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New Insight into Regulation of α IIb β 3 Integrin Signaling by Filamin A

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New insight into regulation of α IIb β 3 integrin signaling by filamin A

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Abstract

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. Here, we report on the regulation of platelet integrin α IIb β 3 signaling by the FLNa subtype. 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 immunoglobulin-like (Ig) repeat 21 which mediates the FLNa- β 3 interaction; and Del3 lacking the C-terminal Ig repeat 24, responsible for FLNa dimerization and interaction with the small Rho GTPase involved in actin cytoskeleton reorganisation. Fibrinogen binding to HEL cells in suspension and talin- β 3 interaction in cells adherent to immobilized fibrinogen were assessed using the PKC agonist (phorbol 12-myristate 13-acetate) also to activate α IIb β 3. Our results show that FLNa-actin and FLNa- β 3 interactions negatively regulate α IIb β 3 activation. Moreover FLNa-actin interaction represses Rac activation, contributing to the negative regulation of α IIb β 3 activation. In contrast, the FLNa dimerization domain which maintains Rho inactive, was found to negatively regulate α IIb β 3 outside-in signaling. We conclude that FLNa negatively controls α IIb β 3 activation by regulating actin polymerization and restraining activation of Rac, as well as outside-in signaling by repressing Rho.

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 bi-directional 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 talin⁷⁻⁸ and α -actinin associate with 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 $\beta 3$ integrin tail is critical for agonist-induced $\alpha IIb\beta 3$ activation and that the selective disruption of talin- $\beta 3$ interaction protects mice from thrombosis.⁹

FLN another important actin-binding protein ensures the link between integrin and actin cytoskeleton. FLNa is one among three isoforms of FLNs (FLNa, FLNb, FLNc), a family of cytoskeletal proteins with high molecular mass that organize actin filaments into networks and link these actin networks to cell membranes.¹¹ 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 immunoglobulin-like (IgFLN) domains that are interrupted by flexible hinge regions between domains 15 and 16, and 23 and 24.¹¹⁻¹² Dimerization through Ig 24 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.¹³⁻¹⁴ A direct FLNa- $\beta 3$ interaction in resting platelets requiring FLNa repeat 21 (possibly also Ig repeats 9, 12, 17 and 19) and $\beta 3$ amino acids 747-755 awaits direct evidence. Indeed, the concept of constitutive binding of FLNa to $\beta 3$ tail to keep $\alpha IIb\beta 3$ at rest in platelets comes only from binding assays using protein constructs and structural biology data, and not from *in vivo* cellular assays.¹⁵⁻¹⁶ It has been proposed that the activation of integrin $\alpha IIb\beta 3$ requires the dissociation of FLNa from the $\beta 3$ cytoplasmic domain.¹⁵⁻¹⁶ 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.¹⁷ In this model, FLNa claps together αIIb and $\beta 3$ cytoplasmic tails, thereby stabilizing $\alpha IIb\beta 3$ in an inactive state and preventing spontaneous $\alpha IIb\beta 3$ activation. FLNa was proposed to prevent talin interaction with $\beta 3$ and integrin activation. However, this mechanism awaits direct experimental evidence in platelets, in particular because of contradictory observations: basal activation of $\alpha IIb\beta 3$ was not observed in absence of FLNa in Flna-null mice¹⁴ nor in a gain-of-function mutant FLNa in human platelets.¹⁸ One possibility is that FLNa domains other than Ig repeat 21 also regulate $\alpha IIb\beta 3$

activation. For example, the reorganization of the cytoskeleton and the interaction of the C-terminal region of FLNa with actin was not investigated with respect to α IIb β 3 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 GTPases were also unexplored. The focus of this study was therefore to determine whether other FLNa domains are involved in the regulation of α IIb β 3 activation and “outside-in” signaling of α IIb β 3 integrin. We found that FLNa-actin and FLNa- β 3 interaction exercise a negative regulation on α IIb β 3 activation whereas the dimerization domain of FLNa which interacts with the small Rho and Rac GTPases, negatively regulates outside-in signaling of α IIb β 3. We conclude that Rac and Rho GTPases are maintained in an inactive state by FLNa-actin interaction and FLNa-Rho interaction respectively.

Results

Expression of WT and FLNa mutants in HEL cells

To determine which FLNa domains regulate α IIb β 3 activation, wild-type (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 α IIb β 3 activation.¹⁸⁻¹⁹ The choice of the deleted domains was dictated by the importance of these FLNa domains in different cell functions⁸ as previously described (Fig 1A). Del1 lacks the actin-binding domain (ABD) at the N-terminus and is important for actin reorganization. Del2 lacks the Ig repeat 21 domain that interacts with the β 3 domain and appears essential for the regulation of α IIb β 3 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 adeno-associated virus integration site 1 (AAVS1) (Fig 1B). Transfection efficiency was evaluated by western blotting using an antibody specific for FLNa. 14-3-3 ζ which was previously shown to undergo very little biological variation in platelets²⁰ was used as loading control. First, the empty vector-transfected HEL cells exhibit a weak basal level of FLNa (Fig 1C). Comparably high levels of FLNa are expressed in WT- and mutants-transfected cells, reaching 6- to 7-fold the level of FLNa in the control (empty vector). This large increase in FLNa overexpression in WT and mutants transfected cells over endogenous FLNa level allowed us to examine the effect of FLNa WT and mutants on α IIb β 3 activation in the next experiments.

