CHAPTER 7

Scaffold protein paxillin serine 178 phosphorylation determines mammary tumor cell migration and metastasis formation through regulating EGFR expression

Running title: Paxillin serine 178 phosphorylation regulates cell migration through EGFR expression

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ABSTRACT

Paxillin is a multi-domain scaffold protein that localizes at focal adhesions and is involved in regulation of the dynamic changes of matrix adhesion and cytoskeletal reorganization which are required for cell migration. Although EGF stimulation induces JNK-mediated phosphorylation of serine 178 of paxillin, the exact role and mechanism of this modification in tumor cell migration in the context of spontaneous cancer metastasis is unknown. Stable expression of the paxillinS178A mutant in metastatic mammary adenocarcinoma MTLn3 cells significantly decreased EGF-induced migration which was related to impaired focal adhesion turnover. In an orthotopic in vivo breast cancer model, paxillinS178A decreased lung metastasis formation. A decreased expression of EGFR in three independent paxillinS178A clones compared to wt-paxillin clones was observed. Re-expression of wt-EGFR in MTLn3-paxillinS178A fully restored the EGF-driven cell motility and focal adhesion dynamics. Furthermore, in vivo EGFR-MTLn3-paxillinS178A cells spontaneously metastasize from breast to lung. Our data indicate a role for paxillin-Ser178 in the regulation of EGFR and, thereby, in the regulation of EGF-driven cell migration and metastasis formation.

1. Introduction

Breast cancer represents the most common worldwide type of cancers among women. The occurrence of metastases, or secondary tumors, is a critical determinant of the prognosis for breast cancer patients. The formation of secondary tumors involves distinct cellular processes including cell migration, invasion, intra- and extra-vasation and proliferation. These processes are regulated by growth factors, cytokines and cellular matrix molecules[1]. Epidermal growth factor (EGF) stimulates cell proliferation and migration through activation of the receptor tyrosine kinase EGFR, followed by activation of downstream signaling pathways including different mitogen-activated protein kinases (MAPKs) cascades: extracellular signal-regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK) (for review see[2]).

The JNK group of kinases has essential roles in cancer development including regulation of the survival/proliferation balance as well as cell migration [2-6]. Dual Thr and Tyr phosphorylation of JNK by upstream MAP kinase kinases, MKK4 and MKK7, results in JNK activation and nuclear translocation. In the nucleus, JNKs phosphorylates and activates transcription factors including members of the AP-1 family such as c-Jun. The JNK-AP-1 pathway regulates the expression of genes involved in the cell cycle, survival and apoptosis and extracellular matrix homeostasis[3,7-13]. Besides phosphorylation of transcription factors, several cytoskeleton-associated adaptor and signaling proteins have recently been identified as direct JNK substrates. These include the intermediate filament protein keratin 8[14], microtubule-associated proteins (MAPs), such as MAP1B, MAP2, DCX and SCG10 [15-17], the actin-binding protein spir[18], the protein kinase p90RSK[19], the adaptors insulin receptor substrate 1 (Irs-1), p66ShcA[20,21] and β-catenin[22] and paxillin[3]. This suggests that the direct JNK-mediated phosphorylation of these targets may thereby influence cell migration.

Paxillin is a 68 kD multidomain adaptor protein associated with focal adhesions. It functions as a scaffold to integrate multiple signaling pathways involved in matrix adhesion dynamics and cell migration[23-27]. Focal adhesions, the closest contacts between cells and extra-cellular matrix (ECM), are important sites for signaling events[28-30]. Besides five Leu-rich LD motifs and four double zinc-finger LIM domains that mediate protein–protein interactions, paxillin also contains many candidate tyrosine and serine/threonine kinases phosphorylation sites[31]; the phosphorylation of paxillin modulates its interaction with other proteins[27]. Recent studies have identified paxillin as a novel JNK substrate[2,3]. EGF-driven JNK activation results in the efficient JNK-mediated phosphorylation of paxillin at Ser178 thereby affecting migration of NBT-II cells, MDA-MB-231 human breast cancer cells, Chinese hamster ovary (CHO-K1)[3] and corneal epithelial cells[32]. Interestingly, expression of a Ser178 to Ala mutant of paxillin, but not wild type paxillin, significantly inhibits the migration of NBT-II, MDA-MB-231, and CHO-K1 cells[3]. How JNK-mediated phosphorylation of paxillin regulates cell migration and

adhesions is still largely unknown. Moreover it is unclear whether this phosphorylation event is relevant for cancer metastasis formation.

Here we explored the role and mechanism of paxillin-Ser178 phosphorylation in breast tumor progression using the highly metastastic breast tumor cell line MTLn3 as a model. Ectopic expression of paxillin-S178A mutants significantly decreased EGF-induced cell migration in association with decreased activation of the PI3K/AKT and MEK/ERK pathways. In orthotopic mammary gland tumor/metastasis model the JNK-mediated phosphorylation of paxillin at Ser 178 is essential for efficient metastasis of the MTLn3 cells to the lung. While paxillin-S178A expression reduced the EGFR expression levels in the MTLn3 cells, re-expression of EGFR rescued the defected tumor cell migration and metastasis formation. Altogether our data indicate that the serine 178 residue of paxillin is an important player in cell migration of metastasis formation and that this is related to the modulation of the EGFR-signaling pathway.

2. Materials and methods

2.1 Chemicals and antibodies

Alpha modified minimal essential medium without ribonucleosides and deoxyribonucleosides (α-MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin and geneticin (G418 sulphate) were from Life Technologies. Rat tail collagen type I was from Upstate Biotechnology. LipofectAMINE Plus transfection reagents were from Invitrogen. Primary antibodies were anti-paxillin (BD), anti-tubulin, anti-GFP (Sigma, St. Louis, MO), anti-pT183/pY185-JNK (Promega), anti-Phospho-Thr202/Tyr204 ERK1/2, anti-pSer473-AKT (Cell Signaling), anti-pSer178-paxillin (Abcam, Cambridge, UK), anti-EGFR for western blot (rabbit polyclonal, Cell Signaling Technology) and FACS/immunostaining (mouse monoclonal, Calbiochem). All secondary antibodies were from Jackson (GαRb-AP, GαM-HRP, GαRb-HRP or GαM-CY5). The Western-Star immunodetection system (Tropix kit) was from Applied Biosystems and ECL Plus reagent was from Amersham. Hoechst 33258 and rhodamine-phalloidin were from Molecular Probes and Aqua Poly/Mount was from Polysciences. All other chemicals were of analytical grade.

2.2 Cell Culture

MTLn3 cells were cultured as before[33]. To generate stable cell lines, MTLn3 rat mammary carcinoma cells were transfected with GFP-paxillin or GFP-paxillinS178A along with empty vector pcDNA3 using LipofectAMINE plus reagents according to manufacturer's procedures. Stable transfectants were selected using G418 and individual clones were picked and at 100 μg/ml G418 (complete medium). Cells were used for up to 8 passages and expression levels of

GFP tagged proteins remained stable during experiment period. For EGF experiments, 80-90% confluent cells were starved for 4hr followed by exposure to EGF (10 nM).

