CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover

Samantha J. Stehbens1,3, Matthew Paszek2,3, Hayley Pemble1, Andreas Ettinger1, Sarah Gierke1 and Torsten Wittmann1,4

Turnover of integrin-based focal adhesions (FAs) with the extracellular matrix (ECM) is essential for coordinated cell movement. In collectively migrating human keratinocytes, FAs assemble near the leading edge, grow and mature as a result of contractile forces and disassemble underneath the advancing leading edge. We report that clustering of microtubule-associated CLASP1 and CLASP2 proteins around FAs temporally correlates with FA turnover. CLASPs and LL5β (also known as PHLDB2), which recruits CLASPs to FAs, facilitate FA disassembly. CLASPs are further required for FA-associated ECM degradation, and matrix metalloprotease inhibition slows FA disassembly similarly to CLASP or PHLDB2 (LL5β) depletion. Finally, CLASP-mediated microtubule tethering at FAs establishes an FA-directed transport pathway for delivery, docking and localized fusion of exocytic vesicles near FAs. We propose that CLASPs couple microtubule organization, vesicle transport and cell interactions with the ECM, establishing a local secretion pathway that facilitates FA turnover by severing cell–matrix connections.

Cell migration is essential for development, tissue remodelling and wound healing, and requires coordination of intracellular signalling and cytoskeleton dynamics to generate traction forces. These forces are transmitted to the cell exterior by focal adhesions (FAs), stratified protein structures that connect the actin cytoskeleton to the extracellular matrix (ECM). FAs are initiated outside the cell by ECM binding of integrin trans-membrane receptors, promoting integrin clustering and recruitment of intracellular adaptor proteins1. Nascent adhesions either rapidly turn over or connect to the actin cytoskeleton, and grow and mature in response to actomyosin contractile forces. Mature FAs thus mechanically couple the ECM to the actin cytoskeleton and provide anchor points required for cell migration2. However, FAs also have to release and disassemble underneath the cell body for productive forward movement, and an unresolved question is how FA disassembly is spatially and temporally controlled in a migrating cell.

Microtubules regulate FA disassembly3, and repeated targeting by dynamic microtubules promotes FA turnover4. Global FA disassembly is also induced by microtubule regrowth after nocodazole removal, which involves focal adhesion kinase (FAK), Rho GTPases and the endocytic machinery5-7. However, it is not known how FA turnover is locally controlled in migrating cells. Several +TIPs that bind to growing microtubule plus ends8, including the adenomatous polyposis coli protein (APC), the spectraplakin MACF1 (also known as ACF7) and CLASP proteins are closely associated with FAs (refs 9–11). CLASPs promote the stability of peripheral microtubules (refs 11,12), but it is not known why CLASP-mediated microtubule stabilization is important for cell migration.

By using quantitative live-cell imaging of migrating epithelial cells in which FAs show highly coordinated turnover, we find that CLASPs tether microtubules to FAs. CLASPs facilitate disassembly of mature FAs and are required for FA-associated ECM degradation, and we identify FAs as hotspots of exocytosis. On the basis of these data, we propose that FA disassembly can be facilitated through targeted, local exocytosis and ECM degradation releasing integrin–matrix connections. CLASPs are thus central to coupling the organization of intracellular vesicle transport to the remodelling of cell–matrix interactions, highlighting a previously unappreciated molecular pathway controlling FA turnover.

1Department of Cell and Tissue Biology, University of California San Francisco, 513 Parnassus Avenue, San Francisco, California 94143, USA. 2Department of Surgery and Center for Bioengineering and Tissue Regeneration, University of California San Francisco, 513 Parnassus Avenue, San Francisco, California 94143, USA. 3Present addresses: Institute of Health Biomedical Innovation (IHBi), Queensland University of Technology Translational Research Institute, 37 Kent Street, Woolloongabba, Queensland, 4102, Australia (S.J.S.); School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York (M.P.). 4Correspondence should be addressed to T.W. (e-mail: torsten.wittmann@ucsf.edu). Received 14 February 2014; accepted 24 April 2014; published online 25 May 2014; DOI: 10.1038/ncb2975
RESULTS

FA-associated CLASP-decorated microtubule clusters correlate with FA disassembly

Wounding of a confluent HaCaT cell monolayer induces sheet migration in which cells retain cell–cell contacts. FAs labelled by stable expression of paxillin–mCherry showed highly coordinated turnover dynamics at the edge of these migrating cell sheets. In contrast to randomly migrating cells, in which many nascent FAs turn over rapidly, most FAs that appeared near the leading edge of migrating HaCaT cells matured and turned over in a highly coordinated manner as the cells moved forward. Thus, HaCaT sheet migration is a good model for investigating the dynamics of a homogenous FA population involved in cell migration. Transiently expressed enhanced green fluorescent protein (eGFP)–CLASP2 consistently accumulated along microtubules around more than 90% of mature FAs at the border between cell body and lamella/lamellipodia (Supplementary Video 1). These eGFP–CLASP2-decorated microtubules did not overlap with FAs, but instead surrounded and seemed to engulf FAs shortly before FA disassembly (Fig. 1a,b). In addition, eGFP–CLASP2 puncta appeared near the leading edge, flowed inward and accumulated around FAs, and seemed to capture microtubules at areas adjacent to FAs (Fig. 1c). Quantitative analysis of fluorescence intensity dynamics of paxillin–mCherry and eGFP–CLASP2 turnover confirmed that CLASP2-decorated microtubule clusters begin to assemble around mature FAs (Fig. 1d). Further CLASP2 accumulation correlated with FA disassembly, suggesting a role for CLASPs during the disassembly phase of FA turnover, and CLASP2-decorated microtubule clusters disappeared after complete FA disassembly. Both human CLASP isoforms, CLASP1 and CLASP2, similarly and independently localized around FAs, as indicated by immunofluorescence with isoform-specific antibodies (Fig. 1e,f). These data demonstrate a tight spatial and temporal correlation between FA turnover and the accumulation of CLASP-decorated microtubules, suggesting that CLASPs act to target and/or transiently capture microtubules at mature FAs.

CLASPs anchor microtubules near FAs

To test whether CLASPs specifically mediate microtubule interactions with FAs, we generated HaCaT cell lines stably expressing either a control non-targeting short hairpin RNA (shRNA) or shRNAs targeting either CLASP1 or CLASP2. Out of four independent shRNA sequences tested for each CLASP isoform, two resulted in specific reduction of either CLASP1 or CLASP2 protein levels by more than 90% (Supplementary Fig. 1e and Fig. 1f). We were unable to generate CLASP1/2 double-depleted cell lines, probably owing to mitotic defects. Depletion of either CLASP isoform resulted in fragmentation of the Golgi apparatus (Supplementary Fig. 1a,f), indicating significant knockdown of CLASP function in either CLASP1- or CLASP2-depleted cells.

Consistent with previous reports, the peripheral microtubule array was less dense and disorganized in CLASP-depleted cells (Supplementary Fig. 1b)\(^{11,12}\). To determine whether CLASPs specifically mediate microtubule interactions with FAs, we used scanning angle interference microscopy (SAIM), a super-resolution technique that enables measurements of the distance of fluorescent-labelled structures from the ventral cell surface at ~5 nm axial resolution\(^{15}\). Consistent with previous total internal reflection fluorescence (TIRF) microscopy studies, in control cells, microtubules descended towards the ventral cell surface as microtubules approached the cell periphery (Fig. 1g)\(^{16}\). However, axial resolution in TIRF is limited, and the improved resolution achieved by SAIM indicated that microtubules further approached the ventral cell surface in close proximity to FAs (Fig. 1g). The height of these FA-associated microtubules above the substrate–medium interface was often markedly less than 150 nm, indicating axial co-localization of these microtubules with the top of the FA plaque\(^{15,17}\). Strikingly, in CLASP-depleted cells, microtubules did not descend towards FAs and remained significantly above the height of FA-associated microtubules in control cells (Fig. 1g,h), indicating that CLASPs participate in tethering microtubules to FAs.

