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Podosome dynamics and location in vascular smooth muscle cells require CLASP-dependent microtubule bending

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Abstract

Extracellular matrix (ECM) remodeling during physiological processes is mediated by invasive protrusions called podosomes. Positioning and dynamics of podosomes define the extent of ECM degradation. Microtubules are known to be involved in podosome regulation, but the role of microtubule network configuration in podosome dynamics and positioning is not well understood. Here, we show that the arrangement of the microtubule network defines the pattern of podosome formation and relocation in vascular smooth muscle cells. We show that microtubule plus-end targeting facilitates de novo formation of podosomes, in addition to podosome remodeling. Moreover, specialized bent microtubules with plus ends reversed towards the cell center promote relocation of podosomes from the cell edge to the cell center, resulting in an evenly distributed podosome pattern. Microtubule bending is induced downstream of protein kinase C (PKC) activation and requires microtubule-stabilizing proteins known as cytoplasmic linker associated proteins (CLASPs) and retrograde actin flow. Similar to microtubule depolymerization, CLASP depletion by siRNA blocks microtubule bending and eliminates centripetal relocation of podosomes. Podosome relocation also coincides with translocation of podosome-stimulating kinesin KIF1C, which is known to move preferentially along CLASP-associated microtubules. These findings indicate that CLASP-dependent microtubule network configuration is critical to the cellular location and distribution of KIF1C-dependent podosomes.

Introduction

Extracellular matrix (ECM) degradation and remodeling is an essential component of tissue rearrangement occurring in development, regeneration and disease. In most cases, ECM organization is changed by specific protrusive organelles, invadosomes, which consist of

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Author contribution

XZ performed the experiments and analysis of MT pattern/dynamics and actin flow; NE performed experiments and analysis on podosome number and KIF1C movement; CA performed experiments on podosome relocation; SKH provided expertise on Src pathway and wrote the manuscript; IK oversaw the whole project, performed analysis of podosome dynamics, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

polymerizing actin filaments and cell-matrix adhesion components. Invadosomes serve as sites for matrix metalloproteinase (MMP) secretion and promote ECM remodeling through both local ECM degradation and mechanical disturbance of the ECM. Though somewhat variable among cell types, two major categories of invadosomes are generally recognized: small dynamic podosomes typical for developing and regenerating tissues and their pronounced cancer counterparts, called invadopodia, which are responsible for cancer cell invasion. Podosomes and invadopodia differ in their morphology and lifetime, which define the extent of ECM remodeling and distinguish between physiological and malignant ECM remodeling modes. Specifically, elongated, long-lived invadopodia cause deep, site-specific ECM degradation, which is a hallmark of cancer invasiveness. Podosomes, in contrast, cause even and shallow ECM degradation as they are relatively short-lived structures, which relocate throughout the ventral cell surface (Burgstaller and Gimona, 2005; Linder, 2007; Linder et al., 2011). Thus, proper dynamics and cellular distribution of podosomes is essential for physiological ECM remodeling.

Like other components of the actin cytoskeleton, podosomes are tightly regulated by Src family kinases (Destaing et al., 2011; Boateng and Huttenlocher, 2012), Rho family small GTPases (Lener et al., 2006; Ory et al., 2008; van Helden and Hordijk, 2011), and mechanosensitive pathways (Stegemann et al., 2005; Collin et al., 2008; Albiges-Rizo et al., 2009). Cytoskeletal networks that serve as intracellular highways, microtubules (MT), and MT-dependent trafficking are also known to regulate podosomes in a number of cell types, including osteoclasts, macrophages and vascular smooth muscle cells (VSMCs). In osteoclasts, podosome regulation involves MT stabilization and post-translational modification of tubulin (Destaing et al., 2005; Gil-Henn et al., 2007). At the same time, polymerizing plus-ends of dynamic MTs have been associated with podosome regulation in all three above-mentioned cell types. MT plus ends facilitate disassembly and splitting of macrophage podosomes (Kopp et al., 2006) and are necessary for the rearrangement of osteoclast podosomes into the functional podosome belt (Biosse Duplan et al., 2014). It was shown that MT plus end-tracking protein (+TIP) EB1 and kinesin KIF1C, which can be recruited to the MT plus ends by interactions with MT +TIPs CLASPs (Efimova et al., 2014), are major molecular players in podosome regulation in osteoclasts (Biosse Duplan et al., 2014) and macrophages/VSMCs (Kopp et al., 2006; Efimova et al., 2014), respectively.

Several questions remain in this intensively developing field: first, the role of MT plus ends in podosome formation *de novo*, rather than podosome remodeling, has not been addressed; second, it is unclear how MT network configuration is designed to ensure proper localization of MT plus-end-dependent podosome regulation.

To address these questions, we studied highly dynamic podosomes of VSMCs, which form at the cell periphery in close contact with focal adhesions and then relocate centripetally and distribute throughout the ventral cell surface (Kaverina et al., 2003) to provide even ECM remodeling (Lener et al., 2006). In this study we addressed whether MTs are involved in the regulation of these characteristic dynamics.

Our results indicate that MT plus end targeting is necessary for *de novo* formation of podosomes at specific sites, the locations of which depend on the MT network

rearrangement triggered by the podosome induction pathway. This indicates that modification of the MT cytoskeleton is actively involved in the transmission of the regulatory signal. We find that MT-stabilizing +TIPs CLASPs are required to organize MT tracks with specific geometry that define the cellular location of podosomes.

