SUPPLEMENTARY MATERIALS
MATERIALS AND METHODS

Reagents and antibodies. Antibodies 8275 against the last 20 amino acids of the β3 C-terminus, [1] 1B5 against the murine integrin β3 subunit, [2] D57 against human integrin αIIbβ3 [3], PAC1 against activated human αIIbβ3, [4] and antibodies to human and mouse ADAP, [5] and SKAP-HOM (SKAP2), [6] have been described. A kindlin-3 antibody was raised in rabbits. Mouse monoclonal antibody against β-actin and rabbit monoclonal antibodies against kindlin-2, kindlin-3 and the N- or C-terminus of ADAP were from Abcam (Cambridge, MA); FLAG and talin mouse monoclonal antibodies were from Sigma-Aldrich (St. Louis, MO), and rat antibody against murine αIIb, was from BD Biosciences (San Jose, CA). An antibody against the talin NH2 domain was from Santa Cruz Biotechnology (Dallas, TX). Odyssey secondary antibodies were from Licor Biosciences (Lincoln, NE). PAR4 receptor agonist peptide (AYPGKF) was from American Peptide Company (Sunnyvale, CA), and PAR1 peptide (SFLLRN) was from Sigma-Aldrich. Rhodamine-phalloidin, and secondary Alexa-conjugated antibodies were from Molecular Probes/Life Technologies (Eugene, OR). Fibrinogen was from Enzyme Research (Southbend, IN). Proximity Ligation Assay kit was from O-Link Bioscience (Uppsala, Sweden). Additional reagents were from Sigma-Aldrich, Thermo Scientific (USA) or GE Healthcare (San Diego, CA).

Mouse strains. Mice deficient in ADAP and SKAP2 have been described. [7,8] $ADAP^{+/+}$ and $SKAP2^{+/+}$ mice represented littermate controls. No differences in expression levels of αIIbβ3, kindlin-3 or talin were found between platelets from wild type versus knockout mouse strains used. Mice were housed according to IACUC regulations.

Human and mouse platelet preparations. Washed platelets were prepared from human and mouse whole blood drawn into ACD anticoagulant, washed and resuspended in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2.6H_2O$, 3.3 mM $NaH_2PO4.H_2O$, 3.8 mM HEPES, 0.1% bovine serum albumin (BSA), 0.1% dextrose) as described. [9] Human blood was drawn from antecubital veins from consenting normal drug-free donors and mouse blood was drawn by cardiac puncture following euthanasia.

CHO cell culture, transfection and DNA constructs. HEK 293T, NIH 3T3 and CHO K1 cells that stably expressed aIIbβ3 (αIIbβ3-CHO cells) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro, Manassas, VA) with non-essential amino acids, 10% fetal bovine serum (FBS), antibiotics, and L-glutamine. Cells were transiently-transfected using Lipofectamine (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and harvested 48 hours later. αIIbβ3-CHO cells were engineered to conditionally express the

human PAR1 thrombin receptor [10] and mouse talin using a tetracycline-inducible vector [11] (Invitrogen, Carlsbad, CA) [12] Vector pEFBOS/ ADAP, with an inserted N-terminal 5' FLAG tag, [5] was used to express full-length ADAP (residues 1-783) and as a template to prepare ADAP truncation mutants, where the 5' primer containing a SalI site, TTACTGTCGACGGATCTGCCATGGACTAC, was used together with reverse primers that inserted a stop codon and shifted the inherent EcoRV restriction recognition sequence in order to truncate ADAP to desired lengths. The PCR fragment was digested with SalI/EcoRV, and then replaced into similarly digested pEFBOS/ADAP. pEFBOS/ADAP(1-783)Δ498-565 and pEFBOS/ADAP(1-615)Δ498-565 were prepared by digesting the parent vectors with NdeI followed by re-ligation of the cut vector. PGEX4T1-GST/talin and PGEX4T3-kindlin-1 were from Asoka Banno (UC San Diego LA Jolla, CA)[13] and David Calderwood (Yale University, New Haven, CT), [14] respectively. GST constructs were expressed in E. Coli BL21 DE-pLys cells (EMD Biochemicals, San Diego, CA), purified using Glutathione-Sepharose beads, and dialyzed against PBS. pCMV/human kindlin-2 and pcDNA3.1/mouse kindlin-3 were gifts from Cary Wu (University of Pittsburgh, Pittsburgh, PA). EGFPN1 (Clontech, Mountain View, CA) was used as a transfection marker in CHO cell flow cytometry experiments. RIAM in αIIbβ3-PAR1/talin CHO cells was knocked down with an FG12/shRIAM lentiviral construct, [13] and a scrambled ROCK1 sequence was used as a negative shRNA control. [15] RIAM knockdown was verified by Western blotting.

REFERENCES

1. Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH. Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. *J Biol Chem*. 1995;270(44):26146-26151.

2. Lengweiler S, Smyth SS, Jirouskova M, et al. Preparation of monoclonal antibodies to murine platelet glycoprotein IIb/IIIa (alphaIIbbeta3) and other proteins from hamster-mouse interspecies hybridomas. *Biochem Biophys Res Commun*. 1999;262(1):167-173.

3. O'Toole TE, Loftus JC, Du XP, et al. Affinity modulation of the alpha IIb beta 3 integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul*. 1990;1(12):883-893.

4. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem*. 1985;260(20):11107-11114.

