Focal adhesion assembly requires phosphorylation signaling

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Abstract

Integrins link the cytoskeleton to the extracellular matrix for cell movement. Integrin ligation stimulates tyrosine phosphorylation of integrin-associated proteins but the role of phosphorylation signaling in regulating integrin-cytoskeletal linkages and assembly of focal adhesions is unclear. Using spreading or migrating epithelial cells, we provide evidence that phosphorylated Cas (p130Cas, BCAR1), its binding partner, Crk, and inactive focal adhesion kinase (FAK) cluster together with inactive integrins at the cell periphery at sites that develop into focal adhesions containing F-actin, active integrins, active FAK and mechanosensing proteins such as vinculin and talin. Cas, Crk, Src family kinases (SFK) and Rac1 are required for focal adhesion formation and cell spreading, while vinculin is not needed for Cas clustering. Furthermore, Rac1 provides positive feedback onto Cas through reactive oxygen, opposed by negative feedback from the ubiquitin proteasome system. The results suggest that phosphorylation signaling precedes and regulates mechanosensing during focal adhesion formation.

Introduction

Cell migration is tightly regulated during all stages of metazoan life, from gastrulation, through organogenesis, to intestinal homeostasis and wound healing in adults (Mayor et al., 2016, Rorth 2011). Deregulated cell migration contributes to pathological states including fibrosis, inflammation, and cancer. Heterodimeric α-β integrins are the best understood cell adhesion receptors, forming transmembrane bridges between the extracellular matrix (ECM) and internal cytoskeleton (Hynes 2002). Integrins switch between closed-inactive conformations and open-active conformations (Iwamoto et al., 2015, Law et al., 1996, Su et al., 2016). Either ligands outside the cell or signals inside the cell can stabilize the active conformation and induce assembly of transient protein complexes, known as nascent adhesions or focal complexes, on the integrin cytoplasmic tails (Iwamoto et al., 2015, Moser et al., 2009). Over time, nascent adhesions can disassemble or grow to become larger, more stable, focal adhesions (Kanchanawong et al., 2022). Focal adhesions contain nanoclusters of integrins and associated proteins closest to the membrane, followed by force-transducing talin and vinculin, with actin stress fibers and actin-associated proteins on top (Kanchanawong et al., 2010, Legerstee et al., 2021). Mechanosensing plays a key role in focal adhesion maturation. For example, talin unfolds under actin tension, exposing binding sites for vinculin and other structural and regulatory proteins, which provide additional links to the actin cytoskeleton and resist contractile forces to drive cell translocation (Wolfenson et al., 2019).

While mechanosensing explains many aspects of adhesion dynamics, integrin-dependent phosphorylation signaling may also be important. Integrin binding to ECM or antibodies rapidly activates Src-family kinases (SFKs) and focal adhesion kinase (FAK), stimulates phosphorylation of many integrin-associated proteins including Cas (p130Cas, BCAR1) and paxillin, and activates the Rac1 GTPase (Burridge et al., 1992, Harte et al., 1996, Mitra et al., 2006, Nojima et al., 1995, Parsons et al., 2010, Petch et al., 1995, Vuori et al., 1995, Webb et al., 2002, Zaidel-Bar et al., 2003). Cas is a scaffolding molecule that is critical for embryonic development and SFK-mediated cell transformation in vitro (Honda et al., 1999, Honda et al., 1998), regulates cancer in various mouse models, and is associated with poor survival in breast cancer (Cabodi et al., 2010, Honda et al., 1998, Pylayeva et al., 2009, van der Flier et al., 2000, Wallez et al., 2012, Wendt et al., 2009). At the molecular level, Cas localizes to focal adhesions

through an N-terminal SH3 domain and a C-terminal focal adhesion targeting (FAT) domain (Donato et al., 2010, Nakamoto et al., 1997). SFKs and Cas mutually activate, with Cas stabilizing SFKs in their active state and SFKs phosphorylating Cas at up to 15 repeated YxxP motifs (Chodniewicz et al., 2004, Pellicena et al., 2001). After phosphorylation, the pYxxP motifs bind various proteins including Crk-family proteins (Crk and CrkL), which bind and stimulate DOCK180-ELMO, a Rac1 guanine nucleotide exchange factor (GEF), and other proteins (Chodniewicz et al., 2004, Gotoh et al., 1995, Hasegawa et al., 1996, Klemke et al., 1998, Knudsen et al., 1994, Tanaka et al., 1994). Rac1 can then promote actin polymerization and lamellipodial protrusion through the WAVE/Arp2/3 complex, as well as induce focal complexes through unknown mechanisms (Nobes et al., 1995, Stradal et al., 2004, Zaidel-Bar et al., 2003). Cas phosphorylation also stimulates Cas degradation by the ubiquitin-proteasome system, allowing for termination of Cas signaling (Steenkiste et al., 2021, Teckchandani et al., 2016, Teckchandani et al., 2014).

The rapid phosphorylation of Cas following integrin engagement suggests that Cas may regulate focal adhesion dynamics. Indeed, there is general agreement that Cas deletion or inhibition prevents focal adhesion disassembly but the role of endogenous Cas in focal adhesion assembly is controversial, with one report that Cas-deficient mouse embryo fibroblasts (MEFs) have normal adhesion assembly and another showing that assembly is slowed (Meenderink et al., 2010, Webb et al., 2004). Here we report that Cas regulates focal adhesion assembly in normal epithelial cells during spreading or migration on ECM. Cas co-clusters with inactive integrins nearly a minute before the integrins are activated and focal adhesion proteins join the cluster. A positive-feedback loop between SFKs, Cas, Crk, Rac1 and reactive oxygen species (ROS) promotes the formation of these early clusters and subsequent adhesion assembly, opposed by negative feedback from the ubiquitin-proteasome system. The results suggest a key role for SFK-Cas signaling in early stages of focal adhesion formation.

Results

Live imaging shows Cas clusters mark sites of future vinculin clusters

To our knowledge, the detailed kinetics of integrin-dependent phosphorylation signaling relative to focal adhesion assembly have not been measured in any cell type, and there have been few studies of adhesion kinetics in normal epithelial cells. Therefore, we set out to measure kinetics of Cas protein in integrin adhesions using fluorescently tagged Cas in a human epithelial line, MCF10A. To avoid over-expression, which stimulates cell migration (Klemke et al., 1998, Yano et al., 2000), we developed an approach to genetically modify cells to express tagged Cas or other proteins of interest at endogenous level (see Methods). An mScarlet (mSc)-Cas fusion protein was created by inserting an artificial exon into the first intron of the *Cas* gene (Figure 1A, Figure 1 –figure supplement 1A). Genomic sequencing provided evidence for different clones with varied indels at the insertion site, but these errors were corrected by splicing, yielding an mRNA encoding the expected Cas^{mSc} fusion protein (Figure 1 –figure supplement 1B-E). Western blotting revealed similar levels and phosphorylation of Cas and Cas^{mSc} proteins, indicating that most cells are heterozygous (Figure 1B).

To test whether Cas^{mSc} localizes to focal adhesions, we transduced Cas^{mSc} cells to express near-endogenous levels of YFP-tagged vinculin (VCL) and imaged the ventral surface of the cells using total internal reflection (TIRF) microscopy. Cas^{mSc} and YFP-VCL substantially co-localized (Figure 1C). To compare Cas and vinculin dynamics, we performed two-channel time-lapse TIRF imaging as cells attached and spread on collagen. Cas^{mSc} formed clusters at the first points of cell-substrate contact and moved outwards with the spreading edge (Figure 1D, Supplementary Video 1). Vinculin clusters formed later, often overlapping with Cas clusters. Kymographs revealed sites where Cas clusters developed into vinculin clusters (Figure 1D, note transition from magenta to white to green). Similar patterns of Cas and vinculin clustering were observed under spontaneous lamellipodial protrusions generated by fully spread cells (Figure 1E, Supplementary Video 2). Again, kymographs revealed many clusters that initiated with Cas and transitioned to vinculin (Figure 1E). These results together suggest that Cas clustering marks sites of vinculin clustering during spreading and migration.

To quantify cluster formation and avoid possible selection bias, we developed a computational pipeline to delineate regions of interest (ROIs) in which Cas, vinculin, or both Cas and vinculin intensities exceeded thresholds that were automatically set for each frame (see Methods). ROIs were tracked over time and quantified if they exceeded 20 pixels in area (0.5 μm^2) and persisted for at least 3 frames ($\geq$40 s) (Figure 1F, Supplementary Video 3). These thresholds exclude the smallest, shortest-lived nascent adhesions but include larger focal complexes (Kanchanawong et al., 2022). The mean intensity of each channel in each ROI was then quantified over the duration of the recording and the intensities smoothed and normalized to a range of 0 to 1 (Figure 1 – figure supplement 2A). Profiles of individual ROIs showed a strong trend for Cas intensity to increase before vinculin intensity and for Cas intensity to decrease before vinculin intensity. We measured the time delay between Cas and vinculin reaching half-maximal intensity, $\Delta t_{1/2}$ (VCL-Cas) (Figure 1 – figure supplement 2B). Time delays for ROIs in a single cell varied with a non-normal distribution (Figure 1 – figure supplement 2C). However, the probability of vinculin joining a Cas cluster was much greater than Cas joining a vinculin cluster, with median $\Delta t_{1/2}$ (VCL-Cas) significantly greater than zero (median 54.8 s, 95% CI 23-105 s, for 90 ROIs in the cell shown) (Figure 1 – figure supplement 2C). This bolsters the hypothesis that Cas is a leading indicator for ensuing vinculin dynamics. Averaging the median $\Delta t_{1/2}$ (VCL-Cas) across 6 spreading cells in several experiments yielded a time delay of 44.6$\pm$5.0 s (mean and SEM) (Figure 1G). A broad range of $\Delta t_{1/2}$ (VCL-Cas) was also observed under lamellipodia formed by a migrating cell (Figure 1 – figure supplement 2D). Averaging median $\Delta t_{1/2}$ (VCL-Cas) across multiple ROIs under lamellpodia in 8 migrating cells in several experiments yielded a time delay 60.1$\pm$9 s (mean and SEM) (Figure 1H). Vinculin clustering was also delayed relative to Cas in spreading Cas^{mSc} YFP-VCL HeLa cells (mean $\Delta t_{1/2}$ (VCL-Cas) 44.1$\pm$9 s, Figure 1 – figure supplement 2E-F).

Overall, these results show a strong tendency for Cas to form clusters at the edge of spreading or migrating epithelial cells, 45-60 s before being joined by vinculin. For comparison, the time interval between arrival of talin, vinculin and paxillin in nascent adhesions of CHO-K1 cells migrating on fibronectin is ~14 s or ~2 s for non-maturing or maturing adhesions respectively (Han et al., 2021). The replacement of Cas clusters by vinculin clusters suggests that Cas may

spatially coordinate vinculin clustering and adhesion assembly in both spreading and migrating MCF10A and HeLa cells.

Cas clusters precede vinculin clusters independent of ECM

Cells bind to different matrix proteins through distinct integrin heterodimers, such as α5β1 for fibronectin and α2β1 for collagen (Hynes 2002). To test whether Cas clusters co-localize with focal adhesions formed by different integrins, we seeded Cas^{mSc} YFP-VCL MCF10A cells on different matrix proteins (Figure 2A). Cell spreading on fibronectin was inhibited by the integrin α5β1-blocking monoclonal antibody (mAb) P8D4 (Alfandari et al., 2003, Davidson et al., 2002), while spreading on collagen was inhibited by the integrin α2β1-blocking mAb P1E6 (Carter et al., 1990, Tuckwell et al., 1995) (Figure 2A). Mature adhesions, detected with the 9EG7 mAb that binds active integrin β1 (Bazzoni et al., 1995, Lenter et al., 1993), were larger and more intense on collagen than fibronectin (Figure 2B). Nevertheless, TIRF imaging of the ventral surface of cells attaching and spreading on fibronectin showed a similar clustering of Cas before vinculin (Supplementary Video 4). The mean $\Delta t_{1/2}$ (VCL-Cas) was similar for fibronectin and collagen (Figure 2C), suggesting Cas clusters may specify localization of integrin α5β1 and α2β1 adhesions.

Initial Cas clusters mark sites of future integrin clusters

To test whether integrins localize to early or late Cas clusters, we transduced MCF10A Cas^{mSc} cells with a lentiviral vector encoding β1Ecto-pH, a recombinant integrin β1 with a pH-sensitive pHlorin tag inserted in the extracellular domain (Huet-Calderwood et al., 2017). This integrin is cell-surface expressed, localizes to adhesions, exhibits normal integrin activation, and restores adhesion in integrin β1 knockout MEFs (Huet-Calderwood et al., 2017). Live dual-color TIRF imaging of β1Ecto-pH in Cas^{mSc} MCF10A cells revealed that β1Ecto-pH localized to Cas clusters, but, like YFP-VCL, β1Ecto-pH kinetics were significantly delayed relative to Cas, with mean $\Delta t_{1/2}$ (β1-Cas) 63.9$\pm$13.5 s (Figure 3A-B, Supplementary Video 5).

As an independent approach to measure integrin clustering, we edited the *ITGB1* gene in HeLa Cas^{mSc} cells (Figure 3C). Briefly, we targeted the *ITGB1* final intron to insert an artificial exon encoding the C-terminus of integrin β1 fused to GFP through an optimized 18-residue linker

(Parsons et al., 2008), followed by the SV40 virus 3' processing and polyadenylation (pA) signal (Figure 3 – figure supplement 1A). Primary transcripts were expected to terminate at the inserted pA signal and be spliced to create an mRNA encoding C-terminally tagged ITGB1^{GFP}. Following transfection and FACS, the correct integration and mRNA sequence were confirmed by PCR and RT-PCR (Figure 3 – figure supplement 1B-E). Western blotting with GFP antibody showed a fusion protein of the expected mobility (Figure 2D). Live imaging Cas^{mSc} ITGB1^{GFP} cells revealed integrin clustering after Cas with $\Delta t_{1/2}$ (ITGB1-Cas) 77.8$\pm$9.3 s (mean and SEM, n=5 cells) (Figure 3E-F, Supplementary Video 6). Thus, integrin β1 and vinculin are similarly delayed in their arrival in Cas clusters in MCF10A and HeLa cells.

Initial Cas clusters contain Crk

Previous studies showed that integrin clustering or activation by ECM or antibodies stimulates the tyrosine phosphorylation of Cas and other proteins (Arias-Salgado et al., 2003). Our finding that early Cas clusters before integrin led us to wonder whether Cas phosphorylation occurs late in the process. Since Cas phosphorylation cannot be imaged directly, we instead analyzed recruitment of Crk, which binds phosphorylated but not non-phosphorylated Cas and initiates downstream signaling (Chodniewicz et al., 2004). We edited the *CRK* gene in Cas^{mSc} HeLa cells, targeting the last intron to create a C-terminal fusion of Crk to mGreenLantern (mGL) (Figure 4A, Figure 4 – figure supplement 1A). Following transfection and FACS, the expected integration was confirmed by PCR (Figure 4 - figure supplement 1B-D). Western blotting showed approximately equal expression of Crk^{mGL} and Crk, suggesting most cells are heterozygous (Figure 4B). Imaging Cas^{mSc} Crk^{mGL} cells during attachment and spreading revealed rapid recruitment of Crk^{mGL} on Cas^{mSc} clusters (Supplementary Video 7, Figure 4C). The median time delay, $\Delta t_{1/2}$ (Crk-Cas), averaged across several cells, was 5.1$\pm$7.7 s (Figure 4D), much shorter than the time for vinculin or integrin recruitment. Crk recruitment was significantly delayed by treatment with eCF506, a specific SFK inhibitor, indicating that Crk recruitment requires phosphorylation (Supplementary Video 8, Figure 4E) (Fraser et al., 2016). Together these results suggest Cas is phosphorylated and recruits Crk before peak recruitment of integrin and vinculin, raising the possibility that Cas phosphorylation is stimulated by Cas clustering when local integrin density is submaximal.

Spatial distribution of adhesion proteins in Cas clusters

Time-lapse TIRF imaging of spreading Cas^{mSc} YFP-VCL cells showed the continual addition of Cas^{mSc} near the outer edge of the cell, at the head of developing clusters, followed by recruitment of YFP-VCL towards the tail of the cluster (Figure 5A). Thus, the spatial distribution of Cas^{mSc} and YFP-VCL along the length of a cluster reflects their temporal order of assembly. We reasoned that we could estimate the order of arrival and departure of other adhesion proteins from their spatial distribution within Cas clusters. To this end, we fixed and permeabilized Cas^{mSc} YFP-VCL cells after 30 min of spreading and used combinations of different antibodies for TIRF or confocal immunofluorescence imaging. For each antibody combination, intensity profiles were quantified along the length of approximately 20, 4 μm-long clusters from several cells, aligned relative to Cas^{mSc}, normalized, and averaged. Mean normalized intensity profiles of Cas^{mSc} and YFP-VCL are presented in Figure 5B, and results for various antibodies are presented as heat maps in Figure 5C and sample images in Figure 5 – figure supplement 1.

The intensity profiles show several important features. First, phosphorylated (pY410) Cas is maximal at the head of the cluster, ~0.5 μm from the cell edge (Figure 5C, Figure 5 – supplement 1), consistent with the rapid recruitment of Crk^{mGL} to Cas^{mSc} clusters (Figure 4C). The head of the cluster also contained highest amounts of FAK phosphorylated at Y861, an SFK phosphorylation site (Eliceiri et al., 2002). However, levels of FAK protein and kinase-active pY397 FAK (Le Coq et al., 2022), were low at the head of the cluster.

