).
5. Taniguchi, N. & Korekane, H. Branched N-glycans and their implications for cell adhesion, signaling and clinical applications for cancer biomarkers and in therapeutics. *BMB Rep* **44**, 772–781 (2011).
6. Janik, M. E., Litynska, A. & Vereecken, P. Cell migration-the role of integrin glycosylation. *Biochimica et biophysica acta* **1800**, 545–555 (2010).
7. Sperandio, M., Gleissner, C. A. & Ley, K. Glycosylation in immune cell trafficking. *Immunol Rev* **230**, 97–113 (2009).
8. Gu, J., Isaji, T., Sato, Y., Kariya, Y. & Fukuda, T. Importance of N-glycosylation on $\alpha_5\beta_1$ integrin for its biological functions. *Biol Pharm Bull* **32**, 780–785 (2009).
9. Zhao, Y. *et al*. Branched N-glycans regulate the biological functions of integrins and cadherins. *FEBS J* **275**, 1939–1948 (2008).
10. Lertkiatmongkol, P. *et al*. The Role of Sialylated Glycans in Human Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)-mediated Trans Homophilic Interactions and Endothelial Cell Barrier Function. *J Biol Chem* **291**, 26216–26225 (2016).
11. Zhao, Y. Y. *et al*. Functional roles of N-glycans in cell signaling and cell adhesion in cancer. *Cancer Sci* **99**, 1304–1310 (2008).
12. van Kooyk, Y. & Rabinovich, G. A. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol* **9**, 593–601 (2008).
13. Taniguchi, N. & Kizuka, Y. Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Adv Cancer Res* **126**, 11–51 (2015).
14. Pochec, E. *et al*. Aberrant Glycosylation of $\alpha_V\beta_3$ Integrin is Associated with Melanoma Progression. *Anticancer Res* **35**, 2093–2103 (2015).
15. Takahashi, M., Kizuka, Y., Ohtsubo, K., Gu, J. & Taniguchi, N. Disease-associated glycans on cell surface proteins. *Mol Aspects Med* **51**, 56–70 (2016).
16. Hoja-Lukowicz, D., Przybylo, M., Duda, M., Pochec, E. & Bubka, M. On the trail of the glycan codes stored in cancer-related cell adhesion proteins. *Biochimica et biophysica acta* **1861**, 3237–3257 (2017).
17. Kremser, M. E. *et al*. Characterisation of $\alpha_3\beta_1$ and $\alpha_V\beta_3$ integrin N-oligosaccharides in metastatic melanoma WM9 and WM239 cell lines. *Biochimica et biophysica acta* **1780**, 1421–1431 (2008).
18. Hynes, R. O. Integrins: bi-directional, allosteric, signalling machines. *Cell* **110**, 673–687 (2002).
19. Springer, T. A. & Dustin, M. L. Integrin inside-out signaling and the immunological synapse. *Current opinion in cell biology* **24**, 107–115 (2012).
20. Isaji, T. *et al*. N-glycosylation of the β-propeller domain of the integrin α_5 subunit is essential for $\alpha_5\beta_1$ heterodimerization, expression on the cell surface, and its biological function. *J. Biol. Chem.* **281**, 33258–33267 (2006).
21. Sato, Y. *et al*. An N-glycosylation site on the β-propeller domain of the integrin α_5 subunit plays key roles in both its function and site-specific modification by beta1,4-N-acetylglucosaminyltransferase III. *J Biol Chem* **284**, 11873–11881 (2009).
22. Ranjan, A., Bane, S. M. & Kalraiya, R. D. Glycosylation of the laminin receptor ($\alpha_3\beta_1$) regulates its association with tetraspanin CD151: Impact on cell spreading, motility, degradation and invasion of basement membrane by tumor cells. *Exp Cell Res* **322**, 249–264 (2014).
23. Nicolaou, N. *et al*. Gain of glycosylation in integrin α_3 causes lung disease and nephrotic syndrome. *J Clin Invest* **122**, 4375–4387 (2012).
24. Kaszuba, K. *et al*. N-Glycosylation as determinant of epidermal growth factor receptor conformation in membranes. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 4334–4339 (2015).
25. Xiong, J.-P. *et al*. Crystal structure of the extracellular segment of integrin $\alpha_V\beta_3$. *Science* **294**, 339–345 (2001).
26. Xiong, J. P. *et al*. Crystal structure of the complete integrin $\alpha_V\beta_3$ ectodomain plus an α/β transmembrane fragment. *J Cell Biol* **186**, 589–600 (2009).
27. Xiao, T., Takagi, J., Wang, J.-h, Coller, B. S. & Springer, T. A. Structural basis for allostery in integrins and binding of fibrinogen-mimetic therapeutics. *Nature* **432**, 59–67 (2004).