Fibrinogen binding to α IIb β 3 in HEL cells expressing FLNa-WT and FLNa-mutants

The next step was to evaluate the effect of the deletion of FLNa domains on α IIb β 3 activation in HEL cells activated or not with phorbol 12-myristate 13-acetate (PMA: activator of protein kinases C). This model was previously well characterized and used to investigate the regulation of α IIb β 3 activation.¹⁸⁻¹⁹ Because of the reported increased level of β 3 integrin in Flna-null platelets,¹⁴ the first step was to examine the expression of α IIb β 3 integrin in WT- and mutants-transfected cells by flow cytometry (Fig 2A-2D). No difference in α IIb β 3 expression level was observed between empty vector-transfected cells and FLNa WT- or mutants-transfected cells (Fig 2E). Then, α IIb β 3 activation was assessed by flow cytometry by measuring binding of Oregon Green-labeled fibrinogen to PMA-activated (800 nM) HEL cells in suspension (Fig 2F). Specific binding of fibrinogen to α IIb β 3 and not to other integrins was determined by subtracting the residual fibrinogen binding obtained in the presence of the α IIb-blocking monoclonal antibody (P2) from total binding in the absence of P2. First, in the absence of PMA, no difference in fibrinogen binding was observed in the presence or absence of P2 in WT- and mutants-transfected cells indicating that α IIb β 3 integrin activation does not take place in resting cells (Supplemental Fig1). In the presence of PMA, specific fibrinogen binding to α IIb β 3 integrin in WT FLNa-transfected cells, was decreased compared to control-transfected cells though not in a statistically significant manner (Fig 2F). Interestingly, FLNa-Del1 transfection (suppression of FLNa-actin interaction) induced a significant increased binding (3.7-fold; $p=0.03$) compared to FLNa-WT (Fig 2F) strongly suggesting that α IIb β 3 activation was negatively regulated by FLNa-actin interaction. Likewise, Del2 transfection (suppression of FLNa- β 3 interaction) induced a 3.8-fold increase in fibrinogen binding ($p=0.03$) (Fig 2F) consistent with FLNa- β 3 interaction negatively regulating α IIb β 3 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 (Fig 2F) indicating that this C-terminal domain of FLNa does not impact α IIb β 3 activation and does not participate to inside-out signaling. Altogether, these results clearly show that FLNa-actin and FLNa- β 3 interactions negatively regulate α IIb β 3 activation.

FLNa-actin interaction negatively regulates talin- β 3 association 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 $\beta 3$ integrin, a requirement for $\alpha \text{IIb}\beta 3$ activation, in the presence or absence of PMA activation.

First, the adhesion was assessed on a fibrinogen matrix (10 $\mu\text{g}/\text{mL}$) during 30 minutes. Quantification of adherent cells showed a similar adhesion of FLNa-WT and FLNa-Del1 cells in the absence of PMA, an adhesion slightly decreased compared to control cells, though not statistically significant (Fig 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; $p=0.03$) (Fig 3A). Altogether our results suggest that FLNa-actin interaction is involved in the negative regulation of adhesion by activated $\alpha \text{IIb}\beta 3$.

Before considering talin recruitment to $\alpha \text{IIb}\beta 3$, we first examined the expression level of talin, evaluated by western blotting. Similar expression level of talin was observed in WT and Del1-transfected cells compared with control cells (Fig 3B) allowing the quantification of talin- $\beta 3$ complex by PLA (DuoLink assay). First, in the absence of PMA, bright red dots indicated a basal level (around 5 dots/cell) of talin- $\beta 3$ complexes in HEL cells transfected with control or both FLNa constructs (Fig 3C). In the presence of PMA, the level of talin- $\beta 3$ complexes significantly increased in cells expressing FLNa-Del1 (15.0 ± 1.4 dots/cell) compared to FLNa-WT (7.1 ± 0.8 dots/cell) ($p = 0.008$) (Fig 3C-3D). The significant ($p= 0.0007$) increase in talin- $\beta 3$ complexes by PMA (15.0 ± 1.4 dots/cell versus 4.6 ± 0.6 dots/cell without PMA) was only observed in Del1-transfected cells and correlated with a significant decrease in FLNa- $\beta 3$ interaction with PMA (7.2 ± 1.1 dots/cell; $p= 0.002$, versus 17.6 ± 2.3 dots/cell in absence of PMA) (Fig 3E). Note that the dissociation of FLNa from $\beta 3$ after PMA also occurred in WT-transfected cells indicating that FLNa-actin interaction is not involved in the dissociation of FLNa from $\beta 3$ and suggesting that talin recruitment requires FLNa dissociation from $\beta 3$ associated with another event. This is consistent with FLNa-actin interaction acting as a negative regulator of $\alpha \text{IIb}\beta 3$ activation probably by preventing the recruitment of talin to $\beta 3$ integrin. In addition, the level of talin- $\beta 3$ complexes correlates with cytoskeleton assembly: some very spread out FLNa-Del1 cells exhibited very high levels of talin- $\beta 3$ complexes (Fig 3C) correlating with a spatial organization of polymerized actin different from control and FLNa-WT cells: virtually no stress fibers, nor focal adhesion plaques or focal points (Fig 3D). Finally, the level of actin polymerization as measured by total fluorescence intensity/cell was increased in FLNa-Del1 cells (81.0 ± 8.7 A.U.) compared with control cells (69.6 ± 5.7 A.U) and FLNa-WT cells (66.1 ± 6.2 A.U) (Fig 3F), though significance was not reached. However, FLNa-Del1 cells exhibited statistically significant increased areas, higher ($650.1 \pm 30.7 \mu\text{m}^2$) than in FLNa-control cells ($508.0 \pm 26.0 \mu\text{m}^2$) or FLNa-WT cells ($494.0 \pm 23.6 \mu\text{m}^2$; $p=0.008$) (Fig 3G). Altogether, these results indicate that FLNa-actin interaction which seems responsible for the spatial organization of actin is a negative regulator of talin- $\beta 3$ interaction.

FLNa-actin interaction negatively regulates talin- $\beta 3$ association 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- $\beta 3$ association. The role of Rho in talin- $\beta 3$ complex formation in the presence of PMA was assessed using Y-27632, a specific inhibitor of the RhoA kinase ROCK, a RhoA effector. When FLNa-WT cells were pretreated with Y-27632 (10 μM), a tendency ($p=0.06$) to increased talin- $\beta 3$ complexes (10.8 ± 1.7 dots/cell versus 4.0 ± 1.4 dots/cell in the absence of Y-27632) was observed (Fig 4A), suggesting that Rho is activated (Rho-GTP) in FLNa-WT cells, consistent with the large stress fibers observed in these cells (Fig 3D, 3F). Importantly, in FLNa-Del1 cells, talin- $\beta 3$ complexes significantly increased compared to FLNa-WT cells (14.9 ± 2.1 dots/cell vs 4.0 ± 1.4 dots/cell; $p=0.02$) and were 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 regulates talin- $\beta 3$ interaction 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 quantified talin- β 3 complexes using a specific Rac inhibitor (EHop-016). Cell pretreatment with 10 μ M EHop-016 for 15 minutes had no significant effect on talin- β 3 complexes in control cells and FLNa-WT cells (Fig 4B). However, in FLNa-Del1 cells, the high level of talin- β 3 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- β 3 assembly, but is repressed by the FLNa domains CH1-CH2. Paralleling FLNa-dependent talin- β 3 assembly behavior, the area of control cells ($508.2 \pm 26.0 \mu\text{m}^2$) and FLNa-WT cells ($494.0 \pm 23.6 \mu\text{m}^2$), was not affected by the Rac inhibitor (control: $442.9 \pm 26.0 \mu\text{m}^2$ and WT cells: $409.0 \pm 17.8 \mu\text{m}^2$) while the increased cell area in FLNa-Del1 ($650.0 \pm 30.7 \mu\text{m}^2$) returned to basal level after Rac inhibition ($393.0 \pm 9.4 \mu\text{m}^2$) (Fig 4C). 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 sub-membranous Rac-GTP was significantly increased (173%; $p=0.005$) compared with FLNa-WT cells (100%) (Fig 4D, 4E) 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- β 3.