Results

In the absence of MTs, podosomes are restricted to the cell periphery

Podosomes in the rat VSMC line A7r5 can be rapidly induced by PKC-activating phorbol ester PDBu treatment (Hai et al., 2002). Podosomes can be detected as dense actin patches at the ventral cell layer that are positive for a signature podosome marker Tks5 ((Seals et al., 2005; Efimova et al., 2014) and supplemental Fig. S1). We previously showed that in cells where MTs have been depolymerized by nocodazole treatment (as in supplemental Fig. S2), PDBu-induced formation of podosomes is significantly suppressed (Efimova et al., 2014). Approximately 4% of nocodazole-treated cells, however, contained a small number of residual podosomes, indicating that podosome formation, in principle, can occur in the absence of MTs. We speculate that this occurs because during podosome formation, MTs deliver certain essential factors to cellular sites enriched in other podosome components (e.g. actin polymerization machinery). In the absence of MTs, such factors could be distributed throughout the cytoplasm and occasionally accumulate at potential podosome sites, including at the cell periphery, where actin polymerization factors are active. Consistent with this prediction, we found that the residual podosomes accumulated in circular arrangements (rosettes) at the cell periphery (Fig. 1B, C, E, F) rather than scattered throughout the cell (Fig. 1A, C, D).

An alternative method to achieve “permanent” presence of large developed podosomes in A7r5 cells is to express oncogenic Src (Fig. 2A), which constitutively activates the PKC-dependent podosome induction pathway (Tatin et al., 2006). Constitutive podosomes in oncogenic Src-expressing cells are normally larger than PDBu-induced podosomes and are easily recognizable by high actin density and the presence of Tks5 (supplemental Fig. S1). We found that the number of constitutive podosomes was strongly reduced when cells were treated with nocodazole (Fig. 2B, C), resembling our observations in PDBu-treated cells. Residual Src-induced podosomes were also found predominantly at the cell periphery (Fig. 2B).

Centripetal podosome relocation requires MTs

Because the capacity for ECM remodeling depends on podosome dynamics and lifetime (Linder, 2007; Linder et al., 2011), we addressed whether the restricted podosome patterns in nocodazole-treated cells result from a defect in podosome dynamics. Analysis of PDBu-induced podosome dynamics was challenging due to their rarity in nocodazole-treated cells. Therefore, we analyzed dynamics of Src-induced constitutive podosomes, which were suppressed by nocodazole but still present in sufficient numbers. We applied nocodazole to oncogenic Src-transformed cells expressing either DsRed-SM22 (a smooth muscle protein associated with dynamic actin filaments in podosomes (Gimona et al., 2003)) or RFP-cortactin (a well established podosome marker), and used TIRF imaging to follow podosome

dynamics. Live-cell imaging revealed multiple highly dynamic podosomes both near the cell center and at the periphery of control cells (Fig. 2D). In contrast, the center of nocodazole-treated cells was devoid of podosomes (Fig. 2E, F). At the same time, podosomes present at the periphery of nocodazole-treated cells were dynamic, similar to untreated control. In both cases, old podosomes continuously disassembled and new ones assembled; however, there was a striking difference in the positioning of newly formed podosomes in relation to preexisting podosome sites. In control cells, new podosomes formed at new locations (Fig. 2D, H; movies 1 and 2, left); in nocodazole-treated cells, new podosomes remained at the sites where old podosomes had disassembled (Fig. 2E, I; movies 1 and 2, right). To quantitatively assess the positions of new podosomes, we developed a method to describe how much of the cell area dynamic podosomes cover while relocating (including both podosome sliding and formation at the new sites). To do this, we determined the area of original podosomes at the start of the observation period (first frame) and the summarized area covered by the new podosomes elsewhere (where podosomes were detected at least in one of the following movie frames). The ratio between the newly occupied area and the original area covered by podosomes describes how actively podosomes relocate during the recorded period. This “podosome area increase factor” (see Materials and Methods) equals 0 when podosomes do not move (no new area occupied), equals 1 if podosomes relocate once (an equally large new area is occupied by a relocated podosome), equals 2 if podosomes relocate twice to new locations (two new locations of a podosome cover an area two times bigger than the original podosome), etc. We found that the podosome area increase factor was reduced 10-fold in the nocodazole condition as compared to the control condition, indicating that in the absence of MTs, podosomes were not capable of efficient relocation (Fig. 2G). Considering the lack of centrally located podosomes in nocodazole-treated cells and our previous finding that podosomes in VSMCs are normally initiated at the cell periphery and move toward the cell center as they mature (Kaverina et al., 2003), we conclude that centripetal relocation of podosomes is impaired in nocodazole-treated cells. These data indicate that MTs strongly facilitate relocation of podosomes and formation of podosomes at new spatial sites within a cell.

Podosome formation is associated with MT plus ends and MT bending

To understand whether podosome formation is directly driven by MTs, we investigated the spatial relationship between podosomes and polymerizing MT plus ends in live cells. We labelled podosomes using RFP-cortactin and MT ends using EB3-GFP, a +TIP commonly used to track plus ends of growing MTs. Podosomes were stimulated by PDBu treatment and their dynamics recorded by spinning disk microscopy (Fig. 3A-C). Podosome de novo formation was frequently observed immediately after targeting by an EB3 comet (Fig. 3B, C; movie 3). For analysis, all sites of podosome formation were detected, and the relative time of MT targeting of these sites prior to podosome formation was determined (see Materials and Methods). This analysis revealed that the majority of podosomes were formed immediately or within 30 seconds after a polymerizing MT plus end reached a point at the cell cortex that was within 1 μ m of the incipient podosome center (Fig. 3D, left). In contrast, cortex sites randomly distributed in space and time did not correlate with preceding MT targeting (Fig. 3D, right). This analysis provides the first evidence that direct MT plus end targeting stimulates podosome formation. In combination with previous findings suggesting

that MT targeting facilitates podosome splitting/disassembly in macrophages (Kopp et al., 2006) and podosome belt assembly in osteoclasts (Biosse Duplan et al., 2014), these data indicate that factors delivered by MT plus ends facilitate dynamic turnover of podosomes. Because relocation of podosomes in VSMCs occurs in centripetal direction, it is plausible to suggest that MTs deliver podosome-initiating factors from peripheral podosomes to more central sites. Interestingly, we found that centrally located podosomes were often distributed along MT bundles that were bent at the cell periphery and turned with their plus ends toward the cell center, while peripheral podosomes were found close to MT bundles parallel to the cell edge (Fig. 3E, F). In contrast, MTs that radiate from the cell center to the cell periphery were not associated with podosomes. Such localization suggests that factors underlying podosome relocation are delivered toward the cell center in a MT plus end directed manner.

