LETTER TO THE EDITOR

Specific O-glycans in the mechanosensory domain of glycoprotein Ibα are important for its stability and function

Platelets lacking O-glycans exhibit a reduced lifespan and increased clearance in the liver due to defective sialylation.[1,2] Variations in O-glycosylation affect platelet functions and abolish surface expression of glycoproteins, particularly glycoprotein (GP)Ibα that contains the vast majority of sialic acids on the platelet and is implicated in fast clearance of desialylated platelets.[1,3] GPIbα is a major subunit in the GPIb-IX complex that also contains GPIbβ and GPIX.[4] Ligand-mediated unfolding of the mechanosensory domain (MSD) in GPIbα, and consequently the exposure of the Trigger sequence, is the key step in activating GPIb-IX signaling and inducing platelet clearance.[3-5] The isolated MSD is not stable.[4,6] Desialylation of O-glycans could induce spontaneous MSD unfolding.[3] It is not clear which of the 18 serine (Ser) and threonine (Thr) residues in the MSD are actually O-glycosylated (Figure 1A). Here, we characterize GPIbα mutants with altered O-glycosylated MSD in transfected Chinese hamster ovary (CHO) cells, and report evidence for specific MSD O-glycans being important for the stability and signaling function of GPIbα.

GPIbα expressed on transfected CHO cells exhibits a lower molecular weight than that on platelets,[7] indicating that GPIbα O-glycans on platelets are larger and more complex than those in CHO cells. Nevertheless, surface expression and functions of GPIbα, including its expression level affected by the glycosylated MSD, have been faithfully recapitulated in transfected CHO cells,[4,6] suggesting that the CHO cell is an appropriate model system for the study of GPIbα conformation and stability. In order to investigate how O-glycosylation in the MSD affects GPIbα, we arbitrarily divided the MSD into three regions: N-terminal (residues Ala417-Pro442), C-terminal (Val443-Glu471), and the Trigger (Ser472-Phe483). All Ser and Thr residues in each region were mutated to Ala to generate three GPIbα variants, named NΔO, CΔO, and TΔO, respectively. Expression vectors encoding these variants and the wild-type (WT) were transfected into CHO cells that stably expressed human GPIbβ and GPIX (CHO-βIX cells) and characterized as previously described[6,7] (Figure 1; *Online Supplementary Figure S1*).

Compared to that of WT, surface expression of the NΔO variant, but neither CΔO nor TΔO, was significantly reduced (Figure 1B, C; *Online Supplementary Figure S1*). Consistently, the NΔO protein in transfected cells was much less than the WT, and it had a lower molecular weight (*Online Supplementary Figure S1C, D*). In comparison, CΔO had a WT-like molecular weight, and two glycosylated bands were present in CHO-TΔO cells expressing TΔO, one of which even had higher molecular weight than WT (*Online Supplementary Figure S1C, G*). GPIbβ expression was significantly reduced in NΔO and TΔO cells than WT, and GPIX expression was reduced in all three variant cells (Figure 1D, E; *Online Supplementary Figure S2*). Moreover, CΔO formed a non-GPIb complex[8] in addition to the native GPIb complex (*Online Supplementary Figure S2D*). These results indicate that i) the majority of MSD O-glycans are in the N-terminal region, ii) O-glycans in the N-terminal region are critical to GPIbα expression, and iii) O-glycans in the C-terminal region are important for proper GPIb-IX assembly.

Like in circulating platelets, GPIbα is continuously proteolyzed or shed from transfected CHO cells, with its extracellular domain, known as glycocalicin (GC), released into the culture media. The shedding cleavage site is located in the MSD (Figure 1A).[9] CHO cells expressing NΔO (CHO-NΔO cells) produced more GC than WT and two other mutants. Adding GM6001, a broad-spectrum metalloprotease inhibitor, to culture markedly increased GPIbα surface expression in CHO-WT and other mutant cells and reduced GC generation (*Online Supplementary Figure S3A*; Figure 1F, H). However, a significant level of GC was still present in CHO-NΔO cells, suggesting that NΔO could be cleaved by proteases other than metalloproteases. This is consistent with an earlier report that GPIbα variants lacking residues 461-483 could express GC on the surface of transfected CHO cells at a high level without GPIbβ and GPIX.[6] In comparison, the level of GC produced by shedding of the CΔO variant was significantly lower than that from WT GPIbα. In CHO-TΔO cells, both glycosylated variants were shed, although it was difficult to quantitate their extent of shedding separately (Figure 1G, H).

