Podosome-regulating kinesin KIF1C translocates to the cell periphery in a CLASP-dependent manner

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Running title: CLASPs are required for KIF1C transport
Abstract

The kinesin KIF1C is known to regulate podosomes, actin-rich adhesion structures, which remodel the extracellular matrix during physiological processes. Here we show that KIF1C is a player in the podosome-inducing signaling cascade. Upon induction of podosome formation by protein kinase C, KIF1C translocation to the cell periphery intensifies and KIF1C accumulates in the proximity of peripheral microtubules enriched with plus tip-associated proteins CLASPs and around podosomes. Importantly, without CLASPs, both KIF1C trafficking and podosome formation are suppressed. Moreover, chimeric mitochondria-targeted CLASP2 recruits KIF1C, suggesting a transient CLASP-KIF1C association. We propose that CLASP creates preferred microtubule tracks for KIF1C to promote podosome induction downstream of PKC.

Introduction

Microtubules (MTs) serve to deliver and position molecular complexes and organelles within a cell, thereby defining its architecture. An important part of this function is locating the sites of actin cytoskeleton assembly and remodeling (Hoogenraad and Akhmanova, 2010; Kaverina and Straube, 2011; Etienne-Manneville, 2013). Amongst other actin-based structures, MTs regulate invasive protrusions, termed podosomes (Babb et al., 1997; Linder et al., 2000; Destaing et al., 2003; Evans et al., 2003; Destaing et al., 2005; Jurdic et al., 2006; Kopp et al., 2006; Gil-Henn et al., 2007; Purev et al., 2009; McMichael et al., 2010; Biosse Duplan et al., 2014), and their cancer counterparts, invadopodia (Schoumacher et al., 2010; Quintavalle et al., 2011).

Podosomes consist of a core of constantly polymerizing actin filaments and an outer adhesive ring. These structures serve as exocytosis sites for matrix metallo-proteases (MMPs) (Lener et al., 2006; Gimona et al., 2008; Linder et al., 2011; Murphy and Courtneidge, 2011). Podosomes are found in multiple extra-cellular matrix (ECM)-remodeling cells such as osteoclasts, macrophages and synthetic vascular smooth muscle cells (VSMCs). In these cell types, efficient ECM remodeling does not occur if podosomes
are not present. A growing body of evidence implicates podosome-dependent ECM remodeling in cell migration and invasion during morphogenesis (Teti et al., 1991; Lener et al., 2006; Gil-Henn et al., 2007; Proszynski et al., 2009; Rottiers et al., 2009; Quintavalle et al., 2010; Linder et al., 2011; Saltel et al., 2011). For example, the ECM-remodeling capacity of synthetic VSMCs is important for angiogenesis and vascular repair (Lener et al., 2006; Quintavalle et al., 2010; Chen et al., 2013). Also, migration and invasion potentials of VSMCs have a direct impact on atherosclerotic plaque formation and stability (Quintavalle et al., 2010; Lacolley et al., 2012; Chen et al., 2013).

Podosomes are dynamic structures and are induced or disassembled in response to physiological signals (Chambers and Fuller, 2011; Dovas and Cox, 2011; van Helden and Hordijk, 2011; Hoshino et al., 2013). Podosome dynamics are strongly regulated by MTs (Babb et al., 1997; Linder et al., 2000; Destaing et al., 2003; Evans et al., 2003; Destaing et al., 2005; Jurdic et al., 2006; Kopp et al., 2006; Gil-Henn et al., 2007; Purev et al., 2009; McMichael et al., 2010; Biosse Duplan et al., 2014). It has become clear from a number of recent studies that MT-podosome relationships are multifaceted: both stable (acetylated (Destaing et al., 2005; Purev et al., 2009; Biosse Duplan et al., 2014)) and dynamic (Kopp et al., 2006; Biosse Duplan et al., 2014) MT subpopulations are essential for podosome regulation. Moreover, several independent molecular machineries structurally and/or functionally link MTs to podosomes, including tubulin acetylation enzymes (Destaing et al., 2005; Purev et al., 2009; Biosse Duplan et al., 2014), MT plus end-associated protein complexes (EB1 (Biosse Duplan et al., 2014)), actin-dependent molecular motors (myosin-X (McMichael et al., 2010)) and several MT-dependent molecular motors (Kopp et al., 2006; Wiesner et al., 2010; Cornfine et al., 2011). Within the last group, podosome function in ECM remodeling critically depends on kinesins KIF5B, KIF3A/KIF3B and KIF9, which deliver MMPs to podosomes (Wiesner et al., 2010; Cornfine et al., 2011). More intriguingly, the kinesin KIF1C regulates the dynamics of podosomes themselves (Kopp et al., 2006), possibly due to the capacity of this motor to transport essential podosome components, such as integrins (Theisen et al., 2012). In principle, such transportation could be either constitutive or triggered downstream of physiological signals that induce podosome formation. Activation of KIF1C trafficking would be a suitable regulatory step in the
signaling cascade leading to ECM remodeling. However, whether KIF1C transport is regulated by podosome inducing signals, has not been addressed.