FLNa- β 3 interaction negatively regulates talin- β 3 association in adherent HEL cells

The α IIb β 3-FLNa interaction and its role in platelets remain unclear. FLNa was proposed to prevent talin binding to β 3 and to maintain the integrin in a resting state, thus implying that α IIb β 3 activation required the dissociation of FLNa from β 3 prior to its activation.¹⁵⁻¹⁶ However recent data suggested that talin association to β 3 may occur in absence of FLNa release from β 3.¹⁷ We thus explored in HEL cells adhering to a fibrinogen matrix, the recruitment of talin to β 3 integrin in conditions of absence of FLNa- β 3 interaction by overexpressing the mutant FLNa-Del2.

First, cell adhesion was assessed on a fibrinogen matrix (10 μ g/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 slightly but not significantly decreased compared with control cells (16.4 ± 1.0 cells/field) (Fig 5A). In contrast, in the presence of PMA, though statistical significance was not reached, a moderate increase in adhesion was observed with FLNa-Del2 (19.5 ± 1.3 cells/field) compared to FLNa-WT cells (15.2 ± 1.2 cells/field) (Fig 5A) suggesting that FLNa- β 3 interaction could participate to the negative regulation of adhesion to the fibrinogen matrix observed with FLNa-WT. After checking the normal expression of talin in FLNa-Del2 cells (Fig 5B), quantification of talin- β 3 complexes showed after PMA activation a significant increase in FLNa-Del2 cells (21.2 ± 3.2 dots/cell) compared with FLNa-WT cells (8.2 ± 1.2 dots/cell; $p=0.002$) (Fig 5C). Interestingly, a significant increase in talin- β 3 interaction ($p=0.008$) after PMA was only observed in FLNa Del2 cells. These results indicate that FLNa- β 3 interaction exerts a negative regulation on talin- β 3 interaction only after PMA activation (Fig 5C). As expected, a similar profile of FLNa- β 3 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 β 3 in FLNa Del2 cells (Fig 5D). In parallel, actin polymerization was not significantly increased in Del2 cells (88.1 ± 3.6 A.U.) versus WT cells (66.8 ± 6.2 A.U.) (Fig 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 (Fig 5F, 5G). Finally, we examined the role of actin reorganization on talin- β 3 interaction. As already shown in Figure 4A, talin- β 3 interaction in FLNa-WT cells (9.1 ± 2.7 dots/cells) increased but not significantly in the presence of the ROCK inhibitor (10 μ M) (17.7 ± 3.0 dots/cell) (Fig 5H). In contrast, a similar increase in talin- β 3 interaction in FLNa-Del2 cells in both absence and presence of the ROCK inhibitor, was consistent with FLNa- β 3 interaction negatively controlling α IIb β 3 activation independently of

Rho. Altogether, these results indicate that FLNa- β 3 interaction acts as a negative regulator of integrin activation independently of Rho.

Negative regulation of Rho by FLNa-Rho interaction, does not impact α IIb β 3 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 (Fig 2F) suggesting that FLNa-Del3 kept the ability to negatively control α IIb β 3 activation. This shows that the C-terminal domain of FLNa is not involved in α IIb β 3 activation and does not participate to 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 (Fig 6A) indicating that the C-terminal domain of FLNa does not play a major role in adhesion. While the expression level of talin in FLNa Del1 cells as evaluated by western blotting was slightly decreased compared with control cells (Fig 6B), talin- β 3 complex level was similar between FLNa-WT and FLNa-Del3 cells in the presence or absence of PMA (Fig 6C), confirming that the C-terminal region of FLNa does not participate to the signaling pathway (talin- β 3 interaction) involved in α IIb β 3 activation. In contrast, actin polymerization of FLNa-Del3 cells (fluorescence intensity measurement) was markedly increased (147%; $p=0.001$) compared with FLNa-WT cells (100%) (Fig 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) (Fig 6F, 6G) showed a significant increase in FLNa-Del3 cells (147.3%; $p=0.007$) 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 (Fig 6F). Altogether, these results suggest first that the C-terminal region of FLNa does not participate to α IIb β 3 inside-out signaling and second that FLNa-Rho interaction exerts a negative control on α IIb β 3 outside-in signaling by keeping Rho inactive.

Discussion

FLNa plays a major role in platelet functions. FLNa is constitutively bound to α IIb β 3 integrin. It has been proposed that activation of integrin α IIb β 3 requires the substitution of FLNa by talin in the β 3 cytoplasmic domain.¹⁶ Whether FLNa had any active role or not in maintaining α IIb β 3 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.¹⁷ In this model, β 3 integrin-binding domain Ig21 of FLNa claps together α IIb and β 3 cytoplasmic tails, thereby stabilizing α IIb β 3 in an inactive state and preventing spontaneous α IIb β 3 activation. This would not only keep the integrin in a resting state, but also prevent talin interaction with β 3. 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 α IIb β 3 activation and of “outside-in” signaling. Three deleted domains were chosen: Del1 lacking the CH1-CH2 domains at the FLNa N-terminus which mediate interaction of FLNa with actin; Del2 lacking the Ig repeat 21 mediating the FLNa- β 3 interaction, and responsible for the negative regulation of α IIb β 3 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 α IIb β 3 activation in the megakaryocytic HEL cell line, both in adherent cells and in cell suspension. Indeed, both fibrinogen binding to suspended HEL cells and talin- α IIb β 3 interaction in adherent cells were increased after PMA activation in Del1 mutant cells comparatively to WT cells. In parallel, FLNa- α IIb β 3 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 β 3. Moreover, 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. The inhibition of the talin- α IIb β 3 interaction observed with a Rac GTPase inhibitor suggests that the negative regulation of α IIb β 3 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 β 3 subunit in resting platelets through Ig repeat 21 (and possibly Ig repeats 9, 12, 17 and 19). The model of Liu et al¹⁷ proposes that FLNa mediates the retention of the integrin in a resting state thus preventing spontaneous α IIb β 3 activation, but awaits more experimental evidence. In fact some reports are in apparent contradiction with the model, such as for example, Flna-null mice, in which basal activation of platelet α IIb β 3 integrin was not observed despite the over expression of α IIb β 3 integrin suggesting that other mechanisms are involved to prevent integrin activation.¹⁴ Another unresolved issue is whether activation of integrin α IIb β 3 requires the dissociation of FLNa from the β 3 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, in adherent cells talin- α IIb β 3 interaction was largely increased in Del2 mutant-expressing HEL cells compared with WT FLNa-expressing HEL cells indicating that this interaction negatively regulates α IIb β 3 activation. These results are in agreement with those observed by the group of Falet¹⁴ and our group¹⁸ showing that basal activation of α IIb β 3 was not observed in absence of FLNa in Flna-null mice¹³ or with a gain-of-function mutant FLNa in human platelets.¹⁸

