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ORIGINAL ARTICLE

New insights into regulation of αIIbβ3 integrin signaling by filamin A

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Abstract

Background: Filamin (FLN) regulates many cell functions through its scaffolding activity cross-linking cytoskeleton and integrins. FLN was shown to inhibit integrin activity, but the exact mechanism remains unclear.

Objectives: The aim of this study was to evaluate the role of filamin A (FLNa) subdomains on the regulation of integrin αIIbβ3 signaling.

Methods: Three FLNa deletion mutants were overexpressed in the erythro-megakaryocytic leukemic cell line HEL: Del1, which lacks the N-terminal CH1-CH2 domains mediating the FLNa-actin interaction; Del2, lacking the Ig-like repeat 21, which mediates the FLNa-β3 interaction; and Del3, lacking the C-terminal Ig repeat 24, responsible for FLNa dimerization and interaction with the small Rho guanosine triphosphatase involved in actin cytoskeleton reorganisation. Fibrinogen binding to HEL cells in suspension and talin-β3 proximity in cells adherent to immobilized fibrinogen were assessed before and after αIIbβ3 activation by the protein kinase C agonist phorbol 12-myristate 13-acetate.

Results: Our results show that FLNa-actin and FLNa-β3 interactions negatively regulate αIIbβ3 activation. Moreover, FLNa-actin interaction represses Rac activation, contributing to the negative regulation of αIIbβ3 activation. In contrast, the FLNa dimerization domain, which maintains Rho inactive, was found to negatively regulate αIIbβ3 outside-in signaling.

Conclusion: We conclude that FLNa negatively controls αIIbβ3 activation by regulating actin polymerization and restraining activation of Rac, as well as outside-in signaling by repressing Rho.

KEYWORDS
actin, filamin A, integrin, Rho-GTPase, talin

Essentials

- Filamin A (FLNa) is thought to keep integrin αIIbβ3 turned off, preventing platelet adhesiveness.
- We asked if FLNa domains other than the αIIbβ3-binding domain were involved in αIIbβ3 turnoff.
- Domain deletions of FLNa showed first that the FLNa-actin–binding domain also restrained αIIbβ3.
- This also showed that the FLNa dimerization domain blocks αIIbβ3 signals generated by adhesion.

1 | INTRODUCTION

Integrins are a large family of cell-surface receptors that mediate cell adhesion to the extracellular matrix as well as cell-cell interactions. The interaction between integrins and their various ligands involves dynamic processes leading to different responses such as adhesion, spreading, or migration.[1] Integrin receptors are heterodimers of α and β subunits that contain a large extracellular domain responsible for ligand binding, a transmembrane domain and a short cytoplasmic tail. They mediate signal transduction through the cell membrane in both directions.[2] The initial binding of cells to the matrix or the interaction of soluble cell activating agonists (such as thrombin or ADP in platelets) with their cognate receptors initiates intracellular signaling, leading to an increase in affinity of the integrin for extracellular ligands involving a reversible conformational change of the extracellular domain of the integrin heterodimer (inside-out signaling).[3] In turn, binding of ligands to integrins triggers a signal within the cell, resulting in cytoskeletal reorganization (outside-in signaling).[2,3] The cytoplasmic tail of integrins, and notably of β subunits, plays a pivotal role in these bidirectional signaling processes and consequently in integrin functions through interaction with different partners.[4] Understanding the complex molecular basis of integrin regulation requires identification of these integrin intracellular partners and characterization of their activities.

Among these proteins, actin-binding proteins such as filamins (FLNs),[5,6] talin,[7,8] kindlin3, and α-actinin associate with the β-integrin subunit cytoplasmic tail, ensuring many connections between integrins and the actin cytoskeleton. The first cytoplasmic protein shown to directly bind integrins was talin. A number of elegant studies showed that talin is a critical integrin-activating protein and that talin binding to β tail integrins via its FERM (4.1, ezrin, radixin, moesin) domain was an essential final step in integrin activation.[9,10] In platelets, in vitro and in vivo studies have clearly shown that the binding of talin to the β3 integrin tail is critical for agonist-induced αIIbβ3 activation and that the selective disruption of talin-β3 interaction protects mice from thrombosis.[9,10] Kindlin3 is another important regulator of integrin activation. It has been proposed that talin modulates αIIbβ3 affinity, while kindlin-3 modulates clustering and avidity or recruitment of talin to the plasma membrane in the proximity of αIIbβ3.[11]

FLN, another important actin-binding protein, ensures the link between integrin and actin cytoskeleton. FLNa is one among three isoforms of FLNs (filamin A [FLNa], filamin B [FLNb], and filamin C [FLNc]), a family of cytoskeletal proteins with high molecular mass that organize actin filaments into networks and link these actin networks to cell membranes.[12] FLNa and FLNb are ubiquitously expressed, while FLNc is expressed in skeletal and cardiac muscle cells. FLNs are homodimers of two monomers of 280 kDa subunits. Each subunit contains an N-terminal actin-binding domain composed of two calponin homology domains followed by 24 Ig-like (IgFLN) domains that are interrupted by flexible hinge regions between domains 15 and 16, and 23 and 24.[12,13] Dimerization through the Ig24 FLNa domain results in a flexible parallel homodimer that can promote high-angle branching of actin filaments. In addition, IgFLN domains act as scaffolds to numerous transmembrane receptors and cytosolic signaling proteins. In platelets, FLNa is the most abundant isoform.[14,15] A direct FLNa-β3 interaction in resting platelets requiring FLNa repeat 21 (possibly also Ig repeats 9, 12, 17, and 19) and β3 amino acids 747 to 755 awaits direct evidence. Indeed, the concept of constitutive binding of FLNa to the β3 tail to keep αIIbβ3 at rest in platelets comes only from binding assays using protein constructs and structural biology data, and not from in vivo cellular assays.[16,17] It has been proposed that the activation of integrin αIIbβ3 requires the dissociation of FLNa from the β3 cytoplasmic domain.[16,17] However, more recently, based on structural analysis, a new molecular mechanism for FLNa-mediated retention in a resting state of the integrin has been proposed.[18] In this model, FLNa claps together the αIIb and β3 cytoplasmic tails, thereby stabilizing αIIbβ3 in an inactive state and preventing spontaneous αIIbβ3 activation. FLNa was proposed to prevent talin interaction with β3 and integrin activation. However, this mechanism awaits direct experimental evidence in platelets, in particular because of contradictory observations: Basal activation of αIIbβ3 was not observed in the absence of FLNa in Flna-null mice[15] nor in a gain-of-function mutant FLNa in human platelets,[19] suggesting that FLNa does not play a negative role in integrin regulation in platelets.[15] One possibility is that FLNa domains are also mechanically regulated.[20] Indeed, intramolecular interactions such as FlnA 20 to 21 and 18 to 19 domains have been shown to autoinhibit filamin.[20] The reorganization of the actin cytoskeleton regulated by its interaction with FLNa[15] in the C-terminal region was not investigated with respect to αIIbβ3 activation. Also, the role of the dimerization domain of the FLNa N-terminal region and the interaction of different FLNa partners such as the Rac and Rho guanosine triphosphatases (GTPases) were also unexplored. FLNa has also been reported to play roles in integrin regulation other than inactivation. Indeed, FLNa seems to be required for optimal T-cell integrin-mediated force transmission, flow adhesion, and cell trafficking.[21]

The focus of this study was therefore to determine whether other FLNa domains were involved in the regulation of αIIbβ3 activation

and "outside-in" signaling of αIIbβ3 integrin using FLNa constructs overexpressed in erythro-megakaryocytic leukemic (HEL) cells. These constructs have already been used in a previous work evaluating the role of the FLNA subdomains in platelet biogenesis.[22] We found that FLNa-actin and FLNa-β3 interaction exercise a negative regulation on αIIbβ3 activation, whereas the dimerization domain of FLNa, which interacts with the small Rho and Rac GTPases, negatively regulates outside-in signaling of αIIbβ3. We conclude that Rac and Rho GTPases are maintained in an inactive state by FLNa-actin interaction and FLNa-Rho interaction, respectively.