Second, the head of the cluster also contains peak levels of inactive integrin β1, detected with antibody AIIB2 (Takada et al., 1993), suggesting it is not attached to the ECM (Figure 5C, Figure 5 – supplement 1). In contrast, active integrin β1, detected with antibody 9EG7, peaked with YFP-VCL further back in the cluster, ~1.2 μm from the edge. This region also contained highest levels of mechanosensing and structural components of focal adhesions, including kindlin2, talin1, paxillin, kinase-active pY397 FAK, and F-actin (Figure 5C). The presence of mechanosensing proteins and active integrins suggests that the center of the cluster is attached to the ECM and subject to mechanical force.

Overall, while differential antibody access may affect immunofluorescent staining profiles, the spatial patterns suggest a temporal sequence of events in which active phosphorylated Cas initially clusters with inactive integrin β1 and SFK-phosphorylated but kinase-inactive FAK. Since integrin β1 is in its closed conformation it is probably not attached to the ECM. Later, phosphorylated and total Cas levels decrease as integrin β1 adopts the active conformation, binds to ECM, resists force, and recruits mechanosensing proteins to form a mature focal adhesion (model, Figure 5D).

Phosphorylated Cas is enriched in the adhesome

Even though many imaging studies have detected Cas in focal adhesions, Cas is not a member of the consensus adhesome as defined by proteomics or proximity biotinylation (Kanchanawong et al., 2022). However, pY Cas is detected in the phosphoadhesome and total Cas could escape detection if its overall abundance is low (Cas protein copy number is ten-fold lower than integrin β1 in MCF10A and HeLa cells (Kulak et al., 2014, Ly et al., 2018)). We used Western blotting as a sensitive approach to characterize the MCF10A cell adhesome. Cells were seeded on polylysine or collagen for 60 min and incubated with protein-protein cross-linking reagents before lysis and separation of adhesome and supernatant fractions as described (Humphries et al., 2009, Schiller et al., 2011). We then reversed the cross-links and performed Western blotting on the adhesome and supernatant samples. This semi-quantitative approach allowed an estimation of the distribution of each protein between the adhesome and supernatant fractions regardless of abundance. We found that the adhesome contained ~20-27% of total cellular integrin β1, talin, vinculin, paxillin, FAK and active (pY397) FAK, but only ~7% of a control protein, ERK (Figure 5 – figure supplement 2). The adhesome fraction also contained ~25% of total cellular Cas, suggesting it is as enriched in adhesions as bona fide adhesion proteins. Moreover, the adhesome fraction of cells plated on collagen but not polylysine contained 73% of total cellular phospho-Cas. This suggests that the subpopulation of Cas in the adhesome is phosphorylated and able to signal while Cas in the cytosol is mostly inactive. Given that most of the Cas in mature adhesions exchanges with the cytosol on a timescale of a few seconds (Machiyama et al., 2014), Cas may be rapidly phosphorylated when it enters adhesions and dephosphorylated when it leaves while pY397FAK remains active in the cytosol.

Cas regulates vinculin recruitment and focal adhesion assembly

The early arrival of Cas at sites of future focal adhesions suggests it may be regulatory. To test this possibility, we depleted Cas from MCF10A Cas^{mSc} YFP-VCL cells using siRNA (Figure 6 – figure supplement 1A). Cas-depleted cells were non-migratory and their adhesions were immobile (Figure 6 – figure supplement 1B, Supplementary Video 9). Initial cell attachment and spreading were strongly inhibited, with significant decreases in cell area, the number of vinculin clusters, and intensities of Cas, vinculin, and other focal adhesion proteins including talin1, kindlin2 and FAK in the remaining clusters (Figure 6A-B, Figure 6 – figure supplement 1C, Supplemental video 10). By way of comparison, vinculin depletion had little effect on cell spreading, the number of Cas clusters, or the intensity of Cas in those clusters (Figure 6A, B, Figure 6 – figure supplement 1D). This suggests that Cas is limiting for cell spreading, vinculin recruitment and focal adhesion formation but vinculin is not limiting for Cas recruitment, consistent with their order of assembly.

FAK is important to recruit Cas to the edge of spreading fibroblasts (Zhang et al., 2014). FAK is also detected in early Cas clusters in MCF10A cells (Figure 5C). However, depleting FAK had no effect on MCF10A cell spreading, the intensity of Cas or vinculin clusters, or the median time delay between Cas and vinculin recruitment (Figure 6 – figure supplement 2A-C). Kindlin2 and talin1 directly bind integrin tails and play important roles in integrin adhesion in fibroblasts, with kindlin2 interacting with paxillin and talin1 recruiting vinculin and providing attachment for actin, enabling force generation, adhesion maturation and cell spreading (Kanchanawong et al., 2022). Similarly, in MCF10A cells, both kindlin2 and talin1 were important for attachment and spreading. Depleting kindlin2 or talin1 decreased cell spreading, the intensity of vinculin clusters, and increased the time delay between Cas and vinculin recruitment but had no effect on Cas clustering (Figure 6 – figure supplement 2D-F). Thus Cas regulates cell spreading and clustering of talin1, kindlin2 and vinculin, and promotes talin1- and kindlin2-dependent recruitment of vinculin.

Integrins can be activated “inside-out” by proteins binding to their cytoplasmic tails, or “outside-in” by interactions with the ECM or with molecules or ions that stabilize the active open conformation (Hynes 2002). We tested whether Cas is required for outside-in adhesion assembly

by treating control or Cas-depleted cells with Mn^{2+}, which stabilizes the active integrin conformation (Gailit et al., 1988, Lenter et al., 1993). While Mn^{2+} increased the spread area of control cells, it had no effect on the spreading of Cas-depleted cells and did not rescue the intensity of vinculin clusters (Figure 6C). This suggests that Cas is rate-limiting for outside-in as well as inside-out integrin activation.

SFK-Cas-Crk-Rac1 signaling regulates adhesion assembly

Known Cas signaling pathways involve Cas binding to SFKs through the proline region and pYDYV motif, followed by phosphorylation of the repeated YXXP motifs in the substrate domain and recruitment of various downstream effectors through adaptor proteins such as Crk and CrkL (Chodniewicz et al., 2004). We investigated whether Cas phosphorylation and Crk/CrkL binding are involved in vinculin recruitment in MCF10A Cas^{mSc} YFP-VCL cells by use of the SFK inhibitor eCF506 and RNAi. Either inhibiting SFKs or depleting Crk/CrkL approximately doubled the time interval between Cas and vinculin clustering, suggesting phosphorylation-dependent Cas-Crk/CrkL signaling regulates the rate of focal adhesion formation (Figure 7A, Figure 7 – figure supplement 1A-B, Supplementary Videos 11-12). To test whether a specific SFK regulates adhesion assembly, we knocked down each of the major SFKs - Src, Fyn and Yes1 - and measured adhesion and spreading on collagen. Remarkably, cell spreading, adhesion number, vinculin intensity, and Cas phosphorylation were all inhibited by depletion of Yes1 but not Src or Fyn, while Cas cluster formation was normal (Figure 7B, Figure 7 – figure supplement 1B-D). This implies that Yes1 may have a special role in phosphorylating Cas and recruiting Crk/CrkL to stimulate adhesion assembly in MCF10A cells spreading on collagen.

Crk/CrkL bridge between phosphorylated Cas and downstream effectors including DOCK180-ELMO, which serve as guanine nucleotide exchange factors (GEFs) for Rac1 (Chodniewicz et al., 2004). Rac1 regulates lamellipodia protrusion by binding the WAVE regulatory complex (WRC) to drive Arp2/3 complex-mediated actin polymerization (Takenawa et al., 2007). Transient activation of Rac1 also induces formation of nascent adhesions at the cell edge, although the specific mechanism is unclear (Nobes et al., 1995, Zaidel-Bar et al., 2003). We used a Forster resonance energy transfer (FRET) biosensor (Rac1-2G) to measure Rac1 activity

during MCF10A cell adhesion (Fritz et al., 2015). Rac1-2G FRET activity was stimulated around the periphery of cells attaching to collagen and was inhibited by the Rac1-specific inhibitor EHT1864 (Onesto et al., 2008) (Figure 7 – figure supplement 1E). EHT1864 also inhibited cell spreading and reduced and delayed vinculin clustering but had no effect on Cas clustering (Figure 7C, Figure 7 – figure supplement 1F, Supplementary Video 13). Importantly, depleting Cas or Yes1 inhibited Rac1 (Figure 7D-E), consistent with Rac1 activation by the SFK-Cas-Crk/CrkL-DOCK180/ELMO pathway (Chodniewicz et al., 2004). Together, these results suggest that early clustering of phosphorylated Cas with Crk and inactive integrins activates Rac1 to trigger both cell spreading and assembly of vinculin-containing focal adhesions.

Linkage between cell spreading and adhesion assembly through positive and negative feedback

If the sole function of Cas in focal adhesion assembly is to activate Rac1, then activation of Rac1 may bypass the need for Cas. To test this possibility, we over-expressed either wildtype or constitutively-active GFP-Rac1^{Q61L} in MCF10A Cas^{mSc} cells by transient transfection and examined cells during attachment to collagen. As expected, GFP-Rac1^{Q61L} cells spread more than GFP-Rac1^{WT} cells (Figure 8A). Surprisingly, Cas depletion prevented Rac1-induced spreading (Figure 8A). This suggests that, even though Cas requires Rac1 to support normal spreading and adhesion formation, active Rac1 requires Cas to induce spreading, raising the possibility of a positive feedback loop from Rac1 back to Cas.

We investigated which Rac1 effector may be involved in positive feedback on Cas. Rac1 stimulates localized production of reactive oxygen species (ROS) by the Nox1 NADPH-dependent oxidase by binding p47^{phox} (Ushio-Fukai 2006). ROS are short-range signaling molecules, rapidly reacting with and inhibiting protein-tyrosine phosphatases (PTPs) and increasing local tyrosine phosphorylation of various substrates including Cas, SFKs, and p190RhoGAP (Garton et al., 1996, Giannoni et al., 2005, Nimnual et al., 2003, Tonks 2005). Thus, Rac1 potentially activates Cas through a Rac1-ROS-PTP-SFK-Cas pathway. Such a positive feedback loop, where the output signal is fed back as input, can amplify the signal, enhancing the outcome (Brandman et al., 2008). To test whether ROS are involved in positive feedback and adhesion assembly in MCF10A cells, we inhibited Nox1 with

diphenylamineiodonium (DPI) (Reis et al., 2020). DPI inhibited Rac1 (Figure 8B), inhibited cell spreading, and delayed vinculin recruitment to Cas clusters without inhibiting Cas cluster formation (Figure 8C, Supplementary Video 14). This suggests that positive feedback through ROS amplifies and sustains SFK-Cas-Crk/CrkL-Rac1 activity that recruits vinculin.

Positive feedback loops require negative feedback to avoid runaway amplification (Brandman et al., 2008). Phosphorylation-dependent signaling can be inhibited by PTPs or phosphorylation-dependent proteolysis. During collective migration, Cas signaling is inhibited by negative feedback through the $CRL5^{SOCS6}$ ubiquitin ligase complex, which targets only the phosphorylated form of Cas for proteasomal degradation (Teckchandani et al., 2016, Teckchandani et al., 2014). We tested whether $CRL5^{SOCS6}$ regulates focal adhesion assembly during MCF10A cell attachment. Either SOCS6 depletion or a Cullin inhibitor, MLN4924, shortened $\Delta t_{1/2}$ (VCL-Cas) during cluster assembly, consistent with $CRL5^{SOCS6}$ interfering with focal adhesion formation by negative feedback on phospho-Cas signaling (Figure 8D, Supplementary Video 15).

Together, these results suggest a model in which nascent clusters of inactive integrins, phosphorylated Cas, active SFKs and Crk stimulate Rac1 and generate ROS, creating positive feedback that strengthens and maintains signaling, leading to recruitment of talin1, kindlin2, vinculin and other mechanosensing proteins to form a focal adhesion (Figure 8E). Negative feedback from $CRL5^{SOCS6}$, which ubiquitinates phosphorylated Cas, fine-tunes signal strength to ensure proper assembly.

Discussion

The current view of integrin activation and focal adhesion assembly, based largely on studies in fibroblasts, is that mechanosensitive proteins kindlin2 and talin1 bind to and stabilize active integrins, allowing engagement with rearward flowing actin, recruiting vinculin and opening additional binding sites to build the adhesion and generate high ECM traction force (Henning Stumpf et al., 2020, Kanchanawong et al., 2010, Legerstee et al., 2021, Wolfenson et al., 2019, Zhu et al., 2021). The structural components of focal adhesions are recruited in a timespan of a few seconds (Bachir et al., 2014, Choi et al., 2008, Han et al., 2021, Laukaitis et al., 2001). Phosphorylation is stimulated early in the process (Zaidel-Bar et al., 2003), possibly driven by integrin clustering, which could promote trans-phosphorylation of SFKs (Arias-Salgado et al., 2003, Buensuceso et al., 2003, Huang et al., 1993), or by forces between focal complexes and the cytoskeleton (Pelham et al., 1997, Schoenwaelder et al., 1999). Many manipulations that increase tyrosine phosphorylation also stimulate focal adhesion assembly (Burridge et al., 1996). However, the exact roles of tyrosine kinases, their substrates, and Rac1 in adhesion assembly has remained unclear. Focal adhesion assembly rates were reportedly normal in Cas, FAK and SFK knockout MEFs and Cas is not part of the core adhesome (Horton et al., 2015, Robertson et al., 2015, Webb et al., 2004). In addition, RhoA, not Rac1 is thought to stimulate local actin polymerization for focal complex formation (Kanchanawong et al., 2010, Wolfenson et al., 2019). Our results suggest a different process for adhesion assembly, at least in epithelial cells, with critical roles for SFKs, Cas and Rac1 to recruit mechanosensing proteins (Figure 8E). We find that Cas, Crk, and low amounts of integrin β1 and FAK cluster together at sites that develop into focal adhesions. Moreover, Cas, Crk/CrkL, SFKs and Rac1 are required for focal adhesion formation. Vinculin, FAK, kindlin2 and talin1 do not regulate Cas clustering. Instead, kindlin2 and talin1 appear to be needed after Cas clusters form, to recruit vinculin. Integrin is inactive in the early Cas clusters, suggesting it is not engaged with the ECM. Nevertheless, Cas is phosphorylated and associated with Crk. Cas clusters grow and are activated through positive feedback involving SFKs, Cas, Crk/CrkL, Rac1 and ROS, opposed by negative feedback through $CRL5^{SOCS6}$. Subsequently, Cas levels and phosphorylation in the clusters decrease as more integrin β1 and core focal adhesion proteins are recruited and activated. Only during this later stage is FAK activated, consistent with FAK as a mechanosensor (Le Coq et al., 2022). Thus, in

epithelial cells, Cas is phosphorylated before integrins are activated, and phospho-Cas signaling is needed to initiate focal complexes and start recruiting mechanosensing proteins.

A central role for Cas in focal adhesion assembly is unexpected but may be cell type- or integrin-specific. Previous studies that identified key roles for kindlin2 and talin1 made use of fibroblasts binding to fibronectin or blood cells interacting with fibrinogen. Fibroblasts bind fibronectin through integrin αvβ3 as well as α5β1, while blood cells bind fibrinogen through αIIbβ3. Different integrins heterodimers have a different modes of activation and requirements, for example, talin1 binds more strongly to β3 than to β1 (Anthis et al., 2010, Lu et al., 2016). We found that MCF10A cell adhesion to collagen requires integrin α2β1 and attachment to fibronectin requires integrin α5β1. Moreover, MCF10A and HeLa cells express integrin β3 at 10-fold lower level than β1 (Kulak et al., 2014, Ly et al., 2018). The type of integrin may affect the specific SFK involved. In fibroblasts, Src co-localizes with, and is required for, αvβ3 adhesions but not α5β1 adhesions (Felsenfeld et al., 1999), while Fyn and Cas are needed for force-dependent adhesion assembly mediated by integrin αvβ3 on fibronectin (Kostic et al., 2006, von Wichert et al., 2003). In our experiments, Yes1 was limiting for vinculin recruitment during α2β1-mediated attachment to collagen. SFKs vary in their trafficking mechanisms and affinity for different types of membranes (Resh 1999, Sandilands et al., 2008). Yes1 has a higher affinity than Src for integrin β1 cytoplasmic tails (Arias-Salgado et al., 2003) and for liquid-ordered membrane domains associated with integrin clusters (Lietha et al., 2020). Thus, Yes1 may have specific roles in α2β1-mediated adhesions based on its interactions with membrane microdomains.

Even though we only studied epithelial cells, there are indications that Cas also regulates focal adhesion assembly in fibroblasts. Meenderink et al found that expressing wildtype Cas at normal level in Cas-knockout MEFs doubled the rate of adhesion assembly (Meenderink et al., 2010). This was unexpected given that adhesion assembly rates in wildtype and SFK- or Cas-knockout MEFs were previously reported to be equal (Webb et al., 2004). One possibility is that culturing mutant MEFs as monolayers may select for variant cells with strong adhesion, bypassing a requirement for Cas. We avoided potential selection artifacts by use of transient knockdowns,

complemented where possible by chemical inhibitors only during cell spreading or migration. Other researchers reported that chemogenetic induction of Src-Cas complexes but not Src-FAK complexes in fibroblasts is sufficient to drive adhesion assembly (Karginov et al., 2010). Furthermore, Cas localizes adjacent to new adhesion sites at the edge of spreading fibroblasts (Zhang et al., 2014). However, a potential role for Cas in specifying adhesion localization in fibroblasts was not investigated.