In this study we show that protein kinase C (PKC) activation strongly facilitates KIF1C transport to the cell periphery to initiate podosome formation. Moreover, our results reveal that MT-associated proteins CLASPs are necessary for efficient translocation of KIF1C along MTs and are critical components of podosome induction signaling. Together, these findings reveal a new pathway within the multifaceted MT-dependent podosome regulation.

Results

Podosome formation in VSMCs requires MTs

In the rat aortic vascular smooth muscle cell (VSMC) line, A7r5, multiple small podosomes can be rapidly induced by PKC activation via phorbol ester PDBu treatment (Hai et al., 2002). Podosomes in VSMCs contain specific podosome markers such as Tks5 (Fig. 1A), include accumulations of F-actin with a characteristic morphology (Fig. 1A,C) and are enriched in proteins involved in actin polymerization (Hai et al., 2002; Kaverina et al., 2003; Lener et al., 2006) such as cortactin (Fig. 1A,F).

We took advantage of the PDBu-inducible podosome model to address regulation of de novo podosome formation by MTs. To test whether MTs are essential for podosome formation, we completely depolymerized MTs in A7r5 cells by nocodazole (Suppl. Fig. S1A-C) and applied PDBu. We found that the number of podosomes formed was significantly decreased under these conditions (Fig. 1D,G,H) to levels comparable to non-induced cells (Fig. 1B,E). This indicates that MTs are required for podosome formation in VSMCs, as was previously described for macrophages and osteoclasts (Babb et al., 1997; Linder et al., 2000; Destaing et al., 2003; Evans et al., 2003; Destaing et al., 2005; Jurdic et al., 2006; Kopp et al., 2006; Gil-Henn et al., 2007; Purev et al., 2009; McMichael et al., 2010; Biosse Duplan et al., 2014).
Podosome formation in VSMCs requires KIF1C

It has been proposed that MTs exert their control on podosomes by delivering regulatory and structural molecules to podosome sites via MT-dependent transport. Indeed, one of the few identified molecular players essential for podosome turnover is the kinesin KIF1C (Kopp et al., 2006). Interestingly, we found that KIF1C was enriched at podosome sites in A7r5 cells (Fig. 1I). Applying siRNA depletion of KIF1C to A7r5 cells (Fig. 2i, J), we found that the number and size of PDBu-induced podosomes were significantly decreased (Fig. 2A-H). This phenotype was rescued by re-expression of RNAi-resistant KIF1C-GFP (Fig. 2K-N), indicating the specificity of the depletion phenotype. In agreement with this result, expression of dominant-negative mutants of KIF1C (either a truncated cargo-binding tail domain (Fig. 2P) or motor-dead rigor mutant (Fig. 2Q)) mimicked the effect of KIF1C depletion (Fig. 2O-R). The effects of KIF1C loss of function were very significant but milder than the effect of complete MT depolymerization (Fig. 1), suggesting that KIF1C is an essential, though not the only, factor in MT-dependent podosome regulation. These data indicated that KIF1C is required for efficient podosome formation in VSMCs.

PKC pathway facilitates MT-dependent transport of KIF1C to the cell periphery

Next, we questioned whether KIF1C-dependent trafficking is regulated as part of the podosome induction pathway downstream of PKC. Using A7r5 cells stably expressing low levels of KIF1C-GFP (suppl. Fig. S1E), we found that PDBu treatment strongly stimulated KIF1C-GFP translocation to the cell periphery (Fig. 3A-C, Movies 1, 2). In contrast to cell-center localization in control cells, KIF1C accumulated at the cell edge and at the ventral surface of the lamellae in PDBu-treated cells (Fig. 3D-E). This localization was abolished by nocodazole treatment (Figs. 3F-H, Suppl. Fig S1A,B), indicating that KIF1C targeting to the cell periphery was MT-dependent. This result indicates that KIF1C transport is regulated by podosome-inducing signals and is therefore an essential step in the signaling cascade leading to ECM remodeling.
KIF1C moves along CLASP-associated MTs and can be recruited by CLASPs

In agreement with a prior finding of Kopp and colleagues in macrophages (Kopp et al., 2006), we find that in VSMCs KIF1C puncta undergo movements predominantly when associated with the plus ends of polymerizing MTs (Suppl. Fig. 1F-G, Movies 3, 4). This suggests that KIF1C translocates along MTs in close association with MT plus-end tracking protein (+TIP) complex. Because deposition of KIF1C at the cell periphery was dramatically increased by PDBu treatment, we tested if cellular localization of major +TIPs was influenced by PDBu. We were looking for a protein, which in PDBu would localize to MTs stronger than in control, and, therefore, could be a positive regulator of KIF1C transport. Among the proteins tested, certain proteins responded to PDBu treatment by decreased MT plus end association, including EB1 (not shown), which was recently detected as an important podosome regulator (Biosse Duplan et al., 2014); other proteins did not change significantly (e.g. CLIP170, not shown). Importantly, we found a striking change in localization of +TIPs called CLASPs (CLIP-associated proteins), which are known to facilitate MT polymerization and stability (Galjart, 2005; Al-Bassam and Chang, 2011). While normally CLASPs highlighted short MT plus end-tracking comets throughout the whole cell, in PDBu-treated cells CLASPs were arranged in extended patterns only at the cell periphery (Fig. 4A,B); this indicated that CLASP binding to peripheral MT lattice was specifically enhanced by PDBu. We hypothesized that the relocation of CLASPs in response to PKC may be involved in the PDBu-stimulated activation of KIF1C transport to podosomes and concentrated on this protein in this study.

