The VASP-profilin1 (Pfn1) interaction is critical for efficient cell migration and is regulated by cell-substrate adhesion in a PKA-dependent manner

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Running title: PKA-mediated regulation of VASP-profilin1 interaction

Abstract: Dynamic regulation of actin cytoskeleton is an essential feature of cell motility. Action of Enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP), a family of conserved actin-elongating proteins, is an important aspect of regulation of actin cytoskeletal architecture at the leading edge that controls membrane protrusion and cell motility. In this study, we performed mutagenesis experiments in overexpression and knockdown-rescue settings to provide for the first time a direct evidence for the role of actin-binding protein profilin1 (Pfn1) in VASP-mediated regulation of cell motility. We found that VASP’s interaction with Pfn1 is promoted by cell-substrate adhesion and requires down-regulation of protein-kinase (PKA) activity. Our experimental data further suggest that PKA-mediated Ser-137 phosphorylation of Pfn1 potentially negatively regulates the Pfn1-VASP interaction. Finally, Pfn1’s ability to be phosphorylated on Ser-137 residue was partly responsible for the anti-migratory action elicited by exposing cells to a cAMP/PKA agonist. On the basis of these findings, we propose a mechanism of adhesion-protrusion coupling in cell motility that involves dynamic regulation of Pfn1 by PKA activity.

Keywords: Profilin1 (PFN1), vasodilator-stimulated phosphoprotein (VASP), Mena, cell migration, protein kinase A (PKA), phosphorylation, cytoskeleton, adhesion, kinase signaling

INTRODUCTION

Cell motility plays an important role in both physiological and pathological processes ranging from embryonic development to angiogenesis and tumor metastasis (1). It is a highly orchestrated event that can be summarized as a cycle of four fundamental steps: i) membrane protrusion at the leading edge driven by actin polymerization, ii) stabilization of protrusion through integrin-mediated cell-matrix adhesion, iii) cell-body translocation driven by actomyosin contractile force, and finally, iv) rear detachment as a result of the mechanical action of contractile force and/or proteolysis of cell-matrix adhesion components (2). Many of these processes involve dynamic remodeling of the actin cytoskeleton relying on both transcriptional and functional regulation of important structural and regulatory components of the actin cytoskeletal system (3).
Actin nucleators and elongators are major molecular workforces in shaping up the branched actin filament network at the leading edge that lead to lamellipodial protrusion and initiation of cell migration (4). Ena (enabled)/VASP (vasodilator-stimulated phosphoprotein) belong to one such family of actin-binding proteins that consist of three members including Mena (mammalian homolog of Ena), VASP and Evl (Ena/VASP like). These proteins associate with the branched ends of actin filaments and elongate Arp2/3-complex-nucleated actin filaments to drive membrane protrusion (5). Ena/VASP proteins also cooperate with molecular component of WAVE complex to promote Rac-dependent activation of Arp2/3 complex and actin polymerization (6). These proteins localize at the sites of dynamic actin reorganization (e.g. edge of the lamellipodia, filopodial tip) and focal adhesions in motile cells (7) (8,9). All members of Ena/VASP proteins share conserved domain structures. The amino-terminal EVH1 (Ena/VASP homology 1) domain binds to focal adhesion (e.g. vinculin, zyxin) (10) and membrane-associated proteins (e.g. lamellipodin) (11) allowing Ena/VASP to be recruited to specific cellular locations. The central polyproline (PLP) domain enables Ena/VASP to interact with certain SH3-domain bearing proteins (Src, Abl) and profilin (Pfn: a family of G-actin-binding protein and a prominent nucleotide exchange factor of actin that inhibits spontaneous nucleation of actin but promotes barbed-end-directed actin polymerization (12)) (7). The carboxy-terminal EVH2 domain has a G-actin binding site, an F-actin binding region (these interactions are essential for Ena/VASP-driven actin polymerization) and a coiled-coil region (mediates tetramerization of Ena/VASP and in turn allows bundling of actin filaments) (13-15).

Loss of Ena/VASP function inhibits multiple actin-dependent processes including axonal guidance (16-18) and intracellular propulsion of bacterial pathogens (a molecular mimicry of membrane protrusion) (19), and higher Ena/VASP activity at the leading edge positively correlates with the speed of membrane protrusion of motile cells (20,21). Although Ena/VASP proteins promote 3D invasive migration of breast cancer cells (22,23) (an exception to this is Evl which inhibits invasiveness of breast cancer cells (24,25)), the effect of Ena/VASP perturbation on 2D cell motility has been context-specific. Knockout and knockdown of VASP inhibited 2D migration of murine cardiac fibroblasts (26) and MCF7 breast cancer cells (27), respectively. In contrast, random 2D motility of mouse embryonic fibroblast (MEF) was found to be actually enhanced in the absence of Ena/VASP activity (28). The apparent paradox of faster 2D motility of MEF in Ena/VASP-devoid condition was attributed to Ena/VASP’s anti-capping action. Specifically, by displacing capping protein from the barbed end of actin filaments, Ena/VASP activity results in longer actin filaments and faster membrane protrusion, but these protrusions tend to be unstable (as longer actin filaments are prone to bucking) leading to low persistence of protrusion and unproductive global cell motility (29,30). Relevant to protrusion, an intact PLP domain of VASP is necessary for efficient actin polymerization-driven intracellular motility of bacterial pathogens (19). In fact, the rate of actin assembly by VASP is dramatically enhanced by its PLP interaction with Pfn1 (the major isoform of Pfn and a key promoter of membrane protrusion) in vitro (29,31). These findings are also consistent with enriched Pfn1-VASP interaction at the leading edge of motile cells (32).
Surprisingly however, PLP interaction of VASP was found to be dispensable for whole cell motility at least in the case of MEF (33). Specifically, this study showed that re-expression of VASP in Ena/VASP-null fibroblasts reduced the overall speed of cell motility, and this effect required an intact EVH2 but not the PLP domain of VASP (33). Although the underlying reasons for this discrepancy are not clear, a simple explanation could be that whole cell motility is more complex than membrane protrusion alone. Alternatively, the dispensable nature of PLP interaction of VASP in cell motility could be cell-type specific. Another potential issue could be that since VASP also interacts with multiple SH3- and WW-domain proteins using its PLP domain, deletion of the entire PLP domain of VASP is not specific for selectively interfering with its interaction with Pfn1. Therefore, the significance of VASP:Pfn1 interaction in cell motility is yet to be conclusively resolved.

In this study, we for the first time directly demonstrate that VASP regulates cell motility through its interaction with Pfn1, and this interaction is regulated by cell-adhesion in a protein-kinase A (PKA)-dependent manner that likely involves phosphorylation of Pfn1 on its Ser-137 residue.