2 | MATERIAL AND METHODS

2.1 | Material

Fibrinogen was obtained from HYPHEN BioMed SAS (Neuville sur Oise, France). Alexa Fluor 488–labeled phalloidin, Oregon Green 488-labeled fibrinogen, Alexa Fluor secondary antibodies and ProLong Gold antifade reagent were from Molecular Probes (Eugene, OR, USA). The CD41/CD61-PE and REA-PE antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Rac inhibitor (EHop-016) and Rho-associated protein kinase (ROCK) inhibitor (Y-27632) were obtained from Abcam (Cambridge, England). Anti-human β3 chain was purchased from Becton Dickinson (East Rutherford, NJ, USA). The monoclonal anti-active Rac-GTP was from Biomol (Kelayres, PA, USA). The polyclonal antibody directed against integrin αIIb (clone P2) was from Beckman Coulter (Brea, CA, USA). The polyclonal antibodies directed against talin, phosphorylated myosin light chain (MLC-P) and FLNa were obtained from Abcam. Peroxydase-conjugated AffiniPure secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA). Proximity ligation assay (Duolink) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HEL cells [HEL 92.1.7 (ATCC TIB-180)] were from American Type Culture Collection (Manassas, VA, USA).

2.2 | FLNa expression in the HEL cell line

Constructs encoding wild-type (WT), Del1, Del2, and Del3 FLNa cDNAs correspond to those previously described respectively as WT, Del1, Del3, and Del4.[22] Del1 represents a mutant lacking the actin-binding domain (ABD) at the N-terminus, Del2 a mutant lacking the Ig21 domain that interacts with the β3 domain, and Del3 a mutant lacking the dimerization domain involved in the interaction with small GTPases as previously described.[22]The cDNAs were cloned into adeno-associated virus integration site 1 (AAVS1)-SA-2A-puro-pA plasmid under the regulation of the CAG promoter and a zinc finger nuclease-based technology was used to generate stable cell lines, by targeting constructs to the AAVS1 locus as previously described.[22] HEL cells were transfected with the constructs by nucleofection using the Neon transfection system 5 (Invitrogen-ThermoFisher Scientific, Saint-Aubin, France) according to the manufacturer's instructions using 1 μg of plasmid DNA for 10^5 cells. Expression and stability of the mutant FLNa proteins were verified by immunoblotting. Note that HEL cells instead of native megakaryocytes were used in this study. Indeed, this model (HEL cells) was technically easier to control. Identical expression levels of αIIbβ3 integrin and FLNa in WT versus mutants Del1, Del2, and Del3 HEL cells was very difficult to obtain in native megakaryocytes. Moreover, the quantity of megakaryocytes as previously described by our group[22] was relatively low, increasing the difficulty of the experiments.

2.3 | Fibrinogen binding to HEL cells

Activation of αIIbβ3 in FLNa-expressing HEL cells was evaluated by soluble fibrinogen binding using flow cytometry (Accuri C6 Plus flow cytometer; Becton Dickinson). Cells were stimulated with 800 nM phorbol 12-myristate 13-acetate (PMA) for 10 minutes in the presence of 20 μg/mL of fibrinogen (5 μg Oregon Green–labeled fibrinogen + 15 μg cold fibrinogen) as previously described.[19] Specific binding to αIIbβ3 was measured by flow cytometry in the presence or absence of a monoclonal antibody (clone P2; 10 μg/ml) blocking the binding of fibrinogen to αIIbβ3. The difference represents the specific fibrinogen binding to αIIbβ3.

2.4 | Proximity ligation assay

FLNa-expressing HEL cells were stimulated with 20 nM PMA for 5 minutes and allowed to adhere onto fibrinogen coated coverslips (10 μg/mL) for 30 minutes at 37°C. Adherent cells were fixed with 4% paraformaldehyde in cytoskeleton buffer (0.1 M PIPES; 2 M Glycerol; 1 mM EGTA; 1 mM $MgCl_2$; pH 6.9) for 15 minutes, then permeabilized in the same buffer containing 0.2% Triton X-100 for 5 minutes. Fixed cells were incubated overnight with the αIIb-specific P2 mouse monoclonal antibody (2 μg/mL), and a rabbit polyclonal antibody directed against talin (2 μg/mL). Proximity ligation assays were performed according to the manufacturer's instructions (Duolink) using oligonucleotide-coupled secondary antibodies against mouse and rabbit primary antibodies (proximity ligation assay [PLA] probes). Only when a pair of PLA probes have bound two primary antibodies in close proximity (<40 nm), a red fluorescent spot is generated because of the hybridization and circularization of fluorescently labeled oligonucleotides during the amplification reaction. No fluorescence was detected when the cells were subjected to the reaction in the presence of the two complementary oligonucleotides but in the absence of the primary antibodies. The number of spots per cell was quantified using an epifluorescence microscope and Fiji software.

2.5 | Immunoblotting

HEL cells were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 μM

phenylarsine oxide, 1% SDS, 5 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4). The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with various primary antibodies (see Results section). Immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Pierce). Images of the chemiluminescent signal were captured using a G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene, Karnataka, India).

2.6 | Statistical analysis

Results were analyzed using one-way analysis of variance followed by least significant difference multiple comparisons as indicated.

3 | RESULTS

3.1 | Expression of WT and FLNa mutants in HEL cells

To determine which FLNa domains regulate αIIbβ3 activation, WT and mutant FLNa constructs were overexpressed in HEL cells. Indeed, among the hematopoietic cell lines, with megakaryocytic potential, HEL cells have been regularly used to study αIIbβ3 activation.[19,23] The choice of the deleted domains was dictated by the importance of these FLNa domains in different cell functions[14] as previously described (Figure 1A). Del1 lacks the ABD at the N-terminus and is important for actin reorganization. Del2 lacks the Ig repeat 21 domain that interacts with the β3 domain and appears essential for the regulation of αIIbβ3 activation. Del3 lacks the dimerization domain at the C-terminus and the interaction with the small Rho and Rac GTPases required for actin reorganization.

We first targeted insertion of FLNa expression constructs into the AAVS1 (Figure 1B). Transfection efficiency was evaluated by western blotting using an antibody specific for FLNa. 14-3-3ζ, which was previously shown to undergo very little biological variation in platelets,[24] was used as loading control. First, the empty vector-transfected HEL cells (control) exhibit a weak basal level of FLNa (Figure 1C). Comparably high levels of FLNa are expressed in WT- and mutant-transfected cells, reaching six- to sevenfold the level of FLNa in the control (empty vector). This large increase in FLNa overexpression in WT- and mutant-transfected cells over the endogenous FLNa level allowed us to examine the effect of FLNa WT and mutants on αIIbβ3 activation in the next experiments.

3.2 | Fibrinogen binding to αIIbβ3 in HEL cells expressing FLNa WT and FLNa mutants

The next step was to evaluate the effect of the deletion of FLNa domains on αIIbβ3 activation in HEL cells activated or not with

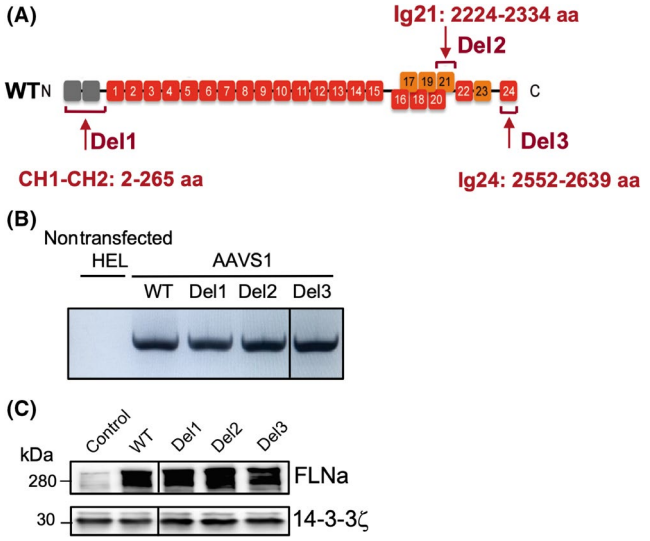

FIGURE 1 Expression of WT and mutants FLNa in HEL cells. (A) Several *FLNA* cDNAs lacking domains essential for cell function were transfected in HEL cells. A mutant lacking the ABD at the N-terminus (Del1), a mutant lacking the Ig repeat 21 domain that interacts with β3 (Del2) and a mutant lacking the dimerization domain involved in the interaction with the small Rho and Rac GTPases (Del3). The full-length *FLNA* cDNA (WT) was introduced as a positive control and the empty vector as a negative control. Diagram adapted from Falet.[14] (B) A zinc finger nuclease-based technology targeting the AAVS1 locus was used. Integration at the AAVS1 locus was checked by polymerase chain reaction. (C) The level of FLNa protein expressed was quantified by immunoblotting using a polyclonal antibody against FLNa. AAVS1, adeno-associated virus integration site 1; ABD, actin-binding domain; FLNa, filamin A; GTPase, guanosine triphosphatase; HEL, erythro-megakaryocytic leukemic; WT, wild-type