We reported previously that unfolding of the MSD helps expose the shedding cleavage site and could increase shedding of GPIbα.[3,5] Monoclonal antibody (MAb) WM23 recognizes a sequence in the sialomucin region of GPIbα, and MAb 5G6 binds a linear epitope containing the ADAM17 shedding cleavage site in the MSD.[10,11] The ratio of 5G6 binding to WM23 binding has been utilized as a proxy for the extent of MSD unfolding.[3-5] In order to understand the mechanism for the increased shedding of the NΔO variant, fluorescently labeled WM23 and 5G6 were incubated with aforementioned CHO cells, and it was observed that binding of WM23 to CHO-NΔO cells was about 50% of those to CHO-WT and other mutant cells (Figure 2A, B). Binding of 5G6 to CHO-NΔO cells was significantly increased compared to WT and others (Figure 2C, E), suggesting that the MSD in the NΔO variant on transfected CHO cells is more

unfolded than that in WT and is therefore more readily cleaved or shed. On the other hand, the MSD is well folded in the CΔO and TΔO variants. Therefore, O-glycans in the N-terminal region of the MSD are important to GPIbα expression and stability.

After transfected CHO cells adhere to immobilized VWF, binding of von Willebrand factor (VWF) in turn could induce signaling into CHO cells and result in filopodia formation.[4,5,12,13] The extent of filopodia formation correlates with GPIb-IX-mediated cellular signaling.[3,5,12,13] In order to assess the effects of O-glycosylation in the MSD on binding and signaling functions of GPIbα, CHO cells expressing WT GPIbα, NΔO, CΔO, and TΔO variants were placed on a VWF-coated slide in the presence of botrocetin and EDTA, cell adhesion and filopodia formation therein were visualized by fluorescence microscopy and quantitated for comparison.[12,13] The number of adherent CHO-CΔO and CHO-TΔO cells was indistinguishable from that of CHO-WT cells, consistent with the observation that they did not affect binding of GPIbα to VWF (Figure 3A, B; *Online Supplementary Figure*