Interestingly, CLASP-associated MTs were located in the podosome-rich regions of the cell, and were frequently found in close contact with podosomes (Fig. 4B) and peripheral accumulations of KIF1C (Fig. 4C). Live-cell imaging indicated that KIF1C at the cell periphery moved predominantly in association with CLASP-rich peripheral MTs and accumulated in the vicinity of their ends (Fig. 4C,D, Suppl. Fig. S1D, Movies 5, 6).

Thus, it is possible that CLASPs promote KIF1C transport, for example by stimulating KIF1C recruitment to MTs. In agreement with this hypothesis, when a chimeric protein, in
which CLASP2 was combined with the mitochondrial component TOM20 (mito-CLASP, (Efimov et al., 2007)), was expressed in cells, a significant fraction of KIF1C-GFP accumulated at the mitochondria indicating that KIF1C-GFP followed the CLASP chimera (Fig. 4 E-H; Suppl. Fig. S2A,B). This recruitment is likely mediated by the tail domain of KIF1C, because the tail domain (Fig. 4 E,F, I,J, Suppl. Fig. S2C,D) but not the KIF1C motor domain (not shown) was significantly recruited to mitochondria by mito-CLASP. Interestingly, full length KIF1C that was recruited to mitochondria by mito-CLASP was likely functional and responsive to PKC activation: this was evident from relocation of mitochondria toward the cell periphery in transfected cells (Fig. 4 K-M; Suppl. Fig. S2E,F). These data indicate that in cells CLASPs interact with a subset of KIF1C molecules via the KIF1C tail domain.

**CLASPs are necessary for KIF1C trafficking and deposition at the cell periphery**

The evidence that KIF1C transiently associates with CLASPs suggests that CLASPs may be important for KIF1C transport to the sites of podosome formation. Thus, we tested whether CLASPs modulate KIF1C targeting to the cell periphery. Strikingly, CLASP depletion, by two alternative siRNA combinations (Fig. 5A, Suppl. Fig. S3), completely abolished KIF1C accumulation at the cell edge and at podosome formation sites (Fig. 5B-E; Suppl. Fig. S4A-C). In CLASP-depleted cells, KIF1C-positive puncta were diffuse throughout the cell body; however, KIF1C protein levels were not affected by CLASP depletion (Suppl. Fig. S3D). Distribution of KIF1C in CLASP-depleted cells was similar to what was observed in nocodazole-treated cells lacking MTs (Fig. 3G,H, Suppl. Fig. S1B), suggesting that without CLASP, MTs cannot support KIF1C transport to the cell periphery. Decreased translocation of KIF1C to the cell periphery might, in principle, be explained by the low MT number in CLASP-depleted cells (Mimori-Kiyosue et al., 2005; Efimov et al., 2007). To test this possibility, we addressed whether KIF1C accumulations could be found at MT plus ends in CLASP-depleted cells. We found that, in sharp contrast with the control cells, no KIF1C accumulations could be detected in association with MTs under these conditions (Fig. 5C,D; Suppl. Fig. S4B,C); this indicates that deficient KIF1C distribution at the cell periphery did not result from the decrease of MT number in CLASP-depleted cells.
Rather, our data suggest that translocation of this motor along MTs is blocked without CLASPs.

To test this directly, we followed KIF1C-GFP motility in CLASP-depleted and control cells by live cell imaging. In control cells, KIF1C-GFP puncta and tubes often underwent fast directional translocations typical of MT-dependent membrane trafficking (Fig. 5F,G, Movie 7 left). Interestingly, in the absence of CLASPs these movements were largely abolished (Fig. 5H-J, Suppl. Fig. S4D-E, Movie 7 right), indicating that CLASPs are required for KIF1C transport along MTs.

CLASPs are necessary for podosome formation

Because the podosome-stimulating kinesin KIF1C cannot translocate to the cell periphery in CLASP-depleted cells, one would predict that CLASP is required for podosome formation. PDBu treatment of cells, depleted of CLASPs by two alternative siRNA combinations, indicated that podosome numbers, detected by cortactin staining, were dramatically reduced as compared to cells treated with non-targeted control siRNA (Fig. 6A-F, K). Podosome numbers in CLASP-depleted cells were efficiently rescued by ectopic expression of CLASP2-RFP, which cannot be silenced by siRNA combination 2 (Fig. 6G-K). Depletion of CLASP1 or CLASP2 separately led to partial podosome suppression (not shown). Thus, it is likely that both CLASPs act redundantly as essential effectors in the podosome induction pathway downstream of PKC.

