Vimentin supports directional cell migration by controlling focal adhesions

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Abstract

Fibroblastic migration is of key importance in wound healing. While the intermediate filament (IF) protein vimentin is required for normal wound healing, we examined whether vimentin-mediated regulation of fibroblast migration could be involved. In wound healing assays triggering cell polarization and directed migration, we observed that vimentin-deficient mouse and rat embryonic fibroblasts lost their directional persistence. We show that vimentin maintains directionality by guiding focal adhesions (FAs) in fibroblasts. Detailed analysis showed that vimentin stabilizes FAs and regulates their disassembly rate. The destabilization of Vim-/- FAs was reflected by smaller FAs. Live cell and super-resolution imaging demonstrate that vimentin interacts dynamically with the key molecules of FAs and, importantly, with FAK, which is crucial for the maturation of FAs. These results demonstrate that vimentin IFs control the maturation, stability, dynamics, arrangement, and overall orientation of FAs, with a net effect on FA coordination during migration.

Introduction

Cell migration is essential to a range of key physiological processes, such as immune response, wound healing, cancer metastasis, and embryonic development (Chernoivanenko et al.,2013; Conway & Jacquemet, 2019). Cell migration is enabled through highly dynamic and concerted interactions between cytoskeletal and adhesion proteins, the joint maneuvering of which will regulate adhesive properties of cells, determine cell polarity, and the speed and direction of cells. Directional cell migration is maintained by the coordinated interplay between different cytoskeletal systems and their interactions with focal adhesions (FAs), which maintains a spatiotemporally sustained movement of the cellular projections at the leading edge (Seetharaman & Etienne-Manneville, 2020).

The intermediate filament (IF) protein vimentin has been shown to be involved in cell migration in a number of instances that relate to wound healing and cancer progression (Battaglia et al., 2018; Cheng et al., 2016; Satelli & Li, 2011). Vimentin is expressed in fibroblasts, endothelial cells, lymphocytes, and several specialized cells, all of which are characterized by active adhesion-dependent migratory functions and all of which arise from the migratory cells of developing embryos. Vimentin has also been established as a hallmark of epithelial to mesenchymal transition in several physiological processes (Cheng et al., 2016; Ivaska et al., 2011; Virtakoivu et al., 2015).

While vimentin knockout experiments initially revealed mild a phenotype in Vim-/- mice (Colucci-Guyon et al., 1994), subsequent studies have shown that these mice exhibit a broad spectrum of distinct phenotypes, involving a wide range of deficiencies related to maintenance of stemness, differentiation, proliferation, mammary gland development, angiogenesis, vascular stiffness, steroidogenesis, glia development, myelination of peripheral nerves, adhesion, migration and invasion (Cheng & Eriksson, 2017). The mice have a prominent defect in wound healing (Cheng et al., 2016; Eckes et al., 2000), which has been shown to be due to strongly impaired fibroblast growth and migration at the wound edge, as well as inhibited TGF-β signaling, and epithelial to mesenchymal transition (Cheng et al., 2016). Furthermore, Vim-/- fibroblasts have exhibited delayed migration towards the wound edge (Cheng et al., 2016; Eckes et al., 2000). Leukocytes, astrocytes, and different types of cancer cells also show improper motility when vimentin is absent (Satelli & Li, 2011).

Directional persistence is important for motile cells to reach a particular destination inside the organism in response to signaling cues (Ridley et al., 2003). A key factor determining cell directionality is the polarity of the cell (Etienne-Manneville et al., 2013; Zhang et al., 2014). Several recent studies have shown that IFs have a key role in determining migration, cell directionality and cell-ECM in fibroblasts (De Pascalis et al., 2018; Seetharaman & Etienne-Manneville, 2020), but the underlying mechanisms for these observations remained to be determined.

Early on, it was shown that vimentin interacts with actin and microtubules (MTs). Vimentin has been shown to associate with MTs through interaction with the motor proteins kinesin and dynein, an interaction which has been postulated to enable vimentin to function as a "template" for MT organization (Gyoeva & Gelfand, 1991; Helfand et al., 2002).

Give the previously indicated multifactorial roles of vimentin in migrating cells and that IFs in general have been implicated in determining cell migration directionality, we wanted to specifically address the role of vimentin in fibroblasts directionality. Fibroblasts have a straightforward IF composition, with vimentin as the only type-III IF protein. As previous studies have focused on interactions with actomyosin complexes and MTs, we wanted to specifically address how vimentin regulates FAs during directional cell migration. Although vimentin has been shown to associate with FAs and in some cell types to interact with integrins (Burgstaller et al., 2010; Ivaska et al. 2005; Strouhalova et al., 2020), the mechanistic links between vimentin cytoskeleton, cell adhesion dynamics, and the cellular forces acting on the ECM remain poorly understood. Here, we show how vimentin regulates directionality of cell migration by regulating the dynamics, orientation, and composition of FAs.

Results

Vimentin regulates directional cell migration

To examine the effects of vimentin on directional persistence required for wound healing in detail, we performed scratch wound assays using WT and Vim KO mouse (MEFs) and rat embryonic fibroblasts (REFs) to replicate the wound healing process at the cellular level in vitro and to induce polarization of cells that become stimulated to migrate (Fig. 1A, B; Video 1A, B). The cells were allowed to migrate for 24 hours, and single cells were then manually tracked. The tracking analysis showed a lack of direction persistence in both Vim-/- MEFs and REFs (Fig.1 C; quantitation of MEF directionality in Fig. S1A). To investigate whether the directional deficiency effect is specific for the polarized cells in wound healing assays, we also performed random migration experiments with sparsely populated cell cultures. We observed a similar lack of directionality with both mouse and rat fibroblasts when random cell migration experiments with sparsely populated cultures were performed (Fig.1 B, D), demonstrating that the loss in directionality is intrinsic and not stimulated. Surprisingly, while the Vim-/- fibroblasts lost direction during scratch wound migration, there was no significant difference in the speed of migration of vimentin knockout fibroblasts compared to their wild type counterparts, although there was a tendency for a slightly higher mean migration speed in Vim-/- fibroblasts (Fig. S1 B).

To study whether cell matrix –mediated guidance can be involved in these observations, we seeded the cells on cell-derived matrix (CDM) that was obtained from WT MEFs. Interestingly, while WT MEFs showed stringent directionality on CDM (Video 2A), Vim-/- MEFs showed no directionality at all when plated on the CDM, in fact, with such lack of directionality that Vim-/- MEFs seemed to disarrange the CDM organization (Video 2B; Fig. S1 C) as compared to WT MEFs (Video 2B), the movements of which were aligned with the organization of the CDM (resulting in further alignment). Taken together, these results demonstrate that vimentin is important in maintaining intrinsic directionality of a migrating cell in different environments/stimulated conditions.

