PPM1F controls integrin activity via a conserved phospho-switch

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Control of integrin activity is vital during development and tissue homeostasis, while derailing of integrin function contributes to pathophysiological processes. Phosphorylation of a conserved threonine motif (T788/T789) in the integrin β cytoplasmic domain increases integrin activity. Here, we report that T788/T789 functions as a phospho-switch, which determines the association with either talin and kindlin-2, the major integrin activators, or filaminA, an integrin activity suppressor. A genetic screen identifies the phosphatase PPM1F as the critical enzyme, which selectively and directly dephosphorylates the T788/T789 motif. PPM1F-deficient cell lines show constitutive integrin phosphorylation, exaggerated talin binding, increased integrin activity, and enhanced cell adhesion. These gain-of-function phenotypes are reverted by reexpression of active PPM1F, but not a phosphatase-dead mutant. Disruption of the ppm1f gene in mice results in early embryonic death at day E10.5. Together, PPM1F controls the T788/T789 phospho-switch in the integrin β1 cytoplasmic tail and constitutes a novel target to modulate integrin activity.

Introduction

Integrins are essential heterodimeric cell surface receptors that mediate extracellular matrix adhesion and instruct animal cells about the chemical and mechanical properties of their microenvironment (Gahmberg et al., 2009; Hynes, 2002; Morse et al., 2014). Accordingly, integrins are instrumental for cell adhesion during development, tissue regeneration, or leukocyte extravasation, but also contribute to pathological processes such as cancer cell invasion and metastasis (Bökel and Brown, 2002; Hamidi and Ivaska, 2018; Nieswandt et al., 2009; Sekine et al., 2012; Vestweber, 2002; Winograd-Katz et al., 2014).

A major regulatory principle of integrins involves an extensive conformational change, which has been termed integrin activation (Calderwood, 2004; Sims et al., 1991; Vinogradova et al., 2002). The active conformation of integrins can be stabilized either by the presence of an extracellular ligand (outside-in activation) or by a characteristic intracellular binding event of the scaffold protein talin to the cytoplasmic tail of the integrin β subunit (inside-out activation; Hughes et al., 1996; Shattil et al., 2010; Vinogradova et al., 2002; Wegener et al., 2007). During inside-out activation, the globular head of talin binds to a conserved NPxY amino acid sequence, thereby spatially separating the α and β subunits and forcing the extracellular domains into the extended, active conformation (Anthis et al., 2009; Calderwood et al., 2002; Wegener et al., 2007). This active conformation is a prerequisite for proper integrin-mediated cell attachment to the extracellular matrix (Harburger and Calderwood, 2009; Moser et al., 2009). Cell adhesion can be further promoted by integrin clustering (Bunch, 2010; Cluzel et al., 2005; van Kooyk and Figdor, 2000), which is supported by kindlin (Li et al., 2017; Ye et al., 2013), an additional binding partner of the integrin β subunit (Bledzka et al., 2012; Harburger et al., 2009; Li et al., 2017). Together, talin and kindlin initiate the formation of large, heteromeric protein complexes at integrin cytoplasmic tails, which are termed focal adhesion sites. These structures can comprise several hundred distinct proteins, the so-called integrin adhesome (Horton et al., 2015; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007).

Besides talin and kindlin as positive regulators of integrin function, several negative regulators of integrin activity such as filaminA, Dok1, Sharpin, or ICAP-1 have been described (Bouvard et al., 2003; Kiema et al., 2006; Liu et al., 2015; Oxley et al., 2008; Rantala et al., 2011). These nonenzymatic proteins are thought to act by competitive binding to the integrin β subunit, where they displace positive regulators of integrin activity. For example, filaminA and talin have overlapping binding sites in the leukocyte-specific integrin subunits β2 and β7, which occupy in a mutually exclusive manner (Kiema et al., 2006; Takala et al., 2008). Interestingly, an evolutionary conserved
threonine motif within the context of the filaminA and talin core binding sites is located in the cytoplasmic tails of most integrin β subunits (T788/T789 in the human integrin β1; Fig. 1 A and Fig. S1 A; García-Alvarez et al., 2003; Gingras et al., 2009; Kiema et al., 2006; Liu et al., 2015; Wegener et al., 2007). Upon cell stimulation, these threonine residues are phosphorylated (Buyon et al., 1990; Chatila et al., 1989; Craig et al., 2009; Hibbs et al., 1991; Hilden et al., 2003), and mutations mimicking Ser/Thr phosphorylation lead to enhanced integrin activity and integrin-based cell adhesion in vitro (Craig et al., 2009; Nilsson et al., 2006). In contrast, alanine substitution of this particular threonine motif severely compromises integrin function, leading to impaired integrin activation and abrogation of cell-matrix adhesion (Fagerholm et al., 2005; Hibbs et al., 1991; Nilsson et al., 2006; Wennerberg et al., 1998). These prior findings indicate that the conserved T788/T789 residues could form a phospho-switch to regulate integrin affinity and, thereby, control integrin-mediated cellular processes. However, the enzymatic machinery operating this phospho-switch within the cell is currently unknown.

Here we report that phosphorylation of the conserved threonine motif in the cytoplasmic tail of the integrin β1 subunit dissociates filaminA to allow access of talin to its canonical NPxY binding site. Using a focused genetic screen, we identify a member of the metal-dependent protein phosphatase (PMP) family, the serine/threonine phosphatase PPM1F, as the critical enzyme responsible for dephosphorylating the threonine motif. Our results uncover the mechanistic details of integrin activity regulation by this conserved phospho-switch and identify the underlying enzymatic machinery, thereby providing a novel access point to modulate integrin activity.

Results
The integrin β1 T788/T789 motif constitutes a conserved phospho-switch to regulate integrin activity
The T788/T789 motif of the β1 integrin cytoplasmic tail, which is located within the context of talin, kindlin, and filaminA binding sites, is highly conserved across species and within different human β subunits (Fig. 1 A and Fig. S1 A). Previous studies using the leukocyte-specific integrins β2 and β7 already suggested that these threonine residues could operate as a phospho-switch to control binding of talin versus filaminA (Kiema et al., 2006; Takala et al., 2008). To evaluate the consequences of integrin β1 T788/T789 phosphorylation for filaminA or talin binding in vitro, we produced recombinant cytosplasmic domains mimicking T788 or T788/T789 phosphorylation (T/D or TT/DD) or harboring nonphosphorylatable alanine residues (TT/AA; Fig. S1, B and C). We also generated a Y783A mutant in the NPxY motif, which impairs talin and filaminA association, and mutated a tyrosine residue outside of the talin or filamin core binding sites to alanine (Y795A; Calderwood et al., 1999; Pfaff et al., 1998). Pull-down experiments with the various integrin cytoplasmic domains showed that His-Small Ubiquitin-Related Modifier (SUMO)-tagged yeast enolase as irrelevant control protein did not associate with any of the integrin β1 cytoplasmic domains (Fig. 1 B). The recombinant His-SUMO-tagged integrin binding domains of filaminA (Ig domain 19–21 of filaminA) and talin (F3 lobe of the talin head domain) bound the integrin β1 WT and TT/AA variants, but not the Y783A variant (Fig. 1 B), in line with previous reports (Calderwood et al., 1999; O’Toole et al., 1995; Pfaff et al., 1998; Tadokoro et al., 2003). Importantly, talin also showed unaltered association with the pseudo-phosphorylated integrin β1 variants T/D and TT/DD, while filaminA binding was reduced (T/D) or completely absent (TT/DD), indicating that filaminA–integrin interaction is controlled by the phosphorylation state of the T788/T789 motif (Fig. 1 B).

Similar results were obtained by solid phase binding assays, where one of the binding partners was immobilized (Fig. 1 C). Again, the pseudo-phosphorylation of T788/T789 led to a complete loss of filaminA binding (Fig. 1 C). Furthermore, pull-down assays with biotinylated, synthetic peptides covering residues 762–798 of integrin β1 in either the unphosphorylated or the phosphorylated (pT788/pT789) form confirmed that phospho-threonine residues at these positions impede filaminA binding (Fig. 1 D). Modeling of the pT788/pT789 β1A integrin peptide based on known structures of the filaminA/integrin β1 complex (PDB2BRQ; Fig. 1 E) or the talin/integrin β1D complex (PDB3G9W; Fig. 1 F) suggested that phosphorylation of T788 and T789 not only sterically obstructs the binding interface with filaminA but also leads to charge repulsion. In contrast, integrin β1 pT788/pT789 phosphate groups do not interfere with the binding interface to talin (Fig. 1 E). These structural models are in line with data reported previously for β2 and β7 (Kiema et al., 2006; Takala et al., 2008) and strongly support our conclusion that phosphorylation of the integrin β1 T788/T789 motif disrupts filaminA binding, but does not impact talin association.

The integrin β1 T788/T789 phospho-switch regulates talin versus filaminA binding in intact cells
To validate our in vitro findings in the cellular context, we performed Opa-protein–triggered integrin clustering (OPTIC) assays (Baade et al., 2019; assay scheme, Fig. S1 D; construct expression, Fig. S1 E). Clustering of the WT integrin β1 cytoplasmic domain at the plasma membrane led to a strong recruitment of GFP-talin, while GFP alone was not enriched (Fig. 2 A). Furthermore, talin was equally well recruited to the pseudo-phosphorylated TT/DD and, to a slightly lesser extent, to the TT/AA variant (Fig. 2 A). Only disruption of the NPxY core binding motif (integrin Y783A) abolished talin recruitment (Fig. 2 A). In contrast, the filaminA integrin binding domain was only recruited in the case of the TT/AA mutant, where phosphorylation of this motif is impossible, while clustering of the WT integrin as well as the phospho-mimicking TT/DD variant did not support filaminA recruitment in the intact cell (Fig. 2 B).

These results demonstrated that the phosphorylation status of the integrin β1 T788/T789 motif dictates the association with integrin activity regulators in intact cells. Moreover, these findings also suggested that filamin can only occupy the talin binding site if the T788/T789 motif in the integrin tail is dephosphorylated. To test this idea, we performed in vitro competition assays with recombinant talin, filaminA, and integrin β1 cytoplasmic domains (Fig. 2 C). While talin was not able to displace filaminA from integrin β1 (Fig. S1 F), increasing levels of
Figure 1. The integrin β1 T788/T789 motif constitutes a conserved phospho-switch to regulate integrin activity. (A) Alignment of cytoplasmic amino acid residues of human integrin β subunits. The conserved threonine motif (red), the proximal NPxY motif (blue), the distal NPxY motif (green), and the binding sites of talin, kindlin-2, and filaminA are marked. (B) Strep-tag-integrin β1 (Strep-ITGB1) cytoplasmic domains in the WT form, with modifications of the T788/T789 motif (T/D, TT/DD, TT/AA), alanine mutation of Tyr-795 (Y795A), or of Tyr-783 (Y783A) were incubated with His-tagged enolase, FLN19-21, or talin-F3. Upon streptactin pulldown, bound His-tagged proteins were detected by WB with α-His antibody or Coomassie staining. 50% of His-tagged proteins were directly loaded (input) for comparison. (C) His-tagged proteins as in B were immobilized in triplicate wells and incubated with the indicated Strep-ITGB1 variants at 4°C. After washing, binding was detected by incubation with streptactin-HRP. Bars represent mean ± SEM of triplicates from a representative experiment. (D) Biotinylated integrin β1 peptides in the nonphosphorylated form (β1-762-798) or phosphorylated at T788/T789 (β1-762-798 pTpT) were bound to streptavidin-agarose (0.5 mg/ml beads) before being incubated with His-tagged enolase, His-talin-F3, GST, or GST-tagged FLN19-21. Beads without peptide loading were used as negative control. Upon pulldown, bound His- and GST-tagged proteins were detected by WB with α-His or α-GST antibody and Coomassie staining. 50% protein amount was directly loaded as input. (E and F) Structural models of filaminA-Ig21/integrin β7 complex (PDB2BRQ) or talin/integrin β1D complex (PDB3G9W). The phosphorylated β1A T788/T789 motif was generated in Coot. The electrostatic surfaces of filaminA-Ig21 and talin-F3 are depicted in red (negative charge, value −5) and blue (positive charge, value 5), β1 is shown in yellow; the phosphorylated threonine residues are shown in ball and stick representation. See also Fig. S1. Coot, Crystallographic object-oriented toolkit; H. sapiens, Homo sapiens; w/o, without.
filaminA led to a sharp drop in talin association with the WT integrin β1 tail (Fig. 2 C). However, in the case of the pseudo-phosphorylated integrin β1 T788D, even a large excess of filaminA was not able to outcompete talin (Fig. 2 C). Identical results were obtained by solid phase binding assays (Fig. S1 G). These biochemical findings support the idea that dephosphorylation of integrin β1, and in particular of the T788/T789 motif, is a prerequisite to allow filaminA to displace talin and to inactivate integrins (Fig. 2 D).

The phosphatase PPM1F regulates integrin activity and integrin-dependent cell adhesion

Based on the observations that a phosphorylated T788/T789 motif impedes filamin binding and that the pseudophosphorylated integrin β1 T788D promotes cell adhesion (Craig et al., 2009; Nilsson et al., 2006), we hypothesized that an integrin-directed protein phosphatase(s) counteracts integrin activation. Accordingly, deletion of such a putative protein phosphatase should lead to a gain of function with regard to integrin-based cell-matrix adhesion. Therefore, we performed a focused genetic knock-down screen with shRNAs individually targeting all protein phosphatases reported in the integrin adhesome (Zaidel-Bar et al., 2007; Fig. S2 A). As the cellular system, we deliberately chose 293T cells, a human cell line exhibiting weak adhesion to extracellular matrix proteins under tissue culture conditions.

Stable knock-down cells were plated on the integrin ligands fibronectin or collagen or on poly-L-lysine, to which cells attach independently of integrins (Fig. 3 A). Compared with control cells, depletion of the protein tyrosine phosphatases (PTPs)
PTP-1B, PTP-PEST, and RPTPa as well as depletion of the serine/threonine phosphatase PPM1F (also known as POPX2 [Koh et al., 2002], Ca2+/calmodulin-dependent protein kinase phosphatase [CaMKP; Ishida et al., 2008], and hFEM2 [Tan et al., 2001]) resulted in enhanced cell adhesion to collagen and/or fibronectin, but not poly-L-lysine (Fig. 3, A and B). PTP-1B, PTP-PEST, and RPTPa dephosphorylate the focal adhesion proteins paxillin, p130Cas, and c-Src, respectively, which could indirectly affect integrin-mediated adhesion (Arias-Salgado et al., 2005; Garton et al., 1996; Shen et al., 2000). As PPM1F, a member of the PPM (Moorehead et al., 2009), dephosphorylates serine/threonine residues and has not been implicated in cell adhesion, we decided to focus on this enzyme. Western blotting (WB) confirmed the depletion of the phosphatase in knock-down cells and demonstrated that levels of several key focal adhesion proteins such as integrin β1, talin, filamin, kindlin-2, focal adhesion kinase, paxillin, vinculin, zyxin, integrin-linked kinase (ILK), ezrin, or α-actinin were not altered (Fig. S2, B). These results indicated that reduction of PPM1F is directly connected to increased integrin-mediated cell adhesion.