2.3 Luciferase reporter assay

MTLn3 cells were transiently transfected with the reporter constructs pGL3-Tata-5xE3AP1, pGL3-Tata-5xcollTRE, pGL3-Tata-5xJun2 (provided by Hans van Dam) using Lipofectamine Plus reagent[33]. After 16 hrs cells were serum starved for 4 hours followed by analysis of luciferase activity using a luminescence plate reader.

2.4 Proliferation, attachment and wound healing assay

For proliferation assay, cells were plated in complete medium on 6 well-plates and cultured for 24, 48, 72 or 96 hrs at which time point the amount of cells was determined by counting. For cell attachment assay, 1 hr serum-starved cells were replated in complete medium on collagen-coated 6-well plates for 30, 60, 90 and 120 minutes, and following a PBS wash, attached cells were trypsinized and counted. For wound healing assays, monolayer cells were scratched using a pipette tip to generate a wound followed by a wash with medium and then incubated in α-MEM supplemented with 1% (v/v) FBS for 20 hrs. Wounds were photographed using phase contrast microscopy with a Nikon Coolpix digital camera directly after scratching and after 20 hrs. Wound closure was determined using Image J software.

2.5 Live cell imaging

2.5.1 Random cell migration

Cells were cultured in glass-bottom plates overnight and starved for 4 hrs followed by visualization with high throughput microscopy for 1 hr on a Nikon TE 2000-E microscope equipped with perfect focus system in a humid climate of 37°C and 5% CO_2. Subsequently, cells were treated with EGF or HGF and the exact same fields were visualized for 1 hr. Movies were captured five minutes per frame with 20x objective. Cell speed was determined by tracking cell center and calculating the distance between two sequential frames. Cell dynamics were measured with cell surface area change between two sequent frames by homemade macro adopted in Image-Pro Plus (version 5.1, Media Cybernetics Inc., Silver Spring, MD).

2.5.2 TIRF and FRAP

Total internal reflection fluorescence (TIRF) microscopy was performed on GFP-paxillin MTLn3 cells and GFP-S178A-paxillin in a climate control chamber. TIRF movies were captured on a Nikon TIRF microscope system (Eclipse TE 2000-E, Nikon with automated stage) with framing every 5 minutes for 4 hrs using NIS-elements AR software (Nikon). To determine the turnover of GFP-tagged paxillin proteins in individual focal adhesions, fluorescence recovery after photobleaching (FRAP) was performed as follows: photobleaching was applied to a small area covering a single focal adhesion for 1 s with laser intensity of 50 µW. Redistribution of fluorescence was monitored with 100 ms time intervals at 7.5µW starting directly after the bleach pulse. Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve for a single experiment. All measurements were performed at 37 °C using a heating stage with feedback temperature control and the experiment was performed on at least three different days. Images were analyzed with Image software (Zeiss). The relative fluorescence intensity of individual focal adhesion was calculated at each time interval as follows: $I_{rel}(t) = (FA_t / FA_0)$, where FA_t is the intensity of the focal adhesion at time point t after bleaching, FA_0 is the average intensity of the focal adhesion before bleaching.

2.6 Gel electrophoresis and immunoblotting

Western blot analysis was performed as described before. Equal protein amounts of cellular lyses (25 µg; Bradford protein assay) were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked in either 0.2% (w/v) casein in TBS-T (for phospho-state specific antibodies) or 5% (w/v) BSA in TBS-T (for other primary antibodies) and probed with primary antibody overnight followed by sufficient washes and incubation with secondary antibodies. Alkaline phosphatase (AP)-conjugated secondary antibodies for phospho-proteins were detected with the Western-Star immunodetection system. For detection of horseradish peroxidase (HRP)-conjugated antibodies, ECL Plus reagent was used and followed by visualization on a Typhoon Imager 9400 (520nm, blue laser).

2.7 Immunofluorescence

Cells were plated on collagen coated glass coverslips. Cells were briefly washed in PBS, followed by fixation in 3.7% formaldehyde for 10 min at room temperature. After washing, coverslips were blocked in TBP (0.1% (w/v) Triton X-100, 0.5% (w/v) BSA in PBS, pH 7.4). Incubation with primary antibodies diluted in TBP containing 0.05% (w/v) NaN_3 was carried out overnight at 4°C. Coverslips were incubated with secondary antibodies conjugated to Cy5. Coverslips were mounted

on glass slides using Aqua Poly/Mount and cells were visualized using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60X Nikon objective.

2.8 In vivo tumor growth and metastasis formation

6-week old Rag2 $^{-/-}$ $\gamma c^{-/-}$ mice were obtained from in house breeding. Animals were housed in individually ventilated cages under sterile conditions containing 3 mice per cage. Sterilised food and water were provided *ad libitum*. To measure spontaneous metastasis, tumor cells (5 x 10^5) were injected into the right thoracic mammary fat pads as described previously[34]. Horizontal (h) and vertical (v) tumor diameters were determined at weekly intervals, and tumor volume (V) was calculated ($V=4/3\pi(1/2[\sqrt{(h*v)}]^3$). After 4 weeks, the animals were sacrificed with Nembutal, the lungs and were excised as well as the primary tumor which was also weighted. For quantification of all GFP-paxillin positive macro- and micro-metastasis, the flat side of the right lung was analysed with the immunofluorescence microscope using a X10 objective lens (NA 0.25). For visualization of the large surface lung metastases, the lungs were injected with Indian inkt in Feketes. Next the right lung and primary tumor were fixated in 4% paraformaldehyde. For both the primary tumors and lungs from each mouse paraffin sections (5 µm) were stained with hematoxylin and eosin followed by histological analysis.

2.9 Statistical Analysis

Student's t test was used to determine significant differences between two means ($p<0.05$).

3. Results

3.1 EGF-induced cell migration of MTLn3 cells is dependent on JNK activity and associated with paxillin Ser178 phosphorylation

First we determined the role of the JNK-paxillin signaling in EGF-induced migration of the highly metastatic MTLn3 rat mammary adenocarcinoma cells. Exposure to EGF causes a rapid onset of lamellipodia formation, random cell migration and scattering of MTLn3 cells (Fig. 1A; and see supplemental movies M1-3). An inhibitor of JNK, SP600125, blocked the migration almost completely which was associated with increased cell clustering of the MTLn3 cells (Fig. 1A). This clustered phenotype was associated with increased localization of β-catenin at cell-cell contacts (Fig. 1B). Quantitative analysis of the movies indicates that inhibition of JNK drastically reduces directional movement (Fig. 1C) and velocity of the cells (Fig. 1D). Next we determined the involvement of paxillin in this process. Treatment with EGF induced the transient phosphorylation of paxillin at

Ser residue 178 in association with JNK activation. Importantly, this phosphorylation was dependent on JNK, since SP600125 prevented the efficient phosphorylation of paxillin at Ser178 (Fig. 1E). These data indicate that in MTLn3 cells EGF-induced JNK-mediated phosphorylation of paxillin at Ser178 regulates cell migration.

