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The Mechanism of Kindlin-mediated Activation of Integrin αIIbβ3

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Abstract

Increased ligand binding to cellular integrins ("activation") plays important roles in processes such as development, cell migration, extracellular matrix assembly, tumor metastasis and hemostasis and thrombosis[1-5]. Integrin activation encompasses both increased integrin monomer affinity and increased receptor clustering[6] and depends on integrin-talin interactions[5]. Loss of kindlins results in reduced activation of integrins[7-13]. Kindlins might promote talin binding to integrins through a cooperative mechanism[5, 14-16]; however, kindlins do not increase talin association with integrins[17]. Here we report that, unlike talin head domain (THD), kindlin-3 caused little effect on the affinity of purified monomeric αIIbβ3, and it didn't enhance activation by THD. Furthermore, studies with ligands of varying valency showed that kindlins primarily increased cellular αIIbβ3 avidity rather than monomer affinity. In platelets or nucleated cells, loss of kindlins markedly reduced αIIbβ3 binding to multivalent but not monovalent ligands. Finally, silencing of kindlins reduced the clustering of ligand-occupied αIIbβ3 as revealed by total internal reflection fluorescence (TIRF) and electron microscopy. Thus, in contrast to talins, kindlins have little primary effect on integrin αIIbβ3 affinity for monovalent ligands and increase multivalent ligand binding by promoting the clustering of talin-activated integrins.

Keywords

integrin; kindlin; talin signal transduction; cell adhesion

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Author Contributions

FY and MHG conceived the project and directed the research. FY, BGP and PA performed experiments. AK and SJS provided essential reagents. MM, RF, and CTL provided kindlin-3 null mouse platelets. FY and MHG wrote the paper which was edited by SJS and RF.

The authors declare that they have no conflict of interest.

Supplemental Information

Supplemental Figures S1-S4 and experimental procedures are available in supplemental information.

Kindlin-3 has a minimal effect on the affinity of monomeric αIIbβ3

To the effect of kindlins on individual integrins, we purified kindlin-3, the isoform in platelets, to monomeric platelet integrin αIIbβ3 inserted in 10-12 nm diameter phospholipid bilayers (nanodiscs)[18]. Since αIIbβ3 clustering can not occur under these conditions, ligand binding measures the affinity of integrin monomers[18]. Recombinant purified kindlin-3 was monomeric (Fig. S1A), and was well-folded as judged by a sharp melting point (48°C) in differential scanning calorimetry (not shown). Kindlin-3, at a concentration that saturated β3 integrin binding [14, 17], was >25 fold less active than talin head domain (THD) in increasing binding of PAC1, activation-specific antibody to αIIbβ3 nanodiscs and it failed to enhance PAC1 binding induced by THD (Fig 1A, S1B).. Indeed, kindlin-3 reduced THD-induced PAC1 binding, possibly due to competition between THD and kindlin-3 for binding to the limited lipid surface present in the nanodiscs, rather than to the β3 integrin[14, 17]., Recombinant kindlin-3 bound to the β3 cytoplasmic domain (Kd=67±9 nM) but not to a kindlin binding-defective β3 mutant (β3(Y759A), Fig S1C). Kindlin-3 also bound αIIbβ3 nanodiscs(Fig S1D). Thus, in contrast to THD, recombinant kindlin-3 did not increase the ligand-binding affinity of monomeric αIIbβ3 in nanodiscs.

Kindlins selectively increase the binding of multivalent ligands to recombinant integrin αIIbβ3

The foregoing results suggest that kindlin-3 may regulate ligand binding to integrins by a mechanism other than modulation of monomer affinity. Platelet activation clusters integrin αIIbβ3 and αIIbβ3 dimerization can cooperate with increased monomer affinity to increase the binding of multivalent but not monovalent ligands [6, 20, 21]. These observations led us to compare the effects of kindlins on the binding of multivalent versus monovalent ligands. Kindlin-1 and 2 are thought to promote αIIbβ3 activation in non-hematopoietic cells via a mechanism similar to that of kindlin-3 in platelets and are thus used to study αIIbβ3 regulation in such cells [11-13, 22]. Kindlin-1 did not synergize with THD to increase monovalent PAC1 Fab binding to integrin αIIbβ3 in HEK293 cells, but dramatically increased THD-induced binding of decavalent PAC1(Fig 1B). To extend this finding, we used the monovalent fibronectin 10^{th} type III repeat(3FN10), an activation-dependent ligand for integrin αIIbβ3[23], because it can be expressed in prokaryotic systems at the high concentrations required for this experiment, can be directly labeled (e.g. with biotin), and binds to murine αIIbβ3. Again, kindlin-1 did not synergize with THD to increase the specific binding of 3FN10 to integrin αIIbβ3, measured as binding inhibitable by an αIIbβ3-specific antagonist, eptifibatide (Fig 1C, S1E). 3FN10 binding was not maximal in the THD-transfected cells because addition of Mn^{2+} markedly increased binding (Fig 1C). Mn^{2+} enhanced THD induced 3FN10 binding, indicating that Mn^{2+} binding to the extracellular domain can synergize with talin to increase integrin monomer affinity. In sharp contrast to results with 3FN10, kindlin-1 markedly synergized[13, 22] with THD in the binding of decavalent PAC1 IgM (Fig 1C, S1E). The increase of PAC1 binding upon THD expression was greater than that observed with monomeric 3FN10, probably reflecting both the clustering[24] and conformational changes[18] of αIIbβ3 induced by THD.