Vimentin interacts with maturing and retracting adhesions

To study whether the observed loss of direction could be due to vimentin interactions with FAs, we examined the interaction of vimentin with FAs during cell migration. To this end, live cell imaging of Vim-/- MEFs transfected with mCherry-tagged vimentin along with either Emerald-tagged paxillin or GFP-tagged FAK was performed. The cells were imaged with a TIRF microscope in random migration assays. Vimentin IFs and unit length filaments (ULFs) were observed to be in proximity with both GFP-FAK and Emerald-Paxillin in FAs (Fig. 2 A, B) residing at both maturing and retracting FAs (Video 3 A, B). Apart from the interactions of FAs in protrusions of lamellipodia, we also found interactions of vimentin with nascent adhesions in filopodia and, importantly, also with FAs at the other end of the cell, i.e., the retracting edge of the migrating cells. These findings are consequential considering the crucial role of FAs in maintaining the net traction force distribution that in turn contributes to the directional persistence of migration. FAs at the retracting edges of a migrating cell are as important as the FAs in the protrusions or the leading edge of a migrating cell. The balanced distribution of the net contractile force generated by actomyosin machinery can be attributed to the control of FAs in all parts of a migrating cell. Interaction of vimentin with FAs in retracting edges along with FAs in leading edge suggests strongly that there must be an active control of net force distribution mediated by vimentin across a migrating cell to maintain the direction. This finding is important since FAK is crucial for the maturation of FAs.

To determine whether vimentin enters the nanostructure of FAs, 3D STED super-resolution microscopy was employed. The STED images showed XY and XZ plane colocalization of vimentin with the FAs components pFAK (Fig. 2C, Fig. S2A), paxillin, vinculin (Fig. 2D; Fig. S2B). The STED images show that vimentin IFs and ULFs both enter the FAs and are also often reaching for the tip of FAs, which corresponds well to results showed in Movie 3 (A, B, C). In this way, it is clear that vimentin filaments enter FAs. Based on the selective nature of the co-localization of vimentin with FAs, it can be inferred that vimentin controls select FAs critical for sustaining net directionality of the migrating cell (Video 4).

Vimentin is required for FA organization and stability

To study whether vimentin affects the orientation and the dynamics of FAs during directional migration, WT and Vim KO fibroblasts transfected with Emerald-Paxillin were live imaged in a scratch wound assay, followed by analysis of time-lapse movies by employing the FA analysis server (FAAS) (Berginski & Gomez, 2013). Leader cells on the scratch wound edge were selected for analysis.

To analyze whether vimentin affects the orientation of FAs, FAs in time-lapse videos (Movie 4) was analyzed using FAAS (Berginski & Gomez, 2013; Wu et al., 2012). Fig. 3A shows representative images of the time-dependent change in FAs, by superimposing all time-lapse images of a single migrating cell. While there was a clearly coordinated spatiotemporal progression of FA development in migrating WT cells, Vim-/- fibroblasts, in contrast, showed seemingly random progression, which is evident from the lack of uniformity of FAs translocation in a consistent direction between initial time point (blue) and final time point (red). A selection of how this effect is manifested in live cells is shown in Movie 4. Hence, Vim-

/- MEFs possess completely disorganized and disoriented FAs during directed migration. Global FA alignment was measured using FAAI as previously described (Wu et al., 2012). FAAI is calculated with the formula 90-(SD of FA angles) where 90 degrees angle is taken as a reference angle to measure the dominant FA angle of a cell.

When the temporal variation of the FA alignment index calculated by formula 90-(SD of FA angles) was analyzed, we observed that the FAAI of Vim-/- MEFs showed a significantly higher degree of variation compared to WT MEFs (Fig. 3 B), which agrees with the analysis in Fig. 3A. The drastically increased variation of FAAI value (SD) indicates a complete loss in the coordinated alignment of FA angles in Vim-/- MEFs across a given sample population. Fig. S3A shows the variation in FAAI of a sample population over time as each cell in the sample migrates directionally. Fig.3C represents the data of Fig. S3A reduced along the time dimension to highlight the observed striking variation in FAAI when vimentin is absent. This is also further supported by analysis of birth angles of newly formed FAs (Fig. S3 B). While WT FAs demonstrate periodicity of birth angles at the instance of their formation, this periodicity is not obvious in Vim-/- MEFs. Thus, the predominantly selective development of FAs at preferential angles from birth in WT seems to be coupled to the active control exerted by vimentin. This preference directly leads to the decrease in FAAI variation observed in WT (Fig. 3B, Fig. S3A). These results conclusively demonstrate that the FA alignment is practically lost in the absence of vimentin, implying that the observed vimentin-FA interaction and interplay is reflected as an overall coordination of the FAs.

In the live cell movies (representative videos; Video 5 A, B), involving analysis of more than 2000 individual FAs, the quantitative analysis of the dynamics of both WT (Video 5A) and Vim-/- MEFs (Video 5B) showed that the focal disassembly rate was significantly higher in Vim-/- MEFs compared to WT MEFs (Fig.3C), although their assembly rate was unaffected (data not shown). This implies that the stability was clearly reduced in Vim-/- MEFs, resulting in altered FA dynamics. To further analyze this effect, the FA stability was quantified by measuring the recovery rate of fluorescence intensity after photobleaching (FRAP) of paxillin using ectopically expressed emerald-paxillin in MEF WT and Vim-/- MEFs. The obtained FRAP curves for MEF WT and Vim-/- MEFs revealed that vimentin deficient cells possess FAs that have a significantly faster recovery rate (Fig. 3D, E). At the same time, the half time for FA recovery after bleaching for MEF WT was significantly higher (median ≈1 sec) compared to Vim-/- MEFs (median≈ 0.5 sec) (Fig. 3 F). Correspondingly, Vim-/- MEFs exhibit a higher mean fold change of FA components during the stability phase (Fig.3G) of FA development gathered from TIRF imaging data. Mean fold change denotes the mean fold increase in FA intensity from the beginning of the stability phase over time. These results demonstrate that Vim-/- FAs exhibit significantly reduced stability, and the faster recovery rate translates into faster turnover of FA components that could be the cause for faster disassembly.

Taken together, these findings demonstrate that vimentin is required to maintain the spatiotemporal dynamics and orientation of FAs towards the scratch wound edge during directional migration. The faster turnover and disassembly of FA observed in Vim-/- cells suggests a loss of sustained global behavior of FAs across the migrating cell which is required to keep up the directional persistence of migration. The faster turnover may also explain the slightly elevated migration speed observed in Vim-/- cells. The disorientation of individual FAs

during the course of migration is likely to be the prime reason for the lost directionality of individual cells.