In contrast, the mutant Del3 lacking the domain of dimerization and of interaction with the Rho and Rac GTPases had no effect on α IIb β 3 activation. Indeed, fibrinogen binding to HEL in suspension observed in FLNa Del3- or FLNa WT-expressing cells was identical. Similarly, talin- α IIb β 3 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- β 3 (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. Overall, these results clearly demonstrate that FLNa is a predominant actor of the regulation of integrin α IIb β 3 activation (Fig 7). Indeed, two domains of FLNa involving FLNa- β 3 and FLNa-actin interaction negatively regulate α IIb β 3 activation. FLNa-actin interaction maintains the Rac GTPase in an inactive state which is required for the negative regulation of α IIb β 3 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.

Material and Methods

Material

Fibrinogen was obtained from HYPHEN BioMed SAS (Andresy, 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). The CD41/CD61-PE and REA-PE antibodies were obtained from Miltenyi Biotec (Paris, France). Rac inhibitor (EHop-016) and ROCK inhibitor (Y-27632) were obtained from Abcam (Amsterdam, Holland). Anti-human $\beta 3$ chain was purchased from Becton Dickinson (Le Pont de Claix, France). The monoclonal anti-active Rac-GTP was from Biomol (Hamburg, Germany). The polyclonal antibody directed against integrin α IIb (clone P2) was from Beckman Coulter (Villepinte, France). The polyclonal antibodies directed against talin, phosphorylated myosin light chain (MLC-P) and FLNa were obtained from Abcam (Cambridge, UK). Peroxydase-conjugated AffiniPure secondary antibodies were obtained from Jackson TM ImmunoResearch Laboratories, Inc. (West Grove, PA). Proximity ligation assay (Duolink) was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). HEL cells [HEL 92.1.7 (ATCC® TIB-180™)] were from American Type Culture collection (Manassas, VA).

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 Del4.²¹ 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 $\beta 3$ domain, and Del3 a mutant lacking the dimerization domain involved in the interaction with small GTPases as previously described.²¹ The cDNAs were cloned into AAVS1-SA-2A-puro-pA plasmid under the regulation of the CAG promoter and a zinc finger nucleases-based (ZFN) technology was used to generate stable cell lines, by targeting constructs to the AAVS1 locus as previously described.²¹ HEL cells were transfected with the constructs by nucleofection using the Neon transfection system (Invitrogen-ThermoFisher Scientific, Saint-Aubin, France) 5 according to the manufacturer's instructions using 1 μ g of plasmid DNA for 10⁵ cells. Expression and stability of the mutant FLNa proteins were verified by immunoblotting.

Fibrinogen binding to HEL cells

Activation of α IIb β 3 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 PMA for 10 minutes in the presence of 20 μ g/mL of fibrinogen (5 μ g Oregon green-labeled fibrinogen + 15 μ g cold fibrinogen) as previously described.¹⁸ Specific binding to α IIb β 3 was measured by flow cytometry in the presence or absence of a monoclonal antibody (clone P2; 10 μ g/mL) blocking the binding of fibrinogen to α IIb β 3. The difference represents the specific fibrinogen binding to α IIb β 3.

Proximity ligation assay (PLA)

FLNa-expressing HEL cells were stimulated with 20 nM PMA for 5 minutes and allowed to adhere onto fibrinogen coated coverslips (10 μ g/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 MgCl₂ ; 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 α IIb-specific P2 mouse monoclonal antibody (2 μ g/mL), and a rabbit polyclonal antibody directed against talin (2 μ g/mL). Proximity ligation assays were performed according to the manufacturer's TM instructions (Duolink) using oligonucleotide-coupled secondary antibodies against mouse and rabbit primary antibodies (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 absence of the primary antibodies. The number of spots per cell was quantified using Fuji software.

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 μ M phenylarsine oxide, 1% SDS, 5 μ g/mL leupeptin, 10 μ g/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, Rockford, IL). 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, Cambridge, UK).

Statistical analysis

Results were analyzed using 1-way ANOVA followed by a least significant difference multiple comparisons as indicated.

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Author contributions: L.L., C.S and M.B. performed experiments; M.B. designed experiments; F.A., C.V, H.R., J-P.R. and M.B wrote the manuscript.

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Competing financial interests: The authors declare no competing financial interests

Figure Legends

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 $\beta 3$ (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. (B) A zinc finger nucleases-based technology targeting the AAVS1 locus was used. Integration at the AAVS1 locus was checked by PCR. (C) The level of FLNa protein expressed was quantified by immunoblotting using a polyclonal antibody against FLNa.

Figure 2: Fibrinogen binding is enhanced in HEL cells expressing mutants lacking FLNa-actin (Del1) and FLNa- $\beta 3$ (Del2) interactions.

(A,B,C,D,E) The expression level of $\alpha IIb\beta 3$ 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 $\alpha IIb\beta 3$) and a REA-PE antibody as control. The bar graph represents the means \pm SEM of three independent determinations. (F) Then HEL cells transfected with plasmids encoding wild-type FLNa (FLNa-WT) or FLNa mutants were stimulated with 800 nM PMA in the presence of Oregon Green 488-labeled fibrinogen (20 mg/mL). The empty vector was transfected as control. Specific fibrinogen binding in the presence or absence of a specific monoclonal antibody (P2) blocking fibrinogen binding to $\alpha IIb\beta 3$ was measured by flow cytometry (BD Accuri C6+ flow cytometer). The graph represents the means \pm SEM of six independent determinations (* $P < 0.05$, 1-way ANOVA followed by Tukey's multiple comparison).

Figure 3: FLNa-actin interaction regulates negatively talin- $\beta 3$ interaction.

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-D-E) Talin- $\alpha IIb\beta 3$ and FLNa- $\alpha IIb\beta 3$ associations were assessed by proximity ligation assay (PLA, see Methods) and fluorescence microscopy. Red spots correspond to PLA positive signals. Non-specific 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 \pm SEM of 3 independent experiments for (E to H) and 8 independent experiments for (A and C) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 1-way ANOVA followed by Tukey's multiple comparison).