PMA; activator of protein kinases C. This model was previously well characterized and used to investigate the regulation of αIIbβ3 activation.[19,23] Because of the reported increased level of β3 integrin in FlnA-null platelets,[15] the first step was to examine the expression of αIIbβ3 integrin in WT- and mutant-transfected cells by flow cytometry (Figure 2A-D). No difference in αIIbβ3 expression level was observed between empty vector-transfected cells (heretofore termed *control cells*) and FLNa WT- or mutant-transfected cells (Figure 2E). Then, αIIbβ3 activation was assessed by flow cytometry by measuring binding of Oregon Green–labeled fibrinogen to PMA-activated (800 nM) HEL cells in suspension (Figure 2F). Specific binding of fibrinogen to αIIbβ3 and not to other integrins was determined by subtracting the residual fibrinogen binding obtained in the presence of the αIIbβ3-blocking monoclonal antibody (P2) from total binding in the absence of P2. First, in the absence of PMA, no specific fibrinogen binding was observed in control WT- and mutant-transfected cells, indicating that αIIbβ3 integrin activation does not take place in resting cells (Figure 2F). In contrast, in the presence of PMA, a specific fibrinogen binding to αIIbβ3 integrin occurred whether cells were transfected or not (Figure 2F). The difference in fibrinogen binding between

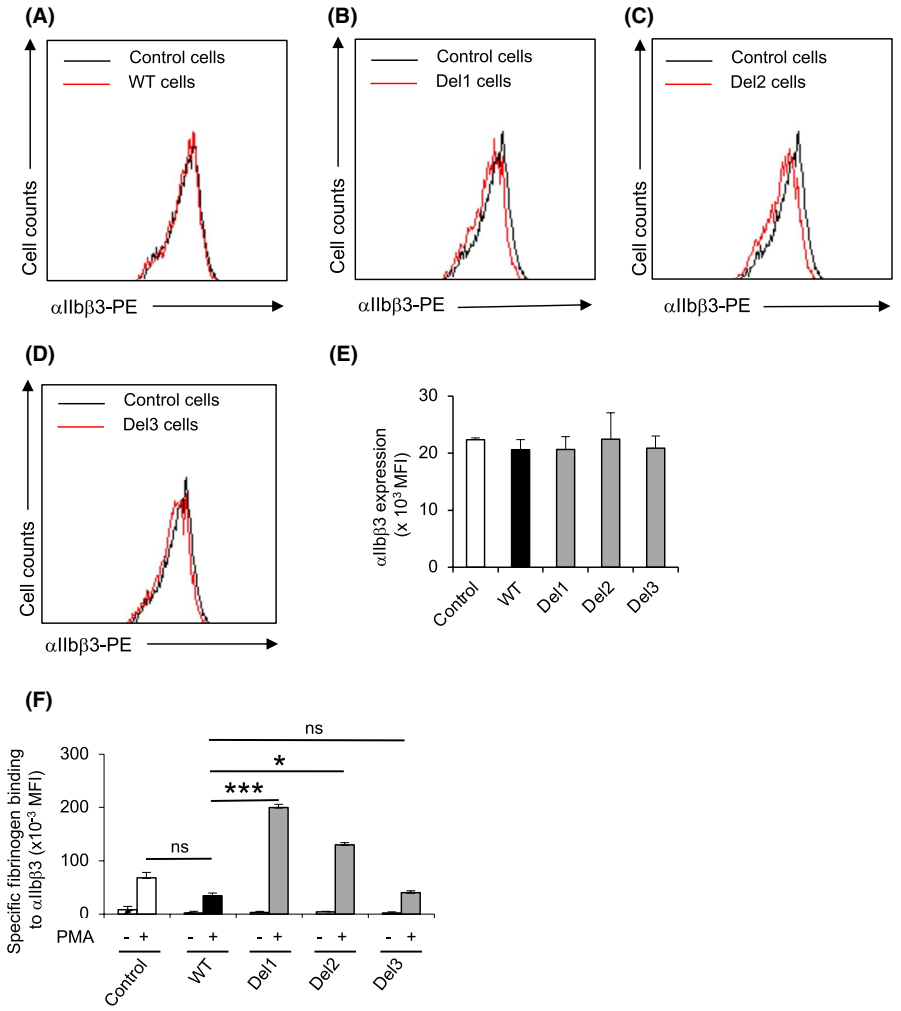

FIGURE 2 Fibrinogen binding is enhanced in HEL cells expressing mutants lacking FLNa-actin (Del1) and FLNa-β3 (Del2) interactions. (A-E) The expression level of αIIbβ3 was quantified in HEL cells transfected with plasmids encoding wild-type FLNa (FLNa WT) or FLNa mutants by flow cytometry (BD Accuri C6+ flow cytometer) using a CD41/CD61-PE antibody (to detect αIIbβ3) and a Isotype control antibodies; clone REA293 (REA-PE) antibody as control. The bar graph represents the means ±SEM of three independent determinations. (F) Then HEL cells transfected with plasmids encoding FLNa WT or FLNa mutants were stimulated with 800 nM PMA in the presence of Oregon Green 488–labeled fibrinogen (20 μg/mL). The empty vector was transfected as control. Specific fibrinogen binding with or without PMA and in the presence or absence of a specific monoclonal antibody (P2) blocking fibrinogen binding to αIIbβ3 was measured by flow cytometry (BD Accuri C6+ flow cytometer). The graph represents the means ±SEM of six independent determinations (*$P < .05$, one-way analysis of variance followed by Tukey's multiple comparison). FLNa, filamin A; HEL, erythro-megakaryocytic leukemic; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean

control and WT-transfected cells was not statistically significant (Figure 2F), suggesting that high expression levels of FLNa do not interfere with the regulation of αIIbβ3 activation. Interestingly, FLNa-Del1 transfection (suppression of the FLNa-actin–binding domain) induced a significant increase in fibrinogen binding (5.8-fold; $P = .001$, n = 6 compared to FLNa WT [Figure 2F]), strongly suggesting that αIIbβ3 activation was negatively regulated by FLNa-actin interaction. Likewise, Del2 transfection (suppression of FLNa-β3 interaction) induced a 3.8-fold increase in fibrinogen binding ($P = .03$, n = 6 [Figure 2F]) consistent with FLNa-β3 interaction negatively regulating αIIbβ3 activation. Finally, Del3 transfection (absence of the FLNa dimerization domain preventing the interactions with Rho/Rac GTPases) yielded low fibrinogen binding similar to FLNa WT (Figure 2F), indicating that this C-terminal domain of FLNa does not impact αIIbβ3 activation and does not participate to inside-out signaling. Altogether, these results clearly show that FLNa-actin and FLNa-β3 interactions negatively regulate αIIbβ3 activation.

3.3 | FLNa-actin interaction with β3 affects negatively the proximity of talin with β3 in adherent HEL cells

Next, using an adhesion assay of HEL to a fibrinogen matrix, we explored how the absence of FLNa-actin interaction (Del1) could affect the recruitment of talin to β3 integrin, a requirement for αIIbβ3 activation, in the presence or absence of PMA activation.

First, the adhesion was assessed on a fibrinogen matrix (10 μg/mL) during 30 minutes. Quantification of adherent cells showed a similar adhesion of FLNa WT and FLNa-Del1 cells in the absence of PMA (Figure 3A). In contrast, in the presence of PMA (20 nM), adhesion of FLNa-Del1 was significantly higher than that observed in the absence of PMA (19.5 ± 0.7 cells/field compared to 11.0 ± 1.2 cells/field; $P = .03$ [Figure 3A]). Altogether, our results suggest that FLNa-actin interaction is involved in the negative regulation of adhesion by activated αIIbβ3.