Vimentin deficiency causes reduced FA area

There are several molecular pathways that work in concerted coordination to keep directional persistence of a cell during migration. To understand which components of the molecular machinery in the FAs are affected by the absence of vimentin, key proteins in the FA complex of the migrating fibroblasts were imaged for quantitative analysis. WT and Vim-/- fibroblasts were fixed after 4h in a scratch wound assay and confocal microscopy was used to determine the number of FAs and their mean area. The quantitation of the FA number and mean FA area was performed in cells immunolabeled for paxillin, as paxillin is an FA protein present in most types of FAs (Fig. 4 A). Image analysis revealed that whereas the number of FAs was similar in both WT and Vim KO fibroblast (Fig. 4 B), the mean FA area was significantly reduced in Vim KO fibroblasts (Fig.4 C). The faster disassembly rate described above (Fig. 3B) seems to be reflected as a reduced FA area and demonstrate that the presence of vimentin is also required to stabilize the size of FAs.

Vimentin guides FAs to maintain the directional persistence

Our results show that vimentin acts to coordinate the FA orientation to yield net directionality of cells. The assumed modus operandi suggest coordination of an FA-based all-wheel drive of migrating fibroblasts to maintain directional persistence (Fig. 5). Previous studies show that vimentin IFs interact with actomyosin complexes of the cell migration machinery (Jiu et al., 2015, 2017) and here we show that vimentin interacts with critically involved FA proteins either directly or indirectly. In summary, the bidirectional interaction of vimentin with, as previously shown, the actin arcs and stress fibers (Jiu et al., 2015, 2017) and, in this study, with the FA complexes, yields the proper contractile and traction forces as well as coordination of the FAs that is required for the net directionality of a migrating cell.

Discussion

The mechanisms through which vimentin controls cell migration and especially cell directionality is still obscure. Here, we show that vimentin is required for directional persistence and that both long filaments and short squiggles, interact with FAs in a way that determines their maturation, stability, dynamics, arrangement, localization, and overall orientation in migrating cells. Vimentin squiggles move in a retrograde flow towards the rear end of the cell to interact with the FAs and in the absence of vimentin, the average size of FAs are dramatically reduced. Together, our results reveal a role for vimentin as a regulator of the dynamics and localization of FAs and also uncover how the interplay between vimentin and FAs controls cell directionality.

The longer lifespan of vimentin IFs has been indicated to be an excellent basis to form a memory that maintains the polarity in a migrating cell (Gan et al., 2016). Vimentin controls cell polarity also by helping to orient and redistribute the traction forces generated by the contraction of actomyosin bundles (Costigliola et al., 2017). FAs have crucial role in this process since the traction stress is exerted on the extracellular matrix (ECM) via actin stress fibers attached to FAs. FAs consist of more than 150 components (Zaidel-Bar et al., 2007). They are indispensable for signaling, cytoskeletal reorganization, and force transduction from and

to ECM (Kanchanawong et al., 2010). Some preceding studies have indicated vimentin to be recruited to a subset of FAs via plectin1f linker protein (Burgstaller et al., 2010). The vimentin-associated FAs have been shown to exhibit higher adhesion strength and this is mediated by the presence of β3 integrin which is crucial for recruitment of vimentin to the FAs (Bhattacharya et al., 2009).

Vimentin may act directly or indirectly via the linker protein plectin to control the recruitment and stabilization of FA proteins and also contribute to the mechanical coupling between FAs and actin stress fibers. Uncoupling vimentin from FAs via plectin depletion hampers directional cell migration (De Pascalis et al., 2018; Gregor et al., 2014). Accordingly, vimentin binds to actin both directly and also indirectly through plectin (Osmanagic-Myers et al., 2015), which, in turn, has been shown to enable a bidirectional interaction between vimentin and transverse actin arcs during retrograde actin flow. This bidirectional interaction will impact the lamellipodial integrity (Jiu et al., 2015). The regulation of FAs by vimentin has also been shown to involve the actin-binding protein filamin A (Kim et al., 2010). Vimentin hitchhikes on actin transverse arcs and this retrograde flow towards nuclear periphery is enabled via plectin1F linker protein and restricted by F-Actin (Jiu et al., 2015). This helps in regulating the turnover of the vimentin filaments and its transport from cell periphery to the nuclear periphery and the rear part of the cell.

Our data show that both vimentin long filaments (Fig.2 A) and squiggles (Fig.2 B) associate with the maturing and disassembling FAs in anterograde and retrograde directions. These FA-vimentin interactions were not limited to leading edge of the migrating cell but were also observed in the retracting edges of the cell. To the best of our knowledge, this kind of interactions have not been reported elsewhere. Maintenance of an active control of FA dynamics and orientation across the cell periphery is crucial for balanced distribution of contractile forces created by stress fibers connected to the FAs. Vimentin precursors have been reported to be immobilized by plectin 1f protein in a subset of FAs (Burgstaller et al, 2012) and that this immobilization helps the assembly of long filaments. It is plausible that there is an intermediate process that links the squiggles or vimentin foci to interact with the FAs on cell periphery, to be further transported to the actin transverse arcs. This cycling of vimentin squiggles/foci might involve: the immobilization to the FAs, further transport to actin transverse arcs, followed by the retrograde flow to the nuclear periphery.

Our data (Fig 3) suggests that FAs of wild type cells have higher stability than the knockout cells lacking vimentin as supported by previous literature (Tsuruta et al, 2003). While the disassembly rate of FAs in knockout cells was significantly higher, vimentin appears to control FA turnover, which is also supported by the data on the FRAP recovery rate and half time recovery measurements. The faster disassembly rate must be creating inefficient duration for holding the FA components in place to maintain the stability that is required for proper directionality, as clearly indicated by the disorientation of FAs when vimentin is absent. The stability and orientation of FAs go hand in hand (Berginski, M. E., & Gomez, S. M, 2013). Hence, a certain degree of FA stability is required for the spatiotemporal localization of the traction forces generated by the stress fibers connected to the FAs (Costigliola et al, 2017). Our study

shows that vimentin has a major impact on these aspects crucial for directional persistence by stabilizing FAs.

While there was no difference in FA numbers between WT and Vim KO fibroblasts, the mean FA area was significantly reduced in Vim KO fibroblasts (Fig 4C). The reduced area links vimentin to FA stability maintenance. It is tempting to assume that the close interactions with FAK could be related to promotion of Y397 autophosphorylation site of FAK. This is supported by the previous literature where it was shown that vimentin is required for the activation of FAK through VAV2-Rac1 pathway in lung cancer cells (Havel et al., 2014). Vimentin has also been associated with adhesion regulated signaling as it is crucial for the PKCε-mediated β1 integrin recycling that maintains cell adhesion (Ivaska et al., 2005). Vimentin can directly interact with β4 integrin containing adhesions leading to recruitment of Rac1 promoting actin polymerization and directional cell migration (Colburn & Jones, 2018). Vimentin also can bind to the cytoplasmic tail of β3 integrin (Bhattacharya et al., 2009) and might directly affect integrin signaling and the connection to actin and MTs. In addition, vimentin can induce the phosphorylation of the MT-associated GEF-H1 at Ser886 leading to RhoA activation and regulating stress fiber assembly (Jiu et al., 2017).