Figure 4: FLNa-actin interaction negatively regulates talin- $\beta 3$ interaction 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 μ M) (A) or the Rac inhibitor EHop-216 (10 μ M) (B-C) were plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488-labeled phalloidin. (A-B) Talin- $\alpha IIb\beta 3$ association 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 \pm SEM of 3 independent experiments and statistical significance was determined by 1-way ANOVA followed by Tukey's multiple comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 5: FLNa- α IIb β 3 interaction negatively regulates talin- β 3 interaction

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- α IIb β 3 association, 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-F-G) Actin polymerization and MLC phosphorylation were visualized and quantified by fluorescence microscopy. (H) Talin- α IIb β 3 association was assessed by PLA in the presence of the ROCK inhibitor Y-27632 (10 μ M). The graphs represent the means \pm SEM of 3 independent experiments for E, F, G, and 8 independent experiments for A and C (* P <0.05, ** P <0.01, *** P <0.001, 1-way ANOVA followed by Tukey's multiple comparison).

Figure 6: FLNa-Rho interaction and FLNa dimerization have no effect on talin- α IIb β 3 interaction 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- α IIb β 3 association 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. The graphs represent the means \pm SEM of 3 independent experiments for E-G and 8 independent experiments for A and C (** P <0.01, *** P <0.001, 1-way ANOVA followed by Tukey's multiple comparison).

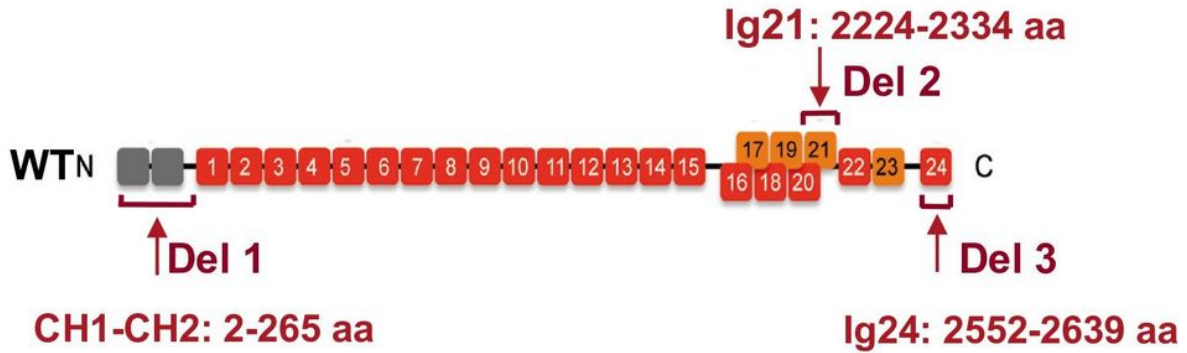
Figure 7: Multiple sites of FLNa regulate α IIb β 3 signaling.

FLNa-actin and FLNa- β 3 interactions exercise a negative regulation on α IIb β 3 activation. In contrast, the dimerization domain of FLNa which interacts with GTPases Rho and Rac, negatively regulates outside-in signaling of α IIb β 3 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 α IIb β 3 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 β 3 lift its negative regulation on Rac and Rho.