Discussion

Based on our data, we propose a model, in which CLASPs act as essential players in the regulation of podosome formation because only CLASP-decorated MTs are capable of supporting KIF1C translocation to putative podosome sites at the cell periphery (Fig. 7A). CLASP enrichment at MTs was previously shown to result from GSK3β inactivation at the cell periphery, which leads to enhanced MT binding of dephosphorylated CLASPs.
In our system, this change in MT binding is likely to be caused by PKC-dependent GSK3β inactivation triggered by PDBu treatment (Goode et al., 1992). In organisms, this pathway may be triggered through extracellular factors that induce podosomes, such as PDGF signaling (Quintavalle et al., 2010).

Our data implicate CLASPs as important regulatory factors for the trafficking function of KIF1C. It is also noteworthy that the effects of KIF1C depletion or inactivation are less striking than the effects of CLASP depletion, suggesting that additional factors facilitates podosome formation in CLASP-dependent manner. It is plausible to suggest that certain molecular motors sharing similarities with KIF1C (e.g. other kinesin-3 family members KIF1B and KIF1A) are involved. However, the nature of additional CLASP-dependent factors is yet unclear: this study is the first direct evidence that CLASPs regulate molecular motor function, which has been previously suggested by the essential role of CLASPs in specific cellular precesses. For example, CLASP-coated Golgi-derived MTs have especially strong capacity for transportation and assembly of Golgi stacks (Miller et al., 2009). Also, CLASP is essential for mitochondria transport or positioning in S. pombe (Chiron et al., 2008), which could be interpreted as a result of CLASP-dependent kinesin regulation in that system.

Because CLASP2 can recruit KIF1C to mitochondria, we propose that MT-bound CLASPs directly stabilize the association of KIF1C with MTs, similar to the recently discovered function of doublecortin-KIF1A cooperation in neurons (Liu et al., 2012) or EB1-KIF17 cooperation in polarizing epithelia (Jaulin and Kreitzer, 2010). A less likely possibility is that CLASP activates KIF1C in an MT-independent manner, similar to kinesin-1 activation by MT-associated protein ensconsin (Barlan et al., 2013). In principle, another possible mechanism could involve the indirect effect of CLASP-dependent increase of MT lifetime and stability (Akhmanova et al., 2001; Mimori-Kiyosue et al., 2005; Drabek et al., 2006; Lansbergen et al., 2006), which was shown to facilitate transport by specific kinesins (Reed et al., 2006; Cai et al., 2009; Hammond et al., 2010). Stable MTs are indeed important for podosome regulation in osteoclasts (Destaing et al., 2005; Purev et al., 2009). However, KIF1C (similar to another kinesin-3 family member KIF1A (Cai et al., 2009)) moves with growing MT plus ends and thus prefers dynamic MT tracks rather than stable ones.
Accordingly, we suggest that dynamic CLASP-associated MTs normally serve as preferred tracks for KIF1C transport, and that relocation of CLASPs to peripheral MTs, upon PDBu treatment, facilitates KIF1C translocation to the lamella and, subsequently, triggers podosome formation (Fig. 7A). This is already the second reported mechanism whereby dynamic, rather than stable, MTs regulate podosome formation and dynamics: it has been shown recently that EB1, a +TIP MT protein which associates only with polymerizing dynamic MT ends, facilitates podosome formation in osteoclasts via an interaction with cortactin (Biosse Duplan et al., 2014). This and our present findings indicate that targeting of podosomes by dynamic MT ends is critical for regulation of these adhesive structures, resembling MT regulation of focal adhesions (Kaverina et al., 1999).

Overall, our data establish CLASPs and KIF1C as sequential molecular players in the signaling cascade downstream of PKC (Fig. 7B) and as critical components of the podosome induction pathway.

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 (MatTek) coated with 10µg/ml fibronectin 24 hours prior to experiments. In 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 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 (except Fig. 2A) 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). The video sequence presented in Fig. 2A was acquired at Nikon Sweptfield Confocal with an Andor Ultra 897 camera, attached to a fully motorized Ti-E with Perfect Focus and Tokai Hit incubation chamber using the 100x 1.45 Lambda objective, and driven by NIS-Elements AR.

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. DeltaVision Elite with Alexa488, Alexa 594 and Alexa647 filter sets (Chroma) and a Coolsnap HQ CCD camera under control of SoftWorx (Applied Precision LLP) was used for acquisition/deconvolution stacks of fixed cells.

Image acquisition and editing

Fig. 1 (B-G), Fig. 2 (A-D,E,F), Fig. 4 (A,B), Fig. 6 (A-F), Fig. S1 (A-D), Fig. S3 (A-C), Fig. S4 (A-C), wide-field fluorescent microscopy acquired as 12bit images.

Fig. 3 (B), Fig. 4 (C), Fig 5 (F-I), Fig. S1 (F-J), FigS4 (D,E): single plane spinning disk confocal microscopy, acquired as 16 bit images. Two-color images are taken in near-simultaneous mode. Single time frames or maximum intensity projections over time are shown as indicated in figure legends.