PPM1F knock-down in normal human dermal fibroblasts (NHDF) recapitulated the phenotype observed in 293T cells leading to enhanced cell adhesion on integrin ligands (Fig. 3, C and D). Depletion of PPM1F did not affect expression of integrin subunits or other cytosolic focal adhesion proteins (Fig. S2, C and D), suggesting that the increased adhesion might be due to alterations in integrin activity. Indeed, PPM1F knock-down NHDFs exhibited elevated levels of active integrin β1 (Fig. 3 E),
PPM1F knock-out (KO) results in constitutive integrin activity and an exaggerated cell adhesion phenotype

PPM1F is ubiquitously expressed, but with high levels in neuronal cells (Ishida et al., 2018). To confirm the phenotype of PPM1F knock-down cells, we disrupted the Ppm1f gene in human glioblastoma A172 cells by CRISPR/Cas9. Compared with A172 WT cells and A172 control cells, which were transduced with a vector lacking the PPM1F sgRNA, the derived A172 PPM1F KO cells completely lacked expression of PPM1F (Fig. 4 A). Expression of focal adhesion proteins was unaltered and surface expression of different integrin subunits was not increased in A172 PPM1F KO cells (Fig. S3, A and B). Similar to PPM1F knock-down 293T and NHDF cells, the A172 PPM1F KO cells showed a 1.5–2-fold increase in cell adhesion compared with A172 WT or control cells at different time points after plating on substrates coated with low, medium, or high concentrations of extracellular matrix ligands (Fig. 4 B; and Fig. S3, C and D). Furthermore, A172 PPM1F KO cells displayed elevated levels of active integrin β1 (Fig. 4 C) and exhibited a prominent increase of active integrin β1 in the form of a peripheral “active integrin belt” (Fig. 4 D), which colocalized with enlarged clusters of talin (Fig. 4 D and Fig. S3 E). This phenotype was seen in around 80–90% of A172 PPM1F KO cells during the first 1–2 h of spreading (Fig. 4 E and Fig. S3 F). In general, PPM1F KO cells did not spread as fast as A172 WT cells and, therefore, covered a smaller area (Fig. 4, F and G; and Fig. S3 G), suggesting that cell spreading might be compromised due to intensified integrin–matrix interaction. This observation also indicates that other PPM1F substrates such as p21-activated kinase (PAK) or mammalian Diaphanous-related formin 1 (mDia1), which promote actin-based cell protrusions and which are negatively regulated by PPM1F, might not be responsible for the spreading defect of PPM1F KO cells (Koh et al., 2002; Parrini et al., 2009; Xie et al., 2008). To further confirm that the increased cell adhesion of PPM1F-deficient cells is connected to filamin-dependent activity regulation of integrins, we performed epistasis experiments. Therefore, A172 control cells and PPM1F KO cells received either a control shRNA or shRNA targeting human filaminA (Fig. 4 H). Similar to the KO of PPM1F and consistent with the known inhibitory role of filaminA (Liu et al., 2015; Takala et al., 2008; Waldt et al., 2018), shRNA-mediated knock-down of filaminA in A172 control cells increased cell adhesion, reduced cell spreading, and elevated integrin activity (Fig. 4, I and J; and Fig. S4, A–D). However, depletion of filaminA in PPM1F KO cells did not further increase the elevated integrin-dependent adhesion or the enhanced integrin activity in these cells, nor did it further reduce cell spreading (Fig. 4, I and J; and Fig. S4, A–D). The results of these epistasis experiments highlight the strong similarities in the phenotype of PPM1F KO cells and filaminA knock-down cells and suggest that PPM1F and filaminA work together in the same pathway controlling integrin activity (Fig. S4 E). Together, the absence of PPM1F results in a gain of function with regard to integrin-based cell adhesion due to enhanced integrin activity, elevated talin recruitment, and reduced filaminA association with integrin β1.

The phosphatase PPM1F regulates the phosphorylation state of the integrin T788/T789 motif

Association of filaminA and talin with the integrin β subunit as well as integrin activity are regulated by phosphorylation of the T788/T789 motif. Therefore, we wondered about the phosphorylation status of these residues in PPM1F-deficient cells. Interestingly, while suspended A172 WT cells showed low levels of integrin β1 T788/T789 phosphorylation, KO of PPM1F resulted in constitutively elevated levels of pT788/pT789 (Fig. 5 A). Upon seeding onto fibronectin, the level of pT788/pT789 increased transiently in WT A172 cells during the initial attachment phase up to 45 min (Fig. 5 B). In contrast, PPM1F KO cells permanently exhibited substantially elevated T788/T789 phosphorylation (Fig. 5 C). Quantification of multiple blots demonstrated a four- to fivefold higher phosphorylation level of integrin β1 T788/ T789 in A172 PPM1F KO cells compared with WT cells (Fig. 5 D). To rigorously demonstrate that this phenotype is due to the lack of PPM1F activity, we complemented the PPM1F KO A172 cells with either WT monomeric (m)Kate-PPM1F or the phosphatase-dead mutant mKate-PPM1F D360A (Fig. S3 H). As seen before, expression of core focal adhesion proteins or surface expression of integrin subunits was not altered by this genetic manipulation (Fig. S3, A and B). However, expression of PPM1F WT, but not expression of PPM1F D360A, reverted the increased phosphorylation of integrin β1 T788/T789 back to levels seen in WT A172 cells (Fig. 5 D). The increased integrin T788/T789 phosphorylation seen in the PPM1F KO cells correlated well with the elevated integrin activity and enhanced cell adhesion to integrin ligands, which was also reverted back to basic levels upon re-expression of WT PPM1F, but not PPM1F D360A (Fig. 5, E–G). Together, these findings are consistent with the idea that PPM1F regulates the phosphorylation state of the T788/T789 motif, thereby controlling association of talin versus filaminA with the cytoplasmic tail of integrin β1 and determining cell-matrix adhesion strength (Fig. 5 H). The uniform phenotype of enhanced integrin activity observed upon depletion or disruption of PPM1F in multiple cell types also indicated that PPM1F might act directly on the integrin β1 subunit.

Recombinant PPM1F dephosphorylates the conserved T788/T789 motif in the integrin β1 cytoplasmic domain

To test the ability of PPM1F to directly dephosphorylate pT788/pT789 of integrin β1, human PPM1F WT and PPM1F D360A were produced in Escherichia coli (Fig. 6 A). Using the generic phosphatase substrate 4-methylumbelliferylphosphate (4-MUP), maximum velocity (Vmax) and Michaelis constant (Km) values of PPM1F were comparable to other Ser/Thr phosphatases (Gee et al., 2015). To rigorously demonstrate that this phenotype is due to the lack of PPM1F activity, we complemented the PPM1F KO A172 cells with either WT monomeric (m)Kate-PPM1F or the phosphatase-dead mutant mKate-PPM1F D360A (Fig. S3 H). As seen before, expression of core focal adhesion proteins or surface expression of integrin subunits was not altered by this genetic manipulation (Fig. S3, A and B). However, expression of PPM1F WT, but not expression of PPM1F D360A, reverted the increased phosphorylation of integrin β1 T788/T789 back to levels seen in WT A172 cells (Fig. 5 D). The increased integrin T788/T789 phosphorylation seen in the PPM1F KO cells correlated well with the elevated integrin activity and enhanced cell adhesion to integrin ligands, which was also reverted back to basic levels upon re-expression of WT PPM1F, but not PPM1F D360A (Fig. 5, E–G). Together, these findings are consistent with the idea that PPM1F regulates the phosphorylation state of the T788/T789 motif, thereby controlling association of talin versus filaminA with the cytoplasmic tail of integrin β1 and determining cell-matrix adhesion strength (Fig. 5 H). The uniform phenotype of enhanced integrin activity observed upon depletion or disruption of PPM1F in multiple cell types also indicated that PPM1F might act directly on the integrin β1 subunit.

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Figure 4. PPM1F KO results in constitutive integrin activity and an exaggerated cell adhesion phenotype. (A) A172 cells expressing Cerulean (WT) were treated with sgRNA against Cerulean (control) or sgRNA against Cerulean and PPM1F (PPM1F KO) combined with Cas9. Clonal cell lines were derived and analyzed by WB with polyclonal α-PPM1F (upper panel) or α-tubulin (lower panel) antibodies. (B) Cells from A were seeded onto 10 µg/ml FNIII9-11 for 30 min in triplicate. Cell adhesion was measured as in A. Adhesion of A172 WT cells was used as reference. Bars represent mean ± SEM from three independent experiments. Statistics was calculated using one-way ANOVA, followed by Bonferroni post hoc test (*, P < 0.05). (C) Cells from A were replated onto 0.1 µg/ml FNIII9-11-coated 96-well plates for 40 min. Samples were stained for active integrin β1 (9EG7), total integrin β1 (AIIB2), or with secondary antibody only as a control. (D) Images of cell adhesion assay. (E) Quantification of the percentage of cells with pericellular integrin belt (30 min). (F) Images of cell adhesion assay. (G) Quantification of the area of the integrin belt (30 min). (H) Western blot analysis of PPM1F and FilaminA expression. (I) A172 cells were treated with 0.4 or 10 µg/ml FN for 20 min, and cell adhesion was measured. (J) A172 cells were treated with 10 µg/ml FN for 60 min, and cell adhesion was measured.
control. The graph shows the distribution of active integrin β1 versus total integrin β1 after background subtraction. Bars represent mean ± SEM from a representative experiment done in triplicate. (D) Cells transfected with GFP-talin were seeded onto FN19-11 for 30 min. Cells were fixed, stained with antibodies against active integrin β1, and analyzed by confocal microscopy; scale bar, 10 μm. Arrowheads point to active integrin β1/talin enrichment. Insets: Higher magnification of boxed area; scale bar, 5 μm. (E) Quantification of peripheral belt formation by active integrin (upper graph) or talin-1 (lower graph) in A172 WT and PPMIF KO cells. Data are shown in percentages of all cells analyzed; n ≥ 2, derived from ≥ 2 independent experiments. (F) A172 WT, control, and PPMIF KO cells were seeded onto 2 μg/ml FN19-11 coated coverslips for 30 min or 1.5 h before fixation and staining with DAPI and phalloidin-CysS. Representative pictures are shown; scale bar, 25 μm. (G) Quantification of cell area from cells in F; n ≥ 100 cells from two or more independent experiments; mean values and 95% confidence intervals are shown, outliers are represented as dots, and statistics was performed using one-way ANOVA with post hoc Bonferroni test (***, P < 0.001). (H) A172 WT and PPMIF KO cells were transduced with lentiviral particles harboring shRNA against human filaminA or empty pLKO.1 vector as a control. After puromycin selection, lysates were prepared and subjected to WB analysis using indicated antibodies. (i) Cell adhesion assays were performed with starved A172 cells from H for 20 or 60 min using 0.4 or 10 μg/ml FN19-11 as integrin-dependen matrix. After a washing step, adherent cells were fixed and stained with crystal violet. Staining was quantified and normalized to the total number of seeded cells. Bar graphs show mean ± SEM of four independent experiments done in triplicate referenced to WT cell adhesion (=1); one-way ANOVA and Bonferroni post hoc test (***, P < 0.001; ***, P < 0.01; *, P < 0.05). (j) Cell lines from H were kept in suspension for 45 min and incubated for 15 min with 10 μg/ml FN19-11 before staining for total (AIIB2) or active integrin β1 (9EG7). Samples were analyzed by flow cytometry, 10,000 counts; unstained cells and an IgG-matched irrelevant antibody served as negative controls. The mean fluorescence intensity (MFI) ratio of active to total integrin β1 was calculated and normalized to the WT sample (=1). Bars represent mean ± SEM from three independent experiments; one-way ANOVA and Bonferroni post hoc test (**, P < 0.01; *, P < 0.05). See also Fig. S3 and Fig. S4. FN, FN19-11.

Kindlin2 association with the phosphorylated integrin β1 cytoplasmic tail requires the presence of talin

The T788/T789 motif is also at the core of the kindlin binding site in integrin β1 (Fig. 1 A) and kindlin, together with talin, is the major positive regulator of integrin function (Harburger et al., 2009; Li et al., 2017; Theodosiou et al., 2016). Therefore, we wondered if the phosphorylation state of T788/T789 also influences kindlin association with integrin β1. In vitro, recombinant kindlin2 bound the WT integrin β1 cytoplasmic domain and the Y783A mutant with the disrupted NPXY talin binding motif, but not the Y795A variant, which disrupts the core NPXY kindlin binding motif (Li et al., 2017; Fig. 7 A). Kindlin2 also did not associate with the A788/A789 variant, which is in line with previous studies showing that the hydroxyl-groups of the conserved threonine residues are involved in H-bonds between kindlin and integrin β1 (Li et al., 2017). Unexpectedly however, kindlin2 did not bind the pseudo-phosphorylated integrin β1 variants T/D and TT/DD (Fig. 7 A), and the same results were obtained by solid phase binding assays (Fig. 7 B). Furthermore, pull-down assays with biotinylated, phosphorylated (pT788/pT789), or unphosphorylated integrin β1 peptides confirmed that phosphorylation of these threonine residues prevents kindlin2 binding (Fig. 7 C). Similar to the situation with talin, kindlin2 was readily displaced from the integrin β1 tail in vitro by the integrin-binding domain of filaminA (Fig. 7 D and Fig. S1 H). These puzzling results would imply that kindlin2 is outcompeted by filaminA, when the integrin β1 tail is unphosphorylated, but kindlin2 could also not bind on its own, when the T788/T789 motif is phosphorylated. As kindlin2 cooperates with talin to modulate integrin function (Theodosiou et al., 2016; Ye et al., 2013), we wondered whether the capability of talin to associate with the phosphorylated integrin tail might enable binding of kindlin2. Therefore, recombinant full-length human kindlin2 and His-tagged talin F3 domain were employed in pull-down assays with various Twin-StrepII-tag (strept)-tagged integrin β1 cytoplasmic domains (Fig. 7 E). As before, kindlin2 alone bound the unphosphorylated integrin β1 cytoplasmic domain, but failed to associate with the pseudo-phosphorylated TT/DD variant (Fig. 7 F). Importantly, the presence of the recombinant