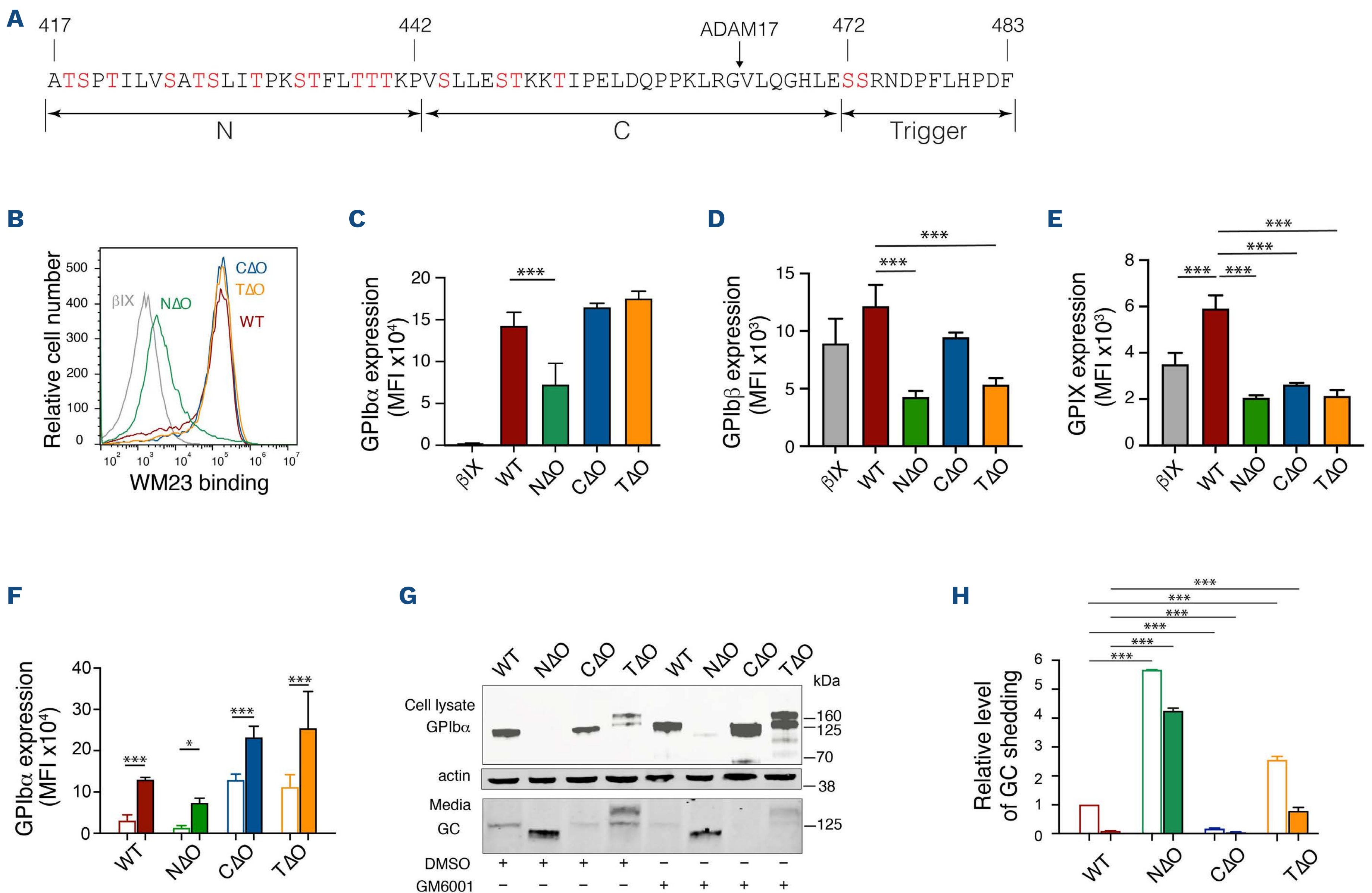

Figure 1. O-glycans in the N-terminal region of the mechanosensory domain are important to GPIbα expression and shedding. (A) The sequence of human GPIbα mechanosensory domain (MSD) includes residues A417-F483. Putative O-glycosylation sites, serine or threonine residues, are colored red. The N-terminal region (A417 to P442), the C-terminal region (V443 to E471), and the Trigger region (S472 to F483) are marked. The ADAM17 cleavage site is denoted by the arrowhead. (B) Overlaid flow cytometry histograms showing surface expression of GPIbα variants in stably transfected CHO cells that expressed wild-type GPIbβ and GPIX. βIX: cells transfected with an empty vector. NΔO, CΔO, and TΔO denote the GPIbα constructs that have mutations of O-glycosylation sites. GPIbα expression was detected by flow cytometry with anti-GPIbα WM23 antibody.[6] (C-E) Bar plots of (C) GPIbα variant, (D) GPIbβ, and (E) GPIX surface expression in noted stably transfected CHO cells. GPIbβ and GPIX was detected with monoclonal antibodies RAM.1 and FMC25, respectively. The mean fluorescence intensity (MFI) was quantified and plotted (mean ± standard deviation, n=3). Comparison of result was performed by one-way ANOVA. *P<0.05; ***P<0.001. (F) Bar plots of GPIbα expression level in each transfected CHO cell treated with vehicle (unfilled bar graph) or GM6001 (color-filled bar graph), quantified by MFI. (G) Representative western blots showing cellular expression of GPIbα in noted stably transfected CHO cells and glycocalicin (GC) released from these cells into the culture media. Cell lysates of denoted cells were resolved in SDS gels and transferred to the PVDF membrane, which was blotted by WM23 or anti-β-actin antibody. Molecular weight markers are labeled on the right. (H) Bar plot of relative GC levels from noted cells after treatment of vehicle (open bar) or GM6001 (filled bar). The GC band was quantitated by densitometry and normalized against that from CHO-WT cells before GM6001 treatment. Data are shown as mean ± standard deviation, n=3. Comparison of results was performed by one-way ANOVA with *post hoc* tukey test *P<0.05; ***P<0.001.