5. Musci MA, Hendricks-Taylor LR, Motto DG, et al. Molecular cloning of SLAP-130, an SLP-76-associated substrate of the T cell antigen receptor-stimulated protein tyrosine kinases. *J Biol Chem*. 1997;272(18):11674-11677.

6. Marie-Cardine A, Verhagen AM, Eckerskorn C, Schraven B. SKAP-HOM, a novel adaptor protein homologous to the FYN-associated protein SKAP55. *FEBS Lett*. 1998;435(1):55-60.

7. Peterson EJ, Woods ML, Dmowski SA, et al. Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science*. 2001;293(5538):2263-2265.

8. Togni M, Swanson KD, Reimann S, et al. Regulation of in vitro and in vivo immune functions by the cytosolic adaptor protein SKAP-HOM. *Mol Cell Biol*. 2005;25(18):8052-8063.

9. Kasirer-Friede A, Cozzi MR, Mazzucato M, De Marco L, Ruggeri ZM, Shattil SJ. Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors. *Blood*. 2004;103(9):3403-3411.

10. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*. 1999;103(6):879-887.

11. Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum Gene Ther*. 1998;9(13):1939-1950.

12. Watanabe N, Bodin L, Pandey M, et al. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin αIIbβ3. *J Cell Biol*. 2008;181(7):1211-1222.

13. Banno A, Goult BT, Lee H, Bate N, Critchley DR, Ginsberg MH. Subcellular localization of talin is regulated by inter-domain interactions. *J Biol Chem*. 2012;287(17):13799-13812.

14. Harburger DS, Bouaouina M, Calderwood DA. Kindlin-1 and -2 directly bind the C-terminal region of beta integrin cytoplasmic tails and exert integrin-specific activation effects. *J Biol Chem*. 2009;284(17):11485-11497.

15. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science*. 2003;302(5642):103-106.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Calpain-mediated cleavage of talin, kindlin-3 and β3. Human (**A-C**) or mouse (**D-F**) unstirred platelets were stimulated for the indicated times with SFLLRN (PAR1 agonist peptide) or AYPGKF (PAR4 agonist peptide), respectively, then lysed and western blotted with antibodies recognizing talin head domain (**A,D**), β3 tail (**B,E**) and kindlin3 (**C,F**) cleavage products. As a positive control, platelets were activated with 0.5 U/ml thrombin under stirring conditions for 4 minutes at 37^{o} C, as indicated.

Figure S.2. His-kindlin-3 pull-down of ADAP. Resting human platelet lysate was incubated with His-kindlin-3 overnight, and pull-downs were Western blotted for ADAP and for β3, as described for Figure 1. Gels were stained with Coomassie Brilliant Blue to assess protein loading. Results shown are representative of two experiments.

Figure S.3. Controls for proximity ligation assays. A-F. Washed human platelets were stimulated with SFLLRN, plated on fibrinogen-coated coverslips for one hour, and processed as described in Methods for proximity ligation assay (PLA) detection of protein-protein interactions. Specific PLA signals appeared as bright red dots when ADAP-talin (**A**) and ADAP-kindlin (**E**) primary antibodies were used (arrows), but not when control pairs of IgG and primary antibody against ADAP (**B**), talin (**C**), kindlin-3 (**F**), or IgG/IgG (**D**) were used. Platelets were counterstained with FITC-phalloidin (green pseudocolor).

Figure S4. GST-talin pull-down of ADAP from RIAM knockdown and control CHO cells expressing PAR1, talin and αIIbβ3. A. Cells were transduced to express RIAM shRNA or

control shRNA. After transient-transfection with ADAP or vector cDNA (Mock), cells were analyzed for RIAM expression by Western blotting. **B**. Cell lysates were incubated with GST-talin, and the presence of ADAP in the pull downs was assessed by Western blotting. Gels were stained with Coomassie to assess protein loading. Results shown are representative of three experiments.

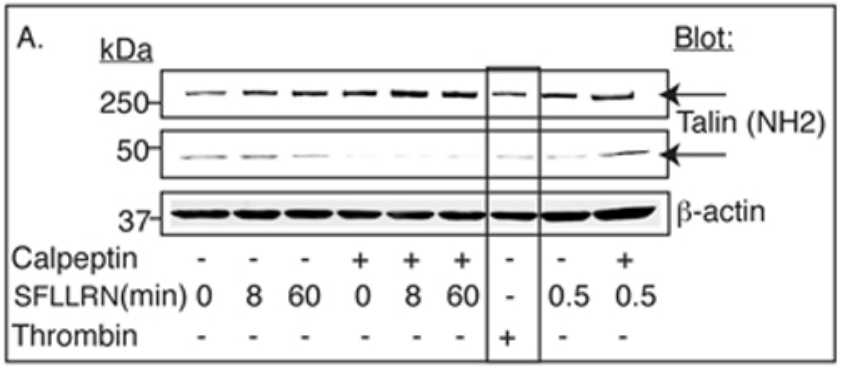
Figure S5. ADAP associates with kindlins in CHO cells expressing PAR1, talin and αIIbβ3. Cells were transiently-transfected with ADAP and kindlin-2 (or kindlin-3), plated on fibrinogen and processed for immunofluorescence microscopy. As indicated, cells were stained for ADAP and kindlins, and with rhodamine phalloidin to label F-actin. ADAP and kindlin-2 (top panels) were highly co-localized, especially at cell peripheries and in focal-adhesion structures, often together with actin (arrows). ADAP also co-localized with kindlin-3, but was more prevalent in cytoplasmic, punctate structures (middle panels). Bottom panels show staining with control IgG antibodies. Results are representative of three experiments.