et al., 1999), while PPMIF D360A was inactive (Fig. 6 B). PPMIF also dephosphorylated synthetic peptides spanning the integrin β1 T788/T789 motif (Fig. 6, C and D). Though the doubly phosphorylated peptide (β1-pT788/pT789) served as a suitable substrate for PPMIF, dephosphorylation was more effective with the mono-phosphorylated integrin β1 peptides β1-pT788 or β1-pT789 (Fig. 6 D). PPMIF acted specifically on integrin pT788, since a phospho-peptide derived from myosin light chain (MLC; pMLC) was not dephosphorylated (Fig. 6, C and D). In a complementary approach, PPMIF was overexpressed and purified from human cells. Again, we observed dephosphorylation of synthetic integrin phospho-peptides by WT GST-PPMIF isolated from human cells, but not by GST-PPMIF D360A (Fig. S5, A–E). In a further approach, we used purified Ca2+/calmodulin-dependent kinase II (CaMKII) β, which has been reported as a potential kinase of integrin β1 (Suzuki and Takahashi, 2003), to phosphorylate the recombinant cytoplasmic domain of integrin β1 in the presence of ATP, Ca2+, and calmodulin. In contrast to WT integrin β1, the T788A/T789A mutant was not phosphorylated, demonstrating that CamKIIβ selectively acts on the integrin β1 T788/ T789 motif in vitro (Fig. S5 F). Recombinant WT PPMIF, but not PPMIF D360A, dephosphorylated the resulting integrin β1 pT788/ pT789 (Fig. 6 E). To check if other protein phosphatases also act on the T788/T789 motif, we recombinantly expressed several enzymes, including integrin-linked kinase-associated protein (ILKAP), an additional member of the PPM family present at integrin adhesion sites; Leung-Hagesteijn et al., 2001), PP5, and PTP1B (Fig. 6 F), and verified the activity of the purified proteins with 4-MUP (Fig. S5 G). Next, PPMIF, ILKAP, PP5, and PTP1B were incubated with the doubly phosphorylated β1-pT788/pT789 peptide. While PTP1B, PP5, and ILKAP failed to dephosphorylate integrin β1 pT788/pT789 to a significant extent, PPMIF was highly active (Fig. 6 F). Moreover, ILKAP did not dephosphorylate the GST-β1 integrin cytoplasmic domain phosphorylated in vitro by CaMKIIβ, while PPMIF was active against this phospho-protein substrate (Fig. 6, G and H). Therefore, PPMIF is the integrin phosphatase that specifically controls the phosphorylation state of the conserved T788/T789 motif in the cytoplasmic domain of integrin β subunits.

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PPMIF is the integrin phosphatase

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Figure 5. The phosphatase PPM1F regulates the phosphorylation state of the integrin T788/T789 motif in intact cells. (A) Starved A172 WT and PPM1F KO cells were kept in DMEM plus 0.25% BSA for the indicated time periods before WCLs were prepared and subjected to WB analysis with indicated antibodies. (B and C) Starved A172 WT (B) or PPM1F KO cells (C) were seeded onto 2 µg/ml FNIII9-11 for the indicated time periods before being lysed and analyzed by WB as in (A). (D) Graph showing the fold change in pT788/T789 integrin/β1-integrin expression in WT and PPM1F KO cells. (E) Bar graph showing integrin activity measured by SEG7/A1B2. (F) Phospho-T788 integrin staining in WT and PPM1F KO cells at 60 min. (G) Bar graph showing cell adhesion fold change in WT and PPM1F KO cells under different conditions. (H) Schematic diagram illustrating the regulatory roles of PPM1F in integrin phosphorylation and cell adhesion.

PPM1F is the integrin phosphatase.
analyzed by immunoblotting as in A; PPMIF KO cells were used as a control in B to directly compare integrin β1 phosphorylation levels. (D) PPMIF KO cells were reconstituted with either WT human PPMIF (PPMIF KO + hPPMIF-mKate2) or the phosphatase-dead mutant PPMIF D360A (PPMIF KO + hPPMIFD360A-mKate2). Furthermore, PPMIF KO cells were stably transduced with mKate2 (PPMIF KO + mKate2). As a comparison, A172 WT cells and A172 cells transduced with a vector lacking the PPMIF sgRNA (control) were used. The cells were seeded onto 2 µg/ml FNIII9-11 for 45 min and WCLs subjected to WB with indicated antibodies (left panels). Bar graphs show densitometric quantification of band intensities from pT788/pT789-β1 versus total β1 integrin antibody signal for the indicated samples from three independent experiments; WT was set to 1 (right graph). Statistics was performed using one-way ANOVA, followed by Bonferroni post hoc test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). (E) Indicated A172 cell lines were kept in suspension for 45 min and incubated for 15 min with 10 µg/ml FNIII9-11 before being stained for total (AIIB2) or active integrin β1 (9E10G), Samples were analyzed by flow cytometry, 10,000 counts. The mean fluorescence intensity ratio of active to total integrin β1 was calculated and normalized to the WT sample (= 1). Bars represent mean fluorescence intensity ± SEM of four independent experiments; statistics was performed using one-way ANOVA and Bonferroni post hoc test (***, P < 0.001; *** P < 0.01). (F and G) Cell adhesion assays were performed with starved A172 cell lines for 20 or 60 min using 0.4 or 10 µg/ml FNIII9-11; 2% BSA-coated wells were used as a negative control. After a washing step, adherent cells were fixed and stained with crystal violet. Staining was quantified and referenced to WT cell adhesion (= 1). (F) Representative pictures after 60 min adhesion on 10 µg/ml FNIII9-11; scale bar, 150 µm. (G) Bar graphs show mean ± SEM of four independent experiments pipetted in triplicate; statistics was performed using one-way ANOVA and Bonferroni post hoc test (***, P < 0.001; ***, P < 0.01). (H) Model summarizing effects of PPMIF KO on integrin activity. In PPMIF KO cells, the balance between active and inactive integrins is shifted toward the active conformation by constitutive phosphorylation of integrin β1 at T788/T789, thereby prohibiting filaminA binding, while promoting increased talin association and cell adhesion. DA, phosphatase-dead mutant of PPMIF D360A; FN, FNIII9-11; P, phospho-; T, threonine.

Kindlin2 and talin recruitment are dictated by integrin β1 phosphorylation and PPMIF activity

To follow the consequences of integrin β1 T788/T789 phosphorylation for kindlin2 recruitment in intact cells, we employed integrin β1 chimeras and their phospho-mimicking mutants as well as deletion of PPMIF. In agreement with the in vitro binding data, GFP-kindlin2 clustered at WT integrin β1 tails, but was not enriched at the integrin β1 TT/AA or the integrin β1 Y795A variant (Fig. 8 A). However, in contrast to the situation with purified components, GFP-kindlin2 was also strongly enriched at pseudo-phosphorylated integrin β1 in intact cells (Fig. 8 A). The recruitment of GFP-kindlin2 in this situation was critically dependent on talin binding, as mutation of the core talin binding site in the regular (Y783A) or in the pseudophosphorylated (TT/DD + Y783A) integrin β1 tail abolished the increased association (Fig. 8, A and B).

The capability of kindlin2 to associate with full-length phosphorylated integrin β1 was also reflected by the fact that PPMIF KO cells, which display elevated levels of pT788/pT789, showed not only a peripheral ring of active integrin but also accumulation of kindlin2 at these sites (Fig. 8 B). The accumulation of kindlin2 in these structures mimicked the accumulation seen for talin in the PPMIF KO cells (Fig. 8 B). Importantly, reexpression of active, WT PPMIF, which reduces the increased integrin β1 T788/T789 phosphorylation, also prevented kindlin2 and talin accumulation, while in PPMIF KO cells reexpressing inactive PPMIF D360A, the massive peripheral ring of active integrin and the exaggerated presence of kindlin2 and talin persisted (Fig. 8 B). These data illustrate how phosphorylation of the integrin β1 subunit enforces cooperation between the integrin activators talin and kindlin2 to overcome filamin-mediated inhibition of integrins. Moreover, these results point to the critical position of PPMIF during integrin activity regulation as the major protein phosphatase directed toward the conserved T788/T789 motif.

PPMIF activity determines the interaction of talin and filaminA with the integrin β1 cytoplasmic tail in intact cells

To test whether PPMIF activity toward the conserved threonine motif in the integrin cytoplasmic tail dictates integrin activity, we again employed the chimeric receptor proteins in the pseudophosphorylated (WT) or pseudo-phosphorylated (T788D/T789D) forms. These integrin β1 chimeras were coexpressed with PPMIF WT or PPMIF D360A and together with either GFP-talin (Fig. 8 C) or the GFP-tagged integrin binding domain of filamin (GFP-FLN19-21; Fig. 8 D). Upon PPMIF overexpression, talin recruitment to the clustered WT β1 integrin tail was strongly impaired, while overexpression of inactive PPMIF D360A did...
not compromise talin association (Fig. 8 C). As predicted from the in vitro binding assays, talin also associated with integrin β1 T788D/T789D, and this association could not be diminished by overexpression of PPM1F (Fig. 8 C). Vice versa, GFP-filaminA was only enriched around clustered WT integrin β1 i ns i t u -tions where WT PPM1F, but not the D360A mutant, was...
Figure 7. Kindlin-2 association with the phosphorylated integrin β1 cytoplasmic tail requires the presence of talin. (A) Strep-tag integrin β1 (Strep-ITGB1) cytoplasmic domains in the WT form, with modifications of the T788/T789 motif (T/D, TT/DD, TT/AA), alanine mutation of Tyr-795 (Y795A), or of
Tyr-783 (Y783A) were incubated with His-kindlin-2. Upon streptavidin pulldown, bound kindlin-2 was detected by WB with α-His antibody or Coomassie staining. 50% of His-kindlin-2 was directly loaded (input) for comparison. (B) His-tagged kindlin-2 was immobilized and incubated with the indicated Strept-ITGB1 variants at 4°C in triplicate wells. After washing, binding was detected by incubation with streptavidin-HRP. Bars represent mean ± SEM of triplicates from one representative experiment. (C) Biotinylated integrin β1 peptides with or without phosphorylated T788/T789 were bound to streptavidin-agarose (0.5 mg/ml beads), before incubation with His-kindlin-2. Upon streptavidin pulldown, bound kindlin-2 was detected as in A. (D) Kindlin-2 is the N-terminal kindlin-2 fragment, which is also detected by α-His antibody and interacts with integrin tails. (E and F) strept-tag-integrin β1 (Strep-ITGB1) cytoplasmic domains in the WT form, with modifications of the T788/T789 motif (T/D, TT/DD, TT/AA), alanine mutation of Tyr-795 (Y795A), or of Tyr-783 (Y783A) together with or without T788D/T789D mutation were incubated with kindlin-2 and talin-F3 alone or together in a 1:1 ratio. Upon streptavidin pulldown, bound His-tagged proteins were detected by WB using α-His antibody or Coomassie staining. Input is shown in E, pulldown in F; *Kindlin-2 as in C. (G) Indicated biotinylated integrin β1 peptides were bound to streptavidin-agarose (0.25 mg/ml beads) before incubation with His-tagged talin-F3 (1×) and kindlin-2 (1×) with or without GST-tagged FLN19-21 (5× molar amount). Upon streptavidin pulldown, bound His- and GST-tagged proteins were detected by WB using α-His or α-GST antibody and Coomassie staining (right panels); left panels show protein input (100%); *Kindlin-2 as in C. (H) Schematic view of talin and kindlin-2 versus filaminA association with the integrin β1 cytoplasmic tail depending on T788/T789 (pseudo-)phosphorylation. See also Fig. S1, P, phospho-; T, threonine; w/o, without; wt, wild type.

Homozygous disruption of the ppmIf gene results in embryonic lethality, and ppmIf+/− fibroblasts show constitutive T788/T789 phosphorylation, elevated integrin activity, and increased cell adhesion

Integrin β1 and its key interaction partners, such as talin, kindlin, filaminA, paxillin, and focal adhesion kinase, are all essential for mammals (Fassler and Meyer, 1995; Ilic et al., 1995; Stephens et al., 1995; Monkley et al., 2000; Hagel et al., 2002; Feng et al., 2006; Montanez et al., 2008). Furthermore, compromising integrin activity regulation via targeted mutations in the integrin β1 cytoplasmic domain abrogates integrin function in the intact tissue (Czuchra et al., 2006; Meves et al., 2013). Accordingly, if PPMIF indeed serves a critical function in integrin activity regulation, one would expect a severe phenotype upon inactivation of the ppmIf gene.

To test this prediction, we employed mice containing a gene-trap insertion in exon 4 of the ppmIf gene resulting in disruption of the ppmIf gene (Fig. 9 A). The genotype of the mice containing the disrupted ppmIf allele was verified by PCR (Fig. 9 B). While crosses between WT (ppmIf+/+) and heterozygous (ppmIf+/−) mice led to the expected 50:50 ratio of ppmIf+/+ and ppmIf+/− offspring, crosses between heterozygous ppmIf+/− animals yielded no homozygous ppmIf−/− pups, suggesting that PPMIF-deficient embryos die before or around E10.5 in utero. To unambiguously determine their genotype, we isolated primary murine embryonic fibroblasts (MEFs) from E10.5 embryos. PCR on genomic DNA of isolated fibroblasts confirmed that the small malformed embryos harbor two dysfunctional PPMIF alleles (ppmIf−/−), while the normal-sized embryos were either homozygous for the WT allele or heterozygous (ppmIf+/−; Fig. 9 E). Primary fibroblasts from ppmIf−/− mouse embryos and WT littermates were immortalized by retroviral transduction with SV40 large T antigen, and WB with polyclonal anti-murine PPMIF (mPPMIF) antibodies demonstrated the absence of the full-length enzyme or any truncated ppmIf gene products in ppmIf−/− KO fibroblasts (MEF ppmIf−/−; Fig. 9 F). Already during regular cell culture, MEF ppmIf−/− showed enhanced cell attachment to the culture dish, and it took longer to detach these cells during passaging. When placed onto fibronectin, the primary ppmIf−/− cells showed pronounced accumulation of active integrin β1 and talin at peripheral focal adhesion sites (Fig. 9 G). Most importantly, phosphorylation of the threonine motif in the integrin β1 cytoplasmic tail was strongly elevated in the MEF ppmIf−/− cells compared with the WT cells (Fig. 9 H). The enhanced integrin phosphorylation in MEF ppmIf−/− cells correlated with elevated levels of active integrin β1 (Fig. 9 I) and translated into increased adhesiveness on low (0.4 µg/ml) and high (10 µg/ml) concentrations of the integrin ligand fibronectin (Fig. 9 J). Furthermore, the enhanced integrin-mediated cell adhesion directly translated into reduced cell migration velocity and migration distance of MEF ppmIf−/− cells (Fig. 9 K). These results highlight the severe consequences of PPMIF deletion on integrin function in primary cells. Together, our data further confirm a critical and nonredundant role of PPMIF as a negative regulator of integrin activity during embryonic development and during physiological integrin-dependent processes such as cell adhesion, spreading, and migration.