PDBu induces changes in MT configuration in an actin- and CLASP-dependent manner

When MT plus-end associated factors are translocated from old podosome sites to new ones, this occurs in the direction of MT growth (plus end propagation). However, in a radial microtubule array, which is considered the most common interphase microtubule network organization, microtubule minus ends are concentrated at microtubule-organizing centers (MTOCs) in the cell center, while MT plus ends extend toward the cell periphery. In this radial MT array, podosomes would move with MT plus end propagation from the cell center to the cell periphery. However, they translocate inwards. Thus, we suggested that the MT network is reorganized so that some MT plus ends propagate toward the cell center. To test this, we investigated whether podosome-inducing signaling affects the MT network. We found that PDBu treatment indeed causes a significant change in MT orientation and dynamics. Specifically, the MT network as revealed by tubulin immunostaining, became less organized and cells developed prominent arrays of bent MTs (compare Fig. 4A to B). Live cell imaging of MT plus tip markers EB3-GFP or GFP-CLIP170 (Fig. 4E, F; movie 4) revealed that PDBu treatment resulted in ~3-fold increase in the number of MTs growing toward the cell center (Fig. 4J). At the same time, the number of MT plus ends at the cell periphery increased only ~2-fold (Fig. 4I), indicating that PDBu treatment specifically increased the percentage of reversed MT tracks (Fig. 4K), while enhancing MT polymerization in general.

Several +TIPs can modulate MT dynamics and organization (Lansbergen and Akhmanova, 2006; Morrison, 2007; Gouveia and Akhmanova, 2010). We recently showed that activation of pathways that stimulate podosome formation also enhanced MT-binding of CLASPs (Efimova et al., 2014) which facilitate MT polymerization and stability (Galjart, 2005). As expected, CLASP depletion resulted in decreased MT density as compared to control, but MT organization and dynamics in CLASP-depleted cells were not significantly changed by PDBu treatment. PDBu did not induce the characteristic disorganized and bundled arrays normally associated with podosome formation (Fig. 4C, D). Moreover, live-cell imaging revealed that the number and directionality of growing MT plus ends changed very little upon PDBu treatment of CLASP-depleted cells (Fig. 4G-K; movie 4). Strikingly, the number of reversed MTs increased only ~4% compared to ~13% increase in control (Fig. 4J, K). These data allow us to conclude that CLASP is required for MT reorganization triggered

by PDBu treatment; thus, PDBu-triggered changes in MT configuration likely involve altered CLASP binding to peripheral MTs.

It is known that MTs at the cell periphery can be dragged toward the cell center by retrograde actin flow-produced forces (Waterman-Storer and Salmon, 1997; Salmon et al., 2002; Schaefer et al., 2008). We have imaged a MT plus end marker GFP-CLIP170 and an actin marker mCherry-Utrophin (Burkel et al., 2007) in PDBu-treated cells. Consistently with previous findings, MT plus ends were often displaced by retrograde actin movement, resulting in a change of the direction of MT propagation (Fig. 5A-C, movie 5). We have also observed that retrograde actin flow in A7r5 cells was enhanced upon PDBu treatment (Fig. 5D, movie 6 left). To test whether retrograde flow is required for PDBu-induced MT reversal, we used an inhibitor cocktail designed previously to efficiently block actin dynamics in actively protruding cells (Peng et al., 2011). We have optimized this inhibitor cocktail (see Materials and Methods) to completely stop the actin flow in PDBu-treated A7r5 cells (Fig. 5E, movie 6 right). Under these conditions, PDBu failed to induce MT reversal (Fig. 5F-M, movie 7), indicating that actin flow-applied forces are necessary for MT bending and for the change in MT growth direction. Together, these data suggest that actin flow actively bends CLASP-associated MTs.

CLASPs are required for centripetal podosome relocation

If our hypothesis that bent MTs with their plus ends directed toward the cell center are required for centripetal podosome relocation is correct, CLASP-dependent MT remodeling must be important for podosome positioning. To test this hypothesis, we examined constitutive podosomes induced by oncogenic Src in CLASP-depleted cells, immunostained for actin or cortactin (Fig. 6A-E). The pattern of Src-induced podosomes resembles the effect of MT depolymerization: while the number of podosomes is decreased (Fig. 6G), all the remaining podosomes are localized at the cell periphery (Fig. 6C, E). This pattern was similar to the effect of MT depolymerization by nocodazole, consistent with our previous finding that podosome formation in response to PDBu is similarly diminished in CLASP-depleted and nocodazole-treated cells (Efimova et al., 2014). Furthermore, we visualized Src-induced podosomes in CLASP-depleted cells by live-cell TIRF microscopy and analyzed their location and dynamics similarly to Fig. 2D-I. These analyses determined that the center of CLASP-depleted cells was devoid of podosomes (Fig. 6I), in contrast to control cells (Fig. 6H, L). The podosomes in both control and CLASP-depleted cells were dynamic and underwent efficient assembly and disassembly. In control cells, new podosomes assembled at multiple new sites (relocated, Fig. 6H, J), while in CLASP-depleted cells they predominantly assembled at the same locations (Fig. 6I, K; movies 8 and 9). Quantification of the podosome area increase factor showed that CLASP-depleted cells have defects in centripetal podosome relocation similar to the defects observed in cells lacking MTs (Fig. 6M).

Overall, the effects of CLASP depletion on podosome relocation were very similar to those of complete MT depolymerization, indicating that CLASPs are essential players in the MT-dependent regulation of podosome positioning, and the CLASP-dependent change in MT configuration is necessary for centripetal podosome relocation.