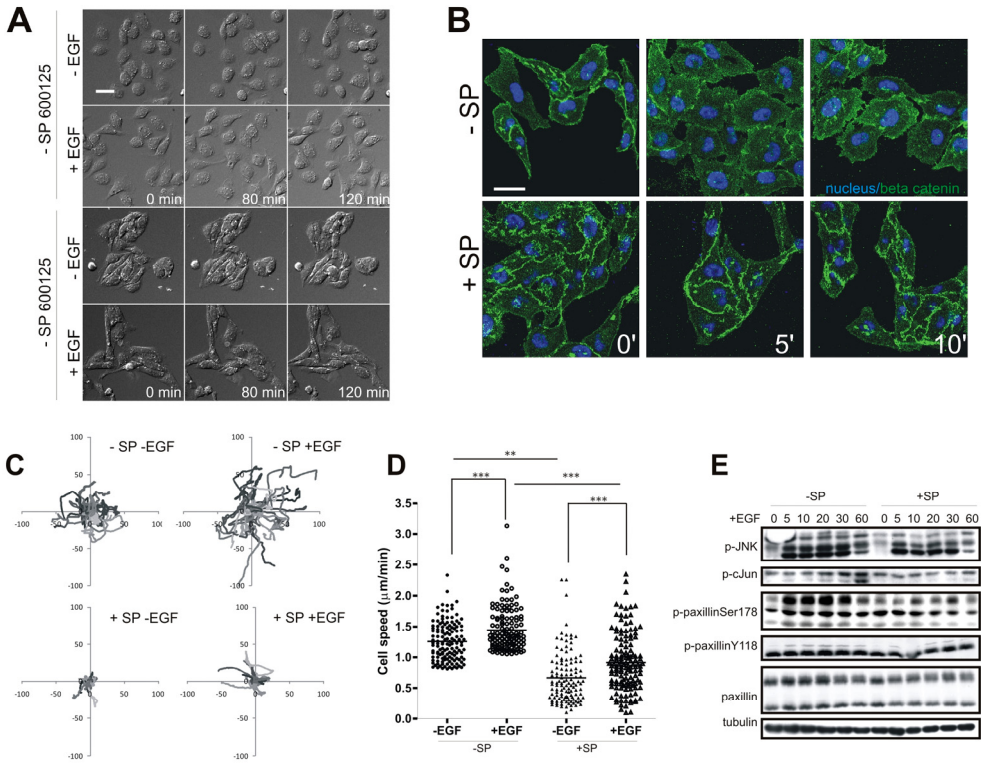

Figure 1: JNK-mediated phosphorylation of paxillin S178 plays a role in tumor cell migration. (A) MTLn3 cells are followed with DIC microscopy after EGF stimulation (10 nM) or not for 3hours with or without JNK inhibitor SP600125 (20 µm). Cells were starved in serum free medium for 3 hours and the inhibitor was added 30 min prior to imaging. The cells exposed to SP6000125 show clearly cell clustering in both control and EGF stimulated situations. Snapshots of the time-lapse made for 2 hours are shown, scale bar is 50 µm. (B) Inhibition of JNK results in localization of β-catenin at cell-cell contact, MTLn3 cells were fixed 0,5 and 10 min after EGF stimulation with or without SP600125 and stained for the nucleus (blue) and β-catenin (green). Scale bar is 20 µm. (C) JNK inhibition reduced the random movement of MTLn3 cells. Plots represent the overall trajectories of individual cells of representative time-lapse movies of the 4 conditions (+/-EGF and +/- SP600125). X and Y axis are in µm. Cell tracking was done with Image Pro software. (D) JNK inhibition reduced cell speed of MTLn3 cells in both control and EGF treatments ($p<0.001$). (E) Inhibition of JNK results in the absence of c-Jun phosphorylation after EGF stimulation and significantly reduces phosphorylation of paxillin Ser178. The loading of the Western Blot was controlled with total paxillin and tubulin staining.

3.2 Paxillin Ser178Ala mutant inhibits MTLn3 cell motility in vitro

To further investigate the role of paxillin Ser178 in cell migration, we generated MTLn3 cell lines stably expressing either GFP-tagged wt-paxillin or mutant GFP-paxillin in which the serine residue 178 was replaced by alanine (further referred to as paxillin-S178A). Three wt-paxillin clones and three paxillin-S178A clones were selected for further experiments. MTLn3 clones stably expressing GFP-wt-paxillin or GFP-paxillin-S178A were evaluated by flow cytometry (see quantification in Fig.S1A), western blotting (Fig. S1A) and immunofluorescence (Fig. 2A). Expression levels were approximately equal in all three wt clones; one S178A clone had a lower expression level most likely due to reduced number of GFP-positive cells (Fig S1A). The paxillin S178A cells were smaller and clustered with enhanced cell-cell contact while the wt cells are stretched with large lamellipodia and almost no cell-cell contact (Fig. 2A and Fig.S1B). In contrast to wt-paxillin, paxillin S178A cells formed cluster with strong β-catenin and E-cadherin positive cell-cell junctions (Fig. 2B and supplemental data 1C), reminiscent to the effect of SP600125 in control cells (compare Fig. 1B). Given the prominent role of paxillin in focal adhesion formation and dynamics, we next examined the effect of paxillin-S178A expression on MTLn3 cell attachment and spreading. Significantly less paxillin-S178A cells attached compared to wt-paxillin cells (Fig. 2C-a). Furthermore, while most of wt-paxillin cells had already spread, most of the S178A-paxillin cells remained rounded and presented a smaller surface area even after three hours of spreading (Fig. 2C-b). Next we determined the effect of paxillin-S178A on directed cell migration in an artificial wound healing assay. The percentage of wound closure was determined after 20 hrs (Fig. 2D and Fig.S1D). While wt-paxillin cells had closed the wound by 83 %, in contrast, paxillin-S178A cells had only closed 25% of the wound.

