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The Focal Adhesion-Associated Signaling Adaptor Paxillin is Required for Breast Cancer Metastasis by Regulating Directed Cell Migration

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Chapter 6

Abstract
Directed cell migration requires the coordination of cell-matrix adhesion and cell-cell signaling and is fundamental during tumor metastasis formation. Here, we show that the focal adhesion adaptor protein paxillin has a crucial role in the regulation of breast cancer cell metastasis. Cells depleted of paxillin by shRNA-interference displayed a scattered and less polarized phenotype resulting in reduced persistence and velocity during 2D cell migration. Furthermore, knockdown cells failed to invade collectively a 3D collagen environment compared to control cells. Paxillin depletion almost completely inhibited the efficient colonization of the lungs in a breast cancer 4T1 orthotopic mouse tumor metastasis model. Genome-wide expression profiling indicated that Rac levels were significantly reduced while Cdc42 levels were increased in cells devoid of paxillin, which was confirmed by Western blotting. Although RhoA levels were unaffected, a reduction in contractile force through reduced RhoA/ROCK signaling-dependent non-muscle myosin II activity was observed. We suggest that paxillin expression determines the levels of Rho GTPases expression and activity and thereby contributes to the mode of cell motility and metastasis formation.
Introduction

Cell migration is essential for normal embryonic development, immune system function and tissue repair, but it also contributes to inflammatory diseases and tumor cell invasion [1,2,3]. Directional migration (i.e. cell motility in one direction) is a multi-step process that involves actin-driven protrusion of the plasma membrane, designation of a leading edge, formation of new extracellular matrix (ECM) adhesions, contraction of the cytoskeleton and disassembly of rearward adhesions [4,5]. This orchestrated process appears to be controlled by multiple mechanisms, including microtubules, Rho GTPases [6,7] and integrin signaling [8]. In addition, cell-ECM adhesion-mediated tumor cell migration often takes place in collaboration with cadherin-based cell-cell adhesion, and a number of papers have documented a variety of events where the coordinated regulation of these cell-ECM and cell-cell adhesions occurs [9]. In the case of the breast carcinoma 4T1 cell line, used in our study, both of these adhesions are simultaneously involved in cell migration (Truong et al., in preparation). Collective migration observed in 4T1 cells is also observed in invasion and metastases of carcinomas such as lobular breast cancer [10].

Paxillin is a multidomain focal contact adaptor protein involved in integrin signaling [11] and it functions as a molecular scaffold for the coordination of Rho GTPase signaling during cell migration in 2D [12,13]. The NH2 terminus of paxillin contains five leucine-rich domains, termed LD motifs, which mediate protein-protein interactions [14,15]. The LD4 motif of paxillin binds a complex of proteins known to be implicated in actin cytoskeletal regulation. These include Cdc42/Rac guanine nucleotide exchange factor PIX, PAK and the SH2-SH3 adaptor protein Nck. This complex is linked to paxillin through the ARF-GTPase-activating protein paxillin kinase linker (PKL). Consequently paxillin serves as a platform in the control of actin cytoskeleton dynamics by both the Rho and ARF family GTPases.

Regulation of the actin cytoskeletal dynamics that occur after the activation of integrins via engagement with the ECM is effected primarily by the Rho family of small GTPases, i.e. Cdc42, Rac and Rho [6,16]. Activation of Cdc42 and Rac initiates the formation of filopodia, lamellipodia and peripheral membrane ruffles respectively, as well as focal contacts [17,6]. In particular, activation of the small GTPase Rac and actin-driven membrane protrusion has been reported to occur in the close proximity to focal adhesions (FAs) in several cell types. However, the molecular mechanism by which FA position is spatially coupled to Rac activation and lamellipodia extension remains unclear. More recently, Rho GTPases have also been identified as critical determinants of cancer cell migration through 3D ECM environments. In this context,
elevated Rac1 activity promotes the elongated mesenchymal morphology and motility [18], whereas elevated RhoA activity drives the amoeboid mode of invasion by simulating membrane blebbing through Rho kinase (ROCK) and non-muscle myosin II activity and, thereby, actomyosin contractility [19-21]. Cdc42 appears to regulate both amoeboid and mesenchymal invasion strategies in melanoma cells [22], but the mechanisms by which the activity of Rho family GTPases are coordinated and counterbalanced are poorly understood.