To directly test the role of ligand valency in 3FN10 binding, we chemically cross-linked 3FN10 and isolated a mixed population of oligomers. In contrast to monovalent 3FN10 or GST-3FN10, kindlin-1 and kindlin-2 markedly increased the capacity of THD to enhance the specific binding of multivalent GST-3FN10 to αIIbβ3 (Fig. 1D). Mutation of RGD sequence in 3FN10 to AAA abolished the binding of both monomeric and oligomeric 3FN10 (Fig S1F), confirming its specificity. The kindlin-induced increase in multivalent ligand binding was independent of an intact actin cytoskeleton or myosin II-mediated contractility, as latrunculin A, an inhibitor of actin polymerization, or blebbistatin, a myosin

II ATPase inhibitor, was without effect (Fig S1G). Thus, kindlins synergized with THD in stimulating the binding of multivalent but not monovalent ligands to αIIbβ3 and this effect required neither an intact actin cytoskeleton nor myosin II-driven contractility.

Silencing of kindlin-2 selectively inhibits the binding of multivalent ligands to recombinant integrin αIIbβ3

We then asked whether depletion of kindlins selectively impairs binding of integrin ligands. HEK 293 cells express both kindlin-1 and kindlin-2 [25], making it difficult to perform siRNA-mediated silencing. Thus we turned to CHO cells, which express only kindlin-2 [13, 17]. In CHO cells expressing an active αIIbβ3(D723R) mutant[26], silencing kindlin-2 inhibited the binding of oligomeric 3FN10, but it had no effect on the binding of monovalent 3FN10. This was a specific effect as re-introducing shRNA-resistant kindlin-2 restored the binding of oligomeric 3FN10 (Fig 2A). Moreover, kindlin-2 depletion resulted in similar selective inhibition of oligomeric but not monomeric 3FN10 binding to an active integrin chimera expressed in CHO cells[27, 28](Fig S2A). Furthermore, adhesion of αIIbβ3-expressing CHO cells to surface-bound 3FN10, which is functionally multivalent, was reduced in kindlin-2 silenced cells (Fig S2B). Thus, kindlin-2 silencing inhibits binding of multivalent but not monovalent ligands to αIIbβ3 expressed in nucleated cells.

Kindlin-3 deficient platelets are primarily defective in binding to multivalent but not monovalent αIIbβ3 ligands

Next we sought to determine how kindlins function in regulation of αIIbβ3 in platelets. Mice lacking kindlin-3 suffer embryonic and perinatal lethality, recurrent infections, and erythrocyte defects in addition to reduced αIIbβ3 functions[8, 11, 29]. To examine the consequences of loss of kindlin-3, encoded by the *Fermt3* locus, in healthy adult mice, we reconstituted irradiated mice with kindlin-3 or talin null hematopoietic cells mixed with wild type hematopoietic cells expressing DsRed (Fig 2B). Intracellular staining of isolated platelets indicated that kindlin-3 or talin was depleted from the respective (DsRed negative) platelet population (Fig 2B).

PAR4 thrombin receptor agonist peptide stimulated similar binding of monovalent 3FN10 to αIIbβ3 in both kindlin-3 null and wild type platelets, whereas loss of platelet talin-1 significantly inhibited 3FN10 binding(Fig 2C). In contrast and as expected [11, 30, 31], loss of either kindlin-3 or talin-1 impaired the binding of multivalent fibrinogen, which has at least 4 potential αIIbβ3 binding sites(Fig 2D). Furthermore, loss of kindlin-3 had little effect on either fibrinogen or 3FN10 binding when platelets were activated exogenously by Mn^{2+}, consistent with previous reports that Mn^{2+} can promote both affinity increase [32, 33] and integrin clustering [24, 34] (Fig S2C). Deletion of talin reduced binding of 3FN10 but not fibrinogen to Mn^{2+} stimulated platelets (Fig S2C), suggesting that talin binding synergizes with Mn^{2+} in increasing integrin monomer affinity. At higher agonist concentration (1 mM PAR4 peptide), the defect in fibrinogen binding to kindlin-3 null platelets was even less pronounced. To better quantify this result, we examined binding of various concentrations of 3FN10 to wild-type or kindlin-3 null platelets. The binding isotherms of 3FN10 to wild-type and kindlin-3 null platelets virtually overlapped (Fig S2D,F) indicating that a lack of kindlin-3 does not change the affinity of 3FN10 for αIIbβ3 on activated platelets or the capacity of a thrombin receptor agonist peptide to stimulate increased binding of a monovalent ligand to αIIbβ3 (Fig. S2E,F). Therefore, in agonist-stimulated platelets as in nucleated cells, lack of kindlin-3 has a major effect on the binding of multivalent but not monovalent αIIbβ3 ligands, whereas, lack of talin has major effects on both ligand types.