We note that SFK-Cas-Crk/CrkL-Rac1-ROS signaling not only initiates focal adhesion formation but also cell spreading. It was not possible to test whether Cas induces adhesion assembly and spreading through parallel or sequential pathways because of positive feedback: inhibiting any step in the loop inhibited both adhesion assembly and spreading. This makes it challenging to identify the step that simulates adhesion assembly. However, Rac1-induced actin polymerization may drive adhesion assembly, for example by creating retrograde flow and activating mechanosensing proteins. Indeed, seminal studies showed that transient expression of active Rac1 induces focal complexes, but these only mature into focal adhesions if RhoA is activated (Nobes et al., 1995). Thus, the loss of Cas from clusters may be important to locally decrease Rac1 activity and allow focal adhesion maturation. Unfortunately, the Rac1 sensor used did not allow high resolution imaging of Rac1 activity at the level of individual adhesions.

Our results raise the question of how Cas clusters form and grow. Even though integrin β1 density is lower in initial Cas clusters than in later focal adhesions, inactive integrin β1 was readily detected with antibody to inactive β1. Given that integrin β1 is 10-times more abundant than Cas in these cells (Kulak et al., 2014, Ly et al., 2018), there may be similar numbers of inactive integrin β1 and Cas molecules in the initial clusters, and integrin-Cas interaction may somehow initiate clustering. However, direct Cas-integrin binding has not been detected. The Cas SH3 and FAT domains respectively bind focal adhesion proteins FAK and vinculin (Janostiak et al., 2014, Polte et al., 1995) and paxillin, ajuba and BCAR3 (Mace et al., 2011, Pratt et al., 2005, Zhang et al., 2017), but Cas clustering did not require vinculin, paxillin or FAK. Alternatively, as mentioned above, Yes1 may not only phosphorylate Cas but also regulate Cas localization to liquid-ordered membrane domains that contain β1 integrins (Lietha and Izard 2020). Growth of initial clusters could then be driven by networks of phospho-Cas with Crk,

CrkL and multivalent downstream effectors, such as DOCK180. Indeed, recent studies elegantly showed that phospho-Cas undergoes phase separation when mixed with an SH2-SH3 protein, Nck, and an Nck-binding protein, N-WASP (Case et al., 2022). These protein condensates synergize with FAK-paxillin condensates and kindlin to cluster integrins in vitro. While our results differ in which specific proteins are involved, it is possible that similar protein condensates form from multivalent networks of phospho-Cas with adaptors, effectors and integrins. These condensates could then grow in space and time through Rac1-induced actin polymerization, trans-phosphorylation of Cas-Yes complexes, and positive feedback through ROS. Further understanding of the precise mechanism will require additional in vivo and in vitro analysis.

Materials and Methods

Plasmids

pMSCVpuroEYFPvinculin was described previously (Teckchandani et al., 2016). EGFP-Rac1^{WT} and EGFP-Rac1^{Q61L} were gifted by K. Wennerberg, University of North Carolina, Chapel Hill, NC (Arthur et al., 2004). The following vectors were provided by the indicated investigators: pmScarlet-i_C1 (gifted from Dorus Gadella, Addgene plasmid # 85044), pORANGE cloning template vector (gifted from Harold MacGillavry, Addgene # 131471), pCE-mp53DD (gifted from Shinya Yamanaka, Addgene # 41856), pLenti-Rac1-2G (gifted from Olivier Pertz, Addgene # 66111), pcDNA3.1-mGreenLantern (gifted from Gregory Petsko, Addgene # 161912) (Campbell et al., 2020), pMD2.G and psPAX2 (Didier Trono, Addgene #12259 and 12260). pLenti Ecto-pHluorin β1 integrin with 4-residue linkers was kindly provided by David A. Calderwood (Yale University School of Medicine, USA) (Huet-Calderwood et al., 2017).

Gene editing

We inserted fluorescent protein tags into endogenous genes by homology-independent intron targeting (Serebrenik et al., 2019, Zhong et al., 2021), with modifications. Intron targeting has several advantages over exon targeting. First, the exact position of artificial exon within the intron is unimportant, allowing insertion at the single guide RNA (sgRNA) SpCas9 target site with highest predicted efficiency. Second, the exon does not need to be inserted precisely, since errors should be corrected by RNA splicing. Thus, homology arms are unnecessary, shortening the donor sequence. Third, by inserting a fluorescent protein open-reading frame lacking an initiation codon, the majority of fluorescent cells should be correctly targeted and can be isolated by FACS without need for single-cell cloning.

We used the pORANGE vector which encodes, on a single plasmid, an sgRNA, SpCas9, and a polylinker for subcloning the donor sequence (Willems et al., 2020). Target sites were identified in introns of interest using the CHOPCHOP sgRNA designer (Labun et al., 2019). Corresponding sgRNA sequences were synthesized (Integrated DNA Technologies), annealed and inserted into pORANGE using combined restriction digestion and ligation at the BbsI sites. Donor sequences were then inserted at the HindIII (5') and XhoI or BamHI at (3') sites. For N-

terminal tagging, the donor contained: (a) a canonical splice acceptor (SA) sequence (Smith 1997); (b) a fluorescent tag sequence, lacking initiation codon; (c) a canonical splice donor (SD) sequence. For C-terminal tagging, the donor contained: (a) the SA sequence; (b) the last exon coding sequence followed by the fluorescent tag and stop codon; (c) the SV40 3' processing and polyadenylation sequence (Connelly et al., 1988). The ends of donor were sandwiched between inverted sites for the gene targeting sgRNA, ensuring donor excision by SpCas9 (Connelly et al., 1988) (see Supplementary Figures for plasmid designs). The donor sequence was either PCR amplified (Q5 High-Fidelity DNA polymerase, New England Biolabs) or ordered as a gBlock (Integrated DNA Technologies), digested with restriction enzymes (HindIII-HF and XhoI-HF or BamHI-HF, New England Biolabs), gel purified, and cloned into pORANGE containing the corresponding sgRNA.

The targeting vectors were transiently transfected with Lipofectamine 2000 into MCF10A epithelial cells or HeLa cells together with pCE-mp53DD, an episomal plasmid encoding a dominant-negative mutant of TP53, to avoid apoptosis due to DNA damage responses (Haapaniemi et al., 2018, Ihry et al., 2018). Fluorescent cells were selected by FACS two weeks after transfection and characterized.

Oligonucleotides for *Cas* gene editing

Name	**Sequence and notes**
Cas sgRNA	BbsI-**TARGET**
Cas gRNA Fw	caccg**ATCAGCGGTGTTCACTCAAG**
Cas gRNA Rv	aaac**CTTGAGTGAACACCGCTGAT**c
Cas mScarlet PCR	**HindIII-TARGET-<u>PAM</u>**-*SPLICE DONOR*-mScarlet
mSc Donor HindIII Fw	ata**aagcttATCAGCGGTGTTCACTCAAG<u>GGG</u>***CTAATCTCCTCTC TTCTCCTCTCTCCAG*gtgagcaagggcgaggcagt
	XhoI-<u>PAM</u>-TARGET-*SPLICE ACCEPTOR*-mScarlet
mSc Donor XhoI Rv	ata**ctcgag<u>CCC</u>CTTGAGTGAACACCGCTGAT***AACCAATACTTAC* cttgtacagctcgtccatgcc
Cas Genomic PCR	
a	CACCTCTACATTCTAGCCTGGG

b	GAACCTGCAACCCAAAACAC
c	GCCCCGTAATGCAGAAGAAG
d	GCATGAACTCCTTGATCACTGC
Cas cDNA PCR	
a	TCGGAGCCCCGAGGGCACGCG
b	CACGATGCCCTGGCGCCCATG
c	CCGCGGCACCAACTTCCCTCC
d	CGGGGATGTCGGCGGGGTGCT

Oligonucleotides and gBlocks for *Crk* gene editing

Name	Sequence and notes
Crk sgRNA	BbsI-**TARGET**
Crk gRNA Fw	CACCG**CCCTGCGGCTGGACTTACGT**
Crk gRNA Rv	AAAC**ACGTAAGTCCAGCCGCAGGG**C
Crk Genomic PCR	
a	TGACCCATACAGTGACTTCAGG
b	TTATGCATCTGGGCTTGTACTG
c	GAGCAAAGACCCCAACGAGAA
d	GCTGAACTTGTGGCCGTTTAC
Crk cDNA PCR	
a’	CTGATTGGAGGTAACCAGGAG
b’	GCAGATGAACTTCAGGGTCAG
Crk^{mGL} gBlock (Donor)	**HindIII-PAM-TARGET**-*SPLICE ACCEPTOR*-3’ORF-*GGGS-mGL-stop*-spacer-*SV40polyA*-**PAM-TARGET-BamHI**
	agcata**aagcttCCAACGTAAGTCCAGCCGCAGGG***CTAATCTCCTCTCTTCTCCTCTCTCCAG*GTCGGTGAGCTGGTAAAGGTTACGAAGATTAATGTGAGTGGTCAGTGGGAAGGGGAGTGTAATGGCAAACGAGGTCACTTCCCATTCACACATGTCCGTCTGCTGGATCAACAGAATCCCGATGAGGACTTCAGC*ggcgctagcatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctgg*

	*acggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacc tacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggccca ccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaa gcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttct tcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctg gtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggca caagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaaga acggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctc gccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaa ccactacctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcaca tggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaag taa*agcgctccatggccc*AACTTGTTTATTGCAGCTTATAATGGTTACAA ATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTC ACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTT ATCATGTCTGGATCTC***CCA*AACGTAAGTCCAGCCGCAGGG ggatcc**tatgca

Oligonucleotides and gBlocks for *ITGB1* gene editing

Name	Sequence and notes
ITGB1 sgRNA	BbsI-**TARGET**
ITGB1 gRNA Fw	CACCG**GCGCCTTCTGTTCACGATAA**
ITGB1 gRNA Rv	AAAC**TTATCGTGAACAGAAGGCG**C
ITGB1 Genomic PCR	
a	AGTAACTTCCGTAGGAGACCCC
b	CATTCTTGAGTCCTTCCTCCAC
c	AACGGCATCAAGGTGAACTTC
d	GTAGGTCAGGGTGGTCACGAG
ITGB1 cDNA PCR	
a’	GTGTGGTTGCTGGAATTGTTC
b’	GTAGGTCAGGGTGGTCACGAG

ITGB1^{GFP} gBlock (Donor)	**HindIII-PAM-TARGET-***SPLICE ACCEPTOR*-3'ORF-*linker-GFP-stop*-spacer-*SV40polyA*-**PAM-TARGET-BamHI**
	agcata**aagcttCCTTTATCGTGAACAGAAGGCGC***CTAATCTCCTCTCTTCTCCTCTCTCCAG*GGTGAAAATCCTATTTATAAGAGTGCCGTAACAACTGTGGTCAATCCGAAGTATGAGGGAAA*Aggagggggggggcccggaggcgggggggaggcgggggatccaccggtcgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccacccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtaa*agcgct*CCATGGCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTC**CCTTTATCGTGAACAGAAGGCGC ggatcc**tatgca

Cell lines, transfection and infection

MCF10A cells were originally obtained from Dr. J. Brugge (Harvard Medical School) and confirmed by STR profiling. Cells were cultured in DMEM/F12 growth media (Thermo Fisher Scientific) supplemented with 5% horse serum, (Thermo Fisher Scientific), 10 μg/ml insulin (Thermo Fisher Scientific), 0.1 μg/ml cholera toxin (EMD Millipore), 0.5 μg/ml hydrocortisone

(Sigma-Aldrich), and 20 ng/ml EGF (Thermo Fisher Scientific) and passaged using trypsin/EDTA or Accutase (Sigma-Aldrich A6964). For experiments, cells were detached with Accutase and resuspended in assay media (DMEM/F12, 2% horse serum, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0 ng/ml EGF).

HeLa cells were initially obtained from ATCC and confirmed by STR DNA profiling. Cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (both 100U/ml) and passaged with trypsin/EDTA. For experiments, cells were detached using Accutase and resuspended in DMEM without serum.

Retro- and lentiviruses were generated by transfecting 293FT cells with viral vector, pMD2.G and psPAX2 in 2:1:2 ratio with PolyJet transfection reagent (SignaGen Laboratories). Media were harvested two days later and added to recipient cells with 1 μg/ml polybrene (Sigma) for 8-16 hours. Expressing cells were selected using 1 μg/ml puromycin or FACS, depending on the vector.

Antibodies

Following antibodies were used: mouse anti-Cas (610271) (BD Biosciences); rabbit-phospho-Y410Cas (4011S) and phospho-Y249Cas (4014S) (Cell Signaling Technology); mouse anti-vinculin (V9131, Sigma-Aldrich); sheep anti-paxillin (AF4259, R and D Systems); rabbit anti-pY31 paxillin (44-720G, Biosource); rat anti-integrin-β1-activated (9EG7, 553715) and mouse anti-paxillin (610051) (BD Biosciences); rabbit anti-Talin1 (A14168-1-AP), rabbit-anti Kindlin2 (11453-1-AP) and mouse anti-FAK (66258-1-Ig) (Proteintech); mouse anti-integrin β1 (12G10, MAB2247) (Millipore); rat anti-integrin-β1-inactive (AIIB2), mouse anti-integrin α5β1 (P8D4) and mouse anti-integrin α2β1 (P1E6) (Developmental Studies Hybridoma Bank); AlexaFluor 488 goat anti-rabbit IgG (H+L), AlexaFluor 488 goat anti-mouse IgG (H+L), AlexaFluor 647 goat anti-mouse IgG (H+L), AlexaFluor 647 goat anti-sheep IgG (H+L) and AlexaFluor 633 goat anti-rat IgG (H+L) (Invitrogen); IRDye 680RD goat anti-mouse and IRDye 800CW goat anti-rabbit (LI-COR).

Inhibitors

Inhibitor	Source	Concentration
DPI	EMD Millipore, Cat: 300260-10MG	10 μM
eCF506	Cayman Chemical, Cat: 19959	100 nM
EHT1864	ApexBio, Cat: B5487	10 μM
MLN4924	Fisher, Cat: 50161353	5 μM

siRNA transfection

Cells were suspended in growth media and added to dishes with 50 pmol pooled siRNA and RNAiMAX (Invitrogen) as per the manufacturer's instructions. Transfection was repeated two days later and cells analyzed after a further two days.

Negative control siRNA	Qiagen, Cat: 1027280	AAT TCT CCG AAC GTG TCA CGT
siFyn	Dharmacon, Cat: L-003140-00-0005	J-003140-11: CGG AUU GGC CCG AUU GAU A J-003140-12: GGA CUC AUA UGC AAG AUU G J-003140-13: GAA GCC CGC UCC UUG ACA A J-003140-14: GGA GAG ACA GGU UAC AUU C
siCrk	Dharmacon, Cat: M-010503-03-0005	D-010503-02: GGA GAC AUC UUG AGA AUC C D-010503-03: UCC CUU ACG UCG AGA AGU A D-010503-04: GGA CAG CGA AGG CAA GAG A D-010503-19: GGG ACU AUG UGC UCA GCG U
siCrkL	Dharmacon, Cat: M-012023-02-0005	D-012023-01: CCG AAG ACC UGC CCU UUA A D-012023-02: GAA GAU AAC CUG GAA UAU G D-012023-05: AAU AGG AAU UCC AAC AGU U D-012023-18: AGU AAA ACU UAA CGG ACU U
siSOCS6	Qiagen, Cat: GS9306	SI03068359: CAG CTG CGA TAT CAA CGG TGA SI00061383: TAG AAT CGT GAA TTG ACA TAA SI00061376: CGG GTA CAA ATT GGC ATA ACA SI00061369: TTG ATC TAA TTG AGC ATT CAA
siYes1	Qiagen, Cat: GS7525	SI02223942: GAG GCT CCT GCT TAT TTA TAA SI02223935: CCA GCC TAC ATT CAC TTC TAA SI00302218: AAT CCC TCC ATG AAT TGA TGA

		SI02635206: AAG TAT AAT GCA GTA CAT TAA
siBCAR1 (Cas)	Qiagen, Cat: GS9564	SI02757741: AAG CAG TTT GAA CGA CTG GAA SI02757734: CTG GAT GGA GGA CTA TGA CTA SI04438280: CCA GGA ATC TGT ATA TAT TTA SI04438273: CAA CCT GAC CAC ACT GAC CAA
siSrc	Qiagen, Cat: GS6714	SI02664151: CTC CAT GTG CGT CCA TAT TTA SI02223928: CGG CTT GTG GGT GAT GTT TGA SI02223921: AAG CAG TGC CTG CCT ATC AAA SI03041605: ACG GCG CGG CAA GGT GCC AAA
siVinculin	Dharmacon, Cat: L-009288-00-0005	J-009288-05: UGA GAU AAU UCG UGU GUU A J-009288-06: GAG CGA AUC CCA ACC AUA A J-009288-07: GCC AAG CAG UGC ACA GAU A J-009288-08: CAG CAU UUA UUA AGG UUG A
siPTK2 (FAK)	Dharmacon, Cat: L-003164-00-0005	J-003164-13: GCG AUU AUA UGU UAG AGA U J-003164-14: GGG CAU CAU UCA GAA GAU A J-003164-15: UAG UAC AGC UCU UGC AUA U J-003164-16: GGA CAU UAU UGG CCA CUG U
siPaxillin	Dharmacon, Cat: L-005163-00-005	J-005163-05: CAA CUG GAA ACC ACA CAU A J-005163-06: GGA CGU GGC ACC CUG AAC A J-005163-07: CCA AAC GGC CUG UGU UCU U J-005163-08: UGA CGA AAG AGA AGC CUA A
siFERMT2 (Kindlin2)	Dharmacon, Cat: L-012753-00-0005	J-012753-05: GCC CAG GAC UGU AUA GUA A J-012753-06: CUA CAU AUU UCU CUC AAC A J-012753-07: GAA CUG AGU GUC CAU GUG A J-012753-08: AAU GAA AUC UGG CUU CGU U
siTalin1	Dharmacon, Cat: L-012949-00-005	J-012949-05: GAA GAU GGU UGG CGG CAU U J-012949-06: GUA GAG GAC CUG ACA ACA A J-012949-07: UCA AUC AGC UCA UCA CUA U J-012949-08: GAG AUG AGG AGU CUA CUA U

Cell lysis and immunoblotting

Cells were harvested after 30 min of attachment. Cells were washed three times with cold phosphate-buffer saline (PBS) followed by lysis in RIPA buffer (1% Triton X-100, 1% sodium

deoxycholate, 0.1% SDS, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EGTA) with freshly added protease and phosphatase inhibitors (10 µg/mL Aprotinin, 1 mM PMSF, 1 mM sodium vanadate) on ice. The lysates were collected after 30 min of incubation on ice and centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were collected and adjusted to equal protein concentration using the Pierce BCA protein assay kit.