CLASPs facilitate FA turnover during epithelial sheet migration

The striking spatiotemporal correlation between CLASP cluster formation and the disassembly phase of FA turnover indicated a role for CLASP-decorated microtubules during FA disassembly. To test this hypothesis, we analysed FAs by immunofluorescence of endogenous paxillin. Depletion of either CLASP1 or CLASP2 resulted in a three- to fivefold increase in FA size (Supplementary Fig. 1d, g) associated with thick F-actin bundles with increased myosin phosphorylation (Supplementary Fig. 1b–d), and CLASP-depleted cells appeared larger and flatter. Because these findings are consistent with an FA turnover defect, we compared FA dynamics in control and CLASP1- or CLASP2-depleted, paxillin–mCherry-expressing, migrating HaCaT keratinocytes by time-lapse spinning disk confocal microscopy (Fig. 2a and Supplementary Video 2). FAs that matured at the base of the lamella appeared both larger and longer lived in CLASP-depleted cells. To quantify the dynamics of these FAs, paxillin–mCherry fluorescence intensity profiles as a function of time were fitted with a logistic function during the assembly phase, and a single exponential decay during the disassembly phase (Fig. 2b)\(^{18–20}\). FA lifetime was defined as the time during which the paxillin–mCherry fluorescence intensity remained above the half maximum. Whereas the FA assembly rate was unchanged, the disassembly rate was significantly decreased and FA lifetime was increased about twofold in CLASP-depleted cells (Fig. 2c). Consistent with an FA disassembly defect, other aspects of FA dynamics were aberrant in CLASP-depleted cells. Individual FAs often underwent cycles of failed disassembly and reassembly, and large, mature FAs often started to slide towards the cell centre (Fig. 2d), eventually undergoing disassembly once they appeared to reach a critical tension threshold. Such extensive FA sliding was not observed in control cells. Coordinated FA turnover is important for productive cell migration, and the directionality of migrating CLASP-depleted cells was reduced when compared with control cells, which consistently moved in nearly straight paths perpendicular to the edge of the cell monolayer (Fig. 2e and Supplementary Fig. 2). Taken together, these data demonstrate that CLASPs are key players in controlling the disassembly phase of FA dynamics.

FAs direct CLASP cluster assembly

We next analysed what determined the formation and maintenance of cortical FA-associated CLASP accumulation. Notably, microtubules were not required. On treatment with 3.3 μM nocodazole, which depolymerized nearly all microtubules and increased contractility...
Figure 1 Mature FAs recruit CLASP2-decorated microtubules. (a) Spinning disk confocal microscopy of a migrating HaCaT epithelial cell expressing eGFP–CLASP2 (black) and paxillin–mCherry (magenta). In this and subsequent figures, images from live time-lapse sequences are contrast inverted to better visualize intracellular protein dynamics. See Methods for details. (b) eGFP–CLASP2 and paxillin–mCherry dynamics at the advancing cell edge in the region indicated in a. Arrows indicate the birth of two FAs that are engulfed by CLASP2-decorated microtubules before disassembly. Elapsed time is in minutes. (c) Continuation of the same sequence showing only the eGFP–CLASP2 channel to highlight eGFP–CLASP2 particles that appear near the leading edge, increase in intensity while flowing retrograde and often seem to capture growing microtubules (red arrows). (d) Turnover dynamics of paxillin–mCherry-labelled FAs and surrounding eGFP–CLASP2. Fluorescence intensity profiles measured as a function of time were normalized to the maximum paxillin–mCherry fluorescence intensity for each FA and aligned relative to one another (n=20 FAs). The solid line is an exponentially modified Gaussian curve fit, and dashed lines 95% confidence intervals. (e) Immunofluorescence of endogenous CLASP1 and CLASP2. Insets show only the CLASP channel. In this and subsequent figures, single channels are shown with inverted contrast for improved clarity. (f) Immunofluorescence demonstrating isoform-specific shRNA-mediated CLASP depletion. (g) SAIM of microtubule height above the substrate in control and CLASP2-depleted cells. The bottom panels show areas from different cells at higher magnification and a shallower height map. Note the decrease of microtubule height specifically near FAs in control cells that is largely absent from CLASP2-depleted cells. (h) Quantification of microtubule height near FAs larger than 1 µm² in control and CLASP-depleted cells. n=40 (control shRNA); 28 (CLASP1 shRNA); 28 (CLASP2 shRNA) cells from two experiments. The box-and-whisker plot shows median, first and third quartile (box) and 95% confidence intervals (notches) with whiskers extending to the furthest observations within ±1.5 times the interquartile range. Dots are individual data points, and source data are included in Supplementary Table 3. P values were calculated by non-parametric Kruskal–Wallis analysis of variance with Bonferroni error correction.
CLASP1 and CLASP2 are essential for FA disassembly in migrating epithelial cells.

(a) Time-lapse sequences of paxillin–mCherry dynamics in control and CLASP-depleted migrating HaCaT cells. The regions indicated are shown at higher magnification. The maximum intensity projections (MIPs) over the entire 3 h time-lapse sequences on the right further illustrate FA turnover defects and sliding. Elapsed time is in minutes. (b) Examples of turnover dynamics of representative FAs in control and CLASP-depleted cells used for calculation of FA dynamics parameters in c. Data points are three-frame running averages of FA fluorescence intensity. The green solid line is a logistic fit of the FA assembly phase, the red line a single exponential decay fit of the disassembly phase. The dashed arrow indicates FA lifetime as defined by fluorescence intensity above the half-maximum of the fit. (c) Analysis of FA assembly rates, lifetime and disassembly rates in control and CLASP-depleted migrating HaCaT cells. n = 54 (control shRNA); 53 (CLASP1 shRNA); 55 (CLASP2 shRNA) FAs from three experiments. (d) Length of FA sliding measured from MIPs. n = 405 (control shRNA); 217 (CLASP1 shRNA); 404 (CLASP2 shRNA) FAs. Representative data set of three experiments. Only outliers are shown as individual data points. (e) Quantification of directed migration of control and CLASP-depleted HaCaT cells at the edge of a cell monolayer. n = 62 (control shRNA); 71 (CLASP1 shRNA); 71 (CLASP2 shRNA) cells. Representative data set of three experiments. The box-and-whisker plots show the median, first and third quartiles (boxes) and 95% confidence intervals (notches) with whiskers extending to the furthest observations within ±1.5 times the interquartile range. Dots are individual data points and source data for c–e are included in Supplementary Table 3. P values were calculated by non-parametric Kruskal–Wallis analysis of variance with Bonferroni error correction.
Figure 3  CLASP clusters around FAs do not depend on microtubules. (a) Time-lapse sequence of eGFP–CLASP2 (black) and paxillin–mCherry (magenta) dynamics in HaCaT epithelial cells treated with 3.3 μM nocodazole at $t=0$ min. (b) Average dynamics of individual paxillin–mCherry-labelled FAs and surrounding eGFP–CLASP2 in nocodazole-treated cells ($n=16$ FAs). The solid line is an exponentially modified Gaussian curve fit, and dashed lines are 95% confidence intervals. (c) Time-lapse sequence of eGFP–CLASP2 and paxillin–mCherry dynamics after nocodazole washout, illustrating microtubule repolymerization and capture at FA-associated CLASP clusters, and subsequent FA disassembly. (d) Time-lapse sequence of eGFP–CLASP2 and paxillin–mCherry dynamics in HaCaT cells in which microtubules were depolymerized by 3.3 μM nocodazole for 90 min before the addition of 10 μM Rho-kinase inhibitor Y-27632, resulting in disassembly of both FAs and associated CLASP clusters. (e) Scatter plot of the correlation between FA size and microtubule-independent eGFP–CLASP2 clusters. (f) Structured illumination super-resolution microscopy of eGFP–CLASP2 and paxillin–mCherry-expressing nocodazole-treated HaCaT cells, illustrating close intercalation of CLASP clusters and FAs. (g) Localization of endogenous CLASP1 (left) and CLASP2 (right) around FAs in 3.3 μM nocodazole-treated HaCaT cells. (h) HaCaT cells expressing paxillin–mCherry and either phosphomimetic eGFP–CLASP2 8xS/D (left) or non-phosphorylatable eGFP–CLASP2 9xS/A (right). (i) Time-lapse sequence of eGFP–CLASP2 and paxillin–mCherry dynamics in HaCaT epithelial cells treated with 10 μM Y-27632 at $t=0$ min. (j) Average dynamics of individual paxillin–mCherry-labelled FAs and surrounding eGFP–CLASP2 in 10 μM Y-27632-treated cells ($n=20$ FAs). The solid line is an exponentially modified Gaussian curve fit, and dashed lines are 95% confidence intervals. All elapsed time is in minutes.
nocodazole washout, eGFP–CLASP2 at FAs rapidly reassOCIated with newly growing microtubules before FA disassembly (Fig. 3c and Supplementary Video 4), suggesting that FA-associated CLASP clusters participate in microtubule capture to promote FA disassembly. Endogenous CLASP1 and CLASP2 formed similar clusters around FAs in nocodazole-treated cells (Fig. 3g), demonstrating that these microtubule-independent CLASP clusters were not artefacts of eGFP–CLASP2 overexpression. In addition, mutations of glycin synthase kinase 3β (GSK3β) phosphorylation sites that alter CLASP microtubule binding11 had little effect on FA association. Phosphomimetic eGFP–CLASP2 8xS/D, which has reduced microtubule-binding activity, accumulated in punctate clusters around FAs (Fig. 3h). Non-phosphorylatable eGFP–CLASP2 9xS/A-decorated microtubules also accumulated around FAs (Fig. 3h), indicating that CLASP2 accumulation around FAs is independent of GSK3β phosphorylation and microtubule association. Next, we tested whether CLASP clusters depended on FAs. Maintenance of mature FAs requires myosin-mediated contractility22. Contractility inhibition by the Rho-kinase inhibitor Y-27632 resulted in synchronized, rapid FA disassembly. eGFP–CLASP2-decorated microtubules immediately filled the area previously occupied by the FA (Fig. 3i and Supplementary Video 5), and eGFP–CLASP2-decorated microtubule clusters disappeared ∼15 min after Y-27632 induced FA disassembly (Fig. 3j). Microtubules were not required for CLASP cluster disassembly, as Y-27632 also rapidly induced FA and associated eGFP–CLASP2 disassembly in cells in which microtubules had first been depolymerized with nocodazole (Fig. 3d). Together, these data show that FAs recruit and maintain associated CLASP clusters independent of CLASP interactions with microtubules.