Kindlins promote clustering of occupied integrins

Integrin clustering increases binding of multivalent ligands without affecting the affinity of integrin αIIbβ3 monomers[6], suggesting that integrin clustering might account for most of kindlins' effects. We used TIRF microscopy, an established method for studying clustering[24, 35], to measure αIIbβ3 clustering at the sub-micron scale. Initial cell adhesion to immobilized fibrinogen is αIIbβ3 activation-independent [36, 37], possibly due to increased ligand density and altered fibrinogen conformation [38], enabling us to examine effects of kindlins independent of their effects on integrin activation, *per se*. Silencing of kindlin-2 with two different shRNAs significantly reduced both the brightness and size of integrin puncta (Fig 3A,B,D), even though it had little or no effect on integrin expression (Fig. 3A,C). Furthermore, silencing of kindlin-2 by these shRNAs did not affect the distance of the plasma membrane from the substrate, since fluorescence intensity of a membrane-intercalated dye was equal in control and kindlin-2 silenced cells (Fig S3 A,B) in TIRF images.

To assess integrin clustering at the nanoscale, we used electron microscopy to examine ventral membranes of fibrinogen-adherent αIIbβ3-expressing CHO cells[39]. In control shRNA-transduced cells, we observed numerous clusters, each containing >5 gold particles and usually less than 200 nm in size, i.e. they account for the point sources seen in the TIRF images (Fig 4A, left panel). In sharp contrast, in kindlin-2 silenced cells gold particles were present; however, there were much fewer clusters (Fig 4A, middle panel, 4B). The total number of gold particles was also reduced, likely reflecting an overall decreased abundance of integrins in the ventral membrane. Only occasional random gold particles were seen in irrelevant IgG-stained membranes (Fig 4A right panel). Thus, kindlin-2 promotes the clustering of ligand-occupied αIIbβ3 at the nanoscale.

Kindlins could increase αIIbβ3 clustering either before or after ligand binding. We reasoned that in the latter case, kindlin-dependent increases in ligand binding would be prevented by fixing the cells before addition of ligand. When cells were fixed before adding ligands to αIIbβ3-expressing HEK293 cells, THD induced a near 3 fold increase in PAC1 binding but kindlin-1 failed to synergize with THD in inducing PAC1 binding (Fig S4, A). In contrast, kindlin-1 dramatically increased THD-induced PAC1 binding in cells fixed after ligand addition (Fig S4, A). Furthermore, consistent with earlier reports that clustering requires occupancy of the integrin [40], when the ability of αIIbβ3 to bind ligand was blocked by a β3 Asp^{119} mutation the integrin remained diffusely distributed as judged by TIRF microscopy (Fig S4, B). Thus, the effect of kindlins on multivalent ligand binding to integrin αIIbβ3 is mediated by their capacity to promote the clustering of ligand-occupied αIIbβ3.

Discussion

These results pinpoint the locus of kindlin action in mediating integrin αIIbβ3 activation. Earlier studies in invertebrates[41, 42] and cultured cells[43, 44] showed that kindlins mediate formation of integrin-mediated adhesions and foreshadowed studies showing that kindlins regulate the capacity of integrins to bind soluble ligands[7, 8, 10, 11, 13] and to promote integrin signals that regulate the cytoskeleton[12]. Here, we found that the kindlins increase soluble ligand binding to αIIbβ3 primarily by increasing avidity for multivalent ligands due to kindlin's ability to promote clustering of ligand-occupied integrins. Our results now explain why kindlins alone have little apparent effect on soluble ligand binding to cellular integrins in the absence of talin-mediated activation and comport with the finding that kindlin-3 does not induce changes in β3 transmembrane domain topology, which induces integrin αIIbβ3 activation [45].

We utilized 3FN10 as a monovalent ligand because it binds to both murine and human αIIbβ3 and for technical ease of cross-linking. Previous studies showed that kindlin-3 over-expression increased 3FN(7-10) (7th to 10th type III fibronectin repeats) [11] binding to integrin α5β1 in RAW 264.7 cells. In contrast to 3FN10, 3FN(7-10) contains a free cysteine [46] and two integrin binding sites, one in 3FN9 and the other in 3FN10 [47], and is thus not monovalent. In addition there may be cell specific, kindlin-specific and integrin-specific mechanisms of regulation, as 1) kindlin-1 and kindlin-2 synergize with THD to activate αIIbβ3 but inhibit THD-induced α5β1 activation in CHO cells [22]; 2) over-expressed kindlin-3 is functional in RAW 264.7 cells but not in CHO cells [11]; and 3) kindlin-3 is required for αLβ2 but not α4β1 increased cell adhesiveness under fluid shear stresses[9]. Secondary antibodies can oligomerize monovalent PAC1 Fab under appropriate conditions, converting it into a multivalent ligand [48]. We formaldehyde-fixed the cells before adding secondary antibody or streptavidin, as recommended by Bunch[48], to avoid potential oligomerization of the 3FN10. Indeed, when highly concentrated PAC1 Fab was used to overcome its low affinity as a monovalent ligand[48], we obtained results comparable to those using 3FN10 (Fig 1B). Thus, when secondary antibody-induced oligomerization is avoided, kindlins have little effect on the binding of an authentic monovalent ligand to integrin αIIbβ3.