Lysates were adjusted to SDS sample buffer, heated at 95°C, and resolved on SDS-PAGE using 15% polyacrylamide/0.133% bis-acrylamide or 12.5% acrylamide/0.1% bis-acrylamide gels, and transferred on to nitrocellulose membrane. Blocking was performed in Odyssey blocking buffer (LI-COR Biosciences) supplemented with 5% BSA for 30 min. After blocking, membrane was probed with the primary antibody overnight, washed in Tris-buffered saline (TBS) 0.1% Tween 20, followed by incubation for 45 min with IRDye 800CW goat anti-rabbit or 680RD goat anti-mouse conjugated secondary antibodies. Images were collected using the Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified using ImageJ.

Cell spreading and migration assays

Cells were treated with siRNA using the double transfection method as described above. Cells were starved overnight in assay media (MCF10A) or DME (HeLa), then detached with Accutase and resuspended in assay media or DME. Cells were incubated for 60 min in 5% CO_2 at 37°C in suspension before adding to glass-bottom dishes (FluoroDish, FD35-100, World Precision Instruments) or 12-mm diameter coverslips (Fisherbrand 1254580) that had been previously coated with 50 µg/ml collagen-I (Advance Biomatrix, #5056) or 5 µg/ml fibronectin (Sigma, #F1141) for at least 3 hr at 37°C and washed with PBS. Pharmacological agents were added just before seeding cells for imaging.

Live-imaging

Dual-color imaging of live cells was performed for a 30-min time period, either immediately after plating the cells, to record attachment and spreading, or approximately 24 hr after plating, to record spontaneous lamellipodia formation. Images were recorded every 20 s in 4-5 fields of view on a fully automated TIRF microscope (Nikon Ti, 100x/1.49 CFI Apo TIRF oil immersion objective) equipped with Perfect Focus, motorized x-y stage, fast piezo z stage, stage-top

incubator with temperature and CO_2 control, and Andor iXon X3 EMCCD camera with 512 x 512-pixel chip (16 micron pixels). The images were acquired using Nikon NIS Elements software and processed using ImageJ. Slight drift in the dual color time lapse images was corrected using ImageJ registration tool Image Stabilizer.

Cell migration was imaged once every 15 min for 12 hr using phase-contrast microscopy on an IncuCyte S3 and analyzed using ImageJ.

Immunofluorescence

MCF10A Cas^{mSc} cells, either expressing YFP-vinculin or not, were plated on glass-bottom dishes or coverslips and allowed to attach for 30 min before fixation with 4% paraformaldehyde in PBS for 15 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked for an hour in 5% normal goat serum, 2% BSA in PBS, all at room temperature. The cells were incubated with different combinations of primary antibodies (1:200 dilution) for either 2-3 hours at room temperature or overnight at 4 °C, then washed gently three times. Alexa Fluor-conjugated secondary antibodies were added at 1:200 dilution for 1 hr at room temperature. Alexa Fluor 488 or 647-conjugated phalloidin was used for F-actin. Glass-bottom dishes were left in PBS for TIRF microscopy as above. Coverslips were mounted in ProLong Gold (Invitrogen) for confocal microscopy.

Confocal microscopy

Fixed, immunostained coverslips were imaged on the Dragonfly 200 High-speed Spinning disk confocal imaging platform (Andor Technology Ltd.) on a Leica DMi8 microscope stand equipped with iXon EMCCD and sCMOS Zyla camera. The images on Dragonfly were acquired on 100x/1.4 oil immersion objective by using Fusion Version 2.3.0.36 (Oxford Instruments) software together with Imaris simultaneous deconvolution. The most ventral image was used for quantification.

Immunofluorescence quantification

Macros were written in ImageJ for uniform image processing and analysis. Backgrounds were subtracted and intensities adjusted equally across each image set.

For analysis of the spatial distribution of the proteins and phosphoproteins within Cas/vinculin clusters, a line (5x25-pixels width x length, 0.16 micron/pixel) was plotted along the major axis of a Cas^{mSc} cluster, starting from the outer edge and moving inwards. The intensity profile for each antibody along this line was quantified in ImageJ and aligned using Cas^{mSc} as a reference point. The mean normalized intensity profile for each antibody was then calculated across 20-25 regions from several cells. A heat map for the mean intensity profile of the normalized value was generated using GraphPad Prism.

To analyze the number and intensity of clusters containing either Cas or vinculin, or both, images were first spit into individual channels and then summed using the 'Image calculator' command in ImageJ. This summed image was used to generate a binary image mask by applying the manual threshold through Yen method. The number, areas, and median intensity of each channel in regions of interest ≥20 pixels (0.52 μm^2) was quantified for each cell analyzed. Cell area was quantified either manually by drawing around the cell or from a binary mask created by thresholding through the Triangle method, setting minimum size as 50 pixels.

Quantification of cluster kinetics from two-color TIRF videos

TIRF image datasets were exported as two-channel time-series hyperstacks in TIF format. Quantification of the time shift between red (Cas^{mSc}) and green (YFP-VCL, β1-EctopH, $ITGB1^{GFP}$ or Crk^{mGL}) cluster formation was performed in MATLAB (R2021b). The pipeline involves the following steps: drift correction, image preprocessing and denoising, focal adhesion segmentation and tracking, intensities extraction and normalization, and signal analysis.

Drifts between timeframes were corrected by registering the image of one frame to the image of the previous frame using a translation transformation. Drift corrected timeseries were then denoised with a median filter and their background was equalized with a top-hat transform. Clusters were segmented separately in each channel at each timeframe by intensity thresholding and size filtering. Threshold values were arbitrarily defined as a quarter of the intensity value of the 50th brightest pixel of the dataset for each channel, and the union of the binary masks of the two channels with an area greater than 20 pixels (0.52 μm^2) was used to define clusters. Clusters

were tracked over time by creating a 3D stack (XYT) and by computing the resulting connected components. Only clusters tracked in 3 or more frames (≥40 s) were quantified. For each tracked cluster, the average mean intensity of each channel over time was extracted by first measuring the mean intensity within the mask at each timeframe from time zero to the end, resulting in a number of intensity traces equal to the number of timeframes where the cluster was tracked.

To calculate the time shift between the red and green channel intensity profiles for each tracked cluster, average mean intensity traces were rescaled to the [0 1] value range, and after smoothing the signal by applying a moving average filter, the time at which the rescaled intensity reaches 0.5 ($t_{1/2}$) was interpolated. The time shift was finally calculated by subtracting red (Cas) $t_{1/2}$ from green (vinculin, integrin or Crk) $t_{1/2}$.

Adhesome isolation

Adhesome samples were isolated as described with minor modifications (Schiefermeier et al., 2014). MCF10A cells were detached from near-confluent 5-cm plates, resuspended in assay media, incubated in suspension for 30 min, then re-seeded onto an equal number of 5-cm plates that had been precoated with 50 μg/ml collagen. After one hour at 37°C, one dish was washed and lysed in 400 μl RIPA buffer to provide a sample of total protein (T) and the other dishes were washed gently with room temperature PBS and incubated with 2 ml freshly diluted 0.5 mM dithiobis(succinimidyl propionate) (DSP, EMD Millipore 322133) 0.05 mM (1,4-di [3'-(2'-pyridyldithio)propionamido] butane) (DPDPB, Sigma 16646) in PBS for 5 min at room temperature. Cross-linking was terminated by washing twice with 50 mM Tris 140 mM NaCl pH7.4 before transferring to ice. 400 μl RIPA buffer was added and the plates were rocked at 4°C for 1 hr. Supernatants (S) were collected in a 2-ml microtube (Axygen MCT-200-L-C). The plates were washed twice with PBS, drained, and 400 μl RIPA buffer added. 2-Mercaptoethanol was added to 2% concentration to all samples, and all microtubes and dishes were sealed and incubated at 50°C for 1 hr. After cooling to room temperature, the dish was scraped and the adhesome (A) fraction was transferred to a 2-ml microtube. Total, supernatant and adhesome fractions were sonicated (10 s, microtip) to shear DNA and the proteins were precipitated by adding 1.6 ml acetone and placing at -20°C for 1 hr. After centrifugation (10 min, 14,000 rpm), pellets were drained, dried in a stream of air, and incubated with 25 μl 5x concentrated SDS

sample buffer at 95°C for 5 min, then 100 μl RIPA buffer was added to all tubes. Samples were centrifuged and equal volumes analyzed by SDS PAGE, typically on 10% acrylamide/0.13% bisacrylamide gels, followed by Western blotting. The procedure was scaled up as needed to run replicate blots for probing with different antibody combinations. On occasion, dilutions of the T sample were loaded to estimate detection sensitivity.

Ratiometric FRET imaging

Rac1-2G reporter was expressed in MCF10A cells by lentiviral transduction. After siRNA or inhibitor treatment, cells were allowed to attach to collagen for 30 min then fixed and mounted. Coverslips were imaged on a Leica SP8 confocal microscope using Leica HCX Plan Apo 63X/ 1.40 oil immersion objective. Excitation and emission wavelengths as follows: donor (mTFP1) excitation 440 nm, emission 450-510 nm: FRET excitation 440 nm, emission 515-600 nm; acceptor (mVenus) excitation 514 nm, emission 515-600 nm. All channels were collected on the HyD detectors. Images were processed with the Lightening deconvolution (Leica LASX software) and FRET ratio in the most ventral plane quantified using ImageJ as described (Kardash et al., 2011).

Statistics

Distributions were analyzed using GraphPad Prism. Median and 95% confidence intervals were calculated for non-normal distributions. Data from multiple cells or biological replicates were assumed to follow normal distributions allowing calculation of mean and standard deviation (SD), or standard error of the mean (SEM) in cases where data from multiple cells in biologically independent experiments were combined. Comparisons between control and experimental populations were made using the non-parametric Mann Whitney U-test except where noted. In some experiments, a single control was compared with multiple experimental conditions. If the experimental conditions tested alternative hypotheses, we used the non-parametric Kruskal-Wallis ANOVA followed by Dunn’s multiple comparison test.

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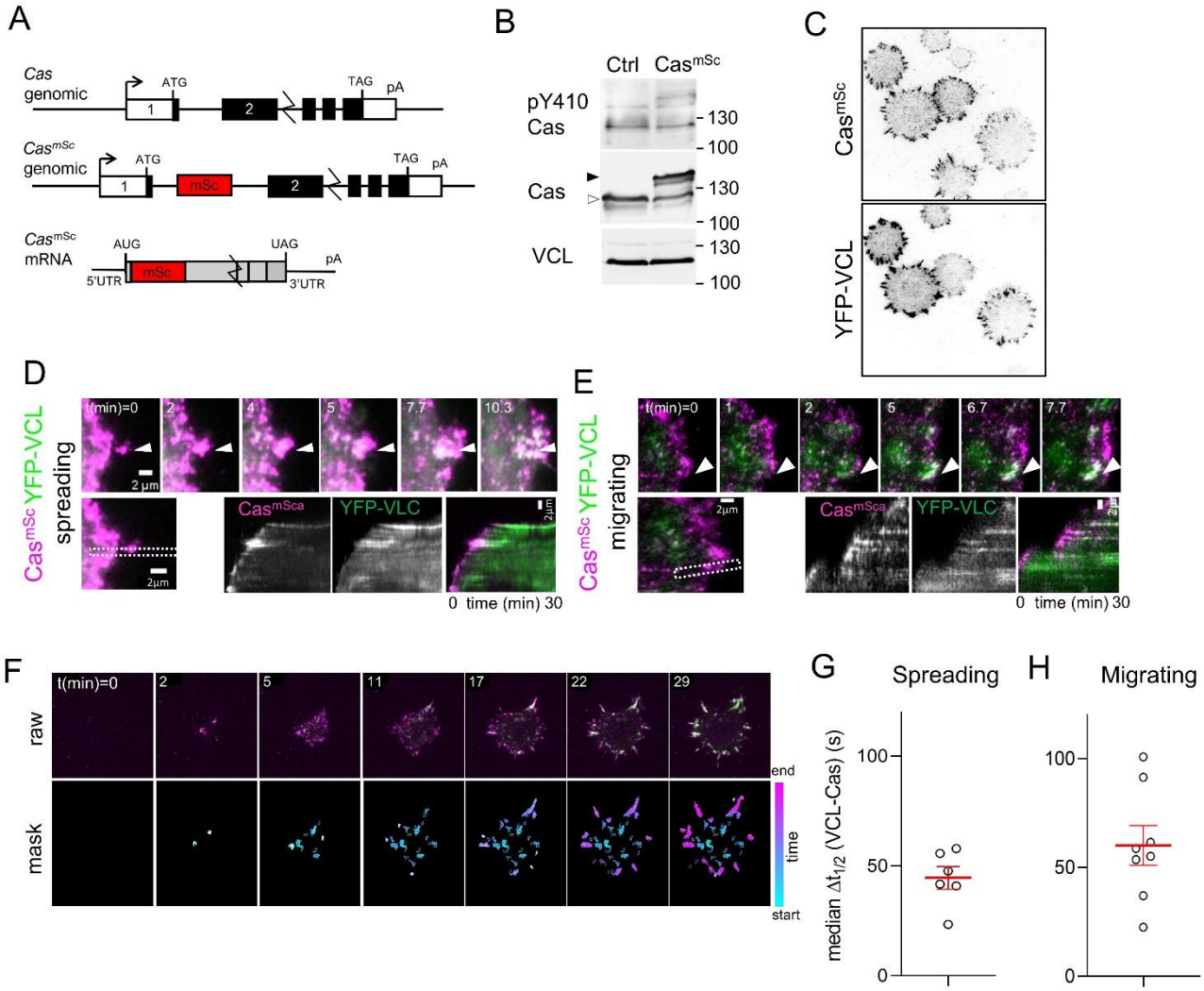
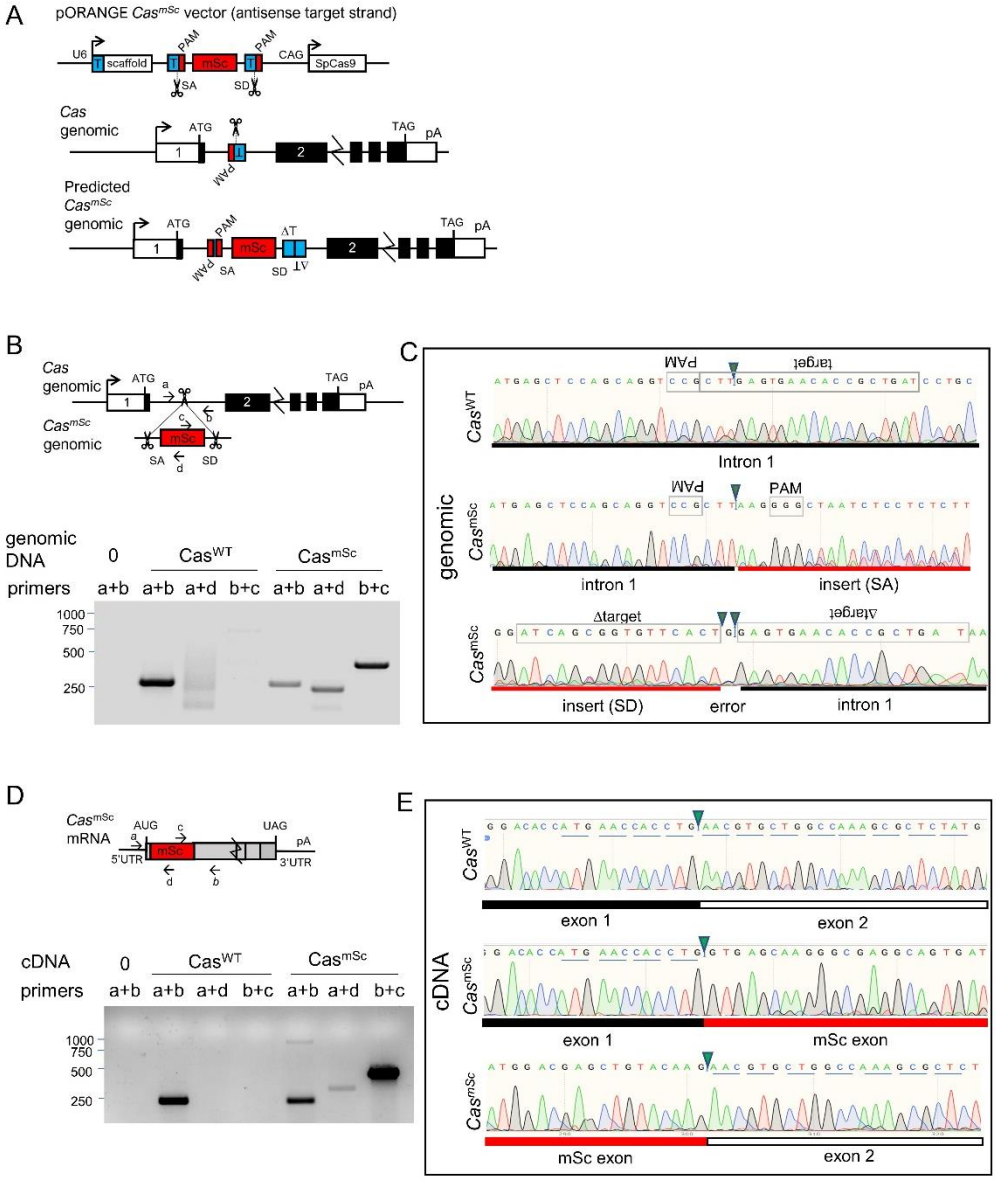
Figure 1