Fig. 2 (A): single plane swept-field confocal microscopy, acquired as 16 bit. Single time frames are shown.
Fig. 1 (I), Fig. 3 (D-G), Fig. 4 (G-L), Fig. 5 (B-D), Fig. 6 (G-J), Fig. S2: laser-scanning confocal 8-bit images, all channels acquired in sequential mode to avoid cross-talk. Single slices or maximum intensity projections are shown as indicated in figure legends.

Fig. 1 (A), Fig. 2 (K,L,O-Q): wide-field deconvolution microscopy, acquired as 16 bit. Maximum intensity projections of deconvolved image stacks are shown.

For all multi-color images, single channels were contrasted independently; gamma-adjustment was used to visualize minor details. Kymograph (Fig.2H) was built along a freehand line using the ImageJ "reslice" function.

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 of 5µM for 30 minutes in fixed cell experiments or was added to the medium during live cell imaging.

siRNA and expression constructs

Two different combinations of mixed siRNA oligonucleotides against CLASP1 and CLASP2 were used. Combination 1 (Mimori-Kiyosue et al., 2005): CLASP1 siRNA targeted sequence: 5’-GGATGATTTACAAGACTGG-3’; CLASP2 siRNA targeted sequence: 5’-GACATACATGGGTCTTAGA-3’. Combination 2 (custom design, Sigma): CLASP1 siRNA targeted sequence: 5’-CGGGAUUGCAUCUUUGAAA-3’; CLASP2 siRNA targeted sequence: 5’-CUGAUAGUGUCUGUUGGUU-3’. KIF1C siRNA oligo targeted sequence was 5’-GUGAGCUAUAGGAGAGCU-3’. Non-targeting siRNA (Dharmacon) was used for controls.

The following plasmid constructs were used: RFP–cortactin (a gift from Marko Kaksonen, EMBL, Heidelberg, Germany), KIF1C-GFP and KIF1C-mCherry (Theisen et al., 2012),
EB3-mCherry lentiviral construct (a gift from Dr. Al Reynolds, Vanderbilt University, TN), mCherry-dTOM20 (Drosophila outer mitochondrial protein) (a gift from Dr. Ethan Lee, Vanderbilt University, TN), Tks5-GFP ((Oikawa et al., 2008), a gift from Dr. Tsukasa Oikawa, Kobe University, Japan), GFP-CLASP2 and RFP-CLASP2 (gifts from Dr. Anna Akhmanova, Utrecht University, the Netherlands).

Mito-CLASP (dTOM20 fused with the N-terminal end of CLASP2a in a pCS2 vector) was used for CLASP mislocalization to mitochondria (Efimov et al., 2007).

Cloning of the dominant negative mutations was based on the FLAG-tagged rescue construct of human Kif1C p4xFLAG-Kif1C$^{\text{RIP1}}$, which has been described previously (Theisen et al. Dev Cell 2012). The motor and neck domain (amino acids 1-359) was deleted by amplifying the Kif1C tail using PCR with primers atGaAttCTAtgGCCCGGCTGATTAGAGAGC and GTggatcCACAGCTGCCCCACTCTC and digestion with EcoRI and BamHI. The G102E rigor mutation was introduced into the Kif1C motor domain using a 3-step mutagenesis PCR with upstream primer GgaatTCTGGAGCTATGGCTGGTG, downstream primer ACTGACCTTCTCCGAGTCC and mutagenesis primer TGGTATAGGATTTCtCAGCCC. A fragment comprising of the N-terminal half of the motor domain containing the mutation was replaced in p4xFLAG-Kif1C$^{\text{RIP1}}$ using EcoRI and BsiWI.

The Kif1C rescue plasmid protected against the Kif1C siRNA used in this study was generated on the basis of pKif1C-eGFP described previously (Theisen et al. Dev Cell 2012). 5 silent point mutations were introduced in the RNAi target site using a 3-step PCR with upstream and downstream primers as for G102E rigor and mutagenesis primer CTGTGGAGGTGtctTAcATGGAaATCTACTGTGAGCG. The fragment containing the mutation was exchanged with EcoRI and BsiWI to generate pKif1C$^{\text{RIP2}}$-eGFP. Deletion of the Kif1C tail beyond the first two coiled coil domains was done by introducing a BamHI restriction site after amino acid position V490 in Kif1C using primers GgaatTCTGGAGCTATGGCTGGTG and GAAGggatCCACAGTTCCCCCATCCTC and replacing the BsiWI-BamHI fragment in pKif1C$^{\text{RIP2}}$-eGFP with the truncated fragment to create pKif1C$^{\text{RIP2}}$(1-490)-eGFP.
Transfection, infection and stable lines

For transient transfection of plasmid DNA, Fugene6 (Roche) or Amaxa nucleofection (Lonza) (X-001 program) were used according to the manufacturer's protocols. Experiments were conducted 18-24 hours after transfection. For siRNA oligonucleotide transfection, HiPerFect (Qiagen) was used according to the manufacturer's protocol. Experiments were conducted 72 hr after transfection, as at this time minimal protein levels were detected. For viral infection, supernatant containing lentiviral particles was collected from HEK293T cells transfected with the lentiviral expression vectors and second generation packaging constructs (Invitrogen). A7r5 cells were infected with supernatant containing lentiviral particles in the presence of 8 µg/ml polybrene overnight.