**LL5β is required for CLASP-mediated FA turnover**

We next determined whether LL5β, a peripheral membrane protein that binds CLASPs and is required for CLASP recruitment to the cell cortex23, showed FA-associated dynamics similar to CLASPs. In contrast to non-motile HeLa cells, in which cortical LL5β is static23, LL5β clusters in migrating HaCaT keratinocytes were highly dynamic, and appeared and grew in the vicinity of maturing FAs (Fig. 4a and Supplementary Video 6). Similar to CLASPs, FA-associated LL5β fluorescence intensity reached a maximum when mCherry–paxillin fluorescence peaked and the FA had started to disassemble. In addition, small, punctate LL5β particles seemed to be released from these FA-associated sites and underwent retrograde flow towards the cell body, during which they slowly disappeared. Similar to CLASPs, cortical FA-associated LL5β clusters depended on FAs, as they quickly disappeared in Y-27632-treated cells (Supplementary Fig. 3a). In contrast, LL5β clusters did not require microtubules or phosphoinositide 3-kinase activity. eGFP–CLASP2 and endogenous LL5β staining partially overlapped around FAs (Fig. 4c), which was further enhanced when CLASP binding to microtubules was eliminated either by microtubule depolymerization or in cells expressing eGFP–CLASP2 8xS/D (Fig. 4c). In addition, both eGFP–LL5β and eGFP–CLASP2 localized to similar punctate FA-associated structures as shown by TIRF microscopy (Fig. 4b). Overall, the localization and dynamics of these LL5β clusters were similar to the spatiotemporal correlation of CLASPs with FAs.

If LL5β recruits CLASPs to FAs, we expected LL5β to also be required for normal FA disassembly dynamics. To test this hypothesis, we generated LL5β-depleted HaCaT cell lines using two independent shRNA sequences that reduced LL5β expression levels by ∼90% (Supplementary Fig. 3b,c). As expected, LL5β depletion resulted in loss of FA-associated CLASPs (Supplementary Fig. 3d). In contrast, LL5β accumulation around FAs was not affected in CLASP-depleted cells (Supplementary Fig. 3e), confirming that LL5β recruits CLASPs to the cell cortex23. Cytoskeleton phenotypes (Supplementary Fig. 3f, g) and FA turnover defects in LL5β-depleted cells were similar to what we observed in CLASP-depleted cells. Compared with control, FAs were larger in LL5β-depleted cells, and showed two- to threefold increased lifetimes and decreased disassembly rates (Fig. 4d,e). In contrast to CLASP-depleted cells, LL5β-depleted cells also exhibited a moderate FA assembly defect, suggesting further CLASP-independent functions. Together, these data demonstrate that LL5β-mediated CLASP recruitment around FAs facilitates FA turnover.

**CLASPs are required for FA-associated ECM degradation**

ECM degradation mediated by the membrane-associated matrix metalloprotease MT1–MMP occurs in the vicinity of FA sites in invasive cancer cells24. To determine if FA-associated ECM degradation is a normal cell process and a mechanism that could control FA turnover dynamics, we examined whether FA-associated ECM degradation occurred in non-transformed migrating epithelial cells plated on fluorescently labelled gelatin. HaCaT cells spread slowly on gelatin-coated coverslips and exhibited little ECM degradation at early times, consistent with low invasiveness of precancerous HaCaT cells25. After 24 h, however, in addition to punctate ECM degradation underneath the cell centre, peripheral areas of ECM degradation sharply delineated FAs, creating a pattern of footprints of many generations of FAs during cell spreading. Time-lapse microscopy and analysis of fluorescence intensities further revealed a strong spatial and temporal correlation of paxillin–mCherry FA turnover and Alexa 488–gelatin degradation (Fig. 5a,b and Supplementary Video 7), similar to CLASP accumulation around FAs. In CLASP-depleted cells, residual gelatin degradation localized to the cell centre and did not correlate with FAs, demonstrating that CLASPs are required for local FA-associated ECM degradation (Fig. 5c,d).

To determine if proteolytic ECM degradation near FAs is a mechanism by which CLASPs facilitate FA disassembly, we treated cells with BB-94, a broad-spectrum MMP inhibitor26. As expected, BB-94 completely blocked FA-associated Alexa 488–gelatin degradation24 (Fig. 5e). In addition, BB-94 inhibited FA disassembly (Fig. 5f), similar to what we observed in CLASP- or LL5β-depleted cells (Figs 2c and 4e). This demonstrates that, similarly to CLASPs, extracellular MMP activity facilitates FA turnover. Because inhibition of actomyosin contractility induces rapid and simultaneous disassembly of mature FAs, we asked to what extent this depended on CLASPs or MMP activity. Y-27632 still induced FA disassembly in either BB-94-treated or CLASP-depleted cells at a slightly reduced rate comparable with normal FA disassembly in migrating cells (Fig. 5g). This indicates that, although FA-associated ECM degradation facilitates FA disassembly, inhibition
Figure 4 LL5β is required for CLASP-mediated FA turnover. (a) Time-lapse sequence of eGFP-LL5β (black) and paxillin–mCherry (magenta) dynamics in a migrating HaCaT epithelial cell at the edge of a cell monolayer. The image on the right shows an MIP of only the LL5β channel over the entire 3 h sequence, illustrating the complex dynamics of LL5β particles near FAs. Elapsed time is in minutes. (b) TIRF microscopy of eGFP–LL5β and eGFP–CLASP2 near paxillin–mCherry labelled FAs, demonstrating a similar punctate pattern at the ventral cell surface. (c) LL5β immunofluorescence in HaCaT cells expressing the indicated eGFP–CLASP2 constructs, indicating increased overlap of LL5β and CLASPs in the absence of CLASP-microtubule binding. (d) Time-lapse sequences of paxillin–mCherry dynamics in control and LL5β-depleted migrating HaCaT cells. The regions indicated are shown at higher magnification. Elapsed time is in minutes. (e) Analysis of FA assembly rates, lifetime and disassembly rates in control and LL5β-depleted migrating HaCaT cells. n = 33 (control shRNA); 21 (LL5β shRNA 28); 32 (LL5β shRNA 39) FAs from three experiments. The box-and-whisker plots show median, first and third quartiles (boxes) and 95% confidence intervals (notches) with whiskers extending to the furthest observations within ±1.5 times the interquartile range. Dots are individual data points and source data are included in Supplementary Table 3. P values were calculated by non-parametric Kruskal–Wallis analysis of variance with Bonferroni error correction.
Figure 5 CLASPs are required for FA-associated ECM degradation. (a) Time-lapse sequence of Alexa-488–gelatin degradation in a paxillin–mCherry-expressing cell illustrating spatiotemporal correlation with FA turnover. The region indicated is shown at higher magnification. Areas of matrix degradation appear as dark areas in the Alexa-488–gelatin images. Elapsed time is indicated in minutes. (b) Average turnover dynamics of Alexa-488–gelatin at individual paxillin–mCherry-labelled FAs. Fluorescence intensity profiles measured as a function of time were normalized to the maximum Alexa-488–gelatin and paxillin–mCherry fluorescence intensities, and aligned relative to the half maximum of FA assembly \((n = 20 \text{ FAs})\). Because FA lifetime is highly variable in spreading HaCaT cells, the FA disassembly phase was not included in this quantification. Solid lines are a logistic fit for FA assembly and a single exponential decay for ECM degradation. Dashed lines are 95% confidence intervals. (c) Matrix degradation by control and CLASP-depleted HaCaT cells after 24 h of spreading on Alexa-488–gelatin-coated coverslips (green). Cells were also immunostained for paxillin (magenta). (d) Quantification of the area of gelatin degradation in the periphery of control and CLASP-depleted cells. \(n = 64\) (control shRNA); 50 (CLASP1 shRNA); 39 (CLASP2 shRNA) cells from three experiments. (e) Alexa-488–gelatin degradation is inhibited in BB-94-treated cells. (f) Analysis of FA assembly rates, lifetime and disassembly rates in control and BB-94-treated migrating HaCaT cells. \(n = 48\) (control); 53 (BB-94) FAs from three experiments. (g) Analysis of Y-27632-induced (10 \(\mu\)M) FA disassembly in control, BB-94-treated and CLASP-depleted HaCaT cells. \(n = 43\) (control); 43 (BB-94); 37 (CLASP1 shRNA); 22 (CLASP2 shRNA) FAs. The box-and-whisker plots show median, first and third quartiles (boxes) and 95% confidence intervals (notches) with whiskers extending to the furthest observations within \(\pm 1.5\) times the interquartile range. Dots are individual data points and source data for (d), (f) and (g) are included in Supplementary Table 3. \(P\) values were calculated by non-parametric Kruskal–Wallis analysis of variance with Bonferroni error correction.