Live imaging shows Cas clusters mark sites of future vinculin clusters

(**A**) *Cas* wildtype and *Cas^mSc^* genomic organization and *Cas^mSc^* mRNA structure. (**B**) Representative immunoblot showing the pY410Cas, total Cas and vinculin (VCL) in control (Ctrl) and Cas^mSc^ MCF10A cells. (**C**) Cas^mSc^ co-localization with YFP-VCL. Cas^mSc^ MCF10A cells expressing YFP-VCL plated on COLI-treated glass-bottom dish for 30 min and visualized by TIRF microscopy. (**D-E**) Cas^mSc^ clusters form before vinculin clusters. TIRF microscopy of Cas^mSc^ YFP-VCL cells. Upper panels: individual time frames from (**D**) spreading or (**E**) migrating cells. Arrowheads indicate a Cas^mSc^ cluster (magenta) that is later joined by YFP-VCL (green). Lower panels: kymographs. (**F**) Pipeline for tracking ROIs. Upper panels: Raw images, false-colored Cas^mSc^ magenta and YFP-VCL green, from TIRF microscopy of spreading Cas^mSc^ YFP-VCL cells. Lower panels: masks showing tracked ROIs, color coded by time of onset. (**G**). Median $\Delta t_{1/2}$ (VCL-Cas) of multiple ROIs from n=6 spreading cells. Error bars show mean (44.6 s) and SEM (5 s). (**H**) Median $\Delta t_{1/2}$ (VCL-Cas) of multiple ROIs from n=8 spreading cells. Error bars show mean (60.1 s) and SEM (9 s).

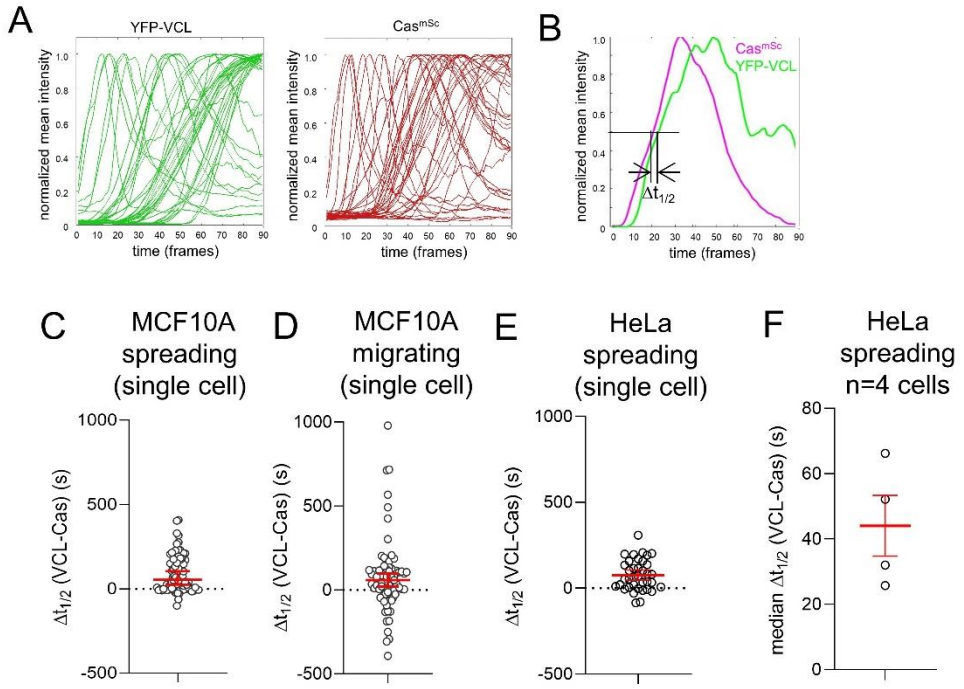
Figure 1 – figure supplement 1



Vector design and validation of Cas^{mSc} tagging

(**A**) Overview of the modified pORANGE-based system for tagging endogenous *Cas* at the N-terminal end by inserting an artificial exon. T, target sequence; PAM, protospacer adjacent motif; SA, splice acceptor; SD, splice donor, SpCas9, *Streptococcus pyrogenes* Cas9. (**B**) Primer positions and PCR products amplified from *Cas^{WT}* and *Cas^{mSc}* genomic DNA. (**C**) Sanger sequencing of *Cas^{WT}* (amplified with primers a+b) and 5' and 3' junctions of *Cas^{mSc}* (amplified with a+d and c+b respectively) genomic DNA. Note poor sequencing and inserted base indicative of imprecise insertion. (**D**) Primer positions and PCR products amplified from *Cas^{WT}* and *Cas^{mSc}* cDNA. (**E**) Sanger sequencing of RT-PCR amplified products from exon-exon junctions of *Cas^{WT}* (primers a+b) and *Cas^{mSc}* (primers a+d and c+b respectively) cDNA. Note in-frame *mSc* sequence between *Cas* codons 4 and 5.

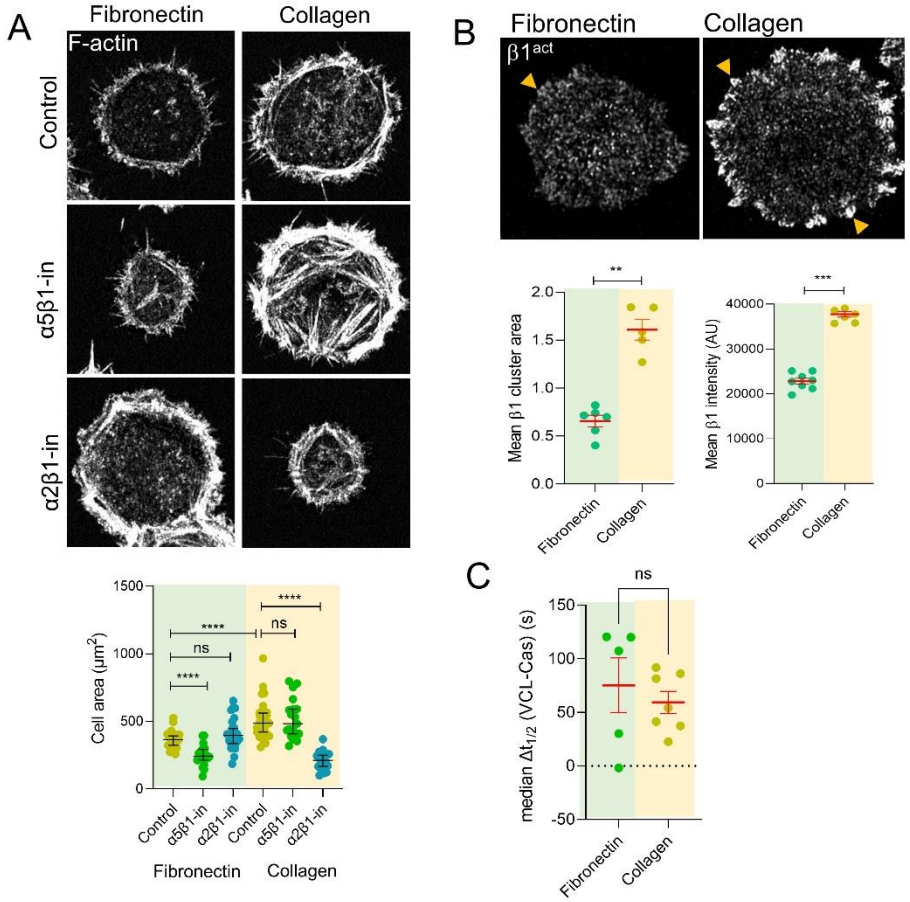
Figure 1 – figure supplement 2



Time interval between Cas and vinculin cluster assembly

(**A**) Normalized intensity profiles of multiple YFP-VCL and Cas^{mSc} ROIs tracked over 30 min of spreading in a single cell. Frame interval 20s. Each line tracks mean intensity of an ROI ≥20 pixels (0.5 μm^2) in area. (**B**) Paired intensity profiles for YFP-VCL and Cas^{mSc} in a single tracked ROI. $\Delta t_{1/2}$ was calculated by subtracting $t_{1/2}$ of normalized intensity for YFP-VCL from Cas^{mSc} of the same ROI. (**C**) $\Delta t_{1/2}$ (VCL-Cas) for 73 ROIs in a single spreading Cas^{mSc} YFP-VCL MCF10A cell. Error bars indicate median (58.1 s) and 95% confidence interval (19.7-96.9 s). (**D**) $\Delta t_{1/2}$ (VCL-Cas) for 90 ROIs in a single migrating Cas^{mSc} YFP-VCL MCF10A cell. Error bars indicate median (54.8 s) and 95% confidence interval (23-105 s). (**E**) $\Delta t_{1/2}$ (VCL-Cas) for 37 ROIs in a single spreading Cas^{mSc} YFP-VCL HeLa cell. Error bars indicate median (66.2 s) and 95% confidence interval (21-114 s). (**F**) Median $\Delta t_{1/2}$ (VCL-Cas) for n=4 spreading Cas^{mSc} YFP-VCL HeLa cells. Error bars indicate mean (44.1 s) and SEM (9 s).

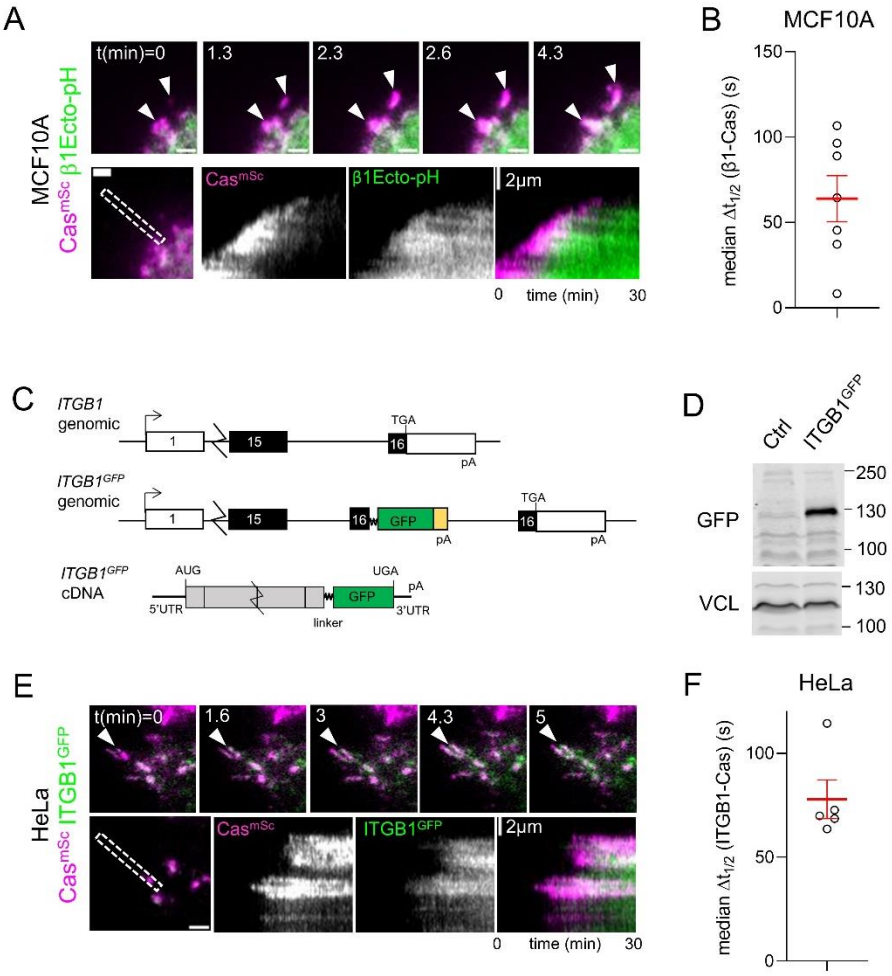
Figure 2



Cas clusters precede vinculin clusters independent of ECM

(**A**) Integrin specificity of different ECM. Representative images and mean spread areas of Cas^{mSc} MCF10A cells plated for 30 min on fibronectin- or COLI-coated surface and stained with phalloidin. Cas^{mSc} MCF10A cells were treated with blocking antibodies α5β1 (P8D4) and α2β1 (P1E6) during spreading on fibronectin- or collagen-coated glass for 30 min. Cells were then fixed and stained with phalloidin to visualize F-actin. (**B**) Staining for active-β1 using conformation-specific antibodies 9EG7. Ventral section. Mean β1 cluster area and intensity were calculated from 5-6 cells containing >50 clusters of ≥20 pixels area. (**C**) Median $\Delta t_{1/2}$ (VCL-Cas) n=5-7 spreading Cas^{mSc} YFP-VCL MCF10A cells on fibronectin or collagen. Error bars indicate mean and SEM. ns, nonsignificant by Mann-Whitney test.

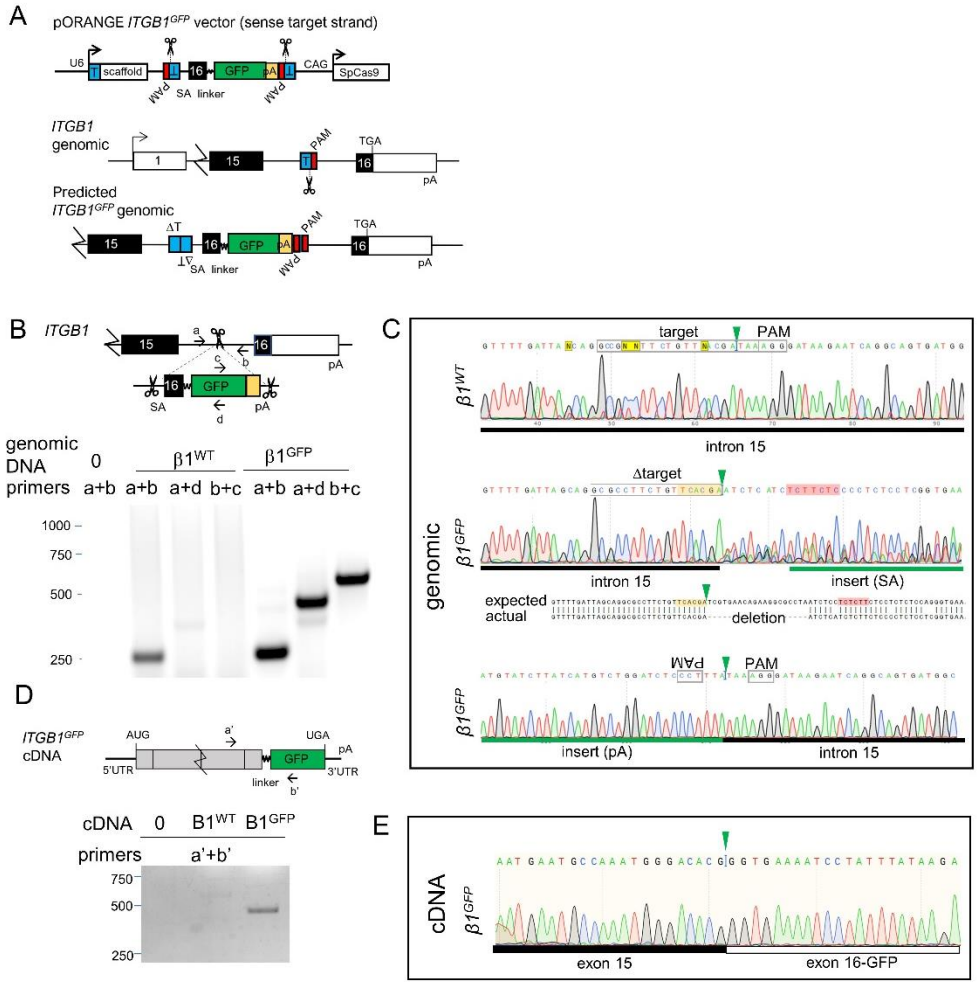
Figure 3



Live imaging shows Cas clusters mark sites of future integrin clusters

(**A**) Cas^{mSc} clusters form before β1Ecto-pH clusters. TIRF microscopy of spreading Cas^{mSc} β1Ecto-pH MCF10A cells. Upper panels: individual time frames. Arrowheads indicate a Cas^{mSc} cluster (magenta) that is later joined by β1Ecto-pH (green). Lower panels: kymographs. (**B**) Median $\Delta t_{1/2}$ (β1-Cas) of multiple ROIs from seven spreading Cas^{mSc} β1Ecto-pH MCF10A cells. Error bars show mean (63.9 s) and SEM (13 s). (**C**) *ITGB1* wildtype and *ITGB1^{GFP}* genomic organization and *ITGB1^{GFP}* mRNA structure. (**D**) Immunoblot showing expression of $ITGB1^{GFP}$ protein in Cas^{mSc} $ITGB1^{GFP}$ HeLa cells. (**E**) Cas^{mSc} clusters form before $ITGB1^{GFP}$ clusters. TIRF microscopy of Cas^{mSc} $ITGB1^{GFP}$ HeLa cells. Upper panels: individual time frames. Arrowheads indicate a Cas^{mSc} cluster (magenta) that is later joined by $ITGB1^{GFP}$ (green). Lower panels: kymographs. (**F**). Median $\Delta t_{1/2}$ (ITGB1-Cas) of multiple ROIs from five spreading Cas^{mSc} $ITGB1^{GFP}$ HeLa cells. Error bars show mean (77.9 s) and SEM (21 s).