Before considering talin recruitment to αIIbβ3, we first examined the expression level of talin, evaluated by western blotting. Similar expression levels of talin were observed in WT- and Del1-transfected cells compared with control cells (Figure 3B) allowing the quantification of talin-β3 closeness by proximity ligation assay (PLA; DuoLink assay). First, in the absence of PMA, bright red dots indicated a basal level (around 5 dots/cell) of talin-β3 complexes in HEL cells transfected with control or both FLNa constructs (Figure 3C). In the presence of PMA, the level of talin-β3 complexes significantly increased in cells expressing FLNa-Del1 (15.0 ± 1.4 dots/cell) compared to FLNa-WT (7.1 ± 0.8 dots/cell; $P = .008$ [Figure 3C-D]). The significant ($P = .0007$) increase in talin-β3 complexes by PMA (15.0 ± 1.4 dots/cell vs 4.6 ± 0.6 dots/cell without PMA) was observed only in Del1-transfected cells and correlated with a significant decrease in FLNa-β3 interaction with PMA (7.2 ± 1.1 dots/cell; $P = .002$ vs 17.6 ± 2.3 dots/cell in absence of PMA [Figure 3E]). Note that the dissociation of FLNa from β3 after PMA was similar in Del1 and WT cells, indicating that FLNa-actin interaction is not involved in the dissociation of FLNa from β3 and suggesting that talin recruitment requires FLNa dissociation from β3 associated with another event. This is consistent with FLNa-actin interaction acting as a negative regulator of αIIbβ3 activation probably by preventing the recruitment of talin to β3 integrin. In addition, talin-β3 vicinity was correlated with cytoskeleton assembly: some very spread-out FLNa-Del1 cells exhibited very high levels of talin-β3 complexes (Figure 3C) correlating with a spatial organization of polymerized actin different from control and FLNa WT cells: virtually no stress fibers or focal adhesion plaques or focal points (Figure 3D, Figure S1). Indeed, in FLNa WT cells, cortical actin appears distributed at the periphery of the cytoplasm and stress fibers are well organized (Figure S1). In contrast, a disorganization of stress fibers was observed in FLNa-Del1 cells associated with less pronounced labeling of cortical actin. These results suggest strongly that cortical cytoskeleton is differently regulated in FLNa-Del1 cells allowing easier recruitment of talin. Finally, the level of actin polymerization as measured by total fluorescence intensity/cell was not significantly increased in FLNa-Del1 cells (81.0 ± 8.7 AU) compared with control cells (69.6 ± 5.7 AU) and FLNa WT cells (66.1 ± 6.2 AU [Figure 3F]). However, FLNa-Del1 cells exhibited statistically significant increased areas, higher (650.1 ± 30.7 μm^2) than in FLNa-control cells (508.0 ± 26.0 μm^2) or FLNa WT cells (494.0 ± 23.6 μm^2; $P = .008$; Figure 3G). Altogether, these results indicate that FLNa-actin interaction, which seems responsible for the spatial organization of actin, negatively affects talin-β3 proximity.

3.4 | FLNa-actin interaction negatively controls talin-β3 proximity via Rac inactivation

Because the spatial organization of actin is dependent on the small Rho and Rac GTPases, we next evaluated the role of these GTPases in talin-β3 proximity. The role of Rho in talin recruitment to β3 in the presence of PMA was assessed using Y-27632, a specific inhibitor of the ROCK, a RhoA effector. When FLNa WT cells were pretreated with Y-27632 (10 μM), a tendency ($P = .06$) to increased talin-β3 complexes (10.8 ± 1.7 dots/cell vs 4.0 ± 1.4 dots/cell in the absence of Y-27632) was observed (Figure 4A), suggesting that Rho is activated (Rho-GTP) in FLNa WT cells, consistent with the large stress fibers observed in these cells (Figure 3D). Importantly, in FLNa-Del1 cells, the recruitment of talin by β3 significantly increased compared to FLNa WT cells (14.9 ± 2.1 dots/cell vs 4.0 ± 1.4 dots/cell; $P = .02$) and was not affected by the ROCK inhibitor (13.2 ± 1.0 dots/cell in the presence of Y-27632). Altogether, these results indicate that FLNa-actin interaction counteracts the proximity of talin and β3 independently of Rho activation.

Because FLNa-Del1 cells were larger and thus more spread out compared with FLNa-WT cells, we examined a potential role of Rac, another small Rho-family GTPase known to control actin polymerization of branched cytoskeleton involved in spreading. We thus next assessed talin-β3 proximity using a specific Rac inhibitor (EHop-016). Cell pretreatment with 10 μM EHop-016 for 15 minutes had no effect on talin-β3 proximity in control cells and FLNa WT cells (Figure 4B), However, in FLNa-Del1 cells, the high level of talin-β3 complexes (20.4 ± 1.6 dots/cell) was strongly reduced by the Rac inhibitor (4.4 ± 0.4 dots/cell; $P = 3.6 \times 10^{-7}$). This suggests that, contrary to Rho, Rac may positively regulate talin-β3 proximity but is repressed by the FLNa domains CH1 to CH2. Paralleling FLNa-dependent talin-β3 assembly behavior, the increased cell area observed in FLNa-Del1 (650.0 ± 30.7 μm^2) compared with control cells (508.2 ± 26.0 μm^2; $P = .03$) returned to basal level after Rac inhibition (393.0 ± 9.4 μm^2; $P = .005$; Figure 4C and Figure S2). Finally, the activation of Rac (Rac-GTP) in FLNa-Del1 and FLNa WT cells was quantified by immunofluorescence assay with an antibody recognizing specifically the active form of Rac. In FLNa-Del1 cells, mainly submembranous Rac-GTP was significantly increased (173%; $P = .005$) compared with FLNa WT cells (100% [Figure 4D, E]) consistent with FLNa-actin maintaining Rac in an inactive state. All these results indicate that the inactive state of Rac (Rac-GDP), maintained by the FLNa-actin interaction, is required for the negative regulation of talin-β3.

3.5 | FLNa-β3 interaction negatively regulates talin-β3 proximity in adherent HEL cells

The αIIbβ3-FLNa interaction and its role in platelets remain unclear. FLNa was proposed to prevent talin binding to β3 and to maintain the integrin in a resting state, thus implying that αIIbβ3 activation required the dissociation of FLNa from β3 before its activation.[16,17] However, recent data suggested that talin association to β3 may occur in the absence of FLNa release from β3.[18] We thus explored

