Regulation of directional cell migration by membrane-induced actin bundling

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Summary

During embryonic development and in metastatic cancers, cells detach from the epithelium and migrate with persistent directionality. Directional cell migration is also crucial for the regeneration and maintenance of the epithelium and impaired directional migration is linked to chronic inflammatory diseases. Despite its significance, the mechanisms controlling epithelial cell migration remain poorly understood. Villin is an epithelial-cell-specific actin modifying protein that regulates epithelial cell plasticity and motility. In motile cells villin is associated with the highly branched and the unbranched actin filaments of lamellipodia and filopodia, respectively. In this study we demonstrate for the first time that villin regulates directionally persistent epithelial cell migration. Functional characterization of wild-type and mutant villin proteins revealed that the ability of villin to self-associate and bundle actin as well as its direct interaction with phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] regulates villin-induced filopodial assembly and directional cell migration. Our findings suggest that convergence of different signaling cascades could spatially restrict villin activity to areas of high PtdIns(4,5)P_2 and F-actin concentration to assemble filopodia. Furthermore, our data reveal the ability of villin to undergo actin- and PtdIns(4,5)P_2-induced self-association, which may be particularly suited to coalesce and reorganize actin bundles within the filopodia.

Key words: Villin, Actin, Bundling, PtdIns(4,5)P_2, Metastasis, Epithelia, Filopodia, Cell migration

Introduction

Directional cues provided by the filopodia regulate wound healing and re-epithelialization and loss of this function is associated with serious inflammatory diseases (Schäfer and Werner, 2008). Additionally, metastatic cells have abundant filopodia and the number of filopodia correlates with their invasiveness (Machesky, 2008). Since a majority of organs in mammals are established by epithelial cells and ~90% of human cancers are derived from epithelial cells, understanding the cellular-molecular mechanism of filopodial assembly and function is of direct relevance to the understanding of not only regeneration but also metastases of the epithelium (Vignjevic et al., 2007). In epithelial cells, filopodia display other unique functions including the regulation of cell-cell interactions and the alignment of cells correctly to close the gap between epithelial sheets (Khurana and George, 2011). Filopodia also regulate the adhesion, spread and survival of bacterial and viral pathogens in host epithelial cells (Berger et al., 2009; Bulgin et al., 2010). Even though filopodia are ubiquitous actin-based structures the mechanism of filopodial assembly and their function during development, physiological stresses, injury and disease still remains largely unknown.

In this study we demonstrate for the first time, that tissue-specific actin bundling proteins like villin can assemble filopodia and regulate epithelial cell migration. *In vivo* villin is important for the reorganization of the actin cytoskeleton in response to both physiological and pathological stresses thus, playing a major role in regulating actin dynamics during changes in cellular plasticity, cell motility, cell morphogenesis and wound repair (Athman et al., 2003; Ferrary et al., 1999; Revenu et al., 2007; Tomar et al., 2006; Tomar et al., 2004). Here we provide evidence that the ability of villin to self-associate and bundle actin filaments, as well as its ability to bind phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] drives the emergence of filopodia to regulate directional cell migration. Our study highlights how a tight spatial and temporal regulation of actin dynamics and membrane reorganization by villin is crucial for the generation of cellular protrusions in epithelial cells.

Results

Actin bundling by villin regulates epithelial cell migration

Previous studies have shown that villin is a major regulator of epithelial cell migration (Athman et al., 2003; Tomar et al., 2006; Tomar et al., 2004). Villin is an actin-capping, -nucleating, -severing and -bundling protein, but how the different actin-regulating functions of villin affect cell migration remains to be characterized (Khurana, 2006; Khurana and George, 2008). In motile cells, villin localizes to the lamellipodia, filopodia and microspikes (George et al., 2007; Khurana et al., 2008). We reasoned that the ability of villin to bundle actin filaments may describe its localization in filopodia as well as its function in the assembly of filopodia. To assess the role of villin in actin bundling and in the regulation of epithelial cell migration, we monitored cells expressing SEYFP-tagged full-length villin (VIL/WT) as well as a villin mutant that fails to dimerize and therefore fails to bundle actin (VIL/Δ21–67/112–119; supplementary

material Fig. S1A) (George et al., 2007). Cell motility was measured using an *in vitro* wound assay in the absence or presence of the motogen lysophosphatidic acid (LPA; 2 μM) as described by us previously (Khurana et al., 2008; Tomar et al., 2006). VIL/NULL cells treated with LPA migrated faster than untreated cells (30±2.2%, $*P<0.001$, $n=12$; Fig. 1A). In the presence of villin, LPA treatment further enhanced cell migration (88±2.5%, $^{\#}P<0.001$, $n=12$). Cells expressing the actin bundling mutant of villin VIL/Δ21–67/112–119 migrated significantly slower than cells expressing VIL/WT ($^{\ddagger}P<0.001$, $n=12$). There was also a significant inhibition of migration in MDCK cells expressing VIL/Δ21–67/112–119 in the presence (15±1.1%, $^{\dagger}P<0.05$, $n=12$) of LPA compared to VIL/NULL cells. In contrast, there was no significant difference in the rate of cell migration in cells expressing VIL/Δ21–67/112–119 in the absence or presence of LPA. These data demonstrate that MDCK cells expressing mutant villin fail to respond to external stimuli, in this case LPA, suggesting that directional cell migration in response to external cues may be lost in villin mutant cells. We speculate that overexpression of mutant villin protein, which retains the binding properties to PtdIns(4,5)P_2 and F-actin like wild-type villin could compete with other endogenous proteins required for cell migration thus, inhibiting basal cell migration rates. Similar findings were made using a cell migration assay developed by Platypus Technologies (Kam et al., 2008). Fig. 1B shows representative wells and provides a quantitative measure of rates of migration to demonstrate that cells expressing VIL/WT migrate faster than VIL/NULL cells ($P<0.001$) and that deletion of the villin dimerization site significantly retards villin-induced cell migration ($P<0.001$ compared to VIL/WT).

Villin regulates directional cell migration

Using time-lapse microscopy we measured the migration of single VIL/NULL MDCK cells or cells expressing VIL/WT or VIL/Δ21–67/112–119. For these studies cells were plated at very low density to minimize cell-cell contact. VIL/WT cells migrated in the same direction for a sustained period of time without turning even in the absence of a chemotactic gradient, thus showing an intrinsic directionality or persistence of migration (Fig. 2A). By contrast, VIL/Δ21–67/112–119 and VIL/NULL cells exhibited a complete loss of directionality. These mutant cells exhibited unstable cell protrusive activity in multiple directions. Therefore, while the wild-type villin cells displayed a polarized morphology with an elongated polygonal shape, VIL/NULL and the mutant villin cells adopted a non-polarized, rounded shape. To quantify this loss of cell polarity, we calculated the elliptical factor (EF), the ratio between the longest and the shortest axis of the cell (EF >2 is indicative of a polarized morphology). In VIL/WT cells, the EF was significantly higher than VIL/NULL or VIL/Δ21–67/112–118 cells (VIL/WT cells had an average EF of 2.99 and mutant villin had an average EF of 1.2 and there was ~60% decrease in the EF

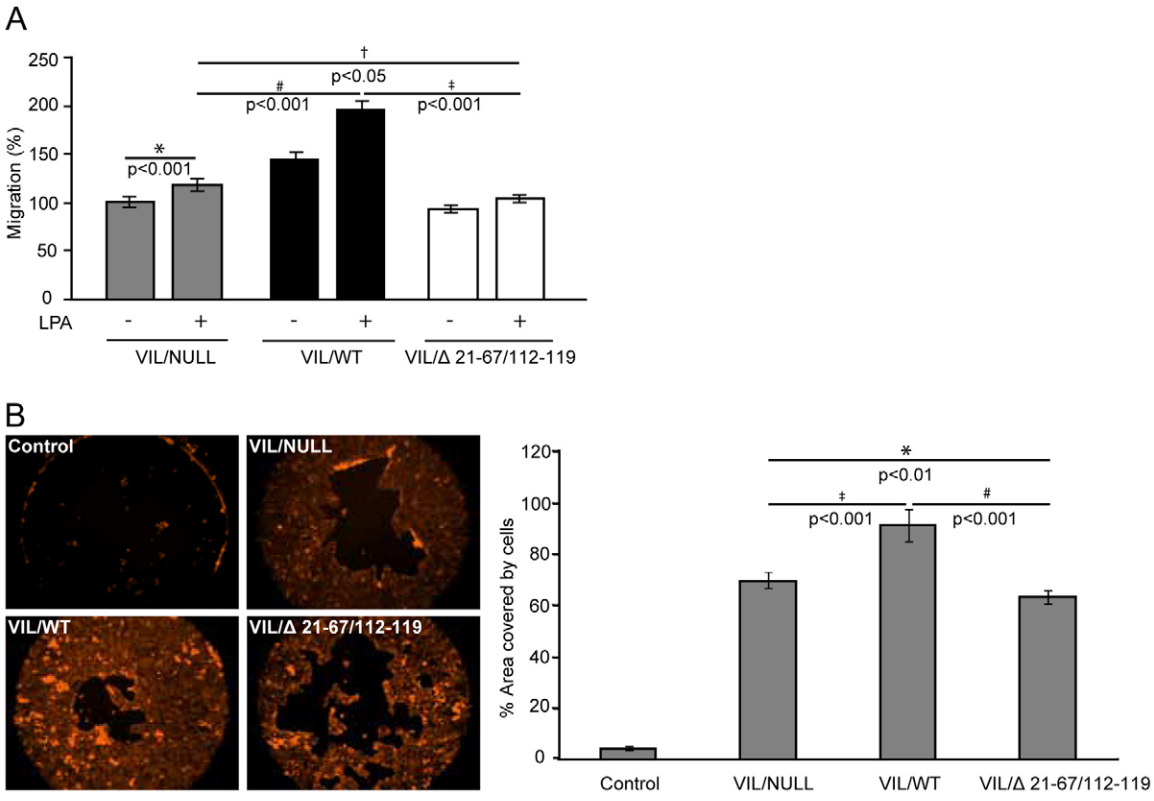

Fig. 1. Villin dimers regulate cell migration. (**A**) MDCK cells without ectopic protein expression (VIL/NULL), stably expressing wild-type villin (VIL/WT) or stably expressing the dimerization mutant of villin (VIL/Δ21–67/112–119) were subjected to a wound healing assay in the presence or absence of LPA (2 μM). Data are normalized to the control values (which were set as 100) and expressed as the percent change in migration compared to control. Control refers to cell migration 7 h post-wounding in VIL/NULL cells in the absence of LPA. (**B**) The rate of migration of wild-type and mutant villin cells was also determined using the Oris cell migration assay, as described by the manufacturer. Values are means ± s.e.m.