In summary, several studies have indicated vimentin having a multifactorial role in migrating cells. Furthermore, as IFs in general have been associated with cell migration directionality, we wanted to specifically address this question in fibroblasts, as they are dynamically migrating cells that play a key role in wound healing, which is seriously affected in vimentin-deficient mice. Fibroblasts have a straightforward IF composition, with vimentin as the only type-III IF protein. As previous studies have focused on interactions with actomyosin complexes and MTs, we wanted to specifically address the roles of vimentin IF in determining the performance and orientation of FAs during directional cell migration. Our results reveal functional interplay between the FAs and vimentin filaments during directional migration of fibroblast cells in a stimulated directional migration setting. Both vimentin long filaments and squiggles affect the dynamics, localization, and the orientation of FAs. Vimentin do not appear to affect the assembly rate since the assembly rate was similar in vimentin-deficient cells. Nevertheless, there is a significant influence of vimentin in maintenance of FA stability, size and orientation as Vim KO fibroblasts exhibited higher FA disassembly rate and lower average FA area. We propose a new model, as depicted in Figure 5, that vimentin acts as an active nano driver of the FA wheels across the cell body of a migrating fibroblast that helps to maintain the directional persistence via balanced distribution of the net contractile force. it Our results indicate that that vimentin IFs stabilize especially the axial arrangement of distinct FA layers. In this way, IFs and FAs would jointly form a signaling and mechanotransduction hub to generate cell-wide synchronization of FA organization to enable directional persistence, similarly as vimentin has been suggested to have memory function to maintain the polarity of a migrating cell (Gan et al., 2016).

In the future, it will be important to examine the exact role of vimentin IF networks and their interplay with FA complexes, in cell directionality maintenance in a three-dimensional environment, as well as to uncover the detailed molecular signaling and other mechanisms underlying vimentin-mediated FA formation, disassembly, and the arrangement of FA

proteins in different vertical compartments such as integrin signaling layer, force transduction layer and actin regulatory layer.

Materials and Methods

Cell lines, antibodies and plasmid constructs

MEF WT and Vim-/- cells were isolated from wild type mice and vimentin knockout mice respectively in our laboratory (Virtakoivu et al., 2015b). They had previously been immortalized with SV40 transfection lentiviral vector. REF WT and Vim-/- were prepared using CRISPR Cas9 system as described earlier (Vakhrusheva et al., 2019). All the cells were cultured in DMEM media with high glucose (# D5671, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (Biowest), 2 mM L-glutamine, 100 U ml-1 penicillin, 100 µg ml-1/ streptomycin. Plasmid constructs of emerald-Paxillin and GFP-FAK were obtained as. mCherry empty vector (EV), vimentin wild-type (WT) and vimentin S71A plasmids were kind gifts from Hong-Chen Chen (Department of Life Sciences and Agricultural Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan) (Pan, Chen, & Chen, 2011). Antibodies used were: vimentin chicken polyclonal (# 919101, Biolegend), phosho-FAK (Y397) rabbit polyclonal (Cat# 44-624G, Thermo Fischer Scientific), phosho-FAK (Y397) rabbit monoclonal (Cat# ab81298, Abcam), Anti-fibronectin antibody (Sigma-Aldrich, Cat# F3648) Anti-collagen I alpha 1 antibody (Novus Biologicals, Cat# NB600-408), mouse monoclonal vinculin (Sigma Aldrich, Cat# V9131), rabbit monoclonal paxillin (Abcam, Cat# ab32084), rabbit monoclonal FAK (Thermo Fischer Scientific, Cat# 701094), Anti- rabbit Alexafluor488, Anti-rabbit Alexafluor555, Anti-rabbit Alexafluor647, alpha-tubulin rabbit polyclonal (CST), Alexafluor Abberior Star Phalloidin.

Scratch wound assay

For measuring the speed and directional persistence of the migrating cells, WT and vimentin KO fibroblasts were plated in 6 wells plate (Falcon) till confluency. A scratch was made using 10 µl pipette tip and the cells were allowed to migrate for 24 hrs at 37 C and 5% CO2. The scratch area was imaged every 10 mins using CellIQ live imaging machine from Chipman Technologies, Finland. The time-lapse movies obtained were used to track the individual cells for obtaining the single cell trajectories, speed, and directionality.

Transfections

Transient transfections for all the cell lines were performed using Xfect transfection reagent from Takara (Cat# 631318) in 6 well plates (Falcon) as per manufacturer's recommendations.

Cell Derived Matrix generation and Directional Migration Assay Cell derived matrix was generated from WT MEFs in 24 well plate format as described (Kaukonen et al., Nature Methods, 2017).

Immunolabeling and immunofluorescence

Cells were grown on glass coverslips until confluency. Using 10µl pipette tip confluent cell layer was scratched and allowed the cells to migrate. Cells were fixed using 4% PFA for 10 min at 4 hr time points. Cells were washed with PBS 3X and then permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT, washed in PBS 3X, blocked with 5% Goat serum in PBS for 1 hour in RT. Then the coverslips were incubated on top of 100ul drops of primary antibody in 5% Goat serum in PBS for 1 hour at RT, dip washed 10X in PBS, incubated on 100ul drops of secondary antibody for 1 hour at RT, dip washed the coverslips using forceps 10X in PBS, rinsed in MilliQ water 3X and mounted with Mowiol mounting media. Samples were imaged with 3i CSU-W1 spinning disk microscope equipped with a Hamamatsu sCMOS Orca Flash4.0 camera using 60X /1.4NA plan-apochromat oil immersion objective and Abberior STED microscope equipped with a Photo Multiplier Tube using 100x/1.4NA Olympus UPLSAPO oil immersion objective.

Live cell imaging

For investigating the FA turnover, WT and Vim-/- fibroblasts were transfected with emerald-paxillin and allowed to reach confluency on a 22 mm coverslip. Upon reaching confluency and after 48 hrs of transfection, the coverslip was transferred to Atto Fluor (Thermo Fischer Scientific) live cell imaging chamber. Complete DMEM growth media was added and a scratch was made using 10ul pipette tip. This media was replaced with live imaging media (1ml) from Invitrogen supplemented with L-glutamine, Pencillin, Streptamycin and 10% FBS and kept in the incubator for about 1h to allow the cells to begin migration. TIRF live imaging was performed on DeltaVision OMX with a Ring-TIRF system equipped with 3x Front illuminated sCMOS using 60x/1.49 NA Olympus APO N TIRF objective using 488 laser excitation. Imaging was done for 5-8 cells per imaging sessions of 1 hr with an interval of 20 secs, exposure time of 100-150ms, laser power at 1%, frequency of 96.5 Mhz, and image size of 1024x1024 pixels.