RESULTS

Ena/VASP modulates cell motility through its interaction with Pfn1

VASP contains three distinct PLP regions: a single GPPPPP (GP₅) site within amino acids (aa) 116-135, a repeat of three GP₅ sites within aa 160-194, and a 2⁰²GPPPAPPLP²¹⁰ site (the aa numbers correspond to the human VASP sequence). A previous X-ray crystallography study on VASP suggested that the last GPPPAPPLP segment of VASP has a nearly 10-fold higher binding affinity for Pfn1 compared to GP₅ sites, and L209 in this segment makes a critical hydrophobic interaction with Y6 residue of Pfn1 (34). To selectively impair VASP:Pfn1 interaction without causing any major structural perturbation of VASP, we set out an initial strategy to introduce a glutamic acid (E) substitution on the third proline residue in each of the first four GP₅ clusters and L209 in the last PLP segment in a cumulative manner. By co-immunoprecipitation assay in HEK-293 cells, we confirmed that P119E mutation in the first GP5 region superimposed with L209E mutation in the loading region was sufficient to drastically reduce (by ~80%) VASP’s binding to Pfn1 (Fig 1A-B), and therefore did not need to introduce any additional mutation in the other GP₅ regions. Since SH3 domain proteins typically bind to XPPXP motif, we predicted that our mutation strategy will selectively reduce VASP’s interaction with Pfn1 without affecting its binding to SH3-domain bearing proteins. As a proof-of-concept, we confirmed that P119E/L209E mutation did not reduce VASP’s interaction with Abl, a known SH3-ligand of VASP (supplementary Fig S1). Since EVH1 domain is responsible for VASP’s targeting to membrane and focal adhesions, and EVH2 domain of VASP contains binding sites for G-actin, F-actin and coiled-coiled regions (allows VASP to bundle actin-filaments), we predicted that P119E/L209E mutations in the PLP regions should not interfere with VASP’s ability to localize at the leading edge, focal adhesions and actin stress-fibers. Immunofluorescence images of murine NR6 fibroblasts (we chose these fibroblasts as these cells form robust actin stress-fibers and focal adhesions) following transient transfection of either GFP-VASP or GFP-VASP-P119E/L209E confirmed localization of both forms of GFP-VASP at all of those sites (Fig 1C).
Next, to examine whether interfering with Pfn1’s interaction has any effect on VASP’s ability to regulate actin cytoskeleton, focal adhesions and cell motility, we generated stable clones of MDA-MB-231 (MDA-231: a highly motile metastatic human breast cancer cell line) overexpressing either GFP-VASP or GFP-VASP-P119E/L209E (stable GFP expressers served as control). We estimated ~3.5 fold-overexpression of VASP in GFP-VASP cell line compared to the control GFP cells, and comparable expression levels of GFP-VASP and GFP-VASP-P119E/L209E in the respective cell lines (Figs 2A-B). Note that the expression of endogenous VASP was not affected by GFP-VASP overexpression (Fig 2A). Rhodamine-phalloidin staining of these cells revealed that overexpression of both forms of GFP-VASP increased the total F-actin content, the abundance of actin stress-fibers and F-actin at the leading edge relative to control cells (Figs 2C-F), and this is consistent with F-actin elongating and bundling functions of VASP. Interference with Pfn1’s interaction did not affect VASP-induced increase in total F-actin content. However, F-actin at the leading edge was more prominent in GFP-VASP (~2-fold increase over control) than GFP-VASP-P119E/L209E expressers (~1.5-fold increase over control) suggesting that VASP:Pfn1 interaction promotes actin polymerization at the leading edge. There was only marginal difference in actin stress-fiber density between the two VASP cell lines. Immunostaining for vinculin (a marker for focal adhesions) also revealed an increase in both abundance and the average area of focal adhesions upon overexpression of either VASP or P119E/L209E-VASP relative to control cells but there was no statistically significant difference in either of those focal adhesion parameters between the two VASP cell lines (supplementary Fig S2). Therefore, VASP:Pfn1 interaction does not appear to play a role in focal adhesion formation.

We next performed random 2D cell motility assays with MDA-231 cells in both stable and transient overexpression settings of GFP-VASP and GFP-VASP-P119E/L209E using GFP-transfectants as control. First, in stable transfection settings, overexpression of GFP-VASP resulted in a 50% reduction in the average speed of MDA-231 cells (in terms of both average speed and the net distance traveled), and this effect was completely mitigated upon P119E/L209E substitution (Fig 3A-B; Supplementary movies M1-M3 show representative random motility of GFP, GFP-VASP and GFP-VASP-P119E/L209E expressers). From the time-lapse images, we also calculated the change in the direction of centroid movement (Δθ) as a function of time and further computed the standard deviation of Δθ (a higher value indicates larger fluctuation in the directionality of movement) as we had done before (35). VASP overexpression resulted in larger standard deviation of Δθ (consistent with frequent turning movement of these cells – a feature that is counterproductive for efficient cell motility) when compared to control cells, and this effect was abolished upon the P119E/L209E substitution suggesting that VASP’s negative effect on directional persistence is dependent on its interaction with Pfn1 (Fig 3C). Our overall motility results obtained with stable overexpressers of VASP were further confirmed in transient overexpression settings (represent mixed pool of cells with varying degrees of VASP overexpression) which also revealed slower migration of MDA-231 cells upon overexpression of VASP and reversal of this effect upon P119E/L209E substitution (supplementary Fig S3A). Based on these overexpression results, we suspected that inhibition of cell motility upon VASP
overexpression would be less pronounced in a background of Pfn1 that is unable to interact with PLP ligands. To test this, we transiently silenced endogenous Pfn1 in stable GFP and GFP-VASP expressers by siRNA treatment resulting in mean knockdown efficiency of Pfn1 to be ~90% (supplementary Figs S3B-C) and then rescuing with siRNA-resistant forms of either WT or PLP-binding deficient H133S mutant of Pfn1 (36) as CFP-tagged proteins. Motility analyses of CFP-positive cells (which displayed a range of CFP-Pfn1 expression in transfected cells as judged by their fluorescence intensity) revealed that in WT-Pfn1 rescued background, stable GFP-VASP overexpression led to ~50% reduction in the average cell speed of MDA-231 cells (supplementary Fig S3D). However, in a PLP-binding deficient Pfn1 background (H133S-Pfn1), VASP-induced suppression of cell motility (now by only ~20%) is significantly reduced. Note that the slight 20% reduction in the speed of GFP-VASP expressers in H133S-Pfn1 compared to WT-Pfn1 background is not surprising since H133S substitution disrupts Pfn1’s bindings to all PLP-domain containing proteins including other important actin cytoskeletal regulators (e.g. formins). Finally, to compare the effects of deletion of the entire PLP domain vs selective interference of Pfn1’s interaction of VASP-induced changes in cell motility, we analyzed MDA-231 cell motility in stable overexpression settings of either GFP-VASP or PLP-deleted form of GFP-VASP (denoted as GFP-VASP-ΔPRO as previously described (37)) or GFP (control). By immunoblot analyses, we confirmed comparable expression levels of GFP-VASP and GFP-VASP-ΔPRO (by ~3.5 fold) in the respective cell lines (Fig 3D). Conforming to our earlier results, these experiments also showed the general feature of partial reversal of VASP-induced inhibition of cell motility in the absence of its PLP domain (50% vs 20% reduction of speed in GFP-VASP vs GFP-VASP-ΔPRO overexpression settings, respectively) (Fig 3E). Persistence analyses also revealed that control GFP expressers had better directional persistence (whether calculated based on time or standard deviation of Δθ – only time-based data is shown here) than the other two cell lines (Fig 3F). Although GFP-VASP-ΔPRO overexpressers showed a trend of better directional persistence than GFP-VASP overexpressers, the difference was not statistically significant (unlike the difference between GFP-VASP and GFP-VASP-P119E/L209E expressers). These minor differences between our results in ΔPRO and P119E/L209E settings of VASP were not surprising given that ΔPRO-VASP is not only deficient in its interaction with Pfn1 but also other SH3 domain proteins some of which might play a role in influencing directional persistence of motility. In summary, our results from all of these overexpression studies demonstrate that VASP elevation inhibits cell motility utilizing its PLP domain interaction with Pfn1.