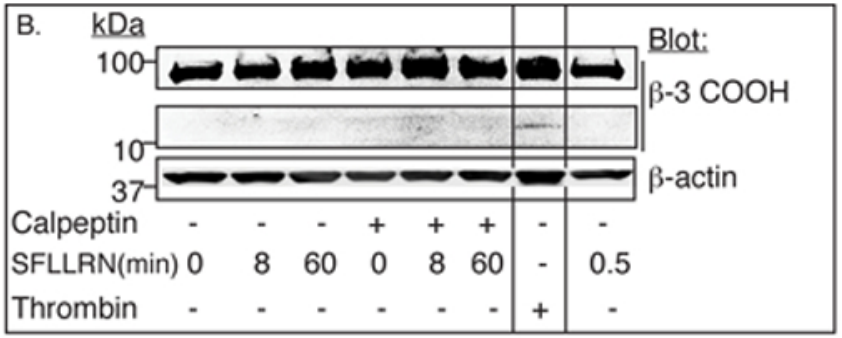
Figure S.6 Primary data for PAC-1 binding to transfected CHO cells. A,B. CHO cells expressing PAR1, talin and αIIbβ3 were transiently-transfected with mock DNA, ADAP and/or kindlin-2 (**A**), or kindlin-3 (**B**) along with GFP as a transfection marker, and PAC-1 binding to transfected cells was assessed by flow cytometry as described in the Methods section. Results are presented from a single experiment showing specific PAC-1 binding normalized only for αIIbβ3 expression.

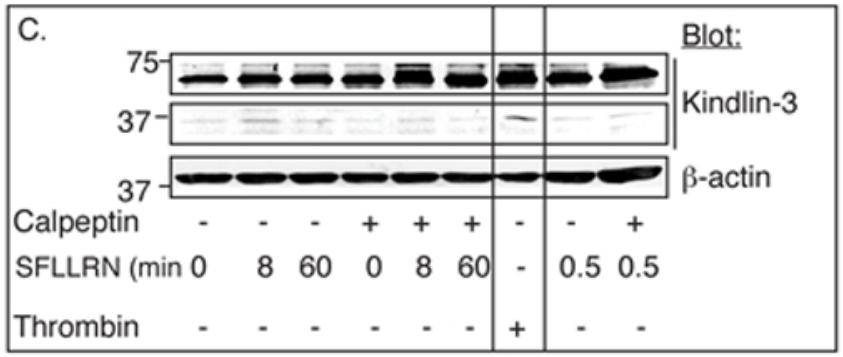
Figure S.7. αIIbβ3 and ADAP expression levels in transfected CHO cells utilized for PAC-1 binding studies. CHO cells expressing PAR1, talin and αIIbβ3 were transiently-transfected with mock DNA, ADAP and/or kindlin-2, or kindlin-3 along with GFP as a transfection marker. **A,B.** Densitometry of ADAP expression levels in CHO cells transfected with ADAP alone or together with kindlin-2 (**A**) or kindlin-3 (**B**). Average αIIbβ3 expression on transfected cells, determined by flow cytometry was similar between transfectants (**C**). Results represent the mean ± SEM of at least five experiments.

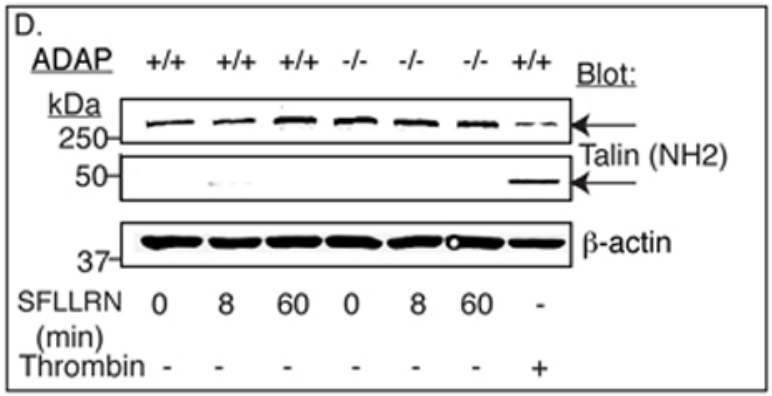
Figure S.8. Talin co-immunoprecipitation of β3 and kindlin-3 from ADAP$^{+/+}$ and ADAP$^{-/-}$ mouse platelet lysate. Resting or AYPGKF-stimulated mouse platelets were lysed and immunoprecipitated using an antibody against talin, and Western blots were prepared to detect β3 and kindlin-3. ADAP deletion was verified by blotting ADAP$^{+/+}$ and ADAP$^{-/-}$ platelet lysate with an antibody against murine ADAP.

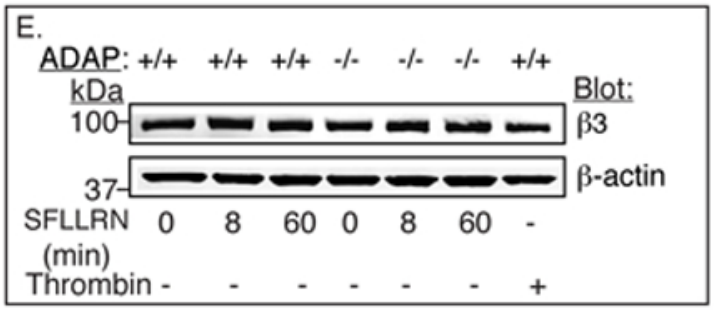
Table 1. P values for experiments shown in Figure 6 A,B. Student's unpaired t-test was used to determine probability values for FITC-fibrinogen binding to $ADAP^{+/+}$ (WT) and $ADAP^{-/-}$ (KO) platelets in the presence or absence of POW-2.