S3B). With little GPIbα expression on the surface, very few CHO-NΔO cells adhered to the VWF surface. Consequently, only filopodia in CHO-CΔO and CHO-TΔO cells were counted (Figure 3A, C, D). Compared to CHO-WT cells, fewer filopodia and smaller area coverage per cell was observed in CHO-CΔO cells, indicating less robust signaling activity in the CΔO mutant. These results suggest that the CΔO variant, while retaining its ability to bind VWF, is largely devoid of its ability to transmit signals into the cell. The underlying mechanism remains to be defined. A possible explanation could be that removing O-glycans in the C-terminal region stabilizes the MSD and increases the unfolding force threshold such that VWF tether-mediated tension cannot adequately pull open the MSD and induce signaling. Since part of CΔO existed in the cell as non-GPIb complex,[8] it also suggests that the non-GPIb complex may not have the signaling ability. In comparison, adherent CHO-TΔO cells produced more filopodia than CHO-WT cells (Figure 3), suggesting that the GPIbα signaling function may be enhanced by mutations in the TΔO variant. However, it was not clear which TΔO form, or both, may alter the signaling function of GPIbα.

Overall, our results demonstrate that O-glycans at different locations in the MSD exhibit different, or even opposite, effects on GPIbα expression and function. A recent study comparing donor platelets from different ABO blood groups reported that type O platelets form less stable interactions with VWF at arterial shear than non-O platelets.[14] From our results, it is conceivable that a type O blood group in the C-terminal region may reduce GPIb-IX signaling and thereby affect platelet interaction or adhesion with VWF under shear, although a solid link between MSD dynamics and VWF-mediated platelet translocation remains to be established. In addition, a patient diagnosed with the platelet-type von Willebrand disease was reported to miss nine residues (PTILVSATS) in the N-terminal region of the MSD.[15] The GPIbα expression level on platelets is normal in this patient, suggesting that this sequence, including any O-glycans therein, may not significantly affect the stability of the MSD. It remains unclear how missing this 9-residue sequence affects the VWF-binding activity.

Our results indicate that not all Ser/Thr residues in the MSD are glycosylated. The exact O-glycosylation sites in this domain remain to be defined. A striking feature of the TΔO variant is its two distinct glycosylated forms (Figure 1D; *Online Supplementary Figure S1C*). One of them had a WT-like molecular weight, and the other had a higher molecular weight, suggesting different glycosylation pattern.