of FA-associated contractile forces can still trigger dissociation of the intracellular FA plaque in the absence of MMP activity and ECM degradation.

CLASPs direct FA-associated exocytosis

Our results implicate FA-associated ECM degradation as a potential mechanism by which CLASPs facilitate FA turnover.
We thus asked how CLASPs could guide local MMP activity. Because CLASP clusters connect microtubules to mature FAs, we hypothesized that intracellular vesicle transport to and/or from FAs along FA-associated microtubule tracks may participate in CLASP-mediated FA disassembly. Recent reports implicated endocytosis in FA disassembly in fibroblasts\(^\text{23,29,30}\). However, we could not detect obvious overlap of eGFP–CLASP2 localization with clathrin heavy chain or dynamin II immunofluorescence in either control migrating HaCaT cells, nocodazole-treated cells in which CLASPs maximally accumulated around FAs, or cells in which FA disassembly was acutely induced by nocodazole washout (Supplementary Fig. 4), indicating that CLASP clusters are probably not involved in an endocytosis-mediated mechanism of FA turnover.

To test whether CLASP-stabilized microtubule tracks towards FAs could instead serve as anterograde transport pathways mediating local ECM degradation through FA-associated MMP exocytosis, we analysed MT1–MMP–eGFP dynamics in relation to mCherry–pauxillin FA turnover\(^\text{14}\). A large fraction of MT1–MMP–eGFP localized to the plasma membrane, indicating that the carboxy-terminal cytoplasmic eGFP does not disrupt MT1–MMP exocytosis. In addition, membrane-bound MT1–MMP–eGFP as well as MT1–MMP–eGFP in smaller intracellular vesicles was enriched along mature FAs, reminiscent of FA-associated CLASP clusters (Fig. 6a). We next used TIRF microscopy to better visualize MT1–MMP–eGFP exocytosis. Although membrane-bound MT1–MMP–eGFP largely obfuscated vesicle trafficking, MT1–MMP–eGFP vesicles became more visible after extensive photobleaching of the plasma membrane pool. This enabled us to observe examples of transport, docking and sudden disappearance of MT1–MMP–eGFP vesicles near FAs characteristic of exocytosis (Fig. 6b,c and Supplementary Video 8). In an attempt to improve visualization of the vesicular pool of MT1–MMP, we tested a different MT1–MMP reporter construct\(^\text{24}\), MT1–MMP–mCherry–TM (transmembrane domain) brightly localized to the lumen of large intracellular compartments that evolved through macropinocytosis, during which mCherry fluorescence was released from the membrane (Supplementary Fig. 5a,b), thus probably representing endosomes or lysosomes. Comparatively little MT1–MMP–mCherry–TM localized to the plasma membrane, and we were unable to directly observe MT1–MMP–mCherry–TM exocytosis.

To quantify CLASP-mediated FA-associated exocytosis, we next analysed eGFP–RAB6A dynamics on the basis of the rationale that CLASP and LL5\(^\alpha\) bind factors required for RAB6/RAB8-mediated exocytosis\(^\text{23,29,30}\). Although RAB6 was originally implicated in retrograde Golgi-to-endoplasmic reticulum transport\(^\text{11}\) and endoplasmic reticulum tubules are continuously pulled towards the leading edge of migrating HaCaT cells, we did not observe a specific enrichment of endoplasmic reticulum around FAs (Supplementary Fig. 5c). To test if RAB6-positive vesicles are delivered to FAs, we next analysed eGFP–RAB6A dynamics in HaCaT cells expressing paxillin–mCherry by TIRF microscopy. This enabled us to clearly visualize eGFP–RAB6A vesicle dynamics and exocytosis near the ventral cell surface (Fig. 7a,b and Supplementary Video 9). In control cells, eGFP–RAB6A vesicles moved along linear tracks, often stopped at membrane domains adjacent to FAs and after a short period of pause rapidly disappeared (Fig. 7b). eGFP–RAB6A vesicle disappearance in most cases was not accompanied by a track leaving the FA, and was often associated with a brief burst of plasma membrane-associated fluorescence (Supplementary Fig. 6a). Docked eGFP–RAB6A vesicles disappeared within \(\sim 500\) ms, during which the eGFP–RAB6A signal spread laterally, but the total amount of fluorescence did not decrease (Supplementary Fig. 6b), strongly indicating two-dimensional diffusion of eGFP–RAB6A characteristic for vesicle fusion with the plasma membrane\(^\text{30,32}\).

### DISCUSSION

Here, we identify CLASPs as links connecting microtubules to regulated FA turnover. Mature FAs recruit CLASPs independent of microtubules through direct interactions with LL5\(^\beta\) (ref. 23). FAs are thus probably the source of a biochemical or mechanical signal mediating LL5\(^\beta\) and CLASP recruitment. Although the pleckstrin homology domain of LL5\(^\beta\) binds phosphatidylinositol 3,4,5-triphosphate\(^\text{35}\), phosphoinositide 3-kinase inhibition does not abolish FA-associated LL5\(^\beta\) clusters in HaCaT or in HeLa cells\(^\text{23}\). In migrating HaCaT cells, LL5\(^\beta\) and CLASP particles appear near the leading edge and move towards the cell interior at a rate similar to F-actin retrograde flow, consistent with LL5\(^\beta\) binding to filamins\(^\text{33–35}\). Similar to LL5\(^\beta\), the laminin receptor \(\alpha\)3\beta1 integrin localizes to a zone adjacent to FAs in primary keratinocytes\(^\text{36}\), and cortical LL5\(^\beta\) accumulation depends on \(\alpha\)3\beta1 integrin engagement\(^\text{37}\). Thus, the formation of FA-associated LL5\(^\beta\) and CLASP clusters probably requires a complex interplay of signalling, adhesion and F-actin dynamics. In any case, the time delay between FA assembly and LL5\(^\beta\)/CLASP recruitment establishes a feedback mechanism facilitating disassembly of large, mature FAs required for controlled cell movement.