Discussion

Dynamic modulation of integrin activity is indispensable for the normal functioning of animal cells, especially with regard to cell attachment, spreading, and migration (Morse et al., 2014; Shattil et al., 2010). Integrin activity is controlled by protein–protein overexpression (Fig. 8 D). These results indicate that continuous activity of PPMIF toward the integrin β1 T788/T789 motif is needed to enable filaminA association, which nicely explains the elevated integrin activity in PPMIF-deficient as well as filaminA-deficient cells. Together, these data illustrate the consequences of PPMIF activity on protein–protein interactions occurring at the integrin β1 subunit in intact cells and suggest that this phosphatase holds a key position to set integrin activity levels.
Figure 8. Kindlin2 and talin versus filaminA recruitment are dictated by integrin β1 phosphorylation and PPM1F activity in intact cells. (A) 293T cells were cotransfected with indicated CEA3-integrin β1 cytoplasmic tail fusion proteins together with GFP-kindlin-2. After 48 h, cells were infected with
interactions occurring at the cytoplasmic tail of the integrin β subunit, and the fine-tuning of cell adhesion requires regular transitions from talin/kindlin-bound active to filaminA-associated inactive integrin. Here we identify the molecular machinery that executes this integrin activity switch: the cytoplasmic metal-dependent protein phosphatase PPM1F. This serine/threonine phosphatase acts on a conserved threonine motif located in the cytoplasmic tail of most integrin β subunits and thereby controls filamin binding. Accordingly, this phosphatase represents the first enzyme known to directly regulate integrin activity.

PPM1F belongs to the PPMs, which, in contrast to the family of phospho-protein phosphatases, do not rely on regulatory subunits for subcellular localization and substrate recognition (Moorhead et al., 2009; Stern et al., 2007). Instead, these phosphatases harbor additional domains involved in protein–protein interaction and phosphatase activity regulation (Ishida et al., 2018). The tertiary structure of PPM1F is not known, but a protein interaction and phosphatase activity regulation (Ishida et al., 2018). The tertiary structure of PPM1F is not known, but a

CEACAM-binding bacteria (Ngo) for 1 h, fixed, and stained for CEACAM3. Micrographs (left panel) show representative infection sites of bacterial attachment (blue) and CEACAM3 integrin β1 clustering (red). Recruitment of GFP-kindlin-2 (green) to clustered integrin cytoplasmic tails is indicated (white arrowhead). Scale bar, 1 μm. Kindlin-2 recruitment was quantified with the enrichment ratio (ER) indicating the -fold enrichment of GFP intensity at bacterial attachment sites versus the overall cellular GFP level (middle panel). Data from GFP-transfected cells (Fig. 2 A) were used as negative control for statistical calculations. Dots represent individual ER values from 30–60 recruitment sites from n ≥ 2 independent experiments. Horizontal lines indicate mean values and 95% confidence intervals (whiskers). The red line indicates the threshold of positive recruitment. The bar graph (right panel) depicts the percentage of cells showing a ratio of kindlin-2 recruitment ER ≥ 2. Statistically significant differences were evaluated using one-way ANOVA, followed by Bonferroni post hoc test (***, P < 0.001; **, P < 0.01). (b) A72 WT, PPM1F KO, or PPM1F KO cells repressing mKate-tagged PPM1F or PPM1F D360A were seeded directly or 48 h after transfection with GFP-talin-1 onto 2 μg/ml FN(8.11) for 1.5 h. Cells were fixed, stained with antibodies against active integrin β1 (9EG7) and optionally against kindlin-2, and analyzed by confocal microscopy; scale bar, 10 μm. Arrowheads point to active integrin β1/talin or kindlin-2 recruitment. Insets: Higher magnification of boxed area; scale bar, 5 μm. (C and D) 293T cells were cotransfected with indicated CEAC3-integrin β1 cytoplasmic tail fusion protein in the WT (CEA3-WT) or T788D/T789D (CEA3- TT/DD) form together with GFP, GFP-talin-1 (C), or GFP-FLN19-21 (D). After 48 h, cells were infected with CEACAM-binding bacteria (Ngo) for 1 h, fixed, and stained for CEACAM3. Micrographs (left panel) show representative infection sites of bacterial attachment (blue) and CEAC3-integrin β1 clustering (red). Recruitment of GFP proteins (green) to integran cytoplasmic tails is indicated (white arrowhead). Scale bar, 1 μm. Protein recruitment was quantified with the ER indicating the fold enrichment of GFP intensity at bacterial attachment sites versus the overall cellular level (middle panel). Dots represent individual ER values of 60 recruitment sites from three independent experiments. Horizontal lines indicate mean values and 95% CIs (whiskers). The red line indicates the threshold of positive recruitment. The bar graph (right panel) depicts the percentage of cells showing a ratio of protein recruitment ER ≥ 2. Statistically significant differences were evaluated using one-way ANOVA, followed by Bonferroni post hoc test (***, P < 0.001; *, P < 0.05). See also Fig. S1.
Figure 9. Homozygous disruption of the ppm1f gene results in embryonic lethality and ppm1f−/− fibroblasts show constitutive T788/T789 phosphorylation, elevated integrin activity, and increased cell adhesion. (A) Schematic representation of the WT and targeted ppm1f locus. Insertion of a

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lacz-neomycin-resistance cassette into exon 4 resulted in gene disruption and expression of β-galactosidase under the control of the ppm1f gene promoter. The primers used for genotyping (blue) and the resulting PCR fragments (red) are shown. E, exon number; P1, gene specific primer forward; P2, gene specific primer reverse; P3, targeted primer forward. (b) DNA extracts from tail biopsies were genotyped by PCR using primers indicated in A to result in a 250-bps product (WT allele, P1, P2) or a 450-bps product (targeted allele, P2, P3). (c) ppm1f−−/− and ppm1f+/− mice were mated as depicted, and the offspring was genotyped after weaning by PCR using DNA extracts from tail biopsies. (d) Close-up view of head morphology at E10.5 from WT, ppm1f−/−, and ppm1f+/− embryos. Ppm1f−/− embryos are smaller in size and exhibit a stunted telencephalon (white arrow) and reduced development of branchial arches (black arrowhead). Scale bars, 1 mm. (e) Two ppm1f−/− mice were mated, and genomic DNA was extracted from fibroblasts isolated from ppm1f−/− (WT), ppm1f−/− (heterozygous), and ppm1f−−/− mouse embryos at E10.5 (MEFs). Genotyping PCR identified WT, heterozygous, and homozygous ppm1f KO embryos. (f) WCLs from MEFs isolated from WT (ppm1f+/−) or from ppm1f−/− embryos were probed with polyclonal α-mPPM1F antiserum (upper panel) or monoclonal α-tubulin (lower panel). PPM1F−/− cells do not express truncated variants of PPM1F. (g) MEFs as in F were seeded onto 1 μg/ml FNIII9-12 for 2 h. Samples were fixed and stained for talin (left row) or the active integrin β1 (right row) before analysis by confocal microscopy; scale bar, 20 μm. Insets show higher magnification of boxed areas; scale bar, 5 μm. Arrowheads point to active integrin β1 or talin enrichment. (h) MEFs as in F were seeded onto 2 μg/ml FNIII9-12 for 45 min, and WCLs were subjected to WB with indicated antibodies (left panels). Bar graphs (right panels) show densitometric quantification of band intensities from pT788/pT789-β1 versus total β1 integrin antibody signal from four independent experiments; WT was set to 1. Statistics was performed using one-way ANOVA, followed by Bonferroni post hoc test (**, P < 0.01). (i) MEFs as in F were seeded in triplicate onto fibronectin-coated wells for 20 min, and cell adhesion was quantified. Bars represent mean ± SEM of five independent experiments; statistics was performed using unpaired Student’s t test (**, P < 0.01). (j) MEFs as in F were seeded in triplicate onto fibronectin-coated wells for 20 min, and cell adhesion was quantified. Bars represent mean ± SEM of five independent experiments; statistics was performed using unpaired Student’s t test (**, P < 0.01; *, P < 0.05). (k) Serum-starved MEFs were stimulated by addition of 10% FCS, and cell migration was monitored every 30 min for 12 h using time-lapse microscopy. Cell tracks were evaluated for velocity and covered distance. Bars show mean ± SEM of three independent experiments. Samples were done in duplicate, each n = 15; unpaired t test (**, P < 0.001).

Phosphorylated integrin tail unclips the integrin α and β subunits and leads to extension of the bend extracellular domains (Fig. 10 C). In this situation, talin binding is required before kindlin2 can associate with the membrane distal NPXY motif, as the T788/T789 motif is phosphorylated (Fig. 10 D). Upon kindlin2 binding, the active conformation of integrins is further stabilized, and integrin-mediated adhesion is further promoted by receptor clustering (Fig. 10 D). The phosphorylation-guided cooperation between talin and kindlin, as suggested by our in vitro and in vivo data, could be a means to safeguard against a kindlin2-mediated inside-out activation of integrins in the absence of prior talin binding. In retrospect, these findings now provide a mechanistic explanation for the previously observed adhesion phenotype of integrin β1 T789D and of integrin β1 T788/789AA-expressing cells, which appear locked in either the active or inactive situation (Nilsson et al., 2006; Wernerberg et al., 1998). Our novel observations also nicely combine with previous results in a coherent picture of integrin activity regulation from within the cell (inside-out signaling). It has been noted before that overexpression of the isolated talin FERM domain can trigger inside-out signaling of integrin β1 and β3, while kindlin overexpression on its own is not sufficient, but rather intensifies talin-initiated integrin activity (Harburger et al., 2009; Li et al., 2017; Ma et al., 2008; Ye et al., 2010). On the other hand, kindlin critically contributes to integrin activity when cells respond to an integrin ligand and reorganize their cytoskeleton during outside-in signaling (Böttcher et al., 2017; Montanez et al., 2008; Theodosiou et al., 2016).

Interestingly, clustering of the WT integrin β1 chimera in intact cells, a situation mimicking the unclipped integrin, does not lead to filaminA recruitment. This finding could indicate that the threonine motif is mainly phosphorylated when the integrin β subunit is separated from the integrin α subunit. Intriguingly, upon phosphorylation of PPM1F, but not PPM1F D360A, filaminA accumulates at the WT integrin β1 cytoplasmic tail. This observation suggests that the activity of the overexpressed phosphatase can override a potential default phosphorylation of the threonine motif in the unclipped integrin β subunit to allow filaminA binding. It is interesting to speculate that a default phosphorylation of the conserved threonine motif in the isolated WT integrin β1 tail would not only promote displacement of the negative regulator filamin but also prohibit kindlin2 from driving integrin inside-out signaling in the absence of talin. This scenario is in line with the observation that kindlin overexpression does not lead to integrin inside-out activation (Ma et al., 2008; Harburger et al., 2009; Ye et al., 2010; Li et al., 2017).

A major remaining question is where and when the conserved threonine motif in integrin β subunits is phosphorylated and which kinase(s) are involved in this regulatory step. Phosphorylation of the conserved integrin threonine motif has been most intensely studied for the integrin β2 subunit, where T758/T759 form part of the Kx2TTTV motif in the cytoplasmic tail. In this case, stimulation of G-protein-coupled receptors or the T cell receptor leads to phosphorylation of T758 in integrin β2 and enhanced integrin-mediated cell attachment (Chatila et al., 1989; Fagerholm et al., 2005; Takala et al., 2008; Uotila et al., 2014; Valmu et al., 1991). Application of PKC inhibitors abrogates T758 phosphorylation, and the corresponding synthetic peptides of the integrin cytoplasmic domain are phosphorylated in vitro by conventional and unconventional PKC enzymes (Fagerholm et al., 2002). However, additional kinases such as CaMKII have been shown to associate with the integrin β1 subunit in breast tumor cells (Takahashi, 2001), and inhibitors of CaMKII prevent the increase in T789 phosphorylation driven by constitutive active Ndr1 kinase, an abundant kinase in differentiating neurons (Rehberg et al., 2014). Our kinase assays indicate that multiple serine/threonine kinases can relay signaling inputs, eventually originating from different extracellular and/or intracellular cues, toward the integrin β1 cytoplasmic domain.
Our finding of a strong elevation of integrin phosphorylation upon deletion of a single phosphatase was therefore unexpected. However, in several distinct human cell types and in different scenarios, such as matrix-attached or suspended cells, PPM1F deficiency leads to constitutive T788/T789 phosphorylation. It has to be noted that apart from the integrin β1 subunit, PPM1F acts on additional substrates such as kinases, cytoskeletal proteins, and apoptosis regulators (Zhang et al., 2013; Ishida et al., 2018). Therefore, it remains to be determined whether the abortion of embryonic development seen in PPM1F KO mice is a direct consequence of alterations in integrin activity and to what extent deregulation of other PPM1F substrates may play a role. However, the early embryonic lethality observed upon disruption of genes encoding integrin β1, talin, kindlin, filaminA, and PPM1F in mammals and the functional interplay of these proteins in intact cells strongly argue for a critical role of PPM1F-mediated integrin activity regulation in vivo.

It is easily conceivable that PPM1F is ideally suited to serve as key control for the T788/T789 phospho-switch, as it dephosphorylates the integrin β cytoplasmic domain, and this phosphatase is also able to reverse the auto-phosphorylation of CaMKII at Thr286 (Harvey et al., 2004; Ishida et al., 1998). Thus, PPM1F could shift the balance toward the unphosphorylated, inactive integrin by acting on both an integrin-directed serine/threonine kinase and the integrin T788/T789 motif itself. Taken together, our study identifies PPM1F as the enigmatic integrin phosphatase that acts on the highly conserved threonine motif in the integrin β cytoplasmic domain (Gahmberg et al., 2014). Thereby, this widely expressed protein phosphatase functions as an essential constituent of the integrin off-switch. In contrast to other negative regulators of integrins, such as ICAP-1α, Sharpin,
or Dok1, PPM1F has a defined enzymatic activity that can serve as a target for small molecule modulators. Given the important role of fine-tuning integrin activity in thrombus formation, immune cell motility, or wound healing, agonists as well as antagonists of PPM1F could provide novel access points to adjust integrin activation thresholds.