For investigating the FA interaction with vimentin, Vim-/- fibroblasts were transfected with emerald-paxillin/GFP-FAK and mcherry-vimentin. Vim-/- fibroblasts were chosen instead of WT fibroblasts to avoid vimentin overexpression artifacts. TIRF imaging was performed for 15 min with an interval of 3 secs, exposure time of 100-150ms, laser power at 1%, frequency of 96.5 Mhz, and image size of 1024x1024 pixels. Excitation wavelengths of 488nm and 568 nm were used for emerald-paxillin/GFP-FAK and mcherry-vimentin, respectively. For confocal live imaging, a Zeiss LSM880 equipped with 32-channel Airyscan Hexagonal element using 40x/1.2 NA Zeiss LD LCI Plan-Apochromat water immersion objective using 488 and 561 laser lines for Emerald-paxillin and mcherry-vimentin, respectively. The samples were imaged for 1h at 1 min intervals with optimal z spacing of 0.18 microns. All live imaging was performed in a temperature-controlled chamber at 37 C and 5% CO2, equipped with a humidifier.

For measuring the turnover of FA components, Emerald-paxillin transfected WT and Vim-/- cells were imaged with Zeiss LSM880 scope with 40x/1.2 NA Zeiss LD LCI Plan-Apochromat water immersion objective. Imaging was performed 24-48 h post transfection with 488nm laser line at 2% power. The settings including zoom and rotation were adjusted to include FAs, background, and cytoplasmic staining in each field of view and ensure that the time period of

scanning was 100ms. Imaging was performed for 30 iterations with 2 pre-bleach and 28 post bleach scans, using rectangular areas on FAs for bleaching with 100% laser intensity. Intensities of bleached, background and reference regions over time were measured in ImageJ. Cytoplasmic region devoid of any FAs were used as reference region. The analysis was performed using easyFRAP standalone software (Rapsomaniki et al., 2012). The data was fitted with a single exponential fit.

Image Analysis and Statistics

Fixed cell images were analyzed using ImageJ (Collins, 2007; Schindelin et al., 2012) and custom-made macros. TIRF movies were analyzed using FA Analysis Server (FAAS) (Berginski & Gomez, 2013). GraphPad Prism was used for statistical analysis. Analysis was performed using Welch's t-test and Wilcoxon rank-sum test.

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Author contributions

Experimental design was prepared by Arun P Venu and John E Eriksson. Scratch wound experiments and image analysis were performed by Arun P Venu. TIRF live imaging experiments were performed by Arun P Venu, Mayank Modi, and related data analysis were performed by Arun P Venu and Mayank Modi. Spinning Disc imaging and related image analysis were performed by Mayank Modi. STED imaging was performed by Arun P Venu with assistance from Elena Tcarenkova and Ujjwal Aryal. Alexander Minin provided the rat fibroblasts and some of the data with those fibroblasts. Advice and assistance were provided by Guillaume Jacquemet, Yaming Jiu, and Fang Cheng. The manuscript was written by Arun P Venu, Mayank Modi, and John E Eriksson. All authors revised and edited the manuscript. John E Eriksson acquired the funding.

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Figure Legends

Fig.1. Vimentin is required for fibroblasts directional persistence. (A) Representative images of scratch wound assays and (B) random migration assays performed using mouse (MEF WT and MEF Vim KO) and rat (REF WT and REF Vim KO) fibroblasts. Single cell trajectories are shown with varying colors (A, B). (C) Quantification of directionality of MEF WT and MEF KO cells measured from CellIQ Imagen software. Numbered trajectories are shown for different individual cells in the scratch wound assay demonstrate complete loss of directionality in Vim-/- MEFs (quantitation in Fig. S1) and REFs. (D) The same effect is observed in the single cell trajectories of individual cells in random migration assays. N= 8-12 cells, repeats=3.

Fig 2. Vimentin interacts with maturing and retracting adhesions during directional migration. (A, B). Representative timeframes from live imaging of MEF Vim KO cells transfected with mCherry vimentin filaments (in magenta) and FAK-GFP (in green) interacting with each other in both maturing and disassembling FAs Imaged every 3 secs for 15 mins using Deltavision OMX TIRF microscope. (C)Representative images of endogenous vimentin colliding with paxillin and FAK in MEF WT cells captured using Abberior STED microscope. Upper row in (C) shows the confocal overview and the lower row shows the STED image of the FA marked by the square box in confocal overview. Similarly, in HDF WT cells vimentin colliding with vinculin and paxillin is shown (D). N=8-12 cells, repeats=3. Scale bar: 1 µm.

Fig 3. Vimentin-deficiency disorganizes and destabilizes FAs during directional migration. (A) Representative images of superimposed frames of time-lapse movie of MEF WT and MEF Vim KO cells migrating in a scratch wound edge. The figure represents a cumulative illustration of the spatiotemporal coordination of FA growth and maturation during directional migration, with the change in color from blue to red indicating the progression from start to end of the time-lapse period. Imaging was performed every 20 secs for 1 hour using TIRF microscopy. (B) Distribution disassembly rates of FAs of MEF WT and MEF Vim-/- cells. Number of adhesions > 2000 for both MEF WT and MEF Vim-/-; number of cells=30, repeats=3. (C) SDs of FA Alignment Index (FAAI) of multiple cells at every time point during time-lapse imaging represented in the box plot labelled temporal variation in FAAI. Mean FRAP recovery curves of individual FAs as shown for MEF WT (D) and Vim-/- (E), respectively. (F) Distribution of Half time of recovery post-bleaching as evident from the FRAP curves, N=10 cells.(G) Distribution of FA stability phase half time (in min) calculated by the difference between the half time of the peak intensity of assembly and disassembly phases for individual FAs.

Fig 4: Vimentin deficiency causes reduced FA area.(A)Representative spinning disk confocal images of FAs of MEF WT and MEF Vim KO cells in the scratch wound edge. FAs are immunostained by paxillin antibody. Each image is a maximum intensity projection of all the z-stacks. Scale bar: 10 µm. (B) Quantification of FA count and mean FA area (µm2) of MEF WT (N=68) and MEF Vim KO cells (N=73).

Fig 5: Vimentin steers FAs. Illustration summarizing the role of vimentin in the maintenance of directional persistence by modulating the orientation, size and arrangement of FAs. FAs (in dark orange) assemble and disassemble in coordinated fashion in required locations for the perpetual maintenance of net traction force created from the contractility of actomyosin

stress fibers (5B, in blue). This organization and the orientation of FAs are directly or indirectly modulated by vimentin (in magenta) as shown in 5B. Both long vimentin filaments and unit length filaments (ULF) interact with FAs as in 5B. The interaction of vimentin with actomyosin stress fibers enables the buffering of traction force exerted on FAs directionally from ECM to cytosol and vice versa. This is important to maintain the net force required for the forward movement of the cell through stable protrusions and consequent lamellipodia formation. In the absence of vimentin this buffering or steering control of the FAs is affected (as in 5A) and the associated net traction forces through the FAs gets irregular and thus leads to loss of directional persistence. Faster disassembly rate of FAs also leads to lack of organizational control of FA-actomyosin stress fiber-traction force-lamellipodia axis which is crucial for a moving fibroblast to reach from A to B.