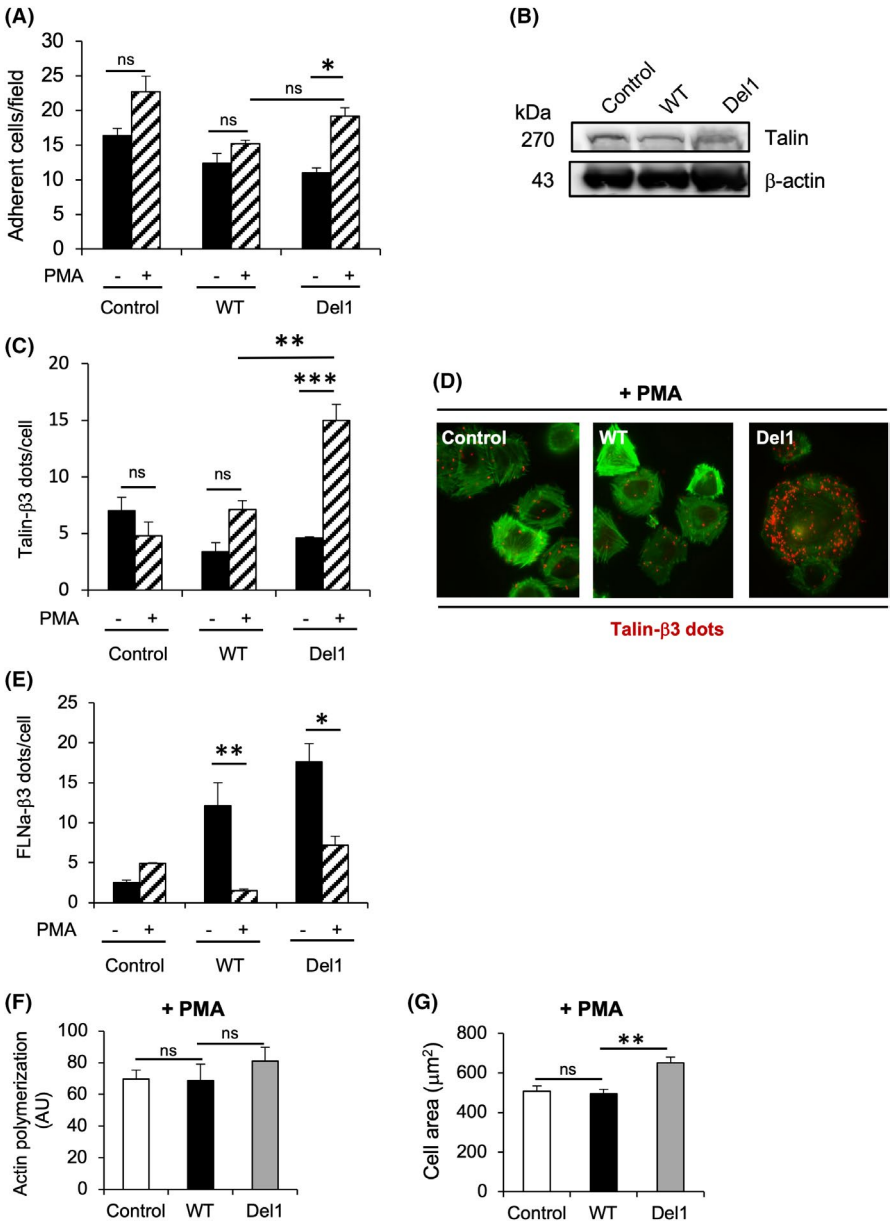

FIGURE 3 FLNa-actin interaction regulates negatively talin-β3 proximity. Transfected cells (WT or Del1 mutant) were stimulated or not by PMA (20 nM), plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488-labeled phalloidin. (A) Cell adhesion was visualized by fluorescence microscopy and quantified using the Fiji software. (B) Talin expression level in control, WT and Del1 cells was assessed by immunoblotting. (C-E) Talin-αIIbβ3 and FLNa-αIIbβ3 proximity were assessed by proximity ligation assay (PLA; see Methods) and fluorescence microscopy. Red spots correspond to PLA positive signals. Nonspecific signal was detected when HEL cells were incubated without primary antibodies ("negative control"). Red spots per cell were quantified using the Fiji software. For each experiment, at least 200 cells per condition were analyzed. (F-G) Actin polymerization and cell spreading were visualized with Alexa Fluor 488–labeled phalloidin and quantified. The graphs represent the means ±SEM of three independent experiments for (E to G) and 8 independent experiments for (A and C) (*$P < .05$, **$P < .01$, ***$P < .001$, one-way analysis of variance followed by Tukey's multiple comparison). Note that experiments in Figures 3, 5, and 6 were run in parallel. Thus, CTRL and WT are identical in all three figures. FLNa, filamin A; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean

in HEL cells adhering to a fibrinogen matrix, the recruitment of talin to β3 integrin in conditions of absence of FLNa-β3 interaction by overexpressing the mutant FLNa-Del2.

First, cell adhesion was assessed on a fibrinogen matrix (10 μg/mL) during 30 minutes. In the absence of PMA, the similar level of adhesion observed with FLNa WT (12.4 ± 1.4 cells/field) and FLNa-Del2 cells (9.0 ± 0.7 cells/field) was not significantly decreased compared with control cells (16.4 + 1.0 cells/field; Figure 5A). In contrast, stimulation with PMA induced a significant increase in adhesion of FLNa-Del2 cells (from 9.0 ± 0.7 to 19.5 ± 1.3 cells/field; $P = .01$) consistent with inhibition of adhesion by FLNa bound to β3. After checking the normal expression of talin in FLNa-Del2 cells (Figure 5B), assessment of talin-β3 proximity after PMA activation indicated a significant increase in

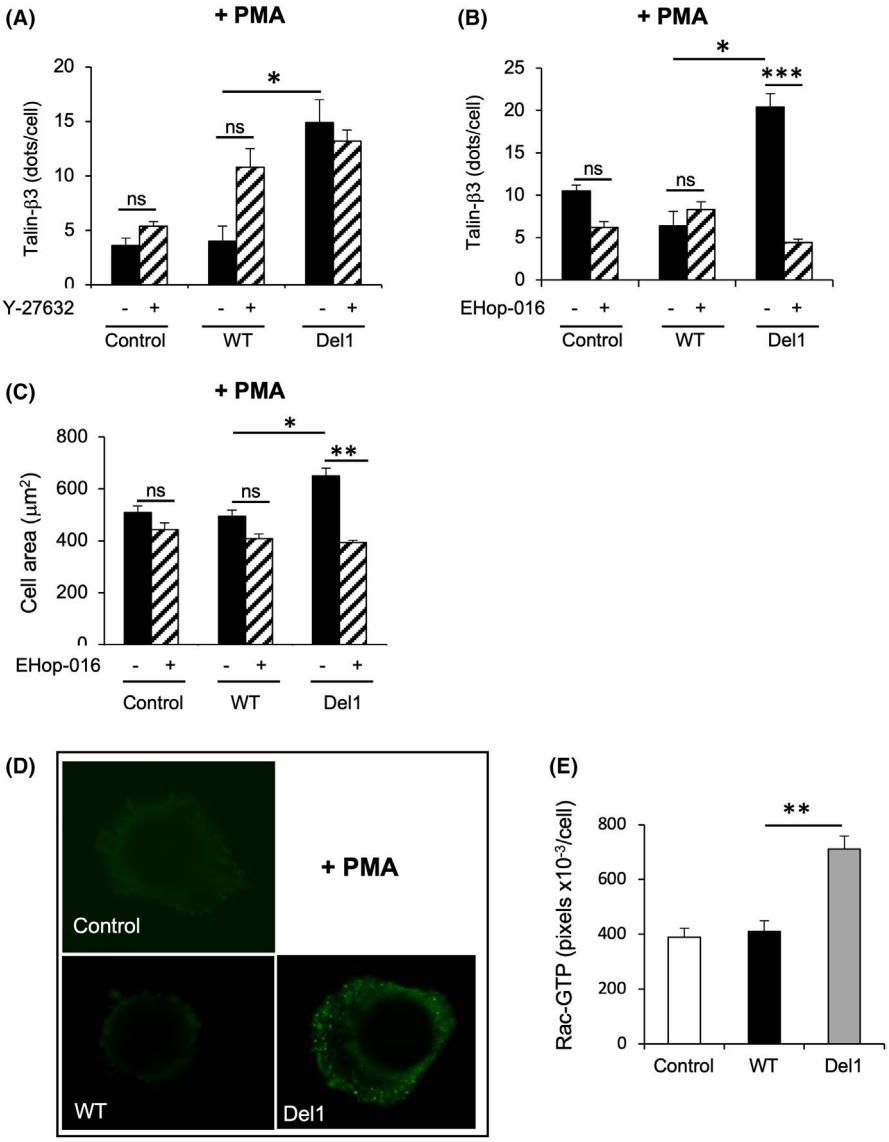

FIGURE 4 FLNa-actin interaction negatively regulates talin-β3 proximity via Rac inactivation. Transfected cells (WT or Del1 mutant) stimulated by PMA (20 nM) in the presence or absence of the ROCK inhibitor Y-27632 (10 μM) (A) or the Rac inhibitor EHop-216 (10 μM) (B-C) were plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488–labeled phalloidin. (A-B) Talin-αIIbβ3 proximity was assessed by PLA and dots visualized by fluorescence microscopy. (C) Spreading was visualized with Alexa Fluor 488-labeled phalloidin and quantified. (D-E) Activated Rac (Rac-GTP) was visualized and quantified with a fluorescence microscope. For each experiment, at least 200 cells per condition were analyzed. The graphs represent the means ±SEM of 3 independent experiments and statistical significance was determined by one-way analysis of variance followed by Tukey's multiple comparison (*$P < .05$, **$P < .01$, ***$P < .001$). FLNa, filamin A; PLA, proximity ligation assay; PMA, phorbol 12-myristate 13-acetate; ROCK, Rho-associated protein kinase; SEM, standard error of the mean