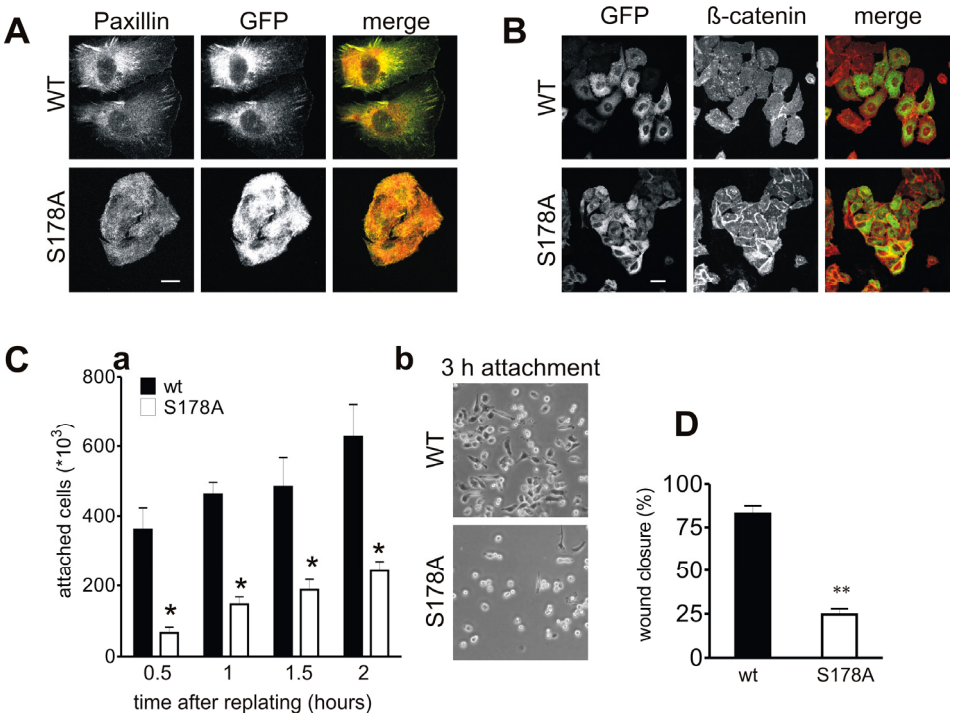

Figure 2: Expression of paxillin-S178A mutant decreases cell spreading and directed cell migration. 3 different clones for GFP-wt-paxillin and GFP-paxillin S178A MTLn3 cells were selected for further research. (A) Stable expression of mutant GFP-paxillinS178A in MTLn3 cells results in cell clustering and reduction of the cell area. Immunostaining of paxillin (red) colocalizes with ectopic expression of GFP-wt-paxillin and GFP-paxillinS178A. Scale bar is 10 µm. (B) Expression of mutant paxillin induces cell clustering through strong localization of β-catenin at cell-cell contact. Immunostaining of β-catenin (red) and GFP-paxillin protein (green). Scale bar is 20 µm. (C) Expression of paxillinS178A results in reduced cell adhesion (a) and spreading even after 3 hours (b). (D) The directed cell migration is also altered in the mutant cells. In a scratch assay, the wound was almost closed after 20 hours in the wt cells, while the mutant cells closed only 25% of the wound.

3.3 Paxillin-Ser178Ala affects EGF-induced cell migration and focal adhesion dynamics

Next we investigated the effect of S178A on the dynamics of cell migration after EGF stimulation. While upon EGF stimulation wt-paxillin cells rapidly formed lamellipodia and became highly motile, paxillin-S178A cells showed decreased cell motility and did not respond at all to EGF stimulation (Fig. 3A and supplemental movies M5-8). Since paxillin-S178A most likely acts as a dominant negative construct in these cells, it may compete for the localization of endogenous paxillin

at focal adhesions and prevents the phosphorylation of endogenous paxillin on Ser178 by JNK. Indeed, EGF stimulation hardly activated phosphorylation of endogenous paxillin whereas in wt-paxillin cells, both endogenous and GFP-wt-paxillin were phosphorylated at Ser178 after EGF treatment (Fig.S2). Importantly, EGF-induced mobility shift of endogenous paxillin was observed in both wt and S178A cell lines, indicating that most of the other paxillin modifications were unaffected (Fig.S2).

To understand the mechanism of the inhibitory effect of paxillin-S178A on cell migration, we determined the dynamics of focal adhesions in wt and mutant cells using TIRF microscopy. MTLn3 cells expressing wt paxillin show already in the control situation a high focal adhesion turnover which was enhanced upon EGF stimulation. In contrast, mutant cells show a much slower rate of FAs disassembly either in the presence or absence of EGF (Fig. 3B and supplemental movies M9-12). This decreased focal adhesion dynamics was associated with reduced mobility of GFP-paxillin-S178A as determined by fluorescence recovery after photobleaching (FRAP) experiments. Although under serum free conditions the rates and percentages of fluorescence recovery of the wild type GFP-paxillin and GFP-paxillin-S178A were similar, an increase in the mobile fraction of GFP-paxillin was measured in cells exposed to EGF after 30 min (Fig. 3C-a); this was not observed for GFP-paxillin-S178A (Fig. 3C-b). This suggests that P-paxillinS178 modifies its affinity for binding partners at FA resulting in decreased stability of paxillin when MTLn3 cells are stimulated with EGF.

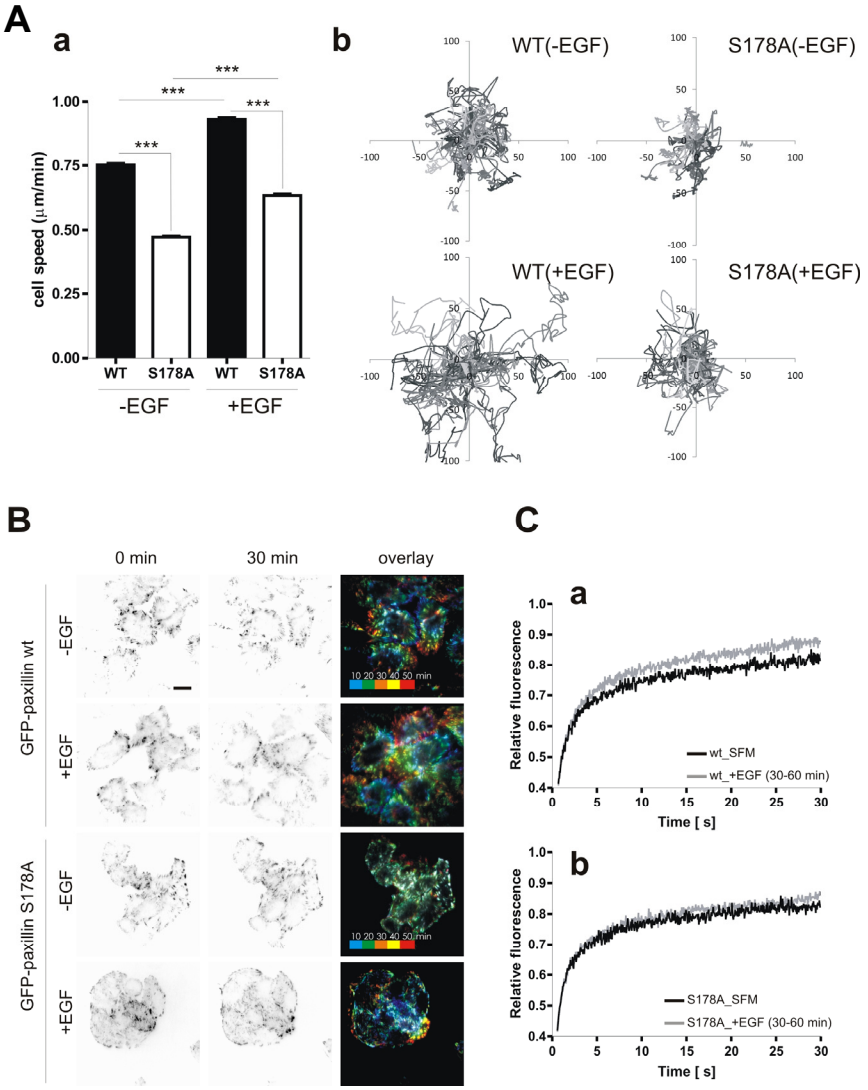

Figure 3: EGF-driven cell migration is inhibited in paxillin S178A cells because of an impaired focal adhesion turnover. (A) Paxillin S178A reduces cell speed (a) and directional cell movement of MTLn3 cells (b). Cells were followed for approximately 10 hours with epi-fluorescence. Cell tracking was done with Image Pro software. ($p<0.001$). (B) Matrix adhesions dynamics in MTLn3 cells was visualized with TIRF microscopy. Overlay of images at different times in red, green and blue produces a white/grey image if no change of focal adhesion positions. The turnover is impaired in mutant cells and is not responsive to EGF stimulation. Scale bar is 20 µm. (C) Protein dynamics was measured with the spot bleaching technique (25 focal adhesions bleached per experiment, n=2). In control situation, the protein kinetic was similar in both WT and S178A cells (see wt_SFM and S178A_SFM in a and b). In contrast, only wt-paxillin responded to EGF stimulation; thus increased matrix adhesion turnover correlates with reduced immobile fraction of wt-paxillin at FA (see wt_+EGF and S178A_+EGF in a and b).