EB3-mCherry A7r5 stable line was generated using lentiviral constructs and maintained in the same conditions as A7r5. KIF1C-GFP stable A7r5 cell line was produced by G418 antibiotic selection (500µg/ml). HEK293T cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum.

Antibodies and immunofluorescence

The following antibodies were used: rabbit polyclonal anti-CLASP2 VU- 83 (Efimov et al., 2007), rabbit polyclonal anti-CLASP1 (Epitomics), rat monoclonal anti-CLASP2 KT69 (Fitzgerald), mouse monoclonal anti-α-tubulin DM1a (Sigma), rabbit polyclonal anti-tubulin (Abcam), rabbit polyclonal anti-KIF1C (Cytoskeleton), mouse monoclonal (Upstate), rabbit polyclonal (Cell Signaling) anti-cortactin and mouse monoclonal anti-FLAG® M2 (SIGMA) antibodies. Alexa 350, 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, 568 or 647 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)
for 10 min at room temperature, or in 4% paraformaldehyde plus 0.1% Glutaraldehyde and 0.3% Triton in CB buffer for 10 min at room temperature, or for 5 min in methanol (for anti-CLASP2 staining) 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 hr, and 40 min with the secondary antibodies. All antibodies were diluted in blocking solution prior to 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 blot analysis

Western blotting was performed with the Protein Electrophoresis and Western Blotting System (Bio-Rad). Briefly, cells collected from a 10cm dish were spun down and resuspended in Laemmli Sample Buffer (Bio-Rad). 30µg of total protein samples were applied to a 10% polyacrylamide gel and processed for electrophoresis. 0.45uM nitrocellulose membrane was used for protein blotting. The membrane was incubated with primary and then secondary (LI-COR, IRDye™ 800 or 700) antibodies 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

Podosome number per cell (Fig. 1H, Fig. 2G,M,R, Fig. 6K) and average podosome size (Fig. 2H) were quantified using ImageJ particle analysis or ImagePro Analyzer (Media Cybernetics) on thresholded images of cortactin stainings visualized by wide field or deconvolved (DeltaVision) fluorescent microscopy. Objects were verified by comparing with actin staining and wrongly merged objects split manually.

Fold increase of KIF1C-GFP intensity at the cell edge (Fig. 3C) 6µm (20px) was quantified using singe plane spinning disk confocal movies of KIF1C-GFP-expressing cells. For the
first and last frames of a movie, a cell outline was drawn and a band of 6µm (20px) width was taken for quantification. After background subtraction, the summarized fluorescence intensity within these bands was measured and the ratio between the last and the first frames was taken as the fold increase.

Accumulation of KIF1C in lamellae (Fig. 3H) was quantified using maximal intensity projections of confocal stacks of immunostained KIF1C. A cell outline was drawn and a band of ~20µm (150px) width was taken as the cell lamella. Area of lamella and area taken by KIF1C accumulations were quantified using ImageJ particle analysis tool, and the percentage of area taken by KIF1C was quantified.

Number of KIF1C movement events co-localized with MT plus ends was determined using spinning disk confocal sequences (5 sec/frame) of KIF1C-GFP and EB3-mCherry-expressing cells. First, all events of directional KIF1C displacement for 4 pixel or more were marked in the GFP channel. Then, moving KIF1C particles were superimposed at mCherry channel in real or flipped (control) orientation. The number of KIF1C particles moving together with EB3 comets was detected, and percentage of co-localized movements out of the overall movement events was quantified.

Number of KIF1C movement events co-localized with CLASP-coated MTs was quantified similarly, with RFP-CLASP2 in the red channel; flipped red channel was used as a control. The number of KIF1C particles moving along CLASP-rich MTs was detected, and percentage of co-localized movements out of the overall movement events was quantified.

KIF1C trafficking (Fig. 3J) was determined using spinning disk confocal sequences (5 sec/frame) of KIF1C-GFP expressing cells. KIF1C puncta were manually tracked using the MTrackJ plugin of ImageJ. Particle displacements longer than 0.2µm (microscope resolution) were isolated. Trajectories were summed and divided by the total number of particles analyzed per cell; this calculation gives the distance travelled per particle. This analysis is modified from a previously used approach (Barlan et al., 2013).

Co-localization of KIF1C and mitochondria (Fig., 4E,F) was quantified as Manders coefficients using JACoP plugin of ImageJ. Mitochondria images were thresholded
automatically; KIF1C images were thresholded for the equal number of pixels above background.