A.
kDa
Blot:
250
Talin (NH2)
50
37
β-actin
Calpeptin - - - + + + - - +
SFLLRN(min) 0 8 60 0 8 60 - 0.5 0.5
Thrombin - - - - - - + - -


B.
kDa
Blot:
100
β-3 COOH
10
37
β-actin
Calpeptin - - - + + + - -
SFLLRN(min) 0 8 60 0 8 60 - 0.5
Thrombin - - - - - - + -


C.
Blot:
75
Kindlin-3
37
37
β-actin
Calpeptin - - - + + + - - +
SFLLRN (min 0 8 60 0 8 60 - 0.5 0.5
Thrombin - - - - - - + - -


D.
ADAP +/+ +/+ +/+ -/- -/- -/- +/+
Blot:
kDa
250
Talin (NH2)
50
37
β-actin
SFLLRN (min) 0 8 60 0 8 60 -
Thrombin - - - - - - +


E.
ADAP: +/+ +/+ +/+ -/- -/- -/- +/+
kDa
Blot:
100
β3
37
β-actin
SFLLRN (min) 0 8 60 0 8 60 -
Thrombin - - - - - - +

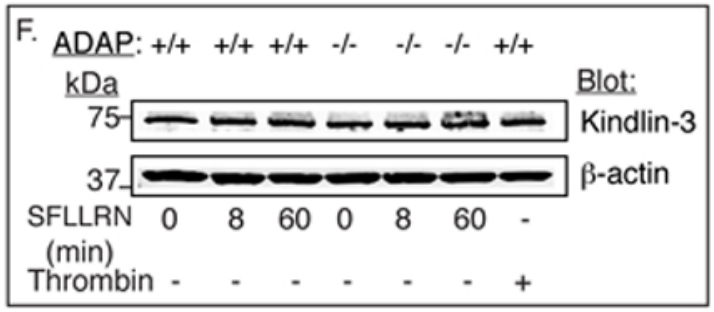

F.
ADAP: +/+ +/+ +/+ -/- -/- -/- +/+
kDa
Blot:
75
Kindlin-3
37
β-actin
SFLLRN (min) 0 8 60 0 8 60 -
Thrombin - - - - - - +

Figure S1

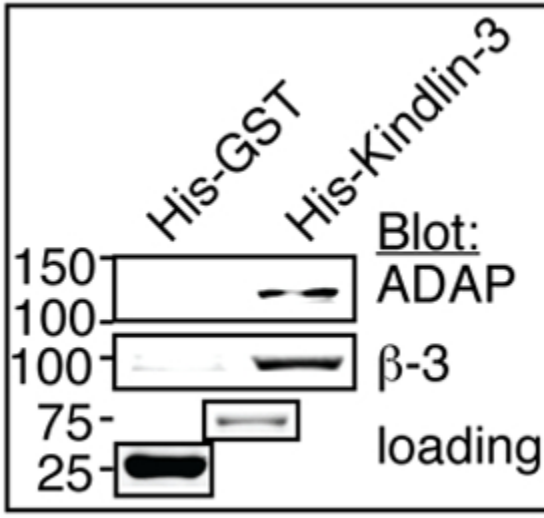
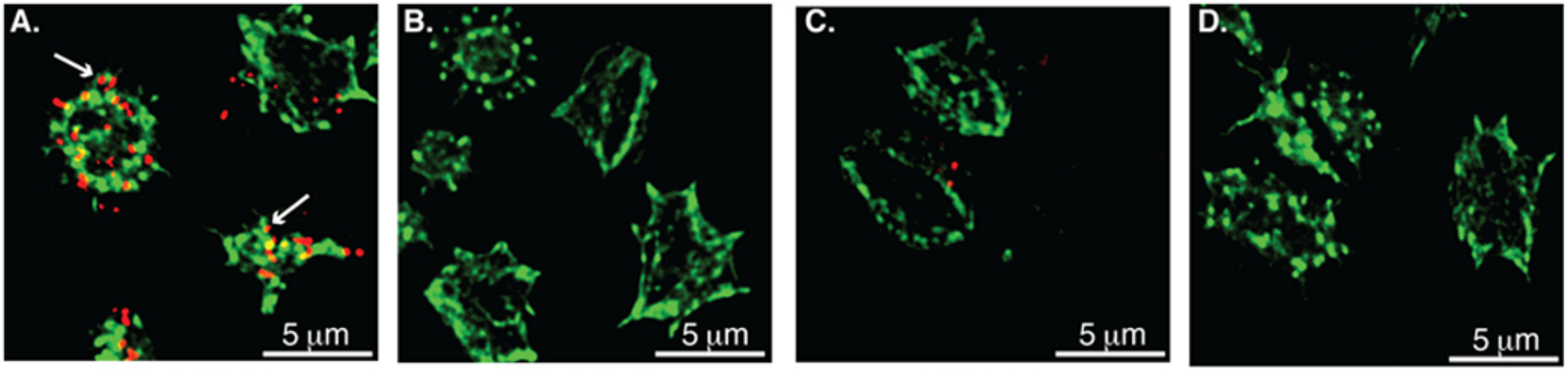