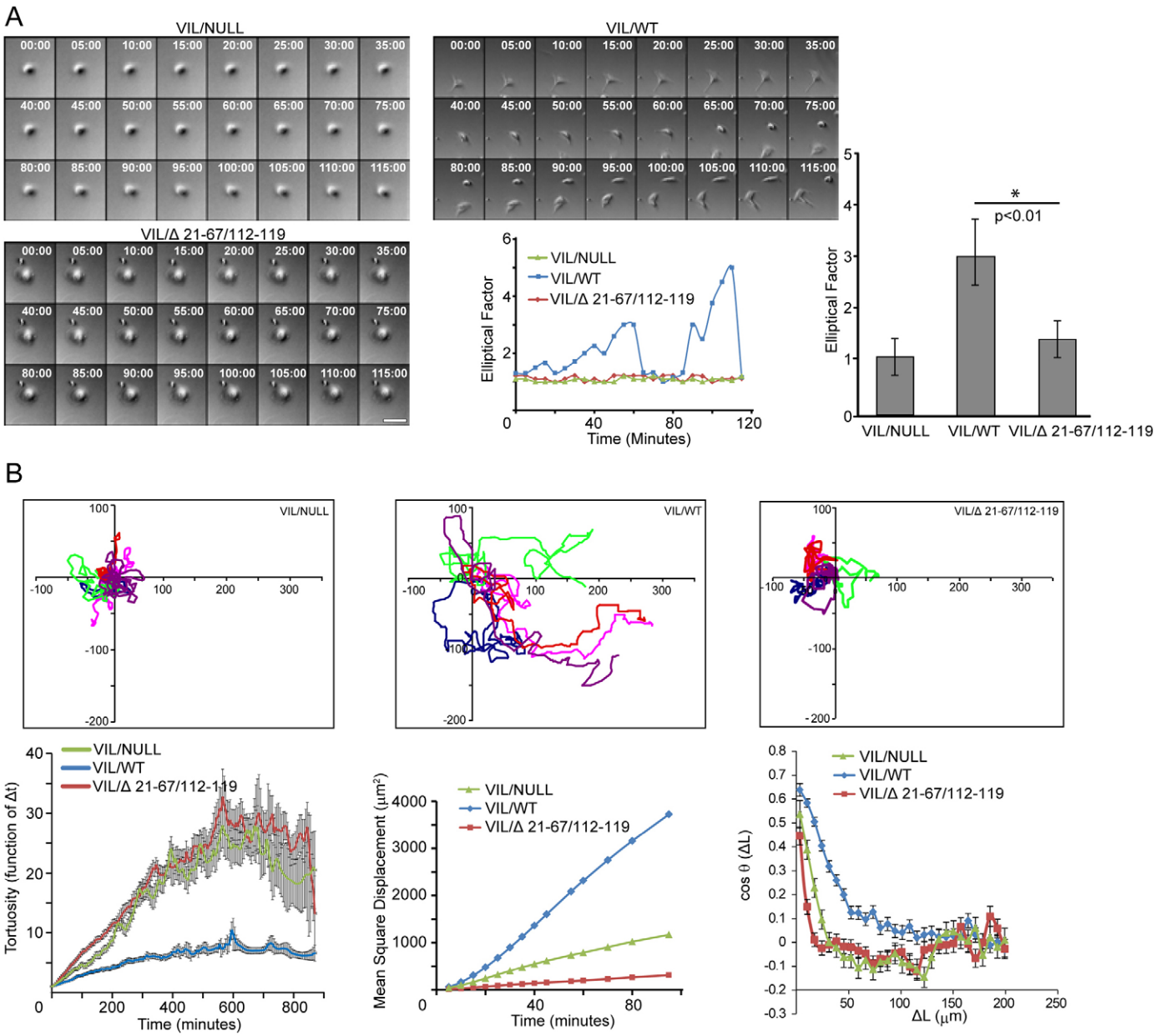

Fig. 2. Villin regulates directionally persistent cell migration in isolated cells. (**A**) Time-lapse images of MDCK Tet-Off VIL/NULL cells and cells expressing VIL/WT or VIL/Δ21–67/112–119 were collected over a period of 120 min. The elliptical factor was calculated as a measure of cell polarization. Values are means ± s.e.m. (n=15). (**B**) The path of migration of individual VIL/NULL, VIL/WT or VIL/Δ21–67/112–119 cells was tracked using the cell track function in Metamorph software. The track of individual cells were consolidated and re-plotted from the origin. Cell migration directionality was quantified by measuring the average angle between velocity vectors $\langle\cos[\theta(L)]\rangle$ as a function of distance along the trajectory L, the average tortuosity as a function of time, and the mean and square displacement as a function of time. All data were scored from 30 individual cells. The error bars indicate 95% confidence intervals.

of mutant villin compared to wild-type villin, $P<0.01$. VIL/NULL cells had an EF of 1.0). This loss of cell polarity, we suggest, leads to the non-persistent mode of translocation of VIL/NULL or mutant villin cells compared to cells expressing wild-type villin. The position of the individual cell nuclei was tracked in time-lapse image series to quantify directional cell movement (Fig. 2B). The directional persistence of VIL/WT cells measured by $\langle\cos[\theta(L)]\rangle$ was longer than that of VIL/NULL or VIL/Δ21–67/112–119 ($P<0.05$). VIL/NULL and mutant villin cells also demonstrated higher tortuosity and lower mean square displacement values compared to VIL/WT confirming the loss of persistent directional cell migration. Thus, villin enhances both the length of time and the distance that cells persistently move in the same direction.

In order to characterize the molecular mechanism of villin-induced epithelial cell migration, we also tracked the migration of individual cells at the leading edge of the wound as described previously (Koch et al., 2009). For cells expressing wild-type villin, tracks of individual cell trajectories were very directional, oriented towards the wound and had fewer kinks and bends in

their tracks (Fig. 3A). These results indicated that the VIL/WT expressing cells had lower change in direction and therefore were more persistent. By contrast, the migration trajectories of individual cells expressing mutant villin protein appeared random. Cell velocity (μm/h) was also significantly higher for VIL/WT cells (85 μm/h) compared to VIL/NULL cells (65 μm/h; $*P<0.01$) and significantly lower for VIL/Δ21–67/112–119 cells (50 μm/h) compared to VIL/WT cells ($^{\ddagger}P<0.001$). The directional persistence of migrating VIL/Δ21–67/112–119 cells as measured by $\langle\cos[\theta(L)]\rangle$ showed a decrease compared to VIL/WT. Tortuosity of VIL/WT was also significantly lower than that of VIL/Δ21–67/112–119 or VIL/NULL cells ($P<0.05$). The increase in tortuosity and decrease in persistent directional migration correlates with reduced wound closure in VIL/Δ21–67/112–119 and VIL/NULL cells compared to VIL/WT cells (Fig. 1). To directly measure cell polarity stimulated by a directional stimulus, we assayed for microtubule-organizing center (MTOC) polarization in epithelial cells migrating out of a wounded monolayer. In MDCK cells expressing VIL/WT, MTOC reorient towards the leading edge (Fig. 3B). This was substantially diminished in mutant villin cells (70% decrease compared with VIL/WT, $*P<0.001$). Collectively these results demonstrate a defect in the establishment of cell polarity in villin mutant cells that fail to self-associate and fail to bundle actin. Thus, both single cells as well as a population of cells expressing full-length villin maintain persistent directed cell migration over longer periods of time compared to villin mutants that fail to bundle actin filaments. To explore directional migration stimulated by external stimuli, we performed chemotaxis assays using a modified Boyden chamber. Cell chemotaxis towards HGF (10 ng/ml) an important physiological regulator of directed epithelial cell migration during wound repair, was reduced 91% in mutant villin cells compared with cells expressing wild-type villin ($^{\ddagger}P<0.001$; Fig. 3C). As noted previously, expression of full-length villin significantly enhanced cell chemotaxis compared to VIL/NULL cells (4.69-fold increase, $*P<0.001$) (Wang et al., 2007). The increase in maximum distance from origin and the area covered by a moving cell is due to changes in average cell speed and/or cell turning. Together our data demonstrate that cells expressing villin are capable of modulating both these components namely, directional migration and migration rates.