Mitochondria in lamella (Fig. 4M) was quantified using ImageJ particle analysis on thresholded images of mCherry-TOM20 visualized by wide field fluorescent microscopy, after exclusion of 20µm-wide area of compacted mitochondria around the nucleus.

Conflict of interest

The authors declare that they have no conflict of interest.

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References


Figure legends

Figure 1. Podosome formation in A7r5 cells requires MTs. A, Immuno-fluorescence visualization of podosomes in a PDBu-treated A7r5 cell by Tks5 (red), actin (phalloidin, green) and cortactin (blue). The boxed region from the overview (left) is enlarged at the right and shows Tks5-positive podosomes and their co-localization with actin and cortactin. Maximal intensity projection of a confocal stack. B-G. Wide field fluorescent microscopy of actin (phalloidin, B-D,) and cortactin (E-F) in A7r5 cells. Multiple podosomes are spread throughout a cell after 40 min of PDBu treatment (C, F) in contrast to untreated cells (B, E) or cell pre-treated with nocodazole for 2 hours before PDBu application (D, G). H, Podosome numbers based on cortactin staining (similar to (E-G). N=40±10. Student’s unpaired two-tailed t-test. Asterisks, p<1*E-06. I, KIF1C (red) accumulates at the cell edge and podosomes (arrows). Phalloidin, green. The boxed region from the overview (top) is enlarged below. Arrows, podosomes.

Figure 2. Podosome formation in A7r5 cells depends on KIF1C. Arrows, podosomes. A-F, Immuno-fluorescence visualization of podosomes by actin (phalloidin, green, A,B) and cortactin (green, L,M). KIF1C (red) is shown in (J,K) for cells in (H,I). B, D, F, After KIF1C depletion only few immature podosomes are detected. Remaining KIF1C is detected in the cell center (D). G, Podosome numbers based on data similar to (L,M). N=32. Student’s unpaired two-tailed t-test. Asterisk, p<0.01. H, Average of mean podosome area per cell (µm²) based on data similar to (E,F). N=32. Student’s unpaired two-tailed t-test. Asterisk, p<1*E-06. I, Western blotting indicates significant depletion of KIF1C. Actin, loading control. J, Quantification of KIF1C levels detected by Western blotting. N=3. Student’s unpaired two-tailed t-test. Asterisk, p<0.001. K,L, Re-expression of KIF1C-GFP (red) in KIF1C-depleted cell (L) rescues podosome formation as compared with GFP-expressing KIF1C-depleted cell (K). GFP, pseudo-colored red. Cortactin, green. M, Podosome numbers in KIF1C-depleted and rescued cells. N=45. Student’s unpaired two-tailed t-test. Asterisks, p<0.05. N, Western blotting indicates KIF1C-GFP expression in control and KIF1C-depleted cells. Actin, loading control. O-Q, Expression of dominant-negative FLAG-tagged Kif1C cargo-binding domain (P) or rigor motor mutant (Q) suppresses podosome formation.
as compared to control (O). Cortactin, green. R, Podosome numbers in dominant negative construct-expressing cells. N=45. Student’s unpaired two-tailed t-test. Asterisks, p<0.05.

**Figure 3.** Podosome induction signaling facilitates MT-dependent KIF1C deposition in lamellae. A, PDBu facilitates accumulation of KIF1C-GFP (green) to the cell periphery. Frames from a single plane confocal image sequence at 30 sec (left) and 24 min (right) of PDBu treatment. Pseudo-colored map of KIF1C intensity (purple/low to white/high). See Movie 1. B, Deposition of KIF1C-GFP (green) in cell lamella before (left) and after (right) 90 minute PDBu treatment. See Movie 2. C, Fold increase of KIF1C-GFP intensity in cell lamellae in live cells with or without PDBu treatment. Based on data as in (B). Student’s unpaired two-tailed t-test. Asterisk, p<0.01. N=6-12. D-G, Immunostained KIF1C (red, arrows) at the cell periphery. KIF1C modestly localizes to lamellae of untreated cells (D), and accumulates in lamella of a cell after 40 minute PDBu treatment (E). No KIF1C accumulations are found in nocodazole-treated (F) and nocodazole-pretreated plus PDBu-treated (G) cells. Maximal intensity projection of confocal stacks. H, Percent of area in cell lamellae taken by KIF1C accumulation. Based on data as in (D-G). N=10. Student’s unpaired two-tailed t-test. Asterisk, p<0.001.