FLNa-Del2 cells (21.2 ± 3.2 dots/cell) compared with FLNa WT cells (8.2 ± 1.2 dots/cell; $P = .002$ [Figure 5C]). Interestingly, a significant increase in talin-β3 proximity ($P = .008$) after PMA was observed only in FLNa-Del2 cells. These results indicate that inhibition of talin-β3 proximity by FLNa-β3 interaction is dependent on PMA activation (Figure 5C). As expected, a similar profile of FLNa-β3 interaction was observed in control (empty vector) and FLNa-Del2 cells in the presence or absence of PMA confirming that only endogenous FLNa interacts with β3 in FLNa-Del2 cells (Figure 5D). In parallel, actin polymerization was similar in Del2 cells (88.1 ± 3.6 AU) and WT cells (66.8 ± 6.2 AU [Figure 5E]). In the same way, the phosphorylation of the myosin light chain (MLC) reflecting the activation of the GTPase Rho, did not increase in Del2 cells compared with FLNa WT cells (Figure 5F, 5G). Finally, we examined the role of actin reorganization on talin-β3 proximity. As already shown in Figure 4A, talin-β3 proximity in FLNa WT cells (9.1 ± 2.7 dots/cells) did not significantly increase in the presence of the ROCK inhibitor (10 μM) (17.7 ± 3.0 dots/cell) (Figure 5H). In contrast, a similar increase in talin-β3 proximity in FLNa-Del2 cells in both the absence and presence of the ROCK inhibitor, was consistent with FLNa-β3 interaction negatively controlling αIIbβ3 activation independently of Rho. Altogether, these results indicate that FLNa-β3 interaction acts as a negative regulator of integrin activation independently of Rho.

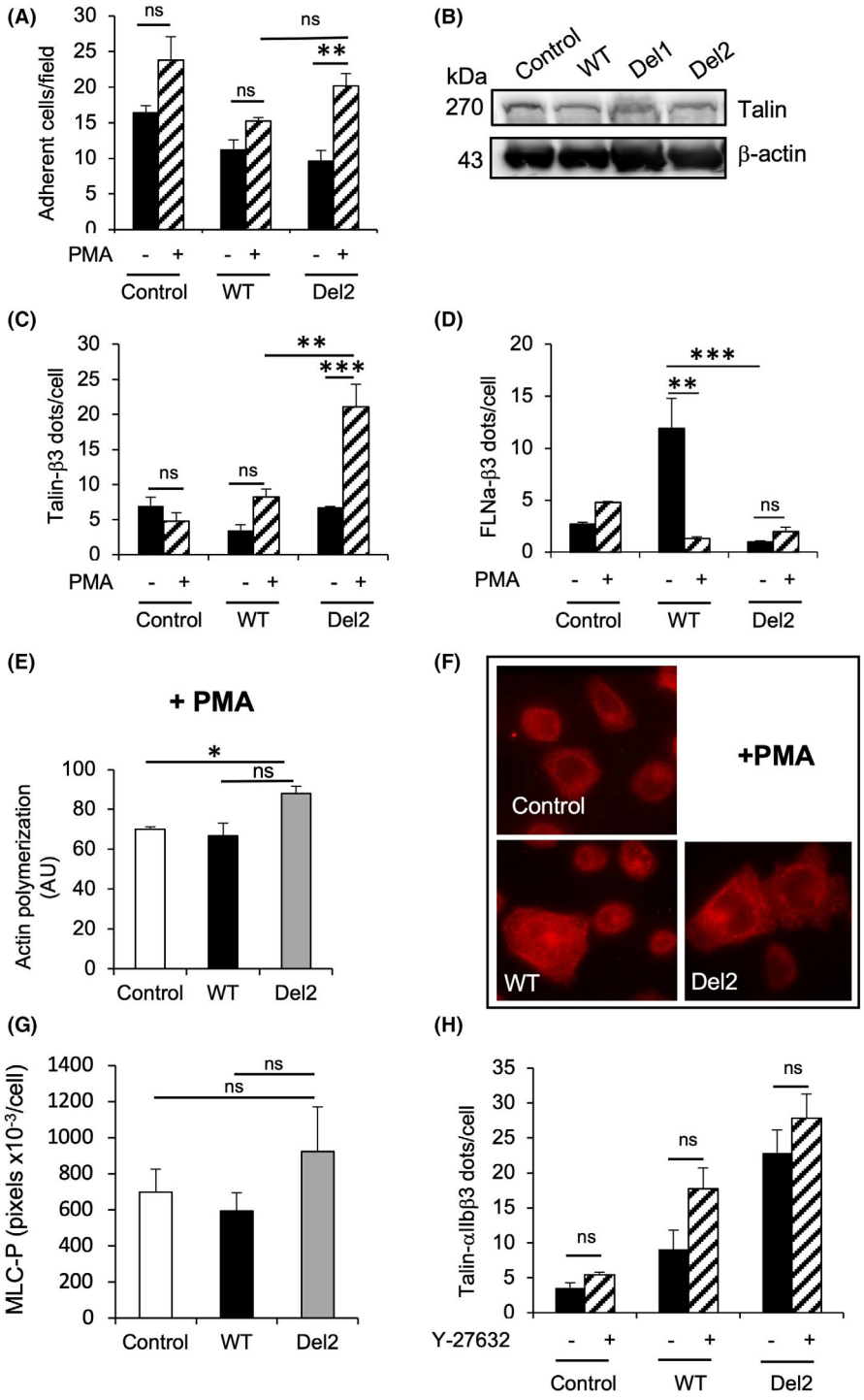

FIGURE 5 FLNa-αIIbβ3 interaction negatively regulates talin-β3 proximity. Transfected cells (WT or Del2 mutant) stimulated or not by PMA (20 nM) were plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488–labeled phalloidin. (A) Cell adhesion was visualized by fluorescence microscopy and quantified using the Fiji software. (B) Talin expression level was assessed by immunoblotting. (C-D) Talin-and FLNa-αIIbβ3 proximity, respectively, was assessed by PLA and fluorescence microscopy. Red spots per cell were quantified using the Fiji software. For each experiment, at least 200 cells per condition were analyzed. (E-G) Actin polymerization and MLC phosphorylation were visualized and quantified by fluorescence microscopy. (H) Talin-αIIbβ3 proximity was assessed by PLA in the presence of the ROCK inhibitor Y-27632 (10 μM). The graphs represent the means ±SEM of 3 independent experiments for E, F, G, and 8 independent experiments for A and C (*$P < .05$, **$P < .01$, ***$P < 0.001$, one-way analysis of variance followed by Tukey''s multiple comparison). Note that experiments in Figures 3, 5, and 6 were run in parallel. Thus, CTRL and WT are identical in all three figures. MLC, myosin light chain; PLA, proximity ligation assay; PMA, phorbol 12-myristate 13-acetate; ROCK, Rho-associated protein kinase; SEM, standard error of the mean; WT, wild-type