28. Zhu, J. *et al*. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Mol. Cell* **32**, 849–861 (2008).
29. Dong, X. *et al*. $\alpha_V\beta_3$ Integrin Crystal Structures and their Functional Implications. *Biochemistry* **51**, 8814–8828 (2012).
30. Zhu, J., Zhu, J. & Springer, T. A. Complete integrin headpiece opening in eight steps. *J Cell Biol* **201**, 1053–1068 (2013).
31. Coller, B. S. & Shattil, S. J. The GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood* **112**, 3011–3025 (2008).
32. Coller, B. S. $\alpha_{IIb}\beta_3$: structure and function. *Journal of thrombosis and haemostasis: JTH* **13**(Suppl 1), S17–25 (2015).
33. Atkinson, S. J., Ellison, T. S., Steri, V., Gould, E. & Robinson, S. D. Redefining the role(s) of endothelial $\alpha_V\beta_3$-integrin in angiogenesis. *Biochemical Society transactions* **42**, 1590–1595 (2014).
34. Seguin, L., Desgrosellier, J. S., Weis, S. M. & Cheresh, D. A. Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance. *Trends Cell Biol* **25**, 234–240 (2015).
35. Shattil, S. J., Kim, C. & Ginsberg, M. H. The final steps of integrin activation: the end game. *Nature reviews. Molecular cell biology* **11**, 288–300 (2010).
36. Tadokoro, S. *et al*. Talin binding to integrin β tails: a final common step in integrin activation. *Science* **302**, 103–106 (2003).
37. Calderwood, D. A. *et al*. The talin head domain binds to integrin β subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* **274**, 28071–28074 (1999).
38. Hughes, P. E., O'Toole, T. E., Ylanne, J., Shattil, S. J. & Ginsberg, M. H. The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J. Biol. Chem.* **270**, 12411–12417 (1995).
39. Liu, J., Wang, Z., Thinn, A. M., Ma, Y. Q. & Zhu, J. The dual structural roles of the membrane distal region of the α-integrin cytoplasmic tail during integrin inside-out activation. *J Cell Sci* **128**, 1718–1731 (2015).
40. Zhu, J. *et al*. The structure of a receptor with two associating transmembrane domains on the cell surface: integrin $\alpha_{IIb}\beta_3$. *Mol. Cell* **34**, 234–249 (2009).
41. Kim, C. *et al*. Basic amino-acid side chains regulate transmembrane integrin signalling. *Nature* **481**, 209–213 (2012).
42. Zhu, J., Boylan, B., Luo, B.-H., Newman, P. J. & Springer, T. A. Tests of the extension and deadbolt models of integrin activation. *J. Biol. Chem.* **282**, 11914–11920 (2007).
43. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K. & Pierce, M. Aberrant N-glycosylation of β_1 integrin causes reduced $\alpha_5\beta_1$ integrin clustering and stimulates cell migration. *Cancer Res* **62**, 6837–6845 (2002).
44. Isaji, T., Sato, Y., Fukuda, T. & Gu, J. N-glycosylation of the I-like domain of β_1 integrin is essential for β_1 integrin expression and biological function: identification of the minimal N-glycosylation requirement for $\alpha_5\beta_1$. *J Biol Chem* **284**, 12207–12216 (2009).
45. Mitchell, W. B., Li, J., French, D. L. & Coller, B. S. $\alpha_{IIb}\beta_3$ biogenesis is controlled by engagement of α_{IIb} in the calnexin cycle via the N15-linked glycan. *Blood* **107**, 2713–2719 (2006).