3.4 GFP-paxillin-S178A expression impairs cells metastases formation of MTLn3 cells in an orthotopic breast tumor model

Next we determined whether GFP-paxillin-S178A affect the spontaneous metastasis formation of MTLn3 cells. The MTLn3 cell line is an excellent model to study metastasis formation from mammary gland tumors to the lung[34]. We injected wt and mutant cells in the mammary fat pad of immunodeficient Rag2$^{-/-}$ $\gamma^{-/-}$ mice and after three weeks mice were sacrificed for the analysis of the primary mammary gland tumor as well as lung metastases. All primary tumors were still GFP positive, indicating expression of wt or mutant GFP-paxillin. The edges of the wt-GFP-paxillin tumors were more invasive-like then those of GFP-paxillin-S178A tumors (Fig. 4A). Yet, the size of the primary tumor was not significantly altered by GFP-paxillin-S178A (Fig. 4B). GFP-paxillin-S178A MTLn3 cells formed significantly less spontaneous lung metastases than wt-GFP-paxillin MTLn3 cells (Fig. 4C and D). Interestingly, while wt-paxillin cells formed relatively more small micro-metastatic lesions compared to mutant cells, also these metastases had a more scattered phenotype (Fig. 4A). These data indicate an essential role of the residue S178 of paxillin in breast cancer progression.

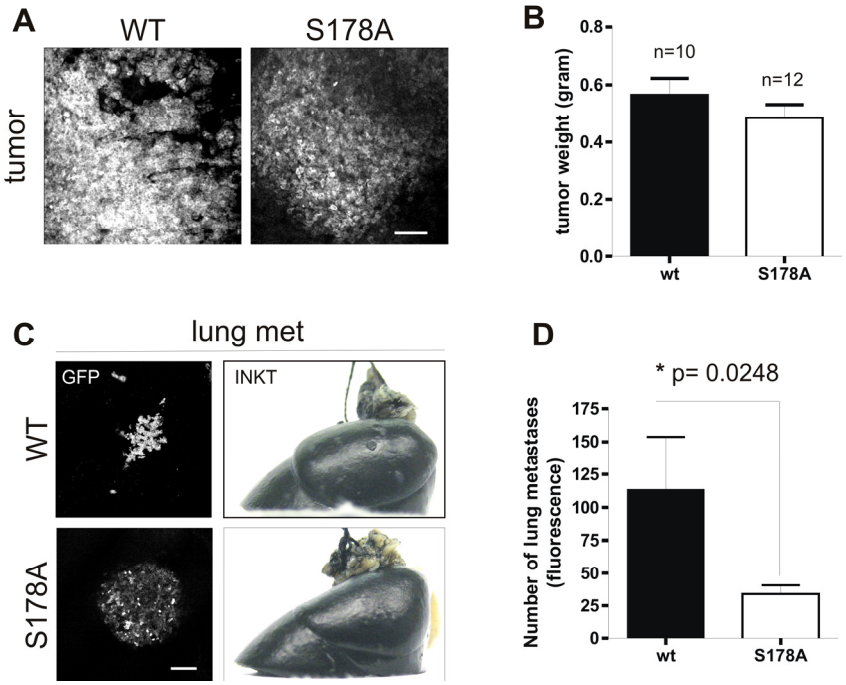

Figure 4: Expression of paxillin-S178A mutant impairs lung metastases formation in a mouse *in vivo* model. (A). Clones nr 2 of GFP-wt-paxillin and GFP-paxillin S178A MTLn3 cell-lines were injected orthopically in 6 weeks young female Rag2$^{-/-}\gamma c^{-/-}$ mice. Two-photon intravital imaging confirmed that the primary tumor were highly GFP-positive. Scale bar is 100 µm. (B) Approximately 28 days later, the

mice were sacrificed and the fresh tumor was weighted. No significant difference was measured between the wt group mice (n=10) and S178A group mice (n=12). (C) The number of surface GFP positive lung metastases were counted using epi-fluorescent microscope. Two-photon intravital microscopy revealed that there were many more metastases in the wt group in general small and invasive like while in the mutant group very few and large in size lung metastases were detectable. The lungs stained with black inkt show the same results. Scale bar is 100 µm. (D) The number of GFP-positive lung metastases was significantly higher in the wt group than in the mutant group.

3.5 Paxillin S178A overexpresion results in EGFR down regulation at mRNA and protein levels

Next we sought to determine the possible mechanism by which paxillin-S178A affects tumor cell migration and metastasis formation. EGFR signaling is regulated and trans-activated at focal adhesions by both integrins[35,36] and FAK[37,38]. Therefore we reasoned that possibly paxillin-S178A would disturb the EGFR signaling pathway. Interestingly, all three mutant S178A MTLn3 clones had much lower level of EGFR than in wt clones (Fig. 5A). This was associated with reduced mRNA expression of EGFR in paxillin-S178A cells (Fig. 5B). As a consequence downstream EGFR signaling towards Akt and ERK were also affected in S178A clones (Fig. 5C). While EGF caused clear activation of Akt in wt-paxillin cells, proper Akt activation by EGF was inhibited in paxillin-S178A. In a similar fashion ERK activation was reduced in paxillin-S178A cells compared to wt-paxillin cells after EGF treatment (Fig. 5C). Since EGF also caused JNK activation in control MTLn3 cells (see figure 1), we also determined the activation of JNK signaling pathway in these cells. Indeed paxillin-S178A expression also inhibited EGF-induced JNK activation (Fig. 5C), which was associated with reduced transactivation of the c-Jun transcriptional activity as determined by luciferase reporter assays (Fig. 5D). While the activity of the negative control TATA-luc and the positive control construct 5xCollagenase were unaffected, the activity of the c-Jun reporter construct was significantly decreased in paxillin-S178A mutant cells. These data indicate that S178A-paxillin affects the expression of EGFR possibly through the regulation of c-Jun-mediated EGFR expression[39,40], thereby disturbing downstream signaling pathways that are essential in the cell migration process.