Actin bundling by villin is required for filopodial assembly in epithelial cells

To begin to elucidate the role of villin in the regulation of directional cell migration, we elected to evaluate the association of villin with filopodia. Overexpression of SEYFP-tagged villin induced numerous filopodia-like protrusions in MDCK cells (Fig. 4A). Furthermore villin localized to the entire length of all filopodia in these cells. In Caco-2 BBe1 cells, which express endogenous villin, villin was likewise localized along the entire length of the filopodia and colocalized with fascin, a known marker of filopodia (Fig. 4B). A quantitative analysis of filopodia in MDCK VIL/NULL cells and villin-expressing (VIL/WT) cells was obtained from confocal images for 30 typical cells of each cell line. Most cells expressing full-length human villin contained significantly higher (9.25-fold increase, $*P<0.001$) number of filopodia compared with VIL/NULL cells. We have previously shown that the villin mutant VIL/Δ21–67/112–119 fails to self-associate and bundle actin filaments (George et al., 2007). We now show that the villin mutant VIL/Δ21–67/112–119 also fails to assemble filopodia ($P<0.001$ compared to VIL/WT). Based on these data, we hypothesized that filopodial assembly by villin may be required for directionally persistent cell migration. To test this hypotheses, we probed the morphological phenotype and villin distribution in MDCK cells expressing SEYFP-tagged VIL/WT or VIL/Δ21–67/112–119. As shown in Fig. 4C, cells expressing VIL/WT extend filopodia from an active region of the cell surface (see arrowhead) which subsequently develops into lamellipodia from these sites leading to both cell spread and cell translocation. The restriction of lamellipodial formation to an active site on the cell surface indicates a functional polarization of VIL/WT cells (Winklbauer and Selchow, 1992). By contrast, cells expressing VIL/Δ21–67/112–119 failed to initiate filopodial assembly or generate similarly active areas on the cell surface (Fig. 4Cb). Although, VIL/Δ21–67/112–119 cells developed large lamellipodia these lamellipodia extended throughout the cell surface and were not restricted to a polarized leading edge (Fig. 4Cb). Interestingly then, in MDCK cells expressing VIL/Δ21–67/112–119 despite the loss of filopodia, lamellipodia were unaffected and the mutant villin protein localized to the lamellipodia similar to wild-type villin (Fig. 4C; supplementary material Fig. S1B,C) and in fact, significantly stimulated lamellipodial assembly all over the cell perimeter (supplementary material Fig. S1D). There was no significant difference in the F-actin-binding property of recombinant VIL/Δ21–67/112–119 proteins compared to VIL/WT (supplementary material Fig. S1E). Likewise, there was no significant difference in the actin-polymerizing property of VIL/Δ21–67/112–119 compared to VIL/WT (supplementary material Fig. S1F). Together these findings demonstrate that localization of villin to filopodia and lamellipodia can be functionally separated and that the actin-bundling activity of villin is not required for localization of villin to the lamellipodia (Athman et al., 2003; Tomar et al., 2006). To further test our hypothesis that actin-bundling by villin regulates filopodial assembly in epithelial cells, SEYFP–villin and cerulean–villin were co-expressed in MDCK Tet-Off cells and villin dimerization was analyzed by FRET as described previously (George et al., 2007). The FRET activity was noted along the cell margins and most significantly in filopodia (Fig. 4D, arrowheads). The average FRET efficiency within the filopodia was between 35 and 45%, $P<0.01$, $n=15$. These data confirm that villin dimers localize to the filopodia and are involved in filopodium formation. It should be noted that the presence of villin dimers in this assay is an underestimate as we cannot account for dimers formed between two SEYFP-tagged villin molecules or two cerulean-tagged villin molecules. Together these data demonstrate that villin dimers are associated with filopodia (Fig. 4D) and that deletion of the villin dimerization site prevents villin-induced filopodial assembly (Fig. 4A) in cells. Furthermore, knockdown of endogenous villin (supplementary material Fig. S2A,B) significantly retards both the assembly of filopodia (supplementary material Fig. S2C) and rates of cell migration (supplementary material Fig. S2D) despite the expression of other filopodia-associated actin-bundling proteins like fascin (supplementary material Fig. S2A). Collectively, these results demonstrate a defect in the establishment of cell polarity in villin mutant cells that fail to self-associate, fail to bundle actin and therefore fail to assemble filopodia, thereby establishing a

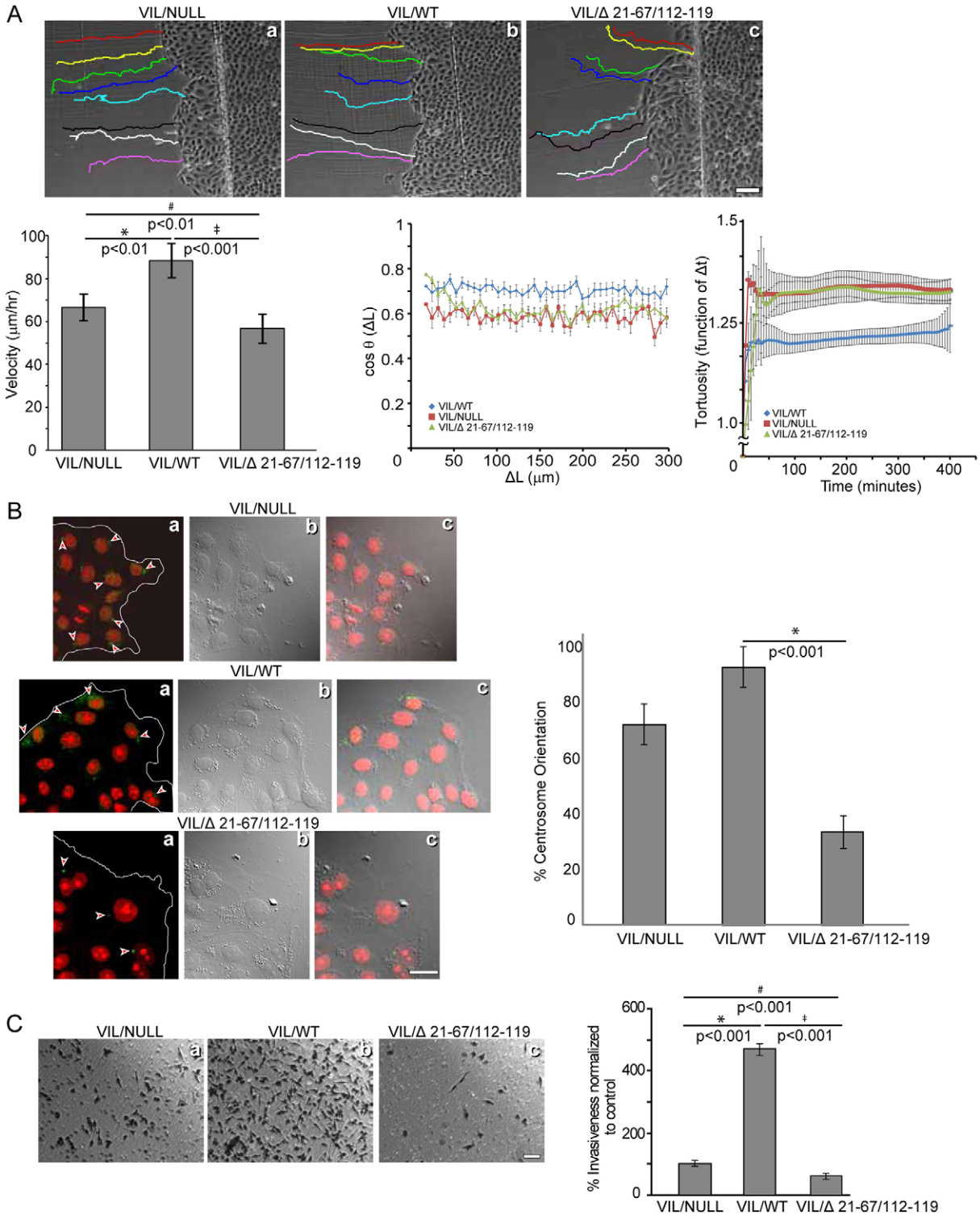

Fig. 3. Villin regulates directionally persistent cell migration in confluent epithelial monolayers. (**A**) Directional migration of MDCK cells expressing SEYFP (a), SEYFP-tagged VIL/WT (b) or SEYFP-tagged VIL/Δ21–67/112–119 (c) was determined by tracing the path of migration of cells present at the wound edge over a period of 7 h using the cell track function in Metamorph software. The velocity of cell migration was calculated and compared for 30 individual cells. Values are the measured means ± s.e.m. Cell migration directionality was quantified by measuring $\langle\cos[\theta(L)]\rangle$ as a function of distance along the trajectory L, and the average tortuosity as a function of time t, that were scored for 30 individual cells. The error bars indicate 95% confidence intervals. (**B**) The directional migration of MDCK VIL/NULL cells and cells expressing VIL/WT or VIL/Δ21–67/112–119 was measured by determining the orientation of the centrosome compared to the cell boundary. Centrosomes were identified by staining for pericentrin. Propidium iodide was used for nuclear staining (a), and the phase-contrast images (b) were merged (c) to determine the leading edge of motile cells. The percentage of cells with the centrosome oriented towards the leading edge was quantified. Values are means ± s.e.m. (n=15). (**C**) Directional cell migration in the presence of external stimuli was measured using a modified Boyden chamber. VIL/NULL cells (a) were compared with VIL/WT (b) and VIL/Δ21–67/112–119 (c) cells. Values are means ± s.e.m. (n=15).