Reduced soluble ligand binding alone might not account for the defective platelet aggregation and increased bleeding observed in kindlin-3 null mice and patients. The combined effects of reduced surface integrin expression [11], defective integrin clustering and impaired integrin outside-in signaling [10-12, 43] may all make important contributions to the hemostatic defect. Similarly, the effect of kindlin-mediated clustering on avidity can be a major contributor to kindlin-3's capacity to support the function of β1 or β2 integrins that mediate increased resistance of leukocytes to detachment under flow[8, 19, 49]. Kindlin-mediated leukocyte arrest is associated with an additional conformational change in αLβ2 that can be driven by shear stress on the integrin[9, 19]. Thus, in addition to effects on soluble ligand binding, it is clear that kindlin-3 makes multiple contributions to the adhesive functions of blood cells.

Integrin-bound kindlins might promote integrin clustering by redistributing integrins between different pools, such as those on the cell surface, in storage pools, and in recycling pathways [50, 51]. Indeed, loss of kindlin-3 is associated with reduced αIIbβ3 surface expression in murine platelets by about 25% [11] (and our unpublished results), and over-expression of kindlins can increase integrin expression in a CHO cell clone [22]. Alternatively, kindlins can recruit migfilin [43] and integrin-linked kinase (ILK) [12] to occupied integrins, thereby enabling the further recruitment of actin-binding proteins such as filamins, PINCHs and parvins [52] that could promote cooperative integrin clustering. Kindlins can also bind to polyphosphoinositides [53-55], thereby localizing integrins in membrane domains that might favor clustering. The present work will enable further studies to evaluate the precise roles of kindlin-binding proteins and phospholipids in the capacity of kindlins to increase multivalent ligand binding to and clustering of integrins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Kindlins increase ligand binding by clustering talin-activated and ligand-occupied integrins
- In contrast, kindlins have little direct effect on purified αIIbβ3 integrin monomer affinity
- These data reveal how kindlin and talin cooperate to activate integrins