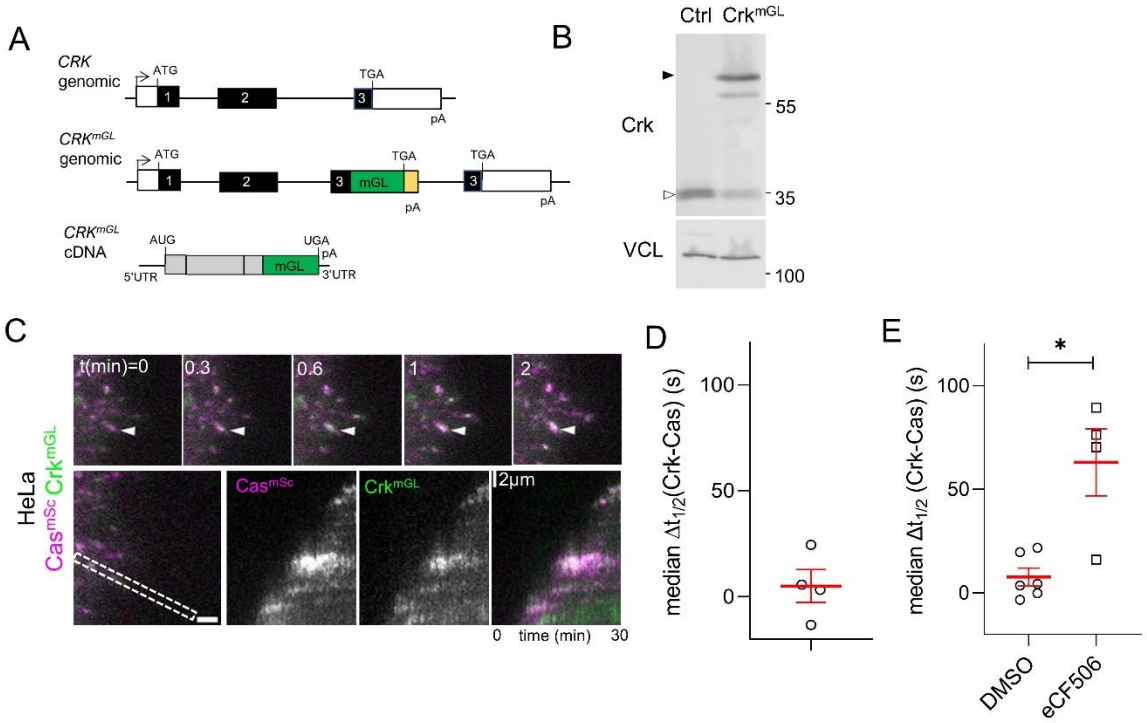
Figure 3 – figure supplement 1



Vector design and validation of ITGB1^{GFP} tagging

(**A**) Overview of the modified pORANGE-based system for tagging endogenous *ITGB1* at the C-terminal end by inserting an artificial exon and 3' end signal. T, target sequence; PAM, protospacer adjacent motif; SA, splice acceptor; SD, splice donor, SpCas9, *Streptococcus pyrogenes* Cas9; pA, SV40 3' end and poly A signal sequence. (**B**) Primer positions and PCR products amplified from *ITGB1^{WT}* and *ITGB1^{GFP}* genomic DNA. (**C**) Sanger sequencing of *ITGB1^{WT}* (amplified with primers a+b) and 5' and 3' junctions of *ITGB1^{GFP}* (amplified with a+d and c+b respectively) genomic DNA. Note poor sequencing and deletion indicative of imprecise insertion. (**D**) Primer positions and PCR products amplified from *ITGB1^{WT}* and *ITGB1^{GFP}* cDNA. (**E**) Sanger sequencing of RT-PCR amplified product from exon-exon junction of *ITGB1^{GFP}* (primers a'+b') cDNA. Note in-frame *ITGB1* sequence between exon 15 and the introduced exon 16-GFP fusion.

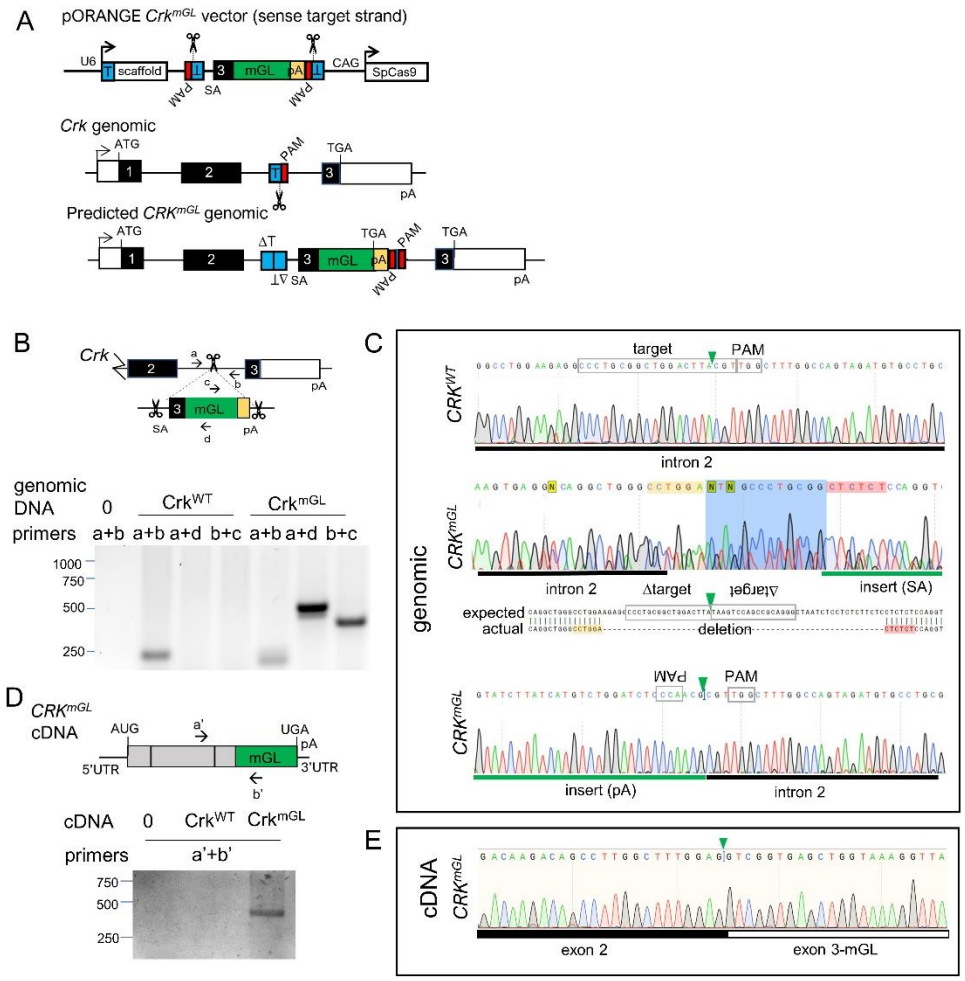
Figure 4



Initial Cas clusters contain Crk

(**A**) *Crk* wildtype and *Crk*^{mGL} genomic organization and *Crk*^{mGL} mRNA structure. (**B**) Immunoblot showing expression of Crk^{mGL} protein in Crk^{mGL} HeLa cells. (**C**) Crk^{mGL} clusters form shortly after Cas^{mSc} clusters. TIRF microscopy of Cas^{mSc} Crk^{mGL} HeLa cells. Upper panels: individual time frames. Arrowheads indicate a Cas^{mSc} cluster (magenta) that is rapidly joined by Crk^{mGL} (green). Lower panels: kymographs. (**D**) Median $\Delta t_{1/2}$ (Crk-Cas) of multiple ROIs from four spreading Cas^{mSc} Crk^{mGL} HeLa cells. Error bars show mean (5.1 s) and SEM (7.7 s). (**E**) Median $\Delta t_{1/2}$ (Crk-Cas) of multiple ROIs from 4-6 spreading Cas^{mSc} Crk^{mGL} HeLa control and eCF506-treated cells. Error bars show mean and SEM. *, P<0.05 by Mann Whitney U test.

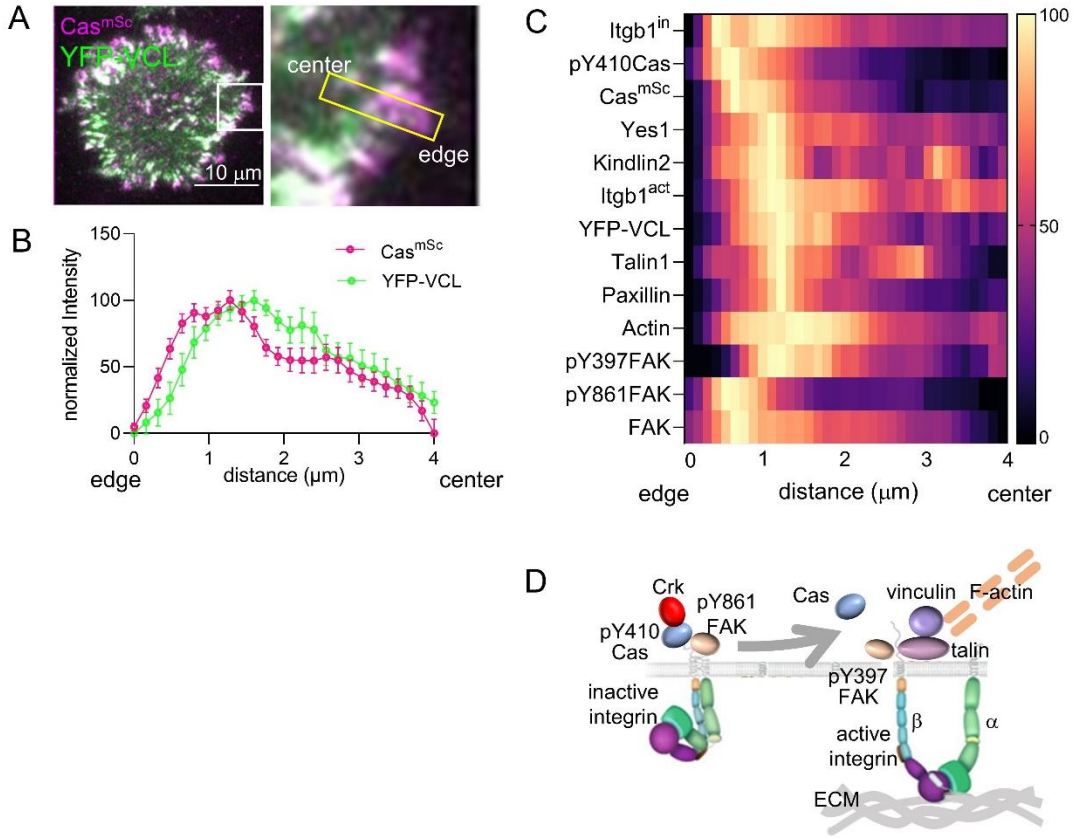
Figure 4 – figure supplement 1



Vector design and validation of Crk^{mGL} tagging

(**A**) Overview of the modified pORANGE-based system for tagging endogenous *Crk* at the C-terminal end by inserting an artificial exon and 3' end signal. T, target sequence; PAM, protospacer adjacent motif; SA, splice acceptor; SD, splice donor, SpCas9, *Streptococcus pyrogenes* Cas9; pA, SV40 3' end and poly A signal sequence. (**B**) Primer positions and PCR products amplified from *Crk^{WT}* and *Crk^{mGL}* genomic DNA. (**C**) Sanger sequencing of *Crk^{WT}* (amplified with primers a+b) and 5' and 3' junctions of *Crk^{mGL}* (amplified with a+d and c+b respectively) genomic DNA. Note poor sequencing and deletion indicative of imprecise insertion. (**D**) Primer positions and PCR products amplified from *Crk^{WT}* and *Crk^{mGL}* cDNA. (**E**) Sanger sequencing of RT-PCR amplified product from exon-exon junction of *Crk^{mGL}* (primers a'+b') cDNA. Note in-frame *Crk* sequence between exon 2 and the introduced exon 3-mGL fusion.