Materials and methods

Antibodies used

The following antibodies were used with the corresponding dilutions for WB, immunofluorescence (IF), immunohistochemistry, or integrin activity assay (IA): α-actinin (BM75.2; mouse anti-human/anti-mouse; Abcam; 1:1,000 WB), αi-integrin (TS2/7; mouse anti-human/anti-mouse; Abcam; 1:50 IF), αv-integrin (6E1; mouse anti-human/anti-mouse; DSHB; 1:60 IF), αv-integrin (P1B5; mouse anti-human/anti-mouse; DSHB; 1:60 IF), αv-integrin (BIIIG2; rat anti-human; DSHB; 1:10 IF; and MR5; rat anti-mouse; BD Pharmingen; 1:300 IF), αv-integrin (PE-P2W7; mouse anti-human/anti-mouse; sc-9969; 1:300 IF), β1-integrin (AIIB2; IgG1, rat anti-human; DSHB; 1:600 IA; and HMβ1-1, armenan hamster anti-mouse; BioLegend; 1:300 IF/IA; and D2E5; rabbit anti-human; Cell Signaling; 1:1,000 WB), active β1-integrin (9EG7; G1, rat anti-human/anti-mouse; generous gift of D. Vestweber, Max-Planck-Institut for Molecular Medicine, Münster, Germany; 1:300 IA/IF), p788/789 β1-integrin (44-872G; rabbit anti-human; Thermo Fisher Scientific; 1:1,000 WB), β1-integrin (2C9.G3; Armenian hamster anti-human/anti-mouse; ebioscience; 1:300 IF), β1-integrin (AST-3T; mouse anti-human; BioLegend; 1:150 IF; and KN-52; mouse anti-mouse/human; ebioscience; 1:300 IF), carboxyermobryn antigen-related cell adhesion molecule (CEACAM) 1,3,4,5,6 (D14HD11; Aldevron; 1:6,000 WB, 1:200 IF), Ezrin (MAB3822; mouse anti-human; Millipore; 1:200 WB), FAK (77; mouse anti-human; BD Biosciences; 1:250 WB), ILK (EPI593Y; rabbit anti-human; Epitomics; 1:800 WB), Kindlin-2 (3A3; mouse anti-human; Millipore; 1:200 WB, 1:250 IF), paxillin (5H11; mouse monoclonal; Thermo Fisher Scientific; 1:1,000 WB), PPM1F (I7020-1-AF; rabbit anti-human; Protein-Tech; 1:1,000 WB), mPPM1F (1147; rabbit anti-human; Cell Signaling; 1:1,000 WB, 1:250 IF), HRP-conjugated goat anti-rabbit IgG (Santa Cruz; 1:250), HRP-conjugated goat anti-rabbit IgG (Jackson; 1:3,000 WB), streptactin-HRP (IBA Lifesciences; 1:10,000 WB, 1:1,000 ELISA), murine endoglin (CD105; rat anti-mouse; MJ/18; DSHB), and unspecific control IgG (anti-mouse 96/1; generated at the Tierforschungsanlage, University of Konstanz).

Recombinant DNA

For the construction of His-Sumo–tagged talin F3 subdomain of the FERM domain (residues 309–411), the murine talin1 cDNA (kindly provided by R. Fassler, Max-Planck-Institut Biochemistry, Martinsried, Germany) was amplified with the following primers: mTalin1 F3-forward: 5’-GAGCTTCTACACCTGGTGCTTCTCTTTTCTAGTC-3’ and mTalin1 F3-reverse: 5’-GTCTCTAGATCACCGCAATGGTGCTTCTGCTG-3’. The resulting PCR fragment was cloned into pET24a His-Sumo bacterial expression vector (Andréasson et al., 2008) via Bsal and Xhol restriction sites. For construction of GFP-tagged talin, cDNA of human talin-1 (kindly provided by R. Fassler) was cloned into pDNR Dual (Clontech) using the following primers and restriction digest with SalI/AgeI: hTalin1_SalI sense: 5’-GGATCCCATGGCTCTGGACGGGATAAG-3’ and hTalin1_AgeI anti: 5’-GTCTATACCGG TTATTAGTCTC ATCTCGAAGCTCTG-3’. Cre/LoxP recombination was used to move sequences from pDNUR1 into pEGFP-C1-loxP for eukaryotic expression.

Human kindlin2 cDNA (ID4547604 in pOTB7; Source Biology) was used as a template for PCR amplification with the following primers: hKindlin2_SalI sense: 5’-ATCGTGCAACGCCTGGACGGGATAGATG-3’ and hKindlin2_BamHI anti: 5’-TACCGGATCTCCACCCACACCTGTAAG-3’. The product was cloned into pDNR Dual via SalI/BamH1 restriction digest. The kindlin2 cDNA was transferred to pEGFP-C1-loxP vector by Cre-recombination. Mouse kindlin2 cDNA (pCMV-SPORT6; Source Biology) was used as a template for PCR amplification with the following primers: mKindlin2_LIC sense: 5’-AATCTCTCCCGCCCATGGCTTGGAGGATAAGATG-3’ and mKindlin2_LIC anti: 5’-GCCAATAACCCGTCACACACCTGTAAG-3’. The product was cloned into pDNR Dual-LIC vector by ligation-independent cloning (LIC; Adrian et al., 2019). The kindlin2 insert was inserted into pET24a-His-SUMO by PCR amplification with the following primers: mKindlin2_BamHI sense: 5’-TATAGGTATCCAGGTCCTGAGGAGGATGTAAG-3’ and mKindlin2_BamHI anti: 5’-TATACCTCGAATGACCCACACCTGTAAG-3’ and by restriction digest with BamH1 and Xhol enzymes.

The integrin-binding part of filamentA (FilamentA I91-21 [FLN1g19-21]) was generated by amplifying human filamentA (plasmid 8982; Addgene) with the following primers: FLN1g19-forward 5’-AATCTCTCCCGCCCATAGGATCCAGGCAATTGGAAT-3’ and FLN1g19-reverse 5’-CCCCACATACCCACACCTGCCAAATCAGGGAAG-3’. It was cloned into pDNR-Dual-LIC. FLN1g19 cDNA was subsequently transferred to pEGFPC1-loxP by Cre-mediated recombination, generating GFP-FLN1g19-21 for mammalian expression, and to pGEX4T1 loxP (Schmitter et al., 2007) for bacterial expression of GST-FLN1g19-21.

For His-Sumo–tagged FLN1g19-21, the respective filamentA sequence was amplified by PCR with the primer pair FLN1g19-forward: 5’-GACGCTTCAAGTGGGATGCGAGTCTG-3’.
TCGGCTCTCG-3' and FLNig21-reverse: 5'-CTGATGCTCGAGTTA
AGACGGGAAAGCCACAG-3'. The resulting PCR fragment was
cloned into pET24a-His-Sumo via BsaI and XhoI restriction
sites.

cDNA of control protein yeast enolase-1 was obtained by
 genomic DNA extraction (PureLink Genomic DNA Mini Kit;
Thermo Fisher Scientific) from Candida albicans SC5314 (gen-
erous gift of J. Morschhäuser, University of Würzburg, Würz-
burg, Germany) and PCR amplification using the following
primers: enolase-1 BamHI sense: 5'-ATAGAATTCCTGGAGCCCTT
TTAATGATAATTCAGT
CTGCTAGCCTGTGCTGGCTTCTTTGACGTCG-3'
and enolase-1 XhoI NEF anti: 5'-ATATCTCT
AGTTTGCTAAATTTGAAAAGGAGAAATG
and 6xHis-SUMO-tagged or GST-tagged TalinF3, Kindlin-2,
FLNig21-21, enolase-1, human 7xHis-TEV ILKAP (plasmid 34817;

CEACAM3-integrin β1 fusion constructs were generated via
PCR amplification using the cDNA of human integrin β1 WT, 
T788/789D, or T788/789A synthetic sequences as a template
with following primers: integrin β1_E762-K798 BamHI sense:
5'-GGGGCTATGATCCGAGTTTCTAAATTTGAAAGAGA
AAATG-3' and integrin β1_E762-K798_XhoI anti: 5'-TATCTCT
GAGTCTATTTCCCTCATATCTGGC-3'. PCR products were
cloned into pcDNA3.1 CEACAM3ACT using BamHI/XhoI restric-
tion sites (Baade et al., 2019; Schmitter et al., 2004). For the
β1 integrin Y783A construct, pcDNA3.1 CE3-β1 WT integrin-
containing plasmid was used to perform site-directed muta-
genesis using the following primers: integrin β1 Y783A sense:
5'-CAGGGTGAAGATCTTCTGGAAGAGTGCTGAACAC
TTTTCCACCGTGTCG-3' and integrin β1 Y783A anti: 5'-CACAGGTTTACGGCACT
CTTAGCAAATAGGATTTTCACCAGTGTCG-3'.

For the generation of mCherry- and GST-tagged PPM1F, the
cDNA of human PPM1F (I.M.A.G.E. cDNA clone IRAU6p96F0116D)
was obtained from Source BioScience. The following primers
were used for amplification: hPPM1F-IF-sense: 5'-GAAGTTATACGT
CGACCACCACCTCCTGAGACCCC-3' and hPPM1F-IF-anti: 5'-ATG
GCTGAGAAGATCGCTACTCTTTGAGG-3'. The resulting
PCR fragment was cloned into pDR-CMV using the In-Fusion
dry-down PCR Cloning Kit (Clontech). The sequence-veri-
ﬁed pDR-CMV hPPM1F was used as donor vector, and the insert
was transferred by Cre-mediated recombination into the ac-
ceptor vector pmCherry-C1-loxP (mammalian expression) and
gEX-4T1-loxP (bacterial expression) generating N-terminal-
tagged pmCherry-hPPM1F and gEX GST-hPPM1F. For the genera-
tion of mCherry- or GST-tagged hPPM1F D360A, the phosphatase
dead mutant of PPM1F (Harvey et al., 2004), site-directed
mutagenesis was performed with the hPPM1F-containing vec-
tors using primers PPM1F-D360A-forward: 5'-GACTACCTCG
CTGCTAGCCTGGCTGGCTTCTTTGAGCCTG-3' and PPM1F
D360A-reverse: 5'-GTCAAAAGAGGCAGCAGCAGCTAGACG
GTAATCGCC-3'.

For expression of PTP1B (a kind gift from W. Hofer, Uni-
versity of Konstanz, Konstanz, Germany), the cDNA was cloned
into pDRN-Dual-LIC after PCR amplification using the primers
PTP1B LIC sense: 5'-ACTCTCTCCCCGGCTGAGGATGAGAAGG
AGTTCC-3' and PTP1B LIC anti: 5'-CCCCTACTCCGCTATG
TTGGCTGTTGACAGG-3', followed by Cre-lox recombination into
gEX4T1 loxP vector for bacterial expression. All recom-
binant constructs were verified by sequencing.

Expression of recombinant proteins
Strep-tagged or GST-tagged integrin β1 cytoplasmic domains
and 6xHis-SUMO-tagged or GST-tagged TalinF3, Kindlin-2,
FLNig21, enolase-1, human 7xHis-TEV ILKAP (plasmid 34817;
Addgene; pQTEV vector by K. Buessow, Protein Structure Factory, Berlin, Germany), GST-tagged human PTPιβ and human Trx-His-S-PPS (a kind gift of D. Dietrich, University of Konstanz, Konstanz, Germany), and GST-tagged fibronectin type III repeats 9–11 (FNIII9–11, gift of M.A. Schwartz, Yale University, New Haven, CT) were expressed in E. coli as described for PPMIF. Proteins were purified using His- or GST-Trap FF column (GE Healthcare) or Streptactin Superflow column (IBA Lifesciences) depending on the protein and dialyzed against appropriate buffers.

**Pulldown assays with integrin cytoplasmic domains**

2.5 µg of Strep-tagged integrins or 10 µg of biotin-integrin phospho-peptide β1-762-798 pTyrT (Biotin-EFAKFEKEMNA KWDTGENPIYKSA)pT[pT]VNPKYEK-OH or biotin-integrin peptide β1-762-798 (Biotin-EFAKFEKEMNAKWDTGENPIYKSA) VTVTPNPKYEK-OH; Novopep Limited) were loaded onto Streptactin Sepharose beads (50% suspension; IBA Lifesciences) or streptavidin agarose beads (50% suspension; IBA Lifesciences) or streptavidin agarose beads (50% suspension; IBA Lifesciences) and phosphatase saturating substrate (para-nitrophenolphosphate and sodium orthovanadate, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml Pefabloc, 5 µg/ml pepstatin, and 10 µM benzamidine) and inhibitors (10 mM sodium pyrophosphate, 100 mM NaF, 1 mM EGTA, 0.1% wt/vol SDS, and 1% vol/vol deoxycholic acid) supplemented with freshly added Na3VO4 (200 mM) and Na4P2O7 (200 mM) for 30 min at RT under continuous rotation. After centrifugation (2,700 × g, 2 min, 4°C), samples were washed three times with IP buffer. Then integrin-loaded beads were suspended in bait protein solution (corresponding amount of protein diluted in IP buffer) and incubated 2 h at 4°C under constant rotation. Samples were centrifuged (2,700 g, 2 min, 4°C) and washed again three times with IP buffer. Streptactin samples were eluted under native conditions by adding 30 µl of buffer BXT (50 mM Tris, pH 8, 150 mM NaCl, 50 mM biotin; IBA Lifesciences). After 10 min incubation at RT under constant rotation, samples were centrifuged. Supernatants were mixed with 4× SDS and boiled for 5 min at 95°C before they were subjected to WB. Streptavidin agarose beads were directly mixed with 2× SDS and boiled for 10 min at 95°C to elute proteins from biotin-integrin peptides before they were subjected to WB.

**Solid phase binding assay**

96 wells (high-binding; 655061; Greiner Bio-One) were coated with 1 µM of His-Sumo-tagged or GST-tagged proteins in PBS (1.37 M NaCl, 26.8 mM KCl, 14.7 mM KH2PO4, and 78.1 mM Na2PO4) overnight at 4°C in triplicate. Wells were washed three times with PBS and blocked with 200 µl/well PBS plus 2% BSA for 1 h at RT before incubation with 0.5 µM of Strep-tagged integrin β1 proteins in PBS plus 0.05% Tween overnight at 4°C. For competitive assays with FLN19-21 and TalinF3 domain, Strep-tagged integrin β1 cytoplasmic domains were immobilized first before incubation with FLN19-21 and/or TalinF3. Wells were washed three times with PBS plus 0.05% Tween and blocked again for 1 h at RT. Streptactin-HRP (IBA Lifesciences) or mouse-α-His antibodies followed by HRP-conjugated goat α-mouse IgG antibodies for competitive assays were added in blocking buffer for 1 h at RT, respectively. Finally, wells were intensively washed with PBS plus 0.05% Tween, and 100 µl/well of tetramethylbenzidine (TMB) solution was added (9.5 ml of 2.4 mg/ml TMB in 1:9 aceton:ethanol plus 20 µl/10 ml H2O2 plus 0.5 ml 30mM potassium citrate, pH 4.1). The reaction was stopped after 20 min by adding 100 µl/well 2 M H2SO4, and samples were measured at 450 nm in a microplate reader (Varioscan; Thermo Fisher Scientific).

**Cell culture and transient transfection**

Human embryonic kidney 293T cells (293T; American Type Culture Collection CRL-3216) and A172 glioblastoma cells (American Type Culture Collection CRL-1620) were grown in DMEM supplemented with 10% calf serum. NHDFs were obtained from PromoCell and cultured in PromoCell fibroblast growth medium. All cells were maintained at 37°C, 5% CO2, and subcultured every 2–3 d.