Podosomes relocate with KIF1C accumulations

We recently showed that CLASP-dependent podosome formation depends on molecular motor protein KIF1C, which preferentially moves at CLASP-associated MTs (Efimova et al., 2014). Because relocation of podosomes is associated with the presence of CLASP, we tested whether KIF1C was involved in MT- and CLASP-dependent podosome relocation. Live-cell imaging of cells co-expressing KIF1C-GFP and RFP-cortactin showed that the deposition of KIF1C-GFP at distinct cortical sites often preceded formation or relocation of podosomes (Fig. 7). Often, KIF1C-GFP mass relocated from an existing podosome to a new podosome formation site and then extended around the cortactin-rich core of an emerging podosome, forming a ring-like structure (Fig. 7B, F; movies 10 and 12), which we described previously (Efimova et al., 2014). Relocating KIF1C accumulations were of tubular or vesicular shape, suggesting that KIF1C was associated with membrane structures. Furthermore, we observed frequent movement of KIF1C-GFP from the cell periphery toward the cell center (Fig. 7C-I), in agreement with the preferential podosome relocation direction. Initial podosomes were formed when KIF1C accumulations at the protruding cell edge were relocated slightly (2-3 μ m) backward through a centripetal movement and accumulated around an emerging podosome core (Fig. 7C, D; movie 11). When podosomes were already prominent and surrounded by KIF1C, KIF1C-rich tubes often extended from the podosome rim toward the cell center, and new inner podosomes initiated at these extensions (Fig. 7E, F; movie 12).

To test whether KIF1C movement is potentially involved in centripetal podosome relocation along reversed MTs, we addressed whether KIF1C moves along MTs with their plus ends extending toward the cell center. Imaging of KIF1C-GFP together with MT plus-end marker EB3-mCherry revealed that KIF1C was indeed moving along MTs of reversed polarity (Fig. 7G, I; movie 13) in MT plus end-directed mode. This suggests that reversed MTs are necessary for the relocation of KIF1C from peripheral to inner podosome formation sites (see model in Fig. 8).

Discussion

Previous data from several groups including ours indicate that MTs regulate podosome formation and dynamics in physiological inducible systems (such as activation of macrophages and osteoclasts or VSMC phenotypic plasticity). Here, we show that constitutive podosomes induced by oncogenic Src also strongly depend on the presence of MTs (Fig. 2), indicating that uncontrolled Src activity only partially compensates for MT regulation and that, at least in part, MTs act downstream of Src and PKC activation. At the same time, active Src is sufficient to facilitate podosome formation at the cell periphery, suggesting that all other essential factors are available at this location but not throughout the cell. We hypothesize that centripetal podosome relocation requires MT-dependent transport of molecular complexes (including components of actin polymerization and adhesion machinery) to new membrane sites, which initially lack actin polymerization and/or adhesion.

Importantly, we show here that a large part of MT response to PKC activation is remodeling of the MT network from a classic radial array with MT plus ends directed toward cell

periphery to a complex network with multiple reversed MTs (Fig. 4). Our data implicate this reversed MT network in the even distribution of podosomes over the ventral cell surface. This even distribution is thought to be important for physiological uniform ECM remodeling by VSMCs rather than site-specific degradation typical of cancer cells (Burgstaller and Gimona, 2005; Linder, 2007; Linder et al., 2011).

Formation of reversed MTs is a CLASP-dependent process and is likely due to PDBu-facilitated binding of CLASPs to MTs, which we previously demonstrated (Efimova et al., 2014). CLASP binding to MTs is known to be negatively regulated by GSK3 β phosphorylation (Wittmann and Waterman-Storer, 2005; Kumar et al., 2009; Kumar et al., 2012). We propose that because PKC is capable of GSK3 β inactivation (Goode et al., 1992), PKC activator PDBu likely stimulates MT binding of dephosphorylated CLASPs.

Others have shown that CLASPs efficiently regulate MT organization and dynamics through their capacity to promote MT rescues (switches from shrinkage to growth) (Komarova et al., 2002; Al-Bassam et al., 2010), decrease MT polymerization rates (Mimori-Kiyosue et al., 2005a; Drabek et al., 2006; Nakano et al., 2010), and facilitate persistent growth and capture of MTs at the cell edge (Mimori-Kiyosue et al., 2005a; Lansbergen et al., 2006; Kumar et al., 2009). Likely, CLASPs facilitate MT reversal because they promote persistent growth of MTs, which then turn rather than depolymerize upon reaching the cell boundary. We also show that MT reversal is driven by retrograde actin flow, which pulls polymerizing MTs toward the cell center (Waterman-Storer and Salmon, 1997; Salmon et al., 2002; Schaefer et al., 2008). It is possible that CLASPs bound to the MT lattice prevent buckling MTs from breaking, either directly or via modulation of other MT-lattice binding proteins (Grimaldi et al., 2014). Additionally, MT reversal might benefit from CLASP-dependent capture of MTs at the cell cortex via LL5 β or ELKS, two proteins that form an anchor for CLASPs to the plasma membrane (Lansbergen et al., 2006). In this scenario, retrograde actin flow carries growing MTs inward, while CLASP-associated MT portions remain anchored at the cell edge; the combination of these activities results in complex morphology of the MT network.

We propose that the functional significance of MT remodeling is to build a network where multiple MT plus ends propagate from the cell periphery toward the cell center. This network is required for efficient relocation of plus-end associated factors from the cell edge throughout the cell. In the case of podosome formation in VSMCs, which occurs MT plus end targeting sites and along reversed MTs, it is likely that molecular complexes are relocated from the initial podosomes at the cell edge (Fig. 8). In contrast, during podosome belt formation in migrating macrophages (Bhuwania et al., 2012) and osteoclasts (Biosse Duplan et al., 2014), podosomes relocate in a MT-dependent manner toward the cell edge, and MT plus ends likely bring podosome-stimulating factors outwards. According to our model, MT reversal in these systems should not be required, and, indeed, has not been observed. However, it is plausible to suggest that MT plus ends relocate essential factors from old to new podosome sites in these cell types via common mechanisms.