Although FA turnover may involve proteolytic cleavage of intracellular FA components\(^\text{18,38}\) and endocytosis\(^\text{16,5}\), mechanisms that disassemble mature FAs underneath the advancing cell body are
incompletely understood. For example, it is unlikely that endocytosis initiates FA turnover, as this would require internalization of active, ECM-engaged integrin molecules. In contrast, FAs rapidly disassemble when contractile forces are released, and we hypothesize that integrin detachment from the ECM can trigger FA disassembly. Recent findings show that MT1–MMP mediates ECM degradation near FAs in invasive cancer cells\(^{24,40}\), and is required for directional cell migration\(^{41}\). Our data demonstrate that sharply delineated FA-associated ECM degradation also occurs during non-invasive HaCaT cell spreading, consistent with the activity of a membrane-bound protease. This indicates that FA-associated ECM degradation is a universal process during normal and pathological tissue remodelling.
Figure 7 Targeting of RAB6A-mediated exocytosis to FAs depends on CLASPs. (a) TIRF image of control HaCaT cell expressing eGFP–RAB6A (black) and paxillin–mCherry (magenta). (b) Kymograph and time-lapse sequence of the region in a. Pausing vesicles generate vertical and moving vesicles diagonal tracks. Coloured arrowheads correlate specific vesicles in the kymograph and time-lapse sequence. Red arrow: burst of eGFP–RAB6A diffusion characteristic for a plasma membrane fusion event. (c) Map of all observed (~200) eGFP–RAB6A fusion events in a 10 min time-lapse sequence overlaid onto the paxillin–mCherry channel, illustrating clustering near FAs. (d) Histogram of eGFP–RAB6A fusion distance to the nearest FA, compared with a uniform distribution (200 eGFP–RAB6A fusion events in a 10 min time-lapse sequence overlaid onto the paxillin–mCherry channel, illustrating clustering near FAs. (e) Immunofluorescence showing endogenous RAB6 vesicles around FAs. (f) TIRF image of CLASP1-depleted cell expressing eGFP–RAB6A and paxillin–mCherry. (g) Kymographs and time-lapse sequences of the regions in f, illustrating different types of aberrant eGFP–RAB6A vesicle dynamics. Black arrowhead: paused FA-associated vesicle that fails to fuse and continues to move on. (h) Analysis of eGFP–RAB6A vesicle number in regions surrounding FAs (left; n=40 (control shRNA); 48 (CLASP1 shRNA); 52 (CLASP2 shRNA)) or identically sized regions between FAs (right; n=27 (control shRNA); 43 (CLASP1 shRNA); 38 (CLASP2 shRNA)) from three experiments. (i) Analysis of the distance of fusion events from the nearest FA. n=399 (control shRNA); 347 (CLASP1 shRNA); 300 (CLASP2 shRNA) fusion events from three cells per condition. Six extreme outliers (>5× interquartile range from median) were removed from this data set. (j) Quantification of vesicle dwell time, the duration between an eGFP–RAB6A vesicle stopping near an FA and vesicle disappearance, determined by measuring the length of vertical kymograph track segments. n=254 (no shRNA); n=298 (control shRNA); 234 (CLASP1 shRNA); 149 (CLASP2 shRNA) vesicles from two experiments. The box-and-whisker plots show median, first and third quartiles (boxes) and 95% confidence intervals (notches) with whiskers extending to the furthest observations within ±1.5 times the interquartile range. Dots are individual data points, outliers only in i and j. Source data for h,i and j are included in Supplementary Table 3. P values were calculated by non-parametric Kruskal–Wallis analysis of variance with Bonferroni error correction.
In support of the hypothesis that FA-associated ECM degradation facilitates FA turnover, we find that FA-associated ECM degradation depends on CLASPs, and that either CLASP depletion or inhibition of MMP activity elicits strikingly similar inhibition of FA turnover. In addition, without MT1–MMP, cells cannot internalize integrins42, indicating that this requires cleavage of extracellular ECM–integrin interactions. As expected, FA disassembly is still triggered by actomyosin contractility inhibition that bypasses detachment from the ECM. However, because MMPs have other targets in addition to ECM components, alternative mechanisms are possible. For example, MT1–MMP activates other MMPs (ref. 43), and alters the extracellular signalling environment by releasing latent ECM-bound growth factors44. MT1–MMP also inhibits ADAM-dependent cleavage of FGF2, preventing ectodomain shedding, which reduces downstream signalling45. To what extent such MMP modulation of signalling networks contributes to FA dynamics remains to be determined.

Our results raise the question of how CLASP-tethered microtubules promote FA-associated ECM degradation. Although MMPs are crucially important for normal and pathological ECM remodelling46,47, surprisingly little is known about how MMP activity is spatially and temporally controlled. MT1–MMP may be retained or activated by direct interactions with FA components48, but it is not known how MT1–MMP is transported to FAs. Although we were able to observe examples of eGFP–MT1–MMP exocytosis near FAs, complex intracellular dynamics and plasma membrane localization precluded quantitative analysis. We used eGFP–RAB6A as a different marker to test whether an FA-directed exocytosis pathway exists, because the RAB6-docking protein ELKS that is biochemically linked to CLASPs and LL5b. Remarkably, we find that RAB6A vesicle docking and fusion with the plasma membrane is highly enriched near FAs, demonstrating FA-directed exocytosis in migrating cells. Interestingly, accumulation of RAB6 vesicles around FAs is evident in the original paper implicating RAB6 in retrograde Golgi-to-endoplasmic reticulum transport13. Although we cannot exclude that RAB6-mediated retrograde transport occurs near FAs, we did not observe specific endoplasmic reticulum–FA interactions. RAB6A and RAB8 localize to the same exocytic vesicles39, and MT1–MMP secretion requires RAB8 (ref. 48). RAB6A is thus an appealing candidate for mediating MT1–MMP exocytosis. Although a recent screen of MT1–MMP–mCherry–TM vesicle associated Rab GTPases did not identify RAB6A (ref. 49), this may be due to MT1–MMP–mCherry–TM prominently labelling endosomal and lysosomal compartments, making it difficult to distinguish smaller and dimmer exocytic vesicles. This highlights the necessity for improved reporters of MT1–MMP dynamics, and the importance of future work examining the spatial distribution of other Rab GTPases potentially involved in MT1–MMP exocytosis, and how secretory pathways to FAs and invadopodia are related28,30.

In any case, we demonstrate that membrane domains surrounding FAs are exocytosis hotspots and it is likely that FA-associated exocytosis underlies CLASP-mediated FA turnover and ECM degradation. Interestingly, MACF1 (also known as ACF7), a +TIP that guides microtubules along F-actin fibres, also facilitates FA turnover. Thus, CLASPs and MACF1 may participate in different aspects of the same process, but their functional relationship is not understood, and little is known of how MACF1 is involved in secretory vesicle trafficking. Collectively our data support a mechanism in which targeted MT1–MMP delivery to FAs requires CLASP-stabilized FA-associated microtubule tracks, and promotes FA turnover through local release of cell–matrix interactions. Although our main readout in the current study is an FA turnover defect, FA-associated exocytosis may be more broadly important for ECM remodelling and play central roles in normal and pathological tissue dynamics. In addition, other exocytic cargo is probably transported along these FA-associated microtubule tracks, and it will be important to determine to what extent this pathway is involved in more physiological three-dimensional cell migration systems in which directed vesicle trafficking to the protruding cell tip depends on +TIP functions35.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.J.S. and T.W. conceived the project. S.J.S., M.P., H.P., A.E., S.G. and T.W. generated reagents, conducted experiments and analysed data. M.P. contributed SAIM data and analysis. S.J.S. and T.W. wrote the manuscript and assembled figures.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cell culture. Immortalized HaCaT cells\(^1,2\), HEK-293FT (Invitrogen, R700-07) and HEK-293A cells were cultured in DMEM, 10% fetal bovine serum (Invitrogen, 26140), 100 l.u. ml\(^{-1}\) penicillin, 100 μg ml\(^{-1}\) streptomycin (Invitrogen, 15070063), 2 μM β-mercaptoethanol and 200 μM non-essential amino acids (Invitrogen, 11140-050). 293FT cells were maintained in 0.5 mg ml\(^{-1}\) geneticin/G418 (Invitrogen, 10131-035). According to NIH recommendations, the identity of HaCaT cells was established by short tandem repeat profiling (DDC Medical). The profile below is identical to a recently published profile of the HaCaT cell line\(^3\) indicated in bold except for the two underlined loci: D5S1558 (16); TH01 (9.3); D21S11 (28, 30.2); D16S539 (9, 12); D1S2769 (9, 12); D21S11 (12, 13); D13S317 (10, 12); D1S2769 (10, 9, 11); D4S447 (9, 11); D3S1358 (9, 12); AMEL (x); vWA (16, 17); D8S1179 (14); TPOX (11, 14); FGA (24); D19S433 (13, 14); D5S1338 (17, 25).