For transient transfection of 293T cells, cells were seeded at 25% confluence the day before and transfected using the standard calcium phosphate method with a total amount of 5 µg plasmid DNA/dish. For transient transfection of A172 cells, Lipofectamine 3000 was used (Thermo Fisher Scientific) according to the provided protocol after seeding 2.5 × 105 cells for 2 h into six-well plates.

**OPTIC**

The detailed OPTIC protocol, fluorescence microscopy, and evaluation of protein recruitment were described elsewhere (Baade et al., 2019). Briefly, HEK293T cells were transfected with pcDNA3.1 CEACAM3-integrin β1 fusion constructs together with GFP, GFP-FLNig19-21, GFP-talin-1, or GFP-kindlin-2 and optionally mCherry-PPMIF or mCherry-PPMIFD360A. 48 h after transfection, cells were seeded onto 10 µg/ml poly-L-lysine–coated coverslips in suspension medium (DMEM plus 0.25% BSA). After 2 h, adherent cells were infected with Pacific Blue–stained N90 (Opn4ζ– expressing, nonpiliated Neisseria gonorrhoeae MS11; Baade et al., 2019) at MOI 20 for 1 h in DMEM plus 0.25% BSA. Infection medium was aspirated, and cells were immediately fixed with 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and stained with α-CEACAM antibody (clone D14HD11; Aldevron) in blocking solution (10% calf serum [CS] in PBS) after washing with PBS and blocking. Wells were again washed with PBS and blocked followed by a second antibody staining. Coverslips were mounted on glass slides using Dako mounting medium (Dako). Samples were imaged at RT on a Leica SP5 confocal microscope equipped with a 63.0×/1.40 NA oil HCX PL APO CS UV objective and analyzed using LAS AF Lite software. Protein recruitment at one bacterial infection site was quantified per cell (n = 60 cells per sample).

**Whole cell lysates (WCLs) and WB**

To obtain WCLs, equal cell numbers were lysed by treatment with radioimmunoprecipitation assay buffer (1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 0.1% wt/vol SDS, and 1% vol/vol deoxycholic acid) supplemented with freshly added protease and phosphatase inhibitors (10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml Pefabloc, 5 µg/ml pepstatin, and 10 µM benzamidine) and phosphatase saturating substrate (para-nitrophenolphosphate [pNPP]; Sigma-Aldrich; 10 mM). Chromosomal DNA and cell debris were pelleted by addition of Sepharose beads and
centrifugation (13,000 rpm, 30 min, 4°C). Supernatant was supplemented with 2× or 4× SDS sample buffer (2 or 4% wt/vol SDS, 20% wt/vol glycerol, 125 mM Tris-HCl, 10/20% vol/vol β-mercaptoethanol, and 1% wt/vol Bromophenol blue, pH 6.8) and boiled for 5 min at 95°C. The protein amount was adjusted via the bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific) according to the manufacturer’s protocol and analyzed by WB using a prestained marker as protein size control (26619; Thermo Fisher Scientific). Briefly, proteins were resolved on 8–18% SDS-PAGE. After separation, the proteins were transferred to a polyvinylidine fluoride membrane (Merck Millipore), followed by blocking in 2% BSA containing 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5 (TBS-T) buffer. The membrane was incubated with primary antibody in blocking buffer overnight at 4°C, washed three times with TBS-T, and incubated with HRP-conjugated secondary antibody in TBS-T for 1 h at RT. The chemiluminescent signal of each blot was detected with ECL substrate (Thermo Scientific) on the Chemidoc Touch Imaging System (Bio-Rad) in signal accumulation mode. Acquired images were processed in Adobe Photoshop CS4 by adjusting illumination levels of the whole image.

shRNA constructs and cloning

For the generation of recombinant, shRNA-expressing lentiviral particles, the shRNA vector system pLKO.1 developed by Stewart et al. (2003) was applied. The different shRNAs were designed by using the AAN19 algorithm and siRNA selection program of the Whitehead Institute for Biomedical Research (http://sirna.wi.mit.edu/). According to the prediction of the siRNA selection program, two complementary oligos were synthesized. The sequences of all shRNA-oligos used in this study are provided in Table 1. The oligos were annealed and cloned via AgeI and EcoRI restriction sites into plasmid pLKO.1 puro (plasmid 8453; Addgene), which provides puromycin resistance for selection of stable knock-down cells. The correct insertion of the shRNA cassettes was verified by sequencing.

sgRNA constructs and cloning

sgRNAs against Cerulean and human PPM1F were designed according to an appropriate gRNA target site selected by CHOP-CHOP (http://chopchop.cbu.uib.no/; Montague et al., 2014). The following sgRNA encoding complementary oligos were synthesized and annealed: hPPM1F-sgRNA_sense: 5′-CAACGCGACCGA CCAGACTTTTCT-3′ and hPPM1F-sgRNA_anti: 5′-AAGCAGAACAA CATCTGTCGGTGC-3′ as well as Cerulean-sgRNA_sense: 5′-CACGCCCTGCAAGCTAGAAGCAA-3′ and Cerulean-sgRNA_anti: 5′-AAGCAGAACAAATCTGTCGGTGC-3′. The resulting sgRNA-Cerulean oligo was inserted into the BbsI restriction site of vector pSpCas9(BB)-2A-GFP (plasmid 48138; Addgene; a gift from F. Zhang, Broad Institute, Cambridge, MA). Using the primer pair CRISPR-U6-sgRNA_KpnI_sense: 5′-ATAGGTACCGTGAGGGCCATTATTTCCC-3′ and CRISPR-U6-sgRNA_KpnI_anti: 5′-ATAGGTACCGTGAGGGCCATTATTTCCC-3′, the complete U6 promoter-driven sgRNA-Cerulean-containing cassette was amplified and inserted into the KpnI site of plentiCRISPR v2 vector (plasmid 52961; Addgene; a gift from F. Zhang), resulting in plentiCRISPR v2 sgRNA-Cerulean. Using the BsmBI/Esp3I restriction sites, the sgRNA-PPM1F oligo was inserted in plentiCRISPR v2 sgRNA-Cerulean, resulting in plentiCRISPR v2 sgRNA-Cerulean-sgRNA-PPM1F. The correct insertion of the Cerulean sgRNA cassette and of the PPM1F sgRNA was verified by sequencing.

Lentiviral production and generation of stable cell lines

Lentiviral particles were produced as described previously (Muenzner et al., 2010). Briefly, 293T cells were transfected by standard calcium phosphate coprecipitation using 3.5 µg pMD2.G (packaging cassette), 5 µg psPAX2 (viral envelope expression cassette), and 6.5 µg pLKO.1, plentiCRISPR v2, or pLL3.7 containing the desired shRNA, sgRNA, or cDNA, respectively. After 72 h, virus-containing culture supernatant was collected and ultra-centrifuged, and target cells were infected with virus concentrate by spinfection (1 h, 800 g, RT) with 8 µg/ml polybrene following incubation for 24 h at 37°C. Control cells were generated by transducing cells with virus harboring empty pLKO.1, pLL3.7-LIC-mKate, or plentiCRISPRv2 containing sgRNA against Cerulean. After 48 h recovery time, cells transduced with pLKO.1 or plentiCRISPRv2 were selected with puromycin (0.8 µg/ml, 6 d). A172 cells transduced with pLL3.7 mCerluean or pLL3.7-LIC-mKate-derived vectors were selected for fluorescence protein expression by flow cytometry. Single cell clones were expanded by adding 20% conditioned medium to the regular growth medium supplied with 20% FCS and penicillin/streptomycin.

Generation of PPM1F KO A172 cells

For the generation of A172 PPM1F KO cells, A172 cells were first stably transduced with a lentiviral vector encoding mCerulean cDNA. Using A gel/BsrG restriction sites, the GFP cDNA in vector pLL3.7 (plasmid 11795; Addgene) was exchanged for mCerulean cDNA derived from plasmid pmCerulean-Ci (kind gift of D. Piston, Vanderbilt University Medical Center, Nashville, TN). A172 cells stably expressing mCerulean were used as a basis for CRISPR/Cas9-mediated disruption of the Cerulean cDNA (as a sign for successful Cas9 expression and activity) and of the PPM1F gene. Accordingly, the cells were transduced with plentiCRISPR v2 sgRNA-Cerulean (A172 control cells) or plentiCRISPR v2 sgRNA-Cerulean-sgRNA-PPM1F. After puromycin selection, single Cerulean-negative cells were sorted by flow cytometry, and clonal cell lines were grown up using addition of 20% conditioned medium to the regular growth medium supplied with 20% FCS and penicillin/streptomycin. The derived cells lack Cerulean expression (A172 control) or lack Cerulean and PPM1F expression (A172 PPM1F KO cells) as verified by WB and sequencing of the Cas9-disrupted genomic Ppm1f locus.

Complementation of PPM1F KO A172 cells

For the reexpression of mKate2-PPM1F and mKate2-PPM1F D360A in A172 PPM1F KO cells, the pLL3.7 LIC mKate2 vector was created. First, mKate2 cDNA (pmKate2-c vector; plasmid FP181; Evrogen) was amplified using the primers pLL3.7_mKate2_Agel_sense: 5′-ACTACCGGTATGGTGCAGCGATGATTAA G-3′ and pLL3.7_mKate2_EcoRI_anti: 5′-GTCGAATTCTTACCTT

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PPM1F is the integrin phosphatase
Table 1. Lentiviral plasmids encoding desired shRNA for stable protein knock-down

| Vector | shRNA       | Primer                           |
|--------|-------------|----------------------------------|
| pLKO.1 | hANKRD28    | 5'-ccggaAGCCTTAGTCTATTCAATctcgagTATGAAATAGGACCTAGGACcttttttg-3' |
|        |             | 5'-aattccccccccAGCCTTAGTCTATTCAATctcgagTATGAAATAGGACCTAGGACtt-3' |
| pLKO.1 | hβ1 Integrin| 5'-ccggaAAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hFilaminA   | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hILKAP      | 5'-ccggaAAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPPM1F      | 5'-ccggaACCACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPP2A       | 5'-ccggaAAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGAAGGTTGTAAGGCGCTCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPTP1B      | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPTP-PEST   | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPTPRF      | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hPTPRO      | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
| pLKO.1 | hRPTPo      | 5'-ccggaACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttttttttg-3' |
|        |             | 5'-aattccccccccAGACACACCTACTTTTGAGATCctcgagGAGGCTTAAAGGATGCTGAttt-3' |
Table 1. Lentiviral plasmids encoding desired shRNA for stable protein knock-down (Continued)

| Vector | shRNA   | Primer                                   |
|--------|---------|------------------------------------------|
| pLKO.1 | hSHP1   | hSHP1_shRNA_sense                         |
|        |         | 5'-ccggaaCCCTTCTCCTTGTACTcgagATTACAAGGAAGAGGtttttttg-3'|
|        |         | hSHP1_shRNA_anti                          |
|        |         | 5'-aattcaaaaaaCCCTTCTCCTTGAAATctcgagATTACAAGGAAGAGGtttttttg-3'|
| pLKO.1 | hSHP2   | hSHP2_shRNA_sense                         |
|        |         | 5'-ccggaaCAGACGCAAGAAAACTTTATctcgagAATAAACCTTTCTGGCCTGGTtttttttg-3'|
|        |         | hSHP2_shRNA_anti                          |
|        |         | 5'-aattcaaaaaaCAGACGCAAGAAAACTTTATctcgagAATAAACCTTTCTGGCCTGGTtttttttg-3'|
| pLKO.1 | hTCPTP  | hTCPTP_shRNA_sense                        |
|        |         | 5'-ccggaaCCTGCACCTTGAATAAGCActcgagTGCGTTATATCAAGTGCAAGtttttttg-3'|
|        |         | hTCPTP_shRNA_anti                         |
|        |         | 5'-aattcaaaaaaCCTGCACCTTGAATAAGCActcgagTGCGTTATATCAAGTGCAAGtttttttg-3'|

GTGCCAGGTTTCTAGG-3’. The resulting mKate-encoding PCR fragment was cloned into pLL3.7 (plasmid 11795; Addgene) via AgeI/EcoRI restriction digest to replace the GFP cDNA to yield the pLL3.7-mKate2 vector. Subsequently, pLL3.7-LIC-mKate2 vector was created by replacing the multiple cloning site with a LIC sequence (primers 5’-CTAGCGACTCTCCGCAGTTAGGGGCGA-3’ and 5’-CCGTTGCCCACTAAACCGGAGAGTCG-3’) via NheI/AgeI restriction digest to replace the GFP cDNA in pmCherry were modified by site-directed mutagenesis with primers Rescue_sgRNA-hPPM1F_for: 5’-CGAACAGATCAAATGTTCCTGAGGAAAGCCAAGAGAGCG-3’ and Rescue_sgRNA-hPPM1F_rev: 5’-CCTCGGAGGGAACATTTGATCTGTTCGCCGGAAAGCTTCTCTGAGGG-3’ to inactivate the sgRNA-hPPM1F target sequence by silent mutations. The resulting sgRNA-resistant cDNAs of hPPM1F and hPPM1F D360A were amplified with primers PPM1F_LIC_for: 5’-CGAAPGACGACGAAATGTTCCTGAGGAAAGCCAAGAGAGCG-3’ and PPM1F_LIC_rev: 5’-CCTCGGAGGGAACATTTGATCTGTTCGCCGGAAAGCTTCTCTGAGGG-3’ and inserted via LIC (Aslanidis and de Jong, 1990) into pLL3.7-LIC-mKate2 to result in pLL3.7-mKate2-hPPM1F and pLL3.7-mKate2-hPPM1F D360A.

**IF staining for FACS analysis**

Cells were detached with trypsin/EDTA, pelleted, and suspended in FACS buffer (0.1% NaAzide and 5% FCS in PBS). After centrifugation (800 rpm, 3 min), cells were washed with FACS buffer, and 3 × 10^5 cells were transferred into Eppendorf tubes, centrifuged (2,500 rpm, 2 min, 4°C), and incubated with primary antibody in FACS buffer at the desired concentration for 1 h at 4°C under constant rotation. Cells were washed thrice with FACS buffer and incubated with secondary antibody in FACS buffer at desired concentrations for 30 min at 4°C under constant rotation in the dark. After washing with PBS, cells were analyzed by flow cytometry (BD LSRII, FACSDiva software; BD Biosciences).