Mechanisms of MT plus end-dependent podosome relocation likely include more than one factor. Our previous data indicate that MTs relocate podosome-inducing factors via the plus-end directed motor KIF1C, which moves preferentially along CLASP-rich MTs (Efimova et

al., 2014). KIF1C trafficking is facilitated by CLASP binding to MTs upon PDBu treatment (Efimova et al., 2014). KIF1C is essential for podosome formation (Kopp et al., 2006; Efimova et al., 2014) and can be found at the outer podosome ring without direct contact with MTs (Efimova et al., 2014). The fact that KIF1C-positive complexes/membranes can relocate from an “old” to “new” podosome site (Figs. 7, 8) allows us to suggest that MT-dependent podosome relocation involves KIF1C-driven transport of essential molecules (e.g. integrins (Theisen et al., 2012)) from initial podosomes to new podosome formation sites. Because KIF1C often moves close to the MT plus end, (Fig. 7 and (Kopp et al., 2006)), a new podosome arising upon MT plus end targeting (Fig. 3) might be a consequence of KIF1C relocation. In addition, it is very likely that other MT plus end-associated processes facilitate podosome induction; for example, the +TIP EB1 can facilitate podosome formation and dynamics via interaction with cortactin (Biosse Duplan et al., 2014).

Materials and methods

Cells

A7r5 rat smooth muscle cells (ATCC) were grown in low glucose (1000 mg/l) Dulbecco's modified Eagle's medium (DMEM) without Phenol Red supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Cells were plated on glass coverslips or glass-bottom dishes (MatTech) coated with 10 µg/ml fibronectin 24 hours prior to experiments. In all live-cell experiments cells were maintained on the microscope stage at 37°C under mineral oil for media equilibrium maintenance.

Microscopy

Wide-field fluorescence imaging was performed using a Nikon 80I microscope with a CFI APO 60x oil lens, NA1.4, and CoolSnap ES CCD camera (Photometrics).

Single-plane confocal live cell video sequences were taken using a Yokogawa QLC-100/CSU-10 spinning disk head (Visitec assembled by Vashaw) attached to a Nikon TE2000E microscope with a Perfect Focus System using a CFI PLAN APO VC 100x oil lens NA 1.4, a back-illuminated EM-CCD camera Cascade 512B (Photometrics) driven by IPLab software (Scanalytics).

Leica TCS SP5 confocal laser scanning microscope with an HCX PL APO 100x oil lens NA 1.47 was used for taking confocal stacks of fixed cells, except of the images showing Tks5 localization. Nikon A1R confocal laser scanning microscope with a TIRF 100x 1.49 NA oil lens was used for Tks5 images.

TIRF live-cell videos were acquired on a Nikon TE2000E microscope with Nikon TIRF2 System using a TIRF 100x 1.49 NA oil lens, and Cascade 512B camera (Photometrics) and IPLab software (Scanalytics).

Treatments

For MT depolymerization, nocodazole (2.5 µg/ml) was added to the culture media for 2 hours. For stimulation of podosome formation Phorbol 12,13-dibutyrate (PDBu) (Sigma) was used at final concentration 5 µM for 30 minutes in fixed cell experiments or added to the

medium during live cell imaging. To arrest actin dynamics, a pharmacological inhibitor cocktail was used (modified from (Peng et al., 2011)). The inhibitors were added simultaneously with PDBu to a final concentration of 4µm Y27632 (Sigma), 1.6µm jasplakinolide (Santa Cruz Biotechnology) and 1µm latrunculin B (Sigma).

siRNA, DNA expression constructs and transfection protocols

Two different combinations of mixed siRNA oligonucleotides against CLASP1 and CLASP2 were used. Combination 1 (Mimori-Kiyosue et al., 2005b) included the CLASP1-targeted siRNA sequence 5'-CCUACUAAAUGUUCUGACC-3' and the CLASP2-targeted siRNA sequence 5'-CUGUAUGUACCCAGAAUCU-3'. Combination 2 (custom design, Sigma) included the CLASP1-targeted siRNA sequence 5'-CGGGAUUGCAUCUUUGAAA-3' and the CLASP2-targeted siRNA sequence 5'-CUGAUAGUGUCUGUUGGUU-3'. The KIF1C-targeted siRNA sequence was 5'-GUGAGCUAUAUGGAGAUCU-3'. Non-targeting siRNA (Dharmacon) was used for controls. For siRNA oligonucleotide transfection, HiPerFect (Qiagen) was used according to the manufacturer's protocol. Experiments were conducted 72 h after transfection, as at this time minimal protein levels were detected.

The following plasmid DNA constructs were used: DsRed SM22 (a gift from Dominique Brandt, University of Hannover, Germany, (Gimona et al., 2003)), RFP-cortactin (a gift from Marko Kaksonen, EMBL, Heidelberg, Germany), KIF1C-GFP (a gift from Anne Straube, Warwick University (Theisen et al., 2012)), oncogenic GFP-Src-527F (a gift from Dr. Margaret Frame, Edinburgh, UK), GFP-CLIP170 and EB3-GFP (a gift from Anna Akhmanova, University of Utrecht, the Netherlands). mCherry-UtrCH (utrophin) was a gift from William Bement (Addgene plasmid # 26740 (Burkel et al., 2007)). For transient transfection of plasmid DNA, Fugene6 (Roche) or Amaxa nucleofection (Lonza) (X-001program) was used according to the manufacturer's protocols. Experiments were conducted 18-24 hours after transfection.

Antibodies, immunofluorescence and Western blotting

The following antibodies were used: rabbit polyclonal antibodies anti-CLASP2 VU-83 (Efimov et al., 2007), rabbit polyclonal anti-CLASP1 (Epitomics), rabbit polyclonal anti-tubulin (Abcam), rabbit polyclonal anti-KIF1C (Cytoskeleton), mouse monoclonal (Upstate), rabbit polyclonal (Cell Signaling) anti-cortactin antibodies, and rabbit anti-Tks5 antibody (Santa Cruz Biotechnology). Alexa647, Alexa488 and Alexa568-conjugated highly cross-absorbed goat anti-mouse and anti-rabbit IgG antibodies (Invitrogen/Molecular Probes) were used as secondary antibodies. Actin cytoskeleton was visualized by Alexa Fluor® 350, 488 or 568 Phalloidin (Invitrogen/Molecular Probes).