**Figure 4.** CLASP associates with KIF1C-rich MTs. A,B, CLASPs (green, arrows) marks MT plus ends in a non-treated cell (A), but upon PDBu treatment (B) accumulate at the lattice of MTs in close contact with podosomes (cortactin, red). Immunostaining, wide-field fluorescence. C, KIF1C-mCherry (red, yellow arrows) moves with the end of a GFP-CLASP2 (green, white arrows)-associated MT in a PDBu-treated cell. Frames from a video sequence. Enlarged from the box in Suppl. Fig. S1F. See also Movies 5 and 6. D, KIF1C (red) relocation events occurring at the ends of MTs associated with CLASP2 (green), as compared to the same events superimposed on a spatially shifted RFP-CLASP2 video sequence. Based on data as in Movies 5, 6. Student’s t-test: p<0.001. N=4 cells (14-19 events/cell.) E, F, Co-localization of ectopically expressed KIF1C with mitochondria in cells with (mitoCL) or without (ctr) mito-CLASP expression. Mander’s coefficients M1 (E) and M2 (F) for thresholded images are shown. Both full length (left) and (right) and tail domain constructs are recruited to mitochondria by CLASP. G, H. KIF1C-GFP (red) co-localizes with CLASP (green, immunostained) and mitochondria (mCherry-TOM20, blue) in cell with (H) but not without (G) mito-CLASP. I, J, FLAG-tagged Kif1C tail (red) co-localizes with
CLASP (green, immunostained) and mitotracker (blue) in cell with (J) but not without (I) mito-CLASP. See also Suppl. Fig S2A-D. K,L, Mitochondria in mCherry-TOM20 alone (K) or mCherry-TOM20 and mito-CLASP co-transfected (L) cells. See also Suppl. Fig S2F,G. In applicable panels, yellow dotted lines indicate cell borders. M, Number of mitochondria in lamellae in cells transfected with mCherry-TOM20 or mito-CLASP. Based of the data as in K,L, N=28-36. Student’s unpaired two-tailed t-test. Asterisk, p<0.01.

**Figure 5.** MT-dependent transport of KIF1C to podosomes requires CLASPs. A, CLASP1 and CLASP2 depletion levels identified by Western blotting in cells depleted by siRNA combinations 1 and 2. Student’s unpaired two-tailed t-test. Asterisks, p<0.01. B-D, KIF1C (red, arrows) accumulates around podosomes in the lamellae of PDBu-treated non-targeted control cells (B), but is missing from the lamellae of CLASP-depleted cells (C,D). Boxes outlined on the left are enlarged on the right. Phalloidin, blue. Tubulin, green. Immunostaining. Maximal intensity projections of confocal stacks. E, Percent of area in cell lamellae taken by KIF1C accumulation. Based on data as in (B-D). N=10. Control populations same as in Fig. 2C,D. Student’s unpaired two-tailed t-test. Asterisk, p<0.001. F-I, KIF1C-GFP (green) trafficking visualized by a single plane confocal image sequence. Single frame cell overviews are shown of the left. Video sequences from the boxed regions are shown on the right as enlarged maximal intensity projections over time. Arrows indicate tracks of KIF1C particle movement in non-targeted control cells (F,G) and lack of directional movement in CLASP-depleted cells (H,I), in PDBu-treated (G,I), or untreated (F,H) cells. See Movie 7. J. Directional movement of KIF1C puncta is enhanced by PDBu in control, but not CLASP-depleted, cells. Based on data as in F-I and Suppl. Figure S4D,E. N=10-17 cells. Student’s unpaired two-tailed t-test. Asterisk, p<0.05.

**Figure 6.** Podosome formation in A7r5 cells requires CLASPs. A-F, Cortactin immunostaining of vehicle-treated (A-C) and PDBu-treated (D-F) cells. Arrows, podosomes. Transfected siRNA: A,D, non-targeted control; B,E, anti-CLASPs siRNA combination 1; C,F, anti-CLASPs siRNA combination 2. Podosomes are formed in NT control (D, arrows), but not CLASP-depleted cells (E,F). Wide-field fluorescence microscopy. G-J, In cells treated anti-CLASPs siRNA combination 2, podosome formation in PDBu (I) is rescued by expression of siRNA2-insensitive RFP-CLASP2 (H,J). Cortactin immunostaining (G,I). K,
Podosome numbers based on data similar to (A-J). N=45±15. Student’s unpaired two-tailed t-test. Asterisks, p<1E-5.

**Figure 7. Models.** **A,** A model of podosome regulation by CLASP-dependent KIF1C transportation. Upon PDBu treatment, CLASP is recruited to MTs and stimulates KIF1C binding to MTs. This leads to KIF1C-dependent transport of essential factors to podosome formation sites. **B,** A schematic representation of proposed signaling cascade triggered by PDBu treatment.
Figure 5

A) CLASP depletion efficiency in Western

B) PDBu

C) CLASPsi1

D) CLASPsi2

E) KIF1C at lamellae (% area)

F) control

G) PDBu

H) CLASPsi1

I) CLASPsi1

J) Distance traveled per vesicle (μm)
Figure 6

(A) control, (D) PDBu, (G) CLsi2/resc/contr, (H) CLsi2/resc/contr, (B) CLsi1, (E) CLsi1/PDBu, (I) CLsi2/resc/PDBu, (J) CLsi2/resc/PDBu, (K) Number of Podosomes per Cell

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Figure 7

A  CLASP1s and KIF1C recruitment to MT

KIF1C delivery and podosome formation

B  Proposed pathway

PDBu
PKC
GSK3β
CLASP1&2
KIF1C
integrins or other cargo
podosomes

- CLASPs
- CLASPs at MT tip
- podosome
- KIF1C