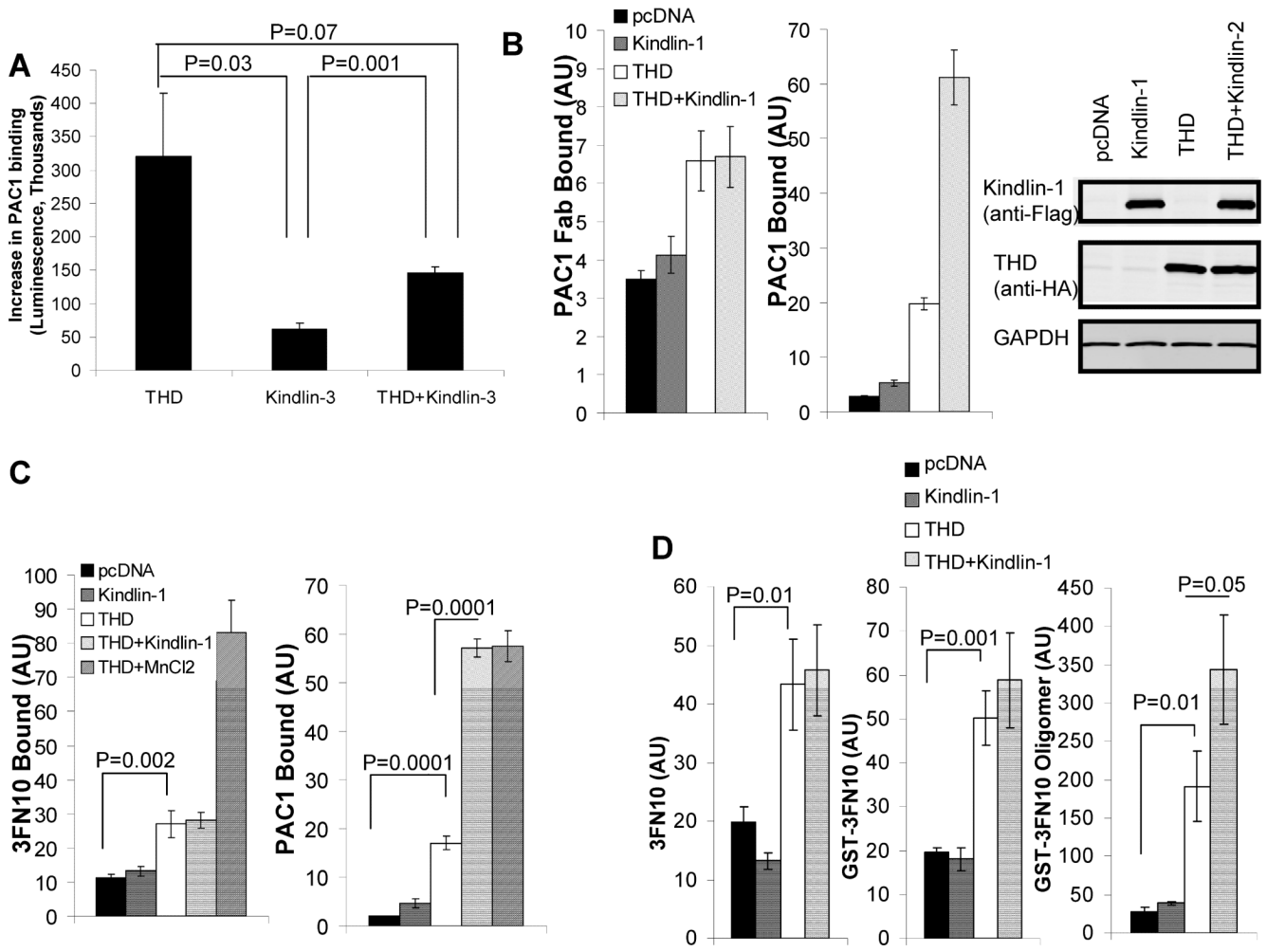

Figure 1.

Kindlins selectively increase the binding of multivalent ligands to recombinant integrin αIIbβ3 cells but not to monomeric integrins in nanodiscs. **(A)** Activation of αIIbβ3 in nanodiscs was assayed by PAC1 binding as described in the supplemental information. Increase in integrin activation is calculated as (L_i-L_0-), where L_i is the Eptifibatide inhibitable PAC1 binding (luminescence) in the presence of THD(1 μM), kindlin-3(2.5 μM) or THD+kindlin-3, L_0 is PAC1 binding to integrin nanodiscs alone, which was 535,000 arbitrary units. The concentration of THD was that inducing half the maximal response observed at 5 μM THD. Error bars indicate ±SEM of three independent determinations. **(B)** HEK293 cells stably expressing αIIbβ3 were transfected with cDNA encoding kindlin-1, THD, or kindlin-1+THD. Twenty-four hr. after transfection, cells were harvested, split into 3 groups, and stained separately with PAC1, PAC1Fab, or D57 (anti-αIIbβ3). Cells were then fixed in 3.7% formaldehyde. The quantity of PAC1 (decavalent), PAC1Fab (monovalent) or D57 bound was assayed by APC-conjugated anti-IgM (μ chain specific) and anti-IgG, and analyzed by flow cytometry. The αIIbβ3-specific ligand binding was calculated as described in the supplemental information. Right panel shows the western blots confirming the abundance of expressed proteins in the transfected cells. Error bars indicate ± SEM of 3 independent experiments. PAC1 Fab was used at a concentration at least 25 fold higher than that used by Bunch [48]. **(C)** HEK293 cells stably expressing αIIbβ3 were transfected with cDNA encoding kindlin-1, THD, or kindlin-1+THD. The cells were

processed as described in (B), and the binding of PAC1 (decavalent), D57, or 3FN10 (GST-removed, monovalent) bound was assayed. The αIIbβ3-specific ligand binding was calculated as described in the supplemental information. Error bars indicate ± SEM of 4 independent experiments. **(D)** HEK293 cells, stably expressing αIIbβ3, were transfected with cDNA encoding kindlin-1, THD, or kindlin-1+THD. The cells were harvested and then the binding of D57, biotinylated 3FN10 monomer (with GST removed), GST-3FN10, or oligomeric GST-3FN10 was assayed. Depicted is the αIIbβ3-specific ligand binding as described in supplemental information. Error bars indicate ± SEM of four independent experiments.