For immunofluorescence microscopy, cells on coverslips were fixed in 4% paraformaldehyde plus 0.3% Triton in cytoskeleton (CB) buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl₂, pH 6.1), or in 4% paraformaldehyde plus 0.1% Glutaraldehyde and 0.3% Triton in CB buffer for 10 minutes at room temperature, or in methanol (for anti-CLASP2 staining) for 5 minutes at -20°C. Fixed cells were soaked in PBS for at least one hour. Then, non-specificity was blocked with

blocking solution (1% horse serum, 0.1% BSA in PBS) for 30 min. Incubation with the primary antibodies was 1 h, and with the secondary antibodies 40 min. All antibodies were diluted in blocking solution prior use. Immunostaining was performed at room temperature. After washing, samples were mounted into ProLong® Gold Antifade Reagent (Invitrogen/Molecular Probes) on glass slides and stored at -20°C .

Western blotting was performed with the Protein Electrophoresis and Western Blotting System (Bio-Rad). Briefly, cells collected from 10cm dish were spun down and resuspended in Laemmli Sample Buffer (Bio-Rad). 30 μg of total protein samples were applied to 10% polyacrylamide gel and processed for electrophoresis. 0.45 μM nitrocellulose membrane was used for protein blotting. The membrane was incubated with primary and then secondary (LI-COR, IRDye™ 800 or 700) antibody diluted in Odyssey Blocking Buffer. 0.2% Tween-20 was added to lower background. Odyssey Infrared Imaging System (LI-COR) was used for membrane scanning.

Quantitative analyses and image processing

Podosome number per cell (Figs. 2C, 6G) were quantified using ImageJ particle analysis on thresholded images of cortactin or phalloidin staining visualized by wide field fluorescent microscopy.

Cells with different podosome patterns (Fig. 1C): cells with more than 10 podosomes were considered podosome positive. Podosomes in circular arrangements within 10 μm^2 on lamella area were considered rosettes.

To analyze the area taken by relocating podosomes in the cell center (Figs. 2F, 6L), a cell border outline was drawn over a maximal intensity projection of a TIRF imaging sequence (100 frames, 20 sec/frame). Then, the outline was moved toward the cell center by 8 μm (see the dotted line in Fig. 2E, D and Fig. 6H, I). The area taken by podosomes inside and outside this border was quantified using ImageJ analysis tool on a thresholded image.

To analyze podosome relocation, an area increase factor was introduced (Figs. 2G, 6M). TIRF imaging sequences (100 frames, 20 sec/frame, as in Fig. 2E, D and Fig. 6H, I) were analyzed using ImageJ analysis tool. In the thresholded first frame of the movie, the summarized area of all podosomes was measured (S_1). Then, the selection was extended by 3.2 μm (20 pixels) to cover the typical area of a single podosome size fluctuation during a movie. This area, taken by initial podosomes and their fluctuations, was subtracted from the time projection of the movie. The area taken by podosomes on the remaining area of the time projection was measured (S_t). The area increase factor was calculated as S_t/S_1 . The area increase factor equals 0 if podosomes did not move more than 3.2 μm .

To analyze podosome formation after MT targeting (Fig. 3D), double channel spinning disk confocal sequences (5 sec/frame) of cells co-expressing RFP cortactin with EB3-GFP were used. All events of podosome formation were identified on a single channel visualizing RFP-cortactin. For controls, the same number of random locations within a cell was introduced. Random number pairs were generated and automatically put as coordinates on an image by a custom macro in ImageJ. If a randomly generated location co-localized with a

podosome site, it was excluded from the analysis, and the macro was run to generate another random spot. Circles of $\sim 1.5\mu\text{m}$ (10 pixels) radius corresponding to a small podosome size were considered as location sites for analysis. Then, both groups of cell sites were plotted on the second channel of the movie sequence representing EB3-GFP. The closest time point of EB3 targeting of these sites prior to the event was determined. Events were grouped according to the time between the EB3 targeting or KIF enrichment and podosome formation event or random event time.

Number and direction of MT growth tracks (Fig. 4I-K, 5M) was determined by analyzing spinning disk confocal sequences (5 sec/frame) of GFP-CLIP170 expressing cells by Imaris software particle tracking tool. Alternatively, a color-coded time projection of the image sequences was generated by 'temporal-color code' function in ImageJ. Direction of a track was determined by comparison of the track direction with the direction of cell radius (from the cell center to the periphery). Track with an angle of 90 degrees or below were considered direct. Tracks with an angle higher than 90 degrees were considered reversed. The comparison was run by a custom MatLab script or manually. Number of MTs was normalized per unit area for (Fig. 4I,J). The same cells before and 15/30 minutes after treatment were analyzed. Percentage of reversed MTs out of all growing MTs per cell was calculated, and then increase in this percentage in the same cell after PDBu treatment was calculated (Fig. 4K, 5M).

To analyze retrograde actin flow (Fig. 5 D,E), mCherry-utrophin-expressing cells were imaged by single-plane spinning disk confocal microscopy at 1 min/frame rate. Kymograph was generated by the reslice function in Image J.

For all fluorescence images presented, brightness, contrast and gamma settings for each individual fluorescence channel were adjusted to make small structural features visible. Image analysis was done with Image J, Imaris, and MatLab. All the statistics were performed with Excel software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MTs are required for podosome formation and scattering upon PKC activation
Wide-field immuno-fluorescence microscopy. Actin (phalloidin). Podosomes are indicated by arrows. A, D. Multiple podosomes are spread throughout a cell after 40 min of PDBu treatment. B, E, F. In cells pre-treated with nocodazole for 2 hours before PDBu application, podosomes are few and form circular accumulations (rosettes). A, B, Cell overviews. D, E, F, Examples of podosome distribution in cell lamellae. C, Distribution of podosome patterns in cell populations. Left pie shows percentage of cells with and without podosomes in PDBu-treated cells. Right pie shows percentage of cells with scattered versus rosette-like podosomes within podosome-bearing cell sub-population. Control cell population, top. Nocodazole-treated cell population, bottom. N=100 cells. Two-tailed Fisher's exact test, $P < 0.0001$.