HaCaT cell migration assays were carried out as previously described\(^4\). Briefly, no 1.5 coverslips (64-0713, Warner Instruments) were cleaned by being heated and sonicated for 20 min in Versa-Clean dishwashing detergent (04-324, Fisher) diluted in ddH\(_2\)O followed by 20 min sonication in ddH\(_2\)O. Coverslips were rinsed multiple times in ddH\(_2\)O then stored in 100% ethanol and flame-sterilized before coating with fibronectin (11051407001, Roche, 10 μg ml\(^{-1}\)). HaCaT cells were seeded on fibronectin-coated coverslips at a density to ensure confluency within 24–48 h. Cells were rinsed and incubated in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS for 5 min to loosen cell–cell contacts. Coverslips were placed, cell side up, onto Parafilm. A stainless-steel, flat-edged razor blade was used to slice half the coverslip, removing cells from one side of the monolayer to create a monolayer edge. Wounded monolayers were then incubated with 0.25% trypsin–EDTA for 10 s, and rinsed five times with media to remove cell debris. For live-cell microscopy, coverslips were mounted into sealed, custom-made imaging chambers with media containing 20 mM HEPES at pH 7.5 and left to recover overnight at 37°C, 5% CO\(_2\) before imaging.

DNA constructs, adenoviral and lentiviral vectors. Adenovirus particles for transient expression of wild-type eGFP–CLASP2 (340–1362) and the non-phosphorylatable eGFP–CLASP2 (340–1362) 9xS/A mutant were as described\(^5\). The phosphomimetic eGFP–CLASP2 (340–1362) 8xSxD construct was subcloned into pENTR/D-TOPO and recombined into pAD/CMV/VS-DEST following the manufacturer’s instructions (Invitrogen no K2400-20, 42-0200). High-titre adenovirus stocks were produced and purified by CsCl, density gradient ultracentrifugation\(^6\), and cells were infected with adenovirus as described previously\(^7,8\). In brief, the adenovirus titre was adjusted for optimal transduction levels in HaCaT cells and cells were infected for 5 h, after which the tissue culture medium was replaced.

To generate stable fluorescent protein expressing HaCaT cell lines, paxillin–mCherry\(^9\), eGFP–RAB6A (ref. 9) and eGFP–LL35 (ref. 33) were subcloned into pENTR/D-TOPO and recombined into pLenti6/V5-DEST (Invitrogen, 35-1277). Lentivirus particles were packaged using ViraPower (Invitrogen, 44–2835), and HaCaT cells were transduced with pLenti lentivirus particles following the manufacturer’s instructions as previously described\(^8\). Briefly, HaCaT cells were seeded to be 70% confluent on the day of infection in a 35 or 60 mm dish. Cells were incubated in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS for 5 min at 37°C, 5% CO\(_2\) to loosen cell–cell contacts before the addition of virus particles. Infected HaCaT cells were selected and cultured in DMEM (Invitrogen, 11–0650), and 0.1 mM MβCD (Biochemical) was added to the medium for 7 days and FACS sorted to select for low expression levels. Dual eGFP–RAB6A/paxillin–mCherry and eGFP–LL35/paxillin–mCherry cell lines were made by transducing HaCaT cells stably expressing paxillin–mCherry with the respective eGFP–protein/pLenti6 virus and FACS sorted for cells expressing both eGFP and mCherry simultaneously. Cells were maintained under blasticidin selection, which was removed 24 h before experimental use.

Antibodies, immunofluorescence and immunoblotting. Primary antibodies used for immunofluorescence (and immunoblotting) including specific dilutions used are listed in Supplementary Table 2. All secondary fluorescently labelled antibodies were from Invitrogen except highly cross-absorbed secondary antibodies from Jackson ImmunoResearch (Table 1). Secondary antibody dilutions were adjusted linearly to enable comparison of protein of interest to loading controls. Samples were heated to 100°C for 5 min, centrifuged, and left to recover overnight at 4°C before loading. 20 μg of protein was loaded per lane onto a 7.5% Tris–glycine gel. Gels were run at 100 V. Gels were stained with Coomassie blue and destained with 10 mM methanol and 10 mM acetic acid. Stained gels were imaged using a FluorChem Q gel documentation system (92-14116-00, Alpha Innotech), cropped in Adobe Photoshop CS5 and assembled in Adobe Illustrator CS5. Images were adjusted linearly to enable comparison of protein of interest to loading controls.

Gelatin degradation assay. Gelatin-coated coverslips were prepared essentially as described\(^10,11\). In brief, 0.2% porcine gelatin (G-1890, Sigma) was labelled with Alexa 488 succinimidyl ester (A20000, Invitrogen). Covered slides were coated with a solution of 0.1% w/v poly-L-lysine (P4707, Sigma) for 20 min at 22°C, and subsequently washed three times with PBS before incubating with 0.5% glutaraldehyde (16220, Electron Microscopy Services) for 15 min at 22°C. Coverslips were rinsed three times with PBS then inverted onto drops of Alexa 488–gelatin preheated to 37°C (diluted 1:4 with unlabelled 0.2% gelatin) for 10 min at 22°C. Gels on the coverslips were then placed in tissue culture dishes and crosslinked with 0.5% glutaraldehyde for 15 min to prevent tearing of the gelatin resulting from contractile forces generated by adherent cells. Glutaraldehyde was quenched in 5 mM NaBH\(_4\) (452882, Sigma–Aldrich) for 15 min at 22°C. Coverslips were rinsed three times in PBS before dipping in 70% ethanol for sterilization. Coated coverslips were then incubated in DMEM after 45 min at room temperature. Secondary antibodies and phallolidin were diluted in blocking buffer. Coverslips were rinsed three times in blocking buffer for 5–10 min each before incubating in secondary antibodies (1:500) for 45 min at room temperature. Secondary antibodies and phallolidin were diluted in blocking buffer. Coverslips were rinsed three times in PBS, 5–10 min each, then mounted in either Mowiol mounting medium (0.1 M Tri-s HCl pH 8.5, 25% glycerol, 10% Mowiol 4–88, 475994, Calbiochem) or mounted in 0.1% n-propyl gallate (P-3130, Sigma) in PBS for TIRF imaging and stored at 4°C.

For immunofluorescence of CLASP1 and CLASP2, samples were fixed in –20°C methanol for 5 min, before three rehydration washes with PBS for 10 min each. Samples were blocked overnight at 4°C in blocking buffer before processing for immunofluorescence as described above.

For immunoblotting, cells were lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and protease inhibitors) containing phosphatase inhibitors (50 mM NaF; 1 mM NaVO\(_4\), 1 mM EGTA, 10 mM sodium pyrophosphate, 1 mM β-glycerol phosphate and 10 mM calyculin A) for 5 min on ice, scraped off the tissue culture dish, transferred to an Eppendorf tube and incubated on ice for a further 5 min. Cell lysates were centrifuged at 13,000 rpm for 5 min and supernatants transferred either to a new tube for a bicinchoninic acid assay or to one containing SDS–polyacrylamide gel electrophoresis sample buffer. Samples were heated to 100°C for 5 min, and centrifuged before loading. 20 μg of protein was loaded per lane onto a 7.5% Tris–glycine gel. Gels were run at 100 V. Gels were stained with Coomassie blue and destained with 10 mM methanol and 10 mM acetic acid. Stained gels were imaged using a FluorChem Q gel documentation system (92-14116-00, Alpha Innotech), cropped in Adobe Photoshop CS5 and assembled in Adobe Illustrator CS5. Images were adjusted linearly to enable comparison of protein of interest to loading controls.
in 0.1% w/v crystalline trypsin (T0303, Sigma) in Hanks balanced salt solution (14025-076, Invitrogen) for 5 min at 37 °C. The detached cells were transferred into 5 ml Hanks balanced salt solution and centrifuged at 2,000 rpm at 22 °C for 5 min. The supernatant was aspirated and the cell pellet resuspended in 1 ml complete medium. The resuspended cell population was added to the coverslips in a 12-well dish containing 1 ml of DMEM per well and left to adhere at 37 °C in a 5% CO2 incubator. Cells were fixed with 4% paraformaldehyde in BRB80 at indicated times and processed for immunofluorescence.