Paxillin expression and mutations have been implicated in the poor prognosis of various tumors including breast [23,24] and lung [25,26] suggesting that paxillin is important for controlling cell migration and invasion in living tissues. Although the function of paxillin has been extensively studied in cell migration, a direct role for paxillin in breast cancer metastasis formation has not yet been demonstrated. Here we show that depletion of paxillin decreases not only velocity and directionality of 4T1 breast cancer cells in 2D but also invasion in a 3D environment and metastasis formation in vivo. This inhibition was accompanied by a change in cell shape due to reorganization of the actin cytoskeleton, cell-ECM and cell-cell contacts. We show that an imbalance in Rho GTPases expression and activity is associated with these changes. In conclusion, paxillin is a critical regulator in metastatic breast cancer progression.

Materials and Methods

Cell culture
4T1 cells (ATCC-CRL-2539) and variants were cultured in RPMI (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (PAA, Pasching, Austria) and 100 International Units/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were maintained in a 5% CO₂ humidified chamber at 37ºC.

Generation stable paxillin knockdown cell lines
Stable paxillin knockdown cell lines were generated using lentiviral MISSION shRNA vectors (Sigma Aldrich, St. Louis, MO, USA). The mouse sequences were (TRCN0000097194) CCGGGAACTACATTTCAGCCCTCAACTCGAGTTGAGGGCTGAAATGTAGTTCTTTTTG and (TRCN0000097196) CCGGGTCGTAAAGATTACTTCGGCATCTCGAGATGTCGAAGTAATCTTTACGATTTTG. A non-targeting vector was used as control.
Antibodies and inhibitors
Mouse anti-paxillin antibody was purchased from BD Transduction. Mouse anti-FAK (clone 4.47) was purchased from Upstate. Rabbit anti-FAK Y397 and rabbit anti-paxillin Y118 were purchased from Biosource (Invitrogen, Carlsbad, CA, USA). Mouse anti-β-catenin, mouse anti-E-cadherin and mouse anti-N-cadherin were purchased from BD Transduction (Franklin Lakes, NJ, USA). Rabbit anti-ZO1 was purchased from Zymed (Invitrogen). Rhodamin phalloidin was purchased from Molecular Probes (Invitrogen). Inhibitors used in this study are ML-7 (Santa Cruz Biotechnology), 688000 Y-27632 (Calbiochem®) and 553502 RacI (Calbiochem®).

Proliferation assay
A sulforhodamine B (SRB) assay was performed with the 4T1 cell lines to quantify their proliferation rate. Four cell concentrations per cell line were plated in a 96 wells plate. Two different cell densities of 4T1 cells were used in the assay (250–1,000 cell/well). After 24 hr of adhesion the cells were exposed to medium containing 1% FBS. Every 24 hr one 96-wells cell plate was fixated with 30 µl 50% trichloroacetic acid (TCA) solution per well (adding the TCA directly to the assay medium) leaving the plate 1 hr at 4 ºC. The plates were washed five times with tap water and then dried in the flow hood. Cells were stained with 60 µl 0.4% (w/v) SRB in 1% acetic acid and shaken for 30 min on a plate shaker. The plates were washed five times with 1% acetic acid and dried in the flow hood. To measure the absorbance, bound SRB was dissolve in 100 µl 10mM unbuffered Tris solution (pH>10) through shaking for 10 min. Absorbance at 530 nm was measured with a Fluostar plate reader.

Soft agar colony assay
Cells were plated in 1.5 mL top agar (0.34% w/v LMP agarose in complete medium) on top of 2.5 mL bottom agar (0.66% w/v LMP agarose in complete medium) in a 6-well plate (Corning, New York, NY, USA). After 7 days, 150 uL of a 5mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma Aldrich) was added and incubated overnight at 4 ºC. Digital images of the well were taken with a Canon IXUS digital camera. Colonies were counted and size was determined using a particle analysis macro in ImageJ (NIH, Bethesda, MD, USA).