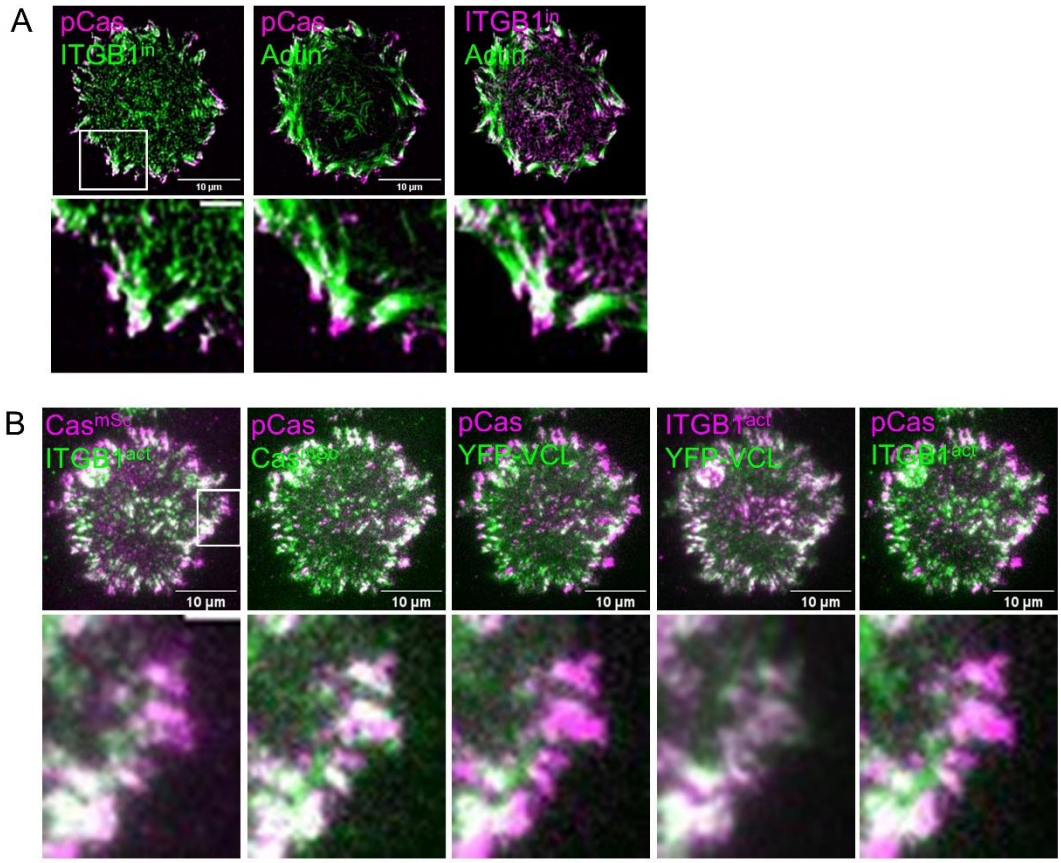
Figure 5



Spatial distribution of integrin adhesion proteins in Cas clusters

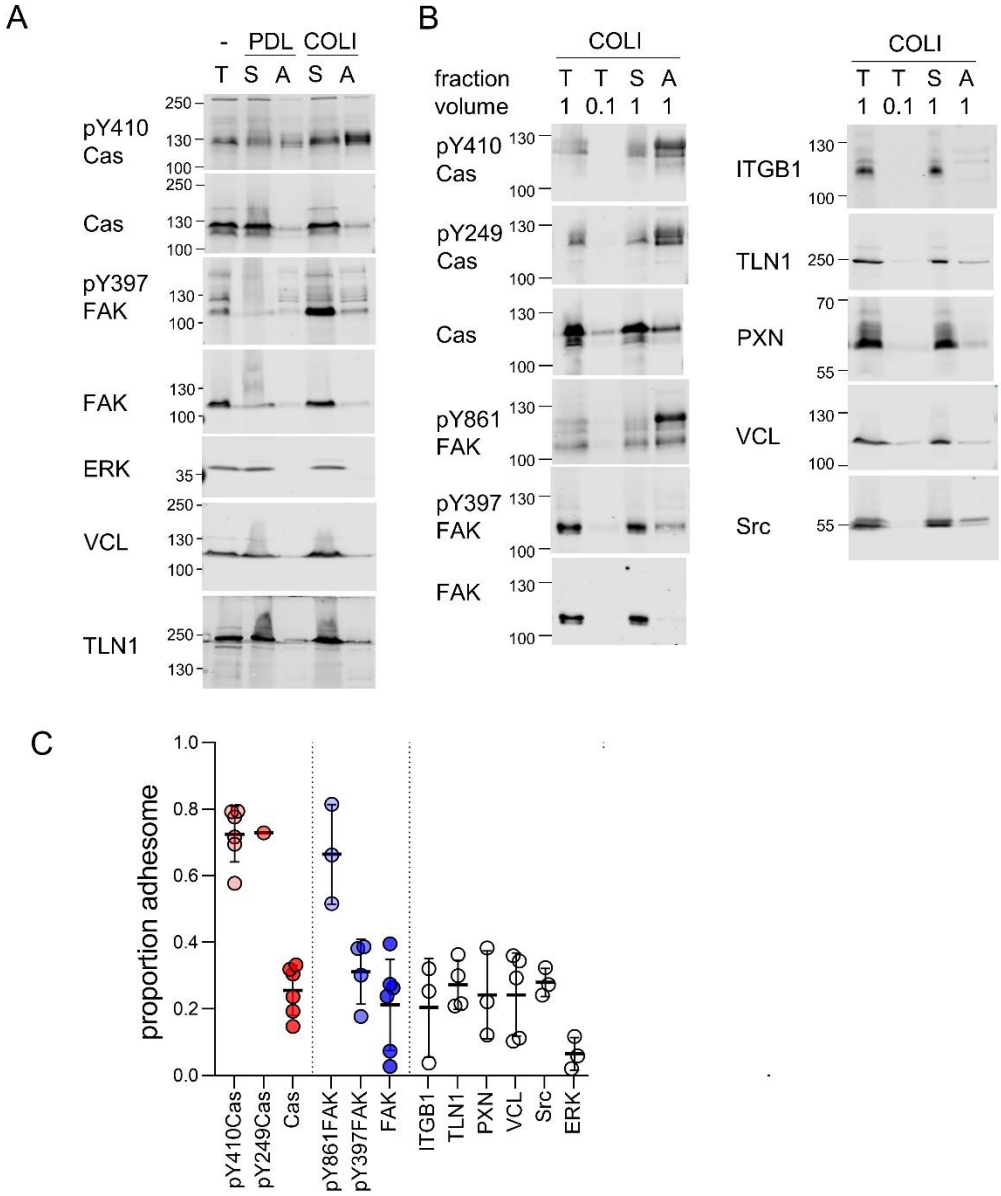
(**A**) Representative image used for measuring spatial distribution of proteins and phosphoproteins across developing focal adhesions. Cas^{mSc} YFP-VCL MCF10A cells were fixed and imaged by TIRF after 30 min spreading. Inset shows 4 x 0.8 µm region, starting from cell edge to towards cell center, used to graph mean intensity against distance. (**B**) Mean intensity against distance plot for Cas^{mSc} and YFP-VCL across ≥20 regions from several cells. Error bars indicate mean and SEM. (**C**) Heat map of normalized mean immunofluorescence intensity against distance for various antigens in Cas^{mSc} MCF10A stained with indicated antibodies. (**D**) Model showing inferred progression from initial nascent adhesions or focal complexes containing high levels of pY410Cas, inactive integrin and pY861FAK, progressing to become focal adhesions containing low levels of Cas and high levels of active integrin, vinculin, pY397FAK, F-actin, talin, paxillin and kindlin.

Figure 5 – figure supplement 1



Representative images showing spatial distribution of adhesion proteins in Cas clusters
(A) Cas^{mSc} MCF10A cell stained for pY410Cas, inactive integrin β1, and F-actin. (B) Cas^{mSc} YFP-VCL MCF10A cell stained for pY410Cas and active integrin β1.

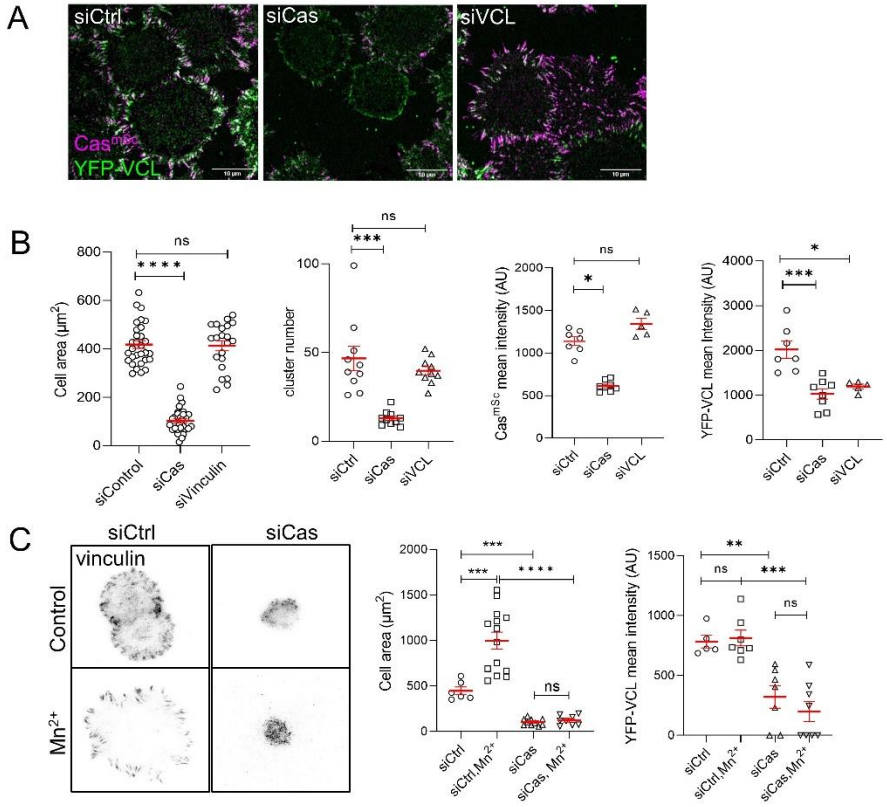
Figure 5 – figure supplement 2



Phosphorylated Cas is enriched in the adhesome

(A-B) Representative results from two independent experiments. Western blots of total (T), supernatant (S), and adhesome (A) fractions from cells plated on different ECM for 60 min were probed with the indicated antibodies. PDL, poly-D-lysine; COLI, collagen-I. (**C**) Quantification of multiple independent experiments. Proportion adhesome was calculated from A/(S+A). Error bars indicate mean and SD.

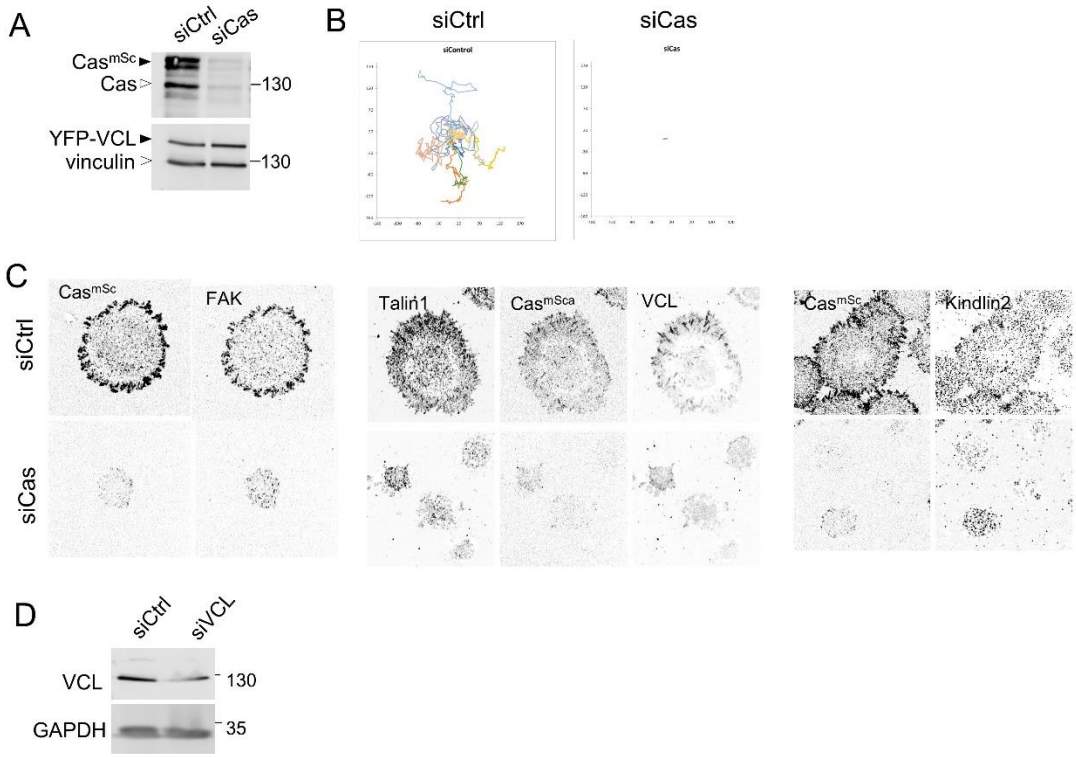
Figure 6



Cas regulates vinculin recruitment and focal adhesion assembly

(**A**) Representative images (ventral section) of Cas^{mSc} YFP-VCL MCF10A cells treated with control, Cas or vinculin siRNA and fixed after 30 min of spreading. (**B**) Quantification of mean cell area and the number, mean Cas intensity, and mean vinculin intensity of individual Cas and/or vinculin clusters. Error bars show mean and SEM for n=10-50 cells from three biological repeats. ns, non-significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 by Kruskal-Wallis followed by Dunn's multiple comparison test. (**C**) Representative images of MCF10A expressing YFP-VCL cells treated with control or Cas siRNA and fixed after spreading in the absence or presence of Mn^{2+} for 30 min. Graphs show the mean cell spread area and mean intensity of YFP-VCL clusters. Error bars show mean and SEM for n=6-20 cells from two biological repeats. P values from pairwise Mann Whitney U tests, comparing each test condition to the control shown.

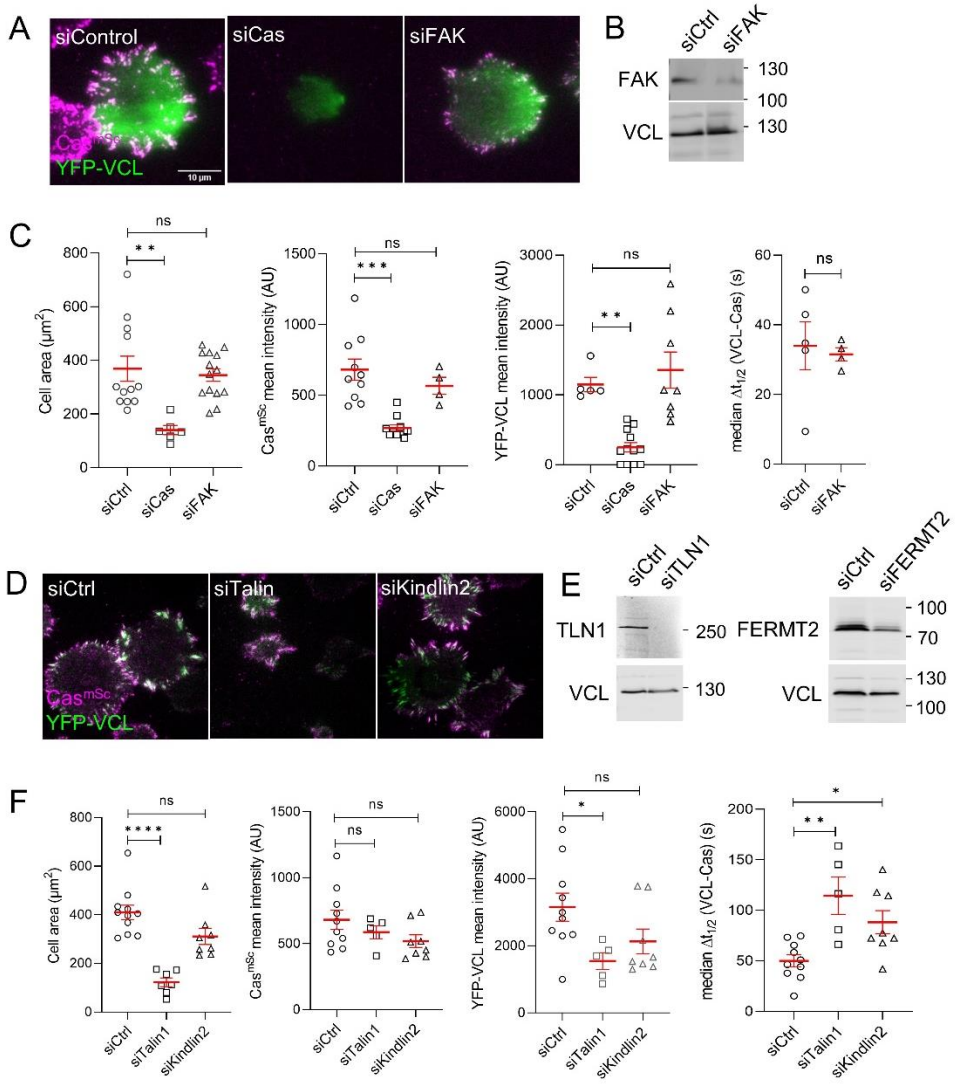
Figure 6 – figure supplement 1



Cas depletion inhibits MCF10A cell migration

(**A**) Immunoblot of cell lysates, (**B**) wind-rose plots depicting total path traveled (12 hrs), and (**C**) representative images (ventral section), of Cas^{mSc} YFP-VCL MCF10A cells treated with control or Cas siRNA. (**D**) Immunoblot of cell lysates harvested from Cas^{mSc} MCF10A cells treated with control or VCL siRNA.

Figure 6 – figure supplement 2

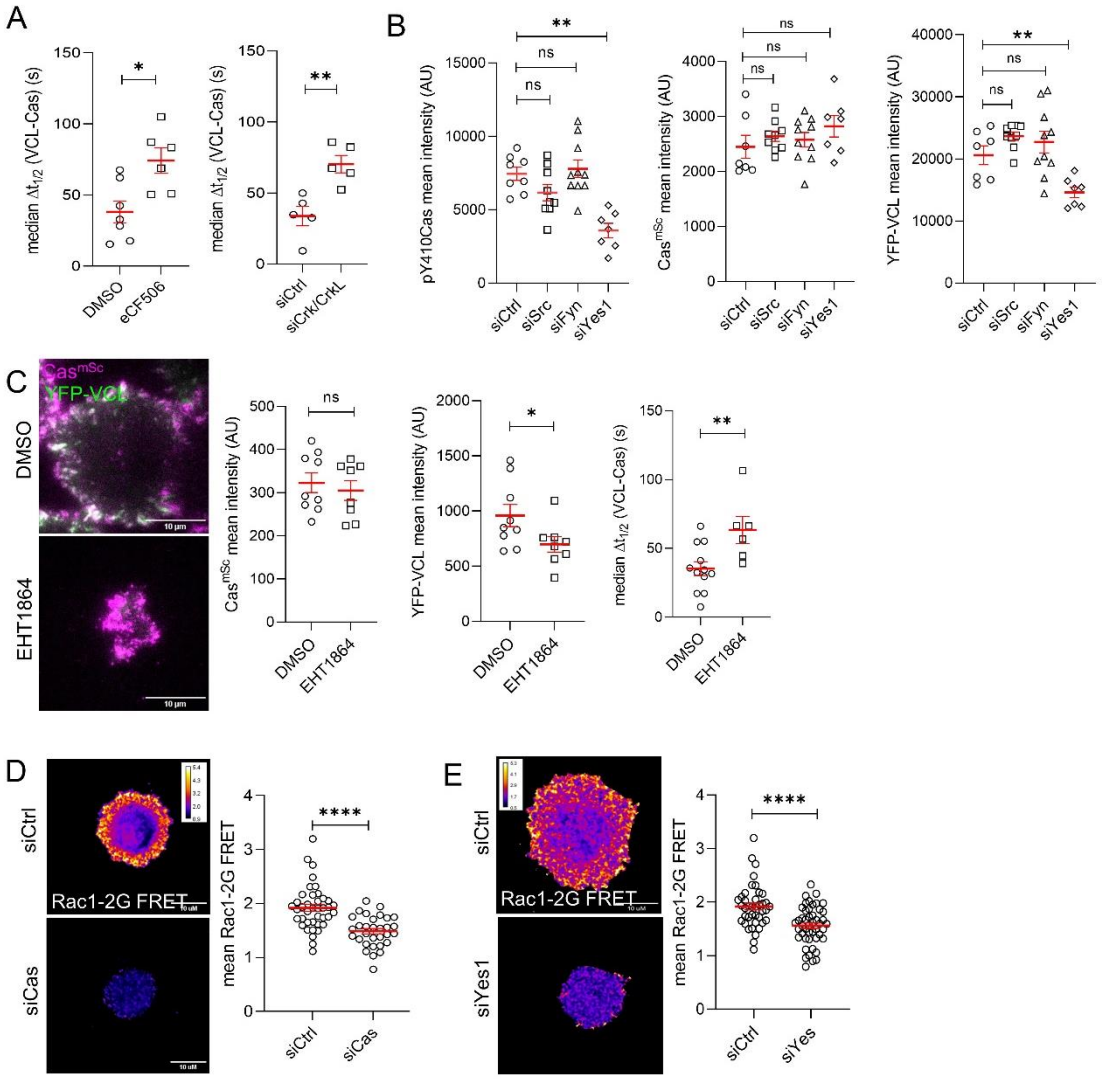


(**A**) Representative endpoint dual-color time-lapse TIRF micrographs of spreading Cas^{mSc} YFP-

Controls and additional data showing Cas regulates vinculin recruitment and focal adhesion assembly

VCL MCF10A cells treated with control, Cas or FAK siRNA; (**B**) Immunoblot showing FAK depletion. (**C**) Mean cell area, mean Cas^{mSc} and YFP-VCL intensity, and median $\Delta t_{1/2}$ (VCL-Cas) from 4-12 cells in three biological repeats. Error bars show mean and SEM. (**D**) Representative endpoint dual-color time-lapse TIRF micrographs of spreading Cas^{mSc} YFP-VCL MCF10A cells treated with control, talin1 or kindlin2 siRNA. (**E**) Immunoblot showing talin1 and kindlin2 depletion. (**F**) Mean cell area, mean Cas^{mSc} and YFP-VCL intensity, and median $\Delta t_{1/2}$ (VCL-Cas) from 5-12 cells in three biological repeats. ns, non-significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 by Kruskal-Wallis followed by Dunn's multiple comparison test.