Figures

Figure 1

A



B



C

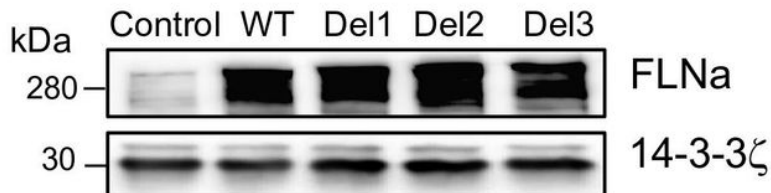


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Figure 2

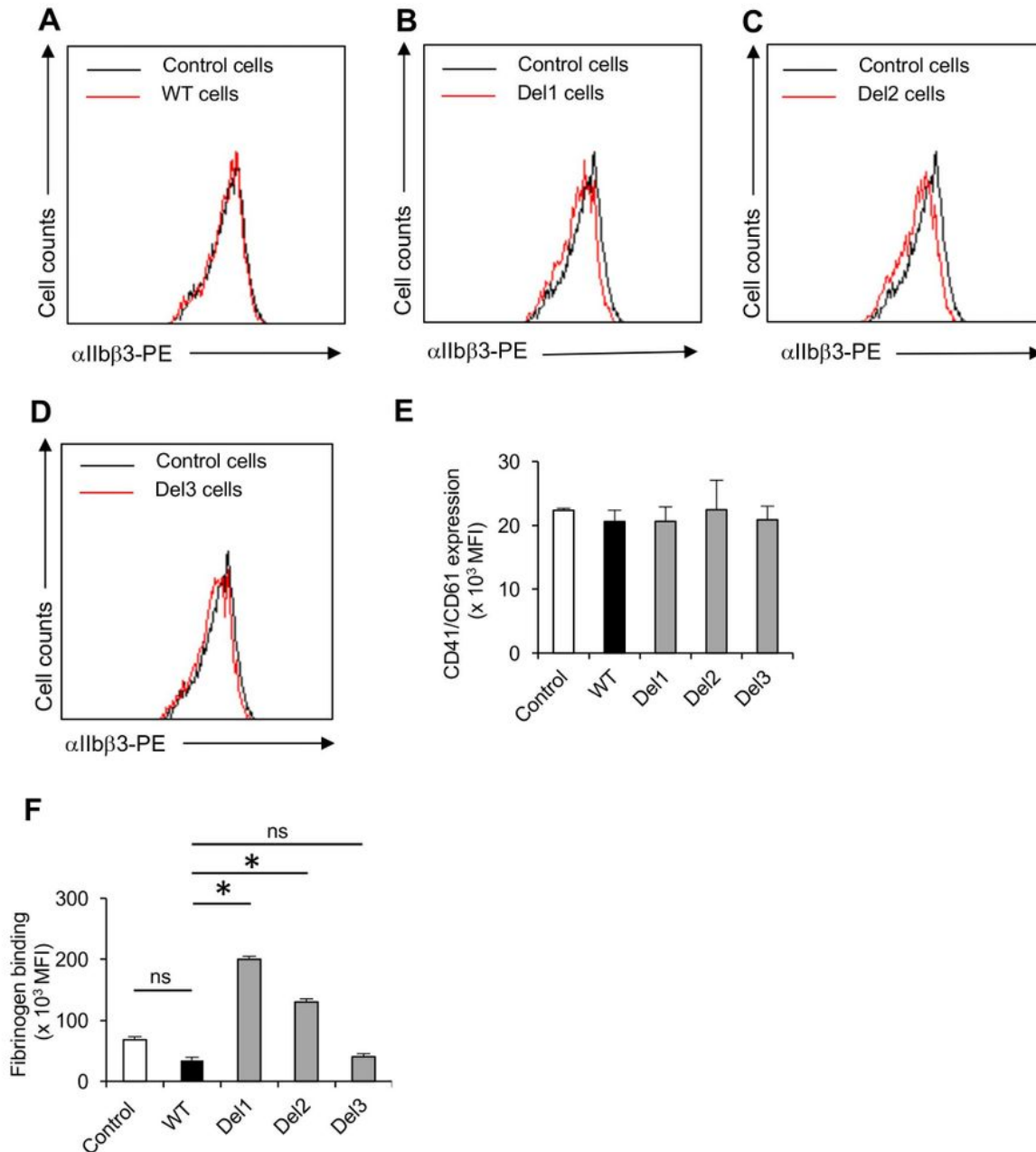


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Figure 3

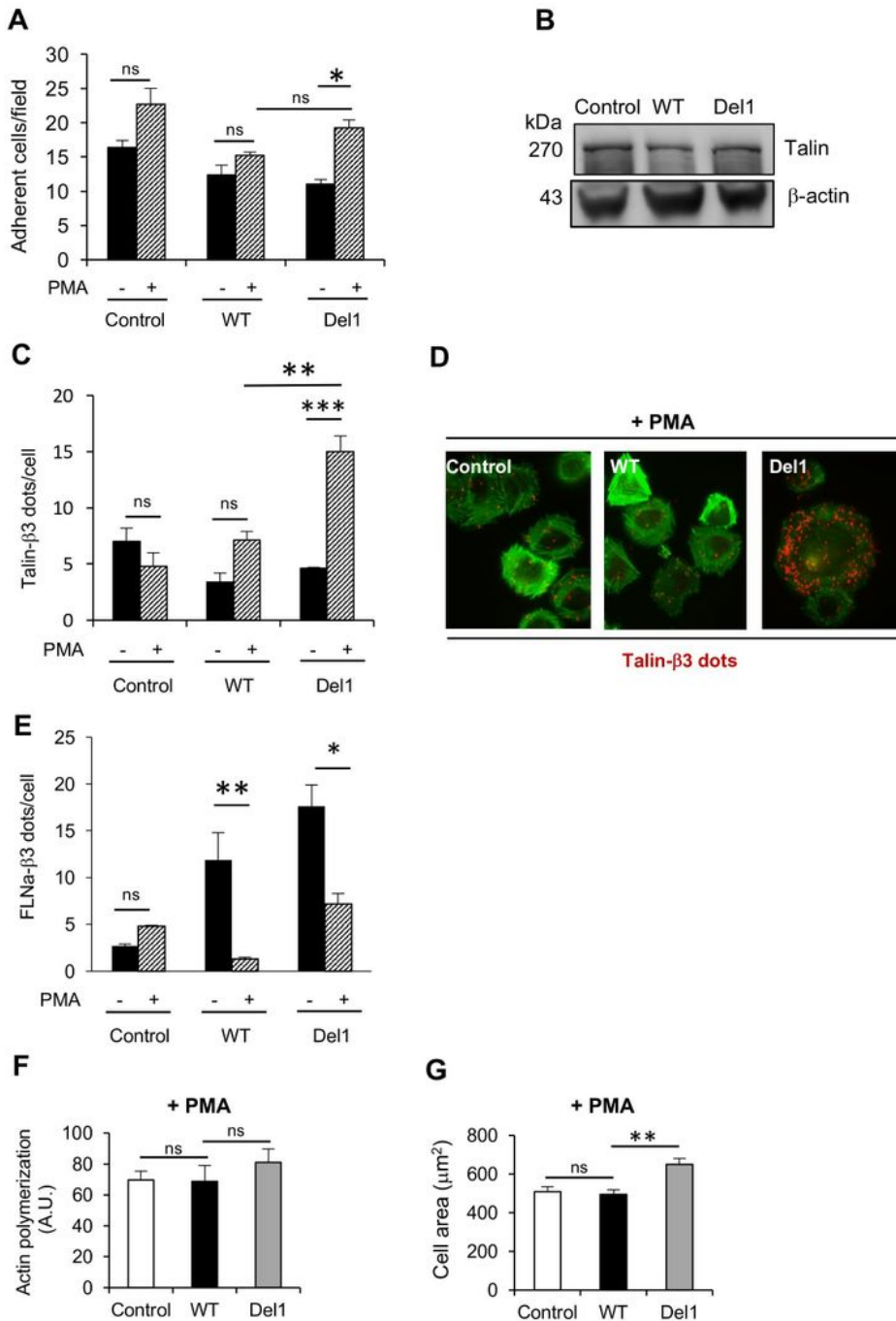


Figure 3

FLNa-actin interaction regulates negatively talin-β3 interaction. 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-D-E) Talin-αIIbβ3 and FLNa-αIIbβ3 associations were assessed by