To further determine whether VASP:Pfn1 interaction plays a role in regulating membrane protrusion dynamics, we performed kymography analyses of membrane protrusions of GFP, GFP-VASP and GFP-VASP-P119E/L209E overexpressers of MDA-231 cells (Fig 4A shows representative kymography scans of these different groups of cells). The most prominent changes induced by overexpression of fully functional VASP were increased membrane ruffling (a feature that is counterproductive for cell motility and represented by dark lines in kymography scans [Fig 4A]) and ~20% higher protrusion frequency (Fig 4B) than the other two cell lines. Because of the
natural heterogeneity of membrane protrusions, we performed a histogram analysis of protrusion distance, which revealed that compared to either GFP or GFP-VASP-P119E/L209E expresser, GFP-VASP expressers displayed a somewhat higher % of protrusion events biased toward lower range of protrusion distance (Fig 4C). Collectively, these data demonstrate that a) hyperactivity of VASP increases the propensity but decreases the stability of membrane protrusions (as judged by prominent ruffling) and b) VASP regulates protrusion dynamics through its interaction with Pfn1.

As a complementary strategy to our overexpression experiments, we also evaluated the effect of downregulation of Ena/VASP activity on MDA-231 cell motility by selective knockdown of either VASP or Mena or both (as control, cells were transfected with non-targeting control siRNA). We achieved ~90% mean reduction of Mena and VASP expression in MDA-231 cells 72 hours after the respective siRNA transfection (when both siRNAs were used, the mean knockdown efficiency of Mena and VASP was >=80%) (Figs 5A-B). Phenotypic analyses revealed that a significant fraction (~50%) of MDA-231 cells subjected to dual knockdown of Mena and VASP exhibited a highly elongated morphology lacking the characteristic well-spread lamellipodial structure. This phenotype was also seen in cells subjected to either VASP or Mena knockdown (more prominently in the latter), but not as pronounced as seen in cells where expressions of both Mena and VASP were silenced (Figs 5C-D). These observations are consistent with Ena/VASP’s critical importance in lamellipodial protrusion as previously revealed in other cell types (11,21,30,38). When analyzed for random motility, VASP knockdown alone had no statistically significant effect on the average speed of randomly migrating MDA-231 cells (Fig 5E). Silencing Mena alone reduced the average speed by about 15% (p<0.05). Although dual knockdown of Mena and VASP induced a strong morphological phenotype, surprisingly, the average speed decreased by only 25% (p<0.01) thus suggesting a rather modest inhibitory effect of Mena/VASP knockdown on the overall speed of MDA-231 cells (the possible reasons are discussed later). Next, we performed rescue experiments where we co-silenced endogenous Mena and VASP expressions in MDA-231 cells (since VASP knockdown alone had no discernible effect on overall cell motility) and then rescued by transient transfection of either GFP-VASP or GFP-VASP-P119E/L209E-encoding plasmids. Use of an siRNA that targets the 3’-UTR of VASP avoided siRNA-mediated targeting of exogenous VASP constructs. We achieved 90% or higher efficiency of Mena and VASP knockdown (Fig 5F). As additional groups, cells transfected with either control siRNA or Mena/VASP knockdown cells were transfected with GFP plasmid. We observed ~40% slower migration of MDA-231 cells when Mena and VASP expressions were simultaneously downregulated (see supplementary movies M4-M5), and this overall motility phenotype was rescued by ectopically expressed GFP-VASP but not GFP-VASP-P119E/L209E (Fig 5G). These data provide further evidence that VASP regulates cell migration through its interaction with Pfn1.

Furthermore, qualitative similarity between the overall effects of Mena/VASP knockdown and VASP overexpression on MDA-231 cell motility suggests that an optimum level of Ena/VASP activity promotes migration of MDA-231 cells.

**VASP:Pfn1 interaction is regulated by cell-cell adhesion in a PKA-dependent manner**
Ena/VASP proteins are prominently regulated by serine/threonine phosphorylation at multiple sites (e.g. Ser 157, Ser 239, Thr 278 for VASP) by the actions of cAMP- and cGMP-dependent protein kinases including PKA and protein kinase G (PKG) (29,39,40). Phosphorylations of VASP on S239 and T278 residues in its EVH2 domain reduce its affinity to G-actin while phosphorylation of S157 in the PLP domain of VASP abrogates its interactions with various SH3-domain bearing proteins (Abl, Src) (29,41). Interestingly, S157 phosphorylation does not affect VASP’s intrinsic binding to Pfn1 at least in a purified protein mixture setting (39). Therefore, whether and how VASP’s interaction with Pfn1 is regulated in cells is unclear. We next performed a series of biochemical experiments to obtain insight into what cellular pathway could potentially regulate VASP:Pfn1 interaction in cells. We chose HEK-293 cells for these biochemical experiments mainly due to high efficiency of transfection of this cell line. An important insight came from experiments where we examined VASP’s interaction with ectopically expressed myc-Pfn1 in HEK-293 cells in attached vs suspended states. Specifically, co-immunoprecipitation experiments revealed that VASP’s interaction with myc-Pfn1 is dramatically reduced when cells were switched from adherent to suspended conditions (Figs 6A-B). Conversely, VASP’s interaction with myc-Pfn1 was prominently upregulated when cell culture condition was changed from a low adhesive to a high-adhesive condition (created by increasing the coating density of collagen-I on tissue-culture plate before seeding the cells) (Figs 6C-D). PKA activity is modulated by cell-substrate adhesion (specifically, cell detachment promotes PKA activity) (41). VASP phosphorylation (marked by a phosphatase-sensitive electrophoretic mobility shift) is a characteristic signature of PKA activation, which we also observed in HEK-293 cells upon their detachment from the underlying substrate (Fig 6E). Furthermore, cell-detachment induced downregulation of VASP:Pfn1 interaction was completely blocked when cells were pretreated with H89, a pharmacological inhibitor of PKA (Fig 6F-G), suggesting PKA’s involvement in cell-adhesion-dependent modulation of VASP:Pfn1 interaction. Consistent with these findings, acute treatment of cells with forskolin (FSK: a cAMP agonist and a potent activator of PKA) (42) also dramatically reduced VASP:Pfn1 interaction in cells (Fig 6H-I). PKA activation was confirmed by the characteristic electrophoretic mobility shift of VASP associated with its phosphorylation in response to FSK treatment (Fig 6H). Although S157 phosphorylation has no effect on the intrinsic VASP:Pfn1 binding in vitro (39), since S157 phosphorylation promotes membrane localization of VASP (43), one cannot totally exclude the possibility of PKA-induced changes in the localization of VASP indirectly affecting its interaction with Pfn1. Therefore, we performed co-immunoprecipitation experiments to assess myc-Pfn1 binding to various forms (WT or phosphodead (S157A) or phosphomimetic (S157E)) of GFP-VASP in HEK-293. Consistent with the previously reported findings of in vitro binding studies (39), we also found no effect of S157E substitution of VASP on its interaction with Pfn1 (Fig 6J). Collectively, these results demonstrate that VASP:Pfn1 interaction can be negatively regulated by loss of cell-substrate adhesion through PKA activation but at least not involving S157 phosphorylation of VASP.