Live cell imaging random cell migration assay
Glass bottom 96-well plates (Greiner Bio-one, Monroe, NC, USA) were coated with 20 µg/µL collagen type I (isolated from rat tails) for 1 hr at 37ºC. Cells were
plated and after 48 hr, cells were pre-exposed to 0.1 µg/uL Hoechst 33342 (Fisher Scientific, Hampton, NH, USA) to visualize nuclei. After refreshing the medium, cells were placed on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 20x objective (0.75 NA, 1.00 WD) automated stage and perfect focus system. Automatically, 3 positions per well were defined and the Differential Interference Contrast (DIC) and Hoechst signal was acquired every 10 min for a total imaging period of 12 hr. After the imaging period, plates were fixated using 4% paraformaldehyde and stored for later immunostaining. All data was converted and analyzed using custom made ImagePro Plus macros. Cell migration was quantified by tracking nuclei in time [27].

Total Internal Reflection Fluorescence (TIRF) imaging of actin dynamics
CELLview glass bottom dishes with four compartments were coated for 1 hr at 37°C with 20 µg/µL collagen type I (isolated from rat tails). Transient expression of eGFP-actin in both Ctrl shRNA and PXN shRNA cells was done 24 hr prior to imaging directly in the glass bottom dishes. Dishes were mounted on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 60x oil objective (1.49 NA, 0.12 WD) and perfect focus system. Actin cytoskeleton was visualized using TIRF with the 488 laser for one hr, with one-min interval.

Western Blotting
Cells were scraped in ice-cold TSE (10 nM Tris-HCL, 250 mM sucrose, 1mM EGTA pH 7.4) supplemented with inhibitors. After sonication of cell lysates, protein concentration was determined by Bio-Rad protein assay (Hercules, CA, USA) using IgG as internal standard. 30 µg of total cellular protein was separated on 7.5-10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA). Blots were blocked in 5% w/v bovine serum albumin in TBST (0.5 M NaCl, 20 mM Tris-HCl, 0.05% v/v Tween20 pH 7.4) and probed with primary antibody (overnight, 4°C) followed by incubation with secondary antibody either Dylight-649 or Horseradish peroxidase-coupled (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Dylight-649 was directly visualized by scanning on Typhoon imaged 9400 (Amersham Biosciences, Uppsala, Sweden). HRP probes were first activated by incubation with Enhanched Chemiluminescence reagent (Amersham Biosciences) and detected using film.
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**Immunofluorescence staining**

Cells were cultured on 12-mm 10 µg/uL collagen coated coverslips and fixated in 4% paraformaldehyde in PBS for 10 min at room temperature or with MeOH -20°C for 20 min and then 15 min with 4,0% formaldehyde (pH 7.0-7.2) for the pericentrin and tubulin staining. Coverslips were blocked in TBP (0.1 % v/v Triton-X and 0.5% w/v bovine serum albumin in PBS) for 1 hr at room temperature and overnight incubated with primary antibody, followed by 1 hr incubation of fluorescently labeled secondary antibody. Next, coverslips were incubated with 2 µg/mL Hoechst 33258 (Sigma Aldrich) to visualize nuclei and mounted in Aqua Polymount (Polysciences, Warrington, PA, USA). Cells were imaged using a Nikon Eclipse TE 2000-E confocal microscope fitted with a 20x objective (0.75 NA, 1.00 WD) and 4 times digital zoom.

**3D invasion**

3D invasion assay was conducted as described previously [28]. In short, collagen type I solution was obtained from Upstate-Milipore. Collagen was diluted to a working concentration of about 2 mg/ml in PBS containing 1xDMEM (stock 10x, GIBCO), 44 mM NaHCO3 (stock 440 mM), 0,1 M Hepes (stock 1M, Biosolve). Cell suspensions derived from trypsin-detached adherent cultures were filtered to remove clumps, centrifuged, and washed twice with PBS. About 7x10⁶ cells were re-suspended in 30 µl PBS containing 2% polyvinylpyrrolidone (PVP; Sigma Aldrich). Cell suspensions in 2% PVP were microinjected (~1x10⁶/droplet) with a microinjector into solidified collagen gels in 8 well µslides (IBIDI).