46. Luo, B.-H., Springer, T. A. & Takagi, J. Stabilizing the open conformation of the integrin headpiece with a glycan wedge increases affinity for ligand. *Proc. Natl. Acad. Sci. USA* **100**, 2403–2408 (2003).
47. Kamata, T. *et al*. Structural requirements for activation in $\alpha_{IIb}\beta_3$ integrin. *J Biol Chem* **285**, 38428–38437 (2010).
48. Li, J. *et al*. Conformational equilibria and intrinsic affinities define integrin activation. *EMBO J* **36**, 629–645 (2017).
49. Arnaout, M. A., Mahalingam, B. & Xiong, J. P. Integrin structure, allostery, and bidirectional signaling. *Annu. Rev. Cell Dev. Biol.* **21**, 381–410 (2005).
50. Mor-Cohen, R. *et al*. Unique disulfide bonds in epidermal growth factor (EGF) domains of β_3 affect structure and function of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins in different manner. *J Biol Chem* **287**, 8879–8891 (2012).
51. Kamata, T., Handa, M., Sato, Y., Ikeda, Y. & Aiso, S. Membrane-proximal α/β stalk interactions differentially regulate integrin activation. *J. Biol. Chem.* **280**, 24775–24783 (2005).
52. Hang, Q. *et al*. A key regulator of cell adhesion: Identification and characterization of important N-glycosylation sites on integrin α_5 for cell migration. *Mol Cell Biol* **37**, e00558–16 (2017).
53. Hang, Q. *et al*. N-Glycosylation of integrin α_5 acts as a switch for EGFR-mediated complex formation of integrin $\alpha_5\beta_1$ to $\alpha_6\beta_4$. *Sci Rep* **6**, 33507 (2016).
54. Hou, S. *et al*. Importance of membrane-proximal N-glycosylation on integrin β_1 in its activation and complex formation. *FASEB J* **30**, 4120–4131 (2016).
55. Nagae, M. *et al*. Crystal structure of $\alpha_5\beta_1$ integrin ectodomain: Atomic details of the fibronectin receptor. *J Cell Biol* **197**, 131–140 (2012).
56. Liu, Y., Pan, D., Bellis, S. L. & Song, Y. Effect of altered glycosylation on the structure of the I-like domain of β_1 integrin: a molecular dynamics study. *Proteins* **73**, 989–1000 (2008).
57. Zhang, C. *et al*. Modulation of integrin activation and signaling by α1/α1'-helix unbending at the junction. *J Cell Sci* **126**, 5735–5747 (2013).
58. Bouaouina, M., Lad, Y. & Calderwood, D. A. The N-terminal domains of talin cooperate with the phosphotyrosine binding-like domain to activate β_1 and β_3 integrins. *J Biol Chem* **283**, 6118–6125 (2008).
59. Shattil, S. J., Hoxie, J. A., Cunningham, M. & Brass, L. F. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem* **260**, 11107–11114 (1985).
60. Kouns, W. C. *et al*. Further characterization of the loop structure of platelet glycoprotein IIIa: partial mapping of functionally significant glycoprotein IIIa epitopes. *Blood* **78**, 3215–3223 (1991).
61. Coller, B. S., Peerschke, E. I., Scudder, L. E. & Sullivan, C. A. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J. Clin. Invest.* **72**, 325–338 (1983).

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Author Contributions

X.Cai, A.M.M.Thinn, Z.Wang, H.Shan, and J.Zhu performed the experiments and analyzed the data. J.Zhu designed the study and wrote the manuscripts. All the authors contributed to the manuscript preparation.

Additional Information

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