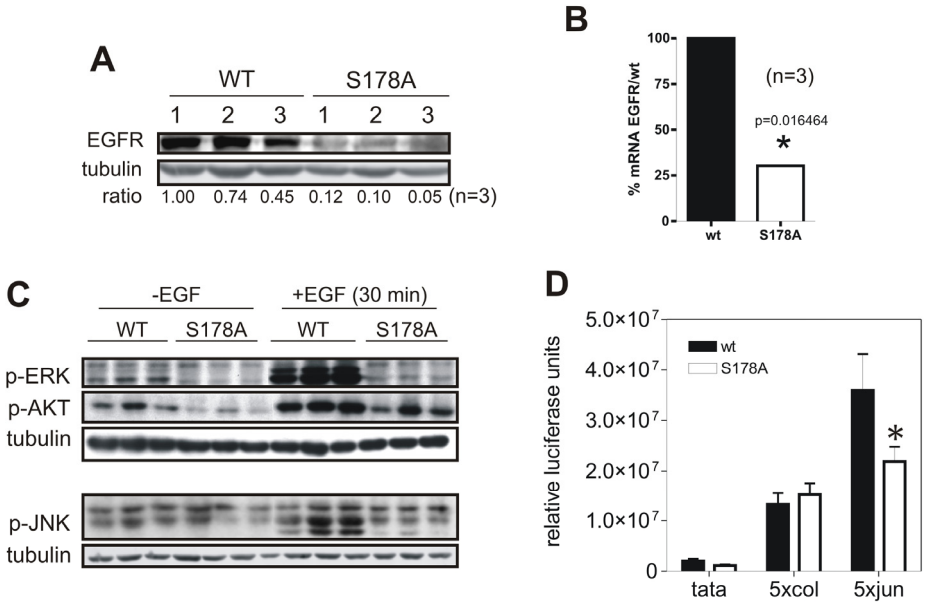

Figure 5: EGFR and EGFR-signalling is downregulated in paxillinS178A mutant cells. (A) Paxillin S178A overexpresion results in EGFR down regulation at protein levels in all three different mutant clones. Ratios of EGFR levels calculated with respect to WT1 clone are indicated below. (B) Lower EGFR at protein levels in mutant cells correlates with its significant downregulation at mRNA level. (C) EGFR downstream signaling towards Akt, ERK and JNK was also affected in S178A clones. (D) The altered JNK signaling in S178A results in reduced activity of AP1 member c-Jun. AP1- induced transcription was determined using a TATA-luciferase reporter assay in all the different clones in starved medium conditions.

3.6 Re-expression of wt-EGFR in paxillin-S178A cells restores EGF-driven cell motility and lung metastasis formation.

Finally, we determined the role of the downregulation of EGFR in the reduced cell motility and metastasis of paxillin-S178A cells. For this purpose we re-expressed EGFR in paxillin-S178A using retroviral transfection followed by FACS sorting (Fig. 6A and Fig. S3A). EGFR-paxillin-S178A cells showed a more spread phenotype than the mutant cells only (see Fig. 6A and also Fig.S3B). Both EGF-driven cell migration was rescued as well as the protein turnover of paxillin-S178A at focal adhesions upon EGF stimulation (Fig. 6B and Fig. S3C; compare with Fig. 3C). This was associated with a sustained activation of both JNK and ERK after EGF exposure (Fig. S3D). Next we determined whether EGFR re-expression also restored the capacity of MTLn3 paxillin-S178A to metastasize to the lungs. For this purpose we injected paxillin-S178A cells and EGFR-GFP-paxillin-S178A cells in the mammary fat pad of immunodeficient Rag2$^{-/-}$ $\gamma^{-/-}$ mice. The tumor growth of paxillin S178A and paxillinS178A EGFR cells was similar in the tested animal (Fig. 6C-b) and all tumor cells in the primary tumors were GFP positive (Fig. 6C-a).

Importantly, there was an approximately three fold increase in the lung tumor burden in GFP-paxillin-S178A cells that expressed EGFR (Fig. 6D). These data indicate that the reduced metastasis formation of GFP-paxillin-S178A cells is directly related to the expression levels of the EGF receptor.

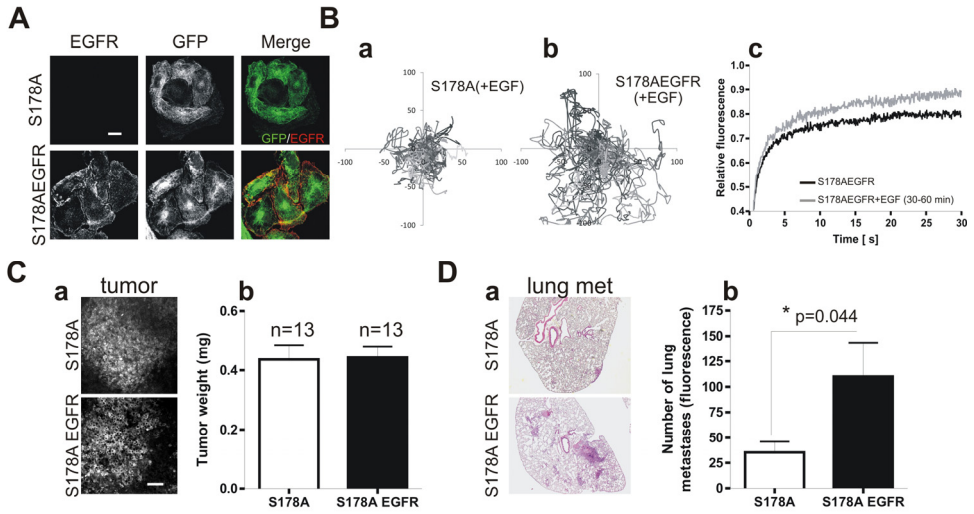

Figure 6: Re-expression of wt-EGFR fully restores EGF-driven cell motility and lung metastasis formation. (A) Re-expression of wt-EGFR (human) rescues partially the cell phenotype: more spread and less cell-cell contact. Immunostaining with a specific mouse monoclonal antibody against human confirmed the ectopic expression of EGFR in S178A cells; paxillinS178A (green) and human EGFR (red). Scale bar is 10 µm. (B) Re-expression of wt-EGFR restores EGF-driven cell motility and protein dynamics. S178A and S178AEGFR cells were followed for 6 hours with epi-fluorescence microscopy. Trajectories of the cell movements are plotted in (a) and (b) (X and Y are in µm). Protein dynamics was quantified with spot bleaching technique (c). Fluorescence recovery was followed in approximately 25 focal adhesions per experiments (ns=2). (C) Pictures of S178A and S178AEGFR primary tumors confirmed that they were still highly GFP-positive (a). Scale bar is 100 µm. There was no significant difference in fresh tumor weight (b) between S178A group (n=13) and S178AEGFR group (n=13). (D) EGFR re-expression restores capacity of MTLn3 paxillin S178A to metastasize to the lungs. In (a), representative H&E staining of lung coupes of both groups are shown. In (b), the number of GFP-positive counted in the fresh lungs at time of sacrifice is shown.

4. Discussion

Here we investigated the role and mechanism of growth factor-induced and JNK-mediated paxillin-Ser178 in tumor cell migration *in vitro* and metastasis formation *in vivo*. For this purpose we used the highly motile and metastatic cell line MTLn3[41]. Our findings indicate that: 1) EGF-induced cell migration of MTLn3 cells is dependent on the activity of JNK and associated with a JNK-mediated phosphorylation of Ser178 of paxillin; 2) expression of paxillin-S178A mutant prevents cell migration in association with a reversal of a scattered phenotype and

re-formation of E-cadherin-based cell-cell junctions; 3) paxillin-S178A reduces the expression EGFR and consequently diminishes the proper activation of downstream signaling pathways; and 4) the paxillin-S178A mutant prevents spontaneous lung metastasis formation which is rescued by re-introduction of the EGFR. Together our data suggest an intricate link between EGFR signaling, JNK activation and paxillin-phosphorylation on Ser178, which then acts to sustain this signaling pathway by modulating EGFR expression.