Figure 2. Proper distribution and relocation of oncogenic Src-induced constitutive podosomes requires MTs

A, B, Wide-field immuno-fluorescence microscopy. Actin (phalloidin). C, Podosome numbers based on data similar to (Fig. 1 A and Fig. 2A, B). $N=40 \pm 10$. Student's unpaired two-tailed t-test. Asterisks, $p < 0.001$. Error bars indicate SEM. D-I, Analysis of podosome dynamics in cells co-expressing caSrc and a podosome marker visualized by TIRF microscopy. D, E, Visualization of DsRed-SM22. Single frames (left) are compared to the maximal intensity projections over 16 minute time lapse sequences (right). In untreated cell (D), area taken by podosomes over time extends beyond sites of initial podosome location (arrows) and often spreads to the cell center (inside of the dotted line). In nocodazole (E), podosomes formed throughout the sequence remain at their initial locations (arrows) and do not move into the cell center. F, podosome area (μm^2) in the cell center. Based on maximal intensity projections of TIRF sequences, as in (D, E). $N=10$. Student's unpaired two-tailed t-test, $p < 0.001$. G, Area increase factor illustrating podosome relocation. The factor equals the area taken by newly formed podosomes divided by the area taken by podosomes in the first frame of the sequence. Based on data as in (D, E). $N=8$. Student's unpaired two-tailed t-test, $p < 0.001$. Error bars indicate SEM. H, I. TIRF video frames representing RFP-cortactin-marked podosome dynamics in caSrc-expressing cells. In untreated cell (H), podosomes completely disassemble (arrows), while new podosomes are continuously formed at new sites (chevrons). In nocodazole (I), podosomes either remain in place (arrows), or re-form at the same locations as disassembled ones (chevron).

Figure 3. Podosomes form after MT plus end targeting and distribute along reversed MTs
 A, Maximal intensity projection over a time-lapse single plane confocal sequence visualizing a cell expressing RFP-cortactin (red) to highlight PDBu-induced podosomes and EB3-GFP. EB3-GFP highlights MT growth tracks over 2.5 minutes (green), and over the following 20 seconds (magenta) to indicate direction of MT polymerization. B, Video frames from the area boxed in (A). Arrows indicate the podosome formation site. C, A kymograph along the direction of MT growth shown in B over 3 minutes. Note podosome formation upon MT targeting. D, Spatial correlation of podosome formation with preceding MT targeting. Based on image sequences as in (A-C). E, F, PDBu-induced podosomes (yellow arrows) are found along MT, which are parallel to the edge or reversed but not extended from the cell center to periphery (white arrows). Phalloidin, red. Tubulin, green. Wide-field immuno-fluorescence microscopy. In (F), the boxed region from (E) is shown, and the red channel (actin) is artificially over-contrasted for clear visualization of podosomes and MTs.

Figure 4. PDBu-induced MT rearrangement depends on CLASP

A-D, Maximal intensity projection of a confocal stack visualizing tubulin immunostaining (green) in control and CLASP-depleted cells. A, B, In cells transfected with non-targeted control siRNA, PDBu treatment disturbs MT pattern (B) as compared to untreated cells (A). C, D, In CLASP-depleted cells, MT pattern does not differ between untreated (C) and PDBu-treated (D) cell. E-H, Projections over time illustrating tracks of GFP-CLIP170 movement before (E, G) and after (F, H) PDBu treatment in a non-targeted control (E, F) and a CLASP-depleted cell (G, H). GFP-CLIP170 movement tracks over 2.5 minutes are shown in green, and over the following 20 seconds in magenta to indicate direction of MT polymerization. MT track pattern is changed by PDBu treatment in control (E, F) but not CLASP-depleted (G, H) cell. Single-plane confocal live-cell imaging. I-K, PDBu-induced changes in growing MTs based on data similar to those in (E-H). I, Increase in number of growing MTs depends on CLASP. GFP-CLIP170 comet number is shown. N=4. Student's paired two-tailed t-test. Black asterisks, $p < 0.05$. Outline X, $p > 0.09$. Student's unpaired two-tailed t-test. Black X, $p > 0.1$. J, Increase in number of reversed MTs depends on CLASP. Number of GFP-CLIP170 comets moving at angle $> 90^\circ$ to the cell radius is shown. N=4. Student's paired two-tailed t-test. Black asterisk, $p < 0.02$. Student's unpaired two-tailed t-test. Black X, $p > 0.1$. K, Increase in percentage of reverted MTs within the growing MT population in single cells before and after treatment. N=4. Student's unpaired two-tailed t-test. Asterisk, $p < 0.02$. Error bars indicate SEM.