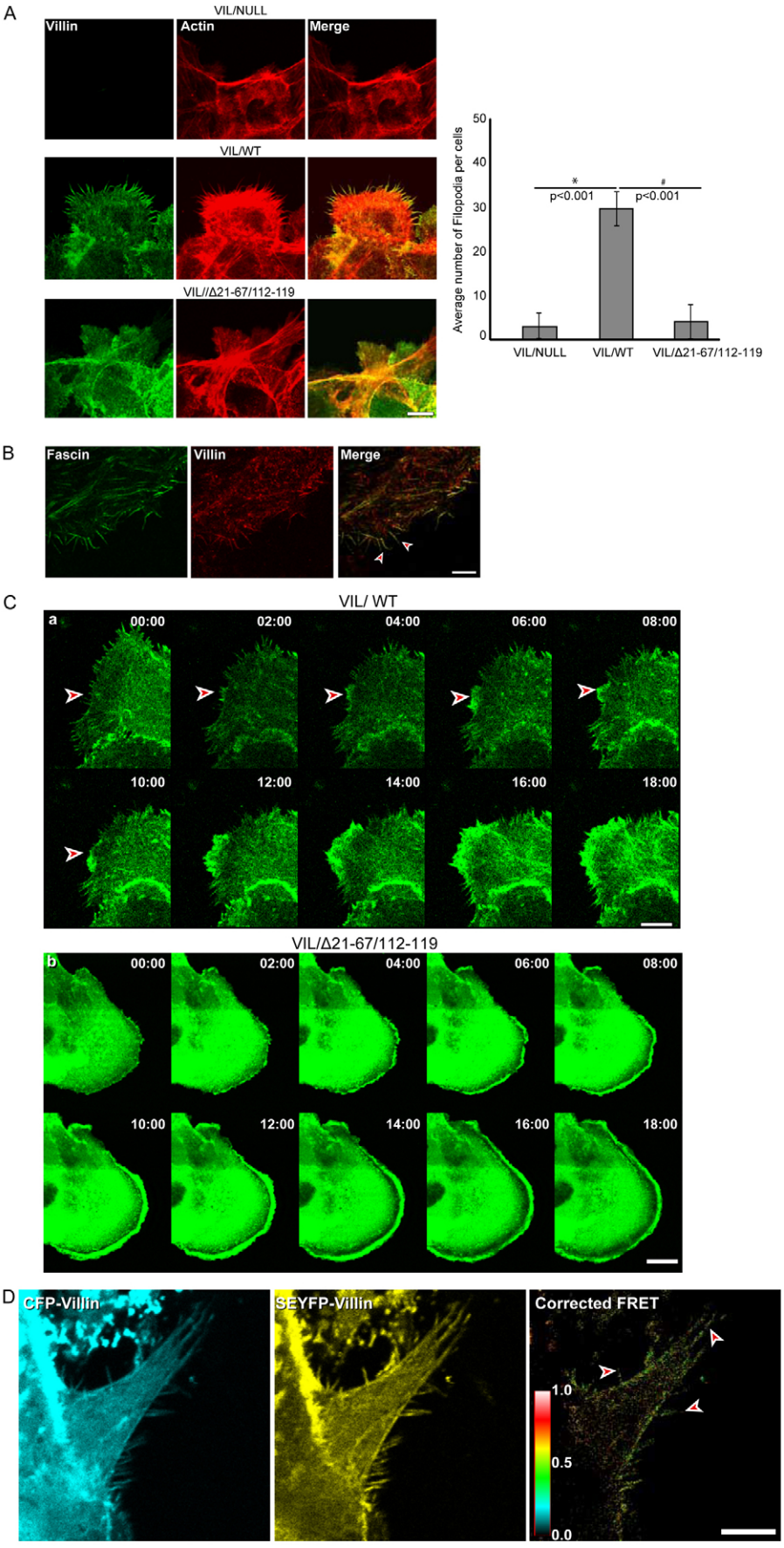

Fig. 4. Villin dimers are required for filopodial assembly. MDCK Tet-Off VIL/NULL, VIL/WT and VIL/Δ21–67/112–119 cells were examined by fluorescence microscopy to assess filopodial assembly. The intracellular distribution of villin and actin was determined in these cells and merged images show colocalization of villin and actin in filopodia. Scale bar: 10 µm. The number of filopodia in these cells was quantified, and values are means ± s.e.m. (n=20). (**B**) Caco-2 BBe1 cells were stained for fascin (green) and villin (red). Scale bar: 5 µm. (**C**) The distribution of SEYFP–VIL/WT (a) and SEYFP–VIL/Δ21–67/112–119 (b) in LPA (2 µM)-treated MDCK Tet-Off cells was observed over a period of 20 min. The arrowhead indicates the location of filopodia which mature into an emerging lamellipodium. The images are representative of 15 cells with similar results. Scale bars: 5 µm. (**D**) MDCK Tet-Off cells co-expressing cerulean–villin (CFP–villin) and SEYFP–villin were treated with LPA (2 µM) and FRET analysis was done on migrating cells. FRET intensity is shown in pseudo-color mode, and the color scale represents the relationship between color and pixel value. Scale bar: 10 µm.

correlation between the function of villin in filopodial assembly and its ability to regulate directional cell migration.

The association of Villin with PtdIns(4,5)P_2 localizes villin dimers to filopodia

We have previously reported the direct association of villin with PtdIns(4,5)P_2 (Kumar et al., 2004). Furthermore, we have demonstrated that *in vitro* PtdIns(4,5)P_2 enhances the actin-bundling function of villin (Kumar et al., 2004). Since the actin bundling property of villin is regulated by dimerization, and villin co-localizes with PtdIns(4,5)P_2 within the filopodia (Fig. 5A; supplementary material Fig. S3), we speculated that PtdIns(4,5)P_2 could promote actin bundling by enhancing villin self-association. To test this hypothesis, full-length recombinant human villin protein (20 nM) was incubated with increasing concentrations of PtdIns(4,5)P_2 (0–80 μM) containing unilamellar vesicles. To characterize villin dimers we used a short spacer length cross-linker (3 Å), 1,5-difluoro-2,4-dinitrobenzene (DFDNB) as described by us previously (George et al., 2007). Sub-optimal amounts of DFDNB were used to highlight the increase in villin dimers if any, in the presence of vesicles containing PtdIns(4,5)P_2. As shown in Fig. 5B, consistent with our hypothesis, increasing concentrations of PtdIns(4,5)P_2 increased the concentration of villin dimers. It may be noted that the mobility of cross-linked villin dimers on 8% SDS-PAGE is in excess of their predicted masses as reported by us earlier (George et al., 2007). We also determined that other phospholipids that do not bind villin such as phosphatidylcholine (PC) had no effect on villin self-association (supplementary material Fig. S4A) (Kumar et al., 2004).

To understand how villin-induced filopodia generate and maintain their shape, we investigated filopodia initiation in cells that overexpress PtdIns(4,5)P_2. For these studies, MDCK cells were infected with adenovirus to overexpress HA-tagged Iγ635 isoform of phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase), which has been shown previously to increase the cellular levels of PtdIns(4,5)P_2 (Ishihara et al., 1998) (Fig. 5C). The overexpression of PIP5-kinase in the absence of villin in MDCK Tet-Off cells had no effect on filopodial assembly (VIL/NULL; Fig. 5E,F). In contrast, overexpression of PIP5-kinase in the presence of villin (VIL/WT) increased the number of filopodia per cell. Quantification of filopodia in these cells revealed a 9.6-fold increase ($^*P<0.001$) in filopodia in MDCK cells expressing VIL/WT compared to cells that do not express villin (Fig. 5F). More importantly, in VIL/WT cells overexpressing PIP5-kinase Iγ635 there was an even more significant increase (10.2 fold, $^*P<0.001$) in the average number of filopodia assembled per cell compared with VIL/NULL cells overexpressing PIP5-kinase. MDCK VIL/NULL cells did not show any statistically significant increase in filopodium formation even in the presence of higher intracellular PtdIns(4,5)P_2 levels, which suggested to us that a direct interaction of villin with PtdIns(4,5)P_2 was responsible for the increased number of filopodia noted in VIL/WT cells. In contrast, overexpression of PIP5-kinase in cells expressing mutant villin proteins that cannot bundle F-actin (VIL/Δ21–67/112–119) or lack any one of the two major PtdIns(4,5)P_2 binding site (ΔPB2 or ΔPB5; Fig. 5D) prevented filopodial assembly (Fig. 5E,F). MDCK cells overexpressing the PtdIns(4,5)P_2 phosphatase SigD showed a significant decrease in the number of filopodia assembled ($P<0.001$, $n=30$) compared to cells expressing the phosphatase-deficient mutant SigD (C462S; Fig. 5G) (Mason et al., 2007). In contrast, MDCK cells treated with the PLC-γ1 inhibitor U73122 showed as significant increase ($P<0.001$, $n=30$) in the number of filopodia assembled compared to cells treated with the negative control U73343 (Fig. 5H).

Likewise, we noted that in Caco-2 BBe1 cells, which endogenously express villin, overexpression of PIP5-kinase Iγ635 (Fig. 6A) significantly increased (2.4-fold increase; $^*P<0.001$) the number of filopodia assembled per cell (Fig. 6B). Increase in intracellular PtdIns(4,5)P_2 levels in Caco-2 cells also increased villin self-association which was accompanied by a concomitant decrease in the monomer concentration of villin (Fig. 6C). The changes in dimer formation were normalized with the actin loading control and quantified. A 1.17-fold and 1.22-fold increase in dimer formation was observed in Caco-2 cells upon the expression of the Iβ and Iγ635 isoforms of PIP5-kinase, respectively. Uncross-linked cell extracts (−DFDNB) showed only villin monomers and no villin self-association. Similar increase in villin dimers were noted in MDCK cells overexpressing PIP5-kinase (data not shown). Time and dose response studies for DFDNB-induced villin dimerization are shown in supplementary material Fig. S4B,C. Together these data provide an understanding of how PtdIns(4,5)P_2 functions as a local signal to regulate actin reorganization in villin expressing cells.

PtdIns(4,5)P_2 exposes a hidden dimerization site within villin

To further deduce the mechanism of PtdIns(4,5)P_2 regulated dimerization of villin, we prepared 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-tagged fluorescent lipid vesicles containing different combinations of PtdIns(4,5)P_2 and PC and incubated them with villin proteins. Our previous studies have shown that villin has the ability to bind to PtdIns(4,5)P_2 but not to PC (Kumar et al., 2004). Incubation of pure PC containing vesicles in the presence or absence of VIL/WT did not cause any change in the distribution of these vesicles (Fig. 6D). 88% of vesicles remained as individual vesicles and only 12% formed 1–2 attachments. However, incubation of VIL/WT with PtdIns(4,5)P_2 containing vesicles [99% PC, 1% PtdIns(4,5)P_2] caused an aggregation of these vesicles. Only 10% vesicles remained as individual vesicles, 50% formed one or two attachments, while 40% formed three or more attachments. However, when PtdIns(4,5)P_2 containing vesicles were incubated with VIL/Δ21–67/112–119 protein, no clustering of vesicles was observed. 80% vesicles remained as individual vesicles, while 20% formed 1–2 attachments. Taken together with the data presented above, we suggest that binding of villin to PtdIns(4,5)P_2 could result in a conformational change (Kumar et al., 2004) that exposes the dimerization site in villin allowing villin self-association but also inducing a clustering of PtdIns(4,5)P_2.