Video Legends

Video 1 A, B. Migration following scratch wound assay of REF cells (related to Figure 1; similar videos obtained with MEFs; see data in Fig. 1). Time-lapse imaging of WT (Video 1A) and Vim-/- (Video 1B) REF cells after making a scratch. Images were acquired every 5 mins. The display rate is 25 frames/s, and total Movie duration is 24hrs. Please note that the wild type cells of both MEF and REF migrate in a well-coordinated manner to close the wound, whereas Vim-/- cells of both MEF and REF migrate with lost direction and coordination.

Video 2 A, B. Migration assay of MEF cells on WT MEF CDM, Related to Figure 1. Time-lapse imaging of WT (Video 2A) and Vim-/- (Video 2B) MEF cells on WT MEF CDM. Cells are numbered for tracking their trajectories. Images were acquired every 5 mins. The display rate is 25 frames/s, and total Movie duration is 24hrs. Please note that the WT MEF cells migrates directionally being aligned to the CDM fibrils and the fibrils are clearly visible. Vim-/- cells are migrating with no directional persistence and not in alignment with the CDM fibrils, which lack proper organization after the migration of Vim-/- MEFs.

Video 3 A,B. Dynamic association of vimentin with FAs (related to Figure 2). Time-lapse imaging of Vim-/- cells expressing mCherry-vimentin and GFP-FAK. Video 3 A: Duration was 11.32 min, and the recording time interval was 10 s/frame. Please note that vimentin filaments (magenta) associate dynamically with FAs (green) at the cell edge and those particular FAs mature or disassemble. Video 3 B: Duration was 6.72 min, and the recording time interval was 10 s/frame. Please note that vimentin ULFs or squiggles (magenta) associate dynamically with FAs (green) at the cell edge in a retrograde movement pattern and those particular FAs mature or disassemble.

Video 4: Dynamic association of vimentin with FAs (related to Figure 2). Time-lapse imaging of Vim-/- cells expressing mCherry-vimentin and GFP-FAK.Duration was 2.63 min, and the recording time interval was 1min/frame. Please note that vimentin filaments (magenta) associate dynamically with FAs (cyan) at the cell periphery and controls the direction of those FAs in the direction of the movement of the cell.

Video 5A, B: Dynamics of FAs in MEF cells (related to Figure 3). Time-lapse imaging of WT (Video 5A) and Vim-/- (Video 5B) MEF cells expressing emerald-paxillin. Duration was 10.71 min and 7.15 min for WT and Vim-/- cells respectively, and the recording time interval was 20

s/frame. Please note that the FAs (cyan) of WT MEF cells progress in a steady smooth fashion whereas FAs of Vim-/- MEF cells lack that, instead they are more rapidly changing their progression direction and speed.

Supplementary figure legends

Fig. S1 (related to Fig.1): (A, B) Graphs showing the quantitation of the directionality and the speed of migration of MEF WT and MEF Vim KO cells. There is significant difference in the directionality whereas the speed of the migration is similar between the cell types. (C, D) Single cell tracking data of the cell migration of MEF WT and MEF Vim KO cells on CDM obtained from MEF WT cells. Migration was tracked for 24 hrs with 5 min intervals via manual tracking using Cell IQ Imagen software.

Fig. S2 (related to Fig. 2): Representative images of different FAs where vimentin interacts with different FA molecules MEF WT cells. (A) Vimentin (VIM) and phospho-FAK, (B) Vimentin and vinculin (Vin), (C, D) vimentin (Vim) and paxillin (Pax). Images were obtained using Abberior STED microscope, XY plane of the FA was captured using 2D STED mode of the microscope and XZ plane of the FA was captured using 3D STED mode of the microscope. Scale bar: 1 µm.

Fig. S3 (related to Fig. 3): Different graphs showing relative differences between WT and Vim-/- MEFs undergoing directional migration. (A) Mobile fraction of an FA post-recovery during FRAP. (B) Distribution of mean fold change in FA intensity during stability phase of FA life cycle for individual adhesions. (C) Line plots with SD error bars of FAAI at each time point during the time lapse of imaging individual cells over 180 timepoints. (D) The frequency histogram shows the distribution of FA angles at birth for WT (black) and Vim-/- (blue) MEFs. Y-axis stands for median of count of FAs with the given birth angle on a per cell basis. The bias in FAs of WT fibroblasts at birth is apparent from the defined periodicity in the angular distribution which lacks in case of Vim-/- fibroblasts. Individual FAs were tracked over time in time-lapse movies and the angle of the first occurrence of a given FA was recorded as the birth angle. (E) Distribution of duration (in mins) of the assembly and disassembly phases during FA lifetime. (F) Distribution of assembly rates of FAs of MEF WT and MEF Vim-/- cells. Number of adhesions > 12000 for both samples.

Fig 1. Vimentin is required for directional persistence

A.

WT | Vim -/-

MEF

REF

B.