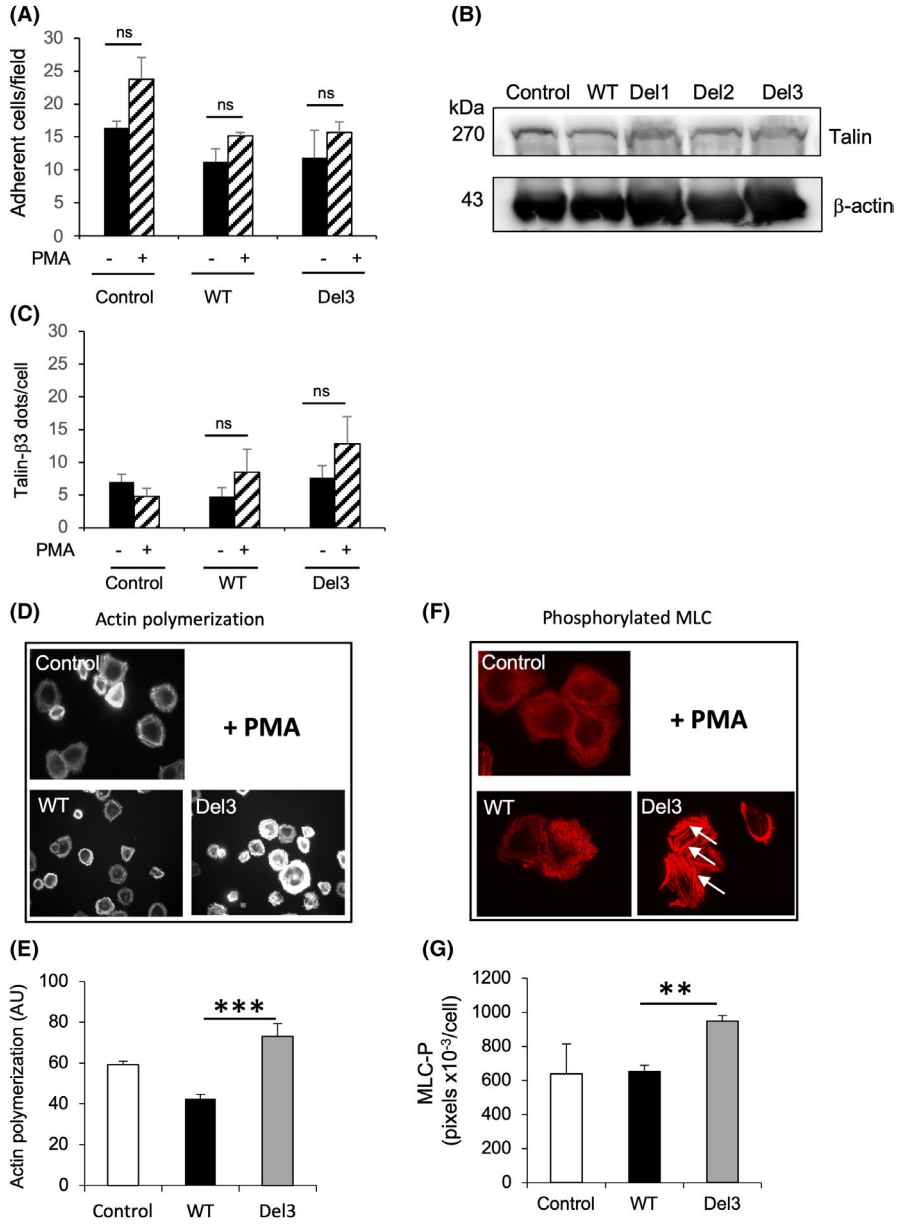

FIGURE 6 FLNa-Rho interaction and FLNa dimerization have no effect on talin-αIIbβ3 proximity but maintain Rho in an inactive state. Transfected cells (WT or Del3 mutant) stimulated or not by PMA (20 nM) were plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488–labeled phalloidin. (A) Cell adhesion was visualized by fluorescence microscopy and quantified using the Fiji software. (B) Talin expression level was assessed by immunoblotting. (C) Talin-αIIbβ3 proximity was assessed by PLA and fluorescence microscopy and quantified using the Fiji software. For each experiment, at least 200 cells per condition were analyzed. (D-E) Actin polymerization and (F-G) MLC phosphorylation was visualized and quantified with a fluorescence microscope (white arrows indicate large stress fibers in Del3 cells). The graphs represent the means ±SEM of three independent experiments for E-G and eight independent experiments for A and C (**$P < .01$, ***$P < .001$, one-way analysis of variance followed by Tukey's multiple comparison). Note that experiments in Figures 3, 5, and 6 were run in parallel. Thus, CTRL and WT are identical in all three figures. FLNa, filamin A; MLC, myosin light chain; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean; WT, wild-type

3.6 | Negative regulation of Rho by FLNa-Rho interaction, does not impact αIIbβ3 activation

The last step was to investigate the role of the dimerization domain and of interaction with Rho and Rac in the C-terminal region of FLNa using the Del3 deletion. In FLNa-Del3 cells, fibrinogen binding was low and similar to that of FLNa WT cells (Figure 2F), suggesting that FLNa-Del3 kept the ability to negatively control αIIbβ3 activation. This shows that the C-terminal domain of FLNa is not involved in αIIbβ3 activation and does not participate in inside-out signaling. We next addressed the question of the impact of the Del3 mutant on cell adhesion. In the presence or absence of PMA, adhesion of FLNa-Del3 cells was comparable to the adhesion observed with FLNa WT cells (Figure 6A), indicating that the C-terminal domain of FLNa does not play a major role in adhesion. The expression level of talin in FLNa-Del3 cells evaluated by western blotting was

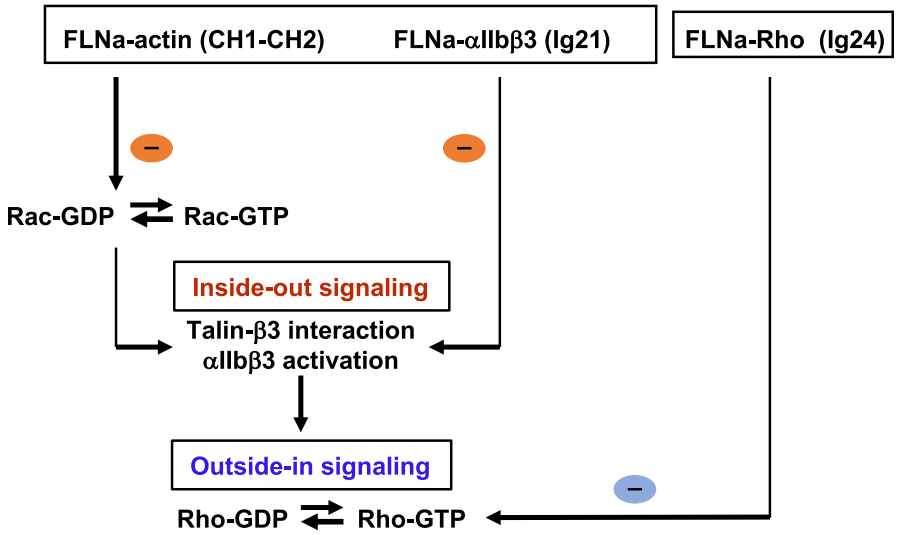

FIGURE 7 Multiple sites of FLNa regulate αIIbβ3 signaling. FLNa-actin and FLNa-β3 interactions exercise a negative regulation on αIIbβ3 activation. In contrast, the dimerization domain of FLNa, which interacts with GTPases Rho and Rac, negatively regulates outside-in signaling of αIIbβ3 integrin. The GTPases Rac and Rho are involved in these processes. Rac is maintained inactive by the interaction of FLNa with actin via the CH1-CH2 N-terminal domain, contributing to the negative regulation of αIIbβ3 activation, and Rho, which is involved in the regulation of outside-in signaling, is maintained in an inactive state by its interaction with the FLNa C-terminal domain. Platelet activation and FLNa uncoupling from β3 lift its negative regulation on Rac and Rho. FLNa, filamin A; GTPase; guanosine triphosphatase

normal (Figure 6B), and the talin-β3 complex level was similar between FLNa WT and FLNa-Del3 cells in the presence or absence of PMA (Figure 6C), confirming that the C-terminal region of FLNa does not participate in the signaling pathway (via talin-β3 association) involved in αIIbβ3 activation. In contrast, actin polymerization of FLNa-Del3 cells (fluorescence intensity measurement) was markedly increased (147%; $P = .001$) compared with FLNa WT cells (100%) (Figure 6D, 6E), suggesting that the C-terminal FLNa dimerization domain encompassed by Del3 deletion negatively controls Rho activation. Indeed, assessment of MLC phosphorylation (fluorescence intensity measurement [Figure 6F, G]) showed a significant increase in FLNa-Del3 cells (147.3%; P=.007) compared with FLNa WT cells (100%). Note that MLC phosphorylation exhibited a marked striped pattern at the cell periphery, mimicking stress fibers in all FLNa-Del3 cells (Figure 6F). Altogether, these results suggest first that the C-terminal region of FLNa does not participate in αIIbβ3 inside-out signaling and second that FLNa-Rho interaction exerts a negative control on αIIbβ3 outside-in signaling by keeping Rho inactive.