The association of villin with F-actin also promotes dimerization

Self-association induced by actin binding has been shown to affect the actin cross-linking activities of many proteins including scruin, VASP and vinculin (Bachmann et al., 1999; Johnson and Craig, 2000; Sanders et al., 1996). Consistent with that observation, we speculated that the dimeric state of villin could likewise be modulated by the other ligand of villin, F-actin. To

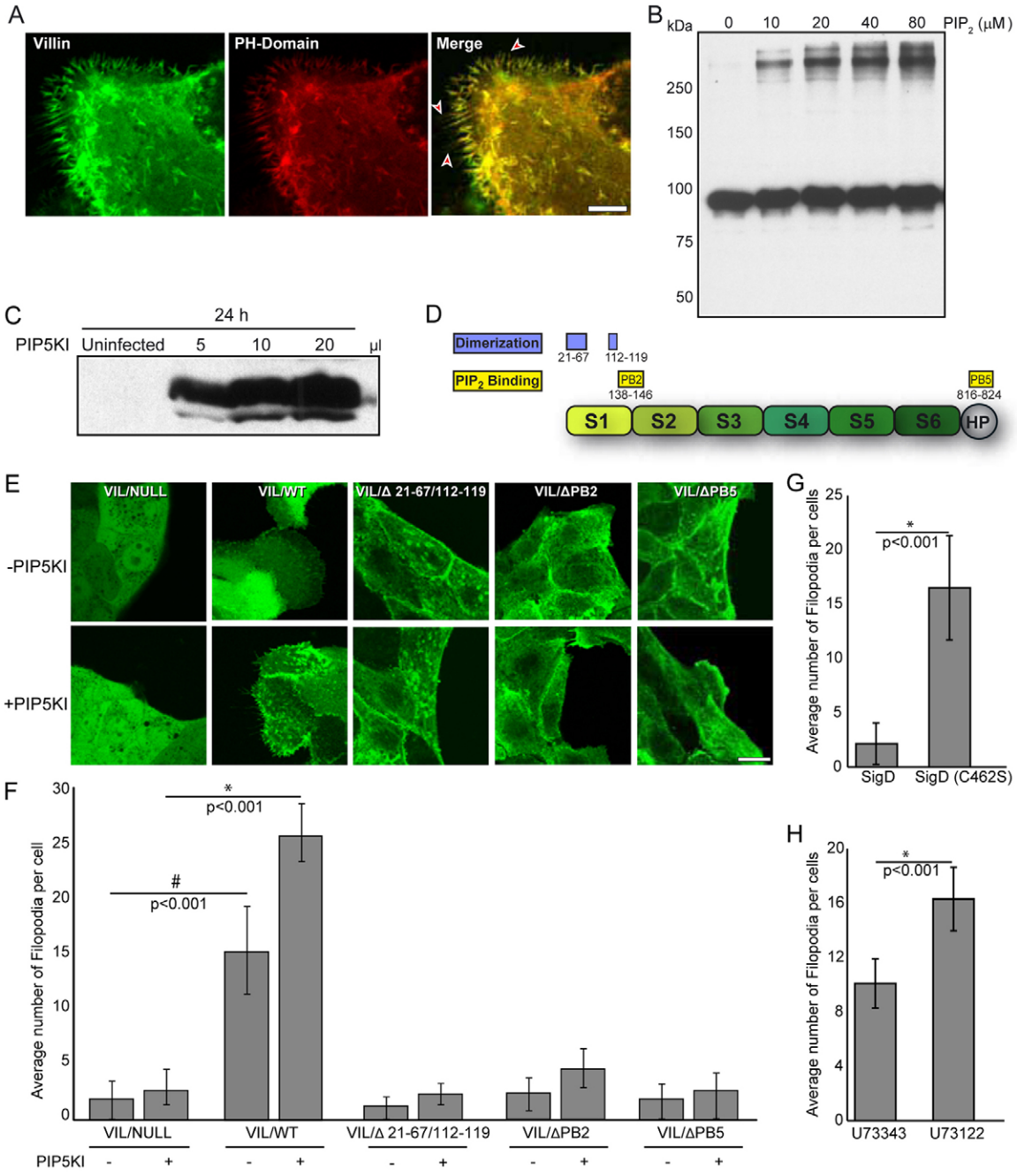

Fig. 5. PtdIns(4,5)P_2 regulates villin self-association and villin-induced filopodial assembly. (**A**) Co-localization of SEYPF–villin and CFP–PH-domain in MDCK Tet-Off cells. Scale bar: 10 μm. (**B**) Recombinant villin was incubated with increasing concentrations of PtdIns(4,5)P_2 (PIP_2; 0–80 μM) followed by cross-linking with a sub-optimal concentration (10-fold molar excess) of DFDNB for 1 hour at room temperature. The cross-linked proteins were separated by 8% SDS-PAGE and villin dimers were identified by western analysis. (**C**) The optimal concentration of adenovirus required to express HA-tagged PIP5-kinase I (PIP5KI) in MDCK Tet-Off cells was determined by infecting cells with different aliquots of recombinant adenovirus for 16 h followed by incubation for 24 h for protein expression. PIP5-kinase expression was detected by western analysis using an HA antibody. (**D**) Schematic diagram shows the dimerization [amino acids (a.a.) 21–67 and 112–119] and the two PtdIns(4,5)P_2 binding (PB2: a.a. 138–146; PB5: a.a. 816–824) sites in human villin protein. (**E**) Filopodial assembly in MDCK Tet-Off cells (VIL/NULL), MDCK Tet-Off cells expressing SEYFP-tagged villin (VIL/WT) or mutant villin proteins (VIL/Δ21–67/112–119 or VIL/ΔPB2 or VIL/ΔPB5) were infected with adenovirus to express EGFP or PIP5-kinase I. Scale bar: 10 μm. (**F**) Quantification of filopodia in MDCK Tet-Off VIL/NULL and VIL/WT cells expressing EGFP (−) or PIP5-kinase (+). There was a significant increase in filopodial assembly in cells expressing VIL/WT compared to VIL/NULL cells ($^{\#}P<0.001$, $n=20$) and a more significant increase in VIL/WT cells compared to VIL/NULL cells when they expressed higher levels of intracellular PtdIns(4,5)P_2 ($^{*}P<0.001$). Overexpression of PIP5-kinase had no effect on filopodial assembly in the mutant villin cell lines. Values are means ± s.e.m. (**G**) MDCK Tet-Off cells expressing VIL/WT and the PtdIns(4,5)P_2 phosphatase SigD show a significant decrease ($P<0.001$, $n=30$) in the average number of filopodia assembled per cell compared to cells overexpressing the phosphatase-deficient mutant SigD (C462S). (**H**) MDCK Tet-Off cells expressing VIL/WT treated with the PLC-γ1 inhibitor U73122 show a significant increase ($P<0.001$, $n=30$) in the average number of filopodia assembled per cell compared to cells treated with the negative control (U73343).

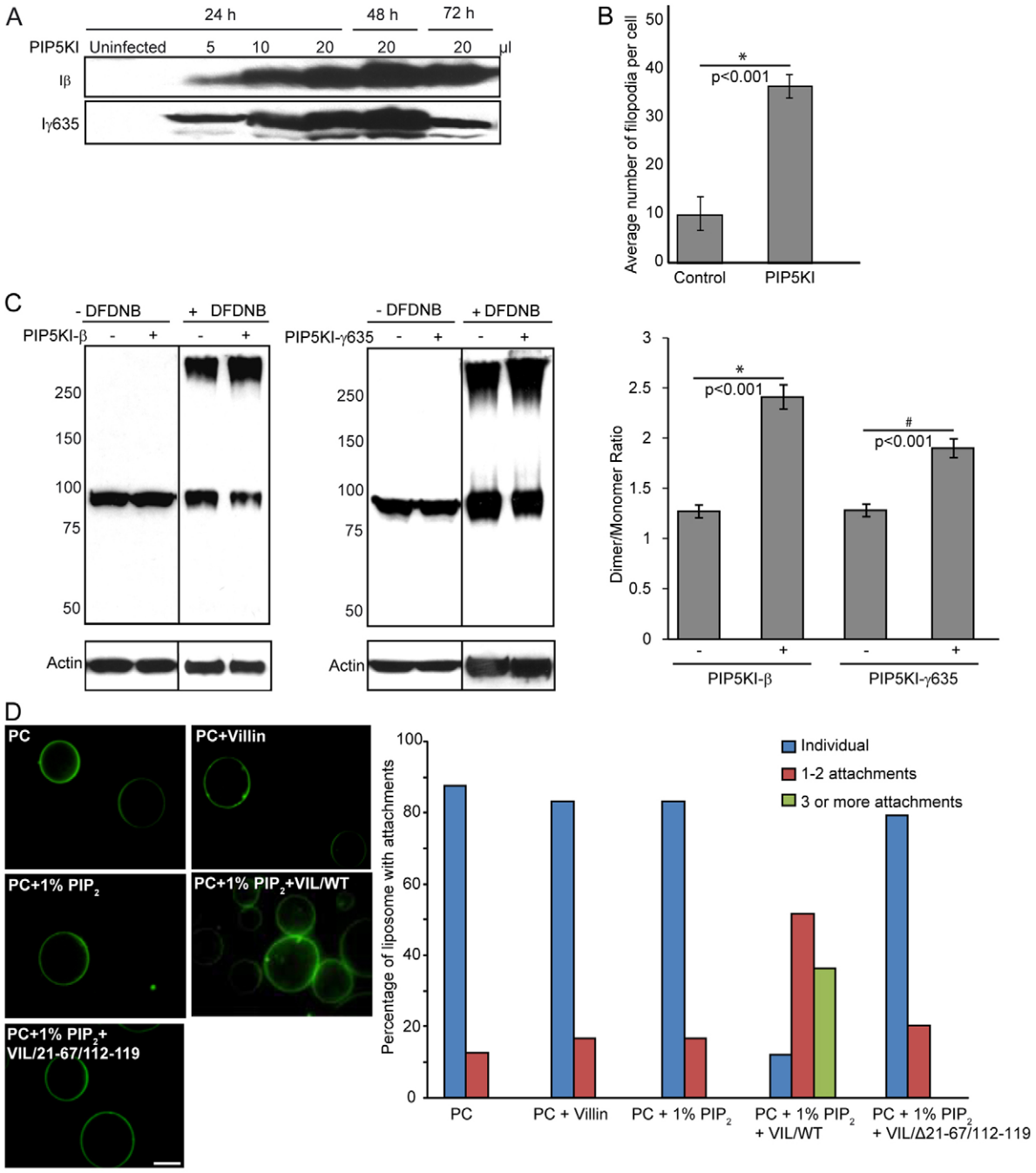

Fig. 6. Cellular PtdIns(4,5)P_2 levels regulate dimerization of villin and filopodial assembly in Caco-2 BBe1 cells. (**A**) The optimal concentration of adenovirus required to express HA-tagged PIP5-kinase I in Caco-2 BBe1 cells was determined by infecting cells with different amounts of recombinant adenovirus for 16 h, followed by incubation for 24–72 h for protein expression. PIP5-kinase expression was detected by western blot analysis using an HA antibody. (**B**) Quantification of filopodia in Caco-2 cells expressing vector alone (control) or PIP5-kinase I shows significant increase in the number of cells expressing filopodia compared to control cells (*P<0.001 n=20). Values are means ± s.e.m. (**C**) The effect of increased intracellular PtdIns(4,5)P_2 (PIP$_2$) levels on villin dimerization was determined in Caco-2 BBe1 cells expressing either control Ad-EGFP or HA-tagged PIP5-kinase Iβ and Iγ635 for 48 h at 37℃. Villin protein was cross-linked by incubating cells with 2.5 mM DFDNB for 1 h at 37℃. Villin monomers and dimers were identified by western blot analysis. This is a representative of four experiments with similar results. (**D**) Binding of PtdIns(4,5)P_2 to villin results in the exposure of dimerization site. Vesicles containing 100% PC were incubated with either buffer or with villin (VIL/WT) for 30 min at room temperature. Similarly, vesicles containing 99% PC, 1% PtdIns(4,5)P_2 were incubated with buffer, 2 µM wild-type villin (VIL/WT) or 2 µM mutant protein VIL/Δ21–67/112–119. The vesicles were made fluorescent by the incorporation of NBD-PC. Scale bar: 10 µm.