MEF

REF

C. WT | Vim -/-

D WT | Vim -/-

MEF

REF

Fig 2. Vimentin interacts with maturing and retracting adhesions

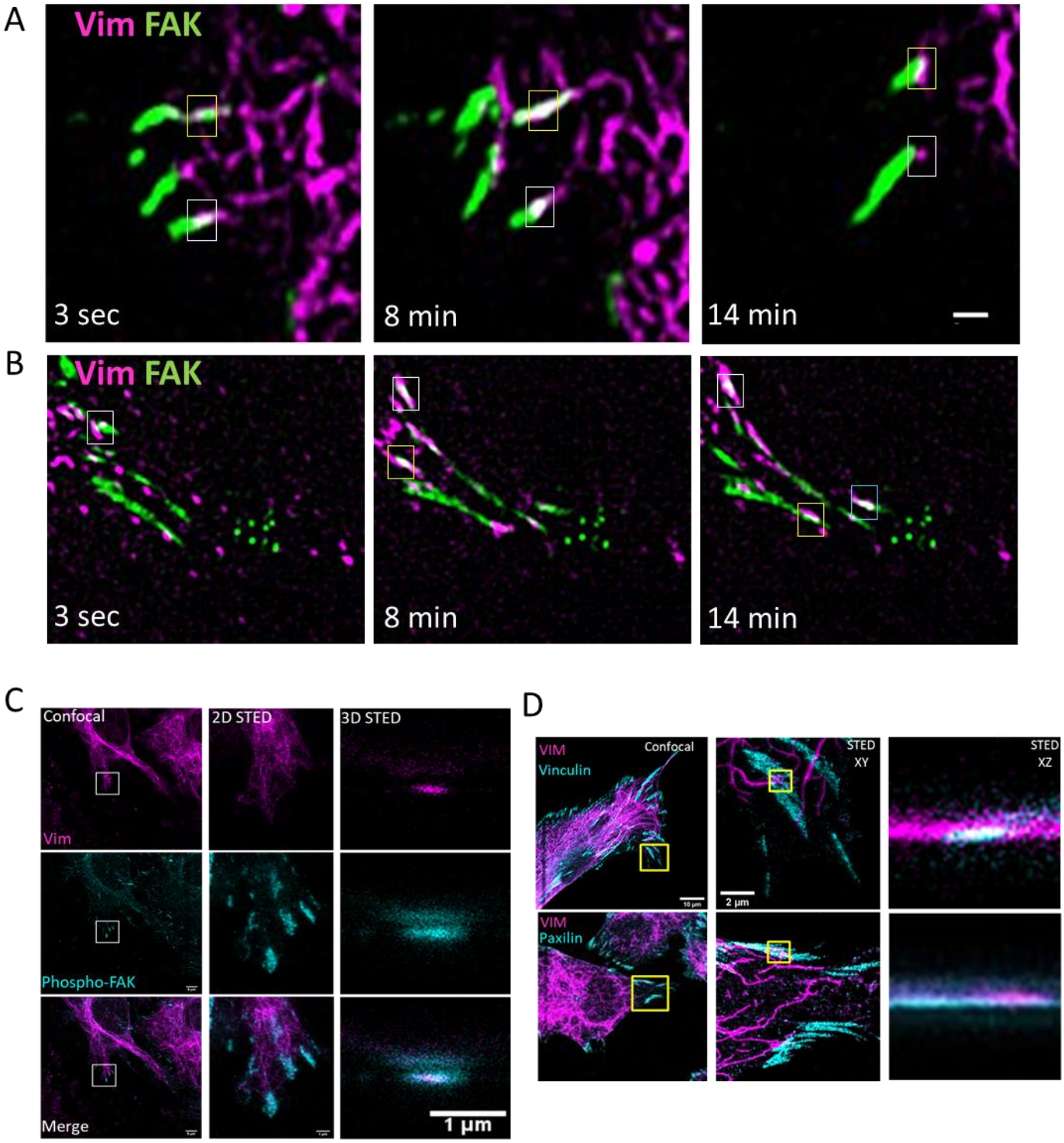

Fig 3. Vimentin-deficiency disorganizes and destabilizes FAs during directional migration