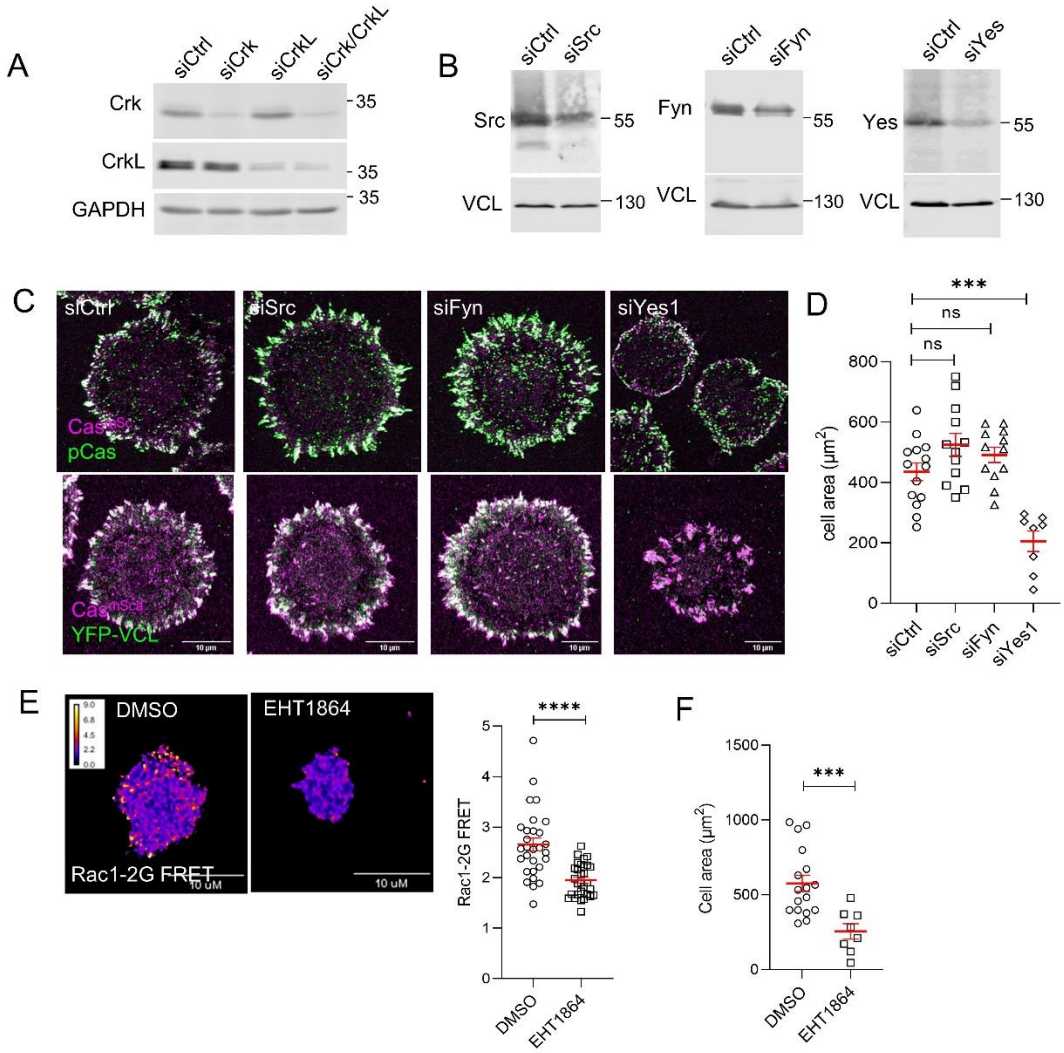
Figure 7



SFK-Cas-Crk-Rac1 signaling regulates adhesion assembly

(**A**) Median $\Delta t_{1/2}$ (VCL-Cas) from 5-8 time-lapse dual-color TIRF micrographs of spreading Cas^{mSc} YFP-VCL MCF10A cells treated with DMSO or SFK inhibitor eCF506 (left), or with control or Crk/CrkL siRNA (right). (**B**) Mean cluster intensity of the pY410Cas, Cas^{mSc} and YFP-VCL in Cas^{mSc} YFP-VCL MCF10A cells treated with control, Src, Fyn or Yes1 siRNA and fixed after 30 min of spreading. Error bars show mean and SEM from n=7-10 cells from three biological repeats. (**C**) Representative endpoint dual-color time-lapse TIRF micrographs (left) and quantification (right) of spreading Cas^{mSc} YFP-VCL MCF10A cells treated with DMSO or Rac1 inhibitor EHT1864. Error bars show mean and SEM of 6-12 cells in 3 biological repeats. (**D-E**) Representative FRET ratio images (left) and quantification (right) of Rac1-2G biosensor MCF10A cells treated with (**D**) control or Cas, or (**E**) control or Yes1 siRNA and fixed after 30 min of spreading. Error bars show mean and SEM from >30 cells from 3 biological replicates. All P values by Mann Whitney U tests.

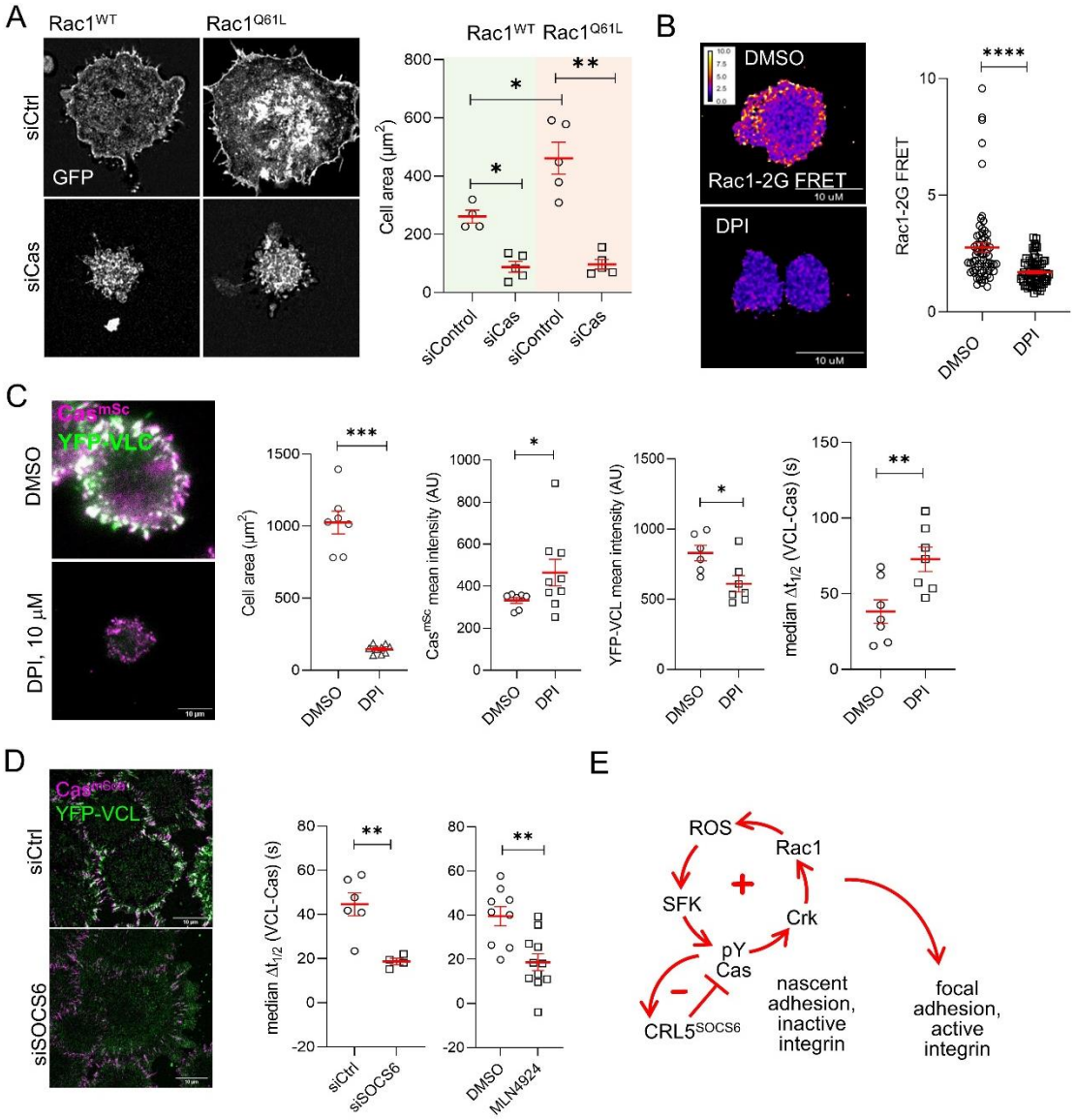
Figure 7 – figure supplement 1



Controls and additional data showing SFK-Cas-Crk-Rac1 signaling regulates adhesion assembly

(**A-B**) Representative immunoblots of cell lysates from Cas^{mSc} MCF10A cells treated with (**A**) control, Crk, CrkL or Crk/CrkL siRNA or (**B**) control, Src, Fyn or Yes1 siRNA. (**C**) Images (ventral section) of Cas^{mSc} YFP-VCL MCF10A cells treated with control, Src, Fyn, or Yes1 siRNA and fixed after 30 min of spreading. Staining for Cas^{mSc} and pY410Cas (above) or Cas^{mSc} and YFP-VCL (below). (**D**) Mean areas of siSFK cells in (**C**). (**E**) Representative FRET ratio images (left) and quantification (right) of Rac1-2G biosensor MCF10A cells treated with DMSO or Rac1 inhibitor EHT1864 and imaged after 30 min spreading. (**F**) Mean areas of EHT1864-treated cells. All error bars represent mean and SEM and all P values by Mann Whitney U tests.

Figure 8



Linkage between cell spreading and adhesion assembly through positive and negative feedback

(**A**) Rac1 requires Cas to induce cell spreading. Representative images (ventral section) (left) and area quantification (right) of MCF10A cells transiently expressing EGFP-Rac1WT or -Rac1Q61L that were treated with control or Cas siRNA and fixed after 30 min of spreading. (**B-C**) NADPH-dependent oxidase inhibitor DPI inhibits Rac1 activation, cell spreading and vinculin recruitment to Cas clusters. (**B**) Representative FRET images (left) and quantification (right) of Rac1-2G biosensor MCF10A cells treated with DMSO or DPI and fixed after 30 min of spreading. (**C**) Representative endpoint dual color TIRF micrographs (left) and quantification (right) of 30 min spreading Cas^{mSc} YFP-VCL MCF10A cells treated with control or DPI. Graphs show mean cell area, mean Cas^{mSc} and YFP-VCL intensity, and median $\Delta t_{1/2}$ (VCL-Cas) from 7-

12 cells in three biological repeats. (**D**) Inhibiting Cullins accelerates vinculin recruitment to Cas clusters. Representative endpoint dual color TIRF micrographs (left) and quantification (right) of 30 min spreading Cas^{mSc} YFP-VCL MCF10A cells treated with control or SOCS6 siRNA or with DMSO or Cullin Neddylation inhibitor MLN4924. Graphs show median $\Delta t_{1/2}$ (VCL-Cas) from 4-10 cells in three biological repeats. All error bars represent mean and SEM and all P values by Mann Whitney U tests. (**E**) Model showing nascent adhesions as sites of Cas clustering with inactive integrin, which leads to SFK-dependent phosphorylation of Cas, recruitment of Crk/CrkL, activation of Rac1 and ROS and positive feedback that strengthens and maintains signaling and recruits talin1, kindlin2 and other proteins to form a focal adhesion. This positive feedback is opposed by negative feedback resulting from SOCS6/cullin-mediated ubiquitination of phosphorylated Cas.

Supplementary videos

Supplemental video 1 Cas (magenta) and vinculin (green) dynamics during attachment and spreading. Cas^{mSc} YFP-VCL MCF10A cell spreading on collagen. 20 s time intervals. Playback 7 frames/s.

Supplemental video 2 Cas (magenta) and vinculin (green) dynamics during lamellipodia extension by a migrating cell. Cas^{mSc} YFP-VCL MCF10A cell cultured on collagen 24 hr.

Supplemental video 3 Quantification of Cas and vinculin cluster dynamics. MCF10A Cas^{mSc} YFP-VCL MCF10A cell spreading on collagen. (Left) Raw data. (Right) Regions of interest defined as regions of 20 pixels (0.5 μm^2) or greater in which either or both channel intensities exceed threshold in three consecutive frames. Regions are color coded according to the first frame in which the region is first detected, from cyan to magenta. See Methods.

Supplemental video 4 Effect of ECM. Cas (magenta) and vinculin (green) dynamics during attachment and spreading on collagen (left) and fibronectin (right).

Supplemental video 5 Integrin clustering in MCF10A cells. Cas (magenta) and integrin (green) dynamics during attachment and spreading. Cas^{mSc} β1Ecto-pH MCF10A cell.

Supplemental video 6 Integrin clustering in HeLa cells. Cas (magenta) and integrin (green) dynamics during attachment and spreading. Cas^{mSc} $ITGB1^{GFP}$ HeLa cell.

Supplemental video 7 Crk clustering. Cas (magenta) and Crk (green) dynamics during attachment and spreading. Cas^{mSc} Crk^{mGL} HeLa cell.

Supplemental video 8 SFK inhibition delays Crk (green) recruitment to Cas (magenta). Cas^{mSc} Crk^{mGL} HeLa cells spreading on collagen in presence of DMSO (left) or SFK inhibitor eCF506 (right).

Supplemental video 9 Vinculin dynamics in control (left) and Cas-depleted (right) Cas^{mSc} YFP-VCL cells, 24 hr after plating on collagen.

Supplemental video 10 Cas requirement for vinculin clustering. Cas (magenta) and vinculin (green) dynamics in control (left) and Cas-depleted (right) Cas^{mSc} YFP-VCL MCF10A cells during attachment and spreading on collagen.

Supplemental video 11 SFK inhibition delays vinculin (green) recruitment to Cas (magenta). Cas^{mSc} YFP-VCL MCF10A cells treated with DMSO (left) or eCF506 (right) during attachment and spreading on collagen.

Supplemental video 12 Crk/CrkL depletion delays vinculin (green) recruitment to Cas (magenta). Cas^{mSc} YFP-VCL MCF10A cells treated with control (left) or Crk/CrkL (right) siRNA.

Supplemental video 13 Rac1 inhibition delays vinculin (green) recruitment to Cas (magenta). Cas^{mSc} YFP-VCL MCF10A cells treated with DMSO (left) or EHT1864 (right).

Supplemental video 14 ROS regulates vinculin (green) recruitment to Cas (magenta). Cas^{mSc} YFP-VCL MCF10A cells treated with DMSO (left) or DPI (right).

Supplemental video 15 SOCS6 delays vinculin (green) recruitment to Cas (magenta). Cas^{mSc} YFP-VCL MCF10A cells treated with contol (left) or SOCS6 (right) siRNA.