test this hypothesis, we elected to examine the effect of F-actin on villin self-association. For these studies recombinant villin protein was incubated with increasing concentrations of F-actin (0–200 nM) followed by cross-linking with DFDNB. The cross-linked proteins were examined for villin dimers. As shown in Fig. 7A, *in vitro* F-actin is a potent regulator of villin dimerization. Higher order actin–actin- or actin–villin-associated bands were not detected as shown in Fig. 7B. To validate these findings, we incubated Caco-2 BBe1 cells with increasing concentrations of the actin depolymerizing agent latrunculin A (0–8 μM) followed by cross-linking with DFDNB. As expected, decreasing the concentration of F-actin inhibited villin dimerization in Caco-2 cells (Fig. 7C). The possibility of the presence of villin–actin cross-linked proteins was ruled out as no higher order proteins was detected while probing for actin (Fig. 7D). We have previously reported that villin oligomerization with multiple cross-linkers including non-cleavable cross-linkers like DSS and EGS assembles villin dimers (George et al., 2007). It may be noted that these non-cleavable cross-linkers also show no villin–actin self-association in Caco-2 BBe1 cells (supplementary material Fig. S4D). DFDNB treatment also does not alter the intracellular distribution of villin or actin in Caco-2 BBe1 cells (supplementary material Fig. S4E). Together, these data allow us to conclude that F-actin can induce villin self-association both *in vitro* and in cells. Based on these data and those shown above, we suggest that PtdIns(4,5)P_2 could serve to recruit villin to the membrane which together with actin bound to villin could transform actin reorganization into functional cell surface structures such as the filopodia (Fig. 8).

Villin dimers are negatively regulated by tyrosine phosphorylation of villin but not by calcium

Calcium and tyrosine phosphorylation regulate the actin-modifying functions of villin as well as its ligand-binding properties (Panebra et al., 2001; Zhai et al., 2001). To further characterize the regulation of villin in filopodial assembly, we elected to examine the effect of tyrosine phosphorylation and Ca^{2+} on villin dimerization. Interestingly, we determined that tyrosine phosphorylation of villin prevented its ability to self-associate (Fig. 7E). In contrast, increasing concentrations of the cross-linker DFDNB increased dimerization of non-phosphorylated villin protein. Furthermore, we noted that PtdIns(4,5)P_2 enhanced the cross-linking of non-phosphorylated villin (VIL/WT), but had no effect on self-association of tyrosine phosphorylated villin (pTyr-VILT/WT; Fig. 7F). Since sub-optimal levels of DFDNB were used for these studies, dimers of non-phosphorylated villin were seen only in the presence of PtdIns(4,5)P_2. Together these data suggest that direct association of villin with PtdIns(4,5)P_2 and F-actin could enhance its actin-bundling function thus, enhancing filopodial assembly by villin. By contrast, tyrosine phosphorylation of villin may serve to prevent formation of actin bundles within the filopodia, thus serving as an antagonist of filopodial assembly. These data support our previous findings that *in vitro*, tyrosine phosphorylation of villin inhibits actin-bundling by villin and promotes actin severing (Zhai et al., 2001). These data also demonstrate another molecular mechanism for the separation of the actin bundling and actin severing functions of villin. Calcium had no effect on villin self-association (supplementary material Fig. S5A). The absence or presence of calcium together with PtdIns(4,5)P_2 also had no effect on villin dimerization (supplementary material Fig. S5B).

Discussion

We have previously shown that dimerization of villin is a prerequisite for F-actin bundling by villin (George et al., 2007). We now demonstrate that dimerization of villin regulates filopodial assembly. We speculate that villin dimerization may serve to recruit villin from a large cytoplasmic pool to well defined membrane sites, in response to external cell stimuli to polarize the cell and induce membrane protrusion. Villin also directly interacts with phospholipids including PtdIns(4,5)P_2 which localizes villin to the plasma membrane (Kumar et al., 2004). We now show that *in vitro* and in cells PtdIns(4,5)P_2 promotes villin dimerization. There are several examples in published literature of actin-binding proteins that dimerize following PtdIns(4,5)P_2 binding (Fukami et al., 1992; McGough et al., 1997; Spudich et al., 2007; Tacon et al., 2004). It may be noted that dimers of Myosin X are recruited to the filopodia (Bohil et al., 2006) and more recent studies have shown that localization of Myo 1B to the filopodia requires PtdIns(4,5)P_2 binding by Myo 1B (Komaba and Coluccio, 2010). Other studies have described how PtdIns(4,5)P_2 binding promotes self-assembly of filopodia by clustering of actin regulatory proteins which is initiated by the direct interaction of these proteins with PtdIns(4,5)P_2 (Lee et al., 2010). Most importantly, PtdIns(4,5)P_2 has been identified as a local regulatory factor in actin dynamics and consistent with that function its distribution in cells is localized to highly dynamic and F-actin rich areas, colocalizing with membrane ruffles and filopodia (Tall et al., 2000; van Rheenen and Jalink, 2002). Based on these and data provided in this study we suggest that the association of villin with F-actin and the plasma membrane could promote villin self-association, thus increasing both the local F-actin, PtdIns(4,5)P_2 as well as villin concentration in localized membrane microdomains. This we suggest could facilitate actin bundling and filopodial assembly by villin (Fig. 8).

Actin-binding proteins like scruin have been shown to bind F-actin, which regulates not only the ability of scruin to self-associate but also its actin cross-linking function (Sanders et al., 1996). This suggests that the oligomeric state of monomeric actin cross-linking proteins can be modulated by F-actin in ways that affects their actin cross-linking activities (Johnson and Craig, 2000). In this study we demonstrate for the first time that like scruin and vinculin, the ability of villin to bind F-actin regulates its dimerization and its actin-bundling function, both of which determine its role in filopodial assembly in epithelial cells. Since villin possesses both PtdIns(4,5)P_2 and F-actin binding activity, it could serve as a scaffolding protein and thereby enable the assembly and localized restriction of PtdIns(4,5)P_2 and F-actin. Since these macromolecules also affect the actin bundling function of villin, villin could also serve as an effector of the signaling pathway that restrict these macromolecules to localized membrane microdomains thus, regulating cellular protrusion and directional cell migration. Since overexpression of villin potently induces cellular filopodia, we suggest that the PtdIns(4,5)P_2-binding function of villin may in fact induce membrane evaginations. We speculate that the interaction of plasma membrane PtdIns(4,5)P_2 with villin could result in the clustering of PtdIns(4,5)P_2 which could result in the bending of the membrane through electrostatic interactions. We suggest that