S137 phosphorylation of Pfn1 plays a role in PKA-dependent regulation of VASP:Pfn1 interaction and cell motility.
We and others previously demonstrated increased phosphorylation of Pfn1 in cells in response to FSK treatment in a PKA-dependent manner (44,45). We further showed that Pfn1 is a direct phosphorylation substrate of PKA and identified several PKA phosphorylation sites of Pfn1 by mass-spectrometry (S56/S57, T89, S91/T92) (44). Phosphorylation of S57 or S91/T92 does have any effect on Pfn1’s ability to interact with either actin or PLP ligands (such as VASP) while T89 phosphorylation elicits changes in Pfn1 that are hallmarks of proteins folded into alternative three-dimensional conformations including detergent insolubility, protein aggregation and accelerated proteolysis. In silico analyses predicted that T89 phosphorylation is likely to enhance Pfn1’s interaction with actin. However, since protein coverage was incomplete in our previous study, we could not rule the possibility of additional phosphorylation sites of Pfn1 by PKA. Two bioinformatics programs (NetphosK, Kinasephos) predicted that PKA can potentially phosphorylate Pfn1 on S137, a residue that can be also phosphorylated by other AGC kinases (e.g. PKC, ROCK) leading to reduced Pfn1’s PLP interaction (46,47). In fact, among all the predicted phosphorylation sites, S137 residue has the maximum likelihood of phosphorylation of Pfn1 by PKA. Therefore, we further explored whether PKA-mediated S137 phosphorylation of Pfn1 could be a potential mechanism of negative regulation of Pfn1:VASP interaction. Although Pfn1 does not have the consensus PKA phosphorylation RRXS/T motif, it has an RRS motif involving S137 residue. First, to test whether PKA can phosphorylate Pfn1 on S137, we performed an in vitro kinase assay where recombinant GST-tagged Pfn1 or GST (control) were treated with the catalytic subunit of PKA and the kinase assay products were analyzed by immunoblotting with a phospho-PKA substrate antibody (recognizes the RRX(S/T)p motif). Immunoreactivity of the kinase assay product of GST-Pfn1 but not GST with phospho-PKA substrate antibody confirmed that PKA is capable of phosphorylating GST-Pfn1 (Fig 7A). Substitution of S137 residue of Pfn1 by non-phosphorylatable alanine (S137A) dramatically abrogated PKA’s ability to phosphorylate Pfn1 in vitro as demonstrated by loss of immunoreactivity of phospho-RRXS antibody of the kinase assay product (Fig 7B). We also performed a similar in vitro PKA phosphorylation assay with membrane-immobilized 13-mer C-terminal peptide of Pfn1 (CYEMASHLRRSQY – this peptide is restricted to only one consensus site [S137] of PKA phosphorylation), which also showed evidence of PKA-induced phosphorylation of the peptide (Fig 7C). These data are consistent with the notion that S137 is a potential PKA phosphorylation site of Pfn1. Unfortunately, we were unable to confirm S137 as an in vivo PKA phosphorylation site of Pfn1 by mass-spectrometry of immunoprecipitated Pfn1 from FSK-treated cell lysate, possibly owing to low abundance of S137 phosphorylated form of Pfn1 (note that a direct in vivo demonstration of S137 phosphorylation of Pfn1 by mass-spectrometry is lacking in the literature to date).

Next, we performed immunoprecipitation experiments in HEK-293 cells to examine VASP’s binding to either wild-type (WT) or S137A (phosphodead) or S137E (phosphomimetic) variants of myc-Pfn1, which showed that Pfn1:VASP interaction is almost abolished upon S137E substitution of Pfn1 (Fig 7D-E). Interestingly, S137A substitution of Pfn1 (which prevents Pfn1’s ability to be phosphorylated on S137 residue) also
reduced the basal Pfn1:VASP interaction in these cells to some extent but the effect was not as dramatic as seen for the S137E mutant of Pfn1 (possible reasons and the implication of these results are discussed later). However, actin binding of Pfn1 was not affected by any of these mutations (Fig 7F), suggesting that S137 residue is not involved in actin interaction of Pfn1. Next, when we compared FSK-induced changes in VASP’s interaction with WT- vs S137A-Pfn1, we found that FSK-induced attenuation of VASP:Pfn1 interaction was much more pronounced for WT-Pfn1 (~76%) than S137A-Pfn1 (~32%) (these numbers are based on the average of 2 experiments) (Fig 7G). Collectively, these data are consistent with a scenario that S137 phosphorylation of Pfn1 is at least partially involved in PKA-dependent negative regulation of Pfn1:VASP interaction.

Finally, to determine whether S137 phosphorylation of Pfn1 has any relevance in cell motility response to PKA agonist, we assessed MDA-231 cell motility following acute stimulation of FSK (DMSO-treated cells served as control). In these experiments, cells were subjected to endogenous Pfn1 knockdown and rescued with either WT or non-phosphorylatable S137A forms of myc-Pfn1 by transient transfection (these constructs also contained an IRES-GFP-coding region). Analyses of time-lapse images of GFP-positive cells revealed that FSK stimulation caused a prominent 40% reduction in the speed of cells expressing WT-Pfn1. In the basal state, S137A-Pfn1 expressers exhibited a trend of slightly slower speed compared to the WT-Pfn1 expressers although the difference was not statistically significant; importantly however, FSK stimulation did not reduce the speed of S137A-Pfn1 expressers any further (Fig 7H). These data imply that S137 phosphorylation of Pfn1 may play a role in PKA-dependent modulation of cell motility.

DISCUSSION

Ena/VASP proteins are major players in regulating actin cytoskeletal geometry, membrane protrusion and cell motility (10,18,19,21,28,30). Based on evidence that Pfn1 profoundly enhances the F-actin elongating capability of Ena/VASP (29), and that both Pfn1 and the PLP region of VASP are required for efficient actin-driven intracellular motility of bacterial pathogens (19,29), it has been widely postulated that Ena/VASP’s interaction with Pfn1 plays a role in membrane protrusion and cell motility. However, a direct experimental proof of this postulate is still lacking in the literature. In fact, a previous study in fibroblasts that utilized a deletion mutant of VASP lacking the entire PLP domain indicated that PLP-domain interactions of VASP may be dispensable for cell motility (33). As PLP domain deletion also abrogates VASP’s interaction with SH3- and WW-domain proteins, we introduced minimal point mutations in the PLP region of VASP to query the effect of specific downregulation of VASP:Pfn1 interactions on VASP’s ability to modulate actin cytoskeleton, focal adhesions, membrane protrusion dynamics and cell motility using either overexpression or knockdown or knockdown/rescue strategies. Our studies in overexpression settings showed that Pfn1’s interaction plays a role in VASP-stimulated actin polymerization at the leading edge of migrating cells. Clearly, these data provide a functional relevance of previously reported enriched Pfn1:VASP interaction at the leading edge of motile cells including MDA-231 cells (32) (48). We further showed that VASP stimulates focal adhesion formation but this does not require Pfn1’s interaction. This is not surprising given that VASP’s targeting to focal adhesion is dependent on
its EVH1 rather than PLP domains. Our kymography studies also provide for the first time a direct evidence of VASP’s ability to modulate membrane protrusion dynamics utilizing its interaction with Pfn1. We showed that VASP overexpression stimulated protrusion frequency and ruffling (indicator of unstable protrusions – a feature that has been previously related to anti-capping activity of Ena/VASP (29,30)), and these effects are mitigated upon interference of its interaction with Pfn1. These protrusion features are consistent with reduced directional persistence of protrusion and slower overall speed of migration of MDA-231 breast cancer cells upon VASP overexpression (similar to anti-migratory effect of VASP in fibroblasts (28)), and reversal of motility-related phenotypes when Pfn1 binding capability of VASP was downregulated. Similar results were also obtained when the general PLP-ligand interaction capability of Pfn1 was abrogated, although the phenotypic reversion in this case was not complete - this was not unexpected as Pfn1 also interacts with many other PLP domain proteins besides VASP.