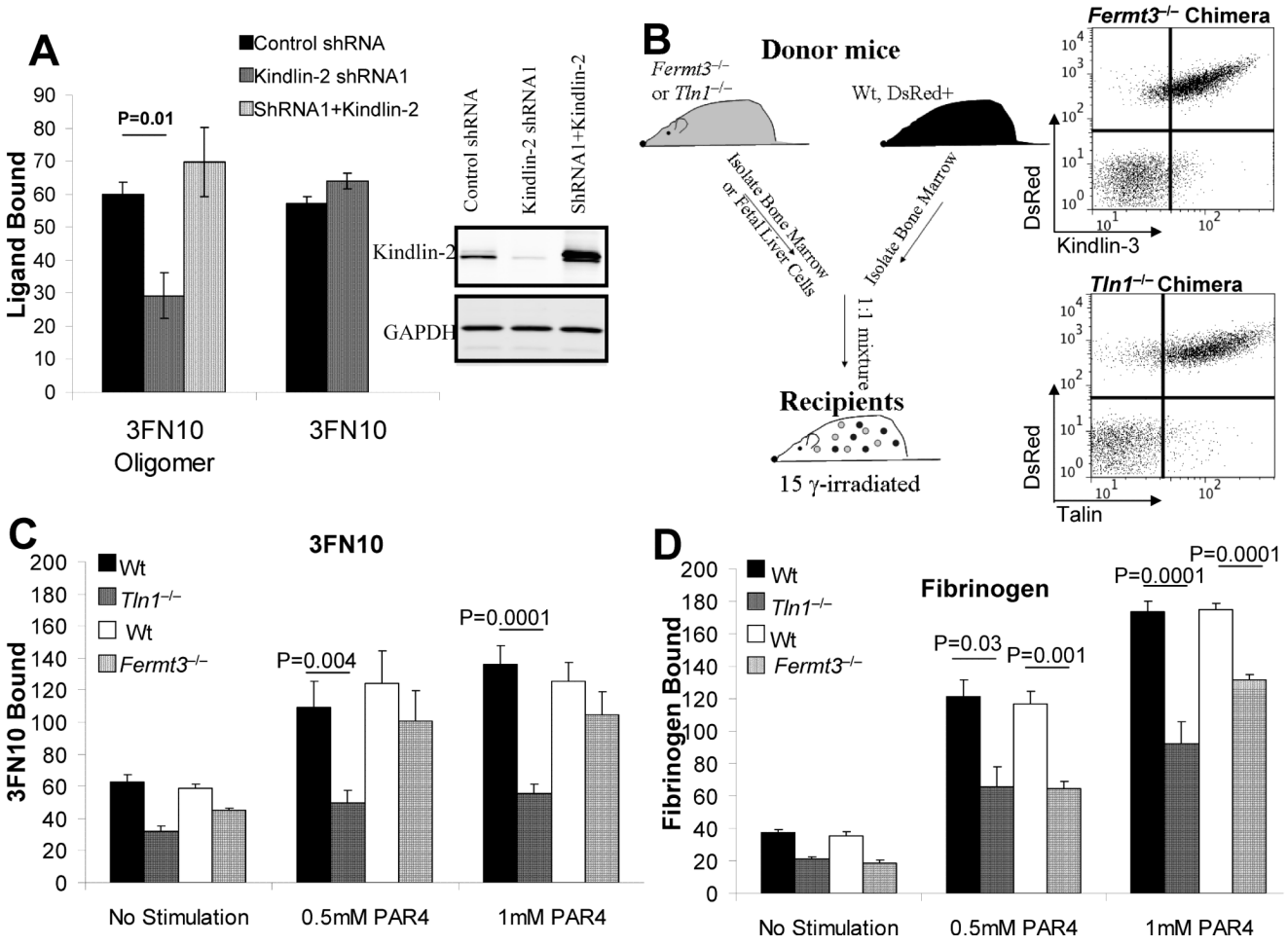

Figure 2.

Depletion of kindlins primarily inhibits the binding of multivalent ligands to integrin αIIbβ3 in nucleated cells and in mouse platelets. **(A)** Cells expressing an active αIIbβ3(D723R) mutant were transduced with lentivirus expressing a GFP marker and either a scrambled shRNA (control) or kindlin-2 shRNA1. To confirm the specificity of previously characterized kindlin-2 knock-down constructs [17], shRNA1-transduced cells were transiently co-transfected with an shRNA-resistant human kindlin-2 and tdTomato as a transfection marker (at a ratio kindlin-2:tomato=50:1). The cells were then harvested and binding of anti-αIIbβ3 (D57), monomeric 3FN10 (with GST removed) or GST-3FN10 oligomers was used to measure the αIIbβ3-specific ligand binding as described in the supplemental information. Kindlin2 expression level was assessed by immunoblotting as shown in the right panel. The western blot was performed with total cells whereas the only transduced (GFP positive) and transfected (tomato positive) cells were analyzed for ligand binding by flow cytometry. **(B)** Generation of chimeric mice as described in the supplemental information. For intracellular flow cytometry, platelets were fixed with 2% formaldehyde, permeabilized and stained with rabbit anti-kindlin-3 polyclonal antibody or biotinylated anti-talin monoclonal antibody (clone 8d4). After washing, bound antibodies were detected with either FITC-anti-rabbit secondary antibody or APC-strepavidin respectively. The dot plots show the reduced expression of kindlin-3 and talin1 in DsRed negative *Fermt3*$^{-/-}$, and *Tln1*$^{-/-}$ platelets respectively. **(C)** PAR4-stimulated binding of 3FN10 to platelets. Platelets from irradiated mice reconstituted with mixtures of DsRed-

expressing wild-type (Wt) and either *Fermt3*$^{-/-}$ or *Tln1*$^{-/-}$ hematopoietic stem cells were incubated in the presence of the indicated concentration of PAR4 agonist peptide or buffer. Eptifibatide-inhibitable specific binding of 3FN10 (with GST removed) to αIIbβ3 on DsRed-negative *Fermt3*$^{-/-}$ or *Tln1*$^{-/-}$ or DsRed positive wild-type platelets was assessed by FACS. Cells were separately stained with anti-CD41 (αIIb) to calculate ligand binding as described in the supplemental information. 3FN10 at 100 μg/ml (9.4 μM) was used. **(D)** PAR4-stimulated binding of fibrinogen to platelets. In an experiment conducted on the same day as that described in (C), 3FN10 was replaced by FITC-conjugated fibrinogen. In both C and D, error bars indicate standard error of the mean (7 chimeric mice for each genotype).