**Spontaneous metastasis assay**

Experiment with 4T1 was done as previously described. In short, female Rag2⁻/⁻ γc⁻/⁻ mice were orthotopically injected with 100,000 4T1 cells in the fourth mammary gland (6-8 animals per group). Tumor growth was monitored over a 3-weeks period and then mice were sacrificed and tumor and lungs were isolated. Tissue was processed for immuno histochemical analysis. Lungs were injected with Indian ink to count surface metastasis.

**Statistics**

Student’s t test was used to determine significant differences between two means (P<0.05 or P<0.01). Values are represented as mean ± sem. Significant differences are marked in the graphs.
Results

Paxillin is required for 4T1 cancer cell migration and invasion

In order to determine whether paxillin is required for the process of breast cancer metastasis we used the mouse breast carcinoma cell line 4T1 that is a well established cell line to study spontaneous metastasis formation in an orthotopic mouse breast cancer model [29]. We created knockdown cell lines using two different shRNA lentiviral vectors targeting paxillin. Western blotting of both PXN shRNA cell lines confirmed the efficient knockdown of paxillin in those cells (Fig 1A). A proliferation assay using SRB showed no difference at all in cell proliferation between control and paxillin knockdown in monolayer culture or soft agar assay (SFig 1).

Figure 1: Paxillin mediates breast cancer cell 2D migration and invasion through 3D collagen gels.
A) Representative Western blot of total cell lysates from 4T1 Ctrl cells and PXN depleted cells. Phase contrast pictures of Ctrl and PXN shRNA cells. PXN shRNA cells exhibit long tail-like retraction fibers (see arrows). B) Snapshots of time-lapse imaging of a spreading assay of both 4T1 Ctrl and knockdown cell lines. C) Quantification of velocity of 4T1 Ctrl and PXN shRNA cells after automated tracking of nuclei in a random cell migration assay of 12 hr [27]. (t-test, *P<0.01 and **P<0.001; error bars, SEM from all individual cells of a representative experiment). Diagrams represent the migration trajectories covered in 12 hr of 4T1 Ctrl and PXN shRNA cells. D) Snapshots of time-lapse imaging of spheroids containing either Ctrl or PXN shRNA cells invading through a 3D collagen gel in the first 24 hr. E) Quantification of Ctrl and PXN shRNA spheroids (n=8 per cell line) area after 48 hr of invasion in a 3D collagen gel. (t-test, **P<0.001; error bars, SEM from all individual spheroids of a representative experiment).
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Next we investigated the effect of paxillin depletion in a cell spreading assay. Although 4T1 PXN shRNA and Ctrl shRNA cells did spread on a collagen coated surface, PXN depletion strongly affected the speed of this process (Fig 1B). Furthermore, time-lapse live cell imaging showed that both PXN knockdown cell lines migrated less efficiently than control cells (Fig 1C). While the effect of paxillin knockdown on cell migration is mainly studied in 2D, still little is known about its role in 3D ECMs, which is more physiologically relevant for cancer metastasis. We therefore carried out a 3D invasion assay using tumor cell spheroids [28]. Ctrl spheroids showed more invasive behavior than PXN shRNA cell lines (Fig 1D) and manual quantification of

**Figure 2:** Paxillin knockdown in 4T1 cells does not affect tumor growth but prevents metastasis to the lungs in an orthotopic mouse tumor metastasis model. A) Representative H&E staining of Ctrl and PXN shRNA tumor tissue. B) Primary tumor weight at the end point of the spontaneous metastasis assay. Primary tumor growth measured by caliper in both Ctrl and PXN shRNA injected groups. C) Immunofluorescence staining of paxillin, Ki67 and E-cadherin respectively of both Ctrl and PXN shRNA tumor tissue sections. D) After approximately 3 weeks, lungs were isolated, injected with ink (right lobes) and lung (left lobe) sections were stained with H&E. Number of lung macro-metastases at the end of the spontaneous metastasis assay (t-test, *P<0.05 and **P<0.001; error bars, SEM from all individual animals, n=7 per group).
the spheroids size demonstrated that PXN shRNA spheroids showed significantly less surface area with less branches than control spheroids (Fig 1E).