Our motility results in three different settings of Ena/VASP perturbation including VASP overexpression (represents artificially induced highest Ena/VASP activity), Mena/VASP knockdown (lowest Ena/VASP activity) and Mena/VASP knockdown with VASP rescue (modest Ena/VASP activity) suggest that either too high or too low Ena/VASP activity can be counterproductive for cell migration. A few points are worth discussing in this context. First, given our finding that Pfn1’s interaction was critical for VASP’s ability to rescue the motility defect of Mena/VASP-depleted MDA-231 cells clearly suggest that there is likely a concentration range of Ena/VASP for which Ena/VASP promotes cell migration through its interaction with Pfn1. Second, slower MDA-231 cell migration upon Mena/VASP depletion as shown here contrasts the previous finding of increased motility of MEF in Mena/VASP-depleted condition (28). This suggest that effect of loss-of-function of Mena/VASP on cell motility can be cell-type specific. Third, although the effect of Mena/VASP knockdown on MDA-231 cell morphology was striking, the overall motility phenotype was much less severe (resulted in 25-40% reduction in overall speed). There could be several possible explanations. For example, the effect of Mena/VASP knockdown could be partly mitigated by a compensatory action of Evl, the third member of the Ena/VASP protein family. We think it is unlikely because a) Evl has an anti-migratory effect on breast cancer cell motility (this action is related to Evl-induced stimulation of contractility and suppression of membrane protrusion utilizing its interaction with Pfn2 (the minor isoform of Pfn) (25), and b) we previously showed that inhibiting the action of all members of Ena/VASP proteins by mitochondrial sequestration had no effect on the overall speed of MDA-231 cells (which may also indirectly suggest opposite effects of Mena/VASP and Evl on breast cancer cell motility) (35). Second, since various members of Ena/VASP proteins have shared ligands but with different affinities (13) (25), it is possible that in the near absence of Mena/VASP, Evl might promote cell motility through its interactions with certain ligands that are otherwise preferred for Mena and/or VASP when all three members are present. Although membrane protrusion is an obligatory step for cell migration, random protrusions in all directions is counterproductive for cell migration. Therefore, another simple explanation could be that by reducing the overall flare of protrusion (including lateral protrusions), Mena/VASP depletion could enhance the polarized feature of cells (a feature that
promotes cell motility), and partly offset the protrusion-related motility defect.

Although Pfn1 is an important ligand for VASP, what biochemical pathways regulate Ena/VASP:Pfn1 interaction in cells are not known. In this study, we demonstrate for the first time that VASP:Pfn1 interaction is negatively regulated by PKA most likely through PKA-mediated S137 phosphorylation of Pfn1. Our kinase assay data showing 1) PKA’s ability to phosphorylate Pfn1 as well as C-terminal peptide of Pfn1 that only contains a single PKA consensus site of phosphorylation (S137), and importantly, 2) abrogation of PKA-mediated Pfn1 phosphorylation upon S137A substitution, provide a reasonable in vitro evidence of PKA-mediated S137 phosphorylation of Pfn1. However, since we do not have a direct mass-spectrometry-based evidence of S137 phosphorylation of Pfn1 in FSK-treated cells, we acknowledge that our results are suggestive of but not definitive with regard to S137 phosphorylation of Pfn1 being responsible for PKA-dependent negative regulation of Pfn1:VASP interaction. In fact, our experiments showed that the basal VASP:Pfn1 interaction is somewhat lower for S137A-Pfn1 compared to WT Pfn1, and furthermore, S137A substitution does not completely prevent FSK-induced downregulation of Pfn1:VASP interaction in cells. One possibility is that S137A substitution may open Pfn1 to phosphorylation of some other neighboring residues that can also reduce Pfn1’s affinity for PLP ligands. Future studies are needed to address these issues.

Another key finding of this study is the regulation of Pfn1:VASP interaction by cell-substrate adhesion through PKA. These results open the possibility of a new mechanistic model of adhesion-protrusion coupling during cell migration involving cycles of PKA activity as schematized in Fig 8. Specifically, we propose that stabilization of membrane protrusions through adhesions to the underlying substrate triggers downregulation of PKA activity and reduced phosphorylation of Pfn1 (on S137) and VASP. Since S137 phosphorylation of Pfn1 inhibits its PLP interaction with VASP and phosphorylation of VASP reduces its actin binding (29,39), downregulation of these phosphorylation events as a consequence of cell attachment could potentially stimulate VASP’s interactions with both actin and Pfn1, driving actin polymerization and a new round of membrane protrusion. Given that S137D substitution inhibits Pfn1’s interaction with huntingtin (a PLP ligand of Pfn1) (47), Pfn1’s interaction with other PLP domain-bearing actin cytoskeleton regulators besides VASP might be also sensitive to S137 phosphorylation. In fact, we have preliminary indication of reduced mDia1 (a formin family protein and a known ligand of Pfn1) binding to S137E-Pfn1 compared to S137A-Pfn1 (supplementary Fig S4). Therefore, in our model, we further propose that while the protrusion remains unattached, a local rise of PKA activity could act as a brake on VASP:Pfn1 (and/or formin:Pfn1) mediated actin assembly limiting the protrusion until new adhesions are formed.

Finally, PKA influences different facets of actin cytoskeleton-regulatory processes including modulation of activities of a) Rho-family GTPases (Rho, Rac and Cdc42), b) actin-binding proteins (e.g. VASP, myosin), and c) kinases which indirectly control the function of actin-binding proteins (e.g. p21-activated kinase) (49). PKA’s role in cell migration is complex and context-specific. There are pharmacological and genetic evidence of PKA’s negative regulation of
cell motility, and in other cellular contexts, cAMP/PKA signaling has a pro-migratory role (49-52). The present study showed that cAMP agonist FSK suppresses breast cancer cell motility, and FSK-induced inhibition of cell motility is partly dependent on Pfn1’s ability to be phosphorylated on S137 residue. As a key consequence of S137 phosphorylation of Pfn1 is downregulation of its PLP interactions (as seen with VASP, formins), our data imply that negative regulation of Pfn1’s PLP interaction could be an important mechanism of the anti-migratory action of PKA. Although this study is specifically focused on VASP, it will be interesting to determine in future studies whether Pfn1’s interaction with other PLP ligands that are relevant in the context of cell motility are also affected by PKA or other kinases activated downstream of PKA through phosphorylation of Pfn1.