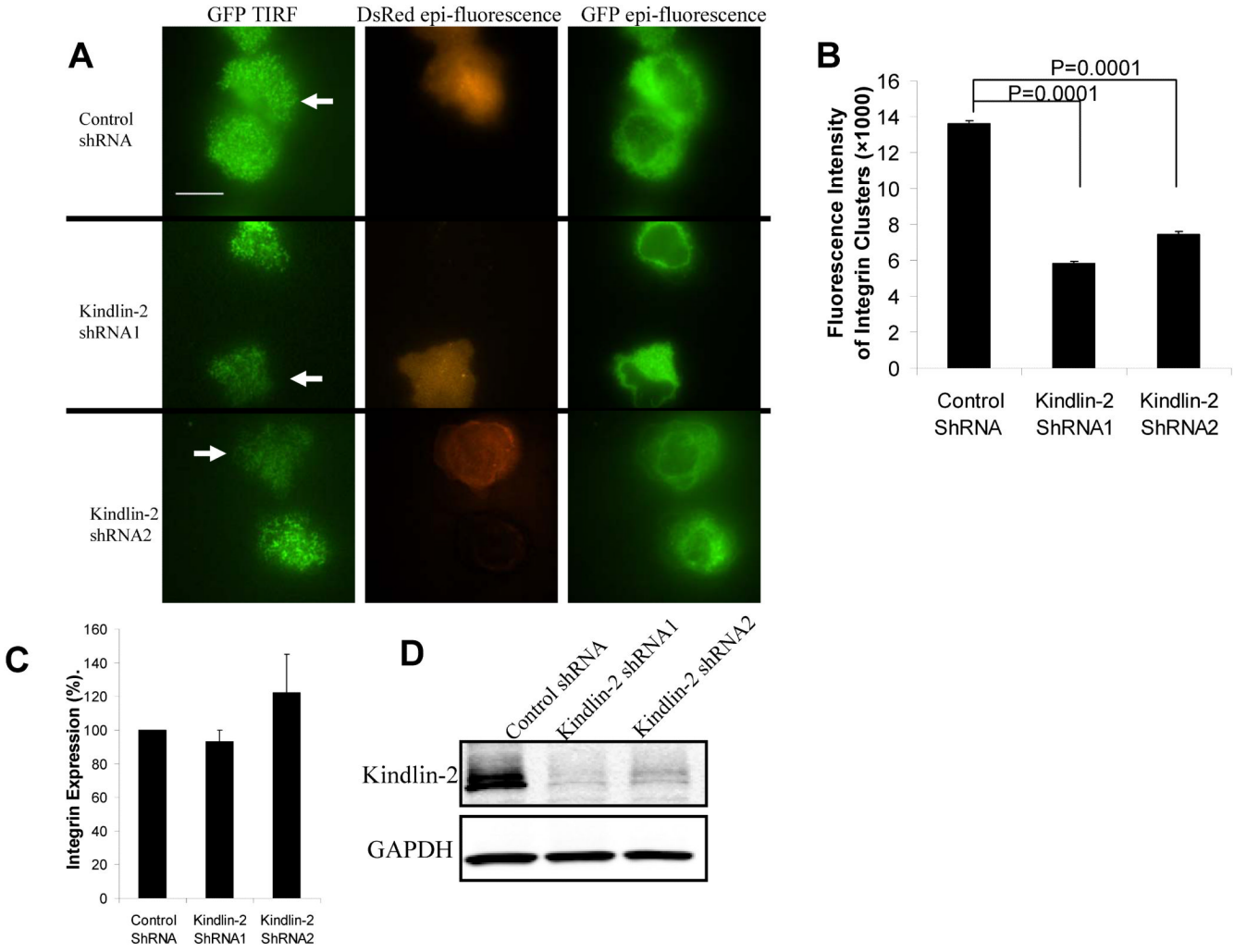

Figure 3.

Kindlins promote clustering of occupied integrins. **(A)** Cells expressing αIIb-GFPβ3 transduced with lentivirus encoding both kindlin-2 shRNA and DsRed were mixed with uninfected cells and plated on fibrinogen-coated cover slips. The left panels are TIRF images of the distribution of αIIb-GFPβ3 at the cell-substrate interface in uninfected cells (DsRed negative) and shRNA-transduced cells (DsRed positive, white arrows). DsRed epi-illuminated fluorescence images in the middle panel indicate shRNA lentiviral transduction. GFP epi-illuminated fluorescence images in the right panels indicate comparable αIIbβ3 fluorescence in uninfected control cells and kindlin-2 shRNA-expressing cells. Scale bar is 10 μm. **(B)** Fluorescence intensities of integrin puncta were measured and averaged as described in supplemental information. Error bars indicate standard error of n=1223, 1204 and 1008 puncta in uninfected cells or those transduced with kindlin-2 shRNA1 or shRNA2 respectively. **(C)** Cells from the experiment depicted in Panel (A) were stained with anti-αIIbβ3 (D57) and analyzed by FACS to assess αIIbβ3 surface expression. Data are expressed as percent of αIIbβ3 expressed in control shRNA-infected cells. **(D)** DsRed-positive shRNA-transduced cells were isolated by FACS and kindlin-2 expression was assessed by western blotting.