Images were acquired by spinning disk confocal microscopy as described below, and regions of interest (ROIs) were selected using the ‘Simple ROI editor’ and the ‘Bezier’ tool in NIS-Elements to outline individual adhesions. If the FA significantly changed size and location and moved out of the ROI over time, ROIs were redrawn to include all fluorescence using the ‘redraw’ function in the NIS-Elements ROI tool. Fluorescence intensity as a function of time was measured from the unprocessed image data using the ‘Time measurement’ tool in NIS-Elements. Cytoplasmic background was subtracted by using duplicated ROIs adjacent to FAs. Fluorescence intensity as a function of time was similarly measured in dual-wavelength experiments, demonstrating spatiotemporal correlation of paxillin–mCherry and eGFP–CLASP2 turnover.

To quantify FA turnover dynamics, time-lapse fluorescence intensity data were smoothed with a three-frame moving average to reduce noise. The assembly phase was fitted with a logistic function, in which \( f_{\text{max}} \) is the maximum fluorescence intensity, \( k_a \) is the rate constant and \( k_d \) the time at half maximum:

\[
y_{\text{assembly}}(t) = \frac{f_{\text{max}}}{1 + e^{-\left(\frac{t - t_{\frac{1}{2}}}{k_a}\right)}}
\]

The disassembly phase was fitted with a single-exponential decay with the rate constant \( k_d \):

\[
y_{\text{disassembly}}(t) = f_{\text{max}} e^{-k_d(t-t_{\frac{1}{2}})}
\]

Least square curve fitting was done using the Solver function in Excel (Microsoft). FA lifetime was defined as the time during which the fluorescence intensity remained above the half maximum and was calculated from the assembly and disassembly curve fits:

\[
t_{\frac{1}{2}} = a - \frac{\ln \left( \frac{f_{\text{max}}}{2} \right)}{k_d} - t_{\frac{1}{2}}
\]

Of note, we probably underestimate FA disassembly defects because FAs that did not assemble and disassemble within the 3 h time-lapse sequence were not included in our analysis.

RAB6A vesicle number. Random frames were selected from eGFP–RAB6A TIRF time-lapse sequences. ROIs were drawn using the Bezier tool in NIS-Elements of approximately 15–35 µm² (ref. 2), around the largest FA in each cell, then duplicated and placed over FAs and adjacent cortical areas that did not contain an FA. Areas were selected in the paxillin–mCherry channel independent of the eGFP–RAB6A channel to not bias region selection. Vesicles were then counted in each ROI. The number was normalized to the ROI area and multiplied by a factor to calculate vesicles per 100 µm².

RAB6A kymographs and analysis of vesicle dwell time. eGFP–RAB6A TIRF time-lapse sequences were rotated such that FAs to be analysed were aligned with the horizontal image axis. Images were filtered as described above to enhance contrast of RAB6A vesicles. The image sequence was then cropped closely around FAs as indicated by the dashed box in Supplementary Fig. 7a. The vertical width of the crop (y axis) was 20 pixels. A sequence of x–t kymographs was generated in the ‘Show Slices View’ in NIS-Elements by right-clicking the x-t view and using the ‘Switch Axes and Create New Document’ command. The resulting image sequence is shown in Supplementary Fig. 7b and corresponds to 20 kymographs for each of the horizontal (x axis) lines of pixels in the cropped time-lapse sequence. To better visualize the tracks of vesicles that moved at paths that were not parallel to the horizontal axis, maximum intensity projections were generated of either the entire x–t kymograph sequence (Supplementary Fig. 7c) or only of selected planes. eGFP–RAB6A vesicle dwell time was defined as the time from when a vesicle stopped moving until it disappeared, and was measured as the length of vertical eGFP–RAB6A tracks in the kymographs using the NIS-Elements Annotations and Measurements’ vertical measurement tool.

Repeatability of experiments. Figures 1e, 1f, 3c, 3d, 3f–h, 4a–c, 6 and 7e contain representative images of live cell or immunofluorescence experiments that are not otherwise quantified. Each of these experiments was carried out at least three times.
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**Supplementary Figure 1** Validation and phenotypes of CLASP depletion.

(a) Representative images of Golgi fragmentation in CLASP-depleted cells stained for GM130 to identify the Golgi apparatus, and Sytox orange as a nuclear stain. (b) Cytoskeleton phenotypes of CLASP depletion. Peripheral microtubules are sparser and disorganized. F-actin stress fibres anchoring into FAs are more predominant in CLASP-depleted cells. (c) Immunofluorescence of phospho-myosin (ppMLC) and E-cadherin indicate that CLASP depletion increases cell contractility. (d) Representative images of migrating HaCaT cells at the edge of a cell monolayer stained for paxillin (magenta) and F-actin (green), expressing control (non-targeting), CLASP1 or CLASP2 shRNA. Only one of the two shRNA sequences used for each CLASP isoform is shown. Single channels of the indicated regions are displayed with inverted contrast. (e) Immunoblot of lysates from cells expressing different shRNA constructs after 7 days of puromycin selection. Tubulin was used as loading control. Blots were probed with isoform-specific antibodies to either CLASP1 or CLASP2. Uncropped blots are shown in Supplementary Figure 8. (f) Quantification of the degree of Golgi fragmentation around the nucleus in HaCaT cells migrating at the edge of a cell monolayer. The Golgi fragmentation angle $\alpha$ was defined as the angle through the center of the nucleus that encompasses all Golgi structures. $n = 73$ (control shRNA); 35 (CLASP1 shRNA #32); 47 (CLASP1 shRNA #33); 65 (CLASP2 shRNA #55); 43 (CLASP2 shRNA #58) cells. Representative data set of three experiments. (g) Quantification of FA size in CLASP-depleted cells. Each data point is the average FA size from one image which contained 3-5 cells. $n = 28$ (control shRNA); 32 (CLASP1 shRNA #32); 35 (CLASP1 shRNA #33); 27 (CLASP2 shRNA #55); 34 (CLASP2 shRNA #58) images. Representative data set of three experiments. Box-and-whisker plots show median, 1st and 3rd quartile (box), and 95% confidence intervals (notches) with whiskers extending to the furthest observations within ±1.5 times the interquartile range. Dots are individual data points.
**Supplementary Figure 2** CLASPs are required for directional migration. (a) Example phase images of HaCaT cells migrating at the edge of a cell monolayer expressing control, CLASP1 or CLASP2 shRNAs. (b) Plots of representative cell migration paths in control and CLASP-depleted cells from 20 cells per condition. Data is representative of 3 independent experiments. Phase contrast time lapse sequences were rotated such that the wound edge was aligned with the vertical image axis, and cells are migrating to the right. The positions of dark features in the nucleus (nucleoli) were tracked over time with the “Time Measurements” function in NIS Elements using “Auto Detect ROI”. Migration paths were normalized to the starting position.
Supplementary Figure 3 Validation and phenotypes of LL5β depletion.
(a) Localization of endogenous LL5β (green) around paxillin-labeled FAs (magenta) in control HaCaT cells, and cells treated with the indicated drugs. Insets show only the LL5β channel of the indicated regions with inverted contrast. (b) Immunoblot of lysates from cells expressing different shRNA constructs after 7 days of puromycin selection. Tubulin and GAPDH were used as loading controls. Uncropped blots are shown in Supplementary Figure 8. (c) Immunofluorescence of LL5β and paxillin, in wound-edge HaCaT cells expressing control (non-targeting), or the indicated LL5β shRNAs. (d) Immunofluorescence of CLASP2, in HaCaT cells expressing control or CLASP2 shRNA. LL5β-depletion in HaCaT cells does not affect LL5β localization to adhesion sites. (e) Immunofluorescence of paxillin and LL5β in HaCaT cells expressing control or CLASP2 shRNA. CLASP-depletion in HaCaT cells abolishes peripheral CLASP accumulations. (f) Immunofluorescence of tubulin or (g) F-actin (phalloidin) in HaCaT cells expressing control or LL5β shRNA. Cytoskeleton phenotypes of LL5β depletion are qualitatively similar to CLASP-depletion. Peripheral microtubules are sparser and disorganized. F-actin stress-fibers anchoring into FAs are more predominant in LL5β-depleted cells.
Supplementary Figure 4 CLASPs do not co-localize with endocytic vesicles. (a) Nocodazole washout experiment in cells expressing EGFP-CLASP2. Cells were pre-treated for 90 minutes with 3.3 μM nocodazole prior to washout, and immunostained for endocytic markers clathrin heavy chain (HC) or dynamin II. Images were acquired by spinning disc confocal microscopy. No obvious colocalization is observed between CLASP2 and either clathrin HC or dynamin II. (b) Nocodazole washout experiment in cells immunostained for clathrin HC and paxillin. Cells were pre-treated for 90 minutes with 3.3 μM nocodazole prior to washout. Samples were imaged using total internal reflection microscopy (TIRF).
Supplementary Figure 5 MT1-MMP-mCherry-TM and ER dynamics in migrating HaCaT cells. (a) Diagram of fluorescently tagged MT1-MMP constructs used. SP, signal peptide; TM, transmembrane domain; HPX, hemopexin domain. (b) Spinning disk confocal microscopy of a migrating HaCaT epithelial cell at the edge of a cell monolayer expressing MT1-MMP-mCherry-TM. Red arrowhead indicates the evolution of a bright, luminal labelled vesicle through macropinocytosis indicative of cleavage of the mCherry tag from the transmembrane domain. (c) Dynamics of AcGFP-tagged Sec61 as an endoplasmic reticulum marker in a migrating HaCaT cell expressing paxillin-mCherry. Although ER tubules are dynamically pulled forward into the leading lamella, there was no obvious correlation between ER dynamics and FA turnover. Elapsed time is in minutes.
Supplementary Figure 6 EGFP-Rab6A vesicle fusion with the plasma membrane. (a) High speed TIRF imaging of EGFP-Rab6A vesicles. Red arrowheads indicate two independent fusion events associated with lateral spreading of EGFP-Rab6A signal in the plasma membrane. Elapsed time is in seconds. (b) Analysis of fluorescence intensity across EGFP-Rab6A vesicles at different time points during fusion normalized to vesicle intensity before fusion (n = 11 vesicles). Solid lines are Gaussian fits. The integrated intensity remains high while the signal spreads in the TIRF plane demonstrating lateral diffusion of EGFP-Rab6A indicative of fusion with the plasma membrane and thus exocytosis. Error bars are 95% confidence intervals.
Supplementary Figure 7 Workflow of generating kymographs to quantify EGFP-Rab6A vesicle transport. (a) Rotated raw and filtered data, cropped region around a FA (dashed box), and single x-t kymograph at the orange line. (b) Set of all twenty x-t kymographs for each horizontal row of pixels in the cropped image. (c) Maximum intensity projection of all twenty x-t kymographs. See methods for additional details.
**Supplementary Figure 8** Uncropped immunoblots shown in supplementary Figures 1 and 3. Membranes were cut along the dotted lines before incubation with primary antibodies in order to probe shRNA targets and loading controls (GAPDH and tubulin) on the same blots.
Supplementary Video Legends