**Experimental Procedures**

**Plasmid and siRNAs:** WT-Pfn1 and H133S-Pfn1 were sub-cloned into ECFP-vector at Hind3 and BamH1 sites. The S137A and S137E mutants of myc-Pfn1 (on an IRES-GFP backbone vector) were created by site-directed mutagenesis using the following primers: S137A: (sense: 5’-CTCACCTGCAGCAATGCCTACTG-3’) and S137E (sense: 5’-CTCACCTGCAGCAATGCCTACTG-3’). Pfn1 constructs were made Pfn1-siRNA-resistant by placing a silent mutation in the siRNA targeting region without changing the peptide encoding (targeting region of Pfn1-siRNA has been previously described (53)). VASP and Mena siRNAs, targeted to the untranslated region (GAGUGAAUCUGCGCGGAGA) and the open reading frame (GAGAGAGACGCGAGAACAU), respectively, were purchased from Dharmacon (Lafayette, CO). Sequential point-mutations of EGFP-VASP were performed on regulatory GP5 site P120 and loading site L210 residues using PCR-based site-directed mutagenesis. The forward and reverse primers for P120E mutant were 5’-GGA GGT GGG CCC CCT GAA CCC CCA GCA CTT CCC - 3’ and 5’-GGG AAG TGC TGG GGG TTC AGG GGG CCC ACC TCC - 3’ respectively. The forward and reverse primers for L210E mutant were 5’-CCC CTT GCA CCC CCT GAA CCG GCA GCA CAG GGC - 3’ and 5’-GCC CTG TGC TGC CGG TTC AGG GGG TGC AGG GGG - 3’ respectively. EGFP-VASP-ΔPRO (lacks the entire PLP domain of VASP) was a generous gift of Dr. Frank Gertler (MIT).

**Cell culture and transfection:** HEK-293 cells were cultured in DMEM-F12 media supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen - Carlsbad, CA). MDA-MB-231 (MDA-231) breast cancer cells were cultured in EMEM media and supplemented with 10% FBS, sodium pyruvate and antibiotics. NR6 mouse fibroblasts were cultured in alpha-MEM supplemented with 7.5% FBS, 1x non-essential amino acids, 1x L-glutamine, 1x sodium pyruvate, and antibiotics. MDA-231 cells stably transfected with EGFP or various VASP constructs using Lipofectamine 2000 reagent (Invitrogen - Carlsbad, CA) were cultured in the media described above with an additional supplement of 500 μg/ml G418. SiRNAs were transfected at a working concentration of 100 nM using a transfection reagent commercially available from Dharmacon (Lafayette, CO) following the manufacturer’s protocol. All silencing-based experiments were performed 72 hours after transfection.

**Immunoblotting:** Total lysates were prepared by extracting cells with modified RIPA buffer (25mM Tris—HCl (pH 7.5),...
150mM NaCl, 1% (v/v) NP-40, 5% (v/v) glycerol, 1mM EDTA, 50mM NaF, 1mM sodium pervanadate, and protease inhibitors). The lysates were clarified at 13,000 rpm for 10 min at 4 °C and the protein concentration was determined using a Bradford protein assay kit (Pierce; Rockford, IL). For protein electrophoresis, equal amounts of protein samples (ranged from 10 (for HEK-293) – 20 (for MDA-231) µg) were loaded on an SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% non-fat dry milk in TBST for 1 hour at room temperature, immunoblotting was performed overnight with the appropriate antibodies. After extensive washing with TBST, the blot was incubated with the appropriate secondary antibody (1:1000 dilution, Pharmingen, San Diego, CA) and washed 3 times with TBST before performing chemiluminescence for the visualization of protein bands.

**Immunostaining/phalloidin staining:** For immunostaining, cells were washed with PBS, fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 5 min and then blocked with 10% goat-serum for 1 h at room temperature. After incubating with the primary antibodies (vinculin at 1:100 dilution) for 1 h at room temperature, cells were washed two times with PBS containing 0.02% tween and 2 times with PBS and then incubated with secondary antibodies (source: Jackson Immunoresearch, West Grove, PA). Vinculin-stained cells were imaged with a 60X objective on a Nikon A1 confocal microscope. For rhodamine-phalloidin staining, cells were washed with PBS, fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 5 min and then stained with rhodamine-phalloidin following manufacturer’s protocol. Stained cells were washed two times with PBS containing 0.02% tween, two times with PBS, and then once with distilled water before mounting on slides for imaging using a 60× oil-immersion objective on an Olympus IX71 inverted microscope. Quantitative fluorescence analyses of images were performed by Metamorph software.

**Immunoprecipitation:** Cell extracts (500-100 µg) were precleared by constant mixing with Protein G Plus/Protein A-conjugated Agarose Beads (Calbiochem) at 4°C for 1 hour. Precleared lysates were incubated with the appropriate antibody overnight followed by Protein G Plus/Protein A-conjugated Agarose Beads for 2 hrs at 4°C on a rotor. The beads were washed with the lysis buffer before eluting the immunoprecipitated protein complex by boiling the beads in the presence of 2-mercaptoethanol. The eluate was directly loaded for SDS-PAGE for immunoblotting.

**In vitro Kinase assay:** Bacterial expression and purification of GST and GST-Pfn1 and the PKA kinase assay were performed as previously described (44). Kinase assay products were run on an SDS-PAGE and probed with phospho-RRXS antibody. For peptide kinase assay, different quantities of C-terminal Pfn1 peptide (CYEMASHLRRSQY – synthesized by the peptide synthesis facility at the University of Pittsburgh) was spotted on a pre-soaked (in 5% BSA TBST solution) nitrocellulose membrane and allowed to dry briefly before subjecting the membrane to PKA kinase assay as previously described (44). The membrane was washed with TBST thoroughly before probing with the phospho-RRXS antibody.

**Phosphatase assay:** After total lysate was prepared, lambda phosphatase (NEB) was added to the lysate and incubated according
to manufacturer’s protocol for 30 minutes at 30°C.

**Antibodies:** Monoclonal GFP, VASP and vimentin antibodies were obtained from Pharmingen (San Diego, CA). Polyclonal GFP antibody was obtained from Abcam (Cambridge, MA). Polyclonal Pfn1 and monoclonal Mena antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal GAPDH antibody was obtained from Abd Serotec (Raleigh, NC). Polyclonal myc (1:2000) antibody was obtained from Sigma Aldrich (St. Louis, MO). Phospho-RRXS antibody was a product of Cell Signaling (Danver, CO). Vinculin and mDia1 antibodies were obtained from Cell Signaling (Danver, CO). Monoclonal tubulin antibody was purchased through Sigma Aldrich (St. Lousi, MO). Abl antibody was kindly provided by Dr. Alan Howe (University of Vermont). The concentrations of different antibodies for immunoblotting were: GFP (1:1000), VASP (1:1000), Pfn1 (1:1000), Mena (1:200), GAPDH (1:2000), myc (1:2000), tubulin (1:3000), Abl (1:1000), mDia1(1:1000) and phospho-RRXS (1:1000).

**Single-cell migration assay**
MDA-231 cells were sparsely plated on a 35 mm plastic tissue-culture dish coated with collagen-I. After overnight incubation, time-lapse videomicroscopy of randomly migrating cells were performed simultaneously at multiple fields of observations with a 10x objective at an interval of 1 minute for a total duration of 120 minutes. Cell trajectory was built via frame-by-frame analyses of the centroid positions (x, y) of cell-nuclei. Protrusion direction was determined by creating a vector from the centroid of a cell-nuclei and the farthest point of a protrusion before protrusion is retracted. Persistence was calculated using a non-overlapping interval random walk model as previously described (54). The acquired images were analyzed using the NIH ImageJ software.

**Kymography:** Short-duration (10 min) time-lapse images of cells were recorded at a 10-sec time-interval. Kymographs marking the beginning to the end of protrusion were constructed based on 1-pixel wide (0.3 μm) lines drawn at multiple locations (3–4) across the protruding membrane. All images were acquired and analyzed using Metamorph and NIH ImageJ softwares, respectively, as previously described by us (35).