Figure S2

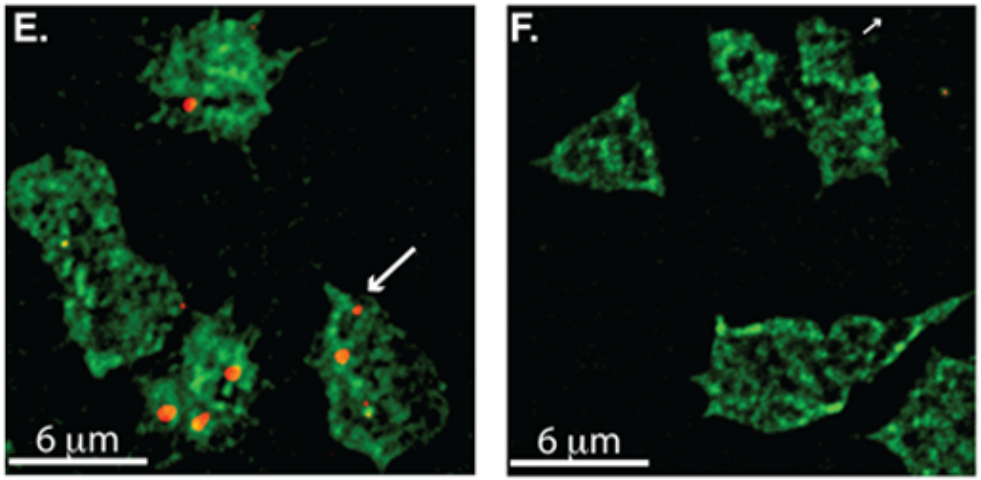


ADAP/ Talin
Proximity Signal
F-actin

ADAP/ IgG
Proximity Signal
F-actin

IgG/ Talin
Proximity Signal
F-actin

IgG/ IgG
Proximity Signal
F-actin



ADAP/ Kindlin-3
Proximity Signal
F-actin

IgG/ Kindlin-3
Proximity Signal
F-actin

Figure S3

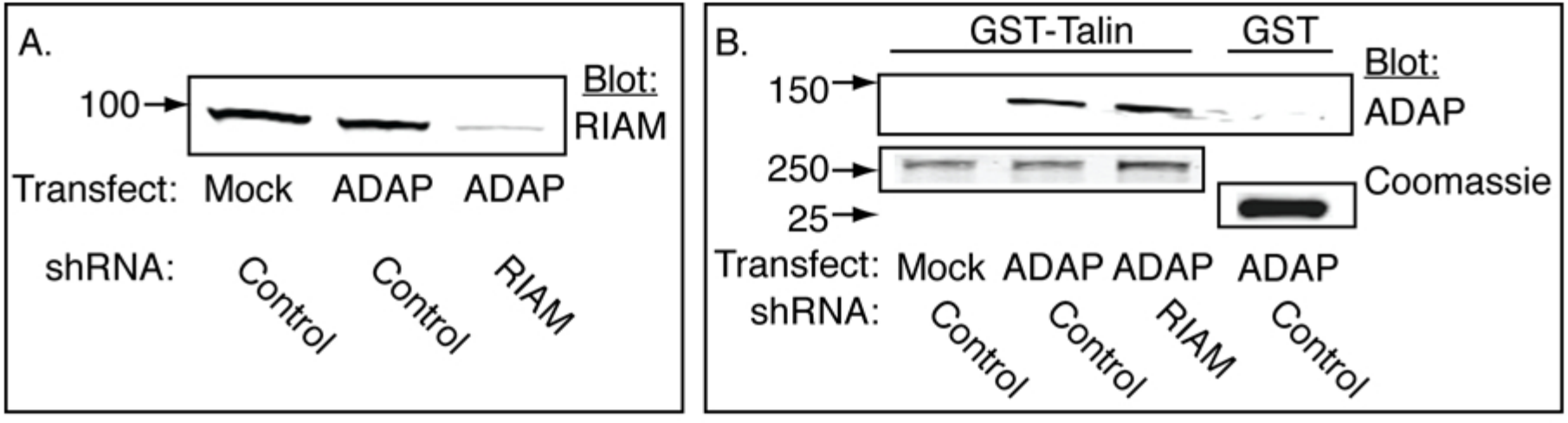

A.
100
Blot:
RIAM
Transfect: Mock ADAP ADAP
shRNA: Control Control RIAM
B.
GST-Talin
GST
Blot:
150
ADAP
250
Coomassie
25
Transfect: Mock ADAP ADAP ADAP
shRNA: Control Control RIAM Control