Our data indicate that EGF-induced JNK activation is essential for MTLn3 cell migration. EGF caused the rapid activation of JNK in association with increased cell migration and inhibition of JNK by SP600125 prevented EGF-driven cell migration. Phosphorylation of paxillin on Ser178 was induced upon EGF stimulation in a manner sensitive to the JNK inhibitor, and expression of the Ser178Ala mutant form of paxillin inhibited cell motility and EGF-driven MTLn3 cell migration[32,42]. By immunofluorescence we observed that active phosphorylated JNK co-localizes with paxillin at focal adhesions in MTLn3 (data not shown) as was previously observed in renal epithelial cells[43]. Also in keratinocytes, bladder epithelial cells and human breast tumor cells expression of paxillin-S178A inhibits cell migration[3]. These results implicate a JNK-paxillin signaling axis in the regulation of MTLn3 cell migration but the mechanism of these effects remained so far unclear. We now demonstrate that the JNK-paxillin axis regulates both the dynamics of focal adhesions through modulation of paxillin protein dynamics at focal adhesions as well as the dynamics of E-cadherin-based adherence junction formation. We do not yet know how exactly the phosphorylation at Ser-178 affects paxillin dynamics, but it is conceivable that Ser-178 phosphorylation induces a conformational change affecting paxillin interactions with other focal adhesion components. Alternatively, the decreased EGFR expression in paxillin-S178A cells may affect downstream signaling pathways that indirectly affect focal adhesion dynamics as well as adherence junction stability. Indeed, re-introduction of EGFR in paxillin-S178A cells reversed the epithelial-like phenotype and made paxillin-S178A again less immobile at focal adhesions, suggesting that this is not an intrinsic characteristic of paxillin-S178A, but rather due to altered signaling in cells mediated by downstream EGFR signaling pathways. This needs further investigation.

Here, for the first time, we demonstrate that the serine residue 178 of paxillin is essential for spontaneous metastasis formation. While at the moment of sacrifice the orthotopic mammary gland tumors of wt-paxillin and paxillin-S178A MTLn3 cells were equal in weight, the lung burden in the mutant group animals was significantly reduced than in wt group. Although a role for paxillin-S178 in cell migration has been established in several tumor cell lines[44-47], its role in cancer metastasis remained so far completely unknown. Several signaling pathways can activate JNK, including p21-activated protein kinase (Pak1) and the G protein coupled receptor proteinase-activated receptor 2 (PAR2) which is associated with enhanced cell migration. Under both conditions overexpression of paxillin-S178A

mutant inhibits the cell migration[48,49]; so far no *in vivo* data on the role of paxillin-S178 in the metastasis formation are presented. While we demonstrate a role for paxillin-S178 in breast cancer metastasis, further studies are required to establish this role in metastasis formation in other tumor models.

Our findings suggest a role of the JNK-paxillin pathway in the regulation of the expression of EGFR, thereby affecting cell migration and metastasis formation. In three independent paxillin-S178A MTLn3 clones we observed a significant reduction in EGFR mRNA and protein expression levels. These reduced levels decreased the efficient EGF-induced activation of signaling pathways downstream of EGFR; nevertheless, an equal mobility shift of endogenous paxillin protein was observed by western blotting, suggesting only partial defects in the EGFR signaling cascade. Given the fact that EGFR is overexpressed in advanced breast cancer, and that EGFR antagonists inhibit spontaneous metastasis formation of MTLn3 cells, we anticipated that the reduced EGFR expression was an essential component of the inhibited cell migration and metastasis formation. Indeed, re-expression of the wt-EGFR in the mutant cells did rescue the migratory phenotype of MTLn3-paxillin-S178A cells as well as their metastatic capacity under *in vivo* conditions. This regulation of the EGFR by paxillin phosphorylation has not been described before, neither there has been evidence for the regulation of the expression of other receptors by paxillin. It is unclear how in our model paxillin regulates EGFR expression. In MCF7 cells stable overexpression of c-Jun induces an increase in EGFR expression suggesting that AP-1 transcription factors can regulate EGFR transcription levels[40]; such a regulation is also observed in keratinocytes[39]. Our data indicate that paxillin-S178A MTLn3 cells also have reduced c-Jun transcriptional activity, which could thereby explain the reduced expression of EGFR, which underline a role of the JNK-c-Jun signaling pathway in the regulation of EGFR at transcriptional level. Our preliminary data in MCF7 cells that have increased ectopic expression of EGFR demonstrate that paxillin is involved in EGFR recycling and downstream signaling (de Graauw, unpublished observations). Thus, an alternative explanation may be that JNK-mediated phosphorylation of Ser178 of paxillin is required for efficient EGFR endocytosis and recycling, an essential component in its activation[50]. Hence this will affect proper JNK activation and AP-1 transcription factor activation thereby modulating EGFR expression. Alternatively, paxillin may affect the expression of EGFR by its known role in the regulation of gene expression through its interaction with Erk[27], poly-A-binding protein[51], Abl[52] and steroid receptors, or through its own ability to undergo nucleocytoplasmic shuttling[27,53].

Our findings indicate that the JNK-paxillin axis modulates the scattered phenotype of MTLn3 cells. Both inhibition of JNK as well as ectopic expression of paxillin-S178A mutant reverted a scattered phenotype towards a more epithelial-like morphology with the formation of E-cadherin/β-catenin cell-cell junctions. This morphological switch was observed under serum-starved and EGF treatment. The (in)direct tyrosine phosphorylation of β-catenin by EGFR and other receptor

tyrosine kinases such as c-Met is known to destabilize its binding to E-cadherin. Because of the low EGFR expression in the paxillin mutant cells, we anticipate that this results in de-phosphorylation of β-catenin leading to its localization to the cell-cell contact. Intriguingly, HGF treatment of S178A cells reverted the epithelial phenotype which was blocked by SP600125 (data not shown). These data suggest that paxillin-S178A does not affect the intrinsic molecular components and machinery required for the scattered phenotype and support that the effect of paxillin-S178A is rather related to the defects in the EGFR signaling pathways. Consequently, the defects in *in vivo* metastasis formation of MTLn3-paxillin-S178A cells are most likely largely due to defects EGF signaling, while tumors cells that do escape may use other pathways, such as c-Met-driven cell migration. This fits with our observations that ectopic EGFR expression itself is sufficient to again allow metastasis formation of paxillin-S178A cells.

In summary, our data provide insight into the crucial role of JNK-mediated phosphorylation of paxillin Ser residue 178 in tumor cell migration and metastatic spread, which is directly related to maintenance of EGFR expression. Given the essential role of Ser178 phosphorylation in the migration and metastasis formation, monitoring this paxillin phosphorylation in tumor samples from patients may be indicative of the activation of this pro-metastatic pathway and possibly predictive for the disease prognosis. Moreover, with the further development of specific JNK inhibitors it is anticipated that novel targeted therapies may be used in the clinic in the future. Further work in this area is required.