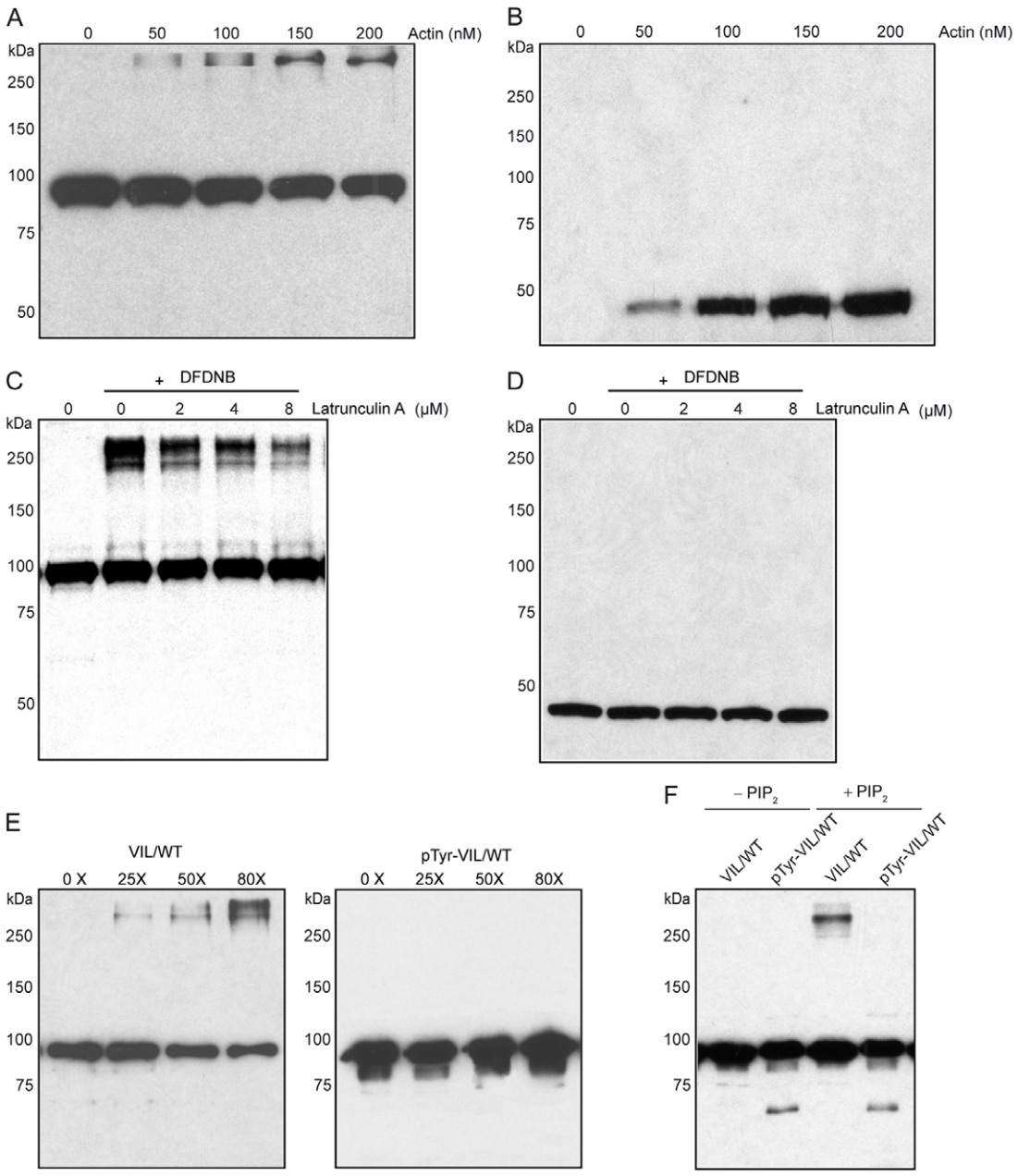

Fig. 7. F-actin regulates dimerization of villin. (**A**) Recombinant villin protein was incubated with increasing concentrations of actin (0–200 nM) followed by cross-linking with sub-optimal concentrations (10-fold molar excess) of DFDNB. Cross-linked proteins were separated on 8% SDS-PAGE and villin monomers and dimers were identified by using villin antibodies. (**B**) The presence of villin–actin cross-linked proteins was checked by reprobing the blot with actin antibodies. (**C**) The effect of F-actin on villin self-association was examined in Caco-2 BBe cells incubated with varying concentrations of latrunculin A (0–8 μM; 1 h at room temperature). Cross-linked proteins (2.5 mM DFDNB, 1 h at 37℃) were separated on 8% SDS-PAGE and villin dimers were identified by western blot analysis. (**D**) The presence of villin–actin cross-linked proteins was ruled out by reprobing the blot with actin antibodies. (**E**) 20 nM recombinant tyrosine-phosphorylated villin (pTyr-VIL/WT) and non-phosphorylated villin (VIL/WT) proteins were incubated with increasing concentrations of DFDNB (0- to 80-fold molar excess) for 1 hour at room temperature. Cross-linked proteins were separated on 8% SDS-PAGE and villin monomers and dimers were identified by using villin antibodies. (**F**) VIL/WT or pTyr-VIL/WT recombinant proteins (20 nM) were incubated with or without PtdIns(4,5)P_2 (40 μM) for 30 min at 4℃ followed by cross-linking with DFDNB (10-fold molar excess). Cross-linked proteins were analyzed by 8% SDS-PAGE and western blot analysis. The blot is representative of four experiments with similar results.

a tight spatiotemporal coordination of the molecular mechanisms controlling membrane bending and actin dynamics may be crucial for the generation of filopodia. We predict that proteins such as villin that are simultaneously capable of regulating actin dynamics and binding the plasma membrane may have a prominent role in this process.

Filopodia are highly dynamic structures that respond to extracellular stimuli, including signals originating from the

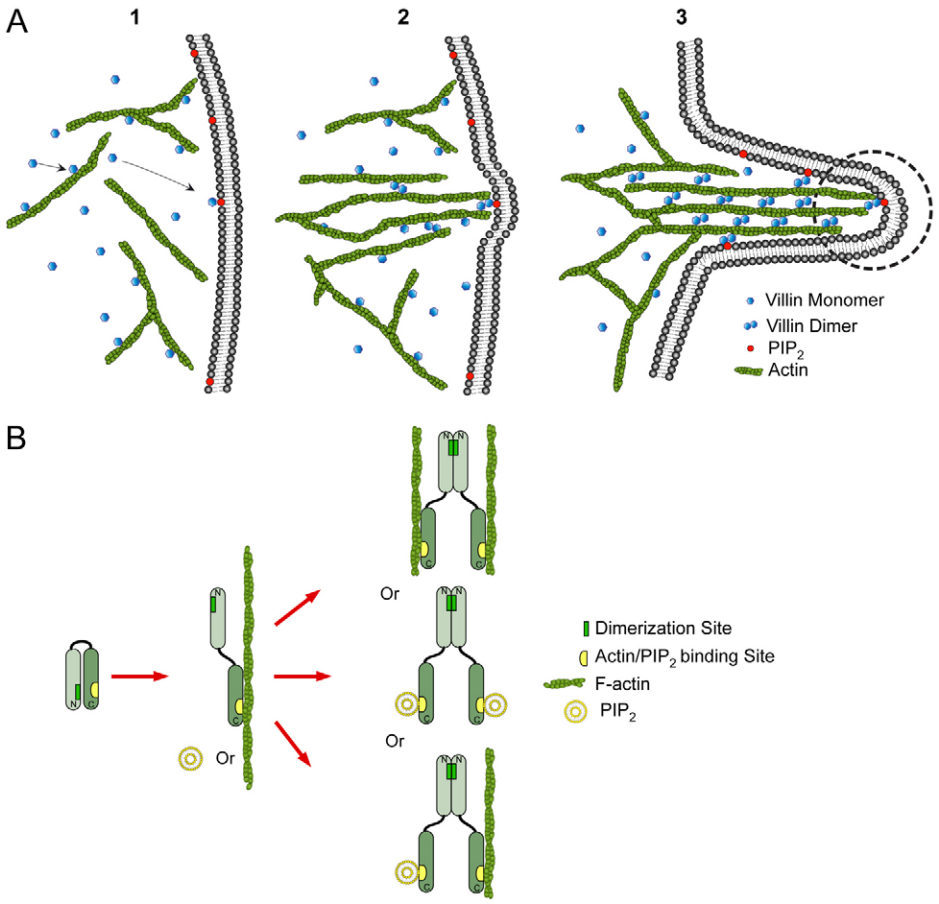

Fig. 8. Model of villin-induced filopodial assembly in epithelial cells. (**A**) We suggest that the direct interaction of villin with plasma membrane PtdIns(4,5)P_2 localizes villin to specific membrane microdomains (1). We speculate further that the interaction of villin with membrane PtdIns(4,5)P_2 and the underlying actin cytoskeleton unmasks the villin dimerization site resulting in villin self-association (2). We hypothesize that this leads to increased local concentrations of PtdIns(4,5)P_2, F-actin and villin in membrane microdomains. Actin bundling by villin in these microdomains generates a new membrane protrusion as a filopodium (3). (**B**) The binding of actin and PtdIns(4,5)P_2 to villin induces a conformational change resulting in the exposure of the villin dimerization site, which promotes actin bundle assembly and/or clustering of PtdIns(4,5)P_2 to promote membrane deformation and filopodial assembly.

extracellular matrix and other surrounding cells. The ability of villin to regulate its self-association in response to changes in PtdIns(4,5)P_2, F-actin and tyrosine phosphorylation suggests that villin could regulate how the turnover of filopodia is regulated in response to extracellular signaling. The ability of villin to bundle actin filaments in filopodia could provide the rigidity required for filopodial assembly and maintenance. By contrast, the regulatory mechanisms that modify the ability of villin to bundle actin filaments in the filopodia could regulate the remodeling, turnover or inhibition of filopodial assembly within the cell. We have previously demonstrated that tyrosine phosphorylation of villin enhances actin severing but inhibits actin bundling by villin (Zhai et al., 2001). Thus, tyrosine phosphorylation of villin may be a signaling cue to activate the actin-severing, but inhibit the actin-bundling function of villin. We speculate that this may in fact be a physiologically pertinent molecular mechanism to retain non-phosphorylated villin within the filopodia and the tyrosine phosphorylated villin within the lamellipodia. Tyrosine phosphorylation of villin mediates conformational changes in villin and we speculate that these conformational changes may limit villin dimerization (Kumar et al., 2004). We suggest that changes in response to receptor activation leading to tyrosine phosphorylation of villin could provide a physiologically relevant mechanism to regulate the inhibition of villin self-association and therefore, filopodial assembly by villin. It may be noted that tyrosine phosphorylation and regulation of actin-severing by phosphorylated villin have been shown to regulate lamellipodial assembly and rates of epithelial cell migration (Athman et al., 2003; Tomar et al., 2006; Tomar et al., 2004). In this study we demonstrate that localization of villin to filopodia and lamellipodia can be functionally separated and that the actin bundling activity of villin is not required for the localization of villin to the lamellipodia. Changes in the actin modifying functions of villin from an actin-bundling to an actin-severing protein could shift the filopodial protrusion to lamellipodial protrusion and also redistribute villin between these two developing leading edge protrusions. Other functions of villin that are also regulated by PtdIns(4,5)P_2 such as the inhibition of actin-capping could also shift lamellipodial protrusion to filopodial protrusion (Mejillano et al., 2004). Together our data suggest that the function of villin may be in membrane protrusion and assembly of the filopodia when it functions as a ‘guide’, probing space ahead of the lamellipodium. However, villin may also be part of the filopodium which functions as a mechanical device ‘penetrating’ the environment and serving as a robust scaffold for the lamellipodial protrusion. This is consistent with our finding that villin regulates directionally persistent cell migration. This versatility may provide villin with the tools to regulate both the exploratory behavior of carcinoma cells and the fast moving speeds of metastatic cancer cells. The tissue-specific distribution of villin in epithelial cells suggests that these unique properties of villin may be particularly suitable for regulation of re-epithelialization and metastases.

Fascin is the best characterized actin bundling protein that is associated with filopodia. Most mammalian cells express additional actin bundling proteins many of which form parallel actin bundles and localize to the filopodia (Khurana and George, 2011). It remains unclear if fascin is sufficient for filopodium

formation or if multiple actin bundlers share this function in eukaryotic cells. In this study we demonstrate for the first time that villin shares the ability to assemble filopodia and regulate directional cell migration in epithelial cells that do not express fascin but also in transformed epithelial cells that express both fascin and villin. Since fascin is absent from normal adult epithelial cells and only expressed in some carcinomas, our study underscores the role of actin cross-linkers other than fascin in the assembly of filopodia in specialized cell types such as epithelial cells (Khurana and George, 2011). Since villin is more widely distributed in epithelial cells it is unlikely that fascin and villin have overlapping, redundant functions in filopodial assembly and regulation (Khurana and George, 2011). In this study we demonstrate that transformed epithelial cells that do express both villin and fascin such as colon carcinomas, downregulation of endogenous villin inhibits filopodial assembly and cell migration. In this context, it is noteworthy that overexpression of villin in fascin downregulated cells can rescue cell migration (Chen et al., 2010). The molecular mechanism of villin- and fascin-mediated cell migration appears different in these cells (Chen et al., 2010). We believe that together these findings have important implications for the role of villin in filopodial assembly in both normal epithelial cells that do not express fascin as well as in carcinomas that either lack fascin or express both villin and fascin. Compelling data supporting our hypotheses come from studies done in *Drosophila* where both fascin and villin have been shown to have non-overlapping functions and both proteins have been shown to be genetically required for actin bundle assembly (Guild et al., 2005).