Figure S4

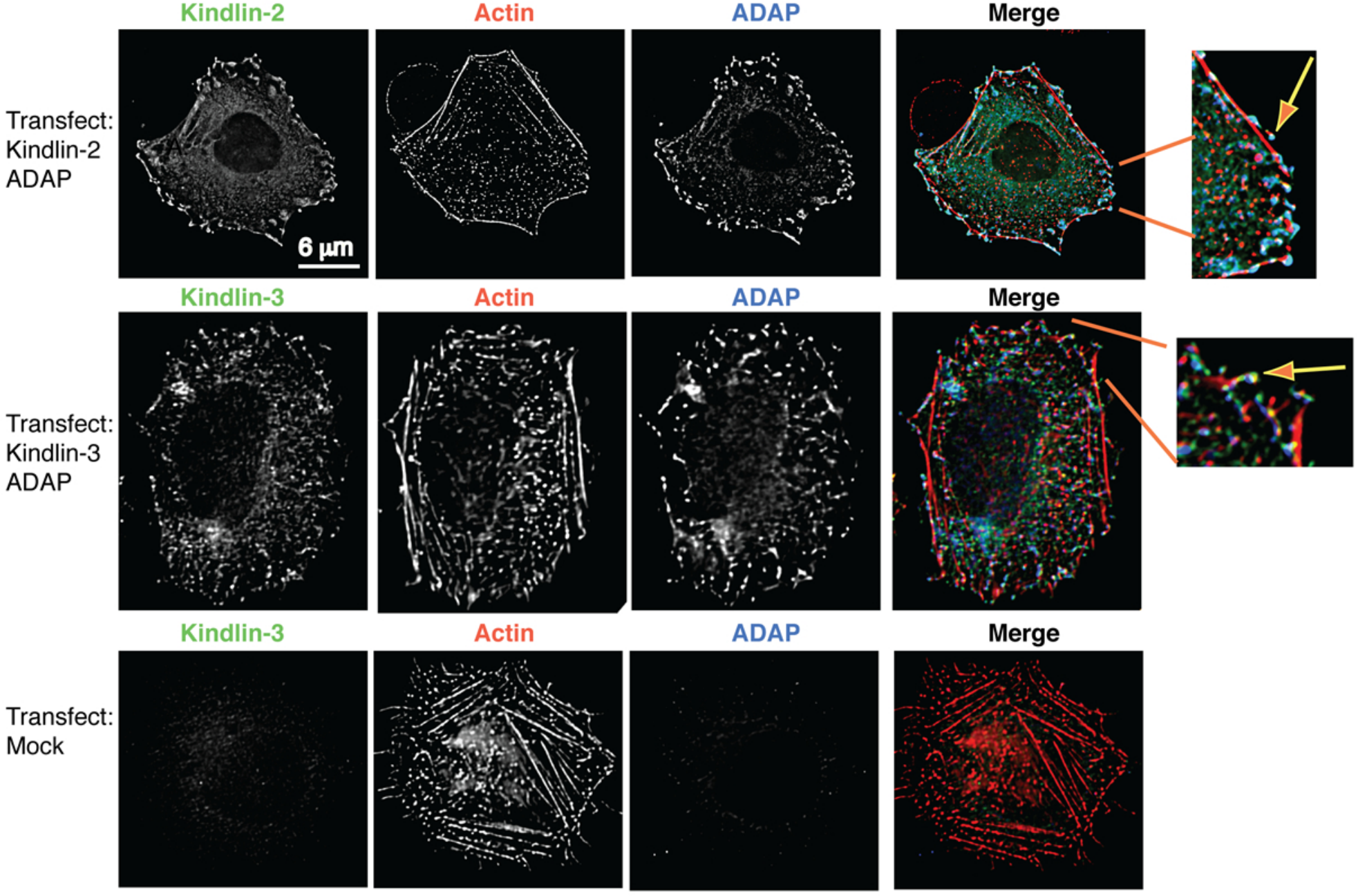

Kindlin-2
Actin
ADAP
Merge
Transfect:
Kindlin-2
ADAP
6 µm
Kindlin-3
Actin
ADAP
Merge
Transfect:
Kindlin-3
ADAP
Kindlin-3
Actin
ADAP
Merge
Transfect:
Mock