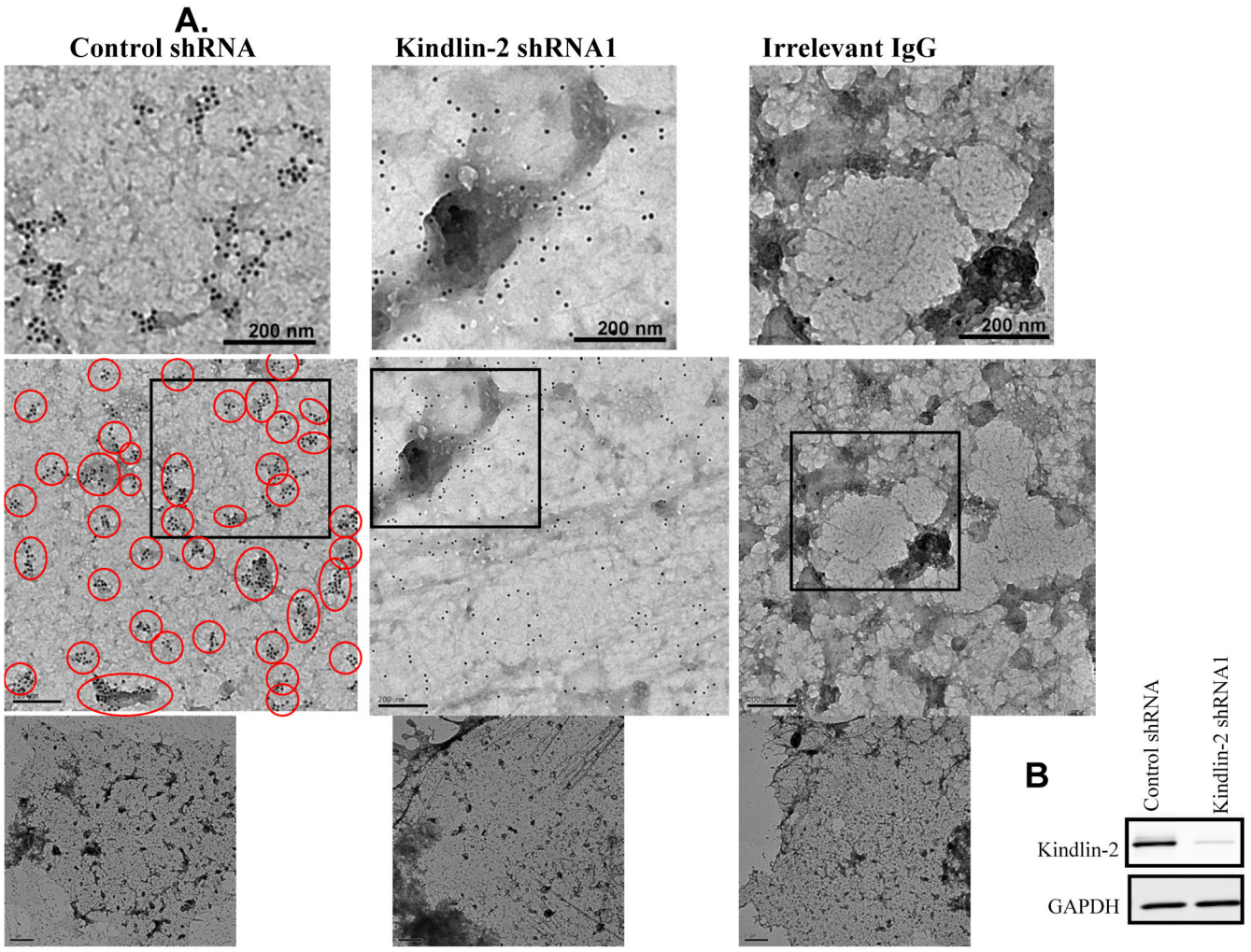

Figure 4.

Loss of kindlin-2 reduces clustering of αIIbβ3 integrins. **(A)** EM images of colloidal gold-labeled αIIbβ3 in adherent cell ventral membrane. CHO cells stably expressing αIIbβ3 were transduced with lentivirus expressing control shRNA or kindlin-2 shRNA1, adhered to fibrinogen-coated EM grids, swollen in a hypotonic buffer and subjected to a flow of low salt buffer to remove the cell body. The remaining ventral membrane sheet was stained with an anti-β3 tail antibody followed by colloidal gold adsorbed 2° antibody. Left, middle and right columns are control shRNA cells, kindlin-2 knockdown cells, and irrelevant IgG-stained control shRNA cells at differing magnifications. Many clusters of colloidal gold containing >5 particles per cluster are present in control shRNA cells and such clusters are absent in kindlin-2 silenced cells. Red circles indicate examples of integrin clusters. Scale Bars are 200 nm for the images in top and middle row and 2 μm for the images in bottom row. The images in the middle and lower rows are ventral membranes at different magnifications. Images in the top row show enlarged views of selected areas from those in the middle row. **(B)** Cells in (A) were lysed and analyzed with anti-kindlin-2 or anti-GAPDH by western blotting.