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SUPPLEMENTAL DATA

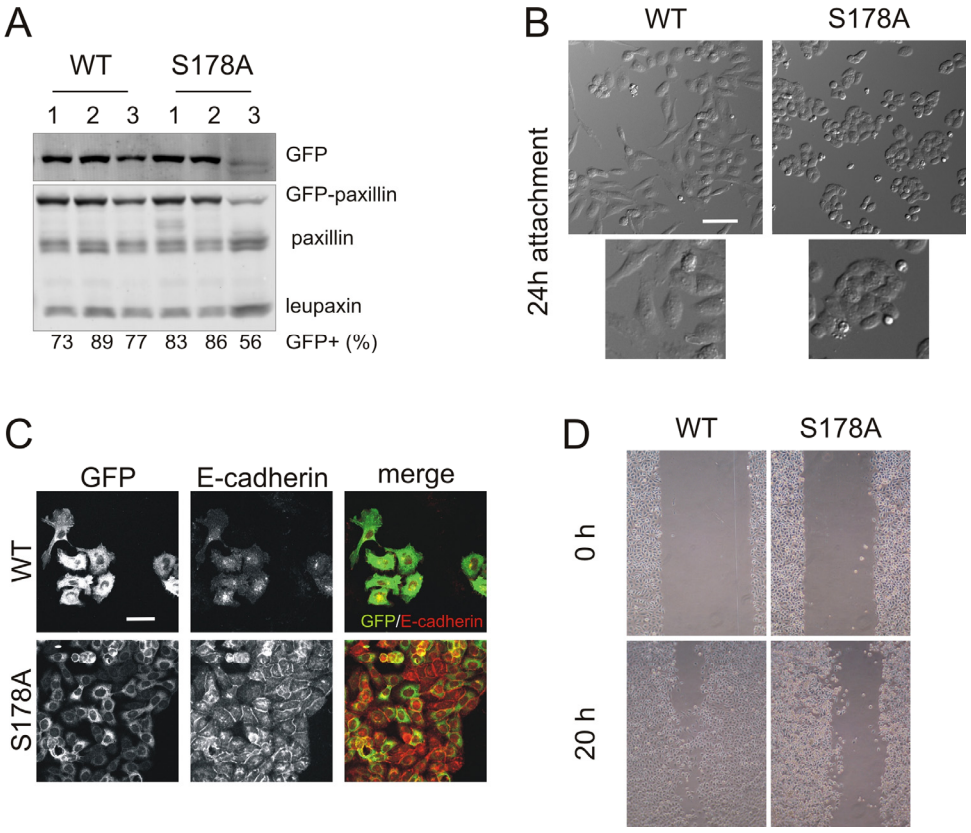

Figure S1: Expression of paxillin-S178A mutant decreases cell spreading and directed cell migration. (A) Cell lysates for each cell line were collected and analyzed for GFP-paxillin expression by western blotting. The GFP signal was also detected by FACS analysis; percentage of positive cells is given below the western blot. (B) wt and S178A cells after 24 hours attachment on glass coverslips coated with collagen (DIC imaging). Wt-paxilln cells are spread and do not show cell-cell contacts while S178A cells are smaller in size and show increased cell clustering. Scale bar is 50 µm. (C) Expression of mutant paxillin induces cell clustering through strong localization of E-cadherin at cell-cell contact. Immunostaining of E-cadherin (red) and GFP-paxillin protein (green). Scale bar is 20 µm. (D) Snapshots of wound closure of wt and S178A cells in the beginning and 20 hours later after the scratch was made.

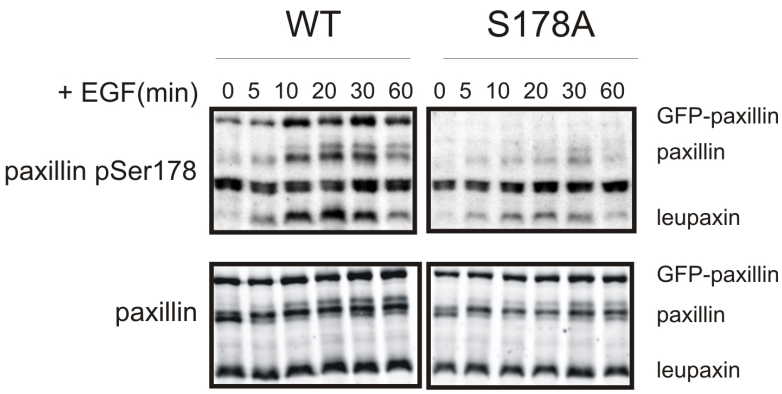

Figure S2: Phosphorylation of endogenous paxillin Ser 178 after treatment with EGF for 60 minutes. Paxillin-S178A mutant overexpression impairs phosphorylation of endogenous paxillin at Ser 178 EGF treatment for 0, 5, 10, 20, 30 and 60 minutes.

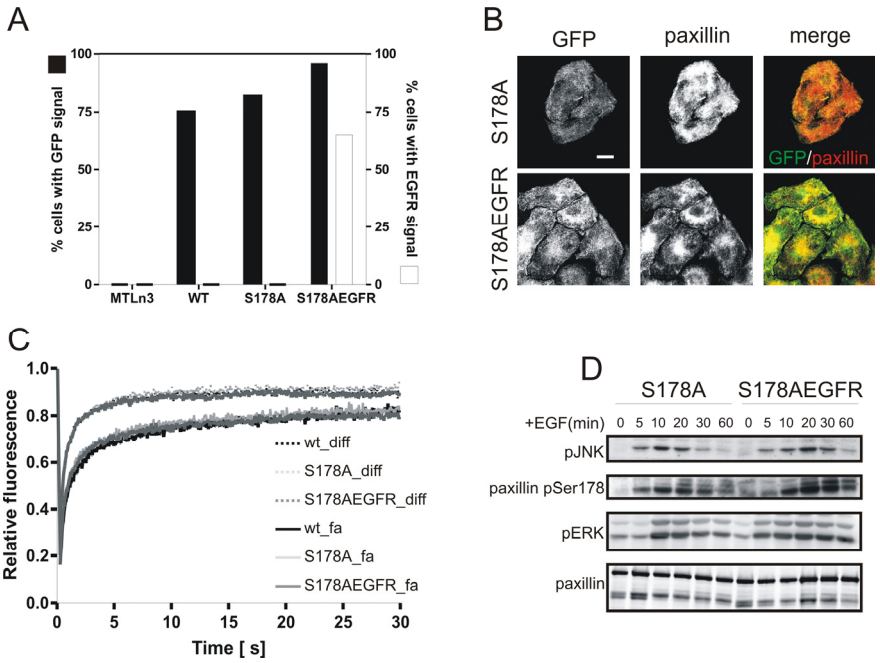

Figure S3: Re-expression of wt-EGFR restores wt cell phenotype and activation of ERK and JNK signaling. (A) Detection of human wt-EGFR in mutant cells with flow cytometry (FACS). (B) Mutant cells with EGFR re-expression show a more spread phenotype which is comparable with the wt cells. Immunostaining of GFP-paxillinS178A (green) and total paxillin (red). Scale bar is 10 µm. (C). There is no difference in protein dynamics in cytoplasm (_diff) or at focal adhesions (_fa) in starved conditions between the three cell-types: wt, S178A and S178AEGFR. (D). Re-expression of wt-EGFR restores JNK and ERK activitation upon EGF treatment.

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