Figure 5. PDBu-induced MT rearrangement is driven by retrograde actin flow

A-C, Single-plane spinning disk confocal images of a PDBu-treated cell (~30 minutes), co-expressing mCherry-utrophin (actin marker, cyan) and GFP-CLIP170 (MT plus end marker, red). A, an overview. In (B), the boxed region from (A) is shown. The direction of the actin flow is illustrated as a difference between the maximum intensity projection of utrophin images within the first 50 seconds on the movie (blue) and the projection within the subsequent 50 seconds of the movie (green). The CLIP170 tracks are shown as a time projection throughout the whole time sequence (1 minute 40 seconds, red). In (C), the single utrophin time frames (cyan) and CLIP170 propagation tracks (red) are shown for the boxed region from (B). D-E, Single-plane spinning disk confocal images of mCherry-utrophin-expressing cells 5 minutes before and 10 minutes after treatment. Red lines in the “before” images indicate positions where kymograms (right images) were generated. In the kymograms, red dotted lines indicate the time of the drug application. Green fine-dotted lines illustrate the slopes of the flow. In (D) PDBu only was added resulting in accelerated actin flow. In (E) Inhibitor cocktail (4 μ m Y27632, 1.6 μ m jasplakinolide, and 1 μ m latrunculin B) was added together with PDBu, blocking actin flow (vertical kymogram slopes). F-H, Maximal intensity projection of a confocal stack visualizing tubulin immunostaining (green). PDBu-only treatment disturbs MT pattern (G) as compared to untreated cells (F); in cells co-treated with PDBu and the inhibitor cocktail (H), MT pattern is close to non-treated cells (F). Arrows indicate typical MT patterns. I-L, Projections over time illustrating tracks of GFP-CLIP170 movement before (I, K) and after (J, L) treatment with PDBu only (I, J) or PDBu and the inhibitor cocktail (K, L). GFP-CLIP170 movement tracks over 2.5 minutes are shown in green, and over the following 20 seconds in magenta to indicate direction of MT polymerization. MT track pattern is changed by PDBu-only

treatment (I, J) but not in combination with the inhibitors (K, L). Single-plane spinning disk confocal live-cell imaging. M, Increase in percentage of reverted MTs within the growing MT population in single cells before and after treatment. Analysis based on live cell imaging similar to (I-L). N=7. Student's unpaired two-tailed t-test. Asterisk, $p < 0.02$. Error bars indicate SEM.

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Figure 6. Proper distribution and relocation of constitutive podosome requires CLASPs
A-E, Wide-field immuno-fluorescence microscopy. Arrows indicate podosomes. A-C, Actin (phalloidin). D, E, Cortactin. Non-targeted control siRNA-transfected (A), non-targeted control siRNA and GFP-caSrc-transfected (B, D) and GFP-caSrc-transfected and anti-CLASPs siRNA combination 1-transfected (C) cells. F, Western blotting indicating CLASP1 and CLASP2 depletion levels in cells treated by siRNA combinations 1 and 2. G, Podosome numbers based on data similar to (A-E). N=40±10. Student's unpaired two-tailed t-test. Asterisks, p<0.001. Error bars indicate SEM. H-K, siRNA-transfected cells co-expressing caSrc and an actin-associated podosome marker DsRed-SM22 visualized by TIRF microscopy. H-I, Single frames (left) are compared to the maximal intensity projections over 16 minute time lapse sequences (right). In non-targeted control siRNA-transfected cell (H), area taken by podosomes over time extends beyond sites of initial podosome location (arrows), and often spreads to the cell center (inside of the dotted line). In CLASP-depleted cell (I), podosomes formed throughout the sequence remain at their initial locations (arrows) and do not move into the cell center. J, K. TIRF video frames representing DsRed-SM22-marked podosome dynamics in caSrc-expressing cells. In non-targeted control siRNA-transfected cell (J), podosomes completely disassemble (arrows), while new podosomes are continuously formed at new sites (chevrons). In CLASP-depleted cell (K), podosomes either remain in place (1'-10'), or re-form (31') at the same locations as disassembled ones

(arrows). L, podosomes area (μm^2) in the cell center. Based on maximal intensity projections of TIRF sequences, as in (H, I). N=10. Student's unpaired two-tailed t-test, $p<0.005$. M, Area increase factor illustrating podosome relocation. Based on data as in (H, I). N=10. Student's unpaired two-tailed t-test. $p<0.001$. Error bars indicate SEM.

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Figure 7. Podosomes relocate with KIF1C moving along MTs

A-E, Single plane confocal images of live cells expressing KIF1C-GFP (green) and RFP-cortactin (red). A, B, An example of podosome formation stimulated by forward KIF1C relocation. A, cell overview. B, Video frames of the region boxed in (A). Yellow arrow indicates a site where a podosome is formed after a KIF1C tube extension. White arrow indicates another podosome which relocates together with KIF1C density. Asterisks indicate positions of initial (at 15", shown in 1'20") and final (at 1'20", shown in 15" frame) podosome location. A maximal intensity projection of the time sequence is shown on the right to illustrate tracks of KIF1C movement (parallel to green arrow) and coordinated relocation of KIF1C density and the second podosome (parallel to green-and-pink arrow). C-F, Examples of podosome formation stimulated by centripetal KIF1C relocation. C, E, cell overviews. D, Video frames of the region boxed in C. KIF1C accumulations (yellow arrows) move from the cell edge to surround a site in lamella (white arrow) where subsequently a podosome is formed. A maximal intensity projection of the time sequence is shown on the right to illustrate tracks of KIF1C movement (parallel to green arrows). F, Video frames of the region boxed in E. A projection extends backward from a KIF1C accumulation (yellow arrows), which surrounds a large podosome, and a new podosome forms in association with this projection (white arrow). A maximal intensity projection of the time sequence is shown on the right to illustrate tracks of KIF1C movement (parallel to green arrow). G-I, Single plane confocal images of a live cell expressing KIF1C-GFP (green) and EB3-mCherry (red). G, cell overview. H, I, Video frames of regions boxed in G. KIF1C is delivered backward by EB3-marked MTs growing toward the cell center. In H, A KIF1C tube is pulled by a reversed MT tip. In I, a reversed MT tip facilitates movement of a KIF1C particle and its fusion with a larger KIF1C accumulation. In both cases, MTs continue to grow after KIF1C deposition. Maximal intensity projections of the time sequence are shown on the right to

illustrate movement tracks of EB3 (parallel to pink arrows) and KIF1C (parallel to green arrows).

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Figure 8. Model of CLASP-dependent MT reversal and podosome relocation downstream of PKC

Recruitment of CLASP to MTs promotes actin-dependent MT buckling and reversal at the cell periphery and, at the same time, facilitates KIF1C trafficking. KIF1C delivers podosome-promoting molecules to and between podosome formation sites. Such delivery along curved reversed MTs facilitates centripetal relocation of podosomes and their even distribution across the ventral surface of a cell.