In summary, our data demonstrate for the first time how actin bundling proteins such as villin serve as crucial molecules that function to converge distinct signaling cascades and thus, contribute to the sustained activation of cell surface protrusion. While the basic mechanisms of cell motility are reasonably well understood, how these mechanisms are coupled to the navigational mechanism that integrates extracellular signals with cytoskeletal remodeling to induce directional, persistent migration remains unclear. Our study reveals new modes and mechanisms for actin bundling proteins and their participation in the transduction of signals leading to the acquisition of this directionally persistent migratory phenotype. Moreover, they demonstrate that PtdIns(4,5)P_2-binding actin-bundling proteins could serve as scaffolds upon which new membrane deformations are built to initiate cell migration. The results of our study also highlight the role of tissue-specific actin regulatory proteins. Our study allows us to discover the true diversity of the actin-binding proteins suggesting that characterization of tissue- and cell-specific proteins will identify new members that assemble such structures in different cell types. We predict that a more comprehensive appreciation of the heterogeneity and plasticity inherent to epithelial cell motility will allow the prediction of the pathologic course taken by metastatic cells which would eventually assist in devising therapeutic strategies to circumvent metastasis.

Materials and Methods

Materials

MDCK Tet-Off and HT-29/19A cells were a kind gift from Dr Keith Mostov (University of California, San Francisco) and Dr A. P. Naren (University of Tennessee, Memphis, TN), respectively. Caco-2 BBe1 cells were purchased from ATCC (Rockville, MD) and HeLa Tet-Off cells from Clonetech. Ad-EGFP was a kind gift from Dr A. Hassid (University of Tennessee, Memphis, TN); adenovirus expressing recombinant HA-tagged PIP5-kinase Iβ and Iγ635 were a kind gift from Dr Hisamitsu Ishihara (Tohoku University Graduate School of Medicine, Sendai, Japan), PH-domain of human PLC-δ1 was a kind gift from Dr Edward Tall (State University of New York at Stony Brook), and SigD and mutant SigD (C462S) were a generous gift from Dr Sergio Grinstein (University of Toronto, Canada). Antibodies for villin and actin were purchased from BD Transduction Laboratories, pericentrin antibody from Abcam, HA antibody from Roche, fascin antibody from Millipore and, PtdIns(4,5)P_2 antibody from Assay Design. Phosphatidylcholine (PC), NBD (4-nitrobenzo-2-oxa-1,3-diazole)-PC and PtdIns(4,5)P_2 were purchased from Avanti polar lipids and lysophosphatidic acid (LPA) from Sigma-Aldrich. Matrigel invasion chambers were purchased from BD Biosciences. Oris cell migration assay kit was obtained from Platypus Technologies (Madison, WI), Alexa-Fluor-555-conjugated wheat germ agglutinin (WGA) was from Invitrogen and CyTrak from Biostatus. Replication-defective lentivirus transduction particles encoding control and villin shRNA were obtained from Sigma-Aldrich.

Preparation of unilamellar lipid and fluorescent NBD-tagged lipid vesicles

Unilamellar lipid vesicles were prepared using the procedure described earlier (Kumar et al., 2004). Fluorescent liposomes were prepared according to the method of Pontani et al. and Takiguchi et. al. (Pontani et al., 2009; Takiguchi et al., 2009).

Purification of recombinant villin

Glutathione *S*-transferase (GST)-tagged non-phosphorylated (VIL/WT) or tyrosine phosphorylated (pTyr-VIL/WT) recombinant villin were prepared and purified using the method described previously (Panebra et al., 2001).

Chemical cross-linking of recombinant villin and villin in cells

In vitro cross-linking of recombinant villin protein (20 nM) with DFDNB (0- to 100-fold molar excess) was performed as described by us previously (George et al., 2007; Kobayashi and Hearing, 2007; Tatu and Helenius, 1997) Villin in Caco-2 BBe1 and MDCK Tet-Off cells was cross-linked by incubating cells at room temperature with DFDNB (2.5 mM) for 30 min as described by us previously (George et al., 2007).

Stable transfection of cells

Preparation of stable clones of MDCK Tet-Off cells expressing SEYFP or cerulean-tagged VIL/WT or VIL/Δ21–67/112–119 has been described by us previously (George et al., 2007). Villin shRNA lentiviral particles were used to transduce HT-29/19A or Caco-2 BBe1 cells at a multiplicity of infection (MOI) of 1 for 16 h, followed by expression for 48 h. Transduced cells were selected in medium containing puromycin (2 μg/ml). For all studies with transfected cells, multiple clones of each cell line with similar protein expression levels or a mixed clone were used to avoid clonal variations. MDCK Tet-Off and Caco-2 BBe1 cells were cultured as described previously (Tomar et al., 2006; Wang et al., 2007).

Overexpression of PIP5-kinase and SigD

Replication deficient PIP5-kinase Iβ and Iγ635 adenovirus were amplified and purified from HEK 293 cells and optimum viral titer for expression was determined by infecting confluent Caco-2 BBe1 and MDCK cells with different aliquots of virus for varying time intervals followed by western blot using an HA antibody. Additionally, control cells were mock infected with Ad-EGFP. Sub-confluent MDCK cells were co-transfected with EGFP-actin and SigD or EGFP-actin and phosphatase-deficient mutant SigD (C462S) at a 5 to 1 ratio of SigD/SigD (C462S) to EGFP-actin. Transfected cells were identified by the expression of the fluorescent protein products.

Microscopic analysis

Fluorescence and phase contrast images were obtained by confocal laser scanning microscopy (LSM5 Pascal and Zeiss 700, Carl Zeiss, Thornwood, NY) using a 40× NA 1.3 (Plan NeoFluor) or 100× NA 1.4 oil (Plan Apochromat) objectives. Calculation of corrected FRET (FRETc) was carried out by using the Zen software and N-FRET (Xi) module. Appropriate controls were included to account for the bleed through of cerulean and SEYFP through the FRET filter channel as described previously (George et al., 2007). Images are displayed in pseudocolor mode, where white and black areas display high and low values of FRET in the range of 0–200 relative light units, respectively. FRET was calculated from at least 10 cells in three independent experiments. FRET was performed on cells in the absence of DFDNB.

Cell motility assay

Cell motility was studied using the Oris cell migration assay as described by the manufacturer (Platypus Technologies, LLC., Madison, WI). Cells were stained using Alexa-Fluor-555-labeled wheat germ agglutinin (WGA555) and images were taken 7 h post-migration. Control refers to samples in which the plug was not removed. In addition, cell motility was measured using a wound healing assay for epithelial cells (MDCK and HT-29) as described in our previous studies (Wang

et al., 2007). Cells were stimulated by the addition of LPA (2 μM). Images were collected at the initial time of wounding and at various intervals up to 7 h with a Nikon Eclipse TE2000-U inverted microscope, equipped with a CoolSnap ES charge-coupled device (CCD) camera, an optiscan motorized stage system. Images were analyzed by using Metamorph image analysis software as described previously (Tomar et al., 2006). The modified Boyden chamber assays were performed as previously described (Wang et al., 2007).

Persistent migration assay

Time-lapse images of MDCK Tet-Off cells expressing wild type and mutant villin proteins were collected post wounding at 5-minute intervals over a period of 16 h. Cell positions were recorded every ten frames using Track Object tool in Metamorph. Velocity of cells was determined according to the method of Pankov et al. (Pankov et al., 2005). To directly measure directional persistence the correlation between the angles of velocity vectors was calculated as a function of their separation along the trajectory L. The velocity at each point in the trajectory was calculated as the net displacement divided by time, $\mathbf{v}_i=(\mathbf{x}_{i+1}-\mathbf{x}_i)/\Delta t$. The angle θ between two velocity vectors $\mathbf{v}_i$ and $\mathbf{v}_j$ was calculated from the dot product, $\cos\theta=\mathbf{v}_i\cdot\mathbf{v}_j/\|\mathbf{v}_i\|\|\mathbf{v}_j\|$, as a function of the distance L between their positions ($\mathbf{x}_i$ and $\mathbf{x}_j$). For trajectories in which correlation in the direction of motion is lost, the average angle between velocity vectors (cosθ) decreases exponentially with distance along the trajectory L, i.e. $\cos\theta=\exp^{-L/L_p}$, where L_p is defined as the persistence length of the trajectory. To quantify the impact of loss of directional persistence on the shape of trajectories, the tortuosity of a trajectory segment was calculated as a function of the time interval Δt between segment end points (Pankov et al., 2005). Calculations were carried out using routines written in IDL (ITT VIS, Boulder, CO). Error bars for both calculations represent the 95% confidence interval for each measurement; statistics were generated by averaging over intervals of L or Δt over multiple trajectories for each strain. Finally for isolated cells that exhibited nearly diffusive-like motion the mean-square displacement $\langle\Delta x^2(t)\rangle=\langle(x(t+\tau)-x(\tau))^2\rangle$ was calculated as a function of time t; the average is taken over all starting times τ.

Quantification of the formation of lamellipodia and filopodia

Filopodium initiation rates were determined from randomly chosen cell peripheries over a period of 20 min after the addition of LPA (2 μM). Newly formed filopodia were marked and counted; 30 cells were counted for each group.

Determination of microtubule-organizing center polarization

Cells were seeded on coverslips, and wounded monolayers were fixed 7 h after wounding. The localization of the MTOC was determined by immunolabeling using pericentrin antibody. The cell nucleus was stained by CyTRAK following the manufacturer's protocol. The wound edge was observed by phase-contrast images. Cells in which MTOC was contained within the quadrant facing the wound were scored as positive (Palazzo et al., 2001).

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http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.116244/-/DC1

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