**Paxillin is required for breast cancer metastasis**

Given the role of paxillin in the regulation of 3D invasion, we next examined the metastatic potential of these highly invasive 4T1 cells in an orthotopic mouse tumor metastasis model. Both Ctrl and PXN knockdown cells were able to form a primary tumor at site of injection with similar overall tumor histology (Fig 2A). Also no difference in tumor growth rate between the different groups was observed (Fig 2B), which was confirmed by a similar percentage of proliferation marker Ki67 positive cells in either Ctrl shRNA or PXN groups (Fig 2C). In sharp contrast, we observed a significant strong reduction in lung tumor burden for both PXN shRNA 4T1 cell lines compared to Ctrl shRNA cells, which was observed at macroscopic inspection by counting surface lung metastasis as well as microscopic analysis by evaluating the lung metastases in H&E sections (Fig 2D). These data indicate that paxillin depletion affects the ability of 4T1 cells to spontaneously metastasize from the primary tumor to the lungs. Interestingly, a further detailed analysis of the surrounding tissue of the primary tumor in H&E sections revealed the presence of micro-metastases in Ctrl shRNA group; these were not at all observed in the PXN shRNA group (Fig 2A, see arrows). These data show for the first time that paxillin is essential for the metastatic process, without affecting overall primary tumor outgrowth.

**Paxillin is required for efficient directional migration in 2D**

Interestingly, PXN knockdown cell lines showed a scattered phenotype compared to control 4T1 cells (Fig 1A; Fig 3A DIC inserts), suggesting perturbation of cell-cell adhesion junctions. Although the protein expression levels of core adherence junctions, including β-catenin, E-, N- and Pan-cadherin, were not affected, immunofluorescence staining revealed a clear delocalization of these proteins from cell-cell contacts to cytoplasm (Fig 3A), indicating a regulation of cell adherence junctions in 4T1 cells by paxillin. Also the expression level of cell-ECM adhesion molecules was not changed at the protein level, but the immunostaining for the focal adhesion marker vinculin showed a clear enlargement of cell-ECM adhesion structures in PXN shRNA cells in the cell periphery compared to Ctrl shRNA cells (Fig 3B). Paxillin loss and mutations have been reported to induce migration defects including impaired movement in a 2D wound healing assay [30]. Therefore, next we analyzed the effect of paxillin knockdown in directional migration in 2D using
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Automated tracking of 4T1 cell migration paths over a period of 12 hr after wounding confluent cell monolayers confirmed that PXN shRNA cells had a lower migration speed, and, importantly the persistence of cell migration was significantly affected (Fig 3C). Persistent migration requires the coordination of protrusive activity at the leading edge of the cell combined with rear retraction to enable efficient, polarized movement [1,31,17]. Polarized membrane protrusion as well as the reorientation of the centrosome towards the leading edge is a feature of directional cell migration. Examination of the cells at the wound edge showed that Ctrl cells exhibited a typical polarized morphology with a significant lamellipodium facing

Figure 3: Loss of paxillin expression affects cell-cell contact and directed cell migration.
A) Immunofluorescence images of 4T1 Ctrl and PXN knockdown cells stained for cell-cell contact proteins including β-catenin, ZO-1 and E-cadherin. Protein levels of the same proteins measured by western blot analysis. B) Representative immunofluorescence images of 4T1 Ctrl and PXN shRNA cells stained for the focal adhesion marker vinculin. Equal level of vinculin in both cell-lines was confirmed using western blot analysis. C) Effect of paxillin knockdown on cell migration in a wound healing assay. Confluent Ctrl and PXN shRNA cells were scratched and recorded using combined DIC and epifluorescent imaging: time-lapse images were captured every 5 min for 6 hr. Representative images taken at 5 min and 6 hr after wounding are shown. Quantification of migration after wound healing. Migration of individual cells at the wound edge was determined by nucleus tracking [27]. Representative tracks of Ctrl and PXN shRNA cells are shown. Cell migration velocity was also calculated. (t-test, *P<0.01; error bars, SEM from all individual cells of a representative experiment).
D) Edge cells were scored as being orientated if their centrosome (in green: pericentrin staining) was distal from cell-cell contacts relative to the nucleus (white arrows: orientated; yellow arrows: not orientated).
**Figure 4: Paxillin knockdown results in an altered actin dynamics through reduced non-muscle myosin II activity.** A) Immunofluorescence images of 4T1 Ctrl and PXN knockdown cells stained for total Rac1 (red) and paxillin (green). Representative Western blot analysis of RhoA, Rac1 and Cdc42 expression in both control and knock down cells. B) Effect of paxillin knockdown on membrane dynamics of 4T1 cells. Membrane ruffling was imaged using DIC imaging: time-lapse images were captured every 1 min for 10 min. C) Effect of paxillin knockdown on actin dynamics. Ctrl and PXN shRNA cells were transiently transfected with GFP-actin and imaged using TIRF imaging: time-lapse images were captured every min for 30 min. D) Immunofluorescence analysis of F-actin (red) and pMLC (green) in Ctrl and PXN knockdown cell lines, which was confirmed by Western blot analysis.
Paxillin is required for metastasis by regulation of directed cell migration into the wound (Fig 3C arrows). Yet PXN shRNA cells showed reduced lamellipodia activity (Fig 3C). Furthermore, in PXN shRNA cells the centrosome was not oriented per se towards the wound of the 2D culture; this was the case for Ctrl shRNA cells (Fig 3D). In conclusion, these data underlie a role for paxillin in the directional cell migration of 4T1 cells in relation to defected orientation of the centrosome.