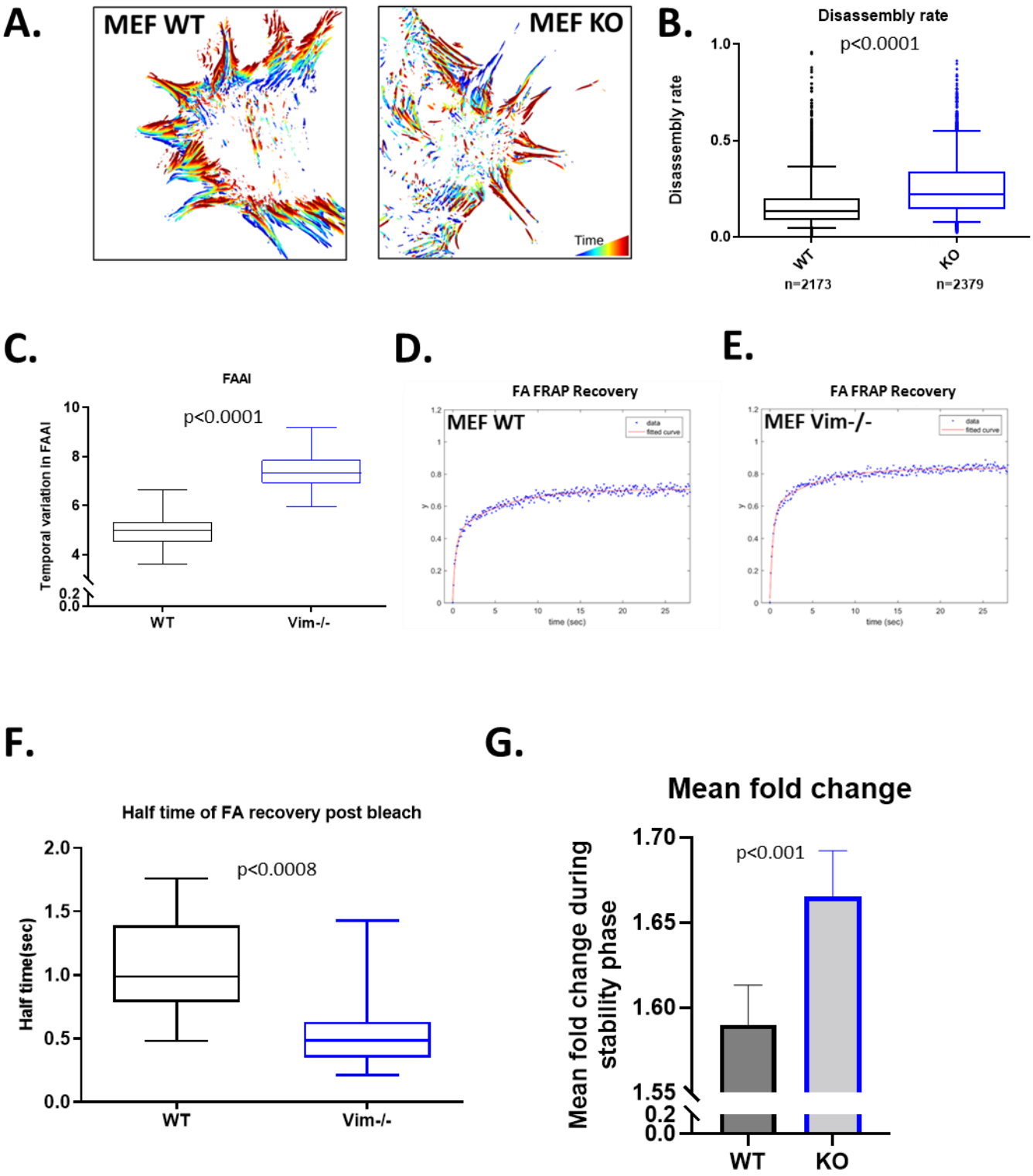

Fig 4. Vimentin deficiency causes reduced focal adhesion area

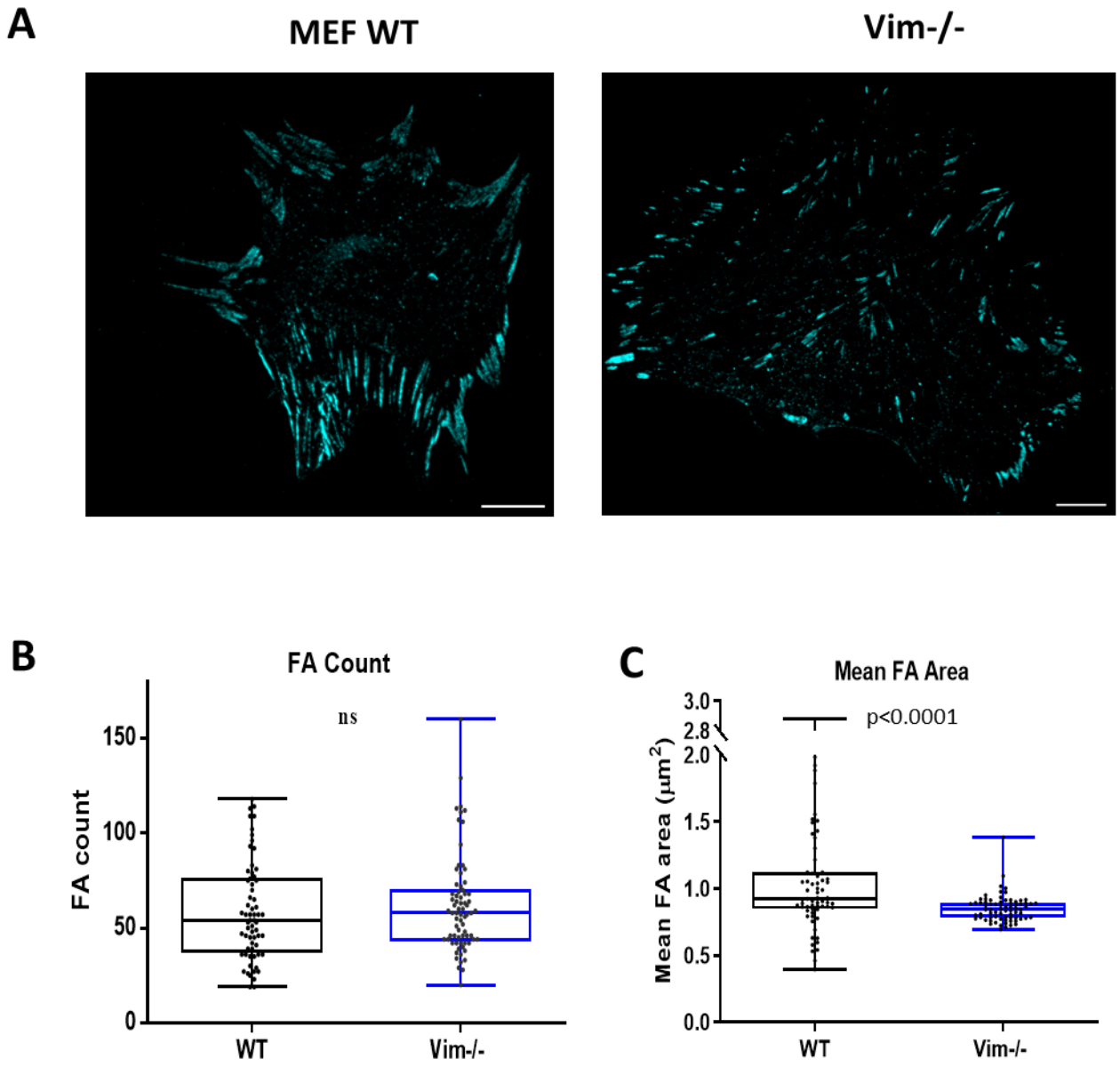
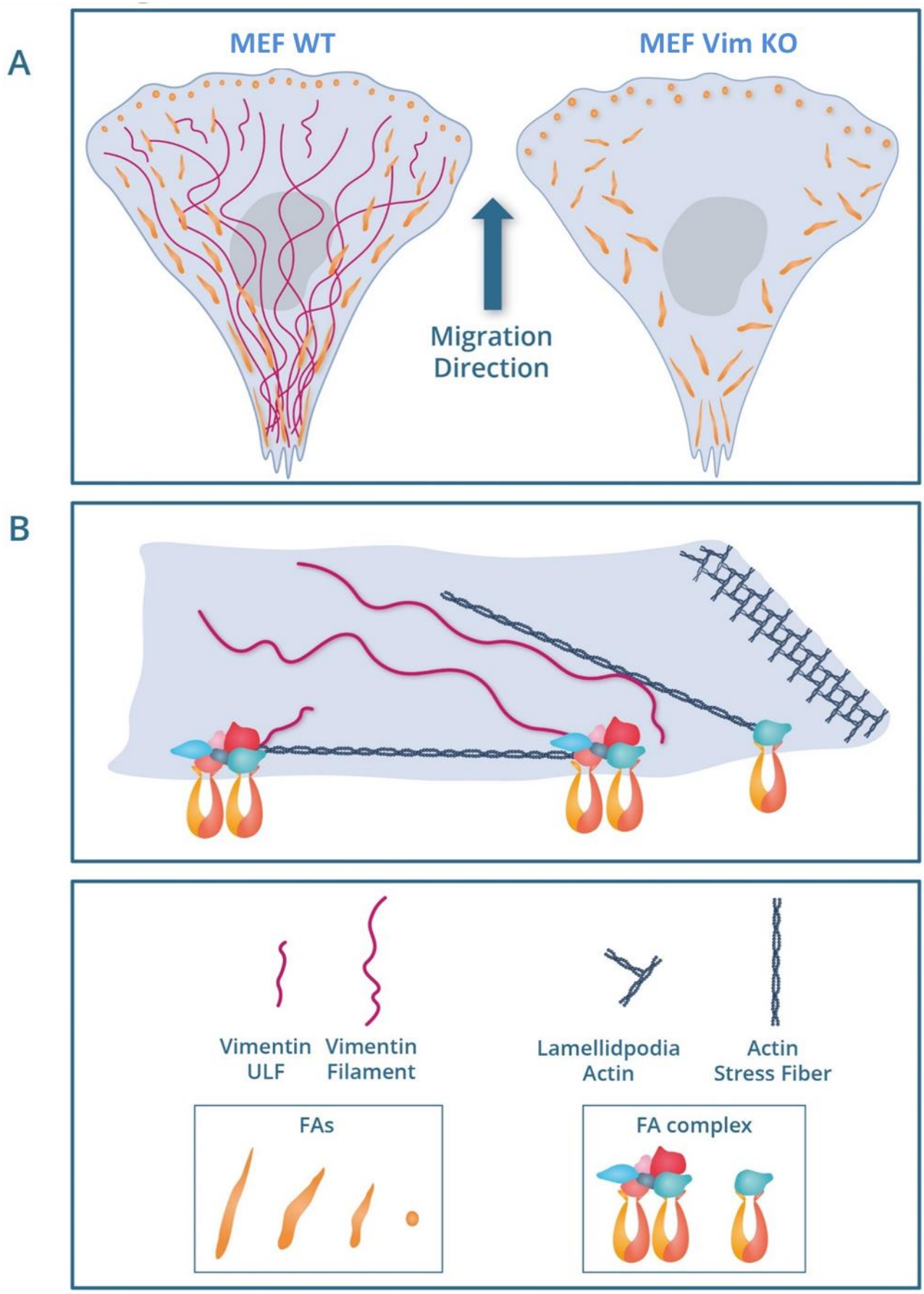
Fig 5. Vimentin steers focal adhesions