Figure S5

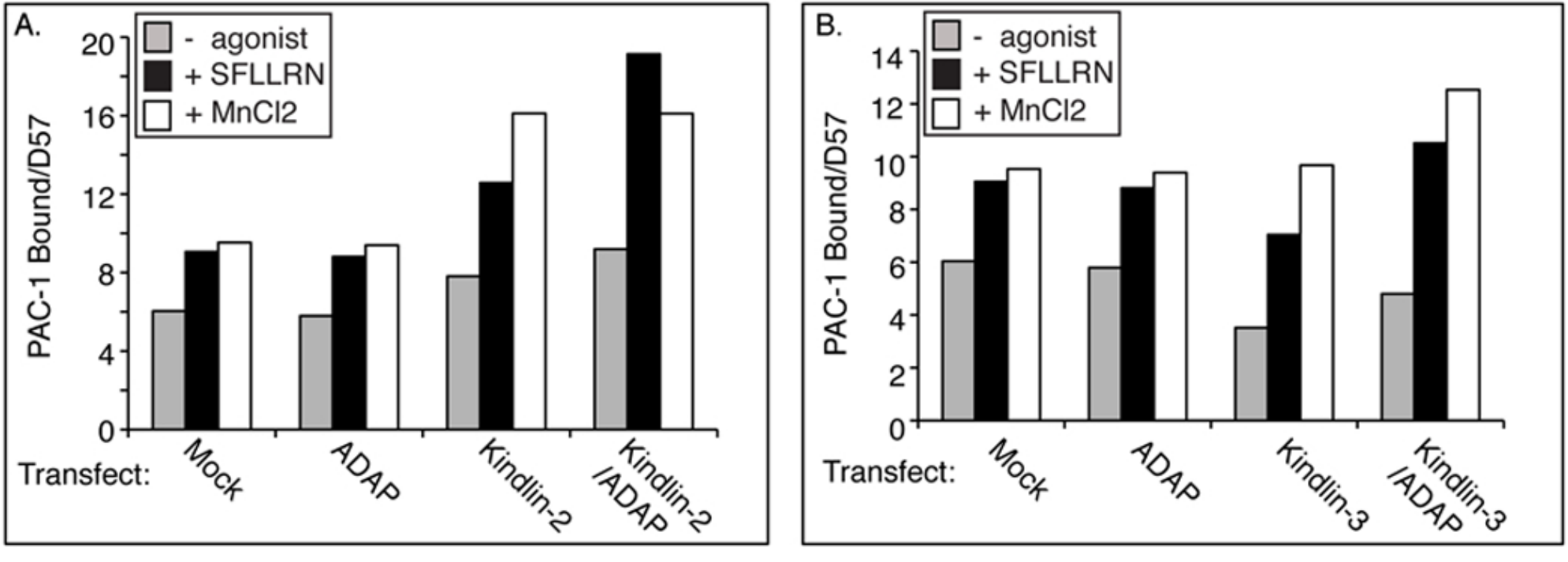
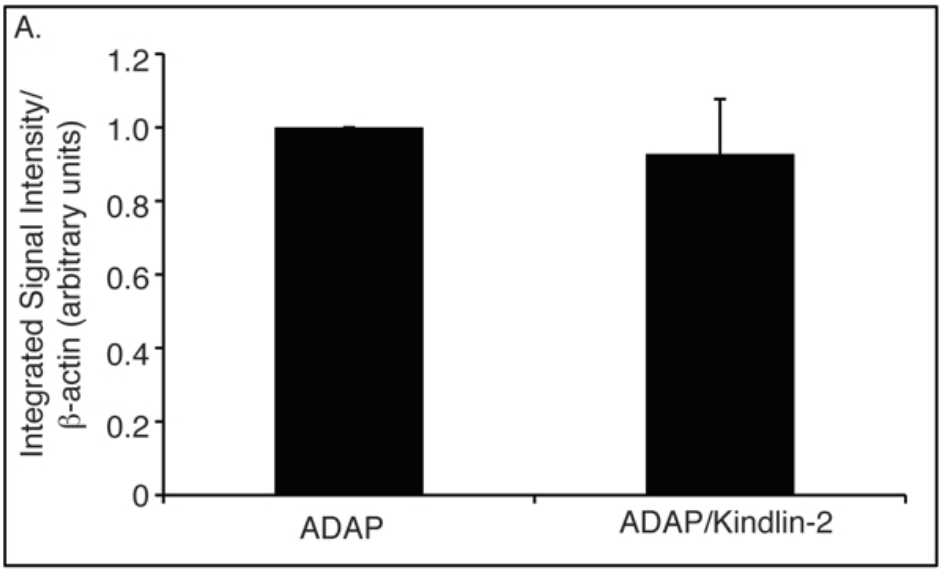
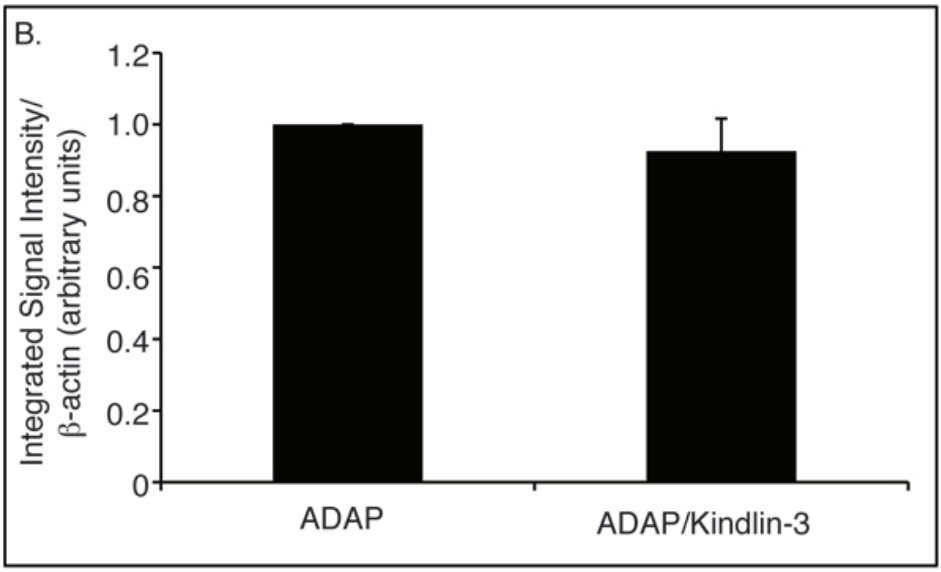

A.
- agonist
+ SFLLRN
+ MnCl2
PAC-1 Bound/D57
0
4
8
12
16
20
Transfect:
Mock
ADAP
Kindlin-2
Kindlin-2 /ADAP
B.
- agonist
+ SFLLRN
+ MnCl2
PAC-1 Bound/D57
0
2
4
6
8
10
12
14
Transfect:
Mock
ADAP
Kindlin-3
Kindlin-3 /ADAP