4 | DISCUSSION

FLNa plays a major role in platelet functions. FLNa is constitutively bound to αIIbβ3 integrin. It has been proposed that activation of integrin αIIbβ3 requires the substitution of FLNa by talin in the β3 cytoplasmic domain.[17] Whether FLNa had any active role or not in maintaining αIIbβ3 in a resting state remained an open question. Recently, evidence for FLNa-mediated active retention of the integrin in a resting state has been provided.[18] In this model, β3 integrin-binding domain Ig21 of FLNa claps together αIIb and β3 cytoplasmic tails, thereby stabilizing αIIbβ3 in an inactive state and preventing spontaneous αIIbβ3 activation. This would not only keep the integrin in a resting state but also prevent talin interaction with β3. However, the latter mechanism remains to be assessed experimentally. This is what the present study addresses, through examination of the role of different domains of FLNa in the regulation of αIIbβ3 activation and of "outside-in" signaling. Three deleted domains that we have already used in a previous study to evaluate the role of the FLNa subdomains in platelet biogenesis[22] were chosen: Del1, lacking the CH1 to CH2 domains at the FLNa N-terminus, which mediate interaction of FLNa with actin; Del2, lacking the Ig repeat 21, mediating the FLNa-β3 interaction, and responsible for the negative regulation of αIIbβ3 activation; and, finally, Del3, lacking Ig repeat 24, and the FLNa dimerization domain at the C-terminus, which also interacts with the small Rho and Rac GTPases involved in the reorganization of the actin cytoskeleton.

For the first time, this study shows that the N-terminal domain of FLNa, including FLNa-actin interaction, is involved in the negative regulation of αIIbβ3 activation in the megakaryocytic HEL cell line, both in adherent cells and in cell suspension. Both fibrinogen binding to suspended HEL cells and talin-αIIbβ3 proximity in adherent cells were increased after PMA activation in Del1 mutant cells compared to WT cells. Note that the tendency of PMA-treated control HEL cells to adhere to fibrinogen better than the WT and Del1 mutant cells is not statistically significant. This absence of significance is strengthened by the fact that talin-β3 proximity is not increased in control cells. In parallel, FLNa-αIIbβ3 dissociation, which occurred after PMA activation in Del1 mutant cells but also in WT cells, indicates that FLNa-actin interaction is not involved in this dissociation and that this event is not sufficient to recruit talin to β3. Moreover, the similar dissociation of FLNa from β3, which is observed both in WT and in Del1 cells after PMA activation, indicates that the FLNa-β3 interaction is not artificially affected by the mutation. The

surface area of Del1 mutant-expressing cells was found enhanced compared to WT-expressing cells, exhibiting also a significant increase in spreading and Rac activation. Our results are in agreement with the results obtained by Falet et al[15] in FlnA-null platelets, where the bulk of the filaments composing the cytoskeleton are lost in large platelets. Moreover, the inhibition of the talin-αIIbβ3 proximity observed with a Rac GTPase inhibitor suggests that the negative regulation of αIIbβ3 supported by FLNa-actin interaction occurs through Rac inactivation. These unexpected results may be explained by FLNa-actin interaction at the N-terminal region of FLNa interfering with the Rac negative regulation by the C-terminal domain (Ig repeat 24). An alternative explanation is that FLNa indirectly regulates Rac activation through reorganization of the actin cytoskeleton and/or control of an intermediate partner. Finally, we cannot exclude that FLNa-Rac interaction may also involve an unknown domain close to the FLNa-actin interaction domain.

FLNa is constitutively linked to the cytoplasmic domain of the integrin β3 subunit in resting platelets through Ig repeat 21 (and possibly Ig repeats 9, 12, 17, and 19). The model of Liu et al[18] proposes that FLNa mediates the retention of the integrin in a resting state, thus preventing spontaneous αIIbβ3 activation, but awaits more experimental evidence. In fact, some reports are in apparent contradiction with the model, such as Flna-null mice, in which basal activation of platelet αIIbβ3 integrin was not observed despite the overexpression of αIIbβ3 integrin, suggesting that other mechanisms are involved to prevent integrin activation.[15] Another unresolved issue is whether activation of integrin αIIbβ3 requires the dissociation of FLNa from the β3 cytoplasmic domain. In our experiments, fibrinogen binding induced by PMA in HEL cells in suspension was largely increased in HEL cells transfected with Del2 mutant (lacking Ig repeat 21) compared with cells transfected with FLNa WT. Consistent with this result, cell adhesion and talin-αIIbβ3 proximity were largely increased in Del2 mutant-expressing HEL cells compared with WT FLNa-expressing HEL cells, indicating that this interaction negatively regulates αIIbβ activation. These results are in agreement with those observed by Falet et al[15] and our group[19] showing that basal activation of αIIbβ3 was not observed in the absence of FLNa in Flna-null mice[15] or with a gain-of-function mutant FLNa in human platelets.[19] In the future, it would be interesting to test other domains and other associated proteins. Associated proteins such as migfilin and kindlin3 have been reported to regulate integrin activation.[25-30] It has been proposed that migfilin binding to FLNa would lead to FLNa release from β3 integrin, leaving β3 binding sites free for interaction with talin.[25,26] However, migfilin was not detected in proteomics analysis of human platelets,[27] and only low levels of migfilin messenger RNA and protein have been reported.[26] The question of the role of migfilin in platelets remains open. Kindlin3 is a critical factor in integrin regulation, and its binding site overlaps with FLNa. Kindlin3 and talin seem to synergistically coactivate αIIbβ3.[28-30] It has been proposed that talin modulates αIIbβ3 affinity, while kindlin-3 modulates clustering and avidity or recruitment of talin to the plasma membrane in the proximity of αIIbβ3.[11] The current model is that talin and kindlin3 compete with FLNa for β3 interaction, but this is not in agreement with our results, at least for talin, showing FLNa dissociation from αIIbβ3 not correlated with talin binding: This is probably also the case for kindlin3 but remains to be confirmed. New complete studies would be required to examine the role of these proteins in αIIbβ3 regulation.

In contrast to the other mutants, the mutant Del3 lacking the domain of dimerization and of interaction with the Rho and Rac GTPases had no effect on αIIbβ3 activation. Indeed, fibrinogen binding to HEL in suspension observed in FLNa-Del3– or FLNa WT–expressing cells was identical. Similarly, talin-αIIbβ3 was maintained at a low level in adherent FLNa-Del3 HEL cells. In these conditions, actin polymerization and MLC phosphorylation were largely increased, indicating that similar to the FLNa Del2 mutant, disruption of FLNa-β3 (Del2) or of FLNa-Rho (Del3) interaction increased Rho activity in outside-in signaling. We thus conclude that FLNa maintains Rho in an inactive state and negatively regulates outside-in signaling. FLNa is known to interact with different GTPase-activating or guanine nucleotide exchange factor proteins,[31] which regulate the activity of GTPase Rho. It would thus be interesting to identify which of these partners is able to modulate RhoA activity in the absence of dimerization domain.

We cannot exclude that the model of HEL cells may not be a perfect platelet replicate and that the expression of αIIbβ3 integrin and the overexpression of WT and mutants FLNa may differ in platelets and HEL cells. Moreover, in platelets the expression of FLNa (5 μM in mice) allows the interaction of FLNa with several receptors, including GPIbα and αIIbβ3 and signaling proteins (Syk, STIM1, FilGAP and Rho GTPases). Whether these interactions actually take place in HEL cells remains to be established. The HEL model thus requires further assessment in the future.

Overall, these results clearly demonstrate that FLNa is a predominant actor of the regulation of integrin αIIbβ3 activation (Figure 7). Indeed, two domains of FLNa involving FLNa-β3 and FLNa-actin interaction negatively regulate αIIbβ3 activation. FLNa-actin interaction maintains the Rac GTPase in an inactive state, which is required for the negative regulation of αIIbβ3 activation. In contrast, the C-terminal domain of FLNa which contains the domain of FLNa dimerization and of interaction with GTPases (Rho, Rac) is involved in the regulation of outside-in signaling by maintaining Rho in an inactive state.

RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

LL, CS, and MB performed experiments; MB designed experiments; FA, CV, HR, JPR, and MB wrote the manuscript.

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SUPPORTING INFORMATION

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