Loss of paxillin results in more protrusion dynamics via an altered balance of the Rho GTPases

The restricted spatiotemporal regulation of the small GTPase Rac1 seems essential for both lamellipodia and cell-cell contact formation. Paxillin acts as a scaffold protein for various Rac signaling complexes such as CrkII/Dock180/ELMO and PKL-PIX-PAK. We anticipated that the loss of cell-cell contact and directional migration of PXN shRNA cells could be related to altered regulation of Rho-GTPases including Rac. We first investigated the localization of Rac1 by immunofluorescence in Ctrl and PXN shRNA cells and revealed lower Rac1 signals in PXN shRNA cells, which was confirmed by Western blot analysis (Fig 4A). Interestingly, while RhoA levels were hardly affected, the Cdc42 levels were strongly increased in PXN shRNA cells compared to control (Fig 4A). RhoGTPases have different roles in the regulation of the actin cytoskeletal network: RhoA induces actin stress fiber formation, Rac1 induces active actin dynamics in the lamellipodia and Cdc42 induces filopodia formation. Detailed observation of membrane dynamics using DIC imaging at a higher magnification showed that PXN shRNA cells demonstrated multiple random membrane protrusions instead of a continuous lamellipodium in control cells (Fig 4B). We next followed the actin dynamics using transient transfection with GFP-actin in both Ctrl and PXN shRNA cells in combination with TIRF imaging. While control cells showed the well-distributed formation of actin polymerization at the leading edge and stable stress fibers at the rear of the cells, PXN knockdown cells demonstrated loss of these organized stress fibers and the formation of more individual small protrusion, likely mimicking filapodia (Fig 4C). Loss of more stable RhoA dependent stress fibers was further confirmed by the depletion of contractile P(Ser 18)-MLC positive actin stress fibers; these were abundantly present in control cells but not at all in PXN shRNA cells. This was confirmed by Western blotting. Altogether these data suggest a relation between the decreased motility and invasion of breast cancer cells lacking paxillin and altered coordinated dynamics of the actin cytoskeleton, most likely through deregulation of the expression of the Rho GTPases Rac1 and Cdc42.
Chapter 6

Discussion

Cancer cell invasion and subsequent metastasis is associated with increased malignancy and decreased patient survival. Therefore, understanding the intracellular mechanisms controlling metastasis formation is highly relevant. Using a suitable breast cancer cell line for both in vitro and in vivo models [29], in this study we established a crucial role of paxillin in breast cancer metastasis formation: paxillin does not affect tumor growth but is essential for the efficient dissemination of breast tumor cells. Directional migration is a multi-step process that involves 1) actin-driven protrusion of the plasma membrane, 2) designation of a leading edge, 3) formation of new extracellular matrix (ECM) adhesions, 4) contraction of the cytoskeleton and 5) disassembly of rearward adhesions [5,4]. Herein, we demonstrated that paxillin knockdown affects most of these steps including 1) disturbed focused lamellipodia protrusion activity, 2) defected cell polarization in a wound healing assay, 3) slower adhesion turnover, and 4) reduced RhoA-based cytoskeleton contractility.