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Figure 4

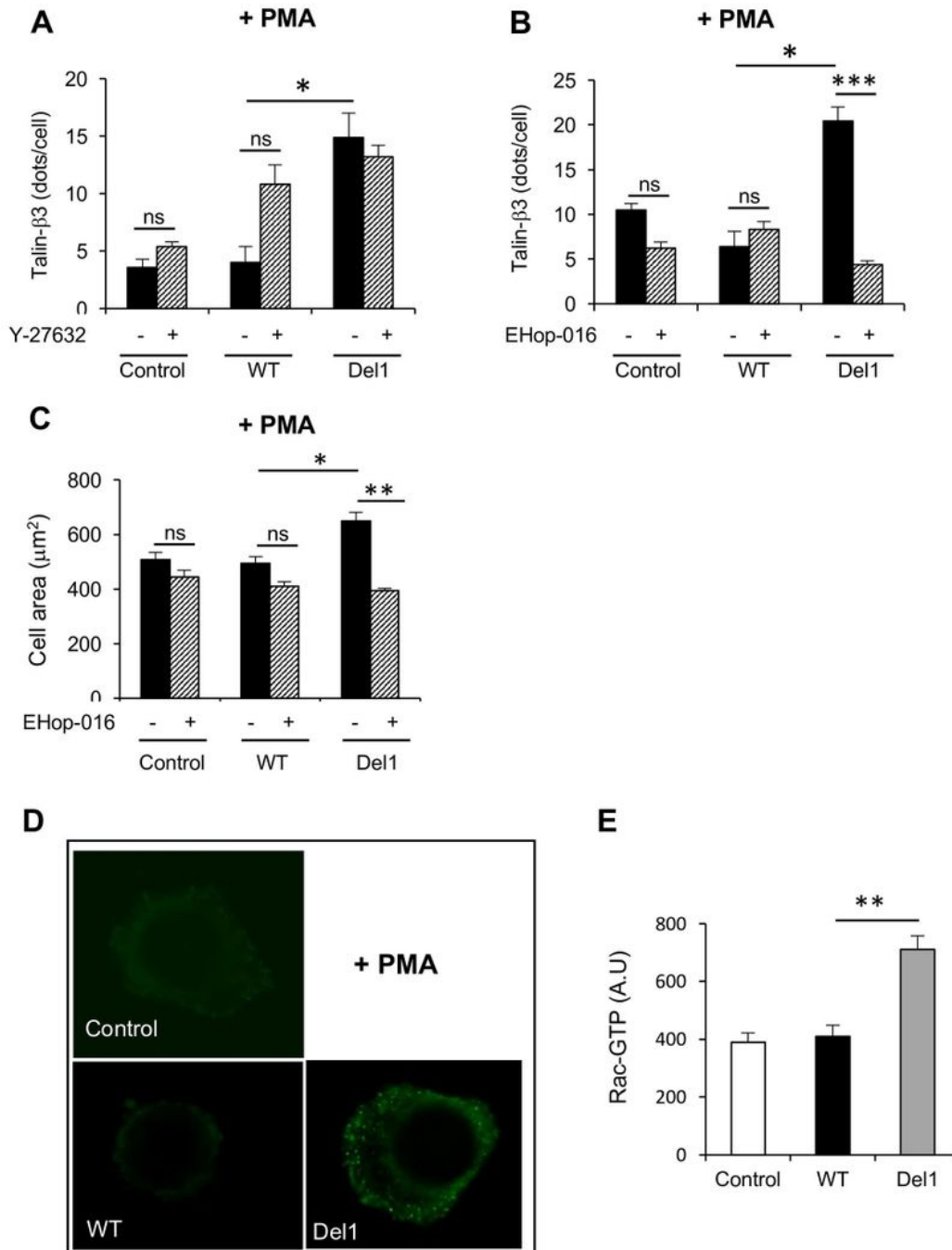


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FLNa-actin interaction negatively regulates talin- β 3 interaction via Rac inactivation. Transfected cells (WT or Del1 mutant) stimulated by PMA (20 nM) in the presence or absence of the ROCK inhibitor Y-27632 (10 μ M) (A) or the Rac inhibitor EHop-216 (10 μ M) (B-C) were plated onto fibrinogen-coated coverslips for 30 minutes and then stained with Alexa Fluor 488-labeled phalloidin. (A-B) Talin- α IIb β 3 association 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 \pm SEM of 3 independent experiments and statistical significance was determined by 1-way ANOVA followed by Tukey's multiple comparison (*P <0.05, **P<0.01, ***P<0.001)