**Replating assay for pT788/pT789 β1 integrin analysis in intact cells**

10-cm dishes were coated with 2 μg/ml FN_119-11 in PBS overnight at 4°C. Cells were starved with DMEM plus 0.5% FCS overnight at 37°C. The next day, dishes were blocked with DMEM plus 0.25% BSA for 1 h at 37°C. In parallel, cells were trypsinized, trypsin-inactivated with soybean inhibitor, counted, and kept in PBS overnight at 4°C. After washing thrice with PBS, blocking for another 20 min, coverslips were treated with secondary antibody in blocking solution and optionally with phalloidin-Cy5 or DAPI for 1 h at RT in the dark. Finally, cells were washed thrice with PBS, and mounted with Dako fluorescent mounting medium (Dako). Samples were imaged on an Leica SP5 confocal microscope equipped with a 63.0×/1.40 NA oil HCX PL APO CS UV objective and acquired in xy mode with a 1,024 × 1,024 pixel format and 100 Hz scanning speed at 8-bit resolution. All images were analyzed in ImageJ Software. For spreading assays, a macro was set up together with the Bioimaging Center at the University of Konstanz and used for quantitative picture analysis. Unrecognized cells were analyzed manually in the Leica LAS AF Lite software.

**IF staining for confocal microscopy and cell spreading analysis**

Sterile coverslips were coated with 0.4–10 μg/ml FN_119-11 in PBS overnight at 4°C in a 4-well plate, and cells were starved with DMEM plus 0.5% FCS for 15 h. The next day, coating solution was removed, and wells were blocked with suspension medium (DMEM plus 0.25% BSA). In parallel, cells were trypsinized, trypsin-inactivated with soybean trypsin inhibitor (12.5 mg in 50 ml DMEM, sterile filtered; AppliChem), counted, and kept in suspension medium for 45 min at 37°C. 2.5 × 10^4 cells were seeded onto coverslips and allowed to adhere for corresponding time periods. Cells were fixed with 4% PFA supplemented with 0.1% Triton X-100 for 5 min at RT and again without Triton X-100 for 20 min. Coverslips were washed thrice with PBS (0.9 mM CaCl_2 and 0.5 mM MgCl_2 in 1× PBS) and blocked for 20 min with blocking solution (10% CS in PBS). Primary antibody solution was added for 1 h at RT. After washing thrice with PBS and blocking for another 20 min, coverslips were treated with secondary antibody in blocking solution and optionally with phalloidin-Cy5 or DAPI for 1 h at RT in the dark. Finally, cells were washed thrice with PBS, and mounted with Dako fluorescent mounting medium (Dako). Samples were imaged on a Leica SP5 confocal microscope equipped with a 63.0×/1.40 NA oil HCX PL APO CS UV objective and acquired in xy mode with a 1,024 × 1,024 pixel format and 100 Hz scanning speed at 8-bit resolution. All images were analyzed in ImageJ Software. For spreading assays, a macro was set up together with the Bioimaging Center at the University of Konstanz and used for quantitative picture analysis. Unrecognized cells were analyzed manually in the Leica LAS AF Lite software.

PPM1F is the integrin phosphatase
concentrations of collagen I (Sigma-Aldrich), FNIII9-11, or poly-L lysine (SERVA) as an integrin-independent control overnight at 4°C. Wells were blocked with DMEM plus 0.25% BSA for 1 h at 37°C. In parallel, cells were trypsinized and kept in suspension medium for 45 min. 2 × 10^4 cells/well were seeded and allowed to adhere for the indicated time periods at 37°C. After incubation, nonadherent cells were removed by gently washing with PBS^+ thrice. Adherent cells were fixed with 4% PFA in PBS for 15 min, washed with PBS, and stained with 0.1% crystal violet in 0.2 M borate buffer (pH 8.5) for 30 min. After intense washing, the color was unhinged from cells with 10 mM acetic acid, and the absorption was measured at 590 nm using a spectrophotometer.

Cell adhesion assay
96-well plates were coated with PBS containing corresponding concentrations of collagen I (Sigma-Aldrich), FNIII9-11, or poly-L lysine (SERVA) as an integrin-independent control overnight at 4°C. Wells were blocked with DMEM plus 0.25% BSA for 1 h at 37°C. In parallel, cells were trypsinized and kept in suspension medium for 45 min. 2 × 10^4 cells/well were seeded and allowed to adhere for the indicated time periods at 37°C. After incubation, nonadherent cells were removed by gently washing with PBS^+ thrice. Adherent cells were fixed with 4% PFA in PBS for 15 min, washed with PBS, and stained with 0.1% crystal violet in 0.2 M borate buffer (pH 8.5) for 30 min. After intense washing, the color was unhinged from cells with 10 mM acetic acid, and the absorption was measured at 590 nm using a spectrophotometer.

For ELISA, 96-well plates were coated with 0.1 µg/ml FNIII9-11 in PBS overnight at 4°C. The next day, wells were blocked with 0.25% BSA in DMEM. In parallel, cells were trypsinized and kept in suspension for 1 h. Then, 5 × 10^4 cells were seeded in triplicate and allowed to adhere for 40 min. Then cells were transferred onto ice, fixed with 4% PFA, washed with PBS, and permeabilized with 0.15% Triton X-100 for 15 min. Afterward, cells were blocked with 2% BSA in PBS for 20 min and stained with 9E7 or AIIB2 antibodies. After incubation with the primary antibody, cells were washed with PBS, blocked for 20 min, and incubated with the secondary HRP-conjugated goat anti-rat IgG antibody for 1 h at RT. Finally, cells were intensively washed, and 100 µl of substrate solution was added (10 ml of 2.4 mg/ml TMB in 10% acetic/90% ethanol with 0.5 ml of 30 mM potassium citrate, pH 4.1). The enzymatic color reaction was stopped by adding 100 µl/well 2 M H_2SO_4, and the absorption was detected via spectrophotometric measurement at 450 nm. Controls were stained with secondary antibody only.

For FACS, starved cells were trypsinized and kept in suspension (2% BSA and 5 mM glucose in PBS) for 45 min before they were stimulated with 10 µg/ml FNIII9-11 for 15 min at 37°C or kept unstimulated by adding double-distilled H_2O. Cells were put on ice and split into two fractions, which were stained for either active integrin β1 (9E7; 1:600) or total integrin β1 (HMβ1-1; 1:300, or AIIB2; 1:600) for 1 h on ice in PBS plus 2% BSA. Cells were washed thrice with PBS and incubated with Rhodamine-Red conjugated secondary antibody for 45 min on ice in the dark. Cells were washed, and fluorescence intensity was measured by flow cytometry (BD LSRII, FACSDiva software; BD Biosciences).

Expression and purification of GST-tagged hPPM1F and hPPM1F D360A in 293T cells and E. coli
293T cells were transfected by standard calcium phosphate coprecipitation using plasmid DNA encoding for either GST-hPPM1F or GST-hPPM1F D360A. 48 h after transfection, cells were lysed in lysis buffer (50 mM Tris, pH 8, 1% Triton X-100, 1 mM EDTA, and 0.1% β-mercaptoethanol). Cleared lysates were incubated with glutathione-Sepharose beads for 3 h under constant rotation at 4°C. Beads were pelleted via centrifugation and washed three times in lysis buffer and once in GST buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM DTT, and 5 mM MgCl_2). For the elution of the GST-fusion proteins, beads were incubated in GST buffer supplemented with 10 mM reduced L-glutathione twice for 20 min at 4°C under constant rotation. Aliquots were transferred to liquid nitrogen, and long-term storage occurred at −80°C.

For the production of recombinant GST-tagged hPPM1F or GST-hPPM1F D360A, the corresponding sequence-verified construct was transformed into competent E. coli BL21 Rosetta (DE3). Bacteria were cultured in lysogeny broth medium containing appropriate antibiotics at 37°C with constant shaking at 200 rpm. Expression was induced at OD_580 = 0.6 with 0.5 mM IPTG at 30°C and 220 rpm for 3 h, and bacteria were pelleted by centrifugation at 4,700 rpm, 20 min, RT. Afterward, bacteria were resuspended in PBS (pH 7.4) supplemented with 5 mM EDTA, protease inhibitors (10 µg/ml Pefabloc, 10 µg/ml aprotinin, 1 µM PMSF, and 5 µg/ml leupeptin) and 2.5 mM DTT and sonicated at 4°C three times for 2 min. Cleared lysates were put onto a GST-Trap FF column (GE Healthcare) and washed with PBS, pH 7.4. Finally, GST-tagged proteins were eluted with 50 mM Tris, pH 8.0, supplemented with 10 mM reduced L-glutathione and dialyzed against 25 mM sodium phosphate, pH 8.0, supplemented with 150 mM NaCl and 1 mM EDTA or against 50 mM Tris pH 8.0 supplemented with 150 mM NaCl and 1 mM Tris(2-carboxyethyl)phosphine (TCEP). The amount and the purity of proteins were analyzed via SDS-gel electrophoresis and aliquots supplemented with 10% glycerol frozen at −80°C.

In vitro phosphorylation and phosphatase assays
Human recombinant CaMKIIβ (FV4205; Thermo Fisher Scientific) was resuspended in kinase buffer (50 mM Tris-HCl, pH 7.7, 10 mM MgCl_2, 5 mM MnCl_2, 1 mM TCEP, and 0.05% Triton X-100) either supplemented with 200 µM ATP, 1.2 µM calmodulin (Sigma-Aldrich), and 2 mM CaCl_2 or without supplementation and incubated for 10 min at 30°C. The kinase assay was started by adding 2 µg of purified GST-fusion protein and incubated for 60 min at 30°C under constant shaking at 750 rpm. The reaction was stopped via the addition of SDS sample buffer.

For the in vitro phosphatase assay, the CaMKIIβ phosphorylated cytoplasmic tail of β1 integrin was incubated with 2 µg of recombinant GST-PPM1F or PPM1F D360A or corresponding amounts of ILKAP in phosphate buffer (50 mM Tris-HCl, pH 8, 10 mM MnCl_2, and 0.01% Tween 20) for 1 h at 30°C under constant shaking at 750 rpm. The reaction was stopped by the addition of either SDS sample buffer or the same volume of malachite green solution (54 mM NH_4Mo and 0.9 mM malachite...
green in 1 M HCl) and analyzed by WB or photometric measurement with OD_{630nm}.

Phosphatase assays with phospho-peptides were conducted using peptides synthesized by Pepscan: β2T788pT789: (Biotin-Abx-TGEPYKSAvg[pt][pT]TVNVPKYE Gordon-OG), β1-22pT788: (H-TG ENPYKSA-V[pT]TVNVPKYE Gordon-OG), and β1-22pT789: (H-TG ENPYKSA-V[pT]TVNVPKYE Gordon-OG), and ML2-20pT10: (H-MSSKRAKAK [pT]TTKRPQRATS-OG). Depending on the assay, recombinant, E. coli-expressed GST-tagged PPMIF, PPMIFD360A, PTPIB, 7xHis-TEV ILKAP, Trx-His-S-PP5, or GST-PPMIF and GST-PPMIF D360A in phosphatase buffer for 1 h at 30°C. The reaction was stopped by adding the same volume of malachite green solution, and OD_{630nm} was measured. To determine PPMIF kinetics, GST-tagged PPMIF or PPMIFD360A was expressed in 293 cells and purified by glutathione agarose. Proteins were inhibited by 100 mM EDTA, 20 mM NaF, or 250 µM PTP1B, respectively, for either K2HPO4 or 4-methylumbelliferone was measured in parallel and used to determine the kinetic parameters K_{m} and V_{max} by directly fitting the data to the Michaelis–Menten equation \( \frac{V}{S} = \frac{V_{max}}{K_{m}} + \frac{S}{V_{max}} \), where reaction rate \( v \), maximum velocity \( V_{max} \), substrate concentration \( [S] \), and \( [S] \) are indicated. All statistical significances were determined using a two-tailed t-test. All data are presented as mean ± SEM or mean ± SD as indicated. Statistics

Husbandry and genotyping of mice

Mice were kept in accordance with relevant institutional and national guidelines and regulations in the central animal care facility of University of Konstanz. The B6.129P2-PPMIFtmDgen/ J (PPMIF−/−) mouse strain was obtained from The Jackson Laboratory. The targeted pmm1f gene was created by Deltagen by inserting a Lac0-SA-ires-Neo555G/Kan cassette via homologous recombination into the pmm1f locus, allowing the endogenous promoter to drive expression of β-galactosidase. The PPMIF+/+ mice have been backcrossed at least 20 generations to C57BL/6 mice. 3-wk-old mice or embryos were genotyped by amplification of DNA extracted from tissue biopsies or isolated from mouse embryonic fibroblast. The following PCR primers were used: primer 1, WT forward: 5’-CAACTTCTCATCATGCCCCATCAG-3’; primer 2, common reverse: 5’-AAGCATAGGGGACGATGTCGTCCTGTC-3’; and primer 3, targeted allele forward: 5’-GGTGGGATTACGATAAATGCTGC TCT-3’. For genotyping, a PCR with 32 cycles was performed with an annealing temperature of 59°C and an elongation time of 40 s at 72°C, yielding a 200-bps and 450-bps PCR fragment for the WT and the targeted allele, respectively (see also Fig. 9 A).

Derivation of MEFs

For the generation of pmm1f−/− MEFS, heterozygous mice were mated, and 10.5 d after coitus, the female mice were anesthetized and sacrificed. The uterus was dissected and cut between the implantation sites along the uterine horns into pieces containing single embryos. The embryos were isolated by removing the enveloping tissue, washed in sterile fibroblast growth medium (Promocell) supplemented with penicillin, streptomycin, and ciprofloxacin, and minced via up and down pipetting. The tissue homogenates were plated onto gelatin (0.1%) and human fibronectin (2 µg/ml)-coated dishes. After the second passage, primary fibroblasts were immortalized via transduction with pBabeZeo SV40 large T (plasmid 1779; Addgene; Hahn et al., 2002). MEFS were cultured in DMEM supplemented with 10% FCS, nonessential amino acids, and sodium pyruvate.

Generation of polyclonal anti-PPM1F antibody

The cDNA of mPPM1F was obtained from Source BioScience (I.M.A.G.E. Full Length cDNA clone IARVp968A0987D; sequence accession BC042570) and was amplified with primers mPPMIF-BamHI-forward: 5’-GCTTTAGATCAATGCTCTGGAGCCGC ACAGAAC-3’ and mPPMIF-HindIII-reverse: 5’-GCGCCGTCTAGCTCTTCTGAGTATTAC-3’. The resulting PCR fragment was cloned into the pET24a-His-Sumo bacterial expression vector (Andréasson et al., 2008) via BamHI and HindIII restriction sites. The sequence-verified construct was transformed into competent E. coli BL21 (DE3), and expression of the recombinant protein was induced at OD_{580} = 0.67 with 0.5 mM IPTG at 30°C for 4.5 h. His-Sumo-tagged mPPMIF was purified on a HisTrap FF column and eluted with 50 mM sodium phosphate buffer, pH 8, 0.5 M NaCl, and 0.5 M imidazole before removal of the His-Sumo tag by Ulp1 protease (Andréasson et al., 2008). 100 µg of purified recombinant mPPMIF were used for immunization of a New Zealand White rabbit in accordance with relevant institutional and national guidelines and regulations in the central animal care facility of University of Konstanz.