Supplementary Video 1 EGFP-CLASP2 and paxillin-mCherry dynamics in a migrating HaCaT cell. Spinning disk confocal microscopy time lapse sequence of a migrating HaCaT epithelial cell at the edge of a cell monolayer expressing EGFP-CLASP2 (black) and paxillin-mCherry (magenta). EGFP-CLASP2-decorated microtubules engulf FAs before FA disassembly underneath the advancing cell body. Images were acquired every 2 minutes. The video plays at 15 frames s⁻¹ and is thus accelerated 1800 times.

Supplementary Video 2 Focal adhesion turnover dynamics in control and CLASP-depleted cells. Spinning disk confocal microscopy time lapse sequences of migrating HaCaT epithelial cells at the edge of a cell monolayer expressing paxillin-mCherry. Left panel: Control shRNA; middle panel: CLASP1 shRNA; right panel: CLASP2 shRNA. FA disassembly underneath the advancing cell body is disrupted in CLASP-depleted cells. Images were acquired every 3 minutes. The video plays at 15 frames s⁻¹ and is thus accelerated 2700 times.

Supplementary Video 3 Focal adhesion-associated CLASP clusters are microtubule independent. Spinning disk confocal microscopy time lapse sequence of a HaCaT epithelial cell expressing EGFP-CLASP2 (black) and paxillin-mCherry (magenta). 3.3 µM nocodazole was added after 20 minutes, when EGFP-CLASP2-labeled growing microtubule plus ends abruptly disappear. Images were acquired every 2 minutes. The video plays at 15 frames s⁻¹ and is thus accelerated 1800 times.

Supplementary Video 4 Focal adhesion-associated EGFP-CLASP2 dynamics after nocodazole washout. Spinning disk confocal microscopy time lapse sequence of a HaCaT epithelial cell expressing EGFP-CLASP2 (black) and paxillin-mCherry (magenta). 3.3 µM nocodazole was washed out at the beginning of the time lapse sequence. Re-growing microtubules associate with CLASP clusters prior to FA disassembly. Note that other FAs that did not have associated CLASP clusters do not disassemble. Images were acquired every 30 seconds. The video plays at 15 frames s⁻¹ and is thus accelerated 450 times.

Supplementary Video 5 CLASP clusters depend on focal adhesions. Spinning disk confocal microscopy time lapse sequence of a HaCaT epithelial cell expressing EGFP-CLASP2 (black) and paxillin-mCherry (magenta). 10 µM Y-27632 was added after 20 minutes, which induces FA and subsequent EGFP-CLASP2 cluster disassembly. Images were acquired every 2 minutes. The video plays at 15 frames s⁻¹ and is thus accelerated 1800 times.

Supplementary Video 6 EGFP-LL5β and paxillin-mCherry dynamics in a migrating HaCaT cell. Spinning disk confocal microscopy time lapse sequence of a migrating HaCaT epithelial cell at the edge of a cell monolayer expressing EGFP-LL5β (black) and paxillin-mCherry (magenta). Similar to CLASPs, EGFP-LL5β punctae surround FAs before FA disassembly. Images were acquired every 3 minutes. The video plays at 15 frames s⁻¹ and is thus accelerated 2700 times.

Supplementary Video 7 Focal adhesion-associated matrix degradation in a spreading HaCaT cell. Spinning disk confocal microscopy time lapse sequence of a paxillin-mCherry (magenta) expressing HaCaT epithelial cell spreading on Alexa488-labeled gelatin. Images were acquired every 5 minutes. The video plays at 10 frames s⁻¹ and is thus accelerated 3000 times.

Supplementary Video 8 MT1-MMP-EGFP vesicle dynamics near focal adhesions. Total internal reflection microscopy time lapse sequence of HaCaT epithelial cells expressing MT1-MMP-EGFP (black) and paxillin-mCherry (magenta) after initial photobleaching of membrane-bound signal. The same three fusion events as shown in Fig. 6c are highlighted by colored squares. Images were acquired every 0.3 seconds. The video plays at 24 frames s⁻¹ and is thus accelerated 7 times.

Supplementary Video 9 EGFP-Rab6A vesicle dynamics in a migrating HaCaT cell. Total internal reflection microscopy time lapse sequence of HaCaT epithelial cells expressing EGFP-Rab6A (black) and paxillin-mCherry (magenta). A number of closely FA-associated membrane fusion events are highlighted in the magnified region on the right. Images were acquired every second. The video plays at 30 frames s⁻¹ and is thus accelerated 30 times.

Supplementary Video 10 EGFP-Rab6A vesicle dynamics in CLASP-depleted cells. Total internal reflection microscopy time lapse sequence of HaCaT epithelial cells expressing EGFP-Rab6A (black) and paxillin-mCherry (magenta). Left panel: CLASP1 shRNA; right panel: CLASP2 shRNA. Images were acquired every second. The video plays at 30 frames s⁻¹ and is thus accelerated 30 times.

Supplementary Tables Legends

Supplementary Table 1: CLASP and LL5β shRNA sequences.

Supplementary Table 2: List of primary antibodies and other reagents used for immunofluorescence and immunoblotting.

Supplementary Table 3: Raw data and statistics.