**Statistics and data representations:** All statistical tests were performed with ANOVA followed by Tukey-Kramer post-hoc test analysis. P value less than 0.05 was considered to be statistically significant. In box and whisker plots, crosses represent the mean, middle lines of the box indicates median, top of the box indicates 75th percentile, bottom of the box measures 25th percentile and the two whiskers indicate the 10th and 90th percentiles, respectively. In bar graphs, the data were represented as mean ± standard deviation.
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CONTRIBUTIONS: DG and WV were involved in design and execution of experiments and preparing the manuscript. SS was involved in intellectual contribution. PR was involved in the overall planning and execution of the study and preparation of manuscript.
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Figure Legends

Fig 1. Effect of P119E/L209E substitution on Pfn1 interaction and cellular localization of VASP. A-B) Total lysates and myc-Pfn1 immunoprecipitates of HEK-293 cells co-transfected with myc-Pfn1 and either GFP-VASP or GFP-VASP-L119E/L209E immunoblotted with GFP and myc antibodies (panel A). The bar graph in panel B summarizes the relative binding of GFP-VASP and GFP-VASP-P119E/L209E to myc-Pfn1 immunoprecipitates (N=3 experiments; **: p<0.01). C) Representative fluorescence images of NR6 fibroblasts transfected with GFP-VASP (left panel) or GFP-VASP-P119E/L209E (right panel) (scale bar - 25 µm). Thin arrows, thick arrows and arrowheads indicate VASP localization to the leading edge, focal adhesion and stress fibers, respectively.

Fig 2. Effect of stable overexpression of GFP-VASP and GFP-VASP-L119E/L209E on actin cytoskeleton in MDA-231 cells. A) GFP (left) and VASP (right) immunoblot of lysates of GFP and GFP-VASP expressers of MDA-231 cells (vimentin blot: loading control). B) Representative GFP immunoblot of lysates of MDA-231 cells stably expressing GFP, GFP-VASP, and GFP-VASP-P119E/L209E constructs (GAPDH blot: loading control). C) Representative fluorescence images (acquired with a 60X objective) of MDA-231 stably expressing GFP, GFP-VASP, or GFP-VASP-P119E/L209E and stained with DAPI (nucleus; blue) and rhodamine-phalloidin (F-actin; red). The lower most panels are zoomed insets of phalloidin images to reveal actin stress-fibers (scale bars in the upper and lower panels are 50 µm and 16 µm, respectively). D-F) Scatter plots of fluorescence intensities of rhodamine-phalloidin (D-E; panel D: total; panel E: leading edge; bar indicates the mean) and box and whisker plot summarizing stress-fiber abundance (F) of various groups of cells relative to GFP expressers of MDA-231 cells (data summarized from 25-32 cells from 2 experiments; *p<0.05; **p<0.01).

Fig 3. VASP overexpression inhibits MDA-231 cell motility through its PLP interaction of Pfn1. A-C) Box-and whisker plots summarizing the average speed (panel A), net distance (panel B) and standard deviation of Δθ (panel C) analyzed from time-lapse motility of the indicated sublines of MDA-231 cells. ‘n’ indicates the number of cells analyzed for each group pooled from 3 independent experiments (*: p<0.05; **: p<0.01). D) Representative GFP immunoblot of lysates of MDA-231 cells stably expressing GFP, GFP-VASP and GFP-VASP-ΔPRO constructs (vimentin blot: loading control). Note that the immunoblot in this panel represents the same experimental data shown in the left panel of Fig 2A with an additional lane of GFP-VASP-ΔPRO cell lysate shown alongside. E) Box and whisker plot showing the relative speed of migration of GFP, GFP-VASP and GFP-VASP-ΔPRO expressing MDA-231 cells. F) Box and whisker plot summarizing the angular persistence of motility of GFP, GFP-VASP, and GFP-VASP-ΔPRO expressers. ‘n’ indicates the number of cells analyzed for each group pooled from 3 independent experiments (*: p<0.05; **: p<0.01).

Fig 4. VASP regulates protrusion dynamics through its interaction of Pfn1. A) Representative kymograph images revealing protrusion dynamics of MDA-231 cells stably expressing either GFP or GFP-VASP or GFP-VASP-P119E/L209E (horizontal axis: time (total duration: 10 min); vertical axis: distance (saw tooth pattern indicates individual protrusion and retraction events of the membrane; ruffles are indicated by arrow). B) Box and whisker plot summarizing the protrusion frequency (number of protrusions recorded within a 10 min time
period) of different groups of cells relative to GFP expressers. C) Relative occurrence of protrusion events for different ranges of protrusion distance (as indicated in pixels) of various groups of cells. These data are based on analyses of all protrusions detected at ~3 different locations at the leading edge per cell from at least 15 cells per group (*p<0.05).

Fig 5: Effect of Ena/VASP knockdown on MDA-231 cell motility. A-B) Representative Mena, VASP and vimentin (loading control) immunoblots of lysates of MDA-231 transfected with the indicated siRNAs (panel A); bar graph in panel B shows the relative expression levels (mean +/- std. dev) of Mena and VASP for these transfection conditions (n=3 experiments). C-D) Phase contrast images comparing the morphology of MDA-231 cells for different transfection conditions (panel C: cells with elongated phenotype are indicated by arrow; bar – 20 µm); the bar graph panel D summarizes the % of cells with elongated phenotype for each of the transfection conditions. E) Box and whisker plot representing the average speed of MDA-231 cells for the various transfection conditions. In panels D and E, ‘n’ indicates the number of cells analyzed for each group pooled from 3 independent experiments (*: p<0.05; **: p<0.01). F-G) Representative Mena, VASP and GAPDH (loading control) immunoblots (panel F) of lysates of MDA-231 transfected with the indicated siRNAs in the rescue experiments (number within the parentheses indicate the knockdown efficiencies of Mena and VASP from each of the 2 experiments). Box and whisker plot (panel G) representing the average speed of MDA-231 cells for the indicated siRNA and plasmid transfection conditions. ‘N’ indicates the number of cells analyzed for each group pooled from 2 independent experiments (*: p<0.05; **: p<0.01).

Fig 6: VASP:Pfn1 interaction is regulated by cell-adhesion involving the action of PKA: A-D) Myc-Pfn1 immunoprecipitates from the lysates of HEK-293 cells expressing myc-Pfn1 in adherent vs suspended state (panel A) and low-adhesive vs high-adhesive culture conditions (panel C) probed with VASP and myc antibodies (collagen coating concentration: 10 µg/ml (low adhesive) and 50 µg/ml (high adhesive)). Panels B and D quantify the relative binding of VASP to myc-Pfn1 for the indicated experimental conditions (N=3 experiments). E) Total lysates of HEK-293 cells in attached vs detached states (+/- phosphatase treatment) were run on a 10% SDS-PAGE and probed with the VASP antibody to demonstrate detachment-induced VASP phosphorylation. The numbers in the parentheses indicate the individual phospho-VASP band intensity in phosphatase-treated relative to untreated condition from each of the two experiments with the average value indicated below. F-G) Myc-Pfn1 immunoprecipitates from HEK-293 cells expressing myc-Pfn1 from adherent vs suspended (with or without pretreatment of H89) immunoblotted with VASP and myc antibodies (panel F); the relative binding of VASP to myc-Pfn1 for these different conditions are summarized in panel G (N=3 experiment). H-I) Total cell lysates (TCL - run on a 10% SDS-PAGE to reveal VASP phosphorylation) and myc-Pfn1 immunoprecipitates (run on a 15% SDS-PAGE) of DMSO vs 50 µM FSK-treated HEK-293 cells expressing myc-Pfn1 probed with the indicated antibodies (panel H; these treatments were done for 15 min). Relative binding of VASP to myc-Pfn1 in DMSO vs FSK-treated conditions are summarized in panel I. J) Myc-Pfn1 immunoprecipitates of HEK-293 cells that were co-transfected with myc-Pfn1 and the various indicated VASP constructs (GFP-tagged) probed with GFP and myc antibodies.