Figure 5

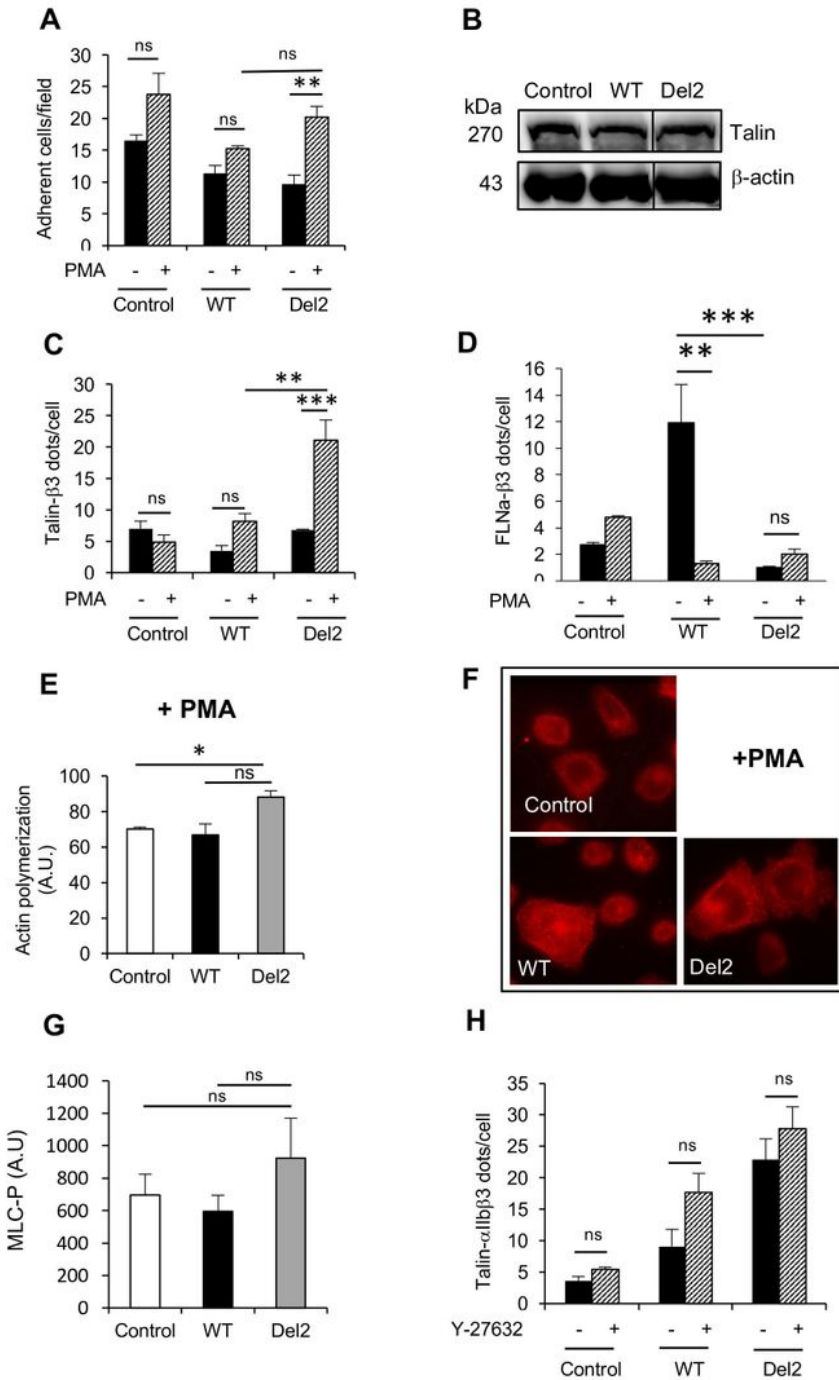


Figure 5

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Figure 6

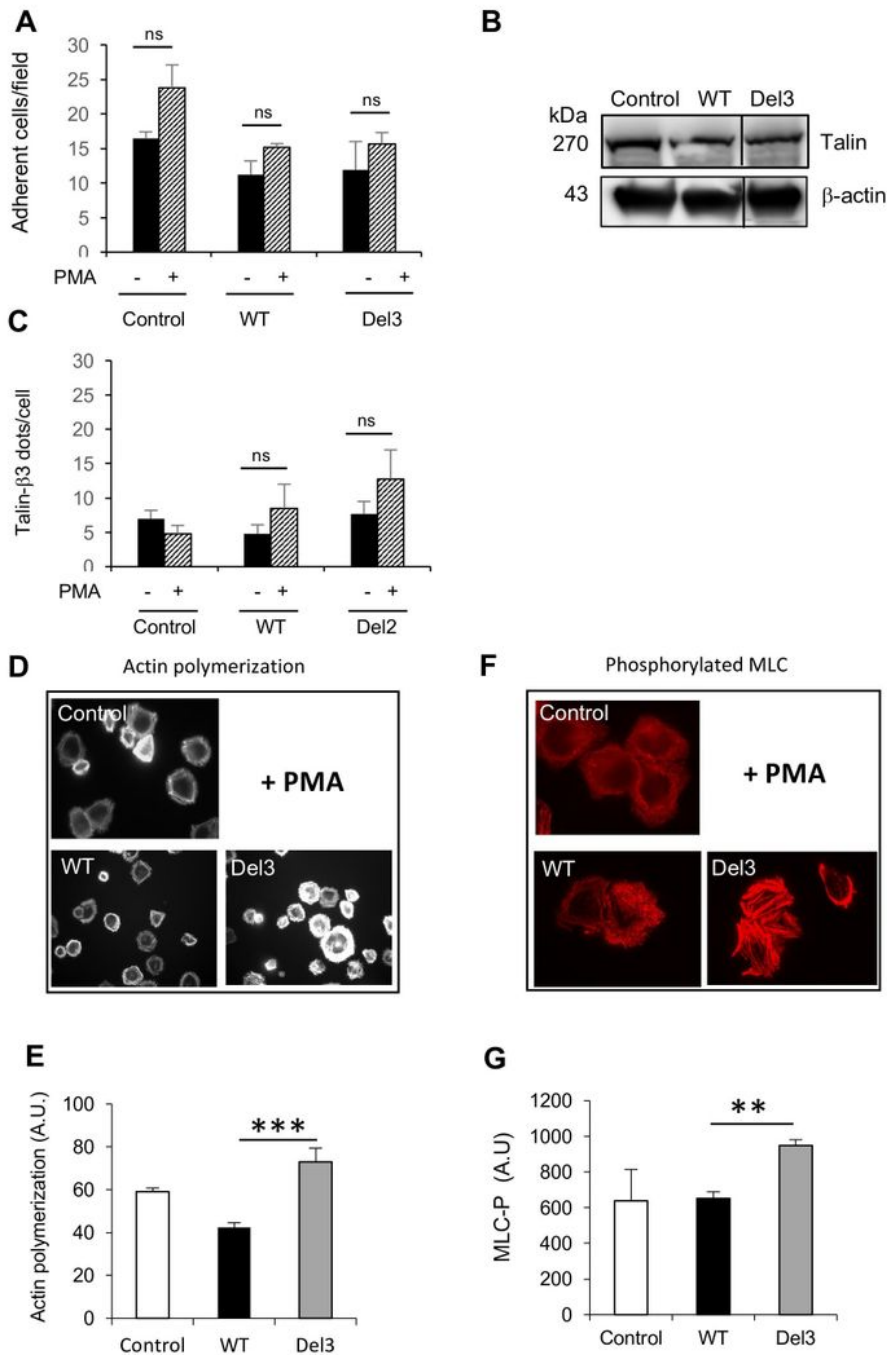


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Figure 7

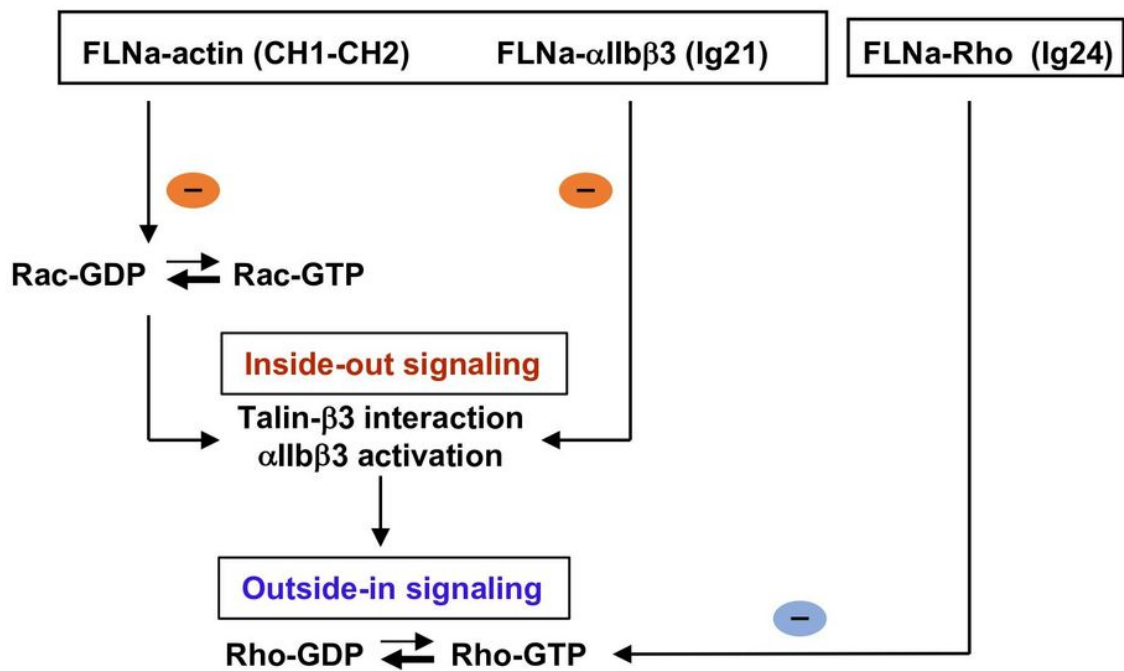


Figure 7

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