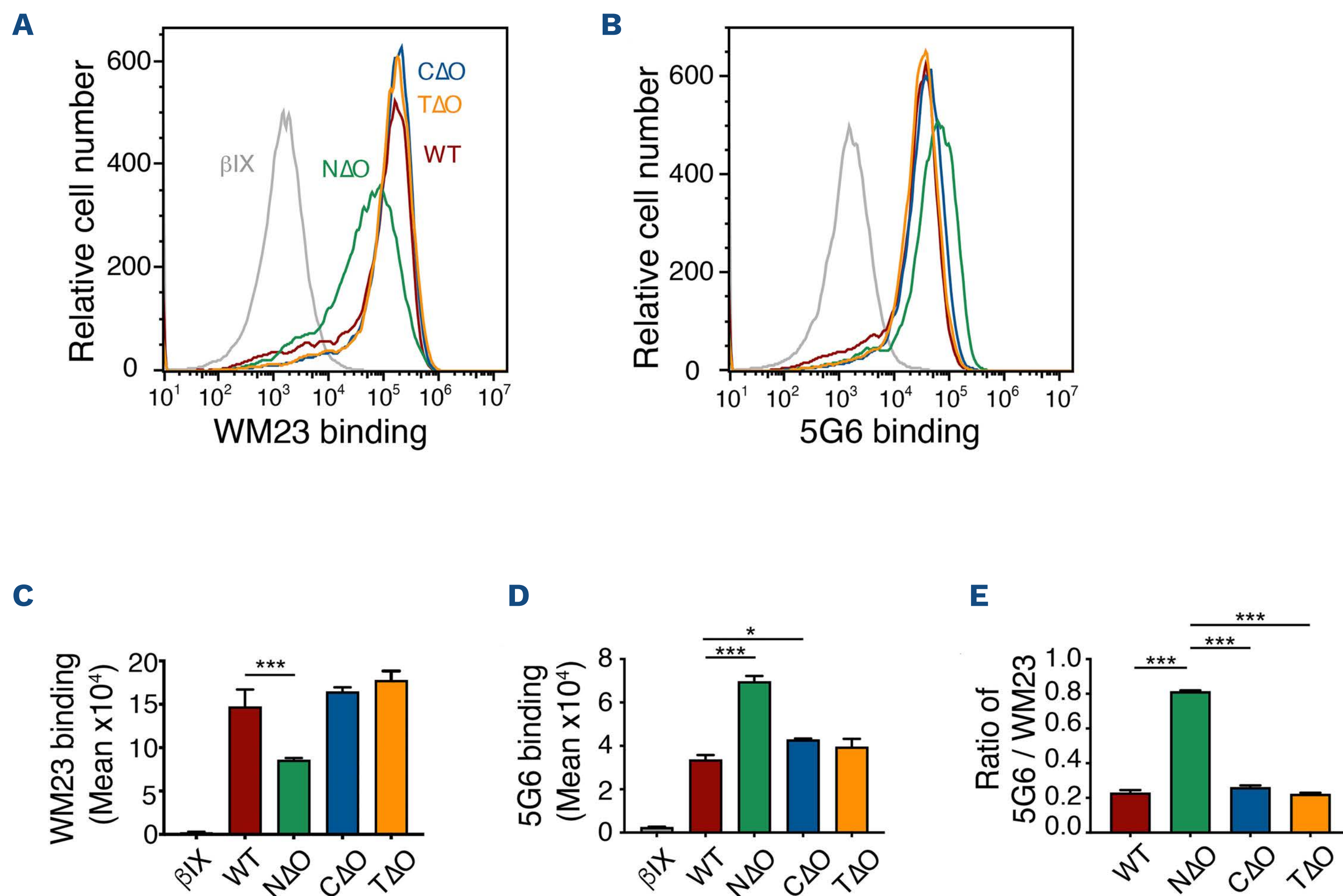

Figure 2. Absence of O-glycans in the N-terminal region increased unfolding of the mechanosensory domain. (A, B) Overlaid flow cytometry histograms showing binding of (A) WM23 antibody and (B) 5G6 antibody in noted stably transfected CHO cells. Each antibody binding level was quantitated by mean fluorescence intensity as described[3,5] and in the *Online Supplementary Appendix*. (C-E) Bar plots showing (C) quantitated WM23 binding level, (D) 5G6 binding level, and (E) ratio of 5G6/WM23 binding in noted variant CHO cells. Data are shown as mean ± standard deviation, n=3. Comparison of results was performed by one-way ANOVA with *post hoc* tukey test. *$P<0.05$; ***$P<0.001$.