Figure S6


A.
Integrated Signal Intensity/
β-actin (arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0
ADAP
ADAP/Kindlin-2


B.
Integrated Signal Intensity/
β-actin (arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0
ADAP
ADAP/Kindlin-3

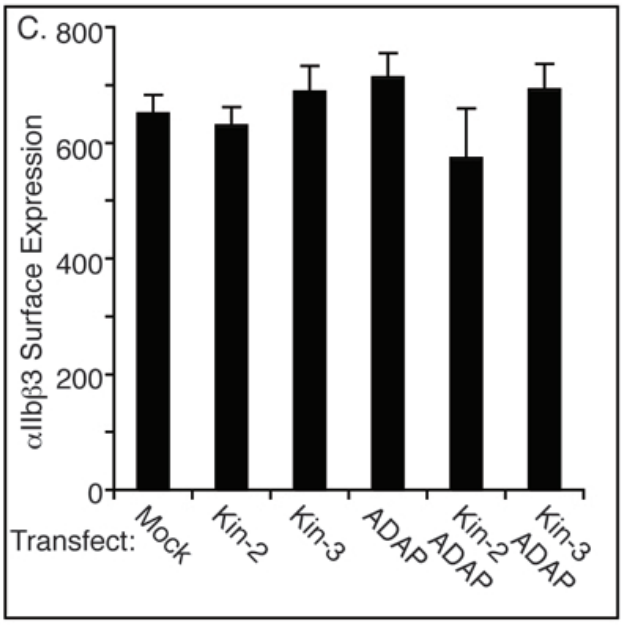

C.
αIIbβ3 Surface Expression
800
600
400
200
0
Transfect:
Mock
Kin-2
Kin-3
ADAP
Kin-2 ADAP
Kin-3 ADAP

Figure S7

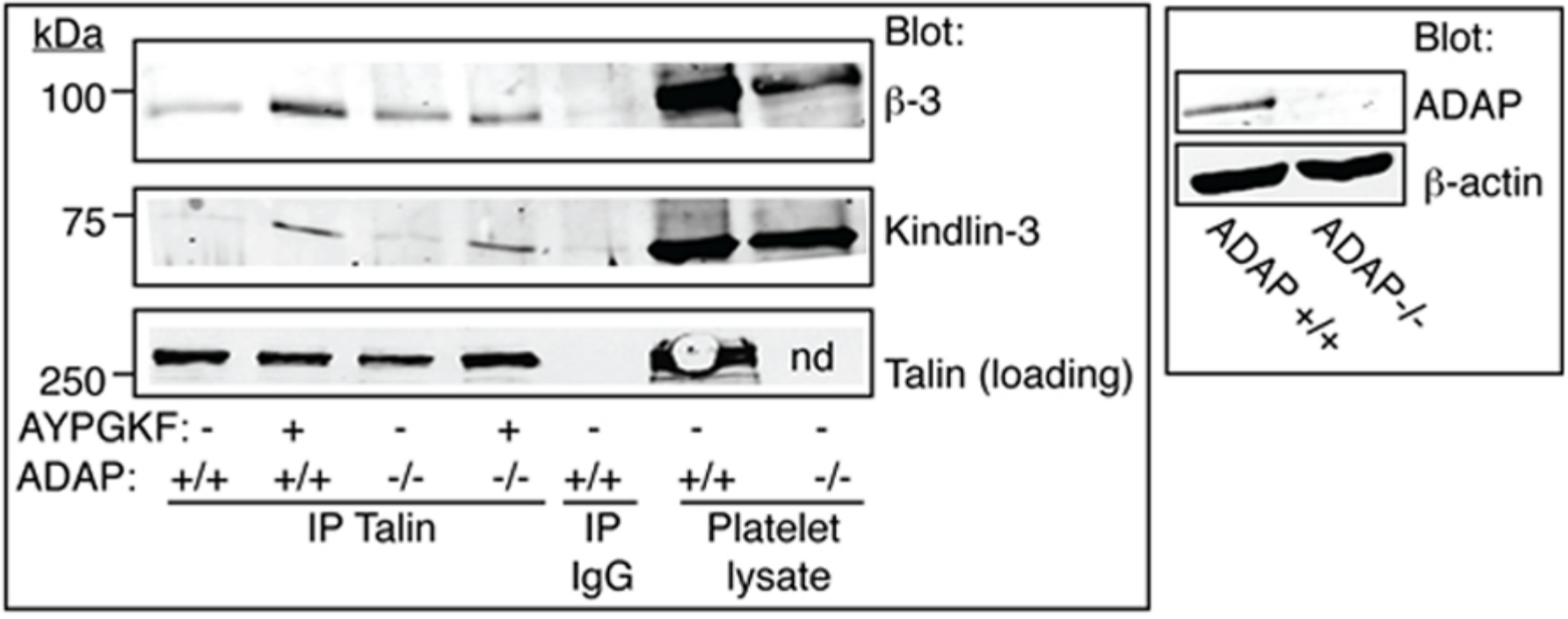

kDa
100
75
250
Blot:
β-3
Kindlin-3
nd
Talin (loading)
AYPGKF: - + - + - - -
ADAP: +/+ +/+ -/- -/- +/+ +/+ -/-
IP Talin
IP IgG
Platelet lysate
Blot:
ADAP
β-actin
ADAP +/+
ADAP-/-

Figure S8

	P Values						
Par 4 (μM)	0	50	100	160	250	500	1000
WT -POW-2: KO -POW-2	0.35	0.03	0.003	0.004	0.04	0.04	0.02
WT -POW-2: WT +POW-2	0.31	0.03	0.005	0.002	0.02	0.07	0.07
KO -POW-2: KO +POW-2	0.06	0.16	0.03	0.004	0.03	0.11	0.22
WT +POW-2: KO +POW-2	0.32	0.46	0.19	0.18	0.07	0.01	0.03

Table S1