We demonstrated that 4T1 breast cancer cells devoid of paxillin exhibited a reduction in velocity and persistence (Fig 1). This is consistent with previous 2D analyses in which cell migration was reduced upon paxillin knockdown [32,30,33]. We further demonstrate that the typical 3D collective invasion of 4T1 cells in a collagen matrix was severely impaired in paxillin depleted cells (Fig 1). This fits with observations in the mesenchymal human breast cancer MDA-MB-231 cell line [34]. Efficient migration on 2D substrate requires the scaffold function of paxillin to coordinate adhesion disassembly [33]. Indeed, while knockdown of paxillin failed to affect the localization of FAK and vinculin to focal adhesions, the size, number and distribution of the adhesions was altered in comparison with control cells (larger and more peripheral, Fig 3), which is in agreement with slower adhesion turnover as shown previously [33].

Persistent migration requires moderate levels of restricted Rac1 activity [4]. Pankov and colleagues found that a relatively small change in total Rac activity can serve as a switch between directional and random cell migration in multiple cell-types [4]. Slightly lowering Rac1 activity resulted in suppression of peripheral protrusions, which promoted persistent migration of a cell in one direction due to absence of new peripheral protrusions that could produce a change in the direction of cell migration. In our study, we observed unstable protrusion activity in paxillin knockdown cells, so the lack of persistence observed in paxillin knockdown cells may be in part due to altered Rac1 activity (probably increased levels of active Rac1 although total Rac1 are decreased) (Fig 4). Furthermore, paxillin depleted cells
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exhibited a significant decrease in the activation of non-muscle myosin II (Fig 4) suggesting a significant reduction of the Rho/ROCK pathway. Indeed, inhibition of the Rho/ROCK pathway leads to decreased cell motility but enhanced protrusion in several cell types [35]. Similar to our observations of paxillin depleted cells during 2D migration, unstable deregulated protrusion and adhesion formation has also been reported in cells expressing the LD4 deletion mutant of paxillin [36]. Therefore it is possible that paxillin scaffold function through its LD4 motif is necessary for Rac-driven adhesion and protrusion stabilization in 2D. Ectopic expression of the LD4 motif or the dominant negative form of Rac1 would add more insight into the mechanism. Finally in addition to enhanced protrusiveness, paxillin knockdown cells exhibit long tail-like retraction fibers suggestive of defect in rear release. Efficient release is necessary for cell migration [37,38], which may also explain the reduced cell motility of paxillin depleted cells. Interestingly, we observed a clear increase in the levels of Cdc42 protein in PXN shRNA cells. A polarizing signal can be triggered by cell-cell adhesion, the actin cytoskeleton and Cdc42 [39]. Moreover, Cdc42 inducing more filodipodia like extension in PXN shRNA cells. Likewise, a possible increased Cdc42 in concert with decreased Rac1 and RhoA activity is a likely explanation for the defects in cell migration. This would lead to loss of polarized cell protrusion formation and consequently deregulated cell migration. Further experiments to establish the activity of individual Rho-GTPase family members as well as expression of dominant negative or constitutive activated Cdc42 or Rac1 should shed more light on the mechanism of defected actin reorganization and migration of PXN knockdown cells.

In conclusion our observations demonstrate that paxillin is critical to the regulation of the actin cytoskeleton changes that accompany integrin engagement with the ECM and subsequent cell spreading, motility and invasion. This is reflected in the poor metastatic potential of breast tumor cells that lack paxillin. Further detailed investigation on the individual role of RhoGTPases in the control of migration in paxillin knockdown cells will be required.
**Supplemental figure 1: Paxillin knockdown in 4T1 does not affect cell proliferation.** A) A SRB assay was used to quantify the proliferation rate of Ctrl and PXN shRNA cells using two cell densities (t-test, not significant; error bars, SEM from three different experiments). B) A soft agar assay was used to verify that paxillin knockdown in 4T1 cells does not affect anchorage independent growth. Representative images of Ctrl and paxillin depleted 4T1 cells colonies grown in agar are shown.
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