Single cell tracking

MEFS were seeded in 24-well plates and incubated for 24 h. Cells were starved in DMEM supplemented with 0.5% BSA for 12 h, and afterward stimulated with serum-containing growth medium and imaged for 12 h (30 min/frame). Single cells were tracked manually using the ImageJ particle tracking plugin and analyzed using the chemotaxis and migration tool (Ibidi GmbH).

Statistics

All data are presented as mean ± SEM or mean ± SD as indicated. All statistical significances were determined using a two-tailed Student’s t test or one-way ANOVA followed by Bonferroni post hoc test with Prism5 (GraphPad). Significance is indicated with *, P < 0.05; **, P < 0.01; ***, P < 0.001; or ns, not significant.

Online supplemental material

Fig. S1 shows that the T788/T789 motif in the integrin β1 cytoplasmic tail is evolutionary conserved and that its pseudo-phosphorylation regulates the association with talin and filamin. Fig. S2 demonstrates that knock-down of PPMIF in 293T cells or NHDFs does not alter expression of core focal adhesion proteins and does not affect integrin surface levels. Fig. S3 shows that PPMIF KO in A172 cells does not alter integrin

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surface levels or expression of core focal adhesion proteins, but strongly affects integrin-dependent processes. Fig. S4 shows that filaminA knock-down pheno-copies integrin-dependent effects of PPM1F depletion in A172 cells. Fig. S5 shows that PPM1F purified from 293T cells dephosphorylates the conserved T788/T789 motif in the integrin β1 cytoplasmic domain.

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Author contributions: T.M. Grimm, N.I. Dierdorf, and C.R. Hauck conceived the study and designed the experiments; T.M. Grimm and N.I. Dierdorf conducted the experiments and evaluated the data; C. Paone conducted cell migration assays; K. Betz performed structure modeling; T.M. Grimm and C.R. Hauck wrote the paper. All authors read and approved the final manuscript.

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Figure S1. Related to Fig. 1, Fig. 2, Fig. 7, Fig. 8, and Fig. 9: The T788/T789 motif in the integrin β1 cytoplasmic tail is evolutionarily conserved, and its phosphorylation regulates association with filaminA and talin. (A) Alignment of cytoplasmic amino acid residues of integrin β subunits derived from different species. The conserved threonine residues (red), the proximal NPxY motif (blue), and the distal NPxY motif (green) are marked. (B) Alignment of amino acid residues of strep-tagged integrin β1 cytoplasmic tails with indicated binding sites of talin, filaminA, and kindlin-2. The threonine residues (red) and the proximal (blue) and distal (green) NPxY motifs are marked. Point mutations are marked by black boxes. (C) The indicated strep-tag-integrin β1 (Strep-ITGB1) cytoplasmic domains and His-tagged enolase, talinF3 domain, FLN19-21, or kindlin-2 were expressed as soluble proteins in BL21 DE3 bacteria and equal amounts detected by WB and Coomassie staining. (D) Schematic model of the OPTIC principle. OPTIC fusion constructs consist of integrin β1 cytoplasmic domains (ITGB-C) and the transmembrane and extracellular domain of CEACAM3 (CEA3). Receptor clustering is triggered by binding of Opa52 protein expressing Neisseria gonorrhoeae (N. gonorrhoeae) to CEACAM3, thereby potentially recruiting an intracellular protein of interest (POI). (E) 293T cells were transiently transfected with the indicated expression constructs and WCL subjected to WB; CEACAM1 protein served as positive control for the CEACAM antibody, and nontransfected 293T cell lysate served as negative control. (F) GST-FLN19-21 and Strep-ITGB1 WT were incubated with increasing amounts of His-tag. Upper panels show the input proteins. Upon streptactin pulldown, proteins bound to ITGB1 WT were visualized by Coomassie staining (bottom panel), WB with α-GST antibody to detect FLNIg19-21 (second panel), or with α-His to detect talinF3 (third panel). Coomassie staining also verified similar amounts of precipitated Strep-ITGB1 WT (lowest panel). (G) Strep-ITGB1 WT or the T788D/T789D variant was immobilized in triplicate wells and incubated with His-tagged talinF3 and increasing amounts of GST-FLN19-21 at 4°C. After washing, talinF3 binding was detected by incubation with α-6xHis antibody and secondary HRP-coupled antibody. Bars represent mean ± SEM of triplicates from a representative experiment. Coomassie staining verified similar amounts of input FLN19-21 (upper panel), talinF3 (middle panel), and Strep-ITGB1 WT and TT/DD (lowest panel). (H) GST-FLN19-21 and Strep-ITGB1 WT were incubated with increasing amounts of His-kindlin2. Left panels show the input proteins. Upon streptactin pulldown, proteins bound to ITGB1 WT were visualized by Coomassie staining (right; top panel), WB with α-His to detect kindlin2-2 (second panel), or with α-GST antibody to detect FLNIg19-21 (third panel). Coomassie staining also verified similar amounts of precipitated Strep-ITGB1 WT (lowest panel). C. owczarzaki, Capsaspora owczarzaki; D. melanogaster, Drosophila melanogaster; D. rerio, Danio rerio; G. gallus, Gallus gallus; H. sapiens, Homo sapiens; M. musculus, Mus musculus; X. laevis, Xenopus laevis; mCh, mCherry.

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Figure S2. Related to Fig. 3: knock-down of PPM1F in 293T cells or NHDFs does not alter expression of core focal adhesion proteins and does not affect integrin surface levels. (A) Overview of protein phosphatases present in the human integrin adhesome (Zaidel-Bar et al., 2007). These enzymes have been targeted individually by specific shRNA-encoding lentiviral particles. Control cells were treated with lentiviral particles lacking shRNA. (B) Immunoblotting of WCL from PPM1F knock-down (shPPM1F) and control 293T cells probed with antibodies against the indicated focal adhesion proteins. Monoclonal α-tubulin antibody was used as loading control. (C) Immunoblotting of WCLs from control and PPM1F knock-down (shPPM1F) NHDF probed with antibodies against core focal adhesion proteins. Probing with monoclonal α-tubulin antibody confirmed equal loading of WCLs. (D) Integrin surface expression levels of control and shPPM1F NHDF were analyzed by flow cytometry. Cells were stained with the indicated monoclonal integrin-specific antibodies. As a comparison, cells were stained the fluorescent labeled second antibody only (second Ab), or remained unstained; count ≥10,000 cells. (E) FilaminA was depleted in NHDF with a shRNA encoding lentivirus. WCLs were analyzed by WB with α-filaminA (upper panel) and α-tubulin (lower panel) antibodies. (F) FilaminA-depleted NHDFs were seeded for 1.5 h onto 1 µg/ml FNIII9-11-coated coverslips. After fixation, cells were stained with indicated antibodies and analyzed by confocal microscopy; scale bar, 20 µm. Insets: Higher magnification of boxed areas; arrowheads point to enrichment of active integrin β1 and filaminA; scale bar, 10 µm. (G) Integrin β1 was depleted in NHDF with a shRNA encoding lentivirus. WCLs were analyzed by WB with α-integrin β1 (upper panel) and α-tubulin (lower panel) antibodies. Control and integrin β1-depleted NHDF were seeded for 1.5 h onto 1 µg/ml FNIII9-11-coated coverslips. After fixation, cells were stained with a monoclonal antibody against active integrin β1 (9EG7) and phalloidin. Additional samples were stained with the second antibody only; scale bar, 20 µm.
Figure S3. Related to Fig. 4: PPM1F KO in A172 cells does not increase integrin surface levels or alter expression of core focal adhesion proteins, but strongly affects integrin-dependent processes. (A) A172 cells expressing Cerulean (WT) were treated with sgRNA against Cerulean combined with Cas9 or with sgRNAs against Cerulean and PPM1F combined with Cas9. Clonal Cerulean-negative (Control) and clonal Cerulean/PPM1F-negative cell lines (hPPM1F KO) were derived. The hPPM1F KO cells were stably transduced with mKate2 encoding lentivirus (hPPM1F KO plus mKate2), or lentivirus encoding hPPM1F WT (PPM1F KO plus hPPM1F-mKate2), or lentivirus encoding PPM1F D360A (PPM1F KO plus hPPM1F DA-mKate2). WCL from the different cell lines were analyzed by WB with antibodies against indicated core focal adhesion proteins. Monoclonal α-tubulin antibody was used as loading control. (B) A172 cell lines as in A were analyzed by flow cytometry for surface expression levels of indicated integrins by staining with integrin-specific antibodies. IgG control: WT cells receiving an isotype-matched control antibody. In the case of integrin β3, NIH3T3 cells served as positive controls. Count ≥10,000 cells. WT, hPPM1F-mKate2; DA, hPPM1F D360A-mKate2. (C) A172 WT, control, or hPPM1F KO cells were seeded onto 0.4 or 2 µg/ml FNIII9-12 for 30 min in triplicate. Cells were washed, fixed, and stained with crystal violet. Crystal violet staining was quantified and normalized to A172 WT cells. Bars represent mean ± SEM from three independent experiments. Significance was calculated using one-way ANOVA, followed by Bonferroni post hoc test (*, P < 0.05). For cell adhesion on 10 µg/ml FN, see Fig. 2 H. (D) A172 WT, control, or PPM1F KO cells were transiently transfected with GFP-talin-1 before seeding onto 2 µg/ml FNIII9-11 for 1.5 h. Fixed cells were stained with antibodies against active integrin β1 (9EG7) and analyzed by confocal microscopy. Scale bars, 10 µm. Arrowheads point to clusters of active integrin β1/talin. Insets: Higher magnification of boxed area; scale bar, 5 µm. (F) Quantification of cells from E showing a peripheral active integrin belt (upper graph) or peripheral talin-1 clustering (lower graph). Data are shown in percentages of all cells analyzed by confocal microscopy; n ≥ 30 derived from two or more independent experiments. (G) A172 WT, control, or PPM1F KO cells were seeded onto 0.4 µg/ml FNIII9-12 coated coverslips (left side) or onto 10 µg/ml FNIII9-12 coated coverslips (right side) for 30 min (upper panels) or 1.5 h (lower panels) before fixation and staining with DAPI and phalloidin. Cells were analyzed by confocal microscopy. Representative pictures are shown; scale bar, 25 µm. Quantification of cell areas was done in ImageJ using a custom plugin. Cells not recognized by the plugin were manually analyzed with Leica LAS AF Lite software; n ≥ 100 cells from two or more independent experiments; box plots depict means with 95% CIs (whiskers) and outliers (dots). Significance was determined using one-way ANOVA with post hoc Bonferroni test (**, P < 0.01; ***, P < 0.001). (H) A172 hPPM1F KO and derived reconstituted cell lines were lysed and subjected to WB with α-PPM1F (upper panel) and α-tubulin (lower panel) antibodies to test for PPM1F expression levels. The two bands correspond to the hPPM1F-mKate2 fusion protein and the hPPM1F protein, which results from proteolytic separation of the mKate tag. FN, FNIII9-12.
Figure S4. Related to Fig. 4: filaminA knock-down in A172 cells pheno-copies integrin-dependent effects of PPM1F depleted cells. (A) Cell adhesion assays were performed with indicated A172 cell lines using 10 µg/ml FNIII9-11. Shown are representative pictures of crystal violet stained wells after 20 min adhesion; scale bar, 150 µm. (B) Indicated cell lines were seeded onto 5 µg/ml FNIII9-11 for 2 h, fixed, and stained against active integrin β1 before analysis by confocal microscopy; scale bar, 10 µm. Insets show higher magnification of boxed areas. Arrowheads point to active integrin β1, which accumulates at the cell periphery in PPM1F KO and filaminA knock-down cells; scale bar, 5 µm. (C and D) Indicated cell lines were seeded onto 10 µg/ml FNIII9-11 for 30 min or 2 h, fixed, and stained with DAPI and phalloidin-Cy5. Samples were imaged using confocal microscopy. (C) Representative images from cells at indicated time points; scale bar, 25 µm. Cell spreading was quantified in D. Bars show mean with 95% CIs from two independent experiments; n > 80 cells. Statistics was performed using one-way ANOVA, followed by Bonferroni post hoc test (***, P < 0.001). (E) Model summarizing effects of filaminA knock-down on integrin activity. In filaminA knock-down cells, the balance between active and inactive integrins is shifted toward the active conformation. Talin-integrin association is increased due to the lack of the counterregulator filaminA, thereby promoting cell adhesion, reducing cell spreading and pheno-copying effects of PPM1F KO cells. FilaminA KD, filaminA knock-down; P, phospho-; shFilA, short-hairpin RNA targeting filaminA; T, threonine.

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Related to Fig. 6: PPM1F purified from 293T cells dephosphorylates the conserved T788/T789 motif in the integrin β1 cytoplasmic domain. (A) GST-tagged PPM1F or PPM1FD360A was expressed in 293 cells and affinity-purified from lysates with glutathione-coupled beads. Equal amounts of beads were subjected to WB analysis using α-GST antibody. (B) The integrin β1 derived synthetic peptide β1-pT788/pT789 was incubated with increasing amounts of cell-purified GST-PPM1F, with 200 ng GST-PPM1F D360A (PPM1F DA), or 100 ng calf intestine alkaline phosphatase (CIAP) for 1 h at 30°C. Released phosphate was detected by malachite green solution. Background values (buffer plus malachite green) were subtracted; w/o, peptide without phosphatase. Shown are mean ± SD of three independent experiments; unpaired t test, *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) β1-pT788/pT789 was incubated with 100 ng of cell-purified GST-PPM1F or GST-PPM1F DA for indicated time periods at 30°C. Released phosphate was detected as in B. (D) 50 ng PPM1F or PPM1F DA were incubated with the indicated phospho-peptides for 60 min and released phosphate measured as in B. (E) Dephosphorylation of increasing β1-pT788/pT789 peptide concentrations by 150 ng cell-purified PPM1F was analyzed after 60 min incubation at 30°C as in B. The indicated curve was obtained by direct fit of the data to the Michaelis–Menten equation, and V_max and K_m values were determined. (F) 120 ng His-tagged CaMKIIβ was incubated with recombinant GST-tagged WT β1 integrin cytoplasmic domain, the nonphosphorylatable integrin β1 TT/AA mutant, or without any substrate. As indicated, samples received calmodulin (CaM), Ca^2+, and ATP. After 60 min, reactions were stopped via addition of SDS sample buffer and subjected to WB using the indicated antibodies. Samples without kinase or ATP/Ca^2+/calmodulin served as additional controls. (G) 200 ng of the indicated recombinant phosphatases were employed in enzyme assays using the fluorogenic substrate 4-MUP. The increase in fluorescence of 4-MU was recorded over 30 min for each enzyme (green squares), while samples without phosphatase (black dots) or including the phosphatase together with a phosphatase inhibitor (orange triangles) served as controls. Depicted is a representative experiment.