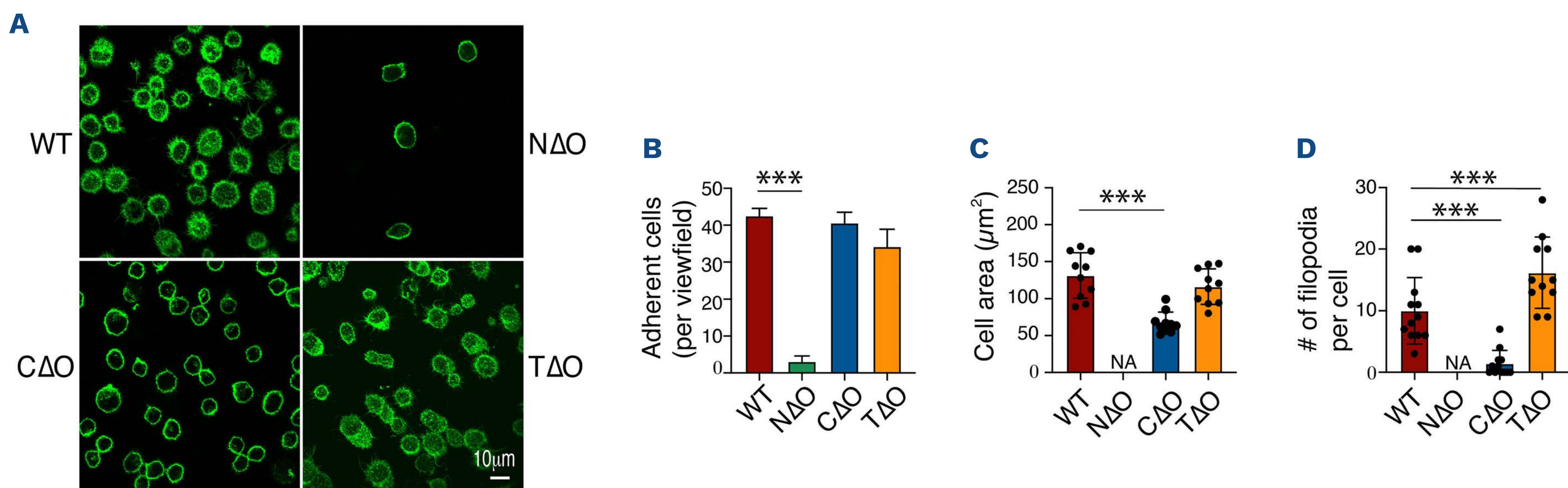

Figure 3. Differential effects of specific O-glycans in the mechanosensory domain on filopodia formation of transfected CHO cells. (A) Representative confocal microscopy images of noted transfected CHO cells that are adhered to von Willebrand factor (VWF) in the presence of botrocetin and ethylenediaminetetraacetic acid (EDTA). Briefly, a glass slide was coated with human VWF at 10 μg/mL in phosphate-buffered saline (PBS) at 4 °C overnight and blocked with 1% bovine serum albumin in PBS for 1 hour at 22°C. CHO cells stably expressing wild-type and mutant GPIb-IX were resuspended in modified Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.35) containing 5 mM EDTA at the concentration of 1×10^6 cells/mL. The cells were placed on VWF-coated slides in the presence or absence of 1 μg/mL botrocetin for 30 minutes (min) at 37 °C. The adherent cells on the slide were washed with PBS buffer, fixed with 4% paraformaldehyde for 10 min, and stained with TRITC-conjugated phalloidin in PBS containing 0.1% Triton X-100 for 30 min. Images were collected on an Olympus Fluo View FV1000 confocal microscope. Images collected in 10-12 viewfields from 2 independent experiments were analyzed using Fiji (ImageJ) and a macro written for quantitative analysis of filopodia.[13] (B-D) Bar plots of (B) number, (C) average surface area, and (D) number of filopodia of adhered CHO cells. Error bars represent mean ± standard deviation. Significance determined by one-way ANOVA with *post hoc* Tukey test. ***$P<0.001$.

In other words, one or both of these Ser residues may not be O-glycosylated. They are important for proper folding of the MSD. Mutating them to alanine may alter the MSD conformation and expose an otherwise shielded Ser/Thr residue or residues for new O-glycosylation. It may also alter GPIbα interaction with GPIbβ/GPIX and form non-GPIb complex that does not contain GPIX (Figure 1D, E; *Online Supplementary Figure S2D*). Also, such an effect may not be the same in transfected CHO cells as in platelets. This complication makes it difficult for the approach of Ser/Thr-scanning mutagenesis, since it cannot be assumed that the effect on O-glycosylation induced by a site-specific mutation in the MSD is limited to that specific site.

Authors

Yingchun Wang and Renhao Li

Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

Correspondence:
R. LI - renhao.li@emory.edu

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Contributions

YW designed and performed research, analyzed results, prepared figures and wrote the manuscript. RL designed research, analyzed results, prepared figures and wrote the manuscript.

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Data-sharing statement

For the original data set, please contact the corresponding author.

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