Fig 7: S137 phosphorylation of Pfn1 is involved in PKA-dependent regulation of VASP:Pfn1 interaction and cell motility. A) In vitro PKA-kinase assay products of GST
(control) and GST-Pfn1 probed with pRRXS antibody (reactions without ATP or PKA served as additional controls). Coomassie staining in parallel confirms comparable loading of GST and GST-Pfn1 for the various experimental conditions. B) PKA-kinase assay products of GST-Pfn1 (WT) and GST-Pfn1-S137A probed with pRRXS antibody [coomassie staining confirms comparable loading of WT and S137 mutant forms of GST-Pfn1]. C) Dot blot of various amounts of membrane-immobilized C-terminal Pfn1 peptide treated with PKA and ATP, and probed with pRRXS antibody. D-F) Myc-Pfn1 immunoprecipitates of HEK-293 cells expressing the indicated various myc-tagged Pfn1 constructs probed with VASP (panel D), actin (panel F) and myc (panels D and F) antibodies. Panel E quantifies the relative binding of VASP to the various myc-Pfn1 constructs (N=3 experiments). G) Myc-Pfn1 immunoprecipitates from the lysates of myc-Pfn1- and myc-Pfn1-S137A-expressing HEK-293 cells following treatment with either DMSO or FSK (50 µM, 15 min) probed with VASP and myc antibodies. The numbers in the parentheses indicate relative VASP binding to the WT and S137A mutant form of myc-Pfn1 in DMSO vs FSK-treated conditions from 2 individual experiments with the mean of the two numbers indicated on the top (for any given transfection, the VASP band intensity for the corresponding DMSO treatment was used for normalization purpose). H) Box and whisker plot showing the speed of migration of MDA-231 cells following transient transfection of either myc-Pfn1 or myc-Pfn1-S137A constructs and treated with either DMSO or FSK (endogenous Pfn1 expression was silenced in these cells by siRNA treatment; ‘N’ = number of cells from 3 experiments; *: p<0.05; **: p<0.01 and NS: not significant).

Fig 8. A proposed schematic model of adhesion-protrusion coupling during cell migration:
Cycles of localized changes in PKA activity in response to deadhesion and adhesion (marked by triangles) regulates Pfn1:VASP-mediated actin (marked by ovals) polymerization and membrane protrusion through modulating Pfn1 and VASP phosphorylation (see discussion for further description). The hypothetical model also incorporates a possible scenario of Pfn1’s interaction with formin proteins being modulated by phosphorylation of Pfn1.
Gau et al. Fig 1

A) Immunoblot analysis showing the expression of GFP-VASP and myc-Pfn1 after different transfections. The bands for GFP-VASP and myc-Pfn1 are indicated.

B) Graph showing VASP: Pfn1 binding. The binding is normalized to input with a bar graph indicating the relative binding levels.

C) Images showing GFP-VASP and GFP-VASP-P119E/L209E transfected cells. Arrows indicate specific features of the transfected cells.
Gau et al. Fig 2

A) GFP-VASP and VASP blots with molecular weights (kD) of 55 and 35.

Cell line: GFP, GFP-VASP

B) GFP-VASP and GAPDH blots with molecular weights (kD) of 55 and 35.

Cell line: GFP, GFP-VASP, GFP-VASP (P119E/L209E)

C) Fluorescence images showing GFP, GFP-VASP, and GFP-VASP (P119E/L209E) in cells.

D) Bar graph showing fluorescence intensity (total) relative to GFP cells.

E) Bar graph showing fluorescence intensity (loading edge) relative to GFP cells.

F) Box plot showing stress fiber abundance relative to GFP.
Gau et al. Fig 4

A) GFP  GFP-VASP  GFP-VASP-P119E/L209E

Distance

Time

B) Protrusion frequency (relative to GFP cells)

C) Fraction of total number of protrusions

Individual protrusion distance (pixels)

GFP  GFP-VASP  GFP-VASP
P119E/L209E
Gau et al. Fig 5

A) siRNA: control VASP Mena Mena + VASP
   Mena
   VASP
   Vimentin

B) Expression (relative to control)

C) siRNA: control VASP
   Mena Mena + VASP

D) % cells with elongated phenotype

E) Average speed (relative to control)

F) Mena VASP GAPDH
   siRNA: Ctrl VASP/Mena

G) Average speed (relative to control siRNA/GFP plasmid transfectants)

plasmid: GFP GFP GFP-VASP GFP-VASP- P119E/L209E
siRNA: control Mena+VASP
Gau et al. Fig 6

A) IP: anti-myc

VASP  
myc-Pfn1

Adherent Suspended

B) VASP-Pfn1 binding (relative to adherent state)

C) IP: anti-myc

VASP  
myc-Pfn1

Collagen: coating density

D) VASP-Pfn1 binding (relative to control)

E) phospho-VASP - VASP

phosphatase:

GAPDH  

Adh Susp +

F) IP: anti-myc

VASP  
myc-Pfn1

H) 

IP: myc-Pfn1

phospho-VASP - VASP

TCL

IP: myc-Pfn1

GFP-VASP

J) IP: anti-myc

GFP-VASP: WT S157A S157E
Gau et al. Fig 7

A) ATP: - + +
   PKA: - - +
   pRRXS
   GST (coomassie)
   pRRXS
   GST-Pfn1 (coomassie)

B) (kD)
   pRRXS
   GST-Pfn1 (coomassie)
   GST-Pfn1: WT S137A

C) Pfn1 C-term peptide (ng):
   1000 500 100 10 1

D) IP: myc-Pfn1
   VASP
   Myc
   myc-Pfn1: WT S137A S137E

E) VASP/Pfn1 binding (relative to WT)
   myc-Pfn1: WT S137A S137E

F) (kD)
   Actin
   Myc
   GAPDH
   Myc-Pfn1: WT S137A S137E TL

G) (kD)
   VASP
   myc-Pfn1
   myc-Pfn1: WT S137A

H) ** NS
   NS
   ** NS
   Average speed (relative to control)
   DMSO FSK
   myc-Pfn1 (control) (10) (50)
   myc-Pfn1-S137A

n=41 n=50 n=54 n=52 n=47
Gau et al., Fig 8

Low PKA activity
Low Pfn1 phosphorylation
attachment

Increased VASP:Pfn1 and VASP:actin interactions through downregulation of site-specific phosphorylations of Pfn1 and VASP, respectively

Additional possibility: increased Pfn1:formin interaction

High PKA activity
High Pfn1 phosphorylation
detachment

Low PKA activity
Low Pfn1 phosphorylation
attachment

protrusion
The VASP-profilin1 (Pfn1) interaction is critical for efficient cell migration and is regulated by cell-substrate adhesion in a PKA-dependent manner

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